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Vitamin D Status in Cow's Milk Protein Allergy

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Abstract
Introduction: Cow's milk protein allergy (CMPA) affects 1.9 - 4.9% of children worldwide and can be IgE-mediated or non-IgE mediated. Studies have highlighted the increased risk of inadequate vitamin D intake in children with CMPA on elimination diets, with potential effects on bone health, respiratory health and immune function. This study aims to investigate whether children with CMPA are at a higher risk of vitamin D insufficiency than their healthy counterparts.
Methods: This was an observational, cross-sectional study carried out at the specialist Paediatric Allergy Clinic, Bon Secours Hospital Cork and the Department of Paediatrics, Bon Secours Hospital Cork. 52 children with CMPA and 26 healthy controls, aged 6 to 24 months, were included. Exclusion criteria included known bone metabolism disorders, chronic renal and gastrointestinal disorders, and chronic infectious diseases. A retrospective chart review of children with CMPA was conducted to collect data on patient demographics, clinical presentation, and management. Serum 25(OH)D levels of children with CMPA (n=18) and healthy controls (n=26) were quantified and compared. Caregivers of children with CMPA were administered an online questionnaire to assess compliance to vitamin D supplementation.
Results: The serum 25(OH)D levels of children with CMPA were significantly lower than that of healthy controls (47.5 vs 62.5 nmol/L, p = 0.030). Among children with CMPA, children with IgE-mediated CMPA (p=0.041) and children with multiple concomitant IgE-mediated food allergies (p=0.011) had significantly lower serum 25(OH)D levels than their healthy counterparts. Among children with CMPA, breastfeeding status, reported vitamin D supplement use, age and gender were not significantly associated with serum 25(OH)D levels. The questionnaire revealed only 44.4% of caregivers to be fully adherent to vitamin D supplementation guidelines.
Conclusion: Children with CMPA are at higher risk of serum 25(OH)D insufficiency than healthy controls. Closer monitoring is required to ensure that vitamin D supplements are administered from birth to 12 months, at the recommended frequency.

Keywords: cow's milk protein allergy; vitamin D insufficiency; supplementation; children; infants

Introduction

3.1 Cow's milk protein allergy

Cow's milk protein allergy (CMPA) is an immune-mediated food hypersensitivity that affects between 1.9% and 4.9% [1-2] of young children worldwide. CMPA can be IgE or non-IgE mediated. IgE-mediated CMPA presents predominantly with immediate symptoms such as vomiting, acute urticaria, angioedema and wheezing while non-IgE mediated CMPA tends to present with a delayed onset of diarrhoea, constipation, bloody stools and atopic dermatitis. The dietary elimination of cow's milk proteins (CMP) is key to CMPA management. In exclusively breastfed infants, mothers should consume a CMP-free diet with vitamin D supplements [1, 3]. In non-breastfed children aged less than 24 months, a CMP-free diet and replacement with a hypoallergenic formula is recommended [2]. Most children, after 9 months of age, are suitable for home-based reintroduction of extensively heated, "baked milk", products to promote CMP-tolerance acquisition [1, 4]. At ages above 24 months, solid foods and liquids free of CMP are thought to provide a nutritionally adequate elimination diet [3].

3.2 Vitamin D insufficiency/deficiency in CMPA

Several paediatric research societies have highlighted a higher risk of vitamin D (serum 25(OH)D) deficiency in CMPA and recommend supplementation for the duration of the CMP-elimination diet [5-6]. However, serum 25(OH)D concentrations that constitute deficiency in children remain contentious [7-9] (Appendix A). The Institute of Medicine's serum 25(OH)D classification [9], adopted in this study, considers levels <30 nmol/L deficient and levels between 30 and 50 nmol/L insufficient (Table 2). In general, low serum 25(OH)D levels in children can arise from low dietary intake, inadequate sun exposure, lack of supplementation and maternal hypovitaminosis [10]. In early childhood, however, cow's milk consumption and vitamin D supplements (VDS) are thought to be the greatest determinants of serum 25(OH)D status [11]. Hence, the elimination of dairy is

particularly likely to cause nutritional deficiencies in young children with CMPA [12]. Failure to correct low serum 25(OH)D levels with VDS and fortified formula can compromise bone health [13], respiratory health and immune system function [14] in this group. In fact, inappropriately managed CMPA has resulted in children developing rickets[15] and multiple bone fractures [16] in the past.

On the contrary, a growing body of evidence suggests inadequate serum 25(OH)D levels may cause food allergen sensitisation [17], particularly IgE-mediated CMPA [18]. Low serum 25(OH)D levels prevent differentiation of naive T lymphocytes into regulatory T cells (Treg), which typically act to suppress harmful allergic responses mediated by Th1 and Th2 cells [19]. Adequate serum 25(OH)D levels can increase Treg levels, and may even promote the acquisition of tolerance in CMPA [20-21].

3.3 Recommendations for vitamin D supplementation

Despite guidelines emphasising the need for formal nutritional assessment [2, 5-6], children with CMPA are currently not recognised as a high-risk group for serum 25(OH)D insufficiency in Ireland, and do not require screening [22]. Generic supplementation protocols are thought to sufficiently support children with CMPA to achieve the recommended dietary allowance for vitamin D. The Irish Health Service Executive (HSE) recommends that all infants, both breastfed and formula-fed, receive a daily 5μg vitamin D supplement from birth to 12 months of age [23]. However, the rates of VDS use in children with CMPA and the efficacy of generic supplementation protocols in achieving serum 25(OH)D sufficiency in CMPA have not been established.

Hence, this study aims to investigate whether children with CMPA are at higher risk of serum 25(OH)D insufficiency than the general paediatric population, assess their use of vitamin D supplementation, and ultimately, identify areas for improvement in the nutritional management of CMPA.

Methods
4.1 Study design

This was an observational, cross-sectional study carried out in the specialist Paediatric Allergy Clinic and Department of Paediatrics, Bon Secours Hospital Cork from October 2018 to January 2020. The study consisted of a retrospective chart review of children with CMPA and a follow-up questionnaire on VDS use for caregivers.

4.2 Study participants

Children aged between 6 and 24 months attending the Paediatric Allergy Clinic with an established diagnosis of IgE-mediated or non-IgE mediated CMPA were included (n = 52). The diagnosis was based on a suggestive clinical history and confirmed to be: (i) IgE-mediated CMPA in the case of a positive skin-prick test and/or raised serum CMP-specific IgE level, or (ii) non-IgE mediated CMPA in the case

of symptom resolution with CMP elimination and recurrence on reintroduction. Patients with no evidence of CMPA or other food allergies, who have had serum 25(OH)D levels quantified between 6 and 24 months of age, were eligible for inclusion as controls (n=26). The exclusion criteria for both cases and controls were (i) age < 6 months or > 24 months, (ii) known bone metabolism disorders, (iii) renal or gastrointestinal disorders, and (iv) chronic infectious diseases.

4.3 Study procedures

A chart review of referral letters and clinic visit proformas, both written and electronic, of children with CMPA (n=52) was undertaken to obtain information on demographic and clinical variables. For patients identified as cases, measurement of serum 25(OH)D levels was advised and values obtained. The serum 25(OH)D levels, age and gender of selected controls (n=26) were obtained from the Bon Secours Hospital laboratory. Letters containing the URL of the online questionnaire were posted to study participants in February (n=38) and December 2019 (n=43).

4.4 Study measures
4.4.1 Clinical and demographic data

Data on variables (Table 1) associated with demographic characteristics, clinical presentation and clinical management of children with CMPA were obtained from chart review.

4.4.2 Serum 25(OH)D quantification

For the analysis of serum 25(OH)D levels, a blood sample was collected by a trained professional and analysed using accredited methods such as mass spectrometry or automated immunoassay in the Bon Secours Hospital Laboratory or a HSE laboratory. Serum 25(OH)D levels of 18 children with CMPA and 26 healthy controls were obtained. For the purpose of this study, the internationally recognised Institute of Medicine's vitamin D classification, which is most similar to the HSE laboratory testing guidelines, was chosen (Table 2).

4.4.3 Assessment of vitamin D supplementation usage

Frequency, duration and dose of VDS use were assessed via a short questionnaire (Appendix B). Full compliance was defined as daily usage of VDS, at the recommended dose of 5μg, from birth to the time of study, for children under 12 months of age, or from birth to 12 months of age, for children between 12 to 24 months of age. Non-compliance was deemed to be a complete lack of VDS for the first year of life. The difference between reported rates of VDS use, documented by a paediatrician in the patient's chart, and actual rates of adherence, based on questionnaire responses, was investigated. Caregivers' knowledge surrounding serum 25(OH)D deficiency and the HSE VDS recommendations was assessed using true-false statements.

4.5 Data analysis

Statistical analysis was performed using SPSS IBM version 25. Continuous variables were tested for normality using the Shapiro-Wilk test. Descriptive statistics were expressed as means with standard deviations for normally distributed data, and medians with interquartile ranges for skewed distributions. The independent t-test, non-parametric Mann-Whitney U test and Kruskal-Wallis test with post-hoc Bonferroni-correction were utilised to assess associations between CMPA and continuous variables, while Pearson's chi-squared test and Fisher's exact test were used to assess associations between CMPA and categorical variables. Cases with missing serum 25(OH)D levels were excluded from inferential statistical analysis. An exact binomial test was used to assess the difference between reported and actual compliance to VDS. For all tests, the significance level was set at 0.05.

Table 1. Clinical and Demographic Variables		
Demographic	**Clinical Presentation**	**Clinical Management**
Age	Type of CMPA	Type of hypoallergenic formula
Gender	Symptoms on allergen exposure	Breastfeeding status
Ethnicity	Concomitant IgE-mediated food allergies	Vitamin D supplement use
	Presence of eczema	Solid food intake
	Atopic family history	Home-based CMP reintroduction
	Skin-prick test results	Acquisition of tolerance
	CMP-specific serum-IgE	Dietitian referral

Table 2. Institute of Medicine Vitamin D Classification		
Serum 25(OH)D level		**Health Status**
Description	**nmol/L**	
Deficiency	< 30	Risk of deficiency; may lead to rickets in infants and children
Insufficiency	30 - 50	Generally considered inadequate for bone and overall health
Sufficiency	≥ 50	Generally considered adequate for bone and overall health

Results

5.1 Review of CMPA presentation and management

The mean age of CMPA diagnosis was 9.13 months (n=52). 73.1% (n=38) of children had non-IgE mediated CMPA and 26.9% (n=14) had IgE-mediated CMPA. At the point of chart review, children with CMPA were older (median: 16 months, Q1: 14.00, Q3: 24.00) than controls (11 months, Q1: 8.25, Q3: 14.00). All children were of Caucasian ethnicity.

Gastrointestinal manifestations, such as vomiting and diarrhoea, were the most frequently reported (76.9%, n=40) presenting symptoms. Gastrointestinal manifestations were significantly more common in non-IgE mediated CMPA (86.7% vs 50.0%, p=0.010) while skin symptoms were more likely in IgE-mediated CMPA (92.9% vs 23.7%, p<0.001). Patients with IgE-mediated CMPA were significantly more likely than peers with non-IgE mediated CMPA to have concomitant IgE-mediated allergies (71.4% vs 15.8%, p<0.001), be breastfed (71.4% vs 31.6%, p=0.001), and have eczema (85.7% vs 55.3%, p=0.043) (Table 3).

At the time of study, all children had been started on complementary feeding with solids. Of the 92.3% of patients who were eligible for home-based CMP reintroduction, 45.8% (n=22) demonstrated steady progress on the "iMAP Milk Ladder" while 6.3% (n=3) developed allergic reactions on attempting CMP reintroduction.

Table 3. Demographic and Clinical Characteristics of Children with CMPA

	Non-IgE mediated CMPA (n=38) n (%)	IgE-mediated CMPA (n=14) n (%)	CMPA (n=52) n (%)	Controls (n=26) n (%)	*p*-value
Gender					0.405[1]
Female	19 (50.0)	8 (57.1)	27 (51.9)	12 (46.2)	
Male	19 (50.0)	6 (42.9)	25 (48.1)	14 (53.8)	
Age					0.003[1]
≤12 months	20 (52.6)	7 (50.0)	27 (51.9)	4 (15.4)	
13 – 24 months	18 (47.4)	7 (50.0)	25 (48.1)	22 (84.6)	
Skin symptoms					< 0.001[2]
Urticaria	9 (23.7)	13 (92.9)	22 (42.3)		
Gastrointestinal symptoms	33 (86.7)	7 (50.0)	40 (78.4)		0.010[2]
Vomiting	13 (34.2)	3 (21.4)	16 (30.8)		
Diarrhoea	16 (42.1)	1 (7.1)	17 (32.7)		
Constipation	10 (26.3)	2 (14.3)	12 (23.1)		
Irritability	9 (23.7)	0 (0.0)	9 (17.3)		
Bloating	4 (10.5)	0 (0.0)	4 (7.7)		
Poor weight gain	3 (7.9)	1 (7.1)	4 (7.7)		
PR bleeding	3 (7.9)	0 (0.0)	3 (5.8)		
Respiratory symptoms	2 (5.3)	1 (7.1)	3 (5.8)		0.391[2]
Rhinitis	1 (2.6)	0 (0.0)	1 (1.9)		
Wheeze	1 (2.6)	1 (7.1)	2 (3.8)		
Concomitant food allergies (IgE)	6 (15.8)	10 (71.4)	16 (30.7)		<0.001[2]
Eczema	21 (55.3)	12 (85.7)	10 (19.6)		0.043[2]
First-degree relative with atopy	18 (47.4)	6 (42.9)	24 (46.2)		0.745[2]
Feeding patterns					0.001[2]
Partial breastfeeding	12 (31.6)	10 (71.4)	22 (42.3)		
Extensively hydrolyzed formula	13 (34.2)	2 (14.3)	15 (28.8)		
Amino acid formula	8 (21.1)	0 (0.0)	8 (15.4)		
Soy formula/plant-based milk/goat milk	6 (15.8)	1 (7.1)	7 (13.5)		

[1] *p* value from Pearson's chi-squared test comparing children with CMPA and healthy controls.
[2] *p* value from Pearson's chi-squared test/Fisher's exact test comparing children with IgE-mediated CMPA and non-IgE mediated CMPA.

5.2 Vitamin D status of children with CMPA compared to healthy controls

Serum 25(OH)D levels of 18 patients with CMPA and 26 healthy controls were obtained (Appendix D). The median serum 25(OH)D level of children with CMPA was significantly lower than that of healthy controls (47.50 vs 62.5 nmol/L, U = 143.5, p = 0.030, r = 0.10634) (Figure 1).

There was also a higher frequency of serum 25(OH)D insufficiency observed in those with CMPA compared to controls (55.6% vs 30.8%, p=0.091) (Figure 2). A similar pattern was observed when an alternative definition of serum 25(OH)D insufficiency (<75 nmol/L) [8] was utilised for analysis (88.9% vs 61.6%, p=0.134) (Appendix A).
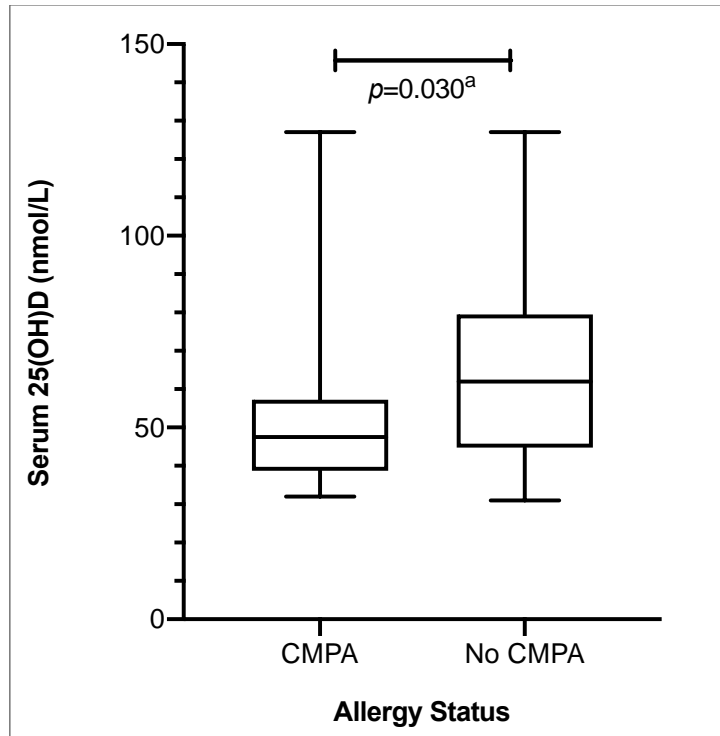
Figure 1. Box-and-whisker plot comparing serum 25(OH)D levels of children with CMPA and healthy controls; [a] Mann-Whitney U test.
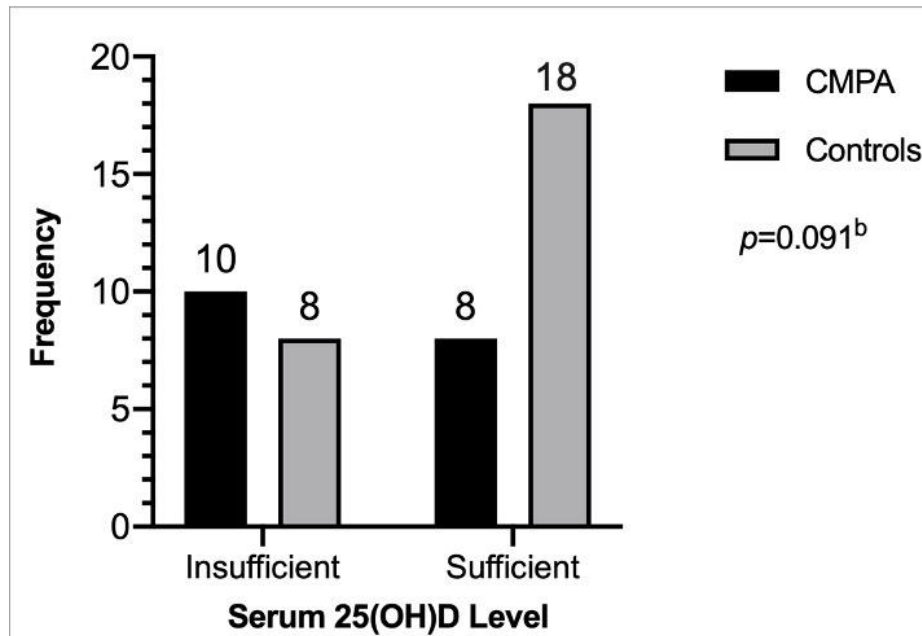

Figure 2. Bar chart showing frequency of serum 25(OH)D insufficiency in CMPA and controls; [b] Pearson's chi-squared test.
Serum 25(OH)D insufficiency: 30 – 50 nmol/L
Serum 25(OH)D sufficiency: ≥ 50 nmol/L

5.3 Factors affecting Vitamin D levels in children with CMPA

Further sub-group analysis revealed that children with IgE-mediated CMPA (p=0.041), and children with CMPA and multiple IgE-mediated allergies (p=0.011) had significantly lower serum 25(OH)D levels than their counterparts. A one-way Kruskal-Wallis test with post-hoc Bonferroni correction revealed that children with CMPA and concomitant IgE-mediated allergies had a significantly

lower serum 25(OH)D level than controls (39.00 vs 62.00 nmol/L, p=0.004). However, no significant difference in serum 25(OH)D levels was detected between participants with CMPA alone and controls (55.00 vs 62.00 nmol/L, p=1.0000*) (Figure 3). Interestingly, feeding patterns (breastfeeding vs hypoallergenic formula use), reported VDS use, age and gender were not significantly associated with serum 25(OH)D levels (Table 4).
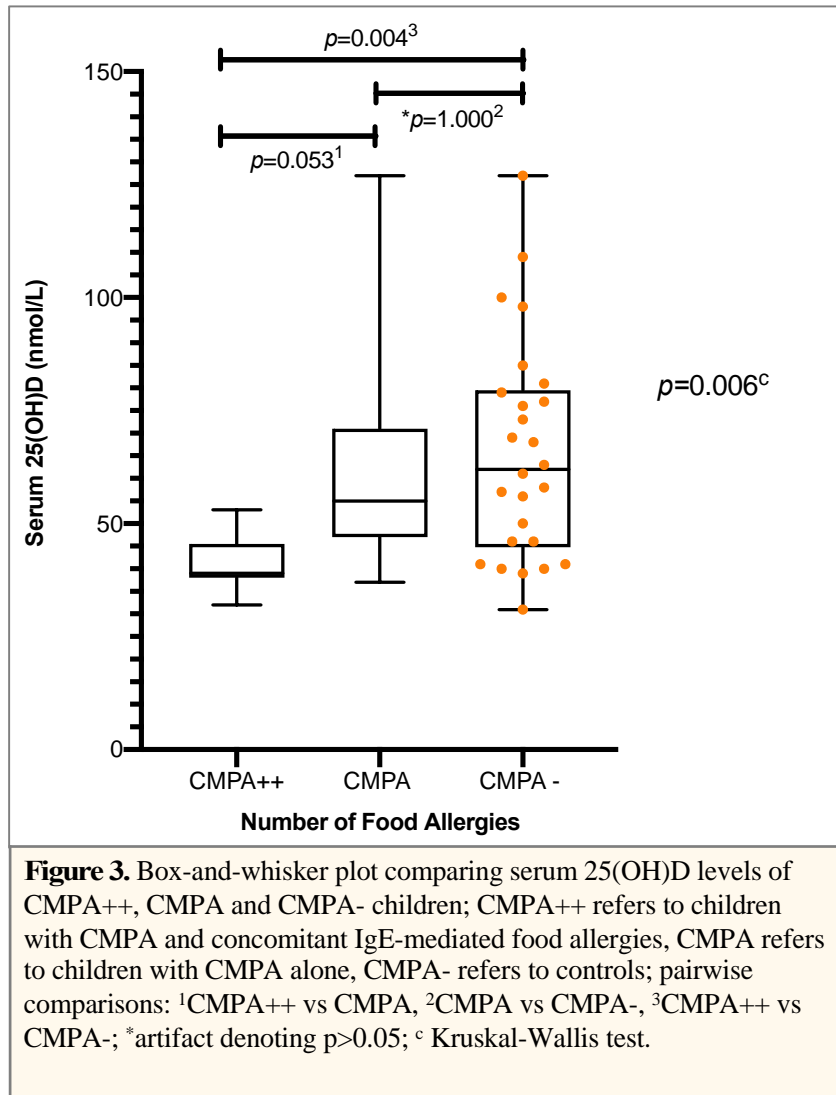

Figure 3. Box-and-whisker plot comparing serum 25(OH)D levels of CMPA++, CMPA and CMPA- children; CMPA++ refers to children with CMPA and concomitant IgE-mediated food allergies, CMPA refers to children with CMPA alone, CMPA- refers to controls; pairwise comparisons: [1]CMPA++ vs CMPA, [2]CMPA vs CMPA-, [3]CMPA++ vs CMPA-; *artifact denoting p>0.05; [c] Kruskal-Wallis test.

Table 4. Clinical Characteristics Affecting Serum 25(OH)D Levels in CMPA		
	Median (IQR)[1] / Mean (±SD)[0] serum 25(OH)D (nmol/L)	p-value[2]
Gender		0.318
Male (n=7)	39.00 (38.00-57.00)[1]	
Female (n=11)	50.00 (41.00-58.00)[1]	
Age	44.00 (37.00-48.00)[1]	0.063
≤12 months (n=11)	53.00 (39.00-62.00)[1]	
13 – 24 months (n=7)		
Feeding patterns		0.134[3]
Breastfeeding (n=11)	44.91 (±9.50)[0]	
Formula-feeding (n=7)	66.71 (±32.96)[0]	
Type of CMPA		0.041
IgE-mediated (n=7)	39.00 (38.00-47.00)[1]	
Non-IgE mediated (n=11)	53.00 (44.00-62.00)[1]	
Number of allergies		0.006[4]
CMPA with concomitant allergies (n=8)	39.00 (38.00-45.50)[1]	
CMPA only (n=10)	55.00 (47.00-71.00)[1]	
No known food allergies (n=26)	62.00 (44.75-79.50)[1]	
Reported VDS use		0.291
Compliant (n=12)	49.00 (39.50-57.75)[1]	
Partial/non-compliant (n=6)	41.00 (37.75-55.25)[1]	

[1] Median (IQR); [0] Mean (±SD);
p value obtained from: [2] Mann-Whitney U test unless otherwise stated; [3] t-test; [4] Kruskal-Wallis test

5.4 Rates of adherence to Vitamin D supplementation

80.8% (42/52) of children with CMPA reported full compliance to VDS at doctor's appointments. However, on closer scrutiny through the study questionnaire, only 44.4% (8/18) were using VDS at the correct duration and frequency (Table 5). An exact binomial test revealed a statistically significant difference in the rates of full compliance to VDS when reported in clinic compared to when assessed via the questionnaire (83.3% vs 44.4%, p=0.039). The main reasons for partial compliance were starting VDS late (n=5, 27.8%), frequent or rare use (n=6, 33.3%), and stopping supplementation prematurely (n=2, 11.1%). Children fully compliant to VDS, based on questionnaire responses, were not more likely than partially compliant or non-compliant peers to have sufficient serum 25(OH)D levels (100% vs 33.3%, p=0.119).

Table 5. Summary of Questionnaire Responses

	Questionnaire compliance			Overall (n=18)
	n (%)			n (%)
	Compliance	Partial compliance	Non-compliance	
Reported compliance				
Fully compliant	7 (38.9)	8 (44.4)	0 (0.0)	15 (83.3)
Partially compliant	1 (5.6)	1 (5.6)	0 (0.0)	2 (11.1)
Non-compliant	0 (0.0)	0 (0.0)	1 (5.6)	1 (5.6)
Frequency				
Daily	8 (44.4)	3 (16.7)	–	11 (61.1)
Frequently (2-3/week)	–	5 (27.8)	–	5 (27.8)
Rarely	–	1 (5.5)	–	1 (5.6)
Never	–	–	1 (5.6)	1 (5.6)
Duration				
0-12 months	8 (44.4)	2 (11.1)	–	10 (55.6)
Stopped prematurely	–	2 (11.1)	–	2 (11.1)
Started late	–	5 (27.8)	–	5 (27.8)
Not started	–	–	1 (5.6)	1 (5.6)
Knowledge				
Serum 25(OH)D importance[1]	17 (94.4)	1 (5.6)	0 (0.0)	17 (94.4)
HSE recommendations[2]	16 (88.9)	1 (5.6)	1 (5.6)	16 (88.9)

[1] Answered *True* to "C1: All children ≤ 12 months old living in Ireland should receive a Vitamin D supplement"

[2] Answered *True* to "C2: Inadequate Vitamin D levels reduce bone health and can lead to rickets"

Discussion

6.1 Serum 25(OH)D levels of children with CMPA worldwide

This study is one of the first studies in Ireland to investigate the serum 25 (OH)D levels of children with CMPA. The mean serum 25(OH)D level of children aged ≤ 24 months with CMPA was found to be lower in this study (53.4 nmol/L, SD = 23.6) than levels reported internationally (range: 74 - 88.5 nmol/L) [10, 21, 24, 25]. Considerable variation in the percentage of children with CMPA with "insufficient" serum 25(OH)D levels exists in current literature, ranging from 20.3 % [10] to 80.0% [20]. However, heterogeneous definitions of serum 25(OH)D insufficiency in these studies hindered meaningful comparison. The lack of a consensus may reflect the current lack of understanding on the serum 25(OH)D level that exerts pathogenic effects on child health [26].

6.2 Serum 25(OH)D levels of children with CMPA compared to controls

Children with CMPA were found to have significantly lower serum 25(OH)D levels than healthy controls in our study (47.5 nmol/L vs 62.5 nmol/L, p=0.031), reinforcing the widely reported tendency of food allergic children to have lower serum 25(OH)D levels [17-18, 27-29] than their peers. It must be noted that most of these studies [17-18, 27-29] had only include children with IgE-mediated CMPA. This is potentially because of the growing interest in the scientific, in-vitro association [20-21] between low serum 25(OH)D levels, low Treg cell counts, and increased IgE sensitisation.

Of two recent studies including children ≤ 24 months with both IgE and non-IgE mediated CMPA, Silva et. al. [10] corroborated our finding while Ercan et. al.[24] found no statistically significant difference in serum 25(OH)D levels between children with CMPA and controls. It is important to note that while none of the children in Silva et. al. were taking VDS, 100% of children and mothers in Ercan et. al. had been fully compliant to VDS in infancy and pregnancy respectively. Similar studies among older, pre-pubertal children [30-32] have reported no significant differences in serum 25(OH)D levels between CMPA and non-allergic groups. The varying findings of these existing studies highlight the relatively more important role that dietary vitamin D sources, including supplements, play in maintaining serum 25(OH)D levels in early childhood in comparison to later childhood and adolescence, when skin sunlight exposure becomes more determinant of [11] serum 25(OH)D levels. By including children with non-IgE mediated allergy, our study findings suggest that cow's milk exclusion can render children with either form of CMPA at risk of serum 25(OH)D insufficiency, underscoring their need for VDS.

6.3 Factors affecting serum 25(OH)D levels among children with CMPA

6.3.1 Multiple concomitant IgE-mediated allergies

Children with CMPA and concomitant IgE-mediated allergies had significantly lower serum 25(OH)D concentrations than controls. This may seem intuitive since children with multiple food allergies have more restrictive elimination diets and are reportedly [33] at higher risk of insufficient nutritional intake. However, low serum 25(OH)D levels may, in fact, be predisposing children to multiple food allergies. Several studies have suggested that serum 25(OH)D insufficiency among children ≤ 24 months renders them at higher risk of IgE sensitisation [28] and in particular, of developing multiple IgE-mediated food allergies [17-18, 27]. This is further supported by our study findings, which suggested a significantly lower serum 25(OH)D level in IgE-mediated CMPA compared to non-IgE mediated CMPA, despite both groups being on similar CMP elimination diets.

6.3.2 Breastfeeding status & Vitamin D supplementation

Previous studies have indicated a heightened risk of low serum 25(OH)D in patients with CMPA that are breastfed and not on fortified hypoallergenic formulas [10, 34-35]. Surprisingly, in our study, partially breastfeeding children did not have significantly lower serum 25(OH)D levels than peers on hypoallergenic formulas. Given that hypoallergenic formulas used by children in our study (e.g. Nutramigen, Neocate) [35-36] are expected to meet daily vitamin D intake requirements, our findings cast doubt on the efficacy of enteral vitamin D absorption in children with CMPA. In fact, the systemic inflammatory response mounted following allergen exposure may inhibit gastrointestinal absorption of fat-soluble vitamins, such as vitamin D [37-38]. This may be the reason that studies evaluating the effect of generic doses of enteral VDS in CMPA, including ours, have failed to find a correlation between supplementation and serum 25(OH)D levels in CMPA [39]. This calls for larger studies to investigate the appropriate dose, frequency and route of VDS administration for children with CMPA.

6.4 Vitamin D supplementation among children with CMPA in Ireland

The reported (80.8%) and actual (44.4%) rates of adherence to VDS among children with CMPA in Ireland fell within the range reported by previous studies [10, 39, 40] among children ≤ 24 months of age. National recommendations seem to play a limited role in encouraging VDS use. For instance, a lower rate of VDS use was reported in an English study (0%) [40] compared to a Norwegian study (86%) [39], despite national recommendations in both countries. Instead, monitoring of adherence at health surveillance visits, and encouragement of supplementation independent of the mode of feeding have emerged as factors significantly promoting VDS adherence [41]. In fact, in our study, out of caregivers who

provided a reason for stopping VDS prematurely (n=2) or after 12 months (n=2), 75.0% (n=3) cited confusion surrounding the need for VDS when their child stopped breastfeeding and/or started solid food intake. As such, it is important for healthcare professionals to emphasise that supplements be continued regardless of changes in feeding patterns, especially for children ≤ 12 months of age.

6.5 Limitations and future directions

Serum 25(OH)D insufficiency is a complex and multifactorial problem. Our retrospective, cross-sectional study design and small sample size did not allow for confounding variables such as prenatal VDS, maternal hypovitaminosis, and sunlight exposure to be controlled for. The recruitment of controls on the basis of availability of serum 25(OH)D levels may also have introduced selection bias as clinical suspicion of insufficiency may have been the very reason for quantification of serum 25(OH)D, resulting in levels unrepresentative of the healthy paediatric population. However, our strict exclusion criteria for renal and gastrointestinal diseases, and bone metabolism disorders should have mitigated this. Finally, participant bias may have affected the results of VDS questionnaires, with participants having a higher likelihood of recognising the importance of VDS than non-participants.

In the future, a prospective cohort study with repeated serum 25(OH)D level measurements at birth and at multiple time points i.e. pre-diagnosis, post-diagnosis and post-tolerance acquisition can be useful to better understand the temporal relationship between low serum 25(OH)D levels and immune-mediated food hypersensitivities, especially CMPA. Furthermore, multi-centre studies can be conducted to achieve the sample size necessary to run an accurate multiple regression analysis to ascertain whether there exists an independent association between CMPA and serum 25(OH)D insufficiency. Finally, more scientific studies are essential to generate a more robust classification of serum 25(OH)D deficiency, insufficiency and adequacy. As long as the optimal serum 25(OH)D level needed to prevent negative health consequences remains nebulous, it is difficult to justify screening or craft special supplementation protocols for high-risk groups.

Conclusions

This study has shown that more than half of the children with CMPA had insufficient serum 25(OH)D levels. Despite high rates of VDS and fortified hypoallergenic formula use, children with CMPA on elimination diets appear to have significantly lower serum 25(OH)D levels than healthy controls. Notably, children with multiple concomitant allergies or the IgE-mediated form of CMPA may be particularly vulnerable to lower serum 25(OH)D levels, warranting closer monitoring. Maintenance on fortified hypoallergenic formulas, compliance to VDS and age > 12 months were not significantly related to higher serum 25(OH)D levels in children with CMPA. Despite moderate

rates of full compliance to VDS, caregivers exhibited a high degree of awareness of the value of vitamin D for health and the existence of the national HSE VDS recommendation. Thus, targeted patient counselling on the duration and frequency of supplement use is likely to be a promising method of improving VDS adherence and efficacy.

The findings of our study highlight that children with CMPA are at higher risk of serum 25(OH)D insufficiency, and act as an impetus for further research into optimising the nutritional management of CMPA, especially with respect to vitamin D.

List of Abbreviations
CMPA: cow's milk protein allergy
CMP: cow's milk proteins
IgE: Immunoglobulin E
Serum 25(OH)D: Serum 25-hydroxyvitamin D / vitamin D
VDS: vitamin D supplement
Treg: regulatory T cells
HSE: Heatlh Service Executive

Conflicts of Interest
The author declares no conflict of interest.

Ethics Approval and/or Participant Consent
This research project was approved by the Clinical Research Ethics Committee (CREC) in July 2018 (Appendix C).

Authors' Contributions
SGP: designed study, collected and analysed data, drafted the manuscript
IM: designed study, collected data, reviewed manuscript

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Appendix A

Table comparing the frequency of serum 25(OH)D sufficiency, insufficiency and deficiency between children with CMPA and healthy controls using 2 different classification systems: (i) Institute of Medicine Classification and (ii) Endocrine Society Classification.

Table 6. Comparison of the Serum 25(OH)D Levels of Children with CMPA & Control Group			
	CMPA group (nmol/L)	**Control group (nmol/L)**	**p-value**
	Median (IQR)	**Median (IQR)**	
Overall	47.5 (Q1: 38.75, Q3: 57.25)	62.5 (Q1: 44.75, Q3: 79.50)	0.031[1]
	n (%)	**n (%)**	
Institute of Medicine Classification			0.091[2]
Serum 25(OH)D insufficiency (30-50nmol/L)	10 (55.6)	8 (30.8)	
Serum 25(OH)D sufficiency (≥50nmol/L)	8 (44.4)	18 (69.2)	
Endocrine Society Classification			0.134[3]
Serum 25(OH)D deficiency (<50nmol/L)	10 (55.6)	8 (30.8)	
Serum 25(OH)D insufficiency (50-75nmol/L)	6 (33.3)	8 (30.8)	
Serum 25(OH)D sufficiency (>75nmol/L)	2 (11.1)	10 (38.5)	

[1] p value from Mann-Whitney U test; [2] p value from Pearson's chi-squared test; [3] p value from Fisher's exact test.

Appendix B
Questionnaire on Vitamin D supplement use administered to caregivers of children with CMPA.

Vitamin D Supplement Use in Ireland

This questionnaire aims to assess your child's use of vitamin D supplements. Thank you for taking the time to fill out this questionnaire. It is expected to take less than 10 minutes of your time.

1. **What is your child's date of birth?**

_____

SECTION A

2. **Question A1: Are you currently giving your child a vitamin D supplement?**
   Mark only one oval.

   ◯ Yes

   ◯ No

If you answered "No" to Question A1, please move on to Section B. If not, please complete Questions A2, A3 and A4.

3. **Question A2: At what age (in months) did your child start taking a vitamin D supplement?**

_____

4. **Question A3: What is the dose of vitamin D supplement (in micrograms) that your child takes per day?**

_____

5. **Question A4: How often do you give your child a vitamin D supplement?**
   Mark only one oval.

   ◯ Daily

   ◯ Frequently (i.e. once in every 2-3 days)

   ◯ Once a week

   ◯ Rarely

   ◯ Other: _____

If you have filled in Section A completely, please skip Section B and move on to Section C.

SECTION B

NOTE: Only answer questions in Section B if you HAVE NOT answered Questions A2, A3 and A4!

6. **Question B1: Have you ever given your child a vitamin D supplement in the past?**
   Mark only one oval.

   ◯ Yes

   ◯ No

7. **Question B2: What is the dose of the vitamin D supplement (in micrograms) that your child took per day?**

   _____

8. **Question B3: At what age (in months) did you start giving your child a vitamin D supplement?**

   _____

9. **Question B4: How often did you give your child a vitamin D supplement?**
   Mark only one oval.

   ◯ Daily

   ◯ Frequently (i.e. once every 2-3 days)

   ◯ Once a week

   ◯ Rarely

   ◯ Other: _____

10. **Question B5: How many months did your child take vitamin D supplements for?**

    _____

11. **Question B6: What was your reason for stopping vitamin D supplementation for your child?**

_____

_____

_____

_____

_____

SECTION C

These questions are meant to assess your understanding of the need for Vitamin D supplementation. Please select either "True" or "False" for each of the statements below. All survey participants are required to complete questions C1 and C2.

12. **Question C1: All children ≤ 12 months old living in Ireland should receive a vitamin D supplement.**
    Mark only one oval.

    ◯ True

    ◯ False

13. **Question C2: Inadequate levels of vitamin D reduce bone health and can lead to Rickets.**
    Mark only one oval.

    ◯ True

    ◯ False

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Appendix C
This project was approved by the Clinical Research Ethics Committee on 27/07/18.

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Committee

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ECM 6 (ee) 14/08/18 Medical School Co-Ordinator: Dr Eileen Duggan

Date: 31st July 2018

Dr Ioana Maris
Consultant Paediatrician
Bon Secours Hospital
College Road
Cork

Study Title: Vitamin D status in children with cow's milk protein allergy.

The above application received at CREC on:

27th July 2018

is

Approved	Not Approved due to the following:
✓	

The following documents have been approved:

Submission Document	Approved	Version	Date
Application Form	Yes		20th March 2018
Data Collection Sheet	Yes		
Questionnaire/Survey	Yes		
Invitation Letter	Yes		
Participant Information Leaflet	Yes		
Consent Form	Yes		
Interview/Focus Group Guide	None		

The co-investigator(s) involved in the project will be:

Medical Student Name	Student Number	Other
Sumitha Grace Pandiaraja	115106390	

Full approval is granted to carry out the above study.

Yours sincerely

Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
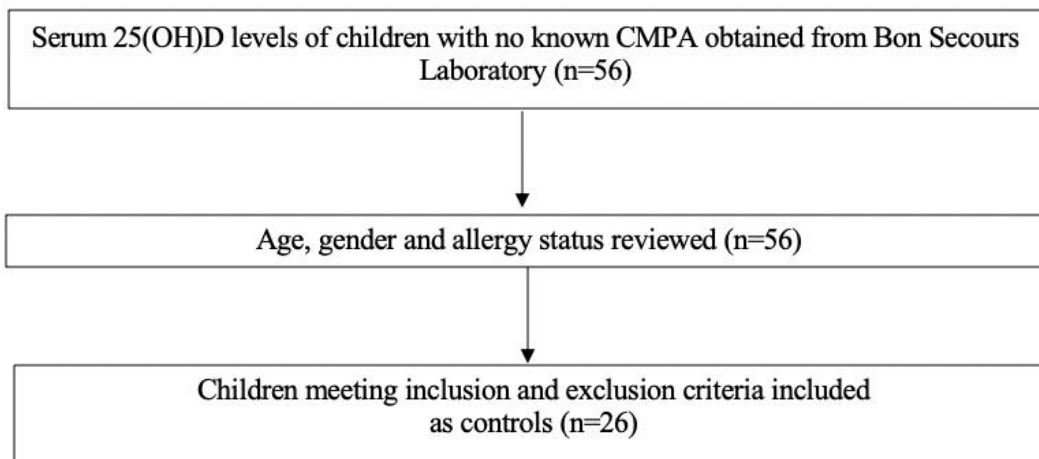Of the Cork Teaching Hospitals

Ollscoil na hEireann, Corcaigh - National University of Ireland, Cork.

Appendix D

Flow chart representing numbers of children with CMPA (cases) at different stages of the study.
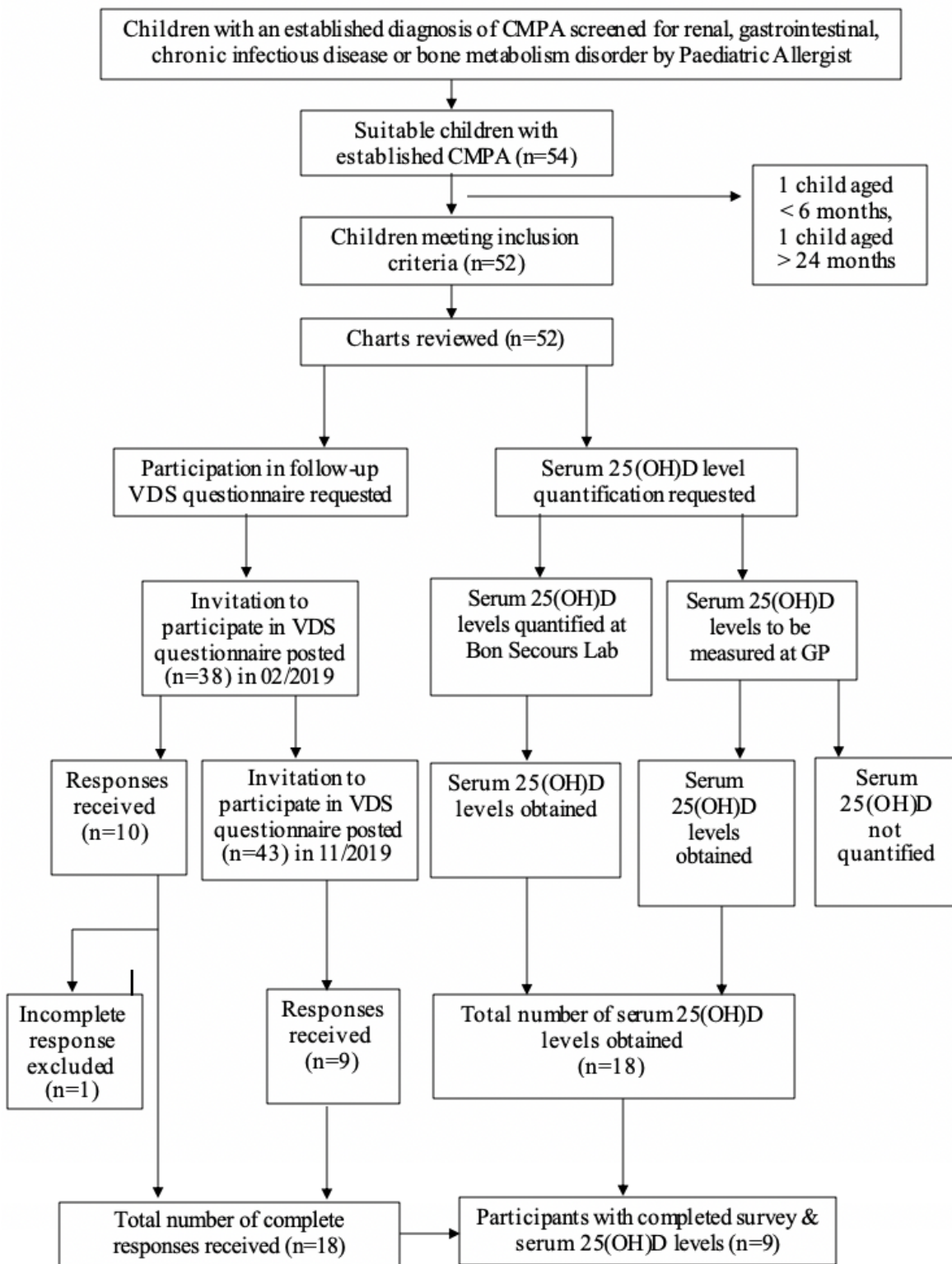
Appendix E

Flow chart representing numbers of children with no evidence of CMPA (controls) at different stages of the study.

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