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Review

Are vitamins A and D important in the development of food allergy and how are they best measured?

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ABSTRACT

Food allergy has a dramatic impact on a child's (and their family's) quality of life and places a major financial burden on the community. It has been hypothesized that the increase in food allergy may relate to the concordant rise in prevalence of vitamin D insufficiency. More recently a second hypothesis has implicated vitamin A sufficiency in the development of immune tolerance. Together, these hypotheses have prompted investigation into the circulating levels of vitamins A and D in relation to food allergy prevalence. This review aims to examine the relationship between vitamins A and D and food allergy.

The first part of this review presents the available epidemiological data which proposes a dramatic increase of food allergy and related anaphylaxis during the last two decades. There is some indirect evidence that variation in food allergy prevalence within countries might be linked with ambient ultra violet radiation exposure and thus potentially with vitamin D levels. Only a few studies to date have directly examined the relationship between measured serum vitamin D levels and either food sensitization or allergy. The significance of vitamin A in food allergy prevalence is only provided through a hypothetical association due to its role in the immune system.

The second part of this review discusses the relevant aspects of the analytical methods to assess vitamin A and D levels in children. The primary methods utilized relate to measuring the main circulating forms of vitamins A and D in blood i.e. retinol and 25-hydroxy-vitamin-D₃ respectively. Chromatographic separation coupled with mass spectrometric detection is considered the gold standard method for both vitamins. These analytical methods should be fully validated for the use in pediatric populations to ensure they are fit for their clinical purpose.

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Introduction

Food allergy has a dramatic impact on a child and family's quality of life [1] and places a major financial burden on the community [2]. Of further concern, hospitalizations for food allergy-related anaphylaxis, the most severe expression of a food allergic reaction and a life threatening condition, have dramatically increased since the early 1990s. Recent studies demonstrate that 8–10% of one year old infants living in Melbourne, Australia have challenge-proven IgE-mediated food allergy [3]. This is the highest reported prevalence of challenge-proven food allergy in the world [4] (Fig. 1). The cause of this epidemic is unknown and identifying contemporary environmental factors that may underlie the increase in food allergy is actively being investigated [4,5].

The increase in food allergy may relate to the concordant rise in prevalence of vitamin D¹ insufficiency (VDI) [6]. Previous studies have shown that greater than one third (39%) of Australian women of child bearing age have VDI [7]. The high rate of VDI may relate to a number of factors including reduced sun exposure, low dietary intake of foods that are naturally rich in vitamin D, as well as lack of routine vitamin D supplementation in this country – unlike in the US and Europe. A number of recent findings suggest an association between early life VDI and food allergy. This is an important hypothesis with clear public health implications. The initial findings of a latitude gradient in the prescription of adrenaline auto-injectors for the treatment of food allergy anaphylaxis (higher rates further from the equator) have recently been extended by a large cross-sectional study which found a clear link between low vitamin D status (which was directly measured) and proven food allergy in young infants of Australian born parents [8].

Vitamin A has an important role in immune function [9]. The health impact of vitamin A deficiency in developing countries has been recognized for some time; this includes vitamin A's association with increased risk of mortality in malnourished infants due to impaired immune system function associated with vitamin A deficiency [10]. Hence, vitamin A supplementation in association with vaccination programs has been established to combat deficiency in preschool children in these countries [11]. Recent evidence suggests that neonatal supplementation of vitamin A is associated with an increase in atopic sensitization and atopic disease [12]. In addition, it has also been proposed that retinoic acid (one of the three forms of vitamin A) may influence the acquisition of immune intolerance [13]. Indeed vitamin A appears to play a key role in enabling humans to consume a wide range of foods without stimulating an adverse immune reaction [9].

Together, these hypotheses have prompted further investigation into the circulating levels of vitamins A and D in relation to food allergy prevalence. While there is no clear evidence examining an interaction between vitamins A and D in association with food allergies, it is expedient to be able to measure these vitamins together. Ideally, the analytical methods utilized for the measurement of these vitamins are both precise and accurate. We also desire methods that are sensitive and specific. The higher order methods which are reported to achieve these desired method characteristics are based on chromatographic techniques for both vitamins.

The chromatographic method group, while considered by many to be of a higher order, is technically challenging and has been shown in recent years to not always be as robust as hoped [14,15]. Whereas the alternative immunoassay method group, which is widely utilized for vitamin D analysis, is highly automated and technically less challenging and considered by many to be acceptable for routine analysis [16]. However, often a limitation of both groups (chromatography and immunoassay) remains their inability to distinguish the various forms or precursors of vitamins D and A [17].

This review aims to examine the relationship between vitamin A and vitamin D and food allergy, and includes a discussion of the measurement techniques, to provide the precise and accurate data required to assess vitamin status in pediatric populations.

What is the link between vitamins A & D and food allergy?

Generally food allergy is defined as a maladaptive immune response to an inoffensive food related antigen [18]. Available epidemiological data propose a dramatic increase in food allergy and related anaphylaxis during the last two decades [19]. There are several studies available which provide indirect and direct evidence for vitamin D and suggestive evidence for vitamin A levels in relation to food allergy prevalence.

Vitamin D: the indirect evidence

There is some evidence that variation in food allergy prevalence within countries might be linked with ambient ultra violet radiation (UVR) exposure and thus potentially with vitamin D levels. Although few studies internationally have measured food allergy prevalence in population-based samples using the gold standard of oral food challenges, several have assessed the correlation between proxy markers of food allergy status and latitude. Four studies have been published which report that areas further from the equator with lower ambient UVR levels may have more food allergy [18,20–22]. In Australia, parent-reported egg and peanut allergy are more common in southern states i.e. further from the equator, which have less year round sunlight. Also in Australia, prescriptions of hypoallergenic formulas which are used for the treatment of food allergy are more common in southern states, as are prescriptions for epinephrine auto-injectors for the treatment of anaphylaxis [21]. Similar trends in prescription of auto-injectors have been described in the US, with these being more common in areas further from the equator [23].

Comparisons between countries with varying ambient UVR levels are theoretically possible. In practice, such studies are hampered by the lack of standardized protocols for measuring food allergy. This limits the ability to make accurate comparisons between studies. The EuroPrevall study which does use standardized protocols to measure food allergy across multiple countries may provide further insight into potential links between UVR and food allergy prevalence [24]. However, even these comparisons are complicated by differences between countries in sun exposure behavior, such as the use of sunscreen and time spent outside. Such comparisons are further complicated by the varying recommendations for maternal and infant vitamin D supplementation and food chain fortification with vitamin D. In addition to a potential link between UVR exposure on a population level and food allergy,

¹ The generic Vitamin D term is used when it refers to the whole vitamin D analog group.

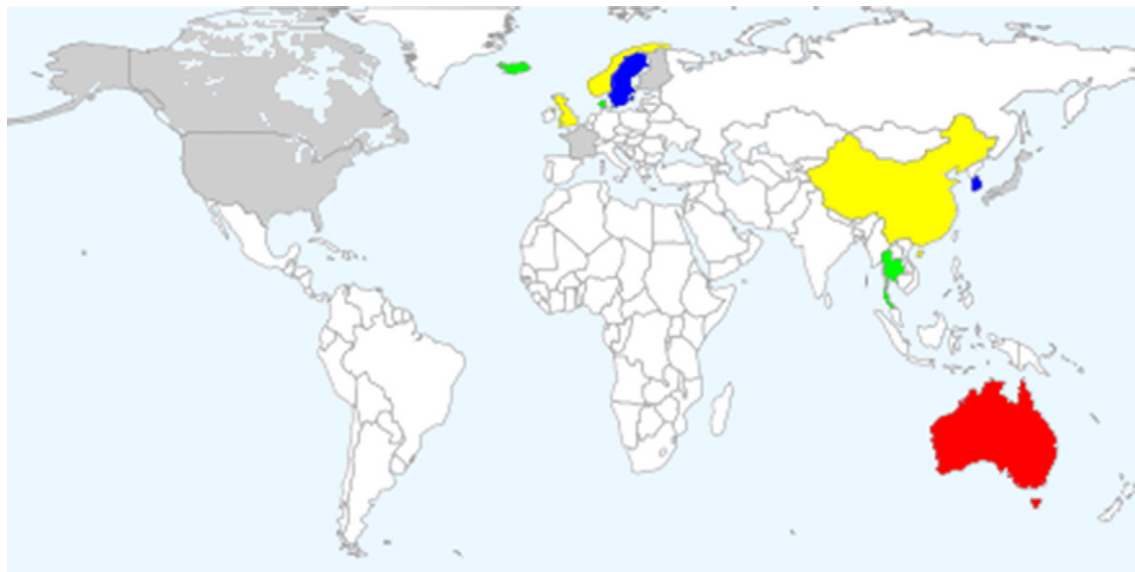


Fig. 1. Variation in the documented global prevalence of food allergy in children ≤ 5 years of age. This map represents information from 14* countries globally and is derived from a mixture of sources which includes parent reports, symptoms and sensitization and oral food challenges (OFCs). This data is adapted from information provided through the World Allergy Organization and information provided in reference no. [4]. The map was developed using online software from <http://www.aneki.com/map.php>. The prevalence, based on percentage reports in the population: ■ OFC high prevalence ($>8\%$) – i.e. Australia ■ OFC medium prevalence (4–8%) – i.e. China, Norway, and United Kingdom. ■ OFC low prevalence ($<4\%$) – i.e. Denmark, Iceland, and Thailand. ■ Non-OFC proven food allergy. Korea, Sweden, Taiwan* have data based on sensitization studies. The documented incidence range is from 3.2 to 11%. ■ Non-OFC proven food allergy. Canada, Finland, France, Hong Kong*, Japan and the USA have data based on parent reports. The documented incidence range is from 4.1 to 9.2%. □ Information not available or not provided. *Note: the software used to generate this map does not distinguish Taiwan and Hong Kong from mainland China.

there is some indirect evidence to support a link between individual UVR exposure and food allergy [21,22].

Food allergy is widely believed to have increased over recent decades and the available evidence, although scarce and mixed, seems to overall support an increase. Examination of changing vitamin D exposure and food allergy prevalence at the population level over time may support a causal link between vitamin D and food allergy, although the evidence is not clear. The indirect evidence for and against is:

- 1) **Supporting evidence.** Two studies have shown that patients with food allergy were more likely to have been born in autumn/winter when ambient UVR levels are lower and vitamin D stores may therefore be lower [20,21]. In addition, there has been a contemporaneous decrease in vitamin D levels in many parts of the world due to changes in behavior leading to less sun exposure over time [25].
- 2) **Contrary evidence.** On the other hand, time trends over a longer period may not support a link between vitamin D and food allergy [26]. To this effect, Wjst argues that in the 1900s when low vitamin D levels were common in England, seen in high rates of rickets, allergic disease in general and presumably food allergy also appears to have been uncommon [27]. This observation, while important, is of course complicated by a wide range of other environmental factors that have changed over the last century. It has been argued, for instance, that low vitamin D status may only be a risk factor for food allergy in the presence of a 'Westernized' gut microbiota [28].

Vitamin D: the direct evidence

Only a few studies to date have examined the relationship between measured serum vitamin D levels and either food sensitization or allergy. These studies are:

- 1) In the US-based National Health and Nutrition Examination Survey (NHANES 2005–2006), children with total 25-hydroxy-vitamin D (25-OH-VitD) <37.5 nmol/L in blood were more likely (odds ratio,

2.39; 95% CI, 1.29–4.45) to have allergic sensitization to peanuts than children with total 25-OH-VitD levels >75 nmol/L [29].

- 2) A separate study found that low vitamin D at birth (defined as cord blood 25-OH-VitD level <27.5 nmol/L i.e. <11 ng/mL [30]) was associated with food sensitization in children with four specific genotypes while this association was not present in children with other genotypes [31]. The genes involved were IL4 (rs2243250), MS4A2 (rs512555), FCER1G (rs2070901), and CYP24A1 (rs2762934); which are involved in IgE synthesis and regulation as well as vitamin D metabolism. This finding of a gene–vitamin D interaction provides some evidence to support a causal relationship between vitamin D levels and food sensitization.
- 3) In a recent Australian population-based cohort study of one year old infants, those with vitamin D insufficiency were three times more likely to be egg allergic and eleven times more likely to be peanut allergic compared to those with sufficient serum vitamin D levels [8]. Among sensitized infants, those with VDI were six times more likely to be food allergic. This association was only present among infants of Australian-born parents, suggesting a possible gene–environment interaction.
- 4) Some studies have also suggested that high vitamin D levels or oral supplementation with vitamin D may be associated with an increased risk of allergic disease in general, although these have generally not examined food allergy specifically [25].
- 5) One recent small study found that higher maternal and cord blood vitamin D levels were associated with a higher risk for food sensitization and allergy in the first two years of life [32]. This study, however, was limited by significant loss to follow-up.

These apparently conflicting findings might be explained by a differential immune effect of oral vitamin D supplementation compared with vitamin D derived from UVR exposure. Alternatively, there is some evidence to support a 'u-shaped association' whereby both high and low serum vitamin D levels may increase the risk of allergic disease. The timing of adequate vitamin D exposure may also be important, and

longitudinal studies investigating the effect of serum vitamin D levels at different time periods from birth through the first years of life are still required to investigate this.

Vitamin A: is it worth studying as a potential risk factor?

To date no study has looked at the role of vitamin A in the development of food allergy, although there is reasonable evidence that it plays a role in immune development. Therefore the investigation of the role of vitamin A alongside vitamin D in the risk of food allergy development is a worthwhile line of investigation — especially since many foods such as dairy, fish and eggs contain reasonable quantities of both of these fat soluble vitamins. The role of vitamin A in immune health is reviewed below.

- 1) **Role in the gut.** Vitamin A plays a vital role in epithelial cell differentiation and immune system development [33,34] and has a well-established role in gut mucosal immune system development [9]. There is emerging evidence that vitamin A may also play a role in immune tolerance and tolerance to food proteins through imprinting of gut-homing specificity on T cells [35]. Since the body mucosal surface is the front line barrier exposed to food allergens, the development of mucosal immune tolerance is essential to normal development of food tolerance.
- 2) **Role in immune response.** Vitamin A appears to play a key role in maintaining an adequate number of peripheral naive T cells. To achieve oral tolerance, vitamin A is likely to be essential in enhancing conversion of T cells to regulatory T cells by a TGF mediated mechanism [36–38]. There is also some evidence regarding an association of elevated circulatory vitamin A levels and increased risk of IgE mediated sensitization [39]. This may be due to skewing of the immune system towards a T helper-2 cell response [40].
- 3) **Vitamin A supplementation.** One study reported a direct association between vitamin supplementation in early childhood and a higher risk of food allergy [41], however it is not clear whether an increase in vitamin A intake could help to explain this association. Vitamin A deficiency is common in developing countries and to prevent clinical consequences, vitamin A is supplemented in early infancy [42]. Although water based preparations of vitamin A are available, fat soluble based preparations such as peanut oil are usually advocated because of improved preparation and storage. Moreover, peanut oil contains omega-3 and omega-6 fatty acids [42] which are recommended to reduce autoimmune disease and prevent allergy [43,44]. It has been reported in young children that the water-soluble form of vitamin A and D supplementation increases the odds of developing allergic diseases compared with fat-soluble forms possibly because it is so poorly absorbed [42].
- 4) **Potential interplay with vitamin D.** The antagonist effect of vitamin A on vitamin D action is illustrated by a former study [45]. The main relevance of this is that preparations containing both vitamins (such as cod liver oil) may behave differently to pure forms of either [46]. Indeed, this is a factor that should be considered when evaluating the observation that increasing cod liver oil supplementation over the 20th century was associated with an increase in allergic disease [27].

Further research needs to be conducted to establish evidence for or against the role of vitamin A in food allergies.

What is the biological plausibility of vitamin D in relation to food allergy?

Vitamin D plays a role in a number of aspects of immune function that are likely to be important in promotion or maintenance of food tolerance [28,47,48]. Vitamin D is involved in maintaining intestinal mucosal barrier integrity, thus vitamin D deficiency might lead to increased food antigen uptake potentially increasing the risk of food allergy. Vitamin D also promotes the expression of IL-10-secreting T-regulatory cells [49],

which are involved in maintenance of immune tolerance. Vitamin D deficiency might also impair immunological tolerance by leading to a pro-sensitization immune imbalance [50]. Overall, it is proposed that there is biological plausibility for a true link between vitamin D and food allergy, due to the significant role of vitamin D in maintaining mucosal immunity, healthy gastro-intestinal microbiota and allergen tolerance [18].

How are these vitamins measured?

Vitamins A and D are classified as fat soluble vitamins and both are recognized to have hormonal like actions on nuclear receptors in target tissues. As both compounds are hydrophobic they are transported in the circulation by their respective carrier proteins. To exert their effect both vitamins require conversion to their active forms. To assess the levels of vitamins A and D in blood, the main circulating forms (i.e. retinol and 25-OH-VitD₃ respectively) are routinely measured on serum or plasma samples. There has been extensive discussion in the literature relating to the lack of agreement between methods for these vitamins. The following section briefly outlines the method characteristics for the measurement of the main circulating form of these vitamins.

Vitamin A methods

In pediatric laboratories in developed countries², serum vitamin A levels are most commonly measured to monitor cystic fibrosis patients and to screen newly arrived immigrants from developing countries. By contrast, in developing countries vitamin A is almost exclusively measured to assess clinical deficiency, which is supported by World Health Organization (WHO) programs to combat the vitamin A deficiency epidemic observed in low Gross Domestic Product (GDP) economies. The lower reference limit for serum vitamin A is clearly defined by the WHO as 0.7 µmol/L (with clinical signs of deficiency at 0.35 µmol/L) in children [10]. The upper reference limit is less clearly defined with variations seen between laboratories [51]. Analysis of this vitamin is almost exclusively confined to chromatographic separation techniques in laboratories worldwide [51–53].

Measurement of vitamin A, due to its low circulatory concentration and structural similarity of related analogs remains a challenge. Most laboratories use high performance liquid chromatography with spectrophotometric detection to measure retinol [51,54]. More recently mass spectrometry based detection methods have been reported [55–57]. Prior to sample analysis, the serum is prepared by liquid–liquid extraction techniques and includes a protein precipitation step to remove vitamin A from its binding protein. In all assays an internal standard is added to correct for losses in the preparation technique. Commercial calibrators available for vitamin A are usually based on the National Institute for Standards and Technology (NIST) reference material; currently NIST SRM 968e.

While the NIST reference material is available and utilized by commercial calibrator manufacturers, agreement between laboratories for vitamin A results remains a problem [14]. This hinders harmonization efforts in laboratory medicine, and presents problems with the transportability of results. In addition, currently there is no reference method listed for retinol measurement [58]. However, liquid chromatography coupled with tandem mass spectrometry can be considered as a prospective nominee for a retinol measurement reference method, as it is able to meet precision and accuracy requirements [59].

Further confounding the interpretation of vitamin A results is the problem that serum retinol levels do not always accurately reflect liver retinyl ester levels (i.e. the storage form of vitamin A) [60]. However once the vitamin A liver supply is diminished, circulatory levels will diminish too. Hence, serum retinol concentration is still of value to

² According to the International Monetary Fund, advanced economies comprise 65.8% of global nominal GDP and 52.1% of global GDP (PPP) in 2010. IMF GDP data September 2011.

dichotomize deficient from replete patients. Retinol levels are also relevant for acute toxicity but have been reported to be of less value in isolation for chronic toxicity [61]. In addition, blood collection is the least invasive collection method to assess vitamin A status.

Vitamin D methods

In general vitamin D refers to two major groups of metabolites; ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Ergocalciferol is basically derived from plant sources which are typically ingested in supplementation form. Cholecalciferol is the endogenous form of vitamin D, that is formed from conversion of 7-dehydrocholesterol to pre-vitamin D₃ due to skin tissue UV exposure. Liver hydroxylation results in the 25-hydroxyvitamin form of both D₂ and D₃. The bio-active form is generated by the kidney as; 1- α ,25-dihydroxy for either D₂ or D₃ in combination with the insignificant 24,25-(OH)₂-VitD₃ analog³ [62–64].

25-OH-VitD₃ has a higher circulatory concentration and longer half-life compare with 1,25-(OH)₂-VitD₃. 25-OH-VitD₃ is considered the appropriate analyte to measure in commercial assays [65]. The early measurement techniques of competitive binding protein and radio immunoassay were developed to evaluate the circulating levels of 25-OH-vitamin D [66–70]. The purported advantage of these assays was their ability to provide equal recognition of 25-OH-VitD₂ and 25-OH-VitD₃. Due to the structural similarity of the vitamin D analogs (Fig. 2), even with sample solvent extraction pre-treatment, the specificity and sensitivity of immunoassay methods have been critically questioned [68]. Still immunoassay based technology for the measurement of 25-OH-VitD₃ is the most broadly used [16].

Epimerization of vitamin D at position three of the structure results in a shift of the hydrogen atom relative to the “plane of paper”. While this isomerization occurs with a number of compounds in the vitamin D pathway, this process is particularly noteworthy for 25-OH-VitD₃, when the 3-epi-25-OH-VitD₃ is produced. This 3-epi-25-OH-VitD₃ epimer is expressed more significantly in younger children rather than adults [71], with prominent amounts of 3-epi observed in pediatric serum samples [72]. The clinical significance of this epimer is still to be fully elucidated. However, 3-epi-25-OH-VitD₃ when present (due to its structural similarity to 25-OH-VitD₃) interfere in 25-OH-VitD methods resulting in the over estimation of 25-OH-VitD₃. Hence, the presence of this epimer cannot be neglected when determining a 25-OH-VitD₃ concentration; especially in children [73]. In addition, 3-epi-1,25(OH)₂-VitD₃ may be formed from 3-epi-25-OH-VitD₃ hydroxylation. Although in general 3-epi-25-OH-VitD₃ is considered as a biologically inactive isomer of cholecalciferol, it is suggested that some of the biological activity of cholecalciferol may relate to 3-epi-1,25(OH)₂-VitD₃ [74,75].

Many of the current immunoassay techniques are now fully automated and do not require any sample pre-treatment e.g. extraction. The manufacturers' designed anti-25-OH-VitD₃ antibodies may cross-react with 25-OH-VitD₂ and other forms of vitamin D (such as; 24,25-(OH)₂-VitD and 25,26-(OH)₂-VitD or 25,26-(OH)₂-VitD-26,23-lactone) [76,77]. Thus, measured vitamin D has been reported to be over-estimated by 10–20% using either competitive binding or immunoassay techniques [78]. These interferences, which vary between immunoassay methods, contribute significantly to the problem of standardization with established trueness for vitamin D.

Ideally in the routine diagnostic environment, the adoption of assays that are accurate and precise which have a low labor involvement and are cost effective with excellent turnaround time is the ideal. Currently such an assay is not available. However, chromatography separation

coupled with mass spectrometry is considered by some as the most promising approach for vitamin D metabolite measurement [65,79,80]. Still standardization with traceability of this method group has also proved challenging [81]. This impacts on our ability to clinically interpret vitamin D levels and is a significant barrier to establishing the optimum level of vitamin D for health [80,82]. This partially explains why the literature reports significant differences in the optimal level of vitamin D [8,29,83,84].

Liquid chromatography coupled with MS/MS system, potentially provides exceptional specificity in terms of different analyte separation and measurement [85,86]. Utilization of the multiple reaction monitoring mode (MRM) delivers the greatest specificity, along with liquid chromatography separation of the structurally related isomers. However, with regard to vitamin D measurement, separation of the 25-OH-VitD₃ from epi-25-(OH)-VitD₃ is still challenging [77,87]. When clear separation of the vitamin D epimers is achieved, LC-MS/MS is generally considered the working “gold standard” method, for quantitation of 25-OH-VitD₂ and 25-OH-VitD₃ and epi- 25-OH-VitD₃ [88,89].

Recently a more sensitive method utilizing LC-MS/MS has been described for 3-epi-25-OH-VitD₃ measurement. This method, which uses a two dimensional ultra-performance liquid chromatography (UPLC) separation technique, coupled with tandem mass spectrometry (UPLC-MS/MS) clearly separates and quantitates 25-OH-VitD₃, epi-25-OH-VitD₃, and 25-OH-VitD₂, requiring a small sample volume (50 μ L) with 2 nmol/L (0.8 ng/mL) analytical sensitivity [17]. This method also separates the 25-OH-VitD₃ isobar 1- α -OH-VitD₃ as well as 7- α -OH-4-cholesten-3-1. The assay sensitivity is reported to be 0.62 nmol/L (0.25 ng/mL). Thus, this two dimensional LC-MS/MS technique is potentially the approach of choice to quantitate multiple fractions of vitamin D simultaneously [17].

The two dimensional LC-MS/MS approach is reported to separate potentially interfering phospholipids [17]. These phospholipids are known to cause alterations in ion intensity [85]. Such interference from phospholipids is known to reduced method sensitivity and compromise the accuracy of the result. While an isotope matched internal standard helps to minimize the effects, the ideal is to optimize the LC-MS/MS method to either remove or at least fully separate chromatographically from the compound of interest; i.e. vitamins D and A. As these lipids are present in each blood sample, potentially to varying degrees, the monitoring of their presence (usually through MRMs of 184 $\rightarrow$ 184 and 104 $\rightarrow$ 104) is recommended as part of routine LC-MS/MS analysis [85].

A final consideration of particular importance for pediatric samples is the volume of blood required for analysis [90]. To ensure fitness for the intended clinical purpose, which includes achieving the desired sensitivity, some methods can require large sample volumes. Generally, this also means a large amount of blood is collected and stored in deep freezers when population based studies are conducted. Such volume requirements are considered a disadvantage for pediatric population-based assessment of the fat soluble vitamins. Recently, an alternative approach collecting samples on Guthrie cards has been proposed by a few groups. Vitamin D has been successfully derived from Guthrie cards by some groups [91–95] and the optimization of vitamin A measurement has been reported by others [96–98].

Combined vitamins A and D analysis

Given the potential for vitamin A, in addition to vitamin D, to have a role in relation to the prevalence of food allergies, it would be ideal to measure these analytes simultaneously. This would be advantageous in terms of laboratory efficiency plus also for the determination the pattern of vitamin A alongside vitamin D. Any such method would still require clear separation of the carbon-3-epimer. Recently, such a method has been presented, indicating that simultaneous measurement is possible [99] (Fig. 2).

³ For note, in the earlier clinical section of this document we referred to the term vitamin D to encompass the generic meaning of the word. For the discussion of vitamin D methods the specific names are used for clarity.

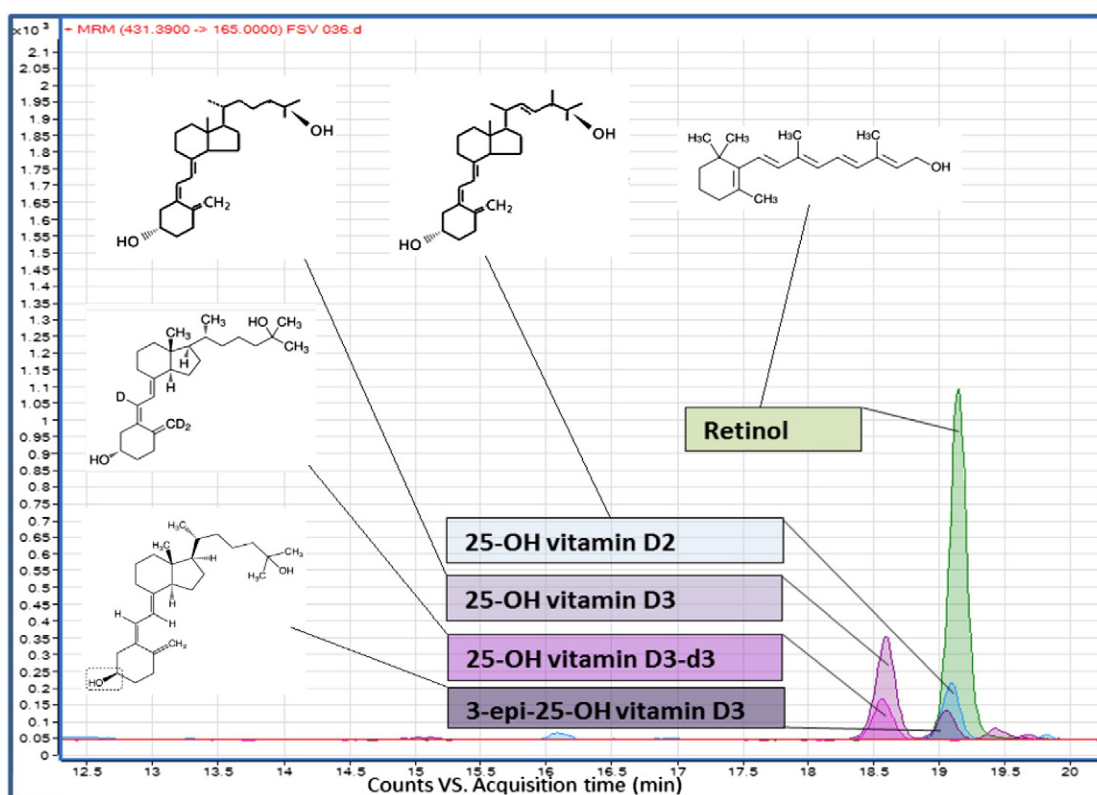


Fig. 2. LC-MS/MS-MRM chromatogram of 25-OH-vitamin D analogs and retinol separation in a human serum sample. 25-OH-vitamin D₃-d₃ is the deuterated analog that is added to the sample as the internal standard. The x axis represents the acquisition time (min) and the y axis is the ion counts. Each of the vitamin D analogs is a secosteroid i.e. steroid-like chemical structure with the second ring opened. Vitamin A, measured as retinol, is a cyclohexene ring attached to a tetra-isoprene chain. This figure is modified from Ref. [99].

Alternative sample collection – Guthrie cards

Classically, analytical methods for vitamin A and vitamin D utilize either serum or plasma separated from whole blood. Mostly, in large population-based studies, especially involving children and infant participants, we face some uncertainties in terms of sample collection and storage. Blood spot card (Guthrie's) are suggested as the alternative sampling method (dried capillary blood spot) to store and undertake further blood analyses for large population-based studies [100]. These studies already utilize chromatography/mass spectrometry measurement methods [91,98,101]. It has been demonstrated that retinol, 25-OH-VitD₂ and 25-OH-VitD₃ are stable in dried blood spot card and measurable, utilizing such methods [94,96,97].

Several studies report a high level of agreement for the cholecalciferol and retinol concentrations between venous blood samples and Guthrie's. Venous blood samples have a higher hematocrit than capillary samples [102]. The implication of this is that dried blood sample may have a slightly lower serum fraction than samples derived from venepuncture. Accordingly, it is expected that lower values for either retinol or cholecalciferol will be obtained from Guthrie's compare to serum/plasma, unless adjusted for sample used for analysis. Therefore, in the estimation of vitamins in dried blood samples such as Guthrie cards, assessment of blood spot size and hemoglobin concentration needs to be taken into account [95].

Where to from here?

In summary, the increase of food allergy may be related to a concordant rise in prevalence of vitamin D insufficiency. In addition, vitamin A is linked to immunity and it is plausible that it may also have a role in the risk of developing food allergies. However, further studies need to

be conducted to investigate the impact of vitamin A and D insufficiency or excess at birth on food allergy in early childhood. Furthermore, understanding the adverse effects of a high prevalence of VDI in the general population is an important public health initiative, since VDI may be corrected with the simple and inexpensive intervention of dietary vitamin D supplementation. Before this can be achieved however, the optimum level of vitamin D for reducing the risk of allergic disease and maintaining overall health will need to be established.

Chromatographic separation coupled with mass spectrometric detection is considered the higher order method for both vitamins. These analytical methods should be fully validated for the use in pediatric populations to ensure they are fit for their clinical purpose, including chromatographic separation of the 25-OH-VitD₃ from its epimer. To utilize blood spots for the investigation of these vitamins at birth, the LC-MS/MS methods will require further optimization in order to evaluate the small volume samples derived from dried blood spot cards.

Finally, it is essential to develop integral population data for the status of blood vitamin D and vitamin A in order to decide if the hypothesized relationships do exist in early life.

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