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ORIGINAL ARTICLE

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Maternal vitamin D and E intakes during early pregnancy are associated with airway epithelial cell responses in neonates

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Summary

Background Antenatal factors including maternal diet may predispose to airway disease, possibly by impacting on fetal airway development.

Objective This cohort study tested the hypothesis that maternal vitamin D and E status in early pregnancy is associated with airway epithelial cell (AEC) responses in new born infants and examined constitutive and TNFα/IL-1β, house dust mite (HDM) extract or lipopolysaccharide (LPS)-stimulated neonatal AEC responses *in vitro*.

Methods Maternal dietary vitamin D and E intakes (plasma 25[OH]D_3 or α-tocopherol) were characterized at 10–12 weeks gestation. Neonatal nasal AECs were collected soon after birth and cultured to tertiary passage. Constitutive and stimulated – TNFα/IL-1β, HDM extract or LPS – secretory responses (VEGF, RANTES, MCP-1, IL-17A, IFN-γ, GM-CSF, eotaxin, MIP1-α, MIP1-β, ICAM, IL-6, IL-8, IL-10, TNF) in 139 AEC cultures were quantified.

Results AEC mediator release was greater following TNF-α/IL-1β, HDM or LPS stimulation compared to constitutive release. Increased maternal dietary vitamin D was associated with significant increases in IL-10 release by AEC after stimulation with TNF-α/IL-1β ($P = 0.024$) or HDM ($P = 0.049$). Maternal plasma α-tocopherol at 10–12 weeks gestation was positively associated with MIP1α (Spearman's rho 0.242, $P = 0.009$) and IL-3 (ρ 0.189, $P = 0.043$) responses after TNF-α/IL-1β stimulation and negatively associated with TNF (ρ -0.404, $P = 0.011$) and MIP1β (ρ -0.322, $P = 0.046$) responses after LPS stimulation.

Discussion Neonatal AECs respond to pro-inflammatory and allergenic stimuli *in vitro* demonstrating their potential to function as components of the innate immune response. Our findings suggest that associations exist between maternal micronutrient intake during early pregnancy and aspects of stimulated neonatal airway epithelial cell secretory function that may in turn impact on the development of asthma and/or allergic rhinitis in later life.

Keywords vitamin D, vitamin E, neonate, airway epithelial cells
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Introduction

The airway epithelium plays critical roles in the initiation, orchestration and perpetuation of the airway immune and inflammatory responses of asthma. There is much evidence demonstrating it to be structurally and functionally abnormal in asthma [1]. However, what is not known is when these abnormalities develop, but given the importance of early life factors, it seems likely that they develop early, possibly *in utero*. Such developmental programming of airway epithelial cell (AEC) function at a time of rapid lung growth and remodelling has the potential to establish a life course of abnormal airway epithelial cell function, airway structure and function.

The potential importance of maternal diet during pregnancy has been highlighted by studies reporting associations between reduced maternal vitamin D [2–6]

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and vitamin E [7–9] intake during pregnancy with increased risk of childhood asthma and/or wheezing illness [10]. Both micronutrients have the potential to exert immune-modulatory effects [11, 12]; however, given the importance of AEC in asthma pathogenesis, it is plausible that these antenatal exposures might impact on fetal AEC development and neonatal AEC function.

Conventionally, lower respiratory tract AECs are obtained bronchoscopically; however, in neonates, such an approach is precluded by practical and ethical issues. In contrast, the nose is a relatively non-invasive, readily accessible source of AEC, and these have been validated as surrogates for bronchial AEC [13, 14]. More recently, we developed a method to harvest and culture AEC from the noses of neonates within the 48 hours of birth [15]. These AECs, which are essentially 'naïve' and yet to be exposed to the modifying effects of inhaled environmental pollutants and pathogens, provide a unique tool to gain insight into the role of antenatal influences on airway development and the pathogenesis of asthma and allergic rhinitis. This study tested the hypothesis that maternal vitamin D and E intake during pregnancy is associated with neonatal AEC function. To do this, *in vitro* constitutive and stimulated inflammatory mediator release by cultured neonate nasal AEC was quantified and related to maternal vitamin D and E intake during pregnancy.

Methods

Subjects and study design

In this cohort study, maternal vitamin D and E intake and plasma status in early pregnancy were related to the airway epithelial cell responses of their infants soon after birth. Healthy pregnant women attending Aberdeen Maternity Hospital for a routine 'dating' ultrasound scan at 10–12 weeks gestation were invited to participate. Women were recruited regardless of their smoking status or personal/family history of asthma or atopic disease. An interviewer administered a questionnaire to establish smoking history, personal/family history of asthma and atopic disease and socio-economic status (SES, Scottish Index of Multiple Deprivation). Women were invited to complete semi-qualitative food frequency questionnaire (FFQ, version 6.6 of the Scottish Collaborative Group Food Frequency Questionnaire) [16]. In women of childbearing age, the correlation coefficients between intakes of vitamin D and E derived by this questionnaire and 4-day weighed records were 0.38 ($P < 0.001$] and 0.52 ($P < 0.001$], respectively [16].

A non-fasted venous blood sample was collected. Plasma α-tocopherol (adjusted for cholesterol) was quantified by HPLC, [17] total 25-hydroxyvitamin D_3 ($25[OH]D_3$) by HPLC–tandem mass spectrometry [18] and cotinine (an objective measure of tobacco smoke exposure) by liquid chromatography-tandem mass spectrometry [19].

The study was approved by the North of Scotland Research Ethics Service (ref 10/S0802/55), and all women provided written informed consent.

Neonatal AEC sampling and culture

Nasal AECs were harvested from healthy, non-sedated neonates at the earliest opportunity after birth. Exclusion criteria for sampling were premature delivery (i.e. <36 weeks gestation), admission to the neonatal unit and presence of group B Streptococcus on maternal cervical swab. Nasal AECs were sampled by brushing both nostrils with an interdental brush and cultured to tertiary confluent monolayers and their epithelial phenotype confirmed as previously described [15]. Cultured cells were used for a variety of applications. Semi-quantitative membrane arrays were first used to determine which cytokines or chemokines were secreted by neonatal AEC constitutively or following stimulation (data not shown). From these experiments, a panel of cytokines and chemokines deemed to be of relevance was chosen for quantitative measurement in AEC supernatants. Mediator release was measured in cell supernatants as in our earlier studies using either ELISA (IL-8) or cytometric bead array (CBA) analysis (VEGF, RANTES, MCP-1, IL-17A, IFN-γ, GM-CSF, eotaxin, MIP1-α, MIP1-β, ICAM, IL-6, IL-10, TNF). Constitutive/basal mediator release was determined from AEC at rest together with measurement of mediator release from AEC exposed *in vitro* to pro-inflammatory, environmental and allergic stimuli for 24 hours using previously reported protocols [13–15]. Briefly, confluent nasal AEC tertiary monolayers in 3.5 cm^2 wells were left unstimulated or treated for 24 h with one of the following: interleukin 1β (IL-1β) in combination with tumour necrosis factor-α (TNF-α) (both at 10 ng/mL, R&D, Abingdon, UK), 25 μg/mL house dust mite extract (HDM) (Greer Laboratories, Lenoir, NC, USA) or 100 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich Ltd., Poole, UK). All results were normalized to cellular protein content of lysed monolayers quantified using the Bradford assay as previously described [13–15].

Statistics

The primary outcome was nasal AEC cytokine and chemokine release constitutively and after exposure to the various described stimuli. Responses to stimuli were computed by subtracting the constitutive unstimulated values. The distribution of virtually all of the cytokine and chemokine values did not approximate to normal or log-normal distributions; consequently, nonparametric methods were used.

The primary exposures of interest were maternal vitamin D and E intakes. For vitamin D and E intakes, dietary and supplement intakes were summated, energy adjusted [20] and divided into thirds. Secondary exposures were plasma 25[OH]D_3, α-tocopherol, maternal asthma, atopic disease or smoking status. Plasma α-tocopherol and 25[OH]D_3 were logarithmically transformed and adjusted for gestational age; α-tocopherol was also adjusted for cholesterol. Nasal AEC constitutive and stimulated cytokine and chemokine release was related to maternal vitamin D and E intake and plasma indices using the Jonckheere–Terpstra test and Spearman correlation coefficients, respectively. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0. (Armonk, NY, USA).

Results

In total, 269 pregnant women were recruited and nasal samples were obtained from 192 (71%) neonates. Details on the maternal, paternal and neonate characteristics together with nasal AEC culture success rate are presented in Table 1. There were no significant biases in the maternal characteristics of the neonates sampled and the neonates from whom AECs were successfully cultured. Neonates sampled and those with successful AEC culture had greater birthweight. AEC culture was more successful where samples were taken in younger neonates (Table 1). The median age at AEC sampling was 26 h (IQR, 16–45). Of the 192 nasal AEC samples seeded, primary monolayers were established from 164 (85%) samples, with 139 (72%) of samples successfully cultured to confluence at third passage. Of the mediators quantified, we observed relatively high constitutive secretion of GM-CSF, ICAM-1, IL-6, IL-8 and VEGF with relatively low levels of MCP-1 and RANTES by neonate AEC. AECs from 134 infants were exposed to TNF-α/IL-1β, 106 to HDM and 53 to LPS. Mediator release was greater after culture with all three stimuli compared to basal/constitutive release (Tables 2–4).

No significant associations were found between secretion of any of the mediators measured from neonatal AEC and maternal history of asthma or atopic disease, maternal smoking status or plasma cotinine, or mode of delivery (data not shown).

Vitamin D

Estimates of maternal vitamin D intake were positively associated with maternal plasma 25[OH]D_3 levels (Spearman's rho 0.278, $P = 0.001$). Women were recruited year round, and although plasma 25[OH]D_3 levels varied with season, being lowest in the winter

Table 1. Maternal, paternal and neonatal characteristics of recruited women and the neonates sampled and those from whom AECs were successfully cultured and analysed

	Recruited (n = 269)	Neonates sampled (n = 192)	P*	AEC cultured (n = 139)	P†
Maternal					
Age (mean 95% CI)	31.0 (30.5–31.4)	31.1 (30.5–31.6)	0.664	30.9 (30.2–31.5)	0.618
First pregnancy (n, %)	158 (59%)	110 (57%)	0.379	76 (55%)	0.139
Smoker (n, %)	14 (5.2%)	9 (4.7%)	0.548	5 (3.6%)	0.275
SIMD (median, IQR)	5283 (5491–5993)	5290 (4766–6044)	0.800	5291 (4670–5925)	0.803
Age leaving full-time education (median IQR)	21 (18–23)	21 (17–23)	0.253	21 (18–23)	0.932
Asthma	67 (25%)	48 (25%)	1.000	34 (25%)	0.832
Eczema	48 (18%)	33 (17%)	0.624	20 (14%)	0.119
Hayfever	100 (37%)	72 (38%)	0.920	49 (35%)	0.469
Vitamin D intake (ug/d mean 95% CI)‡	17.8 (15.3–20.3)	18.6 (15.4–21.8)	0.296	17.3 (14.8–19.7)	0.645
Vitamin E intake (mg/d mean 95% CI)‡	17.3 (16.0–18.6)	17.7 (16.2–19.3)	0.249	17.6 (15.8–19.4)	0.594
Plasma 25 OH vitamin D (nmol/L mean 95% CI)	55.8 (52.7–58.9)	56.3 (52.6–60.0)	0.821	55.6 (51.4–59.7)	0.748
Plasma α-tocopherol (umol/L mean 95% CI)	16.6 (15.2–18.0)	16.3 (14.7–17.9)	0.575	15.6 (15.4–19.6)	0.180
Paternal					
Asthma	56 (21%)	41 (21%)	0.781	26 (19%)	0.424
Eczema	30 (11%)	21 (11%)	0.804	14 (10%)	0.529
Hayfever	77 (29%)	52 (27%)	0.535	39 (28%)	0.613
Neonate					
Female (n, %)	135 (50%)	96 (50%)	0.693	70 (50%)	0.894
Gestational age at delivery (days, median, IQR)	282 (276–288)	281 (277–287)	0.238	282 (277–288)	0.251
Weight (g, mean, 95% CI)	3527 (3466–3587)	3571 (3504–3638)	0.001	3604 (3530–3679)	0.003
Age when sampled (hrs, median, IQR)		26 (16–45)		24 (15–40)	0.015

*Comparator, those that were not sampled, Chi^2, unpaired t-tests, Mann–Whitney U-test.

†Comparator, those with unsuccessful AEC culture, Chi^2, unpaired t-tests, Mann–Whitney U-test.

‡Energy adjusted.

Table 2. Airway epithelial cell release of cytokines/chemokines before (constitutive) and after exposure to TNF-α/IL1-β (n = 134)

Mediator	Constitutive expression† (pg/mg)† (median, IQR)	Expression following TNF-α/IL1-β exposure† (pg/mg)† (median, IQR)	P*
Eotaxin	0 (0–0)	0 (0–2)	<0.001
GM-CSF	32.1 (7.8–171)	693 (267–1582)	<0.001
ICAM	72.9 (0–216)	1268 (674–2064)	<0.001
IFN- γ	0 (0–1)	0 (0–3)	0.025
IL-3	0 (0–2)	0 (0–1)	0.241
IL-6	3434 (613–10 260)	27 670 (9874–56 920)	<0.001
IL-8	6690 (3570–14 150)	107 600 (69 320–156 700)	<0.001
IL-10	0 (0–0.9)	0 (0–1.26)	0.561
IL-17A	0 (0–0.2)	0 (0–2)	0.010
MCP-1	3.4 (0–5.7)	909 (272–2727)	<0.001
MIP-1α	0 (0–4)	0 (0–20)	0.014
MIP-1β	0 (0–0)	55.9 (18–184)	<0.001
RANTES	2.7 (0–6.1)	120.9 (30.9–36.6)	<0.001
VEGF	2067 (1020–3739)	4193 (2381–5775)	<0.001

*Wilcoxon signed-rank test.
†Values stated are median (IQR) mediator release expressed as pg per mg protein content of the confluent cellular monolayer.

Table 3. Airway epithelial cell release of cytokines/chemokines before (constitutive) and after exposure to LPS (n = 53)

Mediator	Constitutive expression† (pg/mg)† (median, IQR)	Expression following LPS exposure† (pg/mg)† (median, IQR)	P*
Eotaxin	0 (0–0)	0 (0–2.8)	<0.001
GM-CSF	31.9 (4.7–259.5)	108.2 (18.8–454)	<0.001
ICAM	59.1 (0–192.9)	206.5 (31.1–610.9)	<0.001
IFN- γ	0 (0–0)	1. 6 (0–6.0)	<0.001
IL-3	0 (0–0)	2.2 (0.2–9.6)	<0.001
IL-6	5191 (91.7–14 226)	9045 (1036–28 700)	<0.001
IL-8	6310 (1690–14 290)	17 510 (6580–37 025)	<0.001
IL-10	0 (0–0)	1.6 (0–4.4)	<0.001
IL-17A	0 (0–0)	1.1 (0–5.7)	<0.001
MCP-1	5.3 (0–168)	36.1 (8.1–207.7)	0.011
MIP-1α	0 (0–0)	1.0 (0–11.2)	<0.001
MIP-1β	0 (0–0)	0 (0–1.1)	<0.001
RANTES	3.9 (2.3–6.8)	3.1 (1.2–7.3)	0.859
TNF	0 (0–0)	10.7 (4.8–18.6)	<0.001
VEGF	3277 (1896–5203)	2944 (1827–4309)	0.428

*Wilcoxon signed-rank test.
†Values stated are median (IQR) mediator release expressed as pg per mg protein content of the confluent cellular monolayer.

Table 4. Airway epithelial cell release of cytokines/chemokines before (constitutive) and after exposure to house dust mite (n = 106)

Mediator	Constitutive expression† (pg/mg)† (median, IQR)	Expression following HDM exposure† (pg/mg)† (median, IQR)	P*
Eotaxin	0 (0–0)	0 (0–0)	0.133
GM-CSF	30.6 (6.7–145.8)	73.1 (9.9–330)	<0.001
ICAM	57.5 (0–142.5)	271.4 (48.7–668)	<0.001
IFN- γ	0 (0–0.8)	0 (0–0.3)	0.920
IL-3	0 (0–1.2)	3.3 (0.2–7.1)	<0.001
IL-6	2350 (493–9629)	12 706 (699–26 212)	<0.001
IL-8	5720 (3107–10 175)	15 355 (5695–30 713)	<0.001
IL-10	0 (0–0.9)	0 (0–0.8)	0.460
IL-17A	0 (0–0.3)	0 (0–0.1)	0.648
MIP-1α	0 (0–4.2)	0 (0–3.2)	0.264
MIP-1β	0 (0–0)	0.4 (0–8.6)	<0.001
MCP-1	1.3 (0–22.8)	29.1 (3.8–136.8)	<0.001
RANTES	3.4 (0.5–6.4)	12.4 (2.9–71.7)	<0.001
TNF	0 (0–1.5)	30.1 (3.0–124.8)	<0.001
VEGF	2002 (1020–3579)	2340 (1066–3651)	0.372

*Wilcoxon signed-rank test.
†Values stated are median (IQR) mediator release expressed as pg per mg protein content of the confluent cellular monolayer.

50.7 nmol/L (95% CI 39.6–61.8) and highest in the summer 60.4 nmol/L (95% CI 52.1–68.6), this was not statistically significant (P = 0.174). There were no associations between maternal plasma 25[OH]D_3 at 10–12 weeks gestation and constitutive AEC mediator release; however, increasing maternal dietary vitamin D intake was associated with decreasing IL-3 release, $P < 0.001$ (Fig. 1).

There were no associations between maternal plasma 25[OH]D_3 at 10–12 weeks gestation and AEC mediator release after stimulation with LPS; however, increasing maternal vitamin D intake was associated with significant increases in IL-10 release by neonate nasal AEC after stimulation with TNF-α/IL-1β (P = 0.024) or HDM (P = 0.049) (Fig. 2).

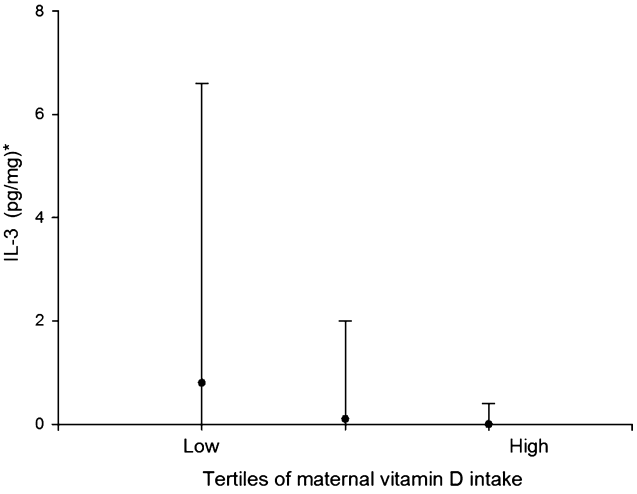

Fig. 1. Association between maternal vitamin D intake and constitutive IL-3 release. P trend<0.001 (Jonckheere–Terpstra). *Values stated are median (IQR) mediator release expressed as pg per mg protein content of the confluent cellular monolayer.

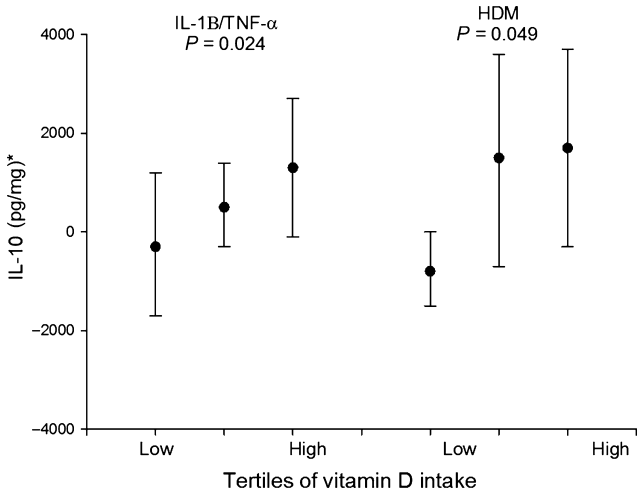

Fig. 2. Association between maternal vitamin D intake and IL-10 release after stimulation with TNF-α/IL-1β and HDM. *P*-values computed using Jonckheere–Terpstra test. *Values stated are median (IQR) mediator release expressed as pg per mg protein content of the confluent cellular monolayer.

Vitamin E

Estimates of maternal vitamin E intake were positively associated with maternal plasma α-tocopherol levels (Spearman's rho 0.206, $P = 0.019$). There were no associations between maternal dietary vitamin E intake and constitutive mediator release. Maternal plasma α-tocopherol at 10–12 weeks gestation was associated with decreasing constitutive release of TNF (Spearman's ρ -0.225, $P = 0.014$), MIP-1α (ρ -0.242, $P = 0.008$), MIP1β (ρ -0.204, $P = 0.027$) and IL-3 (ρ -0.198, $P = 0.032$).

There were no significant associations seen between maternal vitamin E intake and mediator release by neonate nasal AEC after stimulation. Maternal plasma α-tocopherol at 10–12 weeks gestation was, however, associated with increasing MIP1α (Spearman's ρ 0.242, $P = 0.009$) and IL-3 (ρ 0.189, $P = 0.043$) responses after stimulation with TNF-α/IL-1β and with decreasing TNF (ρ -0.404, $P = 0.011$) and MIP1β (ρ -0.322, $P = 0.046$) responses after stimulation with LPS.

Discussion

We have recently described a safe, minimally invasive and reliable method of culturing nasal AEC from neonates [15]. Here, we extend these observations demonstrating constitutive release of and up-regulation of inflammatory mediator release in response to TNFβ/IL-1α, HDM extract or LPS stimuli by AEC cultured from substantial numbers of neonates. These findings demonstrate essentially naive AEC potentially function as components of the innate immune response and contribute to adaptive immune responses. We explored determinants of neonatal AEC mediator release with a focus on maternal dietary vitamin D and E exposures during early pregnancy. The main finding was that maternal vitamin D and E parameters were associated with AEC responses in the setting of encountering common environmental exposures. We observed significant associations between increasing vitamin D intake and decreasing constitutive release of the pro-inflammatory cytokine IL-3 and increased secretion of the anti-inflammatory cytokine IL-10 by IL-1β/TNF-α- or HDM-stimulated neonate nasal AEC. Taken together, these findings suggest that an antenatal environment deficient in dietary vitamin D may lead to an exaggerated AEC inflammatory response in the context of post-natal infection and allergen exposure, leading to increased risk for asthma. Observational studies have consistently reported lower maternal vitamin D intake during pregnancy to be associated with an increased risk of wheeze, asthma, eczema and atopic sensitization in children up to the age of 5 years [2–4]. In contrast, maternal and/or umbilical cord $25[OH]D_3$ has been reported to be either not associated [21], adversely associated [22] or beneficially [5, 6] associated with respiratory and/or atopic outcomes up to the age of 9 years. These apparently inconsistent associations may be explained by our study where an interaction between antenatal (dietary) 'priming' plus post-natal (infective or allergen) exposures are relevant to the later development of symptoms. Vitamin D is thought to influence both innate and adaptive immune responses [23] and may also exert anti-inflammatory effects in asthma as *in vitro* $1,25(OH)_2D3$ has been shown to increase IL-10 secretion by human B cells [24], regulatory T cells [25] and dendritic cells [26]. Here, we show for the first time a potential *in vivo* anti-inflammatory effect by vitamin D on IL-10 secretion by neonate AEC that may be relevant to the development of immunological tolerance upon first exposure of the airways to environmental insults [27, 28]. Previous work has demonstrated that vitamin D is important in fetal lung alveolarization and surfactant production in rats [29, 30]. Vitamin D also has complex effects on surfactant production in alveolar [31] or fibroblast cell lines [32]. Our findings suggest that vitamin D influences human fetal AEC development.

The associations we present between maternal α-tocopherol and AEC mediator release are less straightforward than those for vitamin D intake but may give insight into interactions between ante and post-natal exposures. In the context of simulated viral infection (TNFα/IL1β simulation), maternal plasma α-tocopherol was positively correlated with release of the pro-inflammatory mediators MIP1α and IL-3. In contrast, increasing plasma α-tocopherol was associated

with a reduced pro-inflammatory response to simulated bacterial infection (LPS), an association concordant with the observation that early life endotoxin exposure is associated with reduced likelihood of subsequent asthma and atopic disease [33, 34]. Lower maternal dietary vitamin E intake during pregnancy is associated with an increased risk of childhood wheezing [7, 8] and asthma [9]. An association between maternal vitamin E intake and *in vitro* responses by cord blood mononuclear cell responses [35] suggests that maternal vitamin E may influence fetal immune development. Our novel finding that plasma α-tocopherol at 10–12 weeks gestation modulates neonatal AEC mediator release suggests effects on fetal AEC development, providing a further mechanistic explanation for the relationship between maternal dietary vitamin E intake and childhood asthma.

In this study, maternal vitamin D intake, but not plasma 25[OH]D_3, was associated with neonatal AEC inflammatory mediator release. This is analogous with the epidemiological data outlined above whereby maternal vitamin D intake but not plasma 25[OH]D_3 is consistently associated with childhood outcomes [2–6, 21, 22]. Dietary intake typically contributes about 10% to UK vitamin D status, and whilst studies of plasma 25[OH]D_3 truly reflect associations with vitamin D status, perhaps studies of vitamin D intake should be interpreted more broadly. Vitamin D is never naturally consumed in isolation, but as foods containing many nutrients; indeed, it may be the combination of naturally associated nutrients that is important and should perhaps be replicated in intervention studies. Trials of vitamin D supplements during pregnancy are underway; given the inconsistencies between dietary and plasma vitamin D, these may not provide clear answers. In the current study, maternal plasma α-tocopherol, but not maternal vitamin E intake, was associated with neonatal AEC inflammatory mediator release. A possible explanation for this is that dietary vitamin E comprises natural isoforms, for example α- and γ-tocopherol, and there is evidence that these stereoisomers have different biological effects; for example, in murine eosinophilic allergic lung inflammation, α-tocopherol is anti-inflammatory, whereas γ-tocopherol is pro-inflammatory [36].

The current prospective study is the first to culture neonate AEC in substantial numbers and to relate mediator release to antenatal exposures. Although the study was hypothesis driven, the study was somewhat exploratory with many inflammatory mediators being quantified. The study has several limitations. The absence of previous neonatal studies precluded meaningful consideration of statistical power, and because we investigated a wide range of potentially relevant mediators, the reported associations may reflect the number of tests of association conducted. The AECs cultured were nasal in origin and not from the lower airways. However, it is not ethically or physically possible to sample neonatal bronchial epithelium. The micronutrient status of the pregnant women was well characterized; however, the inherent limitations of FFQ represent a null bias. In addition, maternal nutrient status was quantified 30 weeks prior to the nasal AEC samples being obtained. This and the time and processes taken to culture the AEC to tertiary passage constitute further null bias. We were unable to demonstrate associations between AEC mediator release and maternal smoking and parental asthma, reflecting possibly the small number of women (n = 5) who smoked or had a history of asthma (n = 34). Although we demonstrate associations with several mediators, the quantities of some of these mediators were small and IL-3 release was not significantly increased by TNF-α/IL1-β exposure. The current study had several strengths. Sample size was larger than most studies of AEC in adults and older children; moreover, by sampling healthy neonates within days of birth, the AEC had not been influenced by post-natal exposures such as pollution or infection. Further studies will be needed to support or refute our findings and to establish clinical relevance. Follow-up of the cohort is envisaged to investigate whether the AECs of neonates who develop childhood asthma differ from AECs of children who do not. If so, it may be possible to use neonatal nasal AECs in the clinical setting as a predictive biomarker to quantify subsequent risk of asthma.

In summary, we have developed a reliable and reproducible method to sample and culture nasal AECs from substantial numbers of neonates. In addition, we have demonstrated that neonatal AECs have the potential to function as components of the innate immune response, possibly directing adaptive immune responses to pathogens and allergens. Our findings are the first to demonstrate associations between maternal micronutrient intake during early pregnancy and aspects of stimulated neonatal airway epithelial cell secretory function that may in turn impact on the development of asthma and/or allergic rhinitis in later life.

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Conflict of interest

All of the authors declare that no conflict of interest exists.

References

1 Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 2011; **242**:205–19.

2 Camargo CA, Rifas-Shiman SL, Litonjua AA *et al*. Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 years of age. *Am J Clin Nut* 2007; **85**:788–95.

3 Maslova E, Hansen S, Jensen CB, Thorne-Lyman AL, Strøm M, Olsen SF. Vitamin D intake in mid-pregnancy and child allergic disease – a prospective study in 44,825 Danish mother-child pairs. *BMC Preg Childbirth* 2013; **13**:199.

4 Devereux G, Litonjua AA, Turner SW *et al*. Maternal vitamin D intake during pregnancy and early childhood wheezing. *Am J Clin Nutr* 2007; **85**:853–9.

5 Jones AP, Palmer D, Zhang G, Prescott SL. Cord blood 25-hydroxyvitamin D3 and allergic disease during infancy. *Pediatrics* 2012; **130**:e1128–35.

6 Camargo CA, Ingham T, Wickens K *et al*. Cord-blood 25-hydroxyvitamin D levels and risk of respiratory infection, wheezing, and asthma. *Pediatrics* 2011; **127**:e180–7.

7 Litonjua AA, Rifas-Shiman S, Ly NP *et al*. Maternal antioxidant intake in pregnancy and wheezing illnesses in children at 2 y of age. *Am J Clinical Nutr* 2006; **84**:903–11.

8 Martindale S, McNeill G, Devereux G, Campbell D, Russell G, Seaton A. Antioxidant intake in pregnancy in relation to wheeze and eczema in the first two years of life. *Am J Respir Crit Care Med* 2005; **171**:121–8.

9 Devereux G, Turner SW, Craig LCA *et al*. Reduced maternal vitamin E intake during pregnancy is associated with asthma in 5-year-old children. *Am J Respir Crit Care Med* 2006; **174**:499–507.

10 Nurmatov U, Devereux G, Sheikh A. Nutrients and foods for the primary prevention of asthma and allergy: systematic review and meta-analysis. *J Allergy Clin Immunol* 2011; **127**:724–33.

11 Muehleisen B, Gallo RL. Vitamin D in allergic disease: shedding light on a complex problem. *J Allergy Clin Immunol* 2013; **131**:324–9.

12 Li-Weber M, Giasisi M, Trieber MK, Krammer PH. Vitamin E inhibits IL-4 gene expression in peripheral blood T-cells. *Eur J Immunol* 2002; **32**:2401–8.

13 McDougall CM, Blaylock MG, Douglas JG, Brooker RJ, Helms PJ, Walsh GM. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. *Am J Respir Cell Molec Biol* 2008; **39**:560–8.

14 Pringle EJ, Richardson HB, Miller D *et al*. Nasal and bronchial airway epithelial cell mediator release in children. *Pediatr Pulmonol* 2012; **47**:1215–25.

15 Miller D, Turner SW, Spiteri-Cornish D *et al*. Culture of airway epithelial cells from neonates sampled within 48-hours of birth. *PLoS One* 2013; **8**: e78321.

16 Masson LF, McNeill G, Tomany JO *et al*. Statistical approaches for assessing the relative validity of a food-frequency questionnaire: use of correlation coefficients and the kappa statistic. *Public Health Nutr* 2003; **6**:313–21.

17 Driskell WJ, Neese JW, Bryant CC, Bashor MM. Measurement of vitamin A and vitamin E in human serum by high-performance liquid chromatography. *J Chromat* 1982; **231**:439–44.

18 Tolppanen AM, Sayers A, Fraser WD, Lawlor DA. Association of serum 25-hydroxyvitamin D3 and D2 with academic performance in childhood: findings from a prospective birth cohort. *J Epidemiol Comm Health* 2012; **66**:1137–42.

19 Dunlop AJ, Clunie I, Stephen DW, Allison JJ. Determination of Cotinine by LC–MS-MS with automated solid-phase extraction. *J Chromatogr Sci* 2014; **52**:351–6.

20 Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *Am J Clin Nutr* 1997; **65**(suppl):1220S–8S.

21 Pike KC, Inskip HM, Robinson S *et al*. Maternal late-pregnancy serum 25-hydroxyvitamin D in relation to childhood wheeze and atopic outcomes. *Thorax* 2012; **67**:950–6.

22 Gale CR, Robinson SM, Harvey NC *et al*. Maternal vitamin D status during pregnancy and child outcomes. *Eur J Clin Nutr* 2008; **62**:68–77.

23 Mann EH, Chambers ES, Pfeffer PE, Hawrylowicz CM. Immunoregulatory mechanisms of vitamin D relevant to respiratory health and asthma. *Ann N Y Acad Sci* 2014; **1317**:57–69.

24 Heine G, Niesner U, Chang HD, Radbruch A, Worm M. 1,25-dihydroxyvitamin D(3) promotes IL-10 production in human B cells. *Eur J Immunol* 2008; **38**:2210–8.

25 Urry Z, Xystrakis E, Richards DF *et al*. Ligation of TLR9 induced on human IL-10–secreting Tregs by 1α,25-dihydroxyvitamin D3 abrogates regulatory function. *J Clin Invest* 2009; **119**:387–98.

26 Penna G, Adorini L. 1 Alpha, 25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* 2000; **164**:2405–11.

27 Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nature Immunol* 2001; **2**:725–31.

28 Bohle B, Kinaciyan T, Gerstmayr M, Radakovicsa A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10–producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007; **120**:707–13.

29 Gaultier C, Harf A, Balmain N, Cuisinier-Gleizes P, Mathieu H. Lung mechanics in rachitic rats. *Am Rev Respir Dis* 1984; **130**:1108–10.

30 Nguyen M, Guillozo H, Garabedian M, Balsan S. Lung as a possible additional target organ for vitamin D during fetal life in the rat. *Biol Neonate* 1987; **52**:232–40.

31 Rehan VK, Torday JS, Peleg S *et al*. 1Alpha,25-dihydroxy-3-epi-vitamin D3, a natural metabolite of 1alpha,25-dihydroxy vitamin D3: production and biological activity studies in pulmonary alveolar type II cells. *Mol Genet Metab* 2002; **76**:46–56.

32 Lunghi B, Meacci E, Stio M *et al*. 1,25-dihydroxyvitamin D3 inhibits proliferation of IMR-90 human fibroblasts and stimulates pyruvate kinase activity in

confluent-phase cells. *Mol Cell Endocrinol* 1995; **115**:141–8.
33 Karvonen AM, Hyvarinen A, Gehring U *et al*. Exposure to microbial agents in house dust and wheezing, atopic dermatitis and atopic sensitization in early childhood: a birth cohort study in rural areas. *Clin Exp Allergy* 2012; **42**:1246–56.
34 Gehring U, Strikwold M, Schram-Bijkerk D *et al*. Asthma and allergic symptoms in relation to house dust endotoxin: phase two of the International Study on Asthma and Allergies in Childhood (ISAAC II). *Clin Exp Allergy* 2008; **38**: 1911–20.
35 Devereux G, Barker RN, Seaton A. Antenatal determinants of neonatal immune responses to allergens. *Clin Exp Allergy* 2002; **32**:43–50.
36 Cook-Mills JM, Abdala-Valencia H, Hartert T. Two faces of Vitamin E in the lung. *Am J Respir Crit Care Med* 2013; **188**:279–84.