ASTHMA DIAGNOSIS, PHENOTYPES AND SEVERITY, AND INDOOR MICROBIAL EXPOSURE AMONG URBAN AND RURAL CHILDREN IN SASKATCHEWAN, CANADA

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ABSTRACT

Background: Childhood asthma is less common in rural compared to urban settings. This could be linked to possible asthma under-diagnosis in rural children. Furthermore, asthma presents with multiple phenotypes and degrees of severity; and may have varied associations with indoor microbial exposures.

Objectives: i) to investigate if rural children experience more asthma under-diagnosis compared to urban children; ii) to investigate the relationship between endotoxin and beta-(1→3)-D-glucan (BDG) with atopic asthma and exercise-induced bronchospasm (EIB); and iii) to examine the associations between endotoxin and BDG with asthma severity.

Methods: In 2015, following a 2013 cross-sectional study, we approached those who gave consent for further testing and repeated the survey and completed clinical assessments. The 2015 study included 335 schoolchildren (aged 7–17 years) in Saskatchewan, Canada. Play and mattress area settled dust sample collection was also completed. Asthma was identified based on survey responses and then based on a validated asthma algorithm. Children with confirmed asthma using the asthma algorithm (n = 116) formed the study population for the second (asthma phenotypes) and third (asthma severity) objectives. We evaluated asthma phenotypes based on skin prick testing and exercise challenge testing and asthma severity based on standard guidelines. Endotoxin and BDG were measured from dust samples using *limulus* amoebocyte lysate assay.

Results: The study population was comprised of 73.4% (large urban, LU), 13.7% (small urban, SU) and 12.8% (rural, R). The proportions of participants with survey-based vs. algorithm-based asthma classification were: 28.5% vs. 33.3% (LU), 34.8% vs. 41.3% (SU), and 20.9% vs. 34.9% (R). Among the algorithm-based asthma cases, 71.1% were atopic, 22.4% had EIB, 75.9% had

mild asthma, and 24.1% had moderate/severe asthma. Play area endotoxin was inversely associated with atopic asthma while mattress endotoxin was positively associated with EIB. Furthermore, mattress endotoxin was positively associated with moderate/severe asthma and decreased lung function while play area BDG was inversely association with moderate/severe asthma.

Conclusion: The study revealed evidence of asthma under-diagnosis in rural children. Furthermore, the study provided evidence of varied associations between indoor microbial exposures and asthma phenotypes as well as asthma severity.

CO-AUTHORSHIP

This dissertation contains three separate manuscripts which were completed and written by Mr. Oluwafemi Oluwole in collaboration with his supervisor, Dr. Joshua A. Lawson from the Canadian Centre for Health and Safety in Agriculture (CCHSA) and Department of Medicine, College of Medicine, University of Saskatchewan, and dissertation advisory committee members: Drs. Donna C. Rennie (CCHSA and College of Nursing, University of Saskatchewan), Roland Dyck (CCHSA and Department of Medicine, College of Medicine, University of Saskatchewan), and Senthilselvan Ambikaipakan (Department of Public Health Sciences, University of Alberta). Other non-committee member co-authors include Drs. Darryl Adamko (Department of Pediatrics, College of Medicine, University of Saskatchewan), Shelley Kirychuk, George Katselis (CCHSA and Department of Medicine, College of Medicine, University of Saskatchewan), and Mrs. Anna Afanasieva (CCHSA, College of Medicine, University of Saskatchewan).

"*Asthma diagnosis among children along an urban-rural gradient*"

Mr. Oluwole conceptualized and designed the study, conducted data collection and management, interpreted the data, and prepared and revised the manuscript; Dr. Joshua A. Lawson, Mr. Oluwole's PhD supervisor, contributed to the study concept, design, data management and interpretation, reviewed and revised the manuscript; Dr. Donna C. Rennie contributed to the study concept, data collection, results interpretation, reviewed and revised the manuscript; Dr. Ambikaipakan Senthilselvan contributed to the study design, data analysis methods, results interpretation, reviewed and revised the manuscript; Dr. Roland Dyck contributed to study design, reviewed and revised the manuscript; Mrs. Anna Afanasieva contributed to data collection, results interpretation, and reviewed the manuscript; Dr. Darryl Adamko contributed to the study methodology, results interpretation, reviewed and revised the manuscript.

"*The association between endotoxin and beta-(1→3)-D-Glucan in house dust with asthma phenotypes among schoolchildren*"

Mr. Oluwole conceptualized and designed the study, conducted data collection and management, interpreted the data, and prepared and revised manuscript; Joshua A. Lawson contributed to the study concept, design, data management and interpretation, reviewed and revised the manuscript; Dr. Donna C. Rennie contributed to the study concept, data collection, results interpretation, reviewed and revised the manuscript; Dr. Ambikaipakan Senthilselvan contributed to the study design, data analysis methods, results interpretation, reviewed and revised the manuscript; Dr. Roland Dyck contributed to study design, reviewed and revised the manuscript; Mrs. Anna Afanasieva contributed to data collection, results interpretation, and reviewed the manuscript.

Drs. Shelley Kirychuk and George Katselis contributed to samples preparation, laboratory analysis of dust samples, results interpretation, reviewed and revised the manuscript.

"*The association between endotoxin and beta-(1→3)-D-Glucan in house dust with asthma severity among schoolchildren*"

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DEDICATION

This dissertation is dedicated to:

i) God for seeing me through the most rewarding experience of my career so far.

ii) Children with asthma and other respiratory-related diseases, especially those who live, play and work in rural settings. It is my hope that the findings of this study will lead to a better understanding of the burden of childhood asthma in rural settings and improved delivery of healthcare.

iii) All who are in search of knowledge.

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LIST OF ABBREVIATIONS

AHR:	Airway hyperresponsiveness
ALEX:	Allergy and Endotoxin Study
ALSPAC:	Avon Longitudinal Study Parents and Children
ANOVA:	Analysis of variance
ATS:	American Thoracic Society
BALF:	Bronchoalveolar lavage fluid
BDG:	Beta-(1→3)-D-Glucan
BHR:	Bronchial hyperresponsivesness
CAC:	Canadian Asthma Consensus
CCHSA:	Canadian Center for Health and Safety in Agriculture
CI:	Confidence interval
CTS:	Canadian Thoracic Society
DV-PEF:	Diurnal variability in peak expiratory flow
ECT:	Exercise challenge testing
ECRHS:	European Community Respiratory Health Survey
EIB:	Exercise-induced bronchospasm
ERS:	European Respiratory Society
ETS:	Environmental tobacco smoke
EU:	Endotoxin Units
FEV_1:	Forced Expiratory Volume in 1 second
FEF:	Forced expiratory flow
FVC:	Forced Vital Capacity
GEE:	Generalizing Estimating Equations
GINA:	The Global Initiative for Asthma
GLI:	Global Lung Function Initiative
GM:	Geometric Means
GSD:	Geometric Standard Deviation
HBSC:	Health Behavior in School-aged Children
IFN-γ:	Interferon-gamma
IL:	Interleukin

IOM:	Institute of Medicine
ISAAC:	International Study of Asthma and Allergies in Childhood
LAL:	Limulus *Amoebocytes* Lysates
LCA:	Latent cluster analyses
LFT:	Lung function testing
LPS:	Lipopolysaccharides
LRTI:	Lower respiratory tract illness
MAAS:	Manchester Asthma and Allergy Study
MCT:	Methacholine challenge test
NAEPP:	National Asthma Education and Prevention Program
NAIHL:	National Agricultural and Industrial Hygiene Laboratory
NHLBI:	National Heart, Lung, and Blood Institute
NLSCY:	National Longitudinal Survey of Children and Youth
OASIS:	Ontario Asthma Surveillance Information System
OH FA:	Hydroxyl fatty acid
OR:	Odds ratio
PEF:	Peak Expiratory Flow
PEFR:	Peak Expiratory Flow Rate
qPCR:	Quantitative Polymerase Chain Reaction
RH:	Relative Humidity
SAGE:	Study of Asthma, Genes and Environment
SD:	Standard Deviation
SPT:	Skin Prick Test
SRHS:	Saskatchewan Rural Health Study
TH cell:	T-helper type cell
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TRAIL:	Tracking Adolescents' Individual Lives Survey
WHO:	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 General introduction

Asthma is less common in rural compared to urban children,[1] prompting the conclusion that rural living may protect against the development of childhood asthma. While the protective effect of rural living for childhood asthma may be true, there is other evidence of increased frequency of asthma symptoms in rural compared to urban children[2–4] suggesting that the often reported lower prevalence of asthma in rural children may be due, in part, to asthma under-diagnosis in children living in rural settings.

The indoor environment is an important factor in the management and risk of childhood asthma. The relationships between indoor microbial exposures and childhood asthma have been shown to be inconsistent with protective as well as risk effects reported.[5] The reported opposing associations could be linked to the different presentations of asthma in children with the disease; as asthma is a multifactorial condition with multiple presenting phenotypes and differing degrees of severity.[6] To guide asthma management, characterization of asthma phenotype and severity is necessary and understanding its relationship with indoor microbial exposures could identify biological agents that aggravate asthma among children. Making use of this knowledge may eventually aid attempts to reduce asthma morbidity.

Despite the general understanding that there is geographic variation in asthma prevalence and severity, urban-rural asthma diagnostic patterns as well as the relationships between indoor

microbial exposures and asthma phenotypes and severity have not been well studied. This gap is the focus of my dissertation.

1.2 Background

Asthma is a chronic inflammatory disease of the airways characterized by airflow obstruction and symptoms such as wheeze, cough, shortness of breath, and chest tightness.[7] It is one of the most common chronic diseases among children,[8,9] and a leading cause of medical expenses.[10–12] More than 13% of children had been diagnosed with asthma in Canada[13,14] and the average direct cost from asthma exacerbations in 2013 was estimated to be around $883.48 per patient per year.[15] Knowledge of the etiology of asthma is currently less clear as asthma is a multifactorial disease with multiple presenting phenotypes and varied degree of severity.[16,17] While asthma can affect individuals of all ages, it is more common in children.[18]

It is generally believed that childhood asthma is less common in rural compared to urban settings.[1,19,20] In a nationwide prospective cohort study investigating asthma incidence among children in Canada, rural children had lower prevalence of asthma compared to their urban counterparts.[21] Similar urban-rural variations have also been reported in cross-sectional studies in Saskatchewan[2] and Manitoba,[22] Canada with childhood asthma (defined as doctor-diagnosed asthma) reported to be significantly higher in urban compared to rural children.

Some of these studies of urban-rural childhood asthma prevalence are of particular interest because in addition to demonstrating evidence of lower asthma prevalence in the rural areas,[19,20] they have also shown that children living in rural areas, and possibly farming environment, are less often atopic and have less allergic diseases than non-rural, non-farm children. This was demonstrated in a Canada-wide longitudinal study [the National Longitudinal Survey of Children and Youth (NLSCY)] involving 13,524 asthma-free children, aged 0–11

years old.[23] Participants were drawn from the original cycle conducted in 1994/1995 (Cycle 1) and followed for two years to the second cycle in 1996/1997 (Cycle 2). Children were categorized into three groups based on their locations; rural farming, rural non-farming, and non-rural children. Cumulative incidence of asthma (defined as physician-diagnosed asthma) for the two year follow-up period was 2.3% for rural farming, 5.3% for rural non-farming, and 5.7% for non-rural children. The Cycle 8 of the NLSCY study was completed in (2008/2009) among 10,941 of the participants who were followed up over a 14 year period. Similar to the results obtained in Cycle 2, the cumulative incidence of asthma was 10.2% rural farming, 13.1% for rural non-farming, and 16.5% for non-rural children;[21] further suggesting that farm and/or rural environments is protective of asthma.

There have been several potential explanations for the observed differences in asthma prevalence based on location of dwelling. One of these explanations has been that exposure to multiple environmental microbial agents protects against the development of asthma and atopy.[24,25] Recent renditions of the microbial exposure hypothesis suggest that inflammation associated with the development of allergic diseases such as asthma is often driven by an imbalance between T-helper type 1 [T_H1 (anti-inflammatory)] cells and T_H2 (pro-inflammatory) cells depending on the influence of environmental exposures and allergens on these cells.[25] That is, decreased microbial exposures early in life results in insufficient production of T_H1 cells, which in turn, results in persistent production of T_H2 cells. The decreased microbial exposure helps to skew the immune response away from T_H1 toward T_H2 or suppress the $T_H1$cytokine producing cells and thus, increases the tendency to develop asthma later in life.[25]

Prenatal exposures in rural and farm environments have further reinforced the protective effects of microbial exposures on allergic diseases through production of certain cytokines. Of

note is T_H1 cell-associated cytokines such as interleukin-12 (IL-12) and interferon-gamma (IFN-γ), which have been found to be significantly higher in cord blood cells of farm compared to non-farm infants whereas the T_H2 cell-associated cytokines such as IL-5, and IL-10 (which are allergic inflammatory cytokines) were unaffected.[25,26] This evidence demonstrates that stimulating T_H1 cells during pregnancy and in early childhood might suppress T_H2 immune responses and associated allergic diseases. Furthermore, the lower levels of T_H2 cytokine secretion that have been observed in children from farming families further supports the protective role of rural and farm exposures.[27]

While environmental exposures may explain some of the urban-rural asthma prevalence differences, there may be other factors that also help explain the differences. Compared to urban children, rural children may have reduced or limited access to healthcare services for asthma symptoms reporting, diagnosis, and management. These barriers to healthcare services may lead to failure to properly diagnose asthma and subsequently lead to lower asthma prevalence in the rural areas. For example, in a nationwide cross-sectional study in Canada, asthma prevalence was observed to be significantly lower in rural compared to urban-metro children whereas there was no statistically significant difference in asthma symptoms or hospitalization due to wheeze in the past 12 months across location of dwelling (urban-metro, non-metro-adjacent, and rural locations).[28] The evidence suggests that differences in diagnosing patterns could be another potential explanatory factor for the previously observed lower prevalence of asthma in rural children. Therefore, research focusing on specific rural conditions, such as consequences of lack of access, patient reporting differences, and differences in asthma diagnostic patterns, rather than simply rural residential status may further our understanding on the asthma-protective dogma associated with rural living.

Although there is no "gold standard" for assessing childhood asthma diagnosis, clinical symptom history in combination with airway obstruction as measured by pulmonary function testing remains the recommended standard protocol.[29,30] Due to accessibility difficulties, it is possible that rural and farm children with underlying symptoms of asthma may have limited access to pulmonary specialists leading to under-diagnosis of asthma and potential biases in the estimation of urban-rural asthma outcomes and differences in management strategies for asthma.[31] These barriers to accessing pulmonary specialists may particularly contribute to asthma diagnostic disparities suffered among asymptomatic rural children whose asthma conditions may only manifest in the presence of triggers.

Irrespective of location of dwelling, the indoor environment is considered an important factor in the management and risk of childhood asthma. Children are exposed to a complex variety of microbial agents in the indoor environment, mostly derived from fungal or bacterial origin.[32,33] However, an exposure that has received attention in recent years, partly because of its potential roles in the development or exacerbation of asthma, is endotoxin which is used as a surrogate for gram-negative bacterial exposure in house dust.[32,34] Mold derived components such as beta-(1→3)-D-glucan is another exposure that has received attention and is used as surrogate for indoor fungal exposure.[32–35] While indoor microbial exposures, particularly endotoxin, have been observed to reduce the risk of childhood asthma in both rural farming and rural non-farming children,[34] the evidence is inconsistent as some studies have reported increased risk[36,37] or no association.[38,39] This could be linked to different presentation characteristics of asthma in children with the disease. Thus, characterization of asthma phenotypes is important when investigating asthma in relation to microbial exposures. Also, while endotoxin[34,40,41] and beta-(1→3)-D-glucan[35,42,43] exposures may be thought to prevent asthma development, these

exposures may worsen asthma conditions and increase severity of the disease in children with asthma since endotoxin[44,45] and beta-(1→3)-D-glucan[46,47] are also pro-inflammatory in nature.

1.3 Purpose of the study

The purpose of this dissertation is to examine: 1) differences in asthma diagnostic patterns between rural and urban children and to see if rural children are likely to experience more asthma under-diagnosis compared to urban children; 2) the relationship between asthma phenotypes and endotoxin and beta-(1→3)-D-glucan levels in house dust; and 3) associations between asthma severity, as measured by recommended guidelines, and endotoxin and beta-(1→3)-D-glucan exposures in house dust.

1.4 Organization of the dissertation

A manuscript-style approach was used for this dissertation. The objectives were investigated through three separate manuscripts. Manuscript I: The aim of the study reported in Manuscript I was to identify if the previously reported lower prevalence of asthma found with rural children was related to asthma under-diagnosis in rural children. For Manuscripts II and III, only children identified as positive for asthma from the study conducted in Manuscript I were considered as the study population. This selection allows for a strong asthma definition for the study population. Manuscript II describes the findings regarding asthma phenotypes as assessed by atopic status and bronchial hyperresponsivesness (BHR) and their associations with indoor endotoxin and beta-(1→3)-D-glucan exposures. Manuscript III reports a similar approach used in Manuscript II assessing the role of domestic endotoxin and beta-(1→3)-D-glucan exposure levels for asthma phenotypes but in this study asthma severity is examined using categories

determined according to the National Asthma Education and Prevention Program (NAEPP) guidelines[17] as well as the relationship with lung function.

Chapter 2 details the relevant literature describing asthma in general, operational definitions, asthma phenotypes and severity, urban-rural asthma differences in asthma morbidity and reported associated risk factors. Chapter 3 describes the study populations and the research methodology in general. Chapters 4, 5, and 6 present Manuscript I, II, and III, respectively. Chapter 7 reports the conclusions from the dissertation based on the three manuscripts and brings the three manuscripts together for discussion. Finally, the recommendations resulting from the study and future research directions are presented in Chapter 8.

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CHAPTER 2

LITERATURE REVIEW

2.1 General scope of literature review

The overall aim of this study was to investigate urban-rural asthma diagnostic patterns and the relationship between indoor microbial exposures and asthma phenotypes and severity in children with asthma. This chapter describes the disease, asthma, its pathophysiology and natural history as well as the operational definitions of asthma that are commonly used in epidemiological studies. Asthma phenotypes, and severity as well as asthma prevalence (international and local) are also described. The urban versus rural asthma phenomenon was also reviewed to show geographical variation in asthma prevalence and morbidity. Literature providing explanations to the observed urban-rural difference in childhood asthma and the associated risk factors are presented. Finally, characteristics of studies that have investigated associations between microbial exposures and childhood asthma and asthma-related symptoms are also provided.

2.2 Methods

The literature review for this study was conducted using information from multiple sources including peer-reviewed journal articles, textbooks, review articles, consensus guidelines, conference attendance, and internet resources. Updated searches were completed in June and July 2017 and the literature review was updated as appropriate. Searches were completed using PubMed, Embase, Google Scholar, Science Direct, Web of Science and the University of Saskatchewan Library search engines to identify studies that evaluated rural and farming

environment and asthma. The search was broadened to include asthma severity, phenotypes and diagnostic patterns. Search terms included combinations of key words such as: “rural”, “urban”, “farming or agriculture” “endotoxin” “asthma”, “severity” “phenotypes”, “diagnostic patterns”, “lung function”, “FEV_1”, “FVC”, “asthma risk factors”, “burden of asthma”, “asthma care and management”, “access to care”, “environment”, “children”, among others as well as combinations of these. Bibliographies of all relevant articles were also screened to find other appropriate articles based on their appearance in the previously read scientific articles. Selected articles were mostly peer-reviewed articles but technical reports, executive summaries and proceedings were also considered if they contained important information. Selected articles were evaluated based on the following criteria: 1) studies written in the English Language, 2) studies that include data and information pertinent to any of the research objectives, 3) studies that were published after 1990. Most of the selected publications used a cohort or cross sectional study design, and originated from different countries.

2.3 Pathophysiology and pathogenesis of asthma, and its natural history

This section describes asthma, the processes that lead to asthma manifestations, its complications, and its natural history. The understanding of these processes is important in establishing the rationale for investigating specific risk factors and to help identify a suitable study population.

2.3.1 Pathophysiology and pathogenesis of asthma

Asthma is a multifactorial disease of the bronchial airway that typically presents with a high-pitched whistling sound (wheezing), which is heard during breathing in individuals suffering from the disease.[1] The word “asthma” comes from a Greek word meaning “panting” or

"gasping" and was first described by the Ancient Greek physician Hippocrates.[2] From the ancient times to the present day, asthma has puzzled and confused physicians with symptoms of asthma sharing similarities or overlapping with other respiratory and allergic reaction symptoms such as bronchiolitis and croup.[1] According to the Global Strategy for Asthma Management and Prevention, three main features define asthma: *chronic inflammation*, *bronchial hyperresponsiveness* (BHR), and *airway obstruction*.[3] These terms form the basis of the pathological, physiological and clinical features of asthma and defined asthma as a common chronic inflammatory disorder of the airways characterized by variable and recurring symptoms, airflow obstruction, and BHR.[3] The interaction of these three features of asthma determines the clinical manifestations of the disease.

The concept of asthma pathophysiology and pathogenesis has been described and continues to evolve since asthma is a complex, multifactorial disease with multiple presenting phenotypes.[4] However, irrespective of the phenotypic patterns of asthma, airway inflammation has remained the predominant feature underlying the pathophysiology of asthma.[5] The resultant effect of the inflammation on the airway structure and function leads to the development of asthma (Figure 2–1); which often manifests as symptoms of recurrent episodes of wheezing, shortness of breath, chest tightness, and coughing.

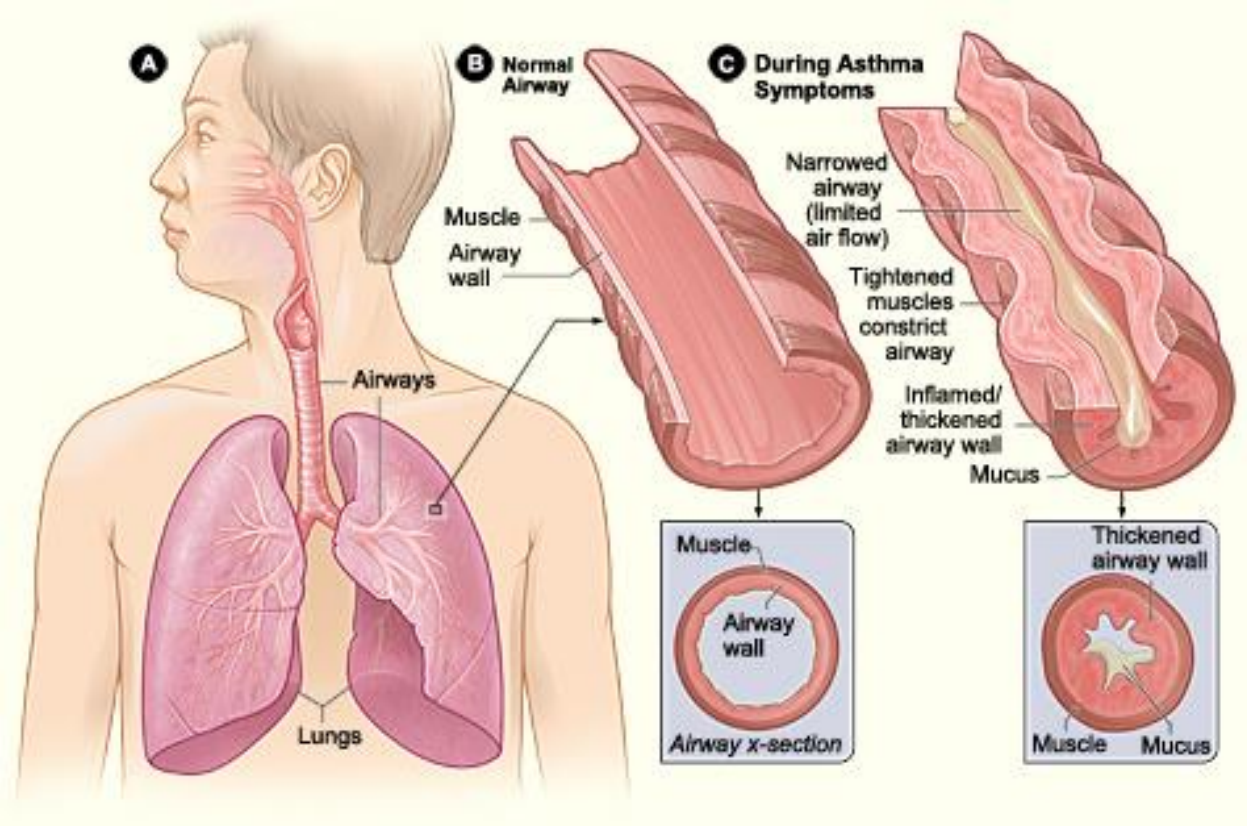

Figure 2–1: Pathophysiology of asthma [Used with permission (Appendix 1) from the National Heart, Lung, and Blood Institute; National Institutes of Health, U.S. Department of Health and Human Services. Link: https://www.nhlbi.nih.gov/health/health-topics/topics/asthma/].
(A) Location of the lungs and airways within the body; (B) Cross-section of airway of a person without asthma: the muscles around the airway are relaxed and open and there is no swelling inside the airway; (C) Cross-section of airway of a person with asthma: the inside of the airways is swollen, filled with mucus, and the muscles around the airways narrowed or tightened.

The airway inflammatory process in the pathophysiology of asthma is a complex multicellular process. In a susceptible individual, inhalation of allergens and/or other irritants initiate the release of mast cells, eosinophils, and T_H2 lymphocytes triggering a cascade of inflammation and systemic inflammatory responses such as acute bronchoconstriction.[6] The

airway responds to inhaled allergen through the T_H2 response pathway with the release of T_H2-associated cytokines or key mediators such as IL-4, IL-5, and IL-13 as well as the antibody IgE which are more specific to and regulate many aspects of allergic inflammation.[7]

The early inflammatory response to allergen is mast cell proliferation, promoted by IL-13, which induces rapid release of mediators such as histamine, leukotrienes, and prostaglandins.[8] These mediators are responsible for the contraction of smooth muscle cells and mucous secretion which result in severe airway obstruction in patients with asthma.[9] While the allergen activation through the T_H2-dependent pathway and IgE receptors is likely the most common occurrence in the pathophysiology of asthma, sensitized mast cells may also be activated by osmotic stimuli to induce bronchoconstriction which is usually seen in the case of exercise-induced bronchospasm (EIB)[10] The mast cell mediators are also responsible for the late phase cellular responses characterized by influx of inflammatory cells, eosinophils, and neutrophils which are associated with swelling of the bronchial wall and increased airway hyperresponsiveness (AHR).[11]

Maturation of eosinophils is stimulated by IL-5[12] with eosinophils identified as the major contributing cells implicated in allergic asthma as well as airway dysfunction.[13] These cells contain inflammatory mediators which induce airway epithelial cell damage, AHR, and airway remodeling that are constantly seen in patient with allergic asthma[14] with the exception of patients with severe asthma who may demonstrate a combination of eosinophilic and neutrophilic inflammation or, in some cases, neutrophilic inflammation alone.[14,15] As such, "eosinophilic asthma" is currently used to characterize a subclass of asthma phenotype with high influx of eosinophils in the bronchial airways.[16,17] The use of anti-IL-5 monoclonal antibody in

patients with asthma has demonstrated greater efficacy in reducing eosinophils in the airway[18] further confirming the role of eosinophils in the pathophysiology of asthma.

Contrary to eosinophils, the pathological role of neutrophils remains uncertain but neutrophils have been found to be the dominant inflammatory leucocyte in the airways and sputum of person with severe asthma[19] and have been found to be associated with severe airway obstruction in patients with asthma.[20]

In addition to inflammation and AHR, persistence of chronic inflammation through increased production of IL-13 may also induce epithelial damage, leading to airway remodeling in individuals with asthma.[5] Airway remodeling is believed to occur due to aberration in the process of injury-repair mechanism which leads to reconstruction of the epithelial wall of the airways.[21] The resultant effect of thickening of the basement membrane is another morphological hallmark of asthma and is found to be common in patients with atopic compared to non-atopic asthma.[22]

While the understanding of the pathophysiology of asthma continues to evolve, confidence in the fundamental role of T_H2 cytokines and pattern of inflammation exists. The T_H2 cytokines have been found to be significantly elevated in the bronchoalveolar lavage (BAL) of individuals with asthma leading to suggestion that asthma is a T_H2-cell-dependent, IgE-mediated allergic disease.[23]

2.3.2 Natural history of asthma and wheeze

The natural course of manifestation of symptoms of asthma over time, either by remission, relapse or increasing severity, is commonly referred to as the natural history of the disease.[24] From the available longitudinal studies, it appears that the manifestations of asthma and wheezy disorders have temporal patterns; varying considerably over time in the course of life. Studies

have shown that children who experienced asthma symptoms (e.g. wheeze) early in life may have different experience of the condition later in life:[4,25] In some cases the condition may either completely resolve (often known as remission), temporally resolve and recur again (known as relapse), or persist into adolescence and adulthood (known as persistent).[24] Each of these categories has differing risk factors, albeit with some degree of overlap between categories.

Based on the above life course patterns of asthma, longitudinal studies investigating the natural history of asthma and wheeze have identified several phenotypes depending on the onset of wheeze and asthma. One of these studies is the 1980 to 1984 population-based Tucson Children's respiratory birth cohort study from Arizona, USA.[4] Participants for this study were 826 children who had complete follow-up data at both three and six years of age from the original 1,246 newborns between 1980 and 1984. Depending on their history of wheezing, children were observed to fall into one of four clinically distinct wheezing categories (or three temporal patterns): never wheeze (51.5%); transient wheezing [(19.9%) defined as children who had ≥1 lower respiratory tract illness (LRTI) with wheezing during the first 3 years but no wheeze at 6 years of age]; late-onset wheezing [(15%) defined as children with no episodes of wheeze before the first 3 years of life but had wheeze at the age of 6 years]; and persistent wheezing [(13.7%) defined as children who had wheezing before the first 3 years of life and continued to wheeze at 6 years of age]. The study also observed that compared to never wheeze children, persistent wheezing children were more likely to have allergic sensitization, maternal smoking, and mothers with history of asthma during the first year of life, whereas transient wheezing children were more likely to have mothers who smoked but not mothers with history of asthma. In two other cohort studies, one from each of Canada[26] and the United Kingdom,[27] similar results were observed where majority of children were likely to wheeze early in life

(preschool age) and outgrow the conditions by school age, although those with persistent wheezing were more likely to develop asthma at school age.

While the majority of infants with wheeze are transient wheezers and may outgrow the conditions by school age, other evidence from the Tucson study suggests that after infancy, both transient wheezing and persistent wheezing may continue to experience a significant decrease in lung function into adolescence signaling negative respiratory outcome and predisposition to asthma later in life.[25] In this study nested within the Tucson study, children were further monitored from age 6 to 16 years.[25] The results showed that both late-onset and persistent wheezers were more likely to continue to wheeze from age 8 to 16 years compared with never wheezing [RRs = 3.12; 95%CI: 2.5–3.9 (late-onset wheezing), and 3.8; 95%CI: 3.1–4.7 (persistent wheezing)]. The diminished lung function which was originally observed in both transient early wheezing and persistent wheezing at age 6 years in the Martinez *et al* study[4] persisted at age 11 and 16 years with these groups of children experiencing significantly lower lung function compared with never wheezing.[25] Additionally, persistent wheezers continued to be more atopic at ages 11 and 16 years as earlier observed when they were at age 6 years.[4]

Cumulatively, these studies revealed a number of temporal patterns of asthma-related symptoms from preschool age to adulthood and suggest different pathogenesis for wheezing and asthma among children. The general consensus from the studies is that, although asthma may begin at any time in life, most asthma-related symptoms (e.g wheeze) are experienced in the first few years of life, mostly at infancy and may be associated with allergic sensitization while wheezing conditions after preschool age are more likely to be non-atopic.[4]

2.4 Asthma diagnosis

Accurate asthma diagnosis is the first step towards effective treatment and management of the disease. However, asthma presents with a variety of features with different phenotypic expressions. As such, establishing diagnosis in children may be difficult as there is currently no "gold standard". In addition, different guidelines suggest slightly different criteria that should be applied.

The Global Initiative for Asthma (GINA),[28] National Heart, Lung, and Blood Institute (NHLBI),[29] European Respiratory/American Thoracic Societies (ERS/ATS),[30] and the Canadian Thoracic Society (CTS)[31] guidelines specifically addressed the challenges of diagnosing asthma in children and described key features for assessing the possibility of asthma in this particular age group, most notably, the assessment of symptoms history. The features are not exclusive to asthma alone, but those that increase the probability of asthma. According to these guidelines, features include symptoms such as wheeze, shortness of breath, chest tightness and cough, particularly if these symptoms occur: 1) at night or early in the morning; 2) when exposed to cold air or common allergens; and 3) when engaged in vigorous exercise. Other features such as sensitization to common environmental allergens, and the presence of sputum eosinophils may also be used to assist in asthma diagnosis.[31] While the presence of a combination of these multiple key symptoms may increase the probability of asthma, objective lung function assessment as determined by spirometry is also recommended to improve diagnostic accuracy.[31] This section provides a background of methods commonly used to identify the presence of asthma for epidemiological studies.

2.4.1 Methods used to evaluate the presence of asthma in epidemiological studies

Two methods are generally used to aid diagnosis of asthma in epidemiological studies: questionnaire report of symptoms and assessment of lung function.

2.4.1.1 Questionnaire report of symptom history

A physician diagnosis of asthma in children should be based on a comprehensive and careful review of current and past clinical symptoms (such as wheeze or cough), frequency and duration of symptoms, timing of symptoms (day or night), family and personal history of atopy, as well as response to previous treatments. While family and personal history of allergic disease are strong risk factors for predicting asthma and should be taken into consideration in arriving at a diagnosis for asthma,[32] most diagnoses of asthma using symptoms is based on a history of recurrent wheeze and/or cough,[33] especially if these symptoms improved in children following the use of asthma medications.

In epidemiological studies, questionnaires incorporating asthma-related symptoms such as wheeze, cough, chest tightness or shortness of breath are the most frequently and widely used tools in studies investigating the prevalence, incidence, and severity of asthma.[34] The questionnaire developed for the International Study on Asthma and Allergies in Childhood (ISAAC) study team for the prevalence of respiratory symptoms and asthma in children ages 6–7 and 13–14 years has been the most widely and commonly used tool worldwide.[35] The ISAAC questionnaire is comprised of four "core" questions for assessing asthma and asthma symptoms (Ever wheeze: history of wheezing ever; Current wheeze: wheezing in the last 12 months; wheezing upon exertion/vigorous exercise, and dry cough at night), three questions on the severity of symptoms (number of wheezing episodes or attacks in a year, wheezing at night and

difficulty in completing sentences due to wheezing), and one question on the physician diagnosis of asthma.

Several validation studies have reported good agreement between questionnaire report of physician-diagnosed asthma and clinical assessment of asthma in children.[34,36–38] In a study among 2,845 children in Melbourne, Australia, the ISAAC questionnaire demonstrated high sensitivity (85%) and specificity (81%) when compared with physician diagnosis of asthma.[37] In Sweden, a study among 6,295 children (aged 1–6 years) validated three of the core ISAAC questions against clinically diagnosed asthma and found high validity (Ever asthma: sensitivity = 76.9%, specificity = 97.5%; Ever wheeze: sensitivity = 84.5%, specificity = 77.4%; Current wheeze: sensitivity = 86.3%, specificity = 84.1%).[39] Similarly, in Norway, the ISAAC questionnaire report of ever asthma had a sensitivity of 96% and specificity of 87% among 729 children (aged 7–14 years) when compared with physician assessment of asthma.[40] Also, in Finland, current wheeze in the past 12 months showed high agreement when validated against clinical assessment of current asthma (sensitivity = 78% and specificity = 97%) among 1,633 children (7–12 years).[34] In the same study, ever asthma had a sensitivity of 88% and specificity of 97%. A study conducted in Canada also found high sensitivity (83.6%) and specificity (93.6%) for parental report of childhood asthma when validated against diagnosis of asthma using health claim data.[36] Therefore, based on the good agreement and validity between questionnaire responses and clinical assessment of asthma across populations as noted above, questionnaire report of asthma and asthma symptoms remains a powerful tool for identifying those with asthma and assessing asthma prevalence in epidemiological studies, especially where cost and practical limitations of working with large populations are present.

2.4.1.2 Spirometry

Measurement of lung function is made possible with the use of spirometry conducted by blowing into a spirometer which measures how quickly full lungs can be emptied of inhaled air and the total volume of air exhaled in the process.[41] The obtained lung function variables such as forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, peak expiratory flow rate (PEFR), and forced expiratory flow between 25% and 75% of FVC ($FEF_{25\%–75\%}$) provide objective assessment of the degree of severity of airway obstruction and help to confirm the diagnosis of asthma.[28,29] The most important variables from the spirometry measurements are the FVC (the volume of air expired as forcefully as possible following full inspiration) and FEV_1 (volume of air expired in one second of an FVC manoeuvre) as they are more repeatable.[42] While FEV_1/FVC ratio and $FEF_{25\%–75\%}$ are also important variables, they are dependent on the validity of expiratory effort measured by the FEV_1 and FVC.[42]

Guidelines for performing and interpreting pulmonary function to aid asthma diagnosis have been published by the ATS, CTS, and ERS.[41–43] Spirometry results can be expressed as absolute values and as a percentage of predicted values.[42] The predicted values (also known as the reference values) are obtained from a comparable population of healthy and asymptomatic subjects matched for age, gender, height, and, on occasion, ethnicity. The FEV_1, expressed as a percent of predicted values, is used to grade the degree of severity of the abnormality in airflow obstruction (e.g. FEV_1 >80% = mild, 60% – 80% = moderate, and <60% = severe).[29] However, FEV_1 is generally an insensitive measure for asthma diagnosis as this has been shown to be normal in approximately 90% of children with asthma, regardless of level of severity, especially if the asthma condition is stable and well managed.[44–46]

Peak flow measurement assessed as peak expiratory flow (PEF) is also used to assist in the diagnosis of asthma. However, the sensitivity of PEF in assessing the presence of asthma in children is limited (sensitivity = 50% and specificity = 72%) compared to FEV_1 (sensitivity = 45% and specificity = 95%).[47] In addition, PEF requires serial assessment of lung function over a period of time (days or weeks) where a change in PEF value of >12% and >200 mL demonstrates variability in lung function and may be useful in establishing asthma diagnosis.[48]

Pulmonary function assessments are useful steps in asthma diagnosis but they are often effort dependent with some degree of insensitivity,[48] especially in individuals with stable asthma. In addition, while spirometry may be useful to assess symptomatic asthma, they may not be useful to assess subjects with intermittent or non-symptomatic asthma.[49] Further diagnostic tests are needed to establish a diagnosis of asthma in such individuals. One method to improve the diagnosis of asthma is to induce bronchoconstriction to access the degree of BHR.

2.4.1.3 Bronchial hyperresponsiveness (BHR) testing

In many asymptomatic children with relatively mild, controlled or stable asthma, FEV_1/FVC can be normal.[43] Such children are further screened through bronchial provocation testing to assess AHR. In most cases, a challenge test with inhaled methacholine [methacholine challenge test (MCT)] is used but an exercise challenge test (ECT) can also provide similar information. The response is assessed with spirometry. The spirometry variable mostly used for BHR testing is FEV_1 because it is repeatable and the exhalation time can be shortened to 2 seconds to assess BHR at other stages of the spirometry procedure compared to standard 6 seconds used at baseline.[43]

BHR tests such as ECT stimulates the release of histamine from mast cell and other inflammatory cytokines to induce inflammation, swelling of airway tissues, and subsequent narrowing of the airway.[50] The acute airway narrowing resulting from ECT with a fall of 10%–15% in baseline predicted FEV_1 values in response to vigorous exercise (ECT) is indicative of possible asthma[51] and is referred to as exercise-induced bronchospasm (EIB).[52]

Several epidemiological studies have validated the use of MCT[53,54] or ECT[55–57] as methods to identify children with asthma. All of these studies demonstrated moderate sensitivity and high specificity [MCT: sensitivity (49%–50%)[53,54] and specificity (84%–99%);[53,54] ECT: sensitivity (27%–57%)[55–57] and specificity (90%–95%)[55–57]]. In a study among 8–11 years children to demonstrate whether ECT is a suitable measure for BHR, Haby *et al*[55] showed ECT had low sensitivity (27%) but high specificity (95%) when validated against physician-diagnosed asthma. Another study in Australia among 393 children (aged 13–15 years) demonstrated sensitivity of 57% and specificity of 90% for ECT.[57] Similar validity results have been observed for MCT in New Zealand (sensitivity = 50% and specificity = 84%).[54]

These results suggest that BHR, especially ECT, has a limited sensitivity but is highly specific in establishing asthma diagnosis.

2.5 Assessment of asthma severity

Asthma severity can be measured using a combination of clinical symptoms, and lung function variables.[58] While spirometry, as a "standard method", may assist in diagnosing asthma, the use of spirometry and clinical symptoms, separately, to diagnose asthma has been reported to result in significant under-classification of asthma severity in children[59] and therefore, should be used together to assess severity. Based on this, an asthma severity classification has been created by the National Asthma Education and Prevention Program (NAEPP) which recommends that, in

children older than five years, the initial determination of asthma severity be based on a combination of current daytime and nighttime symptoms as well as on objective evaluation of lung function by spirometry or peak expiratory flow (FEV_1 or PEF).[58] This scheme classifies asthma into four levels at diagnosis: mild intermittent asthma, mild persistent asthma, moderate persistent asthma, and severe persistent asthma (Table 2–1).

Table 2–1: Criteria for classification of asthma severity according to the NAEPP guidelines[58]

Severity categories	Daytime symptoms	Nighttime symptoms	FEV_1 or PEF (% of Predicted Normal)
Mild intermittent asthma	≤2 days/week	≤2 nights/month	≥80
Mild persistent asthma	>2 days/week	3–4 night/month	≥80
Moderate persistent asthma	Daily	≥5 nights/month	>60 – 80
Severe persistent asthma	Continuously	Frequent	≤60

The effectiveness and the accuracy of the NAEPP guideline for classifying asthma severity have been assessed. In a cohort study from the USA: The National Cooperative Inner-City Asthma Study (Cohort 1) and the Inner-City Asthma (Cohort 2), Stout *et al* examined 640 children (aged 8–11 years) with asthma (Cohort 1: n = 257 children and Cohort 2: n = 383 children) to determine whether addition of lung function testing to clinical history contained in the NAEPP guidelines significantly changes asthma severity classification.[60] Results from the study showed that a combination of clinical symptoms and spirometry results could improve the accuracy of asthma severity classification. Specifically, when daytime or nighttime symptoms alone were used to classify children into severity categories, 47.9% and 38.6% of children were classified to have mild intermittent asthma while 33.5% and 42.6% were classified to have moderate or severe persistent asthma in Cohort 1 and Cohort 2 respectively. However, the

addition of spirometry variables (either FEV_1 or PEF) to clinical symptoms reclassified 22.8% and 27.7% of children originally classified as intermittent asthma into moderate or severe asthma in Cohort 1 and Cohort 2, respectively. Similarly, 31.2% and 33.3% of children with symptoms consistent with mild persistent asthma were reclassified as having moderate or severe asthma in Cohort 1 and Cohort 2, respectively.[60]

The above results demonstrated that symptoms history alone is likely to underestimate asthma severity and further confirmed the clinical application of the NAEPP guidelines in asthma severity assessments. However, one of the fundamental components of asthma guidelines has been the actual assessment of disease severity to guide treatment recommendations and management of asthma conditions.[61] As such, the NAEPP asthma severity guidelines were meant to be used to categorize asthma severity in patients not already receiving treatment or therapy.[62] However, this is not often the case, as the guidelines have also been used to assess severity in patients already on treatment.[63] For this reason, asthma severity guidelines were updated. According to the first updates of the GINA guidelines,[28] it is important to recognize that asthma severity not only involves frequency of symptoms and the underlying lung function impairments but also based on frequency of medication use and response to treatment. This is the additional definition requirement to the clinical features already proposed by the NAEPP guidelines for assessing asthma severity[58] and was subsequently endorsed by the ATS/ERS Task Force.[63] The medication use and response to treatment criteria have also been incorporated into the CTS guidelines[31] for asthma control.

Both the NAEPP and GINA guidelines have been validated against physician assessment of asthma severity with GINA showing better agreement compared to NAEPP guidelines.[62] This could be as a result of the inclusion of medication use in the GINA compared to NAEPP

guidelines. However, GINA guidelines also raised another concern in that it may be assessing asthma control as opposed to asthma severity because the criteria included medication use and response to treatment therapy. It is possible that physicians may label patients with severe asthma condition as less severe asthma, especially if their asthma conditions are well controlled under intensive medication regimen.[62] Furthermore, not all patients with asthma or asthma-related symptoms have access to effective medications and respiratory specialists, especially if they live in settings with limited access to healthcare services.[61] They may not have received a diagnosis of their asthma conditions or been prescribed appropriate medications for treatment. Therefore, to help disease management and allow for appropriate epidemiological assessments of asthma severity in a population, it is necessary that disease severity be determined in the absence of treatment therapy, especially if patients are currently untreated.[61] Based on these reasons, the NAEPP guidelines is currently being used as a simple tool in epidemiological studies to assess asthma severity and to identifying people at risk of severe exacerbation.[61,62] This will allow for initial asthma management plans which could be supplemented with step-by-step treatment procedures for effective asthma control.[61,64]

2.6 Asthma phenotypes

The NAEPP/GINA guidelines for asthma severity suggest that if an individual with asthma meets any one criterion for a specific severity category, the subject is assigned to that category despite potential disease heterogeneity within each category.[58] The major assumption with these schemes is that all subjects within a specific asthma severity category share similar disease characteristics. However, asthma is a heterogeneous disease with multiple phenotypes.[4,65] Patients with asthma differ with respect to factors that triggered attacks, the clinical presentation,[66,67] and patterns of inflammatory responses.[65]

The early classification of asthma phenotypes described two distinct phenotypes: allergic (atopic) and non-allergic (non-atopic) asthma, based on positive skin test to common allergens or the presence of specific IgE antibodies against common allergens.[68,69] Although allergic sensitization remains the basis of atopic asthma phenotype, not all children with asthma are atopic and most children with atopy do not develop asthma.[68] The prevalence of atopy among children with asthma appears to be mainly determined by the general prevalence of atopy in the population.[70] While atopic status remains the most commonly used parameter for classifying asthma phenotypes, the recognition of other phenotypes based on triggers (e.g. infection[71,72] and exercise,[65,73]), clinical or physiological expression (e.g severity-defined,[74,75] treatment resistant,[76,77] and age at onset[4,78]), and type of inflammation (e.g eosiniphilic and neutrophilic[23,78]) has demonstrated that the development and manifestation of the disease is beyond allergic sensitization alone. The recognition of these differences in asthma presentation has led to its description as a heterogeneous disorder.[79]

EIB is also currently being recognized as another asthma phenotype and has been found to be useful for identifying children at risk of asthma.[51] A large proportion of patients with asthma demonstrated BHR following ECT. This can also occur in individuals without a known asthma diagnosis.[80] For this reason, there are current debates on the use of ECT as a unique entity asthma phenotype.[80] However, the resulting AHR or bronchoconstriction induced by ECT is considered a marker of asthma or showed evidence that exercise may trigger asthma and should be regarded as a pathological process that leads to symptom expression and clinical evidence of asthma in children.[10,80,81]

Classifying asthma into unique phenotype categories is difficult because of overlaps between phenotype groups. However, epidemiological methods (statistical methods) and

symptom-based methods (clinical methods) have been used to classify the disease into unique clusters within a population of asthma patients.[82] Using cluster analyses which attempt to eliminate bias in categorizing asthma phenotypes by avoiding definition of the asthma conditions (i.e. placement of patients into asthma severity categories) before analysis, three large studies performed in Europe[83–85] and one in the United States[86] identified distinct phenotype clusters based on age of onset of asthma and duration, allergic status, sex, clinical symptoms, medication use, healthcare utilization, lung function, airway inflammation and other clinical characteristics that varied between the studies. Despite differences in study designs, variables that were analyzed, and studied populations from these studies, no asthma phenotype class achieves all the requirements for a distinct or discrete phenotype. There were clear overlaps in phenotypes identified.

2.7 Asthma prevalence

2.7.1 Global asthma prevalence

In 1998, after the completion of the ISAAC Phase I (1993–1997), global asthma prevalence for children was reported to be 11.2%.[87] From 2000–2003, Phase III of the project was repeated to assess changes over time. While there were some differences in results across centers (some with increased prevalence and some with decreased prevalence) , the time trend analysis showed that, overall, the percentage of children and adolescents reported to have asthma increased significantly with global asthma prevalence increasing from 11.2% to 13.5%; which indicates an annual increase of 0.28% overall.[87] Currently, the prevalence of asthma-related symptoms (particularly wheeze) has reached 20% or more in some developed parts of the world, including Canada.[88] Using combined data from the ISAAC Phase I and the European Community

Respiratory Health Survey (ECRHS) for children (6–7 years and13–14 years) Masoli *et al* estimated that prevalence of "clinical asthma" defined by current wheezing (self-reported wheezing in the past 12 months) increases globally by 50% every decade while BHR plus current wheezing is around 40%–60%.[89] Based on this figure, the study projected that in addition to the currently estimated 300 million people (both children and adults) who suffer from asthma worldwide; there may be an additional 100 million more cases in 2025 as countries become more urbanized.[89] This is evidenced from the observed decreases in international differences in asthma prevalence. In the ISAAC Phase I study, asthma was reported to be more common in high-income and industrialized countries but much lower in low-income and developing countries.[90] In Phase III, however, it became clearer that a high prevalence of asthma symptoms is not restricted to the high-income countries alone. The majority of countries that originally had low asthma prevalence in Phase I reported increases in asthma prevalence in Phase III.[87] This suggests that while the overall global prevalence of asthma continues to increase, the global prevalence disparities are at the same time decreasing, possibly reflecting greater awareness of asthma, improved diagnostic practices, and increased environmental exposure or a combination of these factors.

2.7.2 Childhood asthma prevalence in Canada

Over 3 million people (including children and adults) are already diagnosed with asthma in Canada.[91] Using data from the NLSCY study, changes in asthma prevalence among children aged 0–11 years were examined from 1994/1995 through 2000/2001.[92] Reports from the study showed that in the mid-1990s, 11% of Canadian children were diagnosed with asthma. However, over a period of five years (by 2000/2001), the prevalence had risen to more than 13.4%, an increase of nearly 70,000 cases over the five year period, at a rate of 14,000 cases per year.[92]

Also, among 2–7 year olds, the prevalence of asthma increased between 1994/1995 (11.5%) and 2000/2001 (13.2%) but later decreased between 2006/2007 (11.5%) and 2008/2009 (9.8%).[93] Using a broader age category (0–19 years), Statistics Canada reported that asthma prevalence among Canadian children and adolescents increased steadily from 2.3% to 12.2% between 1978 and 1996 and later stabilized in the late 1990s and early 2000s (15.5% in 1998, 15.6% in 2000, 16% in 2003).[94] While this report and another study[95] suggested that asthma prevalence stabilized in the later years of 1990s, the overall trends in asthma prevalence have been increasing in different provinces in Canada. For examples, in the province of Ontario, which has one third of Canada's population (nearly 13 million), a population-based cohort study suggested that age- and sex-standardized asthma prevalence increased from 8.5% to 13.3% between 1996 and 2005,[96] In British Columbia (BC) and Prince Edward Island (PEI), the proportions of doctor-diagnosed asthma also increased from 7.1% to 8.3% between 2002 and 2007 in BC,[97] and from 7.4% to 10.1% between 2002 and 2008 in PEI.[98] The recent overall asthma prevalence from the NLSCY over a 14-year period from Cycle 1 (1994/1995) to Cycle 8 (2008/2009) is 15.9% with highest prevalence (18%) in the Atlantic provinces (Nova Scotia, New Brunswick, and Prince Edward Island), followed by Quebec (17%), Prairie provinces (Alberta, Manitoba, and Saskatchewan; 15.6%), Ontario (15.2%), and British Columbia (14.9%).[99] This suggests increasing asthma prevalence among children in Canada.

2.7.3 Childhood asthma prevalence in Saskatchewan

The prevalence of asthma has also been shown to follow increasing trends over time in the province of Saskatchewan although there has been some indication of stabilization as well.[95] Using physician billing data from the Medical Claim Insurance Branch (MCIB) database, the prevalence of asthma among school-age children (5–14 years) in Saskatchewan was reported to

increase from 2.6% in 1981 to 4.4% in 1990.[100] This is consistent with the results of the national asthma trends as reported by the Statistics Canada where asthma prevalence among children <20 years in Canada also increased from 3.2% in 1984 to 11.5% in 1994.[94] Similarly, using the Saskatchewan Health databases, asthma prevalence among children (aged 5–14 years) in Saskatchewan also increased between 1991 and 1995 (increasing from approximately 5.3% to 6.2%, respectively). Thereafter, it either decreased or stabilized between 1996 and 1998 (6.1% in 1996, 6.0% in 1997, and 5.9% in 1998).[95] After the stabilization period, asthma prevalence among children in Saskatchewan has continued to rise. Among adolescents aged ≥12 years, the asthma prevalence increased from 7.7% in 1997[101] to 8.1% in 2003[102] in two separate reports from the Statistics Canada databases.

According to the Saskatchewan Ministry of Health reports, the overall asthma prevalence among children and adolescents in Saskatchewan has increased by three to four folds since 2002 to 2011.[103] Among children (age 5–9 years old) current asthma prevalence was reported to be 16% while among adolescents (10–14 years old) the prevalence is approximately 21%.[103] In addition, the report also showed that among children, asthma appeared to be more prevalent in males compared to females in both age groups (18.9% vs. 13% and 24.1% vs. 16.7%, respectively). Recent report from the NLSCY study, also shows that the overall cumulative incidence of asthma over a 14-year period from Cycle 1 (1994/1995) to Cycle 8 (2008/2009) in children (0–11 years) in the Prairies which comprised of Alberta, Manitoba and Saskatchewan is 15.6%.[99]

2.8 Place of residence and asthma prevalence and severity

The importance of "place" to health status has become increasingly evident as places where people live and work can have enormous impact on their health. This is also the case with childhood asthma.

2.8.1 The urban versus rural asthma phenomenon

The prevalence of asthma among children appears to differ depending on places of residence.[104] Data from the ISAAC Phases I and III studies suggest that the prevalence of asthma and asthma-related symptoms are higher among children and adolescents living in urban compared to children in rural settings.[105] Within Canada, regional variation in asthma and asthma-related symptoms have also been reported. In a nationwide cross-sectional survey of schoolchildren (aged 11–15 years) participating in the Health Behaviour in School-aged Children study, asthma prevalence was reported to be higher in urban metro areas compared to rural regions (17.6% vs. 14.8%) with adolescents from rural areas having a lower risk of current asthma compared with participants from large metro regions (OR = 0.76; 95%CI: 0.61–0.95).[106] Similarly, in two separate surveys among 3,564 children (7 year-old) in Manitoba, Canada; Korzyskyj and Becker also observed prevalence of both atopic asthma and asthma to be higher in children living in urban center compared to children living in southern and northern parts of rural Manitoba (atopic asthma: 9% in urban, 5% in southern rural, and 4% in northern rural;[107] asthma: 14% in urban, 10% in southern rural, and 8% in northern rural[108]).

Several other studies from different countries have also investigated urban-rural differences in childhood asthma. While results may varies and inconsistent across these studies with some showing higher asthma prevalence in rural areas, urban locations tend to have higher

proportion of children with asthma.[109] Characteristics of the various studies that have compared urban-rural differences in childhood asthma and asthma morbidity are presented in Table 2–2.

Table 2–2: Characteristics and results of studies investigating asthma prevalence and asthma-related symptoms in urban and rural populations among school-age children

First Author[Reference#] (Year published) Location(s)	Study design	Study population (Sample size)	Operational definition of asthma used in study	Urban vs. rural asthma findings	Other related findings or strength of the association
Lawson JA[110] (2017) Canada	Cross-sectional	5–14 years (3509)	1) Reported lifetime physician-diagnosed asthma 2) Current asthma: Ever asthma plus positive response to wheeze, asthma episodes, breathing medication, or healthcare utilization for asthma in the past 12 months	Prevalence of both ever asthma (15.1% vs. 20.7%) and current asthma (10.9% vs. 14.9%) were significantly lower in rural compared to urban children. The prevalence of ever wheeze (27.4% vs. 27.2%) and current wheeze (13.1% vs. 14.0%) were similar and not statistically different between rural and urban children	The risk of >3 wheezing episodes in the past 12 months was higher among rural children with asthma (aOR = 2.93; 95%CI: 1.26–6.86) compared to urban children despite lower prevalence in the rural children
Brozek G[111] (2016) Belarus Poland Ukraine	Multicenter cross-sectional	7–13 years (n = 12548)	1) Reported physician-diagnosed asthma 2) Current wheeze: Wheezing in the past 12 months	Asthma prevalence was lower in rural compared to urban children in the three countries involved: Belarus: (1.4% vs. 1.5%) Poland: (3.5% vs. 4.1%) Ukraine: (1.4% vs. 2.1%). Similar results were observed for current wheeze: Belarus: (10.7% vs. 10%) Poland: (4.8% vs. 5.2%) Ukraine: (11.5% vs. 13%). Results were not statistically significant within country	Further analysis of ratio of wheeze symptoms (current wheeze) to report of diagnosed asthma showed evidence of asthma under-diagnosis among rural children in all three countries [Rural vs. Urban: 10.9:1 vs. 8.1:1 (Belarus); 17.3:1 vs. 7.3:1 (Ukraine); and 2.4:1 vs. 1.9:1 (Poland)]

Zhu W[112] (2015) China	Cross-sectional	≤14 years (n = 20722)	1) Reported lifetime physician-diagnosed asthma 2) On-the-spot physician confirmation of asthma following positive responses to any or a combination of asthma-related symptoms	Prevalence of asthma was 1.3% for rural and 3.7% for urban. On-the-spot diagnosis of asthma was 48.7% for rural and 73.9% for urban. Also, 28.9% of physician confirmed asthma (28.9% of 48.7%) were rural children originally incorrectly diagnosed with bronchitis compared to 12.9% of urban children (12.9% of 73.9%).	The overall asthma prevalence based on screening questionnaire and on-the-spot physician examination was 2.83%. Due to the apparent asthma misdiagnosis in rural children, only 35.6% of rural children with confirmed asthma received prescription drug for asthma management compared to 56.5% of urban children
Vlaski E[113] (2014) Macedonia	Cross-sectional	12–16 years (n = 5507)	Reported lifetime physician-diagnosed asthma	Prevalence of asthma was lower in rural compared to urban children (1.2% vs. 1.9%; p=0.26). Prevalence of current wheeze was lower in rural compared to urban children (4.9% vs. 7.2%; p=0.03)	After adjusting for potential confounders, rural dwelling remained protective for current wheeze (OR = 0.74) and asthma (OR = 0.97) but not significant.
Lawson JA[114] (2014) Canada	Prospective cohort	12–18 years (n = 956)	Reported lifetime physician-diagnosed asthma	Over a 12-year follow-up period of 21,274,890 person-years, the incidence of asthma was lower among rural compared to urban adolescents (6.4 vs. 10.7 cases per 1000 person-years).	Overall incidence of asthma over the follow-up period was 10.2 cases per 1000 person-years and was higher in females compared to male adolescents (13.2 vs. 6.6 per 1000 person-years
Kausel L[115] (2013) Chile	Cross-sectional	13–14 years (n = 3363)	Reported current asthma symptoms (Had wheezing or	A significant dose-response effect was observed along an urban-rural gradient for current	Both rural and semiurban location were inversely associated with current

			whistling in the chest in the past 12 months)	asthma with lowest prevalence observed in rural (6%) compared to semiurban (10.1%) and urban (16%) children.	asthma (ORs = 0.4 and 0.6, respectively) but the association was only significant for rural location
Guner SN[116] (2011) Turkey	Cross-sectional	6–18 years (n = 607)	Reported lifetime physician-diagnosed asthma	No statistical difference in the prevalence of asthma was observed between urban and rural location of residence (10.5% vs. 7.1%; *p*=0.16)	Urban-rural asthma prevalence difference was also not significant among children with family history of atopy (31.4% vs. 25.7%; *p*=0.71)
Kolokotroni O[117] (2011) Cyprus	Cross-sectional at two point intervals (1999–2000 and 2007–2008)	7–8 years (n = 4944 for 1999–2000 survey and n = 2216 for 2007–2008 survey)	Reported lifetime asthma (Has your child ever had asthma?) Current wheeze (wheezing in the past 12 months)	This study assessed temporal changes in the prevalence of asthma in urban and rural areas between two intervals. In the first interval (2000), prevalence of both asthma and current wheeze were significantly lower in rural compared to urban areas (asthma: 9.7% vs. 11.9%; current wheeze: 5.4% vs. 7.5%, respectively). This was reversed in the second interval (2008) with the prevalence of both asthma and current wheeze higher in rural compared to urban areas (asthma: 18.4% vs. 17.1%; current wheeze: 9.7% vs. 8.4%, respectively).	Between 2000 and 2008, the prevalence of current wheeze was almost significantly doubled in rural areas (5.4% vs. 9.7% with OR = 1.81). No apparent significant change was observed for urban areas (7.5% vs. 8.4% with OR = 1.08); suggesting that recent increases in the prevalence of asthma and asthma-related symptoms may appear to be more pronounced in rural Cyprus children
Lawson JA[106] (2011) Canada	Cross-sectional	11–15 years (n = 4726)	Reported lifetime physician-diagnosed asthma (Has a doctor	A significant dose response of lessening risk of asthma across an urban-rural gradient.	Lower risk of asthma was associated with rural locations (OR = 0.81). However, prevalence of

			ever said you have asthma?)	Asthma prevalence was lowest in rural regions (14.8%), followed by non-metro adjacent (15.6%) and metro areas (17.7%)	current wheeze was similar and non-significant across the three locations.
Valet RS[118] (2011) USA	Prospective cohort	Children followed up from birth until the age of 5.5 years (n = 117080)	Validated algorithm that required an ICD-9 asthma diagnosis code 493	This study recruited children across an urban-rural gradient: urban, semiurban, and rural locations. Using the ICD-9 asthma code, the prevalence of asthma was 13% in rural, 12% in semiurban, and 11% in urban children from the ages of 4–5.5 years (p<0.001). Overall asthma prevalence was 11.8%	Rural and semiurban children had greater number (mean) of outpatient visits for any reason (15.7 and 14.6; respectively) compared to urban children (11.0). Urban compared to rural children with asthma had greater use of prescribed asthma medications (2.0 vs. 1.0)
Pesek RD[119] (2010) USA	Cross-sectional	4–17 years (n = 6376)	Physician provider diagnosis of asthma based on validated asthma algorithm	No apparent difference in provider-diagnosed asthma between urban and rural children (20% vs. 19%) but rural compared to urban children were more likely to be identified as "at-risk" (having asthma-related symptoms without provider-diagnosed asthma) for asthma (27.8% vs. 24.6%).	Among children identified as "at-risk-for-asthma", rural compared to urban children were also more likely to be classified as having moderate to severe persistent asthma (45.9% vs. 34.5%)
Ma Y[120] (2009) China	Cross-sectional	13–14 years (n = 7077)	Reported lifetime physician-diagnosed asthma	Prevalence of asthma was significantly lower in rural compared to urban area (1.1% vs. 6.3%).	Prevalence of current wheeze was also significantly lower in rural compared to urban area (1.0% vs. 7.2%). The strength of the associations

					were very strong both for asthma (OR = 6.1) and current wheeze (OR = 7.5)
Solé D[121] (2007) Brazil	Cross-sectional	13–14 years old from two cities: Caruaru (n = 3026) and Santa Maria (n = 6123)	Wheeze symptom in the last 12 months	Prevalence of asthma was significantly lower in rural adolescents from Caruaru compared to their urban counterparts (12.5% vs. 18.6%). No urban-rural differences were observed in Santa Maria (16.7% vs. 15.3%)	Rural living was significantly associated with asthma among Caruaru adolescents after adjusting for potential confounders (OR = 1.60)
Kozyrskyj A[107] (2006) Canada	Cross-sectional	0–7-years (n = 3564)	Atopic asthma	Children from both northern and southern rural areas compared to urban children had significantly lower prevalence of atopic asthma (4% and 5%, respectively vs. 9%)	Children with family history of allergy (atopic) were more likely to develop atopic asthma (OR = 1.87)
El-Sharif N[122] (2002) Palestine	Cross-sectional	6–12 years (n = 3623)	Reported lifetime physician-diagnosed asthma	Asthma prevalence was significantly higher in children from urban refugee camps compared to children from rural villages (15.6% vs. 8.1%). Similar results were observed for urban-rural prevalence of wheezing in past 12 months (12.6% vs. 8.2%; respectively).	Prevalence odds ratio (POR) for urban vs. rural asthma was 1.48. Severity of wheezing attacks (≥12 attack of wheezing in the past 12 months) was significantly higher in urban compared to rural children (POR = 2.67).

While many of the studies in Table 2–2 showed lower prevalence of asthma in rural compared to urban children, symptoms suggestive of asthma were often higher in rural compared to urban children. This can be seen from three specific studies.

The cross-sectional study from Saskatchewan, Canada, showed that rural children had significantly reduced risk of current asthma compared to their urban counterparts (OR = 0.58; 95%CI: 0.42–0.99).[110] However, the prevalence of ever wheeze (27.4% vs. 27.2%) and current wheeze (13.1% vs. 14.0%) were similar and not statistically different between rural and urban children.[110] Also, among those with asthma, 24.8% of rural compared to 12.3% of urban had severe asthma symptoms (>3 episodes of wheeze in the past 12 months).[110]

A second study by Valet *et al* from Tennessee, USA[118] further showed evidence of asthma under-treatment in rural compared to urban children with urban children having greater proportion of one or more prescription fillings for asthma medication (35% vs. 31%; $p<0.001$) despite rural children having greater asthma morbidity.

A third study by Pesek *et al* from Arkansas, USA[119] also showed that asthma morbidity (measured by frequency of asthma symptoms, and medication use) was significantly higher in the rural compared to urban children, even though the use of healthcare services appeared to be similar between the two groups (19% vs. 20%). Furthermore, a higher proportion of children in the rural group were classified as being "at-risk-for-asthma" compared to urban children (27.8% vs. 24.6%). "At-risk-for-asthma" children in this study was defined as children who had symptoms and frequency of medication use consistent with asthma diagnosis but had never been diagnosed of their asthma conditions either by a physician or other healthcare professional workers.

Findings from these studies not only suggest higher asthma-related symptoms and morbidity in rural children but also show evidence that rural children may be under-diagnosed for asthma, thus contributing to the lower asthma prevalence estimates observed in the rural settings.

Multiple factors may be used to explain the urban-rural asthma patterns described in Table 2–2. This may include environmental factors (particularly farm exposure in the rural areas), lower or lack of hospital report of symptoms consistent with asthma in rural children, limited access to healthcare facilities among others.

2.9 Farm environment exposure as potential explanation for urban-rural asthma phenomenon

One distinct factor between rural and urban areas which has been observed to have an association with asthma is exposure to a farming environment among the majority of rural populations. Exposure to farming environment may protect against allergic diseases in childhood such that children who grow up on farms are often less atopic, have less allergic disease, and often have less asthma compared to non-farm children.[123] Although, the specific factors of the farming environments that may be responsible for the protection of allergic diseases among farm children are still not clear, it appears that high microbial exposure either through contact with farm animals (through animal feeding and cleaning of animal pens)[124,125] or consumption of unpasteurized farm milk[125,126] are possible explanations (Figure 2–1).

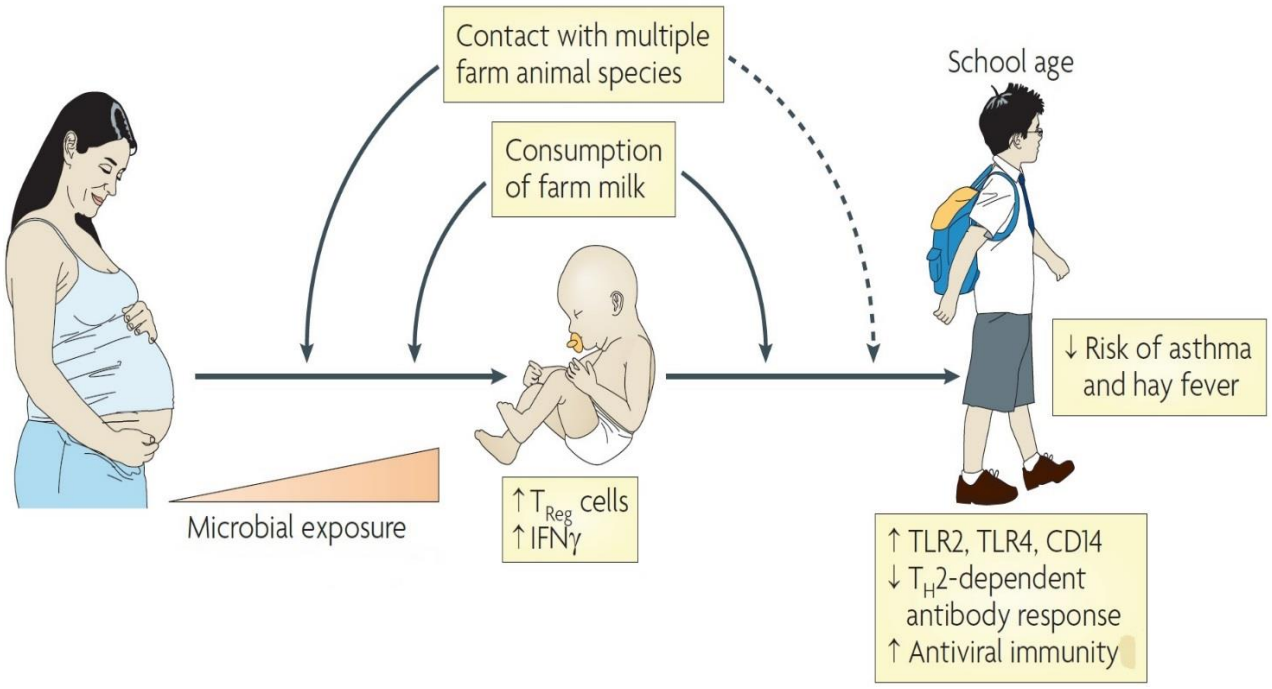

Figure 2–1: Farm exposures and the development of childhood allergic diseases[127] (used with permission from Nature Publishing Group. Permission License: Appendix 2).

The mechanisms of the protective effects of farming exposure and childhood asthma are still not well defined, but are likely to be associated with the developing immune system and exposures around the time of birth.[127,128] As depicted in Figure 2–1, pregnancy and early life stages appear to represent the most important biological window of opportunity for shaping the immune responses in farm-exposed children. Specifically, contact with farm animals and/or consumption of unpasteurized farm milk results in increased microbial exposure of women engaging in farming activities during pregnancy. This programs the exposed child's immune responses at birth by enhancing regulatory cell (Treg) and interferon (IFN)-γ to induce polarization of T_H dependent cells. Further exposure of child to animals and/or consumption of unpasteurized farm milk in early childhood activates the innate immune pathways through expression of Toll-like receptors [specific for microbial products (TLRs)] and CD-14 that

upregulates and promotes T_H1 cell-dependent cells has the potential to suppress the development of T_H2 dependent allergic diseases including asthma at school age.[129]

2.10 Beyond urban-rural environmental exposure differences: urban-rural asthma diagnostic patterns

Environmental exposures have mostly been implicated for the urban-rural asthma prevalence differences.[123] While childhood asthma may be disproportionately common in urban compared to rural children,[109,130] asthma may be under-diagnosed in rural children with recent studies showing similar or increased asthma-related symptoms in rural compared to urban children.[110,118,119] Furthermore, many children in rural settings who reported absence of physician-diagnosed asthma upon screening by a questionnaire have been observed to have asthma when examined by a physician.[131] This is not surprising as rural children who might otherwise be eligible for asthma care must also have the means to travel to the location of care before they can access healthcare services. Since triggers of asthma exacerbation, as well as healthcare access issues are common in rural communities, urban-rural differences in diagnosing patterns or access to healthcare services for symptoms reporting could also contribute to the observed asthma prevalence difference between rural and urban communities.

There is evidence that rural children could be less likely to become diagnosed with asthma, even when they experience symptoms suggestive of asthma[132,133] as supported by a study that investigated childhood asthma prevalence among 6–14 years old children in two rural Iowa counties in the USA.[131] Chrischilles *et al* reported that, overall, 13.8% of children in the two rural counties (Keokuk and Louisa) of Iowa reported frequent symptoms of asthma. Of these children, less than half (41.6%) reported ever been given a positive diagnosis of asthma by a physician. Similarly, of the 4.9% that reported severe asthma symptoms (defined as wheezing

limiting child's speech to only 1 or 2 words between breaths or if the child had any visits to the emergency department or hospitalizations because of asthma, bronchospasm, or wheezing in the past 12 months), only 67.5% had received asthma diagnosis by a physician. A recent cross-sectional study in Saskatchewan, Canada, also showed lower asthma prevalence in rural (15.1%) compared to urban (20.7%) children but similar prevalence of ever wheeze (27.4% vs. 27.2%) and current wheeze (13.1% vs. 14.0%) between settings.[110]

Data from these studies showed that asthma in rural and farming children may be under-diagnosed and may explain some of the observed differences in asthma prevalence. A focus on urban-rural asthma diagnostic pattern as a potential explanation for urban-rural asthma prevalence differences is thus warranted.

2.11 Risk factors for asthma

In addition to place of residence as a risk factor for asthma, several studies have implicated a number of other risk factors in the development of childhood asthma. Reports from these other studies suggest that asthma appears to be a multifactorial disease where a number of personal/host factors (e.g. sex, gender, family history of allergic diseases) and environmental exposures (e.g. air pollution, dampness, mold, tobacco smoke exposure, microbial exposures, allergen exposure etc) play significant roles in its etiology. Many of these risk factors may also be distributed differently across rural and urban populations such that the geographical variation in the prevalence of asthma and asthma-related symptoms may be closely related to the differential distribution of these factors. As such, it is important to consider these other factors while investigating childhood asthma along an urban-rural gradient.

2.11.1 Personal or host risk factors for asthma

2.11.1.1 Gender and age

As mentioned earlier in the discussion of the natural history of asthma, the vast majority of asthma starts in early childhood in which children with asthma experienced their first episodes before the age of 6 years.[134] During this stage of life, both incidence and prevalence of asthma are higher in males than in females. This trend continues until around puberty and reverses in adolescence, with higher prevalence of asthma occurring in females.[135,136] The mechanisms underlying the gender shift in asthma prevalence are unclear but an increase in asthma incidence coupled with a decrease in remission of asthma in females compared with males during adolescence have been suggested as some of the possible explanations.[137]

The Tracking Adolescents' Individual Lives Survey (TRAILS) study in the Netherlands assessed the associations of puberty stages and transition through puberty with the prevalence, incidence and remission of asthma in 2,230 subjects followed from birth until 24 years of age.[137] Three survey data were collected on the presence of asthma at mean age. 11.1, 13.6, and 16.3 years. Results showed prevalence of asthma to be similar in boys and girls at mean age of 11.1 years (7.7% vs. 7.4%) and 13.6 years (6.0% vs. 5.8%). However, at a mean age of 16.3 years, the prevalence of asthma was significantly higher in females compared to males (6.2% vs. 4.3%). In addition, incidence of asthma was observed to be higher (2.8% vs. 1.4%) and remission was lower (2.1% vs. 3.4%) in female compared with male subjects at mean age of 16.3 years; suggesting that the observed shift in the prevalence of asthma was most likely related to both increased incidence and decreased remission of asthma in female subjects compared with males during puberty-adolescent transition stage. Similarly, a population-based study in Saskatchewan, Canada also revealed sex switchover in asthma predominance where asthma prevalence was

significantly greater in males compared to females for preschool age (0–4 years: OR = 1.57; 95%CI: 1.54–1.60) and school-going children (5–14 years: OR = 1.39; OR = 1.36–1.40).[95] However, at age 15–34 years, a sex switchover from male to female predominance was observed with lower prevalence of asthma in males compared to females (OR = 0.90; 95%CI = 0.89–0.90).

2.11.1.2 Family history of asthma and allergy

Children with a family history of asthma are more likely to develop asthma themselves. In an international systematic review of 33 studies, Burke *et al* observed that family history of asthma was consistently identified as a strong predictor of asthma when one or more first-degree relatives has asthma, with most ORs ranging between 2 and 4.[138] Similarly, in another study, London *et al* demonstrated that having parents or siblings with history of asthma and allergy increases the risk of developing different asthma phenotypes with a prevalence ratio (PR) of 12.1 for early-onset persistent, 7.51 for early-onset transient, and 5.38 for late-onset asthma.[139] This familial aggregation of asthma disease suggests that a positive family history might be used to identify children at risk of developing asthma.

2.11.1.3 Obesity

Associations between obesity and asthma have been observed in adults and adolescents[140–142] as well as school-age children.[143] A study by Gilliland *et al* in Southern California followed up 3,792 children for 5 years (1993–1998) and found overweight and obesity to be associated with increased risk of incident asthma with a RR of 1.5 (95%CI: 1.14–2.03) for overweight and 1.60 (95%CI: 1.08–2.36) for obese children.[143] Similarly, a study in Germany found prevalence of doctor-diagnosed asthma to be 2.5% for normal weight, 5.8% for overweight and 10.3% for

obese children.[144] In Canada, Sharma *et al* also found obesity to be associated with airway hyperresponsiveness (OR = 1.37, 95%CI: 1.02–1.82).[145]

Obesity may be particularly important for severe asthma as studies have reported that asthma in obese individuals tend be more severe and more difficult to control.[146,147] A recent systematic review of asthma and obesity showed that apart from increasing the severity of asthma, overweight and obesity was associated with poorer asthma control and poorer response to therapy when compared with normal weight individuals.[148]

2.11.1.4 Race/ethnicity and Socio-economic status

Several studies have linked ethnicity as another possible risk factor for asthma. The prevalence, morbidity, and severity of asthma are believed to be higher in children who belong to certain ethnicity or group. In a National Health Survey study in the USA, being black was significantly associated with the likelihood of having current asthma among children <18 years of age (RR = 1.47, 95%CI: 1.35–1.60) and ED visit for asthma in past 12 months (RR = 3.11, 95%CI: 2.72–3.56).[149] In another study among 0–19 years children, black children compared to white were likely to have severe asthma symptoms measured as frequency of ED visit (OR = 2.34, 95%CI: 1.99–2.77).[150]

Poor socio-economic status (SES) among the black population has further led to suggestion that the relationship between race and childhood asthma could be confounded by SES resulting in significant asthma burden in the less privileged group. This was confirmed in a cross-sectional study among 14,244 children (aged <18 years) in the USA where black children were at higher risk of asthma compared to white children (OR = 1.20, 95%CI: 1.03–1.40)[151] but when the analysis was stratified by income status, only black children in which family income were less than half of federal poverty level showed increased risk for asthma (OR = 1.99,

95%CI: 1.09–3.64). This result suggests that while certain ethnic groups may be disproportionately affected by asthma, understanding how the disparities in childhood asthma occurred may play an important role in accurately assessing the risk of asthma among children.

In Canada, study investigating the role of race/ethnicity in childhood asthma compared Aboriginal and non-Aboriginal children. The NLSCY study showed the prevalence of asthma to be lower in Aboriginal (5.7%) compare to non-Aboriginal children (10%).[152] In the First Nations Regional Health Survey involving 238 First Nation communities from 10 Canadian provinces, the prevalence of asthma was reported to be 14.6% among 0–11 years children living on reserves.[153] The population studied were drawn from the national population of children self-identified as Aboriginal[154]

2.11.2 Environmental risk factors for asthma and asthma severity

Asthma can be triggered or exacerbated by exposure to many environmental factors.[155,156]

2.11.2.1 Pet exposures

The associations between pet exposures and asthma are inconsistent. Some studies suggest pet ownership is protective for asthma[155,156] while others demonstrate a risk factor for asthma.[157] A combined analysis of 11 European birth cohort studies found no association between keeping a pet early in life and asthma in school-age children.[158] Similarly, a systematic review of 17 and 13 birth cohort studies of cat and dog exposures, respectively, found that cat and dog exposures during infancy had no effect on the development of asthma or wheezing symptoms.[159] In addition, dog exposure during infancy was found to protect children from developing sensitization against aeroallergens.[159] In a large cross-sectional study from 35 centers in 16 different countries, Roost *et al* found that early cat exposure was positively associated with

allergic sensitization to cat and wheezing.[160] The difficulties in establishing consistent associations between pet ownership and respiratory disease may be as a result of potential selection bias or failure to assess interaction effects. A meta-analysis of data from 12 European birth cohort studies on asthma and allergy showed that family history of allergy to cat or dog significantly reduced the odds of owning both animals (OR = 0.91; 95%CI: 0.85–0.99 for cat and OR = 0.90; 95%CI: 0.86–0.94 for dog). In addition, high parental education level had even more pronounced effects on cat (OR = 0.84; 95%CI: 0.71–0.98) and dog ownership (OR = 0.61; 95%CI: 0.54–0.70).[161]

While pet exposures may show inconsistent results for asthma development, they are strong risk factors for asthma severity in sensitized children with asthma. A cohort study among 4–12 years old children examined the relationship of common household allergens from cats and dogs and asthma severity (quantified using the GINA guidelines of both frequency of symptoms and medication use) in atopic and non-atopic children with asthma.[162] After adjusting for potential confounders, children living in homes with detectable levels of dog allergen and who were tested positive to dog allergen were two to nearly three times as likely to suffer from severe asthma (OR = 2.52; 95%CI: 1.24–5.08). Similar results were observed for children living in homes with detectable levels of cat allergen and who were also sensitized to cat allergens (OR = 2.18; 95%CI: 1.09–4.35).

2.11.2.2 Environmental tobacco smoke (ETS) exposure

Studies have reported an association between ETS and childhood asthma. In one of these studies investigating maternal smoking during pregnancy and asthma, the risk of developing asthma during the first 7 years of life was 25% higher in children whose mother smoked less than 10 cigarette per day during pregnancy and 36% higher in children whose mother smoked more than

10 cigarettes per day compared to non-smokers.[163] The risk of developing asthma has been found to be even stronger if the grandmother of a child had smoked during the mother's own fetal period.[164] A case-control study nested within the Children's Health Study in Southern California showed utero exposure to maternal smoking to be associated with increased risk of asthma in children during the first 5 years of life and persistent asthma thereafter (OR: 1.5, 1.0–2.3 for both).[164] The risk was particularly increased if the grandmother of a child had also smoked during the child mother's fetal development period (OR = 2.1, 95%CI: 1.4–3.2).[164]

In a meta-analysis of longitudinal studies, the incidence of asthma or wheezing was related to maternal smoking with a stronger effect in the first 5–7 years of life (OR = 1.31, 95%CI: 1.22–1.41) and during the school years (OR = 1.13, 95%CI: 1.04–1.22).[165] Similarly, in a cross-sectional study of children and adolescents aged 6–7 years and 13–14 years respectively, tobacco smoke exposure was positively associated with wheeze, current asthma, exercise-induced asthma and severe asthma, particularly if mother or both parents smoke.[166]

In addition to being a risk factor for the development of asthma, ETS can also exacerbate asthma conditions in children with the disease. This can exacerbate asthma conditions in children who already have the disease leading to more severe symptoms, decreased lung function, more asthma-related doctor visits, and a poorer response to asthma therapy.[165,167] Using objective measures and biomarkers of ETS exposure, a study among children aged 4–16 years with physician-diagnosed asthma correlated smoke exposure as indicated by serum cotinine levels with pulmonary function test and clinical outcomes and observed that children with high serum cotinine levels (>0.63 mg/mL) were more likely to have frequent asthma exacerbations (aOR = 2.7; 95%CI: 1.1–6.5).[168]

The risk of asthma following exposure to maternal smoking during pregnancy may be a result of the adverse influence on the development of fetal respiratory system, as suggested by findings of a relation between maternal smoking in pregnancy and lung function impairment in newborns,[169,170] which may be increased when combined with postnatal maternal smoke exposure.[163]

2.11.2.3 Biological exposures

Biological exposures include a wide variety of biological agents commonly found in indoor environments such as allergens (e.g. house dust mite, cockroach, and mouse), bacteria (e.g. endotoxin), and fungi (e.g. mold); and have been recognized to have associations with respiratory disorders.[171] Common household allergens that have been identified as risk factors for asthma and asthma severity include dust mite, mold, cockroaches, and animal dander allergens (from pets, mice, rats).[162] While the specific roles of allergens in the development and exacerbation of asthma are currently not fully understood, indoor microbial agents (endotoxin and beta-(1→3)-D-glucan) are also independent risk and protective factors for asthma and asthma-like symptoms in children as detailed below:

2.11.2.3.1 Endotoxin and its association with asthma and asthma severity

Endotoxin is a lipopolysaccharide (LPS) which makes up a major component of the outer membrane of gram-negative bacteria[172] and has the capacity to induce the production of T_H1 cells such as IFN-γ and IL-12 which are anti-inflammatory cytokines.[172] The first observational study to report that environmental exposure to endotoxin protects against allergic sensitization was documented in the USA in 2000.[173] In this study, 61 asthma-prone infants (aged 6–24 months) with at least three physician-diagnosed episodes of wheezing were recruited and

concentrations of house dust endotoxin and allergens were examined in the infants' homes. The results of the study demonstrated that the homes of allergen-sensitive infants contained significantly lower concentration of endotoxin [measured in endotoxin units (EU) per mL] compared to homes of non-sensitive infants (468 vs. 1,035 EU/mL; p=0.01). In addition, a high level of house dust endotoxin concentrations was also associated with increased production of IFN-γ-producing T_H cells (CD14 T cells; r = 0.59; p=0.01) which are capable of inducing T_H1 immune responses[173] prompting the general believe that endotoxin exposure early in life is potentially protective against allergic diseases, including asthma.

Since the Gerada *et al* study in the USA, there have been some inconsistencies. Some studies have reported that endotoxin may modulate or protect against asthma development[174,175] while others have shown evidence of increased risk,[176,177] and no association;[178,179] making endotoxin a subject of continuous research. For example, Braun-Fahrlander *et al*[180] reported that exposure to endotoxin levels in mattress dust were inversely associated with atopic asthma (OR = 0.48; 95%CI: 0.28–0.81) and atopic sensitization (OR = 0.76; 95%CI: 0.58–0.98) among schoolchildren (aged 6–13 years) in Germany, Austria, and Switzerland. Contrary to this report, Thorne *et al* demonstrated that exposure to high endotoxin concentration levels from bedroom floor (OR = 1.57; 95%CI: 0.76–3.22), mattress (OR = 1.88; 95%CI: 0.90–3.93), and family room floor (OR = 1.98; 95%CI: 0.99–3.94) increased the risk of physician-diagnosed asthma.[177] Similarly, in a case-control study among schoolchildren (aged 6–18 years) in Saskatchewan Canada, mattress endotoxin concentration (OR = 0.44; 95%CI: 0.20–0.98) and load (OR = 0.38; 95%CI: 0.20–0.75) were inversely associated with being a case with a greater effect in children ≤12 years and without a personal history of allergic disease.[181] These results mirrored the protective effects observed in a two European birth cohort studies: The German LISA (Lifestyle

Related Factors on Immune System and the Development of Allergies in Childhood), and the GINI (German Infant Nutritional Intervention) where endotoxin from children's mattresses' dusts was also found to be associated with a lower risk of physician-diagnosed asthma among 6 years old children (OR = 0.55; 95%CI: 0.31–0.97).[182] A summary of studies investigating associations between endotoxin and childhood asthma is presented in Table 2–3.

Table 2–3: Characteristics and results of studies investigating the association between endotoxin and presence of asthma and asthma-related symptoms among school-age children

First author (Year published) Location(s)	Study design	Study population (Sample size)	Method of dust sample collection and levels of endotoxin exposure	Operational definition of outcomes	Findings and strength of association
Tischer C[183] (2015) Germany Spain The Netherlands	Birth cohort	Children followed from birth to 10 years of age (n = 1429)	Method of dust sample collection: Vacuum. Dust samples were collected at 2–3 months of age Living room GM beta-endotoxin levels: Concentration (EU/mg): 11.76 Load (EU/m^2): 1.16	Physician-diagnosed asthma at age 10 years: Defined as report of doctor-diagnosed asthma ever within the 10 year period. Current asthma at age 6 years and at age 10 years Defined as meeting ≥2 of 3 conditions: 1) doctor-diagnosed asthma ever, 2) wheezing in the past 12 months, 3) asthma medication use in the past 12 months	Higher endotoxin concentrations were significantly and positively associated with current asthma at 6 years of age (OR = 1.96) in the Germany cohort while higher endotoxin load was inversely associated with doctor-diagnosed ever asthma in the Spain cohort (OR = 0.39).
Kavonen AM[184] (2012) Austria Finland France Germany Switzerland	Prospective cohort study	Birth cohort of rural children followed from birth to 2 years of age (n = 1133)	Method of dust sample collection: Vacuum Living room floor GM endotoxin levels (overall mean from the 5 locations): Load (EU/m^2): 17,007 (rug), 2.582 (smooth floor) Mother's mattress GM endotoxin level (overall mean from the 5 locations):	Asthma: ≥1 parental report of doctor-diagnosed asthma and/or doctor-diagnosed asthmatic bronchitis >1 during follow-up period. Wheezing: Parental report of wheeze symptoms at any time-points between 2 and 24 months of age	Living room floor endotoxin load was inversely and significantly associated with incidence of asthma (OR = 0.71) and wheeze (OR = 0.72) during the first 2 years of life. Similar protective effect was seen for mattress endotoxin load and incidence of asthma (OR = 0.79). There was effect modification by

			Load (EU/m^2): 1,637		farming status. When the data was stratified by farming status, the significant protective effects of mattress endotoxin was only seen in non-farmers' children (ORs = 0.68 for asthma and 0.71 for wheezing apart from cold)
Lawson JA[181] (2012) Canada	Case-control	6–18 years (n = 310)	Method of dust sample collection: Vacuumed Play area GM levels: Concentration (EU/mg): Cases (51.8), Controls (40.8) Load (EU/m^2): Cases (868.2, Controls (817.3) Mattress GM levels: Concentration (EU/mg): Cases (19.6), Controls (21.1) Load (EU/m^2): Cases (240.5), Controls (376.2)	Asthma cases: Report of doctor-diagnosed asthma or wheeze in the past year	Play area endotoxin concentration (OR = 1.64) and load (OR = 1.10) increased the risk of being a case but not statistically significant. Mattress endotoxin concentration (OR = 0.44) and load (OR = 0.38) were significantly inversely associated with being a case only in children who were ≤12 years. Also, among children without a personal history of allergy, there were statistically significant inverse associations between mattress endotoxin concentration (OR = 0.20) and load (OR = 0.22) in children ≤12 years

Lawson JA[176] 2011 Canada	Cross-sectional	6–18 years (n = 98)	Method of dust sample collection: Vacuum Play area GM level Concentration (EU/mg): No wheeze (45.0), wheeze (83.2) Load (EU/m^2): No wheeze (790.0), wheeze (1257.5) Mattress GM levels: Concentration (EU/mg): No wheeze (18.9), wheeze (19.7) Load (EU/m^2): No wheeze (282.5), wheeze (272.1)	Report of wheeze in the previous 12 months	There was an increased likelihood of self-reported wheeze with higher endotoxin level. Play area endotoxin concentration was associated with increased risk of wheeze (OR = 4.41) with a borderline significance (p=0.08).
Tischer C[182] (2011) Germany The Netherlands	Multicenter birth cohort study	Children followed up from birth to age 6 years (n = 696)	Method of dust sample collection: Vacuum Levels of endotoxin in the two locations were: **Germany** Play area median levels: Concentration (EU/g): 19,400 Load (EU/m^2): 3,749 Mattress median levels: Concentration (EU/g): 12,222 Load (EU/m^2): 3,053 The Netherlands Play area median levels: Concentration (EU/g): 18,196	Definitions of asthma outcome differed in the two locations **Asthma** Germany: Physician-diagnosed asthma in the last 6 months between the 5^{th} and 6^{th} year of life. The Netherlands: Ever diagnosed asthma plus child had asthma past 12 months Definition of wheeze outcome was similar and defined as wheeze or	In the German study, mattress endotoxin concentration (OR = 0.55) and load (OR = 0.46) were significantly associated with reduced risk of physician-diagnosed asthma. Similar associations were observed for wheezing but this was not statistically significant. There were trends towards increased risk of asthma and wheeze in the Netherland study OR ranging from 1.11 to 1.51

			Load (EU/m^2): 2,299 Mattress median levels: Concentration (EU/g): 10,608 Load (EU/m^2): 2,356	whistling in the chest past 12 months	but the associations were not statistically significant.
Rosenbaum PF[185] (2010) USA	Cohort study	Infants at risk for asthma (due to maternal history of asthma) followed from birth until 1 year of age (n = 103)	Method of dust sample collection: Vacuum Play area GM endotoxin levels: Concentration (EU/mg): 76.1	Wheeze in the first year of life defined as healthcare provider diagnosis of wheeze. Clinical assessments of wheeze was undertaken at 3, 6, 9 and 12 months of age	High endotoxin levels (≥100 EU/mg) were significantly associated with increased risk of wheeze in the first year of life (Unadjusted OR = 2.62). Similar association trend was observed when endotoxin was used as continuous variable. OR = 1.13 for each 20 EU/mg increase.
Iossifova[186] (2009) USA	Prospective Cohort	Children followed up from birth to age 3 years (n = 483)	Method of dust sample collection: Vacuum Living room endotoxin concentration interquartile end point: 38.90–165.0 EU/g	Children classified as having high future asthma risk based on a validated Asthma Predictive Index (API) index score. Positive API if they reported recurrent wheezing at age 3 years and met at least 1 of 3 major criteria (parental history of asthma, allergic sensitization to ≥1 aeroallergens, and eczema) or 2 of 3 minor criteria (wheezing	When expressed as continuous or categorical variables in quartiles) endotoxin exposure was associated with a slight increased risk of wheezing in children with atopy at the age of 3 years (OR = 1.37). Similar association was observed for API at age 3 years (OR = 1.37). These associations were not statistically significant.

				without a cold, physician-diagnosed allergic rhinitis, and allergic sensitization to milk or egg)	
Gehring U[178] (2008) ISAAC Multi-centre study Albania Italy NewZealand Sweden United Kingdom	Cross-sectional	9–12 years (n = 840)	Method of dust sample collection: Vacuum. Living room floor GM endotoxin levels: Concentration (EU/g): Lowest to highest = 6,532 (Sweden) – 35,581 (Italy) Load (EU/m^2): Lowest to highest = 684 (Italy) – 3602 (Sweden)	Asthma ever: Report of ever had asthma Current wheeze: Wheezing or whistling in the chest past 12 months	In a combined analysis across all countries, high living room floor endotoxin load levels were significantly associated with reduced risk of asthma ever (OR = 0.29) and current wheeze (OR = 0.77)
Rennie DC[187] (2008)	Case-control	6–13 years (n = 197 including 89 cases matched to 107 healthy controls based on age and sex)	Method of dust sample collection: Vacuum. Play area GM endotoxin levels: Concentration (EU/mg): 17.31 Load (EU/m^2): 6,536 Mattress GM endotoxin levels: Concentration (EU/mg): 8.82 Load (EU/m^2): 2,498.63	Asthma case: Report of physician-diagnosed asthma and/or wheeze without a cold in the past 12 months Control: No asthma or wheeze	Mattress endotoxin (OR = 0.90) and play area (OR = 0.92) endotoxin concentration were not significantly associated with being a case.
Campo P[188] (2006) USA	Birth cohort	Children followed up from birth to 1st year of life (n = 532)	Method of dust collection: Vacuum Play area GM endotoxin levels: Concentration (EU/mg): 77.8 if pets present in	Asthma: Parental report of physician-asthma Recurrent wheezing: ≥2 wheezing episodes in the past 12 months	High play endotoxin exposures (≥10 EU/mg) were significantly associated with reduced risk of recurrent wheezing (OR = 0.4) and any

			home and 58.7 if no pets present in home	Any wheezing: ≥1 wheezing episodes in the past 12 months. Allergic wheezing: ≥2 wheezing episodes in the past 12 months and a positive SPT response to at least 1 of 15 aeroallergens tested	wheeze (OR = 0.3) only in the presence of two or more dogs in the home. This suggests an interaction between endotoxin exposure and pet ownership in the relationship between endotoxin exposure and childhood asthma.
Douwes J[189] (2006) The Netherlands	Birth Cohort	Children of atopic mothers followed up from birth to 4th year of life (n = 696)	Method of dust sample collection: Vacuum Play area (living room floor) median endotoxin load (EU/m^2): 217 (smooth floor), 9,503 (carpet floor), 27,481 (rug floor) Mattress median endotoxin load (EU/m^2): 856	Asthma: Report of physician-diagnosed asthma at any time in the past 4 years (Ever asthma) Wheeze symptoms: ≥1 episodes in the first 3 years	Mattress levels of endotoxin was not statistically associated with asthma and wheeze. However, play area endotoxin levels were inversely and significantly associated with doctor-diagnosed asthma (OR = 0.40) at four years of age suggesting microbial endotoxin exposure in early life might protect against asthma.
El-Sharif N[190] (2006) Palestine	Case-control	6–12 years (n = 375). However, n=132 (66 cases and 66 controls were randomly selected for dust sampling and matched 1:1 for	Method of dust sample collection: Vacuum Median play area floor endotoxin level: Concentration (EU/mg): 48.51 Median mattress endotoxin concentration (EU/mg): 25.722	Cases: Report of wheeze past 12 months Controls: No report of ever wheeze and no physician-diagnosed asthma	High living room endotoxin concentration was significantly associated with reduced risk of being a case among sensitized cases compared to non-sensitized controls (ORs = 0.04).

		school location, class, and sex)	Endotoxin exposure were categorized into tertiles: 1st tertile (Low): <16.02 EU/mg 2nd tertile (Medium): 16.021–41.754 EU/mg 3rd tertile (High): >41.754 EU/mg		Similarly, medium levels of mattress endotoxin concentration was associated with lower odds of being a case among non-sensitized cases compared to non-sensitized controls (OR = 0.13)
Gillespie J[191] (2006) New Zealand	Birth cohort	Children followed up from birth to 15 months of age (n = 881)	Method of dust sample collection: Vacuum Mattress floor GM endotoxin level: Concentration (EU/g): 9,244. Endotoxin levels were categorized in quartiles	Wheeze: Report of wheezing or whistling in the chest at any time during the 15 months monitoring period (Ever wheeze)	Exposure to higher level of endotoxin concentration (within the 4th quartile) at 3rd month of age was positively and significantly associated with wheezing at 15 months of age (OR = 1.54). The association was particularly stronger and remained significant in children with parental history of allergic disease (OR = 1.67)
Horick N[192] (2006) USA	Prospective Cohort	Children followed up from birth to 6–8 months of age (n = 4044)	Method of dust sample collection: Vacuum and airborne dust sampling at 2–3 months of age; Airborne sampling at 6–8 months of age Mean play area endotoxin levels: Vacuumed dust endotoxin: Concentration (EU/mg): 93.1	Wheezing: Any wheezing episodes corresponding to one or more wheezing events in the first year of life. Uncorrected estimate: Represent the relative increase in risk of wheeze associated with an increase over the	After adjusting for potential confounders, the model corrected for measurement error showed a significant larger effect of endotoxin exposure. The uncorrected RR of 1.45 increased to RR of 5.56 after correction for measurement error, suggesting that correction

			Airborne endotoxin: Concentration (EU/m^3): 0.81. Both airborne and dust endotoxin levels were measured to correct for measurement errors by accounting for t error induced by using house dust endotoxin exposure as a surrogate measure for airborne endotoxin	interquartile range in dust endotoxin exposure Corrected estimate: Represent relative risk (RR) for an interquartile range increase in airborne endotoxin exposure.	for measurement error has a large impact on the point estimate of the effect of increased endotoxin exposure and respiratory diseases.
Perszanowski MS[179] (2006) USA	Prospective Cohort	Children followed up from birth to 3 years of age (n = 301)	Method of dust sample collection: Vacuum Bedroom floor GM endotoxin level: Concentration (EU/mg): 75.9 Load (EU/m^2): 3,892	Wheezing: Parental report of wheezing during at least one of 12, 24, and/or 36 months of life at which interview was conducted.	Higher endotoxin exposure was significantly associated with increased risk of wheezing at 2 years of age (OR = 1.34) with the association stronger in children with maternal history of asthma. However, when wheeze was considered as a longitudinal variable, endotoxin concentration was not associated with the presence of wheeze over time
Tavarnier GOG[193] (2005) United Kingdom	Case-control	4–17 years (n = 200 including 90 matched pairs of asthmatic and healthy controls	Method of dust sample collection: Vacuum Play area median endotoxin levels: Concentration (EU/mg): 36.11	Report of physician-diagnosed asthma.	The study suggests endotoxin as a risk factor for asthma. Play area endotoxin concentration was significantly associated with increased

		based on age, sex, and sibship)	Mattress median endotoxin level; Concentration (EU/mg): 10.99		risk of asthma (OR = 1.88). The association was not seen for mattress endotoxin concentration.
Thorne PS[177] (2005) USA	Cross-sectional	Nationwide sample comprising of adults and children (n = 2456)	Method of dust sample collection: Vacuumed Play area geometric mean (GM) levels: Concentration (EU/mg): 63.9 Load (EU/m^2): 17,600 Mattress levels GM Concentration (EU/mg): 18.7 Load (EU/m^2): 4,160	Asthma: Physician-diagnosed asthma, Asthma symptoms: Any asthma-related symptoms (e.g. cough) past years Wheeze: i) Current wheeze: Wheeze past 12 months ii) Ever wheeze: Report of wheeze ever	High level of mattress endotoxin (>19.6 EU/mg) increased the risk of current wheeze (OR = 2.05) and ever wheeze (OR = 2.01). High level of play area endotoxin (>33.9 EU/mg) also increased the risk of asthma (OR = 1.98), medication use (OR = 2.11), and ever wheeze (1.35), p>0.05. After stratification by age (<18 years vs. ≥18 years), the significant associations were only seen in adults and absent in children.
Braun-Fahrländer C[180] (2002) Austria Germany Switzerland	Multicenter cross-sectional	6–13 years (n = 812)	Method of dust sample collection: Vacuum. Dust samples was collected from mattress of children rural locations stratified by farming households and non-farming households. GM endotoxin levels: Concentration (EU/mg): Farming households	Atopic asthma: Report of physician-diagnosed asthma plus positive test for specific IgE ≥3.5 kU per liter otherwise, they are considered non-atopic asthma. Atopic wheeze: Report of wheeze or whistling in the chest during the previous 12 months plus	Mattress endotoxin loads was significantly associated with reduced risk of atopic asthma (OR = 0.48) and atopic wheeze (OR = 0.62) but not non-atopic asthma (OR = 1.13) and non-atopic wheeze (OR = 1.14) in the total population. The study further showed that

			(37.8), non-farming households (22.8) Load (EU/m^2): Farming households (29,897), non-farming households (14,456)	positive test for specific IgE ≥3.5 kU per liter otherwise, they are considered non-atopic wheeze	exposure to farming environment during the first year of life and current endotoxin exposure significantly reduced the risk of atopic asthma (ORs = 0.42 and 0.52; respectively) but not non-atopic asthma.
Gerada JE[173] (2000) USA	Cross-sectional	9–24 months (n = 61)	Method of dust sample collection: Vacuum. Dust samples was collected from living room floor, kitchen floor, and participant's mattress and in a single vacuum bag. GM level: Concentration: 912 EU/mL. Range: 104 EU/mL–10000 EU/mL.	Allergen sensitization: Skin prick testing to common indoor inhalant allergens and food allergens.	Sensitized infants had significantly lower concentrations of endotoxin in their homes compared to non-sensitized infants (GM = 468 vs. 1035 EU/mL, respectively; p=0.01). Furthermore, increased endotoxin concentration correlated with increased production of IFN-γ CD4 T cells (r = 0.59; p=0.01).

Individuals exposed to endotoxin may also demonstrate acute pulmonary responses that may indicate symptoms of asthma severity. In a case-control study among children in Humboldt, Saskatchewan, exposure to higher levels of endotoxin, particularly, mattress endotoxin, were significantly associated with a lower lung function (FEV_1), especially in female children with asthma or wheeze (beta = –0.25, $p<0.01$).[176] Similarly, endotoxin load in the play areas of children with asthma or wheeze was significantly associated with greater variability in the diurnal peak expiratory flow (DV-PEF) (OR = 2.42; 95%CI: 1.03–5.67).[194] Another study among 148 schoolchildren (aged 7–11 years) in the Netherlands also showed association between house dust endotoxin exposure and greater PEF variability.[195] However, after adjusting for beta-(1→3)-D-glucan exposure in the indoor environment, the association was lost suggesting that the acute inflammatory effects of indoor endotoxin exposure may be equally related to other microbial biomarkers in the indoor environments. A summary of studies investigating associations between endotoxin and lung function as well as asthma severity indicators is presented in Table 2–4.

Table 2–4: Characteristics and results of studies investigating the association between endotoxin and asthma severity indicators and lung function among children and adults

First author (Year published) Location(s)	Study design	Study population (Sample size)	Method of dust sample collection and levels of endotoxin exposure	Operational definition of outcomes	Findings and strength of association
McSharry C[196] (2015) Scotland	Cross-sectional	16–60 years (n = 55) All participants have asthma)	Method of dust sample collection: Vacuum. Median living room floor endotoxin levels: Concentration (EU/g): 10.4 Median bedroom room floor endotoxin levels: Concentration (EU/g): 10.0	Primary outcome: Lung function assessed with FEV_1 before the use of a bronchodilator	Living room endotoxin concentration levels were correlated with decreased FEV_1 but was marginally significant (p=0.063).
Lawson JA[176] (2011) Canada	Case-control	6–18 years (n = 309)	Method of dust sample collection: Vacuum Median endotoxin levels: Play area load (EU/m^2): 1011.3 Mattress load (EU/m^2): 402.5	Lung function measures: FEV_1, FVC, FEV_1/FVC, and $FEF_{25\%–75\%}$	Higher mattress endotoxin load was associated with lower FEV1 (beta = -0.25) only in female cases. There was a significant interaction between outcome, mattress endotoxin and gender. Among female cases, higher mattress endotoxin exposure was associated with lower FEV_1 while the association was similar for between male and female controls.
Lawson JA[194] (2011) Canada	Cross-sectional	6–18 years (n = 98)	Method of dust sample collection: Vacuum Play area GM level:	Clinical measures of diurnal variation (morning and evening) in Peak Expiratory Flow	There was a greater DV-PEF associated with higher endotoxin levels during a two week

			Concentration (EU/mg): Low DV-PEF (44.14), High DV-PEF (60.0) Load (EU/m^2): Low DV-PEF (705.3), High DV-PEF (1090.8) Mattress GM levels: Concentration (EU/mg): Low DV-PEF (15.7), High DV-PEF (23.2) Load (EU/m^2): Low DV-PEF (248.4), High DV-PEF (315.9)	variability (DV-PEF) over a two week monitoring period. DV-PEF was categorized as high DV-PEF and low DV-PEF.	monitoring period. Play area endotoxin load was significantly associated with high DV-PEF (OR = 2.42). Similar trend was seen for play area endotoxin concentration but was not significant (2.54).
Rennie DC[187] (2008)	Case-control	6–13 years (n = 197 including 89 cases matched to 107 healthy controls based on age and sex)	Method of dust sample collection: Vacuum. Play area GM endotoxin levels: Concentration (EU/mg): 17.31 Load (EU/m^2): 6,536 Mattress GM endotoxin levels: Concentration (EU/mg): 8.82 Load (EU/m^2): 2,498.63	Report of physician-diagnosed asthma and/or wheeze without a cold in the past 12 months. Severity indicator: >3 days of being kept at home for chest illness	Mattress endotoxin concentration was significantly associated with being kept at home for chest illness for more than 3 days in the past 12 months (β = 1.05). The association was not observed in non-atopic cases, atopic controls or non-atopic controls.
Iossifova YY[197] (2007) USA	Cohort study	Children followed up from birth to age 2 years (n = 574)	Method of dust sample collection: Vacuum Play area endotoxin range; Concentration (EU/mg): 6.0–800.0. Exposure assessed in quartiles: (1^{st}: 6.0–38.8; 2^{nd}: 38.9–78.8; 3^{rd}: 78.9–165.0; 4^{th}: 165.1–800.0)	Recurrent wheeze: ≥2 wheezing episodes in the past 12 months Recurrent wheezing combined with allergen sensitization: Recurrent wheezing plus positive test (≥3mm) to at least	There were no significant association between endotoxin exposures and studied outcomes although there was a positive trend towards increased risk of recurrent wheeze with allergen sensitization (OR = 1.60)

				one of 15 common allergens	
Rabinovitch N[198] (2005) USA	Two interval particulate-exposure monitoring design	6–13 year (n = 24: Interval 1 n = 10; Interval 2 n = 14). All subjects were children with asthma attending school specifically for asthma children	Method of dust sample collection: Personal exposure monitoring of dust samples with personal exposure monitor calibrated at flow rate of 2 L/min. Interval 1: 10 consecutive days of personal exposure monitoring and indoor monitoring for $PM_{2.5}$ Interval 2: 2 consecutive days completed 3 times of personal exposure monitoring and indoor monitoring from PM^{10} Median personal $PM_{2.5}$ endotoxin level (Interval 1): Concentration (EU/m^3): 0.08 Median personal PM_{10} endotoxin level (Interval 2): Concentration (EU/m^3): 0.37	All children had asthma. Primary outcome: FEV_1. Morning FEV_1 performed immediately after 24-hr personal monitoring period and evening FEV_1 performed 9–12 hrs after monitoring interval. Asthma severity score: Based on the 5-point (0–4) severity of asthma symptoms. 0 = No symptoms; 4 = Symptoms severe enough to prevent play or sleep. asthma symptoms scores	Higher level personal endotoxin exposure at interval 2 was significantly associated with decreased FEV1 (-316 mL per EU/m3). Similar trend was observed for interval 1 but not statistically significant (p=0.15). This associations were not seen with indoor endotoxin concentration (p=0.80). In addition, during interval 2, personal endotoxin exposure was significantly associated with increased risk of reporting asthma symptoms severe enough to prevent sleep (OR = 2.04, p=0.04)
Thorne PS[177] (2005) USA	Cross-sectional	Nationwide sample comprising of adults and children (n = 2456)	Method of dust sample collection: Vacuumed Play area geometric mean (GM) levels: Concentration (EU/mg): 63.9 Load (EU/m^2): 17,600 Mattress levels GM Concentration (EU/mg): 18.7 Load 4,160	Medication use: Current asthma medication use	Unadjusted analysis showed significantly elevated odds ratios between high bedroom floor endotoxin concentration (>16.6 EU/mg) and current asthma medication use (OR = 2.42). Similar

					results was observed for mattress endotoxin concentration (OR = 1.72). After adjustment, the ORs were still elevated (2.28 for bedroom floor endotoxin and 1.83 for mattress endotoxin) but not significant.
Douwes J[195] (2000) The Netherlands	Cross-sectional	7–11 years (n = 148)	Method of dust sample collection: Vacuum Play area GM levels: Concentration (EU/g): Asthmatics (11,588), Non-symptomatic (10,915), Symptomatic (12,642) Load (EU/m^2): Asthmatics (2,493), Non-symptomatic (2,082), Symptomatic (2,443) Mattress GM levels: Concentration (EU/g): Asthmatics (3,983), Non-symptomatic (4,772), Symptomatic (5,696) Load (EU/m^2): Asthmatics (1,202), Non-symptomatic (1,820), Symptomatic (2,082)	PEF variability (morning and evening) over a 16 week monitoring period. Asthma symptoms: Report of respiratory symptoms such as recent wheeze, shortness of breath, or dry cough, and/or doctor-diagnosed asthma ever. Atopic: Positive test (≥3 mm wheal diameter) to at least one allergen from a panel of six common allergens.	In unadjusted analysis, levels of play area endotoxin load was associated with PEF-variability over the 16 week monitoring period (OR = 1.43). The association was significant particularly in atopic children with asthma symptoms (OR = 1.66) However, the significant association was lost after adjusting for potential confounders. No association was found for mattress endotoxin levels both in the univariate and adjusted models.
Rizzo MC[199] (1997) Brazil	Case-control	6–16 years	Method of dust sample collection: Vacuum	Cases: Physician-diagnosed asthma	Study demonstrate that endotoxin exposure exacerbates asthma

		(n = 20 including 10 controls)	Monthly samples (from February 1993 to February 1994) were taken from bedroom floor and from mattress. Levels of endotoxin: Not provided as a single unit but highest levels were observed in the summer months (Dec-Feb) and lowest levels were observed in April and Aug.	Controls: No personal history of allergic disease and no respiratory symptoms. Symptoms severity score among cases were established based on guidelines: "0" = none and '5" = severe enough to incapacitate	symptoms in children with asthma. There was a positive correlation between endotoxin exposure and clinical asthma severity scores (r = 0.63, p<0.05). The association appeared to be similar all year round.
Michel O[200] (1991) Belgium	Cross-sectional	19–61 years (n = 28)	Method of dust sample collection: Vacuum. Samples from mattress and bedroom floor were pooled as a single lot. Median LPS levels: Concentration (ng/ml): 5.6. Exposure was assessed as categorical (Low: ≤5.6 ng/ml; and High >5.6 ng/ml)	Asthma: Defined as recommended by ATS. That is, presence of clinical picture, associated with either an increase of ≥20% in FEV_1 post bronchodilator or ≥20% decrease in FEV_1 following histamine inhalation dose of <480 μg. In addition all subjects with asthma present with Dpt allergy (based on clinical history, RAST and SPT) or non-allergic asthma with perennial clinical presentation. Patients also had ≥4 visits to the clinic in the past 1 year.	FEV1/FVC was significantly lower in subjects with high LPS exposure compared to low LPS exposure (67.0% vs. 84.5%). Similarly, compared with patients exposed to low LPS concentration, high LPS group showed significantly bronchodilator (8.0 vs 4.0), increased oral corticosteroid intake (13.5 vs. 0 and treatment score (44.3 vs. 14.0)

				Primary outcome: Lung function determined with FEV_1 and FEV_1/FVC	
Michel O[201] (1989) Belgium	Case-control laboratory inhalation and responsiveness study	23–62 years (n = 14 including 6 healthy controls)	Method of dust sample collection: N/A Subjects were challenged with saline (as placebo) on day 1 and LPS (as treatment) on day 8.	Asthma cases (n = 8): Subjects with complains of dyspnea and/or wheezing and demonstrated airway obstruction after histamine challenge Controls (n = 6): No allergic antecedents, no family history of atopy and not taking medication	In control subjects, there was no significant change in FEV_1 following challenge with 20 μg of LPS after comparison with placebo. However, among those with asthma, there was a significant reduction in FEV_1 following inhalation of 20 μg of LPS compared with placebo group. The decrease in FEV_1 was observed within 15 min after LPS inhalation and lasted for at least 5 hours. The mean decrease in FEV_1 during the 5 hours after LPS inhalation was 6.7%.

2.11.2.3.2 Beta glucan and its association with asthma and asthma severity

The association between beta-(1→3)-D-glucan and respiratory health is currently less well investigated than that with endotoxin. In a German birth cohort study,[182] exposure to beta-(1→3)-D-glucan from children's mattresses was associated with a lower risk of asthma (OR = 0.76; 95%CI: 0.40–1.45) and wheeze (OR = 0.78; 95%CI: 2.35–11.54). In a case-control study among 422 children that participated in the population-based Study of Asthma, Genes and Environment (SAGE) birth cohort in Manitoba, Canada, Maheswaran *et al* observed that children who were exposed to high level of beta-(1→3)-D-glucan in home dust at age 7–10 years developed persistent atopic asthma at age 11–14 years (OR = 1.79; 95%CI: 1.14–2.81).[202] The results of the study further showed that, in children without asthma, exposure at age 7–10 years increased the risk of BHR at adolescence (OR = 1.74; 95%CI: 1.05–2.89). A summary of studies investigating associations between beta-(1→3)-D-glucan and childhood asthma and asthma symptoms is presented in Table 2–5.

Table 2–5: Characteristics and results of studies investigating the association between beta-(1→3)-D-glucan and presence of asthma and asthma-related symptoms among school-age children

First author (Year published) Location(s)	Study design	Study population (Sample size)	Method of dust sample collection and levels of endotoxin exposure	Operational definition of outcomes	Findings and strength of association
Tischer C[183] (2015) Germany Spain The Netherlands	Birth cohort	Children followed from birth to 10 years of age (n = 1429)	Method of dust sample collection: Vacuum. Dust samples were collected at 2–3 months of age Living room GM beta-(1→3)-D-glucan levels: Concentration (μg/mg) = 1.75	Physician-diagnosed asthma at age 10 years: Defined as report of doctor-diagnosed asthma ever within the 10 year period. Current asthma at age 6 years and at age 10 years Defined as meeting ≥2 of 3 conditions: 1) doctor-diagnosed asthma ever, 2) wheezing in the past 12 months, 3) asthma medication use in the past 12 months	Beta-(1→3)-D-glucan exposure was not significantly associated with current asthma at 6 years of age (OR = 1.04), at 10 years of age (OR = 0.96). While beta-(1→3)-D-glucan appeared to have reduced effects on doctor-diagnosed asthma ever at 10 years of age (OR = 0.84), the association was not significant.
Blatter J[203] (2014) Puerto Rico	Case-control	6–14 years (n = 317)	Method of dust sample collection: Vacuum. Dust samples from mattress surfaces, living room and kitchen areas were combined as a single lot for microbial analysis. Beta-(1→3, 1→6)-D-glucan range: Concentration (μg/mg): 0.01–23.0. Exposure as assessed in quartiles: (1st Quartile: 0.01–	Asthma cases: Physician-diagnosed asthma and wheeze in the prior year Control: No asthma or wheeze.	There was no significant association between beta-(1→3, 1→6)-D-glucan and being a case for asthma.

			0.05; 2nd Quartile: 00.05–0.14; 3rd Quartile: 0.14–0.29; 4th Quartile: 0.30–23.0)		
Maheswaran D[202] (2014) Canada	Prospective cohort	Children followed up from birth to age 14 years (n = 422)	Method of dust sample collection: Vacuum. Play area and mattress dust samples were combined into a single lot for analysis. Mean beta-(1→3)-D-glucan levels: Concentration (μg/g): 36.58 (Winter), 53.90 (Spring), 63.08 (Autum), 79.38 (Fall)	Asthma (assessed at age 7–10 years and ages 11–14 years): Physician-diagnosed asthma confirmed by pediatric allergist according to the Canadian Asthma Consensus guidelines. BHR (assessed at age 7–10 years and ages 11–14 years): Assessed with methacholine challenge test. PC20 ≥8mg/mL considered as positive. Atopy asthma: Physician-diagnosed asthma plus positive test to at least 1 of 16 tested allergens.	At ages 7–10, beta-(1→3)-D-glucan levels in house increased the risk of asthma (OR = 1.15), and atopic asthma (OR = 1.21), albeit non-significant. However, after adjusting for potential confounders, including endotoxin exposure, beta-(1→3)-D-glucan exposure at age 7–10 was significantly associated with persistent atopic asthma (OR = 1.79) and BHR (OR = 1.87) at age 11–14 in children with existing asthma conditions. In children without asthma at age 7–10, high beta-(1→3)-D-glucan exposure at ages 7–10 years also significantly predicted BHR at ages 11–14 years (OR = 1.80).
Tischer C[182] (2011) Germany The Netherlands	Multicenter birth cohort study	Children followed up from birth to age 6 years (n = 696)	Method of dust sample collection: Vacuum Median beta-(1→3)-D-glucan levels: **Germany**	Definitions of asthma outcome differed in the two locations **Asthma**	In both the German and Dutch studies, no significant associations were observed between exposure to beta-(1→3)-

			Living room floor: Concentration (μg/g): 2,229 Load (μg/m^2): 445 Mattress: Concentration (μg/g): 1,859 Load (μg/m^2): 421 The Netherlands Living room floor: Concentration (μg/g): 2,137 Load (μg/m^2): 177 Mattress: Concentration (μg/g): 1,662 Load (μg/m^2): 380	Germany: Physician-diagnosed asthma in the last 6 months between the 5th and 6th year of life. The Netherlands: Ever diagnosed asthma plus child had asthma past 12 months **Wheeze** Definition of wheeze outcome was similar and defined as wheeze or whistling in the chest past 12 months **Dry cough** Germany: Ever have nocturnal chesty cough without a cold or bronchitis The Netherlands: Presence of nocturnal cough without a cold or an infection in the chest past 12 months.	D-glucan and asthma and wheeze. However, in children with parental history of allergy, exposure to mattress beta-(1→3)-D-glucan load was significantly associated with decreased risk of dry cough in the German study (OR = 0.65).
Iossifova YY[186] (2009) USA	Birth cohort	Children followed up from birth to age 3 years (n = 483)	Method of dust sample collection: Vacuum Living room beta-(1→3)-D-glucan range: Concentration (μg/g): 0.35–960 Exposure was assessed in quartiles (1st Quartile: 0.35–	Children classified as having high future asthma risk based on a validated Asthma Predictive Index (API) index score. Positive API if they reported recurrent	The study showed different results at low and high beta-(1→3)-D-glucan exposure levels. Low beta-(1→3)-D-glucan exposure (<22 μg/g) was associated with increased risk of positive API (OR = 3.4)

			22 μg/g; 2nd Quartile: 22.1–60.0 μg/g; 3rd Quartile: 60.1–133.0; 4th Quartile: 133.1–960 μg/g)	wheezing at age 3 years and met at least 1 of 3 major criteria (parental history of asthma, allergic sensitization to ≥1 aeroallergens, and eczema) or 2 of 3 minor criteria (wheezing without a cold, physician-diagnosed allergic rhinitis, and allergic sensitization to milk or egg)	whereas at high beta-(1→3)-D-glucan exposure (>133 μg/g) children had reduced risk of positive API (OR = 0.6) at 3 years of age. Similar trends of associations were observed for wheezing with atopy at the age of 3 year. These associations were not significant
Iossifova YY[197] (2007) USA	Cohort study	Children followed up from birth to age 2 years (n = 574)	Method of dust sample collection: Vacuum Play area beta-(1→3)-D-glucan range: Concentration (μg/g): 3–900 Exposure was assessed in quartiles (1st Quartile: 3–22 μg/g; 2nd Quartile: 23–60 μg/g; 3rd Quartile: 61–133; 4th Quartile: 134–900 μg/g)	Recurrent wheeze: ≥2 wheezing episodes in the past 12 months Recurrent wheezing combined with allergen sensitization: Recurrent wheezing plus positive test (≥3mm) to at least one of 15 common allergens	Exposure to high beta-(1→3)-D-glucan (within the 4th quartile) was significantly associated with reduced risk of recurrent wheezing (OR = 0.39) as well as recurrent wheeze with allergen sensitization (OR = 0.13). The association was reversed for lower exposure (within the 1st quartile). Recurrent wheezing (OR = 3.04) and recurrent wheezing without allergic sensitization (OR = 4.89) were significantly and positively associated with

					low (1→3)-D-glucan exposure level
Douwes J[189] (2006) The Netherlands	Birth Cohort	Children of atopic mothers followed up from birth to 4th year of life (n = 696)	Method of dust sample collection: Vacuum Play area (living room floor) median beta-(1→3)-D-glucan load: Load (μg/m^2): 90 (smooth floor), 686 (carpet floor), 1,005 (rug floor) Mattress median beta-(1→3)-D-glucan load: μg/m^2: 90	Asthma: Report of physician-diagnosed asthma at any time in the past 4 years (Ever asthma) Wheeze symptoms: ≥1 episodes in the first 3 years	High play area and mattress beta-(1→3)-D-glucan (load or concentration) were not associated with doctor-diagnosed asthma and wheeze. Further adjustment for other important confounders rendered models highly unstable.
Schram-Bijkerk D[204] (2005) Austria Germany The Netherlands Sweden Switzerland	Case-control	5–13 years (n = 14,893)	Method of dust sample collection: Vacuum Mattress GM beta-(1→3)-D-glucan levels: Cases Concentration (μg/g): 2,662 Load (μg/m^2): 402 Controls Concentration (μg/g): 2,959 Load (μg/m^2): 519	Wheeze: Parental report of wheeze in the past 12 months or wheeze ever. Atopic: Positive SPT Cases: atopic and non-atopic wheezers Controls: Non-atopic non-symptomatic.	Overall, mattress beta-(1→3)-D-glucan load was associated with reduced risk of atopic wheeze with a borderline significance. Similar reduced risk was observed for each country but was not statistically significant. The protective effects of beta-(1→3)-D-glucan were also observed in both farm and non-farm reference children.

Exposure to beta-(1→3)-D-glucan has also been found to be associated with lower lung function. In a study among 148 schoolchildren (aged 7–11 years) in Amsterdam, The Netherlands, high levels of (1→3)-β-D-glucan from living room floor dust samples was associated with 1.6-fold increase in peak expiratory flow (PEF) variability, particularly in atopic children with asthma symptoms.[195] The lower lung function and higher PEF variability may suggest exacerbation of asthma conditions following exposure, after asthma has develop. That is, among subjects with asthma, biological contaminants may be associated with greater asthma severity by enhancing pre-existing allergic and non-allergic inflammation. A summary of studies investigating associations between beta-(1→3)-D-glucan and lung function as well as asthma severity indicators is presented in Table 2–6.

Table 2–6: Characteristics and results of studies investigating the association between beta-(1→3)-D-glucan and asthma severity indicators and lung function among children and adults

First author (Year published) Location(s)	Study design	Study population (Sample size)	Method of dust sample collection and levels of endotoxin exposure	Operational definition of outcomes	Findings and strength of association
McSharry C[196] (2015) Scotland	Cross-sectional	16–60 years (n = 55) All participants have asthma)	Method of dust sample collection: Vacuum. Median living room floor beta-(1→3)-D-glucan levels: Concentration (ng/g): 435 Median bedroom room floor beta-(1→3)-D-glucan levels: Concentration (ng/g): 435	Primary outcome: Lung function assessed with FEV_1 before the use of a bronchodilator	While high living room and bedroom beta-(1→3)-D-glucan concentrations demonstrated trends toward decreased FEV_1 values (ρ = -0.173 and -0.107, respectively), the correlations were not significant, p>0.10 in both cases.
Tischer C[183] (2015) Multicenter across Europe	Cross-sectional	20–44 years (n = 956)	Method of dust sample collection: Vacuum Median mattress beta-(1→3)-D-glucan level: Concentration (μg/mg): 0.87	Primary outcome: Lung function assessed with FEV_1 and FVC	There was no evidence of association between beta-(1→3)-D-glucan exposure and kung function. While high beta-(1→3)-D-glucan exposure appeared to decrease FEV_1 (-10 mL/s) and FVC (-10 mL), the associations were not significant.
Blatter J[203] (2014) Puerto Rico	Case-control	6–14 years (n = 317)	Method of dust sample collection: Vacuum. Dust samples from mattress surfaces, living room and kitchen areas were combined as a single lot for microbial analysis.	Asthma cases: Physician-diagnosed asthma and wheeze in the prior year Control: No asthma or wheeze. i) Primary outcomes: Lung function assessed	Beta-(1→3, 1→6)-D-glucan was significantly associated with decreased FEV_1 with a dose-response trend (1^{st} Quartile = 2.06 L/s, 2^{nd} Quartile = 2.03 L/s, 3^{rd} Quartile = 1.92

			Beta-(1→3, 1→6)-D-glucan range: Concentration (μg/mg): 0.01–23.0. Exposure as assessed in quartiles: (1st Quartile: 0.01–0.05; 2nd Quartile: 00.05–0.14; 3rd Quartile: 0.14–0.29; 4th Quartile: 0.30–23.0)	with FEV_1, FVC, and FEV_1/FVC. ii) Asthma severity indicator: ≥1 ED/urgent care visit for asthma past 12 months	L/s, 4th Quartile = 1.78 L/s; p=0.02). Furthermore, among children with asthma, high beta-(1→3, 1→6)-D-glucan exposure level (in the 4th quartile) was significantly associated with increased odds of 1 or more visits to the ED/urgent care for asthma in the past 12 months (OR = 8.76) after adjustment for potential confounders (OR = 8.76)
Rylander R[205] (2006) Sweden	Case-control exposure study	(n = 82) Exposed: Poultry worker = 42. Mean age = 45.2 years. Unexposed: non-poultry worker = 40. Mean age = 38.5	Method of dust sample collection: Stationary dust sampling with Isopore filter calibrated at 2 l/m airflow for 30–60 min sampling period. Dust samples were taken in the poultry house only. Mean airborne beta-(1→3)-D-glucan level: Concentration (ng/m^3): 20	Primary outcome: Pulmonary function assessed with FEV1, FVC, and FEV1/FVC. Bronchial responsiveness assessed with methacholine challenge test (MCT)	Baseline FEV1 was significantly lower in poultry workers compared to controls (101.1 vs. 110.7, p<0.05). Following MCT, the average % decrease in FEV_1 in poultry workers was significantly larger compared to controls (-9.5% vs. -3.4%, p<0.001).
Douwes J[195] (2000) The Netherlands	Cross-sectional	7–11 years (n = 148)	Method of dust sample collection: Vacuum Play area GM beta-(1→3)-D-glucan levels: Concentration (μg/g): Asthmatics (743), Non-	Primary outcome: PEF variability (morning and evening) over a 16 week monitoring period in children with asthma and asthma symptoms.	Univariate analysis showed that play area levels of play area beta-(1→3)-D-glucan load was significantly associated with PEF-variability over the 16 week monitoring

			symptomatic (612), Symptomatic (754) Load (μg/m^2): Asthmatics (167), Non-symptomatic (126), Symptomatic (169) Mattress GM beta-(1→3)-D-glucan levels: Concentration (μg/g): Asthmatics (903), Non-symptomatic (718), Symptomatic (792) Load (μg/m^2): Asthmatics (283), Non-symptomatic (276), Symptomatic (293)		period in asthmatic (OR = 1.45) and symptomatic (OR = 1.33) children. The associations were stronger particularly in atopic children (ORs = 1.63 for asthmatic and 1.58 for symptomatic). The association remained significant after adjusting for potential confounders, including endotoxin exposure levels (OR > 1.5). No association was found for mattress beta-(1→3)-D-glucan levels.
Rylander R[206] (1996) Sweden	Laboratory inhalation challenge study	Adults	Method of dust sample collection: N/A Subject exposed to aerosol of beta-(1→3)-D-glucan for 4 hours.	Primary outcomes: Airway responsiveness assessed with MCT and respiratory symptoms	Beta-(1→3)-D-glucan caused an increase in the severity of respiratory symptoms determined by throat and chest irritation. Also, significant airway responsiveness was observed following exposure to beta-(1→3)-D-glucan.

2.12 Summary of literature review and restatement of research rationale

Asthma is the most common chronic disease among children. The pathophysiology of asthma is complex and involves multicellular processes resulting in phenotypic heterogeneity of the disease. Central to the various phenotypic patterns is the presence of underlying airways inflammation.

Furthermore, markers of asthma can vary between children, although these markers may sometime overlap as asthma is a multifactorial disease.

The NAEPP guidelines[58] recommend that asthma severity be assessed using a combination of frequency of clinical respiratory symptoms (day- and night-time symptoms) and objective lung function criteria (determined with forced expiratory volume in one second [FEV_1]).

Asthma appears to be less common in rural compared to urban children. Environmental factors including endotoxin exposures in rural settings have mostly been implicated for the urban-rural asthma differences. However, reports from Australia, Canada, Europe, and USA suggest that respiratory symptoms suggestive of asthma could be higher and even worse in children living in rural compared to those in urban settings.[116,118,207,208] From this, it can be inferred that there may be under-diagnosis of asthma in rural areas.

Methods to evaluate the presence or absence of asthma include symptoms history and lung function assessment. However, this is not often conducted in many epidemiological studies investigating childhood asthma due to cost and convenience.

Epidemiological studies on asthma and microbial exposures have reported conflicting evidence with some showing indoor endotoxin exposures have protective effects while others showed risk or no association effects. Characterization of objective measures of asthma

phenotypes and their associations with objectively measured indoor microbial exposures are important as they may help explain some on the discrepancies.

Similarly, studies examining the relationships between asthma severity and indoor microbial exposures have often focused on reports of symptom frequency by questionnaire without completing clinical evaluation to assess degree of asthma severity. Characterization of objective measures of asthma severity indicators and their associations with objectively measured indoor microbial exposures should also be investigated.

By addressing these research gaps, data from the study will aid in identifying issues around asthma diagnosis along an urban-rural gradient and address the indoor microbial exposure associated with asthma severity and phenotypic expression among children with asthma. This could be important in improving asthma outcomes and patient care in children with asthma.

2.13 Research objectives

The overall aim of this study is to examine urban-rural asthma diagnostic pattern, as well as to investigate the relationships between indoor microbial exposures [endotoxin and beta-(1→3)-D-glucan as biomarkers of bacterial and fungal exposure, respectively] and asthma phenotypes and severity among children with asthma in Saskatchewan. Toward this goal, the following specific objectives are proposed:

2.13.1 Objective 1: To assess asthma diagnostic patterns along an urban-rural gradient by investigating difference in proportion of diagnosed asthma based on survey-based and algorithm-based asthma classification.

2.13.1.1 Hypothesis*:* Rural children will experience under-diagnosis of asthma more compared to urban children.

2.13.2 Objective 2: To examine associations between asthma phenotypes and endotoxin and beta-(1→3)-D-glucans of exposure.

2.13.2.1 Hypothesis: Endotoxin and beta-(1→3)-D-glucan exposure will be associated with reduced risk of atopic asthma compared to non-atopic asthma but will be associated with increased risk of exercise-induced bronchospasm compared to non-exercise-induced bronchospasm.

2.13.3 Objective 3: To examine associations between asthma severity and endotoxin and beta-(1→3)-D-glucans of exposure.

2.13.3.1 Hypothesis 1: Endotoxin and beta-(1→3)-D-glucans exposure will be associated with increased risk of moderate/severe asthma compared to mild asthma.

2.13.3.2 Hypothesis 2: Endotoxin and beta-(1→3)-D-glucans exposure will be associated with .decreased lung function in children with asthma.

2.14 References

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CHAPTER 3

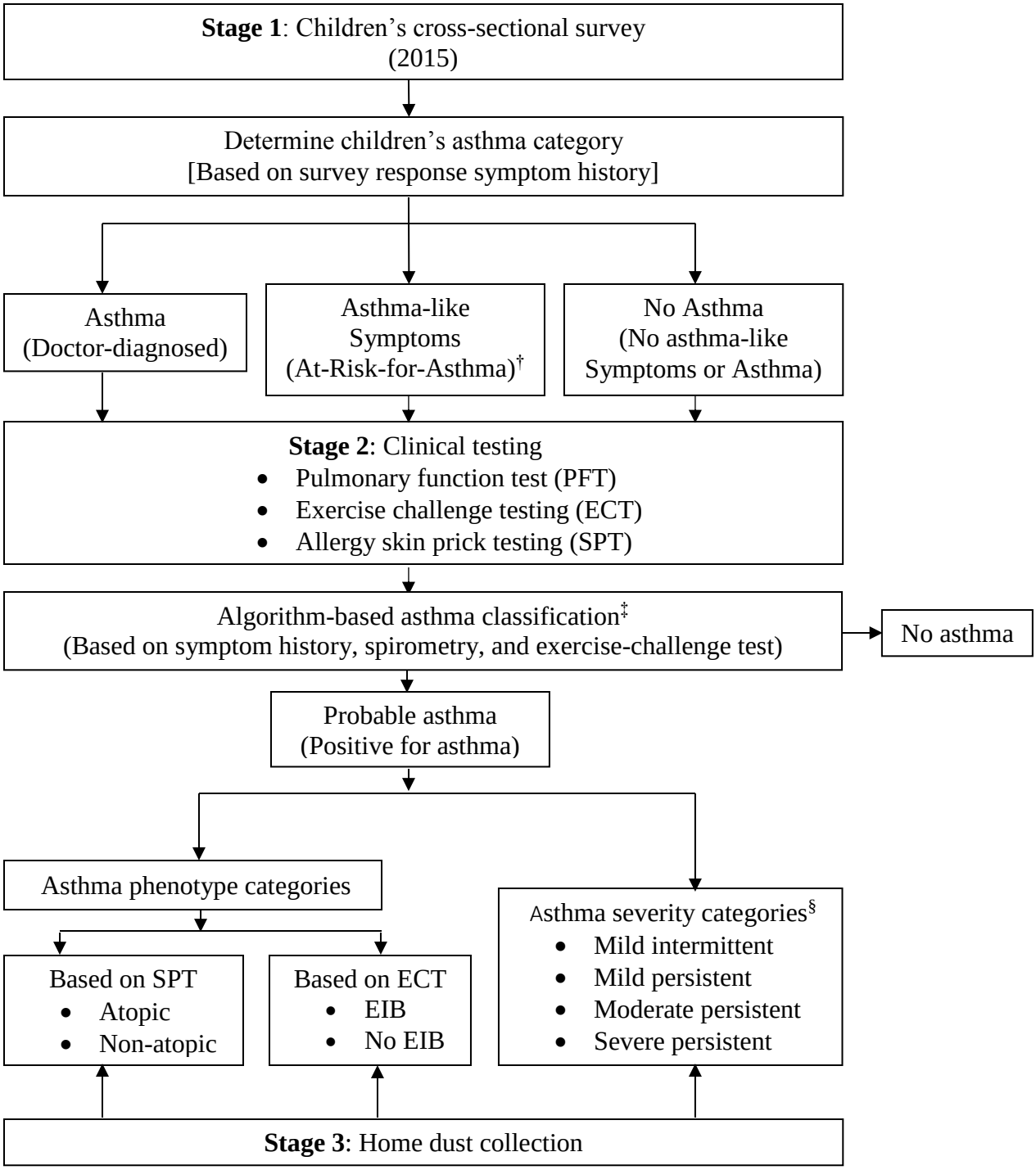
METHODOLOGY

3.1 Overview

This section describes the general methodology for the study including the study design, study population, data collection methods, and dust sample preparation and analysis procedures. Since this is a manuscript-style dissertation, methodologies specific to Objectives 1, 2, and 3 are further described in their manuscript sections (Chapters 4, 5 and 6, respectively).

3.2 Study design

This study used a cross-sectional design and incorporated three stages of data collection that included cross-sectional survey, clinical assessment, and home dust collection **(**Figure 3–1**)**. Data from Stages 1 and 2 formed the basis of Objective 1 (Asthma diagnosis). The asthma population identified in Objective 1 and data from Stages 1, 2 and 3 formed the basis of Objectives 2 (asthma phenotypes) and 3 (asthma severity).



†No report of physician-diagnosed asthma but a positive response to any or a combination of asthma-related symptoms.
‡Based on a 3-stage asthma case-detection algorithm.[1]
§Based on NAEPP asthma severity classification guidelines.[2]
EIB: Exercise-induced bronchospasm.

Figure 3–1: Flow chart of study design and data collection procedures.

3.3 Study location

The study was conducted in the province of Saskatchewan, Canada. Children were recruited from Regina (approximately population size of 200,000[3]), Prince Albert (approximately population size of 35,000[4]) and the rural or farm towns surrounding Prince Albert (approximately population size <2,500) (Figure 3–2). These areas were chosen based on their population size and density as well as the lack of previous asthma research in these areas. For this study, location of dwelling for children were considered as "Large Urban", "Small Urban" or "Rural" depending on if a child lives in Regina, Prince Albert or the small towns, farm or acreages outside of Prince Albert; respectively. The urban-rural gradient chosen for the study parallels Statistics Canada definitions based on modified Beale codes where the definitions of large urban, small urban, and rural in this study match those of small metropolitan (urban settlements of 50,000 to 249,999 people), non-metropolitan small city zone (20,000-49,999 people) and rural (<2,500 people).[5]

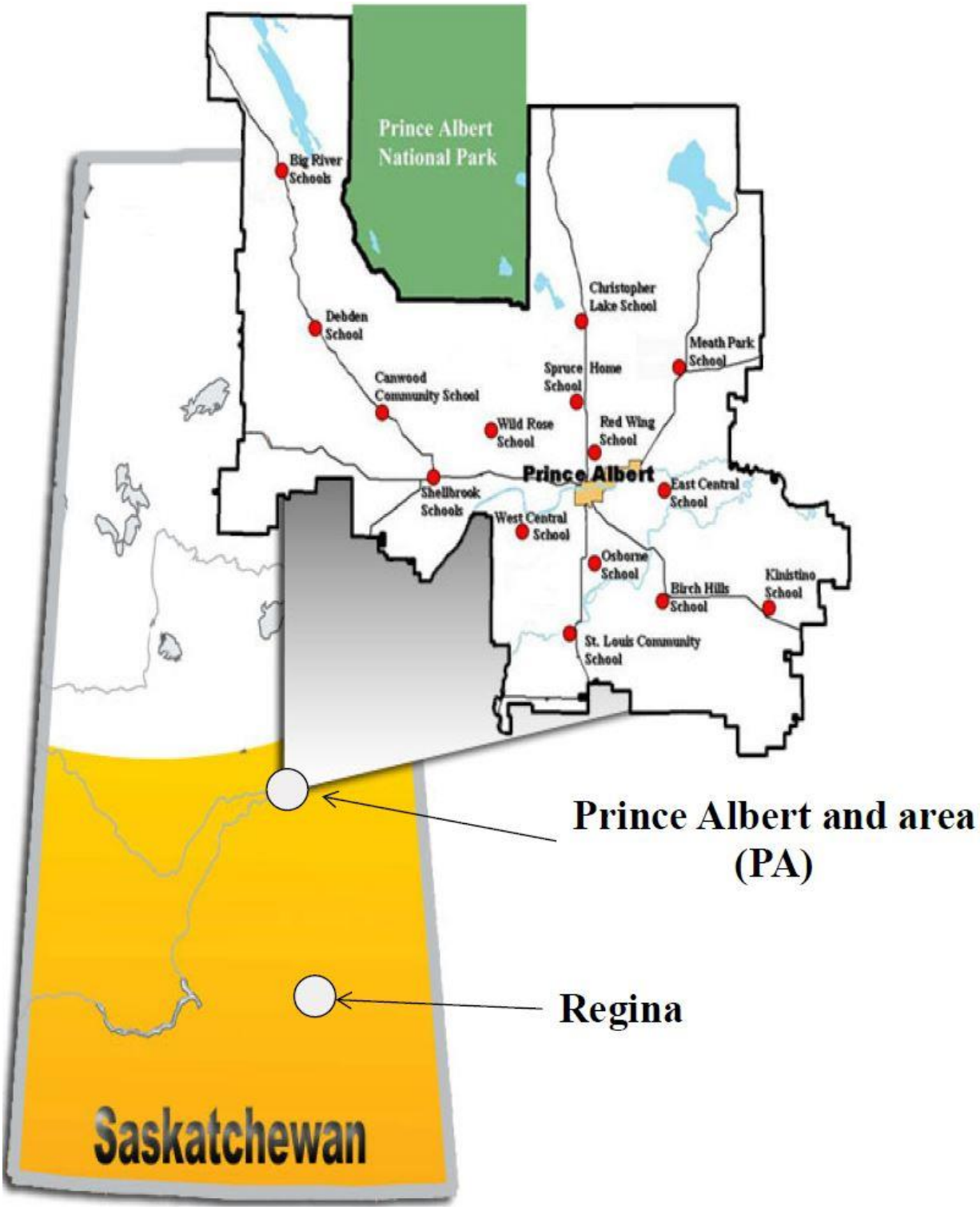


Figure 3–2: Map of Saskatchewan showing Regina, Prince Albert, and towns around Prince Albert as study locations (Source: Saskatchewan Rivers Public School Division Website: https://www.srsd119.ca/?page_id=483).

3.4 Data collection

This study was conducted during spring, fall and winter seasons (May 2015 to April 2016). The survey component was conducted between May 2015 and November 2015. Clinical testing (spirometry, exercise challenge testing, and skin prick testing) and home dust sample collection were completed concurrently between December 2015 and April 2016.

3.4.1 Subject recruitment and study population

In 2013, children from Kindergarten to Grade 8 (approximately 5–14 years) were initially recruited into a cross-sectional survey from schools using an urban-rural gradient (n = 3,509) as previously described.[6] Meetings were held with the school division directors followed by communication with school principals prior to any data collection. Schools in the large urban center were randomly selected (35 schools) due to the large number of schools. All schools in the Prince Albert Region (12 schools) and the surrounding areas (9 schools) under the same school division administration were selected for small urban and rural settings. Children had the option of participating in further survey and clinical testing. In 2015, we re-approached those who gave the consent (n = 1,348) and repeated the same cross-sectional survey followed by a clinical component (spirometry, exercise challenge testing, allergy skin testing) and environmental home dust collection component in those children and parents who consented to each of the procedure. All children, now from Grade 2 to 10 (approximately 7–17 years), were eligible to participate. Study packages, including an information letter, survey and pre-paid return envelope, were mailed to parents for completion.

3.4.2 Survey instrument and operational definitions of asthma

The survey questionnaire used standardized questions from the International Study of Asthma and Allergy in Childhood (ISAAC),[7,8] the American Thoracic Society Children's Respiratory Disease,[9] and the questionnaires used previously in Saskatchewan Lung Health studies.[10,11] The questionnaires included the core asthma, allergy, and respiratory symptoms questions as well as information on general health, parental health history, environmental exposure, and sociodemographic factors as well as housing characteristics (Appendix 3). Based on responses to the questionnaire, children were classified into one of three categories as follows (Figure 3–1):

i) **Diagnosed asthma**: Defined as a positive response on the questionnaire to either of the following questions: "Has this child ever been diagnosed as having asthma by a doctor?", "In the past 12 months, has this child taken medicine that your doctor prescribed for a breathing problem"

ii) **At-risk-for-asthma**: Defined as no report of diagnosed asthma but a positive response to any or a combination of the following questions: "Has this child ever had wheezing or whistling in the chest at any time in the past?", "Has this child ever had wheezing or whistling in the chest in the past 12 months?", "Has your child had a dry cough at night apart from a cough associated with a cold or chest infection?", "Has this child woken up at night because of cough?"

iii) **No asthma**: Defined as no reports of physician-diagnosed asthma and no asthma-like symptoms or taking medication for breathing.

3.4.3 Pulmonary function assessment

All children in the three asthma categories who consented to lung function testing performed pulmonary function testing using the PC-based Easy-On ultrasonic spirometer (ndd Medical Technologies, Zurich, Switzerland) in accordance with the American Thoracic Society recommendations for children.[12] After withholding short acting inhaled bronchodilator therapy in children with asthma for at least 6 hours before test, children completed at least three, but not more than seven, maneuvers in a sitting position while wearing a nose clip. All tests were performed in the child's school. However, children who were absent from school on the test date or whose parents wanted to be present during testing had testing completed at home. Children were tested by experienced technicians who were blinded to the asthma status of each child.

The best of 3 acceptable and reproducible efforts of FVC, FEV_1, FEV_1/FVC, and $FEF_{25\%–75\%}$ were recorded for each child. Reference equations based on the recently developed all-age, multi-ethnic Global Lung Function Initiative (GLI) was used to determine predicted values.[13] Spirometry was not performed on subjects who answered yes to questions regarding any of the following conditions in the past 3 months: "heart or abdominal surgery," and "hospitalization for any heart problems." As part of the pulmonary function testing, anthropometric data (height, weight, and waist circumference) was also obtained. Height was measured against a wall using a fixed tape measure with subjects standing in socks and in their normal indoor clothing. Weight was measured with subjects standing on a calibrated flat scale with their socks on and dressed in normal indoor clothing. Waist circumference was measured between the lowest rib and iliac crest, horizontally through the narrowest part of the torso.

3.4.4 Exercise challenge testing (ECT)

In order to consider a marker of BHR, all children who performed spirometry also performed ECT if they consented. The ECT involves stepping up and down on a 6 inch Aerobic Stepper (Merrybody Sport, China). The level of exercise intensity was selected to maintain a heart rate (HR) ≈150–200 beats per minute while stepping continuously for 5 minutes after reaching the target heart rate. HR was monitored throughout the exercise with a Polar heart rate monitor (Polar Electro, Woodbury, NY) attached to the chest wall by a strap. If necessary, study personnel also stepped with the children to provide encouragement. Spirometry was repeated 3 and 10 minutes after cessation of the exercise. These times have been reported to coincide with the predicted maximal decrease in FEV_1 and $FEF_{25\%–75\%}$ and the expected recovery period in children.[2] To determine whether EIB has occurred, percent change in FEV_1 or $FEF_{25\%–75\%}$ values from the baseline values after the exercise was computed. Children with >15% decrease in FEV_1 or a ≥25% decrease in $FEF_{25\%–75\%}$ from baseline at any of the post exercise testing intervals (3 or 10 minutes) were considered to have EIB based on recommended guidelines.[14,15]

3.4.5 Allergy skin prick testing (SPT)

Skin prick test (SPT) reactivity to six common, non-food allergens, was also completed. The allergens included *Alternaria, Cladosporium, Aspergillus*, house dust mite mix, local grass, and cat dander (Omega Laboratory, Montreal, QC, Canada). Two controls including a histamine positive control and a saline negative control were used to reduce false positives and false negatives. SPT was performed on the volar side of the child's forearm with the standardized allergen extracts according to recommended protocol of practice.[16] The wheal size diameter was measured after 15 minutes. Subjects was considered positive for atopy if a positive reaction to at

least one of the applied allergens is raised ≥3 mm compared to the saline control. All SPTs in the study were performed by trained technicians who were blinded to the asthma status of each child.

3.4.6 Home dust collection

Dust samples were collected from the floor of child's play area as well as the mattress on which the child slept. Dust was used to measure endotoxin and beta-glucan [beta-(1→3)-D-glucan] exposure. Dust was collected by using one of two Solaris Turbo Plus vacuum cleaners (Model: Miele S514, Germany). The power for the two cleaners was set at 950 W which exceeds the minimum power recommended according to the ISAAC protocol.[17] Prior to data collection, the two vacuum cleaners (VC) were calibrated for flow rate and static pressure at the College of Engineering, University of Saskatchewan. The flow rate vs. static pressure curves for each of the two cleaners were found to be similar prior to data collection (VC 1: $R^2 = 0.9996$; VC 2: $R^2 = 0.9999$) and post data collection (VC 1: $R^2 = 0.9998$; VC 2: $R^2 = 0.9986$).

The X-Cell-100 dust collection filter socks (Midwest Filtration LLC, OH, USA; Figure 3–3) were used to obtain dust samples. The pore size of the filter was between 4.0 and 12.3 microns. The filter socks were in sterile condition from the manufacturer. Prior to data collection, each filter sock was placed into individual Ziploc bags which were labeled with a unique identification number (ID) and no other information (for confidentiality and blinding reasons). Each filter bag was then weighed using the Adventurer Balance (Model AR1530, Ohaus Corp, Pine Brook, NJ, USA) at the Canadian Center for Health and Safety in Agriculture's National Agricultural and Industrial Hygiene Laboratory (CCHSA's–NAIHL) in the D-Wing of the Health Sciences Academic Complex at the University of Saskatchewan.

Figure 3–3: The X-Cell 100 Dust Sampling Sock used for dust sample collection (Source: Midwest Filtration LL website: http://www.midwestfiltration.com/dust-sampling.php).

In each home and for different locations within the home (play area or mattress), a new filter sock was placed into the distal end of the extension tube of vacuum cleaners and sealed with a clean crevice device tool that was placed over the distal end of the extension tube for dust collection. Sampling area and time for dust collection followed the standardized ISAAC protocol.[17] Floors with wall to wall carpet had $2m^2$ vacuumed for 4 minutes while smooth floor with at least $4m^2$ of carpet had $2m^2$ vacuumed for 4 minutes. Completely smooth floor or floor with one or two small carpets had $4m^2$ vacuumed for 4 minutes. For mattresses, dust sample collection was completed with the bottom sheet (the sheets that the child slept on) in place over

the mattress during vacuuming with comforters, pillows, and duvets removed. The length and width of the mattress were measured and the whole area of the mattress was vacuumed for 2 minutes. In order to correct for any modifying factors during the dust sampling process, a blank sample was collected for every sixth house visited according to recommended protocol.[17]

Following dust sample collection, filter socks were placed back in the Ziploc bag and transported to CCHSA's–NAIHL for further processing. At the CCHSA's–NAIHL laboratory, the filter socks containing the dust samples were weighed after dust sample collection by the same person that weighed them prior to data collection, using the same scale. To minimize errors in dust weight, pre- (filter socks only) and post-data collection (filters socks with dust sample) weights were completed in triplicate and the average weight recorded. Differences in pre- and post-data collection weights were recorded and the samples were stored desiccated in a fridge at 4^{o}C until extraction and analysis.

3.4.7 In-home assessment

During home visit for dust sample collection, the technician conducted a brief inspection of the home to visually assess indoor home characteristics. The technician walked through the major rooms in the house including the child's bedroom, living room, kitchen, bathroom, and dining areas. In each location, the presence of mold or dampness was noted. In addition, the technician noted the presence of a mildew odor or musty smell, presence of pets and the presence of air quality equipment such as heating sources (firewood or natural gas), air conditioners, humidifier, dehumidifier, or heat recovery ventilator (HRV) system.

3.5 Dust sample extraction procedures

The step-by-step procedures for dust extraction are shown in Appendix 4. Dust samples were sieved through a 300 μm mesh sieve, weighed and stored desiccated at 4^0C. Prior to extraction, samples were brought to room temperature and 10 mg (0.010 g) of sieved dust sample was measured into a 50 mL conical tube for extraction. Dust samples were extracted with 20 mL 0.05% Tween-20 solution (GE Healthcare Bio-Science, Mississauga, ON, Canada) in pyrogen-free distilled water and shaken at 325 revolution per minute (RPM) for 2 hours using the Thermo Scientific MaxQ 2000 Bench Top Shaker (ThermoFisher Scientific, Mississauga, ON, Canada). The extracted solution was then centrifuged at 1,000 g (gravity force; g-force) for 15 minutes using the Sorvall ST 16R centrifuge (ThermoFisher Scientific, Mississauga, ON, Canada) to obtain supernatants. The supernatants were then aliquoted in approximately 1.0 mL into 1.5 mL microcentrifuge tubes and stored at -80ºC pending endotoxin and beta-(1→3)-D-glucan analyses.

3.6 Microbial endotoxin and beta-(1→3)-D-glucan analysis procedures

Frozen aliquots were allowed to attain room temperature before analysis.

An aliquot of the supernatant was diluted 1 in 10, and was used to measure endotoxin. Endotoxin analysis was performed using the *Limulus* Amoebocyte Lysate (LAL) assay according to manufacturer's recommendations (Appendix 4). Briefly, 100 μL of five endotoxin standards (range from 0.005–50 EU/mL) extracts and the LAL reagent water blank were dispensed into a 96-well plate (Appendix 5) and used to construct a standard curve from which the endotoxin activity of the samples were calculated. After 10 minutes incubation at 37ºC, 100 μL of the Kinetic-QCL was added to each of the well to activate enzymatic reaction. The absorbance was monitored at 405 nm for 2 hours, using the ELx808 spectrophotometric plate reader (BioTek, Winooski, VT, USA). The

activated enzyme releases p-nitroaniline (pNA) from a synthetic substrate, producing a yellow color. Using the initial absorbance reading of each well as its own blank, the time required before the appearance of a yellow color was determined and considered as the reaction time. The amount of endotoxin present is inversely proportional to the reaction time. The concentration of endotoxin in unknown samples was referenced to a standard curve and computed using linear correlations. The kinetic LAL assay is optimized to be linear from 0.005 EU/mL to 50.0 EU/mL. Endotoxin levels were expressed as concentration [endotoxin units per milligram of dust sample (EU/mg)] and as load [EU per square meter of sampled area (EU/m^2)].

Soluble beta-(1→3)-D-glucan levels in the second aliquot were measured with the Glucatell assay kit based on the Kinetic Onset Time protocol according to manufacturer's specifications (Associate of Cape Cod, East Falmouth, MA) (Appendix 4). Similar to endotoxin analysis, 100 μL of six beta-(1→3)-D-glucan standards (range from 3.125–100 μg/mg) extracts and the Glucatell reagent water blank were dispensed into a 96-well plate (Appendix 5) and used to construct a standard curve from which the beta-(1→3)-D-glucan activity of the samples were calculated. In contrast to the endotoxin analysis, the absorbance of the beta-(1→3)-D-glucan was monitored at 405 nm for 1 hour using the ELx808 spectrophotometric plate reader (BioTek, Winooski, VT, USA). The Glucatell assay is a modified LAL assay but is based upon the same principles described for endotoxin. The only difference is that, rather than activating factor C originally used for endotoxin detection, the Glucatell assay activates factor G leading to a series of enzymatic reactions.[18] Due to the removal or disabling of factor C from the enzymatic reaction pathways, the glucan-specific LAL assay does not cross-react with endotoxin allowing for beta-(1→3)-D-glucan detection while avoiding false positive results.[19,20] Similar to endotoxin, beta-(1→3)-D-glucan levels were expressed as concentration [per gram of sampled dust (μg/g)] and loading [per square meter of sampled area ($\mu g/m^2$)].

3.7 General statistical analysis considerations

Statistical analyses were completed with the Statistical Package for the Social Science (SPSS) Version 24 (SPSS Inc. Armonk, NY: IBM Corp.) and the Statistical Analysis System (SAS) Version 9.4 (SAS Institute Inc., Cary, NC, USA). Statistical significance was defined by an alpha level of 0.05. For each objective, descriptive analyses using frequencies and proportions for categorical variables and means with standard deviations for continuous variables were completed.

Following descriptive analyses, multiple logistic regression and multiple linear regression models were fitted as appropriate to examine association of endotoxin and beta-(1→3)-D-glucan with asthma phenotypes or severity as appropriate (Objectives 2 and 3, respectively). Strengths of association were assessed by the odds ratio (OR) and 95% confidence interval (95%CI) if logistic regression model was used and by beta coefficient (standard error) if linear regression model was used.

3.7.1 Analysis for Objective 1

The main outcomes for Objective 1 were survey-based asthma classification (report of physician-diagnosed asthma) and algorithm-based asthma classification [positive for asthma based on combinations of survey symptoms report and clinical testing (spirometry and ECT)]. Proportions of children "positive for asthma" by survey-based and algorithm-based methods were compared for each location using the McNemar test for correlated proportions. Lung function variables were also compared by location of residence using one-way between-group analysis of variance (ANOVA) and analysis of covariance (ANCOVA) as appropriate. A more detailed description of the statistical analysis is located in Chapter 4.

3.7.2 Analysis for Objective 2

The outcomes for Objective 2 were asthma phenotypes assessed, separately, as atopic vs. non-atopic asthma (based on atopic sensitization) and EIB vs. no EIB (based on results of ECT). Multiple logistic regression models were fitted to test the association between endotoxin and beta-(1→3)-D-glucan exposure with asthma phenotypes. Separate models were fitted for play area and mattress endotoxin and beta-(1→3)-D-glucan exposure variables. Variables were entered into the model based on the purposeful selection procedure by Hosmer to account for potential confounders.[21] A more detailed description of the statistical analysis is located in Chapter 5.

3.7.3 Analysis for Objective 3

The outcomes for Objective 3 were asthma severity categories (mild persistent asthma vs. moderate/severe persistent asthma) as determined by the NAEPP asthma severity classification guidelines[2] as well as lung function. Similar analyses and models for Objective 2 were used for Objective 3 to examine the relationships between endotoxin and beta-(1→3)-D-glucan exposure with asthma severity. In addition, multiple linear regression models were fitted to examine associations between endotoxin and beta-(1→3)-D-glucan exposure and lung function variables. A more detailed description of the statistical analysis is located in Chapter 6.

3.8 Sample size and power calculation summary

The main purpose of the study was to investigate urban-rural asthma diagnosis patterns, as well as the relationships between indoor microbial exposures [endotoxin and beta-(1→3)-D-glucan as

biomarkers of exposure] and asthma phenotypes and severity among children with asthma in Saskatchewan. The sample size to achieve these objectives was calculated using the G*Power statistical software (Version 3.1.7). All sample size estimates were calculated based on two-tailed analysis with alpha level set at 0.05 while power was set at 80% (1-β).

The initial sample size calculation for this study suggested a total sample size of n = 540 for Objective 1, n = 125 for Objective 2, and n = 85 for Objective 3 (Appendix 6). In the end, we had n = 335 for Objective 1, n = 99 for Objective 2, and n = 102 for Objective 3. While the sample size obtained (n = 335) was below the initial estimated sample size (n = 540), statistically significant associations were observed for each objective suggesting that the power of the study was sufficient for some of the associations investigated. Despite this, there were some relatively strong strengths of association observed in the analyses for Objective 3 that did not reach statistical significance. This could be an indication that power was not sufficient for all associations considered.

3.9 Ethical approval

Ethical approval was obtained from the University of Saskatchewan Biomedical Research Ethics Board (Bio # 14–162). The original ethical approval for the study is Bio # 11–03 with annual progress and amendment completed (Appendix 7). In addition to these approvals, the local Catholic School Board and Public School Board in each location of the study approved the study. Finally, prior to taken part in the study, parents/legal guardians/legal caregivers were required to complete a consent form and children were required to complete an assent form (Appendix 8). These forms were included as extra pages in the survey questionnaires mailed to the address of participants. Completion and return of questionnaire including the consent and assent pages implied voluntary consent by the children and their custodians. During the clinical assessment

and home dust collection, parents/guardians/caregivers and the participating children were further asked to confirm their voluntary participation in the study.

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CHAPTER 4

ASTHMA DIAGNOSIS AMONG CHILDREN ALONG AN URBAN-RURAL GRADIENT

(MANUSCRIPT I)

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4.1 Abstract

Background: Studies have reported lower asthma prevalence in rural compared to urban areas. While environmental factors have mostly been implicated for these differences, the lower asthma prevalence could also be linked to asthma under-diagnosis in rural children.

Objectives: We investigate if rural children experience under-diagnosis of asthma more compared to urban children.

Methods: In 2013, we conducted a cross-sectional survey of schoolchildren (5–14 years) across an urban-rural gradient in Saskatchewan, Canada. In 2015, we approached those who gave consent for further testing (now age 7–17 years) to repeat the survey, and to conduct clinical testing (spirometry and exercise challenge testing). Based on survey responses, children were classified into "no asthma", "at-risk-for-asthma", and "diagnosed asthma". We then classified asthma status as either "no asthma" or "probable asthma" based on a validated asthma algorithm.

Results: The study population of 335 schoolchildren was comprised of 73.4% from large urban, 13.7% from small urban and 12.8% from rural. Proportion with report of physician-diagnosed asthma was 28.5% (Large urban), 34.8% (Small urban), and 20.9% (Rural). Mean percent predicted FEV_1 and $FEF_{25\%–75\%}$ were lower in rural compared to small urban and large urban children ($p<0.05$). Among those not classified as diagnosed asthma by the survey, the algorithm further identified the presence of asthma in 5.5% large urban, 8.1% small urban, and 18.8% rural children ($p=0.03$).

Conclusion: The study revealed evidence of asthma under-diagnosis in rural areas and further supports the use of objective measures in addition to symptoms history when investigating asthma across urban-rural gradients.

Key words: Asthma algorithm, asthma diagnosis, pulmonary function, schoolchildren, urban-rural gradient.

4.2 Introduction

More than 13% of school-age children are estimated to have diagnosed asthma in Canada,[1] with childhood asthma being a leading cause of morbidity and medical expenses among children.[2,3] While the burden of asthma is high among children,[4] its prevalence varies geographically with most studies reporting lower asthma prevalence in rural compared to urban areas.[5–10] Environmental factors have mostly been implicated for these differences. However, the association could also be linked to possible under-diagnosis of asthma in rural children.

In a national sample of Canadian adolescents, asthma prevalence was lower in rural compared to urban children but no differences were observed in the prevalence of wheeze symptoms.[7] Rural US children had a lower report of diagnosed asthma compared to urban children despite increased report of asthma-related symptoms among rural children.[11] In our recent study in Saskatchewan, Canada, similar results were observed where rural children had a lower prevalence of asthma despite increased prevalence of wheeze among children with asthma.[12] Results from these studies suggest that diagnostic differences may be contributing to the lower asthma prevalence among rural children.

While there are clinical assessment methods such as spirometry and fractional exhaled nitric oxide (F_ENO) that may aid the diagnosis of asthma, no one test alone is considered as standard diagnostic test for asthma in children.[13,14] To date, assessments are largely based on history and response to pharmacotherapy.[15] However, while the expression of recurring symptoms of wheeze may provide evidence of asthma among children,[13,16] objective lung function assessment is recommended to improve diagnostic accuracy.[17,18] It is uncommon in population-based epidemiological studies when investigating geographic variations in asthma prevalence to conduct clinical investigations. The use of symptom history in combination with

clinical assessment may help in assessing the true burden of childhood asthma along an urban-rural gradient.

Our overall aim was to investigate if rural children experience more asthma under-diagnosis compared to urban children. We hypothesized that the addition of an objective clinical test would better identify cases of true asthma. As part of this investigation, we investigated differences in lung function and exercise challenge test (ECT) results along an urban-rural gradient and whether the addition of clinical measures (lung function testing and ECT) improved the diagnostic classification of asthma.

4.3 Methods

4.3.1 *Study design and location*

This was a cross-sectional study conducted across an urban-rural gradient in the province of Saskatchewan, Canada. The study locations included Regina (population approximately 200,000[19]), Prince Albert (population approximately 35,000[20]), and the rural area (small town, farm and non-farm) in the region around Prince Albert (population <2,500 people). Location of dwelling was classified as "Large Urban", "Small Urban", or "Rural" based on whether the child lived in Regina, Prince Albert or the rural farm and non-farm locations surrounding Prince Albert, respectively. The urban-rural gradient parallels Statistics Canada definitions based on modified Beale codes where definitions of large urban, small urban, and rural match those of small metropolitan (urban settlements of 50,000 to 249,999 people), non-metropolitan small city zone (20,000–49,999 people), and rural (<2,500 people).[21]

4.3.2 *Study population, selection and recruitment*

Participants in this study were from an initial 2013 cross-sectional study of schoolchildren attending Kindergarten to Grade 8 as previously described.[12] The 2013 cross-sectional survey was conducted to investigate the prevalence of childhood asthma and asthma-related symptoms in the region. At this time, children had the option of participating in further survey and clinical testing. In 2015, we approached those who gave previous consent for follow-up, repeated the survey, and conducted clinical assessments. The current study population was comprised of children in Grades 2 to 10 (approximately 7–17 years). Study packages, including an information letter, survey and pre-paid return envelope, were mailed to parents for self-completion.

In order to obtain accurate information on current respiratory symptoms that correspond to lung function values in our studied population, the results presented in the current study were based on data from the 2015 survey only, which was completed just prior to lung function testing.

The study was approved by the University of Saskatchewan Biomedical Research Ethics Board (Bio #: 14–162). Completion and return of the survey implied voluntary consent for the questionnaire portion. All children and a parent provided written assent and consent, respectively, prior to clinical testing. All school divisions involved approved the study.

4.3.3 *Data collection instruments*

4.3.3.1 *Survey instrument and operational definition*

Surveys were based on standardized questionnaires from the International Study of Asthma and Allergy in Childhood (ISAAC),[22,23] the American Thoracic Society Children's Respiratory Disease,[24] and questionnaires used previously in Saskatchewan lung health

studies.[25,26] The ISAAC questionnaire has been shown to have high validity when compared to physician assessment of asthma[16,27] and have been used across a range of pediatric groups.[28,29] Questions about respiratory and allergic symptoms, general health, parental health history, environmental exposures, and socio-demographic factors as well as housing characteristics were included. Children were classified into one of 3 asthma categories based on questionnaire responses (survey-based asthma classification): "diagnosed asthma", "at-risk-for-asthma", or "no asthma". Physician-diagnosed asthma (probable asthma) was defined as a positive response to: "Has this child ever been diagnosed as having asthma by a doctor?" and/or a positive response to: "Has this child taken prescribed asthma medication in the past 12 months?" At-risk-for asthma was defined as positive responses to wheeze or whistling symptoms or other respiratory symptoms such as cough and shortness of breath but no diagnosed asthma or asthma medication. Furthermore, the definition of at-risk-for-asthma in this study was based on symptoms report but not on risk factors such as parental history of asthma or allergy. Similar definition of at-risk-for-asthma in this study has also been used in previous studies.[11,30]

4.3.3.2 *Pulmonary function assessment*

Of the 335 children who participated in the study, a total of 288 (86%) performed spirometry testing. Spirometry assessment was completed according to recommended standards for children[17,18] using the Easy-on-PC spirometer (ndd Medical Technologies, Zurich, Switzerland). Some subjects were excluded from testing because they were unable to perform the test due to existing medical conditions (n = 3). Subjects who were unwilling to perform the test (did not consent to testing) were also excluded (n = 44). There were no significant differences across diagnosis groups or urban-rural gradient among the 44 subjects that refused to participate in pulmonary function tests (p>0.05 for both). Tests were performed in the child's school or at

home within normal indoor temperature (range: 21ºC–28ºC) and relative humidity (range: 35%–45%). Children were tested by experienced technicians who were blinded to the asthma status of each child. Data were assessed for quality and completeness by the same technicians. At least three successful and repeatable maneuvers were performed for each child. Lung function values for forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1/FVC ratio, and forced expiratory flow between 25% and 75% of FVC ($FEF_{25\%-75\%}$) were obtained. Predicted values were based on the all-age, multi-ethnic Global Lung Function Initiative (GLI) reference equation.[31]

4.3.3.3 *Exercise Challenge Testing (ECT)*

Because children with stable asthma could produce normal spirometry results,[32] all children were further tested with ECT[33] shortly after spirometry assessment to help identify children with a positive indication of asthma. Tests were also performed in the same location where the child performed spirometry testing (i.e. either child's school or at home). Of the 288 subjects who performed spirometry testing, 281 (98%) further completed the ECT by stepping up and down on a 6 inch Aerobic Stepper (Merrybody Sport, China). After attaining exercise intensity level (stepping rate) that placed their heart rate between 150–200 beats per minute, children were required to step continuously for 5 minutes at the target heart rate. Heart rates were monitored throughout the exercise with a Polar heart rate monitor (Polar Electro, Woodbury, NY). Spirometry was repeated 3 and 10 minutes after cessation of exercise. These times have been reported to coincide with the predicted maximal decrease in FEV_1 and $FEF_{25\%–75\%}$ and the expected recovery period in children.[34]

4.3.3.4 *Algorithm-based asthma classification*

Following questionnaire responses and clinical testing, we used the validated asthma algorithm developed by Gerald *et al*[30] (Appendix 9) to identify children positive for asthma. All children who had FEV_1/FVC ratio <80% upon spirometry testing; or if they demonstrated a >15% decrease in FEV_1 or a ≥25% decrease in $FEF_{25\%–75\%}$ from baseline at any of the post exercise testing intervals (3 or 10 minutes) were considered to be positive for asthma (probable asthma).[32,35] The spirometry and ECT results were finally used to classify children into two distinct asthma groups: "positive for asthma" or "no asthma".

4.3.4 *Statistical analysis*

Statistical analyses were completed with the Statistical Package for the Social Sciences (SPSS) Version 24 (SPSS Inc. Armonk, NY: IBM Corp.) and the Statistical Analysis System (SAS) Version 9.4 (SAS Institute Inc., Cary, NC, USA). Categorical demographics, environmental and respiratory symptoms were compared between locations of dwelling using the independent samples chi-squared (χ^2) and Fisher's exact tests as appropriate. A one-way between-group analysis of variance (ANOVA) was conducted to compare differences in mean values for percent predicted lung function variables between locations of dwelling. Scheffe pairwise *post hoc* comparisons were used to assess if there were statistically different levels of lung function between locations following the overall ANOVA assessment. These analyses were also repeated after stratification by asthma status. Finally, proportions of children "positive for asthma" by survey-based and algorithm-based methods were compared for each location using the McNemar test for correlated proportions. Finally, to confirm if rural children are more likely to be misclassified for asthma based on survey report of physician-diagnosed asthma, we created a

variable for asthma misclassification using the survey-based and algorithm-based asthma classification. Asthma misclassification was given if the subject had no affirmative response to physician-diagnosed asthma question on the survey but identified as positive for asthma based on the asthma case-detection algorithm. Logistic regression analysis was performed to assess associations between location of dwelling and asthma misclassification adjusting for age, sex, ethnicity, smoke exposure, parental education level, and parental history of allergy.

4.4 Results

4.4.1 *Study population characteristics*

A total of 335 children participated in the current study and differed significantly in some characteristics (age, parental education, tobacco smoke exposure, and parental history of allergy) compared to the original group in 2013 survey (Table 4–1). Of the 335 children that participated in 2013, most (73%) were large urban, 14% were small urban, and 13% were rural residents. The socio-demographic, personal and environmental characteristics of the participants by location of dwelling are shown in Table 4–2. Compared to large urban and small urban, rural children, on the average, were approximately 2 years older, 2 kg heavier, and travelled 28 minutes longer to access medical care. Rural children were more likely to be female and Caucasian compared to small urban and large urban children. Small urban children had a higher proportion of parents who smoked, parents with a history of allergic diseases, and were more likely to be exposed to secondhand tobacco smoke compared to children from large urban and rural locations. In terms of indoor environmental characteristics, rural children were more likely to own a pet but less likely to live in homes that have natural gas heating, an air conditioner, or a humidifier compared to urban children.

4.4.2 *Previous self-reported asthma-related symptoms based on questionnaire reports*

Based on the parental response to the questionnaire, proportion with report of physician-diagnosed asthma, at-risk-for-asthma and absence of asthma was 28.4%; 36.1% and 35.5%, respectively. However, rural children with physician-diagnosed asthma had a higher proportion of chronic bronchitis compared to large urban children while small urban at-risk-for-asthma children had a higher proportion of cough symptoms compared to large urban children (Table 4–3). Although results were not statistically significant, a higher proportion of urban compared to rural children reported taking prescribed asthma medications if they had physician-diagnosed asthma (70% vs. 66.7%; p=0.89) and if they were at-risk-for-asthma (22.7% vs. 13.3%; p=0.46); respectively.

4.4.3 *Pulmonary function measures among participants*

Lung function variables differed significantly by location of home (Table 4–4). Overall, mean percent predicted FEV_1 and $FEF_{25\%–75\%}$ were lower in the rural children compared to small urban and large urban groups. However, after stratification by survey-based asthma classification status, the lower mean values for FEV_1 and $FEF_{25\%–75\%}$ seen in the rural group were only found in the at-risk-for-asthma children and not in the diagnosed asthma or no asthma groups. Figure 4–1 presents the comparison of lung function variables indicative of bronchial hyperresponsiveness (FEV_1 and $FEF_{25\%–75\%}$) before and after exercise. Baseline absolute values before exercise and at 3 and 10 minutes after exercise were significantly lower in the at-risk-for-asthma rural children in comparison to at-risk-for-asthma large urban and small urban children (p<0.05 for both FEV_1 and $FEF_{25\%–75\%}$) but not in the diagnosed or no asthma groups. Similarly,

change in baseline FEV_1 and $FEF_{25\%-75\%}$ after cessation of exercise were also significantly lower in at-risk-for-asthma rural children compared to at-risk-for-asthma large urban children.

4.4.4 *Investigation of the algorithm-based asthma classification system*

Figure 4–2 shows the results of the asthma case-detection algorithm procedure. Among those not classified as asthma by survey ("No asthma" and "At-risk-for-asthma" groups), the combination of spirometry and ECT identified 5.5% (large urban), 8.1% (small urban), and 18.8% (rural) more children as positive for asthma; p=0.026. Among those classified as asthma by survey, 31.6% met the spirometry and ECT criteria for asthma diagnosis (Large urban = 57.1%, Small urban = 14.3%, and Rural = 28.6%). Overall, in addition to the 95 children with physician-diagnosed asthma identified by survey questionnaire, the algorithm further identified 21 children positive for asthma for a combined total of 116 children positive for asthma. In each of the location of dwelling, the proportions of children with positive indication of asthma from the 3-stage asthma case detection algorithm (those classified as having probable asthma with the questionnaire and those classified as having probable asthma with spirometry or ECT) were higher compared to those identified as positive for asthma by survey questionnaire alone, and was statistically significant for rural settings (34.9% vs. 20.9%; Figure 4–3).

To ensure that confounding was not the reason for under-diagnosis, we assessed associations between location of dwelling and asthma misclassification using logistic regression analysis. After adjusting for age, sex, ethnicity, parental smoking, parental education level, and parental history of allergy, rural [odds ratio (OR) = 8.19, 95%CI: 2.31–29.10) children were significantly more likely to be misclassified as "no asthma" compared to large urban children when asthma diagnosis was based on survey report of physician-diagnosed asthma.

Finally, since exercise-induced bronchospasm can be thought of as a unique entity, we also completed the analysis after exclusion of those with positive ECT. The results showed that the proportion of children positive for asthma was 30.6% (Large Urban), 37.8% (Small Urban), and 31.3% (Rural) which are similar to and confirm our results when using ECT. Also, after excluding children with positive ECT from the "No asthma" group, the proportion of children with positive indication of asthma using the asthma case-detection algorithm was 31.7% (Large Urban), 40% (Small Urban), and 34.9% (Rural).

4.5 Discussion

The current study revealed potential evidence of asthma under-diagnosis in rural areas. Compared to large urban and small urban children, rural children without a history of diagnosed asthma based on survey responses were more likely to be reclassified as positive for asthma when objective measures (spirometry and ECT) were used. Also, children at-risk-for-asthma in rural areas had lower lung function than other locations.

Many of the previous studies that have investigated urban-rural differences in childhood asthma used survey questionnaire in assessing variations in asthma burden across locations of dwelling. Findings from these studies showed lower asthma prevalence in rural compared to urban children.[6,7,10,36,37] Furthermore, when questionnaire reports of asthma diagnosis and symptoms were both considered, asthma diagnosis has been shown to be lower in rural children despite symptoms consistent with asthma diagnosis being similar across an urban-rural gradient[7,38] or even higher in rural compared to urban children.[11] In the Canadian portion of the International Health Behavior in School-Aged Children (HBSC) study (aged 6–10 years), Lawson *et al* found an urban-rural gradient for asthma prevalence, with lower asthma in rural areas, but the prevalence of wheeze was similar across locations.[7] In a separate study that

compared results from two cross-sectional studies conducted at two different intervals (1994 and 2002) among 6–7 and 13–14 years old children in 8 different centers in Italy, there was a significant increase in doctor-diagnosed asthma among urban compared to rural children despite similar prevalence of asthma-related symptoms between the two locations.[38] Another study among schoolchildren (aged 4–17 years) from Arkansas, USA, showed similar results where rural children had an increased report of asthma-related respiratory symptoms and a slightly lower prevalence of physician-diagnosed asthma compared to urban children.[11] These results suggest that the apparent lower prevalence of childhood asthma in rural locations may be linked to under-diagnosis. This may be particularly important among children with asthma-related symptoms.

In this report, we have confirmed these data using questionnaires but also lung function measures. Among children in the at-risk-for-asthma group, rural children had significantly lower lung function compared to their small urban and large urban counterparts. These results were not seen among the asthma and no asthma groups, which had similar lung function across the urban-rural gradient. This may be due to better asthma management among the asthma group. Furthermore, the values for FEV_1 and FEV_1/FVC (both >80%) among the at-risk-for-asthma rural children indicate relatively good lung function at present. However, the fact that these values were significantly lower in this group compared to large urban and small urban children may indicate possible future decline in lung function particularly if the at-risk-for-asthma rural children continue to be unrecognized for asthma diagnosis resulting in suboptimal management of their respiratory conditions. This is further evidenced from the Dunedin birth cohort study in New Zealand (n = 613) where children with persistent wheeze at age 9 and 13 years had

significantly lower lung function (as measured by FEV_1, FEV_1/FVC, and $FEF_{25\%–75\%}$) at age 26 years relative to those without persistent wheezing.[39]

While urban-rural environmental exposures, particularly farming exposures in the rural areas, has been the most common explanation for low asthma prevalence in rural locations,[40–42] accessibility of healthcare services for diagnosis may also play an important role. We did not access the potential role of accessibility to healthcare services in the current study. However, we found that rural parents travelled approximately 28 minutes longer, on average, compared to large urban and small urban dwelling parents to receive healthcare for their child ($p<0.001$). Similarly, in a US-based cross-sectional study, rural residents were found to travel approximately 15 kilometers longer, on average, compared to urban residents to access healthcare services.[43] Thus, the decision to take a child to a healthcare facility for asthma diagnosis and subsequent treatment/management of asthma conditions could have financial implications in rural settings that are not always seen in the urban areas.

Speculatively, individuals living in rural areas may choose to visit a physician differently than urban populations, which could be due to access issues. This barrier to healthcare services might have contributed to failure to properly diagnose asthma among symptomatic rural children since rural children who might otherwise be eligible for asthma diagnosis must also have the means to travel to the location of care before they can be diagnosed. In a study among 6–14 year olds schoolchildren (n = 3,090) in two rural Iowa counties in USA, asthma prevalence in the rural areas was reported to rival those in large cities.[44] However, approximately 42% of the rural children with frequent symptoms of asthma (night- or day-time cough, wheeze or shortness of breath) reported ever been given a diagnosis of asthma by a physician. We suggest that objective tests are necessary when investigating asthma prevalence, especially across geographical

locations, realizing that differential patient presenting or survey reports of physician diagnosis of asthma may be underestimating the true burden of asthma across urban-rural gradient.

The primary result of this study showed that rural compared to large urban children were more likely to experience asthma under-diagnosis. However, we must also evaluate the possibility of asthma over-diagnosis among the large urban children. Among children with survey-based report of physician-diagnosed asthma, only 30 (31.6%) met the spirometry and ECT criteria for asthma diagnosis (Large urban = 57.1%, Small urban = 14.3%, and Rural = 28.6%; p=0.03). Therefore, it is possible that the higher proportion of survey-based physician-diagnosed asthma in the large urban children may be due to some labeling bias or over-diagnosis of asthma in children living in large urban areas. Due to better access to healthcare services in the large urban children, other respiratory symptoms similar to but not directly related to asthma diagnosis might have been misinterpreted as asthma upon presentation to hospitals, resulting in asthma over-diagnosis in the large urban children. However, since the asthma diagnosis and management guidelines are standardized,[35] asthma management should be similar between urban and rural locations in Canada.[45] The most plausible explanation for the remaining 68% of the survey-based physician-diagnosed asthma children for not meeting the spirometry and ECT criteria for asthma diagnosis could be that their asthma conditions were well controlled. Most of the children in the physician-diagnosed asthma compared to at-risk-for-asthma group (76.3% vs. 26.4%; p<0.001) had been taking inhaled corticosteroid in the past 12 months.

This study has some limitations. The participation rate we experienced was low, especially in the small urban and rural locations. It is important to bear in mind that participants for this kind of cross-sectional field study are frequently hard to reach, especially in the small urban and rural locations where many participants live on farms and only a low number of

participating students are found per school. However, as our results indicated statistical significant differences, the power of the study was sufficient to show the hypothesized effect. Also, we cannot exclude the possibility that there may be response bias in our sample as indicated by differences in some of the participants' characteristics in the current study compared to the original group in 2013. While this may inflate some of the estimates observed in this study, we would expect that this would occur non-differentially between locations of dwelling allowing our interpretation of the results to still remain valid. Also, the potential presence of a biased sample in the current study may not be a major problem since we were interested in asthma diagnostic differences within each location of dwelling as opposed to asthma prevalence differences across location of dwelling. The algorithm used to identify subjects for asthma in the current study incorporated ECT as part of the asthma case-detection procedure.[30] While exercise-induced bronchospasm resulting from ECT can also occur in individuals without asthma, ECT has demonstrated a 57% sensitivity and 90% specificity in identifying children with positive indication of asthma.[46] To ensure that we were not falsely diagnosing asthma in some children, we reanalyzed the data excluding those with positive ECT. The results were similar to what we obtained when using ECT. Finally, participants in this study were from an urban-rural gradient in the province of Saskatchewan, Canada. Since our definition of urban-rural gradient parallels the Statistics Canada definitions which considers population size, density, and distance to metropolitan areas,[21] our findings might also reflect similar urban-rural patterns in asthma diagnosis if children of similar age range were screened for asthma using same asthma algorithm in other provinces.

Our study also had several strengths. We used a standardized and validated survey instrument.[22,23] In addition to this, we used objective measures of lung health (spirometry and

ECT) as part of a well-developed and validated asthma case-detection algorithm[30] across all the regions included in this study. This algorithm has high sensitivity (82%) and specificity (93%) when compared to clinical assessment of asthma by a physician as "gold standard". Finally, all equipment, techniques, and quality control for testing were based on those recommended by standard guidelines[17,18] and were identical across all locations so that bias in the observed urban-rural differences in lung function would be minimized.

In conclusion, there is little published information about an urban-rural gradient in asthma diagnosis. This study provides evidence of rural under-diagnosis of asthma and further supports the use of objective measures in addition to symptom history when investigating asthma across an urban-rural gradient. Rural children with asthma-like symptoms following questionnaire screening who do not have physician-diagnosed asthma nevertheless were found to have reduced lung function. This provides important evidence that the often reported lower prevalence of asthma in rural compared to urban areas may be due, in part, to asthma under-diagnosis in rural locations.

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Table 4–1: Comparison of characteristics between participants in the 2013 baseline survey and those in the 2015 follow-up study

	Did not complete the clinical testing phase (2013) (n = 3338)	Completed the clinical testing phase (2015) (n = 335)	*p*-value
Personal characteristics			
Mean age (±SD), years	9.52 (2.76)	9.03 (2.52)	<0.001
% Female	50.6	48.5	0.46
% > high school (maternal)	73.7	86.1	<0.001
% > high school (paternal)	67.2	78.3	<0.001
Ethnic background			
% Caucasian	62.6	79.8	<0.001
% Others	37.4	20.2	
Tobacco smoke exposure			
% Maternal smoking	18.7	4.6	<0.001
% Paternal smoking	24.3	12.9	<0.001
% Either parent smoking	31.3	14.7	<0.001
Indoor characteristics			
% Pet ownership	52.2	53.2	0.73
% Dampness in home	16.3	19.2	0.18
% Home mold	12.2	11.0	0.55
% Air conditioner	71.2	76.7	0.04
% Air filter	63.2	64.9	0.57
% Humidifier	38.3	41.5	0.29
Parental history of asthma and allergies			
% Parental history of asthma	16.9	20.9	0.07
% Parental history of allergy	37.3	50.1	<0.001

Table 4–2: Socio-demographic, personal and environmental characteristics of the study population (n = 335) by location of dwelling

	Location of dwelling			
	Large Urban (n = 246)	Small Urban (n = 46)	Rural (n = 43)	*p*-value*
Personal characteristics				
Mean age (±SD), years	10.8 (2.6)	11.0 (2.7)	12.7 (2.7)	<0.001
% Female	43.5	56.5	58.1	0.08
Ethnic background				
% Caucasian	78.3	73.9	93.0	0.05
% Others	21.6	26.1	7.0	
% > high school (maternal)	87.2	82.6	83.7	0.63
% > high school (paternal)	81.6	73.8	64.3	0.03
Secondhand tobacco smoke exposure				
% Maternal smoking	4.1	10.9	4.7	0.17†
% Paternal smoking	7.9	20.5	7.1	0.02†
% Either parent smoking	9.0	22.7	9.3	0.02†
Indoor characteristics				
% Pet ownership	11.8	26.1	27.9	0.04
% Dampness in home	20.1	31.8	31.0	0.10
% Home mold	13.5	8.7	19.0	0.36
Heating sources				
% Natural Gas	97.1	100.0	70.7	<0.001†
% Electricity	2.2	0	4.9	
% Others	0.8	0	24.4	
% Air conditioner	82.9	80.4	44.2	<0.001
% Humidifier	37.0	10.9	16.3	<0.001
Parental history of asthma and allergies				
% Parental history of asthma	19.9	30.4	16.3	0.20
% Parental history of allergic disease	34.6	54.3	37.2	0.04
Distance to healthcare				
Time travelled to access healthcare (±SD), minutes	12.7 (16.8)	12.8 (15.5)	40.93 (24.0)	<0.001

**p*-values reflect ANOVA (for continuous variables) or χ^2 test (for categorical variables) comparison for each characteristic.
†Statistical difference assessed by the Fisher's exact test because of small cell sizes (expected values <5).

Table 4–3: Profile of lung health indicators among at-risk-for-asthma and diagnosed asthma groups by location of dwelling

	Location of dwelling			
At-Risk-for-asthma	Large Urban (n = 89)	Small Urban (n = 17)	Rural (n = 15)	*p*-value*
% Ever wheeze	46.9	50.0	60.0	0.64
% Current wheeze	16.1	23.5	33.3	0.26†
% Chronic bronchitis	7.9	0	0	0.23†
% Current cough	23.5	58.8	26.7	0.004
% Cough disturbing sleep	43.8	52.9	46.7	0.78
% Wheeze with exercise	51.7	58.8	73.3	0.28
% Taking breathing medications past 12 months	22.7	11.8	13.3	0.46†
Diagnosed asthma	Large Urban (n = 70)	Small Urban (n = 16)	Rural (n = 9)	*p*-value*
% Current asthma	81.4	87.5	100.0	0.32†
% Ever wheeze	86.4	80.0	100.0	0.37†
% Current wheeze	56.1	53.3	66.7	0.80
% Chronic bronchitis	1.4	6.3	33.3	0.003†
% Current cough	42.9	62.5	55.6	0.31†
% Cough disturbing sleep	48.6	68.8	44.4	0.31
% Wheeze with exercise	78.6	87.5	87.5	0.63†
% Taking breathing medications past 12 months	70.0	75.0	66.7	0.89†

**p*-values reflect χ^2 tests comparison for each variable.

†Statistical difference assessed by the Fisher's exact test because of small cell sizes (expected values <5).

Table 4–4: Baseline mean (±SD) percent predicted lung function variables by location of dwelling and asthma status

Location of dwelling				
Overall	Large Urban (n = 219)	Small Urban (n = 37)	Rural (n = 32)	*p*-value*
FVC (L/s)	98.7 (12.7)	101.2 (9.6)	95.0 (10.9)	0.10
FEV_1 (L/s)§	96.0 (13.3)	98.2 (10.9)	89.3 (12.9)‡	0.011
FEV1/FVC	96.8 (6.9)	96.4 (6.6)	93.6 (10.1)	0.07
$FEF_{25\%–75\%}$ (L/s)§	88.6 (23.1)	91.6 (20.2)	78.8 (20.4)‡‡	0.040
No asthma	Large Urban (n = 77)	Small Urban (n = 10)	Rural (n = 13)	*p*-value*
FVC (L/s)	98.0 (10.1)	97.4 (8.7)	97.6 (9.5)	0.97
FEV_1 (L/s)	96.2 (11.4)	95.8 (13.9)	94.5 (9.7)	0.89
FEV1/FVC	97.6 (5.8)	97.3 (8.5)	96.7 (8.3)	0.88
$FEF_{25\%–75\%}$ (L/s)	90.0 (21.3)	92.2 (24.4)	87.7 (14.9)	0.87
At-Risk-for asthma	Large Urban (n = 75)	Small Urban (n = 14)	Rural (n = 14)	*p*-value*
FVC (L/s)	98.3 (13.9)	100.1 (10.3)	90.9 (12.3)	0.12
FEV_1 (L/s)§	96.7 (13.4)	98.0 (9.6)	82.1 (13.7)‡	<0.001
FEV1/FVC§	98.0 (6.0)	97.3 (5.01)	90.0 (12.3)‡	<0.001
$FEF_{25\%–75\%}$ (L/s)§	92.2 (23.0)	92.0 (17.4)	67.2 (22.2) ‡	<0.001
Diagnosed asthma	Large Urban (n = 67)	Small Urban (n = 13)	Rural (n = 5)	*p*-value*
FVC (L/s)	100.0 (14.1)	105.2 (8.4)	99.8 (7.8)	0.42
FEV_1 (L/s)	95.0 (15.2)	100.2 (10.1)	96.0 (8.7)	0.48
FEV1/FVC	94.5 (8.5)	94.6 (6.7)	95.8 (2.5)	0.94
$FEF_{25\%–75\%}$ (L/s)	83.1 (24.4)	90.7 (21.0)	87.8 (11.9)	0.54

**p*-values reflect ANOVA comparison for each lung function variable across locations of dwelling.
§Scheffe pairwise *post hoc* comparisons: ‡$p<0.05$ (Rural compared to large urban and compared to small urban); ‡‡$p<0.05$ (Rural compared to small urban alone).

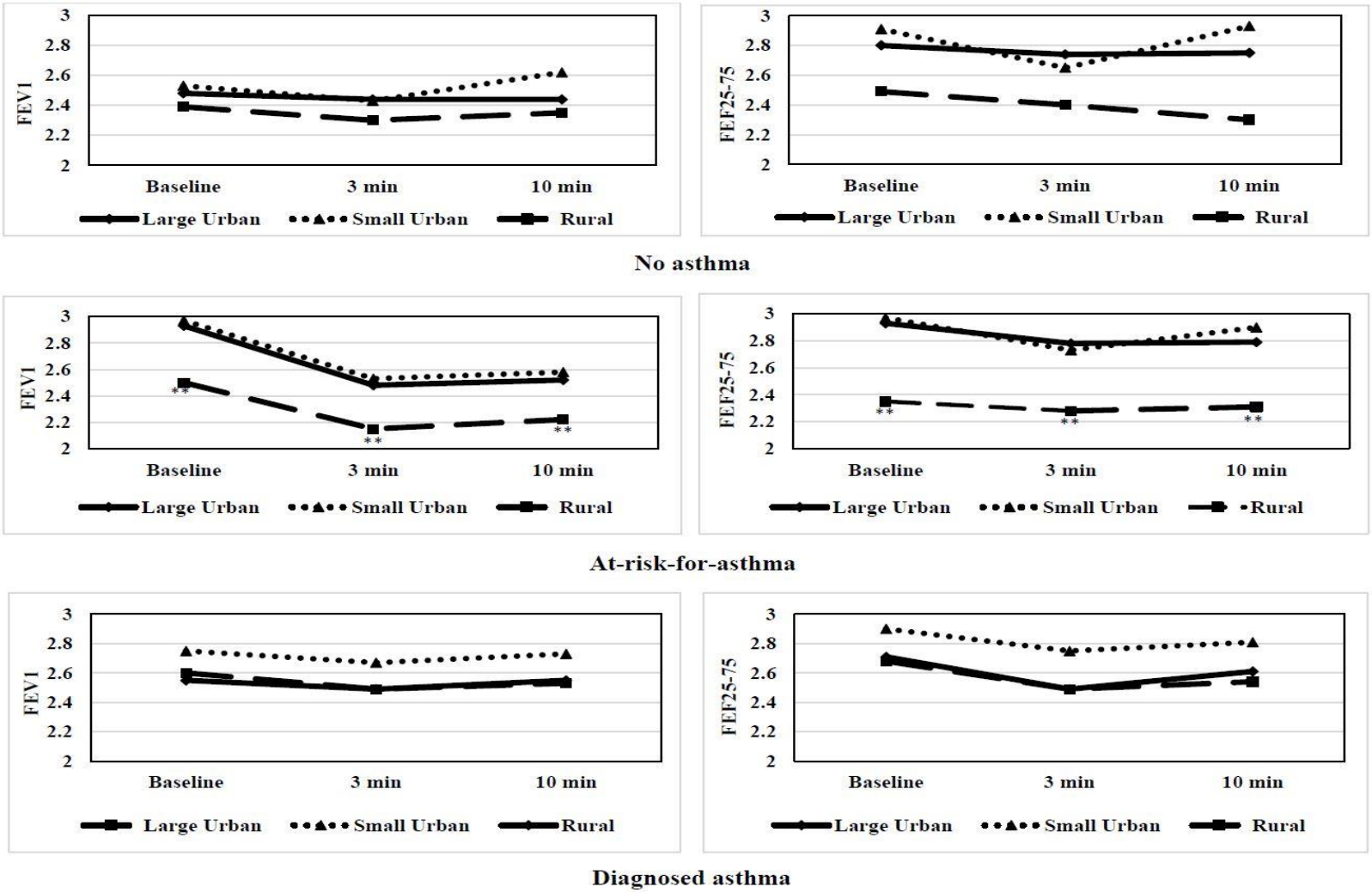
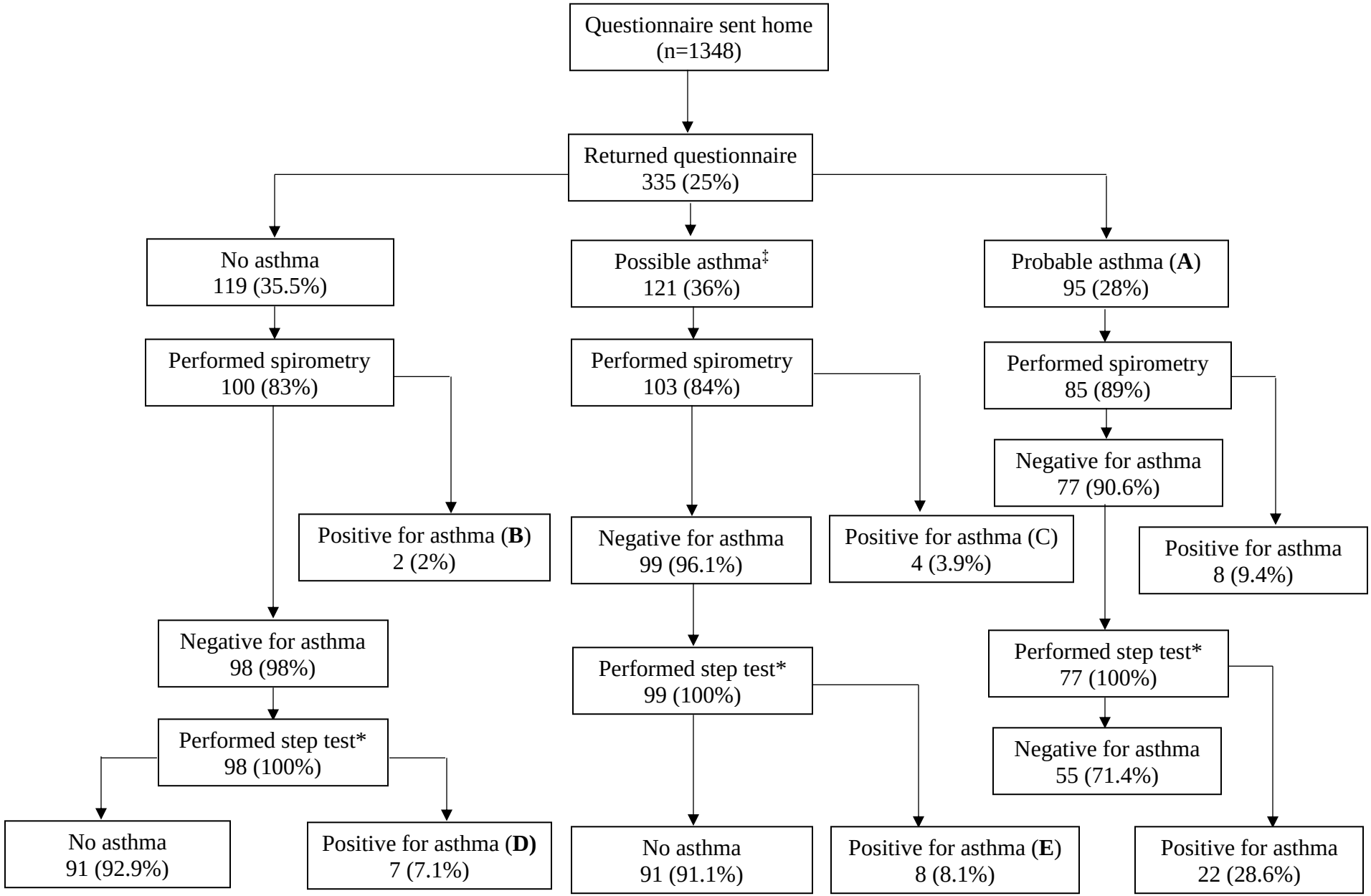


Figure 4–1: Mean lung function variables indicative of bronchial hyperresponsiveness (FEV_1 and $FEF_{25\%–75\%}$) at baseline and after cessation of exercise by location of dwelling and asthma status.
**$p<0.05$ (Scheffe pairwise *post hoc* comparisons): Rural compared to large urban and compared to small urban children.



‡Children with asthma related symptoms but no survey report of physician-diagnosed asthma.
*Positive for asthma based on > 15% decrease in FEV_1 or ≥ 25% decrease in $FEF_{25\%–75\%}$ from baseline.
Total new cases positive for asthma with spirometry and ECT: B + C + D + E = 21 subjects.
Total positive for asthma with questionnaire, spirometry and ECT: A + B + C + D + E = 116 subjects.

Figure 4–2: Asthma case-detection procedure based on the 3-stage asthma case detection algorithm by Gerald *et al*.[30]

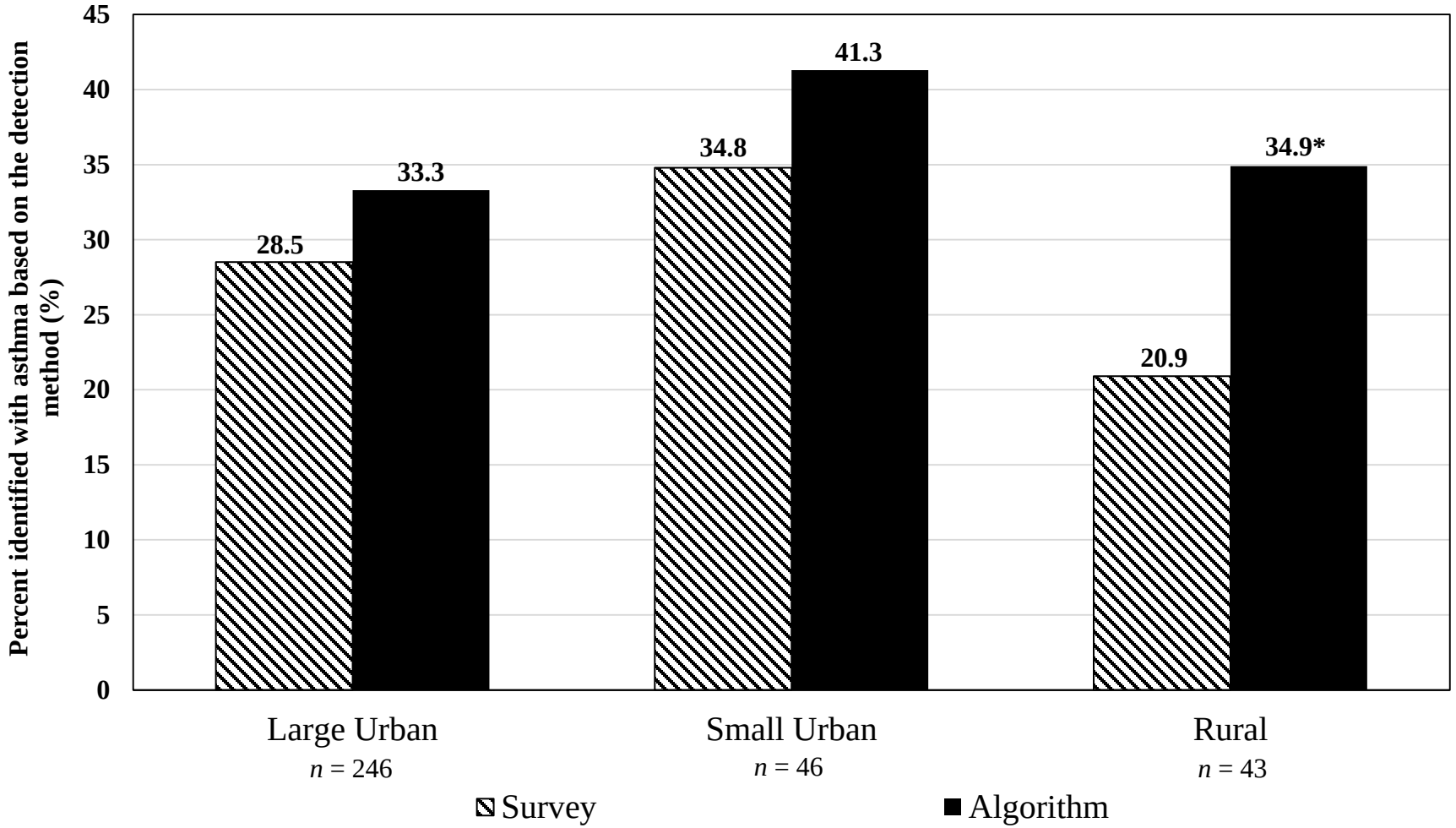


Figure 4–3: Comparison of proportion of survey-based vs. algorithm-based children with a positive indication of asthma by location of dwelling.
*Statistically significant when compared to proportion of cases detected with questionnaire alone ($p<0.05$) based on McNemar's test.

CHAPTER 5

THE ASSOCIATION BETWEEN ENDOTOXIN AND BETA-(1→3)-D-GLUCAN IN HOUSE DUST WITH ASTHMA PHENOTYPES AMONG SCHOOLCHILDREN (MANUSCRIPT II)

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5.1 Abstract

Background: Both protective and risk associations have been reported between microbial exposures and childhood asthma. The paradoxical relationships may be dependent on asthma phenotype of children with the disease.

Objective: We investigated the associations between exposure to endotoxin and beta-(1→3)-D-glucans in house dust with atopic asthma and exercise-induced bronchospasm in children with asthma.

Methods: A clinical cross-sectional study was performed among 335 schoolchildren (aged 7–17 years) in the province of Saskatchewan, Canada. Children with asthma were identified (n = 116/335) by a validated algorithm that included questionnaire diagnosis and clinical testing. Atopic asthma status was determined by skin prick testing while exercise-induced bronchospasm was evaluated by exercise challenge testing. Levels of endotoxin and beta-(1→3)-D-glucans exposures were measured in dust samples from the child's mattress and play area floors. Logistic regression analyses were used to explore associations between endotoxin and beta-(1→3)-D-glucan with each asthma phenotype separately.

Results: Among the 116 children with asthma, 44.4% were atopic and 22.4% had exercise-induced bronchospasm. Exposure to high play area endotoxin concentration [adjusted odds ratio (aOR) = 0.15, 95%CI: 0.02–0.95] and load (aOR = 0.13, 95%CI: 0.02–0.99) were associated with decreased risk of atopic asthma, independent of beta-(1→3)-D-glucan exposure. In contrast, exercise-induced bronchospasm was positively associated with high mattress endotoxin concentration (aOR = 7.80, 95%CI: 1.13–53.69). There were no consistent and significant associations between beta-(1→3)-D-glucan and atopic asthma or exercise-induced bronchospasm.

Conclusion: The study demonstrated that the association with indoor microbial exposure may depend on asthma phenotypes. The lack of association with beta-(1→3)-D-glucan indicates that the effect might be particularly attributable to endotoxin.

Keywords: Asthma phenotypes, Atopic asthma, Exercise-induced bronchospasm, House dust, Endotoxin, Beta-(1→3)-D-Glucan, Children

5.2 Introduction

Indoor microbial exposure has been suggested to influence the presence of respiratory disorders, including childhood asthma.[1] However, the associations are conflicting. Endotoxin has been reported to have protective,[2,3] risk effects,[4–6] and no association[7,8] for childhood asthma. Similar protective[9–11] and risk[12,13] effects have also been observed for beta-(1→3)-D-glucan. Reasons for the paradoxical effects are unclear but could be linked to different presentations of the disease in children with asthma.

This is further justified given that asthma is a complex disease with multiple presenting phenotypes, including allergic and non-allergic asthma. Previous studies of endotoxin have shown more consistent associations with allergic sensitization.[3,14,15] Studies have also shown that exposure to endotoxin is inversely associated with asthma and wheeze among atopic children.[16,17] Beta-(1→3)-D-glucans have also been found to have such reduced effects on recurrent wheezing[10] but positively associated with impaired lung function, primarily among atopic children.[18]

Endotoxin is a component of the outer cell wall of gram-negative bacteria capable of initiating strong immune modulatory and pro-inflammatory responses.[19,20] Similarly, beta-(1→3)-D-glucan, a structural component of cell wall of most fungi, is a potent agent capable of inducing adverse and protective effects for respiratory health effects.[3,18] As such, endotoxin and beta-(1→3)-D-glucan exposures may exhibit varied patterns of associations for asthma, resulting in differential clinical presentation in children who have the disease.

Furthermore, in both allergic and non-allergic asthma, bronchial hyperressponsiveness (BHR) represents a predominant feature of clinical presentation.[21] Exercise-induced bronchospasm (EIB) is one method of assessing BHR, but currently there are limited studies

investigating the effects of house dust endotoxin and beta-(1→3)-D-glucan exposures on EIB among children with asthma.

In the present study, we investigated the association between house dust endotoxin and beta-(1→3)-D-glucan exposure with asthma phenotypes based on atopic sensitization or EIB in children with asthma. We hypothesized that exposure to high levels of house dust endotoxin and beta-(1→3)-D-glucan will be inversely related to atopic asthma but positively related to EIB. This may provide some insight into the clinically relevant effects of indoor microbial exposures and asthma phenotypes among children with asthma for better childhood asthma management.

5.3 Methods

5.3.1 *Study population, selection, and recruitment*

We conducted a cross-sectional study among schoolchildren with asthma (aged 7–17 years) in the province of Saskatchewan, Canada from 2015–2016. Participants in this study were part of an initial 2013 cross-sectional survey of schoolchildren as previously described.[22] In the 2013 survey, those who consented to participate in further testing were re-approached in 2015. At this time, we repeated the survey and completed clinical testing (spirometry, exercise challenge testing, and skin prick test) as well as home dust sample collection. A total of 335 schoolchildren completed the survey.

The study was approved by the University of Saskatchewan Biomedical Research Ethics Board (Bio #: 14–162). Completion and return of the survey implied voluntary consent for the questionnaire portion. All children and a parent provided written assent and consent, respectively, prior to clinical testing and home dust collection. Furthermore, all school divisions involved approved the study.

5.3.2 *Survey questionnaire*

We used standardized and validated questions from the International Study of Asthma and Allergy in Childhood (ISAAC),[23,24] the American Thoracic Society Children's Respiratory Disease,[25] and questionnaires used previously in the Saskatchewan Lung Health studies[2,26] to obtain information on respiratory health (including physician-diagnosed asthma), general health, parental health history, environmental exposure, sociodemographic factors as well as housing characteristics. A total of 335 schoolchildren completed and returned the survey questionnaire.

5.3.3 *Spirometry and exercise challenge testing (ECT)*

Of the 335 subjects with survey responses, 288 (86%) performed spirometry and ECT. During home or school visits, trained field technicians performed spirometry assessments according to recommended standards for children.[27,28] Measurements of forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and forced expiratory flow ($FEF_{25\%–75\%}$) were done using the Easy-on PC spirometer (ndd Medical Technologies, Zurich, Switzerland). Some subjects were excluded from testing because they were unable to perform the test due to existing medical conditions (n = 3).

ECT was also completed based on recommended protocols.[29] Briefly, children stepped up and down on a 6 inch step at a heart rate between 150–200 beats per minute for 5 minutes. Heart rates were monitored throughout the exercise with a Polar heart rate monitor (Polar Electro, Woodbury, NY). Spirometry was repeated 3 and 10 minutes after cessation of exercise.

5.3.4 *Skin prick test (SPT)*

All SPTs were completed at the school or during home visits. Tests were completed on the forearm using a panel of six common and standardized allergen extracts according to recommended protocol:[30] cat, local grass, *Aspergillus, Alternaria, Cladosporium*, and house dust mite (Omega Laboratory, Montreal QC, Canada). Two controls: a histamine positive control and a saline negative control were used to reduce false positives and false negatives. The wheal size diameter was measured after 15 minutes. Subjects was considered positive for atopy if a positive reaction to at least one of the applied allergens is raised ≥3 mm compared to the saline control. All SPTs in the study were performed by trained technicians who were blinded to the asthma status of each child.

5.3.5 *Classification of asthma*

The asthma classification criteria was based on the validated asthma case-detection algorithm.[29] This algorithm has high sensitivity (82%) and specificity (93%) when compared to clinical assessment of asthma by a physician as "gold standard". Based on the parental response to the questionnaire, children were classified as "diagnosed asthma" if they had positive responses to the questions: "Has this child ever been diagnosed as having asthma by a doctor?" and/or "Has this child taken prescribed asthma medication in the past 12 months?" Children who were otherwise classified as "no asthma" based on survey responses but who had FEV_1/FVC ratio <80% upon spirometry testing; or demonstrated a >15% decrease in FEV_1 or a ≥25% decrease in $FEF_{25\%–75\%}$ from baseline after cessation of exercise were considered to be positive for asthma.[31,32] Overall, a total of 116 children were identified to have asthma and formed the study population for the results reported in the current study.

5.3.6 *Classification of asthma phenotypes*

Two phenotype-defined asthma outcomes were considered in the current study. This included phenotype assessment based on: 1) atopic status (atopic vs. non-atopic asthma) for the n = 116 children with asthma, and 2) EIB status also for the n = 116 children using the ECT results (EIB vs. no EIB). Atopic asthma was defined as sensitization (≥3 mm in wheal diameter compared to saline control after 15 minutes) to one or more allergens from SPT in the presence of diagnosed asthma. EIB was defined as >15% fall in FEV1 from baseline after cessation of exercise.

5.3.7 *Collection and analysis of dust samples to quantify endotoxin and beta-(1→3)-D-glucan exposure*

Vacuumed dust samples were obtained from the floor of play area and from mattress surfaces by trained personnel according to standardized protocols.[33] Briefly, carpet floors had 2 m^2 vacuumed while smooth floors had 4 m^2 vacuumed. Dust collection from the mattress surfaces was completed after all duvets, blankets and sheets that the child slept under were removed. The entire surface area of the mattress was then vacuumed for 2 minutes. Dust samples were weighed, sieved through a 300 μm mesh sieve, and stored desiccated at 4°C until extraction and analysis.

Prior to analysis, samples were brought to room temperature and 10 mg of sieved dust was weighed out for extraction. Dust samples were extracted with 20 mL 0.05% Tween 20 (Fisher Scientific, Mississauga, ON, Canada) in pyrogen-free water (GE Healthcare Bio-Science, Mississauga, ON, Canada)[34] and shaken at 325 revolutions per minute (RPM) for 2 hours. The extracted solution was then centrifuged (Sorvall ST 16R, Thermo Fisher Scientific, Mississauga, ON, Canada) at 1000 x g for 15 minutes to obtain supernatant and 1 mL aliquots were stored at -

80ºC until analysis. An aliquot of the supernatant was diluted 1 in 10, and was used to measure endotoxin by the chromogenic *Limulus* Amoebocyte Lysate (LAL) Kinetic QCL assay according to manufacturer's specifications (Lonza, Walkersville, MD, USA). Water soluble fraction of beta-(1→3)-D-glucan was measured in the second aliquot using the Glucatell assay kit with a beta-(1→3)-D-glucan-specific inhibition enzyme based on the Kinetic Onset Time protocol according to manufacturer's specifications (Associate of Cape Cod, East Falmouth, MA, USA).[35]

To quantify endotoxin and beta-(1→3)-D-glucan levels, the absorbance of endotoxin and beta-(1→3)-D-glucan was monitored at 405 nm for 2 hours and 1 hour, respectively, using the Biotek ELx808 plate reader and Gen5 v 2.06 software (BioTek, Winooski, VT, USA). Values were compared to standard curves prepared for endotoxin (0.005 EU/mL–50 EU/mL) and beta-(1→3)-D-glucan (3.125 pg/mL–100 pg/mL). Endotoxin and beta-(1→3)-D-glucan levels were recorded as concentration (per gram of sampled dust) and load (per square meter of sampled area); expressed as EU/mg and EU/m^2 for endotoxin and μg/g and μg/m^2 for beta-(1→3)-D-glucan. Results were reported as such given that concentration and load represent different aspects of indoor microbial exposure (dose and burden).[36] To ensure quality, the assays were conducted using reagents from a single lot. Laboratory technicians were blinded to disease status of each child and to the indoor location where each dust was sampled (i.e. whether sample was from child's play area or mattress surface).

5.3.8 *Statistical analysis*

Categorical variables were compared, separately for atopic vs. non-atopic and EIB vs. no EIB, using the independent samples chi-square (χ^2) and Fisher's exact tests as appropriate while continuous variables were compared using the independent sample Student *t*-test. Mean endotoxin and beta-(1→3)-D-glucan concentration and load for play area and mattress were

expressed as geometric mean (GM). Correlation between log transformed play area and mattress endotoxin and beta-(1→3)-D-glucan was also assessed using Pearson's correlation coefficient (r).

Multiple logistic regression models were then fitted to test the association between endotoxin and beta-(1→3)-D-glucan exposure with asthma phenotypes expressed as either atopic vs. non-atopic asthma (non-atopic asthma as reference) or EIB vs. no EIB (no EIB as reference). Separate independent models were fitted for each of play area and mattress dust including: i) a univariate model for each endotoxin (concentration and load) and beta-(1→3)-D-glucan (concentration and load); ii) main effects model for endotoxin; iii) main effects models for beta-(1→3)-D-glucan; and iv) main effects plus endotoxin and beta-(1→3)-D-glucan. Variables were included in the models based on statistical significance identified in the univariate analyses, clinical importance, and the effect the removal of a variable had on the beta coefficient of other variables in the model.[37] The additional variables included in the model were sex, age, parental education (≤ high school vs. > high school), parental smoking (yes vs. no), parental history of asthma and allergy (yes vs. no), pet ownership (yes vs. no), home dampness (yes vs. no), and location of residence (urban vs. rural). Endotoxin and beta-(1→3)-D-glucan remained, *a priori*, in all models. Levels of endotoxin and beta-(1→3)-D-glucan were modelled as categorical variables in tertiles to define low (1st tertile), medium (2nd tertile), and high (3rd tertile) levels because the relationships with moderate/severe asthma did not meet the linear assumption when log-transformed (ln) endotoxin and beta-(1→3)-D-glucan were used. Low (1st tertile) was used as reference level. Throughout the analyses, generalized estimating equations (GEE) were used to account for clustering within families. The Hosmer-Lemeshow test was used to assess model Goodness-of-Fit.

The strength of the associations were assessed using odds ratios (ORs) and their 95% confidence interval (CI). Statistical significance was defined by an alpha level of $p \leq 0.05$. All analyses were completed with the Statistical Package for the Social Science (SPSS) Version 24 (SPSS Inc. Armonk, NY: IBM Corp.) and the Statistical Analysis System (SAS) Version 9.4 (SAS Institute Inc., Cary, NC, USA).

5.4 Results

5.4.1 *Subject characteristics, respiratory symptoms and exposure characteristics*

Of the 116 children identified with asthma, 99 (85.3%) completed SPT while all had completed ECT. Of these, 71/99 (71.7%) were atopic and 26/116 (22.4%) had EIB. When we considered overlap in phenotype categories, 54 (54.5%) had atopic asthma alone, 6 (6.1%) had EIB alone, 17 (17.2%) had both atopic asthma and EIB, and 22 (22.2%) had non-atopic asthma and no EIB. Table 5–1 presents an overview of the distribution of socio-demographic, home characteristics, and early life characteristics when phenotypes were assessed as atopic vs. non-atopic or as EIB vs. no EIB. The distribution of sex and age were similar between atopic and non-atopic asthma groups. However, compared to children with atopic asthma, children with non-atopic asthma were more likely to live in modern homes (p=0.03). Children with no EIB, on average, were older with a higher BMI and were more likely to have parents with history of allergic disease compared to children with EIB (p<0.05). The distribution of all other characteristics was similar and not statistically significant between the asthma phenotype groups. Respiratory symptoms were also similar among the asthma groups except for ever wheeze which was significantly higher in EIB compared to no EIB group (Table 5–2).

Endotoxin and beta-(1→3)-D-glucan were at detectable levels in all house dust samples (Appendix 10). Overall, endotoxin ranges were as follows: play area (2.20 EU/mg–6.55 EU/mg and 8.19 EU/m^2–12.76 EU/m^2); and mattress (0.69 EU/mg–6.21 EU/mg and 6.02 EU/m^2–12.18 EU/m^2) (Appendix 10). Geometric mean endotoxin and beta-(1→3)-D-glucan levels in play areas and mattresses did not differ when we compared atopic to non-atopic asthma or EIB to no EIB groups (Table 5–3). Play area endotoxin load correlated with play area beta-(1→3)-D-glucan load (r = 0.43, p<0.001). Similarly, mattress endotoxin concentration correlated with mattress beta-(1→3)-D-glucan concentration (r = 0.44, p<0.001). All other correlations, though statistically significant, were generally weak (r<0.3) (Appendix 11).

5.4.2 *Associations between house dust endotoxin and beta-(1→3)-D-glucan exposure levels and atopic asthma*

When asthma phenotypes were assessed based on atopic status only, unadjusted regression analyses showed that high play area endotoxin concentration and load were negatively associated with atopic asthma (OR = 0.25, 95%CI: 0.07–0.91) (Table 5–4). Multiple logistic regression analysis (Model II) showed similar statistically significant associations in the high endotoxin group [endotoxin concentration: adjusted odds ratio (aOR) = 0.15, 95%CI: 0.03–0.86; endotoxin load: aOR = 0.11, 95%CI: 0.02–0.75]. To determine if the associations found for high play area endotoxin concentration and load were independent of beta-(1→3)-D-glucan levels, we adjusted for beta-(1→3)-D-glucan concentration and load as appropriate in addition to covariates included in Model II (Model IV). Independent of play area beta-(1→3)-D-glucan levels, the negative association between atopic asthma and high play area endotoxin concentration (aOR = 0.15, 95%CI: 0.02–0.95) and load (aOR = 0.13, 0.02–0.99) remained significant.

5.4.3 *Associations between house dust endotoxin and beta-(1→3)-D-glucan exposure levels and exercise-induced bronchospasm*

Table 5–5 presents the associations between endotoxin and beta-(1→3)-D-glucan with EIB when phenotypes were assessed based on ECT, irrespective of atopic status. Exposure to high level of mattress endotoxin concentration was significantly associated with EIB both at the univariate level (OR = 4.64, 95%CI: 1.15–18.75; Model I) and after adjusting for potential covariates (aOR = 6.13, 95%CI: 1.12–33.52; Model II). We also determined whether the increased risk of EIB associated with high mattress endotoxin concentration was related to beta-(1→3)-D-glucan levels in the mattress dust (Model IV). The results showed that independently of mattress beta-(1→3)-D-glucan concentration and other potential confounders identified in Model II, the positive association between mattress endotoxin concentration and EIB remained statistically significant and became stronger (aOR = 7.80, 95%CI: 1.13–53.69). The associations for beta-(1→3)-D-glucan exposure levels with EIB were inconsistent and non-significant whether expressed as concentration or as load.

5.5 Discussion

In the current study, high endotoxin measures in play area dust were inversely associated with atopic asthma. When asthma was assessed based on EIB status, this pattern was reversed with EIB positively associated with high mattress endotoxin concentration. Beta-(1→3)-D-glucan levels in house dust showed no significant effect neither on atopic asthma nor EIB.

In the Allergy and Endotoxin Study (ALEX) conducted among children (aged 6–13 years) in Austria, Germany, and Switzerland,[16] Braun-Fahrlander *et al* demonstrated that endotoxin load in samples of dust derived from children's mattresses were inversely associated

with atopic asthma. Consistent with results in this previous study, we also demonstrated an inverse relationship between high endotoxin levels and atopic asthma, but the associations were limited to play area endotoxin levels. It may be difficult to directly compare our results with the ALEX study because the study modelled endotoxin as continuous variable while we modelled it as a categorical variable based on tertiles. However, our study results are similar to the results of a cross-sectional study in Palestine among 6–12 years old children[38] where medium and high (the second and third tertiles) endotoxin levels in play area floor dust were found to be inversely associated with atopic wheeze (report of wheeze in the past 12 months) in a dose response manner.

In the current study, the association between atopic asthma and endotoxin exposure was not consistent between the two locations of dust sampling (play areas and mattress surfaces). While studies have shown that mattress endotoxin levels decreased the risk of allergic sensitization[15,39] and atopic asthma,[16] we expanded on previous studies by showing that, among children with asthma, the associations may be limited to play area endotoxin. Reasons for the observed associations are unclear in this study but may be related to differences in the determinants of endotoxin in different locations in the homes[2,26] (Appendix 12) or differences in endotoxin's length of 3-hydroxyl fatty acids (3-OHFAs) chain.[40] In a US study that characterized the types of endotoxin in house dust samples based on the length of fatty acid chain, Park *et al*[40] showed that shorter-chain 3-OHFAs ($C_{10:0}$, $C_{12:0}$, and $C_{14:0}$) were positively correlated with endotoxin activity while longer-chain groups tend to have negative correlation ($C_{16:0}$) or no correlation ($C_{18:0}$). Furthermore, mattress dust endotoxin contained longer-chain 3-OHFA ($C_{16:0}$) while dusts from family room area floors contained predominantly shorter-chain 3-OHFAs ($C_{10:0}$, $C_{12:0}$, and $C_{14:0}$). These observations may explain some of the inconsistencies in reported

associations between indoor endotoxin levels and respiratory diseases. For example, a study among 2,209 schoolchildren (aged 11–15 years) in China found reduced risk of respiratory symptoms (wheeze and attack of breathlessness) with shorter endotoxin 3-OHFA chain while longer 3-OHFA chain lengths tended to be positively associated with respiratory symptoms.[41] Similar results were also observed for wheeze with shorter lengths of endotoxin 3-OHFA in a Malaysian study.[42] Based on the presence of shorter 3-OHFA chain length in play area floor dust compared to mattress dust [40] coupled with reduced risk of respiratory symptoms associated with shorter endotoxin 3-OHFA in the China[41] and Malaysia[42] studies, it is plausible that the 3-OHFA in our samples may also differ in chain length structures between play area and mattress dust endotoxin. This may assist in explaining our results.

There is evidence that inhaled endotoxin exposure can induce BHR. However, these effects have only been found in adults[43,44] and an animal study.[45] In a clinical bronchial challenge test among adults with asthma in Belgium, Michel *et al*[44] found a significant reduction in FEV_1 (≥10% decrease) which lasted between 15–45 minutes following inhalation of 20 μg of endotoxin extract. Similarly, a study in Australia showed significant BHR (measured as increased influx of neutrophil into bronchoalveolar lavage fluid [BALF]) in rats challenged with lipopolysaccharide (LPS: a major constituent of bacterial endotoxin) if exposure occurs early in the sensitization process.[45] We also found similar trends of associations in the current study, albeit with a different bronchial challenge test (exercise challenge test) and source of endotoxin exposure (settled house dust). High mattress endotoxin level was significantly associated with increased risk of EIB. Furthermore, the result suggests that EIB response to endotoxin may be dose related with a statistically significant and stronger association found for high endotoxin exposure (aOR = 7.80, 95%CI: 1.13–53.69) than for the association at medium endotoxin

exposure (aOR = 2.46, 95%CI: 0.35–17.55) endotoxin exposure levels. This is consistent with dose-response relationship results observed in an endotoxin exposure challenge test study among 77 adults which demonstrated significant bronchoconstriction (decreased in FEV_1) following inhalation of 200 μg of endotoxin extract compared to inhalation of 30 μg.[46]

Associations between play area endotoxin levels and EIB were not found in the present study. Possible explanations for the presence of EIB and high mattress endotoxin concentration and the lack of it for play area endotoxin levels may also be that children come into closer contact with microbial agents while sleeping and or differences in play and mattress areas endotoxin's structure. Mattress dust contains longer-chain 3-OHFA[40] and it is suggested that longer-chain 3-OHFAs ($C_{12:0}$–$C_{14:0}$) may elicit stronger and significant potent immunological effects compared to shorter-chain 3-OHFAs.[47] Furthermore, studies have shown mattress dust to be the most reproducible source of house dust exposure[48] with non-significant variation over a six-month period[49] compared to play areas which may be regularly vacuumed.

Exposure to beta-(1→3)-D-glucan has been suggested to be inversely related to wheezing[10] and atopic sensitization[10,15] and positively associated with atopic asthma[13] and BHR.[13,18] We observed non-significant trend towards reduced risk of atopic asthma linked to high play area beta-(1→3)-D-glucan levels. While this may be due to a low sample size, it is also possible that the relationship between beta-(1→3)-D-glucan exposure and childhood asthma may activate an independent pathway different from that associated with endotoxin exposure. In other words, the relationships may not be based on allergic reactions since, in most cases, beta-(1→3)-D-glucan has been considered as non-immunogenic or non-allergic in humans.[18]

Causation of childhood asthma remains poorly understood and most studies have ignored the distinction between atopic and non-atopic asthma even though these phenotypes may have

distinction mechanisms.[50] The mechanism by which endotoxin is related to childhood asthma and allergy is still unclear but is currently believed to be linked to the imbalance between T_H1 and T_H2 immune response cells. Allergic diseases are typically characterized by predominance of T_H2 cells.[51] However, exposure to high levels of endotoxin inhibits the T_H2 immune response and promotes T_H1 immune responses, preventing atopic immune development and associated diseases.[38,52] On the other hand, endotoxin is also considered as a pro-inflammatory agent potentiating the release of inflammatory mediators such as allergic release of histamine and neutrophils to induce BHR in humans.[53] However, the presence of EIB in non-atopic subjects in the present study (6.1%) may further suggest that response to endotoxin exposure in children with asthma could also be mediated by a non-allergic mechanism. Investigations that demonstrate absence of an immediate skin prick test response to endotoxin extracts in atopic subjects may help to validate the claim for non-allergic mechanism.

Consistent with the paradoxical effects of endotoxin exposure, we demonstrated that while endotoxin may protect from atopic asthma, it could also induce bronchoconstriction in children with existing asthma. It is interesting therefore to speculate that exposure to endotoxin may be involved in different pathways of the innate immune systems and thus different asthma phenotypes. For example, it is possible that associations with EIB represent acute effects of endotoxin exposure in children with asthma while the inverse association with atopic asthma may reflect long-term immune response, possibly from early life exposure, which shifts the immune response away from the atopic T_H2 cells towards the less allergic T_H1 to mitigate allergy and asthma.[2,54] This is further evidenced from a study in Australia which demonstrates that LPS exposure has the potential to prevent allergic disease in rats only if the exposure occurs early (≤6

days) in the sensitization process.[45] Beyond the 6 days period, such exposure further exacerbates allergic response and BHR.

High endotoxin levels in the indoor environment could coexist with high beta-(1→3)-D-glucan levels.[13] Due to this, it may be difficult to consider the exposures independently. To parcel out the independent effects of endotoxin on the study outcomes, we adjusted for beta-(1→3)-D-glucan levels in our endotoxin analysis models. The results showed no indications of stronger effects of endotoxin on either atopic asthma (Table 5–4 Model IV) or EIB (Table 5–5 Model IV) compared to models without beta-(1→3)-D-glucan adjustment (Atopic asthma: Table 5–4 Model II; EIB: Table 5–5 Model II). Similar analyses have been performed in a previous study in the Netherlands investigating the relationships between house dust endotoxin and beta-(1→3)-D-glucan levels and peak expiratory flow (PEF) variability in children.[18] In this study, however, the significant effect of endotoxin exposure on PEF variability from univariate analysis was lost following multivariate analysis which included adjustment for house dust beta-(1→3)-D-glucan level. The methodological differences in defining outcome variables in our study (atopic asthma and EIB) and that of the Netherlands study (PEF variability) might have accounted for the observed varied results.

While house dust beta-(1→3)-D-glucan has been shown to be positively associated with wheezing in infants[10] and atopic sensitization in children (aged 2–4 years),[15] our results suggests that that the associations between indoor endotoxin exposure, and atopic asthma as well as EIB may occur independently of beta-(1→3)-D-glucan levels in the indoor environment. This indicates that endotoxin exposure in the indoor environment may be more important to consider than beta-(1→3)-D-glucan. This finding requires further confirmation and should be interpreted with caution due to relatively small sample size of the study; and also because a measure of

endotoxin does not capture all microbial exposure and qualitative differences in exposure which has been shown to better predict childhood asthma outcomes compared to single microbial marker[55] should be completed.

Limitations of our study should be considered. The participation rate for this study was low. It is important to note that participants in this study were recruited from urban and rural locations and were frequently hard to reach, especially those in rural locations. However, as our results indicated statistical significance for endotoxin, we believe that the power of the study was sufficient. The non-significant findings of beta-(1→3)-D-glucan exposure may partly be explained by other factors such as the non-immunogenic or non-allergic properties of beta-(1→3)-D-glucan in humans[18] or inadequate statistical power. We cannot exclude the possibility of selective avoidance as another potential source of bias in this study. For example, it is possible that allergic parents might tend to keep a cleaner indoor environment that could reduce exposure to dust as previously observed.[56] We feel that there is no indication that this bias affects the results of our study. First, the results of the distribution of parental history of allergic disease were similar between atopic and non-atopic children with asthma (60% vs. 57%; p=0.76). Second, the associations between indoor endotoxin levels and home cleaning habits by parents have been shown to not differ by atopic status in previous studies.[57,58] We used dust samples collected at a single time-point. While seasonal variation in house dust levels and microbial components may exist,[59,60] a single time-point dust sample collection has been the most commonly used method in epidemiological studies due to convenience and cost constraints. In addition, provided sampling procedures are standardized, studies showed that sampling of settled dust is reproducible and that a single dust sampling for endotoxin analysis have little variation over time and reflects longer-term exposure to microbial products for at least 1 year period.[61] We

acknowledge that this may not be true for all populations and there should be caution when comparing the results of this study to other studies that have used dust samples collected at multiple and different time intervals. Data collection for this study was at one point in time and used prevalent asthma cases. Therefore, the cross-sectional observational design of the study precludes us from drawing conclusion about causality.[62] However, support for our findings comes from longitudinal study which showed reduced allergic sensitization in children following endotoxin exposure[3] and animal studies which demonstrate significant BHR in rats challenged with LPS.[45] Finally, the dust extraction analysis procedure used in this study is specific in determining the water soluble fraction of beta-(1→3)-D-glucan[63] which may not represent the most potent fraction of beta-(1→3)-D-glucan compared to alkaline soluble fraction.[64] This may be one of the reasons, in addition to small sample size, beta-(1→3)-D-glucan levels were not statistically associated with neither atopic asthma nor EIB in the current study.

The strengths of our study included the use of objective measures for exposure and outcome assessments thus limiting the possibility of recall bias for the associations reported herein. We also used an established algorithm of case finding to minimize misclassification of asthma status.[29] Clinical data and dust samples were collected by trained technicians using standardized protocols.[27,28,30,33] Laboratory personnel were blinded to asthma status of each child. Furthermore, home dust collection was conducted concurrently with clinical data. Finally, we studied the effects of endotoxin and beta-(1→3)-D-glucan, simultaneously, on objectively measured asthma phenotypes (atopic and EIB) among children with asthma. Current studies that have assessed similar relationships between endotoxin exposures and BHR have only been conducted in adults[43,44] and animal model.[45]

In summary, we demonstrated that environmental exposure to indoor microbial products as assessed by the measurement of house dust endotoxin levels was inversely associated with atopic asthma but positively associated with EIB in children with preexisting asthma conditions. Furthermore, the lack of association with beta-(1→3)-D-glucan levels, either when assessed separately or included in models for endotoxin, may indicate that the effects might be particularly attributable to endotoxin.

5.6 References

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Table 5–1: Characteristics of the study population (n = 116) by asthma phenotype groups

	Atopic asthma (n = 71)*	Non-atopic asthma (n = 28)*	*p*-value	Exercise-induced bronchospasm (n = 26)	Absence of Exercise-induced bronchospasm (n = 90)	*p*-value
Mean age (±SD), years	10.9 (2.7)	11.0 (2.5)	0.86	10.0 (2.8)	11.3 (2.6)	0.04
Body mass index (±SD), kg/m^2	20.3 (5.0)	21.0 (4.8)	0.55	18.3 (2.6)	21.2 (5.3)	<0.001
% Female	32.4	32.1	0.98	26.9	37.8	0.31
Ethnic background						
% Caucasian	81.0	75.0	0.52	79.2	82.1	0.77†
% Others	19.0	25		20.8	17.9	
Physical activity						
% Low	1.4	7.1	0.31	0.0	4.4	0.55
% Moderate	31.0	32.1		34.6	34.4	
% High	67.6	60.7		65.4	61.1	
Parental education level						
% > high school (maternal)	80.0	88.9	0.38	84.0	83.1	1.00†
% > high school (paternal)	76.1	84.6	0.37	91.7	75.6	0.09
Tobacco smoke exposure						
% Parental smoking	13.2	14.3	1.00†	15.4	11.5	0.73†
% Environmental tobacco smoke	7.0	10.7	0.68†	0.0	11.2	0.11
Home characteristics						
% Modern home: 1980–Present	36.9	61.5	0.03	36.0	47.6	0.31
% Gas heating	97.1	92.6	0.61†	96.2	94.2	0.36†
% Home air filter	47.9	50.0	0.85	57.7	48.9	0.43
% Home humidifier	28.2	28.6	0.97	30.8	28.9	0.85
% Pet ownership	57.7	57.1	0.96	57.7	58.9	0.91
% Carpet flooring	58.2	71.4	0.28	70.0	54.9	0.22
% Home dampness	25.7	22.2	0.72	15.4	27.3	0.22
% Visible mold	10.4	7.7	1.00†	12.5	11.6	1.00†
Family history						
% Parental history of asthma	36.6	39.3	0.81	26.9	36.7	0.36
% Parental history of allergic disease	60.6	57.1	0.76	42.3	65.6	0.03

Early life characteristics						
% Breastfed	81.5	96.4	0.10	84.0	88.2	0.73
% Attended daycare	70.8	69.2	0.89	72.0	66.3	0.59
% Consumed raw farm milk	5.6	0	0.5†	3.8	5.6	1.00†
% Contact with farm buildings	16.4	11.1	0.75†	15.4	18.1	1.00†
% Contact with farm animals	15.9	14.8	1.00†	11.5	18.6	0.56†
Intrauterine exposure characteristics						
% Mother consumed farm milk	0	3.6	0.28†	0.0	1.1	1.00†
% Mother contact with farm animals	12.7	14.3	1.00†	3.8	14.4	0.19†
Location of residence						
% Urban	91.5	85.7	0.46†	96.2	84.4	0.19†
% Rural	8.5	14.3		3.8	15.6	

*99/116 of the children identified to have asthma completed SPT.

†Statistical difference assessed by the Fisher's exact test due to small cell sizes (expected values <5).

Table 5–2: Respiratory symptoms among subjects by asthma phenotype status

	Atopic asthma (n = 71)	Non-atopic asthma (n = 28)	*p*-value	EIB (n = 26)	No EIB (n = 90)	*p*-value
Respiratory symptoms						
% Ever wheeze	75.4	80.0	0.64	84.7	50.0	<0.001
% Wheeze past 12 month	51.4	48.0	0.77	52.9	40.0	0.26
% Cough past 12 months	47.9	42.9	0.65	30.8	47.8	0.12
% Chest congestion	13.8	22.2	0.36†	19.2	15.9	0.69
% Chronic bronchitis	1.4	3.6	0.49†	3.8	4.4	1.00†
% Nasal congestion	43.7	33.3	0.35	46.2	40.4	0.60
% Hay fever	9.9	7.1	1.00†	3.8	10.0	0.45†

†Statistical difference assessed by the Fisher's exact test due to small cell sizes (expected values <5).
EIB: Exercise-induced bronchospasm.

Table 5–3: Geometric mean of endotoxin and beta-(1→3)-D-glucan concentration and load in house dust from the play area floor and mattresses by asthma phenotypes

	Atopic asthma (n = 71)	Non-atopic asthma (n = 28)	*p*-value	EIB (n = 26)	No EIB (n = 90)	*p*-value
Play area	GM (GSD)	GM (GSD)		GM (GSD)	GM (GSD)	
Endotoxin concentration (EU/mg)	51.3 (2.3)	63.4 (2.0)	0.30	54.3 (2.3)	52.2 (2.2)	0.84
Endotoxin load (EU/m^2)	20837.3 (2.5)	27783.6 (2.1)	0.20	23746.7 (2.4)	21857.5 (2.4)	0.70
Beta-(1→3)-D-glucan concentration (μg/g)	9.0 (2.1)	9.1 (1.7)	0.92	9.2 (1.8)	8.9 (2.1)	0.85
Beta-(1→3)-D-glucan load (μg/m^2)	102.9 (5.9)	174.2 (4.2)	0.22	113.4 (6.9)	129.3 (6.4)	0.78
Mattress						
Endotoxin concentration (EU/mg)	20.5 (2.3)	20.5 (2.1)	0.99	27.0 (2.6)	19.4 (2.3)	0.12
Endotoxin load (EU/m^2)	9631.6 (2.5)	9596.0 (2.3)	0.99	13242.7 (2.9)	8798.2 (2.5)	0.08
Beta-(1→3)-D-glucan concentration (μg/g)	4.5 (1.9)	4.8 (1.8)	0.66	5.2 (2.5)	4.5 (1.8)	0.33
Beta-(1→3)-D-glucan load (μg/m^2)	45.8 (4.4)	49.9 (4.4)	0.82	58.1 (5.0)	42.4 (4.1)	0.39

GM: Geometric mean; GSD: Geometric standard deviation.
EU: Endotoxin units.

Table 5–4: Multiple logistic regression analyses describing the associations between endotoxin and beta-(1→3)-D-glucan levels[†] and atopic asthma

	Model I OR (95%CI)	Model II* aOR (95%CI)	Model III* aOR (95%CI)	Model IV*[§] aOR (95%CI)
Play area				
Endotoxin concentration (EU/mg)				
Low	1.00	1.00	–	1.00
Medium	0.67 (0.17–2.65)	0.51 (0.10–2.57)	–	0.42 (0.08–2.24)
High	0.25 (0.07–0.91)[‡]	0.15 (0.03–0.86)[‡]	–	0.15 (0.02–0.95)[‡]
Endotoxin Load (EU/m^2)				
Low	1.00	1.00	–	1.00
Medium	0.67 (0.17–2.65)	0.48 (0.08–2.97)	–	0.47 (0.07–3.11)
High	0.25 (0.07–0.91)[‡]	0.11 (0.02–0.75)[‡]	–	0.13 (0.02–0.99)[‡]
Beta-(1→3)-D-glucan concentration (μg/mg)				
Low	1.00	–	1.00	1.00
Medium	1.14 (0.34–3.75)	–	0.77 (0.17–3.45)	0.89 (0.18–4.36)
High	0.91 (0.27–3.05)	–	0.97 (0.23–4.13)	1.54 (0.32–7.55)
Beta-(1→3)-D-glucan load (μg/m^2)				
Low	1.00	–	1.00	1.00
Medium	0.39 (0.11–1.45)	–	0.28 (0.05–1.48)	0.36 (0.06–2.04)
High	0.40 (0.11–1.52)	–	0.26 (0.05–1.48)	0.40 (0.06–2.60)
Mattress				
Endotoxin concentration (EU/mg)				
Low	1.00	1.00	–	1.00
Medium	0.71 (0.22–2.27)	0.53 (0.13–2.18)	–	0.56 (0.13–2.40)
High	0.90 (0.26–3.09)	0.66 (0.13–3.31)	–	0.70 (0.13–3.91)
Endotoxin Load (EU/m^2)				
Low	1.00	1.00	–	1.00
Medium	0.69 (0.21–2.30)	0.60 (0.14–2.59)	–	0.63 (0.14–2.86)
High	0.75 (0.22–2.59)	0.46 (0.10–2.18)	–	0.48 (0.10–2.41)
Beta-(1→3)-D-glucan concentration (μg/mg)				

Low	1.00	–	1.00	1.00
Medium	1.40 (0.39–5.01)	–	1.37 (0.25–7.39)	1.50 (0.27–8.42)
High	0.71 (0.23–2.25)	–	0.88 (0.22–3.61)	1.01 (0.22–4.54)
Beta-(1→3)-D-glucan load (μg/m^2)				
Low	1.00	–	1.00	1.00
Medium	0.86 (0.25–3.00)	–	0.91 (0.21–4.03)	0.92 (0.21–4.09)
High	0.78 (0.23–2.63)	–	0.58 (0.13–2.58)	0.54 (0.11–2.56)

EU: Endotoxin units.

*Statistical comparisons between atopic and non-atopic asthma were completed using logistic regression with GEE to account for clustering within families.

[†]Low, medium, and high levels for endotoxin and beta-(1→3)-D-glucan were determined based on their corresponding tertile values of the exposure distribution, separately, for play and mattress areas: Low (1st tertile), Medium (2nd tertile), and High (3rd tertile).

Model I: Model with no adjustments for atopic asthma; Models II: Adjusted model for atopic asthma with endotoxin as an independent variable; Model III: Adjusted model for atopic asthma with beta-(1→3)-D-glucan as an independent variable.

aOR: Adjusted odds ratio. Models II and III were adjusted for sex, age, parental education, parental smoke, parental history of asthma and allergy, pet ownership, home dampness, and location of residence.

[§]In addition to adjusted variables in Models II and III, Model IV was mutually adjusted for endotoxin or beta-(1→3)-D-glucan as appropriate. That is, model with play area endotoxin concentration as an independent variable was adjusted for play area beta-(1→3)-D-glucan concentration. Model with play area endotoxin load as an independent variable was adjusted for play area beta-(1→3)-D-glucan load. Similar procedure was performed for mattress endotoxin and beta-(1→3)-D-glucan.

[‡]$p<0.05$.

Table 5–5: Multiple logistic regression analyses describing the associations between endotoxin and beta-(1→3)-D-glucan levels[†] and exercise-induced bronchoconstriction

	Model I OR (95%CI)	Model II* aOR (95%CI)	Model III* aOR (95%CI)	Model IV*[§] aOR (95%CI)
Play area				
Endotoxin concentration (EU/mg)				
Low	1.00	1.00	–	1.00
Medium	1.00 (0.31–3.24)	1.09 (0.24–5.03)	–	1.05 (0.22–5.00)
High	0.83 (0.25–2.78)	0.78 (0.14–4.29)	–	0.62 (0.10–3.96)
Endotoxin Load (EU/m^2)				
Low	1.00	1.00	–	1.00
Medium	1.50 (0.43–5.31)	1.96 (0.38–10.23)	–	1.83 (0.30–11.44)
High	1.79 (0.52–6.15)	2.31 (0.37–14.46)	–	2.15 (0.30–15.18)
Beta-(1→3)-D-glucan concentration (μg/mg)				
Low	1.00	–	1.00	1.00
Medium	2.09 (0.62–7.05)	–	5.20 (0.68–39.75)	5.19 (0.62–43.09)
High	1.24 (0.34–4.54)	–	2.38 (0.42–13.64)	2.18 (0.35–13.47)
Beta-(1→3)-D-glucan load (μg/m^2)				
Low	1.00	–	1.00	1.00
Medium	0.67 (0.19–2.35)	–	1.21 (0.23–6.46)	1.11 (0.19–6.41)
High	1.19 (0.38–3.74)	–	1.83 (0.32–10.33)	1.58 (0.23–10.82)
Mattress				
Endotoxin concentration (EU/mg)				
Low	1.00	1.00	–	1.00
Medium	2.77 (0.65–11.75)	2.07 (0.34–12.54)	–	2.46 (0.35–17.55)
High	4.64 (1.15–18.75)[‡]	6.14 (1.12–33.52)[‡]	–	7.80 (1.13–53.69)[‡]
Endotoxin Load (EU/m^2)				
Low	1.00	1.00	–	1.00
Medium	1.94 (0.51–7.38)	0.58 (0.10–3.33)	–	.53 (0.09–3.10)
High	2.70 (0.74–9.83)	2.67 (0.57–12.43)	–	2.42 (0.49–11.84)
Beta-(1→3)-D-glucan concentration (μg/mg)				

Low	1.00	–	1.00	1.00
Medium	0.70 (0.21–2.28)	–	0.52 (0.10–2.67)	0.42 (0.08–2.30)
High	0.70 (0.21–2.28)	–	0.76 (0.18–3.21)	0.44 (0.09–2.21)
Beta-(1→3)-D-glucan load ($\mu g/m^2$)				
Low	1.00	–	1.00	1.00
Medium	0.70 (0.21–2.28)	–	0.62 (0.13–2.83)	0.55 (0.11–2.67)
High	0.70 (0.21–2.28)	–	0.46 (0.09–2.29)	0.38 (0.07–2.13)

EU: Endotoxin units.

*Statistical comparisons between EIB and no EIB groups were completed using logistic regression with GEE to account for clustering within families.

[†]Low, medium, and high levels for endotoxin and beta-(1→3)-D-glucan were determined based on their corresponding tertile values of the exposure distribution, separately, for play and mattress areas: Low (1st tertile), Medium (2nd tertile), and High (3rd tertile).

Model I: Model with no adjustments for EIB; Models II: Adjusted model for EIB with endotoxin as an independent variable; Model III: Adjusted model for EIB with beta-(1→3)-D-glucan as an independent variable.

aOR: Adjusted odds ratio. Models II and III were adjusted for sex, age, parental education, parental smoke, parental history of asthma and allergy, pet ownership, atopic sensitization, home dampness, and location of residence.

[§]In addition to adjusted variables in Models II and III, Model IV was mutually adjusted for endotoxin or beta-(1→3)-D-glucan as appropriate. That is, model with play area endotoxin concentration as an independent variable was adjusted for play area beta-(1→3)-D-glucan concentration. Model with play area endotoxin load as an independent variable was adjusted for play area beta-(1→3)-D-glucan load. Similar procedure was performed for mattress endotoxin and beta-(1→3)-D-glucan.

[‡]$p<0.05$.

CHAPTER 6

THE ASSOCIATION BETWEEN ENDOTOXIN AND BETA-(1→3)-D-GLUCAN IN HOUSE DUST WITH ASTHMA SEVERITY AMONG SCHOOLCHILDREN (MANUSCRIPT III)

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6.1 Abstract

Background: Asthma severity can be affected by microbial exposures. However, less is known about the specific indoor agents aggravating the disease in children. This could aid childhood asthma management strategies and reduce morbidity.

Objective: To examine associations between indoor endotoxin and beta-(1→3)-D-glucan exposures and asthma severity in children with asthma.

Methods: We conducted a clinical cross-sectional study of schoolchildren aged 7–17 years in the province of Saskatchewan, Canada. Children with asthma (n = 116) were identified through a combination of survey responses and objective clinical assessments. Asthma severity categories among the children with asthma were based on recommended guidelines (mild asthma: >2 days/week of daytime asthma symptoms, ≤4 night/month of nighttime asthma symptoms, and ≥80% predicted FEV_1; moderate/severe asthma: Continuous daytime asthma symptoms, frequent nighttime asthma symptoms, and ≤60% predicted FEV_1). Levels of indoor endotoxin and beta-(1→3)-D-glucan were measured in dust samples obtained from play area floors and child's mattresses.

Results: The study population of 116 children with asthma was comprised of 75.9% mild asthma and 24.1% moderate/severe asthma. Exposure to high mattress endotoxin concentration was positively associated with moderate/severe asthma [adjusted odds ratio (aOR) = 11.40, 95% confidence interval (CI): 1.45–89.43] while high beta-(1→3)-D-glucan concentration (aOR = 0.16, 95%CI: 0.03–0.89) and load (aOR = 0.10, 95%CI: 0.02–0.72) in play areas were inversely associated with moderate/severe asthma. Furthermore, among the children with asthma, high mattress endotoxin concentration was significantly associated with lower FVC (p=0.01) and FEV_1 (p=0.03). These associations were not seen for beta-(1→3)-D-glucan.

Conclusion: Our results showed differential effects of microbial exposures on childhood asthma severity and further highlight domestic endotoxin exposure effects on respiratory health outcomes in children with asthma.

Key words: Asthma severity, Lung function, House dust, Endotoxin, Beta-(1→3)-D-glucan, Children.

6.2 Introduction

Asthma is the most common chronic disease[1] and a leading cause of morbidity among children in Canada, accounting for a great deal of economic burden per year.[2] Examination of risk factors for asthma severity could identify exposures that aggravate the disease among children and aid attempts to reduce morbidity and subsequent healthcare utilization and costs. The National Asthma Education and Prevention Program (NAEPP) Expert Panel Report 2 guidelines[3] recommend that asthma severity be assessed using a combination of frequency of clinical respiratory symptoms (day- and night-time symptoms) and objective lung function criteria (determined with forced expiratory volume in one second [FEV_1]).

Exposures to dust mite,[4,5] furred pets,[6,7] and tobacco smoke[8,9] have been shown to be associated with asthma severity. While exposure to indoor mold or dampness have also been reported to worsen asthma symptoms,[10,11] to date, the impact of many indoor microbial components in the exacerbation of asthma remains poorly assessed.

Evidence of associations between endotoxin, a component of gram-negative bacteria,[12] and childhood asthma is controversial with some studies showing protective,[13,14] and adverse[15–17] effects as well as no association.[18,19] Endotoxin is also seen as a pro-inflammatory agent, which means that it could also be associated with worsening asthma conditions.[15–17] Indeed, it has been shown that endotoxin may aggravate asthma conditions in children with the disease, in terms of increased frequency of wheezing and asthma medication use[3,17] suggesting that the indoor environment may play an important role in the management of childhood asthma. Most of the previous studies examining the relationships between asthma severity and endotoxin have focused on reports of the frequency of wheeze[17,20] and asthma medication use.[17] Additional indicators of severity, incorporating objective measures of disease severity, should be

investigated for better understanding of the associations between indoor microbial exposures and asthma severity in children.

While beta-(1→3)-D-glucan represents a marker of both bacterial and fungal exposure,[21] its role in the exacerbation of asthma is also less well investigated with previous studies focusing on endotoxin exposure as a sole marker of indoor microbial exposure.[17,22] Investigating the respiratory effects of endotoxin and beta-(1→3)-D-glucan exposures, in tandem, will further our knowledge of the relationships between indoor microbial exposure and asthma severity in children.

In this study, we examined the relationships between house dust endotoxin and beta-(1→3)-D-glucan exposure levels and asthma severity in schoolchildren with asthma. In addition, we also investigated the relationships between endotoxin and beta-(1→3)-D-glucan exposures and lung function in these children. Identifying specific microbial indoor exposure associated with asthma exacerbations is important as this could help guide asthma management among children and, ultimately, decrease associated morbidity and healthcare costs.

6.3 Methods

6.3.1 *Study population, selection and recruitment*

A cross-sectional study with clinical components was conducted in the province of Saskatchewan, Canada from 2015–2016. The study population consisted of schoolchildren (aged 7–17 years) who were part of an initial 2013 cross-sectional survey previously described.[23] Briefly, study packages, including an information letter, survey and pre-paid return envelope, were mailed to parents for self-completion in 2013. Those who consented to participate in further testing were re-approached in 2015. At this time, we repeated the survey in order to obtain

accurate information on current respiratory symptoms that correspond to lung function values in the participants. Subsequently, clinical testing (spirometry and exercise challenge testing) as well as home dust sample collection was completed in 2015–2016.

The study was approved by the University of Saskatchewan Biomedical Research Ethics Board (Bio #: 14–162). Furthermore, all school divisions involved approved the study. Completion and return of the survey implied voluntary consent for the questionnaire portion. All children and a parent provided written assent and consent, respectively, prior to clinical testing and home dust collection.

6.3.2 *Survey questionnaire*

Parents completed a questionnaire based on the standardized and validated questions from the International Study of Asthma and Allergy in Childhood (ISAAC),[24] the American Thoracic Society Children's Respiratory Disease,[25] and questionnaires used previously in the Saskatchewan Lung Health studies.[13] Questions about respiratory health, general health, parental health history, environmental exposure, sociodemographic factors as well as housing characteristics were included.

6.3.3 *Spirometry and exercise challenge testing (ECT)*

During home or school visits, children performed spirometry assessment according to recommended standards[26] using the Easy-on PC spirometer (ndd Medical Technologies, Zurich, Switzerland). Measurements of forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and forced expiratory flow ($FEF_{25\%–75\%}$) were obtained. Predicted values were based on the all-age, multi-ethnic Global Lung Function Initiative (GLI)

reference equation.[27] Children were excluded from testing if they were unable to perform the test due to existing medical conditions (n = 3).

ECT was also completed based on recommended protocols.[28] Briefly, children stepped up and down on a 6 inch step at a heart rate (HR) between 150–200 beats per minute for 5 minutes. Heart rates were monitored throughout the exercise with a polar Heart Rate monitor (Polar Electro, Woodbury, NY). Spirometry was repeated at 3 and 10 minutes after cessation of exercise.

6.3.4 *Classification of asthma*

Asthma was identified through a combination of survey responses and results from clinical assessments (spirometry and ECT). Children were classified as positive for asthma if they had positive response to the questions: "Has this child ever been diagnosed as having asthma by a doctor?" and/or a positive response to: "Has this child taken prescribed asthma medication in the past 12 months?" (n = 95). Otherwise, they were classified as "no asthma". We then used the validated asthma case detection algorithm developed by Gerald *et al*[28] to further identify children positive for asthma (n = 21). This was based on an FEV_1/FVC ratio less than 80% upon spirometry testing and/or demonstrated a greater than 15% decrease in FEV_1 or 25% or greater decrease in $FEF_{25\%–75\%}$ from baseline after cessation of exercise.[29,30] Overall, from this process, 116 children were identified to have asthma. The algorithm has a high sensitivity (82%) and specificity (93%) when compared to clinical assessment of asthma by a physician.[28]

6.3.6 *Classification of asthma severity*

The 116 children positive for asthma were classified into one of four asthma severity groups based on the NAEPP guidelines[3]: mild intermittent, mild persistent, moderate persistent, and severe persistent asthma.

In the NAEPP guidelines, spirometry does not distinguish between mild intermittent and mild persistent asthma (both with $FEV_1 \geq 80\%$), therefore the two severity groups were collapsed into one single group (mild intermittent/mild persistent asthma).[31] Similarly, because it is difficult to reliably differentiate moderate persistent from severe persistent asthma based on self-reported symptom frequency alone (i.e. differentiating between "daily" and "continual" daytime symptoms or defining "frequent" nights with symptoms),[3,32] the two most severe groups (moderate and severe persistent asthma) were also collapsed into one severity group (moderate/severe persistent asthma). Overall, two asthma severity groups [mild intermittent/mild persistent asthma (mild asthma) and moderate/severe persistent asthma (moderate/severe asthma)] were considered as the primary outcomes for the current study; with mild asthma used as the reference group.

6.3.7 *Collection and analysis of dust samples to quantify endotoxin and beta-(1→3)-D-glucan exposure*

Settled dust samples were vacuumed from the floor of child's play area and mattress surfaces adhering to recommended standardized protocols[33] using pre-weighed X-Cell-100 filter socks with a pore size of approximately 4.0–12.3 microns (Midwest Filtration LLC, OH, USA). Carpet floors had 2 m^2 vacuumed for 4 minutes while completely smooth floors (e.g. hardwood, laminate, or linoleum) had 4 m^2 vacuumed for the same time duration. Dust collection from the mattress surfaces (with the bottom sheet on) was completed after all duvets, blankets and sheets that the child slept under were removed and the entire surface area of the mattress was then

vacuumed for 2 minutes. Dust samples were stored in a desiccator at 4ºC until extraction and analysis.

Samples were brought to room temperature and 10 mg of sieved dust was weighed out for extraction. Dust samples were extracted with 20 mL 0.05% Tween 20 (Fisher Scientific, Mississauga, ON, Canada) in pyrogen-free water[34] (GE Healthcare Bio-Science, Mississauga, ON, Canada) and shaken at 325 revolutions per minute (RPM) for 2 hours. The extracted solution was then centrifuged (Sorvall ST 16R, Thermo Fisher Scientific, Mississauga, ON, Canada) at 1000 x g for 15 minutes to obtain supernatant and 1 mL aliquots were stored at -80ºC until analysis. An aliquot of the supernatant was diluted 1 in 10, and was used to measure endotoxin in the chromogenic *Limulus* Amoebocyte Lysate (LAL) Kinetic QCL assay according to manufacturer's specifications (Lonza, Walkersville, MD, USA). The water soluble fraction of beta-(1→3)-D-glucan was measured in a second aliquot using the Glucatell assay kit with a beta-(1→3)-D-glucan-specific inhibition enzymes based on the Kinetic Onset Time protocol according to manufacturer's specifications (Associate of Cape Cod, East Falmouth, MA, USA).[35]

To quantify endotoxin and beta-(1→3)-D-glucan levels, the absorbance of endotoxin and beta-(1→3)-D-glucan was monitored at 405 nm for 2 hours and 1 hour, respectively, using the Biotek ELx808 plate reader (BioTek, Winooski, VT, USA). Values were compared to standard curves prepared for endotoxin (0.005EU/mL–50 EU/mL) and beta-(1→3)-D-glucan (3.125 pg/mL–100 pg/mL). Endotoxin and beta-(1→3)-D-glucan levels were reported as concentration (per gram of sampled dust) and load (per square meter of sampled area) given that the two measures represent different aspects of indoor microbial exposure (dose and burden, respectively).[36]

6.3.8 *Statistical analysis*

All analyses were completed using the Statistical Package for the Social Science (SPSS) Version 24 (SPSS Inc. Armonk, NY: IBM Corp.) and the Statistical Analysis System (SAS) Version 9.4 (SAS Institute Inc., Cary, NC, USA). Categorical variables were compared between asthma severity groups using the independent samples chi-square (χ^2) and Fisher's exact tests as appropriate while continuous variables were compared using the independent sample Student *t*-test. Mean endotoxin and beta-(1→3)-D-glucan for play area and mattress were expressed as geometric mean (GM) and compared between severity groups. Comparison of absolute values of lung function variables between severity groups were completed using the analysis of covariance (ANCOVA) adjusted for age, sex, and height.

We assessed the associations between endotoxin and beta-(1→3)-D-glucan levels and the dichotomous health outcome of asthma severity (mild asthma or moderate/severe asthma) using multiple logistic regression. Similarly, multiple linear regression models were fitted to assess the associations between endotoxin and beta-(1→3)-D-glucan levels and lung function variables (FVC, FEV_1, FEV_1/FVC, and $FEF_{25\%–75\%}$). Additional variables included in the models were sex, age, height, parental smoking, home dampness, visible mold, asthma medication use, allergen sensitization, and location of residence. These variables were included based on statistical significance from the univariate analyses, clinical/biological importance, or the effect the removal of a variable had on the beta coefficient of other variables in the model.[37,38] Endotoxin and beta-(1→3)-D-glucan remained, *a priori*, in all models. Levels of endotoxin and beta-(1→3)-D-glucan were modelled as categorical variables in tertiles to define low (1st tertile), medium (2nd tertile), and high (3rd tertile) levels because the relationships with moderate/severe asthma did not meet the linear assumption when log-transformed (ln) endotoxin and beta-(1→3)-D-glucan were used. Low, medium, and high levels were determined based on their

corresponding tertile values of the exposure distribution, separately, for play and mattress areas. The reference value was the lower endpoint of each of the tertiles. Separate independent models were fitted for each of play area and mattress dust including: i) a univariate model for each endotoxin (concentration and load) and beta-(1→3)-D-glucan (concentration and load); ii) main effects model for endotoxin; iii) main effects models for beta-(1→3)-D-glucan; and iv) main effects plus endotoxin and beta-(1→3)-D-glucan. Throughout the analyses, generalized estimating equations (GEE) were used to account for clustering within families. The Hosmer-Lemeshow test was used to assess model Goodness-of-Fit.

The strength of the associations were assessed using: i) odds ratio (OR) and their 95% confidence interval (CI) for categorical outcome variables (mild asthma vs. moderate/severe asthma), and ii) beta coefficients with their standard errors for continuous outcome variables (baseline lung function measurements). Statistical significance was defined by an alpha level of 0.05.

6.4 Results

6.4.1 *Characteristics of study population*

The socio-demographics characteristics for the study population are presented in Table 6–1. Distributions for most of the characteristics, including age, were similar between the two asthma severity groups (p>0.05). When comparing respiratory symptoms and asthma severity indicators between the two groups, children with moderate/severe asthma were more likely to experience night cough, wheeze during exercise, have greater than 3 episodes of asthma, speech limitations, and were more likely to miss school days compared to children with mild asthma (Table 6–2). Other asthma indicators were not statistically significant between the two groups.

Baseline lung function differed significantly between severity groups when assessed either as absolute or percent predicted values (Table 6–3). Mean FVC, FEV_1, FEV_1/FVC, and $FEF_{25\%–75\%}$ were all significantly lower in children with moderate/severe asthma compared to children with mild asthma.

6.4.2 *Mean endotoxin and beta-(1→3)-D-glucan exposure levels among asthma severity groups*

There were no statistically significant differences in mean (geometric) endotoxin levels between the two severity groups (Table 6–4). However, children with moderate/severe asthma had significantly lower play area beta-(1→3)-D-glucan concentration and load compared to the mild asthma group.

6.4.3 *Associations between endotoxin and beta-(1→3)-D-glucan exposure levels and asthma severity and lung function measures*

Mattress endotoxin concentration levels were significantly associated with increased risk of moderate/severe asthma in a dose response manner, independent of beta-(1→3)-D-glucan exposure levels (Table 6–5). This pattern was reversed for beta-(1→3)-D-glucan exposure levels. Play area beta-(1→3)-D-glucan levels were significantly associated with reduced risk of moderate/severe asthma independent of endotoxin exposure levels.

To further investigate indicators of severity, we looked at the associations between endotoxin and beta-(1→3)-D-glucan exposure levels and frequency of wheeze in the past 12 months. In contrast to overall severity assessed by the NAEPP guidelines, higher levels of mattress beta-(1→3)-D-glucan concentration was significantly associated with more frequent

wheeze (>3 episodes) in the past 12 months (aOR = 7.58, 95%CI: 1.17–72.76) while no significant association was observed for endotoxin.

Table 6–6 presents the associations between endotoxin and beta-(1→3)-D-glucan exposures with lung function variables among the 116 children with asthma. Similar to the relationships between endotoxin and moderate/severe asthma, mattress endotoxin concentration levels were significantly associated with decreased absolute values for FVC [beta (β) = -0.32, SE = 0.12) and FEV_1 (β = -0.27, SE = 0.12) after adjusting for potential confounders, including beta-(1→3)-D-glucan levels. No such association was observed for beta-(1→3)-D-glucan levels.

6.5 Discussion

This study demonstrated varied associations between indoor microbial exposure and asthma severity outcomes depending on the specific microbial agent. Endotoxin exposure was positively associated with moderate/severe asthma while beta-(1→3)-D-glucan showed inverse associations. Also, endotoxin levels were associated with lower lung function but the association was non-existent for beta-(1→3)-D-glucan. However, beta-(1→3)-D-glucan was associated with increased wheeze frequency. These results suggest that while endotoxin may be more consistently associated with adverse lung health outcomes, beta-(1→3)-D-glucan has also shown associations with some indicators of asthma severity. These differential patterns may be acting through different mechanisms or may reflect different roles as a possible causal agent versus trigger.

The most recent update from the Institute of Medicine (IOM) on the review of indoor environmental exposures and asthma exacerbation showed evidence of associations between endotoxin exposure levels and asthma severity.[39] In a cross-sectional study among schoolchildren with asthma (aged 6–13 years) in the USA, Rabinovitch *et al*[40] demonstrated that

endotoxin levels had significant positive associations with asthma severity indices (measured as asthma symptom scores severe enough to prevent play or sleep in children). However, in this earlier study, endotoxin levels were measured from dust obtained from personal exposure monitoring of schoolchildren versus vacuumed dust sample used in the current study. In the National Survey of Lead and Allergens in Housing study, also in the USA, Thorne *et al*[17] reported that endotoxin levels in bedroom floor dust were significantly associated with increased frequency in the daily need for asthma medication. We add to the previous evidence by showing that high endotoxin levels significantly increased the risk of clinically defined moderate/severe asthma, further highlighting the potential clinical importance of microbial endotoxin exposure and asthma exacerbation in children with asthma.

We also demonstrated an inverse association between high play area beta-(1→3)-D-glucan levels and moderate/severe asthma. A Puerto Rican study among children (aged 6–14 years) showed positive associations between high beta-(1→3, 1→6)-D-glucan exposure and asthma severity (measured as ≥1 visits to the emergency department [ED]/urgent care for asthma).[41] Another study among schoolchildren with asthma in the Netherlands (aged 7–11 years) also reported a significant association between high play area beta-(1→3)-D-glucan and increased peak expiratory flow (PEF) variability as an indicator of asthma severity.[42] While the results of our study are in contrast to these two previous studies, one observational study in Australia found beta-(1→3)-D-glucan to be significantly associated with increased FEV_1 as a measure of severity indicator.[43] A separate study in the USA also showed that high beta-(1→3)-D-glucan exposure level was inversely associated with frequency of recurrent wheezing in infants.[44] These inconsistencies between studies could be explained by methodological discrepancies and/or differences. For example, in the Puerto Rico study, parental reports of ED

visits for asthma care were used as an asthma severity indicator while we used a combination of clinical symptoms and lung function variables based on NAEPP guidelines.[3] Also, the inflammatory potency of beta-(1→3)-D-glucan also appears to be strongly dependent on the type and conformation of glucans in house dust. While we assayed beta-(1→3)-D-glucan in the current study, Blatter *et al* assayed beta-(1→3, 1→6)-D-glucan which has been reported to be a stronger inducer of inflammatory responses compared to beta-(1→3)-D-glucan.[45]

Reasons for the observed inverse association between beta-(1→3)-D-glucan and moderate/severe asthma in the current study are unclear but could be related to the airway inflammatory pattern that may be present in our subjects (eosinophilic or neutrophilic inflammation). For example, an inhalation challenge study in guinea pigs showed that repeated exposures to beta-(1→3)-D-glucan induced a significant increase in eosinophil counts without an increase in neutrophils.[46] While patients with severe asthma may differ from those with mild asthma in having higher eosinophil counts in their airways,[47] studies by Wenzel *et al* showed that not all severe asthma patients have airway eosinophilia[48] and that neutrophil counts are also higher than normal in the airways of subjects with severe asthma.[49] Unfortunately, no measurement of eosinophil and neutrophil counts was undertaken in this study to investigate if the observed associations between beta-(1→3)-D-glucan and moderate/severe asthma was related to airway inflammatory patterns.

Another possible explanation could be related to indoor mold level alteration through remediation strategies such as cleaning. A randomized controlled trial in the US assessed the effects of indoor mold reduction through moisture remediation strategies on asthma exacerbation.[50] Compared to the controlled group, remediation group had a significantly lower rate of asthma exacerbation (measured as frequency of asthma symptoms in days and ≥2 ED visit

or ≥1 hospitalization for asthma past 12 months). In the current study, we observed borderline significant difference in report of indoor visible mold between moderate/severe and mild asthma group (8.4% vs. 22.2%, respectively; p=0.08). Since significant associations have been reported between indoor visible mold,[51] moldy odor,[52] and culturable mold spores[51] with increased beta-(1→3)-D-glucan concentration levels in house dust, it is possible that children with moderate/severe asthma in our study may be cleaning their homes more intensely with a focus on mold reduction. Such a practice might have reduced beta-(1→3)-D-glucan exposure producing the observed inverse association with moderate/severe asthma due to the cross-sectional nature of the study design.

Finally, beta-(1→3)-D-glucan is positively associated with indoor relative humidity (RH)[51] with indoor beta-(1→3)-D-glucan concentration levels significantly higher in summer compared to winter period.[21] Dust sample collection for this study was completed between December 2015 and April 2016. As such, beta-(1→3)-D-glucan levels may be lower than levels required to induce adverse respiratory outcomes in the studied population due to the relatively colder, drier, and lower RH in winter compared to summer period.[53] Alternatively, beta-(1→3)-D-glucan compared to endotoxin may not be as an important measure of microbial activity in relation to childhood asthma in this region of Canada due to the low RH.

We observed an increased risk of moderate/severe asthma with high mattress endotoxin concentration in the current study. While it may be argued that the above explanations for beta-(1→3)-D-glucan should also be applicable to endotoxin exposures, it should be noted that endotoxin is ubiquitous in nature and represents a measure of gram-negative bacteria.[12,52] For these reasons, it has been suggested that endotoxin concentration in dust samples is not necessarily associated with mold exposures in contrast to beta-(1→3)-D-glucan which is a gram-

positive cell wall of most fungi, including mold.[52] Additionally, beta-(1→3)-D-glucan is believed to be a less potent inducer of inflammatory reaction and respiratory symptoms than bacteria endotoxin.[42] Therefore, it is not surprising that endotoxin behaves differently than beta-(1→3)-D-glucan in their association with moderate/severe asthma in the current study.

Individuals with asthma appear to have a heightened response to acute pulmonary effects of endotoxin. In a controlled challenge study among adults, Kitz *et al*[54] found a significant decrease in FEV_1 in those with asthma 90 mins after inhalation of endotoxin extracts compared to healthy controls. While the study showed the effects of acute exposure to endotoxin on lung function in adults, we complemented these results by looking at children with asthma in the current study. We found high mattress endotoxin levels were significantly associated with lower FVC and FEV_1 in children with asthma. These findings are also consistent with a previous study among schoolchildren (aged 6–18 years) in Canada where higher indoor endotoxin level was inversely associated with decreased FEV_1[22] and greater diurnal PEF variability.[55] In another study in the US among schoolchildren (aged 6–13 years), personal endotoxin exposure was also associated with lower daily evening FEV_1.[40] Results from our study further expanded the evidence in the IOM review[39] and showed that the adverse respiratory effects of endotoxin in children with asthma may be independent of other microbial exposure in the indoor environment such as beta-(1→3)-D-glucan. In addition, the effects may not depend on asthma severity status of a child, but that once children develop asthma, they are more likely to be sensitive to the acute inflammatory effects of endotoxin compared to those without asthma. Although we controlled for asthma medication use in our models, further studies are needed in children with poorly controlled asthma to validate the magnitude of lung function declines associated with long-term acute and/or chronic endotoxin exposure in the indoor environment.

We did not find any statistically significant associations between beta-(1→3)-D-glucan and lung function in the current study. However, in a separate analysis that assessed the relationships between indoor microbial exposures and asthma severity indicators, mattress beta-(1→3)-D-glucan concentration was significantly associated with report of more wheeze frequency (>3 episodes) in the past 12 months. The positive association between beta-(1→3)-D-glucan concentration and increased episodes of wheeze (>3 episodes) in the past year and its lack of association with lung function may suggest that, in children with asthma, beta-(1→3)-D-glucan exposure could be related to symptom exacerbation and not necessarily lung function impairment. Beta-(1→3)-D-glucan exposure might result in increased symptom severity but not enough to impair pulmonary function. Alternatively, it could be that beta-(1→3)-D-glucan levels in the homes of children involved in this study were lower than a level required to induce significant lung function impairment.

Limitations of our study should be considered. The participation rate experienced was low. We recruited participants from urban and rural locations and were frequently hard to reach, especially in the rural locations. This might have led to low statistical power as was seen in the case of some strong estimates found for play area endotoxin and mattress beta-(1→3)-D-glucan levels but lacking statistical significance in this study. Dust samples were collected at a single time-point. As such, it is possible that the current measured microbial exposure levels may not reflect the level of exposure present when the reported episode of asthma severity actually happened. However, we feel that the microbial exposure levels reported in the current study may reflect similar exposure patterns that were occurring at the time of event for two reasons. First, studies have suggested that a single dust sampling for endotoxin analysis showed little variation over time[56–58] and reflects longer-term exposure to microbial products for at least a 1 year

period.[59] Second, while some studies might have reported seasonal variability in house dust levels and microbial components,[21,60,61] we obtained similar results of associations between microbial exposures and asthma severity after additional adjustment for seasonality in our models. Finally, the dust extraction analysis procedure used in this study is specific in determining the water soluble (WS) fraction of beta-(1→3)-D-glucan[62] which may not represent the most potent fraction of beta-(1→3)-D-glucan compared to alkaline soluble (AS) fraction.[63] This extraction procedure may be one of the reasons beta-(1→3)-D-glucan levels in the current study were overall lower (10 fold) than the amounts of beta-(1→3)-D-glucan detected in other studies.[14,42,44,64] Furthermore, in addition to small sample size, the lower levels of the WS fraction of beta-(1→3)-D-glucan may also explain the lack of association seen in the case of strong estimates found for mattress beta-(1→3)-D-glucan levels and asthma severity in this study. However, while the AS fraction of beta-(1→3)-D-glucan may represent the most potent fraction of beta-(1→3)-D-glucan[62] and has been the most commonly investigated,[14,42,44,64] we have further demonstrated, in this study, that the WS soluble fraction of beta-(1→3)-D-glucan could also worsen respiratory symptoms in children with preexisting asthma conditions.

Our study also has several strengths. We used objective measures for exposure and outcome assessments and recommended guidelines to define asthma severity,[3] thus limiting the possibility of bias. Furthermore, clinical data and dust samples were collected by trained technicians using standardized protocols.[26,65] Laboratory personnel were blinded to disease status of each child and to source of dust samples (play area or mattress dust). Finally, home dust collection was conducted concurrently with clinical data to eliminate possible bias in dust sampling which could have been influenced by the health status of the child.

In conclusion, we demonstrated that asthma severity in children is associated with indoor microbial exposure. Endotoxin increased the risk of moderate/severe asthma and worsened lung function in children with asthma while beta-(1→3)-D-glucan exposure was sufficient to exacerbate symptom severity but not enough to impair pulmonary function or induce moderate/severe asthma. These results are important as they help clarify the role of endotoxin and beta-(1→3)-D-glucan in childhood asthma morbidity, highlighting that endotoxin may have more detrimental effects on respiratory health outcome in children with asthma. This further supports the notion that asthma severity might not be associated with the same microbial exposures associated with asthma development. For examples, endotoxin[13,14,66] and beta-(1→3)-D-glucan[44,57,67] exposures may prevent asthma development but may also increase severity of existing asthma conditions; warranting the need for indoor microbial exposure avoidance in the management of childhood asthma.

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Table 6–1: Demographic characteristics of study population by asthma severity group

	Mild Asthma (n = 88)	Moderate/Severe Asthma (n = 28)	*p*-value
Mean age (±SD), years	11.0 (2.7)	10.9 (2.6)	0.78
Body mass index (±SD), kg/m^2	20.4 (4.7)	20.9 (5.8)	0.62
% Overweight	14.8	16.0	0.89[†]
% Male	61.4	75.0	0.19
Ethnic background			
% Caucasian	84.1	73.1	0.24[†]
% Others	15.9	26.9	
Physical activity			
% Low	3.4	3.6	0.96[†]
% Moderate	35.2	32.1	
% High	61.4	64.3	
Parental education level			
% > high school (maternal)	81.6	88.9	0.56[†]
% > high school (paternal)	76.5	88.0	0.21
Tobacco smoke exposure			
% Parental smoking	11.8	14.3	0.75[†]
% Environmental tobacco smoke (ETS)	10.2	3.7	0.45
Home characteristics			
% Home with air filter	50.0	53.6	0.74
% Home with humidifier	28.4	32.1	0.71
% Pet ownership	59.1	57.1	0.86
% Dampness in the home	33.3	21.8	0.26
% Home with visible mold	22.2	8.4	0.08
Atopic sensitization			
% Atopic[‡]	72.4	69.6	0.79
% Mold sensitization[‡‡]	53.9	56.5	0.83
Family history			
% Parental history of asthma	35.2	32.1	0.78
% Parental history of allergy	59.1	64.3	0.63

Location of residence			
Urban	88.6	82.1	0.35[†]
Rural	11.4	17.9	

[†]Statistical difference assessed by the Fisher's exact test due to small cell sizes (expected values < 5).

[‡]Children were tested for atopic sensitization using a panel of standardized allergen extracts: cat, local grass, mold (*Aspergillus, Alternaria, Cladosporium*), and house dust mite. Subjects was considered atopic if a positive reaction to at least one of the applied allergens is raised ≥3 mm compared to the saline control.

[‡‡]Sensitization (positive skin prick test) to any of the three tested mold allergens (*Alternaria, Aspergillus, or Cladosporium*).

Table 6–2: Profile of respiratory symptoms, asthma severity indicators, and healthcare accessibility among the study population

	Mild Asthma (n = 88)	Moderate/Severe Asthma (n = 28)	*p*-value
Respiratory symptoms			
% Wheeze past 12 months	39.3	88.5	<0.001
% Night cough past 12 months	38.6	60.7	0.04
% Wheeze during/after exercise	65.9	89.3	0.02
% Sleep disturbance due to cough past 12 months	43.2	60.7	0.11
Asthma severity indicators past 12 months			
% > 3 asthma episodes	27.3	48.1	0.04
% > 3 wheeze episodes	29.5	42.3	0.28
% Speech limit to 1–2 words	6.8	21.4	0.04
School absenteeism			
% Missed school due to breathing problem past 12 months	28.9	38.5	0.01
Mean (±SD) number of school missed days per child in the past 12 months	1.0 (2.6)	3.3 (6.0)	0.07
Median number of school missed days per child in the past 12 months	0.0	2.0	0.002‡
Asthma medication			
% Prescribed breathing medication past 12 months	55.7	71.4	0.14
% Taking asthma medication for at least 2 days per week	23.9	35.7	0.22
% Prescribed antibiotics for respiratory infections past 12 months	39.8	50.0	0.34
Mean (±SD) number of time prescribed antibiotic per child per year	1.6 (0.9)	2.2 (1.8)	0.11
Median number of time prescribed antibiotic per child in the past 12 months	1.0	2.0	0.104‡
Healthcare accessibility			
Time travelled to access basic healthcare (±SD), minutes	15.6 (19.9)	20.7 (21.3)	0.26

Time travelled to access emergency healthcare (±SD), minutes	14.4 (14.7)	18.8 (16.7)	0.21

SD: Standard deviation

‡Test performed with Mann-Whitney U test.

Table 6–3: Comparison of lung function values between asthma severity groups

	Mild Asthma (n = 81)		Moderate/Severe Asthma (n = 25)	
	Absolute Value* Mean (SD)	% Predicted of Normal Mean (SD)	Absolute Value* Mean (SD)	% Predicted of Normal Mean (SD)
FVC (L)	3.15 (0.05)	102.8 (13.0)	2.83 (0.10)‡	92.3 (13.0)†
FEV_1 (L)	2.63 (0.05)	98.8 (12.9)	2.16 (0.09)‡‡	74.0 (9.1)††
FEV_1/FVC	0.84 (0.10)	95.8 (7.6)	0.77 (0.02)‡‡	87.6 (10.9)†
$FEF_{25\%–75\%}$ (L)	2.82 (0.08)	89.8 (21.8)	2.01 (0.15)‡‡	64.2 (26.5)††

SD: Standard deviation; B: Baseline; L: Litre.

*Adjusted for age, sex, and height.

‡$p<0.05$, ‡‡$p<0.001$ (for absolute values); †$p<0.001$; ††$p=0.001$ (for percent predicted values).

Table 6–4: Geometric mean (GSD) of endotoxin and beta-(1→3)-D-glucan concentration and load in house dust from play area floor and mattresses by asthma severity status

	Mild Asthma (n = 78)	Moderate/Severe Asthma (n = 24)	*p*-value
Play area			
Endotoxin concentration (EU/mg)	58.7 (2.3)	49.2 (1.9)	0.64
Endotoxin load (EU/m^2)	23102.6 (2.4)	19559.2 (2.2)	0.41
Beta-(1→3)-D-glucan concentration (μg/g)	9.7 (2.0)	7.1 (2.0)	0.05
Beta-(1→3)-D-glucan load (μg/m^2)	157.4 (5.4)	61.1 (9.3)	0.03
Mattress			
Endotoxin concentration (EU/mg)	20.8 (2.4)	20.4 (2.3)	0.93
Endotoxin load (EU/m^2)	9610.4 (2.5)	9283.6 (2.8)	0.88
Beta-(1→3)-D-glucan concentration (μg/g)	4.6 (2.0)	4.5 (1.7)	0.90
Beta-(1→3)-D-glucan load (μg/m^2)	46.7 (4.1)	40.3 (5.0)	0.67

GSD: Geometric standard deviation.
EU: Endotoxin units.

Table 6–5: Multiple logistic regression analyses describing the associations between endotoxin and beta-(1→3)-D-glucan levels[†] and moderate/severe asthma[††]

	Model I OR (95% CI)	Model II* aOR (95% CI)	Model III* aOR (95% CI)	Model IV*[§] aOR (95% CI)
Play area				
Endotoxin concentration (EU/mg)				
Low	1.00	1.00	–	1.00
Medium	1.61 (0.53–4.88)	2.88 (0.61–13.47)	–	4.61 (0.77–27.82)
High	1.00 (0.31–3.24)	1.11 (0.21–5.73)	–	2.88 (0.44–19.07)
Endotoxin Load (EU/m^2)				
Low	1.00	1.00	–	1.00
Medium	0.86 (0.29–2.57)	1.00 (0.24–4.14)	–	3.23 (0.54–19.52)
High	0.72 (0.23–2.22)	0.60 (0.11–3.53)	–	1.96 (0.27–14.35)
Beta-(1→3)-D-glucan concentration (μg/mg)				
Low	1.00	–	1.00	1.00
Medium	0.39 (0.13–1.21)	–	0.15 (0.03–0.86)[‡]	0.15 (0.03–0.89)[‡]
High	0.39 (0.13–1.21)	–	0.17 (0.03–0.87)[‡]	0.16 (0.03–0.89)[‡]
Beta-(1→3)-D-glucan load (μg/m^2)				
Low	1.00	–	1.00	1.00
Medium	0.45 (0.14–1.40)	–	0.27 (0.06–1.29)[‡]	0.23 (0.04–1.20)
High	0.54 (0.18–1.63)	–	0.13 (0.02–0.75)[‡]	0.10 (0.02–0.72)[‡]
Mattress				
Endotoxin concentration (EU/mg)				
Low	1.00	1.00	–	1.00
Medium	1.49 (0.46–4.86)	4.40 (0.82–23.55)	–	7.05 (1.07–46.55)[‡]
High	2.10 (0.67–6.64)	5.36 (1.01–28.67)[‡]	–	11.40 (1.45–89.43)[‡]
Endotoxin Load (EU/m^2)				
Low	1.00	1.00	–	1.00
Medium	1.68 (0.52–5.39)	3.70 (0.76–17.90)	–	4.08 (0.78–21.32)
High	1.68 (0.52–5.39)	2.13 (0.44–10.36)	–	2.41 (0.44–13.09)
Beta-(1→3)-D-glucan concentration (μg/mg)				
Low	1.00	–	1.00	1.00

Medium	1.94 (0.62–6.14)	–	1.40 (0.31–6.34)	1.44 (0.30–6.94)
High	1.44 (0.44–4.70)	–	1.48 (0.36–6.11)	1.48 (0.30–7.22)
Beta-(1→3)-D-glucan load (μg/m^2)				
Low	1.00	–	1.00	1.00
Medium	0.74 (0.25–2.18)	–	1.78 (0.44–7.24)	1.77 (0.43–7.23)
High	0.51 (0.16–1.62)	–	1.08 (0.25–4.67)	0.99 (0.22–4.42)

EU: Endotoxin units.

[†]Low, medium, and high levels for endotoxin and beta-(1→3)-D-glucan were determined based on their corresponding tertile values of the exposure distribution, separately, for play and mattress areas: Low (1st tertile), Medium (2nd tertile), and High (3rd tertile).

[††]Severity category based on the NAEPP guidelines.[3] Mild asthma used as reference category.

*Statistical comparisons between moderate/severe asthma and mild asthma were completed using logistic regression with GEE to account for clustering within families.

Model I: Model with no adjustments for moderate/severe asthma; Models II: Adjusted model for moderate/severe asthma with endotoxin as an independent variable; Model III: Adjusted model for moderate/severe asthma with beta-(1→3)-D-glucan as an independent variable.

aOR: Adjusted odds ratio. Models II and III were adjusted for sex, age, parental smoke, home dampness, visible mold in home, asthma medication use, allergen sensitization, and location of residence.

[§]In addition to adjusted variables in Models II and III, Model IV was mutually adjusted for endotoxin or beta-(1→3)-D-glucan as appropriate. That is, model with play area endotoxin concentration as an independent variable was adjusted for play area beta-(1→3)-D-glucan concentration. Model with play area endotoxin load as an independent variable was adjusted for play area beta-(1→3)-D-glucan load. Similar procedure was performed for mattress endotoxin and beta-(1→3)-D-glucan.

[‡]$p<0.05$.

Table 6–6: Multivariate linear regression analyses* describing the associations between endotoxin and beta-(1→3)-D-glucan levels† and lung function among children with asthma

	FVC β(SE)	FEV_1 β(SE)	FEV_1/FVC β(SE)	$FEF_{25\%–75\%}$ β(SE)
Play area§				
Endotoxin concentration (EU/mg)				
Low	Ref	Ref	Ref	Ref
Medium	-0.19 (0.11)	-0.17 (0.11)	0.01 (0.02)	-0.22 (0.22)
High	0.17 (0.12)	0.08 (0.13)	-0.01 (0.03)	0.02 (0.25)
Endotoxin Load (EU/m^2)				
Low	Ref	Ref	Ref	Ref
Medium	-0.14 (0.12)	-0.12 (0.12)	0.01 (0.02)	-0.15 (0.24)
High	0.20 (0.14)	0.19 (0.14)	0.02 (0.03)	0.20 (0.27)
Beta-(1→3)-D-glucan concentration ($\mu g/mg$)				
Low	Ref	Ref	Ref	Ref
Medium	0.16 (0.12)	0.19 (0.12)	0.02 (0.02)	0.28 (0.22)
High	0.13 (0.12)	0.04 (0.12)	-0.02 (0.02)	-0.03 (0.23)
Beta-(1→3)-D-glucan load ($\mu g/m^2$)				
Low	Ref	Ref	Ref	Ref
Medium	0.05 (0.13)	0.13 (0.13)	0.03 (0.03)	0.33 (0.24)
High	0.23 (0.14)	0.18 (0.13)	0.00 (0.03)	0.19 (0.24)
Mattress§				
Endotoxin concentration (EU/mg)				
Low	Ref	Ref	Ref	Ref
Medium	-0.40 (0.12)‡	-0.32 (0.12)‡	0.00 (0.02)	-0.29 (0.24)
High	-0.32 (0.12)‡	-0.27 (0.12)‡	0.00 (0.02)	-0.25 (0.24)
Endotoxin Load (EU/m^2)				
Low	Ref	Ref	Ref	Ref
Medium	-0.18 (0.12)	-0.22 (0.12)	-0.02 (0.02)	-0.26 (0.22)
High	-0.14 (0.13)	-0.12 (0.12)	0.00 (0.02)	-0.12 (0.24)
Beta-(1→3)-D-glucan concentration ($\mu g/mg$)				
Low	Ref	Ref	Ref	Ref

Medium	0.01 (0.13)	-0.09 (0.12)	-0.03 (0.02)	-0.19 (0.23)
High	0.04 (0.13)	-0.06 (0.12)	-0.02 (0.02)	-0.12 (0.22)
Beta-(1→3)-D-glucan load ($\mu g/m^2$)				
Low	Ref	Ref	Ref	Ref
Medium	-0.00 (0.12)	-0.06 (0.12)	-0.02 (0.02)	-0.06 (0.22)
High	-0.08 (0.12)	-0.11 (0.12)	-0.01 (0.02)	-0.03 (0.22)

EU: Endotoxin units.

*Models adjusted for sex, age, height, parental smoking, home dampness, visible mold in home, allergen sensitization, asthma medication use, and location of residence.

[†]Low, medium, and high levels for endotoxin and beta-(1→3)-D-glucan were determined based on their corresponding tertile values of the exposure distribution, separately, for play and mattress areas: Low (1st tertile), Medium (2nd tertile), and High (3rd tertile).

[§] In addition to adjusted variables, models were also mutually adjusted for endotoxin or beta-(1→3)-D-glucan as appropriate. That is, model with play area endotoxin concentration as an independent variable was adjusted for play area beta-(1→3)-D-glucan concentration. Model with play area endotoxin load as an independent variable was adjusted for play area beta-(1→3)-D-glucan load. Similar procedure was performed for mattress endotoxin and beta-(1→3)-D-glucan.

β(SE): Beta coefficient and standard error for the difference in lung function per levels of endotoxin or beta-(1→3)-D-glucan exposure.

[‡]$p<0.05$.

CHAPTER 7

GENERAL DISCUSSION

7.1 Summary of results and what the results add to the literature

Childhood asthma is understood to differ in prevalence between urban compared to rural children[1–6] despite reports of similar prevalence of asthma-related symptoms between the two locations.[3,7,8] Environmental factors have mostly been implicated for the lower asthma prevalence in rural settings. Investigating urban-rural asthma diagnostic patterns may provide further explanation to the observed prevalence differences.

With children spending most (approximately 90%) of their time indoors,[9] the indoor environment has become an important factor in the management and risk of childhood asthma. Indoor microbial exposures, particularly endotoxin, have been observed to reduce the risk of childhood asthma,[10,11] irrespective of location of dwelling.[12] However, the evidence is inconsistent as other studies have reported increased risk[13–15] as well as no association.[16,17] Similarly, protective[18–20] and risk[21,22] effects have also been observed for beta-(1→3)-D-glucan. An important question to address is whether these differential effects could be associated with asthma phenotypes in children with asthma.

Furthermore, while indoor microbial exposures may protect against childhood asthma in general,[18,23] there are indications that microbial exposure thought to protect against childhood asthma may result in worsened asthma symptoms or asthma severity in children with preexisting asthma but less is known about the specific indoor microbial agent aggravating the disease.

Identifying indoor microbial agents potentiating asthma exacerbation in children with the disease could aid attempts to reduce severity and associated asthma morbidity.

This dissertation showed that the often reported geographical variation in childhood asthma could be, in part, related to diagnostic patterns. The dissertation also provided an alternative explanation to the environmental theory which has been considered in most previous studies[24–26] as a reason for lower asthma prevalence in rural locations. Because the proportion of children classified as having asthma increased by a much greater amount in rural children compared to children in large urban settings when objective clinical measures were considered, the results revealed evidence of asthma under-diagnosis in rural compared to urban settings.

Symptoms consistent with asthma diagnosis can be higher in rural compared to urban children[3,8,27] or similar in both groups,[28] as also observed in this study. Therefore, it is possible that estimates of asthma prevalence in previous epidemiological studies using parent-report of physician-diagnosed asthma across geographic locations might be biased due to differences in diagnosing.

Results from studies investigating the associations between microbial endotoxin exposures and childhood asthma have been inconsistent. Some of the inconsistency may be due to different presenting phenotypes. To bring some clarity to the inconsistencies in the associations, we assessed the relationships between endotoxin and beta-(1→3)-D-glucan with asthma phenotypes. Endotoxin exposure was inversely associated with atopic asthma but positively associated with bronchial hypperresponsiveness (BHR) assessed as exercised-induced bronchospasm (EIB). The results showed that indoor microbial exposures were related to asthma phenotypes in different ways and suggest that these differential relationships may help explain some of the inconsistency in previous reports of microbial exposure to asthma.

Allergen activation through the T_H2-dependent pathway and IgE receptors is likely the most common occurrence in the pathophysiology of atopic asthma.[29] While the mechanism for the paradoxical relationships between endotoxin exposure and atopic asthma and EIB was not investigated in the current study, the results further support the theory of imbalance between T_H1 and T_H2 immune response (for atopic asthma)[30,31] and the release of histamine in the airways to induce bronchoconstriction (for EIB).[32] The inverse association observed for endotoxin exposure and atopic asthma in this study correlates well with the suggestion that asthma is a T_H2-cell-dependent disease[29] and further provides evidence that the association between endotoxin and asthma may be potentially mediated by an effect of endotoxin on atopy. Also, sensitized mast cells may activate cytokines to induce airway bronchoconstriction.[33] Therefore, the increased risk of EIB as observed in the current study further supports the theory that allergic mechanisms may not be the only and/or most important underlying mechanism in the pathophysiology of asthma.[34]

The above results are consistent with our hypothesis that the discrepancies in the associations between endotoxin and childhood asthma, as reported in previous studies, could be linked to different presentations of the disease in children with asthma. While there is evidence that inhaled endotoxin exposure can induce BHR, these effects have only been found in adults[35,36] and an animal studies[37] using endotoxin inhalation challenge test methods. Results from the current study complement these earlier results[35,36,37] by showing similar findings in children with EIB and home based indoor endotoxin exposures from house dust.

Central to the pathophysiology of asthma is underlying airway inflammation which reflects different aspects of the disease severity from mild intermittent to severe persistent.[33] The indoor environment has been reported as a possible source of triggers that could worsen asthma

severity in individuals with asthma.[38] While indoor exposures to dust mite,[39,40] furred pets,[41,42] tobacco smoke,[43,44] and report of visible mold[45,46] have been shown to be associated with asthma symptoms severity, the role of indoor microbial exposure from house dust on clinically assessed degree of asthma severity remains less studied.

Results from the current study further confirm and extends the existing literature on respiratory outcomes in children with asthma following exposure to indoor microbial endotoxin and beta-(1→3)-D-glucan. Asthma severity was associated with indoor endotoxin and beta-(1→3)-D-glucan exposures. However, while beta-(1→3)-D-glucan exposure could only be sufficient to exacerbate asthma symptom frequency it was not enough to impair pulmonary function. Contrary to the results for beta-(1→3)-D-glucan, endotoxin was consistently associated with adverse lung health outcomes.

One of the fundamental components of asthma guidelines has been the assessment of disease severity and associated risk factors to guide treatment recommendations and management of asthma conditions such as avoidance of factors that could trigger and worsen asthma.[47] In line with these guidelines, results from this study highlight that exposure to microbial inflammatory agents, such as endotoxin and beta-(1→3)-D-glucan can increase asthma severity in children with asthma.

To summarize, while differences in the environment may explain some of the observed differences in asthma prevalence between urban and rural areas, presenting and diagnosing patterns should also be considered. Also, while studies have shown protective associations between childhood asthma and microbial exposures (e.g. endotoxin),[10,11] and used this information to help explain the differences in asthma prevalence between urban and rural locations, this study further showed that some of the inconsistencies with these associations with

microbial exposures may be due to differences in phenotypes; and that asthma severity might be associated with the same microbial exposures thought to protect against asthma development, particularly endotoxin.

7.2 Validity of the study

The overall validity of this dissertation and the results are further discussed below.

7.2.1 Internal validity

In epidemiological studies, there is need to determine if observed differences in outcomes or effects of certain exposure on an outcome variable are likely to be due to alternative explanations. The process of ruling out such alternative explanation is referred to as assessing or establishing internal validity and shows the extent to which the findings of the study reflect the actual situation of the study population.[48] Establishing internal validity for this study is based on assessment of research design and/or operational procedures for the study.

7.2.1.1 Research design

7.2.1.1.1 Selection bias

A major selection issue that could impact this study is response bias which occurs when participants differ from non-participants or there is a systematic difference between responders and non-responders. The sampling frame for this study was based on a 2013 cross-sectional survey of schoolchildren as previously described.[27] Of the 3,509 participants who completed the survey in 2013, 1,348 (38.4%) agreed to participate in further survey and clinical testing (clinical testing phase) and were re-approached in 2015. However, only 335 children (24.8%) participated

and formed the study population for this dissertation (Appendix 13). Reasons for the low participation rate were relocation (packages were returned stamped "Moved", n = 154), refusal (subjects were no longer interested in the study, n = 257), and non-response (packages not returned to study center, n = 602). There were significant differences in age (mean age: 9.03 vs. 9.52 years), parental education (maternal: 86.1% vs. 73.7%; paternal: 78.3% vs. 67.2%), parental smoking (14.7% vs. 31.3%), and parental history of allergy (50.1% vs. 37.3%) between those who completed the clinical testing phase (in 2015) and those who did not (only completed the cross-sectional survey in 2013), respectively. Participation of study subjects in 2015 was also driven by the presence of respiratory symptoms (wheeze) and report of physician diagnosis of asthma in the 2013 survey, which may indicate the possibility of response bias and a tendency towards a less healthy population for this study (Appendix 14).

The potential presence of a biased sample is not a major problem for this study because of the study objectives and the outcomes assessed. Manuscript I assessed asthma diagnostic patterns within each location of dwelling as opposed to comparing asthma prevalence across the urban-rural gradient. This allowed each location of dwelling to serve as its own comparison when estimating the changes in proportions of survey-based asthma classification and algorithm-based asthma classification. However, we acknowledge that if the full population (n = 1,348) had participated, the proportion of survey report of physician-diagnosed asthma is likely to be lower than the estimates reported in this study and likely to represent the population prevalence estimates more closely. Also, since only children identified as positive for asthma from Manuscript I formed the study population for results reported in Manuscripts II and III, the associations reported in Manuscripts II and III will be valid given the well-defined study population and objective measures used in selecting these participants.

Finally, while the participants in the current study differed in some characteristics compared to those that participated in the 2013 cross-sectional survey (Appendix 14), the presence of biased sample in this study is expected to have occurred non-differentially between locations of dwelling. For example, the proportion of subjects that did not participate in the 2015 study was equally distributed across locations of dwelling (Large Urban = 90.1%, Small Urban = 92.8%, and Rural = 92.4%). Furthermore, the proportions of parental history of asthma and allergy, parental education levels, child reporting ever being diagnosed for asthma by physician, or ever wheeze were not significantly different across locations of dwelling. This allowed the interpretation of the results to still remain valid.

7.2.1.1.2 Information bias

This study was conducted in a way to attempt minimizing information bias, both for exposures of interest [endotoxin and beta-(1→3)-D-glucan] and outcomes (asthma phenotypes and severity).

In Manuscript I, asthma was initially assessed based on survey report to classify subjects into three distinct groups: physician-diagnosed asthma (if they reported ever being diagnosed for asthma by a physician), at-risk-for-asthma (if they reported asthma-related symptoms but no physician-diagnosed asthma), and no asthma (no physician-diagnosed asthma and no asthma-related symptoms). However, to further validate these responses for the presence or absence of asthma, all consenting subjects further performed spirometry and ECT. Subjects positive for asthma were then identified using a validated asthma case-detection algorithm that combined survey responses and the clinical measures.[49] This procedure improved the asthma diagnostic classification and minimized possible information bias that might have occurred from survey responses.

This study further employed objective clinical assessments to reduce information bias greatly when classifying outcome variables for Manuscript II (atopic asthma and EIB) and Manuscript III (mild asthma vs. moderate/severe asthma). Subjects were classified as atopic vs. non-atopic based on objective SPT or as EIB vs. no EIB based on the results of ECT. The use of asthma severity classification guidelines[50] which combined night- and day-time symptom history with lung function (determined by FEV_1) further enabled us to move beyond the common questionnaire reports of frequency of symptom history as indicators of asthma severity in this study to evidence-based severity classification guided by expert panel recommendations.[50]

The exposure variables for Manuscript II and III were endotoxin and beta-(1→3)-D-glucan. In the current study, endotoxin and water soluble fraction of beta-(1→3)-D-glucan, as markers of microbial exposures, were objectively measured using recommended protocols[51] and appropriate analytical procedures based on the quantitative kinetic chromogenic LAL assay (endotoxin) and the Kinetic Onset Time Glucatell assay [beta-(1→3)-D-glucan], thus eliminating the possibility of exposure misclassification. Finally, all samples were analyzed after questionnaire data collection was completed and in the same batch in the laboratory.

7.2.1.1.3 Dust sample measurement

Handling and measurement of dust samples in the current study were done in accordance to recommended protocol[51] to increase internal validity. In order to correct for any modifying factors during the dust sampling process, a blank sample was collected for every sixth house visited according to recommended protocol.[51] Following dust sample collection, filter socks were placed back in the Ziploc bag and transported to the Canadian Center for Health and Safety in Agriculture's National Agricultural and Industrial Hygiene Laboratory (CCHSA's–NAIHL) for further processing. The filter socks containing the dust samples were weighed after dust sample

collection by the same person that weighed them prior to data collection, using the same scale. To minimize errors and ensure repeatability in dust weight, all pre- (filter socks only) and post-data collection (filters socks with dust sample) weights were completed in triplicate and the average weight recorded. Microbial endotoxin and beta-(1→3)-D-glucan were measured from the aliquots extracted from 10 mg (0.010 g) of sieved dust samples (also weighed three times to ensure repeatability and accuracy of dust weight).

7.2.1.2 Operational procedures

7.2.1.2.1 Confounding factors

Manuscripts II and III for this dissertation used risk factor modeling based upon *a priori* etiological hypothesis rather than exploratory modelling. The models were focal in nature in that they related exposures to endotoxin and beta-(1→3)-D-glucan to specific respiratory outcome assessed as asthma phenotypes and asthma severity. When results are not likely attributable to chance as depicted by $p \leq 0.05$ for the main results of this dissertation, then it is important to assess whether the results could be explained by other factors.

This study observed adverse as well as protective effects of indoor exposure to microbial agents in relation to asthma phenotype and asthma severity. However, there are a number of factors which have to be accounted for in order for the observed associations to be valid. Controlling for potentially confounding variables in this dissertation minimizes the potential for an alternative explanation for the results observed and provides more confidence that the effects of endotoxin and beta-(1→3)-D-glucan on asthma phenotypes and asthma severity are due to the appropriate independent variable. The confounding variables were controlled for using multivariate analyses. We considered and included in our models the common and important

known risk factors and confounders for asthma phenotypes and severity based on literature, biological/clinical importance and statistical significance. However, it is possible that there may be some other unmeasured potential confounders (residual confounders) in this study such as information on household income, age at which asthma was diagnosed, and levels of control. Also, it is possible that other unmeasured constituents in dust samples, such as particulate matter, metals, ergosterol, and/or peptidoglycan were involved in mediating the associations between endotoxin and beta-(1→3)-D-glucan and asthma outcomes observed in the current study. Finally, to avoid reporting spurious associations between studied health outcomes and endotoxin levels, all analyses were adjusted for beta-(1→3)-D-glucan levels and vice versa.

7.2.2 External validity

The participants in this study were school-age children from both urban and rural settings in the province of Saskatchewan, Canada. The definition of urban-rural gradient for this study parallels the Statistics Canada definitions based on modified Beale codes which considers population size, density, and distance to metropolitan areas and is applicable across locations in Canada.[52] For this reason, findings from Manuscript I might also reflect a similar urban-rural pattern in asthma burden in other provinces if children of a similar age range were screened for asthma using similar asthma case-detection procedures.

Similarly, we used a validated asthma case-detection method to identify the study population for Manuscripts II and III. Since healthcare and asthma management are standardized across locations in Canada,[53] we expect that results of Manuscripts II and III will also reflect similar effect patterns among children with asthma in other location if similar procedures are used. However, caution should be taken when comparing these results with populations from other countries where environmental exposures and healthcare management practices may differ

significantly from that obtained in Canada. Also findings from this study, especially results of asthma diagnosis using lung function assessments in Manuscript I, may not be applicable to populations outside of the age range studied (7–17 years old).

7.3 Evaluation of evidence of cause-effect relationships in this study

Showing that endotoxin and beta-(1→3)-D-glucan are associated with asthma phenotypes and asthma severity in this study does not necessarily imply that there is a cause-effect relationship. While the design and analytical procedures for this study might have greatly reduced the potential for systematic and random errors as well as controlled for important confounding variables, the results of this study should be evaluated based on the "Bradford Hill (Hill's) criteria" for assessing evidence of cause-effect relationship.

7.3.1 Temporality

The studies in this dissertation used a cross-sectional design and presents challenges in drawing causal associations due to temporality of events between exposures and respiratory health outcomes. Hill's criteria necessitate an exposure to precede the occurrence of outcomes.[54] In Manuscript II and III for this dissertation, exposures and respiratory health outcomes were determined at the same time preventing from drawing a causal relationship as we were unable to determine which come first: the microbial exposures or the respiratory outcomes. While settled house dust sample may have little variation over time and reflects longer-term exposure to microbial agents,[55] the associations observed in the current study would need to be confirmed in longitudinal cohort studies investigating early indoor microbial exposures and later respiratory disease development.

7.3.2 Strength of association

Manuscript II showed statistically significant decreased risk of atopic asthma and increased risk of EIB for endotoxin exposures. Similarly Manuscript III demonstrated statistically significant decreased risk of moderate/severe asthma for beta-(1→3)-D-glucan but increased risk of moderate/severe asthma for endotoxin exposure. Hill's criteria suggests that strong associations are more likely to be causal than weak associations if confounding factors have been adequately adjusted for in the analyses.[54] The results reported in this study attempted to remove confounding effects and were moderate to strong associations. However, while some of the associations observed in this study were strong, they were not statistically significant, likely due to the small sample size.

7.3.3 Biological gradient (dose-response relationship)

This study also showed a dose-response pattern for endotoxin exposure and decreased risk of atopic asthma (Medium level: OR = 0.42; High level: OR = 0.15) compared to low levels. Similarly, a dose-response pattern was also observed for endotoxin exposure and increased risk of EIB (Medium level: OR = 2.46; High level: OR = 7.80) and asthma severity (Medium level: OR = 7.05; High level: OR = 11.40) compared to low levels. These results are also consistent with other studies that have demonstrated dose-response curves in the relationship between microbial exposure and asthma exacerbation[56] and atopic asthma.[30]

7.3.4 Consistency of associations

The results for endotoxin exposures and asthma phenotypes as well as asthma severity are mostly consistent with what has been previously reported. The ALEX study in Austria, Germany, and

Switzerland provides some of the strongest evidence suggestive of inverse associations between endotoxin exposure in house dust and atopic asthma.[12] The Prevention of Allergy Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study in Austria, Germany, the Netherlands, Sweden, and Switzerland also demonstrated a decreased risk of atopic wheeze associated with mattress dust endotoxin exposure.[57] With regards to asthma severity, previous studies have also shown that asthma severity is positively associated with exposure to microbial contaminants. A study in the Netherlands showed a significantly positive association between endotoxin exposure and increased PEF variability as indicator of asthma severity in children with asthma symptoms.[58] Other studies have also shown an association between endotoxin exposure and increased frequency of wheezing and asthma medication use as severity indicators.[15,50]

Few studies have investigated the relationship between beta-(1→3)-D-glucan and asthma severity. However, the available evidence also suggests positive associations with severity indicators such as frequency of ED visits[56] and PEF variability.[58] In contrast to results from these studies, we observed inverse associations between beta-(1→3)-D-glucan and moderate/severe asthma. The reasons are not clear in this study but it has been shown that acute exposure to beta-(1→3)-D-glucan may not elicit an inflammatory response that had occurred after exposure to endotoxin.[59,60] It is difficult to ascertain if a similar situation occurred in this study. Alternatively, beta-(1→3)-D-glucan might be an inadequate surrogate of house dust microbial exposure in Canada because of the relatively colder and drier environment with lower RH compared to European countries where some of the studies have reported positive association with beta-(1→3)-D-glucan.[58]

Some of the associations observed were not consistent by location of microbial exposure within the indoor environment (play area and mattresses) of the current study. For example, the decreased risk of atopic asthma with endotoxin exposure was only significant for play area and not mattress endotoxin levels. The increased risk of EIB and asthma severity with endotoxin exposure was significant in mattress and not play area endotoxin levels. Reasons for the varied associations are unclear in this study but may be related to differences in the determinants of endotoxin in different locations in the homes,[10,61] proximity to microbial agents in mattress compared to play areas, or differences in endotoxin's structures and potency in the specific home location[62] rather than location itself. For example, mattress dust contains longer-chain 3-OHFA while play area endotoxin contain shorter-chain 3-OHFA[62] and it is suggested that longer-chain 3-OHFAs ($C_{12:0}$–$C_{14:0}$) may elicit stronger and significant potent immunological effects compared to shorter-chain 3-OHFAs.[63]

7.3.5 Biological plausibility and coherence

A number of studies have demonstrated the inflammatory and allergic mechanism of microbial exposures and this is consistent with the multicellular processes involved in the pathophysiology of asthma.[29,33] Endotoxin exposure inhibits the T_H2 and promotes T_H1 immune responses, preventing atopic immune development and associated diseases in humans[30,31,32] as well as in animals.[37] Furthermore, agricultural and domestic house dust extracts induced significant TNF-α inflammatory cytokine in human monocytes.[64] Removal of endotoxin from all dust samples significantly reduced TNF-α responsiveness, suggesting a preferential role for endotoxin in inducing inflammatory responses from airway inflammatory cells. The mechanism for beta-(1→3)-D-glucan is less clear but is believed to also induce increases in airway eosinophil and neutrophils counts.[60] Therefore, findings from this study have some basic biological plausibility

relating microbial exposures to asthma-related outcomes as demonstrated in both humans and animals studies based on the natural history of the disease.

7.4 Other limitations and strengths of the study

Limitations and strengths of the specific objectives for this study have been mentioned in their respective chapters: Chapter 4 (asthma diagnosis along an urban-rural gradient, Manuscript I), Chapter 5 [the association between endotoxin and beta-(1→3)-D-glucan with asthma phenotypes, Manuscript II], and Chapter 6 [the association between endotoxin and beta-(1→3)-D-glucan with asthma severity, Manuscript III]. However, there are some other limitations and strengths regarding the dissertation in general that should be mentioned.

7.4.1 Other limitations

One major limitation of the study is that all data (survey, clinical assessments, and home dust collection) was obtained using a cross-sectional study design. This study design can only identify associations and not necessarily establish causation since exposures and outcomes were measured at the same time point. However, due to practical considerations, cross-sectional studies are common and important and this limitation is considered typical of all cross-sectional studies.[10,14,61]

ECT was used to induce bronchoconstriction in this study. However, there are other challenge tests that have been completed in epidemiological studies to identify children positive for asthma. These include hypertonic saline[65] and methacholine challenge test (MCT)[66] and have been found to show similar validity when assessed against actual physician assessment of asthma as gold standard (Saline test: sensitivity = 54%, specificity = 94%; MCT: sensitivity = 50%, specificity = 84%). These results correspond to the validity obtained for ECT in another study

(sensitivity = 57% and specificity = 90%).[67] This suggests that ECT as used in the current study has similar validity indicators as other asthma challenge methods and should be suitable for the purposes of the current study.

A case-detection protocol may not be as valid as using physician assessment. However, we used a validated asthma case-detection algorithm[49] to identify children positive for asthma in this study. The asthma case-detection algorithm used to identify children for asthma in this study has been validated against clinical physician assessment of asthma and has shown high sensitivity (82%) and specificity (93%).[49] These children formed the study population for investigating the objectives reported in Manuscripts II and III. While we acknowledge that the use of algorithm-based asthma classification may not be as accurate as physician assessment of asthma, due to practical considerations, convenience and cost, physician assessment was not possible in the current study.

The outcome for Manuscript II was asthma phenotypes based on skin prick testing (SPT) and ECT to determine atopic asthma and EIB, respectively. We determined atopy based on a positive skin reaction to at least one of the tested allergens (cat, house dust mite, local grass *Alternaria, Cladosporium, and Aspergillus*). These allergens–though believed to be common in the areas under study– may not necessarily identify all cases of atopy in our study population. Some children may be allergic to other allergens not tested in this study (e.g. dog, horse, and other food allergens) and thus misclassified as non-atopic in this study. Therefore, it is important to also consider atopy defined in terms of total serum IgE to provide an overall estimate of allergic sensitization in our study population of children with asthma.

In the current study, asthma phenotypes were not entirely distinct as there was some overlap between atopic asthma and EIB. For example, 17/116 (17.2%) children with asthma in

this study had both atopic asthma and EIB (Appendix 15). While the study protocol used objective measures to minimize misclassification of asthma phenotypes, analyses for atopic asthma and EIB, separately, comprised of subjects with both phenotype subclass and were not mutually exclusive of each. However, this is not unusual and it should be noted that asthma is an heterogeneous disease with multiple presenting phenotypes[47,68] and no single asthma phenotype class achieves all the requirements for a distinct or discrete asthma phenotype class.[68] Even in latent cluster analyses (LCA) studies which attempt to eliminate bias in categorizing asthma phenotypes by avoiding definition of the asthma conditions before analysis, there were clear overlaps in phenotypes despite differences in study designs, variables that were analyzed, and studied populations.[69–71,72]

Seasonal variation in asthma severity is another inherent limitation in this study. Asthma severity were assessed based on a combination of day- and night-time symptoms as well as objective lung function assessment. While asthma severity is not a stable feature but may change with time, classification by disease severity typically suggests a static feature.[73] It is suggested that asthma severity be assessed over a sufficient period of 6–12 months for accurate prognosis of severity.[73] Therefore, in the assessment of asthma severity based on symptoms and lung function, a single point-in-time classification may be less reliable. To help guide initial disease management and assessment of individuals at risk of asthma exacerbation in a population, planning and results of epidemiological research (especially cross-sectional studies) are currently based on such limited information.[73] In addition, to accurately assess asthma severity in patients with asthma, determination of severity status should be made before the start of treatment therapy.[74] This is to enable practitioners to develop a stepwise management protocol according to

the degree of disease severity.[75] To minimize the effect of this limitation on the estimates reported in this study, we adjusted for asthma medication use in all models for asthma severity.

Although assessment of endotoxin and beta-(1→3)-D-glucan in settled dust is considered to be an objective method, both microbial agents may only represent part of the indoor total microbial exposure comprising other agents such as muramic acid, ergosterol, and peptidoglycan.[76] An index of combined quantity of indoor microbial exposure is suggested to predict asthma better than single microbial marker independently of microbial diversity.[76] Therefore, causal conclusions may be hindered by the variability of the microbial components in the indoor environment. Also while beta-(1→3)-D-glucan represents the major component of cell wall of most fungi,[77] it is also found in certain plant materials as well as some bacteria.[77,78] For this reason, indoor fungal exposure based on beta-(1→3)-D-glucan as a marker of exposure might therefore be overestimated. Furthermore, the dust extraction analysis procedure used in the current study is specific in determining the water soluble fraction of beta-(1→3)-D-glucan[79] which may not represent the most potent fraction of beta-(1→3)-D-glucan compared to alkaline soluble fraction.[77] This may be one of the reasons, in addition to small sample size, that beta-(1→3)-D-glucan levels were not associated with some relatively strong strengths of association observed in the analyses for Objective 3.

All assessments of house dust endotoxin and beta-(1→3)-D-glucan, as markers of microbial exposures, in the current study assume that the endotoxin and beta-(1→3)-D-glucan concentrations measured are a proxy for inhaled endotoxin and beta-(1→3)-D-glucan levels. Endotoxin and beta-(1→3)-D-glucan were assayed from settled house dust samples in this study. This was done by vacuuming predefined areas from mattress and play area floors following standardized protocol.[51] One major criticism of this method is that certain particles found in

settled house dust may be too large or heavy to become airborne and might not be inhalable. It is suggested that air sampling methods, particularly personal exposure monitoring of personal cloud,[80] could be considered to more represent the risk of relevant exposure to inhalable microbial components in the indoor environment.[81] However, air sampling requires large numbers of samples to be collected as temporal variation in airborne concentration is very high.[82] Since these methods are also very costly, work intensive, time consuming and mostly impractical in large epidemiological studies, assessment of microbial exposures in settled house dust by vacuuming currently represents the most convenient, less expensive objective indicator that the indoor environment is out of balance. Another advantage of settled dust sampling over personal exposure monitoring is the presumed integration over time that occurs in deposition of dust on surfaces.[82] Since dust samples were collected on surfaces such as carpets, microbial agents can proliferate sufficiently whereas air sampling may allow only crude measure of dust sampling for airborne microbial concentrations.[82]

7.4.2 Other strengths

Establishing an accurate diagnosis of asthma is important for patient care as it helps guide treatment protocols.[50] This study expanded on previous work by using a validated algorithm, which included symptoms report and clinical measurements to classify asthma status then included those children identified using the algorithm in the subsequent studies (Objectives 2 and 3) allowing for a strong definition of asthma cases.

Separating the independent effects of indoor microbial agents has been one of the major difficulties of earlier studies, this study measured and considered both fungal and bacterial exposures in all models to try and tease out the independent effects of endotoxin and beta-(1→3)-D-glucan on asthma phenotypes and severity.

While few studies have also been conducted to assess relationships between microbial exposures (particularly endotoxin exposures) and BHR, this has only been done in adults[35,36] and animal model[37] studies using inhalation challenge tests. This dissertation expands the findings to child populations for the first time using EIB as indicator of BHR and microbial exposure in house dust. Due to the manuscript-based nature of this dissertation and the involvement of inter-disciplinary teams, the manuscripts presented in Chapters 4, 5, and 6 also benefitted immensely from a variety of feedback and perspectives from several review comments. This may have enhanced the interpretation and presentation of findings reported in this work.

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CHAPTER 8

RECOMMENDATIONS AND CONCLUSIONS

8.1 Recommendations

The results from this dissertation lead to some recommendations.

Establishing an accurate diagnosis of asthma is important in order to guide therapy and improve patient care.[1] One factor that seems to contribute to asthma misdiagnosis is that objective measures are not often utilized in combination with symptom history, especially in community-based epidemiological studies. The use of objective measures in combination with symptom history in diagnosing childhood asthma is necessary in order to comply with recommended guidelines[2] and for accurate assessment of asthma burden for public health planning.

Studies comparing urban-rural asthma prevalence have, to date, implicated environmental factors as a possible explanation to the lower asthma prevalence in rural areas. However, based on the results from this study, the prevalence of asthma should also consider under-diagnosis issues in order not to further underestimate asthma in rural areas. In addition, it is important to use objective measures in rural areas to assess patients and reduce misclassification, which was seen in this study and possibly occurred through lack of access to basic equipment such as spirometers. Overall, because we found that the algorithm-based asthma classification identified more children with asthma compared to survey-based report of physician-diagnosed asthma (34.6% vs. 28.4%, $p<0.001$), Manuscript I has a central message for asthma diagnosis and management: "*if a child is wheezing, coughing, or experiencing shortness of breath, parents*

should insist on having objective clinical assessments completed (spirometry and/or BHR test) for accurate assessment of their child's respiratory condition when they see their physician."

Inadequate treatment and control of asthma as defined by Canadian Asthma Consensus Guidelines,[2] is still present in 26%–45% Canadian children.[3,4] This may be due to lack of diagnosis and appropriate therapy in children with asthma. More accurate diagnosis of asthma should better determine clinical management of the disease and improve quality of life, especially in rural settings. Since rural children have limited access to healthcare compared to urban children as seen in this study and may also have a shortage of healthcare professionals, findings from this study call for a need for school-based screening programs for childhood asthma. This is likely to eliminate the barriers to symptoms reporting and asthma diagnosis; and improve public health planning for childhood asthma across urban-rural locations. This may have direct implications for asthma management in terms of treatment, medication prescription, and asthma education. While school-based asthma screening program will be very helpful in identifying children with asthma in rural areas, it will only be cost effective in locations where there is a high prevalence of children with unrecognized asthma.

Since group and/or location comparisons facilitate our understanding of asthma burden, this study has implications for epidemiological research of asthma prevalence and risk factors. For example, epidemiological research of childhood asthma in Canada has been largely based on parental report of physician-diagnosed asthma.[5–8] Findings from this dissertation suggest that this method of asthma prevalence assessment may result in underestimation of the true prevalence and burden of asthma across locations. Measures that could improve accurate diagnosis of asthma such as supplementing existing healthcare services, especially in rural settings, to include pulmonary specialists and improved healthcare accessibility are needed. These measures could

improve public health planning for disease prevention, intervention or management that are location-specific.

Results from this study also provide understanding of indoor microbial agents associated with asthma phenotype and severity. Given the important role indoor microbial exposures, particularly endotoxin, play in asthma exacerbation and bronchoconstriction as observed in the current study and other studies,[9,10,11] avoidance or elimination of microbial agents in the indoor environment–once a child has been diagnosed with asthma–, could improve clinical asthma outcomes and related asthma morbidity in children and doing so improve patient care. While no intervention studies specifically evaluating the effectiveness of reducing endotoxin or beta-(1→3)-D-glucan concentrations on asthma morbidity have been performed, a randomized trial in the USA showed that reduction of microbial contaminants through moisture remediation strategies (reduction in water infiltration, heating, ventilation/air conditioning alterations) significantly reduced asthma exacerbations in the remediation group compared to the control group.[12] The population attributable fraction of childhood asthma among Canadian children is high for indoor microbial exposures (13%).[13] Also, the average direct cost from asthma exacerbations among children in Canada is estimated to be around $883.48 per patient per year.[14] Current recommendations by the Canadian Asthma Consensus guidelines for asthma control in children acknowledge the use of environmental control measures.[15] Thus, remediation strategies aimed at reducing indoor endotoxin levels and other indoor microbial contaminants are warranted and could form part of the Canadian Asthma Consensus guidelines to assist in controlling childhood asthma, reduce asthma morbidity, and decrease associated healthcare cost.

8.2 Future research directions

The strong associations between microbial exposures [endotoxin and beta-(1→3)-D-glucan] and asthma phenotypes and severity observed in the current study are interesting. However, the study used a cross-sectional design which can only identify associations and not necessarily establish causation. Cohort or longitudinal studies of asthma severity and phenotypes in relation to indoor endotoxin and beta-(1→3)-D-glucan exposures are needed for establishing causation. While early life exposures to microbial agents may protect against asthma development later in life,[16] it is also possible that such early life exposure to endotoxin and beta-(1→3)-D-glucan in children with asthma may have greater effects on asthma severity and morbidity later in life compared to current exposures.

Furthermore, measurements of endotoxin and beta-(1→3)-D-glucan as markers of indoor microbial exposures represent only crude markers of the total microbial exposure in the indoor environment.[17] Exposure to microbial derived components may not be confined to a specific agent but rather the composition and diversity of indoor microbial exposure might play a crucial role than the quantity of specific microbial exposure levels[17] and result in different respiratory outcomes from different microbial profiles. Therefore, studies of microbial exposures characterizations involving molecular techniques quantitative polymerase chain reaction (qPCR) or microbial DNA profiling analyses are needed to improve evidence about the diversity of indoor microbial exposure and associated respiratory health outcomes.

Different species of bacteria can release different types of endotoxin[18] and beta-(1→3)-D-glucan reactivity also appears to be related to specific fungal specie.[19] This might be the reason beta-(1→3)-D-glucan was also inversely associated with moderate/severe asthma in this study. This hypothesis should be evaluated in future studies by identifying specific bacterial and

fungal species in house dust and their associations with asthma respiratory morbidity, including asthma severity. Different markers of endotoxin could have varied health effects.[20,21] Studies that characterize endotoxin based on the length of fatty acid chain and investigate their associations with asthma respiratory outcomes is warranted to further explain some of the results in this study. In addition to speciation and structural components, it is possible that endotoxin exposure levels from different sources could elicit different patterns of inflammation as observed in the current study. Mattresses endotoxin was strongly associated with asthma phenotype (EIB) and asthma severity (moderate/severe asthma) in this study compared to play area endotoxin levels. Assessment of determinants of microbial exposures levels in different indoor environments will allow more specific control measures to be developed and help improve asthma management in children with the disease.

Asthma is a complex multifactorial disease with both the involvement of environment and genetic component. A recent study from the Danish Twins Registry identified that while 76% of the variations in overall asthma symptoms and severity were associated with environmental factors, 24% of the variations were due to genetic factors after adjusting for confounders,[22] suggesting possible effects of gene-by-environment interactions in the development of asthma. Future research should also consider the role of genetic component when investigating the relationships between indoor microbial exposures and respiratory outcomes in children. The tendency of genetic component to influence the impact of microbial exposures might identify reason for the paradoxical relationship between microbial exposure and asthma-related outcomes.

Finally, this was a cross-sectional study with adequate but relatively small sample size. This might have resulted in low statistical power for one of the objectives and explains the

absence of statistical significance for some of the strong estimates observed in this study. Future population-based studies with larger sample size are warranted to further validate the results reported in this study as well as to extend the investigation to potential interactions such as the gene-environment interactions described above. A larger sample, population-based study may also help in assessing the proportion of respiratory outcomes attributable to exposure of interests in this study. The population attributable fraction will be an efficient and powerful tool for planning and setting priorities for prevention and interventions strategies that may help reduce the burden of asthma in the population.

8.3 Conclusions

Several studies have showed evidence suggesting that asthma prevalence is lower in rural compared to urban settings. This study revealed that undiagnosed asthma may be a more common phenomenon in rural compared to urban settings and may explain some of the previously reported lower prevalence of asthma in rural children. This is further evidenced from results of studies which have revealed childhood asthma prevalence in rural locations to be similar with urban setting[23,24] with the rural asthma distribution associated with socioeconomic status and certain environmental factors (such as indoor smoking, pest in home, etc.) rather than geographical location.[25,26] Findings from this dissertation support the use of objective measures in combination with symptom history when evaluating asthma prevalence across geographical locations.

Furthermore, asthma is a heterogeneous disease with multiple presenting phenotypes.[27] A broad review of literature suggests inconsistencies in associations between indoor microbial exposure and childhood asthma and morbidity. This study identified a contrasting effect in the association between endotoxin exposure, as a marker of indoor bacterial exposure, and asthma

phenotypes. Two subtypes of asthma, atopic asthma and EIB, are affected differently by indoor endotoxin exposure; suggesting that the inconsistencies in associations in previous studies could be related to different asthma phenotypes.

With regards to asthma severity and microbial exposure, this study further supports the notion that severity might be associated with the same microbial exposures though to prevent asthma development. For example, with microbial exposure being higher in the indoor environments, such exposure may keep allergic asthma from developing but continuous inhalation may increase the risk of asthma severity and induce both immediate and sustained airway obstruction in individuals with preexisting asthma conditions.

Overall, the results of this study highlight the importance for improving understanding of the urban-rural asthma burden and identifying indoor microbial factors associated with asthma morbidity for planning and developing programs aimed at reducing asthma morbidity among children.

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APPENDIX

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Appendix 3

The Saskatchewan Children's Lung Health Study Questionnaire

PROTECTED WHEN COMPLETED

SASKATCHEWAN CHILDREN'S LUNG HEALTH STUDY

UNIVERSITY OF SASKATCHEWAN

TO PARENTS OR GUARDIANS:

Researchers at the University of Saskatchewan are conducting a study to learn more about the lung health of children in your region. A similar questionnaire was completed for this child in spring 2013. Thank you for taking part in this important study. This questionnaire is a follow-up to the baseline questionnaire.

Instructions for completing this survey

1. The person most familiar with this child's health should complete the questionnaire.
2. Please read each question carefully. Please try to answer all of the questions, but remember you don't have to answer any question if you choose not to.
3. Please complete the Consent/Assent form on the last page of the questionnaire. This is required prior to completing clinical testing.
4. Please be sure to complete the contact information on **Page 1** of this survey.
5. When you have finished, place the questionnaire in the postage paid business reply envelope and mail it back to us at the University.

Thank you

Oluwafemi Oluwole, PhD Candidate

Josh Lawson, Principal Investigator

The University of Saskatchewan

Supported by a grant from the *Saskatchewan Health Research Foundation*

Instructions

The questionnaire can be answered by checking the best answer or by filling in the blank with a number or words.

Example 1: Does your child usually have a cough at night?

☑ Yes ☐ No

Example 2: How many years has your child lived in this home?

__6__ Years

PART ONE: CONTACT INFORMATION:

Name of School _____ Child's Grade in September 2015 ____

Child: First Name _____ Last Name _____

Parent: First Name _____ Last Name _____

Street Address or land location _____

PO Box _____

Telephone No. (Home): _____ (Cell):_____

Person completing questionnaire:

☐ Mother

☐ Father

☐ Other Relationship to child _____

Date Completed:_____ _____ _____
Day Month Year

PART TWO – HEALTH OF THIS CHILD

Breathing symptoms

1. Has your child had a dry cough at night apart from a cough associated with a cold or chest infection? (*Tick all that apply*)

   ☐ Yes, past 12 months

   ☐ Yes, before the last 12 months

   ☐ No

2. Has this child woken up at night because of a cough? (*Tick all that apply*)

   ☐ Yes, past 12 months

   ☐ Yes, before the last 12 months

   ☐ No

3. Does this child usually have congestion in the chest or bring up phlegm or mucus apart from colds?

   ☐ Yes

   ☐ No

   ☐ Don't know

   If **YES**, has this congestion or phlegm been present for as much as 3 months in a row out of the year? (*Tick all that apply*)

   ☐ Yes, past 12 months

   ☐ Yes, before last 12 months

   ☐ No

4. Has this child ever had wheeze or whistling in the chest at any time in the past?

   ☐ Yes

   ☐ No

   ☐ Don't know

   If **YES**, at what age did this child first start to wheeze? _____ years

5. Has this child had wheezing or whistling in the chest **in the past 12 months**?

   ☐ Yes

   ☐ No

   ☐ Don't know

   IF **YES**, CONTINUE TO **QUESTION 6**

   IF **NO**, at what age did this child stop wheezing?
   _____ years GO TO **QUESTION 10**

6. Does the wheezing or whistling in the chest occur:

   ☐ apart from colds?

   ☐ with colds?

   ☐ both apart from colds and with colds?

7. How many attacks of wheezing has this child had **in the past 12 months**?

   ☐ none

   ☐ 1–3

   ☐ 4–12

   ☐ more than 12

8. **In the past 4 weeks**, how often, on average, has your child had episodes of cough, chest tightness, or wheezing **in the morning or during the day**? (*Select <u>best</u> answer*)

   ☐ Never in the past 4 weeks

   ☐ Less than two days per week

   ☐ At least two days per week

   ☐ Every day on most days

   ☐ More than once a day

9. **In the past 4 weeks**, how often, on average, has your child had episodes of cough, chest tightness, or wheezing **at night or while sleeping**? (*Select best answer*)

☐ Never in the past 4 weeks

☐ Less than one night per week

☐ One night per week or more

☐ Every night

10. **In the past 12 months**, has shortness of breath ever been severe enough to limit your child's speech to only one or two words at a time between breaths?

☐ Yes

☐ No

11. Does your child have episodes of cough, chest tightness, trouble breathing, or wheezing, or wheezing during or after exercise/sports? (*Select best answer*)

☐ Never

☐ Occasionally

☐ Often

☐ Always

12. Has this child ever been diagnosed as having asthma by a doctor?

☐ Yes

☐ No

☐ Don't know

If **YES**, at what age was this child diagnosed with asthma?
_____ years

13. **In the past 12 months**, how many times has this child required health care for asthma from the following places?

	# of times	Not Applicable
Hospital inpatient		☐
Emergency room		☐
Doctor's office		☐

14. **In the past 12 months**, how many asthma episodes (e.g. attacks, symptoms such as cough or wheeze or shortness of breath) has your child had?

☐ none

☐ 1-3

☐ 4-12

☐ more than 12

☐ Not Applicable

15. How often has your child's sleep been disturbed by breathing problems **in the past 12 months**?

☐ Never in the past 12 months

☐ At least once in the past 12 months

☐ At least once per month

☐ At least once per week

☐ Every day or nearly every day

16. **In the past 12 months** have you or another family member missed work because of your child's chest illness?

☐ Yes

☐ No

17. **In the past 12 months** how many days of school has your child missed because of breathing problems? (*Best estimate*)
_____ days

18. **In the past 12 months**, has this child's parents' sleep been disturbed because of this child's breathing problems?

☐ Yes

☐ No

☐ Don't know

19. Has this child had a prescription of antibiotics for respiratory infections (chest, ears, or throat) **in the past 12 months**?

☐ Yes If **YES**, how many? _____

☐ No

20. **In the past 4 weeks**, how often, on average, has this child taken medicine (e.g. syrup, an inhaler or a breathing machine) that your doctor prescribed to treat episodes of cough, chest tightness, trouble breathing, or wheezing? (*Select best answer*)

☐ Never in the past 4 weeks

☐ Less than two days per week

☐ Two or more days per week but not everyday

☐ Every day on most days

☐ More than once a day

21. **In the past 12 months**, has this child taken medicine that your doctor prescribed for a breathing problem?

☐ Yes

☐ No

If **NO**, GO TO **QUESTION 22**.

If **YES**, please list the medication and how often it is used on average:

Medicine	Daily	Sometimes (as needed)	Rarely
	☐	☐	☐
	☐	☐	☐
	☐	☐	☐
	☐	☐	☐

22. Has a doctor ever said this child had any of the following illnesses? (*Check all that apply*)

Chronic bronchitis..... ☐
Hay fever..... ☐
Eczema..... ☐
Pneumonia..... ☐
Sinus trouble..... ☐
Croup..... ☐
Whooping cough..... ☐
Tonsillitis..... ☐
Sleep apnea..... ☐
Ear infections..... ☐
Diabetes..... ☐
Urinary incontinence..... ☐
Nervous difficulties..... ☐
ADD/ADHD..... ☐
Anxiety..... ☐
Depression..... ☐
Others..... ☐
*Specify*_____

23. **In the past 12 months**, has your child ever had a problem with sneezing, or a runny, or a blocked nose when he/she did **NOT** have a cold or the flu?

☐ Yes

☐ No

24. Has this child ever had an allergy (e.g. hives, runny nose, sneezing and / or wheezing) to any of the following:

	Yes	No
House dust	☐	☐
Grain dust	☐	☐
Pollen	☐	☐
Trees	☐	☐
Grasses	☐	☐
Mold or mildew	☐	☐
Dog	☐	☐
Cat	☐	☐
Birds/feathers	☐	☐
Farm animals	☐	☐

25. Has this child ever had an allergy (e.g. hives, runny nose, sneezing and / or wheezing) to foods?

☐ Yes

☐ No

If **YES** to foods, what food(s)? _____

26. Has this child ever had any allergy confirmed by skin testing?

☐ Yes

☐ No

☐ Don't know

☐ Not Applicable

27. Has this child ever had anaphylaxis?

☐ Yes

☐ No

☐ Don't know

28. Has this child ever been hospitalized for breathing problems before the age of 3 years?

☐ Yes Number of time _____

☐ No

☐ Don't know

29. Does this child snore?

☐ Yes

☐ No

☐ Don't know

30. Does your child have frequent nasal congestion (mouth breathing)?

☐ Yes

☐ No

31. Has this child had an operation to remove their tonsils or adenoids?

☐ Yes

☐ No

☐ Don't know

32. Does your child bring up stomach contents frequently (have frequent symptoms of heartburn, reflux)?

☐ Yes

☐ No

33. **In the past 12 months**, did you ever experience any difficulties getting routine or on-going healthcare for this child?

☐ Yes

☐ No

☐ Don't know

34. In the **past 12 months**, were you ever unable to get prescription medications this child was supposed to take?

☐ Yes

☐ No

35. **In the past 12 months**, has this child had acetaminophen/paracetamol (e.g. Tylenol)?

☐ Yes

☐ No

☐ Don't know

36. **In the past 12 months**, has this child used any holistic or traditional medicine for breathing problems (e.g. homeopathic, herbal, rat root, etc.)?

☐ Yes

☐ No

☐ Don't know

PART THREE – LIFESTYLE & ENVIRONMENT

37. How long has your child lived in your current home? _____ years

38. Is this child living in the same location as June 2013?

☐ Yes

☐ No

If **YES**, SKIP TO QUESTION 46; IF **NO**, CONTINUE TO QUESTION 39

39. Which best describes the type of housing unit in which you live?

☐ One family house

☐ A building for 2 or more families

☐ Other, please specify: _____

40. How many rooms are there in your home (not including bathrooms, porches, balconies, halls, or entrance ways)?

_____ number

41. How many people live in your home?

_____ number

42. When was your home built?

☐ Between 1980-present

☐ Before 1980

☐ Don't know

43. In your house, what fuel is usually used for heating?

☐ Natural gas/central heating

☐ Electricity

☐ Coal

☐ Wood

☐ Don't know

☐ Other, specify _____

44. How long do you travel (in one direction) to receive routine and on-going medical care for this child? _____ minutes

45. How long do you travel (in one direction) to receive 24 hour emergency health care services for this child? _____ minutes

46. Where does this child receive **MOST** of their medical care?

☐ Emergency department

☐ Regular family physician

☐ Hospital

☐ Medi-clinic, walk-in clinic, minor emergency clinic, ambulatory clinic

47. Does your house have any damage caused by dampness (e.g. wet spots on walls, floors)?

☐ Yes

☐ No

☐ Don't know

48. **During the past 12 months**, has there been water or dampness in your home from broken pipes, leaks, heavy rain, or floods?

☐ Yes

☐ No

☐ Don't know

49. Does your home (including basement) frequently have a mildew odor or musty smell?

☐ Yes

☐ No

50. Are there signs of mold or mildew in any living areas in your home?

☐ Yes

☐ No

☐ Don't know

51. Where is your child currently living?

☐ Farm

☐ Acreage

☐ In town

☐ Reserve

If this child lives on a "FARM", what is **produced for sale** on your farm or ranch? (*Check all that apply*).

☐ Grain

☐ Cattle (beef)

☐ Cattle (dairy)

☐ Pigs

☐ Poultry

☐ Vegetable/fruit

☐ Other (*Please specify*): _____

☐ Nothing for sale

52. **In the past 12 months**, on average, how often has this child spent 1 hour near or in the following activities

	Everyday	At least once a week	At least once a month	Less than once a month	Never
Haying or moving or playing with hay bales					
Feeding livestock					
Cleaning or playing in barns					
Laying or removing straw from pens					
Emptying or filling grain bins					
Cleaning or playing in pens or corrals					
Riding horses					
Pesticide application					

53. If you raise livestock on your farms (e.g. cattle, pig, poultry), do you frequently add antibiotics to livestock feed?

☐ Yes

☐ No

☐ Not Applicable

54. **In the past 12 months**, have you had any problems with mice or pests in your home?

☐ Yes

☐ No

☐ Don't know

55. Do you have any of the following in your home? (*Check all that apply*)

Air conditioners .....☐

Air filter.....☐

Humidifier.....☐

Dehumidifier.....☐

Wood fireplace .....☐

Heat recovery ventilator (HRV) .....☐

56. **In the past 12 months**, have you had any of the following pets living in your home? (*Check all that apply*)

Cat.....☐

Dog .....☐

Bird .....☐

57. On a typical day, what is the main part of this child's journey to school? (*Tick one box only*)

☐ Walking

☐ School bus

☐ Bicycle

☐ Car

☐ Others (*Please specify*): _____

58. Does any person currently smoke **inside** the house?

☐ Yes

☐ No

59. Does this child's father currently smoke?

☐ Yes

☐ No

☐ Don't know

60. Does this child's mother currently smoke?

☐ Yes

☐ No

☐ Don't know

61. Is this child exposed to smoke in a car or in a bus?

☐ Yes

☐ No

☐ Don't know

62. Do any of the friends of this child smoke?

☐ Yes

☐ No

☐ Don't know

63. Has this child ever smoked tobacco?

☐ Yes

☐ No

☐ Don't know

64. Is this child exposed to tobacco smoke from alternate caregivers?

☐ Yes

☐ No

☐ Don't know

65. Is this child's classroom floor at school carpeted?

☐ Yes, fully

☐ Yes, partly

☐ No

66. Do any of the following animals live in the child's classroom at school: turtle, fish, furred animals, bird?

☐ Yes

☐ No

67. How often do trucks pass through the street where you live, on weekdays?

☐ Never

☐ Seldom

☐ Frequently through the day

☐ Almost the whole day

68. During a normal week, how many hours a day (24 hours) does your child watch TV or play video games?

☐ Less than 1 hour

☐ 1 hour but less than 3 hours

☐ 3 hours but less than 5 hours

☐ 5 hours or more

Physical activity is any activity that increases your heart rate and makes you get out of breath some of the time. Physical activity can be done in sports, school activities, playing with friends, or walking to school. For these next two questions, add up all the time this child spends in physical activity each day.

69. **Over the past 7 days**, on how many days was this child physically active for a total of at least 60 minutes per day? ____days

70. Over a **typical or usual** week, on how many days was this child physically active for a total of at least 60 minutes per day? ____days

71. **In the past 12 months**, how often, on average, did your child eat or drink the following? (*Please check*)

	Never/ Occasionally	Once or twice per week	Three or more times per week
Meat (beef, lamb, chicken, pork, etc.)			
Seafood (including fish)			
Fruit			
Vegetables			
Bread or cereal			
Pasta, rice, or potatoes			
Milk			
Unpasteurized milk (raw milk)			
Eggs			
Nuts			
Wild meat or bird (e.g. deer, elk, rabbit, duck)			
Potato chips or sweets (including chocolate)			
Soft drinks or pop			
Fast foods or foods including hamburgers, chicken nuggets, deep dried foods, or French fries			

PART FOUR – THIS CHILD, THE FAMILY, AND EARLY LIFE EXPOSURES

72. Child's sex: Male ☐ Female ☐

73. Date of Birth: ___ ___ ___
Month Day Year.

74. Child's age: ____

75. How tall is this child? (*For best results please use a tape measure against a wall*)
_____ feet, ____ inches OR ___ cm

76. How much does this child weigh? (*For best results please use a scale*)
____ pounds OR ___ kg

77. How often, on average, did this child consume unpasteurized milk (e.g. raw milk, farm milk etc) **in the first year of life**?

☐ Never

☐ Less than once per week

☐ 1–6 times per week

☐ Daily

☐ Don't know

78. Is this child a first born child?

☐ Yes

☐ No

☐ Don't know

79. How many siblings (both brothers and sisters) does this child have?
i) Number of older sibling(s) _____
☐ Don't know
ii) Number of younger sibling(s) _____
☐ Don't know

80. At the end of the month, how much money do you have left over?
☐ Some money
☐ Just enough money
☐ Not enough money

81. Type of household
☐ Single parent home
☐ Two parent /partner home

82. In the past 12 months, has this family ever struggled to meet the basic living requirements (i.e. food, housing, power, heating, water, clothing, school supplies, fees for school events, etc.)?
☐ Yes
☐ No

83. In which period of life was your child involved (**at least once a week**) in the following?

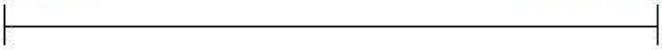
	Never	First year of life	Aged 2–5 yrs	Aged > 5 yrs
Live on the farm with livestock	☐	☐	☐	☐
Live on the farm without livestock	☐	☐	☐	☐
Live on the farm with straw	☐	☐	☐	☐
Contact with farm animals (e.g cattle, pigs, goats, sheep, or poultry)	☐	☐	☐	☐
Contact with farm buildings containing grain or hay	☐	☐	☐	☐
Pesticide application	☐	☐	☐	☐

84. How often, on average, did you have contact with/participate in the following while being pregnant with this child?

	Never	Less than once a week	At least once a week	At least once a day
Consume unpasteurized farm milk	☐	☐	☐	☐
Farm animals (e.g cattle, pigs, goats, sheep, or poultry)	☐	☐	☐	☐
Farm buildings containing grain or hay	☐	☐	☐	☐
Pesticide application	☐	☐	☐	☐

85. How well do you feel like your child is generally supported (socially, emotionally, medically, etc.) by your community (please mark on the line)?

No Support **Extremely Supported**



Comments (*If you have any additional comments, please write your comments on this page*):

Please complete the Consent/Assent form on the next page of the questionnaire

FOR ADMINISTRATIVE USE ONLY (DO NOT COMPLETE THIS SECTION)

Please check which statement applies (to be completed by the person re-affirming the assent):

☐ The child is capable of reading and understanding the assent form and has signed the above documentation of assent to take part in this study.

☐ The child is not capable of reading the assent form, however, the information was explained verbally to the subject who has verbally given assent to take part in this study.

Printed name of person re-affirming assent:	Signature:	Date:
_____	_____	_____

Please, turn over

THANK YOU
for completing the questionnaire.

Place the questionnaire in the postage paid business reply envelope and mail it back to us at the University of Saskatchewan.

Appendix 4

Saskatchewan Children's Lung Health Study Dust Extraction and Analysis Standard Operating Procedures

Weighing and Sieving Samples

Required Equipment, Supplies, and Solutions:

Top Loading Adventurer Balance from OHAUS Corp.

Item Number: AR1530

Polypropylene Low-profile Snap-seal Sample Container, 120 mL (4 oz) from VWR

Catalogue Number: 16126-022

Fisherbrand sieve 50 mesh size sieve (300 µm) from Fisher

Catalogue Number: 361014743

Kimberly-Clark Kimtech Science Kimwipes Delicate Task Wipes (4.4 x 8.4 inches) from Fisher (Health Sciences Supply Centre)

Catalogue Number: 06666-2

70% Ethanol made from 100% Ethanol (purchase at Health Science Supply Centre, 4L)

Dish Soap

Distilled Water

100ml beaker

Weighing Protocol

Wear gloves, mask and lab coat when weighing settled dust samples!

1. Place 100ml beaker into scale and zero the scale
2. Place the Ziplock bag with filter in a 100ml plastic beaker

3. Place this beaker into the scale
4. Wait for the scale to equalize
5. Record weight
6. Repeat Steps 4 and 5 two more times for a total of three trials to consider repeatability
7. Record average dust weight from the three trials
8. Remove the filter

Note: The above procedures were performed exactly the same way for pre- (empty filters) and post-data collection (filters containing dust sample) weights

Sieving Protocol

Wear gloves and mask when dealing with settled dust!
Sieve will need to be cleaned after each sample with soap and water, then rinsed with water and Sprayed with 70% ethanol. Make sure that the sieve is dry before proceeding with the next sample.

9. Label polypropylene containers on the top and the side with Sample ID.
10. Obtain a sterile polypropylene container and zero the balance.
11. Remove the container from the balance and place the Fisherbrand sieve 50 mesh size sieve (300 μm) on top of it.
12. Empty the contents of the sock onto the sieve.
13. Weigh the contents of the polypropylene container to obtain a sieved mass for the sample.
14. Record the weight of the dust in the container in your logbook as post sieved weight.
15. Discard the contents that were larger than the sieve.

16. Clean the sieve between each sample with dish soap and water, rinse with water, and spray with 70% ethanol. Wait until the sieve is dry before proceeding to the next sample. *To aid in the drying of the sieve faster, one can use a Kimwipe and gently pat the sieve and also place the sieve in the fumehood.*
17. Repeat Steps 1-5 for each sample.

Settled Dust Extraction

Required Equipment, Supplies, and Solutions:

Mettler Toledo MX5 microbalance, (max 5.1 g) from Mettler Toledo

Item Number:

OHAUS Top Loading balance

Falcon 50mL conical Polypropylene tubes from Fisher (Health Science Supply Centre)

Catalogue Number: 352070

Hyclone HyPure Cell Culture Grade Water (Pyrogen-free water, 500mL) from Fisher

Catalogue Number: SH30529.02

Tween 20 from Fisher (500mL)

Catalogue Number: BP337-500

Thermo Scientific MaxQ 2000 Bench Top Shaker

Model 4310

Sorvall ST 16R Bench Top Centrifuge from Fisher

0.05% Tween 20 solution (Pyrogen-free) made from 100% Tween 20 and Hyclone Hypure Cell Culture grade water.

Sterile Serological Pipettes from Fisher (Health Science Supply Centre)

Sterile Graduated cylinders

Sterile Nalgene bottles

1.5 mL microcentrifuge tubes (RNase, DNase, and Pyrogen-free) from Fisher (Health Science Supply Centre)

Weighing of dust samples

1. Label 50mL conical tubes with Sample ID before beginning.
2. Weigh out 10 mg (0.010 g) of the sieved settled dust into a 50 mL conical tube. Record the weight of the dust in your logbook.
3. If one is not using the dust immediately after weighing, store the samples at 4°C until ready for extraction.

Preparing Pyrogen-free 0.05% Tween 20

i. ***Make this solution in the biosafety cabinet, to limit any possible contamination of the solution.***

ii. ***Tween 20 is a very viscous solution, pipette slowly when aspirating and dispensing from the pipette.***

iii. ***This solution should be made fresh before each set of extractions.***

1. Turn on the biosafety cabinet, and clean with 70% ethanol. Allow a contact time of 5 minutes before wiping down the hood.
2. Prepare 0.05% Tween 20 (Pyrogen-free) using Tween 20 (Fisher, Cat# BP337-500) and Hyclone HyPure Cell Culture Grade Water (Fisher, Cat# SH30529.02) in a Sterile Nalgene Bottle.
3. Put the lid on the bottle and mix thoroughly.

Extraction of Sieved Settled Dust Samples.

1. If samples have been stored at 4°C, take the appropriate number of samples out of the fridge and allow them to come to room temperature for 15-30 minutes.
2. Add 20mL of 0.05% Tween 20 (Pyrogen-free) to each sample.
3. Label 1.5mL microcentrifuge tubes for the aliquots of dust extracts.
4. Shake the samples at room temperature at 325 RPM for 2 hours on the Thermo Scientific MaxQ 2000 Bench Top Shaker.
5. Centrifuge the samples at 1000 x g for 15 minutes in the Sorvall ST 16R centrifuge.
6. Aliquot the supernatant in ~1.0 mL volumes into 1.5mL microcentrifuge tubes for Endotoxin and water-soluble β-(1→3)-D-glucan analysis.
7. Store the aliquots in -80°C until endotoxin and β-(1→3)-D-glucan analysis is ready to be done.

Endotoxin and Water-soluble β-(1→3)-D-glucan Analysis

Required Equipment, Supplies, and Solutions:

Limulus Amebocyte Lysate Kinetic-QCL kit from Lonza

Catalogue Number: 50-650U

Glucatell Kit made by Associates of Cape Cod, from MJS Biolynx

Catalogue Number: GT002

BioTek ELx808 plate reader from Fisher

Item Number:

Gen5 2.06 Software from Fisher

Endotoxin

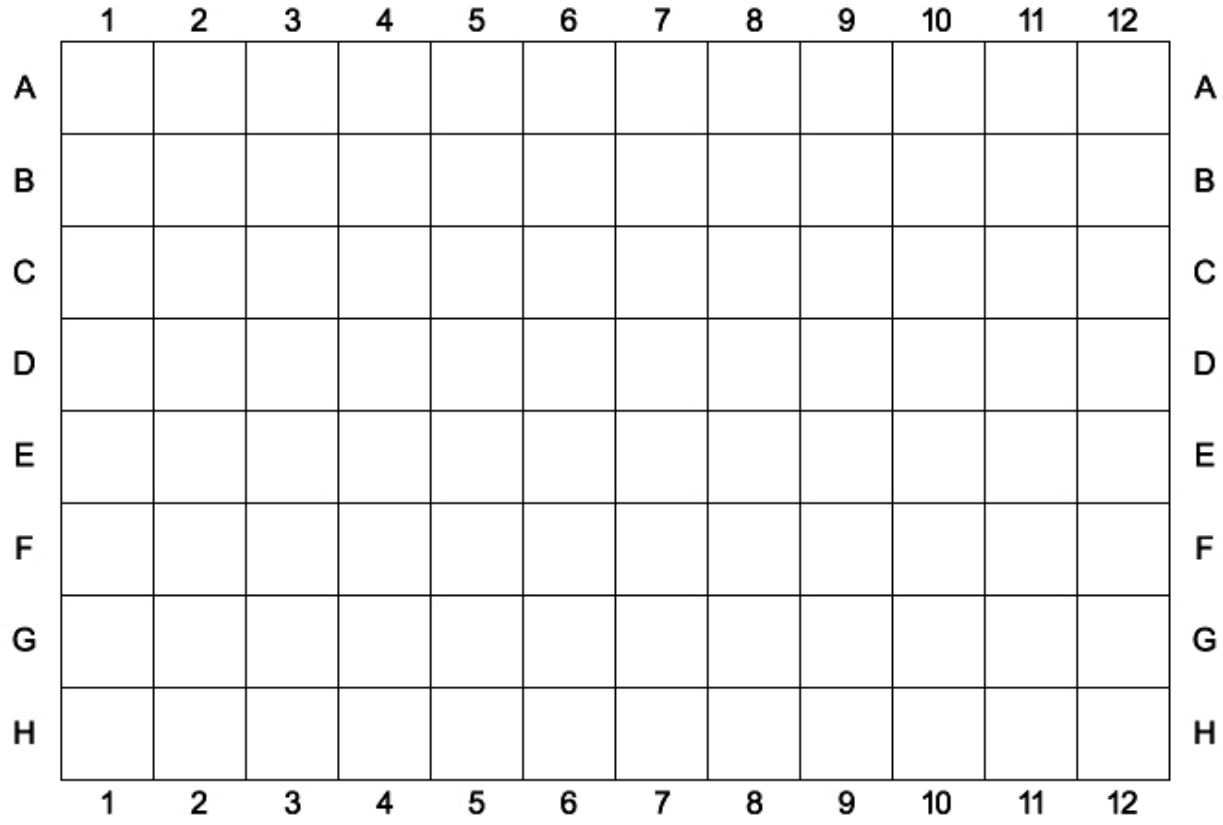
1. Dilute samples *at least* 1:10 with Pyrogen-free water.
2. Perform assay as per the procedure provided with the kit.

Beta-(1→3)-D-glucan

1. Dilute samples *at least* 1:100 with Pyrogen-free water.
2. Perform assay as per the Kinetic – Time of Onset Assay procedure provided with the kit.

Appendix 5

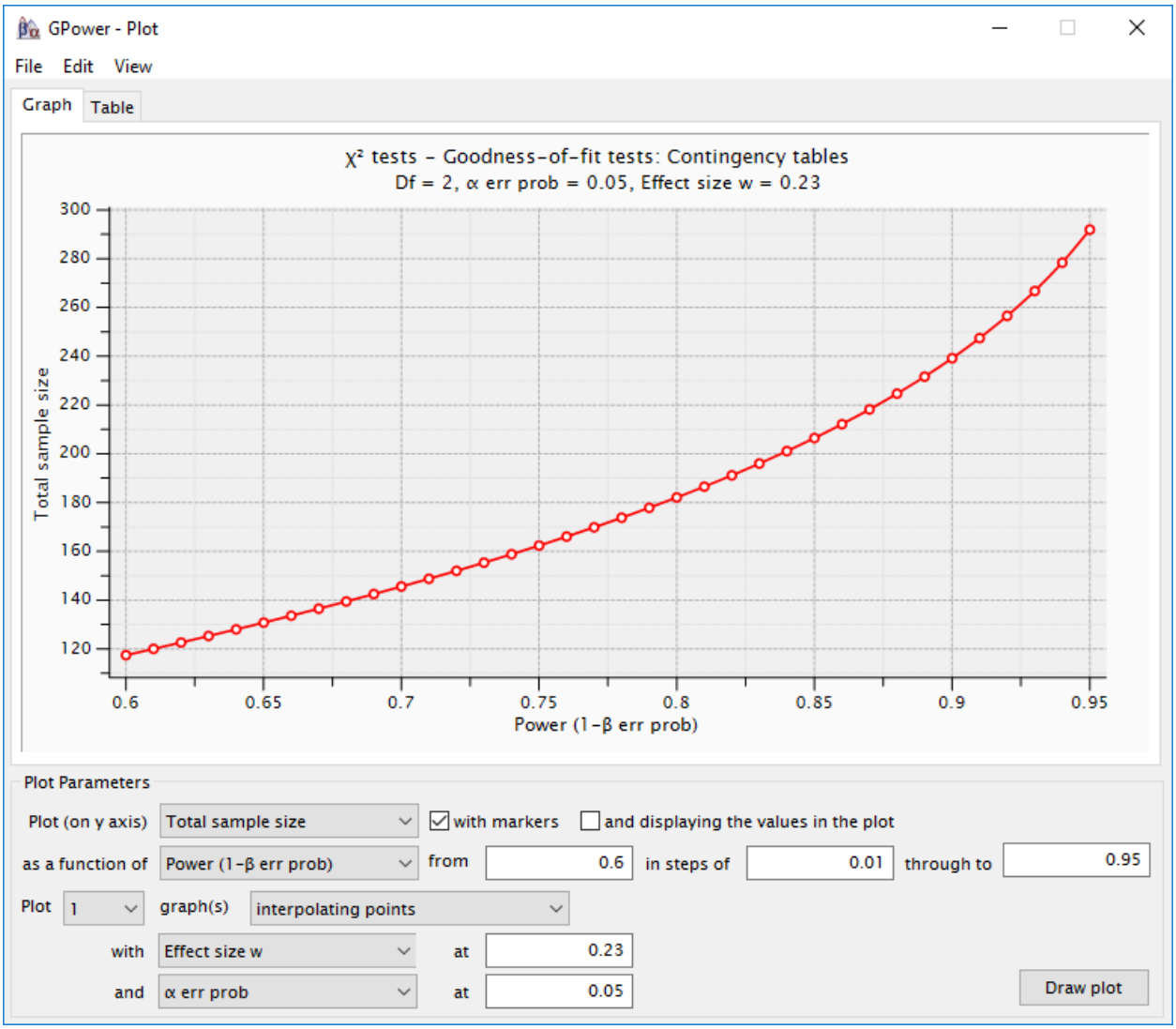
Template of the 96-well plate used for endotoxin and beta-(1→3)-D-glucan analysis



Appendix 6

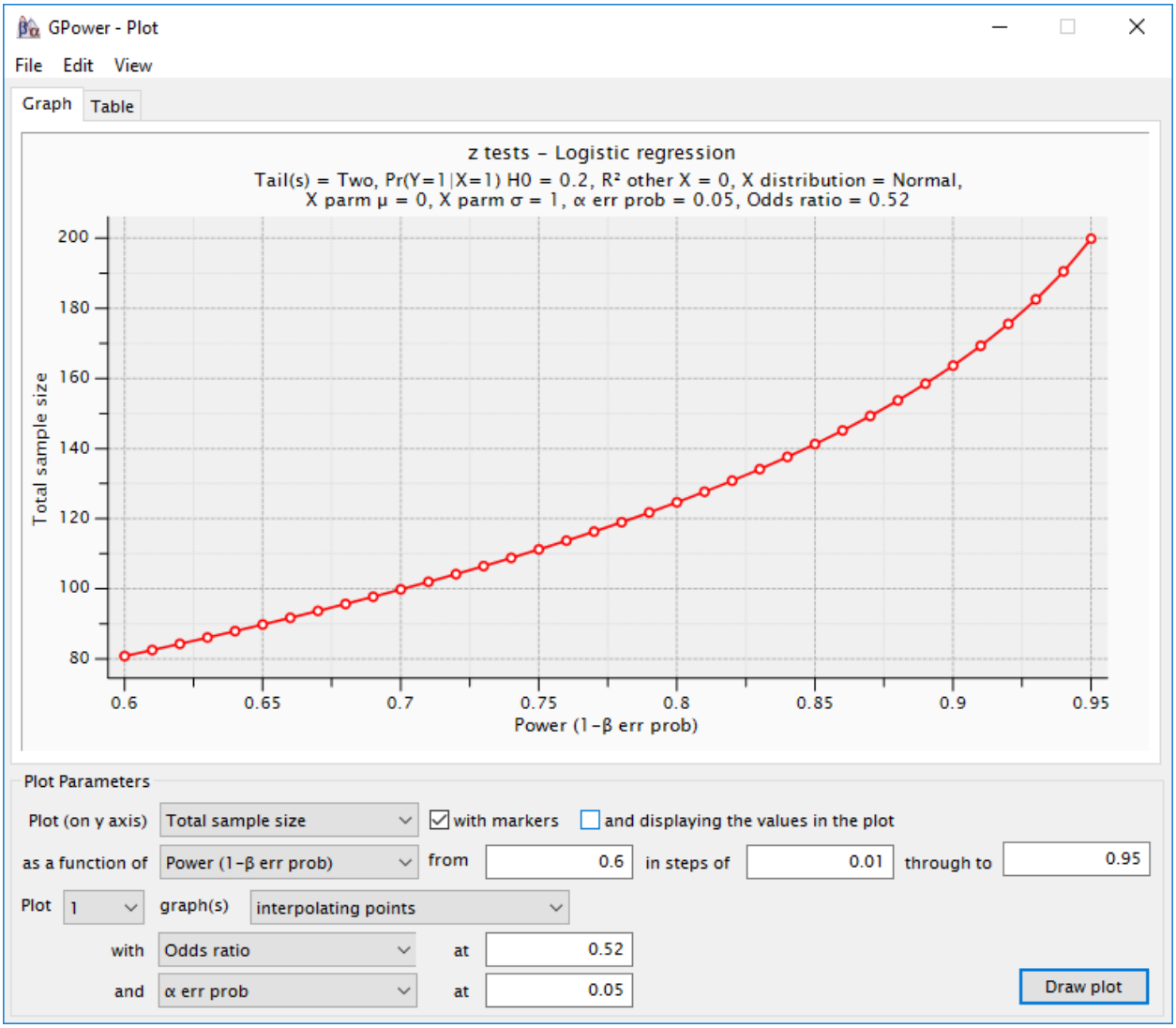
Sample size and power calculation summary

Sample size for Objective 1



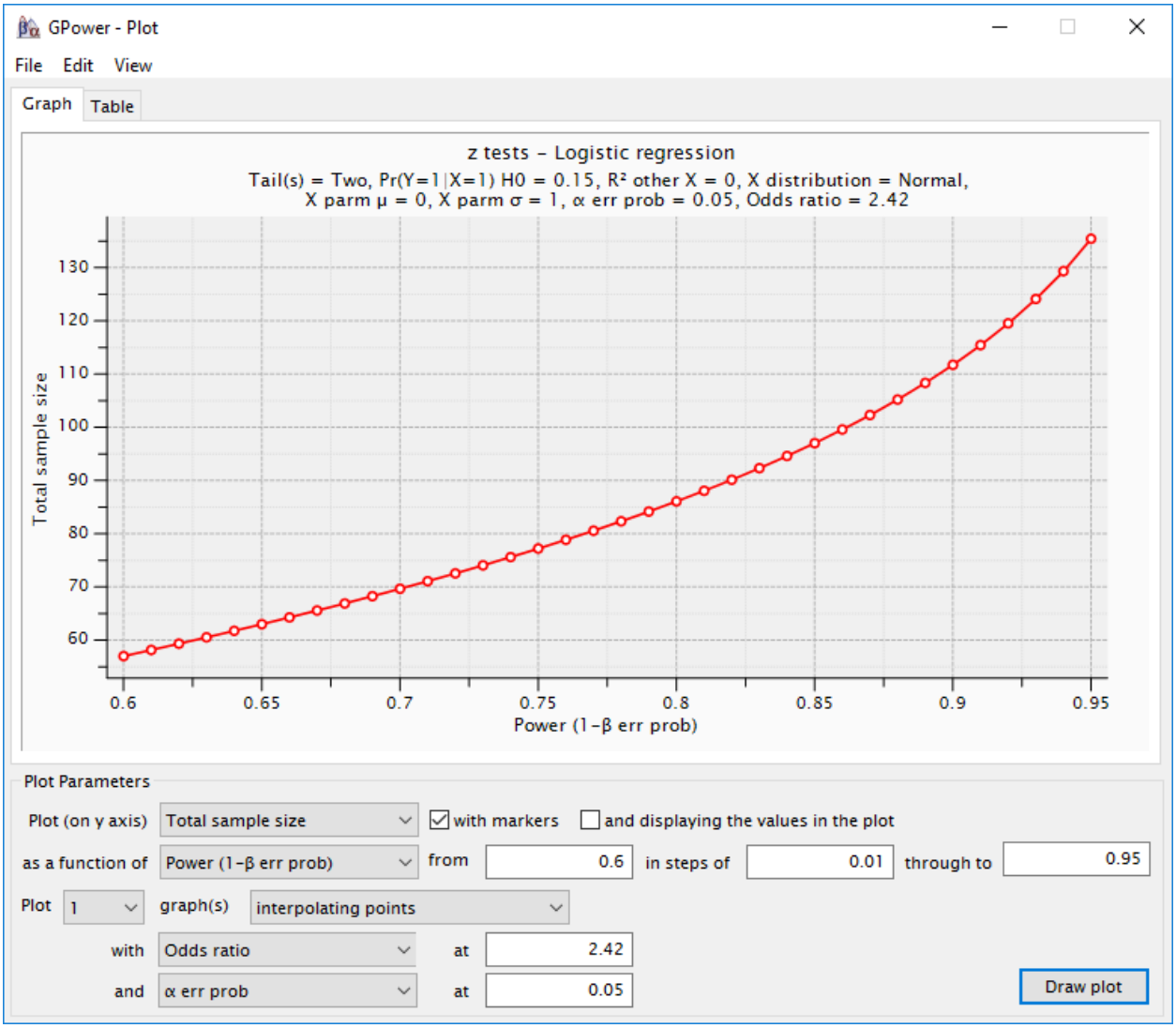
Proportions of survey-based and algorithm-based asthma classification were compared. Based on expected difference of ≥20% if asthma is under-diagnosed in the rural areas, a sample of 180 children was calculated per location of dwelling for a total of 540 children in order to detect significant differences in proportion between survey-based and algorithm-based asthma classification. Power (1-β) was set at 80% and α level was 0.05.

Sample size for Objective 2



Results from the 2002 childhood asthma and endotoxin study by Braun-Fahrlander *et al*. was used to calculate sample size for Objective 2. The study reported that current mattress endotoxin exposures (endotoxin loads in units/mg of dust) in home of 812 children (6–13 years old) showed an inverse association with atopic asthma. Based on the reported OR of 0.52 (95%CI: 0.30–0.90) for atopic asthma in exposed children, a total sample size of 125 children was calculated and found to be sufficient to detect differences in the association between endotoxin exposures and atopic asthma. Power (1-β) was set at 80% and α level was 0.05.

Sample size for Objective 3



Results from the 2011 study by Lawson *et al*. investigating the association between endotoxin and lung function among children and adolescents living in a rural area was used to calculate sample size for Objective 3. During a 2-week monitoring period of DV-PEF among children and adolescent, Lawson et al. reported positive association between endotoxin and greater DV-PEF (OR = 2.42; 95%CI: 1.03–5.67). Based on the Cohen's medium effect size of 0.3, a total of 85 sample was found to be sufficient to detect moderate associations in the relationship between endotoxin and lung function. Power (1-β) was set at 80% and α level was 0.05.

Appendix 7

Ethical approval certificates

(Original, amendments, and re-approval certificates)

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 03-Jul-2014

Notice of Ethical Review

PRINCIPAL INVESTIGATOR
Josh Lawson

DEPARTMENT
Canadian Centre for Health and Safety in Agriculture

Bio #
14-162

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
University of Saskatchewan
Saskatoon SK

SUB-INVESTIGATOR(S)
Donna Rennie, Darryl Adamko

STUDENT RESEARCHER(S)
Oluwafemi Oluwole

FUNDER(S)
CANADIAN INSTITUTES OF HEALTH RESEARCH (CIHR)
SASKATCHEWAN LUNG ASSOCIATION

TITLE
Saskatchewan Lung Health Study - Phase 2 (Environment and Biological Exposure Assessment)

Thank you for submitting the above protocol to the Biomedical Research Ethics Board for review. The REB has reviewed the protocol for your proposed study, and has withheld issuing a Certificate of Approval until the following conditions have been satisfied or information provided:

(Please highlight or underline changes made to the consent form when resubmitting)

Application Form

1. Section 7.3: Please include a statement about how the data will be destroyed (i.e., confidential shredding, permanent deletion, etc.)

Consent Form

1. Page 2 of 6, top of page references the survey. Has this survey not already been completed? If so, it should be referenced in the past tense as something that has been done, and also mention that on that form the parents gave permission for the researchers to contact them again.
2. Page 2 of 6, number 5 (Physician assessment): please give more detail about where and when this assessment will take place, and briefly outline what it will involve.

Please note that your research project cannot begin until you have received a Certificate of Approval from the Biomedical Research Ethics Board.

If you have any questions regarding these requirements, please call:

University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Josh Lawson	Canadian Centre for Health and Safety in Agriculture	14-162

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
University of Saskatchewan
Saskatoon SK

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Donna Rennie, Darryl Adamko

STUDENT RESEARCHER(S)
Oluwafemi Oluwole

FUNDER(S)
CANADIAN INSTITUTES OF HEALTH RESEARCH (CIHR)
SASKATCHEWAN LUNG ASSOCIATION

TITLE
Saskatchewan Lung Health Study - Phase 2 (Environment and Biological Exposure Assessment)

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
02-Jul-2014	08-Sep-2014	Amended Application for Biomedical Research Ethics Review received 17 Jul 2014 Parent Information Letter and Consent Form dated July 15, 2014 Assent Form dated August 19, 2014 Lung Health Study amended survey	07-Sep-2015

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The Bio-REB considered the requirements of section 29 under the Health Information Protection Act (HIPA) and is satisfied that this study meets the privacy considerations outlined therein. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval

Please send all correspondence to:
Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place
Saskatoon, SK Canada S7N 4J8

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Josh Lawson	Canadian Centre for Health and Safety in Agriculture	14-162

and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research projects involving human subjects under section 29 of The Health Information Protection Act (HIPA).

University of Saskatchewan
Biomedical Research Ethics Board

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UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Josh Lawson	Canadian Centre for Health and Safety in Agriculture	14-162

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Saskatoon SK

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Oluwafemi Oluwole

FUNDER(S)
CANADIAN INSTITUTES OF HEALTH RESEARCH (CIHR)
SASKATCHEWAN LUNG ASSOCIATION
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)

TITLE
Saskatchewan Lung Health Study - Phase 2 (Environment and Biological Exposure Assessment)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Addition of New Funder - Saskatchewan Health Research Foundation (SHRF) Invitation Letter (31-Mar-2015)) Survey Questionnaire (25-Mar-2015) Information Letter (09-Apr-2015)	13-Apr-2015	07-Sep-2015

Acknowledgment:
Amendment Request Cover Letter (31-Mar-2015)

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
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University of Saskatchewan
Biomedical Research Ethics Board

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Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR
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Canadian Centre for Health and Safety in Agriculture

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SASKATCHEWAN LUNG ASSOCIATION
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)

TITLE
: Saskatchewan Lung Health Study - Phase 2 (Environment and Biological Exposure Assessment)

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Information Letter (21-Apr-2015) Survey Questionnaire (4-May-2015)	27-May-2015	07-Sep-2015

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

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UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Re-Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Josh Lawson	Canadian Centre for Health and Safety in Agriculture	14-162

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University of Saskatchewan
Saskatoon SK

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SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)

TITLE
Saskatchewan Lung Health Study - Phase 2 (Environment and Biological Exposure Assessment)

RE-APPROVED ON	EXPIRY DATE
08-Sep-2015	07-Sep-2016

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This re-approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This re-approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research projects involving human subjects under section 29 of The Health Information Protection Act (HIPA).

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Oluwafemi Oluwole

FUNDER(S)
CANADIAN INSTITUTES OF HEALTH RESEARCH (CIHR)
THE LUNG ASSOCIATION OF SASKATCHEWAN
SASKATCHEWAN HEALTH RESEARCH FOUNDATION (SHRF)

TITLE
Saskatchewan Lung Health Study - Phase 2 (Environment and Biological Exposure Assessment)

RE-APPROVED ON	EXPIRY DATE
22-Aug-2016	21-Aug-2017

Delegated Review ☐ Full Board Meeting ☒

IRB 1 Registration #00001471 ☒
IRB 2 Registration #00008358 ☐
Not Applicable: ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This re-approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face meeting. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

Please send all correspondence to:
Research Services and Ethics Office
University of Saskatchewan
Room 223 – Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Josh Lawson	Canadian Centre for Health and Safety in Agriculture	14-162

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research studies involving human participants under section 29 of The Health Information Protection Act (HIPA)

Please send all correspondence to:

Research Services and Ethics Office
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Saskatoon, SK Canada S7N 5C9

Appendix 8

Parental consent and child assent form

PARENTAL CONSENT/CHILD ASSENT TO PARTICIPATE

Saskatchewan Children's Lung Health Study Clinical Assessment– Children's Evaluation

- I have read (or someone has read to me) the information in this consent form.
- I understand the **purpose and procedures** and the **possible risks and benefits** of the study for my child.
- I understand that the time to complete the tests will be about **1 hour**.
- I understand that my child is free to **refuse to participate in any part of the study** for any reason before, during, or after testing is complete.
- I understand that **until such time as information about my child is** pooled with other children's data and presented at conferences, meetings or is published, I may **withdraw** any information about my child.
- I understand that the information collected for this study will be stored and summarized in a way that does not identify my child. **I consent** to the use of my child's de-identified information for the purposes of the study.
- I understand that by signing this document **I do not waive** any of my legal rights or those of my child.
- I will be given a **signed copy** of this consent form.
- **I agree** that my child may participate in any or all of the following tests (*Please check all or any*):

	Yes	No
Height, weight, and waist measurements	☐	☐
Breathing test	☐	☐
Breathing test after exercise	☐	☐
Allergy test on the skin	☐	☐
Urine sample collection	☐	☐
Home dust sample collection	☐	☐

I DO / DO NOT give permission for the Saskatchewan Ministry of Health to provide my child's health care information about visits to doctor, hospital stays and prescriptions. If giving permission, please provide your child's Saskatchewan Health Services Number _____. It will be checked against the registry and used to gather your child's information.

Name of child: _____

Printed name of Parent or Caregiver: Signature: Date:

_____ _____ _____

I have read this paper or have had it read to me. I understand what I have to do in this study and I agree to take part in it.

Printed name of Child: Signature: Date:

_____ _____ _____

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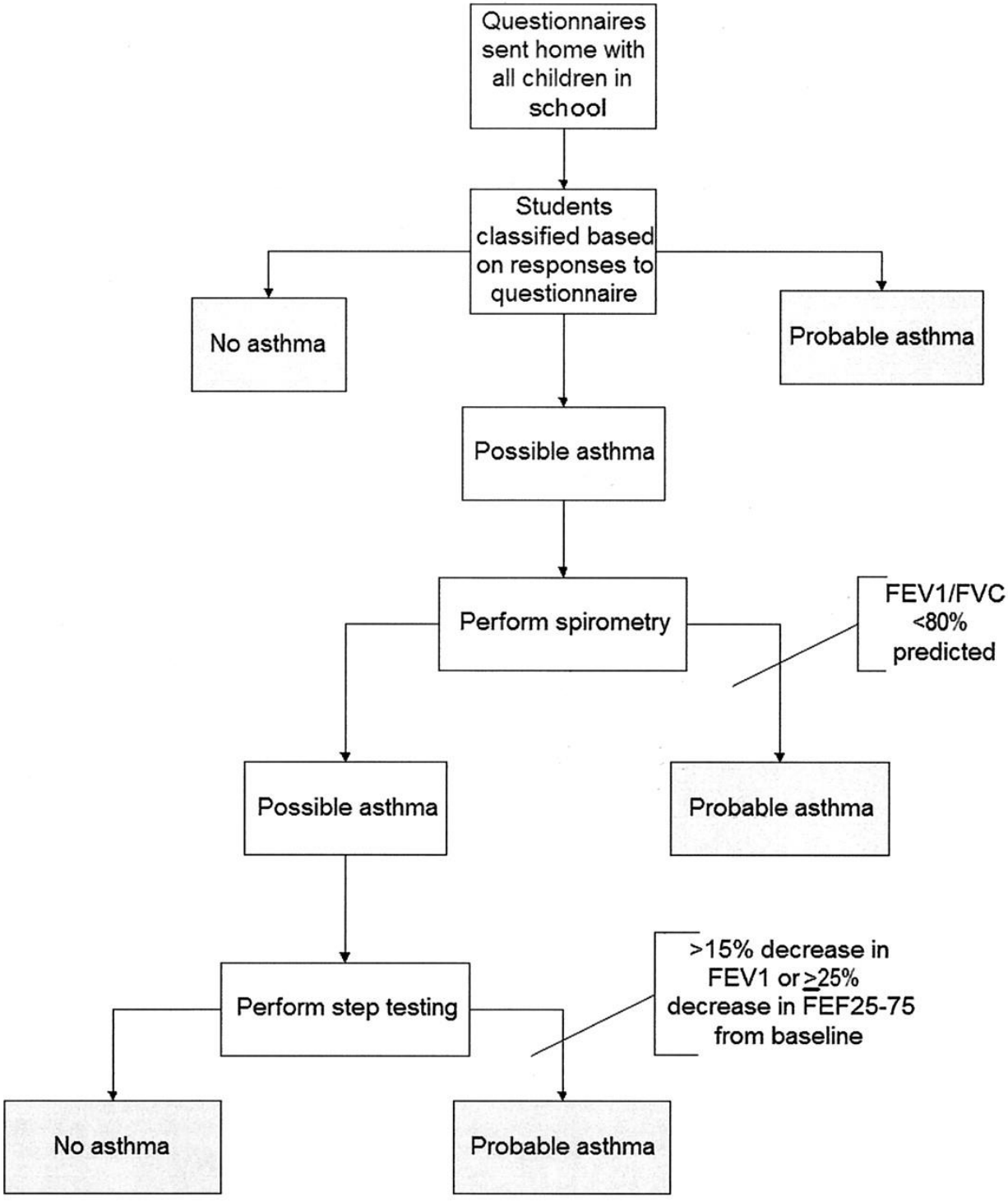
Appendix 9

The 3-Stage asthma-case detection algorithm* used in the study



*Algorithm was developed by the Lung Health Center study team at the University of Alabama at Birmingham

Appendix 10

Mean endotoxin and beta-(1→3)-D-glucan exposure levels in house dust with ranges

Sampling location (no. of detectable samples/total samples)	Mean		Standard Deviation		Minimum		Maximum		Interquartile Range (IQR)	
	Ln	Geometric	Ln	Geometric	Ln	Geometric	Ln	Geometric	Ln	Geometric
Play area (102/102)										
Endotoxin, EU/mg	3.96	52.46	0.79	2.20	2.20	9.03	6.55	699.24	0.98	2.66
Endotoxin, EU/m^2	10.01	22247.84	0.87	2.39	8.19	3604.72	12.76	348014.70	0.96	2.61
Beta-(1→3)-D-glucan, μg/g	2.20	9.03	0.70	2.01	0.31	1.36	5.03	152.93	0.89	2.44
Beta-(1→3)-D-glucan, μg/m^2	4.84	126.47	1.86	6.42	0.69	1.99	8.47	4769.51	2.72	15.18
Mattress (102/102)										
Endotoxin, EU/mg	3.04	20.91	0.82	2.27	0.69	1.99	6.21	497.70	1.01	2.74
Endotoxin, EU/m^2	9.16	9506.06	0.94	2.56	6.02	411.58	12.18	194852.86	1.22	3.39
Beta-(1→3)-D-glucan, μg/g	1.52	4.57	0.65	1.91	0.15	1.16	4.41	82.27	0.84	2.32
Beta-(1→3)-D-glucan, μg/m^2	3.81	45.15	1.46	4.31	0.44	1.55	7.43	1685.81	2.39	10.91

Ln: Natural log transformed.

Appendix 11

Correlation between play area and mattress endotoxin and beta-(1→3)-D-glucan levels

	Play area		Mattress	
Play area	Endotoxin	Beta-(1→3)-D-glucan	Endotoxin	Beta-(1→3)-D-glucan
Endotoxin (EU/mg)	1; ***0.94*****	0.23*, ***0.30****	0.10, ***0.11***	-0.03, ***-0.01***
Endotoxin (EU/m^2)	0.94**, ***1***	0.22*, ***0.43*****	0.08, ***0.11***	-0.06, ***0.00***
Beta-(1→3)-D-glucan (μg/mg)	0.23*, ***0.22****	1, ***0.65****	-0.04, ***-0.01***	0.01, ***0.00***
Beta-(1→3)-D-glucan (μg/m^2)	0.30*, ***0.43*****	0.65*, ***1***	0.05, ***0.09***	-0.00, ***0.09***
Mattress				
Endotoxin (EU/mg)	0.10, ***0.08***	-0.04, ***0.05***	1, ***0.93*****	0.44**, ***0.14***
Endotoxin (EU/m^2)	0.11, ***0.11***	-0.01, ***0.09***	0.93**, ***1***	0.39**, ***0.22****
Beta-(1→3)-D-glucan (μg/mg)	-0.03, ***-0.06***	0.01, ***-0.00***	0.44**, ***0.39*****	1, ***0.53***
Beta-(1→3)-D-glucan (μg/m^2)	-0.01, ***0.00***	0.00, ***0.09***	0.14, ***0.22****	0.53**, ***1***

Values in bold and italic represent indoor load levels for endotoxin and beta-(1→3)-D-glucan as appropriate.
*p<0.05; **p<0.001

Appendix 12

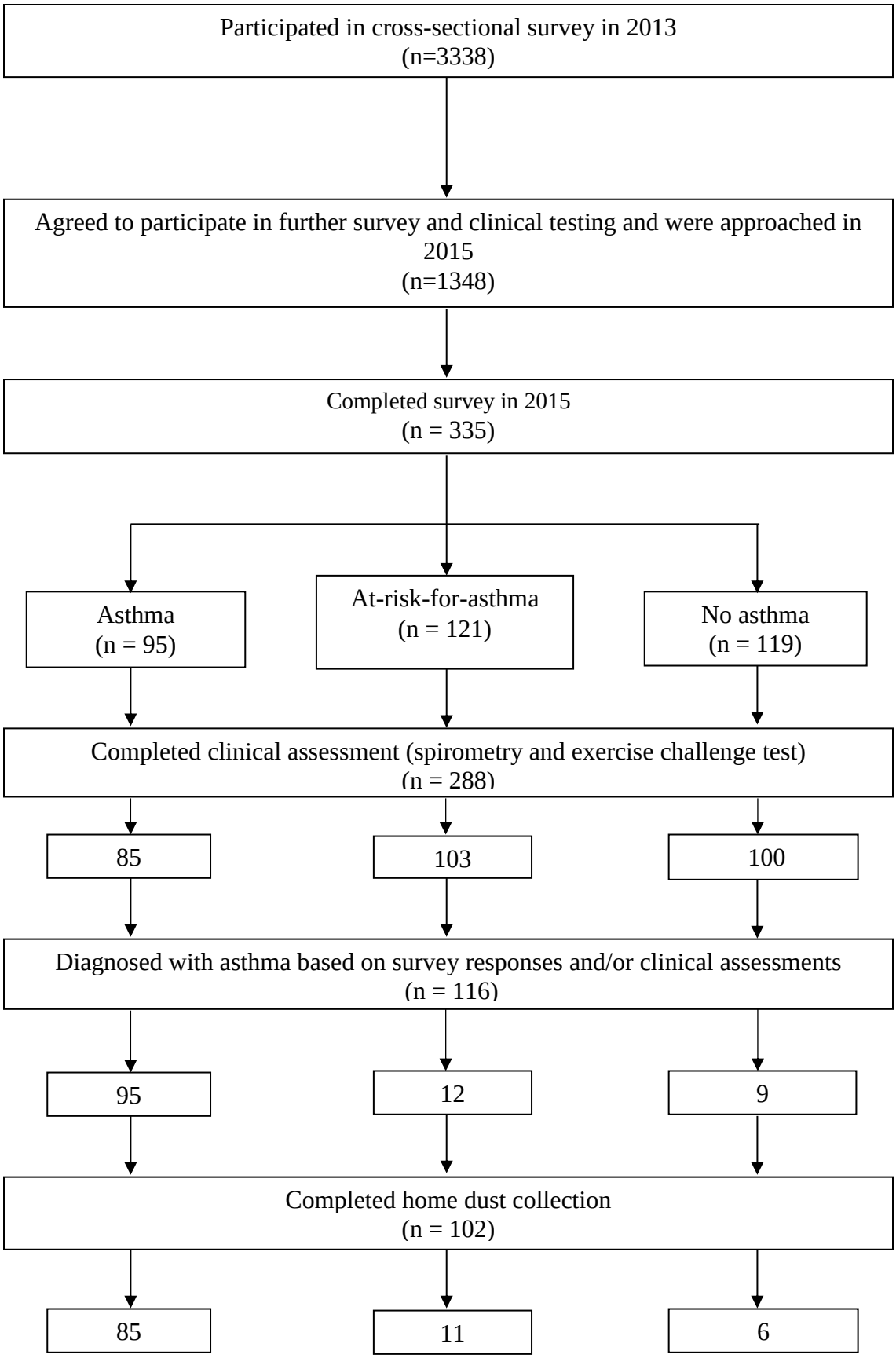
Determinants of indoor endotoxin and beta-(1→3)-D-glucan levels by location within home

	Play area				Mattress			
	Endotoxin (EU/mg) β (SE)	Endotoxin (EU/m^2) β (SE)	BDG (μg/mg) β (SE)	BDG (μg/m^2) β (SE)	Endotoxin (EU/mg) β (SE)	Endotoxin (EU/m^2) β (SE)	BDG (μg/mg) β (SE)	BDG (μg/m^2) β (SE)
Parental smoking (ref: none)	0.19 (0.22)	0.26 (0.24)	0.01 (0.27)	0.19 (0.70)	-0.17 (0.31)	-0.05 (0.34)	-0.04 (0.24)	0.79 (0.53)
Parental education (ref: ≤high school)	0.09 (0.23)	0.06 (0.25)	-0.24 (0.30)	-0.83 (0.74)	0.06 (0.33)	0.09 (0.36)	-0.16 (0.26)	-0.30 (0.57)
Age of home (ref: before 1980)	0.25 (0.15)	0.23 (0.17)	0.20 (0.19)	0.15 (0.50)	0.22 (0.22)	0.18 (0.24)	0.13 (0.17)	0.17 (0.38)
Pet ownership (ref: none)	0.13 (0.15)	0.09 (0.16)	-0.25 (0.19)	-0.76 (0.48)	0.02 (0.21)	0.08 (0.23)	0.08 (0.17)	0.44 (0.36)
Parental history of allergy (ref: none)	0.03 (0.16)	0.07 (0.17)	-0.13 (0.20)	0.23 (0.50)	0.22 (0.23)	0.08 (0.24)	0.46 (0.17)‡	0.57 (0.39)
Humidifier (ref: none)	-0.12 (0.18)	-0.04 (0.20)	-0.24 (0.23)	-0.08 (0.58)	0.23 (0.26)	0.16 (0.28)	-0.29 (0.20)	-0.15 (0.45)
Home dampness (ref: none)	0.20 (0.16)	0.18 (0.18)	-0.15 (0.21)	0.10 (0.53)	0.21 (0.24)	0.30 (0.26)	-0.09 (0.18)	-0.11 (0.40)
Visible mold (ref: none)	-0.22 (0.20)	-0.07 (0.22)	-0.01 (0.26)	0.54 (0.66)	0.03 (0.30)	-0.15 (0.32)	0.39 (0.23)	0.15 (0.50)
Rural home (ref: urban)	-0.84 (0.27)‡	-0.99 (0.29)‡	-0.16 (0.34)	-1.64 (0.86)	-0.47 (0.39)	-0.54 (0.42)	-0.46 (0.30)	-0.82 (0.66)
Season (ref: fall/winter)	-0.71 (0.20)‡	-0.63 (0.21)‡	-0.29 (0.20)	-0.90 (0.63)	0.11 (0.28)	0.31 (0.30)	-0.02 (0.22)	0.44 (0.48)

EU: Endotoxin unit, BDG: Beta-(1→3)-D-glucan, β: beta coefficient, SE: standard error.
Model adjusted for each variable in the table as well as sex, age, dehumidifier, fireplace, environmental tobacco smoke, presence of mice in home, and farm living status. Endotoxin and beta-(1→3)-D-glucan are log transformed.
‡p<0.05.

Appendix 13

Flow chart of study respondents depicting numbers of participants for each phase of the study


Participated in cross-sectional survey in 2013
(n=3338)
Agreed to participate in further survey and clinical testing and were approached in 2015
(n=1348)
Completed survey in 2015
(n = 335)
Asthma
(n = 95)
At-risk-for-asthma
(n = 121)
No asthma
(n = 119)
Completed clinical assessment (spirometry and exercise challenge test)
(n = 288)
85
103
100
Diagnosed with asthma based on survey responses and/or clinical assessments
(n = 116)
95
12
9
Completed home dust collection
(n = 102)
85
11
6

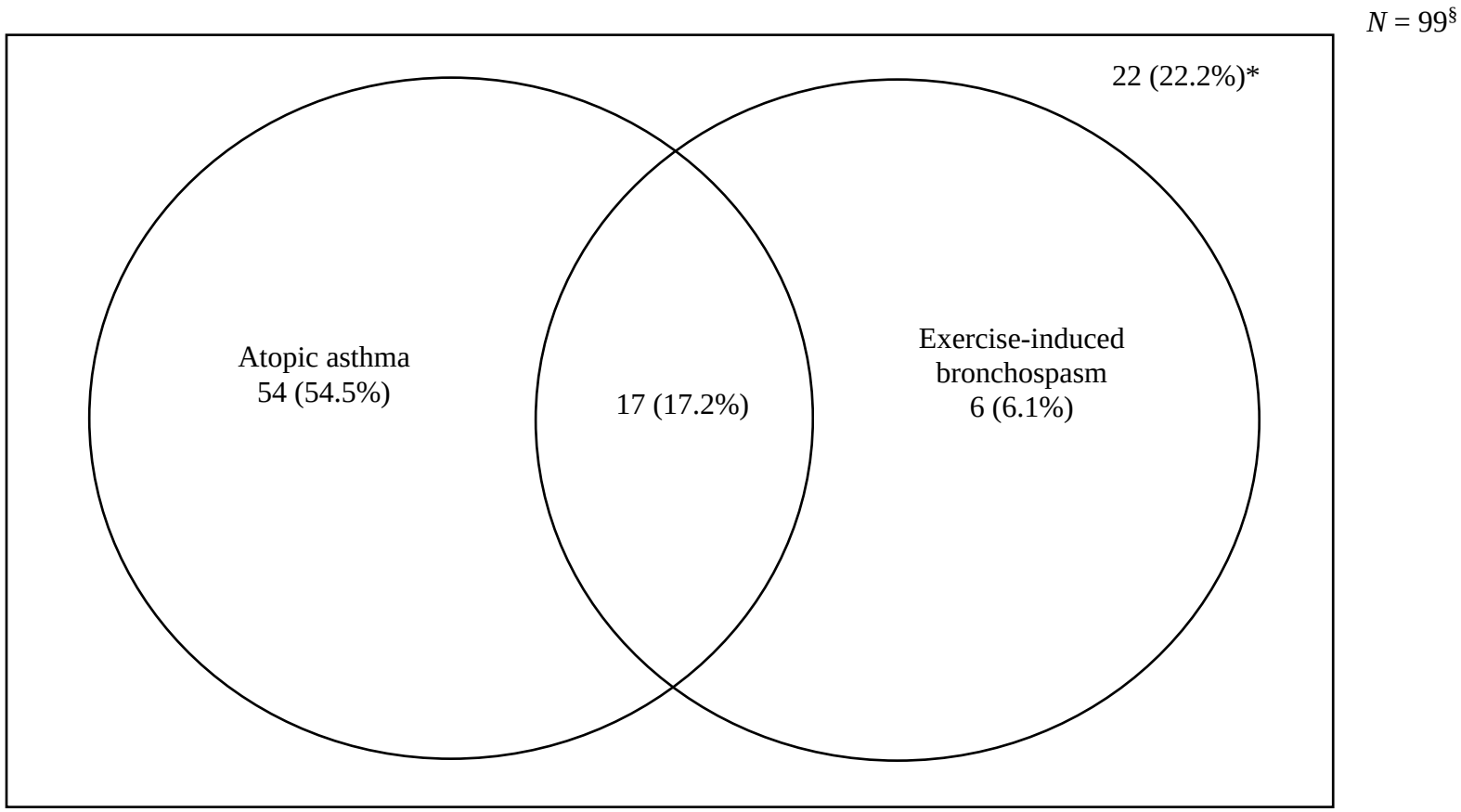
Appendix 14

Comparison of characteristics between participants in the 2013 baseline survey and those in the 2015 follow-up study

	Did not complete the clinical testing phase (2013) (n = 3338)	Completed the clinical testing phase (2015) (n = 335)	*p*-value
Personal characteristics			
Mean age (±SD), years	9.52 (2.76)	9.03 (2.52)	0.001
% Female	50.6	48.5	0.456
% > high school (maternal)	73.7	86.1	<0.001
% > high school (paternal)	67.2	78.3	<0.001
Ethnic background			
% Caucasian	62.6	79.8	<0.001
% Other ethnic background	37.4	20.2	
Tobacco smoke exposure			
% Maternal smoking	18.7	4.6	<0.001
% Paternal smoking	24.3	12.9	<0.001
% Either parent smoking	31.3	14.7	<0.001
Indoor characteristics			
% Pet ownership	52.2	53.2	0.731
% Dampness in home	16.3	19.2	0.182
% Home mold	12.2	11.0	0.546
% Air conditioner	71.2	76.7	0.041
% Air filter	63.2	64.9	0.569
% Humidifier	38.3	41.5	0.292
Parental history of asthma and allergies			
% Parental history of asthma	16.9	20.9	0.07
% Parental history of allergy	37.3	50.1	<0.001
Child's previous history of wheeze and asthma			
% Ever wheeze	7.8	12.2	<0.001
% Ever asthma	8.2	12.6	<0.001

Appendix 15

Venn diagram of asthma phenotypes among children with asthma in the study population showing proportions with overlap in atopic asthma and EIB



*Proportion with no atopic asthma and no EIB.

§17/116 of the children identified to have asthma did not consent to skin prick test.

Supplementary I

Specific variables determined during the study

Variable	Procedure	Phase of assessment	Specific Objective
1. Subject descriptors			
Demographics	SQ	I	
Age, gender, ethnicity	SQ	I	
Health information	SQ	I	
Location of residence (Large Urban, Small Urban, and Rural)	SQ	I	1
2. Asthma diagnostic pattern			
Physician-diagnosed asthma	SQ, CA, ACDA	I, II	1
At-risk-for asthma	SQ, CA, ACDA	I, II	1
No asthma	SQ, CA, ACDA	I, II	1
3. Asthma phenotype categories			
Atopic asthma and non-atopic asthma	SQ, SPT	I	2
EIB and no EIB	LFT, ECT, CA	I	2
4. Asthma severity categories			
Mild intermittent asthma	SQ, LFT, CA	I	3
Mild persistent asthma	SQ, LFT, CA	I	3
Moderate persistent asthma	SQ, LFT, CA	I	3
Severe persistent asthma	SQ, LFT, CA	I	3
5. Pulmonary Function test			
FEV_1 (Liters) % predicted	LFT	I	3
FVC (Liters) % predicted	LFT	I	3
FEV_1/FVC (%)	LFT	I	3
$FEF_{25-75\%}$ (Liters) % predicted	LFT	I	3
PEFR (Liters) % predicted	LFT	I	3
6. Allergy skin test			
Alternaria	SPT	I	2
Cladosporium	SPT	I	2
Aspergillus	SPT	I	2
House dust mite	SPT	I	2
Local grasses	SPT	I	2
Cat dander	SPT	I	2
7. Biological and environmental assessment			
Endotoxin concentration and load	LMA	II	2, 3
Beta-(1→3)-D-Glucan concentration and load	LMA	II	2, 3

Abbreviations: SQ– Survey questionnaire; CA– Clinical assessment; ACDA– Asthma case-detection algorithm; EIB– Exercise-induced bronchospasm; LFT– Lung function testing; ECT– Exercise challenge test; FEV_1– Forced expiratory volume in 1 second; FVC– Forced vital capacity; FEF– Forced expiratory flow; LMA: Laboratory microbiology analysis.

Supplementary II

Relationship between other risk factors examined in the literature review and asthma phenotypes and severity

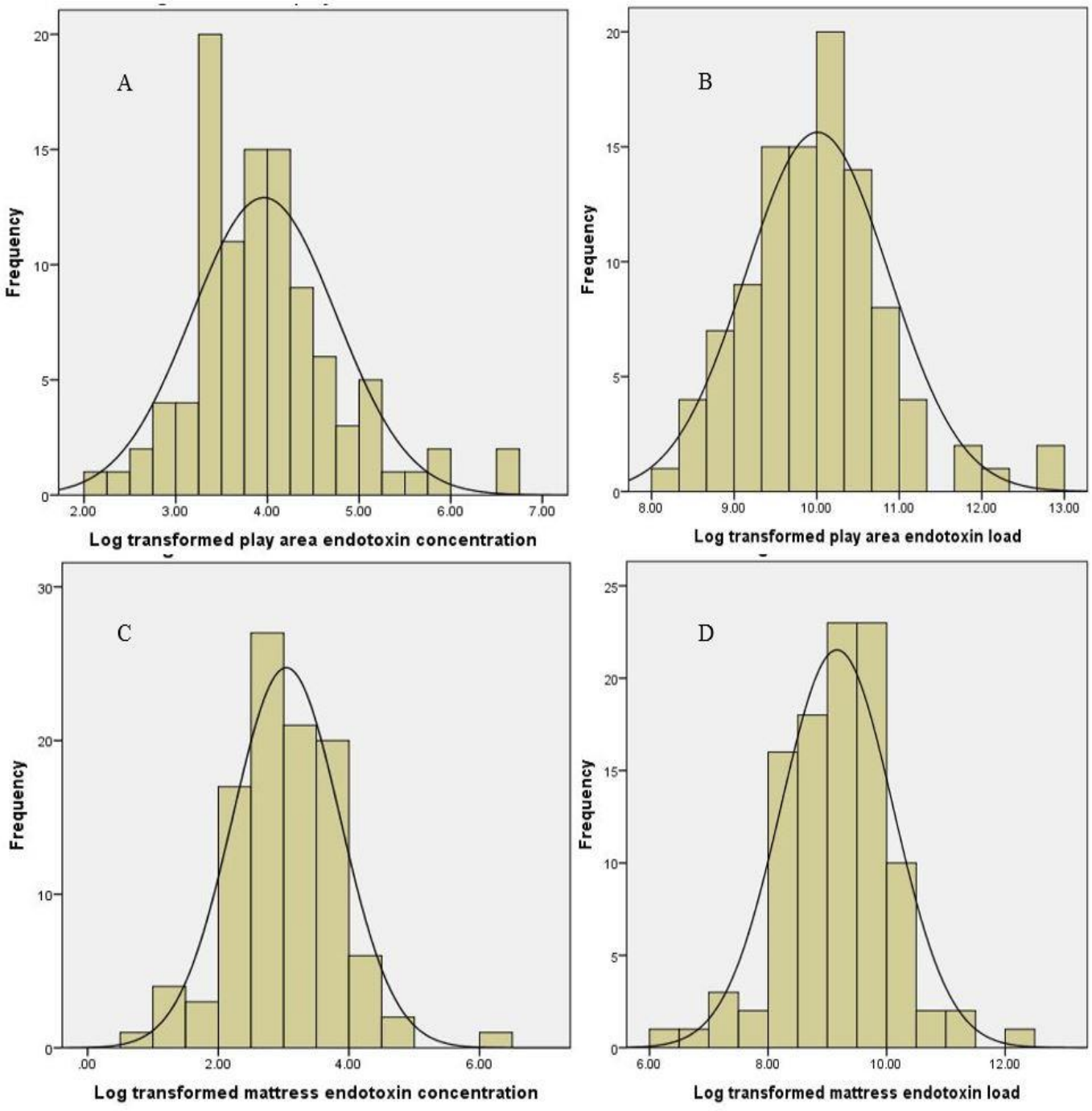
	Atopic asthma OR (95%CI)	EIB OR (95%CI)	Asthma severity* OR (95%CI)
Sex			
Male	1.00	1.00	1.00
Female	1.01 (0.40–2.58)	0.61 (0.23–1.59)	0.53 (0.21–1.38)
Age			
< 12 years	1.00	1.00	1.00
≥ 12 years	0.87 (0.36–2.11)	0.51 (0.20–1.29)	0.99 (0.42–2.33)
Ethnicity			
Caucasian	1.00	1.00	1.00
Others	0.71 (0.24–2.04)	1.21 (0.39–3.76)	1.96 (0.68–5.59)
Obesity			
Not overweight	1.00	1.00	1.00
Overweight	0.75 (0.23–2.44)	0.71 (0.62–0.81)	1.10 (0.32–3.76)
Parental history of asthma			
No	1.00	1.00	1.00
Yes	0.89 (0.36)	0.64 (0.24–1.67)	0.87 (0.35–2.16)
Parental history of allergy			
No	1.00	1.00	1.00
Yes	1.15 (0.47–2.80)	0.39 (0.16–0.94)	1.25 (0.52–3.01)
Pet ownership (Cat)			
No	1.00	1.00	1.00
Yes	2.07 (0.69–6.14)	1.09 (0.42–2.84)	0.44 (0.15–1.28)
Pet ownership (Dog)			
No	1.00	1.00	1.00
Yes	0.58 (0.24–1.41)	1.14 (0.48–2.74)	1.15 (0.49–2.67)
Pet ownership (Cat and Dog)			
No	1.00	1.00	1.00
Yes	0.94 (0.30–2.95)	1.50 (0.52–4.36)	0.47 (0.13–1.72)
Pet ownership (Cat or Dog)			
No	1.00	1.00	1.00
Yes	1.03 (0.42–2.48)	0.85 (0.39–2.31)	0.92 (0.39–2.18)
Paternal smoking			
No	1.00	1.00	1.00
Yes	0.81 (0.22–2.96)	1.65 (0.46–5.89)	0.89 (0.23–3.49)
Maternal smoking			
No	1.00	1.00	1.00
Yes	3.00 (0.35–25.58)	0.45 (0.06–3.99)	0.42 (0.05–3.60)
Father and mother smoking			
No	1.00	1.00	1.00

Yes	1.25 (0.12–12.52)	1.12 (0.11–11.25)	0.74 (0.67–0.83)
Either father or mother smoking			
No	1.00	1.00	1.00
Yes	0.92 (0.26–3.26)	1.40 (0.40–4.90)	1.25 (0.36–4.35)
Dampness in home past year			
No	1.00	1.00	1.00
Yes	1.21 (0.42–3.48)	0.49 (0.15–1.55)	1.79 (0.69–4.62)
Visible mold/mildew in home			
No	1.00	1.00	1.00
Yes	1.4 (0.27–7.23)	1.09 (0.27–4.31)	3.10 (0.94–10.22)

EIB: Exercised-induced bronchospasm.
*Outcome is moderate/severe asthma.

Supplementary IIIA

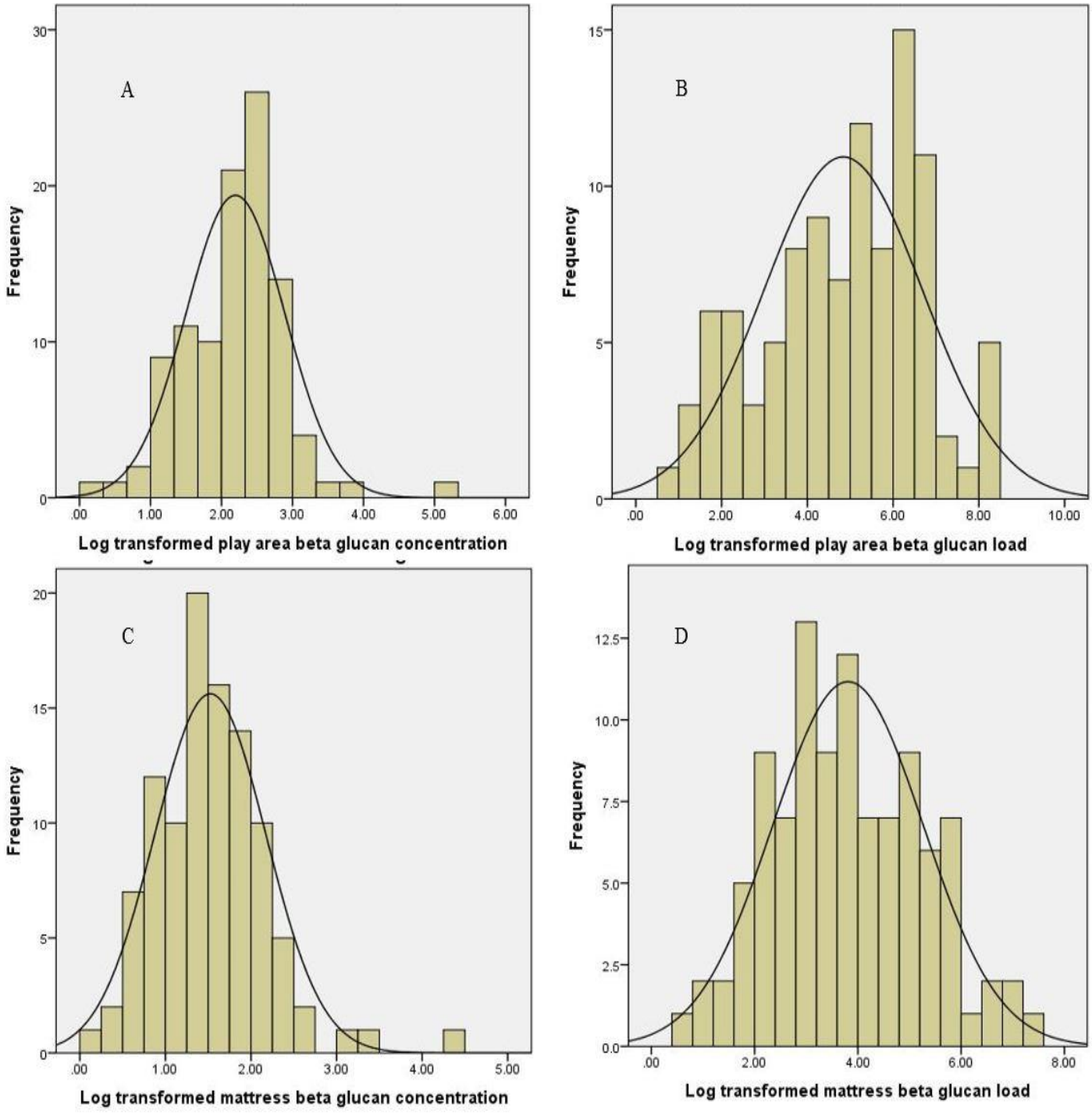
Histogram for log endotoxin values (n = 102)



Histogram for: (A) log play area endotoxin concentration (EU/mg); (B) log play area endotoxin load (EU/m^2); (C) log mattress endotoxin concentration (EU/mg); (D) log mattress endotoxin load (EU/m^2).

Supplementary IIIB

Histogram for log beta-(1→3)-D-Glucan values (n = 102)



Histogram for: (A) log play area beta-(1→3)-D-Glucan concentration (μg/g); (B) log play area beta-(1→3)-D-Glucan load (μg/m^2); (C) log mattress beta-(1→3)-D-Glucan concentration (μg/g); (D) log mattress beta-(1→3)-D-Glucan load (μg/m^2).