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Abstract

Vitamin D deficiency has been widely reported among pregnant women and infants around the world. Women with low sun exposure, high BMI, low vitamin D intakes and socioeconomic disadvantage with poor quality diets are at greatest risk of vitamin D deficiency, leading to very low serum concentrations of 25-hydroxyvitamin D (25(OH)D) in their offspring and an increased risk of nutritional rickets. Many observational studies, supported by compelling in vitro and in vivo data, have generated evidence suggesting that low vitamin D status in pregnancy may also contribute to the risk of adverse perinatal outcomes including hypertensive disorders (e.g., preeclampsia), fetal growth restriction, and preterm birth. However, the few large randomized controlled trials (RCTs) conducted to date have generated conflicting evidence for a role of vitamin D supplementation in improving perinatal outcomes. Vitamin D supplementation policies during pregnancy and implementation of policies vary within and between jurisdictions. Regulatory authorities have cited insufficient evidence to establish pregnancy-specific targets for serum 25(OH)D concentrations or prenatal vitamin D intake that effectively reduce the risks of adverse perinatal and infant outcomes. This paper arises from a Debate on Vitamin D Requirements during Pregnancy, held at the 22nd Vitamin D Workshop, 2019. From varied perspectives, our objectives were to evaluate the evidence for: vitamin D metabolism in pregnancy and the prevalence of gestational vitamin D deficiency worldwide; the translation of laboratory research findings to clinical studies on the role of vitamin D in perinatal health; the challenges of designing and conducting clinical trials to establish prenatal vitamin D requirements; and results to date of major large RCTs of prenatal vitamin D supplementation. Lastly, we explored potential next steps towards generating robust clinical data in this field to address both public health protection and patient care.

Keywords	Vitamin D; Vitamin D Requirements; Pregnancy; Perinatal health; Vitamin D status

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Controversies in Vitamin D

Vitamin D in pregnancy: Where we are and where we should go

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Abstract

Vitamin D deficiency has been widely reported among pregnant women and infants around the world. Women with low sun exposure, high BMI, low vitamin D intakes and socioeconomic disadvantage with poor quality diets are at greatest risk of vitamin D deficiency, leading to very low serum concentrations of 25-hydroxyvitamin D (25(OH)D) in their offspring and an increased risk of nutritional rickets. Many observational studies, supported by compelling *in vitro* and *in vivo* data, have generated evidence suggesting that low vitamin D status in pregnancy may also contribute to the risk of adverse perinatal outcomes including hypertensive disorders (e.g., preeclampsia), fetal growth restriction, and preterm birth. However, the few large randomized controlled trials (RCTs) conducted to date have generated conflicting evidence for a role of vitamin D supplementation in improving perinatal outcomes. Vitamin D supplementation policies during pregnancy and implementation of policies vary within and between jurisdictions. Regulatory authorities have cited insufficient evidence to establish pregnancy-specific targets for serum 25(OH)D concentrations or prenatal vitamin D intake that effectively reduce the risks of adverse perinatal and infant outcomes. This paper arises from a Debate on Vitamin D Requirements during Pregnancy, held at the 22nd Vitamin D Workshop, 2019. From varied perspectives, our objectives were to evaluate the evidence for: vitamin D metabolism in pregnancy and the prevalence of gestational vitamin D deficiency worldwide; the translation of laboratory research findings to clinical studies on the role of vitamin D in perinatal health; the challenges of designing and conducting clinical trials to establish prenatal vitamin D requirements; and results to date of major large RCTs of prenatal vitamin D supplementation. Lastly, we explored potential next steps towards generating robust clinical data in this field to address both public health protection and patient care.

Key-words: Vitamin D; Vitamin D Requirements; Pregnancy; Perinatal health; Vitamin D status

Introduction

Pregnancy and infancy are life-stages for which evidence of low vitamin D status is widespread but the evidence basis for setting dietary requirements for vitamin D is weakest (Institute of Medicine, 2011). Prevention of low vitamin D status is required to reduce the risk of nutritional rickets among children and adolescents and osteomalacia in adults, which can have severe and lasting consequences for bone growth and skeletal integrity (Munns et al 2016). The recent discovery of non-skeletal roles for vitamin D has changed the landscape considerably and mechanistic studies have elaborated the molecular roles of vitamin D in many systems, for example, immunity (Adams and Hewison, 2008, Chun et al., 2014b). Modulation of the intrauterine immune environment by vitamin D has been implicated in adverse perinatal outcomes, including recurrent pregnancy loss, preeclampsia and spontaneous preterm birth (Liu and Hewison, 2012, Tamblyn et al., 2015). Indications are that early pregnancy may be the critical window for prevention of these adverse events.

The definition of healthy vitamin D status during pregnancy, typically indicated by circulating concentrations of 25-hydroxyvitamin D (25(OH)D), is the subject of intense debate, which has been challenged by a lack of data from well-powered, well-controlled trials in pregnant women, particularly among women with low circulating 25(OH)D at baseline. Multiple systematic reviews of the role of vitamin D supplementation during pregnancy have been published but these have lacked data from substantial trials and there is no consensus as to whether physiological requirements for 25(OH)D are higher during pregnancy than in non-pregnant adults (Roth et al., 2017). It is also worth noting that trials and reviews of trials have framed the question around the benefit of *additional* vitamin D supplementation during pregnancy (using a pharmacological model of intervention regardless of baseline status) rather than the role of *sufficient vitamin D to support healthy pregnancy from the outset*.

A Debate on the controversial topic of Vitamin D Requirements during Pregnancy was held at the 22nd Vitamin D Workshop, 2019. Our objectives were to evaluate the current state of the evidence for:

- vitamin D metabolism in pregnancy, and the prevalence of gestational vitamin D deficiency worldwide;
- the translation of laboratory research findings to clinical studies on the role of vitamin D in perinatal health;
- the challenges of designing and conducting clinical trials to establish prenatal vitamin D requirements; and,
- results to date of major large RCTs of prenatal vitamin D supplementation.

The three authors hold some shared and some divergent views on the core issues discussed and provided their perspectives on these issues. Our common goal was to explore potential next steps towards generating robust clinical data in this field to address both public health protection and patient care. In the presentation of the evidence in this paper, we aimed to convey the plurality of divergent viewpoints rather than present a unified or consensus statement.

Low vitamin D status is common among pregnant women and infants

A key question to be addressed is whether the prevalence of 25(OH)D <25-30 and <50 nmol/L is higher among pregnant women than among other population groups. Saraf and colleagues (2016) estimated the global prevalence of 25(OH)D concentrations < 50 nmol/L at 54% among pregnant women and 75% among newborns. Almost one in five pregnant women and one in three newborns were below 25 nmol/L, placing them at increased risk of developing rickets and osteomalacia. A systematic review of 25(OH)D concentrations in low- and middle-income countries (LMIC) by Cashman et al (2019) reported that 29 out of the 83 countries included had 25(OH)D data available. Afghanistan, Pakistan, India, Tunisia, Syria, the West Bank and Gaza and Mongolia were classified as "hot spots" for very low 25(OH)D (<25-30 nmol/L) among women, pregnant women or infants on the basis of having a prevalence in excess of 20%(Cashman et al., 2019). In low income and middle eastern countries in particular, women and girls appear to have lower circulating 25(OH)D than their male counterparts, probably due to clothing practices (Roth et al., 2018a, Lips et al., 2019). Palacios and Gonzales (2014) described a high prevalence (>20%) of 25(OH)D <30 nmol/L among pregnant women and infants, in many countries, including in South Asia and the Middle East; up to 60% of women in India and 86% of infants in Iran had 25(OH)D < 30 nmol/L.

One of the challenges of interpreting global 25(OH)D data is the methodological variability in analytical methods and data reporting, which can be particularly challenging for maternal samples (Best et al 2019). The relatively low uptake, and low rate reporting of laboratory performance of users of quality assurance systems, such as DEQAS (Carter et al 2017), leads to a lack of transparency. Application of the NIH Vitamin D Standardization Protocol (VDSP) should be used to standardize 25(OH)D data if serum aliquots are available for re-analysis by LC-MS/MS, using a validated statistical approach (Durazo-Arvizu et al 2017). The CDC Vitamin D Standardization-Certification Program (VDSCP) is also available as an avenue to achieve accreditation of 25(OH)D analysis.

Within temperate countries, the prevalence of vitamin D deficiency is much higher among persons with darkly pigmented skin tones. Using VDSP, the ODIN project reported the first internationally standardised dataset of vitamin D status and the prevalence of vitamin D deficiency across Europe (Cashman et al., 2016). While the overall prevalence of 25(OH)D < 30 nmol/L was 13%, about twice that of the US (5.9%) (Schleicher et al., 2016) and Canada (7.4%) (Sarafin et al., 2015), persons with more darkly pigmented skin tones were at much higher risk than their white counterparts both in Europe and North America. As part of the ODIN project, Kiely et al. (2016) analysed a pregnancy cohort of 1800 women in Ireland, where there is no maternal vitamin D supplementation policy. Data from CDC-accredited LC/MS-MS analysis of 25(OH)D showed that 17% were below 30 nmol/L at 15 weeks of gestation; this was 49% among women of ethnic minority (Kiely et al., 2016). Among the infants of this cohort, 46% of umbilical cord sera had a 25(OH)D < 30 nmol/L; this was 73% among infants from women of ethnic minority (Kiely et al., 2017b).

Nutritional Rickets

In line with the prevalence data for 25(OH)D among mothers and newborns, it is no surprise to note that the greatest burden of nutritional rickets is in Africa, Asia and the Middle East (Thacher et al., 2006, Prentice, 2013). There is also evidence that while the incidence of nutritional rickets is stable or decreasing among whites, it is increasing in North America and Europe among immigrant populations (Creo et al., 2017). Thacher et al (2016) provided a summary of nutritional rickets in immigrant and refugee children using data collected in the UK, Denmark, Australia, Canada and the US. Populations most at risk were South Asian, African and Middle Eastern African, with incidence rates (per 1000,000/year) up to 24 in young children < 3 years and up to 4.9 among children ≤15 years. The stark contrast between whites and ethnic minorities in western countries is highlighted in the *Global Consensus Recommendations on Prevention and Management of Nutritional Rickets* (Munns et al., 2016). Most clinical insights are gained from hospital admissions; Uday et al (2018) have documented hypocalcemic dilated cardiomyopathy associated with nutritional rickets and in some cases, fatal consequences. These prevalence estimates of maternal and infant 25(OH)D concentrations and the resulting effects elevate vitamin D deficiency among mothers and babies to the status of a serious public health problem that requires urgent action.

Challenges in defining nutritional requirements for vitamin D during pregnancy

To provide the policy context, Mairead Kiely (MK) also summarized current dietary recommendations for vitamin D in in pregnant and lactating women in line with the 25(OH)D concentration targets they are intended to reach. Individual intake recommendations range from 5 to 15 µg/day (200-600 IU) to achieve 25(OH)D targets of ≥ 25 to 50 nmol/L (10 to 20 ng/mL) (Kiely et al., 2017a). Although there is some variation between these recommendations, they were all formulated based on evidence from RCTs of vitamin D status and bone health outcomes. Due to a lack of evidence on which to set nutritional requirements for 25(OH)D during pregnancy specifically, recommendations for vitamin D intake are the same for pregnant and lactating women as non-pregnant adults.

Vitamin D requirements from the perspective of vitamin D biology

Carol Wagner (CW) presented data showing that unlike other times during the lifespan, pregnancy involves the orchestration of events that must protect the mother while allowing growth and development of the fetus. There is a delicate immune balance that nurtures the allogeneic fetus, while maintaining reactivity against pathogens to the outside world (Racicot et al., 2014, Mor and Cardenas, 2010, Vijayendra Chary et al., 2015a). During the first trimester, a proinflammatory state allows for the invasion of the uterine wall by fetal derived cytotrophoblast cells to become the placenta. Following the establishment of the maternal-placental-embryonic unit, there is rapid growth of the fetus, which must occur in a state that is by comparison to the earlier first stage of pregnancy, anti-inflammatory (Racicot et al., 2014, Mor and Cardenas, 2010). This relatively anti-inflammatory state will last well beyond the second trimester and into the third trimester, when the uterine environment becomes more proinflammatory to allow the onset of labour and the expulsion of the fetus from the uterus (Racicot et al., 2014, Mor and Cardenas, 2010). Dysregulation of these tightly controlled

biophenomena at a systemic and placental level have been considered as a potential mechanism mediating pathogenesis of recurrent pregnancy loss (Ji et al., 2019) preeclampsia and spontaneous preterm birth (Weiss et al., 2016, Mirzakhani et al., 2016). It is postulated by many that these adverse events during pregnancy have their origins early-on in pregnancy, with derangement of the critical balance in immune function (Hollis and Wagner, 2017b, Liu and Hewison, 2012, Tamblyn et al., 2015).

$1,25(OH)_2D$ mediates gene expression via VDR and VDREs, and increased concentrations of $1,25(OH)_2D$ could have implication for gene pathways and subsequent function. In mouse models of vitamin D deficiency studied early in gestation, an array of genes in the placenta are upregulated and others downregulated based on maternal vitamin D status (Liu et al., 2011), which find corollaries in human studies, especially following adverse pregnancy outcomes such as preeclampsia (Powe et al., 2010, Robinson et al., 2010, Baker et al., 2010, Bodnar et al., 2014, Mirzakhani et al., 2016). Parallel findings are noted in the upregulation of certain placental genes that have been implicated in preeclampsia (Schulz et al., 2017). There are studies that describe alternation in T cell phenotype associated with preterm birth (Zahran et al., 2018) and recurrent pregnancy loss in women who are vitamin D deficient (Ji et al., 2019, Kwak-Kim et al., 2016, Wu et al., 2018, Sharif et al., 2018). There are also non-genomic calcitriol-dependent biological effects that can take place in cells, involving second messengers generated by membrane-initiated signalling pathways (Novakovic et al., 2009).
Classic vitamin D receptor (VDR) and the membrane-associated rapid response steroid-binding protein (MARRS) found in the cell membrane may bind $1,25(OH)_2D$ and initiate the activation of numerous pathways involving various protein kinases (PKC), MAPK, PKA, phosphatidyl inositol phosphate, and Ca2+ and chloride channels (Olmos-Ortiz et al., 2015). The VDR and regulatory metabolic enzymes are expressed in both placenta and decidua, indicating a potential critical point in the immunomodulation at the maternal-fetal interface (Vijayendra Chary et al., 2015b). Further, activities of vitamin D response element (VDRE)-containing genes can be grouped in quite diverse biological networks: bone and mineral metabolism; cell life and death (comprising proliferation, differentiation and apoptosis); immune function—both innate and adaptive immunity that are thought to be operational during the various stages of pregnancy (Knabl et al., 2017).

During pregnancy, there are major adaptations in vitamin D metabolism. After conception, the fertilized egg undergoes a series of reproductive cell divisions to form the three germ layers—endoderm, mesoderm, and ectoderm) that give rise to different types of cells, many of which express the vitamin D receptor (VDR) (Kaludjerovic and Vieth, 2010). Various cells give rise to different organs through organogenesis that extends from 4 to 10 weeks of gestation with the placental fully formed and functional by 4 weeks of gestation. During this time of immense activity and growth, localized concentrations of vitamin D in the form of 25(OH)D (which crosses the placenta from the maternal side) and $1,25(OH)_2D$ (which is synthesized by the fetus as early as 6-10 weeks of gestation) can interact with various signalling systems to regulate organ development (Kaludjerovic and Vieth, 2010). As mentioned, it is the placenta that permits the transfer of 25(OH)D from the mother to the fetus but also the placenta through its production of 1α-hydroxylase that local synthesis of $1,25(OH)_2D$ occurs. $1,25(OH)_2D$ binds to VDR in specialized cells to induce genomic and nongenomic responses that stimulate organ development.

Focusing on $1,25(OH)_2D$ during pregnancy, $1,25(OH)_2D$ increases more than 2–3 fold in the first weeks of pregnancy that appears to come mainly from maternal kidney synthesis but some is placentally-derived (Bikle et al., 1984, Hollis et al., 2011, Tamblyn et al., 2018, Best et al., 2019). This increase is noted in both animal models and human studies as measured by RIA and LC-Tandem mass spec (LC-MS) and is accompanied by a rise in VDBP, the main carrier of all vitamin D moieties with greater avidity for 25(OH)D (Bikle et al., 1984, Vijayendra Chary et al., 2015a, Hollis et al., 2011)(Hollis and Wagner, 2017a, Novakovic et al., 2009, Best et al., 2019). Despite this, there is no corresponding rise in 25(OH)D during pregnancy (Hollis and Wagner, 2017a).

The increase in $1,25(OH)_2D$ early in the first trimester of pregnancy is linked to higher Cyp27B1 (1-α-hydroxylase) activity in maternal kidney, placental trophoblasts and decidua (Bikle et al., 1984, Olmos-Ortiz et al., 2015, Whitehead et al., 1981, Hollis et al., 2011). Longitudinal human studies also report a progressive increase in both bound and free $1,25(OH)_2D$ concentrations (Best et al., 2019). Previous animal studies in partly and completely nephrectomized rats report that this rise is mainly attributed to increased maternal synthesis *per se*, rather than decreased clearance (Gray et al., 1979). This increase in $1,25(OH)_2D$ occurs at a time during pregnancy before increased fetal calcium requirements manifest, increasing from ~2 mg/day in the first trimester to 100 mg/day in last trimester that is continued throughout lactation. Yet, immediately after delivery, maternal calcitriol concentrations return to pre-pregnancy levels, despite significant calcium requirements needed during lactation (Carneiro et al., 2010, Ritchie et al., 1998). $1,25(OH)_2D$ concentrations by 12 weeks of gestation have already increased to a mean of 180 pmol/L, further increasing to around 300 pmol/L toward the end of pregnancy, compared to nonpregnant control mean concentration of 91 pmol/L (Papapetrou, 2010, Ardawi et al., 1997, Hollis et al., 2011). Calcitriol circulates at low levels in fetal blood, typically less than 50% of the maternal value (Fleischman et al., 1980, Hollis and Pittard, 1984a, Seki et al., 1994, Hillman et al., 1985). Both animal models and human data suggest that 25(OH)D readily crosses the placenta, with cord blood concentrations that are around 70-100% of maternal concentrations (Hollis and Pittard, 1984a, Hollis et al., 2011, Best et al., 2019). Calcitriol synthesis in the fetus is likely suppressed by the high serum calcium, high phosphorus, and low PTH concentrations typically observed in cord blood (Kovacs, 2014).

It is interesting that the rise in maternal $1,25(OH)_2D$ during pregnancy occurs without any effect on serum ionized or corrected calcium and appears to result from uncoupling of $1,25(OH)_2D$ from its well-established actions in the non-pregnant state (Hollis et al., 2011). While VDBP increases concomitantly, it does not increase to the same degree as $1,25(OH)_2D$ (Hollis et al., 2011). In addition, upregulation of *CYP27B1* mRNA in the maternal kidneys, exerts characteristics of “extrarenal” production from an endocrine standpoint, such as relative unresponsiveness to stimulation by PTH (except under conditions of extreme vitamin D deficiency) and lack of feedback inhibition of CYP27B1, and thus, 1-α-hydroxylase by $1,25(OH)_2D$ itself. This increase in $1,25(OH)_2D$ seems to be independent of parathyroid hormone (PTH), which itself remains within the normal adult range throughout pregnancy (Wagner and Hollis, 2011, Haddow et al., 2011, Hollis et al., 2011). Other hormones could

also contribute to the bioregulation of 1-α-hydroxylase such as PTH-rP, estradiol, prolactin, and placental lactogen (Ardawi et al., 1997).

As shown in **Figure 1** below (from Hollis et al, with permission, (Hollis et al., 2011), this kinetic reaction saturation graph of 25(OH)D and $1,25(OH)_2D$ shows that 25(OH)D has direct influence on $1,25(OH)_2D$ concentrations throughout pregnancy, an event which does not occur during any other time during human life. It is apparent in the figure that as lower concentrations of $1,25(OH)_2D$ increase, first order kinetics becomes zero order kinetics, with a plateauing of the graph and an inflection point at 100 nmol/L (40 ng/mL) 25(OH)D—the level required to optimize $1,25(OH)_2D$ production during pregnancy. (**Place Figure 1 near here)**

Based on these findings, it is thought by some that this inflection point of 100 nmol/L may be critical during pregnancy for both maternal and fetal well-being, and that attainment of this threshold early-on may be key. There are data to suggest that vitamin D status early in pregnancy sets the stage for vitamin D's enabling effect on immune function (Mirzakhani et al., 2016, Weiss and Litonjua, 2017). In their *post hoc* analysis using multivariable log-binomial regression of maternal 25(OH)D status during pregnancy, McDonnell et al reported that women attaining maternal 25(OH)D concentrations ≥100 nmol/L compared to ≤50 nmol/L, adjusted for covariates, was associated with a reduction in the risk of preterm birth by 59% (McDonnell et al., 2017).

With more modern scientific technological advancement, vitamin D has been shown to act on innate and structural cells to promote antimicrobial defense through induction of antimicrobial peptides (e.g. cathelicidin, beta-defensins) and autophagy (Liu et al., 2006, Chun et al., 2014a). Bridging both innate and adaptive immunity, vitamin D also acts on myeloid dendritic cells (DC) to promote a tolerogenic antigen presenting cell (APC) phenotype (Penna et al., 2007). In their recent review, Hawrylowicz and Santos provide compelling evidence that vitamin D reduces naïve T cell expansion, and downregulates Th1 and Th17 responses, but has variable effects on Th2 responses linked to allergic disease (Hawrylowicz and Santos, 2019). These interactions result in an increased frequency of human Foxp3 and IL-10 Treg *in vitro* and *in vivo* (Penna et al., 2005, Van Der Aar et al., 2011, Nikolic and Roep, 2013, Urry et al., 2012, Pfeffer and Hawrylowicz, 2018, Hawrylowicz and Santos, 2019, Jeffery et al., 2009). Similarly, Vasiliou et al., in their human dust mite-allergy mouse model, showed that vitamin D deficiency *in utero* led to an increased frequency of Th2 cells and reduced anti-inflammatory IL-10+ T cells in the lungs (Vasiliou et al., 2014), arguing for an *in utero* process that continues after birth. As noted by Hawrylowicz and Santos (Hawrylowicz and Santos, 2019), neonatal and adult immunity differs, and assumptions of how vitamin D promotes protective immunity in pregnancy and neonates may be distinct to effects in adults, as they note that in recently published, independent vitamin D supplementation pregnancy studies that showed such differences (e.g. no correlation with Foxp3) (Hornsby et al., 2018, Chi et al., 2011, Guven et al., 2012). Clearly, more research is needed in this area to inform the design of more meaningful clinical trials.

Translation of laboratory findings to clinical studies

Palacios (2019b) concluded that notwithstanding inconsistent reporting of adverse events, vitamin D supplementation appears safe. Studies presented by CW also showed a high degree of safety in both mother and fetus/neonate and later, in the offspring during longitudinal follow-up studies (Hollis et al., 2011, Wagner et al., 2013b, Wagner et al., 2013a, Al-Garawi et al., 2016, Chawes et al., 2016, Zhang et al., 2016, Litonjua et al., 2016, Wolsk et al., 2017b, Roth et al., 2018b, Hornsby et al., 2018, Stukes et al., 2016, Kelly et al., 2019, Litonjua et al., 2020). During pregnancy, there was no evidence of toxicity from high 25(OH)D concentrations or differences in maternal serum calcium, phosphorus, and urinary calcium/creatinine ratios or adverse pregnancy outcomes among the many women who have been supplemented with various combinations of vitamin D up to 110 μg/day or 1250 μg/week during pregnancy. There are some individuals, however, who could develop toxicity if not previously diagnosed with idiopathic hypercalcemia of childhood associated with Williams Syndrome (a rare genetic disorder that results in hypercalcemia with even modest vitamin D supplementation due to abnormal metabolic processing (Aravena et al., 2002) or from other metabolic genetic defects in vitamin D processing such as CYP24A1 mutations (Macdonald et al., 2020). Some studies show benefit to the pregnant woman and her developing fetus with improved vitamin D status (Rostami et al., 2018) yet others do not (Roth et al., 2018b), but none have shown toxicity with higher dosing within certain inclusion and exclusion entry criteria. Despite the known basic science aspects of vitamin D, clinical trials often fail to note differences in outcomes of supplementation during pregnancy due to a number of factors listed in **Table 1 (place Table 1 near here)**.

Based on animal and clinical studies looking at the effect of vitamin D status at baseline early in pregnancy and pregnancy outcomes (Liu et al., 2013, Dawodu et al., 2013, Chan et al., 2015, Mirzakhani et al., 2016, Hewison, 2018), it would appear that the most profound effects of early supplementation are seen in those with the greatest deficiency states at baseline (Ganguly et al., 2018, Rostami et al., 2018). In addition, it would appear that the effects are seen more notably when total circulating 25(OH)D is used as the biomarker of vitamin D status during pregnancy rather than analysed on an intention-to-treat (ITT) basis. Higher dose effects are more apparent in those who follow the prescribed supplementation regimen and when the analysis is on a *per* protocol basis (Abercrombie et al., 2018). Another consideration is that in a classic drug study with ITT analysis, the concentration of the drug in question starts at 0 in both the control and treatment group(s); yet, in nutrient studies such as vitamin D supplementation trials, no one starts at a concentration of zero. For this reason, Heaney outlined the criteria that should be met for all nutrient studies to avoid this faulty design (Heaney, 2014). The other question that continues to plague vitamin D studies is what is really representative of that nutrient—in this case, vitamin D? Is it the parent compound or a metabolite—25(OH)D, and is that metabolite really indicative of what is going on at the cellular level? Should the metabolite be measured in its bound form or its free state (Tsuprykov et al., 2019, Best et al., 2019)?

Best et al provide a longitudinal examination of the changes in various moieties of vitamin D during adolescent pregnancy and included in their analyses free circulating 25(OH)D measurement as well as VDBP (Best et al., 2019). In their analysis of 58 adolescent pregnant

females, the authors found that regardless of race or visit, total 25(OH)D was a stronger correlate of PTH, 1,25$(OH)_2$D, and of the catabolite 24,25$(OH)_2$D than free circulating 25(OH)D, and neither total nor free 25(OH)D was related to serum calcium. While African American pregnant girls had lower total 25(OH)D ($p < 0.0001$), their free 25(OH)D did not significantly differ by race ($p = 0.2$). Of interest, in these pregnant adolescents, VDBP concentration was elevated and inversely associated with the percent free 25(OH)D, but measured free 25(OH)D provided no advantage over total 25(OH)D as a predictor of PTH, 1,25(OH)2D, 24,25(OH)2D, or calcium. Thus, it appears for the moment that total circulating 25(OH)D concentration is the best indicator of vitamin D status.

Other issues in assessing vitamin D status during pregnancy and its relevance to outcomes includes the question of whether baseline 25(OH)D in a study represents the 25(OH)D concentration circulating around the time of conception. Vitamin D supplementation as a treatment is plagued with nonadherence whereas using 25(OH)D as the biomarker is not. There are also issues of whether or not total circulating 25(OH)D is reflective of vitamin D metabolism within the cell, especially with variable bolus dosing. Perhaps it is baseline 25(OH)D in early pregnancy or even preconception that is more relevant in showing the effect of vitamin D status on the fetal and maternal health, but to date, no trials have addressed circulating 25(OH)D at the time of conception and placental activity.

Systematic reviews and meta-analyses of vitamin D supplementation during pregnancy show mixed results in prevention of preeclampsia and preterm birth, which may reflect timing of supplementation initiation as well as study design in terms of dosing regimen and maternal vitamin D status starting point (as noted in Table 1). The differences between *in vitro* and animal studies results of vitamin D's role as an enabler and the often-null results of randomized controlled trials challenges us to explore alternative hypotheses and to design studies that integrate these divergent findings.

Another factor that could potentially affect study results includes estimation of sample size. On what do we base the sample size of a clinical trial? Is it anticipated treatment effects such as preeclampsia, hypertension, gestational diabetes, or changes in a particular relevant biomarker such as T cell phenotype? Another question is when in pregnancy do you look for effect? What biomarker is most appropriate? Should sample size be based on VDBP genotype, specific SNPs or ethnicity, all of which affect vitamin D metabolism? These questions impact study design but also data analysis. Such questions influence clinical study design and create limitations in our ability to interpret the data in a meaningful manner. As has been the case with the advent of scientific inquiry, the basis for what we prescribe clinically must have a demonstrated rationale that befits the data and the laboratory, and which can be translated to bedside practices with continued meaning and relevance. Without study design that incorporates the nature of the system—in this case a hormonal system that has many functions—we will fail to see benefit and make conclusions that might be premature or biased. Through careful understanding of cellular mechanisms that may be influencing its demonstrated effect and a broader systems-based approach across mother, placenta and fetus, we will move closer to a greater understanding of the dynamic effect of vitamin D during pregnancy on both the mother and her developing fetus.

Alternative perspective based on evidence from published RCTs of prenatal vitamin D supplementation

While acknowledging the well-justified enthusiasm for the potential benefits of improving vitamin D status in the prenatal period, Dan Roth (DR) presented a summary of selected high-quality RCTs showing insufficiently convincing evidence to merit widespread changes to clinical practice guidelines or policy (Roth et al., 2018a). As of the end of 2019, the World Health Organization (WHO) advises that, "vitamin D supplementation is not recommended for pregnant women to improve maternal and perinatal outcomes" (World Health Organization, 2016). While there is little doubt that prenatal vitamin D supplementation improves maternal and fetal vitamin D status in a dose-dependent manner, the clinical benefits to the mother and baby remain a subject of debate (Palacios et al., 2019b, Roth et al., 2017, Palacios et al., 2019a). The question is whether *additional* vitamin D during pregnancy, over and above that required by a non-pregnant woman, is required. To illustrate how prenatal vitamin D RCTs have so far failed to provide robust evidence of clinical benefits, it is instructive to consider five published blinded RCTs that each included at least 500 enrolled women, were generally of high-quality, and for which publication in reputable journals suggests they underwent thorough peer review (**Table 2**) **Place Table 2 near here**. None of these five trials individually demonstrated a beneficial effect of the vitamin D intervention on their primary outcomes, as reported in the original trial publications (Table 3). For other secondary outcomes of interest (e.g., birth weight, gestational age at birth or preterm, neonatal or infant hospitalization, or gestational hypertension or preeclampsia), none of these trials provided strong evidence of benefits of vitamin D (i.e., either the outcome was reported and the effect was null or non-significant, or the outcome was not reported) (Roth et al., 2018b, Litonjua et al., 2016, Cooper et al., 2016, Chawes et al., 2016, Hollis et al., 2011). Importantly, these trials reported adverse event monitoring and none have raised major safety concerns, so the overall inference is that prenatal vitamin D supplementation safely raises 25(OH)D without clearly providing other benefits to pregnant women and babies.

However, further efforts to unpack the results of some of these trials have led to alternative interpretations based on sub-group effects, post-hoc secondary analyses, and longer-term follow-up studies. For example, in their original trial report, Hollis et al. (2011) did not find any significant differences in maternal and neonatal clinical outcomes (Hollis et al., 2011); however, in a post-hoc analysis adjusting for race, published in 2013, Hollis and Wagner wrote that, "the data from our RCT strongly suggest that vitamin D supplementation during pregnancy can significantly decrease complications of pregnancy including primary caesarean section ($p=0.046$), hypertensive disorders of pregnancy ($p=0.05$) and comorbidities of pregnancy ($p=0.03$)" (Hollis and Wagner, 2013). This particular interpretation of the trial continues to be cited as direct evidence of the clinical benefits of high-dose prenatal vitamin D supplementation (Hollis, 2019). In the primary report of the MAVIDOS trial, a planned sub-group analysis indicated that vitamin D supplementation increased bone mineral content in newborns delivered in the winter, in contrast to the overall null findings of the trial (Cooper et al., 2016). Yet, conference presentations, press releases and news stories about MAVIDOS frequently highlighted this sub-group effect and emphasized the potential benefits of vitamin D (Grey and Bolland, 2016). Two of the five major trials (VDAART and

COPSAC) had similar primary outcomes related to early-childhood wheeze and asthma, and the pooled analysis of these trials indicated that vitamin D significantly reduced the incidence of asthma or recurrent wheeze in the offspring up to three years of age (Wolsk et al., 2017a). However, follow-up of the children in one of the trials showed no sustained effects, such that the prevalence of asthma diagnoses by age 6 years was similar between the vitamin D and control groups (Brustad et al., 2019).

Researchers and practitioners who conduct and interpret vitamin D RCTs are faced with a dilemma when the primary results of a trial conflict with – or are less convincing than – other *post-hoc* or alternative analyses of the same data, especially if the latter analyses support their original hypothesis. Retaining the original randomized design, including all participants in the analyses, and applying an intent-to-treat approach are all strategies that serve to reduce biases, but there is often an appeal to dissect the data in other ways that are believed to reveal hidden truths about physiology that may be obscured by the bluntness of the RCT approach (**Figure 2**). Conventional analysis of a RCT is by no means a sure-fire path to clarity, and no trial is without its limitations. For example, the placebo-controlled dose-ranging RCT of prenatal vitamin D in Bangladesh from Roth and colleagues was specifically designed to test whether vitamin D improves infant growth in a setting where stunting is common (Roth et al., 2018b). It was not designed or powered to examine effects on uncommon maternal or perinatal outcomes, and it is possible that the initiation of supplementation in the 2nd trimester was too late to affect some outcomes such as preeclampsia (Roth et al., 2018b). Other aspects of the trial may have limited its generalizability to the broader population of Bangladeshi women (e.g., perhaps trial participants were healthier than average) or to populations outside of Bangladesh (e.g., perhaps the trial participants' concurrent nutritional deficiencies constrained the effects of vitamin D). **Place Figure 2 near here**

Unfortunately, systematic reviews and meta-analyses of RCTs can further confuse rather than clarify the state of the evidence, as their conclusions can only be as robust as the underlying evidence on which they are based (Roth et al., 2017). For example, authors of the recent update of the Cochrane Collaboration systematic review of vitamin D in pregnancy had to rely on mostly small trials of low or questionable quality to draw conclusions about the effects of vitamin D (versus placebo) on maternal and neonatal outcomes (Palacios et al., 2019a). In summarizing one of the meta-analyses, Palacios *et al*. wrote that, "supplementation with vitamin D probably reduces the risk of pre-eclampsia compared to no intervention or placebo (risk ratio (RR 0.48, 95% CI 0.30 to 0.79)" and they considered this to be based on "moderate-certainty evidence" (Palacios et al., 2019a). This conclusion is reiterated prominently in the abstract and plain-language summary, and if true, would be of considerable public health importance. However, scrutiny of the four trials on which this finding was based reveals the weakness of the conclusion. The aggregated sample size was only 499 women (Palacios et al., 2019a), far lower than what would be required in a primary prevention trial designed for precise estimation of effects of a prenatal intervention on preeclampsia. Only one of the four trials reported having any type of prospective ascertainment protocol and standardized case definition for preeclampsia (Behjat Sasan et al., 2017), and all of the published trial reports lack sufficient information to confidently establish the reliability of their findings (Asemi et al., 2013, Sablok et al., 2015, Behjat Sasan et al.,

2017, Naghshineh and Sheikhaliyan, 2016). Furthermore, reviews that incorporated other trials (including those in which control groups received a low-dose of vitamin D) do not support a beneficial effect of additional vitamin D for prevention of preeclampsia, at least not beyond the amount in conventional prenatal multiple micronutrient supplements (5-15 μg/day) (Palacios et al., 2019b, Roth et al., 2017).

To date, there have not been *any* rigorous and adequately powered RCTs of prenatal vitamin D supplementation for the prevention of hypertensive diseases of pregnancy or other adverse maternal outcomes (Palacios et al., 2019b, Roth et al., 2017, Palacios et al., 2019a). Authors of reviews or commentaries may conclude that vitamin D prevents pregnancy complications if their meta-analysis was methodologically flawed (e.g., biased study selection, errors in data abstraction) (Fogacci et al., 2019) or focused entirely on observational studies that cannot provide evidence of causal effects (Tous et al., 2019, Zhou et al., 2017, Yuan et al., 2019). In a recent article, Hollis recently wrote that, "Most of these clinical trials have validated the positive effects of prenatal vitamin D on birth outcomes …," (Hollis, 2019) citing four published articles to support his claim: 1) an Iranian study that reported a lower frequency of preeclampsia, preterm delivery and adverse pregnancy outcomes at a hospital that conducted screening for vitamin D deficiency and provided supplementation, compared to a control hospital in another city (Rostami et al., 2018) (limitations of this study were highlighted in a letter to the editor (Shub and McCarthy, 2019)); 2) a secondary analysis of VDAART that examined asthma/wheeze outcomes but did not report on any birth outcomes (Wolsk et al., 2017b); 3) another secondary analysis of VDAART that found there was no effect of vitamin D supplementation on preeclampsia risk based on the RCT design, but did find an association between 25(OH)D and preeclampsia using a cohort analysis (Mirzakhani et al., 2016); and, 4) an observational study of the association between 25(OH)D and preterm birth (McDonnell et al., 2017). There are certainly many studies suggesting that prenatal vitamin D supplementation *may* have benefits, including some small RCTs; however, an unbiased assessment must take stock of the entire body of evidence, taking into account study design and quality.

A pragmatic approach - setting the maternal vitamin D requirement to safeguard the fetal skeleton by targeting threshold 25-hydroxyvitamin D concentrations

MK proposed the possibility that given the various determinants of perinatal outcomes, including personal, genetic and healthcare factors, as well as overall diet and nutrition, it may be a too tall order to expect a single nutrient intervention to deliver dramatically improved outcomes. This may be true both in low resource settings, where malnutrition is common, or where there are no maternal or neonatal supplementation policies, but also in countries with well-nourished, healthy mothers who have 25(OH)D status at baseline in excess of 50 nmol/L and excellent obstetric care. Irrespective of the potential benefits of *additional* 25(OH)D to enable the establishment and maintenance of a healthy pregnancy, the complete dependence of the fetus and newborn infant on maternal 25(OH)D concentrations has been consistently reported (Hollis and Pittard, 1984b). It is widely accepted that at minimum, very low 25(OH)D (<25-30 nmol/L) during pregnancy should be prevented to protect fetal skeletal development (Munns et al., 2016). As cord 25(OH)D concentration is usually lower than the

corresponding maternal concentration (Hollis and Pittard, 1984b) and others, prevention of maternal 25(OH)D below 25-30 nmol/L may not provide fetal and neonatal protection (Kiely et al., 2016). Current recommendations for vitamin D during pregnancy do not consider the vitamin D requirement of newborn infants and assume the immediate availability of an adequate supply from early life. Therefore, Kiely et al (2017a) proposed prevention of neonatal vitamin D status <25-30 nmol/L, indicated by umbilical cord 25(OH)D, as a potential target for defining maternal vitamin D requirements during pregnancy, consistent with prevention of nutritional rickets (Munns et al., 2016). In a placebo-controlled dose-response RCT, O'Callaghan et al (2018) reported that maintaining maternal 25(OH)D concentrations ≥ 50 nmol/L at the end of pregnancy ensured that cord sera were all > 25 nmol/L. The vitamin D_3 intake required to maintain maternal 25(OH)D > 50 nmol/L in almost all mothers was 30 μg/day (1200 IU) (O'Callaghan et al., 2018), which is twice the current US and EU recommendation of 600 IU. This study confirmed suggestions from two RCTs in Canada (March et al., 2015) and New Zealand (Grant et al., 2014), that although 10 μg vitamin D per day may prevent maternal 25(OH)D falling below 30 nmol/L, a minimum of 25 μg/d might be needed to maintain newborn 25(OH)D at this concentration. Further similar dose-response studies are required in different racial and ethnic groups and in various settings to generate an inclusive estimate of maternal vitamin D requirements for the prevention of neonatal deficiency. Adequate consideration of calcium intake is required in these studies. Calcium is infrequently considered in RCTs of vitamin D and perinatal health although its role in the calcium metabolic system and maintenance of normal PTH concentrations is linked to perinatal health (Hofmeyr et al., 2014, Hemmingway et al., 2018).

Conclusions

Laboratory science is driving our understanding of the role of vitamin D in healthy pregnancy, most recently from the perspective of immune function. At a fundamental level, the role of vitamin D in skeletal development has been accepted for many years, but the particular needs of pregnant women and newborn infants, a high risk sector of the population, have been underserved. While clinical practice guidelines are potentially more responsive to new evidence, public health nutrition recommendations are infrequently updated, creating a lengthy gap between the clinical research evidence base and its translation into guidance. National/international policy implementation is often slow or non-existent, contributing to a wide dichotomy between the state of the art and public policy, manifested in gross inequalities in health care provision and poor outcomes for at risk mothers and babies. This is evidenced by the wide variation in vitamin D status and rates of nutritional rickets among at-risk groups within and between countries.

In the face of such uncertainty, where do we go from here? Even without strong evidence from RCTs, it would be reasonable and safe for public health authorities to take a population approach that aims to eliminate the prevalence of 25(OH)D <30 nmol/L and facilitate achievement of a personal target of 50 nmol/L, including among women of reproductive age (Kiely et al., 2017a; Roth et al., 2018a). In this scenario, fortification of staple foods with vitamin D (Roth et al., 2018a) and scale-up of use of antenatal multiple micronutrient

supplements (Sudfeld and Smith, 2019) (which include modest amounts of vitamin D) will likely achieve this aim and reduce the risk of severe vitamin D deficiency.

To determine if higher intakes of vitamin D are required in pregnancy, the optimal strategy would be to conduct a large-scale RCT of prenatal vitamin D supplementation that goes beyond the scope of any previous or ongoing RCT: multi-country collaboration (including both high- and lower-income settings), early first trimester (or preconception) enrolment, factorial design with calcium, and designed and powered for uncommon maternal outcomes (preeclampsia, intrauterine death), preterm birth, and longer-term child health outcomes (including nutritional rickets). Such a trial would be financially costly and logistically challenging, but should be strongly considered by an international consortium of researchers and funders.

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966 **Table 1. Factors Affecting Clinical Outcomes of Vitamin D Supplementation Trials**
967 **during Pregnancy**

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• Timing of intervention: when in gestation did supplementation occur
• Sample size
• Baseline serum 25(OH)D: e.g., the most profound effects of early supplementation seen in those with the lowest 25(OH)D concentrations at baseline
• Sunshine exposure
• Habitual vitamin D and calcium intake
• Race/ethnicity
• VDBP genotype
• Dosing frequency of vitamin D: daily vs. weekly vs. monthly dosing
• Adherence to dosing regimen
• Types of assays used to measure 25(OH)D concentration
• Type of analysis—*Intention-to-treat* vs. *per protocol*
• Biomarker used for vitamin D status: Is total circulating 25(OH)D concentration really the biomarker of what is going on at the cellular level during pregnancy? Should free 25(OH)D be measured? This is a question that comes up frequently.

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973 **Table 2. Characteristics and primary findings of five randomized controlled trials of**
974 **prenatal vitamin D supplementation***

	Hollis 2011(Hollis et al., 2011)	**Litonjua 2016 (VDAART)(Litonjua et al., 2016)**	**Cooper 2016 (MAVIDOS)** (Cooper et al., 2016)	**Chawes 2016 (COPSAC)(Chawes et al., 2016)**	**Roth 2018 (MDIG)(Roth et al., 2018b)**
Country	USA	USA	UK	Denmark	Bangladesh
Total N randomized	502	881	1134	623	1300
Highest vitamin D dose	4000 IU/day	4400 IU/day	1000 IU/day	2800 IU/day	4000 IU/day (given as 28,000 IU/week)
Control group vitamin D dose	400 IU/day	400 IU/day	Placebo	400 IU/day	Placebo
Primary outcome measure	Serum 25(OH)D concentration	Early-childhood asthma or recurrent wheeze	Neonatal bone mass	Early-childhood persistent wheeze	Infant length (at one year of age)
Effect of vitamin D on primary outcome	Strong positive effect	No significant effect	No significant effect	No significant effect	No significant effect

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976 *Trials selected if they were double-blind and enrolled >500 participants.

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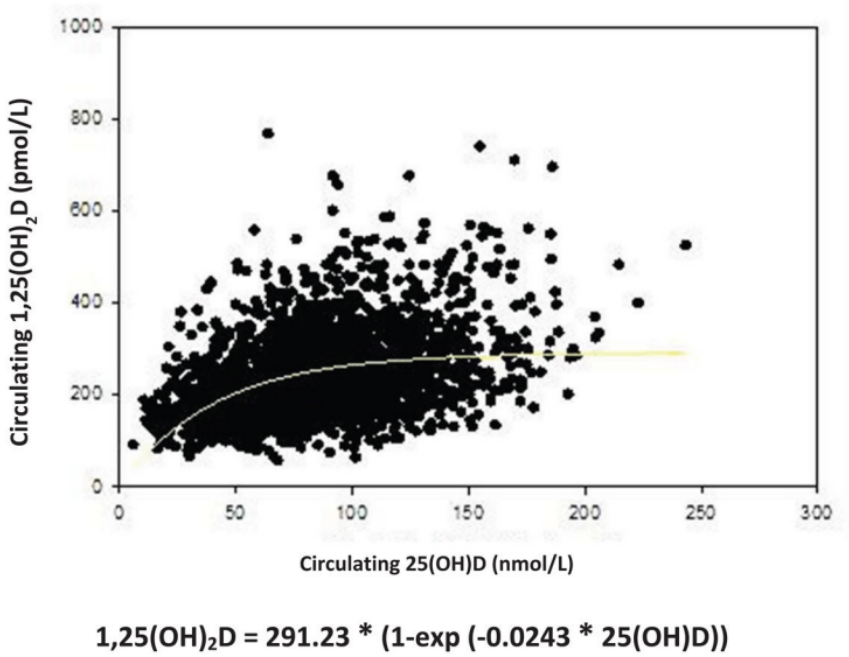

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981 **Figure 1. Substrate-Product Relationships of Vitamin D Metabolites during Pregnancy.**
982 This panel demonstrates the relationship circulating 25(OH)D to control the production of
983 1,25(OH)2D during pregnancy. All data points for all subjects in all groups were included in
984 this analysis.

985 (from Hollis et al, 2011, Figure 3b Panel b, with permission (Hollis, 2011))

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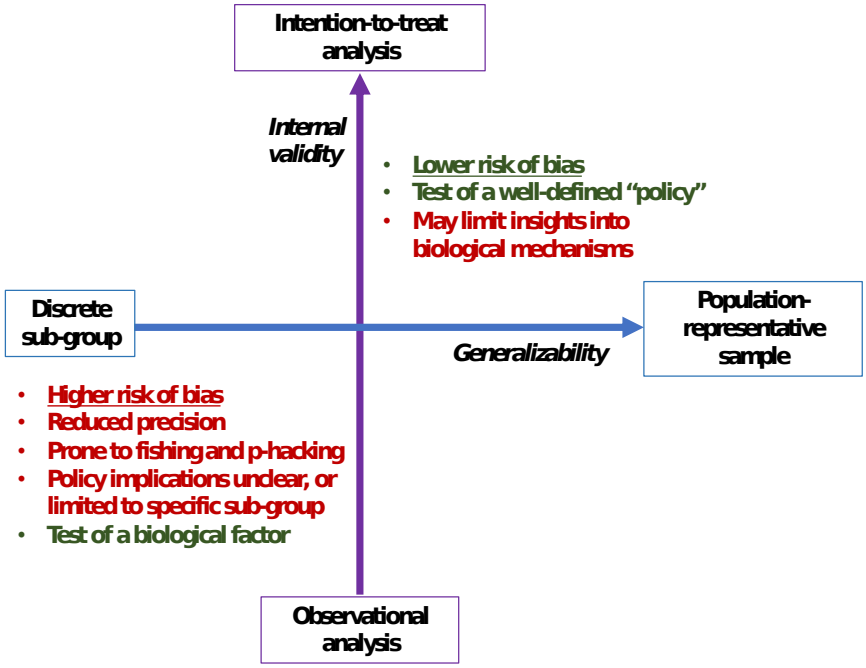

Figure 2. Schematic representation of the advantages and disadvantages of approaches available to investigators when analyzing data from a randomized controlled trial. The horizontal axis reflects the spectrum of choices related to the participant selection criteria. For example, a discrete sub-group analysis may involve restriction to participants with baseline 25(OH)D<30 nmol/L, whereas population-representative sampling would include all participants irrespective of baseline characteristics, adherence, or other non-random factors. The vertical axis reflects the spectrum of choices related to the assignment of the exposure variable. An intention-to-treat approach entails direct comparisons of the experimental versus control groups, relying only on the random assignment regardless of adherence or completeness of follow-up; in contrast, observational analyses usually ignore the random allocation and instead examine the association between an outcome and a non-random indicator of vitamin D status (e.g., 25(OH)D or number of vitamin D doses received).