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Mold-sensitised adults have lower Th2 cytokines and a higher prevalence of asthma than those sensitised to other aeroallergens

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Short title: Lower Th2 cytokine levels in mold-sensitised adults

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ABSTRACT **Words: 244**

Background: Evidence suggests that specific allergen **s**ensitisations are associated with different allergic diseases which may reflect different underlying immune profiles. We aimed to examine the cytokine profiles of individuals sensitised to eight common aeroallergens.

Methods: We used data from the Tasmanian Longitudinal Health Study (TAHS) a population-based cohort study of 45 year olds. Serum cytokines (IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α) were measured in 1,157 subjects using the Lincoplex assays. Participants underwent skin prick testing for house dust mite (HDM), cat, grasses and molds. Multivariable linear regression was used to compare serum cytokine levels between sensitised and non-atopic subjects.

Results: The prevalence of allergic sensitisation to any aeroallergen was 51% (95%CI 47%,54%). Being sensitised to any aeroallergen was strongly associated with current asthma (OR=3.7, 95%CI 2.6,5.3) and being sensitised to molds was associated with a very high risk of current asthma (OR=11.4, 95% 5.4,24.2). The geometric mean (GM) levels of Th2 cytokines (IL-4, IL-5 and IL-6) for adults sensitised to *Cladosporium* were significantly lower than the levels for non-atopic individuals [IL-4 ratio of GMs=0.25, 95%CI 0.10-0.62, p=0.003; IL-5 GM=0.55, 95%CI 0.30-0.99, p=0.05; and IL-6 GM=0.50, 95%CI 0.24-1.07, p=0.07]. Individuals sensitised to other aeroallergens all showed elevated Th2 cytokine levels.

Conclusion: First large population-based study to demonstrate reduced Th2 cytokines levels in people sensitised to mold. Underlying biological mechanisms driving allergic inflammatory responses in adults sensitised to molds may differ from those sensitised to other aeroallergens. These findings suggest that it may be necessary to tailor treatments in individuals sensitised to molds compared with other aeroallergens in order to optimise outcomes.

INTRODUCTION **Words: 3445**

Allergic sensitisation is defined as the predisposition to produce allergen-specific Immunoglobulin E (IgE) antibodies in response to allergens(1). Specific IgE sensitisation can be measured either by the detection of IgE antibodies in serum or by a positive skin prick test(2). The prevalence of allergic sensitisation varies considerably by regions. The European community health study (ECRHS) conducted in 17 countries found that SPTs for seven selected allergens (*house dust mite*, cat, grass, birch, olive pollen, *Alternaria* and *Cladosporium*) enabled the identification of almost all sensitised subjects(3). This study also found that including regional specific allergens for some countries increased the number of sensitised people identified; highlighting the importance of the local environment in determining prevalence of allergic sensitisation.

Allergic sensitisation is a major risk factor for allergic diseases such as atopic dermatitis(4), allergic rhinitis(5) and asthma(2, 6). Different allergens also have different relationships with allergic diseases. For example, sensitisation to HDM is more commonly associated with asthma(7) and sensitisation to pollens with allergic rhinitis(8). Moreover, sensitisation to airborne fungi/molds, potentially the most ubiquitous aeroallergens in the environment, is strongly associated with severe asthma, poor asthma control and a higher likelihood of complications such as bronchiectasis(9-13).

Despite evidence for a strong association between allergic sensitisation and allergic diseases, the underlying biological mechanisms driving these allergic inflammatory responses are not well defined. The majority of studies examining the inflammatory cascade and response to allergens have measured *in vitro*/*ex vivo* cytokine production by peripheral blood mononuclear cells (PBMCs)(14, 15), and cytokine levels in bronchial lavage (BAL)(16) or sputum(17). The majority of these studies have involved a small numbers of subjects or samples, and have been performed using highly selected patients groups and their results may not reflect the relationship seen *in vivo* and in samples from the general population. Additionally, in epidemiological studies of allergic sensitisation there is a tendency to combine allergic sensitisation into “any” allergic sensitisation, which assumes all allergens act in a similar manner and elicit a similar allergic response. To our knowledge no previous study has examined the relationship between peripheral blood cytokine levels and allergic sensitisation in a population-based sample of individuals, and

in particular no studies have focused on investigating cytokine profiles by type of allergen sensitisation (eg pollen vs mold vs HDM).

METHODS

Study Design - Tasmanian Longitudinal Health Study (TAHS)

Participants were part of the Tasmanian Longitudinal Health Study (TAHS) the details of which have been reported elsewhere (18, 19). In brief, TAHS began in 1968 when 8583 children (probands) attending school in Tasmanian and born in 1961 were recruited to participate in a study of asthma. Their parents completed respiratory health questionnaires for the child, who then completed lung function testing. Follow-up surveys were completed in 1974, 1979, 1992 and the most recent starting in 2002. We traced 7,562 (88.1%) of the original 1968 cohort to an address and achieved a response of 5,729 (78.4%) to a postal survey(18). A subgroup of these respondents selected on the basis of their participation in the previous follow-ups, was invited to participate in a detailed laboratory study which ran from 2005 until 2008. Of the 2,373 invited for the laboratory study, 1389 (58.5%) attended the laboratory, 354 (15%) completed a telephone questionnaire only, and 630 (26.5%) withdrew. At the laboratory visit, participants completed a detailed interviewer-administered questionnaire, performed lung function tests, skin prick tests for sensitisation and provided a blood sample. Details of the testing are described below (Figure 1). Ethics approval was obtained from the Human Research Ethics Committee at the University of Melbourne (HREC Number 040375).

Skin Prick Test

Skin Prick Tests (SPTs) were performed for eight aeroallergens (Hollister-Stier, EBOS Groups Pty Ltd, Australia) including *Dermatophagoides Pteronyssinus*(HDM), Cat Pelt (Cat), *Cladosporium Cladosporioides* (CLAD), *Alternaria Tenuis* (ALT), *Penicillium* mix (PEN), *Aspergillus Fumigatus* (ASP), Perennial Rye Grass (Rye), Mixed Grasses #7 (MIXED, which included Kentucky Bluegrass, Orchard, Redtop, Timothy, Sweet Vernal grass, Meadow Fescue, Perennial Rye grass) and histamine as a positive control. Tests were performed using single-tine lancets (Stallergenes, Antony, France) on the volar surface of the forearm. A wheal size of at least 3mm greater than the negative control was regarded to indicate sensitisation.

Blood Sample Processing and Cytokine Measurement

A whole blood sample was collected from participants and stored at room temperature until processing. The average time from blood collection to processing and freezing was 1.8 days. The serum samples were collected in serum tubes and spun at 1328 RCF (2500 RPM) for 15 minutes and aliquoted into four 1ml fractions and immediately stored at -80°C. For cytokine testing a single, previously unthawed serum vial was thawed at 4°C.

Preliminary experiments were performed to determine which cytokines could be detected in the serum samples. Details about these preliminary experiments are available in the online supplement. Based on these results and our a priori hypotheses the cytokines interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and Tumour Necrosis Factor-alpha (TNF-α) were selected for analysis. Cytokines were measured simultaneously according to the manufacturers' instructions. In brief, microspheres, each with a unique red/infrared spectrum signature, covalently coupled to cytokine-specific receptors, were incubated overnight with 50μL of standards, controls and blood serum samples in a 96-well microtitre filter plate at 40ºC. Following incubation, the plate was washed to remove excess reagents and incubated with detection antibody, comprising a mixture of biotinylated mouse/rat anti-human cytokine antibodies directed against each of the 6 cytokines. After one-hour incubation at room temperature, streptavidin-conjugated phycoerythrin was added for 30 minutes. All incubation steps were carried out in the dark. Fluids were removed by gentle vacuum; the beads were washed and resuspended in sheath fluid before assay on the Luminex instrument (Luminex Corporation, Austin, Texas, USA). The 1184 samples were run in 8 batches on the Luminex instrument. Cytokine concentrations were predicted to the log base10 using asymmetrical 5-parameter logistic equation in Graphpad Prism5 software (GraphPad Software, La Jolla, CA 92037, USA) and then were back transformed to obtain concentrations in pg/mL.

The minimum detection limit for the assay was 0.13 pg/mL and the maximum was 2000 pg/mL. Samples with readings below or above these levels were assigned values of 0.5 times the minimum value (0.065 pg/mL) or the maximum value (2000 pg/ml) respectively. The percentages of samples with levels below the 0.13pg/mL detection limit were 20.8%, 16.7%, 5.9%, 0.0%, 21.2%, and 0.4% for IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α respectively. The

percentages of samples with levels above the 2000pg/mL detection limit were 0.5%, 0.0%, 0.3%, 3.5%, 0.6% and 0.0% for IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α respectively. The samples with an undetectable reading were evenly distributed between the batches and outcome groups. As sensitivity analyses including and excluding these undetectable readings did not materially change the results, the data including the assigned values are presented.

For a subgroup of 79 participants we measured the cytokines in duplicate and determined the reliability of the measurements by calculating the Intraclass Correlation Coefficient (ICC) for each cytokine. ICCs for all cytokines were greater than 90%: (IL-4 = 98.9% [95% CI: 98.4% to 99.3%]; IL-5 = 96.3% [95% CI: 94.6% to 97.9%]; IL-6 = 98.6% [95% CI: 98.0% to 99.2%]; IL-8 = 97.7% [95% CI: 96.6% to 98.7%]; IL-10 = 94.1% [95% CI: 91.6% to 96.6%]and TNF-α = 92.7% [95% CI: 89.6% to 95.8%]). In addition, we had two quality controls (QCs) for each cytokine, run in duplicate in each batch. All readings for QCs were within the expected range with coefficients of variation less than 15%. Based on these data, further cytokine analyses were performed in singlicate.

Definitions

Atopic sensitisation was defined as a positive SPT to any of the allergens tested. Those who tested negative to all eight allergens were categorised as non-atopic and served as controls. For the specific allergen sensitisation, participants were categorised into one of three groups (1) Non-atopic, (2) Positive for the specific allergen, (3) Negative for the specific allergen but positive to other allergens. This was done for all specific allergens (HDM, Cat, CLAD, ALT, PEN, ASP, Rye, and MIXED GRASSES). Ever having had asthma in 2004 was defined by a positive response to the question “Have you ever had asthma?” Current asthma was based on self-reported morning, day-time and night time symptoms, and/or flare-ups in the last 12 months.

Statistical analysis

All statistical analyses were performed using Stata version 13 (StataCorp, College Station, Texas, USA). Prevalence estimates were reweighted for the sampling frame using inverse probability weights. Multiple logistic regression analysis was performed to examine the association between sensitisation to any and specific allergens and current asthma. These regression analyses were adjusted for gender, current smoker, inhaled corticosteroid (ICS) use

last year and the season attended at the laboratory. As cytokine concentrations were predicted to the log base 10 geometric means (GM) and 95% confidence interval (CI) are presented as summary statistics. Multiple linear regression models were used to estimate the associations between the serum cytokine concentrations and sensitisation to any and specific allergens. These regression analyses were adjusted for batch variability, gender, current smoking status, current asthma, level of education, season of study attendance, time until blood processing and ICS use. As the distribution of some of the cytokine concentrations (log base 10 units) was slightly skewed, all analyses were repeated using bootstrap methods with 1000 repetitions. The confidence intervals are derived using these methods.

RESULTS

Prevalence of the atopic sensitisation of the study group

In total, 1157 participants were included in this analysis (Figure 1). Of these, over 55% (n=645) were found to be atopic and the proportions sensitised to specific allergens were as follows: 38% (n=497) to HDM; 13% to Cat; 4% (n=49) to *Cladosporium*; 7% (n=105) to *Alternaria*; 2% (n=35) to *Penicillium*; 3% (n=51) to *Aspergillus*; 28% (n=358) to rye grass and 26% (n=341) to mixed grasses.

Figure 2 shows the prevalence of sensitisation to the individual allergens adjusted for the sampling weights. Over 51% (95%CI 47%-54%) of participants were sensitised to at least one allergen with the most common sensitisation being to HDM followed by rye grass and mixed grasses. The most prevalent mold sensitisation was to *Alternaria* at 7% (95%CI 6%-9%).

Personal characteristics by atopic sensitisation

The distributions of several characteristics across specific allergen sensitised groups are summarised in Table 1. The proportion of those with current asthma and hence current inhaled corticosteroid (ICS) use was higher in the atopic group when compared with the non-atopic group. In contrast, the proportion of females in the atopic group was lower (46%) than in the non-atopic group (55%). The proportion of current smokers in atopic and non-atopic groups was similar (Table 1).

Association between specific atopic sensitisation and current asthma

We examined associations between specific allergen sensitisation and participants' current asthma status (Table 2). After controlling for confounders, mold sensitised participants had the strongest associations with current asthma (*Penicillium*-odds ratio (OR) = 11.4, 95%CI 5.4, 24.2; *Aspergillus* OR 9.6, 95%CI, 5.1 to 18.3; *Cladosporium* OR=7.5, 95%CI 3.9, 14.5; Any Mold 6.40, 95% 4.06, 10.1). The odds of having current asthma in participants sensitised to other aeroallergens were also high (Table 2).

Cytokine profiles for atopic and non-atopic groups

Geometric means (GMs) and corresponding 95% CIs for the serum cytokines tested for non-atopic and atopic groups are shown in Table 3 and Figure 3. Significantly higher serum levels of IL-5 were found in the atopic group compared with the non-atopic group [ratio of adjusted GM = 1.37, (95% CI: 1.06 to 1.77), p=0.02]. IL-4 and IL-6 levels were also elevated in the atopic group but were not statistically significant. There were no differences in IL-8, IL-10 and TNF-α concentrations between the atopic and non-atopic groups (Figure 3).

Cytokine profiles in specific allergen sensitised groups

The adjusted geometric mean concentrations and ratio of geometric means between sensitised and non-atopic individuals are presented in Table 3 and Figure 3. The serum IL-4 concentrations for *Cladosporium and Aspergillus*-sensitised individuals were significantly lower than non-atopic individuals [*Cladosporium* ratio of GM = 0.25, (95% CI: 0.10 to 0.62), p=0.003; *and Aspergillus* ratio of GM = 0.32, (95% CI: 0.12 to 0.82), p=0.02]. The IL-5 concentrations for HDM, Rye and Mixed grasses-sensitised groups were significantly increased when compared with the non-atopic group. On the other hand, for individuals sensitised to *Cladosporium*, serum IL-5 levels were significantly lower than for the non-atopic group [ratio of GM = 0.55, (95% CI: 0.30 to 0.99), p=0.05]. For IL-6 individuals sensitised to *Cladosporium* also had reduced concentrations compared to non-atopic [ratio of GM = 0.50, (95% CI: 0.24 to 1.07), p=0.07].

The *Cladosporium*-sensitised group exhibited a different Th2 cytokine pattern, with significantly lower IL-4, IL-5 and IL-6 cytokines, than the non-atopic group. This pattern was different to that observed with the other molds. We found that 80% of *Cladosporium (CLAD*+ n=39/49), 69% of *Penicillium (PEN*+ n=24/35), 71% of *Aspergillus (ASP* + n=36/51) and 44% of *Alternaria (ALT* + n=46/105) were sensitised to other molds. To examine this further we examined the cytokine

profiles in the *Cladosporium*-sensitised individuals, both in the presence and in the absence of other mold sensitisation (Figure 4). For *Cladosporium*-sensitised individuals who were also sensitised to other molds (CLAD +ve / Other MOLD +ve) the levels of IL-4, IL-5 and IL-6 were significantly lower than the levels in non-atopic individuals (Figure 4). For IL-5 the CLAD +ve / Other MOLD –ve group, also had reduced levels which did not reach statistical significance possibly due to the small sample size (n=10).

DISCUSSION

Using a large, population-based adult cohort, we investigated associations between several serum cytokines and specific allergen sensitisations. Contrary to previous reports(20, 21), participants sensitised to *Cladosporium, Penicillium* and *Aspergillus* had reduced serum Th2 cytokine profiles compared with non-atopic subjects. *Cladosporium*-sensitised participants demonstrated the strongest association with reduced Th2 cytokine profiles, with significantly reduced levels of IL-4 and IL-5, and marginally reduced levels of IL-6. Individuals sensitised to the common aeroallergens, HDM, cat pelt and rye and mixed grasses, had significantly elevated levels of IL-5, and moderately increased levels of IL-4 and IL-6 compared with the non-atopic group, consistent with previous studies(22, 23).

The reason why mold sensitised individuals had lower systemic Th2 cytokine levels than individuals sensitised to other aeroallergens in our study is unclear. There is evidence that both antigens and proteases in many allergens including fungi influence the inflammatory immune response. A recent study by Kauffman *et al.*(24) found that cytokine production generally exhibited a bell-shaped dose-response curve suggesting gradual increases in cytokine production at low fungi levels followed by a plateau and diminishing levels of cytokine production as fungi concentrations increased. This suggests that once sensitised to molds an individual's ongoing cytokine response may be influenced by mold levels in the environment. In our study *Cladosporium*-sensitised individuals demonstrated the lowest Th2 levels of all the fungi tested and demonstrated significantly smaller wheal sizes (data not shown). *Cladosporium* is one of the most common fungi in Australia(25), with a study finding *Cladosporium* present in more than 90% of homes(26). The high prevalence of *Cladosporium* in Australia could indicate most people in this study were exposed to high levels in their environment and therefore their cytokine response patterns were different to those of other aeroallergens.

Another possible mechanism to explain associations between mold sensitisation and lower Th2 cytokine is that mold-induced inflammation is driven by the innate immune system rather than the classic Th2 pathway. The innate system relies on the recognition of evolutionarily conserved structures on pathogens [pathogen-associated molecular patterns (PAMPs)], through germ line-encoded pattern recognition receptors (PRRs) including the essential receptors, Toll-like receptors (TLRs)(27) and C-type lectin receptors(28, 29). Molds reaching the airway epithelium activate TLR-equipped epithelial cells, which leads to activation of nuclear factor NFκB and mitogen-activated protein (MAP)-kinase that in turn results in the synthesis of a complex range of molecules including cytokines, chemokines, cell adhesion molecules, and immunoreceptors, which together orchestrate an early host inflammatory response(30). Another mechanism, also not resulting in elevated serum Th2 cytokines, for mold-sensitised inflammation may occur via the Dectin-1 pathway(28). Dectin-1 binds and internalizes β-glucans, which are found in the cell walls of fungi, leading to the production of reactive oxygen species (ROS), activation of NF-κB and subsequent secretion of proinflammatory cytokines including TNF-α(31).

Over a third of our mold sensitised individuals had current asthma, which was higher than the prevalence in those with sensitisation to other aeroallergens. We also found mold sensitisation was associated with a significantly increased risk of current asthma. People sensitised to molds allergens have been shown to be at an increased risk of asthma, and also increased rates of severe asthma, hospital admission and death(12). The reason why mold sensitised individuals tend to have more severe disease than those sensitised to other aeroallergens is unclear. Mold is very common in the environment, and respiratory exposure is almost constant. Mold spores are smaller in size than pollen allowing them to be inhaled deeply into the airways(32). They also have the ability to actively germinate and thus may evoke a stronger inflammatory response than other aeroallergens. Another possible explanation for our finding of a different cytokine profile in those with mold sensitisation could be related to the higher rates of asthma in our mold sensitised subjects. A previous study in individuals with asthma found those with severe poorly controlled asthma expressed lower levels of IL-4, and higher levels of IL-8 and IFN-γ in their bronchial walls than those with moderate asthma under good control(17). The authors suggested that those with severe poorly controlled asthma had a more neutrophilic inflammatory response

similar to that of patients with COPD and cystic fibrosis. It is possible that individuals sensitised to molds have a more neutrophilic inflammation and therefore their cytokine profiles are different to people sensitised to other aeroallergens. Unfortunately we did not have samples from the airways to examine this possibility. We did measure IL-8 levels, which were found to be higher in those sensitised to *Alternaria* and *Cladosporium* but the associations didn't reach statistical significance.

Of the four molds tested in this study the highest prevalence was to *Alternaria* with_7% sensitized followed by *Cladosporium* (4%), *Aspergillus* (3%) and *Pencillium* (2%). In the European Community Respiratory Health Survey I (ECRHS I) a study of European adults aged between 25-45 years the sensitisation pattern to molds was similar to our study with 4.4% sensitised to *Alternaria* and 2.3% sensitised to *Cladosporium*(3). The ECRHS I study found lower prevalences of HDM, grass pollen and cat sensitisation than we did (21%, 17% and 10% respectively in ECRHS). There have been few studies that have examined the relationship between serum cytokine levels in the general population sample. Two studies of cytokine levels in healthy population-based samples(33, 34) have reported levels of IL-6 similar to our levels, IL-4 level well below our levels and IL-5 levels higher in one study(33) and not detected in the other(34). The reasons for these discrepancies may include our method of statistical analysis as our levels were adjusted geometric means while other studies reported raw values. It may also reflect unmeasured or unrecorded disease pathology in the different studies.

Strengths of our study include the large cohort of individuals with allergic status confirmed through SPTs, and true population-based controls not recruited because of complex or severe disease. Our study is also the first to report serum cytokines levels in mold sensitised individuals in contrast to previous studies that measured *in vitro* cytokine levels from peripheral blood mononuclear cells(14, 15, 20) or alveolar epithelial cells lines(24) stimulated with molds. A number of parameters can affect measurements of cytokine levels in biological specimens collected in large scale trials and epidemiological studies including sample handling and storage, and the use of plasma vs serum to measure cytokines (35). A delay in processing samples can possibly lead to changes in cytokine levels due to degradation, absorption, or cellular production of cytokines (36). We therefore adjusted all analyses for time to processing and found no difference in our results. A recent study reported that stability of cytokines may be affected by

duration of sample storage at -80°C, although most cytokines were consistently stable for up to 2-3 years (37). Our samples were in storage for on average 2 years (samples collected between 2004 and 2008, and processed in 2008), so time dependent effects are expected to be minimal. There is some evidence that cytokine levels are lower when measured in serum rather than plasma (35, 38). However since all our samples were measured using serum any effect on cytokine levels would have been consistent across all samples resulting in an overall non-differential effect on the reported associations. Such non-differential misclassification can only lead to an underestimation of the reported associations. There is known to be variation in SPT reactivity with age(39) but all the TAHS participants were aged 45 at this assessment and so age is not likely to be a factor in our study. There is also known to be a lack of standardisation of fungal extracts used for SPTs with varying potency between different brands(40). We used the Hollister-Stier allergens in our study and further studies using alternative brands would be necessary to confirm our findings. We also did not have serum IgE measurement and so there is the possibility of misclassification of some non-sensitised individuals. On the other hand, data from the ECRHS indicated that nearly all sensitised individuals were identified by seven selected allergens (3) and any misclassification in relation to mold sensitisation would have tended to bias the results towards the null.

In summary, our study is the first large population based study to demonstrate reduced Th2 cytokine levels in people sensitised to molds. We also found a much higher odds of current asthma in individuals sensitised to mold compared with individuals sensitised to other aeroallergens. We postulate that differences in the Th2 cytokine profiles may indicate alternate pathogenic mechanisms are associated with mold sensitisation which may suggest alternative therapies may be useful in asthmatics with mold sensitisation compared with other aeroallergens. Further studies are needed to confirm our finding and to clarify the mechanisms of inflammatory disease in mold-sensitised individuals.

AUTHOR CONTRIBUTIONS

Study concept and design: MCM, RKK, MLKT, JAS, JLH, GGG, EHW, SCD

Acquisition of data: MCM, RKK, MLKT, IF, MCS, HT, SM, EHW, SCD

Analysis and interpretation of data: MCM, RKK, MLKT, JAS, SCD

Drafting of the manuscript: MCM, JCR, RKK, MLKT, SCD

Critical revision of the manuscript for important intellectual content: all authors.

Statistical analysis: MCM, RKK, JAS

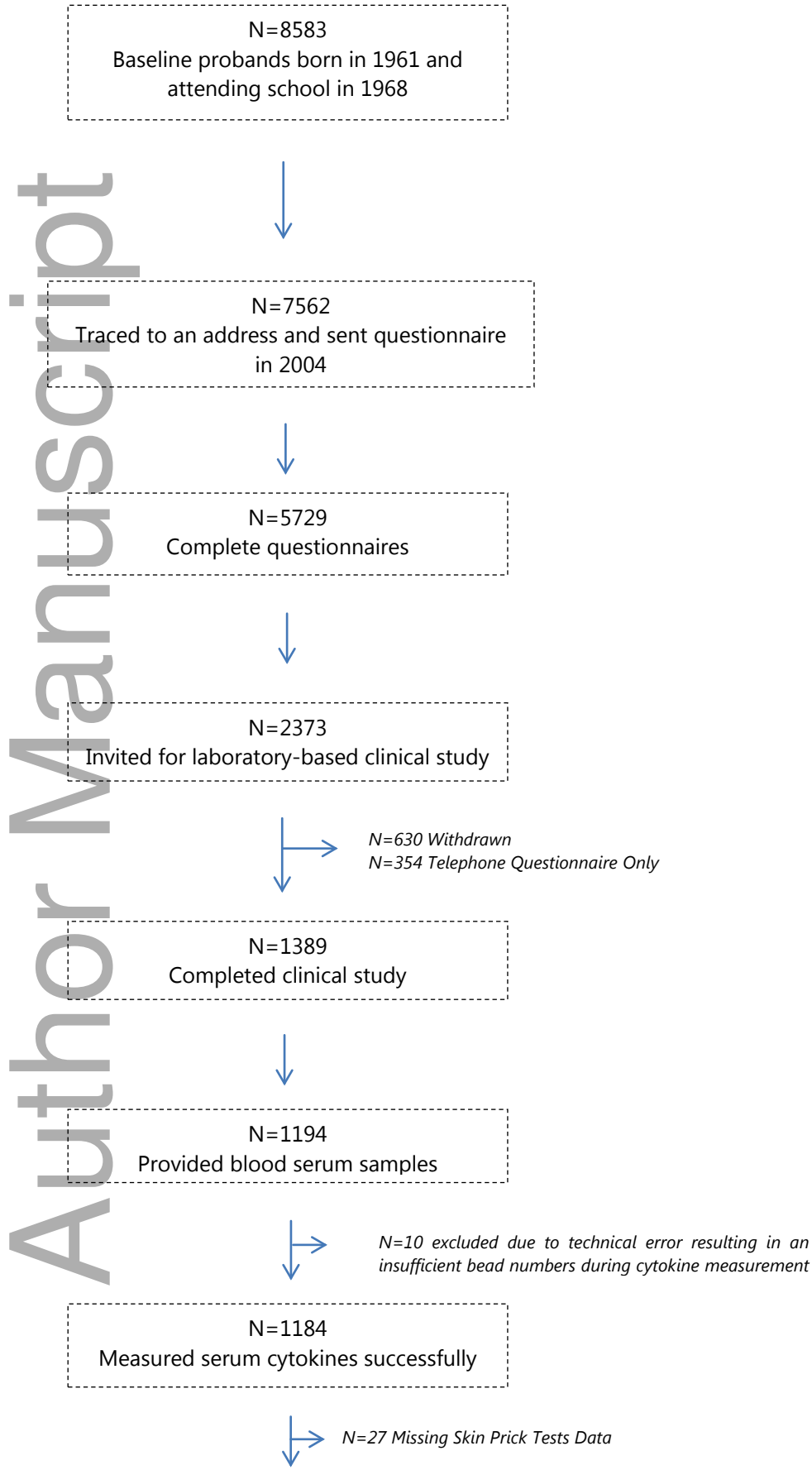
Obtained funding: MCM, JLH, SM, EHW, SCD

Administrative, technical, and material support: MCM, MLKT, SCD

Study supervision: MCM, MLKT, JAS, EHW, SCD.

CONFLICTS OF INTEREST

All authors declare that they do not have any conflicts of interest.



N=1157
Number of participants in the final analysis

Figure 1: Overview of the TAHS study participants in the analysis

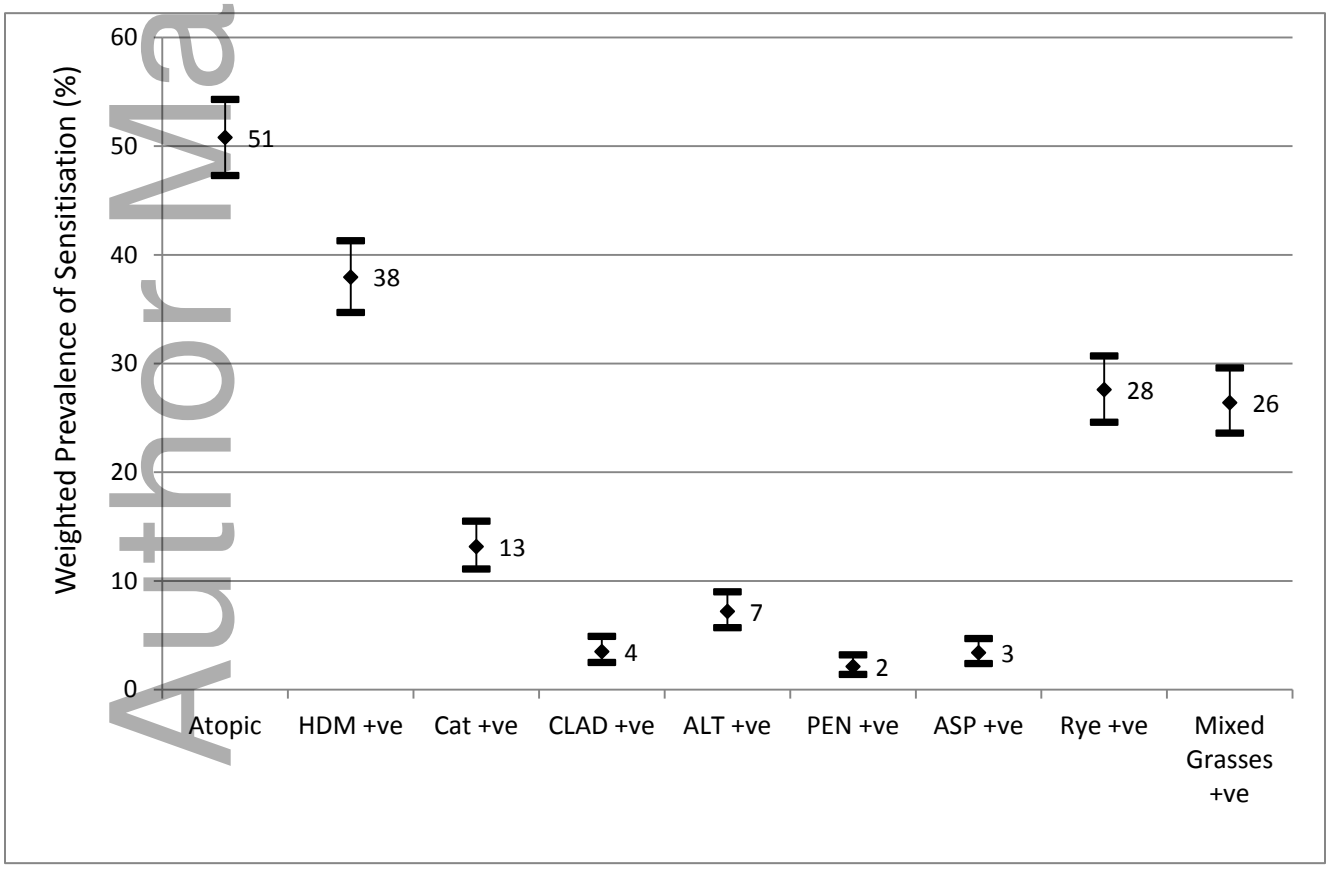


Fig 2: Adjusted prevalence of sensitisation to individual allergens

Table 1: Distribution of demographic characteristics by atopic sensitisation

Characteristics	Non-atopic[1] (n=512)	Atopic[2] (n=645)	HDM + (n=497)	CAT + (n=188)	Mold sensitivity				Grass sensitivity	
					CLAD. + (n=49)	*ALT.* + (n=105)	*PEN.* + (n=35)	*ASP.* + (n=51)	RYE + (n=358)	MIXED GRASSES + (n=341)
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Female gender	282(55%)	299(46%)	217(44%)	103(55%)	25(51%)	50(48%)	15(43%)	19(37%)	173(48%)	162(48%)
Current smoker[3]	149(29%)	175(27%)	135(27%)	47(25%)	12(25%)	24(23%)	9(26%)	11(22%)	87(24%)	81(24%)
Season attended the lab study[4]										
Winter	204(40%)	249(39%)	196(39%)	72(38%)	19(38%)	41(39%)	12(34%)	20(39%)	146(41%)	140(41%)
Summer	44(9%)	59(9%)	48(10%)	17(9%)	7(14%)	16(15%)	4(11%)	7(14%)	30(8%)	31(9%)
Autumn	104(20%)	151(23%)	109(22%)	42(22%)	8(16%)	22(21%)	10(29%)	10(20%)	81(23%)	83(24%)
Spring	106(31%)	185(29%)	143(29%)	57(30%)	15(31%)	26(25%)	9(26%)	14(27%)	100(28%)	86(25%)
Current asthma	48(9%)	167(26%)	138(28%)	78(42%)	20(41%)	34(32%)	17(49%)	23(45%)	102(28%)	98(29%)
Current ICS use	31(6%)	78(12%)	66(13%)	41(22%)	8(16%)	13(12%)	8(23%)	13(26%)	45(13%)	45(13%)
Used Antihistamine	8 (1.5%)	27 (4.2%)	20 (4.0%)	14 (7.5%)	5 (10.2%)	5 (4.8%)	3 (8.6%)	51(9.8%)	14(3.9%)	16(4.7)
Monosensitisation[5]	---	220 (34%)	173(35%)	14(7%)	2(4%)	10(10%)	1(3%)	2(4%)	9(2%)	9(3%)
Polysensitisation[6]	---	425 (66%)	324(65%)	174(93%)	47(96%)	95(90%)	34(97%)	49(96%)	349(98%)	332(97%)

[1]Non-atopic = SPT negative to all aeroallergens

[2]Atopic = SPT positive to any aeroallergen(s)

[3]data missing for 5 participants

[4]data missing for 1 participant

[5]SPT positive to only one (specific) allergen

[6]SPT positive to two or more allergens

Table 2 – Association between current asthma and specific allergen sensitisation when compared with the non-atopic group

Allergen Sensitisation	No (N=942) % n	Yes (N=215) % n	**Adjusted odds ratios[1]**	
			OR(95% CI)	**p-value**
Non-sensitised	49.3% 464	22.3% 48	Ref	
Atopic to any allergen	50.7% 478	77.8% 167	3.73 (2.62, 5.30)	0.0001
HDM and Cat				
HDM -ve / Other Aero +ve	12.6% 119	13.5% 29	2.43 (1.46, 4.03)	0.0001
HDM + ve	38.1% 359	64.2% 138	4.22 (2.93, 6.08)	0.0001
CAT -ve / Other Aero +ve	39.1% 368	41.4% 89	2.57 (1.75, 3.78)	0.0001
CAT + ve	11.7% 110	36.3% 78	7.45 (4.87, 11.4)	0.0001
MOLDS				
CLAD. -ve / Other Aero +ve	47.7% 449	68.4% 147	3.49 (2.44, 4.99)	0.0001
CLAD. + ve	3.1% 29	9.3% 20	7.53 (3.90, 14.5)	0.0001
ALT. -ve / Other Aero +ve	43.2% 407	61.9% 133	3.49 (2.43, 5.02)	0.0001
ALT. + ve	7.5% 71	15.8% 34	5.07 (3.03, 8.48)	0.0001

PEN. -ve / Other Aero +ve	48.8% 460	69.8% 150	3.47 (2.43, 4.95)	0.0001
PEN. + ve	1.9% 18	7.9% 17	11.4 (5.38, 24.2)	0.0001
ASP. -ve / Other Aero +ve	47.8% 450	66.9% 144	3.41 (2.38, 4.87)	0.0001
ASP. + ve	2.9% 28	10.7% 23	9.61 (5.05, 18.3)	0.0001
POLLEN				
RYE -ve / Other Aero +ve	23.6% 222	30.2% 65	3.13 (2.07, 4.73)	0.0001
RYE + ve	27.2% 256	47.4% 102	4.23 (2.88, 6.21)	0.0001
MIXED GRASSES-ve / Other Aero +ve	25.0% 235	32.1% 69	3.12 (2.07, 4.69)	0.0001
MIXED GRASSES + ve	25.8% 243	45.6% 98	4.31 (2.93, 6.35)	0.0001

OR = Odds Ratio; 95% CI = 95% Confidence Interval; [1]Adjusted for gender, current smoker, inhaled corticosteroid use last year and the season attended at the laboratory

Table 3 – Adjusted geometric mean concentrations of IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α cytokines (in pg/ml) by the Atopic Sensitisation group

Cytokine	Atopic Sensitisation group	N	Geometric Mean (95% CI) in pg/mL			Ratios of Geometric Means (95% CI)			P-Value
IL-4	Non- atopic	509	30.25	23.13	39.55	1.00			
	Atopic	641	38.09	29.93	48.48	1.26	0.87	1.82	0.22
	HDM+ve	493	37.37	28.32	49.33	1.23	0.84	1.81	0.28
	Cat +ve	186	36.79	23.25	58.20	1.22	0.70	2.10	0.48
	CLAD +ve	48	7.62	3.25	17.91	0.25	0.10	0.62	0.003
	ALT +ve	105	25.35	13.88	46.27	0.84	0.43	1.61	0.59
	PEN +ve	34	12.70	4.20	38.46	0.42	0.13	1.29	0.13
	ASP +ve	51	9.72	3.98	23.70	0.32	0.12	0.82	0.018
	Rye +ve	354	38.58	27.63	53.85	1.28	0.83	1.96	0.27
	Mixed Grasses +ve	337	37.96	26.54	54.30	1.25	0.80	1.98	0.33
IL-5	Non- atopic	509	0.25	0.20	0.30	1.00			
	Atopic	641	0.34	0.29	0.40	1.37	1.06	1.77	0.016
	HDM+ve	493	0.33	0.27	0.39	1.33	1.02	1.73	0.04
	Cat +ve	186	0.34	0.25	0.47	1.39	0.96	2.01	0.09
	CLAD +ve	48	0.14	0.08	0.24	0.55	0.30	0.99	0.05
	ALT +ve	105	0.30	0.20	0.45	1.20	0.76	1.90	0.44
	PEN +ve	34	0.25	0.11	0.55	0.99	0.43	2.28	0.99
	ASP +ve	51	0.21	0.11	0.39	0.84	0.44	1.62	0.61
	Rye +ve	354	0.33	0.27	0.41	1.35	1.01	1.80	0.05
	Mixed Grasses +ve	337	0.36	0.29	0.45	1.45	1.08	1.95	0.01
IL-6	Non- atopic	509	11.82	9.91	14.10	1.00			
	Atopic	641	14.34	12.32	16.69	1.21	0.96	1.53	0.11
	HDM+ve	493	14.89	12.44	17.81	1.26	0.98	1.63	0.08
	Cat +ve	186	15.26	11.33	20.54	1.29	0.91	1.84	0.15
	CLAD +ve	48	5.94	2.85	12.38	0.50	0.24	1.07	0.07
	ALT +ve	105	13.50	8.76	20.79	1.14	0.72	1.82	0.58

	PEN +ve	34	8.45	3.74	19.10	0.71	0.31	1.65	0.43
	ASP +ve	51	8.82	4.33	17.99	0.74	0.35	1.56	0.43
	Rye +ve	354	13.94	11.28	17.23	1.18	0.89	1.56	0.25
	Mixed Grasses +ve	337	15.29	12.37	18.90	1.30	0.97	1.72	0.08
IL-8	Non- atopic	509	257.63	221.90	299.16	1.00			
	Atopic	641	291.20	255.38	332.04	1.13	0.92	1.39	0.25
	HDM+ve	493	286.91	247.20	333.01	1.11	0.90	1.38	0.33
	Cat +ve	186	256.53	198.29	331.86	0.99	0.73	1.35	0.96
	CLAD +ve	48	330.35	203.18	537.12	1.28	0.76	2.16	0.35
	ALT +ve	105	337.24	240.66	472.57	1.31	0.90	1.90	0.16
	PEN +ve	34	273.58	157.73	474.50	1.06	0.60	1.88	0.84
	ASP +ve	51	238.70	141.22	403.49	0.92	0.53	1.63	0.79
	Rye +ve	354	290.68	244.11	346.15	1.13	0.89	1.43	0.32
	Mixed Grasses +ve	337	308.11	257.07	369.29	1.20	0.94	1.52	0.14
IL-10	Non- atopic	509	3.06	2.43	3.85	1.00			
	Atopic	641	2.87	2.37	3.49	0.94	0.69	1.28	0.69
	HDM+ve	493	3.05	2.43	3.82	1.00	0.72	1.39	1.00
	Cat +ve	186	2.61	1.80	3.77	0.85	0.55	1.32	0.47
	CLAD +ve	48	2.47	1.34	4.55	0.81	0.42	1.56	0.53
	ALT +ve	105	3.53	2.32	5.38	1.16	0.71	1.87	0.55
	PEN +ve	34	3.30	1.65	6.59	1.08	0.52	2.26	0.84
	ASP +ve	51	3.28	1.65	6.49	1.07	0.52	2.23	0.85
	Rye +ve	354	2.60	2.01	3.36	0.85	0.60	1.20	0.36
	Mixed Grasses +ve	337	2.73	2.10	3.56	0.89	0.63	1.27	0.52
TNF-α	Non- atopic	509	6.70	6.26	7.16	1.00			
	Atopic	641	6.67	6.25	7.12	1.00	0.91	1.10	0.93
	HDM+ve	493	7.03	6.55	7.54	1.05	0.95	1.16	0.31
	Cat +ve	186	7.14	6.26	8.14	1.07	0.92	1.24	0.39
	CLAD +ve	48	7.15	5.71	8.96	1.07	0.84	1.35	0.58
	ALT +ve	105	7.00	5.91	8.28	1.05	0.87	1.26	0.64

	PEN +ve	34	6.49	4.71	8.92	0.97	0.70	1.35	0.85
	ASP +ve	51	7.64	6.12	9.54	1.14	0.90	1.44	0.27
	Rye +ve	354	6.87	6.32	7.47	1.03	0.92	1.15	0.64
	Mixed Grasses +ve	337	7.15	6.53	7.83	1.07	0.95	1.20	0.26

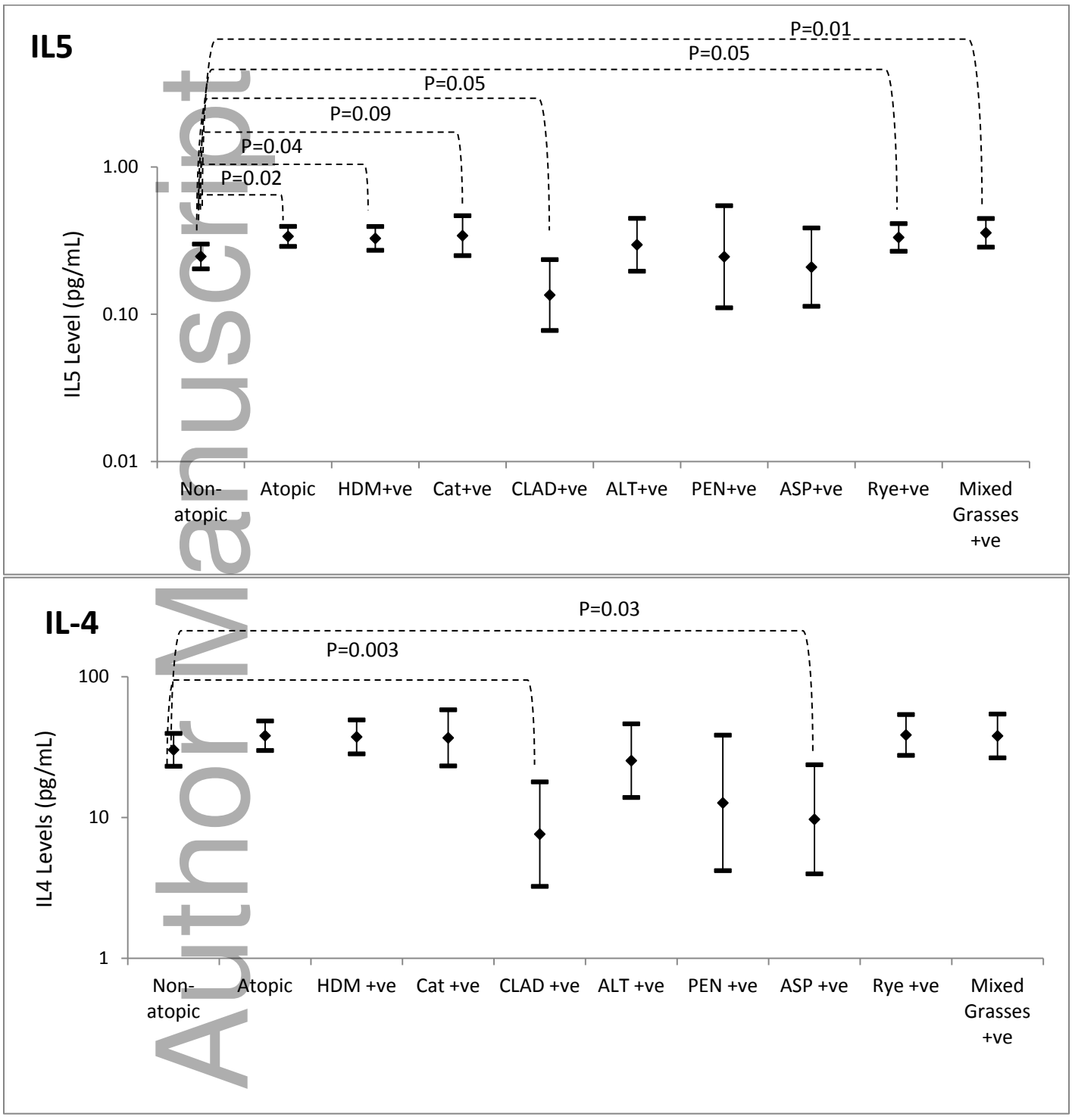


Figure 3 –Adjusted geometric mean concentrations of IL-4, and IL-5 cytokines (in pg/ml) by the atopic sensitisation group.

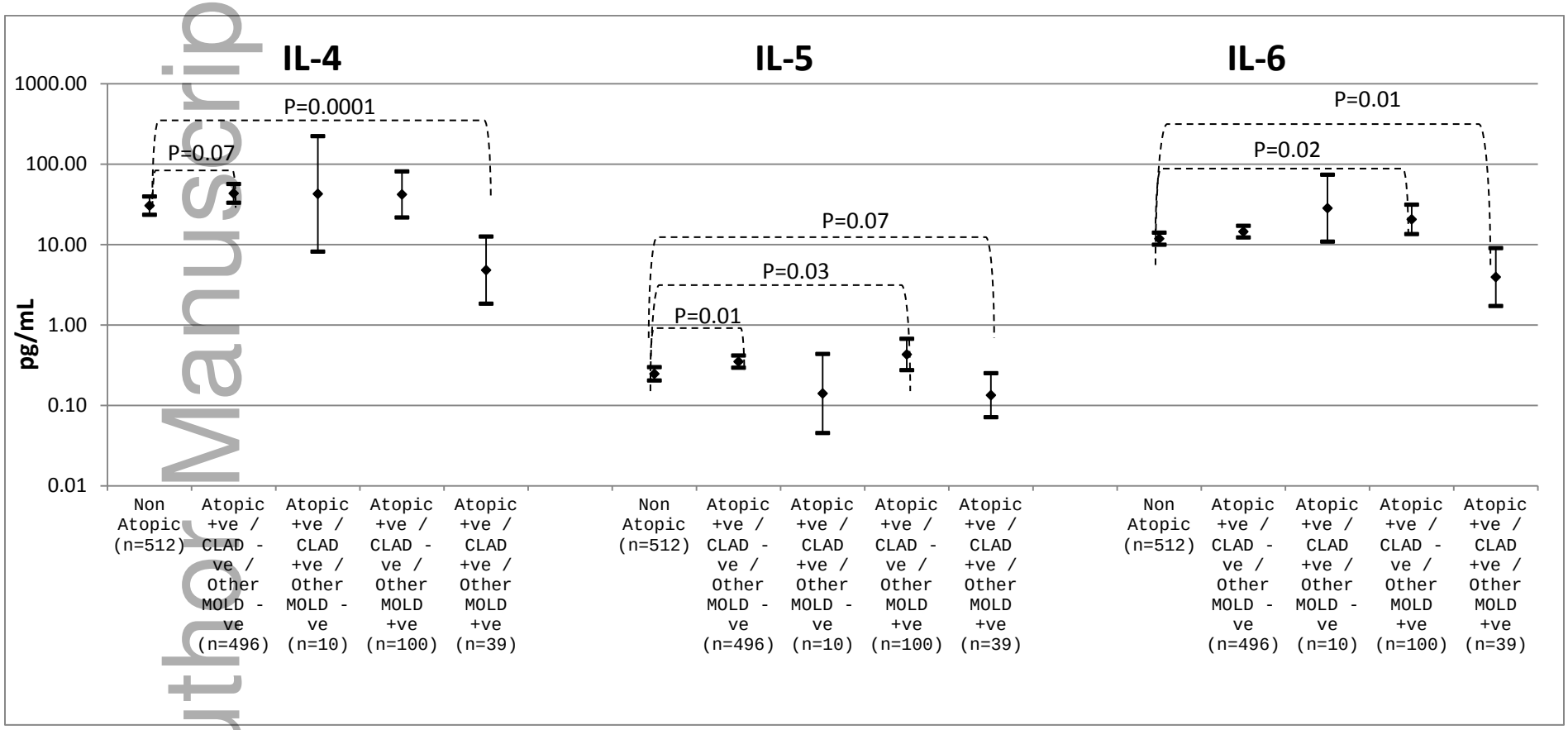


Figure 4 – Adjusted geometric mean concentrations of IL-4, IL-5 and IL-6 cytokines (in pg/ml) by any sensitisation, any mold and Cladosporium sensitisation groups. Atopic individuals (those sensitised to at least 1 allergen) were further divided into four groups based on *Cladosporium* (CLAD) and other mold (Other MOLD) sensitisation status.

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