Anti-bacterial IgE in the antibody responses of house dust mite allergic
children convalescent from asthma exacerbation

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Clinical Mechanisms in Allergic Disease

Summary

Background Atopic sensitization to the house dust mite (HDM) is associated with altered
antibody responses to the nasopharyngeal colonizing bacterium Haemophilus influenzae and
children admitted to the emergency department for asthma exacerbation have reduced IgG
responses to HDM allergens.

Objective To investigate anti-bacterial and anti-allergen antibody responses during
convalescence from asthma exacerbation and differences found in exacerbations associated
with and without viral infection.

Results IgE antibodies to the P6 bacterial antigen increased in 60% of sera during
convalescence and for many children achieved titres as high as IgE titres to allergens. In
contrast IgE anti-HDM titres declined during convalescence. The anti-bacterial IgE titres were
the same in subjects with and without virus infection while the anti-HDM IgE declined more
rapidly in virus-infected subjects. IgG titres to the major HDM allergens showed no consistent
increase and the overall IgG anti-HDM titres even declined in subjects without a virus
infection. Anti-bacterial IgG antibodies in contrast to IgE did not change. Patients with
frequent episodic or persistent asthma had similar IgE anti-bacterial titres to patients with
infrequent asthma during the acute phase, although they had reduced IgG titres to both the
bacteria and the HDM.

Conclusions During the period following an acute exacerbation of asthma there was a marked
and specific increase in anti-bacterial IgE compared with a reduced IgE response to HDM. This
provides further support for the concept of T-helper type 2 responses to bacterial antigens
playing a role in asthma pathogenesis.

Keywords asthma, bacteria, IgE, IgG, rhinovirus

Submitted 14 November 2008; revised 22 February 2009; accepted 24 February 2009

Introduction

The exacerbation of asthma in house dust mite (HDM)-
alergic people usually results from non-allergenic trig-
gers such as a virus infection acting on inflamed airways
rather than an increased response to allergens [1]. Recent
studies of the IgE anti-HDM antibody profile of children
attending an emergency department, however, showed
that while HDM-allergic children with exacerbations had
similar IgE responses to those of allergic children from a
community cohort, they had strikingly reduced IgG1 and
IgG4 antibodies [2]. The prevalence was reduced from
92% to 33%. This suggests the IgG antibodies could have
direct protective effects as shown in experimental models
[3] or be markers for a regulatory response. Allergic
sensitization is also associated with aberrant antibody
responses to other antigens. The authors have shown
decreased IgG1 antibodies to the protective P6 outer
membrane protein antigen of the nasopharyngeal colo-
nizing bacterium Haemophilus influenzae in infancy, and
the production of IgG4 antibodies in some atopic but not
non-atopic people [4]. Asthma exacerbations are asso-
ciated with intensified inflammation and increased IL-6
and T-helper type 2 (Th2) cytokine production with decreases in IL-10 and IFN-γ [5–9] and may influence antibody responses. The study here has accordingly examined anti-allergen antibody responses at exacerbation and during convalescence and possible bystander effects as judged by antibody responses to the P6 outer membrane protein. Because rhinovirus (RV) infection was detected in many of the children, responses in the absence or presence of known virus infection were examined. Convalescence was associated with some decrease in IgE anti-allergen antibodies but there was no increase for IgG. There was also no change in IgG antibody to the P6 antigen even though IgG1 to P6 and HDM was found to be decreased in those subjects with frequent episodic or persistent asthma. Strikingly over 60% of the convalescent children had increased IgE to the anti-P6 outer membrane protein with titres that were often similar to those induced by major allergens.

**Methods**

**Subjects**

Paired acute and convalescent plasma were examined for 53 HDM-allergic children who presented to the Emergency Department of Princess Margaret Hospital for Children (PMH) with acute asthma (Table 1). Peripheral blood samples were obtained as soon as possible after initiation of treatment and within 24 h of presentation to the hospital and plasma were stored at −80°C. A further blood sample was obtained after the acute attack, when the child was clinically well. The median time between acute and convalescent visits was 9.5 weeks (interquartile range 7.2–21.1 weeks). The standard management of children presenting with an asthma exacerbation at PMH included oxygen supplementation if saturations were ≤94%, β2-agonists (salbutamol) and an anticholinergic (ipratropium bromide) via metered dose inhaler with large volume spacer at 20-min intervals for the first hour, and prednisolone 1 mg/kg (maximum 40 mgs) orally. Oral steroids were rarely used beyond 2 days after the exacerbation. Antibiotics were not administered on admission and only one child had any history of recorded antibiotic use either before the acute attack or at convalescence. The severity of the acute asthma attack at presentation was determined using a previously validated scoring system with a possible score of 5–15 determined for each subject: 5–7 mild, 8–11 moderate, 12–15 severe [10]. The pattern of asthma severity was also assessed according to National Asthma Council of Australia guidelines to determine whether children suffered from infrequent episodic, frequent episodic, or persistent asthma exacerbations [11]. Parents of all participants gave informed consent and the Ethics Committee of King Edward Memorial and Princess Margaret Hospitals, Western Australia, approved the study.

**Table 1. Characteristics of the study population**

|                        | HDM-allergic (n = 53) |
|------------------------|-----------------------|
| Mean age in years (SD) | 8.2 (3.5)             |
| Males/females (%)      | 35/18 (66/34)         |
| Oxygen saturation on presentation (%) (SD) | 93.8 (3.5) |
| Asthma severity scores, mean (SD) | 9.4 (2.4) |
| Preceding symptoms of URTI, n (%) | 40 (76) |
| Virus isolated, n (%) | 27 (69)*               |
| Infrequent episodic asthma (%) | 55.8†              |
| Frequent asthma (%)    | 19.2†                 |
| Persistent asthma (%)  | 25†                   |

* n = 39.
† n = 52.

HDM, house dust mite; SD, standard deviation; URTI, upper respiratory tract infection.

**Viral detection**

At acute presentation, a pernasal aspirate (PNA) was collected using an 8-French suction catheter attached to a low-pressure suction unit, with secretions aspirated into a mucous trap. The secretions were eluted in 2 mL of saline and transported immediately to the laboratory for the detection of common viral respiratory pathogens. PNA’s were collected from 39/53 HDM-allergic children at admission to hospital in order to identify the presence of viral infection. Respiratory syncytial virus (RSV), adenovirus (Ad), influenza (InfV) and parainfluenza (PIV) viruses were either identified by direct immunofluorescence (IF) using virus-specific monoclonal antibodies or were isolated in cell culture (LLC–MK2, A549 and MRC5 cell lines) and identified by indirect IF by PMH (Subiaco, Western Australia). RV, human metapneumovirus (MPV) and coronavirus (CoV) were detected by RNA extraction and RT-PCR. RV and CoV were identified by PathWest (QEII Medical Centre, Nedlands, Western Australia) and MPV was identified by PMH. Some samples were re-analysed with the Respiratory MultiCode-PLx Assay (RMA), a new multiplex PCR assay [12], to confirm or detect the presence of RSV, Ad, InfV, PIV, RV, MPV, CoV and enterovirus. The RMA assay, which can detect 20 copies of cDNA, detected 25% more positive RV samples than the PCR assays conducted by PathWest at that time. The lower detection rate by PCR was due to deficiencies in the assay that have subsequently been amended in that samples that were tested more recently gave the same results as the RMA.

**Antigen and allergens**

The P6 outer membrane protein of *H. influenzae* from the Eagen isolate was produced as a fusion polypeptide with
glutathione-S-transferase (GST) and a GST control was produced directly from pGEX-2T [4]. It was also made with a N-terminal hexa-histidine tag (P6–H6) to confirm binding was due to the P6 component and not to the GST tag. Natural Der p 1 was purified from spent mite medium by antibody affinity chromatography. Natural Der p 3 was purified according to Heymann et al. [13] and then further substrate purified with benzamidine Sepharose 6B (Amer- sham Pharmacia Biotech AB, Uppsala, Sweden). Natural Der p 4 was purified by substrate affinity with β-cyclo-
dextrin Sepharose 6B (Amersham Pharmacia Biotech AB). Recombinant Der p 2.0101, Der p 5 and Der p 7 were produced as recombinant polypeptides with a N-terminal hexa-histidine tag and purified by Ni–NTA (Qiagen, Hilden, Germany). All proteins were further purified by high-
resolution size exclusion chromatography and passed over Mustang E filters (Pall Life Sciences, New York, NY, USA) to remove residual endotoxin from the preparations.

**Quantitative antibody binding assays**

DELFIATM (Wallac, Turku, Finland) assays were used to quantify IgE, IgG1 and IgG4 binding to the P6 antigen and to a panel of mite allergens (Der p 1, 2, 3, 4, 5 7). The quantities of antibody were interpolated from a standard curve created with human/mouse anti-Der p 2 chimeric antibodies (Indoor Biotechnologies Ltd., Cardiff, UK) by the procedures of Schuurman et al. [14]. The IgE and IgG DELFIATM assays are described in detail elsewhere [2]. The lower limits of detection for IgE, IgG1 and IgG4 antibody binding are 0.1, 10 and 1 ng/mL, respectively, and consistent with the expected performance of DELFIATM the standard curves all have a 5-log dynamic range. Negative values were given the lower limit of detection for each antibody assay. The results have been given either as the titres to individual allergens or as the summation of the responses of each person to all the allergens (sum of the specificities, SOS) [2].

**Statistical analysis**

As antibody data were highly skewed they were log-
transformed before analysis. Total plasma IgE levels were normalized after transformation and the mean and 95% confidence intervals (95% CI) calculated. Differences in total plasma levels between groups and as for all other normalized data were compared by the paired or unpaired t-test. Differences in the level of specific antibody binding by selected groups were compared either by the non-parametric Mann–Whitney and Wilcoxon signed rank tests or by the χ2 test. All analyses were done using the SPSS 11.5 for Windows (Chicago, IL, USA).

**Results**

**Anti-house dust mite allergen immunoglobulin E and immunoglobulin G subclass**

Paired acute and convalescent plasma from 53 HDM-allergic children were available to measure IgE and IgG antibody titres to specific HDM allergens. As shown by the scatter distributions (Fig. 1) the IgE titres were lower to all allergens in most of the convalescent plasma. This was statistically significant for Der p 1 (P<0.001), Der p 2 (P<0.001), Der p 5 (P<0.001) and Der p 7 (P<0.001).

Antibodies to Der p 1 and Der p 2 make up over half the anti-HDM IgE response and when tracked for each individual were decreased in a majority of the subjects (Fig. 2). On average the titres were reduced two to threefold but were still at high levels.

Most of the IgG antibody responses were directed to the major Der p 1 and Der p 2 allergens. As reported
Previously the prevalence was about 30% and the titres and prevalence showed no consistent change during the convalescent period and the titres of most patients remained undetectable (Fig. 3). There was no concordance between patients with variations in the anti-Der p 1 and Der p 2 titres.

**Anti-P6 outer membrane protein**

As outlined in the introduction young children that develop atopy have reduced IgG1 antibodies to the P6 bacterial antigen and atopic people of all ages have a much greater propensity to make the highly Th2 associated IgG4 subclass of antibodies. IgG1, IgG4 and IgE anti-P6 antibodies were accordingly measured to examine the titres at presentation and if these changed during the convalescence period. The IgG1 antibody was readily detected to P6 and this did not change over the convalescence period (Fig. 4). The IgG4 was detected less frequently and showed no consistent change with convalescence. In contrast IgE antibody showed an increase in 32/53 HDM-allergic plasma during convalescence (P < 0.001). For most children this meant the development of previously undetected responses and for some individuals the responses reached titres from 5 to 15 ng/mL.

**Asthma history**

Children admitted to emergency departments can have a wide range of previous experience with asthma [15] so it might be expected that those with a greater history of disease would have different antibody titres. The antibody responses in the plasma of children who had infrequent episodic asthma exacerbations were compared against the responses of children who had either frequent episodic or persistent asthma exacerbations (Table 2). Children who had frequent or persistent asthma had lower IgG1 response to the P6 bacterial antigen and to the HDM allergens (SOS) (P < 0.05). Both groups had comparable total IgE immunoglobulin and anti-HDM IgE antibody.

**Virus infection**

It is possible that the antibodies titres of children that present with or without a virus infection might identify different reasons for the exacerbation and the virus infection can affect the course and outcome of the convalescence. Nasal aspirates and paired IgE measurements were available for 39 HDM-allergic children, 27 of whom had detectable virus with RV being identified in 24/27 cases. The presentations with viral and non-viral associated exacerbations were distributed throughout the year so no difference in exposure was apparent. Children
with and without virus infection had high levels of total IgE and this decreased during convalescence for both groups but remained high (Table 3). The IgE anti-HDM titres showed the same pattern although the decrease in IgE did not reach significance for the children without virus (P = 0.233). The anti-P6 IgE antibodies were found in 70% of the HDM-allergic children with virus infection and in 67% of the children from whom virus was not detected. Both groups had high and low IgE anti-P6 responders. The prevalence of IgG was the same in both the infected and uninfected groups. The anti-HDM IgG1 titres were the same at the acute phase but the titres of the group without virus declined during convalescence (P < 0.01). Children

with virus, however, had more severe asthma symptom scores on average than those admitted to hospital without detectable virus (asthma severity score at presentation: 9.7 vs. 8.1, P < 0.05).

Table 2. Total and specific antibody titres in acute plasma in children with either infrequent episodic or frequent episodic/persistent asthma exacerbations

|                | Infrequent (n = 29) | Frequent/persistent (n = 23) |
|----------------|---------------------|-----------------------------|
| Total IgE Ig (kU/L) | 538 (275–802)       | 641 (380–902)               |
| IgE HDM (ng/mL)    | 31.3 (7.54–132)     | 81.1 (5.17–237)             |
| IgE P6 (ng/mL)     | 0.1 (0.1–0.52)      | 0.1 (0.1–1.64)              |
| IgG1 P6 (ng/mL)    | 9950 (4139–18 889)  | 4823 (126–6862)*            |
| IgG1 HDM (ng/mL)   | 15 684 (2345–61 339) | 3847 (765–16 853)*          |

Results show the mean of total IgE immunoglobulin with 95% confidence limits in parentheses and the median sum of specific HDM IgE, specific P6 IgE, specific P6 IgG1 and sum of specific HDM IgG1 with the interquartile range in parentheses.

*Children with frequent episodic/persistent asthma exacerbations had lower IgG1 antibody titres to P6 and HDM allergens (P < 0.05) than children with infrequent episodic asthma exacerbations.

Table 3. Total and specific antibody titres in children with or without virus

|                | Acute                  | Convalescent             |
|----------------|------------------------|--------------------------|
| Total IgE Ig (kU/L) | 612 (320–905)         | 358 (204–512)*          |
| IgE HDM (ng/mL)    | 50.2 (5.51–118)       | 7.9 (1.22–53.9)**       |
| IgE P6 (ng/mL)     | 0.1 (0.1–0.56)        | 1.78 (0.1–6.8)*         |
| IgG1 P6 (ng/mL)    | 5028 (1079–10 682)    | 6398 (3079–17 581)      |
| IgG1 HDM (ng/mL)   | 8703 (2721–32 909)    | 10 193 (1437–19 487)    |
| Virus negative (n = 12) |                  |                          |
| Total IgE Ig (kU/L) | 569 (202–937)         | 486 (89.9–882)*         |
| IgE HDM (ng/mL)    | 77.5 (15.6–220)       | 31.6 (4.84–102)         |
| IgE P6 (ng/mL)     | 0.1 (0.1–1.03)        | 2.52 (0.1–5.0)*         |
| IgG1 P6 (ng/mL)    | 7162 (3509–11 906)    | 12 869 (4072–38 100)    |
| IgG1 HDM (ng/mL)   | 9656 (1164–17 295)    | 2017 (60–6947)*         |

*24/27 children were infected with rhinovirus

TABLE 2. Total and specific antibody titres in plasma in children with or without virus.

Acute                Convalescent

|                | Virus positive (n = 27) | Virus negative (n = 12) |
|----------------|------------------------|-------------------------|
| Total IgE Ig (kU/L) | 612 (320–905)         | 569 (202–937)           |
| IgE HDM (ng/mL)    | 50.2 (5.51–118)       | 77.5 (15.6–220)         |
| IgE P6 (ng/mL)     | 0.1 (0.1–0.56)        | 0.1 (0.1–1.03)          |
| IgG1 P6 (ng/mL)    | 5028 (1079–10 682)    | 7162 (3509–11 906)      |
| IgG1 HDM (ng/mL)   | 8703 (2721–32 909)    | 9656 (1164–17 295)      |

*2009 Blackwell Publishing Ltd, Clinical & Experimental Allergy, 39: 1170–1178
Discussion

During convalescence the titres of IgE immunoglobulin and IgE antibody to HDM allergens decreased for the majority of children and this occurred for the major Der p 1 and Der p 2 as well as the mid-potency Der p 5 and 7 allergens. When the difference between the acute and convalescent IgE anti-HDM titres in the virus positive and virus negative groups were analysed separately, the patients without a virus infection showed a twofold reduction in titre while the average titre for the virus positive group were reduced sixfold and this was statistically significant. A possible reason for this is that the RV infection had caused a transient increase before presentation so there was a greater decline in convalescence. The reductions should, however, be viewed in the context that the IgE titres detected during the exacerbations were no higher than those detected for children with stable asthma [2] and that the decreases were usually only to levels still within the range commonly found during the exacerbations. Because the children were administered increased medication after the exacerbation, particularly corticosteroids, the treatment, as reported by some investigators [16–18], could be a possible cause of the decline. However, regardless of the mechanism responsible for the decreased IgE, the reduction would be beneficial for the patient. As well as reducing responses to HDM allergen the decrease in total IgE would reduce the allergen-independent FcεR1 binding that activate mast cells and stabilize receptor expression [19]. It was previously found that the children recruited from the emergency department with an asthma exacerbation had markedly less IgG antibody to mite allergens than children with stable asthma [2] with a prevalence of 33 compared with 92%. While some individual changes of IgG were found in convalescence they were both up and down, with most titres remaining negative. An IgG response was thus not restored over the period of convalescence but this remains an important feature to study for the susceptibility to acute attacks of asthma. When the summated IgG1 titres for all the HDM allergens were analysed they in fact declined during the convalescence period but only for the group without associated RV infection. The potential significance of the low IgG antibody was further underscored by the finding here that the summated IgG1 titres to the HDM allergens were the lowest in the patients with frequent episodic and persistent asthma. Animal studies have demonstrated that there are several mechanisms by which IgG antibodies can ameliorate allergic responses in vivo [3] so the sustained low titres in children with exacerbations and the association of low titres with persistent disease suggests they may be protective for human asthma.

A previous study measured antibodies to the P6 outer membrane protein of *H. influenzae* as a potential mucosal bystander antigen to the responses to allergen [4]. A dysregulation of the response to P6 was found with atopic infants showing a slow development of IgG1 antibodies, then for the most atopic, the subsequent development of IgG4 antibodies, an isotype rarely found in the antibody responses of non-atopic subjects. Although the regulation of IgG subclasses in humans does not show the clear Th1 and Th2 demarcation of cytokine regulation found in mice and is still being investigated [20], IgG4 antibody is strongly associated with Th2 responses and is preferentially up-regulated over IgG1 by the Th2 transcription factor GATA-3 [21]. Our observations showing that the IgG responses did not change in the study period following the exacerbation, and in particular that no IgG4 was induced, do not support the idea of a bystander regulation.

A striking and unexpected finding was however made showing that 60% of the children developed IgE antibody to P6 during convalescence, either increasing from pre-existing titres or appearing from undetectable levels. This is therefore evidence for the possibility that Th2 cytokines produced during the exacerbation to direct an antibacterial response to a Th2-regulated antibody isotype. The presence of IgE antibodies to bacteria, including *H. influenzae*, has been known for some time [22, 23] but the quantitative assays used here reveal not only a very high frequency, but also the antibody levels can be of considerable magnitude. A number of the children developed titres of 5–15 ng of IgE/mL, which raises the possibility that the IgE interacting with the bacterial antigen may even have an effect on prolonging the inflammation. For comparison, the median anti-Fel d 1 IgE titre for people with rhinoconjunctivitis to cats is 6 ng/mL [24, 25]. The anti-P6 titres even reach the level of the responses to the major HDM allergens of about 50% of the HDM-sensitized children in this study and 40% of those measured by Trombone et al. [26]. The bacteria are usually present in concentrations of over 10^3/mL of aspirate [27] so there is reasonable scope for antigen–antibody interactions. Similar responses to other colonizing bacteria must also be considered. It is unlikely that the IgE had a direct role in the exacerbation because the titres were low at that time and it is a matter for speculation as to whether or not the up-regulation required increased exposure to antigen from a recrudescence of an existing infection or a new colonization.

The increase in IgE antibody to P6 in the face of decreasing anti-HDM IgE titres might suggest that the decline in anti-HDM IgE antibody is not due to glucocorticoid administration and indeed there are data showing glucocorticoid has varying effects on IgE production [28]. The effect of corticosteroids depends on the differentiation state of the target cells that are controlling or limited the responses [29]. Steroids for example inhibit early B-cell responses and increase later immunoglobulin switching. The results here are reminiscent of those reported by Diaz-Sanchez et al. [30] showing that steroids markedly...
inhibited the mucosal IgE antibody response of patients challenged with ragweed allergen but at the same time only had a marginal effect on the adjuvant activity of the extract as measured by the increase in total IgE immunoglobulin. The present data are similar in that the anti-allergen response was inhibited to a much greater degree than the IgE immunoglobulin titres and antibodies to the bystander antigen increased.

P6 is the most studied protective antigen of this bacterium with a highly conserved sequence present on all isolates. It induces antibody responses in children during successive colonizations by predominately nontypable organisms, which, as shown by recent studies, can be expected to occur several times a year [27]. The study environment has had universal Hib vaccination since 1993 with an almost complete eradication of type b disease and excellent compliance with the programme [31] so Hib infection or vaccination would not have influenced the anti-P6 results shown here. Moreover, although not studied in Australia, Hib vaccination was shown to almost eliminate the ability to detect type b carriage in England, a country with a similar socioeconomic environment [32].

As well as the possibility that the anti-bacterial IgE could be involved in the inflammation, the IgE may be a marker for the induction of dysregulated immunity that could contribute to the morbidity of asthma and bacterial infection [33]. In the recent study by Bisgaard [34] it was also shown that bacterial colonization, especially with H. influenzae, was a strong risk factor for recurrent wheeze and asthma early in life so it is possible that immune responses to colonizing bacteria may have an important relationship with allergic disease.

Consideration of the pathophysiological significance of the IgE antibody to P6 should include the possibility that similar and parallel responses to other H. influenzae epitopes and antigens of other colonizing bacteria occur and that Gram-negative bacteria including H. influenzae have an active mechanism for constitutively releasing outer membrane vesicles, of 20–200 μm. These would contain the P6 antigen [35] and could be distributed into the airways. Localized IgE-mediated inflammatory responses also can have systemic effects. As an example this has been demonstrated for thymic stromal lymphopoietin (TSLP), a cytokine that can be induced by FceRI-dependent responses of mast cells [36]. It was suggested from the study of Astrakhan that TSLP derived from a local site might impair tolerogenic B-cell responses and promote allergic responses to new specificities [37]. The anti-bacterial IgE produced during convalescence seems to stem from the exacerbation itself because it is produced in children regardless of their clinical history or evidence for a viral infection. Low IgG1 anti-P6 titres were found in subjects with frequent and persistent asthma and were low at presentation and after convalescence so they should be investigated for a role in susceptibility to asthma and as markers for defining susceptible children.

It is becoming apparent that virus and especially RV infection may be the most prevalent cause of exacerbations of asthma and that the infection could drive a cytokine response with a Th2 bias. Detailed virology was performed on the subjects here and it found that 27 (69%) had a detectable infection and 24/27 were RV. The symptoms at presentation were on average worse for the infected subjects but none of the IgE antibody and immunoglobulin measurements at presentation, which were high for both groups, were different. The induction of anti-P6 IgE antibody was also similar between the infected and uninfected groups. The possibility that there are differences was however indicated by the larger decline of the anti-HDM IgE in the infected group and the decrease in the already low IgG anti-HDM antibody titres in the uninfected but not the infected group.

In summary children convalescing from an asthma exacerbation with and without a detectable virus infection developed increased IgE antibodies to the P6 antigen of the bacterium H. influenzae and decreased IgE antibodies to the HDM allergens. The low IgG anti-HDM antibodies previously reported in children admitted to the emergency department for exacerbation did not increase and even decreased in the children who did not have an associated viral infection. The IgE anti-P6 titres that developed could be as high as titres frequently found for allergens and may play a role in maintaining allergic sensitization, or be indicative of a deviated protective immunity.

Acknowledgements

The authors thank the study families, study nurses and personnel for recruitment and follow-up of the families, and Lee Hazell for producing the proteins used in the study.

Funding: This work was supported by the National Health and Medical Research Council of Australia. Ingrid Laing is supported by the Australian Respiratory Council Ann Woolcock Research Fellowship.

Competing interest: All authors declare they have no competing interests.

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