VITAMIN D METABOLISM IN HUMAN PREGNANCY

WITH AN EMPHASIS ON THE PLACENTA AND BONE METABOLISM

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ABSTRACT

Vitamin D is an essential fat-soluble nutrient that plays a critical role in calcium homeostasis among women of childbearing age. Vitamin D has also been linked to pregnancy health outcomes and may have long-term effects on maternal and child health. Nevertheless, the effects of human pregnancy on vitamin D requirements are poorly understood, and very little is known about placental vitamin D metabolism and its putative effects on maternal circulating pool of vitamin D. The impact of maternal vitamin D status on maternal and fetal bone health is also unclear. As such, my dissertation sought to advance understanding of vitamin D metabolism in human pregnancy, to characterize the effect of the placenta on maternal circulating vitamin D metabolites, and to examine the impact of maternal vitamin D status on maternal and fetal bone health.

To achieve these aims, biomarkers of vitamin D status/metabolism were measured in blood and placental tissue obtained from third-trimester pregnant women who participated in a long-term controlled feeding study and consumed equivalent intakes of vitamin D, calcium, and phosphorus. A human placental cell culture model was also used to characterize vitamin D uptake, intracellular metabolism, secretion, and to inform the interpretation of the human study data.

The data showed that pregnant women had 30-100% higher ($P<0.01$) circulating vitamin D metabolites [25(OH)D, $1,25(OH)_2D$, 3-epi-$25(OH)D_3$, and $25(OH)D:24,25(OH)_2D$] than nonpregnant women suggesting that the placenta may be a source of vitamin D metabolites during pregnancy. This notion was supported by the significant associations ($P\leq0.04$) between maternal circulating vitamin D metabolites and placental mRNA abundance of vitamin D machinery (*LRP2, CUBN, CYP2R1, CYP27B1,* and *CYP24A1*), and by the endogenous production and secretion of $25(OH)D_3$ by placental trophoblasts *in vitro*. The data also showed that maternal 25(OH)D, free 25(OH)D, $1,25(OH)_2D$, and epi-$25(OH)D_3$ were inversely associated ($P\leq0.04$) with maternal bone resorption markers (deoxypyridinoline, NTx, and iPTH).

Overall, these data collectively suggest that pregnancy increases circulating pools of maternal vitamin D metabolites, in part, through the placenta which can produce and secrete all forms of vitamin D metabolites including $25(OH)D_3$. These findings also imply that achieving higher maternal concentrations of circulating vitamin D metabolites during pregnancy may attenuate maternal bone loss.

BIOGRAPHICAL SKETCH

Heyjun Park grew up in Seoul, South Korea. She received her B.S. and M.S. in Food and Nutrition from Yonsei University, Seoul, in 2008 and 2010, respectively. During her master's program with a focus on clinical nutrition, she was handpicked as a graduate student researcher by the Brain Korea 21 Project and received a research grant from the National Research Foundation of Korea. Through these accomplishments, she could participate in several national projects on the associations of cardiovascular disease (CVD) with nutritional and genetic biomarkers. Specifically, she measured and analyzed metabolites related to vascular endothelial function and DNA damage markers from patients with CVD.

These experiences deepened Heyjun's interest in human nutrition and led her to the doctoral program in the Division of Nutritional Sciences at Cornell University in the fall of 2011 at which time she joined the laboratory of Dr. Caudill. Heyjun's research projects addressed fundamental biological questions on vitamin D metabolism during pregnancy and the role of the placenta in modulating the vitamin D metabolite profile in maternal circulation. The projects also advanced understanding of the effect of maternal vitamin D status on maternal and fetal bone health. During her doctoral program, she was awarded two nationally competitive external research grants, the 'Gerber Foundation Novice Researcher Award' and the 'USDA NIFA Predoctoral Fellowship,' one of the most prestigious awards for doctoral students in the nutrition field. In addition, although Heyjun is a Korean registered dietitian (RD), she was also enrolled in the Didactic Program in Dietetics (DPD) at Cornell and has completed the DPD requirements to earn the U.S. RD credential. She expects to begin her supervised practice in 2017.

Dedicated to my parents and husband

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Finally, I owe my greatest debt of gratitude to my parents, Sang-Sik Park and Jin-Sook Choi, my sister Heyri Park, and my husband Jason Na who have given me their endless, unconditional love and moral support throughout my life, including my Ph.D. journey at Cornell.

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$1,25(OH)_2D$: 1,25-dihydroxyvitamin D

$1,25(OH)_2D$:25(OH)D: ratio of 1,25-dihydroxyvitamin D to 25-hydroxyvitamin D

$24,25(OH)_2D$: 24,25-dihydroxyvitamin D

25(OH)D:$24,25(OH)_2D$: ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D.

25(OH)D: 25-hydroxyvitamin D

ALP: alkaline phosphatase

CTx: carboxy-terminal cross-linking telopeptide of type 1 collagen

CUBN: cubilin

CYP24A1: mitochondrial 24-hydroxlyase

CYP27A1: mitochondrial 25-hydroxylase

CYP27B1: mitochondrial 1α-hydroxylase

CYP2R1: microsomal 25-hydroxylase

DBP: vitamin D binding protein

DEQAS: Vitamin D External Quality Assessment Scheme

DMEQ-TAD: 4-[2-(6,7-dimethoxy-4-methyl-3-oxoquinoxalin-2-yl)ethyl]-1,2,4-triazole-3,5-dione

DPD: deoxypyridinoline

DRI: dietary reference intakes

EAR: estimated average requirement

Epi-$25(OH)D_3$: C3 epimer of 25-hydroxyvitamin D_3

FBS: fetal bovine serum

GC: vitamin D binding protein gene

GUSB: beta-glucuronidase

HMRU: Human Metabolic Research Unit

IOM: Institute of Medicine

iPTH: intact parathyroid hormone

LC-MS/MS: liquid chromatography tandem-mass spectrometry

LMM: linear mixed-effects model

LOD: limit of detection

LRP2: low density lipoprotein-related protein 2; megalin

NIST: National Institute of Standards and Technology

NTx: amino-terminal cross-linking telopeptide of type 1 collagen

OC: osteocalcin

PTAD: 4-Phenyl-1,2,4-triazoline-3,5-dione

RDA: recommended dietary allowance

RT-PCR: quantitative real-time PCR

RXR: retinoid X receptor

SRM: Standard Reference Material

UVB: ultraviolet B

VDR: vitamin D receptor

VDRE: vitamin D response element

PREFACE

SPECIFIC AIMS

The overarching goal of my dissertation research was to advance current understanding of maternal vitamin D metabolism in human pregnancy with an emphasis on the placenta, and to delineate the effects of maternal vitamin D status on maternal and fetal bone health outcomes.

Aim 1: To test the hypothesis that the reproductive state (i.e., pregnancy and lactation) will alter blood biomarkers of vitamin D metabolism. This aim was achieved by measuring circulating concentrations of 25-hydroxyvitamin D [25(OH)D], 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], vitamin D binding protein (DBP), 24,25-dihydroxyvitamin D [$24,25(OH)_2D$], and C3 epimer of $25(OH)D_3$, and by estimating free 25(OH)D, the 25(OH)D:$24,25(OH)_2D$ ratio, and the $1,25(OH)_2D$:25(OH)D ratio in pregnant, lactating and control women who consumed equivalent amounts of vitamin D and other nutrients. *Results are presented in chapter 1.*

Aim 2: **To test the hypothesis that placenta metabolizes vitamin D and contributes to the changes in maternal circulating vitamin D metabolites.** This aim was achieved by measuring placental gene expression levels of the megalin-cubilin receptor (LRP2 and CUBN), 25-hydroxylase (CYP2R1), 1α-hydroxylase (CYP27B1), and 24-hydroxylase (CYP24A1) as well as the metabolite concentrations of vitamin D metabolites including 25(OH)D from placental tissues. In addition, associations with maternal circulating vitamin D metabolites were examined. *Results are presented in chapter 2.*

Aim 3: To examine the uptake and metabolic fate of vitamin D [i.e., vitamin D, 25(OH)D, $1,25(OH)_2D$, and $24,25(OH)_2D$] in human placental trophoblasts. This aim was achieved by employing a human placental cell culture model using stable isotope methodology. *Results are presented in chapter 2.*

Aim 4: To test the hypothesis that maternal serum 25(OH)D concentrations are associated with maternal and fetal markers of bone metabolism. This aim was achieved by examining associations between maternal markers of vitamin D status [i.e., 25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$, free 25(OH)D, and C3 epimer of $25(OH)D_3$) and maternal/fetal biochemical markers of bone metabolism (i.e., osteocalcin, alkaline phosphatase, carboxy-terminal cross-linking telopeptide of type 1 collagen, amino-terminal cross-linking telopeptide of type 1 collagen, deoxypyridinoline, and parathyroid hormone) in pregnant and control women consuming controlled amounts of vitamin D-related nutrient such as calcium and phosphorus. *Results are presented in chapter 3.*

OUTPUT

This dissertation research yielded two published peer-reviewed articles (Chapter 1 and 3) and one original research manuscript which will be submitted for publication within the next few months (Chapter 2).

BACKGROUND, SIGNIFICANCE AND RATIONALE

Vitamin D requirement and the prevalence of vitamin D inadequacy in pregnant and lactating women

Due to uncertainties about the specific biological roles of vitamin D during pregnancy and lactation, no incremental increases in the vitamin D RDA (relative to women of reproductive age) were made by the Institute of Medicine (IOM) for pregnant and lactating women (1,2). Thus, 600 IU vitamin D/d is currently recommended for all women of childbearing age including those who are pregnant or breastfeeding. Nevertheless, vitamin D inadequacy among pregnant and lactating women is highly prevalent worldwide with the percentage of inadequacy varying by race, ethnicity, or latitude/country of residence (3–5). Moreover, several epidemiologic and case-control studies have shown that low vitamin D status increases the risk for adverse reproductive outcomes such as impaired fetal/neonatal growth and bone development, preeclampsia, higher risk of Cesarean delivery, and bacterial vaginosis (6). **Therefore, it is important to quantify the effect of the reproductive state (i.e., pregnancy and lactation) on vitamin D metabolism and establish vitamin D recommendations for pregnant and lactating women that promote optimal health of both mother and fetus/infant.**

Vitamin D metabolism and its classical endocrine function

Vitamin D is a unique essential nutrient because it has an exogenous (dietary) source and an endogenous source when synthesized by the skin. The body obtains vitamin D in the form of vitamin D_2 and D_3 by the intake of fatty fish, egg yolk, vitamin D fortified foods, or dietary supplements. When exposed to ultraviolet B (UVB) with wavelengths between 290 and 320 nm, 7-dehydrocholesterol is converted to previtamin D_3 in the epidermis, and subsequently thermally isomerized to vitamin D_3 (7). Irrespective of the source, both dietary and endogenously produced

vitamin D are ultimately carried to the liver (1). Dietary vitamin D is absorbed in the intestine and packaged with other lipids into a chylomicron. The chylomicron enters into the systemic circulation via the lymph and is then metabolized to a chylomicron remnant by lipoprotein lipase in the peripheral tissues. In turn, this vitamin D-containing remnant is transported into the liver. On the other hand, endogenously produced vitamin D in the skin diffuses across subcutaneous adipose layer into the blood, binds to vitamin D binding protein (DBP), and is taken up by the liver.

In the liver, vitamin D is hydroxylated to 25-hydroxyvitamin D [25(OH)D] by microsomal 25-hydroxylase, CYP2R1 (the major one) or mitochondrial 25-hydroxylase, CYP27A1. 25(OH)D can then be released into the blood where it mostly binds to DBP; small amounts of free 25(OH)D can also be found in circulation (8). Because 25(OH)D is biologically inert, it must be converted to an active form to perform its classical endocrine function. Renal proximal tubular cells take up DBP-bound 25(OH)D from the glomerular ultrafiltrate through endocytic receptors, the megalin-cubilin receptor complex, and the second hydroxylation to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], also called calcitriol, occurs by mitochondrial 1α-hydroxylase (CYP27B1). Once produced, the biologically active form of vitamin D, 1,25$(OH)_2$D, is released into circulation where it binds to DBP and enters the classical target tissues such as bone, intestine, and kidney. In cells of the target tissues expressing vitamin D receptor (VDR), 1,25$(OH)_2$D binds to VDR in the nucleus and VDR forms a heterodimer with retinoid X receptor (RXR). Next, this 1,25$(OH)_2$D-VDR-RXR heterodimeric complex binds to a vitamin D response element (VDRE) located in vitamin D-dependent genes to regulate the expression of these genes with functions in calcium and phosphate homeostasis (9). Through this action, vitamin D is intimately involved in bone development and maintenance (10) (**Figure P.1**). Other physiologic processes that are influenced

by $1,25(OH)_2D$ include the immune system, insulin secretion, blood pressure, and fetal development (11).

The degradation of $1,25(OH)_2D$ and 25(OH)D is carried out by mitochondrial 24-hydroxlyase (CYP24A1), which is found in all target tissues and produces inactive metabolites such as calcitroic acid and 24,25-dihydroxyvitamin D [$24,25(OH)_2D$], respectively (Figure P.1). When the $1,25(OH)_2D$-VDR-RXR complex binds to VDRE in *CYP24A1* gene, $1,25(OH)_2D$ can stimulate its degradation through activation of the catabolic enzyme CYP24A1. The produced catabolic metabolites are then excreted through the bile in the feces (12).

As $1,25(OH)_2D$ is a hormone, the metabolic step of $1,25(OH)_2D$ synthesis is tightly regulated in the body, and its half-life is about 4 hours. In contrast, the synthesis of 25(OH)D is less regulated than the synthesis of $1,25(OH)_2D$, and it has a longer half-life, approximately three weeks. In addition, its synthesis depends on the levels of the substrate, vitamin D. For these reasons, 25(OH)D is the major circulating form of vitamin D and serum concentration of 25(OH)D is currently the best biomarker of vitamin D status as it reflects the total exposure from the diet and cutaneous synthesis (13).

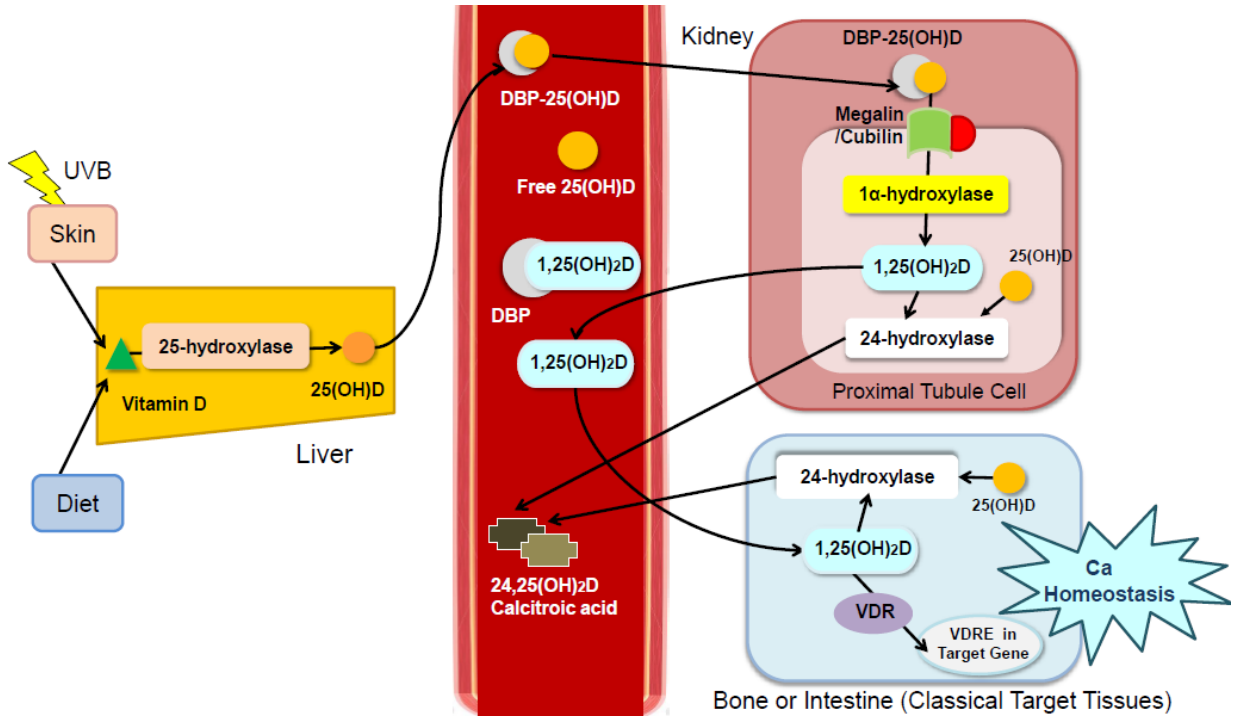

Figure P.1. Overview of vitamin D metabolism and its classical endocrine function. Both endogenously synthesized vitamin D in the skin and absorbed dietary vitamin D in the intestine are transported into the liver and hydroxylated to 25(OH)D by 25-hydroxylase. Released 25(OH)D binds to DBP and travels to the kidney where the second hydroxylation by 1a-hydroxylase to $1,25(OH)_2D$ occurs. $1,25(OH)_2D$ is released into circulation, and then can enter and act on the target cells through a VDR-mediated transcriptional regulation.

Vitamin D metabolism in pregnancy and lactation

Pregnancy is characterized by a dramatic increase in two maternal circulating vitamin D biomarkers: about a 150% increase in DBP (14–16) and a 200% increase in $1,25(OH)_2D$ (14,17–19). However, the underlying mechanisms of these changes during pregnancy are largely unknown.

Data on the effect of pregnancy on maternal circulating 25(OH)D is mixed among studies with reports of no changes (17,15,20), increases (14,18,21,22), or decreases (19,23). A meta-analysis (17) including 24 studies in pregnant and nonpregnant women reported no difference in serum 25(OH)D between these groups. However, most of the studies were conducted prior to current standardized efforts to reduce large inter-laboratory variations in measuring serum 25(OH)D such as National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) for serum 25(OH)D or the Vitamin D External Quality Assessment Scheme (DEQAS). As such, substantial heterogeneity among the studies that used different quantification methods of 25(OH)D raises concerns regarding the validity of these results. Moreover, most studies did not adequately assess maternal vitamin D intake and/or intake of other relevant nutrients (i.e., calcium and phosphorus). Because vitamin D intake affects serum 25(OH)D, a biomarker of exposure to vitamin D, and dietary calcium and phosphorous interact with vitamin D metabolism, the contradictory results of 25(OH)D during pregnancy may have arisen from differences in the dietary intake of these nutrients across the studies. Some studies on the effect of lactation on maternal vitamin D metabolism have reported decreases in circulating $1,25(OH)_2D$, DBP, and 25(OH)D (15,24,25). However, it is unclear if vitamin D status of lactating women differs from that of a nonpregnant, nonlactating woman under conditions of equivalent intakes of vitamin D and related nutrients.

Finally, changes in additional maternal circulating vitamin D metabolites including 24,25$(OH)_2$D in pregnancy and lactation have been less explored compared to 1,25$(OH)_2$D, DBP, and 25(OH)D. **Taken together, human feeding studies which experimentally control the intake of vitamin D and related nutrients are needed to more fully understand the influence of pregnancy and lactation on vitamin D metabolism and requirements.**

Placental vitamin D metabolism and its possible contribution to circulating vitamin D metabolites including 25(OH)D

Very little is known about human placental vitamin D metabolism (26). Moreover, the mechanism leading to alterations in maternal vitamin D metabolism during pregnancy is largely unexplored. However, the placenta is emerging as a potential mediator of the changes in maternal circulating vitamin D metabolites as it expresses all components of the vitamin D metabolic pathway such as the megalin-cubilin receptor complex (LRP2 and CUBN, respectively) (27,28), 1α-hydroxylase (CYP27B1) (29–31), 24-hydroxlyase (CYP24A1) (31,32), and 25-hydroxylase (CYP2R1) (31).

The megalin-cubilin receptor complex was initially identified in the kidney proximal tubules (33). However, it was later found to be located in the membrane of tissues other than the kidney, including the placenta (27,28). In the kidney, DBP-bound 25(OH)D is taken up through the megalin-cubilin receptor-mediated endocytosis and subsequently used to produce 1,25$(OH)_2$D (34). Thus, placental possession of the megalin-cubilin receptors (27,28) along with the findings of elevations of DBP and 1,25$(OH)_2$D during pregnancy (15,16) could suggest that the placenta may be a source of the bioactive form of vitamin D.

The placenta also has several enzymes that comprise the vitamin D metabolic pathway such as CYP27B1 (29–31), CYP24A1 (31,32), and CYP2R1 (31). Furthermore, *in vitro* production of 1,25$(OH)_2$D (29,35) and 24,25$(OH)_2$D (35,36) by CYP27B1 and CYP24A1, respectively, was reported in human placental cells. The placentally produced 1,25$(OH)_2$D was further confirmed to be capable of entering maternal circulation as nephrectomized pregnant rats exhibited placental release of [^{3}H]-1,25$(OH)_2$D into maternal circulation after injecting [^{3}H]-25(OH)D (37), and nephrotic pregnant women who discontinued her oral treatment with 1,25$(OH)_2$D for 3 weeks did not show decreases in serum 1,25$(OH)_2$D concentrations (a half-life of ~4 hours) (38). In contrast to the placental production of 1,25$(OH)_2$D and 24,25$(OH)_2$D which have been established, whether the placenta produces 25(OH)D from vitamin D by CYP2R1 and contributes to the maternal circulating pool of this vitamin metabolite remain unknown. Lastly, it is also unclear about the impact of placental vitamin D metabolism on additional circulating vitamin D metabolites such as 24,25$(OH)_2$D.

In sum, the placenta appears to play a central role in regulating vitamin D metabolism and status during pregnancy. However, additional human studies are needed to better understand placental vitamin D metabolism and its contribution to the modification of maternal circulating vitamin D metabolites. Unfortunately, studies in human pregnancies examining this possibility have left out key measurements including placental concentrations of vitamin D metabolites. In addition, no study has systematically examined uptake and metabolic fate of all forms of vitamin D such as parent vitamin D, 25(OH)D, 1,25$(OH)_2$D, and 24,25$(OH)_2$D in human placental trophoblasts *in vitro*.

The impact of maternal vitamin D status on maternal and fetal bone health outcomes

During pregnancy, ~25-30g of calcium is transferred to the fetus, and 99% of it is used to mineralize the skeleton. To meet most of this demand for calcium, maternal intestinal calcium absorption doubles early in pregnancy (39). Maternal bone resorption may also occur to provide calcium to the circulation and ultimately the fetus, although the extent to which mother's bone resorption contributes calcium to the developing fetus is unclear (40). Because vitamin D is known to influence the aforementioned physiological processes (i.e., intestinal calcium absorption and bone resorption) in a normal nonpregnant state (1), one might expect that ensuring sufficient vitamin D would be critical in meeting the high demands for calcium during gestation. However, whether this classical endocrine function of vitamin D in bone health is operational during pregnancy is uncertain.

In animal studies, vitamin D-deficient pregnant rats and *VDR* knockout pregnant mice exhibited increased calcium absorption in the intestine (41–43). Moreover, fetuses from severely vitamin D-deficient or VDR knockout mice showed normal calcium homeostasis and normal bone mineralization, although the mother mice showed severe hypocalcemia (44,45). Thus, it has been suggested that the adaptations which occur to meet a substantial calcium demand for fetal bone mineralization in pregnancy may occur via a vitamin D-independent route (46).

However, in contrast to the animal studies, results from human studies are mixed regarding the impact of maternal vitamin D status on fetal bone health. Some studies observed associations between maternal vitamin D status and indices of bone health in the fetus (e.g., bone mineral density and biochemical markers of bone metabolism) (22,47–49), whereas others observed no associations (50–52). Moreover, results from a few clinical trials that examined the effect of vitamin D supplementation on maternal benefit for bone health among pregnant women did not

extend beyond raising serum 25(OH)D concentrations (3,40,53). Nevertheless, the majority of the studies did not account for maternal dietary intakes of calcium and phosphorus which could be important confounders and contribute to the inconsistencies across study findings. For example, low calcium intake can disturb bone metabolism (54) and mask the relationship between vitamin D status and bone health. **Thus, human feeding studies that control for dietary intakes of vitamin D-related nutrients during pregnancy are needed to more precisely assess the relationship between maternal vitamin D status and maternal and fetal biomarkers of bone metabolism.**

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CHAPTER 1

Vitamin D metabolism varies among women in different reproductive states consuming the same intakes of vitamin D and related nutrients[1,2]

[1]Park H, Brannon PM, West AA, Yan J, Jiang X, Perry CA, Malysheva OV, Mehta S, Caudill MA. Vitamin D metabolism varies among women in different reproductive states consuming the same intakes of vitamin D and related nutrients. J Nutr. 2016 Aug;146(8):1537-45. doi: 10.3945/jn.116.229971. Regarding the copyright of this article, authors have the right to include the article in their thesis or dissertation.

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ABSTRACT

Background: The impact of the reproductive state on vitamin D metabolism and requirements is uncertain in part because of a lack of studies with controlled dietary intakes of vitamin D and related nutrients.

Objective: We aimed to quantify the impact of the reproductive state on a panel of vitamin D biomarkers among women of childbearing age consuming equivalent amounts of vitamin D and related nutrients.

Methods: Nested within a feeding study providing 2 doses of choline, healthy pregnant (26–29 wk gestation; *n* = 26), lactating (5 wk postpartum; *n* = 28), and control (nonpregnant/nonlactating; *n* = 21) women consumed a single amount of vitamin D (511±48 IU/d: 311±48 IU/d from diet and 200 IU/d as supplemental cholecalciferol) and related nutrients (1.6±0.4 g Ca/d and 1.9±0.3 g P/d) for 10 wk. Vitamin D biomarkers were measured in blood obtained at baseline and study-end, and differences in biomarker response among the reproductive groups were assessed with linear mixed models adjusted for influential covariates (e.g., body mass index, season, race/ethnicity).

Results: At study-end, pregnant women had higher ($P < 0.01$) circulating concentrations of 25-hydroxyvitaminD [25(OH)D; 30%], 1,25-dihydroxyvitamin D [1,25$(OH)_2$D; 80%], vitamin D binding protein (67%), and C3 epimer of 25$(OH)D_3$ (100%) than control women. Pregnant women also had higher ($P < 0.04$) ratios of 25(OH)D to 24,25-dihydroxyvitamin D [24,25$(OH)_2$D; 40%] and 1,25$(OH)_2$D to 25(OH)D (50%) than control women. In contrast, no differences ($P > 0.15$) in vitamin D biomarkers were detected between the lactating and control groups. Notably, the study vitamin D dose of 511 IU/d achieved vitamin D adequacy in most participants (95%) regardless of their reproductive state.

Conclusions: The higher concentrations of vitamin D biomarkers among pregnant women than among control women suggest that metabolic adaptations, likely involving the placenta, transpire to enhance vitamin D supply during pregnancy. The study findings also support the adequacy of the current vitamin D RDA of 600 IU for achieving serum 25(OH)D concentrations > 50 nmol/L among women differing in their reproductive state. This trial was registered at clinicaltrials.gov as NCT01127022.

INTRODUCTION

Worldwide vitamin D inadequacy is common among pregnant and lactating women (1–3), and it is linked in some epidemiologic studies to adverse reproductive outcomes, including impaired fetal/neonatal growth, preeclampsia, and immune disorders (4, 5). In 2011, the Institute of Medicine (IOM) established a vitamin D RDA of 600 IU for women of childbearing age. This recommendation was based on the vitamin D intake projected to achieve circulating concentrations of 50 nmol/L for 25-hydroxyvitaminD [25(OH)D], the primary indicator of vitamin D status. Reproductive state was not considered in the formulation of this RDA because of an absence of 25(OH)D dose-response data among pregnant and/or lactating women (1, 6).

Interestingly, pregnancy is characterized by robust increases in circulating 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] (7–10), the biologically active form of vitamin D, and vitamin D binding protein (DBP) (8, 11, 12), a carrier of vitamin D metabolites in blood. However, the effect of pregnancy on circulating 25(OH)D is less clear with studies reporting no effects (7, 11, 13), increases (9, 14, 15), or decreases (10, 16). These mixed findings across studies may arise from intake differences of vitamin D and relevant nutrients (e.g., calcium and phosphorus), which were either unknown or incompletely assessed. Some studies have shown that circulating concentrations of 1,25$(OH)_2$D, DBP, and 25(OH)D decline during lactation (9, 11, 17, 18); however, it is unclear if vitamin D status differs from that of a nonpregnant, nonlactating woman under conditions of comparable vitamin D intake. Furthermore, little is known about the effect of reproductive state on several additional biomarkers of vitamin D metabolism, including 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], a catabolite of vitamin D; the ratio of 25(OH)D to 24,25$(OH)_2$D [25(OH)D:24,25$(OH)_2$D], a newly proposed sensitive indicator of vitamin D status (19–22); free 25(OH)D (16, 23); and the C3 epimer of 25-hydroxyvitamin D_3 [epi-25$(OH)D_3$], a

recently identified metabolite (24, 25) with unknown biological function.

To advance understanding of the effects of reproductive state on vitamin D status and metabolism, we used samples obtained during a feeding study that provided a single amount of vitamin D and related nutrients (e.g., calcium and phosphorus) to pregnant, lactating, and control (nonpregnant, nonlactating) women across a 10-wk time period. Our aims were as follows: 1) to quantify the impact of reproductive state on a comprehensive panel of blood vitamin D biomarkers; 2) to examine interrelations among biomarkers of vitamin D metabolism; and 3) to ascertain the adequacy of the current RDA for achieving target circulating 25(OH)D concentrations of 50 nmol/L among pregnant and lactating women.

METHODS

Study participants

Pregnant women entering their third trimester (26–29 wk gestation), lactating women (5 wk postpartum), and control women of reproductive age (nonpregnant and nonlactating) were recruited from Ithaca, New York (latitude 42.4˚N) between January 2009 and October 2010 as previously described (26, 27). Inclusion criteria included the following: 1) age of 21–40 y; 2) healthiness as assessed by health-related questionnaire, a blood chemistry profile, and a complete blood count; 3) normal liver and kidney function; 4) willingness to comply with the study protocol; 5) singleton pregnancy without pregnancy-associated complications (pregnant women only); and 6) willingness to breastfeed exclusively during the study period (lactating women only). Exclusion criteria included use of tobacco, drug, or alcohol and use of prescription medications known to affect liver function. The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and the Cayuga Medical Center (the hospital where pregnant participants delivered their infants). Informed consent was obtained from all study participants before study entry. This trial was registered at clinicaltrials.gov as NCT01127022.

Study design, diet, and supplements

This study used biological samples obtained during a feeding study in which healthy pregnant (n = 26), lactating (n = 28), and control (n = 21) women were randomly assigned to 480 or 930 mg choline/d for $\geq$10 wk (26, 27). Throughout the feeding period, consumption of all essential micronutrients was strictly controlled and equivalent across reproductive groups.

A mean total of 511 ± 48 IU vitamin D/d was consumed by participants throughout the study period. Of this total, a mean of 311 ± 48 IU vitamin D/d was provided by the 7-d rotational menu as estimated with the use of the USDA National Nutrient Database for Standard Reference Release 28 (**Supplemental Table S1.1**), and 200 IU cholecalciferol/d came from a daily prenatal multivitamin supplement (Pregnancy Plus; Fairhaven Health LLC). For calcium and phosphorus, mean intakes were estimated to be 1.6 ± 0.4 g Ca/d and 1.9 ± 0.3 g P/d (USDA National Nutrient Database for Standard Reference Release 28). In addition to the prenatal multivitamin supplement, all women consumed a docosahexaenoic acid supplement (200 mg/d; Neuromins; Nature's Way Products) and a potassium and magnesium supplement (3 times/wk; General Nutrition Corp).

Meals were prepared in the Human Metabolic Research Unit (HMRU) at Cornell University. The pregnant and control women consumed ≥ 1 meal/d throughout the week at the HMRU, whereas the lactating women consumed 1 meal/d thrice weekly to lessen the burden of travel to and from the HMRU while caring for an infant. Supplements were consumed with the onsite meal under the supervision of study personnel. All other meals and supplements were provided as carry-out and were consumed off site. Participants were required to 1) refrain from consuming food, beverages (except water), and supplements outside of those provided by study personnel; 2) complete daily checklists of menu items and supplements; and 3) return empty food containers and disposables of their carry-out meals and supplements.

Sample collection and processing

Fasting (10-h) venous blood samples and 24-h urine samples were obtained at study baseline (week 0) and study-end (defined as week 10), processed, and stored at -80˚C (26).

Analytical measurements

25(OH)D. Serum 25(OH)D was quantified with an isotope dilution LC-MS/MS method (24) that was validated in part by our laboratory's achieving the performance target set by the Vitamin D External Quality Assessment Scheme for the October 2012 distribution (i.e., within 14% of All Laboratory Trimmed Mean; within 4% of the values for Vitamin D External Quality Assessment Scheme samples analyzed by the National Institute of Standards and Technology). 25(OH)D was extracted from 150-µL serum samples, calibrators, and control samples (24). The calibrators for 25(OH)D_2, 25(OH)D_3, and epi-25(OH)D_3 were made by diluting ethanol stock solutions with PBS-4% albumin. Internal standard (100 µL) that contained 75 nmol d_3-25(OH)D_3/L and 50 nmol d_3-25(OH)D_2/L (IsoSciences) was then added to all of the samples. Extracts (65 µL) were injected onto a PFP column (PFP Accucore 2.1 X 100, 2.6 mm) with matching guard column at 45˚C and separated by an LC-MS/MS system that consisted of a Surveyer HPLC system(pump and autosampler) and a TSQ Quantum Ultra mass spectrometer operated with XCalibur (2.2 SP1.48) software (ThermoElectron Corp). The analytes of interest were eluted from the column at a flow rate of 150 µL/min under the following conditions: 73% methanol and 27% water (0–9th min), linear gradient from 73% to 100% methanol (9th– 11th min), 100% methanol (11th–12th min), linear gradient from 100% to 73% methanol (12th–13th min), and 73% methanol (13th–18th min). To prevent deposit build-up in the mass spectrometer, the flow was directed into the spectrometer only between the 9th and 16th min. Atmospheric pressure chemical ionization in the positive ion mode with the use of selected reaction monitoring was used for detection. Transition pairs for the analytes of interest were the same as previously described (24). Intra- and inter-assay CVs were 2.1% and 4.7%, respectively, from our in-house control duplicates that consisted of 3 human sera with 25(OH)D concentrations spanning the range of the calibration curve, a bovine serum rich in

25(OH)D_2, and National Institute of Standards and Technology SRM 2972 (within the range of certified values). We herein refer to the sum of 25(OH)D_2 and 25(OH)D_3 as "25(OH)D" which does not include epi-25(OH)D_3.

DBP and 1,25(OH)$_2$D. DBP and 1,25(OH)$_2$D in plasma samples were quantified with ELISA kits [R&D Systems for DBP; Immunodiagnostic Systems, Inc. for 1,25(OH)$_2$D], according to the manufacturers' instructions. Intra- and inter-assay CVs for the DBP assays were 5.7% and 5.6%, respectively, from our in-house controls of human plasma with 3 different DBP concentrations. In the 1,25(OH)$_2$D assays, both intra- and inter-assay CVs were <10% (5.2% and 7.8%, respectively) from kit controls of low and high concentrations. In addition, all measured concentrations of the kit controls fell within the acceptable ranges provided by the manufacturer.

24,25(OH)$_2$D. Quantification of circulating 24,25(OH)$_2$D was performed with the LC-MS/MS method (20) with modifications from our instrumentation. Briefly, 24,25(OH)$_2$$D_3$ calibration curves were created by diluting ethanol stock solutions of 24,25(OH)$_2$$D_3$ with PBS-4% albumin. All plasma samples, calibrators, and control samples were mixed with 5 µL of internal standard solution that contained 1.5 pmol d_6-24,25(OH)$_2$$D_3$/L (Toronto Research Chemicals Inc.). Next, 24,25(OH)$_2$$D_3$ was extracted from 200-µL samples, calibrators, and controls with the use of a liquid-liquid extraction, and the upper layer was dried under nitrogen before derivatization with DMEQ-TAD (20). Extracts (65 µL) were resuspended in 60:40 methanol/water solution and injected onto a PFP column (PFP Accucore 2.1 X 100, 2.6 mm) with matching guard column at 45˚C and separated by the LC-MS/MS system described for serum 25(OH)D quantification. 24,25(OH)$_2$$D_3$ was eluted from the column at a flow rate of 200 µL/min under the following

conditions: 40% acetonitrile and 60% water (0–3rd min), linear gradient from 40% to 60% acetonitrile (3rd–5th min), 60% acetonitrile (5th–10th min), linear gradient from 50% to 70% acetonitrile (10th–12th min), linear gradient 70–80% acetonitrile (12th–15th min), returning to 40% acetonitrile (15th–17th min). To prevent deposit build-up, the flow directed into the mass spectrometer was limited from the 2nd to 10th min. Atmospheric pressure chemical ionization in the positive ion mode with selected reaction monitoring was used for 24,25$(OH)_2D_3$ detection. Two transition pairs were used for 24,25$(OH)_2D_3$ [m/z 762.5 > 468.2 (762.5 > 247.1 qualifier)], whereas a single transition pair was used for d_6-24,25$(OH)_2D_3$ (m/z 768.5 > 247.1). Intra- and inter-assay CVs were 5.7% and 4.9%, respectively, from duplicate measurements of our in-house control samples (human plasma with 2 different 24,25$(OH)_2D_3$ concentrations).

Free 25(OH)D. Free 25(OH)D concentrations were estimated with a previously described equation (28, 29) in which quantified circulating concentrations of 25(OH)D [i.e., 25$(OH)D_2$ and 25$(OH)D_3$], albumin, and DBP were computed. Because of limited availability of serum samples, plasma DBP concentrations were measured and then entered in the equation.

Total calcium. Serum total calcium was quantified by an automated chemistry analyzer (Dimension Xpand Plus; Siemens Healthcare Diagnostics).

Genotyping. The *GC* (vitamin D binding protein gene) rs7041 G > T, *CYP2R1* (25-hydroxylase gene) rs12794714 A > G, and *CYP2R1* rs10741657 A > G genotypes were determined by Endpoint Genotyping on a Roche LightCycler 480 with the use of the Applied Biosystems TaqMan Genotyping Assays (Life Technologies) after DNA extraction and purification with the Qiagen

DNeasy kit.

Statistical analysis

To test for differences in baseline characteristics among the reproductive groups, we used 1-factor ANOVA for normally distributed continuous variables, Kruskal-Wallis test for non-normally distributed continuous variables, and χ^2 tests or Fisher exact tests for categorical variables. To examine the effect of reproductive state on biomarkers of vitamin D metabolism at both study baseline and study-end, we used linear mixed-effects models (LMMs). For each biomarker, the LMM included reproductive state, time, and their interaction term as fixed effects, and a random participant identifier factor. In addition, each LMM considered the following covariates: age, ethnicity/race, prepregnancy/baseline BMI (in kg/m^2), education, season of study entry, multivitamin supplement use before study entry, genetic variants in vitamin D metabolism, and choline intake (480 or 930 mg/d). Covariates that achieved statistical significance ($P < 0.05$) were retained in the final models, and the Bonferroni correction was used for post hoc comparisons. Finally, relations among the biomarkers of vitamin D were assessed with Pearson correlation analysis.

All analyses were performed with JMP Pro 11 (SAS Institute, Inc.). Data that did not meet the normality and homogeneity of variance criteria were ln-transformed. Two influential outliers with studentized residuals >3 were excluded from free 25(OH)D analysis. Because epi- 25(OH)D_3 had values below the limit of detection of 1.0 nmol/L, the limit of detection was used in place of "not detectable" among 47% of control, 31% of pregnant, and 54% of lactating women in the statistical models. Data are presented as arithmetic means $\pm$ SDs or geometric means (95% CI), unless otherwise specified. *P* values were 2-tailed and considered significant at <0.05. Data

derived from the LMMs are “predicted mean concentrations (or ratios)” of the vitamin D biomarkers and account for influential covariates.

RESULTS

Participant characteristics and study baseline measurements (ANOVA)

The characteristics of the study participants (26 pregnant, 28 lactating, and 21 control women) and their study baseline (week 0) measures are shown in **Table 1.1.** No differences in age and prepregnancy/baseline BMI were observed among the reproductive groups. In addition, the distributions of ethnicity/race, season of study entry, and education were balanced across the groups as were the distributions of the vitamin D-related genetic variants, *GC* rs7041 G>T and *CYP2R1* rs12794714 A>G polymorphisms. In contrast, *CYP2R1* rs10741657 A>G polymorphism distribution differed ($P = 0.03$) among the groups with the pregnant women having a lower prevalence of the variant GG genotype than the control and lactating women. Multivitamin supplement use before study entry also differed ($P < 0.001$) among the reproductive groups, with higher use in the pregnant and lactating women than in the control women.

Most vitamin D metabolites varied by reproductive state at baseline (Table 1.1). The pregnant women exhibited 40% higher ($P = 0.01$) serum 25(OH)D concentrations than the control women, and 100–150% higher ($P < 0.001$) DBP concentrations than the control and lactating women. Similarly, the pregnant women had 90% and 130% higher ($P < 0.001$) $1,25(OH)_2D$ concentrations than the control and lactating women, respectively. Although $24,25(OH)_2D$ concentrations did not differ across the groups at baseline, 25(OH)D:$24,25(OH)_2D$ was higher in the pregnant women than in the control (30%; $P = 0.03$) and lactating (61%; $P < 0.001$) women, which did not differ from each other. The pregnant women also had 70% higher ($P = 0.048$) serum epi-$25(OH)D_3$ concentrations than those of the control women and almost double ($P = 0.004$) the concentrations of the lactating women.

The lactating women differed from the control and pregnant women in several variables. The $1,25(OH)_2D$:25(OH)D was lower in the lactating women than in the control (-31%; $P = 0.04$) and pregnant (-47%; $P < 0.001$) women, which did not differ from each other. In addition, the lactating women showed higher concentrations of free 25(OH)D than the control (47%; $P = 0.03$) and pregnant (67%; $P = 0.002$) women, which did not differ from each other. Finally, the lactating women had higher ($P < 0.03$) serum calcium concentrations than the control and pregnant women, which differed from each other with lower ($P < 0.001$) serum calcium concentrations among the pregnant women.

Table 1.1
Baseline characteristics and concentrations of vitamin D metabolites among the pregnant, lactating, and control women[1]

	Pregnant (*n*=26)	Lactating (*n*=28)	Control (*n*=21)	*P*
Age, y	28 ± 3	29 ± 5	29 ± 5	0.82
Ethnicity, *n*				0.81
White	16	20	14	
African American	1	1	2	
Hispanic	4	3	2	
Asian	4	1	1	
Other	1	3	2	
BMI^2, kg/m^2	23 [21, 26]	25 [21, 32]	24 [21, 25]	0.45
Season at study-entry, *n*				0.81
April–September	14	17	11	
October–March	12	11	10	
Multivitamin supplement use				<0.001
before study-entry, *n*	22	21	7	
Education, *n*				0.30
≤ High school	4	8	6	
College	11	13	5	
> College	11	17	10	
GC rs7041 G>T polymorphism, *n*				0.35
GG	9	3	6	
GT	11	16	9	
TT	6	8	6	
CYP2R1 rs10741657 A>G polymorphism, *n*				0.03
AA	2	3	2	
AG	20	12	8	
GG	4	13	11	
CYP2R1 rs12794714 A>G polymorphism, *n*				0.15
AA	5	9	6	
AG	20	13	11	
GG	1	6	4	
Serum 25(OH)D, nmol/L	89 ± 29[a]	73 ± 23[ab]	64 ± 25[b]	0.006
Plasma DBP, μg/mL	405 (319, 515)[a]	165 (136, 199)[b]	204 (164, 254)[b]	<0.001
Plasma $1,25(OH)_2D$, pmol/L	283 (232, 344)[a]	125 (104, 152)[b]	151 (129, 178)[b]	<0.001

Table 1.1 (Continued)

Plasma 24,25$(OH)_2$D, nmol/L	9.6 (7.5, 12.4)	12.7 (10.2, 15.9)	9.1 (6.7, 12.5)	0.13
25(OH)D: 24,25$(OH)_2$D	8.7 (7.5, 10.1)[a]	5.4 (4.8, 6.1)[b]	6.7 (5.8, 7.8)[b]	<0.001
1,25$(OH)_2$D: 25(OH)D	3.4 (2.7, 4.2)[a]	1.8 (1.5, 2.2)[b]	2.6 (2.1, 3.2)[a]	<0.001
Serum epi-25$(OH)D_3$, nmol/L	3.2 ± 2.1[a]	1.5 ± 0.9[b]	1.9 ± 1.2[b]	0.003
Free 25(OH)D, pmol/L	17.3 (13.8, 21.5)[b]	28.8 (24.1, 34.4)[a]	19.5 (14.9, 25.5)[b]	0.002
Serum total calcium, mg/dL	8.7 ± 0.4[c]	9.4 ± 0.3[a]	9.2 ± 0.3[b]	<0.001

[1] Values are geometric means (95% CIs), means±SDs, or medians [IQRs]. Values in a row with a superscript letter indicate significant differences in the vitamin D metabolite among the reproductive groups (i.e., a>b>c), $P < 0.05$. *CYP2R1*, 25-hydroxylase gene; DBP, vitamin D binding protein; epi-25$(OH)D_3$, C3 epimer of 25-hydroxyvitamin D_3; *GC*, vitamin D binding protein gene; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 1,25$(OH)_2$D:25(OH)D, ratio of 1,25-dihydroxyvitamin D to 25-hydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D:24,25$(OH)_2$D, ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D.
[2] Self-reported prepregnancy BMI of the pregnant and lactating women and baseline BMI of the control women.

Effect of reproductive state on blood biomarkers of vitamin D metabolism (covariate-adjusted LMM)

Serum 25(OH)D. Reproductive state did not interact with time ($P = 0.48$) to affect serum 25(OH)D concentrations. However, a 25% increase ($P = 0.02$) in 25(OH)D concentrations was observed among the control women but not among the pregnant and lactating women whose concentrations remained stable ($P \geq 0.21$) throughout the study (**Figure 1.1**A). Similar to study baseline, the pregnant women had ~30% higher ($P < 0.01$) concentrations of 25(OH)D (89 nmol/L) than the control women (69 nmol/L) at study-end (Figure 1.1A), whereas serum 25(OH)D concentrations in the lactating women (75 nmol/L) did not differ ($P \geq 0.15$) from the pregnant and control women. At study-end, predicted mean serum concentrations for all reproductive groups were above the

estimated average requirement and RDA target values of 40 nmol/L and 50 nmol/L, respectively (Figure 1.1A). In addition, all participants, except for 2 pregnant and 2 control women, had unadjusted 25(OH)D concentrations > 50 nmol/L.

Plasma DBP. Reproductive state did not interact with time ($P = 0.13$) to influence circulating DBP concentrations that did not change ($P > 0.9$) among the lactating and control women but tended to decrease (-9%; $P = 0.07$) among the pregnant women (Figure 1.1B). Similar to study baseline, the pregnant women had 67% higher ($P < 0.001$) DBP concentrations (340 µg/mL) than the control women (203 µg/mL) and almost 100% higher ($P < 0.001$) concentrations than the lactating women (173 µg/mL) at study-end. DBP concentrations in the lactating and control women did not differ ($P = 0.42$) from each other (Figure 1.1B).

Plasma 1,25(OH)$_2$D. Reproductive state did not interact with time ($P = 0.88$) to affect circulating 1,25(OH)$_2$D response with stable ($P \geq 0.52$) concentrations of 1,25(OH)$_2$D among all the reproductive groups throughout the study (Figure 1.1C). Similar to study baseline, the pregnant women had 80% and 120% higher ($P < 0.001$) 1,25(OH)$_2$D concentrations (299 pmol/L) than the control (162 pmol/L) and lactating (134 pmol/L) women at study-end, respectively (Figure 1.1C). 1,25(OH)$_2$D concentrations in the lactating and control women did not differ from each other ($P > 0.9$).

Plasma 24,25(OH)$_2$D. Reproductive state interacted with time ($P = 0.003$) to affect circulating 24,25(OH)$_2$D concentrations. Although no changes ($P \geq 0.31$) in 24,25(OH)$_2$D concentrations were observed within the pregnant and lactating women throughout the study, 24,25(OH)$_2$D

tended to increase (22%; P =0.06) among the control women (Figure 1.1D). Interestingly, despite 42% higher ($P < 0.03$) concentrations of 24,25$(OH)_2$D in the lactating women than the pregnant women at baseline, no differences ($P > 0.9$) were detected among the pregnant (8.3 nmol/L), lactating (8.5 nmol/L), and control (9.8 nmol/L) groups at study-end (Figure 1.1D).

25(OH)D:24,25(OH)$_2$D. Reproductive state and time interacted ($P < 0.001$) to influence 25(OH)D:24,25$(OH)_2$D, with increases ($P < 0.001$) observed among the lactating women (Figure 1.1E) but not among the pregnant and control women ($P > 0.9$). Similar to study baseline, the pregnant women exhibited a 40% higher ratio ($P < 0.001$) than the control women (8.8 compared with 6.3) at study-end. However, in contrast to the higher ratio ($P < 0.001$) among the pregnant (compared with lactating) women at study baseline, no difference ($P = 0.17$) between the 2 groups (8.8 compared with 7.4) was detected at study-end (Figure 1.1E).

1,25(OH)$_2$D:25(OH)D. Reproductive state did not interact with time ($P = 0.36$) to influence 1,25$(OH)_2$D:25(OH)D that remained stable ($P \geq 0.56$) among all the reproductive groups across study time points. At study-end, the pregnant women had a 50% higher ratio ($P = 0.04$) than the control women (3.3 compared with 2.2), despite no differences ($P = 0.53$) in this ratio between the 2 groups at study baseline (Figure 1.1F). The pregnant women also had an 83% higher ($P < 0.001$) 1,25$(OH)_2$D:25(OH)D than the lactating women (3.3 compared with 1.8) at study-end, which was similar to study baseline (Figure 1.1F).

Serum epi-25(OH)D$_3$. Reproductive state did not interact with time ($P = 0.92$) to influence serum epi-25$(OH)D_3$ concentrations that did not change ($P > 0.9$) among all reproductive states

throughout the study. Similar to study baseline, epi-25(OH)D_3 concentrations in the pregnant group (3.05 nmol/L) were twice ($P < 0.004$) those of the control (1.51 nmol/L) and lactating (1.31 nmol/L) groups at study-end (Figure 1.1G), which did not differ from each other ($P > 0.9$).

Free 25(OH)D. Reproductive state did not interact with time ($P = 0.65$) to affect free 25(OH)D concentrations that remained stable ($P \geq 0.1$) among the lactating and control women over the course of the study and increased slightly (10%; $P = 0.02$) among the pregnant women (Figure 1.1H). Although the lactating women tended to have higher ($P = 0.05$) concentrations of free 25(OH)D than the control women at study baseline, no difference ($P = 0.15$) was observed among these groups at study-end (31.3 and 24.1 pmol/L for lactating and control women, respectively). Similar to study baseline, the lactating women had 66% higher ($P < 0.001$) concentrations of free 25(OH)D than those of the pregnant women (18.8 pmol/L) at study-end, which did not differ from the control women ($P = 0.18$).

Serum total calcium. Reproductive state did not interact with time ($P = 0.46$) to influence serum calcium concentrations that was stable ($P \geq 0.5$) among the lactating and control women but increased slightly (3%; $P = 0.04$) in the pregnant women throughout the study (Figure 1.1I). Similar to study baseline, the lactating women (9.6 mg/dL) had higher calcium concentrations than those of the control (9.2 mg/dL; $P = 0.003$) and pregnant women (8.9 mg/dL; $P < 0.001$) at study-end. In addition, the pregnant women showed lower ($P = 0.01$) serum calcium concentrations than the control women at study-end.

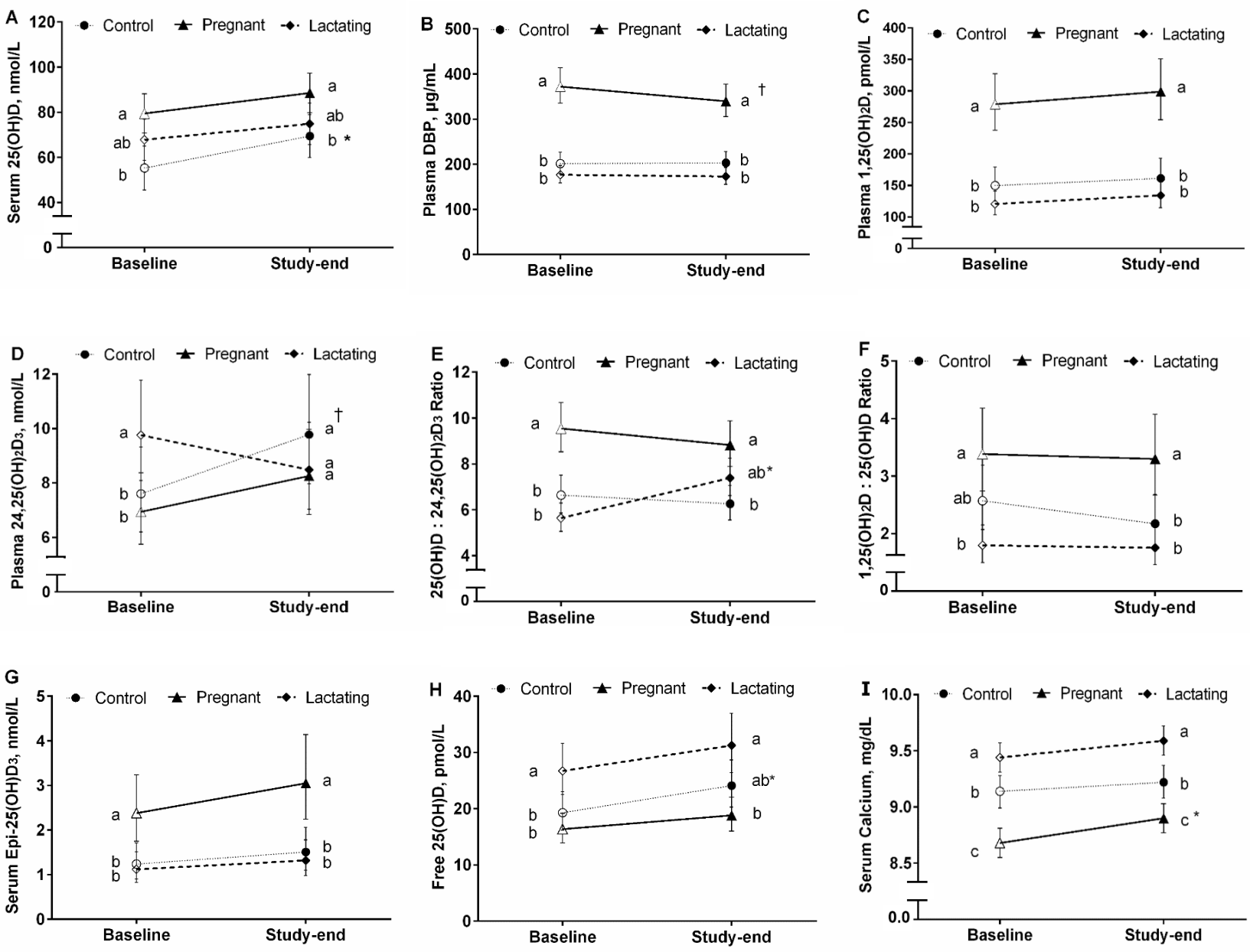
A
Serum 25(OH)D, nmol/L
Control
Pregnant
Lactating
Baseline
Study-end
B
Plasma DBP, µg/mL
C
Plasma 1,25(OH)2D, pmol/L
D
Plasma 24,25(OH)2D3, nmol/L
E
25(OH)D : 24,25(OH)2D3 Ratio
F
1,25(OH)2D : 25(OH)D Ratio
G
Serum Epi-25(OH)D3, nmol/L
H
Free 25(OH)D, pmol/L
I
Serum Calcium, mg/dL

Figure 1.1. Circulating vitamin D metabolites [25(OH)D (A), DBP (B), 1,25$(OH)_2$D (C), 24,25$(OH)_2$D (D), 25(OH)D:24,25$(OH)_2$D (E), 1,25$(OH)_2$D:25(OH)D (F), epi-25$(OH)D_3$ (G), free 25(OH)D (H), and total calcium (I)] among the pregnant (n = 26), lactating (n = 28), and control (n = 21) women who consumed equivalent amounts of vitamin D and related nutrients (e.g., calcium and phosphorus) for 10 wk. All concentrations are predicted geometric means (95% CIs), except 25(OH)D (predicted arithmetic mean), derived from the covariate-adjusted linear mixed models. Means at a time without a common letter differ, $P < 0.05$. *,† Significantly different from baseline: *$P < 0.05$; $^{\dagger}P \leq 0.07$. DBP, vitamin D binding protein; epi-25$(OH)D_3$, C3 epimer of 25-hydroxyvitamin D_3; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 1,25$(OH)_2$D:25(OH)D, ratio of 1,25-dihydroxyvitamin D to 25-hydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D:24,25$(OH)_2$D, ratio of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D.

Associations among circulating vitamin D metabolites in all participants throughout the study

Among all study participants, circulating 25(OH)D correlated positively with 24,25$(OH)_2$D (**Figure 1.2**A), 1,25$(OH)_2$D (Figure 1.2B), DBP (Figure 1.2C), and free 25(OH)D (Figure 1.2D) at both baseline and study-end. 1,25$(OH)_2$D and 24,25$(OH)_2$D were correlated throughout the study (Figure 1.2E) as was 1,25$(OH)_2$D with DBP (Figure 1.2F). No correlations were detected between 1,25$(OH)_2$D and free 25(OH)D at either baseline (P = 0.68) or study-end (P = 0.29); however, free 25(OH)D correlated positively with 24,25$(OH)_2$D at both study time points (Figure 1.2G).

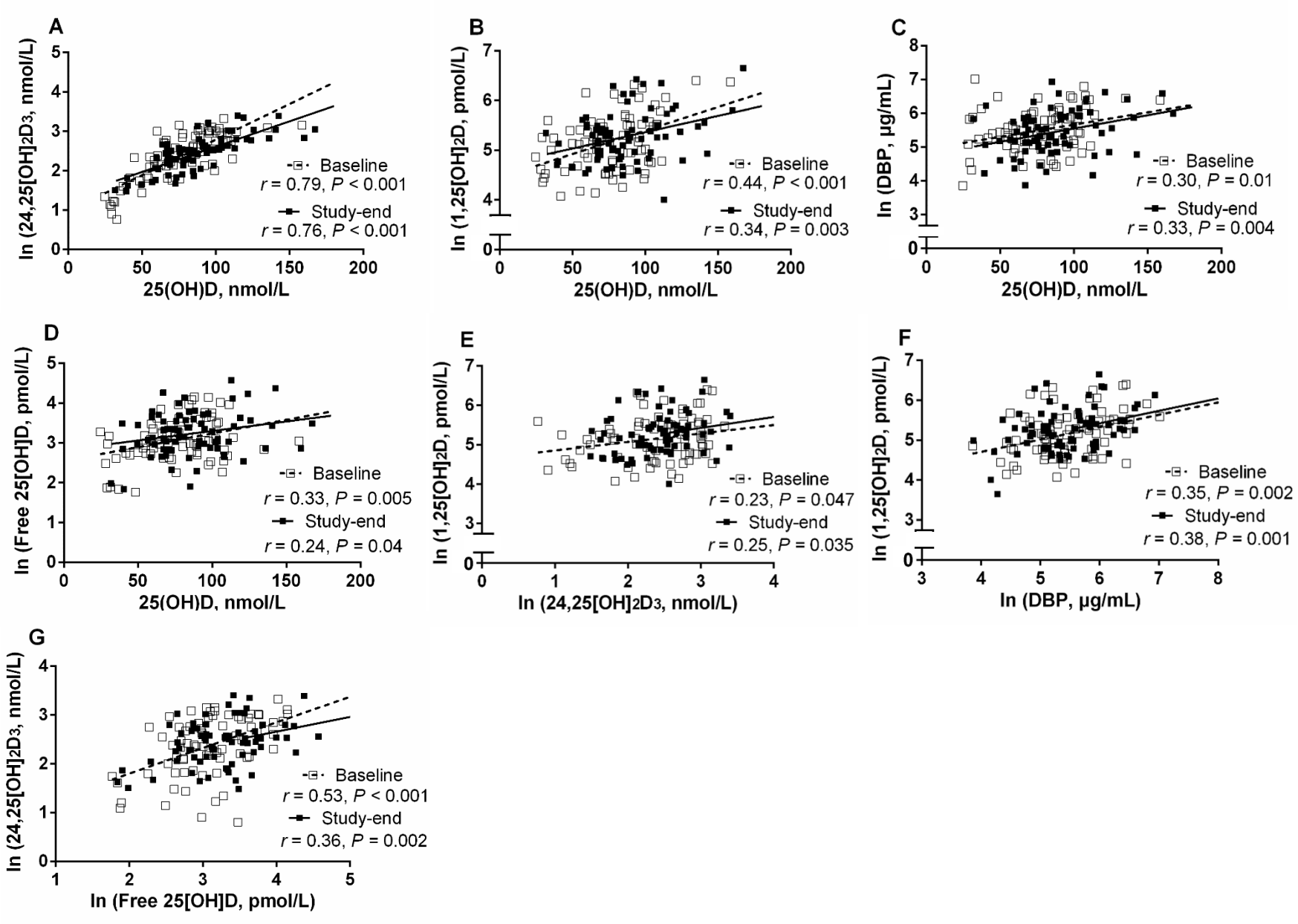
A
ln (24,25[OH]2D3, nmol/L)
25(OH)D, nmol/L
Baseline
r = 0.79, P < 0.001
Study-end
r = 0.76, P < 0.001
B
ln (1,25[OH]2D, pmol/L)
25(OH)D, nmol/L
Baseline
r = 0.44, P < 0.001
Study-end
r = 0.34, P = 0.003
C
ln (DBP, µg/mL)
25(OH)D, nmol/L
Baseline
r = 0.30, P = 0.01
Study-end
r = 0.33, P = 0.004
D
ln (Free 25[OH]D, pmol/L)
25(OH)D, nmol/L
Baseline
r = 0.33, P = 0.005
Study-end
r = 0.24, P = 0.04
E
ln (1,25[OH]2D, pmol/L)
ln (24,25[OH]2D3, nmol/L)
Baseline
r = 0.23, P = 0.047
Study-end
r = 0.25, P = 0.035
F
ln (1,25[OH]2D, pmol/L)
ln (DBP, µg/mL)
Baseline
r = 0.35, P = 0.002
Study-end
r = 0.38, P = 0.001
G
ln (24,25[OH]2D3, nmol/L)
ln (Free 25[OH]D, pmol/L)
Baseline
r = 0.53, P < 0.001
Study-end
r = 0.36, P = 0.002

Figure 1.2. Relations among circulating vitamin D metabolites in all participants (n = 75) by study time point: 25(OH)D and 24,25$(OH)_2$D (A), 25(OH)D and 1,25$(OH)_2$D (B), 25(OH)D and DBP (C), free 25(OH)D and 25(OH)D (D), 1,25$(OH)_2$D and 24,25$(OH)_2$D (E), 1,25$(OH)_2$D and DBP (F), and free 25(OH)D and 24,25$(OH)_2$D (G). All metabolites were ln-transformed, except for 25(OH)D. DBP, vitamin D binding protein; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

Associations among circulating vitamin D metabolites by reproductive state at study-end

At study-end, circulating 25(OH)D correlated positively with 1,25$(OH)_2$D among the control women (**Table 1.2**) and tended to correlate among the pregnant women (r = 0.36, P = 0.07). 25(OH)D was also highly correlated with 24,25$(OH)_2$D, and with epi-25$(OH)D_3$ in all the reproductive groups (Table 1.2). Although 1,25$(OH)_2$D correlated positively with 24,25$(OH)_2$D among the control women, no relation between these metabolites was observed among the pregnant or lactating women.

Epi-25$(OH)D_3$ correlated positively with 24,25$(OH)_2$D in all the reproductive groups and with DBP in the pregnant women at study-end (Table 1.2). In addition, free 25(OH)D correlated with epi-25$(OH)D_3$ and with 24,25$(OH)_2$D among the pregnant and control women at study-end (Table 1.2). However, only the pregnant women showed correlations of free 25(OH)D with 25(OH)D (r = 0.38, P = 0.057) and 1,25$(OH)_2$D (Table 1.2).

Table 1.2. Correlations among circulating vitamin D metabolites in pregnant, lactating and control women at study-end[1]

Correlations between vitamin D metabolites	Pregnant (n = 26)		Lactating (n = 28)		Control (n = 21)	
	r	*P*	*r*	*P*	*r*	*P*
25(OH)D, $1,25(OH)_2D$	-	NS	-	NS	0.48	0.03
25(OH)D, $24,25(OH)_2D$	0.85	<0.001	0.67	<0.001	0.89	<0.001
25(OH)D, DBP	-	NS	-	NS	-	NS
25(OH)D, Epi-$25(OH)D_3$	0.72	<0.001	0.63	<0.001	0.59	0.007
25(OH)D, Free 25(OH)D	-	NS	-	NS	-	NS
$1,25(OH)_2D$, $24,25(OH)_2D$	-	NS	-	NS	0.64	0.002
$1,25(OH)_2D$, DBP	-	NS	-	NS	-	NS
$1,25(OH)_2D$, Free 25(OH)D	0.47	0.02	-	NS	-	NS
$1,25(OH)_2D$, Epi-$25(OH)D_3$	-	NS	-	NS	-	NS
Free 25(OH)D, $24,25(OH)_2D$	0.46	0.02	-	NS	0.48	0.03
Free 25(OH)D, Epi-$25(OH)D_3$	0.49	0.03	-	NS	0.42	0.03
Epi-$25(OH)D_3$, DBP	0.40	0.04	-	NS	-	NS
Epi-$25(OH)D_3$, $24,25(OH)_2D$	0.56	0.003	0.58	0.001	0.49	0.03

[1] Metabolites were ln-transformed, except 25(OH)D and epi-$25(OH)D_3$. NS, $P \geq 0.05$. DBP, vitamin D binding protein; epi-$25(OH)D_3$, C3 epimer of 25-hydroxyvitamin D_3; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D.

DISCUSSION

To the best of our knowledge, this is the first feeding study to control intakes of vitamin D and related nutrients such as calcium and phosphorus that can affect vitamin D status and metabolism. Three main findings emerged as follows: 1) pregnancy induces alterations in vitamin D metabolism, including increases in 25(OH)D, 25(OH)D:24,25$(OH)_2$D, and epi-25$(OH)D_3$; 2) reproductive state modulates the interrelations among circulating vitamin D metabolites; and 3) the RDA for vitamin D is likely adequate for most women of reproductive age, including pregnant and lactating women.

Pregnancy increases the circulating pool of vitamin D

Circulating concentrations of 1,25$(OH)_2$D, DBP, and 1,25$(OH)_2$D:25(OH)D were significantly higher among third-trimester pregnant women than the control women, confirming previous reports (7–12). The pregnant women also exhibited significantly higher circulating concentrations of 25(OH)D than the control women even after adjusting for season, prestudy supplement use, ethnicity/race, BMI, and genetic variants that influence 25(OH)D concentrations. This elevation in 25(OH)D paralleled the rise in DBP among the pregnant women, suggesting that DBP-bound 25(OH)D, rather than free 25(OH)D (which did not differ between the pregnant and control women), contributed to the higher total circulating concentrations of this metabolite. DBP-bound 25(OH)D may be favored over the free form because it is more stable and is taken up in a regulated manner by tissues that express megalin-cubilin receptors such as the placenta (30).

No differences in circulating concentrations of 24,25$(OH)_2$D, a major catabolite of 25(OH)D (22), were detected between the pregnant and control women. However, 25(OH)D:24,25$(OH)_2$D was significantly higher among the pregnant women than the control

women, indicating attenuation of vitamin D catabolism in this reproductive state. In addition, the pregnant women had higher concentrations of epi-25(OH)D_3 than the control women which may be a consequence of the elevated maternal vitamin D pool or could imply a possible role for this metabolite in maternal and fetal health.

In contrast to the robust effects of pregnancy on biomarkers of vitamin D metabolism, no differences were detected at study-end between the lactating (15 wk postpartum) and control women. These findings are consistent with previous reports that examined calcium homeostasis during lactation (9, 11, 31). Of note, circulating 25(OH)D:24,25$(OH)_2$D increased throughout the study among lactating women because of a decrease in their 24,25$(OH)_2$D. Thus, lactating women may achieve vitamin D pools that are similar to nonlactating women by reducing 24-hydoxylase (CYP24A1) activity.

Reproductive state influenced the interrelations among circulating vitamin D metabolites

Data from the present study showed strong positive correlations between 25(OH)D and 24,25(OH)2D at study-end among the pregnant, lactating, and control women, supporting the catabolism of 25(OH)D to 24,25$(OH)_2$D as a means to maintain homeostasis. These findings are consistent with previous reports in healthy adults (19, 20, 22) and pregnant women at term (7), but they deviate somewhat from a study in lactating women which reported significant correlations of 25(OH)D and 24,25$(OH)_2$D at 1 wk postpartum but not at later time points (18). In addition, although 25(OH)D tended (n = 26; r = 0.36; P = 0.07) to be correlated with the bioactive 1,25$(OH)_2$D metabolite among the pregnant women at study-end, the linear relation between these 2 metabolites became significant when the pregnant women with serum 25(OH)D concentrations < 100 nmol/L were examined separately (n =33; r =0.39; P = 0.02). This finding supports prior

work to suggest that substrate-dependent $1,25(OH)_2D$ production from 25(OH)D reaches a plateau at ~100 nmol 25(OH)D/L in pregnant women (32). Pregnant women also exhibited a higher number of significant correlations between free 25(OH)D, a metabolite recently linked to bone health (33, 34), and other metabolites. Specifically, free 25(OH)D was associated with 4 metabolites among pregnant women [i.e., total 25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$, and epi-$25(OH)D_3$], 2 metabolites among control women [i.e., $24,25(OH)_2D$ and epi-$25(OH)D_3$], and zero metabolites among lactating women. This suggests that free 25(OH)D may be a useful indicator of vitamin D status during pregnancy but not during lactation. Notably, the bioactive metabolite $1,25(OH)_2D$, was associated with $24,25(OH)_2D$ concentrations in control women but not in pregnant and lactating women. This finding supports the notion that feedback inhibition of $1,25(OH)_2D$ production (by CYP24A1) is uncoupled during pregnancy (35, 36) and lactation, possibly because of a higher demand for $1,25(OH)_2D$ in these reproductive states.

Vitamin D intake approximating the current RDA achieved adequacy in >90% of the study participants within each reproductive group

This study provided a mean of 511 IU vitamin D/d, falling between the current estimated average requirement (400 IU/d) and the RDA (600 IU/d), through a mixed diet and a prenatal supplement. Although a simulated dose-response curve of serum 25(OH)D concentrations generated by the IOM did not include pregnant and lactating populations (6), it is notable that the study dose readily achieved 25(OH)D concentrations above both the RDA-targeted value and the IOM cutoff for vitamin D adequacy ($\geq$50 nmol/L) in 95% of the participants: 92% in pregnant, 100% in lactating, and 90% in control women after a 10-wk period of controlled feeding. In addition, on examining 25(OH)D:$24,25(OH)_2D$, all participants had values <20 (unadjusted ratios)

at study-end, corresponding to vitamin D sufficiency (20). As such, the RDA of 600 IU/d, which is higher than the vitamin D dose of the present study, would be expected to meet vitamin D requirements of these populations as defined by the IOM (5), although additional dose-response studies are warranted.

Study limitations

The provision of a single dose of vitamin D is the main limitation of this study. We cannot exclude the possibility that the effect of reproductive state on vitamin D biomarkers and their relations might differ under conditions of lower or higher vitamin D intakes. In addition, oral contraceptive use of the control women was not considered in our statistical models. However, inclusion of this variable would be expected to accentuate the difference between the pregnant and control women (8 of whom used oral contraceptives), secondary to the positive relation between oral contraceptive use and serum 25(OH)D concentrations (37).

Conclusions

Pregnancy increases circulating pools of vitamin D metabolites in a manner that is independent of dietary intake, supplement use, season, ethnicity/race, and BMI. The factors contributing to this increase are unclear, but they may involve the placenta that expresses the vitamin D machinery required for the synthesis of 25(OH)D and $1,25(OH)_2D$ (30, 38, 39). The study findings also show that interrelations among vitamin D metabolites are modified by reproductive state, particularly during pregnancy. Finally, our data support the adequacy of the vitamin D RDA (600 IU) for achieving serum 25(OH)D concentrations of 50 nmol/L among women of childbearing age, including those who are pregnant or breastfeeding.

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Supplemental Table S1.1
Daily estimated intakes of vitamin D provided by the 7-day rotational menu[1,2]

Day	Breakfast and Snack	Lunch	Dinner	Vitamin D Intake (IU)
Monday	2 eggs, scrambled (72) 2 slices WW[3] toast (0) 1 fruit cup (peaches; 0) Fruit juice[4] (0) Yogurt[5] (85) V8 juice[6] (0)	Pesto sandwich: 2 slices WW bread (0) Pesto (0) Swiss Cheese (8.9) Romaine lettuce (0) Celery sticks (0) Carrot sticks (0)	Beef & cheese tacos: 3 corn tortillas (0) Ground beef (80% lean; 8.6) Cheddar cheese (7.4) Iceberg lettuce (0) Melon (0) Milk[7] (135)	317
Tuesday	Waffle (0) 1 egg, hard-boiled (44) Fruit juice (0) Yogurt (85) V8 juice (0)	Tuna sandwich: 2 slices WW bread (0) Tuna, canned (light; 102) Cheddar Cheese (7.4) Iceberg lettuce (0) Mayonnaise (1.1) Grapes (0)	Spaghetti: Cooked pasta (0) Tomato sauce (0) Mushrooms (white; 2.1) Parmesan cheese (2.0) Mozzarella cheese (3.5) Milk (135)	329
Wednesday	Fitness Crunch cereal (0) Milk (135) 1 box of raisins (0) 1 medium banana (0) Fruit juice (0) Yogurt (85) V8 juice (0)	Pastrami sandwich: 2 slices WW bread (0) Pastrami (1.0) Swiss cheese (4.9) Romaine lettuce (0) Cucumber (0)	Vegetarian pizza: Dough (0) Tomato sauce (0) Red peppers, jarred (0) Mushrooms (white; 2.1) Spinach (0) Mozzarella cheese (3.5) Apple sauce (0) Milk (135)	370

Supplemental Table S1.1 (Continued)

Day	Breakfast and Snack	Lunch	Dinner	Vitamin D Intake (IU)
Thursday	2 pancakes (0) Blueberries, frozen (0) Fruit juice (0) Yogurt (85) V8 juice (0)	Egg salad sandwich: 2 slices WW bread (0) 1 egg, hard-boiled (44) Mayonnaise (1.4) Romaine lettuce (0) Celery sticks (0) Carrot sticks (0)	Beef & broccoli stir-fry: Beef (sirloin steak; 4.2) Broccoli, frozen (0) Rice (0) Onions, frozen (0) Melon (0) Milk (135)	268
Friday	1 WW bagel (0) 1 fruit cup (oranges; 0) Fruit juice (0) Yogurt (85) V8 juice (0)	Bean Burrito: Black beans, canned (0) Cheddar cheese (7.4) Rice (0) 1 large WW tortilla (0)	Lasagna: 2 WW lasagna noodles, dry (0) Tomato sauce (0) Ground beef (80% lean; 4.9) Cottage cheese (0) Mozzarella cheese (3.5) Parmesan cheese (2.0) Summer squash (0) Zucchini (0) Milk (135)	238
Saturday	2 raspberry muffins (4.2) 1 medium banana (0) Fruit juice (0) Yogurt (85) V8 juice (0)	Turkey Sandwich: 2 slices WW bread (0) Turkey (breast; 2.1) Provolone cheese (4.9) Iceberg lettuce (0) Cucumber (0)	Chicken Quesadilla: Chicken (breast; 3.0) 2 large WW tortillas (0) Cheddar cheese (37) Corn, frozen (0) Milk (135)	296

Supplemental Table S1.1 (Continued)

Day	Breakfast and Snack	Lunch	Dinner	Vitamin D Intake (IU)
Sunday	Oat granola cereal (0) Milk (135) 1 box of raisins (0) 1 fruit cup (peaches; 0) Fruit juice (0) Yogurt (85) V8 juice (0)	Vegetable soup (0) Corn muffin (0) Grapes (0)	Goulash: Cooked pasta (0) Beef (sirloin steak; 4.2) Onion, frozen (0) Red peppers, jarred (0) Tomatoes, canned (0) Tomato puree (0) Potatoes, canned (0) Pineapple, canned (0) Milk (135)	360

[1] Vitamin D intake levels were estimated using the USDA National Nutrient Database for Standard Reference (ref 28).
[2] Values in the parentheses represent amount of vitamin D in international units.
[3] WW, whole wheat
[4] Apple, cranberry-grape, or cranberry juice
[5] Yogurt (vitamin D fortified; raspberry, peach, or vanilla flavored; Dannon Company)
[6] V8 juice (Campbell Soup Company)
[7] Fluid milk (vitamin D fortified; whole, reduced fat, non-fat, or chocolate milk; Cornell Dairy products)

CHAPTER 2

Placental vitamin D metabolism and its associations with circulating vitamin D metabolites in pregnant women[1,2]

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ABSTRACT

Context: Little is known about placental vitamin D metabolism and its impact on the maternal compartment.

Objective: This study sought to advance understanding of placental vitamin D metabolism and its role in modulating maternal circulating vitamin D metabolites.

Design, Setting, and Participants: Nested within a feeding study, 24 pregnant women consumed a single amount of vitamin D (511IU/d) for 10 weeks and provided blood and placental tissue for vitamin D biomarker assessment. In addition, cultured human trophoblasts were incubated with ^{13}C-vitamin D_3 to examine intracellular generation of vitamin D metabolites and their secretion.

Main Outcomes: Placental and circulating vitamin D metabolites; placental mRNA abundance of vitamin D metabolic components; trophoblast ^{13}C-labeled vitamin D metabolites and mRNA abundance.

Results: In placental tissue, 25(OH)D_3 was strongly correlated (r=0.83, P<0.001) with 24,25$(OH)_2D_3$. Moreover, these placental metabolites were strongly correlated ($r \geq 0.85$, $P \leq 0.04$) with their respective metabolites in maternal circulation. Positive associations ($P \leq 0.03$) were also observed between placental mRNA abundance of vitamin D metabolic machinery and circulating vitamin D metabolites (i.e., *LRP2* with 25(OH)D_3 and epi-25(OH)D_3; *CUBN* with 25(OH)D_3; *CYP2R1* with epi-25(OH)D_3; *CYP24A1* with 25(OH)D_3, 24,25$(OH)_2D_3$, epi-25(OH)D_3 and 1,25$(OH)_2D_3$; *CYP27B1* with epi-25(OH)D_3 and 1,25$(OH)_2D_3$. Remarkably, trophoblasts incubated with ^{13}C-vitamin D_3 showed ^{13}C-25(OH)D_3 production and secretion, and *CYP24A1* transcript induction *in vitro*.

Conclusions: The significant associations between many of the placental biomarkers of vitamin D metabolism and circulating vitamin D metabolites among pregnant women, along with evidence

of trophoblast production and secretion of vitamin D metabolites, especially 25(OH)D_3, suggest that the placenta modulates the vitamin D metabolite profile in maternal circulation.

INTRODUCTION

Substantial alterations in circulating concentrations of vitamin D metabolites and related proteins have been observed during human pregnancy. For example, circulating concentrations of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], a bioactive vitamin D metabolite, and vitamin D binding protein (DBP), a carrier for circulating vitamin D metabolites, have been shown to increase by 1.5-2.0 times across gestation (1–4). Similarly, increases in 25-hydroxyvitamin D_3 [$25(OH)D_3$] (2,5,6), the major form of vitamin D in blood, and in C3-epimer of $25(OH)D_3$ [epi-$25(OH)D_3$] (7,8) have also been observed in some studies. Nonetheless, the underlying mechanisms of these pregnancy-induced changes in circulating vitamin D metabolites are largely unknown.

The placenta is an organ of pregnancy that expresses all components of the vitamin D metabolic pathway (i.e., megalin-cubilin receptors (LRP2 and CUBN, respectively) (9,10), 25-hydroxylase (CYP2R1) (11), 1α-hydroxylase (CYP27B1) (12,13), and 24-hydroxlyase (CYP24A1) (11,14)), and thus, could mediate the observed pregnancy-induced alterations in circulating concentrations of the vitamin D metabolites in the maternal compartment. Indeed, placental production of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ by the actions of CYP27B1 and CYP24A1 on $25(OH)D_3$ have been established (12,15,16). Moreover, although the extent to which the placenta contributes to a doubling of circulating $1,25(OH)_2D_3$ as compared to the kidney is still debated, it is unequivocally confirmed in nephrectomized animals that placentally produced $1,25(OH)_2D_3$ can enter maternal circulation (17,18). However, it remains unclear whether placental CYP2R1 produces $25(OH)D_3$ from parent vitamin D_3, and whether this metabolite can enter maternal blood. In addition, because the kidney internalizes DBP-bound 25(OH)D through the megalin-cubilin receptors from the glomerular filtrate (19), presence of this receptor complex in placental membrane along with a dramatic increase in DBP during pregnancy support the possibility that

the placenta takes up 25(OH)D bound to DBP in a tissue-specific manner and regulates the production of several forms of vitamin D metabolites. Lastly, the role of the placenta in mediating circulating vitamin D metabolites such as 24,25$(OH)_2D_3$ and epi-25$(OH)D_3$ is under investigated.

To advance current understanding of placental vitamin D metabolism and its impact on the maternal compartment, this study examined associations between biomarkers of vitamin D metabolism in placental tissue and maternal blood obtained from pregnant women participating in a long-term controlled feeding study. Associations between placental vitamin D metabolites and placental expression of vitamin D metabolizing genes were also examined. In addition, a human placental cell culture model was used to explore placental uptake of ^{13}C-labeled vitamin D_3 and its metabolic fate.

METHODS

Human Feeding Study

Study participants, study design, and sample collection

Twenty-four third-trimester singleton pregnant women (26-29 weeks gestation; ≥ 21yr) in good health status were recruited from Ithaca, New York during 2009-2010 to participate in a 12-week feeding study that featured two choline intake levels and provided an average total intake of 511 IU vitamin D/d [311 IU from a diet and 200 IU from a prenatal multivitamin supplement (Pregnancy Plus; Fairhaven Health LLC)], 1.6 g calcium/d, and 1.9 g phosphorus/d (8,20,21). Venous blood samples obtained at week 0 (study-baseline) and week 10 (representing study-end), along with placental tissue samples collected at delivery, were used for measurements of vitamin D metabolic and genomic parameters. The study protocol was approved by the Institutional Review Board for Human Study Participant Use at Cornell University and at Cayuga Medical Center where babies were delivered. All participants provided their informed consent prior to study entry.

Measurements of vitamin D metabolites in blood and placental tissues

Serum $25(OH)D_3$, serum epi-$25(OH)D_3$, and plasma $24,25(OH)_2D_3$ were quantified using a stable isotope dilution LC-MS/MS methodology as previously described (8). Plasma $1,25(OH)_2D_3$ (Immunodiagnostic Systems) and DBP (R&D Systems) were quantified using ELISA kits. Estimation of free $25(OH)D_3$ concentrations was performed using a previously published equation (22).

Placental $25(OH)D_3$ and $24,25(OH)_2D_3$ were extracted from homogenized placental tissues (0.4 g) in 1 mL of methanol following liquid-liquid extraction (addition of 600 μL acetonitrile,

1 mL MTBE, 25 μL internal standard), solid-phase extraction using Oasis HLB cartridges (3 cc/60 mg), and 0.1 mg/mL DMEQ-TAD derivatization (23, 24). An internal standard solution containing 215 μmol/L d_3-25(OH)D_3 (IsoSciences) and 1.7 μmol/L d_6-24,25$(OH)_2D_3$ (Toronto Research Chemicals Inc.) was added to the homogenized tissues at the beginning of the extraction (24). Extracts were resuspended in 110 μL of 60:40 methanol/water solution and injected onto an LC-MS/MS system to quantify the vitamin D metabolites. LC-MS/MS conditions followed the protocol for circulating 24,25$(OH)_2D_3$ (8).

Quantitative real-time PCR of the vitamin D machinery in placental tissues

RNA was extracted from placental tissue samples using RNeasy Mini kit (Qiagen), and the concentrations were determined using a NanoDrop ND-1000 instrument (Thermo). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), and quantitative real-time PCR (RT-PCR) was conducted using an Applied Biosystems ABI 7300 system. The TaqMan Gene Expression Assays (Thermo Fisher Scientific) were Hs00167999_m1 (*CYP24A1*), Hs00168017_m1 (*CYP27B1*), Hs01379776_m1 (*CYP2R1*), Hs01119018_g1 (*LRP2*), and Hs00153607_m1 (*CUBN*). The reaction conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Based on the $2^{-\Delta\Delta Ct}$ method (25), data were expressed as fold change in mRNA expression of the genes of interest which were normalized to the housekeeping gene [beta-glucuronidase (*GUSB*; Hs99999908_m1)] and relative to the calibrator.

In Vitro Placental Trophoblast Cell Culture Model

HTR-8/SVneo cell culture

HTR-8/SVneo cells which are immortalized human first-trimester placental extravillous trophoblasts were obtained as a gift from Dr. Charles H. Graham (Queen's University, Canada). The cells (passage number 17-21) were plated on a culture dish (BD Biosciences) at a seeding density of 1.39 x 10^6 cells/dish, and cultured in standard RPMI1640 medium which contained 1.25-5% fetal bovine serum (FBS), 2 mM L-glutamine (all from Corning Life Sciences), and each vitamin D treatment [i.e., $^{13}C_2$-vitamin D_3, $^{13}C_5$-25(OH)D_3, unlabeled-vitamin D_3, 25(OH)D_3, or 1,25$(OH)_2D_3$] at 37˚C in a humidified atmosphere of 5% CO_2/95% air. 100% ethanol was added to the standard medium to serve as a control treatment (0.1% ethanol). Cell counts and viability were determined in duplicate using a TC10^{TM} cell counter (Bio-Rad) and a trypan blue exclusion test, respectively. The experiment was repeated three times. Within each replication, each treatment was performed in triplicate.

Unlabeled vitamin D treatment and quantitative RT-PCR of vitamin D metabolic enzymes

To examine effects of vitamin D exposure [vitamin D_3, 25(OH)D_3, 1,25$(OH)_2D_3$] on gene expression response of vitamin D metabolic enzymes in the placenta, HTR-8/SVneo cells were cultured for 72-h with one of the unlabeled vitamin D or vitamin D metabolites. Final vitamin D concentrations of each treatment were 2,500 nM, 5,000 nM, and 10,000 nM of vitamin D_3 (Cayman Chemical), 500 nM of 25(OH)D_3 (Cayman Chemical), and 100 nM of 1,25$(OH)_2D_3$ (Sigma-Aldrich).

From harvested cell pellets, total RNA was extracted (PerfectPure RNA Cell and Tissue kit), quantified and subjected to reverse transcription and quantitative RT-PCR. The TaqMan Gene

Expression Assays for *CYP24A1*, *CYP27B1*, *CYP2R1* and *GUSB*, reaction conditions, and data expression method were the same as those described above in the quantitative RT-PCR protocol used for the placental tissues.

^{13}C-labeled vitamin D treatment [$^{13}C_2$-vitamin D_3, $^{13}C_5$-25(OH)D_3] and measurements of ^{13}C-labeled vitamin D metabolites from cells and media

To explore the metabolic fate of vitamin D_3, HTR-8/SVneo cells were cultured for 24-h, 72-h, and 96-h with either $^{13}C_2$-vitamin D_3 (Cambridge Isotope Laboratories) or $^{13}C_5$-25(OH)D_3 (IsoSciences LLC), resulting in final concentrations of 5,000 nM or 500 nM, respectively. These dosing concentrations of $^{13}C_2$-vitamin D_3 and $^{13}C_5$-25(OH)D_3 were selected using data from the unlabeled vitamin D treatment. At each culture time point, the cells and media from the treatments and control were harvested separately and stored at -80˚C until measurements.

^{13}C-labeled vitamin D metabolites (i.e., ^{13}C-vitamin D_3, ^{13}C-25(OH)D_3, ^{13}C-1,25$(OH)_2D_3$, ^{13}C-24,25$(OH)_2D_3$) were extracted from the cells, which were sonicated in 200 μL deionized water, based on liquid-liquid and solid-phase extractions described for placental tissue vitamin D metabolites, but followed by 50 μL of 1 mg/mL PTAD derivatization (23,26). Media (15 mL) was mixed with 15 mL methanol and 25 μL internal standard, and subsequently underwent the solid-phase extraction and the derivatization. An internal standard solution mix for $^{13}C_2$-vitamin D_3 treatment and ethanol control contained d_6-vitamin D_3, d_3-25(OH)D_3, d_3-1,25$(OH)_2D_3$, and d_6-24,25$(OH)_2D_3$, while an internal standard solution for $^{13}C_5$-25(OH)D_3 treatment contained d_3-25(OH)D_3, d_3-1,25$(OH)_2D_3$, and d_6-24,25$(OH)_2D_3$. 10 μL of extracts diluted with 0.1% formic acid in water (+5 mM methylamine) were injected and separated by an LC-MS/MS system (Ultimate 3000 UHPLC coupled to Thermo Scientific QE-MS) with Waters BEH C18 columns

(2.5 μm; 2.1 x 50mm) (26). Extracts from the ethanol control were injected twice with extracts from either $^{13}C_2$-vitamin D_3 or $^{13}C_5$-25(OH)D_3 treatment for separate comparisons. Elution of the analytes of interest at a flow rate of 250 μL/min was optimized under the following conditions: 40% of 0.1% formic acid in methanol and 60% of 0.1% formic acid in water (+5mM methylamine) at 0 min; linear gradient from 40% to 70% of 0.1% formic acid in methanol (0-5th min); 70% of 0.1% formic acid in methanol (5-9th min); linear gradient from 70% to 40% of 0.1% formic acid in methanol (9-12th min); and 40% of 0.1% formic acid in methanol (12-17th min). The analytes of interest were detected in electrospray ionization (ESI)-positive ion mode. Details about quantification including transition pairs and calibration ranges for the analytes of interest in ^{13}C-labeled vitamin D treatments are provided in the **Table 2.1** and **Supplemental Table S2.1**, respectively.

Table 2.1. Transition pairs for the analytes of interest in the ^{13}C-labeled vitamin D treatments

Analytes of interest	Transition pairs	
	$^{13}C_2$-vitamin D_3 treatment	$^{13}C_5$-25(OH)D_3 treatment
Vitamin D_3	m/z 591.43 (280.1>298.1)	-
d$_6$-vitamin D_3	m/z 597.43 (280.1>298.1)	-
$^{13}C_2$-vitamin D_3	m/z 593.43 (280.1>298.1)	-
25(OH)D_3	m/z 607.42 (280.1>298.1)	m/z 607.42 (280.1>298.1)
d$_3$-25(OH)D_3	m/z 610.42 (283.1>301.1)	m/z 610.42 (283.1>301.1)
$^{13}C_2$-25(OH)D_3	m/z 609.42 (281.1>299.1)	-
$^{13}C_5$-25(OH)D_3	-	m/z 612.42 (280.1>298.1)
24,25$(OH)_2D_3$	m/z 623.42 (280.1>298.1)	m/z 623.42 (280.1>298.1)
d$_6$-24,25$(OH)_2D_3$	m/z 629.42 (280.1>298.1)	m/z 629.42 (280.1>298.1)
$^{13}C_2$-24,25$(OH)_2D_3$	m/z 625.42 (280.1>298.1)	-
$^{13}C_5$-24,25$(OH)_2D_3$	-	m/z 628.42 (280.1>298.1)
1,25$(OH)_2D_3$	m/z 623.42 (296.1>314.1)	m/z 623.42 (296.1>314.1)
d$_3$-1,25$(OH)_2D_3$	m/z 626.42 (299.1>317.1)	m/z 626.42 (299.1>317.1)
$^{13}C_2$-1,25$(OH)_2D_3$	m/z 625.42 (296.1>314.1)	-
$^{13}C_5$ 1,25$(OH)_2D_3$	-	m/z 628.42 (296,1>314.1)

Statistical analysis

Differences between baseline and study-end circulating vitamin D metabolites among pregnant women were tested using a paired *t*-test except for epi-25(OH)D_3 which was analyzed by the Wilcoxon signed-rank test. To examine associations of placental mRNA abundance of the vitamin D machinery with vitamin D metabolites in blood and placental tissues, a linear mixed model (LMM) was constructed. Each placental and circulating vitamin D metabolite were entered into the model as a fixed effect, while the participant identifier was entered as a random effect. Covariates considered in initial models are shown in **Table 2.2** and **Supplemental Table S2.2**, and those with a significance level of $P < 0.2$ were retained in final models. In addition, associations between placental and circulating vitamin D metabolites were assessed using a Pearson's correlation analysis.

To examine changes in concentrations of intracellular and extracellular ^{13}C-labeled vitamin D metabolites from HTR-8/SVneo cells with ^{13}C-labeled vitamin D treatments, the LMM and the Bonferroni correction was used. Culture time (24-h, 72-h, or 96-h), location of the metabolite extraction (cell or medium), and their interaction were fixed effects, and experiment number (1st, 2nd or 3rd experiments) and sample identifier nested within the experiment number were random effects. The LMM was also used to assess changes in product:precursor ratios in the vitamin D metabolic pathway. Differences in enzyme gene expression among the vitamin D treatment groups (unlabeled) in the cells were tested using a 1-factor ANOVA.

All analyses were conducted using JMP Pro 12 (SAS Institute), STATA 14 (StataCorp LP), and SigmaPlot 11 (Systat Software). Data not normally distributed were ln-transformed and are presented as arithmetic means±SDs or geometric means (95% CIs), unless otherwise specified. *P* values were 2-tailed and considered significant at < 0.05.

Table 2.2. Characteristics and concentrations of circulating and placental vitamin D metabolites among the third-trimester pregnant women[1,2]

Characteristics	**Pregnant women (*n* =24)**	
Age, y	29 ± 3	
Prepregnancy BMI, median [IQR], kg/m^2	23 [21, 26]	
Ethnicity, *n*		
Caucasian / African American / Latino / Asian / Other	14 / 1 / 4 / 4 / 1	
Season at study entry, *n*		
April–September / October–March	13 / 11	
Gestational age at birth, weeks	39.9 ± 0.7	
Circulating vitamin D metabolites	**Baseline**	**Study-end**
25(OH)D_3, nmol/L	85.3 ± 28.6[a]	96.8 ± 33.0[b]
1,25(OH)$_2$$D_3$, geometric means (95% CIs), pmol/L	290 (235, 357)[a]	312 (258, 379)[a]
24,25(OH)$_2$$D_3$, nmol/L	11.4 ± 6.1[a]	12.8 ± 5.2[a]
Free 25(OH)D_3, geometric means (95% CIs), pmol/L	15.7 (11.8, 20.9)[a]	19.4 (15.0, 25.1)[b]
Epi-25(OH)D_3, nmol/L	3.2 ± 2.2[a]	4.5 ± 3.5[b]
24,25(OH)$_2$$D_3$: 25(OH)D_3	0.13 ± 0.04[a]	0.13 ± 0.03[a]
DBP, μg/mL	461 ± 247[b]	422 ± 233[a]
Placental vitamin D metabolites	**Delivery**	
25(OH)D_3, pmol/g	20.9 ± 8.2	
24,25(OH)$_2$$D_3$, pmol/g	2.2 ± 1.5	
24,25(OH)$_2$$D_3$: 25(OH)D_3	0.10 ± 0.04	

[1] Values are mean ± SD, unless otherwise indicated. For circulating vitamin D metabolites, values in a row with a superscript letter indicate significant differences between baseline and study-end (i.e., a < b). $P < 0.05$.

[2] DBP, vitamin D binding protein; Epi-25(OH)D_3, C3 epimer of 25-hydroxyvitamin D_3; Free 25(OH)D_3, free 25-hydroxyvitamin D_3; 25(OH)D_3, 25-hydroxyvitamin D_3; 1,25(OH)$_2$$D_3$, 1,25-dihydroxyvitamin D_3; 24,25(OH)$_2$$D_3$, 24,25-dihydroxyvitamin D_3

RESULTS

Characteristics and measures of vitamin D metabolites in blood and placenta among pregnant women

Table 2.2 depicts the characteristics of the third-trimester pregnant women along with their vitamin D metabolite concentrations in blood at baseline and study-end, and in placental tissue. Differences were detected between baseline and study-end for $25(OH)D_3$, free $25(OH)D_3$, epi-$25(OH)D_3$, and DBP, whereas no differences were detected between these study time points for $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, and the $24,25(OH)_2D_3$:$25(OH)D_3$ ratio.

Associations between placental and circulating vitamin D metabolites

A strong positive correlation (r = 0.83, P < 0.001) was detected between placental $25(OH)D_3$ and placental $24,25(OH)_2D_3$ at delivery. Moreover, these placental vitamin D metabolites were significantly correlated with the majority of circulating vitamin D metabolites through time (**Figure 2.1**). Placental $25(OH)D_3$ showed strong positive correlations with circulating $25(OH)D_3$ (Figure 2.1A) and $24,25(OH)_2D_3$ (Figure 2.1B) at both baseline and study-end. Placental $25(OH)D_3$ was also positively correlated with baseline circulating $24,25(OH)_2D_3$:$25(OH)D_3$ ratio (Figure 2.1C), study-end free $25(OH)D_3$ (Figure 2.1D), and study-end epi-$25(OH)D_3$ (Figure 2.1E). In addition, placental $24,25(OH)_2D_3$ showed positive correlations with circulating $25(OH)D_3$ (Figure 2.1F) and $24,25(OH)_2D_3$ (Figure 2.1G) throughout the study, and placental $24,25(OH)_2D_3$ was positively correlated with study-end epi-$25(OH)D_3$ (Figure 2.1H).

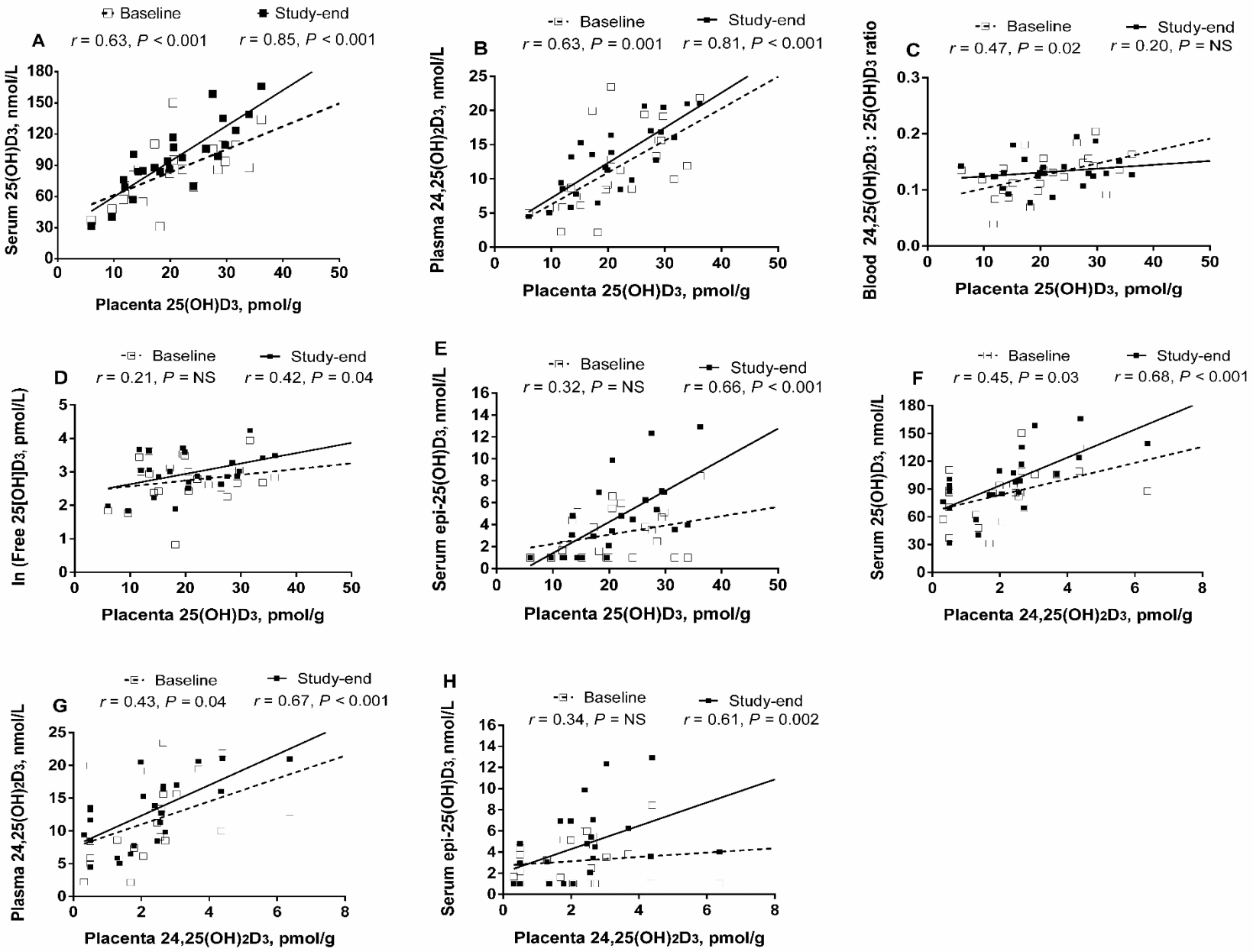

A
Baseline
Study-end
r = 0.63, P < 0.001
r = 0.85, P < 0.001
Serum 25(OH)D3, nmol/L
Placenta 25(OH)D3, pmol/g
B
r = 0.63, P = 0.001
r = 0.81, P < 0.001
Plasma 24,25(OH)2D3, nmol/L
C
r = 0.47, P = 0.02
r = 0.20, P = NS
Blood 24,25(OH)2D3 : 25(OH)D3 ratio
D
r = 0.21, P = NS
r = 0.42, P = 0.04
ln (Free 25[OH]D3, pmol/L)
E
r = 0.32, P = NS
r = 0.66, P < 0.001
Serum epi-25(OH)D3, nmol/L
F
r = 0.45, P = 0.03
r = 0.68, P < 0.001
Placenta 24,25(OH)2D3, pmol/g
G
r = 0.43, P = 0.04
r = 0.67, P < 0.001
H
r = 0.34, P = NS
r = 0.61, P = 0.002

Figure 2.1 Relationships between placental and circulating vitamin D metabolites in pregnant women (n = 24) by study time point: (A) placental 25(OH)D_3 and serum 25(OH)D_3; (B) placental 25(OH)D_3 and plasma 24,25$(OH)_2D_3$ (C) placental 25(OH)D_3 and circulating 24,25$(OH)_2D_3$:25(OH)D_3 ratio; (D) placental 25(OH)D_3 and circulating free 25(OH)D_3; (E) placental 25(OH)D_3 and serum epi-25(OH)D_3; (F) placental 24,25$(OH)_2D_3$ and serum 25(OH)D_3; (G) placental 24,25$(OH)_2D_3$ and plasma 24,25$(OH)_2D_3$; (H) placental 24,25$(OH)_2D_3$ and serum epi-25(OH)D_3. Study-baseline values are presented in open squares with dashed line slopes and study-end values are presented in closed squares with solid line slopes. Free 25(OH)D_3 was ln-transformed.

Associations of placental gene expression of the vitamin D machinery with placental vitamin D metabolites

Placental *LRP2* mRNA abundance was positively associated with placental 24,25$(OH)_2D_3$:25(OH)D_3 ratio [R^2 = 0.62; β = 1873.54 (235.39, 3511.68); P = 0.028] and placental 25(OH)D_3 [R^2 = 0.44; β = 6.51 (1.67, 11.34); P = 0.011]. Further, placental *CYP2R1* and *CYP24A1* mRNA abundance tended to be inversely associated with placental 24,25$(OH)_2D_3$:25(OH) D_3 ratio [For *CYP2R1*, R^2 = 0.52, β = -9.06 (-18.47, 0.34), P = 0.058; For *CYP24A1*, R^2 = 0.20, β = -8.72 (-17.43, -0.0042), P = 0.050].

Associations of placental gene expression of the vitamin D machinery with circulating vitamin D metabolites

Placental *CYP24A1* mRNA abundance was positively associated with circulating 1,25$(OH)_2D_3$ at both baseline and study-end (**Table 2.3**), and with baseline circulating 25(OH)D_3, 24,25$(OH)_2D_3$, free 25(OH)D_3 and epi-25(OH)D_3. Placental *LRP2* mRNA abundance was positively associated with circulating 25(OH)D_3 and epi-25(OH)D_3 at both baseline and study-end, and with study-end free 25(OH)D_3 (Table 2.3). Placental *CUBN* mRNA abundance was positively associated with circulating 25(OH)D_3 and 1,25$(OH)_2D_3$ at both study time points (Table 2.3),

while placental *CYP2R1* mRNA abundance was positively associated with circulating $1,25(OH)_2D_3$ throughout the study, and with baseline epi-$25(OH)D_3$ (Table 2.3). Finally, placental *CYP27B1* mRNA abundance was positively associated with baseline epi-$25(OH)D_3$ and study-end $1,25(OH)_2D_3$, and tended to be associated with baseline $25(OH)D_3$ (Table 2.3).

Table 2.3
Associations of placental gene transcript abundance of the vitamin D metabolic machinery with circulating vitamin D metabolites among pregnant women (n = 24)[1-3]

Placental Genes	Circulating Vitamin D Metabolites	R^2	β (95% CI)	*P* Value
LRP2	**Baseline**			
	$25(OH)D_3$	0.51	3.3 (1.7, 5.0)	< 0.001
	Epi-$25(OH)D_3$	0.58	22 (-0.61, 44)	0.06
	Study-end			
	$25(OH)D_3$	0.64	2.5 (1.2, 3.9)	0.001
	Free $25OHD_3$	0.82	8.9 (5.2, 13)	0.0003
	Epi-$25(OH)D_3$	0.55	16 (5.5, 26)	0.005
CUBN	**Baseline**			
	$25(OH)D_3$	0.31	0.016 (0.0026, 0.029)	0.02
	$1,25(OH)_2D_3$	0.47	0.0035 (0.0015, 0.0052)	0.001
	Study-end			
	$25(OH)D_3$	0.49	0.020 (0.0073, 0.032)	0.004
	$1,25(OH)_2D_3$	0.53	0.0037 (0.0019, 0.0056)	< 0.001
CYP2R1	**Baseline**			
	$1,25(OH)_2D_3$	0.34	0.0025 (0.0009, 0.0042)	0.004
	Epi-$25(OH)D_3$	0.56	0.23 (0.11, 0.34)	< 0.001
	Study-end			
	$1,25(OH)_2D_3$	0.32	0.0021 (0.0007, 0.0034)	0.006
CYP27B1	**Baseline**			
	$25(OH)D_3$	0.54	0.0095 (-0.0007, 0.020)	0.06
	Epi-$25(OH)D_3$	0.40	0.19 (0.079, 0.31)	0.002
	Study-end			
	$1,25(OH)_2D_3$	0.57	0.0022 (0.0007, 0.0037)	0.007
CYP24A1	**Baseline**			
	$25(OH)D_3$	0.43	0.024 (0.010, 0.037)	0.002
	$1,25(OH)_2D_3$	0.40	0.0043 (0.0019, 0.0067)	0.001
	$24,25(OH)_2D_3$	0.21	0.069 (0.0071, 0.13)	0.03
	Free $25(OH)D_3$	0.33	0.075 (0.024, 0.13)	0.007
	Epi-$25(OH)D_3$	0.56	0.38 (0.22, 0.54)	< 0.001

Table 2.3 (Continued)

	Study-end			
CYP24A1	$1,25(OH)_2D_3$	0.45	0.0036 (0.0016, 0.0056)	0.001

[1] Data were derived from the covariate-adjusted linear mixed models.
[2] All placental vitamin D-related gene transcript abundance (dependent variables) were ln-transformed except for *LRP2*.
[3] No association achieved statistical significance between placental gene expression and circulating vitamin D metabolites unless listed in this table.

Effects of vitamin D exposure on placental gene expression of the vitamin D metabolic enzymes in HTR-8/SVneo cell cultures

We examined effects of vitamin D treatment [unlabeled vitamin D_3, $25(OH)D_3$, $1,25(OH)_2D_3$] on gene expression response of vitamin D metabolic enzymes in the placental cells. $1,25(OH)_2D_3$ (100 nM) and $25(OH)D_3$ (500 nM) treatments up-regulated ($P < 0.05$) *CYP24A1* gene expression by 734- and 566-fold, respectively, compared to the ethanol control at 72-h (**Figure 2.2**). Similarly, the higher concentrations of vitamin D_3 treatments yielded a dose-response increase ($P < 0.05$) in *CYP24A1* mRNA abundance (43-fold at 2,500 nM, 114-fold at 5,000 nM, and 142-fold at 10,000 nM compared to the control; Figure 2.2). In contrast, vitamin D_3, $25(OH)D_3$, or $1,25(OH)_2D_3$ treatment did not influence *CYP27B1* and *CYP2R1* mRNA expression (**Supplemental Table S2.3**).

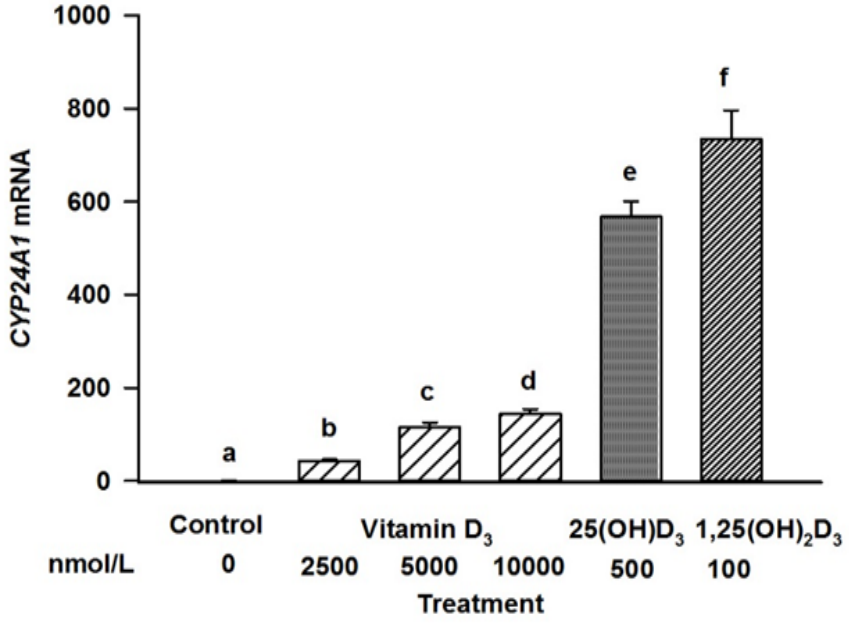
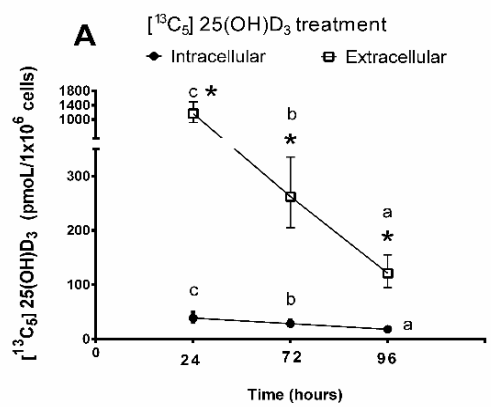


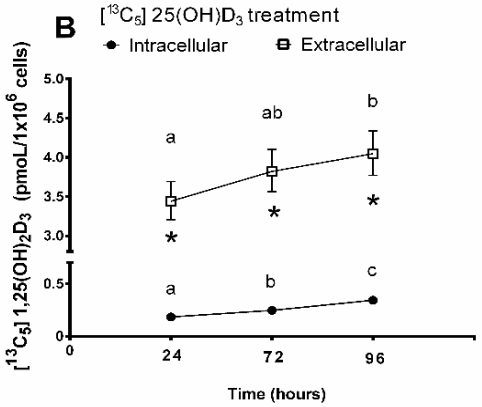
Figure 2.2. Responses of *CYP24A1* mRNA abundance in HTR-8/SVneo cells to vitamin D (unlabeled) treatment at 72-h. Values are means ± SE. Data were obtained from three separate experiments with each experiment containing three replicates per vitamin D treatment, and were analyzed after $\log_{10}$ transformation. Means without a common letter differ, $P < 0.05$.

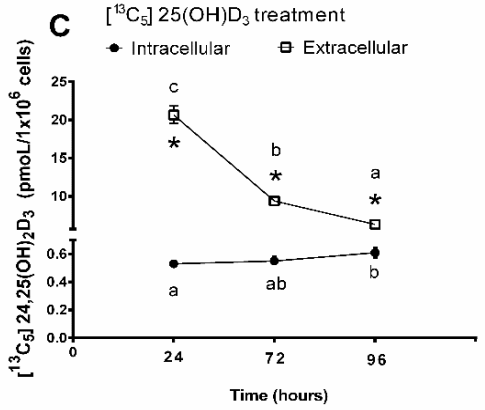
Intracellular and extracellular concentrations of ^{13}C-labeled vitamin D metabolites in HTR-8/SVneo cell cultures

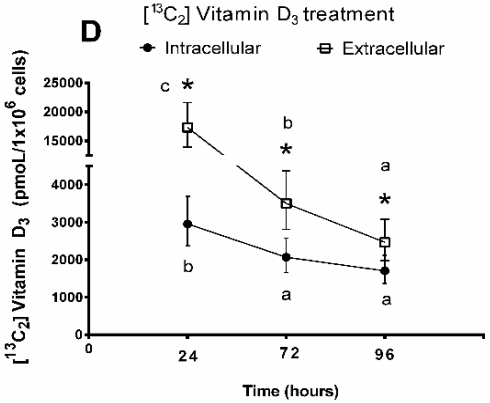
In the $^{13}C_5$-25(OH)D_3 treatment (**Figure 2.3**A-2.3C), intracellular $^{13}C_5$-25(OH)D_3 concentrations decreased by 53% ($P < 0.001$) (Figure 2.3A), and extracellular $^{13}C_5$-25(OH)D_3 concentrations (i.e., treatment concentrations in the media) decreased by 90% ($P < 0.001$) from 24-h to 96-h. In contrast, intracellular and extracellular concentrations of $^{13}C_5$-1,25$(OH)_2D_3$ increased by 86% ($P < 0.001$) and 18% ($P = 0.017$), respectively, from 24-h to 96-h (Figure 2.3B). Although intracellular $^{13}C_5$-24,25$(OH)_2D_3$ concentrations increased slightly (15%; $P = 0.008$) from 24-h to 96-h (Figure 2.3C), extracellular $^{13}C_5$-24,25$(OH)_2D_3$ concentrations decreased (-70%; $P < 0.001$).

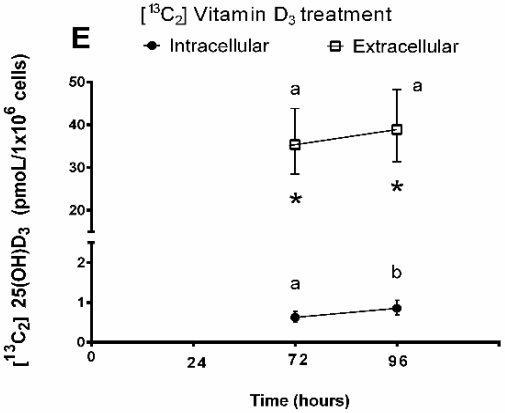
In the $^{13}C_2$-vitamin D_3 treatment (Figure 2.3D-2.3G**)**, intracellular $^{13}C_2$-vitamin D_3 concentrations decreased (-42%; $P < 0.001$) from 24-h to 96-h (Figure 2.3D), as did extracellular $^{13}C_2$-vitamin D_3 concentrations (-86%; $P < 0.001$). In addition, intracellular $^{13}C_2$-25(OH)D_3 concentrations increased by 37% ($P < 0.001$) from 72-h to 96-h (Figure 2.3E), whereas extracellular $^{13}C_2$-25(OH)D_3 concentrations remained stable. Finally, extracellular $^{13}C_2$-1,25$(OH)_2D_3$ and $^{13}C_2$-24,25$(OH)_2D_3$ concentrations increased by 203% ($P < 0.001$) and 75% ($P < 0.001$), respectively, from 72-h to 96-h (Figure 2.3F, 2.3G). None of ^{13}C-labeled vitamin D metabolites were detected in the ethanol control.

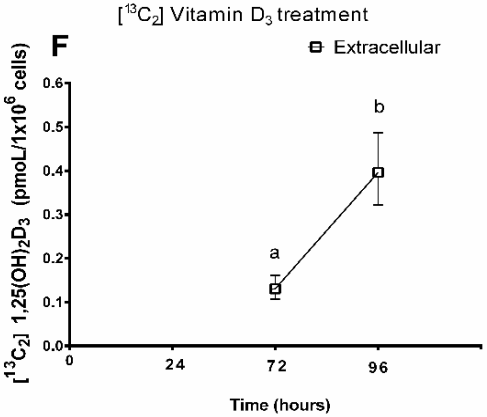

A
[13C5] 25(OH)D3 treatment
Intracellular
Extracellular
[13C5] 25(OH)D3 (pmoL/1x10^6 cells)
Time (hours)


B
[13C5] 25(OH)D3 treatment
Intracellular
Extracellular
[13C5] 1,25(OH)2D3 (pmoL/1x10^6 cells)
Time (hours)


C
[13C5] 25(OH)D3 treatment
Intracellular
Extracellular
[13C5] 24,25(OH)2D3 (pmoL/1x10^6 cells)
Time (hours)


D
[13C2] Vitamin D3 treatment
Intracellular
Extracellular
[13C2] Vitamin D3 (pmoL/1x10^6 cells)
Time (hours)


E
[13C2] Vitamin D3 treatment
Intracellular
Extracellular
[13C2] 25(OH)D3 (pmoL/1x10^6 cells)
Time (hours)


F
[13C2] Vitamin D3 treatment
Extracellular
[13C2] 1,25(OH)2D3 (pmoL/1x10^6 cells)
Time (hours)

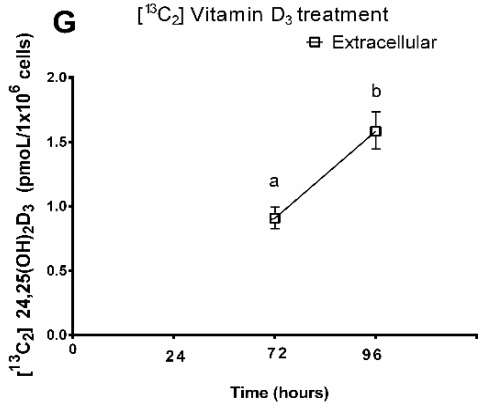

G
[13C2] Vitamin D3 treatment
Extracellular
[13C2] 24,25(OH)2D3 (pmoL/1x10^6 cells)
Time (hours)

Figure 2.3. Intracellular and extracellular concentrations of ^{13}C-labeled vitamin D metabolites from HTR-8/SVneo cells incubated with $^{13}C_5$-25(OH)D_3 (A-C) or $^{13}C_2$-vitamin D_3 (D-G) for 24-h, 72-h, and 96-h: (A) $^{13}C_5$-25(OH)D_3, treatment; (B) $^{13}C_5$-1,25(OH)D_3, product; (C) $^{13}C_5$-24,25(OH)D_3, product; (D) $^{13}C_2$-vitamin D_3, treatment; (E) $^{13}C_2$-25(OH)D_3, product; (F) $^{13}C_2$-1,25(OH)D_3, product; (G) $^{13}C_2$-24,25(OH)D_3, product. All concentrations are predicted geometric means (95% CIs) derived from the covariate-adjusted linear mixed models. Values were obtained from three separate experiments with each experiment containing three replicates per vitamin D treatment at a time point. Different letters denote changes in concentrations through time within cells or media, $P < 0.05$. Asterisks (*) denote differences in concentrations between cells and media at a time, $P < 0.05$. None of ^{13}C-labeled vitamin D metabolites were detected in the ethanol control treatment.

Ratios of product to precursor in the ^{13}C-labeled vitamin D metabolic pathway in HTR-8/SVneo cell cultures

In the $^{13}C_5$-25(OH)D_3 treatment, ratios of intracellular $^{13}C_5$-1,25$(OH)_2D_3$:$^{13}C_5$-25(OH)D_3 and $^{13}C_5$-24,25$(OH)_2D_3$:$^{13}C_5$-25(OH)D_3 increased ($P < 0.001$) from 24-h to 96-h (**Figure 2.4**A). In the $^{13}C_2$-vitamin D_3 treatment, extracellular:intracellular ratios for $^{13}C_2$-1,25$(OH)_2D_3$:$^{13}C_2$-25(OH)D_3 and $^{13}C_2$-24,25$(OH)_2D_3$: $^{13}C_2$-25(OH)D_3 increased ($P < 0.001$) from 72-h to 96-h (Figure 2.4B). At each time point, the 24,25$(OH)_2D_3$:25(OH)D_3 ratio was significantly higher ($P < 0.001$) than the 1,25$(OH)_2D_3$:25(OH)D_3 ratio in both $^{13}C_5$-25(OH)D_3 and $^{13}C_2$-vitamin D_3 treatments.

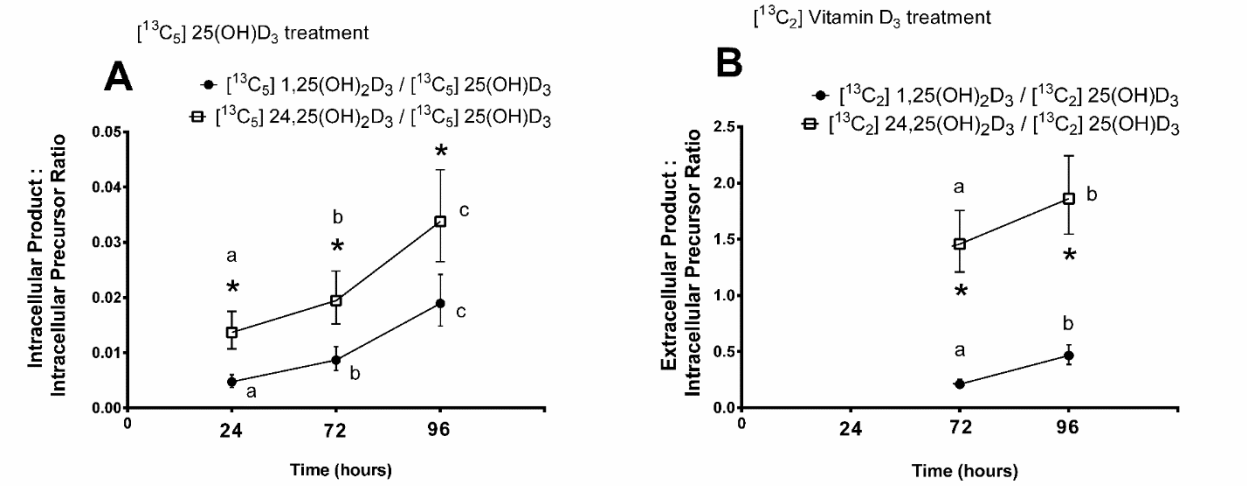


Figure 2.4. Production of ^{13}C-1,25(OH)$_2$D$_3$ and ^{13}C-24,25(OH)$_2$D$_3$ that are normalized to the precursor, ^{13}C-25(OH)D$_3$, in HTR-8/SVneo cells incubated with $^{13}C_5$-25(OH)D$_3$ (A) or $^{13}C_2$-vitamin D$_3$ (B) for 24-h, 72-h, and 96-h. All ratios are predicted geometric means (95% CIs) derived from the covariate-adjusted linear mixed models. Values were obtained from three separate experiments with each experiment containing three replicates per vitamin D treatment at a time point. Different letters denote changes within 1,25(OH)$_2$D$_3$:^{13}C-25(OH)D$_3$ ratio or 24,25(OH)$_2$D$_3$:25(OH)D$_3$ ratio through time, P<0.05. Asterisks (*) denote differences between 1,25(OH)$_2$D$_3$:25(OH)D$_3$ ratio and 24,25(OH)$_2$D$_3$:25(OH)D$_3$ ratio at a time, P < 0.05. None of ^{13}C-labeled vitamin D metabolites were detected in the ethanol control treatment.

DISCUSSION

To the best of our knowledge, this is the first study to comprehensively assess associations between placental vitamin D metabolites and placental gene expression of vitamin D metabolic components as well as their relationships with circulating vitamin D metabolites. Human placental trophoblast cells were also used to investigate vitamin D uptake, metabolism, and secretion in response to treatment with different vitamin D metabolites *in vitro*. Findings from the associations between placental and circulating vitamin D biomarkers of our human study suggest that: (i) the import of 25(OH)D_3 by the placenta occurs mostly through a regulated process; (ii) the placenta partitions the precursor, 25(OH)D_3, between synthetic or catabolic pathways thereby regulating the supply of 1,25$(OH)_2D_3$; and (iii) the placenta plays an active role in the uptake and metabolism of C3-epimeric forms of vitamin D_3. Remarkably, we also demonstrate that placental trophoblast cells produce and secrete not only 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$, but also, for the first time, 25(OH)D_3 from parent vitamin D_3.

Characterization of placental vitamin D metabolism and its putative role in modulating circulating concentrations of vitamin D metabolites

Numerous positive associations between placental and circulating concentrations of vitamin D metabolites were found in the present study (Figure 2.1). Further, and more notably, positive associations ($P < 0.03$) were observed between placental tissue mRNA abundance of vitamin D metabolic components and circulating vitamin D metabolites (i.e., *LRP2* with 25(OH)D_3, free 25(OH)D_3 and epi-25(OH)D_3; *CUBN* with 25(OH)D_3 and 1,25$(OH)_2D_3$; *CYP2R1* with epi-25(OH)D_3 and 1,25$(OH)_2D_3$; *CYP24A1* with 25(OH)D_3, 24,25$(OH)_2D_3$, epi-25(OH)D_3 and 1,25$(OH)_2D_3$; *CYP27B1* with epi-25(OH)D_3 and 1,25$(OH)_2D_3$). Overall, these data are consistent

with a modulatory role of the placenta on maternal circulating concentrations of vitamin D metabolites.

Interestingly, the cellular uptake of 25(OH)D_3, the major form of circulating vitamin D, appears to be mediated through the megalin-cubilin receptor complex and is thus a regulated process. For example, placental 25(OH)D_3 showed a stronger positive correlation with total circulating 25(OH)D_3 (comprised of free and DBP-bound forms), as compared to circulating free 25(OH)D_3, at both baseline and study-end. In addition, placental mRNA abundance of the low density lipoprotein-related protein 2 (*LRP2*, also known as megalin), the main endocytic receptor for DBP, was positively associated with total circulating 25(OH)D_3 throughout the third trimester. Similarly, placental *CUBN* mRNA abundance (cubilin), which interacts with megalin to import DBP-bound vitamin D, was positively associated with total circulating 25(OH)D_3, but not with free 25(OH)D_3. Taken together, these data suggest that placental cellular uptake of 25(OH)D_3 occurs mainly through the megalin-cubilin receptor complex as opposed to simple diffusion.

The placenta also appears to regulate the pool of bioactive vitamin D, 1,25$(OH)_2D_3$, by shunting its precursor, 25(OH)D_3, between pathways which produce the active form [1,25$(OH)_2D_3$] or the catabolic form [24,25$(OH)_2D_3$] of vitamin D. Specifically, placental *CYP27B1* mRNA abundance (encodes 1α-hydroxylase) tended to be positively associated with circulating baseline 25(OH)D_3 ($P = 0.06$) and study-end 1,25$(OH)_2D_3$ ($P = 0.007$), supporting a substrate-dependent placental production of 1,25$(OH)_2D_3$ (13,27). Placental *CYP24A1* mRNA abundance (encodes 24-hydroxylase) was also positively associated with circulating baseline 25(OH)D_3 and 24,25$(OH)_2D_3$. In addition, placental 25(OH)D_3 was strongly correlated with placental 24,25$(OH)_2D_3$ and circulating 24,25$(OH)_2D_3$ throughout the third trimester. These data collectively suggest that the placenta plays an active role in regulating the supply of 1,25$(OH)_2D_3$

by partitioning 25(OH)D_3 between synthetic or catabolic pathways.

A possible role in the uptake and metabolism of C3-epimeric forms of vitamin D_3 by the placenta is also shown. The mRNA abundance of placental *LRP2* was positively associated with circulating epi-25(OH)D_3 at both baseline and study-end, suggesting that placental uptake of epi-25(OH)D_3, which can bind to DBP (28), is through the megalin-cubilin receptor system. In addition, the mRNA abundance of placental *CYP2R1* (encodes 25-hydroxylase), *CYP27B1* (encodes 1α-hydroxylase), and *CYP24A1* (encodes 24-hydroxylase) were positively associated with circulating epi-25(OH)D_3 at baseline. Given that CYP27B1 and CYP24A1 show hydroxylating abilities of C3-epimers *in vitro* (29), these data suggest that the placenta could be a source of several C3-epimers [i.e., 3-epi-25(OH)D_3, 3-epi-1,25$(OH)_2D_3$, and 3-epi-24,25$(OH)_2D_3$] during gestation. The finding that pregnant women have higher concentrations of circulating epi-25(OH)D_3 as compared to nonpregnant subjects (7,8) provide further support for this working hypothesis. Nonetheless, additional work is needed to establish the physiological role of C3-epimers in pregnancy.

Placental trophoblasts produce and secrete 25(OH)D_3 along with 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$

The cell culture component of this study shows that human placental trophoblast cells *in vitro* are capable of cellular uptake of parent vitamin D_3, and more importantly, its use in the synthesis and export of 25(OH)D_3. Specifically, $^{13}C_2$-25(OH)D_3 increased in the cell pellets and media from HTR-8/SVneo cells incubated with $^{13}C_2$-vitamin D_3. Although others have also reported production of 25(OH)D_3 by placental mitochondria incubated with vitamin D_3 (30), our finding of 25(OH)D_3 export by trophoblast cells provides compelling evidence that the placenta

could be an extra-hepatic source of circulating 25(OH)D_3. This notion is further supported by reports of higher serum 25(OH)D concentrations among pregnant women compared to nonpregnant control women consuming equivalent intakes of vitamin D (8). In addition, we show upregulation of *CYP24A1* (24-hydroxlyase) in HTR-8/SVneo cells exposed to parent vitamin D_3. Given that an inductive effect of 1,25$(OH)_2D_3$ on *CYP24A1* transcription has also been shown in human syncytiotrophoblasts (31), data from the present study suggest that both parent vitamin D_3 and 1,25$(OH)_2D_3$ have regulatory roles in placental vitamin D metabolism.

The cell culture component of this study also demonstrates biosynthesis and export of both 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ from parent vitamin D_3. Specifically, HTR-8/SVneo cells incubated with $^{13}C_2$-vitamin D_3 had increased extracellular concentrations of ^{13}C-1,25$(OH)_2D_3$ and ^{13}C-24,25$(OH)_2D_3$. Similar to prior reports (12,15,16,32), we also observed *in vitro* uptake of 25(OH)D_3, and its use for the synthesis and export of 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ in trophoblasts.

Notably, trophoblast cells generate and release greater amounts of 24,25$(OH)_2D_3$ than 1,25$(OH)_2D_3$ from vitamin D_3. When normalized to the precursor ^{13}C-25(OH)D_3, cells produced significantly more ^{13}C-24,25$(OH)_2D_3$ than ^{13}C-1,25$(OH)_2D_3$ at all time points in both $^{13}C_2$-vitamin D_3 and $^{13}C_5$-25(OH)D_3 treatments. One might expect that placental production of 1,25$(OH)_2D_3$ would outweigh that of 24,25$(OH)_2D_3$ due to the weaker expression levels of placental CYP24A1 than CYP27B1 (11,14) as well as methylation of the placental *CYP24A1* gene promoter region (33) which could lead to its downregulation. However, we observed a robust induction of *CYP24A1* transcript abundance after treatments with both parent vitamin D_3 and 25(OH)D_3 which likely contributed to the greater production of 24,25$(OH)_2D_3$ in HTR-8/SVneo cells. In addition, others have shown that CYP24A1 (versus CYP27B1) has a higher affinity for 25(OH)D_3 (34,35) which

would be expected to generate more $24,25(OH)_2D_3$ relative to $1,25(OH)_2D_3$.

Study limitations

Because protein expression levels of placental vitamin D machinery were not quantified in this study, we cannot exclude the possibility that associations of maternal circulating/placental vitamin D metabolites with the protein abundance of the metabolic genes might differ from those with the mRNA abundance. In addition, the HTR-8/SVneo cell culture may not be the best model that could fully represent human placental vitamin D metabolism. However, HTR-8/SVneo cells in the present study showed an extensive range of vitamin D metabolism from vitamin D_3 uptake to $25(OH)D_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ production, thereby elucidating the metabolic fate of vitamin D_3 within the cell as well as its secretory forms.

Conclusions

In this study, the placenta is identified as an extra-hepatic, extra-renal source of all forms of vitamin D metabolites [i.e., $25(OH)D_3$, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$]. As such, these placentally produced metabolites could influence maternal circulating vitamin D pools in a manner that is independent of dietary intake and other confounders. Further, like the kidney, the placenta appears to regulate cellular uptake of $25(OH)D_3$, the major form of circulating vitamin D, through its megalin-cubilin receptor complex. Finally, our data suggest that the placenta may metabolize and be a source of circulating C3-epimers of vitamin D. Taken together, these data provide compelling evidence that the placenta plays an active role in modulating the vitamin D metabolite profile in maternal circulation.

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Supplemental Table S2.1. Calibration ranges for each ^{13}C-labeled vitamin D metabolite in the $^{13}C_2$-vitamin D_3 and $^{13}C_5$-25(OH)D_3 treatments[1]

	$^{13}C_2$-vitamin D_3 treatment		$^{13}C_5$-25(OH)D_3 treatment	
	pmol in media/sample[2]	pmol in cell pellets/sample[2]	pmol in media/sample	Pmol in cell pellets/sample
$^{13}C_2$-vitamin D_3	250-25,000	250-25,000	-	-
$^{13}C_2$-25(OH)D_3	1.25-1,250	2.5-500	-	-
$^{13}C_5$-25(OH)D_3	-	-	100-8,000	2.5-500
$^{13}C_2$-24,25$(OH)_2D_3$	0.5-50	0.5-50	-	-
$^{13}C_5$-24,25$(OH)_2D_3$	-	-	0.5-50	0.5-50
$^{13}C_2$-1,25$(OH)_2D_3$	0.3-30	0.3-30	-	-
$^{13}C_5$-1,25$(OH)_2D_3$	-	-	0.3-30	0.3-30

[1] Unlabeled 25(OH)D_3, 1,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$ were used to quantify $^{13}C_2$-25(OH)D_3, $^{13}C_2$-24,25$(OH)_2D_3$, $^{13}C_5$-24,25$(OH)_2D_3$, $^{13}C_2$-1,25$(OH)_2D_3$, and $^{13}C_5$-1,25$(OH)_2D_3$ because these ^{13}C-labeled vitamin D metabolites were not commercially available.

[2] Each treatment at each culture time was performed in triplicate (i.e., sample 1,2,3), thus ^{13}C-labeled metabolites were extracted and quantified in media and cell pellets of each sample.

Supplemental Table S2.2. Gestational and neonatal outcomes and additional biochemical measurements of the third-trimester pregnant women[1,2]

Gestational and neonatal characteristics	Pregnant women (*n* = 24)
Parity (Primiparas / Multiparas), *n*	11 / 13
Gestational weight gain, kg	15.9 ± 4.6
Mode of delivery (Vaginal / Caesarean Section), *n*	17 / 5
Multivitamin supplement use before entering the study (yes), *n*	20
Choline intake during the study (480mg / 930mg), *n*	12 / 12
GC rs7041 G>T polymorphism (GG / GT / TT) [3], *n*	8 / 10 / 6
CYP2R1 rs10741657 A>G polymorphism (AA / AG / GG) [3], *n*	4 / 18 / 2
CYP2R1 rs12794714 A>G polymorphism (AA / AG / GG) [3], *n*	5 / 18 / 1
Infant gender (Boy / Girl), *n*	17 / 7
Infant birth weight, kg	3.4 ± 0.3
Infant head circumference, cm	34.2 ± 1.1
Infant length, cm	50.3 ± 1.8

Biochemical measures[4]	Baseline	Study-end
Calcium, mg/dL	8.7 ± 0.4[a]	8.9 ± 0.3[b]
Phosphorus, mg/dL	3.4 ± 0.5[a]	3.5 ± 0.5[a]
iPTH, geometric means (95% CIs), pg/mL	15.9 (12.2, 20.9)[a]	21.7 (17.8, 26.5)[b]

[1] Values are mean ± SD, unless otherwise indicated. For biochemical measures, values in a row with a superscript letter indicate significant differences between baseline and study-end (i.e., a < b). $P < 0.05$.
[2] *CYP2R1*, 25-hydroxylase gene; *GC*, vitamin D binding protein gene; iPTH, intact parathyroid hormone.
[3] Genotypes of single nucleotide polymorphisms in the vitamin D-related genes were determined by the Endpoint Genotyping on a Roche LightCycler 480 using the Applied Biosystems TaqMan Genotyping Assays (Life Technologies), as previously described (8).
[4] Serum calcium and phosphorus were quantified using an automated chemistry analyzer (Dimension Xpand Plus), and plasma iPTH was quantified using a Siemens Immulite 2000 automated immunoassay, as previously described (8).

Supplemental Table S2.3. Responses of *CYP27B1* and *CYP2R1* mRNA abundance in HTR-8/SVneo cells to vitamin D (unlabeled) treatment at 72-h[1]

Treatment	*CYP27B1*	*CYP2R1*
Control	1.0 ± 0.1	1.0 ± 0.0
Vitamin D_3 5,000 nM	1.3 ± 0.2	1.1 ± 0.1
25(OH)D_3 500 nM	1.0 ± 0.1	1.2 ± 0.0
1,25$(OH)_2D_3$ 100 nM	1.0 ± 0.1	1.1 ± 0.0

[1] There was no effect of vitamin D_3, 25(OH)D_3, or 1,25$(OH)_2D_3$ on responses of *CYP27B1* and *CYP2R1* mRNA expression.

CHAPTER 3

Maternal vitamin D biomarkers are associated with maternal and fetal bone turnover among pregnant women consuming controlled amounts of vitamin D, calcium, and phosphorus[1,2]

[1]Park H, Brannon PM, West AA, Yan J, Jiang X, Perry CA, Malysheva OV, Mehta S, Caudill MA. Maternal vitamin D biomarkers are associated with maternal and fetal bone turnover among pregnant women consuming controlled amounts of vitamin D, calcium, and phosphorus. Bone. 2016 Dec; 95:183–191. doi: 10.1016/j.bone.2016.12.002. Regarding the copyright of this article, authors have the right to include the article in their thesis or dissertation.

[2]Supported by USDA National Institute of Food and Agriculture, Agriculture and Food Research Initiative Education and Literacy Initiative Predoctoral Fellowship 2016-67011-25174; NIH grant 1R03HD080824-01A1; the Gerber Foundation grant 1843-3882; the American Egg Board/Egg Nutrition Center; and the Beef Checkoff, through the National Cattlemen's Beef Association and Nebraska Beef Council. The funding sources had no role in the study design, interpretation of the data, and/or publication of results.

ABSTRACT

Vitamin D plays a central role in calcium homeostasis; however, its relationship with bone turnover during pregnancy remains unclear due to a lack of studies that have rigorously controlled for vitamin D and other nutrients known to influence bone metabolism. Similarly, prior investigations of the effect of pregnancy on bone turnover relative to the nonpregnant state may have been confounded by varying intakes of these nutrients. Nested within a controlled intake study, the present investigation sought to quantify associations between maternal vitamin D biomarkers and biochemical markers of bone turnover among pregnant (versus nonpregnant) women and their fetuses under conditions of equivalent and adequate intakes of vitamin D and related nutrients. Changes in markers of bone turnover across the third trimester were also examined. Healthy pregnant (26–29 wk gestation; n = 26) and nonpregnant (n = 21) women consumed 511 IU vitamin D/d, 1.6 g calcium/d, and 1.9 g phosphorus/d for 10 weeks while participating in a controlled feeding study featuring two choline doses. Based on linear mixed models adjusted for influential covariates (e.g., BMI, ethnicity, and season), pregnant women had 50–150% higher ($P < 0.001$) concentrations of bone resorption markers than nonpregnant women. Among pregnant women, increases in maternal 25(OH)D across the study period were associated ($P < 0.020$) with lower osteocalcin and deoxypyridinoline at study-end and higher fetal osteocalcin. In addition, maternal free 25(OH)D, 1,25$(OH)_2$D and 24,25$(OH)_2$D tended to be negatively associated ($P \leq 0.063$) with maternal NTx at study-end, and maternal free 25(OH)D and 24,25$(OH)_2$D were positively associated ($P \leq 0.021$) with fetal CTx. Similarly, maternal 3-epi-25$(OH)D_3$ was negatively related ($P \leq 0.037$) to maternal NTx and deoxypyridinoline at study-end. These declines in bone resorption markers resulting from higher vitamin D biomarker concentrations among pregnant women coincided with increases in their albumin-corrected serum

calcium concentrations, indicating that calcium transfer to the fetus was uncompromised. Notably, none of these associations achieved statistical significance among nonpregnant women. Overall, our study findings suggest that achieving higher maternal concentrations of vitamin D biomarkers might attenuate third-trimester bone resorption while ensuring sufficient calcium delivery to the fetus.

INTRODUCTION

Pregnancy is characterized by a high maternal demand for calcium secondary to the laying down of bone in the fetus. This demand for calcium is mostly met by enhanced intestinal absorption of calcium which has been shown to double during pregnancy (1). Increased calcium mobilization from the bone is another mechanism to ensure adequate calcium to the developing fetus. However, maternal bone loss may ensue, particularly when the dominant adaptation of enhanced maternal intestinal calcium absorption does not fully meet calcium demands (2).

Calcium homeostasis is regulated by vitamin D which influences intestinal calcium absorption, bone resorption and renal calcium absorption in a normal nonpregnant state. In pregnancy, a 2–3 times increase in 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] concentrations contributes, in part, to the doubling of intestinal calcium absorption (2). However, whether vitamin D plays a critical role in calcium mobilization from the bone during gestation is unclear. Animal studies have reported normal calcium homeostasis in fetuses from mothers that were either severely depleted in vitamin D (3,4), or deficient in the vitamin D receptor (VDR) (5,6), suggesting that fetal bone mineralization is independent of maternal vitamin D (7). In humans, data are mixed with some, but not all (8–10), studies reporting an impact of maternal vitamin D status (25-hydroxyvitamin D [25(OH)D]) on fetal bone development (11–15) and maternal bone turnover (16,17). These differences across studies may be secondary to diverse maternal intakes of calcium and phosphorus from food and supplements, which like vitamin D, play essential roles in bone biology and thus are important confounders (18–20).

In addition to 25(OH)D, other forms of vitamin D may have critical roles in bone health. 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] has been associated with bone mineralization in chicks and mice recovering from skeletal fracture (21,22). Free 25(OH)D was also shown to be associated

with bone mineral density in nonpregnant populations (23,24). Similarly, the C-3α epimer of $25(OH)D_3$ [3-epi-$25(OH)D_3$] was reported to have calcemic effects in cell culture studies (25,26). Nonetheless, very few studies have examined the relationship between these other biomarkers of vitamin D and markers of bone turnover during human pregnancy.

As part of a controlled feeding study, our research group recently demonstrated that vitamin D metabolism varies among women in different reproductive states consuming adequate and equivalent amounts of vitamin D, calcium, and phosphorus. Specifically, higher circulating concentrations of several vitamin D biomarkers including 25(OH)D, $1,25(OH)_2D$, vitamin D binding protein (DBP), and 3-epi-$25(OH)D_3$ were observed among pregnant women as compared to nonpregnant control women (27). As an extension of this feeding study, the present study sought to examine the relationships between maternal vitamin D biomarkers [i.e., 25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$, free 25(OH)D, 3-epi-$25(OH)D_3$] and biochemical markers of bone turnover among pregnant women, nonpregnant women, and the newborns of the pregnant women. In addition, changes in bone turnover markers across the third trimester were examined in pregnant women who consumed adequate and equivalent intakes of vitamin D, calcium, and phosphorus.

MATERIALS AND METHODS

Participants

The study participants were twenty-six singleton pregnant women who entered the study at 26–29 weeks gestation and twenty-one nonpregnant control women of childbearing age. The women were recruited from Ithaca, New York (latitude 42.4°N) during 2009–2010 (28,29) and were 21–40 years old, non-smokers, and in good general health based on responses to a health-history questionnaire, a blood chemistry profile, and a complete blood count. Pregnant women reported no use of alcohol throughout their prenatal period, and all women abstained from alcohol during the feeding study. The study protocol was reviewed and approved by the Institutional Review Board for Human Study Participant Use at Cornell University and the Cayuga Medical Center (the hospital where pregnant women delivered their infants). Written informed consent was obtained from all participants prior to study entry.

Study design

The current study was nested within a controlled feeding study in which the study participants consumed a 7-day rotational menu, and either 100 or 550 mg/d of supplemental choline (28). In addition, both pregnant and nonpregnant women consumed a prenatal multivitamin supplement on a daily basis containing vitamin D and calcium (Pregnancy Plus [Fairhaven Health LLC]), a DHA supplement (200 mg/d; Neuromins [Nature's Way Products]), and a potassium and magnesium supplement (3 times/wk.; General Nutrition Corp). During the week, pregnant and nonpregnant women consumed one meal each day along with their daily supplements at the onsite location under supervision. For offsite and weekend meals, food, beverages, and supplements were provided as carry-outs.

All pregnant and nonpregnant participants consumed an average daily intake of 511 ± 48 IU vitamin D (311 ± 48 IU from food, 200 IU from a supplement), 1,622 ± 414 mg calcium (1,582 ± 414 mg from food and 40 mg from a supplement), and 1,877 ± 280 mg phosphorus for 10 weeks. Food estimates were based on the USDA National Nutrient Database for Standard Reference Release 28.

Sample collection and processing

Fasting (10-h) EDTA venous blood and serum, as well as 24-h urine samples, were collected from all participants at study baseline (study week 0; corresponding to "the beginning of the third trimester") and at study-end (study week 10; corresponding to "near term" before delivery). Fetal cord blood samples (n = 23) were also obtained at delivery in EDTA-coated tubes. Three cord blood samples were not retrieved: one participant delivered without notifying the research team, and two participants gave birth at home. The samples were processed and stored at −80 °C until analysis (28).

Analytical measurements

Vitamin D biomarkers and calcitropic hormones in pregnant and nonpregnant women

Vitamin D biomarkers and calcitropic hormones were quantified in blood samples from pregnant and nonpregnant women. Serum 25(OH)D (i.e., the sum of 25(OH)D_2 and 25(OH)D_3), serum 3-epi-25(OH)D_3, and plasma 24,25$(OH)_2$D were quantified using isotope dilution liquid chromatography-mass spectrometry methodology (30,31) with modifications based on our instrumentation as previously described (27). Assay precision and accuracy were assessed by using the National Institute of Standards and Technology SRM and participating in the Vitamin D External Quality Assessment Scheme. Measurements of plasma 1,25$(OH)_2$D (Immunodiagnostic

Systems, Inc., Scottsdale, AZ) and DBP (R&D Systems, Minneapolis, MN) were conducted using ELISA kits, and free 25(OH)D was estimated by an equation (32). Plasma intact parathyroid hormone (iPTH) was quantified by a Siemens Immulite 2000 automated immunoassay.

Biochemical markers of bone turnover in pregnant and nonpregnant women

Bone resorption markers

ELISA kits were used to measure carboxy-terminal cross-linking telopeptide of type 1 collagen (CTx; Immunodiagnostic Systems, Inc., Scottsdale, AZ), and amino-terminal cross-linking telopeptide of type 1 collagen (NTx; MyBiosource, San Diego, CA) in plasma samples from pregnant and nonpregnant women. Deoxypyridinoline (DPD) was measured in urinary samples from pregnant and nonpregnant women using ELISA kits (Quidel Corporation, Athens, OH) and was subsequently expressed on the basis of creatinine concentrations.

Bone formation markers

Osteocalcin (OC) was quantified in plasma samples from pregnant and nonpregnant women using ELISA kits (R&D Systems, Minneapolis, MN), while alkaline phosphatase (ALP) was measured in serum samples from pregnant and nonpregnant women using an automated chemistry analyzer (Dimension Xpand Plus; Siemens Healthcare Diagnostics).

Serum calcium and phosphorus

An automated chemistry analyzer was used to measure serum total calcium, albumin, and phosphorus. Then, serum calcium concentrations were corrected for albumin concentrations.

Biochemical markers of bone turnover in neonates (cord blood)

CTx and OC were measured in cord plasma obtained at delivery using ELISA kits mentioned above.

Genotyping in pregnant and nonpregnant women

Genotypes of three SNPs in the vitamin D binding protein gene (i.e., *GC* rs7041) and 1-alpha-hydroxylase gene (i.e, *CYP2R1* rs12794714 and *CYP2R1* rs10741657) that are known to be associated with 25(OH)D concentrations (33) were determined among pregnant and nonpregnant women as previously described (27).

Statistical analysis

Data were analyzed by JMP Pro 11 (SAS Institute, Inc., Cary, NC). Differences in demographic characteristics and concentrations of vitamin D biomarkers between pregnant and nonpregnant women were assessed using independent *t*-tests (normally distributed continuous variables); Wilcoxon Rank Sum tests (non-normally distributed continuous variables); and Chi-squared or Fisher's exact tests (categorical variables). Changes in vitamin D biomarkers throughout the study within each group were tested using paired *t*-tests except for the change in 3-epi-25(OH)D_3 by Wilcoxon Signed-Rank test due to non-normal distribution. Because of different statistical approaches to assess vitamin D biomarkers, values reported in **Table 3.1** are slightly different from the values in our previous report (27).

Linear mixed models (LMMs) were used to examine differences/changes in bone turnover markers between the two groups at baseline and study-end. Reproductive state and time (i.e., baseline and study-end) were included as fixed factors, while participant identifier was entered as

a random factor. Initial models included several covariates such as age, ethnicity/race, BMI (pre-pregnancy or baseline), education, prestudy multivitamin supplement use, season at study entry, genetic variants in vitamin D metabolism, and choline intake (480 or 930 mg/d). Covariates achieving statistical significance ($P < 0.05$) were retained in the final models. Bonferroni corrections were performed for post hoc comparisons between pregnant and nonpregnant groups at each study time point, and between baseline and study-end within each group.

The relationships of maternal vitamin D biomarkers with maternal and fetal bone turnover markers were also assessed using LMMs. Candidate covariates for initial models included the variables mentioned above along with variables related to gestation and neonatal anthropometric outcomes [i.e., gestational age at birth, gestational weight gain, mode of delivery, parity, season at birth, neonate's gender, birthweight, head circumference, and length (**Supplemental Table S3.1**). Final models retained the covariates that achieved statistical significance ($P < 0.05$). LMMs were also used to assess differences in each bone marker between pregnant women and their fetuses.

In all analyses, data that did not satisfy the normality and homogeneity of variance criteria were log (ln)-transformed, and influencing data that had studentized residuals > 3 were excluded (i.e., two ALP values). When covariates were retained in final models, data are presented as predicted means. *P* values < 0.05 for two-tailed tests were considered statistically significant.

Table 3.1
Participant characteristics and concentrations of blood vitamin D biomarkers[1,2]

	Pregnant (n = 26)	Nonpregnant (n = 21)	P-value
Age, y	28 ± 3	29 ± 5	0.791
Pre-pregnancy or baseline BMI, kg/m^2	23.7 ± 3.1	23.5 ± 2.8	0.813
Ethnicity, *n*			0.716
White	16	14	
Non-White	10	7	
Multivitamin supplement uses before study entry, *n*			< 0.001
Yes	22	7	
No	4	14	
CYP2R1 rs10741657 A>G polymorphism, *n*			0.012
AA	2	2	
AG	20	8	
GG	4	11	
CYP2R1 rs12794714 A>G polymorphism, *n*			0.153
AA	5	6	
AG	20	11	
GG	1	4	
GC rs7041 G>T polymorphism, *n*			0.873
GG	9	6	
GT	11	9	
TT	6	6	
Season at study entry, *n*			0.920
April–September	14	11	
October–March	12	10	
Serum 25(OH)D, nmol/L			
Baseline	88.7 ± 28.5	63.9 ± 24.7	0.004
Study-end	97.8 ± 32.1*	78.3 ± 25.3**	0.028

Table 3.1 (Continued)

Plasma $1,25(OH)_2D$, geometric means (95% CIs), pmol/L			
Baseline	283 (232, 344)	151 (129, 178)	< 0.001
Study-end	303 (252, 364)	163 (139, 191)	< 0.001
Plasma $24,25(OH)_2D$, geometric means (95% CIs), nmol/L			
Baseline	9.6 (7.5, 12.4)	9.1 (6.7, 12.5)	0.784
Study-end	11.4 (9.5, 13.8)	11.7 (9.2, 15.0)**	0.867
Plasma DBP, geometric means (95% CIs), μg/mL			
Baseline	405 (319, 515)	204 (164, 254)	< 0.001
Study-end	370 (291, 470)**	205 (166, 255)	< 0.001
Serum 3-epi-$25(OH)D_3$, nmol/L			
Baseline	3.2 ± 2.1	1.9 ± 1.2	0.018
Study-end	4.5 ± 3.6*	2.5 ± 2.2	0.035
Free 25(OH)D, geometric means (95% CIs), pmol/L			
Baseline	16.0 (12.3, 20.8)	19.5 (14.9, 25.5)	0.291
Study-end	19.2 (15.1, 24.4)*	25.7 (20.8, 31.7)*	0.074

[1] Data are presented as means ± SDs, unless otherwise indicated. The *P* value in each row indicates a significant difference between pregnant and nonpregnant women. An asterisk beside a vitamin D biomarker indicates a significant difference between baseline and study-end within either pregnant or nonpregnant women: *, $P < 0.05$. **, $P < 0.01$.

[2] *CYP2R1*, 25-hydroxylase gene; DBP, vitamin D binding protein; 3-epi-$25(OH)D_3$, C3 epimer of 25-hydroxyvitamin D_3; *GC*, vitamin D binding protein gene; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D.

RESULTS

Participant characteristics and concentrations of blood vitamin D biomarkers

Demographic variables and vitamin D measures of the study participants (26 pregnant and 21 nonpregnant women) are presented in Table 3.1. Briefly, no differences between pregnant and nonpregnant groups were detected in age, pre-pregnancy/baseline BMI, ethnicity, or season at study entry, whereas a greater portion of pregnant women had taken multivitamin supplements as compared to nonpregnant women prior to study entry. Pregnant women had higher 25(OH)D, $1,25(OH)_2D$, DBP, and 3-epi-$25(OH)D_3$ concentrations than nonpregnant women at baseline and study-end. Conversely, $24,25(OH)_2D$ and free 25(OH)D concentrations did not differ between pregnant and nonpregnant women at either study time point. Over the course of the study (i.e., baseline to study-end), 25(OH)D, 3-epi-$25(OH)D_3$, and free 25(OH)D increased, and $24,25(OH)_2D$ tended to increase (P = 0.057) among pregnant women. Increases in 25(OH)D, $24,25(OH)_2D$ and free 25(OH)D were observed among nonpregnant women.

Associations of maternal vitamin D biomarkers with bone turnover markers in pregnant women and their fetuses

Serum 25(OH)D was not associated with bone turnover markers at either baseline or study-end. However, increases in serum 25(OH)D across the study period (i.e., concentration at study-end – concentration at baseline) were associated negatively with maternal OC and DPD/Cr at study-end, and positively with fetal OC at delivery (**Table 3.2**). For $1,25(OH)_2D$, positive associations were observed at baseline and study-end with maternal OC and DPD/Cr, whereas negative associations were observed at these study time points with maternal NTx (Table 3.2). No associations with $24,25(OH)_2D$ and maternal or fetal bone markers were detected at baseline.

However, study-end 24,25$(OH)_2$D was positively related to fetal CTx at delivery, and tended to be negatively related to study-end maternal NTx (Table 3.2). Baseline free 25(OH)D was associated negatively with baseline maternal iPTH and ALP, and positively with fetal CTx (Table 3.2). In addition, study-end free 25(OH)D was negatively related to study-end maternal ALP and NTx (Table 3.2). Finally, baseline 3-epi-25$(OH)D_3$ was negatively associated with study-end maternal ALP, while study-end 3-epi-25$(OH)D_3$ was negatively related to study-end maternal DPD/Cr and ALP (Table 3.2). Moreover, the increase in 3-epi-25$(OH)D_3$ through time was negatively associated with study-end maternal NTx (Table 3.2).

Table 3.2
Associations of maternal vitamin D biomarkers with biochemical markers of bone turnover in mothers (n = 26) and their fetuses (n = 23)[1-3]

Maternal vitamin D markers		R^2	β (95% CI)	*P*	Significant Covariates[4,5]
	Maternal bone markers at baseline				
$1,25(OH)_2D$ at baseline	NTx	0.30	−0.002 (−0.0043, −5.0e-6)	0.050	BMI
$1,25(OH)_2D$ at baseline	DPD/Cr	0.38	0.001 (0.0004, 0.002)	0.010	rs10741657
$1,25(OH)_2D$ at baseline	OC	0.34	0.003 (0.0003, 0.006)	0.035	Season, rs10741657
Free 25(OH)D at baseline	iPTH	0.54	−0.020 (−0.040, −0.001)	0.040	Education, rs10741657
Free 25(OH)D at baseline	ALP	0.31	−0.012 (−0.023, −0.0005)	0.042	rs7041
	Maternal bone markers at study-end				
Δ 25(OH)D[6]	DPD/Cr	0.49	−0.009 (−0.016, −0.002)	0.020	Age, rs10741657
Δ 25(OH)D[6]	OC	0.74	−0.031 (−0.046, −0.017)	< 0.001	BMI, gestational weight gain, rs10741657, rs12794714
$1,25(OH)_2D$ at baseline	DPD/Cr	0.60	0.001 (0.0001, 0.002)	0.033	Season, age, rs10741657
$1,25(OH)_2D$ at baseline	OC	0.46	0.004 (0.002, 0.007)	0.002	Season, rs10741657
$1,25(OH)_2D$ at study-end	NTx	0.31	−0.002 (−0.003, 4.4e-5)	0.056	BMI
$24,25(OH)_2D$ at study-end	NTx	0.68	−0.061 (−0.126, 0.004)	0.063	Ethnicity, gestational weight gain, rs10741657
Free 25(OH)D at study-end	NTx	0.30	−0.018 (−0.038, 0.001)	0.062	BMI
Free 25(OH)D at study-end	ALP	0.90	−0.011 (−0.018, −0.004)	0.004	BMI, education, infant weight, infant length, rs7041
3-epi-$25(OH)D_3$ at baseline	ALP	0.92	−0.078 (−0.119, −0.037)	0.001	BMI, ethnicity, season, infant weight, infant length, mode of delivery

Table 3.2 (Continued)

3-epi-25(OH)D_3 at study-end	DPD/Cr	0.56	−0.040 (−0.075, −0.004)	0.031	Age, infant length
3-epi-25(OH)D_3 at study-end	ALP	0.49	−0.058 (−0.093, −0.023)	0.002	BMI
Δ 3-epi-25(OH)D_3[6]	NTx	0.72	−0.104 (−0.020, −0.007)	0.037	BMI, rs10741657, rs7041, infant weight, infant head circumference
	Fetal bone markers at delivery				
Δ 25(OH)D[6]	OC	0.83	0.042 (0.015, 0.068)	0.005	BMI, gestational weight gain, rs10741657, rs7041
24,25$(OH)_2$D at study-end	CTx	0.70	0.031 (0.007, 0.055)	0.018	BMI, ethnicity, gestational weight gain, infant weight, rs10741657, prestudy supplement use
Free 25(OH)D at baseline	CTx	0.53	0.008 (0.001, 0.015)	0.021	Gestational weight gain, infant weight, infant length, rs10741657

[1]Data were derived using linear mixed models which controlled for covariates.
[2]All biochemical markers of bone turnover were log(ln)-transformed except for fetal CTx.
[3]None of these associations achieved statistical significance among nonpregnant women.
[4]Influential covariates which achieved statistical significance ($P < 0.05$) were retained in the final model of each outcome.
[5]Two intake levels of dietary choline of this study did not achieve a statistical significance, and consequently, were not included in any of the final models.
[6]Calculated by subtracting baseline values from study-end values.

Effect of pregnancy on biochemical markers of bone turnover and their response through time

Plasma intact parathyroid hormone

Pregnant women had lower (P = 0.005) iPTH concentrations than nonpregnant women at baseline (**Figure 3.1**A). Reproductive state did not interact with time (P = 0.533) to affect circulating iPTH concentrations. However, iPTH concentrations increased by a mean of 38% (P = 0.054) among pregnant women throughout the study, but not among nonpregnant women whose concentrations remained stable (P = 0.594; Figure 3.1A). Similar to baseline, iPTH concentrations were 31% lower (P = 0.043) among pregnant women (18 pg/mL) than nonpregnant women (26 pg/mL) at study-end after adjustment for covariates (reproductive state, time, and *CYP2R1* rs12794714) (Figure 3.1A).

Plasma carboxy-terminal cross-linking telopeptide of type 1 collagen

CTx concentrations were not different (P > 0.9) between pregnant and nonpregnant women at baseline (Figure 3.1B). Reproductive state interacted with time (P < 0.001) to influence circulating CTx response. Specifically, pregnant women experienced a mean 55% increase in CTx (P < 0.001) throughout the study period (Figure 3.1B), whereas no change (P > 0.9) in CTx was detected in nonpregnant women. Consequently, pregnant women had 50% higher (P = 0.003) CTx concentrations (0.61 ng/mL) than nonpregnant women (0.40 ng/mL) at study-end after adjustment for covariates (reproductive state and time) (Figure 3.1B).

Plasma amino-terminal cross-linking telopeptide of type 1 collagen

Pregnant women had higher (P = 0.006) NTx concentrations than nonpregnant women at baseline (Figure 3.1C). Reproductive state interacted with time (P = 0.006) to affect circulating NTx concentrations, with a mean 28% increase (P < 0.001) observed among pregnant women (Figure 3.1C), but not among nonpregnant women (P = 0.459). Similar to baseline, NTx concentrations in pregnant women (62 ng/mL) were 150% higher (P < 0.001) than nonpregnant women (25 ng/mL) at study-end after adjustment for covariates (reproductive state, time, *CYP2R1* rs12794714, season, BMI, and education) (Figure 3.1C).

Urinary deoxypyridinoline / creatinine

Urinary DPD/Cr values were not different (P = 0.136) between pregnant and nonpregnant women at baseline (Figure 3.1D). Reproductive state interacted with time (P < 0.001) to influence DPD/Cr. Pregnant women exhibited a mean 59% increase (P < 0.001) in DPD/Cr, although no change (P > 0.9) was detected in nonpregnant women (Figure 3.1D). As a result, pregnant women showed approximately 100% higher (P < 0.001) DPD/Cr (8.6 nmol/mmol) than nonpregnant women (4.5 nmol/mmol) at study-end after adjustment for covariates (reproductive state and time) (Figure 3.1D).

Plasma osteocalcin

Pregnant women had lower (P = 0.005) OC concentrations that nonpregnant women at baseline (Figure 3.1E). Reproductive state interacted with time (P = 0.007) to influence circulating OC concentrations. Specifically, nonpregnant women experienced a mean 50% reduction (P < 0.001) in OC throughout the study period, whereas OC remained stable among pregnant women

($P = 0.331$). At study-end, there was no difference ($P = 0.267$) between pregnant (4.7 ng/mL) and nonpregnant (5.9 ng/mL) women after adjustment for covariates (reproductive state and time) (Figure 3.1E).

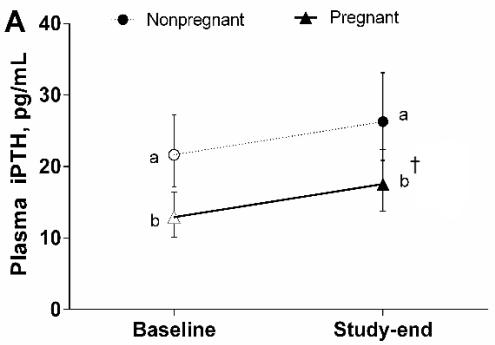
Serum alkaline phosphatase

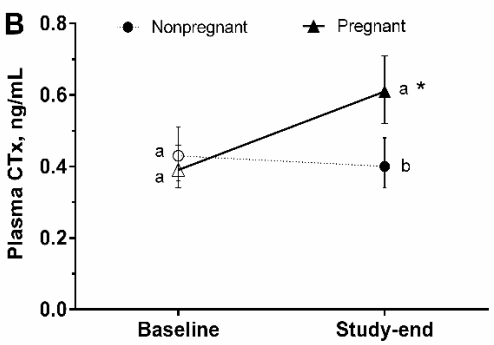
Pregnant women had higher ($P < 0.001$) ALP concentrations than nonpregnant women at baseline (Figure 3.1F). Reproductive state interacted with time ($P < 0.001$) to affect circulating ALP response. ALP concentrations among pregnant women more than doubled ($P < 0.001$) over the course of the study (Figure 3.1F), whereas nonpregnant women showed stable concentrations ($P = 0.705$). Similar to baseline, pregnant women (186 U/L) had 240% higher ($P < 0.001$) ALP concentrations than nonpregnant women (54 U/L) at study-end after adjustment for covariates (reproductive state, time, and *GC* rs7041) (Figure 3.1F).

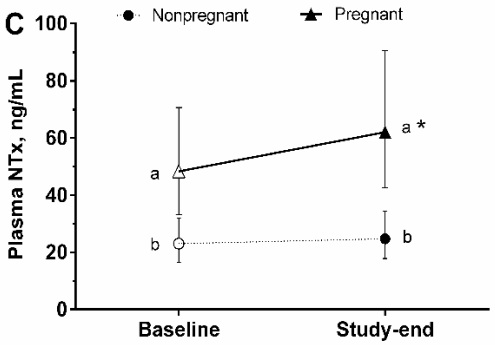
Albumin-adjusted Serum Calcium and Phosphorus

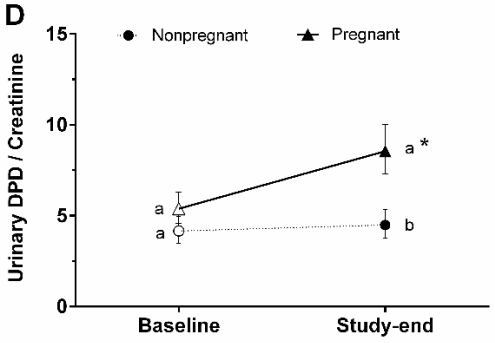
Pregnant women showed higher ($P < 0.001$) albumin-adjusted serum calcium concentrations (Figure 3.1G) and non-different ($P = 0.646$) phosphorus concentrations (Figure 3.1H) as compared to nonpregnant women at baseline. Reproductive state did not interact with time ($P \geq 0.202$) to influence albumin-adjusted serum calcium and phosphorus concentrations. However, pregnant women experienced a significant increase ($P = 0.002$) in albumin-adjusted serum calcium through time (Figure 3.1G),whereas albumin-adjusted serum calcium remained stable ($P > 0.452$) among nonpregnant women. While no change ($P = 0.545$) in serum phosphorus occurred among pregnant women, a significant increase ($P = 0.030$) in phosphorus was detected in nonpregnant women over the course of the study (Figure 3.1H). Similar to baseline, pregnant

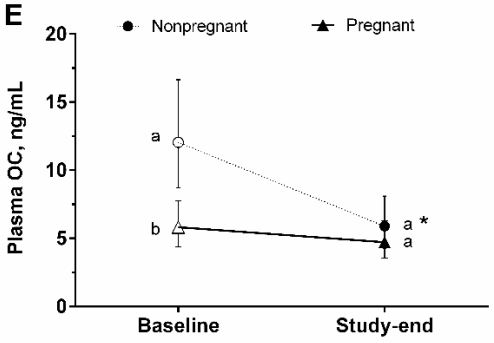
women showed higher ($P < 0.001$) albumin-adjusted serum calcium concentrations than nonpregnant women at study-end after adjustment for covariates (reproductive state and time) (Figure 3.1G), whereas phosphorus did not differ ($P = 0.084$) between the two groups after adjustment for covariates (reproductive state and time) (Figure 3.1H).

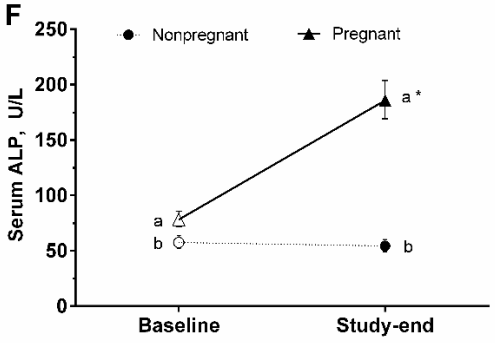

A
Nonpregnant
Pregnant
Plasma iPTH, pg/mL
40
30
20
10
0
a
a
b
b †
Baseline
Study-end

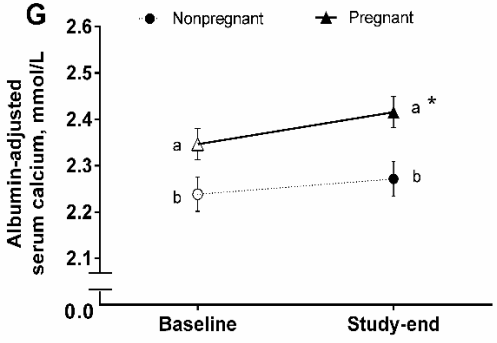

B
Nonpregnant
Pregnant
Plasma CTx, ng/mL
0.8
0.6
0.4
0.2
0.0
a
a
a *
b
Baseline
Study-end


C
Nonpregnant
Pregnant
Plasma NTx, ng/mL
100
80
60
40
20
0
a
b
a *
b
Baseline
Study-end


D
Nonpregnant
Pregnant
Urinary DPD / Creatinine
15
10
5
0
a
a
a *
b
Baseline
Study-end


E
Nonpregnant
Pregnant
Plasma OC, ng/mL
20
15
10
5
0
a
b
a *
a
Baseline
Study-end


F
Nonpregnant
Pregnant
Serum ALP, U/L
250
200
150
100
50
0
a
b
a *
b
Baseline
Study-end


G
Nonpregnant
Pregnant
Albumin-adjusted serum calcium, mmol/L
2.6
2.5
2.4
2.3
2.2
2.1
0.0
a
b
a *
b
Baseline
Study-end

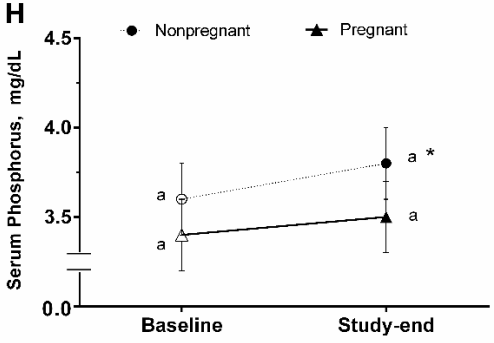

H
Nonpregnant
Pregnant
Serum Phosphorus, mg/dL
4.5
4.0
3.5
0.0
a
a
a *
a
Baseline
Study-end

Figure 3.1. Biochemical markers of bone turnover in pregnant and nonpregnant women consuming equivalent and adequate amounts of vitamin D, calcium, and phosphorus for 10 weeks. Values are predicted means after adjusted for covariates. Baseline values are represented as open shapes, while study-end values are represented as closed shapes. Different letters (i.e., a > b > c) indicate significant differences ($P < 0.05$) in bone markers between pregnant and nonpregnant groups at each study time point. An asterisk (*) indicates a significant change ($P < 0.05$) in the concentrations of bone markers within each group throughout the study, and a long cross symbol (†) indicates a borderline significant change ($P = 0.054$). **(A)** iPTH, intact parathyroid hormone; **(B)** CTx, carboxy-terminal cross-linking telopeptide of type 1 collagen; **(C)** NTx, amino-terminal cross-linking telopeptide of type 1 collagen; **(D)** Urinary DPD/Creatinine, urinary deoxypyridinoline/creatinine; **(E)** OC, osteocalcin; **(F)** ALP, alkaline phosphatase; **(G)** Albumin-adjusted serum calcium; **(H)** Phosphorus.

Comparison of fetal bone markers to maternal markers

Fetal OC concentrations at delivery (33.5 ng/mL) were 6-7 times higher ($P < 0.001$) than OC concentrations in pregnant women at baseline (5.8 ng/mL) and study-end (4.7 ng/mL) (**Figure 3.2**A). Similarly, fetal CTx concentrations (0.77 ng/mL) were 30-100% higher ($P \leq 0.034$) than CTx concentrations in pregnant women at baseline (0.39 ng/mL) and study-end (0.61 ng/mL) (Figure 3.2B).

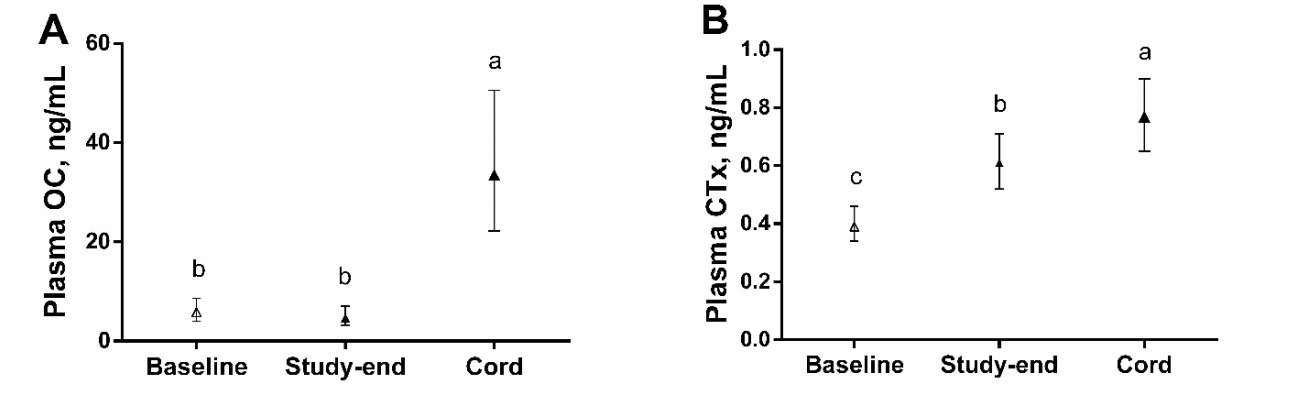


Figure 3.2. (A, B) Bone turnover markers, OC and CTx, in pregnant women (baseline and study-end) and their fetuses (delivery). Values are means (95% CIs). Different letters (i.e., a > b > c) indicate significant differences ($P < 0.05$) in OC and CTx between pregnant women and their fetuses.

DISCUSSION

To the best of our knowledge, this is the first controlled feeding study to assess associations of vitamin D biomarkers with bone turnover during pregnancy. Participants consumed equivalent amounts of vitamin D along with calcium and phosphorus which can influence bone metabolism during pregnancy (19,34). The intake levels of all essential nutrients aligned with, or exceeded, current dietary recommendations (1). Our findings show associations between several vitamin D biomarkers and a panel of biochemical markers of maternal and fetal bone metabolism. Moreover, our data suggest that achieving higher concentrations of maternal vitamin D biomarkers including 25(OH)D (> 90 nmol/L) across the last third of pregnancy may be a nutritional strategy for reducing pregnancy-induced maternal bone loss.

Higher maternal concentrations of vitamin D biomarkers are associated with reduced maternal bone resorption

We found that increases in serum 25(OH)D across the third trimester of pregnancy were associated with decreases in bone turnover markers (i.e., DPD/Cr and OC). In addition, higher concentrations of 24,25$(OH)_2$D, which rise in parallel with 25(OH)D, were associated with lower NTx concentrations among pregnant women at study-end (near term) although statistical significance was not achieved ($P = 0.063$). This latter finding is consistent with previous studies in vitamin D-replete animals showing reduced bone resorption during the administration of pharmacological doses of 24,25$(OH)_2$D (35,36). Of note, free 25(OH)D (but not protein-bound 25(OH)D) was negatively associated with PTH in pregnant women at baseline, suggesting that low concentrations of free 25(OH)D may increase PTH secretion, possibly resulting in greater calcium loss from maternal bone at the beginning of the third trimester. Furthermore, lower

concentrations of free 25(OH)D at study-end were also associated with higher study-end concentrations of the bone resorption marker NTx. Although $1,25(OH)_2D$ was positively associated with DPD/Cr throughout the study period, it was also negatively related to NTx. In addition, higher $1,25(OH)_2D$ concentrations were associated with higher OC concentrations at both baseline and study-end.

Overall, data from these functional bone health outcomes suggest that higher concentrations of maternal vitamin D biomarkers including 25(OH)D (> 90 nmol/L) might reduce calcium mobilization from maternal bone. However, while this reduction may be of benefit to maternal bone, it raises questions as to the adequacy of calcium supply to the developing fetus. Notably, albumin-adjusted serum calcium concentrations increased throughout the third trimester of our pregnant participants, and this increase was not influenced by any of the biomarkers of vitamin D status even after adjustments. These findings suggest that net calcium transfer from the maternal to the fetal compartment was uncompromised by vitamin D-associated declines in maternal bone resorption. A recent randomized controlled trial in Iran that administered a high vitamin D dose (2,000 IU) throughout the third trimester (37) reported no difference in maternal bone measurements between the supplement and placebo groups. However, the supplementation group showed low serum 25(OH)D concentrations (mean of 45 nmol/L) over the course of the study, indicating that the high dose of vitamin D supplementation did not yield sufficient vitamin D status among the pregnant women. These data collectively suggest that concentrations of vitamin D biomarkers [e.g., 25(OH)D], rather than the assigned vitamin D dose, might be an important discriminatory factor when ascertaining the effects of maternal vitamin D on bone health.

Maternal vitamin D biomarkers are associated with fetal bone metabolism

Increases in maternal serum 25(OH)D across the third trimester were associated with increases in fetal OC. This finding suggests that achieving higher maternal 25(OH)D concentrations during pregnancy may promote bone formation in fetal skeleton. Maternal 25(OH)D readily crosses the placenta (38); thus greater transfer of 25(OH)D to the fetal compartment could enhance fetal OC synthesis by raising fetal $1,25(OH)_2D$ which is known to stimulate OC production (39). Alternatively, because achieving higher maternal 25(OH)D concentrations was coincidentally associated with lower maternal OC levels among pregnant women in this study, it is also possible that maternal 25(OH)D has a putative a role in the transplacental movement of maternal OC, which has been shown to cross the placenta (40).

In addition to bone formation, mineralization of the fetal skeleton encompasses bone resorption. In the present study, maternal free 25(OH)D and $24,25(OH)_2D$ were positively associated with fetal CTx, a marker of bone resorption. Thus, a higher maternal vitamin D status appears to promote fetal bone turnover (i.e., bone formation + bone resorption) which may be favorable when mineralization of the fetal skeleton is accelerated. However, due to a lack of calcium and other bone resorption marker measurements in cord blood as well as amniotic fluid, we cannot exclude the possibility that higher fetal bone resorption may be indicating fetal hypocalcemia and fetal skeleton demineralization. Nonetheless, this appears unlikely within the context of this study because fetal demineralization resulting from elevated fetal bone resorption generally transpires secondary to diminished maternal calcium transfer.

In contrast to the other vitamin D biomarkers, maternal 3-epi-$25(OH)D_3$ did not show any relationship with bone markers in fetuses, suggesting that maternal 3-epi-$25(OH)D_3$ may not influence fetal bone metabolism. Unlike 25(OH)D, 3-epi-$25(OH)D_3$ may not readily cross the

placenta to the fetus, and consequently, would not participate in fetal bone development. Indeed, no significant correlation was detected between maternal and fetal 3-epi-25(OH)D_3 in a recently published study (41).

Our findings of associations between maternal vitamin D biomarkers and fetal markers of bone metabolism appear to be inconsistent with two recent randomized controlled trials (42,43) which demonstrated no effects of maternal vitamin D_3 supplementation (1,000 IU/d (43) and 200 IU/d (42)) on fetal bone measurements as compared to a placebo group. The reasons for these inconsistencies are unclear but may be due to differences in the intake of bone-related nutrients. For example, dietary calcium was not reported in one study (43) and the supplementation group had very low vitamin D intake (234 IU/d from food and the supplement) in the other study (42). In addition, genetic variants that might alter bone metabolism were not assessed in either study which may obscure study findings. For example, in the present study, *CYP2R1* rs10741657 A > G polymorphism emerged as the most common significant covariate in the linear mixed models examining associations between vitamin D biomarkers and maternal/fetal bone turnover markers. In addition, others have reported that polymorphisms in the *VDR* gene influenced changes in bone measurements among adolescent pregnant women consuming supplemental vitamin D and calcium (44).

Pregnancy induced a net negative balance of bone metabolism even under conditions of sufficient intake of bone-related nutrients

All of the bone resorption markers (i.e., CTx, NTx, and urinary DPD/Cr) increased by 28-60% throughout the third trimester of pregnancy and were 50-150% higher than those of nonpregnant women consuming equivalent amounts of bone-related nutrients, even after

controlling for another confounding factors such as BMI, ethnicity/race, season, and vitamin D-related genetic variants. In contrast, concentrations of the bone formation marker OC did not change among pregnant women, nor did it differ from those of nonpregnant women at study-end, which aligns with prior work (45–47). This finding of stable and non-different OC concentrations among pregnant women remained ($P > 0.9$) even after removing the effects of hemodilution by adjusting for albumin concentrations. Further, although ALP increased and remained higher among pregnant women, this increase is partly attributable to the placental contribution and may not be an accurate reflection of maternal bone formation. Overall, our findings are consistent with other longitudinal studies that have reported high levels of bone resorption among third-trimester women (45–48).

In agreement with some (47,49) but not all (50,51) previous work, pregnant women maintained lower PTH concentrations than nonpregnant women throughout their third trimester. Moreover, although PTH is well-known for its role in stimulating the production of $1,25(OH)_2D$ in nonpregnant state, $1,25(OH)_2D$ was not associated ($P \geq 0.293$) with PTH at any study time-point among pregnant women. Taken together, the divergent relationship between these hormones supports prior work indicating that $1,25(OH)_2D$ is not under the influence of PTH during pregnancy (52).

Study limitations

This feeding study provided a single dose of vitamin D and thus prohibited comparisons among dosing levels. In addition, the study sample size was relatively small and may have reduced the statistical power of our study. Finally, bone mass parameters, indicative of quantitative bone changes, were not obtained.

Conclusions

Overall data from the present study suggest that higher concentrations of maternal vitamin D biomarkers including 25(OH)D (> 90 nmol/L) might have functional benefits for pregnant women by reducing maternal bone resorption without compromising fetal calcium supply. Large-scale randomized controlled trials with higher doses of vitamin D supplementation are needed to confirm the potential functional benefits (i.e., less reduction in maternal bone mass) within the context of adequate dietary calcium and phosphorus intake.

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Supplemental Table S3.1. Gestational and neonatal outcomes of the third-trimester pregnant women[1]

	Pregnant women (n = 26)
Choline intake (480 or 930 mg choline/d)	13 / 13
Gestational age at birth (weeks)	40.0 [39.8 – 40.0]
Gestational weight gain (kg)	15.4 [12.1 – 18.6]
Mode of delivery (Vaginal / Caesarean Section, n)	19 / 5
Parity (Primiparas / Multiparas, n)	12 / 14
Season at birth (April–September / October–March, n)	18 / 4
Infant gender (Boy / Girl, n)	19 / 7
Infant birth weight (kg)	3.4 $\pm$ 0.3
Infant head circumference (cm)	34.2 $\pm$ 1.1
Infant length (cm)	50.8 [49.2 – 52.1]

[1]Data are presented as mean$\pm$SDs for infant birth weight and infant head circumference; medians [IQRs] for gestational age at birth, gestational weight gain, and infant length; and sample size number.

AFTERWORD

Maternal vitamin D inadequacy, a global health problem, increases the risk of adverse pregnancy outcomes. Yet, many aspects of vitamin D metabolism and requirements in human pregnancy are poorly understood. My dissertation research, which experimentally controlled intakes of vitamin D and related nutrients, advances our fundamental understanding of vitamin D metabolism and requirements in three major ways.

First, the data showed that pregnant women had higher circulating vitamin D metabolites and other indicators of vitamin D status than nonpregnant women under dietary conditions of equivalent vitamin D intake. Of note, these differences remained after adjusting for several confounding factors, suggesting that pregnancy accommodates the demands for vitamin D by increasing the maternal circulating pool of vitamin D in a manner that is independent of dietary intake, season, BMI, and ethnicity/race. Second, the data provide evidence in support of an active role of the placenta in modulating maternal vitamin D metabolism and status. For example, numerous significant associations of circulating vitamin D metabolites with placental vitamin D metabolites and placental mRNA abundance of vitamin D machinery were observed. Moreover, the data from the HTR-8/SVneo cell culture model demonstrated for the first time an ability of human placental trophoblasts to produce and secrete $25(OH)D_3$, illustrating the placenta as a source of all forms of the major vitamin D metabolites [i.e., 25(OH)D, $1,25(OH)_2D$, and $24,25(OH)_2D$]. Lastly, the data also showed that several of the vitamin D biomarkers were inversely associated with bone resorption markers among pregnant women who consumed adequate amounts of calcium and phosphorus. Importantly, these vitamin D-associated declines in maternal bone resorption were not accompanied by a fall in albumin-adjusted serum calcium concentrations, indicating that calcium delivery to fetus was not compromised.

Overall, these data are anticipated to inform future establishment of vitamin D recommendations for pregnant women that promote optimal maternal health including bone protection. Additional studies are needed that administer higher or lower vitamin D intakes than the intake provided in this study and include a period of early pregnancy.

APPENDIX

Pregnant Women
Health History Questionnaire

Demographic Information

Date: _____
Month Day Year

Name: _____
First Last

Phone _____ _____ _____
Home Cell Email

Address: _____

_____

_____

Age: _____ Date of Birth: _____
Mo. Day Year

Education Completed:

_____High School Graduate _____Master's Degree

_____Bachelor's Degree _____Doctoral Degree

Present Work/Student Status:

_____Working Full-time _____Full-time student
_____Working Part-time _____Part-time student
_____Not Employed _____Retired

Pregnancy Information

Gestational Age at Present: _____ (weeks)

Date of Last Menstrual Period: _____ (month/day/year)

Due Date: _____ (month/day/year)

Anthropometric Data

Height (without shoes) _____

Pre-Pregnancy weight (dressed with shoes) _____

Current weight (dressed with shoes) _____

Medical History

Indicate if you have had or currently have any of the following medical problems.
Check all that apply:

Yes	No	
___	___	Alcoholism
___	___	Cardiovascular Disease (Atherosclerosis / Heart Attack/Stroke)
___	___	Eye Problems (ie, glaucoma, cataracts, retinopathy)
___	___	Gall Bladder Disease
___	___	High Blood Cholesterol
___	___	High Blood Pressure
___	___	Intestinal Disorders
___	___	Kidney Disease
___	___	Liver Disease
___	___	Lung Disease

___ ___ Neurologic/Seizure Disorder

___ ___ Obesity / Overweight

___ ___ Thyroid Disease

___ ___ Tumors / Cancer – List type: _____

___ ___ Ulcers

___ ___ Other – Specify: _____

___ ___ Recent surgeries? If yes, specify: _____

Indicate the prescription/nonprescription medicines you currently use on a regular basis. Check all that apply:

___ Allergy Medicines/Antihistamines ___ Antacids

___ Antibiotics ___ Anti-arrhythmics

___ Anti-inflammatory Agents (i.e. Ibuprofen) ___ Aspirin

___ Asthma Medicines ___ Beta Blockers

___ Blood Pressure Medicines ___ Blood Thinners

___ Diabetes Medicines – Specify: _____

___ Diuretics ___ Gout Medicines

___ Heart Medicines – Specify: _____

___ Hormones – Specify: _____

___ Seizure Medicines ___ Thyroid Medicines

___ Medicines interfering with folate metabolism – Specify: _____

___ Other – Specify: _____

Supplement Use

Indicate the supplements you currently use on a regular basis.
Check all that apply.

___ Prenatal Supplement (name/brand)

_____

___ Multivitamin/Minerals ___ Herbs

___ Amino acids ___ Ergogenic Aids (i.e., creatine)

___ Other-Specify: _____

Activity Status

What would best describe your activity level?

____ Level 1: Usual daily activities
____ Level 2: Some form of exercise (i.e., walking, dancing, riding a bike) at least 3 times per week
____ Level 3: Vigorous exercise (i.e., singles tennis, running, aerobics, weight lifting) at least 3 times/ week.

Beverage Consumption

Indicate the amount and frequency with which you consume the following at present:

	Amt. (each)	Never/ Rarely (5x/yr)	Occas. (2-3x/mo.)	Freq. (2-3x/wk.)	Daily
Beer	_____	_____	_____	_____	_____
Liquor	_____	_____	_____	_____	_____
Wine	_____	_____	_____	_____	_____
Wine Coolers	_____	_____	_____	_____	_____
Coffee	_____	_____	_____	_____	_____
Tea	_____	_____	_____	_____	_____
Soda	_____	_____	_____	_____	_____

Lactating Women
Health History Questionnaire

Demographic Information

Date: _____
Month Day Year

Name: _____
First Last

Phone _____ _____ _____
Home Cell Email

Address: _____

_____

_____

Age: _____ Date of Birth: _____
Mo. Day Year

Education Completed:

_____High School Graduate _____Master's Degree

_____Bachelor's Degree _____Doctoral Degree

Present Work/Student Status:

_____Working Full-time _____Full-time student
_____Working Part-time _____Part-time student
_____Not Employed _____Retired

Pregnancy Information

Due Date: _____ (month/day/year)

Delivery Date: _____ (month/day/year)

Health Insurance Provider: _____

Total Weight Gained During Pregnancy: _____

Complications During Pregnancy: _____ Yes _____No

If yes, describe:

_____
_____
_____
_____

Complications During Labor: _____Yes _____No

If yes, describe:

_____
_____
_____
_____

Newborn Information

Date of Delivery: _____
Month Day Year

Mode of Delivery (i.e., vaginal or C-section): _____

Gestational Age: _____

Length: _____

Weight: _____

Head Circumference: _____

Anthropometric Data

Height (without shoes) _____

Pre-Pregnancy weight (dressed with shoes) _____

Current weight (dressed with shoes) _____

Medical History

Indicate if you have had or currently have any of the following medical problems.
Check all that apply:

Yes	No	
___	___	Alcoholism
___	___	Cardiovascular Disease (Atherosclerosis / Heart Attack/Stroke)
___	___	Eye Problems (ie, glaucoma, cataracts, retinopathy)
___	___	Gall Bladder Disease
___	___	High Blood Cholesterol
___	___	High Blood Pressure
___	___	Intestinal Disorders
___	___	Kidney Disease
___	___	Liver Disease
___	___	Lung Disease
___	___	Neurologic/Seizure Disorder
___	___	Obesity / Overweight
___	___	Thyroid Disease
___	___	Tumors / Cancer – List type: _____

___ ___ Ulcers

___ ___ Other – Specify: _____

___ ___ Recent surgeries? If yes, specify: _____

Indicate the prescription/nonprescription medicines you currently use on a regular basis. Check all that apply:

___ Allergy Medicines/Antihistamines ___ Antacids

___ Antibiotics ___ Anti-arrhythmics

___ Anti-inflammatory Agents (i.e. Ibuprofen) ___ Aspirin

___ Asthma Medicines ___ Beta Blockers

___ Blood Pressure Medicines ___ Blood Thinners

___ Diabetes Medicines – Specify: _____

___ Diuretics ___ Gout Medicines

___ Heart Medicines – Specify: _____

___ Hormones (ie, birth control bills) – Specify: _____

___ Seizure Medicines ___ Thyroid Medicines

___ Medicines interfering with folate metabolism – Specify: _____

___ Other – Specify: _____

Supplement Use

Indicate the supplements you currently use on a regular basis.
Check all that apply.

___ Prenatal Supplement (name/brand)

_____

___ Multivitamin/Minerals

___ Herbs

___ Amino acids

___ Ergogenic Aids (i.e., creatine)

___ Other-Specify:

_____

Activity Status

What would best describe your activity level?

____ Level 1: Usual daily activities

____ Level 2: Some form of exercise (i.e., walking, dancing, riding a bike) at least 3 times per week

____ Level 3: Vigorous exercise (i.e., singles tennis, running, aerobics, weight lifting) at least 3 times/ week.

Non-Pregnant Women
Health History Questionnaire

Demographic Information

Date: _____
Month Day Year

Name: _____
First Last

Phone _____ _____ _____
Home Cell Email

Address: _____

_____

_____

Age: _____ Date of Birth: _____
Mo. Day Year

Education Completed:

_____High School Graduate _____Master's Degree

_____Bachelor's Degree _____Doctoral Degree

Present Work/Student Status:

_____Working Full-time _____Full-time student
_____Working Part-time _____Part-time student
_____Not Employed _____Retired

Number of Children: _____

Number of pregnancies: _____

Anthropometric Data

Height (without shoes) _____

Current weight (dressed with shoes) _____

Medical History

Indicate if you have had or currently have any of the following medical problems.
Check all that apply:

Yes	No	
___	___	Alcoholism
___	___	Cardiovascular Disease (Atherosclerosis / Heart Attack/Stroke)
___	___	Eye Problems (ie, glaucoma, cataracts, retinopathy)
___	___	Gall Bladder Disease
___	___	High Blood Cholesterol
___	___	High Blood Pressure
___	___	Intestinal Disorders
___	___	Kidney Disease
___	___	Liver Disease
___	___	Lung Disease
___	___	Neurologic/Seizure Disorder
___	___	Obesity / Overweight
___	___	Thyroid Disease
___	___	Tumors / Cancer – List type: _____
___	___	Ulcers
___	___	Other – Specify: _____
___	___	Recent surgeries? If yes, specify: _____

Indicate the prescription/nonprescription medicines you currently use on a regular basis.
Check all that apply:

___ Allergy Medicines/Antihistamines ___ Antacids

___ Antibiotics ___ Anti-arrhythmics

___ Anti-inflammatory Agents (i.e. Ibuprofen) ___ Aspirin

___ Asthma Medicines ___ Beta Blockers

___ Blood Pressure Medicines ___ Blood Thinners

___ Diabetes Medicines – Specify: _____

___ Diuretics ___ Gout Medicines

___ Heart Medicines – Specify: _____

___ Hormones – Specify: _____

___ Seizure Medicines ___ Thyroid Medicines

___ Medicines interfering with folate metabolism – Specify: _____

___ Other – Specify: _____

Supplement Use

Indicate the supplements you currently use on a regular basis.
Check all that apply.

___ Multivitamin/Minerals ___ Herbs

___ Amino acids ___ Ergogenic Aids (i.e., creatine)

___ Other-Specify:

_____

Activity Status

What would best describe your activity level?

____ Level 1: Usual daily activities
____ Level 2: Some form of exercise (i.e., walking, dancing, riding a bike) at least 3 times per week
____ Level 3: Vigorous exercise (i.e., singles tennis, running, aerobics, weight lifting) at least 3 times/ week.

Beverage Consumption

Indicate the amount and frequency with which you consume the following:

	Amt. (each)	Never/ Rarely (5x/yr)	Occas. (2-3x/mo.)	Freq. (2-3x/wk.)	Daily
Beer	_____	_____	_____	_____	_____
Liquor	_____	_____	_____	_____	_____
Wine	_____	_____	_____	_____	_____
Wine Coolers	_____	_____	_____	_____	_____
Coffee	_____	_____	_____	_____	_____
Tea	_____	_____	_____	_____	_____
Soda	_____	_____	_____	_____	_____

Consent, Pregnant Women

Cornell University
Informed Consent for Research Involving Human Study Participants

You are being invited to participate in a research study. This form is designed to provide you with information about this study. The Principal Investigator or representative will describe this study to you and answer any of your questions.

Project Title:

Effect of maternal choline intake on maternal/fetal biomarkers of choline status

Investigators:

Marie Caudill, PhD, RD; Associate Professor, Division of Nutritional Sciences, Cornell University

Eva Pressman, MD. Director of Obstetrics and Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York

Richard Canfield, PhD. Research Associate, Division of Nutritional Sciences, Cornell University

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Sandy Saintonge, MD. Assistant Professor of Clinical Public Health and Clinical Pediatrics, Weill Cornell Medical College, Attending Physician, New York Hospital Queens, Department of Emergency Medicine

Cydne Perry, PhD. Post-doctoral Associate, Division of Nutritonal Sciences, Cornell University

What the study is about:

Choline, a nutrient found in the diet (i.e., eggs, nuts, and beef), was recently recognized as a required nutrient for humans. The recommended dietary intake level for pregnant women is 450 mg/d. However, at present, it is not known whether this amount is optimal for pregnant women.

Thus, this study seeks to assess the effect of pregnancy on choline status and to examine whether the current recommended dietary intake, 450 mg/d, is enough to optimize choline status during pregnancy.

There is evidence from animal studies that excess choline consumed by the mother during pregnancy may have long lasting beneficial effects on the memory and attention of the newborn. However, no study has examined this in humans.

Thus, this study seeks to assess the effect of extra choline (450 mg/d) for a total choline intake of 900 mg/d during pregnancy on learning, attention and memory in the infant. The choline intake of 900 mg/d is an amount that can be obtained by the diet and is well below the upper level of tolerance, 3500 mg/d.

There is evidence that choline status affects folate (another essential nutrient) and vice versa. *Thus, this study seeks to examine the relationship between choline and folate status.*

What we will ask you to do:

Screening Phase:

First, it is necessary to determine whether you meet the inclusion criteria of this study. This process will take up to 1 hour. The inclusion criteria are as follows:

- I am pregnant *(non-pregnant women are also eligible for the study in order to provide us with a control group)*
- I have not entered my third trimester (i.e., wk 27)
- I am between 21 and 40 years of age.
- I eat meat and other animal products
- I am not a current smoker.
- I am not using recreational drugs
- I am not drinking alcoholic beverages
- I am healthy
- I do not have gestational diabetes
- I do not have anemia.
- I do not have kidney or liver problems.
- I am not chronically taking medication that affects liver function.
- I had a body mass index (BMI) < 34 as my pre-pregnancy weight

During the screening phase, you will have your blood drawn (~ 4 tablespoons) which will be used to assess your health status and measure select vitamins/nutrients. DNA will also be obtained from your blood in order to investigate the effect of nutrients for people of different genetic make-up. You will also complete a health history questionnaire.

Dietary Intervention Phase

If you meet the required criteria, you will be invited to participate in a twelve week feeding study. Throughout this study you will consume a normal mixed diet consisting of breakfast, lunch, dinner and snacks. You will consume at least one meal per day (breakfast, lunch or dinner) in the human metabolic research unit located at Cornell University. The other meals will be provided as takeout meals. You will also consume a prenatal vitamin/mineral supplement to ensure that you are meeting the recommended dietary intake levels for pregnant women of all the essential nutrients. In addition, you will be randomized to a choline supplement to

achieve total choline intakes of 450 or 900 mg/d. To increase the study's validity, the level of choline intake to which you are assigned (i.e., 450 or 900 mg/d) will not be disclosed.

To help us better understand choline requirements and use during pregnancy, a small portion of the choline will be labeled from week 6 to 12. This label (marker) is safe and has been used many times in studies involving babies, young children and pregnant women.

It is important that you eat all of the food that we give you and that you eat nothing else outside of the study food. You will be weighed every week and extra food/beverages will be provided as necessary to ensure appropriate weight gain (~ 1 pound per week).

Blood will be taken by a trained phlebotomist (i.e., a person that is trained to draw blood) before the start of the study and at weeks 3, 6, 9, 10 and 12. The blood will be used to analyze how choline intake, folate intake and/or pregnancy influences biomarkers of status. Also we will use the DNA obtained from the blood to analyze how genetic differences influence the use of choline and/or folate. In addition, you will be required to collect your urine over a 24 hour period at the beginning of the study (week 0) and at weeks 6, 9, and 12.

Time Between End of Dietary Intervention Phase and Delivery

We request that you continue on the choline supplement that you consumed during the intervention study (ie, 100 or 500 mg/d) until you delivery the baby. This is an estimated time of 1 week. We will provide the choline supplement on a weekly basis.

At Delivery

We intend to obtain a final blood sample from you and from the umbilical cord when you deliver the baby. This blood will be used to measure your choline and folate status as well as that of the baby (i.e., umbilical cord). We will also use the cord blood to extract DNA to provide information on the influence of genetic differences on choline/folate status in your baby. In addition, we intend to retain the placenta which is normally discarded after the delivery of your baby. Your placenta will be used to examine how choline intake influences the expression of relevant genes as well as choline utilization. Lastly, we intend to ask you a few

questions (i.e., less than 5 minutes of your time) about your delivery and the baby's height and weight (included in your informational packet).

Samples obtained during your participation on this study may be used by the investigators (or collaborators) for the purposes of learning more about other nutrients (ie., vitamin D and biotin) and how genetic differences affect requirements for these nutrients.

It is possible that other investigators will have access to your blood samples (including DNA), placental tissue, and urine samples; however, the samples will be de-identified (ie, no names will be used).

Cognitive Testing of Infant

We would like to perform cognitive tests that assess your infant's attention and memory when he/she is three, six, nine and twelve months of age. These tests will measure your child's eye movements to changes in pictures/images. Feeding your baby between tests is fine. These tests are non-invasive. Each test is approximately 5-10 minute and will take up to 30 minutes total.

Risks and benefits:

We foresee minimal risks as part of your participation in this study. The choline dosage of 450 mg/d is the recommended amount for pregnant women and is considered to be adequate. The choline dosage of 900 mg/d is considered to be safe (Institute of Medicine, National Academy of Science) and falls within the range of choline intake consumed by the general population. *None-the-less, we will monitor your health status as you progress through this study. Specifically we will obtain a blood chemistry profile and a complete blood count at weeks 0, 3, 6, 9 and 12. These tests provide information on your blood lipids, liver and kidney function, and iron status. We will also take your blood pressure on a weekly basis. Should any problems be identified, we will refer you to your physician who will advise on your continuing to participate in this study.*

As with any blood draw, it is also possible that you will experience some dizziness, ill-feeling and bruising during/after the blood draw. In addition, having blood samples taken always includes a certain low risk of infection. *To minimize this risk, blood will be drawn by a trained phlebotomist at the human metabolic research unit while you are lying down.*

There are no established direct benefits to you or your baby. From this study we hope to draw conclusions regarding the optimal intake levels of choline and folate in pregnant women, potentially benefiting the health of other women and children.

In the event that you should be injured in the course of this research study, you will be provided with necessary medical care in Cayuga Medical Center At Ithaca. This statement does not mean that either such medical care or hospitalization, if necessary, will be free of charge.

Compensation:

Week of Study	Payment
1	$50
2	$60
3	$70
4	$80
5	$100
6	$120
7	$140
8	$160
9	$180

10	$200
11	$220
12	$240

You will be paid the dollar amounts indicated above in two allotments ($480 at week 6 and $1140 at week 12) for a total of $1620. Should you stop participating in the study, you will be compensated for the time you have spent on the study.

You will also be compensated for the blood and information obtained at delivery ($100) and for each cognitive evaluation session ($50 per session x 4 sessions for a total of $200).

Taking part is voluntary:

Your participation in this study is completely voluntary. If you decide to take part, you are free to withdraw at any time; however you will not be eligible to receive monetary compensation after ending the study. You will be able to keep the money you have earned up to that point.

The results of your blood tests, the genotyping and your child's cognitive tests will be kept confidential.

In any sort of report we make public, we will not include any information that will make it possible to identify you. Research records will be kept in a locked file; only the researchers will have access to the records. We will need to videotape the infant's face in order to analyze reaction times based on the infant's eye movements. Only the researchers will have access to the videotape. Upon the conclusion of the data analysis, the videotape will be destroyed (by September 2015).

If you have questions: The main researcher conducting this study is Professor Marie Caudill. Please ask any questions you have now. If you have questions later, you may contact Marie Caudill at mac379@cornell.edu or at 607-254-7456. Should you contact Professor Caudill via email, please be aware that there is a chance that your answers could be read by a third party as internet and email transmissions are neither private or secure.

If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) for human study participants at 607-255-5138 or access their website at http://www.irb.cornell.edu. You may also report your concerns or complaints anonymously through Ethicspoint or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

In addition to agreeing to participate, I also consent to having my child's cognitive function assessed and to having these activities videotaped during the session.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

This consent form will be kept by the researcher for at least five years beyond the end of the study and was approved by University of Cornell's Institutional Review Board for Use of Human Study Participants on June 5 2008 and by Cayuga's Medical Center's Review Board for Use of Human Study Participants on October 16, 2008.

Consent, Lactating Women

Cornell University
Informed Consent for Research Involving Human Study Participants

You are being invited to participate in a research study. This form is designed to provide you with information about this study. The Principal Investigator or representative will describe this study to you and answer any of your questions.

Project Title:

Effect of maternal choline intake on maternal/fetal biomarkers of choline status

Investigators:

Marie Caudill, PhD, RD; Associate Professor, Division of Nutritional Sciences, Cornell University

Eva Pressman, MD. Director of Obstetrics and Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York

Richard Canfield, PhD. Research Associate, Division of Nutritional Sciences, Cornell University

Barbara Strupp, PhD. Professor, Division of Nutritional Sciences, Cornell University

Srisatish Devapatla, MD. Director, Special Care Nursery, Chairman- Dept. of Pediatrics, Cayuga Medical Center at Ithaca, Ithaca, New York.

Sandy Saintonge, MD. Assistant Professor of Clinical Public Health and Clinical Pediatrics, Weill Cornell Medical College, Attending Physician, New York Hospital Queens, Department of Emergency Medicine

Cydne Perry, PhD. Postdoctoral Research Associate, Division of Nutritonal Sciences, Cornell University

What the study is about:

Choline, a nutrient found in the diet (i.e., eggs, milk, and beef), was recently recognized as a required nutrient for humans. However, at present, the amount of choline required during pregnancy and lactation is unknown. Current dietary intakes in the general population range from ~ 100 to 1000 mg/d with an average intake of 300 mg/d. In 1998, an "Adequate Intake" level for choline of 425, 450 and 550 mg/d was established for non-pregnant, pregnant and lactating women respectively. However, these estimates probably exceed actual requirements given that habitual choline intake levels in healthy populations are ~ 300 mg/d.

There is evidence from animal studies that extra choline consumed by the mother during and after pregnancy may have long lasting beneficial effects on the memory and attention of the newborn. However, no study has examined this in humans.

Thus, this study seeks to examine choline metabolism (i.e., use) and requirements in pregnant and lactating women. In addition, it seeks to assess whether higher choline intakes improve biomarkers of choline status (i.e., increase blood concentrations).

Finally, there is evidence that choline status affects folate (another essential nutrient) and vice versa. *Thus, this study seeks to examine the relationship between choline and folate status.*

What we will ask you to do:

Screening Phase:

First, it is necessary to determine whether you meet the inclusion criteria of this study. This process will take up to 1 hour. The inclusion criteria are as follows:

- I am a lactating mom who plans to exclusively breast feed for the duration of the study (ie, the next 10 weeks).
- I can begin the feeding study at the start of my fifth week of lactation.
- I am between 21 and 40 years of age.
- I eat meat and other animal products.
- I am not a current smoker.
- I am not using recreational drugs.
- I am not drinking alcoholic beverages.
- I am healthy.
- I do not have kidney or liver problems.
- I am not chronically taking medication that affects liver function.

During the screening phase, you will have your blood drawn (~ 4 tablespoons) which will be used to assess your health status and measure select vitamins/nutrients. DNA will also be obtained from your blood in order to investigate the effect of nutrients for people of different genetic make-up. You will also complete a health history questionnaire.

Dietary Intervention Phase

If you meet the required criteria, you will be invited to participate in a ten week feeding study. Throughout this study you will consume a normal mixed diet consisting of breakfast, lunch, dinner and snacks. You will consume at least one meal every Monday, Wednesday, and Friday (breakfast, lunch or dinner) in the human metabolic research unit located at Cornell University. The other meals will be provided as takeout meals. You will also consume a prenatal vitamin/mineral supplement to ensure that you are meeting the recommended dietary intake levels for lactating women of all the essential nutrients.

The study diet provides 350 mg/d choline and contains food items that are significant sources of choline (i.e., eggs, beef, and milk). In addition, you will be randomized to a choline supplement to achieve total choline intakes of 450 or 900

mg/d. To increase the study's validity, the level of choline intake to which you are assigned (i.e., 450 or 900 mg/d) will not be disclosed.

To help us better understand choline requirements and use during lactation, a small portion of the choline will be labeled from study week 6 to 10. This label (marker) is safe and has been used many times in studies involving babies, young children and pregnant / lactating women.

It is important that you eat all of the food that we give you and that you eat nothing else outside of the study food. You will be weighed every week and extra food/beverages will be provided as necessary to ensure that calorie needs are being met.

Blood will be taken by a trained phlebotomist (i.e., a person that is trained to draw blood) before the start of the study and at weeks 3, 6, 9, and 10. The blood will be used to analyze how choline intake, folate intake and/or lactation influences biomarkers of status. Also we will use the DNA obtained from the blood to analyze how genetic differences influence the use of choline and/or folate.

In addition, you will be required to collect your urine over a 24 hour period and to provide a breast milk sample at the beginning of the study (week 0) and at weeks 3, 6, 9 and 10. The breast milk will be expressed completely from one breast (either right or left) at the beginning of the second feeding time (ie, 10 am). All supplies/materials needed for this expression will be provided. Samples obtained during your participation on this study may be used by the investigators (or collaborators) for the purposes of learning more about other nutrients (ie., vitamin D and biotin) and how genetic differences affect requirements for these nutrients.

It is possible that other investigators will have access to your blood samples (including DNA) and urine samples; however, the samples will be de-identified (ie, no names will be used).

Cognitive Testing of Infant

We would like to perform cognitive tests that assess your infant's attention and memory when he/she is three, six, nine and twelve months of age. These tests will measure your child's eye movements to changes in pictures/images. Feeding your baby between tests is fine. These tests are non-invasive. Each test is approximately 5-10 minutes and will take up to 30 minutes total.

Risks and benefits:

We foresee minimal risks as part of your participation in this study. The choline dose of 450 mg/d is below the "adequate intake" level of 550 mg/d; however, it is

above the average intake level of 300 mg/d consumed by the general population. In addition, the 450 mg/d choline intake level in this study is comprised of dietary choline obtained from a normal mixed diet (i.e., 350 mg/d) and supplemental choline (100 mg/d). The choline dosage of 900 mg/d is considered to be safe (Institute of Medicine, National Academy of Science) and falls within the upper range of choline intake consumed by the general population. *None-the-less, we will monitor your health status as you progress through this study. Specifically we will obtain a blood chemistry profile and a complete blood count at weeks 0, 3, 6, 9 and 10. These tests provide information on your blood lipids, liver and kidney function, and iron status. We will also take your blood pressure on a weekly basis. Should any problems be identified, we will refer you to your physician who will advise on your continuing to participate in this study.*

As with any blood draw, it is also possible that you will experience some dizziness, ill-feeling and bruising during/after the blood draw. In addition, having blood samples taken always includes a certain low risk of infection. *To minimize this risk, blood will be drawn by a trained phlebotomist at the human metabolic research unit while you are lying down.*

There are no established direct benefits to you or your baby. From this study we hope to draw conclusions regarding the optimal intake levels of choline and folate in lactating women, potentially benefiting the health of other women and children.

Compensation:

Week of Study	Payment
1	$50
2	$60
3	$70
4	$80
5	$100
6	$120
7	$140
8	$160
9	$200
10	$220

You will be paid the dollar amounts indicated above in two allotments ($360 at week 5 and $840 at week 10) for a total of $1200. Should you stop participating in the study, you will be compensated for the time you have spent on the study.

You will also be compensated for each cognitive evaluation session ($50 per session x 4 sessions for a total of $200).

Taking part is voluntary:

Your participation in this study is completely voluntary. If you decide to take part, you are free to withdraw at any time; however you will not be eligible to receive monetary compensation after ending the study. You will be able to keep the money you have earned up to that point.

The results of your blood tests, the genotyping and your child's cognitive tests will be kept confidential.

In any sort of report we make public, we will not include any information that will make it possible to identify you. Research records will be kept in a locked file; only the researchers will have access to the records. We will need to videotape the infant's face in order to analyze reaction times based on the infant's eye movements. Only the researchers will have access to the videotape. Upon the conclusion of the data analysis, the videotape will be destroyed (by September 2015).

If you have questions: The main researcher conducting this study is Professor Marie Caudill. Please ask any questions you have now. If you have questions later, you may contact Marie Caudill at mac379@cornell.edu or at 607-254-7456. Should you contact Professor Caudill via email, please be aware that there is a chance that your answers could be read by a third party as internet and email transmissions are neither private or secure.

If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) for human study participants at 607-255-5138 or access their website at http://www.irb.cornell.edu. You may also report your concerns or complaints anonymously through Ethicspoint or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

In addition to agreeing to participate, I also consent to having my child's cognitive function assessed and to having these activities videotaped during the session.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

This consent form will be kept by the researcher for at least five years beyond the end of the study and was approved by University of Cornell's Institutional Review Board for Use of Human Study Participants on October 15, 2008.

Consent, Nonpregnant Women

Cornell University
Informed Consent for Research Involving Human Study Participants

You are being invited to participate in a research study. This form is designed to provide you with information about this study. The Principal Investigator or representative will describe this study to you and answer any of your questions.

Project Title:

Effect of maternal choline intake on maternal/fetal biomarkers of choline status

Investigators:

Marie Caudill, PhD, RD; Associate Professor, Division of Nutritional Sciences, Cornell University

Eva Pressman, MD. Director of Obstetrics and Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of Rochester School of Medicine, Rochester, New York

Richard Canfield, PhD. Research Associate, Division of Nutritional Sciences, Cornell University

Barbara Strupp, PhD. Professor, Division of Nutritional Sciences, Cornell University

Srisatish Devapatla, MD. Director, Special Care Nursery, Chairman- Dept. of Pediatrics, Cayuga Medical Center at Ithaca, Ithaca, New York

Sandy Saintonge, MD. Assistant Professor of Clinical Public Health and Clinical Pediatrics, Weill Cornell Medical College, Attending Physician, New York Hospital Queens, Department of Emergency Medicine

Cydne Perry, PhD. Postdoctoral Research Associate, Division of Nutritonal Sciences, Cornell University

What the study is about:

This study seeks to provide data about nutrient requirements / utilization during pregnancy (see below). *You are being invited for possible participation in this study in order to examine choline requirements/use in non-pregnant women and to serve as a control for the pregnant women.*

Choline, a nutrient found in the diet, was recently recognized as a required nutrient for humans. The recommended dietary intake level for non-pregnant and pregnant women is 425 or 450 mg/d, respectively. However, at present, it is not known whether 450 mg/d is enough to maintain choline status in pregnant women.

Thus, this study seeks to assess the effect of pregnancy on choline status and to examine whether the current recommended dietary intake, 450 mg/d, is enough to maintain choline status during pregnancy. Your participation in this study will enable us to compare your choline status to that of a pregnant woman and in doing so assess the effect of pregnancy on choline status.

It is possible that choline affects the use of folate, another essential nutrient and vice versa. *Thus, this study seeks to examine the relationship between choline and folate.*

What we will ask you to do:

Screening Phase:

First, it is necessary to determine whether you meet the inclusion criteria of this study. This process will take up to 1 hour. The inclusion criteria are as follows:

- I am not pregnant or lactating
- I am between 21 and 40 years of age
- I eat meat and other animal products
- I am not a current smoker.
- I am not using recreational drugs
- I am willing to refrain from drinking alcoholic beverages during the 12-week study period
- I am healthy
- I do not have anemia.
- I do not have kidney or liver problems.
- I am not chronically taking medication that affects liver function.
- I have a body mass index (BMI) $\leq$ 34

During the screening phase, you will have your blood drawn (~ 4 tablespoons) which will be used to assess your health status and measure select vitamins/nutrients. DNA will also be obtained from your blood in order to investigate the effect of nutrients for people of different genetic make-up. You will also complete a health history questionnaire.

Study Phase

If you meet the required criteria, you will be invited to participate in a twelve week feeding study. Throughout this study you will consume a normal mixed diet consisting of breakfast, lunch, dinner and snacks. You will consume at least one meal per day (breakfast or dinner) in the human metabolic research unit located at Cornell University. The other meals will be provided as takeout meals. You will also consume a customized vitamin/mineral supplement to ensure that you are meeting the recommended dietary levels for pregnant (and non-pregnant) women of all the essential nutrients. In addition, you will be randomized to a choline supplement to acheive total choline intakes of 450 or 900 mg/d. To increase the study's validity, the level of choline intake to which you are assigned (i.e., 450 or 900 mg/d) will not be disclosed.

To help us better understand choline requirements and use during pregnancy, a small portion of the choline or other relevant nutrients will be labeled from week 6 to 12. This label (marker) is safe and has been used many times in studies involving babies, young children and pregnant women.

It is important that you eat all of the food we give you and that you eat nothing outside what is provided. You will be weighed every week and your calorie intake will be modified to ensure that you do not lose or gain more than a couple of pounds.

Blood will be taken by a trained phlebotomist before the start of the study and at weeks 3, 6, 9, 10 and 12. The blood will be used to analyze how choline intake, folate intake and/or pregnancy influenced biomarkers of status. Also we will use the DNA obtained from the blood to analyze how changes in genes influence the use of choline and/or folate. In addition, you will be required to collect your urine over a 24 hour period at the beginning of the study (week 0) and at weeks 6, 9, and 12. Samples obtained during your participation on this study may be used by the investigators (or collaborators) for the purposes of learning more about other nutrients (ie., vitamin D and biotin) and how genetic differences affect requirements for these nutrients.

It is possible that other investigators will have access to your blood (including DNA) and urine samples; however, the samples will be de-identified (ie, no names will be used).

Risks and benefits:

We foresee minimal risks as part of your participation in this study. All essential nutrients will be provided at or above the recommended intake level for non-pregnant women. The choline dosage of 900 mg/d is considered safe (Institute of Medicine, National Academy of Science) and falls within the range of choline intake consumed by the general population.

It is possible that you will experience some dizziness or ill-feeling during the blood draw. In addition, having blood samples taken always includes a certain low risk of infection. *To minimize this risk, blood will be drawn by a trained phlebotomist at the human metabolic research unit while you are lying down.*

There are no established direct benefits to you. From this study we hope to draw conclusions regarding the optimal intake levels of choline and folate for pregnant women, potentially benefiting the health of other women and children.

In the event that you should be injured in the course of this research study, you will be provided with necessary medical care in Cayuga Medical Center At Ithaca.

This statement does not mean that either such medical care or hospitalization, if necessary, will be free of charge.

Compensation:

Week of Study	Payment
1	$50
2	$60
3	$70
4	$80
5	$100
6	$120
7	$140
8	$160
9	$180
10	$200
11	$220
12	$240

You will be paid the dollar amounts indicated above in two allotments ($480 at week 6 and $1140 at week 12) for a total of $1620. Should you stop participating in the study, you will be compensated for the time you have spent on the study.

Taking part is voluntary:
Participating in this study is completely voluntary. If you decide to take part, you are free to withdraw at any time; however you will not be eligible to receive monetary compensation after ending the study. You will be able to keep the money you have earned up to that point.

The results of your blood tests including the genotyping will be kept confidential.

In any sort of report we make public, we will not include any information that will make it possible to identify you. Research records that contain your name (i.e., informed consent, health history questionnaire, and the blood chemistry profiles and complete blood counts) will be kept in a locked filing cabinet in the office of the PI. All other data (ie., blood measurements, DNA sequences) will be identified only by a code (i.e., number).

If you have questions: The main researcher conducting this study is Professor Marie Caudill. Please ask any questions you have now. If you have questions later, you may contact Marie Caudill at mac379@cornell.edu or at 607-254-7456.
Should you contact Professor Caudill via email, please be aware that there is a chance that your answers could be read by a third party as internet and email transmissions are neither private or secure.

If you have any questions or concerns regarding your rights as a subject in this study, you may contact the Institutional Review Board (IRB) at 607-255-5138 or access their website at http://www.irb.cornell.edu. You may also report your concerns or complaints anonymously through Ethicspoint or by calling toll free at 1-866-293-3077. Ethicspoint is an independent organization that serves as a liaison between the University and the person bringing the complaint so that anonymity can be ensured.

You will be given a copy of this form to keep for your records.

Statement of Consent: I have read the above information, and have received answers to any questions I asked. I consent to take part in the study.

Your Signature _____ Date _____

Study Representative Signature _____ Date _____

This consent form will be kept by the researcher for at least five years beyond the end of the study and was approved by University of Cornell's Institutional Review Board for Use of Human Study Participants on June 5 2008 and by Cayuga Medical Center's Review Board for Use of Human Study Participants on October 16 2008.