

The effects of maternal smoking on early mucosal immunity and sensitization at 12 months of age

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With the dramatic rise in asthma and respiratory disease, there is an urgent need to determine the effects of common environmental exposures on early immune development. In this study, we examined the effects of maternal smoking as a major adverse exposure in early life, on mucosal immune function and allergen sensitization in the first year of life. A cohort of 60 smokers and 62 non-smokers was recruited in pregnancy, and followed prospectively at 3 and 12 months of age for saliva collection [for immunoglobulin (Ig) A measurements], urine collection (for cotinine levels) and clinical assessments (for allergy and infection history). Allergen skin-prick tests were also performed at 12 months of age. Specific IgA to common colonizing bacteria was measured on saliva samples, including pneumococcal polysaccharide (PS) serotype 14 and non-typeable *Haemophilus influenza* (NTHI) outer membrane protein 6 (OMP6). Eighty-two mothers and their infants completed the 12-month follow-up period – 56 in the maternal non-smoking group and 26 in the maternal smoking group. Maternal smoking was associated with significantly higher total infant salivary IgA at 12 months of age ($p = 0.026$), and more chronic upper respiratory tract symptoms ($p = 0.012$). However, there were no differences in the level of specific IgA antibodies to common colonizing bacteria (pneumococcal PS serotype 14 and NTHI OMP6). In general, the IgA levels at 12 months were higher in children who had more chest infections in the first year (Kendall's tau b, 0.282; $p = 0.003$). There was also a trend of lower respiratory tract symptoms (wheeze) ($p = 0.142$) in infants of smokers. There were no effects of maternal smoking on the rates of allergen sensitization, atopic dermatitis and food allergy at 12 months of age. In conclusion, maternal smoking did not inhibit the production of anti-microbial IgA, suggesting that other factors are responsible for the increased susceptibility to infection in these infants. The increased mucosal inflammation in these children was not associated with effects on early allergy propensity.

Paul Noakes, Angie Taylor, Jasmine Hale, Liza Breckler, Peter Richmond, Sunalene G. Devadason and Susan L. Prescott

School of Paediatrics and Child Health Research,
University of Western Australia, Perth, Australia

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Susan L. Prescott, School of Paediatrics and Child Health Research, University of Western Australia, Perth, Western Australia
PO Box D184, Princess Margaret Hospital, Perth WA 6001, Australia
Tel.: +61 89 340 8171
Fax: +61 89 388 2097
E-mail: susanp@ichr.uwa.edu.au

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Asthma is the most common chronic disease of childhood. The associated airway inflammation appears to be the culmination of both local epithelial dysfunction and a more generalized immune dysregulation that results in allergic predisposition, although the sequence and

relationship of these events are unclear. A number of antenatal and early postnatal events may contribute to this. Maternal smoking is one of the best documented adverse exposures that is likely to contribute to airway disease through a number of pathways. These include well-recognized

effects on airway development *in utero* (1, 2) and direct irritant damage in the postnatal period. There is also evidence that maternal smoking could have *in utero* effects on immune development, as evidenced by alterations in cord blood immune parameters compared with the newborns of non-smokers (3–5). We have also recently demonstrated that maternal smoking in pregnancy attenuates toll-like receptor (TLR) mediate innate immune responses (Noakes et al., in press). While these effects on immune competence could contribute to the well-documented increased susceptibility to respiratory infection (6), some studies also suggest that maternal smoking also increases the risk of allergic immune responses (7). It is still not clear how these events contribute to chronic airway inflammation, but these observations suggest that the immune effects of smoking are likely to be complex.

The development of local immune function in the respiratory mucosa is of central relevance in the development of airway disease. The mucosal immune system is rapidly stimulated at birth by bacterial colonization. The development of effective mucosal immunity is essential for protection against infection. In addition to this, events at mucosal surfaces may also influence local immune responses during primary mucosal allergen exposure in the postnatal period. Based on observations that adult smokers have lower salivary immunoglobulin (Ig) A levels (8), it has been proposed (9) that similar effects in infants could contribute to the increased respiratory morbidity. However, to our knowledge, the development of pathogen-specific IgA secretion has not been compared in infants of non-smoking and smoking mothers. The main aim of this study was to assess the effects of maternal smoking on the development of mucosal IgA secretion, including total and pathogen-specific IgA [non-typeable *Haemophilus influenza* (NTHI) and *Pneumococcus* species]. We also examined this in relationship to clinical symptoms and the development of sensitization in the first year of life. As most mothers who smoke in pregnancy continue to smoke in the postnatal period, we did not examine the separate effects of pre- and post-natal exposure.

Materials and methods

Overview

This was a prospective pregnancy cohort study (n = 122) designed to compare the effects of maternal smoking on foetal immune development and post-natal immune maturation.

Study population

The original pregnancy cohort was recruited from major maternity hospitals in Perth, and included 60 smokers and 62 non-smokers, who were invited to participate in this follow-up study. Of these, 82 mothers and their healthy, full-term babies completed this 12-month follow-up study, with 56 in the maternal non-smoking group and 26 in the maternal smoking group. The resulting groups were balanced for maternal atopic status and socio-economic status (as defined by educational level) as shown on Table 1.

The infants were initially seen at 3 months of age for the collection of clinical data on respiratory and other symptoms. Infant samples (saliva and urine) were also collected for the measurement of salivary IgA and urinary cotinine (as follows). Parents were given prospective diary cards to be completed (with information regarding feeding practices, food introduction, infection history and other symptoms) and returned at 12 months of age (discussed next).

At 12 months of age, the infants were reviewed for additional clinical history, saliva collection and a physical examination by a paediatrician (described in the following). Allergy skin-prick tests (SPT) were also performed at this visit to assess sensitization status (in the following).

During the study period, all infants were vaccinated following the Australian vaccination schedule, which included *H. influenza* type b (Hib). The schedule only included the seven-valent (4, 6B, 9V, 14, 18C, 19F and 23F) pneumococcal conjugated vaccine (Prevenar) from 1 January 2005; thus, very few children were vaccinated against pneumococcal serotypes (non-smokers n = 4; smokers, n = 6).

Sample collection

Saliva was collected from the buccal cavity of the infants at 3 (n = 89) and 12 (n = 65) months of age, using Specimen Suction Set (Maersk Indoplas Pty Ltd, Sydney, Australia) attached to a suction pump and immediately frozen at –80°C for later analysis of IgA antibody levels. Long-term storage has earlier been reported not to have any effect on the antibody affinity (10), nor does long-term storage and repeated cycles of freezing and thawing alter the molecular weight of IgA (11). Both saliva samples from each infant were assayed in the same test batch to reduce inter-assay variations.

Measurement of total and specific IgA

We measured specific IgA to bacteria commonly found in the upper respiratory tract. Pneumococcal polysaccharide (PS) serotype 14 and non-typeable *H. influenza* (NTHI) outer membrane protein 6 (OMP6) were selected, because these reflect the presence of common colonizing bacteria that can also produce respiratory pathology (12).

Firstly, IgA antibodies to pneumococcal PS serotype 14 (serotype 14; American Type Culture Collection, Manassas, VA, USA) were measured by enzyme immunoassay (EIA) as described previously (13, 14) with a few modifications. Five micrograms of PS in 1 ml of phosphate buffered saline (PBS) were incubated for 5 h at 37°C in the wells of microtitre plates (Greiner Bio-one, Solingen, Germany). An identical background plate coated with PBS only was used to subtract the unspecific binding. The wells were blocked with 10% (v/v) foetal calf serum (Gibco, Paisley, UK) in PBS (FCS-PBS) for 1 h at 37°C. Serum pool 89-SF (15) was applied as a reference. The saliva samples were diluted 1/10 in FCS-PBS containing 10 µg/ml of pneumococcal cell wall polysaccharide (CWPS; Statens Serum Institut, Copenhagen, Denmark) and 5 µg/ml of 22F to neutralize non-specific antibodies and increase specificity, and analysed in triplicates. The plates were incubated at room temperature for 2 h with shaking. Monoclonal anti-human IgA (kind gift from George Carlone) antibody was diluted (1/10,000) in FCS-PBS, and were pipetted into wells and incubated as earlier. After that, alkaline phosphatase conjugated polyclonal rabbit anti-mouse IgG (Biosource International, Camarillo, CA, USA) was added (1/2000), and the plates were incubated overnight at room temperature. The substrate, *p*-nitrophenyl phosphate disodium (Sigma, Castle Hill, Australia) in carbonate buffer (pH = 9.8) was added and incubated for 1 h at 37°C. The absorbances were detected (Biotek Instruments Inc, Winooski, VT, USA) at wavelength 405 nm.

Secondly, for the determination of IgA antibodies NTHI OMP, microtitre plates (NUNC, Roskilde, Denmark) were coated with 5-µg/ml OMP6 antigen [kindly supplied by Professor Wayne Thomas, Molecular Biotechnology Division, Institute of Child Health Research (ICHR)] diluted in carbonate/bicarbonate buffer (pH = 9.6) for 4 h at 37°C. The plates were then blocked with 5% skim/PBS/Tween (w/v) for 1 h at 37°C. The reference sera (pooled in house sera from laboratory volunteers) were serially diluted,

added to the wells, and then incubated for 2 h at 37°C. Alkaline phosphatase conjugated goat anti-human IgA (Biosource International) was diluted 1/500, then added to the wells and incubated for a further 90 min at 37°C. The substrate was added and incubated for a final 2 h at 37°C. Substrate development was stopped with the addition of sodium hydroxide, and the plates were read at 405 nm.

The total IgA was measured by using a conventional ELISA (Probiomix, Eveleigh, Australia). Briefly, Greiner Microlin 200 plates (Greiner Bio-one) were coated with anti-human IgA (Silenus Anti-Human IgA; Chemicon Australia, Boronia, Australia), in imidazole/1% (v/v) bovine serum albumin (BSA). The plates were incubated for 2 h at 37°C. The plates were blocked with 200 µl/well of 0.5% casein, and incubated at 37°C for 45 min. After incubation, the plates were dried for a maximum of 20 min at 37°C, and then stored overnight at 4°C. Human standard serum NOR-04 (Nordic, Tilburg, Netherlands) was used as a standard reference. Saliva samples were diluted 1/2000 in PBS/Tween and added to the wells, along with the standards and blanks (100 µl/well). The plates were incubated at room temperature for 20 min. After washing (PBS/Tween), 100 µl of detection antibody (Silenus Anti-human IgA HRP; Chemicon, Sweden) diluted 1/2,000 in PBS/Tween was added. The plates were incubated for 15 min at room temperature. Detection was performed using the peroxidase substrate tetramethylbenzidine (TMB; Roche, Diagnostics, Castle Hill, Australia). The reaction was stopped (1-M H₃PO₄) and absorbances detected at 450-nm wavelength.

Cotinine measurements

The urinary cotinine levels were measured using the liquid-phase extraction methodology. Briefly, a total of 2.0 ml of infant urine (standard or test sample) and 40 µl of internal standard (2-phenylimidazol, 25 µg/ml) were added to 15-ml screw-capped tubes. After the addition of 1-ml of 5-M potassium hydroxide and 5-ml dichloromethane, the tubes were shaken vigorously and centrifuged at 4000 rpm for 4 min. The supernatants were discarded by aspiration. After evaporation to dryness under gentle stream of nitrogen at ambient temperature, residues were reconstituted in 300 µl of mobile phase. An aliquot (25 µl) was injected into the HPLC system via the automatic sampler. The HPLC system consisted of a C₁₈ reversed-phase cartridge column – an isocratic

mobile phase, which was a mixture of aqueous phase, methanol and acetonitrile in the respective proportions of 81:10:9 (v/v/v) and a flow rate of 1 ml/min.

Clinical assessment at 12 months

Clinical assessment included a detailed history and examination by a paediatrician (SP), who was blinded to smoking status. A diagnosis of atopic dermatitis was made in infants with typical skin lesions (16) responsive to topical steroids. IgE-mediated food allergy was defined as a history of immediate (within 60 min) symptoms following contact with food (such as egg, dairy, nut and so on) and a positive SPT to the implicated food. Symptoms include skin reactions (hives, rash or swelling) and/or respiratory symptoms (cough, wheeze, stridor) and/or gastrointestinal symptoms (abdominal pain, vomiting, loose stools) and/or cardiovascular symptoms (collapse).

Infection data was collected prospectively (diary cards), and based on both physician diagnosed disease (such as 'pneumonia', 'upper respiratory tract infection', 'bronchiolitis'), and parental recording of symptoms (on diary cards), such as 'persistent cough' (for more than 2 wk) and 'wheeze'. Children were recorded as having 'upper respiratory infections', if they had infections that were limited to coryzal symptoms in the absence of significant chest symptoms.

We also collected data on other environmental factors that could confound or influence the relationship of smoking and allergic disease risk. We obtained information about other clinical disease and common exposures (vaccination, daycare, diet and medication use) and the home environment (including carpeting, daycare attendance, sibship, pets, mattress and pillow covers).

Allergen SPT

This was performed using a standardized technique (17) and allergen extracts (milk, peanut, house dust mite, cat; Hollister-Stier Laboratories, Spokane, Australia) or whole egg, and histamine as a positive control and glycerine as a negative control. A wheel diameter of ≥ 3 mm was considered positive.

Statistical analysis

The IgA data were not normally distributed, and were not able to be normalized with natural logarithmic transformations. Comparisons were

made with non-parametric statistical techniques using the Mann-Whitney *U*-test (for unpaired comparison), Wilcoxon signed-rank tests (for paired comparisons) or Spearman correlation coefficients. Group data are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. The Pearson's chi-squared test was employed for categorical variables, and Fisher's exact test was used when the expected frequency for any cell was less than 5. 'The factors that were examined as potential confounding influences were factors that were demonstrated to differ between the groups (including feeding practices, birth order and allergy risk). Relevant factors were entered into multiple regression modeling to calculate adjusted weighted effects for maternal smoking and these variables.' A significance level of $p < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS software (Version 11 for Macintosh).

Ethical approval

Ethical approval for collection of these samples and the associated studies was granted by the Princess Margaret Hospital Research and Ethics Committee.

Results

The characteristics of the population

Most (73.1%) women who smoked in pregnancy continued to smoke in the postnatal period ($p < 0.001$). Although many claimed not to smoke 'near' the infant (83.8%), 'we noted that infant urinary cotinine levels in the maternal smoking group were significantly higher at 3 months of age ($p = 0.002$) compared with the maternal non-smoking group, although this relationship was not evident at 12 months of age' (not shown). The rates of maternal-reported passive (partner or other) smoke exposure were also higher if mothers smoked ($p = 0.117$). As noted in the Methods section, the follow-up rate at 12 months was significantly lower for smokers (26/60) than for non-smokers (56/62) ($p < 0.001$). Although it is difficult to exclude subsequent differences compared with the population that were lost to follow up, there were no differences in the baseline characteristics of the smokers that attended follow up ($n = 26$) compared with those that did not ($n = 34$).

Although there were no differences in the proportion of infants that were breastfed (ever) in the first year of life ($p = 0.329$), mothers who

Table 1. Characteristics (exposures) of infants in the smoking group compared with the non-smokers at 12 months of age†

Characteristics	Mothers smoked in pregnancy (n = 26)	Mothers did not smoke in pregnancy (n = 56)	p value*
Maternal age ± s.e.m. (years)	30.88 ± 0.93	32.49 ± 0.62	0.889
Maternal smoking (current)	19/26 (73.1)	1/56 (1.8)	<0.001
Exposure to passive smoke (partner or other)			
Nil	10/26 (38.5)	35/56 (62.5)	0.117
Light	13/26 (50.0)	18/56 (32.1)	–
Moderate	3/26 (11.5)	3/56 (5.4)	–
Maternal allergy	16/26 (61.5)	27/56 (48.2)	0.261
Socio-economic status‡	18/26 (69.2)	38/51 (74.5)	0.623
Infant feeding			
Breastfed (ever)	26/26 (100)	54/56 (96.4)	0.329
Formula fed (ever)	24/26 (92.3)	39/56 (69.9)	0.024
Length of breastfeeding (months)§	3.25 (0.5, 6.25)	8.0 (4.5, 10.5)	0.03
Weaning (months)§	4.0 (4, 6)	5.0 (4, 6)	0.095
No. siblings			
None	14/26 (55.6)	18/56 (32.1)	0.062
1	8/26 (29.6)	25/56 (44.6)	–
2	3/26 (11.1)	10/56 (17.9)	–
3	1/26 (3.7)	3/26 (5.4)	–
Exposure to pets	7/26 (26.9)	22/56 (39.3)	0.229
Childcare	16/26 (61.5)	44/56 (78.6)	0.167

*p < 0.05 was considered a significant difference between the groups (see values in bold).

†Pearson's chi-square (categorical) or Mann-Whitney U-test (continuous) determined differences between groups. Data represented as number (%), unless otherwise indicated.

‡Data on socio-economic status include 26 smokers and 51 non-smokers only.

§Data represent median (interquartile range).

smoked were likely to give up breast feeding earlier ($p = 0.03$) compared with non-smoking mothers [as also observed by others (18)]. These mothers were therefore also significantly more likely to use infant formula, either for supplementary feeds or in place of breast feeding ($p = 0.024$) (Table 1). Infants of smoking mothers tended to start weaning foods at an earlier age ($p = 0.095$) compared with unexposed infants (Table 1). There were no differences in the age of first exposure to potentially 'allergenic foods', such as hens' egg or cows' milk (not shown). Data on 'clinical reactivity' to any other foods (and milk formulae) were collected, and there were no differences between the groups (not shown). It is possible that these differences in feeding practices could influence allergy risk and IgA levels and, where possible, this was examined as potential confounding factor in subsequent analyses (discussed later).

There were no differences in exposure to pets (cats and dogs) or daycare attendance for infants of smokers vs. non-smokers (Table 1). There was a trend for infants of non-smoking mothers to have

a greater number of older siblings ($p = 0.062$) compared with infants of smoking mothers. Because of previously reported associations between birth order and allergy risk (19), this was also included as a potential confounding influence.

The effects of cigarette smoke exposure on early postnatal mucosal immune responses

We examined the effects of maternal smoking on the development of total IgA levels and specific IgA responses to common bacteria (detected in infant saliva at 3 and 12 months of age). IgA was detectable in all saliva samples. Infants exposed to maternal cigarette smoke in pregnancy and the first year of life had significantly higher total IgA levels ($p = 0.026$) at 12 months compared with those infants who were unexposed (Fig. 1).

As documented in other studies (20), IgA levels decreased with age (Fig. 2) [smokers; $n = 16$, median difference = 2.92 mg/l, interquartile range (IQR) (–4.00, 13.9) ($p = 0.148$) and non-smokers; $n = 42$, median difference = 4.94, IQR (–0.77, 10.71) ($p = 0.002$)].

We also assessed the effects of smoking on specific (microbial) IgA responses. By 3 months of age, most children (regardless of smoke exposure) had detectable IgA specific for *Pneumococcal* serotype 14 (96.7% of all infants) or *Haemophilus* OMP6 (89.6% of all infants). These rates of detection remained the same at 12 months of age, although the absolute levels were slightly lower. There were no differences in detection rates in infants of smokers and non-smokers at 3 months of age (Table 2). Furthermore, despite the higher levels of total IgA in

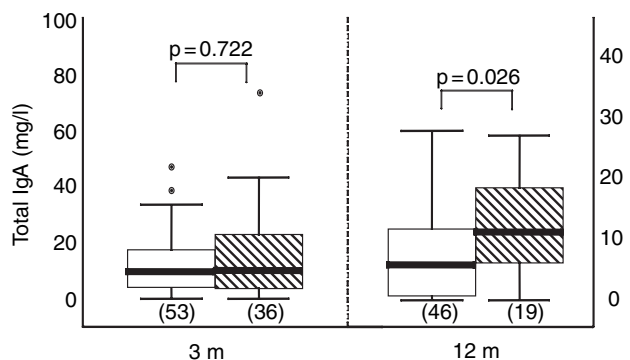


Fig. 1. Comparison of total salivary IgA in infants of smoker and non-smokers. The total immunoglobulin (Ig) A levels (mg/l) in saliva from infants at 3 and 12 months of age, who have been exposed to maternal cigarette smoke (shaded box), compared with those unexposed (clear box). The number of infants is indicated in parentheses. The median, interquartile ranges, 95% confidence intervals and outlying values are shown.

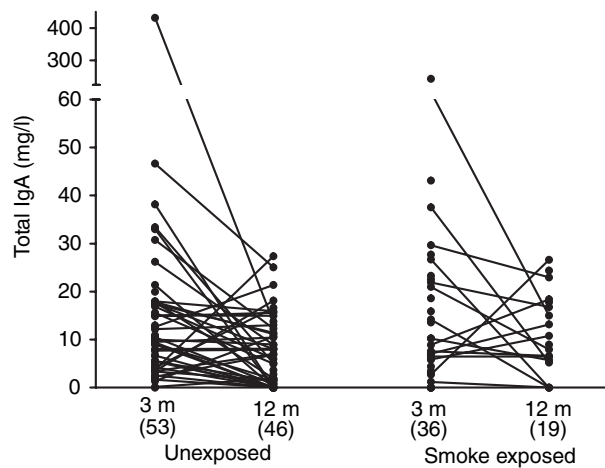


Fig. 2. Individual data for age-related changes in total IgA. Changes in total immunoglobulin (Ig) A (mg/l) in saliva samples between 3 and 12 months of age in infants exposed to passive cigarette smoke compared with those unexposed. The number of infants is indicated in parentheses.

infants of smokers at 12 months, there was no difference in the absolute levels of antibodies to *Pneumococcal* serotype 14 or *Haemophilus* OMP6.

There were also no associations between cotinine levels and total or specific IgA levels at 3 or 12 months of age (not shown).

It is possible that differences in feeding practice may have contributed to the observed differences in IgA levels in the infants of smokers. Because IgA could not be normalized by logarithmic transformation, it was not possible to exclude this effect of feeding on this continuous variable using linear regression. Even if this was possible, many babies received both formulae and breast milk, making the data difficult to analyses in this way, or when using subgroup analyses according to feeding practice.

In summary, maternal smoking was associated with higher total salivary IgA antibody levels at 12 months. However, at 3 months of age, there were no clear relationships between IgA levels and maternal smoking (or cotinine levels).

The relationship between smoking and respiratory symptoms

Infants of smokers also tended to have higher rates (34.6%) of lower respiratory tract symptoms (wheeze) than the non-smoking group (19.6%; $p = 0.142$). Infants of smokers had more than twice the prevalence (19.2%) of bronchiolitis as the non-smokers (8.9%).

Although infants of smokers had similar rates of upper respiratory tract infections (Table 3), these infants were significantly more likely to have persistent nasal congestion and discharge (30.8%) compared with the non-smoking group (8.9%; $p = 0.012$). However, this was no longer statistically significant after the effect of birth order and breast-feeding practices were taken into account using logistic regression [odds ratio (OR) = 2.2, 95% confidence interval (CI), 0.423 to 11.42; $p = 0.349$].

Infants of smokers tended to have higher rates of gastro-intestinal infections ($p = 0.052$), compared with unexposed infants (not shown).

Table 3. Comparison of clinical disease in infants of smoking and non-smoking mothers at 12 months of age†

Symptoms/ diagnosis, n (%)	Smokers (n = 26)	Non-smokers (n = 56)	p value*
Persistent nasal congestion/ discharge (without a cold)	8/26 (30.8)	5/56 (8.9)	0.021
Acute upper respiratory tract infection (URTI)	22/26 (84.6)	52/56 (92.9)	0.242
History of wheeze with chest infection	9/26 (34.6)	11/56 (19.6)	0.142
Diagnosis of bronchiolitis	5/26 (19.2)	5/56 (8.9)	0.275
Persistent cough (>2 weeks)	8/26 (30.8)	14/56 (25.0)	0.583
Diagnosis of asthma	0/26	1/56 (1.8)	1.000
Ear infection (diagnosed)‡	1/26 (3.8)	8/56 (14.3)	0.260
Atopic dermatitis	12/26 (46.2)	29/56 (51.8)	0.635
Food allergy	7/26 (26.9)	18/56 (32.1)	0.633

* $p < 0.05$ was considered a significant difference between the groups (see value in bold).

†Significant differences between the groups for categorical variables were determined by Pearson's chi-square, or Fisher's exact test when expected frequency was <5.

‡Ear disorders included acute infections and serous otitis media (with or without grommets).

Table 2. Specific antibody titres for saliva samples at 3 and 12 months of age to OMP6 of NTHI and pneumococcal serotype 14 in the smoking group compared with non-smokers (controls)

Antibodies	Smokers	Non-smokers	p value*
OMP6 (3 m) (AEU), median (IQR)	0.31 (0.19, 1.15) (29/32)	0.50 (0.22, 1.55) (40/45)	0.357
OMP6 (12 m) (AEU), median (IQR)	0.30 (0.11, 0.56) (13/17)	0.48 (0.17, 1.00) (36/44)	0.232
PS serotype 14 (3 m) (ng/ml), median (IQR)	25.00 (12.5, 40.00) (35/37)	20.00 (12.00, 32.00) (54/55)	0.315
PS serotype 14 (12 m) (ng/ml), median (IQR)	22.00 (16.75, 29.25) (20/22)	15.00 (9.00, 26.5) (48/49)	0.202

*p values result from Mann-Whitney U-tests of differences between the two groups.

The number of responders for each assay is also indicated in brackets (n/n). $p < 0.05$ was considered significant.

NTHI, non-typeable *Haemophilus influenzae*; OMP6, outer membrane protein 6; AEU, arbitrary ELISA units; PS, pneumococcal polysaccharide; IQR, interquartile range.

Table 4. Sensitization patterns of children at 12 months of age: comparison of infants of smokers and non-smoking mothers†

Sensitization	Infants of Smokers (n = 26)	Infants of non-smokers (n = 54)	p value*
Positive skin-prick test, n (%)			
Whole egg (fresh)	5 (19.2)	11 (20.4)	0.905
Cows milk	0 (0)	1 (1.9)	0.485
Peanut	0 (0)	2 (3.7)	0.320
Cat pelt	0 (0)	2 (3.7)	0.320
Rye grass	0 (0)	0 (0)	—
Mould mix	0 (0)	0 (0)	—
House dust mite	0 (0)	2 (3.7)	0.320
Grass mix	0 (0)	0 (0)	—

*p < 0.05 was considered a significant difference between the groups.

†There was no significant difference between the groups for any of the variables determined by Pearson's chi-square.

The relationship between smoking and early evidence of allergic disease

At 12 months, the rates of atopic dermatitis and food allergy (Table 3) were similar in the smoking and non-smoking groups. This was not affected by maternal allergy, feeding practices or birth order in logistic regression modelling.

There were also no differences in the rates of sensitization in the smoking and non-smoking groups at 12 months of age. In the first year of life, the most common allergen sensitization involved food allergens. Twenty-three per cent of this population had a positive SPT to one or more allergen, and the most common of these was to egg (20%) (Table 4). Only two infants (3.7%) showed evidence of aeroallergen (home dust mite) sensitization.

Relationship between IgA levels and clinical symptoms

Children who developed more chest infections in the first year of life had higher total IgA levels at 12 months of age (Kendall's tau b, 0.282, $p = 0.003$). A similar relationship was seen with the number of reported gastro-intestinal infections (Kendall's tau b, 0.248; $p = 0.021$), supporting the notion that mucosal exposure to infectious agents enhances local IgA production. There were no relationships between total (or specific) IgA levels and sensitization patterns at 3 or 12 months of age (not shown).

Discussion

In this study, we have shown that maternal smoking was associated with higher *total* salivary IgA at 12 months (but not 3 months) of age compared with infants of non-smoking mothers. Although it is possible that this may be the result

of the differences in the duration of breast feeding (which was shorter in the smoking group), it may also be attributed to added respiratory infections in the intervening period, as the total IgA levels at 12 months were higher in association with chest infections over the first year (Kendall tau b, 0.282, $p = 0.003$). This is also in keeping with observations that increased antigenic exposure (such as through chronic or recurrent microbial exposure) has been associated with higher levels of salivary IgA (10).

Almost all infants developed specific IgA antibodies to *Pneumococcus* and NTHI responses by 3 months of age. There were similar rates of specific IgA detection in infants of smokers compared with non-smokers, suggesting similar rates of carriage. A recent study by Greenberg et al. (21) demonstrated that exposure of children (1–59 months) to cigarette smoke at home was associated with an increased rate of carriage of *Streptococcus pneumoniae*, compared with children in homes with non-smoking parents. This study also found no difference in the colonization rates for *H. influenzae* between children with smoking parents and those with non-smoking parents. Independent measures of cotinine were not assessed. The differences between our findings and those of Greenberg et al. (21) may be a result of (i) the universal exposure to these bacteria (12) or (ii) the non-specific polyclonal increase in IgA rather than specific exposure (22).

There were also no statistically significant differences in the magnitude of specific IgA responses in infants of smokers and non-smokers at 3 and 12 months. This suggests that smoke exposure did not impair the capacity for local IgA production in this early period.

There was a trend for more respiratory pathology in infants of smoking mothers. This was significant ($p = 0.012$) for chronic upper airway symptoms (nasal congestion and discharge), but was only a trend for lower respiratory symptoms (wheezy chest infections). This is consistent with the literature suggesting increased risk of respiratory infection and wheeze (23–27). Our findings could indicate that smoking increases the early susceptibility to infection, thereby increasing the subsequent IgA responses. Our recent observations that maternal smoking attenuates neonatal TLR-mediated microbial responses support the notion that smoking induces a relative immunodeficiency (Noakes et al., in press). Alternatively, the observations could reflect the direct irritant effects of smoking, which

cause local inflammation, thereby increasing the risk of local infection (mucosal disruption) and IgA production.

Local encounter with noxious environmental factors, such as cigarette smoke and respiratory pathogens, is likely to influence the development of immune networks in the airways (28). Higher mucosal IgA levels in infants of smokers in our study support this. Others have also noted evidence of mucosal dysregulation in infants exposed to cigarette smoke, with increased susceptibility to infection and sudden infant death syndrome (SIDS) (20). This could also reflect increased adherence of respiratory pathogens to buccal epithelial cells with tobacco-smoke exposure (29).

Events at mucosal surfaces can have important effects not only on local responses, but also on responses at other sites (30, 31). Exposure to cigarette smoke at mucosal surfaces has well-documented irritant effects, and can result in local epithelial damage (32) and inflammatory changes with decreased mucous production (33). These local effects are also likely to influence systemic immune programming, including responses to allergens and microbial agents. Despite this, there has been no consistent evidence that parental smoking is associated with an increased risk of sensitization or allergic disease [systematic review in Ref. (34)]. More recently, Kulig et al. (7) observed that maternal smoking is associated with a significantly higher risk of sensitization to food allergens (OR: 2.3, 95% CI: 1.1–4.6) compared with unexposed children. However, although our study suggested that smoking had mucosal effects (higher IgA) we did not see any differences in the rates of allergic sensitization to foods in this study. Egg sensitization is most common at this age, and was seen in almost identical rates in infants of smokers (19.2%) and non-smokers (20.4%), with no trend to suggest that a biologically significant difference may have been missed because of the population size (type 2 error). There were also no differences (or trends) in other early manifestations of the atopic phenotype (namely atopic dermatitis and food allergy). Although food allergen sensitization is a good indicator of the atopic phenotype, this does not exclude potential differences in aeroallergen responses with age. We also saw no associations between allergic sensitization and IgA levels. Although Bottcher et al. (35) noted lower total secretory IgA in the saliva of infants with allergen sensitization or atopic parents (in a similar population size), we did not observe this.

As with all studies that examine the effects of smoking in humans, this study has a number of

limitations related to the ethical inability to conduct blinded randomized controlled trials. Despite efforts to match the groups at recruitment, the smokers went on to choose different infant feeding practices [as also observed by others (18)], and were less likely to return to follow-up assessments. As indicated, it is difficult to exclude subsequent differences compared with the population that failed to follow up, although their baseline characteristics were similar. We also recognize that there are a range of potential variables, such as differences in allergen exposure, lower respiratory illness, day-care attendance and postnatal diet, which may confound or influence the relationship of smoking and allergic disease risk. To assess these factors within the scope and logistics of this study, parents completed prospective calendar diaries which were clarified by interview questionnaires at the clinical visits (see Methods). This enabled us to identify differences in feeding practices in the smoking group which were accounted for in subsequent analyses. Finally, another difficulty in all studies of this nature is the accurate documentation of infectious episodes and respiratory symptoms which can be non-specific. Because of the difficulty relying on parental reporting of infections, we did not rely on one parameter, but examined effects on a range of measures that could reflect increased respiratory pathology (such as the range of symptoms, doctor visits, medication use and specific diagnosis). Collectively, these measures suggest an increase in symptoms and pathology in the smoke exposure group, which is in keeping with many other studies [reviewed in Ref. (36)]. It was not feasible, practical or affordable to conduct more detailed microbial and environmental surveillance in this study.

In summary, maternal smoking was associated with an increase in the total infant salivary IgA by 12 months of age. As IgA levels showed a significant correlation with chest infections, we speculate that the higher IgA levels were secondary to chronic or recurrent airway inflammation in this group. There was no evidence that maternal smoking inhibited the production of anti-microbial IgA, suggesting that other factors, such as chronic irritant effects of tobacco smoke, are responsible for the increased susceptibility to infection in these infants. Differences in feeding practices may have contributed to this directly or indirectly. It is also possible that antenatal effects on cellular immune function (Noakes et al., in press) could contribute to the increased susceptibility to infection in infants of smokers. We did not see any evidence of smoking having effects on early allergen sensitization or atopic dermatitis,

although definitive long-term studies are still needed to examine the effects on the development of aeroallergen sensitization and if this impacts subsequent respiratory disease. However, it is arguably more important that research funding is directed to better strategies for smoking cessation and prevention, with a particular focus on avoiding early life exposure when the adverse effects are greatest.

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