

The Effect of Vitamin D on Airway Reactivity and Inflammation in Asthmatic Children: A Double-Blind Placebo-Controlled Trial

Ronen Bar Yoseph, MD,¹ Galit Livnat, MD,¹ Zeev Schnapp, MD,¹ Fahed Hakim, MD,¹ Husein Dabbah, MD,^{2,3} Aviv Goldbart, MD,⁴ and Lea Bentur, MD^{1,5*}

Summary. Background: Cross-sectional studies have reported an association of vitamin D deficiency with increased asthma prevalence and severity, and with allergies. The effect of vitamin D as sole therapy on airway hyper-reactivity (AHR) and airway inflammation has not been reported. Aim: To evaluate the effect of vitamin D therapy on AHR as assessed by methacholine concentration, causing a 20% reduction in FEV₁ (PC₂₀-FEV₁) and fractional exhaled nitric oxide (FeNO), systemic markers of allergy and inflammation, and exhaled breath condensate cytokines. Patients and Methods: Children aged 6–18 years with a clinical diagnosis of mild asthma currently not receiving anti-inflammatory therapy and with low vitamin D levels were included in this randomized, double-blind, placebo-controlled study assessing the effect of 6 weeks of treatment with oral vitamin D 14,000 units once weekly or placebo. Results: Of the 39 patients included, 20 received vitamin D treatment and 19 received a placebo. Vitamin D replacement resulted in a significant increase in vitamin levels, which remained unchanged in the placebo group ($P < 0.0001$). There was no change in IgE, eosinophil count, high sensitivity C-reactive protein, FeNO levels or PC₂₀-FEV₁ following treatment. Similar values of exhaled breath condensate cytokines (IL4, IL5, IL10, IL17, and γ interferon) were observed in both groups. Conclusions: In our small group of children with mild asthma, no difference could be demonstrated between the effect of vitamin D and placebo, despite significant increases in vitamin D blood levels. Larger interventional studies are needed to fully explore the possible effect of vitamin D in asthma.

Pediatr Pulmonol. © 2014 Wiley Periodicals, Inc.

Key words: vitamin D; airway hyper-reactivity; fractional exhaled nitric oxide; exhaled breath condensate.

Funding source: Novartis, The Israeli Pediatric Society.

INTRODUCTION

Vitamin D deficiency is as a public health problem in westernized countries, even in sun-replete countries.¹ It

has been suggested as a major player in the pathogenesis of allergy and asthma.^{2–6} Cross-sectional studies have reported an association of vitamin D deficiency with increased risks for airway hyper-reactivity (AHR),^{2,7}

¹Pediatric Pulmonology Unit, Meyer Children's Hospital, Rambam Health Care Campus, Haifa, Israel.

²Department of Pediatrics, Western Galilee Hospital, Nahariya, Israel.

³Faculty of Medicine, Galilee Bar-Ilan University, Safed, Israel.

⁴Department of Pediatrics, Soroka University Medical Center, Beer Sheva, Israel.

⁵Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel.

Conflict of interest: None.

The manuscript has been seen and approved by all authors. Dr. Bar-Yoseph wrote the first draft of the manuscript, and the manuscript was edited by Prof. Bentur. No one received an honorarium, or other form of payment to produce the manuscript. The study was supported by investigator initiated research from Novartis and The Israeli Pediatric Society.

*Correspondence to: Prof. Lea Bentur, MD, Pediatric Pulmonology Unit, Meyer Children's Hospital, Rambam Health Care Campus, P.O. Box 9602, Haifa 31092, Israel. E-mail: l_bentur@rambam.health.gov.il

Received 13 January 2014; Accepted 28 May 2014.

DOI 10.1002/ppul.23076
Published online in Wiley Online Library
(wileyonlinelibrary.com).

I hereby confirm that there are no prior publications or submissions with any overlapping information, including studies and patients.

The manuscript has not been and will not be submitted to any other journal while it is under consideration by The Journal of Pediatric Pulmonology.

reduced lung function,^{2,8,9} increased asthma severity,^{2,10} and steroid treatment.⁶ Therefore, improving vitamin D status theoretically holds promise in the primary prevention of asthma, in decreasing exacerbations, and in treating steroid resistance.

Alternatively, vitamin D deficiency may represent a marker of westernized lifestyle with other potentially confounding factors. Western lifestyle is associated with a shift from outdoor to indoor activity, altered nutrition and increasing obesity, exposure to cigarette smoke, ultrafine particles and ozone, and inadequate physical activity. These factors are also linked to increased asthma prevalence and its increased severity. Therefore, before recommending treatment with vitamin D, double-blind placebo-controlled clinical trials are needed to evaluate the clinical relevance of vitamin D supplementation therapy in asthma.

To the best of our knowledge, only one interventional study assessed the role of vitamin D/placebo in asthma. The study evaluated the effect of vitamin D/placebo as adjuvant therapy to inhaled corticosteroids and reported decreased asthma exacerbations triggered by acute respiratory infections in the vitamin D group.¹¹

We were unable to find interventional studies assessing vitamin D as a sole treatment in asthma. The aim of our study was to evaluate the effect of vitamin D on airway reactivity and on the inflammatory and allergic markers in mild asthmatic children.

PATIENTS AND METHODS

The study was approved by the institutional review board of Rambam Health Care Campus (#483-10).

Study Population

Inclusion Criteria

Age 6–18 years, clinical diagnosis of mild asthma, positive methacholine challenge test (PC₂₀-FEV₁ <16 mg/ml),¹² and insufficient (<30 ng/ml) vitamin D levels.^{5,13}

ABBREVIATIONS

FeNO	fractional exhaled nitric oxide
AHR	airway hyper-reactivity
hs-CRP	high sensitivity C reactive protein
FEV ₁	forced expiratory volume in the first second
FVC	forced vital capacity
FEF _{25–75}	forced expiratory flow between 25% and 75% of FVC
PEFR	peak expiratory flow rate
MCT	methacholine challenge test
PC ₂₀ -FEV ₁	methacholine provocation concentration causing 20% reduction in
EBC	exhaled breath condensate
Vitamin D level	serum 25(OH) D level

Exclusion Criteria

FEV₁ < 65% predicted or any acute illness on the day of the MCT, anti-inflammatory treatment (inhaled corticosteroids or montelukast) over the previous 2 weeks, bronchodilator administration during the 24 hr prior to MCT, and systemic corticosteroids in the 2 months prior to enrollment.

Methods

Study Design

The study was a randomized, placebo-controlled, and included three visits. The study was carried out in the Pulmonary Out-Patient Clinic situated in a tertiary university-affiliated medical center.

Visit 1. Written parental consent was obtained. Demographics, child’s asthma/atopy history, and family history of asthma/atopy/smoking were recorded. Blood tests for serum 25(OH) vitamin D (hereafter vitamin D) level, complete blood count (CBC), IgE, and Hs-CRP were performed.

Visit 2. Each patient performed MCT, skin prick tests, FeNO and exhaled breath condensate (EBC) collection. Following a positive MCT and low vitamin D levels, patients received 6 weeks of treatment with either oral vitamin D 14,000 in 2 ml units once weekly (equivalent to 2,000 units per day) or placebo (2 ml of olive oil). The vitamin D and placebo were provided by the local pharmacy in identical, similarly colored, opaque containers.

Visit 3. After 6 weeks of treatment with either oral vitamin D or placebo, each patient was re-evaluated. Spirometry, MCT, FeNO, and collection of EBC were performed, and blood tests for vitamin D, CBC, IgE, and Hs-CRP were analyzed.

Spirometry

Spirometry was performed in accordance with the ATS/ERS (American Thoracic Society/European Respiratory Society) Task Force, using a KoKo spirometer (n-Spire Healthcare, Inc., Longmont CO).¹⁴ Results were expressed as percent predicted derived from Polgar and Quanjer.¹⁵

Methacholine Challenge Test (MCT)

MCT was performed according to published guidelines.¹² The solutions were administered using a pulmonary dosimeter (nSpire Health, Inc.) according to manufacturer’s instructions. On completing the MCT, 200 mcg of albuterol inhaler was given to all patients.

Blood Tests

Blood samples were analyzed for CBC, serum IgE levels, Hs-CRP, and vitamin D. Serum 25(OH) D concentrations were assayed by the Liaison[®] that uses direct competitive chemiluminescence immunoassay (CLIA) technology.

Fractional Exhaled Nitric Oxide (FeNO)

Patients performed three online single breath maneuvers according to international guidelines using the Eco

Medics NO-analyzer (CLD 88 exhalizer Eco Physics AG, Duernten, Switzerland). An animation biofeedback assisted the children in maintaining the flow rate at 50 ml/sec during the total length of the exhalation.¹⁶ The mean value of at least two successful FeNO measurements, according to ATS/ERS guidelines, was entered in the analysis.

Exhaled Breath Condensate (EBC)

EBC samples were collected in RTubes¹⁷ (Respiratory Research, Charlottesville, VA), according to manufacturer's instructions and ERS recommendations. The collected EBCs were stored at -80°C until analysis.¹⁸ Samples were analyzed for IL4, IL5, IL10, IL17 (high sensitivity ELISA kits; R&D Systems, Inc., Minneapolis, MN) and gamma interferon (high sensitivity ELISA kit; eBioscience, San Diego, CA). All samples were assayed in duplicate, at two dilutions, at plate reader absorbance (450 nm for all assays). Results were analyzed with a 4-parameter logistic curve fit. The intra-assay and inter-assay variability were $<10\%$; specificity ranged from 94% to 98%. The limit of detection of the assays was 0.06 pg/ml for IL5, 15 pg/ml for IL17, 0.11 pg/ml for IL4, 0.09 pg/ml for IL10, and 0.15 pg/ml for gamma interferon.

Skin Prick Test (SPT) for Inhaled Allergens

The SPT was performed according to guidelines.¹⁹

Primary end point: Defined as $\text{PC}_{20}\text{-FEV}_1$.

Secondary end points: Fractional exhaled NO (FeNO), inflammatory mediators in exhaled breath condensate (EBC), and systemic markers of allergy and inflammation.

Analysis and Statistics

Sample size was calculated according to $\text{PC}_{20}\text{-FEV}_1$ using Win Episcope 2.0. A sample size of 36 patients was necessary to detect an increase in $\text{PC}_{20}\text{-FEV}_1$ from 4 ± 1 to 5 ± 1 mg/ml, with a power of 80% and confidence level of 95%. Statistical analysis was performed using a software package (SPSS, version 18; SPSS, Inc., Chicago, IL). Normal distribution was assessed by Kolmogorov–Smirnov Z. Paired Student's *t*-test or Mann–Whitney were performed for parametric values according to the distribution, Fisher exact test and Pearson Chi square analysis were used for non-parametric values. Demographics and baseline characteristics were compared using one-way analysis of variance for quantitative variables. For EBC, analysis of variance (ANOVA) was used followed by Neuman–Keuls post hoc tests, whenever appropriate. Multiple regression analysis was performed to evaluate the relationship between MCT and the other variables. Results were expressed as mean $\pm$ standard deviation (SD), or median and range and 95% confidence interval for the difference before and after treatment period as applicable. Cohen's *d* was used to evaluate size effect. $P < 0.05$ was considered statistically significant.

RESULTS

Fifty-six patients had a positive MCT on visit 1, 11 of whom had normal vitamin D blood levels (vitamin D > 30 ng/ml). Six of the 45 patients with low vitamin D levels did not start the treatment period (due to asthma exacerbation, inconvenience of visits scheduled, or vitamin D supplementation before visit 2). Therefore, 39 patients were treated with vitamin D/placebo and one patient dropped out (vitamin D treatment due to exacerbation) (Fig. 1). There was no statistical difference in demographics and baseline characteristics between the two study groups (Table 1).

Pulmonary function tests, $\text{PC}_{20}\text{-FEV}_1$, eosinophil, IgE, and Hs-CRP blood levels were similar in the two study groups (Table 2). Similar levels of FeNO and EBC cytokines were observed at baseline visit (Tables 2 and 3). Following the 6-week trial, vitamin D levels increased in the vitamin D treatment group, but remained unchanged in the placebo group (33.1 ± 2.1 vs. 21.4 ± 2.1 ng/ml, respectively, $P < 0.0001$). However, despite the increase in vitamin D, no change was observed in PFT's, eosinophils, IgE, and FeNo levels. Similarly, no statistically significant increase in $\text{PC}_{20}\text{-FEV}_1$ was observed in either group (Table 2). The number of patients who showed improvement in $\text{PC}_{20}\text{-FEV}_1$ defined by one-fold concentration or by one stage was similar in both groups.

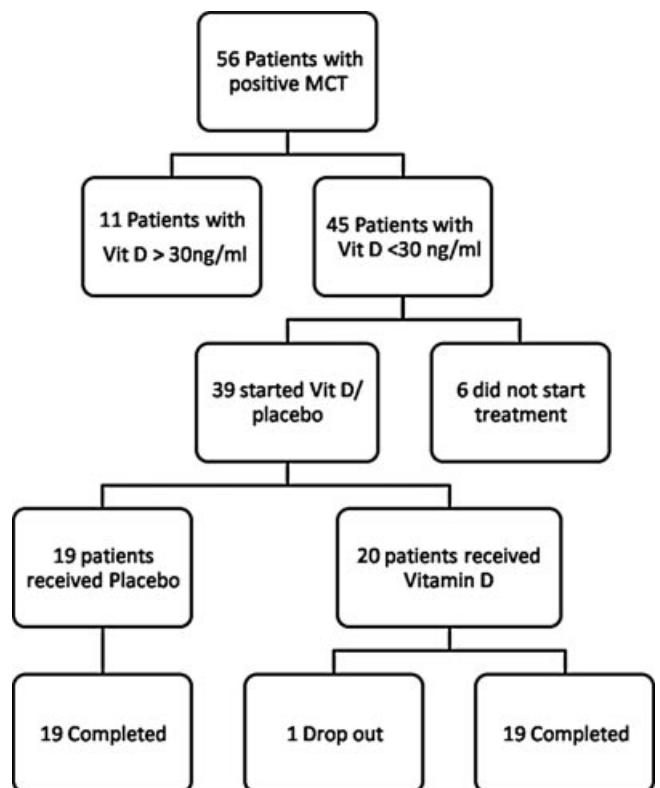


Fig. 1. Flow chart of patients recruited for the study.

TABLE 1—Patient Characteristics

Parameter	Vitamin D (n = 20)	Placebo (n = 19)	P value
Male/female	12/8	13/6	NS
Age (years)	13.5 ± 3.6	12.4 ± 3.6	NS
BMI	19.38 ± 3.29	21.53 ± 3.79	NS
# of patients with passive smoking	7/20	12/19	NS
# of patients with pet (cat/dog) at home	13/20	7/19	NS
# of patients with allergy symptoms	14/20	9/19	NS
# of patients with positive SPT	5/20	6/19	NS

SPT, skin prick test.

#=Number of patients.

EBC cytokines changed following 6 weeks of treatment (increase in IL5, IL10, and γ interferon and decrease in IL17) but similar changes were observed in the placebo group (Table 3).

DISCUSSION

In our small group of children with clinical mild asthma, no difference could be demonstrated between vitamin D as a sole treatment and placebo on multiple parameters (MCT, FeNO, hs-CRP, IgE, airway cytokines, and eosinophils), despite a significant increase in vitamin D blood levels. Observational studies have clearly reported an association between vitamin D deficiency and increased asthma/allergy prevalence and severity. Although association studies are often used for ascertaining causes, clinical trials of vitamin D supplementation are needed to ensure causation.

Previous studies supported correlations between vitamin D blood levels and response to inhaled corticosteroids.^{20–22} One interventional study assessed the role of vitamin D as adjuvant therapy to inhaled steroids in children and reported decreased asthma exacerbations triggered by viral infections.¹¹ However, no increase in vitamin D level was observed, and similar improvements in FEV1 and asthma questionnaire were observed in the placebo group. The similar improvement in both groups in FEV1 and asthma questionnaire may have been due to the co-administration of inhaled steroids resulting in less room for improvement. In the current study we aimed to assess the effect of vitamin D as sole therapy on a wide range of measurements (AHR, FeNO, systemic markers of allergy and inflammation, and EBC cytokines) and tried to quantify the change in aspects of asthma, allergy and inflammation.

Vitamin D insufficiency/deficiency is highly reported in healthy and asthmatic children^{2,10,23–25} In our study, 80% of the children had suboptimal vitamin D levels that increased to a sufficient level in the treatment group.

Several studies have correlated vitamin D blood levels with FVC, FEV₁, FEF_{25–75%}, and FEV₁/FVC.^{2,8,9} In our study, no correlation was found between these parameters and vitamin D or between groups before or after the treatment period (data not shown).

Two previous studies reported correlation between vitamin D level and measure of airway hyper-responsiveness (exercise test, MCT).^{2,7} To the best of our knowledge, no interventional study has assessed the effect of vitamin D supplementation on MCT. In our study, increasing vitamin D did not attenuate non-specific airway inflammation.

Exhaled breath condensate contains airway epithelial lining fluid and airway mediators.²⁶ Several studies

TABLE 2—Vitamin D, IgE, Eosinophil Count, Hs-CRP, FeNO Levels, and PC20-MCT Before and After 6 Weeks of Treatment in the Two Study Groups

Parameter (mean ± SD) median (range)	Vitamin D (n = 19)			Placebo (n = 19)			Vitamin D versus Placebo
	Pre	Post	95% CI P value	Pre	Post	95% CI P value	Cohen's d effect size
Vitamin D (ng/ml)	20.8 ± 6.5 21.5 (9.7–29.8)	33.1 ± 7.9* 33 (20.2–54.8)	8.5–16.2 <0.0001	20.0 ± 7.1 19.8 (6.0–29.6)	21.4 ± 10.2 19.9 (6–41.9)	[(-1.6)–4.3] NS	0.62
IgE (IU/ml)	432.8 ± 465.7 320 (18.1–1650)	398.1 ± 412.6 272 (18.7–1450)	[(-81.3)–11.8] NS	433.8 ± 455.0 302 (15.7–1680)	539.5 ± 651.8 268.5 (9.2–2430)	[(-100.3)–311.6] NS	0.23
Eosinophil (10 ³ /μl)	0.56 ± 0.94 0.33 (0.1–4.3)	0.32 ± 0.18 0.31 (0.1–0.8)	[(-0.72)–0.2] NS	0.39 ± 0.34 0.35 (0.01–1.14)	0.31 ± 0.25 0.22 (0.02–0.9)	[(-0.20)–0.04] NS	0.11
Hs-CRP (mg/L)	1.99 ± 1.0 1.68 (1.1–4.8)	2.29 ± 1.68 1.7 (0.94–6.73)	[(-0.40)–1.01] NS	2.08 ± 1.33 1.61 (0.85–5.75)	2.13 ± 0.88 1.88 (0.94–4.38)	[(-0.39)–0.48] NS	0.11
FeNO (ppb)	36.6 ± 39.1 21.8 (5–153.3)	34.2 ± 26.8 33.0 (2–99.9)	[(-20.5)–15.8] NS	58.6 ± 54.7 38.2 (4.8–165.8)	51.0 ± 40.2 35.0 (5.6–133.0)	[(-19.4)–4.2] NS	0.09
PC20-FEV ₁ (mg/ml)	2.79 ± 3.7 1.1 (0.15–11.6)	5.07 ± 5.5 3.3 (0.05–16)	[(-0.6)–5.15] NS	2.17 ± 2.9 1.06 (0.1–9.13)	4.13 ± 5.7 1.2 (0.01–16)	[(-1.01)–5.03] NS	0.02

*P < 0.0001.

TABLE 3—EBC Cytokines Levels Before and After 6 Weeks of Treatment in the Two Study Groups

Parameter (pg/ml)	Vitamin D (n = 19)			Placebo (n = 19)			Vitamin D versus Placebo
	Pre	Post	95% CI <i>P</i> value	Pre	Post	95% CI <i>P</i> value	<i>P</i> value
IL-4	6.75 ± 4.56	5.82 ± 1.34	[(-3.30–1.41)] NS	7.58 ± 3.81	5.12 ± 1.56	[(-4.54)–(-0.39)] 0.02	0.17
IL-5	1.64 ± 0.3	2.61 ± 0.89	[0.53–1.41] <0.0001	1.62 ± 0.35	2.07 ± 0.55	[0.06–0.84] 0.027	0.30
IL-17	35.81 ± 9.55	28.92 ± 3.54	[(-11.6)–(-2.2)] 0.007	34.49 ± 8.24	30.11 ± 4.04	[(-8.7)–(-0.06)] 0.047	0.14
IL-10	0.95 ± 0.19	1.27 ± 0.45	[0.06–0.58] 0.018	0.96 ± 0.19	1.53 ± 0.53	[0.30–0.82] <0.0001	0.23
INF γ	0.24 ± 0.04	0.28 ± 0.03	[0.01–0.06] 0.001	0.22 ± 0.04	0.29 ± 0.04	[0.05–0.09] <0.0001	0.35

examined airway biomarkers in exhaled breath condensate in asthmatic patients. Elevated IL4, IL5, IL17, and γ interferon levels and decreased IL10 levels were reported in the EBC of asthmatics compared with the control group.^{27–29} In our study, IL-17 and IL-4 were relatively decreased while IL-10 was increased compared to previous studies. The difference may be the result of down regulation of regulatory T cells by vitamin D insufficiency that elaborate anti-inflammatory cytokines, including IL10.³⁰ Alternatively, assay variability and standardization of EBC collection and storage might contribute to these differences.

Patients with severe asthma exhibit increased levels of TH17 associated cytokines IL-17A and IL-22, which are not inhibited by steroids. 1,25(OH)2D3 was found to inhibit TH17 cytokine production in moderate to severe asthmatic patients, identifying it as a novel steroid-enhancing property of vitamin D in asthmatic patients.³¹ We assessed the effect of vitamin D supplementation on airway cytokines. There was a significant increase of TH1 cytokines (IL10, INF gamma) and decrease of TH2 cytokines (IL17 significantly and IL4 only marginally), with increased IL5 (Th2 cytokine). However, these changes were similar in both groups. We are unaware of previous studies evaluating the effect of vitamin D on EBC cytokines. It should be noted that both study groups displayed the same trend (not statistically significant) towards improvement in MCT and airway cytokines. This improvement can theoretically be explained by seasonal variation,³² although no seasonal variation was found in our small sample size study. In addition, both groups received a small amount of olive oil (2 ml) once weekly. Although several studies found that the Mediterranean diet and olive oil have positive effects in preventing asthma, the small amount given here makes this possibility unlikely.³³

Increased FeNO levels are found in patients with allergy and atopy and correlate with sputum eosinophils

and AHR.¹⁶ If vitamin D supplementation attenuated TH2 response, a decrease in FeNO levels would be expected in the treatment group. However, no correlation between vitamin D levels and FeNO and no differences between the groups were observed in our study.

Serum IgE and eosinophils correlate with allergic asthma^{34–36} and vitamin D blood level correlate with IgE and eosinophils.² In our study, no correlation between vitamin D levels and serum IgE and eosinophils was found, and no differences between the groups were observed in this interventional study.

Recent population-based studies showed correlation between increased levels of hs-CRP with a high frequency of AHR and low FEV₁.^{37,38} No study has evaluated CRP in relation to vitamin D levels in asthmatic children. In our study, no correlation between vitamin D level and CRP or differences between study groups were observed.

This double-blind placebo-controlled study assessed the effect of vitamin D therapy on AHR as assessed by PC20-FEV₁ and markers of systemic and airway inflammation and allergy. The major limitation of our study is the small cohort, a sample size of 36 patients, calculated based on MCT, was achieved. However, in view of the wide range of MCT values observed, the possibility of a type 2 error cannot be excluded. We included only mild atopic asthmatics with near normal baseline pulmonary function tests in order to perform MCT and to avoid the possible impact of anti-inflammatory drugs or asthma severity on vitamin D levels. Mild disease may represent a specific genetic polymorphism and the results may not be applicable to higher disease activity. The level of vitamin D and the length of treatment required for an immunomodulatory effect are unknown.

In conclusion, in this double-blind placebo-controlled interventional study, no difference between the effect of vitamin D and placebo on MCT, FeNO, hs-CRP, IgE, airway cytokines, and eosinophils could be demonstrated, despite significant increases in vitamin D blood levels.

Although the results of this study do not support a role for vitamin D as sole therapy in mild asthma, the small sample size precludes any firm conclusion. Larger interventional studies with vitamin D as sole therapy and as add-on to corticosteroids are needed to fully explore the possible effect of vitamin D in asthma.

REFERENCES

- 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–e370.
- Brehm JM, Celedon JC, Soto-Quiros ME, Avila L, Hunninghake GM, Forno E, Laskey D, Sylvia JS, Hollis BW, Weiss ST, Litonjua AA. Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *Am J Respir Crit Care Med* 2009;179:765–771.
- Hughes DA, Norton R. Vitamin D and respiratory health. *Clin Exp Immunol* 2009;158:20–25.
- Lange NE, Litonjua A, Hawrylowicz CM, Weiss S. Vitamin D, the immune system and asthma. *Expert Rev Clin Immunol* 2009;5:693–702.
- Litonjua AA. Childhood asthma may be a consequence of vitamin D deficiency. *Curr Opin Allergy Clin Immunol* 2009;9:202–207.
- 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.
- 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;158:437–441.
- Black PN, Scragg R. Relationship between serum 25-hydroxyvitamin D and pulmonary function in the third national health and nutrition examination survey. *Chest* 2005;128:3792–3798.
- Burns JS, Dockery DW, Neas LM, Schwartz J, Coull BA, Raizenne M, Speizer FE. Low dietary nutrient intakes and respiratory health in adolescents. *Chest* 2007;132:238–245.
- Brehm JM, Schuermann B, Fuhlbrigge AL, Hollis BW, Strunk RC, Zeiger RS, Weiss ST, Litonjua AA. Serum vitamin D levels and severe asthma exacerbations in the Childhood Asthma Management Program study. *J Allergy Clin Immunol* 2010;126:52–58 e55.
- Majak P, Olszowiec-Chlebna M, Smejda K, Stelmach I. Vitamin D supplementation in children may prevent asthma exacerbation triggered by acute respiratory infection. *J Allergy Clin Immunol* 2011;127:1294–1296.
- Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, MacIntyre NR, McKay RT, Wanger JS, Anderson SD, Cockcroft DW, Fish JE, Sterk PJ. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 2000;161:309–329.
- Rosen CJ. Clinical practice. Vitamin D insufficiency. *N Engl J Med* 2011;364:248–254.
- Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J. Standardisation of spirometry. *Eur Respir J* 2005;26:319–338.
- Quanjer PH, Borsboom GJ, Brunekreef B, Zach M, Forche G, Cotes JE, Sanchis J, Paoletti P. Spirometric reference values for white European children and adolescents: polgar revisited. *Pediatr Pulmonol* 1995;19:135–142.
- 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;171:912–930.
- Rosias PP, Robroeks CM, Kester A, den Hartog GJ, Wodzig WK, Rijkers GT, Zimmermann LJ, van Schayck CP, Jobsis Q, Dompeling E. Biomarker reproducibility in exhaled breath condensate collected with different condensers. *Eur Respir J* 2008;31:934–942.
- Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, Becher G, van Beurden WJ, Corradi M, Dekhuijzen R, Dweik RA, Dwyer T, Effros R, Erzurum S, Gaston B, Gessner C, Greening A, Ho LP, Hohlfeld J, Jobsis Q, Laskowski D, Loukides S, Marlin D, Montuschi P, Olin AC, Redington AE, Reinhold P, van Rensen EL, Rubinstein I, Silkoff P, Toren K, Vass G, Vogelberg C, Wirtz H. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 2005;26:523–548.
- Bousquet J, Heinzerling L, Bachert C, Papadopoulos NG, Bousquet PJ, Burney PG, Canonica GW, Carlsen KH, Cox L, Haahtela T, Lodrup Carlsen KC, Price D, Samolinski B, Simons FE, Wickman M, Annesi-Maesano I, Baena-Cagnani CE, Bergmann KC, Bindsløv-Jensen C, Casale TB, Chiriac A, Cruz AA, Dubakiene R, Durham SR, Fokkens WJ, Gerth-van-Wijk R, Kalayci O, Kowalski ML, Mari A, Mullol J, Nazamova-Baranova L, O’Hehir RE, Ohta K, Panzner KP, Passalacqua G, Ring J, Rogala B, Romano A, Ryan D, Schmid-Grendelmeier P, Todo-Bom A, Valenta R, Woehrl S, Yusuf OM, Zuberbier T, Demoly P. Practical guide to skin prick tests in allergy to aeroallergens. *Allergy* 2012;67:18–24.
- Gupta A, Sjoukes A, Richards D, Banya W, Hawrylowicz C, Bush A, Saglani S. Relationship between serum vitamin D, disease severity, and airway remodeling in children with asthma. *Am J Respir Crit Care Med* 2011;184:1342–1349.
- Kreindler JL. Is the best offense a good D-fense? Should we use vitamin D as adjunctive therapy for asthma? *Am J Respir Crit Care Med* 2012;186:470–472.
- Vu LH, Whiteman DC, van der Pols JC, Kimlin MG, Neale RE. Serum vitamin D levels in office workers in a subtropical climate. *Photochem Photobiol* 2011;87:714–720.
- Tylavsky FA, Cheng S, Lyytikäinen A, Viljakainen H, Lamberg-Allardt C. Strategies to improve vitamin D status in northern European children: exploring the merits of vitamin D fortification and supplementation. *J Nutr* 2006;136:1130–1134.
- Oren Y, Shapira Y, Agmon-Levin N, Kivity S, Zafirir Y, Altman A, Lerner A, Shoenfeld Y. Vitamin D insufficiency in a sunny environment: a demographic and seasonal analysis. *Isr Med Assoc J* 2010;12:751–756.
- Brehm JM, Acosta-Perez E, Klei L, Roeder K, Barmada M, Boutaoui N, Forno E, Kelly R, Paul K, Sylvia J, Litonjua AA, Cabana M, Alvarez M, Colon-Semidey A, Canino G, Celedon JC. Vitamin D insufficiency and severe asthma exacerbations in Puerto Rican children. *Am J Respir Crit Care Med* 2012;186:140–146.
- Carraro S, Baraldi E. Exhaled breath condensate in children: present knowledge and future prospects. *J Breath Res* 2008;2:037003.
- Robroeks CM, van de Kant KD, Jobsis Q, Hendriks HJ, van Gent R, Wouters EF, Damoiseaux JG, Bast A, Wodzig WK, Dompeling E. Exhaled nitric oxide and biomarkers in exhaled breath condensate indicate the presence, severity and control of childhood asthma. *Clin Exp Allergy* 2007;37:1303–1311.
- Profita M, La Grutta S, Carpano E, Riccobono L, Di Giorgi R, Bonanno A, Pace E, Bonsignore G, Bousquet J, Vignola AM, Gjomarkaj M. Noninvasive methods for the detection of upper and lower airway inflammation in atopic children. *J Allergy Clin Immunol* 2006;118:1068–1074.
- Shahid SK, Kharitonov SA, Wilson NM, Bush A, Barnes PJ. Increased interleukin-4 and decreased interferon-gamma in exhaled breath condensate of children with asthma. *Am J Respir Crit Care Med* 2002;165:1290–1293.

30. Maalmi H, Berraies A, Tangour E, Ammar J, Abid H, Hamzaoui K, Hamzaoui A. The impact of vitamin D deficiency on immune T cells in asthmatic children: a case-control study. *J Asthma Allergy* 2012;5:11–19.
31. Nanzer AM, Chambers ES, Ryanna K, Richards DF, Black C, Timms PM, Martineau AR, Griffiths CJ, Corrigan CJ, Hawrylowicz CM. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1 α , 25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J Allergy Clin Immunol* 2013;132:297–304, e293.
32. Fruchter O, Yigla M. Seasonal variability of the methacholine challenge test. *J Asthma* 2009;46:951–954.
33. Castro-Rodriguez JA, Garcia-Marcos L, Alfonseda Rojas JD, Valverde-Molina J, Sanchez-Solis M. Mediterranean diet as a protective factor for wheezing in preschool children. *J Pediatr* 2008;152:823–828, 828 e821–822.
34. Gergen PJ, Arbes SJ, Jr., Calatroni A, Mitchell HE, Zeldin DC. Total IgE levels and asthma prevalence in the US population: results from the National Health and Nutrition Examination Survey 2005–2006. *J Allergy Clin Immunol* 2009;124:447–453.
35. Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 1991;325:1067–1071.
36. Ulrik CS. Peripheral eosinophil counts as a marker of disease activity in intrinsic and extrinsic asthma. *Clin Exp Allergy* 1995;25:820–827.
37. Sunyer J, Pistelli R, Plana E, Andreani M, Baldari F, Kolz M, Koenig W, Pekkanen J, Peters A, Forastiere F. Systemic inflammation, genetic susceptibility and lung function. *Eur Respir J* 2008;32:92–97.
38. Deraz TE, Kamel TB, El-Kerdany TA, El-Ghazoly HM. High-sensitivity C reactive protein as a biomarker for grading of childhood asthma in relation to clinical classification, induced sputum cellularity, and spirometry. *Pediatr Pulmonol* 2012;47: 220–225.