Characterization of leukotrienes in a pilot study of older asthma subjects

Sharmilee M Nyenhuis1,2, Elizabeth A Schwantes1 and Sameer K Mathur*1

Introduction
Asthma is a chronic inflammatory disorder of the lower airway that results in mucus secretion, airway edema and reversible airway obstruction [1]. These characteristic features lead to the clinical symptoms of asthma, which include recurrent episodes of wheezing, breathlessness, chest tightness and coughing [2]. The pathophysiology of asthma is not fully elucidated but the current paradigm involves multiple cell types (i.e. eosinophils, neutrophils, mast cells, T-cells) and their mediators.

The most recent estimates of asthma prevalence in those over the age of 65 are between 4 and 8%, which may be an underestimate due to an underdiagnosis of asthma in the elderly [3]. An increase in morbidity and mortality and a reduced response to bronchodilators in the emergency department setting have been shown in older asthmatics compared to their younger counterparts [4-7]. Elderly asthmatics also have a higher rate of severe exacerbations, emergency department visits, and hospitalizations than younger asthmatics [8,9].

Leukotrienes are potent pro-inflammatory lipid mediators that have been shown to have a role in asthma [10]. Leukotrienes are a product of arachidonic acid via the 5-lipoxygenase pathway. Leukotriene B4 (LTB4) is a potent chemotactic agent and activator of neutrophils and is also produced mainly by neutrophils. The presence of LTB4 in the lung results in neutrophil recruitment and activation leading to superoxide anion generation and cholinergic airway hyperresponsiveness [11]. The cysteinyl leukotrienes (CysLTs), LTC4, LTD4, and LTE4, are known for their profound effect on the airway, including increased microvascular permeability and vasodilation, airway smooth muscle contraction, mucous secretion and impaired mucociliary clearance [10]. CysLTs are produced by eosinophils, basophils, mast cells, macrophages and to a lesser degree by T cells and endothelial cells [10,12]. Increased CysLT levels have been detected in the sputum of patients with asthma and have been shown to correlate with symptom severity [13].

Despite older asthmatics often having more severe disease and exacerbations, few studies have been done to characterize their asthma at a cell and/or molecular level. We have previously shown that functional differences exist in eosinophils from older adult asthmatics in vitro; specifically, diminished IL-5 stimulated eosinophil derived neurotoxin (EDN) release and superoxide production [14]. In this pilot study, we sought to compare pro-inflammatory lipid mediator production, specifically leukotrienes LTB4 and CysLT, both in vitro and in vivo in young and older adult asthmatics. Identifying the inflammatory differences seen in older asthmatics may be important for the diagnosis, improving the morbidity and mortality, as well as determining optimal therapies of the disease in this growing population.

Methods
We recruited subjects in two age groups, 20 to 40 years (n = 12) and 50 to 70 years (n = 6), in a study protocol approved by the University of Wisconsin Health Sciences Institutional Review Board. Inclusion criteria included a physician diagnosis of mild to moderate asthma with a provocative concentration of methacholine causing a 20% fall in FEV1 (PC20) of < 8 mg/mL or albuterol reversibility on spirometry of ≥ 12%. Exclusion criteria included history of tobacco use > 5 pack-years or use in the previous year, prednisone use within 1 month, donation of blood (greater than 1/2 pint) in the previous 8 weeks, FEV1 < 60%, severe asthma, upper and/or lower respiratory tract infection in the previous 4 weeks, diabetes, pregnancy, or an active cardiovascular disease other than controlled hypertension.

At the study visit, medical history, spirometry with bronchodilator reversibility, sputum induction, allergy skin prick test, physician exam and phlebotomy were performed. Sputum processing was performed as described previously [15]. LTB4, LTC4 and CysLT levels in culture...
supernates or the sputum samples were measured using the LTB₄, LTC₄ and CysLT competitive EIA kits, respectively (Cayman Chemical, Ann Arbor, MI). Heparinized venous blood was lysed in a hypotonic solution and subjected to gradient centrifugation. Neutrophils were isolated from the granulocyte pellet and were > 95% pure and > 98% viable. Eosinophils were also isolated from the granulocyte pellet by negative selection using anti-CD16, anti-CD14, and anti-CD3 magnetic beads (Miltenyi Biotechnology; Auburn, CA) as described previously [16]. The eosinophils were typically > 99% pure and > 98% viable.

Results

Table 1 shows a comparison of lung function and peripheral blood characteristics for the subjects in the study. Both FEV₁ and FVC were significantly lower in the older group compared to the young subjects, which is consistent with lung volume decreases in the aging population [17]. However, percentage of predicted values, for both FEV₁ and FVC were comparable between young and older groups, suggesting similar disease severity in both age groups. There was no difference in peripheral blood eosinophil counts between young and older asthmatics. Sputum cell differentials revealed a tendency for increased percentage of neutrophils in older asthma subjects, 72(65-76)%, compared to young asthma subjects, 42(21-66)%, p = 0.09 (Figure 1A). This is consistent with our previous cohort of older asthmatics exhibiting a significantly higher percentage of sputum neutrophils [18]. The total number of eosinophils in the sputum were similar in both the young and older group [Young: 0.6 (0-1.3) × 10,000 cells/gm; Older: 0.5 (0.1-3.9) × 10,000 cells/gm; Figure 1B], while the total numbers of neutrophils apparently increased though not statistically significant [Young: 30 (4-138) × 10,000 cells/gm; Older 68 (4-304) × 10,000 cells/gm; Figure 1C].

In vivo levels of LTB₄ and CysLT were measured in the induced sputum samples from young and older asthma subjects. Sputum LTB₄ levels in older asthma subjects, 320 (301-397) pg/mL, were significantly decreased compared to the young subjects, 1235 (706-2259) pg/mL, p = 0.015 (Figure 2A). Sputum CysLT levels in older asthma subjects, 264 (224-269) pg/mL, were lower than in younger asthma subjects, 717 (408-1152) pg/mL; however, this only approached statistical significance, p = 0.06 (Figure 2B).

In order to determine whether these in vivo differences were due to an age-related diminished ability of neutrophils or eosinophils to produce leukotrienes, both puri-

Table 1: Subject Characteristics

|                          | Young (n = 12) | Older (n = 6) |
|--------------------------|---------------|--------------|
| Age (years, range given)* | 27 (20-35)    | 64 (58-70)   |
| Duration of disease      | 17 (6-30)     | 37 (4-65)    |
| FEV₁ (L)*                | 3.35 (+/- 0.81) | 2.24 (+/- 0.28) |
| FEV₁ % Predicted         | 86 (+/- 14.7) | 81 (+/- 16.46) |
| FVC (L)*                 | 4.28 (3.99-5.02) | 3.24 (+/- 0.71) |
| FVC % Predicted          | 100 (+/- 12.15) | 89 (+/- 12.62) |
| WBC (10⁶ cells/mL)       | 5.55 (4.75-6.85) | 5.6 (5.13-6) |
| Absolute Eosinophil Count (cells/mL) | 204 (+/- 127.75) | 114 (+/- 82.95) |
| Positive Allergy Testing* | 11/12         | 6/6          |
| Inhaled Corticosteroid Use | 6/12          | 4/6          |

Data are presented as mean values (+/- standard deviation; t-test) or median values (25%-75% interval; Mann-Whitney Rank-Sum). *Statistically significant difference between young subjects and older subjects (p < 0.05). +At least one positive skin prick test result for grass, mold, ragweed, tree, cat, dog, or dust mite.
fied neutrophils and eosinophils were treated with calcium ionophore to stimulate leukotriene production. As shown in Figure 2C, the neutrophil production of LTB$_4$ from older asthma subjects, 8708 ± 5326 pg/mL, was comparable to the young subjects, 10234 ± 3078 pg/mL, $p = 0.45$. Eosinophil production of LTC$_4$ from young, 5836 (3678-9512) pg/mL, and older asthma subjects, 4766 (2685-8456) pg/mL, was also similar in the two groups, $p = 0.78$ (Figure 2D).

Since GM-CSF can stimulate leukotriene production by neutrophils and eosinophils[11] and changes in GM-CSF mediated signaling have been shown to occur in elderly human subjects in vitro[19,20], we examined GM-CSF production by peripheral blood mononuclear cells (PBMCs) in the older and young asthma subjects. As
shown in Figure 3, GM-CSF production by unstimulated PBMC was similar; however, LPS-stimulated PBMCs from the older asthma subjects, 125 ± 118 pg/mL, produced significantly less GM-CSF than their younger counterparts, 317 ± 136 pg/mL, p = 0.01.

Discussion
To our knowledge, a characterization of leukotriene levels has not been previously performed in an older adult asthma population. In this pilot study of young and older mild-to-moderate asthmatics we found that older asthma subjects had lower in vivo levels of LTB₄ and CysLT in the sputum at baseline disease. This difference in leukotriene levels was not a reflection of fewer eosinophils and neutrophils in the airway as the total number of these cells in the sputum were similar or greater in our older asthmatic group. However, young and older asthma subjects produced comparable amounts of LTB₄ and LTC₄ in vitro when neutrophils and eosinophils, respectively, were stimulated with calcium ionophore. The observation that LPS stimulation of PBMCs resulted in less GM-CSF production in the older asthma subjects provides a potential explanation for the age-related differences in sputum leukotriene levels. Rather than an intrinsic defect in neutrophils and eosinophils, there may be less GM-CSF in the airways of older asthmatics serving as a stimulant to produce leukotrienes. However, it is possible that other age-related changes in the in vivo inflammatory milieu contribute to the diminished levels of leukotrienes in the airway.

There are several limitations to this study including the absence of a control non-asthmatic population, lack of subjects >65 years old, and few total number of subjects enrolled. Though multiple studies have consistently revealed increases in leukotriene production in asthmatic airways compared to controls, we cannot fully gauge the
Glucocorticoids have not been shown to affect LTB4 for-
tory mediators and neutrophil apoptosis [22]. However,
can have multiple effects such as a decrease in inflamma-
tory observations to serve as the focus of future studies.

Figure 3 Production of GM-CSF in PBMCs; Peripheral blood
mononuclear cells (PBMCs) were either not stimulated or stimu-
lated with LPS (0.4 μg/mL) and supernatants analyzed by ELISA
for GM-CSF. Comparisons between the young and older groups were
performed with t test analysis. Statistical significance was defined at p
< 0.05. White bars: young subjects (n = 12); Gray bars: older subjects (n = 6); Mean: --- (dashed) Median: ----- (solid)

magnitude of our findings in the older asthmatic without
non-asthmatic older controls [21]. It is possible though that
our findings would have been more profound if we
had more subjects in an even older (> 65 years old) popu-
lation. Furthermore, as a pilot study, we had a limited
number of subjects enrolled in order to establish prelimi-
ary observations to serve as the focus of future studies.

Calcium ionophore is a potent activator of leukotriene
production in eosinophils and neutrophils. It is possible that
we were unable to detect small differences in leukotri-
ene production in vitro with the use of such a potent
stimulator of leukotriene production. Therefore, a less
potent, physiologically-relevant stimulant (such as GM-
CSF) could reveal a difference in leukotriene production
in vitro.

The use of inhaled glucocorticoids by some subjects in
our study may represent a confounder as glucocorticoids
may actually represent a tendency for decreased respon-
siveness to glucocorticoids as has been observed in the
neutrophil phenotype of severe asthma [26].

Our findings show that aging can result in changes in
the airway environment in asthmatics, specifically an
increase in airway neutrophils and decreases in both
LTB4 and CysLT levels at baseline. This characterization
of leukotrienes in older adult asthmatics reveals signifi-
cant differences that may have clinical relevance not only
in baseline asthma but also during an exacerbation of dis-
ease. Understanding the biological changes of airway
inflammation in the aging population will aid in the
development of future therapies and impact the increased
morbidity and mortality that is associated with this phe-
ton of asthma.

Abbreviations
CysLTs: cysteinyl leukotrienes; LTB4: leukotriene B4; PBMCs: peripheral blood
mononuclear cells; GM-CSF: granulocyte macrophage colony-stimulating fac-
tor; EDN: eosinophil derived neurotoxin; FVC: forced vital capacity; FEV1: forced
expiratory volume in 1 second; LPS: lipopolysaccharide

Competing interests
SMN received a fellowship award co-sponsored by GlaxoSmithKline, which
helped fund this study.

Authors’ contributions
SMN performed the leukotriene and GM-CSF immune assays, analyzed data,
created graphs and drafted the manuscript. EAS performed cell separations,
sputum processing, sputum differentials, assisted with immune assays, and
assisted with data analysis and drafting of the manuscript. SKM conceived of
the study, and participated in its design and coordination and helped to draft
the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgements
We thank Andrea Marquardt and Kristen Fox for assistance with laboratory pro-
cedures. We thank Jurga Zdanaviciene, our research coordinator for this study.
We thank Mary Anne Kennedy, Tina Palas, and Cheri Swenson for administra-
tive assistance. We also thank Michael Evans for his statistical assistance. This
study was funded by T. Franklin Williams Scholar Program, co-sponsored by the
Atlantic Philanthropies, the American Academy of Allergy, Asthma and Immu-
nology, the John A. Hartford Foundation, and the Association of Specialty Pro-
fessors (SKM), Hartford Center of Excellence (SKM), NIH P01 HL088594 (SKM),
GlaxoSmithKline/American Academy of Allergy, Asthma and Immunology
Allergy Fellowship Award (SMN), and Wisconsin Allergy and Immunology
Research Training Program T32 AI07635 (SMN). GlaxoSmithKline had no role
in study design, collection, analysis, interpretation of data, writing of the manu-
script or the decision to submit the manuscript for publication.

Author Details
1Department of Medicine, Section of Allergy, Pulmonary and Critical Care,
University of Wisconsin, Madison, WI, USA and 2Department of Medicine,
Section of Pulmonary, Critical Care, Sleep and Allergy, University of Illinois-
Chicago, Chicago, IL, USA

Abbreviations
CysLTs: cysteinyl leukotrienes; LTB4: leukotriene B4; PBMCs: peripheral blood
mononuclear cells; GM-CSF: granulocyte macrophage colony-stimulating fac-
tor; EDN: eosinophil derived neurotoxin; FVC: forced vital capacity; FEV1: forced
expiratory volume in 1 second; LPS: lipopolysaccharide

Competing interests
SMN received a fellowship award co-sponsored by GlaxoSmithKline, which
helped fund this study.

Authors’ contributions
SMN performed the leukotriene and GM-CSF immune assays, analyzed data,
created graphs and drafted the manuscript. EAS performed cell separations,
sputum processing, sputum differentials, assisted with immune assays, and
assisted with data analysis and drafting of the manuscript. SKM conceived of
the study, and participated in its design and coordination and helped to draft
the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgements
We thank Andrea Marquardt and Kristen Fox for assistance with laboratory pro-
cedures. We thank Jurga Zdanaviciene, our research coordinator for this study.
We thank Mary Anne Kennedy, Tina Palas, and Cheri Swenson for administra-
tive assistance. We also thank Michael Evans for his statistical assistance. This
study was funded by T. Franklin Williams Scholar Program, co-sponsored by the
Atlantic Philanthropies, the American Academy of Allergy, Asthma and Immu-
nology, the John A. Hartford Foundation, and the Association of Specialty Pro-
fessors (SKM), Hartford Center of Excellence (SKM), NIH P01 HL088594 (SKM),
GlaxoSmithKline/American Academy of Allergy, Asthma and Immunology
Allergy Fellowship Award (SMN), and Wisconsin Allergy and Immunology
Research Training Program T32 AI07635 (SMN). GlaxoSmithKline had no role
in study design, collection, analysis, interpretation of data, writing of the manu-
script or the decision to submit the manuscript for publication.

Author Details
1Department of Medicine, Section of Allergy, Pulmonary and Critical Care,
University of Wisconsin, Madison, WI, USA and 2Department of Medicine,
Section of Pulmonary, Critical Care, Sleep and Allergy, University of Illinois-
Chicago, Chicago, IL, USA

The lower levels of CysLTs in the airways of older adults
may have an impact on the effectiveness of CysLT recep-
tor antagonists in the older population. Two studies to
date have examined the efficacy of the CysLT receptor
antagonists in an older adult population and concluded
that their effectiveness might be limited or altered in
older asthmatics [24,25]. Furthermore, the neutrophil
predominance found in the airway of older asthmatics

Read more
References

1. National Asthma Education and Prevention Program: Expert panel report 3: (Source Document) 2007 [http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm]. Bethesda, MD: National Heart, Lung and Blood Institute.

2. Busse W, Kraft M. Cysteinyl leukotrienes in allergic inflammation: strategic target for therapy, Chest 2005, 127(4):S12-26.

3. Enright PL, et al.: Underdiagnosis and Undertreatment of Asthma in the Elderly, Chest 1999, 116(3):603-613.

4. Bellia V, et al.: Prospective multicenter study of acute asthma in younger versus older adults presenting to the emergency department, Journal of the American Geriatrics Society 2006, 54(1):46-55.

5. Oguzulgen IK, et al.: What can predict the exacerbation severity in asthma? Allergy and Asthma Proceedings 2007, 28(3):344-347.

6. Banerji A, et al.: National Surveillance for Asthma --- United States, 1980--2004. MMWR Surveillance Summary 2007, 56(8):1-54.

7. Marks GB, Correll PK, Williamson M. Asthma in Australia 2005, Medical Journal of Australia 2005, 183(9):445-446.

8. Hamid Q, et al.: Relationship of Upper and Lower Airway Cytokines to Outcome of Experimental Rhinovirus Infection, American Journal of Respiratory and Critical Care Medicine 1998, 157(6):S210-S213.

9. Nagata M, Sedgwick JB, Busse WW. Differential-Effects of Granulocyte-Macrophage Colony-Stimulating Factor on Eosinophil and Neutrophil Superoxide Anion Generation, Journal of Immunology 1995, 155(10):4948-4954.

10. Mathur SK, et al.: Age-Related Changes in Eosinophil Function in Human Subjects, Chest 2008, 133(2):412-419.

11. Gern JE, et al.: Relationship of Upper and Lower Airway Cytokines to Outcome of Experimental Rhinovirus Infection, American Journal of Respiratory and Critical Care Medicine 2000, 162(6):2226-2231.

12. Nyenhuis SM, et al.: Airway neutrophil inflammatory phenotype in older subjects with asthma, The Journal of allergy and clinical immunology 2007, 125(3):163-165.

13. Nyenhuis et al.: Effect of age on response to zafirlukast in patients with asthma in the Accolate Clinical Experience and Pharmacoepidemiology Trial (ACCEPT), Annals of Allergy Asthma & Immunology 2000, 84(2):217-225.