University of Groningen

Genetic and environmental interplay in asthma severity and its underlying airway pathology

Ierodiakonou, Despo

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Ierodiakonou, D. (2014). *Genetic and environmental interplay in asthma severity and its underlying airway pathology*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 11-10-2022

Genetic and environmental interplay in asthma severity and its underlying airway pathology

Despo Ierodiakonou

Layout and printing: Lovebird design & printing solutions
www.lovebird-design.com

ISBN (print): 978-90-367-6829-0
ISBN (digital): 978-90-367-6830-6

Cover inspired by Charles Jencks' DNA Double Helix Sculpture, Cambridge UK

Copyright © 2014 by D. Ierodiakonou. All rights reserved. No parts of this book may be reproduced or transmitted in any form or by any means without prior permission of the author.

university of groningen

Genetic and Environmental Interplay in Asthma Severity and Its Underlying Airway Pathology

PhD Thesis

to obtain the degree of PhD at the

University of Groningen

on the authority of the

Rector Magnificus Prof. E. Sterken

and in accordance with

the decision by the College of Deans.

This thesis will be defended in public on

Monday 3 March 2014 at 14:30 hours

by

Despo Ierodiakonou

born on 31 July 1983

in Lefkosia, Cyprus

Supervisors:

Prof. D.S. Postma

Prof. H.M. Boezen

Prof. G.H. Koppelman

Co-Supervisor:

Dr. J.M. Vonk

Assessment committee:

Prof. B. Brunekreef

Prof. S.T. Weiss

Prof. R.J. Sinke

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Asthma Epidemic

Asthma is a chronic inflammatory disorder of the airways, associated with variable structural changes, that affects children and adults of all ages. It is associated with airway hyperresponsiveness (the airways narrow in an exaggerated fashion after being exposed to a trigger) and variable airflow obstruction. Asthma, like other chronic diseases, is a complex disease and heterogeneous in its origins and clinical expression. It results from the interaction of multiple genetic, epigenetic and environmental factors.[1]

Patients with asthma typically experience recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. Asthma attacks are generally triggered by allergens, viral respiratory infections and airborne irritants. The respiratory symptoms are usually associated with airway smooth muscle contraction, mucus production and oedema of the airway wall, leading to airflow obstruction which is often reversible either spontaneously or following treatment. When uncontrolled, asthma can markedly interfere with normal daily activities and seriously impact an individual's quality of life.[2]

The World Health Organisation has estimated that 300 million individuals have asthma worldwide, and that this will reach 400 million by 2025 with current rising trends.[3,4] In the United States, the prevalence of asthma increased from 7.3% (20.3 million persons) in 2001 to 8.2% (24.6 million persons) in 2009, a 12.3% increase.[5] Asthma is the most common disease among children worldwide. The International Study of Asthma and Allergies in Childhood (ISAAC) reported that the prevalence of asthma among school-age children varies across countries (5–10%) and figures are even higher in younger children for asthma related symptoms, such as wheezing. These prevalence trends in children were comparable in adults.[6] Adult asthma includes persistent disease from childhood, reactivated childhood asthma and adult onset asthma. The European Community Respiratory Health Survey (ECRHS) reported that there is a widespread variation in self-reported wheezing in the adults, varying from 4.1% to 32%.[4,7] In the Netherlands ~20% of adults studied reported having wheeze.[7]

The disparity in asthma prevalence among countries is narrowing due to a rising prevalence in low and middle income countries as they adopt a more Western-type lifestyle, and it is plateauing in high income countries.[8-10] In the Netherlands, data from general practitioners and from repeated cross-sectional surveys indicate that a steep rise in the prevalence of childhood asthma since the 1980s (from 1 to 5 %) has been followed by a leveling off or a decreasing trend in the late 1990's; that was similar in adults.[11]

Factors considered to underlie the increase in asthma are poorly understood even though connections with geographical patterns, urbanization and the Western-type lifestyle seem to be a common factor.[12-15] Possible contributing factors include diet and obesity, exposure to certain environmental chemicals and drugs, timing of infections, psychological stress, tobacco smoking, changes in housing type and indoor environment, air pollution and climate changes.[3,12,16,17] However, as not all exposed people develop asthma, it suggests that the individual's genetic susceptibility must play a key role.[1]

Asthma is a disease that generally has a good prognosis for the majority of affected people. However, there seem to be several different subgroups, both in children and adults, with various pathological and clinical phenotypes that influence the disease's severity and trajectory.[18] Nevertheless, the costs of asthma are substantial and include both direct medical costs (hospitalization, emergency room treatment, doctor's visits and medication) and indirect, non-medical costs (time lost from work/school and premature deaths). The countries with the highest death rates due to asthma are those in which controller therapy is not available, such as in the developing word.[3] The number of disability-adjusted life years (DALYs) lost due to asthma worldwide has been estimated to be currently about 15 million per year. Worldwide, asthma accounts for around 1% of all DALYs lost, which is thought to reflect the high prevalence of asthma and its impact on lifestyle.[4,19]

In summary, asthma is a heterogeneous disease with different phenotypes and variable clinical manifestations, which depend on the age, ethnicity, socioeconomic status, genetic background, and environmental influences. Genetic susceptibly probably is the underlying cause of different severities among individuals affected with asthma.[18,20] The complexity of the disease together with lack of a cure for asthma makes this disease a public health burden reflected in the use of health services and the loss of leisure and productivity/work time of asthmatic individuals. Thus, it is essential to identify the genetic and environmental factors involved in asthma development and severity, to understand the underlying clinicopathological mechanisms, and to develop new disease measures of prevention and management.

Asthma: From fetal life to adulthood

Epidemiological studies suggest that asthma, like other common diseases, has at least part of its origins in early life.[21] The developmental plasticity of health and disease (DOHAD) hypothesis suggests that environmental exposures

during developmental periods can cause subtle alterations in physical function which, although physically almost invisible, may increase the risk of disease and dysfunction later in life.[22,23] The window of developmental plasticity extends from preconception to early childhood. *In utero* exposure to smoking and air pollution is well documented to be associated with asthma early in life and they involved epigenetic responses.[24,25] Other examples of known major exposures for development of respiratory disease or low airway function include no or shorter periods of breastfeeding and maternal obesity,[26,27] while early-exposure to some types of farming environments, larger family size and/or daycare attendance, and sufficient Vitamin D levels seem to be protective against asthma development.[28-31] Long-term follow-up studies have shown that impaired respiratory health or lung function in early childhood is associated with asthma and other respiratory diseases in later life.[32] Therefore, identification of perinatal and childhood risk factors for wheezing and low airway function may help find appropriate and effective interventions to prevent onset of asthma in childhood and/or later in life.

Important questions about the prognosis of asthma i.e., whether a child may outgrow the disease, the natural history of asthma and childhood risk factors associated with asthma development and severity in adulthood, have been addressed by several studies in the past:[33-40] Better lung function in childhood is consistently associated with better prognosis of asthma in adulthood, whereas the severity of airway hyperresponsiveness is not a good predictor of asthma outcome. Childhood atopy is associated with the persistence of respiratory symptoms but not with lower lung function in adulthood. Severity and duration of childhood asthma is associated with lung function decrements later in life. There is a switch around puberty in the gender susceptibility to develop asthma and environmental tobacco smoke exposure during childhood is associated with increased prevalence of asthma in adults. The tight relation between early life exposures and asthma development and severity in childhood but also later in life rise the need for longitudinal studies in asthma epidemiology and the identification of those childhood factors linked with asthma severity in adulthood.

An important finding is that early treatment with inhaled corticosteroids may improve lung function and airway hyperresponsiveness in children at least in the short-term period but relapse occurs after stopping of the treatment.[41] In adults, intervention with inhaled corticosteroids is recommended for control of disease and for prevention of progression of asthma and lung function decline.[42] Moreover, mild intermittent asthma is generally associated with

good control in adults when treated with inhaled corticosteroids but there is considerable variety in treatment response between individuals, which possibly can be explained by genetic make-up.[43]

Inside the airways: epithelial barrier, airway wall inflammation and remodeling

Airway epithelium is the natural barrier between environment and the underlying tissue. Its integrity is important for protecting the airways against noxious inhalants like cigarette smoke, airborne irritants and biological agents. Regulation of cell-cell junction stability and dynamics is crucial to maintain tissue integrity and allow normal tissue remodeling and wound healing throughout life.[44,45]

Recent advances in understanding the pathophysiology of asthma development and severity have pointed towards a prominent role of the airway epithelium.[46,47] Histological studies indicate that in bronchial biopsies of asthmatic patients the epithelium is interrupted and fragile.[48-51] A relative new hypothesis is that chronic injury by environmental irritants and/or defective repair of the airway epithelium predisposes airways to abnormal responses to inhaled agents and allergens, and leads to release of cytokines and growth factors.[52-54] The latter may contribute to airway wall remodeling and inflammation as a consequence of complex interaction between affected airway epithelial cells and the underlying mesenchymal and immune cells, known as activation of the Epithelial-Mesenchymal Trophic Unit.

Airway inflammation in asthma is linked to allergic sensitization. Asthma attacks reflect the local recruitment and activation of T_H2 cells, eosinophils, basophils and other leukocytes, and persistent mediator production by resident and recruited cells.[55,56] Mediators that initiate late-phase reactions are thought to be derived from mast cells activated by allergen-induced IgE or from T-cells that recognize allergen derived peptides. Important factors that affect the likelihood of developing clinically significant sensitization include the host genotype[57] and whether exposure occurs together with agents that can enhance the sensitization process such as endotoxin and air pollution.[58-60] Yet, prolonged or repetitive environmental exposures induce persistent inflammation typically characterized not only by increased airway inflammation at the affected site but also by substantial changes in the extracellular matrix and alterations in the number, phenotype and function of structural cells in the affected tissues.[61]

Structural changes taking place in the epithelium and in the submucosal area of the bronchi are termed airway remodeling. It includes enhanced

accumulation of extracellular matrix (collagen I, III, V and fibronectin) with subepithelial fibrosis manifested as basal membrane thickening,[62] hyperplasia of blood vessels,[63] hyperplasia and hypertrophy of goblet cells and subepithelial glands with excess production of mucus,[64] as well as myofibroblast and smooth muscle cell hyperplasia.

In synopsis, the airway epithelium plays a key role in the airway pathology of asthma. Under acute and chronic environmental exposures there is a complex interaction between affected airway epithelial cells and the underlying mesenchymal cells that together are thought to regulate the airway inflammation and tissue remodeling, characteristics of chronic allergic airway disease.

Relation of airway pathology with asthma severity and the role of genetics

Chronic airway inflammation and airway wall remodeling are two important pathological features of asthma, associated with airway hyperresponsiveness.[65] They result in airway wall thickening along with narrowing of the airway lumen. This influences the normal flow of air in and out the lungs, linked with airway obstruction and progressive loss of lung function.[34,66-68] Nevertheless, longitudinal studies have shown that some asthma patients go into remission.[18,69,70] This heterogeneity is resulting from the effect of multiple genetic and interacting environmental factors on the pathophysiology of asthma.[20,71,72]

The latter enforces the idea that investigation of genes encoding adhesion molecules, cytokines and growth factors might be a key for understanding better the underlying mechanisms of asthma severity. This may ultimately lead to the development of therapeutic targets and approaches that help restore epithelial function, reduce chronic airway inflammation and prevent airway wall remodeling.

Glucocorticosteroids constitute the cornerstone of regular asthma treatment. Inhaled corticosteroids (ICS) have been widely used for suppressing inflammation and asthma symptoms. ICS may modulate epithelial repair and airway remodeling,[73,7474] while use of ICS is found to be associated with less accelerated lung function decline at adult age[42] and with improvement of airway hyperresponsivness.[42,74] However, a subpopulation of asthma patients does not respond well to glucocorticosteroids and a marked increase in TNF-alpha production has been implicated in the pathophysiology of corticosteroid refractoriness.[75] Treatment with anti-TNFα therapy has been used as

alternative to steroid-resistant asthma and it has demonstrated improvements in asthma quality-of-life, lung function, hyperresponsivness and reduction in exacerbation frequency.[76] However, there is again marked heterogeneity in response to anti-TNFα therapy suggesting that benefit is likely to be reserved to a small sub-group; whereas recently 5 cases have been described to develop new onset asthma with anti-TNFα therapy.[77] Together, this poses questions about the exact role of individual genetic susceptibility in the anti-inflammatory treatment response.

Aims of the thesis

Asthma is a heterogeneous disease resulting from the effect of multiple genetic and interacting environmental factors on its pathophysiology. This becomes more complicated by epigenetic changes induced by environmental exposures early in its development. Therefore we need studies directed towards determining the mechanisms by which genetic and environmental factors and their interactions affect clinicopathological outcomes in asthma and whether such effects can be modified by anti-inflammatory treatment. Although many studies have investigated the genetic and environmental determinants of asthma susceptibility and severity, longitudinal genetic studies seeking associations with the natural course and asthma outcome are scarce. Similarly, the genetic background of airway pathology is not well documented.

The first aim of the current doctoral thesis is to investigate the role of genes encoding proteins involved in epithelial barrier integrity, chronic airway inflammation and airway wall remodeling. (1a) With a candidate gene approach we investigate whether single nucleotide polymorphisms in *E-cadherin* (*CDH1), Tumor Necrosis Factor alpha and beta* (*TNF)* and Transforming Growth Factor beta-1 (*TGFB1)* genes are associated with chronic airway inflammation and airway wall remodeling in bronchial biopsies of adult asthmatics. (1b) Moreover, we use cross-sectional and longitudinal data to assess whether the same genes are associated with asthma severity outcomes in adults (e.g., airway hyperresponsiveness, lung function level and decline, asthma remission). In this way, we attempt to link the direction of associations with clinical and histological outcomes, so called loose replication. (1c) Finally, we focus on effect modification by inhaled corticosteroids (*CDH1 and TNF* genes) and smoking (*TGFB1 gene)*. Exploration of this aim is presented in 3 separate chapters, one chapter for each of the three candidate genes.

The second aim of this thesis is to examine whether genetic variants modify

long-term effects of air pollution in children with asthma participating in a clinical trial for 4 years. In order to understand the effect modification of genetic variants on air pollution we first (2a) define the primary effects of air pollution on lung function level and the severity of airway hyperresponsiveness in a longitudinal way and then (2b) assess effect modification by anti-inflammatory treatment (budesonide and nedocromil Vs placebo). (2c) Finally, we use a hypothesis-free (genome-wide) analysis to investigate whether genetic variants modify the long-term effects of air pollution on asthma severity in children and based on the genome-wide results a pathway-level approach was introduced as a way to assess the overall evidence of association of pollution interaction with a group of functionally related genes (i.e., a gene set), thus incorporating prior biological knowledge. Aim 2 results in two chapters, one presenting the primary effects of pollution in children with asthma and effect modification by anti-inflammatory treatment, and the other presenting the genome-wide gene by air pollution interaction study (GWIS) and a pathway analysis based on the GWIS results.

REFERENCES

1. Blumenthal MN. Genetic, epigenetic, and environmental factors in asthma and allergy. Ann Allergy Asthma Immunol 2012 Feb;108:69-73.

2. World Health Organization, Asthma Fact Sheet No. 307. May 2011; Available at: http://www.who.int/mediacentre/factsheets/fs307/en/. Accessed April, 2013.

3. World Health Organization. Global surveillance, prevention and control of chronic respiratory diseases: a comprehensive approach, 2007. 2007; Available at: http://www.who.int/gard/publications/GARD%20Book%202007.pdf. Accessed April, 2013.

4. Masoli M, Fabian D, Holt S, Beasley R, Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy 2004 May;59:469-478.

5. Centers of Disease Control and Prevention, Vital Signs: Asthma Prevalence, Disease Characteristics, and Self-Management Education --- United States, 2001--2009. May 2011; Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6017a4.htm. Accessed April, 2013.

6. Pearce N, Sunyer J, Cheng S, Chinn S, Bjorksten B, Burr M, Keil U, Anderson HR, Burney P. Comparison of asthma prevalence in the ISAAC and the ECRHS. ISAAC Steering Committee and the European Community Respiratory Health Survey. International Study of Asthma and Allergies in Childhood. Eur Respir J 2000 Sep;16:420-426.

7. Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). Eur Respir J 1996 Apr;9:687-695.

8. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, Williams H, ISAAC Phase Three Study Group. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006 Aug 26;368:733-743.

9. Pearce N, Ait-Khaled N, Beasley R, Mallol J, Keil U, Mitchell E, Robertson C, ISAAC Phase Three Study Group. Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax 2007 Sep;62:758-766.

10. Sunyer J, Anto JM, Tobias A, Burney P. Generational increase of self-reported first attack of asthma in fifteen industrialized countries. European Community Respiratory Health Study (ECRHS). Eur Respir J 1999 Oct;14:885-891.

11. van Schayck CP, Smit HA. The prevalence of asthma in children: a reversing trend. Eur Respir J 2005 Oct;26:647-650.

12. Janson C, Anto J, Burney P, Chinn S, de Marco R, Heinrich J, Jarvis D, Kuenzli N, Leynaert B, Luczynska C, Neukirch F, Svanes C, Sunyer J, Wjst M, European Community Respiratory Health Survey II. The European Community Respiratory Health Survey: what are the main results so far · European Community Respiratory Health Survey II. Eur Respir J 2001 Sep;18:598-611.

13. Nicolaou N, Siddique N, Custovic A. Allergic disease in urban and rural populations: increasing prevalence with increasing urbanization. Allergy 2005 Nov;60:1357-1360.

14. Marshall GD. Internal and external environmental influences in allergic diseases. J Am Osteopath Assoc 2004 May;104:S1-6.

15. Baiz N, Annesi-Maesano I. Is the asthma epidemic still ascending? Clin Chest Med 2012 Sep;33:419-429.

16. Papoutsakis C, Priftis KN, Drakouli M, Prifti S, Konstantaki E, Chondronikola M, Antonogeorgos

G, Matziou V. Childhood overweight/obesity and asthma: is there a link · A systematic review of recent epidemiologic evidence. J Acad Nutr Diet 2013 Jan;113:77-105.

17. World Allergy Organization (WAO), White Book on Allergy. 2011; Available at: http://www.worldallergy.org/UserFiles/file/WAO-White-Book-on-Allergy_web.pdf, 2013.

18. Holloway JW, Yang IA, Holgate ST. Interpatient variability in rates of asthma progression: can genetics provide an answer · J Allergy Clin Immunol 2008 Mar;121:573-579.

19. Accordini S, Corsico A, Cerveri I, Gislason D, Gulsvik A, Janson C, Jarvis D, Marcon A, Pin I, Vermeire P, Almar E, Bugiani M, Cazzoletti L, Duran-Tauleria E, Jogi R, Marinoni A, Martinez-Moratalla J, Leynaert B, de Marco R, Therapy and Health Economics Working Group of the European Community Respiratory Health Survey II. The socio-economic burden of asthma is substantial in Europe. Allergy 2008 Jan;63:116-124.

20. Holloway JW, Yang IA, Holgate ST. Genetics of allergic disease. J Allergy Clin Immunol 2010 Feb;125:S81-94.

21. Kotecha S, Kotecha SJ. Long term respiratory outcomes of perinatal lung disease. Semin Fetal Neonatal Med 2012 Apr;17:65-66.

22. Brew BK, Marks GB, CAPS (Childhood Asthma Prevention Study) Investigators. Perinatal factors and respiratory health in children. Clin Exp Allergy 2012 Nov;42:1621-1629.

23. Duijts L. Fetal and infant origins of asthma. Eur J Epidemiol 2012 Jan;27:5-14.

24. Wright RJ, Brunst KJ. Programming of respiratory health in childhood: influence of outdoor air pollution. Curr Opin Pediatr 2013 Apr;25:232-239.

25. DiFranza JR, Aligne CA, Weitzman M. Prenatal and postnatal environmental tobacco smoke exposure and children's health. Pediatrics 2004 Apr;113:1007-1015.

26. Sonnenschein-van der Voort AM, Jaddoe VW, van der Valk RJ, Willemsen SP, Hofman A, Moll HA, de Jongste JC, Duijts L. Duration and exclusiveness of breastfeeding and childhood asthma-related symptoms. Eur Respir J 2012 Jan;39:81-89.

27. Lowe A, Braback L, Ekeus C, Hjern A, Forsberg B. Maternal obesity during pregnancy as a risk for early-life asthma. J Allergy Clin Immunol 2011 Nov;128:1107-9.e1-2.

28. Illi S, Depner M, Genuneit J, Horak E, Loss G, Strunz-Lehner C, Buchele G, Boznanski A, Danielewicz H, Cullinan P, Heederik D, Braun-Fahrlander C, von Mutius E, GABRIELA Study Group. Protection from childhood asthma and allergy in Alpine farm environments-the GABRIEL Advanced Studies. J Allergy Clin Immunol 2012 Jun;129:1470-7.e6.

29. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, Weiss G, Nyberg F, van Hage M, Pershagen G, Brunekreef B, Riedler J, Lauener R, Braun-Fahrlander C, von Mutius E, PARSIFAL Study team. Not all farming environments protect against the development of asthma and wheeze in children. J Allergy Clin Immunol 2007 May;119:1140-1147.

30. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. N Engl J Med 2000 Aug 24;343:538-543.

31. Bozzetto S, Carraro S, Giordano G, Boner A, Baraldi E. Asthma, allergy and respiratory infections: the vitamin D hypothesis. Allergy 2012 Jan;67:10-17.

32. Stern DA, Morgan WJ, Halonen M, Wright AL, Martinez FD. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. Lancet 2008 Sep 20;372:1058-1064.

33. Vonk J, Postma D, Boezen H, Grol M, Schouten J, Koeter G, Gerritsen J. Childhood factors associated with asthma remission after 30 year follow up. Thorax 2004 11//;59:925-929.

34. Vonk JM, Jongepier H, Panhuysen CIM, Schouten JP, Bleecker ER, Postma DS. Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up. Thorax 2003 04//;58:322-327.

35. Vonk JM, Boezen HM. Predicting adult asthma in childhood. Curr Opin Pulm Med 2006 Jan;12:42-47.

36. Grol MH, Gerritsen J, Vonk JM, Schouten JP, Koeter GH, Rijcken B, Postma DS. Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. Am J Respir Crit Care Med 1999 Dec;160:1830-1837.

37. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, Cowan JO, Herbison GP, Silva PA, Poulton R. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med 2003 Oct 9;349:1414-1422.

38. Rasmussen F, Taylor DR, Flannery EM, Cowan JO, Greene JM, Herbison GP, Sears MR. Risk factors for airway remodeling in asthma manifested by a low postbronchodilator FEV1/vital capacity ratio: a longitudinal population study from childhood to adulthood. Am J Respir Crit Care Med 2002 06/01/;165:1480-1488.

39. Almqvist C, Worm M, Leynaert B, working group of GA2LEN WP 2.5 Gender. Impact of gender on asthma in childhood and adolescence: a GA2LEN review. Allergy 2008 Jan;63:47-57.

40. Larsson ML, Frisk M, Hallstrom J, Kiviloog J, Lundback B. Environmental tobacco smoke exposure during childhood is associated with increased prevalence of asthma in adults. Chest 2001 Sep;120:711-717.

41. Strunk RC, Childhood Asthma Management Program Research Group. Childhood Asthma Management Program: lessons learned. J Allergy Clin Immunol 2007 Jan;119:36-42.

42. Dijkstra A, Vonk JM, Jongepier H, Koppelman GH, Schouten JP, Hacken NHTt, Timens W, Postma DS. Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex. Thorax 2006 02//;61:105-110.

43. Lima JJ, Blake KV, Tantisira KG, Weiss ST. Pharmacogenetics of asthma. Curr Opin Pulm Med 2009 Jan;15:57-62.

44. Cavey M, Lecuit T. Molecular bases of cell-cell junctions stability and dynamics. Cold Spring Harb Perspect Biol 2009 Nov;1:a002998.

45. Pokutta S, Weis WI. Structure and Mechanism of Cadherins and Catenins in Cell-Cell Contacts. Annu Rev Cell Dev Biol 2007 /11/;23:237-261.

46. Chanez P. Severe asthma is an epithelial disease. Eur Respir J 2005 06/01/;25:945-946.

47. Ricciardolo FLM, Stefano AD, Krieken JHJMv, Sont JK, Schadewijk Av, Rabe KF, Donner CF, Hiemstra PS, Sterk PJ, Mauad T. Proliferation and inflammation in bronchial epithelium after allergen in atopic asthmatics. Clinical & Experimental Allergy 2003;33:905-911.

48. Montefort S, DjukanoviÄ‡ R, Holgate ST, Roche WR. Ciliated cell damage in the bronchial epithelium of asthmatics and non-asthmatics. Clin Exp Allergy 1993 03//;23:185-189.

49. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989 12//;140:1745-1753.

50. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi HM, Vernon-Wilson E, Sammut D, Bedke N, Cremin C, Sones J, Djukanovic R, Howarth PH, Collins JE,

Holgate ST, Monk P, Davies DE. Defective epithelial barrier function in asthma. J Allergy Clin Immunol 2011 Sep;128:549-56.e1-12.

51. Knight DA, Stick SM, Hackett TL. Defective function at the epithelial junction: a novel therapeutic frontier in asthma · J Allergy Clin Immunol 2011 Sep;128:557-558.

52. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001 Nov 15;164:S28-38.

53. Holgate ST. Epithelium dysfunction in asthma. J Allergy Clin Immunol 2007 Dec;120:1233-44; quiz 1245-6.

54. Holgate ST. The airway epithelium is central to the pathogenesis of asthma. Allergol Int 2008 Mar;57:1-10.

55. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nat Med 2012 May 4;18:693-704.

56. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 2010 Feb;125:S73-80.

57. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat Rev Immunol 2008 Mar;8:169-182.

58. Herrick CA, Bottomly K. To respond or not to respond: T cells in allergic asthma. Nat Rev Immunol 2003 May;3:405-412.

59. Saxon A, Diaz-Sanchez D. Air pollution and allergy: you are what you breathe. Nat Immunol 2005 Mar;6:223-226.

60. Carlsten C, Melen E. Air pollution, genetics, and allergy: an update. Curr Opin Allergy Clin Immunol 2012 Oct;12:455-460.

61. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature 2008 Jul 24;454:445-454.

62. Liesker JJ, Hacken NHT, Zeinstra-Smith M, Rutgers SR, Postma DS, Timens W. Reticular basement membrane in asthma and COPD: Similar thickness, yet different composition. Int J Chron Obstruct Pulmon Dis 2009;4:127-135.

63. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. Chest 2005 03//;127:965-972.

64. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, Hotchkiss JA, Zhang Y, Novikov A, Dolganov G, Fahy JV. Mild and Moderate Asthma Is Associated with Airway Goblet Cell Hyperplasia and Abnormalities in Mucin Gene Expression. Am J Respir Crit Care Med 2001 02/01/;163:517-523.

65. Busse WW. The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: its measurement and clinical significance. Chest 2010 Aug;138:4S-10S.

66. Pascual RM, Peters SP. Airway remodeling contributes to the progressive loss of lung function in asthma: An overview. J Allergy Clin Immunol 2005 09//;116:477-486.

67. ten Brinke A. Risk factors associated with irreversible airflow limitation in asthma. Curr Opin Allergy Clin Immunol 2008 Feb;8:63-69.

68. Janson C. The importance of airway remodelling in the natural course of asthma. Clin Respir J 2010 May;4 Suppl 1:28-34.

69. Braman SS. Growing old with asthma: what are the changes and challenges · Expert Rev Respir Med 2010 Apr;4:239-248.

70. Upham JW, James AL. Remission of asthma: The next therapeutic frontier · Pharmacol Ther 2011 Apr;130:38-45.

71. Lemanske RF,Jr, Busse WW. Asthma: clinical expression and molecular mechanisms. J Allergy Clin Immunol 2010 Feb;125:S95-102.

72. Yang IA, Savarimuthu S, Kim ST, Holloway JW, Bell SC, Fong KM. Gene-environmental interaction in asthma. Curr Opin Allergy Clin Immunol 2007 Feb;7:75-82.

73. Benayoun L, Letuve S, Druilhe A, Boczkowski J, Dombret M, Mechighel P, Megret J, Leseche G, Aubier M, Pretolani M. Regulation of Peroxisome Proliferator-activated Receptor gamma Expression in Human Asthmatic Airways . Relationship with Proliferation, Apoptosis, and Airway Remodeling. Am J Respir Crit Care Med 2001 10/15/;164:1487-1494.

74. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, Walters E. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax 2002 04//;57:309-316.

75. Holgate ST, Holloway J, Wilson S, Howarth PH, Haitchi HM, Babu S, Davies DE. Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities. J Allergy Clin Immunol 2006 Mar;117:496-506; quiz 507.

76. Desai D, Brightling C. TNF-alpha antagonism in severe asthma · Recent Pat Inflamm Allergy Drug Discov 2010 Nov;4:193-200.

77. Guilleminault L, Carre P, Beau-Salinas F, Taille C, Dieude P, Crestani B, Diot P, Marchand-Adam S. Asthma unmasked with tumor necrosis factor-alpha-blocking drugs. Chest 2011 Oct;140:1068-1071.

CHAPTER 2

E-CADHERIN GENE POLYMORPHISMS IN ASTHMA PATIENTS USING INHALED CORTICOSTEROIDS

D. Ierodiakonou[1,5], D.S. Postma[2,5], G.H. Koppelman[3,5], H.M. Boezen[1,5], J. Gerritsen[3,5], N. ten Hacken[2,5], W. Timens[4,5] and J.M. Vonk[1,5]

European Respiratory Journal 2011; 38: 1044–1052

1. Department of Epidemiology, University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands.

2. Department of Pulmonology, University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands.

3. Department of Paediatric Pulmonology and Paediatric Allergology, Beatrix Children's Hospital, University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands.

4. Department of Pathology and Medical Biology, University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands.

5. Groningen Research Institute for Asthma and COPD, Groningen, The Netherlands.

ABSTRACT

E-cadherins form intercellular junctions that maintain epithelial integrity. Epithelial integrity is impaired in asthma and can be restored by inhaled corticosteroids (ICSs). Our aim was to investigate the association of CDH1 gene polymorphisms (single-nucleotide polymorphisms (SNPs)) with airway remodelling, inflammation and forced expiratory volume in 1 s (FEV1) decline in asthma patients and assess whether ICSs modulate these effects.

Bronchial biopsies of 138 asthmatics were available (population 1). Associations of 17 haplotype-tagging SNPs with epithelial E-cadherin expression, biopsy parameters and FEV1/vital capacity (VC) ratio were tested. FEV1 and VC data were collected in 281 asthmatics with 30-yr follow-up (population 2). Linear mixed-effect models were used to assess associations of SNPs with FEV1 decline.

Seven out of the 17 SNPs were associated with airway remodelling, three with CD8+ T-cell counts, two with eosinophil counts and seven with FEV1 decline. All associations occurred only in patients using ICS. In general, alleles associated with less remodelling correlated with less FEV1 decline and higher FEV1/VC. Decreased epithelial E-cadherin expression was associated with five SNPs in non-ICS users.

In conclusion, our data show that CDH1 polymorphisms are associated with epithelial E- cadherin expression and suggest that epithelial adhesion is an important contributor to airway remodelling and lung function in asthma. These effects are modified by the use of inhaled corticosteroids.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterised by bronchial hyperresponsiveness (BHR), and pathological changes in the epithelium and submucosal area of the bronchi. Histological studies indicate that the epithelium is interrupted and fragile in bronchial biopsies from asthmatics [1, 2]. Both genetic and environmental factors as well as their interactions are involved in asthma development and its course during lifetime. Recent advances in the understanding of the pathophysiology of asthma have pointed towards a prominent role of the airway epithelium in asthma development and severity [3, 4].

Airway epithelium is the natural barrier between the environment and underlying tissue. Its integrity is important for protecting the airways against noxious inhalants, such as environmental tobacco smoke, particles and biological agents.

Regulationofcell–celljunctionstabilityanddynamicsiscrucialtomaintaining tissue integrity and allowing tissue remodelling throughout development [5, 6]. E-cadherin, the major cadherin expressed in epithelial cells, is an adhesion molecule that is able to establish and stabilise cellular junctions between adjacent cells in the presence of Ca2+ [7] and is coded by the CDH1 gene on chromosome 16q22.1. HIROSAKO et al. [8] suggested that human bronchial intraepithelial lymphocyteshave roles distinct from subsets of other lymphocytes, and that CD8+ cells and CD103+ (ligand of E- cadherin) cells have potentially important functions in the bronchial epithelium.

Longitudinal studies have demonstrated that some asthma patients develop irreversible airway obstruction and progressive loss of lung function [9]. Inhaled corticosteroids (ICSs) have been widely used for suppressing inflammation and asthma symptoms. Early use of ICSs has been found to be associated with less accelerated forced expiratory volume in 1 s (FEV1) decline [10], and with improvement of BHR and airway remodelling [11]. Furthermore, corticosteroids are thought to modulate epithelial repair [12] and there is evidence that they upregulate epithelial E-cadherin expression [13].

We hypothesised that loss of epithelial integrity resulting from loss of epithelial E-cadherin expression or disruption of E-cadherin-mediated cell–cell contacts predisposes airways to abnormal responses to inhaled substances. Subsequent remodelling processes and airway inflammation may lead to airway narrowing and accelerated lung function decline.

Therefore, we investigated the associations between CDH1 polymorphisms and airway remodelling (basement membrane (BM) thickness and subepithelial

vasculature), inflammation and annual FEV1 decline in asthma patients and assessed whether ICS use modulates these associations.

MATERIALS AND METHODS

Populations

Population 1

Population 1 consisted of 138 asthmatics who had participated in cohort studies conducted by our research group in previous years. They were re-examined for lung function and BHR, and underwent bronchoscopy with biopsy collection [14, 15]. The main exclusion criteria were: FEV1 ,1.2 L; bronchiectasis; upper respiratory tract infection (e.g. colds); and/or use of antibiotics or oral corticosteroids within the 2 months before inclusion. DNA samples and biopsy data were available in 137 subjects.

Population 2

Population 2 consisted of 281 asthma patients who had been referred to the asthma clinic at Beatrixoord Hospital in Haren, the Netherlands, over the period 1966–1975. Patients who, at their first visit, were ,45 yrs of age, had 020% fall in FEV1 during a histamine challenge test (provocative concentration causing a 20% fall in FEV1 ≤32 mg·mL^{-1}) and were sympto- matic according to current American Thoracic Society criteria

[16] were re-examined during the period 1991–1999 [9]. At both visits, patients filled in a questionnaire on respiratory symptoms and underwent lung function, histamine challenge and skin-prick tests. The clinical assessment was performed as previously described [10]. Before testing, participants had to be in a stable condition without any exacerbation in the previous 6 weeks. After their first visit, subjects had annual routine check-ups for their asthma. Their medical records provided information on lung function and corticosteroid use during these check-ups. DNA samples have been collected from 253 subjects. 32 subjects in population 1 were derived from population 2. All participants originated from the northern region of the Netherlands. The study was approved by the medical ethics committee of the University Medical Center Groningen (Groningen, the Netherlands), and all participants gave signed, informed consent.

Biopsy collection and processing

Bronchoscopy, collection and processing of the bronchial biopsies were performed as previously described [14]. Immunohistochemistry was performed on 3-mm, formalin-fixed, paraffin-embedded tissue specimens that were deparaffinised with xylene, dehydrated in ethanol and after antigen retrieval, incubated with the primary antibodies: anti-E-cadherin antibody (#610181; BD Bioscience, Breda, the Netherlands) for epithelial E-cadherin expression; anti-eosinophilic peroxidase antibody for eosinophil detection (laboratories of N.A. Lee and J.J. Lee, Mayo Clinic, Scottsdale, AZ, USA); anti-CD8 (DAKO, Heverlee, the Netherlands) antibody for T-cells; and anti-CD31 monoclonal antibody for vessel endothelial cells. BM thickness was calculated by dividing the BM surface area by BM length (mm). The number of positively stained inflammatory cells was counted in a total area of 0.1 mm2 in the submucosa, 100 mm beneath the BM. The number of CD31+ vessels in the submucosal area was counted in the whole section (excluding epithelium, muscle and mucous gland areas); therefore, we measured the number of vessels per area (0.1 mm2). Finally, E-cadherin expression was determined as the percentage of BM covered with E-cadherin-positive intact epithelium (fig. 1). Further details on the immunochemistry and quantification procedures used are presented in the online supplementary material.

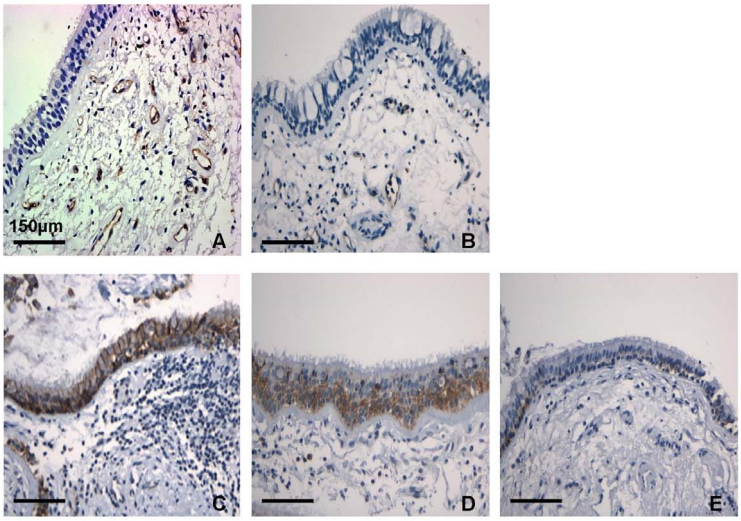


FIGURE 1. Immunostaining of airway wall biopsies of asthma patients. Representative images of a) strong and b) weak CD31 staining of vessels, and c) strong, d) moderate and e) weak epithelial E-cadherin staining. Magnification: objective 20x. Scale bars=150 mm.

Genotyping of single-nucleotide polymorphisms

We genotyped 17 haplotype-tagging single-nucleotide polymorphisms (SNPs) in CDH1 according to HapMap CEU (Utah residents with Northern and Western European ancestry) genotype data (r2,0.8; minor allele frequency .10%). 12 of the SNPs were genotyped by KBioscience Ltd (Hoddesdon, UK) using their competitive allele-specific PCR system (KASPar), and five SNPs were derived from a genome-wide association study on asthma using the 370-kb Illumina chip (Illumina, Eindhoven, the Netherlands) (see figure 2 for the genotyped SNPs).

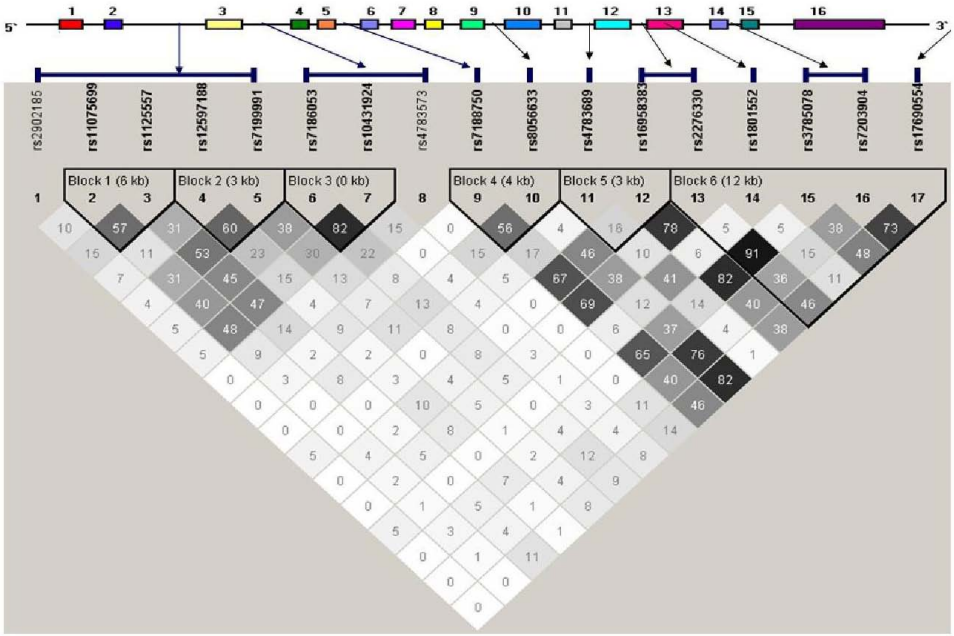


FIGURE 2. Linkage disequilibrium plot and correlation coefficients (100r2) of 17 genotyped CDH1 gene single-nucleotide polymorphisms (SNPs) in population 2 (n5281). The location of SNPs is given for the HapMap (www.hapmap.org).

Statistics

Normalisation of the distribution of the variables was performed by natural logarithm transformation where necessary. Multiple linear regression analysis adjusted for sex, age and smoking was used to assess the effect of the SNPs on airway remodelling parameters (BM thickness and submucosal vessel numbers) and inflammatory cells (eosinophils and CD8+T-cells) in population 1. Additionally, we used FEV1/ vital capacity (VC) ratio post-bronchodilation (BD) adjusted for sex, age, height and smoking as a marker of remodelling in both populations [17]. Since E-cadherin expression is known to be upregulated

by ICSs [13, 18, 19], we tested for interaction between ICS and genotypes by introducing interaction terms into the models. General (heterozygotes versus wild types and homozygous mutants versus wild types) genetic models were used. When the number of subjects with the homozygote mutant genotype was less than five, only dominant (heterozygotes and homozygous mutants pooled versus wild types) models were used. Association between epithelial E-cadherin expression (percentage of BM covered with intact epithelium positive for E-cadherin) and genotypes was assessed with nonparametric tests using dominant genetic models. The association between denuded epithelium (percentage BM covered with denuded epithelium) and epithelial E-cadherin expression was tested with multiple linear regression adjusted for sex and age. In population 2, FEV1 decline was analysed with linear mixed-effect (LME) models as described previously [10]. Only FEV1 measurements after the age of 30 yrs were included, since this is the age where the lung function is in a plateau phase or decline begins in normal subjects [20]. LME models were adjusted for sex, age, first FEV1 after age 30 yrs, height, pack-years of smoking and use of oral corticosteroids. Inclusion criteria required subjects having FEV1 measurements before and after the introduction of ICS, with a minimum of three FEV1 measurements over a period of 02 yrs. Subjects who never used ICSs were excluded. FEV1 levels and annual FEV1 declines were estimated for the two periods (without and with ICS use) by introducing the following variables: time; genotype; ICS use; and their interactions. LME models were conducted in S-plus 7.0 (Insightful Corp., Seattle, WA, USA). All other analyses were conducted with SPSS (version 16; SPSS Inc., Chicago, IL, USA). Two-tailed p-values of <0.05 were considered statistically significant.

RESULTS

Clinical characteristics and genotype distribution

Clinical characteristics of both populations are presented in table 1. All subjects were atopic and hyperresponsive at first clinical assessment. Population 1 had a higher mean lung function and 57 (41.3%) subjects used ICS, while 146 (52%) subjects were on ICS treatment in population 2. For the analysis on FEV1 decline 125 subjects met the inclusion criteria. Table 2 presents details on corticosteroid use in populations 1 and 2. Genotype distributions in the two asthmatic populations are presented in table E1 in the online supplementary material. All SNPs were in Hardy-Weinberg equilibrium (p>0.05). The linkage disequilibrium (LD) plot of the SNPs in population 2 is shown in figure 2.

TABLE 1 Characteristics of populations 1 and 2

	Population 1	Population 2
Asthmatics n	138	281
Year of assessment	2002–2006	1991–1999
Males %	50.7	60.5
Age yrs	47.2±12.7	51.3±9.1
ICS use	57 (41.3)	146 (52.0)
LABA use	37 (26.8)	NA
Non/ex-/current smokers %	44.9/34.1/21.0	40.6/32.0/27.4
FEV_1 % pred		
Pre-BD	91.7±18.0	70.1±23.6
Post-BD	99.9±17.0	82.5±22.4
FEV_1/VC		
Pre-BD	71.2 (8.8)	60.2 (14.2)
Post-BD	75.6 (6.5)	64.8 (13.5)
CD31+ vessels# n	18.0±8.2	NA
BM thickness µm	6.0±1.6	NA
CD8+ cells# n	23.0 (1–204)	NA
EPX+ cells# n	2.0 (0–40)	NA
Epithelial E-cadherin expression¶	88.4 (0–100)	NA

Data are presented in mean±SD, n (%) or median (range), unless otherwise stated. ICS: inhaled corticosteroid; LABA: long-acting β-agonist; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; BD: bronchodilation; VC: vital capacity; BM: basement membrane; EPX: eosinophilic peroxidase; NA: not applicable. #: per 0.1 mm^2 submucosal area; ¶: % BM covered with E-cadherin-positive intact epithelium.

TABLE 2 Characteristics of corticosteroid use in populations 1 and 2

	Population 1	Population 2
Total subjects n	138	281
Subjects using ICSs at final survey	57 (41.3)	146 (52.0)
ICS dose# µg·day^{-1}	800 (100–2000)	800 (50–6000)
Subjects using OCSs at final survey	0	14 (5.0)
Subjects using LABAs among ICS users	31 (54.4)	NA
Subjects included in analysis of FEV_1 decline¶		
Subjects who ever used ICSs		125 (100.0)
Subjects who ever used OCSs		69 (55.2)
Age at start of ICS use yrs		42 (21–70)
Duration of ICS use yrs		13.5 (2.2–25.7)
ICS dose# µg·day^{-1}		694 (179–2400)
Duration of OCS use yrs		9.8 (0.1–34.8)
OCS dose µg·day^{-1}		7.6 (2.1–15.0)

Data are presented as n (%) or median (range), unless otherwise stated. ICS: inhaled corticosteroid; OCS: oral corticosteroid; LABA: long-acting β-agonist; FEV_1: forced expiratory volume in 1 s; NA: not applicable. #: all doses were recalculated as beclomethasone equivalents (100 µg·day^{-1} beclomethasone is equivalent to 100 µg·day^{-1} budesonide (not by Turbuhaler®; AstraZeneca, Zoetermeer, the Netherlands), 50 µg·day^{-1} budesonide by Turbuhaler® or 50 µg·day^{-1} fluticasone); ¶: only ever-ICS users (n=125).

Epithelial E-cadherin expression

Five CDH1 SNPs were significantly associated with epithelial E-cadherin expression (rs8056633, rs16958383, rs2276330, rs3785078 and rs7203904). The minor alleles of these SNPs were significantly associated with lower expression of E-cadherin in individuals without ICS and generally with similar expression as the wild types in those with ICS use (table 3 and fig. 3; table E2 in the online supplementary material). There were no significant differences in epithelial E-cadherin expression between never- and ever- (ex- and current pooled) smokers or among the three smoking categories (data not shown). The association between denuded epithelium with epithelial E-cadherin expression was very close to significance (b=-0.003; p=0.05; natural logarithm scale).

Airway remodelling: BM thickness and subepithelial vasculature

In population 1, seven out of 17 SNPs were associated with BM thickness and subepithelial vasculature in the presence of ICS (rs7188750, rs8056633, rs16958383, rs2276630, rs3785078, rs7203904 and rs17690554). The minor alleles of these polymorphisms were all associated with thinner BM and/or a lower number of vessels in the submucosal area (table 3 and fig. 3). Without ICS use, these effects of the minor alleles were

less prominent or even reversed, but not significantly so. Interactions between these SNPs and ICSs with respect to airway remodelling were significant for rs2276330 and rs3785078 (tables E3.1 and E3.2 in the online supplementary material).

Airway inflammation: submucosal CD8+ T-cell and eosinophil counts

With ICS use, rs11075699, rs2276630 and rs1125557 were significantly associated with CD8+ T-cell counts in the submucosal area. CD8+ T-cell counts were higher in subjects heterozygous for rs11075699 and in carriers of the minor C-allele of rs2276330, while subjects with a homozygous mutant genotype for rs1125557 had lower CD8+T-cell counts compared with wild-types. Minor alleles of rs2902185 and rs10431924 were associated with higher eosinophil counts. These associations were absent in asthmatics without ICS. Interactions between SNPs and ICSs on inflammatory cell counts (CD8+ T-cells and eosinophils) were statistically significant for three out of five SNPs (rs1125557, rs10431924 and rs2276330) and for rs7188750, respectively (fig. 3; tables E4.1 and E4.2 in the online supplementary material).

FEV1 decline and FEV1/VC

Seven out of 17 SNPs were significantly associated with FEV1 decline in the presence of ICSs. The minor alleles for rs8056633, rs16958383, rs7203904 and rs17690554 were associated with less FEV1 decline. For example, during ICS use, FEV1 decline was 20.4 mL $\cdot yr^{-1}$ for subjects with the wild-type genotype of rs16958383, while it was 36.6 mL $\cdot yr^{-1}$ less in homozygous carriers of the minor allele (p50.004; fig. 3). The minor alleles for rs1125557, rs7199991 and rs7186053 were associated with accelerated FEV1 decline. A significant interaction of ICS with CDH1 SNPs on FEV1 decline was present only for rs7199991 and rs3785078. There were no associations with lung function decline in the absence of ICS use (table 3; table E5 in the online supplementary material).

In population 2, SNPs that were associated with less FEV1 decline were also associated with higher post-BD FEV1/VC with ICS use. Additionally, rs4783573, rs7188750 and rs3785078 were associated with higher post-BD FEV1/VC (table 3 and fig. 3). Interactions of these SNPs with ICSs on post-BD FEV1/ VC were significant. In population 1, these SNPs had the same direction of association with post-BD FEV1/VC, but only the interaction with rs7203904 was significant (tables E6.1 and E6.2 in the online supplementary material).

TABLE 3 Summary table of *CDH1* single-nucleotide polymorphisms (SNPs) significantly associated with airway remodelling and lung function decline in the presence of inhaled corticosteroids (ICSs), and their associations with post-bronchodilation (BD) forced expiratory volume in 1 s (FEV_1)/vital capacity (VC) and epithelial E-cadherin expression

CDH1 SNP	Genotype	ICS use					No ICS use
		BM thickness µm	CD31+ vessels per 0.1 mm^2 submucosal area	Post-BD FEV_1/VC %	Change in FEV_1 $mL \cdot yr^{-1}$	Epithelial E-cadherin expression#	Epithelial E-cadherin expression#
		B	B	B	B	median difference¶	median difference¶
rs1125557	AG	0.5	3.9	1.7	-19.7*	-2	-9
	GG	0.2	2.9	-4.2	-17.6*		
rs7199991	AC	-0.3	3.2	0.9	-13.5*	-4	-11
	CC				-5.1		
rs7186053	GA	0.2	2.0	-2.2	-16.7*	-3	-9
	AA			-4.6	-13.7		
rs7188750	GA/AA	-0.9	-6.9*	5.2*	5.6	2	-21
rs8056633	TG	-0.7	-4.7*	7.1*	8.6	-4	-17*
	GG				27.2*		
rs16958383	GA	-1.0*	-6.1*	5.5*	10.5	-3	-48*
	AA				36.6*		
rs2276330	TC/CC	-1.1*	-6.9*	4.5	6.6	-6	-33*
rs3785078	AC/CC	-1.1*	-6.6*	6.1*	10.5	-4	-46*
rs7203904	GC	-0.6	-4.7*	5.1*	1.7	-5	-18*
	CC			9.8*	27.4*		
rs17690554	CG	-1.0*	-2.8	7.5*	15.3*	-3	-14
	GG				26.9*		

BM: basement membrane; B: regression coefficient; median difference: median of heterozygotes and homozygous mutants pooled minus median of wild-types. #: % BM covered with E-cadherin-positive intact epithelium; ¶: significance tested with Mann–Whitney U-test. *: $p<0.05$.

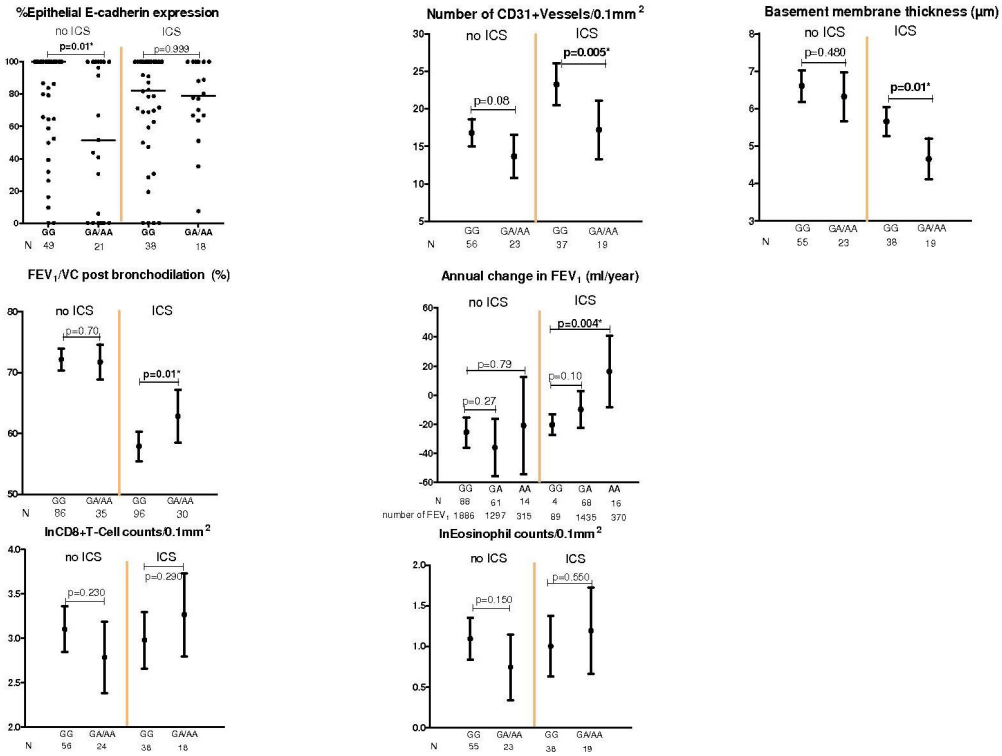


FIGURE 3. CDH1 rs16958383 associations with epithelial E-cadherin expression, airway remodelling, inflammation and lung function decline in adult asthma patients. CDH1 rs16958383 represents single-nucleotide polymorphisms associated with airway remodelling (rs7188750, rs8056633, rs16958383, rs2276330, rs3785878, rs7203904 and rs17690554). General linear models were used to estimate the means of each dependent variable adjusted for sex, age and smoking. a) Epithelial E-cadherin expression, expressed as % basement membrane (BM) covered with E-cadherin-positive intact epithelium. $: subjects; —: median. b) CD31+ vessels per 0.1 mm2 submucosal area. c) BM thickness. d) Post-bronchodilation (BD) forced expiratory volume in 1 s (FEV1)/vital capacity (VC) ratio. e) Annual change in FEV1. f) CD8+ T-cells per 0.1 mm2 submucosal area. g) Eosinophils per 0.1 mm2 submucosal area. $: mean; whiskers: 95% CI. ICS: inhaled corticosteroid. Bold indicates significance.

DISCUSSION

Our results indicate that E-cadherin (CDH1) gene polymorphisms are associated with airway remodelling, inflammation and lung function decline in individuals with asthma. In summary, with ICS use, seven out of the 17 SNPs were associated with airway remodelling, three with CD8+ T-cells counts and two with eosinophil counts in the submucosa, and seven with

FEV1 decline. Consistently, alleles associated with less airway remodelling correlated with less FEV1 decline and higher post-BD FEV1/VC. While all associations occurred during ICS use, associations with epithelial E-cadherin expression were significant for five SNPs in the absence of ICSs. These results may indicate that ICSs may influence the way these polymorphisms express their effect and are consistent with in vitro results showing that administration of glucocorticosteroids can upregulate cadherin expression, improve cell–cell contact and strengthen the epithelial barrier [19, 21, 22].

Altered expression of epithelial junction proteins on bronchial epithelium may contribute to a defective epithelial barrier [1, 23] with subsequent functional and clinical manifestations. In our longitudinal study (population 2), the minor alleles of three SNPs (rs1125557, rs7199991 and rs7186053) were associated with accelerated FEV1 decline during ICS use. rs7199991 is in complete LD (r251 according to www.hapmap.org, version 4.0) with a promoter-region SNP (rs16260), suggesting that this may have functional significance. To date, E-cadherin gene polymorphisms have not been studied in asthma, but recent findings have shown associations of CDH1 SNPs with Crohn's disease [24], another impaired epithelial barrier disorder, which may share pathways with asthma [25, 26]. Moreover, in genome-wide association studies, CDH1 has recently been associated with susceptibility to ulcerative colitis [27] and colorectal cancer [28]. The new associations suggest that changes in the integrity of the intestinal epithelial barrier may contribute to the pathogenesis of these diseases; hence, CDH1 polymorphisms are important for understanding the pathogenesis of impaired epithelial barrier diseases.

Additionally, our study showed that the minor alleles of four other SNPs (rs8056633, rs16958383, rs7203904 and rs17690554) were associated with less FEV1 decline. The latter four alleles were also associated with less airway remodelling in biopsies of asthmatics using ICSs in population 1. The fact that we observed a significant decrease in BM thickness in the presence of ICSs is consistent with the findings of BROEKEMA et al. [15], who previously showed that symptomatic asthmatics using ICS in our population 1 had significantly thinner BMs than asymptomatic asthmatics who did not use ICSs (5.7 and 6.5 mm, respectively; p,0.05). However, our results highlight that this decrease in BM thickness is probably not present in all asthmatics using ICSs, but only in those with a specific CDH1 genotype.

RASMUSSEN et al. [17] used post-BD FEV1/VC as an indirect measure of airway remodelling, and our results point in the same direction, i.e. alleles associated with less airway remodelling (rs7188750, rs8056633, rs16958383,

rs3785078, rs7203904 and rs17690554) were also associated with higher post-BD FEV1/VC and less FEV1 decline in population 2. In population 1, only rs7203904 showed a significant association with FEV1/ VC, which is probably due to lower power: lower number of ICS users (57 (41%) versus 146 (52%) subjects) and less post-BD FEV1/VC variance (SD 6.5 versus 13.5) compared with population 2. In contrast, KOSCIUCH et al. [29] concluded that there is no relationship between BM thickness and lung function tests, such as FEV1, FEV1/VC, residual volume % predicted and total lung capacity % predicted, but they included only asthmatic patients who never used ICS or who were withdrawn from ICSs for 03 months. Our study suggests that discrepant findings may, at least partially, be due to a modulating effect of both ICSs and CDH1 SNPs on airway remodelling and lung function. As previous studies have shown, treatment with ICSs may reduce or control the intensity of airway remodelling, improve FEV1 and reduce loss of lung function [30–32]. It has yet to be elucidated how ICSs prevent these adverse effects. Based on our findings and on evidence that corticosteroids upregulate cadherin expression [18, 33], we speculate that ICS interaction with CDH1 SNPs might be one possible explanation.

The fact that we do not observe significant differences in epithelial E-cadherin expression between the mutant and wild-type alleles during ICS use could be attributed to the fact that the mutant alleles interact with ICSs and promote E-cadherin upregulation to a similar level as the wild-types. However, in the absence of ICS the mutant alleles are not capable of producing the same amount of protein compared to wild types. The nearly significant, inverse association we found between damaged/denuded epithelium and epithelial E-cadherin expression indicates that E-cadherin is involved in epithelial integrity, and it is probable that epithelial damage is increased in subjects with lower E-cadherin expression.

In our study, we also observed that specific CDH1 polymorphisms, in the presence of ICSs, tended to be associated with recruitment of CD8+ T-cells in the submucosa. Previously, SONT et al. [34] found that despite ICS treatment, CD8+ T-cell infiltration might persist in asthma patients and that CD8+, but not CD4+, cells are associated with BHR [34] and lung function decline [35], and recently, HIROSAKO et al. [8] showed that CD8 and CD103 (ligand for E-cadherin) are highly expressed in asthmatic bronchial intraepithelial lymphocytes. In that study, the percentage of CD8+ cells was higher than the percentage of CD4+ cells in intraepithelial lymphocytes in asthma. The expression of CD103 was significantly higher in CD8+ cells compared with CD4+ cells, suggesting that the interaction between E-cadherin (CD103) and

CD8+ cells is the reason for the higher percentage of CD8+ cells in asthmatic intraepithelial lymphocytes [8]. Furthermore, CEPEK et al. [36] demonstrated that T-cells express a member of the cadherin superfamily and this may contribute to T-cell-mediated immune surveillance via heterotypic adhesions with mucosal epithelial cells. Combining these findings, we could speculate that there might be a link between specific CDH1 polymorphisms and expression or binding of E-cadherin on T-cells, a finding that needs further investigation.

One could suggest that the lack of multiple testing correction is responsible for the current results. We decided not to apply a sequential (classical) Bonferroni correction for a number of reasons. First, our choice for the current study was explicitly driven by previous observations suggesting E-cadherin expression is related to epithelial integrity, airway remodelling and disease progression. Secondly, the independent variables in our analyses (e.g. airway remodelling and FEV1 decline) are mutually related, indicating that a rigid statistical procedure, such as Bonferroni correction for multiple testing, would not do justice to their biologically linked nature. Finally, although adjustment for multiple testing will decrease the chance of a type I error, it will also increase the chance of a type II error, so that a true association is not found. This is especially possible in a relatively small study such as ours. Thus, we followed the advice given by PERNEGER [37]: "Simply describing what was done and why, and discussing the possible interpretations of each result, should enable the reader to reach a reasonable conclusion without the help of Bonferroni adjustments".

In our study, the associations between the SNPs and the biopsy parameters in population 1 were confirmed in population 2, where the same risk alleles were associated with FEV1 decline. This is called a loose replication [38]. The observed interaction of CDH1 SNPs with ICS use makes it difficult to find another study that is suitable for replication of our results. First, no other study on adult asthma patients exists with longitudinal data on lung function both with and without ICS use during such a long follow-up period. Secondly, bronchial biopsy studies in asthmatics usually investigate fewer patients than our study, which decreases the power to find associations.

In conclusion, our data show that CDH1 SNPs are associated with epithelial E-cadherin expression and suggest that epithelial adhesion is an important contributor to airway remodelling and lung function in asthma. These effects are modified by the use of ICSs.

ACKNOWLEDGEMENTS

We thank F. Volbeda and A. Dijkstra (Dept of Pulmonology, University Medical Center Groningen (UMCG), Groningen, the Netherlands) for collecting the biopsies, M. Broekema and M. Lodewijk (Dept of Pathology and Medical Biology, UMCG) for processing and staining of the biopsies, and M. Olivier (Dept of Genetics, UMCG) for her help with genotyping.

FINANCIAL SUPPORT

This study was supported, in part, by a grant from the Netherlands Asthma Foundation (AF 3.2.07.15).

REFERENCES

1 Montefort S, Djukanovic´ R, Holgate ST, et al. Ciliated cell damage in the bronchial epithelium of asthmatics and non-asthmatics. Clin Exp Allergy 1993; 23: 185–189.

2 Jeffery PK, Wardlaw AJ, Nelson FC, et al. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989; 140: 1745–1753.

3 Chanez P. Severe asthma is an epithelial disease. Eur Respir J 2005;25: 945–946.

4 Ricciardolo FL, Di Stefano A, van Krieken JH, et al. Proliferation and inflammation in bronchial epithelium after allergen in atopic asthmatics. Clin Exp Allergy 2003; 33: 905–911.

5 Cavey M, Lecuit T. Molecular bases of cell–cell junctions stability and dynamics. Cold Spring Harb Perspect Biol 2009; 1: a002998.

6 Pokutta S, Weis WI. Structure and mechanism of cadherins and catenins in cell–cell contacts. Annu Rev Cell Dev Biol 2007; 23: 237–261.

7 Aberle H, Schwartz H, Kemler R. Cadherin–catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 1996; 61: 514–523.

8 Hirosako S, Goto E, Tsumori K, et al. CD8 and CD103 are highly expressed in asthmatic bronchial intraepithelial lymphocytes. Int Arch Allergy Immunol 2010; 153: 157–165.

9 Vonk JM, Jongepier H, Panhuysen CIM, et al. Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up. Thorax 2003; 58: 322–327.

10 Dijkstra A, Vonk JM, Jongepier H, et al. Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex. Thorax 2006; 61: 105–110.

11 Ward C, Pais M, Bish R, et al. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax 2002; 57: 309–316.

12 Benayoun L, Letuve S, Druilhe A, et al. Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: relationship with proliferation, apoptosis, and airway remodeling. Am J Respir Crit Care Med 2001; 164: 1487–1494.

13 Foty RA, Corbett SA, Schwarzbauer JE, et al. Dexamethasone up- regulates cadherin expression and cohesion of HT-1080 human fibrosarcoma cells. Cancer Res 1998; 58: 3586–3589.

14 Broekema M, ten Hacken NHT, Volbeda F, et al. Airway epithelial changes in smokers but not in ex-smokers with asthma. Am J Respir Crit Care Med 2009; 180: 1170–1178.

15 Broekema M, Timens W, Vonk JM, et al. Persisting remodeling and less airway wall eosinophil activation in complete remission of asthma. Am J Respir Crit Care Med 2011; 183: 310–316.

16 Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am Rev Respir Dis 1987; 136: 225–244.

17 Rasmussen F, Taylor DR, Flannery EM, et al. Risk factors for airway remodeling in asthma manifested by a low postbronchodilator FEV1/vital capacity ratio: a longitudinal population study from child- hood to adulthood. Am J Respir Crit Care Med 2002; 165: 1480–1488.

18 Carayol N, Campbell A, Vachier I, et al. Modulation of cadherin and catenins expression by tumour necrosis factor-a and dexa- methasone in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2002; 26: 341–347.

19 Carayol N, Vachier I, Campbell A, et al. Regulation of E-cadherin expression by dexamethasone and tumour necrosis factor-a in nasal epithelium. Eur Respir J 2002; 20: 1430–1436.

20 Rijcken B, Weiss ST. Longitudinal analyses of airway responsive- ness and pulmonary function decline. Am J Respir Crit Care Med 1996; 154: S246–S249.

21 Foty RA, Corbett SA, Schwarzbauer JE, et al. Dexamethasone up- regulates cadherin expression and cohesion of HT-1080 human fibrosarcoma cells. Cancer Res 1998; 58: 3586–3589.

22 Boivin MA, Ye D, Kennedy JC, et al. Mechanism of glucocorticoid regulation of the intestinal tight junction barrier. Am J Physiol Gastrointest Liver Physiol 2007; 292: G590–G598.

23 De Boer WI, Sharma HS, Baelemans SMI, et al. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. Can J Physiol Pharmacol 2008; 86: 105–112.

24 Muise AM, Walters TD, Glowacka WK, et al. Polymorphisms in E-cadherin (CDH1) result in a mis-localised cytoplasmic protein that is associated with Crohn's disease. Gut 2009; 58: 1121–1127.

25 Bernstein CN, Wajda A, Blanchard JF. The clustering of other chronic inflammatory diseases in inflammatory bowel disease: a population-based study. Gastroenterology 2005; 129: 827–836.

26 Schreiber S, Rosenstiel P, Albrecht M, et al. Genetics of Crohn disease, an archetypal inflammatory barrier disease. Nat Rev Genet 2005; 6: 376–388.

27 UK IBD Genetics Consortium, Barrett JC, Lee JC, et al. Genome- wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. Nat Genet 2009;41: 1330–1334.

28 Houlston RS, Webb E, Broderick P, et al. Meta-analysis of genome- wide association data identifies four new susceptibility loci for colorectal cancer. Nat Genet 2008; 40: 1426–1435.

29 Kosciuch J, Przybylowski T, Gorska K, et al. Relationship between airway basement membrane thickness and lung function tests in patients with asthma. Pneumonol Alergol Pol 2009; 77: 256–263.

30 Olivieri D, Chetta A, Del Donno M, et al. Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-con- trolled study. Am J Respir Crit Care Med 1997; 155: 1864–1871.

31 Chetta A, Zanini A, Foresi A, et al. Vascular component of airway remodeling in asthma is reduced by high dose of fluticasone. Am J Respir Crit Care Med 2003; 167: 751–757.

32 O9Byrne PM, Pedersen S, Busse WW, et al. Effects of early intervention with inhaled budesonide on lung function in newly diagnosed asthma. Chest 2006; 129: 1478–1485.

33 Blecharz KG, Drenckhahn D, Forster CY. Glucocorticoids increase VE-cadherin expression and cause cytoskeletal rearrangements in murine brain endothelial cEND cells. J Cereb Blood Flow Metab 2008; 28: 1139–1149.

34 Sont JK, Han J, van Krieken JM, et al. Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids. Thorax 1996; 51: 496–502.

35 van Rensen ELJ, Sont JK, Evertse CE, et al. Bronchial CD8 cell infiltrate and lung function decline in asthma. Am J Respir Crit Care Med 2005; 172: 837–841.

36 Cepek KL, Rimm DL, Brenner MB. Expression of a candidate cadherin in T lymphocytes. Proc Natl Acad Sci USA 1996; 93: 6567–6571.

37 Perneger TV. What's wrong with Bonferroni adjustments. BMJ 1998; 316: 1236–1238.

38 Holloway JW, Koppelman GH. Identifying novel genes contribut- ing to asthma pathogenesis. Curr Opin Allergy Clin Immunol 2007; 7: 69–74.

CHAPTER 3

Inhaled corticosteroids modify clinicopathological effects of Tumor Necrosis Factor gene in asthma

Despo Ierodiakonou MD[1,5], Dirkje S. Postma MD, PhD[2,5], Gerard H. Koppelman MD, PhD[3,5], Jorrit Gerritsen MD, PhD[3,5], Nick H.T. ten Hacken MD, PhD[2,5], Wim Timens MD, PhD[4,5], H. Marike Boezen PhD[1,5], Judith M. Vonk PhD[1,5]

Submitted

1. Department of Epidemiology, University of Groningen, University Medical Center Groningen

2. Department of Pulmonology, University of Groningen, University Medical Center Groningen

3. Department of Pediatric Pulmonology and Pediatric Allergology-Beatrix Children's Hospital, University of Groningen, University Medical Center Groningen

4. Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen

5. Groningen Research Institute for Asthma and COPD, Groningen, The Netherlands

ABSTRACT

Background: Tumor Necrosis Factor-alpha (TNFα) is implicated in epithelial barrier disruption, airway wall inflammation and remodelling. Inhaled corticosteroids (ICS) suppress TNFα expression and improve epithelial integrity, remodelling and lung function in asthma. We hypothesize that ICS-use modifies *TNF* gene effects in asthma.

Methods: We investigated interactions between *TNF* single nucleotide polymorphisms (SNPs) and ICS-use with denuded epithelium, airway inflammation and airway remodelling in 137 asthmatics. Associations with airway hyperresponsiveness (AHR) and lung function were analyzed in two additional asthma populations (n=244 and 302).

Results: ICS-use modified effects of rs1800630-A, rs1799964-C and rs2229094-C. Without ICS-use, these alleles were associated with increased denuded epithelium (p=0.001-0.03), whereas effects were reversed with ICS-use ($P_{interaction}$=0.02). With ICS-use, the alleles were associated with higher FEV_1%predicted (p=0.006-0.04) and sputum eosinophils (p=0.007-0.03). Only rs909253-C was associated with lower FEV_1 with ICS-use. There were (borderline) significant ICS-interactions with rs1800630-A (increased submucosal eosinophils: p=0.04, macrophages: p=0.03, neutrophils: p=0.04), with rs1799964-C (increased macrophages: p=0.07) and with rs2229094-C (increased macrophages: p=0.06, neutrophils: p=0.05). There were no significant associations with airway remodelling and AHR.

Conclusions: In subjects with specific *TNF* genotypes, improved epithelial integrity with ICS-use is concordant with improved lung function. ICS-use did not modify *TNF* effects on airway remodelling but increased pro-inflammatory effects, a finding requiring further attention.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by airway hyperresponsiveness (AHR) and airway wall changes. The severity of the disease varies, some subjects developing irreversible airflow obstruction and progressive loss of lung function,[1] whereas others reach asthma remission.[2,3] This heterogeneity may result from the interaction of multiple genetic and environmental factors.[4]

Tumor Necrosis Factor-alpha (TNFα) is a pro-inflammatory cytokine highly expressed by monocytes in bronchoalveolar lavage (BAL) fluid and mast cells in airway wall biopsies of asthmatics.[5,6] The biological role of TNFα and findings in animal studies and clinical (anti-TNFα) trials have been well summarized[7,8]**:** TNFα induces AHR, up-regulates adhesion molecules (ICAM-1 and VCAM-1) facilitating the migration of inflammatory cells into the airway wall and activates pro-fibrotic and remodeling mechanisms in the submucosa. In addition, TNFα disrupts tight junctions in the epithelial barrier, increases epithelial permeability[9] and decreases the expression of adhesion molecules responsible for epithelial integrity i.e. E-cadherin and Catenins.[10-12] Given the role of TNFα in asthma pathophysiology, the *TNF* gene (i.e. *TNF alpha and beta*) is a widely investigated candidate gene in asthma. *TNF* single nucleotide polymorphisms (SNPs) are associated with TNF levels (rs1800630, rs909253), risk for AHR (rs1800629) and asthma (rs1800629, rs1800630, rs909253).[13-18]

Inhaled corticosteroids (ICS) constitute the cornerstone of asthma treatment. Of interest, glucocorticosteroids suppress TNFα expression in several tissues including the lung[19] and in alveolar macrophages of BAL from asthmatics.[20] Additionally, they inhibit TNFα-induced epithelial adhesion disruption.[10,11] However, a subpopulation of asthma patients does not respond well to ICS, whereas TNFα has been implicated in the pathophysiology of severe steroid-resistant asthma.[21] This poses questions about the role of *TNF* gene variation in response to ICS.

We hypothesize that the positive effects of ICS on asthma severity and airway pathology[22] might be mediated via interaction with the *TNF* gene. Our aim was to investigate the interactions between ICS and *TNF* SNPs on epithelial integrity, airway inflammation and features of airway remodelling. In addition, we analyzed associations with asthma severity phenotypes i.e. lung function and AHR in two asthma populations.

MATERIALS AND METHODS

Details on study populations and methods used in the present report are provided in an online data supplement.

Subjects

All participants had doctor's diagnosed asthma and originated from the northern region of The Netherlands. In summary: Population 1: 138 asthma patients performed a bronchoscopy and induced sputum in addition to AMP challenge and lung function testing. Populations 2 and 3: 281 and 302 asthma patients, respectively underwent lung function and histamine challenge testing. Ethical approval was obtained from the Medical Ethics Committee of the University of Groningen, and written informed consent was obtained from all participants of the 3 populations.

Statistics

We tested with multiple linear regression for associations of *TNF* SNPs with a. epithelial integrity (length of basement membrane (BM) covered with denuded epithelium in μm), b. inflammatory cell counts (eosinophils, macrophages, neutrophils and mast cells per 0.1mm^2 of submucosa) and c. airway remodeling (BM thickness, submucosal vessel numbers and airway smooth muscle and goblet cell counts) in no-ICS and ICS users group and we assessed ICS by SNPs interactions in population 1. Furthermore we assessed associations of SNPs with epithelial E-cadherin expression in bronchial biopsies and with inflammatory cell counts in induced sputum from no-ICS and ICS users separately (Mann Whitney U-test). In populations 2 and 3 we tested associations with lung function and AHR slope and meta-analyzed the population-specific results.

All regression modeling was conducted with IBM SPSS statistics (version 20; Armonk, NY USA: IBM Corp 2011). Because the number of subjects with the homozygote mutant genotype was relatively low in the 2 subgroups (n≈<10) (see table E1 online repository), only dominant (heterozygotes and homozygotes mutant pooled vs wild types) models were used. Meta-analysis was performed with R-programming language using the meta-package (R version 2.14.1 (2012-03-29)). Two tailed p-values of <0.05 were considered statistically significant and p-values between 0.05 and 0.09 of borderline significance.

RESULTS

The clinical characteristics of the three populations are shown in Table 1. In all 3 populations a substantial proportion of patients used ICS (41%, 51% and 80%, respectively). Table 2 presents the genotyped SNPs. The genotype distributions are shown in Table E1a-d in the Online Repository. All SNPs were in Hardy-Weinberg equilibrium (p>0.05). Figure E1 shows that the linkage disequilibrium (LD) correlations (r^2) among tagging SNPs were ≤ 0.75.

TABLE 1 **Characteristics of the three populations studied**

	Population 1	Population 2	Population 3
n	137	244	302
Males n (%)	70 (51.1)	147 (60.2)	106 (35.1)
Age, years, mean (SD)	47 (12.6)	51 (8.8)	33.9 (8.8)
Lean/Overweight/Obese, n (%)	50/59/29 (36/43/21)	97/126/21 (40/52/8)	166/93/51 (54/30/16)
Never/ex/current smokers, n (%)	61/47/29 (44/34/21)	99/74/71 (41/30/29)	169/91/41 (56/30/14)
Inhaled corticosteroid use, n (%)	57 (41.3)	125 (51.4)	242 (80.1)
Severe asthma, n (%)	17 (12.4)	100 (41.2)	71 (23.7)
Airway hyper-responsiveness, n (%)	72 (52.6) (PC_{20}AMP<320mg/ml)	165 (77.1) (PC_{20}Hist<16mg/ml)	234 (80.4) (PC_{20}Hist<16mg/ml)
FEV_1% predicted pre-BD, mean (SD)	91.7 (18.0)	70.8 (23.6)	86.3 (16.2)
FEV_1/ VC% pre-BD, mean (SD)	70.7 (11.5)	60.5 (14.2)	73.0 (11.3)

n = frequency; SD: standard deviation; FEV_1: forced expiratory volume in 1 second; VC: vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; Severe asthma= pre-BD FEV_1%predicted <80 and inhaled corticosteroid use; Lean: BMI < 25, overweight: 25 ≤ BMI <30, Obese: BMI ≥ 30

TABLE 2 **Minor allele frequencies (MAF) in the populations 1, 2 and 3**

Designation in previous studies	*TNF* tag SNP	Location Chr6	Major: Minor	MAF 1	MAF 2	MAF 3*
G-308A	rs1800629	TNF-α promoter	G:A	0.186	0.238	0.225
C-863A	rs1800630	TNF-α promoter	C:A	0.173	0.186	0.139
T-1031C	rs1799964	TNF-α promoter	T:C	0.204	0.233	0.194
T-495C (Cys13Arg)	rs2229094	LTA Exon-2	T:C	0.283	0.280	0.263
A+252G (Nco I)	rs909253	LTA Inton-1	T:C	0.369	0.393	0.377
none	rs915654**	LTA promoter	A:T	0.348	0.340	0.300

SNPs: single nucleotide polymorphisms
*Genome Wide Association Study on asthma; **imputed SNPs in GWAS
(*TNF* genomic region +/- 5kb; MAF >0.1; HapMap.org)

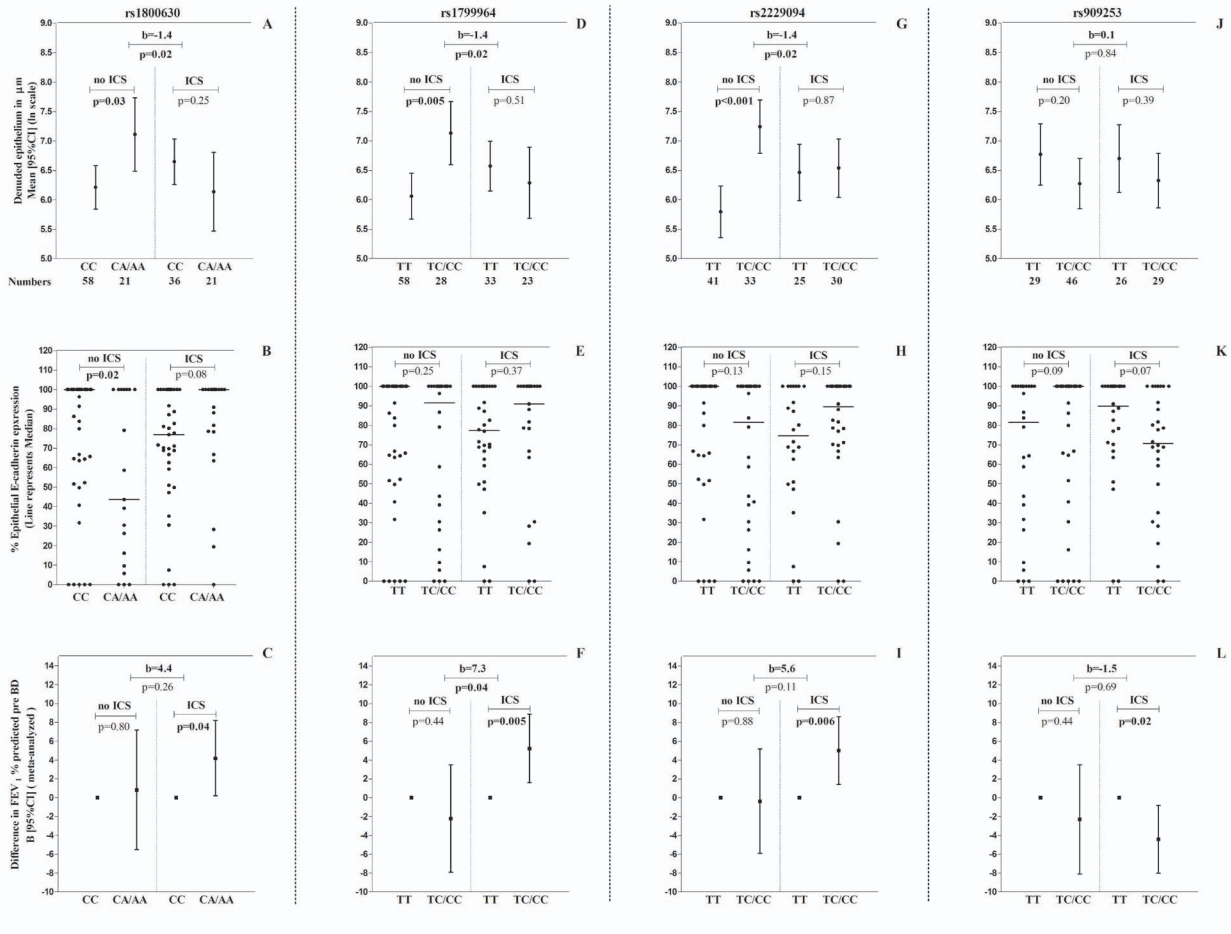


Figure 1: Associations of rs1800630 (A-C), rs1799964 (D-F), rs2229094 (G-I) and rs909253 (J-L) with epithelial integrity and FEV_1% predicted and their interactions with inhaled corticosteroids (ICS). With ICS use the minor alleles of rs1800630, rs1799964 and rs2229094 are associated with improved epithelial integrity and FEV_1, whereas the C-allele of rs909253 is associated with lower FEV_1 and a tendency for impaired epithelial integrity. X-axis indicates the genotype groups for the *TNF* SNPs (homozygotes for major allele versus heterozygotes and homozygotes for minor allele pooled)

Epithelial Integrity

There were no significant associations between ICS use and epithelial integrity defined by length of BM covered with denuded epithelium and epithelial E-cadherin expression (p=0.23 and p=0.50 respectively). Figure 1 shows the interactions between ICS use and *TNF* SNPs on epithelial integrity. The effects of rs1800630-A, rs1799964-C and rs2229094-C were modified by ICS, i.e. in the absence of ICS these alleles were significantly associated with increased (ln) denuded epithelium, whereas with ICS use these effects were reversed, resulting in a significant difference between ICS and no-ICS group (interactions p=0.02). In no-ICS users, rs1800630-A, rs1799964-C and rs2229094-C were significantly associated with increased denuded epithelium (b=0.9, b=1.1 and b=1.4 respectively) but only rs1800630-C was significantly associated with lower (median) E-cadherin expression (p=0.017). In this group rs1800629-A had a significantly higher E-cadherin expression while rs909253-C had a borderline significant association with lower epithelial E-cadherin expression (p=0.02 and p=0.07, respectively).Tables E2 and E3 show results in details.

Airway inflammation in bronchial biopsies

We tested for associations of *TNF* SNPs with submucosal (ln) numbers of eosinophils, macrophages and neutrophils in no-ICS and ICS groups and we assessed ICS by SNPs interactions (Table E4). Rs1800629-A, rs1800630-A, rs909253-C and rs915654-T were significantly associated with increased numbers of eosinophils in ICS users only (b=0.6 (p=0.045), b=0.6 (p=0.05), b=0.7 (p=0.021), b=0.9 (p=0.002), respectively).

Interactions between ICS and rs1800629-A, rs1800630-A, rs909253-C and rs915654-T were also significantly associated with increased eosinophil counts (b=0.8 (p=0.031), b=0.8 (p=0.038), b=1.2 (p=0.002), b=1.3 (p<0.001) respectively). ICS (tended to) modified the associations of rs1800630-A, rs1799964-C and rs2229094-C with the 3 cell types in the submucosa (Figure 2): Interaction between ICS and rs1800630-A was significantly associated with increased macrophages (b=0.6 (p=0.028)) in addition to increased eosinophils. Interactions between ICS and rs1799964-C and rs2229094-C had a similar direction of associations with macrophage numbers but were of borderline significance (b=0.4 (p=0.07) and b=0.5 (p=0.055) respectively). Interactions between ICS and rs1800630-A and rs2229094-C were also associated with increased numbers of neutrophils (b=0.7 (p=0.036) and b=0.6 (p=0.051)). In no-ICS users, the 3 SNPs were significantly associated with decreased

numbers of macrophages (b=-0.4 (p=0.023), b=-0.4 (p=0.025), b=-0.3 (p=0.041), respectively). There were no significant associations with numbers of mast cells (data not shown).

Rs1800630-A, rs1799964-C and rs2229094-C were associated with increased eosinophil counts in induced sputum of ICS users. In sputum of no-ICS users, rs1800629-A and rs2229094-C were significantly associated with increased numbers of macrophages. There were no significant associations with numbers of neutrophils in induced sputum (Table 3).

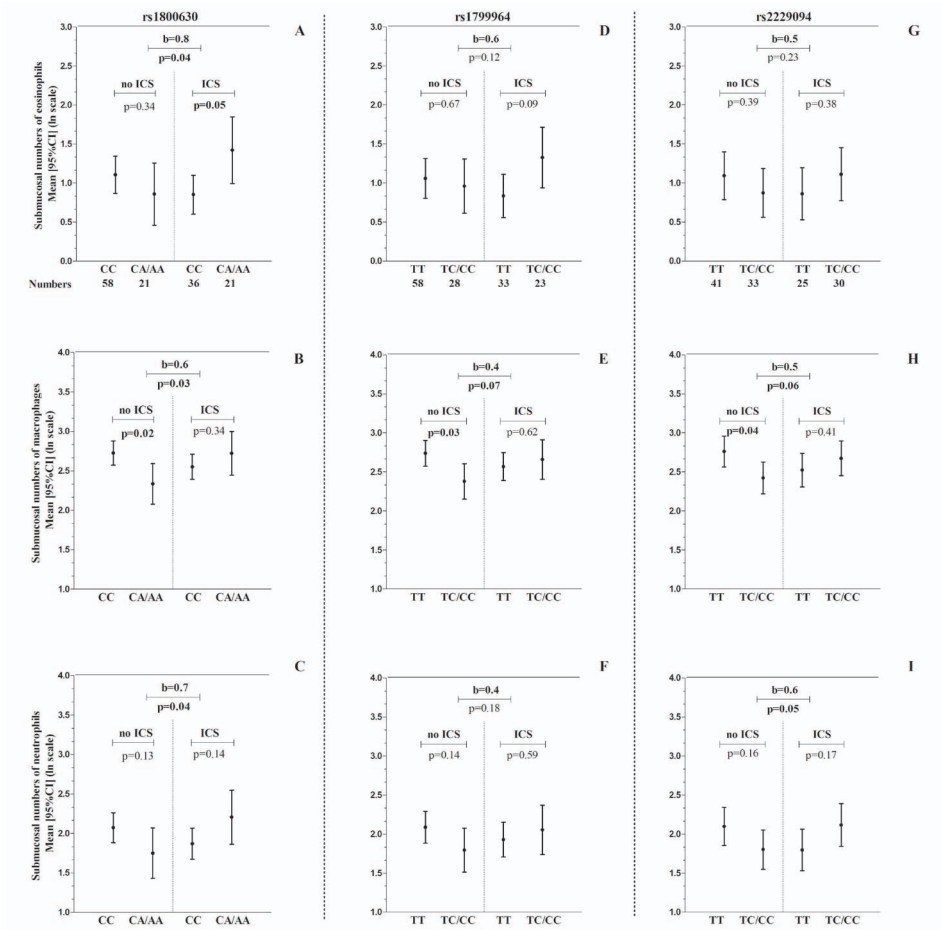


Figure 2: Associations of rs1800630 (A-C), rs1799964 (D-F) and rs2229094 (G-I) with number of submucosal eosinophils, macrophages and neutrophils and their interactions with inhaled corticosteroids (ICS). ICS modify the associations between the minor alleles and numbers of inflammatory cells in the submucosal area, i.e. infiltration of inflammatory cells is more likely in subjects with the specific genotypes who use ICS. X-axis indicates the genotype groups for the *TNF* SNPs (homozygotes for major allele versus heterozygotes and homozygotes for minor allele pooled)

TABLE 3 Associations of *TNF* SNPs with inflammatory cell counts in induced sputum from population 1

Population 1	Inflammatory cells x 10^6 / ml sputum											
TNF SNPs	number of eosinophils				number of macrophages				number of neutrophils			
	no ICS use		ICS use		no ICS use		ICS use		no ICS use		ICS use	
	MD	P-value	MD	P-value	MD	P-value	MD	P-value	MD	P-value	MD	P-value
rs1800629-A	-0.001	0.291	-0.002	0.950	0.004	**0.020**	-0.089	0.616	0.018	0.396	-0.034	0.783
rs1800630-A	0.004	0.186	0.007	**0.007**	0.036	0.662	-0.032	0.473	-0.014	0.973	0.012	0.379
rs1799964-C	0.004	0.746	0.008	**0.002**	0.055	0.084	-0.009	0.358	0.026	0.315	0.013	0.334
rs2229094-C	0.000	0.676	0.003	**0.033**	0.062	**0.037**	0.046	0.142	0.026	0.418	0.021	0.420
rs909253-C	-0.006	0.553	-0.001	0.814	0.004	0.269	-0.082	0.205	-0.041	0.104	-0.090	0.814
rs915654-T	0.000	0.554	0.000	0.063	-0.022	0.412	-0.051	0.840	-0.025	0.953	-0.007	0.579

MD: Median Difference; P- values are given with a Mann Whitney U test; SNPs: single nucleotide polymorphisms;

Airway remodelling

There were no significant interactions between ICS and *TNF* SNPs on investigated airway remodelling parameters. In ICS users, rs909253-C was associated with decreased vessel numbers in the submucosa (b=-4.3; p=0.048; Table E5). In no-ICS users, rs1800630-A and rs915654-T were significantly associated with decreased number of vessels (b=-5.1 (p=0.011) and b=-4.4 (p=0.018) respectively). There were no significant associations between *TNF* SNPs or their interactions with ICS and BM thickness (Table E6), goblet cell numbers or ASM area (data not shown).

Lung function and airway hyperresponsiveness

Table 4 shows the results of the meta-analysis of lung function level of populations 2 and 3. In ICS users, rs1800630-A, rs1799964-C and rs2229094-C were associated with increased and rs909253-T with decreased pre- and post-bronchodilator FEV_1% predicted (Figure 1). Rs1799964-C and rs2229094-C were associated with pre- and post-BD FEV_1/FVC ratio as well. Among the 4 SNPs, only the interaction between ICS and rs1799964-C was significant for pre-BD FEV_1%predicted and borderline significant for post-BD FEV_1%predicted and FEV_1/VC. In addition, the interaction with rs1800629-A had borderline significant associations with increased pre- and post-BD FEV_1/VC.

There were no significant associations with (ln) AHR slope (Table E7). Separate results for the 2 populations are shown in Tables E8-E13 in the online repository.

TABLE 4 **Associations of *TNF* SNPs with lung function: Meta-analysis of populations 2 and 3**

FEV_1% predicted pre BD

TNF SNPs	no ICS use				ICS use				SNP x ICS			
	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value
rs1800629-A	0	-3.55	-9.0 2.0	0.200	77.7*	1.27	-7.4 9.9	0.774	0	5.44	-1.4 12.3	0.121
rs1800630-A	0	0.81	-5.5 7.2	0.802	49	**4.17**	**0.2 8.2**	**0.041**	38	4.44	-3.4 12.3	0.269
rs1799964-C	4	-2.21	-7.9 3.5	0.444	0	**5.21**	**1.6 8.9**	**0.005**	31	**7.33**	**0.3 14.3**	**0.040**
rs2229094-C	0	-0.44	-5.9 5.2	0.877	0	**5.04**	**1.4 8.6**	**0.006**	43	5.61	-1.3 12.5	0.111
rs909253-C	0	-2.26	-8.1 3.5	0.444	0	**-4.39**	**-8.0 -0.8**	**0.018**	0	-1.47	-8.7 5.7	0.688
rs915654-T	72*	3.28	-7.0 13.6	0.532	0	-1.17	-4.7 2.4	0.520	76*	-3.88	-18.0 10.2	0.207

FEV_1% predicted post BD

TNF SNPs	no ICS use				ICS use				SNP x ICS			
	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value
rs1800629-A	0	-4.04	-9.0 0.9	0.112	72.7*	1.01	-6.3 8.4	0.788	0	4.95	-1.3 11.2	0.119
rs1800630-A	0	0.63	-5.2 6.4	0.832	23	**3.11**	**-0.4 6.6**	**0.084**	0	3.63	-3.6 10.8	0.322
rs1799964-C	0	-1.58	-6.8 3.6	0.551	0	**3.99**	**0.8 7.2**	**0.015**	0	**5.73**	**-0.6 12.1**	**0.077**
rs2229094-C	0	-0.53	-5.6 4.6	0.838	0	**3.88**	**0.7 7.0**	**0.015**	0	4.73	-1.5 10.9	0.135
rs909253-C	26	-2.75	-8.1 2.6	0.310	0	**-3.34**	**-6.6 -0.2**	**0.042**	54	-0.62	-7.1 5.9	0.852
rs915654-T	58	3.26	-1.8 8.3	0.203	0	-1.23	-4.4 1.9	0.443	64	-4.81	-14.5 6.6	0.125

FEV_1/VC% pre BD

TNF SNPs	no ICS use				ICS use				SNP x ICS			
	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value
rs1800629-A	0	-1.32	-4.6 2.0	0.432	91*	2.38	-5.9 10.7	0.575	39	**4.20**	**-0.0 8.4**	**0.050**
rs1800630-A	0	-0.03	-3.9 3.8	0.988	0	**2.24**	**-0.3 4.8**	**0.081**	0	2.06	-2.8 6.9	0.404
rs1799964-C	32	-1.43	-4.9 2.1	0.420	0	**2.75**	**0.4 5.1**	**0.021**	0	3.57	-0.8 7.98	0.107
rs2229094-C	3	0.31	-3.1 3.7	0.858	0	**2.49**	**0.2 4.8**	**0.033**	0	1.86	-2.4 6.1	0.395
rs909253-C	0	0.11	-3.4 3.7	0.950	73*	-0.39	-5.3 4.5	0.876	0	-0.07	-4.5 4.3	0.975
rs915654-T	21	0.75	-2.6 4.1	0.660	0	-1.52	-3.8 0.8	0.197	0	-3.22	-7.5 1.0	0.136

FEV_1/VC% post BD

TNF SNPs	no ICS use				ICS use				SNP x ICS			
	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value	I^2	B#	95% CI	p-value
rs1800629-A	0	-1.66	-4.6 1.3	0.264	90*	1.91	-5.1 9.0	0.595	58	**3.57**	**-0.1 7.2**	**0.057**
rs1800630-A	0	-0.76	-4.2 2.6	0.661	0	1.55	-0.5 3.6	0.142	0	1.95	-2.3 6.2	0.364
rs1799964-C	0	-2.05	-5.1 1.0	0.183	0	**2.08**	**0.2 4.0**	**0.033**	0	**3.61**	**-0.2 7.4**	**0.061**
rs2229094-C	0	-0.91	-3.9 2.1	0.548	0	**1.87**	**-0.04 3.8**	**0.055**	0	2.55	-1.2 6.3	0.179
rs909253-C	0	-0.29	-3.4 2.8	0.857	76*	-0.54	-5.1 4.0	0.818	20	-0.21	-4.0 3.6	0.917
rs915654-T	23	0.78	-2.1 3.7	0.602	42	-1.22	-3.1 0.7	0.211	0	-2.97	-6.7 0.7	0.115

SNPs: single nucleotide polymorphisms, ICS: inhaled corticosteroids, B: regression coefficient, CI: confidence interval; SNP x ICS: interaction; FEV_1: forced expiratory volume in 1 second, VC: vital capacity, BD: bronchodilation, I^2 : Index of heterogeneity (in percentages), * P-value of Cochran's Q-test (of heterogeneity) is <0.07
Fixed effects are used. When I^2 is high (>70%) and/or Q-test has a p<0.07 random effects are used

DISCUSSION

TNFα is a cytokine highly expressed in mast cells and present in BAL in asthma.[5,6] It plays a role in AHR and airway pathology i.e. epithelial barrier integrity, airway inflammation and remodelling.[7] ICS have been shown to improve asthma severity and airway pathology,[22] to interfere/compete with TNFα for nuclear localization[23] and to decrease mRNA TNFα expression in alveolar macrophages obtained by BAL from patients with mild to moderate asthma.[20] *TNF* loci are found to be associated with asthma, AHR development and TNFα plasma levels[13-18] but to our knowledge this study is the first investigating the interactions between ICS and *TNF* loci in asthma. We thus show for the first time that interactions between ICS and *TNF* SNPs are significantly associated with epithelial integrity, airway inflammation and lung function, indicating that the genetic variability in the *TNF* gene plays a role in ICS response. Interactions of ICS and rs1800630-A (*TNFα*-promoter), rs1799964-C (*TNFα*-promoter) and rs2229094-C (*TNFβ (LTA)* non-synonymous SNP in exon-2) were significantly associated with improved epithelial integrity defined by the length of BM covered with denuded epithelium, and carriers of these alleles tended to have higher epithelial E-cadherin expression with ICS use. These findings are in agreement with previous studies indicating that ICS inhibit the TNFα induced adverse effects on epithelial barrier.{{57 Carayol,Nathalie 2002; 25 Carayol,N. 2002; 530 Tenenbaum,T. 2008}} Based on our findings we speculate that ICS interact with the *TNF* gene, inhibiting potential TNF expression linked to these SNPs (as these are located on promoter region and exon-1), and ultimately protect against the adverse effect of TNF on the epithelial barrier.

In a previous publication, we suggested that ICS play a role in epithelial integrity, airway remodelling and lung function in asthma, depending on individual genetic variability.[24] Our new findings point in the same direction: the 3 independent (tagging) SNPs were associated with epithelial integrity and additionally with higher lung function in ICS users, but did not have an effect on airway remodelling. Similarly, Barake et al found that fluticasone propionate (FP) significantly decreases mRNA expression of TNFα in alveolar macrophages of asthmatics, in addition to improvements in lung function without effects on airway remodelling as measured by BM thickness.[20] Ward et al showed that the decrease in BM thickness during FP treatment occurred later than the improvement in lung function (12 and 3 months respectively), and that AHR was positively correlated with improvement in BM thickness.[22] Inconsistencies in interventional studies are also seen for the effects of ICS on airway vasculature,[25]

suggesting that the dose and duration of ICS therapy seem to be critical determinants. As the nature of this study is retrospective, we can only speculate that an interaction of ICS with SNPs in *TNF* might influence the expression of TNF and restore epithelial integrity quicker than the time needed to improve remodelling and AHR. A prospective, clinical trial would be more appropriate to further investigate this.

In the same subjects, i.e. carriers of rs1800630-A, rs1799964-C and rs2229094-C, the pro-inflammatory effects of *TNF* SNPs were enhanced with ICS use in the submucosa and in induced sputum, although epithelial integrity and lung function were significantly improved. It is has been shown that TNFα mediates infiltration of inflammatory cells via up-regulation of cellular adhesion molecules (ICAM-1, VCAM-1).[7,8] More recent findings indicate that *TNF* polymorphisms induce expression of adhesion molecules in vascular smooth muscle cells,[14] and that ICS enhance TNFα mediated leukocyte adhesion to airway endothelial cells, potentially facilitating inflammatory cell migration into lung tissue.[26] Although a pro-inflammatory role of ICS in asthma might seem contradicting, we should note that inflammation comprises a series of changes in the vascular bed and in connective tissue with the purpose of eliminating the exogenous irritants and repairing the damaged tissue. Nevertheless, our study suggests that similar to their protective effects on epithelial integrity, the pro-inflammatory effects of ICS are not seen in all subjects but only in asthmatics with specific *TNF* genotypes. Importantly, poor inhalation technique, non-compliance to and/or misclassification of ICS use might dilute the effects of *TNF* SNPs. It is possible that the effect modification by ICS could be dose-dependent, and we acknowledge that the absence of information on the dose of ICS used by asthma patients is a limitation of this study.

Rs909253-C (*LTA*-intron 1) previously associated with asthma,[17] was the only SNP in our study associated with lower epithelial E-cadherin expression and lower lung function in the presence of ICS, as well as with increased submucosal eosinophil counts. It is well established that up-regulation of TNFα is implicated in the pathophysiology of severe steroid-resistant asthma[21] and rs909253-C is known to be associated with increased TNFα expression and serum levels,[14,27] possibly inferring a link with severe refractory asthma. The MAF of this variant was the highest among the tagging SNPs included (approximately 40%), making this variant important for further investigation in severe asthma.

Due to lack of expression data we cannot conclude whether the clinical and pathological effects observed in our study are driven by high or low TNF expression levels and by which cellular sources. Functional studies on *TNF* SNPs have given

contradicting results,[13] and determination of TNF expression can be problematic since effects of *TNF* SNPs on asthma phenotypes are further complicated by the fact that this locus interacts with several environmental factors i.e. environmental tobacco smoke, air pollution, endotoxin, allergens, obesity, that might influence its expression but also alter ICS response in asthma.[28-32]

One could suggest that the lack of multiple testing corrections increases the risk for false positive results. Although generally large studies are needed in order to detect statistically significant gene by environment interactions, we were still able to find significant interactions in population 1 (n=137; ICS user n=57) suggesting that it is unlikely that these associations are spurious. Our decision not to correct for multiple comparisons was not only based on our hypothesis driven study but also on the *a priori* decisions to meta-analyze associations with lung function and AHR in populations 2 and 3. Furthermore, we did so since we attempted to indirectly replicate the results, i.e. direction of associations with clinical and histological outcomes, so called loose replication. Finally, the outcomes in our analyses (clinical and histological phenotypes) are mutually related, suggesting that a conservative statistical approach would not do justice to their biologically linked nature. Thus, we followed the advice given by Perneger: "Simply describing what was done and why, and discussing the possible interpretations of each result, should enable the reader to reach a reasonable conclusion without the help of Bonferroni adjustments".[33]

Our study adds one more gene to the list of genes that can influence ICS response in asthma.[34] We show for the first time that the beneficial effects of ICS use on airway pathology and clinical severity are seen in asthmatics with specific *TNF* genotypes. In these carriers, better epithelial integrity with ICS use was concordant with higher level of lung function. ICS did not modify effects of *TNF* SNPs on airway remodelling but significantly increased pro-inflammatory effects. The potential implication of the latter in tissue repair needs further attention.

ACKNOWLEDGMENTS

We thank F Volbeda and A Dijkstra (Department of Pulmonology) for collecting the biopsies and characterizing the patients, M Broekema and M Lodewijk (Department of Pathology and Medical Biology) for processing and staining of the biopsies, M Olivier (Department of Genetics) for her help with genotyping and S. Scholtens (Department of Epidemiology) for helping with meta-package in R. University Medical Center Groningen (UMCG).

FINANCIAL SUPPORT

The Dutch studies were supported by the Netherlands Asthma Foundation, Stichting Astma Bestrijding, the National Institute of Health and Novartis Pharmaceuticals.

REFERENCES

1.Ten Brinke A. Risk factors associated with irreversible airflow limitation in asthma. Curr Opin Allergy Clin Immunol 2008 Feb**;8**:63-69.

2. Upham JW, James AL. Remission of asthma: The next therapeutic frontier? Pharmacol Ther 2011 Apr**;130**:38-45.

3. Holloway JW, Yang IA, Holgate ST. Interpatient variability in rates of asthma progression: can genetics provide an answer? J Allergy Clin Immunol 2008 Mar**;121**:573-579.

4. Yang IA, Savarimuthu S, Kim ST, Holloway JW, Bell SC, Fong KM. Gene-environmental interaction in asthma. Curr Opin Allergy Clin Immunol 2007 Feb**;7**:75-82.

5. Ying S, Robinson DS, Varney V, Meng Q, Tsicopoulos A, Moqbel R et al. TNF alpha mRNA expression in allergic inflammation. Clin Exp Allergy 1991 Nov**;21**:745-750.

6. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R et al. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. Am J Respir Cell Mol Biol 1994 May**;10**:471-480.

7. Thomas PS. Tumour necrosis factor-alpha: The role of this multifunctional cytokine in asthma. Immunol Cell Biol 2001 04**;79**:132-140.

8. Berry M, Brightling C, Pavord I, Wardlaw A. TNF-alpha in asthma. Curr Opin Pharmacol 2007 Jun**;7**:279-282.

9. Ye D, Ma I, Ma TY. Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. Am J Physiol Gastrointest Liver Physiol 2006 Mar**;290**:G496-504.

10. Carayol N, Campbell A, Vachier I, Mainprice B, Bousquet J, Godard P et al. Modulation of Cadherin and Catenins Expression by Tumor Necrosis Factor-alpha and Dexamethasone in Human Bronchial Epithelial Cells. Am J Respir Cell Mol Biol 2002 03/01/**;26**:341-347.

11. Carayol N, Vachier I, Campbell A, Crampette L, Bousquet J, Godard P et al. Regulation of E-cadherin expression by dexamethasone and tumour necrosis factor-{alpha} in nasal epithelium. Eur Respir J 2002 12/01/**;20**:1430-1436.

12. Tenenbaum T, Matalon D, Adam R, Seibt A, Wewer C, Schwerk C et al. Dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine choroid plexus epithelial cells after infection with Streptococcus suis in vitro. Brain Res 2008 Sep 10**;1229**:1-17.

13. Mekinian A, Tamouza R, Pavy S, Gestermann N, Ittah M, Mariette X et al. Functional study of TNF-alpha promoter polymorphisms: literature review and meta-analysis. Eur Cytokine Netw 2011 Jun**;22**:88-102.

14. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 2002 Dec**;32**:650-654.

15. Castro-Giner F, Kogevinas M, Machler M, de Cid R, Van Steen K, Imboden M et al. TNFA -308G>A in two international population-based cohorts and risk of asthma. Eur Respir J 2008 Aug**;32**:350-361.

16. Sharma S, Sharma A, Kumar S, Sharma SK, Ghosh B. Association of TNF haplotypes with asthma, serum IgE levels, and correlation with serum TNF-alpha levels. Am J Respir Cell Mol Biol 2006 Oct**;35**:488-495.

17. Randolph AG, Lange C, Silverman EK, Lazarus R, Weiss ST. Extended haplotype in the tumor necrosis factor gene cluster is associated with asthma and asthma-related phenotypes. Am J Respir Crit Care Med 2005 Sep 15**;172**:687-692.

18. Li Kam Wa TC, Mansur AH, Britton J, Williams G, Pavord I, Richards K et al. Association between - 308 tumour necrosis factor promoter polymorphism and bronchial hyperreactivity in asthma. Clin Exp Allergy 1999 Sep**;29**:1204-1208.

19. Shima H, Ohshiro K, Taira Y, Miyazaki E, Oue T, Puri P. Antenatal dexamethasone suppresses tumor necrosis factor-alpha expression in hypoplastic lung in nitrofen-induced diaphragmatic hernia in rats. Pediatr Res 1999 Nov**;46**:633-637.

20. Baraket M, Oliver BG, Burgess JK, Lim S, King GG, Black JL. Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study. Respir Res 2012 Feb 2**;13**:11.

21. Holgate ST, Holloway J, Wilson S, Howarth PH, Haitchi HM, Babu S et al. Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities. J Allergy Clin Immunol 2006 Mar**;117**:496-506; quiz 507.

22. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D et al. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax 2002 04//**;57**:309-316.

23. Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. Immunol Cell Biol 2001 Aug**;79**:376-384.

24. Ierodiakonou D, Postma DS, Koppelman GH, Boezen HM, Gerritsen J, Ten Hacken N et al. E-cadherin gene polymorphisms in asthma patients using inhaled corticosteroids. Eur Respir J 2011 May 3.

25. Horvath G, Wanner A. Inhaled corticosteroids: effects on the airway vasculature in bronchial asthma. Eur Respir J 2006 Jan**;27**:172-187.

26. Matsuda A, Orihara K, Fukuda S, Fujinaga H, Matsumoto K, Saito H. Corticosteroid enhances TNF-alpha-mediated leukocyte adhesion to pulmonary microvascular endothelial cells. Allergy 2008 Dec**;63**:1610-1616.

27. McArthur JA, Zhang Q, Quasney MW. Association between the A/A genotype at the lymphotoxin-alpha+250 site and increased mortality in children with positive blood cultures. Pediatr Crit Care Med 2002 Oct**;3**:341-344.

28. Mahajan A, Tabassum R, Chavali S, Dwivedi OP, Chauhan G, Tandon N et al. Obesity-dependent association of TNF-LTA locus with type 2 diabetes in North Indians. J Mol Med (Berl) 2010 May**;88**:515-522.

29. Wu H, Romieu I, Sienra-Monge JJ, del Rio-Navarro BE, Anderson DM, Dunn EW et al. Parental smoking modifies the relation between genetic variation in tumor necrosis factor-alpha (TNF) and childhood asthma. Environ Health Perspect 2007 Apr**;115**:616-622.

30. Lee YL, McConnell R, Berhane K, Gilliland FD. Ambient ozone modifies the effect of tumor necrosis factor G-308A on bronchitic symptoms among children with asthma. Allergy 2009 Sep**;64**:1342-1348.

31. Zhang H, Hang J, Wang X, Zhou W, Sun B, Dai H et al. TNF polymorphisms modify endotoxin exposure-associated longitudinal lung function decline. Occup Environ Med 2007 Jun**;64**:409-413.

32. Sutherland ER, Goleva E, Strand M, Beuther DA, Leung DY. Body mass and glucocorticoid response in asthma. Am J Respir Crit Care Med 2008 Oct 1**;178**:682-687.

33. Perneger TV. What's wrong with Bonferroni adjustments. BMJ 1998 Apr 18**;316**:1236-1238.

34. Lima JJ, Blake KV, Tantisira KG, Weiss ST. Pharmacogenetics of asthma. Curr Opin Pulm Med 2009 Jan**;15**:57-62.

CHAPTER 4

TGF-β1 POLYMORPHISMS AND ASTHMA SEVERITY, AIRWAY INFLAMMATION, AND REMODELING

Despo Ierodiakonou, MD[1,5], Dirkje S. Postma, MD PhD[2,5], Gerard H. Koppelman, MD PhD[3,5], Jorrit Gerritsen, MD PhD[3,5], Nick H. T. ten Hacken, MD PhD[2,5], Wim Timens, MD PhD[4,5], H. Marike Boezen, PhD[1,5], Judith M. Vonk, PhD[1,5]

Journal of Allergy and Clinical Immunology 131(2)

1. Department of Epidemiology, University of Groningen, University Medical Center Groningen

2. Department of Pulmonology, University of Groningen, University Medical Center Groningen

3. Department of Pediatric Pulmonology and Pediatric Allergology-Beatrix Children's Hospital, University of Groningen, University Medical Center Groningen

4. Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen

5. Groningen Research Institute for Asthma and COPD, Groningen, The Netherlands

CAPSULE SUMMARY

Associations of *TGFB1* gene polymorphisms with both clinical and pathological outcomes are linking the *TGFB1* gene with the pathophysiology of asthma severity and progression. The effects of *TGFB1* polymorphisms may be modified by smoking.

To the Editor:

The development and progression of asthma are determined by multiple genetic and environmental factors. A relatively new hypothesis is that chronic injury by environmental irritants and/or defective repair of the airway epithelium leads to the release of cytokines and growth factors.[1] These factors drive chronic airway inflammation and remodeling seen in asthma. Airway inflammation and remodeling are 2 important pathologic features associated with airway hyperresponsiveness (AHR) and fixed airflow obstruction.[2]

TGF-β1 is a cytokine whose level increases in asthma.[3] It is in volved in airway pathology.[4] The release of TGF-β1 in the submucosa, mainly by eosinophils, is stimulated by environmental factors such as allergens and cigarette smoke. Experimental studies show that exposure to cigarette smoke increases *TGFB1* gene expression[5] and decreases eosinophil migration in the airway.[6]

Single nucleotide polymorphisms (SNPs) in the *TGFB1* gene have been associated with asthma development, yet only a few studies have investigated associations with asthma severity and the underlying airway inflammation7 and none with FEV1 decline and airway remodeling in asthma. Our aim was to investigate associations between *TGFB1* SNPs and asthma severity (ie, AHR, FEV1 level, asthma remission, FEV1 decline) and features of airway wall inflammation and remodeling in 2 asthma populations. In addition, we assessed interactions between *TGFB1* SNPs and smoking.

Details on the clinical assessment, outcome definitions, immunochemistry and quantification procedures, genotypic information, and statistical analysis are presented in this article's Online Repository at www.jacionline.org. The study was approved by the medical ethics committee of the University Medical Center Groningen, and participants signed informed consent.

The clinical characteristics of the 2 populations and genotype distributions are shown in Tables E1 and E2, A and B, in this article's Online Repository at www.jacionline.org. All SNPs were in Hardy-Weinberg equilibrium ($P > .05$). Fig E1 in this article's Online Repository at www.jacionline.org shows the linkage disequilibrium plots of the SNPs.

Table I presents a summary of the main results. Rs6957-G associated with more severe AHR and airflow obstruction, increased subepithelial eosinophil and macrophage counts, and increased basement membrane thickness. These associations were more pronounced in ever-smokers. Rs10417924-A associated with less AHR and a trend for higher FEV1 (eversmokers), more asthma remission, and higher subepithelial $CD8^+$ T cells. Rs1800469-T associated with less FEV1 decline and lower FEV1 (ever-smokers) and increased goblet cells.

Rs1800470-C associated with less FEV1 decline and lower FEV1. Rs4803455-A associated with accelerated FEV1 decline.

TABLE 1	Main associations of TGFB1 SNPs with markers of air- way pathology and severity of asthma				
TGFB1 SNPs	Outcome	B or OR	P value	B or OR	P value
rs6957-G 3'UTR	FEV_1 decline[1]	-0.2	.97	7.5	.16*
	FEV_1% predicted pre-BD[1,2]	-7.2	**<.001**	-9.60	**.001**
	FEV_1% predicted post-BD[1,2]	-6.9	**.001**	-8.0	**.006**
	AHR severity[1,2]	1.6	**.04**	3.6	**<.001**
	Asthma remission[1,2]	0.5	**.09**	0.5	.20
	Eosinophils in biopsies (ln)[2]	0.45	**.02**	0.78	**.003†**
	Macrophages in biopsies (ln)[2]	0.26	**.045**	0.48	**.01†**
	Basement membrane thickness[2]	0.39	.17	0.076	**.04**
rs10417924-T 3'UTR	FEV_1 decline[1]	-0.3	.94	2.3	.64
	FEV_1% predicted pre-BD[1,2]	3.7	.40	5.4	**.08**
	FEV_1% predicted post-BD[1,2]	3.5	.40	4.3	.16
	AHR severity[1,2]	0.7	.13	0.5	**.04**
	Asthma remission[1,2]	2.2	**.03**	3.0	**.01**
	$CD8^+$ T-cells in biopsies (ln)[2]	0.37	**.02**	0.46	**.03**
rs1800469-T Promoter	FEV_1 decline[1]	6.3	**.06**	8.9	**.03**
	FEV_1% predicted pre-BD[1,2]	-2.7	.16	-6.4	**.02***
	FEV_1% predicted post-BD[1,2]	-2.7	.19	-5.4	**.05†**
	Severe asthma[1,2]	1.7	.40	1.3	.74
	Goblet cells in biopsies (ln)[2]	0.23	**.03**	0.27	**.08**
rs1800470-C Exon 1	FEV_1 decline[1]	10.7	**.002**	11.00	**.01**
	FEV_1% predicted pre-BD[1,2]	-4.1	**.04**	-6.8	**.01†**
	FEV_1% predicted post-BD[1,2]	-4.7	**.02**	-7.0	**.01**
	Severe asthma[1,2]	1.5	.60	1.4	.74
rs4803455-A Intron 2	FEV_1 decline[1]	-11.4	**.003**	-15.7	**<.001**
	FEV_1% predicted pre-BD[1,2]	3.2	.16	3.8	.22
	FEV_1% predicted post-BD[1,2]	2.1	.33	3.0	.32

Associations of TGFB1 SNPs with FEV1 decline were investigated in population 1.
Associations with cross-setional lung function level and clinical outcomes were assessed in populations 1 (n 5 243) and 2 (n 5 137) and meta-analyzed. Associations with pathologic outcomes were investigated in population 2. Populations are shown as superscript numerals.
Complete asthma remission: FEV1%predicted post-BD > 90%and absence of AHR and symptoms and medication; severe asthma: FEV1%predicted pre-BD< 80%and ICS use. The number of positively stained inflammatory cells was counted in a total area of 0.1mm2 in the submucosa, 100 mm under the basement membrane. Goblet cell numbers were counted on periodic acid–Schiff–stained biopsy sections and expressed per 1000 mm of basement membrane.
Dominant genetic models were used in multiple linear and logistic regression adjusted for sex, age, ICS use, and smoking status (total group). Two tailed P values of<.05 were considered statistically significant and P values between .05 and .09 of borderline significance (boldface text).
AHR, Airway hyperresponsiveness PC20 histamine < 16 mg/mL (population 1) and PC20 AMP < 320 mg/mL (population 2); AMP, adenosine 5-monophosphate; B, regression coefficient; BD, bronchodilation; ICS, inhaled corticosteroids; OR, odds ratio; 39UTR, 3 prime untranslated region; VC, vital capacity. *Significant interaction (P <.05). Interaction of borderline significance (.05 < P < .09).

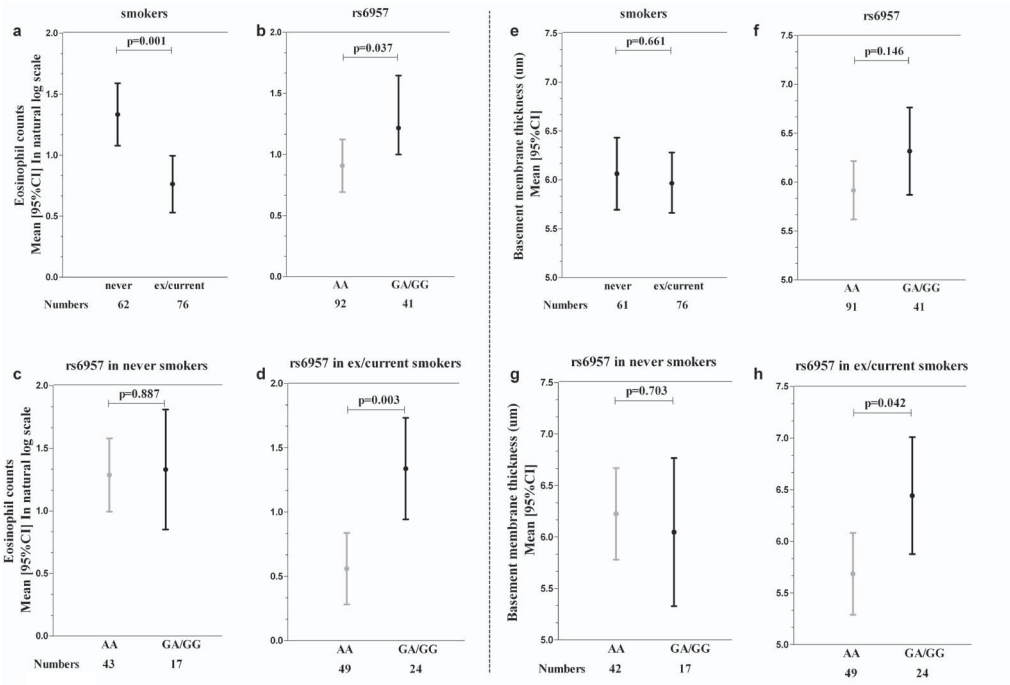


FIG 1. Associations between subepithelial eosinophil counts and smoking status (A), rs6957 (B), rs6957 in never-smokers (C) and in ever-smokers (D). Associations between basement membrane thickness and smoking status (E), rs6957 (F), rs6957 in never-smokers (G) and in ever-smokers (H). Mean (95% CI) eosin- ophil counts are given by generalized linear models adjusted for sex, age, and inhaled corticosteroid use.

TGFB1 by smoking interaction: Significant effects on severity phenotypes were observed mainly in ever-smokers (see Fig 1; also see Fig E2 in this article's Online Repository at www.jacionline.org). Additional significant associations were found in ever-smokers compared with the total group (see Table I). We found a limited number of significant interactions, but several of the significant associations seen in ever-smokers had an interaction of borderline significance (.05 <P <.09). Tables E3 to E14 in this article's Online Repository at www.jacionline.org present detailed results.

Our study shows that *TGFB1* SNPs are significantly associated with asthma severity defined bymore severeAHR, airflowobstruction, and asthma remission, as well as with airway inflammation and remodeling. Of importance, there is indirect replication, because the direction of the SNP associations with asthma severity was consistent with the direction of the SNPassociations with pathologic changes.We confirm results of previous studies showing that rs1800469-T is associated with airflowobstruction.[7] Onthe basis of its association with

increased numbers of goblet cells in bronchial biopsies, we propose that mucus hypersecretion but not basement membrane thickness might be a missing link between the *TGFB1* promoter SNP and airflow obstruction in asthma.

Three (rs6957, rs1800469, and rs1800470) of the 6 SNPs in our study are known to influence TGFβ1 serum levels (see References E14-E16 in this article's Online Repository at www.jacionline.org). However, the pleiotropic nature and multiple sources of this cytokine make it difficult to determine in which compartment/cell type its expression should be measured. Because of lack of expression data, we cannot conclude whether these pathologic changes in our study are driven by high or low TGFβ1 expression and by which cellular sources. Elucidating the effects of TGFβ1 on asthma phenotypes is further complicated by the fact that its functions are controlled not only by inhaled substances and subsequently expression levels in the airways but also by the chronic response of the cells and the interaction with other growth factors and pathways in the immediate microenvironment.

We confirm that smokers have lower eosinophilic inflammation/infiltration than do nonsmokers (Fig 1).[6] However, when accounting for *TGFB1* genotypes, this effect no longer exists.

Asthmatic carriers of the G-allele of rs6957 have significantly higher submucosal eosinophils (and macrophages) than the wild types, an effect that is not significant in nonsmokers. Consistent with the increase in eosinophil counts, basement membrane thickness is significantly increased in carriers of the G-allele in smokers only. Similarly, smoking is not associated with CD81.

T-cells counts or AHR severity, but when accounting for the genotype, smokers carrying the A-allele of rs10417924 have significantly higher $CD8^+$ T cells and less severe AHR (Fig E2), a relationship consistent with an earlier study.[8] These data provide evidence of an interaction between *TGFB1* SNPs and smoking on asthma severity.

Our study is the first investigating the effects of *TGFB1* on FEV1 decline in asthma (see Fig E3 in this article's Online Repository at www.jacionline.org). Rs1800469-Tand rs1800470-C significantly associated with less FEV1 decline and rs4803455-A with accelerated FEV1 decline. Of interest, SNPs that were associated with more airflow obstruction cross-sectionally (rs6957, rs1800469, and rs1800470) were also associated with less rapid decline in ever-smokers of our study. This is compatible with previous observations that show associations with chronic obstructive pulmonary disease protection,[9] suggesting that high TGFb1 levels might be protective for chronic obstructive pulmonary disease, while others support that highTGFβ1 levels increase the risk for asthma.[4]

These findings may infer a distinct role of *TGFB1* between severe asthma and chronic obstructive pulmonary disease.

Because we decided a priori to replicate and meta-analyze the results of our analysis in the 2 asthma populations, we did not adjust for multiple comparisons. Moreover, the outcomes in our analysis (clinical and pathologic phenotypes) are mutually related, suggesting that a conservative statistical approach would not do justice to their biologically linked nature.

We conclude that the TGFB1 gene plays a role in the pathophysiology of asthma severity. We for the first time show associations of *TGFB1* polymorphisms with airway pathology and additionally link TGFB1 with FEV1 decline in asthma. Our data provide suggestive evidence for an interaction with smoking in asthmatic patients.

Acknowledgment

We thank F. Volbeda and A. Dijkstra (Department of Pulmonology, University Medical Center Groningen) for collecting the biopsies and characterizing the patients, M. Broekema and M. Lodewijk (Department of Pathology and Medical Biology, University Medical Center Groningen) for processing and staining of the biopsies, and M. Olivier (Department of Genetics, University Medical Center Groningen) for her help with genotyping.

REFERENCES

1. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001;164:S28-38.

2. ten Brinke A. Risk factors associated with irreversible airflow limitation in asthma. Curr Opin Allergy Clin Immunol 2008;8:63-9.

3. Bosse Y, Rola-Pleszczynski M. Controversy surrounding the increased expression of TGF beta 1 in asthma. Respir Res 2007;8:66.

4. Salib RJ, Howarth PH. Transforming growth factor-beta in allergic inflammatory disease of the upper airways: friend or foe? Clin Exp Allergy 2009;39:1128-35.

5. Guo ZH, Du YC, Xu JY. The effect of cigarette smoke on the expression of transforming growth factor-beta1 mRNA and collagen type III in airways of asthmatic rats. Zhonghua Jie He He Hu Xi Za Zhi 2008;31:42-5.

6. Botelho FM, Llop-Guevara A, Trimble NJ, Nikota JK, Bauer CM, Lambert KN, et al. Cigarette smoke differentially impacts eosinophilia and remodeling in a house dust mite asthma model. Am J Respir Cell Mol Biol 2011;45:753-60.

7. Ueda T, Niimi A, Matsumoto H, Takemura M, Yamaguchi M, Matsuoka H, et al. TGFB1 promoter polymorphism C-509T and pathophysiology of asthma. J Allergy Clin Immunol 2008;121:659-64.

8. Huang TJ, MacAry PA, Kemeny DM, Chung KF. Effect of CD81 T-cell depletion on bronchial hyper-responsiveness and inflammation in sensitized and allergenexposed Brown-Norway rats. Immunology 1999;96:416-23.

9. Wu L, Chau J, Young RP, Pokorny V, Mills GD, Hopkins R, et al. Transforminggrowth factor-beta1 genotype and susceptibility to chronic obstructive pulmonary disease. Thorax 2004;59:126-9

.

CHAPTER 5

Ambient air pollution, lung function and airway responsiveness in children with asthma

Despo Ierodiakonou[1,2], Antonella Zanobetti[3], Brent A. Coull[4], Steve Melly[3], Dirkje S. Postma[5,2], H. Marike Boezen[1,2], Judith M. Vonk[1,2], Paul V. Williams[6], Gail G. Shapiro[6†], Edward F. McKone[7], Teal S. Hallstrand[8], Jane Q. Koenig[9], Jonathan S. Schildcrout[10], Thomas Lumley[11], Anne N. Fuhlbrigge[12,13], Petros Koutrakis[3], Joel Schwartz[3], Scott T. Weiss[12,13], Diane R Gold[3,12]

Submitted

1. Department of Epidemiology, University of Groningen, University Medical Center Groningen, The Netherlands

2. Groningen Research Institute for Asthma and COPD University Medical Center Groningen, The Netherlands

3. Environmental Epidemiology and Risk Program, Harvard School of Public Health, Boston, MA

4. Department of Biostatistics Harvard School of Public Health, Boston, MA

5. Department of Pulmonology, University of Groningen, University Medical Center Groningen, The Netherlands

6. Department of Pediatrics, School of Medicine, University of Washington Seattle, WA

7. Department of Biostatistics, School of Public Health, University of Washington, Seattle, WA

8. Department of Respiratory Medicine, St. Vincent University Hospital, Dublin, Ireland

9. Department of Pulmonary and Critical Care, School of Medicine, University of Washington Seattle, WA

10. Department of Environmental Health, University of Washington School of Medicine Seattle, WA

11. Department of Biostatistics, School of Medicine, Vanderbilt University Nashville, TN

12. Department of Statistics, University of Auckland, New Zealand

13. Channing Laboratory, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, MA

14. for the Childhood Asthma Management Program Research Group

ABSTRACT

Rationale: Short-term air pollution exposures can increase airflow obstruction in asthmatic children. Long-term pollution effects on lung function and airway hyperresponsiveness, and the modifying effects of controller asthma treatment on pollution effects are less understood.

Methods: We examined associations of lung function and methacholine responsiveness (PC_{20}) with ozone, carbon monoxide, nitrogen dioxide and sulfur dioxide levels in 1,003 asthmatic children participating in a 4-year clinical trial. We further investigated whether budesonide and nedocromil (vs. placebo) modified pollution effects. Daily pollutant concentrations were linked to zip/postal code of residence. Linear mixed models tested associations of within-subject pollutant concentrations with FEV_1 and FVC %predicted, FEV_1/FVC and PC_{20}, adjusting for seasonality and confounders.

Results: Increased four-month average concentrations of carbon monoxide and nitrogen dioxide were associated with reduced post-bronchodilator FEV_1 (change per IQR (95%CI):-0.6 (-0.7, -0.1); -0.2 (-0.4, 0.01), respectively) and FVC (-0.2 (-0.4, -0.01); -0.2 (-0.4, -0.1), respectively). Four-month average carbon monoxide and ozone levels were negatively associated with FEV_1/FVC ($p<0.05$). For carbon monoxide, increases in short-term averaged concentrations were also associated with reduced FEV_1 and FVC. Long-term exposure to sulfur dioxide was associated with reduced $\ln PC_{20}$ (% change (95%CI) per IQR:-6 (-11;-1.5)). Treatment only modified the short-term effect of carbon monoxide on PC_{20}.

Conclusions: Air pollution adversely influences lung function and airway hyperresponsiveness in asthmatic children. Regular treatment with controller medications may not protect against the effects of carbon monoxide on airway hyperresponsiveness. This clinical trial design gave us the opportunity to evaluate modification of pollution effects by treatment without confounding by indication.

INTRODUCTION

Over the past thirty years evidence has accumulated demonstrating that ambient air pollution has adverse effects on the respiratory health of asthmatic and non-asthmatic children.[1-4] In observational studies of asthmatic children, higher short-term exposures to air pollution have been associated with more symptoms, increased need for reliever medication, hospital admissions, lung function decrements, and airflow obstruction.[5-9]

Although ambient air pollution has been linked to reduced lung function in normal children, longitudinal analyses of air pollution effects in asthma are lacking. For instance there are no clinical trials that assessed associations of long-term pollution with lung function, airflow obstruction and airway responsiveness (AHR), and modification of putative pollution effects by controller medications. Pollutants induce adverse effects by affecting oxidant signaling pathways and airway inflammation.[10,11] Inhaled corticosteroids (ICS) have been shown to reduce oxidative stress and improve airway function and asthma symptoms.[8,12] However, recent observational studies suggest that asthmatic children using inhaled corticosteroids (ICS) may be more vulnerable to the adverse health effects of air pollution compared to those that are not on ICS.[13,14] These findings may reflect confounding by indication, since children with more symptomatic asthma may be more likely use an ICS. Only evaluation of pollution effects in the context of a clinical trial can test whether ICS increase or decrease susceptibility to air pollution.

The Childhood Asthma Management Program (CAMP) is such a randomized clinical trial involving eight cities in North America (Albuquerque, New Mexico; Baltimore, Maryland; Boston, Massachusetts; Denver, Colorado; San Diego, California; Seattle, Washington; St. Louis, Missouri; and Toronto-Ontario, Canada). Its main goal was to evaluate the long-term effectiveness and safety of daily inhaled anti-inflammatory medication in children diagnosed with mild-to-moderate asthma.[15,16] Using the pre-randomization observational data from this trial, we reported that short-term air pollution exposures increased asthma exacerbations,[6] with carbon monoxide and nitrogen dioxide having the strongest associations with exacerbations.

The current paper, investigates in the same CAMP study whether short- and long-term exposures to four of the Environmental Protection Agency's criteria air pollutants (ozone, carbon monoxide, nitrogen oxide and sulfur dioxide) are associated with lung function level and AHR in children with asthma. In addition, we investigate whether anti-inflammatory treatment with ICS or Nedocromil modifies the effects of pollution on asthma outcomes.

MATERIALS AND METHODS

CAMP study design and methods have been described elsewhere.[16] Additional detail on all methods used in the present report is provided in an online data supplement. In summary, children enrolled in CAMP were 5–12 years of age and were hyperresponsiveness to methacholine at study entry. 1,041 Children entered the randomization phase and 311, 312, 418 children received budesonide, nedocromil, and placebo, respectively. All subjects were treated and followed for four years with visits at two and four months after randomization and at four-month intervals thereafter. Each parent or guardian signed a consent form and participants of 7 years of age and older signed an assent form approved by each clinical center's institutional review board.

Outcomes Measures

Spirometry, before and after the bronchodilator administration, was conducted at randomization (RZ) and at follow up visits (n=13) according to the American Thoracic Society Standards. We considered both pre- and post-BD FEV_1 and FVC as outcomes in this current analysis as we investigated short- and long-term effects of air pollution. Additionally, the FEV_1/FVC ratio was used as another measure of airflow obstruction. Using the Wright nebulizer-tidal breathing technique a methacholine challenge was performed annually during the treatment phase. Spirometry was performed 90 seconds after each challenge until FEV_1 had fallen by 20% or more (PC_{20}).

Air Pollution Exposure Assessment

Monitoring data on 24-hour averages concentrations of 4 gaseous pollutants (ozone, carbon monoxide, nitrogen dioxide, and sulfur dioxide) were obtained for each metropolitan area. The ZIP or postal code centroid coordinates were used to link participants to daily concentrations from the nearest monitor within 50 km that did not have missing data on that day (December 1993 through June 1999).

Statistical Analysis

We fitted a linear mixed model - with random intercepts for each subject - to estimate the associations between lung function (FEV_1 and FVC % predicted and FEV_1/FVC) and (log-transformed) PC_{20} and same day, 1-week and 4-month moving averages of pollution. Number of days from randomization was the

TABLE1	Demographic characteristics
	N= 1003
City; n (%)	
Albuquerque	121 (12.1)
Baltimore	126 (12.6)
Boston	123 (12.3)
Denver	141 (14.1)
San Diego	122 (12.2)
Seattle	136 (13.6)
Saint Louis	133 (13.3)
Toronto	101 (10.1)
Sex; n (%)	
Males/Females	602/401 (60/40)
Treatment Group; n (%)	
Placebo	407 (40.6)
Budesonide	298 (29.7)
Nedocromil	298 (29.7)
Ethnicity; n (%)	
Caucasians	677 (67.5)
African-Americans	137 (13.7)
Hispanics	97 (9.7)
Other	92 (9.2)
Annual Income =>30K USD; n (%)	
Yes/No	728/235 (76/24)
In utero smoking exposure; n (%)	
Yes/No	114/854 (14/86)
Pre bronchodilator lung function at randomization; mean (SD)	
FEV_1 % predicted	93.8 (14.3)
FVC % predicted	104.0 (13.1)
FEV_1/FVC %	79.7 (8.3)
Post bronchodilator lung function at randomization; mean (SD)	
FEV_1 % predicted	103.0 (12.8)
FVC % predicted	106.5 (12.8)
FEV_1/FVC %	85.5 (6.5)

FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; SD: standard deviation; =>30K USD: equal or more than 30,000 United State Dollars

time trend of the model. Potential for confounding factors was considered carefully, basing choice of covariates on prior CAMP experience.[17,18] To estimate associations across all cities, we constructed a model including city as a covariate, but also compared estimates of this model with study-wide estimates from meta-analyzing city-stratified models. We adjusted for "season" by using sine and cosine functions of time[19] and their interactions with city. In addition, we decomposed daily pollution concentrations into between- and within-subject exposures. We report estimates of within-subject exposure effects (on interquartile range scale (IQR)).

To assess potential effect modification of the pollution- outcomes associations by treatment we included a pollutant concentration by treatment interaction into the models while excluding the baseline (RZ) measurements and used ANOVA likelihood ratio to test effect differences across the 3 treatment groups.

We used SAS® software (version 9.2; SAS Institute Inc. 2008, Cary, NC USA) and IBM SPSS statistics (version 20; Armonk, NY USA: IBM Corp 2011) to manage all data. Statistical analysis was performed in IBM SPSS and R programming language (version 2.15.1; 2012-06-22).

TABLE 2	Distribution of 24-hour mean pollution concentrations by city								
Pollutant	City	N		Percentiles					IQR
		Valid	Missing	10	25	50	75	90	
O_3 (ppb)	ALB	1336	358	13	19	28	36	43	17
	BAL	1703	61	7	13	23	33	43	20
	BOS	1660	62	7	13	21	30	38	17
	DEN	1669	305	6	13	23	32	41	19
	SD	1664	44	13	20	27	35	41	15
	SEA	1071	833	7	11	17	22	28	11
	STL	1667	195	7	12	22	32	41	20
	TOR	1350	64	6	10	17	25	33	14
	TOTAL	**12120**	**1922**	**8**	**14**	**22**	**31**	**39**	**18**
CO (ppmx10)	ALB	1343	351	1	3	7	11	14	8
	BAL	1719	45	3	5	7	11	15	6
	BOS	1660	62	6	8	10	13	16	5
	DEN	1684	290	4	5	8	12	17	7
	SD	1664	44	4	5	8	11	17	6
	SEA	1701	203	7	10	14	19	25	9
	STL	1684	178	4	5	7	9	12	4
	TOR	1350	64	2	6	10	12	15	6
	TOTAL	**12805**	**1237**	**4**	**6**	**9**	**12**	**16**	**6**
NO_2 (ppb)	ALB	1307	387	7	11	17	23	30	12
	BAL	1719	45	14	18	24	29	36	11
	BOS	1660	62	14	20	25	32	38	12
	DEN	1577	397	10	20	29	36	44	17
	SD	1664	44	10	13	19	26	34	13
	SEA	1255	649	11	15	19	24	30	9
	STL	1707	155	8	13	18	24	28	11
	TOR	1350	64	13	19	25	32	39	13
	TOTAL	**12239**	**1803**	**11**	**16**	**22**	**28**	**35**	**13**
SO_2 (ppb)	ALB	25	1669	0	0	4	16	24	16
	BAL	1719	45	2	4	6	9	14	6
	BOS	1660	62	2	3	5	9	13	5
	DEN	1571	403	1	2	4	7	10	4
	SD	1454	254	1	2	2	3	5	1
	SEA	1752	152	2	3	5	7	10	4
	STL	1736	126	1	3	5	9	13	6
	TOR	1347	67	0	2	4	6	9	4
	TOTAL	**11264**	**2778**	**1**	**2**	**4**	**8**	**12**	**5**

ALB: Albuquerque, BAL: Baltimore, BOS: Boston, DEN: Denver, SD: San Diego, SEA: Seattle, STL: Saint Louis, TOR: Toronto, O_3: ozone (ppb); CO: carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); N: number of observations; IQR: interquartile range, ppb: part per billion; ppm: parts per million

RESULTS

All subjects considered in this analysis were randomized into CAMP and followed up during the trial period. A total of 1,003 of the 1,041 total children (96.3%) children were studied. At study entry the mean (SD) age was 9 (2.1) and geometric mean (min-max) for PC_{20} was 1.1 (0.02-2.5) mg/ml. Table 1 shows the

main characteristics of the participants. 82.5% of the children attended all visits during the 4 years of the trial (median number of completed visits=14 (range: 1-14)). Participants had a median of 14 (range: 1-14) pre-BD and 10 (range: 1-10) post-BD lung function measurements and 4 (range: 0-4) PC_{20} tests.

TABLE 3	Interquartile ranges (25th to 75th percentile) of pollution concentrations	
IQR	**Overall**	**Within-subject**
O_3 same day	18	15
O_3 1-week m.a	16	14
O_3 4-month m.a	13	11
CO same day	6	5
CO 1-week m.a	6	4
CO 4-month m.a	6	3
NO_2 same day	13	10
NO_2 1-week m.a	10	7
NO_2 4-month m.a	8	4
SO_2 same day	5	4
SO_2 1-week m.a	4	3
SO_2 4-month m.a	3	2

O_3: ozone (ppb); CO: carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); IQR: interquartile range, ppb: part per billion; ppm: parts per million; m.a: moving average

Pollution concentrations during December'93-June'99 are summarized by city in Table 2. We report the number of observations, percentiles and IQR of daily concentrations of the 4 pollutants. Table 3 shows the IQR of the overall and the within-subject concentration of pollutants.

Correlations of 24-hour mean pollution concentrations are shown in Table 4. Overall, ozone was negatively correlated with the other 3 pollutants that were positively correlated with each other. The same pattern of correlation existed in the 8 separate cities (data shown in Table E1 in the online repository). These relationships are expected because ozone is a secondary pollutant of regional origin, whereas the other pollutants are primary and mostly associated with local sources.

TABLE 4	Correlations of 24-hour mean pollution concentrations				
		O_3	CO	NO_2	SO_2
Spearman's rho	O_3	1	-0.4*	-0.3*	-0.2*
	CO	-0.4*	1	0.4*	0.2*
	NO_2	-0.3*	0.4*	1	0.4*
	SO_2	-0.2*	0.2*	0.4*	1

O_3: ozone (ppb); CO: carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb)
* correlation is significant at $p<0.05$ (2-tailed)

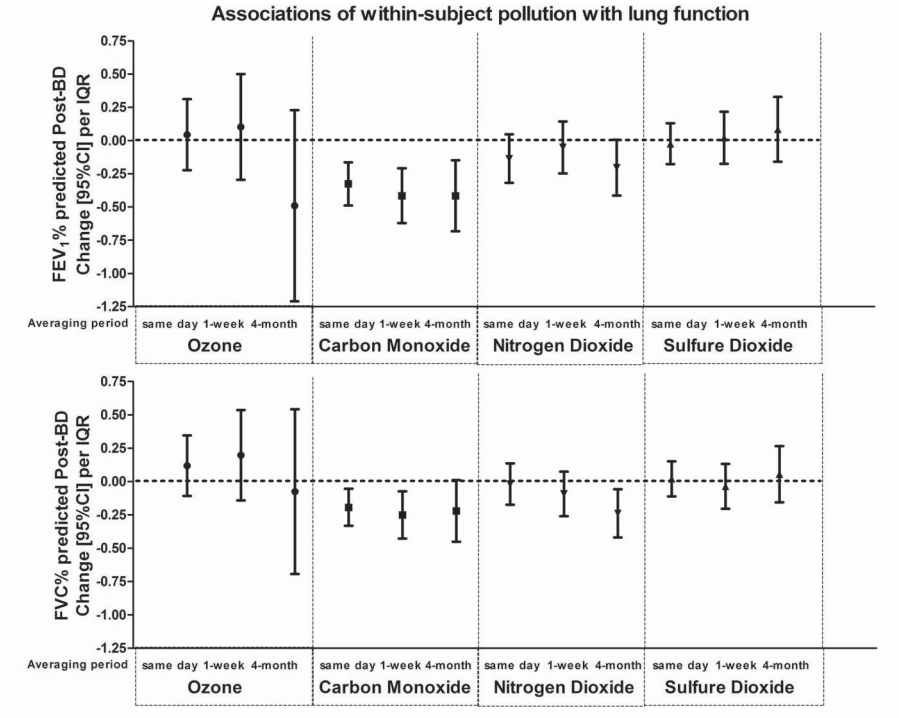


Figure 1. Gaseous pollutants are associated with lung function
Figure 1 presents the associations of pollution with post-bronchodilator (BD) FEV_1 and FVC %predicted. Same day and 1-week and 4-month moving averages of carbon monoxide and 4-month average of nitrogen dioxide had negative associations with post-BD FEV_1 (top graphs panel) and FVC (bottom graphs panel). The evidence for adverse effects on post-BD lung function seems week for 4-month average exposure to ozone (all-months) compared to the traffic pollutants and there were not associations with sulfur dioxide.

Association of pollution with level of lung function

Figure 1 presents the associations of pollution with post-BD FEV_1 and FVC %predicted. Same day and 1-week and 4-month moving averages of carbon monoxide had the most consistent negative associations with post-BD FEV_1 (change(95% CI) per IQR: -0.3(-0.5,-0.2), -0.4(-0.6,-0.2), -0.6(-0.7,-0.1), respectively) and FVC (change(95% CI) per IQR: (-0.2(-0.3, -0.1), -0.3(-0.4, -0.1), -0.2(-0.4,-0.01), respectively). The 4-month average nitrogen dioxide was also negatively associated with post-BD FEV_1 and FVC % predicted (change(95% CI) per IQR: -0.2(-0.4, 0.01) and -0.2 (-0.4,-0.1), respectively). The evidence for negative effects on post-BD lung function seems week for 4-month average exposure to ozone (all-months) compared to the traffic pollutants. Sulfur dioxide was not associated with post-BD FEV_1 and FVC.

TABLE 5 City-specific and city-wide (meta-analysis) associations of within-subject 4-month average pollution concentration with post-BD FEV_1 and FVC %predicted

4-month average	City	IQR	post-BD FEV_1 Change	post-BD FVC Change
O_3 (ppb)	**ALB**	12	-1.69	**-1.78***
	BAL	15	0.31	1.04
	BOS	12	-1.02	-0.65
	DEN	12	0.61	**1.85***
	SD	10	-1.13	-0.14
	SEA	4	-1.35	-1.20
	STL	14	-2.37	-0.52
	TOR	10	0.74	-0.03
	Meta-analysis#	11	**-0.64****	-0.21
CO (ppmx10)	**ALB**	4	-0.35	-0.05
	BAL	3	-0.76	-0.02
	BOS	2	-0.10	0.10
	DEN	3	**-1.64***	**-1.57***
	SD	5	0.18	-0.19
	SEA	4	-0.14	-0.32
	STL	1	0.25	0.24
	TOR	4	-0.49	-0.09
	Meta-analysis#	3	**-0.37***	-0.17
NO_2 (ppb)	**ALB**	6	-0.03	-0.43
	BAL	4	-0.04	-0.05
	BOS	4	**-0.81***	**-0.83***
	DEN	6	**-0.78****	**-0.77***
	SD	7	0.03	0.26
	SEA	3	0.37	0.06
	STL	2	-0.23	-0.06
	TOR	3	-0.26	0.07
	Meta-analysis#	4	**-0.24***	**-0.23***
SO_2 (ppb)	**ALB**	NA	NA	NA
	BAL	3	0.06	-0.03
	BOS	5	-0.49	-0.23
	DEN	1	0.55	0.30
	SD	1	0.08	-0.16
	SEA	2	0.14	0.13
	STL	2	0.30	0.39
	TOR	2	-0.08	-0.06
	Meta-analysis#	2	0.08	0.09

ALB: Albuquerque, BAL: Baltimore, BOS: Boston, DEN: Denver, SD: San Diego, SEA: Seattle, STL: Saint Louis, TOR: Toronto, O_3: ozone (ppb); CO: carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); N: number of observations; IQR: interquartile range, ppb: part per billion; ppm: parts per million; IQR: Interquartile range, ppb: parts per billion; ppm: parts per million; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; BD: bronchodilator; CI: confidence interval; NA: not applicable

Fixed effects are used. When index of heterogeneity is > 50% the random effects are used

* $p<0.05$

** $0.05<=p<0.1$

The city-wide estimates of the meta-analysis were similar to the estimates given by the model with adjustment for city and city by sine/cosine function of time interactions. Table 5 shows the city-specific and meta-analysis estimates for long-term exposures. Meta-analysis estimates of associations of post-BD FEV_1 and FVC % predicted with the 4-month average gas concentrations are comparable to all-cities model estimates in Figure 1.

Table 6 presents the associations of pollution with pre-BD FEV_1 and FVC % predicted. Increases in the average carbon monoxide levels in the 4-months prior to and including the day of the visit were associated with decreases in pre-BD FEV_1 and FVC % predicted. Increases in the 4-month average of nitrogen dioxide also had a borderline significant association with reduced pre-BD FEV_1 and FVC % predicted. Increases in 4-month sulfur dioxide were associated with increases in pre-BD FVC % predicted.

TABLE 6 **Associations of within-subject pollution with pre-BD FEV_1 and FVC %predicted**

		Pre-BD FEV_1 %predicted			Pre-BD FVC %predicted		
Within-subject	**IQR**	**N**	**Change**	**(95%CI)**	**N**	**Change**	**(95%CI)**
O_3 same day	15	11394	-0.02	(-0.27, 0.24)	11393	0.09	(-0.11, 0.30
O_3 1-week m.a	14	11377	-0.15	(-0.53, 0.24)	11376	0.03	(-0.27, 0.33)
O_3 4-month m.a	11	11140	-0.50	(-1.17, 0.17)	11139	-0.25	(-0.78, 0.28)
CO same day	5	12044	-0.13	(-0.29, 0.02)	12043	-0.12	(-0.24; 0.00)
CO 1-week m.a	4	12047	**-0.2****	**-(0.39, 0.00)**	12046	-0.15	(-0.30, 0.01)
CO 4-month m.a	3	12048	**-0.4***	**(-0.62, -0.10)**	12047	**-0.2***	**(-0.42, -0.01)**
NO_2 same day	10	11523	-0.07	(-0.25, 0.10)	11522	-0.003	(-0.14; 0.14)
NO_2 1-week m.a	7	11494	-0.04	(-0.23, 0.15)	11493	0.02	(-0.13; 0.16)
NO_2 4-month m.a	4	11502	**-0.1****	**(-0.31, 0.09)**	11501	**-0.1****	**(-0.23; 0.09)**
SO_2 same day	4	10530	0.10	(-0.05, 0.25)	10529	0.06	(-0.06, 0.17)
SO_2 1-week m.a	3	10537	0.14	(-0.04, 0.33)	10536	0.12	(-0.03;0.26)
SO_2 4-month m.a	2	10540	0.15	(-0.08, 0.39)	10539	**0.23***	**(0.05,0.42)**

O_3: ozone (ppb); CO: carbon monoxide (ppm x10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppb: parts per billion; ppm: parts per million; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; BD: bronchodilator; CI: confidence interval

* $p<0.05$

** $0.05<=p<0.1$

Associations of long-term (4-month average) exposure with FEV_1 /FVC are shown is Figure 2. Reduced post-BD FEV_1 /FVC was associated with 4-month averages of ozone and carbon monoxide, but not with nitrogen dioxide or

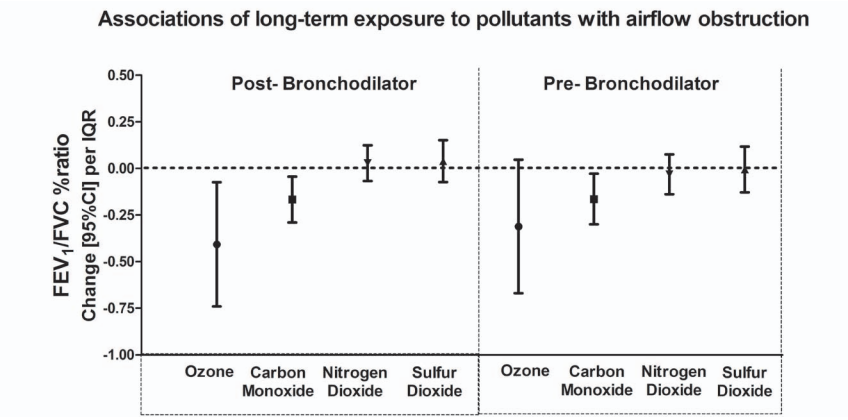


Figure 2: Ambient air pollution is associated with airflow obstruction
Figure 2 shows the associations of long-term (4-month average) exposure with FEV_1 / FVC. Reduced post-bronchodilator (BD) FEV_1/FVC (left graph panel) was associated with 4-month averages of ozone and carbon monoxide, but not with nitrogen dioxide or sulfur dioxide. Carbon monoxide also associated with pre-BD ratio (right graph panel)

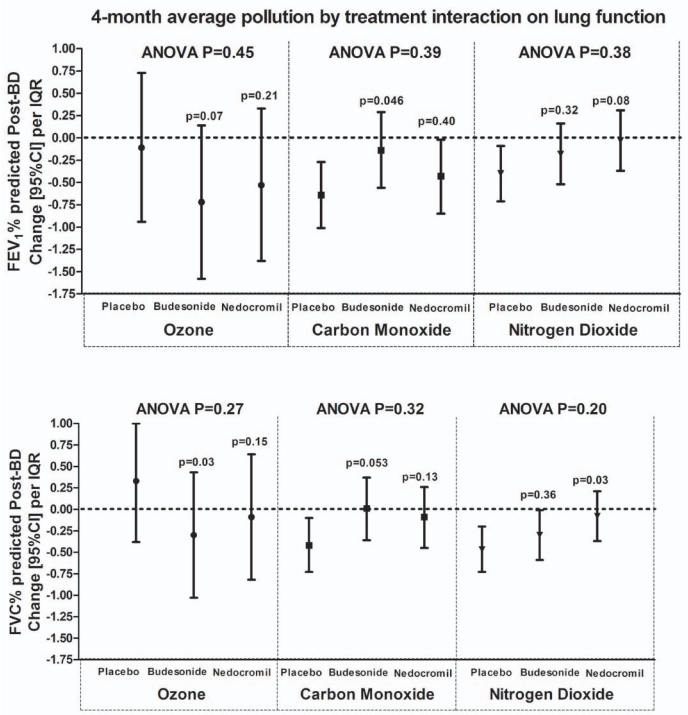


Figure 3: Weak evidence of modification of pollution's effect on lung function by treatment
Figure 3 shows long-term (4-month moving average) pollution effect modification by treatment. Although there were differences in the magnitude of long-term pollution effect between placebo and budesonide or placebo and nedocromil (p-values for interactions), the overall likelihood ratio tests (ANOVA) were not significant .

sulfur dioxide (change (95% CI) per IQR pollution increase:-0.4(-0.8-0.1), -0.2 (-0.3,-0.03), 0.03(-0.1,0.1), 0.03(-0.1,0.1), respectively). Similar associations were found with pre-BD FEV_1/FVC (change(95%CI) per IQR:-0.3(-0.7,0.06), -0.2 (-0.3,-0.02), -0.03(-0.1,0.1), -0.01(-0.1,0.1), respectively).

There was weak evidence of modification of pollution effect on lung function by treatment (Figure 3). Although there were differences in the magnitude of long-term pollution effect between placebo and budesonide or placebo and nedocromil (p-values for interactions ranging from 0.03 to 0.50), the overall likelihood ratio tests were not significant (ANOVA p>0.05; tables E2-E5 in the online repository).

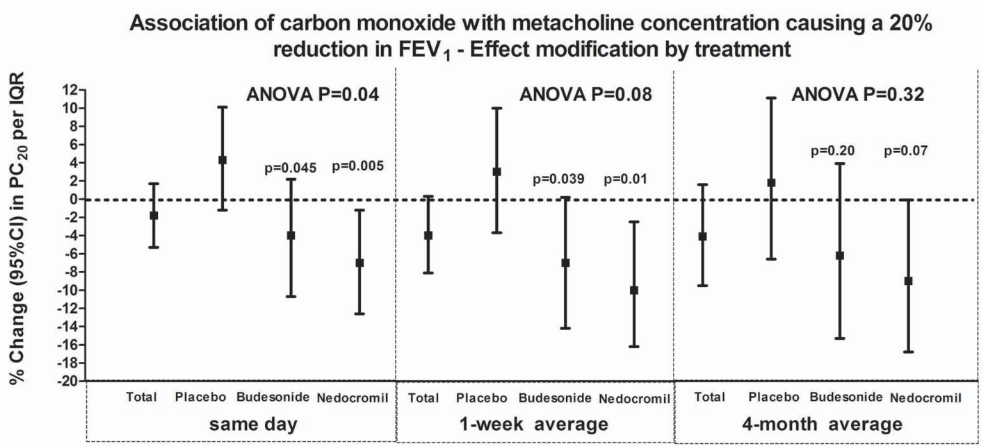


Figure 4 legend: Short-term effect of carbon monoxide on airway hyperresponsiveness is modified by treatment use

Figure 4 shows associations of carbon monoxide with metacholine concentration causing a 20% reduction in FEV_1 (PC_{20}) and effect modification by treatment. Carbon monoxide had a marginal overall effect on PC_{20} for all averaging periods. Compared to children on placebo, children on budesonide and nedocromil had a greater drop in PC_{20} with same day (left graph panel) and 1-week (middle graph panel) average exposures to carbon monoxide (ANOVA p= 0.04 and 0.08, respectively).

Association of pollution with PC_{20}

Overall, the only pollutant that was significantly associated with PC_{20} was the 4-month average sulfur dioxide level (% change(95%CI) per IQR: -6 (-11,-1.5)). Carbon monoxide had a marginal overall effect on PC_{20} for all averaging periods (Figure 4). Compared to children on placebo, children on budesonide and nedocromil had a greater drop in PC_{20} with same day and 1-week average exposures to carbon monoxide (ANOVA p= 0.04 and 0.08, respectively). This was more prominent for nedocromil. Treatment did not modify associations

of sulfur dioxide with PC_{20}. Tables E6 and E7 in the online repository show the associations for all pollutants with PC_{20} and the results of interactions with treatment.

Associations of asthma outcomes with warm-month (May – September) ozone were not statistically significant (Table E8 in the online repository). Two-pollutant models showed similar pollutant-asthma outcomes associations as one-pollutant models (Table E9 in the online repository).

DISCUSSION

Short-term adverse effects of pollution on children's pulmonary health have been extensively studied, meta-analyzed, and systematically reviewed. These studies provide strong evidence that short-term exposures to air pollution can increase airflow obstruction in asthmatic and non-asthmatic children,[20] and that long-term traffic pollution may increase incident asthma and reduce level of lung function in general populations of children.[2,21-24] There are fewer studies considering the effects of long-term exposures to pollution on lung function in asthmatic children,[1,2,20] and none that we know of evaluating long-term effects of pollution on lung function and AHR in asthmatic children in the context of a clinical trial.

In this unique asthma intervention trial, increases in the average long-term (4-month) concentrations of ozone, carbon monoxide and nitrogen dioxide were all associated with reductions in lung function levels consistent with airflow obstruction, and some decrease in vital capacity represented by a drop in FVC. Additionally, an increase in short-term concentration of carbon monoxide, but not for other pollutants, was also associated with reduced lung function.

Endogenous hypoxic-induced carbon monoxide is a mediator of vasodilation and bronchodilation; and in low doses it has been considered as a therapy after organ transplantation because of its beneficial immune effects.[25-29] In asthma, animal studies suggest that acutely inhaled 250-500ppm carbon monoxide decreases allergic inflammation and AHR in mice.[30,31] In contrast, our study shows worsening of AHR and lung function with short-term exposures to carbon monoxide in children with mild-to-moderate asthma. However, the mean concentration of carbon monoxide in our study was much lower (0.9 ppm) than those reported to have beneficial effects and also well below the levels needed to produce high levels of carboxyhemaglobin and tissue hypoxia with clinical symptoms.[32,33] This implies that carbon monoxide effects might

depend on the dose and/or duration of exposure. Similar to our finding, one study in adult asthmatics has reported associations of reduced lung function with short-term exposures to carbon monoxide, but the mechanisms for this association is not known.[34]

Motor vehicles emissions are major sources of carbon monoxide. These source produces many contaminants - such as fine particles and organic compounds - thus in this case it is likely that carbon monoxide is a surrogate for other pollutants,[2,35,36]and that the observed associations might not be due to carbon monoxide per se, but due to other pollutants in traffic emission mixtures. Similarly, nitrogen dioxide may be a marker for complex pollutant mixtures of pollutants emitted by the same sources or related through complex atmospheric reactions. Primary traffic-related pollutants such as elemental/black carbon or freshly emitted primary particles and secondary pollutants, including ozone, are often correlated with nitrogen dioxide.[2,35,36] In the present study, air pollutant levels were correlated such that it was difficult to separate out the contributions of the individual pollutants. All effects estimates on asthma outcomes remained significant after controlling for co-pollutants in multi-pollutant models.

The two pollutants associated with traffic sources—carbon monoxide and nitrogen dioxide—were most strongly and consistently associated with reduced level of lung function and more severe hyperresponsiveness in our children with asthma. Those were the same pollutants that influenced asthma exacerbations pre-randomization in this CAMP trial.[6] However, sulfur dioxide which originates predominately from diesel combustion (diesel fuel content of sulfur was higher during 1990's) and non-traffic fossil fuels (e.g., coal burning power plants and domestic heating), was also associated with enhanced response to metacholine long-term. Most studies investigating the latter association are based on short-term exposures to diesel and they have shown that air pollution may enhance the responsiveness to metacholine as well as to inhaled allergens in sensitized subjects.[37-40] One animal study has suggested an increase in AHR with long-term sulfur dioxide exposure, supporting our findings for this pollutant.[41] However, our study is unique in the investigation of AHR and its relation with short-term as well as long-term in asthmatic children.

We also found a positive association of long-term exposure to sulfur dioxide with pre-BD FVC. Again, experimental and epidemiological studies investigating lung function response to sulfur dioxide focus on acute responses and this makes them difficult to be compared with our finding. Most epidemiologic findings show modest negative or null association of sulfur dioxide with lung

function.[42-44] Generally, it is suggested that after acute exposure to sulfur dioxide the lung function returns to normal after some minutes to hours and that there is a great deal of inter-individual variation in response to sulfur dioxide.[45-47]

Ozone is the most important tropospheric oxidant which is formed through photochemical reactions involving nitrogen dioxide and hydrocarbons. Ozone initiates intracellular oxidative stress and is linked to chronic damage and effects on to the human lung with prolonged exposure.[48] Cumulative exposure is a function of both the rate and duration of exposure and it has been shown that effects of pollution on children's health have greater impact if the children exercise outdoors.[49-52] We also show that longer exposure to ozone is associated with airflow obstruction, indicated by decrease in FEV_1/FVC with increase in 4-month average ozone concentration.

For ozone, associations of reduced lung function with pollution also tended to be stronger for children on budesonide compared to placebo. Two recent studies have suggested that children on ICS were more vulnerable to the adverse effects of ozone and other air pollutants.[13,14] The authors speculated that the observed associations might be explained by the fact that children on ICS are more likely to have worse asthma and that confounding by indication might exist. The design of our trial prevents confounding by indication because of the double-blinded randomized distribution of treatment to children of similar asthma severity. Although the evidence for the interaction of ICS treatment on ozone mediated effects is weak, it is plausible that the children on the ICS had greater exposure to ozone compared to placebo, either because they were more likely to spend more time outside and exercise more due to better control of their asthma,[15,18] or because they had greater minute ventilation because they were able to breathe more deeply when exercising.

In our study, modification of carbon monoxide effects on AHR by anti-inflammatory treatment suggests that use of controller medication may not protect asthmatic children from pollutant effects. The worsening of AHR with short-term exposure to carbon monoxide was stronger for children on budesonide and nedocromil compared to placebo, a finding that needs further investigation.

The present report provides a unique contribution in that it can be considered a meta-analysis of eight large, within-city panel studies. Yet, it does not suffer from many of the challenges associated with meta-analyses in the published literature (e.g., between-study heterogeneity and obvious publication bias). The large and geographically diverse panel of children participating in CAMP trial was followed from December 1993 to June 1999, on average for

4 years. This allowed us to examine the health effects of ambient concentrations of carbon monoxide, nitrogen dioxide, ozone and sulfur dioxide across seasons and geographic regions and results from this study may be applicable to a broad population.

Many studies investigating the long-term effects of pollution have focused on traffic-related exposures and used surrogate measures such as distance to major roads, road density or vehicle density.[2,20,21,23,53,54] In this study we measured daily pollutant concentrations to predict long-term (4-month (but also acute (same day) and intermediate (1-week)) effects on asthma severity in children. We acknowledge that exposure is at the zip/postal code rather than the residence level. However, we limited exposure misclassification bias in two ways: 1) by using zip/postal code level concentrations of pollution instead of averaging monitor-specific concentrations by city; and 2) by restricting the period of interested to the period of the trial for which the great majority of the participants attended all visits. In addition, we investigated pollutants that tend to be regional and we also focus on long-term exposure which is less prone to misclassification.

We conclude that exposure to gaseous pollutants adversely influences level of lung function and AHR in asthmatic children, and treatment use modifies the short-term effects of carbon monoxide on AHR. The longitudinal evaluation of children treated with daily asthma therapy in a clinical trial enabled us to separate the modification of pollution effects by treatment without confounding by indication.

ACKNOWLEDGEMENTS

We dedicate this manuscript to the memory of our friend and colleague Dr. Gail G. Shapiro who passed away unexpectedly during the development of this study. Dr. Shapiro dedicated her life to understanding the causes of childhood asthma and determining the best treatments for asthma. She is deeply missed by her colleagues, patients, and the asthma community.

The Childhood Asthma Management Program trial and CAMP Continuation Study were supported by contracts NO1-HR-16044, 16045, 16046, 16047, 16048, 16049, 16050, 16051, and 16052 with the National Heart, Lung, and Blood Institute and General Clinical Research Center grants M01RR00051, M01RR0099718-24, M01RR02719-14, and RR00036 from the National Center for Research Resources. The CAMP Continuation Study/Phases 2 and 3 were supported by grants U01HL075232, U01HL075407, U01HL075408, U01HL075409, U01HL075415, U01HL075416, U01HL075417, U01HL075419, U01HL075420, andU01HL075408 from the National Heart, Lung, and Blood Institute. The National Jewish Health site was also supported in part by Colorado CTSA grant UL1RR025780 from NCRR/NIH and UL1TR000154 from NCATS/NIH. C:\lm_camp\misc\campdua.wpd June 1, 2013

FINANCIAL SUPPORT

This study was funded by: 5 P01 HL 083069-05 NIH/NHLBI/P01; NIEHS PO1 ES-09825, US EPA RD-83241601, US EPA grant RD 83479801 and the International Initiative for Environment and Public Health Cyprus Program of HSPH. The contents of this publication are solely the responsibility of the grantee and do not necessarily represent the official views of the USEPA. Further, USEPA does not endorse the purchase of any commercial products or services mentioned in the publication.

REFERENCES

1. World Health Organization. Effects of air pollution on children' s health and developement. A review of the evidence. Bonn. 2005; Available at: http://www.euro.who.int/__data/assets/pdf_file/0010/74728/E86575.pdf. Accessed April, 2013.
2. HEI on the Health Effects of Traffic-Related Air Pollution. Traffic-related Air Pollution: A critical Review of the Literature on Emissions, Exposure, and Health Effects. 2010;HEI Special Report 17.
3. Searing DA, Rabinovitch N. Environmental pollution and lung effects in children. Curr Opin Pediatr 2011 Jun;23:314-318.
4. Trasande L, Thurston GD. The role of air pollution in asthma and other pediatric morbidities. J Allergy Clin Immunol 2005 Apr;115:689-699.
5. Tzivian L. Outdoor air pollution and asthma in children. J Asthma 2011 Jun;48:470-481.
6. Schildcrout JS, Sheppard L, Lumley T, Slaughter JC, Koenig JQ, Shapiro GG. Ambient air pollution and asthma exacerbations in children: an eight-city analysis. Am J Epidemiol 2006 Sep 15;164:505-517.
7. Sunyer J, Spix C, Quenel P, Ponce-de-Leon A, Ponka A, Barumandzadeh T, Touloumi G, Bacharova L, Wojtyniak B, Vonk J, Bisanti L, Schwartz J, Katsouyanni K. Urban air pollution and emergency admissions for asthma in four European cities: the APHEA Project. Thorax 1997 Sep;52:760-765.
8. Liu L, Poon R, Chen L, Frescura AM, Montuschi P, Ciabattoni G, Wheeler A, Dales R. Acute effects of air pollution on pulmonary function, airway inflammation, and oxidative stress in asthmatic children. Environ Health Perspect 2009 Apr;117:668-674.
9. Romieu I, Meneses F, Ruiz S, Sienra JJ, Huerta J, White MC, Etzel RA. Effects of air pollution on the respiratory health of asthmatic children living in Mexico City. Am J Respir Crit Care Med 1996 Aug;154:300-307.
10. Kelly FJ. Oxidative stress: its role in air pollution and adverse health effects. Occup Environ Med 2003 Aug;60:612-616.
11. Patel MM, Chillrud SN, Deepti KC, Ross JM, Kinney PL. Traffic-related air pollutants and exhaled markers of airway inflammation and oxidative stress in New York City adolescents. Environ Res 2013 Feb;121:71-78.
12. Delfino RJ, Zeiger RS, Seltzer JM, Street DH, McLaren CE. Association of asthma symptoms with peak particulate air pollution and effect modification by anti-inflammatory medication use. Environ Health Perspect 2002 Oct;110:A607-17.
13. Zora JE, Sarnat SE, Raysoni AU, Johnson BA, Li WW, Greenwald R, Holguin F, Stock TH, Sarnat JA. Associations between urban air pollution and pediatric asthma control in El Paso, Texas. Sci Total Environ 2013 Mar 15;448:56-65.
14. Lewis TC, Robins TG, Mentz GB, Zhang X, Mukherjee B, Lin X, Keeler GJ, Dvonch JT, Yip FY, O'Neill MS, Parker EA, Israel BA, Max PT, Reyes A, Community Action Against Asthma (CAAA) Steering Committee. Air pollution and respiratory symptoms among children with asthma: Vulnerability by corticosteroid use and residence area. Sci Total Environ 2013 Mar 15;448C:48-55.
15. Strunk RC, Childhood Asthma Management Program Research Group. Childhood Asthma Management Program: lessons learned. J Allergy Clin Immunol 2007 Jan;119:36-42.
16. Anonymous. The Childhood Asthma Management Program (CAMP): design, rationale, and methods. Childhood Asthma Management Program Research Group. Control Clin Trials 1999 Feb;20:91-120.
17. Cohen RT, Raby BA, Van Steen K, Fuhlbrigge AL, Celedon JC, Rosner BA, Strunk RC, Zeiger RS, Weiss ST, Childhood Asthma Management Program Research Group. In utero smoke exposure and impaired response to inhaled corticosteroids in children with asthma. J Allergy Clin Immunol 2010 Sep;126:491-497.

18. Anonymous. Long-term effects of budesonide or nedocromil in children with asthma. The Childhood Asthma Management Program Research Group. N Engl J Med 2000 Oct 12;343:1054-1063.

19. Stolwijk AM, Straatman H, Zielhuis GA. Studying seasonality by using sine and cosine functions in regression analysis. J Epidemiol Community Health 1999 Apr;53:235-238.

20. Li S, Williams G, Jalaludin B, Baker P. Panel studies of air pollution on children's lung function and respiratory symptoms: a literature review. J Asthma 2012 Nov;49:895-910.

21. Gao Y, Chan EY, Li LP, He QQ, Wong TW. Chronic effects of ambient air pollution on lung function among Chinese children. Arch Dis Child 2013 Feb;98:128-135.

22. Rosenlund M, Forastiere F, Porta D, De Sario M, Badaloni C, Perucci CA. Traffic-related air pollution in relation to respiratory symptoms, allergic sensitisation and lung function in schoolchildren. Thorax 2009 Jul;64:573-580.

23. Gauderman WJ, Vora H, McConnell R, Berhane K, Gilliland F, Thomas D, Lurmann F, Avol E, Kunzli N, Jerrett M, Peters J. Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. Lancet 2007 Feb 17;369:571-577.

24. Schwartz J. Lung function and chronic exposure to air pollution: a cross-sectional analysis of NHANES II. Environ Res 1989 Dec;50:309-321.

25. Thorup C, Jones CL, Gross SS, Moore LC, Goligorsky MS. Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS. Am J Physiol 1999 Dec;277:F882-9.

26. Cardell LO, Lou YP, Takeyama K, Ueki IF, Lausier J, Nadel JA. Carbon monoxide, a cyclic GMP-related messenger, involved in hypoxic bronchodilation in vivo. Pulm Pharmacol Ther 1998;11:309-315.

27. Kinhult J, Uddman R, Cardell LO. The induction of carbon monoxide-mediated airway relaxation by PACAP 38 in isolated guinea pig airways. Lung 2001;179:1-8.

28. Nakao A, Choi AM, Murase N. Protective effect of carbon monoxide in transplantation. J Cell Mol Med 2006 Jul-Sep;10:650-671.

29. Kourembanas S. Hypoxia and carbon monoxide in the vasculature. Antioxid Redox Signal 2002 Apr;4:291-299.

30. Ameredes BT, Otterbein LE, Kohut LK, Gligonic AL, Calhoun WJ, Choi AM. Low-dose carbon monoxide reduces airway hyperresponsiveness in mice. Am J Physiol Lung Cell Mol Physiol 2003 Dec;285:L1270-6.

31. Chapman JT, Otterbein LE, Elias JA, Choi AM. Carbon monoxide attenuates aeroallergen-induced inflammation in mice. Am J Physiol Lung Cell Mol Physiol 2001 Jul;281:L209-16.

32. Raub JA, Mathieu-Nolf M, Hampson NB, Thom SR. Carbon monoxide poisoning--a public health perspective. Toxicology 2000 Apr 7;145:1-14.

33. Reboul C, Thireau J, Meyer G, Andre L, Obert P, Cazorla O, Richard S. Carbon monoxide exposure in the urban environment: an insidious foe for the heart? Respir Physiol Neurobiol 2012 Nov 15;184:204-212.

34. Canova C, Torresan S, Simonato L, Scapellato ML, Tessari R, Visentin A, Lotti M, Maestrelli P. Carbon monoxide pollution is associated with decreased lung function in asthmatic adults. Eur Respir J 2010 Feb;35:266-272.

35. World Health Organization. Health Aspects of Air Pollution with Particulate Matter, Ozone and Nitrogen Dioxide. Report on a WHO Working Group. 2003; Available at: http://www.euro.who.int/__data/assets/pdf_file/0005/112199/E79097.pdf. Accessed May, 2013.

36. Sarnat JA, Brown KW, Schwartz J, Coull BA, Koutrakis P. Ambient gas concentrations and personal particulate matter exposures: implications for studying the health effects of particles. Epidemiology 2005 May;16:385-395.

37. Jang AS, Choi IS, Lee JH, Park CS, Park CS. Prolonged ozone exposure in an allergic airway disease

model: adaptation of airway responsiveness and airway remodeling. Respir Res 2006 Feb 13;7:24.

38. Svartengren M, Strand V, Bylin G, Jarup L, Pershagen G. Short-term exposure to air pollution in a road tunnel enhances the asthmatic response to allergen. Eur Respir J 2000 Apr;15:716-724.

39. Nordenhall C, Pourazar J, Ledin MC, Levin JO, Sandstrom T, Adelroth E. Diesel exhaust enhances airway responsiveness in asthmatic subjects. Eur Respir J 2001 May;17:909-915.

40. Miyabara Y, Takano H, Ichinose T, Lim HB, Sagai M. Diesel exhaust enhances allergic airway inflammation and hyperresponsiveness in mice. Am J Respir Crit Care Med 1998 Apr;157:1138-1144.

41. Song A, Liao Q, Li J, Lin F, Liu E, Jiang X, Deng L. Chronic exposure to sulfur dioxide enhances airway hyperresponsiveness only in ovalbumin-sensitized rats. Toxicol Lett 2012 Nov 15;214:320-327.

42. Aekplakorn W, Loomis D, Vichit-Vadakan N, Shy C, Wongtim S, Vitayanon P. Acute effect of sulphur dioxide from a power plant on pulmonary function of children, Thailand. Int J Epidemiol 2003 Oct;32:854-861.

43. Peters A, Goldstein IF, Beyer U, Franke K, Heinrich J, Dockery DW, Spengler JD, Wichmann HE. Acute health effects of exposure to high levels of air pollution in eastern Europe. Am J Epidemiol 1996 Sep 15;144:570-581.

44. Hoek G, Brunekreef B. Effects of low-level winter air pollution concentrations on respiratory health of Dutch children. Environ Res 1994 Feb;64:136-150.

45. Lawther PJ, Macfarlane AJ, Waller RE, Brooks AG. Pulmonary function and sulphur dioxide, some preliminary findings. Environ Res 1975 Dec;10:355-367.

46. Winterton DL, Kaufman J, Keener CV, Quigley S, Farin FM, Williams PV, Koenig JQ. Genetic polymorphisms as biomarkers of sensitivity to inhaled sulfur dioxide in subjects with asthma. Ann Allergy Asthma Immunol 2001 Feb;86:232-238.

47. Nowak D, Jorres R, Berger J, Claussen M, Magnussen H. Airway responsiveness to sulfur dioxide in an adult population sample. Am J Respir Crit Care Med 1997 Oct;156:1151-1156.

48. Lodovici M, Bigagli E. Oxidative stress and air pollution exposure. J Toxicol 2011;2011:487074.

49. Millstein J, Gilliland F, Berhane K, Gauderman WJ, McConnell R, Avol E, Rappaport EB, Peters JM. Effects of ambient air pollutants on asthma medication use and wheezing among fourth-grade school children from 12 Southern California communities enrolled in The Children's Health Study. Arch Environ Health 2004 Oct;59:505-514.

50. McConnell R, Berhane K, Gilliland F, London SJ, Islam T, Gauderman WJ, Avol E, Margolis HG, Peters JM. Asthma in exercising children exposed to ozone: a cohort study. Lancet 2002 Feb 2;359:386-391.

51. Spektor DM, Thurston GD, Mao J, He D, Hayes C, Lippmann M. Effects of single- and multiday ozone exposures on respiratory function in active normal children. Environ Res 1991 Aug;55:107-122.

52. Gauderman WJ, Gilliland GF, Vora H, Avol E, Stram D, McConnell R, Thomas D, Lurmann F, Margolis HG, Rappaport EB, Berhane K, Peters JM. Association between air pollution and lung function growth in southern California children: results from a second cohort. Am J Respir Crit Care Med 2002 Jul 1;166:76-84.

53. Brunekreef B, Janssen NA, de Hartog J, Harssema H, Knape M, van Vliet P. Air pollution from truck traffic and lung function in children living near motorways. Epidemiology 1997 May;8:298-303.

54. Perez L, Declercq C, Iniguez C, Aguilera I, Badaloni C, Ballester F, Bouland C, Chanel O, Cirarda F, Forastiere F, Forsberg B, Haluza D, Hedlund B, Cambra K, Lacasana M, Moshammer H, Otorepec P, Rodriguez-Barranco M, Medina S, Kunzli N. Chronic burden of near-roadway traffic pollution in 10 European cities (APHEKOM network). Eur Respir J 2013 Mar 21.

CHAPTER 6

Pathway analysis of a genome-wide gene by air pollution interaction study in asthma

Despo Ierodiakonou[1,2], Brent A. Coull[3], Antonella Zanobetti[4], Steve Melly[4], Dirkje S. Postma[5,2], H. Marike Boezen[1,2], Judith M. Vonk[1,2], Paul V. Williams[6], Gail G. Shapiro[6], Edward F. McKone[7], Teal S. Hallstrand[8], Jane Q. Koenig[9], Jonathan S. Schildcrout[10], Gerard H. Koppelman[2,11], Thomas Lumley[12], Damien D.C. Croteau-Chonka[13], Anne N. Fuhlbrigge[13,14], Petros Koutrakis[4], Joel Schwartz[4], Diane R Gold[4,13], Scott T. Weiss[13,14]

1. University of Groningen, University Medical Center Groningen, Department of Epidemiology, The Netherlands

2. Groningen Research Institute for Asthma and COPD, University of Groningen, University Medical Center Groningen, The Netherlands

3. Department of Biostatistics Harvard School of Public Health, Boston, MA

4. Environmental Epidemiology and Risk Program, Harvard School of Public Health, Boston, MA

5. University of Groningen, University Medical Center Groningen, Department of Pulmonology, The Netherlands

6. Department of Pediatrics, School of Medicine, University of Washington Seattle, WA

7. Department of Respiratory Medicine, St. Vincent University Hospital, Dublin, Ireland

8. Department of Medicine, School of Medicine, University of Washington Seattle, WA

9. Department of Environmental and Occupational Health Sciences, School of Public Health, University of Washington Seattle, WA

10. Department of Biostatistics, School of Medicine, Vanderbilt University Nashville, TN

11. University of Groningen, University Medical Center Groningen , Department of Pediatric Pulmonology and Pediatric Allergology- Beatrix Children Hospital, Groningen, The Netherlands

12. Department of Biostatistics, University of Auckland, New Zealand

13. Channing Laboratory, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, MA

14. for the Childhood Asthma Management Program Research Group

ABSTRACT

Background: Genetics plays a role in the respiratory response of asthmatic children to air pollution, but the genes involved are unknown.

Methods: We investigated whether genes modify effects of nitrogen dioxide (NO_2) and carbon monoxide (CO) on lung function in 522 Caucasian (primary study) and 88 African-American (replication study) asthmatic children from the Childhood Asthma Management Program (CAMP) trial. We used the conditional two-step method for fast linear mixed model (LMM) computations for genome-wide association studies, exploring whether Single Nucleotide Polymorphisms (SNPs) modify the longitudinal relationship between 4-month average pollution and post-bronchodilator FEV1 %predicted values for 4 years. We used the improved gene-set enrichment analysis for genome-wide association studies (i-GSEA4GWAS) for a pathway-level analysis of our genome-wide interaction output.

Results: Three variants (rs13090972, rs958144, rs7041938) showed genome wide statistically significant evidence of interaction with NO_2 on FEV1 %predicted in Caucasians and four additional variants replicated in both Caucasians and African-Americans (rs2720625 (x CO) and rs4672884, rs3769767, rs4378142 (x NO_2)). The top/replicated genome-wide SNPs are associated with redox balance and inflammation. The pathway analysis for NO_2-related FEV1 responses revealed oxidative stress, the Heme oxygenase-1/CO system, calcium homeostasis and metabolic responses. For CO-related FEV1 responses, we also identified association with a pathway linked to the heme oxyganase-1/CO system.

Conclusion: The host lung function response to traffic-related air pollution is linked to genes involved in oxidative stress and inflammatory pathways with subsequent cytotoxicity, while metabolic pathways, calcium homeostasis and the HO-1/CO pathway play a role in cytoprotection. Supported by P01 HL HL083069.

INTRODUCTION

Systematic review of epidemiological studies has demonstrated a strong association between exposure to ambient air pollution and adverse effects on childhood respiratory health.[1-4] Children with underlying chronic lung disease are likely to be more susceptible to the negative effects of air pollution. In asthma, this results in more severe disease, e.g. higher short-term exposure to pollution has been associated with more symptoms, increased need for reliever medication and hospital admissions.[5-8] Moreover, short-term exposure to air pollution is associated with lower lung function levels in asthmatic and non-asthmatic children,[1,9,10] but the long-term effects of pollution on lung function are less well studied in asthmatic children.[9,11-14] We recently investigated the longitudinal relationship between the 4-month average exposure to air pollution and post bronchodilator (BD) forced expiratory volume in 1 second (FEV_1) and showed that long-term exposures to carbon monoxide (CO) and nitrogen dioxide (NO_2) are associated with reduced levels of FEV_1 in children with asthma.(Ierodiakonou et al. unpublished)

Respiratory response to air pollution varies between individuals and genetic susceptibility likely plays a role.[15] Known biological mechanisms by which air pollution can impair health include autonomic dysfunction, oxidative stress, and systemic inflammatory responses.[16-19] In general, the majority of studies have focused on genes involved in anti-oxidant pathways and airway inflammation, and how those may modify responses to ozone.[15,20-22]. A more recent study on lung function growth in children showed that variations in several detoxifying genes modify the adverse effects not only of ozone but also of NO_2, particulate matter, elemental carbon and organic carbon.[23]

In asthma, genes play a role in determining the susceptibility to the harmful effects of air pollution. An example is a recent study showing that asthmatic children in Mexico City with risk alleles in *GSTM1* gene and low intake of Vitamin C may be more susceptible to the acute effects of ozone on their lung function compared to those with the non-risk alleles.[24] The underlying biological mechanisms of air pollution-mediated health effects are not fully understood and the identification of genes and pathways that might be involved warrants further study.

We used a hypothesis-free, genome-wide analysis to investigate whether genetic variants modify the long-term effects of CO and NO_2 on post-BD FEV_1 in children with asthma. We found a set of gene variants interacting with air pollution in two ethnic groups, and with a pathway-level analysis of the genome-wide interaction results we show that we may have identified biological

plausible pathways mediating the effect of CO and NO_2 on lung function in asthmatic children.

MATERIALS AND METHODS

The CAMP study design and methods have been described elsewhere.[25] Additional detail on all methods used in the present report is provided in an online data supplement. In summary, children enrolled in CAMP were 5–12 years of age and were hyperresponsive to methacholine at study entry. 1,041 Children entered the randomization phase and 311, 312, 418 children received budesonide, nedocromil, and placebo, respectively. All subjects were treated and followed for four years with visits at two and four months after randomization and at four-month intervals thereafter. Each parent or guardian signed a consent form and participants of 7 years of age and older signed an assent form approved by each clinical center's institutional review board.

Spirometry, before and after the administration of two puffs of albuterol (bronchodilator) was conducted at randomization (RZ) and at follow up visits (n=13) according to the American Thoracic Society Standards.[26]

Monitoring data on 24-hour average concentrations of CO and NO_2 were obtained for each metropolitan area. The ZIP or postal code centroid coordinates were used to link participants to daily concentrations from the nearest monitor within 50 km that did not have missing data on that day (December 1993 through June 1999).

Genome-wide single nucleotide polymorphisms (SNP) genotyping for CAMP subjects was performed on Illumina's HumanHap550 Genotyping BeadChip (Illumina, Inc., San Diego, CA).

Statistical Analysis

Genome-wide interaction study

In a genome-wide interaction analysis the effort needed to evaluate the effects of hundreds of thousands SNPs on the longitudinally measured trait is prohibitively large with a classical linear mixed model (LMM) approach. We followed the Sikorska et al. conditional two-step approach for fast linear mixed model computations for genome-wide association studies (GWAS),[27] a method to explore whether the longitudinal relationship between 4-month averaged pollution (CO and NO_2) and post-BD FEV_1%predicted is modified by single nucleotide polymorphisms (SNPs) in the human genome. Details are given in the supplementary material.

In summary, in the first step we fitted a LMM with subject-specific (random) intercept and slope for pollution exposure with all SNP terms (main effect and interaction with pollutant) omitted from the model. LMM tests were performed in R programming language (version 2.15.1; 2012-06-22).

At the second step, simple linear regression genome-wide association tests of SNPs with an individual's FEV_1 response to CO and NO_2 (subject-specific/random slopes of pollution as given by LMM in step1), respectively were performed in PLINK (version 1.07; 2009-09-10; Shaun Purcell; http://pngu.mgh.harvard.edu/purcell/plink/),[28] using an additive allelic model. SNPs included in the genome-wide analysis had a minor allele frequency (MAF) > 5%.

We selected the top signals for SNP-pollution interaction in Caucasian CAMP subjects (*P*-value <9.9E-5) as given by the two-step approach and we used the classic LMM including terms of pollution, SNP and SNP by pollution interaction to assess the effect size of the interactions and confirm statistical significance.

Non-Hispanic white (Caucasian) CAMP subjects (n=522) were used as the primary study population and African-American (n=88) CAMP subjects served as the replication study population. We followed this replication approach because we could not find a second study with similar design, population characteristics, genome-wide genotyping and air pollution data.

Pathway-level analysis for the genome-wide SNP by pollutant interaction analysis

To analyze pathway-level SNP- pollutant interactions we used the improved gene-set enrichment analysis for GWAS (*i-GSEA4GWAS*; http://gsea4gwas.psych.ac.cn/inputPage.jsp).[29] GSEA evaluates whether the distribution of genes sharing a biochemical or cellular function is different from the distribution of a ranked genome-wide gene list.[29,30] Details on the *i-GSEA4GWAS* method are given in the supplementary material.

Input data to perform the pathway-level analysis of the SNP-pollution interaction analysis were *P*-values of the two-step genome-wide SNP-pollution interaction analysis in Caucasian CAMP subjects (n=522). To avoid overrepresentation of SNPs in more than one gene we restricted mapping SNPs to +/-20kb around a gene. The server transformed the genome-wide *P*-values to –log (*P*-values), mapped the represented genes and calculated the enrichment score. Next, the canonical pathway method of gene-sets was used for further analysis. These canonical pathways were extracted and curated from Molecular Signatures Database (MSigDB) v2.5 (http://www.broadinstitute.org/gsea/msigdb/).

TABLE 1	Population characteristics
	N= 1003
City; n (%)	
Albuquerque	121 (12.1)
Baltimore	126 (12.6)
Boston	123 (12.3)
Denver	141 (14.1)
San Diego	122 (12.2)
Seattle	136 (13.6)
Saint Louis	133 (13.3)
Toronto	101 (10.1)
Sex; n (%)	
Males/Females	602/401 (60/40)
Treatment Group; n (%)	
Placebo	407 (40.6)
Budesonide	298 (29.7)
Nedocromil	298 (29.7)
Ethnicity; n (%)	
Caucasians	677 (67.5)
African-Americans	137 (13.7)
Hispanics	97 (9.7)
Other	92 (9.2)
Annual Income =>30K USD; n (%)	
Yes/No	728/235 (76/24)
In utero smoking exposure; n (%)	
Yes/No	114/854 (14/86)
Pre bronchodilator lung function at randomization; mean (SD)	
FEV_1 % predicted	93.8 (14.3)
FVC % predicted	104.0 (13.1)
FEV_1/FVC %	79.7 (8.3)
Post bronchodilator lung function at randomization; mean (SD)	
FEV_1 % predicted	103.0 (12.8)
FVC % predicted	106.5 (12.8)
FEV_1/FVC %	85.5 (6.5)

FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; SD: standard deviation; =>30K USD: equal or more than 30,000 United State Dollars

MSigDB included gene-sets denoting canonical pathways integrated from a variety of online resources. We set our analysis to pathways with 5–200 genes.

Significant genes in a pathway are defined as the genes mapped with at least one of the top 5% of all SNPs (0.05* 474,792 = 23,740 SNPs). Each significant gene was represented by the SNP in that gene with the lowest genome-wide SNP-pollutant interaction *P*-value (top SNP per gene). We selected the top SNPs of all given pathways and with classic LMM we estimated the interaction effect size for the gene-sets most significant SNP-pollutant interactions in Caucasians.

RESULTS

All subjects in CAMP considered in this analysis were randomized and followed up during the trial period. A total of 1,003 of the 1,041 randomized children (96.3%) had pollution data available of whom 610 were studied in the genetic analysis. At study entry the mean (SD) age was 9 (2.1) and geometric mean (min-max) PC_{20} 1.1 (0.02-2.5) mg/ml. Table 1 shows the main characteristics of the participants. 82.5% of the children attended all visits during the 4-year trial (median number of completed visits=14 (range: 1-14)). Participants had a median of 10 (range: 1-10) post-BD lung function measurements.

The four-month moving averages of pollutant concentrations during December'93-June'99 are summarized in Table 2. We report the number of observations, percentiles and interquartile range (IQR) of CO and NO_2 concentrations. Table E1 shows the IQR of the overall and the within-subject concentration of pollutants, respectively.

TABLE 2 **Distribution of the 4-month average pollution concentrations**

	4-month average	Carbon Monoxide (ppm x10)	Nitrogen Dioxide (ppb)
N	Valid	12217	12809
	Missing	1825	1233
Percentiles	10	5	13
	25	6	18
	50	9	21
	75	12	26
	90	14	30
IQR	25-75 percentile	5	8

IQR: interquartile range, ppb: part per billion; ppm: parts per million

TABLE 3 **Replicated single nucleotide polymorphisms by pollutant interactions in two ethnicities**

Interacting pollutant	RACE	SNP	Minor allele	CHR	LMM approach CHANGE PER IQR	LMM approach P-value	Mapped gene/nearby gene
Carbon monoxide	AA	rs2720625	A	8	1.24	0.014	58kb 3'-UTR of Magnesium Uptake/Transporter (Tumor Suppressor Candidate 3 -TUSC3) gene
	CAUC				0.84	2.72E-05	
Combined P-value*						5.6E-06	
Nitrogen dioxide	AA	rs4672884	A	2	1.12	0.007	Paroxysmal nonkinesigenic dyskinesia (PNKD) or Myofibrillogenesis regulator 1(MR-1) gene
	CAUC				0.68	1.98E-05	
Combined P-value*						3.3E-06	
Nitrogen dioxide	AA	rs3769767	A	2	1.19	0.036	Methyltransferase like 5 (METTL5) gene
	CAUC				0.91	5.38E-06	
Combined P-value*						1.4E-06	
Nitrogen dioxide	AA	rs4378142	A	23	1.24	0.016	
	CAUC				0.70	2.82E-07	RP11-40F8.2 / ENSG00000233067
Combined P-value*						4.8E-08	

AA: African-American (n=88), CAUC: Caucasian (n=522) Childhood Asthma Management Program subjects; SNP: single nucleotide polymorphism, CHR: chromosome; LMM: linear mixed models; IQR: Interquartile range (carbon monoxide IQR= 0.3 and nitrogen dioxide IQR= 0.004 parts per million)
*MetaP: a program to combine P values (Stuffer's z trend);
Author: Dongliang Ge PhD, Duke University; URL: http://compute1.lsrc.duke.edu/softwares/MetaP/metap.php

Two-step genome-wide SNP by pollutant interaction analysis

Figure 1 presents an overview of our study design and results of the genome-wide SNP-pollutant(s) interaction analysis. After MAF pruning 474,792 SNPs were included in the primary analysis. In Caucasians (n=522) there was suggestive evidence (top signals: *P*-value <9.9E-5) for 47 SNP-CO interactions and 48 SNP- NO_2 interactions (tables E2 and E3, respectively). The quantile-quantile (QQ) plots of the two-step genome-wide SNP by pollutant interaction analysis revealed that the distribution of association *P*-values was similar to that expected for a null distribution and that no *P*-values met the conventional genome-wide statistically significant levels (e.g. Bonferroni corrected minimally significant *P*-value being 0.05/474,792=1.05E-07; Figures E1 and E2).

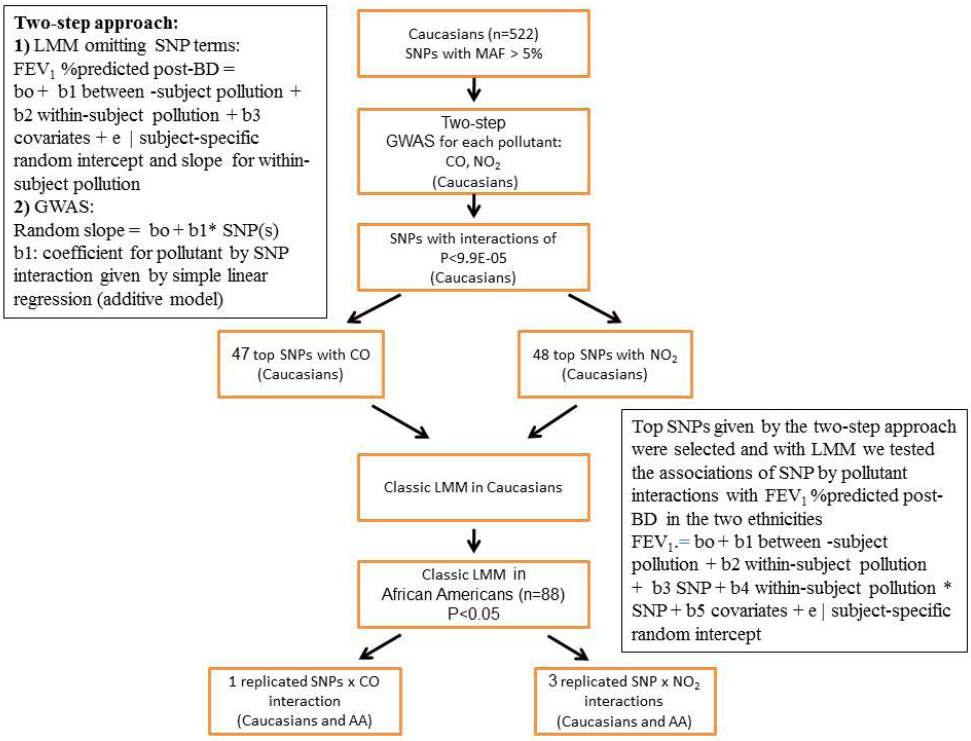


Figure 1 presents the flow chart with the analytic steps and summary of results of the genome-wide gene by pollutant(s) interaction study. Top hit SNPs (P<9.9E-5) interacting with pollutants in Caucasians were selected and with LMM we assessed the interaction effect size in Caucasians and for replication in African-Americans. CO: carbon monoxide; NO2: nitrogen dioxide; LMM: linear mixed model; SNP: single nucleotide polymorphism, GWAS: genome wide interaction study; MAF: minor allele frequency; AA: African Americans; FEV1: forces expiratory volume in 1 second

TABLE 4 **Improved-Gene Set Enrichment Analysis for GWAS suggested pathways for the 2 pollutants in Caucasian CAMP subjects**

CARBON MONOXIDE[1]

	Gene Set Name	Gene Set Description	Gene Set P Value	N*
1	ST_PAC1_RECEPTOR	The signaling peptide PACAP binds to its receptor, PAC1R, which activates adenylyl cyclase and phospholipase C.	0.001	4/6/8

NITROGEN DIOXIDE[2]

	Gene Set Name	Gene Set Description	Gene Set P Value	N*
1	NOS1	Glutamate stimulates NMDA-mediates calcium influx, which promotes nitric oxide synthesis from arginine by neuronal nitric oxide synthase, activating guanylate cyclase.	0.001	12/20/22
2	HSP27	Hsp27 oligomers have molecular chaperone activity and protect heat-stressed cells against apoptosis.	0.002	6/14/16
3	HSA04614_RENIN_ ANGIOTENSIN_SYSTEM	Genes involved in renin-angiotensin system	0.001	8/17/17
4	HSA04514_CELL_ADHESION_MOLECULES	Genes involved in cell adhesion molecules (CAMs)	0.002	58/124/134
5	GS	Activated G-protein coupled receptors stimulate cAMP production and thus activate protein kinase A, involved in a number of signal transduction pathways.	< 0.001	4/6/6
6	RAB	Rab family GTPases regulate vesicle transport, endocytosis and exocytosis, and vesicle docking via interactions with the rabphilins.	0.005	4/9/12
7	FEEDER	Sugars such as mannose, galactose, and fructose are enzymatically converted to glucose via feeder pathways that lead to glycolysis.	0.003	4/8/9
8	AGPCR	G-protein coupled receptors (GPCRs) transduce extracellular signals across the plasma membrane; attenuation occurs by signal molecule degradation or receptor-mediated endocytosis.	0.001	7/10/13
9	HSA05214_GLIOMA	Genes involved in glioma	0.002	26/59/64
10	HSA05218_MELANOMA	Genes involved in melanoma	0.001	29/66/71
11	HEME_BIOSYNTHESIS	NA	0.004	4/8/9
12	RARRXR	RXR and RAR suppress transcription in the absence of ligand and, on binding trans- or 9-cis-retinoic acid, are ubiquitinated to allow transcription to proceed.	0.006	6/14/15

13	IL10	The cytokine IL-10 inhibits the inflammatory response by macrophages via activation of *heme oxygenase 1.*	0.01	6/13/13
14	CALCIUM_REGULATION_IN _CARDIAC_CELLS	NA	0.003	60/125/149
15	HSA00532_CHONDROITIN_SULFATE_BIOSYNTHESIS	Genes involved in chondroitin sulfate biosynthesis	0.005	9/15/22
16	PLCE	Gs-coupled receptors activate adenylyl cyclase, which activates Epac1, leading to the stimulation of PLC and subsequent DAG and IP3 production.	0.005	6/10/12
17	GLYCOSPHINGOLIPID_METABOLISM	NA	0.004	9/24/24
18	VOBESITY	The adipose tissue of obese individuals overexpresses a key glucocorticoid-metabolizing enzyme, activating inactive circulating corticosteroids and inducing insulin resistance.	0.005	4/7/8
19	CREB	CREB is a transcription factor that binds to cAMP-responsive elements (CREs) to activate transcription in response to extracellular signaling.	0.004	13/14/27
20	DNA_REPLICATION_REACTOME	NA	0.009	12/39/49
21	MPR	Progesterone binding to its intracellular receptor activates the MAPK pathway and induces oocyte maturation; binding to membrane receptor inhibits adenylyl cyclase.	0.005	10/21/23
22	TRANSLATION_FACTORS	NA	0.003	10/31/53
23	TEL	Telomerase is a ribonucleotide protein that adds telomeric repeats to the 3' ends of chromosomes.	0.013	8/15/18

[1-2]Details on the genes and mapped top SNPs involved in each pathway can be found in links provided by the *i*-GSEA4GWAS server under the label "view details":
[1]Carbon Monoxide-related gene set/pathway: http://gsea4gwas.psych.ac.cn/getResult.do?result=13F3A972887892430E6A5C369D76FEAD_1372283303807
[2]Nitrogen Dioxide-related gene set/pathway: http://gsea4gwas.psych.ac.cn/getResult.do?result=13F3A972887892430E6A5C369D76FEAD_1372284527739
N*: The number of Significant genes/Selected genes/All genes
Significant genes: genes mapped with at least one of the top 5% of all SNPs; Selected genes: genes included in the *i*-GSEA analysis; All genes: all genes of the gene set.
NA: not applicable (by the *i*-GSEA4GWAS server)
GWAS: genome wide association study;

[1]

Confirmation by classic linear mixed model testing

The top signals of genome-wide SNP- pollutant(s) interactions (*P*-value <9.9E-05) given by the two-step approach were selected and with the LMM approach we assessed the effect size of the interactions. In Caucasians, change in FEV_1 %predicted per IQR increase in pollution level ranged from -1.4 to 1.2 for the 47 SNP-CO interactions (table E2) and from -1.3 to 1.5 (table E3) for the 48 SNP-NO_2 interactions. *P*-values ranged from 7E-07 to 3E-03 (table E2) and from 1E-08 to 7E-04 (table E3), respectively. Three SNP-NO_2 interactions reached genome-wide significance (rs13090972 and rs958144 near *EPHA3* and rs7041938 in *TXNDC8* - in high linkage disequilibrium (r^2=0.8) with rs12684188 in *SVEP1*). One SNP-CO and 3 SNP-NO_2 interactions replicated in African Americans (n=88) (*P*-values < 0.05 and same direction of effect as in Caucasians; tables 3 and E4).

TABLE 5	Summary of Improved-Gene Set Enrichment Analysis for GWAS of gene by pollutant interactions in Caucasian CAMP subjects			
	Number of pathways	Total number of Significant genes/ top SNPs**	Number of significant genes* in > 1 pathway	Top SNP by Pollutant min-max *P*-values$^{\$}$
Carbon monoxide-related	1	4	-	0.001- 0.02
Nitrogen dioxide-related	23	244	34	2.3E-06 – 0.07

GWAS: genome wide association study; SNP: single nucleotide polymorphism; *Significant genes in a gene-set/pathway are the genes mapped with at least one of the top 5% of all SNP by pollutant interactions in the two-step genome-wide analysis; one significant gene might exist in more than one pathway; ** top SNP is the SNP mapped on a significant gene with the lowest genome-wide *P*-value of SNP by pollutant interaction
$^{\$}$ Linear mixed model: FEV_1= b0 + b1 between-subject pollution + b2 within-subject pollution + b3 SNP + b4 SNP x pollutant + b5 covariates + e | subject-specific (random) intercept

Pathway-level analysis for the two-step genome-wide SNP by pollutant interaction analysis

For the *i-GSEA4GWAS* in Caucasian CAMP subjects 474,792 gene variants were imported and 265,485 variants were mapped on genes +/-20kb (total number of genes: 16,854). We identified 1 pathway interacting with CO (gene-set *P*=0.001) and 23 pathways interacting with NO_2 (gene-sets *P*: 0.0001-0.01). Table 4 presents the *i-GSEA4GWAS* suggested pathways for the 2 pollutants. Gene-sets FDR *P*-values ranged 0.08-0.24.

Within each gene-set/pathway there were significant genes (genes mapped with at least one of the top 5% of all SNPs-pollutant interactions in the 2-step

genome-wide analysis). Each significant gene is represented by the SNP in that gene with the lowest genome-wide *P*-value of SNP by pollutant interaction (the top SNP per gene). A significant gene -thus its related top SNP- can be involved in more than one pathway (table 5). Effect sizes of interaction of those top SNPs with pollutants are given in the supplementary material (tables E5 and E6). The set of pathway-analysis top SNPs was unique for each pollutant (tables E5 and E6).

TABLE 6 **Top genome-wide interaction loci and suggested underlying mechanisms**

Top genome-wide interaction locus	Function(s) related to genes*	Pathways linked to those functions
Nitrogen dioxide		
EPHA3	Cell adhesion;	Cell adhesion molecules;
	Immune surveillance;	Glycosaminoglycan (chondroitin) biosynthesis
	Tissue remodeling	Glycosaminoglycan (chondroitin) biosynthesis
TXNDC8 (thioredoxin reductase family)	Cell redox homeostasis	HSP27, iNOS, IL10, Heme biosynthesis–Heme oxyganase-1/CO, Calcium regulation
SEVP1	Cell adhesion; Immune surveillance	Cell adhesion molecules
MR-1	Cell redox homeostasis	HSP27, iNOS, IL10, Heme biosynthesis–Heme oxyganase-1/CO
	Detoxify by-products of metabolism	Glycosaminoglycan (chondroitin) biosynthesis, Glycosphingolipid, Vobesity, Feeder
Carbon monoxide		
TUSC3	Cell redox homeostasis;	PAC1R– Heme oxyganase-1/CO
	Glycolysis (N-glycan biosynthesis)	

* based on coding protein's function(s). Details for each gene are given in the text

DISCUSSION

Most gene–air pollution studies have focused on a few candidate genetic variations and investigated short-term exposures to ozone.[15] Although these small hypothesis-driven studies can contribute to our understanding of specific gene–pollution effects, they often fail to uncover novel disease-causing mechanisms and tend to result in many unreplicated findings.[31] To the best of our knowledge, this is the first hypothesis-free, genome-wide analysis investigating whether genetic variants modify the longitudinal relationship of traffic-related air pollution (CO and NO_2) with FEV_1 in childhood asthma. Three SNPs showed genome-wide statistical evidence of interaction with NO_2 on the change in FEV_1 in Caucasian CAMP subjects. Additionally, one variant showed some evidence for modifying the CO effects and 4 variants for

modifying the NO_2 effects on FEV_1 in both Caucasian and African American children with asthma. The pathway-level analysis suggested that one pathway plays a role in the etiology of CO-related FEV_1 changes and several biologically linked pathways in NO_2-related FEV_1 changes in children with asthma.

Due to the large processing time for a longitudinal genome-wide gene-pollution interaction study with the classic LMM we followed the practical application of the conditional two-step approach by Sikorska et al.[27] This method provides shorter processing time and we confirmed its accuracy, i.e., at a second stage the genome-wide top signals found by the two-step approach were confirmed by LMM testing. Below we discuss the putative genes involved in air pollution effects on lung function in childhood asthma and the pathways involved as identified by our genome-wide association and *iGSEA*4GWAS analyses.

Two loci, the *EPHA3* (receptor tyrosine kinase of Eph family; location 3p11.2) and *TXNDC8* genes (thioredoxin domain containing 8 (spermatozoa) or Spermatocyte/Spermatid-Specific Thioredoxin-3; location 9q31.3) showed genome-wide statistical evidence for interaction with NO_2. The best documented function of the Eph-receptor/ephrin-A signaling is the regulation of cell adhesion and migration processes critical for a wide variety of functions including tissue remodeling and immune surveillance.[32,33] Recent findings suggest that Eph-signaling is involved in pathological conditions such as lung cancer, yet its role in asthma is unknown.[34,35] The fact that receptor tyrosine kinase pathways contribute to aspects of airway inflammation, remodeling and airway hyperresponsiveness in asthma,[36] suggests that we may have identified a novel receptor tyrosine kinases (EPHA3) important for the pathogenesis of asthma in response to NO_2 in Caucasian children. However, we were unable to replicate this locus in African Americans and it would be important to replicate our finding in other Caucasian populations in the future.

The second top signal locus, *TXNDC8*, belongs to the thioredoxin reductase enzymes, a well-characterized subfamily of selenoproteins that perform an essential redox role in immune cells.[37] The genome-wide top hit SNP (rs7041938) in *TXNDC8* found to modify the NO_2 effects on FEV_1 is in high linkage disequilibrium (r^2>0.8) with rs12684188 (LMM *P*-value of interaction=3.9E-07) in *SVEP1* (sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1; location 9q32). In a recent GWAS, a locus containing the *SVEP1* gene showed signals of association with FEV_1 decline in non-asthmatic adults.[38] In our Caucasian asthmatic children the marginal *P*-value of the *SVEP1* variant in the LMM model was not significant. The coding protein of the *SVEP1*, also

called polydom,[39] is recognized as a cell adhesion molecule (CAM) and its particular multidomain structure suggests an important biological role in cellular adhesion and/or in the immune system,[40] but its role in asthma has not been investigated so far.

Three SNP-NO_2 interactions were replicated in African American CAMP subjects (rs4672884 in *MR-1*, rs3769767 in *METTL5* and rs4378142 in a non-coding protein gene /ENSG00000233067). MR-1 shares homology with the family of glyoxylases that functions in the glutathione dependent metabolic pathway, capable of detoxify methylglyoxal, a by-product of glucose, protein and fatty acid metabolism.[41] The latter metabolic cellular processes are activated with hypoxia and oxidative stress, as a way of cells to meet their energy requirement under this condition.[41-43] In addition, methylglyoxal is produced by atmospheric chemical reactions of NO_2.[44] Inside the cell, increased methylglyoxal depletes anti-oxidant enzymes (GSH, SOD) and gives a positive loop to oxidative stress.[42] The functions of Methyl Transferase like 5 (METTL5 or HSPC133) and ENSG00000233067 genes are largely unknown yet.

At SNP-level we also found a suggested CO lung function effect modification by rs2720625 located near the Magnesium Uptake/Transporter (Tumor Suppressor Candidate 3-TUSC3) gene in both ethnic groups. The *TUSC3* locus has been associated with FEV_1/FVC decline in adult asthmatics.[38] The gene product plays a role in N-glycan biosynthesis, cell redox hemostasis, cellular magnesium uptake, and in the N-glycosylation of asparagin protein residues.[38,45] The latter functions suggest that CO might interact with metabolic and oxidative stress pathways via this locus.

At a first glance the top hits and/or replicated signals of our genome-wide interaction study seem not to have a biological plausible connection with one another. Of interest, when zooming out the picture shows that genes with potential metabolic, oxidant/anti-oxidant and inflammatory role (summarized in table 6) might be important for lung function responses to pollution in children with asthma. Potential interaction of NO_2 with metabolic (chondroitin sulfate biosynthesis (glycosaminoglycans biosynthesis), feeder, glycosphingolipid metabolism , vobesity), oxidative stress (HSP27, IL10, Heme Biosynthase-Heme oxygenase/carbon monoxide (HO-1/CO)), inflammatory (nitric oxide synthase (iNOS)) and calcium regulation (Calcium regulation in cardiac cells) pathways were also revealed by our pathway analysis (pathways description/function are shown in table 4). In the supplementary material we describe in more detail how these pathways may be linked to NO_2 exposure and how they are inter-related.

It is plausible that exposure to NO_2 induces oxidative stress with cellular damage and inflammatory responses. Metabolic pathways (i.e. glycolysis) are activated in order to compensate the cellular demands to stress and the HO-1/CO system may protect against oxidative stress and inflammation. Oxidative stress has been associated with calcium influx regulation, two responses observed in our analyses as well.[46,47] Interestingly, a proteomic-based study has shown that allergen-induced early asthma response in rats is associated with glycolysis, calcium binding and mitochondrial activity,[48] supporting our proposed underlying molecular mechanisms for response to environmental toxicants in asthma.

The pathway analysis for the genome-wide SNP-CO interaction output showed that the neuropeptide pituitary adenylate cyclase-activating peptide receptor (PAC1R) pathway is related to CO exposure. The ligand of PAC1R (PACAP) can induce bronchodilation and endogenous regulation of airway tone by means of a CO-dependent, cyclicGMP-related mechanism, thereby providing a link between neurotransmission and local HO-1/CO (but not iNOS/NO) release in the airway smooth muscle.[49-51] PACAP has also pro-inflammatory functions that require calcium regulation.[52-54] Furthermore, mouse studies indicate that this pathway is involved in ventilator responses to hypoxia/hypercapnia and to mild ambient temperature changes.[55-57] The mean concentration of CO in our study was much lower (0.8 ppm) than that to produce high levels of carboxyhemaglobin and tissue hypoxia,[58,59] however the effects on cellular level of low inhaled CO concentrations warrants further study.

Our childhood asthma study had the advantage of having a long follow-up period with high attendance of the subjects, air pollution levels during that period and genome data. There are a few weaknesses as well. Although population stratification is less likely to bias estimates of gene-environment interaction effects,[60] we used as our primary study only Caucasian CAMP subjects. For replication studies, definition and measurement of the exposure and/or outcome is critical to the success of gene–environment investigations, therefore we decided to use the second largest ethnic subgroup of the CAMP as our replication population (although of relative small size), to ensure that the genotyping, outcome and exposure were measured reliably and consistently. Notwithstanding this, we nevertheless found 4 SNP-pollution interactions with similar directions in the two populations even though different linkage disequilibrium patterns exist in the two ethnic groups that might have limited our replication.

After testing for pollution effect modification at the SNP-level, we performed the pathway approach as a way to assess the overall evidence of interaction of pollution with a group of functionally related genes, thus incorporating prior biological knowledge. Our pathway-level analysis of SNP-pollution interactions identified biological plausible mechanisms for pollution-mediated asthma progression in children.

Our findings highlight the promise of pursuing genome-wide gene-environment interaction studies that do not necessarily reach genome-wide significance but prone to be biologically relevant to the effects of or response to the exposures. We conclude that traffic-related air pollution such as with CO and NO_2 is linked to oxidative stress and inflammation, while metabolic pathways, calcium homeostasis and the HO-1/CO pathway play a role in cytoprotection. Our findings may represent the first step for functional research and pharmacological developments for protection against the detrimental effects of air pollution on asthma severity and progression.

ACKNOWLEDGMENT

We dedicate this manuscript to the memory of our friend and colleague Dr. Gail G. Shapiro who passed away unexpectedly during the development of this study. Dr. Shapiro dedicated her life to understanding the causes of childhood asthma and determining the best treatments for asthma. She is deeply missed by her colleagues, patients, and the asthma community.

The Childhood Asthma Management Program trial and CAMP Continuation Study were supported by contracts NO1-HR-16044, 16045, 16046, 16047, 16048, 16049, 16050, 16051, and 16052 with the National Heart, Lung, and Blood Institute and General Clinical Research Center grants M01RR00051, M01RR0099718-24, M01RR02719-14, and RR00036 from the National Center for Research Resources. The CAMP Continuation Study/Phases 2 and 3 were supported by grants U01HL075232, U01HL075407, U01HL075408, U01HL075409, U01HL075415, U01HL075416, U01HL075417, U01HL075419, U01HL075420, andU01HL075408 from the National Heart, Lung, and Blood Institute. The National Jewish Health site was also supported in part by Colorado CTSA grant UL1RR025780 from NCRR/NIH and UL1TR000154 from NCATS/NIH. C:\lm_camp\misc\campdua.wpd June 1, 2013 (2:07pm).

In addition, all work on data collected from the CAMP Genetic Ancillary Study was conducted at the Channing Laboratory of the Brigham and Women's Hospital under appropriate CAMP policies and human subject's protections.

The CAMP Genetics Ancillary Study is supported by U01 HL075419, U01 HL65899, P01 HL083069 R01 HL086601, RC2 HL101543 from the National Heart, Lung and Blood Institute, National Institutes of Health.

FINANCIAL SUPPORT: This study was funded by: 5 P01 HL 083069-05 NIH/NHLBI/P01; NIEHS PO1 ES-09825, US EPA RD-83241601, US EPA grant RD 83479801 and the International Initiative for Environment and Public Health Cyprus Program of HSPH

REFERENCES

1. World Health Organization. Effects of air pollution on children' s health and developement. A review of the evidence. Bonn. 2005; Available at: http://www.euro.who.int/__data/assets/pdf_file/0010/74728/E86575.pdf. Accessed April, 2013.
2. Searing DA, Rabinovitch N. Environmental pollution and lung effects in children. Curr Opin Pediatr 2011 Jun;23:314-318.
3. Trasande L, Thurston GD. The role of air pollution in asthma and other pediatric morbidities. J Allergy Clin Immunol 2005 Apr;115:689-699.
4. Schwela D. Air pollution and health in urban areas. Rev Environ Health 2000 Jan-Jun;15:13-42.
5. Tzivian L. Outdoor air pollution and asthma in children. J Asthma 2011 Jun;48:470-481.
6. Schildcrout JS, Sheppard L, Lumley T, Slaughter JC, Koenig JQ, Shapiro GG. Ambient air pollution and asthma exacerbations in children: an eight-city analysis. Am J Epidemiol 2006 Sep 15;164:505-517.
7. Sunyer J, Spix C, Quenel P, Ponce-de-Leon A, Ponka A, Barumandzadeh T, Touloumi G, Bacharova L, Wojtyniak B, Vonk J, Bisanti L, Schwartz J, Katsouyanni K. Urban air pollution and emergency admissions for asthma in four European cities: the APHEA Project. Thorax 1997 Sep;52:760-765.
8. Romieu I, Meneses F, Ruiz S, Sienra JJ, Huerta J, White MC, Etzel RA. Effects of air pollution on the respiratory health of asthmatic children living in Mexico City. Am J Respir Crit Care Med 1996 Aug;154:300-307.
9. HEI on the Health Effects of Traffic-Related Air Pollution. Traffic-related Air Pollution: A critical Review of the Literature on Emissions, Exposure, and Health Effects. 2010;HEI Special Report 17.
10. Li S, Williams G, Jalaludin B, Baker P. Panel studies of air pollution on children's lung function and respiratory symptoms: a literature review. J Asthma 2012 Nov;49:895-910.
11. Gao Y, Chan EY, Li LP, He QQ, Wong TW. Chronic effects of ambient air pollution on lung function among Chinese children. Arch Dis Child 2013 Feb;98:128-135.
12. Rosenlund M, Forastiere F, Porta D, De Sario M, Badaloni C, Perucci CA. Traffic-related air pollution in relation to respiratory symptoms, allergic sensitisation and lung function in schoolchildren. Thorax 2009 Jul;64:573-580.
13. Gauderman WJ, Vora H, McConnell R, Berhane K, Gilliland F, Thomas D, Lurmann F, Avol E, Kunzli N, Jerrett M, Peters J. Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. Lancet 2007 Feb 17;369:571-577.
14. Schwartz J. Lung function and chronic exposure to air pollution: a cross-sectional analysis of NHANES II. Environ Res 1989 Dec;50:309-321.
15. Yang IA, Fong KM, Zimmerman PV, Holgate ST, Holloway JW. Genetic susceptibility to the respiratory effects of air pollution. Postgrad Med J 2009 Aug;85:428-436.
16. Kelly FJ. Oxidative stress: its role in air pollution and adverse health effects. Occup Environ Med 2003 Aug;60:612-616.
17. Patel MM, Chillrud SN, Deepti KC, Ross JM, Kinney PL. Traffic-related air pollutants and exhaled markers of airway inflammation and oxidative stress in New York City adolescents. Environ Res 2013 Feb;121:71-78.
18. Emmerechts J, Hoylaerts MF. The effect of air pollution on haemostasis. Hamostaseologie 2012;32:5-13.
19. Auerbach A, Hernandez ML. The effect of environmental oxidative stress on airway inflammation. Curr Opin Allergy Clin Immunol 2012 Apr;12:133-139.

20. Kelly FJ, Fussell JC. Air pollution and airway disease. Clin Exp Allergy 2011 Aug;41:1059-1071.

21. Romieu I, Ramirez-Aguilar M, Sienra-Monge JJ, Moreno-Macias H, del Rio-Navarro BE, David G, Marzec J, Hernandez-Avila M, London S. GSTM1 and GSTP1 and respiratory health in asthmatic children exposed to ozone. Eur Respir J 2006 Nov;28:953-959.

22. Takizawa H. Impact of air pollution on allergic diseases. Korean J Intern Med 2011 Sep;26:262-273.

23. Breton CV, Salam MT, Vora H, Gauderman WJ, Gilliland FD. Genetic variation in the glutathione synthesis pathway, air pollution, and children's lung function growth. Am J Respir Crit Care Med 2011 Jan 15;183:243-248.

24. Moreno-Macias H, Dockery DW, Schwartz J, Gold DR, Laird NM, Sienra-Monge JJ, Del Rio-Navarro BE, Ramirez-Aguilar M, Barraza-Villarreal A, Li H, London SJ, Romieu I. Ozone exposure, vitamin C intake, and genetic susceptibility of asthmatic children in Mexico City: a cohort study. Respir Res 2013 Feb 4;14:14-9921-14-14.

25. Anonymous. The Childhood Asthma Management Program (CAMP): design, rationale, and methods. Childhood Asthma Management Program Research Group. Control Clin Trials 1999 Feb;20:91-120.

26. Standardization of Spirometry, 1994 Update. American Thoracic Society. Am J Respir Crit Care Med 1995 Sep;152:1107-1136.

27. Sikorska K, Rivadeneira F, Groenen PJ, Hofman A, Uitterlinden AG, Eilers PH, Lesaffre E. Fast linear mixed model computations for genome-wide association studies with longitudinal data. Stat Med 2013 Jan 15;32:165-180.

28. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007 Sep;81:559-575.

29. Zhang K, Cui S, Chang S, Zhang L, Wang J. i-GSEA4GWAS: a web server for identification of pathways/gene sets associated with traits by applying an improved gene set enrichment analysis to genome-wide association study. Nucleic Acids Res 2010 Jul;38:W90-5.

30. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005 Oct 25;102:15545-15550.

31. Romieu I, Moreno-Macias H, London SJ. Gene by environment interaction and ambient air pollution. Proc Am Thorac Soc 2010 May;7:116-122.

32. Miao H, Wang B. EphA receptor signaling--complexity and emerging themes. Semin Cell Dev Biol 2012 Feb;23:16-25.

33. Arvanitis D, Davy A. Eph/ephrin signaling: networks. Genes Dev 2008 Feb 15;22:416-429.

34. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008 Apr 4;133:38-52.

35. Zhuang G, Song W, Amato K, Hwang Y, Lee K, Boothby M, Ye F, Guo Y, Shyr Y, Lin L, Carbone DP, Brantley-Sieders DM, Chen J. Effects of cancer-associated EPHA3 mutations on lung cancer. J Natl Cancer Inst 2012 Aug 8;104:1182-1197.

36. Guntur VP, Reinero CR. The potential use of tyrosine kinase inhibitors in severe asthma. Curr Opin Allergy Clin Immunol 2012 Feb;12:68-75.

37. Huang Z, Rose AH, Hoffmann PR. The Role of Selenium in Inflammation and Immunity: From Molecular Mechanisms to Therapeutic Opportunities. Antioxid Redox Signal 2012 Apr 01;16:705-743.

38. Imboden M, Bouzigon E, Curjuric I, Ramasamy A, Kumar A, Hancock DB, Wilk JB, Vonk JM, Thun GA, Siroux V, Nadif R, Monier F, Gonzalez JR, Wjst M, Heinrich J, Loehr LR,

Franceschini N, North KE, Altmuller J, Koppelman GH, Guerra S, Kronenberg F, Lathrop M, Moffatt MF, O'Connor GT, Strachan DP, Postma DS, London SJ, Schindler C, Kogevinas M, Kauffmann F, Jarvis DL, Demenais F, Probst-Hensch NM. Genome-wide association study of lung function decline in adults with and without asthma. J Allergy Clin Immunol 2012 May;129:1218-1228.

39. Gilges D, Vinit MA, Callebaut I, Coulombel L, Cacheux V, Romeo PH, Vigon I. Polydom: a secreted protein with pentraxin, complement control protein, epidermal growth factor and von Willebrand factor A domains. Biochem J 2000 Nov 15;352 Pt 1:49-59.

40. Schwanzer-Pfeiffer D, Rossmanith E, Schildberger A, Falkenhagen D. Characterization of SVEP1, KIAA, and SRPX2 in an in vitro cell culture model of endotoxemia. Cell Immunol 2010;263:65-70.

41. Lee HY, Xu Y, Huang Y, Ahn AH, Auburger GW, Pandolfo M, Kwiecinski H, Grimes DA, Lang AE, Nielsen JE, Averyanov Y, Servidei S, Friedman A, Van Bogaert P, Abramowicz MJ, Bruno MK, Sorensen BF, Tang L, Fu YH, Ptacek LJ. The gene for paroxysmal non-kinesigenic dyskinesia encodes an enzyme in a stress response pathway. Hum Mol Genet 2004 Dec 15;13:3161-3170.

42. Desai KM, Chang T, Wang H, Banigesh A, Dhar A, Liu J, Untereiner A, Wu L. Oxidative stress and aging: is methylglyoxal the hidden enemy? Can J Physiol Pharmacol 2010 Mar;88:273-284.

43. Kondoh H, Lleonart ME, Bernard D, Gil J. Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization. Histol Histopathol 2007 Jan;22:85-90.

44. Nishino N, Arey J, Atkinson R. Formation yields of glyoxal and methylglyoxal from the gas-phase OH radical-initiated reactions of toluene, xylenes, and trimethylbenzenes as a function of NO2 concentration. J Phys Chem A 2010 Sep 23;114:10140-10147.

45. Martino AT, Mueller C, Braag S, Cruz PE, Campbell-Thompson M, Jin S, Flotte TR. N-glycosylation augmentation of the cystic fibrosis epithelium improves Pseudomonas aeruginosa clearance. Am J Respir Cell Mol Biol 2011 Jun;44:824-830.

46. Jiang LH, Yang W, Zou J, Beech DJ. TRPM2 channel properties, functions and therapeutic potentials. Expert Opin Ther Targets 2010 Sep;14:973-988.

47. Xu R, Li Q, Zhou XD, Perelman JM, Kolosov VP. Oxidative Stress Mediates the Disruption of Airway Epithelial Tight Junctions through a TRPM2-PLCgamma1-PKCalpha Signaling Pathway. Int J Mol Sci 2013 Apr 29;14:9475-9486.

48. Xu YD, Cui JM, Wang Y, Yin LM, Gao CK, Liu YY, Yang YQ. The early asthmatic response is associated with glycolysis, calcium binding and mitochondria activity as revealed by proteomic analysis in rats. Respir Res 2010 Aug 6;11:107-9921-11-107.

49. Kinhult J, Andersson JA, Uddman R, Stjarne P, Cardell LO. Pituitary adenylate cyclase-activating peptide 38 a potent endogenously produced dilator of human airways. Eur Respir J 2000 Feb;15:243-247.

50. Kinhult J, Uddman R, Cardell LO. The induction of carbon monoxide-mediated airway relaxation by PACAP 38 in isolated guinea pig airways. Lung 2001;179:1-8.

51. Linden A, Cardell LO, Yoshihara S, Nadel JA. Bronchodilation by pituitary adenylate cyclase-activating peptide and related peptides. Eur Respir J 1999 Aug;14:443-451.

52. Kinhult J, Uddman R, Laan M, Linden A, Cardell LO. Pituitary adenylate cyclase-activating peptide inhibits neutrophil chemotaxis. Peptides 2001 Dec;22:2151-2154.

53. El Zein N, Badran B, Sariban E. The neuropeptide pituitary adenylate cyclase activating polypeptide modulates Ca2+ and pro-inflammatory functions in human monocytes through the G protein-coupled receptors VPAC-1 and formyl peptide receptor-like 1. Cell Calcium 2008 Mar;43:270-284.

54. Harfi I, D'Hondt S, Corazza F, Sariban E. Regulation of human polymorphonuclear leukocytes functions by the neuropeptide pituitary adenylate cyclase-activating polypeptide after activation of MAPKs. J Immunol 2004 Sep 15;173:4154-4163.

55. Cummings KJ, Willie C, Wilson RJ. Pituitary adenylate cyclase-activating polypeptide maintains neonatal breathing but not metabolism during mild reductions in ambient temperature. Am J Physiol Regul Integr Comp Physiol 2008 Mar;294:R956-65.

56. Arata S, Nakamachi T, Onimaru H, Hashimoto H, Shioda S. Impaired response to hypoxia in the respiratory center is a major cause of neonatal death of the PACAP-knockout mouse. Eur J Neurosci 2013 Feb;37:407-416.

57. Cummings KJ, Pendlebury JD, Sherwood NM, Wilson RJ. Sudden neonatal death in PACAP-deficient mice is associated with reduced respiratory chemoresponse and susceptibility to apnoea. J Physiol 2004 Feb 15;555:15-26.

58. Raub JA, Mathieu-Nolf M, Hampson NB, Thom SR. Carbon monoxide poisoning--a public health perspective. Toxicology 2000 Apr 7;145:1-14.

59. Reboul C, Thireau J, Meyer G, Andre L, Obert P, Cazorla O, Richard S. Carbon monoxide exposure in the urban environment: an insidious foe for the heart? Respir Physiol Neurobiol 2012 Nov 15;184:204-212.

60. Thomas D. Gene--environment-wide association studies: emerging approaches. Nat Rev Genet 2010 Apr;11:259-272.

CHAPTER 7

Summary and discussion of the thesis – Future perspectives

SUMMARY AND DISCUSSION OF THE THESIS

In **Chapter 1** we have introduced the mainstay hypothesis of the thesis: In asthma the epithelium is more susceptible to damage allowing greater access of environmental allergens, microorganisms, and toxicants to the airway tissue and/or has an altered response to injury by environmental stimuli, with release of cytokines and growth factors by the epithelium itself or by orchestrated immune and mesenchymal cells. The pro-inflammatory response of the epithelium may contribute to local allergen sensitization, chronic and persistent inflammation and airway wall remodeling as a consequence of complex interaction between affected airway epithelial cells and the underlying mesenchymal and immune cells. Airway remodeling and inflammation are associated with the clinical severity of asthma. This hypothesis has been built based on research findings in the literature.[1-6]

Not all asthmatics respond to environmental exposures in the same way and there is heterogeneity in clinical and pathological expression of disease among individuals, both in children and adults with asthma. The individual variation in asthma severity is influenced by genetic and environmental factors,[7-9] and gene-environment interactions add to the complexity of the disease.[10]

This thesis aimed to determine how genetic and environmental factors and their interactions affect asthma heterogeneity. Although many studies have investigated the genetic and environmental determinants of asthma development, fewer studies have so far focused on asthma severity phenotypes or studied asthma phenotypes longitudinally. Despite the fact that there is a distinct link between the clinical expression of asthma and its underlying airway pathology,[5] the genetic background of airway pathology in asthma is not well documented.

The first three studies (chapters 2-4) of the thesis investigated genes with a biologically plausible role in the proposed hypothesis and assessed the associations of genetic variations with aspects of airway pathology (remodeling and inflammation) and asthma severity phenotypes (cross-sectionally and longitudinally) in adult asthmatics. Inhaled corticosteroids (ICS) are the cornerstone of asthma treatment. The severity of asthma and its underlying pathology is improved by inhalation of anti-inflammatory medication,[11-13] whereas tobacco smoke exposure affect these features negatively.[14] Therefore, we studies whether gene by ICS and gene by smoking interactions are associated with asthma severity and pathology.

Chapter 2 presents results of our study on the *CDH1* gene, encoding E-cadherin a key protein of cellular junctions responsible for epithelial integrity[15,16] and immune surveillance of the epithelium.[17] Loss of epithelial E-cadherin disrupts the epithelial barrier that in turn influences resistance of the airway to noxious inhalants,[18] with subsequent pathological and clinical consequences.[2,5,19] Given the evidence for an interrupted and fragile epithelium in bronchial biopsies of asthmatics,[20,21] we investigated the associations of *E-cadherin* (*CDH1)* gene single nucleotide polymorphisms (SNPs) with epithelial E-cadherin expression, airway inflammation and remodeling in bronchial biopsies of asthmatics. Moreover, we assessed associations of SNPs with the severity of airflow obstruction and lung function decline in two asthma cohorts. Furthermore, given a role of glucocorticoids in modulating epithelial repair, E-cadherin expression and clinical severity[22-26] we assessed whether ICS use modifies *CDH1* SNP effects.

We found that a higher epithelial E-cadherin expression was associated with less epithelial denudation in bronchial biopsies of asthmatics. In the absence of ICS, subjects with specific *CDH1* risk alleles did not express the same amount of epithelial E-cadherin compared to wild-types, while in individuals with ICS use the difference in E-cadherin expression between carriers of the risk alleles and wild-types was smaller, suggesting that ICS may improve epithelial E-cadherin expression in subjects with specific *CDH1* genotypes. In subjects using ICS, the same risk alleles were also associated with less airway remodeling and/or airflow obstruction and/or less rapid FEV_1 decline. Another set of *CDH1* SNPs was associated with accelerated FEV_1 decline, a finding only present in asthmatics using ICS, indicating that subjects with such genotypes benefit less from ICS use. The direction of SNP-pathology associations was in line with the direction of SNP-lung function associations, meaning that in subjects using ICS the alleles associated with less airway wall remodeling were also associated with increased post bronchodilator FEV_1/VC and less accelerated FEV_1 decline. A previous study proposed that post bronchodilator FEV_1/VC ratio may be used as an indirect measure of airway remodeling, and our findings tend to support this view.[27] Studies have shown that increased CD8+ T-cells might be present in airway wall biopsies even after administration of ICS with subsequent improvement of clinical severity,[28] and that E-cadherin interacts with CD8+ T-cells via co-expression of the ligand of E-cadherin (CD103) on T-cells. However, in our study we showed that an increased CD8+ T-cell count with ICS use is not observed in all subjects but only in those with specific *CDH1* genotypes, we therefore speculate a role of E-cadherin and epithelium

in immune protection of the asthmatic airway. In conclusion, the findings of this study indicated that there is genetic susceptibility in epithelial E-cadherin expression, hence epithelial integrity in asthma, which may be modulated by ICS use. This genetically determined epithelial integrity also contributes to airway pathology and clinical severity in asthma.

The pro-inflammatory cytokine, Tumor Necrosis Factor (TNF), investigated in the study covered in **Chapter 3** is linked with the first study (Chapter 2) in the following three ways:

a. TNFα disrupts tight junctions in the epithelial barrier, increases epithelial permeability[29] and decreases the expression of adhesion molecules responsible for epithelial integrity i.e., E-cadherin and Catenins.[22,23,30]

b. TNFα induces airway hyperresponsiveness in normal and asthmatic subjects, facilitates the migration of inflammatory cells into the airway wall and activates pro-fibrotic and remodeling mechanisms in the submucosa.[31,32]

c. Glucocorticosteroids inhibit TNFα-induced epithelial adhesion disruption[23,30] and suppress TNFα expression in several tissues, including the lung[33] and the alveolar macrophages of bronchoalveolar lavage fluid from asthmatics.[25]

However, a subpopulation of asthma patients does not respond well to ICS. Furthermore, TNFα has been implicated in the pathophysiology of severe steroid-resistant asthma.[34] We hypothesized that genetic variability in the *TNF* loci may explain the variable response to ICS. We investigated effects of interactions between ICS and *TNF* SNPs on epithelial integrity, airway inflammation and airway remodeling in asthma. In addition, we analyzed effects on asthma severity phenotypes i.e., lung function and airway hyperresponsiveness in two asthma cohorts.

We showed that ICS modified effects of *TNF* SNPs on epithelial denudation: without ICS use, specific alleles were associated with more widespread denuded epithelium, whereas effects were reversed with ICS use. In subjects with those *TNF* genotypes, epithelial integrity was improved with ICS use and that was concordant with improvement in their lung function; a finding similar to the *E-cadherin gene* study (chapter 2). However, in this case ICS did not modify *TNF* effects on airway remodeling or airway hyperresponsiveness, but they increased pro-inflammatory effects (infiltration of inflammatory cells). ICS has been found to enhance TNFα-mediated inflammatory cell migration into

lungs,[35] but we show that the ICS induced pro-inflammatory effects are not present in all subjects but only in asthmatics with specific *TNF* genotypes.

Our findings (chapters 2 and 3) add to further insights in the genetic component of the heterogeneity of asthma severity and response to ICS treatment. Previous candidate gene studies on FEV_1 response to ICS treatment have focused on genes in glucocorticoid (GC) synthesis and metabolism, cellular receptors and transcriptional regulators (i.e. *CRHR1* (GC production), *NR3C1* (GC receptor) *STIP1* (adaptor-chaperon GC-mediated signaling)),[36,37] whereas our studies focused on genes regulated by ICS, thus giving more attention to the downstream transcriptional activities of GC. Recent genome-wide association studies by Tantisira et al. identified two genes of ICS response in asthma (*T* and *GLCCI1)* adding further novel determinants of FEV_1 response to ICS treatment in asthma.[38,39]

Chapter 4 discusses a fibrogenic and immunomodulatory cytokine named Transforming Growth Factor beta (TGFβ1), emerging as a major player in the way the airway epithelium behaves and its ability to repair itself.[40] Variation in the *TGFB1* gene has been previously associated with asthma development and severity, airway inflammation and TGFβ1 serum levels.[41-43] The *TGFB1* mRNA expression in asthma has been shown to be induced by cigarette-smoke.[44] Our study showed that *TGFB1* SNPs were significantly associated with asthma severity defined by airway hyperresponsiveness, airflow obstruction and asthma remission, as well as with airway inflammation and features of airway remodeling. We were the first to show associations of *TGFB1* SNPs with FEV_1 decline in asthma and to find suggestive evidence for an interaction with smoking. Of importance, the direction of the *TGFB1* SNP-associations with asthma severity was consistent with the direction of the SNP-associations with pathological changes. For example, SNP-associations with increased subepithelial numbers of eosinophils and macrophages were in line with SNP-associations with basement membrane thickening and lower lung function, while another SNP-association with increased CD8+ T-cells was in line with SNP-association with asthma remission. In another example, SNP-association with increased goblet cell numbers was in line with more severe airflow obstruction. Our findings confirmed previous findings indicating a role of *TGFB1* gene in airflow obstruction and airway inflammation and in addition linked for the first time *TGFB1* gene variations with airway remodeling and lung function decline in asthma. The associations observed in the total group, became more pronounced in the ever-smoker group, with significant

interactions - yet not in all occasions – suggesting that smoking is a potential *TFGB1* effect modifier.

The last two chapters (Chapters 5 and 6) of the thesis focus on the susceptibility of asthmatic children to ambient air pollution, an unavoidable environmental exposure of the modern world. There is compelling evidence that acute exposure to ambient air pollution increases asthma morbidity and severity in children. [45-49] However, to date we are the first to have assessed associations of long-term air pollution with severity of airflow obstruction and airway responsiveness in a clinical trial, and modification of pollution effects by controller medication used by asthmatic children and by gene variations.

In **Chapter 5** we showed that increased levels (4-month average concentrations) of carbon monoxide (CO) and nitrogen dioxide (NO_2) - and to a smaller extent levels of ozone- were associated with a reduction in post-bronchodilator FEV_1 level. CO also showed short-term (same-day and one-week average concentrations) negative effects on lung function and on airway hyperresponsiveness. Sulfur dioxide was also associated with more severe hyperresponsiveness. The worsening of airway hyperresponsiveness with short-term exposure to CO was stronger for children on ICS and nedocromil compared to placebo, a finding that needs further investigation. Importantly, the longitudinal evaluation of children treated with daily asthma controller therapy in a clinical trial enabled us to separate the modification of pollution effects by treatment without confounding by indication. Because the allocation of treatment in observational studies is not randomized and the indication for treatment may be related to the risk of future health outcomes, the resulting imbalance in the underlying risk profile between treated and comparison groups can generate biased results, as opposed to randomized clinical trials.

Based on the evidence that the respiratory response to air pollution varies between individuals and that genetic susceptibility likely plays a role,[50] we decided to investigate whether genetic variants modify the main pollution effects found in our population, i.e. long-term effects of CO and NO_2 on FEV_1 level. Biological mechanisms by which air pollution can impair health include autonomic dysfunction, oxidative stress, and systemic inflammatory responses.[51-54] However, those mechanisms are not fully understood and the identification of pathways that might be involved warrants further study.

The study in **Chapter 6** includes two parts: 1) a genome-wide gene by air pollution interaction study (GWIS) and 2) a pathway analysis based on the GWIS results. Analysis by a genome-wide scan was preferred above a candidate

gene study, since it has the potential to discover novel genes involved in the genetic susceptibility to pollution. The pathway analysis was performed to assess the overall evidence of association of pollution interaction with a group of functionally related genes (i.e. a gene set), thus incorporating interpretation with prior biological knowledge. For each part we used recently developed statistical methods. We followed the Sikorska et al. conditional two-step approach for fast linear mixed model computations for genome-wide association studies (GWAS),[55] as method to explore whether the longitudinal relationship between 4-month averaged pollution and post-bronchodilator FEV_1 %predicted was modified by SNPs in the human genome, and we used the improved gene-set enrichment analysis for GWAS (*i-GSEA4GWAS*) for the pathway analysis.[56]

The Sikorska et al. method proved to be an accurate surrogate for the classic linear mixed model for a longitudinal G-E study. To the best of our knowledge, this is the first genome-wide analysis to have investigated whether genetic variants modify the longitudinal relationship of traffic-related air pollution with FEV_1 in children with asthma. Three SNPs showed genome-wide significant interactions with NO_2 in Caucasian subjects (rs13090972 and rs958144 in the *EPHA3* (receptor tyrosine kinase of Eph family; location 3p11.2) and rs7041938 in the *TXNDC8* gene (thioredoxin domain containing 8 (spermatozoa) or Spermatocyte/Spermatid-Specific Thioredoxin-3; location 9q31.3) and a set of variants showed evidence for modifying the air pollution effects on FEV_1 in both Caucasian and African American subjects (SNP-NO_2 interactions: rs4672884 in *MR-1*, rs3769767 in *METTL5* and rs4378142 in a non-coding protein gene/ENSG00000233067; SNP-CO interactions: rs2720625 near the Magnesium Uptake/Transporter (Tumor Suppressor Candidate 3 - TUSC3).

The functions of the above genes suggest was that genes with potential metabolic, oxidant/anti-oxidant and inflammatory role might be important for lung function responses to pollution in children with asthma. Potential interactions of NO_2 with metabolic, inflammatory pathways were also revealed by our pathway analysis, whereas a potential interaction with the anti-oxidant heme oxygenase-1/CO system was found with both CO and NO_2 exposures. We conclude that genetics play a role in the respiratory response of asthmatics to traffic-related air pollution by regulating oxidant/anti-oxidant stress cellular mechanisms and inflammation.

Strengths and limitations

In the thesis the populations studied had some distinctive study designs. One of our Dutch adult asthma cohorts had available medical records with more than 2 decades of data on lung function and ICS use for each subject. This unique characteristic of the cohort gave us the opportunity to perform mixed model analysis of lung function decline and in addition assess lung function decline before and after introduction of ICS. The disadvantage of using this unique cohort was that it was difficult to find another study suitable for replicating our results. Cohort studies including bronchial biopsies, as we performed, offer the opportunity of studying both clinical and pathological aspects of asthma. However most of those studies are of smaller size compared to ours and do not have genetic material available as we do, thus we could not replicate SNP-airway pathology associations. Finally, our childhood asthma clinical trial was unique in several ways: it had a long follow-up period and repeated measurements, it included airway hyperresponsiveness not commonly assessed in children with asthma, large numbers of children and cities, short- and long-term pollution exposure and it had genomic data. Yet, for replication studies, definition and measurement of the exposure and/or outcome is critical to the success of investigating G–E interactions. We were not able to detect a study ensuring that the genotyping, outcome and exposure levels were measured reliably and consistently in a longitudinal manner.

FUTURE PERSPECTIVES

In the last 20 years, different approaches have been used to associate genetic polymorphisms to a disease phenotype, resulting in improvement of genotype analyses. The candidate gene approach was the first and most simple method used, based on the *a priori* hypothesis of the involvement of a gene in pathways playing a role in the determination of an intermediate phenotype, such as molecular and cellular functions, or in a clinically evident effect. Genome-wide approaches provided the opportunity to cover the entire genome and have successfully identified numerous loci at which common variants influence disease risk or quantitative traits (www.genome.gov/GWAS).[57] Furthermore, GWA studies gave information on genes involved in previously unsuspected pathways, which could have been never considered in a candidate gene approach.[58] Despite these successes, the variants identified by these studies generally explain only a small fraction of the heritable component of disease risk. [59] This enforces the knowledge that there is not a distinct number of genes

for asthma with moderate-to-small risks, but rather a large number of variations spread over the genome, each with small effects. Such a polygenic model predicts that the more markers are used, the better the disease is predicted and it implies that everybody carries risk variants but patients carrying more risk variants are at higher risk. Multi-locus profiles of genetic risk, so-called "genetic risk scores", have been developed to translate discoveries from candidate gene and GWA studies into tools for population health research.[60] Although most prognostic studies published to date claim significant results and that they found factors that could offer additional predictive value beyond what the standard phenotype based score could achieve, very few translate to clinically useful applications.[61-63]

The mechanisms of action by which associated loci influence disease or quantitative phenotypes are often unclear, because in many cases we cannot pinpoint with certainty the true causal variant(s) at the associated loci, or we do not know through which gene(s) the associated variants exert their effects or the function of these gene(s), or their connection to known disease biology. Thus, finding additional loci that contain causal variants, refining the location and phenotypic consequences of causal variants and progressing from known loci and variants to functional mechanisms are needed.[58,64] This can now be done by assaying gene expression and genetic variation simultaneously on a genome-wide basis in a large number of individuals.[65] Statistical genetic methods can be used to map the genetic factors that underpin individual differences in quantitative levels of expression of many thousands of transcripts in different cell types (expression Quantitative Trait Loci (eQTL)).[66] Integrative genomics and lung eQTLs will serve as an important resource to aid in the understanding of the molecular underpinnings of lung biology and diseases.[65]

Another common obstacle in unfolding asthma pathophysiology is the likelihood that different subsets of genes influence disease susceptibility, disease severity and sub-phenotypes/endotypes (e.g., a minimal subdivision includes atopic asthma, cough variant asthma, brittle asthma, intrinsic asthma, aspirin-intolerant asthma (AIA), and occupational non-IgE-dependent asthma, steroid-resistant asthma, eosinophilic vs neutrophilc asthma etc).[57,67] Since most GWA studies and many candidate gene studies so far have focused on asthma development, there is still genetic ground to be explored in asthma severity, remission and the underlying airway pathology. In addition, study of gene-gene interactions has started providing insight into the pathophysiology of asthma.[68,69]

It is clear that for some genetic variants, the effect of the variant depends on certain environmental exposures, or equivalently, that the effect of an

environmental factor depends on an individual's genetic makeup.[10] Studies of gene-environment interactions and Mendelian randomization approaches have led to increased insights into the importance of environmental triggers for allergic disease.[7,70] Now we are entering the era of epidemiological designs and statistical analysis approaches for studying specific gene-environment interactions.[71] Furthermore, techniques for mining interactions in GWA data are now available and new approaches are being developed for studying entire pathways. Importantly, focus should be given in exploring methods for coupling hypothesis-driven pathway-based approaches with 'agnostic' GWAS.[71,72] These approaches will help to identify underlying mechanisms that might be involved in the variability seen in response to environmental exposures and to asthma treatment.

If a pathway and its related genes are pinpointed and replicated, further insight is needed to determine their role in asthma pathogenesis. Candidate gene studies of pathway-related genes and functional testing may help in this respect as well. For example, eQTL analysis in relevant tissue (association analysis between risk SNPs or haplotypes in asthma patients with mRNA and protein expression in lung tissue); development of in vitro and vivo assays to study gene and its coding protein function are required. Establishing a solid biological role of a gene or set of genes will be the first step to novel therapeutic discoveries.

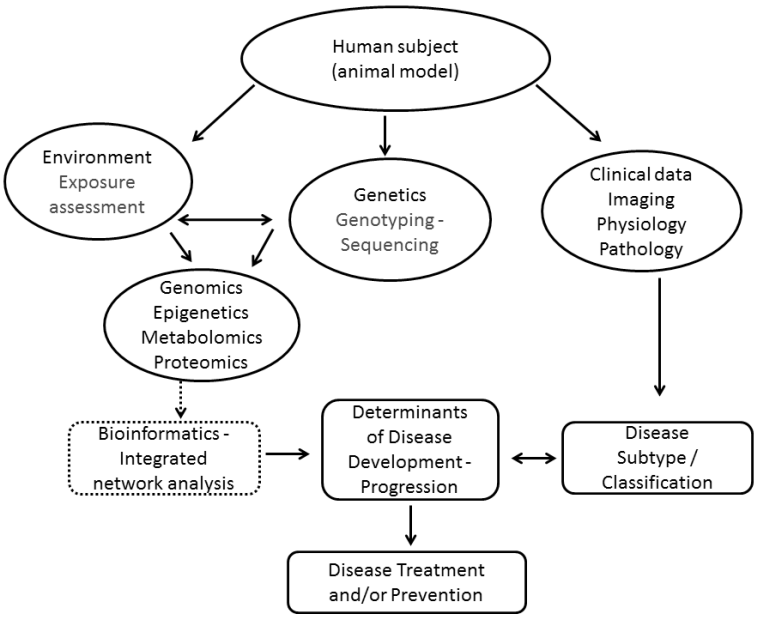
The above means that our pathway analysis of genome-wide gene by air pollution study is only the first step to the journey of exploring the biological mechanisms underlying susceptibility of asthmatics to air pollution and that several steps are needed before we reach new therapeutic targets in asthma. However, we are encouraged by our findings for a prominent role of oxidative-stress, a mechanism that has already been documented by experimental studies to have a role in asthma pathophysiology.[73] Understanding the complex pathways that lead from oxidant stress to asthma development and severity increases potential therapeutic strategies. Future success of antioxidant asthma therapy will require strategies with broad effects on airway redox equilibrium and the selection of appropriate target populations. Furthermore, we cannot discard the epidemiologic evidence that high (diet) intake of antioxidants, such as vitamins A, D, and E; zinc; selenium, fruits and vegetables; and a Mediterranean diet may prevent inflammation and asthma, so experimental studies of these exposures are now warranted.[74,75] However, decreasing exposure to air pollution and other oxidants like (environmental) tobacco smoking, still remains the most clear cut recommendation we can make today.

Proteomic applications have been used as a complimentary technique to verify the suspected candidate proteins involved in asthma. Efforts in asthma proteomics are being made to identify subgroups of disease entities, to establish appropriate biomarkers, and to enhance the understanding of underlying mechanisms in each subgroup.[76] Proteomic studies of animal models of asthma and confirmation of these findings in human tissues will significantly contribute to the understanding of the etiology of asthma and lead to the development of new therapeutic strategies for this highly prevalent disease. Moreover, the study of the proteome in response to environmental change is beginning to generate a number of new hypotheses about how organisms respond and adapt to a variety of stressors.[77]

Last but not least, technology to measure epigenetic marks (DNA methylation, modifications of histone tails, and noncoding RNAs) on a genomic scale and comprehensive approaches to data analysis have recently emerged and continue to improve.[78] Epigenetic mechanisms mediate genomic adaption to the environment and epigenetic alterations can contribute to the development of disease phenotypes, as can genetic variants. Alterations in epigenetic marks have been associated with exposures relevant to asthma, particularly air pollution and tobacco smoke (during pregnancy), as well as asthma phenotypes, in a few population-based studies.[79] Recent studies demonstrate that epigenetic effects of nicotine on asthma and lung function are not only linked to maternal smoking during pregnancy but they can also be multigenerational.[80,81] Epigenetic mechanisms represent a promising line of research to explain part of the hidden inheritance and pathophysiology of asthma and they may open new possibilities for therapeutic intervention.

A great challenge, therefore, as we move forward is to identify constellations of genes that interact to determine risk for specific patients. Such studies should take into account environmental context, as much as possible. These approaches will require very large sample sizes of carefully phenotyped patients, comprehensive genetic surveys of targeted genes or of genome-wide variation for gene-gene and gene-environment interactions, and rigorous statistical approaches that account for multiple comparisons. Collaboration between the different scientific disciplines using epidemiologic, genomic, proteomic and epigenetic information is the key to a network-based approach to human disease,[82,83] and developments in bioinformatics and computational algorithms will help exploit integrative genomic approaches. Better understanding of the implications of cellular interconnectedness on disease progression could lead to identification of disease genes and disease pathways, which, in turn,

could offer better targets for drug development for either prevention or cure of asthma.[84] Network medicine will help discover better and more accurate biomarkers, improve disease classification, and channel personalized therapies and treatment.



Network Medicine

REFERENCES

1. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001 Nov 15;164:S28-38.
2. Holgate ST. Epithelium dysfunction in asthma. J Allergy Clin Immunol 2007 Dec;120:1233-44; quiz 1245-6.
3. Holgate ST. The airway epithelium is central to the pathogenesis of asthma. Allergol Int 2008 Mar;57:1-10.
4. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nat Med 2012 May 4;18:693-704.
5. Bergeron C, Tulic MK, Hamid Q. Airway remodelling in asthma: from benchside to clinical practice. Can Respir J 2010 Jul-Aug;17:e85-93.
6. Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE. The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. Proc Am Thorac Soc 2009 Dec;6:655-659.
7. Holloway JW, Yang IA, Holgate ST. Genetics of allergic disease. J Allergy Clin Immunol 2010 Feb;125:S81-94.
8. Holloway JW, Yang IA, Holgate ST. Interpatient variability in rates of asthma progression: can genetics provide an answer? J Allergy Clin Immunol 2008 Mar;121:573-579.
9. Thomsen SF, van der Sluis S, Kyvik KO, Backer V. A study of asthma severity in adult twins. Clin Respir J 2012 Oct;6:228-237.
10. Blumenthal MN. Genetic, epigenetic, and environmental factors in asthma and allergy. Ann Allergy Asthma Immunol 2012 Feb;108:69-73.
11. Chetta A, Marangio E, Olivieri D. Inhaled steroids and airway remodelling in asthma. Acta Biomed 2003 Dec;74:121-125.
12. de Kluijver J, Schrumpf JA, Evertse CE, Sont JK, Roughley PJ, Rabe KF, Hiemstra PS, Mauad T, Sterk PJ. Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma. Clin Exp Allergy 2005 Oct;35:1361-1369.
13. O'Byrne PM, Pedersen S, Busse WW, Tan WC, Chen Y, Ohlsson SV, Ullman A, Lamm CJ, Pauwels RA. Effects of Early Intervention With Inhaled Budesonide on Lung Function in Newly Diagnosed Asthma*. Chest 2006 06//;129:1478-1485.
14. Fattahi F, Hylkema MN, Melgert BN, Timens W, Postma DS, ten Hacken NH. Smoking and nonsmoking asthma: differences in clinical outcome and pathogenesis. Expert Rev Respir Med 2011 Feb;5:93-105.
15. Aberle H, Schwartz H, Kemler R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 1996 06/15/;61:514-523.
16. Cavey M, Lecuit T. Molecular bases of cell-cell junctions stability and dynamics. Cold Spring Harb Perspect Biol 2009 Nov;1:a002998.
17. Hirosako S, Goto E, Tsumori K, Fujii K, Hirata N, Ando M, Kohrogi H. CD8 and CD103 Are Highly Expressed in Asthmatic Bronchial Intraepithelial Lymphocytes. Int Arch Allergy Immunol 2010 Apr 22;153:157-165.
18. Man Y, Hart VJ, Ring CJA, Sanjar S, West MR. Loss of Epithelial Integrity Resulting from E-Cadherin Dysfunction Predisposes Airway Epithelial Cells to Adenoviral Infection. Am J Respir Cell Mol Biol 2000 11/01/;23:610-617.
19. de Boer WI, Sharma HS, Baelemans SMI, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. Can J Physiol Pharmacol 2008 03//;86:105-112.
20. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An

ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989 12//;140:1745-1753.

21. Montefort S, DjukanoviÄ‡ R, Holgate ST, Roche WR. Ciliated cell damage in the bronchial epithelium of asthmatics and non-asthmatics. Clin Exp Allergy 1993 03//;23:185-189.
22. Tenenbaum T, Matalon D, Adam R, Seibt A, Wewer C, Schwerk C, Galla HJ, Schroten H. Dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine choroid plexus epithelial cells after infection with Streptococcus suis in vitro. Brain Res 2008 Sep 10;1229:1-17.
23. Carayol N, Campbell A, Vachier I, Mainprice B, Bousquet J, Godard P, Chanez P. Modulation of Cadherin and Catenins Expression by Tumor Necrosis Factor-alpha and Dexamethasone in Human Bronchial Epithelial Cells. Am J Respir Cell Mol Biol 2002 03/01/;26:341-347.
24. Foty RA, Corbett SA, Schwarzbauer JE, Steinberg MS. Dexamethasone up-regulates cadherin expression and cohesion of HT-1080 human fibrosarcoma cells. Cancer Res 1998 08/15/;58:3586-3589.
25. Baraket M, Oliver BG, Burgess JK, Lim S, King GG, Black JL. Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study. Respir Res 2012 Feb 2;13:11.
26. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, Walters E. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax 2002 04//;57:309-316.
27. Rasmussen F, Taylor DR, Flannery EM, Cowan JO, Greene JM, Herbison GP, Sears MR. Risk factors for airway remodeling in asthma manifested by a low postbronchodilator FEV1/vital capacity ratio: a longitudinal population study from childhood to adulthood. Am J Respir Crit Care Med 2002 06/01/;165:1480-1488.
28. Sont JK, Han J, van Krieken JM, Evertse CE, Hooijer R, Willems LN, Sterk PJ. Relationship between the inflammatory infiltrate in bronchial biopsy specimens and clinical severity of asthma in patients treated with inhaled steroids. Thorax 1996 05//;51:496-502.
29. Ye D, Ma I, Ma TY. Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. Am J Physiol Gastrointest Liver Physiol 2006 Mar;290:G496-504.
30. Carayol N, Vachier I, Campbell A, Crampette L, Bousquet J, Godard P, Chanez P. Regulation of E-cadherin expression by dexamethasone and tumour necrosis factor-{alpha} in nasal epithelium. Eur Respir J 2002 12/01/;20:1430-1436.
31. Thomas P,S. Tumour necrosis factor-[agr]: The role of this multifunctional cytokine in asthma. Immunol Cell Biol 2001 04;79:132-140.
32. Berry M, Brightling C, Pavord I, Wardlaw A. TNF-alpha in asthma. Curr Opin Pharmacol 2007 Jun;7:279-282.
33. Shima H, Ohshiro K, Taira Y, Miyazaki E, Oue T, Puri P. Antenatal dexamethasone suppresses tumor necrosis factor-alpha expression in hypoplastic lung in nitrofen-induced diaphragmatic hernia in rats. Pediatr Res 1999 Nov;46:633-637.
34. Holgate ST, Holloway J, Wilson S, Howarth PH, Haitchi HM, Babu S, Davies DE. Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities. J Allergy Clin Immunol 2006 Mar;117:496-506; quiz 507.
35. Matsuda A, Orihara K, Fukuda S, Fujinaga H, Matsumoto K, Saito H. Corticosteroid enhances TNF-alpha-mediated leukocyte adhesion to pulmonary microvascular endothelial cells. Allergy 2008 Dec;63:1610-1616.
36. Portelli M, Sayers I. Genetic basis for personalized medicine in asthma. Expert Rev Respir Med 2012 Apr;6:223-236.

37. Tse SM, Tantisira K, Weiss ST. The pharmacogenetics and pharmacogenomics of asthma therapy. Pharmacogenomics J 2011 Dec;11:383-392.

38. Tantisira KG, Lasky-Su J, Harada M, Murphy A, Litonjua AA, Himes BE, Lange C, Lazarus R, Sylvia J, Klanderman B, Duan QL, Qiu W, Hirota T, Martinez FD, Mauger D, Sorkness C, Szefler S, Lazarus SC, Lemanske RF,Jr, Peters SP, Lima JJ, Nakamura Y, Tamari M, Weiss ST. Genomewide association between GLCCI1 and response to glucocorticoid therapy in asthma. N Engl J Med 2011 Sep 29;365:1173-1183.

39. Tantisira KG, Damask A, Szefler SJ, Schuemann B, Markezich A, Su J, Klanderman B, Sylvia J, Wu R, Martinez F, Boushey HA, Chinchilli VM, Mauger D, Weiss ST, Israel E, SHARP Investigators. Genome-wide association identifies the T gene as a novel asthma pharmacogenetic locus. Am J Respir Crit Care Med 2012 Jun 15;185:1286-1291.

40. Salib RJ, Howarth PH. Transforming growth factor-beta in allergic inflammatory disease of the upper airways: friend or foe? Clin Exp Allergy 2009 Aug;39:1128-1135.

41. Ueda T, Niimi A, Matsumoto H, Takemura M, Yamaguchi M, Matsuoka H, Jinnai M, Chin K, Minakuchi M, Cheng L, Shirakawa T, Mishima M. TGFB1 promoter polymorphism C-509T and pathophysiology of asthma. J Allergy Clin Immunol 2008 Mar;121:659-664.

42. Pulleyn LJ, Newton R, Adcock IM, Barnes PJ. TGFbeta1 allele association with asthma severity. Hum Genet 2001 Dec;109:623-627.

43. Zhang Y, Zhang J, Huang J, Li X, He C, Tian C, Peng C, Guo L, Xiao Y, Fan H. Polymorphisms in the transforming growth factor-beta1 gene and the risk of asthma: A meta-analysis. Respirology 2010 05;15:643-650.

44. Guo ZH, DU YC, Xu JY. The effect of cigarette smoke on the expression of transforming growth factor-beta1 mRNA and collagen type III in airways of asthmatic rats. Zhonghua Jie He He Hu Xi Za Zhi 2008 Jan;31:42-45.

45. Tzivian L. Outdoor air pollution and asthma in children. J Asthma 2011 Jun;48:470-481.

46. Schildcrout JS, Sheppard L, Lumley T, Slaughter JC, Koenig JQ, Shapiro GG. Ambient air pollution and asthma exacerbations in children: an eight-city analysis. Am J Epidemiol 2006 Sep 15;164:505-517.

47. Sunyer J, Spix C, Quenel P, Ponce-de-Leon A, Ponka A, Barumandzadeh T, Touloumi G, Bacharova L, Wojtyniak B, Vonk J, Bisanti L, Schwartz J, Katsouyanni K. Urban air pollution and emergency admissions for asthma in four European cities: the APHEA Project. Thorax 1997 Sep;52:760-765.

48. Liu L, Poon R, Chen L, Frescura AM, Montuschi P, Ciabattoni G, Wheeler A, Dales R. Acute effects of air pollution on pulmonary function, airway inflammation, and oxidative stress in asthmatic children. Environ Health Perspect 2009 Apr;117:668-674.

49. Romieu I, Meneses F, Ruiz S, Sienra JJ, Huerta J, White MC, Etzel RA. Effects of air pollution on the respiratory health of asthmatic children living in Mexico City. Am J Respir Crit Care Med 1996 Aug;154:300-307.

50. Yang IA, Fong KM, Zimmerman PV, Holgate ST, Holloway JW. Genetic susceptibility to the respiratory effects of air pollution. Postgrad Med J 2009 Aug;85:428-436.

51. Kelly FJ. Oxidative stress: its role in air pollution and adverse health effects. Occup Environ Med 2003 Aug;60:612-616.

52. Patel MM, Chillrud SN, Deepti KC, Ross JM, Kinney PL. Traffic-related air pollutants and exhaled markers of airway inflammation and oxidative stress in New York City adolescents. Environ Res 2013 Feb;121:71-78.

53. Emmerechts J, Hoylaerts MF. The effect of air pollution on haemostasis. Hamostaseologie 2012;32:5-13.

54. Auerbach A, Hernandez ML. The effect of environmental oxidative stress on airway inflammation. Curr Opin Allergy Clin Immunol 2012 Apr;12:133-139.

55. Sikorska K, Rivadeneira F, Groenen PJ, Hofman A, Uitterlinden AG, Eilers PH, Lesaffre E. Fast linear mixed model computations for genome-wide association studies with longitudinal data. Stat Med 2013 Jan 15;32:165-180.

56. Zhang K, Cui S, Chang S, Zhang L, Wang J. i-GSEA4GWAS: a web server for identification of pathways/gene sets associated with traits by applying an improved gene set enrichment analysis to genome-wide association study. Nucleic Acids Res 2010 Jul;38:W90-5.

57. Sleiman PM, Hakonarson H. Recent advances in the genetics and genomics of asthma and related traits. Curr Opin Pediatr 2010 Jun;22:307-312.

58. Holloway JW, Koppelman GH. Identifying novel genes contributing to asthma pathogenesis. Curr Opin Allergy Clin Immunol 2007 Feb;7:69-74.

59. Weiss ST. What genes tell us about the pathogenesis of asthma and chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2010 Jun 1;181:1170-1173.

60. Belsky DW, Moffitt TE, Sugden K, Williams B, Houts R, McCarthy J, Caspi A. Development and evaluation of a genetic risk score for obesity. Biodemography Soc Biol 2013;59:85-100.

61. Tzoulaki I, Liberopoulos G, Ioannidis JP. Assessment of claims of improved prediction beyond the Framingham risk score. JAMA 2009 Dec 2;302:2345-2352.

62. Talmud PJ, Hingorani AD, Cooper JA, Marmot MG, Brunner EJ, Kumari M, Kivimaki M, Humphries SE. Utility of genetic and non-genetic risk factors in prediction of type 2 diabetes: Whitehall II prospective cohort study. BMJ 2010 Jan 14;340:b4838.

63. Janssens AC, Ioannidis JP, van Duijn CM, Little J, Khoury MJ, GRIPS Group. Strengthening the reporting of Genetic Risk Prediction Studies: the GRIPS statement. Genet Med 2011 May;13:453-456.

64. McCarthy MI, Hirschhorn JN. Genome-wide association studies: potential next steps on a genetic journey. Hum Mol Genet 2008 Oct 15;17:R156-65.

65. Hao K, Bosse Y, Nickle DC, Pare PD, Postma DS, Laviolette M, Sandford A, Hackett TL, Daley D, Hogg JC, Elliott WM, Couture C, Lamontagne M, Brandsma CA, van den Berge M, Koppelman G, Reicin AS, Nicholson DW, Malkov V, Derry JM, Suver C, Tsou JA, Kulkarni A, Zhang C, Vessey R, Opiteck GJ, Curtis SP, Timens W, Sin DD. Lung eQTLs to help reveal the molecular underpinnings of asthma. PLoS Genet 2012;8:e1003029.

66. Bosse Y. Genome-wide expression quantitative trait loci analysis in asthma. Curr Opin Allergy Clin Immunol 2013 Aug 13.

67. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, Lemanske RF,Jr, Wardlaw AJ, Wenzel SE, Greenberger PA. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol 2011 Feb;127:355-360.

68. Chan IH, Tang NL, Leung TF, Huang W, Lam YY, Li CY, Wong CK, Wong GW, Lam CW. Study of gene-gene interactions for endophenotypic quantitative traits in Chinese asthmatic children. Allergy 2008 Aug;63:1031-1039.

69. Howard TD, Koppelman GH, Xu J, Zheng SL, Postma DS, Meyers DA, Bleecker ER. Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am J Hum Genet 2002 Jan;70:230-236.

70. Yang IA, Savarimuthu S, Kim ST, Holloway JW, Bell SC, Fong KM. Gene-environmental interaction in asthma. Curr Opin Allergy Clin Immunol 2007 Feb;7:75-82.

71. Cornelis MC, Tchetgen EJ, Liang L, Qi L, Chatterjee N, Hu FB, Kraft P. Gene-environment interactions in genome-wide association studies: a comparative study of tests applied to empirical studies of type 2 diabetes. Am J Epidemiol 2012 Feb 1;175:191-202.

72. Wei S, Wang LE, McHugh MK, Han Y, Xiong M, Amos CI, Spitz MR, Wei QW. Genome-wide gene-environment interaction analysis for asbestos exposure in lung cancer susceptibility. Carcinogenesis 2012 Aug;33:1531-1537.

73. Dozor AJ. The role of oxidative stress in the pathogenesis and treatment of asthma. Ann N Y Acad Sci 2010 Aug;1203:133-137.

74. Nurmatov U, Devereux G, Sheikh A. Nutrients and foods for the primary prevention of asthma and allergy: systematic review and meta-analysis. J Allergy Clin Immunol 2011 Mar;127:724-33.e1-30.

75. Patelarou E, Giourgouli G, Lykeridou A, Vrioni E, Fotos N, Siamaga E, Vivilaki V, Brokalaki H. Association between biomarker-quantified antioxidant status during pregnancy and infancy and allergic disease during early childhood: a systematic review. Nutr Rev 2011 Nov;69:627-641.

76. O'Neil SE, Lundback B, Lotvall J. Proteomics in asthma and COPD phenotypes and endotypes for biomarker discovery and improved understanding of disease entities. J Proteomics 2011 Dec 10;75:192-201.

77. Tomanek L. Introduction to the symposium „Comparative proteomics of environmental and pollution stress". Integr Comp Biol 2012 Nov;52:622-625.

78. Kabesch M, Adcock IM. Epigenetics in asthma and COPD. Biochimie 2012 Nov;94:2231-2241.

79. Kabesch M, Michel S, Tost J. Epigenetic mechanisms and the relationship to childhood asthma. Eur Respir J 2010 Oct;36:950-961.

80. Rehan VK, Liu J, Naeem E, Tian J, Sakurai R, Kwong K, Akbari O, Torday JS. Perinatal nicotine exposure induces asthma in second generation offspring. BMC Med 2012 Oct 30;10:129-7015-10-129.

81. Leslie FM. Multigenerational epigenetic effects of nicotine on lung function. BMC Med 2013 Feb 4;11:27-7015-11-27.

82. Bhattacharya S, Mariani TJ. Systems biology approaches to identify developmental bases for lung diseases. Pediatr Res 2013 Apr;73:514-522.

83. Silverman EK, Loscalzo J. Network medicine approaches to the genetics of complex diseases. Discov Med 2012 Aug;14:143-152.

84. Barabasi AL, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. Nat Rev Genet 2011 Jan;12:56-68.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL - CHAPTER 2

Inclusion criteria and corticosteroid use in population 1 and 2

In population 1, main inclusion criteria were: FEV_1 ≥1.2 L, absence of bronchiectasis, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within the last 2 months before inclusion. Only 36.8% (21/57) of subjects on ICS treatment had stopped ICS medication for a minimum of four weeks before entering the study.

In population 2, before testing, participants had to be in stable condition without any exacerbation in the last 6 weeks and subjects were allowed to use maintenance medication before testing. Subjects included in the lung function decline analysis required having FEV_1 measurements before and after the introduction of ICS, with a minimum of three FEV_1 measurements over a period of at least 2 years. Subjects who never used ICS were excluded from the performed analysis.

Bronchial biopsies

After local anesthesia, at least 5 bronchial biopsies were obtained from the subsegmental carinas from the left or right lower lobe using a flexible bronchoscope (Olympus BF P20 or BF XT20). Biopsies were fixed in 4% formalin, processed and embedded in paraffin and cut in 3µm thick sections. Macroscopically the best biopsy was selected for processing. Quantification was performed on the largest of three biopsy sections. Morphological features were determined on sections stained with hematoxylin and eosin (HE) and with periodic acid Schiff. Immunohistochemical stainings (except for E-cadherin) were performed using the DAKO autostainer (DAKO, Glostrup, Denmark). The slides were included in a random fashion in each run to avoid group-wise staining. Immunohistochemistry was performed using antibodies directed against CD8+T-cells (CD8, DAKO), CD31+endothelial cells (CD31, DAKO), eosinophilic peroxidase (EPX), epithelial adhesion (E-cadherin, BD Biosciences, Breda, the Netherlands). In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) and the chromogen NovaRED (Vector Labs, Burlingame, USA). EPX was detected via biotinylated anti-mouse IgG1 (Southern Biotech), alkaline phosphatase-labeled conjugate (DAKO) and permanent Red (DAKO). E-cadherin were detected with two peroxidaselabelled conjugates (both DAKO) and 3,3'-Diaminobenzidine

tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands). All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Inflammatory cell numbers were quantified by counting the number of positively stained cells in the submucosal area, 100µm under the basement membrane (BM), in a total area of 0.1mm^2 per biopsy sample. The number of CD31+ vessels in the submucosal area was counted in the whole section (excluding epithelium, muscle and mucus glands areas), hence we measured the number of vessels per area (0.1mm^2). Epithelial layer integrity was assessed on HE-stained biopsy sections and expressed as the percentage of BM covered with 1) normal, intact epithelium (basal and ciliated columnar epithelial cells), 2) denuded epithelium (absence of basal and ciliated cells) and 3) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). The length of basement membrane was variable among patients with a minimum of 973*µm*. Epithelial adhesion was determined by assessment of the percentage of BM covered with E-cadherin+ intact epithelium. The (median [min-max]) length of basement membrane covered with intact epithelium analyzed in each biopsy was 605 [0-3147] µm. BM thickness was calculated based on computer-assisted measurements of BM surface area and BM length. Reproducibility of the measurements was confirmed by a single observer performing repeated measurements in 10% of all cases.

TABLE E1: Genotype distributions in population 1 and 2

CDH1 gene SNPs	Location in gene	Alleles^	Population 1			Population 2		
			wt	htz	hmz	wt	htz	hmz
			n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
rs2902185[G]	Intron 2	T:C	81 (78.6)	22 (21.4)	0 (0)	180 (78.6)	48 (21.0)	1 (0.4)
rs11075699	Intron 2	A:G	44 (32.1)	67 (48.9)	26 (19.0)	67 (27.0)	124 (50.0)	57 (23.0)
rs1125557	Intron 2	A:G	43 (32.1)	67 (50.0)	24 (17.9)	93 (39.1)	106 (44.5)	39 (16.4)
rs12597188	Intron 2	G:A	54 (39.7)	64 (47.1)	18 (13.2)	104 (41.9)	108 (43.5)	36 (14.5)
rs7199991[G]	Intron 2	T:G	50 (48.5)	43 (41.7)	10 (9.7)	125 (54.6)	82 (35.8)	22 (9.6)
rs7186053	Intron 3	G:A	45 (32.8)	72 (52.6)	20 (14.6)	93 (37.2)	119 (47.6)	38 (15.2)
rs10431924	Intron 3	C:T	34 (24.8)	75 (54.7)	28 (20.4)	83 (33.9)	113 (46.1)	49 (20.0)
rs4783573	Intron 3	A:G	56 (41.2)	64 (47.1)	16 (11.8)	107 (43.5)	112 (45.5)	27 (11.0)
rs7188750[G]	Intron 5	G:A	69 (67.0)	29 (28.2)	5 (4.9)	166(72.5)	56 (24.5)	7 (3.1)
rs8056633	Intron 9	T:G	72 (53.7)	56 (41.8)	6 (4.5)	137 (55.7)	90 (36.6)	19 (7.7)
rs4783689	Intron11	C:T	46 (33.8)	64 (47.1)	26 (19.1)	66 (26.6)	121 (48.8)	61 (24.6)
rs16958383	Intron12	G:A	94 (69.1)	38 (27.9)	4 (2.9)	183 (73.8)	58 (23.4)	7 (2.8)
rs2276330[G]	Intron12	T:C	75 (72.8)	24 (23.3)	4 (3.9)	177 (77.3)	50 (21.8)	2 (0,9)
rs1801552[G]	Exon13	C:T	46 (44.7)	48 (46.6)	9 (8.7)	116 (50.7)	91 (39.7)	22 (9.6)
rs3785078	Intron14	A:C	101 (75.4)	31 (23.1)	2 (1.5)	186 (75.0)	59 (23.8)	3 (1.2)
rs7203904	Intron14	G:C	70 (51.1)	58 (42.3)	9 (6.6)	133 (53.8)	91 (36.8)	23 (9.3)
rs17690554	3' UTR	C:G	86 (62.8)	47 (34.3)	4 (2.9)	145 (58.9)	89 (36.8)	12 (4.9)

^major allele fist, [G] Genotyped in genome wide association study (GWAS) on asthma
SNPs= single nucleotide polymorphisms, wt: wild types, htz: heterozygotes and hmz: homozygotes mutant

TABLE E2: Associations of *CDH1* genotypes with epithelial E-cadherin expression in ICS and no ICS group

CDH1 gene SNPs		% epithelial E-cadherin expression#					
		no ICS			ICS		
		N	MEDIAN	^{s}P	N	MEDIAN	^{s}P
rs2902185	TT TC/CC	36 15	100 66	0.09	40 7	79 59	0.51
rs11075699	AA AG/GG	17 54	79 100	0.31	23 33	72 83	0.15
rs1125557	AA AG/GG	20 49	98 100	0.75	21 34	80 89	0.85
rs12597188	GG AG/AA	30 41	98 100	0.61	22 33	81 82	0.88
rs7199991	AA AC/CC	28 23	96 100	0.27	21 26	82 71	0.39
rs7186053	GG GA/AA	25 46	87 100	0.15	18 38	79 88	0.99
rs10431924	CC CT/TT	16 55	76 100	0.10	16 40	81 83	0.65
rs4783573	AA AG/GG	30 40	100 100	0.84	20 36	75 85	0.33
rs7188750	GG GA/AA	35 16	100 79	0.11	30 17	78 80	0.95
rs8056633	TT TG/GG	41 28	100 83	**0.04***	26 29	78 82	0.57
rs4783689	CC CT/TT	31 39	91 100	0.20	11 45	78 82	0.50
rs16958383	GG GA/AA	49 21	100 52	**0.01***	38 18	82 79	0.99
rs2276330	TT TC/CC	38 13	100 67	**0.049***	33 14	78 84	0.43
rs1801552	CC CT/TT	19 32	100 100	0.70	26 21	79 79	0.67
rs3785078	AA AC/CC	55 14	100 54	**0.03***	39 16	83 79	0.95
rs7203904	GG GC/CC	39 32	100 82	**0.04***	27 29	78 83	0.44
rs17690554	CC CG/GG	48 23	100 86	0.11	32 24	79 82	0.36

*p value<0.05, ^{s} significance tested with Mann–Whitney *U* test, # percentage of basement membrane covered with E-cadherin-positive stained intact epithelium, N= number of subjects

Table E3.1: Associations of *CDH1* genotypes with basement membrane thickness in ICS and no ICS group

		Basement membrane thickness (µm)								
***CDH1* gene SNPs**		**no ICS**			**ICS**			**Difference (ICS vs no ICS)#**		
		B	**95% CI**	**P**	**B**	**95% CI**	**P**	**B**	**95% CI**	**P**
rs2902185	TC/CC	-0.63	(-1.59;0.32)	0.19	1.05	(-0.21;2.31)	0.10	**1.68**	**(0.12;3.25)**	**0.036***
rs11075699	AG GG	0.05 -0.12	(-0.72;0.83) (-1.17;0.92)	0.89 0.82	0.60 -0.02	(-0.29;1.49) (-1.07;1.04)	0.18 0.98	0.55 0.11	(-0.62;1.72) (-1.37;1.59)	0.35 0.89
rs1125557	AG GG	-0.67 -0.23	(-1.48;0.13) (-1.31;0.86)	0.10 0.68	0.50 0.22	(-0.38;1.39) (-0.84;1.27)	0.27 0.69	1.17 0.44	(-0.03;2.37) (-1.07;1.95)	0.05 0.56
rs12597188	GA/AA	-0.06	(-0.74;0.63)	0.87	0.03	(-0.76;0.82)	0.94	0.06	(-0.96;1.20)	0.87
rs7199991	AC/CC	-0.14	(-1.01;0.73)	0.75	-0.25	(-1.17;0.67)	0.59	-0.11	(-1.39;1.16)	0.86
rs7186053	GA/AA	-0.45	(-1.17;0.27)	0.22	0.17	(-0.65;1.00)	0.68	0.62	(-0.48;1.72)	0.26
rs10431924	CT TT	-0.73 -0.65	(-1.58;0.12) (-1.72;0.43)	0.09 0.24	0.04 0.22	(-0.87;0.95) (-0.85;1.28)	0.93 0.69	0.77 0.86	(-0.49;2.03) (-0.65;2.37)	0.23 0.26
rs4783573	AG/GG	0.31	(-0.37;1.00)	0.36	-0.04	(-0.86;0.78)	0.92	-0.35	(-1.43;0.73)	0.52
rs7188750	GA/AA	-0.15	(-1.08;0.77)	0.75	-0.86	(-1.79;0.08)	0.07	-0.71	(-2.03;0.61)	0.29
rs8056633	TG/GG	-0.56	(-1.22;0.11)	0.10	-0.67	(-1.43;0.09)	0.09	-0.11	(-1.13;0.90)	0.83
rs4783689	CT TT	0.03 0.11	(-0.73;0.79) (-0.81;1.03)	0.94 0.81	0.04 0.33	(-1.00;1.07) (-0.98;1.65)	0.94 0.62	0.01 0.22	(-1.27;1.29) (-1.41;1.85)	0.99 0.79
rs16958383	GA/AA	-0.25	(-0.97;0.46)	0.48	**-1.05**	**(-1.86;-0.24)**	**0.01***	-0.79	(-1.88;0.29)	0.15
rs2276330	TC/CC	0.09	(-0.90;1.08)	0.86	**-1.08**	**(-2.07;-0.09)**	**0.03***	-1.17	(-2.60;0.26)	0.11
rs1801552	CT/TT	-0.55	(-1.44;0.34)	0.22	0.57	(-0.33;1.48)	0.21	1.12	(-0.14;2.38)	0.08
rs3785078	AC/CC	0.24	(-0.57;1.05)	0.56	**-1.05**	**(-1.88;-0.22)**	**0.01***	**-1.29**	**(-2.44;-0.13)**	**0.03***
rs7203904	GC/CC	-0.14	(-0.81;0.53)	0.68	-0.63	(-1.40;0.14)	0.11	-0.49	(-1.51;0.54)	0.35
rs17690554	CG/GG	-0.07	(-0.77;0.63)	0.84	**-1.00**	**(-1.76;-0.24)**	**0.01***	-0.93	(-1.97;0.11)	0.08

*p value<0.05, #Difference= interaction of ICS with SNP

Table E3.2: Associations of *CDH1* genotypes with subepithelial vasculature in ICS and no ICS group

CDH1 gene SNPs		Number of CD31+vessels per 0.1mm² of submucosal area								
		no ICS			ICS			Difference (ICS vs no ICS)#		
		B	95% CI	P	B	95% CI	P	B	95% CI	P
rs2902185	TC/CC	0.66	(-4.51;5.82)	0.80	-1.01	(-7.88;5.85)	0.77	-1.67	(-10.18;6.84)	0.70
rs11075699	AG	1.20	(-2.94;5.34)	0.57	3.25	(-1.56;8.07)	0.18	2.05	(-4.25;8.35)	0.52
	GG	2.53	(-2.93;7.99)	0.36	3.05	(-2.62;8.71)	0.29	0.52	(-7.35;8.38)	0.90
rs1125557	AG	-1.70	(-5.95;2.55)	0.43	3.93	(-0.84;8.69)	0.11	5.63	(-0.79;12.04)	0.09
	GG	-0.84	(-6.64;4.96)	0.78	2.93	(-2.92;8.78)	0.32	3.77	(-4.43;11.97)	0.36
rs12597188	GA/AA	2.06	(-1.56;5.69)	0.26	4.08	(-0.16;8.32)	0.06	2.02	(-3.52;7.56)	0.47
rs7199991	AC/CC	0.02	(-4.57;4.62)	0.99	3.22	(-1.67;8.11)	0.19	3.20	(-3.54;9.93)	0.35
rs7186053	GA/AA	1.56	(-2.28;5.37)	0.42	2.02	(-2.42;6.46)	0.37	0.47	(-5.41;6.34)	0.88
rs10431924	CT	-0.91	(-5.41;3.59)	0.69	0.81	(-4.19;5.82)	0.75	1.72	(-5.05;8.50)	0.62
	TT	-0.28	(-6.04;5.48)	0.92	0.55	(-5.22;6.31)	0.85	0.83	(-7.27;8.92)	0.84
rs4783573	AG/GG	0.64	(-3.00;4.27)	0.73	1.73	(-2.71;6.16)	0.44	1.09	(-4.73;6.91)	0.71
rs7188750	GA/AA	-3.18	(-7.93;1.56)	0.19	**-6.92**	**(-11.75;-2.09)**	**0.005***	-3.73	(-10.54;3.07)	0.28
rs8056633	TG/GG	-2.82	(-6.37;0.72)	0.12	**-4.65**	**(-8.81;-0.49)**	**0.03***	-1.82	(-7.30;3.65)	0.51
rs4783689	CT	0.04	(-3.93;4.01)	0.98	3.86	(-1.77;9.49)	0.18	3.82	(-3.06;10.69)	0.27
	TT	2.37	(-2.48;7.22)	0.33	5.57	(-1.49;12.62)	0.12	3.19	(-5.49;11.88)	0.47
rs16958383	GA/AA	-3.41	(-7.19;0.37)	0.08	**-6.11**	**(-10.40;-1.83)**	**0.005***	-2.70	(-8.43;3.03)	0.35
rs2276330	TC/CC	1.29	(-3.92;6.49)	0.63	**-6.94**	**(-12.15;-1.73)**	**0.01***	**-8.22**	**(-15.73;-0.71)**	**0.03***
rs1801552	CT/TT	-0.63	(-5.41;4.15)	0.79	0.65	(-4.28;5.59)	0.79	1.28	(-5.53;8.09)	0.71
rs3785078	AC/CC	-0.91	(-5.26;3.45)	0.68	**-6.61**	**(-11.09;-2.13)**	**0.004***	-5.71	(-11.93;0.52)	0.07
rs7203904	GC/CC	-2.27	(-5.76;1.23)	0.20	**-4.66**	**(-8.78;-0.54)**	**0.03***	-2.39	(-7.84;3.05)	0.39
rs17690554	CG/GG	-0.19	(-3.97;3.59)	0.92	-2.83	(-7.02;1.35)	0.18	-2.64	(-8.31;3.03)	0.36

*p value<0.05, #Difference= interaction of ICS with SNP

Table E4.1: Association of *CDH1* genotypes with CD8+T-cells in ICS and no ICS group

CDH1 gene SNPs		Number of CD8+T-cells per 0.1mm² of submucosal area (log-transformed)								
		no ICS			ICS			Difference (ICS vs no ICS)#		
		B	95% CI	P	B	95% CI	P	B	95% CI	P
rs2902185	TC/CC	-0.04	(-0.67;0.59)	0.90	-0.03	(-0.86;0.80)	0.94	0.01	(-1.02;1.04)	0.99
rs11075699	AG GG	-0.02 -0.37	(-0.53;0.49) (-1.04;0.31)	0.93 0.28	**0.62** -0.09	**(0.03;1.22)** (-0.79;0.60)	**0.04*** 0.79	0.65 0.28	(-0.13;1.42) (-0.69;1.25)	0.10 0.57
rs1125557	AG GG	0.47 0.30	(-0.04;0.99) (-0.40;1.00)	0.07 0.39	0.36 **-0.73**	(-0.22;0.94) **(-1.42;-0.05)**	0.22 **0.04***	-0.12 **-1.04**	(-0.89;0.66) **(-2.02;-0.06)**	0.77 **0.04***
rs12597188	GA/AA	0.39	(-0.06;0.84)	0.09	-0.12	(-0.64;0.41)	0.66	-0.51	(-1.20;0.18)	0.14
rs7199991	AC/CC	0.38	(-0.17;0.94)	0.17	-0.29	(-0.88;0.29)	0.32	-0.68	(-1.48;0.13)	0.10
rs7186053	GA/AA	0.48	(0.00;0.95)	0.05	0.13	(-0.43;0.68)	0.65	-0.35	(-1.08;0.38)	0.35
rs10431924	CT TT	0.24 0.32	(-0.31;0.79) (-0.39;1.02)	0.40 0.37	0.53 -0.26	(-0.08;1.14) (-0.96;0.45)	0.09 0.47	0.29 -0.57	(-0.53;1.12) (-1.56;0.42)	0.49 0.25
rs4783573	AG/GG	0.00	(-0.46;0.46)	0.99	0.02	(-0.54;0.57)	0.96	0.01	(-0.72;0.74)	0.97
rs7188750	GA/AA	-0.51	(-1.10;0.08)	0.09	0.53	(-0.07;1.12)	0.08	**1.04**	**(0.20;1.87)**	**0.02***
rs8056633	TG/GG	-0.15	(-0.61;0.32)	0.53	0.05	(-0.49;0.59)	0.86	0.19	(-0.52;0.90)	0.59
rs4783689	CT TT	-0.08 0.42	(-0.57;0.42) (-0.18;1.03)	0.76 0.17	-0.65 -0.42	(-1.34;0.03) (-1.28;0.45)	0.06 0.34	-0.58 -0.84	(-1.42;0.27) (-1.91;0.23)	0.18 0.12
rs16958383	GA/AA	-0.30	(-0.79;0.19)	0.23	0.30	(-0.26;0.87)	0.29	0.60	(-0.15;1.35)	0.11
rs2276330	TC/CC	-0.60	(-1.22;0.03)	0.06	**0.80**	**(0.18;1.42)**	**0.01***	**1.40**	**(0.51;2.30)**	**0.003***
rs1801552	CT/TT	-0.47	(-1.04;0.10)	0.11	0.05	(-0.53;0.64)	0.85	0.53	(-0.29;1.34)	0.20
rs3785078	AC/CC	-0.11	(-0.66;0.45)	0.70	0.36	(-0.22;0.94)	0.23	0.47	(-0.33;1.27)	0.25
rs7203904	GC/CC	-0.08	(-0.53;0.37)	0.73	0.24	(-0.29;0.77)	0.38	0.32	(-0.38;1.01)	0.37
rs17690554	CG/GG	0.01	(-0.46;0.48)	0.97	0.44	(-0.09;0.97)	0.10	0.43	(-0.28;1.14)	0.24

*p value<0.05, #Difference= interaction of ICS with SNPs

Table E4.2: Association of *CDH1* genotypes with eosinophilic peroxidase (EPX)+cells in ICS and no ICS group

CDH1 gene SNPs		Number of EPX+cells per 0.1mm² of submucosal area (log-transformed)								
		No ICS			ICS			Difference (ICS vs no ICS)#		
		B	95% CI	P	B	95% CI	P	B	95% CI	P
rs2902185	TC/CC	-0.01	(-0.65;0.63)	0.97	**0.94**	**(0.11;1.77)**	**0.03***	0.95	(-0.09;1.99)	0.07
rs11075699	AG	0.06	(-0.50;0.61)	0.84	-0.16	(-0.79;0.47)	0.61	-0.22	(-1.05;0.61)	0.60
	GG	-0.06	(-0.78;0.67)	0.87	-0.49	(-1.23;0.26)	0.20	-0.43	(-1.47;0.61)	0.41
rs1125557	AG	-0.01	(-0.57;0.54)	0.96	0.40	(-0.22;1.03)	0.21	0.42	(-0.43;1.26)	0.33
	GG	-0.19	(-0.95;0.57)	0.63	0.56	(-0.18;1.31)	0.14	0.75	(-0.31;1.81)	0.16
rs12597188	GA/AA	0.12	(-0.37;0.62)	0.62	0.21	(-0.36;0.78)	0.46	0.09	(-0.66;0.83)	0.82
rs7199991	AC/CC	0.04	(-0.54;0.63)	0.88	0.07	(-0.54;0.67)	0.83	0.02	(-0.82;0.86)	0.96
rs7186053	GA/AA	0.05	(-0.46;0.55)	0.85	0.43	(-0.15;1.01)	0.14	0.38	(-0.39;1.15)	0.33
rs10431924	CT	-0.09	(-0.68;0.49)	0.75	0.03	(-0.61;0.67)	0.93	0.12	(-0.74;0.99)	0.78
	TT	-0.29	(-1.03;0.45)	0.44	**0.81**	**(0.07;1.55)**	**0.03***	**1.09**	**(0.05;2.14)**	**0.04***
rs4783573	AG/GG	0.10	(-0.38;0.58)	0.68	-0.33	(-0.91;0.24)	0.26	-0.43	(-1.19;0.33)	0.26
rs7188750	GA/AA	-0.24	(-0.86;0.38)	0.45	0.21	(-0.42;0.83)	0.51	0.44	(-0.44;1.33)	0.32
rs8056633	TG/GG	-0.17	(-0.65;0.31)	0.49	-0.04	(-0.59;0.51)	0.89	0.13	(-0.61;0.86)	0.73
rs4783689	CT	-0.02	(-0.55;0.51)	0.94	0.46	(-0.27;1.18)	0.21	0.48	(-0.42;1.37)	0.29
	TT	0.14	(-0.50;0.78)	0.66	0.74	(-0.17;1.66)	0.11	0.60	(-0.54;1.74)	0.30
rs16958383	GA/AA	-0.38	(-0.90;0.14)	0.15	0.16	(-0.42;0.75)	0.58	0.55	(-0.24;1.33)	0.17
rs2276330	TC/CC	-0.20	(-0.87;0.48)	0.57	0.29	(-0.38;0.95)	0.39	0.48	(-0.48;1.45)	0.32
rs1801552	CT/TT	-0.33	(-0.92;0.27)	0.28	-0.16	(-0.77;0.44)	0.60	0.16	(-0.68;1.00)	0.70
rs3785078	AC/CC	0.13	(-0.46;0.73)	0.66	-0.01	(-0.62;0.59)	0.96	-0.15	(-1.00;0.70)	0.73
rs7203904	GC/CC	-0.17	(-0.65;0.31)	0.48	-0.06	(-0.61;0.49)	0.84	0.11	(-0.62;0.84)	0.76
rs17690554	CG/GG	0.15	(-0.35;0.65)	0.56	-0.01	(-0.56;0.54)	0.97	-0.16	(-0.91;0.59)	0.68

*p value<0.05, #Difference= interaction of ICS with SNPs

Table E5: Effect of *CDH1* genotypes on annual change in lung function before and after the introduction of ICS

		FEV_1 annual change in population 2 (ml/year)								
		no ICS			ICS			Difference (ICS vs no ICS)#		
CDH1 gene SNPs		Annual change in WT	Difference in annual change compared to WT B (95%CI)	P	Annual change in WT	Difference in annual change compared to WT B (95%CI)	P	Annual change in WT	Difference in annual change compared to WT B (95%CI)	P
rs2902185	TT	-21.6			-17.4			4.1		
	TC/CC		-17.5 (-38.8;3.8)	0.11		-3.7 (-16.40;8.96)	0.57		13.7 (-10.4;37.9)	0.26
rs11075699	AA	-25.9			-20.3			5.5		
	AG		-1.3 (-20.6;18.1)	0.90		1.0 (-11.77;13.74)	0.35		2.2 (-20.1;24.6)	0.84
	GG		1.8 (-20.8;24.5)	0.87		7.67 (-8.33;23.68)	0.38		5.9 (-21.1;32.8)	0.67
rs1125557	AA	-22.7			-7.6			-7.6		
	AG		-5.4 (-21.8;11.1)	0.52		**-19.7 (-32.0;-7.4)**	**0.002***		-14.3 (-34.5;5.9)	0.16
	GG		-1.1 (-24.2;22.0)	0.93		**-17.6 (-33.0;-2.3)**	**0.024***		-16.6 (-43.7;10.6)	0.23
rs12597188	GG	-23.2			-17.1			6.2		
	GA		-6.5 (-22.8;9.7)	0.43		-1.6 (-13.8;10.7)	0.80		5.0 (-14.7;24.6)	0.62
	AA		-3.2 (-25.0;18.5)	0.77		-2.2 (-18.0;13.7)	0.79		1.1 (-25.0;27.1)	0.94
rs7199991	AA	-29.9			-15.0			14.9		
	AC		7.8 (-9.8;25.4)	0.39		**-13.5 (-25.0;-1.9)**	**0.023***		**-21.3 (-41.3;-1.2)**	**0.04***
	CC		16.5 (-7.0;40.0)	0.17		-5.1 (-20.6;10.4)	0.52		-21.7 (-48.8;5.4)	0.12
rs7186053	GG	-25.8			-8.7			17.1		
	GA		-1.7 (-18.4;15.0)	0.84		**-16.7 (-28.2;-5.1)**	**0.005***		-15.0 (-34.6;4.6)	0.13
	AA		-6.1 (-29.5;17.3)	0.61		-13.7 (-28.8;1.5)	0.08		-7.6 (-34.5;19.3)	0.58
rs10431924	CC	-25.8			-14.0			11.8		
	CT		-2.7 (-20.5;15.1)	0.76		-4.6 (-18.1;8.9)	0.51		-1.9 (-23.3;19.5)	0.86
	TT		-6.0 (-27.6;15.6)	0.58		-12.1 (-26.7;2.5)	0.10		-6.1 (-31.0;18.7)	0.63
rs4783573	AA	-24.9			-20.7			4.3		
	AG		-2.9 (-18.9;13.1)	0.43		4.7 (-6.8;16.2)	0.43		7.6 (-11.4;26.5)	0.43
	GG		10.1 (-15.2;35.5)	0.98		19.2 (-1.5;39.8)	0.07		9.0 (-22.6;40.6)	0.58

rs7188750	GG	-25.8			-19.2			6.6		
	GA/AA		0.4 (-20.7;21.6)	0.97		5.6 (-6.6;17.7)	0.37		5.2 (-18.2;28.5)	0.66
rs8056633	TT	-28.3			-23.0			5.3		
	TG		6.5 (-10. 6;23.6)	0.46		8.6 (-3.9;21.1)	0.18		2.2 (-18.1;22.3)	0.84
	GG		6.2 (-18.5;30.8)	0.62		**27.2 (14.2;40.3)**	**<0.001**		21.1 (-5.6;47.7)	0.12
rs4783689	CC	-21.6			-13.6			7.9		
	CT		-4.8 (-23.6;14.0)	0.62		-6.4 (-20.9;8.1)	0.39		-1.6 (-24.8;21.5)	0.89
	TT		-8.3 (-29.2;12.5)	0.43		-4.1 (-20.5;12.3)	0.62		4.2 (-21.8;30.2)	0.75
rs16958383	GG	-25.3			-20.4			4.9		
	GA		-10.8 (-30.2;8.6)	0.27		10.5 (-2.1;23.2)	0.10		21.4 (-0.8;43.5)	0.06
	AA		4.4 (-28.1;36.9)	0.79		**36.6(11.9;61.3)**	**0.004***		32.2 (-6.4;70.8)	0.10
rs2276330	TT	-25.4			-19.1			6.2		
	TC/CC		-11.1 (-34.9;12.8)	0.36		6.7 (-6.3;19.6)	0.31		17.7 (-8.1;43.6)	0.18
rs1801552	CC	-27.5			-15.0			12.5		
	CT		5.9 (-12.9;24.7)	0.54		-7.5 (-25.8;10.9)	0.44		-13.4 (-34.9;8.1)	0.22
	TT		1.2 (-26.9;29.2)	0.94		-7.2 (-18.5;4.1)	0.21		-86 (-40.6;23.9)	0.61
rs3785078	AA	-24.4			-20.0			4.3		
	AC/CC		-10.6 (-28.6;7.4)	0.25		10.5 (-1.9;22.9)	0.10		**21.1 (0.4;41.8)**	**0.05**
rs7203904	GG	-24.6			-22.3			2.3		
	GC		-3.9 (-20.8;13.1)	0.66		1.7 (-9.7;13.0)	0.78		5.5 (-14.1;25.2)	0.58
	CC		6.0 (-21.4;33.5)	0.67		**27.4 (13.4;41.4)**	**<0.001**		21.4 (-8.6;51.3)	0.16
rs17690554	CC	-25.3			-23.5			1.8		
	CG		-2.2 (-18.8;14.5)	0.80		**15.3 (4.0;26.6)**	**0.008***		17.4 (-1.5;36.2)	0.07
	GG		20.2 (-37.2;77.61)	0.49		**26.9 (7.2;46.5)**	**0.007***		6.7 (-51.4;64.7)	0.82

*p value<0.05, #Difference=interaction of ICS with SNP, FEV_1=forced expiratory volume in one second, WT=wild types

Table E6.1: Associations of *CDH1* genotypes with FEV_1/VC post bronchodilator in ICS and no ICS group in population 1

CDH1 gene SNPs		FEV_1/VC post bronchodilator population 1 (%)								
		no ICS			ICS			Difference (ICS vs no ICS)#		
		B	95% CI	P	B	95% CI	P	B	95% CI	P
rs2902185	TC/CC	-0.59	(-6.30;5.11)	0.84	-6.25	(-13.47;0.98)	0.09	-5.65	(-14.73;3.42)	0.22
rs11075699	AG	-1.37	(-5.91;3.17)	0.55	3.67	(-1.48;8.82)	0.16	5.03	(-1.76;11.83)	0.15
	GG	-3.05	(-8.97;2.87)	0.31	2.32	(-3.76;8.40)	0.45	5.37	(-3.11;13.85)	0.21
rs1125557	AG	2.51	(-2.08;7.11)	0.28	1.01	(-4.09;6.11)	0.70	-1.50	(-8.41;5.40)	0.67
	GG	4.36	(-1.86;10.57)	0.17	-1.74	(-7.83;4.36)	0.57	-6.09	(-14.76;2.58)	0.17
rs12597188	GA/AA	3.11	(-0.91;7.13)	0.13	0.39	(-4.22;4.99)	0.87	-2.72	(-8.79;3.34)	0.38
rs7199991	AC/CC	1.80	(-3.30;6.90)	0.49	0.53	(-4.69;5.75)	0.84	-1.27	(-8.56;6.02)	0.73
rs7186053	GA/AA	2.86	(-1.36;7.07)	0.18	1.01	(-3.76;5.77)	0.68	-1.85	(-8.22;4.53)	0.57
rs10431924	CT	3.94	(-0.87;8.76)	0.11	4.68	(-0.51;9.86)	0.08	0.73	(-6.37;7.83)	0.84
	TT	2.61	(-3.49;8.72)	0.40	-1.57	(-7.61;4.47)	0.61	-4.19	(-12.72;4.35)	0.33
rs4783573	AG/GG	-1.37	(-5.32;2.59)	0.50	0.30	(-4.43;5.02)	0.90	1.66	(-4.58;7.91)	0.60
rs7188750	GA/AA	-2.52	(-7.90;2.85)	0.35	2.61	(-2.73;7.96)	0.33	5.14	(-2.47;12.74)	0.18
rs8056633	TG	-0.62	(-4.63;3.40)	0.76	-1.81	(-6.42;2.80)	0.44	-1.19	(-7.31;4.93)	0.70
	GG	1.21	(-9.11;11.53)	0.82	9.29	(-0.94;19.53)	0.07	8.08	(-6.57;22.73)	0.28
rs4783689	CT	2.73	(-1.61;7.07)	0.22	-4.40	(-10.29;1.49)	0.14	-7.13	(-14.44;0.18)	0.06
	TT	0.40	(-4.87;5.66)	0.88	-5.99	(-13.45;1.47)	0.11	-6.39	(-15.67;2.90)	0.18
rs16958383	GA/AA	0.41	(-3.88;4.70)	0.85	0.53	(-4.27;5.33)	0.83	0.12	(-6.34;6.58)	0.97
rs2276330	TC/CC	-3.86	(-9.61;1.88)	0.18	3.63	(-2.03;9.29)	0.21	7.49	(-0.72;15.70)	0.07
rs1801552	CT/TT	-0.26	(-5.48;4.96)	0.92	1.42	(-3.80;6.65)	0.59	1.68	(-5.63;9.00)	0.65
rs3785078	AC/CC	-0.80	(-5.71;4.11)	0.75	1.92	(-3.07;6.92)	0.45	2.72	(-4.24;9.69)	0.44
rs7203904	GC	0.28	(-3.68;4.23)	0.89	-2.16	(-6.79;2.47)	0.36	-2.44	(-8.54;3.67)	0.43
	CC	-1.18	(-10.24;7.88)	0.80	**8.95**	**(0.82;17.08)**	**0.03***	10.13	(-2.19;22.45)	0.11
rs17690554	CG/GG	-1.87	(-6.03;2.30)	0.38	2.17	(-2.34;6.67)	0.34	4.03	(-2.11;10.17)	0.20

*p value<0.05, #Difference= interaction of ICS with SNP, FEV_1= forced expiratory volume in one second, VC= vital capacity

Table E6.2: Associations of *CDH1* genotypes with FEV_1/VC post bronchodilator in ICS and no ICS group in population 2

CDH1 gene SNPs		FEV_1/VC post bronchodilator population 2 (%)								
		no ICS			ICS			Difference (ICS vs no ICS)#		
		B	95% CI	P	B	95% CI	P	B	95% CI	P
rs2902185	TC/CC	-0.08	(-4.63;4.48)	0.97	-4.63	(-9.52;0.27)	0.06	-4.55	(-11.24;2.15)	0.18
rs11075699	AG	-0.69	(-5.51;4.12)	0.78	2.55	(-1.85;6.96)	0.26	3.25	(-3.25;9.74)	0.33
	GG	-3.45	(-9.25;2.34)	0.24	-0.18	(-5.42;5.05)	0.95	3.27	(-4.54;11.09)	0.41
rs1125557	AG	1.16	(-3.21;5.52)	0.60	1.69	(-2.59;5.96)	0.44	0.53	(-5.51;6.57)	0.86
	GG	3.79	(-2.54;10.12)	0.24	-4.18	(-9.57;1.20)	0.13	-7.97	(-16.28;0.33)	0.06
rs12597188	GA	2.18	(-1.96;6.32)	0.30	1.17	(-3.02;5.37)	0.58	-1.01	(-6.88;4.85)	0.73
	AA	0.21	(-6.67;7.09)	0.95	-0.75	(-6.03;4.54)	0.78	-0.96	(-9.68;7.77)	0.83
rs7199991	AC/CC	1.10	(-2.82;5.01)	0.58	0.89	(-3.07;4.84)	0.66	-0.21	(-5.74;5.31)	0.94
rs7186053	GA	1.78	(-2.32;5.89)	0.39	-2.16	(-6.25;1.93)	0.30	-3.94	(-9.71;1.83)	0.18
	AA	-0.86	(-6.90;5.18)	0.78	-4.59	(-9.98;0.80)	0.09	-3.73	(-11.77;4.31)	0.36
rs10431924	CT	1.39	(-2.92;5.70)	0.53	-1.45	(-5.89;2.99)	0.52	-2.84	(-8.99;3.31)	0.36
	TT	0.20	(-5.62;6.01)	0.95	-1.82	(-6.99;3.35)	0.49	-2.02	(-9.73;5.70)	0.61
rs4783573	AG	-3.22	(-7.35;0.91)	0.13	3.58	(-0.39;7.55)	0.08	**6.80**	**(1.09;12.51)**	**0.02***
	GG	-2.76	(-8.67;3.15)	0.36	**8.36**	**(1.38;15.35)**	**0.02***	**11.12**	**(1.98;20.27)**	**0.02***
rs7188750	GA/AA	-1.84	(-6.00;2.31)	0.38	**5.19**	**(0.70;9.69)**	**0.02***	**7.03**	**(0.95;13.12)**	**0.02***
rs8056633	TG/GG	-2.89	(-6.67;0.88)	0.13	**7.14**	**(3.33;10.94)**	**<0.001***	**10.03**	**(4.64;15.41)**	**<0.001***
rs4783689	CT	-0.39	(-4.97;4.20)	0.87	-3.54	(-8.17;1.08)	0.13	-3.16	(-9.69;3.37)	0.34
	TT	-0.22	(-5.58;5.15)	0.94	-4.67	(-9.95;0.61)	0.08	-4.45	(-11.96;3.05)	0.24
rs16958383	GA/AA	-0.81	(-5.04;3.41)	0.70	**5.50**	**(1.09;9.91)**	**0.01***	**6.31**	**(0.23;12.39)**	**0.042***
rs2276330	TC/CC	-0.84	(-5.24;3.56)	0.71	4.48	(-0.43;9.39)	0.07	5.32	(-1.23;11.87)	0.11
rs1801552	CT/TT	2.21	(-1.62;6.05)	0.26	0.88	(-3.03;4.79)	0.66	-1.34	(-6.83;4.16)	0.63
rs3785078	AC/CC	-1.64	(-5.96;2.67)	0.45	**6.07**	**(1.68;10.45)**	**0.01***	-1.34	(-6.83;4.16)	0.63
rs7203904	GC	-2.45	(-6.48;1.57)	0.23	**5.14**	**(1.12;9.17)**	**0.01***	**7.60**	**(1.88;13.31)**	**0.01***
	CC	-3.24	(-9.76;3.28)	0.33	**9.84**	**(3.08;16.59)**	**<0.001***	**13.08**	**(3.71;22.45)**	**0.01***
rs17690554	CG/GG	-2.63	(-6.37;1.11)	0.17	**7.45**	**(3.66;11.24)**	**<0.001***	**10.08**	**(4.77;15.40)**	**<0.001***

*p value<0.05, #Difference= interaction of ICS with SNP, FEV_1= forced expiratory volume in one second, VC= vital capacity

SUPPLEMENTARY MATERIAL - CHAPTER 3

Subjects

All participants had doctor's diagnosed asthma and originated from the northern region of The Netherlands.

Population 1: 138 asthma patients, previously investigated by our research group were re-examined during the period 2002-2006.[E1] Lung function testing and adenosine 5'-monophosphate (AMP) provocation test were performed followed 1-2 weeks later by a bronchoscopy. DNA samples and bronchial biopsies were available in 137 subjects.

Population 2: 281 asthma patients who entered a cohort study between 1966 and 1975 were re-examined during the period 1991–1999.[E2] Patients filled in a questionnaire on respiratory symptoms and underwent lung function testing, histamine challenge (30 seconds tidal breathing method) and skin prick tests. DNA samples have been collected from 244 subjects. Thirty two subjects of population 2 underwent bronchoscopy and are part of population 1.

Population 3: This asthmatic population was investigated between 1998 and 2000. It consisted of subjects with asthma who were ascertained through local hospitals and media appeals. Available parents, sibs, spouses, and children donated DNA to form trios (i.e. a patient and both parents) for genetic statistical analyses. This population has been extensively described elsewhere.[E3] In the current analyses, we selected the asthma patients only (n=302) (defined by algorithm[E4]) and evaluated AHR (histamine PC_{20} < 32 mg/mL using the 30-second inhalation method) and FEV_1 and VC (%predicted) assessed by water-sealed spirometry.

Ethical approval was obtained from the Medical Ethics Committee of the University of Groningen, and written informed consent was obtained from all participants of the 3 populations.

Biopsy collection and processing (population 1)

Quantification was performed on the largest of three biopsy sections taken. Morphological features were determined on sections stained with hematoxylin and eosin (HE) and with periodic acid Schiff. Immunohistochemistry was performed on 3µm formalin fixed, paraffin embedded tissue specimens that were deparafinized with xylene, dehydrated in ethanol and after antigen retrieval, incubated with the primary antibodies: anti-NP57 (DAKO) for neutrophils, anti-CD68 (DAKO) for macrophages, anti-AA1 (DAKO) for mast cells and anti-EPX

antibody for eosinophils. The slides were included in a random fashion in each run to avoid group-wise staining. All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Basement membrane (BM) thickness was calculated by dividing the BM surface area by BM length (μm), based on computer-assisted measurements. The number of CD31+ (DAKO) vessels in the submucosal area was counted in the whole section (excluding epithelium, muscle and mucus glands areas) therefore we measured the number of vessels per area (0.1mm^2). The number of positively stained inflammatory cells was counted in a total area of 0.1 mm^2 in the submucosa, 100μm under the BM. Goblet cell numbers were counted on PAS-stained biopsy sections and expressed per 1000 μm of BM. Epithelial layer integrity was assessed on HE-stained biopsy sections and expressed as length (μm) of BM covered with 1) normal, intact epithelium (basal and ciliated columnar epithelial cells), 2) denuded epithelium (absence of basal and ciliated cells) and 3) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). Epithelial adhesion was determined by assessment of the percentage of BM covered with E-cadherin+ intact epithelium (Anti-E-cadherin antibody (BD Bioscience #610181, Breda, The Netherlands). Additionally, airway smooth muscle (ASM) area and mast cells within ASM were quantified. A minimal area of 0.05mm^2 ASM was considered sufficient for the assessment of mast cell infiltration. Reproducibility of the measurements was confirmed by a single observer performing repeated measurements in 10% of all cases.

Sputum induction, processing and measurements (population 1)

Sputum was induced by inhalation of 5% hypertonic saline aerosols over three consecutive periods of 5 minutes. Processed whole sputum samples were stained with May-Grünwald-Giemsa to obtain cell differentials by counting total 600 viable, non-squamous cells. Sputum was not used if the percentage of squamous cells was greater than 80% or if the total number of nonsquamous cells was less than 600.

Genotyping of Single Nucleotide Polymorphisms (SNPs)

Six haplotype tagging SNPs in the *loci (TNFα and LTA genes*) (-/+5kb) according to HapMap CEU genotype data (release 24/phase II Nov'08) ($r^2 <$ 0.8) were genotyped by K-Bioscience Ltd (UK) using their competitive allele specific PCR system (KASPar). We concentrated on promoter polymorphisms,

non-synonymous variants, and polymorphisms already linked to asthma and/or TNF levels. For population 3 the SNPs were derived from a genome wide association study on asthma using the 307 and 370Kb Illumina chip. We used BEAGLE software (version 3.1.1) for imputation and CEU samples from HapMap2 and as reference populations.

Statistics

In population 1, normalization of the distribution of the variables was performed by natural logarithm transformation if necessary. Multiple linear regression analysis adjusted for gender, age and smoking was used to assess the effect of the SNPs on a. epithelial integrity (length of basement membrane (BM) covered with denuded epithelium in μm), b. inflammatory cell counts (eosinophils, macrophages, neutrophils and mast cells per 0.1mm^2 of submucosa) and c. airway remodeling parameters (BM thickness, submucosal vessel numbers and goblet cell counts). We tested interaction between ICS and genotypes by introducing interaction terms into the models. We assessed associations of SNPs with numbers of cells in induced sputum (eosinophils, macrophages and neutrophils), with airway smooth muscle (ASM) area and number of mast cells within ASM, and with epithelial E-cadherin expression in the two groups (no ICS and ICS users) with non-parametric tests (Mann-Whitney U test) because normalization of the residuals was not possible in regression models.

In populations 2 and 3, we assessed the main effects of SNPs with multiple linear regression models and tested for associations of the interactions between ICS and SNPs with pre and post bronchodilator (BD) FEV_1%predicted, corrected for smoking. We also assessed associations with FEV_1/VC ratio and (ln) AHR slope corrected for gender, age, height and smoking. We used the beta-coefficients and standard errors of the SNPs and their interactions with ICS to meta-analyze associations with asthma severity phenotypes i.e. AHR slope and lung function in the two populations.

All regression modeling was conducted with IBM SPSS statistics (version 20; Armonk, NY USA: IBM Corp 2011). Because the number of subjects with the homozygote mutant genotype was relatively low in the 2 subgroups (n≈<10) (see table E1 online repository), only dominant (heterozygotes and homozygotes mutant pooled vs wild types) models were used. Meta-analysis was performed with R-programming language using the meta-package (R version 2.14.1 (2012-03-29)). Two tailed p-values of <0.05 were considered statistically significant and p-values between 0.05 and 0.09 of borderline significance.

REFERENCES

E1. Broekema M, ten Hacken NHT, Volbeda F, Lodewijk ME, Hylkema MN, Postma DS, et al. Airway Epithelial Changes in Smokers but Not in Ex-Smokers with Asthma. Am J Respir Crit Care Med 2009 12/15/;**180**:1170-1178.

E2. Panhuysen CI, Vonk JM, Koëter GH, Schouten JP, van Altena R, Bleecker ER et al. Adult patients may outgrow their asthma: a 25-year follow-up study. Am J Respir Crit Care Med 1997 04//;**155**:1267-1272.

E3. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ, Xu J, Koning H, Bruinenberg M, Nolte IM, van Diemen CC, Boezen HM, Timens W, Whittaker PA, Stine OC, Barton SJ, Holloway JW, Holgate ST, Graves PE, Martinez FD, van Oosterhout AJ, Bleecker ER, Postma DS. Identification of PCDH1 as a Novel Susceptibility Gene for Bronchial Hyperresponsiveness. Am J Respir Crit Care Med 2009 11/15/;180:929-935.

E4. Panhuysen CI, Bleecker ER, Koeter GH, Meyers DA, Postma DS. Characterization of obstructive airway disease in family members of probands with asthma. An algorithm for the diagnosis of asthma. Am J Respir Crit Care Med 1998 Jun;157:1734-1742.

Table E1 a-d: genotype distributions of *TNF*

a. Population 1		Total		no ICS users		ICS users	
		n	%	n	%	n	%
rs1800629	GG	88	64.2	50	62.5	38	66.7
	GA	47	34.3	28	35.0	19	33.3
	AA	2	1.5	2	2.5	0	
	Total	137		80		57	
rs1800630	CC	94	69.1	58	73.4	36	63.2
	AC	37	27.2	19	24.1	18	31.6
	AA	5	3.7	2	2.5	3	5.3
	Total	136		79		57	
rs1799964	TT	84	62.2	51	64.6	33	58.9
	CT	47	34.8	25	31.6	22	39.3
	CC	4	3.0	3	3.8	1	1.8
	Total	135		79		56	
rs2229094	TT	66	51.2	41	55.4	25	45.5
	CT	53	41.0	24	32.4	29	52.7
	CC	10	7.8	9	12.2	1	1.8
	Total	129		74		55	
rs909253	TT	55	42.3	29	38.6	26	47.3
	CT	54	41.5	32	42.7	22	40.0
	CC	21	16.2	14	18.7	7	12.7
	Total	130		75		55	
rs915654	AA	55	41.7	35	46.0	20	35.7
	TA	62	47.0	30	39.5	32	57.1
	TT	15	11.3	11	14.5	4	7.1
	Total	132		76		56	

b. Population 2		Total		no ICS users		ICS users	
		n	%	n	%	n	%
rs1800629	GG	142	58.2	67	56.3	75	60.0
	GA	88	36.1	44	37.0	44	35.2
	AA	14	5.7	8	6.7	6	4.8
	Total	244		119		125	
rs1800630	CC	160	67.5	80	69.0	80	54.8
	AC	66	27.8	33	28.4	33	22.6
	AA	11	4.6	3	2.6	8	5.5
	Total	237		116		121	
rs1799964	TT	142	58.6	74	62.7	68	54.8
	CT	87	36.0	41	34.7	46	37.1
	CC	13	5.4	3	2.6	10	8.1
	Total	242		118		124	
rs2229094	TT	122	52.1	63	55.8	59	48.8
	CT	93	39.7	45	39.8	48	39.7
	CC	19	8.0	5	4.4	14	11.5
	Total	234		113		121	
rs909253	TT	89	37.2	39	33.6	50	40.7
	CT	112	46.9	56	48.3	56	45.5
	CC	38	15.9	21	18.1	17	13.8
	Total	239		116		123	
rs915654	AA	103	43.3	49	41.5	54	45.0
	TA	108	45.4	58	49.2	50	41.7
	TT	27	11.3	11	9.3	16	13.3
	Total	238		118		120	

c. Population 2*		Total		no ICS users		ICS users	
		n	%	n	%	n	%
rs1800629	GG	101	60.3	49	63.6	52	60.5
	GA	52	33.1	24	31.2	28	32.5
	AA	10	6.6	4	5.2	6	7.0
	Total	163		77		86	
rs1800630	CC	100	67.0	46	63.9	54	65.8
	AC	48	29.1	24	33.3	24	29.3
	AA	6	3.9	2	2.8	4	4.9
	Total	154		72		82	
rs1799964	TT	90	58.1	41	53.2	49	56.4
	CT	65	36.9	32	41.6	33	37.9
	CC	9	5.0	4	5.2	5	5.7
	Total	164		77		87	
rs2229094	TT	70	45.2	32	43.8	40	48.8
	CT	72	46.5	35	47.9	35	42.7
	CC	13	8.3	6	8.3	7	8.5
	Total	155		73		82	
rs909253	TT	65	38.3	31	40.3	34	40.5
	CT	70	44.6	35	45.5	35	41.6
	CC	26	17.1	11	14.3	15	17.9
	Total	161		77		84	
rs915654	AA	66	43.7	30	40.6	36	41.8
	TA	72	44.1	34	45.9	38	44.2
	TT	22	12.2	10	13.5	12	14.0
	Total	160		74		86	

d. Population 3		Total		no ICS users		ICS users	
		n	%	n	%	n	%
rs1800629	GG	181	59.7	35	58.3	146	60.3
	GA	106	35.3	21	35.0	85	35.1
	AA	15	5.0	4	6.7	11	4.6
	Total	302		60		242	
rs1800630	CC	224	74.3	48	80.0	176	72.7
	AC	72	23.7	11	18.3	61	25.2
	AA	6	2.0	1	1.7	5	2.1
	Total	302		60		242	
rs1799964	TT	196	65.0	40	66.7	156	64.5
	CT	95	31.4	19	31.6	76	31.4
	CC	11	3.6	1	1.7	10	4.1
	Total	302		60		242	
rs2229094	TT	169	56.1	33	55.0	136	56.2
	CT	107	35.3	23	38.3	84	34.7
	CC	26	8.6	4	6.7	22	9.1
	Total	302		60		242	
rs909253	TT	115	38.0	19	31.7	96	39.7
	CT	146	48.5	33	55.0	113	46.7
	CC	41	13.5	8	13.3	33	13.6
	Total	302		60		242	
rs915654	AA	147	48.8	30	50.0	117	48.3
	TA	133	43.8	27	45.0	106	43.8
	TT	22	7.3	3	5.0	19	7.9
	Total	302		60		242	

ICS: Inhaled corticosteroids; n: number of subjects
* Subgroup of population 2 included in the FEV_1 decline analysis: Inclusion criteria required subjects having FEV_1 measurements before and after the introduction of ICS, with a minimum of three FEV_1 measurements over a period of at least 2 years. Subjects who never used ICS were excluded.

Table E2: Associations of *TNF* genotypes with length of basement membrane covered with denuded epithelium (in µm) in population 1

Population 1	Denuded Epithelium (ln-scale)											
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	B	95%CI		P-value	B	95%CI		P-value	B	95%CI		P-value
rs1800629-A	-0.4	-1.2	0.3	0.276	-0.7	-1.7	0.2	0.110	-0.3	-1.5	0.9	0.599
rs1800630-A	0.9	0.1	1.7	**0.031**	-0.5	-1.4	0.4	0.253	-1.4	-2.6	-0.2	**0.022**
rs1799964-C	1.1	0.3	1.8	**0.005**	-0.3	-1.2	0.6	0.514	-1.4	-2.5	-0.2	**0.022**
rs2229094-C	1.4	0.7	2.2	**<0.001**	0.1	-0.7	0.9	0.856	-1.4	-2.5	-0.3	**0.016**
rs909253-C	-0.5	-1.3	0.3	0.200	-0.4	-1.2	0.5	0.396	0.1	-1.0	1.3	0.836
rs915654-T	0.6	-0.2	1.3	0.141	-0.3	-1.2	0.6	0.508	-0.9	-2.1	0.3	0.149

SNPs: single nucleotide polymorphisms, ICS: inhaled corticosteroids, B: regression coefficient, CI: confidence interval; SNP x ICS: interaction

Table E3: Associations of *TNF* genotypes with epithelial E-cadherin expression in population 1

Population 1	% E-cadherin expression			
***TNF* SNPs**	**no ICS use**		**ICS use**	
	MD	**P-value**	**MD**	**P-value**
rs1800629-A	16.0	**0.024**	-10.0	0.482
rs1800630-A	-56.0	**0.017**	28.0	**0.076**
rs1799964-C	-8.0	0.254	13.0	0.370
rs2229094-C	-19.0	0.129	14.0	0.146
rs909253-C	19.0	**0.092**	-20.0	**0.066**
rs915654-T	-14.0	0.203	2.0	0.951

MD: Median Difference; P- values are given by a Mann Whitney U test; SNPs: single nucleotide polymorphisms;

Table E4: Associations of *TNF* SNPs with airway wall inflammation in population 1

Population 1	number of eosinophils / 0.1mm² submucosa (ln-scale)											
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	B	95%CI		P-value	B	95%CI		P-value	B	95%CI		P-value
rs1800629-A	-0.3	-0.7	0.2	0.313	0.6	0.01	1.2	**0.045**	0.8	0.1	1.6	**0.031**
rs1800630-A	-0.2	-0.8	0.3	0.348	0.6	0.0	1.1	**0.050**	0.8	0.04	1.6	**0.038**
rs1799964-C	-0.1	-0.6	0.4	0.686	0.5	-0.1	1.1	0.086	0.6	-0.2	1.3	0.122
rs2229094-C	-0.2	-0.7	0.3	0.388	0.2	-0.3	0.8	0.384	0.5	-0.3	1.2	0.230
rs909253-C	-0.5	-1.0	0.04	**0.033**	0.7	0.1	1.2	**0.021**	1.2	0.4	1.9	**0.002**
rs915654-T	-0.4	-0.9	0.02	0.061	0.9	0.3	1.4	**0.002**	1.3	0.6	2.0	**<0.001**
	number of macrophages / 0.1mm² submucosa (ln-scale)											
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	B	95%CI		P-value	B	95%CI		P-value	B	95%CI		P-value
rs1800629-A	0.1	-0.3	0.4	0.675	-0.1	-0.4	0.3	0.794	-0.1	-0.6	0.4	0.642
rs1800630-A	-0.4	-0.7	-0.1	**0.023**	0.2	-0.2	0.5	0.359	0.6	0.1	1.1	**0.028**
rs1799964-C	-0.4	-0.7	-0.1	**0.025**	0.1	-0.3	0.5	0.627	0.4	0.0	0.9	**0.070**
rs2229094-C	-0.3	-0.7	0.01	**0.041**	0.2	-0.2	0.5	0.417	0.5	0.0	1.0	**0.055**
rs909253-C	0.04	-0.4	0.3	0.787	-0.1	-0.5	0.2	0.493	-0.1	-0.6	0.4	0.737
rs915654-T	-0.2	-0.5	0.1	0.122	0.1	-0.3	0.5	0.546	0.3	-0.1	0.8	0.148
	number of neutrophils / 0.1mm² submucosa (ln-scale)											
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	B	95%CI		P-value	B	95%CI		P-value	B	95%CI		P-value
rs1800629-A	0.2	-0.2	0.6	0.378	0.2	-0.2	0.7	0.313	0.1	-0.6	0.7	0.836
rs1800630-A	-0.3	-0.7	0.1	0.127	0.3	-0.1	0.8	0.144	0.7	0.0	1.3	**0.036**
rs1799964-C	-0.3	-0.7	0.1	0.140	0.1	-0.3	0.6	0.590	0.4	-0.2	1.0	0.176
rs2229094-C	-0.3	-0.7	0.1	0.145	0.3	-0.1	0.8	0.168	0.6	0.0	1.2	**0.051**
rs909253-C	0.1	-0.3	0.5	0.748	0.02	-0.4	0.5	0.925	0.0	-0.6	0.6	0.889
rs915654-T	-0.1	-0.5	0.3	0.555	-0.02	-0.5	0.4	0.935	0.1	-0.5	0.7	0.755

SNPs: single nucleotide polymorphisms, ICS: inhaled corticosteroids, B: regression coefficient, CI: confidence interval; SNP x ICS: interaction

Table E5: Associations of *TNF* genotypes with number of vessels in the submucosal area in population 1

Population 1	number of vessels / 0.1mm²											
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	B	95%CI		P-value	B	95%CI		P-value	B	95%CI		P-value
rs1800629-A	0.9	-2.8	4.6	0.636	-3.9	-8.4	0.5	0.084	-4.8	-10.7	1.1	0.107
rs1800630-A	-5.1	-9.0	-1.2	**0.011**	-0.01	-4.3	4.3	0.995	5.1	-0.8	10.9	0.087
rs1799964-C	-3.0	-6.6	0.6	0.098	0.8	-3.4	5.0	0.703	3.8	-1.7	9.3	0.177
rs2229094-C	-3.0	-6.7	0.7	0.108	1.9	-2.4	6.1	0.390	4.9	-0.9	10.6	0.094
rs909253-C	-0.4	-4.1	3.3	0.828	-4.3	-8.6	-0.04	**0.048**	-3.9	-9.6	1.8	0.177
rs915654-T	-4.4	-8.0	-0.8	**0.018**	-1.7	-6.2	2.8	0.454	2.7	-3.1	8.5	0.356

SNPs: single nucleotide polymorphisms, ICS: inhaled corticosteroids, B: regression coefficient, CI: confidence interval; SNP x ICS: interaction

Table E6: Associations of *TNF* genotypes with basement membrane thickness (in µm) in population 1

Population 1	Basement membrane thickness in µm											
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	B	95%CI		P-value	B	95%CI		P-value	B	95%CI		P-value
rs1800629-A	-0.3	-1.0	0.4	0.349	-0.2	-1.0	0.7	0.684	0.2	-0.9	1.3	0.772
rs1800630-A	-0.2	-0.9	0.6	0.629	-0.6	-1.4	0.2	0.129	-0.4	-1.5	0.7	0.428
rs1799964-C	-0.4	-1.1	0.3	0.234	-0.5	-1.3	0.3	0.193	-0.1	-1.2	1.0	0.837
rs2229094-C	-0.3	-1.0	0.4	0.423	-0.3	-1.1	0.5	0.445	-0.02	-1.1	1.0	0.969
rs909253-C	0.2	-0.5	0.8	0.628	0.3	-0.5	1.1	0.432	0.1	-0.9	1.2	0.788
rs915654-T	0.1	-0.6	0.7	0.849	-0.3	-1.1	0.5	0.510	-0.3	-1.4	0.7	0.531

SNPs: Single nucleotide polymorphisms, ICS: inhaled corticosteroids, B: regression coefficient, CI: confidence interval; SNP x ICS: interaction

Table E7: Associations of *TNF* genotypes with airway hyperresponsiveness: Meta-analysis of populations 2 and 3

Airway hyperresponsiveness (ln of slope)												
TNF SNPs	no ICS use				ICS use				SNP x ICS			
	I^2	$B^{\#}$	95% CI	p-value	I^2	$B^{\#}$	95% CI	p-value	I^2	$B^{\#}$	95% CI	p-value
rs1800629-A	0	-0.16	-0.6 0.3	0.503	0	0.01	-0.3 0.3	0.974	0	0.15	-0.4 0.7	0.602
rs1800630-A	0	0.01	-0.5 0.6	0.972	53	-0.24	-0.6 0.1	0.167	67	-0.34	-1.0 0.3	0.322
rs1799964-C	46	0.15	-0.3 0.6	0.557	0	-0.16	-0.4 0.1	0.245	53	-0.30	-0.9 0.3	0.330
rs2229094-C	72*	0.07	-0.8 0.9	0.879	0	-0.14	-0.4 0.1	0.307	67	-0.29	-0.9 0.3	0.348
rs909253-C	0	-0.17	-0.6 0.3	0.463	0	0.03	-0.3 0.4	0.867	0	0.10	-0.5 0.7	0.744
rs915654-T	0	-0.14	-0.6 0.3	0.516	0	-0.04	-0.3 0.2	0.779	0	0.05	-0.5 0.6	0.863

SNPs: Single nucleotide polymorphism, ICS: inhaled corticosteroids, B: regression coefficient, CI: confidence interval; SNP x ICS: interaction. I^2 : Index of heterogeneity (in percentages), * P-value of Cochran's Q-test (of heterogeneity) is <0.07, # Fixed effects are used. When I^2 is high (>70%) and/or Q-test has a p<0.07 random effects are used

Table E8: Associations of rs1800629 with lung function and airway hyperresponsiveness in populations 2 and 3

rs1800629-A	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value
Population 2 (n=244)	**no ICS (n= 67 vs 52)**					**ICS (n= 75 vs 50)**					**SNP x ICS**				
FEV_1%predicted pre BD	-1.70	3.67	-8.90	5.50	0.65	6.20	3.62	-0.90	13.30	**0.09**	7.90	5.15	-2.20	18.00	0.13
FEV_1%predicted post BD	-3.40	3.57	-10.30	3.60	0.34	5.40	3.52	-1.50	12.30	0.13	8.70	5.05	-1.10	18.60	**0.08**
FEV_1/VC% pre BD	-0.20	2.14	-4.40	4.00	0.93	6.80	2.19	2.60	11.10	**0.002**	7.00	3.06	1.00	13.00	**0.02**
FEV_1/VC% post BD	-1.00	1.99	-4.80	2.90	0.62	5.70	1.99	1.80	9.60	**0.004**	6.70	2.76	1.20	12.10	**0.02**
(ln) AHR slope (n=213)	-0.20	0.31	-0.80	0.40	0.49	-0.30	0.36	-0.90	0.40	0.41	-0.10	0.46	-0.90	0.80	0.88
Population 3 (n=302)	**no ICS (n= 34 vs 25)**					**ICS (n= 146 vs 96)**					**SNP x ICS**				
FEV_1%predicted pre BD	-6.00	4.23	-14.40	2.30	0.16	-2.70	2.14	-7.00	1.50	0.21	3.30	4.80	-6.10	12.70	0.49
FEV_1%predicted post BD	-4.70	3.62	-11.80	2.40	0.19	-2.20	1.84	-5.80	1.40	0.22	2.50	4.08	-5.50	10.50	0.54
FEV_1/IVC% pre BD	-3.10	2.70	-8.50	2.20	0.25	-1.70	1.38	-4.40	1.00	0.23	1.50	3.01	-4.50	7.40	0.63
FEV_1/IVC% post BD	-2.50	2.24	-6.90	1.90	0.27	-1.50	1.12	-3.80	0.70	0.19	0.90	2.55	-4.00	5.90	0.71
(ln) AHR slope (n=295)	-0.10	0.36	-0.80	0.60	0.71	0.10	0.20	-0.20	0.50	0.46	0.30	0.36	-0.50	1.00	0.50

ICS: inhaled corticosteroids; B: regression coefficient; S.E: standard error; CI: confidence interval; FEV_1: forced expiratory volume in 1 second; IVC: inspiratory vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; SNPs: single nucleotide polymorphisms; SNP x ICS: interaction
n= number of subjects (homozygotes for major allele versus carriers of the minor allele)

Table E9: Associations of rs1800630 with lung function and airway hyperresponsiveness in population 2 and 3

rs1800630-A	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value
Population 2 (n=235)	**no ICS (n= 80 vs 35)**					**ICS (n= 79 vs 41)**					**SNP x ICS**				
FEV_1%predicted pre BD	-0.60	4.08	-8.50	7.40	0.89	8.80	3.88	1.30	16.40	**0.02**	9.40	5.61	-1.50	20.40	**0.09**
FEV_1%predicted post BD	0.80	3.93	-6.90	8.50	0.84	6.90	3.78	-0.60	14.30	**0.07**	6.10	5.46	-4.60	16.80	0.27
FEV_1/VC% pre BD	-0.70	2.45	-5.50	4.10	0.77	2.10	2.35	-2.50	6.70	0.38	2.80	3.37	-3.80	9.40	0.41
FEV_1/VC% post BD	-1.20	2.24	-5.50	3.20	0.60	0.80	2.14	-3.40	5.00	0.70	2.00	3.06	-4.00	8.00	0.51
(ln) AHR slope (n=213)	0.20	0.36	-0.40	0.90	0.45	-0.70	0.36	-1.40	0.00	**0.04**	-1.00	0.51	-1.90	0.00	**0.04**
Population 3 (n=302)	**no ICS (n= 47 vs 12)**					**ICS (n= 176 vs 66)**					**SNP x ICS**				
FEV_1%predicted pre BD	3.20	5.31	-7.10	13.60	0.54	2.40	2.40	-2.30	7.10	0.31	-0.80	5.77	-12.20	10.50	0.89
FEV_1%predicted post BD	0.40	4.49	-8.40	9.20	0.94	2.00	2.04	-2.00	6.00	0.32	1.60	4.95	-8.00	11.30	0.74
FEV_1/IVC% pre BD	1.20	3.32	-5.40	7.70	0.72	2.30	1.53	-0.60	5.30	0.12	1.20	3.62	-6.00	8.30	0.75
FEV_1/IVC% post BD	-0.10	2.76	-5.60	5.30	0.96	1.80	1.22	-0.70	4.20	0.16	1.90	3.01	-4.00	7.80	0.53
(ln) AHR slope (n=295)	-0.30	0.46	-1.10	0.60	0.56	-0.10	0.20	-0.50	0.30	0.73	0.20	0.46	-0.70	1.10	0.70

ICS: inhaled corticosteroids; B: regression coefficient; S.E: standard error; CI: confidence interval; FEV_1: forced expiratory volume in 1 second; IVC: inspiratory vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; SNPs: single nucleotide polymorphisms; SNP x ICS: interaction
n= number of subjects (homozygotes for major allele versus carriers of the minor allele)

Table E10: Associations of rs1799964 with lung function and airway hyperresponsiveness in populations 2 and 3

rs1799964-C	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value
Population 2 (n=240)	**no ICS (n= 74 vs 43)**					**ICS (n=67 vs 56)**					**SNP x ICS**				
FEV_1%predicted pre BD	-4.70	3.78	-12.10	2.70	0.21	7.10	3.57	0.10	14.10	**0.05**	11.80	5.15	1.60	21.90	**0.02**
FEV_1%predicted post BD	-2.30	3.67	-9.60	4.90	0.53	5.40	3.52	-1.50	12.30	0.13	7.70	5.10	-2.30	17.70	0.13
FEV_1/VC% pre BD	-3.20	2.30	-7.60	1.30	0.16	2.20	2.14	-2.10	6.40	0.32	5.30	3.16	-0.80	11.50	**0.09**
FEV_1/VC% post BD	-3.30	2.04	-7.30	0.70	0.11	1.40	1.99	-2.50	5.30	0.48	4.70	2.86	-0.90	10.30	0.10
(ln) AHR slope (n=211)	0.40	0.31	-0.20	1.00	0.19	-0.40	0.31	-1.10	0.20	0.20	-0.80	0.46	-1.70	0.10	**0.07**
Population 3 (n=302)	**no ICS (n= 29 vs 20)**					**ICS (n= 156 vs 86)**					**SNP x ICS**				
FEV_1%predicted pre BD	1.30	4.49	-7.40	10.10	0.76	4.50	2.19	0.20	8.80	**0.04**	3.20	4.95	-6.60	12.90	0.53
FEV_1%predicted post BD	-0.80	3.83	-8.20	6.70	0.84	3.60	1.84	-0.10	7.20	**0.06**	4.40	4.18	-3.90	12.60	0.30
FEV_1/IVC% pre BD	1.20	2.81	-4.40	6.70	0.68	3.00	1.43	0.30	5.80	**0.03**	1.90	3.11	-4.30	8.00	0.55
FEV_1/IVC% post BD	-0.40	2.35	-5.00	4.20	0.87	2.30	1.12	0.00	4.50	**0.05**	2.70	2.60	-2.40	7.80	0.30
(ln) AHR slope (n=295)	-0.30	0.41	-1.00	0.50	0.46	-0.10	0.15	-0.50	0.20	0.41	0.10	0.41	-0.70	0.90	0.77

ICS: inhaled corticosteroids; B: regression coefficient; S.E: standard error; CI: confidence interval; FEV_1: forced expiratory volume in 1 second; IVC: inspiratory vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; SNPs: single nucleotide polymorphisms; SNP x ICS: interaction
n= number of subjects (homozygotes for major allele versus carriers of the minor allele)

Table E11: Associations of rs2229094 with lung function and airway hyperresponsiveness in populations 2 and 3

rs2229094-C	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value
Population 2 (n=232)	**no ICS (n= 63 vs 49)**					**ICS (n= 58 vs 62)**					**SNP x ICS**				
FEV_1%predicted pre BD	-2.80	3.78	-10.30	4.60	0.45	8.00	3.62	0.90	15.10	**0.03**	10.80	5.26	0.50	21.10	**0.04**
FEV_1%predicted post BD	-1.30	3.72	-8.50	6.00	0.73	6.20	3.57	-0.70	13.20	**0.08**	7.50	5.10	-2.60	17.50	0.14
FEV_1/VC% pre BD	-1.20	2.30	-5.70	3.30	0.61	2.70	2.19	-1.60	7.00	0.22	3.90	3.16	-2.30	10.10	0.22
FEV_1/VC% post BD	-2.10	2.09	-6.20	2.00	0.32	2.10	1.99	-1.90	6.00	0.31	4.10	2.91	-1.50	9.80	0.15
(ln) AHR slope (n=203)	0.50	0.31	-0.10	1.10	**0.08**	-0.30	0.31	-1.00	0.30	0.30	-0.90	0.46	-1.70	0.00	**0.05**
Population 3 (n= 302)	**no ICS (n= 32 vs 27)**					**ICS (n= 136 vs 106)**					**SNP x ICS**				
FEV_1%predicted pre BD	2.60	4.29	-5.70	11.00	0.54	4.00	2.14	-0.20	8.20	**0.06**	1.40	4.74	-7.90	10.70	0.77
FEV_1%predicted post BD	0.20	3.62	-6.80	7.30	0.95	3.30	1.79	-0.30	6.80	**0.07**	3.00	4.03	-4.90	10.90	0.45
FEV_1/IVC% pre BD	2.40	2.70	-2.90	7.70	0.37	2.40	1.38	-0.20	5.10	**0.07**	0.00	3.01	-5.90	5.90	1.00
FEV_1/IVC% post BD	0.40	2.19	-4.00	4.70	0.87	1.80	1.12	-0.40	4.00	0.12	1.40	2.50	-3.50	6.30	0.57
(ln) AHR slope (n=295)	-0.40	0.36	-1.10	0.30	0.29	-0.10	0.15	-0.50	0.20	0.48	0.20	0.41	-0.50	1.00	0.53

ICS: inhaled corticosteroids; B: regression coefficient; S.E: standard error; CI: confidence interval; FEV_1: forced expiratory volume in 1 second; IVC: inspiratory vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; SNPs: single nucleotide polymorphisms; SNP x ICS: interaction
n= number of subjects (homozygotes for major allele versus carriers of the minor allele)

Table E12: Associations of rs909253 with lung function and airway hyperresponsiveness in populations 2 and 3

rs909253-C	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value
Population 2 (n=237)	**no ICS (n= 38 vs 77)**					**ICS (n= 50 vs 72)**					**SNP x ICS**				
FEV_1%predicted pre BD	-3.00	3.93	-10.70	4.70	0.45	-1.40	3.67	-8.60	5.80	0.70	1.60	5.41	-9.00	12.20	0.77
FEV_1%predicted post BD	-5.90	3.83	-13.50	1.60	0.12	-0.40	3.62	-7.50	6.70	0.91	5.50	5.31	-4.80	15.90	0.30
FEV_1/VC% pre BD	1.00	2.35	-3.50	5.60	0.66	2.40	2.19	-1.90	6.70	0.28	1.30	3.21	-4.90	7.60	0.67
FEV_1/VC% post BD	-0.20	2.14	-4.30	4.00	0.94	2.10	1.99	-1.90	6.00	0.31	2.20	2.91	-3.50	7.90	0.45
(ln) AHR slope (n=207)	-0.30	0.31	-0.90	0.30	0.39	-0.20	0.36	-0.90	0.50	0.58	0.10	0.46	-0.80	1.00	0.86
Population 3 (n=302)	**no ICS (n= 18 vs 41)**					**ICS (n= 96 vs 146)**					**SNP x ICS**				
FEV_1%predicted pre BD	-1.30	4.49	-10.20	7.50	0.77	-5.40	2.14	-9.60	-1.20	**0.01**	-4.10	5.00	-13.90	5.70	0.41
FEV_1%predicted post BD	0.40	3.83	-7.10	7.90	0.92	-4.10	1.84	-7.70	-0.50	**0.03**	-4.50	4.23	-12.90	3.80	0.29
FEV_1/IVC% pre BD	-1.20	2.86	-6.90	4.40	0.67	-2.60	1.38	-5.40	0.10	**0.06**	-1.40	3.16	-7.70	4.80	0.65
FEV_1/IVC% post BD	-0.40	2.40	-5.10	4.30	0.87	-2.60	1.12	-4.80	-0.40	**0.02**	-2.20	2.65	-7.40	3.00	0.41
(ln) AHR slope (n=295)	0.00	0.36	-0.70	0.70	0.98	0.10	0.20	-0.20	0.50	0.49	0.10	0.41	-0.70	0.90	0.78

ICS: inhaled corticosteroids; B: regression coefficient; S.E: standard error; CI: confidence interval; FEV_1: forced expiratory volume in 1 second; IVC: inspiratory vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; SNPs: single nucleotide polymorphisms; SNP x ICS: interaction n= number of subjects (homozygotes for major allele versus carriers of the minor allele)

Table E13: Associations of rs915654 with lung function and airway hyperresponsiveness in populations 2 and 3

rs915654-T	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value	B	S.E	95%CI		P-value
Population 2 (n=236)	**no ICS (n= 49 vs 68)**					**ICS (n= 53 vs 66)**					**SNP x ICS**				
FEV_1%predicted pre BD	-1.80	3.72	-9.20	5.50	0.62	1.70	3.67	-5.60	8.90	0.65	3.50	5.26	-6.80	13.80	0.50
FEV_1%predicted post BD	-0.80	3.67	-7.90	6.40	0.83	1.10	3.62	-6.00	8.20	0.76	1.90	5.10	-8.20	11.90	0.71
FEV_1/VC% pre BD	-0.80	2.19	-5.20	3.50	0.70	-3.40	2.24	-7.70	1.00	0.13	-2.50	3.11	-8.60	3.60	0.42
FEV_1/VC% post BD	-0.80	2.04	-4.70	3.20	0.70	-3.50	1.99	-7.50	0.40	**0.08**	-2.80	2.86	-8.30	2.80	0.33
(ln) AHR slope (n=207)	0.01	0.30	-0.60	0.60	0.97	-0.20	0.31	-0.90	0.40	0.48	-0.20	0.41	-1.10	0.60	0.58
Population 3 (n=302)	**no ICS (n= 30 vs 29)**					**ICS (n= 125 vs 117)**					**SNP x ICS**				
FEV_1%predicted pre BD	8.70	4.18	0.50	16.90	**0.04**	-2.10	2.09	-6.30	2.00	0.31	-10.90	4.74	-20.10	-1.60	**0.02**
FEV_1%predicted post BD	7.10	3.57	0.10	14.10	**0.05**	-1.80	1.79	-5.30	1.70	0.32	-8.90	3.98	-16.70	-1.10	**0.03**
FEV_1/IVC% pre BD	3.10	2.70	-2.20	8.40	0.25	-0.80	1.38	-3.40	1.90	0.57	-3.90	3.01	-9.80	2.00	0.20
FEV_1/IVC% post BD	2.60	2.19	-1.80	6.90	0.25	-0.50	1.12	-2.70	1.70	0.64	-3.10	2.50	-8.00	1.80	0.22
(ln) AHR slope (n=295)	-0.30	0.31	-1.00	0.30	0.31	0.00	0.15	-0.30	0.30	0.98	0.30	0.41	-0.40	1.10	0.38

ICS: inhaled corticosteroids; B: regression coefficient; S.E: standard error; CI: confidence interval; FEV_1: forced expiratory volume in 1 second; IVC: inspiratory vital capacity; BD: bronchodilation; AHR: airway hyperresponsiveness; SNPs: single nucleotide polymorphisms; SNP x ICS: interaction
n= number of subjects (homozygotes for major allele versus carriers of the minor allele)

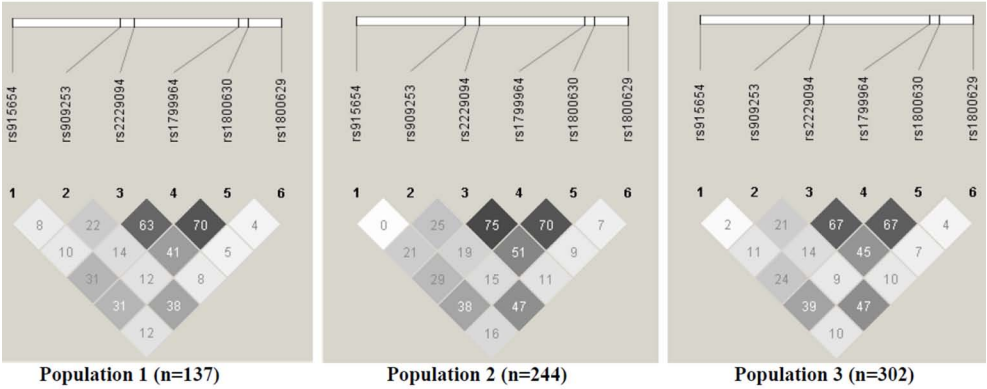


Figure E1: Analysis of the linkage disequilibrium patterns and correlation coefficients (r^2) of 6 genotyped *TNF* gene single nucleotide polymorphisms in the 3 populations using Haploview software. The location of the SNPs is given by HapMap.

SUPPLEMENTARY MATERIAL - CHAPTER 4

Abbreviations used

AA1: mast cell tryptase
AMP: adenosine 5'-monophosphate
BD: bronchodilation
BM: basement membrane
EPX: anti-eosinophilic peroxidase
FEV_1: forced respiratory volume in 1 second
ICS: inhaled corticosteroids
LD: linkage disequilibrium
LME: Linear Mixed Effect models
MAF: minor allele frequency
SNP(s): single nucleotide polymorphism(s)
TGFβ1: Transforming Growth Factor beta-1
TGFB1: Transforming Growth Factor beta-1 gene
VC: vital capacity

METHODS

Subjects

All participants had doctor's diagnosed asthma, were atopic and hyperresponsive at first clinical assessment, and originated from the northern region of The Netherlands.

Population 1: 281 asthma patients who had entered a cohort study between 1966 and 1975 were re-examined during the period 1991–1999.[E1] Before testing, participants had to be in stable condition without any exacerbation in the last 6 weeks and subjects were allowed to use maintenance medication before testing. Patients filled in a questionnaire on respiratory symptoms and underwent lung function testing, histamine challenge (30 seconds tidal breathing method) and skin prick tests. The clinical assessment was performed as previously published.[E2] The subjects' medical records provided information on lung function and corticosteroid use during their annual routine check-ups. DNA samples have been collected from 243 subjects.

Population 2: 138 asthma patients, previously investigated by our research group[E3-5] were re-examined during the period 2002-2006. Main inclusion criteria were: FEV_1 ≥1.2 L, absence of bronchiectasis, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within the last 2 months before inclusion. Only 36.8% (21/57) of subjects on ICS treatment had

stopped ICS medication for a minimum of four weeks before entering the study. Lung function testing and adenosine 5'-monophosphate (AMP) provocation test were performed followed 1-2 weeks later by a bronchoscopy. Subjects with a negative AMP provocation test (PC_{20} AMP > 320 mg/ml) were subjected to a histamine provocation test >3 weeks later.[E6-8] DNA samples and bronchial biopsies were available in 137 subjects. Thirty two subjects of population 2 were derived from population 1.

The study was approved by the medical ethics committee of the University Medical Center Groningen, and all participants signed an informed consent.

Genotyping of Single Nucleotide Polymorphisms (SNPs)

We genotyped 5 haplotype tagging SNPs (rs10417924, rs7254679, rs4803455, rs1800470, rs1800469) in the *TGFB1* gene (+/- 5kb) according to HapMap CEU genotype data (release 24/phase II Nov'08) ($r^2 < 0.8$). The cutoff value used for minor allele frequency (MAF) was 10% but we forced included (MAF<10% according to HapMap) rs1800470, a functional variant on exon-1.[E9] Rs6957 found in previous studies to be associated with decreased lung function in children with asthma[E10] and with decreased TGFβ1 serum levels[E11] was not found in HapMap thus we included it based on its dbSNP (National Center for Biotechnology Information) location. SNPs were genotyped by K-Bioscience Ltd (Herts, Hoddesdon, UK) using their competitive allele specific PCR system (KASPar) (see Figure 1 for the genotyped SNPs).

Bronchial biopsies

After local anesthesia, at least 5 bronchial biopsies were obtained from the subsegmental carinas from the left or right lower lobe using a flexible bronchoscope (Olympus BF P20 or BF XT20). Biopsies were fixed in 4% formalin, processed and embedded in paraffin and cut in 3µm thick sections. Macroscopically the best biopsy was selected for processing. Quantification was performed on the largest of three biopsy sections. Morphological features were determined on sections stained with hematoxylin and eosin (HE) and with periodic acid Schiff. Immunohistochemical stainings were performed using the DAKO autostainer (DAKO, Glostrup, Denmark). The slides were included in a random fashion in each run to avoid group-wise staining. Immunohistochemistry was performed using antibodies: anti-CD8 and anti-CD-4 (DAKO) for T-lymphocytes, anti-CD68 (DAKO) for macrophages, anti-mast cell tryptase (AA1, DAKO) for mast cells and anti-eosinophilic peroxidase

(EPX) antibody for eosinophils. In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) and the chromogen NovaRED (Vector Labs, Burlingame, USA). EPX was detected via biotinylated anti-mouse IgG1 (Southern Biotech), alkaline phosphatase-labeled conjugate (DAKO) and permanent Red (DAKO). All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Inflammatory cell numbers were quantified by counting the number of positively stained cells in the submucosal area, 100µm under the basement membrane (BM), in a total area of 0.1mm² per biopsy sample. Goblet cell numbers were counted on PAS-stained biopsy sections and expressed per 1000 µm of BM. The length of basement membrane was variable among patients with a minimum of 973*µm*. The (median [min-max]) length of basement membrane covered with intact epithelium analyzed in each biopsy was 605 [0-3147] µm. BM thickness was calculated based on computer-assisted measurements of BM surface area and BM length. Reproducibility of the measurements was confirmed by a single observer performing repeated measurements in 10% of all cases.

Statistical Analysis

Normalization of the distribution of the variables was performed by natural logarithm transformation if necessary. Multiple linear regression models were used to test for associations with pre-/post-BD FEV_1%predicted (corrected for smoking status and ICS use) FEV_1/VC (corrected for age, gender, height, smoking status and ICS use), BM thickness and number of goblet and inflammatory cells (corrected for age, gender, smoking status and ICS use). Logistic regression models were used to assess the associations of SNPs with binary clinical outcomes:

1. Airway hyperresponsiveness: PC_{20}_histamine < 16mg/ml (population 1) and PC_{20}_AMP < 320mg/ml (population 2) (corrected for age, gender, smoking status and ICS use)

2. Complete asthma remission: former diagnosis of asthma, PC_{20}_AMP > 320mg/ml and PC_{20}_histamine > 16mg/ml, post-BD FEV_1%predicted > 90%, no asthma symptoms, no asthma medication (corrected for age, gender and smoking status)

3. Severe asthma: pre-BD FEV_1%predicted < 80% and ICS use (corrected for age, gender, and smoking status)

To assess whether there is an interaction of SNPs with smoking we stratified our analysis in 2 groups: never vs ever (ex/current) smokers. A common limitation for detecting gene by environment interactions is study power, therefore we decided *a priori* that we would only test for an interaction (genotype*ever-smokers) if the estimates of the SNPs showed a larger than 40% difference between the strata. We used the estimates (beta-coefficient) and standard errors of the SNPs and their interactions with smoking to meta-analyze associations with asthma clinical outcomes and lung function in the two populations. Fixed effects were used. When index of heterogeneity between studies (I^2) was high (>70) and/or Cochran's Q-test of heterogeneity was nearly significant (p<0.07) then random effects were used. All regression modeling was conducted with SPSS (version 18; SPSS Inc., Chicago, Illinois, USA). Because the number of subjects with the homozygote mutant genotype was relatively low in the 2 subgroups (n≈<10) (see table E1 online repository), only dominant (heterozygotes and homozygotes mutant pooled vs wild types) models were used. Meta-analysis was done with R-programming language using the meta-package (R package 2.8.1. http://cran.r-project.org/bin/windows/base/old/2.8.1/). P-values<0.05 were considered statistically significant and 0.05≤ p-values ≤0.09 of borderline significance (tested 2-sided).

Lung function decline analysis

FEV_1 decline was analyzed with piecewise Linear Mixed Effect models (LME) as described before. [E2] In summary, following the methodology described by Naumova et al,[E12] time was defined as the time relative to the start of inhaled corticosteroid (ICS) treatment in years. Individual variations in the pre and post ICS-period declines and intercepts were accounted for by estimating the random effects for these variables. Sex was incorporated in the model by adding the interaction between time, ICS use, and sex. Other explanatory variables in the model were height (centered at 1.75 m), first available FEV_1 after age 30 (FEV_1 centered at 2.8 L, called "FEV_1 at age 30"), and their interaction with time, pack years of smoking, and oral corticosteroid use. The latter two variables were time varying. Only FEV_1 measurements after the age of 30 years were included since this is the age where the lung function is in a plateau phase or decline begins in normal subjects. [E13]

To assess whether the effect of the SNPs on the course of asthma is modified by cigarette smoking we initially stratified our analysis in 2 groups (ever vs never smokers). For the majority of the SNPs the frequency of the

homozygotes mutants per strata was low (<10) thus dominant (heterozygotes and homozygotes mutant pooled vs wild types) genetic models were used. A common limitation for detecting gene by environment interactions is study power therefore we decided a priori that we will only test for an interaction (genotype*ever-smoker*time) if the estimates of the SNPs show a more than 40% difference between the strata. LME models were conducted in S-plus 7.0 (Insightful Corp., Seattle, Washington, USA). P-values<0.05 were considered statistically significant and 0.05≤ p-values ≤0.09 of borderline significance (tested 2-sided).

Definitions for terms used in tables of the online repository

Strata: never and ever (ex/current)-smokers
Interaction: genotype*ever-smokers
Airway hyperresponsiveness: PC_{20} histamine < 16 mg/ml (population 1) and PC_{20} AMP < 320 mg/ml (population 2)
Complete asthma remission: former diagnosis of asthma, PC_{20} AMP > 320 mg/ml and/or PC_{20} histamine > 16 mg/ml, post-bronchodilation (BD) FEV_1%predicted > 90%, no asthma symptoms, no asthma medication
Severe asthma: pre-BD FEV_1%predicted < 80% and use of inhaled corticosteroids
The number of positively stained inflammatory cells was counted in a total area of 0.1 mm^2 in the submucosa, 100μm under the basement membrane
Goblet cell numbers were counted on PAS-stained biopsy sections and expressed per 1000 μm of basement membrane

REFERENCES

E1. Vonk JM, Jongepier H, Panhuysen CIM, Schouten JP, Bleecker ER, Postma DS. Risk factors associated with the presence of irreversible airflow limitation and reduced transfer coefficient in patients with asthma after 26 years of follow up. Thorax 2003 04//;58(4):322-327.

E2. Dijkstra A, Vonk JM, Jongepier H, Koppelman GH, Schouten JP, Hacken NHTt, et al. Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex. Thorax 2006 02//;61(2):105-110.

E3. Vonk J, Postma D, Boezen H, Grol M, Schouten J, Koeter G, et al. Childhood factors associated with asthma remission after 30 year follow up. Thorax 2004 11//;59(11):925-929.

E4. Grol MH, Gerritsen J, Vonk JM, Schouten JP, Koeter GH, Rijcken B, et al. Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. Am J Respir Crit Care Med 1999 Dec;160(6):1830-1837.

E5. Jansen DF, Schouten JP, Vonk JM, Rijcken B, Timens W, Kraan J, et al. Smoking and airway hyperresponsiveness especially in the presence of blood eosinophilia increase the risk to develop respiratory symptoms: a 25-year follow-up study in the general adult population. Am J Respir Crit Care Med 1999 Jul;160(1):259-264.

E6. Broekema M, Volbeda F, Timens W, Dijkstra A, Lee NA, Lee JJ, et al. Airway eosinophilia in remission and progression of asthma: accumulation with a fast decline of FEV(1). Respir Med 2010 Sep;104(9):1254-1262.

E7. Volbeda F, ten Hacken NH, Lodewijk ME, Dijkstra A, Hylkema MN, Broekema M, et al. Can AMP induce sputum eosinophils, even in subjects with complete asthma remission? Respir Res 2010 Aug 2;11:106.

E8. Broekema M, ten Hacken NHT, Volbeda F, Lodewijk ME, Hylkema MN, Postma DS, et al. Airway Epithelial Changes in Smokers but Not in Ex-Smokers with Asthma. Am J Respir Crit Care Med 2009 12/15/;180(12):1170-1178.

E9. Yamada Y, Miyauchi A, Takagi Y, Tanaka M, Mizuno M, Harada A. Association of the C-509-->T polymorphism, alone of in combination with the T869-->C polymorphism, of the transforming growth factor-beta1 gene with bone mineral density and genetic susceptibility to osteoporosis in Japanese women. J Mol Med 2001 Apr;79(2-3):149-156.

E10. Sharma S, Raby BA, Hunninghake GM, Soto-Quiros M, Avila L, Murphy AJ, et al. Variants in TGFB1, dust mite exposure, and disease severity in children with asthma. Am J Respir Crit Care Med 2009 Mar 1;179(5):356-362.

E11. Saltzman BS, Yamamoto JF, Decker R, Yokochi L, Theriault AG, Vogt TM, et al. Association of genetic variation in the transforming growth factor beta-1 gene with serum levels and risk of colorectal neoplasia. Cancer Res 2008 Feb 15;68(4):1236-1244.

E12. Naumova EN, Must A, Laird NM. Tutorial in Biostatistics: Evaluating the impact of 'critical periods' in longitudinal studies of growth using piecewise mixed effects models. Int J Epidemiol 2001 Dec;30(6):1332-1341.

E13. Rijcken B, Weiss ST. Longitudinal analyses of airway responsiveness and pulmonary function decline. Am J Respir Crit Care Med 1996 12//;154(6 Pt 2):S246-249-S246-249.

E14. Saltzman BS, Yamamoto JF, Decker R, Yokochi L, Theriault AG, Vogt TM, et al. Association of genetic variation in the transforming growth factor beta-1 gene with serum levels and risk of colorectal neoplasia. Cancer Res 2008 Feb 15;68(4):1236-1244.

E15. Silverman ES, Palmer LJ, Subramaniam V, Hallock A, Mathew S, Vallone J, et al. Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. Am J Respir Crit Care Med 2004 Jan 15;169(2):214-219.

E16. Yamada Y, Miyauchi A, Takagi Y, Tanaka M, Mizuno M, Harada A. Association of the C-509-->T polymorphism, alone of in combination with the T869-->C polymorphism, of the transforming growth factor-beta1 gene with bone mineral density and genetic susceptibility to osteoporosis in Japanese women. J Mol Med 2001 Apr;79(2-3):149-156.

Table E1. Characteristics of populations 1 and 2

	Population 1 N = 243	Population 2 N =137
Males	146 (60.1)	70 (51.1)
Age, years	51 (8.8)	47 (12.6)
Never/ex/current smokers	100/74/69(41/31/28)	61/47/29 (44/34/21)
Inhaled corticosteroid use	125 (51.4)	57 (41.6)
Airway hyperresponsiveness	163 (76.9)	72 (52.6)
Complete asthma remission	30 (12.3)	20 (15)
Severe asthma	100 (41.3)	17 (12.5)
FEV_1% predicted	70.6 (23.8)	91.6 (18.0)
FEV_1/ VC%	60.4 (14.3)	70.6 (11.5)

Data are expressed as numbers (%) or mean (SD)
Population 1: longitudinal clinical follow up. Population 2: cross-sectional clinical evaluation and airway wall biopsy collection.

Table E2a: Minor allele frequency

TGFB1 tag SNPs	Chr 19 position	MAF (%) population 1	MAF (%) population 2	Major : Minor
rs6957	3'UTR	0.14	0.16	A:G
rs10417924	3'UTR	0.15	0.17	C:T
rs7254679	3'UTR	0.13	0.13	A:G
rs4803455	Intron 2	0.50	0.46	C:A
rs1800470#	Exon 1	0.38	0.36	T:C
rs1800469*	Promoter	0.30	0.30	C:T

#formerly known as rs1982073
* tags rs2241715, rs2317130, rs1982072 (*TGFB1* genomic region +/- 5kb; MAF <0.1; HapMap.org)

Table E2b: Genotype distribution in populations 1 and 2

		Population 1 (n=243)			Population 2 (n=137)		
***TGFB1* SNPs**	**Genotype**	**Total**	**Never smokers**	**Ever smokers**	**Total**	**Never smokers**	**Ever smokers**
rs6957	AA	178	78	100	92	43	49
	GA	60	21	39	40	17	23
	GG	3	1	2	1	0	1
rs10417924	CC	170	70	100	92	45	47
	TC	56	21	35	37	14	23
	TT	6	1	5	4	1	3
rs7254679	AA	179	77	102	99	46	53
	GA	61	20	41	36	15	21
	GG	1	1	0	0	0	0
rs4803455	CC	58	20	38	37	16	46
	CA	125	58	67	75	35	15
	AA	58	21	37	25	10	0
rs1800470	TT	93	38	55	59	30	29
	TC	110	50	60	57	25	32
	CC	37	9	28	20	6	14
rs1800469	CC	121	53	68	68	31	37
	CT	97	42	55	51	25	26
	TT	25	5	20	15	4	11

Table E3a-E3b: Associations with FEV_1 decline in population 1

E3a		**FEV_1 Decline (ml/year)**			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**
rs6957	AA	reference			
	GA/GG	-0.16	-8.24	7.93	0.97
rs10417924	CC	reference			
	TC/TT	-0.29	-8.32	7.73	0.94
rs1800469	CC	reference			
	CT/TT	6.29	-0.36	12.95	**0.06**
rs1800470	TT	reference			
	TC/CC	10.73	3.81	17.65	**0.002**
rs4803455	CC	reference			
	CA/AA	-11.37	-18.74	-4.01	**0.003**

E3b		FEV$_1$ Decline (ml/year)									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**	**B**	**P-value**
rs6957	AA	reference				reference				reference	
	GA/GG	-14.95	-29.21	-0.69	**0.04**	7.52	-2.97	18.00	0.16	19.13	**0.02**
rs10417924	CC	reference				reference				reference	
	TC/TT	-3.74	-17.60	10.11	0.60	2.33	-7.42	12.08	0.64	5.88	0.47
rs1800469	CC	reference				reference				reference	
	CT/TT	4.89	-7.05	16.83	0.42	8.85	0.78	16.91	**0.03**	3.97	0.56
rs1800470	TT	reference				reference				reference	
	TC/CC	10.27	-1.88	22.42	0.10	10.99	2.41	19.58	**0.01**	NT	NT
rs4803455	CC	reference				reference				reference	
	CA/AA	-4.68	-18.74	9.38	0.51	-15.69	-24.09	-7.29	**<0.001**	-9.68	0.22

Table E4a-E4d: Associations with FEV_1 % predicted in populations 1 and 2

E4a		FEV_1% predicted pre bronchodilation							
		Population 1				Population 2			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	-6.60	-12.26	-0.93	**0.02**	-8.11	-14.66	-1.56	**0.02**
rs10417924	CC	reference				reference			
	TC/TT	-0.60	-6.53	5.34	0.84	8.29	1.66	14.92	**0.02**
rs1800469	CC	reference				reference			
	CT/TT	-1.60	-6.64	3.45	0.53	-4.45	-10.62	1.72	0.16
rs1800470	TT	reference				reference			
	TC/CC	-3.26	-8.45	1.92	0.22	-5.20	-11.20	0.80	**0.09**
rs4803455	CC	reference				reference			
	CA/AA	2.24	-3.66	8.14	0.46	4.42	-2.38	11.22	0.20

E4b		FEV$_1$% predicted pre bronchodilation									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		B	95%CI		P-value	B	95%CI		P-value	B	P-value
***TGFB1* SNPs**	**Genotype**	**Population 1**									
rs6957	AA	reference				reference				reference	
	GA/GG	-3.73	-12.61	5.15	0.41	-8.16	-15.46	-0.86	**0.03**	-3.97	0.50
rs10417924	CC	reference				reference				reference	
	TC/TT	-4.25	-13.44	4.95	0.36	1.58	-6.12	9.27	0.69	-4.08	0.41
rs1800469	CC	reference				reference				reference	
	CT/TT	1.82	-5.58	9.21	0.63	-4.29	-11.18	2.59	0.22	-6.20	0.23
rs1800470	TT	reference				reference				reference	
	TC/CC	-1.09	-8.85	6.68	0.78	-4.98	-11.98	2.02	0.16	-3.70	0.49
rs4803455	CC	reference				reference				reference	
	CA/AA	0.83	-8.42	10.07	0.86	3.21	-4.49	10.91	0.41	2.59	0.68
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	-3.86	-12.65	4.93	0.38	-12.07	-21.63	-2.50	**0.01**	-8.31	0.21
rs10417924	CC	reference				reference				reference	
	TC/TT	4.80	-4.19	13.78	0.29	11.09	1.61	20.58	**0.02**	5.85	0.39
rs1800469	CC	reference				reference				reference	
	CT/TT	1.75	-6.23	9.74	0.62	-9.90	-18.92	-0.88	**0.03**	-11.99	**0.052**
rs1800470	TT	reference				reference				reference	
	TC/CC	0.01	-7.82	7.85	1.00	-9.85	-18.71	-0.99	**0.03**	-10.20	**0.09**
rs4803455	CC	reference				reference				reference	
	CA/AA	3.16	-5.68	12.00	0.48	4.83	-5.19	14.84	0.34	NT	NT

E4c		FEV$_1$% predicted post bronchodilation							
		Population 1				Population 2			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	-5.58	-11.15	-0.02	**0.049**	-8.44	-14.60	-2.27	**0.01**
rs10417924	CC	reference				reference			
	TC/TT	-0.45	-6.28	5.39	0.88	7.75	1.40	14.09	**0.02**
rs1800469	CC	reference				reference			
	CT/TT	-2.56	-7.50	2.38	0.31	-2.47	-8.36	3.41	0.41
rs1800470	TT	reference				reference			
	TC/CC	-4.87	-9.93	0.19	**0.059**	-4.50	-10.23	1.23	0.12
rs4803455	CC	reference				reference			
	CA/AA	2.51	-3.29	8.30	0.40	1.63	-4.85	8.11	0.62

E4d		FEV$_1$% predicted post bronchodilation									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		B	95%CI		P-value	B	95%CI		P-value	B	P-value
TGFB1 SNPs	Genotype	Population 1									
rs6957	AA	reference				reference				reference	
	GA/GG	-2.80	-11.35	5.75	0.52	-6.81	-14.08	0.47	**0.07**	-3.45	0.55
rs10417924	CC	reference				reference				reference	
	TC/TT	-3.08	-11.99	5.83	0.49	0.79	-6.88	8.42	0.84	3.60	0.55
rs1800469	CC	reference				reference				reference	
	CT/TT	0.10	-7.01	7.20	0.98	-4.66	-11.48	2.15	0.18	-4.90	0.34
rs1800470	TT	reference				reference				reference	
	TC/CC	-4.10	-11.47	3.26	0.27	-5.82	-12.77	1.13	0.10	NT	NT
rs4803455	CC	reference				reference				reference	
	CA/AA	0.34	-8.57	9.25	0.94	3.72	-3.93	11.36	0.34	3.65	0.55
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	-7.23	-14.84	0.37	**0.06**	-9.98	-19.35	-0.61	**0.04**	NT	NT
rs10417924	CC	reference				reference				reference	
	TC/TT	4.86	-2.97	12.69	0.22	9.55	0.17	18.93	**0.046**	3.75	0.56
rs1800469	CC	reference				reference				reference	
	CT/TT	2.40	-4.66	9.46	0.50	-6.64	-15.55	2.26	0.14	-9.71	0.10
rs1800470	TT	reference				reference				reference	
	TC/CC	0.30	-6.64	7.24	0.93	-8.84	-17.57	-0.10	**0.047**	-9.76	**0.09**
rs4803455	CC	reference				reference				reference	
	CA/AA	2.08	-5.76	9.92	0.60	1.91	-7.86	11.68	0.70	NT	NT

Table E5a-E5d: Associations with FEV_1/VC in populations 1 and 2

E5a		FEV_1/VC pre bronchodilation							
		Population 1				Population 2			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	0.20	-3.24	3.63	0.91	-1.20	-4.61	2.21	0.49
rs10417924	CC	reference				reference			
	TC/TT	-0.32	-3.85	3.22	0.86	2.00	-1.40	5.41	0.25
rs1800469	CC	reference				reference			
	CT/TT	-0.72	-3.72	2.28	0.64	-1.59	-4.73	1.55	0.32
rs1800470	TT	reference				reference			
	TC/CC	-0.95	-4.07	2.17	0.55	-1.95	-5.03	1.13	0.21
rs4803455	CC	reference				reference			
	CA/AA	0.71	-2.77	4.20	0.69	3.32	-0.08	6.72	**0.056**

E5b		FEV$_1$/VC pre bronchodilation									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		B	95%CI		P-value	B	95%CI		P-value	B	P-value
***TGFB1* SNPs**	**Genotype**	**Population 1**									
rs6957	AA	reference				reference				reference	
	GA/GG	0.36	-5.00	5.72	0.89	-0.14	-4.63	4.35	0.95	-0.64	0.86
rs10417924	CC	reference				reference				reference	
	TC/TT	-1.06	-6.62	4.50	0.71	0.22	-4.36	4.80	0.92	0.88	0.82
rs1800469	CC	reference				reference				reference	
	CT/TT	-0.33	-4.79	4.13	0.88	-1.41	-5.51	2.70	0.50	-1.37	0.66
rs1800470	TT	reference				reference				reference	
	TC/CC	-0.81	-5.61	4.00	0.74	-1.30	-5.48	2.89	0.54	NT	NT
rs4803455	CC	reference				reference				reference	
	CA/AA	0.20	-5.28	5.68	0.94	1.04	-3.52	5.61	0.65	0.96	0.79
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	3.67	-1.76	9.09	0.18	-4.94	-9.70	-0.19	**0.04**	-8.25	**0.02**
rs10417924	CC	reference				reference				reference	
	TC/TT	1.30	-4.03	6.63	0.63	3.05	-1.56	7.66	0.19	1.53	0.66
rs1800469	CC	reference				reference				reference	
	CT/TT	0.14	-4.56	4.84	0.95	-3.68	-8.12	0.77	0.10	-4.15	0.19
rs1800470	TT	reference				reference				reference	
	TC/CC	-1.19	-5.78	3.39	0.60	-2.74	-7.15	1.68	0.22	-1.81	0.56
rs4803455	CC	reference				reference				reference	
	CA/AA	4.08	-1.05	9.21	0.12	1.92	-2.85	6.68	0.43	-2.34	0.50

E5c		FEV$_1$/VC post bronchodilation							
		Population 1				Population 2			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	-0.45	-3.58	2.69	0.78	-1.54	-4.81	1.72	0.35
rs10417924	CC	reference				reference			
	TC/TT	-0.24	-3.47	3.00	0.89	3.09	-0.18	6.36	**0.06**
rs1800469	CC	reference				reference			
	CT/TT	-1.86	-4.58	0.87	0.18	-0.85	-3.87	2.17	0.58
rs1800470	TT	reference				reference			
	TC/CC	-1.90	-4.74	0.94	0.19	-2.13	-5.11	0.86	0.16
rs4803455	CC	reference				reference			
	CA/AA	0.82	-2.38	4.02	0.61	2.37	-0.93	5.67	0.16

E5d		FEV$_1$/VC post bronchodilation									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		B	95%CI		P-value	B	95%CI		P-value	B	P-value
***TGFB1* SNPs**	**Genotype**	**Population 1**									
rs6957	AA	reference				reference				reference	
	GA/GG	-0.27	-5.33	4.78	0.91	-0.63	-4.61	3.35	0.75	-0.26	0.94
rs10417924	CC	reference				reference				reference	
	TC/TT	-0.61	-5.91	4.70	0.82	-0.22	-4.29	3.86	0.92	-0.10	0.98
rs1800469	CC	reference				reference				reference	
	CT/TT	-2.41	-6.60	1.77	0.26	-1.89	-5.52	1.74	0.31	0.19	0.95
rs1800470	TT	reference				reference				reference	
	TC/CC	-2.74	-7.22	1.74	0.23	-1.79	-5.52	1.94	0.34	NT	NT
rs4803455	CC	reference				reference				reference	
	CA/AA	-0.02	-5.24	5.19	0.99	1.20	-2.86	5.26	0.56	1.44	0.67
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	1.15	-3.90	6.19	0.65	-3.98	-8.51	0.54	**0.08**	-4.67	0.17
rs10417924	CC	reference				reference				reference	
	TC/TT	3.52	-1.23	8.27	0.14	3.30	-1.11	7.72	0.14	NT	NT
rs1800469	CC	reference				reference				reference	
	CT/TT	0.82	-3.46	5.09	0.70	-2.11	-6.35	2.13	0.32	-3.84	0.20
rs1800470	TT	reference				reference				reference	
	TC/CC	-1.36	-5.53	2.80	0.52	-2.71	-7.00	1.58	0.21	-1.60	0.59
rs4803455	CC	reference				reference				reference	
	CA/AA	4.08	-1.05	9.21	0.12	1.92	-2.85	6.68	0.43	-2.23	0.50

Table E6: Meta-analysis of associations with lung function in populations 1 and 2

E6 Meta-analysis	*TGFB1 SNP*	Total B [95%CI]; p-value	Never-Smokers B [95%CI]; p-value	Ever-Smokers B [95%CI]; p-value	Interaction B [95%CI]; p-value
FEV_1% predicted pre BD	rs6957-T	**-7.2446[-11.5311; -2.9581] 0.0009**	-3.7962 [-10.0436; 2.4512] 0.2337	**-9.5953 [-15.399; -3.7916] 0.0012**	-5.8673 [-14.4843; 2.7497] 0.182
	rs10417924-G	3.7194 [-4.9841; 12.4229] 0.4023	0.3783 [-6.0475; 6.8042] 0.9081	**5.3534 [-0.6235; 11.3303] 0.0792**	5.7535 [-3.1578; 14.6649] 0.2057
	rs1800469-T	-2.7395 [-6.6455; 1.1665] 0.1692	1.7885 [-3.6371; 7.2141] 0.5182	**-6.358 [-11.83; -0.886] 0.0228**	**-8.6245 [-16.3901; -0.8589] 0.0295**
	rs1800470-C	**-4.0919 [-8.0139; -0.1699] 0.0409**	-0.5421 [-6.0571; 4.9729] 0.8472	**-6.8487 [-12.3411; -1.3564] 0.0145**	**-6.5832 [-14.4199; 1.2534] 0.090**
	rs4803455-A	3.1798 [-1.2756; 7.6352] 0.1619	2.0459 [-4.3418; 8.4335] 0.5302	3.8111 [-2.2951; 9.9173] 0.2212	2.1746 [-6.8552; 11.2045] 0.6369
FEV_1% predicted post BD	rs6957-T	**-6.864 [-10.9958; -2.7323] 0.0011**	-5.2746 [-10.9561; 0.4069] 0.0688	**-7.9979 [-13.7436; -2.2523] 0.0064**	-3.2249 [-11.5682; 5.1185] 0.4487
	rs10417924-G	3.5499 [-4.4768; 11.5766] 0.386	1.4009 [-4.4791; 7.2809] 0.6405	4.2791 [-1.6406; 10.1987] 0.1565	3.6703 [-4.9689; 12.3095] 0.405
	rs1800469-T	-2.5244 [-6.3074; 1.2585] 0.1909	1.2558 [-3.7514; 6.263] 0.623	**-5.3928 [-10.8045; 0.0189] 0.0508**	**-6.9699 [-14.5066; 0.5668] 0.0699**
	rs1800470-C	**-4.7081 [-8.5001; -0.9162] 0.015**	-1.7707 [-6.8211; 3.2797] 0.492	**-6.9913 [-12.4291; -1.5535] 0.0117**	-5.2764 [-12.8497; 2.2968] 0.1721
	rs4803455-A	2.1173 [-2.2027; 6.4373] 0.3368	1.3198 [-4.5649; 7.2044] 0.6602	3.0311 [-2.9906; 9.0529] 0.3239	1.8245 [-6.9163; 10.5653] 0.6825
FEV_1/VC% predicted pre BD	rs6957-T	-0.5075 [-2.9279; 1.9129] 0.6811	1.995 [-1.8175; 5.8074] 0.3051	-2.4011 [-5.6656; 0.8635] 0.1494	**-4.5144 [-9.4247; 0.3960] 0.0716**
	rs10417924-G	0.8864[-1.5654; 3.3383] 0.4786	0.1722 [-3.6753; 4.0196] 0.9301	1.6283 [-1.6215; 4.8782] 0.3261	1.2059 [-3.7477; 6.1594] 0.6333
	rs1800469-T	-1.1365 [-3.3051; 1.0321] 0.3044	-0.1074 [-3.3416; 3.1267] 0.9481	-2.4495 [-5.4622; 0.5632] 0.111	-2.7423 [-7.0703; 1.5857] 0.2143
	rs1800470-C	-1.4575 [-3.6492; 0.7342] 0.1924	-1.0092 [-4.3249; 2.3066] 0.5508	-1.9768 [-5.0156; 1.062] 0.2023	-1.3906 [-5.7747; 2.9936] 0.5342
	rs4803455-A	2.0462[-0.3884; 4.4807] 0.0995	2.2702 [-1.4732; 6.0136] 0.2346	1.4593 [-1.8369; 4.7555] 0.3856	-0.7863 [-5.7239; 4.1513] 0.7549
FEV_1/VC% predicted post BD	rs6957-T	-0.9719 [-3.2302; 1.2864] 0.3989	0.4384 [-3.1324; 4.0092] 0.8098	-2.0899 [-5.0769; 0.8971] 0.1703	-2.3682 [-6.9653; 2.2289] 0.3126
	rs10417924-G	1.4075 [-0.8917; 3.7066] 0.2302	1.6847 [-1.8541; 5.2234] 0.3508	1.4011 [-1.5929; 4.3951] 0.359	-0.6124 [-5.229; 4.0041] 0.7949
	rs1800469-T	-1.4044 [-3.4274; 0.6185] 0.1736	-0.8322 [-3.8189; 2.1546] 0.585	-1.9823 [-4.7398; 0.7751] 0.1588	-1.6922 [-5.718; 2.3336] 0.41
	rs1800470-C	**-2.008 [-4.0656; 0.0496] 0.0558**	-2.0037 [-5.0551; 1.0477] 0.1981	-2.1879 [-5.0027; 0.627] 0.1277	-0.4977 [-4.5964; 3.601] 0.8119
	rs4803455-A	1.5721 [-0.7222; 3.8664] 0.1793	2.0653 [-1.5917; 5.7223] 0.2683	1.4995 [-1.5903; 4.5894] 0.3415	-0.4209 [-5.0432; 4.2014] 0.8584

Table E7a-b: Associations with severity of airway hyperresponsiveness in populations 1 and 2

E7a		Airway hyperresponsiveness							
		Population 1				Population 2			
***TGFB1* SNPs**	**Genotype**	**OR**	**95%CI**		**P-value**	**OR**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	3.31	1.34	8.18	**0.009**	1.18	0.53	2.61	0.68
rs10417924	CC	reference				reference			
	TC/TT	0.69	0.32	1.47	0.33	0.63	0.28	1.40	0.26
rs1800469	CC	reference				reference			
	CT/TT	0.81	0.41	1.58	0.53	1.23	0.60	2.55	0.57
rs1800470	TT	reference				reference			
	TC/CC	0.66	0.32	1.35	0.25	1.08	0.53	2.22	0.84
rs4803455	CC	reference				reference			
	CA/AA	1.01	0.46	2.20	0.99	0.71	0.31	1.59	0.40

E7b		Airway hyperresponsiveness									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		OR	95%CI		P-value	OR	95%CI		P-value	OR	P-value
***TGFB1* SNPs**	**Genotype**	**Population 1**									
rs6957	AA	reference				reference				reference	
	GA/GG	5.84	0.67	51.23	0.11	3.26	1.11	9.53	**0.03**	NT	NT
rs10417924	CC	reference				reference				reference	
	TC/TT	0.47	0.12	1.86	0.28	0.76	0.29	1.96	0.57	1.57	0.59
rs1800469	CC	reference				reference				reference	
	CT/TT	1.07	0.34	3.41	0.91	0.79	0.33	1.89	0.59	0.96	0.96
rs1800470	TT	reference				reference				reference	
	TC/CC	0.38	0.09	1.52	0.17	0.96	0.39	2.39	0.94	3.51	0.13
rs4803455	CC	reference				reference				reference	
	CA/AA	1.88	0.49	7.19	0.36	0.84	0.31	2.27	0.73	0.45	0.33
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	0.20	0.05	0.80	**0.02**	4.74	1.45	15.49	**0.01**	21.44	**0.001**
rs10417924	CC	reference				reference				reference	
	TC/TT	1.58	0.48	5.22	0.45	0.24	0.08	0.72	**0.01**	0.16	**0.03**
rs1800469	CC	reference				reference				reference	
	CT/TT	1.19	0.42	3.36	0.74	1.52	0.57	4.00	0.40	1.34	0.68
rs1800470	TT	reference				reference				reference	
	TC/CC	0.91	0.33	2.53	0.86	1.24	0.46	3.31	0.67	1.42	0.62
rs4803455	CC	reference				reference				reference	
	CA/AA	0.74	0.23	2.41	0.62	1.00	0.35	2.87	0.99	1.43	0.66

Table E8: Meta-analysis of associations with severity of airway hyperresponsiveness in populations 1 and 2

E8 Meta-analysis	*TGFB1 SNP*	Total B [95%CI]; p-value	Never-Smokers B [95%CI]; p-value	Ever-Smokers B [95%CI]; p-value	Interaction B [95%CI]; p-value
AHR	rs6957-T	**1.8512 [1.0197; 3.3607] 0.043**	0.9674 [0.0353; 26.5039] 0.9843	**3.8577 [1.7413; 8.5466] 0.0009**	3.5884 [0.0928; 138.7111] 0.4932
	rs10417924-G	0.6575 [0.3782; 1.1431] 0.1373	0.9373 [0.3812; 2.3046] 0.8878	**0.4631 [0.2256; 0.9506] 0.0359**	0.4977 [0.0533; 4.6485] 0.5405
	rs1800469-T	0.9815 [0.5991; 1.6079] 0.9408	1.1363 [0.5246; 2.4608] 0.746	1.0573 [0.5514; 2.0273] 0.8669	1.1372 [0.4181; 3.0931] 0.8012
	rs1800470-C	0.8417 [0.5072; 1.3968] 0.5049	0.6694 [0.294; 1.5241] 0.339	1.0811 [0.5552; 2.1053] 0.8185	2.0904 [0.7176; 6.09] 0.1765
	rs4803455-A	0.8497 [0.4842; 1.491] 0.5702	1.1134 [0.4599; 2.6951] 0.8118	0.9108 [0.4423; 1.8754] 0.7998	0.8170 [0.2628; 2.5400] 0.7269

Table E9a-b: Associations with complete asthma remission in populations 1 and 2

E9a		Complete asthma remission							
		Population 1				Population 2			
TGFB1 SNPs	Genotype	OR	95%CI		P-value	OR	95%CI		P-value
rs6957	AA	reference				reference			
	GA/GG	0.23	0.06	0.82	**0.02**	0.86	0.28	2.71	0.80
rs10417924	CC	reference				reference			
	TC/TT	2.30	0.96	5.49	**0.06**	2.17	0.78	6.07	0.14
rs1800469	CC	reference				reference			
	CT/TT	1.29	0.57	2.97	0.54	1.38	0.51	3.77	0.53
rs1800470	TT	reference				reference			
	TC/CC	1.46	0.61	3.47	0.39	1.36	0.50	3.69	0.54
rs4803455	CC	reference				reference			
	CA/AA	0.79	0.31	2.01	0.62	0.88	0.30	2.57	0.81

E9b		Complete asthma remission									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		OR	95%CI		P-value	OR	95%CI		P-value	OR	P-value
***TGFB1* SNPs**	**Genotype**	**Population 1**									
rs6957	AA	reference				reference				reference	
	GA/GG	NA	NA	NA	NA	0.29	0.08	1.06	**0.06**	NA	NA
rs10417924	CC	reference				reference				reference	
	TC/TT	1.39	0.20	9.78	0.74	2.55	0.96	6.79	**0.06**	1.62	0.65
rs1800469	CC	reference				reference				reference	
	CT/TT	1.02	0.18	5.98	0.98	1.38	0.54	3.53	0.50	1.25	0.82
rs1800470	TT	reference				reference				reference	
	TC/CC	0.92	0.14	6.09	0.93	1.50	0.57	3.97	0.41	1.35	0.77
rs4803455	CC	reference				reference				reference	
	CA/AA	0.54	0.08	3.64	0.52	0.93	0.32	2.70	0.90	1.74	0.61
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	0.59	0.10	3.36	0.55	1.24	0.27	5.79	0.79	2.24	0.49
rs10417924	CC	reference				reference				reference	
	TC/TT	1.08	0.23	4.96	0.92	4.86	1.03	23.01	**0.046**	4.19	0.19
rs1800469	CC	reference				reference				reference	
	CT/TT	1.56	0.41	6.01	0.52	1.13	0.25	5.12	0.87	0.74	0.77
rs1800470	TT	reference				reference				reference	
	TC/CC	2.00	0.50	7.96	0.32	0.87	0.21	3.69	0.85	0.43	0.40
rs4803455	CC	reference				reference				reference	
	CA/AA	1.98	0.35	11.11	0.44	0.42	0.10	1.82	0.25	0.22	0.18

Table E10: Meta-analysis of associations with complete asthma remission in populations 1 and 2

E10 Meta-analysis	*TGFB1 SNP*	Total B [95%CI]; p-value	Never-Smokers B [95%CI]; p-value	Ever-Smokers B [95%CI]; p-value	Interaction B [95%CI]; p-value
Asthma remission	rs6957-T	**0.4795 [0.2038; 1.1285] 0.0923**	Not applicable	0.5281 [0.1953; 1.4280] 0.2084	Not applicable
	rs10417924-G	**2.2431 [1.1538; 4.3608] 0.0172**	1.1862 [0.3563; 3.9487] 0.7808	**3.0632 [1.3355; 7.026] 0.0082**	2.5732 [0.5715; 11.5868] 0.2183
	rs1800469-T	1.3278 [0.7004; 2.5172] 0.385	1.3356 [0.4575; 3.899] 0.5965	1.3081 [0.5901; 2.8994] 0.5084	1.2231 [0.7937; 1.8849] 0.3613
	rs1800470-C	1.4164 [0.7368; 2.7228] 0.2965	1.5276 [0.5013; 4.6553] 0.4561	1.2679 [0.566; 2.8403] 0.564	1.2306 [0.6979; 2.1700] 0.4734
	rs4803455-A	0.8274 [0.4087; 1.6748] 0.5985	1.1032 [0.3061; 3.9762] 0.8807	0.7086 [0.2998; 1.6746] 0.4324	0.6476 [0.1373; 3.0549] 0.5831

Table E11a-b: Associations with severe asthma in populations 1 and 2

E11a		Severe asthma							
		Population 1				Population 2			
TGFB1 SNPs	Genotype	OR	95%CI		P-value	OR	95%CI		P-value
rs6957	AA	reference				reference			
	GA/GG	1.03	0.55	1.92	0.93	1.79	0.56	5.72	0.33
rs10417924	CC	reference				reference			
	TC/TT	1.23	0.66	2.32	0.52	1.18	0.36	3.84	0.79
rs1800469	CC	reference				reference			
	CT/TT	1.01	0.59	1.74	0.97	3.85	1.09	13.61	**0.04**
rs1800470	TT	reference				reference			
	TC/CC	0.87	0.50	1.54	0.64	3.48	0.90	13.43	**0.07**
rs4803455	CC	reference				reference			
	CA/AA	0.66	0.35	1.25	0.20	0.48	0.15	1.54	0.22

E11b		Severe asthma									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
		OR	95%CI		P-value	OR	95%CI		P-value	OR	P-value
***TGFB1* SNPs**	**Genotype**	**Population 1**									
rs10417924	CC	reference				reference				reference	
	TC/TT	1.53	0.53	4.38	0.43	1.05	0.48	2.31	0.91	1.55	0.50
rs6957	AA	reference				reference				reference	
	GA/GG	0.79	0.29	2.16	0.64	1.22	0.56	2.67	0.62	0.69	0.58
rs1800469	CC	reference				reference				reference	
	CT/TT	1.15	0.50	2.64	0.74	0.96	0.47	1.95	0.90	0.80	0.69
rs1800470	TT	reference				reference				reference	
	TC/CC	1.33	0.55	3.20	0.53	0.61	0.29	1.28	0.19	0.44	0.15
rs4803455	CC	reference				reference				reference	
	CA/AA	0.35	0.12	1.04	**0.058**	0.90	0.41	1.99	0.80	2.48	0.18
		Population 2									
rs6957	AA	reference				reference				reference	
	GA/GG	1.05	0.08	13.92	0.97	1.92	0.53	7.02	0.32	1.56	0.76
rs10417924	CC	reference				reference				reference	
	TC/TT	1.79	0.14	22.33	0.65	1.14	0.30	4.26	0.85	0.71	0.81
rs1800469	CC	reference				reference				reference	
	CT/TT	2.14	0.18	25.69	0.55	3.66	0.89	15.16	**0.08**	1.88	0.66
rs1800470	TT	reference				reference				reference	
	TC/CC	2.07	0.17	24.62	0.57	4.02	0.80	20.26	**0.09**	2.01	0.64
rs4803455	CC	reference				reference					
	CA/AA	0.53	0.04	7.12	0.63	0.40	0.11	1.43	0.16	NT	NT

Table E12: Meta-analysis of associations with severe asthma in populations 1 and 2

E12 Meta- Analysis	*TGFB1 SNP*	Total B [95%CI]; p-value	Never-Smokers B [95%CI]; p-value	Ever-Smokers B [95%CI]; p-value	Interaction B [95%CI]; p-value
Severe Asthma	rs6957-T	1.165 [0.6737; 2.0146] 0.5848	0.8171 [0.3196; 2.0893] 0.6733	1.3773 [0.7051; 2.6902] 0.3487	1.5487 [0.4849; 4.9465] 0.4604
	rs10417924-G	1.2203 [0.6984; 2.1322] 0.4844	1.5638 [0.592; 4.1307] 0.367	1.0705 [0.5435; 2.1086] 0.8439	0.6966 [0.2133; 2.2755] 0.5494
	rs1800469-T	1.7374 [0.4809; 6.2769] 0.3993	1.2226 [0.5568; 2.6844] 0.6166	1.2514 [0.6624; 2.3642] 0.4896	0.8958 [0.3248; 2.4707] 0.8316
	rs1800470-C	1.5143 [0.4020; 5.7034] 0.5397	1.3947 [0.6086; 3.1961] 0.4317	1.3585 [0.2200; 8.3867] 0.7415	0.5332 [0.1854; 1.5333] 0.2433
	rs4803455-A	**0.6149 [0.3522; 1.0735] 0.0872**	**0.3742 [0.1383; 1.0125] 0.0529**	0.7184 [0.3658; 1.4108] 0.3368	2.0748 [0.6217; 6.9242] 0.2352

Table E13a-b: Associations with airway wall inflammation in population 2

E13a		Number of EPX+ eosinophils/0.1mm² (ln)				Number of CD68+ Macropahes/0.1mm² (ln)				Number of AA1+ Mast cells/0.1mm² (ln)			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference				reference			
	GA/GG	0.45	0.07	0.84	**0.02**	0.26	0.01	0.51	**0.045**	-0.05	-0.34	0.23	0.71
rs10417924	CC	reference				reference				reference			
	TC/TT	0.26	-0.14	0.66	0.20	0.14	-0.12	0.40	0.30	0.06	-0.23	0.35	0.68
rs1800469	CC	reference				reference				reference			
	CT/TT	0.19	-0.17	0.55	0.30	0.09	-0.15	0.33	0.46	-0.10	-0.36	0.16	0.46
rs1800470	TT	reference				reference				reference			
	TC/CC	0.21	-0.15	0.57	0.24	0.13	-0.11	0.36	0.28	0.01	-0.25	0.27	0.94
rs4803455	CC	reference				reference				reference			
	CA/AA	-0.15	-0.55	0.25	0.46	-0.07	-0.33	0.19	0.58	0.16	-0.13	0.45	0.27

		Number of CD4+ T-cells /0.1mm² (ln)				Number of CD8+ T-cells/0.1mm² (ln)			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	0.17	-0.19	0.52	0.36	0.15	-0.17	0.48	0.36
rs10417924	CC	reference				reference			
	TC/TT	-0.06	-0.42	0.30	0.74	0.37	0.06	0.69	**0.02**
rs1800469	CC	reference				reference			
	CT/TT	0.02	-0.31	0.35	0.90	-0.01	-0.32	0.29	0.93
rs1800470	TT	reference				reference			
	TC/CC	0.06	-0.26	0.39	0.70	0.04	-0.26	0.34	0.78
rs4803455	CC	reference				reference			
	CA/AA	0.15	-0.21	0.51	0.41	0.07	-0.26	0.41	0.67

E13b		Number of EPX+eosinophils/0.1mm² (ln)									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
TGFB1 SNPs	Genotype	B	95%CI		P-value	B	95%CI		P-value	B	P-value
rs6957	AA	reference				reference				reference	
	GA/GG	0.04	-0.57	0.66	0.89	0.78	0.27	1.29	**0.003**	0.72	**0.07**
rs10417924	CC	reference				reference				reference	
	TC/TT	0.34	-0.27	0.95	0.27	0.25	-0.29	0.79	0.36	NT	NT
rs1800469	CC	reference				reference				reference	
	CT/TT	0.31	-0.21	0.83	0.23	0.10	-0.41	0.61	0.70	-0.25	0.49
rs1800470	TT	reference				reference				reference	
	TC/CC	0.24	-0.28	0.76	0.35	0.25	-0.27	0.77	0.34	NT	NT
rs4803455	CC	reference				reference				reference	
	CA/AA	-0.03	-0.63	0.57	0.92	-0.35	-0.89	0.20	0.21	-0.37	0.37
		Number of CD68+Macropahes/0.1mm² (ln)									
rs6957	AA	reference				reference				reference	
	GA/GG	0.06	-0.25	0.37	0.71	0.48	0.11	0.84	**0.01**	0.47	**0.07**
rs10417924	CC	reference				reference				reference	
	TC/TT	-0.05	-0.36	0.26	0.74	0.32	-0.06	0.70	0.09	0.36	0.17
rs1800469	CC	reference				reference				reference	
	CT/TT	0.10	-0.17	0.36	0.46	0.11	-0.26	0.47	0.56	NT	NT
rs1800470	TT	reference				reference				reference	
	TC/CC	0.05	-0.21	0.31	0.68	0.25	-0.12	0.61	0.18	0.14	0.55
rs4803455	CC	reference				reference				reference	
	CA/AA	-0.01	-0.31	0.29	0.95	-0.18	-0.57	0.21	0.35	-0.24	0.37
		Number of AA1+Mast cells/0.1mm² (ln)									
rs6957	AA	reference				reference				reference	
	GA/GG	-0.01	-0.42	0.41	0.98	-0.09	-0.49	0.32	0.66	-0.07	0.82
rs10417924	CC	reference				reference				reference	
	TC/TT	0.00	-0.43	0.43	1.00	0.06	-0.34	0.46	0.77	NT	NT
rs1800469	CC	reference				reference				reference	
	CT/TT	0.09	-0.28	0.46	0.62	-0.19	-0.57	0.19	0.31	-0.34	0.20
rs1800470	TT	reference				reference				reference	

	TC/CC	0.05	-0.32	0.41	0.80	-0.02	-0.41	0.37	0.91	-0.12	0.66
rs4803455	CC	reference				reference				reference	
	CA/AA	0.20	-0.21	0.61	0.34	0.26	-0.15	0.67	0.20	NT	NT
		Number of CD4+T-cells /0.1mm² (ln)									
rs6957	AA	reference				reference				reference	
	GA/GG	-0.06	-0.59	0.47	0.83	0.31	-0.19	0.80	0.22	0.27	0.46
rs10417924	CC	reference				reference				reference	
	TC/TT	-0.05	-0.57	0.47	0.86	-0.03	-0.53	0.46	0.90	NT	NT
rs1800469	CC	reference				reference				reference	
	CT/TT	-0.07	-0.52	0.39	0.77	0.10	-0.38	0.58	0.68	0.12	0.72
rs1800470	TT	reference				reference				reference	
	TC/CC	-0.12	-0.56	0.32	0.59	0.28	-0.19	0.76	0.24	0.38	0.26
rs4803455	CC	reference				reference				reference	
	CA/AA	0.61	0.13	1.10	**0.01**	-0.32	-0.83	0.18	0.20	-0.97	**0.01**
		Number of CD8+T-cells/0.1mm² (ln)									
rs6957	AA	reference				reference				reference	
	GA/GG	0.07	-0.44	0.58	0.79	0.23	-0.21	0.67	0.31	0.15	0.66
rs10417924	CC	reference				reference				reference	
	TC/TT	0.27	-0.21	0.76	0.27	0.46	0.04	0.88	**0.03**	NT	NT
rs1800469	CC	reference				reference				reference	
	CT/TT	0.20	-0.23	0.64	0.35	-0.17	-0.59	0.26	0.44	-0.44	0.15
rs1800470	TT	reference				reference				reference	
	TC/CC	0.18	-0.25	0.60	0.41	-0.03	-0.46	0.40	0.88	-0.26	0.39
rs4803455	CC	reference				reference				reference	
	CA/AA	0.06	-0.44	0.55	0.83	0.05	-0.41	0.51	0.83	NT	NT

Table E14a-b: Associations with airway wall remodeling in population 2

E14a		**Basement membrane thickness (um)**				**Number of goblet cells per 1000um BM (ln)**			
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**
rs6957	AA	reference				reference			
	GA/GG	0.39	-0.17	0.95	0.17	-0.08	-0.32	0.16	0.52
rs10417924	CC	reference				reference			
	TC/TT	0.00	-0.57	0.57	1.00	0.06	-0.17	0.30	0.58
rs1800469	CC	reference				reference			
	CT/TT	0.22	-0.28	0.71	0.39	0.23	0.03	0.43	**0.03**
rs1800470	TT	reference				reference			
	TC/CC	0.01	-0.50	0.53	0.96	0.07	-0.14	0.28	0.50
rs4803455	CC	reference				reference			
	CA/AA	0.06	-0.52	0.63	0.85	-0.16	-0.39	0.07	0.18

E14b		Basement membrane thickness (um)									
		NEVER SMOKERS				EVER SMOKERS				Interaction	
***TGFB1* SNPs**	**Genotype**	**B**	**95%CI**		**P-value**	**B**	**95%CI**		**P-value**	**B**	**P-value**
rs6957	AA	reference				reference				reference	
	GA/GG	-0.18	-1.11	0.75	0.70	0.76	0.03	1.49	**0.04**	0.86	0.13
rs10417924	CC	reference				reference				reference	
	TC/TT	0.40	-0.50	1.31	0.37	-0.32	-1.06	0.43	0.40	-0.72	0.22
rs1800469	CC	reference				reference				reference	
	CT/TT	0.67	-0.03	1.36	**0.06**	-0.10	-0.81	0.60	0.77	-0.82	0.10
rs1800470	TT	reference				reference				reference	
	TC/CC	0.47	-0.30	1.24	0.22	-0.38	-1.09	0.34	0.30	-0.90	0.10
rs4803455	CC	reference				reference				reference	
	CA/AA	0.18	-0.71	1.07	0.69	0.04	-0.72	0.81	0.91	-0.15	0.80
		Number of goblet cells per 1000um BM (ln)									
rs6957	AA	reference				reference				reference	
	GA/GG	-0.21	-0.64	0.22	0.33	0.07	-0.31	0.44	0.72	0.27	0.33
rs10417924	CC	reference				reference				reference	
	TC/TT	0.26	-0.12	0.63	0.17	-0.14	-0.48	0.20	0.42	-0.38	0.13
rs1800469	CC	reference				reference				reference	
	CT/TT	0.29	-0.04	0.62	**0.08**	0.27	-0.04	0.57	**0.08**	NT	NT
rs1800470	TT	reference				reference				reference	
	TC/CC	0.12	-0.21	0.45	0.47	0.08	0.16	-0.25	0.40	NT	NT
rs4803455	CC	reference				reference				reference	
	CA/AA	-0.16	-0.53	0.21	0.39	-0.04	-0.39	0.31	0.81	0.08	0.74

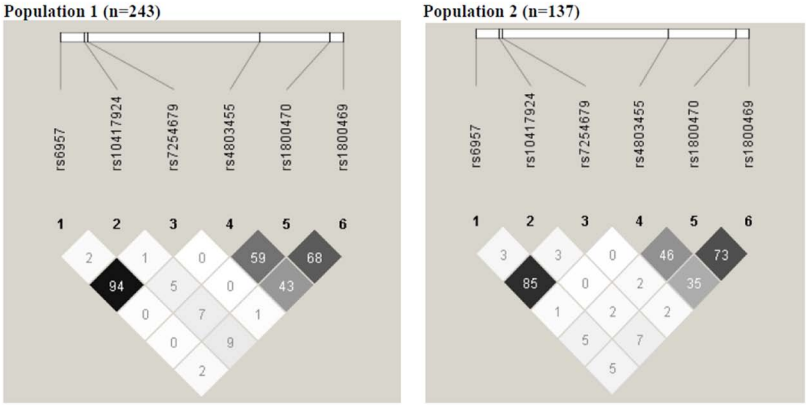


Figure E1: Analysis of the linkage disequilibrium patterns and correlation coefficients (r^2) of 6 genotyped *TGFB1* gene single nucleotide polymorphisms in the two populations using Haploview software. The location of the SNPs is given by HapMap or dbSNP

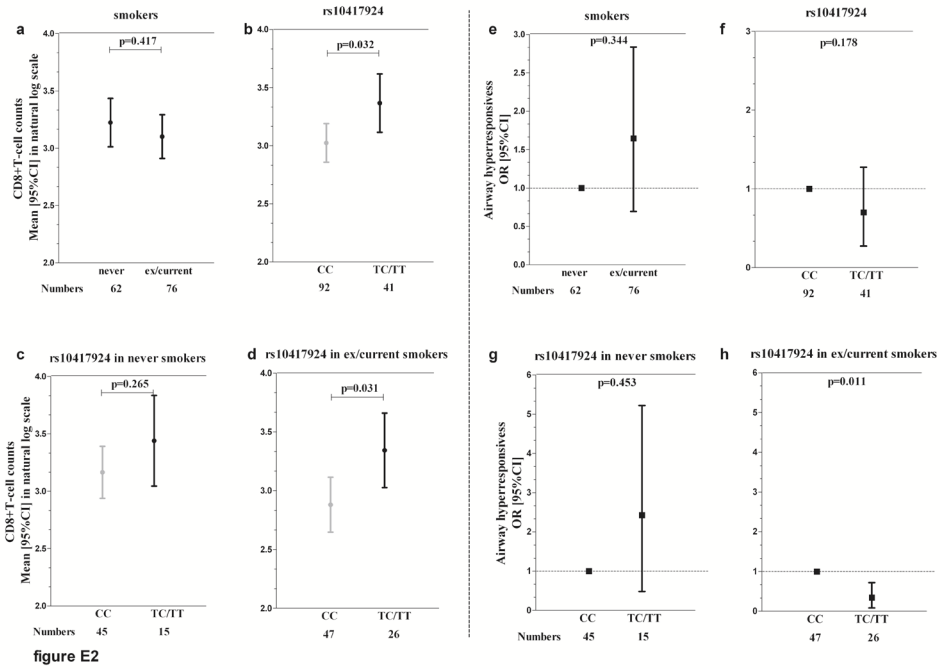


Figure E2 Associations between subepithelial CD8+T-cell counts and (a) smoking status, (b) rs10417924, rs10417924 in never (c) and in ever (d) smokers of population 2. Associations between airway hyperresponsiveness and (e) smoking status, (f) rs10417924, rs10417924 in never (g) and in ever (h) smokers
Odds ratios (95%CI) are given by multiple logistic regression models adjusted for gender, age and inhaled corticosteroids use

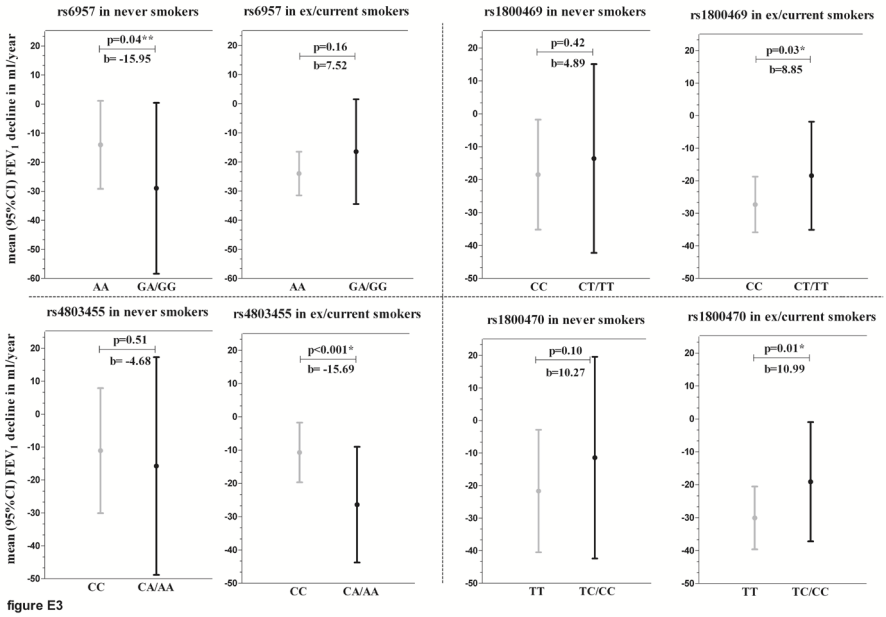


Figure E3 Mean (95%CI) FEV_1 decline (ml/year) per genotype of *TGFB1* SNPs in never and in ever smokers of population 1.
Piecewise linear mixed effect models were used to test the association between *TGFB1* SNPs and FEV_1 decline. Regression Coefficient (beta) and p-value are indicated. *significance level at 0.05; ** significant smoking by SNP interaction

SUPPLEMENTARY MATERIAL - CHAPTER 5

MATERIALS AND METHODS

The Childhood Asthma Management Program (CAMP): is a multi-center, double-blinded clinical trial involving eight clinical centers in North America (Albuquerque, New Mexico; Baltimore, Maryland; Boston, Massachusetts; Denver, Colorado; San Diego, California; Seattle, Washington; St. Louis, Missouri; and Toronto-Ontario, Canada). Its main goal was to evaluate the long-term effectiveness and safety of daily inhaled anti-inflammatory medication in children diagnosed with asthma. Details of the CAMP study design and methods have been described elsewhere.[E1] In summary, children enrolled in CAMP were 5–12 years of age at the initial interview, and completed a 5-16 week pre-randomization screening period (1993-1995). All participants had mild-to-moderate asthma with increased airway responsiveness to methacholine (a provocation concentration causing a 20% reduction in FEV_1 [PC_{20}] ≤ 12.5 mg/ml) at study entry. 1,041 Children entered the randomization phase and 311, 312, 418 children received budesonide, nedocromil, and placebo, respectively. All subjects were treated and followed for four years with follow-up visits at CAMP centers at two and four months after randomization and at four-month intervals thereafter. Each parent or guardian signed a consent form and participants of 7 years of age and older signed an assent form approved by each clinical center's institutional review board.

Outcomes Measures

Spirometry, before and after the administration of two puffs of albuterol, was conducted at randomization (RZ) and at follow up visits (n=13) according to the American Thoracic Society Standards. The focus of the trial on long-term effects of the treatments on lung function was reflected in the choice of post-bronchodilator FEV_1 percentage of predicted (post-BD FEV_1% predicted) as the primary outcome measure.[E2] Use of post-BD FEV_1 rather than the pre-BD measure minimized fluctuations caused by diurnal variation and day-to-day variability of airway tone. However, we considered both pre- and post-BD FEV_1 and FVC as outcomes in this current analysis as we intended to investigate short- and long-term effects of air pollution. Additionally, the FEV_1/FVC ratio was used as another measure of airflow obstruction.

Using the Wright nebulizer-tidal breathing technique a methacholine challenge was performed annually during the treatment phase, at least 4 hours

after the last use of a short-acting bronchodilator and at least 24 hours after the last use of a long-acting bronchodilator. After a control diluent challenge, nine doubling doses of methacholine were nebulized for 2 minutes each at 5-minute intervals. Spirometry was performed 90 seconds after each challenge until FEV_1 had fallen by 20% or more. Methacholine challenge was not performed within 28 days of an upper respiratory tract infection or the use of prednisone for exacerbations of asthma.

Air Pollution Exposure Assessment

Data on concentrations of 4 gaseous pollutants (ozone, carbon monoxide, nitrogen dioxide, and sulfur dioxide) were obtained from the Aerometric Information Retrieval System (www.epa.gov/air/data/index.html) for US cities and from the Ontario Ministry of the Environment (www.ene.gov.on.ca/environment; Air Quality Assessment and Reporting Unit) for Toronto. For each metropolitan area, multiple monitors were used to measure ambient pollution concentrations. Monitor-specific concentrations were 24-hour averages for ozone, carbon monoxide, nitrogen dioxide, and sulfur dioxide. In most cases, for US participants we were able to link ZIP codes of their address at study entry to US Census 2000 ZIP Code Tabulation Areas to identify the latitudes and longitudes of the centroids of those areas. In a few cases ZIP Code centroid coordinates were obtained from www.zip-codes.com. Coordinates for Canadian postal codes were obtained from www.batchgeo.com. The ZIP or postal code centroid coordinates were used to link participants to daily concentrations from the nearest monitor within 50 km that did not have missing data on that day (December 1993 through June 1999). If none of the monitors within 50 km radius was operating the air pollution concentration of that day was set as missing.

Exclusion criteria

Participants were excluded if we were unable to assign their ZIP or postal codes to latitudes and longitudes or if we verified that the centroid of the ZIP or postal code in which they lived was greater than 50 km from the nearest pollutant monitor. For the Canadian participants, a few monitors northwest of Toronto became operational after 2000 and subjects living in postal codes near those monitors were excluded. In total 38 subjects of the 1041 trial participants (3.7%) were excluded.

Statistical Analysis

We examined the relation of level of lung function (FEV_1 and FVC % predicted and FEV_1/FVC ratio) and (log-transformed) PC_{20} with ZIP-code level measures of concentrations of ozone (ppb), carbon monoxide (ppmx10), nitrogen dioxide (ppb) and sulfur dioxide (ppb). To estimate associations across all cities, we constructed a model including city as a covariate, but also compared estimates of this model with study-wide estimates from meta-analyzing city stratified models.[E3] We examined the associations of our outcomes with same day, 1-week and 4-month moving averages of pollution, to assess associations of short-term and longer-term estimated pollution exposures with our 4-monthly (or for PC_{20}, annually) measured outcomes. The moving averages were created by averaging the daily pollution concentrations for the periods of interest on and preceding the clinic visit for lung function and/or PC_{20} measurement. For any given averaging period, we required that 75% of the daily pollution data were available. One and two-pollutant models (ozone –nitrogen dioxide; ozone-carbon monoxide; and carbon monoxide-nitrogen dioxide) were performed.

We fit a linear mixed model - with random intercepts for each subject - to estimate the associations between pulmonary outcomes and ambient air pollutant concentrations. Number of days from randomization was used as the time trend in the model. Potential for confounding by a number of factors was considered carefully, basing choice of covariates on prior CAMP experience[E4,E5]: For lung function, we controlled for gender, ethnicity, *in utero* smoking exposure, annual family income (dichotomized at $15K per annum), treatment group and treatment by time interaction, and for PC_{20}, in addition, we controlled for IgE levels, height and history of parental asthma. We ran the model for PC_{20} with and without adjusting for pre-BD FEV_1.

Confounding due to seasonal factors is a primary consideration in air pollution epidemiology, and we adjusted for "season" by using sine and cosine functions of time[E6] and their interactions with city. In addition, we decomposed daily ambient pollution concentrations into between- and within-subject exposures. Specifically, subject *i*'s exposure to pollutant x_1 on day *t* was decomposed as follows: The between-subject component, is subject *i*'s average exposure during the time in which he or she was observed, and the within-subject component, is the deviation from the average on day *t*. Within-subject effects are likely to be far less confounded by season or by covariates that vary primarily across individual (e.g. in utero smoking exposure, etc) than between-subject effects,[E7] and they are more comparable to the parameters estimated

in a traditional panel study, where all subjects are observed over a single time period. For further discussion regarding the decomposition of time-varying exposures into between- and within-subject components, see the papers by Sheppard[E7] and Neuhaus and Kalbfleisch.[E8] We report estimates of within-subject exposure effects.

To assess potential effect modification of the pollution-asthma outcomes associations by treatment we included an interaction term, i.e., pollutant concentration by treatment into the models while excluding the baseline (RZ) measurements. To compare the magnitude of effects across different pollutants, effect estimates were scaled to interquartile range IQR increases in within-subject pollutant concentrations determined from the distribution of all measurements. Two tailed p-values of <0.05 were considered statistically significant and p-values between 0.05 and 0.09 of borderline significance.

We used SAS® software (version 9.2; SAS Institute Inc. 2008, Cary, NC USA) and IBM SPSS statistics (version 20; Armonk, NY USA: IBM Corp 2011) to manage all data. Statistical analysis was performed in IBM SPSS and R programming language (version 2.15.1; 2012-06-22).

REFERENCES

E1. Anonymous. The Childhood Asthma Management Program (CAMP): design, rationale, and methods. Childhood Asthma Management Program Research Group. Control Clin Trials 1999 Feb;20:91-120.

E2. Strunk RC, Childhood Asthma Management Program Research Group. Childhood Asthma Management Program: lessons learned. J Allergy Clin Immunol 2007 Jan;119:36-42.

E3. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986 Sep;7:177-188.

E4. Cohen RT, Raby BA, Van Steen K, Fuhlbrigge AL, Celedon JC, Rosner BA, Strunk RC, Zeiger RS, Weiss ST, Childhood Asthma Management Program Research Group. In utero smoke exposure and impaired response to inhaled corticosteroids in children with asthma. J Allergy Clin Immunol 2010 Sep;126:491-497.

E5. Anonymous. Long-term effects of budesonide or nedocromil in children with asthma. The Childhood Asthma Management Program Research Group. N Engl J Med 2000 Oct 12;343:1054-1063.

E6. Stolwijk AM, Straatman H, Zielhuis GA. Studying seasonality by using sine and cosine functions in regression analysis. J Epidemiol Community Health 1999 Apr;53:235-238.

E7. Sheppard L. Insights on bias and information in group-level studies. Biostatistics 2003 Apr;4:265-278.

E8. Neuhaus JM, Kalbfleisch JD. Between- and within-cluster covariate effects in the analysis of clustered data. Biometrics 1998 Jun;54:638-645

Table E1. City-specific correlations of 24-hour mean pollution concentrations

CITY CENTER			NO_2	O_3	SO_2	CO
ALB	Spearman's rho	NO_2	1.000	-,488*	NA	,446*
		O_3	-,488*	1.000	NA	-,473*
		SO_2	NA	NA	NA	NA
		CO	,446*	-,473*	NA	1.000
BAL	Spearman's rho	NO_2	1.000	-,160*	,379*	,387*
		O_3	-,160*	1.000	-,311*	-,352*
		SO_2	,379*	-,311*	1.000	,295*
		CO	,387*	-,352*	,295*	1.000
BOSTON	Spearman's rho	NO_2	1.000	-,218*	,378*	,346*
		O_3	-,218*	1.000	-,414*	-,315*
		SO_2	,378*	-,414*	1.000	,273*
		CO	,346*	-,315*	,273*	1.000
DEN	Spearman's rho	NO_2	1.000	-,301*	,469*	,416*
		O_3	-,301*	1.000	-,187*	-,512*
		SO_2	,469*	-,187*	1.000	,343*
		CO	,416*	-,512*	,343*	1.000
SD	Spearman's rho	NO_2	1.000	-,527*	,150*	,743*
		O_3	-,527*	1.000	-,092*	-,446*
		SO_2	,150*	-,092*	1.000	,188*
		CO	,743*	-,446*	,188*	1.000
SEA	Spearman's rho	NO_2	1.000	-,170*	,321*	,512*
		O_3	-,170*	1.000	-.053	-,284*
		SO_2	,321*	-.053	1.000	,203*
		CO	,512*	-,284*	,203*	1.000
STL	Spearman's rho	NO_2	1.000	-,247*	,532*	,535*
		O_3	-,247*	1.000	-,145*	-,223*
		SO_2	,532*	-,145*	1.000	,302*
		CO	,535*	-,223*	,302*	1.000
TOR	Spearman's rho	NO_2	1.000	-,339*	,462*	,533*
		O_3	-,339*	1.000	-,208*	-,296*
		SO_2	,462*	-,208*	1.000	,324*
		CO	,533*	-,296*	,324*	1.000

ALB: Albuquerque, BAL: Baltimore, BOS: Boston, DEN: Denver, SD: San Diego, SEA: Seattle, STL: Saint Louis, TOR: Toronto, O_3: ozone (ppb); CO: carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); ppb: part per billion; ppm: parts per million

E2. Treatment group modification of within-subject pollution effect on Pre-BD FEV_1 %predicted

			Placebo			Budesonide			Nedocromil		
Within-subject	**N**	**IQR**	**Coefficient**	**95%CI**		**Coefficient**	**95%CI**		**Coefficient**	**95%CI**	
O_3 same day	10531	15	0.02	-0.33	0.37	0.03	-0.37	0.43	-0.13	-0.52	0.26
O_3 1-week m.a	10531	14	-0.12	-0.59	0.36	0.15	-0.36	0.66	-0.18	-0.68	0.32
O_3 4-month m.a	10515	11	-0.48	-1.23	0.28	-0.20	-0.97	0.57	-0.63	-1.39	0.14
CO same day	10299	5	-0.03	-0.26	0.20	-0.24	-0.52	0.03	-0.19	-0.47	0.08
CO 1-week m.a	11141	4	-0.16	-0.44	0.12	-0.33	-0.65	-0.01	-0.23	-0.54	0.09
CO 4-month m.a	11142	3	-0.45	-0.79	-0.10	-0.29	-0.68	0.09	-0.36	-0.74	0.02
NO_2 same day	11143	10	-0.06	-0.33	0.21	-0.19	-0.52	0.13	0.04	-0.27	0.34
NO_2 1-week m.a	11144	7	0.03	-0.24	0.31	-0.28	-0.60	0.05	0.02	-0.29	0.34
NO_2 4-month m.a	10740	4	-0.10	-0.38	0.18	-0.28	-0.60	0.03	-0.03	-0.34	0.28
SO_2 same day	10734	4	0.12	-0.10	0.34	0.15	-0.12	0.42	-0.04	-0.30	0.22
SO_2 1-week m.a	10707	3	0.29	0.04	0.55	0.04	-0.27	0.34	-0.02	-0.33	0.28
SO_2 4-month m.a	10709	2	0.21	-0.08	0.51	0.02	-0.32	0.37	0.14	-0.20	0.48

O_3: ozone (ppb); carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppm: parts per million; FEV_1: forced expiratory volume in 1 second; BD: bronchodilator; CI: confidence interval

* P-value for placebo vs budesonide or placebo vs nedocromil <0.10

E3. Treatment group modification of within-subject pollution effect on Post-BD FEV_1 %predicted

			Placebo			Budesonide			Nedocromil		
Within-subject	**N**	**IQR**	**Coefficient**	**95%CI**		**Coefficient**	**95%CI**		**Coefficient**	**95%CI**	
O_3 same day	7325	15	0.12	-0.26	0.49	-0.06	-0.50	0.37	0.12	-0.31	0.55
O_3 1-week m.a	7311	14	0.25	-0.26	0.76	0.23	-0.34	0.80	0.24	-0.31	0.80
O_3 4-month m.a	7184	11	-0.11	-0.94	0.73	**-0.72***	**-1.58**	**0.14**	-0.53	-1.38	0.33
CO same day	7741	5	-0.23	-0.49	0.03	-0.35	-0.65	-0.06	-0.34	-0.63	-0.05
CO 1-week m.a	7741	4	-0.37	-0.68	-0.07	-0.47	-0.82	-0.12	-0.49	-0.84	-0.14
CO 4-month m.a	7742	3	-0.64	-1.01	-0.27	**-0.14***	**-0.56**	**0.29**	-0.43	-0.85	-0.02
NO_2 same day	7439	10	-0.14	-0.43	0.15	-0.16	-0.51	0.19	0.05	-0.28	0.38
NO_2 1-week m.a	7418	7	-0.02	-0.32	0.28	-0.21	-0.57	0.15	0.05	-0.29	0.39
NO_2 4-month m.a	7414	4	-0.40	-0.71	-0.09	-0.18	-0.52	0.16	**-0.03***	**-0.37**	**0.31**
SO_2 same day	6760	4	0.08	-0.16	0.32	0.00	-0.29	0.30	-0.15	-0.43	0.13
SO_2 1-week m.a	6761	3	0.21	-0.06	0.49	-0.05	-0.40	0.30	-0.04	-0.37	0.29
SO_2 4-month m.a	6764	2	0.09	-0.22	0.41	0.17	-0.22	0.55	0.17	-0.20	0.54

O_3: ozone (ppb); carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppm: parts per million; FEV_1: forced expiratory volume in 1 second; BD: bronchodilator; CI: confidence interval

* P-value for placebo vs budesonide or placebo vs nedocromil <0.10

E4. Treatment group modification of within-subject pollution effect on Pre-BD FVC %predicted

			Placebo			Budesonide			Nedocromil		
Within-subject	**N**	**IQR**	**Coefficient**	**95%CI**		**Coefficient**	**95%CI**		**Coefficient**	**95%CI**	
O_3 same day		15	0.23	-0.04	0.50	-0.02	-0.33	0.29	-0.02	-0.32	0.28
O_3 1-week m.a		14	0.18	-0.19	0.55	0.11	-0.29	0.51	0.04	-0.35	0.42
O_3 4-month m.a		11	-0.08	-0.67	0.51	0.03	-0.57	0.63	-0.27	-0.87	0.33
CO same day		5	-0.07	-0.25	0.11	-0.23	-0.45	-0.01	-0.06	-0.27	0.16
CO 1-week m.a		4	-0.15	-0.37	0.07	-0.21	-0.46	0.04	-0.16	-0.41	0.09
CO 4-month m.a		3	-0.30	-0.58	-0.03	-0.18	-0.49	0.13	-0.18	-0.48	0.12
NO_2 same day		10	0.00	-0.21	0.22	-0.20	-0.45	0.05	0.15	-0.09	0.39
NO_2 1-week m.a		7	0.05	-0.17	0.26	-0.21	-0.47	0.04	0.07	-0.18	0.31
NO_2 4-month m.a		4	-0.18	-0.40	0.04	-0.29	-0.54	-0.04	0.07	-0.18	0.31
SO_2 same day		4	0.02	-0.16	0.19	0.08	-0.13	0.29	0.04	-0.17	0.24
SO_2 1-week m.a		3	0.12	-0.08	0.32	0.06	-0.19	0.30	0.12	-0.11	0.36
SO_2 4-month m.a		2	0.21	-0.02	0.45	0.05	-0.23	0.32	0.23	-0.03	0.50

O_3: ozone (ppb); carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppm: parts per million; FVC: forced vital capacity; BD: bronchodilator; CI: confidence interval

* P-value for placebo vs budesonide or placebo vs nedocromil <0.10

E5. Treatment group modification of within-subject pollution effect on Post-BD FVC %predicted

			Placebo			Budesonide			Nedocromil		
Within-subject	**N**	**IQR**	**Coefficient**	**95%CI**		**Coefficient**	**95%CI**		**Coefficient**	**95%CI**	
O_3 same day	7325	15	0.24	-0.07	0.56	-0.12	-0.49	0.26	0.17	-0.19	0.54
O_3 1-week m.a	7311	14	0.41	-0.02	0.84	0.14	-0.34	0.62	0.36	-0.11	0.83
O_3 4-month m.a	7184	11	0.33	-0.38	1.04	**-0.30***	**-1.03**	**0.43**	-0.09	-0.82	0.64
CO same day	7741	5	-0.15	-0.37	0.07	-0.23	-0.48	0.02	-0.18	-0.43	0.07
CO 1-week m.a	7741	4	-0.26	-0.52	0.00	-0.26	-0.56	0.04	-0.30	-0.59	0.00
CO 4-month m.a	7742	3	-0.42	-0.73	-0.10	**0.01***	**-0.36**	**0.37**	-0.09	-0.45	0.26
NO_2 same day	7439	10	-0.05	-0.30	0.20	-0.13	-0.43	0.16	0.07	-0.21	0.35
NO_2 1-week m.a	7418	7	-0.09	-0.35	0.16	-0.26	-0.57	0.04	-0.07	-0.36	0.22
NO_2 4-month m.a	7414	4	-0.47	-0.73	-0.20	-0.30	-0.59	-0.01	**-0.08***	**-0.37**	**0.21**
SO_2 same day	6760	4	0.07	-0.13	0.28	0.01	-0.24	0.27	-0.04	-0.28	0.20
SO_2 1-week m.a	6761	3	-0.03	-0.27	0.21	-0.04	-0.34	0.26	0.00	-0.28	0.29
SO_2 4-month m.a	6764	2	-0.04	-0.31	0.23	0.06	-0.26	0.39	0.14	-0.18	0.45

O_3: ozone (ppb); carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); N: number of observations; IQR: Interquartile range, ppm: parts per million; FVC: forced vital capacity; BD: bronchodilator; CI: confidence interval

* P-value for placebo vs budesonide or placebo vs nedocromil <0.10

E6. Association of within-subject pollution with metacholine concentration causing a 20% reduction in FEV_1

			PC_{20}		
Within-subject	**N**	**IQR**	**%Change**	**95%CI**	
O_3 same day	2880	15	-1.5	-7.7	5.2
O_3 1-week m.a	2878	14	-3.8	-12.8	6.1
O_3 4-month m.a	2809	11	3.6	-11.7	21.7
CO same day	3039	5	-1.8	-5.3	1.7
CO 1-week m.a	3040	4	**-4.0***	**-8.1**	**0.3**
CO 4-month m.a	3040	3	-4.1	-9.5	1.6
NO_2 same day	2959	10	-1.0	-4.9	3.2
NO_2 1-week m.a	2946	7	-1.2	-5.3	3.0
NO_2 4-month m.a	2955	4	-0.9	-5.2	3.7
SO_2 same day	2660	4	-2.1	-5.2	1.1
SO_2 1-week m.a	2661	3	-3.4	-7.3	0.7
SO_2 4-month m.a	2661	2	**-6.0***	**-10.9**	**-1.5**

O_3: ozone (ppb); carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppm: parts per million; PC20: Metacholine provocation concentration (mg/dl) causing a 20% reduction in FEV_1; bronchodilator; CI: confidence interval
* p<0.05 * 0.05<=p<0.1

E7. Treatment group modification of within-subject pollution effect on metacholine concentration causing a 20% reduction in FEV_1

			Placebo			Budesonide			Nedocromil		
Within-subject	**N**	**IQR**	**%Change**	**95%CI**		**%Change**	**95%CI**		**%Change**	**95%CI**	
O_3 same day	2880	15	-6.9	-15.5	2.5	0.6	-9.8	12.2	3.2	-7.0	14.5
O_3 1-week m.a	2878	14	-7.3	-18.9	6.0	5.0	-8.9	21.0	-8.7	-21.0	5.6
O_3 4-month m.a	2809	11	-3.3	-20.4	17.5	4.7	-14.6	28.5	11.3	-9.3	36.6
CO same day	3039	5	4.3	-1.2	10.1	**-4.6***	**-10.7**	**2.2**	**-7.0***	**-12.6**	**-1.2**
CO 1-week m.a	3040	4	3.0	-3.7	10.0	**-7.2***	**-14.2**	**0.2**	**-10.0***	**-16.2**	**-2.5**
CO 4-month m.a	3040	3	1.8	-6.6	11.1	-6.2	-15.3	3.9	**-9.0***	-16.8	-0.1
NO_2 same day	2959	10	2.2	-4.3	9.1	-3.2	-10.1	4.3	-2.6	-9.4	4.7
NO_2 1-week m.a	2946	7	3.9	-2.6	10.9	**-5.1***	**-12**	**2.4**	-4.1	-11.1	3.4
NO_2 4-month m.a	2955	4	2.6	-4.1	9.6	-3.6	-11.0	4.4	-2.8	-9.9	4.9
SO_2 same day	2660	4	0.1	-4.8	5.2	-2.0	-7.6	3.8	-5.3	-10.8	0.5
SO_2 1-week m.a	2661	3	-1.6	-7.5	4.7	-1.4	-8.2	5.9	-7.9	-14.4	-0.9
SO_2 4-month m.a	2661	2	-6.9	-13.1	-0.2	-5.1	-13.1	3.7	-6.7	-14.4	1.8

O_3: ozone (ppb); carbon monoxide (ppm x 10); NO_2: nitrogen dioxide (ppb); SO_2: sulfur dioxide (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppm: parts per million; PC20: Metacholine dose Metacholine provocation concentration (mg/dl) causing a 20% reduction in FEV_1; BD: bronchodilator; CI: confidence interval
* P-value for placebo vs budesonide or placebo vs nedocromil <0.10

E8a-c. Associations of warm-months (May-September) within-subject Ozone with lung function and PC_{20}:

8a.		Pre-BD FEV1 %predicted				Post-BD FEV1 %predicted			
Within-subject	**IQR (ppb)**	**N**	**Change**	**95%CI**		**N**	**Change**	**95%CI**	
O_3 same day	13	5199	-0.036	-0.341	0.270	4036	-0.016	-0.298	0.266
O_3 1-day lag	13	5199	-0.030	-0.343	0.284	4036	0.011	-0.282	0.305
O_3 1-week m.a	9	5197	-0.021	-0.393	0.351	4035	0.035	-0.311	0.381

8b.		Pre-BD FVC %predicted				Post-BD FVC %predicted			
Within-subject	**IQR (ppb)**	**N**	**Change**	**95%CI**		**N**	**Change**	**95%CI**	
O_3 same day	13	5199	0.046	-0.198	0.291	4036	0.040	-0.205	0.285
O_3 1-day lag	13	5199	-0.007	-0.258	0.243	4036	-0.034	-0.289	0.221
O_3 1-week m.a	9	5197	0.061	-0.237	0.358	4035	-0.002	-0.302	0.299

8c.			PC20		
Within-subject	**IQR (ppb)**	**N**	**%Change**	**95%CI**	
O_3 same day	13	1067	0.959	0.887	1.036
O_3 1-day lag	13	1067	0.997	0.921	1.080
O_3 1-week m.a	9	1067	0.965	0.878	1.061

O_3: ozone (ppb); m.a: moving average; N: number of observations; IQR: Interquartile range, ppm: parts per million; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; PC20: Metacholine dose (mg/ml) at which 20% drop in forced expiratory volume in 1 second is achieved; BD: bronchodilator; CI: confidence interval

* P interaction<0.05

* 0.05<= P interaction<0.1

Table E9. Comparison of between one- and two-pollutant model analysis of associations of 4-month average pollution with asthma outcomes

		One-pollutant model		Two-pollutant model	
	4-month average	change per IQR	p value	change per IQR	p value
FEV_1 % predicted post BD	ozone	-0.49	0.18	-0.50	0.17
	nitrogen dioxide	-0.20	0.06	-0.23	0.04
FEV_1 % predicted post BD	carbon monoxide	-0.42	0.002	-0.40	0.004
	nitrogen dioxide	-0.20	0.06	-0.17	0.13
FEV_1 % predicted post BD	carbon monoxide	-0.42	0.002	-0.52	<0.001
	ozone	-0.49	0.18	-0.71	0.05
FVC % predicted post BD	ozone	-0.08	0.80	-0.14	0.66
	nitrogen dioxide	-0.24	0.01	-0.23	0.01
FVC % predicted post BD	carbon monoxide	-0.21	0.06	-0.28	0.02
	ozone	-0.08	0.80	-0.20	0.53
FVC % predicted post BD	carbon monoxide	-0.21	0.06	-0.21	0.09
	nitrogen dioxide	-0.24	0.01	-0.22	0.02
PC_{20} (%change)	ozone	3.65	0.66	2.73	0.74
	nitrogen dioxide	-0.85	0.71	-0.77	0.74
PC_{20} (%change)	carbon monoxide	-4.11	0.16	-3.01	0.23
	ozone	3.65	0.66	2.15	0.79
PC_{20} (%change)	carbon monoxide	-4.11	0.16	-3.01	0.26
	nitrogen dioxide	-0.85	0.71	-0.46	0.84

IQR: Interquartile range, ppm: parts per million; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; PC20: Metacholine dose (mg/ml) at which 20% drop in forced expiratory volume in 1 second is achieved; BD: bronchodilator; CI: confidence interval

SUPPLEMENTARY MATERIAL - CHAPTER 6

MATERIALS AND METHODS

The Childhood Asthma Management Program (CAMP): is a multi-center, double-blinded clinical trial involving eight clinical centers in North America (Albuquerque, New Mexico; Baltimore, Maryland; Boston, Massachusetts; Denver, Colorado; San Diego, California; Seattle, Washington; St. Louis, Missouri; and Toronto-Ontario, Canada). Its main goal was to evaluate the long-term effectiveness and safety of daily inhaled anti-inflammatory medication in children diagnosed with asthma. Details of the CAMP study design and methods have been described elsewhere.[E1] In summary, children enrolled in CAMP were 5–12 years of age at the initial interview, and completed a 5-16 week pre-randomization screening period (1993-1995). All participants had mild-to-moderate asthma with increased airway responsiveness to methacholine (a provocation concentration causing a 20% reduction in FEV_1 [PC_{20}] ≤ 12.5 mg/ml) at study entry. 1,041 Children entered the randomization phase and 311, 312, 418 children received budesonide, nedocromil, and placebo, respectively. All subjects were treated and followed for four years with follow-up visits at CAMP centers at two and four months after randomization and at four-month intervals thereafter. Each parent or guardian signed a consent form and participants of 7 years of age and older signed an assent form approved by each clinical center's institutional review board.

Outcomes Measures

Spirometry, before and after the administration of two puffs of albuterol, was conducted at randomization (RZ) and at follow up visits (n=13) according to the American Thoracic Society Standards.[E2] The focus of the trial on long-term effects of the treatments on lung function was reflected in the choice of post-bronchodilator FEV_1 percentage of predicted (post-BD FEV_1% predicted) as the primary outcome measure.[E3] Use of post-BD FEV_1 rather than the pre-BD measure minimized fluctuations caused by diurnal variation and day-to-day variability of airway tone.

Air Pollution Exposure Assessment

Data on concentrations of 2 gaseous pollutants (carbon monoxide and nitrogen dioxide,) were obtained from the Aerometric Information Retrieval System (www.epa.gov/air/data/index.html) for US cities and from the Ontario

Ministry of the Environment (www.ene.gov.on.ca/environment; Air Quality Assessment and Reporting Unit) for Toronto. For each metropolitan area, multiple monitors were used to measure ambient pollution concentrations. Monitor-specific concentrations were 24-hour averages for carbon monoxide and nitrogen dioxide. In most cases, for US participants we were able to link ZIP codes of their address at study entry to US Census 2000 ZIP Code Tabulation Areas to identify the latitudes and longitudes of the centroids of those areas. In a few cases ZIP Code centroid coordinates were obtained from www.zip-codes.com. Coordinates for Canadian postal codes were obtained from www.batchgeo.com. The ZIP or postal code centroid coordinates were used to link participants to daily concentrations from the nearest monitor within 50 km that did not have missing data on that day (December 1993 through June 1999). If none of the monitors within 50 km radius was operating the air pollution concentration of that day was set as missing.

Exclusion criteria

Participants were excluded if we were unable to assign their ZIP or postal codes to latitudes and longitudes or if we verified that the centroid of the ZIP or postal code in which they lived was greater than 50 km from the nearest pollutant monitor. For the Canadian participants, a few monitors northwest of Toronto became operational after 2000 and subjects living in postal codes near those monitors were excluded. In total 38 subjects of the 1041 trial participants (3.7%) were excluded.

Genotyping and Quality Control

Genome-wide single nucleotide polymorphisms (SNP) genotyping for CAMP subjects was performed on Illumina's HumanHap550 Genotyping BeadChip (Illumina, Inc., San Diego, CA). CAMP samples and markers passed stringent quality-control standards; 6,257 markers were removed as a result of low clustering scores. Markers whose flanking sequences did not map to a unique position on the HG17 reference genome sequence were removed (n = 1,329). Further quality control was performed with PLINK (version 1.07; 2009-09-10). The average completion rate for each marker was more than 99%. Monomorphic markers (n = 3,790) and those with five or more Mendel errors (n = 2,445) were removed. The average genotyping completion rate for each subject was 99.75%

The practical application of the conditional two-step approach is to be used as a LMM surrogate, hence we performed the genome-wide scan for hundreds of thousands SNPs in a fast manner, combining high accuracy and the shortest central processing unit (*CPU*) time.[E4] In the first step, we estimated the subject-specific slope (with at least two available observations) from a LMM with a subject-specific (random) intercept and slope of pollutant exposure, adjusting for potential confounders. In this step, all SNP terms (main effect and interaction with pollutant) are omitted from the model. In the second step, the estimates of the random slopes of pollution exposure computed by the LMM were used as the outcome in a GWAS. Using PLNIK software (version 1.07; 2009-09-10; Shaun Purcell; http://pngu.mgh.harvard.edu/purcell/plink/),[E5] with simple linear regression we fitted the subject-specific pollutant slopes to SNPs. Thus the beta coefficient and *P*-value of each SNP represent the effect and significance of the interaction of the SNP(s) with the pollutant, respectively.

Step 1: Linear Mixed Models

We examined the relation between the level of post-BD FEV_1 %predicted and ZIP-code level 4-month moving average concentrations of carbon monoxide and nitrogen dioxide. The moving averages were created by averaging the daily pollution concentrations for the 4-month intervals between the clinic visits for lung function measurement. For any given averaging period, we required that 75% of the daily pollution data were available.

We fitted a LMM, with random intercepts and random slope for pollutant for each subject, to estimate the longitudinal relation between FEV_1 and ambient air pollutant concentrations. The number of days from randomization was used as the time trend in the model. To estimate associations across all cities, we constructed a model including city as a covariate. Potential for confounding by a number of factors was considered carefully, basing choice of covariates on prior CAMP experience[E6]: we controlled for gender, ethnicity, *in utero* smoking exposure, annual family income (dichotomized at $15K per annum), treatment group and treatment by time interaction. Confounding due to seasonal factors is a primary consideration in air pollution epidemiology, and we adjusted for "season" by using sine and cosine functions of time[E7] and their interactions with city. In addition, we decomposed daily ambient pollution concentrations into between- and within-subject exposures. Specifically, subject *i*'s exposure to pollutant x_1 on day *t* was decomposed as follows: The between-subject component, is subject *i*'s average exposure during the time in which he or she was observed, and the within-

subject component, is the deviation from the average on day *t*. Within-subject effects are likely to be far less confounded by season or by covariates that vary primarily across individual (e.g. in utero smoking exposure, etc) than between-subject effects,[E8] and they are more comparable to the parameters estimated in a traditional panel study, where all subjects are observed over a single time period. For further discussion regarding the decomposition of time-varying exposures into between- and within-subject components, see the papers by Sheppard[E8] and Neuhaus and Kalbfleisch.[E9] We used estimates of within-subject exposure effects.

We used SAS® software (version 9.2; SAS Institute Inc. 2008, Cary, NC USA) and IBM SPSS statistics (version 20; Armonk, NY USA: IBM Corp 2011) to manage all data. Statistical analysis was performed in IBM SPSS and R programming language (version 2.15.1; 2012-06-22).

Pathway-level analysis for the genome-wide SNP by pollutant interaction analysis

With the *i-GSEA4GWAS* method[E10] the maximum –log (*P*-value) of all SNPs mapped to (and around) a gene is used to represent the gene. Then, all genome-wide represented genes are ranked by decreasing values and their distribution is compared to the distribution of genes sharing a biochemical or cellular function. Kolmogorov-Smirnov like statistics gives to each gene set an enrichment score that reflects the trend that genes of a gene set tend to be located at the top of the entire genome-wide gene list. Then, *i-GSEA4GWAS* performs SNP-label permutations to assess the significance of the pathway-based enrichment score and to give the false discovery rate (FDR) for multiple testing correction. Pathways/gene-sets with FDR < 0.25 were regarded as mild confidence that the genes interacting with pollutants were enriched in a pathway/gene-set; FDR < 0.05 were regarded as high confidence that the genes modifying pollution effects were enriched in a gene-set/ pathway. *i-GSEA4GWAS* focuses on the pathways or gene-sets with the highest proportions of significant genes instead of relying solely on the total significance generated from either a few or many significant genes, thus improving the sensitivity to identify pathways/gene sets that represent the combined effects of all possibly modest SNPs/genes.[E10]

Discussion of the pathway analysis of NO2 effect modification by gene variants

The lung is rich in nitric oxide synthases (iNOS), and NO is normally present in the exhaled air (FeNO). Nitrogen dioxide is a pollutant tightly linked with

increased exhaled nitric oxide in children with asthma and is considered as a pro-inflammatory mediator associated with airway inflammation and reduced lung function.[E11,E12] Nitrogen dioxide has long been found to injure the airway epithelial lining, leading to decrements in barrier and active ion transport properties,[E13-E15]and findings indicate a role for the iNOS/NO pathway in nitrogen dioxide-mediated cytotoxicity characterized by interruption of tight junctions/adhesion molecules[E14,E16,E17] and increased cytokine and ICAM-1 production with influx of inflammatory cells[E16,E18]and late phase apoptosis.[E17] Oxidative stress, through production of reactive oxygen species, triggers disturbance in intracellular calcium homeostasis, which has been identified as an important factor in the tight junction defects and the pathogenesis of inflammatory diseases.[E19-E21]

In turn, interluekin-10 is an anti-inflammatory cytokine induced by nitrogen dioxide exposure[E18] found to alleviate the mucosal injury[E22] probably via p38/MAPK activation of the heme oxygenase-1 (HO-1) pathway.[E17,E23,E24] HO-1 catalyzes the oxidation of heme to carbon monoxide (CO) and biliverdin, which play a concerted action in cytoprotection against oxidative stress and in the modulation of cell proliferation and differentiation dependent on the modulation of the p38/MAPK-signaling pathway.[E25] Ryter, Alam and Choi extensively review the HO-1/CO (heme oxygenase/carbon monoxide) system and reported that among other, glucose and lipids metabolism, agents promoting ROS generation, Angiotensine II, hypoxia, hyperthermia, heat shock proteins and a number of cytokines including IL10 induce HO-1/CO production for protection in oxidative tissue injury such as lung injury models.[E25,E26] The HO-1 pathway has also been found to interact with the iNOS pathway in the control of cellular homeostasis.[E27]

REFERENCES

E1. Anonymous. The Childhood Asthma Management Program (CAMP): design, rationale, and methods. Childhood Asthma Management Program Research Group. Control Clin Trials 1999 Feb;20:91-120.

E2. Standardization of Spirometry, 1994 Update. American Thoracic Society. Am J Respir Crit Care Med 1995 Sep;152:1107-1136.

E3. Strunk RC, Childhood Asthma Management Program Research Group. Childhood Asthma Management Program: lessons learned. J Allergy Clin Immunol 2007 Jan;119:36-42.

E4. Sikorska K, Rivadeneira F, Groenen PJ, Hofman A, Uitterlinden AG, Eilers PH, Lesaffre E. Fast linear mixed model computations for genome-wide association studies with longitudinal data. Stat Med 2013 Jan 15;32:165-180.

E5. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007 Sep;81:559-575.

E6. Anonymous. Long-term effects of budesonide or nedocromil in children with asthma. The Childhood Asthma Management Program Research Group. N Engl J Med 2000 Oct 12;343:1054-1063.

E7. Stolwijk AM, Straatman H, Zielhuis GA. Studying seasonality by using sine and cosine functions in regression analysis. J Epidemiol Community Health 1999 Apr;53:235-238.

E8. Sheppard L. Insights on bias and information in group-level studies. Biostatistics 2003 Apr;4:265-278.

E9. Neuhaus JM, Kalbfleisch JD. Between- and within-cluster covariate effects in the analysis of clustered data. Biometrics 1998 Jun;54:638-645.

E10. Zhang K, Cui S, Chang S, Zhang L, Wang J. i-GSEA4GWAS: a web server for identification of pathways/gene sets associated with traits by applying an improved gene set enrichment analysis to genome-wide association study. Nucleic Acids Res 2010 Jul;38:W90-5.

E11. Holguin F, Flores S, Ross Z, Cortez M, Molina M, Molina L, Rincon C, Jerrett M, Berhane K, Granados A, Romieu I. Traffic-related exposures, airway function, inflammation, and respiratory symptoms in children. Am J Respir Crit Care Med 2007 Dec 15;176:1236-1242.

E12. Flamant-Hulin M, Caillaud D, Sacco P, Penard-Morand C, Annesi-Maesano I. Air pollution and increased levels of fractional exhaled nitric oxide in children with no history of airway damage. J Toxicol Environ Health A 2010;73:272-283.

E13. Persinger RL, Poynter ME, Ckless K, Janssen-Heininger YM. Molecular mechanisms of nitrogen dioxide induced epithelial injury in the lung. Mol Cell Biochem 2002 May-Jun;234-235:71-80.

E14. Robison TW, Kim KJ. Dual effect of nitrogen dioxide on barrier properties of guinea pig tracheobronchial epithelial monolayers cultured in an air interface. J Toxicol Environ Health 1995 Jan;44:57-71.

E15. Gordon RE, Solano D, Kleinerman J. Tight junction alterations of respiratory epithelium following long-term NO2 exposure and recovery. Exp Lung Res 1986;11:179-193.

E16. Ayyagari VN, Januszkiewicz A, Nath J. Pro-inflammatory responses of human bronchial epithelial cells to acute nitrogen dioxide exposure. Toxicology 2004 Apr 15;197:149-164.

E17. Ayyagari VN, Januszkiewicz A, Nath J. Effects of nitrogen dioxide on the expression of intercellular adhesion molecule-1, neutrophil adhesion, and cytotoxicity: studies in human bronchial epithelial cells. Inhal Toxicol 2007 Feb;19:181-194.

E18. Pathmanathan S, Krishna MT, Blomberg A, Helleday R, Kelly FJ, Sandstrom T, Holgate ST,

Wilson SJ, Frew AJ. Repeated daily exposure to 2 ppm nitrogen dioxide upregulates the expression of IL-5, IL-10, IL-13, and ICAM-1 in the bronchial epithelium of healthy human airways. Occup Environ Med 2003 Nov;60:892-896.

E19. Xu R, Li Q, Zhou XD, Perelman JM, Kolosov VP. Oxidative Stress Mediates the Disruption of Airway Epithelial Tight Junctions through a TRPM2-PLCgamma1-PKCalpha Signaling Pathway. Int J Mol Sci 2013 Apr 29;14:9475-9486.

E20. Simet SM, Wyatt TA, DeVasure J, Yanov D, Allen-Gipson D, Sisson JH. Alcohol increases the permeability of airway epithelial tight junctions in Beas-2B and NHBE cells. Alcohol Clin Exp Res 2012 Mar;36:432-442.

E21. Jiang LH, Yang W, Zou J, Beech DJ. TRPM2 channel properties, functions and therapeutic potentials. Expert Opin Ther Targets 2010 Sep;14:973-988.

E22. Emami CN, Chokshi N, Wang J, Hunter C, Guner Y, Goth K, Wang L, Grishin A, Ford HR. Role of interleukin-10 in the pathogenesis of necrotizing enterocolitis. Am J Surg 2012 Apr;203:428-435.

E23. Drechsler Y, Dolganiuc A, Norkina O, Romics L, Li W, Kodys K, Bach FH, Mandrekar P, Szabo G. Heme oxygenase-1 mediates the anti-inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 2006 Aug 15;177:2592-2600.

E24. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 2002 Mar;8:240-246.

E25. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev 2006 Apr;86:583-650.

E26. Ryter SW, Otterbein LE, Morse D, Choi AM. Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. Mol Cell Biochem 2002 May-Jun;234-235:249-263.

E27. Foresti R, Motterlini R. The heme oxygenase pathway and its interaction with nitric oxide in the control of cellular homeostasis. Free Radic Res 1999 Dec;31:459-475.

Table E1. Interquartile ranges (25th to 75th percentile) of pollution concentrations

IQR 4-month average	Overall	Within-subject
Carbon Monoxide (10 x ppm)	5	3
Nitrogen Dioxide (ppb)	8	4

IQR: interquartile range, ppb: part per billion; ppm: parts per million

Table E2: Top signals of genome-wide SNP by carbon monoxide interactions and post-BD FEV_1 %predicted in Caucasian CAMP subjects

Carbon monoxide by SNP_minor allele	2-Step approach P value	LMM P value	LMM Change per IQR[5]
rs13121814_G	1.28E-05	7.23E-07	-0.92
rs6569651_C	9.23E-06	9.69E-07	0.88
rs883398_A	2.04E-06	2.11E-06	-0.98
rs2237113_A	2.66E-05	2.22E-06	0.83
rs5742743_A	1.19E-05	3.76E-06	1.22
rs6545694_G	1.01E-05	4.17E-06	0.83
rs1861415_A	8.88E-06	4.41E-06	0.82
rs1770011_G	1.42E-05	4.48E-06	1.00
rs6446330_G	5.50E-05	4.70E-06	-0.92
rs1507559_T	1.37E-05	5.13E-06	0.85
rs4915866_G	5.63E-06	5.16E-06	-0.94
rs12130070_A	1.64E-05	5.20E-06	1.00
rs2878079_G	1.64E-05	5.20E-06	1.00
rs9388766_T	4.07E-05	7.50E-06	0.83
rs6899976_G	5.41E-05	1.06E-05	0.81
rs6679072_G	2.84E-05	1.10E-05	1.09
rs2403547_A	5.87E-06	1.17E-05	0.79
rs1986254_G	7.65E-06	1.26E-05	0.83
rs9317963_A	2.51E-05	1.33E-05	-0.76
rs2041813_A	9.78E-05	1.46E-05	-0.87
rs2518023_T	3.86E-05	1.46E-05	-1.36
rs9388769_G	9.41E-05	1.48E-05	0.79
rs1476162_G	5.24E-05	1.52E-05	-1.33
rs10174379_T	9.71E-05	1.55E-05	-1.07
rs576874_C	5.47E-05	1.71E-05	-0.80
rs2720625_A	4.21E-05	2.72E-05	0.84
rs11078734_T	6.69E-05	2.86E-05	-1.21

rs1605461_G	6.97E-05	3.46E-05	0.75
rs2039056_T	6.49E-05	3.63E-05	-0.73
rs4388690_C	5.95E-05	3.85E-05	0.77
rs9811139_C	7.17E-05	4.05E-05	1.09
rs13413446_C	9.02E-05	4.28E-05	1.00
rs10204566_G	2.56E-05	4.65E-05	0.74
rs3848726_T	6.09E-05	4.77E-05	-0.73
rs11904039_G	6.17E-05	4.80E-05	0.90
rs7519097_A	4.92E-05	5.00E-05	-0.91
rs4677169_A	9.96E-05	5.27E-05	0.83
rs799974_C	4.18E-05	6.38E-05	0.78
rs1470148_A	6.98E-05	6.47E-05	-1.09
rs11126981_A	8.74E-05	7.52E-05	0.76
rs6759519_A	8.06E-05	9.18E-05	0.72
rs6660832_A	8.93E-05	9.92E-05	-0.78
rs2194654_A	8.85E-05	1.21E-04	0.73
rs7600221_A	6.29E-05	1.42E-04	0.69
rs10241629_G	6.59E-05	2.00E-04	0.70
rs10491310_C	7.83E-05	4.66E-04	-0.94
rs11621122_T	8.50E-05	2.57E-03	0.72

SNP: single nucleotide polymorphisms, LMM: linear mixed model FEV_1= b0 + b1 between-subject pollution + b2 within-subject pollution + b3 SNP(additive) + b4 SNP x pollutant + b5 covariates + e | subject-specific (random) intercept, IQR: interquartile range of 4-month average carbon monoxide concentration (0.3 parts per million)
[s]direction of association same as in 2-step approach

Table E3: Top signals of genome-wide SNP by nitrogen dioxide interactions and post-BD FEV_1 %predicted in Caucasian CAMP subjects

Nitrogen dioxide by SNP_minor allele	2-Step P values	LMM P value	LMM Change per IQR[s]
rs13090972_T	1.37E-06	1.33E-08	-1.33
rs958144_T	4.81E-06	2.94E-08	-1.28
rs7041938_G	7.35E-06	1.04E-07	1.14
rs4571247_G	6.31E-06	1.08E-07	-1.25
rs1875137_G	1.85E-05	1.13E-07	0.91
rs11656155_T	6.35E-06	1.66E-07	0.91
rs4704701_G	4.43E-05	2.29E-07	1.02
rs4378142_A	2.35E-05	2.82E-07	0.70
rs12684188_T	3.01E-05	3.88E-07	1.20
rs1205047_A	8.08E-05	3.98E-07	-0.97
rs11159689_T	2.67E-05	7.33E-07	0.84

rs6733550_G	4.14E-05	1.09E-06	0.81
rs10782467_C	3.73E-05	1.17E-06	0.82
rs1510646_T	6.57E-05	1.23E-06	1.19
rs2621949_A	3.61E-05	2.52E-06	1.49
rs711173_C	1.97E-05	2.63E-06	0.79
rs4875101_A	7.17E-05	3.08E-06	-0.79
rs12566794_G	5.98E-05	3.38E-06	-0.72
rs4896716_T	5.62E-05	4.05E-06	1.05
rs8039508_T	8.58E-05	5.01E-06	0.71
rs3769767_A	5.19E-05	5.38E-06	0.91
rs17780310_T	9.73E-05	5.72E-06	-0.73
rs1844723_G	4.71E-05	5.74E-06	-0.85
rs2142301_T	6.64E-05	5.97E-06	-0.73
rs2490591_G	9.10E-05	6.95E-06	0.84
rs7356801_G	9.90E-05	7.02E-06	1.00
rs7642606_T	4.81E-05	8.20E-06	-1.16
rs939590_A	8.45E-05	8.46E-06	-0.78
rs13157529_T	5.54E-06	8.46E-06	-1.08
rs10984545_A	5.17E-05	8.73E-06	-1.20
rs12486049_G	6.45E-05	1.39E-05	-1.13
rs12119540_C	6.17E-05	1.42E-05	0.73
rs10512256_G	9.91E-05	1.52E-05	-1.16
rs1059003_G	9.91E-05	1.52E-05	-1.16
rs10984516_T	9.91E-05	1.52E-05	-1.16
rs4742708_A	9.91E-05	1.52E-05	-1.16
rs10058249_A	1.26E-05	1.57E-05	-1.05
rs3793490_G	9.44E-05	1.64E-05	1.12
rs9323351_G	6.01E-05	1.65E-05	-1.03
rs2157401_C	9.53E-05	1.84E-05	0.53
rs4672884_A	6.20E-05	1.98E-05	0.68
rs897877_A	7.85E-05	2.42E-05	0.67
rs6009565_A	9.42E-05	3.43E-05	0.99
rs831208_T	7.34E-05	6.78E-05	-0.61
rs9528794_C	6.24E-05	7.62E-05	0.81
rs12943384_G	8.62E-05	9.50E-05	-0.80
rs9988260_T	8.38E-05	2.87E-04	-0.83
rs10485399_T	9.69E-05	6.59E-04	-1.03

SNP: single nucleotide polymorphisms, LMM: linear mixed model: FEV_i=b0 + b1 between-subject pollution + b2 within-subject pollution + b3 SNP(additive) + b4 SNP x pollutant + b5 covariates + e | subject-specific (random) intercept
, IQR: interquartile range of 4-month average nitrogen dioxide concentration (0.004 parts per million)
[5]direction of association same as in the 2-step approach

Table E4: Replicated single nucleotide polymorphisms by pollutant interactions in two ethnicities

					LMM approach		2-STEP approach		
Interacting pollutant	**RACE**	**SNP**	**Minor allele**	**CHR**	**CHANGE PER IQR**	**P-value**	**BETA**	**P-value**	**Mapped gene/nearby gene**
Carbon monoxide	AA	rs2720625	A	8	1.24	0.014	0.57	0.042	58kb 3'-UTR of Magnesium Uptake/Transporter (Tumor Suppressor Candidate 3 -TUSC3) gene
	CAUC				0.84	2.72E-05	0.48	4.21E-05	
Combined P-value*						5.6E-06		1.21E-05	
Nitrogen dioxide	AA	rs4672884	A	2	1.12	0.007	57.68	0.108	Paroxysmal nonkinesigenic dyskinesia (PNKD) or Myofibrillogenesis regulator 1(MR-1) gene
	CAUC				0.68	1.98E-05	42.48	6.20E-05	
Combined P-value*						3.3E-06		2.5E-05	
Nitrogen dioxide	AA	rs3769767	A	2	1.19	0.036	77.99	0.105	Methyltransferase like 5 (METTL5) gene
	CAUC				0.91	5.38E-06	52.69	5.19E-05	
Combined P-value*						1.4E-06		2.1E-05	
Nitrogen dioxide	AA	rs4378142	A	23	1.24	0.016	75.96	0.200	RP11-40F8.2 / ENSG00000233067
	CAUC				0.70	2.82E-07	61.19	2.345E-05	
Combined P-value*						4.8E-08		1.2E-05	

AA: African-American (n=88), CAUC: Caucasian (n=522) Childhood Asthma Management Program subjects; SNP: single nucleotide polymorphism, CHR: chromosome; LMM: linear mixed models; IQR: Interquartile range (carbon monoxide IQR= 0.3 and nitrogen dioxide IQR= 0.004 parts per million)
*MetaP: a program to combine P values (Stuffer's z trend);
Author: Dongliang Ge PhD, Duke University; URL: http://compute1.lsrc.duke.edu/softwares/MetaP/metap.php

Table E5: Pathway analysis top SNPs and effect modification of the long-term effects of carbon monoxide on post-BD FEV_1 %predicted in Caucasian CAMP subjects

Top SNPs _minor allele	Change per IQR[s]	P-value[s]
rs11130199_C	-0.55	**0.002**
rs17515291_G	-0.64	**0.001**
rs3768405_A	0.66	**0.001**
rs7035689_A	0.45	**0.022**

Caucasian Childhood Asthma Management Program subjects (n=522); [s] Linear mixed model: FEV_1=b0 + b1 between-subject pollution + b2 within-subject pollution + b3 SNP(additive) + b4 SNP x pollutant + b5 covariates + e | subject-specific (random) intercept
IQR: interquartile range of 4-month average carbon monoxide concentration = 0.3 parts per million

Table E6: Pathway analysis top SNPs and effect modification of the long-term effects of nitrogen dioxide on post-BD FEV_1 %predicted in Caucasian subjects

Top SNP_minor allele	Change per IQR[s]	P-value SNP by nitrogen dioxide[s]
rs1521481_C	0.78	2.32E-06
rs3755377_G	-0.74	3.61E-06
rs12119540_C	0.73	1.42E-05
rs7304453_T	0.71	1.64E-05
rs13219957_A	0.90	2.02E-05
rs13077495_C	-1.27	3.62E-05
rs10151613_C	0.67	4.86E-05
rs1800896_C	0.66	5.29E-05
rs11820322_T	1.77	1.13E-04
rs8_T	-0.73	1.16E-04
rs717099_A	-0.60	1.18E-04
rs4899406_T	-1.04	1.59E-04
rs3087474_T	-1.12	1.61E-04
rs17409602_C	0.67	1.67E-04
rs4952447_C	0.57	1.70E-04
rs1732664_T	0.62	2.15E-04
rs1200138_C	0.64	2.19E-04
rs6904263_T	-0.74	2.21E-04
rs12144820_A	-0.57	2.45E-04
rs7178_G	-1.01	2.46E-04
rs1868289_G	0.63	2.86E-04
rs1025298_C	-0.61	2.90E-04

rs1642763_A	0.66	3.10E-04
rs658132_G	0.66	3.97E-04
rs4899329_C	-0.57	4.41E-04
rs13164785_G	0.67	4.41E-04
rs1981429_G	-0.58	4.54E-04
rs199933_A	-0.65	4.57E-04
rs8102695_A	-0.68	4.88E-04
rs1805352_C	0.59	5.07E-04
rs3774463_T	1.12	5.16E-04
rs478839_G	-0.58	5.30E-04
rs6956460_G	1.05	5.43E-04
rs2581_T	0.54	5.43E-04
rs2025818_T	-0.54	5.79E-04
rs9300729_C	-0.82	5.81E-04
rs1131857_G	0.76	6.42E-04
rs4678009_T	0.60	7.00E-04
rs12695902_G	0.88	7.15E-04
rs3830180_G	0.59	7.91E-04
rs181997_A	-0.57	9.48E-04
rs13229270_A	-0.53	1.01E-03
rs8036393_T	-0.51	1.03E-03
rs2850971_T	-0.58	1.18E-03
rs165979_G	-0.52	1.20E-03
rs2849380_T	-0.63	1.22E-03
rs3790112_T	0.55	1.30E-03
rs17676564_A	-1.01	1.31E-03
rs4254322_G	0.59	1.40E-03
rs629079_C	0.57	1.46E-03
rs4724420_T	-0.52	1.47E-03
rs2236561_G	0.52	1.49E-03
rs797999_T	-0.59	1.51E-03
rs12026958_C	-0.84	1.61E-03
rs11198973_G	0.52	1.63E-03
rs1485237_C	-0.52	1.64E-03
rs9842138_G	0.68	1.68E-03
rs2443502_G	-0.48	1.71E-03
rs10259426_C	-0.65	1.72E-03
rs17041884_T	-0.57	1.80E-03
rs10515521_A	0.65	1.83E-03
rs1489024_G	-0.51	1.98E-03

rs4783446_G	-0.99	2.06E-03
rs2835370_C	0.84	2.10E-03
rs733299_T	-0.52	2.15E-03
rs2281390_T	-0.59	2.17E-03
rs3767248_G	0.48	2.21E-03
rs454006_C	0.60	2.25E-03
rs13422838_C	-0.83	2.27E-03
rs12086956_G	-1.55	2.30E-03
rs12423712_A	0.95	2.52E-03
rs3806206_T	0.74	2.77E-03
rs12589478_A	-0.53	2.84E-03
rs11258248_A	-0.90	2.84E-03
rs7306482_A	-0.48	2.91E-03
rs173681_T	-0.59	2.94E-03
rs16916456_T	0.92	2.97E-03
rs2575710_C	0.46	3.03E-03
rs2292719_A	-0.53	3.13E-03
rs4405588_C	0.46	3.22E-03
rs2531992_A	0.58	3.30E-03
rs6743452_C	-0.46	3.32E-03
rs10066754_T	-0.77	3.32E-03
rs3897583_C	0.48	3.32E-03
rs7623208_T	-0.82	3.39E-03
rs6797758_G	-0.59	3.41E-03
rs7255066_C	0.52	3.55E-03
rs12626592_A	-0.98	3.64E-03
rs744143_A	0.72	3.68E-03
rs3783834_A	-0.49	3.69E-03
rs17172446_A	0.56	3.69E-03
rs3785905_A	-0.73	3.70E-03
rs1819741_C	-0.56	3.86E-03
rs10423648_G	0.90	3.95E-03
rs11122574_T	-1.05	3.98E-03
rs10042299_G	-0.44	4.01E-03
rs12471357_T	0.45	4.12E-03
rs11216689_A	0.98	4.14E-03
rs558190_T	0.52	4.14E-03
rs2104977_A	0.60	4.17E-03
rs3766074_A	0.70	4.19E-03
rs4670185_G	0.88	4.41E-03

rs7623610_A	-0.44	4.51E-03
rs4339559_T	-0.68	4.61E-03
rs2859228_G	-0.47	4.67E-03
rs252649_C	-0.91	4.79E-03
rs260849_G	-0.56	4.83E-03
rs944260_G	-0.52	4.83E-03
rs11666282_T	0.51	4.93E-03
rs2049050_T	-0.48	4.97E-03
rs730005_C	-0.57	5.07E-03
rs16909222_G	-0.79	5.13E-03
rs7515374_C	0.46	5.21E-03
rs17407577_C	-1.04	5.34E-03
rs869789_A	-0.64	5.43E-03
rs2075067_A	-0.74	5.45E-03
rs237484_A	0.44	5.50E-03
rs16923198_A	-0.56	5.73E-03
rs4665716_G	0.87	5.85E-03
rs8068961_C	-0.46	5.88E-03
rs11927393_G	-0.77	5.97E-03
rs982804_C	-0.45	6.06E-03
rs2517912_T	0.45	6.06E-03
rs9904113_A	0.46	6.25E-03
rs352165_A	0.45	6.34E-03
rs6891913_T	-0.47	6.36E-03
rs10487841_C	0.92	6.53E-03
rs16875961_G	-0.66	6.57E-03
rs2876981_C	0.43	6.67E-03
rs6425689_C	-0.47	6.76E-03
rs6885505_G	0.46	6.90E-03
rs3773729_C	0.43	6.98E-03
rs6709175_C	0.52	7.01E-03
rs2393374_T	-0.46	7.22E-03
rs7886134_C	-0.35	7.52E-03
rs1033908_C	0.53	7.66E-03
rs7298903_C	0.61	7.84E-03
rs6804441_G	0.54	7.95E-03
rs1886700_T	0.59	8.12E-03
rs893952_T	0.45	8.27E-03
rs3760055_A	-0.48	8.34E-03
rs9526475_T	-0.47	8.35E-03

rs4072067_G	-0.54	8.42E-03
rs4316697_A	0.44	8.74E-03
rs2007044_G	-0.44	8.75E-03
rs1009897_A	0.43	8.86E-03
rs6092704_C	0.70	9.07E-03
rs999297_A	-0.47	9.13E-03
rs10025791_C	0.42	9.71E-03
rs10032688_A	0.60	9.77E-03
rs1956918_A	0.41	9.81E-03
rs10413199_A	0.55	9.86E-03
rs11636875_G	-0.42	1.01E-02
rs17208112_A	-0.45	1.02E-02
rs733018_G	-0.47	1.04E-02
rs651084_G	0.50	1.04E-02
rs10796316_A	2.63	1.04E-02
rs10799902_A	0.42	1.04E-02
rs583720_A	0.51	1.05E-02
rs6982126_T	0.44	1.05E-02
rs2475193_A	0.52	1.08E-02
rs1784304_A	-0.49	1.09E-02
rs2352865_C	0.50	1.10E-02
rs1619379_T	-0.43	1.10E-02
rs10491087_T	-0.72	1.11E-02
rs12125947_C	-0.41	1.12E-02
rs11247963_A	-0.50	1.13E-02
rs1367728_A	-0.54	1.14E-02
rs527834_A	0.80	1.17E-02
rs2289059_G	0.84	1.20E-02
rs367689_A	-0.74	1.20E-02
rs739999_G	-0.70	1.21E-02
rs12477594_A	1.18	1.29E-02
rs10820938_A	0.74	1.33E-02
rs263238_A	-0.53	1.34E-02
rs4698804_C	-2.14	1.34E-02
rs7066678_A	-0.34	1.41E-02
rs8091758_T	-1.03	1.42E-02
rs919699_A	-0.63	1.43E-02
rs12982646_A	-0.41	1.45E-02
rs932757_A	-0.37	1.47E-02
rs2271026_C	-0.89	1.48E-02

rs899480_G	-0.81	1.48E-02
rs477549_C	-0.61	1.48E-02
rs3767498_A	-0.41	1.49E-02
rs11861415_G	-0.41	1.50E-02
rs7111082_A	0.39	1.52E-02
rs578130_A	0.41	1.52E-02
rs715849_A	-0.41	1.52E-02
rs3002208_G	0.48	1.53E-02
rs1122356_G	-0.43	1.53E-02
rs2922666_T	-0.51	1.58E-02
rs13191198_G	0.38	1.62E-02
rs2524005_A	-0.50	1.69E-02
rs4855074_T	-0.52	1.75E-02
rs10496444_G	0.40	1.75E-02
rs2993577_G	1.79	1.84E-02
rs7167430_C	0.54	1.85E-02
rs742403_G	0.37	1.86E-02
rs9958503_A	-0.64	1.87E-02
rs10823343_G	-0.41	1.89E-02
rs745966_C	-0.61	1.92E-02
rs206187_A	-0.60	1.94E-02
rs1956908_A	0.38	1.97E-02
rs3170368_G	0.55	2.01E-02
rs1693892_G	0.39	2.01E-02
rs715643_T	-0.71	2.14E-02
rs2665742_G	0.36	2.16E-02
rs6429206_T	-0.37	2.24E-02
rs17126224_G	-0.80	2.29E-02
rs7207345_C	-0.39	2.29E-02
rs785520_C	-0.35	2.50E-02
rs197770_G	-0.56	2.59E-02
rs3730064_A	-0.62	2.60E-02
rs3746821_T	-0.57	2.67E-02
rs1014982_T	0.28	2.76E-02
rs1572057_T	0.47	2.86E-02
rs4925479_C	0.39	2.89E-02
rs2652423_T	-0.39	2.93E-02
rs7277902_T	0.54	2.94E-02
rs1398259_C	-0.35	2.96E-02
rs7550839_T	-0.46	2.96E-02

rs10489612_A	-0.54	3.03E-02
rs263955_T	-0.35	3.05E-02
rs9616802_T	-0.34	3.20E-02
rs900781_T	0.56	3.26E-02
rs7255050_A	-0.33	3.31E-02
rs2307373_T	-0.34	3.34E-02
rs7776857_G	0.34	3.38E-02
rs2273814_G	-0.34	3.51E-02
rs6790988_A	-0.35	3.66E-02
rs16833157_A	0.81	3.87E-02
rs2826877_T	-0.32	4.05E-02
rs5030390_A	-0.56	4.07E-02
rs7401414_A	0.47	4.09E-02
rs6949887_A	-0.72	4.64E-02
rs11179589_C	-0.55	4.69E-02
rs270_A	0.41	4.95E-02
rs2494738_A	0.59	5.34E-02
rs12820100_C	-0.32	5.43E-02
rs13323993_A	0.33	5.56E-02
rs1443237_A	-0.35	5.68E-02
rs679305_T	-0.56	6.20E-02
rs1449264_C	0.30	6.66E-02

[5] Linear mixed model: FEV_1=b0 + b1 between-subject pollution + b2 within-subject pollution + b3 SNP(additive) + b4 SNP x pollutant + b5 covariates + e | subject-specific (random) intercept
IQR: interquartile range of 4-month average nitrogen dioxide concentration = 0.004 parts per million

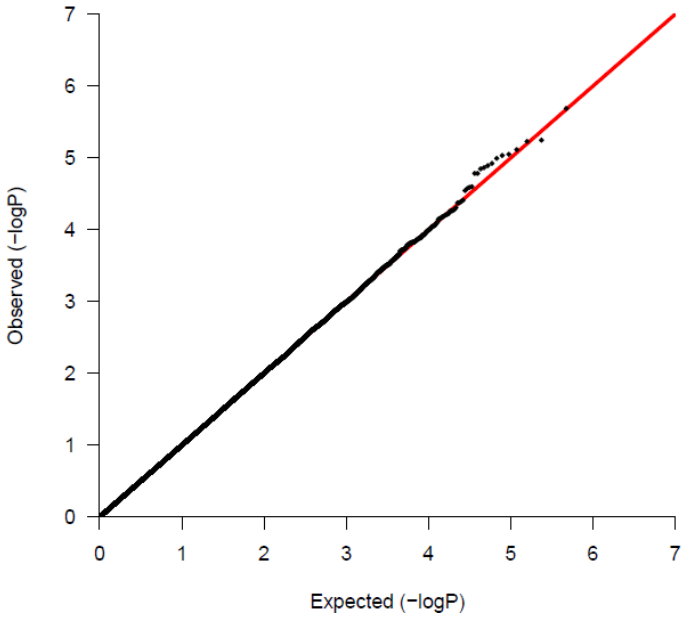


Figure E1: The quantile-quantile (QQ) plot of the genome-wide SNP by carbon monoxide interaction analysis in Caucasian CAMP subjects

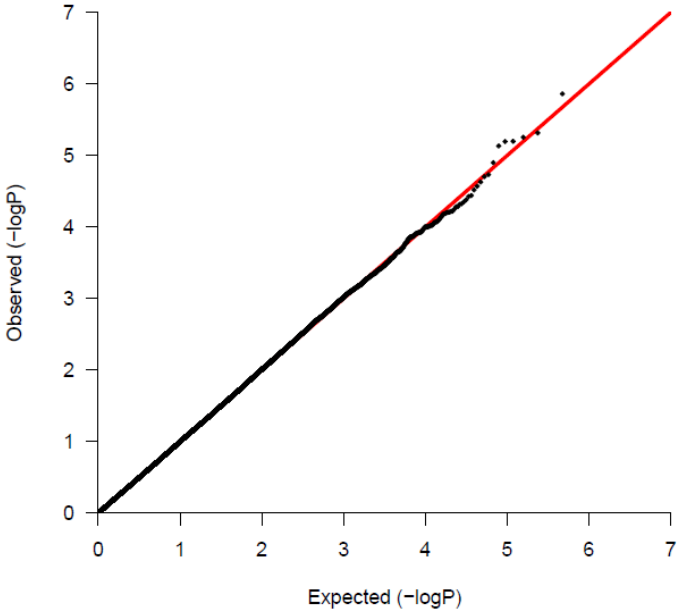


Figure E2: The quantile-quantile (QQ) plot of the genome-wide SNP by nitrogen dioxide interaction analysis in Caucasian CAMP subjects

Thesis Summary

Thesis summary

Asthma is a chronic inflammatory disorder of the airways that affects children and adults of all ages. The World Health Organisation has estimated that 300 million individuals have asthma worldwide, and that this will reach 400 million by 2025 with current rising trends. Patients with asthma typically experience recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. Asthma attacks are generally triggered by allergens, viral respiratory infections and airborne irritants such as tobacco smoke and air pollution. In asthma, the airways narrow in an exaggerated fashion after being exposed to a trigger (a phenomenon called airway hyperresponsiveness) and asthmatics indicate variable airflow obstruction which is often reversible either spontaneously or following treatment, while some patients outgrow their asthma over time. Inhaled corticosteroids constitute the cornerstone of regular asthma treatment. The clinical severity of asthma is associated with airway wall inflammation and structural changes named airway wall remodeling. Inhaled corticosteroids have been associated with suppression of inflammation and asthma symptoms.

Recent advances in understanding the pathophysiology of asthma development and severity have pointed towards a prominent role of the airway epithelium. A relative new hypothesis is that the airway epithelium in asthma is more susceptible to damage allowing greater access of environmental allergens, microorganisms, and toxicants to the airway tissue and/or has an altered response to injury by environmental stimuli, with a pro-inflammatory response of the epithelium contributing to local allergen sensitization, chronic and persistent inflammation and airway wall remodeling.

Nevertheless, not all asthmatics respond to environmental exposures in the same way and there is heterogeneity in clinical and pathological expression of disease among individuals, both in children and adults with asthma. The individual variation in asthma severity is influenced by genetic and environmental factors, and gene-environment interactions add to the complexity of the disease. Similarly, genetics play a role in the individual response to anti-inflammatory treatment.

Studies directed towards determining the mechanisms by which genetic and environmental factors and their interactions affect clinicopathological outcomes in asthma and whether such effects can be modified by anti-inflammatory treatment are needed. Although many studies have investigated the genetic and environmental determinants of asthma susceptibility and

severity, longitudinal genetic studies seeking associations with the natural course and asthma outcome are scarce. Similarly, the genetic background of airway pathology is not well documented.

In this doctoral thesis we investigated the role of genes encoding proteins involved in epithelial barrier integrity, chronic airway inflammation and airway wall remodeling in asthma and showed that genetic variation determines epithelial integrity, the extent of the airway wall inflammation and remodeling, as well as the subsequent clinical severity (i.e. airway hyperresponsiveness, lung function level and decline over time, asthma remission) of adult individuals with asthma. We showed that gene by inhaled corticosteroids and gene by smoking interactions also play a role in the clinical and pathological expression of the disease.

We further focused on the susceptibility of asthmatic children to ambient air pollution, an unavoidable environmental exposure of the modern world. We showed associations of long-term air pollution (carbon monoxide and nitrogen dioxide) exposure with severity of airflow obstruction and airway hyperresponsiveness in children with asthma. We did not find strong evidence of modification of pollution effects by controller medication used in a clinical trial. However, we showed that genetics play a role in the respiratory response of asthmatic children to air pollution by potentially regulating oxidant/anti-oxidant cellular mechanisms and inflammation.

With this thesis we aimed to understand better the underlying mechanisms of asthma severity and we added to the current knowledge of asthma pathophysiology. This may ultimately offer better targets for drug development for either prevention or cure of asthma. Collaboration between the different scientific disciplines using epidemiologic, genomic, proteomic and other molecular information is the key to a network-based approach to human disease that will help discover better and more accurate biomarkers, improve disease classification, and channel personalized therapies and treatment.

Samenvatting

Astma is een chronische inflammatoire aandoening van de luchtwegen, die kinderen en volwassenen van alle leeftijden treft. De WHO schat dat wereldwijd 300 miljoen mensen astma hebben en dat dit, met de huidige stijgende trend, zal oplopen tot 400 miljoen patiënten in 2025. Astmapatiënten ervaren meestal terugkerende aanvallen van piepen, kortademigheid, beklemming op de borst en hoesten, vooral 's nachts of in de vroege ochtend. Astma-aanvallen worden over het algemeen veroorzaakt door allergenen, virale infecties van de luchtwegen en luchtweg irriterende stoffen, zoals tabaksrook en luchtvervuiling. Bij astma vernauwen de luchtwegen zich na blootstelling aan een prikkel (een fenomeen dat luchtweg hyperreactiviteit wordt genoemd). Personen met astma geven aan dat de mate van benauwdheid varieert, en dat die spontaan of na een behandeling kan verdwijnen. Sommige patiënten groeien over hun astma heen. De klinische ernst van astma is geassocieerd met ontsteking van de luchtwegwand en met structurele veranderingen van de luchtwegwand, zogenaamde luchtwegwand-remodellering. Inhalatiecorticosteroïden vormen de basis van de behandeling van astma. De therapie met inhalatiecorticosteroïden leidt tot onderdrukking van ontsteking in de luchtwegen en vermindering van de astmasymptomen.

Recente inzichten in de pathofysiologie van de ontwikkeling en de ernst van astma laten zien dat luchtwegepitheel een belangrijke rol speelt. Met luchtwegepitheel bedoelen we de cellen, die de binnenkant van de luchtwegen bekleden. Een nieuwe hypothese is dat het luchtwegepitheel bij patiënten met astma gevoeliger is voor schade die ontstaat o.a. door het inhaleren van allergenen, micro-organismen en toxische stoffen. Deze schade leidt tot een verhoogde doorlaatbaarheid van het epitheel en een veranderde reactie op deze prikkels uit de omgeving. Hierbij stimuleert het epitheel inflammatie, wat op zich weer bijdraagt aan de lokale reactie op allergenen en chronische en blijvende ontstekingsprocessen en remodellering van de luchtwegwand.

Toch reageren niet alle astmapatiënten op dezelfde manier op de blootstelling aan omgevingsprikkels en bestaat er variatie in zowel de klinische als de pathologische uiting van de ziekte, zowel bij kinderen als bij volwassenen met astma. Deze variatie betreft de ernst van de ziekte wordt beïnvloed door genetische en omgevingsfactoren. En de interactie tussen deze genetische en omgevingsfactoren leidt tot een grote complexiteit en variatie van astma. Daarnaast spelen genetische factoren een rol bij de individuele reactie op de ontstekingsremmende behandeling met inhalatiesteroïden.

Om deze complexiteit te ontrafelen zijn studies nodig naar de mechanismen van genetische en omgevingsfactoren en hun interactie. Daarnaast moet

onderzocht worden welke kenmerken van astma beïnvloed kunnen worden door ontstekingsremmende behandeling. Hoewel veel studies tot nu toe de genetische en de omgevingsfactoren hebben onderzocht voor wat betreft de ontwikkeling en ernst van astma, zijn er nauwelijks studies die deze effecten over de tijd hebben uitgezocht en het natuurlijk beloop van astma hebben bestudeerd. Ook is de genetische achtergrond van de pathologische veranderingen in de luchtwegen niet goed gedocumenteerd. In dit proefschrift onderzochten wij de rol van genen die eiwitten coderen die betrokken zijn bij de integriteit van de epitheliale barrière van de luchtwegwand en de chronische luchtwegontsteking en remodellering van de luchtwegwand bij astma. Wij toonden aan dat genetische variatie mede de epitheliale integriteit bepaalt en ook de uitgebreidheid van de luchtwegwandontsteking en remodellering en daarop volgend de klinische ernst van astma (b.v. luchtweg hyperreactiviteit, het niveau van de longfunctie, de achteruitgang van de longfunctie in de tijd en astma remissie). Wij onderzochten dit bij volwassen personen met astma. We toonden daarnaast aan dat een interactie tussen bepaalde genen en inhalatiesteroïden, en tussen bepaalde genen en roken, een rol speelt bij de klinische en pathologische expressie van astma.

Naast het onderzoek bij volwassenen hebben we ons gericht op de gevoeligheid van kinderen met astma voor luchtverontreiniging, een onvermijdelijke omgevingsblootstelling in de huidige moderne wereld. We toonden aan dat er een verband bestaat tussen langdurige blootstelling aan luchtverontreiniging (koolmonoxide en stikstofdioxide) en de ernst van luchtwegobstructie en hyperreactiviteit bij kinderen met astma. We vonden geen sterk bewijs dat de ontstekingsremmende medicatie, die in deze trial werd onderzocht, de effecten van luchtverontreiniging kon veranderen. Daarentegen vonden we wel dat de genetische factoren een rol spelen bij de respons van kinderen met astma op luchtverontreiniging doordat deze genen mogelijk de oxidant/anti-oxidant mechanismen en ontsteking konden beïnvloeden.

Met dit proefschrift hebben we geprobeerd een beter inzicht te krijgen in de onderliggende mechanismen van de ernst van astma en hierbij hebben we nieuwe kennis toegevoegd aan de bestaande inzichten in de oorzakelijke mechanismen van astma. Dit onderzoek kan uiteindelijk bijdragen aan een betere ontwikkeling van geneesmiddelen voor preventie of genezing van astma. Samenwerking tussen verschillende wetenschappelijke disciplines zoals epidemiologische, klinische, genomische, proteomische en andere moleculaire informatie is de sleutel tot een geïntegreerde benadering van ziektes bij de mens. Deze samenwerking zal helpen om betere en nauwkeurigere biomarkers te ontdekken, en zal ook de classificatie van de diversiteit in astma helpen te verbeteren. Dit moet uiteindelijk leiden tot individueel aangepaste therapie en behandeling van astma.

Περίληψη

Το άσθμα είναι μια χρόνια φλεγμονώδης διαταραχή των αεραγωγών που επηρεάζει παιδιά και ενήλικες όλων των ηλικιών. Ο Παγκόσμιος Οργάνισμός Υγείας εκτιμά ότι τριακόσια εκατομμύρια άτομα παγκοσμίως έχουν άσθμα, αριθμός που υπολογίζεται μέχρι το 2025 να φτάσει με τις τρέχουσες αυξανόμενες τάσεις τα τετρακόσια εκατομμύρια. Ασθενείς με άσθμα συνήθως βιώνουν επαναλαμβανόμενα επεισόδια συριγμού και δύσπνοιας, νιώθουν ένα σφίξιμο στο στήθος και έχουν συχνά βήχα, ιδιαίτερα τη νύχτα ή νωρίς το πρωί. Οι κρίσεις άσθματος συνήθως προκαλούνται από αλλεργιογόνα, ιογενείς λοιμώξεις του αναπνευστικού συστήματος και αερομεταφερόμενες ερεθιστικές ουσίες όπως ο καπνός του τσιγάρου και η ρύπανση του αέρα. Στην ουσία πρόκειται για μία υπεραντίδραση των αεραγωγών στους διάφορους παράγοντες, με αποτέλεσμα οι μυϊκές ίνες να συσπώνται (βρογχόσπασμος) προκαλώντας στένωση του αυλού των αεραγωγών. Ως εκ τούτου, οι ασθματικοί παρουσιάζουν απόφραξη των αεραγωγών η οποία είναι συχνά αναστρέψιμη είτε αυθόρμητα ή μετά από θεραπεία, ενώ μερικοί ασθενείς μπορούν να απαλλαχτούν από το άσθμα με την πάροδο του χρόνου. Η κλινική βαρύτητα του άσθματος συνδέεται με φλεγμονή και ιστολογικές αλλοιώσεις /αναδιαμόρφωση του τοιχώματος των αεραγωγών (airway remodeling). Τα εισπνεόμενα κορτικοστεροειδή έχουν συσχετιστεί με την καταστολή της φλεγμονής και των συμπτωμάτων του άσθματος, και αποτελούν τον ακρογωνιαίο λίθο της τακτική θεραπείας της νόσου.

Πρόσφατες πρόοδοι στην κατανόηση της παθοφυσιολογίας της ανάπτυξης και της βαρύτητας του άσθματος έχουν επισημάνει έναν εξέχοντα ρόλο του επιθηλίου των αεραγωγών, το οποίο είναι η πρώτη γραμμή άμυνας σε ότι εισβάλλει μέσω του εισπνεόμενου αέρα. Μια σχετικά νέα υπόθεση είναι ότι το επιθήλιο της αναπνευστικής οδού των ασθενών με άσθμα είναι πιο επιρρεπές σε βλάβες, επιτρέποντας μεγαλύτερη πρόσβαση σε αλλεργιογόνα, μικροοργανισμούς και τοξικές ουσίες, και/ή έχει μια τροποποιημένη απόκριση στον τραυματισμό από περιβαλλοντικά ερεθίσματα που οδηγούν σε με μια προ-φλεγμονώδη απόκριση του επιθηλίου που συμβάλλει στην τοπική ευαισθητοποίηση σε αλλεργιογόνα, στην χρόνια και επίμονη φλεγμονή και αναδιαμόρφωση των τοίχωμάτων των αεραγωγών.

Παρ 'όλα αυτά, όλοι οι ασθματικοί δεν ανταποκρίνονται στα αερομεταφερόμενα ερεθίσματα κατά τον ίδιο τρόπο, με αποτέλεσμα να υπάρχει ετερογένεια στην κλινική και παθολογοανατομική έκφραση της νόσου μεταξύ των ατόμων με άσθμα, είτε πρόκειται για παιδιά όσο είτε για ενήλικες. Η διακύμανση στην βαρύτητα του άσθματος μεταξύ ασθενών επηρεάζεται

από γενετικούς και περιβαλλοντικούς παράγοντες, καθώς οι αλληλεπιδράσεις μεταξύ γονιδίων και περιβάλλοντος συμβάλλουν στην πολυπλοκότητα της νόσου. Παρομοίως, οι γενετικοί παράγοντες παίζουν βασικό ρόλο στην εξατομικευμένη ανταπόκριση στην αντι-φλεγμονώδη θεραπεία.

Αρκετές μελέτες κατευθύνονται προς τον προσδιορισμό των μηχανισμών με τους οποίους γενετικοί και περιβαλλοντικοί παράγοντες και οι αλληλεπιδράσεις τους επηρεάζουν τις κλινικο-παθολογοανατομικές εκδηλώσεις της νόσου και κατά πόσον αυτές μπορούν να τροποποιηθούν με τη χρήση αντι-φλεγμονώδους θεραπείας. Αν και υπάρχει πληθώρα μελετών που έχουν διερευνήσει τους γενετικούς και περιβαλλοντικούς παράγοντες οι οποίοι καθορίζουν την προδιάθεση και τη βαρύτητα του άσθματος, ωστόσο δεν συμβαίνει το ίδιο με τις μακροχρόνιες γενετικές μελέτες που αναζητούν τη συσχέτιση των γονιδίων με τη φυσική πορεία και την έκβαση του άσθματος. Αξίζει τέλος να σημειωθεί πως ούτε το γενετικό υπόβαθρο της ιστοπαθολογίας των αεραγωγών δεν είναι καλά τεκμηριωμένο.

Σε αυτήν τη διδακτορική διατριβή διερευνάται ο ρόλος των γονιδίων που κωδικοποιούν πρωτεΐνες που εμπλέκονται στην ακεραιότητα του επιθηλιακού φραγμού, τη χρόνια φλεγμονή και την αναδιαμόρφωση του τοιχώματος των αεραγωγών στο άσθμα. Καταδεικνύεται ότι οι γενετικοί πολυμορφισμοί καθορίζουν την επιθηλιακή ακεραιότητα, την έκταση της φλεγμονής και της αναδιαμόρφωση των αεραγωγών, καθώς και την μετέπειτα κλινική βαρύτητα, για παράδειγμα την υπεραντιδραστικότητα των αεραγωγών, την πνευμονική λειτουργία και την φυσική πορεία των ενήλικων ατόμων με άσθμα. Φαίνεται επίσης πως οι αλληλεπιδράσεις των γονιδίων με τα εισπνεόμενα κορτικοστεροειδή ή με το κάπνισμα επίσης διαδραματίζουν ρόλο στην κλινική και ιστολογική έκφραση της νόσου.

Περαιτέρω έμφαση δίδεται στην επιρρέπεια των ασθματικών παιδιών στη ρύπανση του ατμοσφαιρικού αέρα, μια αναπόφευκτη περιβαλλοντική έκθεση του σύγχρονου κόσμου. Διαπιστώνεται ότι μακροχρόνια υψηλά επίπεδα ατμοσφαιρικής ρύπανσης (με μονοξείδιο του άνθρακα και διοξειδίου του αζώτου) συσχετίζονται με τη βαρύτητα της υπεραντιδραστικότητας και απόφραξης των αεραγωγών σε παιδιά με άσθμα. Δεν αποδεικνύεται προστασία από την ρύπανση με τη φαρμακευτική αγωγή. Εντούτοις όμως έγινε σαφές πως το γονιδίωμα παίζει ρόλο στην αναπνευστική απόκριση των ασθματικών παιδιών στη ρύπανση του αέρα με γονίδια που δυνητικά ρυθμίζουν οξειδωτικούς/αντι-οξειδωτικούς κυτταρικούς μηχανισμούς και τη φλεγμονή.

Αυτή η διατριβή είχε στόχο την καλύτερη κατανόηση των υποκείμενων μηχανισμών της βαρύτητας του άσθματος και την ενίσχυση της τρέχουσα

γνώσης της παθοφυσιολογίας του άσθματος. Τα ευρήματά μας μπορούν να βοηθήσουν στις μελέτες για την δημιουργία φαρμάκων για την πρόληψη ή/και τη θεραπεία του άσθματος. Η συνεργασία μεταξύ των διαφόρων επιστημονικών κλάδων που χρησιμοποιούν επιδημιολογικές, γενετικές και άλλες μοριακές πληροφορίες είναι το κλειδί για μια πολύεδρική προσέγγιση της νόσου, η οποία θα συμβάλλει στη βελτίωση της κλινικο-παθολογοανατομικής κατάταξης της και την ανακάλυψη καλύτερων και πιο ακριβών βιοδεικτών, αλλά και εξατομικευμένων θεραπειών.

ACKNOWLEDGMENTS

Carrying out the essential work and then writing this thesis was, undoubtedly, the most arduous task I have undertaken. However, one of the joys of having completed the thesis is looking back at everyone who has helped me over the past four years. Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this thesis possible and because of whom my graduate experience has been one that I will cherish forever.

I would like to begin by thanking my three promotors and my co-promotor: Professors Dirkje Postma, Marike Boezen, Gerard Koppelman and Dr. Judith Vonk, respectively. It is an often used cliché, but in this case it is no overstatement to say that without the consistent guidance, tutelage, support, unparalleled knowledge, and encouragement of those four, this thesis would never have existed.

In particular, my deepest gratitude is to my first promotor, Prof. Dirkje Postma (Dep. Pulmonology, UMCG), for the patient guidance, encouragement and advice she has provided throughout my time as her student. I have been extremely lucky to have a promotor who cared so much about my work and my personal development, and who responded to my questions and queries so promptly. I have been amazingly fortunate to have a promotor who gave me the freedom to explore on my own, and at the same time the guidance to recover when my steps faltered. She taught me how to question thoughts and express ideas. I am also thankful to her for encouraging the use of correct writing and for carefully reading and commenting on countless revisions of the manuscripts. I hope that one day I would become as a good mentor to my students as Dirkje has been to me.

I want to thank Prof. Marike Boezen who has been always there to listen and give advice. I am deeply grateful to her for providing me with an excellent atmosphere for doing research at her department, for her teaching and for our discussions that helped me sort out the technical and financial details of my work. I am indebted to her for her trust, continuous encouragement and guidance.

Prof. Gerard Koppelman's insightful comments and constructive criticisms at different stages of my research were thought-provoking and they helped me focus my ideas. I am grateful to him for holding me to a high research standard and enforcing strict validations for each research result, and thus teaching me how to do research.

I must offer my profoundest gratitude to Dr. Judith Vonk whose office door was always open whenever I ran into a trouble spot or had a question about statistics, my research or writing. From finding an appropriate subject in the beginning of the process for writing thesis, Judith offered her unreserved help and guidance and lead me to finish my thesis step by step. Without her kind and patient instructions, it now seems impossible for me finishing this thesis. One simply could not wish for a better or friendlier supervisor.

Moreover, I would like to thank Dr. Jorrit Gerritsen who initially offered the stipend for hiring a new PhD student, i.e., me, and for his contribution to our monthly group discussions and comments on the manuscripts. In addition, I would like to acknowledge Prof. Wim Timens and Dr. Nick ten Hacken for offering the bronchial biopsies data and supporting the writing of the papers.

I would like to thank the Graduate School of Medical Sciences - Research Institute GUIDE, not only for providing the funding for courses which allowed me to better undertake this research, but also for giving me the opportunity to attend conferences and meet so many interesting people. Most importantly, I thank my promotors who gave me the great opportunity to collaborate with research groups outside UMCG and for trusting me for a significant collaboration with Professors Diane Gold and Scott Weiss at Harvard University during the last year of my doctoral studies.

I thank those two important scientists and beautiful people for embracing me within their research groups at the Channing Laboratory and Harvard School of Public Health. No words can adequately express my deep gratitude and appreciation to Professor Gold who with her patience, care and support helped me overcome many crisis situations. Her great coordinating skills assisted finish this dissertation on time. I thank her for her guidance and kindness and for being available and responsive to all my questions and requests. Professor Gold not only gave me generously of her time and expertise while I was studying in Boston, but she also remained involved when I returned to the Netherlands/Cyprus. I would like to express my profound gratitude to Prof. Weiss for his extremely useful suggestions in the preparation of chapters 5 and 6 of my thesis. He has been a strong and supportive advisor to me and I am grateful for the academic support and the facilities provided to carry out the research work. Thank you both very much for your guidance, your trust and for believing in me.

A project of this nature, based on two continents and with a limited amount of time for preparing two manuscripts, was only possible with the help of many

people in Boston but I would like particularly to thank Dr. Antonella Zanobetti and Dr. Brent Coull to whom I owe much of the analysis presented in Chapters 5 and 6. They generously assisted me with the statistics and were incredibly patient with my lack of knowledge in R. My time at Harvard School of Public Health was very productive and working there was a fantastic experience.

My thanks also go to the members of my thesis reading committee (Prof. B. Brunekreef, Prof. S.T. Weiss and Prof. R.J. Sinke) for spending their time on carefully reading of my thesis as well as for their valuable comments.

I should also like to thank fellow graduate students (in Groningen and Boston) primarily for giving me someone to moan at when work wasn't progressing according to plan and for all the support we shared during our studies. Furthermore, I would like to thank all the members of the Groningen Research Institute for Asthma and COPD, the department of Epidemiology-UMCG, the Harvard School of Public Health and the Channing Laboratory for the inspiring presentations and for sharing our ideas and scientific knowledge.

Getting through my dissertation required more than academic support, and I have many, many people to thank for listening to and, at times, having to tolerate me over the past four years. My friends (in Groningen, Boston, New York and back home in Cyprus) have helped me stay sane through the ups and downs of writing my PhD thesis. Their support and care helped me overcome setbacks and stay focused on my graduate study. I greatly value the friendship of my Greek/Cypriot friends, the fun we shared and I deeply appreciate their belief in me. I am also grateful to Diomis family for their warm hospitality and love and for offering me a home away from home during my stay in US.

Last but not least my parents Christoula and Pavlos, and my sister Maria, receive my deepest gratitude and love for their dedication and the many years of support during my studies that provided the foundation for this work. Most importantly, none of this would have been possible without the love and patience of my family. My immediate family, to whom this dissertation is dedicated to, has been a constant source of love, concern, support and strength all these years. Although they hardly understand what writing a thesis is and what I research on, they were willing to support any decision I made. I would like to express my heart-felt gratitude for your unwavering belief in me. Without you, I would not be the person I am today.

Αφιερωμένο

Οι γονείς μου Χριστούλα και Παύλος, και η αδελφή μου Μαρία, λαμβάνουν τη βαθύτατη ευγνωμοσύνη και την αγάπη μου για την αφοσίωση και στήριξη τους στη διάρκεια των πολυετών σπουδών μου. Αποτέλεσαν τη βάση μου, και ήταν πρόθυμοι να στηρίξουν κάθε απόφαση που πήρα και κάθε προσπάθεια μου.

Τίποτα δεν θα ήταν δυνατόν χωρίς εσάς, και είμαι ευγνώμων για την αταλάντευτη πίστη σας σε μένα. Η διατριβή μου αυτή είναι αφιερωμένη σε εσάς. Χωρίς εσάς δεν θα ήμουν ο άνθρωπος που είμαι σήμερα.

Ιθάκη

Κωνσταντίνος Καβάφης

Σα βγεις στον πηγαιμό για την Ιθάκη,
να εύχεσαι νάναι μακρύς ο δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.
Τους Λαιστρυγόνας και τους Κύκλωπας,
τον θυμωμένο Ποσειδώνα μη φοβάσαι,
τέτοια στον δρόμο σου ποτέ σου δεν θα βρεις,
αν μέν' η σκέψις σου υψηλή, αν εκλεκτή
συγκίνησις το πνεύμα και το σώμα σου αγγίζει.
Τους Λαιστρυγόνας και τους Κύκλωπας,
τον άγριο Ποσειδώνα δεν θα συναντήσεις,
αν δεν τους κουβανείς μες στην ψυχή σου,
αν η ψυχή σου δεν τους στήνει εμπρός σου.

Να εύχεσαι νάναι μακρύς ο δρόμος.
Πολλά τα καλοκαιρινά πρωιά να είναι
που με τι ευχαρίστησι, με τι χαρά
θα μπαίνεις σε λιμένας πρωτοειδωμένους•
να σταματήσεις σ' εμπορεία Φοινικικά,
και τες καλές πραγμάτειες ν' αποκτήσεις,
σεντέφια και κοράλλια, κεχριμπάρια κ' έβενους,
και ηδονικά μυρωδικά κάθε λογής,
όσο μπορείς πιο άφθονα ηδονικά μυρωδικά•
σε πόλεις Αιγυπτιακές πολλές να πας,
να μάθεις και να μάθεις απ' τους σπουδασμένους.

Πάντα στον νου σου νάχεις την Ιθάκη.
Το φθάσιμον εκεί είν' ο προορισμός σου.
Αλλά μη βιάζεις το ταξείδι διόλου.
Καλλίτερα χρόνια πολλά να διαρκέσει•
και γέρος πια ν' αράξεις στο νησί,
πλούσιος με όσα κέρδισες στον δρόμο,
μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.

Η Ιθάκη σ' έδωσε τ' ωραίο ταξείδι.
Χωρίς αυτήν δεν θάβγαινες στον δρόμο.
Αλλα δεν έχει να σε δώσει πια.

Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.
Ετσι σοφός που έγινες, με τόση πείρα,
ήδη θα το κατάλαβες η Ιθάκες τι σημαίνουν.

(Από τα Ποιήματα 1897-1933, Ικαρος 1984)

http://www.kavafis.gr/poems/content.asp?id=81&cat=1

Ithaka

By Constantinos Cavafy

As you set out for Ithaka
hope the voyage is a long one,
full of adventure, full of discovery.
Laistrygonians and Cyclops,
angry Poseidon—don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians and Cyclops,
wild Poseidon—you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope the voyage is a long one.
May there be many a summer morning when,
with what pleasure, what joy,
you come into harbors seen for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind—
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to gather stores of knowledge from their scholars.

Keep Ithaka always in your mind.
Arriving there is what you are destined for.
But do not hurry the journey at all.
Better if it lasts for years,
so you are old by the time you reach the island,
wealthy with all you have gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you will have understood by then what these Ithakas mean.

(C.P. Cavafy, Collected Poems. Translated by Edmund Keeley and Philip Sherrard. Edited by George Savidis. Revised Edition. Princeton University Press, 1992)
http://www.cavafy.com/poems/content.asp?cat=1&id=74