University of Alberta

Exposure to Swine Farming and the Lungs: Gene-Environment Interactions

by

Zhiwei Gao

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Epidemiology

Department of Public Health Sciences

Spring 2013
Edmonton, Alberta

Dedication

This dissertation is dedicated to my loving wife, Yun Zheng, for her support, patience, and encouragement throughout my PhD program.

Also to my mother, Jiahua Sun, my father, Hanpin Gao, and my parent-in-law, Wenrong Wang and Jinshu Zheng for their support and help you have provided to me.

Abstract

Occupational exposures in swine operations are associated with increased risk of respiratory problems. Although the etiology is not fully understood, genetic and environmental factors play important roles. The primary objective of this thesis is to explore effects of polymorphisms in the Toll-like receptor 2 (TLR2), TLR4 and Nitric Oxide Synthase 3 (NOS3) genes on lung function among workers in swine operations (a high exposure group) and non-farming rural residents (a low exposure group).

The studies considered in this thesis were a cross-sectional study of 374 full-time workers and 411 non-farming rural dwellers in 2003/04, and a longitudinal study of 302 workers and 261 non-farming rural dwellers who were initially studied in 1990/91 and were followed up in 1994/95 (217 workers and 171 rural dwellers) and in 2003/04 (173 workers and 119 rural dwellers). Information on demographic and lifestyle factors and lung function measurements were collected at the three time points and blood samples for genotyping were obtained in 2003/04.

The important findings in this study were: (1) Workers with TLR2-16933T/A polymorphism (AA) had greater mean values of lung function than workers with the wild-type (AT+TT) (FEV_1 (L): 3.7 vs. 3.5, p=0.009; $FEF_{25\%-75\%}$ (L/s): 3.7 vs. 3.3, p=0.003); similar associations were also observed for the TLR2Arg677Trp polymorphism among workers; (2) Workers with NOS3-786T/C polymorphism (CC) had greater mean values of lung function than workers with the wild-type (TC+TT) (FEV_1 (L): 3.75 vs. 3.55, p=0.009; FVC (L): 4.79 vs. 4.56, p=0.03); similar associations were also observed for the NOS3Glu298Asp polymorphism among workers; (3) Workers with NOS3-786T/C polymorphism (CC) had a lower annual decline rates in FEV_1 (-30.5 ml/year vs. -58.0 ml/year, p=0.005) and FVC (-16.7 ml/year vs. -50.9 ml/year, p=0.01) than workers with TT genotype; and (4) None of these associations were observed in non-farming rural dwellers.

In conclusion, TLR2 and NOS3 polymorphisms had significantly protective effects on lung function among workers in swine operations. The results from this study will increase our understanding of environmental and genetic determinants of respiratory dysfunction and ultimately lead to better prevention and intervention measures to improve the respiratory health of workers in swine operations.

Acknowledgements

This research was funded by the CIHR Strategic Training Program in Public Health and the Agricultural Rural Ecosystem (PHARE) and CIHR Partner Institutes including the Institute of Health Services and Policy Research, Institute of Circulatory and Respiratory Health, Institute of Infection and Immunity, and the Institute of Population and Public Health. The study was supported by a grant from the Canadian Institutes of Health Research (grant MOP-57907).

First, I am grateful to my supervisor, Dr. Ambikaipakan Senthilselvan, for giving me the opportunity to be part of this research project and leading me through every step of my PhD program with his tremendous support, encouragement, suggestions and comments. Without his guidance, this thesis would not have been possible.

Also, I wish to express my sincere gratitude to my thesis committee members: Drs. Donna Rennie, Jeremy Beach, and Heather McDermid for their incredible mentorship and suggestions which lead to the success of this project.

Special thanks to Dr. James Dosman for his continued help on this research through my thesis project. I also wish to express my thanks to Drs. David Schwartz, and Ivana Yang for their valuable comments and time spent on reviewing my papers.

I acknowledge with gratitude the individual swine workers and rural dwellers who participated in the study and the rural towns in Saskatchewan who provided us with their taxation rolls. I recognize the assistance of Ms. Sandra Halliday, RN, and Ms. Natalie Delmaire, RN, who performed many of the tests. The contribution of E. Barber, PhD, C. Rhodes DVM, SaskPork, and the swine production companies in Saskatchewan to the planning and execution of the project is acknowledged and appreciated. Hospitals and communities in rural Saskatchewan have been helpful in providing facilities for testing, and I thank them for their collaboration.

Table of Contents

APPENDICES

List of Tables

List of Tables (continued)

List of Figures

List of Abbreviations

AIC	Akaike information criterion
AD	Atopic dermatitis
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BHR	Bronchial hyper-responsiveness
BTNL2	Butyrophilin-like 2 gene
COPD	Chronic obstructive pulmonary disease
cNOS	Constitutive nitric oxide synthase
CD14	Cluster of differentiation 14
CO_2	Carbon dioxide
CSF	Colony stimulating factor
DCs	Dendritic cells
eNOS	Endothelial nitric oxide synthase
EU	Endotoxin unit
FcεRI	High affinity IgE receptor
FEV_1	Forced expiratory volume in the first second
$FEF_{25\%-75\%}$	Forced expiratory flow between 25% and 75% of FVC
FVC	Forced vital capacity
HLA	Human leukocyte antigen
HNMT	Histamine N-methyltransferase-encoding gene
H_2S	Hydrogen sulfide
HWE	Hardy-Weinberg equilibrium
GEE	Generalized estimation equations

List of Abbreviations (continued)

GM-CSF	Granulocyte-macrophage- colony stimulating factor
GST	Glutathione S-transferase encoding genes
IFN	Interferon
IKB	Inhibitor of kappa B
IKK	Inhibitor of kappa B Kinase
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor
LBP	LPS binding protein
LD	Linkage disequilibrium
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
mCD14	Membrane CD14
MYD88	Myeloid differentiation primary response gene 88
NF-κB	Kuclear factor-κB
NH_3	Ammonia
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PAF-AH	Platelet-activating factor-acetylhydrolase
PAMP	Pathogen-associated molecular pattern
PM	Particulate matter
PGN	Peptidoglycan
ppm	Parts per million

List of Abbreviations (continued)

PRR	Pattern recognition receptors
sCD14	Soluble CD14
SPT	Allergy skin prick tests
SNP	Single-nucleotide polymorphism
TGF-β_1	Transforming growth factor β_1
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TRAF6	TNF receptor associated factor 6
Treg	Regulatory T cell
tSNP	Tagging SNP

CHAPTER 1

INTRODUCTION

1.1 Statement of the problem

Worker in swine facilities is exposed to high concentrations of dust particles, endotoxin, microorganisms (Gram-positive and Gram negative bacteria) and gases including NH_3 and H_2S.[1] All of these exposures could contribute to a risk of excess respiratory symptoms,[2] reduced lung functions,[3] and increased asthma severity.[4] Endotoxin appears to be the major determinant of lung dysfunction.[5] Large indoor swine production facilities pose a higher risk of adverse respiratory health problems to those who work inside these facilities presumably due to higher concentration of indoor contaminants and longer duration of daily-exposure.

Human populations can also be exposed to low concentrations of airborne contaminants in non-occupational settings, for example, endotoxin can be found in homes (i.e. < 100 EU/m3), especially those houses with animals[6] and cigarette smoking.[7] Asthma severity and frequency in both adults and children have been reported to be associated with endotoxin concentration in house dust. [6] Although there is considerable evidence showing occupational exposure to airborne contaminants lead to lung disease, the etiology is not fully understood. Extensive animal and human population studies over the years have shown that individual susceptibility, in addition to timing and dose of exposure, plays an important role in the development of respiratory diseases. It is clear that many genetic and environmental factors and their interactions including gene-environment, gene-gene, and environment-environment interactions play a role.

Endotoxins are soluble complex lipopolysaccharides (LPS) from gram-negative bacteria. Although endotoxin's role in the development of lung dysfunction and respiratory diseases is not well established, high level of endotoxin exposure has been associated with increased Th1 and

decreased Th2 responses in animal models,[8] lower risk of atopic sensitization in children, [9-11] pro-inflammatory responses,[12,13] reduced lung function[3] and asthma severity in adults. [4]

Toll-like receptors (TLRs) recognize constituents of microbial cell walls or pathogen-specific nucleic acids. Stimulation of the TLRs on these cells leads to initiation of an innate immune response, which includes recruiting immune cells to sites of infection through cytokines, activating the complement cascade and removing of foreign substances by specialized white blood cells, as well as activating the adaptive immune system through an antigen presentation process, which leads to secretion of Th2 cytokines, such as IL-4 and IL-5.[14]

In response to endotoxin exposure, human innate immunity initiates its reactions through Toll-like receptor 4 (TLR4)/CD14 complex.[14] Therefore, the effects of TLR4 polymorphisms, including two polymorphisms in the coding region of the TLR4 gene (Asp299Gly and Thr399Ile polymorphisms), have been the focus in many studies of lung dysfunction and respiratory diseases due to endotoxin exposure. Many population studies have consistently shown that polymorphisms of the TLR4 gene result in hypo-responsiveness and reduced airway response to endotoxin exposure among healthy adults who were challenged by LPS.[15,16] However, in these studies, not all subjects who were hypo-responsive to LPS had mutations in the TLR4 gene, and not everyone with TLR4 polymorphisms was hypo-responsive to LPS.[17] These findings suggest the possibility of involvement of other genes in the responses to LPS exposure.

In the TLR family, the TLR2 and TLR4 genes are the most studied genes and their functions are best understood. TLR2 mainly responds to cell wall structure components from Gram-positive bacteria, such as peptidoglycan (PGN).[18,19] In swine operations, the concentration of Gram-positive bacteria is much higher than that of Gram-negative bacteria,[20] which potentially makes it more relevant to the adverse respiratory health problems reported among workers in swine

operations. Experimental studies using human cells or animal models consistently show that hog confinement dust up-regulates TLR2, rather than TLR4, in human bronchial epithelial cells,[21] and TLR2-deficiency is associated with a reduced immune response, pulmonary inflammation and injury when exposed to bleomycin or organic dust extract.[22-24]

In addition to the TLR family, there is increasing evidence showing that nitric oxide (NO) plays a key role in physiological and pathophysiological events of the lungs.[25-30] It acts as vasodilator, neurotransmitter and inflammatory mediator,[25,26] and induces non-specific toxic effects in responses to infection with bacteria, viruses or parasites.[27] Increased levels of NO in exhaled air was observed among asthmatics,[13,28] healthy naïve volunteers after 5-hour exposure in a pig confinement building,[29] and in workers from swine confinement operations.[30] NO can be generated during the conversion of the amino acid L-arginine to L-citrulline by Nitric Oxide Synthase (NOSs).[31] There are two types of constitutive NOS (cNOS) including neuronal NOS (nNOS) and endothelial NOS (eNOS), and a single type of inducible NOS (iNOS). In humans, each is encoded by distinct genes: nNOS by the *NOS1* gene; iNOS by the *NOS2* gene; and eNOS by the *NOS3* gene. The three human *NOS* genes, *NOS1*, *NOS2* and *NOS3*, are located at 12q24, 17q11.2-q12 and 7q36, respectively, which also harbour many loci associated with asthma.[32,33] Polymorphisms in the NOS3 gene have been associated with atopy, bronchial hyper-responsiveness (BHR), total and specific IgE in asthmatics, and atopic asthma in many studies.[34-36]

To date, results are from either experimental studies using human cells and animal models, or from human studies using naïve healthy adults. There has been limited study of the relationship between polymorphisms in the TLR2, TLR4 and NOS3 genes and lung function responses in workers in swine operations.

1.2 Study objectives

The primary objective of this study is to investigate whether the polymorphisms in the TLR2 and NOS3 genes could modify the effects of occupational exposures in swine operations on lung function. The specific objectives of this study are: firstly, to investigate the effects of Arg753Gln, Arg677Trp and -16933T/A polymorphisms in the TLR2 gene and Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene on lung function in workers from swine operations and non-farming rural dwellers; secondly, to examine the effects of -786T/C, Glu298Asp and -922A/G polymorphisms in the endothelial NOS (NOS3) gene on lung function among workers in swine operations and non-farming rural dwellers and finally, to explore the effects of -786T/C, Glu298Asp and -922A/G polymorphisms in the NOS3 gene on the longitudinal changes in lung function in male workers in swine facilities and male non-farming rural dwellers.

1.3 Thesis submitted for partial fulfillment of PhD

This thesis consists of a comprehensive literature review on all TLRs in the TLR family, genes in the TLR signaling pathway, genes which are associated with asthma and COPD, and genes which are associated with occupational respiratory disease. It is followed by three studies designed to address each of the three specific objectives.

In Chapter 2, a detailed description of the role of TLRs and the polymorphisms in each of the TLR genes in the allergic diseases, such as asthma and allergic rhinitis, is presented.

In Chapter 3, candidate genes for asthma, allergy, COPD, and genes associated with occupational respiratory disease are summarized.

A summary of exposures in swine facilities and effects on respiratory health is presented in Chapter 4. This chapter includes a detailed description of particulate matter, endotoxins, and

gases including hydrogen sulfide, ammonia and carbon dioxide, and health effects due to exposure to them.

A summary of population and methods from two previously conducted studies including a cross-sectional study and a longitudinal study in Saskatchewan are presented in Chapter 5. This chapter includes the overall study designs, study population, data collection, selection of candidate genes and their SNPs, and quality control regarding the genotyping information.

In Chapter 6, results of the first study are presented. In this chapter, the effects of polymorphisms of the TLR2 and TLR4 genes on lung function parameters among workers in swine operations and non-farming rural dwellers are examined using the subjects from a cross-sectional study.

In Chapter 7, results of the second and third studies are presented. The effects of polymorphisms of the NOS3 genes on lung function parameters and lung function annual decline rates among workers in swine operations and non-farming rural dwellers are examined in a cross-sectional and a longitudinal population, respectively.

General discussion and conclusions are presented in the final chapter (Chapter 8). This chapter includes an overview of the thesis research, a summary of the results from the three studies, discussion of the impact of studies, strength and limitations of the studies, and plans for future research.

1.4 References

(1) Crook B, Robertson JF, Glass SA, Botheroyd EM, Lacey J, Topping MD. Airborne dust, ammonia, microorganisms, and antigens in pig confinement houses and the respiratory health of exposed farm workers. Am Ind Hyg Assoc J 1991 Jul;52(7):271-9.

(2) Senthilselvan A, Chenard L, Ulmer K, Gibson-Burlinguette N, Leuschen C, Dosman JA. Excess respiratory symptoms in full-time male and female workers in large-scale swine operations. Chest 2007 Apr;131(4):1197-204.

(3) Kirychuk S, Senthilselvan A, Dosman JA, Zhou C, Barber EM, Rhodes CS, et al. Predictors of longitudinal changes in pulmonary function among swine confinement workers. Can Respir J 1998 Nov;5(6):472-8.

(4) Michel O, Ginanni R, Duchateau J, Vertongen F, Le BB, Sergysels R. Domestic endotoxin exposure and clinical severity of asthma. Clin Exp Allergy 1991 Jul;21(4):441-8.

(5) Vogelzang PF, van der Gulden JW, Folgering H, Kolk JJ, Heederik D, Preller L, et al. Endotoxin exposure as a major determinant of lung function decline in pig farmers. Am J Respir Crit Care Med 1998 Jan;157(1):15-8.

(6) Park JH, Spiegelman DL, Gold DR, Burge HA, Milton DK. Predictors of airborne endotoxin in the home. Environ Health Perspect 2001 Aug;109(8):859-64.

(7) Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. Chest 1999 Mar;115(3):829-35.

(8) Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002 Dec 16;196(12):1645-51.

(9) Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, et al. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. Clin Exp Allergy 1999 Jan;29(1):28-34.

(10) Gereda JE, Leung DY, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 2000 May 13;355(9216):1680-3.

(11) Gehring U, Bischof W, Fahlbusch B, Wichmann HE, Heinrich J. House dust endotoxin and allergic sensitization in children. Am J Respir Crit Care Med 2002 Oct 1;166(7):939-44.

(12) Michel O, Dentener M, Corazza F, Buurman W, Rylander R. Healthy subjects express differences in clinical responses to inhaled lipopolysaccharide that are related with inflammation and with atopy. J Allergy Clin Immunol 2001 May;107(5):797-804.

(13) Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 1994 Jan 15;343(8890):133-5.

(14) Lee SG, Kim BS, Kim JH, Lee SY, Choi SO, Shim JY, et al. Gene-gene interaction between interleukin-4 and interleukin-4 receptor alpha in Korean children with asthma. Clin Exp Allergy 2004 Aug;34(8):1202-8.

(15) Schwartz DA. TLR4 and LPS hyporesponsiveness in humans. Int J Hyg Environ Health 2002 Apr;205(3):221-7.

(16) Senthilselvan A, Dosman JA, Chenard L, Burch LH, Predicala BZ, Sorowski R, et al. Toll-like receptor 4 variants reduce airway response in human subjects at high endotoxin levels in a swine facility. J Allergy Clin Immunol 2009 May;123(5):1034-40, 1040.

(17) Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000 Jun;25(2):187-91.

(18) Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, Cardell LO. Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. Respir Res 2005;6:100.

(19) Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999 Oct;11(4):443-51.

(20) Nehme B, Letourneau V, Forster RJ, Veillette M, Duchaine C. Culture-independent approach of the bacterial bioaerosol diversity in the standard swine confinement buildings, and assessment of the seasonal effect. Environ Microbiol 2008 Mar;10(3):665-75.

(21) Bailey KL, Poole JA, Mathisen TL, Wyatt TA, Von Essen SG, Romberger DJ. Toll-like receptor 2 is upregulated by hog confinement dust in an IL-6-dependent manner in the airway epithelium. Am J Physiol Lung Cell Mol Physiol 2008 Jun;294(6):L1049-L1054.

(22) Razonable RR, Henault M, Paya CV. Stimulation of toll-like receptor 2 with bleomycin results in cellular activation and secretion of pro-inflammatory cytokines and chemokines. Toxicol Appl Pharmacol 2006 Feb 1;210(3):181-9.

(23) Yang HZ, Cui B, Liu HZ, Chen ZR, Yan HM, Hua F, et al. Targeting TLR2 attenuates pulmonary inflammation and fibrosis by reversion of suppressive immune microenvironment. J Immunol 2009 Jan 1;182(1):692-702.

(24) Poole JA, Wyatt TA, Kielian T, Oldenburg P, Gleason AM, Bauer A, et al. Toll-like receptor 2 regulates organic dust-induced airway inflammation. Am J Respir Cell Mol Biol 2011 Oct;45(4):711-9.

(25) Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991 Jun;43(2):109-42.

(26) Birrell MA, McCluskie K, Hardaker E, Knowles R, Belvisi MG. Utility of exhaled nitric oxide as a noninvasive biomarker of lung inflammation in a disease model. Eur Respir J 2006 Dec;28(6):1236-44.

(27) Liew FY, Cox FE. Nonspecific defence mechanism: the role of nitric oxide. Immunol Today 1991 Mar;12(3):A17-A21.

(28) al-Ali MK, Eames C, Howarth PH. Exhaled nitric oxide; relationship to clinicophysiological markers of asthma severity. Respir Med 1998 Jul;92(7):908-13.

(29) Sundblad BM, Larsson BM, Palmberg L, Larsson K. Exhaled nitric oxide and bronchial responsiveness in healthy subjects exposed to organic dust. Eur Respir J 2002 Aug;20(2):426-31.

(30) Von Essen SG, Scheppers LA, Robbins RA, Donham KJ. Respiratory tract inflammation in swine confinement workers studied using induced sputum and exhaled nitric oxide. J Toxicol Clin Toxicol 1998;36(6):557-65.

(31) Barnes PJ, Belvisi MG. Nitric oxide and lung disease. Thorax 1993 Oct;48(10):1034-43.

(32) Barnes PJ. Neurogenic inflammation in the airways. Respir Physiol 2001 Mar;125(1-2):145-54.

(33) Ober C, Cox NJ, Abney M, Di RA, Lander ES, Changyaleket B, et al. Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. Hum Mol Genet 1998 Sep;7(9):1393-8.

(34) Bourgain C, Hoffjan S, Nicolae R, Newman D, Steiner L, Walker K, et al. Novel case-control test in a founder population identifies P-selectin as an atopy-susceptibility locus. Am J Hum Genet 2003 Sep;73(3):612-26.

(35) Lee YC, Cheon KT, Lee HB, Kim W, Rhee YK, Kim DS. Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma. Allergy 2000 Oct;55(10):959-63.

(36) Holla LI, Buckova D, Kuhrova V, Stejskalova A, Francova H, Znojil V, et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. Clin Exp Allergy 2002 Aug;32(8):1193-8.

CHAPTER 2

TOLL-LIKE RECEPTOR (TLR) GENE POLYMORPHISMS*

Abstract

Over the years, studies have found that allergic diseases including asthma are associated with immunological responses to antigens driven by a Th2-mediated immune response. Since Toll-like receptors (TLRs) are involved in both innate and adaptive immune responses to a broad variety of antigens, the association between polymorphisms of TLRs and allergic diseases has been the focus in many animal and human studies. Although the etiology of allergic diseases is still unknown, extensive research over the years has confirmed that the underlining causes are due to many genetic and environmental factors along with the interactions between them, which include gene-environment, gene-gene, and environment-environment interactions. Currently, there is great inconsistency between studies likely due to differences in genetic backgrounds and unique gene-environment interactions in different populations. In this thesis, I provide a review of studies focusing on the association between TLR polymorphisms and allergic diseases which would help researchers to have a better understanding of the role of TLR polymorphisms in the development of allergic diseases, and ultimately lead to developing more efficient therapeutic interventions.

**A version of this chapter 2 has been published in The Application of Clinical Genetics:*

Gao Z, Rennie D, Senthilselvan A (2010). Allergic rhinitis and genetic components: Focus on toll-like receptors (TLRs) gene polymorphism. The Application of Clinical Genetics. 2010. Nov; 2010(3):109-120.

2.1 Introduction

Allergic diseases are the sixth leading chronic disease in the United States.[1] Extensive research in the last two decades has confirmed that the underlying causes of allergic diseases are associated with not only many genetic and environmental factors, but also with the interactions between them, which include gene-gene, environment-environment, and gene-environment interactions. Over the years, more than 100 genes have been linked to atopic diseases, with no single gene contributing to more than 5% to the observed phenotype.[2] The Hygiene Hypothesis, proposed by Strachan in 1989, has been used to explain the increase in allergic diseases reported in the developed countries.[3] According to this hypothesis, allergic diseases are caused by immunological responses to antigens driven by a Th2(T helper type 2)-mediated immune response, which is usually down-regulated by a Th1-mediated immune response. Insufficient stimulation of the Th1 arm due to a lack of exposure to specific infections, or perhaps endotoxin during early life, could lead to an overactive Th2 arm, which is associated with the development of allergic diseases.[4] A family of pattern recognition receptors (PRRs) including TLRs have become the focus over recent years since they are directly involved in human innate immune recognition and regulation of proper adaptive immune responses. The innate approach to microbial recognition by PRRs fundamentally differs from the developmental process of adaptive immune systems, in which T and B lymphocytes are involved. Adaptive immunity is not inherited and is 'trained' over time.[5] Activation of antigen-specific adaptive immunity needs information about the origin of the antigen and the type of response to be induced. All these actions require the involvement of the innate immune system. Improper responses from innate immunity may lead to attacking self-antigens, or responses to harmless persistent environmental antigens.[6] The important role of TLRs in both innate and adaptive immunity has led to

considerable research on the effects of their structures and genetic variations on allergic diseases. In the review part of this study, I aim to summarize our current knowledge of TLRs, polymorphisms of the TLR genes and their roles in the development of allergic diseases.

2.2 TLRs recognition and activation

TLRs are a type of pattern recognition receptors and must be able to distinguish between "self", such as apoptotic cells generated by normal tissue, and "invaders", such as virus and bacteria. A Toll receptor was first found in the fruit fly Drosophila melanogaster with its essential role in activating the synthesis of antimicrobial peptides against fungal infection.[7] The first human Toll-like receptor was reported in 1994.[8] Since then, 10 TLRs (TLR1 to TLR10) have been identified in the human genome. These 10 TLR-encoding genes are located on four autosomes (chromosome 1, 3, 4 and 9) and chromosome X, and are not evenly distributed across these chromosomes. Five TLR-encoding genes are located on chromosome 4 with the TLR1, TLR6 and TLR10 genes on p arm and the TLR2 and TLR3 genes on q arm. The TLR7 and TLR8 genes are located on chromosome X p22. The TLR4, TLR5 and TLR9 genes are located on chromosome 9, 1 and 3 respectively. Based on their cellular localization and pathogen-associated molecular pattern (PAMP) ligands, the 10 human TLRs can be divided into two groups.[9] The first group of TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) recognizes mainly microbial membrane components such as lipids, lipopolysaccharides (LPS) and lipoproteins. The second group of TLRs (TLR3, TLR7, TLR8 and TLR9) recognizes microbial nucleic acids and is expressed exclusively in intracellular vesicles such as endosomes, lysosomes, and endolysosomes. The ligands to TLR10 in human have not yet been identified.[10] TLRs are type I transmembrane proteins with leucine-rich repeats (LRRs) as ectodomain which mediates the

recognition of PAMPs; transmembrane domains; and intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains which is responsible for downstream signal transduction.[11] A unique feature of TLRs is that they recognize a broad diversity of ligands, which include microbial cell wall components (endotoxin from gram-negative bacteria) by TLR4, peptidoglycan and lipoproteins (from gram-positive bacteria) by TLR2 in combination with TLR1 or TLR6,[12] bacterial flagellin by TLR5,[13] unmethylated CpG sequences (from microbial DNA) by TLR9,[14] viral double-stranded RNA by TLR3[15] and viral single-stranded RNA by TLR7 and TLR8.[16] Other genes, such as nucleotide-binding oligomerization domain1 (NOD1) and NOD2 have been found to be involved in signalling initiated by pattern recognition receptors (PRRs),[17,18] but their roles relative to TLR-dependent pathways are still unknown.

TLRs recognize constituents of microbial cell walls or pathogen-specific nucleic acids. Most of the molecules recognized by TLRs are essential to the basic survival of microbes or viruses (integrity, function or replication), and therefore microbial alterations would not be able to escape detection by TLRs.[19] Once microbes penetrate an epithelial barrier, they will be targeted by tissue macrophages, mast cells and immature dendritic cells. Stimulation of the TLRs on these cells leads to initiation of an innate immune response, which includes recruiting immune cells to sites of infection through cytokines, activating the complement cascade and removing of foreign substances by specialized white blood cells, as well as activating the adaptive immune system through an antigen presentation process. The antigen presentation process begins with the engulfing of allergens by antigen-presenting cells (APCs), such as dendritic cells. Following digestion, APCs present fragments of allergen to allergen-specific $CD4^+$ T cells. $CD4^+$ cells secrete Th2 cytokines, such as IL-4 and IL-5.[20,21] Although the innate immune response is an immediate defense against infection, it is less efficient (not antigen

specific), and does not last long. The adaptive immune response has the ability to recognize and remember specific pathogens, and to mount stronger attacks upon re-exposure.

2.3 TLRs signaling pathway of immune response

The signals from TLRs have to be tightly controlled and their over-activation leads to inflammatory diseases.[22] Although there is a limited number of TLRs, they have the capacity to recognize a wide spectrum of ligands. Studies have showed TLRs specificity results from cooperation between TLRs by forming hetero or homodimer.[23] The heterodimers of TLR2 and TLR6 lead to the reorganization of peptidoglycan (a gram-positive pathogen) and activation of tumour necrosis factor-alpha (TNF-α) in the production in macrophages. TLR2 alone recognizes another component, bacterial lipopeptide. TLR4 invokes immune responses by forming a homodimer. These TLR dimers are in a low-affinity complex before ligand binding. The binding of specific ligands leads to a structural re-organization of the cytosolic TIR domains, which is used as a signalling platform for adaptor recruitment.[24]

TLRs may also depend on co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS, which requires MD-2. CD14 and LPS Binding Protein (LBP) are known to facilitate the presentation of LPS to MD-2.[25,26] As well, TLRs need to recruit adaptor proteins to propagate signals within the cytoplasm of cells, which ultimately leads to the activation of transcription factors, such as nuclear factor-κB (NF-κB) and members of the interferon (IFN)-regulatory factor (IRF) family. Detailed information on all TLR signalling pathways and five adaptor proteins to the TIR domain of TLRs has been well summarized and reviewed in other papers.[27,28]

2.4 TLR SNPs and allergic diseases

The TLR-encoding genes are highly polymorphic and play an important role in both innate and adaptive immunity.[29] It is not surprising that SNPs of these genes would have influences on the development of allergic diseases.[30,31] A total of 909 SNPs on 10 human TLR genes are listed on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov; accessed on July 06, 2010). As described at the NCBI website, the majority of studies on the function of TLRs have focused on the TLR2, TLR4 and TLR9 genes. In total, 17 out of the 909 SNPs have been associated with allergic diseases. These seventeen SNPs are comprised of 2 on the TLR1 gene, 4 on the TLR2 gene, 2 on the TLR4 gene, 3 on the TLR6 gene, 1 on the TLR7 gene, 2 on the TLR8 gene, 1 on the TLR9 gene and 2 on the TLR10 gene. Although expressions of the TLR3 and TLR5 genes have been linked with allergic rhinitis[32,33] and asthma,[34] none of the SNPs on the TLR3 and TLR5 genes has been shown to be associated with allergic diseases.

2.4.1 Prevalence of TLR SNPs associated with allergic diseases

Since allele frequency of one gene can vary between different populations, minor allele frequencies of TLR SNPs, which have been associated with allergic diseases including allergic rhinitis, were stratified by ethnic groups, and patient and control groups in Table 2-1.

As shown in Table 2-1, there are two SNPs on the TLR1 gene (rs5743595, rs4833095) which have been associated with atopic asthma. The first SNP (rs5743595) is located in the intron region of the TLR1 gene and the second (rs4833095), a missense mutation, is located in the coding region of the TLR1 gene. The HapMap study (http://www.genecards.org; accessed on July 06, 2010) showed that the prevalence of minor allele T in rs4833095, in African American, Chinese, Japanese, and C allele in Caucasian was 13%, 31%, 36%, and 30%,

respectively. For the SNP rs5743595, prevalence of the minor allele C in Caucasian, G in Chinese and A in Japanese was 27%, 43% and 47%, respectively in the HapMap. A population study of 1,872 German children aged 9 to 11 years showed that the prevalence of the minor allele C allele in rs5743595 and T allele in rs4833095 was 17% and 19%, respectively.[35]

There are four SNPs on the TLR2 gene, which have been associated with allergic rhinitis, asthma and atopy (Table 2-1). Three of them (rs3804099, rs3804100, and rs5743708) are in the coding region and the fourth (rs4696480) is located in the intron region of the TLR2 gene. The HapMap study showed that for rs3804099, the prevalence of minor allele, C was 44% in Caucasian, 36% in Chinese and 28% in Japanese populations and the prevalence of T was 37% in African American population. The prevalence of C allele in rs3804100 was 5% in Caucasian, 7% in African American, 33% in Chinese and 23% in Japanese and the prevalence of A allele in rs5743708 and rs4696480 was less than 5% in the HapMap. A case-control study of 440 adults with allergic rhinitis as cases and 528 healthy adults as controls from Korea showed the prevalence of minor allele C in rs3804099 and rs3804100 was 32% and 31% respectively in cases, and 31% and 29% respectively in controls.[36] Another case-control study of 108 children with allergic asthma and 496 controls from Norwegian showed that the prevalence of minor allele C in rs3804100 was 8% in cases and 11% in controls.[37] A cross-sectional study of 3,099 German children aged 9 to 11 years showed the prevalence of minor allele A in rs5743708 was 3%.[38] Another cross-sectional study of 609 children aged 6 to 11 years from German and Austria showed that the prevalence of minor allele T in rs4696480 was 52% among children living on farms and 49% among those not living on farms.[39]

As shown in Table 2-1, there are two SNPs (rs4986790 and rs4986791) on the TLR4 gene, which have been associated with allergic rhinitis, atopy, and atopic asthma. Both SNPs are

located in the coding region of the TLR4 gene and cause missense mutation. The HapMap study showed that the prevalence of minor allele G in rs4986790 and T allele in rs4886791 was about 3% in Caucasian and African American populations. A study of 915 university students from Saskatchewan, Canada showed that the prevalence of minor allele G in rs4986790 and T in rs4986791 was 5% and 4.7%, respectively.[40] A study of 613 asthma patients aged 6 to 18 years from Turkey showed the prevalence of minor allele G in rs4986790 and T in rs4986791 was 5.1%.[41] A study of 115 children aged 8 to 14 years from Sweden showed that the prevalence of minor allele G in rs4986790 was 9.6%.[42]

Three SNPs on the TLR6 gene (rs5743789, rs5743810 and rs2381289) have been shown to be associated with allergic rhinitis and atopic asthma (Table 2-1). Two SNPs (rs5743789 and rs2381289) are located in the promoter region and the second SNP, rs57438100, is located in the coding region of the TLR6 gene. The HapMap study showed that the prevalence of minor allele A in rs5743789 was 4.3% in African American, 15% in Caucasian and 31% in Asian populations. The prevalence of T allele in rs2381289 was 22% in an African American population and 41% in Caucasian and 48% in an Asian population. The prevalence of T allele in rs5743810 was 41% in Caucasian in the HapMap. A study of 1,872 children aged 9 to 11 years from Germany showed that the prevalence of minor allele A in rs5743789 and T in rs5743810 was 18% and 40%, respectively. [4335] A case-control study of 132 children aged 2 to 15 years with asthma and 212 healthy controls from Germany showed that the prevalence of minor allele T in rs5743810 was 49% in cases and 40% in controls.[44] Another case-control study from China (318 asthmatic cases and 352 non-asthmatic controls) showed the prevalence of minor allele T in rs2381289 was 48% in cases and 41% in controls.[45]

One SNP on the TLR7 gene (rs179008) and two SNPs on the TLR8 gene (rs5741883 and rs2407992) have been associated with allergic rhinitis, atopy and allergic dermatitis (Table 2-1).[46] Two of these SNPs, rs179008 and rs2407992, are located in the coding regions of TLR7 with the first one being a missense mutation and the second being synonymous, whereas rs5741883 is intergenic. The HapMap study showed that the prevalence of minor allele G in rs2407992 was 4% in African American, 18% in Chinese, and 20% in Japanese populations, while the prevalence of the C allele was 35% in Caucasian populations. The prevalence of T allele in rs179008 was 16% in Caucasian and 19% in African American populations and the prevalence of T allele in rs5741883 was 20% in a Caucasian population, 48% in an African American population, and less than 5% in an Asian population in the HapMap. A study of two family samples from Demark showed that the prevalence of minor allele T in rs179008 was 21% and 24%, C in rs2407992 was 38% and 42%, and T in rs5741883 was 20% and 25% in the two samples, respectively.[46]

The SNP (rs5743836) on the TLR9 gene has been associated with atopic eczema. The HapMap study showed that the prevalence of C allele on the SNP of rs5743836 was 16% in Caucasian, 25% in African American and 3% in Asian. Novak et al reported in a case-control study from Germany (274 atopic eczema cases and 252 controls) that the prevalence of minor allele C in rs5743836 was 13% in cases and 11% in controls.[47]

There are two SNPs in the TLR10 gene (rs4129009and rs11466651) which have been associated with allergic rhinitis or atopic asthma. Both SNPs are located in the coding region of the TLR10 gene and cause missense mutations. The HapMap study showed that prevalence of minor allele G on the SNP of rs4129009 was 26% in Caucasian, 40% in Japanese and 44% in Chinese populations; A allele on the SNP of rs11466651was 7% in Chinese, 17% in Japanese,

and less than 5% in Caucasian and African populations. A study of 1,872 Germany children aged 9 to 11 years showed that the prevalence of minor allele G in rs4129009 was 16%.[35] A case-control study from Chinese (318 cases with asthma and 352 non-asthmatic controls) showed the prevalence of minor allele A in rs11466651 was 7% in cases and 9% in controls.[45]

2.4.2 Relationship between TLR SNPs and allergic diseases

TLR1 participates in the innate immune response to microbial agents. It cooperates with TLR2 to mediate the innate immune response to bacterial lipoproteins or lipopeptides leading to an inflammatory response. A case-control study of children aged 9 to 11 years from Germany, which included 624 cases with asthma and/or bronchial hyperresponsiveness (BHR) and 1,248 controls without asthma and BHR, showed that people with the wild-type on two SNPs of the TLR1 gene (rs4833095, rs5743595) were protective against atopic asthma (rs4833095: OR=0.59, 95% CI: 0.41-0.83, p=0.003; rs5743595: OR=0.54, 95% CI: 0.37-0.81, p=0.002) after adjusting for multiple comparisons.[35]

As noted above, TLR2 cooperates with TLR1 to mediate the innate immune response to bacterial lipoproteins or lipopeptides. In a cross-sectional study of children aged 6 to 13 years from rural areas in Austria and Germany, the authors reported that children living in farms (n=229) carrying a T allele in rs4696480 of the TLR2 gene were significantly less likely to have current hay fever symptoms, atopic sensitization, asthma or current asthma symptoms than non-farmers' children living in rural areas (n=380).[39] In a case-control study from Korea of 440 patients with allergic rhinitis and 528 controls with no allergic symptoms and negative on allergy testing, allergic rhinitis was more common in the carriers of the C alleles on both rs3804099 and rs3804100 and C-C haplotype of the TLR2 gene.[36] In a case-control study from Norway, which included 108 allergic asthma cases and 494 controls, T allele on rs3804100 of the TLR2 gene

was significantly associated with allergic asthma (OR=3.40, p=0.009) and rs3804099 of the TLR2 gene had a non-significant positive association with allergic asthma.[37] A large cross-sectional study of 3,099 subjects from Germany showed that the minor allele of rs5743708 of the TLR2 gene was significantly associated with atopy determined by skin test (OR=1.53, 95% CI: 1.06-2.19, p=0.023) and specific serum inhalative allergens (OR=1.57, 95% CI: 1.12-2.20, p=0.009) after adjusting for sex, age and environmental tobacco smoke.[38] Niebuhr et al showed that the cytokine production by monocytes from atopic dermatitis patients carrying minor allele on rs5743708 of the TLR2 gene was significantly higher than those carrying wild type.[48] Abhmad-Nejad et al studied 78 patients with mild to severe atopic dermatitis (AD) and found that genotypes on rs5743708 of the TLR2 gene were associated with AD severity, which was measured by SCORAD. AD patients carrying mutant allele showed higher score than those carrying no mutant allele (median: 55.8 vs. 44.8).[49]

In addition to these SNPs on the TLR2 gene, different types of polymorphisms on TLR2 have been associated with allergic diseases and cancers. A Japanese study of 32 asthmatics by Noguchi et al showed that an insertion/deletion polymorphism in the 5' untranslated region of the TLR2 gene *in virto* has reduced transcriptional activity of the TLR2 gene than the wild-type alleles, but none of the 16 SNPs or haplotypes of the TLR2, 3, 4 and 9 genes were associated with IgE or asthma.[50]

Both TLR2 and TLR4 can recognize microbial membrane structure and have been associated with allergic diseases. A case-control study from Sweden, which included 42 patients with intermittent allergic rhinitis and 27 healthy volunteers, showed an increase in protein expression for TLR2, TLR3 and TLR4 in the nasal mucosa from the patient group and raised the possible involvement of these toll receptors in allergic airway inflammation.[33] TLR4 has been

implicated in signal transduction events induced by LPS found in most gram-negative bacteria. The two missense SNPs (rs4986790, rs4986791) have been extensively studied and associated with allergic rhinitis, airway responsiveness and asthma. Senthilselvan et al studied 915 non-smoking university students from Saskatchewan, Canada and found that the risk of allergic rhinitis in people carrying minor allele on both SNPs was reduced by 88% in comparison to the TLR4 wild type group (p=0.009) and atopy was associated with TLR4 polymorphism only among females.[40] The same group of researchers also studied the effects of these two misssense SNPs on respiratory responses to swine barn exposure in healthy non-smoking and non-allergic volunteers. In this study, 29 persons carrying the minor allele and 29 persons carrying the wild-type allele on both SNPs were exposed for 5 hours to natural swine barn environments. They found that TLR4 variants are significantly associated with reduced airway responsiveness, exhibiting a possible protective effect of TLR4 variants.[51] A case-control study among Turkish children aged from 6 to 18 years, in which 613 asthmatic children and a comparison group of 327 non-asthmatic children were included, showed that both misssense SNPs were significantly associated with atopic asthma in Turkish population.[41] Another study of the SNP, rs4986790, in a Swedish population, which included 115 Swedish children aged 8 to 14 years showed that the children in the polymorphic group were associated with 4 times more likely to have asthma and 7 times more likely to be atopic than wild-type group after adjusting for potential confounders.[42] The authors of the Swedish paper speculate that genetic differences in populations as well as differences in gene-environment interaction might contribute to these discrepancies in the results observed between different countries.

SNPs (rs5743789, rs5743810 and rs2381289) on the TLR6 gene are known to contribute to the innate immune response to Gram-positive bacteria and fungi leading to inflammatory

responses. A population based case-control study among children aged 9 to 11 years from Germany, in which 624 cases with asthma and/or BHR and 1,248 controls without asthma or BHR were included, showed that people with homozygote wild-type allele (T) on rs5743789 was significantly associated with reduced risk of atopic asthma (OR=0.54, 95% CI: 0.37-0.79, p=0.003), whereas people with homozygote wild-type allele (C) on rs5743810 was associated with increased risk of atopic asthma (OR=1.79, 95% CI: 1.24-2.58, p=0.002).[35] Another case-control study from Germany, in which 200 asthma cases (68 adult, 132 children) and 212 controls are included, showed that minor allele on rs5743810 was weakly associated (not significant after Bonferroni correction) with atopic asthma.[44] In a case-control study from China, which included 318 cases with asthmatics and 352 non-asthmatics as controls, showed that the T allele on rs2381289 in TLR6 was significantly associated with increased risk of allergic rhinitis among asthmatic patients (OR=1.79, 95% CI: 1.10-2.91, p=0.025).[45]

The TLR7 and TLR8 genes are activated by single stranded RNA and have similar signalling pathway. A family study of 235 families in Demark showed that the SNPs on TLR7, rs179008 and rs2407992, were significantly associated with allergic rhinitis, asthma, atopic dermatitis and increased specific IgE. The SNP of rs5741883 on the TLR8 gene was also associated with allergic rhinitis among girls in a sub-sample of 135 families.[46]

The ligands to TLR9 are unmethylated CpG dinucleotides from bacteria and virus. TLR9 is expressed by immune cell rich tissues, such as spleen, lymph node, bone marrow and peripheral blood leukocytes. A study of 32 patients with seasonal allergic rhinitis and 18 healthy controls from Sweden found a widespread expression of TLR9 in epithelial cells from nasal mucosa and on all leukocytes derived from bone marrow, and concluded that TLR9 was associated with the development of allergic airway inflammation.[52] Novak et al reported the

results from a family study (a total of 483 parent-affected offspring trios) and a case-control study (274 atopic eczema cases and 252 controls) in Germany. In this study, the minor allele on rs5743836 was significantly associated atopic eczema in the family study, but the association was not significant in the case-control study.[47]

The ligands and specific functions of TLR10 are still unknown. A case-control study from Germany, which included 624 cases with asthma and/or bronchial hyperresponsiveness (BHR) and 1,248 controls without asthma and BHR among children aged 9-11 years showed that the wild-type of rs4129009 in the TLR10 gene was associated with reduced risk of atopic asthma (OR=0.58, 95% CI: 0.39-0.86, p=0.006).[35] Another case-control study from China, which included 318 asthmatic patients aged 14 to 75 years and 352 nonasthmatic controls aged 16 to 74 years, showed a protective association between the A allele on rs11466651 and allergic rhinitis among asthmatic patients (OR=0.49, 95% CI: 0.26-0.95, p=0.046).[45]

2.4.3 Genomic linkage of TLR residing regions to allergic rhinitis

Genomic searches followed by fine mapping and positional cloning are very useful tools for investigating possible genes related to a disease. To date, most research studies have focused on asthma and a limited number of studies on genomic searches for genes related to allergic rhinitis have been conducted. These studies are summarized in Table 2-2.

A linkage analysis was carried among 424 individuals from 100 sib-pair families in Danmark[53.] The authors analyzed 97 polymorphic markers in 11 selected regions on chromosomes 3p, 3q, 4p, 4q, 5q, 6p, 9p, 12q, 12qter, 19q and Xp. Allergic rhinitis was linked to the following regions: 4p15-p14 where the TLR1, TLR6 and TLR10 genes reside, 4q32 where the TLR2 gene resides, and Xp21-p11 which is adjacent to the region where the TLR7 and TLR8 genes reside. In 2005, a genome screen was conducted in a sample of 295 French families with

at least one asthmatic subject.[54] They linked the following regions to allergic rhinitis: 3p24-p14 where TLR9 resides, and 9q22-q34, where TLR4 resides. A genomic linkage analysis of 250 families among a Swedish population confirmed the linkage of chromosome 9q33-q34, where TLR4 resides, to allergic rhinoconjunctivitis, which was also reported in a French study.[55] They also reported that a linkage of region 4q34-q35, where TLR3 resides, to allergic rhinoconjunctivitis.

2.5 Gene-environment interaction

The associations between TLRs and allergic diseases might be modified by environmental factors. Eisenbarth et al. showed in an animal model that LPS induced either Th1- or Th2-skewed immune responses when delivered at different doses. High doses of exposure are more likely to promote Th1 responses whereas low doses of exposure promote Th2 responses.[56] Kaisho et al showed that when activated by LPS, Myeloid differentiation primary response gene 88 (MyD88)-deficient dendritic cells induce Th2-cell differentiation, whereas wild-type dendritic cells induce Th1-cell differentiation.[57] The results from the two studies suggest that low concentrations of LPS exposure activate Th2-cell differentiation through MyD88 independent signaling pathway, whereas high concentrations of LPS exposure activate Th1-cell differentiation through a MyD88 dependent signaling pathway. Epidemiological studies conducted in human populations have confirmed the results observed in animal models. A study of 609 children aged 6 to 13 years from rural areas from Austria and Germany showed that among farmers' children, those carrying a T allele in the TLR2/-16934 polymorphism compared with children with genotype AA were significantly less likely to have current allergic rhinitis symptoms (3% vs. 14%, p=0.01), diagnosis of asthma (3% vs. 13%, p=0.012) and atopic

sensitization (14% vs. 27%, p=0.023).[39] A Swiss study of children aged from 6 to 15 years living in rural communities also showed that after adjustment for potential covariates, farming as a parental occupation was significantly associated with lower rates of sneezing attacks during pollen season (adjusted OR 0.34, 95% CI 0.12-0.89) and atopic sensitization (adjusted OR 0.31, 95% CI 0.13-0.73). In this study, the risk of atopic sensitization was lower in children from full-time farmers (adjusted OR 0.24, 95% CI 0.09-0.66) than from part-time farmers (adjusted OR 0.54, 95% CI 0.15-1.96).[58]

2.6 Treatment of allergic diseases and future perspectives

The hygiene hypothesis enables us to connect the reported increasing prevalence of Th2 associated allergic diseases in developed countries with reduced microorganism exposure. According to this hypothesis, microorganism exposure can help to avoid Th2 associated allergic diseases by inducing either immune deviation in favour of Th1 response or the development of regulatory T cell (Treg) in responses to allergens by TLRs on antigen-presenting cells, such as dendritic cells. Such associations have been demonstrated by the observed association between TLR polymorphisms and allergic diseases in many human and animal studies. However dose, type and timing of allergen exposures likely play an important role in allergic sensitization.

Current therapies of allergic diseases include allergen avoidance, medications (antihistamines, leukotriene inhibitors, oral corticosteroids), and immunotherapy (achieving allergen-specific tolerance, anti-IgE). In order to achieve allergen-specific tolerance, exposure to high-dose allergen is needed to deviate a Th2 immune response in favour of a Th1, to generate IL-10-producing T cells and $CD4^+CD25^+$ T cells and to switch B cell response in favour of IgG. Human $CD4^+CD25^+$ T cells can suppress T cell activation due to allergen exposure; IL-10-

producing T cells can also inhibit IL-4-producing T cells; IgG can down-regulate IgE-dependent Th2 lymphocyte responses.[59] Treatment with corticosteroids achieves anti-inflammatory effects for allergic diseases through increasing IL-10 production by T cells and macrophages. However, symptoms recur following discontinuation of the treatment. In contrast, allergen immunotherapy modulates Th2 responses and benefit can persist for at least 3-4 years after the treatment is discontinued.[60]

The fact that polymorphisms of TLRs and microbial exposure in early childhood are protective against allergic diseases provides us a new potential mechanism of therapy, using well defined bacterial products as immunomodulators in treatment. The above findings have led to the development of a Toll-like receptor 9 vaccine against allergic rhinitis. Animal models have shown inhibiting effects of Th2 cytokine responses following administration of the TLR9 ligand CpG DNA. Conjugating the allergen to CpG DNA dramatically enhances the immune responses to the allergen rather than administering the allergen and the CpG DNA separately (mean of Anti-Amb a 1 IG1: allergen alone=60; Amb a 1 alone=82,269; allergen+Amb a 1=784,852).[61] In a randomized, double-blinded, placebo-controlled phase 2 trial of ragweed-Toll-Like Receptor 9 agonist vaccine for allergic rhinitis, patients with allergic rhinitis who received a six-injection regimen of the vaccine treatment showed significant improvement over placebo in allergic symptoms during the subsequent ragweed season.[62]

Future studies, with particular emphasis on the areas of gene-gene and gene-environment interaction are very much needed. The results of these studies will hold the promise for treatment of allergic rhinitis, as well as other Th2 associated allergic diseases.

2.7 Discussion

Polymorphisms of all the TLR-encoding genes, except the TLR3 and TLR5 genes, have been associated with allergic diseases. Six of them, the TLR2, TLR4, TLR6, TLR7, TLR8 and TLR10 genes have been linked with allergic rhinitis as either protective or harmful factors. Polymorphisms protecting against allergic rhinitis are: T allele in rs4696480 of the TLR2 gene, G allele in rs4986790 and C allele in rs4986791 of the TLR4 gene and A allele in rs11466651 of the TLR10 gene. Polymorphisms increasing the risk of allergic rhinitis are: C allele in rs3804099 and rs3804100 of the TLR2 gene; T allele in rs2381289 of the TLR6 gene, A allele in rs179008 of the TLR7 gene; G allele in rs2407992 and C allele in rs5741883 of the TLR8 gene. These observed associations were obtained from doing either linkage analysis (the TLR7and TLR8 genes) or association analysis (the TLR2, TLR4, TLR6 and TLR10 genes) in certain populations.

The polymorphisms on the TLR-encoding genes have been shown to influence the expression and function of TLRs and associate with different immunity responses. Studies of the direct functional effects of these SNPs showed that homozygotes with the A allele in rs4696480 of the TLR2 gene were associated with increased expression of IL-6 following TLR2 stimulation.[63] In comparison, homozygotes with the minor allele C in rs5743595 of the TLR1 gene, A in rs5743789 of the TLR6 gene, and G in rs4129009 of the TLR10 gene were associated with increased TLR1, TLR6 and TLR10 mRNA expression, higher levels of proinflammatory cytokine TNF-α and Th1-related cytokines IL-12 and IFN-γ, and decreased Th-2-related IL-2 expression.[35]

Identification of the possible genes responsible for a disease can be done in many different ways.[64] Association and linkage studies are commonly used to find genes associated with a

disease in large populations. The most commonly used design in association studies is the case-control, where people with a disease ('cases') and people without disease ('controls') are chosen from the population. Information on demographic and lifestyle factors and other potential confounders is usually collected retrospectively using questionnaires. Although a case-control study usually suffers from recall-bias due to the differences in reporting past events between cases and controls, it is less problematic for genotype variables since they are less likely to change over time. Genotyping is conducted in both cases and controls for genes of interest. Selection of candidate genes is usually based on the results from other studies, where positive associations were identified, or biological functions of genes, such as genes in the signaling pathway.[65] In family-based linkage studies, association between genetic markers and a disease is measured by transmission from parent to affected offspring in family pedigrees.[66] If an allele is associated with a disease, then it is expected to be transmitted from parent to affected offspring more frequently than unaffected offspring. Replication of the results from several studies are required to make robust conclusions on the association between genotypes and phenotypes.[67]

Allergic diseases are associated with exposure to outdoor allergens, such as pollens (grass, trees, and weeds), indoor allergens (house dust mites, pets, and mold) and other environmental factors (air pollution, endotoxin, and bacterial/viral infection), and exposure to livestock.[68-70] However, the roles of these environmental factors in the development of allergic rhinitis are not fully understood. Predisposition to atopy involves a complex immune response process, including gene-gene and gene-environment interactions. The quantity, timing and type of exposure influence the development of allergic diseases. Recent studies have shown that CD14 is required for recognition of 'smooth LPS' (with long O-polysaccharide chains) through TLR4 for MyD88 dependent signaling pathways, but not for 'rough LPS' (without long O-

polysaccharide chains). However, CD14 is required for recognition of 'rough LPS' through TLR4 for MyD88-independent signaling pathways.[71] In a mouse model, differences in the timing of LPS exposure led to totally opposite responses: protecting against sensitization if exposed early in the sensitization process; exacerbating inflammation if exposed after the sensitization process.[72] Another animal model in mice showed that a low dose of LPS exposure to mice induced a Th2 response, while high doses of LPS exposure promoted a Th1 response.[56] Vercelli et al proposed the 'endotoxin switch' hypothesis, which suggests a bimodal relationship between environmental exposures to bacterial products and immune responses (being Th2-like at low and Th1-like at high exposures), to explain the complex dose-dependent relationship of environment-gene interactions.[73]

The increasing prevalence of allergic diseases including allergic rhinitis in the last 20 years can hardly be explained by DNA sequence changes. Other inheritance mechanisms might contribute to the etiology of allergic rhinitis. Epigenetics, which studies inherited changes in phenotype caused by mechanisms other than changes in the underlying DNA sequences, has been associated with allergic diseases. Loss of DNA methylation and histone modification at the Th2 locus have been associated with over-expression of the Th2 associated transcription factor GATA3, and increased expression of Th2 cytokines, IL-4, IL-5 and IL-13.[74] Allergic diseases have a hereditary component as shown in many studies, but do not exhibit a Mendelian hereditary pattern. This suggests the need to look into other mechanisms, like epigenetics, which is heritable. Shin et al reported that human T cells treated with a DNA methyltransferase inhibitor and truncation of methylation sites in the proximal regulatory regions of the STAT4 promoter showed increased expression of STAT4, a T cell-specific transcription factors inducing IFN-γ transcription.[75] A study of FoxP3, a master control transcription factor for controlling

Treg cells, showed decreased FoxP3 expression following methylation of a CpG island, and increased expression by Transforming Growth Factor-β, which in turn decreases methylation of the CpG island.[76] Allergic diseases including allergic rhinitis mostly show imprinting feature with an affected mother significantly more likely to transmit the disease than an affected father,[77] which is compatible with epigenetic mechanisms. Hollingsworth et al showed in mice model that in utero exposure to a diet rich in methyl donors enhanced airway hyperreactivity, lung lavage eosinophilia and IL-13, and higher serum IgE concentrations which were trans-generationally inheritable.[78] The results indicated that environmental factors, such as diet, can modify the risk of allergic airway disease through epigenetic mechanisms.

2.8 Conclusions

TLRs are an interesting group of receptors in the human genome, which can recognize a broad diversity of ligands and are involved in both innate and adaptive immune responses. Although mechanisms on how the TLR-encoding genes interact with each other, with other genes, and with environmental factors in the development of allergic diseases are not fully understood. Animal models and human genetic studies over the years have confirmed the involvement of TLRs in the etiology of allergic diseases.

Looking for genes associated with allergic diseases has been the focus in numerous studies. However general conclusions could not be made because of inconsistent results between studies and lack of replication of the results due to differences in the genetic background across ethnic groups and differences in gene-environment interactions in these studies. The chance of finding new genes associated with allergy might be limited with the completion of human genome project. However, it is believed that allergic diseases are polygenic, which requires examining

simultaneously several genes with each gene likely contributing only a small effect in the relationship with allergic diseases. This leads to a sample size issue for many genetic association studies. A large sample size is required to detect the small contribution of each gene, and an even larger sample is needed to test the gene-gene and gene-environment interactions. Adequately powered studies are the key to ensure successful replication of genetic association studies and detection of small effects of each gene, as well as reducing bias from misclassification of outcomes or/and genotypes. Haplotypes, which combine several SNPs together, should be studied, rather than any single SNP since the observed association between a single SNP and outcomes may be due to the linkage disequilibrium with a nearby polymorphism, which has the true effect on the outcomes. Haplotype analysis can also help to deal with multiple comparison issues, which is inevitable in genetic association studies using large number of SNPs.

It is clear that allergic diseases are influenced by both genetic predisposition and environmental exposure. However, results from epidemiological studies conducted in different populations are not consistent. This might be related to the differences in ethnic background and strength, route and timing of exposure in these studies. Consideration of gene-gene and gene-environment interactions and epigenetics in future studies will provide us with extra insight and ultimately lead to more efficient therapeutic treatments.

Table 2-1. TLR SNPs with significant association with allergic diseases including allergic rhinitis from different populations

TLR	Ethnic group	Design (Sample size)	Age	RS	Allele	Minor allele Frequency (%)	Allergic diseases		
							Inheritance	Allergic rhinitis	Others
TLR1	Germany[35]	Case-control (cases: 624, controls: 1,248)	9-11	rs4833095	C/T	19	Dominant		Atopic asthma
				rs5743595	T/C	17	Dominant		Atopic asthma
TLR2	Germany[38]	Cross-sectional (n=3099)	9-11	rs5743708	G/A	3	Dominant		Atopy
	Germany/Austria[39]	Cross-sectional (n=609)	6-13	rs4696480	A/T	49.7	Dominant	Yes*	Atopy*; Asthma*
	Korean[36]	Case-control (cases: 440, control: 528)	adults	rs3804099	T/C	Cases:32 Controls:31	Haplotype (C-C)	Yes	Atopic asthma
				rs3804100	T/C	Cases:31 Controls:29			
	Norwegian[37]	Case-control (cases: 108, controls: 496)	children	rs3804100	T/C	Cases:8 Controls:11	Dominant		Atopic asthma
TLR4	Canadian[40]	Cross-sectional (n=915)	adults	rs4986790	A/G	5	Dominant	Yes	Atopy†
				rs4986791	C/T	4.7			
	Turkish[41]	Cross-sectional (n=613)	6-18	rs4986790	A/G	5.1	Dominant		Atopic asthma
				rs4986791	C/T	5.1	Dominant		
	Swedish[42]	Cross-sectional (n=115)	8-14	rs4986790	A/G	9.6	Dominant		Atopic asthma
TLR6	Germany[35]	Case-control (cases: 624, controls:1,248)	9-11	rs5743789	T/A	18	Dominant		Atopic asthma
				rs5743810	C/T	40	Dominant		Atopic asthma
	Germany[44]	Case-control (cases: 132, controls: 212)	2-15 22-87	rs5743810	C/T	Cases: 49 Controls:40	Dominant		Atopic asthma
	Chinese[45]	Case-control (cases: 318; controls: 352)	16-74	rs2381289	C/T	Cases:48 Controls:41	Dominant	Yes**	
TLR7	Denmark[46]	Cross-sectional (Samples A:135, B: 100 families)	15-45	rs179008	A/T	Sample A:24 Sample B:21	Recessive & Additive	Yes¶	Atopy^{T}
TLR8	Denmark[46]	Cross-sectional (Samples A:135, B: 100 families)	15-45	rs2407992	G/C	Sample A:42 Sample B:38	Recessive & Additive	Yes¶	Atopy^{T};dermatitis^{T}
				rs5741883	C/T	Sample A:25 Sample B: 20	Recessive	Yes‡	
TLR9	Germany[47]	Cross-sectional & Case-control (n=483, cases: 274, controls: 252)	trios	rs5743836	T/C	Cases:13 Controls:11	TDT$^{\xi}$		Atopic eczema
TLR10	Germany[35]	Case-control (cases: 624, controls: 1,248)	9-11	rs4129009	A/G	16	Dominant		Atopic asthma
	Chinese[45]	Case-control (cases: 318, controls: 352)	16-74	rs11466651	G/A	Cases:7 Controls:9	Dominant	Yes**	

*: among farmers' children; †: among females; ‡: among girls in sample A; ξ: transmission disequilibrium test (TDT); **: among asthmatic patients
¶: sample A + sample B

Table 2-2. Genomic linkage of chromosomal regions to allergic rhinitis

Toll-like receptors (TLRs)			Genomic studies in allergic rhinitis		
TLRs	Chromosome	Region	Ethnic group	Family number	Region
1	4	p14	Danish[53]	100	p14-p15
2	4	q32	Danish[53]	100	q32
3	4	q35	Swedish[55]	250	q34-q35
4	9	q32-q33	Swedish[55]	250	q33-q34
			French[54]	295	q22-q34
5	1	q41-q42	*		
6	4	p14	Danish[53]	100	p14-p15
7	X	p22	Danish[53]	100	p11-p21
8	X	p22	Danish[53]	100	p11-p21
9	3	p21.3	French[54]	295	p14-p24
10	4	p14	Danish[53]	100	p14-p15

* No report available

2.9 References

(1) Meltzer EO. The prevalence and medical and economic impact of allergic rhinitis in the United States. J Allergy Clin Immunol 1997 Jun;99(6 Pt 2):S805-S828.

(2) Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. Genes Immun 2006 Mar;7(2):95-100.

(3) Strachan DP. Hay fever, hygiene, and household size. BMJ 1989 Nov 18;299(6710):1259-60.

(4) Folkerts G, Walzl G, Openshaw PJ. Do common childhood infections 'teach' the immune system not to be allergic? Immunol Today 2000 Mar;21(3):118-20.

(5) Liu AH. Innate microbial sensors and their relevance to allergy. J Allergy Clin Immunol 2008 Nov;122(5):846-58.

(6) Peng Y, Martin DA, Kenkel J, Zhang K, Ogden CA, Elkon KB. Innate and adaptive immune response to apoptotic cells. J Autoimmun 2007 Dec;29(4):303-9.

(7) Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996 Sep 20;86(6):973-83.

(8) Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S, et al. Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. DNA Res 1994;1(1):27-35.

(9) Kawai T, Akira S. TLR signaling. Cell Death Differ 2006 May;13(5):816-25.

(10) Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol 2005 Mar 1;174(5):2942-50.

(11) Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004 Jul;4(7):499-511.

(12) Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 2006 May;117(5):979-87.

(13) Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001 Apr 26;410(6832):1099-103.

(14) Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000 Dec 7;408(6813):740-5.

(15) Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001 Oct 18;413(6857):732-8.

(16) Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004 Mar 5;303(5663):1526-9.

(17) Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001 May 31;411(6837):603-6.

(18) Inohara N, Ogura Y, Chen FF, Muto A, Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. J Biol Chem 2001 Jan 26;276(4):2551-4.

(19) Medzhitov R, Janeway C, Jr. The Toll receptor family and microbial recognition. Trends Microbiol 2000 Oct;8(10):452-6.

(20) Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000 Aug 17;406(6797):782-7.

(21) Clark GJ, Angel N, Kato M, Lopez JA, MacDonald K, Vuckovic S, et al. The role of dendritic cells in the innate immune system. Microbes Infect 2000 Mar;2(3):257-72.

(22) O'Neill LA. Targeting signal transduction as a strategy to treat inflammatory diseases. Nat Rev Drug Discov 2006 Jul;5(7):549-63.

(23) Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 2000 Dec 5;97(25):13766-71.

(24) Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 3. Proc Natl Acad Sci U S A 2006 Jun 6;103(23):8792-7.

(25) Dziarski R, Gupta D. Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. J Endotoxin Res 2000;6(5):401-5.

(26) Kitchens RL. Role of CD14 in cellular recognition of bacterial lipopolysaccharides. Chem Immunol 2000;74:61-82.

(27) O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 2007 May;7(5):353-64.

(28) Cook DN, Pisetsky DS, Schwartz DA. Toll-like receptors in the pathogenesis of human disease. Nat Immunol 2004 Oct;5(10):975-9.

(29) Lazarus R, Vercelli D, Palmer LJ, Klimecki WJ, Silverman EK, Richter B, et al. Single nucleotide polymorphisms in innate immunity genes: abundant variation and potential role in complex human disease. Immunol Rev 2002 Dec;190:9-25.

(30) Yang IA, Holgate ST, Holloway JW. Toll-like receptor polymorphisms and allergic disease: interpreting the evidence from genetic studies. Clin Exp Allergy 2004 Feb;34(2):163-6.

(31) Sabroe I, Parker LC, Wilson AG, Whyte MK, Dower SK. Toll-like receptors: their role in allergy and non-allergic inflammatory disease. Clin Exp Allergy 2002 Jul;32(7):984-9.

(32) Mansson A, Fransson M, Adner M, Benson M, Uddman R, Bjornsson S, et al. TLR3 in human eosinophils: functional effects and decreased expression during allergic rhinitis. Int Arch Allergy Immunol 2010;151(2):118-28.

(33) Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, Cardell LO. Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. Respir Res 2005;6:100.

(34) Lun SW, Wong CK, Ko FW, Hui DS, Lam CW. Expression and functional analysis of toll-like receptors of peripheral blood cells in asthmatic patients: implication for immunopathological mechanism in asthma. J Clin Immunol 2009 May;29(3):330-42.

(35) Kormann MS, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. J Allergy Clin Immunol 2008 Jul;122(1):86-92, 92.

(36) Kang I, Oh YK, Lee SH, Jung HM, Chae SC, Lee JH. Identification of polymorphisms in the Toll-like receptor gene and the association with allergic rhinitis. Eur Arch Otorhinolaryngol 2010 Mar;267(3):385-9.

(37) Bjornvold M, Munthe-Kaas MC, Egeland T, Joner G, Dahl-Jorgensen K, Njolstad PR, et al. A TLR2 polymorphism is associated with type 1 diabetes and allergic asthma. Genes Immun 2009 Mar;10(2):181-7.

(38) Kormann MS, Ferstl R, Depner M, Klopp N, Spiller S, Illig T, et al. Rare TLR2 mutations reduce TLR2 receptor function and can increase atopy risk. Allergy 2009 Apr;64(4):636-42.

(39) Eder W, Klimecki W, Yu L, von ME, Riedler J, Braun-Fahrlander C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol 2004 Mar;113(3):482-8.

(40) Senthilselvan A, Rennie D, Chenard L, Burch LH, Babiuk L, Schwartz DA, et al. Association of polymorphisms of toll-like receptor 4 with a reduced prevalence of hay fever and atopy. Ann Allergy Asthma Immunol 2008 May;100(5):463-8.

(41) Sackesen C, Karaaslan C, Keskin O, Tokol N, Tahan F, Civelek E, et al. The effect of polymorphisms at the CD14 promoter and the TLR4 gene on asthma phenotypes in Turkish children with asthma. Allergy 2005 Dec;60(12):1485-92.

(42) Fageras BM, Hmani-Aifa M, Lindstrom A, Jenmalm MC, Mai XM, Nilsson L, et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. J Allergy Clin Immunol 2004 Sep;114(3):561-7.

(43) Kormann MS, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. 2008;122(1).

(44) Hoffjan S, Stemmler S, Parwez Q, Petrasch-Parwez E, Arinir U, Rohde G, et al. Evaluation of the toll-like receptor 6 Ser249Pro polymorphism in patients with asthma, atopic dermatitis and chronic obstructive pulmonary disease. BMC Med Genet 2005 Sep 28;6:34.

(45) Qian FH, Zhang Q, Zhou LF, Jin GF, Bai JL, Yin KS. Polymorphisms in the toll-like receptor 2 subfamily and risk of asthma: a case-control analysis in a Chinese population. J Investig Allergol Clin Immunol 2010;20(4):340-6.

(46) Moller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA, Borglum AD. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax 2008 Dec;63(12):1064-9.

(47) Novak N, Yu CF, Bussmann C, Maintz L, Peng WM, Hart J, et al. Putative association of a TLR9 promoter polymorphism with atopic eczema. Allergy 2007 Jul;62(7):766-72.

(48) Niebuhr M, Langnickel J, Draing C, Renz H, Kapp A, Werfel T. Dysregulation of toll-like receptor-2 (TLR-2)-induced effects in monocytes from patients with atopic dermatitis: impact of the TLR-2 R753Q polymorphism. Allergy 2008 Jun;63(6):728-34.

(49) Ahmad-Nejad P, Mrabet-Dahbi S, Breuer K, Klotz M, Werfel T, Herz U, et al. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. J Allergy Clin Immunol 2004 Mar;113(3):565-7.

(50) Noguchi E, Nishimura F, Fukai H, Kim J, Ichikawa K, Shibasaki M, et al. An association study of asthma and total serum immunoglobin E levels for Toll-like receptor polymorphisms in a Japanese population. Clin Exp Allergy 2004 Feb;34(2):177-83.

(51) Senthilselvan A, Dosman JA, Chenard L, Burch LH, Predicala BZ, Sorowski R, et al. Toll-like receptor 4 variants reduce airway response in human subjects at high endotoxin levels in a swine facility. J Allergy Clin Immunol 2009 May;123(5):1034-40, 1040.

(52) Fransson M, Benson M, Erjefalt JS, Jansson L, Uddman R, Bjornsson S, et al. Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. Respir Res 2007;8:17.

(53) Haagerup A, Borglum AD, Binderup HG, Kruse TA. Fine-scale mapping of type I allergy candidate loci suggests central susceptibility genes on chromosomes 3q, 4q and Xp. Allergy 2004 Jan;59(1):88-94.

(54) Dizier MH, Bouzigon E, Guilloud-Bataille M, Betard C, Bousquet J, Charpin D, et al. Genome screen in the French EGEA study: detection of linked regions shared or not shared by allergic rhinitis and asthma. Genes Immun 2005 Mar;6(2):95-102.

(55) Bu LM, Bradley M, Soderhall C, Wahlgren CF, Kockum I, Nordenskjold M. Genome-wide linkage analysis of allergic rhinoconjunctivitis in a Swedish population. Clin Exp Allergy 2006 Feb;36(2):204-10.

(56) Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002 Dec 16;196(12):1645-51.

(57) Kaisho T, Hoshino K, Iwabe T, Takeuchi O, Yasui T, Akira S. Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. Int Immunol 2002 Jul;14(7):695-700.

(58) Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, et al. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. Clin Exp Allergy 1999 Jan;29(1):28-34.

(59) Robinson DS, Larche M, Durham SR. Tregs and allergic disease. J Clin Invest 2004 Nov;114(10):1389-97.

(60) Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999 Aug 12;341(7):468-75.

(61) Santeliz JV, Van NG, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. J Allergy Clin Immunol 2002 Mar;109(3):455-62.

(62) Creticos PS, Schroeder JT, Hamilton RG, Balcer-Whaley SL, Khattignavong AP, Lindblad R, et al. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. N Engl J Med 2006 Oct 5;355(14):1445-55.

(63) Oh DY, Schumann RR, Hamann L, Neumann K, Worm M, Heine G. Association of the toll-like receptor 2 A-16934T promoter polymorphism with severe atopic dermatitis. Allergy 2009 Nov;64(11):1608-15.

(64) Gao PS, Huang SK. Genetic aspects of asthma. Panminerva Med 2004 Jun;46(2):121-34.

(65) Davila I, Mullol J, Ferrer M, Bartra J, del CA, Montoro J, et al. Genetic aspects of allergic rhinitis. J Investig Allergol Clin Immunol 2009;19 Suppl 1:25-31.

(66) Clayton D, Jones H. Transmission/disequilibrium tests for extended marker haplotypes. Am J Hum Genet 1999 Oct;65(4):1161-9.

(67) Vercelli D, Martinez FD. The Faustian bargain of genetic association studies: bigger might not be better, or at least it might not be good enough. J Allergy Clin Immunol 2006 Jun;117(6):1303-5.

(68) Krishna MT, Mudway I, Kelly FJ, Frew AJ, Holgate ST. Ozone, airways and allergic airways disease. Clin Exp Allergy 1995 Dec;25(12):1150-8.

(69) Eder W, von ME. Hygiene hypothesis and endotoxin: what is the evidence? Curr Opin Allergy Clin Immunol 2004 Apr;4(2):113-7.

(70) Senthilselvan A, Dosman JA. Risk factors for allergic rhinitis in farmers. Agricultural health and safety: workplace, environment, sustainability. CRC Press Inc.; 1995. p. 75-8.

(71) Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, et al. CD14 is required for MyD88-independent LPS signaling. Nat Immunol 2005 Jun;6(6):565-70.

(72) Tulic MK, Wale JL, Holt PG, Sly PD. Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol 2000 May;22(5):604-12.

(73) Vercelli D. Learning from discrepancies: CD14 polymorphisms, atopy and the endotoxin switch. Clin Exp Allergy 2003 Feb;33(2):153-5.

(74) van PN, Le GG, McConnell MJ. Epigenetic regulation of Th2 cytokine expression in atopic diseases. Tissue Antigens 2008 Aug;72(2):91-7.

(75) Shin HJ, Park HY, Jeong SJ, Park HW, Kim YK, Cho SH, et al. STAT4 expression in human T cells is regulated by DNA methylation but not by promoter polymorphism. J Immunol 2005 Dec 1;175(11):7143-50.

(76) Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J Exp Med 2007 Jul 9;204(7):1543-51.

(77) Litonjua AA, Carey VJ, Burge HA, Weiss ST, Gold DR. Parental history and the risk for childhood asthma. Does mother confer more risk than father? Am J Respir Crit Care Med 1998 Jul;158(1):176-81.

(78) Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. J Clin Invest 2008 Oct;118(10):3462-9.

CHAPTER 3

GENES ASSOCIATED WITH ASTHMA, ATOPY, COPD AND OCCUPATIONAL RESPIRATORY DISEASE

In addition to the ten TLR-encoding genes, 15 genes, which have been associated with asthma, atopy or COPD in at least two independent studies, were genotyped as part of the studies reported in this thesis (Table 3-1). They are located across 10 chromosomes and include IL-10, HNMT, CSF2, IL-13, IL-4, TNF-α, TAP1, PON1, NOS3, FcεRIβ, GSTP1, SERPINA3, IL-4Rα, PAF-AH, and TGFB1 genes. Genetic mapping studies reveal that chromosome 5q31-q33 is of particular interest because it contains the cytokine gene cluster including IL-4, IL-5, IL-9, IL-13 and CSF2 genes. The functions of these genes are described in the following sections.

3.1 Cytokine-encoding genes

Although the inflammatory responses to numerous allergic agents are complex, clinical and experimental studies have strongly suggested the involvement of T helper type 2 (TH2) lymphocytes, and the cytokines that they produce, in the development of allergic diseases, such as asthma. Six cytokine-encoding genes and one cytokine receptor-encoding (IL-4Rα) gene are reviewed in this section. Based on their cellular and mediator effects, these 6 cytokine-encoding genes are grouped into four subgroups: lymphokine-encoding genes (IL-4, IL-13); inhibitory cytokine-encoding genes (IL-10); pro-inflammatory-encoding genes (TNF-α, GM-CSF); growth factor-encoding gene (TGF-B1).

3.1.1 Lymphokine-encoding genes

The protein encoded by IL-4 gene (IL-4 cytokine) is mainly produced from TH2 derived T lymphocytes, eosinophils, basophil and mast cells. Experimental studies have shown that IL-4

also drives the differentiation of naive T helper lymphocytes (TH0) into TH2 lymphocytes,[1,2] and promotes survival of resting T cells in vitro.[3] Aerosol antigen challenge of ovalbumin-sensitized mice showed that IL-4 knockout mice had a significant attenuation of inflammation in the airway.[4.]

The protein encoded by the IL-4-receptor gene consists of two chains, an α chain (a specific high affinity chain) and a second chain that can be either the common γ chain or an α chain of the IL-13 receptor (IL-13Rα).[5] The polymorphisms on the IL-4-receptor gene (Arg551Gln, Ile50Val) have been associated with atopy.[6,7]

The protein encoded by the IL-13 gene (IL-13 cytokine) is a cytokine secreted by many cells, TH1, TH2 and TH0, but especially TH2 cells. IL-13 cytokine plays a central role in many allergic lung disease features, which include airway hyperresponsiveness, and mucus hypersecretion. All of these lead to airway obstruction. Although IL-4 drives TH2 cell development, studies have shown that IL-13 cytokine is critical in allergen-induced AHR than IL-4 cytokine.[8] Similar to IL-4, IL-13 suppresses the products of TH1 cells by decreasing the transcription of IFN-γ and IL-12, therefore favouring the development of TH2 cells. Animal models showed that IL-13a2-IgGfc suppresses the increase in mucus secretion, eosinophilia and bronchial hyperresponsiveness following allergen exposure in mice since IL-13a2-IgGfc, a soluble piece of fusion protein, is designed specifically to block IL-13 expression without affecting IL-4 expression.[9]

3.1.2 Inhibitory-encoding gene

The protein encoded by the IL-10 gene (IL-10 cytokine) is an inhibitory cytokine, produced primarily by monocytes. It can down-regulate the products of not only pro-inflammatory cytokines like IFN-γ, IL-2, IL-3, TNF-α and CSF but also chemokines, adhesion

molecules and antigen-presenting and costimulatory molecules in monocytes/macrophages, neutrophils and T cells.[10] Studies showed that IL-10 controls inflammatory processes by inhibiting NF-κB, which transcriptionally controls the production of pro-inflammatory proteins.[11] In addition to its role in anti-inflammation, it also acts on B cells to enhance immunoglobulin secretion.[12,13] A study by Jeannin et al showed that IL-10 cytokine has a differential effect on IgE vs. IgG4 production by peripheral blood mononuclear cells.[12] It decreases production of IgE induced by IL-4 cytokine, but enhances IgE production in B cells that are already switched to produce IgE. Due to its anti-inflammatory property, IL-10 cytokine is considered as a potential therapy for allergic diseases.[13]

3.1.3 Proinflammatory-encoding genes

In responses to infection, the products of some cytokine-encoding genes are up-regulated during inflammation. Pro-inflammatory cytokines are important mechanisms of defense against pathogenic microorganisms. These cytokines have the ability to kill pathogenic organisms and to inhibit or induce apoptosis of those damaged cells.[14,15] The release of inflammatory cytokines will also recruit and activate many additional inflammatory cells and cytokines into the damaged sites, thus amplifying the inflammation process. Many cytokines including TNF-α have been associated with many respiratory diseases such as asthma.[16-18]

The human TNF gene contains 4 exons with the last exon coding for more than 80% of the protein. The protein encoded by the TNF gene (TNF cytokine) has two major forms, TNF-α and TNF-β. TNF cytokine is mainly produced by macrophage cells as a type II trans-membrane protein in the form of homotrimers.[19] TNF-α is also expressed by other cells, such as T lymphocytes, mast cells and epithelial cells. Kampf et al showed that exposure of human

bronchial epithelia cells to TNF- α, INF-γ and IL-β can cause cell damage and induce both necrosis and apoptosis.[20]

Another proinflammatory-encoding gene is colony stimulating factor (CSF). The protein encoded by the CSF gene (CSF cytokine) is produced by several airway cells such as macrophages, eosinophils, T lymphocytes, airway smooth muscle cells, and epithelial cells.[21] Granulocyte-Macrophage-CSF (GM-CSF) can promote production of inflammatory cells, such as granulocytes (neutrophils, eosinophils, and basophils) and monocytes, and prolong the survival of eosinophils in culture.[22,23] An increased GM-CSF level was observed in bronchoalveolar lavage (BAL) and bronchial epithelial cells of asthma patients.[24,25] Over-expression of GM-CSF is only seen among asthma patients since it is not detectable in sputum from COPD patients,[26] and occurs at lower level in biopsy tissue from chronic bronchitis patients.[27.] GM-CSF also plays an important role in maturation of Dentritic cells by increasing antigen-specific B- and T-cell.[28,29]

3.1.4 Transforming Growth factor encoding gene (TGF-β_1)

The protein encoded by the TGFB1 gene (TGF-β_1 cytokine) is multifunctional and involved in cell proliferation and differentiation. Dysregulation of TGF-β_1 activation and signalling leads to apoptosis.[30] TGF-β_1 is secreted by most of immune cells, including lymphocytes, macrophages, and dendritic cells, and plays an important role in controlling the differentiation, proliferation, and activation of these immune cells. Increased TGF-β_1 expression was found in bronchoalveolar lavage fluid, bronchial biopsies, and plasma from patients with asthma.[31-33] TGF-β_1 has been associated in several aspects of fibrosis, including the deposition of extracellular matrix proteins such as collagens and fibronectin.[34,35] A promoter polymorphism at C-

509T has been shown to be associated with elevated plasma TGF-β_1 levels,[36] total IgE,[37] and asthma risk.[38,39]

3.2 Other genes

3.2.1 FcεRIβ-encoding gene

The high-affinity IgE receptor, FcεRI, is a tetrameric receptor complex consisting of one alpha (FcεRIα), one beta (FcεRIβ), and two gamma chains (FcεRIγ). It is expressed on mast cells and basophils, and is involved in the mediation of allergic responses.[40] Free IgE binds with high affinity to the alpha subunit of the receptor, whereas beta and gamma subunits are involved with signal transduction. In transfected cells and transgenic mice, the presence of the beta subunit in the receptor has been shown to increase IgE-triggered signalling events by 5 to 7 times[41,42] and to amplify the expression of the surface IgE receptor.[43] The Beta subunit has been associated with atopy in genomic studies possible due to an amplification function.[44] A common variant of the FcεRIβ gene, Ile181Leu within the 4th transmembrane domain showed a strong association with atopy.[45]

3.2.2 Glutathione S-transferase encoding genes (GST)

Hundreds of genetic association studies on asthma-related phenotypes have been conducted. However, there are only a limited number of genes that have been associated with asthma in at least one study.[46] The GSTP1 gene has been associated with asthma, IgE and SPT in a British population,[47,48] with Isocyanate-induced asthma in an Italian population,[49] and with lung function growth in an American population.[50] Glutathione S-transferase (GST) is involved in the biotranformation of xenobitics, such as drugs and poisons by conjugating these compounds with reduced glutathione to facilitate dissolution. The protein encoded by the GSTP1 gene is the

most common isoform of GST in lung epithelium, and the polymorphism of the GSTP1 gene has shown to protect against developing asthma possibly because of a role in protection against oxidative stress.[47,51]

3.2.3 Histamine N-methyltransferase-encoding gene (HNMT)

Histamine is an important mediator in the allergic responses. It functions as a potent bronchoconstrictor in human lungs, and produced from mast cells and basophils after cross-linking of surface-bound IgE by allergen.[52] The protein encoded by the HNMT gene (HNMT) is involved in the metabolism of histamine by forming N-methylhistamine. It is not surprising that polymorphisms of the HNMT gene has been linked with allergic diseases since the role of histamine in the allergic responses. A SNP (C314T) of the HNMT gene, a functional polymorphism resulting in an amino acid change (Thr105Ile), is associated with decreased enzyme activity (up to 50%).[53] A population study showed that Caucasian children with genotypes conferring reduced HNMT activity were 2 times more likely to have atopic dermatitis than those who were homozygous for the C314 allele (OR=2.3, 95% CI: 1.1-4.6).[54] A positive association of this SNP and asthma was also reported in another study in Caucasian population.[55] However, studies carried out in a German,[56] a Japanese populations,[57] and an Indian populations,[58] failed to confirm this association.

3.2.4 Nitric oxide synthases3 encoding gene (NOS3)

Nitric Oxide (NO) is an intercellular messenger synthesized from L-arginine by NOS. There is increasing evidence showing that NO plays a key role in physiological and pathophysiological events of the lungs. [59-63] It acts as a vasodilator, neurotransmitter and inflammatory mediator,[59,60] and induces non-specific toxic effect in responses to the infection with bacteria, viruses and parasites.[61] Studies have found that asthmatics are more likely to have

increased level of NO in their expired gas.[62,63] There are two types of constitutive NOS (cNOS) including neuronal NOS (nNOS) and endothelial NOS (eNOS), and a single type of inducible NOS (iNOS). In humans, each is encoded by distinct genes: nNOS by the *NOS1* gene; iNOS by the *NOS2* gene; and eNOS by the *NOS3* gene. The three human *NOS* genes, *NOS1*, *NOS2* and *NOS3* genes, are located at 12q24, 17q11.2-q12 and 7q36, respectively, which harbour many loci associated with asthma.[64,65] Polymorphisms in the NOS3 gene have been associated with atopy, bronchial hyper-responsiveness (BHR), total and specific IgE in asthmatics, and atopic asthma in many studies.[66-68] A population study showed that a misssense mutation in the NOS3 gene (G895T) was associated with decreased NO levels in exhaled air in adults with asthma.[69]

3.2.5 Platelet-activating factor-acetylhydrolase encoding gene (PAF-AH)

Platelet-activating factor (PAF) is a strong pro-inflammatory phospholipid which is involved in the signaling and activation of many proinflammatory cells including platelets, neutrophils, and macrophages.[70] The release and degradation of PAF is controlled by the PAF-AH gene.[71] Low activity of PAF-AH has been linked with asthma among Japanese children.[72] The gene for plasmatic PAF-AH is located at 6p21.2-p12.[73] Two common variants in exon 9 of the PAF-AH gene, V279F and Gln281Arg, have been linked to severe asthma in Japanese population.[74] Other common variants, Ile198Thr in exon 7, and Ala379Val in exon 11 of the PHF-AH gene have also been associated increased risk of atopy and asthma in a Caucasian population.[75]

3.3 Genes involved in LPS signalling

In addition to the TLRs, there are many co-receptors which are required for full ligand sensitivity, such as CD14 in the case of TLR4's recognition of LPS, and adaptor proteins to

propagate signals within cytoplasm of cells, such as MyD88, which ultimately leads to the activation of transcription factor, such as nuclear factor-kappa B (NF-κB1, NF-κBIA, NF-κBIB, IKBKG, REL, RELA).

All TLRs and interleukin 1 receptors (IL-1Rs) have a considerable homology in their cytoplasmic domain known as Toll-IL-1R (TIR), which is also presented on MyD88, an important adaptor protein used by all TLRs. After ligand binding, MyD88 is recruited to the receptor complex by the TIR domain. The death domain of MyD88 is used to recruit members of IL-1 receptor-associated kinase (IRAK) family. IRAK1 is phosphorylated and leaves the membrane to activate tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). Transforming growth factor-β-activated kinase (TAK-1) is then activated by a complex of ubiquitinated TRAF6 and TAK-1 binding protein 2 (TAB2). Activated TAK-1 can activate the IkBα kinase complex (IKK), which consists of IKKα, IKKβ and IKKγ. The phosphorylation of IkBα leads to its degradation, the release of NF-κB, and the activation of NF-κB-dependent genes, such as TNF-α, IL-1 and IL-6. The MyD88-dependent signalling pathway is shared by all members of TLR family and results in inflammatory responses.

3.3.1 CD14 encoding gene (Cluster of differentiation 14)

The gene encoding CD14 is located on 5q31 with 1.5kb span and separated by a short intron.[76] It has two distinct forms: membrane CD14 (mCD14) and soluble CD14 (sCD14). The mCD14 is mainly expressed by macrophages, monocytes and neutrophils, [77]whereas sCD14 appears to derive primarily from monocytes.[77] CD14 transfers LPS and other ligands to the TLR4/MD-2 signaling complex.[78] The activation of these cells through CD14 increases production of IL-12, which is believed to have a key role in induction of Th1 immune responses.[79] A common single nucleotide polymorphism at the promoter region of the CD14

gene, -159C/T, has been associated with increased sCD14 levels in serum and decreased serum IgE level in children with TT homozygotes than those with CT or CC genotypes in an American population,[80] and in an adult Dutch population.[81]

3.3.2 MYD88 encoding gene (myeloid differentiation primary response gene 88)

The adapter protein encoded by the MYD88 gene is involved in TLRs and IL-1 receptor signalling pathways, which ultimately lead to activation of transcription factors, such as nuclear factor-kB (NF-κB) and members of the interferon (IFN)-regulatory factor (IRF) family.[82] This pathway regulates expression of many pro-inflammatory cytokines and chemokines, which all contain the kB site for NF-κB.[83,84] Therefore, the MYD88 gene plays a central role in the innate and adaptive immune response. Studies showed that mice with MYD88-deficience are highly susceptible to a broad range of pathogens at least 36 pathogens (19 bacteria, 7 viruses, 5 parasites, 4 fungi)[85] and do not produce TNF or IL-6 when exposed to IL-1 or TLR ligands.[86,87] Humans with MYD88-deficience are also at increased risk of infection, which is restricted to pyogenic bacterial diseases including invasive pneumococcal disease (IPD).[88] Although this infection is lethal in children (younger than 10 years old), it seems less important for survival in older children and adults possible due to the maturation of TIR-independent innate immunity and adaptive immunity.[85]

3.3.3 NF-κB encoding gene (Nuclear Factor-KappaB)

NF-κB gene plays a central role in immunological processes since its ability to transcriptionally regulate many pro-inflammatory genes.[89] NF-κB/Rel transcription family is composed of homo- and heterodimers of five members of the Rel family including NF-κB1(p50), NF-κB2(p52), RelA(p65), RelB, c-Rel (Rel) with the most common form of a NF-κB1 or NF-κB2 subunit and RelA.[90] NF-κB is usually inactive in cytoplasm by a regulatory protein,

inhibitor of κB (IκB) and phosphorylation of IκB by a protein IκB kinase (IKK) leads to activation of NF-κB.[90] The IKK family consists of IKK-α, IKK-β and a regulatory subunit IKK-γ.[91,92] NF-κB can be activated by many stimuli, including stress-induced, immune, and proinflammatory cytokines (such as IL-1β and TNF-α).[93] Studies showed that NF-κB was significantly increased, by about 2.5 fold in induced sputum of asthma patients than normal projects, especially in epithelial cells and macrophages.[94] Many pro-inflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α), chemokines (RANTES, eotaxin), adhesion molecules and inflammatory enzymes (iNOS) are significantly over expressed in asthmatic airways.[90]

3.4 Genetic susceptibility of occupational respiratory disease

Although exposure factors including types of the respiratory hazards, timing, intensity and duration of exposure are critical in the etiology of respiratory diseases in workers, individual genetic make-up plays an important role in determining the ways our body interacts with and responds to these hazards, which often results in very different outcomes among workers in the same work place with apparently similar exposures. Over the years, many genes have been associated with susceptibility to one or many occupational respiratory diseases. In this section, I describe the genes which have been recently reported to be associated with occupational respiratory disease.

3.4.1 Human leukocyte antigen DPB1encoding gene (HLA-DPB1)

The proteins encoded by the HLA genes comprise the human major histocompatibility complex (MHC), a family of proteins recognised to be important in immunity that are found on cell surfaces of many animals including humans. HLA-DP is part of MHC Class II family. It has

two subunits, DPα and DPβ, which are encoded by the HLA-DPA1 and the HLA-DPB1 genes on human chromosome 6, respectively.

Studies showed that among individuals exposed to beryllium at work, those carrying at least one copy of glutamic acid at position 69 of the DPB1 gene (heterozygous Glu^{69} and homozygous Glu^{69}) were significantly associated with higher risk of developing chronic beryllium disease.[95,96] Furthermore, among workers with presence of Glu^{69}, individuals also carrying Lysine at position 11(Lys^{11}) and Aspartic acid at position 55 (Asp^{55}) were significantly associated with additional risk of beryllium hypersensitivity.[97] The presence of Glu^{69} on the DPB1 gene has also been associated with increased risk of interstitial lung disease (hard metal lung disease) in a case-control study, in which individuals carrying Glu^{69} were found in 19 out of 20 cases, as compared to 17 out of 35 in controls who were exposed but not affected ($p=0.0014$).[98]

3.4.2 Human leukocyte antigen DQB1encoding gene (HLA-DQB1)

The protein encoded by the HLA-DQ gene is also a member of human major histocompatibility complex Class II family. The HLA-DQ gene product is a cell surface receptor, which is found on antigen presenting cells including Dendritic cells (DCs) and Macrophages. It has two chains α and β, which are encoded by the HLA-DQA1 and the HLA-DQB1 genes, respectively. Both genes are located on chromosome 6 (6p21.3) and next to each other. They play an important role in the immune system.

Silicosis is an occupational lung disease caused by inhalation of crystalline silica dust. Small silica dust particles can deeply embed in the distal parts of the lungs, which results in a fibrotic reaction. HLA-DQB1*0402 has been associated with an increased risk of silicosis in a study from Japan.[99] This gene also seems important in asthma. One study of occupational

asthma induced by isocyanates found the condition was negatively associated with HLA-DQB1*0501 and positively associated with HLA-DQB1*0503.[100] A higher frequency of HLA-DQB1*0603 and DQB1*0302 alleles has been observed among patients with red cedar asthma than those healthy controls exposed to red cedar dust in a study from Canada.[101]

3.4.3 Human leukocyte antigen DRB1 encoding gene (HLA-DRB1)

The protein encoded by the HLA-DR gene is also a member of MHC class II cell surface receptor. HLA-DR is a heterodimer of α and β subunits, which are anchored in the cell membrane. It is thought the primary function of HLA-DR is to present extracellular proteins by antigen presenting cells. HLA-DR α and β subunits are encoded by the HLA-DRA and the HLA-DRB genes, respectively.

In a study of western red cedar asthma in Canada, which included 56 patients with proven red cedar asthma and 63 healthy controls subjects exposed to red cedar dust, the frequency of the DRB1*0401-DQB1*0302 haplotype was found to be over-represented in the patients, while, the frequency of the DRB1*0101-DQB1*0501 haplotype was reduced in patients compared to the control subjects.[101] In another study of toluene diisocyanate (TDI)-induced occupational asthma (TDI-OA) in Koreans, which included 84 TDI-OA patients and 47 asymptomatic exposed controls (AECs), the frequency of the HLA-DRB1*1501-DQB1*0602-DPB1*0501 haplotype was significantly higher in the TDI-OA patients than in the AEC.[102] In addition to occupational asthma, HLA-DRB1 allele is also associated with sarcoidosis due to occupational exposure to Beryllium, organic antigens, zirconium, aluminum and titanium.[103] HAL-DRB1*0803 was significantly associated with increased risk of sarcoidosis in a study from Japan (OR=2.43, $p<0.0001$).[104]

3.4.4 Butyrophilin-like 2 encoding gene (BTNL2)

Butyrophilins encoded by the Butyrophilin-like (BTN) gene are type I membrane proteins belonging to the immunoglobulin (Ig) superfamily, with two extracellular Ig-like domains, which are similar to the Ig V (variable) and Ig C (constant) domains. The extracellular domains of BTN are structurally similar to the B7 family, which plays an essential role in the regulation of T cells.[105,106] The human BTN family has 13 genes. Some BTN genes, such as the BTNL2 gene, have been shown to control T cells by inhibiting the proliferation of CD(+4) T cells.[107] A study of 947 independent cases of sarcoidosis in Germany showed that a missense polymorphism of the BTNL2 gene (rs2076530), which resulted in a transition of G→A, was significantly associated with sarcoidosis (OR=2.75 in homozygotes; OR=1.60 in heterozygotes).[108]

3.4.5 Tumor necrosis factors encoding gene (TNF)

The TNF gene was described in detail in section 3.1.3. A prospective cohort study of coal workers' pneumoconiosis (CWP) in 253 coal miners showed a strong gene-environment interaction between a promoter polymorphism at the -308 position (G>A) in the TNF-α gene and occupational exposure to coal dust.[109] This association was also observed in another study of 78 ex-coal miners from the Belgian coal mining industry, in which the A^{-308} genotype in TNF-α was significantly higher in coal miners with a diagnosis of CWP (50%) compared with miners without CWP (25%).[110] In a case-control study of silicosis among black south African gold miners, two polymorphisms in the promoter region of TNF-α (A^{-238} and A^{-376}) were significantly over-represented in gold miners with severe silicosis compared with gold miners without silicosis (A^{-238}: 33% vs. 6%; A^{-376}: 33% vs. 5%).[111]

3.4.6 Glutathione S- transferase encoding gene (GST)

The GST gene was described in detail in section 3.2.2. One of the GST proteins, GSTM1, encoded by the GSTM1 gene (null allele), which results in an inability to produce of the protein,

also leads to decreased antioxidant capability. Polymorphisms at position + 105 in the GSTP1 gene are associated with altered enzyme kinetics. [112]

A study of 182 workers exposed to diisocyanate (109 workers with diisocyanate-induced asthma (DIA); 73 exposed workers without diagnosis of DIA) showed that workers with null genotype in the GSTM1 gene had an increased risk of DIA (OR=1.89; 95%CI: 1.01-3.52).[112]

3.4.7 Toll-like receptors encoding genes (TLRs)

TLRs were described in detail in the Chapter 2. The study described in Chapter 5 of this thesis was the first to show that TLR2-16933T/A and Arg677Trp polymorphisms, rather than TLR4 Asp299Gly and Thr399Ile polymorphisms, were significantly associated protective effects on lung function among workers in swine operations. An experimental study also showed the involvement of TLR2, rather than TLR4, in human bronchial epithelial cells when exposed to hog confinement dusts.[113] However, another study of 381 bakery workers from Korea showed that workers with TLR4 variants (-2027A>G and -1608T>C) had a significantly lower prevalence of work-related lower respiratory symptoms than those with wild-type under recessive inheritance model, which was also confirmed by haplotype analysis.[114]

3.4.8 Nitro Oxide Synthesis encoding genes (NOS)

NOS genes were described in detail in section 3.2.4. Increased levels of NO in exhaled air have been reported among people with asthma,[115,116] among healthy naïve volunteers after 5-hour exposure in a pig confinement building[117] and swine confinement workers,[118] and among coal miners (both symptomatic and asymptomatic workers).[119] The study described in Chapter 6 of this thesis was the first to show that polymorphisms in the NOS3 gene were significantly associated with protective effects on lung function among workers in swine operations. This association was observed in two independent cohorts, a cross-sectional study and a longitudinal study.

3.5 Summary

Exposure to high level of respiratory hazards at work has been associated with increased risk of respiratory disorders in many epidemiological studies. Although the etiology is still not clear, individual genetic-makeup plays an important role. Many experimental studies and human studies using naïve healthy adults have shown the involvement of the TLR-encoding genes, pro-inflammatory cytokine-encoding genes, genes on the TLR signaling pathway and in the pathophysiological process. Many genes have been associated with susceptibility to one or many occupational respiratory diseases. Now, it is clear that these respiratory disorders are polygenic and multifactorial disorders, which involves many genes, environmental exposure factors and interplays between them including gene-gene and gene-environment interactions. So far, there is little work on effects of polymorphisms in candidate genes on respiratory inflammation and lung function in workers in swine operations.

Although many genes and environmental factors collectively contribute to the respiratory problems in workers in swine operations, the polymorphisms in the TLR2, TLR4 and NOS3 genes appear pertinent to pulmonary events in the workers. The respiratory hazards in swine operations include high concentration of dust, Gram-positive and Gram-negative bacteria and many gases. Endotoxin, mainly from Gram-negative bacteria, plays a significant role in lung dysfunction.[120] The ligand of TLR4 is endotoxin; therefore polymorphisms in the TLR4 gene could be significantly associated with lung dysfunction in workers in swine operations. In addition to Gram-negative bacteria, high concentration Gram-positive bacteria, whose membrane structure, peptidoglycan is the ligand to TLR2, was reported in the indoor air in swine operations.[121] Therefore, the polymorphisms in the TLR2 gene may be relevant to the increased

risk of respiratory diseases in workers in swine operations. Many studies showed that increased levels of NO in exhaled air among workers in swine operations and healthy naïve volunteers after 5-hour exposure in pig confinement building.[122,123] The polymorphisms in the NOS3 gene could be an important factor contributing to the increased risk of respiratory disease in the workers. Only polymorphisms in the TLR2, TLR 4 and NOS3 genes were considered in this study.

Table 3-1 Chromosome position of the candidate genes

Chromosome	Number of genes	**Genes**(*Region*)
1	2	**IL-10**(*q31*)
2	2	**REL**(*p12-p13*), **HNMT**(*q22*)
3	2	**MYD88**(*p22*)
4	6	**NF-κB1**(*q24*)
5	4	**CD-14**(*q22-q32*), **CSF2, IL-4, IL-13**(*q31*)
6	2	**TNF-α, TAP1**(*p21*)
7	2	**PON1**(*q21*), **NOS3**(*q36*)
11	3	**FcεRIβ, GSTP1, RELA**(*q13*)
14	2	**NF-κBIA**(*q13*), **SERPINA3**(*q32*)
16	1	**IL-4Rα**(*p12*)
17	1	**PAF-AH**(*p13*)
19	2	**TGFB1, NF-κBIB**(*q13*)
X	2	**IKBKG**(*q28*)

3.6 References

(1) Seder RA, Paul WE, Davis MM, Fazekas de St GB. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J Exp Med 1992 Oct 1;176(4):1091-8.

(2) Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. Proc Natl Acad Sci U S A 1992 Jul 1;89(13):6065-9.

(3) Vella A, Teague TK, Ihle J, Kappler J, Marrack P. Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: stat6 is probably not required for the effect of IL-4. J Exp Med 1997 Jul 21;186(2):325-30.

(4) Coyle AJ, Le GG, Bertrand C, Tsuyuki S, Heusser CH, Kopf M, et al. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. Am J Respir Cell Mol Biol 1995 Jul;13(1):54-9.

(5) Jiang H, Harris MB, Rothman P. IL-4/IL-13 signaling beyond JAK/STAT. J Allergy Clin Immunol 2000 Jun;105(6 Pt 1):1063-70.

(6) Izuhara K, Shirakawa T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. Int J Mol Med 1999 Jan;3(1):3-10.

(7) Shirakawa I, Deichmann KA, Izuhara I, Mao I, Adra CN, Hopkin JM. Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunol Today 2000 Feb;21(2):60-4.

(8) Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999 Mar;103(6):779-88.

(9) Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998 Dec 18;282(5397):2261-3.

(10) Moore KW, de Waal MR, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

(11) Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy--review of a new approach. Pharmacol Rev 2003 Jun;55(2):241-69.

(12) Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. J Immunol 1998 Apr 1;160(7):3555-61.

(13) Pretolani M, Goldman M. IL-10: a potential therapy for allergic inflammation? Immunol Today 1997 Jun;18(6):277-80.

(14) Raitano AB, Korc M. Tumor necrosis factor up-regulates gamma-interferon binding in a human carcinoma cell line. J Biol Chem 1990 Jun 25;265(18):10466-72.

(15) Wu AJ, Kurrasch RH, Katz J, Fox PC, Baum BJ, Atkinson JC. Effect of tumor necrosis factor-alpha and interferon-gamma on the growth of a human salivary gland cell line. J Cell Physiol 1994 Nov;161(2):217-26.

(16) Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. J Allergy Clin Immunol 1992 May;89(5):958-67.

(17) Gosset P, Tsicopoulos A, Wallaert B, Joseph M, Capron A, Tonnel AB. Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. Am Rev Respir Dis 1992 Sep;146(3):768-74.

(18) Hallsworth MP, Soh CP, Lane SJ, Arm JP, Lee TH. Selective enhancement of GM-CSF, TNF-alpha, IL-1 beta and IL-8 production by monocytes and macrophages of asthmatic subjects. Eur Respir J 1994 Jun;7(6):1096-102.

(19) Tang P, Hung M-C, Klostergaard J. Human pro-tumor necrosis factor is a homotrimer. Biochemistry 1996 Jun 25;35(25):8216-25.

(20) Kampf C, Relova AJ, Sandler S, Roomans GM. Effects of TNF-alpha, IFN-gamma and IL-beta on normal human bronchial epithelial cells. Eur Respir J 1999 Jul;14(1):84-91.

(21) Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G. The allergic cascade: review of the most important molecules in the asthmatic lung. Immunol Lett 2007 Oct 31;113(1):6-18.

(22) Hallsworth MP, Litchfield TM, Lee TH. Glucocorticoids inhibit granulocyte-macrophage colony-stimulating factor-1 and interleukin-5 enhanced in vitro survival of human eosinophils. Immunology 1992 Feb;75(2):382-5.

(23) Hallsworth MP, Giembycz MA, Barnes PJ, Lee TH. Cyclic AMP-elevating agents prolong or inhibit eosinophil survival depending on prior exposure to GM-CSF. Br J Pharmacol 1996 Jan;117(1):79-86.

(24) Sousa AR, Poston RN, Lane SJ, Nakhosteen JA, Lee TH. Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. Am Rev Respir Dis 1993 Jun;147(6 Pt 1):1557-61.

(25) Marini M, Vittori E, Hollemborg J, Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. J Allergy Clin Immunol 1992 May;89(5):1001-9.

(26) Betz R, Kohlhaufl M, Kassner G, Mullinger B, Maier K, Brand P, et al. Increased sputum IL-8 and IL-5 in asymptomatic nonspecific airway hyperresponsiveness. Lung 2001;179(2):119-33.

(27) Vignola AM, Chanez P, Chiappara G, Siena L, Merendino A, Reina C, et al. Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis. J Allergy Clin Immunol 1999 Apr;103(4):563-73.

(28) De ST, Butz E, Smith J, Maldonado-Lopez R, Pajak B, Moser M, et al. CD8alpha(-) and CD8alpha(+) subclasses of dendritic cells undergo phenotypic and functional maturation in vitro and in vivo. J Leukoc Biol 2001 Jun;69(6):951-8.

(29) Nguyen CL, Bui JT, Demcheva M, Vournakis JN, Cole DJ, Gillanders WE. Sustained release of granulocyte-macrophage colony-stimulating factor from a modular peptide-based cancer vaccine alters vaccine microenvironment and enhances the antigen-specific T-cell response. J Immunother 2001 Sep;24(5):420-9.

(30) Neptune ER, Frischmeyer PA, Arking DE, Myers L, Bunton TE, Gayraud B, et al. Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet 2003 Mar;33(3):407-11.

(31) Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, et al. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. Am J Respir Crit Care Med 1997 Aug;156(2 Pt 1):642-7.

(32) Kokturk N, Tatlicioglu T, Memis L, Akyurek N, Akyol G. Expression of transforming growth factor beta1 in bronchial biopsies in asthma and COPD. J Asthma 2003 Dec;40(8):887-93.

(33) Joseph J, Benedict S, Badrinath P, Wassef S, Joseph M, Abdulkhalik S, et al. Elevation of plasma transforming growth factor beta1 levels in stable nonatopic asthma. Ann Allergy Asthma Immunol 2003 Nov;91(5):472-6.

(34) Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1989 Mar 11;1(8637):520-4.

(35) Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest 1997 Aug 15;100(4):768-76.

(36) Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, et al. Genetic control of the circulating concentration of transforming growth factor type beta1. Hum Mol Genet 1999 Jan;8(1):93-7.

(37) Hobbs K, Negri J, Klinnert M, Rosenwasser LJ, Borish L. Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. Am J Respir Crit Care Med 1998 Dec;158(6):1958-62.

(38) Pulleyn LJ, Newton R, Adcock IM, Barnes PJ. TGFbeta1 allele association with asthma severity. Hum Genet 2001 Dec;109(6):623-7.

(39) Nagpal K, Sharma S, Rao C, Nahid S, Niphadkar PV, Sharma SK, et al. TGFbeta1 haplotypes and asthma in Indian populations. J Allergy Clin Immunol 2005 Mar;115(3):527-33.

(40) Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. Annu Rev Immunol 1999;17:931-72.

(41) Lin S, Cicala C, Scharenberg AM, Kinet JP. The Fc(epsilon)RIbeta subunit functions as an amplifier of Fc(epsilon)RIgamma-mediated cell activation signals. Cell 1996 Jun 28;85(7):985-95.

(42) Dombrowicz D, Lin S, Flamand V, Brini AT, Koller BH, Kinet JP. Allergy-associated FcRbeta is a molecular amplifier of IgE- and IgG-mediated in vivo responses. Immunity 1998 Apr;8(4):517-29.

(43) Donnadieu E, Jouvin MH, Kinet JP. A second amplifier function for the allergy-associated Fc(epsilon)RI-beta subunit. Immunity 2000 May;12(5):515-23.

(44) Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, et al. A genome-wide search for quantitative trait loci underlying asthma. Nature 1996 Sep 19;383(6597):247-50.

(45) Shirakawa T, Li A, Dubowitz M, Dekker JW, Shaw AE, Faux JA, et al. Association between atopy and variants of the beta subunit of the high-affinity immunoglobulin E receptor. Nat Genet 1994 Jun;7(2):125-9.

(46) Hoffjan S, Nicolae D, Ober C. Association studies for asthma and atopic diseases: a comprehensive review of the literature. Respir Res 2003;4:14.

(47) Fryer AA, Bianco A, Hepple M, Jones PW, Strange RC, Spiteri MA. Polymorphism at the glutathione S-transferase GSTP1 locus. A new marker for bronchial hyperresponsiveness and asthma. Am J Respir Crit Care Med 2000 May;161(5):1437-42.

(48) Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 1998 Feb;19(2):275-80.

(49) Mapp CE, Fryer AA, De MN, Pozzato V, Padoan M, Boschetto P, et al. Glutathione S-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. J Allergy Clin Immunol 2002 May;109(5):867-72.

(50) Gilliland FD, Gauderman WJ, Vora H, Rappaport E, Dubeau L. Effects of glutathione-S-transferase M1, T1, and P1 on childhood lung function growth. Am J Respir Crit Care Med 2002 Sep 1;166(5):710-6.

(51) Spiteri MA, Bianco A, Strange RC, Fryer AA. Polymorphisms at the glutathione S-transferase, GSTP1 locus: a novel mechanism for susceptibility and development of atopic airway inflammation. Allergy 2000;55 Suppl 61:15-20.

(52) Horton JR, Sawada K, Nishibori M, Zhang X, Cheng X. Two polymorphic forms of human histamine methyltransferase: structural, thermal, and kinetic comparisons. Structure 2001 Sep;9(9):837-49.

(53) Preuss CV, Wood TC, Szumlanski CL, Raftogianis RB, Otterness DM, Girard B, et al. Human histamine N-methyltransferase pharmacogenetics: common genetic polymorphisms that alter activity. Mol Pharmacol 1998 Apr;53(4):708-17.

(54) Kennedy MJ, Loehle JA, Griffin AR, Doll MA, Kearns GL, Sullivan JE, et al. Association of the histamine N-methyltransferase C314T (Thr105Ile) polymorphism with atopic dermatitis in Caucasian children. Pharmacotherapy 2008 Dec;28(12):1495-501.

(55) Yan L, Galinsky RE, Bernstein JA, Liggett SB, Weinshilboum RM. Histamine N-methyltransferase pharmacogenetics: association of a common functional polymorphism with asthma. Pharmacogenetics 2000 Apr;10(3):261-6.

(56) Deindl P, Peri-Jerkan S, Deichmann K, Niggemann B, Lau S, Sommerfeld C, et al. No association of histamine- N-methyltransferase polymorphism with asthma or bronchial hyperresponsiveness in two German pediatric populations. Pediatr Allergy Immunol 2005 Feb;16(1):40-2.

(57) Sasaki Y, Ihara K, Ahmed S, Yamawaki K, Kusuhara K, Nakayama H, et al. Lack of association between atopic asthma and polymorphisms of the histamine H1 receptor, histamine H2 receptor, and histamine N-methyltransferase genes. Immunogenetics 2000 Mar;51(3):238-40.

(58) Sharma S, Mann D, Singh TP, Ghosh B. Lack of association of histamine-N-methyltransferase (HNMT) polymorphisms with asthma in the Indian population. J Hum Genet 2005;50(12):611-7.

(59) Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991 Jun;43(2):109-42.

(60) Birrell MA, McCluskie K, Hardaker E, Knowles R, Belvisi MG. Utility of exhaled nitric oxide as a noninvasive biomarker of lung inflammation in a disease model. Eur Respir J 2006 Dec;28(6):1236-44.

(61) Liew FY, Cox FE. Nonspecific defence mechanism: the role of nitric oxide. Immunol Today 1991 Mar;12(3):A17-A21.

(62) Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 1994 Jan 15;343(8890):133-5.

(63) Barnes PJ. NO or no NO in asthma? Thorax 1996 Feb;51(2):218-20.

(64) Barnes PJ. Neurogenic inflammation in the airways. Respir Physiol 2001 Mar;125(1-2):145-54.

(65) Ober C, Cox NJ, Abney M, Di RA, Lander ES, Changyaleket B, et al. Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. Hum Mol Genet 1998 Sep;7(9):1393-8.

(66) Bourgain C, Hoffjan S, Nicolae R, Newman D, Steiner L, Walker K, et al. Novel case-control test in a founder population identifies P-selectin as an atopy-susceptibility locus. Am J Hum Genet 2003 Sep;73(3):612-26.

(67) Lee YC, Cheon KT, Lee HB, Kim W, Rhee YK, Kim DS. Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma. Allergy 2000 Oct;55(10):959-63.

(68) Holla LI, Buckova D, Kuhrova V, Stejskalova A, Francova H, Znojil V, et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. Clin Exp Allergy 2002 Aug;32(8):1193-8.

(69) Storm van's GK, Wechsler ME, Grasemann H, Silverman ES, Le L, Palmer LJ, et al. Association of a missense mutation in the NOS3 gene with exhaled nitric oxide levels. Am J Respir Crit Care Med 2003 Jul 15;168(2):228-31.

(70) Prescott SM, Zimmerman GA, McIntyre TM. Platelet-activating factor. J Biol Chem 1990 Oct 15;265(29):17381-4.

(71) Tjoelker LW, Wilder C, Eberhardt C, Stafforini DM, Dietsch G, Schimpf B, et al. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. Nature 1995 Apr 6;374(6522):549-53.

(72) Miwa M, Miyake T, Yamanaka T, Sugatani J, Suzuki Y, Sakata S, et al. Characterization of serum platelet-activating factor (PAF) acetylhydrolase. Correlation between deficiency of serum PAF acetylhydrolase and respiratory symptoms in asthmatic children. J Clin Invest 1988 Dec;82(6):1983-91.

(73) Stafforini DM, Satoh K, Atkinson DL, Tjoelker LW, Eberhardt C, Yoshida H, et al. Platelet-activating factor acetylhydrolase deficiency. A missense mutation near the active site of an anti-inflammatory phospholipase. J Clin Invest 1996 Jun 15;97(12):2784-91.

(74) Stafforini DM, Numao T, Tsodikov A, Vaitkus D, Fukuda T, Watanabe N, et al. Deficiency of platelet-activating factor acetylhydrolase is a severity factor for asthma. J Clin Invest 1999 Apr;103(7):989-97.

(75) Kruse S, Mao XQ, Heinzmann A, Blattmann S, Roberts MH, Braun S, et al. The Ile198Thr and Ala379Val variants of plasmatic PAF-acetylhydrolase impair catalytical

activities and are associated with atopy and asthma. Am J Hum Genet 2000 May;66(5):1522-30.

(76) Ferrero E, Goyert SM. Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. Nucleic Acids Res 1988 May 11;16(9):4173.

(77) Haziot A, Chen S, Ferrero E, Low MG, Silber R, Goyert SM. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J Immunol 1988 Jul 15;141(2):547-52.

(78) da Silva CJ, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J Biol Chem 2001 Jun 15;276(24):21129-35.

(79) Holt PG, Sly PD, Bjorksten B. Atopic versus infectious diseases in childhood: a question of balance? Pediatr Allergy Immunol 1997 May;8(2):53-8.

(80) Baldini M, Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FD. A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am J Respir Cell Mol Biol 1999 May;20(5):976-83.

(81) Koppelman GH, Reijmerink NE, Colin SO, Howard TD, Whittaker PA, Meyers DA, et al. Association of a promoter polymorphism of the CD14 gene and atopy. Am J Respir Crit Care Med 2001 Mar;163(4):965-9.

(82) O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 2007 May;7(5):353-64.

(83) Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-kappa B in pulmonary diseases. Chest 2000 May;117(5):1482-7.

(84) Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest 2001 Jan;107(2):135-42.

(85) von BH, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. Science 2008 Aug 1;321(5889):691-6.

(86) Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.

(87) Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 1998 Jul;9(1):143-50.

(88) Ku CL, von BH, Picard C, Zhang SY, Chang HH, Yang K, et al. Selective predisposition to bacterial infections in IRAK-4-deficient children: IRAK-4-dependent TLRs are otherwise redundant in protective immunity. J Exp Med 2007 Oct 1;204(10):2407-22.

(89) Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649-83.

(90) Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001 Jan;107(1):7-11.

(91) Zandi E, Chen Y, Karin M. Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science 1998 Aug 28;281(5381):1360-3.

(92) Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, et al. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell 1998 Jun 26;93(7):1231-40.

(93) Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997 Apr 10;336(15):1066-71.

(94) Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. Am J Respir Crit Care Med 1998 Nov;158(5 Pt 1):1585-92.

(95) Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. Science 1993 Oct 8;262(5131):242-4.

(96) Wang Z, White PS, Petrovic M, Tatum OL, Newman LS, Maier LA, et al. Differential susceptibilities to chronic beryllium disease contributed by different Glu69 HLA-DPB1 and -DPA1 alleles. J Immunol 1999 Aug 1;163(3):1647-53.

(97) McCanlies EC, Kreiss K, Andrew M, Weston A. HLA-DPB1 and chronic beryllium disease: a HuGE review. Am J Epidemiol 2003 Mar 1;157(5):388-98.

(98) Potolicchio I, Mosconi G, Forni A, Nemery B, Seghizzi P, Sorrentino R. Susceptibility to hard metal lung disease is strongly associated with the presence of glutamate 69 in HLA-DP beta chain. Eur J Immunol 1997 Oct;27(10):2741-3.

(99) Ueki A, Isozaki Y, Tomokuni A, Ueki H, Kusaka M, Tanaka S, et al. Different distribution of HLA class II alleles in anti-topoisomerase I autoantibody responders between silicosis and systemic sclerosis patients, with a common distinct amino acid sequence in the HLA-DQB1 domain. Immunobiology 2001 Dec;204(4):458-65.

(100) Mapp CE, Balboni A, Baricordi R, Fabbri LM. Human leukocyte antigen associations in occupational asthma induced by isocyanates. Am J Respir Crit Care Med 1997 Oct;156(4 Pt 2):S139-S143.

(101) Horne C, Quintana PJ, Keown PA, Dimich-Ward H, Chan-Yeung M. Distribution of DRB1 and DQB1 HLA class II alleles in occupational asthma due to western red cedar. Eur Respir J 2000 May;15(5):911-4.

(102) Choi JH, Lee KW, Kim CW, Park CS, Lee HY, Hur GY, et al. The HLA DRB1*1501-DQB1*0602-DPB1*0501 haplotype is a risk factor for toluene diisocyanate-induced occupational asthma. Int Arch Allergy Immunol 2009;150(2):156-63.

(103) Glazer CS, Newman LS. Occupational interstitial lung disease. Clin Chest Med 2004 Sep;25(3):467-78, vi.

(104) Suzuki H, Ota M, Meguro A, Katsuyama Y, Kawagoe T, Ishihara M, et al. Genetic Characterization and Susceptibility for Sarcoidosis in Japanese Patients: Risk Factors of BTNL2 Gene Polymorphisms and HLA Class II Alleles. Invest Ophthalmol Vis Sci 2012;53(11):7109-15.

(105) Linsley PS, Peach R, Gladstone P, Bajorath J. Extending the B7 (CD80) gene family. Protein Sci 1994 Aug;3(8):1341-3.

(106) Henry J, Miller MM, Pontarotti P. Structure and evolution of the extended B7 family. Immunol Today 1999 Jun;20(6):285-8.

(107) Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, et al. BTNL2, a butyrophilin/B7-like molecule, is a negative costimulatory molecule modulated in intestinal inflammation. J Immunol 2007 Feb 1;178(3):1523-33.

(108) Valentonyte R, Hampe J, Huse K, Rosenstiel P, Albrecht M, Stenzel A, et al. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. Nat Genet 2005 Apr;37(4):357-64.

(109) Nadif R, Jedlicka A, Mintz M, Bertrand JP, Kleeberger S, Kauffmann F. Effect of TNF and LTA polymorphisms on biological markers of response to oxidative stimuli in coal miners: a model of gene-environment interaction. Tumour necrosis factor and lymphotoxin alpha. J Med Genet 2003 Feb;40(2):96-103.

(110) Zhai R, Jetten M, Schins RP, Franssen H, Borm PJ. Polymorphisms in the promoter of the tumor necrosis factor-alpha gene in coal miners. Am J Ind Med 1998 Oct;34(4):318-24.

(111) Corbett EL, Mozzato-Chamay N, Butterworth AE, De Cock KM, Williams BG, Churchyard GJ, et al. Polymorphisms in the tumor necrosis factor-alpha gene promoter may predispose to severe silicosis in black South African miners. Am J Respir Crit Care Med 2002 Mar 1;165(5):690-3.

(112) Piirila P, Wikman H, Luukkonen R, Kaaria K, Rosenberg C, Nordman H, et al. Glutathione S-transferase genotypes and allergic responses to diisocyanate exposure. Pharmacogenetics 2001 Jul;11(5):437-45.

(113) Bailey KL, Poole JA, Mathisen TL, Wyatt TA, Von Essen SG, Romberger DJ. Toll-like receptor 2 is upregulated by hog confinement dust in an IL-6-dependent manner in the airway epithelium. Am J Physiol Lung Cell Mol Physiol 2008 Jun;294(6):L1049-L1054.

(114) Cho HJ, Kim SH, Kim JH, Choi H, Son JK, Hur GY, et al. Effect of Toll-like receptor 4 gene polymorphisms on work-related respiratory symptoms and sensitization to wheat flour in bakery workers. Ann Allergy Asthma Immunol 2011 Jul;107(1):57-64.

(115) al-Ali MK, Eames C, Howarth PH. Exhaled nitric oxide; relationship to clinicophysiological markers of asthma severity. Respir Med 1998 Jul;92(7):908-13.

(116) Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 1994 Jan 15;343(8890):133-5.

(117) Sundblad BM, Larsson BM, Palmberg L, Larsson K. Exhaled nitric oxide and bronchial responsiveness in healthy subjects exposed to organic dust. Eur Respir J 2002 Aug;20(2):426-31.

(118) Von Essen SG, Scheppers LA, Robbins RA, Donham KJ. Respiratory tract inflammation in swine confinement workers studied using induced sputum and exhaled nitric oxide. J Toxicol Clin Toxicol 1998;36(6):557-65.

(119) Castranova V. Role of nitric oxide in the progression of pneumoconiosis. Biochemistry (Mosc) 2004 Jan;69(1):32-7.

(120) Vogelzang PF, van der Gulden JW, Folgering H, Kolk JJ, Heederik D, Preller L, et al. Endotoxin exposure as a major determinant of lung function decline in pig farmers. Am J Respir Crit Care Med 1998 Jan;157(1):15-8.

(121) Nehme B, Letourneau V, Forster RJ, Veillette M, Duchaine C. Culture-independent approach of the bacterial bioaerosol diversity in the standard swine confinement buildings, and assessment of the seasonal effect. Environ Microbiol 2008 Mar;10(3):665-75.

(122) Sundblad BM, Larsson BM, Palmberg L, Larsson K. Exhaled nitric oxide and bronchial responsiveness in healthy subjects exposed to organic dust. Eur Respir J 2002 Aug;20(2):426-31.

(123) Von Essen SG, Scheppers LA, Robbins RA, Donham KJ. Respiratory tract inflammation in swine confinement workers studied using induced sputum and exhaled nitric oxide. J Toxicol Clin Toxicol 1998;36(6):557-65.

CHAPTER 4

EXPOSURES IN SWINE CONFINEMENT BUILDINGS AND RESPIRATYORY HEALTH OF WORKERS

Working in swine confinement buildings has been associated with respiratory problems, including excess respiratory symptoms,[1] reduced lung function,[2] and increased asthma severity.[3] Large swine confinement buildings pose a higher risk to subjects working inside due to increased animal density and longer duration of exposure in these enclosed spaces. The airborne contaminants in swine operation buildings are complex and include high concentration of dust particles, endotoxin, microorganisms (Gram-positive and Gram negative bacteria) and gases such as hydrogen sulfide, ammonia, carbon dioxide and odors.[4] The complex airborne contaminants could be grouped into three categories: particulate matter, endotoxin and gases.

4.1 Particulate matter

The first group of airborne contaminant consists of a wide variety of particulate matter (PM). The size of suspended particulate matter has a wide range, from 0.001 to 100 micrometers (μm).[5] The large particulate matter (more than 100 μm) do not stay suspended in air long and are usually not inhalable, so are usually not treated as PM; fine particulate matter (less than 0.001 μm) are more likely to act as gas, and thus are also not usually considered as PM.[5] Smaller particles, which can easily go into deep parts of the lungs, pose the biggest threaten to the respiratory system when exposed. Particles of size less than 10 μm are classified as respirable dust. The two most commonly used PM measurements are $PM_{2.5}$ and PM_{10}, which refer to particles with size smaller than 2.5 μm and 10 μm in diameter, respectively. Under Alberta's Occupational Health and Safety Code 2009, occupational exposure limits are 10 mg/m^3 for total particles and 3 mg/m^3 for respirable particles.[6]

A study dust characterization from swine confinement buildings in Iowa (n=21) and in Sweden (n=31) showed that the main constituents of dusts include components from animal feeds (starch granules, grain meal, corn silk), components from animal wastes (bacteria, gut epithelium, undigested feed) and other components (swine dander, mold pollen, insect parts, mineral ash), with a total dust levels of 6.3 mg/m^3 (0.5 mg/m^3 of respirable dust) and 4.6 mg/m^3 (0.33 mg/m^3 of respirable dust) in Iowa and Sweden, respectively.[7] Another study of airborne dust in pig barns from Northern Europe reported that the overall mean inhalable and respirable dust concentrations were 2.19 mg/m^3 and 0.23 mg/m^3, respectively.[8] A study from Sweden showed that during a 3 hour period of cleaning of a swine confinement room using high pressure water, dust aerosols with inhalable dust level of was 0.97 (0.74-1.55) mg/m^3 and respirable dust level was 0.56 (0.51-0.63) mg/m^3.[9] A study of air quality as measured by area monitoring at the breathing zone (1.5 meters from the floor) in 173 buildings on 50 pig farms from Saskatchewan reported that the highest respirable dust concentration was 0.21 mg/m^3 in the nursery area.[10] In a study of 8 swine confinement buildings in Quebec showed that the mean concentration of total dust was 3.54 mg/m^3 ranging between 2.15 and 5.60 mg/m^3.[11]

4.2 Health Effect of Particulate Matter

Health effects from elevated levels of particulate matter are well established. Exposure to PM in swine operation facilities have been associated with irritation of the eyes, nose and throat and a dry cough, many respiratory symptoms (cough, phlegm, wheezing and chest tightness), increased risk of respiratory tract infection and reduced lung function. [1,9,12-14]

A study of healthy adult subjects from Sweden who were exposed for 3 hours during cleaning of a swine confinement room showed that exposure to dust aerosols was associated with increased bronchial responsiveness to methacholine and an acute inflammation response

(neutrophilic granulocytes, monocytes, lymphocytes and IL-6).[9] In Canada, several studies of swine farmers and rural-dwellers in Saskatchewan showed swine farmers had significantly lower FEV_1, accelerated lung function decline rates in FEV_1, FVC and $FEF_{25\%-75\%}$, and excess respiratory symptoms including chronic and usual cough, and chronic and usual phlegm than rural dwellers.[1,13,14]

4.3 Endotoxin

Endotoxin originates from cell wall components of Gram-negative bacteria and appears to be the major determinant of lung dysfunction.[15]

Subjects are exposed to low concentration of endotoxin at their homes (e.g. < 100 EU/m^3), especially those houses with animals,[16] and cigarette smoking.[17] High level of endotoxins in swine operations have been reported in many studies.[11,18,19] A study of 207 workers in swine operations in Iowa showed that the total endotoxin from personal-sampled total dust was 203 EU/m^3.[18] Another study from Saskatchewan reported that the endotoxin concentration from personal-sampled dust could reach 4,035EU/m^3 during a 3-hour exposure of healthy subjects who rode a stationary bike 18km/hour to mimic daily works of workers in swine operations.[19] Even higher endotoxin concentration (4.9×10^3 EU/m^3) was reported in another study from Quebec, where the airborne dust was measured by area monitoring at 1 meter height from the floor.[11]

4.4 Health effect of Endotoxin

Low concentration of endotoxin in homes has been associated with asthma symptoms and severity in adults and children.[20-23] A cross-sectional study of 49 patients with asthma reported that the concentration of house dust endotoxin was significantly associated with the severity of asthma, inversely correlated with FEV_1, FEV_1/FVC, and associated with daily need for oral and

inhaled corticosteroids and daily need for beta 2 agonists.[20] A significant association between endotoxin exposure and respiratory symptoms in asthmatic children was also reported in another study of asthmatic children from Brazil.[21] Since Gram-negative bacteria favor areas of dampness, many studies showed a significant association between respiratory symptoms (chronic wheeze, wheeze with shortness of breath, and allergy) and housing.[22] Another study of children with asthma, dampness at home was also a significant risk factor for the persistence of bronchial hyperreactivity and respiratory symptoms.[23]

Adaptation or tolerance to endotoxin exposure can be developed among subjects with repeated exposures since workers with repeated exposure in animal confinement buildings have been shown to have a smaller decline in lung function, less hyperresponsiveness on methacholine change test, and fewer inflammatory cells from the lower respiratory tract than naïve subjects with no prior exposure.[24]

4.5 Gases

Several hundred gaseous compounds have been found in animal housing buildings.[25] The concentration of these gases can vary significantly in different swine operations. At high concentration, they are recognised to have adverse effects on workers' health. In swine confinement buildings, three major gases, hydrogen sulfide (H_2S), ammonia (NH_3) and carbon dioxide (CO_2), have been monitored and reported frequently in studies from different countries.[26-29] Occupational exposure limits recommended by National Institute for Occupational Safety and Health (NIOSH) and Alberta's Occupational Health and Safety Code (AOHSC) 2009 are summarized in Table 4-1.

4.5.1 Ammonia

Ammonia (NH_3) is a colourless gas with a characteristic pungent smell. NH_3 is a strong irritant, lighter than air and highly water soluble. NH_3 is naturally produced from decay processes of nitrogenous animal and vegetable waste. Under Alberta's Occupational Health and Safety Code 2009, occupational exposure limits for ammonia are 25 parts per million (ppm) for an 8 hour occupational exposure and 35 ppm for a 15 minute or ceiling occupational exposure.[6] The maximum concentration level recommended by NIOSH for NH_3 is 25 ppm.

In swine confinement buildings, NH_3 is mainly produced from decomposition of urine on the floor. In swine confinement buildings, NH_3 concentrations differ significantly from one area to another. Several studies of air quality in pig farms in US and Canada showed that NH_3 concentrations were highest in the farrowing area (19 to 42 ppm) and lowest in the finisher area (15 ppm) as the NH_3 concentrations were measured by area monitoring, which were set at the height of breathing zone.[10,30] NH_3 concentrations can be higher in winter than in summer in Canada.[10,11] A study of 8 swine confinement buildings from Canada showed that the concentration of NH_3 was 19.6 ppm ranging from 1.9 to 25.9 ppm, and the concentration was significantly lower in summer than in winter.[11]

4.5.2 Health effect of NH_3 exposure

Ammonia gas is a strong respiratory irritant; it can be noticeable by smell at a very low concentration of 0.6 ppm, at concentration of 50 ppm it causes irritation of the nose and throat; and at the concentration of 1500 ppm, it can cause tightness in the chest and difficulty breathing; fatality due to massive ammonia inhalation has been reported.[31]

Exposure to NH_3 can lead to damage of the respiratory system, which has been reported in several studies.[32-35] As ammonia is water-soluble, it can be rapidly absorbed in the upper airway and damage airway epithelia cells and inhibit lung cilia from moving out dust particules.[32]

Donham et al in a study of 207 swine operations using intensive housing systems showed that significant correlation between pulmonary function and ammonia exposures, with a greater than 3% decrement in FEV_1 when ammonia level exceeding 7.5 ppm.[18] Another study of 194 Dutch pig farmers also reported strong associations between ammonia exposure and FEV_1, $FEF_{25\%-75\%}$ and PEF, and the associations were stronger in symptomatic farmers than asymptomatic farmers.[19] It was reported that exposure of high concentration of ammonia exposure, as short as 2 minutes, could lead to bronchiolitis obliterans and restrictive lung disease.[35]

4.5.3 Hydrogen sulfide

Hydrogen sulfide (H_2S) is a colorless and flammable gas, heavier than air and soluble in water. It has the characteristic odor of rotten eggs. It usually comes from anaerobic digestion, a process of breakdown of organic matter without oxygen present, such as in swamps and sewers. Under Alberta's Occupational Health and Safety Code 2009, occupational exposure limits for hydrogen sulfide are 10 ppm for an 8 hour occupational exposure and 15 ppm for a 15 minute or ceiling occupational exposure.[6] The maximum concentration level recommended by NIOSH for H_2S is 10 ppm.

Liquid manure storage pits, which consistently collect feces, urine, and spilled feed from pig rooms, are the primary sources of H_2S in pig barns. The concentration of H_2S is usually at low level in swine confinement buildings.[30] Donham et al in a study of air quality as measured by area monitoring at breathing zone from 21 confinement operations from US showed that the average of H_2S concentration was 1.4 ppm ranging from 0.4 to 1.7 ppm in the farrowing area, and 0.5 to 1.3 ppm in the nursery area.[30]

However, large amounts of H_2S can be released whenever the liquid manure slurry of swine barns is agitated. Patni et al in a study of H_2S concentrations as measured by area

monitoring during the slurry mixing operations in three swine barns showed an immediate and rapid rise in H_2S concentration in the pit exhaust air with a peak record of 222 ppm in a 4-minute period when the air was blown into the bottom of a pit to mix the manure.[36] Chenard et al in a study of H_2S concentration as measured by area monitoring at height 1 meter in 4 swine barns in Saskatchewan showed a maximum of 1,000 ppm was recorded after manure agitation and removal workplace activities, at which concentration adverse health effects on those exposed persons could be inevitable. [37]

4.5.4 Health effects of H_2S exposure

Exposure of H_2S at different concentration levels has been associated with many adverse health effects, low concentration of H_2S acute exposure ,< 20 ppm, may cause possible nausea, tearing of the eyes or headaches; at concentration of 20-50 ppm, H_2S can lead to lung irritation, digestive upset; at a concentration of 500-1000 ppm, H_2S can result respiratory paralysis, irregular heart beat; at a concentration of more than 1000 ppm, rapid collapse and death may happen.[38,39]

Workers are usually exposed to high concentration of H_2S when entering into the pits to get animals or do repair and maintenance work. In a study of 77 fatal cases related to on-farm manure storage and handling facilities, the largest percentage (34%) of deaths occurred to persons entering a pit during or soon after the emptying process to conduct repair or maintenance activities or clean out solids, and the second largest percentage of deaths (22%) were persons who attempted to rescue another person.[39]

4.5.5 Carbon dioxide

Carbon dioxide (CO_2) is colorless and heavier than air. At low concentration, CO_2 is odorless; however, a sharp acidic odor is noticeable by smell at higher concentrations. Under

Alberta's Occupational Health and Safety Code 2009, occupational exposure limits for CO_2 are 5,000 parts per million (ppm) for an 8 hour occupational exposure and 30,000 ppm for a 15 minute or ceiling occupational exposure.[6] The maximum concentration level recommended by NIOSH for CO_2 is 5,000 ppm.

CO_2 is the primary by-products of respiration of all living animals and plants. Normally, CO_2 concentrations in swine confinement operations are not a problem. A study of five swine farms from Ireland also showed peak CO_2 concentrations were in a normal range, from 430 to 4780 ppm.[40] However, CO_2 concentration varies between different operations within a swine farm. The study from Ireland reported a big variation in the peak CO_2 concentration ranging from 1600 ppm in weaning areas, to 4700 ppm in general farm areas.[40] Donham et al in a study of 21 swine confinement operations from US reported that the mean concentration of CO_2 as measured by area monitoring at 1.5 meter was 1,640 ppm, and the highest CO_2 concentration was in the farrowing area (1,838 to 2,452 ppm), and lowest CO_2 concentration was in the finisher areas (1,000 to 1,338 ppm).[30] A study of air quality as measured by area monitoring at 1.5 meter in 173 buildings on 50 pig farms from Saskatchewan showed the nursery area had the highest CO_2 concentration (4,524 ppm), and the finisher area was the lowest (3,954 ppm), and the CO_2 concentration was lower in summer (May to September) than in winter (November to April) because of the low ventilation to save heating costs in winter.[10]

4.5.6 Health effect of CO_2 exposure

Short-term exposure to CO_2 concentration lower than 20,000 ppm has no known harmful effects.[41] However, a potential health hazard can occur if a high concentration of carbon dioxide is present in the barn. At concentration of 33,000-54,000 ppm, it leads to low concentration of oxygen deficiency, which causes increased depth of breathing; at a concentration of 75,000 ppm,

workers start to develop increased pulse rate, headache, dizziness, disorientation; at a concentration of more than 100,000 ppm, workers usually develop difficulty in breathing, impaired hearing, nausea and vomiting; at a concentration of more than 300,000 ppm, exposure to CO_2 can quickly result in unconsciousness and convulsions.[41]

Special attention should be given to persons with certain health conditions even when CO_2 concentration is within the occupational exposure limits. Persons with pulmonary and coronary diseases and heart failure should avoid exposure to CO_2 since it can cause increased pulmonary and systemic blood pressure.[42] In addition, alcohol and many drugs such as morphine can supress respiratory center stimulation by CO_2, which can reduce the initiation of compensatory protection mechanisms in the presence of high CO_2 levels.[43]

4.6 Healthy worker effect in occupational epidemiological studies

Healthy worker effect states that an individual must be relatively healthy in order to be employable in a workforce.[44] As a result, morbidity and mortality rates due to harmful exposures at work might be partially masked.[44] The excluding process of "unhealthy individuals" from the workforce leads to differences in health status between workers and the general population, which has been considered as a source of selection bias. In addition to selection process at employment, healthy workers are more likely to stay in the workforce than those who are weak, which also leads to a healthier occupational cohort.[45] Moreover, some regulations set by industries may restrict some risk factors at work, such as no smoking at workplaces, which leads to significantly lower rates of smoking related diseases, such as lung cancer. A study of1,207 men employed in a Canadian oil refinery showed the workers had significantly deficit of lung cancers (observed 7 vs. expected 15.8).[46] A previous study of workers in swine operations showed a significant healthy worker effects among workers who

continued to work, as compared to those dropped out, which could be due to "acclimatization" to continued exposure to endotoxin and other substances in the swine confinement facilities, and workers genetic-makeup could play an important role.[47]

4.7 Summary of respiratory hazards in swine operations

Exposure to these airborne contaminants in the working environment has resulted in many acute and chronic adverse health effects.[1-3] Airborne contaminants are complex and include high concentration of dust particles, endotoxin, microorganisms (Gram-positive and Gram negative bacteria) and gases including NH_3 and H_2S. In order to investigate health effects due to occupational exposure to these respiratory hazards in swine operations, precise measurement of exposure level is crucial. However, it is still a challenging task to accurately measure exposure level of these contaminants.

Exposure level of airborne contaminants is associated with many factors: firstly, individual related factors, such as duration of time each worker spends inside the building, intensity of the work, and concentration of these harmful agents; and secondly, confinement buildings' physical factors, such as maintenances, cleanliness, ventilation and heating system, number of animals and room size, and seasonal environmental factors, such as seasonal temperature and humidity, all influence airborne contaminant concentration inside the buildings. Increased dust deposition and humidity level in some old and poorly maintained buildings could facilitate microbial growth. Less frequent collection of waste from animals could also increase airborne contaminant concentration; thirdly, different sampling methods can also make significant differences in the concentrations of airborne dust.[26] A study of 57 workers from 30 swine farms in southern Sweden showed that personal sampling method had higher dust (total and respirable) and endotoxin (total and respirable) concentrations than area sampling. [26] Studies investigating

effects of occupational exposures to respiratory hazards need to have accurate measurement of exposures. Moreover, extra caution should be given to potential bias caused by healthy worker effect in occupational epidemiological studies.

Table 4-1 Occupational exposure limits recommended by National Institute for Occupational Safety and Health (NIOSH) and Alberta's Occupational Health and Safety Code (AOHSC) 2009

	NIOSH*	AOHSC
Dust		
Total particles	Not listed	10 mg/m^3
Respirable particles	Not listed	3 mg/m^3
Endotoxin		
Total particles	Not listed	Not listed
Respirable particles	Not listed	Not listed
Ammonia		
Time weighted average	25 ppm	25 ppm
Hydrogen sulfide		
Time weighted average	10 ppm	10 ppm
Carbon dioxide		
Time weighted average	5,000 ppm	5,000 ppm

* Taken from http://www.public-health.uiowa.edu/ehsrc/CAFOstudy/CAFO_8.pdf

4.8 References

(1) Senthilselvan A, Chenard L, Ulmer K, Gibson-Burlinguette N, Leuschen C, Dosman JA. Excess respiratory symptoms in full-time male and female workers in large-scale swine operations. Chest 2007 Apr;131(4):1197-204.

(2) Kirychuk S, Senthilselvan A, Dosman JA, Zhou C, Barber EM, Rhodes CS, et al. Predictors of longitudinal changes in pulmonary function among swine confinement workers. Can Respir J 1998 Nov;5(6):472-8.

(3) Michel O, Ginanni R, Duchateau J, Vertongen F, Le BB, Sergysels R. Domestic endotoxin exposure and clinical severity of asthma. Clin Exp Allergy 1991 Jul;21(4):441-8.

(4) Crook B, Robertson JF, Glass SA, Botheroyd EM, Lacey J, Topping MD. Airborne dust, ammonia, microorganisms, and antigens in pig confinement houses and the respiratory health of exposed farm workers. Am Ind Hyg Assoc J 1991 Jul;52(7):271-9.

(5) APTI(Air Pollution Training Institute). Particulate Matter Sampling. United States Environmental Protection Agency; 1983.

(6) Government of Alberta. Occupational Health and Safety Code 2009.Edmonton: Alberta Queen's Printer; 2009.

(7) Donham KJ, Scallon LJ, Popendorf W, Treuhaft MW, Roberts RC. Characterization of dusts collected from swine confinement buildings. Am Ind Hyg Assoc J 1986 Jul;47(7):404-10.

(8) Takai H, Pedersen S, Johnsen JO, Metz JHM, Groot Koerkamp PWG, Uenk GH, et al. Concentrations and Emissions of Airborne Dust in Livestock Buildings in Northern Europe. J Agric Engng Res 1998;70:59-77.

(9) Larsson BM, Larsson K, Malmberg P, Palmberg L. Airways inflammation after exposure in a swine confinement building during cleaning procedure. Am J Ind Med 2002 Apr;41(4):250-8.

(10) Barber EM, Rhodes CS, Dosman JA. A survey of air quality in Saskatchewan pig buildings. 1991.

(11) Duchaine C, Grimard Y, Cormier Y. Influence of building maintenance, environmental factors, and seasons on airborne contaminants of swine confinement buildings. AIHAJ 2000 Jan;61(1):56-63.

(12) Rylander R, Donham KJ, Hjort C, Brouwer R, Heederik D. Effects of exposure to dust in swine confinement buildings--a working group report. Scand J Work Environ Health 1989 Oct;15(5):309-12.

(13) Dosman JA, Graham BL, Hall D, Pahwa P, McDuffie HH, Lucewicz M, et al. Respiratory symptoms and alterations in pulmonary function tests in swine producers in Saskatchewan: results of a survey of farmers. J Occup Med 1988 Sep;30(9):715-20.

(14) Senthilselvan A, Dosman JA, Kirychuk SP, Barber EM, Rhodes CS, Zhang Y, et al. Accelerated lung function decline in swine confinement workers. Chest 1997 Jun;111(6):1733-41.

(15) Vogelzang PF, van der Gulden JW, Folgering H, Kolk JJ, Heederik D, Preller L, et al. Endotoxin exposure as a major determinant of lung function decline in pig farmers. Am J Respir Crit Care Med 1998 Jan;157(1):15-8.

(16) Park JH, Spiegelman DL, Gold DR, Burge HA, Milton DK. Predictors of airborne endotoxin in the home. Environ Health Perspect 2001 Aug;109(8):859-64.

(17) Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. Chest 1999 Mar;115(3):829-35.

(18) Reynolds SJ, Donham KJ, Whitten P, Merchant JA, Burmeister LF, Popendorf WJ. Longitudinal evaluation of dose-response relationships for environmental exposures and pulmonary function in swine production workers. Am J Ind Med 1996 Jan;29(1):33-40.

(19) Dosman JA, Senthilselvan A, Kirychuk SP, Lemay S, Barber EM, Willson P, et al. Positive human health effects of wearing a respirator in a swine barn. Chest 2000 Sep;118(3):852-60.

(20) Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, et al. Severity of asthma is related to endotoxin in house dust. Am J Respir Crit Care Med 1996 Dec;154(6 Pt 1):1641-6.

(21) Rizzo MC, Naspitz CK, Fernandez-Caldas E, Lockey RF, Mimica I, Sole D. Endotoxin exposure and symptoms in asthmatic children. Pediatr Allergy Immunol 1997 Aug;8(3):121-6.

(22) Rennie D, Chen Y, Lawson J, Dosman J. Differential effect of damp housing on respiratory health in women. J Am Med Womens Assoc 2005;60(1):46-51.

(23) Nicolai T, Illi S, von ME. Effect of dampness at home in childhood on bronchial hyperreactivity in adolescence. Thorax 1998 Dec;53(12):1035-40.

(24) Von ES, Romberger D. The respiratory inflammatory response to the swine confinement building environment: the adaptation to respiratory exposures in the chronically exposed worker. J Agric Saf Health 2003 Aug;9(3):185-96.

(25) Schiffman SS, Bennett JL, Raymer JH. Quantification of odors and odorants from swine operations in North Carolina. Agricultural and Forest Meteorology 2001;108:213-40.

(26) Donham K, Haglind P, Peterson Y, Rylander R, Belin L. Environmental and health studies of farm workers in Swedish swine confinement buildings. Br J Ind Med 1989 Jan;46(1):31-7.

(27) Chang CW, Chung H, Huang CF, Su HJ. Exposure assessment to airborne endotoxin, dust, ammonia, hydrogen sulfide and carbon dioxide in open style swine houses. Ann Occup Hyg 2001 Aug;45(6):457-65.

(28) Duchaine C, Grimard Y, Cormier Y. Influence of building maintenance, environmental factors, and seasons on airborne contaminants of swine confinement buildings. AIHAJ 2000 Jan;61(1):56-63.

(29) Zejda JE, Barber E, Dosman JA, Olenchock SA, McDuffie HH, Rhodes C, et al. Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels. J Occup Med 1994 Jan;36(1):49-56.

(30) Donham KJ, Popendorf WJ. Ambient levels of selected gases inside swine confinement buildings. Am Ind Hyg Assoc J 1985 Nov;46(11):658-61.

(31) Price SK, Hughes JE, Morrison SC, Potgieter PD. Fatal ammonia inhalation. A case report with autopsy findings. S Afr Med J 1983 Dec 3;64(24):952-5.

(32) Merchant J, Kline J, Donham K, Bundy D, Hodne C. Human Health Effects. 2012. http://www.ehsrc.uiowa.edu/CAFOstudy/CAFO_6-3.pdf (accessed on December 29, 2012)

(33) Donham KJ, Reynolds SJ, Whitten P, Merchant JA, Burmeister L, Popendorf WJ. Respiratory dysfunction in swine production facility workers: dose-response relationships of environmental exposures and pulmonary function. Am J Ind Med 1995 Mar;27(3):405-18.

(34) Preller L, Heederik D, Boleij JS, Vogelzang PF, Tielen MJ. Lung function and chronic respiratory symptoms of pig farmers: focus on exposure to endotoxins and ammonia and use of disinfectants. Occup Environ Med 1995 Oct;52(10):654-60.

(35) de la Hoz RE, Schlueter DP, Rom WN. Chronic lung disease secondary to ammonia inhalation injury: a report on three cases. Am J Ind Med 1996 Feb;29(2):209-14.

(36) Patni NK, Clarke SP. Transient hazardous conditions in animal buildings due to manure gas released during slurry mixing. Applied Engineering in Agriculture 1991;7(4):478-84.

(37) Chenard L, Lemay SP, Lague C. Hydrogen sulfide assessment in shallow-pit swine housing and outside manure storage. J Agric Saf Health 2003 Nov;9(4):285-302.

(38) Work Safe Alberta. Workplace Health and Safety Bulletin. Government of Alberta . 2012. http://employment.alberta.ca/documents/WHS/WHS-PUB-CH029.pdf (accessed on December 29, 2012)

(39) Beaver RL, Field WE. Summary of documented fatalities in livestock manure storage and handling facilities--1975-2004. J Agromedicine 2007;12(2):3-23.

(40) Mc Donnell PE, Coggins MA, Hogan VJ, Fleming GT. Exposure assessment of airborne contaminants in the indoor environment of Irish swine farms. Ann Agric Environ Med 2008 Dec;15(2):323-6.

(41) Canadian Centre for Occupational Health and Safety. Carbon Dioxide Gas. 2012. http://www.ccohs.ca/oshanswers/chemicals/chem_profiles/carbon_dioxide/health_cd.html (accessed on December 29, 2012)

(42) Cooper ES, West JW, Jaffe ME, Goldberg HI, Kawamura J, Mchenry LC, Jr. The relation between cardiac function and cerebral blood flow in stroke patients. 1. Effect of CO 2 inhalation. Stroke 1970 Sep;1(5):330-47.

(43) Camporesi EM, Nielsen CH, Bromage PR, Durant PA. Ventilatory CO2 sensitivity after intravenous and epidural morphine in volunteers. Anesth Analg 1983 Jul;62(7):633-40.

(44) McMichael AJ, Spirtas R, Kupper LL. An epidemiologic study of mortality within a cohort of rubber workers, 1964-72. J Occup Med 1974 Jul;16(7):458-64.

(45) Li CY, Sung FC. A review of the healthy worker effect in occupational epidemiology. Occup Med (Lond) 1999 May;49(4):225-9.

(46) Theriault G, Provencher S. Mortality study of oil refinery workers: five-year follow-up. J Occup Med 1987 Apr;29(4):357-60.

(47) Chenard L, Senthilselvan A, Grover VK, Kirychuk SP, Lawson JA, Hurst TS, et al. Lung function and farm size predict healthy worker effect in swine farmers. Chest 2007 Jan;131(1):245-54.

CHAPTER 5

METHODS

Two previously conducted population studies from Saskatchewan were used to address my research questions: effects of polymorphisms in the TLR2, TLR4 and NOS3 genes on lung function among workers in swine operations. The two independent studies included a cross-sectional study of 374 full-time workers in swine operations and 411 non-farming rural dwellers and a longitudinal study of 302 male workers in swine operations and 261 male non-farming rural dwellers who participated in the initial study in 1990/91 (Cycle 1) and were followed-up in 1994/95 (Cycle 2) and in 2003/04 (Cycle 3). Several papers have been published from the cross-sectional study[1,2] and the longitudinal study.[3-8]

5.1 The cross-sectional study

5.1.1 Recruitment of workers in swine operations

The study was conducted over two years from 2003 to 2004 in rural Saskatchewan by Canadian Centre for Health and Safety in Agriculture (CCHSA), University of Saskatchewan, Canada. The recruitment of workers in swine operations was conducted in collaboration with the Pork Producers' Association of Saskatchewan (SaskPork) and large swine production companies in Saskatchewan. A brief description of the study was posted in the SaskPork newsletter. Researchers from the study also made visits to the managements of large swine operations to encourage participation. The companies scheduled their workers into prearranged time slots so that they could have the opportunity to go to the evaluation clinics in nearby towns and villages.

To participate in the study, workers in swine operations had to be 17 years or older and have worked in a confinement building for at least four days per week with a total work-duration

of 20 hours or more per week. A total of 374 workers from 38 large swine operations were included in this study. The number of workers from each operation varied from 3 to 37. The participation rate from the large swine operations was approximately 70%.

5.1.2 Recruitment of non-farming rural dwellers

Non-farming rural dwellers were recruited from all residents living in close proximity (within 100 km radius) to a swine production site. Taxation lists provided by rural towns was used to locate them. A letter inviting them was mailed to all persons on the list. To participate in the study, they had to be older than 17 years. Non-farming rural dwellers who were involved in mining, grain handling, metal and auto-body work were excluded because of their potential exposure to respiratory hazards. A total of 411 non-farming rural dwellers were included in this study.

5.1.3 Data collection

Person-to-person interviews and testing were conducted by a trained technician in hospitals and community centers near the swine operation sites and residence of the non-farming rural dwellers. The study was approved by the Biomedical Research Ethnics Board of the University of Saskatchewan and University of Alberta (Appendix A). Informed written consent was obtained from all participants before the interview. Information collected in the cross-sectional study was shown in Table 5-1.

The information of respiratory health and occupational history was collected from the participants using a detailed questionnaire (Appendix B), which had been well validated in previous studies, and included anthropomorphic data, respiratory symptoms, smoking history, past illnesses, occupational history in swine operations.[2]

A current smoker was defined by the questionnaire as a person currently smoking cigarettes at average of 1 or more per day. An ex-smoker was defined as a person who has smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime but was not currently smoking. A life-time non-smoker was defined as a person who had not smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime.

Lung function testing was obtained by trained technicians using a volume displacement spirometer (model 1022; SensorMedics; Yorba Linda, CA) following the American Thoracic Society recommendations.[2] These measurements include forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and forced expiratory flow between 25% and 75% of FVC ($FEF_{25\%-75\%}$). Height (cm), weight (kg) and systolic and diastolic pressure (mmHg) measurements were obtained prior to the lung function testing.

Solutions for allergens (Alternaria sp, swine, house dust mite, cat dander, mixed grass allergens) for the allergy skin prick tests (SPT) were purchased from Western Allergy Services Ltd. Burnaby, BC, Canada, and the procedure for SPT was described in another study reporting the reciprocal association between atopy and respiratory symptoms using the same cohort.[1] Histamine and saline were also included in the SPT as the positive and negative control. The SPT was conducted on the same occasion as the pulmonary function testing using methods in previous studies.[9,10] The SPT was performed on the normal skin on each subject's forearms and the distance between two SPTs was 2 cm to avoid cross-contamination. Participants were considered positive if one or more skin prick test had a raised wheal $\geq$ 3 mm in comparison to the saline control.[11] The technicians conducting the SPT were blinded to the genotypes of each subject.

Blood samples from all subjects were also collected for genotyping, which was conducted at Dr. David Schwartz's laboratory at the National Jewish Hospital in Denver, Colorado. Blood samples were collected in Qiagen PAXgene tubes with blood cards spotted for each sample (S&S, catalog #10538414).[12] A Gentra Autopure robot (Qiagen Corporation, Hilden, Germany) was used to isolate DNA which was quantified by UV spectrophotometry, and stored at -80 °C. Working dilutions of DNA at a concentration of 10 ng/ul were prepared for genotyping. Human TLR4 Asp299Gly and Thr399Ile polymorphisms were genotyped using TaqMan assays and standard protocols on the ABI 7900 Sequence Detection System. Plasmids carrying mutant and wild type sequences were included as controls.[13] All candidate genes were genotyped as a part of a 96-plex GoldenGate genotyping assay on Veracode beads. Assays were performed according to the manufacturer's protocols and scanned on a BeadXpress reader (Illumina, Inc., San Diego, CA, USA). Raw genotyping data were clustered and cleaned in the BeadStudio software (Illumina® GenomeStudio).

5.2 The longitudinal study

The longitudinal study consisted of workers in swine facilities and non-farming rural dwellers who participated in the initial study in 1990/91 (Cycle 1), and were followed-up in 1994/95 (Cycle 2) and in 2003/04 (Cycle 3), respectively (Figure 5-1).

5.2.1 Recruitment of workers in swine operations

The longitudinal study included all swine operations with annual sales of at least 200 hogs in 1988 in the central part of the province of Saskatchewan (Lloydminster in the West, Carrot River in the East, Prince Albert in the North and Moose Jaw in the South) between 1990 and 1991. The information on annual sales of swine operations was provided by the Saskatchewan

Pork Producers Marketing Board. Due to different operations in Hutterite colonies, Hutterite colonies were excluded. After excluding swine operations with less than 200 pigs and swine operations with a daily duration of work inside the barn of less than 2 hours, 412 swine producers were eligible to participate in the study a. Of these, 331 agreed to participate in the study and 302 male workers had pulmonary function measurements and were included in the current study

5.2.2 Recruitment of non-farming rural dwellers

Based on the age distribution and residences of male workers in swine operations, 270 male non-farming rural dwellers were selected from the Saskatchewan hospital services plan lists, school board lists, and bank and post office employees. Of these, 261 non-farming rural dwellers had pulmonary function measurements and were included in the current study.

5.2.3 Follow-up

Of the 302 workers and 261 non-farming rural dwellers studied in Cycle 1, 217 workers and 171 non-farming rural dwellers participated in Cycle 2, which resulted in a 4-year follow-up of 71.9% in the workers and 65.5% in the non-farming rural dwellers (Figure 5-1). An effort was made to contact all subjects previously studied. Of the 217 workers and 171 non-farming rural dwellers who participated in Cycle 2, 163 workers and 118 non-farming rural dwellers participated in Cycle 3, which resulted in a follow-up of 75% in the workers and 69% in the non-farming rural dwellers (Figure 5-1).

5.2.4 Data collection

A technician-administered questionnaire was used to collect information on respiratory and general health status (Appendix C). The same questionnaire was used on all three cycles with the exception that additional questions were added in Cycle 2 and Cycle 3 to elicit changes in health

status and employment status that might have occurred since the previous observations. Respiratory symptoms were obtained using the questions in the Standard American Thoracic Society (ATS) questionnaire.[14] The study was approved by the Biomedical Research Ethnics Board of the University of Saskatchewan and University of Alberta (Appendix A). Informed written consent was obtained from all participants before the interview. Information collected in the longitudinal study was shown in Table 5-1.

A current smoker was defined by the questionnaire as a person currently smoking cigarettes at average of 1 or more per day. An ex-smoker was defined as a person who had smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime but was not currently smoking. A life-time non-smoker was defined as a person who had not smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime.

Years of swine work during the study period was determined by the time intervals between the cycles. For workers who reported that they quit swine farming in response to a question "Have you quit hog farming since the last study" in the Cycle 3, their years of swine work were adjusted by the response to a subsequent question "if yes, in what year did you quit pig farming".

Pulmonary function testing was performed using a volume-displacement spirometer (model 922 SensorMedics). Measurements include forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and forced expiratory flow between 25% and 75% of FVC ($FEF_{25\%-75\%}$). These measurements were obtained by trained technicians following the American Thoracic Society recommendations.[2]

In Cycle 3, blood samples from all subjects were collected for genotyping, which was conducted at Dr. David Schwartz's laboratory at the National Jewish Hospital in Denver, Colorado. Blood samples were collected in Qiagen PAXgene tubes with blood cards spotted for

each sample (S&S, catalog #10538414).[12] A Gentra Autopure robot (Qiagen Corporation, Hilden, Germany) was used to isolate DNA which was quantified by UV spectrophotometry, and stored at -80 °C. Working dilutions of DNA at a concentration of 10 ng/ul were prepared for genotyping. Human TLR4 Asp299Gly and Thr399Ile polymorphisms were genotyped using TaqMan assays and standard protocols on the ABI 7900 Sequence Detection System. Plasmids carrying mutant and wild type sequences were included as controls. All candidate genes were genotyped as a part of a 96-plex GoldenGate genotyping assay on Veracode beads. Assays were performed according to manufacturer's protocols and scanned on a BeadXpress reader (Illumina, Inc., San Diego, CA, USA). Raw genotyping data were clustered and cleaned in the BeadStudio software (Illumina® GenomeStudio).

5.3 Candidate genes

As shown in Table 5-2, the selected 32 candidate genes are located on most of human chromosomes. These candidate genes are involved in LPS signaling pathway, significantly associated with asthma, atopy and COPD from previous studies, and included some novel genes identified in mouse models. The associations of these selected genes have been confirmed in at least two independent populations.

5.3.1 Types of the candidate genes

The first group of candidate genes involved genes, which are thought to be important in the LPS signaling pathways: TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, TLR10) and genes in the TLR4 pathway (CD14, MYD88, NF-κB1, NF-κBIA, NF-κBIB, IKBKG, REL, RELA);

The second group of candidate genes comprises genes, which have been associated with asthma, atopy and COPD: CSF2, IL-10, IL-13, IL-4, IL-4Rα, TNF-α, FcεRIβ, GSTP1, HNMT, NOS3, PAF-AH, TAP1, TGFB1,SERPINA, PON1.

The third group of candidate genes includes genes, which are novel innate immune genes identified in mouse models: ADCY9, CASP4 and E2F1 genes.

SNP data information was downloaded from the following sources: UCSC Genome Browser Server (SNP location, alleles, function and validation); NCBI (two 100 bp flanking sequences for each SNP); or The HapMap project (allele frequency and genotyping data).

5.3.2 Selection of SNPs

The SNPs for each of the 32 candidate genes were selected based on the following criteria:

(i) The association between SNPs with one or more diseases phenotypes were confirmed in several studies;

(ii) Tagging SNPs (representative SNPs in a region with high linkage disequilibrium) for these genes from high density SNP maps were generated by the HapMap project. SNP selector, a web-based tool for SNPs selection was used to select tagging SNPs for these genes.[15] The tagging SNPs were those in high linkage disequilibrium (LD, $r^2 \geq 0.8$).[16,17] SNPs with low minor allele frequency (< 10%) were be excluded and not considered as tagging SNPs.[37,18]

5.3.3 SNP scoring and prioritization

SNP scoring was based on the following criteria. SNP prioritization was done by ranking the assigned scores on each of the following scores.

(i) LD score: it reflects how informative each of the selected SNPs is. The higher the LD score, the more informative the SNP is.

(ii) Quality score: a SNP is considered as "high quality" if the SNP has allele frequency information available or been verified by other studies.

(iii) Function score: a higher score is assigned to SNPs which might affect gene transcript structure or protein product, such as SNPs at a coding region or a splicing site.

(iv) Regulatory score: a higher score is given to the SNPs, which might have higher regulatory potential, such as SNPs at the CpG island or MicroRNA.

(v) Repetitive score: a higher score is assigned to a SNP overlapping with a simple repeat region which is annotated in UCSC.

(vi) Illumina pre-assay score: it is based on the call rate of genotyping a SNP using the Illumina bead platform.

5.3.4 Quality assessment of genotyping

Illumina GenCall Data analysis Software® was used to call genotypes of each SNP. The data for each array was self-normalized to remove back-ground differences and possible cross-talk between the dyes. The SNP genotype was based on cluster formation in scatter plots with the signal intensity fraction on the x-axis, $\text{Signal}_{\text{allele2}}/(\text{Signal}_{\text{allele1}}+\text{Signal}_{\text{allele2}})$, and the logarithm of the signals from both alleles on the y-axis, $\log(\text{Signal}_{\text{allele1}} + \text{Signal}_{\text{allele2}})$. GenTrain score were calculated to reflect the distance from each data point in a cluster to all other data points within the same cluster and to all data points in the closest cluster. Finally, the GenCall score was calculated. It was a product of the GenTrain score and a data-to-model fit score. As recommended by Illumina, calls with GenCall score ≤ 0.15 generally represented failed genotypes, and those with score above 0.7 usually reported well-behaving genotyping.[19]

Figure 5-1: The follow-up of the workers and the rural dwellers from 1990/91 to 2003/04 (number of workers: number of rural dwellers)

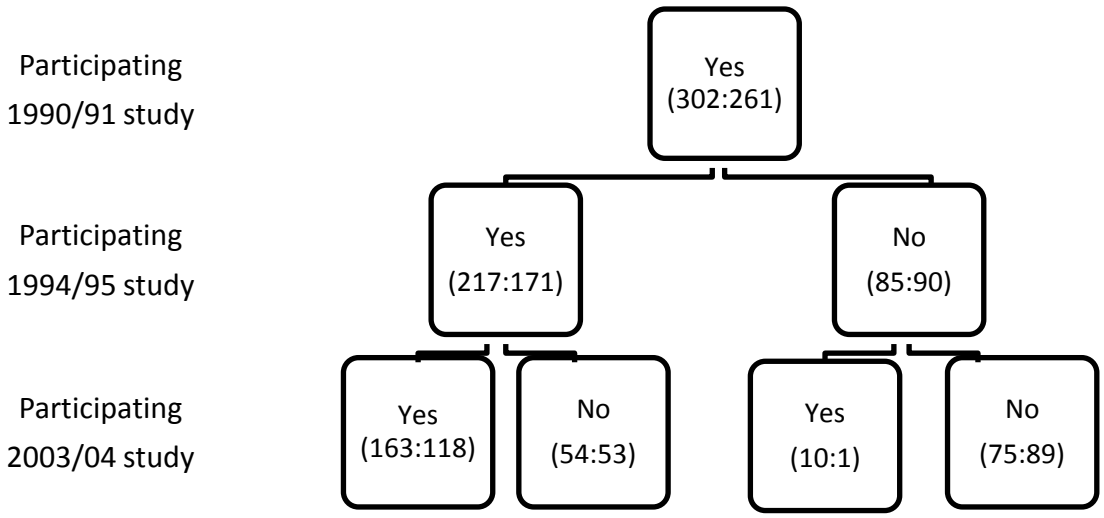


Table 5-1 Information collected for the cross-sectional and longitudinal studies

	Cross-sectional study	Longitudinal study (Cycles 1 to 3)
Questionnaire	✓	✓
Lung function	✓	✓
Skin prick test	✓	-
Blood for genotyping	✓	✓*

* Only at Cycle 3

Table 5-2 Chromosome position of the candidate genes in human

Chromosome	Number of genes	**Genes**(*Region*)
1	2	**IL-10**(*q31*), **TLR5**(*q41-q42*)
2	2	**REL**(*p12-p13*), **HNMT**(*q22*)
3	2	**TLR9**(*p21*), **MYD88**(*p22*)
4	6	**TLR1, TLR6, TLR10**(*p14*), **NF-κB1**(*q24*), **TLR2**(*q32*), **TLR3**(*q35*)
5	4	**CD-14**(*q22-q32*), **CSF2, IL-4, IL-13**(*q31*)
6	2	**TNF-α, TAP1**(*p21*)
7	2	**PON1**(*q21*), **NOS3**(*q36*)
9	1	**TLR4**(*q32-q33*)
11	3	**FcεRIβ, GSTP1, RELA**(*q13*)
14	2	**NF-κBIA**(*q13*), **SERPINA3**(*q32*)
16	1	**IL-4Rα**(*p12*)
17	1	**PAF-AH**(*p13*)
19	2	**TGFB1, NF-κBIB**(*q13*)
X	2	**TLR7**(*p22*), **IKBKG**(*q28*)

5.4 References

(1) Dosman JA, Chenard L, Rennie DC, Senthilselvan A. Reciprocal association between atopy and respiratory symptoms in fully employed female, but not male, workers in swine operations. J Agromedicine 2009;14(2):270-6.

(2) Senthilselvan A, Chenard L, Ulmer K, Gibson-Burlinguette N, Leuschen C, Dosman JA. Excess respiratory symptoms in full-time male and female workers in large-scale swine operations. Chest 2007 Apr;131(4):1197-204.

(3) Zejda JE, Hurst TS, Rhodes CS, Barber EM, McDuffie HH, Dosman JA. Respiratory health of swine producers. Focus on young workers. Chest 1993 Mar;103(3):702-9.

(4) Zejda JE, Barber E, Dosman JA, Olenchock SA, McDuffie HH, Rhodes C, et al. Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels. J Occup Med 1994 Jan;36(1):49-56.

(5) Senthilselvan A, Dosman JA, Kirychuk SP, Barber EM, Rhodes CS, Zhang Y, et al. Accelerated lung function decline in swine confinement workers. Chest 1997 Jun;111(6):1733-41.

(6) Kirychuk S, Senthilselvan A, Dosman JA, Zhou C, Barber EM, Rhodes CS, et al. Predictors of longitudinal changes in pulmonary function among swine confinement workers. Can Respir J 1998 Nov;5(6):472-8.

(7) Chenard L, Senthilselvan A, Grover VK, Kirychuk SP, Lawson JA, Hurst TS, et al. Lung function and farm size predict healthy worker effect in swine farmers. Chest 2007 Jan;131(1):245-54.

(8) Senthilselvan A, Chenard L, Grover V, Kirychuk SP, Hagel L, Ulmer K, et al. Excess longitudinal decline in lung function in grain farmers. J Agromedicine 2010 Apr;15(2):157-65.

(9) Dosman JA, Chenard L, Rennie DC, Senthilselvan A. Reciprocal association between atopy and respiratory symptoms in fully employed female, but not male, workers in swine operations. J Agromedicine 2009;14(2):270-6.

(10) Senthilselvan A, Rennie D, Chenard L, Burch LH, Babiuk L, Schwartz DA, et al. Association of polymorphisms of toll-like receptor 4 with a reduced prevalence of hay fever and atopy. Ann Allergy Asthma Immunol 2008 May;100(5):463-8.

(11) The use of standardized allergen extracts. American Academy of Allergy, Asthma and Immunology (AAAAI). J Allergy Clin Immunol 1997 May;99(5):583-6.

(12) Schwartz DA. TLR4 and LPS hyporesponsiveness in humans. Int J Hyg Environ Health 2002 Apr;205(3):221-7.

(13) Pahwa P, Karunanayake CP, Rennie DC, Chen Y, Schwartz DA, Dosman JA. Association of the TLR4 Asp299Gly polymorphism with lung function in relation to body mass index. BMC Pulm Med 2009;9:46.

(14) Ferris BG. Epidemiology Standardization Project (American Thoracic Society). Am Rev Respir Dis 1978 Dec;118(6 Pt 2):1-120.

(15) Xu H, Gregory SG, Hauser ER, Stenger JE, Pericak-Vance MA, Vance JM, et al. SNPselector: a web tool for selecting SNPs for genetic association studies. Bioinformatics 2005 Nov 15;21(22):4181-6.

(16) Andreas Ziegler, Inke R.Konig. A Statistical Approach to Genetic Epidemiology. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA; 2006.

(17) Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. Am J Hum Genet 2004 Jan;74(1):106-20.

(18) Cupples LA, Arruda HT, Benjamin EJ, D'Agostino RB, Sr., Demissie S, DeStefano AL, et al. The Framingham Heart Study 100K SNP genome-wide association study resource: overview of 17 phenotype working group reports. BMC Med Genet 2007;8 Suppl 1:S1.

(19) Ritchie ME, Liu R, Carvalho BS, Irizarry RA. Comparing genotyping algorithms for Illumina's Infinium whole-genome SNP BeadChips. BMC Bioinformatics 2011;12:68.

CHAPTER 6

ASSOCIATION OF TOLL-LIKE RECEPTOR 2 GENE POLYMORPHISMS WITH LUNG FUNCTION IN WORKERS IN SWINE OPERATIONS*

6.1 Abstract

Background: Workers in swine operations are exposed to indoor dusts and gases and are at increased risk of respiratory problems. Toll-Like Receptor 2 (TLR2) recognizes ligands from Gram- positive bacteria, while Toll-Like Receptor 4 (TLR4) responds to endotoxin from Gram-negative bacteria.

Objective: To investigated the effects of TLR2 and TLR4 polymorphisms on lung function in workers from swine operations and non-farming rural dwellers.

Methods: 374 full-time workers from large swine operations and 411 non-farming rural dwellers from Saskatchewan were included. Information on demography, lifestyle and occupation, lung function measurements and blood samples for genotyping were obtained from the participants. Multiple regression analysis and Bonferroni correction were used in the statistical analysis.

Results: Workers with TLR2-16933T/A polymorphism (AA) had significantly greater mean values of lung function than workers with wild-type (AT+TT) after controlling for potential confounders (FEV_1 (L): 3.7 vs. 3.5, p=0.009; $FEF_{25\%-75\%}$ (L/s): 3.7 vs. 3.3, p=0.003; Predicted FEV_1(%): 100.3 vs. 95.6, p=0.005; $FEF_{25\%-75\%}$(%): 92.4 vs. 83.4, p=0.009). These results were also observed for TLR2Arg677Trp polymorphism among the workers. No such significant differences were observed among non-farming rural dwellers. For Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene, no significant differences were observed in the

mean lung function values between the polymorphic and wild-type groups in both workers and rural dwellers.

Conclusions: This study is the first to report protective effects of TLR2 polymorphisms on lung function among workers in swine operations and raises the possibility that TLR2 polymorphisms are protective of airway disease in subjects exposed to Gram-positive organisms in the inhaled airborne dust.

** A version of this Chapter 6 has been published in Annals of Allergy Asthma and Immunology*

Gao Z, Dosman JA, Rennie DC, Schwartz DA, Yang IV, Beach J, Senthilselvan A. Association of Toll-like receptor 2 gene polymorphisms with lung function in workers in swine operations. *Ann Allergy Asthma Immunol. 2013 Jan:110(1):44-50.*

6.2 Introduction

Working in swine operations is associated with increased risk of many respiratory health problems including accelerated lung function decline,[1] bronchitis-like symptoms,[2] increased risk of developing chronic obstructive pulmonary disease (COPD),[3] and asthma.[4,5] Gram-positive and Gram-negative bacteria, dusts, and gases (H_2S and ammonia) are found in the indoor air in swine operations and likely contribute to respiratory dysfunction among people who are exposed during work.[6,7] Concentration of Gram-positive bacteria was reported to be much higher than that of Gram-negative bacteria in swine operations.[8]

The etiology of these respiratory health problems is not fully understood. However, individual susceptibility plays an important role in the respiratory health status of the workers. TLRs are pattern recognition receptors, highly polymorphic and play an important role in both innate and adaptive immunity.[9-12] TLR2 mainly responds to cell wall structure components from Gram-positive bacteria, such as peptidoglycan, and TLR4 mainly recognizes microbial membrane components from Gram-negative bacteria, such as LPS or endotoxin.[13,14]

TLR4 polymorphisms have been associated with reduced risk of allergic rhinitis, atopy and airway responsiveness in several studies.[15-17] The reduced immune responses of TLR2 polymorphisms have also been investigated in experimental studies using human cells or animal models.[18-20] However, to date there is limited study of the relationship between polymorphisms in TLR2 gene and lung function in human subjects.

This study extended these observations from experimental studies using human cells and animals, and human population studies using naïve healthy adults to full-time workers. The objective of this study was to investigate the effects of TLR2 and TLR4 polymorphisms on lung

function in full-time workers in swine operations and non-farming rural dwellers, who are exposed to high and low levels of respiratory hazards, respectively.

6.3 Materials and methods

6.3.1 Recruitment and data collection

This study was conducted in rural Saskatchewan, Canada. The workers in swine operations were recruited from the Pork Producers' Association in Saskatchewan. Large swine production companies were contacted and requested to circulate a description of the study to their workers. To participate in the study, workers in swine operations had to be 17 years or older and had to work in a confinement building for at least four days per week with a total work-duration of 20 hours or more per week. The participation rate from the large swine operations was approximately 70%.

Non-farming rural dwellers were recruited from non-farming rural dwellers residing in close proximity (within 100 km radius) to a swine production site by mail using taxation lists provided by rural towns. Non-farming rural dwellers who were involved in mining, grain handling, metal and auto-body work were excluded because of their potential exposure to respiratory hazards.

All interviews and pulmonary function tests were conducted in communities located in close approximation to swine production sites and the residences of the non-farming rural dwellers. The study was approved by the Biomedical Research Ethics Board of the University of Saskatchewan. Informed written consent was obtained from all subjects.

A previously validated questionnaire [1,2,16,21] was used to collect information comprising: anthropomorphic data, respiratory symptoms, smoking history, past illnesses, and occupational

history. Each participant also had measurements of height (cm), weight (kg) and systolic and diastolic blood pressure (mmHg).

Pulmonary measurements included forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and forced expiratory flow between 25% and 75% of FVC ($FEF_{25\%-75\%}$). These measurements were obtained using a volume displacement spirometer (model 1022; SensorMedics; Yorba Linda, CA) by trained technicians who followed the American Thoracic Society recommendations.[22] For each subject, the lung function measurements were taken in the sitting position with a nose-clip in place. The technology has been used in our previous studies.[2,23,24] Percentage predicted values for FEV_1, FVC, FEV_1/FVC ratio, and $FEF_{25\%-75\%}$ were obtained using the equations developed by Crapo et al.[25]

A current smoker was defined from the questionnaire as a person currently smoking cigarettes. An ex-smoker was defined from the questionnaire as a person who had smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime but was not currently smoking. A life-time non-smoker was defined from the questionnaire as a person who had not smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime.

Allergy skin prick tests (SPT) were carried out for five allergens (Aleternaria sp., swine, mixed grass allergens, house dust mite and cat dander from Western Allergy Services Ltd. Burnaby, BC, Canada), as described in another study reporting the reciprocal association between atopy and respiratory symptoms using the same cohort.[26] Histamine and saline were also included in the SPT as the positive and negative control. The tests were conducted on the same occasion as the pulmonary function testing using methods in previous studies. [17,26] The SPT was performed on the normal skin on each subject's forearms and the distance between two SPTs was 2 cm to avoid cross-contamination. Participants were considered positive if one or

more skin prick test had a raised wheal ≥ 3 mm in comparison to the saline control.[27] The technicians conducting the SPT were blinded to the genotypes of each subject.

6.3.2 DNA isolation and genotyping

Blood sample collection and genotyping methods have been previously described.[28] Briefly, blood samples were collected in Qiagen *PAXgene* tubes with blood cards spotted for each sample (*S&S, catalog #10538414*). A Gentra Autopure robot (*Qiagen Corporation, Hilden, Germany*) was used to isolate DNA. DNA for genotyping was quantified by PicoGreen assays. Human TLR4 Asp299Gly and Thr399Ile polymorphisms were genotyped using TaqMan assays and standard protocols on the ABI 7900 Sequence Detection System. Plasmids carrying mutant and wild type sequences were included as controls. TLR2 SNPs were genotyped as a part of a 96-plex GoldenGate genotyping assay on Veracode beads. Assays were performed according to manufacturer's protocols and scanned on a BeadXpress reader (Illumina, Inc., San Diego, CA, USA). Raw genotyping data were clustered and cleaned in the BeadStudio software (Illumina® GenomeStudio).

The selection of TLR2 SNPs was based on the following criteria: (1) We selected ones that have been associated with one or more disease phenotypes in multiple cohorts or have been shown to alter gene function in biological assays; (2) We used high density single nucleotide polymorphism (SNP) maps generated by the HapMap project and selected sets of tagging SNPs (tSNPs) for TLR2 using the SNPSelector software.[29] Using these criteria, three SNPs of TLR2 gene (TLR2-16933T/A, Arg753Gln and Arg677Trp) were genotyped. However, TLR2 Arg677Trp polymorphism failed to satisfy the Hardy-Weinberg equilibrium (HWE) in the workers and was excluded from this study.

6.3.3 Statistical methods

Differences in genotype frequencies or descriptive characteristics between workers in swine operations and non-farming rural dwellers were determined by chi square test or by Fisher's exact test when the expected count for any cell was less than five. For each genotype, HWE was tested, and only those SNPs which follow HWE were included. Three inheritance models (additive, dominant and recessive) were fitted to determine the best-fitted model with the smallest Akaike information criterion (AIC) values.

Multiple linear regression analysis was used to test the differences in the mean values of lung function parameters between genotypes of each SNP after controlling for age, sex, height, weight and smoking habit. In the multiple regression analysis, interaction between exposure groups (workers, non-farming rural dwellers) and genotypes of each SNP was included and post-hoc contrasts were used to test the differences in the mean lung function parameters between polymorphic and wild-type groups in workers and non-farming rural dwellers, respectively. The Bonferroni correction was used to adjust the p-values from the multiple regressions for testing multiple TLR2 SNPs. The effect of years of exposure in swine operation on the results was tested by including the interaction between years of exposure and the dichotomous variable indicating polymorphic or wild-type group in the multiple regression of four lung function measurements. We considered years of exposure as both continuous variable and a dichotomous variables (< 10 years vs. ≥10 years of swine work). SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis.

6.4 Results

Table 6-1 shows the characteristics of workers in swine operations and rural dwellers. Workers in the swine operations were significantly younger for both males and females and shorter in height in males than non-farming rural dwellers. Mean weight was similar between the two groups in both males and females. There were more males among the workers (64.2%) than rural dwellers (44.8%). Smoking was more prevalent among the workers than among the rural dwellers. The mean values of lung function parameters were similar between the two groups in both males and females, except mean values of percent-predicted FEV_1, which was significantly lower in the workers than rural dwellers. Atopy was less common in workers in swine operations than non-farming rural dwellers (35.3% vs. 42.1%), but the difference was not statistically significant (Table 6-1).

As shown in Table 6-1, the genotype distributions of Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene and -16933T/A and Arg753Gln polymorphisms in the TLR2 gene were similar between the workers and non-farming rural dwellers. The two SNPs in the TLR4 gene were in high linkage disequilibrium (D'=0.97).

The recessive model (AA vs. AT+TT) of TLR2-16933T/A had the best fit with the smallest AIC values in the regression analysis of all the four lung function parameters in comparison to the additive (AA=2, AT=1 and TT=0) and dominant models (AA+AT vs. TT) (data not shown). Under the recessive model, as shown in Table 6-2 and Figure 6-1, workers with AA genotype (polymorphic group) of -16933T/A polymorphism in the TLR2 gene had significantly greater mean values of lung function parameters than workers with AT or TT genotypes (wild-type) after applying the Bonferroni correction and controlling for age, sex, height, weight, and smoking habit (FEV_1(L): 3.7 vs. 3.5, p=0.009; $FEF_{25\%-75\%}$(L/s): 3.7 vs. 3.3,

p=0.003; predicted FEV_1(%): 100.3 vs. 95.6, p=0.005; predicted FVC (%): 105.4 vs. 101.9, p=0.03; predicted $FEF_{25\%-75\%}$ (%): 92.4 vs. 83.4, p-0.009). Among non-farming rural dwellers, no such associations were observed.

As shown in Table 6-3 and Figure 6-2, workers with AG genotype (polymorphic group) of Arg753Gln polymorphism in the TLR2 gene had significantly greater mean values of lung function parameters than workers with GG genotype (wild-type) after applying the Bonferroni correction and controlling for age, sex, height, weight, and smoking habit (FEV_1/FVC(%): 80.9 vs. 77.8, p=0.03; $FEF_{25\%-75\%}$(L/s): 4.0 vs. 3.3, p=0.004; predicted FEV_1 (%): 103.4 vs. 96.3, p=0.02; predicted FEV_1/FVC (%): 84.1 vs. 82.7, p=0.02; predicted $FEF_{25\%-75\%}$ (%): 102.5 vs. 84.6, p=0.003). Among non-farming rural dwellers, no significant associations were observed between Arg753Gln genotypes and lung function measurements.

As shown in Table 6-4, the effects of interaction term between TLR2-16933T/A polymorphism and an exposure group variable indicating workers with more than 10 years of swine work and non-farming rural dwellers were not significant in the multiple regression analysis of all lung function parameters except $FEF_{25\%-75\%}$ (p=0.03) and percentage predicted $FEF_{25\%-75\%}$ (p=0.05). Among workers with more than 10 years of swine work, workers with TLR2-16933T/A polymorphism had significantly better mean values of $FEF_{25\%-75\%}$ (4.0 vs. 3.3 L/s, p=0.02) and percentage predicted $FEF_{25\%-75\%}$ (98.5 vs. 82.0 %, p=0.04) than workers with wild-type after controlling for age, sex, height, weight and smoking habit. However, none of these associations remained significant after adjusting for Bonferroni correction. As shown in Table 6-5, none of the interaction terms between TLR2 Arg753Gln polymorphism and the exposure group variable was significant in the multiple regression models.

Among workers in swine operations, when the variable of years of swine work was treated as a continuous variable in the interaction term with each of the TLR2 SNPs, none of the interaction terms was significant in the multiple regression analyses (data not shown). .

No significant associations were observed between lung function parameters and TLR4 polymorphisms (Asp299Gly and Thr399Ile) either in the workers or in the non-farming rural dwellers (data not shown).

6.5 Discussion

In our study, Arg753Gln and -16933T/A polymorphisms in the TLR2 gene, rather than Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene, were significantly associated with better lung function in full-time workers in swine operations. In contrast, these findings were not observed in non-farming rural dwellers, a group likely exposed to low levels of respiratory hazards.

Although there is no hypothesis suggesting that inhalation is more relevant than contact through the skin or ingestion, results from several studies indicate that inhalation is relevant for acute and chronic health effects in subjects exposed to the indoor environment in the swine facilities.[1-5,21,23,30] In a study investigating the acute inflammation reactions associated with bacteria concentration in the airborne dust in healthy naïve subjects exposed in a swine facility for three hours, endotoxin (from Gram negative bacteria) and peptidoglycan (mainly from Gram-positive bacteria) concentrations in the airborne dust were associated with bronchial hyperresponsiveness and lower vital capacity , and increased blood granulocyte concentration and body temperature, respectively.[26] In a study of investigating the effect of using respiratory protective device in swine operations in Saskatchewan showed that healthy naïve subjects

wearing respiratory protective devices for five hours had decreased acute symptoms including cough, chest tightness and phlegm and 10% decrease in lung function in comparison to those not wearing respiratory protective devices.[25]

However, individual susceptibility to these exposures seems to be quite variable. Toll-like receptors, a family of immune receptors, which are involved in recognition of pathogen-association molecular patterns, play a pivotal role in the modulation of both innate and adaptive immune responses.[10-12] Human immune responses are important mechanisms of defense against pathogenic microorganisms by release of inflammatory cytokines. However, many cytokines, such as TNF-α, INF-γ have been associated with many respiratory diseases such as asthma.[31-33]

TLR2 has been found to respond to a wide range of ligands of bacterial products including Gram-positive bacteria,[34] whereas, TLR4 seems to respond only to LPS, a major component of the outer cell membrane of Gram-negative bacteria. In an animal model, Hoshino et al showed that TLR4-deficient mice are hypo-responsive to LPS.[35] Arbour et al provided the first direct evidence that polymorphisms in the TLR4 gene in healthy adults were associated with hyporesponsiveness when exposed to a total of 41.5μg LPS (daily LPS exposure level of an agricultural worker).[36] In a study of healthy adults, Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene were associated with a blunted response to inhaled LPS.[15] Another study of healthy young adults showed that subjects with polymorphisms in the TLR4 gene had less reduction in the percentage across-shift change in FEV_1 from baseline than did those with wild-type when they were exposed for 5 hours in a swine operation.[16] Our study showed that among full-time workers in swine operations, Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene were not significantly associated with lung function, raising the possibility that full-time workers might acclimatize to repeated endotoxin exposure in the workplace.

Workers in swine operations are subjected to long-term workplace exposure to a complex indoor environment which includes high concentration of Gram-positive bacteria.[8] Experimental studies showed reduced immune responses in TLR2-deficient macrophages when exposed to cell walls of Gram-positive bacteria,[14] and in human embryonic kidney (HEK) 293 cells when exposed to bleomycin (BLM) in vitro.[18] A study using an animal model showed the expression of CD86 on lung cells (a protein expressed on antigen-presenting cells for T cell activation and survival) and tissue-infiltrating M1 macrophages (pro-inflammatory phenotype) were completely inhibited during the entire BLM-induced pulmonary inflammation stage in TLR2-deficiency mice; pDC (plasmacytoid dendritic cells), M2 macrophages and $FoxP3^+$ Tregs (T regulatory cells) were significantly blocked at the later stage of pulmonary inflammation in TLR2-deficiency mice.[19] In this study, significant protection from apoptosis of pulmonary cells was also observed in TLR2-deficiency mice.[19] A recent study showed TLR2 knock-out mice had reduced TNF-α and IL-6 expression from lung macrophages, and significant reductions in total cellularity, neutrophil influx, TNF-α, IL-6, and CXCL1 from bronchoalveolar lavage fluid and lung tissue when exposed to swine facility dust extract.[20]

There are few published studies reporting the effects of polymorphisms in the TLR2 gene on respiratory systems in human population. A recent study of 916 children from Netherlands showed that children with minor allele A on -16933T/A polymorphism in the TLR2 gene were significantly less likely to have doctor diagnosed asthma in comparison to children with homozygotes of T allele (TA vs. TT: OR=0.61; AA vs. TT: OR=0.53), and polymorphisms in the TLR4 gene (Asp299Gly and Thr399Ile) were not significantly associated with doctor diagnosed asthma in this study.[37] In a study of 229 farmers' children and 380 non-farmers' children in Austria and Germany, the TLR2 -16933T/A polymorphisms were significantly

associated with a diagnosis of asthma, current asthma symptoms, atopic sensitization, and current hay fever symptoms among farmers' children, but not among children from the same rural communities not living on farms.[38] No significant association between polymorphisms of the TLR4 gene and any asthma and asthma-related symptoms was observed in either farmers' children or non-farmers' children.[38] This study concluded that the TLR2 gene was a major determinant of asthma in children of European farmers.[38]

Our study extended the current knowledge from experimental studies using human cells and animal models and human studies using naïve healthy adults to full-time workers who are exposed to high concentration of indoor respiratory hazards at their workplace. Our findings were the first to provide direct evidence that TLR2 Arg753Gln and -16933T/A polymorphisms, rather than TLR4 Asp299Gly and Thr399Ile polymorphisms, are associated with protective effects on lung function among full-time workers in swine operations possibly due to their reduced pro-inflammation responses to their workplace exposure through TLR2 pathway. This also explains the importance of Gram-positive bacteria in causing the adverse health effects among workers in swine operations.

In our study, workers with TLR2-16933T/A polymorphism had statistically significant greater mean values of lung function than workers with wild-type (160 mL in FEV_1 and 390 mL/s in $FEF_{25\%-75\%}$), and a bigger difference in $FEF_{25\%-75\%}$ (670 mL/s) between the polymorphism and wild-type of TLR2 Arg753Gln. A longitudinal study of 217 swine confinement workers and 179 non-farming rural dwellers from 1990/91 to 1994/95 showed that swine confinement workers had excess annual decline of 26.1 mL in FEV_1 and 42.0 mL/s in $FEF_{25\%-75\%}$ over non-farming rural dwellers.[1] In comparison to the annual decline, the observed

differences in FEV_1 and $FEF_{25\%-75\%}$ between polymorphic and wild-type groups are clinically relevant for the respiratory health of workers in swine operations.

There were some limitations in our study. Lack of objective measurement of exposures including endotoxin levels associated with Gram-negative bacteria, and peptidoglycan levels associated with Gram-positive bacteria in our study limits our ability to examine the dose-response relationship between exposure and lung function among workers with polymorphisms and wild-type in the TLR2 and the TLR4 genes. However, inclusion of non-farming rural dwellers in our study allowed us to examine the effects of these polymorphisms in the TLR2 and the TLR4 genes at low level exposure.

In conclusion, this study is the first to report the airways protective effects of TLR2 polymorphisms among workers in swine operations. These findings raise the possibility that TLR2 polymorphisms are protective of airway disease in subjects exposed to Gram-positive organisms in the inhaled airborne dust and genetic testing might provide useful information for identifying workers at higher risk of respiratory problems when working in swine operations.

6.6 References

(1) Senthilselvan A, Dosman JA, Kirychuk SP, Barber EM, Rhodes CS, Zhang Y, et al. Accelerated lung function decline in swine confinement workers. Chest 1997 Jun;111(6):1733-41.

(2) Senthilselvan A, Chenard L, Ulmer K, Gibson-Burlinguette N, Leuschen C, Dosman JA. Excess respiratory symptoms in full-time male and female workers in large-scale swine operations. Chest 2007 Apr;131(4):1197-204.

(3) Eduard W, Pearce N, Douwes J. Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents. Chest 2009 Sep;136(3):716-25.

(4) Dosman JA, Lawson JA, Kirychuk SP, Cormier Y, Biem J, Koehncke N. Occupational asthma in newly employed workers in intensive swine confinement facilities. Eur Respir J 2004 Oct;24(4):698-702.

(5) Dosman JA, Lawson JA, Kirychuk SP, Cormier Y, Biem J, Koehncke N. Three new cases of apparent occupational asthma in swine confinement facility employees. Eur Respir J 2006 Dec;28(6):1281-2.

(6) Crook B, Robertson JF, Glass SA, Botheroyd EM, Lacey J, Topping MD. Airborne dust, ammonia, microorganisms, and antigens in pig confinement houses and the respiratory health of exposed farm workers. Am Ind Hyg Assoc J 1991 Jul;52(7):271-9.

(7) Schwartz DA, Landas SK, Lassise DL, Burmeister LF, Hunninghake GW, Merchant JA. Airway injury in swine confinement workers. Ann Intern Med 1992 Apr 15;116(8):630-5.

(8) Nehme B, Letourneau V, Forster RJ, Veillette M, Duchaine C. Culture-independent approach of the bacterial bioaerosol diversity in the standard swine confinement buildings, and assessment of the seasonal effect. Environ Microbiol 2008 Mar;10(3):665-75.

(9) Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010 May;11(5):373-84.

(10) Lazarus R, Vercelli D, Palmer LJ, Klimecki WJ, Silverman EK, Richter B, et al. Single nucleotide polymorphisms in innate immunity genes: abundant variation and potential role in complex human disease. Immunol Rev 2002 Dec;190:9-25.

(11) Sabroe I, Parker LC, Wilson AG, Whyte MK, Dower SK. Toll-like receptors: their role in allergy and non-allergic inflammatory disease. Clin Exp Allergy 2002 Jul;32(7):984-9.

(12) Yang IA, Holgate ST, Holloway JW. Toll-like receptor polymorphisms and allergic disease: interpreting the evidence from genetic studies. Clin Exp Allergy 2004 Feb;34(2):163-6.

(13) Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, Cardell LO. Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. Respir Res 2005;6:100.

(14) Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999 Oct;11(4):443-51.

(15) Schwartz DA. TLR4 and LPS hyporesponsiveness in humans. Int J Hyg Environ Health 2002 Apr;205(3):221-7.

(16) Senthilselvan A, Dosman JA, Chenard L, Burch LH, Predicala BZ, Sorowski R, et al. Toll-like receptor 4 variants reduce airway response in human subjects at high endotoxin levels in a swine facility. J Allergy Clin Immunol 2009 May;123(5):1034-40, 1040.

(17) Senthilselvan A, Rennie D, Chenard L, Burch LH, Babiuk L, Schwartz DA, et al. Association of polymorphisms of toll-like receptor 4 with a reduced prevalence of hay fever and atopy. Ann Allergy Asthma Immunol 2008 May;100(5):463-8.

(18) Razonable RR, Henault M, Paya CV. Stimulation of toll-like receptor 2 with bleomycin results in cellular activation and secretion of pro-inflammatory cytokines and chemokines. Toxicol Appl Pharmacol 2006 Feb 1;210(3):181-9.

(19) Yang HZ, Cui B, Liu HZ, Chen ZR, Yan HM, Hua F, et al. Targeting TLR2 attenuates pulmonary inflammation and fibrosis by reversion of suppressive immune microenvironment. J Immunol 2009 Jan 1;182(1):692-702.

(20) Poole JA, Wyatt TA, Kielian T, Oldenburg P, Gleason AM, Bauer A, et al. Toll-like receptor 2 regulates organic dust-induced airway inflammation. Am J Respir Cell Mol Biol 2011 Oct;45(4):711-9.

(21) Zejda JE, Hurst TS, Rhodes CS, Barber EM, McDuffie HH, Dosman JA. Respiratory health of swine producers. Focus on young workers. Chest 1993 Mar;103(3):702-9.

(22) Standardization of spirometry--1987 update. Statement of the American Thoracic Society. Am Rev Respir Dis 1987 Nov;136(5):1285-98.

(23) Dosman JA, Senthilselvan A, Kirychuk SP, Lemay S, Barber EM, Willson P, et al. Positive human health effects of wearing a respirator in a swine barn. Chest 2000 Sep;118(3):852-60.

(24) Senthilselvan A, Zhang Y, Dosman JA, Barber EM, Holfeld LE, Kirychuk SP, et al. Positive human health effects of dust suppression with canola oil in swine barns. Am J Respir Crit Care Med 1997 Aug;156(2 Pt 1):410-7.

(25) Crapo RO, Morris AH, Gardner RM. Reference spirometric values using techniques and equipment that meet ATS recommendations. Am Rev Respir Dis 1981 Jun;123(6):659-64.

(26) Dosman JA, Chenard L, Rennie DC, Senthilselvan A. Reciprocal association between atopy and respiratory symptoms in fully employed female, but not male, workers in swine operations. J Agromedicine 2009;14(2):270-6.

(27) The use of standardized allergen extracts. American Academy of Allergy, Asthma and Immunology (AAAAI). J Allergy Clin Immunol 1997 May;99(5):583-6.

(28) Pahwa P, Karunanayake CP, Rennie DC, Chen Y, Schwartz DA, Dosman JA. Association of the TLR4 Asp299Gly polymorphism with lung function in relation to body mass index. BMC Pulm Med 2009;9:46.

(29) Xu H, Gregory SG, Hauser ER, Stenger JE, Pericak-Vance MA, Vance JM, et al. SNPselector: a web tool for selecting SNPs for genetic association studies. Bioinformatics 2005 Nov 15;21(22):4181-6.

(30) Zhiping W, Malmberg P, Larsson BM, Larsson K, Larsson L, Saraf A. Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. Am J Respir Crit Care Med 1996 Nov;154(5):1261-6.

(31) Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. J Allergy Clin Immunol 1992 May;89(5):958-67.

(32) Gosset P, Tsicopoulos A, Wallaert B, Joseph M, Capron A, Tonnel AB. Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. Am Rev Respir Dis 1992 Sep;146(3):768-74.

(33) Hallsworth MP, Soh CP, Lane SJ, Arm JP, Lee TH. Selective enhancement of GM-CSF, TNF-alpha, IL-1 beta and IL-8 production by monocytes and macrophages of asthmatic subjects. Eur Respir J 1994 Jun;7(6):1096-102.

(34) Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999 Nov 19;274(47):33419-25.

(35) Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 1999 Apr 1;162(7):3749-52.

(36) Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000 Jun;25(2):187-91.

(37) Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. Thorax 2010 Aug;65(8):690-7.

(38) Eder W, Klimecki W, Yu L, von ME, Riedler J, Braun-Fahrlander C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol 2004 Mar;113(3):482-8.

Table 6-1. Characteristics of workers in swine operations and non-farming rural dwellers

		Workers Mean ± SD	Rural dwellers Mean ± SD	p value
Age, yr	Male	36.2 ± 11.9	40.1 ± 13.5	0.002
	Female	34.9 ± 10.7	38.7 ± 12.4	0.004
Height, cm	Male	176.8 ± 6.1	178.3 ± 6.7	0.02
	Female	164.4 ± 5.8	164.7 ± 6.5	0.67
Weight, kg	Male	88.1 ± 16.6	90.4 ± 17.8	0.17
	Female	75.5 ± 15.0	75.6 ± 16.9	0.96
Male, no (%)		240 (64.2)	184 (44.8)	< 0.0001
Smoking		No (%)	No (%)	< 0.0001
Current smoker		117 (31.3)	74 (18.0)	
Ex-smoker		101 (27.0)	115 (28.0)	
Nonsmoker		156 (41.7)	222 (54.0)	
Atopic, no (%)		123 (35.3)	169 (42.1)	0.06
Observed FEV_1, L	Male	4.1 ± 0.7	4.1 ± 0.8	0.63
	Female	3.1 ± 0.6	3.1 ± 0.6	0.76
FVC, L	Male	5.3 ± 0.8	5.3 ± 1.0	0.63
	Female	3.9 ± 0.6	3.9 ± 0.6	0.68
FEV_1/FVC, %	Male	77.8 ± 6.6	77.9 ± 6.5	0.88
	Female	80.0 ± 6.2	80.1 ± 6.1	0.87
$FEF_{25\%-75\%}$, L	Male	3.8 ± 1.2	3.8 ± 1.3	0.90
	Female	3.2 ± 1.0	3.1 ± 1.0	0.73
Predicted (%) FEV_1		96.9 ± 12.6	99.3 ± 13.3	0.01
FVC		102.4 ± 12.0	103.9 ± 13.7	0.10
FEV_1/FVC		82.9 ± 2.4	82.6 ± 2.6	0.10
$FEF_{25\%-75\%}$		86.9 ± 25.6	90.1 ± 25.0	0.08
TLR4 Asp299Gly *	GG	0 (0.0)	2 (0.7)	0.20†
(rs4986790)	AG	35 (12.8)	29 (9.8)	
	AA	238 (87.2)	266 (89.6)	
Thr399Ile *	CC	0 (0)	0 (0)	0.91
(rs4986791)	CT	33 (12.1)	35 (11.8)	
	TT	240 (87.9)	262 (88.2)	
LD (D')†		0.97	0.97	
TLR2-16933T/A*	AA	68 (24.5)	66 (20.9)	0.58
(rs4696480)	AT	144 (51.8)	172 (54.4)	
	TT	66 (23.7)	78 (24.7)	
Arg753Gln *	AA	0 (0)	0 (0)	0.99
(rs5743708)	AG	18 (6.6)	20 (6.4)	
	GG	257 (93.4)	294 (93.6)	
LD (D')†		0.55	0.47	

*: HWE was followed in both workers in swine operations and non-farming rural dwellers;
†: Linkage Disequilibrium between the two SNPs in the TLR4 and TLR2 genes, respectively.

Table 6-2. Differences in mean values of lung function parameters between TLR2 -16933T/A polymorphism (AA) and wild type (AT+TT) for workers in swine operations and non-farming rural dwellers in the recessive inheritance model

	Workers in swine operations (n=278)			Non farming rural dwellers (n=316)		
Lung function measurements	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)
Observed vales *						
FEV_1, (L)	3.7 (3.6-3.8)	3.5 (3.5-3.6)	0.2 (0.0-0.3)†	3.6 (3.5-3.7)	3.6 (3.6-3.7)	0.0 (-0.1:0.1)
FVC, (L)	4.7 (4.6-4.8)	4.6 (4.5-4.6)	0.1 (-0.0:0.3)	4.6 (4.5-4.8)	4.6 (4.6-4.7)	0.0 (-0.1:0.2)
FEV_1/FVC, (%)	79.0 (77.5-80.3)	77.7 (76.8-78.5)	1.3 (-0.3:2.9)	78.5 (77.1-78.0)	78.8 (78.1-79.6)	-0.3 (-1.9:1.3)
$FEF_{25\%-75\%}$, (L/s)‡	3.7 (3.4-3.9)	3.3 (3.1-3.4)	0.4 (0.1:0.6)†	3.5 (3.3-3.7)	3.5 (3.4-3.5)	0.0 (-0.2:0.3)
Predicted values (%) **						
FEV_1‡	100.3 (97.4-103.2)	95.6 (93.9-97.2)	4.8 (1.4:8.1)†	98.3 (95.4-101.3)	98.2 (96.7-99.8)	0.1 (-3.2:3.4)
FVC	105.4(102.6-108.3)	101.9(100.2-103.5	3.6 (0.3:6.8)†	103.9(101.0-106.8)	103.4(101.8-104.9)	0.5 (-2.7:3.7)
FEV_1/FVC	83.1 (82.6-83.7)	82.7 (82.3-83.0)	0.5 (-0.2:1.2)	82.5 (82.0-83.1)	82.3 (82.0-82.6)	0.3 (-0.4:0.9)
$FEF_{25\%-75\%}$‡	92.4 (86.5-98.3)	83.4 (80.0-86.8)	9.0 (2.2:15.8)†	87.7 (81.6-93.7)	88.0 (84.8-91.2)	-0.3 (-7.1:6.4)

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit.

**: p-value from the multiple regression analysis after adjusting for smoking habit.

†: significant after the Bonferroni correction for multiple comparisons ($p < 0.025 = 0.05/2$).

‡: the p value (Type III) of the interaction term from the multiple regression analysis is significant.

Table 6-3. Differences in mean values of lung function parameters between TLR2 Arg753Gln polymorphism (AG) and wild type (GG) for workers in swine operations and non-farming rural dwellers

	Workers in swine operations (n=275)			Non farming rural dwellers (n=314)		
	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)
Observed values *						
FEV_1, (L)‡	3.8 (3.6-4.0)	3.6 (3.5-3.6)	0.2(0.0:0.4)	3.5 (3.3-3.7)	3.6 (3.6-3.7)	-0.1 (-0.3:0.1)
FVC, (L)	4.7 (4.5-5.0)	4.6 (4.5-4.6)	0.1(-0.1:0.4)	4.5 (4.3-4.7)	4.6 (4.6-4.7)	-0.1 (-0.4:0.1)
FEV_1/FVC, (%)	80.8 (78.1-83.6)	77.8 (77.1-78.6)	3.1(0.2:5.9)†	78.4 (75.8-81.0)	78.8 (78.1-79.5)	-0.3 (-3.0:2.3)
$FEF_{25\%-75\%}$, (L/s)‡	4.0 (3.6-4.4)	3.3 (3.2-3.4)	0.7(0.2:1.1)†	3.4 (2.9-3.8)	3.5 (3.4-3.6)	-0.1 (-0.6:0.3)
Predicted values (%) **						
FEV_1‡	103.4 (97.8-109.0)	96.3 (94.8-97.8)	7.1 (1.3:12.9)†	96.2 (90.9-101.6)	98.4 (97.0-99.9)	-2.2 (-7.7:3.3)
FVC	106.2(100.7-111.8)	102.4(100.9-103.9)	3.8 (-1.9:9.5)	101.3(96.1-106.6)	103.6(102.2-105.1)	-2.3 (-7.7:3.1)
FEV_1/FVC‡	84.1 (82.9-85.2)	82.7 (82.4-83.0)	1.4 (0.2:2.5)†	81.6 (80.5-82.6)	82.4 (82.1-82.7)	-0.8 (-1.9:0.3)
$FEF_{25\%-75\%}$‡	102.5 (91.0-114.0)	84.6 (81.6-87.7)	17.9 (6.0:29.8)†	86.8 (75.8-97.7)	88.1 (85.1-91.1)	-1.3 (-12.6:9.9)

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit

**: p-value from the multiple regression analysis after adjusting for smoking habit.

†: significant after the Bonferroni correction for multiple comparisons ($p < 0.025 = 0.05/2$).

‡: the p value (Type III) of the interaction term from the multiple regression analysis is significant.

Table 6-4. Differences in mean values of lung function parameters between TLR2 -16933T/A polymorphism (AA) and wild type (AT+TT) for workers in swine operations (≥ 10 years of swine work) and non-farming rural dwellers

Lung function parameters	Workers in swine operations (≥ 10 years of swine work)			Non-farming rural dwellers			p value (Interaction term)
	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)	
Observed *							
FEV_1, (L)	3.9 (3.7-4.1)	3.6 (3.5-3.8)	0.3 (0.0:0.5)	3.6 (3.5-3.7)	3.6 (3.6-3.7)	0.0 (-0.1:0.1)	0.11
FVC, (L)	4.9 (4.6-5.2)	4.7 (4.5-4.9)	0.2 (-0.2:0.5)	4.6 (4.5-4.8)	4.6 (4.6-4.7)	0.0 (-0.1:0.2)	0.46
FEV_1/FVC, (%)	80.0 (76.7-83.2)	77.4 (75.5-79.4)	2.6 (-1.2:6.3)	78.5 (77.1-78.0)	78.8 (78.1-79.6)	-0.3 (-1.9:1.3)	0.16
$FEF_{25\%-75\%}$, (L/s)	4.0 (3.43-4.48)	3.3 (2.9-3.6)	0.7 (0.1:1.3)	3.5 (3.3-3.7)	3.5 (3.4-3.5)	0.0 (-0.2:0.3)	**0.03**
Predicted (%) **							
FEV_1	104.6 (98.0-111.2)	97.0 (93.1-101.0)	7.5 (-0.2:15.3)	98.3 (95.4-101.3)	98.2 (96.7-99.8)	0.1 (-3.2:3.4)	0.09
FVC	109.0 (102.5-115.5)	103.9 (100.0-107.8)	5.0 (-2.5:12.6)	103.9(101.0-106.8)	103.4(101.8-104.9)	0.5 (-2.7:3.7)	0.30
FEV_1/FVC	82.2 (80.9-83.5)	81.4 (80.6-82.2)	0.8 (-0.7:2.3)	82.5 (82.0-83.1)	82.3 (82.0-82.6)	0.3 (-0.4:0.9)	0.57
$FEF_{25\%-75\%}$	98.5 (84.9-112.2)	82.0 (73.8-90.1)	16.6 (0.7:32.4)	87.7 (81.6-93.7)	88.0 (84.8-91.2)	-0.3 (-7.1:6.4)	**0.05**

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit.

**: p-value from the multiple regression analysis after adjusting for smoking habit.

†: significant after the Bonferroni correction for multiple comparisons ($p < 0.0125 = 0.05/4$).

Table 6-5. Differences in mean values of lung function parameters between TLR2 Arg753Gln polymorphism (AG) and wild type (GG) for workers in swine operations (≥ 10 years of swine work) and non-farming rural dwellers

Lung function parameters	Workers in swine operations (≥ 10 years of swine work)			Non-farming rural dwellers			p value (Interaction term)
	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)	Polymorphism Mean (95%CI)	Wild-type Mean (95%CI)	Comparison Diff (95%CI)	
Observed *							
FEV_1, (L)	4.0 (3.39-4.61)	3.7 (3.6-3.8)	0.3 (-0.3:0.9)	3.5 (3.3-3.7)	3.6 (3.6-3.7)	-0.1 (-0.3:0.1)	0.19
FVC, (L)	5.3 (4.5-6.0)	4.7 (4.6-4.9)	0.5 (-0.2:1.3)	4.5 (4.3-4.7)	4.6 (4.6-4.7)	-0.1 (-0.4:0.1)	0.09
FEV_1/FVC, (%)	77.1 (69.0-85.3)	78.2 (76.4-80.0)	-1.1 (-9.4:7.3)	78.4 (75.8-81.0)	78.8 (78.1-79.5)	-0.3 (-3.0:2.3)	0.90
$FEF_{25\%-75\%}$, (L/s)	3.5 (2.2-4.9)	3.5 (3.2-3.7)	0.1 (-1.3:1.4)	3.4 (2.9-3.8)	3.5 (3.4-3.6)	-0.1 (-0.6:0.3)	0.74
Predicted (%) **							
FEV_1	111.8 (94.9-128.6)	98.6 (95.0-102.1)	13.2 (-4.0:30.4)	96.2 (90.9-101.6)	98.4 (97.0-99.9)	-2.2 (-7.7:3.3)	0.10
FVC	119.8 (103.2-136.3)	104.7 (101.2-108.1)	15.1 (-1.8:32.0)	101.3(96.1-106.6)	103.6(102.2-105.1)	-2.3 (-7.7:3.1)	0.06
FEV_1/FVC	83.3 (80.0-86.7)	81.5 (80.8-82.2)	1.8 (-1.6:5.2)	81.6 (80.5-82.6)	82.4 (82.1-82.7)	-0.8 (-1.9:0.3)	0.16
$FEF_{25\%-75\%}$	94.2 (59.7-128.7)	86.4 (79.1-93.6)	7.8 (-27.3:43.0)	86.8 (75.8-97.7)	88.1 (85.1-91.1)	-1.3 (-12.6:9.9)	0.64

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit

**: p-value from the multiple regression analysis after adjusting for smoking habit.

†: significant after the Bonferroni correction for multiple comparisons ($p < 0.0125 = 0.05/4$).

Figure 6-1. Boxplot of lung function between TLR2-16933T/A polymorphic and wild-type groups in rural dwellers and workers

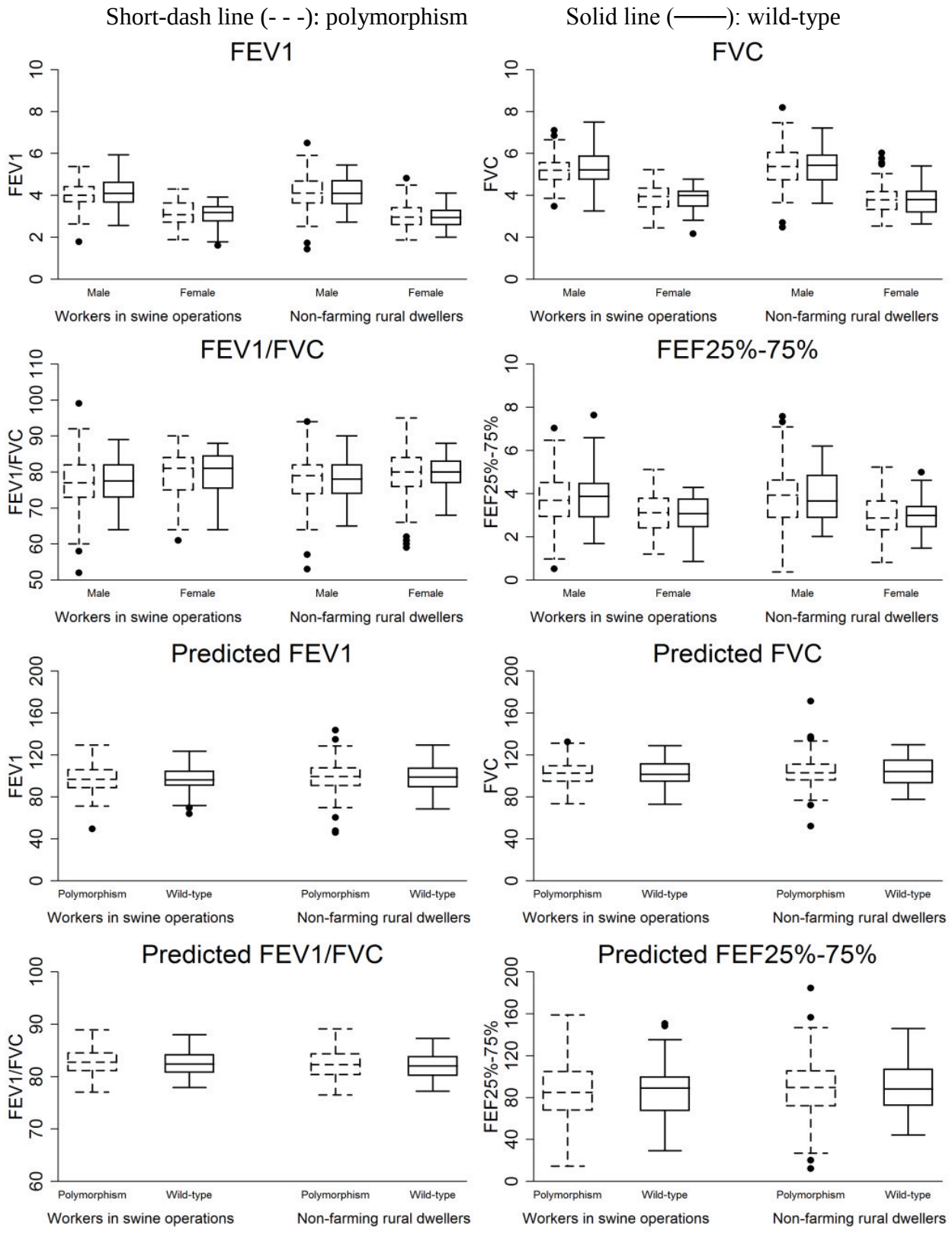
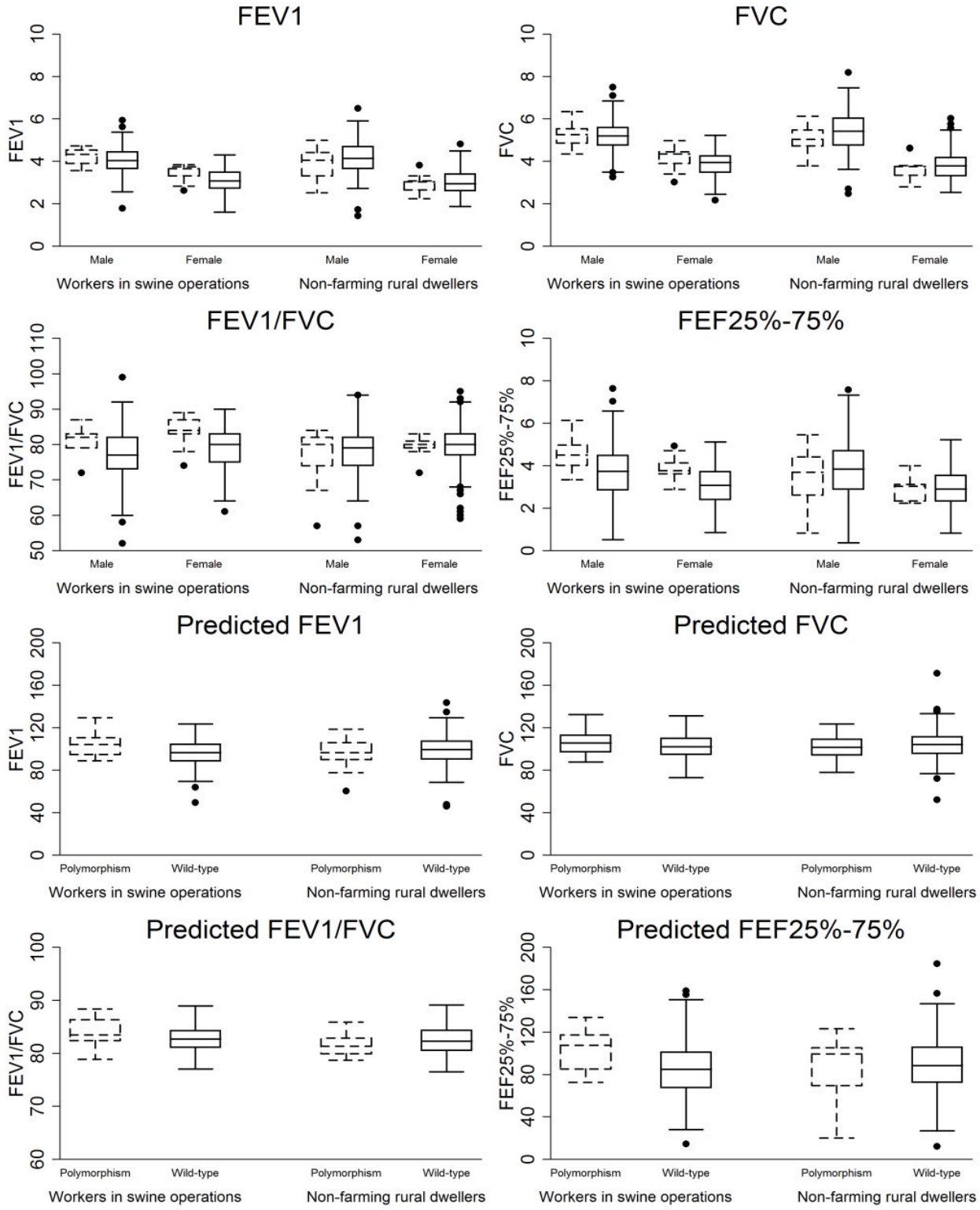


Figure 6-2 Boxplot of lung function between TLR2 Arg753Gln polymorphic and wild-type groups in rural dwellers and workers

Short-dash line (- - -): polymorphism Solid line (——): wild-type



CHAPTER 7

EFFECT OF NOS3 GENE POLYMORPHISMS ON LUNG FUNCTION IN WORKERS IN SWINE OPERATIONS

7.1 ABSTRACT

Occupational exposures to respiratory hazards in swine operations have been associated with increased risk of respiratory disorders. Nitric Oxide is involved in physiological and pathophysiological processes in the lungs. The effects of polymorphisms in the NOS3 gene on lung function among workers in swine operations and non-farming rural dwellers were examined in two independent studies from Saskatchewan.

The first cohort is a cross-sectional study of 374 full-time workers and 411 non-farming rural dwellers in 2003/04. The second cohort is a longitudinal study of 302 male workers and 261 non-farming rural dwellers who participated the initial study in 1990/91 and were followed-up in 1994/95 (217 workers and 171 rural dwellers) and in 2003/04 (173 workers and 119 rural dwellers). Information on demographic and lifestyle factors, lung function measurements and blood samples for genotyping were obtained. Multiple linear regression analysis was used for the statistical analysis of the cross-sectional and longitudinal studies with the addition of generalized estimating equation (GEE) for the longitudinal study. The Bonferroni correction was used to adjust the p-values from the MLRs for testing multiple SNPs in the NOS3 gene.

Workers with NOS3-786T/C polymorphism (CC) had greater mean lung function than those with the wild-type (TC+TT) in the cross-sectional study (FEV_1(L): 3.75 vs. 3.55, p=0.009; FVC(L): 4.79 vs. 4.56, p=0.03) after controlling for potential confounders and adjusting for multiple comparison. In the longitudinal study, workers with NOS3-786T/C polymorphism

(CC) had lower mean annual decline rates in FEV_1 (-30.5 ml/year vs. -58.0 ml/year, p=0.005) and FVC (-16.7 ml/year vs. -50.9 ml/year, p=0.01) than those with the TT genotype after controlling for potential confounders and adjusting for multiple comparison. However, no such associations were observed in non-farming rural dwellers.

This study is the first to report protective effects of polymorphisms in the NOS3 gene on lung function in workers in swine operations in independent cross-sectional and longitudinal studies, suggesting the important role of the *NOS3* gene in the development of airway disease in workers exposed to high concentration of respiratory hazards.

7.2 INTRODUCTION

Studies have demonstrated that working in swine operations is associated with many respiratory health problems including accelerated lung function decline,[1] asthma-like symptoms such as wheezing and shortness of breath,[2] bronchitis-like symptoms such as cough and phlegm production,[3] increased risk of developing chronic obstructive pulmonary disease (COPD)[4] and asthma.[5] Bacteria, dusts and gases (H_2S and ammonia) are found in the indoor air in swine operation and likely to contribute to respiratory dysfunction among people who are exposed during work.[6]

The etiology of these respiratory health problems among workers is not fully understood. However, there is increasing evidence showing that nitric oxide (NO) plays a key role in physiological and pathophysiological events of the lungs.[7] It appears pertinent to pulmonary events in workers in swine operations, increased levels of NO in exhaled air being observed among healthy naïve volunteers after 5-hour exposure in a pig confinement building[8] and swine confinement workers.[9]

NO is generated during the conversion of the amino acid L-arginine to L-citrulline by Nitric Oxide Synthase (NOSs).[10] There are two types of constitutive NOS(cNOS) including neuronal NOS(nNOS) and endothelial NOS(eNOS), and a single type of inducible NOS(iNOS). In humans, each is encoded by distinct genes: nNOS by the *NOS1* gene; iNOS by the *NOS2* gene; and eNOS by the *NOS3* gene.

The three human *NOS* genes, the *NOS1 gene*, the *NOS2* gene and the *NOS3* gene, are located at 12q24, 17q11.2-q12 and 7q36, respectively, which harbour many loci associated with asthma.[11,12] Polymorphisms in the *NOS3* gene have been associated with atopy, bronchial hyper-responsiveness (BHR), total and specific IgE in asthmatics, and atopic asthma in many studies.[13] Moreover, an

experimental study showed possible regulatory role of NO derived from the *NOS3* gene on the magnitude of activation of the *NOS2* gene in response to lipopolysaccharide (LPS) exposure.[14] However, to date there is limited research on the relationship between polymorphisms in the *NOS3* gene and lung function in human subjects.

In view of the important role that NO plays in physiological and pathophysiological events in the lungs, this study aimed to investigate the effects of polymorphisms in the *NOS3* gene on lung function among workers in swine operations and non-farming rural dwellers in independent cross-sectional and longitudinal studies.

7.3 METHODs

7.3.1 Methods for the cross-sectional study

The study was conducted in rural Saskatchewan, Canada. The workers in swine operations were recruited from the Pork Producers' Association in Saskatchewan. Large swine production companies were contacted and requested to circulate a description of the study to their workers. To participate in the study, workers in swine operations had to be 17 years or older and had to work in a confinement building for at least four days per week with a total work-duration of 20 hours or more per week. The participation rate from these swine operations was approximately 70%.

Non-farming rural dwellers were recruited from subjects residing in close proximity (within 100 km radius) to a swine production site by mail using taxation lists provided by rural towns. Non-farming rural dwellers who were involved in mining, grain handling, metal and auto-body work were excluded because of their potential exposure to respiratory hazards.

All interviews and pulmonary function tests were conducted in communities located in close approximation to swine production sites and the residences of the non-farming rural dwellers. The study was approved by the Biomedical Research Ethics Board of the University of Saskatchewan. Informed written consent was obtained from all subjects.

A previously validated questionnaire [1-3,15] was used to collect information comprising: anthropomorphic data, respiratory symptoms, smoking history, past illnesses, and occupational history. Each participant also had measurements of height (cm), weight (kg) and blood pressure of both systolic and diastolic blood pressures (mmHg).

Pulmonary measurements included forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and forced expiratory flow between 25% and 75%

of FVC ($FEF_{25\%-75\%}$). These measurements were obtained by trained technicians who followed the American Thoracic Society recommendations.[16] Percentage predicted values for FEV_1, FVC, FEV_1/FVC ratio, and $FEF_{25\%-75\%}$ were obtained using the equations developed by Crapo et al.[17] Obstructive airway abnormality is defined as a reduced FEV_1/FVC ratio below the 5th percentile of the predicted value.[18]

Allergy skin prick tests (SPT) were carried out for five allergens (Aleternaria sp., swine, mixed grass allergens, house dust mite and cat dander from Western Allergy Services Ltd. Burnaby, BC, Canada). Histamine and saline were also included in the SPT as the positive and negative control. The tests were conducted on the same occasion as the pulmonary function testing using methods in previous studies.[19,20] The SPT was performed on the normal skin on each subject's forearms and the distance between two SPTs was 2 cm to avoid cross-contamination. Participants were considered positive if one or more skin prick test had a raised wheal $\geq$ 3 mm in comparison to the saline control.[21] The technicians conducting the SPT were blinded to the genotypes of each subject.

A current smoker was defined from the questionnaire as a person currently smoking one or more cigarettes a day. An ex-smoker was defined from the questionnaire as a person who had smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime but was not currently smoking. A life-time non-smoker was defined from the questionnaire as a person who had not smoked more than 400 cigarettes (or equivalent amount of tobacco) in his/her lifetime.

7.3.2 Statistical Methods for the cross-sectional study

Differences in genotype frequencies or descriptive characteristics between workers in swine operations and non-farming rural dwellers were determined by chi square test or by

Fisher's exact test when the expected count for any cell was less than five. For each genotype, Hardy-Weinberg equilibrium (HWE) was tested. Linkage disequilibrium was calculated.

Multiple linear regression analysis was used to test the differences in the mean values of lung function parameters between genotypes of each SNP after controlling for age, sex, height, weight, and smoking habit. Three inheritance models (additive, dominant and recessive) were fitted to determine the best-fitted model with the smallest Akaike information criterion (AIC) value. In the multiple regression analysis, interactions between exposure groups (workers, non-farming rural dwellers) and genotypes of each SNP were included and post-hoc contrasts were used to test the differences in the mean lung function parameters between polymorphic and wild-type groups in workers and non-farming rural dwellers, respectively. The Bonferroni correction was used to adjust the p-values from the multiple regressions for testing multiple *NOS3* SNPs. SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis.

7.3.3 Methods for the longitudinal study

The participants were identified from the Saskatchewan Pork Producers Marketing Board registry and were male swine producers aged between 16 and 60 years who worked at least 2 hours per day in an enclosed swine facilities with an annual production of at least 200 pigs per year for at least one year prior to the initial investigation in 1989.[22] Non-farming rural dwellers who were 17 years or older and not involved in occupations with a risk of occupational lung disease, such as mining, grain handling, metal and auto-body work, were selected from local residences within 100 KMs radius of the swine production sites.

Of the 302 workers in swine operations and 261 non-farming rural dwellers who participated in a cross-sectional study in 1990/91 (Cycle 1), 217 workers and 171 rural dwellers were followed-up in 1994/95 (Cycle 2). Of these, 173 workers 119 rural were followed-up again

in 2003/04 (Cycle 3, Figure 1). In total, 153 workers and 107 rural dwellers participated in all three cycles. In Cycle 3, 226 subjects (143 workers and 83 rural dwellers) and 221 subjects (136 workers and 85 rural dwellers) had valid genotyping information for NOS3-786T/C and Glu298Asp polymorphisms, respectively.

A technician-administered questionnaire was used to collect information on respiratory and general health status. The questionnaire was identical on all three cycles with the exception that additional questions were added to elicit changes in health status at the second and third cycles, and employment status at the last cycle that might have occurred since the previous observations.

Years of swine work during the study period was determined by the time intervals between the cycles. For workers who reported that they quit swine farming in response to a question "Have you quit hog farming since the last study" at Cycle 3, their years of swine work were adjusted by the response to a subsequent question "if yes, in what year did you quit pig farming".

At all three cycles, lung function measurements were obtained by trained technicians using a volume displacement spirometer (model 1022; SensorMedics: Yorba Linda, CA) and followed the standards set by the American Thoracic Society.[23] Lung function test variables obtained were forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and forced expiratory flow between 25 and 75 percent of FVC ($FEF_{25\%–75\%}$).

7.3.4 Statistical Methods for the longitudinal study

Frequencies and percentages, and means and standard deviations (SDs) were used to describe categorical and continuous variables respectively. For each genotype, Hardy-Weinberg equilibrium (HWE) was tested. Linkage disequilibrium was calculated.

To examine the differences in annual decline rates in lung function between genotypes of each SNP, an interaction term between each of the *NOS3* SNPs and years of swine work during

the study period was introduced in the multiple regression analysis using GEE (generalized estimation equations) with autoregressive structure for the working correlation. Auto-regressive structure was selected as the working correlation structure to meet the longitudinal nature of this study. Potential confounders such as age, height, weight and smoking habit were controlled in the multiple linear regressions. The Bonferroni correction was used to adjust the p-values from the multiple regressions for testing several *NOS3* SNPs.

7.3.5 *DNA isolation and genotyping*

Blood sample collection and genotyping methods have been previously described.[24] Briefly, blood samples were collected in Qiagen *PAXgene* tubes with blood cards spotted for each sample (*S&S, catalog #10538414*). A Gentra Autopure robot (*Qiagen Corporation, Hilden, Germany*) was used to isolate DNA. DNA for genotyping was quantified by PicoGreen assays. *NOS3* SNPs were genotyped as a part of a 96-plex GoldenGate genotyping assay on Veracode beads. Assays were performed according to manufacturer's protocols and scanned on a BeadXpress reader (Illumina, Inc., San Diego, CA, USA). Raw genotyping data were clustered and cleaned in the BeadStudio software (Illumina® GenomeStudio).

The selection of *NOS3* SNPs was based on the following criteria: (1) We selected ones that have been associated with one or more disease phenotypes in multiple cohorts or have been shown to alter gene function in biological assays; (2) We used high density single nucleotide polymorphism (SNP) maps generated by the HapMap project and selected sets of tagging SNPs (tSNPs) for *NOS3*. The tSNPs of *NOS3* were selected using the SNPSelector software which prioritizes SNPs on their tagging for linkage disequilibrium, SNP allele frequencies and source, function, regulatory potential and repeat status.[25] Using these criteria, three SNPs, *NOS3*-

786T/C(rs2070744), *NOS3* Glu298Asp(rs1799983) and *NOS3*-922A/G(rs1800779) were genotyped.

7.4 RESULTS

7.4.1 Results from the cross-sectional study

Table 7-1 shows the characteristics of workers in swine operations and non-farming rural dwellers. Workers in swine operations were significantly younger and taller than non-farming rural dwellers. Mean weight of the two groups was similar. There were more males among the workers (64.2%) than among the rural dwellers (44.8%). Smoking was more prevalent among the workers than among the rural dwellers. Subjects with positive skin prick test were common in non-farming rural dwellers than workers. Mean values of FEV_1 and FVC were significantly greater in the workers than in the rural dwellers.

As shown in Table 7-1, the genotype distributions of the three SNPs in the *NOS3* gene (*NOS3*-786T/C, Glu298Asp and -922A/G) were similar between the workers and non-farming rural dwellers. Hardy-Weinberg equilibrium (HWE) was followed by all the three SNPs in both exposure groups. *NOS3*-786T/C and -922A/G polymorphisms were in linkage disequilibrium (r=0.94 in the workers and r=0.98 in the rural dwellers) and the latter was removed from further analysis in the cross-sectional study.

The results of *NOS3*-786T/C polymorphism from multiple linear regressions are shown in Table 7-2. The recessive model of this SNP (CC vs. TC+TT) had the best fit with the smallest AIC value in the regression analysis of all the four lung function parameters in comparison to the additive model (CC=2, TC=1 and TT=0) and dominant model (CC+TC vs. TT) (data not shown). Under the recessive model, as shown in Table 7-2 and Figure 7-2, the interaction term of this SNP and a dichotomous variable indicating two exposure groups (workers vs. rural dwellers)

was significant in the multiple linear regression of FEV_1(p=0.001) and FVC (p=0.007), respectively. Workers with CC genotype (polymorphic group) of *NOS3*-786T/C polymorphism had significantly greater mean values of FEV_1 and FVC than workers with TC or TT genotypes (wild-type group) after controlling for age, sex, height, weight, and smoking habit and adjusting for Bonferroni correction (FEV_1(L): 3.75 vs. 3.55, p=0.009; FVC (L): 4.79 vs. 4.56, p=0.02). Among non-farming rural dwellers, people with CC genotype (polymorphic group) had significantly lower mean values of FEV_1, FEV_1/FVC ratio than those with TC or TT genotypes (wild-type group) after controlling for potential confounders. However, the associations between FEV_1/FVC ratio and genotypes among non-farming rural dwellers was not significant after adjusting for multiple comparisons using Bonferroni correction. Similar associations between percent predicted values and the *NOS3*-786T/C polymorphism were observed in workers in swine operations and non-farming rural dwellers, respectively.

The results of *NOS3* Glu298Asp polymorphism from multiple linear regressions are shown in Table 7-3. The recessive model of this SNP (TT vs. TG+GG) had the best fit with the smallest AIC value in the regression analysis of FEV_1 and FVC in comparison to the additive (TT=2, TG=1, GG=0) and dominant models (TT+TG vs. GG), however, the dominant model of this SNP (TT+TG vs. GG) showed the best fit with the smallest AIC value in the regression analysis of FEV_1/FVC ratio and $FEF_{25\%-75\%}$ (data not shown). As shown in Table 7-3 and Figure 7-2, the interaction term of this SNP and a dichotomous variable indicating two exposure groups (workers vs. rural dwellers) was significant in the multiple linear regression of FEV_1(p=0.04), FVC (p=0.04) and FEV_1/FVC (p=0.04), respectively. Under the recessive model of this SNP, workers with TT genotype (polymorphic group) of *NOS3* Glu298Asp polymorphism had significantly greater mean values of FEV_1 and FVC than workers with TG or GG genotypes

(wild-type group) after controlling for age, sex, height, weight, and smoking habit, and adjusting for Bonferroni correction (FEV_1(L): 3.72 vs. 3.55, p=0.02; FVC (L): 4.79 vs. 4.56, p=0.01); under the dominant model of this SNP, no significant differences were observed in the mean values of FEV_1/FVC ratio and $FEF_{25\%-75\%}$ between workers with TT or TG genotypes (polymorphic group) and workers with GG genotype (wild-type group). Among non-farming rural dwellers, no significant differences were observed in the mean values of FEV_1, FVC (in the recessive model), FEV_1/FVC ratio and $FEF_{25\%-75\%}$ (in the dominant model) between the polymorphic and wild-type groups of *NOS3* Glu298Asp polymorphism. Similar associations between percent predicted values and the *NOS3* Glu298Asp polymorphism were observed in workers in swine operations and non-farming rural dwellers, respectively.

7.4.2 Results from the longitudinal study

The comparisons of baseline characteristics showed there were no significant differences between the workers in swine operations (n=173) and non-farming rural dwellers (n=119) in the means of height, weight, and distributions of *NOS3*-786T/C, Glu298Asp and -922A/G polymorphisms (Table 7-4). The workers were younger and currently smoking less than rural dwellers. In Cycle 3 (2003/04), more than half (69%) workers indicated that they no longer worked in swine facilities. The mean years of swine work during the study period was 8.4 years. The HWE was followed by the three polymorphisms in both workers and rural dwellers. The minor allele frequency, C of NOS3-786T/C, T of NOS3Glu298Asp, G of NOS3-922A/G, was 35%, 32% and 34% in the workers, and 40%, 29% and 40% in rural dwellers, respectively. *NOS3*-786T/C ***polymorphism*** was in high linkage disequilibrium with *NOS3*-922A/G polymorphism in both workers (r=0.98) and non-farming rural dwellers (r=1.00), and the latter was removed from further analysis in the longitudinal study

In the multivariate analysis, mean annual decline rates in FEV_1 and FVC in workers with CC genotype in *NOS3*-786T/C polymorphism were significantly lower than those in workers with TT genotype (FEV_1: 30.5 vs. 58.0 ml/year, p=0.005; FVC: 16.7 vs. 50.9 ml/year, p=0.011) after controlling for all confounders and adjusting for multiple comparisons using Bonferroni correction (Table 7-5 and Figure 7-3). Similar associations (borderline significant) between percent predicted values and the *NOS3*-786T/C polymorphism were observed in the works. However, no significant differences were observed in the mean annual decline rates in lung function and percent predicted values between genotypes of *NOS3*-786T/C polymorphism among non-farming rural dwellers.

No significant differences in mean annual decline rates in lung function and percent predicted values were observed between the genotypes of *NOS3*Glu298Asp polymorphism in both workers in swine operations and non-farming rural dwellers (Table 7-6 and Figure 7-3).

The results were not modified when an additional variable indicating the work status (quit or stayed) in the swine operations was included in the multiple regression analysis of the four lung function measurements (data not shown).

The prevalence of obstructive lung disease significantly increased over time during the study period in both workers in swine operations and non-farming rural dwellers (Table 7-7). There was no significant difference in the prevalence of wheeze without cold over time during the study period in both exposure groups (Table 7-7). However, the prevalence of respiratory symptoms including usual/chronic cough and usual/chronic phlegm significantly decreased over time during the study period in workers in swine operations, which was not observed in non-farming rural dwellers (Table 7-7).

7.5 DISCUSSION

To the best of our knowledge, this study is the first to report significant interactions between polymorphisms in the *NOS3* gene and environmental exposures on lung function in human populations. In this study, among workers in swine operations, a group exposed to high levels of respiratory hazards, polymorphisms in the *NOS3* gene were significantly associated with better lung function in the cross-sectional study and lower annual declining rates of lung function in the longitudinal study than those with wild-type. Interestingly, these associations were not observed among non-farming rural dwellers, a group exposed to low levels of respiratory hazards. In addition, the protective effects of polymorphisms in the NOS3 gene on lung function were observed in two independent studies, which make the results of these associations robust.

NOS3-786T/C and Glu298Asp polymorphisms in the *NOS3* gene play important roles in the activity and functionality of the *NOS3* gene. The NOS3 Glu298Asp polymorphism, a coding variant in exon 7, which results in a Glu to Asp substitution at position 298, has an impact on the function of the eNOS protein.[26,27] A study examining the functionality of NOS3-786T/C polymorphism showed that people with $T^{-786}\rightarrow C$ mutation had significantly reduced activity in the *NOS3* gene as assessed by luciferase reporter gene assays.[28] A further study of underlying molecular mechanisms for the *NOS3*-786T/C polymorphism related reduction in NOS3 gene transcription, showed that inhibition of replication protein A1 (RPA1), which specifically binds to the mutant allele, can restore transcription activity due to $T^{-786}\rightarrow C$ mutation in the promoter of *NOS3* gene, whereas, overexpression of RPA1 can reduce it.[29]

Workers in swine operations are at increased risk of respiratory dysfunction due to exposure to a variety of microbes such as gram-positive and gram-negative bacteria, virus, dusts and gases such as NH_3, H_2S and CO_2.[30,31] An elevated level of exhaled NO has been observed

among those with asthma,[32,33] healthy naïve volunteers challenged in a pig confinement building,[8] and swine confinement workers with repeated exposure of respiratory hazards at work.[9] Moreover, NO has been linked with increased plasma exudation, asthmatic inflammation and asthma severity.[34,35]

A number of studies have shown that NO plays an important role in maintaining physiological functions in the lungs, such as pulmonary vascular and airway smooth muscle tone, neurotransmission, airway inflammatory and host defence mechanisms, and bronchodilation.[10,36] However, to date, only a few studies have examined the association between polymorphisms in the *NOS3* gene and respiratory dysfunction, and the results these studies are inconsistent f.[13,37,38] A study of polymorphism in the *NOS3* gene (a 604bp DNA fragment vs. a 573 bp DNA fragment in intro 4) among 310 patients with bronchial asthma and 121 healthy subjects conducted in Korea reported that 604bp DNA fragment was significantly more common among those with bronchial asthma than in normal controls.[38] In contrast, a study from a Czech population reported no significant association between the polymorphism in the *NOS3* gene, specifically a 27bp tandem repeat polymorphism in intro4, and atopic asthma.[13] This study also failed to show a significant association between *NOS3* Glu298Asp polymorphism and atopic asthma.[13] Another study of 300 subjects with atopic asthma and 300 healthy controls from a British population failed to show a significant association between the *NOS3* Glu298Asp polymorphism and atopic asthma.[37] In this study, the non-coding variant (*NOS3*-786T/C), which is located in within 2kb of 5' of the *NOS3* gene and a coding variant, *NOS3* Glu298Asp, were significantly associated with better lung function and slower declining rates in FEV_1 and FVC among workers in swine operations, a group likely exposed to high level of respiratory hazards. No such significant associations were observed among non-farming rural dwellers, a group likely exposed to low level of respiratory hazards.

These results indicating a potential gene-environment interaction may partly explain the discrepancies in the associations reported in different studies. These findings emphasise the importance of considering exposure in studies examining the effects of polymorphisms in the *NOS3* gene on lung dysfunction.

The findings in this study are further supported by several experimental studies using animal models. [39,40] A recent animal study showed in mice with ventilator-induced lung injury, *NOS3*-deficient mice had significantly reduced concentrations of protein and cytokines in broncho-alveolar lavage fluid (BALF) and oxidative stress, and increased lung compliance in comparison to wild-type mice.[39] Another animal study showed that *NOS1*-knockout mice and *NOS1*&*NOS3*-double knockout mice had significantly reduced airway responsiveness in comparison to wild-type mice.[40] Interestingly, there were no significant differences in airway responsiveness between *NOS2*-knockout and wild-type mice. Many studies have shown that NO derived from *NOS2* plays an important role in many pathophysiological processes due to its detrimental effects in the airway.[7,10] Expression of *NOS2* is presented in response to exposure of LPS or cytokines, and not usually presented in un-stimulated cells.[41] An experimental study using bone marrow-derived macrophages from *NOS3*-knockout and wild-type mice showed that in response to LPS exposure, the expression of *NOS2* was significantly reduced in macrophages from *NOS3*-knockout mice compared with the macrophages from wild-type mice, suggesting a regulatory role of *NOS3*-derived NO on *NOS2* expression, which is mediated, at least in part, via NF-κB.[14] The results from this study support the pivotal role of *NOS3* in pro-inflammatory responses, which was observed in this experimental study. This study extended these findings related to the *NOS3* gene from experimental studies to humans by showing significant protective effects of polymorphisms in the *NOS3* gene on lung

function among swine confinement workers with continued exposure of high concentration of respiratory hazards.

Workers in swine operation showed significantly reduced prevalence of respiratory symptoms including usual/chronic cough and usual/chronic phlegm over time during the study period, not in non-farming rural dwellers, suggestive of an adaptation response due to repeat occupational exposure. Adaptation is defined as a reduced injury response due to previous exposure than a single exposure alone.[42] The observed adaptation phenomenon among the workers in this study was consistent with many experimental studies. [43-45] A study using monocyte-derived macrophage (MDM) showed that organic dust extract (ODE) challenged MDMs had significantly reduced cytokine responses (TNF-α, IL-6 and IL-10) after repeat challenge with high-dose ODE.[43] Another study using human peripheral blood monocytes showed that repeated exposure of organic dust attenuated inflammatory responses (TNF-α and IL-6), which was due to inhibition of protein kinase C (PKCα and PKCε).[44] Animal model using mice also showed airway adaptation response to repetitive intranasal organic dust exposure.[45]

There were some limitations in this study. The lack of objective measurement of workplace exposures such as concentrations of dust, gram-positive and gram-negative bacteria and virus limits the ability to examine the dose-response relationship between exposure and lung function among workers with and without polymorphisms in the *NOS3* gene. However, the inclusion of non-farming rural dwellers as a comparison group in this study allowed for examining the effects of these polymorphisms in the *NOS3* gene at a lower exposure level than what is typically found intensive in swine operations.

In conclusion, this study is the first to show variation in the association of polymorphisms in the *NOS3* gene and lung function in human populations exposed to high and low respiratory hazards

raising the possibility of gene-environment. This study also extends our knowledge of the role of the *NOS3* gene in respiratory system from experimental studies to human, suggesting the relevance of *NOS3* gene in the airway disease among populations exposed to high levels of respiratory hazards.

7.6 Reference

(1) Senthilselvan A, Dosman JA, Kirychuk SP, Barber EM, Rhodes CS, Zhang Y, et al. Accelerated lung function decline in swine confinement workers. Chest 1997 Jun;111(6):1733-41.

(2) Zejda JE, Hurst TS, Rhodes CS, Barber EM, McDuffie HH, Dosman JA. Respiratory health of swine producers. Focus on young workers. Chest 1993 Mar;103(3):702-9.

(3) Senthilselvan A, Chenard L, Ulmer K, Gibson-Burlinguette N, Leuschen C, Dosman JA. Excess respiratory symptoms in full-time male and female workers in large-scale swine operations. Chest 2007 Apr;131(4):1197-204.

(4) Eduard W, Pearce N, Douwes J. Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents. Chest 2009 Sep;136(3):716-25.

(5) Dosman JA, Lawson JA, Kirychuk SP, Cormier Y, Biem J, Koehncke N. Three new cases of apparent occupational asthma in swine confinement facility employees. Eur Respir J 2006 Dec;28(6):1281-2.

(6) Zejda JE, Barber E, Dosman JA, Olenchock SA, McDuffie HH, Rhodes C, et al. Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels. J Occup Med 1994 Jan;36(1):49-56.

(7) Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991 Jun;43(2):109-42.

(8) Sundblad BM, Larsson BM, Palmberg L, Larsson K. Exhaled nitric oxide and bronchial responsiveness in healthy subjects exposed to organic dust. Eur Respir J 2002 Aug;20(2):426-31.

(9) Von Essen SG, Scheppers LA, Robbins RA, Donham KJ. Respiratory tract inflammation in swine confinement workers studied using induced sputum and exhaled nitric oxide. J Toxicol Clin Toxicol 1998;36(6):557-65.

(10) Barnes PJ, Belvisi MG. Nitric oxide and lung disease. Thorax 1993 Oct;48(10):1034-43.

(11) Barnes PJ. Neurogenic inflammation in the airways. Respir Physiol 2001 Mar;125(1-2):145-54.

(12) Ober C, Cox NJ, Abney M, Di RA, Lander ES, Changyaleket B, et al. Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. Hum Mol Genet 1998 Sep;7(9):1393-8.

(13) Holla LI, Buckova D, Kuhrova V, Stejskalova A, Francova H, Znojil V, et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. Clin Exp Allergy 2002 Aug;32(8):1193-8.

(14) Connelly L, Jacobs AT, Palacios-Callender M, Moncada S, Hobbs AJ. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. J Biol Chem 2003 Jul 18;278(29):26480-7.

(15) Senthilselvan A, Dosman JA, Chenard L, Burch LH, Predicala BZ, Sorowski R, et al. Toll-like receptor 4 variants reduce airway response in human subjects at high endotoxin levels in a swine facility. J Allergy Clin Immunol 2009 May;123(5):1034-40, 1040.

(16) Standardization of spirometry--1987 update. Statement of the American Thoracic Society. Am Rev Respir Dis 1987 Nov;136(5):1285-98.

(17) Crapo RO, Morris AH, Gardner RM. Reference spirometric values using techniques and equipment that meet ATS recommendations. Am Rev Respir Dis 1981 Jun;123(6):659-64.

(18) Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. Eur Respir J 2005 Nov;26(5):948-68.

(19) Dosman JA, Chenard L, Rennie DC, Senthilselvan A. Reciprocal association between atopy and respiratory symptoms in fully employed female, but not male, workers in swine operations. J Agromedicine 2009;14(2):270-6.

(20) Senthilselvan A, Rennie D, Chenard L, Burch LH, Babiuk L, Schwartz DA, et al. Association of polymorphisms of toll-like receptor 4 with a reduced prevalence of hay fever and atopy. Ann Allergy Asthma Immunol 2008 May;100(5):463-8.

(21) The use of standardized allergen extracts. American Academy of Allergy, Asthma and Immunology (AAAAI). J Allergy Clin Immunol 1997 May;99(5):583-6.

(22) Senthilselvan A, Dosman JA, Kirychuk SP, Barber EM, Rhodes CS, Zhang Y. Accelerated lung function decline in swine confinement workers. 1997;111(6).

(23) Standardization of spirometry--1987 update. Statement of the American Thoracic Society. Am Rev Respir Dis 1987 Nov;136(5):1285-98.

(24) Pahwa P, Karunanayake CP, Rennie DC, Chen Y, Schwartz DA, Dosman JA. Association of the TLR4 Asp299Gly polymorphism with lung function in relation to body mass index. BMC Pulm Med 2009;9:46.

(25) Xu H, Gregory SG, Hauser ER, Stenger JE, Pericak-Vance MA, Vance JM, et al. SNPselector: a web tool for selecting SNPs for genetic association studies. Bioinformatics 2005 Nov 15;21(22):4181-6.

(26) Novoradovsky A, Brantly ML, Waclawiw MA, Chaudhary PP, Ihara H, Qi L, et al. Endothelial nitric oxide synthase as a potential susceptibility gene in the pathogenesis of emphysema in alpha1-antitrypsin deficiency. Am J Respir Cell Mol Biol 1999 Mar;20(3):441-7.

(27) Persu A, Stoenoiu MS, Messiaen T, Davila S, Robino C, El-Khattabi O, et al. Modifier effect of ENOS in autosomal dominant polycystic kidney disease. Hum Mol Genet 2002 Feb 1;11(3):229-41.

(28) Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, et al. T-786-->C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 1999 Jun 8;99(22):2864-70.

(29) Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, et al. Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T-->C mutation associated with coronary spastic angina. Hum Mol Genet 2000 Nov 1;9(18):2629-37.

(30) Crook B, Robertson JF, Glass SA, Botheroyd EM, Lacey J, Topping MD. Airborne dust, ammonia, microorganisms, and antigens in pig confinement houses and the respiratory health of exposed farm workers. Am Ind Hyg Assoc J 1991 Jul;52(7):271-9.

(31) Schwartz DA, Landas SK, Lassise DL, Burmeister LF, Hunninghake GW, Merchant JA. Airway injury in swine confinement workers. Ann Intern Med 1992 Apr 15;116(8):630-5.

(32) al-Ali MK, Eames C, Howarth PH. Exhaled nitric oxide; relationship to clinicophysiological markers of asthma severity. Respir Med 1998 Jul;92(7):908-13.

(33) Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 1994 Jan 15;343(8890):133-5.

(34) Barnes PJ. NO or no NO in asthma? Thorax 1996 Feb;51(2):218-20.

(35) Lim S, Jatakanon A, John M, Gilbey T, O'connor BJ, Chung KF, et al. Effect of inhaled budesonide on lung function and airway inflammation. Assessment by various inflammatory markers in mild asthma. Am J Respir Crit Care Med 1999 Jan;159(1):22-30.

(36) Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. Am J Respir Crit Care Med 1994 Feb;149(2 Pt 1):538-51.

(37) Gao PS, Kawada H, Kasamatsu T, Mao XQ, Roberts MH, Miyamoto Y, et al. Variants of NOS1, NOS2, and NOS3 genes in asthmatics. Biochem Biophys Res Commun 2000 Jan 27;267(3):761-3.

(38) Lee YC, Cheon KT, Lee HB, Kim W, Rhee YK, Kim DS. Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma. Allergy 2000 Oct;55(10):959-63.

(39) Vaporidi K, Francis RC, Bloch KD, Zapol WM. Nitric oxide synthase 3 contributes to ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 2010 Aug;299(2):L150-L159.

(40) De Sanctis GT, MacLean JA, Hamada K, Mehta S, Scott JA, Jiao A, et al. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. J Exp Med 1999 May 17;189(10):1621-30.

(41) de Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris SM, Jr., et al. Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. Proc Natl Acad Sci U S A 1996 Feb 6;93(3):1054-9.

(42) Elder AC, Finkelstein J, Johnston C, Gelein R, Oberdorster G. Induction of adaptation to inhaled lipopolysaccharide in young and old rats and mice. Inhal Toxicol 2000 Mar;12(3):225-43.

(43) Poole JA, Alexis NE, Parks C, MacInnes AK, Gentry-Nielsen MJ, Fey PD, et al. Repetitive organic dust exposure in vitro impairs macrophage differentiation and function. J Allergy Clin Immunol 2008 Aug;122(2):375-82, 382.

(44) Poole JA, Wyatt TA, Von Essen SG, Hervert J, Parks C, Mathisen T, et al. Repeat organic dust exposure-induced monocyte inflammation is associated with protein kinase C activity. J Allergy Clin Immunol 2007 Aug;120(2):366-73.

(45) Poole JA, Wyatt TA, Oldenburg PJ, Elliott MK, West WW, Sisson JH, et al. Intranasal organic dust exposure-induced airway adaptation response marked by persistent lung inflammation and pathology in mice. Am J Physiol Lung Cell Mol Physiol 2009 Jun;296(6):L1085-L1095.

Table 7-1. Characteristics of workers in swine operations and non-farming rural dwellers

	Workers Mean ± SD	Rural dwellers Mean ± SD	p value
Age, yr	35.8 ± 11.5	39.3 ± 13.0	< 0.0001
Height, cm	172.3 ± 8.4	170.8 ± 9.5	0.01
Weight, kg	83.6 ± 17.1	82.2 ± 18.8	0.29
Male, no (%)	240 (64.2)	184 (44.8)	< 0.0001
Smoking	No (%)	No (%)	< 0.0001
Current smoker	117 (31.3)	74 (18.0)	
Ex-smoker	101 (27.0)	115 (28.0)	
Nonsmoker	156 (41.7)	222 (54.0)	
Atopy			0.06
Yes	123 (35.3)	169 (42.1)	
No	225 (64.7)	232 (57.9)	
Lung function tests			
FEV_1	3.7 ± 0.8	3.6 ± 0.9	0.002
FVC	4.8 ± 1.0	4.5 ± 1.1	0.0003
FEV_1/FVC	78.6 ± 6.6	79.1 ± 6.4	0.25
$FEF_{25\%-75\%}$	3.6 ± 1.2	3.4 ± 1.2	0.10
NOS3 -786 T/C (rs2070744)*			0.61
TT	105 (38)	132 (42)	
TC	138 (50)	146 (46)	
CC	35 (13)	38 (12)	
Total	278 (100)	316 (100)	
Glu298Asp (rs1799983)*			0.24
GG	110 (40)	142 (45)	
TG	119 (43)	134 (43)	
TT	45 (16)	38 (12)	
Total	274 (100)	314 (1000	
-922 A/G (rs1800779)*			0.78
AA	109 (39)	133 (42)	
AG	134 (48)	145 (46)	
GG	35 (13)	38 (12)	
Total	278 (100)	316 (100)	
Linkage Disequilibrium (r)			
-786T/C vs. Glu298Asp	0.34	0.46	
-786T/C vs. -922A/G	0.94	0.98	
Glu298Asp vs. -922A/G	0.35	0.46	

* Hardy-Weinberg equilibrium (HWE) was followed in both workers and rural dwellers

Table 7-2. Differences in mean values of lung function parameters between *NOS3*-786 T/C (rs2070744) polymorphic and wild type groups for workers in swine operations and non-farming rural dwellers*

	Workers(n=278)		Rural dwellers (n=316)		Interaction term Type III p value
	Polymorphism vs. Wild		Polymorphism vs. Wild		
	Diff (SE)	p value	Diff (SE)	p value	
Observed					
FEV_1, (L)	0.21(0.08)	**0.009**†	-0.17(0.08)	**0.03**	**0.001**
FVC, (L)	0.22(0.10)	**0.02**†	-0.14(0.09)	0.13	**0.007**
FEV_1/FVC, (%)	0.64(1.06)	0.55	-2.02(1.02)	**0.048**	0.071
$FEF_{25\%-75\%}$, (L/s)	0.21(0.17)	0.22	-0.24(0.17)	0.14	0.059
Percent predicted (%)					
FEV_1	5.89(2.18)	**0.007** †	- 5.39 (2.08)	**0.01**†	**0.0002**
FVC	5.59 (2.15)	**0.01** †	- 3.60 (2.05)	0.08	**0.002**
FEV1/FVC	0.14 (0.44)	0.75	- 0.43 (0.42)	0.31	0.35
$FEF_{25\%-75\%}$	4.85 (4.49)	0.28	- 6.92 (4.30)	0.11	**0.06**

*: Recessive model (CC vs. TC+TT) has the smallest AIC in the regression model of FEV_1, FVC, FEV_1/FVC and $FEF_{25\%-75\%}$;
p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit
†: significant after applying Bonferroni correction for multiple comparisons ($p< 0.03=0.05/2$).

Table 7-3. Differences in mean values of lung function parameters between *NOS3* Glu298Asp (rs1799983) polymorphic and wild type groups for workers in swine operations and non-farming rural dwellers*

	Workers (n=274)		Rural dwellers (n=314)		Interaction term Type III p value
	Polymorphism vs. Wild		Polymorphism vs. Wild		
	Diff (SE)	p value	Diff (SE)	p value	
Observed					
FEV_1, (L)‡	0.17(0.07)	**0.02**†	-0.05(0.08)	0.54	**0.044**
FVC, (L)‡	0.22(0.09)	**0.01**†	-0.05(0.09)	0.63	**0.038**
FEV_1/FVC, (%)§	0.84(0.72)	0.25	-1.23(0.67)	0.07	**0.036**
$FEF_{25\%-75\%}$, (L/s)§	0.15(0.12)	0.21	-0.13(0.11)	0.24	0.087
Percent predicted (%)					
FEV_1‡	3.93 (2.02)	**0.05**	1.66 (2.12)	0.43	0.06
FVC‡	4.39 (1.98)	**0.027**†	-0.94 (2.07)	0.65	0.06
FEV_1/FVC§	0.176(0.30)	0.60	0.33 (0.28)	0.23	0.67
$FEF_{25\%-75\%}$§	4.09 (3.08)	0.18	-3.25 (2.82)	0.25	0.08

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit
†: significant after applying Bonferroni correction for multiple comparisons ($p < 0.03 = 0.05/2$).
‡: Recessive model (TT vs. TG+GG) has the smallest AIC in the regression model of FEV_1 and FVC.
§: Dominant model (TT+TG vs. GG) has the smallest AIC in the regression model of FEV_1/FVC and $FEF_{25\%-75\%}$.

Table 7-4 Comparison of baseline characters between swine workers and non-farming rural dwellers.

	Swine workers Mean(SD)	Non-farming rural dwellers Mean(SD)	p value
No. of workers	173	119	
Age (years)	37.5(10.8)	41.6(9.9)	0.001
Height (cm)	177.1(6.1)	177.3(6.8)	0.78
Weight (kg)	84.3(13.7)	84.4(15.2)	0.99
Years of swine work (years)			
during the study	8.4(4.2)		
Smoke	No (%)	No (%)	0.001
Current	20(11.6)	29(24.4)	
Former	44(25.4)	39(32.8)	
None	109(63.0)	51(31.9)	
Quit swine work*			
Quit	119 (69)		
Stay	54 (31)		
NOS3 786T/C(rs2070744)			0.30
TT	57 (39.9)	31 (37.4)	
TC	72 (50.4)	38 (45.8)	
CC	14 (9.8)	14 (16.9)	
HWE (p value)	0.20	0.69	
Glu298Asp(rs1799983)			0.29
GG	65 (47.8)	40 (47.1)	
TG	55 (40.4)	40 (47.1)	
T/T	16 (11.8)	5 (5.9)	
HWE (p value)	0.41	0.22	
922A/G(rs1800779)			0.31
AA	59 (41.8)	31 (37.4)	
AG	68 (48.2)	38 (45.8)	
GG	14 (9.9)	14 (16.9)	
HWE (p value)	0.38	0.69	
Linkage Disequilibrium (r)			
786T/C vs. Glu298Asp	0.48	0.48	
786T/C vs. 922A/G	0.98	1.00	
Glu298Asp vs. 922A/G	0.49	0.48	

*: in cycle 3 (2003/04 study)

Table 7-5. Lung function decline rate per year and genotypes of *NOS3*-786 T/C (rs2070744) polymorphism for workers in swine operations and non-farming rural dwellers

	NOS3-786 T/C genotypes			Differences between genotypes			
	CC	TC	TT	CC vs. TT		TC vs. TT	
	Slope†(SE)	Slope†(SE)	Slope†(SE)	Diff(SE)	p value	Diff(SE)	p value
Workers in swine operations (n=143)							
Observed							
FEV_1, (L)	-0.03(0.01)	-0.05(0.00)	-0.06(0.01)	0.03(0.01)	**0.005**‡	0.00(0.01)	0.56
FVC, (L)	-0.02(0.01)	-0.04(0.01)	-0.05(0.01)	0.03(0.01)	**0.01**‡	0.01(0.01)	0.30
FEV_1/FVC, (%)	-0.39(0.11)	-0.43(0.06)	-0.35(0.05)	-0.04(0.12)	0.76	-0.08(0.08)	0.33
$FEF_{25\%-75\%}$, (L/s)	-0.07(0.02)	-0.09(0.01)	-0.10(0.01)	0.04(0.02)	0.08	0.02(0.02)	0.32
Percent predicted (%)							
FEV_1	0.03(0.43)	-0.81(0.13)	-0.83(0.14)	0.86(0.45)	**0.05**	0.03(0.19)	0.88
FVC	0.41(0.51)	-0.48(0.13)	-0.59(0.14)	1.00(0.53)	0.06	0.11(0.19)	0.56
FEV_1/FVC	-0.30(0.12)	-0.34(0.07)	-0.25(0.07)	-0.05(0.14)	0.70	-0.09(0.09)	0.33
$FEF_{25\%-75\%}$	-0.73(0.67)	-1.41(0.25)	-1.65(0.33)	0.92(0.75)	0.22	0.24(0.41)	0.56
Non-farming rural dwellers (n=84)							
Observed							
FEV_1, (L)	-0.05(0.01)	-0.06(0.01)	-0.05(0.01)	0.00(0.01)	0.91	0.00(0.01)	0.83
FVC, (L)	-0.03(0.01)	-0.04(0.01)	-0.04(0.01)	0.01(0.01)	0.16	0.00(0.01)	0.99
FEV_1/FVC, (%)	-0.54(0.10)	-0.43(0.07)	-0.35(0.07)	-0.19(0.12)	0.10	-0.08(0.09)	0.36
$FEF_{25\%-75\%}$, (L/s)	-0.13(0.02)	-0.09(0.01)	-0.09(0.01)	-0.03(0.02)	0.12	0.00(0.02)	0.87
Percent predicted (%)							
FEV_1	-0.82(0.16)	-0.90(0.14)	-0.84(0.17)	0.03(0.23)	0.89	-0.06(0.22)	0.80
FVC	-0.26(0.15)	-0.56(0.11)	-0.56(0.16)	0.30(0.22)	0.18	-0.00(0.20)	0.99
FEV_1/FVC	-0.52(0.13)	-0.37(0.08)	-0.26(0.09)	-0.25(0.15)	0.10	-0.11(0.12)	0.36
$FEF_{25\%-75\%}$	-2.45(0.44)	-1.62(0.30)	-1.61(0.38)	-0.84(0.58)	0.15	-0.01(0.49)	0.98

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit.

†: annual lung function decline rate during the follow-up period

‡: significant after applying Bonferroni correction for multiple comparisons ($p < 0.0125 = 0.05/4$).

Table 7-6. Lung function decline rate per year and genotypes of *NOS3*Glu298Asp (rs1799983) polymorphism for workers in swine operations and non-farming rural dwellers

	*NOS3*Glu298Asp genotypes			Differences between genotypes			
	TT	TG	GG	TT vs. GG		TG vs. GG	
	Slope†(SE)	Slope†(SE)	Slope†(SE)	Diff(SE)	p value	Diff(SE)	p value
Workers in swine operations (n=136)							
Observed							
FEV_1, (L)	-0.05(0.01)	-0.06(0.01)	-0.05(0.01)	0.00(0.01)	0.79	-0.01(0.01)	0.32
FVC, (L)	-0.03(0.01)	-0.05(0.01)	-0.04(0.01)	0.01(0.01)	0.54	0.00(0.01)	0.78
FEV_1/FVC, (%)	-0.43(0.12)	-0.45(0.06)	-0.33(0.06)	-0.10(0.13)	0.42	-0.12(0.08)	0.15
$FEF_{25\%-75\%}$, (L/s)	-0.10(0.02)	-0.10(0.01)	-0.08(0.01)	-0.01(0.02)	0.52	-0.02(0.02)	0.25
Percent predicted (%)							
FEV_1	-0.61(0.21)	-0.92(0.15)	-0.56(0.17)	-0.05(0.27)	0.86	-0.36(0.22)	0.11
FVC	-0.26(0.22)	-0.57(0.15)	-0.33(0.19)	0.07(0.29)	0.80	-0.24(0.24)	0.31
FEV_1/FVC	-0.33(0.14)	-0.36(0.07)	-0.22(0.07)	-0.11(0.15)	0.46	-0.14(0.10)	0.14
$FEF_{25\%-75\%}$	-1.62(0.57)	-1.66(0.35)	-1.06(0.25)	-0.57(0.63)	0.37	-0.61(0.43)	0.16
Non-farming rural dwellers (n=85)							
Observed							
FEV_1, (L)	-0.04(0.01)	-0.05(0.00)	-0.06(0.01)	0.01(0.01)	0.15	0.01(0.01)	0.47
FVC, (L)	-0.03(0.01)	-0.04(0.01)	-0.04(0.01)	0.01(0.01)	0.24	0.01(0.01)	0.40
FEV_1/FVC, (%)	-0.39(0.10)	-0.44(0.07)	-0.42(0.06)	0.03(0.12)	0.81	-0.02(0.09)	0.83
$FEF_{25\%-75\%}$, (L/s)	-0.07(0.02)	-0.10(0.01)	-0.10(0.01)	0.03(0.02)	0.19	0.00(0.02)	0.96
Percent predicted (%)							
FEV_1	-0.54(0.26)	-0.80(0.12)	-0.97(0.14)	0.43(0.30)	0.14	0.17(0.19)	0.37
FVC	-0.24(0.21)	-0.43(0.12)	-0.60(0.12)	0.36(0.25)	0.14	0.18(0.17)	0.31
FEV_1/FVC	-0.30(0.13)	-0.39(0.08)	-0.34(0.07)	0.05(0.15)	0.75	-0.05(0.11)	0.68
$FEF_{25\%-75\%}$	-1.10(0.53)	-1.79(0.30)	-1.80(0.31)	0.70(0.61)	0.25	0.01(0.44)	0.98

*: p-value from the multiple regression analysis after adjusting for age, sex, height, weight, and smoking habit.

†: annual lung function decline rate during the follow-up period

Table 7-7. Prevalence of obstructive lung disease, wheeze without cold and respiratory symptoms during the study period among workers in swine operations and non-farming rural dwellers.

	Workers in swine operations			Non-farming rural dwellers		
	Cycle 1 (1990/91)	Cycle 2 (1994/95)	Cycle 3 (2003/04)	Cycle 1 (1990/91)	Cycle 2 (1994/95)	Cycle 3 (2003/04)
Number of subjects	173	163	173	119	118	119
Obstructive (%)*	9.3	14.1	20.2	3.4	10.2	14.3
p value†		0.002			0.001	
Wheeze without cold (%)	26.0	20.9	22.5	16.0	13.6	22.7
p value‡		0.187			0.061	
Usual cough (%)	19.7	20.3	9.8	10.1	8.5	6.7
p value‡		0.007			0.827	
Chronic cough (%)	16.8	16.0	8.7	10.1	10.2	7.6
p value‡		0.029			0.885	
Usual phlegm (%)	31.2	20.3	18.5	11.8	7.6	10.9
p value‡		0.011			0.347	
Chronic phlegm (%)	26.6	16.0	15.0	9.2	8.5	10.9
p value‡		0.007			0.716	

*: Obstructive airway abnormality is defined as a reduced FEV_1/FVC ratio below the 5^{th} percentile of the predicted value.[18]

†: type III p value from logistic regression with GEE adjusting for smoking habit.

‡: type III p value from logistic regression with GEE adjusting for age, height, weight, smoking habit.

Figure 7-1 The follow-up of the workers and the rural dwellers from 1990/91 to 2003/04 (number of workers: number of rural dwellers)

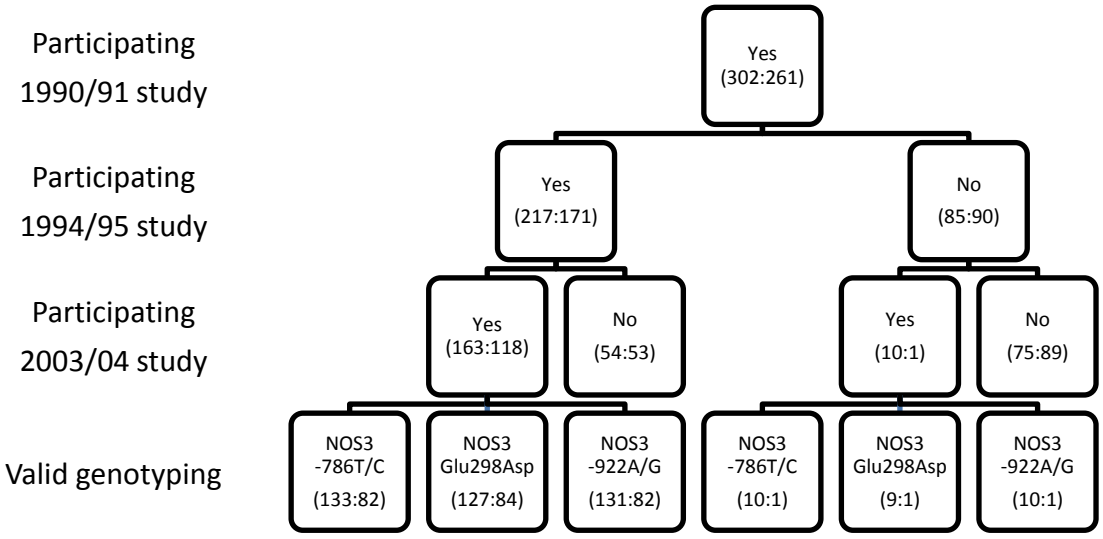
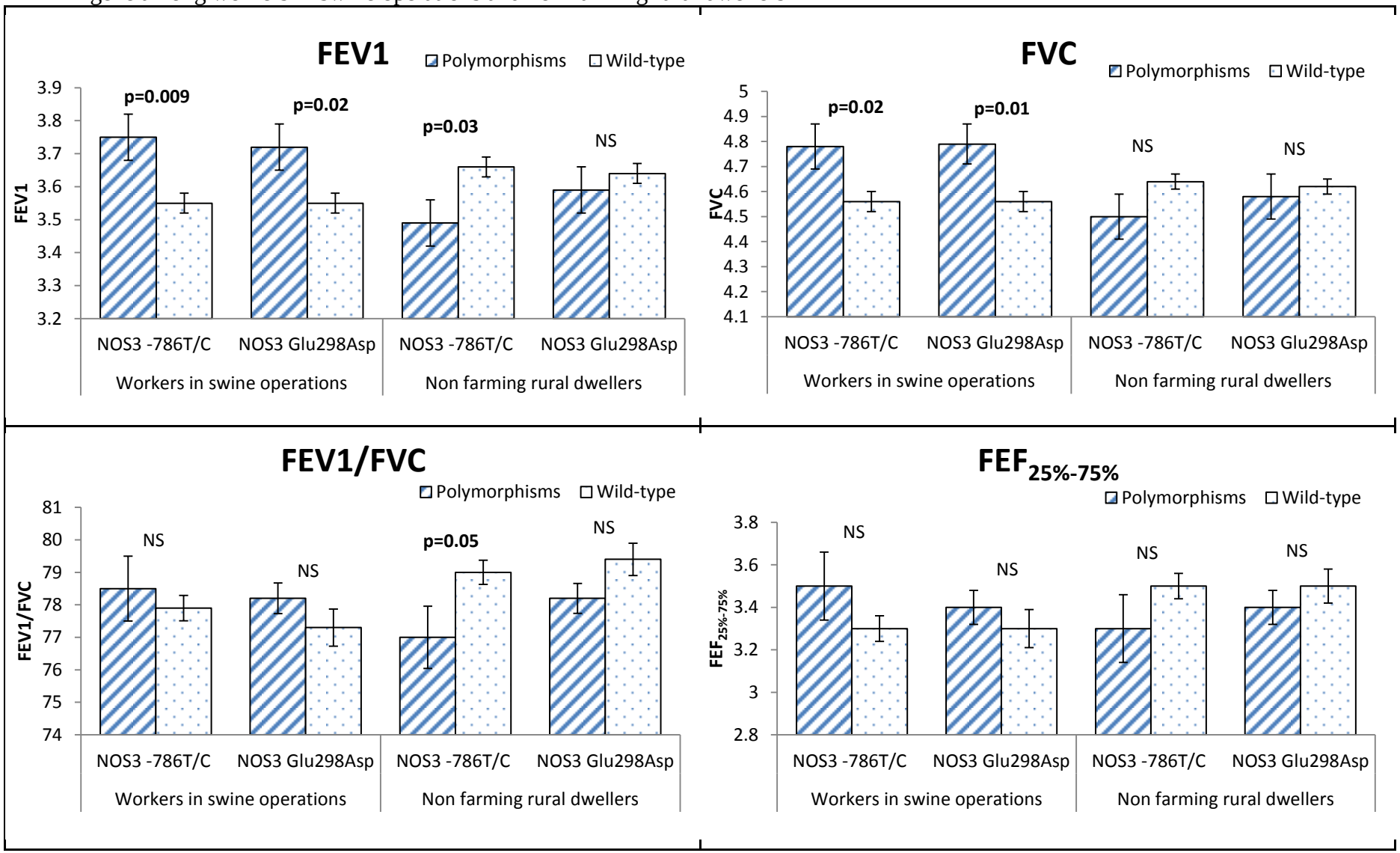
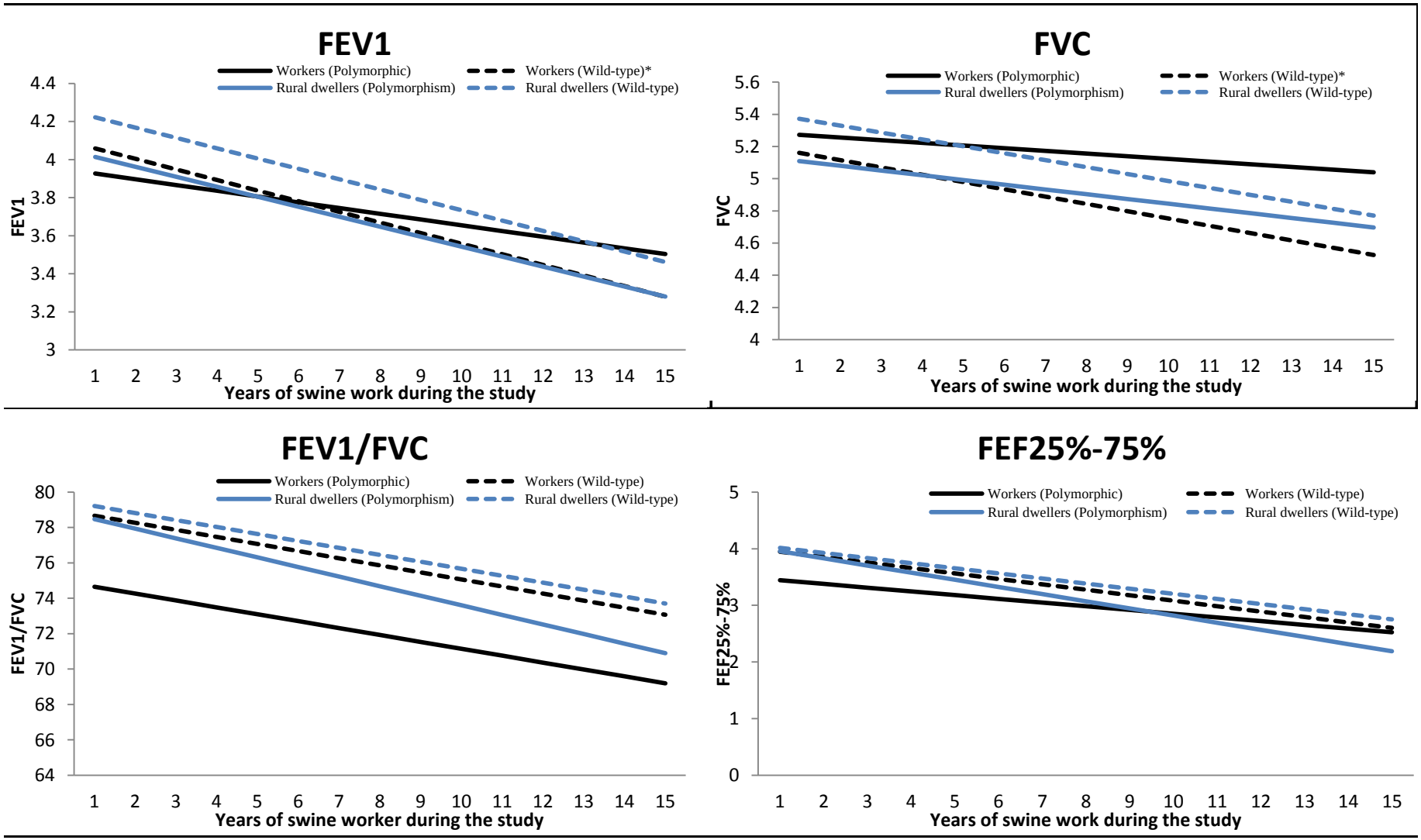


Figure 7-2 Comparisons of mean values of lung function parameters between polymorphic and wild-type groups of polymorphisms in the *NOS3* gene among workers in swine operations and non-farming rural dwellers



NS: not significant at $p<0.05$

Figure 7-3 Comparisons of mean annual decline rates in lung function between polymorphic and wild-type groups of the NOS3-786T/C polymorphism among workers in swine operations and non-farming rural dwellers



CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 Summary of Research

The main goal of the research studies in this thesis was to understand the etiology of respiratory problems experienced frequently by workers in swine operations. Not all workers exposed to high concentration of respiratory hazards will develop airway diseases or suffer from the same severity of respiratory symptoms. It is clear that individual susceptibility plays an important role in the development of adverse respiratory outcomes. To date, involvement of many genes in this process has been extensively explored mainly in experimental studies using animal models or using naïve healthy adults exposed in swine barns for a short period or challenged by limited dose of inhalable dusts. This study builds on the findings of others by exploring the effects of polymorphisms in these genes among human subjects with continued occupational exposure in swine operations for years.

The current study is a secondary data analysis of the data from two studies conducted by the Canadian Centre for Health and Safety in Agriculture (CCHSA), University of Saskatchewan which included a cross-sectional study and a longitudinal study of Saskatchewan rural population. The two studies included two exposure groups. The first exposure group was comprised of workers in swine operations, a group exposed to high levels of respiratory hazards. The second exposure group was comprised of non-farming rural dwellers, a group exposed to low levels of respiratory hazards. This study examined the effects of polymorphisms in the TLR2, TLR4 and NOS3 genes on lung function in the two exposure groups using the cross-sectional study, and the effects of polymorphisms in the NOS3 gene on lung function decline rates using the longitudinal study.

8.2 Summary of results

TLRs are highly polymorphic and play an important role in both innate and adaptive immunity.[1-3] In the TLR family, TLR2 and *TLR4* are the most studied and their functions are best understood.[4,5] The first study examined the effects of TLR2 and TLR4 polymorphisms on lung function in full-time workers in swine operations and non-farming rural dwellers. This study provided direct evidence that Arg753Gln and -16933T/A polymorphisms in the TLR2 gene, rather than Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene, were significantly associated with protective effects on lung function among workers in swine operations possibly due to reducing pro-inflammation responses to the high concentration of respiratory hazards in swine barns through a TLR2 pathway. These results also raise the possibility of an important role of Gram-positive bacteria contributing to the airway diseases among workers in swine operations, as TLR2 pathways mostly respond to Gram positive bacteria.

In addition to the TLRs, there is increasing evidence showing that nitric oxide (NO) plays a key role in physiological and pathophysiological events of the lungs.[6-12] Polymorphisms in the nitric oxide synthase (NOS) genes are pertinent for the development of airway diseases: especially, polymorphisms in the NOS3 gene have been associated with atopy, bronchial hyper-responsiveness (BHR), total and specific IgE in asthmatics, and atopic asthma in studies.[13-15] The second study, a cross sectional study, examined the effects of polymorphisms in the NOS3 gene on lung function among workers in swine operations and non-farming rural dwellers. Among workers in swine operations, a group exposed to high levels of respiratory hazards, those with polymorphisms in the NOS3 gene had better FVC and FEV_1 than those with the wild-type. Interestingly, this association was not observed among non-farming rural dwellers, a group exposed to low levels of respiratory hazards. Similar results were also observed in the third study, a longitudinal study.

In the longitudinal study, workers with the CC genotype of NOS3-786T/C polymorphism had lower annual decline in FEV_1 (30.5 ml/yr vs. 58.0 ml/year p=0.005) and FVC (16.7 ml/yr vs. 50.9 ml/year, p=0.01) when compared with workers with the CC genotype (polymorphism) after controlling for all potential confounders. This association was not observed among non-farming rural dwellers. The results from the cross-sectional and longitudinal studies suggest that NOS3 gene plays an important role in the etiology of airway disease among workers exposed to high concentration of respiratory hazards.

8.3 Significance of the research

The observed associations between polymorphisms of several genes (TLR2, TLR4 and NOS3) and lung function in this study provide direct evidence that the increased risk of respiratory disorders among workers in swine operations are polygenic disorders. This type of disorders is due to multiple genes with each gene having a small effect. Polygenic disorders do not usually have a clear-cut pattern of inheritance. The results from this study showed the importance of the TLR2, TLR4 and NOS3 genes in the development of pulmonary outcomes in human subjects with exposure to high level of respiratory hazards.

In addition to the complexity caused by the involvement of many genes, exposure to high levels of respiratory hazards introduces the possibility of gene-environment interactions. The observed differences in the results in workers in swine operations and non-farming rural dwellers raise the possibility of gene-environment interaction which will help us to better understand the etiology of respiratory diseases. The results from this study also highlight the importance and necessity of including both genetic and environmental factors when examining respiratory diseases subject exposed to high levels of respiratory hazards.

8.4 Strength of the research

The strength of the research in the thesis comes from several aspects: inclusion of population based cross-sectional and longitudinal studies with subjects exposed high and low levels of respiratory hazards; selection of genes and their SNPs; and application of appropriate statistical methods.

In the cross-sectional study, 374 full-time workers in swine operations, a group more likely to be exposed to high concentration of respiratory hazards, were recruited from rural Saskatchewan with a participation rate of approximately 70%. Another group of 411 non-farming rural dwellers, a group more likely to be exposed to low concentration of respiratory hazards, were recruited from areas in close proximity (within 100 km radius) to a swine production site. Subjects with potential exposure to respiratory hazards were excluded from the non-farming rural dwellers. A total of 785 subjects in two exposure groups gave this study sufficient statistical power to study the possible effects of gene and environment interactions on airway diseases and pulmonary function. The inclusion of non-farming rural dwellers in this study enabled us to examine the effects of gene polymorphisms at a lower level of exposure. The exclusion of subjects with possible exposure to the respiratory hazards from the non-farming rural dwellers helps to guarantee the robustness of the results from this study. In addition, the selection of non-farming rural dwellers residing in close proximity to the swine production sites reduced the effects of many potential confounders such as differences in social economic status and ethnicity.

The study also benefits from the strict criteria on selection of genes and their SNPs. The selected candidate genes have been associated with asthma, atopy or COPD in at least two independent populations. The selection of SNPs were based on strict criteria: (1) genes associated with one or more disease phenotypes in multiple cohorts or have been shown to alter

gene function in biological assays; (2) use of high density single nucleotide polymorphism (SNP) maps generated by the HapMap project; and (3) selection of tagging SNPs using the SNPSelector software which prioritizes SNPs on their tagging for linkage disequilibrium, SNP allele frequencies and source, function, regulatory potential and repeat status.[16]

8.5 Limitations

Although new insight has been added to our understandings of the etiology and prevention of airway diseases in human exposed to high concentration of respiratory hazards from this study, there were some limitations in this study.

Lack of objective measurement of exposures including endotoxin levels associated with Gram-negative bacteria, and peptidoglycan levels associated with Gram-positive bacteria in this study limits the ability to examine the dose-response relationship between exposure and lung function among workers with polymorphisms and wild-type in the TLR2 and the TLR4 genes. However, inclusion of non-farming rural dwellers in this study allowed us to examine the effects of these polymorphisms in the TLR2 and the TLR4 genes at low level exposure.

Another limitation comes from the recruitment of workers for the longitudinal study. When the 163 workers were recruited for the longitudinal study at the first cycle in 1990/91, the workers have had worked in swine operations for an average of 12.2 years. With continuous exposure of high level of respiratory hazards over a long period of time, they were no longer naïve workers and were more likely to adapt to the adverse environmental hazards in their workplaces. It would be of interest in a future study to recruit workers who have just started to work in swine operations and monitor their respiratory health over time. This study will benefit us to understand both individual susceptibility and adaptation to the indoor environment.

Loss to follow-up is a limitation in most longitudinal studies and often leads to bias in the results. However, the longitudinal study of workers from swine operations and non-farming rural dwellers considered in this study had acceptable follow-up rates. In addition, a previous study using the data from this longitudinal study reported no significant differences in the mean lung function values and in the distributions of confounders between subjects who participated at all three cycles and those who did not participate.[17,18]

Population stratification might occur in population-based genetic association studies due to the genetic differences in the subpopulations.[19] Violation of HWE test is an indication of population stratification.[20] However, it has minimal effect in this study since only SNPs which followed HWE were included in this study.

Respiratory disorders due to occupational exposure involve many genes and possible interaction between them. However, the sample size was not adequate to examine gene-gene interactions in this study.

8.6 Implications for future research

This study was the first to report the airway protective effects of TLR2 and NOS3 polymorphisms among workers in swine operations. However, like most genetic studies, these variants, individually or collectively, explain only a small proportion of outcome variation. This is referred to as the "missing heritability".[21] Finding the "missing heritability" of complex diseases will have profound effects on genetic studies in the future. Researchers have suggested several approaches to examine the missing heritability. While some researchers suggest, similar to the approach taken in my study, identifying common variants and examining gene by

environment interactions, other researchers suggest examining rare variants and structural variants[22-27] and/or investigating new inheritance mechanisms such as epigenetics.[22,28]

By deep-sequencing more people thoroughly than ever before, researchers have affirmed that rare variants are abound in human genome and more likely to affect the structure or function of proteins, and therefore to have an important role in human health.[29,30] In a recent study conducted in 2012, over half a million SNPS were identified from deep-sequencing of 15,585 human protein-coding genes with an average median depth of 111 times in 2,440 individuals.[29] In this study, 85% of the SNPs were rare variants with a minor allele frequency (MAF) of less than 0.5%, and 95.7% of the SNPs which were predicted to be functionally important were rare variants.[29] The wide-spread rare variants in human genome were also reported in another study of sequencing 202 genes in 14,002 people.[30] These findings suggest future studies should examine the possible roles of low frequency variants (0.5% < MAF <5 %) and rare variants (MAF<0.5%) in the etiology of complex diseases including respiratory diseases associated with occupational exposure to high concentration of respiratory hazards.

Human genomes have many forms of genetic variations, ranging from large, microscopically visible chromosome anomalies to single nucleotide changes. Genomic alterations that involve segments of DNA that are larger than 1kb are usually referred to as structural variants.[31] Deletion, inversion, duplication and translocation can all lead to copy number variants (CNVs). Application of comparative genomic hybridization (CGH) to analyze human genomes revealed that 11,700 CNVs overlapping 1000 genes, and CNVs may account for 13% of the human genome.[23,32] Many complex diseases including autism, Alzheimer disease

and emphysema have been associated with CNVs.[33] However, the discovery of this type of variation has been understudied[27] and should be considered in future studies.

Epigenetics has added more complexity in the etiology of complex diseases.[34] Epigenetics is the study of changes produced by gene expression caused by mechanisms other than changes in DNA sequence such as DNA methylation and histone modification. Epigenetic inheritance is due to DNA modifications, chromatin modification, regulatory RNA molecules, and other mechanisms that are independent of the genetic codes but affect gene expression. [28] An animal study using mice showed that supplementation with methyl donors in utero led to alteration of locus-specific DNA methylation and a skewing T lymphocyte differentiation toward Th2.[35] More importantly, supplementation with demethylating agents during a vulnerable period of fetal development in mice could reverse epigenetically controlled phenotype.[35] A study of bronchial biopsies from normal subjects and subjects with asthma showed increased histone acetyltransferase (HAT) activity and decreased histone deacetylase activity (HDAC) in asthmatics, and subjects with asthma treated with inhaled steroids have reduced HAT activity.[36]

The possible implication of genetic screening for disease risk, such as respiratory disease risk among workers in swine operations mainly depends on the following three criteria: genetic contribution of disease risk; sensitivity and specificity; and positive and negative predictive values of a genetic test.[37] Small or mild effect sizes of common alleles, which have been observed in most genomic-wide association studies under the common disease and common variant hypothesis, limit the possible usefulness of genetic screening. However, the common disease and rare variant hypothesis states that common polygenic diseases are strongly influenced by a large number of rare variants in the same gene (allelic heterogeneity) or multiple

genes (locus heterogeneity) with large effect sizes, which sheds light on the application of genetic screening for disease risk.[38] Nevertheless, the broad spectrum of polymorphisms due to large number of rare variants, some rare variants could be observed in only few people, requires new statistical analysis techniques to increase the power of the test, such as collapsing methods, which have been reviewed in other papers.[39] Another challenge for genetic screening is the low positive predicted value (PPV) of the test, which is the proportion of positive test results that are true positives. It depends on the prevalence of the outcome. In order to reduce false positive, conducting genetic screening among subgroups of people at higher risk, such as workers with respiratory symptoms, can significantly reduce the false positive and increase the PPV.

Another implication from my research is to examine these associations using the data from the Humboldt study which included 2,090 adult residents from the rural town of Humboldt, Saskatchewan which included subjects (71.9%) who have previously lived on a farm (high exposure) and those (28.1%) who have not previously lived on a farm (low exposure).

In summary, future genetic association studies should investigate rare variants and other structural variants, such as copy number variants (deletion, duplication, inversion) and explore other heritable mechanism such as epigenetics.

8.7 Conclusion

The findings from this study raise the possibility that the polymorphisms in the TLR2 and NOS3 genes are protective of airway disease in subjects exposed to the inhaled airborne dust and genetic testing might provide useful information for identifying workers at higher risk of respiratory problems when working in swine operations.

8.8 Reference

(1) Lazarus R, Vercelli D, Palmer LJ, Klimecki WJ, Silverman EK, Richter B, et al. Single nucleotide polymorphisms in innate immunity genes: abundant variation and potential role in complex human disease. Immunol Rev 2002 Dec;190:9-25.

(2) Sabroe I, Parker LC, Wilson AG, Whyte MK, Dower SK. Toll-like receptors: their role in allergy and non-allergic inflammatory disease. Clin Exp Allergy 2002 Jul;32(7):984-9.

(3) Yang IA, Holgate ST, Holloway JW. Toll-like receptor polymorphisms and allergic disease: interpreting the evidence from genetic studies. Clin Exp Allergy 2004 Feb;34(2):163-6.

(4) Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, Cardell LO. Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. Respir Res 2005;6:100.

(5) Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999 Oct;11(4):443-51.

(6) Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991 Jun;43(2):109-42.

(7) Birrell MA, McCluskie K, Hardaker E, Knowles R, Belvisi MG. Utility of exhaled nitric oxide as a noninvasive biomarker of lung inflammation in a disease model. Eur Respir J 2006 Dec;28(6):1236-44.

(8) Liew FY, Cox FE. Nonspecific defence mechanism: the role of nitric oxide. Immunol Today 1991 Mar;12(3):A17-A21.

(9) al-Ali MK, Eames C, Howarth PH. Exhaled nitric oxide; relationship to clinicophysiological markers of asthma severity. Respir Med 1998 Jul;92(7):908-13.

(10) Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet 1994 Jan 15;343(8890):133-5.

(11) Sundblad BM, Larsson BM, Palmberg L, Larsson K. Exhaled nitric oxide and bronchial responsiveness in healthy subjects exposed to organic dust. Eur Respir J 2002 Aug;20(2):426-31.

(12) Von Essen SG, Scheppers LA, Robbins RA, Donham KJ. Respiratory tract inflammation in swine confinement workers studied using induced sputum and exhaled nitric oxide. J Toxicol Clin Toxicol 1998;36(6):557-65.

(13) Bourgain C, Hoffjan S, Nicolae R, Newman D, Steiner L, Walker K, et al. Novel case-control test in a founder population identifies P-selectin as an atopy-susceptibility locus. Am J Hum Genet 2003 Sep;73(3):612-26.

(14) Lee YC, Cheon KT, Lee HB, Kim W, Rhee YK, Kim DS. Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma. Allergy 2000 Oct;55(10):959-63.

(15) Holla LI, Buckova D, Kuhrova V, Stejskalova A, Francova H, Znojil V, et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. Clin Exp Allergy 2002 Aug;32(8):1193-8.

(16) Xu H, Gregory SG, Hauser ER, Stenger JE, Pericak-Vance MA, Vance JM, et al. SNPselector: a web tool for selecting SNPs for genetic association studies. Bioinformatics 2005 Nov 15;21(22):4181-6.

(17) Senthilselvan A, Dosman JA, Kirychuk SP, Barber EM, Rhodes CS, Zhang Y, et al. Accelerated lung function decline in swine confinement workers. Chest 1997 Jun;111(6):1733-41.

(18) Chenard L, Senthilselvan A, Grover VK, Kirychuk SP, Lawson JA, Hurst TS, et al. Lung function and farm size predict healthy worker effect in swine farmers. Chest 2007 Jan;131(1):245-54.

(19) Andreas Ziegler, Inke R.Konig. A Statistical Approach to Genetic Epidemiology. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA; 2006.

(20) Sha Q, Zhang S. A test of Hardy-Weinberg equilibrium in structured populations. Genet Epidemiol 2011 Nov;35(7):671-8.

(21) Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, et al. Finding the missing heritability of complex diseases. Nature 2009 Oct 8;461(7265):747-53.

(22) McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, et al. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat Rev Genet 2008 May;9(5):356-69.

(23) Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, et al. Global variation in copy number in the human genome. Nature 2006 Nov 23;444(7118):444-54.

(24) Cooper GM, Zerr T, Kidd JM, Eichler EE, Nickerson DA. Systematic assessment of copy number variant detection via genome-wide SNP genotyping. Nat Genet 2008 Oct;40(10):1199-203.

(25) Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, et al. Large-scale copy number polymorphism in the human genome. Science 2004 Jul 23;305(5683):525-8.

(26) Shaw CJ, Lupski JR. Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. Hum Mol Genet 2004 Apr 1;13 Spec No 1:R57-R64.

(27) Han K, Lee J, Meyer TJ, Wang J, Sen SK, Srikanta D, et al. Alu recombination-mediated structural deletions in the chimpanzee genome. PLoS Genet 2007 Oct;3(10):1939-49.

(28) Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003 Mar;33 Suppl:245-54.

(29) Tennessen JA, Bigham AW, O'Connor TD, Fu W, Kenny EE, Gravel S, et al. Evolution and functional impact of rare coding variation from deep sequencing of human exomes. Science 2012 Jul 6;337(6090):64-9.

(30) Nelson MR, Wegmann D, Ehm MG, Kessner D, St JP, Verzilli C, et al. An abundance of rare functional variants in 202 drug target genes sequenced in 14,002 people. Science 2012 Jul 6;337(6090):100-4.

(31) Feuk L, Carson AR, Scherer SW. Structural variation in the human genome. Nat Rev Genet 2006 Feb;7(2):85-97.

(32) Conrad DF, Pinto D, Redon R, Feuk L, Gokcumen O, Zhang Y, et al. Origins and functional impact of copy number variation in the human genome. Nature 2010 Apr 1;464(7289):704-12.

(33) Stankiewicz P, Lupski JR. Structural variation in the human genome and its role in disease. Annu Rev Med 2010;61:437-55.

(34) Yang IV, Schwartz DA. Epigenetic mechanisms and the development of asthma. J Allergy Clin Immunol 2012 Dec;130(6):1243-55.

(35) Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. J Clin Invest 2008 Oct;118(10):3462-9.

(36) Ito K, Caramori G, Lim S, Oates T, Chung KF, Barnes PJ, et al. Expression and activity of histone deacetylases in human asthmatic airways. Am J Respir Crit Care Med 2002 Aug 1;166(3):392-6.

(37) Pare PD. Genetic testing for respiratory disease: are we there yet? Can Respir J 2012 Jul;19(4):246-8.

(38) Cirulli ET, Goldstein DB. Uncovering the roles of rare variants in common disease through whole-genome sequencing. Nat Rev Genet 2010 Jun;11(6):415-25.

(39) Dering C, Hemmelmann C, Pugh E, Ziegler A. Statistical analysis of rare sequence variants: an overview of collapsing methods. Genet Epidemiol 2011;35 Suppl 1:S12-S17.

APPENDIX A: ETHICS APPROVAL FOR THE STUDY FROM THE UNIVERSITY OF ALBERTA

Notification of Approval (Renewal)

Date:	September 25, 2012
Amendment ID:	Pro00018692_REN2
Principal Investigator:	Ambikaipakan Senthilselvan
Study ID:	MS2_Pro00018692
Study Title:	Exposure to Endotoxin and the Lung: Gene-Environment and Gene-Gene Interactions
Sponsor/Funding Agency:	CIHR - Canadian Institutes for Health Research CIHR
Approval Expiry Date:	November 5, 2013

Thank you for submitting this renewal application. Your application has been reviewed and approved.

This re-approval is valid for another year. If your study continues past the expiration date as noted above, you will be required to complete another renewal request. Beginning at 30 days prior to the expiration date, you will receive notices that the study is about to expire. If you do not renew on or before the renewal expiry date, you will have to re-submit an ethics application.

All study related documents should be retained so as to be available to the Health REB upon request. They should be kept for the duration of the project and for at least 5 years following study completion.

Sincerely,

Dr. Jana Rieger
Chair, Health Research Ethics Board - Health Panel

Note: This correspondence includes an electronic signature (validation and approval via an online system).

Appendix B: Questionnaire for the cross-sectional study

QUESTIONNAIRE

RESPIRATORY HEALTH PROGRAM
SWINE FARMERS, and NON FARMING CONTROLS

Institute of Agricultural Rural and Environmental Health
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 0W8
306-966-8286

Date:_____ Interviewer:_____ Subject N

Instructions: when a choice is presented, please circle the appropriate answer.

1. Date of Birth: mm_____dd_____yy_____ 2. Sex: M F

3. Ethnic Origin: In which country or province, if Canadian, were each of your grandparents born?

Father's: father _____ mother _____
Mother's: father _____ mother _____

4. Highest grade completed in school: _____

5. Present Occupation: _____

6. Are you presently working in a swine confinement building? 1.yes 2.no (if no go to 41)

HOG/SWINE FARMING

7. How long have you worked in a swine confinement unit? _____years

8. Do you hog farm on a full-time basis? 1.yes 2.no

9. How many days a week and hours a day do you spend in the barn?

_____ days/week _____ hours/day

10. Did you work in the swine barn today? 1.yes 2.no

11. If yes, how many hours ago did you leave the barn? _____

12. If no, when did you last work in the barn? 1.yesterday 2. _____ days ago

13. How many pigs are usually in the barn?

1 = less than 500
2 = 501-1000
3 = 1001-2000
4 = 2001-3500
5 = 3501-5000
6 = more than 5000 specify number: _____

14. In which operations do you work in?

Operation or barn section	Hrs/day	Number of pigs
1. Farrowing		
2. Nursery		
3. Grower / Finisher		
4. Breeding / Gestation		
5. Other (specify):		
6. Other (specify):		

15. What type of confinement unit flooring is installed in each operation sections you work in? (please circle type 1=solid, 2=partly slatted [give percentage slatted], 3=fully slatted)

1. Farrowing: - type 1, 2, 3 _____ %
2. Nursery: - type 1, 2, 3 _____ %
3. Grower/finishing: - type 1, 2, 3 _____ %
4. Breeding/gestation: - type 1, 2, 3 _____ %

16. What type of air inlets are utilized in each room you work in? (please circle type, 1=Continuous inlet, 2= modular inlet [discontinuous], 3=natural ventilation)

1. Farrowing: - type 1, 2, 3
2. Nursery: - type 1, 2, 3
3. Grower/finishing: - type 1, 2, 3
4. Breeding/gestation: - type 1, 2, 3

17. Are those inlets controlled automatically? 1.yes 2.no

18. What type of feed distribution system is used in the rooms you work in? (please circle type, 1= dry feeders (unrestricted), 2=restricted feed, 3=drop – floor or wide open through, 4=wet/dry, 5=liquid feeder)

1. Farrowing: - type 1, 2, 3, 4, 5
2. Nursery: - type 1, 2, 3, 4, 5
3. Grower/finishing: - type 1, 2, 3, 4, 5
4. Breeding/gestation: - type 1, 2, 3, 4, 5

19. What type of feed is used in the rooms you work in? (please circle type, 1=pelleted/crumbles, 2= dry mash/ground feed, 3=liquid)

1. Farrowing: - type 1, 2, 3
2. Nursery: - type 1, 2, 3
3. Grower/finishing: - type 1, 2, 3
4. Breeding/gestation: - type 1, 2, 3

20. Is oil added to the feed? 1.yes 2.no

21. What type of manure storage/collection is used in the rooms you work in? (please circle type, 1=gutters with plugs/pumping, 2=floor scraper, 3=continuous flow gravity, 4=flush, 5=deep storage)

1. Farrowing: - type 1, 2, 3, 4, 5 storage time in gutter _____ days
2. Nursery: - type 1, 2, 3, 4, 5 storage time in gutter _____ days
3. Grower/finishing: - type 1, 2, 3, 4, 5 storage time in gutter _____ days
4. Breeding/gestation: - type 1, 2, 3, 4, 5 storage time in gutter _____ days

22. Do you wear a mask/respirator when working in the barn? 1.yes 2.no (if no go to 29)

23. If yes, specify: 1 = occasionally
2 = most of the time
3 = all the time

24. Why did you start wearing a mask? (please, circle the most important one)

1. had problems breathing (cough, wheeze, chest tightness, nasal stuffiness) while in the barn
2. had problem breathing (cough, wheeze, chest tightness, nasal stuffiness) after work
3. to prevent future breathing problems
4. advice from physician or other professional
5. barn owner's policy
6. other: _____

25. What type of mask do you use in the barn?

1. disposable with one strap
2. disposable with nose clip and two straps
3. ½ mask with disposable cartridges
4. Other : _____

26. Do you wear a mask to *prevent* symptoms such as cough and/or wheeze? 1.yes 2.no

27. Do you wear a mask to *reduce* symptoms? 1.yes 2.no

28. How many years have you been using masks? _____ years

29. Please indicate the frequency with which you experience the following symptoms when working in the swine barn : (please circle the appropriate response; 1=never, 2=occasional, 3=often, 4=very often)

1. headache	1, 2, 3, 4
2. muscle aches and pains	1, 2, 3, 4
3. fever	1, 2, 3, 4
4. plugged, popping ears	1, 2, 3, 4
5. burning/watering eyes	1, 2, 3, 4
6. runny nose	1, 2, 3, 4
7. scratchy throat	1, 2, 3, 4
8. sputum or phlegm	1, 2, 3, 4
9. cough	1, 2, 3, 4
10. shortness of breath	1, 2, 3, 4
11. wheezing	1, 2, 3, 4
12. tightness in chest	1, 2, 3, 4
13. skin rash or hives	1, 2, 3, 4
14. ringing in the ears	1, 2, 3, 4
15. other (specify) _____	1, 2, 3, 4

30. How soon do the symptoms marked above occur after entering the barn? (please circle)

1 = immediately 2 = within 2 hours, 3 = within 2-4 hours,
4 = within 4-8 hours, 5 = more than 8 hours later, 6 = not applicable

31. How long do these symptoms last after leaving the barn? (please circle)

1 = two hours or less, 2 = 2-4 hours, 3 = 4-8hours,
4 = more than 8 hours, 5 = not applicable

32. Do any other farm-related activities cause any of the symptoms checked above? 1.yes 2.no

If yes, please specify the type of activity and symptoms involved:

activity _____ symptom _____
activity _____ symptom _____
activity _____ symptom _____

33. Do you have access to instruments to evaluate the barn air? 1.yes 2.no

34. If yes, specify for which contaminant (circle all applicable)

1. Hydrogen sulphide [H_2S]
2. ammonia
3. carbon monoxide
4. Dust
5. other: _____

35. Do you perform manure management tasks?

1. none 2. plug pulling 3. cleaning manure pits 4. both

36. How often do you perform these tasks?

1. weekly how many plugs pulled: _____ pits cleaned: _____
2. every other week how many plugs pulled: _____pits cleaned: _____
3. monthly how many plugs pulled: _____pits cleaned: _____
4. other frequency: _____ how many plugs pulled: _____pits cleaned: _____

37. Do you power wash? 1. yes 2. no

38. How many hours/week? _____or hours/month? _____

39. Do you wear an H_2S monitor while power washing? 1. yes 2. no

40. Do you sometimes lose your sense of smell for an hour or more during or after working in the barn? 1.yes 2.no

FARMING

41. Have you ever grown grain? (please circle) 1.yes 2.no

42. Do you currently grow grain? 1.yes 2.no

43. For how many years have you been actively involved in grain farming? _____ years

44. Do you take care of any livestock other than pigs? (please circle) 1.yes 2.no

If yes, 1 = dairy 2 = beef cattle 3 = both
4. = other (specify) : _____

45. How long have you had livestock? ____ years

46. How many heads of livestock do you have? _____

47. Is the livestock kept outdoors all the time? 1.yes 2.no

48. If no, how many months of the year is it kept indoors? _____. months

49. Have you ever been exposed to any of the following in the workplace? (please circle as many as apply)

1. Mining please specify: _
2. Diesel exhaust
3. Grain dusts
4. Solvent fumes
5. Asbestos
6. Agricultural chemicals (herbicides, pesticides, fungicides)
7. Welding fumes
8. Other please specify: _____
9. None

COUGH

50. Do you currently have a cough? 1.yes 2.no

51. Do you usually have a cough? 1.yes 2.no
(Count a cough with first smoke or on first going outside. Do not count clearing the throat.)

52. Do you usually cough as much as 4-6 times a day, 4 or more days out of the week?
1.yes 2.no

53. Do you usually cough like this on most days for 3 consecutive months or more during the year?
1.yes 2.no

54. For how many years have you had this cough? ___ years

55. Is your cough caused or made worse by exposure to: (please circle as many as apply)
1. grain dust
2. barn dust
3. cigarette smoke
4. contact with animals
5. plants, pollens, weeds
6. cold air
7. exercise
8. none of the above

PHLEGM

56. Do you currently bring up phlegm from your chest? 1.yes 2.no

57. Do you usually bring up phlegm from your chest? 1.yes 2.no
(Count phlegm with first smoke or on first going outside)

58. Do you usually bring up phlegm like this as much as twice a day, 4 or more days a week?
1. yes 2.no

59. Do you usually bring up phlegm at all on getting up, or first thing in the morning?
1.yes 2.no

60. Do you usually bring up phlegm like this on most days for 3 consecutive months or more during the year? 1.yes 2.no

61. How long have you had problems with phlegm? _____ years

62. Is this problem caused or made worse by exposure to: (circle as many as apply)
1. grain dust
2. barn dust
3. cigarette smoke
5. contact with animals
6. plants, pollen, weeds
7. cold air
8. exercise
9. none of the above

WHEEZING

63. Does your chest currently sound wheezy or whistling? 1.yes 2.no

Does your chest ever sound wheezy or whistling:

64. when you have a cold? 1.yes 2.no

65. occasionally apart from colds? 1.yes 2.no

66. most days or nights? 1.yes 2.no

67. For how many years has this been present? _____ years

68. Is your chest wheezing caused or made worse by exposure to:

(please circle as many as many as apply)

1. grain dust
2. barn dust
3. cigarette smoke
4. contact with animals
5. plants, pollens, weeds
6. cold air
7. exercise
8. none of the above

SHORTNESS OF BREATH

69. Are you troubled by shortness of breath when hurrying on the level or walking up a slight hill? 1.yes 2.no

70. Do you get short of breath during or after exposure to barn or grain dust? 1.yes 2.no

NASAL IRRITATION

71. Do you currently have nasal stuffiness, runny nose, sneezing and/or nasal itchiness?

1.yes 2.no

Does your nasal stuffiness, runny nose, sneezing and/or nasal itchiness ever occur:

72. when you don't have a cold? 1.yes 2.no

73. most days or nights? 1.yes 2.no

74. How many years have those symptoms been present? _____ years

75. Is your nasal stuffiness, runny nose, sneezing and/or nasal itchiness caused or made worse by exposure to: (please circle as many as apply)

1. grain dust
2. barn dust
3. cigarette smoke
4. contact with animals
5. plants, pollens, weeds
6. cold air
7. none of the above

76. Do you have chronic sinus problems? 1.yes 2.no

EYE IRRITATION

77. Do you currently have any itching, irritation, tearing and/or redness of the eye (s)?

1. yes 2.no

78. Do you usually get eye irritation for more than 3 months a year? 1.yes 2.no

79. For how many years has this been present? _____ years

80. Is your itching, irritation, tearing and/or redness of the eye(s) caused or made worse by exposure to: (please circle as many as apply)

1. grain dust
2. barn dust
3. cigarette smoke
4. contact with animals
5. plants, pollens, weeds
6. cold air
7. none of the above

FEVER, CHILLS and/or FLU-LIKE ILLNESS

81. Have you ever had fever, chills or flu-like illness during exposure or after being exposed to barn environment? 1.yes 2.no 3. not applicable

or grain dust? 1.yes 2.no 3. not applicable

82. In what year did you have this kind of illness? _____

83. How many times have you had this kind of illness? _____

84. Did having this kind of illness make you more susceptible to having a cough or chest tightness after working in the barn? 1.yes 2.no

CHEMICALS OR PESTICIDES?

85. Have you ever used fungicides? 1.yes 2.no

86. Have you ever used insecticides? 1.yes 2.no

87. Have you ever used cleaning/disinfectant products other than household type? 1.yes 2.no

88. Have you ever spilled these chemicals or pesticides on your hands or clothing? 1.yes 2.no

89. Have you ever breathed in fumes/dust from those products directly into your lungs?

1.yes 2.no

90. During or immediately after exposure to the chemicals or pesticides have you ever had any health problems or symptoms? 1. yes 2.no

91. What kind of problems did you have? (please circle as many as apply)
    1. weakness
    2. faintness
    3. dizziness
    4. headache
    5. convulsions
    6. trouble breathing
    7. nausea and/or vomiting
    8. stomach pain
    9. diarrhea
    10. muscle twitching, cramps
    11. blurred vision
    12. jaundice
    13. nervousness
    14. other – specify: _____

92. How many times have you had these problems? _____

93. Have you ever been so ill following the exposure that you couldn't work? 1.yes 2.no

94. Have you ever had to go, or be taken, to a doctor or hospital because of these problems?
    1.yes 2.no

95. Do you ever wear the following when using insecticides?
    i. rubber gloves or clothing 1.yes 2.no
    ii. a respirator or mask 1.yes 2.no
    iii. disposable coveralls 1.yes 2.no
    iv. specify any other protective devices used: _____

MEDICAL HISTORY

96. Do you currently have a cold? 1.yes 2.no

97. Have you had a cold within the last 6 weeks? 1.yes 2.no

98. If you get a cold, does it usually (more than half the time) go to your chest? 1.yes 2.no

99. During the past 3 years, have you had any chest illnesses that have kept you off work, indoors at home or in bed? 1.yes 2.no If yes, how many _____ times in 3 years

100. Have you ever had any of the following? (please circle as many as apply)
    1. Chronic bronchitis
    2. Pneumonia
    3. Hay fever
    4. Farmer's lung
    5. Known allergy (ies) Specify: _____

101. Has a doctor ever told you that you had heart trouble? 1.yes 2.no

102. Have you had any treatment for heart trouble in the past 10 years? 1.yes 2.no

103. Has a doctor ever told you that you have high blood pressure? 1.yes 2.no

104. Has a doctor ever told you that you have asthma? 1. yes 2.no

105. In which year? _____

106. Have you ever taken any treatment for asthma? 1.yes 2.no

107. Are you currently on any medications? 1.yes (circle the appropriate medications) 2.no (go to 108)

1. Inhaled bronchodilator (β_2 Agonists)
   - i. Berotec (Fenoterol).
   - ii. Ventolin (Salbutamol) and other similar
   - iii. Bricanyl Turbuhaler (Terbutaline)
   - iv. Oxeze (Formoterol)
   - v. Serevent (Salmeterol)
   - vi. Others : _____
2. Inhaled Corticosteroids
   - i. Nu-Beclomethasone (Beclomethasone)
   - ii. Qvar (Beclomethasone)
   - iii. Pulmicort (Budesonide)
   - iv. Flovent (Fluticasone)
   - v. Advair (Fluticasone and Salmeterol)
   - vi. Symbicort (Budesonide and Formetrol)
   - vii. Others : _____
3. Nasal Corticosteroids
   - i. Rhinicort (Budesonide)
   - ii. Flonase (Fluticasone)
   - iii. Nasonex (Mometasone)
   - iv. Others : _____
4. Oral Corticosteroids
   - i. Prednisone (Prednisolone)
   - ii. Others : _____
5. Oral Broncodilator
   - i. Phyllocontin (Aminophylline)
   - ii. Choledyl (Oxtriphylline)
   - iii. Uniphyl (Theophylline)
   - iv. Others : _____
6. Other Inhalers
   - i. Combivent (Ipratropium and Salbutamol)
   - ii. Intal (Sodium cromoglycate)
   - iii. Tilade (Nedocromil)
   - iv. Others : _____
7. Pills
   - i. Singulair (Montelukast)

ii. Accolate (Zafirlukast)

iii. Others : _____

8. Allergy medications

9. Heart pills

10. Antibiotics

11. Blood pressure pills

12. Other – specify _____

Do you have blood related family members that have

108. asthma 1.yes 2.no

109. other respiratory illness 1.yes 2.no

SMOKING HISTORY

110. Have you ever smoked cigarettes? 1.yes 2.no If no, skip to 118

(No, means less than 20 packs, or, 400 cigarettes, or, 12 oz. of tobacco in lifetime, or less than 1 cigarette a day for a year)

111. Do you currently smoke cigarettes? (As of one month ago) 1.yes 2.no

I. Current smokers:

112. How old were you when you first started regular cigarette smoking? ____ (age)

113. How many cigarettes do you smoke per day now? ____ cig/day

114. On average for the entire time you have smoked, how many cigarettes have you smoked per day? ____ cig/day

II. Ex-smokers:

115. How old were you when you first started regular cigarette smoking? _____ (age)

116. How old were you when you stopped smoking cigarettes completely? ____ (age)

117. On average for the entire time you smoked, how many cigarettes did you smoke per day? _____cig/day

SKIN RASHES

118. Have you ever suffered from skin rashes lasting longer than 2 weeks? 1.yes 2.no

HOUSING CONDITIONS

119. During the past 12 months, has there been water or dampness in your home from broken pipes, leaks, heavy rain, or floods? 1.yes 2.no

120. Does your home frequently have a mildew odour or musty smell? 1.yes 2.no

121. Do you have pets that are currently living in your home?

1. None
2. Cat
3. Dog
9. Others - specify: _____

THE QUESTIONNAIRE IS FINISHED, THANK YOU VERY MUCH!

QUESTIONNAIRE

FARMERS RESPIRATORY HEALTH STUDY
SWINE AND GRAIN FARMERS, and NON FARMING CONTROLS

Institute of Agricultural Rural and Environmental Health
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 0W8
306-966-8286

Date:_____ Interviewer:_____ Subject No.:_____

1. Height (cm): _____ 2. Weight (kg): _____

3. Date of Birth: mm_____dd_____yy_____ 4. Age: _____yr. 5. Sex: M F

6. Marital Status: Single___ Married___ Divorced___ Widowed___

7. Racial Origin: In which country or province, if Canadian, were each of your grandparents born?

   Father's: father _____ mother _____

   Mother's: father _____ mother _____

8. Highest grade completed in school: _____

9. Present Occupation: _____

If you have now or in the past been farming and don't or didn't raise swine, skip to no.11.

If you are a non farming rural dweller and don't raise any livestock, skip to no. 12.

a. Have you quit hog farming since the last study? 1.yes 2.no (If no, Skip to e) quit hog
b. If yes, in what year did you quit pig farming? _____ Yr. went
c. What are the reason for leaving (select the most important one):
1 = retirement
2 = medical condition, specify: _____
3 = change of occupation
4 = other, specify: _____ other)
d. For how many years were you an active hog farmer?_____ years Skip to 11
e. How long have you worked in a swine confinement unit? _____years - yrs worked
f. Do you hog farm on a full-time basis? 1.yes 2.no
g. If no, how many hours a week do you spend in the barn? _____ hours/week
h. Is your current hog operation similar to the operation you ran or worked-in in 1995/1996?
1.yes 2.no
i. If no, what is different? (Select most important one affecting your work in the operation)
1 = increase herd size
2 = decrease herd size
3 = more barns
4 = fewer barns
5 = different job function in barn
6 = more administrative work outside animals' enclosure
7 = new or renovated building
8 = working for someone else
9 = became owner
10 = other, specify: _____

10. HOG/SWINE FARMING
a. How many days a week do you work in the barn? _____days/week
b. How many pigs are usually in the barn? (please circle)
1 = less than 200
2 = 201-400
3 = 401-600
4 = 601-800
5 = 801-1000
6 = 1001-4000
7 = more than 4000
c. How many people work with or have regular access to these swine?
(include yourself, family members and employees) _____ pers.
d. How much time (average hours per day) do you spend inside the confinement unit? -
_____ hours/day
e. In which operations do you work in?

1. Farrowing	hrs/day _____	no. pigs _____
2. Nursery	hrs/day _____	no. pigs _____
3. Grower	hrs/day _____	no. pigs _____
4. Finisher	hrs/day _____	no. pigs _____
5. Breeding	hrs/day _____	no. pigs _____
6. Other (specify)	hrs/day _____	no. pigs _____
7. Total	hrs/day _____	no. pigs _____

f. What type of confinement unit flooring utilized by each operation sections? (please circle type and give percent, 1=solid, 2=partly slatted, 3=fully slatted)

1. Farrowing - type 1, 2, 3; _____ %
2. Nursery: - type 1, 2, 3; _____ %
3. Grower/finishing: - type 1, 2, 3; _____ %
4. Breeding/gestation: - type 1, 2, 3; _____ %

g. What type of ventilation is utilized by each operation? (please circle type, 1=Continuous inlet, 2= modular inlet [discontinuous], 3=natural ventilation)
1. Farrowing - type 1, 2, 3
2. Nursery: - type 1, 2, 3
3. Grower/finishing: - type 1, 2, 3
4. Breeding/gestation: - type 1, 2, 3

h. What type of feed distribution system is used? (please circle type, 1= dry feeders (unrestricted), 2=restricted feed, 3=drop – floor or wide open through, 4=wet/dry)
1. Farrowing - type 1, 2, 3, 4
2. Nursery: - type 1, 2, 3, 4
3. Grower/finishing: - type 1, 2, 3, 4
4. Breeding/gestation: - type 1, 2, 3, 4

i. What type of feed is used? (please circle type, 1=pelleted/crumbles, 2= dry mash/ground feed, 3=liquid)
1. Farrowing - type 1, 2, 3
2. Nursery: - type 1, 2, 3
3. Grower/finishing: - type 1, 2, 3
4. Breeding/gestation: - type 1, 2, 3

j. Is oil added to the feed? (please circle) 1.yes 2.no

k. What type of manure storage/collection is used? (please circle type, 1=stop and flow gravity, 2=floor scraper, 3=continuous flow gravity, 4=flush)
1. Farrowing - type 1, 2, 3, 4 storage time in gutter _____ days
2. Nursery: - type 1, 2, 3, 4 storage time in gutter _____ days
3. Grower/finishing: - type 1, 2, 3, 4 storage time in gutter _____ days
4. Breeding/gestation: - type 1, 2, 3, 4 storage time in gutter _____ days

l. Do you usually wear a mask or respirator when working in the confinement unit? (please circle) 1.yes 2.no (if no go to m)
if yes, specify: 1 = never
2 = occasionally
3 = most of the time
4 = all the time

i) Why did you start wearing a mask? (please, circle the most important one)
1. had problems breathing while in the barn
2. was having symptoms after work
3. for precaution
4. advice from physician or other professional
5. other: _____

ii) What type of mask do you use in the barn?
1. disposable with one strap
2. disposable with nose clip and two straps
3. ½ mask with disposable cartridges
4. charcoal
5. gas
6. hood

iii) Do you wear a mask to prevent symptoms? 1.yes 2.no

iv) Do you wear a mask to alleviate symptoms? 1.yes 2.no

v) Are masks successful in use? 1.yes 2.no

vi) How many years have you been using masks? _____ years

m. Do you have a dust control system in your confinement unit? (please circle) 1.yes 2.no

n. Have you improved the environment since last testing? 1.yes 2.no (if no go to o)

i) Describe improvements on the environment (select the most important one):
1. better ventilation system
2. better manure management system in the barn
3. changes in flooring
4. changes in the feed to lower dust or ammonia
5. other improvements: _____

o. Have you improved the hygiene in your operation since last testing? 1.yes 2.no (if no go to p)

i) Describe the improvement on the hygiene (select the most important one):
1. started all-in all-out
2. more frequent washing and cleaning
3. other improvements: _____

p. Please indicate the frequency with which you experience the following symptoms when working with swine in confinement: (please circle; 1=never, 2=occasional, 3=often, 4=very often)

1. headache	1, 2, 3, 4
2. weakness	1, 2, 3, 4
3. dizziness	1, 2, 3, 4
4. fainting or blackout	1, 2, 3, 4
5. muscle aches and pains	1, 2, 3, 4
6. fever	1, 2, 3, 4
7. nausea or vomiting	1, 2, 3, 4
8. plugged, popping ears	1, 2, 3, 4
9. hearing problems	1, 2, 3, 4
10. burning/watering eyes	1, 2, 3, 4
11. runny nose	1, 2, 3, 4
12. scratchy throat	1, 2, 3, 4
13. sputum or phlegm	1, 2, 3, 4
14. cough	1, 2, 3, 4
15. shortness of breath	1, 2, 3, 4
16. wheezing	1, 2, 3, 4
17. tightness in chest	1, 2, 3, 4
18. skin rash or hives	1, 2, 3, 4
19. other (specify) _____	1, 2, 3, 4

q. How soon do these symptoms occur after entering the barn? (please circle)
1 = immediately 2 = within 2 hours, 3 = within 2-4 hours,
4 = within 4-8 hours, 5 = more than 8 hours later

r. How long do these symptoms last after leaving the barn? (please circle)
1 = two hours or less, 2 = 2-4 hours, 3 = 4-8hours, 4 = more than 8 hours

s. Do any other farm-related activities cause any of the symptoms checked above? (please circle) 1.yes 2.no
If yes, please specify the type of activity and symptoms involved:
activity _____ symptoms _____
activity _____ symptoms _____
activity _____ symptoms _____

11. GRAIN FARMING

a. Have you ever grown grain? (please circle) 1.yes 2.no
If no, please skip to 12.

b. Do you currently grow grain? 1.yes (If yes, Skip to f) 2.no

c. If no, reason for leaving:
1 = retirement
2 = medical condition, specify: _____
3 = change of occupation
4 = other, specify: _____

d. How long has it been since you quit grain farming? _____ years

e. For how many years were you an active grain farmer? _____ years Skip to h

f. How long have you been grain farming? _____ years

g. How many acres:
i. do you have under cultivation? _____ acres
ii. did you have seeded last year? _____ acres
iii. do you normally seed per year? _____ acres
iv. of wheat did you have last year? _____ acres
v. of barley did you have last year? _____ acres
vi. of canola did you have last year? _____ acres
vii. of other crops? Crop _____ acres _____
Crop _____acres _____

h. Do you have any cattle? (please circle) 1.yes 2.no (If no, skip to no. 12)
If yes,
1 = dairy
2 = beef cattle
3 = both

i. how long have you had cattle? ____ years
ii. how many cows do you have? _____ no. cows
iii. how many cattle do you keep outdoors? _____ no. cows
iv. how many cattle do you keep indoors? _____ no. cows
v. how many months of the year are they indoors? _____ months

12. Have you ever been exposed to any of the following in the workplace? (please circle as many as apply)
1. Mining please specify: _____
2. Diesel exhaust
3. Grain dusts
4. Solvent fumes
5. Asbestos
6. Agricultural chemicals (herbicides, pesticides, fungicides)
7. Welding fumes
8. Other please specify: _____
9. None

13. COUGH (please circle yes or no)
a. Do you currently have a cough? 1.yes 2.no cCurr
b. Do you usually have a cough? 1.yes 2.no cusual
(Count a cough with first smoke or on first going outside.)
If no to a and b, skip to 14.
c. Do you usually cough as much as 4-6 times a day, 4 or more days out of the week?
1.yes 2.no cfreq*
d. Do you usually cough at all on getting up or first thing in the morning? 1.yes 2.no cmorn
e. Do you usually cough like this on most days for 3 consecutive months or more during the year? 1.yes 2.no cdurn*
f. For how many years have you had this cough? _____ years cyears.
g. Is your cough caused or made worse by exposure to: (please circle as many as apply)
1. grain dust
2. barn dust, silage
3. cigarette smoke
4. farm chemicals
5. contact with animals
6. plants, pollens, weeds
7. cold air
8. exercise
9. none of the above

14. PHLEGM (please circle yes or no)
a. Do you currently bring up phlegm from your chest? 1.yes 2.no PCurr*
b. Do you usually bring up phlegm from your chest? 1.yes 2.no Pusual
(Count phlegm with first smoke or on first going outside.) If no to a and b, skip to 15.
c. Do you usually bring up phlegm like this as much as twice a day, 4 or more days a week?
1.yes 2.no Pfreq*
d. Do you usually bring up phlegm at all on getting up, or first thing in the morning? Pmorn
1.yes 2.no
e. Do you usually bring up phlegm like this on most days for 3 consecutive months or more during the year? 1.yes 2.no Pdurn*
f. How long have you had this problem with phlegm? _____ years PYears

g. Is this problem caused or made worse by exposure to: (please circle as many as apply)
   1. grain dust
   2. barn dust, silage
   3. cigarette smoke
   4. farm chemicals
   5. contact with animals
   6. plants, pollen, weeds
   7. cold air
   8. exercise
   9. none of the above

h. In your opinion, which grain dusts are most likely to cause cough and/or phlegm, or make it worse? (please circle as many as apply)
   1. wheat
   2. oats
   3. barley
   4. flax
   5. rape
   6. mustard
   7. other – specify _____

15. WHEEZING (Please circle yes or-no)

a. Does your chest currently sound wheezy or whistling? 1.yes 2.no

b. Does your chest ever sound wheezy or whistling:
   i. when you have a cold? 1.yes 2.no
   ii. occasionally apart from colds? 1.yes 2.no
   iii. most days or nights? 1.yes 2.no
   If no to a and b, skip to 21.

c. For how many years has this been present? ____ years

d. Is your chest wheezing caused or made worse by exposure to:
   (please circle as many as many as apply)
   1. grain dust
   2. barn dust, silage
   3. cigarette smoke
   4. farm chemicals
   5. contact with animals
   6. plants, pollens, weeds
   7. cold air
   8. exercise
   9. none of the above

e. In your opinion, which grain dusts are most likely to cause wheezing, or make it worse? (please circle as many as apply)
1. wheat
2. oats
3. barley
4. flax
5. rape
6. mustard
7. other - specify: _____

16. SHORTNESS OF BREATH (please circle yes or no)

a. Are you troubled by shortness of breath when hurrying on the level or walking up a slight hill? 1.yes 2.no If no, skip to 17.

b. Do you get short of breath during or after exposure to grain dust? 1.yes 2.no

c. In your opinion, which grain dusts are most likely to cause shortness of breath, or make it worse? (please circle as many as apply)
1. wheat
2. oats
3. barley
4. flax
5. rape
6. mustard
7. other - specify: _____

17. NASAL IRRITATION (please circle yes or no)

a. Do you currently have nasal stuffiness, runny nose, sneezing and/or nasal itchiness? 1.yes 2.no

b. Do these symptoms ever occur:
i. when you have a cold? 1.yes 2.no
ii. occasionally apart from colds? 1.yes 2.no
iii. most days or nights? 1.yes 2.no
If no to a and b, skip to 23.

c. For how many years has this been present? ____ years

d. Is your nasal stuffiness, runny nose, sneezing and/or nasal itchiness caused or made worse by exposure to: (please circle as many as apply)
1. grain dust
2. barn dust, silage
3. cigarette smoke
4. farm chemicals
5. contact with animals
6. plants, pollens, weeds
7. cold air
8.exercise
9. none of the above

e. In your opinion which grain dusts are most likely to cause nasal stuffiness, runny nose, sneezing and/or nasal itchiness or make it worse? (please circle as many as apply)
1. wheat
2. oats
3. barley
4. flax
5. rape
6. mustard
7. other - specify: _____

18. EYE IRRITATION (please circle yes or no)
a. Do you currently have any itching, irritation, tearing and/or redness of the eye (s)?
1. yes 2.no
b. Do you usually get these symptoms for more than 3 months a year? 1.yes 2.no
If no to a and b, skip to 19.
c. For how many years has this been present? _____ years
d. Is your itching, irritation, tearing and/or redness of the eye(s) caused or made worse by exposure to: (plelase circle as many as apply)
1. grain dust
2. barn dust, silage
3. cigarette smoke
4. farm chemicals
5. contact with animals
6. plants, pollens, weeds
7. cold air
8. exercise
9. none of the above
e. In your opinion, which grain dusts are most likely to cause itching, irritation, tearing and/or redness of the eye(s) or make it worse? (please circle as many as apply)
1. wheat
2. oats
3. barley
4. flax
5. rape
6. mustard
7. other - specify: _____

IF IN YOUR WORK YOU ARE NOT EXPOSED TO GRAIN DUST, PLEASE SKIP TO QUESTION 20

19. FEVER AND/OR CHILLS (please circle yes or no)
a. Have you ever had fever and/or chills during exposure or after being exposed to grain dust?
1.yes 2.no
b. During exposure to grain dust have you ever had:
i. burning, watering or itchy eyes? 1.yes 2.no
ii. stuffy nose? 1.yes 2.no
iii. sore or burning throat? 1.yes 2.no

c. During or immediately after exposure to grain dust have you ever had itchy skin?
1.yes 2.no

20. PESTICIDES AND HERBICIDES (please circle yes or no)

a. Have you ever been exposed to pesticides or herbicides? 1.yes 2.no
If no, skip to 21.

b. Have you ever spilled these chemicals on your hands or clothing? 1.yes 2.no
If yes, please specify the chemicals using the list in 20i. _____

c. Have you ever breathed in fumes from pesticides or herbicides directly into your lungs?
1.yes 2.no
If yes, please specify the chemicals using the list in 20i. _____

d. During or immediately after exposure to pesticides or herbicides have you ever had any health problems or symptoms?
1.yes 2.no
If no, skip to 20j.

e. What kind of problems did you have? (please circle as many as apply)
1. weakness
2. faintness
3. dizziness
4. headache
5. convulsions
6. trouble breathing
7. nausea and/or vomiting
8. stomach pain
9. diarrhea
10. muscle switching, cramps
11. blurred vision
12. jaundice
13. nervousness
14. other – specify: _____

f. How many times have you had these problems? _____ times per year

g. Have you ever been so ill following the exposure to pesticides or herbicides that you couldn't work? 1.yes 2.no

h. Have you ever had to go, or be taken, to a doctor or hospital because of these problems?
1.yes 2.no

i. Which herbicides, fungicides, seed treatments or insecticides caused you to have symptoms? (Please name the worst chemicals by encircling the appropriate numbers.)

Herbicides

1. 2,4-D
2. 2,4-DB
3. Absolute
4. Accord
5. Achieve 80 DG / Achieve Liquid
6. Achieve Extra Gold
7. Advance
8. Afolan F
9. Alley
10. Assert 300 SC
11. Assure II
12. Attain
13. Avadex
14. Banvel II
15. Bonanza
16. Buctril
17. Caliber 400
18. Champion^{120} Extra
19. Champion Plus
20. Credit
21. Curtail M
22. Dyvel
23. Dyvel DS
24. Eclipse
25. Edge
26. Embutox 625
27. Estamines
28. Estaprop
29. Everest
30. Express Pack
31. Factor
32. FlaxMax
33. Freedom Gold
34. Frontline
35. Frontline + 2,4D
36. Fusion
37. Glean
38. Glyfos
39. Glyphosate
40. Gramoxone
41. Harmony Total
42. Heritage 5G
43. Hoe Grass
44. Horizon
45. Horizon BTM
46. K2
47. Koril 235
48. Laser DF
49. Liberty
50. Lontrel
51. Maverick
52. MCPA
53. MCPB + MCPA
54. Mextrol 400 M
55. Odyssey
56. Pardner
57. Pea Pack
58. Poast Ultra
59. Prepass
60. Prestige
61. Prevail
62. Puma^{120} Super
63. Pursuit
64. Pursuit Ultra
65. Refine Extra Toss-N-Go
66. Reflex
67. Reglone Desiccant
68. Renegade
69. Reward
70. Rival
71. Roundup Transorb/Roundup
72. Roundup Dry/Original
73. Rustler
74. Select
75. Sencor
76. Spectrum
77. Stampedes
78. Sundance
79. Sweep
80. Sword
81. Target
82. Thumper
83. Torch
84. Touchdown 600/Touchdown iQ
85. Treflan
86. Trifluralin
87. Triumph Plus
88. Trophy
89. Tropotox Plus
90. Turboprop
91. Vantage / Vantage Plus
92. Venture
93. Victor
94. Other herbicide : _____

Foliar Fungicides

1. Benlate
2. Bravo 500
3. Dithane DG Rainshield NT
4. Quadris
5. Ronilan EG
6. Rvovral Flo
7. Tilt 250E
8. Other fungicide : _____

Seed Treatments

1. Allegiance FL
2. Apron FL
3. Charter
4. Crown
5. Dividend XL RTA
6. Foundation Lite
7. Gaucho CS FL
8. Helix
9. Helix Xtra
10. Raxil 250 FL
11. Raxil FL
12. Thiram 75WP
13. Vitavax products
14. Other seed treatment : _____

Insecticides

1. Counter
2. Cygon
3. Decis
4. Dipel
5. Dylox
6. Eco bran bait
7. Furadan
8. Fyfanon
9. Gastroxin
10. Lagon
11. Lannate
12. Lorsban
13. Malathion
14. Matador
15. Nufos
16. Pounce
17. Protect It
18. Pyrinex
19. Sevin
20. Other – specify: _____

j. Do you ever wear the following when using herbicides, fungicides, seed treatments or insecticides?
   i. rubber gloves or clothing 1.yes 2.no
   ii. a respirator or mask 1.yes 2.no
   iii. a rubber apron 1.yes 2.no
   iv. disposable coveralls 1.yes 2.no

k. How long do you wear clothing after using pesticides and before your clothing is washed? (please circle)
   1. up to 4 hours
   2. 4 to 12 hours
   3. 12 to 24 hours
   4. longer than 24 hours

Subject number: _____ (repeat it here)

21. MEDICAL HISTORY(please circle yes or no)

a. Do you currently have a cold? 1.yes 2.no

b. If you get a cold, does it usually go to your chest? 1.yes 2.no
   (Usually means more than half the time.)

c. During the past 3 years, have you had any chest illnesses that have kept you off work, indoors at home or in bed? 1.yes If yes, how many? ____ times in 3 years 2.no

d. Have you ever had any of the following? (please circle as many as apply)
   1. Chronic bronchitis
   2. Pneumonia
   3. Emphysema
   4. Asthma
   5. Hay fever
   6. Farmer's lung
   7. Chest operations
   8. Chest injuries
   9. Other chest problems
   10. Known allergy (ies) Specify: _____

e. Has a doctor ever told you that you had heart trouble? 1.yes 2.no
   If no, skip to g.

f. Have you had any treatment for heart trouble in the past 10 years? 1.yes 2.no

g. Has a doctor ever told you that you have high blood pressure? 1.yes 2.no

h. Are you currently on any medications? 1.yes 2.no
   (Please circle the appropriate numbers)
   1. Inhaled bronchodilator (β_2 Agonists)
      i. Berotec (Fenoterol)
      ii. Ventolin (Salbutamol) and other similar
      iii. Bricanyl Turbuhaler (Terbutaline)
      iv. Oxeze (Formoterol)
      v. Serevent (Salmeterol)
      vi. Others : _____
   2. Inhaled Corticosteroids
      i. Nu-Beclomethasone (Beclomethasone)
      ii. Qvar (Beclomethasone)
      iii. Pulmicort (Budesonide)
      iv. Flovent (Fluticasone)
      v. Advair (Fluticasone and Salmeterol)
      vi. Symbicort (Budesonide and Formetrol)
      vii. Others : _____
   3. Nasal Corticosteroids
      i. Rhinicort (Budesonide)
      ii. Flonase (Fluticasone)
      iii. Nasonex (Mometasone)
      iv. Others : _____
   4. Oral Corticosteroids
      i. Prednisone (Prednisolone)
      ii. Others : _____
   5. Oral Broncodilator
      i. Phyllocontin (Aminophylline)
      ii. Choledyl (Oxtriphylline)
      iii. Uniphyl (Theophylline)
      iv. Others : _____

6. Other Inhalers
   i. Combivent (Ipratropium and Salbutamol)
   ii. Intal (Sodium cromoglycate)
   iii. Tilade (Nedocromil)
   iv. Others : _____
7. Pills
   i. Singulair (Montelukast)
   ii. Accolate (Zafirlukast)
   iii. Others : _____
8. Allergy medications
9. Heart pills
10. Antibiotics
11. Blood pressure pills
12. Other – specify _____

i) Do you have blood related family members that have
   1) asthma 1.yes 2.no
   2) respiratory illness 1.yes 2.no

22. SMOKING HISTORY(please circle yes or no)

a. Have you ever smoked cigarettes? 1.yes 2.no If no, skip to k.
(No, means less than 20 packs, or, 400 cigarettes, or, 12 oz. of tobacco in lifetime, or, less than 1 cigarette a day for a year)

b. Do you currently smoke cigarettes? (As of one month ago) 1.yes 2.no

I. Current smokers:

c. How old were you when you first started regular cigarette smoking? ____ years old
d. How many cigarettes do you smoke per day now? ____ cig/day
e. On average for the entire time you have smoked, how many cigarettes have you smoked per day? ____ cig/day
f. Do you inhale the cigarette smoke? (Please circle the appropriate number.)
   1. Not at all
   2. Slightly
   3. Moderately
   4. Deeply

   Skip to k.

II. Ex-smokers:

g. How old were you when you first started regular cigarette smoking? ___ years old
h. How old were you when you stopped smoking cigarettes completely? ___ years old
i. On average for the entire time you smoked, how many cigarettes did you smoke per day? _____ cig/day
j. Did you inhale the cigarette smoke? (Please circle the appropriate number)
   1. Not at all
   2. Slightly
   3. Moderately
   4. Deeply

III-Cigar/pipe smokers:

k. Have you ever smoked a Pipe and/or cigars regularly?
(Yes means more than 12 oz. of tobacco in lifetime or more than 1 cigar/week for 1 year.)
1.yes 2.no If no, skip to 23.

l. For how long? __ years

m. Are you currently smoking cigars or pipes? 1.yes 2.no

n. If yes, how much? _____ cigars/week _____ tobacco pouches/week

23.SKIN RASHES (please circle yes or no)

a. Have you ever had a serious skin rash in infancy? 1.yes 2.no

b. Have you ever suffered from skin rashes? 1.yes 2.no

c. Have you ever suffered from skin rashes lasting longer than 2 weeks? 1.yes 2.no

24.HOUSING CONDITIONS

a. What is the main heating source in your home? Is it
1. Radiators (steam or hot water)
2. Gas-heated forced air (vents)
3. Electric-heated forced air (vents)
4. Gas stove/fireplace/wall furnace
5. Electric space heater
6. Kerosene space heater
7. Wood burning stove/fireplace
8. Some other source Specify: _____
9. No source of heat
10. Don't know

b. During the past 12 months, has there been water or dampness in your home from broken pipes, leaks, heavy rain, or floods? 1.yes 2.no

c. Does your home frequently have a mildew odour or musty smell? 1.yes 2.no

d. Do you have pets that are currently living in your home?
1. None
2. Cat
3. Dog
4. Hamster
5, Gerbil
6. Guinea pig
7. Rabbit
8. Bird
9. Others?
10. Specify: _____
11. Don't know

YOU ARE FINISHED NOW, THANK YOU VERY MUCH!

Appendix D: SAS outputs

Table 6-2:

FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.8640	0.0427	3.7803	3.9478	8176.13	<.0001
SEX	F		1	-0.5837	0.0542	-0.6899	-0.4776	116.14	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0288	0.0016	-0.0319	-0.0258	338.73	<.0001
ht_mean			1	0.0390	0.0031	0.0329	0.0450	159.31	<.0001
wt_mean			1	-0.0015	0.0012	-0.0038	0.0008	1.71	0.1906
SMOKE	2		1	-0.1254	0.0452	-0.2141	-0.0368	7.69	0.0055
SMOKE	3		1	-0.0041	0.0447	-0.0918	0.0836	0.01	0.9266
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.1051	0.0425	0.0218	0.1884	6.12	0.0134
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.1609	0.0615	0.0403	0.2815	6.84	0.0089
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-0.1586	0.0864	-0.3280	0.0108	3.37	0.0666
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.4392	0.0127	0.4149	0.4649		

FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	4.9451	0.0517	4.8438	5.0464	9160.78	<.0001
SEX	F		1	-0.7598	0.0655	-0.8881	-0.6314	134.59	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0251	0.0019	-0.0288	-0.0214	176.01	<.0001
ht_mean			1	0.0545	0.0037	0.0472	0.0618	213.24	<.0001
wt_mean			1	-0.0009	0.0014	-0.0037	0.0019	0.40	0.5253
SMOKE	2		1	-0.0631	0.0547	-0.1703	0.0440	1.33	0.2481
SMOKE	3		1	0.0275	0.0541	-0.0785	0.1336	0.26	0.6108
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.0638	0.0514	-0.0369	0.1645	1.54	0.2145
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.1263	0.0744	-0.0195	0.2721	2.88	0.0896
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-0.1198	0.1045	-0.3247	0.0850	1.31	0.2516
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.5310	0.0154	0.5017	0.5621		

FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	78.2529	0.5705	77.1348	79.3711	18814.9	<.0001
SEX	F	1	0.4138	0.7231	-1.0035	1.8311	0.33	0.5672
SEX	M	0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean		1	-0.2049	0.0209	-0.2458	-0.1639	96.03	<.0001
ht_mean		1	-0.0785	0.0412	-0.1593	0.0023	3.63	0.0569
wt_mean		1	-0.0090	0.0157	-0.0398	0.0218	0.33	0.5657
SMOKE	2	1	-1.8821	0.6038	-3.0655	-0.6987	9.72	0.0018
SMOKE	3	1	-0.5387	0.5974	-1.7095	0.6322	0.81	0.3672
SMOKE	4	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0	1	1.1715	0.5673	0.0596	2.2835	4.26	0.0389

SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	1.2752	0.8213	-0.3345	2.8850	2.41	0.1205
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-1.5634	1.1540	-3.8251	0.8984	1.84	0.1755
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	5.8634	0.1701	5.5393	6.2064		

$FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.6051	0.0920	3.4247	3.7854	1534.86	<.0001
SEX	F		1	-0.5049	0.1166	-0.7335	-0.2763	18.74	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0455	0.0034	-0.0521	-0.0389	182.17	<.0001
ht_mean			1	0.0248	0.0066	0.0117	0.0378	13.89	0.0002
wt_mean			1	-0.0016	0.0025	-0.0066	0.0034	0.40	0.5262
SMOKE	2		1	-0.2422	0.0974	-0.4330	-0.0513	6.18	0.0129
SMOKE	3		1	-0.0241	0.0964	-0.2130	0.1647	0.06	0.8022
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.2088	0.0915	0.0295	0.3882	5.21	0.0225
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.3887	0.1325	0.1290	0.6483	8.61	0.0033
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-0.3682	0.1861	-0.7330	-0.0034	3.91	0.0479
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.9457	0.0274	0.8935	1.0011		

Predicted FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	97.2373	0.9959	95.2853	99.1893	9532.29	<.0001
SMOKE	2		1	-3.9270	1.2386	-6.3547	-1.4994	10.05	0.0015
SMOKE	3		1	-1.0789	1.2053	-3.4411	1.2834	0.80	0.3707
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	2.8671	1.1403	0.4321	4.9021	5.47	0.0193
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	4.7572	1.6919	1.4411	8.0732	7.91	0.0049
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-4.6543	2.3778	-9.3147	0.0061	3.83	0.0503
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	12.0983	0.3510	11.4296	12.8062		

Predicted FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	102.3767	0.9778	100.4603	104.2931	10962.5	<.0001
SMOKE	2		1	-1.7043	1.2161	-4.0877	0.6791	1.96	0.1611
SMOKE	3		1	0.1538	1.1833	-2.1653	2.4730	0.02	0.8966
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	1.4948	1.1195	-0.6994	3.6891	1.78	0.1818
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	3.5751	1.6611	0.3195	6.8307	4.63	0.0314
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-3.0687	2.3345	-7.6442	1.5067	1.73	0.1887
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	11.8778	0.3446	11.2213	12.5728		

Predicted FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	82.9958	0.2009	82.6021	83.3895	170726	<.0001
SMOKE	2		1	-0.1189	0.2498	-0.6085	0.3707	0.23	0.6341
SMOKE	3		1	-0.9170	0.2431	-1.3935	-0.4406	14.23	0.0002
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.3890	0.2300	-0.8198	0.0617	2.57	0.1086
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.4964	0.3412	-0.1724	1.1651	2.12	0.1458
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-0.2318	0.4796	-1.1717	0.7082	0.23	0.6289
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	2.4400	0.0708	2.3052	2.5828		

Predicted $FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	86.5219	2.0422	82.5192	90.5246	1794.90	<.0001
SMOKE	2		1	-7.1599	2.5399	-12.1379	-2.1818	7.95	0.0048
SMOKE	3		1	-2.1934	2.4714	-7.0373	2.6506	0.79	0.3748
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	4.5964	2.3383	0.0134	9.1794	3.86	0.0493
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	9.0332	3.4693	2.2335	15.8330	6.78	0.0092
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	0	0	1	-9.3761	4.8758	-18.9324	0.1803	3.70	0.0545
SWINEW*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	24.8083	0.7198	23.4369	26.2599		

Table 6-3:

FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.8863	0.0414	3.8052	3.9674	8821.74	<.0001
SEX	F		1	-0.5822	0.0545	-0.6890	-0.4754	114.15	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0288	0.0016	-0.0319	-0.0257	330.82	<.0001
ht_mean			1	0.0391	0.0031	0.0330	0.0452	158.07	<.0001
wt_mean			1	-0.0017	0.0012	-0.0040	0.0006	1.99	0.1581
SMOKE	2		1	-0.1230	0.0457	-0.2126	-0.0334	7.24	0.0071
SMOKE	3		1	-0.0015	0.0449	-0.0894	0.0865	0.00	0.9735
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.0888	0.0392	0.0119	0.1657	5.12	0.0237
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	0.2209	0.1081	0.0091	0.4327	4.18	0.0409
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743709	0	A/G	1	-0.3411	0.1489	-0.6330	-0.0492	5.25	0.0220
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.4412	0.0129	0.4167	0.4671		

FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	4.9659	0.0501	4.8677	5.0641	9825.54	<.0001
SEX	F		1	-0.7581	0.0660	-0.8874	-0.6288	132.04	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0252	0.0019	-0.0289	-0.0214	172.52	<.0001
ht_mean			1	0.0545	0.0038	0.0471	0.0619	209.01	<.0001
wt_mean			1	-0.0010	0.0014	-0.0038	0.0018	0.52	0.4714
SMOKE	2		1	-0.0663	0.0554	-0.1738	0.0432	1.39	0.2380
SMOKE	3		1	0.0232	0.0543	-0.0833	0.1297	0.18	0.6691
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.0542	0.0475	-0.0389	0.1473	1.30	0.2540
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	0.1253	0.1308	-0.1311	0.3817	0.92	0.3383
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	0	A/G	1	-0.2561	0.1803	-0.6095	0.0973	2.02	0.1555
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.5341	0.0158	0.5045	0.5655		

FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	78.3414	0.5516	77.2603	79.4224	20172.3	<.0001
SEX	F		1	0.4113	0.7264	-1.0124	1.8349	0.32	0.5713
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.2028	0.0211	-0.2442	-0.1615	92.45	<.0001
ht_mean			1	-0.0739	0.0415	-0.1552	0.0074	3.17	0.0749
wt_mean			1	-0.0099	0.0158	-0.0409	0.0211	0.39	0.5312
SMOKE	2		1	-1.8006	0.6094	-2.9950	-0.6062	8.73	0.0031
SMOKE	3		1	-0.4312	0.5982	-1.6037	0.7413	0.52	0.4710
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.9827	0.5231	-0.0426	2.0079	3.53	0.0603
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	3.0731	1.4405	0.2498	5.8963	4.55	0.0329
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.

SWINEW*rs5743708	0	A/G	1	-3.4201	1.9853	-7.3112	0.4711	2.97	0.0849
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	5.8809	0.1713	5.5545	6.2265		

$FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.6506	0.0890	3.4761	3.8250	1682.21	<.0001
SEX	F		1	-0.5019	0.1172	-0.7316	-0.2722	18.33	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0452	0.0034	-0.0518	-0.0385	176.01	<.0001
ht_mean			1	0.0256	0.0067	0.0125	0.0387	14.60	0.0001
wt_mean			1	-0.0019	0.0025	-0.0069	0.0031	0.53	0.4661
SMOKE	2		1	-0.2253	0.0983	-0.4180	-0.0325	5.25	0.0220
SMOKE	3		1	-0.0012	0.0965	-0.1904	0.1880	0.00	0.9901
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	0.1632	0.0844	-0.0022	0.3286	3.74	0.0532
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	0.6696	0.2324	0.2141	1.1252	8.30	0.0040
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	0	A/G	1	-0.8010	0.3204	-1.4289	-0.1731	6.25	0.0124
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.9490	0.0276	0.8963	1.0047		

Predicted FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	97.8873	0.9474	96.0105	99.7241	10671.8	<.0001
SMOKE	2		1	-3.8311	1.2519	-6.2847	-1.3775	9.37	0.0022
SMOKE	3		1	-1.0035	1.2086	-3.3722	1.3653	0.69	0.4064
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	2.1701	1.0476	0.1168	4.2233	4.29	0.0383
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	7.1189	2.9668	1.3040	12.9337	5.76	0.0164
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	0	A/G	1	-9.3096	4.0848	-17.3156	-1.3036	5.19	0.0227
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	12.1577	0.3542	11.4829	12.8722		

Predicted FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	102.9635	0.9323	101.1363	104.7907	12197.6	<.0001
SMOKE	2		1	-1.7203	1.2319	-4.1348	0.6943	1.95	0.1626
SMOKE	3		1	0.1011	1.1893	-2.2299	2.4321	0.01	0.9322
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	1.2156	1.0309	-0.8049	3.2362	1.39	0.2383
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	3.7958	2.9195	-1.9264	9.5180	1.69	0.1936
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	0	A/G	1	-8.1180	4.0197	-13.9985	1.7605	2.32	0.1280
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	11.9641	0.3486	11.3000	12.6672		

Predicted FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	83.0248	0.1892	82.6538	83.3955	192528	<.0001
SMOKE	2		1	-0.1288	0.2500	-0.6188	0.3613	0.27	0.6065
SMOKE	3		1	-0.8996	0.2414	-1.3727	-0.4265	13.89	0.0002
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.2785	0.2092	-0.6886	0.1316	1.77	0.1832
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	1.3760	0.5926	0.2147	2.5374	5.39	0.0202
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	0	A/G	1	-2.2068	0.8158	-3.8058	-0.6077	7.32	0.0068
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	2.4283	0.0707	2.2935	2.5710		

Predicted $FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	87.4348	1.9369	83.6385	91.2312	2037.70	<.0001
SMOKE	2		1	-6.7169	2.5595	-11.7334	-1.7004	6.89	0.0087
SMOKE	3		1	-1.7014	2.4709	-6.5444	3.1416	0.47	0.4911
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	3.4610	2.1419	-0.7370	7.6590	2.61	0.1061
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	17.8992	6.0658	6.0105	29.7878	8.71	0.0032
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	0	A/G	1	-19.2237	8.3515	-35.5923	-2.8551	5.30	0.0213
SWINEW*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	24.8570	0.7242	23.4773	26.3178		

Table 6-4

FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.9589	0.0772	3.8077	4.1102	2631.92	<.0001
SEX	F		1	-0.5689	0.0543	-0.6753	-0.4624	109.77	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0293	0.0016	-0.0323	-0.0262	346.49	<.0001
ht_mean			1	0.0393	0.0031	0.0332	0.0453	162.74	<.0001
wt_mean			1	-0.0016	0.0012	-0.0039	0.0007	1.80	0.1794
SMOKE	2		1	-0.1132	0.0453	-0.2020	-0.0244	6.24	0.0125
SMOKE	3		1	-0.0081	0.0447	-0.0958	0.0795	0.03	0.8556
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	0.0020	0.0793	-0.1535	0.1574	0.00	0.9802
swinewn	1		1	-0.1253	0.0813	-0.2847	0.0341	2.37	0.1235
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.2572	0.1429	-0.0229	0.5372	3.24	0.0719
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-0.2546	0.1548	-0.5580	0.0488	2.71	0.1000
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-0.1227	0.1581	-0.4326	0.1871	0.60	0.4376
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.4372	0.0127	0.4130	0.4628		

FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	5.0818	0.0933	4.8989	5.2648	2963.81	<.0001
SEX	F		1	-0.7436	0.0657	-0.8724	-0.6149	128.21	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0256	0.0019	-0.0294	-0.0219	182.00	<.0001
ht_mean			1	0.0548	0.0037	0.0475	0.0621	216.39	<.0001
wt_mean			1	-0.0009	0.0014	-0.0037	0.0018	0.44	0.5062
SMOKE	2		1	-0.0494	0.0548	-0.1568	0.0581	0.81	0.3677
SMOKE	3		1	0.0254	0.0541	-0.0806	0.1315	0.22	0.6385
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	-0.0825	0.0959	-0.2705	0.1055	0.74	0.3898
swinewn	1		1	-0.1775	0.0984	-0.3703	0.0153	3.26	0.0711
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.1566	0.1728	-0.1822	0.4954	0.82	0.3649
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-0.1501	0.1872	-0.5171	0.2169	0.64	0.4228
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-0.0429	0.1912	-0.4177	0.3319	0.05	0.8225
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.5288	0.0153	0.4996	0.5598		

FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	78.0146	1.0345	75.9871	80.0421	5687.54	<.0001
SEX	F	1	0.4509	0.7278	-0.9757	1.8774	0.38	0.5356
SEX	M	0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean		1	-0.2051	0.0211	-0.2464	-0.1638	94.79	<.0001
ht_mean		1	-0.0769	0.0413	-0.1578	0.0040	3.47	0.0625
wt_mean		1	-0.0091	0.0157	-0.0399	0.0217	0.33	0.5638

SMOKE	2		1	-1.8585	0.6075	-3.0493	-0.6678	9.36	0.0022
SMOKE	3		1	-0.5802	0.5997	-1.7555	0.5952	0.94	0.3333
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	1.3973	1.0631	-0.6863	3.4809	1.73	0.1887
swinewn	1		1	0.2725	1.0902	-1.8642	2.4092	0.06	0.8026
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	2.5526	1.9154	-1.2016	6.3068	1.78	0.1826
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-2.8365	2.0749	-6.9033	1.2303	1.87	0.1716
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-1.5683	2.1194	-5.7223	2.5856	0.55	0.4583
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	5.8808	0.1700	5.5368	8.2035		

$FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.5957	0.1867	3.2690	3.9225	465.19	<.0001
SEX	F		1	-0.4809	0.1173	-0.7208	-0.2610	17.52	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0457	0.0034	-0.0524	-0.0391	181.55	<.0001
ht_mean			1	0.0252	0.0067	0.0122	0.0383	14.40	0.0001
wt_mean			1	-0.0016	0.0025	-0.0066	0.0033	0.41	0.5199
SMOKE	2		1	-0.2321	0.0979	-0.4240	-0.0402	5.62	0.0178
SMOKE	3		1	-0.0345	0.0966	-0.2239	0.1550	0.13	0.7214
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	0.2120	0.1713	-0.1237	0.5478	1.53	0.2158
swinewn	1		1	0.0036	0.1757	-0.3408	0.3479	0.00	0.9838
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.6947	0.3087	0.0896	1.2997	5.06	0.0244
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-0.6732	0.3344	-1.3286	-0.0178	4.05	0.0441
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-0.3776	0.3416	-1.0470	0.2919	1.22	0.2690
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.9445	0.0274	0.8923	0.9998		

Predicted FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	98.6934	2.0806	94.6547	102.7322	2293.88	<.0001
SMOKE	2		1	-3.7204	1.2430	-6.1586	-1.2842	8.96	0.0028
SMOKE	3		1	-1.2437	1.2094	-3.6140	1.1266	1.06	0.3038
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	1.2121	2.1520	-3.0058	5.4299	0.32	0.5733
swinewn	1		1	-1.7891	2.2165	-6.1334	2.5552	0.65	0.4198
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	7.5476	3.9393	-0.1733	15.2684	3.67	0.0554
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-7.4227	4.2673	-15.7866	0.9411	3.03	0.0820
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-3.4741	4.3594	-12.0183	5.0701	0.64	0.4255
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	12.0721	0.3502	11.4048	12.7785		

Predicted FVC

Analysis Of Maximum Likelihood Parameter Estimates

				Standard	Wald 95%		Wald

Parameter			DF	Estimate	Error	Confidence Limits		Chi-Square	Pr > ChiSq
Intercept			1	104.4302	2.0229	100.4855	108.3950	2665.15	<.0001
SMOKE	2		1	-1.4903	1.2202	-3.8818	0.9012	1.49	0.2219
SMOKE	3		1	0.0237	1.1872	-2.3031	2.3506	0.00	0.9840
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	-0.5679	2.1126	-4.7084	3.5727	0.07	0.7881
swinewn	1		1	-2.5225	2.1759	-6.7871	1.7422	1.34	0.2463
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	5.0380	3.8670	-2.5413	12.6172	1.70	0.1926
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-4.5127	4.1891	-12.7232	3.6977	1.16	0.2814
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-1.8547	4.2794	-10.2422	8.5328	0.19	0.6647
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	11.8507	0.3438	11.1956	12.5441		

Predicted FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	81.7529	0.4116	80.9462	82.5596	39453.2	<.0001
SMOKE	2		1	-0.2111	0.2483	-0.6977	0.2755	0.72	0.3951
SMOKE	3		1	-0.8878	0.2416	-1.3611	-0.4142	13.50	0.0002
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	0.8845	0.4298	0.0420	1.7270	4.23	0.0398
swinewn	1		1	1.5264	0.4427	0.6586	2.3941	11.89	0.0006
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	0.8019	0.7868	-0.7402	2.3440	1.04	0.3081
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-0.5428	0.8523	-2.2134	1.1277	0.41	0.5242
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-0.3440	0.8707	-2.0506	1.3626	0.16	0.6928
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	2.4112	0.0700	2.2780	2.5523		

Predicted $FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	85.1247	4.2307	76.8326	93.4168	404.83	<.0001
SMOKE	2		1	-7.0381	2.5520	-12.0399	-2.0363	7.61	0.0058
SMOKE	3		1	-2.4584	2.4830	-7.3250	2.4082	0.98	0.3221
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	6.0365	4.4183	-2.6232	14.6963	1.87	0.1719
swinewn	1		1	1.7139	4.5508	-7.2055	10.6333	0.14	0.7065
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs4696480_t	0		1	16.5775	8.0878	0.7258	32.4293	4.20	0.0404
rs4696480_t	1		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	0	0	1	-16.8912	8.7613	-34.0831	0.2807	3.72	0.0539
swinewn*rs4696480_t	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	1	0	1	-9.2544	8.9503	-26.7966	8.2878	1.07	0.3011
swinewn*rs4696480_t	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	0	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs4696480_t	2	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	24.7854	0.7191	23.4153	26.2357		

Table 6-5

FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	4.0152	0.0696	3.8788	4.1515	3331.49	<.0001
SEX	F		1	-0.5687	0.0548	-0.6757	-0.4616	108.45	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0293	0.0016	-0.0324	-0.0261	339.82	<.0001
ht_mean			1	0.0393	0.0031	0.0333	0.0454	160.81	<.0001
wt_mean			1	-0.0017	0.0012	-0.0040	0.0006	2.10	0.1474
SMOKE	2		1	-0.1109	0.0458	-0.2006	-0.0212	5.87	0.0154
SMOKE	3		1	-0.0016	0.0447	-0.0891	0.0859	0.00	0.9713
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	-0.0488	0.0717	-0.1893	0.0916	0.46	0.4858
swinewn	1		1	-0.1683	0.0733	-0.3121	-0.0246	5.27	0.0217
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	0.3104	0.3178	-0.3124	0.9332	0.95	0.3287
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	-0.4276	0.3339	-1.0819	0.2268	1.64	0.2003
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	-0.0901	0.3375	-0.7516	0.5714	0.07	0.7895
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.4390	0.0128	0.4146	0.4648		

FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	5.1033	0.0842	4.9384	5.2683	3675.91	<.0001
SEX	F		1	-0.7453	0.0661	-0.8748	-0.6158	127.22	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0257	0.0019	-0.0295	-0.0219	179.19	<.0001
ht_mean			1	0.0546	0.0038	0.0472	0.0619	211.31	<.0001
wt_mean			1	-0.0010	0.0014	-0.0038	0.0018	0.54	0.4634
SMOKE	2		1	-0.0511	0.0554	-0.1596	0.0575	0.85	0.3563
SMOKE	3		1	0.0225	0.0540	-0.0834	0.1284	0.17	0.6771
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	-0.0917	0.0867	-0.2617	0.0782	1.12	0.2901
swinewn	1		1	-0.1791	0.0888	-0.3530	-0.0051	4.07	0.0436
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	0.5460	0.3845	-0.2076	1.2997	2.02	0.1556
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	-0.6735	0.4040	-1.4853	0.1183	2.78	0.0955
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	-0.4822	0.4084	-1.2626	0.3382	1.28	0.2577
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.5312	0.0155	0.5017	0.5624		

FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	78.7097	0.9310	76.8849	80.5345	7147.06	<.0001
SEX	F	1	0.4713	0.7309	-0.9611	1.9038	0.42	0.5190
SEX	M	0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean		1	-0.2037	0.0212	-0.2453	-0.1621	91.98	<.0001
ht_mean		1	-0.0714	0.0415	-0.1528	0.0099	2.96	0.0863

wt_mean			1	-0.0103	0.0158	-0.0413	0.0206	0.43	0.5125
SMOKE	2		1	-1.7835	0.6125	-2.9840	-0.5829	8.48	0.0036
SMOKE	3		1	-0.4235	0.5977	-1.5949	0.7480	0.50	0.4786
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	0.5792	0.9591	-1.3005	2.4589	0.36	0.5459
swinewn	1		1	-0.4865	0.9817	-2.4106	1.4375	0.25	0.6201
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	-1.0799	4.2531	-9.4158	7.2560	0.06	0.7996
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	0.7399	4.4684	-8.0181	9.4979	0.03	0.8685
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	4.7044	4.5169	-4.1485	13.5573	1.08	0.2976
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	5.8751	0.1712	5.5490	6.2204		

$FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.7744	0.1502	3.4800	4.0688	631.61	<.0001
SEX	F		1	-0.4858	0.1179	-0.7167	-0.2548	16.97	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0455	0.0034	-0.0523	-0.0388	176.69	<.0001
ht_mean			1	0.0261	0.0067	0.0129	0.0392	15.14	<.0001
wt_mean			1	-0.0019	0.0025	-0.0069	0.0030	0.58	0.4450
SMOKE	2		1	-0.2163	0.0988	-0.4099	-0.0226	4.79	0.0286
SMOKE	3		1	-0.0001	0.0964	-0.1890	0.1889	0.00	0.9994
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	0.0295	0.1547	-0.2738	0.3327	0.04	0.8490
swinewn	1		1	-0.1625	0.1584	-0.4729	0.1478	1.05	0.3047
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	0.0844	0.6861	-1.2603	1.4290	0.02	0.9021
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	-0.2131	0.7208	-1.6258	1.1996	0.09	0.7675
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	0.6689	0.7286	-0.7591	2.0970	0.84	0.3586
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.9477	0.0276	0.8951	1.0034		

Predicted FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	100.1311	1.8609	96.4838	103.7783	2896.35	<.0001
SMOKE	2		1	-3.6239	1.2551	-6.0838	-1.1640	8.34	0.0039
SMOKE	3		1	-1.0744	1.2081	-3.4384	1.2896	0.79	0.3730
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	-0.1146	1.9248	-3.8870	3.6579	0.00	0.9525
swinewn	1		1	-2.8143	1.9875	-6.7097	1.0811	2.01	0.1568
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	13.2017	8.7816	-3.9708	30.3742	2.27	0.1319
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743709	0	A/G	1	-15.3814	9.1978	-33.4087	2.6460	2.80	0.0945
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	-6.6008	9.3067	-24.8416	11.6400	0.50	0.4782
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	12.1263	0.3533	11.4532	12.8389		

Predicted FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	105.1499	1.8285	101.5660	108.7337	3306.83	<.0001
SMOKE	2		1	-1.4981	1.2333	-3.9153	0.9190	1.48	0.2244
SMOKE	3		1	0.0218	1.1852	-2.3011	2.3448	0.00	0.9853
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	-0.9922	1.8913	-4.6991	2.7147	0.28	0.5998
swinewn	1		1	-2.7232	1.9529	-6.5509	1.1045	1.94	0.1632
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	15.1061	8.6093	-1.7679	31.9801	3.08	0.0793
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	-17.4163	9.0379	-35.1303	0.2976	3.71	0.0540
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	-12.4864	9.1449	-30.4101	5.4374	1.86	0.1721
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	11.9155	0.3472	11.2541	12.6157		

Predicted FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	81.8941	0.3686	81.1718	82.6165	49354.5	<.0001
SMOKE	2		1	-0.2183	0.2486	-0.7056	0.2690	0.77	0.3800
SMOKE	3		1	-0.8710	0.2389	-1.3393	-0.4027	13.29	0.0003
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	0.8615	0.3813	0.1142	1.6088	5.11	0.0238
swinewn	1		1	1.4022	0.3937	0.6305	2.1739	12.68	0.0004
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	1.7871	1.7356	-1.6147	5.1888	1.06	0.3032
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	-2.8225	1.8220	-6.1936	0.9486	2.07	0.1501
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	-0.5812	1.8436	-4.1945	3.0322	0.10	0.7526
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	2.4021	0.0700	2.2688	2.5433		

Predicted $FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	89.1529	3.8128	81.6799	96.6259	546.74	<.0001
SMOKE	2		1	-6.6191	2.5716	-11.6583	-1.5799	6.63	0.0101
SMOKE	3		1	-1.7262	2.4713	-6.5699	3.1175	0.49	0.4849
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn	0		1	1.7307	3.9437	-5.9988	9.4602	0.19	0.6808
swinewn	1		1	-2.1221	4.0722	-10.1036	5.8593	0.27	0.6023
swinewn	2		0	0.0000	0.0000	0.0000	0.0000	.	.
rs5743708	A/G		1	7.8365	17.9520	-27.3489	43.0218	0.19	0.6625
rs5743708	G/G		0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	0	A/G	1	-9.1560	18.8457	-46.0929	27.7808	0.24	0.6271
swinewn*rs5743708	0	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	1	A/G	1	11.4947	19.0688	-25.8795	48.8690	0.36	0.5466
swinewn*rs5743708	1	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	A/G	0	0.0000	0.0000	0.0000	0.0000	.	.
swinewn*rs5743708	2	G/G	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	24.8459	0.7239	23.4688	26.3080		

Table 7-2:

FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter				DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept				1	4.0900	0.0796	3.9340	4.2460	2639.51	<.0001
SEX	F			1	-0.5889	0.0542	-0.6952	-0.4826	117.97	<.0001
SEX	M			0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean				1	-0.0287	0.0018	-0.0318	-0.0257	340.00	<.0001
ht_mean				1	0.0390	0.0031	0.0330	0.0450	160.28	<.0001
wt_mean				1	-0.0016	0.0012	-0.0039	0.0007	1.87	0.1719
SMOKE	2			1	-0.1238	0.0452	-0.2123	-0.0353	7.52	0.0061
SMOKE	3			1	0.0014	0.0445	-0.0859	0.0886	0.00	0.9756
SMOKE	4			0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0			1	-0.2678	0.1030	-0.4697	-0.0660	6.76	0.0093
SWINEW	1			0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0			1	-0.2076	0.0793	-0.3631	-0.0521	6.85	0.0089
nos3_786ct_gg	1			0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0		1	0.3773	0.1101	0.1615	0.5932	11.74	0.0006
SWINEW*nos3_786ct_gg	0	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1		0	0.0000	0.0000	0.0000	0.0000	.	.
Scale				1	0.4386	0.0127	0.4143	0.4642		

FVC:

Analysis Of Maximum Likelihood Parameter Estimates

Parameter				DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept				1	5.1806	0.0968	4.9909	5.3704	2863.77	<.0001
SEX	F			1	-0.7624	0.0659	-0.8916	-0.6332	133.70	<.0001
SEX	M			0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean				1	-0.0247	0.0019	-0.0284	-0.0210	170.11	<.0001
ht_mean				1	0.0551	0.0037	0.0477	0.0624	215.99	<.0001
wt_mean				1	-0.0011	0.0014	-0.0039	0.0017	0.58	0.4451
SMOKE	2			1	-0.0664	0.0549	-0.1740	0.0413	1.46	0.2269
SMOKE	3			1	0.0300	0.0541	-0.0760	0.1361	0.31	0.5790
SMOKE	4			0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0			1	-0.2907	0.1253	-0.5362	-0.0452	5.39	0.0203
SWINEW	1			0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0			1	-0.2229	0.0965	-0.4120	-0.0338	5.34	0.0209
nos3_786ct_gg	1			0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0		1	0.3636	0.1339	0.1012	0.6261	7.37	0.0066
SWINEW*nos3_786ct_gg	0	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1		0	0.0000	0.0000	0.0000	0.0000	.	.
Scale				1	0.5333	0.0155	0.5038	0.5645		

FEV_1/FVC:

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	79.1086	1.0629	77.0253	81.1920	5538.94	<.0001
SEX	F	1	0.3078	0.7240	-1.1114	1.7265	0.18	0.6709
SEX	M	0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean		1	-0.2083	0.0208	-0.2491	-0.1676	100.23	<.0001
ht_mean		1	-0.0855	0.0411	-0.1661	-0.0049	4.32	0.0377
wt_mean		1	-0.0070	0.0157	-0.0378	0.0238	0.20	0.6563
SMOKE	2	1	-1.8000	0.8030	-2.9818	-0.6182	8.91	0.0028
SMOKE	3	1	-0.5007	0.5942	-1.6653	0.6638	0.71	0.3994
SMOKE	4	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0	1	-1.5095	1.3752	-4.2049	1.1860	1.20	0.2724
SWINEW	1	0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0	1	-0.6386	1.0594	-2.7148	1.4377	0.36	0.5467
nos3_786ct_gg	1	0	0.0000	0.0000	0.0000	0.0000	.	.

SWINEW*nos3_786ct_gg	0	0	1	2.6577	1.4704	-0.2242	5.5397	3.27	0.0707
SWINEW*nos3_786ct_gg	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	5.8558	0.1699	5.5321	6.1984		

$FEF_{25\%-75\%}$:

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	3.8831	0.1722	3.5456	4.2207	508.31	<.0001
SEX	F		1	-0.5111	0.1173	-0.7410	-0.2811	18.98	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0459	0.0034	-0.0525	-0.0393	185.18	<.0001
ht_mean			1	0.0241	0.0067	0.0111	0.0372	13.12	0.0003
wt_mean			1	-0.0017	0.0025	-0.0067	0.0033	0.45	0.5047
SMOKE	2		1	-0.2353	0.0977	-0.4268	-0.0438	5.80	0.0160
SMOKE	3		1	-0.0071	0.0963	-0.1958	0.1816	0.01	0.9416
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.2815	0.2228	-0.7182	0.1553	1.60	0.2065
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0		1	-0.2085	0.1717	-0.5449	0.1279	1.48	0.2245
nos3_786ct_gg	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0	1	0.4508	0.2383	-0.0164	0.9176	3.58	0.0586
SWINEW*nos3_786ct_gg	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.9488	0.0275	0.8964	1.0043		

Predicted FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	103.5572	2.1006	99.4401	107.6743	2430.34	<.0001
SMOKE	2		1	-3.8199	1.2334	-6.2373	-1.4025	9.59	0.0020
SMOKE	3		1	-0.8486	1.1975	-3.1958	1.4985	0.50	0.4785
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-8.4105	2.8234	-13.9443	-2.8767	8.87	0.0029
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0		1	-5.8868	2.1790	-10.1577	-1.6160	7.30	0.0069
nos3_786ct_gg	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0	1	11.2741	3.0162	5.3625	17.1857	13.97	0.0002
SWINEW*nos3_786ct_gg	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	12.0490	0.3498	11.3829	12.7540		

Predicted FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	108.2219	2.0699	104.1650	112.2789	2733.57	<.0001
SMOKE	2		1	-1.6888	1.2154	-4.0709	0.6932	1.93	0.1647
SMOKE	3		1	0.3532	1.1800	-1.9596	2.6660	0.09	0.7647
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-7.3943	2.7821	-12.8472	-1.9414	7.06	0.0079
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0		1	-5.5947	2.1472	-9.8031	-1.3863	6.79	0.0092
nos3_786ct_gg	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0	1	9.1934	2.9721	3.3682	15.0185	9.57	0.0020

SWINEW*nos3_786ct_gg	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.	.
SWINEW*nos3_786ct_gg	1	0	0	0.0000	0.0000	0.0000	0.0000		.	.
SWINEW*nos3_786ct_gg	1	1	0	0.0000	0.0000	0.0000	0.0000		.	.
Scale			1	11.8728	0.3445	11.2165	12.5675			

Predicted FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	83.1397	0.4255	82.3057	83.9737	38178.3	<.0001
SMOKE	2		1	-0.0517	0.2498	-0.5414	0.4379	0.04	0.8360
SMOKE	3		1	-0.8217	0.2428	-1.2972	-0.3463	11.48	0.0007
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.8424	0.5719	-1.9633	0.2786	2.17	0.1408
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0		1	-0.1416	0.4414	-1.0067	0.7235	0.10	0.7483
nos3_786ct_gg	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0	1	0.5666	0.6110	-0.6308	1.7641	0.86	0.3537
SWINEW*nos3_786ct_gg	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	2.4406	0.0708	2.3057	2.5834		

Predicted $FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	92.9335	4.3323	84.4423	101.4247	460.15	<.0001
SMOKE	2		1	-6.9673	2.5438	-11.9529	-1.9816	7.50	0.0062
SMOKE	3		1	-1.9421	2.4698	-6.6828	2.9986	0.56	0.4558
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-8.1143	5.8231	-19.5273	3.2987	1.94	0.1635
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
nos3_786ct_gg	0		1	-4.8474	4.4941	-13.6556	3.9609	1.16	0.2808
nos3_786ct_gg	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	0	0	1	11.7641	6.2206	-0.4280	23.9561	3.58	0.0586
SWINEW*nos3_786ct_gg	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*nos3_786ct_gg	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	24.8499	0.7210	23.4763	26.3039		

Table 7-3:

FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	4.0499	0.0742	3.9045	4.1952	2981.04	<.0001
SEX	F		1	-0.5724	0.0549	-0.6801	-0.4647	108.56	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0288	0.0016	-0.0319	-0.0257	332.59	<.0001
ht_mean			1	0.0395	0.0031	0.0334	0.0456	160.13	<.0001
wt_mean			1	-0.0019	0.0012	-0.0043	0.0004	2.59	0.1075
SMOKE	2		1	-0.1259	0.0480	-0.2160	-0.0357	7.49	0.0062
SMOKE	3		1	-0.0168	0.0458	-0.1065	0.0729	0.13	0.7136
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.1242	0.1003	-0.3208	0.0725	1.53	0.2159
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_aa	0		1	-0.1698	0.0738	-0.3144	-0.0252	5.30	0.0214
rs1799983_aa	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	0	0	1	0.2163	0.1073	0.0061	0.4265	4.07	0.0437
SWINEW*rs1799983_aa	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.4425	0.0129	0.4179	0.4685		

FVC:

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	5.1790	0.0893	5.0040	5.3540	3364.57	<.0001
SEX	F		1	-0.7468	0.0661	-0.8764	-0.6172	127.52	<.0001
SEX	M		0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean			1	-0.0249	0.0019	-0.0287	-0.0212	172.40	<.0001
ht_mean			1	0.0557	0.0038	0.0484	0.0631	220.00	<.0001
wt_mean			1	-0.0017	0.0014	-0.0045	0.0011	1.44	0.2296
SMOKE	2		1	-0.0683	0.0554	-0.1768	0.0402	1.52	0.2173
SMOKE	3		1	0.0112	0.0551	-0.0968	0.1192	0.04	0.8394
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.2098	0.1208	-0.4465	0.0269	3.02	0.0823
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_aa	0		1	-0.2232	0.0888	-0.3973	-0.0492	6.32	0.0119
rs1799983_aa	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	0	0	1	0.2684	0.1291	0.0154	0.5215	4.32	0.0376
SWINEW*rs1799983_aa	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	0.5326	0.0155	0.5030	0.5639		

FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	78.7085	0.6151	77.5030	79.9140	16376.1	<.0001
SEX	F	1	0.6410	0.7249	-0.7797	2.0616	0.78	0.3766
SEX	M	0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean		1	-0.2048	0.0208	-0.2454	-0.1641	97.28	<.0001
ht_mean		1	-0.0806	0.0410	-0.1609	-0.0002	3.86	0.0495
wt_mean		1	-0.0023	0.0157	-0.0330	0.0284	0.02	0.8830
SMOKE	2	1	-1.8489	0.6049	-3.0345	-0.6633	9.34	0.0022
SMOKE	3	1	-0.6784	0.5948	-1.8441	0.4873	1.30	0.2540
SMOKE	4	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0	1	-0.0184	0.6644	-1.3206	1.2838	0.00	0.9779
SWINEW	1	0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_a	0	1	-0.8377	0.7212	-2.2512	0.5759	1.35	0.2454

rs1799983_a	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	0	0	1	2.0650	0.9852	0.1340	3.9959	4.39	0.0361
SWINEW*rs1799983_a	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	5.8137	0.1695	5.4907	6.1556		

$FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	3.7390	0.1003	3.5424	3.9357	1388.83	<.0001
SEX	F	1	-0.4734	0.1182	-0.7051	-0.2416	16.03	<.0001
SEX	M	0	0.0000	0.0000	0.0000	0.0000	.	.
age_mean		1	-0.0454	0.0034	-0.0520	-0.0387	179.48	<.0001
ht_mean		1	0.0250	0.0067	0.0119	0.0381	14.00	0.0002
wt_mean		1	-0.0011	0.0026	-0.0061	0.0039	0.17	0.6766
SMOKE	2	1	-0.2376	0.0987	-0.4310	-0.0442	5.80	0.0160
SMOKE	3	1	-0.0304	0.0970	-0.2205	0.1598	0.10	0.7542
SMOKE	4	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0	1	-0.0002	0.1064	-0.2126	0.2123	0.00	0.9988
SWINEW	1	0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_a	0	1	-0.1490	0.1176	-0.3796	0.0816	1.60	0.2054
rs1799983_a	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	0	0	0.2757	0.1607	-0.0393	0.5907	2.94	0.0863
SWINEW*rs1799983_a	0	1	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	1	0.0000	0.0000	0.0000	0.0000	.	.
Scale		1	0.9484	0.0277	0.8957	1.0041		

Predicted FEV_1

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	101.8486	1.9769	97.9739	105.7233	2654.11	<.0001
SMOKE	2	1	-3.9418	1.2619	-6.4151	-1.4685	9.76	0.0018
SMOKE	3	1	-1.3700	1.2366	-3.7937	1.0537	1.23	0.2679
SMOKE	4	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0	1	-3.3245	2.7229	-8.6614	2.0124	1.49	0.2221
SWINEW	1	0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_aa	0	1	-3.9317	2.0245	-7.8996	0.0362	3.77	0.0521
rs1799983_aa	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	0	0	5.5924	2.9437	-0.1771	11.3619	3.61	0.0575
SWINEW*rs1799983_aa	0	1	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	1	0.0000	0.0000	0.0000	0.0000	.	.
Scale		1	12.2097	0.3560	11.5314	12.9278		

Predicted FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter		DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept		1	107.2652	1.9335	103.4755	111.0548	3077.61	<.0001
SMOKE	2	1	-1.7402	1.2342	-4.1591	0.6788	1.99	0.1586
SMOKE	3	1	-0.1299	1.2094	-2.5004	2.2405	0.01	0.9144
SMOKE	4	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0	1	-4.0125	2.6631	-9.2321	1.2072	2.27	0.1319
SWINEW	1	0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_aa	0	1	-4.3910	1.9800	-8.2718	-0.5102	4.92	0.0266
rs1799983_aa	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	0	0	5.3280	2.8790	-0.3148	10.9708	3.42	0.0642
SWINEW*rs1799983_aa	0	1	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_aa	1	1	0.0000	0.0000	0.0000	0.0000	.	.

Scale		1	11.9415	0.3482	11.2782	12.6439

Predicted FEV_1/FVC

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	83.1505	0.2280	82.7036	83.5973	133020	<.0001
SMOKE	2		1	-0.1237	0.2539	-0.6213	0.3739	0.24	0.6261
SMOKE	3		1	-0.8818	0.2455	-1.3629	-0.4007	12.90	0.0003
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.3263	0.2703	-0.8561	0.2035	1.46	0.2274
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_a	0		1	-0.1595	0.3036	-0.7545	0.4356	0.28	0.5994
rs1799983_a	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	0	0	1	-0.1743	0.4125	-0.9828	0.6342	0.18	0.6727
SWINEW*rs1799983_a	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	2.4540	0.0716	2.3177	2.5983		

Predicted $FEF_{25\%-75\%}$

Analysis Of Maximum Likelihood Parameter Estimates

Parameter			DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept			1	90.1855	2.3093	85.6593	94.7116	1525.15	<.0001
SMOKE	2		1	-7.2455	2.5718	-12.2857	-2.2053	7.94	0.0048
SMOKE	3		1	-2.4100	2.4864	-7.2832	2.4632	0.94	0.3324
SMOKE	4		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW	0		1	-0.7132	2.7380	-6.0795	4.6531	0.07	0.7945
SWINEW	1		0	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983_a	0		1	-4.0874	3.0751	-10.1145	1.9396	1.77	0.1838
rs1799983_a	1		0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	0	0	1	7.3394	4.1793	-0.8500	15.5288	3.09	0.0790
SWINEW*rs1799983_a	0	1	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	0	0	0.0000	0.0000	0.0000	0.0000	.	.
SWINEW*rs1799983_a	1	1	0	0.0000	0.0000	0.0000	0.0000	.	.
Scale			1	24.8568	0.7248	23.4759	26.3188		

Table 7-5:

FEV_1

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		3.9288	0.1998	3.5372	4.3205	19.66	<.0001
age_n		-0.0353	0.0044	-0.0439	-0.0266	-7.98	<.0001
height_c		0.0239	0.0072	0.0098	0.0379	3.33	0.0009
weight_c		-0.0085	0.0025	-0.0133	-0.0036	-3.44	0.0006
smok	1	-0.0614	0.1040	-0.2653	0.1424	-0.59	0.5548
smok	2	0.0012	0.0646	-0.1254	0.1278	0.02	0.9853
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	0.1736	0.2067	-0.2314	0.5787	0.84	0.4009
NOS3_786CT	AG	0.0960	0.2095	-0.3145	0.5065	0.46	0.6467
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0302	0.0083	-0.0464	-0.0139	-3.64	0.0003
duration_*NOS3_786CT	AA	-0.0278	0.0098	-0.0471	-0.0085	-2.83	0.0047
duration_*NOS3_786CT	AG	-0.0236	0.0092	-0.0417	-0.0055	-2.56	0.0106
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		4.0123	0.1341	3.7494	4.2752	29.91	<.0001
age_n		-0.0327	0.0057	-0.0438	-0.0215	-5.73	<.0001
height_c		0.0216	0.0076	0.0066	0.0365	2.82	0.0048
weight_c		-0.0089	0.0021	-0.0131	-0.0047	-4.16	<.0001
smok	1	0.1546	0.0895	-0.0209	0.3300	1.73	0.0842
smok	2	0.0012	0.0907	-0.1766	0.1791	0.01	0.9892
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	0.2099	0.1649	-0.1133	0.5331	1.27	0.2030
NOS3_786CT	AG	0.2105	0.1515	-0.0865	0.5075	1.39	0.1647
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0524	0.0058	-0.0638	-0.0410	-9.03	<.0001
duration_*NOS3_786CT	AA	-0.0009	0.0086	-0.0177	0.0159	-0.11	0.9148
duration_*NOS3_786CT	AG	-0.0027	0.0079	-0.0181	0.0127	-0.34	0.7325
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Predicted FEV_1

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		95.8912	5.0242	86.0439	105.7385	19.09	<.0001
smok	1	-0.0448	2.6886	-5.3144	5.2248	-0.02	0.9867
smok	2	0.5400	1.6527	-2.6993	3.7793	0.33	0.7439
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	4.3913	5.2082	-5.8127	14.5954	0.84	0.3990
NOS3_786CT	AG	3.8656	5.1907	-6.3079	14.0392	0.74	0.4564
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		0.0311	0.4265	-0.8049	0.8671	0.07	0.9419

duration_*NOS3_786CT	AA	-0.8647	0.4502	-1.7471	0.0176	-1.92	0.0548
duration_*NOS3_786CT	AG	-0.8369	0.4457	-1.7105	0.0368	-1.88	0.0605
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		99.0592	3.3275	92.5374	105.5810	29.77	<.0001
smok	1	4.5910	2.1454	0.3861	8.7959	2.14	0.0324
smok	2	0.1849	2.3922	-4.5038	4.8735	0.08	0.9384
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	2.5206	3.9755	-5.2713	10.3125	0.63	0.5261
NOS3_786CT	AG	1.9320	3.4793	-4.8873	8.7513	0.56	0.5787
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.8158	0.1559	-1.1214	-0.5103	-5.23	<.0001
duration_*NOS3_786CT	AA	-0.0330	0.2328	-0.4892	0.4231	-0.14	0.8871
duration_*NOS3_786CT	AG	-0.0882	0.2131	-0.5059	0.3296	-0.41	0.6791
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		5.2737	0.1846	4.9118	5.6356	28.56	<.0001
age_n		-0.0389	0.0050	-0.0487	-0.0290	-7.74	<.0001
height_c		0.0310	0.0078	0.0157	0.0463	3.97	<.0001
weight_c		-0.0099	0.0031	-0.0159	-0.0039	-3.24	0.0012
smok	1	0.2047	0.0993	0.0101	0.3993	2.06	0.0392
smok	2	0.1401	0.0812	-0.0190	0.2993	1.73	0.0844
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	-0.1122	0.1948	-0.4941	0.2696	-0.58	0.5645
NOS3_786CT	AG	-0.1133	0.1939	-0.4933	0.2667	-0.58	0.5589
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0168	0.0118	-0.0400	0.0064	-1.42	0.1560
duration_*NOS3_786CT	AA	-0.0341	0.0134	-0.0603	-0.0079	-2.55	0.0108
duration_*NOS3_786CT	AG	-0.0247	0.0128	-0.0498	0.0004	-1.93	0.0540
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		5.1102	0.1289	4.8574	5.3629	39.63	<.0001
age_n		-0.0252	0.0066	-0.0381	-0.0123	-3.82	0.0001
height_c		0.0390	0.0105	0.0184	0.0596	3.71	0.0002
weight_c		-0.0128	0.0033	-0.0193	-0.0063	-3.86	0.0001
smok	1	0.1782	0.1099	-0.0372	0.3937	1.62	0.1050
smok	2	-0.0012	0.1173	-0.2311	0.2287	-0.01	0.9918
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	0.2689	0.1774	-0.0789	0.6166	1.52	0.1297
NOS3_786CT	AG	0.2636	0.1595	-0.0490	0.5761	1.65	0.0984
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0294	0.0060	-0.0412	-0.0177	-4.92	<.0001
duration_*NOS3_786CT	AA	-0.0135	0.0095	-0.0323	0.0052	-1.42	0.1560
duration_*NOS3_786CT	AG	-0.0134	0.0081	-0.0293	0.0024	-1.66	0.0972
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Predicted FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		103.3832	4.0264	95.4916	111.2748	25.68	<.0001
smok	1	5.3019	2.2235	0.9440	9.6598	2.38	0.0171
smok	2	3.6440	1.7418	0.2300	7.0580	2.09	0.0364
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	-1.6904	4.1323	-9.7896	6.4088	-0.41	0.6825
NOS3_786CT	AG	-0.0542	4.0451	-7.9824	7.8741	-0.01	0.9893
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		0.4083	0.5052	-0.5818	1.3984	0.81	0.4189
duration_*NOS3_786CT	AA	-0.9976	0.5251	-2.0268	0.0315	-1.90	0.0574
duration_*NOS3_786CT	AG	-0.8846	0.5226	-1.9088	0.1395	-1.69	0.0905
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		101.4824	2.9808	95.6401	107.3247	34.05	<.0001
smok	1	4.6545	2.1391	0.4620	8.8471	2.18	0.0296
smok	2	0.6317	2.3857	-4.0442	5.3077	0.26	0.7912
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	2.9135	3.6758	-4.2909	10.1179	0.79	0.4280
NOS3_786CT	AG	2.6227	3.1355	-3.5228	8.7681	0.84	0.4029
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.2623	0.1472	-0.5508	0.0263	-1.78	0.0748
duration_*NOS3_786CT	AA	-0.2974	0.2219	-0.7323	0.1376	-1.34	0.1803
duration_*NOS3_786CT	AG	-0.2987	0.1882	-0.6678	0.0702	-1.59	0.1125
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

FEV_1/FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		74.7040	2.1878	70.4160	78.9921	34.15	<.0001
age_n		-0.1293	0.0514	-0.2301	-0.0286	-2.52	0.0119
height_c		-0.1211	0.0625	-0.2436	0.0014	-1.94	0.0528
weight_c		-0.0217	0.0278	-0.0762	0.0329	-0.78	0.4364
smok	1	-4.0799	1.1924	-6.4170	-1.7427	-3.42	0.0006
smok	2	-2.2480	0.8354	-3.8853	-0.6107	-2.69	0.0071
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	4.8152	2.3530	0.3033	9.5270	2.09	0.0387
NOS3_786CT	AG	3.2255	2.3530	-1.3863	7.8373	1.37	0.1704
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.3864	0.1085	-0.5952	-0.1776	-3.63	0.0003
duration_*NOS3_786CT	AA	0.0362	0.1190	-0.1971	0.2695	0.30	0.7613
duration_*NOS3_786CT	AG	-0.0412	0.1202	-0.2767	0.1944	-0.34	0.7319
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		78.4455	1.5967	75.3161	81.5749	49.13	<.0001
age_n		-0.2484	0.0498	-0.3459	-0.1508	-4.99	<.0001
height_c		-0.2287	0.0841	-0.3934	-0.0639	-2.72	0.0065
weight_c		-0.0224	0.0344	-0.0898	0.0450	-0.65	0.5146
smok	1	-0.1345	1.0264	-2.1463	1.8772	-0.13	0.8957
smok	2	-0.5582	1.0588	-2.6335	1.5170	-0.53	0.5980
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	0.8904	1.7682	-2.5753	4.3561	0.50	0.6146
NOS3_786CT	AG	0.5827	1.6533	-2.6577	3.8231	0.35	0.7245
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.5420	0.1022	-0.7423	-0.3417	-5.30	<.0001
duration_*NOS3_786CT	AA	0.1942	0.1189	-0.0387	0.4272	1.63	0.1022
duration_*NOS3_786CT	AG	0.1113	0.1167	-0.1174	0.3400	0.95	0.3402
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Predicted FEV_1/FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		92.5749	2.6972	87.2884	97.8613	34.32	<.0001
smok	1	-4.9794	1.4015	-7.7263	-2.2326	-3.55	0.0004
smok	2	-2.6253	1.0292	-4.6425	-0.6081	-2.55	0.0107
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	6.0787	2.9236	0.3486	11.8087	2.08	0.0376
NOS3_786CT	AG	4.0015	2.9104	-1.7028	9.7059	1.37	0.1692
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.3001	0.1234	-0.5420	-0.0581	-2.43	0.0151
duration_*NOS3_786CT	AA	0.0538	0.1407	-0.2219	0.3295	0.38	0.7021
duration_*NOS3_786CT	AG	-0.0387	0.1416	-0.3163	0.2388	-0.27	0.7845
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		97.8747	1.8828	93.9846	101.3648	51.88	<.0001
smok	1	0.1020	1.2826	-2.4118	2.6158	0.08	0.9368
smok	2	-0.7337	1.3787	-3.4360	1.9685	-0.53	0.5946
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	-0.0716	2.1165	-4.2199	4.0768	-0.03	0.9730
NOS3_786CT	AG	-0.5156	1.8883	-4.2165	3.1853	-0.27	0.7848
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.5181	0.1257	-0.7645	-0.2718	-4.12	<.0001
duration_*NOS3_786CT	AA	0.2541	0.1532	-0.0462	0.5545	1.66	0.0972
duration_*NOS3_786CT	AG	0.1463	0.1467	-0.1412	0.4338	1.00	0.3185
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

$FEF_{25\%-75\%}$

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter	Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|

Intercept		3.4519	0.4044	2.6594	4.2444	8.54	<.0001
age_n		-0.0429	0.0092	-0.0610	-0.0248	-4.65	<.0001
height_c		0.0040	0.0137	-0.0228	0.0308	0.29	0.7699
weight_c		-0.0078	0.0060	-0.0195	0.0038	-1.32	0.1882
smok	1	-0.5602	0.2153	-0.9822	-0.1382	-2.60	0.0093
smok	2	-0.3590	0.1479	-0.6489	-0.0692	-2.43	0.0152
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	0.6861	0.4338	-0.1642	1.5363	1.58	0.1138
NOS3_786CT	AG	0.3454	0.4306	-0.4986	1.1893	0.80	0.4225
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0652	0.0195	-0.1034	-0.0270	-3.34	0.0008
duration_*NOS3_786CT	AA	-0.0395	0.0227	-0.0840	0.0049	-1.74	0.0815
duration_*NOS3_786CT	AG	-0.0238	0.0214	-0.0659	0.0182	-1.11	0.2663
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		3.9554	0.3907	3.1896	4.7212	10.12	<.0001
age_n		-0.0595	0.0108	-0.0806	-0.0384	-5.53	<.0001
height_c		-0.0073	0.0157	-0.0380	0.0235	-0.46	0.6425
weight_c		-0.0124	0.0051	-0.0224	-0.0023	-2.41	0.0158
smok	1	0.1771	0.2093	-0.2332	0.5873	0.85	0.3976
smok	2	-0.0277	0.1827	-0.3858	0.3303	-0.15	0.8793
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	0.0975	0.4227	-0.7310	0.9260	0.23	0.8176
NOS3_786CT	AG	0.0308	0.4111	-0.7750	0.8366	0.07	0.9403
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.1260	0.0179	-0.1610	-0.0909	-7.04	<.0001
duration_*NOS3_786CT	AA	0.0345	0.0224	-0.0094	0.0783	1.54	0.1234
duration_*NOS3_786CT	AG	0.0373	0.0208	-0.0034	0.0781	1.80	0.0722
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Predicted $FEF_{25\%-75\%}$

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		85.2037	9.8643	65.8700	104.5375	8.64	<.0001
smok	1	-12.2217	5.2191	-22.4509	-1.9925	-2.34	0.0192
smok	2	-7.8282	3.7472	-15.1725	-0.4838	-2.09	0.0367
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	16.1524	10.7450	-4.9074	37.2121	1.50	0.1328
NOS3_786CT	AG	8.3674	10.6236	-12.4545	29.1892	0.79	0.4309
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.7291	0.6707	-2.0437	0.5854	-1.09	0.2770
duration_*NOS3_786CT	AA	-0.9177	0.7492	-2.3861	0.5507	-1.22	0.2206
duration_*NOS3_786CT	AG	-0.6796	0.7187	-2.0881	0.7289	-0.95	0.3443
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		97.9056	8.7959	80.6659	115.1453	11.13	<.0001
smok	1	5.7601	5.0687	-4.1744	15.6946	1.14	0.2558
smok	2	-1.2556	4.9604	-10.9779	8.4666	-0.25	0.8002
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
NOS3_786CT	AA	-2.0470	9.5844	-20.8321	16.7380	-0.21	0.8309

NOS3_786CT	AG	-4.9002	8.9674	-22.4759	12.6756	-0.55	0.5848
NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-2.4471	0.4427	-3.3148	-1.5794	-5.53	<.0001
duration_*NOS3_786CT	AA	0.8375	0.5847	-0.3085	1.9835	1.43	0.1520
duration_*NOS3_786CT	AG	0.8272	0.5473	-0.2455	1.8999	1.51	0.1307
duration_*NOS3_786CT	GG	0.0000	0.0000	0.0000	0.0000	.	.

Table 7-6:

FEV_1

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		4.1103	0.0823	3.9490	4.2716	49.94	<.0001
age_n		-0.0354	0.0045	-0.0442	-0.0265	-7.82	<.0001
height_c		0.0237	0.0062	0.0075	0.0398	2.87	0.0041
weight_c		-0.0081	0.0025	-0.0130	-0.0032	-3.25	0.0012
smok	1	-0.0736	0.1067	-0.2827	0.1355	-0.69	0.4903
smok	2	-0.0247	0.0680	-0.1579	0.1084	-0.36	0.7157
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-0.2155	0.1358	-0.4816	0.0506	-1.59	0.1124
rs1799983	AC	-0.0892	0.1053	-0.2957	0.1173	-0.85	0.3970
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0498	0.0056	-0.0607	-0.0389	-8.94	<.0001
duration_s*rs1799983	AA	0.0027	0.0102	-0.0173	0.0227	0.26	0.7915
duration_s*rs1799983	AC	-0.0074	0.0075	-0.0222	0.0073	-0.99	0.3233
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		4.2567	0.0953	4.0699	4.4435	44.66	<.0001
age_n		-0.0323	0.0054	-0.0429	-0.0218	-6.03	<.0001
height_c		0.0246	0.0077	0.0095	0.0397	3.20	0.0014
weight_c		-0.0081	0.0022	-0.0123	-0.0039	-3.75	0.0002
smok	1	0.1186	0.0901	-0.0599	0.2931	1.30	0.1952
smok	2	-0.0277	0.0907	-0.2054	0.1500	-0.31	0.7599
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-0.3325	0.1674	-0.6605	-0.0045	-1.99	0.0469
rs1799983	AC	-0.1161	0.1181	-0.3476	0.1154	-0.98	0.3256
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0575	0.0055	-0.0682	-0.0468	-10.53	<.0001
duration_s*rs1799983	AA	0.0147	0.0101	-0.0051	0.0345	1.46	0.1454
duration_s*rs1799983	AC	0.0052	0.0073	-0.0090	0.0195	0.72	0.4701
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Predicted FEV_1

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		100.5065	1.9424	96.6994	104.3136	51.74	<.0001
smok	1	-0.5668	2.7029	-5.8643	4.7308	-0.21	0.8339
smok	2	-0.1425	1.7556	-3.5833	3.2984	-0.08	0.9353
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-4.7398	3.4680	-11.5370	2.0573	-1.37	0.1717
rs1799983	AC	-0.7083	2.4139	-5.4374	4.0248	-0.29	0.7698
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.5638	0.1677	-0.8925	-0.2351	-3.36	0.0008
duration_s*rs1799983	AA	-0.0480	0.2653	-0.5680	0.4720	-0.18	0.8564
duration_s*rs1799983	AC	-0.3573	0.2249	-0.7981	0.0835	-1.59	0.1121
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		103.0183	2.1722	98.7608	107.2757	47.43	<.0001
smok	1	3.9165	2.2027	-0.4007	8.2338	1.78	0.0754
smok	2	-0.3201	2.3808	-4.9864	4.3463	-0.13	0.8931
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-4.4191	4.1664	-12.5850	3.7469	-1.06	0.2889
rs1799983	AC	-3.8726	2.7214	-9.2064	1.4612	-1.42	0.1547
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.9699	0.1432	-1.2505	-0.6892	-6.77	<.0001
duration_s*rs1799983	AA	0.4335	0.2971	-0.1488	1.0157	1.46	0.1445
duration_s*rs1799983	AC	0.1705	0.1920	-0.2058	0.5467	0.89	0.3746
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		5.2728	0.1024	5.0722	5.4734	51.51	<.0001
age_n		-0.0386	0.0051	-0.0485	-0.0286	-7.56	<.0001
height_c		0.0291	0.0091	0.0112	0.0470	3.19	0.0014
weight_c		-0.0097	0.0031	-0.0157	-0.0037	-3.17	0.0015
smok	1	0.2148	0.1065	0.0061	0.4235	2.02	0.0437
smok	2	0.1238	0.0861	-0.0449	0.2927	1.44	0.1504
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-0.0870	0.1915	-0.4623	0.2884	-0.45	0.6498
rs1799983	AC	-0.1832	0.1235	-0.4252	0.0589	-1.48	0.1380
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0425	0.0070	-0.0561	-0.0288	-6.09	<.0001
duration_s*rs1799983	AA	0.0087	0.0143	-0.0193	0.0368	0.61	0.5416
duration_s*rs1799983	AC	-0.0026	0.0092	-0.0206	0.0154	-0.29	0.7750
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		5.4120	0.1275	5.1621	5.6619	42.45	<.0001
age_n		-0.0240	0.0061	-0.0360	-0.0119	-3.90	<.0001
height_c		0.0429	0.0102	0.0229	0.0630	4.19	<.0001
weight_c		-0.0123	0.0031	-0.0183	-0.0063	-4.00	<.0001
smok	1	0.1632	0.1099	-0.0521	0.3785	1.49	0.1373
smok	2	-0.0113	0.1180	-0.2425	0.2198	-0.10	0.9233
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-0.2689	0.1930	-0.6473	0.0894	-1.50	0.1344
rs1799983	AC	-0.1683	0.1514	-0.4652	0.1285	-1.11	0.2663
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0442	0.0055	-0.0550	-0.0335	-8.09	<.0001
duration_s*rs1799983	AA	0.0133	0.0114	-0.0090	0.0356	1.17	0.2436
duration_s*rs1799983	AC	0.0065	0.0078	-0.0088	0.0218	0.84	0.4033
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Predicted FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		103.8157	1.9111	100.0701	107.5613	54.32	<.0001
smok	1	5.4548	2.3083	0.9307	9.9790	2.36	0.0181
smok	2	3.2530	1.8848	-0.4407	6.9468	1.73	0.0843
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-1.5722	3.6084	-8.6448	5.5001	-0.44	0.6630
rs1799983	AC	-1.5954	2.2702	-6.0449	2.8541	-0.70	0.4822
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.3302	0.1879	-0.6985	0.0382	-1.76	0.0790
duration_s*rs1799983	AA	0.0738	0.2917	-0.4979	0.6456	0.25	0.8002
duration_s*rs1799983	AC	-0.2446	0.2411	-0.7172	0.2279	-1.01	0.3103
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		105.8445	2.2882	101.3597	110.3294	46.26	<.0001
smok	1	4.6239	2.1248	0.4595	8.7884	2.18	0.0295
smok	2	0.7005	2.3701	-3.9447	5.3458	0.30	0.7676
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-2.1593	4.4596	-10.8999	6.5813	-0.48	0.6282
rs1799983	AC	-4.3491	2.6812	-9.6041	0.9059	-1.62	0.1048
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.6038	0.1249	-0.8487	-0.3590	-4.83	<.0001
duration_s*rs1799983	AA	0.3622	0.2477	-0.1233	0.8476	1.46	0.1437
duration_s*rs1799983	AC	0.1766	0.1747	-0.1659	0.5190	1.01	0.3123
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

FEV_1/FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		78.0945	0.8853	76.3593	79.8298	88.21	<.0001
age_n		-0.1357	0.0537	-0.2410	-0.0304	-2.53	0.0116
height_c		-0.1012	0.0640	-0.2266	0.0241	-1.58	0.1135
weight_c		-0.0209	0.0311	-0.0817	0.0400	-0.67	0.5024
smok	1	-4.5408	1.1007	-6.6982	-2.3834	-4.13	<.0001
smok	2	-2.4348	0.8381	-4.0774	-0.7922	-2.91	0.0037
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-2.3347	1.8554	-5.9712	1.3018	-1.26	0.2083
rs1799983	AC	0.7049	1.0768	-1.4056	2.8154	0.65	0.5127
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.3270	0.0585	-0.4417	-0.2123	-5.59	<.0001
duration_s*rs1799983	AA	-0.1032	0.1267	-0.3515	0.1450	-0.82	0.4151
duration_s*rs1799983	AC	-0.1206	0.0833	-0.2840	0.0427	-1.45	0.1477
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter	Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept	79.3612	1.0239	77.3544	81.3681	77.51	<.0001
age_n	-0.2595	0.0485	-0.3547	-0.1644	-5.35	<.0001
height_c	-0.1921	0.0887	-0.3659	-0.0182	-2.17	0.0303

weight_c		-0.0170	0.0373	-0.0901	0.0560	-0.46	0.6481
smok	1	-0.6025	1.0404	-2.6416	1.4365	-0.58	0.5625
smok	2	-1.0148	1.0682	-3.1082	1.0789	-0.95	0.3422
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-2.9983	1.6487	-6.2296	0.2331	-1.82	0.0690
rs1799983	AC	-0.1520	1.1393	-2.3851	2.0810	-0.13	0.8938
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.4180	0.0838	-0.5427	-0.2933	-8.57	<.0001
duration_s*rs1799983	AA	0.0275	0.1159	-0.1997	0.2547	0.24	0.8125
duration_s*rs1799983	AC	-0.0185	0.0870	-0.1889	0.1520	-0.21	0.8318
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Predicted FEV_1/FVC

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		96.8122	1.0211	94.8110	98.8135	94.81	<.0001
smok	1	-5.5785	1.2935	-8.1137	-3.0433	-4.31	<.0001
smok	2	-2.8761	1.0406	-4.9156	-0.8365	-2.76	0.0057
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-2.7508	2.3105	-7.2793	1.7776	-1.19	0.2338
rs1799983	AC	0.7712	1.3095	-1.7954	3.3379	0.59	0.5559
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.2195	0.0692	-0.3552	-0.0838	-3.17	0.0015
duration_s*rs1799983	AA	-0.1129	0.1533	-0.4134	0.1875	-0.74	0.4614
duration_s*rs1799983	AC	-0.1447	0.0987	-0.3381	0.0486	-1.47	0.1424
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		97.7474	1.3024	95.1948	100.3001	75.05	<.0001
smok	1	-0.4074	1.3320	-3.0182	2.2033	-0.31	0.7597
smok	2	-1.2248	1.3856	-3.9406	1.4910	-0.88	0.3767
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-2.4377	1.5441	-5.4641	0.5888	-1.58	0.1144
rs1799983	AC	-0.1694	1.4441	-2.9998	2.6610	-0.12	0.9066
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.3418	0.0717	-0.4824	-0.2012	-4.76	<.0001
duration_s*rs1799983	AA	0.0458	0.1460	-0.2404	0.3320	0.31	0.7539
duration_s*rs1799983	AC	-0.0455	0.1114	-0.2639	0.1730	-0.41	0.6834
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

$FEF_{25\%-75\%}$

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		3.9886	0.1601	3.6548	4.2824	24.79	<.0001
age_n		-0.0437	0.0096	-0.0625	-0.0250	-4.58	<.0001
height_c		0.0045	0.0140	-0.0230	0.0320	0.32	0.7475
weight_c		-0.0072	0.0064	-0.0198	0.0054	-1.11	0.2650
smok	1	-0.6397	0.1912	-1.0145	-0.2649	-3.35	0.0008
smok	2	-0.4385	0.1489	-0.7303	-0.1467	-2.94	0.0032

smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-0.4868	0.2829	-1.0412	0.0676	-1.72	0.0853
rs1799983	AC	-0.0726	0.2084	-0.4811	0.3360	-0.35	0.7277
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0808	0.0108	-0.1019	-0.0596	-7.52	<.0001
duration_s*rs1799983	AA	-0.0144	0.0225	-0.0586	0.0297	-0.64	0.5212
duration_s*rs1799983	AC	-0.0187	0.0163	-0.0507	0.0133	-1.14	0.2527
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		4.0521	0.1799	3.6994	4.4047	22.52	<.0001
age_n		-0.0626	0.0105	-0.0832	-0.0420	-5.96	<.0001
height_c		-0.0067	0.0158	-0.0372	0.0239	-0.43	0.6680
weight_c		-0.0106	0.0054	-0.0212	0.0000	-1.96	0.0501
smok	1	0.0623	0.2030	-0.3356	0.4602	0.31	0.7589
smok	2	-0.1121	0.1805	-0.4659	0.2417	-0.62	0.5345
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-0.8295	0.3411	-1.4981	-0.1609	-2.43	0.0150
rs1799983	AC	0.0389	0.2169	-0.3863	0.4641	0.18	0.8577
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-0.0981	0.0121	-0.1219	-0.0743	-8.08	<.0001
duration_s*rs1799983	AA	0.0265	0.0204	-0.0135	0.0664	1.30	0.1939
duration_s*rs1799983	AC	-0.0008	0.0164	-0.0330	0.0314	-0.05	0.9828
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Predicted $FEF_{25\%-75\%}$

Swine workers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		97.1478	3.8833	89.5366	104.7590	25.02	<.0001
smok	1	-14.6032	4.6709	-23.7580	-5.4484	-3.13	0.0018
smok	2	-9.8865	3.7836	-17.3021	-2.4709	-2.61	0.0090
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-11.0429	7.1416	-25.0402	2.9544	-1.55	0.1220
rs1799983	AC	-0.7836	5.0864	-10.7528	9.1855	-0.15	0.8778
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-1.0559	0.2542	-1.5541	-0.5577	-4.15	<.0001
duration_s*rs1799983	AA	-0.5659	0.6298	-1.8004	0.6686	-0.90	0.3689
duration_s*rs1799983	AC	-0.6064	0.4275	-1.4443	0.2314	-1.42	0.1560
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.

Non-farming rural dwellers

Analysis Of GEE Parameter Estimates
Empirical Standard Error Estimates

Parameter		Estimate	Standard Error	95% Confidence Limits		Z	Pr > |Z|
Intercept		96.5839	4.5383	87.6890	105.4787	21.28	<.0001
smok	1	3.5142	5.1151	-6.5112	13.5396	0.69	0.4921
smok	2	-3.0089	4.9383	-12.6877	6.6699	-0.61	0.5423
smok	3	0.0000	0.0000	0.0000	0.0000	.	.
rs1799983	AA	-13.4480	6.9293	-27.0292	0.1331	-1.94	0.0523
rs1799983	AC	-1.1699	5.5780	-12.0927	9.7728	-0.21	0.8353
rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.
duration_sw_c		-1.8035	0.3117	-2.4144	-1.1926	-5.79	<.0001
duration_s*rs1799983	AA	0.7008	0.6132	-0.5010	1.9026	1.14	0.2531
duration_s*rs1799983	AC	0.0116	0.4386	-0.8479	0.8714	0.03	0.9786
duration_s*rs1799983	CC	0.0000	0.0000	0.0000	0.0000	.	.