Vitamin D biomarkers for Dietary Reference Intake development in children: A systematic review and meta-analysis

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Data Share Statement:

Data described in the manuscript, code book, and analytic code, will be made available upon request to the Principle Investigator pending publication of the paper.

***Alphabetized footnote of abbreviations*:**

25(OH)D, 25-hydroxyvitamin D

25(OH)D_2, 25-hydroxyergocalciferol

25(OH)D_3, 25-hydroxycholecalciferol

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

Bio-25(OH)D, bioavailable 25(OH)D;

C3-epimer, C3-epimer of 25(OH)D.

DRI, Dietary reference intakes

EFSA, European Food Safety Agency;

IOM, Institute of Medicine;

RCT, Randomized controlled trial;

PTH, parathyroid hormone

Abstract

Background: Circulating 25-hydroxyvitamin D (25(OH)D) has been the accepted vitamin D exposure/intake biomarker of choice within recent Dietary Reference Intake (DRI) exercises, but use of other vitamin D-related biomarkers as well as functional markers have been suggested. These may be of value in future vitamin D DRI exercises, such as the Food and Agriculture Organization-World Health Organisation's one for young children.

Objective: To systematically review the usefulness of circulating 25(OH)D, parathyroid hormone (PTH), free and bioavailable 25(OH)D, C3-epimer-25(OH)D, vitamin D_3, 24,25-dihydroxyvitamin D (24,25$(OH)_2$D) as well as bone turnover markers and calcium absorption as vitamin D biomarkers for DRI development in children.

Design: Methods included structured searches of published articles, full-text reviews; data extraction; quality assessment; meta-analysis and random-effects meta-regression.

Results: Fifty-nine vitamin D supplementation randomized controlled trials (RCTs) were included (39 in infants/children as the priority group and the remainder in adults where pediatric studies were absent/limited). Vitamin D supplementation significantly raised circulating 25(OH)D in infants and children, but the response was highly heterogeneous [weighted mean difference (WMD): 27.7 nmol/L; 95% CI: 22.9, 32.5; 27 RCTs; I^2=93%). Meta-regression suggested an increase by 1.7 nmol/L (95% CI: 0.7, 2.6) in serum 25(OH)D per each 100 IU increment in vitamin D intake (*P*=0.0005). Vitamin D supplementation had a significant effect on circulating 24,25$(OH)_2$D (WMD: 3.4 nmol/L; 95% CI: 2.4, 4.5; 13 RCTs; I^2=95%), with a dose-response relationship (+0.15 nmol/L per 100 IU; 95% CI: -0.01, 0.29). With circulating PTH, while there was a significant effect of vitamin D on WMD (*P*=0.05),

there was no significant dose-response relationship (*P*=0.32). Pediatric data were too limited in relation to the usefulness of the other biomarkers.

Conclusions: Circulating 25(OH)D may be a useful biomarker of vitamin D exposure/intake for DRI development in infants and children. Circulating $24,25(OH)_2D$ also showed some promise, but further data are needed, especially in infants and children.

Key words: Vitamin D exposure; biomarker; 25-hydroxyvitamin D, Systematic review; children

Introduction

Vitamin D deficiency has considerable implications for human health throughout life and impacts on healthy growth and development as well as successful ageing. Vitamin D (along with calcium and zinc) has been prioritized by the Food and Agriculture Organization (FAO) together with the World Health Organisation (WHO) as part of their update of nutrient requirements for children aged 0 to 36 months [1]. Dietary Reference Intakes (DRI) for vitamin D, as estimates of the dietary requirements for the vitamin, are crucial from a public health perspective in providing a framework for prevention of vitamin D deficiency and optimizing vitamin D status of individuals [2]. Within the risk assessment framework of setting DRIs, there is a need to clarify the relationship between the nutrient intake and the reference level of the critical indicator(s) of health outcomes for nutrient adequacy (contextualized in **Supplemental Figure 1**), taking into consideration sex, life-stage and vulnerable group [3]. Indicators of exposure within the DRI process are measures that correlate with dietary intake of a nutrient, such as nutrient biomarkers, nutritional status, or markers of nutritional status [4]. In relation to vitamin D, serum or plasma 25-hydoxyvitamin D [25(OH)D] has been used as an indicator of vitamin D exposure because it reflects combined dietary supply and cutaneous production [2]. However, given the multitude of vitamin D metabolites (such as vitamin D_3, free and bioavailable 25(OH)D, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], C3-epimer of 25(OH)D, vitamin D binding protein [VDBP]), there have been recent calls to investigate the possibility of inclusion of these as potential biomarkers of vitamin D status, either on their own or as part of a vitamin D panel [5-7].

Our previous systematic review and meta-analysis in 2009 assessed the efficacy of circulating 25(OH)D and parathyroid hormone (PTH), as well as more functional markers

(such as calcium absorption and bone markers), as vitamin D biomarkers [8]. Of note, this previous review of a total of 36 randomized controlled trials (RCTs) in all age-groups, only included data from a few RCTs of infants (*n*=1-2, depending on biomarker) or children (*n*=2-3, depending on biomarker), a factor that impeded a decision on whether 25(OH)D or PTH was useful in infants or children [8]. More recently, the European Food Safety Authority (EFSA) considered the other vitamin D metabolites as potential biomarkers of vitamin D status for use in their DRI review exercise [9], but these were considered for the entire population as opposed to for infants and children only. The WHO in their handbook for guideline development suggest that it is not always necessary to commission a new systematic review but an update of relevant, high-quality systematic reviews could be used as these are usually less expensive and time-consuming than new reviews [10].

Thus, the aim of this systematic review and meta-analysis was to update and expand on our previous review such that circulating 25(OH)D, PTH and other newer potential vitamin D biomarkers that could be considered in terms of their use in defining dietary requirements for vitamin D in children.

Methods for systematic review and meta-analysis

The systematic review and meta-analysis was registered with the PROSPERO International Prospective Register of Systematic Reviews (registration number: CRD42021234329). The methodology in the present work followed the general methods for systematic reviews in the area of serum 25(OH)D as applied by us previously [8,11-14] and again very recently [15], with brief specific details as follows:

Inclusion and exclusion criteria

The present review used a Population Intervention Comparison Outcome (PICO) framework which was aligned closely with that of the *Vitamin D or Calcium and Health Outcomes in Infants and Children Less than Three Years Old* systematic review as part of the FAO-WHO exercise (**Supplemental Table 1**). The *populations of interest* in this study were specified as generally healthy male and female infants and children (for all vitamin D biomarkers), but excluding studies in critically ill, premature infants (≤32 weeks gestational age) or very low birth weight infants (≤1500 grams). Generally healthy populations are defined as having ≤20% of the study population with disease at the study's baseline. Nutritional deficiencies are not considered to be diseases. While the FAO-WHO exercise is focussed on children aged 0-36 months, it has allowed for collection of data from older children (up to 9 years) in some circumstances where it was expected that data would be limited in the 1-3 year olds. This systematic review took a similar approach. While emphasis was on use of data from RCTs in infants/children, wherever possible, for some of the vitamin D biomarkers (e.g., vitamin D_3 and vitamin D metabolites) not covered in our previous review [8], RCT data from all age groups were also considered. This was in recognition of the limited data on such vitamin D biomarkers for infants/children.

In terms of *Interventions or exposures of interest*, studies were RCTs of vitamin D (with or without calcium) supplementation or vitamin D-fortified foods in apparently healthy humans that fulfilled all of the following characteristics: 1) in the case of children, vitamin D_3 or D_2 <63 μg/d (2500 IU, 1 μg = 40 IU), or as daily equivalents, in line with the US Tolerable Upper Intake Levels (ULs) for children 1-3 y [16], administered orally alone or with calcium. In the case of adults, vitamin D_3 or D_2 <100 μg/d (4000 IU), as per US and EU ULs [16,17], or

as daily equivalents, administered orally alone or with calcium, unless RCT data for a particular vitamin D biomarker only existed at higher doses; 2) taken daily, weekly or monthly (which have been shown to be comparable [18]; RCTs which supplied vitamin D less frequently than monthly (e.g., quarterly, annually) were excluded in line with recent DRI exercises [9,16]); 3) reported serum or plasma 25(OH)D after supplementation in at least one vitamin D intervention group and one control group, which could be a placebo group or a standard care dose of vitamin D; 4) no other co-administered hormones or pharmaceutical agents; and 5) 6-week minimum duration (vitamin D status has been shown to reach an equilibrium after 6-8 weeks of vitamin D supplementation, at least in adults (18-85 y) [19]).

Search strategy

The electronic searches for circulating 25(OH)D and PTH, originally performed on Ovid MEDLINE, EMBASE and Cochrane CENTRAL from inception to 25 September 2007 as part of our previous systematic review of biomarkers of vitamin D status [8], were updated for infants and children (aged 1 to 9 years). The update used the same structured search strategy as previously used [8] but in infants and children only, and until 25 September 2020. Electronic searches for biomarkers beyond circulating 25(OH)D and PTH were performed from inception to 25 February 2021. Relevant vitamin D systematic reviews were checked and any potential additional articles collected and assessed for inclusion.

Data collection

Screening of titles and abstracts for collection, screening full-text articles for inclusion, and data extraction from included studies all were performed by a single reviewer with independent duplicate assessment of 100% by a second reviewer.

Data synthesis

We extracted the number of participants included (and assessed) in each arm of each RCT plus means and SDs of the baseline and final values in the treatment and control arms for each vitamin D dose. In a small number of cases (5 studies) where the data on the biomarkers were only presented in graphical form within the paper, the observed means and SDs were estimated using a ruler divided in units of 1 mm, taking care with measurements.

In cases in which there were 2 or more intervention arms and 1 common control group (placebo or a standard care dose of vitamin D), only the highest dose of supplement and the control arm were used for the primary analysis. For subgrouping (e.g., by dose) and meta-regression analysis, the various arms compared with control were included as long as the arms fell into different dose range subgroups. In cases in which data were presented for 2 groups separately (e.g., data for boys and girls, white and dark-skinned, low-calcium and high-calcium intake groups, normal weight and obese, contraceptive use and non-contraceptive use, without and with calcium intervention, fortified milk and fortified orange juice), these were treated as 2 studies in the analysis.

An assessment of the risk of bias in the included RCTs in infants and children aged 1-9 y was performed using the Cochrane Collaboration's original Risk of Bias tool [20] and the following criteria: (i) random sequence generation, (ii) allocation concealment, (iii) blinding of participants and personnel and (iv) blinding of outcome assessment, (v) incomplete outcome data, and (vi) selective outcome reporting. Emphasis was placed on assessment with the priority markers of the present review in mind.

Egger's test for asymmetry of the funnel plot was used to test for detection of publication

bias [21]. This was only applied for biomarkers where there were at least 10 studies.

Definitions of free and bioavailable 25(OH)D

Approximately 85-90% of the total circulating 25(OH)D is tightly bound to its carrier protein, VDBP, which transports it between tissues but restricts access of 25(OH)D (and other vitamin D metabolites) to most cells. Thus, VDBP-bound 25(OH)D is considered biologically inactive [22]. Another 10-15% of 25(OH)D exists in a loose albumin-bound state, and <1% is present as free-circulating 25(OH)D [23]. This free 25(OH)D form is able to enter cells, where it can be further activated to $1,25(OH)_2D$ and perform biological actions, and thus is considered a bioavailable form. The loose albumin-bound 25(OH)D is able to dissociate rapidly in the circulation and is also biologically available for tissues. Thus, bioavailable 25(OH)D is generally recognized as 25(OH)D that is not bound to VDBP (i.e., free plus albumin-bound 25(OH)D).

Outcome measures

Primary outcomes were circulating total 25(OH)D, PTH, free 25(OH)D, bioavailable 25(OH)D, C3-epimer-25(OH)D, vitamin D_3, and $24,25(OH)_2D$ as well as bone turnover markers and calcium absorption. Secondary outcomes were circulating VDBP and $1,25(OH)_2D$, and as such, these markers were not searched specifically, but we took the opportunity to use available data on them from the collection of RCTs identified for the priority markers.

Statistical analysis

For the primary meta-analysis, we performed comparisons using all eligible studies with a random effects model [8] using Review Manager (RevMan) Version 5.4 (The Cochrane

Collaboration, 2020). Weighted mean differences (WMD) along with the 95% confidence intervals (CIs) were reported and presented as forest plots, where appropriate. An estimate of intervention effect was considered statistically significant based on *P* values less than or equal to an alpha level of 0.05, using two-sided tests. Heterogeneity between the studies was assessed using the I^2 statistic, where low, moderate, and high levels of heterogeneity were taken to be approximately correspond to I^2 values of 25%, 50%, and 75%, respectively [8]. If substantial heterogeneity was identified within a primary analysis, it was investigated using subgroup analyses and random effects meta-regression, where there was sufficient data. Meta-regression should generally not be considered when there are fewer than ten studies in a meta-analysis [24]. Subgroup analyses were performed using RevMan, whereas random effects meta-regression was conducted using R version 4.0.3 (R Core Team, Vienna, Austria, 2020) with the extension package "metafor".

Subgroup analysis and investigation of heterogeneity

Subgroup analyses were performed on the basis of vitamin D dose, pediatric population subgroup (infants, children aged 1-3 y, children aged 4-9 y), study design (i.e., vitamin D *v.* control [placebo or vitamin D *v.* standard care dose vitamin D]), baseline 25(OH)D (i.e., < or ≥ 30 nmol/L), latitude (i.e., < or ≥ 40^{o}), duration of study (6-14 wks, 15-37 wks, or ≥42 wks), and analytic techniques for assessment of 25(OH)D (competitive protein-binding assay [CPBA], immuno-based assay, or chromatography-based assay), depending on the biomarker. Where the number of studies allowed (i.e., ten or more), linear random effects meta-regression analysis of response of vitamin D biomarker to vitamin D dose was performed unadjusted and also in the case of some biomarkers, adjusted for covariates [24]. Selection of covariates for inclusion in the adjusted meta-regression models was informed

by subgroup analyses. The covariates for the adjusted meta-regression models with 25(OH)D were baseline 25(OH)D concentration (in nmol/L), latitude (in degrees), age (in months) as well as analytical method (categorical variable), and for PTH were latitude (in degrees) and calcium co-administered (in mg).

Sensitivity analyses

Sensitivity analyses were performed on the basis of presence of high risk of bias in any of the 7 domains within RCTs of infants and children aged 1-9 y, and also on the basis of non-assessment of compliance. The effects on results by excluding such RCTS was examined.

Categorization of biomarkers

The approach towards categorization of a vitamin D biomarker was an adaptation and evolution of that outlined previously by Hooper *et al*. [25] and used in our previous systematic review and meta-analysis [8]. Hooper *et al*. [25] categorized biomarkers as effective or ineffective on the basis of statistically significant and insignificant pooled effect size, respectively, when the pooling of data included ≥3 studies and ≥50 participants overall. In the present work, this was expanded this to incorporate the dose-response of vitamin D biomarker to increased vitamin D intake. Specificity and sensitivity are also important considerations in relation to nutritional biomarkers. Within the present work, specificity was accounted for by adjustment for covariates within the assessment of the dose-response of vitamin D biomarker. Biomarker sensitivity was considered from the perspective of noting any RCTs which reported biomarker values were below the limit of detection (LoD)/quantification (LoQ). Each vitamin D biomarker was deemed 'useful' in the context of DRI development based on a significant pooled effect size and evidence of a dose-response

even following adjustment for covariates, where the data permitted. In the case of sufficient data but a non-significant pooled effect size and lack of evidence of a dose-response, the vitamin D biomarker was deemed 'not useful'.

Results

In total, 1457 titles and abstracts were screened and the number of full papers assessed, as well as included/excluded in the review, is shown in **Figure 1**. For the update of circulating total 25(OH)D and PTH in infants and children aged 1-9 years, 27 RCTs were included [26-52]. Of the 27 studies, 15 RCTs were in infants and 12 RCTs in children aged 1-9 years (of which 4 were studies of children aged 1-3 years); all 27 studies provided extractable data on serum or plasma total 25(OH)D and 15 studies provided extractable data on serum/plasma PTH. For the other potential vitamin D biomarkers, only 6 studies were in pediatric subgroups (3 studies in infants and 3 studies in children aged <9 years) and thus the collection of RCTs was widened by inclusion of studies in other age groups (older children (>9 years), adults and older adults) [53-84]. Of the combined 32 studies of biomarkers beyond total 25(OH)D and PTH, 15 reported on circulating free 25(OH)D data, 4 on circulating bioavailable 25(OH)D data, 13 on circulating $24,25(OH)_2D$ data (and of which 4 also expressed data on the ratio of $24,25(OH)_2D$ to 25(OH)D), 6 on circulating C3-epimer of 25(OH)D data, 3 on circulating vitamin D_3 concentration, 9 on bone turnover marker data, as well as three studies included for investigation of calcium absorption.

Details of included RCTs (including some criteria of quality) are shown in **Table 1** and **Supplemental Table 2**. Of the combined 59 studies, 1 was in males only and 12 in females only (the remainder were mixed; 9 did not specify); 39 studies were in infants or children

and 20 studies in older age groups. Most studies (n = 53) provided vitamin D_3 supplementation, and 4 provided vitamin D_2; 2 did not specify. Five studies gave <400, 11 gave 400-600, 16 gave 800-1000, 16 gave 1200-2000, 7 gave 2001–4000, and 2 gave >4000 IU/d supplemental vitamin D (2 did not specify total dose).

Of the 59 RCTs, all had similar vitamin D biomarker concentrations in intervention and control arms at baseline, with the exception of serum 24,25$(OH)_2$D at baseline in one study [57]. For studies of free and bioavailable 25(OH)D, C3-epimer-25(OH)D, vitamin D_3, 24,25$(OH)_2$D as well as bone turnover markers and calcium absorption, circulating 25(OH)D was shown to be significantly increased by vitamin D supplementation in all studies, with the exception of one RCT where the significant increase evident at 3 months was not evident at endpoint (6 months) [32]. Therefore, the analyses for C3-epimer of 25(OH)D was performed including and excluding data from this study.

There was a wide range of participants per study arm (5-759 subjects). The studies were of adequate duration to reflect a change in vitamin D status. Approximately 37% and 73% of studies reported verification of the supplementation dose and reported compliance, respectively. There was a wide range in percentage of subject dropout (0-50% within a study arm) in these studies. In general, with the exception of the latter limitations, the included studies were of reasonable validity (**Supplemental Table 3** and **Supplemental Table 4**). The summary assessments of risk of bias across domains for the included RCTs of infants and children are shown in **Supplemental Figure 2** in 'Online Resource'. The majority of RCTs had either a low (57-61%) or unclear (36-39%) risk of selection bias (random sequence generation and allocation concealment), with only 4% having high risk in both domains. In relation to performance and detection bias, a majority of RCTs had either low or unclear risk

of bias for blinding of participants and personnel (68% and 25%, respectively) and outcome assessment (61% and 39%, respectively), with only 7% and 0% of RCTs having high risk in these domains, respectively. In relation to attrition bias, risk of bias in relation to incomplete outcome data was low for 46% of RCTs, unclear for 14% and high for 39%. Risk of bias for selective reporting was low (32%) or unclear (68%) in all RCTs.

A regression asymmetry test for bias risk of publications showed no significant evidence of bias (P>0.45, in all cases where there were ten or more studies for a biomarker).

Circulating total 25(OH)D in infants and children

The primary analysis was highly heterogeneous, but point estimates for 21 out of 27 studies in infants and children showed a statistically significant effect of vitamin D supplementation on circulating total 25(OH)D [random-effects weighted mean difference (WMD): 27.7 nmol/L; 95% CI: 22.9, 32.5; 27 studies; 4571 participants; I^2 = 93%; **Figure 2**]. Subgroup analysis on the basis of dose of vitamin D is shown in **Table 2** and suggested a statistically significant effect on circulating total 25(OH)D (treatment effects, P<0.0001 [except for the <400 IU/d dose, P=0.06] and between sub-group difference, P<0.0002), but with significant heterogeneity (I^2 = 84-94%). The relation between treatment effect for circulating 25(OH)D and dose of vitamin D is shown in **Figure 3**. A linear random effects meta regression analysis showed a significant association between dose of vitamin D and circulating 25(OH)D treatment effect (B=0.017; 95% CI: 0.007, 0.026, P=0.0005). There was no evidence of a non-linear response, albeit the dataset was limited (data not shown).

Subgroup analyses on the basis of pediatric population subgroup (infants, children aged 1-3 years, or children aged 4-9 years), study design (vitamin D *v.* placebo or vitamin D *v.*

standard dose vitamin D control) or duration of study (6-14 wks, 15-37 wks, or ≥42 wks), did not show significant between sub-group differences (*P*=0.09-0.30) (Table 3). There were significant between sub-group differences for baseline 25(OH)D < *v.* ≥ 30 nmol/L (*P*<0.01), latitude < *v.* ≥ 40^{o} (*P*<0.01), and analytic techniques for assessment of 25(OH)D (*P*<0.00001) and these did not generally reduce the level of heterogeneity substantially (Table 2). Inclusion of these covariates within a random effects meta regression model did not significantly influence the effect of dose on 25(OH)D (B= 0.021, 95% CI: 0.014, 0.028, *P*<0.0001). The analysis showed that serum 25(OH)D concentration increased by 2.1 nmol/L for every 100 IU of vitamin D intake after adjustment for baseline serum 25(OH)D, latitude, and analytic technique.

Sensitivity analysis in which any RCT with an adjudged high risk in any domain was excluded (*n*=12) showed a modest strengthening of the effect of vitamin D [random-effects WMD: 32.6 nmol/L; 95% CI: 23.7, 41.4; 14 studies; 2243 participants; *P*<0.00001, I^2 = 95%]. Exclusion of those RCTs which did not assess compliance (*n*=8) had little impact on the outcome [random-effects WMD: 28.4 nmol/L; 95% CI: 22.2, 34.6; 19 studies; 3838 participants; *P*<0.00001, I^2 = 93%]. None of the RCTs reported circulating total 25(OH)D concentrations below the LoD or LoQ, two which used LC-MS/MS for its measurement reported that serum 25(OH)D_2 concentrations were below the LoD or LoQ in some participants [36,75].

Using an update of the approach towards categorization of a vitamin D biomarker, as originally proposed by Hooper *et al*. [25], but which included the key element of dose-response of the biomarker to increased vitamin D intake, serum/plasma total 25(OH)D

appeared to be a useful vitamin D biomarker in infants and children, even though heterogeneity remained high.

Circulating PTH in infants and children

Eleven studies compared supplemental vitamin D with placebo and 4 RCTs compared supplemental vitamin D with standard care dose vitamin D control on circulating PTH. Combining all of the 15 studies suggested a statistically significant effect on circulating PTH [random-effects WMD: -0.20 pmol/L; 95% CI: -0.39, -0.00; 15 studies; 1613 participants; I^2 = 52%; **Figure 4**]. While a significant effect overall (*P*=0.05), only four of the 15 point estimates showed a statistically significant effect of vitamin D supplementation on circulating PTH. Subgroup analysis on the basis of dose of vitamin D is shown in **Table 3** and suggested a statistically significant sub-group difference (*P*<0.006). Only vitamin D doses in the range 800-1000 IU/d, but not <400, 400-600 or 1200-2000 IU/d, had a significant effect on circulating PTH (*P*<0.0001). A linear random effects meta regression analysis showed that for vitamin D dose was not a significant predictor of circulating PTH response (*P*=0.32). There was no evidence of a non-linear response, albeit the dataset was limited (data not shown).

Further subgroup analyses suggested there were no significant between sub-group differences when subgrouped on the basis of pediatric population (infancy or children aged 1-9 years [there was only 1 RCT in children aged 1-3 years]) (*P*=0.86) or study design (*P*=0.35) (Table 3). There were borderline significant sub-group differences when subgrouped by calcium co-administration (addition of calcium in both arms *v.* no calcium) and latitude (*P*=0.08 in both cases) (Table 3). Inclusion of these covariates within the linear random effects meta regression model showed that vitamin D dose remained non-

significant (*P*=0.22).

Sensitivity analysis showed that exclusion of the two RCTs which did not assess compliance had little impact on the outcome [random-effects WMD: -0.22 pmol/L; 95% CI: -0.43, -0.01; 13 studies; 1480 participants; *P*=0.04, I^2 = 57%], whereas exclusion of any RCT with an adjudged high risk in any domain (*n*=5) suggested a lack of significant effect of vitamin D [random-effects WMD: -0.11 pmol/L; 95% CI: -0.33, 0.11; 14 studies; 1340 participants; *P*=0.34, I^2 = 58%]. Two RCTs reported baseline circulating PTH concentrations below the LoD in 80% of infants [40] and a minority of children [43]. One child had a circulating PTH concentration below the LoD at the endpoint visit (group not indicated) in the RCT by Mortensen *et al*. [43].

Overall, serum/plasma PTH did not appear to be an effective vitamin D biomarker in infants or children aged 1-9 years.

Circulating 24,25(OH)$_2$D and 24,25(OH)$_2$D to 25(OH)D ratio

Thirteen studies (11 RCTs, of which 2 each had two distinct intervention groups beyond just differing vitamin D dose) reported on the effect of vitamin D supplementation on circulating 24,25(OH)$_2$D concentrations [random-effects WMD: 3.45 nmol/L; 95% CI: 2.35, 4.54; 13 studies; 818 participants; I^2 = 95%; **Supplemental Figure 3A**]. Removal of the RCT in which baseline serum 24,25(OH)$_2$D concentrations differed at baseline between placebo and intervention group [56] has no material impact on the significant effect of vitamin D supplementation [random-effects WMD: 3.29 nmol/L; 95% CI: 2.16, 4.42; 12 studies; 756

participants; I^2 = 94%] (data not shown). Subgroup analysis on the basis of dose of vitamin D suggested a statistically significant effect on circulating 24,25$(OH)_2$D concentrations (treatment effects, *P*<0.0001 for all subgroups, and between sub-group difference, *P*=0.0002) but with significant heterogeneity (I^2 = 89-93%). A linear random effects meta regression analysis showed a borderline significant association between dose of vitamin D and circulating 24,25$(OH)_2$D treatment effect (B=0.0015; 95% CI: -0.0001, 0.0029, *P*=0.051). Five of the 13 RCTs were in pediatric subgroups (3 studies in infants and 2 studies in children; random-effects WMD: 3.51 nmol/L; 95% CI: 0.02, 7.01; 5 studies; 211 participants; I^2 = 91%) and subgroup analysis on the basis of population subgroup showed that there was no significant between sub-group difference (*P*=0.84) and high heterogeneity (I^2 = 91-93%).

Four studies reported the ratio of 24,25$(OH)_2$D to 25(OH)D and inclusion of that data suggested a significant (*P*<0.00001) effect of vitamin D supplementation [random-effects WMD: 0.02; 95% CI: 0.01, 0.02; 4 studies; 307 participants; I^2 = 44%; **Supplemental Figure 3B**]. None of the studies were in infants or children.

Overall, there was some evidence that circulating 24,25$(OH)_2$D concentration was an effective biomarker of vitamin D overall in the population, and likely in infants or children, even though heterogeneity was high. Data were too limited in relation to usefulness of the ratio of 24,25$(OH)_2$D to 25(OH)D as a vitamin D biomarker.

Circulating free 25(OH)D

Fifteen studies (11 RCTs, of which 4 each had two distinct intervention groups beyond just differing vitamin D dose) reported on the effect of vitamin D supplementation on circulating free 25(OH)D (either measured directly via immunoassay and/or calculated) [random-

effects WMD: 8.31 pmol/L; 95% CI: 6.23, 10.40; 15 studies; 934 participants; I^2 = 92%; **Supplemental Figure 4**]. Subgroup analysis on the basis of dose of vitamin D suggested a statistically significant effect on circulating free 25(OH)D (treatment effects (*P*<0.0001 for all subgroups), but no significant between sub-group difference (*P*=0.46) and with significant heterogeneity (I^2 = 86-96%). A linear random effects meta regression analysis showed that vitamin D dose was not a significant predictor of circulating free 25(OH)D response (*P*=0.19). There was no evidence of a non-linear response, albeit the dataset was limited (data not shown). None of the RCTs were in infants or children and thus subgrouping by population group was not performed.

Two of the 11 RCTs (both with two distinct intervention groups each) reported circulating free 25(OH)D as a percentage of total 25(OH)D and analysis of this limited dataset suggested a lack of significant (*P*=0.45) effect of vitamin D supplementation [random-effects WMD: -0.00 %; 95% CI: -0.00, 0.00; 4 studies; 384 participants; I^2 = 61%] (data not shown).

Overall, serum/plasma concentration of circulating free 25(OH)D did not appear to be an effective vitamin D biomarker in adults. Data were too limited in relation to serum/plasma circulating free 25(OH)D expressed as a percentage of total 25(OH)D. There was a lack of data for both indices for infants and children.

Circulating bioavailable 25(OH)D

Four RCTs reported on the effect of vitamin D supplementation on circulating bioavailable 25(OH)D (calculated) [random-effects WMD: 2.32 nmol/L; 95% CI: 1.19, 3.46; 4 studies; 591 participants; I^2 = 95%; **Supplemental Figure 5**]. None of the RCTs were in infants or children.

There were too few studies to perform sub-group analysis by vitamin D dose or random effects meta regression analysis. Data were too limited in relation to the usefulness of circulating bioavailable 25(OH)D as a vitamin D biomarker in adults and there was a lack of data for infants and children.

Circulating C3-epimer of 25(OH)D

Six RCTs reported on the effect of vitamin D supplementation on circulating C3 epimer of 25(OH)D [random-effects WMD: 6.16 nmol/L; 95% CI: 3.56, 8.75; 6 studies; 260 participants; I^2 = 97%; **Supplemental Figure 6**]. One of these RCTs was in pregnant women [75], but exclusion of that data from the analysis did not change the overall outcome [random-effects WMD: 9.40 nmol/L; 95% CI: 5.64, 13.15; 5 studies; 176 participants; I^2 = 97%; P<0.00001] (data not shown). There were too few studies to perform sub-group analysis by vitamin D dose or random effects meta-regression analysis. One RCT reported baseline serum C3 epimer of 25(OH)D concentrations below the LoD in 2 out of 142 pregnant women [75]. Data were too limited in relation to the usefulness of circulating C3 epimer of 25(OH)D as a vitamin D biomarker.

Circulating vitamin D_3

Three RCTs reported on the effect of vitamin D supplementation on circulating vitamin D_3 [random-effects WMD: 24.7 ng/ml; 95% CI: 3.0, 46.4; 3 studies; 108 participants; I^2 = 92%; **Supplemental Figure 7**]. There were too few studies to perform sub-group analysis by vitamin D dose or random effects meta-regression analysis. One RCT reported baseline circulating vitamin D_3 concentrations below the LoQ in 16 out of 62 postmenopausal women [57]. Data were too limited in relation to the usefulness of circulating vitamin D_3 as a

vitamin D biomarker in adults and there was a lack of data for infants and children. It should also be noted that the intervention dose used in two of the RCTs was above the UL (at 6400 IU/d [64] and 11,0000 IU/d [81] respectively) and also one of which was in lactating women [81].

Circulating VDBP and $1,25(OH)_2D$

Ten studies (5 RCTs, of which 3 each had two or more distinct intervention groups beyond just differing vitamin D dose) reported a non-significant (P=0.16) effect of vitamin D supplementation on circulating VDBP concentrations [random-effects WMD: -5.81 nmol/L; 95% CI: -13.97, 2.36; 10 studies; 1046 participants; I^2 = 0%] (data not shown). Subgrouping by type of antibody (monoclonal *v.* polyclonal) used for measurement of VDBP suggested no significant (P=0.24) sub-group difference. None of the studies were in infants or children. Three RCTs in infants/children reported a non-significant (P=0.51) effect of vitamin D supplementation on circulating $1,25(OH)_2D$ concentrations [random-effects WMD: 1.16 pmol/L; 95% CI: -2.31, 4.64; 3 studies; 155 participants; I^2 = 0%] (data not shown).

Bone markers

Eight and 7 RCTs assessed the effect of vitamin D supplementation on serum osteocalcin and serum bone-specific alkaline phosphatase (markers of bone formation), respectively, while 2 RCTs accessed the effect on serum carboxy-terminal telopeptide (CTx), 3 on urinary pyridinoline (Pyr), and 4 on urinary deoxypyridinoline (Dpyr) (markers of bone resorption). Some of these RCTs had more than one distinct interventions groups. Meta-analysis of

these studies in children showed a non-significant (P>0.22, in all cases) effect of vitamin D supplementation on circulating bone biomarker concentrations (**Table 4**). Subgrouping serum osteocalcin and serum bone-specific alkaline phosphatase by vitamin D dose also failed to show any significant effects (P>0.44, for both). There were too few studies to perform random effects meta regression analysis. Data were too limited in relation to the usefulness of bone markers as a vitamin D biomarker in children.

Intestinal calcium absorption

Pooling data from 3 studies in children (one with two distinct intervention groups) showed a non-significant (P=0.49) effect of vitamin D supplementation on intestinal calcium absorption [random-effects WMD: 1.3 %; 95% CI: -2.4, 4.9; 3 studies; 230 participants; I^2 = 0%] (data not shown). There were too few studies to perform sub-group analysis by vitamin D dose or random effects meta regression analysis. Data were too limited in relation to the usefulness of intestinal calcium absorption as a vitamin D biomarker in children.

Discussion

The present work which focussed on infants and children as WHO priority age-groups, updates and extends our previous systematic review of existing and potentially novel vitamin D biomarkers [8], which only included data from a few RCTs of infants or children and, consequently, impeded us at the time from making a judgment of the usefulness of circulating total 25(OH)D or PTH. The present meta-analysis, including unadjusted and adjusted random effects meta-regression, showed that circulating 25(OH)D responded to

increased vitamin D intake in infants (*n*=15 studies) and children (*n*=12 studies), in line with the findings of other systematic reviews in the wider population [8,11,85,86].

Circulating 25(OH)D concentration was used as an indicator of vitamin D exposure/status by the US Institute of Medicine [18] and several European authorities [11,87-89] in their respective vitamin D DRI exercises since 2011. It should be noted, however, that these agencies established the vitamin D intake-serum 25(OH)D relationship from RCTs performed in winter and at latitudes where UVB availability for vitamin D synthesis in the skin was limited [9,16,87-89]. As the WHO vitamin D DRI exercise for children has a global focus, we included RCTs irrespective of season and latitude, and in this sense, it is noteworthy that circulating 25(OH)D was associated with increased vitamin D intake even in the likely presence of appreciable UVB availability in some RCTs. Latitude, together with differences in other RCT characteristics (such as baseline 25(OH)D, dose, and analytical methods), likely contributed to the high level of heterogeneity evident in our analyses, in common with that observed in other systematic reviews [8,11,85,86,90]. Aside from that, all other *a priori* criteria as defined by Hopper *et al*. [25] to claim that circulating 25(OH)D is an effective marker, but also, importantly, evidence of dose-response, were met in infants and children. Circulating PTH concentration and its relationship with 25(OH)D concentration (via the $1,25(OH)_2D$ pathway) has been suggested as a possible biomarker of vitamin D status [9,87]. As plasma 25(OH)D concentration increases, plasma PTH falls. The present review, unlike our previous one [8], had sufficient studies (*n*=15) to allow us make a judgment that circulating PTH was not useful as a vitamin D biomarker in pediatric population subgroups. There was a lack of dose-response in the meta-analysis and the random effects meta-regression, even accounting for potential confounding effects of simultaneously altered

calcium intake, and no indication of a non-linear response of PTH which might occur due to potential threshold effects with increasing vitamin D intake. It has been suggested that while circulating PTH concentration can be indicative of clinical vitamin D deficiency, its use as a marker of vitamin D status is hindered by a number of uncertainties, such as the nature of the 25(OH)D-PTH relationship [16] and several non-vitamin D-related determinants of PTH concentration [87]. For such reasons, the EFSA vitamin D DRI Panel in 2016 concluded that from a population-wide perspective, serum PTH concentration is not a biomarker of vitamin D intake and in healthy subjects it is not a useful biomarker for vitamin D status as assessed by serum 25(OH)D concentration [9].

The ratio of serum 24,25$(OH)_2$D to 25(OH)D concentration has also been suggested as an indicator of vitamin D deficiency, including suggested thresholds [72,91,92]. The current meta-analyses and random effects meta-regression (unadjusted) suggested that circulating 24,25$(OH)_2$D concentration responded to increased vitamin D intake, albeit again with large heterogeneity. However, of the 13 available studies, only five were in infants or children and thus too limited to investigate the dose response in these pediatric populations. In addition, there was insufficient RCT data to explore the dose-response of 24,25$(OH)_2$D:25(OH)D to increased vitamin D intake. Thus, additional RCT data on the response of circulating 24,25$(OH)_2$D and 24,25$(OH)_2$D:25(OH)D to increased vitamin D supplementation would be of value in clarifying the usefulness of this potential biomarker for pediatric population subgroups, as well as other age-groups.

Serum free 25(OH)D, bioavailable 25(OH)D, C3-epimer of 25(OH)D and vitamin D_3 have also been suggested as potential biomarkers of vitamin D status based on their involvement in the vitamin D metabolic pathways, and some having potential direct physiological vitamin D

effects [8, 56, 93,94]. While the present review found evidence of significant treatment effects of vitamin D supplementation in the respective meta-analyses for these four vitamin D biomarkers, there was either no significant dose-response effect (free 25(OH)D) or insufficient RCT data to investigate dose response effects by random effects meta-regression analysis (remaining 3 biomarkers). In addition, there was significant heterogeneity in all cases, and there were either no or a very limited number of RCTs with available data for infants and children. Furthermore, unlike serum 25(OH)D, PTH and, to a lesser extent, $24,25(OH)_2D$, where threshold concentrations linked to vitamin D deficiency or health outcomes have been proposed [5,9,16,87-89,91,92], these have not been proposed, as yet, for these other vitamin D biomarkers. Thus, their ability to distinguish individuals with and without vitamin D deficiency remains unclear. Threshold concentrations for these other vitamin D biomarkers would be critical in deriving DRI values from the vitamin D intake-biomarker dose response relationships. While intestinal calcium absorption and bone markers have also been suggested as potential functional markers of vitamin D status in adults [8,56,93-95], the present meta-analysis highlighted a lack of effect of vitamin D supplementation on intestinal calcium absorption or bone markers in children and insufficient RCT data to investigate dose effects by random effects meta-regression analysis. The insufficient data also negated any investigation of potential confounding factors that may speak to the specificity of these biomarkers. Thus, at this point in time, these newer vitamin D biomarkers are likely not to be of use in vitamin D DRI development and more research is needed to fill data gaps.

The present work had some limitations, especially in relation to the lack of RCTs in infants and children which reported data on some of the newer vitamin D biomarkers. The lack of

sufficient RCT data limited an investigation of dose-response in several cases. The present work was also hindered by limitations of some of the included RCTs in terms of their design, which likely contributed to the heterogeneity (as discussed elsewhere [8,25]). Depending on the domain, only between one and two-thirds of RCTs were deemed to be of low risk of bias, and over a third had high risk of bias in terms of incomplete outcome data. Sensitivity analysis showed a modest strengthening of the effect of vitamin D on circulating total 25(OH)D when these RCTs were excluded. Over a quarter of the RCTs did not assess or report on compliance, however, sensitivity analysis showed that exclusion of these RCTs had little impact on the outcome.

In conclusion, following consideration of several vitamin D biomarkers for potential use for DRI development in infants and children, the present analyses suggest that circulating total 25(OH)D concentration can be regarded as a useful vitamin D biomarker in infants and children. It should be noted, however, that the value of serum 25(OH)D concentration as an indicator of vitamin D exposure and status is limited by a number of other factors, as described in detail elsewhere [18,96,97]. Such factors can be accounted for in the meta-regression analysis of the relationship between serum 25(OH)D concentration and total vitamin D intake within vitamin D DRI process [9,15]. Finally, the choice of measurement methodology can influence the absolute quantification of circulating concentrations of total 25(OH)D [98]. The availability of certified standard reference material (SRM) for 25(OH)D in human serum from the US-based National Institute of Standards and Technology has helped laboratories bench-mark their analysis against the SRM's assigned values [99]. This SRM has become a key component in the standardization of 25(OH)D assays [100]), an initiative

which could prove enormously beneficial in the derivation of DRI for vitamin D going forward.

Authors' contributions to the manuscript: AC, MK and KDC: performed the electronic searches, assessed the studies for inclusion, extracted data, and assessed validity and risk of bias; KDC: conducted meta-analyses and tabulated data; CR conducted the meta regression analyses; KDC: wrote the draft manuscript; AC, MK, CR and KDC approved the final manuscript.

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Table 1. Characteristics of the included studies of infants and children aged 1-9 years reporting circulating 25-hydroxyvitamin D and other vitamin D biomarkers in some cases.

Study [Ref no.]	Population			Intervention dose	Biomarkers measured	Analytical method for 25(OH)D
	Country	Age/age group	Sex			
Abrams, 2013 [26]	USA	4.0–8.9 y	NR	1000 IU/d vitamin D_3	25(OH)D,PTH, $1,25(OH)_2D$	Immunoassay
Aglipay, 2017 [27]	Canada	2.7 ± 1.5 y	M/F (42%/58%)	2000 IU/d vitamin D_3	25(OH)D,	Immunoassay
Akkermans, 2017 [28]	Germany, Netherland, UK	1–3 y	M/F (57%/43%)	68 IU vitamin D/100 mL	25(OH)D,	Immunoassay
Ala-houhala, 1985 [29]	Finland	Infant	NR	1000 IU/d vitamin D_3	25(OH)D,	CPBA
Ala-Houhala, 1986 [30]	Finland	Infant	NR	400 IU/d vitamin D_2	25(OH)D, PTH, $24,25(OH)_2D$	HPLC
Ala-Houhala, 1988 [31]	Finland	8–10 y	M/F (45%/55%)	400 IU/d vitamin D_2	25(OH)D, PTH, $24,25(OH)_2D$, $1,25(OH)_2D$	CPBA
Brett, 2016 [32]	Canada	5.1 ± 1.9 y	M/F (55%/45%)	600 IU/d vitamin D_3	25(OH)D, PTH	Immunoassay
Brett, 2018 [33]	Canada	5.2 ± 1.9 y	M/F (53%/47%)	400 IU/d vitamin D_3	$25(OH)D_3$, PTH, C3-epimer 25(OH)D	Immunoassay
Chandy, 2016 [34]	India	Infant	NR	400 IU/d vitamin D_3	25(OH)D, PTH	Immunoassay
Economos, 2014 [35]	USA	8.0 ± 1.9 y	M/F (61%/39%)	200 IU/d vitamin D_3	25(OH)D, PTH	Immunoassay
Gallo, 2013 [36]	Canada	Infant	M/F (58%/42%)	1600 IU/d vitamin D_3	25(OH)D, PTH, $24,25(OH)_2D_3$; C3-epimer 25(OH)D	LC-MS/MS
Grant, 2014 [37]	New Zealand	Infant	M/F (49%/51%)	800 IU/d vitamin D_3	25(OH)D,	LC-MS/MS
Greer, 1981 [38]	USA	Infant	M/F (54%/46%)	400 IU/d vitamin D_2	25(OH)D, PTH	HPLC
Greer & Marshall, 1989 [39]	USA	Infant	M/F (44%/56%)	400 IU/d vitamin D_2	25(OH)D, PTH, $1,25(OH)_2D$	CPBA
Holmlund-Suila, 2012 [40]	Finland	Infant	M/F (50%/50%)	1600 IU/d vitamin D_3	25(OH)D, PTH	Immunoassay
Hower, 2013 [41]	Germany	3.7 (2–6) y	M/F (56%/45%)	114 IU vit D/100 mL	25(OH)D,	Immunoassay
Loeb, 2019 [42]	Vietnam	8.6 ± 3.9 y	M/F (48%/52%)	2000 IU/d vitamin D_3	25(OH)D,	Immunoassay
Mortensen, 2016 [43]	Denmark	6.6 ± 1.5 y	M/F (47%/53%)	800 IU/d vitamin D_3	25(OH)D, PTH	LC-MS/MS
Ohlund, 2017 [44]	Sweden	5–7 y	M/F (46%/54%)	1000 IU/d vitamin D_3	25(OH)D, PTH	LC-MS/MS
Pittard, 1991 [45]	USA	Infant	M/F (53%/47%)	800 IU/d vitamin D_3	25(OH)D,	CPBA
Ponnapakkam, 2010 [46]	USA	Infant	NR	200 IU/d vitamin D_3	25(OH)D,	Immunoassay

Reuter, 2019 [47]	Australia	Infant	M/F (53%/47%)	400 IU/d vitamin D_3	25(OH)D,	Immunoassay
Rosendahl, 2018 [48]	Finland	Infant	M/F (50%/50%)	1200 IU/d vitamin D_3	25(OH)D, PTH	Immunoassay
Rothberg, 1992 [49]	South Africa	Infant	NR	1200 IU/d vitamin D_3	25(OH)D, PTH	CPBA
Siafarikas, 2011 [50]	Germany	Infant	NR	500 IU/d vitamin D_3	25(OH)D,	Immunoassay
Zhou, 2018 [51]	China	Infant	M/F (52%/48%)	1200 IU/d vitamin D_3	25(OH)D,	NS
Ziegler, 2014 [52]	USA	Infant	NR	800 IU/d vitamin D_3	25(OH)D, PTH	Immunoassay
Wicklow, 2015 [53]	Canada	Infant	M/F (56%/44%)	1200 IU/d vitamin D_3	C3-epimer 25(OH)D_3, 24,25$(OH)_2D_3$	LC-MS/MS

NR, data not reported; NS, not specified; 25(OH)D, 25-hydroxyvitamin D, PTH, parathyroid hormone; 24,25$(OH)_2$D, 24,25- dihydroxyvitamin D; C3-epimer 25(OH)D_3, C3-epimer of 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; CPBA, competitive protein binding assay; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry.

Table 2. Systematic review subgrouping results for serum/plasma total 25-hydroxvitamin D [25(OH)D] in infants and children[1]

25(OH)D analysis	RCTs included	Mean effect, WMD (95% CI)	*P* value[2]	I^2
	no. (total no. of participants)	*nmol/L*		*%*
All studies (primary outcome)	27 (4571)	27.7 (23.0, 32.5)		93
Pediatric subpopulation:				
Infants	15 (1130)	32.4 (24.0, 40.9)		91
Children (1-3 years)	4 (1898)	26.1 (19.0, 33.3)		84
Children (4-9 years)	8 (1543)	22.2 (11.5, 32.8)	0.30	96
Design:				
Vitamin D vs. placebo	18 (2359)	25.5 (19.0, 32.1)		92
Vitamin D vs. standard care dose vitamin D	9 (2212)	32.7 (24.0, 41.3)	0.20	93
Vitamin D dose:				
<400 IU/d	4 (484)	9.0 (-0.4, 18.5)		69
400-500 IU/d	10 (546)	26.4 (17.5, 35.4)		86
800-1000 IU/d	7 (483)	29.5 (18.6, 40.4)		88
1200-2000 IU/d	6 (3058)	36.0 (28.3, 43.7)	0.0002	96
Baseline 25(OH)D:				
<30 nmol/L	3 (70)	36.9 (30.7, 43.2)		13
≥30 nmol/L	22 (4342)	26.8 (21.5, 32.0)	0.01	93
Latitude:				
<40^o	9 (1930)	20.4 (14.8, 26.0)		86
≥40^o	18 (2641)	32.3 (24.9, 39.7)	0.01	93
Treatment duration:				
6-14 weeks	8 (386)	38.5 (23.5, 53.5)		94
15-37 weeks	15 (3502)	23.5 (17.4, 29.6)		94
≥42 weeks	4 (1030)	26.5 (19.8, 33.2)	0.09	56
Analytical technique:				
CPBA	5 (191)	35.2 (30.8, 39.6)		0
Immunoassay-based	15 (3677)	21.0 (14.9, 27.1)		91
Chromatography-based	6 (371)	43.2 (30.2, 56.1)	0.00001	90

[1] RCTs, randomized controlled trials; CPBA, competitive protein-binding assay; WMD, weighted mean difference; I^2, heterogeneity

[2] Via test for subgroup differences within meta-analysis. Test for overall effect within meta-analysis resulted in $P<0.00001$ in all cases, except for vitamin D dose <400 IU/d and Treatment duration ≥42 weeks where $P=0.06$ and <0.0001, respectively.

Table 3. Systematic review subgrouping results for serum/plasma parathyroid hormone [PTH] in infants and children[1]

PTH analysis	RCTs included	Mean effect, WMD (95% CI)	*P* value[2]	I^2
	no. (total no. of participants)	*pmol/L*		*%*
All studies (primary outcome)	15 (1613)	-0.20 (-0.39, -0.00)	0.05	52
Pediatric subpopulation:				
Infants	7 (353)	-0.16 (-0.65, 0.33)	0.52	63
Children (1-9 years)	8 (1260)	-0.21 (-0.41, -0.01)	0.04	44
			0.86[3]	
Design:				
Vitamin D vs. placebo (+/- Ca)	11(636)	-0.27 (-0.52, -0.02)	0.04	45
Vitamin D vs. standard care dose vitamin D	4 (977)	-0.03 (-0.46, 0.40)	0.89	72
			0.35[3]	
Calcium:				
Vitamin D + Ca vs. placebo + Ca	4 (253)	0.02 (-0.22, 0.26)	0.87	
Vitamin D + placebo (- Ca)	11 (1360)	-0.28 (-0.53, -0.24)	0.02	50
			0.08[3]	
Vitamin D dose:				
<400 IU/d	3 (166)	0.08 (-0.19, 0.34)	0.57	0
400-600 IU/d	5 (274)	-0.33 (-0.90, 0.23)	0.25	43
800-1000 IU/d	4 (260)	-0.54 (-0.81, -0.28)	<0.0001	0
1200-2000 IU/d	3 (913)	0.15 (-0.42, 0.72)	0.60	79
			0.006[3]	
Latitude:				
<40^o	3 (180)	-0.55 (-1.00, -0.11)	0.01	43
≥40^o	12 (1433)	-0.11 (-0.32, 0.10)	0.30	49
			0.08[3]	

[1] RCTs, randomized controlled trials; WMD, weighted mean difference; I^2, heterogeneity
[2] Via test for overall effect within meta analysis.
[3] Via test for subgroup differences within meta analysis.

Table 4. Systematic review subgrouping results for bone markers in children[1]

Bone marker analysis	RCTs included	Mean effect, WMD (95% CI)	*P* value[2]	I^2
	no. (total no. of participants)			
Serum osteocalcin (ng/mL)	8[3] (1105)	0.49 (-1.61, 2.58)	0.65	48
Serum bone-specific alkaline phosphatase (μg/L)	7[4] (665)	0.62 (-2.01, 3.26)	0.64	0
Serum CTx (ng/mL)	2[5] (280)	0.01 (-0.09, 0.12)	0.81	0
Urinary Pyr (nmol/mmol creatinine)	3 (309)	-4.60 (-13.4, 4.2)	0.30	0
Urinary Dpyr (nmol/mmol creatinine)	4 (396)	-1.62 (-4.2, 1.0)	0.22	0

[1] RCTs, randomized controlled trials; WMD, weighted mean difference; I^2, heterogeneity
[2] Via test for overall effect within meta analysis.
[3]Four of the RCTs had two distinct sets of RCT arms (12 studies)
[4]Three of the RCTs had two distinct sets of RCT arms (7 studies)
[5]One of the RCTs had two distinct sets of RCT arms (3 studies)
Pyr, urinary pyridinoline; Dpyr, urinary deoxypyridinoline; CTx; serum carboxy-terminal telopeptide.

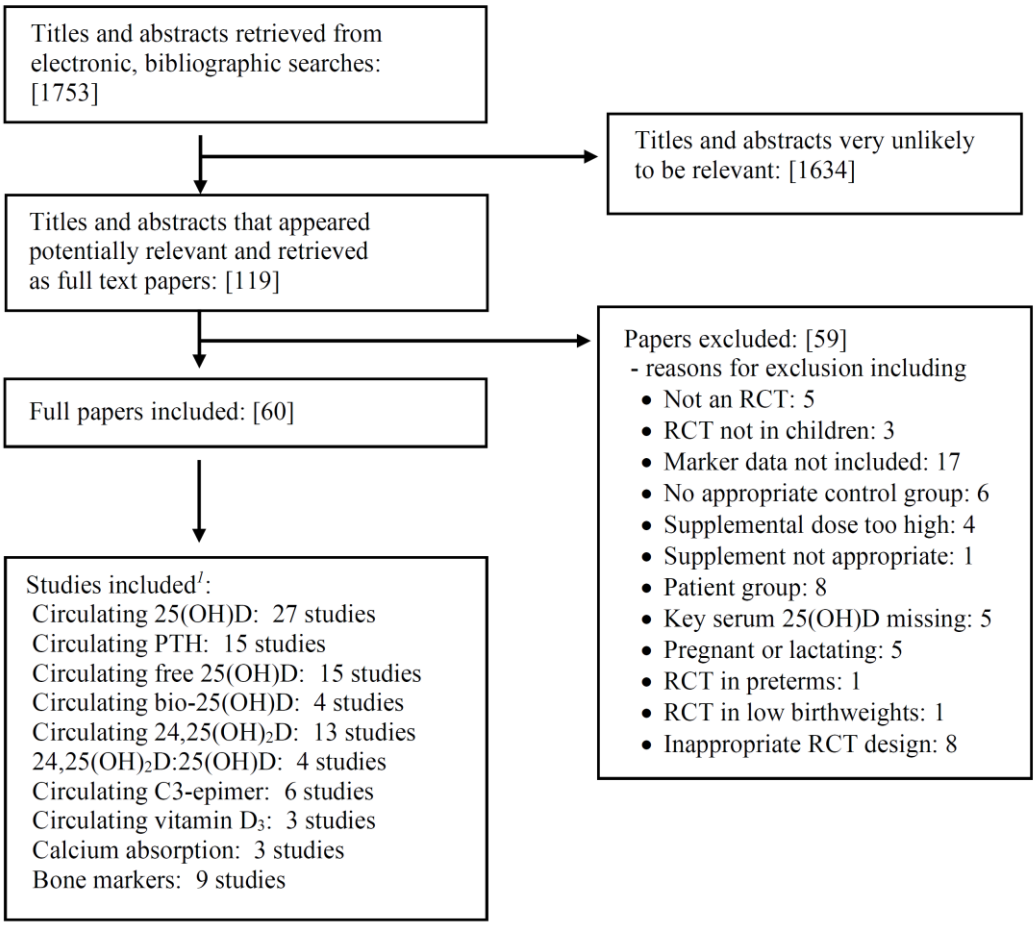

Figure 1. Flow diagram for systematic review of vitamin D biomarkers in infants and children as well as adults for some vitamin D metabolites. [1]In cases in which data were presented for 2 groups separately within a randomized controlled trial, these were treated as 2 studies in the analysis. Some papers provided more than one marker.

25(OH)D, 25-dihydroxyvitamin D; PTH, parathyroid hormone; bio-25(OH)D, bioavailable 25(OH)D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D; C3-epimer, C3-epimer of 25(OH)D.

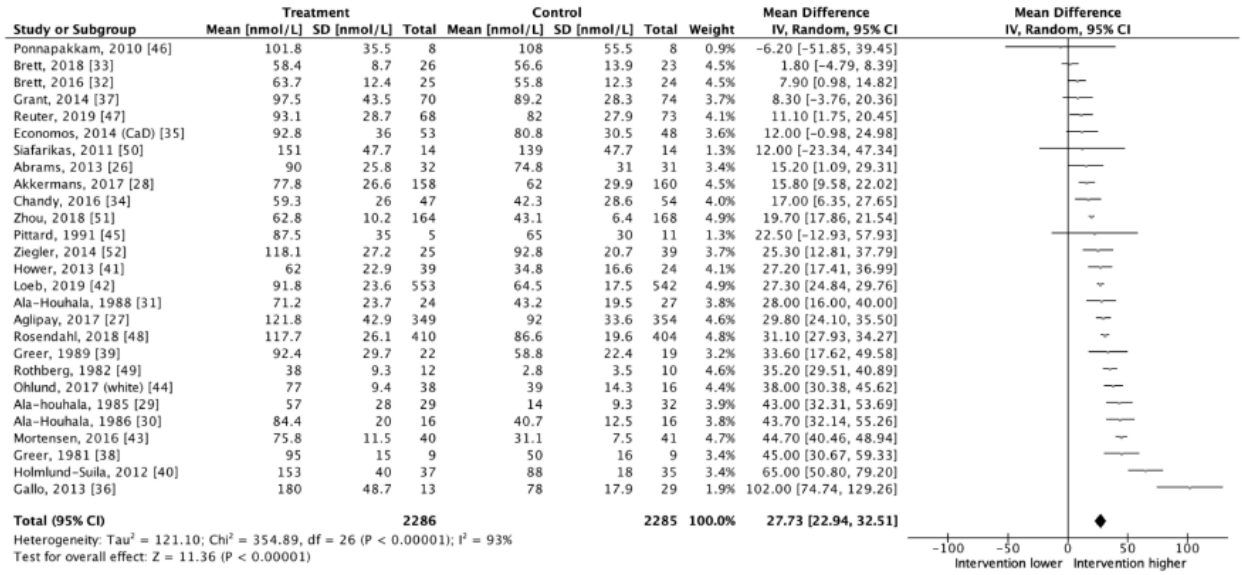

Figure 2. Forest plot of the effect of vitamin D supplementation compared with control on mean change in circulating 25 hydroxyvitamin D concentration (nmol/L) in infants and children.

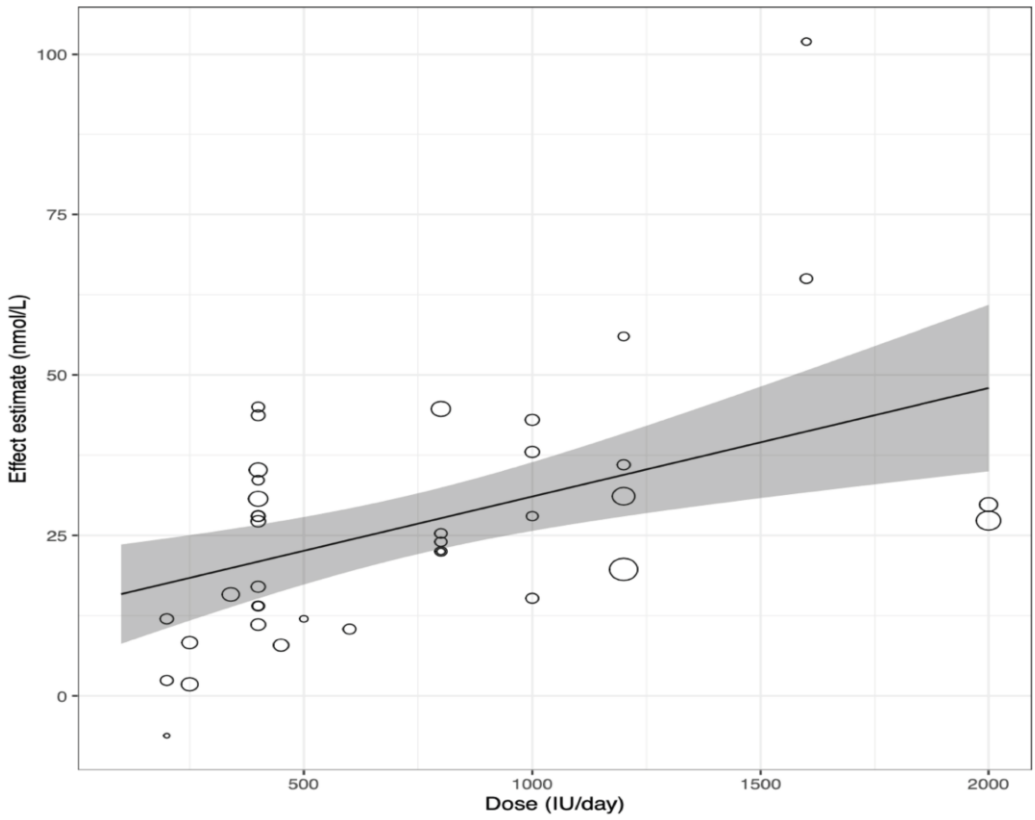

Figure 3. Association of daily vitamin D dose (IU/d) with treatment effect of circulating 25-hydroxyvitamin D in infants and children: a random-effect meta-regression analysis. Increase per 100 IU/day is 1.7 nmol/L; 95% CI: 0.7, 2.6 nmol/L (*P*=0.0005). The fitted meta regression line is shown as the solid black line with corresponding 95% confidence band shown in grey. The circles represent effect estimates from the individual studies included in the meta regression. Sizes of circles reflect study weights as entered in the meta regression model (inverse standard errors).

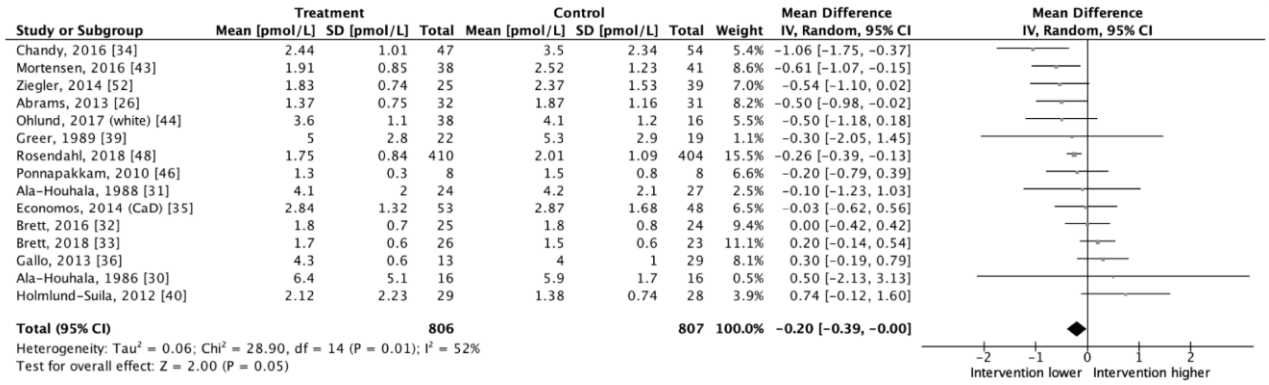

Figure 4. Forest plot of the effect of vitamin D supplementation compared with control on mean change in circulating parathyroid hormone concentration (pmol/L) in infants and children.