

Prenatal Vitamin D Supplementation and Child Respiratory Health: A Randomised Controlled Trial

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Abstract

Background: Observational studies suggest high prenatal vitamin D intake may be associated with reduced childhood wheezing. We examined the effect of prenatal vitamin D on childhood wheezing in an interventional study.

Methods: We randomised 180 pregnant women at 27 weeks gestation to either no vitamin D, 800 IU ergocalciferol daily until delivery or single oral bolus of 200,000 IU cholecalciferol, in an ethnically stratified, randomised controlled trial. Supplementation improved but did not optimise vitamin D status. Researchers blind to allocation assessed offspring at 3 years. Primary outcome was any history of wheeze assessed by validated questionnaire. Secondary outcomes included atopy, respiratory infection, impulse oscillometry and exhaled nitric oxide. Primary analyses used logistic and linear regression.

Results: We evaluated 158 of 180 (88%) offspring at age 3 years for the primary outcome. Atopy was assessed by skin test for 95 children (53%), serum IgE for 86 (48%), exhaled nitric oxide for 62 (34%) and impulse oscillometry of acceptable quality for 51 (28%). We found no difference between supplemented and control groups in risk of wheeze [no vitamin D: 14/50 (28%); any vitamin D: 26/108 (24%) (risk ratio 0.86; 95% confidence interval 0.49, 1.50; $P = 0.69$)]. There was no significant difference in atopy, eczema risk, lung function or exhaled nitric oxide between supplemented groups and controls.

Conclusion: Prenatal vitamin D supplementation in late pregnancy that had a modest effect on cord blood vitamin D level, was not associated with decreased wheezing in offspring at age three years.

Trial Registration: Controlled-Trials.com ISRCTN68645785

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Introduction

Several observational studies have suggested a protective effect of higher maternal vitamin D intake during pregnancy on risk of wheezing [1–3], asthma and allergic rhinitis [4], and eczema [3] in offspring. Dietary intake of vitamin D makes a relatively small contribution to overall vitamin D status, and apparent effects of vitamin D intake may be confounded by other correlated nutrients. These studies did not report outcomes in relation to maternal serum 25-hydroxyvitamin D (25(OH)D) concentration, which takes account of skin synthesis, our primary source of vitamin D, and is the gold standard measure of vitamin D status [5,6]. Observational studies of maternal or cord 25(OH)D levels and child health have yielded conflicting results, with higher 25(OH)D levels associated with increased risk of eczema and asthma [7], reduced risk of respiratory infection and preschool

wheezing [8], and no association with asthma [8] or lung function [9] all reported.

There are plausible biological mechanisms for an association between prenatal 25(OH)D status and wheezing. Developmental programming of lung function and immune responses occurs during pregnancy [10,11]. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D, has been shown in animal and *in vitro* models to have potent immune effects [12] and a role in early lung development [13]. Current recommendations in the United Kingdom are for provision of 10 µg/day (400 IU/day) to all pregnant women [14]. Vitamin D insufficiency is nevertheless very common during pregnancy [15–17], and it has been proposed that prenatal vitamin D supplementation may prevent childhood wheezing and asthma [18]. We assessed children whose mothers had taken part in a randomised controlled trial of prenatal vitamin D supplementation, to determine whether

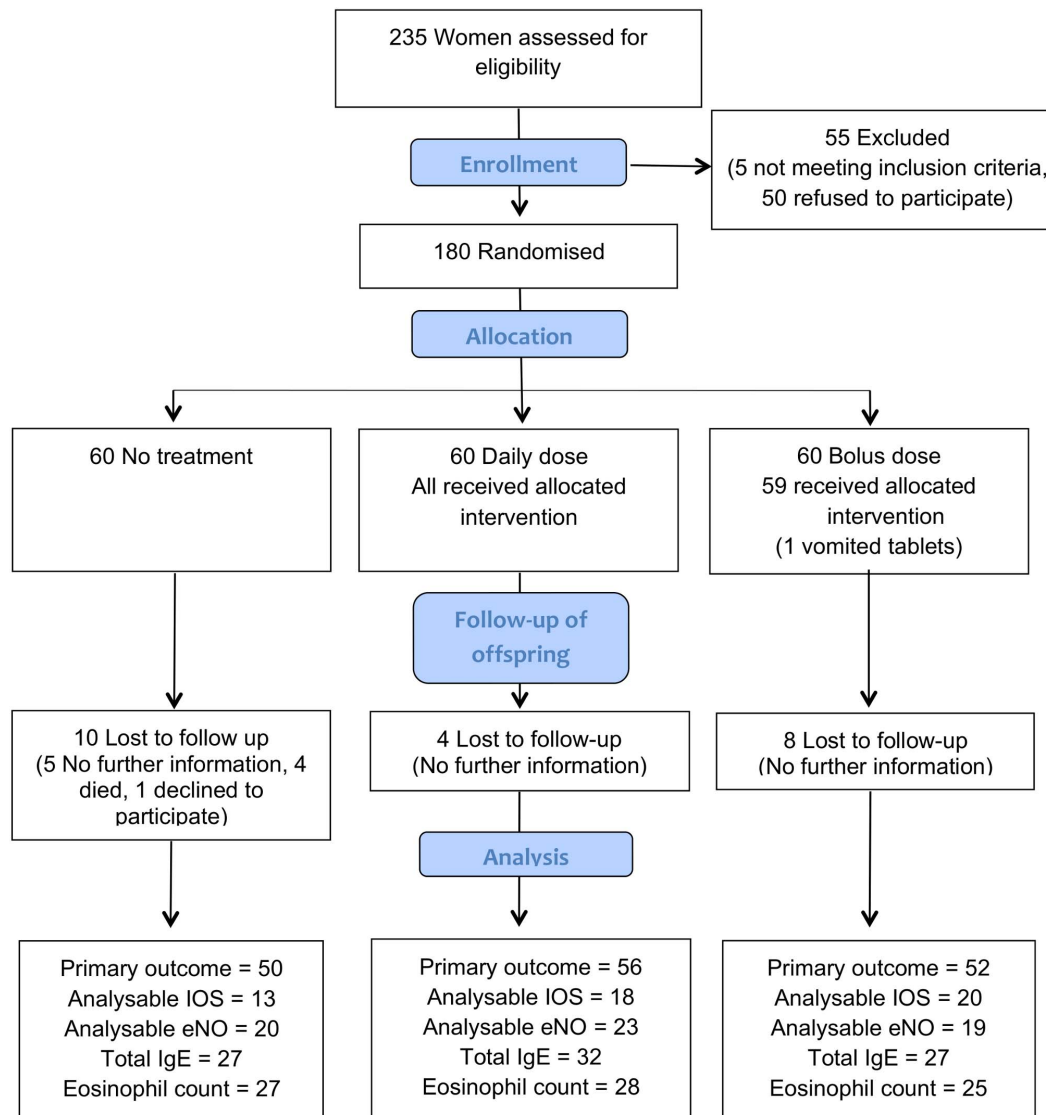


Figure 1. Recruitment and follow up of study participants.

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supplementation prevented wheezing or allergic disease in the first 3 years of life [19].

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Ethics Statement

St. Mary's Hospital Research Ethics Committee approved the follow up study (10/H0712/13) and women gave written informed consent to participation for themselves and their child. Where women only consented to interview over the telephone, verbal consent was taken in place of written consent and this was approved by the Ethics Committee. For each participant who gave verbal consent, this was taken and documented by 2 separate members of the research team.

Study Subjects

This was a prospective follow up study of the offspring of women who took part in an ethnically stratified, parallel group, randomised controlled trial of vitamin D supplementation in pregnancy at St Mary's Hospital London, a university-affiliated hospital prenatal clinic, between April and November 2007. The original trial was conducted to determine the effect of supplementation on 25(OH)D status of mothers in 4 specific ethnic groups, and their babies, at delivery [19]. Eligible participants were women presenting at 27 weeks gestation for routine glucose challenge test from the following ethnic groups: Asian, Middle Eastern, Black and White. Ethnic group was assigned based on participant self-report. Exclusion criteria were known sarcoidosis, osteomalacia, renal dysfunction or tuberculosis.

Study Design

Women were randomised at 27 weeks gestation to no treatment (control), 800 IU ergocalciferol until delivery (daily), or a single oral dose of 200,000 IU cholecalciferol (bolus). The randomisation

Table 1. Characteristics of study participants followed up at age three years.

	Control	Daily vitamin D	Bolus vitamin D	Combined vitamin D
	(n = 50)	(n = 56)	(n = 52)	(n = 108)
Baseline maternal 25(OH)D <25 nmol/L, n/N (%)	24/50 (48)	25/56 (45)	22/52 (42)	47/108 (44)
Male sex, n/N (%)	27/50 (54)	32/56 (57)	26/52 (50)	58/108 (55)
Birth weight (g), mean (SD)	3268 (585)	3321 (525)	3290 (467)	3307 (497)
Ethnicity, n (%)				
Asian	12 (24)	15 (27)	13 (25)	28 (26)
Middle Eastern	13 (26)	14 (25)	14 (26)	28 (26)
Black	12 (24)	14 (25)	13 (25)	27 (25)
White	13 (26)	13 (23)	12 (24)	25 (23)
GA at delivery (weeks), mean (SD)	40 (1)	39 (2)	40 (1)	39 (2)
Nulliparous mother, n/N (%)	23/50 (46)	20/56 (36)	21/52 (40)	41/108 (38)
Vaginal delivery, n/N (%)	29/50 (58)	35/56 (63)	28/52 (54)	63/108 (58)
Maternal smoking during pregnancy, n/N (%)	2/48 (4)	3/55 (5)	0/46 (0)	3/101 (3)
Presence of household smokers, n/N (%)	16/48 (33)	15/55 (27)	17/46 (37)	32/101 (32)
Number of children in household, mean (SD)	2 (1)	2 (1)	2 (1)	2 (1)
Age mother left full time education, mean (SD)	20 (3)	20 (4)	21 (4)	21 (4)
Child in nursery, n/N (%)	27/48 (56)	36/55 (65)	20/45 (44)	57/101 (56)
Cat or dog in household, n/N (%)	4/48 (8)	5/54 (9)	4/46 (9)	9/100 (9)
Maternal Fitzpatrick skin score grade 3–6, n/N (%)	28/44 (64)	33/49 (67)	28/43 (65)	61/92 (66)
Maternal regular vitamin intake at three years, n/N (%)	9/46 (20)	16/53 (30)	16/45 (36)	32/98 (33)
At least one parent with allergic disease, n/N (%)	23/45 (51)	35/53 (66)	21/45 (47)	56/98 (57)
Exclusively breast-fed for 4 months, n/N (%)	22/48 (46)	20/54 (37)	23/44 (52)	43/98 (44)
Any child vitamin supplementation, n/N (%)	30/48 (63)	34/55 (62)	30/46 (65)	64/101 (63)
Completed immunizations to date, n/N (%)	48/49 (98)	54/55 (98)	43/47 (91)	97/102 (95)
Age at time of assessment (months), median (IQR)	37.9 (36.9, 39.9)	37.1 (36.5, 38.8)	37.4 (36.5, 39.5)	37.3 (36.5, 39.0)
Child Fitzpatrick skin score grade 3–6, n (%)	29/42 (69)	34/48 (71)	29/43 (67)	63/91 (69)
Child BMI Z score age 3, mean (SD)	0.51 (1.48)	0.35 (1.15)	0.62 (1.15)	0.47 (1.15)
Child outdoors >1 hour/day, n/N (%)	32/48 (67)	34/53 (64)	33/45 (73)	67/98 (68)
Child TV/computer >2 hours/day, n/N (%)	24/48 (50)	18/53 (34)	17/45 (38)	35/98 (36)
Child 25(OH)D (nmol/L) age 3, median (IQR)	42 (27, 68)	35 (21, 66)	42 (30, 93)	41 (26, 72)

Data shown are for all participants analysed.

IQR = interquartile range, SD = standard deviation, GA = gestational age, SCBU = Special Care Baby Unit, BMI = Body mass index.

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sequence was generated by an independent researcher using computer generated random number lists in blocks of 15, stratified by 4 ethnic groups in a 1:1:1 ratio. The researcher gave participants a study number on entry to the trial, and treatment was allocated from the hospital pharmacy. Women were given instructions to swallow the tablets whole and to avoid other multivitamin supplements containing vitamin D. This trial was conducted before national guidance on routinely providing advice on vitamin D intake during pregnancy was introduced in March 2008 [14]. Investigators blind to original treatment allocation assessed offspring at three years of age using a validated health questionnaire and clinical assessment.

Outcome Assessments

The primary outcome was prevalence of ‘wheeze ever’ as defined by the International Study of Asthma and Allergies in Childhood (ISAAC) [20]. Secondary outcomes were recurrent wheezing (≥ 2 episodes of reported wheezing since birth), wheeze in the year prior to assessment (ISAAC), wheeze with a positive

asthma predictive index (loose criteria) [21], reported history of bronchodilator use, eczema ever (ISAAC), eczema in the year prior to assessment (ISAAC), allergic rhinitis, history of doctor diagnosed food allergy, history of lower respiratory tract infection (LRTI) (any of bronchiolitis, bronchitis, croup, pneumonia or an otherwise unspecified chest infection) and history of >4 episodes of upper respiratory tract infections (URTI) per year (defined as a positive answer to the question ‘how often does your child have an upper respiratory tract infection, with at least two of the following symptoms: cough, runny nose and fever?’).

Exhaled nitric oxide (eNO) was measured using an offline, tidal breathing technique adherent to ATS/ERS guidelines [22]. Impulse oscillometry (IOS) was performed in accordance with current international guidelines [23], before and 15 minutes after inhalation of 400 mcg salbutamol sulphate via spacer (Volumatic, Allen and Hanburys, Middlesex, UK). Resonant frequency, area under the reactance curve, and resistance at 10 and 20 Hz were chosen for data analysis. Bronchodilator responsiveness for each parameter was measured as the percentage change seen post-

Table 2. Clinical outcomes at age three years. Combined vitamin D groups versus control.

	Control	Combined vitamin D	RR	OR	P	aOR [†]	P
	(%)	(%)	(95% CI)	(95% CI)		(95% CI)	
Wheeze ever	14/50 (28)	26/108 (24)	0.86 (0.49, 1.50)	0.82 (0.38, 1.74)	0.60	0.73 (0.33, 1.65)	0.45
Recurrent wheezing	7/50 (14)	17/108 (16)	1.12 (0.50, 2.54)	1.15 (0.44, 2.97)	0.77	1.14 (0.42, 3.13)	0.80
Wheezing in the last year	8/50 (16)	17/108 (16)	0.98 (0.46, 2.13)	0.98 (0.39, 2.45)	0.97	0.95 (0.36, 2.51)	0.92
Wheeze with positive API	7/50 (15)	15/107 (14)	1.00 (0.44, 2.30)	1.00 (0.38, 2.64)	1.00	1.06 (0.37, 3.02)	0.91
Any bronchodilator use	4/49 (8)	24/104 (23)	2.83 (1.04, 7.71)	3.37 (1.10, 10.34)	0.03*	3.14 (0.96, 10.28)	0.06*
Eczema ever	15/49 (31)	30/102 (29)	0.96 (0.57, 1.61)	0.75 (0.35, 1.62)	0.47	0.72 (0.32, 1.61)	0.42
Eczema in the last year	7/49 (14)	20/103 (19)	1.36 (0.62, 3.00)	1.34 (0.52, 3.47)	0.55	1.52 (0.55, 4.21)	0.42
Atopy	7/27(26)	11/68 (16)	0.62 (0.27, 1.44)	0.56 (0.19, 1.66)	0.30	0.29 (0.07, 1.16)	0.08
Allergic rhinitis	7/49 (14)	11/101 (12)	0.76 (0.31, 1.85)	0.63 (0.22, 1.78)	0.38	0.69 (0.22, 2.13)	0.52
Food allergy diagnosis	3/49 (6)	12/102 (12)	1.92 (0.57, 6.50)	1.81 (0.48, 6.85)	0.38	1.93 (0.45, 8.29)	0.38
>4 URTI/year	7/50 (14)	20/103 (19)	1.39 (0.63, 3.06)	1.43 (0.55, 3.68)	0.46	1.34 (0.49, 3.68)	0.57
LRTI ever	11/50 (22)	31/101 (31)	1.40 (0.77, 2.54)	1.47 (0.64, 3.37)	0.37	1.60 (0.67, 3.85)	0.29
Primary health care records							
Recurrent wheeze	3/31 (10)	10/70 (14)	1.48 (0.44, 5.00)	1.56 (0.40, 6.10)	0.52	1.47 (0.33, 6.55)	0.61
Eczema	6/31 (19)	10/70 (14)	0.74 (0.29, 1.85)	0.46 (0.23, 2.12)	0.52	0.63 (0.18, 2.16)	0.46
Food allergy	2/31 (6)	2/70 (3)	0.44 (0.07, 3.00)	0.43 (0.06, 3.18)	0.58	0.14 (0.00, 4.39)	0.26

RR = risk ratio, OR = unadjusted odds ratio, aOR = adjusted odds ratio, API = Asthma predictive index, URTI = upper respiratory tract infection, LRTI = lower respiratory tract infection.

[†]Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the household, age mother left full-time education and baseline concentration of 25(OH)D in maternal blood. n = 139 for primary outcome measure.

*Adjusted p-value for multiple testing non-significant at 20% level.

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Table 3. Clinical outcomes at age three years. Daily vitamin D versus control.

	Control	Daily Vitamin D	RR	OR	P	aOR [†]	P
	(%)	(%)	(95% CI)	(95% CI)		(95% CI)	
Wheeze ever	14/50 (28)	11/56 (20)	0.70 (0.35, 1.40)	0.63 (0.26, 1.55)	0.31	0.56 (0.20, 1.57)	0.27
Recurrent wheezing	7/50 (14)	8/56 (14)	1.02 (0.40, 2.61)	1.02 (0.34, 3.06)	0.97	1.11 (0.32, 3.92)	0.87
Wheezing in the last year	8/50 (16)	8/56 (14)	0.89 (0.36, 2.20)	0.88 (0.30, 2.54)	0.81	0.88 (0.27, 2.90)	0.84
Wheeze with positive API	7/50 (14)	6/56 (11)	0.77 (0.28, 2.13)	0.74 (0.23, 2.36)	0.61	0.65 (0.15, 2.82)	0.57
Any bronchodilator use	4/49 (8)	10/56 (18)	2.23 (0.75, 6.67)	2.45 (0.72, 8.37)	0.15	2.24 (0.54, 9.33)	0.27
Eczema ever	15/49 (31)	15/54 (28)	0.91 (0.50, 1.66)	0.87 (0.37, 2.04)	0.75	0.61 (0.23, 1.67)	0.34
Eczema in the last year	7/49 (14)	11/55 (20)	1.40 (0.59, 3.33)	1.50 (0.53, 4.23)	0.44	1.31 (0.38, 4.54)	0.67
Atopy	7/27 (26)	4/36 (11)	0.41 (0.13, 1.27)	0.36 (0.09, 1.38)	0.13	0.36 (0.05, 2.94)	0.34
Allergic rhinitis	7/49 (14)	7/55 (13)	0.89 (0.34, 2.36)	0.88 (0.28, 2.70)	0.82	0.63 (0.17, 2.36)	0.49
Food allergy diagnosis	3/49 (6)	8/55 (15)	2.38 (0.67, 8.46)	2.61 (0.65, 10.45)	0.16	4.53 (0.52, 39.33)	0.17
>4 URTI/year	7/50 (14)	11/55 (20)	1.43 (0.60, 3.40)	1.54 (0.55, 4.33)	0.42	1.39 (0.41, 4.68)	0.60
LRTI ever	11/50 (22)	14/54 (26)	1.18 (0.59, 2.35)	1.24 (0.50, 3.07)	0.64	1.00 (0.35, 2.91)	1.00
Primary health care records							
Recurrent wheeze	3/31 (10)	4/36 (11)	1.15 (0.28, 4.74)	1.17 (0.24, 5.67)	0.85	1.43 (0.21, 9.89)	0.71
Eczema	6/31 (19)	5/36 (14)	0.72 (0.24, 2.12)	0.67 (0.18, 2.46)	0.55	0.61 (0.13, 2.86)	0.53
Food allergy	2/31 (7)	2/36 (6)	0.86 (0.13, 5.76)	0.85 (0.11, 6.44)	0.88	-	-

RR = Risk ratio, OR = unadjusted odds ratio, aOR = adjusted odds ratio, API = Asthma predictive index, URTI = upper respiratory tract infection, LRTI = lower respiratory tract infection.

[†]Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the household, age mother left full-time education and baseline concentration of 25(OH)D in maternal blood.

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Table 4. Clinical outcomes at age three years.

	Control	Bolus Vitamin D	RR	OR	<i>p</i>	aOR [†]	<i>p</i>
	(%)	(%)	(95% CI)	(95% CI)		(95% CI)	
Wheeze ever	14/50 (28)	15/52 (29)	1.03 (0.56, 1.91)	1.04 (0.44, 2.47)	0.93	1.17 (0.44, 3.10)	0.75
Recurrent wheezing	7/50 (14)	9/52 (17)	1.24 (0.50, 3.07)	1.29 (0.44, 3.77)	0.65	1.91 (0.53, 6.75)	0.31
Wheezing in the last year	8/50 (16)	9/52 (17)	1.08 (0.45, 2.58)	1.10 (0.39, 3.12)	0.86	1.29 (0.40, 4.15)	0.66
Wheeze with positive API	7/50 (14)	9/51 (18)	1.26 (0.51, 3.12)	1.32 (0.45, 3.86)	0.62	2.01 (0.58, 6.99)	0.27
Any bronchodilator use	4/49 (8)	14/48 (29)	3.57 (1.27, 10.09)	4.63 (1.40, 15.34)	0.008*	5.43 (1.39, 21.20)	0.02*
Eczema ever	15/49 (31)	15/48 (31)	1.02 (0.56, 1.85)	1.03 (0.44, 2.44)	0.95	0.86 (0.32, 2.28)	0.76
Eczema in the last year	7/49 (14)	9/48 (19)	1.31 (0.53, 3.24)	1.39 (0.47, 4.08)	0.55	1.97 (0.47, 8.27)	0.36
Atopy	7/27 (26)	7/32 (22)	0.84 (0.34, 2.11)	0.80 (0.24, 2.66)	0.72	0.31 (0.05, 1.83)	0.20
Allergic rhinitis	7/49 (14)	4/46 (9)	0.61 (0.19, 1.94)	0.57 (0.16, 2.10)	0.40	0.91 (0.20, 4.11)	0.90
Food allergy diagnosed	3/49 (6)	4/47 (9)	1.36 (0.32, 5.78)	1.43 (0.30, 6.75)	0.65	1.31 (0.24, 7.18)	0.76
>4 URTI/year	7/50 (14)	9/48 (19)	1.34 (0.54, 3.31)	1.42 (0.48, 4.17)	0.53	1.72 (0.49, 6.04)	0.39
LRTI ever	11/50 (22)	17/47 (36)	1.64 (0.86, 3.14)	2.01 (0.82, 4.92)	0.12	2.87 (1.03, 8.03)	0.05*
Primary health care record							
Recurrent wheeze	3/31 (10)	6/34 (18)	1.82 (0.50, 6.68)	2.00 (0.46, 8.80)	0.35	1.85 (0.36, 9.66)	0.47
Eczema	6/31 (19)	5/34 (15)	0.76 (0.26, 2.24)	0.72 (0.20, 2.64)	0.62	0.63 (0.11, 3.59)	0.60
Food allergy	2/31 (7)	0/34 (0)	0.0 (-, -)	-	-	-	-

Bolus vitamin D versus control.

RR = Risk Ratio, OR = unadjusted odds ratio, aOR = adjusted odds ratio, API = Asthma predictive index, URTI = upper respiratory tract infection, LRTI = lower respiratory tract infection.

[†]Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the household, age mother left full-time education and baseline concentration of 25(OH)D in maternal blood.

*Adjusted p-value for multiple testing non-significant at 20% level.

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bronchodilator. See File S1 for description of IOS quality control and exhaled nitric oxide assessment.

Allergic sensitisation was defined as a skin prick test wheal at least 3 mm greater than the negative control (Glycerine) to one or more of the aeroallergens tested at 15 minutes, in the context of an appropriate response to the positive control (Histamine 10%). House dust mite, alternaria, cladosporium, cat, dog, grass pollen, silver birch pollen, peanut, milk and egg were tested (Stallergenes, Antony, France).

Serum total IgE (ImmunoCAP, Phadia, Uppsala, Sweden), 25(OH)D and eosinophil count were determined on the day of the child's assessment. 25(OH)D was measured in mothers prior to randomisation, and in offspring at birth (cord blood) using a radio-immunoassay (DiaSorin, Stillwater, MN) in a clinical biochemistry laboratory that participates in the international Vitamin D external quality assessment program (DEQAS). Vitamin D deficiency was defined as 25(OH)D <25 nmol/L [24] and sufficiency ≥ 50 nmol/L [24,25].

Primary health care records were obtained from participants' general practitioners and reviewed by a single investigator (RJB), blinded to treatment allocation. Children were categorised as having 'recurrent wheezing' where ≥ 2 episodes of either wheezing, or respiratory distress treated with bronchodilator were recorded; 'eczema' if they had ≥ 2 attendances separated by ≥ 6 months where either topical corticosteroids were prescribed for treatment of an itchy skin rash, or a doctor's diagnosis of eczema was made; and 'food allergy' if this diagnosis was recorded in any part of the primary healthcare record.

Statistical Analysis

In the original study it was calculated that at least eight women in each ethnic group for each arm of treatment would be needed to demonstrate a significant difference in vitamin D levels at delivery in the no treatment group vs. the supplemented groups (power 90%, test of significance at 5% level). To account for drop-outs, preterm delivery, and delivery at another hospital, 15 women were allocated to each arm of treatment within each ethnic group ($n = 180$).

With 180 participants and 80% successful follow up, this study had 80% power with 2-sided alpha of 0.05 to detect a reduction in wheeze from 34% in the children of non-supplemented mothers [21] to 13% in the supplemented group. This level of risk reduction would be consistent with some observational studies [2,21]. For primary analyses, bolus and daily vitamin D supplemented groups were analysed together and compared with the control group. We also report separate analyses of bolus and daily groups versus control, and of children born to mothers with vitamin D deficiency at enrolment (defined as 25(OH)D <25 nmol/L). Binary outcomes were analysed using risk ratios and odds ratios for unadjusted analysis, and logistic regression for adjusted analyses. Continuous outcomes were analysed using linear regression. Potential confounding factors used in the adjustment model were mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the household, age mother left full-time education and baseline concentration of 25(OH)D in maternal blood. For all analyses,

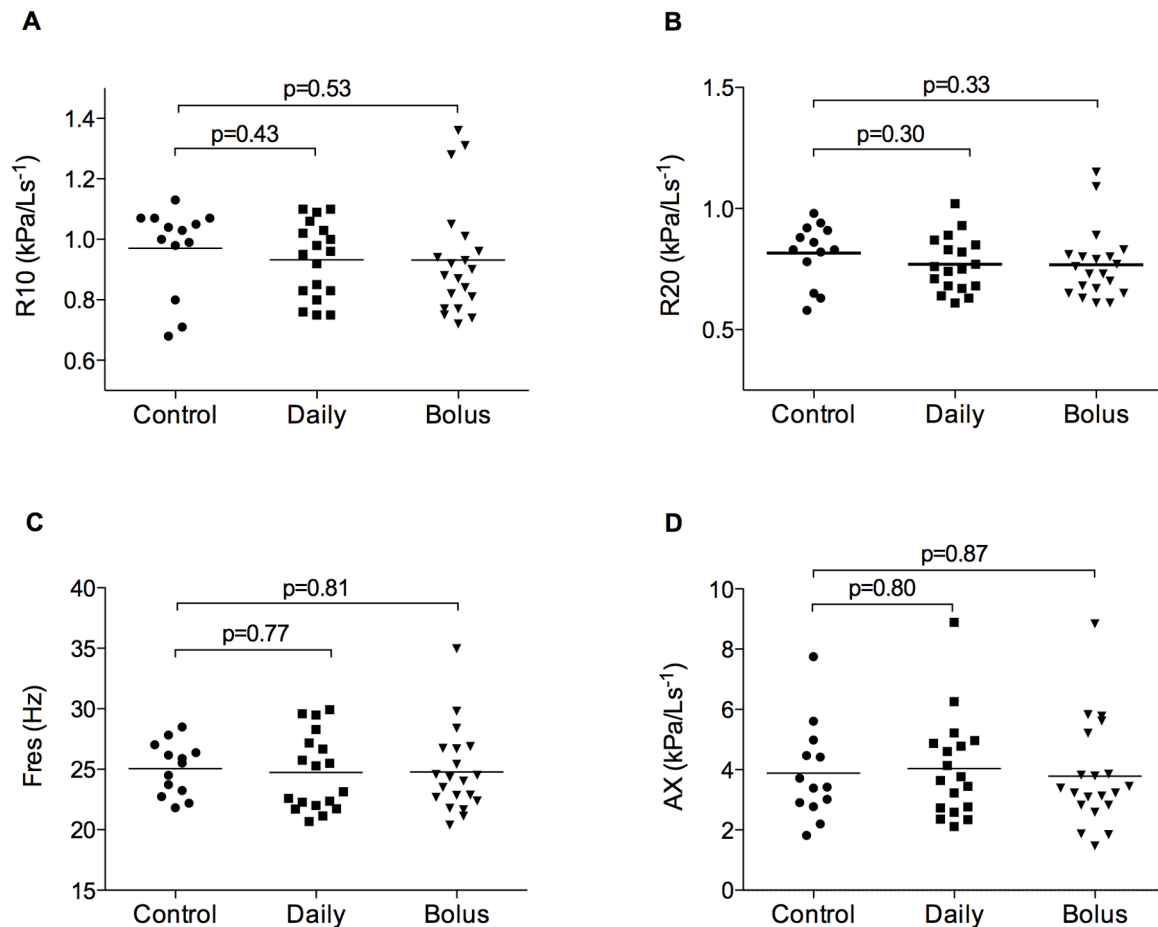


Figure 2. Baseline respiratory resistance at 10 Hz (R10, A), 20 Hz (R20, B), resonant frequency (F_{res} , C) and area under the reactance curve (AX, D) for control (n = 13), daily (n = 18), and bolus (n = 20) vitamin D groups at age three years. Horizontal bars represent means. No significant difference between groups. doi:10.1371/journal.pone.0066627.g002

participants were analysed in the treatment groups to which they were randomised.

Adjustment for multiple testing was performed using the Benjamini and Hochberg method [26]. This works by evaluating all the p-values from multiple analyses in order of significance, and determining whether each one can truly be counted as significant or not. For our analyses, there were 28 secondary clinical outcomes (14 for the daily group, and 14 for the bolus group) and the false discovery rate was controlled at 20%. All analyses were performed using SPSS version 19.0 (IBM, Chicago USA). Details of data handling and adjusted analysis are available in File S1.

Results

Participant flow is shown in Figure 1. There was one twin pregnancy of whom only the first born was included in analysis. There were 4 deaths, all in the control group: one stillbirth at 41 weeks, one death aged two days (no further details available), one death aged 16 hours from meconium aspiration and one child with congenital abnormalities died post-operatively aged 17 months. We successfully assessed the primary outcome measure in 158 (88%) of the cohort at age 3 years, between July 2010 and February 2011. The follow up was stopped after all participants had been successfully contacted or were considered lost to follow

up. 129 (72%) agreed to a review of their primary care record. Atopy was assessed by skin test for 95 children (53%), acceptable IOS data were available for 51 (28%), IgE level for 86 (48%), eNO level for 62 (34%) and eosinophil count for 80 (44%). Characteristics of the children and their mothers in each randomisation group were similar at baseline, as shown in Table 1. As previously reported [19], median cord 25(OH)D levels at delivery were significantly higher in supplemented children compared to the control group [control 17 nmol/l (interquartile range (IQR) 14–22); daily dose 26 nmol/l (IQR 17–45); $P < 0.001$; bolus dose 25 nmol/l (IQR 18–34); $P < 0.001$].

Effect of Prenatal Vitamin D Supplementation on Parent Reported Wheezing, Allergic Disease and Respiratory Infections

We found no significant difference in the primary outcome of 'wheeze ever' between treatment groups (any vitamin D vs. control Table 2, daily vs. control Table 3, bolus vs. control Table 4). There was also no difference between groups in prevalence of eczema or atopy. We found significantly increased bronchodilator use and higher prevalence of LRTI's in the offspring of the bolus group, but this did not remain significant after adjusting for multiple testing at a 20% threshold. File S1 shows complete analyses of these outcomes for the offspring of mothers with

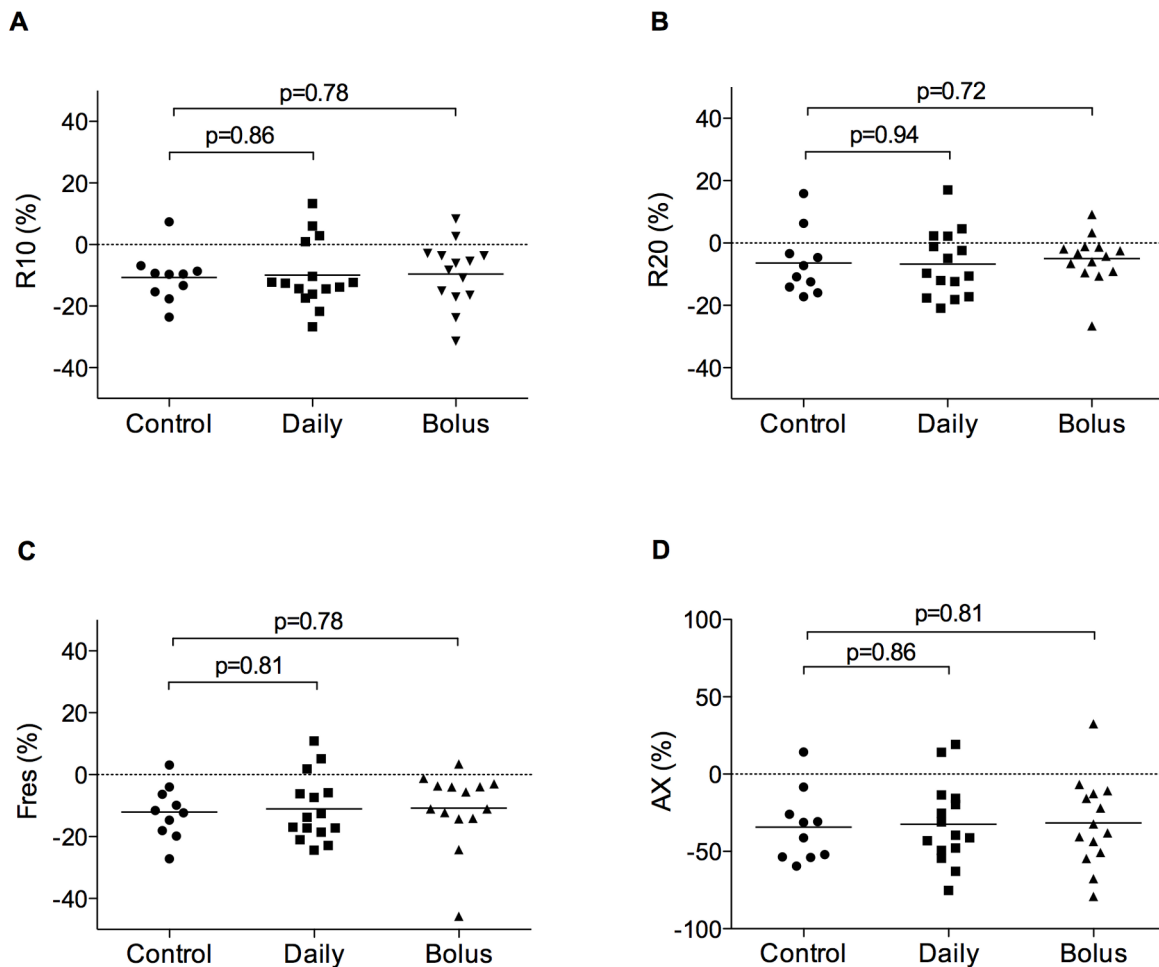


Figure 3. Bronchodilator response for respiratory resistance at 10 Hz (R10, A), 20 Hz (R20, B), resonant frequency (F_{res} , C) and area under the reactance curve (AX, D) for control (n = 10), daily (n = 15), and bolus (n = 14) vitamin D groups at age three years. Horizontal bars represent means. No significant difference between groups.
doi:10.1371/journal.pone.0066627.g003

vitamin D deficiency at baseline (any vitamin D vs. control, Table S1 in File S1, daily vs. control, Table S2 in File S1, bolus vs. control, Table S3 in File S1). We found no significant difference in the primary outcome of ‘wheeze ever’, or for any secondary outcomes between these groups.

Since 22 offspring were not followed up, sensitivity analyses were performed to determine if this influenced the result for the primary outcome. Two scenarios were considered – the first where no drop-outs wheezed and the second where all drop-outs wheezed (Table S4 in File S1). There was no significant difference between groups in wheezing prevalence for either of these scenarios.

For 122 children assessed at 3 years, cord blood 25(OH)D levels were available. We found no difference between the natural logarithm (Ln) of cord blood 25(OH)D levels in children with and without a history of wheeze, atopy, eczema, any LRTI or >4 episodes of URTI per year (Table S5 in File S1). There was also no correlation between Ln cord blood 25(OH)D level and Ln Total IgE at age three years for 68 children (Beta coefficient = -0.05, $P = 0.72$).

Effect of Prenatal Vitamin D Supplementation on Lung Function and Bronchodilator Responsiveness

Fifty-one of 180 (28%) children provided acceptable IOS data for analyses. We found no significant difference between groups in baseline respiratory resistance at 10 or 20 Hz, resonant frequency, area under the reactance curve or percentage response of these parameters to bronchodilator, when data were analysed for the two forms of prenatal vitamin D supplementation combined (Table S6 in File S1), or separately (Figures 2 and 3).

Effect of Prenatal Vitamin D Supplementation on Objective Measures of Allergic Inflammation

Total IgE levels, eNO and eosinophil counts were available for 106/180 (59%), 62/180 (34%) and 80/180 (44%) of offspring. We found no significant difference between groups in these outcomes assessed at age 3, when analysed for the two forms of prenatal vitamin D supplementation combined (Table S7 in File S1) or analysed separately (Figure 4).

Adverse Events

No adverse events related to treatment were reported in the offspring.

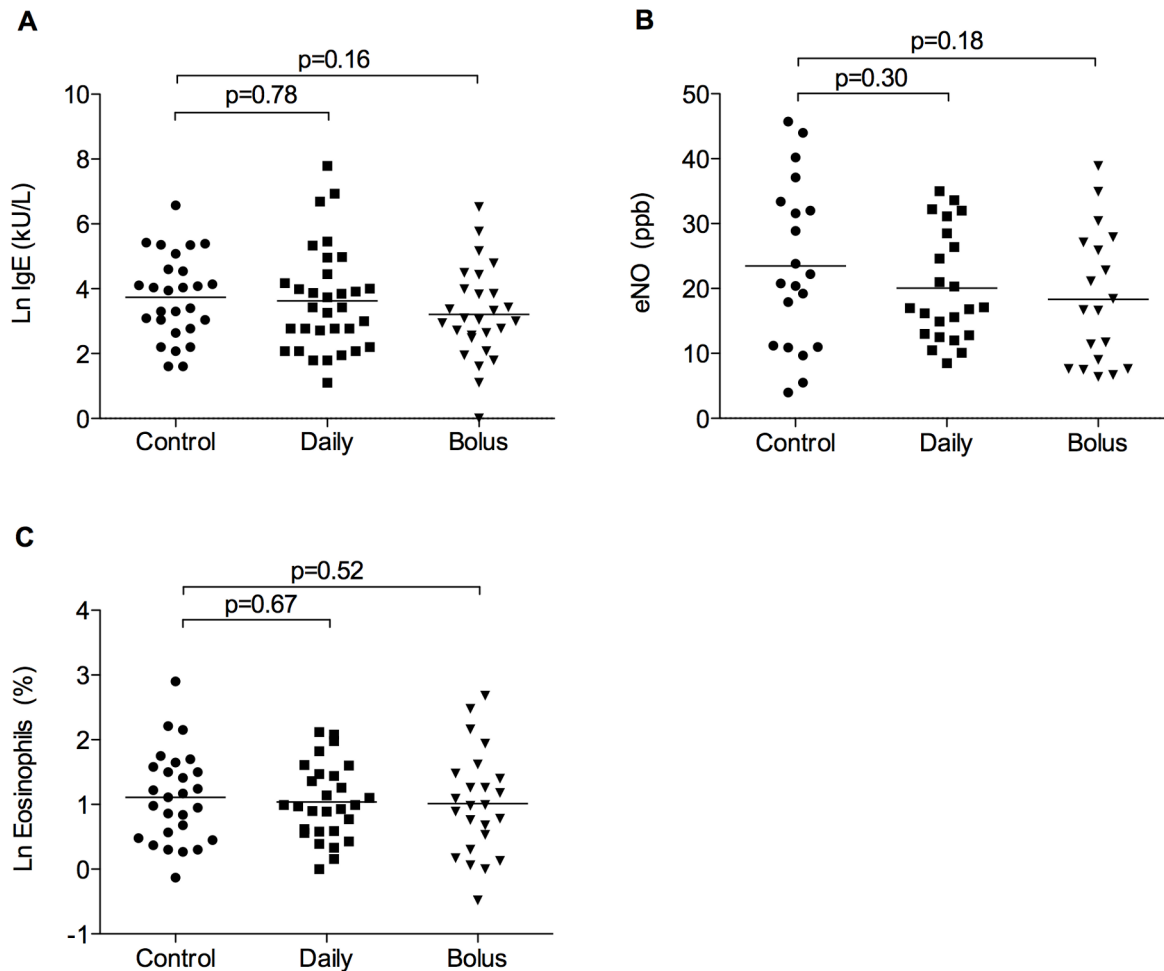


Figure 4. Measures of allergic inflammation at age three years. Ln IgE (A; n = 86), eNO (B; n = 62), and Ln eosinophil count (C; n = 90). Control versus daily and bolus vitamin D. Horizontal bars represent means. No significant difference between groups.
doi:10.1371/journal.pone.0066627.g004

Discussion

In this randomised controlled trial of vitamin D supplementation in late pregnancy in a relatively deficient mixed race population, daily supplementation with 800 IU ergocalciferol or a single bolus dose of 200,000 IU cholecalciferol from 27 weeks gestation did not prevent wheezing, allergic disease or influence measures of lung function or allergic inflammation in offspring in the first three years of life. We were unable to confidently exclude an effect on some secondary outcome measures such as atopy at 3 years, where statistical power was limited. Our results suggest that the most likely explanation for the finding of decreased wheezing risk in offspring of mothers with a higher vitamin D intake during pregnancy in observational studies [1–4], is confounding by other dietary or lifestyle factors associated with vitamin D intake.

Our study has a number of limitations. First, we relied on a subjective primary outcome measure and participants were not blind to treatment allocation, which introduces a risk of reporter bias. Investigators were, however, kept blind to treatment allocation until database lock. Moreover the results of subjective primary outcome measures were supported by the results of both our secondary objective outcome assessments and a blinded assessment of prospectively recorded healthcare records. Second, this was a small study with limited statistical power, particularly for

detecting differences in objective outcomes. Our power calculation was based on the study by Camargo et al [2], which estimated a risk reduction for recurrent wheezing from 34% to 13% comparing mothers with the highest quartile of daily vitamin D intake (median 724 IU) to those in the lowest quartile of intake (median 356 IU). However the prevalence of wheeze in our control group was 28% thus reducing the power of the study. The 95% confidence interval for our primary outcome measure was 0.49 to 1.50, so we are unable to confidently exclude effects of prenatal vitamin D supplementation on risk of wheezing, or on the development of atopy especially in mothers treated with daily vitamin D. Third, we did not assess vitamin D receptor polymorphisms or other potentially important genotypic variations. It is possible that vitamin D supplementation is effective for preventing wheezing or atopy in the presence of specific maternal or infant genotypes, as suggested by others [27]. Fourth, women from four ethnic groups were enrolled in this study, with significant differences in baseline vitamin D levels between Caucasian women and all other ethnic groups (data not shown). Previous observational studies have predominantly studied Caucasians [2]. Although randomisation in this trial was stratified by ethnicity, and we included baseline vitamin D in our adjusted model, it is possible that this source of heterogeneity could have masked positive findings. Fifth, supplementation with vitamin D

only started at 27 weeks of gestation. It is known that immune cells develop much earlier in fetal life [28], and that airway development to the respiratory bronchioles is complete by 16 weeks gestation, [29]. It may be that supplementation earlier in pregnancy, or indeed pre-conception, is necessary for protection against childhood wheezing.

Finally, we are also unable to exclude the possibility that vitamin D supplementation at a higher dose, might protect against early childhood wheezing. Although the vitamin D doses used in this trial were greater than the current recommended intake for pregnant women of 400 IU/day during pregnancy in the United Kingdom [14,17], and 600 IU in the United States [25], only a small percentage of offspring had cord 25(OH)D levels in the sufficient range (13% daily group, 3% bolus group). It has recently been shown that doses of 4000 IU of vitamin D daily are safe and effective for short term treatment of vitamin D deficiency during pregnancy [30], although the long term effects on child health have not been studied. Two on-going trials, the Vitamin D Prenatal Asthma Reduction Trial (NCT00920621), and ABCvitaminD (NCT00856947) are specifically exploring the effects of earlier and higher dose prenatal vitamin D supplementation on child health.

Two recent observational studies that documented prenatal vitamin D status by measuring cord blood 25(OH)D concentration, found relationships with allergic sensitisation and/or wheezing in early childhood [8,31]. When we analysed our own data on cord blood 25(OH)D concentration and wheezing, atopy, total IgE level and frequency of respiratory infection in the first three years, no significant relationship was found. While our interventions did result in at least 50% higher cord blood 25(OH)D concentrations compared with no treatment, cord blood levels were still significantly lower in the intervention groups than in these studies. The results of our randomised controlled trial do not support a strong causal relationship between low prenatal vitamin D status within the deficient/insufficient range and increased risk of allergic sensitization or wheezing in early childhood. We are unable to confidently exclude a similar relationship in more vitamin D sufficient populations [8].

The findings of our study are specific to a population of vitamin D deficient women (half had baseline 25(OH)D levels below 25 nmol/L) and two specific forms of prenatal vitamin D supplementation. The doses of supplementation were chosen

pragmatically, were not stratified based on individual vitamin D status or genotype, and response to treatment was not monitored before delivery. The study population was not selected for allergy or asthma risk and, as a mixed race urban population, is representative of many populations of pregnant women worldwide [32]. Although these findings cannot automatically be generalised to other populations of pregnant women, one might expect prenatal vitamin D supplementation to show its clearest clinical effects in a deficient population such as this.

In summary we found no evidence for a protective effect of prenatal vitamin D supplementation from 27 weeks gestation on childhood wheezing, allergic disease, lung function or markers of airway inflammation in the first 3 years of life. Given the modest effects on cord blood vitamin D achieved by these interventions, the safety and efficacy of higher dose prenatal vitamin D supplementation strategies need to be explored.

Supporting Information

Checklist S1 CONSORT Checklist.
(DOC)

File S1 Supporting Information.
(DOC)

Protocol S1 Trial Protocol.
(DOCX)

Acknowledgments

We are grateful to our research nurses Heather Hanna, Katherine Phillips, Bridget Catterall, Louise Cross and Imperial College London undergraduates Megan Griffiths, Upama Bannerjee, Klara Vosicka, Anjali Thakari and Harisita Patel for their help with follow up assessments, to the late Michael Goldman for his advice on IOS quality control and to all the children and families for contributing their time to participate in this study.

Author Contributions

Conceived and designed the experiments: RJB JOW CJG SOS ARM. Performed the experiments: STG SP. Analyzed the data: STG RH JCK. Contributed reagents/materials/analysis tools: JS SR CY. Contributed to the writing of the paper: CG AM SR CY SP JK JS RLH SS JW. Wrote the paper: STG RB.

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CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported in section
Title and abstract			
	1a	Identification as a randomised trial in the title	Title
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	Abstract
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	Introduction
	2b	Specific objectives or hypotheses	Introduction
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	Study design
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	N/A
Participants	4a	Eligibility criteria for participants	Study subjects
	4b	Settings and locations where the data were collected	Study subjects
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Study design
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	Outcome assessments
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	Statistical analysis
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Study design
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Study design
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Study design
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Study design

Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Study design
	11b	If relevant, description of the similarity of interventions	N/A
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	Statistical analysis
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Supporting information – statistical methods
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Figure 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	Figure 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Study subjects
	14b	Why the trial ended or was stopped	Study subjects
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Table 2
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Table 2
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Table 2
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Tables 3-4 and Supporting Information
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Adverse effects
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Discussion
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Discussion
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Discussion
Other information			

Registration	23	Registration number and name of trial registry	Abstract
Protocol	24	Where the full trial protocol can be accessed, if available	Submitted with manuscript
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Details submitted online

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming; for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Prenatal vitamin D supplementation and child respiratory health: a randomised controlled trial

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Supporting Information

Methods

Impulse oscillometry (IOS) quality control

Impulse oscillometry (IOS) was performed in accordance with ERS/ATS task force guidelines [1], using the Jaeger IOS system (Wurzburg, Germany), before and 15 minutes after inhalation of 400mcg salbutamol sulphate via a large volume spacer (Volumatic, Allen and Hanbury, Middlesex, UK). In addition, the following online quality assurance protocol was adopted (personal communication from the late Michael Goldman): Support of the cheeks to ensure no movement of the mouth (chewing, talking etc.) and thereby minimise upper-airway compliance; observation of the tidal volume time-based trace to ensure it was stable, free from drift with no hyper- or hypo- ventilation; observation of the impedance at 5Hz trace to ensure consistency throughout the measurement; ensuring no obvious spikes in impedance due to swallowing, glottic closure or cough were present. If these criteria were not met, the measurement was stopped and the technique reviewed.

On completion of all data collection for the study, two trained operators (STG, JCK) assessed all readings. Essential acceptance criteria for a reading to be included in analyses were as follows: at least 10 seconds in duration with a minimum of four tidal breaths; stable, regular tidal breaths, free from drift; no obvious abnormalities, recording appears 'physiologically plausible' (i.e. physiological implausible data identified and excluded); coherence >0.4 at 5Hz and >0.7 at 10 Hz. Results were

rejected if there was evidence of tongue position artefact, (noted by a parallel increase in resistance at all frequencies).

For each child, the reported result was the mean of 3 to 5 “acceptable” (as defined above) measurements [1].

Exhaled nitric oxide equipment

Exhaled nitric oxide was measured using an offline, tidal breathing technique in accordance with the principles recommended by the ATS/ERS [2] as follows: measurements were made before other lung function tests and before administration of salbutamol; subjects breathed through a two-way non-rebreathing mask with partitioned nose and mouth chambers (Series 7975, PED SM M/F Mask Yshp, Han Rudolph, Kansas City, USA); subjects breathed normally from a source of nitric oxide free air (<5ppb) (NO scrubber, Sievers reorder P/N: AFL 01410-01, NORTH part no. N7500-2, USA, 40700842 rev E) and exhaled against 5cm H₂O resistance (Model 7100R, flow range 0 to 2 L/sec, SN 710-1227, Han Rudolph, Kansas City, USA) to prevent contamination of the sample with nasal NO; the initial 10 exhalations were discarded to allow for washout of dead space of the lungs; [3] 5 exhalations were collected into a NO-inert bag (Maximum pressure 12 cmH₂O, Series 6000, P/N CR1735, 10x10 outside measurement, 1L capacity, Hans Rudolph, Kansas City, USA) connected via a three way valve (2100 series 3 way stopcock, Han Rudolph, Kansas City, USA); additional samples were collected after 30 seconds of tidal breathing in ambient air; 3 samples were collected ideally, with the median value of at least two measurements was used for data analysis; samples were analysed within 1 hour of collection using the NIOX flex (Aerocrine, Sweden)

with the following settings - 7 seconds collection time, flow rate of 50mls/s [3], calculation segment 50% to 100% of measurement.

Statistical methods

All data were double entered onto a database and underwent range checks, data cleaning and checking with source documents before database lock. A statistical analysis plan was agreed between all the investigators prior to unblinding. Where necessary data were natural log transformed for analysis. All analyses presented here were specified in the statistical analysis plan, and no post-hoc analyses were undertaken.

For adjusted analyses, treatment effects were adjusted for variables thought to influence risk of wheezing or postnatal vitamin D status. These were mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the household, age mother left full-time education, baseline concentration of 25(OH)D in maternal blood. Analyses were based on all participants with complete data.

91 **Tables**

92 Table S1. Clinical outcomes at age three years in offspring of mothers with baseline 25(OH)D <25nmol/L.

93 Combined vitamin D versus control.

	Control	Combined vitamin D	RR	OR	<i>P</i>	aOR [†]	<i>P</i>
	(%)	(%)	(95% CI)	(95% CI)		(95% CI)	
Wheeze ever	7/24 (29)	11/47 (23)	0.80 (0.36, 1.80)	0.74 (0.25, 2.25)	0.60	0.55 (0.15, 2.05)	0.38
Recurrent wheezing	4/24 (17)	8/47 (17)	1.02 (0.34, 3.05)	1.03 (0.28, 3.82)	0.97	0.97 (0.23, 4.02)	0.97
Wheezing in the last year	5/24 (21)	8/47 (17)	0.82 (0.30, 2.23)	0.78 (0.23, 2.71)	0.69	0.68 (0.17, 2.67)	0.58
Wheeze with positive API	4/24 (17)	6/46 (13)	0.78 (0.24, 2.51)	0.75 (0.19, 2.96)	0.68	0.68 (0.15, 3.07)	0.62
Any bronchodilator use	2/24 (8)	9/44 (21)	2.46 (0.58, 10.46)	2.83 (0.56, 14.33)	0.20	2.76 (0.37, 20.54)	0.32
Eczema ever	8/24 (33)	14/43 (33)	0.98 (0.48, 1.99)	0.97 (0.33, 2.79)	0.95	0.51 (0.15, 1.80)	0.30
Eczema in the last year	4/24 (17)	10/44 (23)	1.36 (0.48, 3.89)	1.47 (0.41, 5.31)	0.56	1.09 (0.24, 4.99)	0.91
Atopy	4/13 (31)	4/27 (15)	0.48 (0.14, 1.63)	0.39 (0.08, 1.91)	0.24	-	-
Allergic rhinitis	3/24 (13)	4/44 (9)	0.73 (0.18, 2.99)	0.70 (0.14, 3.42)	0.66	0.44 (0.06, 3.47)	0.43

Food allergy diagnosis	2/24 (8.3)	5/44 (11.4)	1.36 (0.29, 6.51)	1.41 (0.25, 7.88)	0.69	0.06 (0.001, 3.80)	0.18
> 4 URTI/year	3/24 (13)	12/44 (27)	2.18 (0.68, 6.99)	2.63 (0.66, 10.43)	0.16	2.15 (0.45, 10.32)	0.34
LRTI ever	7/24 (29)	13/43 (30)	1.04 (0.48, 2.24)	1.05 (0.35, 3.15)	0.93	0.97 (0.27, 3.51)	0.96
Primary health care record							
Recurrent wheeze	1/17 (6)	5/29 (17)	2.93 (0.37, 23.05)	3.33 (0.36, 31.26)	0.27	-	-
Eczema	3/17 (18)	6/29 (21)	1.17 (0.34, 4.09)	1.22 (0.26, 5.67)	0.80	0.80 (0.10, 6.56)	0.83
Food allergy	0/17 (0)	1/29 (3)	-	-	-	-	-

94 RR=risk ratio, OR=unadjusted odds ratio, aOR= adjusted odds ratio, API = Asthma predictive index, URTI = upper

95 respiratory tract infection, LRTI = lower respiratory tract infection

96 †Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive

97 breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the

98 household, age mother left full-time education and baseline concentration of 25(OH)D in maternal blood. n=139 for

99 primary outcome measure

100

101

102 Table S2. Clinical outcomes at age three years in offspring of mothers with baseline 25(OH)D <25nmol/L.

103 Daily vitamin D versus control.

	Control	Daily vitamin D	RR	OR	<i>P</i>	aOR [†]	<i>P</i>
	(%)	(%)	(95% CI)	(95% CI)		(95% CI)	
Wheeze ever	7/24 (29.2)	4/25 (16)	0.60 (0.20, 1.77)	0.46 (0.12, 1.85)	0.27	0.23 (0.04, 1.33)	0.10
Recurrent wheezing	4/24 (16.7)	3/25 (12.0)	0.72 (0.18, 2.89)	0.68 (0.14, 3.43)	0.64	0.40 (0.06, 2.76)	0.35
Wheezing in the last year	5/24 (20.8)	3/25 (12.0)	0.58 (0.15, 2.15)	0.52 (0.11, 2.46)	0.40	-	-
Wheeze with positive API	4/24 (16.7)	3/25 (12.0)	0.72 (0.18, 2.89)	0.68 (0.14, 3.43)	0.64	0.35 (0.04, 2.96)	0.34
Any bronchodilator use	2/24 (8.3)	4/25 (16.0)	1.92 (0.39, 9.54)	2.10 (0.35, 12.67)	0.42	2.12 (0.12, 36.28)	0.60
Eczema ever	8/24 (33.3)	7/24 (29.2)	0.88 (0.38, 2.03)	0.82 (0.24, 2.80)	0.76	0.16 (0.02, 1.21)	0.08
Eczema in the last year	4/24 (16.7)	5/25 (20.0)	1.20 (0.37, 3.94)	1.25 (0.29, 5.35)	0.76	0.59 (0.09, 4.02)	0.59
Atopy	4/13 (30.8)	1/14 (7.1)	0.23 (0.03, 1.82)	0.17 (0.02, 1.82)	0.11	-	-
Allergic rhinitis	3/24 (12.5)	3/25 (12.0)	0.96 (0.21, 4.30)	0.96 (0.17, 5.27)	0.96	0.68 (0.05, 9.10)	0.77
Food allergy diagnosis	2/24 (0.0)	3/25 (8.0)	1.44 (0.26, 7.88)	1.50 (0.23, 9.87)	0.67	-	-

> 4 URTI/year	3/24 (12.5)	6/25 (24.0)	1.92 (0.54, 6.82)	2.21 (0.48, 10.09)	0.30	1.03 (0.04, 24.96)	0.99
LRTI ever	7/24 (29.2)	6/24 (25.0)	0.86 (0.34, 2.18)	0.81 (0.23, 2.90)	0.75	0.52 (0.10, 2.64)	0.43
Primary health care record							
Recurrent wheeze	1/17 (5.9)	2/16 (12.5)	2.13 (0.21, 21.24)	2.29 (0.19, 28.00)	0.51	-	-
Eczema	3/17 (17.6)	2/16 (12.5)	0.71 (0.14, 3.70)	0.67 (0.10, 4.62)	0.68	-	-
Food allergy	0/17 (0.0)	1/16 (6.3)	-	-	-	-	-

104 RR= Risk ratio, OR=unadjusted odds ratio, aOR= adjusted odds ratio, API = Asthma predictive index, URTI = upper

105 respiratory tract infection, LRTI = lower respiratory tract infection

106 †Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy,

107 exclusive breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number

108 of children in the household, age mother left full-time education and baseline concentration of 25(OH)D in

109 maternal blood.

110 Table S3. Clinical outcomes at age three years in offspring of mothers with baseline 25(OH)D <25nmol/L.

111 Bolus vitamin D versus control.

	Control	Bolus vitamin D	RR	OR	<i>P</i>	aOR [†]	<i>P</i>
	(%)	(%)	(95% CI)	(95% CI)		(95% CI)	
Wheeze ever	7/24 (29.2)	7/22 (31.8)	1.09 (0.46, 2.61)	1.13 (0.32, 3.98)	0.85	1.32 (0.23, 7.63)	0.76
Recurrent wheezing	4/24 (16.7)	5/22 (22.7)	1.36 (0.42, 4.44)	1.47 (0.34, 6.37)	0.61	9.95 (0.49, 203.97)	0.14
Wheezing in the last year	5/24 (20.8)	5/22 (22.7)	1.09 (0.36, 3.27)	1.12 (0.28, 4.54)	0.88	2.24 (0.29, 17.58)	0.44
Wheeze with positive API	4/24 (16.7)	3/21 (14.3)	0.86 (0.22, 3.40)	0.83 (0.16, 4.24)	0.83	2.19 (0.15, 31.74)	0.57
Any bronchodilator use	2/24 (8.3)	5/19 (26.3)	3.16 (0.69, 14.52)	3.93 (0.67, 23.10)	0.11	5.52 (0.36, 83.79)	0.22
Eczema ever	8/24 (33.3)	7/19 (36.8)	1.11 (0.49, 2.50)	1.17 (0.33, 4.12)	0.81	0.66 (0.11, 3.88)	0.65
Eczema in the last year	4/24 (16.7)	5/19 (26.3)	1.58 (0.49, 5.08)	1.79 (0.41, 7.86)	0.44	0.21 (0.001, 32.76)	0.54
Atopy	4/13 (30.8)	3/13 (23.1)	0.75 (0.21, 2.71)	0.68 (0.12, 3.87)	0.66	-	-
Allergic rhinitis	3/24 (12.5)	1/19 (5.3)	0.42 (0.05, 3.73)	0.39 (0.04, 4.07)	0.42	-	-
Food allergy diagnosis	2/24	2/19	1.26 (0.20, 8.16)	1.29 (0.17, 10.15)	0.81	-	-

> 4 URTI/year	3/24 (12.5)	6/19 (31.6)	2.53 (0.72, 8.81)	3.23 (0.69, 15.20)	0.13	11.76 (0.43, 323.56)	0.15
LRTI ever	7/24 (29.2)	7/19 (36.8)	1.26 (0.54, 2.98)	1.42 (0.39, 5.11)	0.59	1.94 (0.36, 10.46)	0.44
Primary health care record							
Recurrent wheeze	1/17 (5.9)	3/13 (23.1)	3.92 (0.46, 33.52)	4.80 (0.44, 52.76)	0.17	-	-
Eczema	3/17 (17.6)	4/13 (30.8)	1.74 (0.47, 6.47)	2.07 (0.37, 11.53)	0.40	-	-
Food allergy	0/17	0/13	-	-	-	-	-

112 RR=Risk ratio, aOR= adjusted odds ratio, API = Asthma predictive index, URTI = upper respiratory tract infection,

113 LRTI = lower respiratory tract infection

114 †Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive

115 breast-feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in

116 the household, age mother left full-time education and baseline concentration of 25(OH)D in maternal blood.

117 Table S4. Sensitivity analysis using imputation for missing primary outcome data.

	Control	Treatment group	RR	OR	P
	(%)	(%)	(95% CI)	(95% CI)	
Scenario assuming no drop-outs wheezed					
Control vs Combined	14/60 (23)	26/120 (22)	0.93 (0.52, 1.64)	0.91 (0.43, 1.90)	0.80
Control vs Daily	14/60 (23)	11/60 (18)	0.79 (0.39, 1.59)	0.74 (0.30, 1.79)	0.50
Control vs Bolus	14/60 (23)	15/60 (25)	1.07 (0.57, 2.02)	1.10 (0.48, 2.53)	0.83
Scenario assuming all drop-outs wheezed					
Control vs Combined	24/60 (40)	38/120 (32)	0.79 (0.53, 1.19)	0.70 (0.37, 1.32)	0.27
Control vs Daily	24/60 (40)	15/60 (25)	0.63 (0.37, 1.07)	0.50 (0.23, 1.09)	0.08
Control vs Bolus	24/60 (40)	23/60 (38)	0.96 (0.61, 1.50)	0.93 (0.45, 1.94)	0.85

118 RR=Risk ratio, OR=Odds ratio, CI= Confidence intervals

120 Table S5. Cord vitamin D levels and clinical outcomes at age 3 years

Outcome	n	Outcome absent Mean ± SD	n	Outcome present Mean ± SD	Unadjusted Mean difference (95% CI)	p	Adjusted ¹ mean difference (95% CI)	p
Wheeze	88	3.20 ± 0.52	34	3.07 ± 0.43	-0.13 (-0.33, 0.07)	0.19	-0.07 (-0.26, 0.11)	0.43
Atopy	59	3.19 ± 0.49	14	3.28 ± 0.44	0.08 (-0.20, 0.37)	0.56	0.10 (-0.17, 0.37)	0.47
Eczema	82	3.14 ± 0.51	34	3.17 ± 0.48	0.03 (-0.23, 0.17)	0.74	0.05 (-0.13, 0.23)	0.56
Any LRTI	84	3.17 ± 0.52	31	3.16 ± 0.45	-0.007 (-0.20, 0.22)	0.95	-0.10 (-0.29, 0.09)	0.30
> 4 URTI/year	95	3.19 ± 0.52	22	3.03 ± 0.41	-0.16 (-0.40, 0.07)	0.17	-0.11 (-0.32, 0.11)	0.33

121 LRTI (Lower respiratory tract infection), URTI (Upper respiratory tract infection).

122 ¹Adjusted for treatment group, mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four
123 months, any parental allergic history, any child vitamin supplementation, number of children in the household, and age mother left full-time education.

124

125 Table S6. Lung function at age three years. Combined vitamin D groups versus control.

Outcome	n	Control	n	Combined	Unadjusted Mean	<i>P</i>	Adjusted Mean	<i>P</i>
		Mean ± SD		Vitamin D	difference		difference [†]	
				Mean ± SD	(95% CI)		(95% CI)	
R10 (kPa/(L/s))	13	0.97 ± 0.14	38	0.93 ± 0.16	-0.04 (-0.14, 0.06)	0.44	-0.05 (-0.17, 0.06)	0.38
R20 (kPa/(L/s))	13	0.82 ± 0.12	38	0.77 ± 0.13	-0.05 (-0.13, 0.04)	0.26	-0.05 (-0.14, 0.05)	0.31
F _{res} (Hz)	13	25.0 ± 2.17	38	24.8 ± 3.28	-0.28 (-2.25, 1.68)	0.77	-0.49 (-2.66, 1.68)	0.65
AX (kPa/L)	13	3.89 ± 1.59	38	3.90 ± 1.69	0.02 (-1.06, 1.10)	0.97	-0.19 (-1.35, 0.97)	0.74
R10% (%)	10	-10.66 ± 8.12	29	-9.74 ± 10.50	-0.91 (-8.33, 6.50)	0.80	-0.55 (-8.50, 7.38)	0.89
R20% (%)	10	-6.37 ± 10.52	29	-5.89 ± 9.31	-0.48 (-7.62, 6.67)	0.89	-1.36 (-8.49, 5.78)	0.70
F _{res} % (%)	10	-12.07 ± 8.59	29	-10.92 ± 11.22	-1.15 (-9.05, 6.76)	0.77	0.96 (-8.70, 10.61)	0.84
AX% (%)	10	-34.20 ± 23.30	29	-31.94 ± 26.92	-2.26 (-21.64, 17.12)	0.81	3.80 (-21.39, 28.99)	0.76

126 R10 and R20 = resistance at 10 and 20 Hz, F_{res} = resonant frequency, AX = area under the reactance curve. Variables labelled ‘post’

127 refer to repeat lung function assessment taken 15 minutes after 400 mcg inhaled salbutamol. All lung function data were acquired

128 using impulse oscillometry.

129 †Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-
130 feeding to four months, any parental allergic history, any child vitamin supplementation, number of children in the household, age
131 mother left full-time education, baseline concentration of 25(OH)D in maternal blood

132 Table S7. Measures of allergic inflammation at age three years. Combined vitamin D groups versus control.

Outcome	n	Control	n	Combined vitamin D	Unadjusted mean	<i>P</i>	Adjusted mean	<i>P</i>
		Mean ± SD		Mean ± SD	difference		difference [†]	
					(95% CI)		(95% CI)	
Ln IgE	27	3.74 ± 1.29	59	3.44 ± 1.52	-0.30 (-0.97, 0.37)	0.38	-0.39 (-1.15, 0.36)	0.30
IgE [‡] (kU/L)	27	42.1 ± 3.62	59	31.20 ± 4.57	0.74 (0.38, 1.45)	0.38	0.68 (0.32, 1.43)	0.30
eNO (ppb)	20	23.50 ± 12.71	42	19.30 ± 9.28	-4.18 (-9.88, 1.52)	0.15	-3.34 (-9.43, 2.75)	0.28
Ln Eos	27	1.11 ± 0.70	53	1.01 ± 0.69	-0.10 (-0.43, 0.22)	0.53	-0.25 (-0.61, 0.11)	0.18
Eos [§] (%)	27	3.03 ± 2.01	53	2.75 ± 1.99	0.90 (0.65, 1.25)	0.53	0.78 (0.54, 1.12)	0.18

133 eNO = exhaled nitric oxide, Eos = eosinophil count as a % of total white cell count

134 [†]Model adjusted for mother's ethnic group, presence of household smokers, maternal smoking in pregnancy, exclusive breast-feeding to four months, any
135 parental allergic history, any child vitamin supplementation, number of children in the household, age mother left full-time education, baseline
136 concentration of 25(OH)D in maternal blood.

137 [‡]Ln IgE result transformed into geometric mean ± SD and multiplicative mean difference with 95% CI138 [§]Ln Eos result transformed into geometric mean ± SD and multiplicative mean difference with 95% CI

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148

149

**Effects of prenatal vitamin D supplementation on respiratory and allergic phenotypes, and bone density in
the first three years of life**

Version 3.1

21st October 2010

MAIN SPONSOR: Imperial College London

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STUDY COORDINATION CENTRE: Imperial College London

REC reference: 10/H0712/13

Protocol authorised by

Name and role

Date

Signature

Robert Boyle, Chief investigator

Stephen Goldring, Co-investigator

Professor Warner, Co-investigator

This protocol describes the research study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

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1. Project Management

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Funder: Asthma UK Grant 09/036

- TITLE** Effects of prenatal vitamin D supplementation on respiratory and allergic phenotypes, and bone density in the first three years of life
- DESIGN** Prospective follow up of participants of a randomised controlled trial
- AIMS** To evaluate the effect of prenatal vitamin D supplementation on health outcomes in the first three years of life
- OUTCOME MEASURES** The primary outcome is the presence or absence of wheezing in the first three years of life. Secondary outcomes include wheezing frequency, atopic sensitisation, allergic disease, bone density, lung function, healthcare utilisation, and markers of immune regulation.
- POPULATION** Our study population is the offspring of 180 women who attended antenatal clinic at St Mary's hospital in the third trimester of pregnancy and were enrolled in a randomised trial of two methods of vitamin D supplementation(1). (Approved by St Mary's Ethics committee: Ref 06/Q0702/172) In this trial 60 women received 800IU of vitamin D daily from 27 weeks gestation, 60 women received a single injection of 120,000IU of vitamin D at 27 weeks gestation, and 60 women received no vitamin D supplementation. The children were born between 1st June 2007 and 21st November 2007 and we will study this population of children at age three years.
- ELIGIBILITY** All children whose mothers were enrolled in the prenatal vitamin D study.
- DURATION** The study involves a single assessment at age 3 years.
- KEYWORDS** Vitamin D; asthma; child; allergy; wheeze

Asthma is the commonest chronic disease of childhood in the United Kingdom. In a recent study the prevalence of asthma in the UK was 20.9% in children aged 6-7 years, and 24.7% in young people aged 13-14 years old (2). Asthma is not curable once it has developed, and in most cases has its origins in early childhood. There is a justified focus on understanding the early life origins of asthma, with a view to developing primary prevention strategies(3).

One possible way to prevent asthma from developing in young children is to give pregnant women vitamin D supplements. There is a plausible rationale for the efficacy of such an approach (4). Four separate cohort studies have found vitamin D intake during pregnancy to be inversely associated with risk of early childhood wheezing in the offspring (5-8). In a North American study, mothers with the highest quartile of vitamin D intake during pregnancy had children with reduced odds for recurrent wheeze (OR 0.39, $P < 0.001$) (5). There was a decrease in these odds of 19% for each 100IU/day increase in estimated daily vitamin D intake during pregnancy, whether such intake was dietary or through supplements. These findings were robust in analyses controlling for a wide variety of potential confounding variables. A separate study in Scotland had remarkably similar findings – vitamin D intake during pregnancy was estimated at 32 weeks gestation. Those mothers with the highest quartile of vitamin D intake had children with decreased risk of persistent wheeze at age 5 compared with those in the lowest quartile (OR 0.33, $P = 0.01$) (6). A third prospective cohort study in Japan of 763 mother infant pairs also described an inverse relationship between maternal vitamin D intake during pregnancy and wheezing at 2 years, with children whose mothers had consumed more than 4.3 micrograms/day of vitamin D having a significantly reduced risk of wheeze and eczema with adjusted odds ratio and 95% confidence intervals of 0.64 [0.43-0.97] and 0.63 [0.41-0.98] respectively (7). Finally, in a cohort study of 1669 children from Finland, maternal intake of vitamin D from food was negatively related to risk of asthma and allergic rhinitis at 5 years of age, independent of several potential confounders (8).

The Vitamin D receptor is expressed by dendritic cells, T cells and activated B cells, and the immunological effects of vitamin D are diverse, but include an important role in promoting regulatory T cell IL-10 secretion (9). The enzyme for conversion of vitamin D to its active form is expressed on dendritic cells, suggesting a possible role for this vitamin in programming the immune response to environmental antigens (10). Vitamin D also plays an important role in fetal lung development (11-12). If the relationship between vitamin D intake in pregnancy and childhood respiratory outcome is causal then vitamin D may promote respiratory health through either its effects on immune development, its effects on lung development or both.

In view of these associations between vitamin D deficiency and respiratory health, some authors have proposed that vitamin D deficiency may explain the rise in asthma prevalence seen in the past 50 years (13). Certainly vitamin D deficiency is widespread in pregnant women in both the United Kingdom and United States (14) (15). Some of the epidemiology of vitamin D deficiency closely parallels that of asthma epidemiology – for example vitamin D deficiency is common in developed countries, in African-Americans, in recent immigrants to developed countries, in obese individuals and in high latitude countries (4). We are therefore presented with the tantalising prospect of an easily reversible major risk factor for asthma. However the potential role of vitamin D in preventing asthma development is controversial, and some observational studies contradict those cited above(16-18). There is a need for a definitive study to evaluate whether vitamin D supplementation during pregnancy can reduce asthma risk or not.

Prenatal vitamin D also plays a role in postnatal bone mineral accrual (19). In a longitudinal cohort study from Finland (20), risk of hip fracture in adulthood was increased in those born short, and in those who had a low rate of childhood growth, suggesting bone growth is sensitive to adverse intrauterine and early childhood influences (19). Furthermore, reduced concentrations of maternal vitamin D during pregnancy were significantly associated with reduced whole body and lumbar spine bone mineral content in children at 9 years of age in an observational cohort study of children from Southampton (21). In this study, the only two predictors of maternal vitamin D status were estimated sunlight exposure and maternal vitamin D supplementation, which both correlated with whole body bone mineral content at age 9 years in the offspring. Interestingly, the same group have shown that infant feeding practices themselves do not influence bone mass at 4 years of age (22), strengthening the argument that maternal vitamin D status during pregnancy is crucial for programming long term skeletal health. As such, intervention studies to explore the effect of prenatal vitamin D supplementation on bone mineral accrual in children are needed.

4. Research questions

Does prenatal vitamin D supplementation reduce the risk of wheezing illness, atopic disease and healthcare utilisation in offspring at three years of age?

Does prenatal vitamin D supplementation increase bone area and bone density in children at three years of age?

5. Hypothesis

In an ethnically mixed population of women attending for antenatal care in an inner city hospital, vitamin D supplementation during the last trimester of pregnancy promotes fetal lung and immunoregulatory development, causing reduced risk of wheezing illness, atopic disease and healthcare utilisation in offspring at three years of age.

6. Study objectives

The primary objective is to measure the effect of prenatal Vitamin D supplementation on the risk of wheezing in children during the first three years of life.

The secondary objectives of this study are to measure the effect of prenatal vitamin D supplementation at three years of age on the following areas of child health:

1. Lung function
2. Allergic disease (Asthma, eczema, rhinitis, food allergy, atopic sensitisation)
3. Bone density and growth parameters
4. Immune development (Measurement of immune regulation markers)
5. Primary and secondary healthcare utilisation

A further objective is to measure the effect of vitamin D supplementation during pregnancy on maternal vitamin D levels three years after delivery.

7. Study design

A randomised trial of two different methods of vitamin D supplementation during the last trimester of pregnancy has recently been completed by co-investigator Dr Robinson (1). This trial evaluated the relative effectiveness of two different methods of vitamin D supplementation during pregnancy on increasing vitamin D levels at delivery in both the mother and the newborn. 180 women were recruited from an ethnically diverse population attending antenatal clinics at St Mary's Hospital, Imperial College Healthcare NHS Trust. After informed consent, women at 27 weeks gestation were randomly allocated into one of three arms: daily supplementation with 800IU of vitamin D (n=60), a single injection of 120,000IU vitamin D (n=60), and no intervention (n=60). The children were born between 1st June 2007 and 21st November 2007. The results of this study have been published and show statistically significant increases in both maternal and neonatal 25-hydroxyvitamin D levels in the intervention groups (1). For the recruited women and their newborns, this trial ended at delivery.

This follow up study aims to assess the effect of pre-natal vitamin D supplementation during pregnancy on child health by inviting the mothers who participated in the above trial to bring their children to Imperial College Paediatric Research Unit at St. Mary's Hospital for a single clinical assessment. We will carry out the assessments at three years of age.

Each child's assessment will include:

1. Clinical history using a structured questionnaire
2. Clinical examination including growth assessment and assessment of skin darkness
3. Lung function tests
4. Skin tests
5. Nasal secretion sampling for inflammatory markers
6. Blood test for immune function markers and RNA/ DNA extraction for future genetic and epigenetic work
7. DXA (Dual-energy X-ray Absorptiometry) scan to measure bone size and density
8. Buccal (mouth) swab for DNA in those who do not consent to a blood test

In addition, each mother will be invited to have the following investigations

1. Assessment of skin darkness
2. Blood test to assess their vitamin D status
3. Blood test for DNA extraction with a view to future testing to evaluate whether certain genetic polymorphisms modify any effects of prenatal vitamin D supplementation on outcomes in mother or child.

4. Buccal (mouth) swab for DNA in those that do not consent to a blood test

9. Primary outcome measure

1. Percentage (%) of children with any wheezing episode in the first 3 years of life.

10. Secondary outcome measures

1. % of children with any wheezing episode in the preceding 12 months
2. % of children using inhaled bronchodilators in the last 12 months
3. Total number of all wheezing episodes since birth
4. % of children with doctor diagnosed asthma
5. % of children with doctor diagnosed eczema either prior to or at the time of the assessment
6. % of children with doctor diagnosed rhinitis either prior to or at the time of the assessment
7. % of children with doctor diagnosed food allergy
8. Total number of upper and lower respiratory tract infections since birth
9. % of children with positive skin prick test responses to one or more of a panel of common allergens
10. Pulmonary airflow resistance at a range of frequencies using impulse oscillometry
11. Change in pulmonary airflow resistance after salbutamol inhalation, using impulse oscillometry
12. Rebreathing exhaled nitric oxide level (in parts per billion) at study assessment
13. Whole body minus head bone area, bone mineral content, areal bone mineral density and estimated volumetric bone mineral density using DXA instrument
14. Sitting and standing height, weight and head circumference
15. 25-hydroxyvitamin D and PTH levels
16. Fasting blood for serum CTX/P1NP
17. Nasal secretions for inflammatory (e.g. IL4, IL5, IL13, eotaxin, RANTES TNF-alpha) and anti-inflammatory (e.g. IL-10, TGF-beta) mediators
18. Total and specific IgE
19. Eosinophil count
20. Total IgG, A, M and IgG subclass levels
21. Tetanus and Hib IgG antibody levels
22. CD4 T cell proliferative response to recombinant DerP1
23. Soluble ICAM-1 and Serum Soluble IL-2R
24. Dendritic cell numbers and phenotype, and FoxP3^{hi} CD4 T cell numbers by flow cytometry
25. Infant RNA/DNA (Store)
26. Maternal vitamin D and PTH levels and maternal DNA

11. Participant entry

Pre-registration evaluation	No tests are required before a participant can enter the study
Inclusion criteria	All children born of mothers enrolled in the previous trial (1) are eligible to be enrolled in the study
Exclusion criteria	Severe congenital or developmental abnormalities likely to significantly affect respiratory health or lung function eg congenital thoracic dystrophy
Withdrawal criteria	None – the study consists of a single clinical assessment. Parents and children who wish to end the assessment may do so at any time.

12. Study assessment

The study consists of a single assessment of the child. Parents and their children who agree to take part in the study will be invited to attend the Paediatric Research Unit at St Mary's hospital at age three years. After informed consent is taken from the parent/guardian a member of the research team will perform the assessment as follows, which is expected to take less than two hours.

11.1 Structured interview

The research clinician will take a medical history from the child's parent/guardian using a structured questionnaire to obtain information on primary and secondary clinical outcomes. This is expected to take approximately 20 minutes. The primary outcome measure is a history of wheezing at any time during the first 3 years of life. We will support this by demonstration of a child wheezing in a short video.

In addition, parent(s) will also answer a validated questionnaire about their own respiratory and allergic health, their risk factors for vitamin D deficiency, and environmental risk factors for wheezing in their child.

11.2 Clinical examination

Children will have their height, weight and head circumference measured and have a clinical examination, including examination of the skin for eczema using the UK Working Party definition for eczema diagnosis. Where eczema is present its severity will be documented using a validated scoring system. The chest and nose will be examined for signs of lung disease and rhinitis. Skin darkness will be measured using a handheld

colorimeter probe placed gently against the skin. This probe measures melanin levels which are known to be a major determinant of vitamin D absorption and status (23).

11.3 Lung function tests

Children will have their lung function measured using impulse oscillometry before and 15 minutes after administration of salbutamol via a spacer. This technique has been shown in several studies to be reliable and valid in children as young as 2 years, (24-26), and is able to detect significant bronchodilator responses which are predictive of asthma (27).

To perform the test the child will be seated comfortably with their head in a neutral position. Using an adjustable arm, the mouthpiece is brought level with the child's mouth. A nose clip is placed on the subject and the child will be encouraged to breathe normally through the mouthpiece. To minimise interference from vibrations in the upper airways the child's cheeks will be firmly supported by their parent/ guardian. Data acquisition should cover several normal breathing cycles like this, usually between 8 and 16 seconds. The mean value of three to five technically acceptable measurements is reported, and usually takes about one minute to acquire (28). 15 minutes after administration of salbutamol delivered via spacer device fitted with a face mask, measurements will be repeated.

11.4 Measurement of exhaled nitric oxide (eNO)

Children will have their exhaled nitric oxide level measured using an offline tidal volume technique, which has been shown in several studies to be a reliable and valid method in children as young as two years (26, 29).

12.5 Allergy skin tests for atopic sensitisation

Children will undergo allergy skin testing to a panel of common allergens including house dust mite, cat dander, grass pollen, alternaria and cladosporium, silver birch pollen, cow's milk, egg and peanut. Atopy is defined as a positive response to at least one allergen, and a positive response is defined as a wheal diameter ≥ 3 mm in the presence of appropriate responses to the negative and positive control.

11.6 Collection of blood samples

If parents/ guardians consent, a blood sample will also be taken from each child. To reduce discomfort a local anaesthetic cream will be applied. A total of 10mls of blood will be taken – 1.5ml EDTA for future DNA extraction, 3ml Tempus tube for future RNA extraction, 2mls for serum separation, and 3.5mls for flow cytometry and cell culture on fresh mononuclear cells.

11.7 Collection of nasal secretions

If parents/ guardians consent, a sample of nasal secretions will also be taken using a filter paper technique established in our department, for evaluation of inflammatory cytokines IL4, IL5, IL13, eotaxin and RANTES (30). For this procedure a small piece of synthetic absorptive matrix (SAM) will be placed in the nostrils of participating children and left in position for 2 minutes to absorb nasal epithelial lining fluid. SAM are subsequently spin filtered and eluate is analysed by multiplex cytokine assay for inflammatory mediators (Luminex Corporation, Austin, TX).

11.8 DXA scan for bone density

A DXA scan uses low energy x-rays from two different sources to measure the density of the bone being tested. To perform this test, children and their parents will be taken to the radiology department of St Mary's hospital accompanied by the research clinician. Children will all be asked to wear similar light clothing, to reduce any artefactual increase in bone density attributable to their clothes. During the test, the child lies still on their back on a couch while an x-ray detector arm (the 'scanner') sweeps over the area to be tested. The whole scan takes 3-4 minutes. During this time age appropriate distraction (for example a video or book) will be provided to maintain cooperation of the child. 'Whole body minus head' bone area, bone mineral content, areal bone mineral density and estimated volumetric bone mineral density will be assessed – in previous studies by co-investigators Nick Harvey and Cyrus Cooper, 95% of children in this age group were able to cooperate sufficiently well to yield a good quality bone mineral density measurement. The radiation exposure related to this procedure for the child is equivalent to 4 days of normal daily background radiation exposure.

11.9 Review of healthcare records

Permission will be sought from each participant to review their child's primary healthcare records held by their General Practitioner, in order to assess healthcare utilisation in relation to respiratory illness and the total number of unplanned healthcare attendances either to a General Practitioner or an Emergency Department during the first 3 years of life.

11.10 Assessment of mothers

If the mother consents, we will assess their skin darkness using the colorimeter probe and collect a blood sample for vitamin D status and DNA extraction.

11.11 Home or telephone assessments

If families are unable to visit for these assessments, they will be offered a home visit by the research clinician to obtain clinical outcomes. If families decline, questionnaires will be administered over the telephone in order to maximise data acquisition for the outcome measures.

11.12 Permission will also be sought to take a buccal swab from both mother and child for DNA analysis. This test involves using a special cotton bud to gently stroke the inside of the mouth to collect cells from the mouth which can then be used to extract DNA. This is especially useful for any mothers/ children who would rather not have a blood test. The test does not hurt.

13. Pilot study

Prior to enrolment of the first patient we will run a pilot study in 60 children with and without a history of pre-school wheezing recruited from the community and from paediatric outpatients at St Mary's hospital. The aim of this pilot study will be to establish local reference values for respiratory resistance and bronchodilator response to salbutamol measured using impulse oscillometry, as advised by the American Thoracic society/ European Task Force statement on pulmonary function testing in pre-school children (28). Given that the majority of published reference ranges for impulse oscillometry in young children have obtained measurements in white children (31), with small numbers of three year olds, it is important that a representative sample in terms of age and ethnicity is obtained. To better interpret these data we will also perform a short questionnaire based interview regarding respiratory symptoms, measure height and weight, skin darkness using the colorimeter probe, inflammatory cytokines in nasal secretions using the filter paper technique and atopic sensitisation by performing an allergy skin test to common allergens.

14. Follow up

Although the study consists of a single assessment at 3 years of age, will ask mothers if they would be willing to be contacted in the future. If we find evidence to suggest that vitamin D supplementation is associated with a reduction in wheezing in the offspring, it would be of value to follow-up the children three years later in order to whether the effects of prenatal vitamin D persist beyond the first 3 years of life.

To minimise bias, all investigators and research staff involved in data collection or analysis for this follow up study will be blinded to the prenatal treatment allocation of the study participants, and participants will be asked not to mention their treatment allocation to the research team at their visit. Staff will also be blind to the results of the maternal and cord blood vitamin D levels until primary analyses have been completed, and any

episodes of inadvertent unblinding prior to data analysis due to maternal comments will be documented and reported in publications.

14. Adverse events

14.1 Definitions

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject which occurs during the assessment visit.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- Results in death
- Is life-threatening – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*
- Requires hospitalisation, or prolongation of existing inpatients' hospitalisation
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

All adverse events will be reported, and the reporting procedures below will be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

14.2 Non serious AEs

All such events, whether expected or not, should be recorded.

14.3 Serious AEs

An SAE form should be completed and faxed to the Chief Investigator within 24 hours.

All SAEs will be reported to the St Mary's Hospital Research and Ethics Committee where in the opinion of the Chief Investigator, the event was:

- 'related', i.e. resulted from the administration of any of the research procedures; and
- 'unexpected', i.e. an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs will be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES form for non-IMP studies.

Local investigators will also report any SAEs to the Imperial College Joint Research Office and local Research Ethics Committee.

Contact details for reporting SAEs

Fax: 0207 594 3984, attention Robert Boyle

Please send SAE forms to: Robert Boyle

Tel: 0207 594 3990 (Mon to Fri 09.00 – 17.00)

15. Statistics and data analysis

An estimated 80% of study participants will be successfully evaluated in this study for the primary outcome measure, with an estimated wheeze prevalence of 34% at 3 years in the children of placebo-treated mothers (32). We will therefore have 80% power to detect a reduction in risk of wheeze to 13% (risk ratio of 0.38) in the children of vitamin D treated mothers at the 5% level of significance. This level of risk reduction would be consistent with that seen in the observational studies (5, 32). Primary analyses will evaluate the effects of either form of vitamin D supplementation during pregnancy on outcome measures using available case analysis without imputation. The strength of any positive findings will be explored using imputation for missing data if greater than 10% of subjects are lost to follow up for the primary outcome measure. Secondary analyses will evaluate the relationship between cord blood 25-hydroxyvitamin D levels and outcome measures of interest. Analyses will use logistic regression to evaluate the effects of the intervention or cord blood levels on binary outcome measures, and linear regression to analyse continuous outcome measures. If there is chance imbalance between the arms with respect to potential confounding factors we will control for these in the regression models.

Data and all appropriate documentation will be stored for a minimum of 10 years after the completion of the study.

16. Regulatory issues

16.1 Ethics approval

The Chief Investigator has obtained approval from the St Mary's Hospital Research Ethics Committee (REC reference 10/H0712/13). The study has also been submitted for Site Specific Assessment (SSA) at Imperial College Healthcare NHS Trust. The Chief Investigator will require a copy of the SSA approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

16.2 Consent

Consent to enter the study will be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Consent will be obtained from a parent or guardian of each child enrolled in the study. This will be signed consent for children attending the paediatric research unit for a study assessment. Where parents or guardians agree to answer the questionnaire over the telephone, but not to attend for a health assessment, verbal consent to take part in the study, witnessed by a second researcher will be obtained and recorded on the consent form. The right of the parent or participant to refuse to participate without giving reasons will be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All parents and participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

16.3 Confidentiality

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

16.4 Indemnity

Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

Imperial College London will act as the main sponsor for this study. Delegated responsibilities will be assigned to the NHS trust taking part in this study.

16.6 Funding

Asthma UK is funding this study (grant 09/036).

Participant travel and parking expenses will be reimbursed for return travel by public transport or personal vehicle to the Paediatric Research Unit, St. Mary's Hospital but taxi travel will not be reimbursed.

16.7 Audit and inspection

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

17. Study management

The day-to-day management of the study will be co-ordinated through Dr Stephen Goldring, Clinical Research Fellow in the Department of Paediatrics, Imperial College London St. Mary's campus. No formal Data or Safety Management Board has been established for this non-interventional study. However, the study team will meet on a regular basis to review its progress.

18. Publication policy

Results of this study will be published in the scientific peer-reviewed literature, and presented at national and international congresses relevant to asthma and allergic disease. Results will also be disseminated to asthma patients via the funder Asthma UK.

19. References

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20. Appendices

Appendix 1	Invitation letter to participants (Main study)
Appendix 2	Participant information sheet (Main study)
Appendix 3	Consent form for mother (Main study)
Appendix 4	Consent form for parents/guardian of child (Main study)
Appendix 5	GP letter (Main study)
Pilot study	
Appendix 6	Participant information sheet (Pilot study)
Appendix 7	Consent form for parents/ guardian of child (Pilot study)
Appendix 8	GP letter (Pilot study)