

# Final report for CRC for Asthma 2006

## Asthma Screening – Phenotype Studies and Markers of Airway Inflammation in Induced Sputum

### SUMMARY

It is increasingly recognised that asthma requires further phenotypic classification in order to understand mechanisms, improve diagnosis and inform treatment decisions. The overall aim of this project was to develop screening tools based on clinical history, examination, responses to anti-asthma treatment and measurement of various biological markers that will facilitate a better definition of the various subtypes of clinical asthma.

A number of specific studies were undertaken in order to achieve the broad overall objectives of the project. These included use of questionnaires and clinical examination to better define the phenotype and disease severity in asthma patients, studies on the prevalence and characterisation of particular phenotypes, including aspirin-intolerant asthma and wine-sensitive asthma, measurement of biological markers in urine and induced sputum, identification of potential markers of eosinophilic and non-eosinophilic airway inflammation in induced sputum of asthmatic patients, and assessment of the effects of a leukotriene receptor antagonist on asthma control.

The prevalence of respiratory symptoms triggered by use of aspirin or non-steroidal anti-inflammatory drugs was 10-11% in patients with asthma and 2.5% in non-asthmatics. Aspirin-intolerant asthma was associated with more severe asthma, nasal polyposis, atopy, sulfite sensitivity, and sensitivity to wine. Aspirin sensitivity appears to be a significant problem in the community and further investigations are required into the mechanisms of these responses and their possible link with other food and chemical sensitivities. In a wine challenge study of wine-sensitive asthmatic patients, clinically significant changes in bronchial hyperresponsiveness, in the absence of changes in FEV<sub>1</sub>, were observed in four of eight subjects. Urinary 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> was identified as a potential marker of reactivity to wine in asthmatic patients.

Baseline urinary leukotriene E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> concentrations were found to be of limited value in discriminating between patients with different severities of asthma. However, urinary 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> concentration was inversely correlated with asthma severity and with % predicted FEV<sub>1</sub>, suggesting an adverse effect of chronic prostaglandin D<sub>2</sub> production on lung function in asthma. Most patients presenting with acute asthma exacerbations did not have increased levels of leukotriene E<sub>4</sub> or 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> in their urine, compared with the levels measured after recovery from the acute episode.

In a study of inflammatory markers in induced sputum, cysteinyl leukotriene (cysLT) concentrations were higher in moderately severe asthmatic patients and in patients with eosinophilic asthma. In contrast prostaglandin(PG) E<sub>2</sub> concentrations and the ratio of PGE<sub>2</sub>: cysLT were lower in patients with eosinophilic asthma. Therefore, increased cysLT production and diminished production of anti-inflammatory PGE<sub>2</sub> may adversely affect lung function and exacerbate airway inflammation in patients with eosinophilic asthma. Patients with persistent asthma were found to develop tolerance to the cysLT receptor antagonist, zafirlukast, with a rebound clinical deterioration on cessation of this drug.

Interleukin (IL)-8, elastase, MMP-9, and LTB<sub>4</sub> were identified as candidate inflammatory markers of non-eosinophilic asthma and cysLT, 8-isoprostane and nitrate were identified as potential markers of eosinophilic asthma. The concentrations of these inflammatory markers were measured in induced sputum supernatants from ~75 patients with different asthma phenotypes. Expression of

the genes for the leukotriene pathway enzymes and receptors was assessed in induced sputum cells of asthmatic patients. There was high expression of the leukotriene pathway genes in sputum inflammatory cells of some, but not all, asthmatic patients. However, this increased gene expression did not appear to be related to the percentage of sputum eosinophils.

The Markers of Disease Activity Project within the CRC for Asthma and Airways will build on the results of Project 6a, using known pathways, immune transcriptome analysis, and proteomics to identify novel asthma biomarkers as targets for the development of novel asthma diagnostic tools.

## **BACKGROUND & OVERVIEW**

Asthma, a disease with multiple phenotypes is characterised by varying degrees of airway inflammation and remodelling. It is increasingly recognised that asthma requires further phenotypic classification in order to understand mechanisms, improve diagnosis and inform treatment decisions. At any one time an individual may have multiple asthma phenotypes and each phenotype may cause asthma symptoms via different mediator pathways and respond to different treatments. The overall aim of this project was to develop screening tools based on clinical history, examination, responses to anti-asthma treatment and measurement of various biological markers that will facilitate a better definition of the various subtypes of clinical asthma. It is hoped that better characterisation of the asthma phenotype will help in the diagnosis of asthma patients, in making appropriate treatment decisions, and assessing response to treatment and control of asthma symptoms. In addition insight will be gained into the mechanisms underlying the different asthma phenotypes. This will be important because future therapies will target specific biochemical pathways, which may be relevant only for certain asthma phenotypes.

The first objective of this project was to use clinical history and examination to categorise each patient's asthma phenotype. This involved the use of questionnaires to collect detailed histories, skin prick testing for determination of atopic status and lung function assessment. The asthma phenotypes that could potentially be identified in this way included atopic/allergic asthma with sensitivity to defined allergens, aspirin-intolerant asthma, exercise or cold air induced asthma, asthma associated with pregnancy or hormonal changes, asthma with a predominantly neural aetiology, occupational asthma induced by defined chemicals or triggers, and asthma induced by defined foods or food additives (eg. peanuts, wine, metabisulphite).

Currently asthma is monitored by measuring airway calibre, not by measures of inflammation. Therefore a second objective of this project was to evaluate the potential usefulness of biochemical and immunological markers of inflammation in urine and induced sputum as adjuncts to clinical history/examination in the assessment of asthma phenotypes. Mediators derived from arachidonic acid (cysteinyl leukotrienes and prostaglandins) are important products of eosinophils, mast cells and the airway epithelium and may mediate many pro- and anti-inflammatory processes in the airways. A major focus of this project was to develop methods and assess the usefulness of measuring metabolites of cysteinyl leukotrienes (LTE<sub>4</sub>) and prostaglandin D<sub>2</sub> (9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub>) in urine of patients with different asthma phenotypes. Furthermore, measurement of biochemical markers in induced sputum may provide a more direct assessment of airway inflammation in asthma. Therefore cysteinyl leukotrienes and prostaglandin E<sub>2</sub>, as well as 8-isoprostane and nitric oxide metabolites, as well as the expression of cysteinyl leukotriene pathway genes, have been measured in induced sputum of patients with different asthma phenotypes. It was envisaged that development of the capacity to monitor asthma by the measurement of these inflammatory markers would result in enhancement of asthma care for the individual patient.

## **KEY INVESTIGATORS**

A/Prof Philip Thompson (In-kind, part time), *Project Leader*; 1999 – 2005  
Prof Peter Gibson (In-kind, part time), *Project Leader*; 2004 – 2005  
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## **OVERALL OBJECTIVES**

The overall objectives of this project were to better define the various subtypes of clinical asthma. The asthma phenotype is heterogeneous with respect to the causative mechanisms, the natural history of the disease and responsiveness to therapy. Therefore the objective was to develop screening tools based on clinical history, examination, responses to anti-asthma treatment and measurement of various biological markers. These screening tools could then be used to categorise the various phenotypes of asthma patients and to facilitate diagnosis and treatment decisions for these patients.

## **SPECIFIC AIMS OF PROJECT 6A**

- 1) To develop screening tools based on clinical history, examination, responses to anti-asthma treatment, responses to various challenges and measurement of various biological markers that will facilitate better definition and diagnosis of the various subtypes of clinical asthma.
- 2) To determine whether the clinical phenotype of asthma patients correlates with markers of eosinophil and mast cell activation such as leukotriene E<sub>4</sub> (LTE<sub>4</sub>), and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> in urine.
- 3) To determine whether the clinical phenotype of asthma patients correlates with markers of eosinophil and mast cell activation (cysteinyl leukotrienes, prostaglandin D<sub>2</sub>, tryptase, eotaxin) in induced sputum.
- 4) To determine whether 8-isoprostane concentrations will provide an indication of whether more severe asthma or asthma characterised by chronic inflammation are associated with increased oxidative stress.
- 5) To identify potential markers of airway inflammation (cytokines, chemokines, proteases, leukotrienes, nitric oxide metabolites) in induced sputum of patients with asthma, and to evaluate the validity of these markers in the phenotypic classification of asthma.
- 6) To assess and compare mRNA expression for the leukotriene synthesis enzymes and receptors in peripheral blood leukocytes and induced sputum cells of patients with different asthma phenotypes and severity of disease.

## **EXPECTED OUTCOMES**

Measurement of biochemical and immunological markers of airway inflammation, combined with clinical assessment can be used to identify the asthma phenotype of individual patients, to monitor disease activity, assess and optimise treatment options, and to develop a point-of-care or laboratory based diagnostic test or management tool.

## **ACHIEVEMENTS AGAINST AIMS AND ORIGINAL MILESTONES**

**Aim: To characterise the prevalence and phenotype of aspirin intolerant asthma (AIA) in Australian asthmatic patients based on clinical history**

**Milestone:** Years 1, 2 – Clinical characterisation of cohorts of aspirin-intolerant asthmatic patients.

*Background:* Aspirin intolerant asthma (AIA) is a clinically distinct syndrome characterised by the precipitation of asthma attacks following the ingestion of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). The prevalence of AIA among Australian asthmatic patients has not previously been reported.

*Methods:* Three populations were surveyed to establish the prevalence of AIA among Australian asthmatics. Two surveys were completed in patients recruited from the metropolitan area in Perth, Western Australia, one comprising 150 patients recruited from hospital based sources (hospital cohort) and the second comprising 366 patients from the membership of the Asthma Foundation of Western Australia (Asthma Foundation cohort). In a third study 1,298 individuals were randomly selected from the rural community of Busselton in Western Australia.

*Results:* The prevalence of AIA in the hospital and Asthma Foundation cohorts was 10.7% and 10.4%, respectively. Univariate analyses in the Asthma Foundation cohort indicated that AIA was associated with more severe asthma (OR = 2.4, 95% CI 1.18 to 4.86), nasal polyposis (OR = 3.19, 95% CI 1.52 to 6.68), atopy (OR = 2.96, 95% CI 1.48 to 5.89), sulfite sensitivity (OR = 3.97, 95% CI 1.87 to 8.41), and sensitivity to wine (OR = 3.27, 95% CI 1.65 to 6.47). Multivariate analyses indicated that atopy (OR = 2.80, 95% CI 1.38 to 5.70), nasal polyposis (OR = 3.39, 95% CI 1.57 to 7.29), and the number of asthma attacks in the previous 12 months (OR = 1.20, 95% CI 1.02 to 1.42) were independent predictors for AIA, as was wine sensitivity (OR = 2.20, 95% CI 1.02 to 4.72). The prevalence of AIA among asthmatic patients in the Busselton cohort was 10.9%. In addition, 2.5% of non-diagnosed asthmatics in this cohort reported asthma symptoms following aspirin ingestion.

*Conclusions:* The prevalence of respiratory symptoms triggered by aspirin/NSAID use was 10-11% in patients with asthma and 2.5% in non-asthmatics. Aspirin sensitivity appears to be a significant problem in the community and further investigations of the mechanisms of these responses and the possible link between this syndrome and other food and chemical sensitivities are required.

**Aim: To determine whether the clinical phenotype of asthmatic patients correlates with the inflammatory markers, leukotriene E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> in urine**

**Milestones:** Years 1, 2 – Clinical characterisation of groups of patients with mild, moderate or severe asthma or aspirin-intolerant asthma. Validation of urinary LTE<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> assays and assessment of within individual variation.

Years 3, 4 – measurement of biological markers (urinary LTE<sub>4</sub>, 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub>) in groups of patients with mild, moderate, severe, and aspirin-intolerant asthma.

*Objective:* To compare urinary leukotriene (LT)E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin (PG)F<sub>2</sub> concentrations in large groups of mild, moderate and severe asthmatic patients and healthy control subjects.

*Methods:* Asthma severity, treatment and aspirin sensitivity were assessed by questionnaire in 168 asthmatic patients. Basal LTE<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> concentrations were measured in urine samples from these patients and from 175 control subjects, using enzyme immunoassays.

*Results:* Urinary LTE<sub>4</sub> was correlated with 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> in both control subjects and asthmatic patients ( $P < 0.002$ ). Median LTE<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> concentrations in patients with severe asthma were significantly reduced compared with mild asthmatic patients ( $P < 0.05$  and  $P < 0.001$ ,

respectively). Urinary  $9\alpha,11\beta$ -PGF<sub>2</sub>, but not LTE<sub>4</sub> was lower in asthmatic patients using inhaled corticosteroids ( $P < 0.02$ ). Multiple regression analysis indicated that urinary  $9\alpha,11\beta$ -PGF<sub>2</sub> concentration was negatively correlated with asthma severity ( $P = 0.003$ ) and also with % predicted FEV<sub>1</sub> ( $P = 0.005$ ).

*Conclusions:* Baseline urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> concentrations are of limited value in discriminating between patients with different severities of asthma. Reduced urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> in patients with severe asthma suggest direct or indirect effects of high dose corticosteroid therapy or that severe asthma is more closely associated with factors other than eicosanoid production. However, the negative association of urinary  $9\alpha,11\beta$ -PGF<sub>2</sub> with lung function suggests an adverse effect of chronic PGD<sub>2</sub> production on lung function in asthma.

**Aim: To measure urinary and plasma inflammatory markers in patients presenting to the Emergency Department with acute asthma**

**Milestones:** Years 3, 4 – measurement of biological markers (urinary LTE<sub>4</sub>,  $9\alpha,11\beta$ -PGF<sub>2</sub>, 8-isoprostane) in patients with acute asthma

*Background:* In acute asthma, eosinophils and mast cells in the lungs are activated to release inflammatory mediators that contribute to the acute asthmatic response and to the inflammation that persists after the acute episode. Measurement of urinary levels of LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> as well as urinary and plasma levels of 8-isoprostane during and after an acute asthma episode will provide an indication of eosinophil and mast cell activation in acute asthma and may provide objective measures for monitoring disease activity and assessing treatment options.

*Methods:* Patients with acute asthma provided urine and blood samples on presentation in the Emergency Department ( $n = 14$ ), and again 6-8 weeks later, after recovery from the acute episode ( $n = 10$ ). Urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub>, as well as 8-isoprostane concentrations in urine and plasma were measured using specific enzyme immunoassays.

*Results:* Urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> concentrations were not significantly different during acute episodes of asthma compared with measurements made 6-8 weeks after recovery. Urinary 8-isoprostane levels during acute asthma exacerbations (1,135 pg/mg creatinine) were higher compared with the levels in samples obtained 6-8 weeks after recovery (758 pg/mg creatinine), but the difference did not reach statistical significance ( $P = 0.08$ ). There was no correlation between urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> concentrations at the time of acute exacerbation or after recovery. Similarly, urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> concentrations were not correlated with urinary 8-isoprostane concentrations during asthma exacerbations or after recovery. Plasma 8-isoprostane concentrations were not significantly different during acute asthma exacerbations compared with post-recovery measurements.

*Conclusions:* Most patients presenting with acute asthma did not have increased levels of LTE<sub>4</sub> or  $9\alpha,11\beta$ -PGF<sub>2</sub> in their urine, compared with the levels measured after recovery from the acute episode. There was a tendency for urinary 8-isoprostane levels to be higher during acute asthma episodes. Urinary LTE<sub>4</sub> and  $9\alpha,11\beta$ -PGF<sub>2</sub> concentrations may be suppressed by high dose inhaled corticosteroid treatment prior to presentation at the Emergency Department.

**Aim: To determine the concordance between the prevalence of aspirin-intolerant asthma (AIA) as determined by clinical history with that determined by use of inhaled lysine aspirin challenges**

**Milestones:** Years 1, 2 – Clinical characterisation of cohorts of aspirin-intolerant asthmatic patients.

Years 3, 4 – Challenge of aspirin-intolerant asthmatic patients and measurement of biological markers before, during and after challenge.

*Background:* Worldwide there is very little data describing the true prevalence of AIA. Previous studies, including our own, based on questioning asthmatic patients about their sensitivity to aspirin-containing medicines and non-steroidal anti-inflammatory drugs (NSAIDs) suggest that AIA affects ~ 10% of adults with asthma. However, it has been suggested that the prevalence may be much higher if assessment is based upon formal challenges. The lysine-aspirin challenge model provides a safe and effective means of identifying aspirin-intolerant asthmatics and can be used as a general screening tool for estimating the prevalence of AIA.

*Methods:* An inhaled lysine-aspirin challenge protocol based on that used by the European Network of Aspirin-Induced Asthma was written and ethics approval obtained. A suitable dosimeter-controlled jet nebuliser was purchased and a sufficient quantity of lysine-aspirin was imported from Italy. We planned to recruit and challenge 100 selected asthma patients of whom a significant number (> 30) were likely to react to inhaled lysine-aspirin challenge.

*Results:* Sixty individuals were pre-screened by telephone interview and 11 subjects willing to undergo inhaled lysine-aspirin challenge were identified. At the time this project was terminated, 7 subjects had completed the challenge protocol and urine samples had been obtained for measurement of biological markers. The project was terminated, following resignation of a staff member and lack of resources for continuation of the project.

**Aim: To determine whether wine challenge of wine sensitive asthmatic patients results in changes in bronchial hyperresponsiveness and urinary eicosanoids**

**Milestones:** Years 3, 4 & 5 – Clinical characterisation and challenge of wine-sensitive asthmatic patients and measurement of biological markers before and after challenge.

*Background:* Previous studies suggest that measurement of changes in forced expiratory volume in 1 s (FEV<sub>1</sub>) following challenge may not detect reactivity to wine in asthmatic patients who report such sensitivities.

*Objective:* The aim of this study was to assess whether changes in bronchial hyperresponsiveness (BHR) or urinary metabolites of cysteinyl leukotrienes and prostaglandin D<sub>2</sub> may be a more useful indication of wine sensitivity in asthmatic patients.

*Methods:* Eight self-reporting wine sensitive asthmatic patients completed double-blind challenges with *high sulfite* and *sulfite free* wine on separate days. FEV<sub>1</sub> and histamine PC<sub>20</sub> were measured before and 60 min after consumption of 150 mL of wine. Pre- and post-challenge urine samples were assayed for leukotriene E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub>.

*Results:* None of the eight subjects demonstrated a clinically significant ( $\geq 15\%$ ) change in FEV<sub>1</sub> following challenge with either *high sulfite* or *sulfite free* wine. However, clinically or borderline significant increases in BHR were observed after wine challenges in three of the eight subjects. Mean histamine reactivity was increased after challenge with *high sulfite* and *sulfite free* wine, although the changes were not statistically significant. Importantly, median urinary 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> concentration increased significantly after *high sulfite* wine challenge ( $P < 0.01$ ), while the increase after *sulfite free* wine did not reach statistical significance ( $P = 0.08$ ). Median urinary leukotriene E<sub>4</sub> concentrations did not change significantly after either wine challenge.

*Conclusions:* Although increases in BHR, in the absence of changes in FEV<sub>1</sub>, were observed in three asthmatic patients following wine challenge, BHR measurements may be of limited usefulness in assessing wine sensitivity. However, urinary 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> may be a marker of reactivity to wine in asthmatic patients and deserves further assessment.

**Aim: To determine whether the clinical phenotype of asthmatic patients correlates with inflammatory markers in induced sputum, including cysteinyl leukotrienes, prostaglandin E<sub>2</sub> and 8-isoprostane**

**Milestones:** Years 3, 4 – Characterisation of patients with different asthma phenotypes and severity of asthma.

Years 5, 6 – Measurement of biological markers in induced sputum (cysLT, PGE<sub>2</sub>) and urine (LTE<sub>4</sub>, 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub>) of these patients.

*Background and objective:* Airway inflammation in asthma is associated with production of cysteinyl leukotrienes (cysLT), prostaglandin (PG)E<sub>2</sub>, 8-isoprostane and nitric oxide, but the link between these mediators and asthma phenotype and severity is unclear. Sputum mediator concentrations, as well as urinary leukotriene (LT)E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin (PG)F<sub>2</sub> in patients with differing asthma severity, and associations with eosinophilic airway inflammation were investigated.

*Methods:* Inflammatory cells were assessed in sputum from 13 control subjects and 12 mild, 14 moderate and 12 severe asthmatic patients. CysLT, PGE<sub>2</sub>, 8-isoprostane, LTE<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -PGF<sub>2</sub> concentrations were measured using enzyme immunoassays. Nitrate was measured using a chemiluminescence analyser.

*Results:* Sputum cysLT concentrations were higher in moderate severity patients ( $P < 0.05$ ). Sputum cysLT ( $P < 0.05$ ) and urinary LTE<sub>4</sub> ( $P < 0.005$ ) were higher in eosinophilic (sputum eosinophils  $\geq 2\%$ ,  $n = 10$ ) than non-eosinophilic asthma patients ( $n = 28$ ). Sputum PGE<sub>2</sub> was higher in moderate and severe patients ( $P < 0.01$ ) but in eosinophilic asthma patients, PGE<sub>2</sub> ( $P = 0.02$ ) and the ratio of PGE<sub>2</sub> to cysLT ( $P = 0.002$ ) were lower than in non-eosinophilic asthma patients. FEV<sub>1</sub> was inversely correlated with sputum eosinophils in all asthmatic patients ( $r_s = -0.5$ ,  $P = 0.002$ ), and cysLT correlated with eosinophils in patients with eosinophilic asthma ( $r_s = 0.64$ ,  $P < 0.05$ ). There were no significant differences in sputum 8-isoprostane or nitrate concentrations.

*Conclusions:* Increased cysLT production and diminished production of anti-inflammatory PGE<sub>2</sub> may adversely affect lung function and exacerbate airway inflammation in patients with eosinophilic asthma.

**Aim: To assess the effects of a cysLT receptor antagonist on asthma control and sputum cysLT and prostaglandin E<sub>2</sub> concentrations**

**Milestones:** Years 5, 6 – Assessment of biological markers as potential indicators of asthmatic patients who are clinically responsive to anti-leukotriene therapy, and clinical trials of anti-leukotriene drugs.

*Background:* Zafirlukast is a potent cysteinyl leukotriene (cysLT) receptor antagonist but few studies have assessed its efficacy over periods greater than six weeks. We assessed whether zafirlukast improves clinical control over three months in patients with persistent asthma.

*Methods:* This was a double blind, placebo-controlled, parallel-group study assessing the clinical effects of zafirlukast, 20 mg twice daily, in 21 asthmatic subjects using  $\beta_2$ -agonists only (Group I), and in 26 subjects with persistent symptoms despite daily inhaled corticosteroid therapy (Group II). Secondary end-points included changes in sputum and peripheral blood cell numbers, sputum cysLT and prostaglandin E<sub>2</sub> concentrations, exhaled nitric oxide levels and airway hyperresponsiveness.

*Results:* Treatment with zafirlukast compared with placebo caused significant early improvements in morning peak expiratory flow (PEF) and FEV<sub>1</sub> in Group I ( $P < 0.01$ ) and FEV<sub>1</sub> in Group II ( $P < 0.05$ ). There were initial within-treatment group improvements in morning waking with asthma in Group I ( $P < 0.05$ ) and in morning PEF,  $\beta_2$ -agonist use and asthma severity scores in Group II ( $P <$

0.05). However, initial improvements with zafirlukast disappeared after 12 weeks, in Group I and to a lesser extent in Group II. Following withdrawal of zafirlukast there were significant deteriorations in evening PEF and FEV<sub>1</sub> in Group I ( $P \leq 0.05$ ), and in FEV<sub>1</sub> in Group II ( $P < 0.05$ ), compared with placebo. There were within-group deteriorations in morning PEF in Group I ( $P < 0.001$ ), and in evening PEF ( $P < 0.005$ ) and nocturnal awakenings ( $P < 0.05$ ) in Group II. There was no evidence of anti-inflammatory effects with zafirlukast but peripheral blood neutrophils increased in Group I following drug withdrawal ( $P=0.005$ ). CysLT and PGE<sub>2</sub> concentrations at baseline and after zafirlukast treatment were not related to any of the clinical outcomes in these patients.

*Conclusions:* Patients with persistent asthma develop tolerance to zafirlukast, with a rebound clinical deterioration on cessation of this drug.

A new research proposal was developed and approved for Project 6a in 2004-2005. This involved collaboration with A/Prof Peter Gibson, John Hunter Hospital, Newcastle, on induced sputum studies in asthma patients with different phenotypes.

**Aim: To identify potential markers of airway inflammation (cytokines, chemokines, proteases, leukotrienes, NO reaction products) in induced sputum of patients with asthma and to evaluate the validity of these markers in determining the phenotype of patients with eosinophilic and non-eosinophilic asthma.**

**Milestones:** Year 1 – Recruitment of patients and measurement of markers of airway inflammation in induced sputum from patients with different asthma phenotypes.

*Background:* In order to understand mechanisms, improve diagnosis and inform treatment decisions, better phenotypic classification of asthma is required. The pattern of airway inflammation is a potentially important variable in the response to treatment. In particular eosinophilic asthma responds well to inhaled corticosteroids, while non-eosinophilic asthma responds poorly to inhaled corticosteroid, but responds well to long acting  $\beta_2$ -agonists. Basing treatment upon airway inflammatory phenotype has yielded good outcomes, and there is therefore a need to develop easily measurable markers of airway inflammation in induced sputum. In this project we aimed to investigate candidate inflammatory markers of eosinophilic and non-eosinophilic asthma, using induced sputum from patients with different asthma phenotypes.

*Methods:* In Newcastle, over 50 induced sputum samples were collected for assessment of mediators. In Perth sputum samples were obtained from 24 asthmatic and non-asthmatic subjects. Cytospins were prepared for all these samples in order to assess whether the patients had eosinophilic or non-eosinophilic asthma.

*Results:* Assays for IL-8, elastase, MMP-9, IL-5, and LTB<sub>4</sub> were developed and validated, and analyses of these markers of non-eosinophilic airway inflammation were completed for all sputum supernatants. Analysis of the markers of eosinophilic airway inflammation, cysLT, 8-isoprostane and nitrate were also completed for all sputum supernatants. In order to validate promising inflammatory markers, spiking experiments and assessment of the effects of dithiothreitol were completed for 8-isoprostane, MMP-9, LTB<sub>4</sub> and cysLT. This data is currently being analysed for preparation of a manuscript on “Markers of eosinophilic and non-eosinophilic airway inflammation in induced sputum of asthma patients”.

**Aim: To assess and compare mRNA expression for the leukotriene synthetic enzymes and receptors, and chemokines in peripheral blood leukocytes and induced sputum cells of patients with different asthma phenotypes and severity of disease.**

**Milestones:** Year 1 – Establishment and optimisation of methods for RNA extraction and quantitative real-time RT-PCR analysis of gene expression in peripheral blood leukocytes and induced sputum cells.

Year 2 – Assessment of gene expression for leukotriene synthesis enzymes and receptors in peripheral blood leukocytes and induced sputum cells from phenotypically different asthmatic patients.

*Background:* Activation of the 5-lipoxygenase (5-LO) pathway in airway eosinophils and mast cells of asthmatic patients results in the production of cysteinyl leukotrienes (cysLT) that cause bronchoconstriction, mucus production, oedema and airway remodelling. The aim of the study was to quantify mRNA expression for 5-LO, 5-lipoxygenase-activating protein (FLAP), LTC<sub>4</sub> synthase, and cysLT1 and cysLT2 receptors in induced sputum cells of asthmatic patients and to assess whether expression differs in patients with compared to those without sputum eosinophils.

*Methods:* Hypertonic saline (4.5%) was used to induce sputum in 36 asthmatic patients. RNA was extracted from sputum plugs and reverse transcribed to cDNA. 5-LO pathway gene expression was quantified by duplex real-time PCR using TaqMan primer/probes for the gene of interest with 18S rRNA as a housekeeping gene.

*Results:* Overall, FLAP mRNA expression was greater than that for LTC<sub>4</sub> synthase ( $P=0.013$ ) and cysLT2 receptor expression was greater than that for cysLT1 receptor ( $P<0.0001$ ). 5-LO expression was strongly correlated with FLAP and LTC<sub>4</sub> synthase expression ( $P<0.0001$ ), and weakly correlated with cysLT1 receptor expression ( $P<0.025$ ). Sputum eosinophils were  $\geq 2.5\%$  in 14 patients and  $< 2.5\%$  in 22 patients, but there were no significant differences in 5-LO pathway gene expression between these groups of patients.

*Conclusion:* There is concomitantly high expression of 5-LO pathway genes in sputum inflammatory cells of some, but not all, asthmatic patients. This increased gene expression does not appear to be related to percentage of sputum eosinophils. The cysLT2 receptor may play an important role in asthmatic airway inflammation.

This data and the data for leukotriene pathway gene expression in peripheral blood leukocytes is currently being analysed for preparation of a paper on “Leukotriene pathway gene expression in patients with different asthma phenotypes and severity of disease”.

## **FUTURE DIRECTIONS**

The work achieved in Project 6a of the CRC for Asthma will be extended and further developed in the Advanced Diagnosis and Monitoring Project: Markers of Disease Activity, in the CRC for Asthma and Airways.

Airways diseases such as asthma and COPD are responsible for a large and increasing burden of illness. The better application of therapy will lead to large economic gains for Australia as a result of improved functional status of people with airway disease and reductions in the costs associated with inappropriate drug usage.

The Markers of Disease Activity project will build on the results of Project 6a, and the established strong scientific evidence that therapy for airway disease can be better applied when guided by knowledge of the status of eosinophilic airway inflammation. Therefore novel asthma biomarkers will be identified and used as targets for diagnostic test development, using a broad approach involving known pathways, immune transcriptome analysis, and proteomics. Targets identified from these searches will be paired with appropriate test platforms and the novel asthma diagnostic tools created will be validated by direct study in relevant patient populations.

## **PUBLICATIONS**

**Vally H**, Taylor ML, **Thompson PJ**. The prevalence of aspirin intolerant asthma (AIA) in Australian asthmatic patients. *Thorax* 2002; 57: 569-574.

**Misso NLA**, **Aggarwal S**, **Phelps S**, **Beard R**, **Thompson PJ**. Urinary leukotriene E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> concentrations in mild, moderate and severe asthma, and in healthy subjects. *Clinical and Experimental Allergy* 2004; 34: 624-631.

**Vally H**, **Thompson PJ**, **Misso NLA**. Bronchial hyperresponsiveness following high and low sulfite wine challenge in wine-sensitive asthmatic patients. Submitted to *International Archives of Allergy and Immunology*.

**Aggarwal S**, Moodley YP, **Thompson PJ**, **Misso NL**. Increased cysteinyl leukotrienes and decreased prostaglandin E<sub>2</sub> in sputum of patients with eosinophilic asthma. Submitted to *Thorax*.

**Aggarwal S**, **Thompson PJ**, **Misso NL**. Urinary leukotriene E<sub>4</sub>, 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub>, and 8-isoprostane concentrations in asthmatic patients during and after an acute exacerbation. Manuscript in preparation.

Reid DW, **Misso NL**, **Aggarwal S**, **Thompson PJ**, Walters EH. Leukotriene antagonist use in persistent asthma: evidence for tolerance on treatment and rebound exacerbation on cessation. Manuscript in preparation.

**Vally H**, **Aggarwal S**, **Thompson PJ**, **Misso NL**. Changes in urinary leukotriene E<sub>4</sub> and 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2</sub> following high and low sulfite wine challenges in wine-sensitive asthmatic patients. Manuscript in preparation.

**Simpson JL**, **Misso NL**, **Aggarwal S**, **Thompson PJ**, **Gibson PG**. Markers of eosinophilic and non-eosinophilic airway inflammation in induced sputum of asthma patients. Manuscript in preparation.

**Aggarwal S**, **Grissell TV**, **Timmins N**, **Thompson PJ**, **Gibson PG**, **Misso NL**. Leukotriene pathway gene expression in patients with different asthma phenotypes and severity of disease. Manuscript in preparation.

### **Conference publications**

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Reid D, Misso N, Thompson P, Walters EH. Induced sputum cysteinyl leukotriene concentrations in asthma. Thoracic Society of Australia & New Zealand, Cairns, 24-27 March 2002. *Respirology* 2002; 7 (Suppl): A3.

Aggarwal S, Misso N, Thompson P. Assessment of cysteinyl leukotriene and prostaglandin E<sub>2</sub> concentrations in induced sputum of asthmatic patients and control subjects. Thoracic Society of Australia & New Zealand, Adelaide, 4-9 April 2003. *Respirology* 2003; 8 (Suppl): A20.

Reid D, Misso N, Wen YD, Thompson P, Feltis B, Walters EH. Inflammatory mediator profile in subjects with persistent asthma despite inhaled corticosteroids. Thoracic Society of Australia & New Zealand, Adelaide, 4-9 April 2003. *Respirology* 2003; 8 (Suppl): A19.

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Thompson PJ, Aggarwal S, Misso NL. Increased leukotriene and lower prostaglandin E<sub>2</sub> concentrations in induced sputum of patients with eosinophilic asthma. European Respiratory Society, Copenhagen, 17-21 September 2005. *Eur Respir J* 2005; 26 (Suppl. 49): 733s.

Aggarwal S, Grissell T, Timmins N, Gibson P, Thompson P, Misso N. 5-Lipoxygenase pathway mRNA expression in sputum cells of patients with eosinophilic or non-eosinophilic asthma. Thoracic Society of Australia & New Zealand, Canberra, 26-29 March 2006. *Respirology* 2006; 11 (Suppl. 2): A27.

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