NUTRITION RESEARCH 82 (2020) 99–109

Available online at www.sciencedirect.com

ScienceDirect

www.nrjournal.com



Sufficient vitamin D status positively modified ventilatory function in asthmatic children following a Mediterranean diet enriched with fatty fish intervention study

Maria M. Papamichael[a], *Catherine Itsiopoulos*[a,e,*], *Katrina Lambert*[b], *Charis Katsardis*[c], *Dimitris Tsoukalas*[d], *Bircan Erbas*[b]

[a] *La Trobe University, School of Allied Health, Human Services &Sport, Department of Dietetics, Nutrition & Sport, Melbourne, Australia*
[b] *La Trobe University, School of Psychology & Public Health, Department of Public Health, Melbourne, Australia*
[c] *National & Kapodistrian University of Athens, Athens, Greece*
[d] *European Institute of Nutritional Medicine, Rome, Italy*
[e] *Murdoch University, College of Science, Health, Engineering & Education, Perth, Australia*

ARTICLE INFO

Article history:
Received 10 October 2019
Revised 30 June 2020
Accepted 10 August 2020

Keywords:
asthma
children
Mediterranean diet
pulmonary function
spirometry
vitamin D

ABSTRACT

Asthma in children is the most prevalent allergic disease worldwide that has become a major public health priority. The objective of this study was to examine the relationship between baseline serum vitamin D status, spirometry, and fractional exhaled nitric oxide levels (FeNO) in 64 Greek children with 'mild asthma' aged 5 to 12 years (51.6% male) in a dietary intervention study. We hypothesized that baseline serum vitamin D levels modify the beneficial response of fatty fish intake on pulmonary function in asthmatic children following a Mediterranean diet. The intervention group consumed 2 fatty fish meals/week (≥150 g cooked filleted fish/meal) as part of the Mediterranean diet for six months, and the control group consumed their usual diet. Baseline serum 25(OH)D was determined using enzyme-linked immunoassay and defined as sufficient levels of 25(OH)D ≥25 ng/mL. Only 36% of children were graded as sufficient in 25(OH)D levels on entry into the study with a higher proportion of girls insufficient than boys (61% vs 39% respectively). Participants with sufficient levels of serum 25(OH)D at baseline, consuming the intervention diet increased FEV_1/FVC by 4.89 units (β = 4.89; 95%CI: 1.19-8.61; $p = 0.013$) and $FEF_{25-75\%}$ by 12.83 units (β = 12.83; 95%CI: 4.27-21.40; $p = 0.006$) compared to controls. No significant differences in pulmonary function or FeNO were observed for those with insufficient levels of 25(OH) D in the intervention or control groups. In conclusion, sufficient serum vitamin D levels enhanced ventilatory function in response to a dietary intervention in asthmatic children.
© 2020 Elsevier Inc. All rights reserved.

Abbreviations: FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEV_1/FVC, ratio of forced expiratory volume in 1 second and forced vital capacity; PEF, peak expiratory flow; $FEF_{25-75\%}$, forced expiratory flow at 25-75% of the pulmonary volume; FeNO, fractional exhaled nitric oxide.

* *Corresponding author at:* College of Science, Health, Engineering and Education, Murdoch University, 90 South Street, Murdoch, Western Australia. Tel.: +61 8 9360 7696.

E-mail addresses: M.Papamichael@latrobe.edu.au (M.M. Papamichael), catherine.itsiopoulos@murdoch.edu.au (C. Itsiopoulos), k.lambert@latrobe.edu.au (K. Lambert), xkatsardis@med.uoa.gr (C. Katsardis), dtsoukalas@einum.org (D. Tsoukalas), b.erbas@latrobe.edu.au (B. Erbas).

https://doi.org/10.1016/j.nutres.2020.08.004
0271-5317/© 2020 Elsevier Inc. All rights reserved.

1. Introduction

There is general consensus that asthma is the most prevalent allergic disease affecting 6.3 million children worldwide [1]. It is a chronic inflammatory disorder of the airways associated with increased morbidity, patient burden, and poor quality of life as well as substantial health care and societal costs, rapidly becoming a public health priority [2]. Studies show that asthma control is poor in children with fewer than 50% of children adhering to prescribed asthma therapy [3].

Asthma is unequivocally caused by a complex combination of genetic and environmental factors [2]. Poor nutrition has been implicated as one of these factors, contributing to increasing asthma prevalence along with vitamin D deficiency in children [4,5]. It is hypothesized that the increasing prevalence of vitamin D deficiency may be linked to the asthma epidemic which has stimulated research interest in the role of vitamin D in asthma pathophysiology. Recent studies have demonstrated that airway epithelia, lung fibroblasts, airway smooth muscle cells, and immune cells (T and B cells, macrophages, monocytes, dendritic cells) contain vitamin D receptors and high levels of the enzyme, 1a-hydroxylase [6]. Vitamin D receptors regulate the transcription of various genes implicated in inflammation and immunomodulation of respiratory epithelium [1]. Vitamin D suppresses pro-inflammatory cytokines Interleukin-17 (IL-17) and IL-13 and promotes anti-inflammatory cytokines such as IL-10 [1]. Thus, vitamin D has a number of biological and immunomodulatory effects that might be important in regulating key mechanisms in asthma.

Low vitamin D status has been reported to be common among children with asthma in a variety of settings [1,7,8]. According to a recent systematic review and meta-analysis undertaken by Jat et al (2017), emerging evidence from observational studies suggest a link between vitamin D deficiency and asthma in children [9]. Data analysis of 23 relevant studies including 13,160 children under 18 years revealed that vitamin D deficiency and insufficiency was prevalent in 28.5% and 26.7% of asthmatic children, respectively. Asthmatic children had lower 25(OH)D levels as compared to non-asthmatic children. Although mixed results were reported for correlations between 25(OH)D and asthma incidence, asthma control, and lung function. Plausible explanations for diversity amongst studies included heterogeneity in study designs, techniques used to measure serum vitamin D, sample size, age, population variability, geographic location, asthma diagnosis at variable ages, and partial controlling for confounders. Similar findings have been published in another systematic review [10], reporting a 36% decreased risk of asthma exacerbation in children. In addition, a beneficial effect of vitamin D supplementation on asthma exacerbations in children has been documented in systematic reviews and meta-analyses [11] .

In our latest randomized controlled trial (RCT) designed to examine the efficacy of a Mediterranean diet supplemented with fatty fish, we found that increased fatty fish intake reduced bronchial inflammation in asthmatic children [12]. The objective of this study was to examine the relationship between baseline serum vitamin D status, spirometry, and fractional exhaled nitric oxide levels (FeNO) in children with 'mild asthma' following a Mediterranean diet enriched with fatty fish intervention study versus the control with a habitual diet. Using data from this trial, we conducted a sub-analysis testing the hypothesis that baseline serum vitamin D levels modify the beneficial response of fatty fish intake on pulmonary function in asthmatic children following a Mediterranean diet and extend further to our work on diet and lung function in childhood asthma.

2. Methods and materials

2.1. Study design

The current study used baseline and 6-month follow-up data from a single-center randomized controlled parallel intervention study that tested the efficacy of fatty fish intake in the context of the Greek Mediterranean diet on asthma in children that took place in Athens, Greece from November 2016 to September 2017 [12]. The trial design and methodology have been published in detail elsewhere [13], and only key features are described here. The study protocol is registered with the Australian and New Zealand Clinical Trial Registry (www.ANZCTR.org.au/ACTRN12616000492459p). The present study was conducted according to the ethical principles of the Declaration of Helsinki (1989), and all procedures involving patients were approved by the Human Ethics Committee of La Trobe University, Australia (HEC 16-035). All parents gave written informed consent prior to recruitment of children into the study.

The primary outcomes of this study are pulmonary function as assessed by spirometry (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$) and bronchial inflammation biomarker fractional exhaled nitric oxide (FeNO).

2.1.1. Subjects/intervention

Seventy-two children were recruited from a pediatric asthma clinic in the greater city of Athens, Greece. Children were 5 to 12 years of age with physician-diagnosed "mild" asthma as defined by GINA (Global Initiative for Asthma guidelines) [14] and willing to comply to the dietary intervention including "fatty fish" in the context of a Mediterranean diet for a period of six months. Following recruitment, children were randomized to the dietary intervention or control group with a 1:1 allocation ratio using an internet platform (http://www.randomization.com) (Fig. 1). The intervention group was instructed to consume two fatty fish meals per week (at least 150g filleted fish/meal), as part of the Greek Mediterranean diet for a period of six months, and the control group consumed their habitual diet. Fatty fish included salmon, sardines, trout, mackerel, and anchovies, fresh and frozen. The following assessments were made at baseline and at the completion of the 6-month trial: health and lifestyle questionnaire, physical activity, medical history, dietary intake using a validated food frequency questionnaire and KIDMED questionnaire, anthropometry, biochemical tests, and lung function using spirometry and FeNO.

2.2. Assessments

2.2.1. Anthropometry

We measured standing height to the nearest 0.1 cm using a SECA stadiometer after shoes and heavy clothing had been removed and children were positioned in the standard

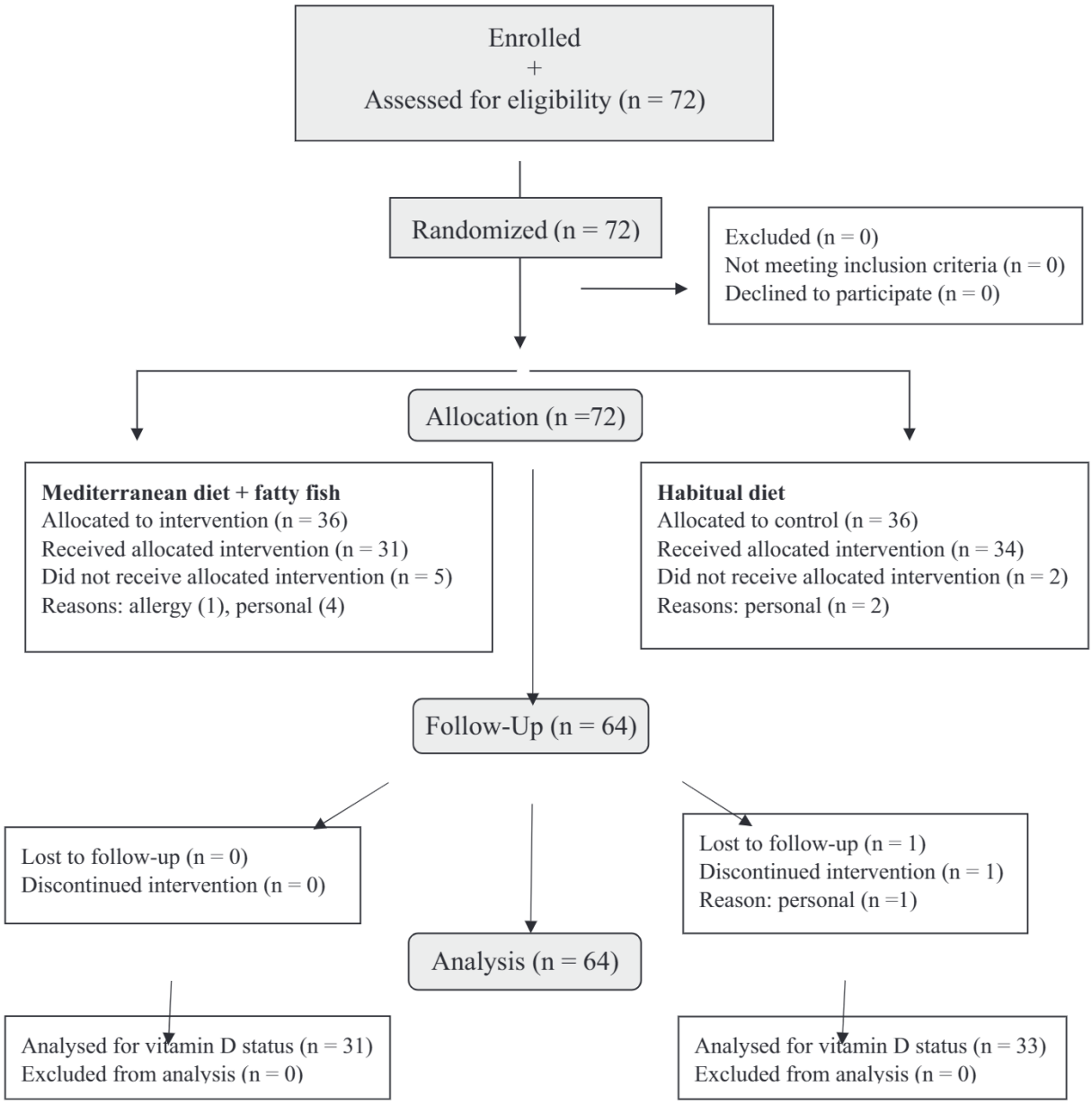


Fig 1 – Selection of participants in the study.

Frankfort horizontal plane [15]. Body weight was measured to the nearest 0.1 kg on calibrated electronic scales (SECA, Hanover, MD, USA). Children's body mass index (BMI) was calculated as weight divided by height squared (kg/m^2) and classified as normal weight, overweight, or obese based on the Hellenic pediatric growth charts [16].

2.2.2. *Spirometry*

Pulmonary function was measured in the standing position with a nose-clip using a portable spirometer (MIR Spirobank II, MIR Inc. USA) in accordance to European Respiratory Society protocol [17]. The highest spirometry value ('best test') of three efforts was used for the analyses. Normal pulmonary function in healthy children was considered spirometry parameter values (FEV_1, FVC, PEF) >80% predicted, $FEF_{25-75\%}$ >60% to 65% and FEV_1: FVC ratio above 80% [18,19]. A variation in FEV_1 of 10% to 12% is considered to be clinically significant in children [18,19].

2.2.3. *Fractional exhaled nitric oxide analysis*

Fractional exhaled nitric oxide was used as a marker for bronchial inflammation [20]. FeNO was measured using NO Breath (Benfont Inc, UK) by use of a single-breath method, according to European Respiratory Society guidelines [20]. No lung inflammation and good asthma control was represented by FeNO values less than 20 ppb [21] .

2.2.4. *Physical activity level*

Physical activity status was estimated using the International Study of Asthma and Allergies in Childhood (ISAAC) Phase 3

Environmental Questionnaire [22]. Regular physical activity was considered to be exercise more than or equal to three times per week (Supplemental S1).

2.2.5. *Dietary assessment*

A Food Frequency Questionnaire (FFQ) based on the validated semi-quantitative PANACEA-FFQ for Greek children aged 10-12 years [23] was administered to all children who participated in the study and was completed by their parents during medical consultations at baseline and six-months. Daily consumption of each food item (in grams per day) was assessed from FFQs.

2.2.6. *Adherence to the Mediterranean diet*

Adherence to the Mediterranean diet intervention was assessed utilizing the KIDMED questionnaire which was created to estimate adherence to the Mediterranean dietary pattern in children and young adults. The KIDMED index is based on the principles sustaining the Mediterranean dietary pattern and those that undermine it [24]. The KIDMED questionnaire comprises of 16 yes or no questions. Twelve questions denote a positive connotation with respect to the Mediterranean diet and are assigned a value of +1; 4 questions denote a negative connotation and are assigned a value of -1. If the sum of the values of the questionnaire range from 0-3, adherence to the Mediterranean diet is categorized as 'low', 4-7 'moderate', and 8-12 as ' high' [24] .

In order to maintain motivation and adherence to the dietary intervention, all participants were reviewed fortnightly by the dietician via face-to-face consultation, e-mail, text, and telephone. During baseline and six-month interviews, adherence to the Mediterranean dietary pattern and fish intake was assessed using the KIDMED questionnaire, which was also used to validate dietary details from FFQs. In order to optimize health benefits, the importance of complying with dietary instructions was emphasized regularly to parents.

2.2.7. *Serum 25-hydroxyvitamin D levels*

At baseline and 6 months, venous samples (4 mL) were collected from children according to WHO guidelines following a 2-hour fast [25]. The samples were centrifuged and the serum was decanted from the supernatant and stored at -20°C until analysis within 24 hours to avoid degradation. In case of hemolysis, blood collection was repeated. Vitamin D status was measured using serum 25(OH)D as it has a half-life of 2–3 weeks, and it is the best indicator of vitamin D exposure from diet and dermal synthesis [26]. Determination of 25(OH)D from blood serum was undertaken using enzyme-linked immuno assay (ELISA) (IDS, Tyne & Wear, UK) [27]. The intra- and inter-assay variability was less than 1.9% and 4.6%, respectively. The performance of the assay for the quantification of 25(OH)D was assessed in co-operation with Vitamin D External Quality Assessment Scheme Advisory (DEQAS), UK [28]. The blood level of 25(OH)D that is defined as vitamin D deficiency remains somewhat controversial. Vitamin D deficient has been variably defined as serum 25 (OH)D concentration <10 to <30 ng/mL (<25 to <75 nmol/L) [29], insufficient 21 to 29 ng/mL (52-72 nmol/L), and sufficient ≥30 ng/mL (≥75 nmol/L), which is based on bone health [30]. There is no general consensus on the threshold of vitamin D for respiratory health. For the purpose of this study, we used the 50th percentile cut-off as a surrogate for 'insufficient' (25(OH)D < 25 ng/mL) and 'sufficient' vitamin D levels (25 (OH)D ≥25 ng/mL) in the present population, which is in accordance with various studies that have defined vitamin D insufficiency values from 10 ng/mL to 35 ng/mL (25-80 nmol/L, respectively) [8,31,32].

2.3. *Statistical analyses*

Sample size was based on spirometry parameter FEV_1 and calculated applying G*Power analysis software [33]. A sample of at least 52 participants was sufficient to generate a power of 90% with a moderate effect size of 0.4 [34] and to evaluate 2-sided hypotheses regarding a difference in FEV_1 between the groups at an $\alpha < .05$. Then, allowing for an attrition rate of 20%, a final sample of at least 64 participants was produced.

Data were analyzed using the Statistical Package for the Social Sciences (IBM SPSS 20.0, IBM Corp, Armonk, NY, USA) and interactions tested for using STATA release 14.1 (College Station, TX, USA) software. Continuous variables were checked for normality applying the Shapiro-Wilks test and are presented as means and standard deviations. Differences between groups were compared using *t* tests and Mann-Whitney test when assumptions of normality were not satisfied. Categorical variables were analyzed using χ^2 test and are presented as frequencies. Vitamin D [25(OH)D] concentrations were used as the continuous variable and 'categorized as 'insufficient' or 'sufficient' (≥25 ng/mL). After stratification of data according to two 25(OH) D categories at baseline (insufficient [<25 ng/mL] and sufficient ≥25 ng/mL), multiple linear regression model was applied to compare mean change in pulmonary function between the groups from baseline to six months. Based on previous research, factors affecting spirometry, FeNO, and vitamin D status, namely age, sex, BMI, and regular physical activity [19,21,35], were used as adjustments and retained in the regression model if they changed the exposure effect size by 10% or were statistically significant. The results from the regression model are presented as unstandardized β coefficients. The level of statistical significance was defined at $P < .05$. Differences in the effect of the intervention on lung function in children stratified by baseline serum vitamin D status were investigated using an interaction analysis and $P < .1$ were considered significant in order to capture important interaction effects. To further explore the association of serum 25(OH)D with spirometry and FeNO, data was sub-divided to 4 strata: deficient ≤10 ng/mL (≤25 nmol/L), insufficient 10 to 20 ng/mL (25-50 nmol/L), inadequate 20 to 30 ng/mL (50-75 nmol/L), and desirable ≥30 ng/mL (>75 nmol/L) [36,37]. Multivariate regression as well as the interaction analysis was also performed, taking confounding factors into consideration.

3. Results

Out of the 72 children recruited, 64 (intervention n = 31, control n = 33) completed baseline and six-month assessments, of which 51.6% (33) were male and 48.4% (31) female with a mean age 8.00 ± 2.00 years. At recruitment, 40.6% (26) of children were overweight/obese according to the Hellenic pediatric growth charts. Only 36% (23) of children were

Table 1 – Socio-demographic and clinical characteristics for intervention groups according to insufficient/sufficient serum Vitamin D at baseline

	Vitamin D baseline						Vitamin D 6 months					
	Insufficient 25(OH)D <25 ng/mL			Sufficient 25(OH)D ≥25 ng/mL			Insufficient 25(OH)D <25 ng/mL			Sufficient 25(OH)D ≥25 ng/mL		
	Group			Group			Group			Group		
Variable	Intervention	Control	P[a]	Intervention	Control	P[a]	Intervention	Control	P[a]	Intervention	Control	P[a]
Age (years)	8.00 ± 2.00	8.00 ± 2.00	0.76	8.00 ± 2.00	9.00 ± 2.00	0.72	8.08 ± 2.31	8.33 ± 2.55	0.79	8.06 ± 2.01	9.06 ± 2.11	0.16
Male (%)	30.40%	50.0%	0.20[b]	62.5%	80%	0.36[b]	41.7%	66.7%	0.19[b]	33.3%	62.5%	0.09[b]
BMI (kg/m^2)	19.00 ± 3.94	19.37 ± 3.69	0.76	17.54 ± 4.04	16.96 ± 3.63	0.73	19.35 ± 3.55	18.07 ± 2.88	0.28[c]	17.98 ± 4.03	19.33 ± 4.70	0.41[c]
Overweight/ obese	56.5%	44.4%	0.34[b]	25%	20%	0.60[b]	50.00%	42.9%	0.72[b]	35.3%	46.7%	0.51[b]
Regular Physical Activity %	34.80%	50.00%	0.33[b]	62.5%	66.7%	0.84[b]	25.00%	46.7%	1.00[b]	55.6%	62.5%	0.07[b]
Medication Yes %	91.30%	77.80%	0.22[b]	62.5%	86.7%	0.18[b]	41.7%	73.3%	0.09[b]	50.0%	50.0%	1.00[b]
Pulmonary function (% pred)												
FVC	94.04 ± 9.27	97.28 ± 11.89	0.33	96.25 ± 6.98	95.13 ± 10.37	0.79	99.33 ± 10.14	95.67 ± 11.27	0.39	95.56 ± 8.87	97.13 ± 8.02	0.59
FEV_1	97.26 ± 9.74	99.00 ± 11.74	0.61	97.13 ± 5.84	99.20 ± 9.32	0.57	103.75 ± 10.66	100.87 ± 10.51	0.49	97.83 ± 8.10	99.44 ± 7.12	0.55
FEV_1/FVC	102.61 ± 4.46	101.17 ± 8.64	0.49	100.13 ± 3.91	103.87 ± 6.58	0.16	103.92 ± 2.23	104.93 ± 4.83	0.51	101.89 ± 4.13	101.88 ± 6.04	0.99
PEF	93.17 ± 20.81	93.11 ± 15.88	0.99	97.63 ± 14.65	93.93 ± 22.37	0.68	108.92 ± 28.62	106.60 ± 25.44	0.83	95.61 ± 12.51	95.50 ± 18.82	0.98
$FEF_{25-75\%}$	101.96 ± 19.47	101.22 ± 24.43	0.91	95.50 ± 11.48	107.20 ± 17.55	0.10	107.17 ± 8.59	106.93 ± 21.14	0.97	100.39 ± 15.72	100.25 ± 17.83	0.98
FeNO (ppb)	17.87 ± 19.17	7.06 ± 5.12	0.59[c]	18.13 ± 13.20	13.87 ± 7.64	0.88[c]	11.83 ± 12.17	21.27 ± 40.30	0.98[c]	16.06 ± 17.09	16.31 ± 17.42	0.56[c]
25(OH)D (ng/mL)	20.35 ± 3.48	18.62 ± 4.85	0.58[c]	31.64 ± 8.66	29.63 ± 5.00	0.97[c]	19.06 ± 2.89	19.99 ± 4.41	0.30[c]	34.01 ± 9.43	32.99 ± 6.55	0.98[c]
KIDMED score	5.78 ± 1.70	5.44 ± 2.06	0.53[c]	4.00 ± 2.33	5.00 ± 2.00	0.32[c]	6.58 ± 1.68	4.93 ± 2.25	**0.03**[c]	5.94 ± 1.16	5.31 ± 1.89	0.13[c]
KIDMED category												
Low	8.7%	22.2%	0.46[b]	37.5%	33.3%	0.85[b]	8.3 %	33.3%	0.11[b]	5.6%	18.8%	0.34[b]
Moderate	69.6%	55.6%		50.0%	60.0%		58.3%	60.00%		88.9%	68.8%	
High	21.7%	22.2%		12.5%	6.7%		33.3%	6.7%		5.6%	12.5%	

P values significant at 5% level in bold.
Values shown are means ± standard deviations.
Baseline: n(25(OH)D <25 ng/mL) = 41; n(Intervention group) = 23; n(Control group) = 18.
Baseline: n(25(OH)D ≥25 ng/L) = 23; n(Intervention group) = 8; n(Control group) = 15.
Six-months: n(25(OH)D <25 ng/mL) = 27; n(Intervention group) = 12 ; n(Control group) = 15.
Six-months: n(25 (OH)D ≥25 ng/L) = 34; n(Intervention group) = 18 ; n(Control group) = 16.
Abbreviations: %pred, % predicted; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEV_1/FVC, ratio of forced expiratory volume and forced vital capacity, PEF, peak expiratory flow, $FEF_{25-75\%}$, mid expiratory flow between 25% and 75% vital capacity; FeNO, fractional exhaled nitric oxide.
[a]P values shown were calculated using *t* test; [b]χ^2 test; [c]Mann-Whitney test.

classified as 'sufficient' [25(OH)D ≥25 ng/mL] with more girls 'insufficient' in 25(OH)D than boys (61% [25] vs 39% [16], respectively; *P* = .007). Approximately half of the children exercised regularly (≥3 times/week), and 83% were taking daily asthma therapy. In addition, children with 25(OH)D <25 ng/mL were more likely to be overweight/obese (*P* = .02), physically inactive (*P* = .07), and taking more asthma medication (*P* = .47) than those sufficient.

Most children (94%) were not taking dietary supplements. One child was taking folic acid, and three were taking a multivitamin containing 5 μg of vitamin D. Adherence to the Mediterranean diet at baseline was 'moderate' in both groups (KIDMED score: intervention 5.32 vs control 5.24, *P* = .72) with 19.4% of children in the intervention group having 'high' adherence versus 15.2% in the control (*P* = .55).

Examination of vitamin D status revealed that serum 25 (OH)D concentrations increased by 20.87% in the intervention group at 6 months and marginally in the control group by 11.53% [(Mean 25(OH)D baseline vs 6 months): Intervention group 23.19 vs 28.03 ng/mL (*P* = .002); control group: 23.95 vs 26.70 ng/mL (*P* = .07)] (crude analysis). More specifically, at baseline 56.1% (23) of children in the intervention group were 'insufficient' in 25(OH) D, which decreased to 44.4% (12) by 6 months (*P* = .007). In comparison, 43.9% (18) in the control group were insufficient in 25(OH)D at baseline and 55.6% (15) at 6 months (*P* > .05). No differences were observed in KIDMED scores, spirometry, or FeNO between intervention or control groups at baseline nor at the end of the trial (*P* > .05).

As the focus of this study was to evaluate changes in pulmonary function and relevant biomarkers by vitamin D status, the baseline and 6 months socio-demographic, KIDMED categories, and bioclinical characteristics are presented in Table 1.

Data on food group intake stratified by vitamin D status (insufficient < 25 ng/mL and sufficient ≥ 25 ng/mL) are shown in Table 2. Mediterranean diet adherence based on vitamin D status is shown in Supplemental Table S1. From Table 2, it is apparent that during the six-month period fatty fish intake increased markedly in the intervention group for all vitamin D levels as compared to the control (p < 0.001).

Stratification by vitamin D level revealed that in children insufficient in 25(OH)D (< 25 ng/mL) at baseline, there was no effect of the dietary intervention for spirometry parameters and bronchial inflammation indicator (FeNO) in the univariate and multivariate analyses (*p* > 0.05). There was a trend towards significance in the ratio of FEV_1/FVC in the univariate model (*p* = 0.044), which became borderline in the multivariate model (*p* = 0.06). However, among children with sufficient

Table 2 – Intake of food groups (g/d) at baseline and 6 months according to vitamin D status per intervention group.

	Vitamin D baseline						Vitamin D 6-months					
	25 (OH)D <25 ng/mL			25(OH)D ≥25 ng/mL			25(OH)D <25 ng/mL			25(OH)D ≥25 ng/mL		
	Group			Group			Group			Group		
Food Group/ Item (g/d)	Intervention	Control	P[c]	Intervention	Control	P[c]	Intervention	Control	P[c]	Intervention	Control	P[c]
Dairy	530.25 ± 290.04	500.89 ± 283.83	0.75	362.32 ± 320.10	527.99 ± 339.46	0.09	362.75 ± 229.97	507.53 ± 302.12	0.19	524.56 ± 307.30	471.47 ± 271.23	0.52
Fruit	313.90 ± 135.45	224.25 ± 113.11	**0.03**	191.59 ± 112.53	285.17 ± 224.05	0.46	328.63 ± 252.95	241.07 ± 137.11	0.48	224.69 ± 123.34	251.54 ± 220.69	0.65
Vegetables	182.87 ± 141.65	170.15 ± 119.34	0.86	124.23 ± 74.91	180.13 ± 152.85	0.44	179.96 ± 95.34	254.53 ± 187.31	0.39	169.68 ± 114.32	162.12 ± 116.69	0.86
Legumes	65.05 ± 36.45	65.23 ± 35.76	0.94	66.75 ± 34.16	45.08 ± 28.40	0.09	71.50 ± 39.84	55.34 ± 34.84	0.25	53.00 ± 25.31	40.78 ± 29.27	**0.03**
Starch	120.96 ± 150.77	67.34 ± 25.31	0.12	91.94 ± 42.71	66.63 ± 26.74	0.12	95.99 ± 70.18	73.74 ± 29.07	0.49	95.28 ± 54.14	76.25 ± 21.24	0.51
Meat	85.74 ± 53.50	80.09 ± 45.45	0.68	79.13 ± 21.15	82.17 ± 29.40	0.94	75.00 ± 28.14	91.02 ± 59.90	0.55	63.87 ± 31.93	78.29 ± 30.76	0.19
Seafood	6.80 ± 12.32	3.96 ± 6.25	0.60	7.65 ± 7.34	5.48 ± 7.73	0.41	6.85 ± 8.11	7.43 ± 6.11	0.67	4.52 ± 6.32	7.65 ± 7.09	0.17
Lean fish	18.20 ± 12.86	11.02 ± 13.51	**0.02**	15.64 ± 8.02	12.55 ± 14.20	0.23	8.76 ± 15.78	13.83 ± 12.76	0.06	18.32 ± 29.60	12.96 ± 8.19	0.28
Fatty fish	16.76 ± 10.26	12.18 ± 13.41	0.08	18.39 ± 16.19	8.28 ± 9.02	0.09	45.75 ± 14.92	11.82 ± 14.01	**0.00**	45.31 ± 22.72	8.96 ± 7.51	**0.00**
Fish	34.96 ± 19.95	23.20 ± 24.94	**0.02**	34.03 ± 18.75	20.83 ± 19.79	0.08	54.51 ± 22.44	25.65 ± 25.18	**0.00**	63.62 ± 44.34	21.92 ± 12.74	**0.00**
Nuts	1.93 ± 2.52	3.71 ± 5.71	0.50	6.11 ± 11.99	6.81 ± 7.08	0.25	5.22 ± 9.77	5.16 ± 6.82	0.92	3.08 ± 4.40	3.92 ± 4.69	0.48
Olive oil	18.26 ± 14.07	17.54 ± 13.11	0.98	11.18 ± 2.05	21.50 ± 16.73	0.07	18.03 ± 15.45	21.50±14.84	0.63	18.58 ± 15.37	17.27 ± 14.17	0.44
Fats	20.73 ± 14.52	22.27 ± 16.29	0.97	18.98 ± 14.88	30.19 ± 22.55	0.16	27.27 ± 24.05	28.30 ± 16.49	0.43	22.64 ± 16.45	22.60 ± 16.19	0.94
Fast food	16.84 ± 15.58	34.61 ± 47.78	0.20	28.03 ± 19.18	17.45 ± 9.97	0.17	19.48 ± 7.52	20.47 ± 17.93	0.85	19.22 ± 15.81	24.45 ± 20.26	0.43
Sweets	24.39 ± 18.44	26.19 ± 16.67	0.61	29.90 ± 14.74	29.94 ± 19.27	0.97	17.83 ± 15.03	27.44 ± 25.05	0.54	23.32 ± 14.85	34.04 ± 33.95	0.31
Savory snacks	24.68 ± 34.66	30.75 ± 22.39	0.10	34.53 ± 21.72	59.84 ± 53.93	0.39	31.53 ± 23.94	37.88 ± 41.35	0.88	27.99 ± 25.98	42.62 ± 32.48	0.07
Soft drinks	5.19 ± 12.49	27.51 ± 46.50	0.05	18.11 ± 21.64	33.16 ± 50.13	0.89	56.20 ± 104.14	26.32 ± 43.06	0.85	22.05 ± 41.23	46.55 ± 50.49	0.06

In bold are shown statistically significant *P* values at 5% level.
Values shown are means ± standard deviations.
Baseline: n(25(OH)D <25 ng/mL) = 41; n(Intervention group) = 23; n(Control group) = 18
Baseline: n(25(OH)D ≥25 ng/mL) = 23; n(Intervention group) = 8; n(Control group) = 15
Six-months: n(25(OH)D <25 ng/mL) = 27; n (Intervention group) = 12 ; n(Control group) = 15
Six-months: n (25 (OH)D ≥25 ng/mL) = 34; n (Intervention group) = 18 ; n (Control group) = 16
[c]P value computed using Mann–Whitney test.

25(OH)D levels (≥ 25 ng/mL), a significant change in spirometry parameters FEV_1/FVC ($p = 0.01$) and $FEF_{25-75\%}$ ($p < 0.001$) was observed in the univariate analysis (Table 3).

When applying multiple linear regression model adjusted for age, sex, regular physical activity, and BMI, significant mean changes in FEV_1/FVC ($\beta = 4.89$, 95% CI: 1.19-8.61; $p = 0.013$) and $FEF_{25-75\%}$ ($\beta = 12.83$, 95% CI: 4.27- 21.40; $p = 0.006$) were observed in the intervention group with sufficient 25(OH) D levels (Table 4). Comparable results were verified by the interaction analysis, FEV_1/FVC ($p_{interaction} = 0.002$) and $FEF_{25-75\%}$ ($p_{interaction} = 0.033$). No significant change was evident for FeNO or other spirometry variables.

Table 3 – Mean change in pulmonary function parameter estimates from baseline in the intervention and control groups stratified by 25(OH) D levels at baseline

Vitamin D		Group		Difference		
	Mean score change	Intervention	Control	Mean	95% CI	P[a]
Sufficient (25(OH)D ≥25 ng/mL)	FVC*	-3.62	0.27	-3.89	-9.79, 2.00	0.18
	FEV_1	-0.75	-1.00	0.25	-4.43, 4.93	0.91
	FEV_1/FVC	3.62	-1.33	4.96	1.60, 8.31	**0.01**
	PEF	-1.37	5.20	-6.57	-25.04, 11.89	0.47
	$FEF_{25-75\%}$	5.62	-6.87	12.49	4.99, 19.99	**0.00**
	FeNO (ppb)	-2.37	11.40	-13.77	-45.44, 17.88	0.38
Insufficient (25(OH)D <25 ng/mL)	FVC	4.56	-0.44	5.01	-1.00, 11.02	0.10
	FEV_1	4.09	1.94	2.14	-3.82, 8.10	0.47
	FEV_1/FVC	-0.48	2.56	-3.03	-5.98, 0.08	**0.04**
	PEF	8.65	8.61	0.04	-13.00, 13.08	0.99
	FEF_{25-75}	1.22	3.44	-2.23	-11.71, 7.26	0.64
	FeNO (ppb)	-4.35	5.33	-9.68	-21.01, 1.65	0.09

In bold are shown statistically significant *P* values at 5% level.
Abbreviations: FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEV_1/FVC, ratio of forced expiratory volume and forced vital capacity; PEF, Peak Expiratory Flow; $FEF_{25-75\%}$, mid expiratory flow between 25% and 75% vital capacity; FeNO, fractional exhaled nitric oxide.
* Spirometry parameters as percent predicted.
[a] Univariate analysis; *t* test.

Table 4 – Multiple linear regression model indicating mean change in FEV1/FVC and $FEF_{25-75\%}$ for children with sufficient 25 (OH) D (≥25 ng/mL) at baseline

Baseline 25(OH)D ≥25 ng/mL		Difference		
Mean score change	Variable	β	95% CI	P [#]
FEV_1/FVC [*]	Group	4.89	1.19, 8.61	**0.013**
	Sex	-1.39	-5.53, 2.73	0.48
	Age	-0.46	-1.46, 0.53	0.34
	BMI	0.22	-0.37, 0.81	0.44
	Regular physical activity	-0.35	-4.25, 3.56	0.85
$FEF_{25-75\%}$	Group	12.83	4.27, 21.40	**0.006**
	Sex	-1.26	-10.79, 8.28	0.78
	Age	0.31	-1.98, 2.60	0.77
	BMI	-0.28	-1.64, 1.08	0.67
	Regular physical activity	-3.57	-12.59, 5.45	0.41

In bold are shown statistically significant *P* values at 5% level.
Abbreviations: FEV_1/FVC, ratio of forced expiratory flow in 1 second on forced vital capacity; $FEF_{25-75\%}$, mid expiratory flow between 25% and 75% vital capacity.
[*] Spirometry parameters as percent predicted.
[#] *P* value for group analysis using multiple linear regression model adjusted for (baseline) age, sex, regular physical activity and BMI; β, unstandardized beta.

Subdivision of 25(OH)D to four strata [≤ 10 ng/mL (≤ 25 nmol/L), 10-20 ng/mL(25-50 nmol/L), 20-30 ng/mL (50-75 nmol/L), and ≥ 30 ng/mL (> 75 nmol/L)] did not alter the results. The adjusted regression analysis showed a positive effect of the intervention on FEV_1/FVC (p = 0.023; β = 23.3; 95%CI: 12.5-34.0) for children with sufficient serum 25(OH) D ≥ 30 ng/mL (≥ 75 nmol/l), although not in the interaction analysis (p = 0.26) (Supplemental Table S2).

Examination of serum 25(OH)D as a continuous variable yielded a significant result for $FEF_{25-75\%}$ (p =0.04) but not for FEV_1, FVC, FEV_1/FVC, PEF, or FeNO (p > 0.05) (Supplemental Table S3). Results are presented pictorially in Supplemental Figure S1 and Figure S2.

4. Discussion

We sought to understand the role of vitamin D status on pulmonary function in children with 'mild asthma' following a Mediterranean diet enriched with fatty fish. The findings of this study support our hypothesis that baseline serum vitamin D levels modify the beneficial response of fatty fish intake on pulmonary function in asthmatic children following a Mediterranean diet.'

Two important findings were highlighted in this study. Firstly, the majority of children (64%) were insufficient in vitamin D predominating in girls [38]. This is consistent with European data documenting the pandemic problem of hypovitaminosis D in children, particularly within the age groups of 1-6 and 7-14 years [29]. This also further supports the findings of recent studies on the relationship between low vitamin D levels and childhood asthma [8,39]. The fact that the majority of our patients were insufficient in vitamin D is alarming, although the thresholds used for vitamin D sufficiency was based on bone metabolism, thus proposing that concentrations > 30 ng/mL may be essential for optimal immune functioning and respiratory health.

Secondly, in children with sufficient serum 25(OH)D at baseline, following a Mediterranean diet enriched with fatty fish was associated with enhanced pulmonary function and airway flow in small airways as indicated by an increase in FEV_1/FVC as well as $FEF_{25-75\%}$, which is an indicator of small airways caliber. No significant effect was observed for bronchial inflammation biomarker, FeNO. This may have clinical significance in managing childhood asthma because FEV_1/FVC has been proposed as a sensitive measure of airway obstruction and $FEF_{25-75\%}$ as a more sensitive marker of obstruction in small airways than FEV_1 or FVC [18,19]. Also, $FEF_{25-75\%}$ reflects early disease [18,19] and has been found to be reduced in children with uncontrolled asthma even when FEV_1 is normal [40]. However, there is a debate about its usefulness because the above parameter is a highly variable measure that is influenced by expiratory time and change in FVC [41]. Low levels of $FEF_{25-75\%}$ and FEV_1/FVC have been associated with steroid use, hospitalization, asthma exacerbations, and asthma severity as compared to children with normal spirometry [40]. Given that the majority of children have normal FEV_1, finding that another spirometry parameter ($FEF_{25-75\%}$) is associated with the presence of airway obstruction, poor asthma outcome, and increased risk of future asthma exacerbation has important implications for health professionals performing spirometry in asthmatic children. It is interesting to note that we did not find an association between vitamin D and FEV_1 and FVC, both of which are measures of total lung capacity. Although the reason for this discrepancy is yet to be elucidated, one could speculate that the impact of vitamin D on lung function may be mediated through changes in airway obstruction which affects FEV_1/FVC and $FEF_{25-75\%}$ rather than via total lung capacity.

The outcome of this study is comparable to other studies in asthmatic children reporting direct correlations between vitamin D status and lung function test measurements including FEV_1/FVC [8,31,39,42] but not for FeNO [8,39]. However, there was no correlation between FVC, FEV_1, FEV_1/FVC, PEF, and

$FEF_{25-75\%}$ in the studies by Brehm (2012) [43], Chinellato (2011) [8], Krobtrakulchai (2012) [44], and Yao (2014) [39].Two studies of vitamin D status in asthma were undertaken in the Mediterranean region [8,31], one in Iran [42], and one in Taiwan [39]. Children's age ranged from 4 to 18 years old. Overall, studies documented a high percentage of vitamin D deficiency in asthmatic children (≥40%) and a dose-response relationship between serum vitamin D and spirometry parameters (FEV_1, FVC, FEV_1/FVC) but not for FeNO [8,39], which is consistent with our findings and suggests a potentially important role for vitamin D in respiratory health. Regarding $FEF_{25-75\%}$, only one out of the four studies examined the effect of vitamin D level on $FEF_{25-75\%}$ in asthmatic children, reporting no effect [39]. A major limitation of these studies is the cross-sectional design which precludes the ability for causal inferences. A feasible explanation for the inconsistency among studies regarding vitamin D and lung function tests is that vitamin deficiency might represent a marker of environmental and lifestyle factors such as dietary, dress habits, time spent indoors and less sun exposure, and availability as well as fortification policies. Additionally, heterogeneity among study methodologies, such as variability in sample size, methods used for vitamin determination, threshold for vitamin D deficiency/sufficiency, lack of data on dietary and vitamin D supplementation, geographic location (latitude), ethnicity, skin melanin content, population variability in vitamin D metabolism, absorption and cutaneous synthesis of vitamin D, genetic variants in vitamin D receptors [6], and season that blood tests were undertaken, are plausible factors that might have led to spurious results. It has been suggested that levels higher than 40 ng/mL may be necessary for optimal immune functioning and overall health [45].

Apart from inflammation, asthma is associated with structural changes in the lungs referred to as airway remodeling [46]. The results of this study suggest that vitamin D could affect tissue remodeling and might be interrelated with the development of airway limitation. One of the pleiotropic effects of vitamin D is its action on the development and mechanics of lung function. Gupta demonstrated that vitamin D levels were inversely correlated to airway smooth muscle mass and lung function in children with severe asthma [46]. However, our study showed the benefits of sufficient serum vitamin D status on lung function even in 'mild' asthmatic children. According to a study in mice undertaken by Zosky et al, lung size and volume were significantly smaller in vitamin D deficient mice compared with replete mice, which influenced lung mechanics [47]. So, sufficient vitamin D levels in children could have resulted in inhibition of smooth muscle proliferation, improved cell growth, and lung structure resulting in less resistance in small airways and airway obstruction, and thus improving airflow as evident by the improvement in the ratio of FEV_1/FVC and $FEF_{25-75\%}$. This is significant because long-term treatment of asthma emphasizes the control of airway inflammation and not the prevention of airway remodeling.

Nonetheless, in line with these findings, no effect of vitamin D was observed on FeNO values in our patients. This is not surprising because FeNO detects eosinophilic airway inflammation in asthma [21], and vitamin D may not be a determinant of eosinophilic inflammation [39]. Although the exact mechanisms underlying the link between vitamin D status and lung function remain unclear, one possibility is that vitamin D could improve lung function by influencing airway remodeling and affecting smooth muscle cell movement, growth and contractility, collagen synthesis, and by inhibiting matrix metalloproteinase and fibroblast proliferation, as well as protection from respiratory infections and enhancing asthma therapy [48]. Other plausible mechanisms include the action of vitamin D on regulating airway inflammation such as control of inflammatory innate and adaptive immune response, induction of antimicrobial mechanisms to efficiently resolve infection, and promotion of regulatory responses, both indirectly by impairment of antigen presenting cell function and directly via effects on T lymphocytes, namely Th1 to Th2, switch and production of anti-inflammatory cytokines (IL10, TGFβ) from Treg cells [1,49].

Another property of vitamin D worth noting, is its ability to enhance the response to asthma therapy by inducing the production of IL-10, a potent anti-inflammatory cytokine from regulatory T-cells [50-52]. We observed an additive effect of vitamin D with regards to the intervention on medication therapy, thus suggesting a dual effect. At baseline, 62.5% of children in the intervention group with sufficient vitamin D level were taking asthma medication compared to 91.3% insufficient in vitamin D. By the end of the study period, there were fewer children in the intervention group with insufficient vitamin D levels and taking medication (41.7%) as compared to baseline. As for the intervention group with sufficient vitamin D levels, only 50% of children were on asthma therapy. By the end of the study trial, serum vitamin D levels increased in the intervention group by approximately 21% as compared to 11% in the control. Similarly, Searing and Brehm [51,53] documented an inverse relationship between vitamin D levels in asthmatic children and use of steroids and anti-leukotriene therapy. A likely explanation could be that vitamin D interacts synergistically and ameliorates the action of medication as was manifested by the improvement in FEV_1/FVC in the intervention group. In addition, having adequate serum vitamin D levels enhanced the effect of the dietary intervention in asthmatic children. Fatty fish is a rich source of vitamin D as well as a variety of other nutrients, antioxidants, and bioactive molecules, including omega 3 fatty acids, selenium, iodine, potassium, and B-vitamins, that when combined these components interact synergistically, having positive effects on pulmonary function [54]. Small dietary modifications such as increased fatty fish intake and sun exposure could improve airway conduction and thereby reduce asthma burden in children.

With respect to insufficient vitamin D levels in children, especially in those who are overweight/ obese, this may be the result of lifestyle factors. Extra-curriculum activities, more time spent indoors watching TV, playing video games and computer-time, and including both parents in the work-force are some of the barriers to daily physical activity in children [55]. We found that, in general, only 50% of children engaged in regular physical activity. Furthermore, children who are overweight sustain less physical exertion than their lean counterparts and therefore are less likely to exercise or play outdoors, which is consistent with our study [56]. From another perspective, obesity-related vitamin D insufficiency

is probably caused by decreased bioavailability from cutaneous and dietary sources due to deposition of vitamin D in adipose tissue [57]. In addition, poor dietary habits and abandonment of the Traditional Mediterranean diet by children [58] as was evident by the low percentage of children with high KIDMED scores, collectively might have contributed to hypovitaminosis D. Another potential limiting factor is reverse causation where children with poor asthma control are less likely to engage in outdoor activities due to difficulty in breathing [59]. Also, since respiratory infections trigger asthma exacerbations in children [60], parents may confine children indoors especially during winter months and as a result, children are exposed to less sunlight. Nonetheless, some studies have reported significant positive associations between vitamin D status and physical activity, independent of sun-exposure [61]. Then again, hypovitaminosis in asthmatic children could be a consequence of inflammatory disease and not the cause [62].

Several factors need to be considered in interpretation of our results. There is a lack of published data on the relationship between vitamin D status and lung function tests in asthmatic children. To the authors' knowledge, this study is the first clinical intervention trial to address the impact of vitamin D status in asthmatic children consuming a Mediterranean diet enriched with fatty fish. Previous publications evaluating vitamin D status and lung function in children have examined the association of serum 25(OH)D or vitamin D supplementation with FEV_1 and FVC [8,11]. A novel element of this study was the investigation of 25(OH)D in relation to FEV_1/FVC and $FEF_{25-75\%}$. Our findings present new evidence suggesting that in children following a Mediterranean diet enriched with fatty fish intervention, those with sufficient serum vitamin D concentrations had better lung function even in small airways and reduced airway obstruction represented by $FEF_{25-75\%}$ and FEV_1/FVC. Nevertheless, future clinical trials are recommended to corroborate these findings. A potential strength is that pulmonary function and bronchial inflammation were assessed quantitatively using spirometry and exhaled nitric oxide analysis, which enforces the accuracy, validity, and reliability of our findings [14]. Innovative to asthma research is the use of metabolomics to assess biochemical markers in children. We used ELISA to quantify serum 25(OH)D as an independent biomarker of vitamin D status compared to studies which rely on dietary intake assessment of vitamin D using self-reported diet techniques that are prone to recall bias [63,64]. In addition, serum concentration of 25 (OH)D is considered to be the most reliable measure of overall vitamin D status and thus can be used to determine whether a patient is vitamin D sufficient [62]. In terms of vitamin D supplements, only three children were taking multivitamins containing 5μg of vitamin D, thus increasing the validity and credibility of our findings.

A possible limitation in our study is the cut-off used for vitamin D status, although we have applied the threshold most commonly used in European pediatric populations [29]. Currently, there is no universal consensus on the concentration of 25(OH)D that defines vitamin D deficiency in children [29] and optimal levels for respiratory conditions such as asthma remain unknown. Another drawback is the small sample size and short-study duration employed for practical reasons such as minimizing study costs and patient burden. The promising findings could be due to chance (small sample size) and this warrants further investigation. A larger sample size would have increased our statistical power to detect a greater effect size. Nonetheless, the statistical power for all analyses was satisfactory based on the power and sample size calculations of the RCT. Seasonal differences could also explain variance in serum 25 (OH)D between intervention groups and time-points. At baseline, blood tests were undertaken in November–January, encompassing typical winter months, and in May-August at follow-up, typical summer months [35,65]. Although at follow-up, 25(OH)D levels increased substantially in the intervention group as compared to the control, most likely due to increased fatty fish intake. Other possible determinants could be daily sun exposure, diet, and social economic status that were not included as potential confounders in the regression model. Nevertheless, the present research focused solely on the effect of serum vitamin D on lung function, and the regression model allowed for the controlling of known confounders such as BMI, age, and physical activity [35].

In conclusion, vitamin D insufficiency in sun-replete countries, including Greece, is common in asthmatic children, especially in girls. Sufficient serum vitamin D levels (≥ 25 ng/mL) enhanced ventilatory function in response to a Mediterranean diet enriched with fatty fish intervention in asthmatic children. Health professionals should emphasize the importance of a healthy lifestyle for children that includes a diet rich in dietary sources of vitamin D such as fatty fish and fortified foods in combination with outdoor play-time for ample sun exposure. Normalization of vitamin D status could reduce cost and asthma burden in children.

Acknowledgment

The authors are grateful to Basilopoulos and Sklavenitis supermarkets for their support to economically-disadvantaged families. The authors declare no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The first author is supported by an Australian Post Graduate Award PhD Scholarship administered by La Trobe University. All co-authors declare that we have seen and approved the final version of the manuscript being submitted. The authors confirm the article is the authors' original work, has not received prior publication, and is not under consideration for publication elsewhere. CK, DT, and MP were responsible for data collection. MP is the principal author of the paper. CI, BE, CK contributed in revising the first and final draft of the manuscript. BE and KL guided the statistical analysis.

Supplemental materials

Supplementary material for this article was provided and include: Assessments (physical activity level, dietary asssessment, adherence to the Mediterranean diet, and serum 25-Hydroxyvitamin D levels); Table S1, KIDMED questionnaire according to vitamin D status per intervention group at baseline and six-months follow-up; Table S2, Adjusted interaction analysis indicating the effect of the

intervention on pulmonary function and FeNO per 25(OH)D strata; Table S3, Interaction analysis for serum 25(OH)D and pulmonary function tests; Figure S1, The effect of the dietary intervention on change in FEV1/FVC by baseline serum 25(OH)D level; and Figure S2, The effect of the dietary intervention on change in FEF 25-75% by baseline serum 25(OH)D level. These supplemental materials can be found online at https://doi.org/10.1016/j.nutres.2020.08.004

REFERENCES

[1] Hall SC, Agrawal DK. Vitamin D and bronchial asthma: an overview of data from the past 5 years. Clin Ther. 2017;39:917–29. https://doi.org/10.1016/j.clinthera.2017.04.002.

[2] Asher I, Pearce N. Global burden of asthma among children. Int J Tuberc Lung Dis. 2014;18:1269–78. https://doi.org/10.5588/ijtld.14.0170.

[3] Thomas M. Why aren't we doing better in asthma: time for personalised medicine? NPJ Prim Care Respir Med. 2015;25:15004. https://doi.org/10.1038/npjpcrm.2015.4.

[4] Papamichael MM, Itsiopoulos C, Susanto NH, Erbas B. Does adherence to the Mediterranean dietary pattern reduce asthma symptoms in children? A systematic review of observational studies. Public Health Nutr. 2017;20:2722–34. https://doi.org/10.1017/s1368980017001823.

[5] Papamichael MM, Shrestha SK, Itsiopoulos C, Erbas B. The role of fish intake on asthma in children: a meta-analysis of observational studies. Pediatr Allergy Immunol. 2018;29:350–60. https://doi.org/10.1111/pai.12889.

[6] Huang H, Porpodis K, Zarogoulidis P, Domvri K, Giouleka P, Papaiwannou A, et al. Vitamin D in asthma and future perspectives. Drug Des Devel Ther. 2013;7:1003–13. https://doi.org/10.2147/DDDT.S50599.

[7] Ehlayel MS, Bener A, Sabbah A. Is high prevalence of vitamin D deficiency evidence for asthma and allergy risks? Eur Ann Allergy Clin Immunol. 2011;43:81–8.

[8] Chinellato I, Piazza M, Sandri M, Peroni D, Piacentini G, Boner AL. Vitamin D serum levels and markers of asthma control in Italian children. J Pediatr. 2011;158:437–41. https://doi.org/10.1016/j.jpeds.2010.08.043.

[9] Jat KR, Khairwa A. Vitamin D and asthma in children: a systematic review and meta-analysis of observational studies. Lung India. 2017;34:355–63. https://doi.org/10.4103/0970-2113.209227.

[10] Cassim R, Russell MA, Lodge CJ, Lowe AJ, Koplin JJ, Dharmage SC. The role of circulating 25 hydroxyvitamin D in asthma: a systematic review. Allergy. 2015;70:339–54. https://doi.org/10.1111/all.12583.

[11] Pojsupap S, Iliriani K, Sampaio TZ, O'Hearn K, Kovesi T, Menon K, et al. Efficacy of high-dose vitamin D in pediatric asthma: a systematic review and meta-analysis. J Asthma. 2015;52:382–90. https://doi.org/10.3109/02770903.2014.980509.

[12] Papamichael MM, Katsardis Ch, Lambert K, Tsoukalas D, Koutsilieris M, Erbas B, et al. Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomised controlled trial. J Hum Nutr Diet. 2019;32:185–97. https://doi.org/10.1111/jhn.12609.

[13] Papamichael MM, Katsardis C, Tsoukalas D, Erbas B, Itsiopoulos C. A clinical trial of Mediterranean diet enriched with fatty fish in pediatric asthma: study protocol. J Pharmacy Pharmacol. 2018;6:225–39. https://doi.org/10.17265/2328-2150/2018.03.004.

[14] GINA. Global Strategy for Asthma Management and Prevention. 2016.: Global Initiative for Asthma (GINA); 2016www.ginasthma.org. [Accessed 15 February 2019].

[15] WHO. Training Course on Child Growth Assessment. . Geneva: World Health Organization; 2008. http://www.who.int/childgrowth/training/en/. [Accessed 14 Mar 2019]

[16] Hellenic Institute of Child Health (ICH). Hellenic pediatric growth charts in Child health book Athens. Greece. 2017:86–8.

[17] Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005;26:319–38. https://doi.org/10.1183/09031936.05.00034805.

[18] Czovek D. Pulmonary function tests in infants and children. In: Kendig's. Disorders of the respiratory tract in children. 9th ed: Elsevier; 2019. p. 174-211.

[19] Katsardis C, Alexandraki S, Paraskakis E. Chapter 2: Spirometry in children 6-16 years old *In:* Katsardis Ch, Koumbourlis A, Anthracopoulos M, Paraskakis E editors. Paediatric Pulmonary Function Testing Indications and Interpretation. New York, USA: NOVA Biomedical; 2015. p. 15-42.

[20] ATS/ERS. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med. 2005;171:912–30. https://doi.org/10.1164/rccm.200406-710ST.

[21] Paraskakis E, Katsardis C, Chatzimichail A. Chapter 17: Exhaled NO. In: Katsardis Ch, Koumbourlis A, Anthracopoulos M, Paraskakis E, editors. Paediatric Pulmonary Function Testing Indications and Interpretation. New York, USA: NOVA Biomedical; 2015. p. 235–48.

[22] International Study of Asthma and Allergies in Childhood (ISAAC). ISAAC Phase 3 Environmental Questionnaire. http://isaac.auckland.ac.nz/phases/phasethree/environmentalquestionnaire/environmentalquestionnaire.html; 2012. [Accessed February 2019].

[23] Antonogeorgos G, Grigoropoulou D, Papadimitriou A, Priftis KN, Anthracopoulos M, Nikolaidou P, et al. Validation of a food frequency questionnaire designed for children 10-12 years: the PANACEA-FFQ. Perioperative Nursing. 2013;2:40–54.

[24] Serra-Majem L, Ribas L, Garcia A, Perez-Rodrigo C, Aranceta J. Nutrient adequacy and Mediterranean Diet in Spanish school children and adolescents. Eur J Clin Nutr. 2003;57:S35–9. https://doi.org/10.1038/sj.ejcn.1601812.

[25] WHO. Chapter 6: Paediatric and neonatal blood sampling. WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy. Geneva: World Health Organization; 2010https://www.ncbi.nlm.nih.gov/books/NBK138647/. [Accessed February 2019].

[26] Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011;96:1911–30. https://doi.org/10.1210/jc.2011-0385.

[27] IDS. OCTEIA 25-Hydroxy-vitamin D. London, UK: Immunodiagnostic Systems (IDS) Inc; 2018www.idsltd.com. [Accessed February 2019].

[28] DEQUAS. Vitamin D External Quality Assessment Scheme. London, UK: Vitamin D External Quality Assessment Scheme; 2019. http://www.deqas.org/. [Accessed Feb 2019]

[29] Cashman KD, Dowling KG, Skrabakova Z, Gonzalez-Gross M, Valtuena J, De Henauw S, et al. Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr. 2016;103:1033–44. https://doi.org/10.3945/ajcn.115.120873.

[30] Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357:266–81. https://doi.org/10.1056/NEJMra070553.

[31] Elnady HG, Hameed EA. Fouda E SA, Refaat E, E B. Serum Vitamin D levels and markers of childhood asthma severity in Greater Cairo INTENSIVE CARE MEDICINE. 2011;37:S355 S6.

[32] Rovner AJ, O'Brien KO. Hypovitaminosis D among healthy children in the United States: a review of the current evidence. Arch Pediatr Adolesc Med. 2008;162:513–9. https://doi.org/10.1001/archpedi.162.6.513.

[33] Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39:175–91. https://doi.org/10.3758/bf03193146.
[34] Lee SC, Yang YH, Chuang SY, Huang SY, Pan WH. Reduced medication use and improved pulmonary function with supplements containing vegetable and fruit concentrate, fish oil and probiotics in asthmatic school children: a randomised controlled trial. Br J Nutr. 2013;110:145–55. https://doi.org/10.1017/S000711451200469.
[35] Valtuena J, Gonzalez-Gross M, Huybrechts I, Breidenassel C, Ferrari M, Mouratidou T, et al. Factors associated with vitamin D deficiency in European adolescents: the HELENA study. J Nutr Sci Vitaminol. 2013;59:161–71. https://doi.org/10.3177/jnsv.59.161.
[36] Hilger J, Friedel A, Herr R, Rausch T, Roos F, Wahl DA, et al. A systematic review of vitamin D status in populations worldwide. Br J Nutr. 2014;111:23–45. https://doi.org/10.1017/s0007114513001840.
[37] Wahl DA, Cooper C, Ebeling PR, Eggersdorfer M, Hilger J, Hoffmann K, et al. A global representation of vitamin D status in healthy populations. Arch Osteoporos. 2012;7:155–72. https://doi.org/10.1007/s11657-012-0093-0.
[38] Lapatsanis D, Moulas A, Cholevas V, Soukakos P, Papadopoulou ZL, Challa A. Vitamin D: a necessity for children and adolescents in Greece. Calcif Tissue Int. 2005;77:348–55. https://doi.org/10.1007/s00223-004-0096-y.
[39] Yao T-C, Tu Y-L, Chang S-W, Tsai H-J, Gu P-W, Ning H-C, et al. Serum 25-hydroxyvitamin D levels in relation to lung function and exhaled nitric oxide in children. J Pediatr. 2014;165:1098–1103.e1. https://doi.org/10.1016/j.jpeds.2014.08.048.
[40] Rao DR, Gaffin JM, Baxi SN, Sheehan WJ, Hoffman EB, Phipatanakul W. The utility of forced expiratory flow between 25% and 75% of vital capacity in predicting childhood asthma morbidity and severity. J Asthma. 2012;49:586–92. https://doi.org/10.3109/02770903.2012.690481.
[41] Kercsmar C, Mcdowell K. Chapter 45: wheezing in older children: asthma. Kendig's Disorders of the respiratory tract in children. 9th ed. Elsevier; 2019. p. 686–721.
[42] Alyasin S, Momen T, Kashef S, Alipour A, Amin R. The relationship between serum 25 hydroxy vitamin d levels and asthma in children. Allergy, Asthma Immunol Res. 2011;3:251–5. https://doi.org/10.4168/aair.2011.3.4.251.
[43] Brehm JM, Acosta-Perez E, Klei L, Roeder K, Barmada M, Boutaoui N, et al. Vitamin D insufficiency and severe asthma exacerbations in Puerto Rican children. Am J Respir Crit Care Med. 2012;186:140–6. https://doi.org/10.1164/rccm.201203-0431OC.
[44] Krobtrakulchai W, Praikanahok J, Visitsunthorn N, Vichyanond P, Manonukul K, Pratumvinit B, et al. The effect of vitamin D status on pediatric asthma at a university hospital, Thailand. Allergy Asthma Immunol Res. 2013;5:289–94. https://doi.org/10.4168/aair.2013.5.5.289.
[45] Hollis BW, Wagner CL, Drezner MK, Binkley NC. Circulating vitamin D3 and 25-hydroxyvitamin D in humans: an important tool to define adequate nutritional vitamin D status. J Steroid Biochem Mol Biol. 2007;103:631–4. https://doi.org/10.1016/j.jsbmb.2006.12.066.
[46] Gupta A, Sjoukes A, Richards D, Banya W, Hawrylowicz C, Bush A, et al. Relationship between serum vitamin D, disease severity, and airway remodeling in children with asthma. Am J Respir Crit Care Med. 2011;184:1342–9. https://doi.org/10.1164/rccm.201107-1239OC.
[47] Zosky GR, Berry LJ, Elliot JG, James AL, Gorman S, Hart PH. Vitamin D deficiency causes deficits in lung function and alters lung structure. Am J Respir Crit Care Med. 2011;183:1336–43. https://doi.org/10.1164/rccm.201010-1596OC.
[48] Ali NS, Nanji K. A review on the role of vitamin D in asthma. Cureus. 2017;9:e1288 e https://doi.org/10.7759/cureus.1288.
[49] Kerley CP, Elnazir B, Faul J, Cormican L. Vitamin D as an adjunctive therapy in asthma. Part 1: a review of potential mechanisms. Pulm Pharmacol Ther. 2015;32:60–74. https://doi.org/10.1016/j.pupt.2015.02.004.
[50] Xystrakis E, Kusumakar S, Boswell S, Peek E, Urry Z, Richards DF, et al. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. J Clin Invest. 2006;116:146–55. https://doi.org/10.1172/jci21759.
[51] Searing DA, Zhang Y, Murphy JR, Hauk PJ, Goleva E, Leung DY. Decreased serum vitamin D levels in children with asthma are associated with increased corticosteroid use. J Allergy Clin Immunol. 2010;125:995–1000. https://doi.org/10.1016/j.jaci.2010.03.008.
[52] Wu AC, Tantisira K, Li L, Fuhlbrigge AL, Weiss ST, Litonjua A. Effect of vitamin D and inhaled corticosteroid treatment on lung function in children. Am J Respir Crit Care Med. 2012;186:508–13. https://doi.org/10.1164/rccm.201202-0351OC.
[53] Brehm JM, Celedon JC, Soto-Quiros ME, Avila L, Hunninghake GM, Forno E, et al. Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. Am J Respir Crit Care Med. 2009;179:765–71. https://doi.org/10.1164/rccm.200808-1361OC.
[54] Weichselbaum E, Coe S, Buttriss J, Stanner S. Fish in the diet: a review. Nutr Bull. 2013;38:128–77. https://doi.org/10.1111/nbu.12021.
[55] Kumar J, Muntner P, Kaskel FJ, Hailpern SM, Melamed ML. Prevalence and associations of 25-hydroxyvitamin D deficiency in US children: NHANES 2001-2004. Pediatrics. 2009;124:e362–70. https://doi.org/10.1542/peds.2009-0051.
[56] Lang JE. Obesity, nutrition, and asthma in children. Pediatr Allergy Immunol Pulmonol. 2012;25:64–75. https://doi.org/10.1089/ped.2011.0137.
[57] Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr. 2000;72:690–3. https://doi.org/10.1093/ajcn/72.3.690.
[58] Grosso G, Galvano F. Mediterranean diet adherence in children and adolescents in southern European countries. NFS Journal. 2016;3:13–9. https://doi.org/10.1016/j.nfs.2016.02.004.
[59] Black PN, Scragg R. Relationship between serum 25-hydroxyvitamin d and pulmonary function in the third national health and nutrition examination survey. Chest. 2005;128:3792–8. https://doi.org/10.1378/chest.128.6.3792.
[60] Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention possible? Lancet. 2015;386:1075–85. https://doi.org/10.1016/s0140-6736(15)00156-7.
[61] Fernandes MR, WDRJ Barreto. Association between physical activity and vitamin D: a narrative literature review. Rev Assoc Med Bras (1992). 2017;63:550–6. https://doi.org/10.1590/1806-9282.63.06.550.
[62] Scientific Advisory Committee on Nutrition SACN. Vitamin D and Health. U.K: Scientific Advisory Committee on Nutrition; 2016https://www.gov.uk/government/groups/scientific-advisory-committee-on-nutrition. [Accessed February 2019].
[63] Bozzetto S, Carraro S, Giordano G, Boner A, Baraldi E. Asthma, allergy and respiratory infections: the vitamin D hypothesis. Allergy. 2012;67:10–7. https://doi.org/10.1111/j.1398-9995.2011.02711.x.
[64] Shim JS, Oh K, Kim HC. Dietary assessment methods in epidemiologic studies. Epidemiol Health. 2014;36:e2014009. https://doi.org/10.4178/epih/e2014009.
[65] Rajakumar K, Holick MF, Jeong K, Moore CG, Chen TC, Olabopo F, et al. Impact of season and diet on vitamin D status of African American and Caucasian children. Clin Pediatr. 2011;50:493–502. https://doi.org/10.1177/0009922810397334.