Is there a threshold at which vitamin D status during pregnancy optimizes maternal and neonatal health outcomes?
A focus on bone and gestational diabetes

Negar Tabatabaei

School of Dietetics and Human Nutrition

McGill University, Montréal

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ABSTRACT

Low vitamin D status, defined by circulating 25-hydroxy vitamin D (25(OH)D) <50 or <75 nmol/L is prevalent among pregnant women and has been associated with neonatal rickets and gestational diabetes mellitus (GDM). Osteocalcin (OC), a bone formation biomarker, linking the bone to glucose metabolism is stimulated by 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active form of vitamin D. In the first study, maternal serum 25(OH)D and OC were explored at early and mid-gestation in GDM (case, n=48) vs healthy (control, n=48) Caucasian pregnant women. Maternal serum 25(OH)D was not different (p=0.80) while OC was higher (p=0.006) in those with GDM vs controls across all trimesters. There are no vitamin D dose-response studies on glucose tolerance and bone outcomes in human and animals. Animal studies commonly use isoflurane, an anaesthetic, which may affect OC and glucose concentrations. Therefore, in the second study, the effect of isoflurane exposure was studied on bone biomarkers in guinea pigs (n=10) at 5, 9 wk of age and glucose at postpartum (26 wk). Isoflurane increased OC during rapid growth (p<0.001) and increased glucose at postpartum (p<0.0001). In the third study, the dose-response effect of maternal dietary vitamin D was investigated on maternal glucose tolerance. Female 4 mo old guinea pigs (n=45) were randomized to isocaloric diets containing different dosages of vitamin D_3 (0, 0.25, 0.5, 1 and 2 IU/g diet) from mating to delivery. The results revealed no differences in AUC (area under the curve) for maternal glucose from the OGTT (oral glucose tolerance test) across groups during pregnancy. However, an inverse association was observed between pre-mating 25(OH)D and AUC for glucose. In the fourth study, the dose-response effect of dietary vitamin D was studied on maternal and neonatal bone outcomes. In the sows, a positive dose-response (p<0.0001) was observed in plasma 25(OH)D but

$1,25(OH)_2D$ reached a plateau once dietary vitamin D was >0.5 IU/g diet. No differences in areal (aBMD) or volumetric bone mineral density (vBMD) or biomarkers were observed among maternal groups. In the 2 d guinea pig neonate: both 25(OH)D and $1,25(OH)_2D$ followed a dose-response ($p<0.0001$) to maternal diet, femoral aBMD was 10 % higher in the 2 IU vs all groups except the 0 IU ($p=0.04$). At the neonatal distal femur and proximal tibia, response to maternal diet was U-shaped for trabeculae (Tb.) vBMD. Expansion of the femur growth plate was observed in the 0 IU vs all other groups. In conclusion, vitamin D status may be more important at pre-mating than mid-gestation in protecting against pregnancy induced glucose intolerance. To reflect normal metabolism, measurements of OC and glucose prior to isoflurane anaesthesia are recommended. Maternal vitamin D status below recommendations may result in features similar to rickets in the offspring but status above recommendations may not be advantageous to the bone in the guinea pig.

RÉSUMÉ

Un taux faible en vitamine D, soit une concentration inférieure à 50-75 nmol/L de 25-hydroxy vitamine D (25(OH)D) dans le sang, est fréquent chez les femmes enceintes et est associée au rachitisme néonatal et au diabète gestationnel (GDM). L'ostéocalcine (OC), un biomarqueur qui relie le métabolisme du glucose à la formation osseuse, est stimulée par la forme active de la vitamine D (1,25-dihydroxyvitamine D ($1,25(OH)_2D$)). La première étude de cette thèse a identifié les taux sérique de 25(OH)D et d'OC chez les femmes enceintes blanches du Québec, en santé (n=48), ou atteintes de GDM (n=48), au début et au milieu de leur gestation. Les résultats ont révélé que chez les personnes atteintes de GDM vs en santé, les taux sériques maternels de 25(OH)D ne sont pas différents (p=0.80) mais les taux d'OC sont plus élevés (p=0.006) durant tous les trimestres. Aucune étude n'a examiné simultanément les doses en vitamine D et ses effets sur la tolérance au glucose et la formation osseuse. De plus, les études précédentes sur modèles animaux ont utilisé de l'isoflurane comme anesthésique, un produit qui peut affecter les concentrations d'OC et de glucose dans le sang. Afin d'étudier ces critères, une examination chez les cochons d'indes (n=10) à 5 et 9 semaines de gestations, l'effet de l'isoflurane (20-25 min) sur les biomarqueurs osseux (OC, désoxypyridinoline totale (tDPD)) ainsi que les niveaux de glucoses après l'accouchement (26 semaines) a été exécutée. Les résultats démontrent que l'isoflurane augmente les taux d'OC pendant la croissance rapide ($p<0.001$), et accrois les niveaux de glucose après l'accouchement ($p<0.0001$). Le troisième projet consiste d'étudier les effets des doses alimentaires en vitamine D et la tolérance au glucose des femelles cochon d'indes enceintes. Des femelles âgées de quatre mois (n=45) ont été soumises à une diète de différentes doses de vitamine D_3 (0, 0,25, 0,5, 1 et 2 IU/g d'aliments) pendant leur grossesse. Les résultats révèlent

aucune différence de l'ASC (l'aire sous la courbe) pour le glucose ainsi que les concentrations d'OC. Cependant, une association inverse a été observée entre les niveaux de 25(OH)D et l'ASC du glucose avant accouplement. La dernière étude examine chez les mères enceintes et nouveaux-nés, l'effet des doses en vitamine D provenant de l'apport alimentaire sur les densités des os. Chez les femelles cochon d'indes, une réponse positive ($p<0.0001$) aux doses de vitamine D alimentaire a été observée sur les niveaux de 25(OH)D dans le plasma, tandis que les taux en $1,25(OH)_2D$ ont atteint un plateau lorsque les doses alimentaires en vitamine D était plus grande que 0.5 IU/g d'aliments. Aucune différences de superficie ou volume de la densité minérale osseuse (sDMO – vDMO), ou des niveaux de biomarqueurs, ont été observées entre les groupes maternels. Chez les nouveaux-nés de 2 jours, bien que les taux de 25(OH)D et $1,25(OH)_2D$ ont suivie une réponse par rapport à l'alimentation de la mère ($p<0.0001$), le sDMO fémorale était 10% plus élevé dans le groupe 2 IU/g par rapport à tous les autres groupes sauf le groupe 0 IU/g d'aliments ($p=0.04$). De plus, au niveau du fémur distal et du tibia proximal des nouveaux-nés, la réponse au régime alimentaire de la mère était en forme de trabécule. Les résultats d'histologie suggèrent une expansion de croissance du fémur dans le groupe 0 IU/g d'aliments vs tous les autres groupes. Dans l'ensemble, cette thèse suggère que les niveaux en vitamine D sont plus importants avant la grossesse que pendant car ils génèrent une protection à l'intolérance au glucose. Elle recommande aussi de mesurer les niveaux d'OC et de glucose avant l'utilisation d'isoflurane dans les modèles animaux de GDM. De plus, les études démontrent que les taux de vitamine D en dessous des recommandations peuvent entraîner chez la progéniture des caractéristiques ressemblant au rachitisme. Par contre, un apport en dessus des doses recommandées n'est pas avantageux pour les os.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. Original contribution to knowledge

The following are the contributions from this thesis to knowledge in the field of maternal and infant nutrition:

- Vitamin D status is not different while osteocalcin (OC), a bone derived protein, is significantly elevated in women with gestational diabetes mellitus (GDM) compared to healthy Caucasian women throughout pregnancy. Therefore, this may be an early compensatory mechanism independent of vitamin D to increase insulin secretion, but which cannot be accomplished because of pancreatic β-cell defects in women with GDM or this could be a phenomenon completely independent of both vitamin D and glucose status.
- The gas anaesthetic, isoflurane, significantly increases OC during rapid growth to at least 9 wk, but not following pregnancy. However, isoflurane increases glucose at postpartum in guinea pigs. Measurements prior to anaesthesia are recommended to better reflect normal metabolism with the added values of improved consistency and comparability in research. Maternal glucose tolerance and concentration is not affected by dietary vitamin D intake and status in the guinea pig model during pregnancy. However, high pre-pregnancy vitamin D status (~250 nmol/L) (assessed by chemiluminescence, Liaison) which is also inversely related to body and abdominal fat may be protective against pregnancy induced glucose intolerance in the guinea pig.
- Maternal dietary vitamin D ranging from deficient to more than recommended in guinea pigs results in a parallel response in maternal vitamin D status, but is not associated with maternal mineral or bone mineral

outcomes. However, the femoral mid-diaphysis stiffness was higher in the 1 IU/g vs the 0 group, only ($p=0.04$). In addition, pregnancy resulted in an increase in maternal long bone and decrease in lumbar vertebrae 3 (3^{rd} vertebrae) areal BMD in adolescent guinea pigs. Thus, the data suggest that immediate demands for Ca homeostasis draw upon trabeculae (Tb.) bone of vertebrae whereas mineral stores for later use in lactation might reside in long bone.

- At birth, the offspring of guinea pig sows fed dietary vitamin D ranging from deficient to more than recommended had a dose-response in vitamin D status but maintained plasma mineral homeostasis at the expense of changes in neonatal bone mineral density (BMD) and microarchitecture. These responses were differential and sex-dependent at the cortical femur with lower tissue mineral density (TMD) in the male neonate at the low and high maternal vitamin D intakes and lower TMD in the female neonate of the deficient group only. In addition, at the neonatal distal femur and proximal tibia, response to maternal diet was U-shaped for trabeculae (Tb.) vBMD with a phenotype suggesting expansion of the growth plate in the 0 IU vs all other groups. However, bone quality assessed by biomechanical testing was unaffected. These data reinforce that maternal vitamin D intakes and status below of recommendations may compromise bone health in the offspring but that intakes and status above recommendations are not advantageous.

2. Research manuscripts

Negar Tabatabaei, Yves Giguère, Jean-Claude Forest, Celia J. Rodd, Richard Kremer, Hope A. Weiler. Osteocalcin but not vitamin D status is higher across pregnancy in Caucasian women with gestational diabetes mellitus. (Chapter 3: Manuscript 1; unpublished at time of thesis submission)

Negar Tabatabaei, Celia Rodd, Richard Kremer, Hope Weiler. Osteocalcin but not deoxypyridinoline increases in response to isoflurane induced anaesthesia in young female guinea pigs. (Chapter 4: Manuscript 2; unpublished at time of thesis submission)

Negar Tabatabaei, Celia Rodd, Richard Kremer, Hope Weiler. High vitamin D status at pre-mating but not during pregnancy may improve maternal glucose tolerance in guinea pigs. (Chapter 5: Manuscript 3; unpublished at time of thesis submission)

Negar Tabatabaei, Celia Rodd, Richard Kremer, Zohreh Khavandgar, Monzur Murshed, Hope Weiler. Varying dosages of vitamin D during pregnancy do not affect maternal bone outcomes but result in differential and sex-dependent effect on neonatal femur trabeculae and cortical bone mineral tissue. (Chapter 6: Manuscript 4; unpublished at time of thesis submission)

3. Abstracts and presentations

- Negar Tabatabaei, Yves Giguère, Marc Charland, Jean-Claude Forest, Celia Rodd, Richard Kremer, Hope Weiler. Osteocalcin is significantly higher in Caucasian pregnant women with gestational diabetes mellitus. Experimental Biology, San Diego, CA, April 21-25, 2012. (Poster presentation)
- Negar Tabatabaei, Yves Giguère, Marc Charland, Jean-Claude Forest, Celia Rodd, Richard Kremer, Hope Weiler. Osteocalcin is significantly higher in Caucasian pregnant women with gestational diabetes mellitus. CIHR Meeting, Ottawa, ON, December 12, 2011. (Oral presentation)
- Negar Tabatabaei, Celia Rodd, Richard Kremer, Hoper Weiler. An Appropriate Animal Model for Studying Maternal Bone Changes during Pregnancy: A Model Resembling Winter Pregnancy in Youth. American Society of Bone and Mineral Research, San Diego, CA, September 16-20, 2011. (Poster presentation)
- Negar Tabatabaei, Celia Rodd, Richard Kremer, Hoper Weiler. The development of gestational diabetes mellitus is not affected by the dosage of vitamin D supplementation during pregnancy in pigmented guinea pigs. Canadian Nutrition Society, Guelph, ON, June 2-4, 2011. (Poster presentation)
- Negar Tabatabaei, Celia Rodd, Richard Kremer, Hoper Weiler. Doubling the dosage of vitamin D supplementation during pregnancy reduces bone mineral content adjusted for body weight in male guinea pig offspring at birth. American Society of Bone and Mineral Research, Toronto, Ontario, October 15-19, 2010. (Poster presentation)
- Negar Tabatabaei, Celia Rodd, Richard Kremer, Hoper Weiler. Significant increase in plasma osteocalcin in response to isoflurane induced anaesthesia in guinea pigs. Experimental Biology, Anaheim, CA, April 24-28, 2010. (Poster)

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS

This thesis consists of four manuscripts prepared for submission to scientific journals, all of which were co-authored with my supervisor, Dr. Hope Weiler. For the guinea pig study, I was involved in the Canadian Institutes of Health Research (CIHR) grant-writing which was approved. I performed all experimental designs, interventions, sample collections, biochemical analyses, statistical analysis of the data and interpretation of the results within the context of the available literature. For the human study, I was responsible for the study design, all biochemical analyses, statistical analysis of the data as well as interpretation of the results within the context of the available literature. I was responsible for writing all four manuscripts as well as the thesis. Dr. Weiler was responsible for the CIHR grant-writing for the guinea pig study and was involved in the animal experimentation as well as human study design, provided guidance on the interpretation of the data and was extremely helpful in critical revision of the manuscripts and the thesis. Dr. Celia Rodd and Dr. Richard Kremer, my thesis committee members, were co-author of the manuscripts. They were both involved in the CIHR grant-writing for the guinea pig study and provided valuable comments in the revision of the manuscripts. Dr. Yves Giguère and Dr. Jean-Claude Forest were co-author of one of the manuscripts and with Dr. Weiler they designed the prospective cohort study of "*Pregnancy disorders and impact on child development and wellbeing: maternal, placental and fetal considerations*" on Quebec pregnant women and provided valuable comments in revising the manuscripts. Dr. Zohreh Khavandgar and Dr. Monzur Murshed at the Faculty of Dentistry at McGill University have performed the histology and histomorphometric analyses of the animal study and have assisted in the interpretation of the data.

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DEDICATION

I dedicate this thesis to my mother, father and husband for all the sacrifices they did for me in their lives and to my grandfather, Mr. Shokrollah Marhamat. My grandfather kept encouraging me about science and was eager to listen to my short scientific talks when I visited him. He was so happy that I was his first grandchild pursuing a PhD degree in the family. My grandfather passed away on March 14, 2011.

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aBMD: Areal bone mineral density

ADA: American Diabetes Association

AI: Adequate Intake

ALP: Alkaline phosphatase

APGAR: Appearance pulse grimace activity and respiration score

AUC: Area under the curve

BA: Bone area

BMC: Bone mineral content

BMD: Bone mineral density

BMI: Body mass index

BMP: Bone morphogenic protein

BV/TV: Bone volume to total volume

Ca: Calcium

Ca^{2+}: Ionized calcium

Calbidin-D_{28k}: Vitamin D-Ca binding protein

CPS: Canadian Paediatric Society

CCHS: Canadian Community Health Survey

Conn. Den: Connectivity density

CDA: Canadian Diabetes Association

CFI: Canadian Foundation for Innovation

CI: Confidence interval

CIHR: Canadian Institutes of Health Research

Cl: Chloride

Cr: Chromium

CRC: Canada Research Chair

CSA: Cross sectional area

CYP450: Cytochrome P450

CYP2R1: Vitamin D 25-hydroxylase gene

CYP24A1: 24-hydroxylase gene

CYP27A1: sterol 27-hydroxylase gene

CYP27B1: 25-hydroxyvitamin D 1-α-hydroxylase gene

d: Day

DA: Degree of anisotropy

DKK1: Ddickkopf-related protein 1

DRI: Dietary Reference Intakes

DXA: Dual-energy X-ray absorptiometry

EAR: Estimated Average Requirement

FGF2: Fibroblast growth factor 2

FGF23: Fibroblast growth factor 23

FGFR3: Fibroblast growth factor receptor 3

FPG: Fasting plasma glucose

FSI: Femur splaying index

g: Gram

GA: Gestational age

GDM: Gestational diabetes mellitus

GEE: Generalized estimating equation

GPW: Growth plate width

H: Hour

HOMA-IR: Homeostatic model assessment-insulin resistance

HSC: Hematopoietic stem cells

Ihh: Indian hedgehog

IGF-1: Insulin-like growth factor-1

IOM: Institute of Medicine

K: Potassium

LC-MS: Liquid chromatography–mass spectrometry

LIPC: Lypochech immunoassay plus control

LRP5: Low density lipoprotein receptor like protein-5

M-CSF: Macrophage-colony stimulating factor

μCT: Micro-computed tomography

Min: Minute

MSC: Mesenchymal stem cells

MMP13: Matrix metallopeptidase 13

Na: Sodium

NHANES-III: National Health and Nutrition Examination Survey-III

No: Number

OGTT: Oral glucose tolerance test

OC: Osteocalcin

OPG: Osteoprotegerin

OR: Odds ratio

PCOS: Polycystic ovary syndrome

PG: Plasma glucose

PMA1b: Plasma membrane Ca pump

P1CP: Procollagen 1 carboxyterminal peptide

PO_4: Phosphate

PPAR-γ: Peroxisome proliferator-activated receptor-γ

PTH: Parathyroid hormone

PTHrP: Parathyroid hormone-related protein

QUICKI: Quantitative insulin-sensitivity check index

RANK: Receptor activator of nuclear factor kappa-B

RANKL: Receptor activator of nuclear factor kappa-B ligand

RCT: Randomized clinical trial

RDA: Recommended Dietary Allowance

RUNX 2: Runt-related transcription factor 2

RXR: Retinoid X receptor

Sep: Separation

SES: Social economic status

SFRP2: Secreted frizzled-related protein 2

SMI: Structural model index

SOX9: SRY (sex determining region Y)-box 9

Tb: Trabeculae

Th: Thickness

tALP: Total alkaline phosphatase

tDPD: Total deoxypyridinoline

TGF-β: Transforming growth factor-β

TMD: Tissue mineral density

TNF-α: Tumor necrosis factor-α

TRPV5: Transient receptor potential V5

TRPV6: Transient receptor potential V6

UL: Tolerable Upper Intake Levels

vBMD: Volumetric bone mineral density

VDR: Vitamin D receptor

VDRE: Vitamin D response element

Vertebrae 3: 3rd vertebra

wk: Week

WT: Wild type

Yr: Year

25(OH)D: 25-hydroxyvitamin D

1,25$(OH)_2$D: 1,25-dihydroxyvitamin D

24,25$(OH)_2$D: 24,25-dihydroxyvitamin D

CHAPTER 1. INTRODUCTION

1.1. Background and rationale

Vitamin D intake and status during pregnancy, the later defined by circulating 25-hydroxyvitamin D (25(OH)D) concentration, is an area of debate among different scientific societies [1-3]. The debate on vitamin D status during pregnancy relates to inconsistent and unknown thresholds of circulating 25(OH)D associated with pregnancy outcomes such as maternal and fetal bone health and maternal glucose homeostasis and tolerance. Vitamin D status is defined by circulating 25(OH)D concentration with values >50 nmol/L recommended as adequate for bone health by the Institute of Medicine (IOM) [1] whereas >75 nmol/L is considered ideal for pregnant women by the Canadian Paediatric Society (CPS) [2]; values below either target are prevalent among pregnant women [4-9]. As 25(OH)D concentrations drop from 100 down to 80 nmol/L, concentrations of the active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) may remain normal or increase [10, 11]. However, if vitamin D deficiency (25(OH)D < 30 nmol/L) develops, $1,25(OH)_2D$ reaches about 33% of its normal values [11, 12] or even undetectable concentrations [13] due to limited substrate. Such values are of consequence since normal physiological concentration of $1,25(OH)_2D$ has beneficial effects on bone [14, 15] and may also improve glucose homeostasis by enhancing insulin synthesis, secretion and sensitivity [16] which may be challenged during pregnancy. In addition, low maternal-fetal transfer of 25(OH)D may adversely be associated with neonatal anthropometry and appearance, pulse, grimace, activity and respiration (APGAR) outcomes [17-19] and body composition at birth [20].

Gestational diabetes mellitus (GDM), a state of hyperglycemia due to decreased insulin sensitivity and pancreatic β-cell insulin response to glucose during

pregnancy, has a worldwide prevalence of 1 to 10% and is associated with higher risk of adverse maternal and neonatal outcomes [21]. Recently, low vitamin D status has been associated with GDM [22-24]. Of the seven studies published to date [22-28], all observed lower serum 25(OH)D concentration in GDM vs healthy subjects at mid-gestation [22-24, 26], but not at early [25] and late pregnancy [27]. However, some of these observational human studies have shown that 25(OH)D concentration at early gestation is inversely associated with 1 h [28] and 2 h [25] glucose concentration from the oral glucose tolerance test (OGTT). Similar negative relationships were observed between 25(OH)D at mid and late gestation with fasting [26] and 30 min glucose concentrations from the OGTT [27]. In women with established GDM, injection and oral consumption of alpha-calcidiol resulted in a negative association between fasting glucose and $1,25(OH)_2D$ concentrations while glucose concentration from the OGTT measured after the ingestion of glucose was unaffected [29]. This vitamin D analogue has less calcemic and phosphoric actions than $1,25(OH)_2D$ [30] and in contrast to cholecalciferol is not regulated by renal vitamin D metabolism [31]. Differences among studies on the relationship between vitamin D status or treatment on glucose tolerance, may partly be due to uncontrolled factors such as plasma calcium (Ca) [32] and phosphate (PO_4) [33] which are positively associated with insulin secretion, pre-pregnancy body mass index (BMI) and central fat (visceral) which are adversely related to glucose tolerance [34]. All these covariates (plasma minerals, weight and adiposity) are also related to vitamin D metabolism [22, 35, 36].

In non-pregnant rats [37] and rabbits [38], a vitamin D deplete diet resulted in undetectable 25(OH)D and less than 50% of baseline $1,25(OH)_2D$ concentrations. In such animal models, injection of $1,25(OH)_2D$ prior to OGTT increased glucose induced insulin secretion [37, 38] and improved glucose tolerance 3 h after delivery

of a glucose load in rats [37] while the latter effect was observed in rabbits only by the injection of 25(OH)D for 2 wks [38]. However, no interventional study has investigated the dose-response effect of oral vitamin D_3 intake on glucose concentration and tolerance during pregnancy.

The implications of low vitamin D status in GDM extend beyond insulin metabolism in the pancreas and include alteration in gene expression of other proteins that have a positive vitamin D response element (VDRE) located on their promoter [39]. Osteocalcin (OC) is one such protein which is secreted from osteoblasts in response to stimulation by $1,25(OH)_2D$ [40]. Its uncarboxylated form has recently been associated with glucose homeostasis by increasing β-cell proliferation, glucose tolerance and sensitivity in mice [41]. Recent evidence suggests that total OC is also secreted from adipocytes [42], and is positively associated with insulin sensitivity in humans [43-46] and negatively related to adiposity features such as BMI and fat mass in mice [47] and humans [48-51]. These associations have not been investigated in interventional trials during pregnancy.

Considering the connection between the bone and endocrine metabolism, recently, two human studies have looked at serum total OC concentration at mid-gestation (24-28 wk) in GDM vs healthy pregnant women [52, 53]. In contrast to the authors' expectations, serum OC concentration was higher (128-148%) in GDM compared to control subjects, suggesting that this may represent a compensatory mechanism to increase insulin secretion in women with GDM [52]. In an attempt to clarify this relationship, a positive association was observed between circulating OC and area under the curve (AUC) for glucose from OGTT in one study [52] yet no correlation was observed between plasma OC and fasting plasma glucose (FPG) in another study [53]. These studies thus yielded inconsistent results and both were

limited by lack of sampling early in pregnancy. Further work is required to clarify if elevated OC has a role in GDM or if it could be used as a biomarker in the endocrine function of bone on glucose metabolism.

The other detrimental outcome of maternal low vitamin D status during pregnancy is neonatal rickets. However, there is insufficient evidence whether fetal vitamin D status and bone outcomes have a dose-response relationship with maternal vitamin D status at both low (<50 nmol/L) and high (>125 nmol/ L) concentration of circulating 25(OH)D. The circulating 25(OH)D <50 nmol/L is considered "insufficient" and > 125 nmol/L is associated with "no evidence of additional benefits" as defined by the IOM in 2011 [1]. Investigating such effects on bone, although possible is not readily feasible and ethical in humans due to possible detrimental consequences. Therefore, an animal model is required for such research. Furthermore, considering the link between the bone and glucose metabolism [54], selection of an appropriate animal model for investigating both bone and glucose tolerance during pregnancy is required. The guinea pig is an appropriate model due to its similarity to human pregnancy regarding changes in serum $1,25(OH)_2D$ and OC concentrations [55] and peak in blood glucose concentrations at 40-60 min after oral glucose ingestion on an OGTT during pregnancy [56].

Assessment of bone density and microarchitecture in laboratory animals requires the use of general anaesthesia for immobilization purpose. Inhalation anaesthetics such as isoflurane are more controllable and result in rapid recovery as compared to injectable anaesthesics [57, 58]. However, there is inconsistent evidence that isoflurane may influence biochemical outcomes related to bone such as OC [59], mineral [57, 59-61] and glucose [57, 62] which are commonly measured in bone studies, leading to inconsistency and lack of comparability among studies. If these

bone and glucose related biomarkers are affected by isoflurane, biochemical measurements should be performed prior to the effect of isoflurane for better consistency and comparability in research.

Deeper understanding of maternal-fetal vitamin D transfer and consequences is required to clarify whether it is advisable to exceed normal recommendations for vitamin D intake. More is understood about low vitamin D intakes and status. Observation human studies (cross-sectional and longitudinal) have reported thresholds for low maternal circulating 25(OH)D concentration ranging from 27.5 to 50 nmol/L of 25(OH)D during pregnancy in relation to fetal bone health. In the UK, pregnant women with low (< 50 nmol/L) serum 25(OH)D concentrations at 34 wk gestation had a 5% increase in fetal metaphyseal femur splaying index (FSI), a feature consistent with congenital vitamin D dependent rickets [63]. Similarly, maternal serum 25(OH)D <28 nmol/L at 28-32 wk gestation was associated with a 3 % decrease in postnatal knee-heel length in Australian neonates, only at a threshold below 40 nmol/L [64]. Regarding mineral content of bone, vitamin D deficiency (25(OH)D <27.5 nmol/L) in Canadian infants was associated with lower whole body and femur, but not lumbar spine, bone mineral content (BMC) relative to body weight [19]. However, in other studies with few deficient subjects, there was no association between maternal supplementation and plasma 25(OH)D with whole body and forearm BMC in Turkish [65] and Asian [66] neonates, respectively. Therefore, the available data is still conflicting regarding the effect and thresholds of maternal 25(OH)D in association with fetal and neonatal bone health outcomes.

Clinical trials of vitamin D supplementation during human pregnancy have included daily supplements of 0 vs 400 [67] or 1000 IU/d [18, 66, 68] and assessed biochemical and anthropometric outcomes. There are two dietary forms of vitamin D:

ergocalciferol (vitamin D_2) from plant sources and cholecalciferol (vitamin D_3) from animal sources [69]. The available vitamin D clinical trials during pregnancy have mainly investigated the effect of dietary D_3 on pregnancy outcomes [10, 18, 66-68]. The only vitamin D dose-response pregnancy trial (400, 2000 and 4000 IU/d) caused a dose-response increase in maternal plasma 25(OH)D while plasma $1,25(OH)_2D$ reached a plateau with the two higher dosage intakes [10], but bone health outcomes have yet to be reported.

Vitamin D status has been accepted to have functional outcomes on bone in infants, children, adolescents and elderly [70-72], with less informative data available in middle-aged adults [71]. Of relevance to this thesis, vitamin D deficiency may lead to rickets in infants [70] and vitamin D is essential for bone development and higher bone mineral density (BMD) in adolescents [71]. Recommended intakes in pregnancy do not differ from non-pregnant adults since changes in bone are assumed to return to pre-pregnancy states if vitamin D status is adequate. Descriptive studies suggest that during pregnancy, women lose 2.1 to 5% of bone mass at trabeculae (Tb.) rich sites [73-75] with almost 3 % greater bone loss in adolescent vs adult pregnancy [75]. In one study, 20-34 y old mothers with the greatest calcaneal bone loss had infants with 9.7% higher (areal bone mineral density) aBMD [76]. However, there is a gap of knowledge on the effect of maternal vitamin D status during pregnancy on bone health particularly at ages where peak bone mass has not yet been established, (i.e. adolescence).

In adolescent rats receiving a vitamin D deplete (0-0.3 IU/d) diet, 2 mo prior to pregnancy (from weaning to mating) as well as during pregnancy, lower maternal metaphyseal femur Tb. volumetric bone mineral density (vBMD) and mid-diaphysis cortical mineralized area [77], lower endosteal bone formation and femur length were

observed in the deplete compared to a replete group (10-25 IU/d) [78]. This suggests that Ca is mobilized from the bone by vitamin D independent pathways, but vitamin D is required for normal bone mineralization. Also, in adolescent guinea pigs, vitamin D deplete (0 IU/g) diets during pregnancy resulted in maternal hypophosphatemia, lower total femur aBMD and distal femur cortical but not Tb. mineral density, wider growth plates and higher osteoid thickness at the proximal tibia compared to guinea pigs on the replete (2.4 IU/g) diet [13]. In this study, deficient vs replete fetuses had hypercalcemia, hypophosphatemia, lower plasma OC, no differences in whole body BMC/weight, but higher hypertrophic chondrocyte area and osteoid thickness at the proximal tibia , suggesting impaired mineralization [13]. In a similar guinea pig model, a deplete (0 IU/g) vs standard vitamin D (1.2 IU/g) diet during pregnancy resulted in lower neonatal plasma OC, whole body and tibia BMC and strength, but no difference in whole body and femoral BMD and strength [79]. Whether there is a threshold at which vitamin D status during pregnancy affects bone health in the mother and offspring, cannot be established from the available data, thus showing the need for a vitamin D dose-response study during pregnancy.

A proper animal model is required for studying the effect of vitamin D intake and status on bone and glucose tolerance during pregnancy. The guinea pig is an appropriate model for studying this [10, 11], since it has a haemomonochorial placenta, similar to humans [55]. Also in this species, changes in serum $1,25(OH)_2D$ and OC concentrations during pregnancy are comparable to humans [55]. Furthermore, fetal guinea pig concentrations of $1,25(OH)_2D$ are proportionate and lower than maternal levels and fetal OC concentrations are higher than maternal concentration [55] which is also similar to humans.

Based on the knowledge to date, the conceptual framework diagram of this thesis is shown in **Figure 1.1.** There seems to be a dose-response effect of maternal dietary vitamin D on circulating 25(OH)D and $1,25(OH)_2D$ and therefore maternal and neonatal health outcomes. Both low (<50 nmol/L) and high (>125 nmol/L) maternal circulating 25(OH)D concentration may lead to low maternal circulating $1,25(OH)_2D$ concentrations. The former occurs as a result of substrate (25(OH)D) insufficiency and the latter due to down-regulation of $1,25(OH)_2D$ (calcitriol). The health consequences associated with low maternal $1,25(OH)_2D$ are imbalanced circulating Ca and PO_4 concentrations, detrimental effects on maternal and fetal bone mass and architecture, the latter as a result of insufficient maternal-fetal mineral transfer to the bone. Low calcitriol may also directly decrease insulin sensitivity, secretion and synthesis and may decrease the stimulation of OC secretion from osteoblasts which will also indirectly lead to decreased insulin secretion and sensitivity. Therefore, maternal vitamin D status associated with improved maternal glucose tolerance and maternal and neonatal bone health outcomes is hypothesised to be between 50-125 nmol/L and that concentrations below and above these values lead to adverse health outcomes.

As summarized in this chapter, vitamin D status and the bone formation biomarker OC have not been studied simultaneously at early gestation in association with GDM in human studies controlled for the main confounding factors. The assessment of bone outcomes in animal models requires the use of anaesthetics which may affect bone and mineral and glucose metabolism. In addition, there is a lack of knowledge on the dose-response effect of dietary vitamin D in a pregnant model on maternal and neonatal glucose homeostasis and bone health outcomes. The following objectives were set to address these gaps of knowledge.

1.2. Thesis objectives and hypotheses

Four objectives and hypotheses are at the core of the work contained in this thesis:

1. **Objective:** To determine 25(OH)D and OC concentrations simultaneously at early and mid-gestation in healthy pregnant women and those with GDM; to investigate the predictive role of early concentrations of these biomarkers and their percent change from early to mid-getsation in the development of GDM. Also, to determine these biomarkers in the cord serum of healthy pregnant women and those with GDM and their association with neonatal anthropometry and APGAR scores and placenta weight (Chapter 3: Manuscript 1).

   Hypothesis: Maternal serum vitamin D status is lower and OC concentration is higher at early gestation in GDM vs healthy pregnant women and they may have a predictive role in the development of GDM. Cord serum 25(OH)D and OC concentrations are associated with neonatal anthropometry and APGAR scores and placenta weight (Chapter 3: Manuscript 1).

2. **Objective:** To investigate the effect of isoflurane on bone metabolism biomarkers from development to maturity in the guinea pig and to measure blood minerals and glucose along with the bone metabolism biomarkers at maturity. The latter are implemented to explain possible mechanisms involved in changes in bone biomarkers and to investigate whether glucose concentrations are affected by the anaesthetic (Chapter 4: Manuscript 2).

   Hypothesis: Exposure to the isoflurane anaesthetic significantly increases OC and affects tDPD (total deoxypyridinoline) from development to maturity as well as blood glucose and minerals at maturity in guinea pigs. Measurements prior to anaesthesia are recommended to better reflect normal metabolism

with the added values of improved consistency and comparability in research (Chapter 4: Manuscript 2).

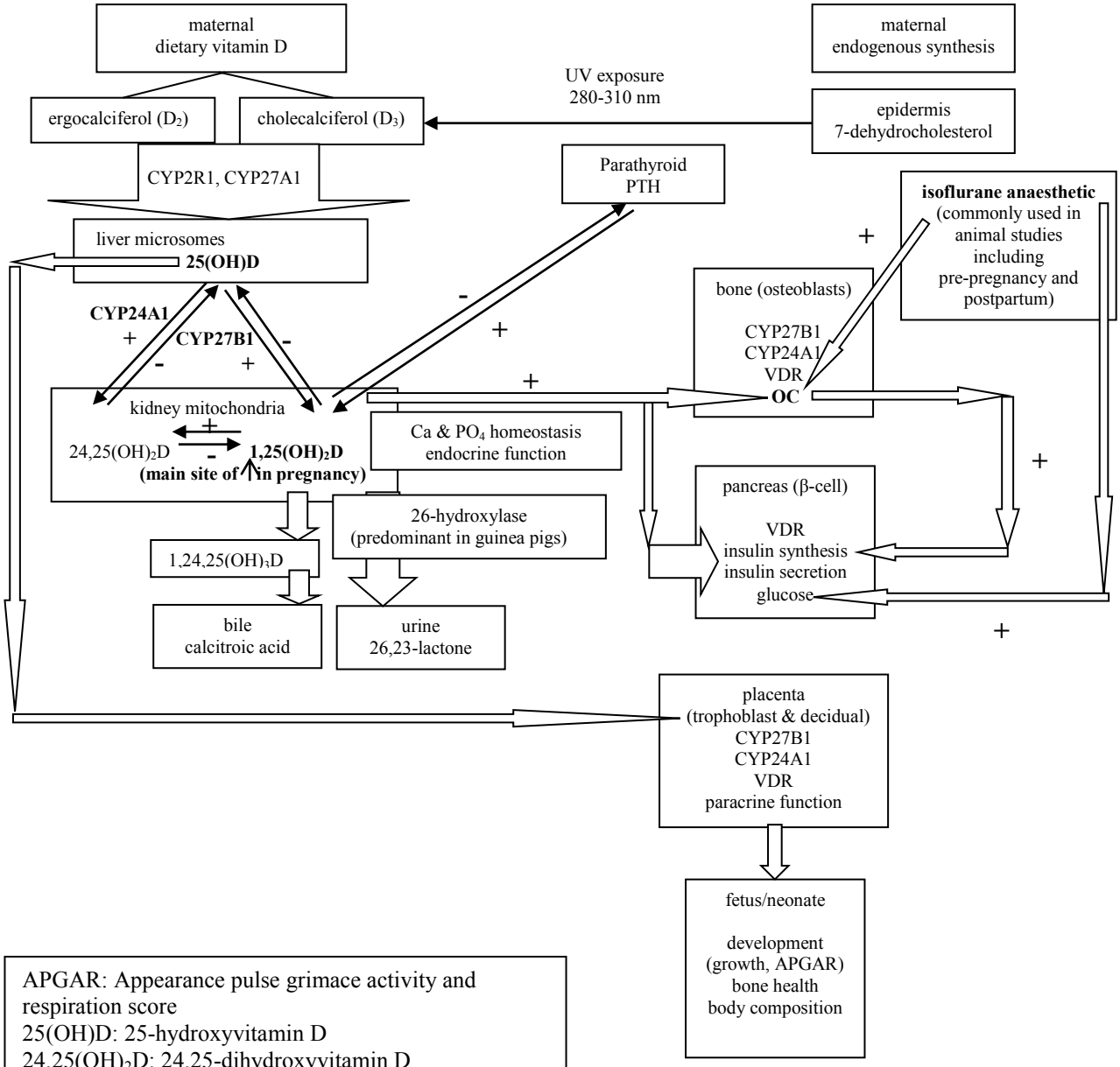
3. **Objective:** To explore the dose-response effect of dietary vitamin D_3 in pregnancy on maternal glucose tolerance as well as maternal and neonatal glucose homeostasis and body composition in association with plasma OC in a pregnant guinea pig model (Chapter 5: Manuscript 3).

   Hypothesis: Maternal glucose tolerance and maternal and neonatal glucose concentration and body composition are affected by vitamin D status, $1,25(OH)_2D$ and OC concentrations; the latter are impacted by the dose-response effect of maternal dietary vitamin D intake during pregnancy in guinea pigs (Chapter 5: Manuscript 3).

4. **Objetive:** To establish the dose-response effect of dietary vitamin D_3 in a pregnant guinea pig model to study maternal and neonatal bone health outcomes including measures of plasma minerals, vitamin D metabolites and bone biomarkers as well as assessment of whole body and regional BMC, BMD, microarchitecture and biomechanics (Chapter 6: Manuscript 4).

   Hypothesis: The hypothesis was that both low (<50 nmol/L) and high (>125 nmol/ L) maternal 25(OH)D concentrations would impair bone development and mass while moderate concentrations (50-125 nmol/L) would result in optimal maternal and fetal bone health by analyses of plasma minerals, vitamin D metabolites and bone biomarkers as well as assessment of whole body and regional BMC, BMD, microarchitecture and biomechanics (Chapter 6: Manuscript 4).

Figure 1. 1. Thesis conceptual framework diagram.



APGAR: Appearance pulse grimace activity and respiration score
25(OH)D: 25-hydroxyvitamin D
24,25$(OH)_2$D: 24,25-dihydroxyvitamin D
1,25$(OH)_2$D: 1,25-dihydroxyvitamin D
CYP2R1: Vitamin D 25-hydroxylase gene
CYP24A1: 24-hydroxylase gene
CYP27A1: sterol 27-hydroxylase gene
CYP27B1: 25-hydroxyvitamin D 1-α-hydroxylase gene
OC: Osteocalcin
PTH: Parathyroid hormone
VDR: Vitamin D receptor

References: [16-19][41][57][59]

CHAPTER 2. LITERATURE REVIEW

2.1. Definition of vitamin D status

Vitamin D status is defined by circulating 25(OH)D concentration [80]. This vitamin D metabolite is not subject to tight homeostatic regulation and has the highest stability as compared to other vitamin D metabolites due to its high affinity for vitamin D binding globulin and resulting long half-life of 2-3 wk [80].

There is reasonable agreement regarding the 25(OH)D concentration associated with vitamin D deficiency and rickets (20-30 nmol/L), but there is still debate regarding the desirable (target) 25(OH)D concentration suggested by different scientific societies as 50 or 75-80 nmol/L across all ages [1-3]. The IOM in 2011 suggested serum 25(OH)D < 30 as “deficient”, 30-50 as ”risk of inadequacy” and ≥ 50 nmol/L as sufficient with no evidence of additional benefits from serum 25(OH)D concentration >75 nmol/L and concentration >125-150 nmol/L was felt to be associated with risk of adverse effects [1]. These cut-offs were set based on bone health outcomes (e.g. increased BMD and intestinal Ca absorption, minimized risk of rickets and osteomalacia) [1]. However, the Endocrine Society suggests a higher concentration of 25(OH)D at 75-80 nmol/L as an optimal vitamin D status [3]. This higher cut-off was mainly based on increased Ca absorption [81], suppression of secondary hyperparathyroidism and increased bone density [82].

2.1.1. Pregnancy and neonate

According to the IOM report, the definition of Estimated Average Requirement (EAR) for vitamin D (400 IU/d) is set to meet the requirements of 50% of the population and is to align with the status target of 40 nmol/L of 25(OH)D, which is not different for pregnancy as compared to non-pregnancy state [1]. The IOM defined a Recommended Dietary Allowance (RDA) of 600 IU/d to align with

25(OH)D concentration of 50 nmol/L to meet recommendations of 97.5 % of the population, which again is similar in pregnancy and non-pregnancy states [1]. The cut-off for vitamin D deficiency is now lower (25(OH)D <30 nmol/L) than the previous one recommended in 1997 (25(OH)D <37.5 nmol/L) for adults but not the one for infants (25(OH)D<27.5 nmol/L) [83]. These definitions are important to consider when examining existing evidence on the topic of vitamin D intakes and status in reproduction. For example, recently Hollis et al, in their study on pregnant women observed that in order to reach maximum serum $1,25(OH)_2D$, serum 25(OH)D should reach 100 nmol/L as substrate and serum 25(OH)D of ~ 75 nmol/L is required to reduce the excretion of Ca in the urine [10]. The latter is similar to results on serum 25(OH)D required for maximum intestinal Ca absorption [81]. Hollis et al. defined serum 25(OH)D <50 nmol/L as "deficient", 50-80 nmol/L as insufficient and >80 nmol/L as sufficient and suggested that 25(OH)D ≥375 nmol/L should be considered as hypervitaminosis D for pregnant women [10]. Other researchers also have similar definitions for vitamin D status according to 25(OH)D as <25 as "deficient", 25-50 as "insufficient", 50-70 as "borderline" and > 70 nmol/L as "sufficient" [63].

The available data is limited for establishing the normal 25(OH)D range in infancy. The fetus is completely dependent on their mother's vitamin D supply which should be sufficient to provide both maternal and neonatal needs [2]. Previous recommendations set by the 1997 IOM set circulating 25(OH)D < 27.5 nmol/L as deficient levels for infants and children [83] which was lower than the new recommended cut-off for deficient 25(OH)D concentration (<30 nmol/L) and that assumed to confer bone health (25(OH)D ≥ 50 nmol/L) in all age groups including infants [1]. The CPS suggests an optimal 25(OH)D concentration from 75-225

nmol/L with possible adverse effects at 25(OH)D >250 nmol/L and toxicity at >500 nmol/L in all age groups including infants [2]. Overall, it has been suggested that perhaps normal plasma 25(OH)D concentrations might differ in infants being 60-70% of maternal levels [84] but further research is required to investigate normal neonatal vitamin D status and the response to maternal dietary intake.

2.2. Dietary Reference Intakes, actual intakes and status of vitamin D

The new IOM (2011) Dietary Reference Intakes (DRI) include an EAR of 400 IU/d for after 1 y of age and a RDA of 600 IU/d for 1-70 y of age, 800 IU/d for those over 70 y and Adequate Intake (AI) of 400 IU/d from birth to 1 y of age [1]. These recommendations are higher than the previously recommended AI of 200 IU from birth to 50 y of age, 400 IU from 50-70 and similar to 600 IU/d for >70 y of age. Even though the DRI values were set based on existing evidence, there is still debate.

The Endocrine Society recommends 1500-2000 IU/d for adults aged 19–50 y in order to reach serum 25(OH)D >75 nmol/L[3]. The previous IOM set the Tolerable Upper Intake Levels (UL) at 1,000 IU/d for infants and 2,000 IU/d for all other ages, but recently increased it to 4,000 for adults [1] including women during their reproductive years. Still, some researchers believe that the UL for dietary vitamin D should be set at 10,000 IU/d [85]. Vitamin D intake ≥ 10,000 IU/d for 5 mo did not increase 25(OH)D to ≥225 nmol/L [85]. This has not been tested for safety during reproduction which spans 9 mo in humans. It is therefore too soon to conduct randomized trials with high intakes.

2.2.1. Canada and the U.S.A

Data from the National Health and Nutrition Examination Survey-III (NHANES-III) conducted from 1988 to 1994 suggest that vitamin D intakes in the US are lower than the previous AI (200 IU) in females older than 12 y and men older

than 50 y of age [86]. Canadian intakes are likely similar to the US although there is a lack of national Canadian data in this regard. A study in women of childbearing ages in Toronto showed that only 25 % consumed supplements containing vitamin D, 46 % did not consume milk, 34 % consumed neither of these vitamin D sources and only 32 % achieved 200 IU/d [87]. The Canadian Community Health Survey 2.2 (CCHS 2.2) from 2004 in 30,000 Canadians included a 24-h recall and suggests that most men and women consume less than 2 servings of milk [88]. Considering the new recommendations by the IOM, it is clear that vitamin D intake from food is much lower than the suggested DRIs. Accordingly, a recent report considering vitamin D intake from food alone documented that the majority (90 %) of Canadian men and women (19-50 y) had vitamin D intakes below 400 IU EAR [89]. However, the 2007-2009 CCHS suggests that Canadian men and women (19-39 y) have an average year-round 25(OH)D concentration of ~ 65 nmol/L and only ~ 10% of have vitamin D deficiency (25(OH)D < 37.5 nmol/L) [90], 31 % < 50 nmol/L [91] but these values would increase to ~ 75 % if the target of 25(OH)D < 75 nmol/L was considered [90]. Concentration of 25(OH)D in men and women (20-39 y) taking vitamin D supplements are about 15 nmol/L higher than those that do not. The prevalence of 25(OH)D < 50 nmol/L almost doubles from 15.4 to 30.4 % among 6-79 y subjects if supplements are not taken [91]. Therefore, supplement use is significantly associated with better vitamin D status (>50 nmol/L) [91]. Overall, population level assessments suggest that dietary vitamin D is not aligned with current recommendations. However, exposure to ultra violet beta (UBV) radiation for endogenous synthesis is likely contributory, yet difficult to quantify.

2.2.2. Pregnancy

The recent RDA set by the IOM has suggests a daily intake of 600 IU in the absence of UVB exposure, which is higher than the previous AI of 200 IU/d and the UL has increased to 4,000 IU/d from the previously recommended 2,000 IU/d [1]. The CPS recommends an intake of 2,000 IU/d during pregnancy and lactation [2]. The Endocrine Society suggests a minimum intake of 600 IU/d of dietary vitamin D for pregnant and lactating women but recommends an intake of 1500-2000 IU/d in order to achieve blood levels of 75 nmol/L [3].

2.3. Metabolism of vitamin D and other calcitropic hormones

The endogenous synthesis of vitamin D occurs in the skin following photoactivation of 7-dehydrocholesterol at 280-310 nm UVB light. The double bond of this precursor then undergoes photochemical isomerisation forming previtamin D_3 which is then converted to vitamin D_3 [92]. Endogenous vitamin D_3 synthesized from the skin and the exogenous plant form of vitamin D (ergocalciferol, vitamin D_2) and animal form (vitamin D_3) are transported in the blood stream to the liver bound to a vitamin D binding protein or to albumin. The first step of hydroxylation takes place via cytochrome P450 (CYP450) enzymes, microsomal and mitochondrial vitamin D 25-hydroxylase, that are regulated by CYP2R1 and CYP27A1 genes, respectively, resulting in the production of 25(OH)D (calcidiol) in the liver. Afterwards, 25(OH)D is transported to the kidney where it undergoes another hydroxylation mainly in the proximal tubules via 25-hydroxyvitamin D 1-α-hydroxylase encoded by the CYP27B1 gene which produces $1,25(OH)_2D$. This active form of vitamin D is considered a sterol hormone by virtue of its mechanism of action similar to other steroid hormones [93]. It binds to the vitamin D receptor (VDR) in target tissues and also requires dimerization to another receptor called retinoid X receptor (RXR) which

then activates or represses gene expression following binding to VDREs on target genes [93]. Interestingly, recent studies have shown that CYP27B1 is also expressed in extrarenal tissues. However the contribution of extrarenal production of $1,25(OH)_2D$ to its circulating levels under normal conditions is debatable. Its main source is still considered the kidney whereas extra-renal production is believed to play an autocrine/paracrine role [92] such as the role of placental production of $1,25(OH)_2D$ on regulation of implantation and prevention of placental inflammation [94].

The catabolism of vitamin D metabolites is through a 24-hydroxylase encoded by CYP24A1 gene occurring mainly in the kidney mitochondria but it is also present in extra-renal tissues with VDRs including bone and intestine [95]. Calcitriol binding to the VDR/RXR complex enhances CYP24A1 and down regulates CYP27B1 gene expression, leading to increase in 24-hydroxylase and decrease in 1α-hydroxylase, respectively. These are important feedback regulatory mechanisms in the metabolism and function of vitamin D metabolites [96]. Both 25(OH)D and $1,25(OH)_2D$ are substrates for 24-hydroxylase although the affinity of CYP24A1 is 10-fold higher for $1,25(OH)_2D$ compared to 25(OH)D. The main degradation product of $1,25(OH)_2D$ is calcitroic acid which is derived from $1,24,25\ (OH)_3D$ and found in the bile. Similarly, 26,23-lactone is another metabolite of $1,25(OH)_2D$ but secreted in the urine in humans. Interestingly, in different species such as guinea pigs the preferred catabolic product of $1,25(OH)_2D$ is 26,23-lactone. These metabolites have low affinity to vitamin D binding protein. Although, it was previously thought that 24,25-dihydroxyvitamin D ($24,25(OH)_2D$), derived from degradation of 25(OH)D, had no function and the bone mineralization defects observed in CYP24A1-null mice

were thought to be solely as a result of $1,25(OH)_2D$ toxicity, recent evidence suggests that $24,25(OH)_2D$ has a role in bone repair [92].

Vitamin D toxicity following excess intake can be dangerous since it is stored in adipose tissue and can last for months to years in humans. Its conversion to 25(OH)D may then lead to vitamin D intoxication [92]. Circulating 25(OH)D >500 nmol/L has been associated with adverse outcomes such as hypercalcemia, hypercalciuria leading to severe dehydration and kidney damage as well as hyperphosphatemia, vomiting, anorexia, fever, constipation, calcification of soft tissues such as kidney and blood vessels [97].

2.3.1. Pregnancy and postnatal metabolism of vitamin D and other calcitropic hormones

2.3.1.1. Maternal

Plasma 25(OH)D concentration is positively correlated with maternal dietary vitamin D intake during human pregnancy [98]. Throughout human pregnancy, plasma 25OHD remains similar or lower than in the non-pregnant state and could remain unchanged if pregnancy begins with severe vitamin D deficiency (<25 nmol/L) [18].

In humans and animals, plasma $1,25(OH)_2D$ increases significantly in normal pregnancy, even after taking into account the increase in vitamin D binding protein accompanied with normal gestation [99, 100]. A 2-3 fold increase in total $1,25(OH)_2D$ occurs in the first trimester while only the free $1,25(OH)_2D$ increases during the third trimester [101]. In addition, intestinal Ca absorption doubles from early pregnancy [102] and is accompanied by an increase in urinary Ca excretion [101]. This suggests that the doubling in intestinal Ca absorption may be as a result of doubling of $1,25(OH)_2D$ concentrations during pregnancy. However, the former takes

place much sooner than the latter and studies in vitamin D deficient animals and those lacking VDR have shown the same magnitude of increases in Ca absorption as controls, ruling out the necessity of $1,25(OH)_2D$ in this process [101]. The placental lactogen and prolactin may be responsible for the increase in the active form of vitamin D during pregnancy [101]. Although, recently Hollis et al. have suggested that in human pregnancy calcitonin may be responsible for activating 1-α-hydroxylase independent of Ca [10]. The increase in $1,25(OH)_2D$ concentrations has been not been attributed to hyperparathyroidism, since parathyroid hormone (PTH) levels are either normal or fall to the low-normal range during early pregnancy when calcitriol increases [101]. However, PTH levels increase from mid to late gestation [101]. Parathyroid hormone-related protein (PTHrP) also increases during pregnancy [102] which in turn stimulates the production of $1,25(OH)_2D$ by extrarenal sources such as decidual and trophoblastic cells in the placenta [100]. However, animal studies have shown that in anephric pregnant models, the increase in $1,25(OH)_2D$ is much lower than in controls, suggesting an underlying and significant role of maternal kidney in the synthesis of the active form of vitamin D [101], which is not compensated for by the placenta. The human fetal skeleton (chondrocytes) may be another site of $1,25(OH)_2D$ synthesis [103], but this metabolite would not likely traverse the maternal-fetal unit and likely play a local role.

One of the classic functions described for $1,25(OH)_2D$ in pregnancy relates to enhanced maternal Ca balance. Although an increase in intestinal Ca absorption is the main source of providing Ca for the fetus during pregnancy, there is good evidence that Ca is also mobilized from maternal bone during pregnancy [104, 105]. Plasma total Ca decreases at the beginning of pregnancy reaching its lowest levels about the second month due to pregnancy induced hypoalbuminemia. However, blood ionized

calcium (Ca^{2+}) does not change significantly during pregnancy [100]. In humans, 30 g Ca is transferred from the mother to the fetus, 99% of which accumulates in the developing skeleton. In humans, this transfer occurs mainly (80 %) in the last trimester and by active transport of 150 mg/d through the placenta [101]. In rats and mice with a gestational period of 21 and 19 d respectively, the major (95%) transfer of mineral content to 8-12 fetuses per litter occurs during the last 4-5 d of gestation. In contrast to human pregnancy, in rats and mice, a decrease in plasma Ca^{2+} is observed in this period accompanied by an increase in PTH at late gestation [101].

In humans postpartum, there is a decrease in estradiol and progesterone and prolactin stimulates the production of PTHrP from the mammary gland. The combination of low estradiol and high PTHrP results in increased bone resorption during lactation [101]. Calcitriol concentrations drop shortly after delivery and remain normal during lactation in humans, but increase during lactation in rat and mice until weaning. Circulating 25(OH)D is either stable or decreases slightly during lactation [101]. These studies and species differences underscore the importance of selecting suitable animal models in furthering knowledge related to vitamin D metabolism towards human health.

2.3.1.2. Fetal

Serum 25(OH)D concentrations are typically lower in the neonate as compared to their mother in both animals and humans [100]. In humans, cord serum 25(OH)D concentrations have been reported to be 65-70% [106] or 50% [107] of maternal values. The fetus depends entirely on the mother's sources of Ca, 25(OH)D and $24,25(OH)_2D$, explaining the high correlation of these metabolites between the mother and fetus [100], but there are inconsistent results regarding the correlation of $1,25(OH)_2D$ between the mother and fetus. Fetal $1,25(OH)_2D$ concentration are lower

than maternal levels in rats, mice, sheep and humans suggesting that $1,25(OH)_2D$ does not readily cross the placenta during pregnancy [101]. The fetal-placental Ca transport system is regulated by PTHrP which is produced in many fetal tissues including the growth plates of long bones, but primarily in the placenta [108]. Serum total Ca, Ca^{2+} and PO_4 are higher in the developing fetus than the mother, while PTH and calcitriol are lower. The combined high Ca and PO_4 as well as the low PTH levels result in suppression of the renal and placental 1α-hydroxylase keeping calcitriol at low levels [1]. It would appear that only at pharmacological concentrations of $1,25(OH)_2D$, this metabolite may cross human and animal (ovine) placenta and therefore most of this metabolite is of fetal renal origin [100].

Postnatally, mineral homoeostasis changes quickly in the neonate [100]. The neonate relies on the intestinal sources of minerals since the placental transfer has been cut. After birth PTH but not PTHrP plays an important role in mineral homeostasis. Total and ionized blood Ca levels decrease 2-3 d after birth in the human neonates and return to normal within 5-10 d. The decrease in Ca^{2+} early after birth is accompanied by an increase in serum PTH which leads to an increase in $1,25(OH)_2D$ synthesis around 5 days after birth [100]. Serum PO_4 concentrations are reported to be at their lowest at birth and increase 2 wk postpartum in the human neonate [109].

2.4. Dietary sources of vitamin D

In addition to endogenous synthesis, vitamin D can be provided by exogenous sources that are available through two dietary forms: ergocalciferol (vitamin D_2) from plant sources and cholecalciferol (vitamin D_3) from animal sources. In Canada, most food sources and supplements have the D_3 form. Vitamin D_3 appears to be more potent than D_2 in increasing circulating adult 25(OH)D (1.7-3.0 times) [110].

However, both isoforms have similar effects in sustaining 25(OH)D when consumed daily [69]. Main food sources of vitamin D include natural sources from fish, eggs, mushrooms and beef and fortified foods such as cow's milk, margarine, soy beverages, rice beverages, orange juice, and yogurt [111]. A pregnant woman who drinks 2 cups of fortified milk would receive only 200 IU/d. Additional intake of 0.5 to 1 cup of fortified orange juice and regular consumption of eggs and fatty fish once weekly would elevate total intake to > 400 IU/d [111] but likely would still be below the 600 IU RDA for adults and pregnant women. Even though beef and eggs are consumed regularly and represent a source of vitamin D intake, the daily intake is usually < 40 IU/d [112]. Additionally, adult women may consume less fortified orange juice and do not regularly eat fish, especially during pregnancy [111]. Therefore, supplements have an important role in contributing to sufficient vitamin D intakes. Most prenatal supplements contain 400 IU of vitamin D_3 per suggested daily dose; this intake has been shown to contribute to a significant improvement in vitamin D status [91].

As previously stated the neonate relies on maternal supplies of vitamin D [2] Breast milk is low in vitamin D containing 25 IU/L and not sufficient for the infant [113]. All infant formula sold in Canada contain 400 IU/L vitamin D_3 [114]. Therefore sources of vitamin D typical to the exclusively breastfed infant diet include the mother's milk, vitamin D drops/syrup, and perhaps cod liver oil, although the vitamin A content of liver oils should be considered due to the risk of toxicity [111].

2.5. Factor affecting 25(OH)D and 1,25$(OH)_2$D

2.5.1. Factors affecting 25(OH)D

Stimulatory factors affecting circulating 25(OH)D are sun exposure, dietary/supplement intake and weight loss [115]. The latter has been associated with

increases in circulating 25(OH)D concentrations from storage of vitamin D in adipose tissue and its release during weight loss [116]. Drugs such as anticonvulsant drugs and glucocorticoids can have an inhibitory effect on 25(OH)D by inducing hepatic oxidase activity through the microsomal enzymes (P450) in the liver [117], which would enhance excretion. Age is another inhibitory factor, with aging there is a decrease in the absorption, transport or liver hydroxylation of vitamin D and therefore lower vitamin D status [118]. Increased skin pigmentation is associated with decreased endogenous synthesis of vitamin D_3 due to photochemical degradation of previtamin D_3 and melanin pigmentation [119]. This suggests that ethnicity can be a confounding factor in vitamin D studies due to difference in endogenous synthesis of vitamin D. In addition, it can affect vitamin D status at the beginning of a study which is a common confounder in the response to dietary intakes. Low dietary Ca [120], and /or PO_4 [121] result in increased activity of 1-α-hydroxlase, increased utilization of 25(OH)D and therefore decrease in circulating 25(OH)D concentration. As opposed to weight loss, weight gain and high BMI can negatively affect vitamin D status due to decreased bioavailability of vitamin D from cutaneous and dietary sources because of its deposition in body fat compartments [122]. Higher prevalence of 25(OH)D <50 nmol/L (61 vs. 36%) has been observed among obese compared to pregnant women of healthy weight at 4–22 wk gestation [36] suggesting sequestration of vitamin D in adipose tissue [123]. Seasonal variation in circulating 25(OH)D is well known with lower concentrations in healthy subjects in winter vs summer due to lower biologically ineffective UVB (2 vs 7 %) [124]. This has also been documented among pregnant women [125]. In the later study, social economic status (SES) was positively correlated with circulating 25(OH)D concentration at late gestation [125].

2.5.2. Factors affecting $1,25(OH)_2D$

There are many factors that have a stimulatory effect on $1,25(OH)_2D$ production such as estrogen, PTH and PTHrP [115]. Estrogen and PTHrP increase during pregnancy and have been proposed as possible hormonal factor causing the increase in blood calcitriol concentrations during pregnancy [101]. As previously stated, low dietary Ca [126] and/or PO_4 [127] can increase the conversion of 25(OH)D to $1,25(OH)_2$ D and therefore increase the concentrations of this metabolite in the circulation. Obesity may have a stimulatory effect on $1,25(OH)_2D$ due to secondary hyperparathyroidism (caused by lower circulating 25(OH)D concentration) which is associated with enhanced renal tubular reabsorption of Ca and increased circulating $1,25(OH)_2D$ [128]. It is well known that PTH activates 1-α-hydroxylation and therefore increases circulating $1,25(OH)_2D$ [115]. Aging results in decreased conversion of 25(OH)D to $1,25(OH)_2D$ by decreasing 1-α-hydroxylation [129]. The use of drugs such as glucocorticoids may have an inhibitory effect on $1,25(OH)_2D$ by increasing renal vitamin D 24-hydroxylase and decreasing renal 1-α-hydroxylase [130]. Finally, fibroblast growth factor 23 (FGF23) inhibits renal 1α-hydroxylase activity [39].

2.6. Prevalence of vitamin D deficiency in pregnancy and neonatal phase

2.6.1. Canada

In northern latitudes, including Canada, maternal vitamin D deficiency is prevalent and is a major risk factor for neonatal vitamin D deficiency [2]. Pregnant women from three First Nations communities residing in northern Manitoba had serum 25(OH)D concentrations ranging from undetectable to 63 nmol/L, with mean concentrations of 18, 21 and 24 nmol/L in each of the three communities [131]. In the same region it has been reported that 76% of women and 43% of the children (3–24

months) had 25(OH)D < 25 nmol/L [114]. In Winnipeg, Manitoba also located at a high latitude (49.54°N), 46 % of healthy mothers from mixed ethnic backgrounds had plasma 25(OH)D < 37.5 and 36% of their neonates had plasma 25(OH)D < 27.5 nmol/L, the latter was assessed using cord blood. Overall 78% of women reported taking multi-nutrient supplements during pregnancy [19]. Lower intakes of supplements (61 vs 93 %) and dietary vitamin D (149 vs 242 IU/d) were reported among deficient (< 37.5 nmol/L) compared to non-deficient women [19]. A study in a multi-ethnic population of pregnant women living in Vancouver (49°N) showed that mean 25(OH)D measured at 25-30 wk gestation was 67 (95% CI 64-69) nmol/L. Only 1% of women had a 25(OH)D concentration <25 nmol/L. Even though, 80% of women took vitamin D supplements containing ≥ 400 IU/d, 24% and 65% of these women had 25(OH)D <50 and <75 nmol/L, respectively [8]. Very few Canadian women of reproductive age achieve the RDA of 600 IU/d for vitamin D intake [1]. However, over 80% of pregnant women consume a multivitamin supplement at some point during pregnancy [132]. It is yet unclear whether the amount of vitamin D provided in prenatal supplements, typically 400 IU, is sufficient to achieve targeted 25(OH)D concentrations.

2.6.2. World wide

Worldwide, the prevalence of vitamin D deficiency (<37.5 nmol/L) among pregnant women is 8% in Western Netherlands, 18% in UK, 25% in UAE, 42 % in Northern India, 50% in South Wales in non-European ethnic minority, 59-84 % in dark skin pigmented individuals in Netherlands, 61 % in New Zealand and 80 % in Iran [133]. However, these measurements of circulating 25(OH)D were performed by different methods and at various time-points throughout pregnancy making it difficult to compare the prevalence of vitamin D deficiency among countries.

2.7. Detrimental effects of vitamin D deficiency in pregnancy

Maternal vitamin D deficiency has been shown to be a major risk factor for rickets in infants. Infants of vitamin D deficient mothers cannot reach optimal vitamin D status without higher supplementation dosages, showing the importance of following the dietary recommendations and/or excellent compliance in supplement intake of vitamin D during pregnancy [113].

2.7.1. Vitamin D deficiency and rickets

The necessity for dietary vitamin D and/or sun exposure was recognized for the prevention of rickets in children as early as 1921 [134]. Rickets is a disease of failure of organic matrix mineralization of the bone, resulting in reduced bone strength and skeletal malformation which will be later discussed in detail [135]. The old child and adult counterpart of rickets is osteomalacia [135].

2.7.1.1. Prevalence of rickets

2.7.1.1.1. Canada

Rickets was a devastating skeletal disease in industrialized counties including North America in early 1900s [136]. Even in the late 20th century, studies in Toronto (1988-1993) [137] and Manitoba (1987) [114] have reported the presence of rickets among children. The Canadian survey from 2002-2004 has reported an annual incidence rate of 2.9 case per 100,000 for rickets among children showing that it is still persistent in Canada. Higher incidence was observed in the Northern Territories. The incidence age was 1.4 ± 0.9 y, most living in urban areas (65%) with intermediate to dark skin (89%) and being breast fed (94%). Rickets was related to absence of sun exposure, but mostly lack of vitamin D supplement intake during pregnancy and infancy [113, 137].

2.7.1.1.2. Worldwide

Even in areas with abundant sunlight such the Middle East (Iran, Pakistan and Kuwait) [138] and other sunny areas such as Greece [139] and Texas, US [140] cases of rickets have been reported. This has been mainly related to social and religious customs that prevent sun exposure in the Middle East population and in general in infants of mothers with darker skin pigmentation who did not receive vitamin D supplements during pregnancy and in exclusively breastfed infants [138-140].

2.7.1.2. Biochemical, histological and clinical signs of vitamin D deficient rickets

As previously stated, one of the adverse outcomes of maternal vitamin D deficiency is neonatal rickets. The biochemical signs accompanying rickets include: normal or low serum Ca concentrations (the latter occurs when Ca stores in the skeleton begin to decrease), hypophosphatemia, increased alkaline phosphatase (ALP), secondary hyperparathyroidism, low urinary Ca and very low serum 25(OH)D concentrations (<25 nmol/L) but normal or even high serum $1,25(OH)_2D$ due to hyperparathyroidism [135, 137]. Abnormalities in the bone include: widening of the epiphyses and metaphyses of rapid growing long bones e.g. tibia and femur as well as enlarged structure and delayed closing of the fontanelles [135]. Cranitobes (softening of the skull), rachitic rosary, Harrison groove, bowing of weight bearing extremities are also signs observed in rickets [135]. In histological assessments, vitamin D deficient rickets has been associated with widened growth plates characterized by an enlarged area of hypertrophic chondrocytes, with a disorganized and greater number of cells and loss of the classic columnar pattern expected of the growth plate [141].

In addition to the classical consequences of vitamin D deficiency during pregnancy which will be further discussed in detail, vitamin D deficiency can also lead to non-classical outcomes during pregnancy [102]. In the mother,

hypovitaminosis D is postulated to contribute to insulin resistance and GDM during pregnancy [102, 142].

2.7.2. Gestational diabetes mellitus

GDM is a state of carbohydrate intolerance that is recognized for the first time during pregnancy [143]. The prevalence of GDM varies from 1-10% worldwide, based on the population, geographic location and diagnostic criteria [21]. Gestational diabetes mellitus serves as a good model for studying the early changes that develop in insulin resistance and type 2 diabetes since both have risk factors in common e.g. excess weight (overweight and obesity) [52], which will be further discussed below.

2.7.2.1. Prevalence and diagnosis criteria

2.7.2.1.2. Canadian Diabetes Association

In the latest report by the Canadian Diabetes Association (CDA) in 2008, the prevalence of GDM was reported as 3.7 % in non-Aboriginal and 8-18 % in Aboriginal pregnant women [144]. In Canada, a screening test at 24-28 wk of pregnancy is done by ingestion of a 50-g glucose followed by measurement of plasma glucose (PG) 1 h later. If PG is <7.8 mmol/L it is considered normal and reassessment is required at different pregnancy trimesters only if multiple risk factors of GDM are present. If PG is 7.8–10.2 mmol/L, OGTT is performed and if PG is ≥10.3 mmol/L, GDM is diagnosed. The OGTT is performed by consumption of 75-g of glucose solution at 24-28 wk gestation. GDM is diagnosed if 2 of the following PG values exceed the thresholds: fasting >5.3 mmol/L, 1 h >10.6 mmol/L, 2 h >8.9 mmol/L [145].

2.7.2.2. Risk factors

In a retrospective cohort study performed in Canada, the risk factors for GDM were maternal age >35 y, previous history of neonatal death, caesarean section

and obesity, but after adjustments for confounders, other traditional risk factors such as multiparity, maternal smoking, history of premature delivery and fetal anomaly were no longer associated with GDM [21]. According to the CDA, criteria risk factors for GDM include previous history of GDM or delivery of a macrosomic infant, member of a high-risk ethnicity (Aboriginal, Hispanic, South Asian, Asian, African), age ≥35 y, BMI 30 ≥kg/m^2, polycystic ovary syndrome (PCOS), acanthosis nigricans and corticosteroid use [145]. Similarly, according to the American Diabetes Association (ADA) the following categories are considered high-risk for GDM: obesity family history (i.e., first-degree relative) of diabetes, previous personal history of GDM or glycosuria [146].

2.7.2.3. Pathophysiology

Insulin resistance during pregnancy has been related to non-insulin hormonal changes such as increased human placental lactogen, human placental growth hormone, progesterone, cortisol and prolactin or perhaps increase in TNF-α or decrease in adiponectin. Therefore, insulin resistance is a physiological process that develops even during a normal pregnancy probably to increase glucose and fatty acid levels as energy substrates for the fetus which is constrained by increases in the secretion of insulin in the mother [147]. Endogenous hepatic glucose production increases by 16–30 % and insulin action decreases to 50–70% of non-pregnant state in late normal pregnancy (despite increased insulin secretion) [148]. When the pancreatic insulin response to glucose stimulus is insufficient, GDM develops [149]. The alteration in carbohydrate metabolism in GDM has been suggested to be identical to type 2 diabetes [150]. Insulin sensitivity changes during pregnancy trimesters, it increases to a small extend at the first trimester while it decreases during the rest of the pregnancy and is at its least at the third trimester, which is why GDM usually

develops in the second half of during pregnancy [147]. Women with GDM have a decrease ability of insulin receptor β subunit to undergo tyrosine phosphorylation which results in 25% lower glucose transport activity in these women as compared to healthy pregnant women [143]. The result of the decrease in insulin sensitivity to all nutrients is a greater availability of nutrients which can result in fetal overgrowth [143].

2.7.2.4. Detrimental outcomes

2.7.2.4.1. Maternal

Even though the majority of women with GDM regain normal glycemia after delivery, they have a 20-50 % higher risk for developing type 2 diabetes in the first 5 to 10 y following pregnancy [151]. This risk increases in women with obesity and those diagnosed with GDM prior to 24 wk of pregnancy [152]. Women that have GDM are at higher risk of caesarean section [153], preterm delivery, premature rupture of membrane, pre-eclampsia and caesarean section [21].

2.7.2.4.2. Fetal

The detrimental effects of GDM are not only limited to maternal health outcomes but also include adverse effects on neonatal health. There is a strong increased risk for macrosomia (>90th percentile for gestational age (GA), adjusted for ethnicity and parity), fetal hyperinsulinemia (assessed by cord C-peptide concentration which is the connecting peptide in the proinsulin used as a marker of insulin secretion) and neonatal increased fat mass (obesity) (>90th percentile for GA) and a risk of neonatal hypoglycaemia [153]. In addition, lower APGAR scores at 1 and 5 min post birth have been observed in neonates of mothers with GDM [21].

Specific mechanisms explaining these alterations are the maternal hyperglycemia transmitted to the infant, resulting in hyperinsulinemia in the fetus

which leads to accumulation of fetal body fat (Pedersen hypothesis) [154]. Accordingly, the insulin resistance in GDM mothers has been shown to be positively correlated with neonatal fat mass at birth [143].

There are no data available on the effects of GDM on fetal bone health, but the following studies suggest that diabetes during pregnancy may cause defects in neonatal bone health. Infants of mothers with insulin-dependent diabetes have lower BMC as compared to infants of healthy mothers and infant distal radius BMC is negatively related to maternal glucose concentration in the first trimester [155]. This effect may be related to decreased transplacental mineral transfer to the fetus, decreased fetal bone accretion or increased resorption in women with diabetes compared to those without [155]. *In vitro* studies, have also suggested an increased risk of defect in the development of the embryonic skeleton under elevated vs physiological concentration levels of glucose due to impaired differentiation of osteoblasts and osteoclasts [156]. However, other studies do not show any difference in whole body BMC in neonate of mothers with diabetes as compared to those of healthy mothers[157].

The similarity between detrimental effects of GDM on neonatal anthropometry and possibly bone outcomes and those of maternal vitamin D deficiency raises the question whether this is a coincidence or that vitamin D deficiency during pregnancy is associated with GDM.

2.8. Classical roles of vitamin D

2.8.1. Calcium and phosphate homeostasis

The classical role of vitamin D is maintaining serum Ca and PO_4 concentrations. This is maintained by the effect of $1,25(OH)_2D$ on intestine, kidney and bone. In the intestine, $1,25(OH)_2D$ increases Ca absorption both transcellularly

(saturable) at the proximal intestine and paracellularly (nonsaturable) at all sites of the intestine by increasing the formation of Ca transporter, transient receptor potential V6 (TRPV6), calbindin and a basolateral Ca pump. Calcitriol also increases PO_4 absorption mainly in the proximal intestine [39]. The absorption of PO_4 in the intestine is dependent on the dietary PO_4 and therefore $1,25(OH)_2D$ may not necessarily increase PO_4 absorption. The role of vitamin D in increasing Ca reabsorption from the kidney is mainly at the distal site along a stimulatory effect on the action of PTH on increasing epithelial Ca channel transient receptor potential V5 (TRPV5), vitamin D Ca binding proteins (calbidin-D_{28k} and calbidin-D-$_{9k}$) and plasma membrane Ca pump (PMA1b). The effect of $1,25(OH)_2D$ in increasing the renal PO_4 absorption has been suggested, but this role of vitamin D on renal PO_4 absorption is dependent on PTH status since PTH blocks its reabsorption and causes PO_4 diuresis. Fibroblast growth factor 23 (FGF23) is secreted from osteoblasts, which like PTH lowers serum PO_4 by suppressing its reabsorption in proximal tubules of the kidney. The active form of vitamin D, $1,25(OH)_2D$ induces an increase in FGF23 while FGF23 in turn inhibits $1,25(OH)_2D$ synthesis. Therefore, there is a $1,25(OH)_2D$–FGF23 axis regulating PO_4 which is as important as the $1,25(OH)_2D$–PTH axis that regulates Ca [39]. It seems that the role of $1,25(OH)_2D$ on FGF23 is the opposite under hypophosphatemic conditions [39].

The effect of $1,25(OH)_2D$ on increasing bone resorption is accompanied by PTH. A decrease in serum Ca concentration triggers the secretion of PTH from the parathyroid glands which increases 1-α-hydroxylase in the kidneys leading to increased $1,25(OH)_2D$ production. Calcitriol decreases PTH through a negative feedback loop [39].

2.8.2. Bone

2.8.2.1. Remodeling

The physiological role of vitamin D on bone include development and turnover of bone, differentiation and survival of distinct bone cells and its classical role in maintaining serum Ca and PO_4 concentrations (to prevent rickets/osteomalacia) and bone homeostasis (balance between bone formation and resorption) by controlling gene expression.

The inorganic component (80 %) of the bone consists of crystals of hydroxyapatite [15]. Deposition of this mineral is partly dependent on $1,25(OH)_2D$. On the one hand, $1,25(OH)_2D$ binds to VDR in osteoblasts which activates genes that promote osteoclast differentiation and bone resorption, on the other hand, calcitriol targets genes that code for bone matrix protein to replace the resorbed bone with new tissue synthesized by the osteoblast. The inactive osteoblasts are bone lining cells responsive to $1,25(OH)_2D$ and PTH. When osteoblasts are embedded in the mineralized bone, they are changed to osteocytes that have mechanosensor activity. Ninety percent of the protein mass of the bone matrix (from the 20 % organic component) consists of collagen type I protein in adults and the remaining 10% is non-collagenous proteins that either promote or inhibit mineralization. These include bone sialoprotein, osteopontin (only found during growth and mineralization stage), OC (mineralization stage) and ALP (matrix maturation); these proteins all have genes with VDRE in their promoters. The genes of these proteins may be either positively or negatively controlled by $1,25(OH)_2D$ depending on physiological levels of the metabolite, differentiation stage of the cell and the interaction of calcitriol with VDR complex on target genes. Physiological concentrations of $1,25(OH)_2D$ lead to normal bone formation and resorption. Some studies suggest that physiological

concentrations of $1,25(OH)_2D$ not only do not stimulate bone resorption but even suppress PTH induced receptor activator of nuclear factor kappa-B ligand (RANKL) [158] mRNA expression which then stimulates bone mineralization [14]. Only pharmacological doses of $1,25(OH)_2D$ mobilizes bone and has bone resorptive properties through increasing RANKL on osteoblasts leading to osteoclastogenesis [14]. Unlike osteoclasts, osteoblasts have VDRs as well as the ability to express CYP27B1 and CYP24A1 which are anabolic and catabolic regulators of $1,25(OH)_2D$, respectively [15]. The active form of vitamin D, $1,25(OH)_2D$ stimulates the VDR-RXR binding to CYP24A1 and osteopontin target gene promoters in osteoblasts [159].

Calcitriol also regulates bone resorption by osteoclasts both indirectly by its effect on osteoblasts that secrete factors that are critical for osteoclastogenesis and directly by differentiating osteoclasts from mononuclear hematopoietic stem cells (HSC) (progenitors can be from marrow, spleen and blood), monocytes and macrophages. Regarding osteoclastogenesis, $1,25(OH)_2D$ increases the receptor activator of nuclear factor kappa-B (RANK) expressed on the monocyte and osteoclasts. Receptor activator of nuclear factor kappa-B ligand on osteoblast and macrophage-colony stimulating factor (M-CSF) produced by osteoblasts are required for conversion of HSC to osteoclasts [15]. The coupling of RANKL on osteoblast with RANK on osteoclast is required for further differentiation of osteoclasts. In addition, osteoprotegerin (OPG) in osteoblasts inhibits osteoclast differentiation by binding to RANK. Osteoprotegerin is down regulated by $1,25(OH)_2D$ in mature osteoblasts favoring bone resorption *in vitro*. Calcitriol enhances the synthesis of proteins involved in the attachment of osteoclast to the bone sealing zone such as osteopontin and integrin $\alpha v\beta 3$. In addition, it increases the matrix metallopeptidase 13

(MMP13) protein, secreted by osteoblasts that remove osteoids from the bone surface which is required for adherence of osteoclast to the bone. After the bone resorption phase by osteoclasts, $1,25(OH)_2D$ stimulates the synthesis of cytokines and release of factors from bone matrix such as transforming growth factor-β (TGF-β) that couple bone resorption to formation and recruit pre-osteoblasts and increase the formation of osteoblast proteins and inhibit osteoclast differentiation. This demonstrates how $1,25(OH)_2D$ connects the cycles of bone formation to resorption and therefore has a role in bone remodeling [15].

Calicitriol has antiproliferative and stimulatory effects on differentiation of different cell types including bone cells. It is important to note that in addition to the concentration of $1,25(OH)_2D$, the stage of differentiation at which the bone cell is at also affects the way this active metabolite influences cells. *In vitro* studies have shown that if osteoblasts are at their preconfluent stage, $1,25(OH)_2D$ at pharmacological levels has an antipoliferative effect and inhibits osteoblast maturation while if at their proconfluent stage, $1,25(OH)_2D$ results in osteoblast maturation and mineralization. *In vivo* studies have shown that $1,25(OH)_2D$ has an anabolic effect on mature mice osteoblasts leading to increased cortical and Tb. bone [15]. Although $1,25(OH)_2D$ has pro apoptosis effects on different cells, it does not have this role in osteoblasts. Calcitriol may also have a protective effect on mature osteoblast pre-osteocytes from their transition into osteocytes in the bone matrix [160].

2.8.2.1. Development

Previous research on VDR-null or 1-α-hydroxylase null mice, did not reveal the importance of $1,25(OH)_2D$ in bone development, since the fetal skeleton was normal at birth; although the mice developed rickets after birth which could be treated

by Ca supplements [161]. However, recent studies have shown that $1,25(OH)_2D$ enhances the Wnt signalling pathway which has a bone formation effect through increase in OPG and decrease in RANKL in osteoblasts (therefore decreasing osteoclastogenesis). This signaling pathway, also fosters osteoblast activity by suppressing the production of adipocytes and chondrocytes from mesenchymal stem cells (MSC) [113] in favor of osteoblast differentiation, proliferation and mineralization and preventing osteoblast apoptosis [162]. The enhancing effect of $1,25(OH)_2D$ on Wnt signalling is by enhancing the formation of low density lipoprotein receptor like protein-5 (LRP5) which is a putative Wnt receptor and inhibits the Wnt signalling antagonists (dickkopf-related protein 1 (DKK1) (unregulated by tumor necrosis factor-α (TNF-α)) and secreted frizzled-related protein 2 (SFRP2)) resulting in an anabolic effect on bone formation. This is also in line with studies showing that VDR null mice have higher mRNA expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) which is essential for differentiation of adipocytes and inhibiting bone promoting properties of Wnt signalling [160].

In summary, $1,25(OH)_2D$ has an important role in maintaining serum Ca and PO_4 concentrations and balance between bone formation and resorption. At physiological levels, $1,25(OH)_2D$ leads to normal bone formation and resorption while pharmacological levels can lead to bone resorption. Calcitriol increases the synthesis of OC and LRP5; which are all anabolic and increases RANKL which is catabolic to bone [39].

2.9. Non-classical roles of vitamin D

In addition to the classical role of vitamin D on mineral and bone metabolism, recent evidence is suggestive of non-classical roles of vitamin D on immune

responses, keratinocytes, cancer and insulin secretion by detection of VDRs on the targeted tissues [163]. The detection of VDRs in the pancreas led to investigating a possible role of $1,25(OH)_2D$ in glucose metabolism.

2.9.1. Possible mechanisms for action of vitamin D on glucose metabolism

The pathophysiology of GDM is similar to type 2 diabetes which includes impaired pancreatic β-cell function, insulin resistance and systemic inflammation [164]. Therefore, the possible mechanisms explaining the role of vitamin D in the pathogenesis of GDM may be similar to type 2 diabetes mellitus as was previously described and for which more research has been conducted.

Calcitriol is known to have both direct and indirect effects on glucose metabolism. Its direct effect is through stimulation of insulin synthesis in the pancreatic islet β-cells as shown in animal and human studies [164, 165], which increases the insulin response to glucose and not basal insulin concentrations [166]. Circulating $1,25(OH)_2D$ can bind to nuclear receptors in many types of cells including β-cells of the pancreas. In addition, 1-α-hydroxylase is also expressed in the β cells [164, 167]. Vitamin D-Ca binding protein (Calbidin-D_{28k}) is localized in pancreatic β-cells and plays an important role in controlling intracellular Ca^{2+} and therefore insulin release from pancreatic β-cells [168]. The fact that it is expressed indicates that $1,25(OH)_2D$ is involved in pancreatic β-cell function. In addition, calcitriol has a role in regulating extracellular Ca concentration and flux through the cell membrane of pancreatic β-cells and peripheral insulin target tissues such as adipose tissue and muscle [164]. Insulin secretion is Ca-dependent, for example, endopeptidase which is required for conversion of proinsulin to insulin is Ca-dependent [16] and $1,25(OH)_2D$ accelerates this conversion *in vitro* [169]. Therefore, insufficient Ca or vitamin D intake may change the intracellular and extracellular

balance in β-cell Ca pool and adversely affect insulin release [166]. This fact also affects peripheral target tissues of insulin which may lead to peripheral insulin resistance [166].

The other indirect pathway is through increasing insulin sensitivity [165]. This role of $1,25(OH)_2D$ can be through up-regulation of insulin receptor and/or by activating PPAR-δ which regulates fatty acid metabolism as a transcription factor in muscle and adipose tissue [164]. Systematic inflammation has been linked to insulin resistance and β-cell dysfunction [166]. $1,25(OH)_2D$ may have a role in promoting β-cell survival through modulating the production and action of cytokines [166]. Another proposed indirect pathway through which $1,25(OH)_2D$ regulates glucose metabolism is by its association with PTH, meaning that studies have shown that hyperparathyroidism is inversely related to insulin sensitivity. Since vitamin D deficiency leads to secondary hyperparathyroidism, it is postulated that some of the effect of $1,25(OH)_2D$ on glucose homeostasis may be through its effect on PTH [165]. Since *in vitro* and *in vivo* studies in rats have shown that administration of PTH can increase intracellular Ca concentrations in adipocytes leading to impaired glucose transport protein 4 (GLUT-4) which is a protein responsible for insulin-regulated glucose transport into the adipose and muscle cells, making insulin stimulated glucose uptake less capable [170]. Lastly, the interaction of the active form of vitamin D with its receptor is also important in its effect on glucose metabolism, therefore VDR gene polymorphism as well as vitamin D-binding protein may affect changes in glucose and insulin secretion irrespective of vitamin D status and $1,25(OH)_2D$ levels contributing to the role of genetics [16, 165, 171].

2.10. Cross-talk between bone and energy metabolism

2.10.1. Osteocalcin

Osteocalcin is the most abundant non-collagenous protein in the bone [172] with a low molecular weight (5700 D) hormone-like peptide, thought to be specifically produced by the osteoblasts and secreted into the circulation [41]. However, recent studies have suggested its secretion from human adipose tissue, at least *in vitro* [42].

During posttranslational modification, OC glutamic acid residues are carboxylated by a vitamin k-dependent γ-carboxylation and Gla protein (γ - carboxyglutamic acid) is formed [41]. The carboxylated OC which is the main constituent of total OC has a high affinity for hydroxyapatite as compared to the uncarboxylated form. Ninety percent of OC is bound to hydroxyapatite in mice [41]. It appears that the uncarboxylated form regulates glucose homeostasis in mice, since only uncarboxylated OC induced an increase in the synthesis of adiponectin in adipocytes, and of insulin in islets [41]. Osteocalcin is decarboxylated during bone resorption by osteoclasts [173]. Human studies have shown similar mechanisms in controlling OC decarboxylation [172]. However, recent research suggests that the role of the uncarboxylated OC in glucose homeostasis may only be in human subjects with abnormal regulation of glucose [43]. In addition, recent studies have also shown a role of the total [44-46] and carboxylated form of OC in the up-regulation of insulin sensitivity in humans [43]. It has been hypothesized that carboxylated OC has a role in insulin sensitivity while the uncarboxylated form is mostly involved in β-cell function [43]. In addition, cell culture studies in rat adipocytes have shown that both carboxylated and uncarboxylated OC stimulate basal as well as insulin stimulated glucose transport in these cells [174].

Even though OC is best known as a bone formation biomarker [175], some research suggest that it may not have a role in bone formation. The $OC^{-/-}$ knock-out mice had higher bone mass as compared to WT [176, 177]; OC can be released into the blood circulation in times of bone resorption as well as bone formation [43]. Furthermore, OC is implicated in soft tissue calcification [178]. As such it is possible that OC has an endocrine function in Ca homeostasis [179, 180]. Indeed it could be a component of a feedback loop following activation of $1,25(OH)_2D$ with an additional function to sequester Ca^{2+} upon its release from the bone to replenish pancreatic β-cell Ca when circulating Ca reach low concentrations.

2.10.2. Animal

Studies showing that adiposity protects against osteoporosis, have led to the understanding that bone remodeling is known to be affected by adipocyte-derived hormones, such as leptin, adiponectin (an adipokine enhancing insulin sensitivity) and more recently OC. In other words, since hormones regulate bone cell functions, it was thought that there must be a feedback regulation of endocrine system by the bone [41]. Lee et al. observed a decrease in β-cell proliferation and increase in glucose intolerance, insulin resistance and visceral fat in mice lacking the osteoblast-secreted molecule ($OC^{-/-}$), OC as compared to wild type (WT) [174] mice and that *in vivo* OC increases glucose tolerance [174]. Results from another study showed that *ex vivo* OC stimulates insulin synthesis in β-cell and adiponectin in adipocytes [41]. The regulation of insulin sensitivity is partly through its effect on adiponectin and independent of its effect on insulin secretion [41]. Adponectin increases insulin sensitivity through increasing fatty acid oxidation in muscles, inhibiting gluconeogenesis, reducing muscle and liver triglyceride and decreasing plasma free fatty acids [51]. The authors concluded that OC is a bone derived hormone that is also

involved in energy metabolism [41]. In addition, another study demonstrated daily injection of 3 or 30 ng/g/d of OC significantly increased β-cell mass and insulin secretion and improved glucose tolerance and insulin sensitivity in mice fed normal or high fat diets suggesting that OC can improve glucose tolerance and prevent type 2 diabetes and also prevented obesity in high fat fed mice [181]. However, no animal study has explored the effect of OC on maternal glucose homeostasis and tolerance during pregnancy.

2.10.3. Human

In healthy states in adult men, a positive significant association was observed between total OC and insulin sensitivity which was stronger in lean compared to overweight and obese subjects but did not reach statistical significance. In addition, a positive association was also shown between OC and insulin secretion but only in lean subjects after accounting for age, BMI and waist circumference [44]. Both forms of OC may be involved in glucose homeostasis in humans. The uncarboxylated OC has been positively associated with β-cell function and the carboxylated OC has been positively related to insulin sensitivity in children with prediabetes [43] and healthy adult men [182].

There is controversy regarding circulating total OC concentrations at early stages of type 2 diabetes with one study showing lower concentrations in newly diagnosed compared to healthy adults and total OC was positively associated with β-cell function, but not insulin resistance [183]. In contrast, another study showed higher plasma total OC in adults with newly diagnosed type 2 diabetes as compared to those with normal glucose homeostasis. There was a significant positive correlation between plasma total OC and glucose concentration at 2 h on a 75g-

OGTT but not FPG [184]. The authors suggested that total OC is independently related to impaired glucose tolerance in early stages of type 2 diabetes [184].

Cross-sectional human studies mostly show that circulating total OC concentration is negatively associated with FPG, hemoglobin A1C, homeostatic model assessment-insulin resistance (HOMA-IR), BMI, fat mass and percent body fat in middle-aged adults [51, 185] and elderly [46, 186] men and women with type 2 diabetes. Whether OC is similarly associated with glucose and body composition during the physiological state of pregnancy considering its association with vitamin D as stated below is an area of research requiring further investigation.

2.10.4. Osteocalcin and vitamin D

The existence of VDRE on promoter of OC gene has been detected in humans and animals. In many species, $1,25(OH)_2D$ stimulates the synthesis of OC [40] which has even been observed during the fetal period in rats [187]. Nevertheless, the injection of $1,25(OH)_2D$ did not change OC concentrations in male rats with diabetes [188]. A decrease in serum $1,25(OH)_2D$ accompanied by a decrease in serum OC has been reported in patients with type 1 diabetes while this was not observed in patients with type 2 diabetes [189]. All of this evidence suggests a potential link between vitamin D status and OC during the physiological state of pregnancy.

2.10.5. Osteocalcin in pregnancy

2.10.5.1. Maternal

Osteocalcin concentrations decrease during pregnancy in healthy women and reach low or undetectable levels by the second trimester and the end of pregnancy which is hypothesized to be due to it being trapped in the placenta [190-192]. Other studies have reported the same decrease in early pregnancy but increase toward the end of human pregnancy [52], reaching its maximum levels at 35 wk, after which it

starts to decrease by 13% until the end of human pregnancy [193]. Studies have also been controversial regarding changes in OC at postpartum showing both decrease and increase in serum OC postpartum in human pregnancy [100]. Similar to non-pregnancy states, the expression of OC gene has shown to be regulated by VDR genotype during human pregnancy [194]. Therefore, changes in OC concentration during pregnancy are important to be considered when investigating the association between vitamin D status and circulating OC during this physiological state.

2.10.5.1.1. Gestational diabetes mellitus

Studies investigating the association between circulating OC concentration and GDM are relatively recent [52, 53]. Maternal serum OC was significantly higher in women with GDM vs healthy pregnant women at mid-gestation (24-28 wk), independent of age and BMI [52]. However, in another study the higher circulating OC concentrations at mid-gestation were no longer significant after adjustment for age and BMI (24-28 wk) in GDM vs healthy control pregnant women [53].

Plasma OC concentrations positively correlated with plasma glucose, C-peptide, basal and total insulin secretion, AUC for insulin in both GDM and healthy controls [52]. In contrast, the hepatic insulin clearance was negatively associated with OC also suggesting that the increase in OC may be related to a decrease in insulin clearance. No correlation was found between OC and HbA1C. In addition, plasma OC concentrations were not different in GDM women receiving diet therapy or diet and insulin therapy [52]. However, in the study by Hossein-Nezhad et al., serum OC had no significant correlation with FPG and HOMA-IR index in GDM and healthy pregnant women [53].

In late gestation (33-38 wk), women diagnosed with GDM had a 39.5% increase in OC concentrations as compared to mid-gestation (24-28 wk). At 3 mo

postpartum, plasma OC concentration increased in both healthy and GDM groups with no difference among groups [52] and there were no longer significant correlations between serum OC concentrations and AUC for glucose, insulin and FPG [52].

It was suggested that the higher plasma OC concentrations in the GDM vs control group may be an early compensatory mechanism of the body to increase insulin secretion in an insulin resistance state such as GDM which cannot be accomplished because of pancreatic β-cell defects in these women [52, 184]. However, it should be considered that since insulin can also increase OC concentrations, this increase may have resulted due to the effect of hyperinsulinemia [52]. Nonetheless, this possible role of OC in association with circulating 25(OH)D and $1,25(OH)_2D$ has not yet been investigated in glucose tolerance during pregnancy.

2.10.5.2. Fetal

Cord OC concentrations seem to reflect the active fetal mineralization at the end of pregnancy and is 2.5-7 fold higher in human fetuses [193] and 4.2 fold higher in fetal guinea pig [55] as compared to maternal OC concentrations at term. There is no maternal-fetal transfer of OC in human or sheep pregnancy [100] suggesting that high OC levels are of fetal and not maternal origin. Osteocalcin concentrations tend to increase significantly from birth to d 5 of age in human neonates. There is a positive correlation between serum $1,25(OH)_2D$ and OC concentration in cord serum and at d 5 of age in the human neonate [100, 195]. In addition, fetal OC concentration seems to be dependent on maternal vitamin D status and uteroplacental blood flow to the fetus [193]. Therefore, vitamin D status and the active form of vitamin D during pregnancy may be associated with fetal OC concentrations which are important to be considered in neonatal bone outcomes studies.

2.11. Bone and reproduction

2.11.1. Bone and pregnancy

Even though studies have evaluated the change of bone biomarkers during pregnancy, these biomarkers can be affected by changes in renal filtration, hemodilution and may be of fetal or placental origin [196]. Bone formation biomarkers, OC, procollagen 1 carboxyterminal peptide (P1CP), ALP decline in early pregnancy, but may increase at late pregnancy. Bone resorption biomarkers, pyridiniolyne and tDPD increase during pregnancy. Overall, increased bone turnover rate has been suggested during pregnancy [197].

Recent studies have suggested a 1 to 4 % decrease in BMD in maternal spine (2.1 %), hip and ultradistal radius (3.8 %) (which are rich in Tb. bone) when comparing pre-conception (3-9 mo) and (1-6 wk) postpartum [101, 196]. Therefore, most bone changes during pregnancy are suggested to be at bone sites containing Tb. [76]. Other studies have either suggested no change in BMD or an increase in cortical bone during pregnancy [196].

2.11.2. Bone and development

Of relevance to this thesis, fetal bone development during pregnancy involves embryonic and fetal stages of development which are important to understand when investigating neonatal bone health outcomes in association with vitamin D status during pregnancy.

2.11.2.1. Embryonic bone development

During embryogenesis, MSC are converted to either chondrocytes or osteoblasts. Two types of ossification occur during bone development: intramembranous ossification during which MSC [113] are differentiated into osteoblast to form membranous bone and endochondral ossification during which

MSC are differentiated into chondrocytes to produce cartilage which provides a growth template for the future bone.

There are several pathways that regulate skeletal formation and cell fate determination, proliferation and maturation in skeletal development. Some of which are: Wnts signalling, Ihh (Indian hedgehog), bone morphogenic proteins (BMPs) and FGFs. SRY (sex determining region Y)-box 9 (Sox 9) and RUNX 2 (runt-related transcription factor 2) are transcription factors that determine chondrocyte and osteoblast cell fates, respectively. These transcription factors are controlled by cell-cell signalling particularly by Wnt and Ihh. Increased canonical Wnt signalling enhances RUNX 2 gene expression and therefore bone formation, but inhibits Sox9 gene expression and therefore chondrocyte differentiation. Indian hedgehog signalling results in osteoblast differentiation by enhancing RUNX 2 gene expression during endochondral ossification. Bone morphogenic proteins are secreted growth proteins that can promote the differentiation of both chondrocytes and osteoblasts from MCS. Fibroblast growth factor receptor 3 (FGFR3) controls the proliferation and hypertrophy of chondrocytes. Fibroblast growth factor signalling can directly or indirectly (through Wnt and BMP signalling) both enhance or inhibit osteoblast proliferation and differentiation depending on the cell context [198].

2.11.2.2. Fetal and neonatal bone development

During endochonral ossification, in which MSC are differentiated into chondrocytes to produce cartilage, chondrocytes inside the cartilage undergo a regulated proliferation and hypertrophy and become oriented along the longitudinal axis which is required for bone elongation. Prehypertophic chondrocytes express Ihh which stimulates the perichondrium (layer of connective tissue surrounding the cartilage) to induce osteoblast differentiation. The speed of chondrocyte hypertrophy

is controlled by the effect of Ihh on the gene expression of PTHrP on cartilage. The receptor for PTH and PTHrP are the same and higher concentrations of the receptors are found in pre hypertrophic chondrocytes. Parathyroid hormone related-protein is required to prevent early chondrocyte hypertrophy. In addition, PTHrP also regulates Ihh in controlling chondrocyte hypertrophy. Therefore, the Ihh-PTHrP forms a negative-loop that determines whether the chondrocyte should keep proliferating or become hypertrophic [198].

Wnt signalling either canonical or β-catenin-independent (noncanonical) controls chondrocyte proliferation and hypertrophy. Fibroblast growth factor 2 (FGF2) signaling negatively controls chondrocyte proliferation and differentiation, whereas in osteoblasts it positively regulates osteoblast proliferation. In addition, BMPs also increase proliferation of chondrocytes in part through Ihh [198].

The interruption of the placental transfer of hormones and nutrition immediately after birth and the change in mechanical load results in a new environment for the skeleton. The first few days of life there may be a decrease in bone formation and an increase in bone resorption biomarkers in the neonate, but once the regular phase of growth initiates, both bone formation and resorption increase [108].

2.12. Vitamin D, bone density, architecture and metabolism biomarkers during pregnancy and at birth

2.12.1. Animal

Mice and rats have large litter sizes and neonates have poorly mineralized bone at birth, therefore are not ideal for studying the effect of nutrition during pregnancy on neonatal bone health at birth. In rabbits and sheep, maternal and fetal 25(OH)D are well correlated [199-201]. Data regarding vitamin D status and fetal

mineralization (more mineralized than mice and rats) is available in the guinea pig. The guinea pig has a haemomonochorial placenta, similar to humans [55]. This is an important fact when studying the effect of vitamin D status during pregnancy because maternal and fetal 25(OH)D and $1,25(OH)_2D$ are highly correlated in guinea pigs [13, 202] and fetal $1,25(OH)_2D$ originates mainly from transplacental transport rather than fetal kidney synthesis [13] while this is not the case in rabbits [199]. In addition, changes in serum $1,25(OH)_2D$ and OC concentrations during pregnancy and fetal vs maternal levels of these parameters in guinea pigs are comparable to humans, whereas these changes are different in sheep [55].

Most studies on the effect of maternal vitamin D deficiency during pregnancy on bone outcomes have been performed on rats [77, 78, 203] that were receiving vitamin D deficient diets since weaning and that had reached undetectable levels of 25(OH)D and $1,25(OH)_2D$ prior to mating. Maternal plasma Ca was lower [78, 203, 204] accompanied by higher femur volume and lower femur Ca content/femur volume in deplete vs replete rats at late gestation (d 20 of 21) [203]. Maternal femoral bone developed changes similar to rickets including a higher perimeter to area ratio and lower metaphyseal femur Tb. vBMD and mid-diaphysis cortical mineralized area [77], endosteal bone formation and femur length [78], in vitamin D deplete vs replete rats during pregnancy [77, 78]. Thus, these results suggested that vitamin D is required for normal bone mineralization in pregnancy in rats [78]. Pregnancy caused an increase in osteoclastic bone resorption and indicators of bone formation which were independent of 25(OH)D and $1,25(OH)_2D$ concentrations [78]. Other studies in rats initiated vitamin D deficiency after mating, but vitamin D status still reached undetectable levels by late gestation [205]. Maternal plasma mineral homeostasis

were maintained in the studies in rats which initiated vitamin D deficient diets only after mating while bone outcomes were not investigated [205].

In 2-3 mo old (adolescent) guinea pigs, fed vitamin D deficient (0 IU/g) vs replete (2.4 IU/g) diets from mating until late gestation, undetectable vs 350 nmol/L maternal 25(OH)D were achieved at late gestation, respectively [13]. However, these differences in vitamin D status did not result in any difference in maternal plasma Ca^{2+} and OC concentrations and distal femur Tb. BMD but resulted in lower plasma PO_4, total femur BMD and distal femur cortical tissue mineral density (TMD) but not Tb. BMD [13]. Maternal proximal tibia histomorphometry showed a higher growth plate width (GPW), osteoid thickness but no difference in Tb. bone volume to total volume (BV/TV) in the deficient vs replete vitamin D group [13]. Therefore, maternal vitamin D deficiency may be associated with features similar to rickets and osteomalacia in developing and mature pregnancy models, respectively. Even though, the detrimental effects of vitamin D deficiency are known on bone health, consequences of a vitamin D dose-response study during pregnancy on maternal bone health outcomes have not been studied. Such a study would be important in order to investigate whether there is a threshold at which maternal vitamin D status during pregnancy would optimize maternal bone health outcomes.

Studies in different species, have also investigated the neonatal bone and mineral outcomes of maternal vitamin D deficiency. In rats, even though vitamin D deficiency caused hypocalcemia [206-208] in the neonate at birth, it did not impair the fetal total body Ca content in one study [206], but resulted in lower fetal body [208] and femoral Ca content [208] in another study. However, normal mineral homeostasis was observed in pups born to rats which consumed vitamin D deficient diets only after mating [205]. In the same species, no change in metaphyseal and

epiphyseal mineralized tissues and longitudinal bone growth were observed in pups at birth but detrimental effects of vitamin D deficiency on these bone outcomes were observed later by weaning [207]. In the two available studies on the effect of maternal vitamin D deficient vs replete diet, neonatal bone outcomes were as follows [13, 79]: in the first study, the fetal guinea pigs born from vitamin D deficient (undetectable) vs replete (~350 nmol/L) sows had hypercalcemia, hypophosphatemia, lower plasma OC, no difference in plasma tDPD, but lower whole body BMC but not BMC/kg [13]. In the same study, histomorphometry of the proximal tibia revealed increased hypertrophic chondrocytes, osteoid surface and thickness in the vitamin D deficient vs replete guinea pig fetus [13]. However, in the second study, guinea pigs mated at 2-3 mo of age were fed vitamin D deficient (0 IU/g) vs normal (1.2 IU/g) diets [79], resulting in maternal plasma 25(OH)D concentration of ~20 nmol/L vs 118 nmol/L at late gestation. In the guinea pig neonate, lower plasma OC, whole body and tibia BMC, tibia young's modulus, maximum load and flexure but no difference in femur biomechanical outcomes and whole body, femur and spine BMD were observed in the vitamin D deficient vs replete group [79]. Whether there is a threshold at which vitamin D status during pregnancy affects bone health, cannot be distinguished from the available data, showing the need for a vitamin D dose-response study during pregnancy.

Pregnant mice models lacking VDR (VDR-null) [209] or 1-α-hydroxylase ablated mice [210] did not lead to fetal bone abnormalities. Fetuses from these models had similar whole body BMC and plasma mineral homeostasis as compared to WT models. Signs of skeletal disorders such as rickets and hypocalcemia and hypophosphatemia only became evident until 2 wk after birth. The reason for this later manifestation is that at the same time at postpartum, intestinal Ca absorption

matures shifting from the nonsaturable passive form facilitated by lactose to the active, saturable process which depends on $1,25(OH)_2D$ [101]. Whether the lack of fetal bone abnormalities in these model is due to low bone mineralization at birth or this being a species specific effect is in mice [101] or that $1,25(OH)_2D$ does not have a major role in fetal mineralization is still controversial [105].

2.12.2. Human

2.12.2.1. Observational

Similar to the non-pregnant state, if women develop vitamin D deficiency during pregnancy, in addition to osteomalacia they are at risk of hypocalcemia and secondary hyperparathyroidism which may lead to bone loss. Data have not supported higher risk for vitamin D deficiency in pregnant as compared to non-pregnant women [102]. Maternal plasma 25(OH)D < 80 nmol/L during pregnancy may be associated with maternal bone loss [211]. However, this association has not been rigorously investigated.

Serum 25(OH)D concentrations <25 vs >75 nmol/L at 34 wk gestation in UK pregnant women was associated with higher distal femur metaphyses cross-sectional area (CSA) and FSI (femur metaphases CSA/femur length), but not femur length as early as 19 and 34 wk of pregnancy [63]. This study shows the importance of vitamin D sufficiency in bone mineralization of the offspring early in its uterine life [212]. Maternal serum 25(OH)D < 35 vs > 35 nmol/L resulted in lower neonatal whole body and femur BMC relative to body weight in Winnipeg, Canada [19]. In Finland, maternal serum 25OHD concentrations <42.6 nmol/L were associated with reduced neonatal tibia BMC and CSA [213] by 13.8 % and 16.1 %, respectively [214].

Studies on the effect of season of birth on neonatal BMC have shown that US neonates born in summer have 12 % lower BMC at the 1/3 distal radius and higher

OC and $1,25(OH)_2D$ concentrations and lower Ca concentrations compared to those born in winter despite there not being a difference in serum 25(OH)D among neonates at birth [215, 216]. In contrast, the same research group reported 6% lower total body BMC, lower cord serum 25(OH)D and $1,25(OH)_2D$ in winter vs summer born Korean neonates probably due to lower maternal vitamin D status during winter [217]. The difference observed between these studies may be due to different neonatal serum 25(OH)D concentrations in the US and Korean population or other uncontrolled confounders such as smoking, SES and etc.. In addition, it is still unknown whether the seasonal effects at early or late pregnancy have a more significant impact on neonatal bone health outcomes.

2.12.2.2. Interventional

Most clinical vitamin D trials have used supplementation intakes between 400-1000 IU/d [18, 66-68] during pregnancy. Vitamin D deficiency in general would result in hypocalcemia and secondary hyperparathyroidism in non-pregnant adults. However, if vitamin D deficiency is not severe during pregnancy, serum Ca concentration is usually maintained within the normal range [218]. Overall, previous studies suggest that vitamin D supplementation (400-1000 IU/d) vs no supplementation during pregnancy does not affect maternal PTH and $1,25(OH)_2D$ concentrations [68, 101, 219], but increases 25(OH)D and either decreases [18, 67, 101, 196, 220] or does not affect ALP concentrations [219].

The IOM has mentioned the need for a vitamin D dose-response study during pregnancy to investigate its effects on bone health outcomes. A dose-response-study would help to distinguish a threshold for the beneficial effects of vitamin D status on maternal and neonatal bone health outcomes. To date, only one clinical trial has explored the dose-response effect of dietary vitamin D_3 (400, 2000 and 4000 IU/d)

during pregnancy which has only reported its effects on vitamin D metabolites, minerals and calciotrophic hormones, but not bone outcomes. The average dietary vitamin D intake was almost 200 IU/d in all groups and no significant difference was observed among groups. Serum 25(OH)D values were significantly different between diets at mid-gestation 79.1 ± 29.5, 94.4 ± 26.1 and 110.8 ± 28.3 nmol/L, respectively which continued until delivery 78.9 ± 36.5, 98.3 ± 34.2 and 111.0 ± 40.4 nmol/L, respectively. The authors considered serum 25(OH)D >80 nmol/L as sufficient vitamin D status and reported that 52.3% , 79.5 % and 83.9% of women achieved this level in the different supplemented groups, respectively at delivery. Serum 25(OH)D had a positive association with $1,25(OH)_2D$ during pregnancy, being significantly higher in the 2000 IU and 4000 IU groups compared to 400 IU. However, this dose-response in vitamin D metabolites did not result in significant differences in maternal serum mineral concentrations, urinary Ca excretion and PTH [10]. Cord serum 25(OH)D concentrations were significantly different among groups at delivery, 45.5±25.3 nmol/L, 57.0±24.5 and 66.3±25.8 nmol/L, respectively. If considering the IOM definition of vitamin D sufficiency (≥ 50 nmol/L), 39.7%, 58.2% and 78.6% of neonates were in this range, respectively. There were no significant differences among groups regarding birth weight and the authors suggested that for neonatal cord serum 25(OH)D to be ≥50 nmol/L, maternal should reach concentrations >80 nmol/L [10].

Most interventional human studies have not investigated the effect of vitamin D supplementation during pregnancy on fetal and neonatal bone health outcomes, but the biochemical outcomes were higher neonatal serum 25(OH)D, $1,25(OH)_2D$ and Ca and lower ALP concentrations in supplemented vs non-supplemented groups [18, 67, 68, 218, 220] . Prevalence of hypocalcemia and defects in ossification (larger

fontanelles) were lower in the vitamin D supplemented vs non-supplemented groups during pregnancy [18]. Some studies did not even observe any differences in neonatal serum $1,25(OH)_2D$, Ca [221] and PTH [68] in maternal vitamin D supplemented vs control groups, perhaps this may be due to the fact that their mothers were randomized to these diets only during the last trimester of pregnancy. The one study that has reported the effect of maternal vitamin D supplementation during pregnancy on fetal bone health has reported no difference in forearm BMC in neonates born from mothers receiving vitamin D supplementation vs the non-supplemented groups [66]. All of these studies were designed on the basis of too few dosages of vitamin D or low supplementation dosages that likely were not sufficient to raise maternal vitamin D status and consequently have not been shown to be beneficial to the fetus and newborn.

2.13. Vitamin D and anthropometry measures during pregnancy and at birth

2.13.1. Animal

No differences in maternal weight gain have been reported during pregnancy in vitamin D deplete compared to replete rats [206]. Neonatal birth weight was also reported to be similar in vitamin D deficient and replete rats [206]. Vitamin D deficient rat pups had normal body weights at birth, but they demonstrated growth failure by 1 wk of age despite normal plasma Ca levels. This may show that vitamin D is not essential for normal Ca homeostasis in the fetal rat, but has an important role in neonatal development [205, 222]. Alternatively, cessation of growth (weight) is a compensatory mechanism to preserve Ca homeostasis. Lower body weight, but no difference in body length was observed in fetal and newborn guinea pigs of vitamin D deficient vs replete sows [13, 79]. The available data are controversial regarding the effect of vitamin D status during pregnancy on neonatal body weight and length,

which might be due to differences in dosages of supplementation and experimental models. A vitamin D dose-response study during pregnancy would provide further information in this regard.

2.13.2. Human

2.13.2.1. Observational

The effect of maternal vitamin D status on neonatal body weight was first described in studies that observed an effect of season of birth on neonatal anthropometry outcomes. Australian infants born in October where the seasons are reversed compared to the northern hemisphere, were 25 g heavier and at their peak limb length as compared to those born in May [223]. However, studies remain inconsistent in terms of the effect of maternal vitamin D status on neonatal body weight and length at birth, showing a positive association [17, 224], no relationship [225] or a negative association [19].

Maternal serum 25(OH)D <28 nmol/L at 28-32 wk of gestation was associated with a shorter gestation length by 0.7 wk (95% CI -1.3 to -0.1) as well as shorter knee-heel length of 4.3 mm (95% CI -7.3 to -1.3) in Australian infants at birth. These associations remained significant after accounting for possible confounders such as smoking, maternal age and height. No other effects of maternal vitamin D deficiency were observed on birth weight, crown-heel length, head circumference but a decrease in mid-upper arm circumference 0.4 cm (-0.8 to -0.04) was observed suggesting that low maternal vitamin D status is associated with decreased neonatal limb lean tissue. This study suggests that maternal vitamin D deficiency was associated with reduced long bone growth and shorter GA [64].

2.13.2.2. Interventional

The observational studies above may be confounded by other factors including dietary intake of the mother as well as seasonal variations. However, the positive relationship between vitamin D status and growth is also observed in some randomized clinical trials (RCT). For example, higher pregnancy weight gain of 71g/d compared to 46g/d was observed in women who received 1000 IU/d vitamin D as compared to placebo during pregnancy [18, 226]. In the human neonate, maternal vitamin D supplementation vs non-supplementation during pregnancy resulted in higher birth weight ranging from 80-410 g in some studies [220, 226, 227] and 50 % lower prevalence of small for gestational age [18, 226] while did not affect birth weight in another study [18, 221, 225]. No effect of maternal vitamin D supplementation during pregnancy on linear growth was detected in infants at birth [18]. A study on adolescents during pregnancy showed that combined supplementation of vitamin D and Ca as compared to Ca alone resulted in higher neonatal body weight but similar length and head circumference [228].

2.14. Vitamin D and glucose homeostasis

As previously stated, low vitamin D status during pregnancy may also be related to other pregnancy complications such as GDM. Nevertheless, such investigations are limited to a few observational studies during pregnancy. Thus the role of vitamin D on glucose metabolism in non-pregnant states will also be discussed below in animal and human models for a better understanding of possible effects.

2.14.1. Animal

The possible role of vitamin D on glucose homeostasis was mainly investigated in Dr. Norman's laboratory in vitamin D deficient rat models [229, 230]. In vitamin D deficient rat models (*in vitro* and *in vivo*), both first and second phases

of pancreatic insulin secretion, which are rapid (stimulated by glucose) and slow (independent of glucose) respectively, were impaired [230, 231]. This role of vitamin D in regulating insulin secretion was independent of dietary energy and Ca intake or serum total Ca, Ca^{2+} and PO_4 concentrations [230, 231]. However, other researchers suggested that the impairment of insulin secretion in vitamin D deficient rats may be due to decreased food intake and to a lesser extent hypocalcemia [232].

Nevertheless, the effect of the injection of 25(OH)D and/or $1,25(OH)_2D$ was investigated in non-pregnant rats [37] and rabbits [38] that had undetectable 25(OH)D and less than 50 % of their baseline $1,25(OH)_2D$ concentrations. In such models, injection of $1,25(OH)_2D$ prior to OGTT increased glucose induced insulin secretion [37, 38] and improved glucose tolerance at 3 h post glucose load in rats [37]. In contrast to the rapid response observed in rats improved glucose tolerance was observed in rabbits only after the injection of 25(OH)D for 2 wk and not after the injection of $1,25(OH)_2D$ or infusion of Ca [38].

However, no interventional study has investigated the dose-response effect of oral vitamin D intake on glucose concentration and tolerance during pregnancy in an appropriate animal model. The guinea pig is a model which has been used for investigating glucose tolerance during pregnancy [56, 233] and has a peak of blood glucose concentrations around 40-60 min after oral glucose ingestion on an OGTT (1 g/kg body weight) during pregnancy which is similar to humans [56].

2.14.2. Human

2.14.2.1. Observational

The existing evidence on the beneficial role of vitamin D on type 2 diabetes is mainly derived through observation studies and post-hoc analyses which have not all considered the possible confounding factors; e.g. adiposity, physical activity and

ethnicity in this association [166]. The cross-sectional studies make it impossible to understand the cause or effect between vitamin D deficiency and diabetes, since serum 25(OH)D measurements are usually after the diagnosis of diabetes or impaired glucose tolerance at a single time-point. The outcomes of case-control studies mostly show that patients with type 2 diabetes have lower serum 25(OH)D as compared to healthy controls [166]. Circulating 25(OH)D has been negatively associated with fasting, 1 h and 2 h PG concentration from OGTT as well as glucose induced-insulin response in healthy men and women [234]. However, this negative association was only observed between vitamin D status and fasting glucose, but not glucose concentrations post glucose load in Caucasian healthy men and women from the Framingham Offspring study [235]. In the NHANES-III, which is a national US population study of multi-ethnic origin over than 20 y of age, lower risk was observed for diabetes (FPG > 7.0 nmol/L) in the highest (≥ 81.0 nmol/L) vs the lowest (≤ 43.9 nmol/L) serum 25(OH)D quartiles in non-Hispanic whites and African Americans, but not in non-Hispanic blacks. This study suggests that the relationship between 25(OH)D and glucose metabolism may differ among different ethnicities [236]. Population-based prospective studies in healthy Caucasian [237] and Finnish [238] men and women 40-69 y old, baseline serum 25(OH)D was inversely associated with the incidence of type 2 diabetes but this did not remain significant after adjustment for confounding factors such as baseline glucose, PTH, Ca concentrations, BMI, smoking, education, social class and physical activity. This highlights the importance of accounting for such confounders in investigating this association. However, the inverse association between baseline serum 25(OH)D and 2 h glucose on a 75-g OGTT remained significant after adjusting for possible confounders [237].

In the large cohort study of Women's Health in middle aged and older women, total intake ≥ 511 IU/d vs ≤ 159 IU/d of vitamin D was associated with lower risk of incidence of type 2 diabetes (2.7 vs 5.6%, respectively). However, this association was no longer significant after adjustment for Ca intake. It should be stated that only 15% of the population consumed vitamin D supplements [239]. In addition, in the Nurses' Health Study in which adult women were followed up for 20 y for the incidence of type 2 diabetes, there was no association between total vitamin D intake and type 2 diabetes. However, vitamin D supplement intake in the higher (≥ 400 IU/d) vs lower (≤ 100 IU/d) category was related to 13 % lower risk of type 2 diabetes. A combined daily intake of 800 IU vitamin D with 1200 mg Ca was associated with a 33% lower risk of type 2 diabetes as compared with an intake of 400 IU vitamin D with 600 mg Ca. This study concluded that vitamin D and Ca intake may both have a beneficial effect on type 2 diabetes, but the association for vitamin D and type 2 diabetes was attenuated after adjustment for Ca intake [240]. In addition, women who consumed < 1 compared to ≥ 3 serving per day of dairy food servings had 11% lower risk of type 2 diabetes, but this did not remain significant after adjustment for total vitamin D and Ca intake [240].

Observational human studies that have investigated the role of vitamin D in GDM have reported lower serum 25(OH)D concentration in GDM vs healthy subjects at mid-gestation [22-24, 26], but not later in pregnancy [27]. Studies investigating circulating 25(OH)D at early gestation have reported controversial results with two studies suggesting no difference [25, 241] and another showing lower concentrations in GDM vs healthy pregnant women [28]. A negative association has been observed between circulating 25(OH)D at early gestation with 1 h [28] and 2 h [25] glucose concentrations from the OGTT. Accordingly, negative relationships were observed

between 25(OH)D at mid and late gestation with fasting [26] and 30 min glucose concentrations [27] from the OGTT, respectively.

At mid-gestation, maternal 25(OH)D insufficiency (<50 nmol/L) was significantly higher in GDM (33%) as compared with controls (14%). After adjustment for confounding variables such as BMI, vitamin D deficiency was associated with a 2.66 higher (OR (95% CI): 2.66 (1.01–7.02)) risk of GDM [22]. Similarly, in a population with higher prevalence of low vitamin D status defined as plasma 25(OH)D <50 nmol/L at mid-gestation, 83.3% of GDM compared to 71.2% of the control group were had low vitamin D status [24]. Nonetheless, the odds of developing GDM in women with serum 25(OH)D <50 nmol/L at mid-gestation did not reach statistical significance (1.92, CI 0.89–4.17). However, the prevalence of GDM was significantly higher (44.2%) among pregnant women with 25(OH)D <12.5 nmol/L as compared to those with 25(OH)D >12.5 nmol/L (23.5%) in this population [26]. At such low concentrations of 25(OH)D, $1,25(OH)_2D$ is likely undetectable, suggesting a critical threshold where deficiency may impact upon pancreatic function. The difference among studies might be due to differences in populations, vitamin D status and other confounders such as adiposity, physical activity, family and self-history of diabetes that were not accounted in all studies.

2.14.2.2. Interventional

The available interventional human studies are of short duration and small sample size, using different metabolites as vitamin D supplements [166] and only one of these studies has been performed during pregnancy which will be discussed later. Non-pregnant women with hypovitaminosis D (circulating 25(OH)D concentration not specified) did not show any difference in AUC for glucose after receiving 2000 IU/d of dietary vitamin D, but an increase in AUC for insulin was observed post vs

pre supplementation, suggesting that vitamin D improves pancreatic β-cell response to glucose [242]. In another human trial, in Australasian men and women (non-pregnant) without diabetes, serum 25(OH)D concentration doubled (from~ 40 to 90 nmol/L) after two supplements of 100,000 IU D_3 were given to subjects 2 wks apart. However, such an increase in serum 25(OH)D concentration did not affect serum glucose, insulin and quantitative insulin-sensitivity check index (QUICKI) as assessed by 75 g-OGTT. These results suggests that correction of vitamin D status at least in short term (1 mo) did not affect glucose homeostasis in non-diabetic healthy patients [243].

The only clinical study investigating the effect of vitamin D supplementation on glucose tolerance during pregnancy was performed in women with established GDM. In these women, injection and oral consumption of Etalpha (alpha-calcidiol) at mid-gestation resulted in a negative association between FPG and $1,25(OH)_2D$ concentrations while glucose concentration post glucose load were unaffected [29]. No difference in Ca^{2+} and PTH concentrations were observed upon the injection or oral supplementation of alpha-calcidiol. This vitamin D analogue has less calcemic and phosphoric actions than $1,25(OH)_2D$ [30] and in contrast to cholecalciferol metabolites is not regulated by renal metabolism [31]. The inverse association between FPG and $1,25(OH)_2D$ concentrations could not be related to increase in insulin levels but is probably due to increase in insulin sensitivity by increasing cellular transport of glucose directly or by enhancing the effect of insulin [29].

To examine the interrelationships between vitamin D status, bone metabolism and glucose during pregnancy a suitable animal model is required. As mentioned previously, the guinea pig is appropriate for these purposes since it has changes in circulating OC and $1,25(OH)_2D$ comparable to human pregnancy. However, in order

to obtain measures of bone density and microarchitecture, anaesthetics such as isoflurane are often used. Nonetheless, there is evidence that isoflurane may influence biochemical outcomes related to bone, mineral and glucose which are commonly measured in bone studies, leading to inconsistency and lack of comparability among studies.

2.15. Effect of isoflurane on biomarkers of bone, mineral and glucose metabolism

The use of general anaesthesia is required to immobilize laboratory animals for bone density and microarchitecture assessment. Inhalation anaesthetics such as isoflurane are more controllable and result in rapid recovery as compared to injectable anaesthesics [57, 58]. The effect of isoflurane on the bone formation biomarker; OC has been investigated in a few studies. Some studies have shown a decrease in plasma OC 2 h after isoflurane-induced anaesthesia in young and adult horses [244] and postmenopausal women [245], but these studies involved the use of different intravenous anaesthetics along with isoflurane and accompanied invasive surgeries which may have confounded the results. To date, only one study has investigated the effect of isoflurane alone on serum OC concentrations which was performed on female adult cynomolgus monkeys and reported doubling serum OC concentrations 30 min after the induction of isoflurane as compared to prior to anaesthesia [59]. However, no study has simultaneously investigated the effect of isoflurane on biomarkers of bone formation, such as OC, and resorption, such as tDPD.

Recent evidence has linked bone to glucose metabolism via the bone formation biomarker, OC [54], therefore investigating the effect of isoflurane on glucose concentration seems reasonable in bone studies. However, there are

inconsistent results regarding the effect of isoflurane on glucose concentrations. Some studies suggest that isoflurane decreases blood glucose in neonatal mice [62] while others report its increase in adult males rats although this trend is not significant among females [57].

Plasma mineral concentrations that are also among the main outcomes in bone studies, may change upon isoflurane induced anaesthesia as demonstrated by a significant decrease in Ca^{2+} in adult cynomolgus monkeys [59], which may be a possible mechanism for increased OC concentration after exposure to isoflurane. Studies in neonate mice [60], rats [57] and adult rabbits [61] have been controversial in regards to the effect of isoflurane on plasma mineral. Further studies are required to clarify the effect of isoflurane exposure on bone and mineral biomarkers and glucose concentrations to reflect normal metabolism and improve comparability in research.

BRIDGE 1

From reviewing the literature, it is evident that low vitamin D status is prevalent among pregnant women worldwide, including Canada [8]. A decrease in circulating 25(OH)D results in normal or increased concentrations of the active metabolite, $1,25(OH)_2D$, but if severe vitamin D deficiency (25(OH)D< 25 nmol/L) evolves, $1,25(OH)_2D$ reaches low [11, 12] or undetectable concentrations [13]. Such low values may be of consequence since physiological $1,25(OH)_2D$ levels may improve glucose homeostasis by enhancing insulin synthesis, secretion and sensitivity [16] which may be challenged during pregnancy. In addition, low maternal transfer of 25(OH)D may adversely associate with neonatal anthropometry and APGAR outcomes [17-19].

The implications of low vitamin D status in GDM extend beyond insulin metabolism in the pancreas, such as its role in stimulation of OC, a protein secreted from osteoblasts [40]. Data are inconsistent regarding vitamin D status at early gestation in GDM vs healthy pregnant women [25, 28], but lower vitamin D status [22-24, 26] and higher OC concentration [52, 53] at mid-gestation have been associated with GDM [22-24, 26]. Nonetheless, no study has investigated both 25(OH)D and OC simultaneously in the development of GDM. Therefore, a nested case (GDM)-control (healthy) study, was designed to investigate 25(OH)D and OC concentrations simultaneously at early gestation prior to the onset of GDM and their changes from early to mid-gestation between healthy pregnant women and those with GDM, as well as their predictive role in the development of GDM. The other objective was to investigate 25(OH)D and OC concentrations simultaneously in the cord serum along with neonatal general health outcomes and placenta weight. The study is reported in Chapter 3.

CHAPTER 3. MANUSCRIPT 1

Osteocalcin but not vitamin D status is higher across pregnancy in Caucasian women with gestational diabetes mellitus

Negar Tabatabaei[1], Yves Giguère[2], Jean-Claude Forest[2], Celia J. Rodd[3], Richard Kremer[4], Hope A. Weiler[1].

Author affiliation:

[1]Dietetics & Human Nutrition, McGill University, Ste. Anne de Bellevue, QC, Canada

[2]CHU de Québec Research Center, and Faculty of Medicine, Université Laval, Québec, QC, Canada

[3]Pediatrics, Montreal Children's Hospital, Montreal, QC, Canada

[4]Medicine, Calcium Research Laboratory, McGill University, Montreal, QC, Canada

Corresponding author:

Dr. Hope Weiler, Tel.: 514-398-7905, Fax: 1 514 398 7739,

e-mail: hope.weiler@mcgill.ca

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3.1. Abstract

The objective was to investigate 25(OH)D and OC concentrations in maternal and cord serum of Caucasian pregnant women with GDM compared with controls and whether changes in concentration of these biomarkers can predict the development of GDM. This was a nested case-control study in 48 GDM and 48 control pregnant women (matched for age, season of conception, pre-pregnancy BMI and pregnancy length). Serum 25(OH)D was measured by chemiluminescence and OC by ELISA in mothers throughout pregnancy (11-13 wk, 24-28 wk and pre-delivery) and in the cord serum at delivery. Differences between groups were compared by mixed model repeated measures ANOVA. Predictors of development of GDM were explored using the generalized estimating equation model. Cord serum concentrations of 25(OH)D and OC plus anthropometry and APGAR scores were compared between groups. Serum 25(OH)D was not different (54.6±2.4 vs 54.5±2.9 nmol/L, $p>0.05$) while OC was higher (1.7±0.3 vs 1.4±0.1 nmol/L, $p<0.05$) in GDM vs controls at 11-13 wk, respectively. Both biomarkers increased with time across pregnancy. Neither serum 25(OH)D and OC at 11-13 wk nor the change in their concentrations from early to mid-gestation predicted the development of GDM. There were no significant differences in cord serum 25(OH)D, OC, anthropometry and APGAR scores in neonates of controls and cases. Vitamin D status is similar, but plasma OC is higher throughout pregnancy in Caucasian women with GDM vs controls but neither predicted GDM. Larger observational studies are required to explore the association between bone biomarkers and GDM, the mechanisms and implications.

Key Words: vitamin D, osteocalcin, gestational diabetes

3.2. Introduction

Gestational diabetes mellitus (GDM), a state of hyperglycemia due to decreased insulin sensitivity and pancreatic β-cell insulin response to glucose during pregnancy, has a worldwide prevalence of 1 to 10% and is associated with higher risk of adverse maternal and neonatal outcomes [21]. Recently, low vitamin D status, defined by circulating 25(OH)D concentration <50 nmol/L [1] or <75 nmol/L [2] has been reported among pregnant women worldwide [133] and is associated with GDM [22-24]. Circulating concentrations of the active metabolite, $1,25(OH)_2D$, is regulated by dietary and hormonal factors other than vitamin D [246], and may be normal or sometimes elevated when circulating 25(OH)D concentrations are low. If severe vitamin D deficiency evolves, $1,25(OH)_2D$ eventually becomes low due to limited substrate [11]. Such low values may be of consequence since observational studies suggest that $1,25(OH)_2D$ both directly and indirectly contributes to glucose homeostasis by enhancing insulin synthesis, secretion and sensitivity [16]. A protective effect of this nutrient has been suggested in non-pregnant insulin resistant states [247]. Of the seven pregnancy studies published to date [22-28], all observed lower serum 25(OH)D concentration in GDM vs healthy subjects at mid-gestation [22-24, 26], but not at early [25] or late pregnancy [27].

The implications of low vitamin D status in GDM extend beyond insulin metabolism in the pancreas and include alteration in gene expression of other proteins that are regulated by vitamin D through specific VDRE [39] such as the OC promoter. The secretion of OC from osteoblasts is under the control of many factors [248], including the stimulatory effect of $1,25(OH)_2D$ [40]. Its uncarboxylated form has recently been associated with glucose homeostasis by increasing β-cell proliferation, glucose tolerance and sensitivity [41]. Considering this connection

between the bone and endocrine metabolism, two studies have looked at serum total OC concentration at mid-gestation (24-28 wk) in GDM vs healthy pregnant women [52, 53]. In contrast to their expectations, serum OC concentration was higher (128-148 %) in GDM compared to control subjects, suggesting that this may be a compensatory mechanism to increase insulin secretion in women with GDM [52]. However, these studies were limited by lack of sampling early in pregnancy.

The objective of this nested case-control study was to examine vitamin D status and OC concentrations simultaneously at early gestation prior to the onset of GDM and the changes from early to mid-gestation in healthy pregnant women and those with GDM, as well as establish if either predicts the development of GDM. These biomarkers were also investigated during the last trimester to better understand the possible effect of GDM management. In addition, the same biomarkers in the cord serum were investigated along with general neonatal health outcomes and placenta weight in relation to maternal 25(OH)D concentration to evaluate if low maternal transfer of 25(OH)D adversely affected other pregnancy outcomes [17-19].

3.3. Materials and methods

This nested case-control study is derived from a prospective longitudinal cohort study, "*Pregnancy disorders and impact on child development and wellbeing: maternal, placental and fetal considerations*", of pregnant women recruited during their routine perinatal visit at Hôpital Saint-Francois d'Assise (HSFA) and Centre Hospitalier de l'Université Laval (CHUL) in Quebec City, Quebec, Canada. Inclusion criteria were healthy pregnant Caucasian women and those with GDM having singleton and term pregnancies with gestation duration of 37-42 wk. Exclusion criteria at recruitment for all participants included a history or new diagnosis of corticosteroid treated asthma, liver disease, renal disease, Crohn's disease, ulcerative

colitis, celiac disease, medication known to affect bone metabolism and history of type 1 or 2 diabetes. Screening was performed at 24-28 wk gestation and GDM was diagnosed in most cases (67.5 %) based on the CDA criteria [249]. Additional cases (32.5 %) were included on the basis of high plasma glucose according to the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) in high risk subjects (obesity and previous history of GDM) [249] and thereafter maintained on insulin treatment. The prevalence of GDM was 5.0 % in the total cohort. Cases and controls were matched based on age (± 4 y), pre-pregnancy BMI (±2 kg/m^2), season of conception (within same season) and gestation length (± 1wk). The study protocol was approved by the CHUQ ethics review board and all participants gave informed consent.

3.3.1. Data collection

3.3.1.1. Maternal data

The following information was gathered through a self-administered questionnaire: age, pre-pregnancy BMI (kg/m^2), reproductive information (season of conception, gestation length and parity) and socio-economic status (marital status, education level, annual income and occupation), smoking (non-smoker, ex-smoker, and smoker) and physical activity (frequency during the last three months of pregnancy). Nutrition information for assessment of dietary vitamin D intake was limited to the information provided by the total serving intake per day of dairy products (milk, yogurt and cheese) and type of prenatal supplements (Centrum Materna, Pregvit®) and their use (yes, no, unknown) during pregnancy. Pregnancy weight was measured at each trimester and recorded for weight gain assessment.

3.3.1.2. Neonatal data

Neonatal information on birth weight, recumbent length, head circumference and APGAR scores at 1 and 5 minutes was collected from the hospital chart. Weight for gestational age at birth (WAZ), length for gestational age at birth (LAZ) and head circumference for gestational age at birth (HCZ) z-scores were calculated according to the WHO child growth standards using WHO Anthro software (version 3.3.3, Geneva, Switzerland).

3.3.2. Blood collection

Blood samples were drawn from pregnant women at 11-13 wk (first trimester), 24-28 wk (second trimester) and at pre-delivery (end of third trimester) in non-fasting states between 0800 and 1600 h. A blood sample was also drawn from the cord at delivery to study neonatal biochemistry. Samples were centrifuged and separated within 2 h of collection and serum was kept at -80°C until biochemical analysis.

3.3.3. Biochemical analyses

Serum 25(OH)D was measured by a chemiluminescence assay using an auto analyzer (Liaison, Diasorin Inc., USA) with intra-assay variability <8.2 % and inter-assay variability <5.5 % for Biorad LIPC (Lypochech Immunoassay Plus Control) level 3 (lot#40230, 25(OH)D: 41.5-77.3 nmol/L). Serum total OC was measured by N-MID, Osteocalcin ELISA (Immunodiagnostic Systems Nordic A/S Herlev, Denmark) with intra-assay variability <7.0 % and inter-assay variability <13.8 %.

3.3.4. Sample size

The sample size estimate was based on detection of a difference of 15 nmol/L in serum 25(OH)D concentration at early pregnancy (16 wk gestation) between cases and controls [22], considering α =0.05, power=80 % and allocation ratio=1 was n=37

per group. In addition, in order to detect a difference of 0.5 nmol/L in serum OC concentration at mid-gestation between cases and controls [52], considering α=0.01, power=80 % and allocation ratio=1, a sample size of n=48 was estimated per group. Therefore, for this study a sample size of n=48 were used for each case and control group.

3.3.5. Statistical analyses

Any set of data deemed non-normal within groups or with unequal variances among groups were first transformed by use of logarithm, squaring, etc. Data are presented as mean ± SEM. Unpaired t-test and Chi-square were used for comparing continuous and categorical variables between the groups, respectively. Fisher's exact test was used for comparison of categorical variables (if n<5 per group). Differences in maternal and neonatal serum biomarkers and neonatal anthropometry and APGAR scores were tested in cases and controls by a MIXED model ANOVA (with repeated measures for maternal time-across pregnancy) for continuous variables after adjusting for confounders (pregnancy weight gain, history of GDM and smoking, servings of dairy intake, frequency of physical activity, prenatal supplement intake and SES). *Post hoc* testing for significance was conducted using a Tukey's test with a $p< 0.05$. Despite the fact that simple ANOVA model requires the independence assumption, this assumption can be dropped in the mixed model ANOVA [250]. In addition, the GEE (generalized estimating equation) logistic model is consistent with the conditional likelihood model for matched case-control studies [251]. The relationships between continuous variables were tested using Pearson correlations. Cord vs maternal biomarker concentrations were compared by paired t-test. Multiple logistic regression GEE model was used to estimate odds ratios (OR) and the 95% confidence interval (CI) for the binomial outcome of GDM. Manual step-wise reverse

logistic regression was performed such that all main and confounding variables were put into the model along with their possible biological interactions and if excluding a variable did not affect the significance of the outcome variable, it was omitted from the model and the model with the least AICs was chosen as the final model. All data were analyzed using SAS (version 9.2, SAS, Inc., Cary, NC., USA).

3.4. Results

3.4.1. Maternal characteristics

By design, characteristics by which control and GDM Caucasian pregnant women were matched were not different between controls and cases **(Table 3.1)**. While previous personal history of GDM was higher in the case group, family history of GDM was not different between groups. In addition, the GDM group had a higher proportion of smokers (current or past) compared to controls **(Table 3.1)**. Weight gain did not differ between controls and cases during pregnancy **(Table 3.1)**. Among women with GDM, 71% received insulin therapy during pregnancy. However, there was no significant difference in weight gain by insulin treatment, either during the last trimester (Insulin (n=34): 4.3±0.5 vs None (n=14): 3.9±0.7 kg, p=0.68) or the entire pregnancy (Insulin (n=34): 14.0±2.2 vs None (n=14): 15.0±2.1 kg, p=0.13).

3.4.2. Maternal biochemistry

There were no significant differences between groups in the prevalence of maternal serum 25(OH)D <50 and <75 nmol/L in control and GDM subjects (data not shown). Overall, the proportion of serum 25(OH)D concentration below 50 nmol/L was 41.7 % (n=40) at early-gestation, 27.1 % (n=26) at mid-gestation and 23.6 % (n=21) at late-gestation. The proportion of maternal serum with 25(OH)D concentration below 75 nmol/L was 84.4% (n=81) at early-gestation, 76.0 % (n=73) at mid-gestation and 73.5% (n=64) at late-gestation in all the population.

Maternal serum 25(OH)D concentration was not different between control and cases throughout pregnancy (p=0.80) **(Figure 3.1 (a))**. However, values increased from the end of the first trimester to the second and remained stable thereafter **(Figure 3.1 (a))** but the increase did not differ across groups (p=0.07). There were no significant differences in percent change of maternal serum 25(OH)D concentration from early to mid-gestation in GDM vs control pregnant women (case 20.5±5.2 and control 15.1±5.9 %, p=0.59). Additionally, serum 25(OH)D was not different in the insulin treated vs non-treated subjects with GDM (Insulin (n=34): 66.9±3.5 vs None (n=14): 61.0±4.2 nmol/L, p=0.29).

There was a significant difference in serum OC between controls and GDM cases with higher values in GDM at all time-points (p=0.006) **(Figure 3.1 (b))**. In addition, values increased across time in pregnancy **(Figure 3.1 (b))** but the increase was not different between controls and cases (p=0.45). There were no significant differences in percent change of maternal serum OC concentration from early to mid-gestation in controls and GDM cases (-3.8±3.2 and 3.2±5.9 % p=0.19, respectively). Insulin treatment for GDM did not result in different OC concentrations (Insulin (n=34): 2.9±0.4 vs None (n=14): 3.6±1.4 nmol/L, p=0.54). Maternal serum 25(OH)D and OC were not associated with smoking history, overall as well as within the control and GDM groups at any time across pregnancy (data not shown). There was no significant correlation between maternal serum 25(OH)D and OC, overall as well as within the control and GDM groups at any time across pregnancy (data not shown).

3.4.3. Predictors of GDM

Vitamin D status and OC concentrations at early pregnancy (11-13 wk gestation) and the percent change from early to mid-gestation did not predict the odds

for development of GDM, both in univariate (data not shown) and multivariate analyses **(Table 3.2)**. However, personal history of GDM and smoking were both significant risk factors for development of GDM **(Table 3.2).**

3.4.4. Neonatal characteristics

There were no statistically significant differences in neonatal anthropometric characteristics between case and control groups (**Table 3.3).** In addition, neonatal general health outcomes did not differ by maternal development of GDM (maternal health), sex or their interaction **(Table 3.3).** No significant differences in anthropometric measurements and placenta weight were detected between cases of GDM treated with insulin and GDM cases not treated with insulin (data not shown). Interestingly, there was a significantly higher APGAR score at 1 min at birth in neonates from GDM pregnant women who were treated with insulin compared to those not treated with insulin (Insulin (n=34): 8.9±0.1 vs None (n=14): 8.0±0.5, p=0.04) but this was not significant at 5 min (Insulin (n=34): 9.7±0.1 vs None (n=14): 9.4±0.3, p=0.17).

3.4.5. Neonatal biochemistry

There were no significant differences between groups in proportion of neonates with cord serum 25(OH)D <50 nmol/L (control n=9, 18.7% vs cases n=2, 4.2%, p>0.05) and <75 nmol/L (control n=20, 41.7% vs cases n=18, 37.5%, p>0.05)**.** Overall, 11.5 % and 39.6% had cord serum 25(OH)D concentrations below 50 nmol/L and 75 nmol/L, respectively at delivery.

There were no differences in cord serum 25(OH)D and OC from control and GDM pregnant women according to maternal development of GDM (maternal health), sex with no interactions **(Table 3.3).** Within the GDM subgroup, insulin treatment did not affect cord 25(OH)D concentrations (Insulin (n=34): 84.4±3.8, vs

None (n=14): 83.6±5.5 nmol/L, p=0.91). Neither was cord OC concentration different among insulin treated and non-treated subjects with GDM (Insulin (n=34): 10.5±1.0, vs None (n=14): 10.3±1.7 nmol/L, p=0.91).

There was no correlation between cord serum 25(OH)D and any neonatal anthropometric measurements and APGAR scores at 1 and 5 min (data not shown). No correlations were observed between cord serum 25(OH)D and OC, overall (r=0.01, p=0.91, n=95) as well as within the control (r=-0.03, p=0.81, n=47) and GDM groups (p=0.08, p=0.59, n=48).

3.4.6. Maternal and neonatal correlations and ratios

Maternal and cord values for serum 25(OH)D were positively correlated overall (r=0.82, p<0.0001) as well as within the control (r=0.91, p<0.0001) and GDM groups (r=0.71, p<0.0001); values were higher in cord samples overall (30.3 %, 95% CI [26.2-40.1 %], p<0.0001). No significant correlation was observed between maternal serum 25(OH)D and neonatal anthropometry and APGAR scores (data not shown). Cord serum OC concentrations were significantly higher than maternal pre-delivery serum OC concentrations overall (37.0 %, 95% CI [33.0-42.0 %], p<0.0001).

3.5. Discussion and conclusions

In this study, vitamin D status was not different while OC, a bone derived protein, was significantly elevated in GDM compared to healthy women throughout pregnancy. Importantly, adjustments were made for a comprehensive set of confounding variables that were not taken into account in previous studies of vitamin D status in GDM [22-28] such as pre-pregnancy BMI, history of smoking, family history of diabetes and physical activity. Nonetheless, early pregnancy and change in maternal vitamin D status and OC levels from early to mid-gestation was not associated with GDM at mid-gestation, despite the fact that the model in this study fit

well with the standard GDM risk factors, history of GDM [252] and smoking [253], respectively.

It is critical to underscore the importance of there being similar risk factors contributing to GDM and vitamin D deficiency (25(OH)D <50 nmol/L). Maternal pre-pregnancy BMI >27 kg/m^2 [254] is associated with increased risk for GDM. Similarly, pregnant women who are obese compared to those of healthy weight have higher prevalence of 25(OH)D <50 nmol/L (61 vs. 36%) at 4–22 wk gestation [36] . These studies suggest that increased sequestration of vitamin D in adipose tissue [123] may be a coincidental outcome with the development of GDM. Prior to this work, GDM was thought to be associated with plasma 25(OH)D <50 nmol/L at mid-gestation [22] in a non-Hispanic white population in the US, even after adjustment for pre-pregnancy BMI. More recently this association was also observed at values <73.5 nmol/L at early gestation [28], but matching or adjustment for pre-pregnancy BMI was not performed. Neither of these vitamin D status targets was associated with GDM in this exclusive Caucasian population that was matched for pre-pregnancy BMI. Nonetheless, the results from this study are similar to studies in early pregnancy in multi-ethnic pregnant women in the UK [25] and late gestation in pregnant Indian women [27] with adjustment for pre-pregnancy BMI or fat mass. These results that span pregnancy suggest that low vitamin D status and development of GDM may be coincidental events with potential for adverse pregnancy outcomes.

The increase in serum 25(OH)D observed in this study has been previously reported during pregnancy [9]. However, it cannot be confirmed to what extent this increase was related to sunlight exposure and/or exogenous sources of vitamin D. Whether redistribution of body fat during pregnancy [255] may have resulted in

release of vitamin D from fat stores [123] and therefore increase in circulating 25(OH)D concentration requires further investigation.

In addition to vitamin D status, the association between serum OC concentration at early pregnancy and the development of GDM was also explored which was higher in GDM vs controls throughout pregnancy. This is in agreement with findings at mid-gestation in Australian and Iranian pregnant women [52, 53], but novel since higher concentrations were even observed at early gestation prior to the diagnosis of GDM. Therefore, this may be an early compensatory mechanism independent of $1,25(OH)_2D$ to increase insulin secretion which cannot be accomplished because of pancreatic β-cell defects in women with GDM [52]. However, higher OC concentrations may also be a phenomenon completely independent of both $1,25(OH)_2D$ and glucose status. Even though insulin itself is a factor known to increase OC concentration [52], plasma OC was not different between insulin treated and untreated GDM patients. Unfortunately insulin levels were not measured in this study mainly because sampling was performed in a non-fasting state.

The consequences of higher serum OC concentrations in GDM vs healthy pregnant women have not been investigated. Recent evidence suggest that higher serum OC are accompanied by higher serum bone resorption biomarker concentrations such as serum C-terminal telopeptide, suggestive of increased bone turnover in GDM vs healthy pregnant women [53, 256]. In line with this, the only longitudinal study investigating the effect of GDM on changes in BMD during pregnancy reported 50 % higher bone loss from the calcis bone in GDM vs healthy pregnant women [257]. In addition, the increase in serum OC at late gestation is suggestive of increased osteoblast activity at late pregnancy and consistent with

previous reports [105]. Even though, the total circulating OC measured in this study is also known to have a role in regulation of insulin sensitivity in humans [46], the uncarboxylated form of OC is thought to be the main regulator of glucose homeostasis [41]. It would be useful to investigate this specific form of OC to further explain its role in gestational diabetes and the bone changes accompanying it.

In this study, the lack of difference in cord serum OC in GDM vs control pregnant women is in accordance with the absence of differences in neonatal vitamin D status and anthropometric measurements. Neither maternal nor cord serum 25(OH)D concentrations were correlated with any of the neonatal anthropometry measurements and APGAR scores which is similar to a previous report [258] but contrasts with studies investigating vitamin D intakes vs status [17] or in populations with lower vitamin D status [18, 19].

The prevalence of low maternal vitamin D status in this study (46°N in Quebec City) is similar to cohort studies conducted in Newfoundland (46º N) and Labrador (53 º N), Canada, with 44 % <50 nmol/L and 80% <75 nmol/L for first trimester serum 25(OH)D [259]. However, the prevalence of low vitamin D status in cord serum samples from the current study was much lower than those reported in this population with 39.2 % <50 nmol/L and 86.3 % <75 nmol/L [259]. In the present study, cord serum 25(OH)D was 30% higher compared to maternal pre-delivery concentrations; this was greater than expected since cord serum 25(OH)D concentrations are usually equal to or 80 % of maternal concentrations [101], although a higher gradient has been previously reported [260]. These results are more similar to cord serum 25(OH)D status in white pregnant women living in Pittsburgh (40ºN), US with 9.7 % <37.5 nmol/L and 66.1% <80 nmol/L [9]. In addition, it should be taken into consideration that pregnant women in the present study were

exclusively Caucasians and matched based on the main confounding variables and therefore may not be comparable to the stated cohorts in terms of prevalence of low vitamin D status.

This study is not without limitations. Its case-control design and possible uncontrolled confounding factors should be considered when interpreting the results. For example, the surprisingly higher rate (62.5 vs 25%) of smoking history in GDM vs controls may be a result of differences in socio economic status between groups. Some researchers speculate that smoking may reduce circulating 25(OH)D concentrations [261] but smoking did not affect 25(OH)D concentrations in this study which is similar to a recent report [262]. In addition, it is important to consider that summer was the season of conception for the majority of the total population (44 %) in this study which may have affected the change in circulating 25(OH)D with time across pregnancy. The prevalence of insulin treatment in women with GDM (71 %) was similar to that observed in the total GDM population (67 %) in this study, but almost double that of the general GDM population [263], suggesting a possibility of selection bias. Plasma $1,25(OH)_2D$ was not measured and may be helpful to further explain the association between OC and glucose metabolism. Measurement of other bone formation biomarkers such as procollagen type 1 N-terminal propeptide (P1NP) and bone resorption biomarkers such as C-telopeptide would also help explain changes in bone metabolism in GDM vs healthy pregnant women.

Overall, the results suggest that vitamin D status is similar while OC, a bone derived protein, is significantly higher in GDM vs healthy pregnant women throughout pregnancy. However, maternal vitamin D status and OC in early-gestation and changes early to mid-gestation did not predict the development of GDM. Larger observational studies are required to further explore the association between bone

biomarkers and the risk of development of GDM, possible mechanisms involved and bone health consequences associated with it. A randomized controlled study aimed at achieving normal 25(OH)D concentrations in a vitamin D supplemented group will be needed to clarify the effect of vitamin D in pregnancy and GDM.

3.6. Acknowledgements

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NT contributed to the design of this study, performed biochemical assessments, interpretation of data, statistical analyses and article writing. YG who is a clinical research scholar from the Fonds de la Recherche du Québec–Santé (FRQ-S) performed a critical review of the manuscript. JCF is in charge of the large prospective cohort study. With YG, he designed the large prospective study and they were responsible for clinical recruitment. CR reviewed the manuscript and provided feedback and consultation on statistical analyses. RK reviewed the manuscript and provided feedback on interpretation of the results. HW contributed to the design of the large prospective study as well as the original thought and design of this nested case-control study and undertook supervision of the biomarker measures and critical review of the manuscript.

3.7. Figure legend

Figure 3.1. Maternal serum a) 25(OH)D and b) osteocalcin concentrations in healthy control and GDM Caucasian pregnant women during pregnancy. Values for serum 25(OH)D across time-points are shown in box plots where the upper and lower quartiles, and the median is represented by the horizontal line within each box and the minimum and maximum values are depicted by the vertical lines. Values for serum osteocalcin are mean±SEM analyzed using mixed model ANOVA with fixed effect of maternal health, time across pregnancy and maternal health*time across pregnancy interaction. Adjusted for pregnancy weight gain, history of GDM and smoking, servings of dairy intake, frequency of physical activity, prenatal supplement intake and SES. Statistical significance, $p<0.05$. Significant effect of maternal health is represented by (a, b, ...) and significant effect of time across pregnancy is presented by (A, B,..). Sample sizes are in parentheses above each data-set.

Table 3.1. Characteristics of healthy (control) and GDM Caucasian pregnant women.

Maternal Characteristics	Control	n	GDM	n	All	n	p-value
^Age (y)	30.0 ± 0.6	48	30.8 ± 0.7	48	30.4 ±0.5	96	-
^Pre-pregnancy BMI (kg/m^2)							
18.0-24.99	35.4%	17	35.4%	17	35.4%	34	
25.0-29.99	25.0%	12	27.1%	13	26.0%	25	-
30.0-35.0	22.9%	11	20.8%	10	21.9%	21	
>35	16.7%	8	16.7%	8	16.7%	16	
^Season of conception							
Fall	16.7%	8	12.5%	6	14.6%	14	-
Winter	12.5%	6	14.6%	7	13.5%	13	
Spring	27.1%	13	27.1%	13	27.1%	26	
Summer	43.7%	21	45.8%	22	44.8%	43	
Weight gain (kg)							
Overall	13.0 ± 1.7	12	14.4 ± 1.5	24	13.93 ± 1.17	36	0.57
Early gestation to pre-delivery	11.1 ±0.7	43	10.3 ± 0.7	45	10.67 ± 0.47	88	0.41
Parity							
≤1	81.2%	39	87.5%	42	84.4%	81	0.40
≥2	16.7%	8	12.5%	6	14.6%	14	
Unknown	2.1%	1	0.0%	0	1.0%	1	
Marital Status							
Married	20.8%	10	16.7%	8	18.8%	18	0.46
Unmarried	75.0%	36	83.3%	40	79.1%	76	
Unknown	4.2%	2	0.0%	0	2.1%	2	
Education							
≤secondary	29.2%	14	39.6%	19	34.4%	33	0.28
≥college	70.8%	34	60.4%	29	65.6%	63	
Family income							
<$60,000	31.3%	15	50.0%	24	40.6%	39	0.09
>$60,000	62.5%	30	45.8%	22	54.2%	52	
Unknown	6.2%	3	4.2%	2	5.2%	5	
Maternal history of GDM							
Yes	2.1%	1	16.7%	8	9.4%	9	0.002
No	72.9%	35	79.1%	38	76.0%	73	
Unknown	25.0%	12	4.2%	2	14.6%	14	

Family History of GDM							
Yes	62.6%	30	75.0%	36	68.7%	66	0.24
No	31.2%	15	16.7%	8	24.0%	23	
Unknown	6.2%	3	8.3%	4	7.3%	7	
Prenatal supplement intake							
Yes	87.5%	42	91.7%	44	89.6%	86	0.36
Materna	50.0%	21	68.2%	30	59.3%	51	
Pregvit®	42.8%	18	25.0%	11	33.7%	29	
Both	7.2%	3	6.8%	3	7.0%	6	
No	8.3%	4	2.1%	1	5.2%	5	
Unknown	4.2%	2	6.2%	3	5.2%	5	
Dairy product intake (serving/d)							
<1	6.3%	3	8.4%	4	7.4%	7	0.37
1-2.99	16.7%	8	14.6%	7	15.6%	15	
3-4.99	43.7%	21	58.3%	28	51.0%	49	
≥5	33.3%	16	18.7%	9	26.0%	25	
Physical activity (30 min/wk)							
0	12.6%	6	14.6%	7	13.5%	13	0.36
<1	18.7%	9	20.8%	10	19.8%	19	
1	35.4%	17	18.7%	9	27.1%	26	
2-3	27.1%	13	31.3%	15	29.2%	28	
≥4	6.2%	3	14.6%	7	10.4%	10	
Smoking history							
Yes	25.0%	12	62.5%	30	43.7%	42	0.001
No	75.0%	36	37.5%	18	56.3%	54	

Values are mean±SEM (standard error of mean) for continuous variables and proportion for categorical variables. Unpaired-t-test and chi-square were used for comparing continuous and categorical variables, respectively. Statistical significance, $p<0.05$. ˉ P-values were no calculated for selected matched variables. ^Case and controls were matched for selected variables.

Table 3.2. Multivariate odds ratios (OR) for development of GDM according to maternal serum 25(OH)D and osteocalcin concentrations at early-gestation and their percent change from early to mid-gestation.

Maternal variables	Odds of developing GDM		
	OR	95% CI	p-value
Serum 25(OH)D			
Early-gestation (per 10 nmol/L)	0.96	0.73-1.26	0.77
Early to mid-gestation (per % change)	1.00	0.99-1.02	0.37
Serum osteocalcin			
Early-gestation (per 1 nmol/L)	2.00	0.79-5.03	0.14
Early to mid-gestation (per % change)	1.01	0.99-1.03	0.18
Personal history of GDM	3.90	1.71-6.09	<0.001
Current or past smoking history	1.84	1.07-2.91	0.001

GEE logistic regression model, Statistical significance, $p<0.05$, n=48.

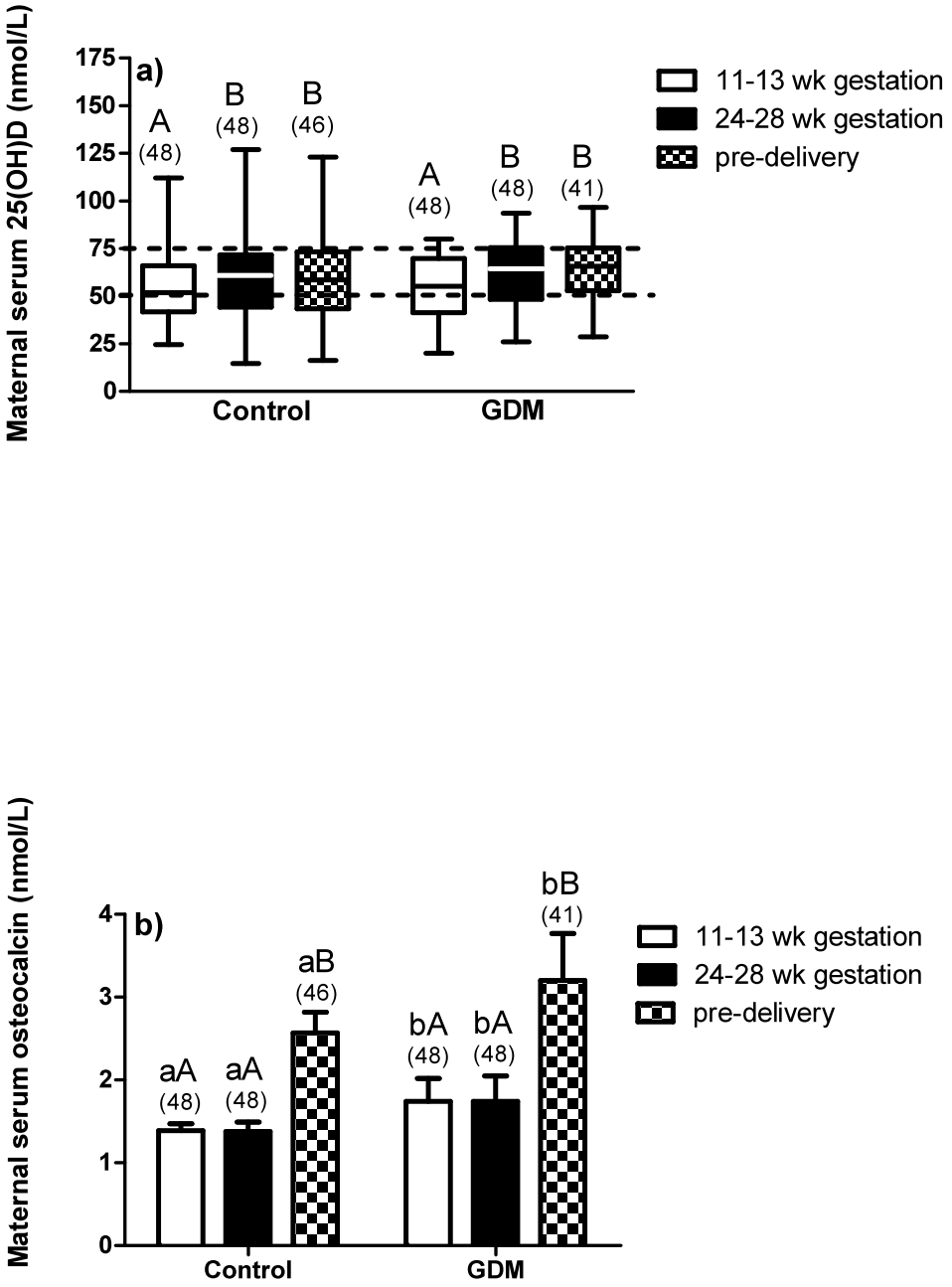
Table 3.3. Neonatal characteristics, general health outcomes and cord serum biomarker concentrations of control and GDM pregnant women.

Neonatal Outcomes	Control	n	GDM	n	All	n	Mixed model ANOVA; p-value		
							Maternal health	Sex	Maternal health* Sex
#Boy (%)	52.1%	25	50.0 %	24	51.0%	49	0.84	-	-
#Delivery Type									
Vaginal	83.4 %	40	77.0 %	37	80.2%	77	0.44	-	-
C-section	16.6 %	8	23.0 %	11	19.8%	19			
^Gestation age (wk)	38.8±0.2	48	38.8±0.1	48	38.8±0.1	96	-	-	-
Weight (g)	3539±80	48	3569±73	48	3554±54	96	0.70	0.29	0.98
WAZ	0.5±0.7	48	0.6±0.1	48	0.5±0.1	96	0.69	0.84	0.94
Length (cm)	51.3±0.4	37	50.9±0.5	37	51.1±0.3	74	0.38	0.16	0.27
LAZ	0.86±0.20	37	0.82±0.21	36	0.84±0.15	73	0.49	0.77	0.94
Head circumference (cm)	34.5±0.2	37	34.3±0.3	37	34.4±0.2	74	0.84	0.36	0.52
HCZ	0.19±0.19	37	0.02±0.26	37	0.11±0.16	74	0.84	0.99	0.52
Placental weight (g)	507.1±15.9	46	538.6±18.6	47	523.0±12.3	93	0.75	0.83	0.36
APGAR score (1 min)	9[6-10]	48	9[6-10]	48	9[6-10]	96	0.22	0.63	0.74
APGAR score (5 min)	10[7-10]	48	10[6-10]	48	10[6-10]	96	0.26	0.40	0.35
Cord serum 25(OH)D (nmol/L)	80.0±4.2	48	84.1±3.1	48	82.1±2.6	96	0.39	0.21	0.25
Cord serum osteocalcin (nmol/L)	10.9±0.8	47	10.4±0.9	48	10.7±0.6	95	0.52	0.10	0.12

Values are mean±SEM for continuous variables and proportion for categorical variables.

Categorical variables are compared by chi-square. #Unadjusted statistics. ^Case and controls were matched for the selected variable. Mixed model ANOVA with fixed effect of maternal health, sex and maternal health*sex interaction. Adjusted for pregnancy weight gain, history of GDM and smoking, servings of dairy intake, frequency of physical activity, prenatal supplement intake and SES (socioeconomic status). Statistical significance, $p<0.05$. WAZ=weight for gestational age at birth z-score, LAZ=length for gestational age at birth z-score, HCZ=head circumference for gestational age at birth z-scores

Figure 3. 1.


a)
Maternal serum 25(OH)D (nmol/L)
175
150
125
100
75
50
25
0
A
(48)
B
(48)
B
(46)
A
(48)
B
(48)
B
(41)
Control
GDM
11-13 wk gestation
24-28 wk gestation
pre-delivery
b)
Maternal serum osteocalcin (nmol/L)
4
3
2
1
0
aA
(48)
aA
(48)
aB
(46)
bA
(48)
bA
(48)
bB
(41)
Control
GDM
11-13 wk gestation
24-28 wk gestation
pre-delivery

BRIDGE 2

In Chapter 3, it was shown that vitamin D status was not different while OC was significantly elevated in GDM vs healthy women throughout pregnancy, but neither was associated with the development of GDM. This may be an early compensatory mechanism independent of $1,25(OH)_2D$ to increase insulin secretion which cannot be accomplished because of pancreatic β-cell defects in women with GDM. However, this study had a cross-sectional design with possible selection bias and uncontrolled confounding factors; therefore for better control of confounders, before initiating a randomized clinical trial of vitamin D dose-response in human pregnancy, an appropriate animal model is required for such research. The guinea pig is a model which has been used for investigating glucose tolerance during pregnancy [56]. Nonetheless, since there seems to be a cross-talk between the bone and glucose metabolism via the bone formation biomarker, OC [54], these studies usually involve bone scans which require the use of general anaesthesia such as isoflurane to immobilize laboratory animals for bone density assessment. From reviewing the literature, there is evidence that isoflurane may influence biochemical outcomes related to bone, such as OC [244, 245], mineral [57, 59-61] and glucose [57, 62] which are commonly measured in bone studies, leading to lack of comparability among studies when measurements are performed after exposure to anaesthesia. Therefore the objective was to investigate the effect of isoflurane on bone metabolism biomarkers across maturation and on blood minerals and glucose at maturity in the guinea pig. The study is presented in Chapter 4.

CHAPTER 4. MANUSCRIPT 2

Osteocalin but not deoxypyridinoline increases in response to isoflurane induced anaesthesia in young female guinea pigs

Negar Tabatabaei[1], Celia Rodd[2], Richard Kremer[3], Hope Weiler[1]

Author affiliation:

[1]Dietetics & Human Nutrition, McGill University, Ste. Anne de Bellevue, QC, Canada

[2]Pediatrics, Montreal Children's Hospital, Montreal, QC, Canada

[3]Medicine, Calcium Research Laboratory, McGill University, Montreal, QC, Canada

Corresponding author:

Dr. Hope Weiler, Tel.: 514-398-7905, Fax: 1 514 398 7739,

e-mail: hope.weiler@mcgill.ca

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4.1. Abstract

The effect of the inhaled anaesthetic, isoflurane was investigated on bone biomarkers, during maturation and on minerals and glucose postpartum. Female guinea pigs (n=10) were anaesthetized during maturation (5 and 9 wk) and postpartum (26 wk of age) with isoflurane during whole body and regional dual-energy x-ray absorptiometry scanning. Blood collection was performed at all ages, before and after anaesthesia for measurement of plasma osteocalcin (OC) and total deoxypyridinoline (tDPD). At 26 wk measurement also included: blood ionized calcium (Ca^{2+}), Na^{+}, Cl^{-} and glucose; plus plasma total Ca, inorganic phosphate (PO_4), total alkaline phosphatase (tALP) and albumin. Plasma OC concentration almost doubled after exposure to isoflurane at 5 wk (30.1± 5.0 to 57.9±11.2 nmol/L, $p<0.001$) and at 9 wk (29.1±7.5 to 62.9±15.9 nmol/L, $p<0.001$), but did not change postpartum (3.7 ± 3.3 to 4.3 ± 3.9 nmol/L, $p=0.88$). There was no effect of isoflurane exposure on plasma tDPD at any age, but the ratio of OC/tDPD almost doubled after isoflurane exposure at 5 wk and at 9 wk but not at 26 wk. Blood ionized Ca^{2+}, Na^{+} and plasma total Ca^{2+} did not change, whereas plasma albumin decreased and PO_4 and Cl^{-} increased upon exposure to isoflurane. Isoflurane modestly decreased tALP (43.2 ± 6.6 to 40.2 ± 5.9 IU/L, $p=0.01$) and increased glucose (7.5±0.6 to 10.9±1.7 mmol/L, $p<0.0001$) postpartum. Isoflurane increases OC during rapid growth, but not following pregnancy whereas it increased glucose postpartum. Measurements prior to anaesthesia are recommended to reflect normal metabolism and improve comparability in research.

Key words: isoflurane, osteocalcin, deoxypyridinoline, , guinea pig

4.2. Introduction

The use of general anaesthesia is required to immobilize laboratory animals for bone density and microarchitecture assessments. Inhalation anaesthetics such as isoflurane are more controllable and result in rapid recovery as compared to injectable anaesthetics [57, 58]. However, there is evidence that isoflurane may influence biochemical outcomes related to bone, mineral and glucose metabolism which are commonly measured in experimental studies relating to human health, leading to inconsistency and lack of comparability among studies and species.

The effect of isoflurane on the bone formation biomarker osteocalcin (OC) has been investigated in a few studies. Some studies have shown a decrease in plasma OC 2 h after isoflurane induced anaesthesia in young and adult horses [244] and postmenopausal women [245]. However, these studies involved the use of different intravenous anaesthetics along with isoflurane and accompanied invasive surgeries which may have confounded the results. To date, only one study has investigated the effect of isoflurane alone on serum OC concentrations which was performed on adult female cynomolgus monkeys and reported a 2-fold increase in serum OC concentrations 30 min after exposure to isoflurane possibly confounded by a reduction in ionized Ca^{2+} [59]. No study has simultaneously investigated the effect of isoflurane on both biomarkers of bone formation, such as OC, and bone resorption, such as total deoxypyridinoline (tDPD).

Recent evidence has linked bone to glucose metabolism via the bone formation biomarker, OC [54]. Therefore, studies investigating OC may simultaneously explore glucose concentration. However, both of these biomarkers

may be affected by isoflurane which will result in misinterpretation on their association if measurements are performed after exposure to isoflorane. Similar to OC, there are inconsistent results regarding the effect of isoflurane on glucose concentrations. Some studies suggest that isoflurane decreases blood glucose in neonatal mice [62], while others report its increase in adult male rats although this trend is not significant among females [57].

These studies demonstrate that the circulating biomarkers of bone, mineral and glucose metabolism may respond differently to isoflurane depending on species, exposure time, age and sex and no study has looked at the effect of isoflurane anaesthesia on all these biomarkers simultaneously in a controlled manner. The main objective of this study was to investigate the effect of isoflurane on bone metabolism biomarkers during maturation (5 and 9 wk) and postpartum (26 wk of age; as a part of a pregnancy study) in the pigmented guinea pig. The guinea pig was chosen since it is a model used in studies on maternal-fetal bone metabolism [13, 79] and is similar to humans in terms of Ca (g/kg) content in the bone [202]. Whereas, other small rodent models (rat and mice) have larger litter size and chemically and physiologically immature bones at birth [187]. In addition, the guinea pig model has also be used in studies of glucose metabolism [56, 233]. In this study, blood minerals and glucose were also measured along with the bone metabolism biomarker 2 d following pregnancy (26 wk) to explain possible mechanisms involved in changes in bone biomarkers and to investigate whether glucose concentrations are affected by the anaesthetic.

4.3. Methods and Material

4.3.1. Animal husbandry

Ethical approval was obtained from the McGill University Animal Care Committee. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care [264]. Female (4 week (wk) old) pigmented guinea pigs (n=10) were purchased from Elm Hill Laboratories (MA, US) and housed singly with room temperature maintained at 21°C, lighting cycle from 0600 to 1800 h.

4.3.2. Diet

The guinea pigs were fed *ad libitum* a purified standard diet in pellet form, purchased from Tekland Global High Fiber Guinea Pig Diet (#2041, Harlan laboratories). The animals were maintained on this diet from 5 to 16 wk. Thereafter, they were randomized to a similar diet until 26 wk which was 2 d post-partum as a part of a larger study involving reproduction (unpublished). Body weight was measured weekly.

4.3.3. Blood sampling and dual-energy x-ray absorptiometry (DXA)

At 5, 9 and 26 wk of age, guinea pigs were kept at a non-fed state for 3 h from 0600 to 0900 h followed by blood sampling from the saphenous vein prior to exposure to isoflurane. Anaesthesia was induced at 5% in an induction chamber followed by maintenance at 2% which was delivered using a cone mask; this lasted 20-25 min. Since there is a circadian variation in OC concentrations [265], all blood sampling was performed consistently from 0600 to 0900 h. In addition, keeping animals in a non-fed state is essential when studying bone biomarkers and glucose concentrations, given that both OC [266] and glucose decrease in the

fasting vs non-fasted state. During the anaesthetized period, whole body and regional (lumbar spine (L1-L4), tibia and femur) scans were performed on the animals using DXA small animal software (Hologic 4500, QDR Version 12.3, Hologic, Bedford, Mass., USA) to obtain measures of body composition, bone mineral content (BMC) and density (BMD). All CV% were < 0.5% for DXA QC on the lumbar spine phantom. All DXA inter-assay and intra-assay variability were < 10% and < 5 % (typically < 2%) for the duration of the study. Nose-rump length was measured using a measuring tape to the nearest 0.1 cm; performed under anesthesia. Afterwards, post-anaesthetic blood samples were collected by saphenous vein at 5 and 9 wk and by cardiac puncture at 26 wk of age. Samples were collected into lithium heparinized tubes for biochemistry or capillary tubes for blood gas. The latter were collected only at 26 wk. All blood samples were kept on ice and centrifuged at 2000 g for 30 min at 4°C. Plasma was collected and stored at -80 °C until analyses. Since one of the criticisms in evaluating the effect of anaesthetics on plasma biomarkers has been the effect of repeated blood sampling and the change in plasma dilution [267], normal saline injections equivalent to the volume of blood sample collected were performed after each saphenous vein sample collection.

4.3.4. Biochemistry

Plasma concentrations of total Ca, PO_4 albumin and tALP were measured using an autoanalzyer (Beckman DxC600 California, USA) at the Montreal Children's Hospital, CAN. All CV% were < 3.1% and typically 1 to 2 %. Osteocalcin and tDPD were measured using ELISA kits (Metra Osteocalcin, Quidel Corporation, San Diego, CA., USA) with known cross-reactivity in guinea

pig plasma. All samples for each guinea pig including all ages (5, 9 and 26 wk) and exposure to anaesthetic (before and after) were measured on the same ELISA kit to exclude the inter-assay variability for each subject. All intra-assay CV% for variability were < 7.8 % and < 6.5 for OC and tDPD, respectively. Blood Ca^{2+}, Na^{+}, Cl^{-} and glucose were measured with an ABL 825 series blood gas analyzer (Radiometer America, Copenhagen, Denmark). All CVs were < 4.2% for all assays and typically 1 to 2 %.

4.3.5. Statistical analysis

Normality of data was assessed using the Kolmogorov-Smirnov test and equal variances examined using the Levene test. Data that failed assumptions for normality were log transformed prior to testing. Data were analyzed for main effects of isoflurane exposure and age and their interaction on bone biomarkers and for the effect of age only on body weight, length, DXA measurements using a mixed model repeated measured ANOVA (SAS Institute, 9.2). Post hoc testing was conducted when interactions were significant using Tukey-Kramer multiple comparison test. Data were analysed by paired-t-test for the effect of isoflurane exposure on all biomarkers measured at 26 wk of age. Data were expressed as mean ± SD. Differences were considered significant if $p<0.05$. The graphs were prepared by Graphpad Prism version 5.01.

4.3.6. Sample size

The sample size required for this study was based on the primary outcome of circulating OC assessed before and after exposure to isoflurane in monkeys [59]. For a 100% increase in plasma OC a sample size of n=7 was calculated with

a β= 0.95 and α= 0.01. To account for potentially smaller changes in the growing guinea pig (i.e. high values at 5 wk), a sample of n=10 was used.

4.4. Results

4.4.1. Anthropometry and body composition characteristics (5, 9 and 26 wk)

All anthropometric measures increased significantly with age ($p<0.0001$) except for fat (%). Whole body BMC and BMD increased significantly across all ages whereas bone area was only different between 5 vs 26 wk of age ($p<0.001$). All regional (lumbar spine (L1-L4), tibia and femur) area, BMC and BMD increased significantly with age ($p<0.001$) **(Table 4.1).**

4.4.2. Plasma biomarkers of bone metabolism (5, 9 and 26 wk)

Overall, plasma OC was not different at 5 and 9 wk of age but was lower 2 d postpartum (from 44.0±3.7 at 5 wk and 46.0±4.7 at 9 wk to 4.0±0.8 at 26 wk, nmol/L) ($p<0.0001$). At 5 and 9 wk, when OC concentrations were highest; plasma OC concentrations almost doubled after exposure to isoflurane ($p<0.0001$) **(Figure 4.1 (a))**. Plasma tDPD was only higher postpartum at 26 wk vs during development at 5 wk of age (12.4±1.1 vs 9.4±0.6 nmol/L, respectively, $p=0.05$), not affected by exposure to isoflurane ($p=0.33$) and without statistical interactions between exposure to isoflurane and time of measurement ($p=0.12$) **(Figure 4.1 (b))**. Plasma OC/tDPD also decreased postpartum compared to the younger non-pregnant measures (0.3±0.1 at 26 wk vs 5.0±0.5 at 5 wk and 4.5±0.6 at 9 wk, molar ratio, $p<0.0001$). This variable almost doubled after exposure to isoflurane (from 3.7 to 6.2 at 5 wk and from 2.6 to 6.3 at 9 wk, molar ratio, $p<0.0001$) only when the values were high and representative of non-pregnant states ($p<0.007$) **(Figure 4.1 (c))**.

4.4.3. Biomarkers at 26 wk

Blood glucose concentrations significantly increased after exposure to isoflurane at 26 wk ($p<0.0001$). There was no difference in total Ca, Ca^{2+} and Na^{+} before and after isoflurane exposure. Plasma PO_4 ($p=0.003$) and blood Cl^{-} ($p=0.007$) concentrations significantly increased with isoflurane; whereas plasma albumin ($p<0.001$) and tALP ($p=0.01$) significantly decreased after isoflurane exposure **(Table 4.2)**.

4.5. Discussion and conclusions

The administration of isoflurane for 20-25 min results in an almost doubling of circulating OC concentrations during rapid growth, but not at 2 d postpartum when values are low. Isoflurane, however, induces an increase in blood glucose concentrations, at least in the early postpartum period. Furthermore, significant changes in electrolytes were also observed. Consequently, measurements of these biomarkers prior to anaesthesia are recommended to better assess metabolic changes and improve consistency and comparability in research protocols.

The significant increase in plasma OC after exposure to isoflurane was observed in young (5 and 9 wk) guinea pigs which is consistent with results obtained in adult cynomolgus monkeys [59]. However, the effect of isoflurane on plasma OC disappeared in adult (26 wk) guinea pigs at 2 d postpartum which might be due to the already low concentrations [268]. The latter is a well known phenomenon as a result of decrease in bone formation with age [269] or following reproduction [268]. However, there is evidence that the growth plates have not yet been closed at this latter time-point in the guinea pig and it is still growing [270].

The lack of effect of isoflurane on OC at 26 wk (postpartum) might also be species specific since isoflurane induced significant changes on OC levels in mature cynomolgus monkeys when high OC concentrations persist at maturity [59].

In contrast to our marker of osteoblast activity, there were no significant effects of isoflurane exposure on osteoclast activity as evidenced by no changes in tDPD at any age. The trend in increase in tDPD upon exposure to isoflurane at 5 and 26 wk might be a reflection of our small sample size, requiring confirmation in subsequent studies. However, overall this bone resportion biomarker increased at 9 and 26 wk of age compared to the 5 wk baseline. The ratio of OC/tDPD, which reflects bone turnover, decreased early postpartum at 26 wk vs values obtained in non-pregnant states at 5 and 9 wk suggesting that bone resorption predominates postpartum. The isoflurane induced changes on plasma OC/tDPD were similar to OC. In general, the decrease in plasma OC, OC/tDPD and increase in tDPD at 26 wk vs 5 wk may be due to post-partum hormonal changes that accompany the initiation of lactation and consequently increases bone resorption during this physiological state [268].

In addition to age and isoflurane-induced-anaesthesia, stress might be another factor modulating OC concentration. Immobilization under a conscious state increases circulating OC to 200% of baseline values after 20 min in young male rats [271]. This type of acute stress is more similar to the anaesthetic induced stress that starts with the animal's physical effort to escape from the chamber and is followed by its immobilization [271]. Increases in OC are most likely due to the release of OC from the surface of bone, rather than increases in

synthesis by osteoblasts, in response to stress hormones such as corticosterone [271]. Significant increases in both serum cortisol and corticosteroid have been reported 1 min to 2 h after exposure to isoflurane in adult rabbits [272]. This suggests that plasma corticosteroid levels may have increased in the guinea pig model after the induction of isoflurane anaesthesia as a result of stress, isoflurane or both. Studies suggesting that *in vivo* administration of glucocorticoids may decrease OC and therefore bone formation have investigated long-term effects of administration of glucocorticoids [273-275]. Considering that the short-term effect of corticosteroids on OC concentrations [272] are in the opposite direction of long-term effects, the increase in corticosteroids induced by the stress caused through exposure to isoflurane may be a possible mechanism explaining the increase in OC, but requires further investigation.

In contrast to adult mature monkeys, guinea pigs at maturity and postpartum did not experience any decrease in blood ionized Ca^{2+} following isoflurane administration in this study. In the monkeys it was proposed that the increase in OC concentration after exposure to isoflurane was, at least in part, the consequence of an increase in plasma parathyroid hormone (PTH) [59]. The lack of change in Na^{+} in the adult guinea pig were similar to previous reports after exposure to isoflurane in monkeys [59], rabbits [61] and rats [58]. The significant increase in plasma PO_4 and blood Cl^{-} is the same as results in adult rabbits [9] that had a similar period of exposure to the anaesthetic. The mechanism explaining the increase in these minerals may be due to the transient effects of isoflurane on glomerular and tubular functions leading to their decreased excretion [61]. The decrease in plasma albumin and tALP is also consistent with results after

exposure to isoflurane in rats and miniature pigs [57, 276]. The decrease in tALP upon exposure to isoflurane at 26 wk is in contrast to plasma OC results, but in accordance with the decrease (insignificant) in the plasma OC/tDPD ratio as a reflection of bone turnover at this time-point. In addition, we should consider that alkaline phosphatase is a biomarker representative of early osteoblast differentiation and OC is a biomarker of later osteoblastic differentiation stage [277]. However, none of the isoflurane induced changes in circulating minerals and albumin were biologically significant and all values were within expected ranges for guinea pigs except for albumin and tALP which were lower than the suggested non-pregnancy ranges [278]. Such changes however are important in scientific inquiry and are of sufficient magnitude to mask changes in physiological response to treatments.

Osteocalcin as a bone formation biomarker may not only be altered in response to stress, but has also been associated with alterations in glucose metabolism. Recent studies linking energy metabolism to bone have found that the uncarboxylated OC normalizes glucose metabolism by increasing both insulin secretion and sensitivity [54]. Therefore, the effect of isoflurane on both OC and glucose was investigated at 26 wk of age. Although at 26 wk of age, no changes were observed in plasma OC concentration after exposure to isoflurane, a significant increase in blood glucose was observed which exceeded the expected values for guinea pigs [278]. The latter may be as a result of the effect of isoflurane in reduction of the ATP sensitivity of KATP channels and therefore impaired glucose-stimulated insulin release as reported in rats [279]. Thus sampling prior to anesthesia, at least at post-partum, is required for glucose

assessments. The increase in blood glucose after exposure to isoflurane is similar to that observed in adult male rats [57] and humans [280]. Nonetheless, these results are in contrast to studies showing a decrease in blood glucose in neonatal mice [62] and miniature pigs [276] and no change in blood glucose in American Kestrel neonates [267] upon the induction of isoflurane anaesthesia. This suggests that the effect of isoflurane on glucose may be age-dependent. Nonetheless, the effect of isoflurane exposure on glucose is preliminary and warrants further research.

There are a number of challenges and limitations to this study. Since at 26 wk of age, samples were collected 2 d postpartum as a part of a larger reproduction study, these differences might be due to post-partum physiology rather that age per se [268]. Moreover, at this age the routes of blood collection were by saphenous vein prior to the effect of the anaesthetic and by cardiac puncture afterwards. Sampling from different routes may affect circulating Na^+, tALP and glucose concentrations [281-283]. Therefore, caution must be taken when interpreting changes in minerals induced by the anaesthetic if blood sampling is done from different sites. Measurement of PTH levels and stress hormones such as corticosteroids are suggested to further explain the mechanisms responsible for changes in OC concentration. Finally, measurement of insulin and C-peptide concentrations are required to further explain changes in blood glucose concentration upon the induction of isoflurane anaesthesia.

In summary, the results from this study demonstrate that administration of isoflurane significantly elevates blood OC levels during rapid growth in guinea pigs, but not thereafter during the early postpartum period, whereas blood glucose

concentrations are elevated by isoflurane postpartum. Measurements of these biomarkers prior to administration of isoflurane are recommended to better interpret metabolic changes and improve consistency and comparability in research protocols.

4.6. Acknowledgments

There are no conflicts of interests related to this article. NT contributed in the design of this study, performed biochemical assessments, interpretation of data, statistical analyses and article writing. CR and RK performed critical review of the manuscript and provided helpful comments on interpretation of results. RK is in receipt of the Canadian Institutes of Health Research MT10839. HW contributed to the original thought and design of this study and undertook supervision of the biomarker measures and critical review of the manuscript. She is in receipt of a CRC (Canada Research Chair) *tier II* in Nutrition, Development and Aging and acknowledges CFI (Canadian Foundation for Innovation) infrastructure support for the DXA. Dr. Weiler wrote the grant along with CR, RK and NT which was approved and funded by CIHR (Canadian Institutes of Health Research). Acknowledgements are also to Nandita Perumal for her assistance during this animal study at the School of Dietetic and Human Nutrition at McGill University.

4.7. Figure legend

Figure 4.1. Plasma (a) osteocalcin (nmol/L) (b) total deoxypyridinoline (nmol/L) concentrations and (c) OC/tDPD molar ratio before and after the induction of isoflurane anaesthesia at 5, 9 and 26 wk of age in female guinea pigs. The effect of isoflurane exposure and age assessed by repeated measured ANOVA. Values are mean ± SD, n=10. Values with different superscripts are significantly different ($p<0.05$).

Table 4. 1. Characteristics of female guinea pig from 5 to 26 wk of age.

Characteristics	5 wk	9 wk	26 wk/ 2 d postpartum
Whole body			
Weight (g)	415.6 ± 35.9[a]	618.3 ± 31.6[b]	902.1 ± 69.4[c]
Length (cm)	23.3 ± 0.9[a]	26.5 ± 0.3[ab]	30.9 ± 0.5[b]
Lean Mass (g)	344.7± 30.4[a]	506.1± 22.9[b]	740.6 ± 64.3[c]
Fat (%)	14.6 ± 2.9[a]	15.1 ± 1.4[a]	14.2 ± 2.1[a]
Fat (g)	61.4 ± 16.8[a]	92.8 ± 9.5[b]	126.6 ± 21.8[c]
Bone Area (cm^2)	64.4 ± 5.0[a]	79.8 ± 3.3[ab]	108.8 ± 4.2[b]
BMC (g)	11.29 ± 1.46[a]	15.92 ± 0.89[b]	24.20 ± 1.68[c]
BMD (g/cm^2)	0.175 ± 0.009[a]	0.199 ± 0.006[b]	0.222 ± 0.009[c]
Lumbar spine (L1-L4)			
Area (cm^2)	1.6 ± 0.1[a]	1.9 ± 0.1[b]	2.1 ± 0.1[c]
BMC (g)	0.28 ± 0.03[a]	0.40 ± 0.03[b]	0.54 ± 0.03[c]
BMD (g/cm^2)	0.182 ± 0.012[a]	0.209 ± 0.013[b]	0.256 ± 0.014[c]
Tibia			
Area (cm^2)[1]	1.6 ± 0.1[a]	1.9 ± 0.1[b]	2.2 ± 0.1[c]
BMC (g)[1]	0.30 ± 0.04[a]	0.43 ± 0.02[b]	0.59 ± 0.05[c]
BMD (g/cm^2)[1]	0.185 ± 0.014[a]	0.233 ± 0.015[b]	0.274 ± 0.014[c]
Femur			
Area (cm^2)[1]	1.3 ± 0.0[a]	1.5 ± 0.1[b]	1.7 ± 0.1[c]
BMC (g)[1]	0.39 ± 0.04[a]	0.58 ± 0.05[b]	0.83 ± 0.08[c]
BMD (g/cm^2)[1]	0.296 ± 0.027[a]	0.377 ± 0.037[b]	0.480 ± 0.050[c]

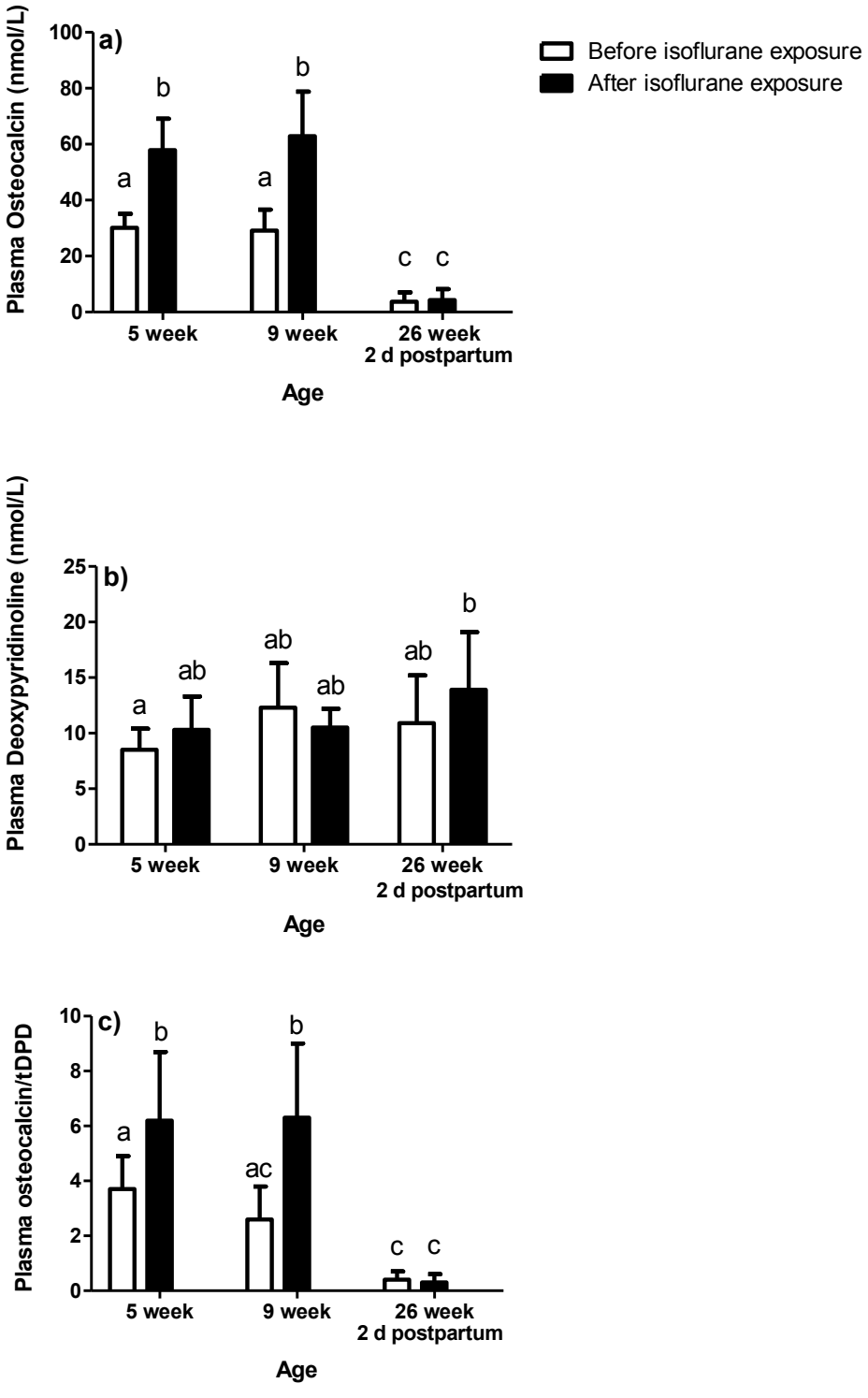
Values are mean ± SD. The effect of age on guinea pig characteristics assessed by repeated measures ANOVA. Values with different upper superscripts are significantly different within each row ($p<0.001$), n=10.[1]Values are average of right and left tibia or femur values.

Table 4. 2. Circulating minerals, albumin, total alkaline phosphatase and glucose concentrations before and after the induction of isoflurane anaesthesia at 26 wk of age (2d postpartum) in female guinea pigs.

Plasma/blood biomarkers	Normal guinea pig ranges [278]	Before exposure to isoflurane	After exposure to isoflurane	p-value
Total Ca (mmol/L)	2.30-2.85	2.44 ± 0.09^{a}	2.44 ± 0.09^{a}	0.93
Ca^{2+} (mmol/L)	-	1.42 ± 0.06^{a}	1.42 ± 0.11^{a}	0.45
PO_4 (mmol/L)	1.29-2.58	1.9 ± 0.3^{a}	2.4 ± 0.5^{b}	0.003
Na^{+} (mmol/L)	133-142	135.6 ± 1.6^{a}	135.9 ± 1.7^{a}	0.34
Cl^{-} (mmol/L)	100-112	104.5 ± 3.6^{a}	106.7 ± 3.3^{b}	0.007
Albumin (g/L)	25-40	16.7 ± 1.2^{a}	15.0 ± 1.6^{b}	0.0001
tALP (IU/L)	80-350	43.2 ± 6.6^{a}	40.2 ± 5.9^{b}	0.01
Glucose (mmol/L)	4.16-7.22	7.5 ± 0.6^{a}	10.9 ± 1.7^{b}	<0.0001

Values are mean ± SD, n=10. The effect of isoflurane exposure assessed by paired t-test. For each measurement, values with different superscripts within each row are significantly different ($p<0.05$).

Figure 4. 1.


a)
Plasma Osteocalcin (nmol/L)
100
80
60
40
20
0
a
b
a
b
c
c
5 week
9 week
26 week
2 d postpartum
Age
Before isoflurane exposure
After isoflurane exposure
b)
Plasma Deoxypyridinoline (nmol/L)
25
20
15
10
5
0
a
ab
ab
ab
ab
b
5 week
9 week
26 week
2 d postpartum
Age
c)
Plasma osteocalcin/tDPD
10
8
6
4
2
0
a
b
ac
b
c
c
5 week
9 week
26 week
2 d postpartum
Age

BRIDGE 3

In Chapter 4, it was shown that there was an increase after exposure to isoflurane in circulating OC concentration during development and glucose concentration at maturity in the guinea pig. These results strongly suggest that measurement of these biomarkers prior to isoflurane anaesthesia is recommended to better reflect normal metabolism and improve consistency and comparability in research. Therefore, all blood collections were performed prior to exposure to isoflurane for all bone biomarkers, mineral and glucose measurements in the guinea pig studies stated in Chapter 5 and 6. In Chapter 3, it was stated that vitamin D status was not different while OC was significantly elevated in GDM vs healthy women throughout pregnancy, but neither were associated with the development of GDM. However, this study had a cross-sectional design with possible uncontrolled confounding factors, selection biases and plasma $1,25(OH)_2D$, the active form of vitamin D was not measured which is required to further explain the association between OC and glucose metabolism. Therefore for better control of confounding variables in human studies, an appropriate controlled animal model was selected [56] and the dose-response effect of dietary vitamin D_3 in pregnancy was investigated on maternal oral glucose tolerance, concentration and body composition in association with plasma OC during pregnancy. In this interventional animal study, the objective was also to explore if maternal vitamin D status, its active form and OC at pre and mid-gestation (d 42) or their changes between these time-points were associated with blood glucose and OGTT results. Since there is evidence that low maternal transfer of vitamin D may adversely affect body composition at birth [20], the dose-response effect of maternal dietary vitamin D was also investigated on neonatal glucose concentration and body composition at postpartum. This study is presented in Chapter 5.

CHAPTER 5. MANUSCRIPT 3

High vitamin D status at pre-mating but not during pregnancy may improve maternal glucose tolerance in guinea pigs

Negar Tabatabaei[1], Celia Rodd[2], Richard Kremer[3] and Hope Weiler[1]

Author affiliation:

[1]Dietetics and Human Nutrition, McGill University, Ste-Anne-de-Bellevue, QC, Canada

[2] Pediatrics, Montreal Children's Hospital, McGill University, Montreal, QC, Canada

[3] Medicine, Calcium Research Laboratory, McGill University, Montreal, QC, Canada

Corresponding author:

Dr. Hope Weiler, Tel.: 514-398-7905, Fax: 1 514 398 7739,

e-mail: hope.weiler@mcgill.ca

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5.1. Abstract

This study explored the dose-response effect of dietary vitamin D_3 during pregnancy on maternal oral glucose tolerance and maternal and neonatal glucose concentration and body composition in relation to plasma osteocalcin (OC). Female pigmented guinea pigs (n=45, 4 mo) were randomized to five dosages of vitamin D_3 (0, 0.25, 0.5, 1 or 2 IU/g diet) from mating to delivery. Plasma vitamin D metabolites, minerals, OC and blood glucose were measured at pre-mating and d 42 (mid-gestation) in sows and at 2 d postpartum in sows and neonates. At d 50 of pregnancy, an oral glucose tolerance test (OGTT; 2 g/kg) was performed with blood glucose measured at 0 to 180 min. Whole body and abdominal composition were measured at pre-mating and 24-36 h postpartum in sows and body composition was assessed at 24-36 h postpartum in pups. A positive dose-response to dietary vitamin D_3 was observed for change in maternal plasma 25-hydroxyvitamin D (25(OH)D) through pregnancy and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) was higher in the 1 vs 0 IU/g group by mid-gestation but not by 2 d postpartum. There were no differences in glucose tolerance and changes in glucose, minerals, OC concentrations and body composition between groups during pregnancy. However, maternal pre-mating plasma 25(OH)D was negatively correlated with whole body and abdominal fat (g) and fat (%) but not lean mass. In addition, an inverse association was observed between pre-mating 25(OH)D with 3 h AUC for blood glucose from OGTT as well as abdominal lean mass with 3 h blood glucose and AUC from the OGTT. In the 2 d neonates, although both 25(OH)D and $1,25(OH)_2D$ followed a dose-response to maternal diet, glucose, OC, minerals and body composition were not different among groups. However, the associations between vitamin D metabolites, OC and body composition in the neonate differed from the sow. Maternal glucose tolerance and

concentration is unaffected by dietary vitamin D intake and status during pregnancy, but higher pre-pregnancy vitamin D status which is also inversely related to body and abdominal fat may be protective against pregnancy induced glucose intolerance.
Key words: Vitamin D, dose-response, pregnancy, glucose tolerance, osteocalcin

5.2. Introduction

Low vitamin D status, defined by circulating 25(OH)D concentration <50 nmol/L [1] or <75 nmol/L [2] is prevalent among pregnant women [4-9]. Circulating concentrations of the active metabolite, $1,25(OH)_2D$, is regulated by dietary and hormonal factors other than vitamin D [246], and may be normal or sometimes elevated when circulating 25(OH)D concentrations are low. If severe vitamin D deficiency evolves, $1,25(OH)_2D$ eventually becomes low due to substrate–dependence [11]. Such low values may be of consequence since observational studies suggest that $1,25(OH)_2D$ both directly and indirectly contributes to glucose homeostasis by enhancing insulin synthesis, secretion and sensitivity [16]. A protective effect of this nutrient has been suggested in non-pregnant insulin resistant states [247]. However, evidence from recent clinical trials is not conclusive regarding the effect of vitamin D supplementation on the risk of GDM [247].

Observational human studies have shown that 25(OH)D in early gestation is negatively associated with 1 h [28] and 2 h [25] glucose concentration from the OGTT. Similar negative relationships were observed between 25(OH)D at mid and late gestation with fasting [26] and 30 min glucose concentrations[27] from the OGTT. In women with established GDM, injection and oral consumption of alpha-calcidiol (alpha-hydroxy-cholecalciferol) resulted in a negative association between fasting glucose and $1,25(OH)_2D$ concentrations while OGTT results were unaffected [29]. This vitamin D analogue has less calcemic and phosphoric actions than $1,25(OH)_2D$ [30] and in contrast to cholecalciferol is not regulated by renal vitamin D metabolism [31]. These controversies are partly due to uncontrolled factors such as plasma Ca [32] and PO_4 [33] which are positively related to insulin secretion and pre-

pregnancy BMI and central fat which are adversely related to glucose tolerance [34]. All these covariates are also related to vitamin D metabolism [22, 35, 36].

Nevertheless, the effect of injection of 25(OH)D and/or $1,25(OH)_2D$ was investigated in non-pregnant rats [37] and rabbits [38] that had undetectable 25(OH)D and less than 50 % of their baseline $1,25(OH)_2D$ concentrations. In such models, injection of $1,25(OH)_2D$ prior to OGTT increased glucose induced insulin secretion [37, 38] and improved glucose tolerance at 3 h post glucose load in rats [37]. In contrast to the rapid response observed in rats, improved glucose tolerance was observed in rabbits only after the injection of 25(OH)D for 2 wk and not after the injection of $1,25(OH)_2D$ or infusion of Ca [38]. However, no interventional study has investigated the dose-response effect of oral vitamin D_3 intake resulting in circulating 25(OH)D ranging from < 25 to >125 nmol/L on glucose concentration and tolerance during pregnancy using an appropriate animal model. The guinea pig can be used for studying glucose intolerance during pregnancy since it is similar to humans in regard to the peak of blood glucose concentrations around 40-60 min after oral glucose ingestion on an OGTT during pregnancy [56].

The implication of low vitamin D status in GDM extends beyond insulin metabolism in the pancreas and includes alteration in expression of genes that have VDRE [39]. Osteocalcin (OC) is one such protein secreted from osteoblast which is stimulated by $1,25(OH)_2D$ [40] and its uncarboxylated form can have beneficial effects on glucose homeostasis in mice [41]. Recent evidence suggests that total OC is also secreted from adipocytes [42], and is positively associated with insulin sensitivity in humans [43-46] and negatively related to adiposity features such as BMI and fat mass in mice [47] and human [48-51]. However, these associations have not been investigated in interventional trials during pregnancy.

Considering the connection between the bone and endocrine metabolism, recently, studies have looked at serum OC concentration at mid-gestation in GDM vs healthy pregnant women [52, 53]. In addition, the former study observed a positive association between circulating OC and AUC for glucose on OGTT, suggesting that this may be an early compensatory mechanism to increase insulin secretion in women with GDM [52]. However, in the later study no correlation was observed between plasma OC and FPG [53]. These studies were limited by lack of sampling early in pregnancy.

Therefore, the main objective was to explore the dose-response effect of dietary vitamin D_3 in pregnancy on maternal oral glucose tolerance and body composition in association with plasma OC. A secondary objective was to study the association between vitamin D metabolites, OC and glucose at all pregnancy time-points and their changes during pregnancy. In addition, to explore if maternal vitamin D status, $1,25(OH)_2D$ and OC at pre and mid-gestation (d 42) or their changes between these time-points are associated with glucose and OGTT results. The effect of maternal dietary vitamin D_3 was also investigated on neonatal glucose and body composition at postpartum since there is evidence that low maternal transfer of vitamin D may adversely affect body composition at birth [20].

5.3. Method and Materials

5.3.1. Animal husbandry

Ethical approval was obtained from the McGill University Animal Care Committee. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care [264]. In addition, this model is similar to humans in regards to haemomonochorial placenta, serum OC and $1,25(OH)_2D$ changes during pregnancy and maternal and fetal correlation of vitamin D metabolites [13, 55, 202].

Pigmented (4 week old) guinea pigs (n=45 female, n=20 male) were purchased from Elm Hill Laboratories (MA, US) and housed singly with room temperature maintained at 21°C, lighting cycle from 0600 to 1800 h in a UVB free environment during the entire experiment to prevent endogenous synthesis of vitamin D.

5.3.2. Diet

The guinea pigs were fed a purified standard diet containing 1.2 IU/g diet vitamin D from 4 weeks to 16 weeks of age at which time they were mated. Decreased fertility associates with vitamin D deficiency in rats and VDR null mice [209, 284], therefore dietary deficiency was induced only after mating. At mating sows were randomized to 1 of 5 diets using random numbers tables. The diets were isoenergetic from *Test Diet* (Division of LandO'Lakes Purina Feed) and equal in all ingredients except for vitamin D (0, 0.25, 0.5, 1 and 2 IU/g diet). The 1 IU/g vitamin D diet is the control diet [285]. For details regarding the diet refer to (Manuscript 4, unpublished). All researchers and animal care staff were blinded to the diets by use of letter coding throughout the study. Diets were in pellet form and food and water were fed *ad libitum*. Food intake was measured weekly using a digital scale.

5.3.3. Protocol

Body weight was measured weekly using a digital scale with animal weighing program (Mettler Toledo SB8001, MA, USA). Nose-rump length was measured using a measuring tape to the nearest 0.1, while the animal was anaesthetized. Blood was sampled in a 3-h non-fed state (0900 h) from the saphenous vein of sows 1 d prior to mating, d 21 (end 1st trimester) and d 42 (end 2nd trimester, closest to d 50 when OGTT was performed) of pregnancy and 24-36 h post-delivery and pups at 24-36 h postpartum. Whole blood was used for glucose and Ca^{2+} and heparinized blood was used for 25(OH)D, $1,25(OH)_2D$, mineral and OC measurement. An OGTT was

performed after 8 h of non-fed state on day 50 of pregnancy by administering a 70% glucose solution orally (2 g/kg body weight). Blood glucose was measured at 0 (baseline), 15, 30, 60, 90, 120, 150 and 180 min after ingestion of the glucose solution. Area under the curve (AUC) for blood glucose concentration at 1 h, 2 h and 3 h after oral glucose ingestion was calculated (Prism software, version 5.1, CA, USA). Necropsy was conducted at 24-36 h postpartum in sows and at 24-36 h postpartum in pups after exsanguination under isoflurane anaesthetic and using cardiac puncture. The blood sample was collected into lithium heparin tubes. All blood samples were kept on ice and centrifuged at 2000 g for 30 min at 4°C. Plasma samples were collected and stored at -80 °C until analyses.

5.3.4. DXA (whole body and abdominal composition)

Anaesthesia was induced using isoflurane AErrane [286] at 5% in an induction chamber followed by maintenance at 2% delivered using a cone mask. During the anaesthetization period, whole body scans were performed on the animals using DXA and small animal software (Hologic 4500, QDR Version 12.3, Hologic, Bedford, Mass., USA), 1 d prior to mating and 24-36 h post-delivery in sows and at 24-36 h postpartum in pups. Abdominal composition assessment was performed by analyzing the lumbar vertebrae (1-4) region. These assessments provided *in vivo* data for fat (g), fat (%) and lean (g) for the whole body and abdominal region. All CV% were < 2.9 %.

5.3.5. Biochemistry

Plasma total 25(OH)D was measured using an auto analyzer (total 25(OH)D, Liaison, Diasorin Inc.). This assay has a sensitivity of 10 nmol/L with CV% <5.3%. Plasma total $1,25(OH)_2D$ was measured using TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Warnex Bioanalytical Service, Laval, Canada). Samples were

extracted using a proprietary procedure which includes a Diels-Alder derivatization. Intra-assay variability was ≤7.0 and inter-assay variability was ≤12.3 % for the selected control samples of guinea pig plasma calcitriol (80.6, 236.6, 608.4 and 1258.4 pmol/L). This assay has a sensitivity of 50 pmol/L. Plasma concentrations of total Ca and PO_4 were measured using an autoanalzyer (Beckman DxC600 California, USA). All CV% were <3.1%. Plasma Ca^{2+} was measured with an ABL 825 series blood gas analyzer (Radiometer America, Copenhagen, Denmark) with all CVs were < 4.2 % for all assays. Blood glucose was measured by OneTouch glucometer, all CVs were < 9.7 %. Osteocalcin was measured using ELISA kits (Metra Osteocalcin, Quidel Corporation, San Diego, CA., USA) with known cross-reactivity in guinea pig plasma. This assay has a sensitivity of 0.08 nmol/L. In order to exclude inter-assay variability each ELISA kit was ran with all samples from n=1 sow belonging to each of the five diets, including all their time points (pre-mating, d 21, d42 and post-mating) and their relevant pup samples. All CV% for intra-assay variability were <5% for OC.

5.3.6. Statistical analyses

5.3.6.1. Sample size

The selected sample size of n=9 pregnancies per diet group was based on an alpha of 0.01 and power of 80 and plasma 25(OH)D and glucose concentration for sows. The sample size based on difference in plasma 25(OH)D of 83.6 % between the vitamin D deficiency vs normal diet group in the same model was very low (n=1) [79]. The sample size to detect a difference in glucose concentration (3 h after glucose load) of 22.2 % [37] was n=6 for the sows. We studied 9 in the event of unsuccessful pregnancy or delivery complications.

5.3.6.2. Data Analysis

All data were audited for accuracy and further screened using scatter plots to identify potential outliers and for normality testing using SAS (version 9.2, SAS, Inc., Cary, NC., USA). If normality testing failed using the Kolmogorov and Smirnov test, data were transformed by use of logarithm, squaring, inverse values etc. Differences between the groups were tested using the fixed effects of diet and time in the sow and diet and sex in the pup in a MIXED model ANOVA adjusting for the effect of block and litter size. *Post hoc* testing for significance was conducted using a Tukey's test with a $p \leq 0.05$. In optimizing the model, maternal baseline whole body and abdominal fat (g), fat (%) or lean mass (g) values, sow and pup plasma OC, 25(OH)D, $1,25(OH)_2D$ were explored as covariates. Pearson correlations were performed to investigate the relationship between variables while adjusting for litter size and block.

5.4. Results

5.4.1. Pregnancy, delivery and body composition

All sows delivered healthy newborn pups except for 1 sow (2 IU/g vitamin D diet) that died during mating. There were no differences among diet groups in weekly weight gain during all pregnancy (43.1±1.8 g/wk, n=44, p=0.74). In addition, no significant differences were observed between diet groups in weekly food intake (305.2±8.3 g/wk, p=0.34) during all pregnancy and weekly food intake per body weight (0.3±0.0 g/kg/wk, p=0.37) during all pregnancy. There were no significance differences in percent change in maternal body length, weight and composition among groups from pre-mating to 2 d postpartum **(Table 5.1)**. There were no significant differences among groups in percent change in maternal abdominal fat (g) (-39.03±5.18, n=44, p=0.38), lean mass (g) (19.14±2.29, n=44, p=0.52) and fat (%)

(-44.96±4.43, n=44, p=0.45) from pre-mating to 2 d postpartum. Growth during pregnancy continued resulting in an increase in body length and weight as well as whole body and abdominal lean mass but a decrease in whole body and abdominal fat mass and percent fat as shown above and **Table 5.1**.

The pregnancies led to delivery of n=125 healthy pups and n=13 stillborn. There were no significant differences across the groups in the number of healthy (median of 25, range 18-30) and stillborn (median of 3, range 1-5) neonates (p=0.26). There were no differences in litter size among diet groups (median of 3, range 1-5). There were no significant effects of diet or diet*sex interaction for body length, weight and composition at 2 d postpartum, but male pups weighed more than females (118.8±2.0 vs 111.2±1.9 g, p=0.02) with higher lean mass (91.0±1.6 vs 85.9±1.5 g, p=0.04) and were longer (16.3±0.1 vs 16.0 ±0.1 cm, p=0.02) **(Table 5.1)**.

5.4.2. Plasma 25(OH)D and 1,25(OH)$_2$D

The details regarding plasma 25(OH)D and 1,25(OH)$_2$D dose-repose to dietary vitamin D_3 during pregnancy are reported elsewhere (Manuscript 4, unpublished). There were no differences among groups in plasma 25(OH)D (252.1±7.9 nmol/L, n=44, p=0.74) and 1,25(OH)$_2$D (209.2±15.4 pmol/L, n=44, p=0.70) at pre-mating (baseline). There was a significant effect of diet on percent change in maternal plasma 25(OH)D from pre-mating to d 42 ($p<0.0001$) and 2 d postpartum ($p<0.0001$). Change in maternal plasma 25(OH)D was statistically different between all groups except the 0, 0.25 and 0.5 IU/g groups by d42 and was statistically different between all groups except in the 0 vs 0.25 and 0.25 vs 0.5 by 2 d postpartum (**Table 5.2)**. Pregnancy was associated with a significant decrease in maternal plasma 25(OH)D in all diet groups except in the 2 IU/g group by d 42 and a decrease in all groups by 2 d postpartum **(Table 5.2).** Change in maternal plasma

$1,25(OH)_2D$ was significantly lower in the 0 vs 1 group by d 42 but no difference was observed among groups by 2 d postpartum **(Table 5.2)**. Pregnancy was associated with a decrease in plasma total $1,25(OH)_2D$ in the 0 group but increase in all other groups by d42 and 2 d postpartum **(Table 5.2)**.

There was a significant dose-response increase in neonatal plasma 25(OH)D ($p<0.0001$) among all groups except the 0 vs 0.25 groups and in neonatal plasma $1,25(OH)_2D$ ($p<0.0001$) among all groups except the 0.5 vs 1 at 2 d postpartum **(Table 5.2)**. There were no significant differences between the sexes and no diet*sex interaction for plasma 25(OH)D and $1,25(OH)_2D$ **(Table 5.2)**. There was a significant negative correlation between plasma 25(OH)D and $1,25(OH)_2D$ at pre-mating ($r=-0.33$, $p=0.03$) but this correlation was significantly positive at d 42 in the sow ($r=0.46$, $p=0.002$) and at 2 d postpartum in the sow ($r=0.43$, $p=0.004$) and the pup ($r=0.36$, $p<0.0001$).

5.4.3. Blood glucose, plasma minerals and osteocalcin

Change in maternal plasma total Ca, Ca^{2+} and PO_4 from pre-mating to d 42 and 2 d postpartum did not differ among groups (**Table 5.1)**. Pregnancy resulted in a decrease in maternal plasma total Ca and PO_4 by d 42 and decrease in all plasma minerals by 2 d postpartum (**Table 5.1)**. Neonatal plasma total Ca, Ca^{2+} and P were not significantly different according to diet, sex and diet*sex interaction at 2 d postpartum as shown in **Table 5.1**.

No differences were observed among groups in change in maternal blood glucose concentration from pre-mating to d 42 and 2 d postpartum. Even though, blood glucose concentrations reached lower than pre-mating concentrations by d 42, they increased by 2 d postpartum **(Table 5.1)**. In addition, there were no significant

effects of diet (p=0.42), sex (p=0.18), diet*sex (p=0.26) on blood glucose concentration at 2 d postpartum **(Table 5.1)**.

Diet did not affect change in maternal plasma OC from pre-mating to d42 and 2 d postpartum but there was a decrease in plasma OC by d 42 which continued to decrease further by 2 d postpartum **(Table 5.1)**. In addition, there were no significant effects of diet (p=0.58) and diet*sex (p=0.64) on plasma OC concentrations but this biomarker was significantly higher (p=0.02) in male vs female pups at 2 d postpartum **(Table 5.1)**.

5.4.4. Maternal oral glucose tolerance test (OGTT)

Blood glucose concentration at 0, 30, 60, 90, 120, 150 and 180 min did not differ among dietary groups **(Figure 5.1 (a))** and AUC for blood glucose 60, 120 and 180 min after ingestion of the oral glucose solution at d 50 of pregnancy **(Figure 5.1 (b)).** Further adjustment for maternal pre-mating whole body and abdominal fat (g), fat (%), lean mass (g), plasma OC, 25(OH)D and $1,25(OH)_2D$ or their percent change during pregnancy did not significantly affect the results. The peaks in blood glucose concentrations were between the 60-90 min after oral glucose ingestion in all groups and glucose concentrations were still higher than baseline values at 3 h on the OGTT **(Figure 5.1(a)).**

There was a negative correlation between maternal plasma 25(OH)D at pre-mating and AUC for blood glucose (r=-0.31, p=0.05) (**Figure 5.2(a))** at 3 h post glucose load and this association remained significant after adjustment for pre-mating whole body and abdominal compositions. Whereas the inverse association between maternal plasma 25(OH)D at pre-mating and blood glucose concentration at 3 h post glucose load (r=-0.34, p=0.02) was no longer significant after exclusion of 3 outliers (r=-0.19, p=0.25). However, there were no associations between blood glucose

concentrations or AUC from the OGTT with plasma 25(OH)D, $1,25(OH)_2D$ or OC concentrations at d42 or their change from pre-mating to d42 pregnancy (data not shown).

There was a negative correlation between maternal lean mass (g) at the abdominal region at pre-mating but not 2 d postpartum with the blood glucose concentrations (r=-0.39, p=0.01) and AUC for blood glucose (r=-0.33, p=0.03) **(Figure 5.2(b))** at 3 h post glucose load but no significant correlations were observed with maternal abdominal fat (g) or (%) composition (data not shown). No significant correlations were observed between blood glucose concentrations or AUC from the OGTT at d 50 pregnancy with any of the whole body compositions at pre-mating and 2 d postpartum or their percent change during pregnancy (data not shown)

5.4.5. Biochemical correlations

There were no significant correlations between maternal plasma 25(OH)D, $1,25(OH)_2D$, OC and blood glucose concentrations at any pregnancy time-point or their changes during pregnancy (data not shown).

There was a negative correlation between neonatal plasma $1,25(OH)_2D$ and OC (r=-0.35, p<0.0001) **(Figure 5.3)** and plasma OC and blood glucose concentration (r=-0.21, p=0.02) at 2 d postpartum. The later was no longer significant after adjustment for plasma $1,25(OH)_2D$ concentrations (r=-0.14, p=0.13). However, a positive correlation was observed between neonatal plasma $1,25(OH)_2D$ and blood glucose concentration (r=0.24, p=0.006) at 2 d postpartum which persisted after adjustment for plasma OC concentrations and body and abdominal composition. There were no significant associations between neonatal plasma 25(OH)D, OC and blood glucose concentration at 2 d postpartum.

5.4.6. Anthropometry correlations

Maternal plasma 1,25$(OH)_2$D and body weight were positively correlated (r=0.38, p=0.01) at d42. Also, change in maternal plasma 1,25$(OH)_2$D by 2 d postpartum was positively correlated (r=0.44, p=0.005) with weekly food intake but this association did not remain significant after adjustment for body weight (r=0.29, p=0.06).

Plasma 25(OH)D, 1,25$(OH)_2$D and OC were significantly correlated with body and abdominal composition measurements at pre-mating but not 2 d postpartum. There was a negative correlation between maternal plasma 25(OH)D and body weight (g), fat (g) and fat (%), but not lean mass (g) at pre-mating **(Figure 5.4(a, c, e, g))**. Similarly, maternal plasma 25(OH)D was negatively correlated with abdominal fat (g) (r=-0.59, p<0.0001) and fat (%) (r=-0.64, p<0.0001) but not lean mass (g) (r=0.07, p=0.67) at pre-mating. Also, maternal plasma 1,25$(OH)_2$D was positively correlated with body weight (g), fat (g), but not fat (%) and lean mass (g) at pre-mating **(Figure 5.4(b, d, f, h))**. Similarly, maternal plasma 1,25$(OH)_2$D was positively correlated with abdominal fat (g) (r=0.31, p=0.05) but not fat (%) (r=0.24, p=0.13) and lean mass (g) (r=0.17, p=0.50) at pre-mating. In addition, there was a positive correlation between maternal plasma OC and body weight (g) (r=0.40, p=0.008), fat (g) (r=0.30, p=0.05), and lean mass (r=0.33, p=0.03) but not fat (%) (r=0.17, p=0.27) at pre-mating. However, no significant associations were observed between maternal plasma OC and any abdominal composition component at pre-mating and 2 d postpartum. There were no significant correlations between maternal glucose concentration and body composition at pre-mating and 2 d postpartum or their changes during pregnancy (data not shown).

There was a positive correlation between neonatal plasma OC and body weight (g) at birth (r=0.21, p=0.02) and also at 2 d postpartum (r=0.19, p=0.02). In addition, a significant correlation was observed between neonatal plasma OC and lean mass (g) (p=0.18, p=0.05) but not fat (g) (r=0.02, p=0.82), fat (%) (r=-0.04, p=0.66) at 2 d postpartum. The neonatal blood glucose concentration was positively correlated with body weight (g) at birth (r=0.18, p=0.05), body mass (g) (r=0.19, p=0.04) and lean mass (g) (r=0.20, p=0.03) but not fat (g) (r=0.04, p=0.65), fat (%) (r=-0.04, p=0.69) at 2 d postpartum. However, neither plasma 25(OH)D nor $1,25(OH)_2D$ correlated with any neonatal body composition measurements at 2 d postpartum (data not shown).

5.5. Discussion and conclusions

Maternal glucose concentration and tolerance is unaffected by dietary vitamin D intake and status during pregnancy in a controlled animal model that initiated pregnancy with high 25(OH)D concentrations (~250 nmol/L). However, vitamin D status at pre-mating was negatively associated with the with the AUC for blood glucose concentration 3 h post glucose load, suggesting that such high vitamin D status at pre-pregnancy, which are much higher than normal values in human pregnancy [10], may have a protective effect on pregnancy induced glucose intolerance. In addition, higher body and abdominal fat is associated with lower vitamin D status at pre-pregnancy, the former also being a risk factor for GDM.

Despite the fact that change in maternal plasma 25(OH)D followed a dose-response to dietary vitamin D_3, plasma $1,25(OH)_2D$ did not follow a dose-response and the decrease in $1,25(OH)_2D$ concentrations in the 0 IU group did not lead to undetectable levels which may be the reason for lack of differences in plasma minerals, OC, fasting glucose and OGTT outcomes among diet groups. These results

are different from the improved insulin secretion and glucose tolerance [37, 38] observed after injection of 25(OH)D or 1,25$(OH)_2$D prior to OGTT in non-pregnant vitamin D deficient rats [37] and rabbits [38] . This may be due to the fact that in the guinea pig model vitamin D status reached deficient, but not undetectable levels only by mid-gestation while undetectable 25(OH)D concentrations had been reached prior to the OGTT in the later studies [37, 38].

The model in this study resembled an autumn pregnancy in which baseline vitamin D status are usually at their highest concentration. Such high 25(OH)D concentration at pre-mating was negatively associated with 3 h glucose concentration and AUC from OGTT. Previous human studies that did not assess vitamin D status at early pregnancy, reported a negative association between maternal 25(OH)D at mid and late-gestation and fasting glucose [26] and glucose tolerance from OGTT [27]. The results from this animal study are in accordance with human studies showing that early gestation circulating 25(OH)D are negatively associated with 1 h [28] and 2 h [25] glucose concentration from the OGTT. Overall, results suggest that in this model, vitamin D status at pre-mating but not mid-gestation may be protective against pregnancy induced glucose intolerance. The lack of such associations between plasma 1,25$(OH)_2$D and glucose tolerance may be related to uncontrolled factors affecting plasma 1,25$(OH)_2$D due to its lower half-life and higher hormonal regulation as compared to 25(OH)D [246], which requires further research.

Pregnancy in the guinea pig resulted in a decrease in body fat whether examined on a whole body or abdominal basis as well as an increase in lean mass which is different from the increase in all these compositions observed at late pregnancy as compared to pre-pregnancy in lean subjects [287] and also postpartum in normal weight women [288, 289]. This may be due to the effect of growth

hormone [290] and the fact that the model in the present study had not reached its mature size at the time of gestation [291]. Nonetheless, human studies have also suggested that body fat reaches a peak at the end of the second trimester which is mainly stored subcutaneously in the thigh and abdomen and is mobilized afterwards toward the end of gestation to provide the needs of the rapidly growing fetus and later on during lactation [292]. Since in the present study only pre and post pregnancy whole body and abdominal composition are available, it is not possible to interpret their changes during different pregnancy trimesters.

A dose-response was observed in both neonatal plasma 25(OH)D and $1,25(OH)_2D$ in response to maternal dietary vitamin D_3, which surprisingly resulted in a positive association between neonatal plasma $1,25(OH)_2D$ and blood glucose concentration measured 3 h after food withdrawal at postpartum. This might be related to the higher neonatal vs maternal $1,25(OH)_2D$ and OC concentrations at postpartum in guinea pigs (Manuscript 4, unpublished) but also requires further investigation. In addition, a negative association was observed between plasma $1,25(OH)_2D$ and OC in the neonate which is in contrast to the positive relationship in human and rat [293] and may be a result of interaction of $1,25(OH)_2D$ with bone-derived growth factors [294]. The negative association observed between plasma OC and blood glucose concentration measured 3 h after food withdrawal in the neonate is in accordance with animal [41] and humans [43-46] reports. However, this was no longer significant after adjusting for plasma $1,25(OH)_2D$, again suggesting the possibility of interaction of calcitriol with growth factors [294].

Interestingly, the negative association between maternal vitamin D status and body weight, as well as body and abdominal fat mass and percent fat were only observed at pre-mating, prior to the vitamin D dose-response intervention probably

due to sequestering of 25(OH)D in body fat [36, 123]. There are no studies on the effect of vitamin D status during pregnancy on body composition, but similar results have been reported in a study on overweight and obese patients [295]. The inverse relationship between change in plasma 25(OH)D and change in BMI and fat mass during a 4 y vitamin D supplementation trial in postmenopausal women suggested a possible effect of vitamin D on body composition [296]. Even though, previous studies have suggested that maternal vitamin D status affects neonatal body fat and leans mass at birth [20, 297], no association was observed between vitamin D status and body composition at postpartum which is similar to results in human neonates [225].

The role of the active form of vitamin D on growth and adipogenesis is controversial. *In vitro* studies suggest stimulatory effects at physiological doses and inhibitory effects at pharmacological levels [298]. In addition, both higher [128, 299, 300] and lower [301, 302] circulating $1,25(OH)_2D$ have been reported in obese vs normal weight humans. In the present study, maternal plasma $1,25(OH)_2D$ and body weight as well as body and abdominal fat mass, but not percent fat and lean mass were positively associated at pre-mating but no associations were observed in the neonate, suggesting that the effect of $1,25(OH)_2D$ on growth and adipose metabolism may be dependent on physiological state.

In contrast to the negative association reported between OC and adiposity features such as BMI and fat mass in animals [47] and humans [48-51], a positive relationship between this bone formation biomarker and all body composition components except percent body fat were observed at pre-mating but no such associations were observed at the abdominal region. In addition, a positive association between plasma OC and body weight and lean mass but not fat mass and

percent fat was observed in the neonate at 2 d postpartum. These interactions between OC and body composition is complex and can be affected by growth factors such as IGF-1 (insulin-like growth factor-1) which has shown to be directly associated with neonatal body weight, fat and leans mass in humans [303]. Perhaps the reason for a stronger association between plasma OC and all body compositions in the sow vs the neonate, is due to higher maternal vs neonatal IGF-1 [304].

There were no relationship between maternal blood glucose concentration measured 3 h after food withdrawal and body composition at pre-mating and 2 d postpartum. However, a negative association was observed between abdominal but not whole body lean mass at pre-mating and 3 h blood glucose and AUC from OGTT. Abdominal obesity at early gestation has been inversely associated with glucose tolerance at late gestation in women [305]. These results show the importance of the lean mass content of the abdominal vs whole body region against glucose intolerance in the guinea pig. Studies have shown a positive association between glucose concentrations and fetal fat mass in normal pregnancy in sheep [306, 307]. In this guinea pig study, such positive relationship was observed between blood glucose concentrations measured 3 h after food withdrawal and body weight and lean mass but not fat mass and percent fat.

The guinea pig model is similar to human pregnancy in terms of time at which glucose concentrations peaks on OGTT [56], but the peak in blood glucose after oral glucose ingestion had a 30 min delay and blood glucose concentration did not return to baseline values after 3 h on the OGTT, suggesting that perhaps the 2 g/kg of 70 % glucose solution may have been too high for the guinea pig OGTT. In addition, pregnancy initiated with much higher circulating 25(OH)D as compared to usual concentrations in human pregnancy [10]. In addition, in the neonate blood

glucose measurement was limited to 3 h after food withdrawal which is lower than the overnight fasting in previous studies [56, 233] and therefore may not accurately represent glucose homeostasis.

Maternal glucose tolerance and concentration is not affected by dietary vitamin D intake and status in the growing guinea pig model during pregnancy, but very high pre-pregnancy vitamin D status (25(OH)D ~ 250 nmol/L) which is also inversely related to body and abdominal fat may be protective against pregnancy induced glucose intolerance. The associations between vitamin D metabolites, OC and body composition is complex and differs by life stages, requiring further research.

5.6. Acknowledgments

N.-T. contributed in the design of this study, performed biochemical assessments, interpretation of data, statistical analyses and article writing. C.-R. reviewed the manuscript and provided on interpretation of the results. R.-K. reviewed the manuscript and provided comments. H.-W. contributed to the original thought and design of this study and undertook supervision of the biomarker measures and critical review of the manuscript. She is in receipt of a CRC *tier II* in Nutrition, Development and Aging and acknowledges are also towards the CFI infrastructure. Dr. Weiler wrote the grant along with C.-R., R.-K. and N.-T. which was approved and funded by CIHR. Thanks are also due to Dr. Jinping Zhao and Asma Babar for their great assistance during this animal study at the School of Dietetics and Human Nutrition.

5.7. Figure legends

Figure 5.1. Maternal a) blood glucose concentration at 0, 30, 60, 90, 120, 150 and 180 min and b) AUC for blood glucose concentration at 60, 120 and 180 min after ingestion of oral glucose solution at d 50 of pregnancy according to dietary intake of vitamin D. Values are means ± SEM, Mixed model ANOVA, Tukey, *post-hoc* test. Samples size (n) per vitamin D diet group: 0 IU/g (n=9), 0.25 IU/g (n=9), 0.5 IU/g (n=9), 1 IU/g (n=9) and 2 IU/g (n=8). Statistical significance, $p \leq 0.05$. There were no significant differences among diet groups in blood glucose concentration and AUC for blood glucose at any time-points after ingestion of the oral glucose solution.

Figure 5.2. Maternal **(a)** plasma 25(OH)D and **(b)** abdominal lean mass (g) at pre-mating in correlation with AUC for blood glucose concentration 3 h after ingestion of oral glucose solution at d 50 of pregnancy according to dietary intake of vitamin D. Pearson correlation adjusted for litter size and block. Samples size, n=44, statistical significance, $p \leq 0.05$.

Figure 5.3. Neonatal plasma $1,25(OH)_2D$ and osteoalcin correlation at 2 d postpartum according to maternal dietary intake of vitamin D. Pearson correlation adjusted for litter size and block. Statistical significance, $p \leq 0.05$.

Figure 5.4. Maternal plasma 25(OH)D and $1,25(OH)_2D$ correlation with body weight **(a-b) ,** fat mass **(c-d)**, percent fat **(e-f)** and lean mass at pre-mating, respectively. Pearson correlation adjusted for litter size and block. Samples size, n=44, statistical significance, $p \leq 0.05$.

Table 5. 1. Body anthropometry and composition and biomarker concentration in pregnant guinea pig sows and neonates in all vitamin D dietary groups.

Outcomes	Maternal change pre-mating to d 42 (%)	p-values	Maternal change pre-mating to 2 d postpartum (%)	Maternal p-values Mixed ANOVA	Neonatal 2 d postpartum		Neonatal p-values Mixed ANOVA		
	All Diets	Diet	All Diets	Diet	All Diets		Diet	Sex	Diet * Sex
					Female	Male			
Body									
Length (cm)	-	-	5.6±0.4(44)	0.94	16.0 ±0.1^{a}(66)	16.3±0.1^{b}(73)	0.26	0.02	0.18
Whole body									
Weight (g)	22.9±1.4(44)	0.06	9.8±1.1(44)	0.71	111.2±1.9^{a}(66)	118.8±2.0^{b}(73)	0.65	0.02	0.63
Fat (%)	-	-	-31.2±4.4(44)	0.55	20.0±0.6(66)	19.1±0.6(66)	0.06	0.78	0.78
Fat (g)	-	-	-24.6±5.0(44)	0.58	22.6±0.8(66)	22.5±0.8(73)	0.08	0.80	0.53
Lean mass (g)	-	-	18.9±1.7(44)	0.98	85.9±1.5^{a}(66)	91.0±1.6^{b}(73)	0.11	0.04	0.42
Plasma									
Total Ca (mmol/L)	-2.6±0.8(44)	0.78	-1.8±0.9(44)	0.90	2.4±0.0(54)	2.4±0.0(58)	0.06	0.68	0.25
Ca^{2+} (mmol/L)	-	-	-4.6±1.5(30)	0.70	1.4±0.0(42)	1.4±0.0(49)	0.42	0.98	0.97
PO_4 (mmol/L)	-16.4±2.9(42)	0.15	-4.1±2.6(42)	0.45	2.5±0.1(49)	2.5±0.1(53)	0.17	0.54	0.09
OC (nmol/L)	-28.6±4.0(44)	0.66	-66.9±2.0(44)	0.79	49.3±1.6^{a}(61)	52.9±1.4^{b}(64)	0.58	0.02	0.64
Blood									
Glucose (mmol/L)	-1.8±4.8(44)	0.51	9.8±3.5(44)	0.24	6.0±0.1(61)	6.2±0.1(64)	0.42	0.18	0.26

Values are means ± SEM**,** *n* is presented in parenthesis. Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$. Different lower superscripts (a, b) are representative of statistical significance between sexes.

Table 5. 2. Plasma 25(OH)D and 1,25$(OH)_2$D change during pregnancy in guinea pig sows and concentrations at 2 d postpartum in neonates according to dietary intake of vitamin D_3.

Outcomes	**Maternal Diet vitamin D (IU/g)**					
	0	**0.25**	**0.5**	**1**	**2**	**p-value**
Plasma 25(OH)D						
Maternal (% change)						
pre-mating to d 42	-75.9±3.0^{a} (9)	-63.3±2.7^{a}(9)	-58.6±3.3^{a}(9)	-27.4±5.2^{b}(9)	18.6±8.2^{c}(8)	<0.0001
pre-mating to 2 d postpartum	-87.9±2.1^{a} (9)	-80.0±1.9^{ab}(9)	-70.1±1.5^{b}(9)	-46.6±4.0^{c}(9)	-0.3±4.8^{d}(8)	<0.0001
Neonatal (nmol/L)						
2 d postpartum	10.5±1.5^{a}(29)	16.0±1.4^{a}(23)	31.9±2.4^{b}(29)	50.8±4.6^{c}(26)	112.7±11.6^{d}(18)	<0.0001
Plasma 1,25$(OH)_2$D						
Maternal (% change)						
pre-mating to d 42	-43.6±11.0^{a}(9)	44.9±29.5^{ab}(9)	146.3±59.5^{ab}(9)	198.4±105.0^{b}(9)	85.2±24.0^{ab}(8)	0.05
pre-mating to 2 d postpartum	-43.4±9.1(9)	58.5±3.8(9)	129.4±110.2(9)	198.5±121.9(9)	44.5±23.7(8)	0.25
Neonatal (pmol/L)						
2 d postpartum	123.1±13.8^{a}(29)	218.0±24.9^{b}(23)	310.6±33.0^{c}(29)	391.3±43.7^{c}(26)	544.0±53.3^{d}(18)	<0.0001

Values are means ± SEM, *n* is presented in parenthesis. Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test. Statistical significance, p≤0.05. Different lower superscripts (a, b, c, d) are representative of statistical significance between diet groups.

Figure 5. 1.

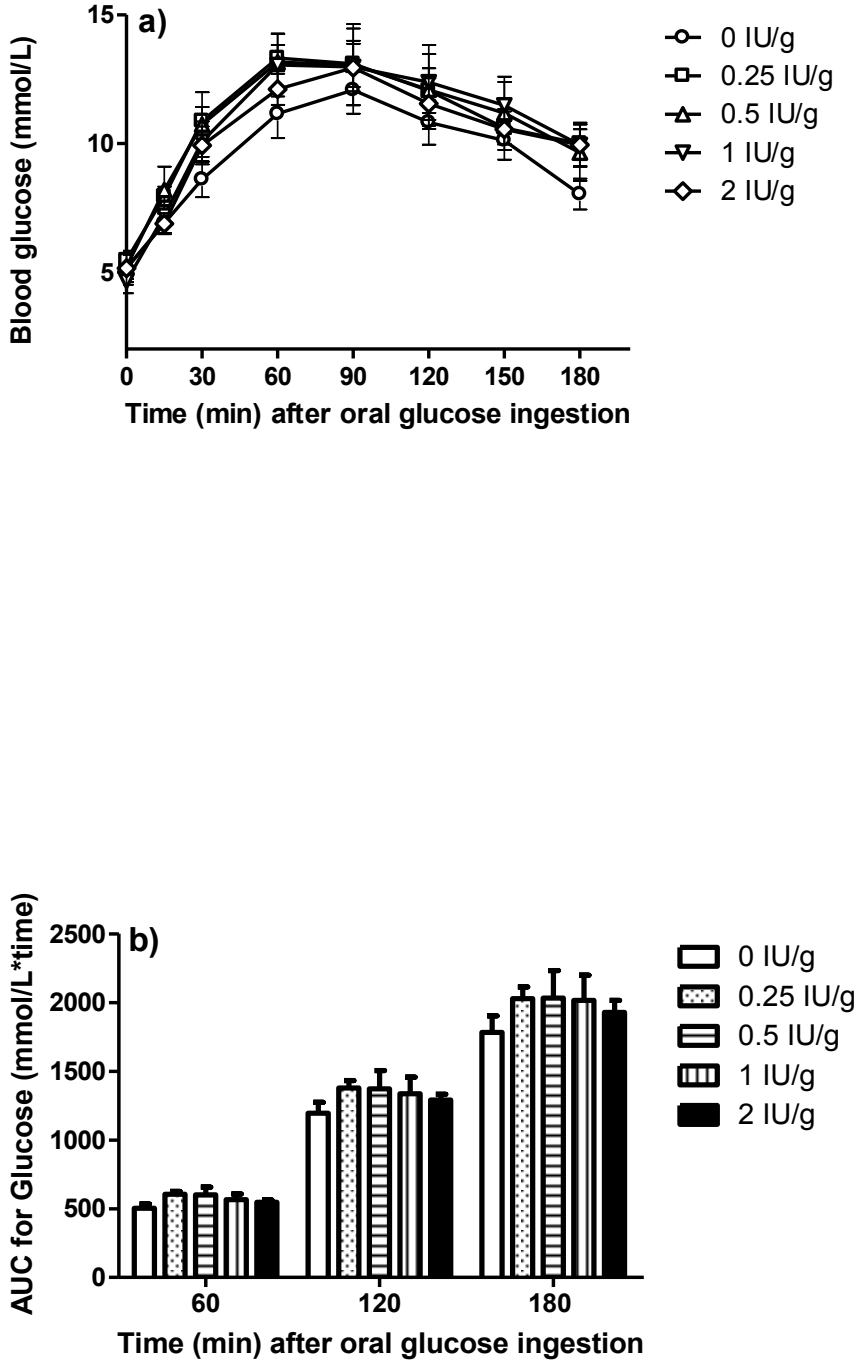

a)
Blood glucose (mmol/L)
15
10
5
0
30
60
90
120
150
180
Time (min) after oral glucose ingestion
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
b)
AUC for Glucose (mmol/L*time)
2500
2000
1500
1000
500
0
60
120
180
Time (min) after oral glucose ingestion
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g

Figure 5. 2.

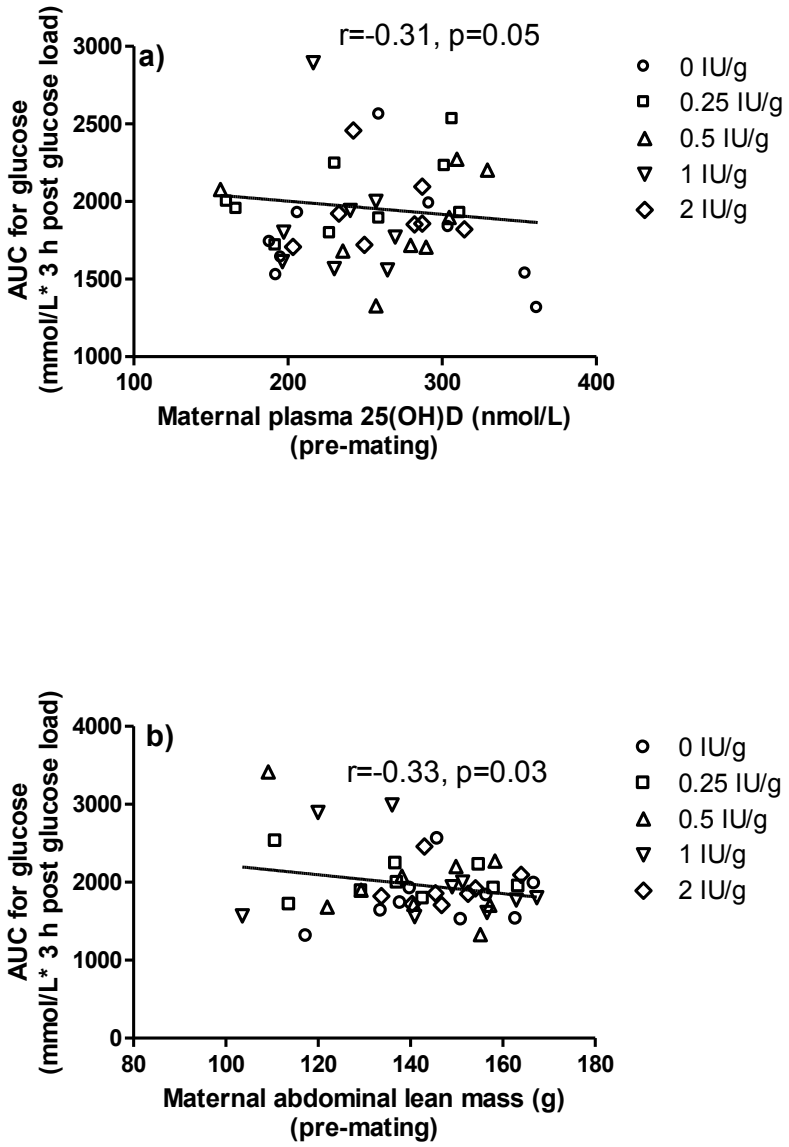

a)
r=-0.31, p=0.05
AUC for glucose (mmol/L* 3 h post glucose load)
3000
2500
2000
1500
1000
100
200
300
400
Maternal plasma 25(OH)D (nmol/L) (pre-mating)
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
b)
r=-0.33, p=0.03
AUC for glucose (mmol/L* 3 h post glucose load)
4000
3000
2000
1000
0
80
100
120
140
160
180
Maternal abdominal lean mass (g) (pre-mating)
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g

Figure 5. 3.

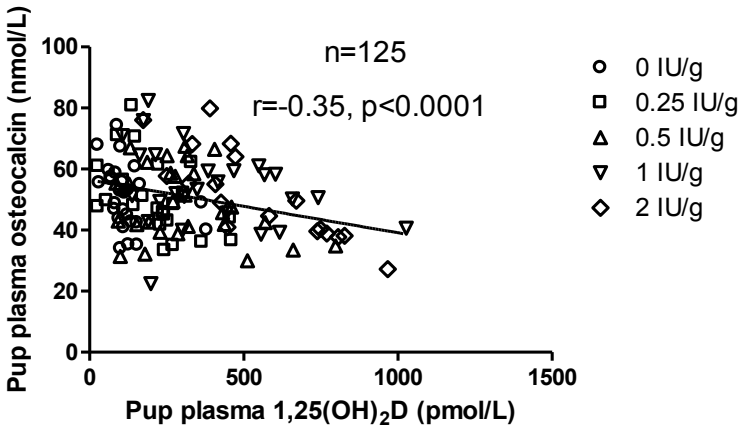
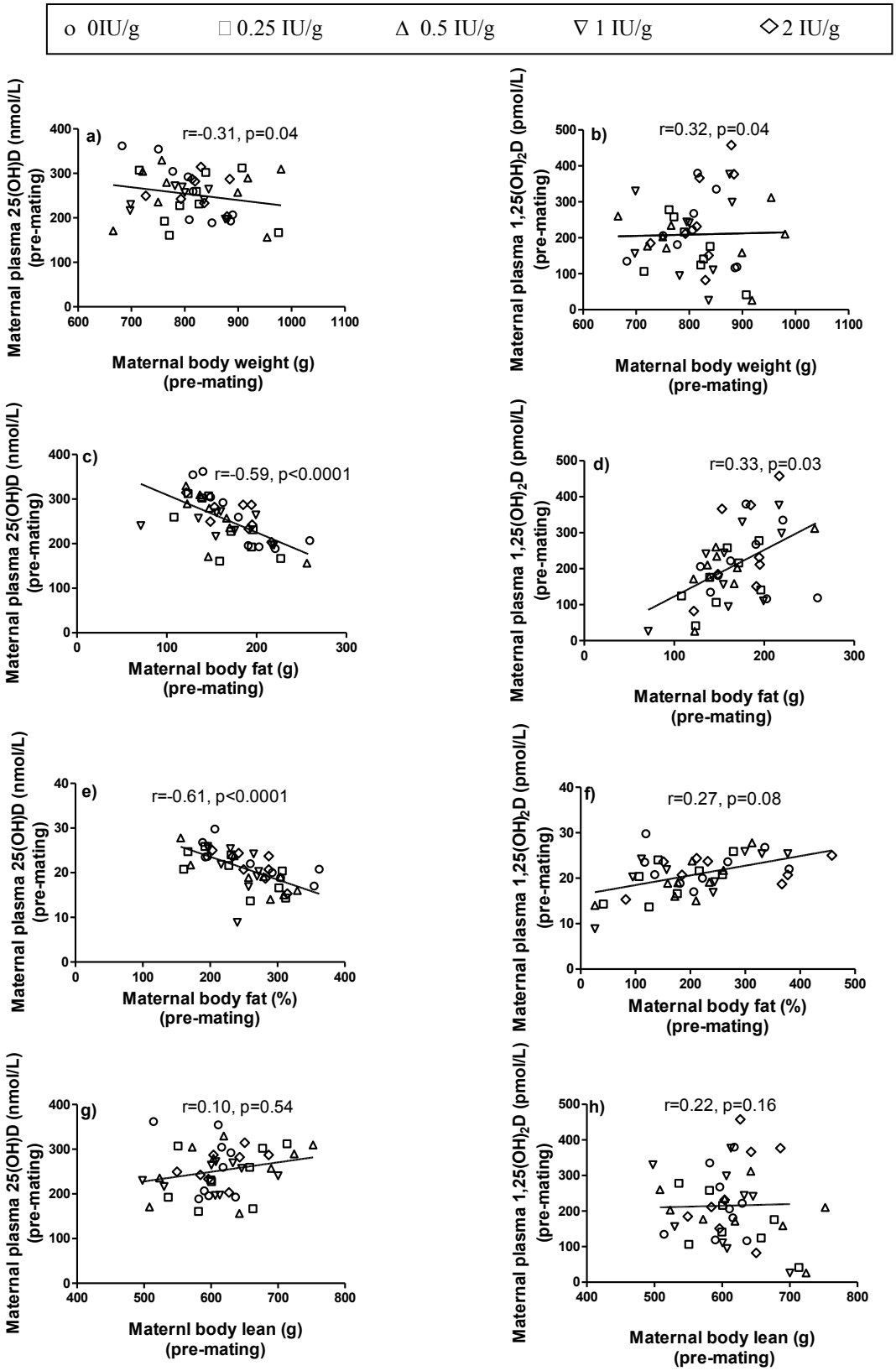

n=125
r=-0.35, p<0.0001
Pup plasma osteocalcin (nmol/L)
Pup plasma 1,25(OH)2D (pmol/L)
0
20
40
60
80
100
0
500
1000
1500
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g

Figure 5. 4.


o 0IU/g
□ 0.25 IU/g
Δ 0.5 IU/g
∇ 1 IU/g
◇ 2 IU/g
a)
r=-0.31, p=0.04
Maternal plasma 25(OH)D (nmol/L) (pre-mating)
Maternal body weight (g) (pre-mating)
b)
r=0.32, p=0.04
Maternal plasma 1,25(OH)2D (pmol/L) (pre-mating)
Maternal body weight (g) (pre-mating)
c)
r=-0.59, p<0.0001
Maternal plasma 25(OH)D (nmol/L) (pre-mating)
Maternal body fat (g) (pre-mating)
d)
r=0.33, p=0.03
Maternal plasma 1,25(OH)2D (pmol/L) (pre-mating)
Maternal body fat (g) (pre-mating)
e)
r=-0.61, p<0.0001
Maternal plasma 25(OH)D (nmol/L) (pre-mating)
Maternal body fat (%) (pre-mating)
f)
r=0.27, p=0.08
Maternal plasma 1,25(OH)2D (pmol/L) (pre-mating)
Maternal body fat (%) (pre-mating)
g)
r=0.10, p=0.54
Maternal plasma 25(OH)D (nmol/L) (pre-mating)
Maternl body lean (g) (pre-mating)
h)
r=0.22, p=0.16
Maternal plasma 1,25(OH)2D (pmol/L) (pre-mating)
Maternal body lean (g) (pre-mating)

BRIDGE 4

In Chapter 5, it was demonstrated that maternal glucose tolerance is unaffected by dietary vitamin D intake and status during pregnancy. However, high pre-pregnancy vitamin D status (25(OH)D concentrations ~ 250 nmol/L) which is also inversely related to whole body and abdominal fat may be protective against pregnancy induced glucose intolerance. According to the IOM report, there is also a lack of knowledge on the dose-response effect of dietary vitamin D during pregnancy on maternal and neonatal bone health. Due to ethical reasons and confounding factors in human pregnancy studies, an animal model is required for such research. The guinea pig model is appropriate for such studies due to having changes in $1,25(OH)_2D$ and OC metabolism similar to human pregnancy [55]. From the literature, in guinea pigs, vitamin D deplete (0 IU/g) vs replete (2.4 IU/g) diets during pregnancy resulted in maternal hypophosphatemia, lower total femur aBMD and distal femur cortical, but not Tb. mineral density, wider growth plates and higher osteoid thickness at the proximal tibia [13]. In this study, deficient vs replete fetus had hypercalcemia, hypophosphatemia, lower plasma OC, no difference in whole body BMC/weight but higher hypertrophic chondrocyte area and osteoid thickness at the proximal tibia [13]. In a similar model, a deplete (0 IU/g) vs standard vitamin D (1.2 IU/g) diet during pregnancy resulted in lower neonatal plasma OC, whole body and tibia BMC and strength, but no difference in whole body and femoral BMD and strength [79]. Whether there is a threshold at which vitamin D status during pregnancy affects bone health, cannot be distinguished from the available data. Therefore, the dose-response effect of dietary vitamin D was investigated on maternal and neonatal bone health outcomes in the pregnant guinea pig model. This study is presented in Chapter 6.

CHAPTER 6. MANUSCRIPT 4

Varying dosages of vitamin D during pregnancy do not affect maternal bone outcomes but result in differential and sex-dependent effects on neonatal femur trabeculae and cortical bone mineral tissue

Negar Tabatabaei[1], Celia Rodd[2], Richard Kremer[3], Zohreh Khavandgar[4], Monzur Murshed[4] and Hope Weiler[1]

Author affiliation:

[1]Dietetics and Human Nutrition, McGill University, Ste-Anne-de-Bellevue, QC, Canada

[2]Pediatrics, Montreal Children's Hospital, McGill University, Montreal, QC, Canada

[3]Medicine, Calcium Research Laboratory, McGill University, Montreal, QC, Canada

[4]Dentistry, McGill University, Montreal, QC, Canada

Corresponding author:

Dr. Hope Weiler, Tel.: 514-398-7905, Fax: 1 514 398 7739, e-mail:

hope.weiler@mcgill.ca

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6.1. Abstract

The dose-response effects of vitamin D status during pregnancy on maternal and neonatal bone health outcomes are not fully known. Therefore, we conducted a dose-response study by randomizing female pigmented guinea pigs (n=45, 4 mo) to five dosages of vitamin D_3 (0, 0.25, 0.5, 1 or 2 IU/g diet) from mating to delivery to establish if mineral homeostasis, bone biomarkers and bone mass across pregnancy and at 2 d postpartum in sows and 2-d old neonates were altered. A positive dose-response to dietary vitamin D was observed for maternal plasma 25-hydroxyvitamin D (25(OH)D) while 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) reached a plateau if vitamin D was ≥0.5 IU vitamin D/g diet. No differences in aBMD or vBMD or biomarkers were observed among maternal groups while the femoral mid-diaphysis stiffness (Young's modulus) was higher in the 1 IU/g vs the 0 group, only. Body weight was not different among neonatal groups whereas both 25(OH)D and $1,25(OH)_2D$ followed a positive dose-response to maternal dietary vitamin D and neonatal femur aBMD was highest in the 2 IU vs all except the 0 IU vitamin D group. Similarly, plasma minerals and OC were not different among neonatal groups, but plasma tDPD was lowest in the 1 and 0.25 groups and plasma total alkaline phosphatase (tALP) had an inverse U-shaped response in female neonates. At the neonatal distal femur and proximal tibia, response to maternal diet was U-shaped for Tb. BV/TV and vBMD with a phenotype suggestive of expansion of the growth plate in the 0 IU vs all groups. Mid-femoral diaphysis cortical TMD was highest in the male 0.5 IU/g diet group while in the females the 0 IU/d diet resulted in the lowest TMD. Femoral mid-diaphysis biomechanical tests were unaffected by diet and sex after adjustment for bone shape and size. A vitamin D dose-response during pregnancy does not significantly affect maternal bone health, but has a differential

and sex-dependent effect on neonatal femur Tb. and cortical bone volume and vBMD. Features similar to congenital vitamin D dependent rickets were observed in the neonatal femur in the 0 IU group. These data reinforce that maternal vitamin D intakes and status below recommendations may compromise bone health in the offspring and that higher intakes (>1 IU) and status are not advantageous.

Key words: vitamin D, dose-response, pregnancy, bone, guinea pig

6.2. Introduction

Vitamin D intake and status during pregnancy, the latter defined by serum 25(OH)D concentration, is an area of debate among different scientific societies [1-3] due to inconsistent and unknown thresholds of vitamin D status associated with fetal and maternal outcomes such as bone health. In the UK, some pregnant women with low (<50 nmol/L) serum 25(OH)D concentrations at 34 wk gestation had a 5 % increase in fetal metaphyseal femur splaying index, a feature consistent with congenital vitamin D dependent rickets [63]. Similarly, maternal serum 25(OH)D <28 nmol/L at 28-32 wk gestation was associated with a 3 % decrease in knee-heel length in Australian neonates [64]. Regarding mineralization of bone, vitamin D deficiency (25(OH)D <27.5 nmol/L) in Canadian infants was associated with lower whole body and femur, but not lumbar spine BMC relative to body weight [19]. However, in other studies with few deficient subjects, there was no association between maternal supplementation and plasma 25(OH)D with whole body and forearm BMC in Turkish [65] and Asian [66] neonates, respectively. Therefore, the available data is still conflicting regarding the effect and thresholds of maternal 25(OH)D in association with fetal and neonatal bone health outcomes, requiring further research.

Clinical trials of vitamin D supplementation during pregnancy have included daily supplements of 0 vs 400 [67] or 1000 IU/d [18, 66, 68] assessing biochemical and anthropometric outcomes. The only vitamin D dose-response pregnancy trial (400, 2000 and 4000 IU/d) caused a dose-response in maternal plasma 25(OH)D while plasma $1,25(OH)_2D$ reached a plateau with the two higher dosage intakes [10], but bone health outcomes have yet to be reported.

Vitamin D deficiency may lead to rickets in infants [70] and vitamin D is essential for bone development and higher BMD in adolescents [71]. Recommended intakes in pregnancy do not differ from non-pregnant adults since changes in bone are assumed to return to pre-pregnancy states if vitamin D status is adequate. Descriptive studies suggest that during pregnancy, women lose 2.1 to 5% of bone mass at Tb. rich sites [73-75] with almost 3-fold greater bone loss in adolescent vs adult pregnancy [75]. In one study, 20-34 y old mothers with the greatest calcaneal bone loss had infants with 9.7% higher aBMD [76]. However, there is a gap of knowledge on the effect of maternal vitamin D status during pregnancy on bone health particularly at ages where peak bone mass is not yet established, (i.e. adolescence).

In adolescent rats, vitamin D deplete (0-0.3 IU/d) diets during pregnancy resulted in lower maternal metaphyseal femur Tb. vBMD and mid-diaphysis cortical mineralized area [77], endosteal bone formation and femur length [78] compared to rats on replete (10-25 IU/d) diets. This suggests that Ca is mobilized from the bone by vitamin D independent pathways but vitamin D is required for normal bone mineralization. In another study in adolescent guinea pigs, vitamin D deplete (0 IU/g) vs high (2.4 IU/g) diets during pregnancy resulted in maternal hypophosphatemia, lower total femur aBMD and distal femur TMD and wider growth plates at the proximal tibia, suggesting impaired mineralization in the 0 IU group [13]. In this study, deficient vs replete fetuses had hypercalcemia, hypophosphatemia, lower plasma OC, no difference in whole body BMC/weight but higher hypertrophic chondrocyte area at the proximal tibia [13]. Vitamin D deficient rickets has been associated with wide growth plates and hypertrophic chondrocytes area, with loss of the columnar pattern [141]. In a similar guinea pig model, a deplete (0 IU/g) vs standard vitamin D (1.2 IU/g) diet during pregnancy resulted in lower neonatal

plasma OC, whole body and tibia BMC and strength, but no difference in whole body and femoral BMD and strength [79]. The threshold at which vitamin D status during pregnancy affects bone health, cannot be distinguished from the available data, showing the need for a vitamin D dose-response study during pregnancy.

Therefore, the objective of this study was to establish a dose-response to dietary vitamin D in a pregnant guinea pig model to study the maternal and neonatal bone health. The hypothesis was that both low (<50 nmol/L) and high (>125 nmol/L) maternal 25(OH)D concentrations would impair bone development while moderate concentrations (50-125 nmol/L) would result in optimal maternal and fetal bone health.

6.3. Method and Materials

6.3.1. Animal husbandry

Ethical approval was obtained from the McGill University Animal Care Committee. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care [264]. The guinea pig was selected due to its similarity to humans in regards to haemomonochorial placenta, Ca content per kg fat-free mass, serum OC and $1,25(OH)_2D$ changes during pregnancy and correlation of maternal and fetal vitamin D metabolites [13, 55, 202]. Pigmented (4 week old) guinea pigs (n=45 female, n=20 male) were purchased from Elm Hill Laboratories (MA, US) and housed singly with room temperature maintained at 21°C, lighting cycle from 0600 to 1800 h in a UVB free environment during the entire experiment to prevent endogenous synthesis of vitamin D.

6.3.2. Diet

The guinea pigs were fed a purified standard diet containing 1.2 IU/g diet vitamin D from 4 weeks to 16 weeks of age at which time they were mated. Decreased fertility

associates with vitamin D deficiency in rats and VDR null mice [209, 284], therefore dietary deficiency was induced only after mating. At mating sows were randomized to 1 of 5 diets using random numbers tables. The diets were isoenergetic **(Table 6.1)** from *Test Diet* (Division of LandO'Lakes Purina Feed) and equal in all ingredients except for vitamin D (0, 0.25, 0.5, 1 and 2 IU/g diet). The 1 IU/g vitamin D diet is the control diet [285]. All researchers and animal care staff were blinded to the diets by use of letter coding throughout the study. Diets were in pellet form and food and water were fed *ad libitum*. Food intake was measured weekly using a digital scale.

6.3.3. Protocol

Body weight was measured weekly using a digital scale (Mettler Toledo SB8001, MA, USA). Blood (heparinized) was sampled in a 3-h non-fed state (0900 h) from the saphenous vein of sows 1 d prior to mating, d 21 (end 1st trimester) and d 42 (end 2nd trimester) of pregnancy and 24-36 h post-delivery and pups at 24-36 h postpartum. Blood samples were collected in blood gas tubes pre-mating and postpartum (24-36 h) in the sows and 24-36 h postpartum in the pups for measurement of Ca^{2+}. Bone mass was measured 1 d prior to mating and 24-36 h postpartum in sows and at 24-36 h postpartum in pups using DXA. Necropsy was conducted at 24-36 h post-delivery in sows and at 24-36 h postpartum in pups after exsanguination under isoflurane anaesthetic and using cardiac puncture. This blood sample was collected equally into lithium heparin and serum tubes. All blood samples were kept on ice and centrifuged at 2000 g for 30 min at 4°C. Plasma and serum were collected and stored at -80 °C until analyses. At necropsy, tibias, femurs and lumbar vertebrae (1-4) were excised. Right-sided-long bones were cleaned of soft tissue, wrapped in normal saline-soaked gauze, and stored at -20°C until microarchitecture or biomechanical testing.

6.3.4. Biochemistry

Blood Ca^{2+} was measured with an ABL 825 series blood gas analyzer (Radiometer America, Copenhagen, Denmark) with all CVs were < 4.2 for all assays. Plasma concentration of total Ca, PO_4 and tALP were measured using an autoanalzyer (Beckman DxC600 California, USA). All CV% were <3.1%. Plasma total 25(OH)D was measured using an auto analyzer (total 25(OH)D, Liaison, Diasorin Inc.). This assay has a sensitivity of 10 nmol/L with CV% <5.3%. Plasma total $1,25(OH)_2D$ was measured using TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Warnex Bioanalytical Service, Laval, Canada). Samples were extracted using a proprietary procedure which includes a Diels-Alder derivatization. Intra-assay variability was ≤7.0 and inter-assay variability was ≤12.3 % for the selected control samples of guinea pig plasma calcitriol (80.6, 236.6, 608.4 and 1258.4 pmol/L). This assay has a sensitivity of 50 pmol/L. Plasma $1,25(OH)_2D$: 25(OH)D was calculated as a proxy for PTH [308]. Osteocalcin and tDPD were measured using ELISA kits (Metra Osteocalcin, Quidel Corporation, San Diego, CA., USA) with known cross-reactivity in guinea pig plasma. These assays have a sensitivity of 0.08 nmol/L and 0.5 nmol/L, respectively. In order to exclude inter-assay variability each ELISA kit was ran with all samples from n=1 sow belonging to each of the five diets, including all their time points (pre-mating, d 21, d42 and post-mating) and their relevant pup samples. All CV% for intra-assay variability were <5% and <8% for OC and tDPD, respectively.

6.3.5. Bone analyses

6.3.5.1. DXA (whole body composition and bone mass)

Anaesthesia was induced by isoflurane AErrane [286] at 5% in an induction chamber followed by maintenance at 2% delivered using a cone mask. During the

anaesthetized period, whole body and regional (lumbar spine vertebrae 1-4, tibia and femur) scans were performed on the animals using DXA and small animal software (Hologic 4500, QDR Version 12.3, Hologic, Bedford, Mass., USA), which provided *in vivo* data for BA, BMC and aBMD for all regions. All CV% were <0.5% for BMC and BMD of the lumbar spine phantom. All DXA intra-assay variability were <0.3% for triplicate values of one guinea pig. Nose-rump length was measured using a measuring tape to the nearest 0.1, while the animal was anaesthetized.

6.3.5.2. Micro-architecture and volumetric BMD (lumbar spine vertebrae 3, right femurs and tibias)

Trabeculae bone micro-architecture (BV/TV, Th. (Thickness), Sep. (Separation), No. (Number), structural model index (SMI), DA (degree of anisotropy), Conn. Den (Connectivity Density)) and vBMD of the distal femur, proximal tibia and vertebrae 3 (3rd vertebra) and the cortical bone (endosteal, periosteum and cortical volume) and TMD of the femur mid-diaphysis was examined using a micro-computed tomography (μCT) scanner (model 1174 SkyScan, Kontich, Belgium). The SMI is structure model index (SMI) which makes it possible to quantify the 3D characteristic of the Tb. structure in terms of the amount of plates and rod composing the structure [309]. The DA provides information on the anisotropic properties of the bone. The Conn. Den characterizes the 3D Tb. network [309]. The SMI and DA may be negatively associated with bone quality while Conn. Den may be positively associated with it. The Image acquisition was performed at 50 kV and 80 μA with a 0.7-degree rotation between frames. In addition to the type of filter to segment bone from background, each set of bone was tested for the best acquired image by manipulating the speed and resolution set up which were used consistently for each bone type. Images were reconstructed by InstaRecon (NRecon, version 1.6).

To delineate the Tb. from cortical bone at each bone site, ROIs were drawn manually and away from the endocortical surface. For each bone type at each site, different adaptive thresholding were tested and the one that would best separate the bone from non-bone tissue was chosen for analyses using the CTAn (1.10.1.3) software. All CVs were < 4% (typically 1-2%).

6.3.5.3. Femur splaying index (FSI)

The FSI of the human fetus has shown to be negatively correlated with maternal vitamin D status during pregnancy [63]. Therefore in order to further investigate the possible appearance of a bone phenotype similar to rickets we explored the neonatal FSI which was calculated as FSI (mm^2/mm) = femur distal metaphyseal cross section area /total femur length [63]. The distal femur metaphyseal CSA (mm^2) was acquired by the μCT from the nearest CSA to the distal metaphysis which had been selected as the first CSA for Tb. assessment on the μCT and the total femur length (mm) was also obtained from the μCT. All CVs were < 4%.

6.3.5.4. Biomechanical strength analyses (right femurs)

Bones were stored at -20°C and thawed to room temperature (23°C) according to standard methods and kept moist in saline-soaked gauze [310]. Cortical bone has an important role in bone strength and perhaps prediction of fracture risks [311]. Therefore, the biomechanical strength test was performed on the femur mid-diaphysis which is rich in cortical bone. Right femur mid-diaphysis was marked at half of the femur total length which was measured using a digital calliper (Fisher scientific,14-648-17) in the sows and pups. Femurs were tested for mechanical bone strength by the 3-point flexure method at the mid-diaphysis using the INSTRON (Model 5544, Incorporated, Canada) equipped with the Bluehill Software (version 2.1). The support span was set at 24 mm and 17 mm and the test extension rate at 1

mm/min and 0.5 mm/min in the sows and pups, respectively. After the data was obtained from the INSTRON, the moment of inertia (MMI) and mid-diaphysis diameter were acquired from the CSA located at the middle of the area analyzed for cortical bone assessment by the μCT. The maximum force (N), deformation (mm) and energy at break (mJ) were directly obtained from the INSTRON without adjusting for bone size. Then after, the stress, strain (%) and Young's modulus were calculated by adjusting the INSTRON data for bone size using the relevant equations [310, 312].

6.3.5.5. Histological assessments (left femurs)

Sows received calcein labeling (a fluorescent label for bone histomorphology; 15 mg/kg s.c in 1 ml) twice per day, 12 h apart, on two separate days, d 58 and d 66 of pregnancy. The calcein was prepared in normal saline, pH adjusted with bicarbonate and filtered for sterility. This dosing is standard practice for dynamic bone histomorphometry in guinea pigs [13, 202, 313]. In pregnancy, calcein in guinea pigs [13, 202] results in labeled fetal bone. This allowed dynamic and static histomorphometry to be conducted according to the standard guidelines of Parfitt in the left femur of guinea pig neonate [314].

For plastic sectioning, the left femur of guinea pig neonates were fixed overnight in 10% formalin, embedded in methyl methacrylate, and sectioned (7-μm thickness), followed by von Kossa, van Gieson as well as safronin-O staining. Images were taken at room temperature using a light microscope (DM200; Leica) with a magnification of 5× for images with von Kossa and van Gieson staining and 10× for those with safronin-O staining. All histological images were captured using a camera (DP72; Olympus), acquired with DP2-BSW software (XV3.0; Olympus), and processed using Photoshop (Adobe).

6.3.6. Statistical analyses

6.3.6.1. Sample size on multiple outcome measures

The selected sample size of n=9 pregnancies per diet group was based on an alpha of 0.05 and power of 80 and whole body and regional BMC and BMD for pups and sows. The sample size based on % change in tibia aBMD of 12.5 % from a previous study (unpublished) was n=7 for the sows. We studied 9 in the event of unsuccessful pregnancy or delivery complications. For the pups, the minimum sample is n=21 based on 0.40 g of whole body BMC [79]; we anticipated an average of 3 pups per litter, which was anticipated to lead to an estimate of n=27.

6.3.6.2. Data analysis

All data were audited for accuracy and further screened using scatter plots to identify potential outliers and for normality testing using SAS (version 9.1, SAS, Inc., Cary, NC., USA). If normality testing failed using the Kolmogorov and Smirnov test, data were transformed by use of logarithm, squaring, inverse values etc. Differences between the groups were tested using diet and time as fixed effects in a MIXED model ANOVA including diet by time interactions across pregnancy for the sow plus the random effect of block, sow/pup and sex of the pups. *Post hoc* testing for significance was conducted using a Tukey's test (adjusted for multiple comparisons) with a $p \leq 0.05$. Pearson and Spearman correlations were performed to investigate the relationship between normal and non-normal variables, respectively. Non-linear regression (dose-response inhibition equation) was used for the relationship between plasma $1,25(OH)_2D$: 25(OH)D and 25(OH)D.

6.4. Results

All sows delivered healthy newborn pups except for 1 in the 2 IU/g vitamin D diet that died at time of mating. There were no differences among diet groups in

weekly weight gain during the first (22.0±1.6 g/wk, p=0.68), second (33.3±2.7 g/wk, p=0.31) or third (73.9±3.4 g/wk, p=0.44) trimester or in total pregnancy weight gain (423.4±15.5 g, p=0.09). Length was not different among groups but increased over pregnancy (5.6 ± 0.4 %, n=44, p<0.0001). In addition, no significant differences were observed between diet groups in weekly food intake (305.2±8.3 g/wk, p=0.34) and weekly food intake per body weight (0.3±0.0 g/kg/wk, p=0.37) during pregnancy.

In total, n=125 healthy pups and n=13 stillborn were born. There were no significant differences among vitamin D containing groups in the number of healthy (median of 25, range 18-30) and stillborn (median of 3, range 1-5) neonates (χ^2,df=5.27,4, p=0.26). There were no differences in litter size among diet groups (median of 3, range 1-5). There were no significant effects of diet or diet*sex interaction for body weight or length 2 d postpartum, but male pups weighed more than females (118.8±2.0 vs 111.2±1.9 g, p=0.02) and were longer (16.3±0.1 vs 16.0 ±0.1 cm, p=0.02).

6.4.1. Vitamin D metabolites

6.4.1.1. Plasma 25(OH)D

There was a significant effect of diet (p<0.0001) and time across pregnancy (p<0.0001) and their interaction (p<0.0001) on maternal plasma 25(OH)D **(Figure 6.1 (a))**. There were no significant differences in maternal plasma 25(OH)D among groups at baseline but pregnancy caused a significant decrease in concentrations as early as d21 with no further decreases at d42. Significantly lower concentrations were observed at 2 d postpartum vs baseline and d21 in all diet groups except 2 IU/g group. Maternal plasma 25(OH)D was significantly lower in the 0 and 0.25 vs 1 IU/g group and all vs the 2 IU/g group at 2 d postpartum (**Figure 6.1(a)).** A similar dose-response (p<0.0001) was observed in neonatal plasma 25(OH)D at 2 d postpartum

among all groups except the 0 vs 0.25 groups **(Figure 6.1 (b))**. There were no significant differences between the sexes (p=0.32) and no diet*sex interaction (p=0.74).

6.4.1.2. Plasma 1,25(OH)$_2$D

There was a significant effect of diet (p<0.0001) and time across pregnancy (p<0.0001) and their interaction (p<0.0001) on plasma 1,25(OH)$_2$D. There were no significant differences in maternal plasma 1,25(OH)$_2$D among groups at baseline but pregnancy resulted in a significant decrease in plasma 1,25(OH)$_2$D in the 0 IU/g group and increase in concentrations in the 0.5 and 1 IU/g groups as early as the end of the first trimester but not until d42 in the 2 IU/g group while no changes were observed in the 0.25 IU/g across these time-points. No further changes were observed at d 42 vs d 21 in any groups but a significant decrease was observed in the 0.5 and 2 IU/g group at 2 d postpartum vs d42 while this decrease was only significant as compared to baseline values in the 0 and 1 IU/g groups and no differences were observed in any groups at 2 d postpartum vs d21. Maternal plasma 1,25(OH)$_2$D was only significantly lower in the 0 vs all other groups 2 d postpartum **(Figure 6.1(c))**. There was a significant dose-response (p<0.0001) in neonatal plasma 1,25(OH)$_2$D at 2 d postpartum among all groups except the 0.5 vs 1 **(Figure 6.1 (d))**. There were no significant differences among the sexes (p=0.48) and no diet*sex interaction (p=0.34).

There was a significant positive correlation between plasma 25(OH)D and 1,25(OH)$_2$D in the sow (r=0.38, p=0.01) and the pup (r=0.41, p<0.0001) at 2 d postpartum. However, the correlation was no longer significant when all pregnancy time-points were included (r=0.11, p=0.16). Maternal and neonatal plasma 25(OH)D

(r=0.87, p<0.0001) and 1,25$(OH)_2$D (r=0.59, p<0.0001) were highly correlated at 2 d postpartum.

Neonatal plasma 25(OH)D were significantly lower (-56 to -69 %), p<0.0001 than maternal concentrations across all groups. However, neonatal plasma total 1,25$(OH)_2$D were mainly higher (-11 to 78 %), p=0.04 than maternal concentrations among diet groups.

6.4.1.3. Plasma 1,25$(OH)_2$D: 25(OH)D (PTH Proxy)

There was a significant inverse relationship between maternal plasma 25(OH)D (nmol/L) and 1,25$(OH)_2$D:25(OH)D ratio (r=-0.72, p<0.0001) at 2 d postpartum **(Figure 6.1(e))**. The same inverse relationship was observed in pups (r=-0.63, p<0.0001) **(Figure 6.1 (f))**.

6.4.2. Plasma minerals and bone biomarkers

There was no significant effect of diet and diet*time interaction on maternal plasma minerals and bone biomarkers, however there was a significant effect of time on all plasma minerals and bone biomarkers as shown in **Supplemental Table 6.1** Maternal plasma total Ca, PO_4, OC significantly decreased by d 42 of pregnancy and plasma total Ca, OC and tALP and blood Ca^{2+} were significantly lower and plasma tDPD was significantly higher at 2 d postpartum vs pre-mating **(Supplemental Table 6.1)**. A weak negative correlation was observed between litter size and maternal plasma total Ca (r=-0.31, p=0.04) but not blood Ca^{2+} (r=-0.27, p=0.14) at 2 d postpartum.

There were no significant correlations between maternal plasma 25(OH)D with plasma mineral concentrations (data not shown) but a significant weak negative correlation was observed between maternal plasma 1,25$(OH)_2$D and PO_4 concentrations (r=-0.31, p=0.04) at 2 d postpartum. There were no significant

correlations between maternal plasma 25(OH)D and $1,25(OH)_2D$ with bone biomarkers at 2 d postpartum (data not shown). However, there was a positive correlation between % change in maternal plasma $1,25(OH)_2D$ and tALP from baseline to 2 d postpartum (r=0.37, p=0.02).

Neonatal plasma minerals were not significantly different according to diet, sex and diet*sex interaction at 2 d postpartum as shown in **Supplemental Table 6.1**. There was no effect of diet on neonatal plasma OC at 2 d postpartum (p=0.58) while it was higher in male vs female neonates (p=0.02) **(Supplemental Table 6.1)**. Plasma tDPD concentrations were significantly lower in neonates of sows on the 1 IU vitamin D diet vs the 0, 0.5 and 2 IU diets (p=0.04) **(Figure 6.2 (a), Supplemental Table 6.1)**. Plasma tALP was significantly higher in male vs female guinea pigs (p=0.002) with a significant interaction between diet and sex (p=0.01) **(Supplemental Table 6.1)** such that female neonates on the 0.5 diet had significantly higher plasma tAPL as compared to other groups **(Figure 6.2(b)).**

There was a significant weak positive correlation between neonatal plasma 25(OH)D with total Ca (r=0.33, p=0.0003) and Ca^{2+} (r=0.27, p=0.01) and a significant negative correlation of neonatal plasma $1,25(OH)_2D$ with PO_4 (r=-0.32, p=0.0009) at 2 d postpartum. There were no significant correlations between neonatal plasma 25(OH)D with bone biomarkers at 2 d postpartum (data not shown). There was a positive correlation between neonatal plasma $1,25(OH)_2D$ with tALP (r=0.35, p=0.0002) and a negative correlation with OC (r=-0.26, p=0.004) at 2 d postpartum**.**

There were no significant correlations between maternal-neonatal plasma minerals and bone biomarkers (data not shown). In addition, neonatal vs maternal, plasma total Ca concentrations were not significantly different (p=0.41) whereas blood Ca^{2+} was significantly lower (1-6 %) (p<0.02) and plasma PO_4 concentrations

were significantly higher (12-52 %) ($p<0.0001$) in neonatal vs maternal plasma at 2 d postpartum. Neonatal plasma OC and tALP concentration were respectively 8.5-10 % and 10-13 % higher than maternal concentrations at 2 d postpartum. There were no significant differences in neonatal vs maternal plasma tDPD concentrations.

6.4.3. Whole body, vertebrae 3, femur and tibia DXA parameters

There were no significant effects of diet and diet*time interactions on maternal whole body, lumbar spine vertebrae 3, femur and tibia area, BMC and aBMD at baseline and at 2 d postpartum **(Supplemental Table 6.2).** In addition, there were no significant effects of diet on maternal percent change of these outcomes from baseline to postpartum (data not shown). While whole body, femur and tibia area and BMC increased over pregnancy, only tibia and femur aBMD were increased. In contrast, vertebrae 3 area increased, BMC was not different and aBMD was lower at 2d postpartum **(Supplemental Table 6.2)**.

There were no significant correlations between maternal plasma 25(OH)D and $1,25(OH)_2D$ with whole body and regional DXA measurements at 2 d postpartum (data not shown). There were no significant effects of diet on neonatal area, BMC and aBMD at the whole body, lumbar spine vertebrae 3 and tibia while femur aBMD was significantly higher in the 2 vs 0.25, 0.5 and 1 diets but not 0 IU/g diet **(Figure 6.2 (c), Supplemental Table 6.2).** In addition, there were no significant effects of sex or diet and their interaction for any of the stated parameters except for a higher tibia area in male vs female pups ($p=0.04$) **(Supplemental Table 6.2)**. Further adjustments for whole body weight, length and lean mass did not significantly change maternal or neonatal whole body aBMD results (data not shown).

There was a significant weak positive correlation between neonatal plasma 25(OH)D and whole body BMC/lean ($r=0.20$, $p=0.03$) and the lumbar vertebrae 3

area (r=0.20, p=0.03)**.** In addition, there was a significant negative correlation between neonatal plasma 1,25$(OH)_2$D and tibia BMD (r=-0.23, p=0.01) and BMC/kg at the whole body (r=-0.25, p=0.006), femur (r=-0.29, p=0.001) and tibia (r=-0.25, p=0.005) but not the vertebrae 3 (r=0.03, p=0.71) as measured by the DXA .

6.4.4. Micro-architecture, volumetric BMD (lumbar spine vertebrae 3, right Femurs and tibias) and femur splaying index

Representative reconstructed μCT images of coronal slice of maternal distal femur and transversal section of distal femur metaphyseal Tb. and mid-diaphysis cortical bone are shown in **Supplemental Figure 6.1(a)** from each diet group. There were no significant differences in maternal vertebrae 3, distal femur and proximal tibia μCT Tb. parameters except for DA at the vertebrae 3 at 2 d postpartum which was significantly higher in the 2 IU group vs all other groups except the 0.25 IU (0 IU, n=9: 2.04±0.70; 0.25 IU, n=9: 3.06±0.76; 0.5 IU, n=9: 1.28±0.99; 1 IU, n=9; 1.94±0.80; 2 IU, n=8: 4.76±0.80, p=0.005)**(Supplemental Table 6.3).** There were no significant differences in maternal femur cortical TMD at mid-diaphysis among diets (p=0.91).

There were no significant correlations between maternal plasma 25(OH)D and 1,25$(OH)_2$D with regional μCT measurements (data not shown) except for a positive correlation between maternal plasma 25(OH)D and DA at the vertebrae 3 Tb. (r=0.38, p=0.01) and femur cortical volume (r=0.35, p=0.02) at 2 d postpartum**.**

There were no differences in neonatal lumbar spine vertebrae 3 μCT Tb. parameters **(Supplemental Table 6.3)**, except for DA which was significantly higher in the 1 IU group vs all groups except the 0.25 IU group (0 IU (n=33): 1.58±0.03; 0.25 IU (n=26): 1.60±0.04; 0.5 IU (n=30): 1.54±0.04; 1 IU (n=27): 1.69±0.04; 2 IU (n=23): 1.60±0.04, p=0.05). In addition, Tb. Sep. at the vertebrae 3 was significantly

higher in male vs female neonates (p=0. 05) **(Supplemental Table 6.3).** At the distal femur a significant U-shaped curve was observed among vitamin D diet groups for Tb. BV/TV, No., Conn. Den. and vBMD and a significant inverse U-shaped curve for SMI among vitamin D diet groups at the distal femur Tb. **(Figure 6.3(a-e), Supplemental Table 6.3)**. The neonatal cortical volume at the femoral mid-diaphysis was significantly higher in the 0 vs 0.25, 0.5 and 1 IU diets but not 2 IU/g diet (p=0.04) **(Figure 6.3(f), Supplemental Table 6.3).** Neonatal femoral mid-diaphysis cortical TMD was significantly different among diet groups (p=0.05) and was significantly higher in female vs male neonates (p=0.01) with a significant diet*sex interaction (p=0.003) such that the females on the 0 IU/d diet had significantly lower TMD vs all other groups while in the male the 0.5 IU/g diet resulted in the highest TMD **(Figure 6.3(g), Supplemental Table 6.3)**.

Representative reconstructed μCT images of coronal slice of distal femur and transversal section of distal femur metaphyseal Tb. and mid-diaphysis cortical bone are shown for female **(Supplemental Figure 6.1(b))** and male **(Supplemental Figure 6.1(c))** neonates. Although no significant effects of diet (24.37±0.10, p=0.68), sex (p=0.25) and diet*sex (p=0.59) were observed on neonatal femur length, there was a significant effect among vitamin D (0-2 IU/g diet) groups (21.80±0.53, 20.10±0.60, 19.18±0.67, 19.61±0.61 and 19.75±0.69, p=0.02, respectively) at the femoral metaphyses CSA which was significantly higher in the 0 vs all groups except the 0.25 group. Femoral metaphyses CSA was significantly higher in males vs females (21.09±0.38 vs 19.09±0.42, p=0.004, respectively). The FSI which is the ratio of these two latter variables was significantly affected by diet (p=0.01) such that it was significantly lower in the 0.5 IU group compared the 0 group only and was also significantly higher in male vs female neonates (p=0.004) (horizontal J-shaped).

There was no interaction between diet and sex (p=0.49) **(Figure 6.3 (h))**. Similar to the distal femur Tb. at the proximal tibia U-shaped trends were observed which were statistically significance for BV/TV and Tb. N. and vBMD **(Figure 6.4 (a-c), Supplemental Table 6.3)**. There were no differences between sexes nor diet*sex interactions for any of the except for those already stated.

There was no significant correlation between neonatal 25(OH)D and μCT outcomes. However, there was a negative association between neonatal plasma $1,25(OH)_2D$ with femur Tb. No. (r=-0.22, p=0.01), DA (r=-0.23, p=0.01) and femur cortical volume (r=-0.20, p=0.03). In addition, there were no significant correlations between neonatal 25(OH)D or $1,25(OH)_2D$ with neonatal FSI (data not shown).

6.4.5. Correlation between DXA and μCT BMD at regional sites

Significant correlations were observed between maternal aBMD and vBMD at vertebrae 3, femur and tibia acquired by the two methods (r=0.30,p=0.045),(r=0.56,p=0.0001) and (r=0.43, p=0.004) respectively. In the neonates, we also observed significant correlations between aBMD values obtained by DXA and vBMD by μCT for vertebrae 3 (r=0.38 and p<0.0001), femur (r=0.72, p<0.0001) and tibia (r=0.59, p<0.0001).

6.4.6. Biomechanical femur strength

There was no significant effect of diet on maternal femur mid-diaphysis 3-point bending strength test features at 2 d postpartum except for a higher Young's modulus (stiffness) in the 1 vs 0 diet group, only (p=0.04) **(Figure 6.5(a), Supplemental Table 6.4)**.

In the neonates, the maximum force at the femur mid-diaphysis was significantly higher in the 0 vs 0.5 and 1 IU groups (p=0.01) at 2 d postpartum **(Figure 6.5(b), Supplemental Table 6.4)** with no effects of sex (p=0.91) and

diet*sex interaction (p=0.74) **(Supplemental Table 6.4)** but the effect of diet was no longer significant as the maximum stress which is the adjusted maximum force for femoral shape and size (p=0.26) and neither were the effects of sex (p=0.39) and diet*sex interaction (p=0.35) **(Supplemental Table 6.4).** In addition, maximum deformation (p=0.001), cross-section diameter (p=0.002) and strain (p<0.0001) were significantly lower and Young's modulus (stiffness) (p=0.02) was higher in female vs male neonates at the femoral mid-diaphysis at 2 d postpartum but there were no effects of diet and sex*diet interaction on these features **(Supplemental Table 6.4).** There were no effects of diet (p=0.86), sex (p=0.08) and diet*sex interaction (p=0.24) on neonatal femur mid-diaphysis energy at break (toughness) **(Supplemental Table 6.4)**.

Maternal femur mid-diaphysis TMD and was positively correlated with maximum stress (r=0.34, p=0.03) and negatively correlated to maximum strain (r=-0.38, p=0.01) at 2 d postpartum. No other significant correlations were observed between maternal femur mid-diaphysis TMD, plasma 25(OH)D and $1,25(OH)_2D$ with any other femur mid-diaphysis 3-point bending test features at 2 d-postpartum (data not shown).

Neonatal femur mid-diaphysis TMD and was positively correlated with maximum force (r=0.56, p<0.0001), stress (r=0.44, p<0.0001) and Young's modulus (r=0.35, p<0.0001) and negatively correlated to maximum deformation (r=-0.37, p<0.0001) and strain (r=-0.24, p<0.01) at 2 d postpartum. There were no significant correlations between neonatal plasma 25(OH)D and femur mid-diaphysis 3-point bending test features at 2 d-postpartum (data not shown). However, neonatal plasma $1,25(OH)_2D$ was positively correlated with energy at break (r=0.27, p=0.003 and negatively correlated with maximum force (r=-0.33, p=0.002) and stress (r=-0.19,

p=0.04) but other correlations with femur mid-diaphysis 3-point bending test features at 2 d-postpartum were not significant (data not shown).

6.4.7. Histological assessments

Representative histological images of coronal slice of distal femur are shown for female **(Figure 6.6(a))** and male **(Figure 6.6(b))** neonates. Visually, there is an expansion of the growth plate in the 0 IU vs all other groups. This phenotype is more evident in female vs male neonates.

6.5. Discussion and conclusions

The maternal and neonatal dose-response of plasma 25(OH)D to dietary vitamin D in this study closely parallels that recently reported in human pregnancy [10]. The striking similarity between maternal and neonatal vitamin D status demonstrates that maternal-fetal transfer is not saturable up to 250 nmol/L of maternal plasma 25(OH)D concentrations in the guinea pig. Even though, neonatal plasma 25(OH)D concentrations were lower than maternal levels, in accordance with previous studies [10, 13, 67, 79, 199, 204], some values at 2 d postpartum reached 125 nmol/L in the 2 IU group. Furthermore, maternal plasma $1,25(OH)_2D$ plateaus at high intakes of vitamin D whereas it follows a dose-response in the neonates. The positive correlation between plasma 25(OH)D and $1,25(OH)_2D$ in the sows as well as in the pups is similar to the recent vitamin D trial in human pregnancy [10] and Korean neonates [217]. Even though plasma $1,25(OH)_2D$ of human neonates [105] and guinea pig fetus [13, 55] are typically lower than maternal concentrations, neonatal plasma $1,25(OH)_2D$ is almost double that of fetal concentrations in guinea pig [55], which may explain the higher pup values observed in this study at delivery. Such elevated concentrations are likely a reflection of renal fetal synthesis of the active form of vitamin D or decrease in its clearance or both [105].

The implication of a high 25(OH)D and $1,25(OH)_2D$ include suppression of PTH [315] and anticipated responses in bone [316]. It should be noted that during pregnancy, there is an increase in circulating maternal and fetal PTHrP which binds to receptors similar to those of PTH on the bone [105]. Using the proxy PTH index, the results suggest that maternal PTH concentration would plateau only at very high vitamin D status, exceeding 200 nmol/L of circulating 25(OH)D but in contrast to previous vitamin D deplete vs replete studies [13, 78], no differences in regional BMC, aBMD, vBMD were observed among diet groups. Interestingly, in the neonate, the proxy for PTH plateaus at much lower 25(OH)D concentrations (<50 nmol/L), especially in the 0 IU group. In contrast to previous reports [79], neonatal whole body BMC and growth were not suppressed in the deficient group. However, a positive association was observed between neonatal plasma 25(OH)D with whole body and regional aBMD and vertebrae 3 and femur vBMD but a negative association was observed between neonatal plasma $1,25(OH)_2D$ with whole body and long bone BMC/kg only. The latter may suggest an anti-proliferative effect of $1,25(OH)_2D$ on growth in the long bone but not the vertebrae, perhaps due to faster appendicular than axial growth during this phase of life [317].

The volumetric and micro architecture assessment of maternal bone was not affected by dietary vitamin D during pregnancy. However, lower neonatal femur cortical TMD possibly due to secondary hyperparathyroidism [318] and higher FSI were observed in the 0 IU group which was also observed as expansion of the growth plate in the histomorphometry images at the distal femur. Such phenotype is consistent with the early phases of congenital rickets and is similar to the higher FSI in fetus of vitamin D deficient (<50 nmol/L) mothers at late gestation [63]. The higher FSI in male vs female, is in accordance with some studies showing higher

prevalence of rickets phenotypes in male infants [319], which may be related to the difference in growth rate rather than sex differences in vitamin D metabolism. The higher distal femur and proximal tibia Tb. vBMD observed in the 0 group may be as a result of the possible secondary hyperparathyroidism in this group [318]. This should therefore not be interpreted as a positive outcome since low $1,25(OH)_2D$ induced a phenotype suggestive of expansion of the growth plate in this group [161, 320]. Low $1,25(OH)_2D$ is associated with high PTH and/or PTHrP which can lead to slow rate of conversion of proliferative to hypertrophic chondrocytes and therefore increased proliferative chondrocyte zone [321] which may have also contributed to expansion of the GPW in the deficient group. However, higher $1,25(OH)_2D$ in the offspring from the 2 IU group was accompanied with higher distal femur and proximal tibia Tb. vBMD which may be a result of possible hypoparathyroidism [322] that is also known to increase the bone Tb. component [318]. The lower femur cortical TMD in the males and not the females of the 2 IU group is also in line with the sex differences observed on the FSI. In addition, the lack of difference in Tb. bone at the lumbar spine may suggest that these effects are mainly observed at in bones with growth plates.

The bone quality is assessed by biomechanical tests and the only study investigating the dose-response effect of vitamin D on bone biomechanics is limited to growing male rats which concluded no effects on tibia strength [323]. The biomechanical data are suggestive of a lower maternal bone quality in the deficient (0) group and beneficial effects on in the 1 IU diet, but not in the higher vitamin D group (2 IU). In addition, in accordance with the neonatal FSI and femur vBMD outcomes in the 0 IU group, the femoral maximum force was higher in the 0 vs 0.5 and 1 IU groups which again is not a positive outcome since it was no longer

significant when adjusted for bone geometry which is similar to previous reports [79]. Also, bone strength data suggest that the femoral flexibility (strain) was lower but stiffness was higher in female vs male offspring, perhaps explained by the lower cross section diameter and higher TMD in the female vs male, respectively. In addition, the femoral cortical mineral density was positively correlated with stress and negatively correlated with strain in both the sows and pups, which is in line with previous reports in long bones of humans [311] and monkeys [324].

In the human neonate assessment of bone health is not readily available resulting in reliance on biochemical measures of Ca and bone metabolism. Despite the neonatal dose-response in plasma vitamin D metabolites, plasma mineral homeostasis was maintained and OC was similar among diet groups which is in contrast to other guinea pig studies using 0 vs 1.2 [79] or 2.4 IU [13] of maternal dietary vitamin D. However, the negative correlation observed between neonatal plasma $1,25(OH)_2D$ and OC at post-natal which is similar to mice [293], may be a result of interaction of $1,25(OH)_2D$ and bone-derived growth factors [294]. Even though, the bone formation biomarker, tALP is not specific to bone, it is known to be highly correlated ($r>0.9$) with the bone-derived ALP in healthy states [325]. There was an inverse U-shaped response only in female plasma tALP and a positive correlation was observed between neonatal plasma $1,25(OH)_2D$ and tALP which is similar to human neonates [326]. Overall, higher plasma tALP and OC in male vs female neonates may explain the observed neonatal sex-dependent bone differences. In addition, the lower neonatal plasma tDPD in the 1 IU and 0.25 IU vs other diet groups is suggestive of lower bone resoprtion in this group. Also, no signs of maternal hypophosphatemia were observed which is in contrast to previous reports in vitamin D deficient models at late-gestation [13]. The weak negative correlation

between plasma $1,25(OH)_2D$ with PO_4 in the sow and neonate at 2 d postpartum may suggest that FGF23 and/or PTH actions along with high plasma $1,25(OH)_2D$ lead to inhibition of PO_4 renal reabsorption [39]. These results show that dose-response to dietary vitamin D during pregnancy results in plasma mineral homeostasis at the expense of changes in neonatal BMD and microarchitecture and with no significant maternal effects.

There are many factors that may explain some of the differences in the observations in this study as compared to other vitamin D deplete vs replete studies in rats [78] and guinea pigs [13, 79]. The dietary Ca and P content of the present study (1.1% Ca and 0.65 % P) was higher than a previous guinea pig study (0.96 % Ca and 0.49 % P) which compensates for some of the detrimental effects of vitamin D deficiency on bone phenotype [13]. In addition, baseline 25(OH)D concentrations which affect the dose-response to vitamin D intakes [327] were similar but high (~250 nmol/L) among all diet groups and the sows were randomized to diets at mating vs several weeks prior to mating [78] to resemble winter pregnancy, therefore plasma 25(OH)D reached the intended concentrations only by late gestation, suggesting that perhaps the ~70 d duration of pregnancy was too short to detect some of the differences in bone.

Similar to humans, pregnancy induced a decrease in maternal vitamin D status along with an increase in $1,25(OH)_2D$ as early as the end of 1^{st} trimester [328]. This was accompanied by a decrease in maternal plasma total Ca similar to human pregnancy [105] but the observed decrease in plasma PO_4 and blood Ca^{2+} is different from humans due to larger litter size in small animals [105]. Similar to previous reports a decrease in plasma OC was observed [55] which may be due to increased uptake of OC by the placenta [329]. However, no changes were observed in tALP and

tDPD during pregnancy. Nonetheless, the increase in plasma PO_4 at 2 d postpartum is similar to previous reports [105, 202] which accompanied a further reduction in OC and increase in tDPD, probably as a result of hormonal changes that initiate upon lactation resulting in increased bone resorption [108]. Also, the decrease in plasma tALP after delivery may be a result of no further placental production [329]. These biochemical changes across pregnancy accompanied an increase in maternal long bone and decrease in vertebrae 3 aBMD, in accordance with these changes in rats [330] and humans [331], suggesting that immediate demands for Ca homeostasis draw upon Tb. bone of vertebrae whereas mineral stores for later use in lactation might reside in long bone in the adolescent pregnant model in this study in which growth plates are not yet closed [270].

The animal model used in this study is not without limitations, including the large litter size (median of 3 [1-5]) and the higher neonatal to maternal whole body Ca content in guinea pigs (~14 %) vs humans (~3 %) [19]. In addition, we could not measure PTH, PTHrP, FGF23 and other metabolites of vitamin D such as $24,25(OH)_2D$ to further explain mechanisms and their possible role in bone health.

In conclusion, providing dietary vitamin D ranging from deficient to more than recommended results in a parallel response in vitamin D status, but is not reflected in maternal mineral or bone health outcomes. The fetus responded with higher Tb. density of long bone at both low and high maternal vitamin D intakes but features of congenital rickets were noted in the deficient group. The neonatal response was differential and sex-dependent at the cortical femur with lower TMD in the male neonates at the low and high maternal vitamin D intakes and lower TMD in the female neonates of the deficient group only while bone quality assessed by strength was unaffected. These data reinforce that maternal vitamin D intakes (<0.5

IU/g) and status (<50 nmol/L) may compromise bone health in the offspring and that higher intakes (>1 IU/g) and status (>125 nmol/L) are not advantageous.

6.6. Acknowledgments

N.-T. contributed in the design of this study, performed biochemical assessments, interpretation of data, statistical analyses and article writing. C.-R. reviewed the manuscript and provided on interpretation of the results. R.-K. reviewed the manuscript and provided comments. H.-W. contributed to the original thought and design of this study and undertook supervision of the biomarker measures and critical review of the manuscript. She is in receipt of a CRC *tier II* in Nutrition, Development and Aging and acknowledges are also towards the CFI infrastructure. Dr. Weiler wrote the grant along with C.-R., R.-K. and N.-T. which was approved and funded by CIHR. Thanks are also due to members of Dr. Weiler's laboratory, Dr. Jinping Zhao, Paula Lavery, Sherry Agellon and Ivy Mak for their great assistance during this animal study.

6.7. Figure legends

Figure 6.1. Plasma 25(OH)D (nmol/L) **(a-b)**, 1,25$(OH)_2$D (pmol/L) **(c-d)** concentrations and in sows randomized to diets containing different vitamin D dosages across pregnancy and their neonates at 2 d postpartum, respectively. Plasma 25(OH)D vs 1,25$(OH)_2$D: 25(OH)D **(e-f)** correlations in sows and neonates at 2 d postpartum. Values are means ± SEM. **Figure a, b, c, d**: Mixed model ANOVA, Tukey, *post-hoc* test. **Figure e, f:** Spearman correlation, adjusted for litter size and block. Statistical significance, $p \leq 0.05$. Sows were randomized to diets containing different vitamin D dosages during pregnancy: 0 IU/g (n=9), 0.25 IU/g (n=9), 0.5 IU/g (n=9), 1 IU/g (n=9) and 2 IU/g (n=8). Neonate, n per diet group is presented on graphs. **Figure a, c:** Different lower superscripts are representative of statistical interaction for diet*time. **Figure b, d:** Different upper superscripts are representative of statistical significance between diet groups. There were no significant effects of sex and diet*sex on plasma 25(OH)D and 1,25$(OH)_2$D.

Figure 6.2. Neonatal **a)** plasma total DPD **b)** plasma tALP **c)** femur aBMD at 2 d postpartum in pups at 2 d postpartum born from sows randomized to diets containing different vitamin D dosages during pregnancy (0, 0.25, 0.5, 1 and 2 IU/g diet). Values are means± SEM statistically adjusted for litter size and block. Statistical analyses were performed by mixed model ANOVA and Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$. n is presented in parenthesis on each bar graph. Different upper superscripts are representative of statistical significance between diet groups and lower superscripts are representative of statistical interaction for diet*sex.

Figure 6.3. Femoral distal metaphysis and mid-diaphysis assessed by μCT in neonates at 2 d postpartum according to dietary intake of vitamin D during pregnancy (0, 0.25, 0.5, 1 and 2 IU/g diet): **a)** metaphysis Tb. BV/TV **b)** metaphysis Tb. N. **c)**

metaphysis Tb. SMI **d)** metaphysis Tb. Conn. Den. **e)** metaphysis Tb. vBMD **f)** mid-diaphysis cortical volume **g)** mid-diaphysis TMD **h)** metaphysis splaying index. Values are means± SEM statistically adjusted for litter size and block. Statistical analyses were performed by mixed model ANOVA and Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$. n is presented in parenthesis on each bar graph. Different upper superscripts are representative of statistical significance between diet groups and lower superscripts are representative of statistical interaction for diet*sex.

Figure 6.4. Proximal tibia metaphysis assessed by μCT in neonates at 2 d postpartum according to dietary intake of vitamin D during pregnancy (0, 0.25, 0.5, 1 and 2 IU/g diet): **a)** Tb. BV/TV **b)** Tb. N. **c)** Tb. vBMD. Values are means± SEM statistically adjusted for litter size and block. Statistical analyses were performed by mixed model ANOVA and Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$. n is presented in parenthesis on each bar graph. Different upper superscripts are representative of statistical significance between diet groups and lower superscripts are representative of statistical interaction for diet*sex.

Figure 6.5. a) Maternal Young's Modulus and **b)** Neonatal Maximum Force (N) from the 3-point bending test bone strength on the femur mid-diaphysis in guinea pig sows and neonates at 2 d postpartum according to dietary intake of vitamin D during pregnancy (0, 0.25, 0.5, 1 and 2 IU/g diet). Values are means ± SEM, n is presented in parenthesis. Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test is vs group 1 IU/g vitamin D in Figure 5(a) but vs all groups in Figure 5(b). Statistical significance, $p \leq 0.05$.

Figure 6.6. Representative histological images of **a)** female and **b)** male neonate coronal slice of distal femur in guinea pigs at 2 d postpartum according to dietary intake of vitamin D during pregnancy (0, 0.25, 0.5, 1 and 2 IU/g diet). *Top:* 5x

magnification, von Kossa and van Gieson staining; *bottom:* 10x magnification, safronin-O staining.

Table 6. 1. Isoenergetic guinea pig grain-based Purina TestDiet

Ingredient	g/kg diet
Carbohydrate	429
Starch	154
Sucrose	223
Fructose	7.3
Lactose	5.6
Glucose	2.7
Fat	60
Cholesterol	0.043
SAFA	17.6
MUFA	19.4
PUFA	17.6
Protein	193
L-Methionine	0.42
Fiber	144
Vitamin[1,2] and mineral premix[3]	174

[1]Premix devoid of ergocalciferol and cholecalciferol, diets differed in their cholecalciferol dosages as follows: (0, 0.25, 0.5, 1 and 2 IU/kg) or (0, 6.2, 12.5, 25, 50 μg/kg) diet.

[2]Contained the following (vitamin mix): carotene, 12.7 mg/kg; vitamin A, 8.5 mg/kg (25 IU/g); DL-a-tocopheryl, 36.1 mg/kg (54 IU/kg); menadione, 5.0 mg/kg; thiamin, 9.0 mg/kg; riboflavin, 6.1 mg/kg; niacin, 62 mg/kg; pantothenic acid, 21 mg/kg; folic acid, 4 mg/kg; pyridoxine, 4 mg/kg; biotin, 0.2 mg/kg; vitamin B-12, 0.01 mg/kg; choline chloride, 1850 mg/kg; and ascorbic acid, 500 mg/kg.

[3]Contained the following (mineral mix): calcium, 11 g/kg; phosphorous, 6.5 g/kg; phosphorous (available), 4.3 g/kg; potassium, 15.5 g/kg; magnesium, 3.6 g/kg; sodium, sulphur, 2.8 g/kg; 3.5 g/kg; chlorine, 7.1 g/kg; fluorine, 26.6 mg/kg; iron, 353 mg/kg; zinc, 78 mg/kg; manganese, 81mg/kg; copper, 14.0 mg/kg; cobalt, 3.4 mg/kg; iodine, 0.88 mg/kg; chromium, 2.0 mg/kg; and selenium, 0.47 mg/kg.

Figure 6. 1.

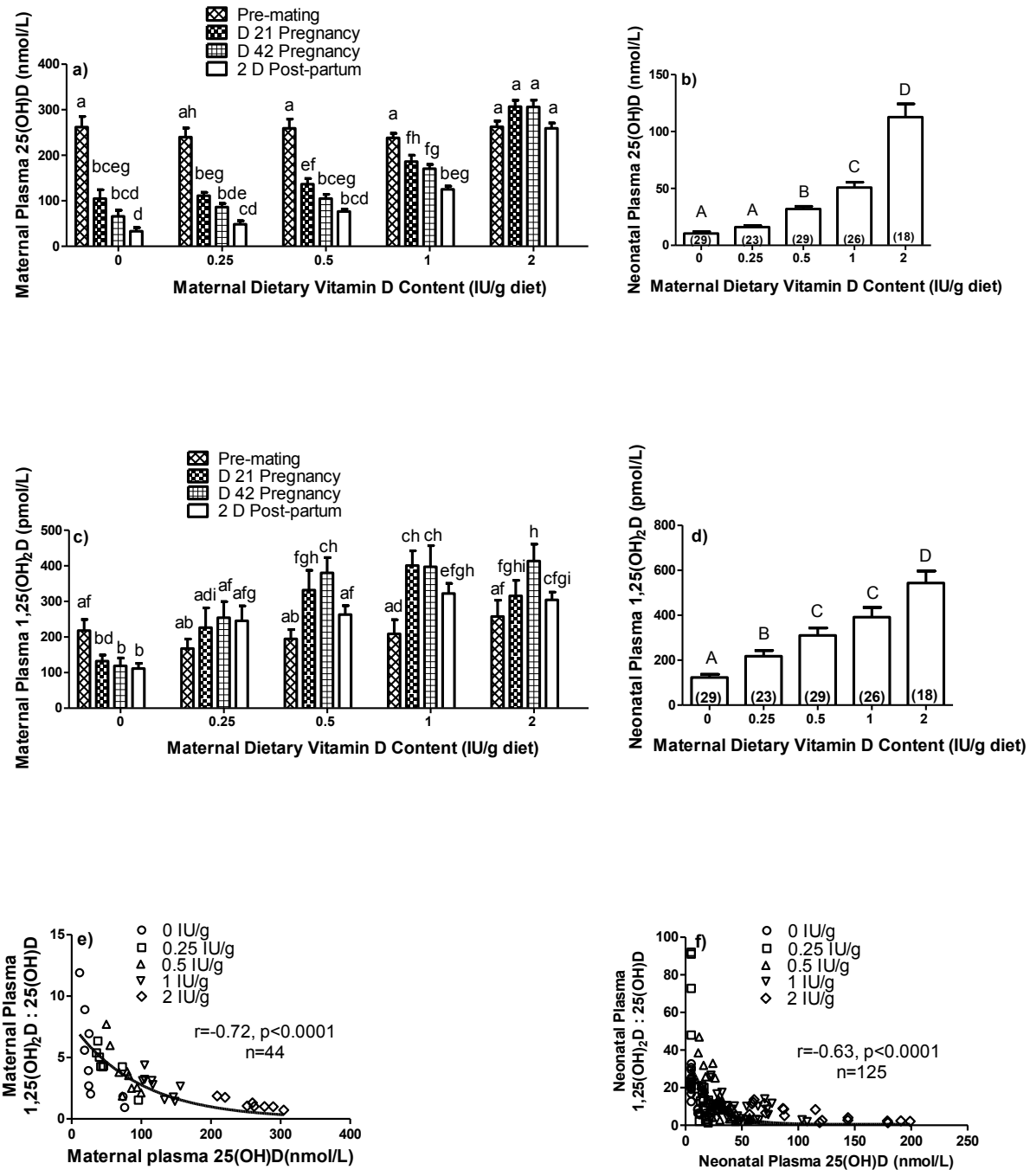

Pre-mating
D 21 Pregnancy
D 42 Pregnancy
2 D Post-partum
a)
Maternal Plasma 25(OH)D (nmol/L)
Maternal Dietary Vitamin D Content (IU/g diet)
b)
Neonatal Plasma 25(OH)D (nmol/L)
Maternal Dietary Vitamin D Content (IU/g diet)
c)
Maternal Plasma 1,25(OH)2D (pmol/L)
Maternal Dietary Vitamin D Content (IU/g diet)
d)
Neonatal Plasma 1,25(OH)2D (pmol/L)
Maternal Dietary Vitamin D Content (IU/g diet)
e)
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
r=-0.72, p<0.0001
n=44
Maternal Plasma 1,25(OH)2D : 25(OH)D
Maternal plasma 25(OH)D(nmol/L)
f)
r=-0.63, p<0.0001
n=125
Neonatal Plasma 1,25(OH)2D : 25(OH)D
Neonatal Plasma 25(OH)D (nmol/L)

Figure 6. 2.

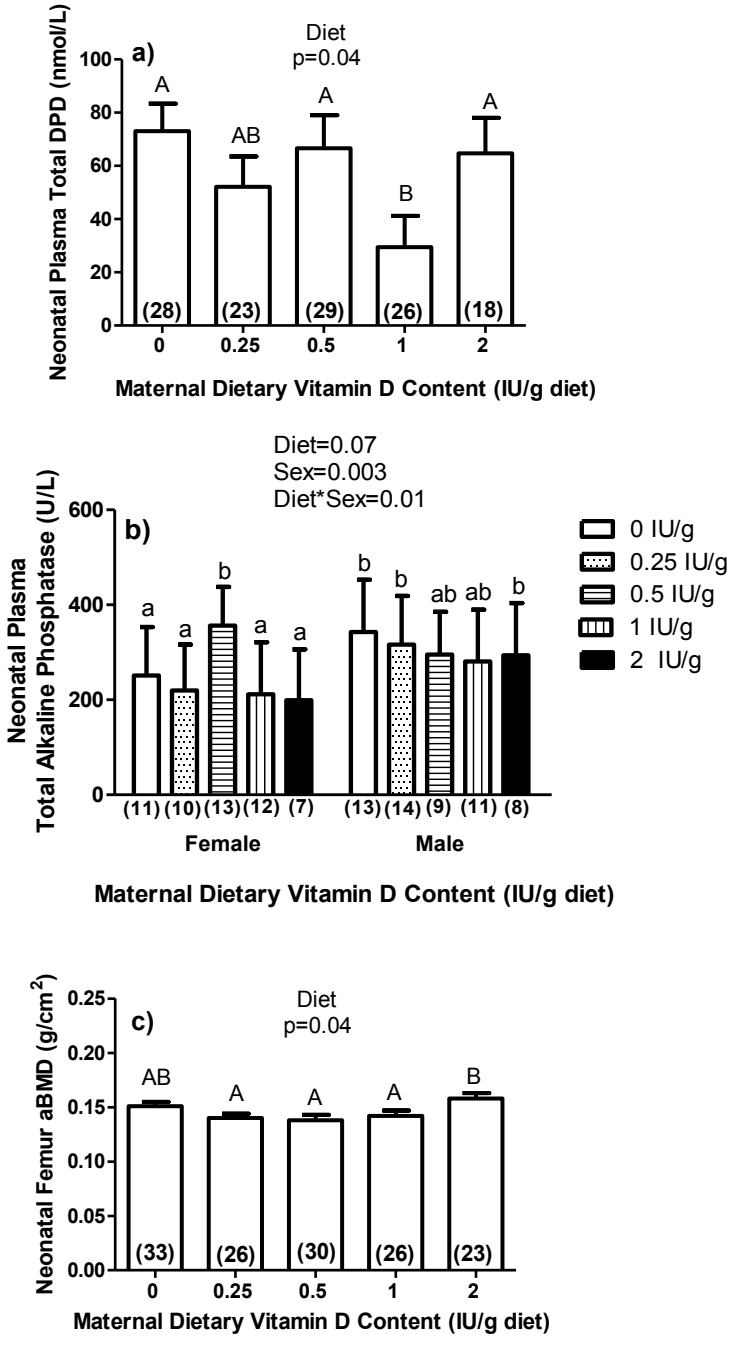

a)
Diet
p=0.04
Neonatal Plasma Total DPD (nmol/L)
100
80
60
40
20
0
A
AB
A
B
A
(28)
(23)
(29)
(26)
(18)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)
b)
Diet=0.07
Sex=0.003
Diet*Sex=0.01
Neonatal Plasma Total Alkaline Phosphatase (U/L)
600
400
200
0
a
a
b
a
a
b
b
ab
ab
b
(11)
(10)
(13)
(12)
(7)
(13)
(14)
(9)
(11)
(8)
Female
Male
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
Maternal Dietary Vitamin D Content (IU/g diet)
c)
Diet
p=0.04
Neonatal Femur aBMD (g/cm2)
0.25
0.20
0.15
0.10
0.05
0.00
AB
A
A
A
B
(33)
(26)
(30)
(26)
(23)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)

Figure 6. 3.

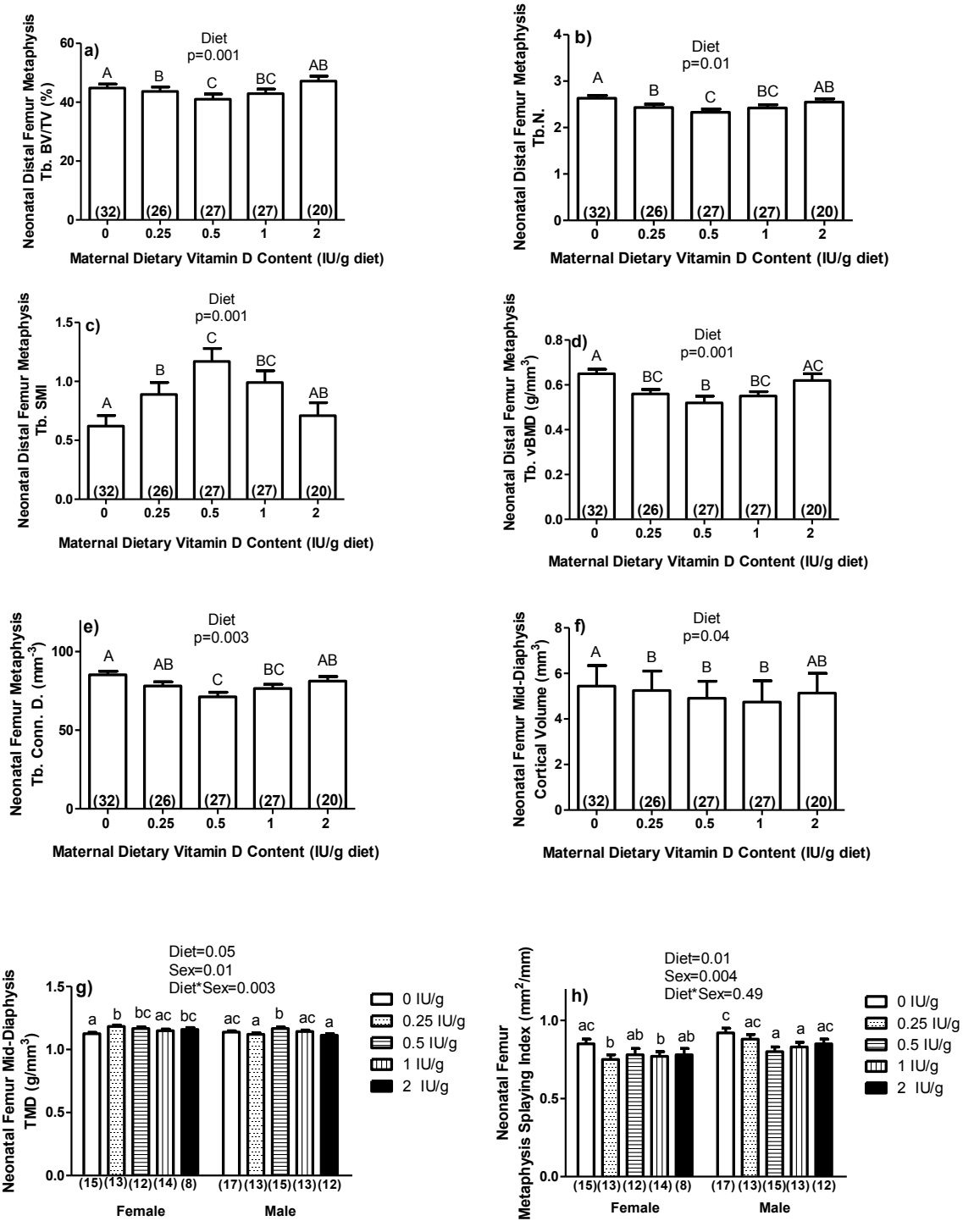
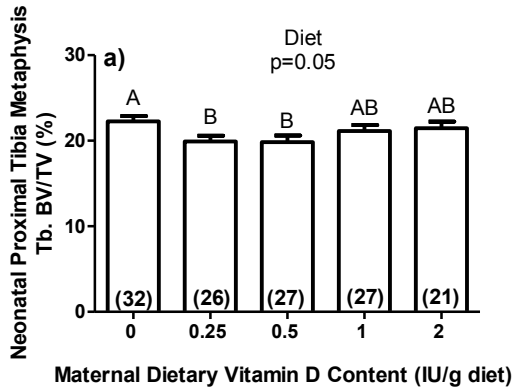
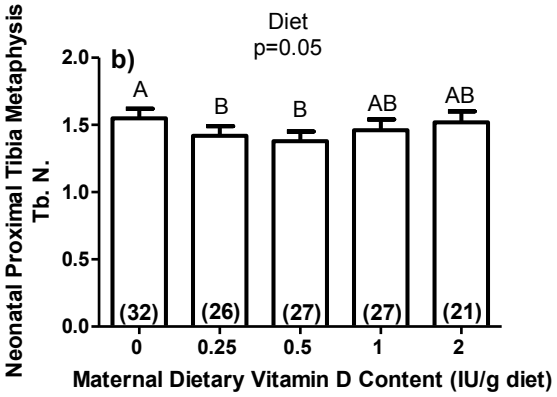

a)
Diet
p=0.001
Neonatal Distal Femur Metaphysis
Tb. BV/TV (%)
A
B
C
BC
AB
(32)
(26)
(27)
(27)
(20)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)
b)
Diet
p=0.01
Neonatal Distal Femur Metaphysis
Tb.N.
A
B
C
BC
AB
(32)
(26)
(27)
(27)
(20)
Maternal Dietary Vitamin D Content (IU/g diet)
c)
Diet
p=0.001
Neonatal Distal Femur Metaphysis
Tb. SMI
A
B
C
BC
AB
(32)
(26)
(27)
(27)
(20)
Maternal Dietary Vitamin D Content (IU/g diet)
d)
Diet
p=0.001
Neonatal Distal Femur Metaphysis
Tb. vBMD (g/mm3)
A
BC
B
BC
AC
(32)
(26)
(27)
(27)
(20)
Maternal Dietary Vitamin D Content (IU/g diet)
e)
Diet
p=0.003
Neonatal Femur Metaphysis
Tb. Conn. D. (mm-3)
A
AB
C
BC
AB
(32)
(26)
(27)
(27)
(20)
Maternal Dietary Vitamin D Content (IU/g diet)
f)
Diet
p=0.04
Neonatal Femur Mid-Diaphysis
Cortical Volume (mm3)
A
B
B
B
AB
(32)
(26)
(27)
(27)
(20)
Maternal Dietary Vitamin D Content (IU/g diet)
g)
Diet=0.05
Sex=0.01
Diet*Sex=0.003
Neonatal Femur Mid-Diaphysis
TMD (g/mm3)
a b bc ac bc
ac a b ac a
(15) (13) (12) (14) (8)
(17) (13) (15) (13) (12)
Female
Male
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
h)
Diet=0.01
Sex=0.004
Diet*Sex=0.49
Neonatal Femur
Metaphysis Splaying Index (mm2/mm)
ac b ab b ab
c ac a a ac
(15) (13) (12) (14) (8)
(17) (13) (15) (13) (12)
Female
Male
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g

Figure 6. 4.


Neonatal Proximal Tibia Metaphysis
Tb. BV/TV (%)
a)
Diet
p=0.05
30
20
10
0
A
B
B
AB
AB
(32)
(26)
(27)
(27)
(21)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)


Neonatal Proximal Tibia Metaphysis
Tb. N.
b)
Diet
p=0.05
2.0
1.5
1.0
0.5
0.0
A
B
B
AB
AB
(32)
(26)
(27)
(27)
(21)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)

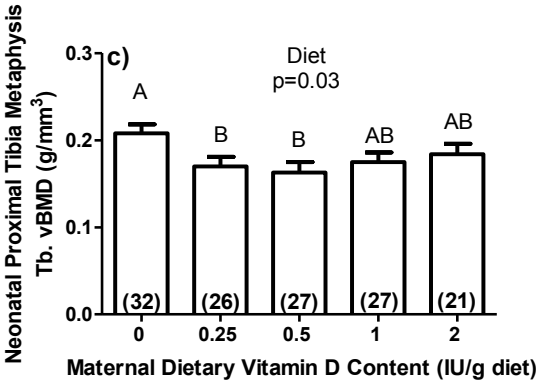
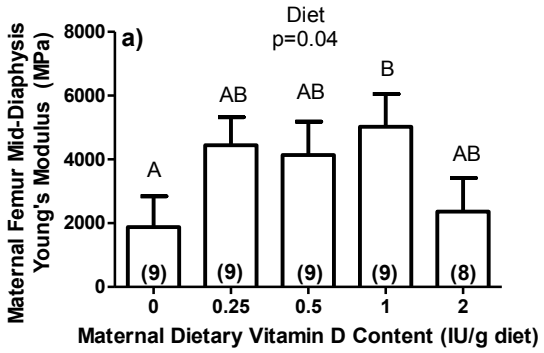

Neonatal Proximal Tibia Metaphysis
Tb. vBMD (g/mm3)
c)
Diet
p=0.03
0.3
0.2
0.1
0.0
A
B
B
AB
AB
(32)
(26)
(27)
(27)
(21)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)

Figure 6. 5.


Diet
p=0.04
a)
Maternal Femur Mid-Diaphysis Young's Modulus (MPa)
8000
6000
4000
2000
0
A
AB
AB
B
AB
(9)
(9)
(9)
(9)
(8)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)

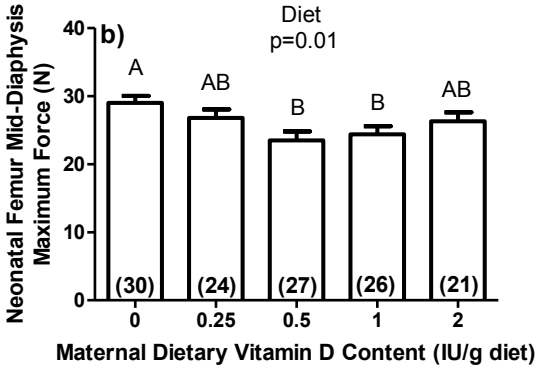
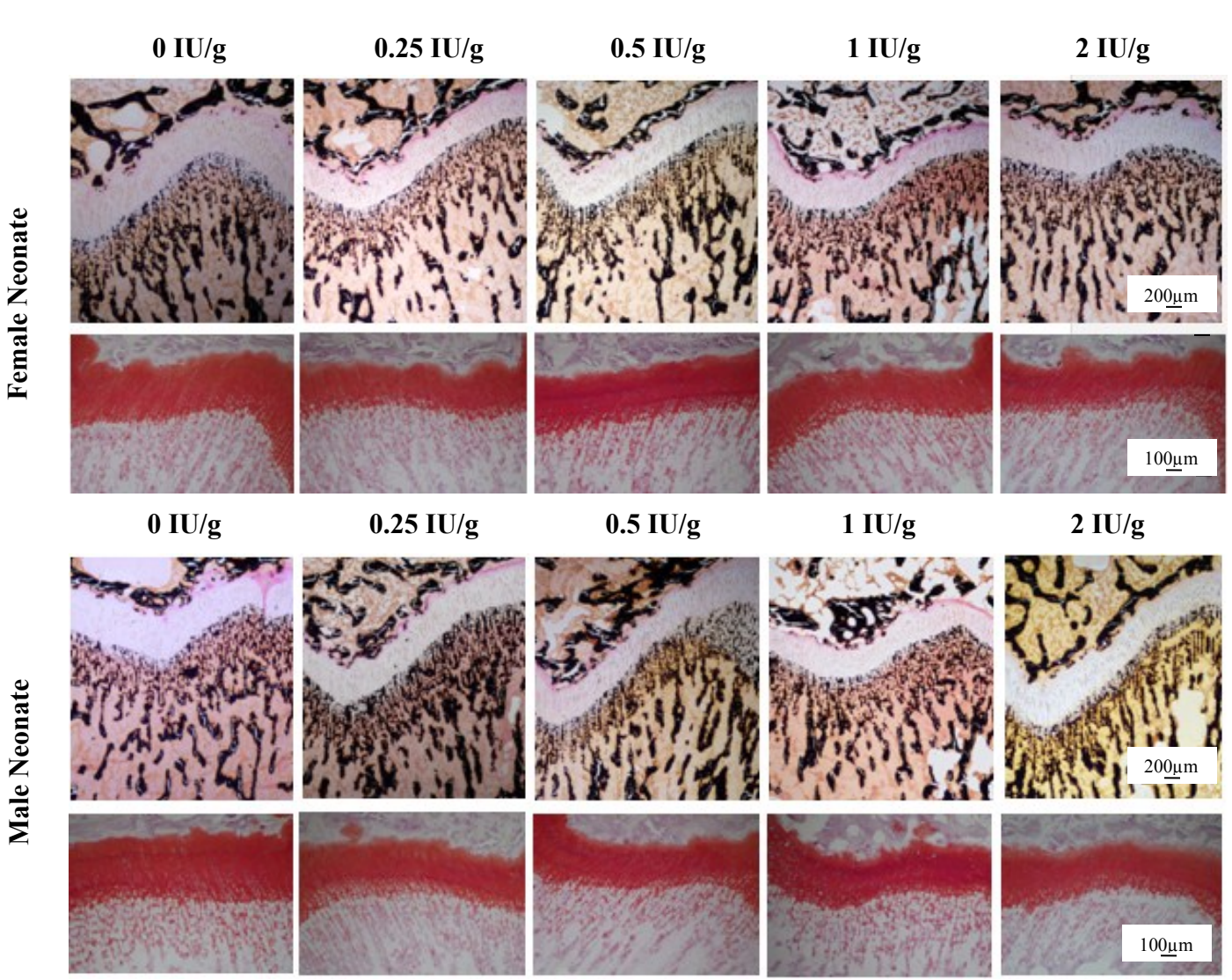

Diet
p=0.01
b)
Neonatal Femur Mid-Diaphysis Maximum Force (N)
40
30
20
10
0
A
AB
B
B
AB
(30)
(24)
(27)
(26)
(21)
0
0.25
0.5
1
2
Maternal Dietary Vitamin D Content (IU/g diet)

Figure 6. 6.


a)
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
Female Neonate
200µm
100µm
b)
0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
Male Neonate
200µm
100µm

6.8. Supplemental Figure legend

Supplemental Figure 6. 1. Representative reconstructed μCT images of a) maternal, b) female and c) male neonate coronal slice of distal femur and transversal section of distal femur metaphyseal Tb. and mid-diaphysis cortical bone in guinea pigs at 2 d postpartum according to dietary intake of vitamin D during pregnancy (0, 0.25, 0.5, 1 and 2 IU/g diet). Images are representative of samples from each group that were closest to the mean (unadjusted for litter size and block).

6.9. Supplemental Tables

Supplemental Table 6. 1. Biomarkers of mineral and bone metabolism in guinea pig sows and neonates in all vitamin D dietary groups**.**

Outcomes	Maternal	Maternal p-values Mixed ANOVA			Neonatal		Neonatal p-values Mixed ANOVA		
	All	Diet	Time	Diet* Time	All		Diet	Sex	Diet* sex
					Female	Male			
Plasma Total Ca (mmol/L)									
Pre-mating	2.51±0.02 (44)[a]								
D21	2.54±0.01 (43)[a]								
D42	2.44±0.01 (44)[b]								
2d Postpartum	2.46±0.02 (44)[b]	0.97	<0.001	0.90	2.42±0.02(54)	2.42±0.02(58)	0.06	0.68	0.25
Blood Ca^{2+} (mmol/L)									
Pre-mating	1.50±0.02(30)[a]								
2d Postpartum	1.44±0.01(33)[b]	0.95	0.003	0.72	1.38±0.02(42)	1.38±0.02(49)	0.42	0.98	0.97
Plasma PO_4 (mmol/L)									
Pre-mating	1.98±0.05(42)[a]								
D21	1.95±0.04(41)[a]								
D42	1.62±0.03(44)[b]								
2d Postpartum	1.87±0.05(42)[a]	0.07	<0.001	0.23	2.53±0.06(49)	2.50±0.06(53)	0.17	0.54	0.09
Plasma OC (nmol/L)									
Pre-mating	16.1±0.7(44)[a]								
D21	14.2±0.5(44)[b]								
D42	10.6±0.4(44)[c]								
2d Postpartum	5.0±0.3(44)[d]	0.62	<0.001	0.86	49.3±1.6(61)	52.9±1.4(64)	0.58	0.02	0.64
Plasma tDPD (nmol/L)									
Pre-mating	15.6±2.6(44)[a]								
D21	16.7±4.1(44)[a]								
D42	20.3±3.6(44)[ab]								
2d Postpartum	31.5±6.6(44)[b]	0.57	0.03	0.97	52.4±8.4(61)	53.1±7.7(63)	0.04	0.24	0.16
Plasma tALP (IU/L)									
Pre-mating	104.1±5.7(44)[a]								
D21	99.0±5.6(42)[a]								
D42	105.0±4.4(44)[a]								
2d Postpartum	44.0±1.4(42)[b]	0.39	<0.001	0.22	489.7±9.5(53)	546.8±15.7(55)	0.11	0.002	0.01

Values are means ± SEM, *n* is presented in parenthesis. Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$. Different lower superscripts (a,b,c,..) are representative of statistical significance among pregnancy time-points. The significant effect of diet on neonatal total DPD is shown in Figure 6.2(a) and the significant effect of diet*sex interaction on plasma tALP is shown in Figure 6.2(b).

Supplemental Table 6. 2. Whole body, lumbar spine vertebrae 3, femur and tibia DXA measurements in pregnant guinea pig sows and neonates of all vitamin D dietary groups.

Outcomes	Maternal Pre-mating	Maternal 2d Postpartum	Maternal p-value Mixed ANOVA			Neonatal 2d Postpartum	Neonatal p-value Mixed ANOVA		
			Diet	Time	Diet*Time		Diet	Sex	Diet* Sex
	All	All				All			
Whole body									
Area (cm^2)	97.01±1.12[a]	106.95±0.97[b]	0.11	<0.001	0.61	29.99±0.30	0.92	0.23	0.82
BMC (g)	22.07±0.34[a]	24.47±0.29[b]	0.55	<0.001	0.64	3.44±0.05	0.97	0.46	0.89
aBMD (g/cm^2)	0.227±0.000[a]	0.229±0.001[a]	0.35	0.11	0.64	0.114±0.001	0.98	0.78	0.83
Vertebrae 3									
Area (cm^2)	0.44±0.00[a]	0.47±0.00[b]	0.94	<0.001	0.68	0.18±0.00	0.63	0.82	0.10
BMC (g)	0.131±0.002[a]	0.132±0.002[a]	0.64	0.78	0.79	0.026±0.005	0.91	0.64	0.56
aBMD (g/cm^2)	0.298±0.003[a]	0.282±0.003[b]	0.33	<0.001	0.15	0.146±0.001	0.95	0.81	0.53
Femur									
Area (cm^2)	1.64±0.02[a]	1.72±0.02[b]	0.13	<0.001	0.43	0.71±0.01	0.22	0.68	0.74
BMC (g)	0.744±0.013[a]	0.83±0.01[b]	0.49	<0.001	0.60	0.103±0.002	0.42	0.17	0.88
aBMD (g/cm^2)	0.455±0.008[a]	0.486±0.007[b]	0.06	0.001	0.54	0.145±0.002	0.04	0.19	0.79
Tibia									
Area (cm^2)	2.03±0.02[a]	2.14±0.02[b]	0.47	<0.001	0.18	0.95±0.01	0.88	0.04	0.32
BMC (g)	0.559±0.008[a]	0.61±0.01[b]	0.86	<0.001	0.38	0.092±0.002	0.29	0.06	0.54
aBMD (g/cm^2)	0.276±0.003[a]	0.284±0.003[b]	0.53	0.008	0.99	0.097±0.001	0.17	0.34	0.42

Values are means ± SEM. Maternal n=44, Neonatal n=138. Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$.

Significant effect of time is shown by different lower superscripts within a row in maternal outcomes. The significant effect of diet on neonatal femur aBMD is shown in Figure 6.2(c).

Supplemental Table 6. 3. Lumbar spine vertebrae 3, distal femur and proximal tibia Tb. and mid-diaphysis femur cortical miroCT measurements in guinea pig sows and neonates at 2d postpartum in all vitamin D dietary groups.

Outcomes		Maternal	Maternal p-value Mixed ANOVA	Neonatal	Neonatal p-value Mixed ANOVA		
		All	Diet	All	Diet	Sex	Diet* Sex
Vertebrae 3	BV/TV(%)	15.58±0.23	0.61	12.30 ± 0.20	0.74	0.27	0.88
	Th(mm)	0.1127±0.0005	0.90	0.877 ± 0.0003	0.21	0.94	0.88
	Sp(mm)	0.324±0.004	0.30	0.229 ± 0.001	0.60	0.05	0.79
	N(mm^{-1})	1.38±0.02	0.46	1.40 ± 0.02	0.77	0.18	0.85
	SMI	1.71±0.02	0.84	1.99 ± 0.02	0.56	0.15	0.72
	D.A.	3.17±0.35	0.005	1.61 ± 0.01	0.05	0.42	0.16
	Conn.Den.(mm^{-3})	37.84±0.94	0.74	89.83 ± 2.32	0.10	0.56	0.45
	vBMD(g/mm^3)	0.351±0.008	0.60	0.243 ± 0.007	0.74	0.27	0.86
Femur Tb.	BV/TV(%)	22.12±0.62	0.57	45.09 ± 0.79	0.001	0.45	0.15
	Th(mm)	0.148±0.001	0.87	0.179 ± 0.001	0.07	0.07	0.16
	Sp(mm)	0.68±0.03	0.25	0.212 ± 0.006	0.09	0.68	0.35
	N(mm^{-1})	1.49±0.03	0.38	2.50±0.03	0.01	0.16	0.52
	SMI	1.57±0.03	0.57	0.87±0.05	0.001	0.21	0.19
	D.A.	1.93±0.02	0.18	1.47 ± 0.01	0.87	0.44	0.31
	Conn.Den.(mm^{-3})	27.40±0.90	0.12	79.74±1.37	0.003	0.79	0.10
	vBMD(g/mm^3)	0.577±0.021	0.77	0.585±0.013	0.001	0.40	0.14
Femur Cortical	Endosteal Vol. (mm)	14.93±0.34	0.17	9.23 ± 0.13	0.07	0.77	0.06
	Periosteum Vol.(mm)	40.12±0.49	0.51	15.43 ± 0.20	0.39	0.63	0.22
	Cortical Vol.(mm)	25.19±0.27	0.31	6.20±0.11	0.04	0.19	0.99
	TMD(g/mm^3)	1.877±0.004	0.91	1.143±0.005	0.05	0.01	0.003
Tibia Tb.	BV/TV(%)	15.23±0.35	0.66	21.33±0.32	0.05	0.63	0.08
	Th(mm)	0.1054±0.0006	0.91	0.1411 ± 0.0005	0.14	0.88	0.33
	Sp(mm)	0.358±0.008	0.58	0.313 ± 0.003	0.10	0.65	0.59
	N(mm^{-1})	1.45±0.03	0.55	1.51±0.02	0.05	0.72	0.14
	SMI	2.11±0.02	0.57	1.47 ± 0.03	0.14	0.11	0.04
	D.A.	2.19±0.02	0.69	1.56 ± 0.01	0.33	0.07	0.33
	Conn.Den.(mm^{-3})	36.13±1.61	0.79	48.24 ± 1.12	0.10	0.66	0.37
	vBMD(g/mm^3)	0.303±0.011	0.67	0.186±0.005	0.03	0.53	0.11

Values are means ± SEM, for the sow n=44 for all variables, for the neonate n=138 at the vertebrae 3, n= 132 at the femur Tb. and cortical and n=133 at the Tibia Tb..

Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$.

The significant effect of diet on neonatal femur Tb. BV/TV, N, SMI, Conn. Den, vBMD are shown in Figure 6.3(a-e) and cortical volume and TMD are shown in Figure 6.3(f-g) and tibia Tb. BV/TV, N and vBMD are shown in Figure 6.4(a-c).

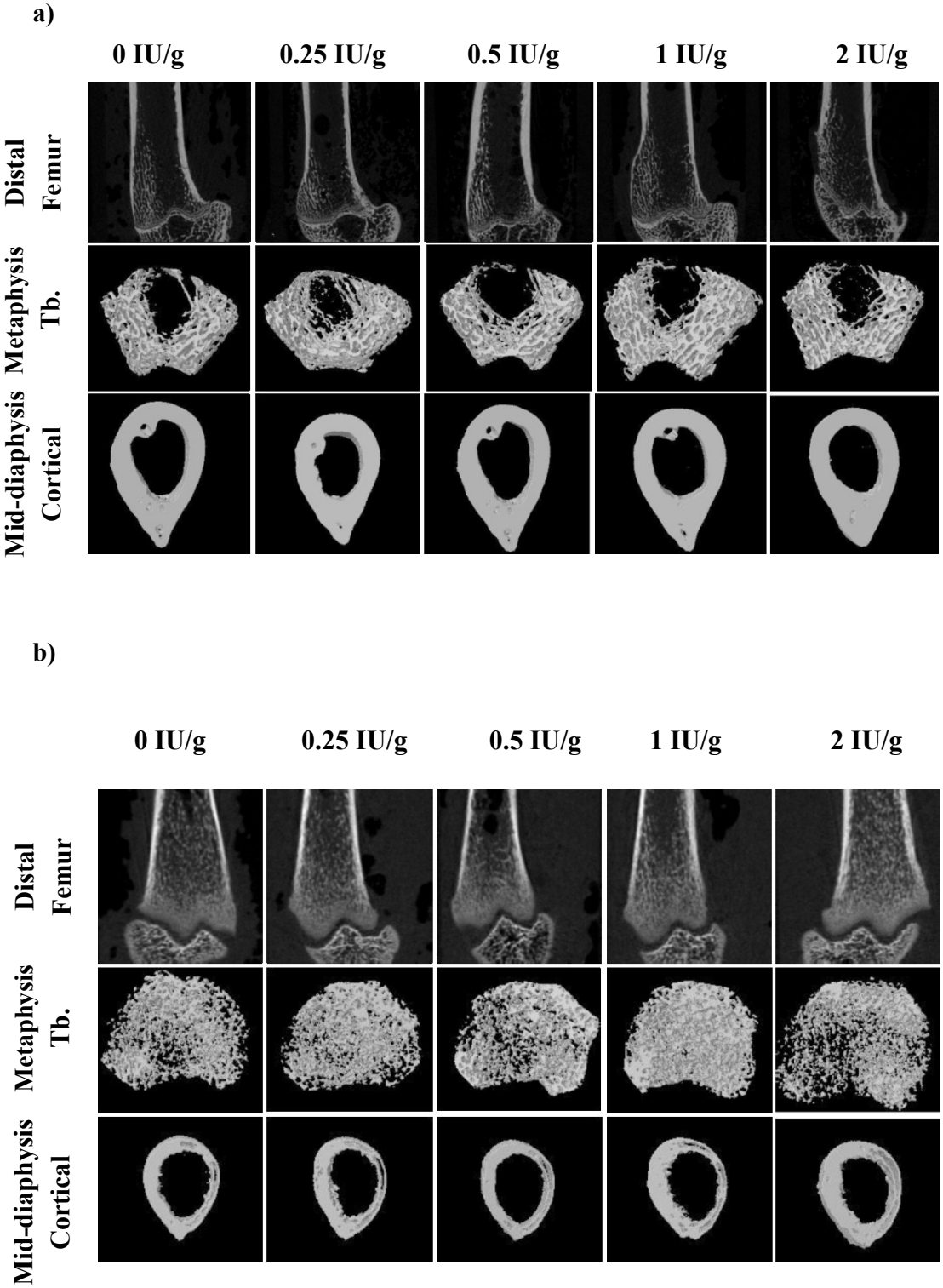
Supplemental Table 6. 4. Femur mid-diaphysis 3-point bending test bone strength in guinea pig sows and neonates at 2 d postpartum according to dietary intake of vitamin D during pregnancy.

Outcomes	Maternal 2 d postpartum	Maternal p-value Mixed ANOVA	Neonatal 2 d postpartum		Neonatal p-value Mixed ANOVA		
			All		Diet	Sex	Diet* Sex
	All	Diet	Female	Male			
Femur mid-diaphysis							
Maximum Force (N)	142.9±4.62(44)	0.70	25.35±0.96(60)	26.38±0.79(68)	0.01	0.91	0.74
Maximum deformation (mm)	0.84±0.03(44)	0.75	0.98±0.02(60)	1.03±0.02(68)	0.78	0.001	0.30
Energy at break (mJ)	52.51±3.22(44)	0.76	23.08±0.85(60)	24.34±1.05(68)	0.86	0.08	0.24
Cross sectional diameter (mm)	3.88±0.03(44)	0.77	2.37±0.02(60)	2.45±0.02(68)	0.87	0.002	0.33
Maximum Stress	82.33±5.01(44)	0.31	32.52±1.47(60)	32.32±1.13(68)	0.26	0.39	0.25
Maximum Strain (%)	3.41±0.13(44)	0.72	14.00±0.27(60)	15.22±0.34(68)	0.81	<0.001	0.23
Young's Modulus	4359.23±374.27(44)	0.04	3194±161.6(60)	2829±154.5(68)	0.36	0.02	0.21

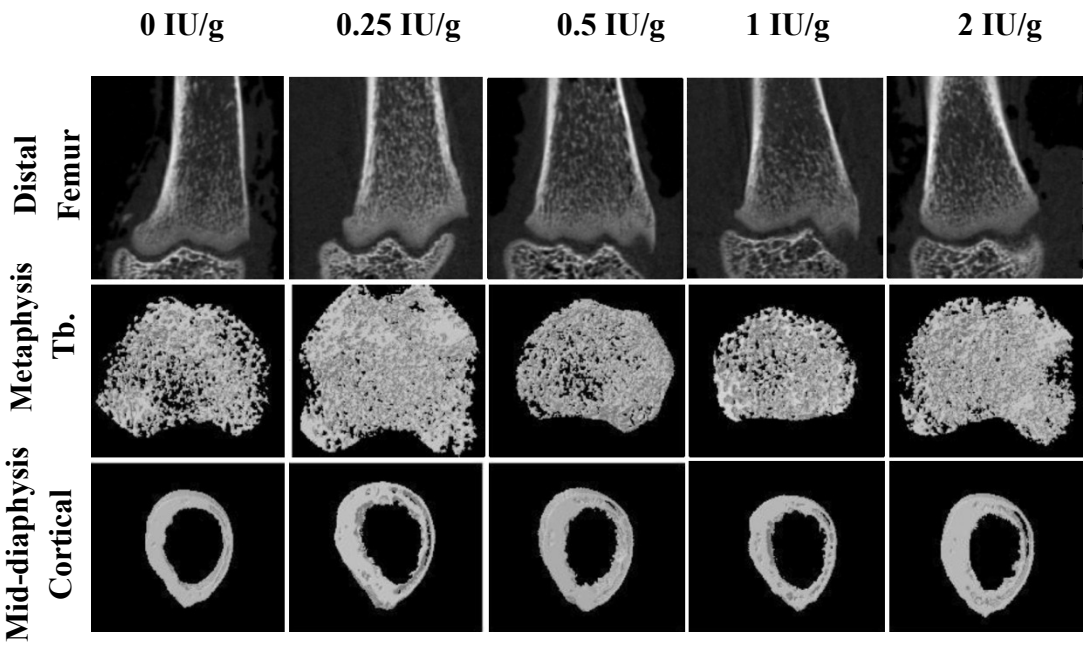
Values are means ± SEM, *n* is presented in parenthesis. Mixed model ANOVA, adjusted for litter size and block. Tukey, *post-hoc* test. Statistical significance, $p \leq 0.05$.

The significant effect of diet on maternal femur mid-diaphysis Young's Modulus is shown in Figure 6.5(a), Tukey, *post-hoc* test are vs diet 1. The significant effect of diet on pup femur mid-diaphysis maximum force is shown in Figure 6.5(b).

Supplemental Figure 6. 1.



c)


0 IU/g
0.25 IU/g
0.5 IU/g
1 IU/g
2 IU/g
Distal Femur
Metaphysis Tb.
Mid-diaphysis Cortical

BRIDGE 5

In Chapter 6 it was shown that maternal and neonatal plasma 25(OH)D show a dose-response to dietary vitamin D during pregnancy in guinea pigs which closely parallels that recently reported in human pregnancy [10]. The striking similarity between maternal and neonatal vitamin D status demonstrates that maternal-fetal transfer is not saturable up to 250 nmol/L of maternal plasma 25(OH)D concentrations in guinea pigs. Even though, neonatal plasma 25(OH)D concentrations were lower than maternal levels, in accordance with previous studies [10, 13, 67, 79, 199, 204], values at 2 d postpartum reached 125 nmol/L in ~30 % of the neonates in the 2 IU/g group. Furthermore, maternal plasma $1,25(OH)_2D$ plateaus at intakes of vitamin D that approximate the recommendations whereas it follows a dose-response in the neonates. In addition, it was revealed that providing such dietary vitamin D ranging from deficient to more than recommended resulted in a parallel response in vitamin D status, but is not reflected in maternal mineral or bone health outcomes. The fetus responded with higher Tb. density of long bone at both low and high maternal vitamin D intakes while features of congenital rickets were apparent in the deficient group. The neonatal response was differential and sex-dependent at the cortical femur with lower TMD in the male neonates at the low and high maternal vitamin D intakes and lower TMD in the female neonates of the deficient group only while bone quality assessed by biomechanical tests was unaffected. These data reinforce that maternal vitamin D intakes and status below the recommendations do not significantly affect maternal bone health but may compromise bone health in the offspring and higher intakes and status are not advantageous.

CHAPTER 7. SUMMARY AND CONCLUSIONS

Six main hypotheses were tested in this thesis which are consecutively discussed in this chapter. The first hypothesis was that maternal serum vitamin D status is lower and OC concentration is higher at early gestation in GDM vs healthy pregnant women; maternal 25(OH)D and OC concentrations at early gestation and the change from early to mid-gestation may have a predictive role in the development of GDM. A nested case (GDM)-control (healthy) study was carried out in pregnant women in Quebec City to test this hypothesis. The first manuscript (Chapter 3) reported the results of this study.

The most novel finding of this study was that OC was significantly elevated in GDM compared to healthy women throughout pregnancy. However, vitamin D status was not different among GDM and healthy pregnancy women across pregnancy. Importantly, adjustments were made for confounding variables that were not all taken into account in previous studies of vitamin D status in GDM [22-28] such as pre-pregnancy BMI, history of smoking, family history of diabetes and physical activity. However, early pregnancy and change in maternal vitamin D status and OC from early to mid-gestation was not associated with GDM.

Therefore the first part of the hypothesis that vitamin D status is lower at early gestation in GDM vs healthy pregnant women is rejected, suggesting that low vitamin D status and development of GDM are coincidental events with potential for adverse pregnancy outcomes. Previous studies that have reported significant lower vitamin D status in GDM vs healthy controls have not adjusted for history of smoking and physical activity [22-28]. However, the second part of the hypothesis that OC concentration is higher at early gestation in GDM vs healthy pregnant women is accepted. This is in agreement with similar findings at mid-gestation in Australian

and Iranian pregnant women [52, 53], but novel since higher concentrations were even observed at early gestation, prior to the outward signs of GDM. Therefore, this may be an early compensatory mechanism independent of $1,25(OH)_2D$ to increase insulin secretion which cannot be accomplished because of pancreatic β-cell defects in women with GDM [52]. However, higher OC concentrations may also be a phenomenon completely independent of both vitamin D and glucose status. Higher OC concentration has been related to higher bone turnover in GDM as compared to normal pregnancy [53, 256]. This may be associated with possible detrimental effects of GDM on bone which is in line with the higher reduction (50 %) of calcis BMD reported in GDM vs healthy pregnant women [257]. Nonetheless, overall the hypothesis that circulating 25(OH)D and OC have a predictive role in the development of GDM was rejected.

Even though the total circulating OC measured in this study is also known to have a role in the regulation of insulin sensitivity in humans [46], the uncarboxylated form of OC is thought to be the main regulator of glucose homeostasis [41]. Therefore, it would be useful to investigate this specific form of OC to further explain the role of this bone biomarker in GDM and the bone changes accompanying it. In addition, measurement of other bone formation biomarkers such as P1NP and bone resorption biomarkers such as C-telopeptide would also help explain changes in bone metabolism in GDM vs healthy pregnant women.

The cause and effect relationship between OC and GDM could also not be determined in this study due to its nested case-control design. Large prospective cohort studies are the next study design recommended to further investigate the causality between circulating OC and GDM in pregnant women and bone health consequences associated with it. Furthermore, the population of pregnant women was

exclusively Caucasians in this study and therefore not representative of other ethnicities. In addition, it is important to consider that summer was the season of conception over represented (i.e. 44% of the population) in this study which may have, even though cases and controls were matched on season, affected the change in circulating 25(OH)D with time across pregnancy. The high variability in circulating 25(OH)D concentrations in the control group suggest that larger sample size is required to increase the power of the study. Therefore, large prospective cohorts across different seasons and ethnicities are required to further explore the association between 25(OH)D and the risk of development of GDM and the possible mechanisms involved. Ideally a randomized controlled study aiming at achieving normal 25(OH)D concentrations in a vitamin D supplemented group will be needed to clarify the effect of vitamin D status and the relationships with the active metabolite $1,25(OH)_2D$ in GDM.

The second hypothesis was that cord serum 25(OH)D concentration is associated with anthropometry measures and APGAR scores in human neonates and placenta weight which was investigated in the same nested-case control study as described above (Chapter 3). In this study it was demonstrated that neither maternal nor cord serum 25(OH)D concentrations was correlated with any of the neonatal anthropometry measurements and placenta weight. These results agree with a recent report with similar vitamin D status as in this nested-case control study [10], but are in contrast to studies in populations with lower vitamin D status [18, 19]. The prevalence of low cord vitamin D status was 11.5 % for 25(OH)D < 50 nmol/L and 39.6% for 25(OH)D < 75 nmol/L, which is lower than those previously reported in neonates in a study in Manitoba [19]. The lack of association between maternal and cord serum 25(OH)D and APGAR scores is similar to a previous report [258], but

contrasts to the lower APGAR scores reported in another study in neonates of mothers with lower vitamin D intakes [17]. This suggests the importance of measuring circulating 25(OH)D concentration vs vitamin D intakes when investigating the association between vitamin D and neonatal APGAR scores. Therefore, the hypothesis that cord serum 25(OH)D concentration is associated with anthropometry and APGAR scores in the neonate and placenta weight is rejected in this exclusive Caucasian population.The homogeneity of the total population due to matching of case and controls for pre-pregnancy BMI, age, GA and the exclusive Caucasian population should be considered in interpretation these results. Whether the results would be different in a population with higher prevalence of low vitamin D status requires further investigation.

Due to difficulty in assessing and controlling for confounding variables in the association between circulating 25(OH)D and pregnancy outcomes (glucose tolerance and bone health) as well as ethical concerns for a vitamin D dose-response study in human studies, the guinea pig model was chosen for the studies in this thesis involving both bone [13, 79] and glucose metabolism [56, 233]. The third hypothesis was thus that exposure to the isoflurane anaesthetic significantly affects OC and tDPD across maturity and changes blood glucose and minerals postpartum in guinea pigs. If so, to better reflect normal metabolism with the added values of improved consistency and comparability in research, measurements prior to anaesthesia would be recommended. The latter is usually not followed in animal studies due to more convenient sample collection after anaesthesia. The results of this study are presented in the second manuscript (Chapter 4). The most novel observation of this study was that administration of isoflurane for 20-25 min almost doubles circulating OC concentrations during rapid growth, but not thereafter and exposure increases blood

glucose concentrations postpartum. Therefore, even though sample collections are usually more convenient after exposure to anaesthetic, measurements of these biomarkers prior to isoflurane anaesthesia are recommended to represent normal metabolism and for consistency and comparability in research. The doubling of plasma OC in guinea pigs after exposure to isoflurane during development is similar to the results obtained in adult cynomolgus monkeys [59]. However, no effect of isoflurane was observed on plasma OC postpartum which might due to the either the observed decrease in bone formation and OC concentrations with increase in age as seen in female horses [269] or following reproduction [108] or both. Additional studies in other species are required prior to generalizing the effect of isoflurane on OC across maturity in different species. The mechanism for increase in OC upon exposure to isoflurane has been suggested to be its release from the bone surface and not increase in its synthesis. This may occur due the effect of stress hormones such as corticosterone [271] which has been shown to increase 1 min to 2 h after exposure to isoflurane in adult rabbits [272]. However, isoflurane exposure did not affect tDPD at any time point suggesting no effect on bone resportion which had never been investigated until now. The increase in tDPD with age may be due to postpartum hormonal changes that accompany the initiation of lactation which result in increased bone resorption during this phase [108]. Exposure to isoflurane did not change blood Ca^{2+}, Na and plasma total Ca at post-partum. However, isoflurane significantly decreased plasma tALP and albumin and increased plasma PO_4 and blood CL at 26 wk, suggesting that measurement of these minerals should be done prior to exposure to isoflurane.

The other objective was to measure the effect of isoflurane on both OC and glucose at post-partum, since OC may be involved in glucose metabolism by increase in both insulin secretion and sensitivity [54]. Although no changes were observed in plasma OC concentration after exposure to isoflurane at maturity, a significant increase in blood glucose was observed which exceeded the expected values for guinea pigs [278]. The latter may be as a result of the effect of isoflurane in reduction of the ATP sensitivity of KATP channels and therefore impaired glucose-stimulated insulin release as reported in rats [279]. Thus it is suggested that sampling prior to anesthesia at mature ages should still be practiced for glucose assessments.

Overall, this study suggests that exposure to isoflurane significantly increases circulating OC during rapid growth (before and after puberty), but not after pregnancy (late adolescence) while tDPD is unaffected. In addition, exposure to isoflurane affects circulating mineral and glucose concentrations postpartum. Therefore, the general hypothesis is accepted which is measurements prior to anaesthesia are recommended for better reflection of normal metabolism and consistency and comparability in research.

However, there are limitations to this research on the effect of isoflurane induced anaesthesia on bone and mineral biomarkers. At 2 d postpartum samples were collected through saphenous vein before exposure to isoflurane but after exposure to isoflurane, the same measurements were done on samples collected through cardiac puncture. Sampling from different routes may affect blood Na, ALP [281] and glucose [282, 283] concentrations. Therefore, caution must be taken when interpreting changes in these parameters induced by the anaesthetic and studies incorporating similar methods of sample collection are required to confirm these results. Measurement of PTH levels and stress hormones such as corticosteroids are

suggested to further explain the mechanisms responsible for changes in OC concentration and measurement of insulin and C-peptide concentrations are required to further explain changes in blood glucose concentration upon the induction of isoflurane anaesthesia.

The fourth hypothesis of this thesis was that maternal glucose concentration and tolerance are affected by dietary vitamin D intake and status during pregnancy. It was also hypothesized that maternal pre and mid-gestation concentrations and percent changes in 25(OH)D, $1,25(OH)_2D$ and OC are associated with OGTT results at mid-gestation; maternal body and abdominal composition at pre-gestation and postpartum are associated with 25(OH)D, $1,25(OH)_2D$ and OC at the same time-points and OGTT results at mid-gestation. These were investigated in the guinea model and the results are presented in Chapter 5 (Manuscript 3). The main outcome of this study was that maternal glucose concentration and tolerance were unaffected by dietary vitamin D intake and status during pregnancy in the guinea pig model and therefore the first section of the hypothesis is rejected. Despite the fact that change in maternal plasma 25(OH)D followed a dose-response to dietary vitamin D_3, plasma $1,25(OH)_2D$ did not follow a dose-response (plateaued once maternal dietary vitamin D intake > 0.5 IU/g). The decrease in $1,25(OH)_2D$ concentrations in the 0 IU/g diet group during pregnancy did not lead to undetectable levels of this active metabolite at late gestation which may be the reason for lack of differences in plasma minerals, OC, fasting glucose and OGTT outcomes among diet groups. These results are different from non-pregnant vitamin D deficient rats [37] and rabbits [38] in which injection of 25(OH)D or $1,25(OH)_2D$ prior to OGTT increased glucose induced insulin secretion [37, 38] and improved glucose tolerance at 3 h post glucose load [37, 38]. This may be due to the fact that in this guinea pig model vitamin D status reached deficient, but

not undetectable levels and only by mid-gestation while undetectable 25(OH)D concentrations had been reached prior to the OGTT in the previous studies in rats and rabbits [37, 38]. This may suggest that low or undetectable 25(OH)D concentration prior to pregnancy may have a detrimental role on glucose tolerance but if pregnancy initiates upon sufficient vitamin D status and reaches deficient levels only at late gestation this may not be associated with detrimental effects on glucose tolerance during pregnancy.

In accordance with these assumptions and despite the lack of difference among diet groups in OGTT results, vitamin D status at pre-mating, but not mid-gestation was negatively associated with AUC for glucose from OGTT. This suggests that vitamin D status may be more important at pre-mating than mid-gestation in protecting against pregnancy induced glucose intolerance. The lack of such associations between plasma $1,25(OH)_2D$ and glucose tolerance may be related to uncontrolled factors affecting plasma $1,25(OH)_2D$, due to its lower half-life and higher hormonal regulation as compared to 25(OH)D [246]. However, it should be considered that pre-mating 25(OH)D concentration (~ 250 nmol/L) were much higher in this guinea pig pregnant model than values normally observed in human pregnancy. Such high circulating 25(OH)D values are despite the fact that the guinea pigs were maintained a on a standard diet containing 1 IU/g diet vitamin D for 3 mo during the adaptation period, prior to mating. In vitamin D metabolism, the main excretory product of $1,25(OH)_2D$ is calcitroic acid which is catalyzed by 24-hydroxylase and excreted in the bile and 26,23-lactone which is another vitamin D degrading product found in the urine. Interestingly, in the guinea pig the preferred catabolic product for $1,25(OH)_2D$ is 26,23-lactone over calcitroic acid [92]. This suggests that $24,25(OH)_2D$ and $1,24,25(OH)_3D$ resulting from 24-hydroxylation of

25(OH)D and 1,25$(OH)_2$D which are then normally converted to calcitroic acid, may accumulate due to less degradation to calcitroic acid [92]. High concentrations of these metabolites cross-react with antibodies for 25(OH)D when assessed by non-chromatographic methods [332] such as chemiluminescence immunoassay (Liaison) which may explain such high 25(OH)D concentrations at baseline. Therefore, more specific methods such as liquid chromatography–tandem mass spectrometry (LC-MS/MS) may be required for detection of the actual concentration of 25(OH)D and other metabolites in such species.

The other outcome of this study on the effect of dietary vitamin D on glucose tolerance during pregnancy was that lower vitamin D status was associated with higher whole body and abdominal fat measured pre-pregnancy, the later also being a risk factor for GDM. Interestingly, this inverse relationship was only observed at pre-mating, prior to the vitamin D dose-response intervention and may be ascribed to sequestering of vitamin D in adipose tissue [36, 123]. There are no studies on the effect of vitamin D status during pregnancy on body composition, but similar results using the active metabolite have been reported in a study on overweight and obese humans [295]. In this guinea pig study, maternal plasma 1,25$(OH)_2$D and body weight as well as whole body and abdominal fat mass, but not percent fat and lean mass were positively associated preconception. Both higher [128, 299, 300] and lower [301, 302] circulating 1,25$(OH)_2$D have been reported in obese vs normal weight humans. Further research is required to clarify the association between the active form of vitamin D and body composition during pregnancy.

The association between OC and adiposity was also investigated in this study. In contrast to the negative association reported between OC and adiposity features

such as BMI and fat mass in animals [47] and humans [48-51], a positive relationship between this bone formation biomarker and all body composition components except percent body fat were observed at pre-mating, but no such associations were observed using the abdominal region. The interaction between OC and body composition is complex and can be affected by growth factors such as IGF-1. Perhaps the reason for a stronger association between plasma OC and all body compositions in the sow vs the neonate is due to higher maternal vs neonatal IGF-1 [304]. This area requires further investigation to clarify the association between OC and body composition (whole body and abdominal) at different ages and physiological states (pregnancy, non-pregnancy).

Abdominal body lean mass was also negatively associated with fasting glucose at pre-mating; however this was not the case with whole body reinforcing that low lean mass in the abdominal region is detrimental to health. Abdominal obesity at early gestation has also been inversely associated with glucose tolerance at late gestation in women [305]. These results show the importance of the lean mass content of the abdominal vs whole body region at pre-gestation against glucose intolerance and the strength of the animal model selected for use in this thesis.

The guinea pig model, however, is not without limitations. Even though, the guinea pig model is similar to human pregnancy in terms of time at which glucose concentration peaks on OGTT [56], the peak in blood glucose after oral glucose ingestion had a 30 min delay and blood glucose concentrations did not return to baseline values after 3 h on the OGTT, suggesting that perhaps the 2 g/kg of 70 % glucose solution may have been too high for the guinea pig OGTT in this study. In addition to plasma $1,25(OH)_2D$ that was a main covariate in the present study, plasma Ca [32] and PO_4 [33] may have a role in regulating glucose metabolism by increasing

insulin secretion. All these covariates were explored in the statistical regression model in this study, but were not significantly associated with glucose concentrations and OGTT results. However, there are other nutrients that may affect glucose metabolism such as Zn [143], chromium (Cr) [143], vitamin K [43], vitamin A [333]. Therefore, future research should also consider the possible role of these nutrients and possibly nutrient-nutrient interactions in the association between vitamin D and glucose tolerance during pregnancy.

The fifth hypothesis of this thesis was that neonatal glucose and body composition postpartum are affected by vitamin D status, $1,25(OH)_2D$ and OC concentrations resulting from the dose-response effect of maternal dietary vitamin D intake and status during pregnancy in guinea pigs and results are presented in Chapter 5 (Manuscript 3). The major result of this study is that a positive dose-response was observed in both neonatal plasma 25(OH)D and $1,25(OH)_2D$ in response to maternal dietary vitamin D_3 which surprisingly resulted in a positive association between neonatal plasma $1,25(OH)_2D$ and blood glucose concentration measured in the non-fed state. This might be related to the higher neonatal vs maternal postpartum $1,25(OH)_2D$ and OC concentrations in guinea pigs as shown in Chapter 6, but requires further investigation. In addition, a negative association was observed between plasma $1,25(OH)_2D$ and OC in the neonate which is in contrast to the positive relationship in humans and rats [293] and may be a result of interaction of $1,25(OH)_2D$ with bone-derived growth factors (IGF-1 and TGF-β) [294]. Also, despite the fact that a negative association was observed between plasma OC and blood glucose concentration in the neonate which is in accordance with animal [41] and human [43-46] reports, this was no longer significant after adjusting for plasma $1,25(OH)_2D$, again suggesting the possibility of interaction of calcitriol with growth

factors [294]. Therefore, overall the section of the hypothesis that neonatal $1,25(OH)_2D$ is associated with glucose was accepted.

Even though some human studies have suggested that maternal vitamin D status affects neonatal body fat and lean mass at birth [20, 297], no association was observed between vitamin D status and body composition 2 d postpartum which is similar to results from another study in human neonates [225]. No associations were observed between the active form of vitamin D and neonatal body weight at birth. Therefore this section of the hypothesis was rejected. Even though previous studies have shown a positive association between glucose concentrations and fetal fat mass in normal pregnancy [306, 307], in this study, this positive relationship was observed between blood glucose concentration and body weight and lean mass, but not fat mass or percent fat. In addition, a positive association between plasma OC and body weight and lean mass was observed in the neonate which is in contrast to previous findings in animals [47] and humans [48-51]. This association is similar to that observed in sows at pre-mating and requires further investigation. Overall, this study suggests that the associations between vitamin D metabolites, OC and body composition is complex and differs by life stages (neonatal vs late adolescence) and physiological states (pregnancy vs non-pregnancy). Further vitamin D interventional human studies are required to clarify the role of vitamin D and osteocalcin on body composition.

There are limitations to this study including that neonate blood glucose measurement was limited to 3 h after food withdrawal which is shorter than the overnight fasting in previous studies [56, 233] and therefore may not accurately represent glucose homeostasis. Studies with longer fasting periods (8-12 h) are required to further investigate these associations.

The sixth hypothesis of this thesis was that both low (<50 nmol/L) and high (>125 nmol/L) maternal 25(OH)D concentrations would impair bone development while moderate concentrations (50-125 nmol/L) would result in optimal maternal and fetal bone health by analyses of plasma minerals, vitamin D metabolites and bone biomarkers as well as assessment of whole body and regional BMC, BMD, microarchitecture and biomechanics. These were investigated in the guinea pig model during reproduction since this animal model is similar to human pregnancy in terms of changes in circulating $1,25(OH)_2D$ and OC and fetal vs maternal levels of these parameters [55]. The results of this section are presented in Chapter 6 (Manuscript 4).

The hypothesized maternal and neonatal plasma 25(OH)D dose-responses to maternal dietary vitamin D were achieved in this study and closely parallels that recently reported in human pregnancy [10]. Maternal plasma 25(OH)D concentration <50 nmol/L was achieved in the 0 IU group at 2 d postpartum and plasma 25(OH)D concentration >125 nmol/L was observed across pregnancy in the 1 and 2 IU groups. However, there was a significant difference between these two groups, since plasma 25(OH)D concentration was ~ 125 nmol/L in the 1 IU group at 2 d postpartum and ≥250 nmol/L at all pregnancy time-points including 2 d postpartum in the 2 IU group. This is while neonatal plasma 25(OH)D measured 2 d postpartum were ≤ 50 nmol/L in all groups except the 2 IU which reached concentrations as high as ~ 125 nmol/L, although still below maternal concentrations in accordance with previous studies [10, 13, 67, 79, 199, 204]. The striking similarity between maternal and neonatal vitamin D status demonstrates that maternal-fetal transfer is not saturable up to 250 nmol/L of 25(OH)D in maternal plasma. Furthermore, maternal plasma $1,25(OH)_2D$ plateaus at high intakes of vitamin D whereas it follows a dose-response in the neonates.

The implication of a high 25(OH)D and $1,25(OH)_2D$ include suppression of PTH [315] and anticipated responses in bone [316]. Using the proxy PTH index, these results suggest that maternal PTH concentration would plateau only at very high vitamin D status, exceeding 200 nmol/L of circulating 25(OH)D. However, no differences in maternal regional BMC, aBMD, vBMD were observed among diet groups. Interestingly, in the neonate, the proxy for PTH plateaus at much lower 25(OH)D concentrations (<50 nmol/L), especially in the 0 IU group. Accordingly, the neonates that had >50 nmol/L, mainly from the 2 IU group, had the highest femur aBMD.

The volumetric and micro architecture assessment of maternal bone was not affected by dietary vitamin D during pregnancy. However, lower neonatal femur cortical TMD possibly due to secondary hyperparathyroidism [318] and higher FSI were observed in the 0 IU group. This observation is consistent with the early phases of congenital rickets and is similar to the higher FSI in fetus of vitamin D deficient (<50 nmol/L) mothers at late gestation [63]. In addition, there were sex-dependent differences in some of the neonatal femoral features such as higher FSI in male vs female. The higher distal femur and proximal tibia Tb. vBMD observed in the 0 group may be due to an increase in the amount of unmineralized bone as a result of the possible secondary hyperparathyroidism in this group [318] and should therefore not be interpreted as a positive outcome. In fact, higher FSI is likely a manifestation of low $1,25(OH)_2D$ and expansion of the growth plate as observed in the histomorphometry of the distal femur growth plate in the 0 IU group [161, 320]. However, higher $1,25(OH)_2D$ in the offspring from the 2 IU group was accompanied with higher distal femur and proximal tibia Tb. vBMD which may be a result of hypoparathyroidism [322] that is also known to increase the bone Tb. component

[318]. Sex differences were observed with lower femur cortical TMD in the males, but not the females of the 2 IU group. Whether these sex-dependent differences are related to growth rate (higher OC and tALP was observed in male vs female) rather than sex differences in vitamin D metabolism should be further investigated. The lack of difference in Tb. bone at the lumbar spine may suggest that sex effects are mainly observed in bones with growth plates.

The observations reviewed above are all static outcomes. Functional outcomes of the bone can be assessed by biomechanical tests. The femoral stiffness assessed as part of the biomechanical tests of this study suggested that the highest vitamin D dosage (2 IU) during pregnancy may not result in higher maternal bone quality. In addition, the femoral tissue mineral density was positively correlated with stress (force adjusted for bone shape and size) and negatively correlated with strain (deformation adjusted for bone shape and size) in both the sows and pups, which is in line with previous reports in long bones of humans [311] and monkeys [324].

The biochemical measures of Ca and bone metabolism suggest that dose-response to dietary vitamin D during pregnancy results in plasma mineral homeostasis at the expense of changes in neonatal BMD and microarchitecture and with no significant maternal effects. The hypothesis, that maternal vitamin D status <50 nmol/L has detrimental effects on fetal bone health is accepted, but higher concentrations should be studied to confirm if an upper range of normal should be set at 125 nmol/L. It should be considered that the chemiluminescence assay used to measure 25(OH)D may have falsely elevated the values due to cross-reactivity of the assay antibody with other vitamin D metabolites. Future vitamin D dose-response studies are recommended to perform measurements of 25(OH)D concentrations using

methodologies such as LC-MS/MS that have less cross-reactivity with other vitamin D metabolites.

There are many other factors that may explain some of the differences in these observations as compared to other vitamin D deplete vs replete studies in rats [78] and guinea pigs [13, 79]. The dietary Ca and P content of this study (1.1% Ca and 0.65 % P) was higher than a previous guinea pig study (0.96 % Ca and 0.49 % P) which has shown to compensate for some of the detrimental effects of vitamin D deficiency on bone phenotype [13]. In addition, baseline 25(OH)D concentrations which affect the dose-response to vitamin D intakes [327] were similar but high (~250 nmol/L) among all diet groups and the sows were randomized to diets at mating vs several weeks prior to mating [78] to resemble conception in summer or early fall to complement human investigations. The plasma 25(OH)D response therefore reached the intended concentrations only by late gestation, suggesting that perhaps the ~70 d duration of pregnancy was too short to cause differences in bone.

Similar to human pregnancy [328], in the guinea pig, pregnancy induced a decrease in maternal vitamin D status along with an increase in $1,25(OH)_2D$ as early as the end of 1st trimester. This was accompanied by a decrease in maternal plasma total Ca similar to human pregnancy [105], but the observed decrease in plasma PO_4 and blood Ca^{2+} is different from humans and likely ascribed to larger litter size in small animals [105]. Similar to previous reports, a decrease in plasma OC was observed [55], which may be due to increased uptake of OC by the placenta [329]. However, no changes were observed in tALP and tDPD during pregnancy. Nonetheless, the increase in plasma PO_4 at 2d postpartum is similar to previous reports [105, 202] which accompanied a further reduction in OC and increase in tDPD, probably as a result of hormonal changes that initiate upon lactation resulting

in increased bone resorption [108]. Also, the decrease in plasma tALP after delivery may be a result of parturition [329]. These biochemical changes across pregnancy accompanied an increase in maternal long bone and decrease in vertebrae 3 aBMD, in accordance with these changes in rats [330] and humans [331]. These bone changes during pregnancy suggest that immediate demands for Ca homeostasis draw upon Tb. bone of vertebrae whereas mineral stores for later use in lactation might reside in long bone in this young pregnant model in which growth plates are not yet closed [270].

Therefore the main outcome of this study is that providing dietary vitamin D ranging from deficient to more than recommended levels results in a positive parallel response in maternal and neonatal vitamin D status, but is not reflected in maternal mineral or bone health outcomes. The fetus responded with higher Tb. density of long bone at both low and high maternal vitamin D intakes, but features of congenital rickets were observed in male and female neonates of the deficient group, suggested by the expansion of the growth plate. The neonatal response was differential and sex-dependent at the cortical femur with lower TMD in the male neonates at the low and high maternal vitamin D intakes and lower TMD in the female neonates of the deficient group only while bone quality assessed by strength was unaffected. These data reinforce that maternal vitamin D intakes and status below recommendations may compromise bone health in the offspring and that higher intakes and status are not necessarily advantageous.

The guinea pig model has limitations for studying maternal and neonatal bone health including the large litter size (median of 3 [1-5]) and the higher neonatal to maternal whole body Ca content in guinea pigs (~14 %) vs humans (~3 %) [19]. In addition, the dosage vitamin D intake IU/kg body weight is not comparable to humans. Measurement of bone strength was performed at the site mainly containing

cortical bone and not beneath the growth plate. Future studies should also investigate biomechanical strength at Tb. rich site of the bone, especially in the neonates. One other limitation of this study is that neonates were not followed after birth but the early signs of rickets in the growth plate reinforce the importance of infant supplementation and its initiation at birth which has also been suggested from results of studies in guinea pigs [79] and in line with recommendations by the IOM [1].

Future studies focusing on the mechanisms involved in the dose-response effect of vitamin D through PTH, FGF23 and other metabolites of vitamin D such as $24,25(OH)_2D$ and the RANK, RANKL, OPG for further explanation of metabolism pathways and bone biology would be useful. In addition, the *in vivo* dose-response effect of a vitamin D during pregnancy on VDR expression in target genes associated with bone health is another interesting area of research which has not been fully investigated.

In conclusion, the studies in this thesis demonstrate that vitamin D status may be more important at pre-mating than mid-gestation in protecting against pregnancy induced glucose intolerance. To reflect normal metabolism, measurements of OC and glucose prior to isoflurane anaesthesia are recommended. Maternal vitamin D status below recommendations may result in features similar to rickets in the offspring, but status above recommendations may not be advantageous to bone as shown in the guinea pig model.

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