

Time-course analysis of 3-epi-25-hydroxyvitamin D₃ shows markedly elevated levels in early life, particularly from vitamin D supplementation in preterm infants

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BACKGROUND: An epimeric form of 25-hydroxyvitamin D₃ (25(OH)D₃) has recently been detected in clinical samples, with relatively high levels in infants. Little is known on 3-epi-25(OH)D₃ formation and physiological function. Our objective was to study dynamics of 3-epi-25(OH)D₃ formation during infancy.

METHODS: 25(OH)D₃ and 3-epi-25(OH)D₃ levels were measured by liquid chromatography-tandem mass spectrometry in 22 preterm (aged 34–37 wk), 15 early preterm (aged <34 wk), and 118 term infants up to 2 y of age. All infants were prescribed vitamin D 400 IU/day after the first week of life.

RESULTS: At birth, 3-epi-25(OH)D₃ levels were 3 (1–7) nmol/l, <10% of total 25(OH)D₃. From the second week to 3 mo of age, both 25(OH)D₃ and 3-epi-25(OH)D₃ increased, with highest 3-epi-25(OH)D₃ contribution in early preterm infants (up to 55% of total 25(OH)D₃ vs. 36% in term infants, $P < 0.0001$). After 3 mo of age, 3-epi-25(OH)D₃ normalized to <10% in all infants.

CONCLUSIONS: At birth, all infants showed low contribution of 3-epi-25(OH)D₃, increasing the week after starting vitamin D supplementation, until 3 mo of age. Highest levels of 3-epi-25(OH)D₃ were found in early preterm infants, supporting the hypothesis that hepatic immaturity plays a role in 3-epi-25(OH)D₃ formation.

Vitamin D has garnered a great deal of attention in recent years due to a global prevalence of vitamin D deficiency associated with an increased risk of a variety of human diseases (1–3). Specifically, hypovitaminosis D in pregnant women is highly common and has important implications for the mother and lifelong health of the child, since it has been linked not only to rickets but also to maternal and child infections, small for gestational age, preterm delivery, pre-eclampsia, gestational diabetes, as well as imprinting on the infant for life chronic diseases (4,5).

Transplacental passage of maternal 25-hydroxyvitamin D₃ (25(OH)D₃) is the sole source of vitamin D in the developing fetus and implicates that infants are wholly reliant on their mother for their vitamin D status. Given the high prevalence of hypovitaminosis D in pregnant women, supplements

are the best way to prevent vitamin D deficiency in infants. Various international committees (e.g., American Academy of Paediatrics Committee on Nutrition or the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)) recommend to provide 400 IU/day of vitamin D to all infants (breastfed, healthy term babies) as a generally safe and efficacious measure to prevent rickets (6,7). For neonatal care, questions remain, such as the optimal vitamin D dose and vitamin D status for preterm, late-preterm, formula-fed, and breastfed term infants (8–10).

It is accepted that the best available marker of vitamin D exposure and vitamin D status is the serum 25(OH)D concentration (11). There is a strong correlation between maternal and infant cord blood 25(OH)D concentrations, cord blood concentration being slightly less than maternal concentrations (12).

The interpretation of serum 25(OH)D concentrations in infants is complicated by the presence of a C₃-epimeric form of 25(OH)D (3-epi-25(OH)D), a vitamin D metabolite that has been shown in infants up to 1 y of age to contribute up to 61% of total 25(OH)D. Serum 3-epi-25(OH)D can be erroneously included as 25(OH)D if immunoassays are used that display cross-reactivity to the C₃-epimer or with high-performance liquid chromatography or liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods that do not resolve both compounds (13).

At present, the source of the C₃-epimer (i.e., diet, supplements, endogenous metabolism) and factors underlying 25(OH)D epimerization are still unclear (14). Also, the physiological importance of the C₃-epimer is heavily debated. *In vitro* work and rodent models show that 3-epi-25(OH)D₃ and the epimeric form of calcitriol (3-epi-1 α ,25(OH)₂D₃) bind vitamin D binding protein at 36–46% and vitamin D receptor at 2–3% as compared with non-epimer 25(OH)D₃ and calcitriol (15). However, 3-epi-1 α ,25(OH)₂D₃ appears nearly as potent as calcitriol in suppressing parathyroid hormone (16), but it has significantly reduced calcemic effects (17). A detailed review of the metabolic pathway and physiological functions of C₃-epimer forms of vitamin D can be found elsewhere (14).

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Table 1. Infant characteristics

	Total (%)
Sex	
Male	78 (48)
Female	85 (52)
Age (d)	
Mean (SD)	138 (205)
0–10 d	68 (42)
11–90 d	44 (27)
91–365 d	24 (15)
>365 d	27 (16)
Gestational age	
Term	118 (72)
Premature 34 wk 0/7 d to 36 wk 6/7 d	22 (14)
Premature < 34 wk 0/7 d	15 (9)
Unknown	8 (5)
Indication of blood sampling	
Infection (C-reactive protein)	70 (43)
Jaundice (bilirubin)	42 (26)
Other	45 (27)
Unknown	6 (4)

Little is known on the time course of C₃-epimer formation over the first years of life. Our objective was to study the dynamics of 3-epi-25(OH)D₃ and 25(OH)D₃ formation during infancy by 25(OH)D₃ and 3-epi-25(OH)D₃ measurement in plasma from preterm and term infants from birth up to 2 y of age.

RESULTS

Baseline characteristics are shown in **Table 1**. **Figure 1** shows the time course of 25(OH)D₃ and 3-epi-25(OH)D₃ formation from birth up to 2 y of age. In 8.5% of all samples, all confined to first week sample collections, 3-epi-25(OH)D₃ concentrations were below the detection limit of 1 nmol/l. 25(OH)D₂ concentrations were below detection limit (2 nmol/l) in all 316 samples.

Time Course of 25(OH)D₃ Concentrations

At birth, mean (range) 25(OH)D₃ levels were 39 (7–112) nmol/l with 77% showing vitamin D insufficiency (<50 nmol/l). Levels remain more or less unchanged in the first week. From the third week of life 25(OH)D₃ levels increase, probably explained by the start of 400 IU/day vitamin D supplementation starting at day 7–10. From the first month to 1 y

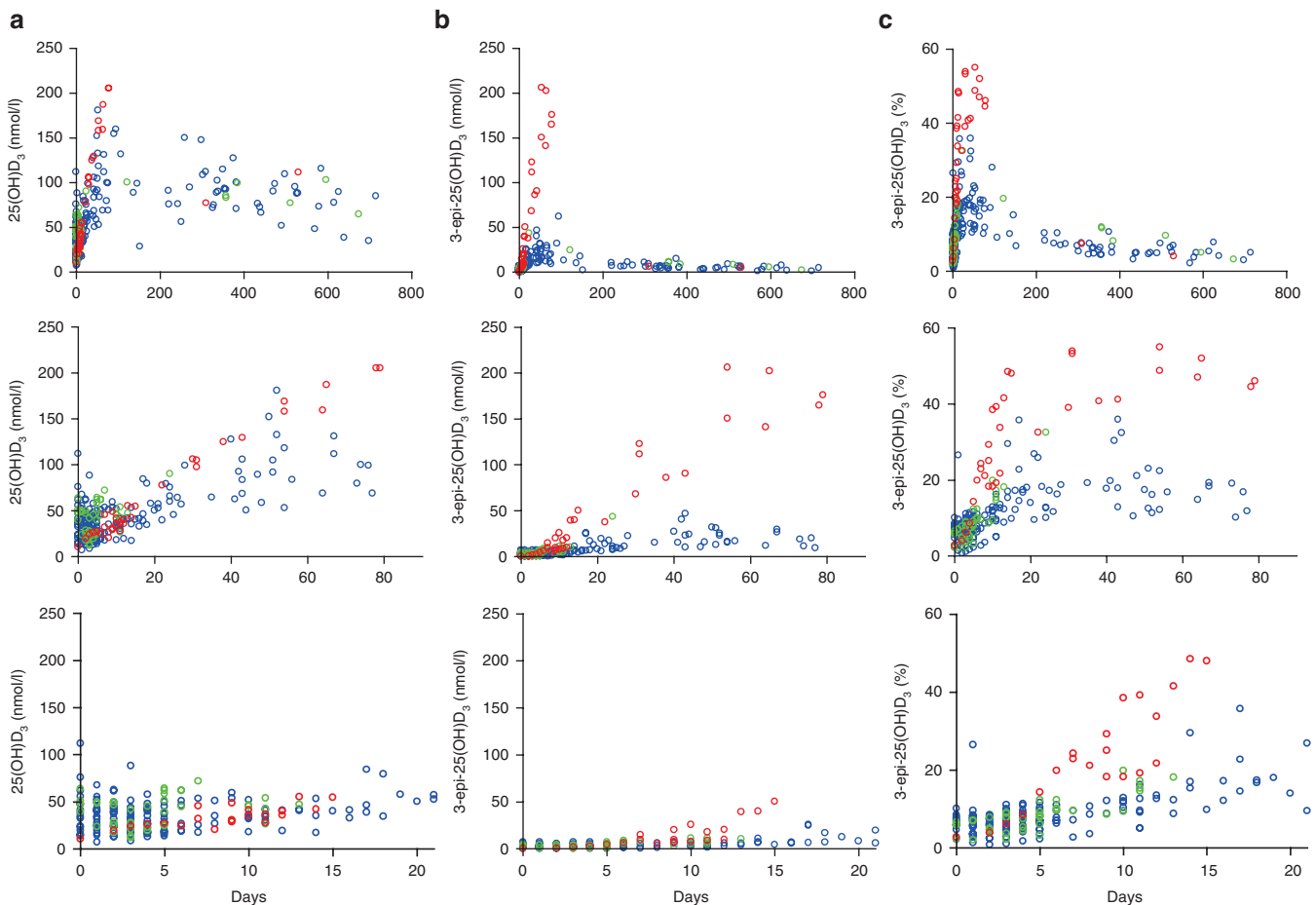


Figure 1. Time course of 25(OH)D₃ and 3-epi-25(OH)D₃ concentrations from birth to 2 y of age in 316 samples from 163 individual infants. (a) 25(OH)D₃ concentrations, (b) 3-epi-25(OH)D₃ concentrations, and (c) relative contribution of 3-epi-25(OH)D₃ to total 25(OH)D₃ (sum of 3-epi-25(OH)D₃ and 25(OH)D₃) are shown for 0–21 d (bottom panels), 0–90 d (middle panels), and 0–2 y (upper panels). Results from term infants (>37 wk of age) are shown in blue, those from preterm infants (34–37 wk of age) are shown in green, and those from early preterm infants (<34 wk of age) are shown in red. 25(OH)D₃, 25-hydroxyvitamin D₃.

of age, most infants (99%) reach 25(OH)D₃ concentrations ≥ 50 nmol/l. Remarkably, some peak up to 200 nmol/l in the third month. From 3 mo of life, a more or less steady-state is achieved with a mean (range) concentration 25(OH)D₃ of 90 (29–160) nmol/l, with a slight tendency to decrease with time (2 y).

Time Course of 3-epi-25(OH)D₃ Concentrations

Plasma 3-epi-25(OH)D₃ concentrations are relatively low in the first week of life (mean (range) of 3 (1–7) nmol/l), with a low relative contribution to 25(OH)D₃ (<10%). 3-epi-25(OH)D₃ concentrations increase from the second week of life with highest levels within the second and third months of age, some reaching as high as 200 nmol/l. Relative 3-epi-25(OH)D₃ contribution to 25(OH)D₃ varied from 15 to 55%. From 3 mo of life, contrary to 25(OH)D₃, a sharp decline in plasma 3-epi-25(OH)D₃ level is seen, thereafter to remain at a relatively constant low level (mean (range) 8 (1–62) nmol/l, <10% of 25(OH)D₃).

Time Course of an Individual Patient

From one patient (gestational age 37 wk and 2 d), plasma samples were obtained over a 3-mo period providing an unique opportunity to follow 25(OH)D₃ and C₃-epimer formation over time within a single individual. As compared with the whole study population, a similar time course is observed with respect to 25(OH)D₃ and C₃-epimer formation (see **Supplementary Figure S1** online). At birth, relative 3-epi-25(OH)D₃ contribution is low (5%). 25(OH)D₃ rises from day 11 on and reaches its maximum at approximately 60 d, to further remain at that level. In this infant, highest C₃-epimer concentration is measured at 47 d of age (16.5 nmol/l (23% of 25(OH)D₃)), after which it declines.

Prematurity as a Determinant of 3-epi-25(OH)D₃ Formation

We speculated that prematurity could be a factor influencing C₃-epimer formation. We therefore categorized the infants into three groups of gestational age: <34 wk preterm, >34 wk

preterm, and term. When observing the first 3 mo, the highest 25(OH)D₃ concentrations belong to the preterm infants born at <34 wk of gestation. Even more striking is the marked increase of the C₃-epimer concentration from day 10 on in this group, as compared with the full term and >34 wk preterm groups (**Figures 1 and 2**), with relative 3-epi-25(OH)D₃ contribution up to 55% in early preterm infants, vs. 36% in full-term infants ($P < 0.0001$; **Figure 1**). Particularly for the early preterm state, an inverse relationship is seen between the gestational age and the 3-epi-25(OH)D₃ concentration (**Figure 3**).

In the term infant population a similar, although less pronounced, rise and fall in C₃-epimer formation is seen. This same trend is seen for birth weight, however, when taking P values for gestational age into account, this trend is clearly related to gestational age rather than birth weight solely (data not shown). No correlation was found between bilirubin value, C-reactive protein (CRP) value, phototherapy, and use of medication regarding height of 3-epi-25(OH)D₃ concentration.

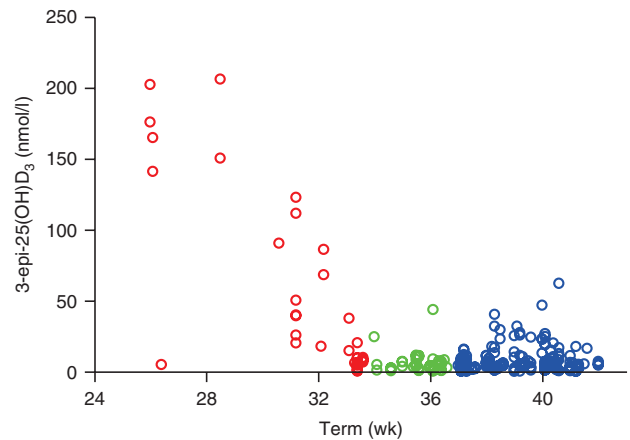


Figure 3. Relationship of plasma 3-epi-25(OH)D₃ concentration (nmol/l) to gestational age (wk). Term infants (>37 wk of age) are shown in blue, preterm infants (34–37 wk of age) are shown in green, and early preterm infants (<34 wk of age) are shown in red. Linear relationship for <34 wk group is $y = -20.0x + 682.7$; $r^2 = 0.64$ ($n = 35$ values). 25(OH)D₃, 25-hydroxyvitamin D₃.

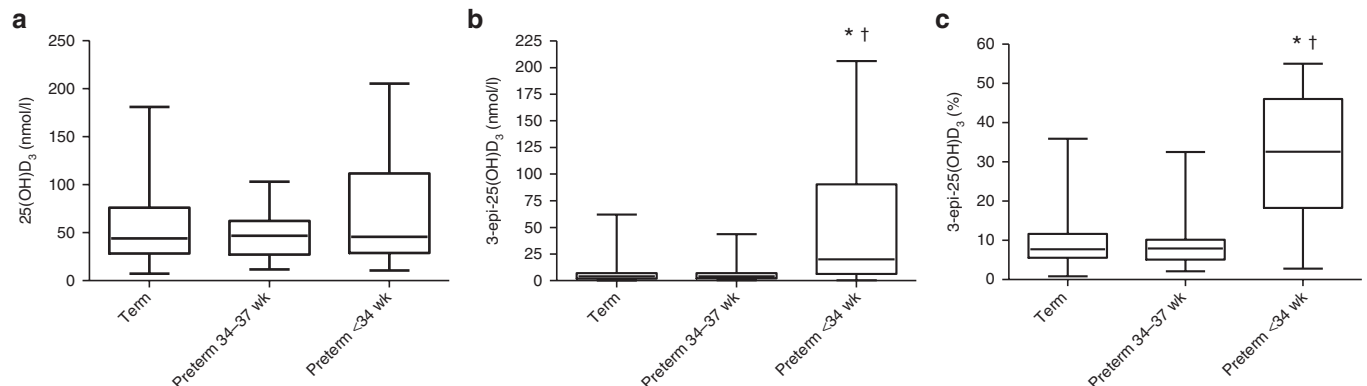


Figure 2. Plasma concentrations (nmol/l) of 25(OH)D₃ and 3-epi-25(OH)D₃ and relative contribution (%) in term, preterm (34–37 wk of age), and early preterm (<34 wk of age) infants. Horizontal lines within each box represent medians, edges of each box represent the 25th and 75th percentiles, and the caps extending from boxes show 1.5 times the interquartile range. (a) 25(OH)D₃, (b) 3-epi-25(OH)D₃, and (c) relative contribution (%) of 3-epi-25(OH)D₃ to total 25(OH)D₃ (sum of 3-epi-25(OH)D₃ and 25(OH)D₃). * $P < 0.0001$ vs. term; † $P < 0.0001$ vs. preterm (34–37 wk of age) (one-way ANOVA). 25(OH)D₃, 25-hydroxyvitamin D₃.

Plasma 25(OH)D₃ Correlation With 3-epi-25(OH)D₃

Plasma 25(OH)D₃ concentrations correlate positively with 3-epi-25(OH)D₃ in a curved-linear relationship. When the three categories of gestational age (<34 wk preterm, >34 wk preterm, and term) are plotted separately, two distinct positive linear relationships can be found: one from the <34 wk group and a pooled one from the term and >34 wk preterm group (the latter groups do not significantly differ from one another (Figure 4a). Relative 3-epi-25(OH)D₃ contribution to 25(OH)D₃ shows a hyperbolic relationship in the <34 wk preterm group (Figure 4b).

Origin of 3-epi-25(OH)D₃

We examined the presence of appreciable amounts of 3-epi-vitamin D₃ in the liquid vitamin D supplements prescribed from the second week of life. The supplement was diluted to result in a concentration of approximately 75 nmol/l. Using the same analytical column facilitating the separation of 25(OH)D₃ and 3-epi-25(OH)D₃, we looked for an epimer signal adjacent to the cholecalciferol signal by monitoring a cholecalciferol-specific mass-to-charge transition by LC-MS/MS. A single prominent peak corresponding to cholecalciferol was observed, but no significant (>5%) C₃-epimer contribution in the cholecalciferol supplement was detected.

DISCUSSION

Time-Course Analysis

Our study provides new information on absolute concentrations of 25(OH)D₃ and 3-epi-25(OH)D₃ at defined time periods and reveals interesting C₃-epimer dynamics over time in early age. At birth and during the first week of life, concentrations of both 25(OH)D₃ and 3-epi-25(OH)D₃ are low. The majority of infants have vitamin D insufficiency (77% <50 nmol/l 25(OH)D₃), reflecting low maternal 25(OH)D₃ concentrations (18). Our data closely match those of another Dutch study measuring the prevalence of vitamin D deficiency in pregnant women and their newborns (19). They found 77% of neonates from

Dutch/European mothers and 98% of neonates from mothers of non-Western origin (mainly Turkish and Moroccan) had vitamin D insufficiency (<50 nmol/l).

3-epi-25(OH)D₃ was detectable in nearly all infants at birth (91.5%) but at low concentration. We can only speculate whether these low 3-epi-25(OH)D₃ levels originate from placental passage or from endogenous fetal production of maternally derived 25(OH)D₃. In a recent cohort study of mother–infant pairs, it was shown that 3-epi-25(OH)D₃ is present at low levels in some pregnant women and fetuses but does not appear to be efficiently transferred transplacentally (20). It was speculated that high C₃-epimer concentrations in infancy are probably due to postnatal formation rather than fetal stores. We can confirm low 3-epi-25(OH)D₃ concentrations in cord blood (unpublished data) lending support to the model that 3-epi-25(OH)D₃ is formed postnatal.

The increases in 25(OH)D₃ and C₃-epimer concentrations after the first week of life coincide with the start of vitamin D supplementation. It is well recognized from pharmacokinetic studies of 25(OH)D₃ formation after single dose administration that highest levels are seen 7–10 d post supplementation (21). Our data show that 3-epi-25(OH)D₃ increases slightly earlier than 25(OH)D₃. By showing the vitamin D supplement not to be a C₃-epimer source, we may conclude that 3-epi-25(OH)D₃ is formed endogenously from the administered vitamin D. This conclusion is corroborated by the finding of increased vitamin D supplementation dosages resulting in higher plasma 3-epi-25(OH)D₃ concentrations (22). Whether C₃-epimer formation is unique to exogenous vitamin D or could also result from dermal vitamin D synthesis remains to be determined.

Despite the continuation of vitamin D supplementation, a pronounced drop in 3-epi-25(OH)D₃ levels is seen after 3 mo of age, which implicates that vitamin D supplementation is not the sole determinant in 3-epi-25(OH)D₃ formation. We assume that the combination of hepatic immaturity during the first 3 mo of age and vitamin D supplementation gives rise to increased 3-epi-25(OH)D₃ levels. The enzyme involved in

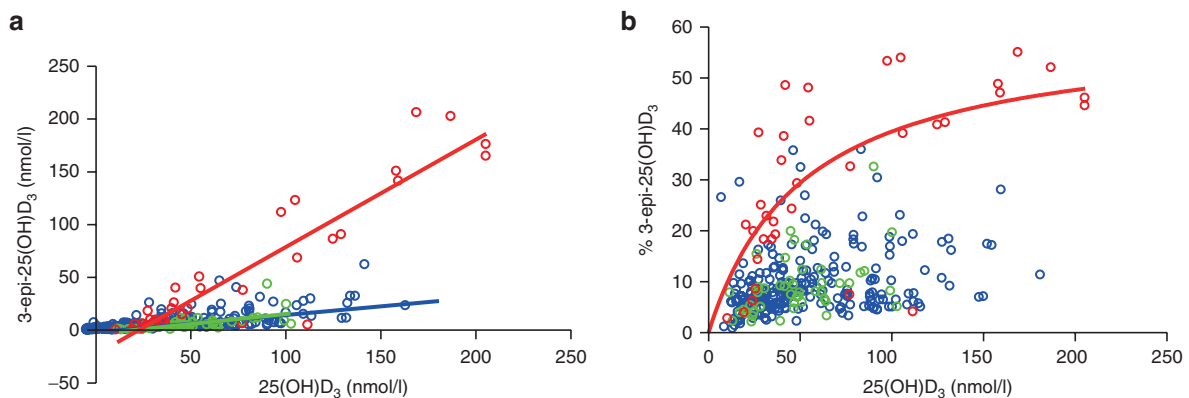


Figure 4. Relationships of 3-epi-25-OH-D₃ to 25(OH)D₃ in plasma infant samples. (a) Relationship of absolute 3-epi-25-OH-D₃ (nmol/l) to 25(OH)D₃ (nmol/l), (b) relationship of relative contribution 3-epi-25-OH-D₃ (%) to 25(OH)D₃ (nmol/l). Term infants (>37 wk of age) are shown in blue, preterm infants (34–37 wk of age) are shown in green, and early preterm infants (<34 wk of age) are shown in red. Linear regression lines in (a) are $y = 1.0x - 22.7$; $r^2 = 0.86$ ($n = 35$ values) for the early preterm infants <34 wk of age and $y = 0.2x - 2.3$; $r^2 = 0.45$ ($n = 273$ values) for the combined term and preterm infants (34–37 wk of age). Hyperbolic relationship for the early preterm infants <34 wk of age in (b) is $y = 60.1x [25(OH)D_3] / 52.1 + [25(OH)D_3]$ ($r^2 = 0.48$; $n = 35$ values). 25(OH)D₃, 25-hydroxyvitamin D₃.

C₃-epimerization of 25(OH)D, as well as the exact conditions favoring epimerization of 25(OH)D, remains to be determined (15). The function of this pathway remains unknown, as well as the explanation for the absence of its activity after the age of 3 mo.

Prematurity as a Major Determinant of C₃-Epimer Formation

Singh *et al.* (13) have shown for the first time that within a group of infants <1 y of age, C₃-epimer formation is inversely correlated with age suggesting that high rates of C₃-epimerization might be a function of possibly hepatic, immature vitamin D metabolism. Our results show a typical rise and fall of C₃-epimer over a 3-mo period of time that is most pronounced in early preterm infants. Our findings differ from those of Granado-Lorencio *et al.* (22) who found higher levels of 25(OH)D₃ and C₃-epimer in term infants compared with preterm infants. The preterm status is likely to be associated with immature vitamin D metabolism that may favor 3-epi-25(OH)D₃ epimerization. Epimerization appears to employ enzymes distinct from the classical hepatic enzyme systems (13). As epimerization appears to be a common mechanism for all major vitamin D metabolites, it will be interesting to investigate C₃-epimer forms of other vitamin D metabolites in plasma from preterm infants in search for similar effects.

Risk of Over-Supplementation

Gallo *et al.* (23) studied the effects of different dosages of oral vitamin D supplementation on vitamin D status in healthy, breastfed infants. In infants supplemented with 400 IU/day, 25(OH)D₃ and 3-epi-25(OH)D₃ concentrations increased to levels similar to ours, with mean peak 25(OH)D₃ and 3-epi-25(OH)D₃ concentrations at approximately 3 mo of 78 and 20 nmol/l, respectively. Interestingly, in the group receiving 1,600 IU/day, they observed peak 25(OH)D₃ concentrations at 3 mo that have been associated with hypercalcemia (mean 180; max 240 nmol/l) with concomitant increases of 3-epi-25(OH)D₃ levels to mean (max) levels of 60 (80) nmol/l (23). Our study adds that in <34 wk born infants, 400 IU/day of vitamin D does result in much higher concentrations of both 25(OH)D₃ and 3-epi-25(OH)D₃ when compared with those born at >34 wk of age. In some infants, when summed, total 25(OH)D reaches as high as 400 nmol/l. As long as we are unaware of the biological significance of 3-epi-25(OH)D₃, these high levels of vitamin D metabolites may potentially be toxic to the preterm infant. It is postulated that high supplementation with vitamin D in the newborn period may confer risk to allergy and asthma later in life (24).

The quantity and effectiveness of vitamin D supplementation in preterm infants remain unclear, they are not always specified in current recommendations or range from 200 to 1,000 IU/day (8). Adding to the confusion is a paucity of high-quality prospective studies and limited information regarding toxicity. Organ system immaturity in preterm infants is connected with long-term complications that have both clinical and public health importance.

A possible explanation for the high 25(OH)D₃ concentrations in preterm infants is their minimal fat mass in which vitamin D and its metabolites are stored. This means that on a body-weight basis, the intake may be 5 to 10 times the amount recommended for term neonates (8). As a consequence, the majority of vitamin D is immediately metabolized to 25(OH)D, remains in the circulation, and may be further metabolized. Our preliminary findings question the safety of current recommendations for vitamin D supplementation in preterm infants. As a precautionary measure, we would like to recommend these to lower levels, in particular in those with (extremely) low fat mass.

The markedly elevated levels of 3-epi-25(OH)D₃, reflecting 3-epi-epimerase activity, may suggest some kind of mechanism to avoid high vitamin D exposure (and/or elevated plasma 25(OH)D concentrations) in preterm infants. Therefore, it would be interesting to have information on 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) levels as an indicator of 25(OH)D inactivation. Unfortunately, we did not measure 24,25(OH)₂D₃. An alternative explanation for the elevated levels of C₃-epimer is increased 3-epimerase activity from hepatic immaturity, for reasons yet unknown. Functionality, as well as the mechanism, leading to C₃-epimer formation remains a topic for further research. It remains to be seen whether 3-epi-25(OH)D₃ could serve as a marker of hepatic immaturity.

Concerns of Assay Methodology

Multiple studies on 25(OH)D₃ formation in infants have used 25(OH)D assays not separating the C₃-epimer from 25(OH)D, so 25(OH)D concentrations may have been seriously overestimated confounding correct interpretation (18,25,26). In fact, a report of unexpectedly high 25(OH)D results (up to 470 nmol/l total 25(OH)D, including the contribution of C₃-epimers) in infants was the clue to the identification of the C₃-epimer interfering with proper measurement of 25(OH)D (13). When reporting as the sum of the C₃-epimer and 25(OH)D₃, we found highly elevated concentrations of total 25(OH)D₃ (up to 400 nmol/l) as well. Therefore, particularly in infants less than half a year of age, serum or plasma 25(OH)D₃ should be measured with an assay that lacks 3-epi-25(OH)D₃ cross-reactivity or chromatographically separates 3-epi-25(OH)D₃ from 25(OH)D₃. The question of whether 3-epi-25(OH)D₃ itself should be routinely quantified needs to be investigated in more detail. Even if the physiological role of the C₃-epimerization pathway has not yet been elucidated, it may become important in the future.

One of the study limitations is that 25(OH)D measurements have been performed on plasma from infants that were referred to our hospital, and as such are not fully representative of a healthy infant population. Limited plasma volume restricted biochemical analysis for calcium homeostasis markers (calcium, parathyroid hormone) or other vitamin D metabolites (e.g., 24,25(OH)₂D₃). When assuming biological activity for 3-epi-25(OH)D₃, it would be interesting to study calcium and parathyroid hormone status in those infant samples that have

highly elevated (up to 400 nmol/l) total 25(OH)D₃ (sum of 25(OH)D₃ and 3-epi-25(OH)D₃).

Compliance is a major issue in supplementation programs. However, the infants involved in this study were not extremely premature and generally in good condition. In the majority of infants, it was therefore possible to administer vitamin D 400 IU/day after the first week of life, but the adherence after hospital discharge may have been suboptimal.

Since the Dutch Health Council advises all children 0–4 y of age 400 IU per day extra starting at day 8 after birth, it is not possible to study infant 25(OH)D₃ and 3-epi-25(OH)D₃ dynamics in the absence of exogenous vitamin D, at least in The Netherlands. Scarce data are available on 3-epi-25(OH)D₃ formation in infants not receiving vitamin D supplementation. The only data we are aware of is a study in Bangladesh infants aged 3–28 wk in whom the absolute concentration of 25(OH)D₃ was observed to increase with age (range 20–120 nmol/l), whereas 3-epi-25(OH)D₃ concentrations remained constant (0–17 nmol/l), resulting in a decline in the relative contribution of the C₃-epimer over time (0–25%) (20). Unfortunately, information on gestational status was lacking. Further studies in infants with and without vitamin D supplementation are needed to provide more conclusive evidence on the role of exogenous vitamin D as a contributing factor of high C₃-epimer formation in infants.

In conclusion, we have shown here that the highest 25(OH)D₃ as well as 3-epi-25(OH)D₃ levels are found in preterm infants within a 30- to 90-d period. Main contributors to C₃-epimer are endogenous formation, most likely depending on vitamin D supplementation as well as early preterm state. This supports the hypothesis that hepatic immaturity may play a role in 3-epi-25(OH)D₃ formation. It also suggests that C₃-epimerization could be a major metabolic pathway for 25(OH)D₃ under certain circumstances. The highly elevated 25(OH)D₃ and 3-epi-25(OH)D₃ concentrations in preterm infants raises concerns on the safety of current vitamin D dosing using 400 IU/day in this particular group and requires further investigation.

METHODS

Sample Collection

Between February and June 2014, we collected left over plasma samples of infants and children between 0 d and 2 y of age. Indications for blood sampling were to determine infection status (CRP), follow-up of jaundice (bilirubin) or thyroid function. In total, 316 samples were collected from 163 individual patients. In 14 cases, two blood samples taken at closely related time points were pooled to obtain sufficient material for vitamin D analysis.

The study was approved by the Canisius-Wilhelmina Hospital Institution Review Board. Parental informed consent was not required as left over plasma samples from routine diagnostics were used. The Dutch Health Council dietary reference intakes and supplementation advice for vitamin D (2012) advises all children 0–4 y of age 400 IU per day extra starting at day 8 after birth, ensuring adequate intake to prevent rickets (27). The guideline does not mention the specific vitamin D needs of preterm infants.

Data of patient characteristics such as age, gestational age, birth weight, indication for blood sampling, bilirubin and CRP value (if known), use of phototherapy, and use of medication were obtained from the patient's file.

Biochemical Measurements

Vitamin D metabolites 25(OH)D₃, 25(OH)D₂, and 3-epi-25(OH)D₃ were measured by a previously described LC-MS/MS method (28). The coefficient of variation of the method was <7.5% for all 25(OH)D metabolites. Lower limits of quantification for 25(OH)D₃, 25(OH)D₂, and 3-epi-25(OH)D₃ were 1, 2, and 1 nmol/l, respectively. Accuracy of our method was verified by measurement of a serum reference panel for 25(OH)D ($n = 20$), with values assigned by the reference measurement LC-MS/MS procedure for 25(OH)D₃ and 25(OH)D₂ (29). Our method closely relates to the reference measurement LC-MS/MS procedure with a mean bias of only -0.38 nmol/l (-0.4%) for 25(OH)D (sum of 25(OH)D₃ and 25(OH)D₂) and -0.2 nmol/l (-3.1%) for 3-epi-25(OH)D₃. Total sample set was determined over four series of measurements.

Statistical Analysis

ANOVA were used for between-group comparisons (25(OH)D₃, absolute and relative 3-epi-25(OH)D₃ vs. gestational age (<34 wk, 34–37 wk, ≥37 wk)). Univariate associations were assessed using Pearson's correlation coefficient. Statistical significance was set at $P < 0.05$. Data were collected and analyzed using the SPSS statistical software for Windows (v.21.0, SPSS inc, Chicago, IL).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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