ORIGINAL ARTICLE

# Antibacterial antibody responses associated with the development of asthma in house dust mite-sensitised and non-sensitised children

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## ABSTRACT

**Background** Infants who develop house dust mite (HDM) allergy and HDM-sensitised children with severe persistent asthma have low antibody responses to the P6 antigen of *Haemophilus influenzae*.

**Objective** To measure the development of antibody to two ubiquitous bacteria of the respiratory mucosa in a prospective birth cohort at high risk of allergic disease and to assess which responses are associated with asthma and atopy.

**Methods** IgG1 and IgG4 antibody to *H influenzae* (P4 and P6) and *Streptoccocus pneumoniae* (PspA and PspC) surface antigens was measured in yearly blood samples of children aged 1—5 years. IgE to the P6 antigen was examined for the 5-year group. The children were stratified based on HDM sensitisation and asthma at 5 years of age.

**Results** HDM-sensitised children had lower IgG1 antibody titres to the bacterial antigens, and early responses (<3 years and before the development of HDM sensitisation and asthma) corrected for multiple antigens were significantly reduced for P4, P6 and PspC ($p=0.008$, $p=0.004$ and $p=0.028$, respectively). Similar associations with asthma were also found ($p=0.008$, $p=0.004$ and $p=0.032$ for P4, P6 and PspC, respectively). The IgG4 antibody titre and prevalence were similar in both HDM-sensitised and non-sensitised groups, but sensitised children had a slower downregulation of the IgG4 response. Children with asthma (27/145 at 5 years) had lower anti-P6 IgE responses ($p<0.05$).

**Conclusions** HDM-sensitised children have early defective antibody responses to bacteria that are associated with asthma. Surprisingly, antibacterial IgE was associated with a reduced risk for asthma.

### Key messages

**What is the key question?**
- Do children who develop asthma and house dust mite sensitisation have altered immune responses to colonising bacteria early in life?

**What is the bottom line?**
- Early impaired antibacterial IgG1 antibody is associated with atopy and asthma. The development of antibacterial IgE antibody is associated with protection from asthma.

**Why read on?**
- Altered immune responses in the development of asthma are not just restricted to viruses.

## INTRODUCTION

There is a strong association between allergy to indoor allergens and the development of asthma, but as many as 50% of children with high IgE antibodies do not develop disease.[1] It is becoming apparent that the development of allergy and asthma is associated with deviated immune responses to respiratory viruses[2 3] and mucosal bacteria[4 5] which, through several proposed mechanisms, could increase susceptibility to sensitisation and disease.[6 7] In keeping with this, children with atopic asthma experience more frequent and severe rhinovirus-induced illnesses[8] and children who develop wheeze have increased bacterial colonisation of the nasopharynx as neonates.[9] Additionally, the airways of people with asthma, once thought to be sterile, carry a larger load of *Haemophilus influenzae* than those of healthy people.[10] The high prevalence of rhinovirus during asthma exacerbations[8] and increased asthma found in children who develop wheezy rhinovirus infections early in life[11] has provided a platform for ongoing investigations. The study of bacteria has not been as extensive, but colonisation with *H influenzae* has been found to precede wheezing and also to be associated with exacerbations and to be independent of viral infection.[12]

In keeping with these associations, IgG1 antibody responses to the protective P6 antigen of *H influenzae* have been found to be decreased in 2-year-old children who developed atopy at 5 years.[4] House dust mite (HDM)-sensitised children with frequent or persistent asthma exacerbations were also shown to have lower anti-P6 IgG1 titres than children with infrequent episodic asthma.[5] One-third of atopic children and adults had the Th2-dependent IgG4 anti-P6 antibody.[4] Antibacterial IgE antibody has also been found in both atopic and non-atopic subjects[4 13] and, counterintuitively, for atopic subjects was inversely related to the risk of asthma.[13]

This paper describes the development of the antibody responses to the P4 and P6 antigens of *H influenzae* and pneumococcal surface proteins A and C (PspA and PspC) of *Streptococcus pneumoniae* in a high-risk cohort of children. The primary aim of the study was to determine if the IgG antibody responses that we had noted previously in our studies with P6[4 5] occurred for different antigens and for different bacteria, and to observe when they occurred. The second aim was to determine if the inverse association of IgE antibody and asthma found in another cohort[13] also occurred in this cohort and if it was present early (5 years).

## METHODS

### Characteristics of the study population

Antibodies were examined in the plasma of children from a prospective birth cohort (Childhood Asthma Study) at 1, 2, 3, 4 and 5 years of age. A total of 263 children at high risk of atopy (at least one parent with a doctor-diagnosed history of hay fever, asthma or eczema) were recruited prenatally between 1996 and 1999.[14] Skin prick tests (SPT) to common allergens were performed. A third of this population was HDM-sensitive[14] with few (<5%) being SPT-positive only to other allergens. The SPT-positive infants were selected solely on the presence of a positive skin test to HDM extract (≥2.5 mm) and the SPT-negative infants were selected on the absence of a skin test response to HDM at 5 years of age (table 1). Children without SPT information at 5 years were not included in the present study, and plasma and SPT data were available for 191, 188, 187, 176 and 145 children, respectively, from years 1 to 5. The selected children were representative of the whole cohort based on variables such as HDM sensitisation, current asthma and gender. Asthma was defined as current doctor-diagnosed asthma and wheeze in the 12 months before the 3-, 4- or 5-year visit. Symptomatic infections, which were mostly viral, were recorded and reported by Kusel *et al*[15] and are summarised in the online supplement. Samples were not collected for bacterial (and viral) carriage.

### Antigens

The bacterial antigens were conserved surface proteins known to elicit protective immune responses that should have similar requirements for antigen processing and T cell responses as the allergens. Details of the antigen preparations for P4 and P6 from *H influenzae*, PspA1, PspA2 and PspC from *S pneumoniae* and the major HDM allergens are outlined in the online supplement.

### Quantification of antibody binding

The IgE, IgG1 and IgG4 antibody isotypes to the bacterial antigens and IgE binding to the major HDM allergens Der p 1 and Der p 2 were assayed by a procedure using humanised chimeric antibodies for absolute quantitation.[16 17] A microtitre plate dissociation-enhanced immunofluorescence assay (DELFIA) was performed where antigen coating was standardised by capturing His-tagged recombinant antigens with anti-His monoclonal antibody.[4] The assay was calibrated by interpolating the results from a titration curve constructed with recombinant Der p 2 captured by the same procedure and a standardised (IU/ml) humanised chimeric IgE, IgG1 or IgG4 anti-Der p 2 antibody (Indoor Biotechnologies, Charlottesville, Virginia, USA). Antibody values below the limits of detection were ascribed a value of half the limit of detection.

Total IgE was quantitated by ImmunoCAP (Phadia, Uppsala, Sweden) in the Immunology Department at Princess Margaret Hospital. IgG1 was quantitated by DELFIA using antibody pairs from BD Biosciences (San Jose, California, USA) and an IgG1 plasma standard curve ranging from 0.02 to 1.64 mg/ml.

### Statistical analysis

Differences in the level of antibody binding by selected groups (SPT+ /SPT− or asthmatic/non-asthmatic at 5 years) were initially compared by Mann–Whitney or $\chi^2$ tests. Correlations were studied using the Spearman rank correlation. In order to investigate the association of asthma and HDM sensitisation at age 5 with the levels of antibody binding in the first 3 years of life and the first 5 years of life, further analysis with generalised estimating equations (GEE) were employed. The analyses accounted for inherent covariance in the same subject. In the GEE regression analyses, levels of antibody binding were log-transformed to approximate a normal distribution. All analyses were performed using GraphPad Prism Software (La Jolla, California, USA) and StataIC V.11 (StataCorp).

## RESULTS

### IgG1 antibody development

IgG1 antibody titres to P4 and P6 of *H influenzae* and PspA (PspA1 and PspA2) and PspC of *S pneumoniae* were measured in samples taken from 1 to 5 years (figure 1, table 2). Atopic children (SPT-positive to HDM at 5 years) showed decreased titres to P4 early in development at years 1 and 2. The mean P6 titres of the atopic group were about half of the non-atopic group for all years. The prevalence in the cohort (figure 2A) shows that responses were slow to develop with only 70% showing detectable titres at year 4. The IgG1 titres of atopic children to the *S pneumoniae* antigens showed reduced responses from years 2 to 3 for PspA (49% of SPT-negative) and from years 1 to 2 for PspC (34% of SPT-negative). There was a significantly reduced prevalence of antibodies at year 2 for PspC but not PspA. PspC induced high titres earlier than the other antigens and reached 10–20-fold higher titres than P4 and PspA at year 5, and these in turn were approximately 10-fold higher than the titres to P6.

**Table 1** Characteristics of the study population

| | 1 year old | 2 years old | 3 years old | 4 years old | 5 years old |
|---|---|---|---|---|---|
| Boy/girls, n (%) | | | | | |
| SPT+ | 44/20 (68/32) | 44/19 (70/30) | 43/19 (69/31) | 41/19 (68/32) | 33/16 (66/34) |
| SPT− | 62/65 (49/51)** | 62/63 (50/50)** | 62/63 (50/50)* | 60/59 (50/50)* | 46/50 (48/52)* |
| Asthma, n (%) | | | | | |
| SPT+ | ND | ND | 5/62 (8.1) | 12/60 (20.0) | 14/49 (28.6) |
| SPT− | ND | ND | 13/125 (10.4) | 11/119 (9.2*) | 13/96 (13.5*) |

The values in parentheses are the percentage of boys/girls or the percentage of children with asthma.
Details of when asthma first started for individual subjects is outlined in the supplementary methods.
*p<0.05, **p<0.001 between SPT-positive and SPT-negative groups.
ND, not determined; SPT, skin prick test.

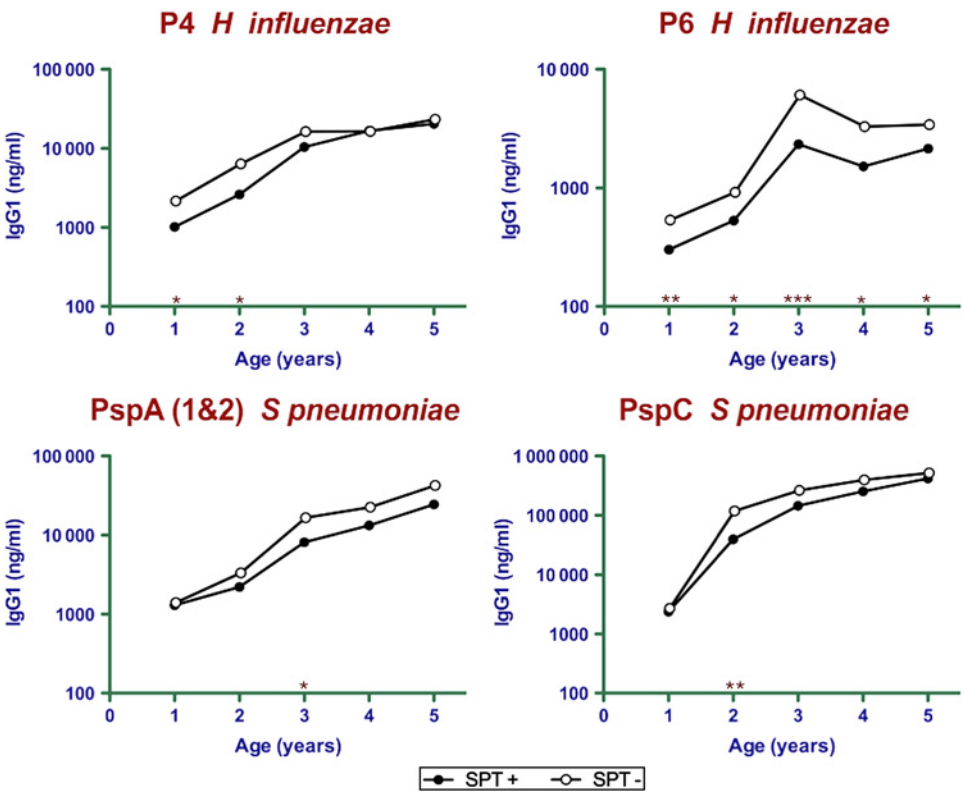


**Figure 1** Development of IgG1 antibody (ng/ml) to the P4 and P6 antigens of *Haemophilus influenzae* and the PspA and PspC antigens of *Streptococcus pneumoniae* in plasma collected from children aged 1—5 years. The mean antibody titre of SPT-positive (filled circles) and SPT-negative (open circles) children is shown. *p<0.05, **p<0.01, ***p<0.001 between-group comparisons (SPT-positive vs SPT-negative). Lower IgG1 titres to P6 at 1, 3 and 5 years and PspC at 2 years were significant after correcting for multiple testing. The geometric mean and 95% CIs are shown in table 2. SPT, skin prick test.

When the children were stratified for asthma at 5 years, the group with asthma had lower anti-P6 IgG1 titres from age 2 years (p<0.05 to p=0.005) and lower anti-P4 at age 5 years (p<0.01, table 3). The mean anti-PspC IgG1 titres at 2 years were 2.5-fold lower in the asthma group, and a similar trend was observed for the anti-PspA titres between the ages of 2 and 4 years and for anti-P4 at age 4 years.

Since the early time points were shown to be important in the initial analyses, we further analysed the associations of HDM sensitisation and asthma at age 5 years with IgG1 antibody responses in the first 3 years of life using GEE regression approaches. The early time points in the first 3 years of life were also before the majority of children developed asthma. Low IgG1 titres to P4, P6 and PspC in the first 3 years of life were significantly associated with HDM sensitisation at age 5, with a significance of p=0.008, p=0.004 and p=0.028, respectively, after correcting for multiple testing. Likewise, low anti-P4, anti-P6 and anti-PspC IgG1 antibody in the first 3 years of life were significantly associated with asthma at age 5, with a significance of p=0.008, p=0.004 and p=0.032, respectively. Similar associations with HDM sensitisation and asthma and low IgG1 antibody responses to P4, P6 and PspC were observed in the first 5 years of life.

Overall, the IgG1 titres to P4 and P6 in different individuals were highly and very significantly correlated (p<0.001), as were the titres to the PspA and PspC antigens (p<0.005; see table 1 in online supplement). However, the titres to the antigens of the

**Table 2** IgG1 antibody titre stratified by HDM sensitisation (SPT-positive or SPT-negative at 5 years of age)

| | 1 year old | 2 years old | 3 years old | 4 years old | 5 years old |
|---|---|---|---|---|---|
| P4 | | | | | |
| SPT+ | 1022 (666 to 1522) | 2617 (1494 to 4583) | 10 458 (6676 to 16 383) | 16 698 (8532 to 32 681) | 20 447 (12 932 to 32 330) |
| SPT− | 2168 (1483 to 3168) | 6393 (4044 to 10 106) | 16 391 (12 020 to 22 353) | 16 423 (10 123 to 26 643) | 23 354 (16 594 to 32 866) |
| p Value | 0.0406 | 0.0353 | 0.1010 | 0.9597 | 0.4891 |
| P6 | | | | | |
| SPT+ | 303 (256 to 358) | 531 (376 to 750) | 2337 (1549 to 3525) | 1521 (977 to 2368) | 2151 (1399 to 3309) |
| SPT− | 538 (425 to 683) | 922 (682 to 1246) | 6086 (4499 to 8233) | 3297 (2295 to 4737) | 3419 (2515 to 4648) |
| p Value | 0.0056 | 0.0434 | <0.001 | 0.0122 | 0.0487 |
| PspA (1 and 2) | | | | | |
| SPT+ | 1310 (997 to 1720) | 2222 (1437 to 3437) | 8144 (4902 to 13 530) | 13 317 (6724 to 26 373) | 24 524 (11 799 to 50 974) |
| SPT− | 1406 (1141 to 1733) | 3347 (2308 to 4854) | 16 657 (11 297 to 24 560) | 22 581 (13 473 to 37 846) | 42 513 (26 510 to 68 174) |
| p Value | 0.7825 | 0.2986 | 0.0234 | 0.2498 | 0.1971 |
| PspC | | | | | |
| SPT+ | 2391 (1632 to 3504) | 39 702 (20 983 to 75 121) | 144 959 (76 288 to 275 444) | 254 361 (151 998 to 425 663) | 419 481 (250 989 to 701 081) |
| SPT− | 2719 (2040 to 3625) | 118 443 (81 380 to 172 387) | 263 382 (182 130 to 380 882) | 396 132 (280 887 to 558 661) | 516 873 (402 468 to 663 797) |
| p Value | 0.6685 | 0.0042 | 0.1644 | 0.1463 | 0.8886 |

The value is the geometric mean of the IgG1 antibody titre (ng/ml) with the CI of antibody binding in parentheses.
Comparisons between SPT-positive and SPT-negative groups by the Mann—Whitney test are shown.
p Values <0.0125 are considered significant after correcting for multiple testing.
HDM, house dust mite; SPT, skin prick test.

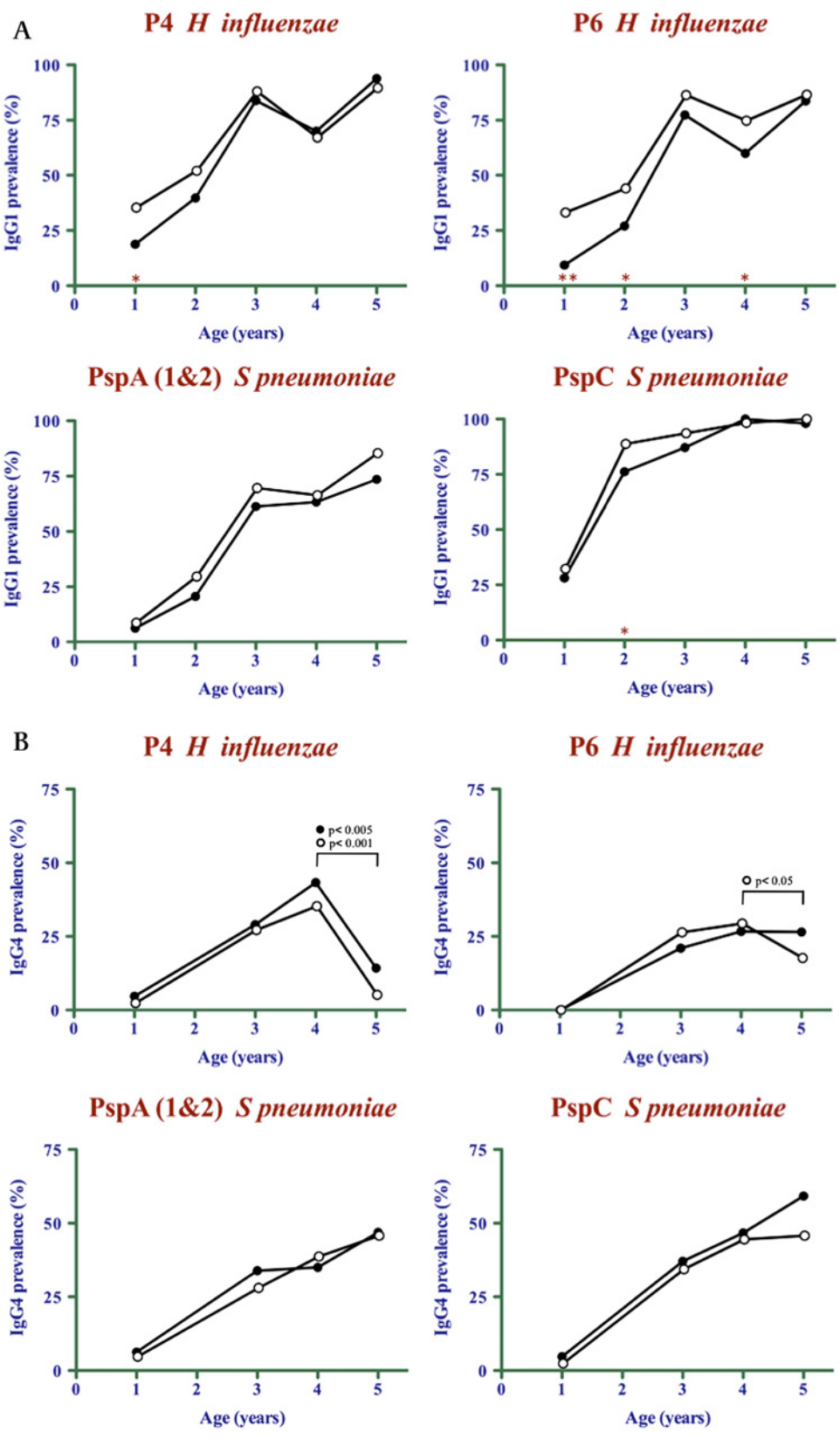


**Figure 2** Prevalence (%) of (A) IgG1 and (B) IgG4 antibody binding to antigens of *Haemophilus influenzae* and *Streptococcus pneumoniae* in plasma collected from SPT-positive (filled circles) and SPT-negative (open circles) children aged 1—5 years. *p<0.05, **p<0.001 between-group comparisons (SPT-positive vs SPT-negative).

different bacteria were not well correlated, although some time points were significant (p<0.05).

### IgG4 development

The titres of IgG4 antibody were low for most subjects (see table 2 in online supplement) and there were many non-responders, so the analysis has been presented as prevalence (figure 2B). No differences between the IgG4 responses of HDM-sensitised and non-sensitised subjects were found for P4, P6, PspA or PspC. There was, however, a gender difference for the response to P6 at 5 years of age with 20/79 boys and 8/66 girls having detectable titres (p<0.05). The

**Table 3** IgG1 antibody titre stratified by asthma at 5 years of age

| | 1 year old | 2 years old | 3 years old | 4 years old | 5 years old |
|---|---|---|---|---|---|
| P4 | | | | | |
| Asthma | 1241 (605 to 2543) | 2771 (1133 to 6777) | 12 938 (6224 to 26 894) | 8334 (2926 to 23 738) | 10 812 (5605 to 20 859) |
| No asthma | 1772 (1292 to 2431) | 5262 (3541 to 7820) | 14 167 (10 764 to 18 645) | 18 648 (12 287 to 28 301) | 26 358 (19 658 to 35 341) |
| p Value | 0.4818 | 0.2706 | 0.7713 | 0.1667 | 0.0081 |
| P6 | | | | | |
| Asthma | 353 (260 to 480) | 423 (257 to 697) | 2499 (1068 to 5847) | 900 (465 to 1741) | 1440 (750 to 2766) |
| No asthma | 461 (380 to 559) | 853 (659 to 1105) | 4881 (3776 to 6308) | 3060 (2253 to 4156) | 3438 (2640 to 4475) |
| p Value | 0.5556 | 0.0248 | 0.0318 | 0.0025 | 0.0099 |
| PspA (1 and 2) | | | | | |
| Asthma | 1235 (800 to 1905) | 1951 (966 to 3941) | 7983 (3254 to 19 584) | 11 159 (3678 to 33 854) | 28 167 (9531 to 83 242) |
| No asthma | 1397 (1167 to 1673) | 3144 (2291 to 4313) | 14 600 (10 449 to 20 401) | 20 777 (13 360 to 32 313) | 37 611 (24 638 to 57 413) |
| p Value | 0.4136 | 0.1648 | 0.1906 | 0.2258 | 0.5736 |
| PspC | | | | | |
| Asthma | 1839 (1104 to 3062) | 38 192 (14 143 to 103 133) | 234 369 (93 344 to 588 457) | 321 962 (153 239 to 676 456) | 43 257 (212 726 to 878 745) |
| No asthma | 2758 (2142 to 3553) | 96 051 (67 665 to 136 346) | 214 956 (151 420 to 305 154) | 345 056 (253 117 to 470 391) | 493 713 (385 567 to 632 192) |
| p Value | 0.2588 | 0.0582 | 0.8011 | 0.7548 | 0.9737 |

The value is the geometric mean of the IgG1 antibody titre (ng/ml) with the CI of antibody binding in parentheses.
Comparisons between SPT-positive and SPT-negative groups by the Mann–Whitney test are shown.
p Values <0.0125 are considered significant after correcting for multiple testing.

responses of the boys and girls to the other antigens were very similar.

The prevalence of IgG4 antibody to P4 and P6 increased to year 4 but then fell—markedly for P4, and the decrease for P6 was only statistically significant for the non-atopic subjects. The prevalence of IgG4 antibody to the *S pneumoniae* antigens did not show this fall and increased progressively from year 1 to 5.

Neither the prevalence of IgG4 antibodies nor the titres were associated with the development of asthma. The titres of IgG1 and IgG4 to P6 and PspA in fact had a consistently positive correlation (r=0.218, p<0.008 and r=0.491, p<0.001 at year 5 for P6 and PspA, respectively; see table 3 in online supplement). The titres to PspC were significantly correlated at some time points.

Overall, the titres of IgG4 to all of the antigens correlated strongly with each other, including comparisons of the *H influenzae* and *S pneumoniae* antigens (p<0.005 to p<0.001; see table 4 in online supplement).

## IgE antibody

IgE antibody assays required low dilutions of serum so they were performed for the year 5 group and measurements were only made with P6. The IgE was detected in 64% of serum samples and there was no difference in titre or the prevalence of responses in the SPT-positive and SPT-negative groups. A number of the higher titres were >10 ng/ml. When analysed for asthma, non-asthmatic children had significantly higher anti-P6 IgE titres (p<0.05; figure 3A). In contrast, the children with asthma had significantly higher IgE titres to the major HDM allergens (p<0.001). No correlation was found between the anti-HDM and anti-P6 IgE titres (r=−0.08, p=0.325) for the whole cohort but, importantly, there was an inverse relationship between HDM and bacterial IgE titres for the SPT-positive group (r=−0.342, p<0.05).

The anti-P6 IgE and IgG1 antibody responses were strongly correlated (figure 3B), but there was no association between the IgE and IgG4 response.

## Immunoglobulin measurements

Total IgE was also measured at age 5 and there was a negative correlation between the IgE immunoglobulin and anti-P6 IgE titre for the SPT-positive group (r=−0.329, p<0.05) and a positive trend was observed for the SPT-negative group (r=0.408, p=0.09). The expected strong correlation between the anti-Der p 1 and Der p 2 IgE titre and total IgE (r=0.644, p<0.001) was found for SPT-positive children.

IgG1 immunoglobulin was measured at age 3 to compare with the early antibody results. Plasma was unavailable at ages 1 and 2. A weak positive correlation between the anti-P4 IgG1 and total IgG1 immunoglobulin was found (r=0.259, p<0.005), but

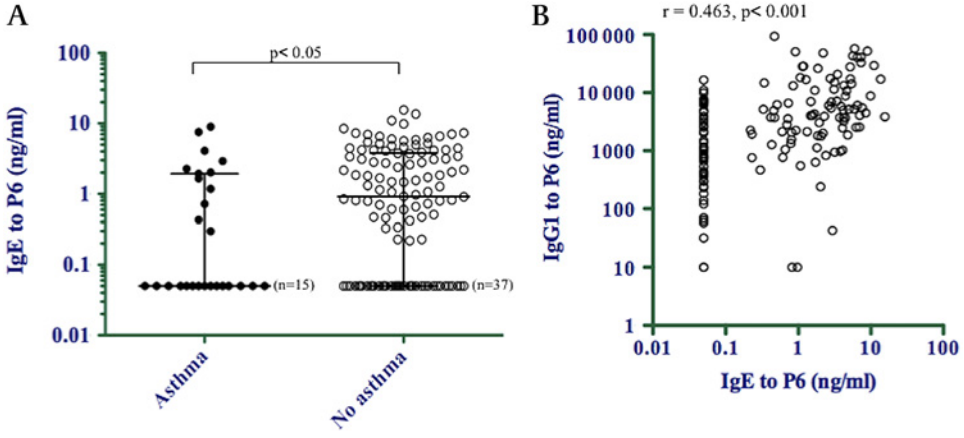


**Figure 3** (A) Anti-P6 IgE antibody production in asthmatic and non-asthmatic 5-year-old children. The number of children with undetectable IgE antibody is shown in parenthesis. (B) Correlation of IgG1 and IgE antibody binding (ng/ml) to P6.

no correlation was found for the other antigens. The IgG1 immunoglobulin levels were similar between the SPT-positive and SPT-negative groups.

## DISCUSSION

The IgG1 results confirm the earlier observation with a subset of this cohort showing that there is an early deficiency in the antibody responses of atopic children to the P6 antigen of *H influenzae*. The results are now extended to show deficiencies in response to the P4 antigen of *H influenzae* and the PspA and PspC antigens of *S pneumoniae*. The most significant differences between the HDM-sensitised and non- sensitised groups were found at either 2 or 3 years, when a rapid increase from infant levels occurred. The decreased titres to the *H influenzae* antigens and the PspC antigen of *S pneumoniae* were associated with the development of asthma. This corroborates the finding with an emergency department cohort showing that children with frequent and persistent exacerbations of asthma have low anti-P6 IgG1 titres.[5] The lower IgG1 titres to P4, P6 and PspC were found at a time that precedes the development of anti-allergen IgE and before asthma was apparent for many children, so they were not the result of bystander effects of prolonged allergic responses. Since IgG switching in humans can be mediated by varying combinations of cytokines produced by Th1, Th2, Th17 and regulatory cells, the deficiencies cannot be easily attributed to a particular T cell lineage.[18] They were not generally associated with reduced immunoglobulin levels, indicating that the defects were only found in certain types of antibody responses.

It was previously shown that about 30% of HDM-sensitised adults and children of school age have IgG4 antibody to the P6 antigen, a response rarely found in non-sensitised subjects.[4] The results here show that the IgG4 titres in the preschool age groups were low, with no significant association with sensitisation or asthma for any of the antigens. There was also no association of asthma severity with IgG4 titres noted in emergency department admissions.[5] It appears that the persistence of the IgG4 antibodies in HDM-sensitised subjects only becomes apparent at an older age and is not associated with disease. The positive association of IgG4 and IgG1 titres is also consistent with this. The production of IgG4 is absolutely dependent on Th2 cell help, so it is a marker for Th2 responses. It is interesting that, for the *H influenzae* antigens, the prevalence of IgG4 fell from 4 to 5 years; this was marked for P4 and was only found in non-sensitised children for P6. Taken together with the results from older subjects,[4] it seems that IgG4 is downregulated in nearly all non-sensitised subjects but persists in some atopic individuals. It is, however, the low IgG1 and not persistent IgG4 that associates with disease. The bias to the male sex found previously for IgG4 antibody to P6 was also evident, but there was no suggestion that it occurred for the other antigens, even P4. It has been shown that P6 (but not P4) stimulates macrophages by a TLR2-dependent mechanism,[19] so there may be a sex difference in some aspect of innate immunity.

The detection of IgE antibody to bacteria has been described many times. The results here provide estimates of absolute quantitation of titres to defined antigens. The responses could reach titres typical of the IgE responses of many people to major allergens. This infers that the IgE may be significant in the pathophysiology, and also that the immunological events that underpin the induction of antibacterial IgE, such as Th2 cytokine production, could be as substantial as those induced by allergen. The responses, however, suggest that IgE is associated with protection from asthma. They corroborate those from an independent large unselected birth cohort where IgE antibody titres to both *H influenzae* and *S pneumoniae* antigens were inversely associated with the risk of asthma,[13] and subjects with the highest titres of antibacterial IgE had the lowest risk of asthma. Although not initially appreciated, the boosting of IgE during convalescence from asthma attacks[5] might be part of a protective response since the antibacterial IgE titres at admission were in fact low. The current study here independently confirms the negative association and also shows that it is evident when asthma first becomes prevalent in children. The protective association might seem paradoxical, but there are at least two general potential pathways. The first is that Th2 cytokines[20] and Th2 cytokine-induced chemokines[21] can have powerful anti-inflammatory effects. The sustained Th2 response to colonising bacteria could downregulate Th1 and Th17 responses which could also have a role in the production of disease.[22] The second is that the IgE antibody itself can enhance antigen capture via Fcϵ receptors on antigen-presenting cells and increase the recruitment of antibacterial T cells which, on balance, have been shown to be polarised to Th1,[23] and these could alter the education of dendritic cells and hence signals to allergen-specific T cells.[24] The inverse association of the IgE to Der p 1 and Der p 2 with the antibacterial IgE points to a downregulation of the anti-allergen Th2 response. The IgE antibodies to both *S pneumoniae* and *H influenzae* are also highly correlated with each other,[13] so there seems to be a group of people with a propensity to make IgE antibody to bacterial antigens and they have a reduced risk of asthma. The recent finding that exposure to a diverse range of microbes is inversely related to the risk of asthma points to the possibility that the protective association of antimicrobial IgE antibody and asthma could extend to other organisms.[25]

The point prevalence rates for the detection of colonisation with *H influenzae* and *S pneumoniae* are conservatively about 30% for each organism,[26 27] with the colonisation episodes typically lasting about 5 weeks. Since the antigens examined have conserved sequences and are found in all isolates, repeated stimulation of the immune response would be expected from the successive waves of colonisation with these organisms every year. Immune responses to P4,[28] P6[29] and to PspA and PspC[30] are associated with protective immunity, so responses against them could have a bearing on colonisation. The reported increased colonisation of *H influenzae* in subjects with asthma[13] would fit the defective IgG1 response reported here.

The low IgG antibody response described here could enhance atopy and asthma by increasing the susceptibility to bacterial infection, and hence tissue damage, by exposure to pharmacologically active bacterial products.[6] It is also possible that underlying immune responses to the bacteria and allergens influence immune responses to each other when they are co-presented at the mucosa to increase the degree of sensitisation. This has been shown, at least for allergens, to be able to increase allergic sensitisation.[31]

It is also possible that the altered antibody responses are just markers that show people with atopy and asthma have alterations in an aspect of their mucosal immune system that extends beyond the response to allergens. These could also extend to virus infections, particularly rhinoviruses which have been implicated as the most frequent virus facilitating both the development and exacerbation of asthma. Recent data have, however, shown that increased bacterial colonisation, independent of viral infection and including both *H influenzae* and *S pneumoniae*, has been associated with susceptibility to asthma[9] and wheezing attacks,[12] and that young children with asthma

have increased carriage of pneumococcus.[32] This is in addition to studies showing that subjects with asthma have an increased susceptibility to invasive bacterial infection, including pneumococcus.[33 34] Bacterial colonisation was not measured in the current cohort but, in keeping with many reports, virus infection is associated with the development of asthma and is especially associated with atopy.[12 15]

The results here are from a high-risk cohort in which at least one parent had an allergy-associated disease. This might limit the interpretation of the results, but it extends previous observations in an emergency department cohort in which reduced IgG antibody was found in children with frequent and persistent asthma exacerbations.[5] The association of IgE antibody with *H influenzae* and *S pneumoniae* was previously ascertained in a large unselected birth cohort.[13]

This study has focused on establishing the association between altered immune responses to colonising bacteria and the development of atopy and asthma. It has done this by extending previous observations with the P6 antigens of *H influenzae* to another protective antigen P4 and to responses to *S pneumoniae*, and by verifying the associations with independent cohorts.[5 13] The development of vaccines against *S pneumoniae* and non-typable *H influenzae* is an area of active research. The *S pneumoniae* polysaccharide-*H influenzae* protein D conjugate vaccine has reduced colonisation with both these organisms,[35] so it might also be used to study how they affect allergy and asthma.

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**Competing interests** None.

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**Contributors** BJH and WRT were responsible for the conception and design of the study and for writing the manuscript. BJH performed the majority of the data analysis, aided by LYC, CEE and LJP. GZ provided expert statistical advice and assisted with the data analysis. BJH, TKH, W-AS and WRT were responsible for the design and validation of the conserved protein antigens used in the study. MMHK, PDS and PGH assisted in interpretation of the analysis and were involved in advice and feedback of the manuscript. All authors gave final approval of the version to be published.

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## SUPPLEMENTARY METHODS

### Characteristics of the Study Population

Virus infections could account for most symptomatic respiratory disease. [15] Twelve viruses (rhinoviruses, coxsackie viruses, echoviruses, enteroviruses, coronaviruses 229E and OC43, respiratory syncytial virus (RSV), influenza A and B, parainfluenza viruses 1-3 (PIF), adenoviruses, human metapneumovirus) and 2 bacteria (*Chlamydia pneumoniae* and *Mycoplasma pneumoniae*) were analysed from nasal aspirates by PCR following a reported respiratory infection in the first year. Rhinoviruses were the most common virus detected (48%), followed by RSV (10.9%), coronaviruses (5.8%), PIF (5.4%) and influenza (4.3%). [15]

44% (12/27) of the children with current asthma at 5 years did not have a history of asthma recorded for previous years. All of the SPT+ children who had doctor-diagnosed asthma at age 3 had asthma at age 4 and 5. In contrast 6/13 SPT- children who were diagnosed with asthma at 3 years of age did not present with asthma later. All the 3-year old asthmatics were followed up at age 4 but five asthmatic children (2 SPT+ and 3 SPT-) in the 4-year group were not followed up at age 5.

### Antigen preparation

Natural Der p 1 was purified from spent mite medium by antibody affinity chromatography using the 4C1 monoclonal antibody. The P4 and P6 outer membrane proteins of *H. influenzae* from the Eagen isolate and Der p 2.0101 were produced as fusion polypeptides with N-terminal hexa-histidine tags in pQE-80L (Novagen, Madison, USA) for P4 and P6 and pET-11d (Novagen) for Der p 2. PspA1 (family 1, clade 2) was derived from the pneumococcal strain Rx1 (aa 1-302), PspA2 (family 2, clade 3) from the V-24 strain (aa 1-410) and PspC (clade B) from the D39 strain (aa 1-445). The Psp proteins were cloned as fusion proteins with a C-terminal six-histidine tag in pET20b (Novagen). The pQE-80L, pET-11d and pET20b-based constructs were expressed in BL21 Star (DE3) pLysS (Novagen) using 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG), in the presence of 100 µg/ml ampicillin and 34 µg/ml chloramphenicol (Invitrogen Corp., Carlsbad, USA). The majority of the expressed recombinant proteins were purified under non-denaturing conditions using $Ni^{2+}$-nitrilotriacetic acid (Ni-NTA) agarose chromatography (Qiagen GmbH, Germany), according to the manufacturer's protocols. Der p 2.0101 was produced under denaturing conditions and refolded prior to Ni-NTA purification. Fractions containing the relevant proteins were pooled, dialyzed into 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mMEDTA, and applied to a Bio-Rad Macro-Prep cation (Der p 2, P4, PspC) or anion (P6, PspA1, PspA2) exchange support (Bio-Rad, Hercules, USA). Elution was achieved with a linear gradient of 100 to 500 mM NaCl in Tris-HCl, pH 7.4, 2 mM EDTA. Fractions containing the relevant protein were pooled and further purified using size exclusion chromatography by applying the samples to either a HiPrep 26/60 Sephacryl HR 100 (Der p 1, Der p 2, P4 and P6) or HR S200 (PspA1, PspA2, PspC) column (GE Healthcare Life Sciences, Buckinghamshire, UK). A single peak was obtained for each of the proteins. Finally, the proteins were sterilized and endotoxin removed using 0.2-µm Mustang E filters (Pall Life Sciences, Portsmouth, UK). The purities of all the proteins were checked on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and the concentrations determined using the optical density at 280 nm (OD280) measurements and extinction coefficients.

**Supplementary table 1** IgG1 antibody correlation

| | 1yo | 2yo | 3yo | 4yo | 5yo |
|---|---|---|---|---|---|
| **P4 vs P6** | 0.531 | 0.663 | 0.652 | 0.628 | 0.552 |
| **p Value** | **< 0.001** | **< 0.001** | **< 0.001** | **< 0.001** | **< 0.001** |
| **PspA (1&2) vs PspC** | 0.201 | 0.341 | 0.234 | 0.367 | 0.331 |
| **p Value** | **0.005** | **< 0.001** | **0.0013** | **< 0.001** | **< 0.001** |
| **P4&P6 vs PspA (1&2)** | 0.002 | 0.242 | 0.034 | 0.166 | 0.118 |
| **p Value** | 0.98 | **< 0.001** | 0.64 | **0.03** | 0.16 |
| **P4&P6 vs PspC** | 0.180 | 0.225 | 0.096 | 0.139 | 0.107 |
| **p Value** | **0.01** | **0.02** | 0.19 | 0.06 | 0.20 |

**Supplementary table 2** IgG4 antibody titre ng/ml (geometric mean and 95% confidence interval)

| | | 1yo | 3yo | 4yo | 5yo |
|---|---|---|---|---|---|
| **P4** | **SPT+** | 5.7 (4.9-6.6) | 20.2 (11.2-36.6) | 33.3 (17.8-62.2) | 8.4 (5.6-12.6) |
| | **SPT-** | 5.3 (5.0-5.7) | 19.4 (12.8-29.2) | 24.6 (16.0-37.6) | 6.0 (5.0-7.3) |
| | **p Value** | 0.3858 | 0.8062 | 0.3719 | 0.0643 |
| **P6** | **SPT+** | 5.0 (5.0-5.0) | 8.6 (6.3-11.9) | 9.6 (6.8-13.4) | 11.2 (7.1-17.7) |
| | **SPT-** | 5.0 (5.0-5.0) | 10.9 (8.4-14.2) | 12.0 (8.8-16.3) | 10.0 (7.0-14.4) |
| | **p Value** | NA | 0.3642 | 0.6180 | 0.2905 |
| **PspA (1&2)** | **SPT+** | 5.7 (4.8-6.7) | 15.8 (10.0-24.9) | 16.6 (10.2-26.8) | 17.8 (11.0-28.9) |
| | **SPT-** | 5.6 (5.0-6.1) | 13.9 (10.1-19.1) | 14.8 (11.0-19.9) | 15.8 (11.7-21.5) |
| | **p Value** | 0.6769 | 0.4866 | 0.9397 | 0.8254 |
| **PspC** | **SPT+** | 1.1 (0.9-1.3) | 5.4 (2.9-9.8) | 12.6 (5.9-26.7) | 18.3 (8.1-41.4) |
| | **SPT-** | 1.1 (0.9-1.2) | 4.9 (3.2-7.6) | 9.0 (5.5-14.8) | 9.8 (5.6-17.2) |
| | **p Value** | 0.1910 | 0.7066 | 0.5049 | 0.2272 |

**Supplementary table 3** IgG1 and IgG4 antibody correlation

| | 1yo | 3yo | 4yo | 5yo |
|---|---|---|---|---|
| **P4** | 0.015 | 0.046 | 0.021 | 0.028 |
| **p Value** | 0.83 | 0.53 | 0.78 | 0.74 |
| **P6** | NA | 0.256 | 0.186 | 0.218 |
| **p Value** | | **< 0.001** | **0.013** | **0.008** |
| **PspA (1&2)** | 0.318 | 0.046 | 0.269 | 0.491 |
| **p Value** | **< 0.001** | 0.53 | **< 0.001** | **< 0.001** |
| **PspC** | 0.284 | 0.0654 | 0.201 | 0.1152 |
| **p Value** | **< 0.001** | 0.37 | **0.007** | 0.17 |

**Supplementary table 4** IgG4 antibody correlation

| | 1yo | 3yo | 4yo | 5yo |
|---|---|---|---|---|
| **P4 vs P6** | NA | 0.628 | 0.447 | 0.284 |
| **p Value** | | **< 0.001** | **< 0.001** | **< 0.001** |
| **PspA (1&2) vs PspC** | 0.223 | 0.208 | 0.289 | 0.285 |
| **p Value** | **0.002** | **0.004** | **< 0.001** | **< 0.001** |
| **P4&P6 vs PspA (1&2)** | 0.374 | 0.720 | 0.609 | 0.259 |
| **p Value** | **< 0.001** | **< 0.001** | **< 0.001** | **0.002** |
| **P4&P6 vs PspC** | 0.279 | 0.204 | 0.267 | 0.384 |
| **p Value** | **< 0.001** | **0.005** | **< 0.001** | **< 0.001** |