

## **Different inflammatory phenotypes in adults and children with acute asthma**

**F. Wang<sup>\*,#,¶</sup>, X.Y. He<sup>#,¶</sup>, K.J. Baines<sup>#,¶</sup>, L.P. Gunawardhana<sup>#,¶</sup>, J.L. Simpson<sup>#,¶</sup>, F. Li<sup>\*</sup> and P.G. Gibson<sup>\*,#,¶</sup>**

\*Dept of Pathogenobiology, Norman Bethune College of Medicine, Jilin University, Changchun, China.

<sup>#</sup>Centre for Asthma and Respiratory Disease, University of Newcastle, Callaghan, NSW, Australia.

<sup>¶</sup>Department of Respiratory and Sleep Medicine, Hunter Medical Research Institute, John Hunter Hospital, New Lambton, NSW, Australia.

### **CORRESPONDENCE**

P.G. Gibson

Respiratory Medicine John Hunter Hospital

Locked Bag 1

Hunter Region Mail Centre

Newcastle

NSW 2310

Australia

Email: Peter.gibson@hnehealth.nsw.gov.au

**ABSTRACT:**

Inflammatory phenotypes are recognised in stable adult asthma, but are less established in childhood and acute asthma. Additionally, *Chlamydothyla pneumoniae* infection as a cause of noneosinophilic asthma is controversial. This study examined the prevalence of inflammatory phenotypes and the presence of current *Chlamydothyla pneumoniae* infection in adults and children with stable and acute asthma.

Adults with stable(n=29) or acute(n=22) asthma, healthy adults(n=11), and children with stable(n=49) or acute(n=28) asthma and healthy children(n=9) underwent clinical assessment and sputum induction. Sputum was assessed for inflammatory cells, and DNA was extracted from sputum cell suspensions and supernatant for *Chlamydothyla pneumoniae* detection using real-time PCR.

Asthma phenotype was predominantly eosinophilic in children with acute asthma (50%) but neutrophilic in adults with acute asthma (82%). Paucigranulocytic asthma was the most common phenotype in both adults and children with stable asthma. *Chlamydothyla pneumoniae* was not detected in 99% of samples.

The pattern of inflammatory phenotypes differs between adults and children, with eosinophilic inflammation being more prevalent in both acute and stable childhood asthma, and neutrophilic inflammation being the dominant pattern of acute asthma in adults. The aetiology of neutrophilic asthma is unknown and is not explained by the presence of current active *Chlamydothyla pneumoniae* infection.

**KEYWORDS:** *Chlamydothyla pneumoniae*, Asthma phenotypes, Children with acute asthma, Adults with acute asthma

## **INTRODUCTION:**

Asthma is a chronic inflammatory airway disease characterised by episodic exacerbations. There is heterogeneity in the airway inflammatory response in asthma that can be related to both exposures, such as allergen and infection, and to treatment[1]. We have previously categorized stable adult asthma into four inflammatory phenotypes based on induced sputum inflammatory cell counts [2]. These include eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic asthma, defined by the presence and/or absence of airway eosinophils and neutrophils. The occurrence and relative frequency of these phenotypes in acute asthma and in childhood asthma is not known. Similarly the relation of specific phenotypes to infectious triggers has not been well studied.

While exposure to allergen in a sensitized individual can elicit an eosinophilic response, the triggers of the noneosinophilic phenotypes have been less well studied. Chronic infection is a possible cause since infections typically elicit a neutrophil response similar to that observed in neutrophilic asthma (NA). This response is mediated via innate immune activation, and we have previously shown evidence of innate immune dysregulation in adults with NA [3]. In that study there was significant upregulation of TLR2 gene expression and a corresponding increase in CXCL-8 (IL-8) in NA. While viral infection is a frequent cause of acute asthma and airway neutrophilia, this is usually mediated by TLR3 or TLR7 activation and does not explain the neutrophilic responses that are seen in stable asthma. These data suggest that other organisms capable of both chronic and acute infection, and eliciting TLR2 mediated responses, may be relevant in noneosinophilic asthma (NEA).

*Chlamydomypha pneumoniae* (Cpn) is a potential cause of NEA since it causes both an acute infection, and a chronic persistent infectious state with reactivation [4]. Cpn infects lung tissue and elicits an innate immune response that is mediated via TLR2 [5] and results in neutrophilic inflammation [4]. Cpn has also been reported to worsen asthma severity [6], and to be a cause of asthma exacerbation [7]. NA occurs typically in older adults, and there is evidence that Cpn infection might precede the onset of adult asthma, as well as persisting in stable chronic asthmatics [8]. Furthermore, the noneosinophilic phenotype of severe asthma is improved by macrolide antibiotics, a class of drugs that are active against Cpn [9]. Cpn detection may be higher in children, or in acute exacerbations of asthma, where there is also heterogeneity of the inflammatory response. Thus, Cpn may play a potential role in modulating the

different inflammatory phenotypes of asthma, and NEA in particular. The potential mechanisms include chronic persistence of Cpn in NEA, or acute infection with clearance of the organism, but persistent immune reprogramming that leads to the immunopathology of NEA [4, 10]. The latter mechanism is biologically plausible and has support from model systems of asthma [4, 10]. Real-Time PCR is an effective method for detecting Cpn using induced sputum [11]. The MOMP gene has previously been shown to be a good genetic marker for CPN in other real time PCR based studies [12, 13].

The aim of this study was to examine the differences in inflammatory phenotype of asthma among adults with stable asthma (ASA), adults with acute asthma (AAA), children with stable asthma (CSA) and children with acute asthma (CAA), and to investigate the role of chronic Cpn infection in the NEA phenotypes. We hypothesized that there would be differences in inflammatory phenotypes between adults and children with asthma, specifically that acute asthma in children and adults would be similar and exhibit a neutrophilic phenotype, whereas in stable asthma the eosinophilic asthma (EA) phenotype would be more prevalent in children compared with adults. We also hypothesized that Cpn infection may be common with NEA phenotypes. To assess these hypotheses we selected subjects who had participated in previous studies [3, 14, 15] and evaluated inflammatory phenotypes from induced sputum cell counts and Cpn using DNA extracted from sputum samples.

## **MATERIALS AND METHODS**

### ***Participants***

There were 4 groups of asthmatics and 2 groups of healthy controls selected for study. Non-smoking ASA (n=29) and AAA (n=22), had asthma defined using the American Thoracic Society criteria, and each had a doctor's diagnosis of symptomatic asthma. CSA (n=49), and CAA (n=28) had asthma diagnosed by a respiratory pediatrician based on clinical and lung function criteria. The subjects with acute asthma were recruited soon after presentation to the John Hunter Hospital emergency department with an acute exacerbation of asthma, as described [3, 14, 15]. Participants were recruited within 4 hours of attendance and they were all pretreated with inhaled  $\beta$ 2 agonist before sputum induction. The stable subjects had no recent (past 4 weeks) respiratory infection, asthma exacerbation or medication change. Two comparison groups were selected comprising non-smoking healthy adults (n=11) and healthy

children (n=9) with no respiratory symptoms and normal lung function. The subjects were selected from the laboratory database based on the selection criteria above, the availability of induced sputum cell counts and stored sputum samples for analysis. Some have been reported in prior research [3, 14]. All participants gave informed consent prior to their inclusion in the study and Hunter New England Area Health Service Human Research Ethics Committees approved this study.

### ***Sputum induction and analysis***

Spirometry (KoKo PD Instrumentation, Louisville CO USA) and sputum induction was performed using hypertonic saline (4.5% for stable asthma and controls; 0.9% for acute asthma) delivered by ultrasonic nebuliser as previously described[16]. The induced sputum was collected, mucocellular portions were selected separated from the whole sputum sample and dispersed using dithiothreitol (DTT) [17]. The suspension was then filtered and a total cell count (TCC) was obtained using trypan blue exclusion. The remaining sample was centrifuged and the cell supernatants were stored at -80°C. The cell pellet was diluted with phosphate buffered saline (PBS) to  $1 \times 10^6$  cells per mL. Cytospins were prepared, stained with May-Grunwald Giemsa and a differential cell count was obtained from 400 non-squamous cells. The remaining cell suspension was stored at -80°C in PBS.

### ***Classification of asthma phenotypes***

Based on previous studies [2, 16], subjects with sputum eosinophils >3% were classified as EA and those with sputum neutrophils >61% and eosinophils <3% were classified as NA. Subjects with sputum neutrophils >61% and sputum eosinophils >3% were classified as mixed granulocytic asthma and those with sputum neutrophils <61% and eosinophils <3% were classified as paucigranulocytic asthma.

### ***Cpn detection***

Genomic DNA was isolated from cell suspensions and cell supernatants of induced sputum using QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA), as per manufacturers instructions for isolation of bacterial DNA. DNA was eluted in 100  $\mu$ L of buffer AE, and stored at -20°C. Quality and quantity of extracted DNA was assessed using the NanoDrop 2000 (Thermo Fisher Scientific Inc, Wilmington, DE, USA). Real-time PCR was performed with the 7500 Real Time PCR system (Applied

Biosystems, Foster City, CA). Cpn was detected using the Quantification of Chlamydomphila pneumoniae Major Outer Membrane Protein (MOMP) gene Advanced kit (PrimerDesign™Ltd) as per manufacturers instructions. Pathogen specific primer/probe mix (FAM labeled), pathogen positive control template and Endogenous β-actin (ACTB) primer/probe mix inside the kit were used to detect Cpn MOMP gene and Endogenous ACTB gene.

### ***Validation of Cpn detection in sputum***

Suitability of sputum processing and DNA extraction methods for recovery of Cpn DNA were assessed in a separate experiment (fig. 3a). Briefly, an induced sputum sample was divided into several aliquots (200ul) and each aliquot were combined with different amounts of CPN positive DNA (PrimerDesign TMLtd). Then aliquots were processed and cell suspension and supernatants were obtained. The processed samples were stored a day in -80 °C to emulate laboratory sample storage conditions before DNA were extractions were performed. The DNA were quantified and subjected to qPCR under identical conditions.

### ***Statistical analysis***

All the data were analysed using Stata 9 (Stata Corporation, College Station, TX, USA). Results were reported as median and interquartile range and the two-sample Wilcoxon Rank Sum test, or Kruskal Wallis test were used to analyse data. Fishers' exact test or chi square test were used to analyse categorical data. Wilcoxon sign rank test was used to analyse paired data such as DNA concentration both in sputum cell suspensions and sputum cell supernatants. Significance was accepted when  $p < 0.05$ . Post hoc analysis significance was accepted as  $p < 0.017$ .

## **RESULTS**

### ***Demographic and clinical characteristics***

The clinical and demographic characteristics of the study subjects are shown in table 1. Compared to healthy controls, adults with asthma exhibited more atopy (90% (stable) and 69% (acute) versus 18%(control),  $p < 0.001$ ) and significantly greater airway obstruction with lower FEV<sub>1</sub> values than healthy controls (71.4% and 73.0% versus 100.8%,  $p < 0.008$ ). Apart from a slightly younger age in the acute asthma group, the two asthmatic groups did not differ with respect to gender, and

maintenance corticosteroid treatment. Children were aged between 7 and 17 years and those with stable asthma had a higher prevalence of atopy than healthy controls (56% *versus* 86%,  $p=0.056$ ). Subjects with CAA had significantly greater airflow obstruction with lower FEV<sub>1</sub>% values than the stable asthma group (53% *versus* 91%,  $p=0.006$ ). Compared to ASA, CSA showed better lung function with higher FEV<sub>1</sub>% values ( $p<0.001$ ). However, subjects with CAA had greater lung function impairment than the AAA. There were fewer ex-smokers in the AAA group (18%), compared to the ASA (38%) and healthy controls ( $p=0.012$ ). There were similar proportions of ICS users in adults with acute and stable asthma, and children with acute and stable asthma (Table 1). The ICS doses were similar also, being  $>1000\mu\text{g}/\text{day}$  in beclomethasone equivalents in adults and around  $480\mu\text{g}/\text{day}$  in children.

#### ***Analysis of inflammatory cells between healthy controls and asthmatic groups in adults and children***

The induced sputum cell quality was good in both adults and children, with similar cell viability and low levels of squamous contamination (median squamous cells  $<4.5\%$ , all groups, data not shown). Induced sputum differential cell counts are shown in table 2. The proportion of neutrophils in the AAA group was significantly higher than that in healthy adults and ASA (fig. 1a). Sputum eosinophils in CAA were significantly higher than in healthy children and CSA (fig. 1d). Both acute asthma groups (adults and children) showed significantly lower percentages of macrophages compared to healthy and stable asthma groups, however numbers of macrophages were not different between groups. CAA had a lower lymphocyte count than CSA (table 2). Total number of cells did not differ between groups.

#### ***Comparison of inflammatory phenotypes between acute and stable asthma in adults and children***

All inflammatory phenotypes were observed in adults and children with stable and acute asthma, and detailed results are shown in table 3. The distribution of phenotypes was similar between ASA and CSA, however there were differences in the relative frequency of the phenotypes between AAA and CAA (fig. 2). In ASA, the most frequent inflammatory phenotype was paucigranulocytic followed by neutrophilic. Similarly, in CSA, the most frequent inflammatory phenotype was paucigranulocytic followed by eosinophilic. In AAA, the inflammatory phenotype was predominantly

neutrophilic, with the remainder being mixed granulocytic. In CAA, the predominant phenotype was eosinophilic followed by the mixed granulocytic. The pattern of inflammatory phenotype in CAA was different to AAA with a significantly higher frequency of the eosinophilic subtype compared to CSA.

### **Real-time PCR for Cpn-MOMP gene**

The differences in inflammatory phenotypes between AAA and CAA indicate different inflammatory processes or aetiological agents. We therefore examined Cpn in these subjects, hypothesising an increased prevalence in acute asthma and NA. Eleven samples containing both sputum cell suspensions and sputum cell supernatant from adult healthy controls, 9 samples containing sputum cell suspensions from healthy children, 26 samples containing both sputum cell suspensions and sputum cell supernatant from ASA, 49 sputum cell supernatant samples from CSA; 22 and 28 sputum cell supernatant samples from AAA and CAA respectively were tested for the presence of Cpn.

DNA was efficiently extracted from both sample types. The DNA concentration of sputum cell supernatant was 14.2 (6.6, 26.6) ng/ $\mu$ l and there was a nonsignificant trend for more DNA to be extracted from sputum cell suspensions 25.2 (13.2, 48.9) ng/ $\mu$ l ( $p=0.062$ ). Amplification plots indicated a typical sigmoid curve for the positive control (data not shown) and flattened baseline bound curves for the negative controls as well as for the samples negatively detected for Cpn. The sigmoid curves for ACTB detected for each sample were within the expected Ct range, the Ct median range was 25.12 (21.38, 34.24), confirming the quality of input DNA. To validate our methods, induced sputum was spiked with serial dilutions of Cpn positive DNA, processed and analysed for Cpn-MOMP DNA by PCR. The assay could detect Cpn-MOMP DNA from induced sputum with a sensitivity of  $2 \times 10^5$  copies/ $\mu$ l, confirming the suitability of our methodology (fig. 3b). However, using this sensitive and validated assay, Cpn was detected from only one sample, a child with acute asthma.



## **DISCUSSION**

This study has identified increased airway inflammation in most cases of acute asthma, but that the inflammatory phenotypes are different between adults and children with acute asthma. These results have implications for the likely mechanisms and triggers of acute asthma in adults and children, and may also impact on treatment responsiveness. We have also found that in stable asthma the paucigranulocytic pattern was most frequent, followed by EA in children and NA in adults. Despite a high frequency of the noneosinophilic phenotypes (neutrophilic and paucigranulocytic), we were unable to confirm a role for current Cpn infection in NEA, despite using a sensitive and specific PCR based assay. This suggests that the potential role for Cpn infection may be to modulate immune responses at the time of infection, which has the potential to induce a long lasting influence on the expression of asthma.

The major finding of this study was the difference in phenotypes between AAA and CAA. Acute asthma is an intensely inflammatory state since the paucigranulocytic phenotype was seen either not at all (adults) or rarely (children, 2%) in this setting. The predominant inflammatory phenotype in AAA was NA (81.8%), whereas in children it was EA . This clearly suggests that there are different inflammatory pathways involved in disease pathogenesis between AAA and CAA. The possibilities for the eosinophil response seen in CAA include allergen exposure, less maintenance corticosteroid therapy, or a different response to triggers of acute asthma. CAA did have less ICS use, but, nonetheless 68% were using maintenance ICS, so this is an incomplete explanation for these effects. Viral respiratory infections are the most frequent cause of acute asthma in adults and children[18]. Acute exacerbations also occur more often in children who are sensitized to aeroallergens [19]. The mixed eosinophil-neutrophil responses in CAA suggest that concurrent exposure to multiple triggers, eg allergen and virus infection, may be an explanation for the inflammatory response observed. This is consistent with the epidemiological observations that a combination of atopic sensitisation, allergen exposure and viral infection are required to precipitate acute asthma in children [20].

Adults exhibit an increase in IL-10 with acute asthma, which is a cytokine with known antieosinophilic effects [21], which may explain the different responses between groups. These differences in inflammatory phenotype were accompanied by differences in clinical effects, as lung function was lower in acute asthma in children than adults. It is interesting to speculate whether the treatment responses might be different between the groups. EA is typically corticosteroid sensitive, whereas the acute response to steroid is either blunted or absent in NEA [22]. These observations have been made in stable asthma, but if they also apply in acute asthma, then it would predict a lesser response to corticosteroid in acute asthma in adults, and the need for other therapies active against neutrophilic responses[23].

The clinical patterns of stable asthma differ between adults and children. Adults with asthma are more likely to exhibit a persistent pattern, whereas episodic asthma is typical in children [24]. Despite these differences in clinical pattern, the inflammatory patterns were similar between adults and CSA. In our study, NEA was common in stable asthma. Gibson et al found that a non-eosinophilic pattern of inflammation was reported in 59% of 56 adults with persistent asthma [25]. The results of our study are similar to Ronchi who found that 28% of subjects showed increased sputum neutrophils in adult stable subjects with asthma [26]. The percentage was much lower in children (20.4%) with stable asthma. But the prevalence of EA was higher in children (30.6%) than in adults (20.7%) with stable asthma in our study, and this was similar with Gibson's results [27]. Furthermore, the specific finding in this study was that the proportion with paucigranulocytic asthma was greatest in ASA (51.0%), and a similar trend was observed in CSA. The paucigranulocytic phenotype may represent controlled airway inflammation, and suggests it may relate to better disease prognosis.

We also sought to examine if Cpn infection could be detected in the NEA phenotypes. To do this we used and validated a sensitive PCR based assay, and examined a wide range of clinical asthma groups. Cpn has been associated with adult onset asthma [28], childhood asthma[29], and asthma severity in adults[6]. It has been postulated that NEA may represent a chronic infectious state, since many of the features mimic the responses elicited by infection, such as neutrophilia, IL-8 release, and activation of toll-like receptors [3]. Infection with Cpn has also been considered a possible cause of NEA[9]. We collected both sputum cell supernatants and suspensions of intact cells in order to maximise the chances of detecting both intracellular and extracellular Cpn,

[11], We included appropriate controls in the assay and the results of positive controls and the ACTB control demonstrate that the methods we used were effective. The detection results were mostly negative in the different clinical populations in this study except one sample in a child with acute asthma. We interpret our results to indicate that current active acute or chronic infection is uncommon in our population. This stands in contrast to other populations, particularly Europe, where Cpn infection is more prevalent. Based on studies on Cpn infection in neonates, we can't exclude the possibility that immunodeviation following resolved Cpn infection in early life may be a mechanism that modulates NEA.

Potential limitations of the study relate to the sample size and the selection methods for subjects. There was good power for the analyses involving neutrophilic asthma in adults and children, and eosinophilic asthma in children (acute and stable) however the small sample size meant limited power to assess the eosinophilic phenotype in adults with acute asthma (power =40%). Similarly, using subjects selected from a database may introduce selection bias. However, the same data collecting methods were used for all participants, where a staff member attended the emergency department to recruit adults and children with acute asthma. As the adult and paediatric emergency rooms are co-located, there was no bias that could arise from differential recruitment. The main limitation to this method was the amount of time that the staff member could spend in the emergency room, ie during working hours. The different FEV1 results between adults and children with acute asthma may reflect timing of measurement after  $\beta$ -agonist dosing. While all subjects had FEV1 measured after  $\beta$ -agonist pretreatment, the number of additional doses before induction was not controlled and may have impacted on comparisons between groups.

In summary, these findings provide evidence for differences in the prevalence of inflammatory phenotypes in AAA and CAA, These differences were associated with clinically significant alterations in lung function and suggest the involvement of different mechanistic pathways in acute asthma in children and adults. CAA showed high eosinophilic inflammatory responses, whereas neutrophilic responses were most frequent in AAA. The paucigranulocytic phenotype was the major pattern observed in both ASA and CSA, although heterogeneity in inflammatory phenotypes was a common feature in stable asthma. Current Cpn infection does not appear to be a

common reason to explain the noneosinophilic phenotype in asthma, nor to increase respiratory acute exacerbation.

ACKNOWLEDGMENT: We thank H. Powell for her statistical assistance and N. Fibbens for data collection.

## REFERENCES

1. ten Brinke, A., et al., *Factors associated with persistent airflow limitation in severe asthma*. Am J Respir Crit Care Med, 2001. **164**(5): p. 744-8.
2. Simpson, J.L., et al., *Inflammatory subtypes in asthma: assessment and identification using induced sputum*. Respirology, 2006. **11**(1): p. 54-61.
3. Simpson, J.L., et al., *Innate immune activation in neutrophilic asthma and bronchiectasis*. Thorax, 2007. **62**(3): p. 211-8.
4. Horvat, J.C., et al., *Chlamydial respiratory infection during allergen sensitization drives neutrophilic allergic airways disease*. J Immunol, 2010. **184**(8): p. 4159-69.
5. Droemann, D., et al., *Disparate innate immune responses to persistent and acute Chlamydia pneumoniae infection in chronic obstructive pulmonary disease*. Am J Respir Crit Care Med, 2007. **175**(8): p. 791-7.
6. Black, P.N., et al., *Serological evidence of infection with Chlamydia pneumoniae is related to the severity of asthma*. Eur Respir J, 2000. **15**(2): p. 254-9.
7. Lieberman, D., et al., *Atypical pathogen infection in adults with acute exacerbation of bronchial asthma*. Am J Respir Crit Care Med, 2003. **167**(3): p. 406-10.
8. Daian, C.M., A.H. Wolff, and L. Bielory, *The role of atypical organisms in asthma*. Allergy Asthma Proc, 2000. **21**(2): p. 107-11.
9. Simpson, J.L., et al., *Clarithromycin targets neutrophilic airway inflammation in refractory asthma*. Am J Respir Crit Care Med, 2008. **177**(2): p. 148-55.
10. Horvat, J.C., et al., *Early-life chlamydial lung infection enhances allergic airways disease through age-dependent differences in immunopathology*. J Allergy Clin Immunol, 2010. **125**(3): p. 617-25, 625 e1-625 e6.
11. Harju, T.H., et al., *Pathogenic bacteria and viruses in induced sputum or pharyngeal secretions of adults with stable asthma*. Thorax, 2006. **61**(7): p. 579-84.
12. Kuoppa, Y., et al., *Quantitative detection of respiratory Chlamydia pneumoniae infection by real-time PCR*. J Clin Microbiol, 2002. **40**(6): p. 2273-4.
13. Apfalter, P., et al., *Comparison of a new quantitative ompA-based real-Time PCR TaqMan assay for detection of Chlamydia pneumoniae DNA in respiratory specimens with four conventional PCR assays*. J Clin Microbiol, 2003. **41**(2): p. 592-600.
14. Wark, P.A., et al., *Chlamydia pneumoniae immunoglobulin A reactivation and airway inflammation in acute asthma*. Eur Respir J, 2002. **20**(4): p. 834-40.
15. Norzila, M.Z., et al., *Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma*. Am J Respir Crit Care Med, 2000. **161**(3 Pt 1): p. 769-74.
16. Simpson, J.L., P. McElduff, and P.G. Gibson, *Assessment and reproducibility of non-eosinophilic asthma using induced sputum*. Respiration, 2010. **79**(2): p. 147-51.
17. Pin, I., et al., *Use of induced sputum cell counts to investigate airway inflammation in asthma*. Thorax, 1992. **47**(1): p. 25-9.
18. Jackson, D.J. and S.L. Johnston, *The role of viruses in acute exacerbations of asthma*. J Allergy Clin Immunol, 2010. **125**(6): p. 1178-87; quiz 1188-9.
19. Sly, P.D., et al., *Early identification of atopy in the prediction of persistent asthma in children*. Lancet, 2008. **372**(9643): p. 1100-6.

20. Green, R.M., et al., *Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study*. *BMJ*, 2002. **324**(7340): p. 763.
21. Grissell, T.V., et al., *Interleukin-10 gene expression in acute virus-induced asthma*. *Am J Respir Crit Care Med*, 2005. **172**(4): p. 433-9.
22. Green, R.H., et al., *Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids*. *Thorax*, 2002. **57**(10): p. 875-9.
23. Johnston, S.L., et al., *The effect of telithromycin in acute exacerbations of asthma*. *N Engl J Med*, 2006. **354**(15): p. 1589-600.
24. Gelfand, E.W. and M. Kraft, *The importance and features of the distal airways in children and adults*. *J Allergy Clin Immunol*, 2009. **124**(6 Suppl): p. S84-7.
25. Gibson, P.G., J.L. Simpson, and N. Saltos, *Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8*. *Chest*, 2001. **119**(5): p. 1329-36.
26. Ronchi, M.C., et al., *Role of sputum differential cell count in detecting airway inflammation in patients with chronic bronchial asthma or COPD*. *Thorax*, 1996. **51**(10): p. 1000-4.
27. Cai, Y., et al., *Persistence of sputum eosinophilia in children with controlled asthma when compared with healthy children*. *Eur Respir J*, 1998. **11**(4): p. 848-53.
28. Hahn, D.L., R.W. Dodge, and R. Golubjatnikov, *Association of Chlamydia pneumoniae (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma*. *JAMA*, 1991. **266**(2): p. 225-30.
29. Cunningham, A.F., et al., *Chronic Chlamydia pneumoniae infection and asthma exacerbations in children*. *Eur Respir J*, 1998. **11**(2): p. 345-9.

**TABLE 1 Subject Characteristics by disease group for adults and children**

	Children						
	Adults	Controls	Stable Asthma	Acute Asthma	Controls	Stable Asthma	Acute Asthma
Subjects, n	11	29	22	9	49	28	
Age yrs <sup>γ</sup>	59 (41, 74)	56 (51, 65)	43 (30, 53)	10 (9, 11)	11 (9, 13)	12 (8, 17)	
Males/females, n	6/5	12/17	7/15	1/8	32/17 <sup>+</sup>	15/13	
Ex-smoker, n(%)	8 (73)	11 (38)	4 (18)	0 (0)	0 (0)	0 (0)	
Atopy, n(%)	2 (18)	26 (90) <sup>+</sup>	11 (69)	5 (56)	42 (86)	N/A	
ICS, n(%)	0 (0)	26 (90) <sup>+</sup>	18 (82) <sup>+</sup>	0 (0)	30 (73) <sup>+</sup>	19 (68) <sup>+</sup>	
BDP equiv ICS daily dose (μg) <sup>#</sup>	0	1537 (958)	1417 (787)	0	484 (325)	N/A	
FEV <sub>1</sub> % pred <sup>γ</sup>	100.8 (91.9, 108.4)	71.4 <sup>¶</sup> (59.5, 87.2)	73.0 <sup>¶</sup> (50.0, 77.0)	92 (86, 103)	91(85, 105)	53 (41,69) <sup>¶</sup>	
FEV <sub>1</sub> /FVC% <sup>γ</sup>	81 (71, 88)	65 <sup>¶</sup> (58, 74)	74 (65, 80)	91 (85, 97)	80 (77, 85)	N/A	

Numbers are n(%), Fisher's exact or Chi-Square test; ICS: inhaled corticosteroid; FEV<sub>1</sub>: forced expiratory volume in 1s; % pred: % predicted; FVC: forced vital capacity; N/A: not assessed; #: Mean(SD), Student's T Test; <sup>γ</sup>: Median (IQR), Kruskal-Wallis test; <sup>¶</sup>Post hoc test p<0.008 versus healthy controls; <sup>+</sup>Post hoc test p<0.001 versus healthy controls.

**TABLE 2 Induced sputum cell counts by disease group for adults and children**

	Adults		Children				P value
	Healthy Control	Stable Asthma	Acute Asthma	Healthy Control	Stable Asthma	Acute Asthma	
N	11	29	22	9	49	28	
TCC x 10 <sup>6</sup> /ml	4.9 (3.2, 6.6)	8.0 (4.2,10.6)	9.4 (3.0,24.7)	3.2 (2.1, 4.4)	3.2 (1.8, 7.0)	5.4 (2.6, 20.9)	0.064
Neutrophils %	30.5 <sup>†</sup> (22.8,61.0)	50.0 <sup>†</sup> (34.5, 69.0)	78.9 (68.7,84.6)	15.8 <sup>†</sup> (6.8,19.5)	27.5 (11.8,58.0)	55.3 (28.5,73.4)	<b>0.004</b>
Neutrophils x 10 <sup>4</sup> /ml	194 <sup>†</sup> (63, 371)	427 (148, 732)	662 (226, 1884)	44 <sup>†</sup> (13, 67)	86 <sup>†</sup> (29, 358)	267 (62, 1440)	<b>0.004</b>
Eosinophils %	0.25 (0, 0.75)	1.00 (0.30, 2.50)	0.64 (0, 2.00)	0.50 <sup>†</sup> (0.25,0.75)	1.75 <sup>†</sup> (0.50, 4.50)	11.75 (5.00, 21.38)	<b>&lt;0.001</b>
Eosinophils x 10 <sup>4</sup> /ml	1.35 (0, 3.29)	6.84 (2.43, 21.87)	6.02 (0, 18.11)	1.5 <sup>†</sup> (0.8, 1.8)	5.5 <sup>†</sup> (1.0, 17.7)	56.1 (21.8, 356.0)	<b>&lt;0.001</b>
Macrophages %	64.3 <sup>†</sup> (33.0, 69.8)	38.3 <sup>†</sup> (23.3,49.5)	15.0 (11.8, 27.3)	81.3 <sup>††</sup> (75.3, 88.0)	58.5 <sup>†</sup> (36.0, 74.0)	18.1 (10.5, 37.4)	<b>&lt;0.001</b>
Macrophages x 10 <sup>4</sup> /ml	228 (134, 314)	207 (147, 357)	143 (86, 251)	193 (174, 373)	166 (100, 272)	126 (63, 302)	0.436
Lymphocytes %	0.25 (0, 1.25)	0.25 (0, 0.50)	0.51 (0, 2.59)	0.75 (0.50, 2.00)	1.00 <sup>†</sup> (0.25, 2.00)	0.25 (0, 0.96)	<b>0.015</b>
Lymphocytes x 10 <sup>4</sup> /ml	1.22 (0, 5.13)	1.8 (0, 5.0)	1.62 (0, 24.7)	3.30 (0.36, 6.19)	3.18 (1.45, 7.22)	1.03 (0, 7.88)	0.275
Columnar epithelial cells %	1.25 (0.25, 2.00)	2.50 (0.25, 5.25)	0.83 (0.26, 2.76)	0.75 (0, 2.75)	2.00 (0.31, 4.25)	3.0 (0.38,4.75)	0.374
Columnar epithelial cells x 10 <sup>4</sup> /ml	6.84 (1.94, 13.95)	11.68 (2.66, 34.02)	5.02 (0.76, 14.93)	2.61 (0, 4.95)	5.72 (2.18, 12.02)	14.33 (3.13, 30)	<b>0.038</b>

Numbers are median (IQR), Kruskal-Wallis test. TCC: TOTAL CELL COUNT; N/A: not assessed; <sup>†</sup>Post hoc test p<0.008 compared to Acute Asthma; <sup>††</sup> Post hoc test p<0.008 compared to Stable Asthma.





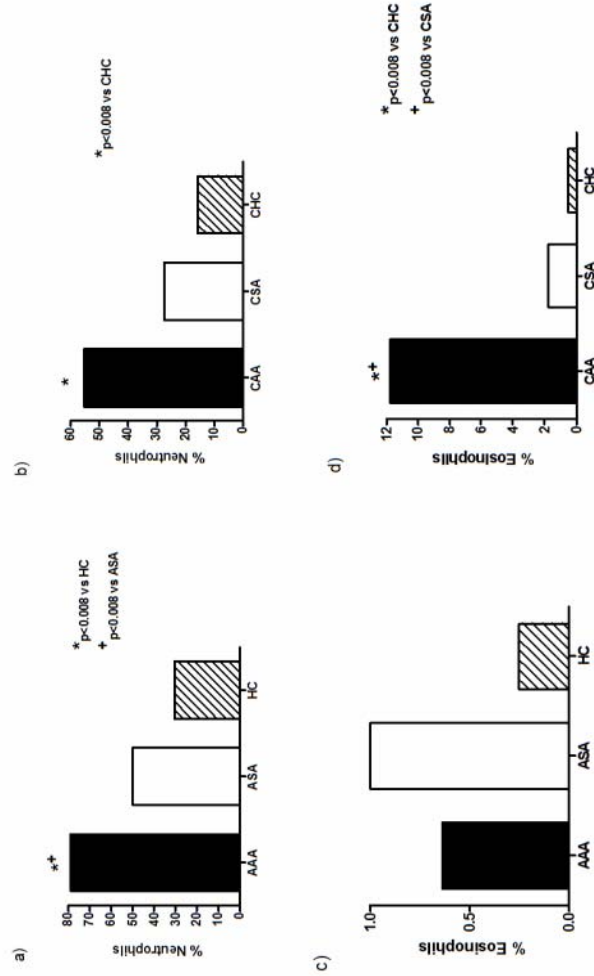
**TABLE 3 Inflammatory Phenotype by disease group for adults and children**

	Adults		Children				P value
	Healthy Control	Stable Asthma	Acute Asthma	Healthy Control	Stable Asthma	Acute Asthma	
N	11	29	22	9	49	28	
Neutrophilic	2 (18.2%)	8 (27.6%)	18 (81.8%)	0	10 (20.4%)	2 (7.1%)	1.820
Eosinophilic	0	5 (17.2%)	0	0	14 (28.6%)	14 (50.0%)	<b>0.011</b>
Mixed Granulocytic	0	1 (3.5%)	4 (18.2%)	0	1 (2.0%)	10 (35.7%)	<b>&lt;0.001</b>
Paucigranulocytic	9 (81.8%)	15 (51.7%)	0	9 (100%)	24 (49.0%)	2 (7.1%)	<b>&lt;0.001</b>

Numbers are n (%), Fisher's exact test.

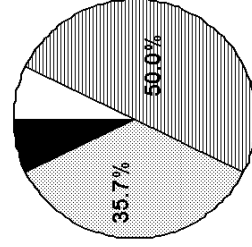
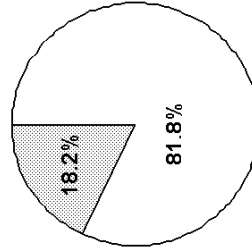
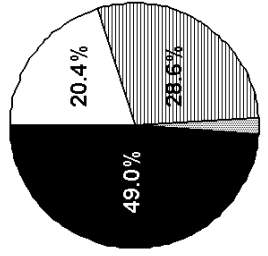
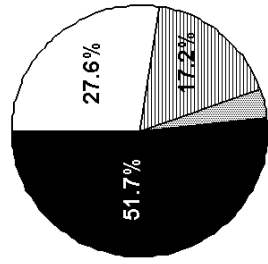
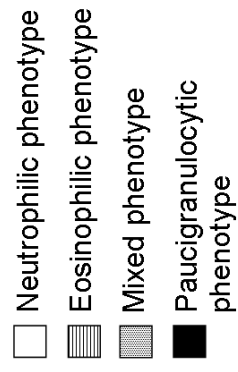
**FIGURE LEGENDS:**

**FIGURE 1.** Proportion of neutrophils and eosinophils for adults and children with asthma. a) Neutrophils in adult populations; b) Neutrophils in paediatric groups; c) Eosinophils in adult populations; d) Eosinophils in paediatric groups. AAA: Adults with acute asthma (n=22); ASA: Adults with stable asthma (n=29); HC: Healthy control (n=11); CAA: Children with acute asthma (n=28); CSA: Children with stable asthma (n=49); CHC: Children healthy control (n=9); a): \*p<0.008 versus HC; +p<0.008 versus ASA. b): \*p<0.008 versus CHC; +p<0.008 versus CSA.



**FIGURE 2.** Inflammatory phenotypes in adults and children with stable and acute asthma. ASA: Adults with stable asthma (n=29); CSA: Children with stable asthma (n=49); CAA: Children with acute asthma (n=28); AAA: Adults with acute asthma (n=22).

Figure 2



**FIGURE 3.** Assessing of the suitability of sample processing and DNA purification strategies for isolation of Cpn DNA. a) Schematic representation outlining sputum spiking with CPN positive DNA and processing. Representative sputum samples were spiked with Cpn positive DNA and processed as described in the materials and methods; b) Comparison of  $c_t$  values of supernatant and corresponding cell suspensions upon qPCR. Presence of CPN DNA was detected in both fractions and assumed correlation of detection threshold to input DNA can be seen.

Figure 3 a)

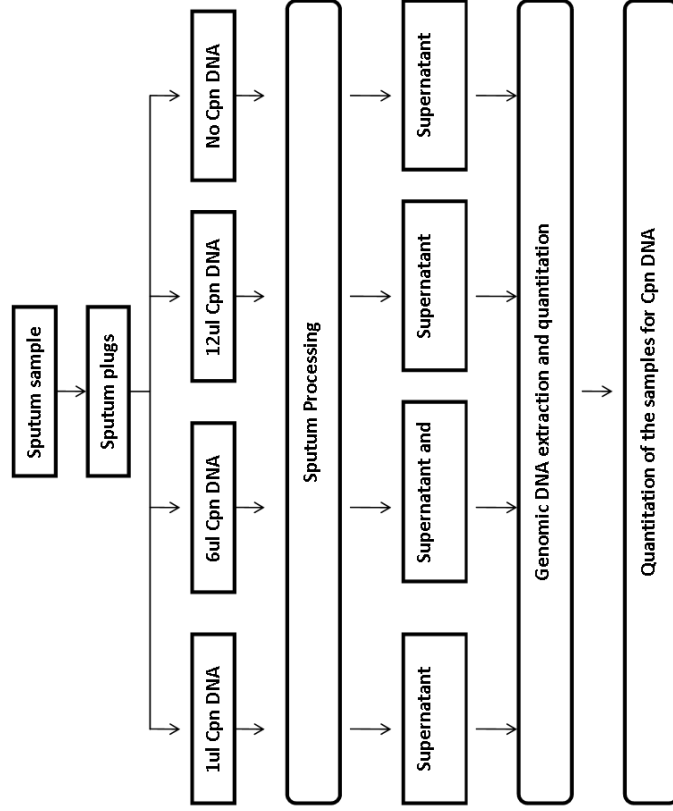


Figure 3 b)

