DEFINING THE ROLE OF THE HUMAN INTESTINAL MICROBIOTA IN CHILDHOOD ASTHMA AND ATOPIC DISEASE

by

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Abstract

Asthma is a chronic inflammatory atopic disease of the airways affecting one in ten children in Westernized countries. The microflora hypothesis of allergic disease proposes the intestinal microbiota as a potential mechanistic link connecting environmental exposures to changes in the developing immune system. Further, animal model studies allude to an early life critical window, during which the immune system is most vulnerable to compositional and functional changes in the intestinal microbiota.

We conducted an epidemiological assessment of early life environmental factors associated with atopy and wheezing at age 1 year and preschool-age asthma in children enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) Study (n = 2,695). Here, we identified early life microflora hypothesis related variables (e.g. pre- and post-natal antibiotic exposure, gestational age, and birth mode) as risk and protective factors for asthma and atopic disease.

Informed by this epidemiological assessment, we used 16S ribosomal RNA sequencing and quantitative polymerase chain reaction to analyze the 3-month and 1-year fecal microbiota of one-year-old CHILD Study subjects positive or negative for atopy and wheezing (n = 319) and among this same cohort, those who were diagnosed with preschool-age asthma or non-atopic non-wheezing controls (n = 76). The fecal microbiota of atopic wheezing subjects compared to controls showed decreases in the abundances of four gut bacterial genera, *Faecalibacterium*, *Lachnospira*, *Rothia*, and *Veillonella*, combined with a reduction in fecal acetate at the 3-month time point only.

Further, we found shifts in the relative abundances of two bacterial taxa in the 3-month fecal microbiota of preschool-age asthmatic children compared to controls; *Lachnospira*

remained decreased among asthmatic children and *Clostridium neonatale* was increased in asthmatics. Quartile analysis at 3-months revealed a negative association between the ratio of these two bacteria (*Lachnospira/Clostridium neonatale*) and asthma risk.

Altogether, this research highlights environmental factors that may be contributing to gut bacterial alterations in subjects with asthma or atopic disease. Additionally, these microbial alterations were no longer present by 1-year of age, suggesting the first 100 days of life as the critical window during which taxa-specific gut bacterial dysbiosis is associated with asthma and atopic disease in humans.

Preface

All work presented here was conducted at the BC Children's Hospital in collaboration with individuals from laboratories in the Michael Smith Laboratories and the Life Sciences Institute at the University of British Columbia, Point Grey Campus and with the Canadian Healthy Infant Longitudinal Development (CHILD) Study. Each parent or legal guardian gave signed informed consent and all research protocols for the following studies in human samples were approved by The University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board (ethics certificate number: H07-03120).

Chapter 1: Versions of **Sections 1.2 – 1.5** and a version of **Figure 1.1** have been previously published in **Stiemsma, L.T.**, Reynolds, L., Turvey, S.E., and Finlay, B.B., The hygiene hypothesis: Current perspectives and future therapies. *Immunotargets Ther.* 2015;4**:**143-57.

I was principally responsible for this manuscript and wrote all sections of this manuscript, except for sections related to helminth parasites (written by Dr. Lisa Reynolds), which are not included in this thesis. Dr. Reynolds also edited the manuscript and Dr. Turvey and Dr. Finlay edited and provided insights on the manuscript. An online version of this manuscript can be found here: https://www.dovepress.com/the-hygiene-hypothesis-current-perspectives-and-future-therapies-peer-reviewed-fulltext-article-ITT.

Versions of **Sections 1.3.2.2 – 1.3.2.4** were previously published in Arrieta, M.C., **Stiemsma, L.T.,** Amenyogbe, N., Brown, E.M., and Finlay, B.B. The intestinal microbiome in early life: health and disease. *Front Immunol.* 2014;5**:**427 in the sections entitled "Mode of

delivery", "Breastfeeding and formula feeding", "Introduction to solid foods", "Antibiotics", and "Asthma and atopy." I wrote these sections for the original manuscript and edited the final version of the overall manuscript. An online version of this manuscript can be found here: http://journal.frontiersin.org/article/10.3389/fimmu.2014.00427/full.

Lastly, versions of **Sections 1.2.1**, **1.4.3,** and **5.1.3 – 5.2.3** are in press as a review article in *Asthma, Allergy, and Clinical Immunology*. **Stiemsma L.T.,** Turvey S.E. Asthma and the microbiome: defining the critical window in early life. *Allergy Asthma Clin Immunol*. 2016.

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I was principally responsible for the study design, data curation, and analyses described in Chapter 2. Dr. Azad advised and assisted in the curation of breast-feeding data. Dr. Lefebvre, David Dai, Dr. Sears, Dr. Subbarao, Dr. Mandhane, Dr. Becker, and The CHILD Study Investigators made CHILD Study data possible and accessible. Dr. Turvey supervised, advised on the study design and analysis, and edited the manuscript.

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K.M., Sears, M.R., Kollmann, T., The CHILD Study Investigators, C.S., Mohn, W.W., Turvey, S.E.$^{\phi}$, and Brett Finlay, B.$^{\phi}$. Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med*. 2015;7(307):307ra152. An electronic version of this manuscript can be accessed here: http://stm.sciencemag.org/content/7/307/307ra152.

This study was carried out in collaboration with Dr. Marie-Claire Arrieta (*co-first author). Contributions from additional co-authors are mentioned below. Specifically, I lead the clinical and epidemiological portion of this study, which included classifying CHILD study subjects into one year atopic/wheezing phenotypes and preschool-age asthma phenotypes based on CHILD Study questionnaires and skin prick test data, calculating risk of asthma for the one year phenotypes, liaising with CHILD study investigators for clinical data and sample acquisition, curating all additional metadata, and conducting all regression analyses on early life environmental factors. In addition, I prepared all stool samples for sequence analysis (DNA extraction, PCR amplification, and quantification) with the help of Lisa Thorson. Dr. Arrieta and I equally contributed to the 16S rRNA sequence and qPCR analyses, to the short chain fatty acid analyses, and in writing the manuscript. I do not include any figures or discuss results from the PICRUSt analysis (lead by Dr. Pedro Dimitriu), the metabolomics analysis (lead by Dr. Arrieta), or the FLVR supplemented mouse model (lead by Dr. Arrieta with the help of Dr. Gold, Dr. Russell, and Heidi Britton) in my thesis, but the mouse model is referenced in the discussion (**Section 3.5**). Dr. Dimitriu also contributed to the optimization of our sequencing strategy and bioinformatic pipeline. Dr. Yurist-Doutsch designed qPCR primers and advised on data interpretation. Boris Kuzeljevik advised on statistical analyses. Dr. Mohn advised on the Illumina sequencing analysis. Dr. McNagny advised on the OVA-mouse model. Dr. Kollmann advised on data interpretation. Dr. Lefebvre, Dr. Sears, Dr. Subbarao, Dr. Mandhane, Dr. Becker,

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I was principally responsible for the study design, data acquisition, and interpretation of results. Due to the longitudinal nature of the CHILD study, this chapter utilizes 16S sequence data from CHILD study subjects previously analyzed in Chapter 3. The subjects were re-classified as asthmatic or controls by age four ($n_{Asthmatic}$ = 39, $n_{Control}$ = 37). Dr. Arrieta and Dr. Dimitriu optimized the sequencing strategy and bioinformatic pipeline (previously used in Chapter 3). Jasmine Cheng conducted qPCRs and extracted DNA from stool samples for additional analyses. Lisa Thorson assisted with stool sample preparation originally analyzed in

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Publications arising from my PhD work to date:

- **Stiemsma L.T.**, Azad M.B., Lefebvre D.L., Dai D., Subbarao P., Mandhand P., Becker A., The Canadian Healthy Infant Longitudinal Development (CHILD) Study Investigators, Sears M.R., Turvey S.E. Associations between early life hygiene hypothesis-related factors and the development of preschool-age asthma and atopic disease. Manuscript in preparation. 2016.

- **Stiemsma L.T.,** Tuvey S.E., Asthma and the microbiome: defining the critical window in early life. *Asthma Allergy Clin Immunol.* In press. 2016.

- **Stiemsma L.T.,** Arrieta M.C., Dimitriu P.A., Cheng J., Thorson L., Lefebvre D.L., Azad M.B., Subbarao P., Mandhane P., Becker A., Sears M.R., Kollmann T.R., The Canadian Healthy Infant Longitudinal Development (CHILD) Study Investigators, Mohn W.M., Finlay B.B.*, Turvey S.E.* Shifts in *Lachnospira* and *Clostridium sp.* in the 3-month stool microbiome are associated with asthma in preschool-age children. Forthcoming: *Clinical Science*. * Both authors contributed equally.

- Arrieta, M.C.*, **Stiemsma, L.T.*,** Dimitriu, P.A., Thorson, L., Russell, S., Yurist-Doutsch, S., Kuzeljevic, B., Gold, M.J., Britton, H.M., Lefebvre, D.L., Subbarao, P., Mandhane, P., Becker, A., Mcnagny, K.M., Sears, M.R., Kollmann, T., The CHILD Study Investigators, C.S., Mohn, W.W., Turvey, S.E.$^{\phi}$, and Brett Finlay, B.$^{\phi}$. Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med*. 2015;7(307):307ra152. * $^{\phi}$ Both authors contributed equally.

- **Stiemsma, L.T**., Reynolds, L., Turvey, S.E., and Finlay, B.B. (2015). The hygiene hypothesis: Current perspectives and future therapies. *Immunotargets Ther.* 2015;4:143-57.

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List of Symbols

Symbol	Definition
≥	Greater than or equal to
>	Greater than
<	Less than
≤	Less than or equal to
%	Percent
ºC	Degrees Celsius

List of Abbreviations

Abbreviation	Definition
AEC	Airway epithelial cells
AW	Atopy + wheeze
BALF	Bronchoalveolar lavage fluid
C-section	Cesarean section
CCL	CC chemokine ligand
CD	Cluster of differentiation
CHILD	Canadian healthy infant longitudinal development study
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
FENO	Fractional exhaled nitric oxide
FEV	Forced expiratory volume
FLVR	*Faecalibacterium, Lachnospira, Veillonella, Rothia*
FOS	Fructo-oligosaccharides
FoxP3	Forkhead box P3
GF	Germ-free
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOS	Galacto-oligosaccharides
GWAS	Genome wide association study
HDAC	Histone deacetylase
HDM	House dust mite
HMO	Human milk oligosaccharide
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interlukin
iNKT	Invariant natural killer T cell
ISAAC	International study of asthma and allergies
kb	Kilobase
L/C	*Lachnospira/Clostridium neonatale*
Lc	Long chain
Ln( )	Natural log
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein -1
min	minutes
mL	Millileter
MLN	Mesenteric lymph node
mm	Millimeter
MyD88	Myeloid differentiation primary response gene 88

NFκB	Nuclear factor kappa-light chain enhancer of activated B cells
ns	Not significant
OR	Odds ratio
OTU	Operational taxonomic unit
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PCA	Principal components analysis
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal deoxyribose nucleic acid
RQ	Relative quantification
rRNA	Ribosomal ribose nucleic acid
Sc	Short chain
SPF	Specific pathogen free
T-reg	T-regulatory cell
T1D	Type 1 diabetes
Th cell	T- helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
ΔC_T	Delta cycle threshold
ηm	Nano molar
µL	Microliter

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To Vancouver,

Thank you for years of hope and months of rain

Chapter 1: Introduction

1.1 Asthma background

1.1.1 Prevalence and global burden

Asthma is an immunoglobulin E (IgE)-mediated chronic inflammatory disease of the airways (1). Other manifestations of IgE-mediated or "atopic" diseases include: atopic dermatitis, allergic rhinitis, and food allergy. These diseases typically manifest in early childhood and can be chronic lifelong burdens for many people. However asthma is often viewed as the most burdensome atopic disorder, due to the prevalence (235 million people worldwide) and associated mortality rates with untreated asthma attacks (an estimated nine asthma-related deaths per day in the United States) (2, 3). Associated with its early life manifestation, asthma has become the most prevalent childhood disease in recent decades, affecting approximately one in ten children worldwide (4). Aside from the obvious danger associated with asthma, this disease is very disruptive to a normal daily lifestyle for children, leading in the cause of emergency room visits and absenteeism from school (5).

Some of the most striking data related to asthma prevalence comes from the United States, where it was reported from 1999 to 2009, that the proportion of people diagnosed with asthma increased by 15% (3). In other Westernized countries (i.e. Canada, Australia, and the UK) the prevalence of this disease was reported to be even higher (up to 30% in some countries), while many countries in Eastern Europe and Asia report a much lower prevalence of this disease (~5%) (4, 6, 7). This rapid increase in prevalence of asthma (and other atopic diseases) as well as the apparent geographical disparity suggests roles for changing environmental exposures and compels more in depth research to better understand the etiology of these diseases.

1.1.2 Clinical presentation

Asthma is characterized by variable airflow obstruction, bronchial hyper-responsiveness, and airway inflammation (8). Typical symptoms include shortness of breath, chest tightness, and wheezing, which are the result of smooth muscle contraction and increased mucus production in the airway and eosinophilic inflammation in the lungs (8, 9). The disease consists of many sub-phenotypes, but airway inflammation remains a central theme, driving airway dysfunction and ultimately increasing its susceptibility to numerous environmental triggers including viruses, air pollution, and common allergens (1).

1.1.3 Atopy and wheezing as early life indicators of asthma

1.1.3.1 The 'atopic march'

Atopy is a term referring to IgE antibody production in response to specific environmental stimuli (10). In infancy and early childhood, the presence of atopy can be determined using allergen skin prick tests. Skin prick tests involve introducing a small prick of purified allergen on the forearm or back of an infant. If a wheal 2mm or greater in diameter (3mm or greater in more stringent clinical tests) forms after this test is performed, the child is determined to be atopic, as they produce IgE for that particular allergen (11).

Typical manifestations of atopy include, asthma, atopic dermatitis, allergic rhinitis, food allergy, and anaphylaxis. Consequently, children diagnosed with one of these diseases often have presented or currently present with other atopic diseases (4). The 'atopic march' refers to the progression of atopic disorders in early life, from atopic dermatitis in infants to asthma in school-age children (12). Asthma can manifest as non-atopic (no evidence of IgE), however this thesis

focuses on atopic or ‘allergic’ asthma (triggered by common allergens; animal hair, dust mites, etc.), which is the most common type of asthma in childhood (13).

1.1.3.2 Wheezing and the asthma predictive index

Wheezing is a coarse whistling sound produced during the expiratory phase of breathing due to enhanced narrowing of the airways (14). Recurrent wheezing (more than one episode during the first 3 years of life) is typically viewed as an indicator among infants and young children for asthma development, however, only 30% of children with recurrent wheezing ultimately go on to develop asthma by school age (15). Consequently, recurrent wheezing has been clinically redefined over the years in an effort to enhance its predictability for future asthma diagnoses (i.e. in the asthma predictive index, which predicts school-age asthma) (16).

1.1.4 Immunological characterization

Here, we will focus on the immunological characterization of allergic asthma as this is the most common type of childhood asthma (13). Allergic asthma and allergies are type I hypersensitivity reactions mediated by the production of IgE (17). This process however involves many immune cells (eosinophils, dendritic cells (DCs), T-helper (Th)-2 cells, mast cells, basophils, *etc*.) that contribute to lung inflammation and produce typical asthma symptoms (symptoms are described in **Section 1.1.4**).

1.1.4.1 Role of airway epithelial cells in allergen sensitization

Atopic diseases are IgE-mediated hypersensitivity reactions to an antigen that would, under normal circumstances, would be harmless to the host (i.e. allergic reaction to peanuts) (18). Airway epithelial cells (AECs) are the first to come in contact with an allergen and consequently, these cells modulate and coordinate immune responses important in the allergen sensitization process (19). Upon recognition of an allergen through various receptors (protease activated receptors, toll-like receptors (TLRs), C-type lectin receptors), AECs initiate an immune cascade through nuclear factor kappa B (NFκB) producing cytokines (e.g. TSLP, GM-CSF, interlukin (IL)-25, IL-33, IL-1β) and chemokines (e.g. C-C ligand (CCL)-2 and CCL20) necessary for DC activation and migration (19). This cytokine production also recruits other immune cells (Th-2 cells, mast cells, basophils), enhancing inflammation at the site of allergen uptake and promoting the onset of allergies and asthma (19).

1.1.4.2 Adaptive immune responses in asthma

In asthma, eosinophilic airway inflammation and bronchial hyperresponsiveness are driven by DC stimulation of Th-2 cells (20). DCs are a specialized population of antigen presenting cells that act at the interface of the innate and adaptive immune systems and play a significant role in antigen (allergen) presentation (1, 21). Following allergen uptake, they enter into local lymph nodes and drive the differentiation of Naïve T-cells into Th-2 cells via the T-cell receptor and the major histocompatibility complex II (1, 21). Th-2 cells produce a number of cytokines to promote IgE isotype switching (IL-4), mucous production and smooth muscle constriction (IL-13), and to recruit eosinophils (IL-5), and mast cells (IL-9) (22). B cells then produce IgE specific to the allergen that is presented (23-27).

Upon secondary interaction with an allergen, crosslinking of allergen-specific IgE is induced on mast cells and basophils, causing these cells to degranulate and release vasoactive mediators (e.g. histamines, leukotrienes) (20). This infiltration of immune cells and degranulation upon secondary interaction with a specific allergen contributes both to the bronchial hyperreactivity and airway inflammation characteristic of asthma and other allergic diseases (20).

Notably however, though allergic asthma is classically Th-2 driven, we now know that many additional T cell subtypes are involved in promoting (Th-17, γδ-T cells, NKT-cells) and modulating (e.g. CD8+ memory T-cells, T-regulatory cells (T-reg)) the allergic response (22, 28-30). Some asthmatic patients display a neutrophil prominent disease, characterized by a Th-1/Th-17 phenotype, in contrast to the eosinophilic Th-2 asthmatic phenotype (20). However, asthma is characterized by overlapping roles of these T-cell subtypes (i.e. IL-17 has been implicated both in the protection and promotion of allergic asthma), and thus, the immunology of this disease can be viewed on a spectrum, with neutrophilic Th-1/Th-17 asthma at one end, eosinophilic Th-2 asthma at the other, and many overlapping immune mechanisms in between (31).

1.1.5 Diagnosis

Asthma is typically diagnosed after five years of age using a combination of parental history, presence of the above symptoms, physical examination, and lung function tests (spirometry) (32). Spirometry testing measures the forced expiratory volume (FEV) before and after treatment with a bronchodilator. Asthmatics usually present with increased FEV

measurements after treatment with the bronchodilator; an increase ≥ 12% in children is an indicator of bronchial responsiveness (32).

A bronchial challenge is used if spirometry testing does not confirm variation in airway obstruction or responsiveness to medications (32). Pharmacological bronchial challenge uses medication (e.g. metacholine, histamine) to provoke the airways and > 20% reduction in FEV is considered indicative of asthma. This test however is often not conducted on young children due to risk of significant bronchial constriction and the need for prolonged cooperation (32).

Other supportive tests for asthma diagnosis include fractional exhaled nitric oxide (FENO) testing, which measures nitric oxide produced in the lungs due to eosinophilic inflammation, and allergy testing by either skin prick or serum IgE testing (32). Often these tests can be employed in support of the tests described above, or potentially for the diagnosis of asthma in preschool-age children (33).

Diagnosis of asthma in preschool-age children is a more elusive process. Children under five years of age do not readily cooperate and often do not adequately control their breathing for successful lung function testing (34). Consequently, physicians rely on parental history of asthma, wheezing episodes, allergy testing, and in more recent years, FENO testing to diagnose children between the ages of three and four (33). There is no cure for asthma, however as with any life-threatening disease, early diagnosis is linked to treatment and ultimately relief from physically taxing symptoms.

1.1.6 Current treatments

Asthma is typically treated using inhaled corticosteroids (suppresses airway inflammation) in combination with a β2-adrenergic agonist (opens bronchial smooth muscle) (20). Oral corticosteroids may be prescribed in cases of severe asthma (35). However, 5 – 10% of patients do not respond to corticosteroid treatment and consequently, other treatments (namely, anti-IgE therapy, mediator antagonists) are also being explored (20).

Anti-IgE therapy, which binds to IgE preventing IgE receptor activation and reducing circulating IgE levels, has proven to be effective in the treatment of allergic asthma in subjects 6 years old and above (36). There are also several cytokine-targeted therapies (IL-5, IL-13, IL-4) being explored, however the results seem to be patient-specific and trials are currently only conducted in adults (37).

Though asthma treatments have improved remarkably over the past decade, there are still no preventative treatments for this disease. Consequently, research is compelled to understand the underlying etiology of asthma to elucidate what factors in early life can potentially be manipulated to prevent the development of this disease altogether.

1.2 Asthma etiology

1.2.1 A multifactorial origin

1.2.1.1 Role of genetic factors in asthma development

The underlying cause of asthma is a complex product of genetic and environmental factors resulting in significant heterogeneity of the disease. Parental history of asthma increases

the likelihood of developing this disease, however assessment of this factor alone is not enough to confirm a person's risk of asthma development (38-40). There is also evidence of a strong link between sex and increased risk of asthma development in children, as boys are more likely to develop childhood asthma than girls (41, 42). Further, genome-wide-association-studies have identified candidate genes that play a role in asthma susceptibility (*ORMDL3* and *SMAD3*) (43). Thus it is clear that genetic components play a role in asthma pathogenesis, however the rapid rise in asthma prevalence suggests changing environmental factors that are potentially playing a bigger role in skewing the developing human immune system toward these hypersensitivity diseases (18).

1.2.1.2 'The post-industrial epidemic'

In addition to the within-generation rise in the prevalence of asthma, there is also an inverse relationship between the incidence of infectious diseases and hypersensitivity diseases (44). Further, the geographical disparity of asthma and atopic diseases is shifting, as developing countries become industrialized and their living conditions become more like the Western world (45). Thus it appears that there may be a link between the development of hypersensitivity diseases and the urbanization or modernization of society (46). Many urban environments have characteristics (lower air quality, higher population density, lower economic status) that predispose populations to asthma; and similar to the geographical disparity of this disease, rural areas with comparable environments do report greater incidences of hypersensitivity diseases (47, 48). There is also the possibility that urbanization does not support proper immune development through a decrease in exposure to environmental microbes as humans shift from an outdoor lifestyle to a more indoor lifestyle (characteristic of urban societies) (49).

This concept of decreased microbial exposure in modern or more urban societies has become a booming research area in the etiology of immune dysregulation. One particular arm of asthma etiology in particular, focuses on factors associated with improved health and a reduction in infectious disease prevalence; for example, improved hygiene, increased antibiotic exposure, and household size (46, 50-55). In particular, David Strachan extensively studied the relationship between household size and atopic disease in the late 1980s, and his initial findings led him to propose the hygiene hypothesis of allergic disease in 1989. This hypothesis sets the stage for the current analyses assessing the role of early life environmental factors in the development of asthma and other hypersensitivity disorders.

1.2.2 The hygiene hypothesis – early life environmental origin

David Strachan proposed the hygiene hypothesis of allergic disease after observing that hay fever was less common among children with older siblings (56). In an assessment of family history, medical records, and allergy skin prick test results in a cohort of 11,765 children, Strachan *et al*. supported this theory as his results indicated that household size was inversely correlated with the development of hay fever (57). He reasoned that children growing up in larger families may experience increased exposure to microbes in early childhood due to inevitable unhygienic contact with older siblings or prenatal exposure from the mother infected by similar unhygienic contact (56). This increased microbial exposure in early life could protect children from developing immune hypersensitivities later in life through appropriate priming of the immune system (56).

1.2.2.1 Immunological support – how 'hygiene' can influence immune development

In 1986, just prior to Strachan's proposal of the hygiene hypothesis, Mosmann and colleagues described the Th-1 and Th-2 cell subtypes, providing an immunological basis for this otherwise observational theory (58). They discovered that fully differentiated murine $CD4^+$ T cells secreted two separate cytokine profiles (Th-1: IFN-γ, IL-2; Th2: IL-4, IL-5) and this produced two different inflammatory responses (58). Th-2 cells play a primary role in the allergen sensitization process (9). Infection with viruses and intracellular bacteria generally stimulates Th-1 immune responses, which suppress Th-2 cytokine activity through the induction of IFN-γ (59, 60). Consequently, the concept of a Th-1 versus Th-2 balance arose whereby a Th-1 dominated immune phenotype (brought on by early life microbial exposures) was thought to inhibit atopic immunopathology (61). Research related to helminth parasites stimulated the need for further explanation beyond this binary view, as these organisms paradoxically induce Th-2 responses while suppressing allergic reactivity (61). T cell plasticity and additional T cell phenotypes (e.g. Th-17, Th-9, and T-reg cells) have more recently been implicated in the control of hypersensitivity disorders (29, 62). Further, many innate cytokines (eg IL-25, IL-33, TSLP) and cell types (eg eosinophils, basophils, mast cells, epithelial cells) also play significant roles in hypersensitivity disease (1). Thus it is clear that specific microbial exposures are involved in the early life priming of the immune system (which is the basis of Strachan's hygiene hypothesis), however since the Th-1 versus Th-2 discovery by Mosmann *et al.*, the immunological foundation of the hygiene hypothesis has been modified to consider the balance between many adaptive and innate immune cell populations. Extending the hygiene hypothesis to account for the role of various parasites (e.g. intestinal helminths) and microbiota compositional shifts provides insight into how early life environmental exposures shape the human immune system (63, 64).

1.2.3 The microflora and old friends hypotheses

The microflora hypothesis is a modern extension of the hygiene hypothesis in that it accounts not only for the effects of external microbes on immune system development but also for the microbes already residing symbiotically within the body (the microbiota). It suggests that early life perturbations (driven by factors such as antibiotic use, infection, or diet) to the bacteria residing in the human intestine (the intestinal microbiota) disrupt the normal microbiota-mediated mechanisms promoting immunological tolerance and consequently bias the immune system toward a state which promotes hypersensitivity disorders (**Figure 1.1**) (64). In support of the microflora hypothesis, a recent study found that uncontacted Amerindians (indigenous peoples of the Americas) exhibited higher levels of bacterial and functional diversity in their skin and fecal microbiota than any other human population previously reported, suggesting that modern societal practices (perturbations) have strong implications on the development of the microbiota (65).

The old friends hypothesis supports the co-evolution of microorganisms and macroorganisms, such as parasitic helminths, with the development of the human immune system and, similar to the hygiene and microflora hypotheses, suggests that the presence of these organisms is required for proper immune development (63, 66, 67). This co-evolution could have occurred in support of human species preservation, with various microbes and parasites selected throughout the human lifespan (68). It is beyond the scope of this thesis to discuss the ever-growing research field of helminths and allergic diseases. However notably, it has been shown in mice that parasitic helminths (namely, *Heligmosomoides polygyrus bakeri*) work together with the intestinal microbiota to modulate allergic inflammation (69). Thus though this thesis focuses specifically on the role of the intestinal bacterial microbiota in asthma and atopic disease, there

are many other micro- and macroorganisms that are likely involved in promoting and protecting against these diseases. Current research in support of these hypotheses focuses on the mechanisms by which the microbiota and parasitic helminths influence immune system development and homeostasis, and potentially confer protection against immune dysregulation.

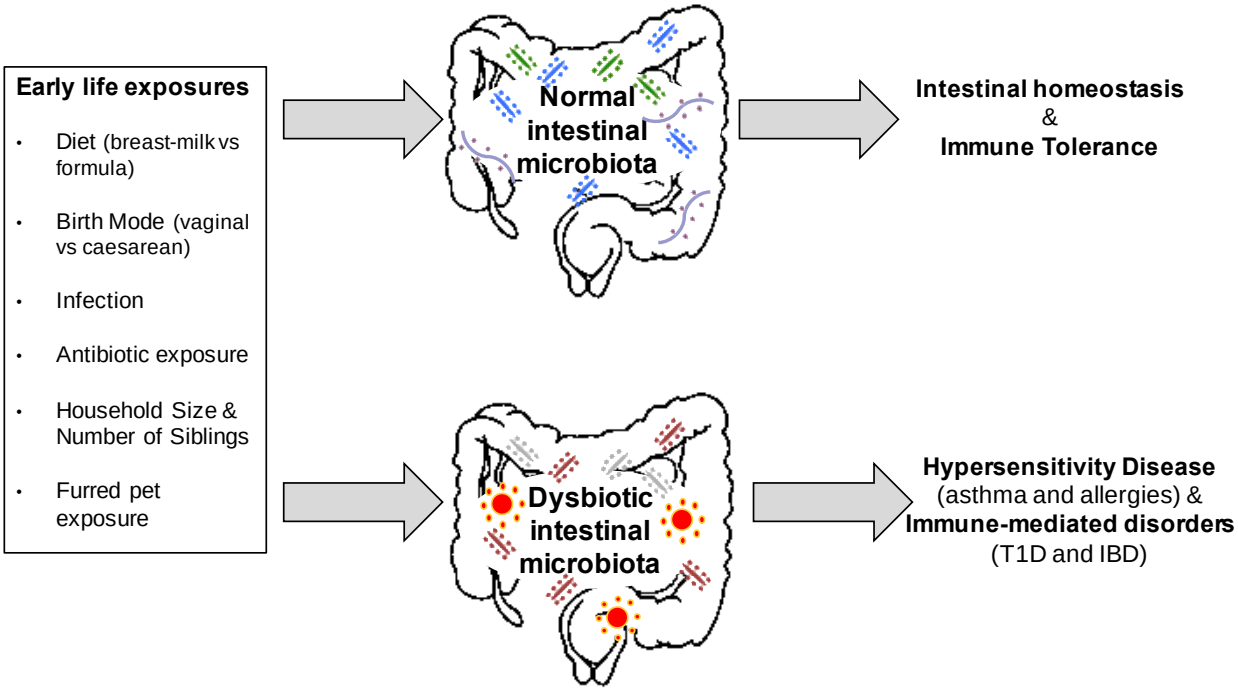

Figure 1.1 The microflora hypothesis

A depiction of the early life environmental exposures differentially associated with promoting a healthy intestinal microbiota, which results in intestinal homeostasis and immune tolerance, and a dysbiotic (unhealthy) intestinal microbiota, which may induce immune dysregulation (printed with permission from Dove Medical Press Ltd, L.T. Stiemsma, *Immunotargets Ther* 2015).

1.3 The gastrointestinal microbiota

1.3.1 Mutualism in the gut

The human microbiota consists of 10 – 100 trillion microbial cells (70). Often the bacteria within the microbiota are described as commensal, but it is clear after much research that these microbes are profoundly integrated with human physiology (71-74). The most populated zone of the human body is by far, the gastrointestinal tract (75). Though there is large interindividual variability in the specific microbial species present, the gastrointestinal tract harbors a diverse microbial community of 500-1000 different bacterial species, among other microbes such as archaea, eukarya, and viruses (75). The juxtaposition of the intestinal microbial genome consisting of 3.3 million non-redundant genes, next to the 22,000 genes within the human genome, is remarkable—further strengthening the concept that these microbes likely play key roles in human health (76).

1.3.2 Techniques to study the microbiota

There are a number of culture dependent and independent techniques used to study the intestinal microbiota. However the most commonly used methods, especially for large cohort human studies, are 16S amplicon sequencing and metagenomic sequencing (77).

1.3.2.1 16S rRNA sequencing

16S rRNA is the core of the 30S subunit of bacterial ribosomes (78). It is ideal for studying the microbiota because it contains both conserved and hypervariable regions (V1 – V9) (78). Amplification of one of these hypervariable regions allows for differentiation between

bacterial taxa, however microbial composition results can vary depending on the region amplified (79). Consequently, among other options (such as the ribosomal 23S subunit or the 16S-23S internally transcribed spacer region) a combination of 16S hypervariable regions is also often used to decrease the likelihood of single hypervariable region bias (80).

Typically, the workflow for 16S community analysis involves DNA or RNA extraction, amplification of a hypervariable region using barcoded primers, standardization, library construction, sequencing, and bioinformatic analysis (81). There are a number of 16S sequencing platforms currently in use (e.g. Illumina Miseq vs. Hiseq, Life Technologies Ion Torrent) (81). Differences between the platforms are centered around the read length and depth of sequencing, i.e. Illumina Hiseq produces 2 base paired-end reads at about 150 base-pairs (bp) in length, while Miseq produces 2 base paired-end reads which are about 300bp in length (80). Longer reads allow for coverage of more than one hypervariable region, enhancing the taxonomic resolution of the sequences (81). However, the depth of sequencing reduces the systematic error rate and the need for significant noise removal during the bioinformatic phase and allows for greater detection of less abundant bacterial taxa (77).

The bioinformatic analysis pipeline used to process and analyze the sequence reads can vary greatly among research groups. However the general process is as follows: sequence reads are typically denoised and quality filtered by size, and clustered into operational taxonomic units (OTUs). These OTUs are then aligned to a reference taxonomy database (e.g. Greengenes or SILVA) after which unclassified or non-bacterial (mitochondrial) reads are removed (82, 83). Classified sequences are then normalized (i.e. by relative abundance) and analyzed using various computational tools. It is very important to consider all of the potential biases associated with sequencing platforms and filtering and clustering techniques when analyzing microbiome data.

16S sequencing technology is changing rather rapidly to account for these biases, however it still does not provide a functional perspective of the microbiota, which ultimately limits the research questions one can ask (84).

1.3.2.2 Metagenomics

16S amplicon sequencing is ideal for analyzing the taxanomic composition of the microbiota, however this does not necessarily translate to functional analyses, making inferring mechanisms to explain associations with specific disease states very difficult in human studies (81). There are tools currently in use to predict the functional metagenome based on 16S amplicon sequencing data (e.g. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PICRUSt) and the compositional data from 16S analysis can be paired with clinical metadata and metabolomic analyses to make informed inferences regarding these mechanisms (85). However whole genome shotgun metagenomics uses sheared genomic DNA to sequence all the available genomes in a sample at a high sequence depth (77). This produces shorter reads that are assembled into longer contigs, which are then compared to databases of known genes (NIH Genbank) and biochemical pathways (Kyoto Encyclopedia of Genes and Genomes (KEGG) (77). This provides researchers with compositional and functional data, but this sequencing sophistication is quite expensive and consequently 16S amplicon sequencing remains the method of choice for many laboratories (81).

1.3.3 Colonization and development of the infant intestinal microbiota

As humans age, so do their microbiota. In fact, the intestinal microbiota does not typically reach full maturity in its composition and functional potential until a child is three years old (86-88). As the intestinal microbiota shifts toward that of adult, there are significant shifts in the abundance of specific bacterial taxa along with a large increase in overall microbial diversity (86). Perhaps unsurprisingly, the 'immature' intestinal microbiota of infancy and early childhood is subject to many perturbations, which may have significant implications with regard to the development of infant immune system (89). Intrauterine development of the gut microbiota and the roles of birth mode, breast-feeding, and antibiotics during infancy are discussed in the following sections.

1.3.3.1 Intrauterine involvement

The major contributor to infant gut colonization is mode of birth (90). However there is evidence of microbes in the placenta, amniotic fluid, umbilical cord, and fetal membranes and thus it is possible that infant gut colonization is initiated during the intrauterine period (91-95). A randomized controlled trial showed that maternal consumption of probiotics during pregnancy resulted in altered expression of TLR genes in the placenta and meconium of neonates (96). Further, oral inoculation of pregnant mice with genetically labeled *Enterococcus faecium* was later isolated from the meconium of the pups after birth by cesarean section (C-section) (97).

Gestational age is also an indicator of prenatal microbiota changes, as one study confirmed an increase in the relative abundance of *Lactobacillus spp*. as a function of gestational age (98). Additionally, one study showed an increase in overall bacterial diversity and in the

proportion of Proteobacteria and Actinobacteria from the first to the third trimester (99). Transfer of these third trimester microbiota to germ free mice induced greater adiposy and inflammation than the first trimester microbiota, suggesting a role of gestational age in metabolic syndrome that is driven by gut microbial changes *in utero* (99).

Additionally, many maternal exposures have been associated with altered infant health (such as maternal diet and antibiotic exposure). For example, maternal malnutrition can result in pre-term birth and maternal obesity has been associated with increased risk of anxiety, depression, and other neuropsychiatric disorders (100, 101). Further, prenatal exposure to maternal antibiotics has been associated with increased risk of asthma in infants (102). Exactly how microbes are transferred *in utero* is currently unknown and the risk of contamination bias is high due to the low biomass associated with intrauterine environments, but there is substantial evidence showing that prenatal exposures (such as maternal antibiotic exposure and diet) have profound effects on infant health and that these effects could be mediated by alterations to the intrauterine microbiota (89).

1.3.3.2 Colonization at birth

The earliest exposure of an infant to post-gestational microbial species occurs through the birth canal, during which a wide variety of microbes that populate the mother (fecal and vaginal microbiota) are transferred to the infant (90, 103). Facultative anaerobes such as *Escherichia coli* and other *Enterobacteriaceae* are among the first to colonize the infant gut post-birth (97). In a few days, these bacteria will have depleted the initial oxygen supplies in the infant gut, paving the way for obligate anaerobes such as *Bifidobacterium, Clostridium,* and *Rumminococcus* (104, 105). As the infant grows, the bacterial populations that colonize various body sites (oral cavity,

gut, skin, vagina) establish niches, making these sites more distinct in the bacterial populations that reside in them (90). For example, *Staphylococcaceae* and *Propionibacteracea* typically populate the skin of adults while *Veillonellaceae* and *Prevotellaceae* dominate the oral cavity, and *Ruminococcaceae* and *Bacteroidaceae* reside in the gut (106).

Approximately 26% of infants born in Canada are born by C-section and the early colonization patterns of cesarean born infants differ greatly from children born vaginally (90, 105, 107). Infants born vaginally exhibit a gut microbiota similar to the mother's vaginal microbiota, while infants born by cesarean show a gut microbiota composition comparable to the mother's skin microbiota (90). In addition to the differences observed in specific taxa abundances (cesarean born infants harbor less *Bifidobacterium* and *Bacteroides*), infants born by cesarean also display lower overall microbial diversity than vaginally born infants (108-110). Further, cesarean births have been correlated with increased risk of asthma and other immune-mediated disorders—supporting the possibility that alterations in natural microbial colonization at birth could result in a hyper-sensitive immune system (55, 111, 112).

1.3.3.3 Maturation of the gut microbiota through breast-feeding

Human milk harbors its own microbiota along with prebiotic substances (human milk oligosaccharides, HMOs) that promote the proliferation of specific gut bacterial taxa (113). If an infant consumes approximately 800 mL of human breast milk a day, he/she is likely ingesting between 1×10^5 and 1×10^7 commensal bacteria, likely conferred from the breast milk itself in addition to contact with the mother's skin microbiota during suckling (114). The transfer of microbes (e.g. *Bifidobacterium, Lactobacillus*) to the neonatal gut has been well established,

indicating that breast-feeding does play a role in neonatal gut colonization (115-118). Further, comparisons between breast-fed and formula fed infants show that breast-fed infants tend to contain a more uniform population of gut microbes (110, 119). For example, *Bifidobacteria* and *Lactobacillus* tend to dominate the guts of breast-fed infants whereas formula-fed infants exhibit higher proportions of *Bacteroides*, *Clostridium*, *Streptococcus*, *Enterobacteria*, and *Veillonell*a *spp.* (119-122). Consequently, a number of studies are enriching infant formula with substances shown to alter the composition of the intestinal microbiota (such as polyamines and dietary nucleotides) in the hopes of shifting formula-fed gut microbial colonization towards that of breast-fed infants (123, 124).

Another area or research regarding formula enrichment is in HMOs and their involvement with the infant gut microbiota. HMOs are considered a prebiotic as they promote the growth and proliferation of beneficial microbiota and consequently, prevent pathogen colonization of the infant gut and exert positive health effects (125). Certain gut-associated bacterial populations such as *Bifidobacterium spp*. possess gene clusters dedicated to the metabolism of these substrates (126, 127). This gut bacterial metabolism of HMOs results in the production of lactate and short chain fatty acids (SCFAs), which are important energy resources for human cells and have been shown to modulate immune cell proliferation and differentiation (128). Though *Bifidobacteria* tend to dominate the guts of breast-fed infants, HMOs are consumed by other bacterial taxa (Bacteroidaceae and Lachnospiraceae) and consequently play a substantial role in the colonization of the infant gut by various microbial species (129, 130). Continued research regarding the role of infant feeding methods in the development of the gut microbiota will shed light on the immune and metabolic mechanisms mediated by the ingestion of human milk, which ultimately promote infant immune health.

1.3.3.4 Early life antibiotics perturb the infant gut microbiota

Prophylactic broad-spectrum antibiotics are commonly prescribed to newborns and infants living in the developed world who are at high risk of infection (131). There is recent evidence that humans are primed for antibiotic resistance due to microbiota that harbor antibiotic resistant genes (collectively known as the 'resistome') (65). In addition to conferring antibiotic resistance, this prophylactic usage of antibiotics in infancy can significantly disrupt the overall ecology of the human microbiota, alter the abundances of resident bacteria, and promote immune dysregulation (132-136).

Though the gut microbiota is rather resilient to disruptive factors like antibiotics, the ecology of this dense microbial population can be severely altered if exposed to antibiotics too early in its development and/or for long periods of time (137, 138). This ecological disruption combined with the decreased microbial diversity (resulting in a microbiota dominated by fewer different bacterial species) of the infant gut can provide opportunities for enteric invaders (139-141). One study showed that the relative risk of children contracting an infection with antibiotic resistant *Escherichia coli* or *Klebsiella pneumoniae* over an infection with an antibiotic susceptible strain of either bacteria was 2.2 times higher if they were exposed to antibiotics 30 days prior to infection (142). Additionally, a study including 53 infants between the ages of 0 and 13 months associated the onset of *Clostridium difficile* infections in infancy with alterations in the infant gut microbiota (141).

Antibiotic usage in early life can also significantly impact the growth of otherwise dominant bacterial phyla in the human gut (138). Infants exposed to ampicillin and gentamycin shortly after birth tended to harbor higher proportions of *Proteobacteria*, *Actinobacteria*, and *Lactobacillus* than unexposed controls for up to 4-weeks after concluding treatment (138).

Further, antibiotic exposure from 6 to 12 months of life was shown to delay maturation of the intestinal microbiota through depletion of specific bacterial taxa (110). These compositional changes were not linked to specific health outcomes, but there are many studies that do link early life antibiotic perturbations to the intestinal microbiota with the development of disease (133, 141, 143-147).

Ultimately of course, the infant intestinal microbiota is shaped by a combination of all these early life exposures (**Fig 1.1**). Much more research is needed to understand the mechanisms facilitating cross-talk between the early life gut microbiota and surrounding immune cells and how alterations to this cross-talk can result in immune-mediated and hypersensitivity diseases.

1.4 The intestinal microbiota in immune development and disease

1.4.1 Impact on immunity

The most striking illustration of the importance of the intestinal microbiota for mammalian immune development comes from studies conducted in germ-free (GF) mice, in which the lack of a microbiota results in reduced Peyer's patches, smaller germinal centers and fewer plasma cells, and increased susceptibility to pathogen invasion when compared to conventionally raised mice (71-74). Specific bacterial species within the microbiota have been shown to induce expression of antimicrobial peptides (e.g. *Bacteroides thetaiotaomicron* induction of regenerating islet-derived 3γ expression by Paneth cells) and mucin production, which ultimately confers protection against pathogen invasion, and combined with regular stimulation of pattern recognition receptors (PRRs), contributes to intestinal homeostasis (148-150). The presence of the microbiota can stimulate $CD4^+$ T cell proliferation, Th-17 cell

differentiation through the induction of IL-1β, and accumulation of colonic T-regs (151-153). Further, gut microbiota also metabolize food components that are indigestible by mammalian enzymes, such as HMOs and dietary fiber (154, 155). This process produces SCFAs, which are essential energy sources for many host tissues and prominent immune modulators (128, 156, 157). These microbiome-immune cell interactions play an elemental role in human immune development; disruption of this interface however can result in the manifestation of immune-mediated and hypersensitivity diseases.

1.4.2 Role of the gut microbiota in immune-mediated diseases

Perturbations to the intestinal microbiota resulting in gut dysbiosis have since been implicated in the development of many immune-mediated disorders, the role of the intestinal microbiota in inflammatory bowel disease (IBD) and type 1 diabetes (TID) is discussed in the following sections.

1.4.2.1 Inflammatory Bowel Disease

IBD is an inflammatory disorder of the gastrointestinal tract encompassing Crohn's disease and ulcerative colitis, both of which are highest in prevalence in North America and Europe (158). The presence of intestinal bacteria appears to be required for the development of experimental colitis, while the composition influences the severity of IBD (159). GF IL-10 deficient mice show no evidence of experimental colitis, while IL-10 deficient mice housed under specific pathogen free (SPF) conditions spontaneously develop the disease (160). Additionally, antibiotics have been shown to attenuate the symptoms of experimental colitis (161-163). Exposure of SPF IL-10 deficient mice to antibiotics displays differential and localized

roles of specific bacteria in mediating experimental colitis (163). For example, treatment of SPF IL-10 deficient mice with vancomycin-imipenem and metronidazole eliminated anaerobic bacteria and reduced colonic injury, while ciprofloxacin and vancomycin-imipenem decreased cecal inflammation and reduced the prevalence of *Escherichia coli* and *Enterococcus faecalis* (163).

Several human studies suggest that early life antibiotic exposure is associated with IBD (164, 165). This discrepancy is likely because antibiotics in murine IBD experiments are typically given as treatment after disease onset, whereas human studies are often retrospective and assess the effects of antibiotic exposure prior to disease onset. In a nested case-control study, children diagnosed with IBD around eight years of age were 2.9 times more likely to have received antibiotics in the first year of life (166). Additionally, antibiotic exposure in the first three months of life was associated with childhood Crohn's disease (165). Similar to the murine studies, antibiotic combination therapy was shown to be effective in treating ulcerative colitis in humans (167).

Diet may also play an important role in IBD through its effects on the intestinal microbiota. Maternal secretory IgA (a component of breast-milk) has been shown to alter the intestinal microbiota composition and the expression of genes associated with intestinal inflammation (168). Additionally, a systematic review negatively correlated breast milk exposure with the development of early-onset IBD in humans, suggesting a protective effect of breast-feeding on IBD development (169).

Together, these results suggest that the development of IBD is driven by early life environmental factors that alter the composition of the intestinal microbiota. Early life diet (breast-feeding) is likely protective against IBD development, while effects of antibiotic

exposure are more complicated. If antibiotics are given in early life, they may result in an intestinal microbiota that promotes IBD development (165, 166). However after disease onset, antibiotics alleviate disease severity by shifting the prevalence of specific microbes that may be promoting the disease (161-163, 165, 167). Regardless, factors related to early life hygiene are involved in IBD development, but there is also evidence that the hygiene and microflora hypotheses are applicable to dysregulatory immune disorders not associated with the gastrointestinal tract, such as T1D.

1.4.2.2 Type 1 Diabetes

Childhood T1D, an autoimmune disorder resulting from T-cell mediated destruction of β-cells in the pancreas, is steadily increasing worldwide (170, 171). Further, developed countries such as Canada and the UK exhibit the highest incidences of the disease (170, 171). Epidemiological evidence supports a link between environmental factors associated with the hygiene hypothesis and the onset of T1D. Having older siblings is negatively correlated with childhood onset T1D, suggesting a protective effect (172). Furred pet exposure seems to also play a role, as one study found in a birth cohort of 3000 children, that children exposed to an indoor dog were less likely to develop T1D than unexposed children (173). Breast-feeding has been associated with protection from T1D and children born by C-section exhibit a higher risk of T1D than children born vaginally (174-176).

Lending support for the microflora hypothesis, a recent study compared the gut microbial compositions of children with T1D and healthy children and concluded that children with T1D showed a significant increase in *Bacteroides*, which was later reduced to that of controls after insulin treatment for two years (177). Oral administration of *Lactobacillus johnsonii* isolated

from BioBreeding diabetes resistant rats was shown to delay the onset of T1D in BioBreeding diabetes prone rats (178). Additionally, MyD88 deficient NOD-mice are protected from disease onset in SPF environments and segmented filamentous bacteria have been reported to protect female NOD mice from disease development (179, 180). Further, a recent study exposed pregnant NOD-mice to antibiotics targeting Gram positive (vancomycin) versus Gram negative (neomycin) microflora (181). The study found that these two antibiotics induced differential immune responses associated with promoting (in the case of vancomycin) or protecting against (in the case of neomycin) T1D in the offspring of the exposed mice, highlighting contrasting roles of bacterial taxa in the development of the neonatal immune system (181).

Additionally, antibiotic therapy has been shown in mice to protect against virus-induced T1D through the alteration of intestinal microbiota composition (182). However in humans the contribution of antibiotics to T1D development is currently unclear, as a population-based human cohort study found no association between T1D and antibiotic exposure in the first eight years of life (183). One human study was able to distinguish T1D infants by changes in their intestinal microbiota, though the role of antibiotics and other early life factors was not assessed (184). Specifically, they found that T1D infants showed reduced alpha diversity characterized by shifts in the abundances of specific bacterial taxa, including increases in pathobiont-like species (*Ruminococcus gnavus* and *Streptococcus infantarius*) and decreases in anti-inflammatory-associated taxa (e.g. Lachnospiraceae and Veillonellaceae) (184). Thus similar to IBD, early life factors common to industrialized countries such as antibiotic exposure, birth mode, and diet are associated with T1D development. However, additional mechanistic research combined with human epidemiological and microbiome analyses are needed to connect these associations with

alterations to the gut microbial composition and subsequent changes in human immune development.

1.4.3 The gut-lung axis

1.4.3.1 Innate immunity microbial crosstalk

Perhaps a less intuitive role of the intestinal microbiota in human health is in gut-mediated lung immunity. The gut-lung axis attempts to mechanistically define how microbes in the gut might influence the immunology in the lung (185). One potential connection is through interactions of the gut microbiota with PRRs of the innate immune system (186). It is well established that pathogen-associated molecular patterns (PAMPs) such as LPS, CpG, and peptidoglycan can stimulate TLR signaling which confers downstream activation of many genes that regulate inflammation and innate immune responses (187). Similar to the antigen-recognition and IgE-mediated hypersensitivity pathways, dendritic cells are also the intermediaries of gut microbiota-immune cell cross-talk, as they regularly sample gut microbes in the intestinal lumen or lymphoid tissues (186). DC sensing of gut microbiota PAMPs promotes immune tolerance in the intestine, but also results in phenotypic changes to DCs and migration to the mesenteric lymph node (MLN) to promote T cell priming (188). In the MLN, T cells also acquire homing molecules (e.g. CCR4, CCR6), which initiate migration to other parts of the body, including the respiratory mucosa (189).

Thus it is possible that interactions with specific gut microbes, via their corresponding PAMPs, could result in varying phenotypic changes in DCs, with downstream effects on lymphocyte priming/homing and ultimately, shifts in anti-inflammatory responses in the airways (189). In a house dust mite (HDM) model of allergic inflammation, chronic intranasal exposure

to endotoxin (bacterial LPS) has been shown to protect mice from HDM-induced asthma (190). The proposed mechanism of this protection is through A20 (ubiquitin modifying enzyme)-mediated inhibition of HDM-induced recruitment of conventional DCs to the lungs and mediastinal lymph nodes (190). Further, prior 2-week treatment of mice with LPS suppressed proliferation and differentiation of adoptively transferred CD4+ HDM-specific 1-DER T cells in the mediastinal lymph nodes into IL-5 and IL-13 –secreting Th-2 cells, highlighting the T-cell priming effects of these DCs (190). Though this is not a gut microbiota mediated pathway, it does highlight the ability of bacterial PAMPs (specifically LPS) to alter DC recruitment to the lungs and protect mice against asthma symptoms.

1.4.3.2 Microbial influences on epigenetics

It is also possible that the intestinal microbiota is linked to lung immunity through microbe-mediated epigenetic modification. Distinct whole blood DNA methylation patterns were associated with two major bacterial phyla, either Firmicutes or Bacteroidetes, and pathway analysis revealed differential methylation (associated with a high or low Firmicute/Bacteroidetes ratio) among genes enriched in functional networks such as, cardiovascular disease, inflammatory responses, obesity, and lipid metabolism (191). Further, production of bacterial methyl groups, cofactors (ie folic acid, B12), and other various substrates (ie choline) and enzymes (ie methyltransferases) can both directly and indirectly affect host DNA methylation and consequently bias cell differentiation toward or against an immune profile that confers tolerance (191). There is also evidence that early life farm microbial exposures may play a role in the methylation of genes related to asthma and allergies (192, 193). Additionally, alluding to the age-sensitive role of the intestinal microbiota, the presence of a conventional gut microbiota in

previously GF neonatal (but not adult) mice decreased hypermethylation of *CXCL16*, which in turn decreased accumulation of invariant natural killer T (iNKT) cells (prominent in the pathogenesis of asthma) in the colon (194).

1.4.3.3 Role of microbial-derived metabolites - SCFAs

Another area of gut-lung axis research involves microbial-derived metabolites, such as SCFAs. SCFAs are direct by-products of bacterial fermentation of carbohydrates and are key energy sources for many host tissues and gut bacterial species (156). There are three major bacterially-produced SCFAs, acetate, propionate, and butyrate which are typically present in the gut in a molar ratio of 60:20:20, respectively (156). These metabolites are known to modify gene expression through inhibition of histone deacetylases (HDACs), cytokine and chemokine production, and cell differentiation, proliferation, and apoptosis (195). With regard to immune tolerance and inflammatory mechanisms, butyrate and propionate induce extrathymic T-reg generation through direct interactions with T cells and indirect interactions through DCs, potentially through the inhibition of HDACs (196). Clostridial species are prominent SCFA producers, and butyrate production by these particular bacteria was associated with the generation of peripheral T-reg cells in the colon (197). In a HDM-model of experimental asthma, both acetate and propionate were capable or reducing cellular infiltration into the airways after HDM exposure (155). Systemic propionate treatment modified bone marrow hematopoiesis and enhanced the generation of DC and macrophage precursors and subsequent recruitment of DCs less effective in promoting Th-2 cell polarization in the lungs (155). In a later study using the same asthma mouse model, maternal intake of acetate was shown to reduce allergic airways disease in the adult offspring of mice (198). Notably, both these studies initially assess the role of

a high fiber diet on the production of SCFA and colonization of intestinal bacteria – highlighting the influence of diet, mediated by gut microbial changes, on the development of the immune system (155, 198). The latter study, however, emphasizes intrauterine effects on the infant immune system mediated by maternal diet, suggesting the need for prenatal prevention strategies using these gut microbial metabolites (198). Ultimately, there is much more to learn regarding the mechanisms of the gut-lung axis, but researchers are working toward a better understanding through improved mouse model and longitudinal human cohort research.

1.5 The intestinal microbiota in atopic disease

1.5.1 Evidence from animal models

Murine model studies mechanistically support a link between the intestinal microbiota and atopic disorders through the experimental manipulation of microbiota compositions. Notably, the absence of a microbiota results in increased allergic responses, including increased lymphocyte and eosinophil inflammation in the airways, increased Th-2 cytokines and IgE production, and increased alveolar macrophages and basophils (199). However many animal studies focus on roles of specific bacterial taxa in atopic disease development.

1.5.1.1 Roles of specific bacterial taxa

In an ovalbumin (OVA)-model of asthma, administration of a common gut pathogen, *E. coli*, to the lung was shown in a TLR4-dependent manner to induce γδ-T cells, decrease activation of lung DCs, and abrogate Th-2 cytokine production to confer protection of mice from allergic airway inflammation (200). Intranasal supplementation of mice, poly-sensitized to birch and grass pollen allergens, with *Bifidobacterium longum* and *Lactobacillus paracasei* at the time

of sensitization resulted in reduced IgE-dependent basophil degranulation in response to allergen challenge (201). Only *Bifidobacterium longum* displayed protective effects when mice were supplemented prior to allergen sensitization (201). Notably however, the bacteria supplemented in these studies were administered intranasally, not orally gavaged, thus though these taxa are common in the gut, these studies highlight the dual roles of the airway and intestinal microbiotas in allergic airway inflammation (200).

Forsythe *et al* show that oral supplementation with live *Lactobacillus reuteri* reduced airway hyperresponsiveness and TNFα, MCP-1, IL-5, and IL-13 level in the bronchoalveolar lavage fluid (BALF), while treatment with *Lactobacillus salivarius* had no effect, indicating that these microbe-driven effects are mediated with specific species of bacteria versus larger bacterial populations (202). Additionally, oral supplementation of mice with *Bifidobacterium longum* protected against airway inflammation, increased Peyer's patches and splenic T-regs, and blocked serum IgE induction in OVA-sensitized animals (203). Conversely, combined oral antibiotic treatment of mice resulted in increased allergic inflammation characterized by increases in serum IgE and circulating basophils (204). This basophil increase was potentially mediated by the microbiota, as conventionally raised mice showed decreased proliferation of bone-marrow resident basophil precursors compared to the antibiotic treated mice (204).

1.5.1.2 Age-sensitive microbial effects

Age is the main driver of compositional and functional differences in the intestinal microbiota (106, 205). Thus it is perhaps unsurprising that many studies assessing the role of the gut microbiota in atopic disease, find the results to be time sensitive. In an OVA-driven model of allergic inflammation, neonatal (but not adult) exposure to a conventional microbiota reduced the

severity of allergic inflammation characterized by decreased accumulation of iNKT cells in the lung, serum IgE, and eosinophils in the BALF (194). Arnold *et al* show in OVA- and HDM-driven mouse models of allergic inflammation that oral infection of neonatal mice with CagA-positive *Helicobacter pylori* prior to OVA- or HDM-challenge resulted in the significant reduction of eosinophils in the BALF, and a decrease in IL-5 and IL-13 cytokine levels when compared to uninfected mice and infected adult mice (143). Notably the role of this bacterium has been supported in humans, as the CagA-positive *H. pylori* strain was inversely associated with asthma in adults and infection with the CagA-negative *H. pylori* strain was shown to be a risk factor for asthma in European school age children (206, 207).

In a study of the airway microbiota, Gollwitzer *et al* show in 2-week old mice, that a shift in the airway microbiota dominant in Gammaproteobacteria and Firmicutes to one more dominant in Bacteroidetes was associated with decreased responsiveness to aeroallergens and the induction of Helios$^-$ T-regs in a manner that required interaction with programmed death ligand 1(208). Though this thesis focuses on the intestinal microbiota, this airway microbiome study emphasizes the early life critical window associated with microbiota alterations and subsequent development of hypersensitivity diseases.

In contrast to the microbiota-mediated studies, Russell *et al* found that perinatal (*in utero* and up to 21 days after birth until weaning) versus strictly prenatal (*in utero*) vancomycin treatment of OVA-challenged mice alters gut microbial composition and exacerbates asthma-related immune responses, which may be driven by increased serum IgE levels and reduced T-reg populations (209). This suggested that the early life critical window for vancomycin treatment in mice was between birth and weaning (209). Interestingly, perinatal treatment with streptomycin did not result in exacerbated disease after OVA-challenge (147). However, in a Th-

1/Th-17-driven model of hypersensitivity pneumonitis, mice treated with streptomycin perinatally showed exaggerated lung inflammation when compared to untreated or vancomycin-treated mice (147, 209). This highlights the ability of altered intestinal microbiota compositions to differentially control disease severity depending on the immunological basis of the disease (147). Altogether, these age-specific mouse studies support the role of an early life 'critical window' during which changes in the microbiota are most influential in protecting or promoting a hyper-sensitive immune system.

1.5.1.3 Prebiotic studies in mice

Prebiotics are chemicals or food components (e.g. inulin, pectin, galacto-oligosaccharides (GOS), fructo-oligosaccarides (FOS)), which are indigestible by pancreatic and intestinal enzymes, but important in the growth and proliferation of the intestinal microbiota (210). Prebiotic substances induce the production of SCFAs by intestinal microbes, which have been shown to promote effector (Th-1 and Th-17) and anti-inflammatory IL-10 producing $FoxP3^+$ and non-$FoxP3^+$ T-cell differentiation (128, 157). Consequently, they continue to be a promising microbe-based therapeutic option to promote immune tolerance. Supplementation of mice with a mixture of short chain (sc)GOS, long-chain (lc)GOS, and pectin-derived acidic oligosaccharides prior to OVA-challenge suppressed airway inflammation and hyperresponsiveness compared to controls (211). Additionally, Trompette *et al* show that a high fiber diet (diet supplemented with 30% pectin) metabolized by the gut microbiota increases the concentrations of circulating SCFAs and decreases allergic inflammation in the lungs of a HDM model of allergic inflammation (155). Studies in mice allow for mechanistic conclusions and inferences regarding the role of pre- and probiotic substances in hypersensitivity diseases. Human studies are

generally correlative, due to ethical and feasibility challenges, however there are informative longitudinal studies that associate global and specific microbial changes with the development of immune-hypersensitivities.

1.5.2 Evidence in humans

1.5.2.1 Pro- and prebiotic studies

Probiotic administration in humans has been shown to protect against allergic rhinitis, peanut allergy, and atopic dermatitis (212-215). However research thus far reveals many gaps in probiotic therapy, likely due to individualized disease phenotypes that may or may not be linked to the specific microbial species' tested (213, 216, 217). Prebiotic oligosaccharide formula supplementation in the first six months of life has been associated with decreased incidences of allergic manifestations until two years of age, supporting the use of these methods in early life in humans (218).

Specific HMOs, scGOS and lcFOS, administered in the first six months of life have been shown to reduce the cumulative incidences of atopic dermatitis, recurrent wheezing, and allergic uticaria (218). Further, a clinical trial using a synbiotic (combined pre- and probiotic) mixture, Immunofortis, found in infants with atopic dermatitis, that supplementation with this mixture for twelve weeks resulted in decreased prevalence of wheezing and asthma medication usage after one year (219). Probiotic studies highlight roles of specific bacteria in conferring protection against asthma and atopic diseases in humans, however longitudinal studies characterizing the microbiota in large human cohorts often initially reveal these potential roles.

1.5.2.2 Longitudinal cohort Studies

Single-strand polymorphism analysis and culture techniques were used to identify microbial exposures among two cohort studies of European children (220). In both cohort studies, researchers found that children growing up on farms in Central Europe encountered a wider range of microbial exposures and had a lower prevalence of asthma and atopy than the reference group (220).

Additional cohort studies use next-generation sequencing techniques to study the human microbiota. One longitudinal study used 16S ribosomal DNA (rDNA) 454 pyrosequencing to analyze the intestinal microbiota compositions of school age asthmatic and non-asthmatic children and found that significant decreases in overall gut microbial diversity at one week and one month of age were correlated with asthma development at school age (221). Another study, using Illumina 16S rDNA sequencing, characterized the gut microbiota of 166 Canadian infants and revealed an increased Enterobacteriaceae/Bacteroidaceae ratio in children sensitized to food allergens at 3-months and 1-year of age compared to non-sensitized children (222). Lower gut microbial richness was observed at 3-months of age only (222). Further, the use of both 16S rDNA and shotgun metagenomic sequencing revealed a strong association between a subspecies of *Faecalibacterium prausnitzii* and atopic dermatitis (223). Atopic dermatitis subjects also showed reduced levels of fecal butyrate and propionate, linking these compositional changes in the intestinal microbiota with functional changes that could affect inflammation related to atopic dermatitis (223). Similar to the age-specific mouse studies, human cohort studies such as these suggest that therapeutic microbial intervention early in life is favorable to prevent later asthma and atopic disease development.

1.5.2.3 Microbiota perturbations associated with asthma and atopic disease in humans

Many human cohort studies lend support for the hygiene and microflora hypotheses by assessing the impact of early life environmental factors known to disturb the intestinal microbiota on atopic disease development later in life. For example, antibiotic usage in the first two years of life has been associated with the development of asthma at 7.5 years of age in a dose dependent manner (52). Additionally, antibiotic usage was reported to precede the manifestation of wheeze in the first two years of life in a questionnaire-based analysis of the KOALA birth cohort in the Netherlands (53). Birth by C-section was associated with lower total microbial diversity, delayed colonization with Bacteroidetes, and decreased Th-1 responses in the first two years of life (108). In line with Strachan's original proposal, one study found that an increased number of older siblings was associated with decreased colonization with *Clostridium difficile* and *Clostridium* cluster 1 and a decreased risk of developing atopic dermatitis (224). Correlative human studies such as these shed light on the environmental factors that may be associated with atopic disease through manipulation of the intestinal microbiota, however research regarding these factors remains controversial and is potentially dependent on confounding effects of variables included in the analyses and the size of the cohorts analyzed.

1.5.3 Major gaps identified

The evidence discussed above highlights the potential role of the intestinal microbiota in the etiology and pathogenesis of atopic diseases. Further, the current research suggests gut microbes and their associated metabolites as therapeutic options for treatment of this disease. Studies have identified a potential early life critical window for microbial therapeutic intervention in mice, however the existence and timing of this opportune window is not yet

characterized in humans (146, 209). Significant improvements over the last decade in the techniques used to analyze the microbiome (i.e. 16S rDNA and shotgun metagenomic sequencing) make it possible to characterize the human microbiome and identify and study bacterial taxa that were previously eclipsed by the small proportion of culturable bacterial groups. If the timing of this early life critical window can be established in humans, it may be possible to use microbe-based therapies as preventative methods for asthma and atopic disease development.

Other gaps in the current research include the lack of accurate and comprehensive assessments of early life clinical variables that may ultimately be affecting the colonization of the infant gut and potentially biasing the infant immune system toward one that confers immune-mediated and hypersensitivity disorders (e.g. antibiotic usage in early life combined with other microbiome-associated factors like mode of birth, breast-feeding, older siblings, etc.). Mechanistic studies in humans are difficult to establish, however epidemiological analyses among longitudinal cohorts provide clinicians, researchers, and the lay population with realistic ways of managing and preventing the development of these diseases. Ultimately, in depth longitudinal human cohort epidemiological and microbiome analyses to complement animal model research aimed at understanding the mechanisms of the gut-lung axis are necessary before any possible preventative methods for childhood asthma can be introduced.

1.6 Study population, hypothesis, and research objectives

This thesis uses stool samples and clinical data (described in Chapter 2 – 4) provided by the Canadian Healthy Infant Longitudinal Development (CHILD) Study to determine the role of the bacterial intestinal microbiota in the development of childhood asthma and atopic disease.

1.6.1 The CHILD Study

The CHILD study is a multi-centre, general population, birth cohort comprised of 3,623 families recruited at four sites across Canada (Vancouver, Edmonton, Manitoba and Toronto); established with the goal of elucidating how various environmental exposures during early life influence the development of asthma and allergic disease (225, 226). Children are enrolled pre-birth and followed until five years of age. During this time, biological samples (e.g. stool, urine, breast milk, blood) and relevant clinical data related to environmental exposures, psychosocial stresses, nutrition, and general health are collected. Ultimately, these data and biological samples are applied in a wide range of research studies related to the development of the immune system in infancy and early childhood. Examples of this research include determining how environmental exposures affect DNA methylation and epigenetics, the role of traffic and air pollution in the development of immune hypersensitivities, and the role of the intestinal microbiota in infant immune development.

1.6.2 Sample CHILD Study populations analyzed

Selection criteria for the subjects analyzed in this thesis are described in the methods sections of the following data Chapters. Briefly, Chapter 2 assesses the role of various early life environmental factors on the development of an early life atopic disease phenotype (1-year atopy + wheeze, AW) and preschool-age asthma. This chapter includes 2,695 subjects from the CHILD cohort to which both a 1- and 3-year clinical phenotype could be assigned. Chapter 3 analyzes the role of the intestinal microbiota among 319 subjects classified with the 1-year clinical phenotype. Of these 319 subjects, 286 had reached 3 years of age and completed the CHILD

Study 3-year clinical assessment. Thus, Chapter 4 analyzes the role of the intestinal microbiota in these children, who were diagnosed with preschool-age asthma and a subset of controls negative for atopy, wheezing, and asthma during the first 3 years of life (n asthma = 39, n control = 37, total = 76; **Figure 1.2**). The sub-cohorts analyzed in Chapters 3 (n = 319) and 4 (n = 76) were determined to be representative of the CHILD cohort in Chapter 2 (n = 2,695) using logistic regression (**Appendix D**). For Chapters 3 and 4, **Figure 1.2** also highlights the subsets used for various analyses (i.e. 13 AW and control subjects for SCFA analysis in Chapter 3). All of these subsets were determined to be representative of the larger cohorts (2,695 – Chapter 2, 319 – Chapter 3, and 76 – Chapter 4) using multivariate logistic regression models (described in **Sections 2.3.4, 3.3.7.1** and **4.3.7.1**, **Appendices A, B, & C**).

1.6.3 Hypothesis and research objectives

The current literature outlined here suggests a role for the intestinal microbiota in asthma and atopic disease pathogenesis. **I hypothesize that specific bacterial populations are involved in protecting or promoting the development of asthma and atopic disease in childhood.**

I tested this hypothesis by completing the following research objectives:

Objective I: Analyze hygiene hypothesis related environmental factors using logistic regression to determine the relationship of these variables with preschool-age asthma and 1-year atopy and wheezing among a large North American cohort of children.

Objective II: Compare the composition and functional potential of the intestinal microbiota among 1-year-old infants positive or negative for atopy and wheezing.

Objective III: Compare the composition of the intestinal microbiota among preschool age children diagnosed with asthma and matched controls negative for atopy, wheezing, and asthma.

1.6.4 Significance

Ultimately, this research will expand our knowledge of the role gut bacteria play in the development of IgE-mediated hypersensitivities in humans. It also highlights the role of early life environmental factors in protecting against or promoting the development of these diseases. Additionally, it sets the stage for more in depth analyses to understand how both positive and negative effects of these environmental factors could be mediated through the intestinal microbiota, while highlighting specific bacteria to be analyzed further mechanistically in animal models. Lastly, the work described here has a profound impact on human health research, as it will inform the development of microbe-based biomarkers and therapeutics to predict and ultimately prevent asthma and other asthma and atopic diseases in children.

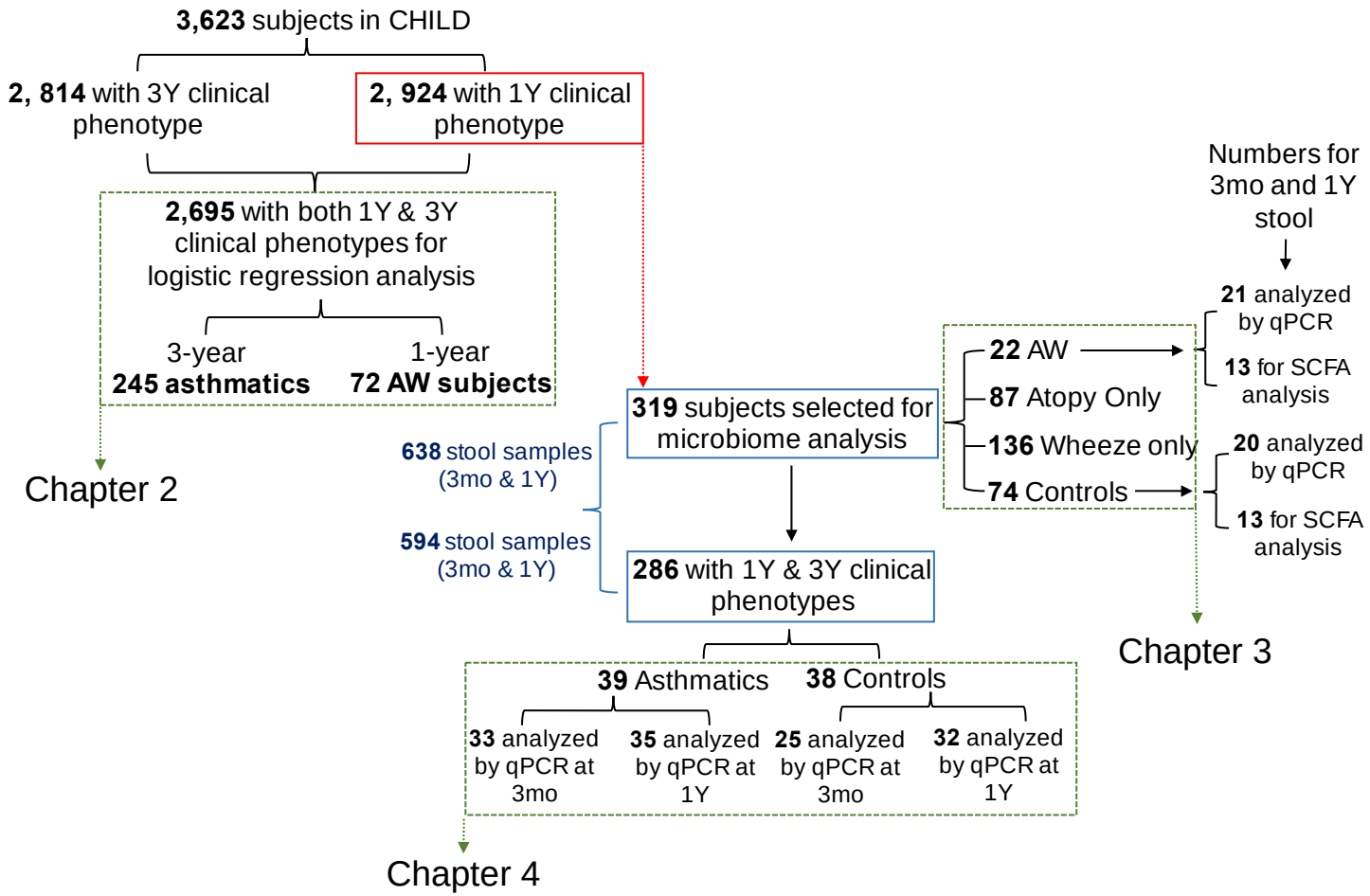

Figure 1.2 Flow chart of CHILD Study subjects analyzed

Chapter 2 includes 2,695 subjects with both a 1- and 3-year clinical phenotype. Chapter 3 includes 319 subjects classified into four clinical phenotypes at 1-year of age (atopy + wheeze (AW), atopy only, wheeze only, and controls). Chapter 4 includes 39 children

diagnosed with preschool-age asthma and 37 controls with no history of atopy, wheezing, or asthma by 3-years of age, which were also analyzed according to the 1-year phenotypes in Chapter 3.

Chapter 2: The relationship between early life environmental factors and the risk of developing childhood asthma and atopic disease

2.1 Synopsis

The etiology of asthma and atopic disease is complex as these IgE-mediated disorders are composite products of genetic and environmental exposures. This epidemiological analysis of early life environmental and genetic factors associated with atopy and wheezing at 1-year and asthma at 3-years among 2,695 subjects enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) Study, highlights roles of hygiene/microflora hypothesis related factors in asthma and atopic disease. Specifically, increased gestational age (in the late third trimester), breast-feeding, and older siblings were determined to be protective against asthma, while pre- and post-natal antibiotic exposure, cesarean birth without labor, and respiratory infections were risk factors for asthma. Further, we identified sex-specific time-sensitive associations between antibiotics and asthma, with males being more likely to develop preschool-age asthma if they are exposed to antibiotics at birth, and females more likely if they were exposed to prenatal antibiotics. Both males and females are more likely to develop asthma if they are exposed to antibiotics from 6-months to 1-year. These findings have strong implications for infant health and in support of the hygiene and microflora hypotheses, suggest the intestinal microbiota as a potential mediator between these early life perturbations and the development of IgE-mediated hypersensitivities.

2.2 Introduction

Asthma and atopy are chronic immune hypersensitivities mediated by a common precursor, IgE (18). These disorders also share many other immune characteristics, such as the release of vasoactive mediators (e.g. histamines) and inflammation due to recruitment of many immune cells (i.e. eosinophils, mast cells, basophils, Th-2 cells) (17). Consequently, there is often an obvious progression of atopic disorders in children, beginning with the development of atopic dermatitis or eczema in infancy and progressing in complexity to asthma by school age—this natural history is generally referred to as the 'atopic march' (12).

The cause of these diseases is difficult to elucidate, as asthma and atopy are composite products of both genetic and environmental factors. There is clear heritability and sex-specific influences increasing the risk of developing IgE-mediated hypersensitivities (39-41). However, there is also a clear geographical disparity and a generational rise in prevalence of these disorders – which cannot be explained by genetics alone (227). The hygiene hypothesis proposes a lack of childhood exposure to various pathogenic and non-pathogenic microorganisms as reason for increased susceptibility to allergic disease (56). This lack of microbial exposure could be aided by common factors, like increased sanitation and antibiotic exposure, associated with growing up in the developed world (53, 228-231).

More recently, research has begun to elucidate the role of the microbiota in human immune development (232, 233). The microflora hypothesis of allergic disease is a modern mechanistic extension of the hygiene hypothesis, as it suggests the intestinal microbiota as the mediator between many early life environmental factors and the subsequent development of asthma and atopic disease (18, 233). Thus, understanding the role of hygiene and microflora hypothesis related variables, along with genetic factors, in the context of the atopic march

(described in **Section 1.1.3.1**) is necessary to understand both the progression of IgE-mediated disease throughout childhood and how these variables can be manipulated to prevent or promote immune dysregulation.

Here we describe an epidemiological analysis of genetic (sex, parental history of asthma, and ethnicity) and environmental (mode of delivery, gestational age, breast-feeding, household size, respiratory infection, pets, and antibiotic exposure) factors in relation to two atopic outcomes, presence of atopy and wheezing at 1 year of age and diagnosis of asthma by 3 years of age, among 2,695 subjects with full data available who were enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) Study. We identify positive and negative associations between hygiene and microflora hypothesis-related factors and the development of asthma and atopic disease. Further, we show that associations with antibiotic exposure are time-sensitive and potentially sex-specific. Collectively, this comprehensive analysis supports the hygiene and microflora hypotheses of allergic disease and suggests the intestinal microbiome as a mediator by which these early life environmental factors protect or promote the development of IgE-mediated hypersensitivities—setting the scene for the detailed gut microbiome analysis presented in the next two chapters of this thesis

2.3 Materials and methods

2.3.1. Study design and population

The CHILD study is a multi-centre, longitudinal, prospective, general population birth cohort including 3,623 pregnant mothers recruited across 4 sites in Canada (Vancouver, Edmonton, Manitoba, Toronto). Of those recruited, 3,299 subjects were enrolled in the 'General'

CHILD Study cohort (**Figure 2.1**). Families are recruited during pregnancy and followed to age 5-years. Detailed characteristics of the CHILD study have been previously described (234, 235). Briefly, the study included pregnant women aged 18 years or older (19 years or older in Vancouver) and infants born after 34 weeks and 4 days gestation with no congenital abnormalities. Questionnaires were administered at recruitment, 36-weeks gestation, at 3, 6, 12, 18, 24, and 30 months, and at 3, 4, and 5-years to collect data related to environmental exposures, psychosocial stresses, nutrition, and overall health. At ages 1, 3, and 5-years, questionnaires validated in the International Study of Asthma and Allergies in Childhood (ISAAC) are completed by the parent or legal guardian and the child was examined by a CHILD Study clinician for evidence of atopic dermatitis, rhinitis or asthma (6). Further, skin prick testing was performed by trained CHILD Study staff using standardized inhalant and common food allergens at 1, 3, and 5-years. 5-year data was not included in this study, as only a third of the subjects enrolled in the CHILD Study have reached age 5 years by mid-2016 when this chapter was prepared.

This analysis includes all subjects from the General CHILD Study cohort that have completed both the 1 and 3-year clinical assessments (2,695 children, **Table A.1**). Of the remaining subjects (n = 717) only 347 could be classified with either phenotype, and those excluded did not appear to systematically differ from the subjects included in the analyses. For comparison, these remaining subjects and their characteristics are included in **Table A.1** (any missing data is imputed with the mode of the data for categorical variables and the mean of the data for continuous variables).

The University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board approved these studies and all participating parents or legal guardians gave signed informed consent.

2.3.2 Clinical phenotypes

2.3.2.1 1-year clinical phenotype

Children were classified at 1-year of age into four clinically relevant phenotypes, atopy + wheeze (n = 72), atopy only (n = 325), wheeze only (n = 402), and controls (n = 1895) based on skin prick testing and wheezing data provided by the CHILD study.

Skin prick test results: Children enrolled in the CHILD study were skin prick tested with 10 allergens (*Alternaria tenuis*, cat hair, dog epithelium, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, German cockroach, peanut, soybean, egg white, and cow's milk) at age 1-year (either on the forearm or back). A child was classified as atopic if after contact with a particular allergen, he/she developed a wheal ≥ 2mm for any of the ten allergens tested. Reaction to histamine was used as a positive control and glycerin as a negative control. Subjects that tested negative to histamine (wheal = 0mm) were not included in this cohort unless they tested positive (with a wheal ≥2mm) for one of the 10 allergens listed above. If the child produced a wheal ≥ 0mm for histamine they were included in this analysis. If a subject tested positive to glycerin, the wheal size for glycerin was subtracted from the wheal size of any positive allergen response. If this resulted in a negative wheal size (e.g. wheal of glycerin = 3mm and wheal of cat hair = 2mm) the subject was not included.

Wheeze questionnaires: Children were assessed for wheezing by their parents and by a CHILD clinician at the 1-year clinical assessment. If the parents recorded wheezing for their

child with or without a cold during the first year of life (recorded via questionnaires at 3, 6, and 12-months), the child was included in the wheezing group. Similarly, children were classified as wheezers if the CHILD clinician heard a wheeze during the 1-year clinical assessment.

For all models, this outcome variable was classified as a categorical covariate defined as AW or non-AW. Reference is non-AW.

2.3.2.2 3-year clinical phenotype

245 children were classified as asthmatic based on either the diagnosis of asthma (142 subjects) or prescription of inhaled corticosteroids or bronchodilators at 3-years of age (103 subjects). The remaining children are classified as controls (n = 2,450).

2.3.3 Definitions of clinical variables

Antibiotic exposure – includes oral and intravenous: continuous covariates (includes number of antibiotic courses for each time point).

Prenatal antibiotics: courses prescribed to the mother between conception and birth of the child. Does not include antibiotic courses prescribed to the mother during labor and delivery (e.g. intrapartum antibiotics).

Maternal antibiotics at birth: antibiotic courses prescribed to the mother during labor and delivery.

Child antibiotics: classified as antibiotic courses prescribed at birth, from birth to 3-months, 3-months to 6-months, and 6-months to 1-year. We also include an additional covariate (birth to 1-year), which is analyzed in **Figures 2.3, 2.4, & 2.6**.

Respiratory infections: includes any respiratory infections reported by parents from birth to 3-months, 3-months to 6-months, and 6-months to 1-year. Covariates are categorically defined with 'yes' for having respiratory infections and 'no' for having no respiratory infections at each time point. Reference is no respiratory infection.

Sex: categorical covariate defined as male versus female. Reference is female.

Gestational Age: continuous covariate, recorded in weeks. Note that gestational age less than 34 weeks and 4 days weeks was an exclusion criterion, thus all CHILD study subjects were born after 34 weeks and 4 days completed gestation.

Parental history of asthma: categorical covariates defined as positive asthma history for at least one parent and negative asthma history for both parents. Reference is negative history for both parents.

Parental ethnicity: grouped as Caucasian or non-Caucasian, categorical covariates defined as at least one Caucasian parent and no Caucasian parents. Reference is no Caucasian parents. Note

that due to the diversity of the CHILD cohort with 10 non-Caucasian ethnicities reported, further classification was not feasible.

Older siblings: categorical covariate defined as at least one older sibling versus no older siblings. Reference is no older siblings.

Dog and cat exposure: categorical covariates defined as 'yes' for having a dog or a cat any time between birth and 1-year of age and 'no' for not having a dog or cat at any time from birth to 1-year of age. Reference is no dog or cat.

Mode of birth: Categorical covariates defined by vaginal delivery, cesarean birth during labor, and cesarean birth without labor. For this study, labor implies membrane rupture, which has significant implications with regard to the colonization of the infants' microbiota (110, 236). Reference is vaginal delivery.

Breast-feeding duration: continuous covariate recorded in months. Includes any breast-feeding (exclusive or non-exclusive) during the first year of life.

Atopic dermatitis at 3-months and 1-year: categorical covariates defined as positive and negative for atopic dermatitis at 3-months or 1-year of age (as determined by a CHILD or non-CHILD physician). Reference is negative for atopic dermatitis.

2.3.4 Multivariate logistic regression analyses

We used the glm2 package in R to conduct logistic regression analyses to determine the associated likelihood of developing asthma by 3-years of age for each 1-year phenotype (**Figure 2.2**) and to analyze potential associations between early life clinical variables and the 1-year AW and 3-year asthmatic phenotypes (**Figures 2.3 – 2.7).** All variables of *a priori* interest were included in the models for **Figures 2.3 – 2.7**. Thus, all odds ratios (OR) and confidence intervals (CI) for these figures are adjusted (versus crude) values. Missing data were imputed with the mode of the variable for all categorical variables and with the mean of the variable for all continuous variables (i.e. for mode of delivery, the mode of this variable was vaginal birth and thus missing values were substituted with vaginal birth; for the breast-feeding variable, the mean number of months was determined for the entire variable and missing values were substituted for this value) (See **Table A.7** for percentages of missing data). ORs above 1 imply an increased likelihood that a child would be classified with the corresponding phenotype, while ORs below 1 imply a decreased likelihood that a child would be classified with the corresponding phenotype (AW or asthma). The 1-year AW phenotype and 1-year atopic dermatitis covariates were included in the logistic regression model for the 3-year phenotype only. The formal interaction test for prenatal antibiotics and sex was also conducted using this same model with the reference for sex changed to male.

A separate model, including only respiratory infection and antibiotic exposure covariates, was constructed for the multiple time points associated with antibiotic exposure in the first year of life to elucidate more accurately, the time point at which antibiotic exposure has the greatest potential to effect the development of the phenotype. Respiratory infection covariates were included in these models as they were identified as significant confounders for one or more of

the antibiotic covariates (resulted in a change of 15% or more to the model coefficient for one or more antibiotic covariates). For these models, crude and adjusted ORs, CIs, and p-values are reported in **Tables A.5** and **A.6**. Graphs include only adjusted ORs and CIs. All graphs were made in GraphPad Prism version 5c. Statistical significance is defined as $p \leq 0.05$.

Tests for trend were conducted using the Spearman correlation in GraphPad Prism version 5c (**Table A.8**).

2.4 Results

2.4.1 Cohort characteristics and establishment of high-risk phenotypes

There are 3,412 subjects enrolled in the general CHILD Study cohort (**Figure 2.1**). 2,924 completed the 1-year clinical assessment. 2,814 completed the 3-year clinical assessment at which the child was assessed for asthma, among other atopic disorders. Of these subjects with either complete 1-year or 3-year clinical assessment data, 2,695 completed both clinical assessments at 1 and 3-years and were included in this study (**Figure 2.1, Table A.1**). These 2,695 subjects were grouped into four clinically relevant phenotypes at 1 year of age; atopy + wheeze (AW, n = 72), wheeze only (n = 402), atopy only (n = 325), and controls (n = 1,896). At 3 years of age, 245 subjects were classified as asthmatic based on a physician diagnosis or if they were prescribed inhaled asthma medications. Children were also classified as asthmatic if they were prescribed inhaled asthma medications (corticosteroids and/or bronchodilators). The asthmatic group was analyzed in comparison to the remaining non-asthmatic subjects (n = 1,896).

Multivariate logistic regression analysis determined the AW group to be 6.48 times more likely than the controls to be diagnosed with asthma by age 3 years (**Figure 2.2, Table A.2**, CI = 3.78 – 11.13). The wheeze only and atopy only phenotypes were 2.9 (CI = 2.07 – 4.01) and 2.8 (CI = 1.95 – 4.01) times more likely to be diagnosed with asthma by age 3 years, respectively. Further, the AW group was 2.3 times more likely to develop preschool-age asthma than the atopy only and wheeze only groups (CI = 1.28 – 4.19 and 1.27 – 4.02, respectively), identifying the AW group as highest risk phenotype (**Figure 2.2, Table A.2**). Based on this identification of the AW group as the highest risk phenotype for asthma, for all analyses conducted on the 1-year clinical phenotypes, we chose to focus exclusively on the AW group versus all remaining subjects (non-AWs, n = 2,623).

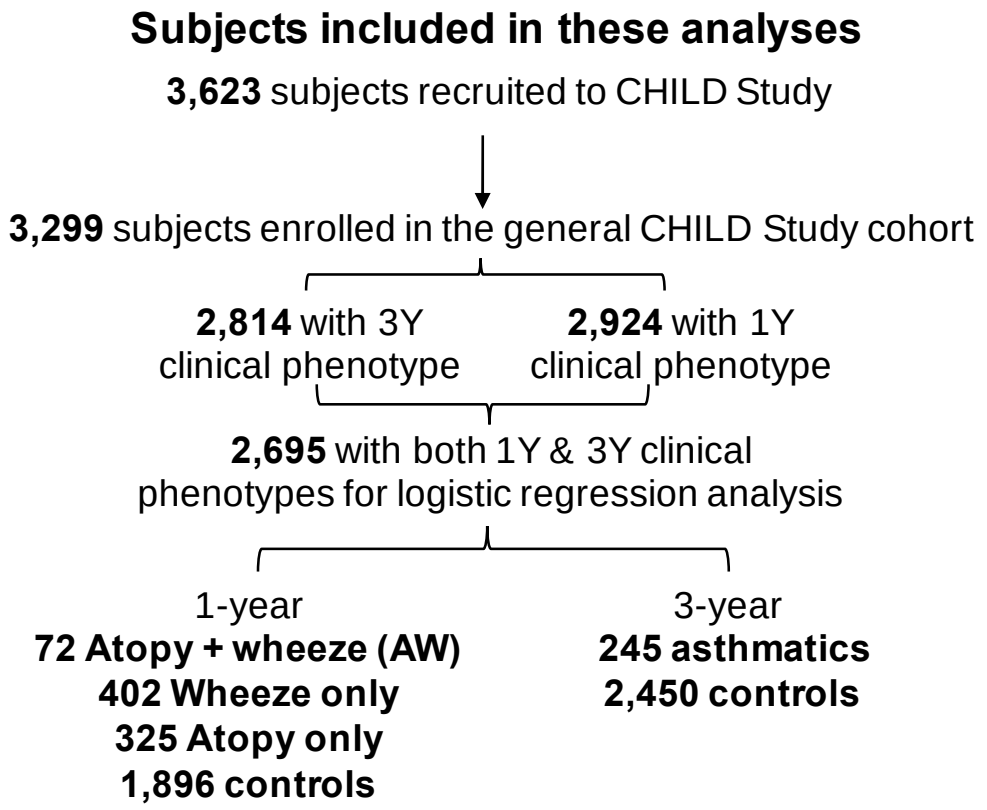

Figure 2.1 Breakdown of subjects analyzed

Of the 3,299 subjects enrolled in the general CHILD Study cohort, 2,695 have complete data for the 1- and 3-year clinical assessments. By 3-years, 245 subjects were diagnosed with asthma or prescribed inhaled asthma medications. The remaining subjects (2,450) were designated as non-asthmatics. At 1-year, 72 subjects were classified into the AW group, 402 in the wheeze only group, 325 in the atopy only group, and the remaining subjects (1,896) were classified as controls (**Table A.1**).

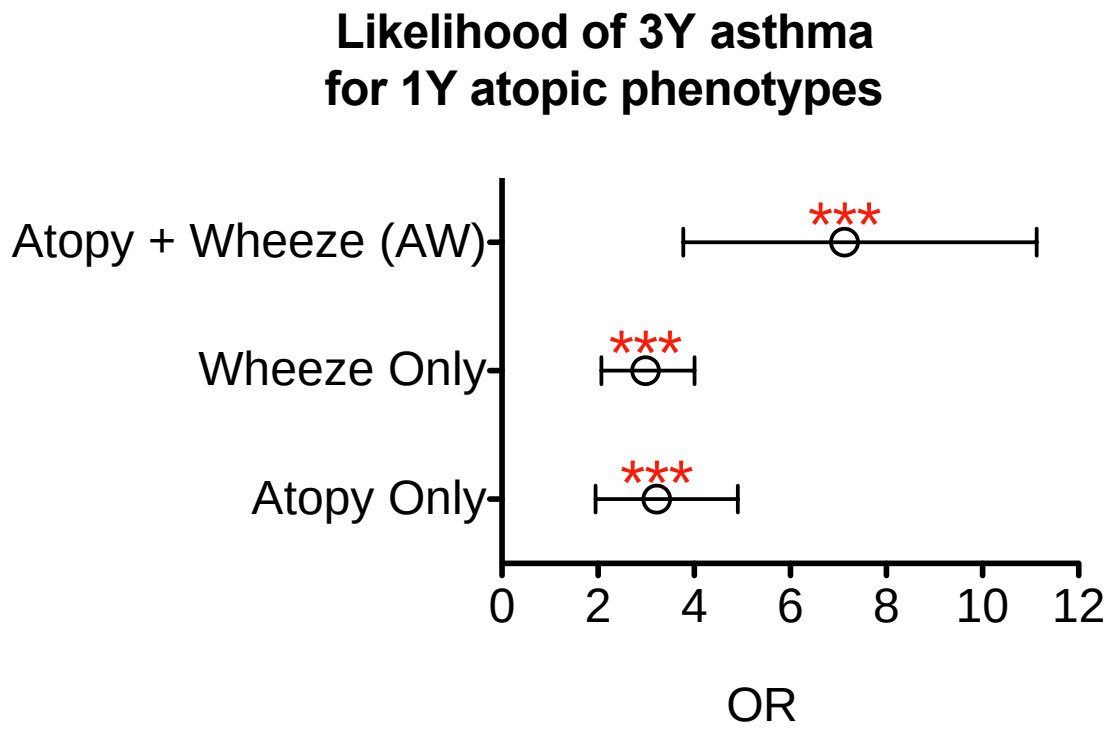

Figure 2.2 Identification of AW group as the 'high-risk' phenotype

Logistic regression identified AW subjects as most likely to develop asthma by 3-years of age (**Table A.2)**. Circles denote ORs and horizontal lines denote CIs. Stars = ***, p ≤0.001.

2.4.2 Factors associated with 1-year atopy and wheezing

The following analyses for both the 1-year AW phenotype and 3-year asthma focused on hygiene/microflora hypothesis related variables within the first year of life (including pre- and post-natal antibiotic exposure, breast-feeding, mode of delivery, older siblings, dog or cat exposure, gestational age, and respiratory infections). Further, additional variables with established associations with asthma and atopic disease (sex, Caucasian vs. non-Caucasian ethnicity, parental history of asthma, and history of atopic dermatitis) were also included. All variables were included in the logistic regression models for **Figures 2.3, 2.4, & 2.6** and **Tables A.3 & A.4**, and thus all ORs and CIs reported for these models are adjusted values.

In line with other epidemiological studies, logistic regression analysis among 1-year-old subjects (n_{AW} = 72, $n_{non\text{-}AW}$= 2,623) identified male sex (OR = 1.78, CI = 1.1 – 3.0), atopic dermatitis in the first three months of life (OR = 3.49,CI = 1.8 – 6.4), parental history of asthma (OR = 1.71, CI = 1.0 – 2.8), and respiratory infections in the first year of life (OR = 3.0, CI = 2.0 – 4.4) as risk factors for atopy and wheezing (**Figure 2.3, Table A.3**) (237). Further, children with at least one Caucasian parent were protected from atopy and wheezing (OR = 0.58, CI = 0.3 – 1.1), though this was not a statistically significant finding (at $p \leq 0.05$; **Figure 2.3, Table A.3**).

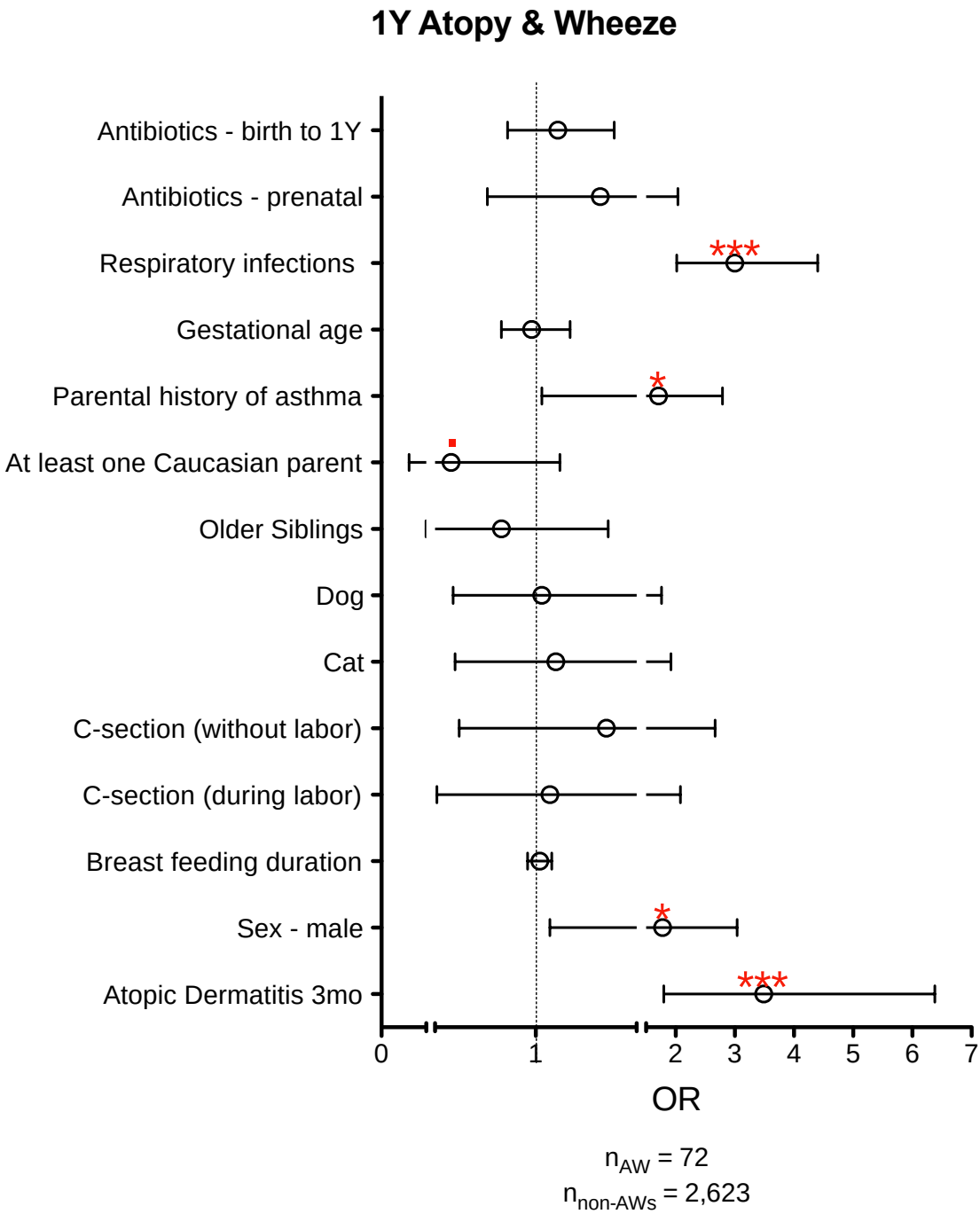

Figure 2.3 Multivariate logistic regression analysis for 1-year AW phenotype

Exact ORs, CIs, and p-values reported in **Table A.3**. Circles denote ORs and horizontal lines denote CIs. The vertical dotted line at 1 highlights CIs that span across 1. CIs that cross 1 are associated with non-significant ORs. Stars = •, $p \leq 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

2.4.3 Hygiene hypothesis related factors are significantly associated with preschool-age asthma development

As a next step, we reviewed the factors associated with the diagnosis of asthma at age 3 years ($n_{asthmatic}$ = 245, $n_{non\text{-}asthmatic}$ = 2,450). Similar to the analysis on the 1-year phenotype, logistic regression identified respiratory infections in the first year of life (OR = 1.46, CI = 1.1 – 1.9), male sex (OR =1.27, CI = 1.0 – 1.7), atopic dermatitis in the first three months of life (OR = 1.96, CI = 1.3 – 3.0), and parental history of asthma (OR = 2.57, CI = 2.0 – 3.4) as risk factors for preschool-age asthma development, though male sex was not statistically significant (at $p \leq 0.05$; **Figure 2.4, Table A.3**). Additionally as expected, atopy and wheezing at 1-year was associated with increased likelihood of an asthma diagnosis (OR = 2.98, CI = 1.7 – 5.2) and Caucasian parental ethnicity was protective against 3-year asthma (OR = 0.60, CI = 0.4 – 0.9; **Figure 2.4, Table A.3**).

Notably, this analysis also revealed roles for additional hygiene and microflora hypothesis related exposures in preschool-age asthma development. Antibiotic exposure in the first year of life was identified as a risk factor for asthma development (OR = 1.16 per additional course of antibiotics, CI = 1.0 – 1.3), along with C-section birth without labor (OR = 1.51, CI = 1.0 – 2.2). Conversely, breast-feeding during the first year of life (OR = 0.97 per additional month of breast-feeding, CI = 0.9 – 1.0), older siblings (OR = 0.75, CI = 0.6 – 1.0), and increasing gestational age (OR = 0.91 per additional week of gestation, CI = 0.8 – 1.0) were identified as protective factors against preschool-age asthma (**Figure 2.4, Table A.3**). All subjects enrolled in the CHILD Study were born after 34 weeks and 4 days gestation. Thus these results suggest that gestational age (even in the late third trimester) has significant implications for the proper immune development of the infant.

In support of these results, we conducted correlation analyses using the Spearman correlation on all continuous variables (**Table A.8**). This analysis identified a weak but significant positive monotonic trend for antibiotic exposure in the first year of life ($r = 0.08$, $p_{adjusted} = 0.0005$) and weak but negative monotonic trends for breast-feeding ($r = -0.05$, $p = 0.02$) and gestational age ($r = -0.05$, $p = 0.05$). Altogether, these results emphasize roles of environmental factors in preschool-age asthma that are predicted to be associated with early life changes to the infant microbiota.

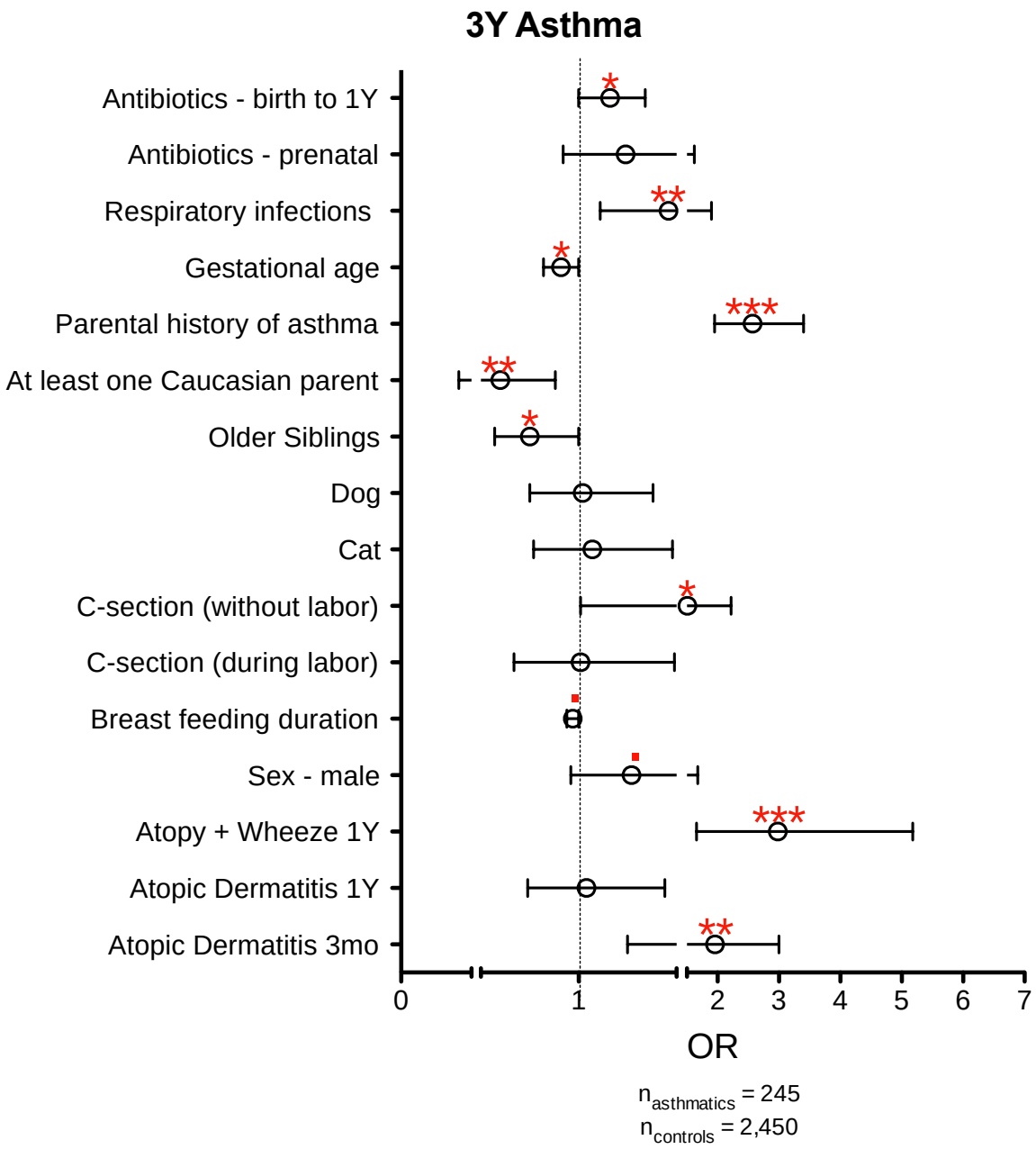

Figure 2.4 Multivariate logistic regression analysis for 3-year asthma

Exact ORs, CIs, and p-values reported in **Table A.3**. Circles denote ORs and horizontal lines denote CIs. The vertical dotted line at 1 highlights CIs that span across 1. CIs that cross 1 are associated with non-significant ORs. Stars = ⋆, $p \leq 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

2.4.4 Infection and exposure to antibiotics at specific time points in early life increases likelihood of preschool-age asthma and atopic disease

In the CHILD study cohort, 22% of subjects (n = 586) received antibiotics at some point throughout the first year of life, with data collected at birth, and at 3- 6- and 12-months of age. Data for prenatal antibiotics and maternal antibiotics at birth was also collected and included in the following analyses. Our analyses suggest that antibiotic exposure in the first year of life is a risk factor for being diagnosed with asthma at age 3 years (**Figure 2.4**). However to determine when during the first year of life antibiotic exposure has the greatest potential to impact the development of childhood asthma and atopic disease, we chose to analyze the association of antibiotic exposure at each of the time intervals described above with the 1- and 3-year phenotypes (**Figure 2.5**). We obtained more precise time-interval data for respiratory infections as well, which we included in these models due to the confounding effects of these two variables on one another (methods, Section 2.3.4). Crude and adjusted values are reported in **Table A.5**, however only adjusted values are reported in **Figure 2.5**.

Multivariate logistic regression analysis on the 1-year AW phenotype identified respiratory infections from 3 months – 6 months (OR = 2.03, CI = 0.9 – 4.1) and 6 months – 1 year (OR= 3.1, CI = 1.9 – 5.1) as risk factors for 1-year atopy and wheezing (**Figure 2.5A, Table A.5**). For preschool-age asthma the results were similar to the model analyzed in the previous section, as respiratory infections (OR = 1.52, CI = 1.1 – 2.1) and antibiotic exposure from 6 months - 1 year (OR = 1.4 per additional course of antibiotics, CI = 1.2 – 1.7) were identified as risk factors for preschool-age asthma (**Figure 2.5B, Table A.5**). However, this analysis also identified antibiotic exposure at birth (antibiotics given to the infant versus

maternal antibiotics at birth; OR= 1.36 per additional course of antibiotics, CI = 1.0 – 1.8) as a risk factor for asthma development (**Figure 2.5B, Table A.5**).

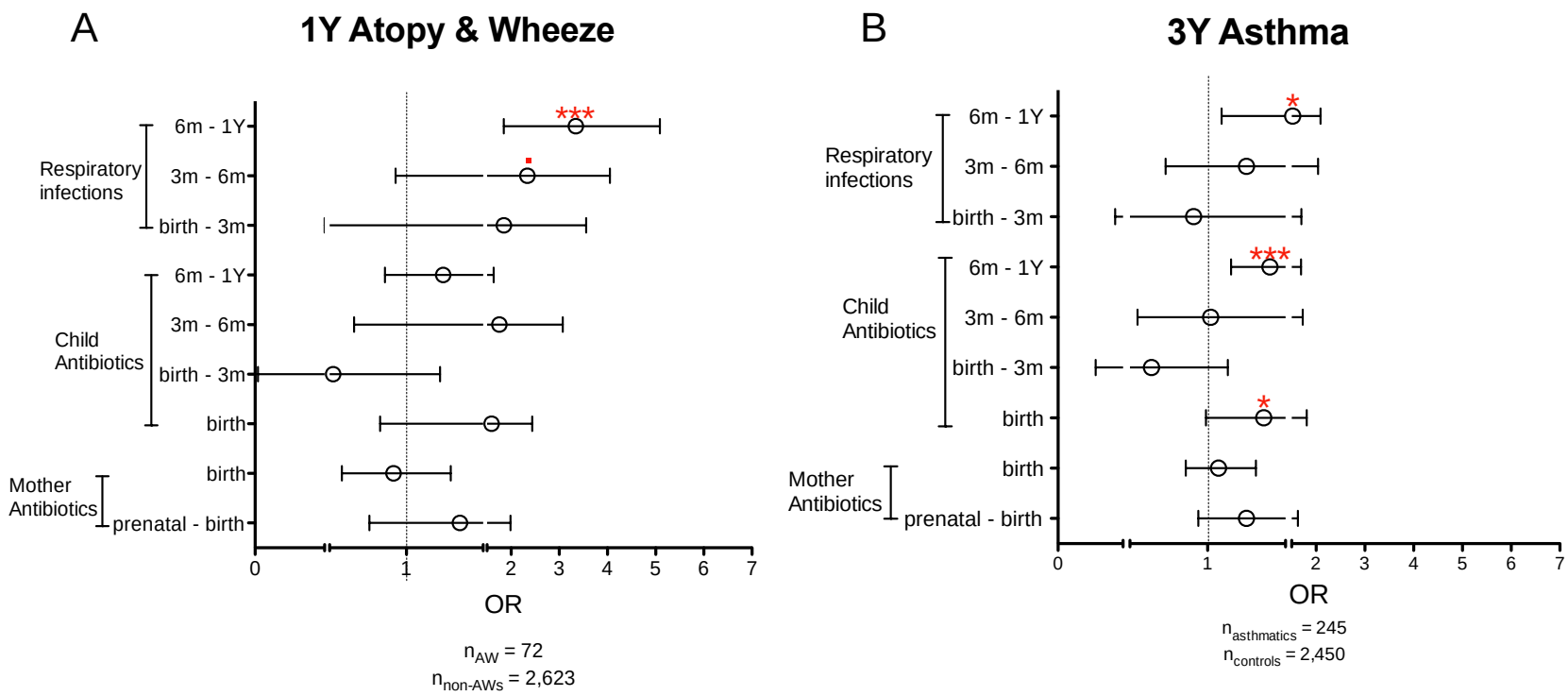

Figure 2.5 Associations of respiratory infections and antibiotics with the A) 1-year and B) 3-year phenotypes

Exact ORs, CIs, and p-values reported in **Table A.5**. Circles denote ORs and horizontal lines denote CIs. The vertical dotted line at 1 highlights CIs that span across 1. CIs that cross 1 are associated with non-significant ORs. Stars = ⋆, $p \leq 0.1$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

2.4.5 Sex-stratified analysis reveals a relationship between specific timing of antibiotic exposure for males versus females and risk of preschool-asthma

The increased likelihood of males to develop 3-year asthma and 1-year atopy and wheezing (**Figures 2.3 & 2.4**) lead us to hypothesize that the roles of other environmental exposures in the first year of life may be sex-specific. We conducted the following logistic regression analyses on the 3-year phenotype only for clinical relevance and because sex-stratification of the 1-year AW population resulted in too few subjects in the AW group.

This sex-stratified analysis revealed shifts in the statistical significance of the genetic and environmental variables analyzed, however the directionality of the majority of the variables (increased versus decreased risk) was similar for both males and females. However interestingly, sex-stratification showed an increased risk of females to develop preschool-age asthma if they were exposed to antibiotics prenatally (OR = 1.79 per additional course of antibiotics, CI = 1.2 – 2.7; **Figure 2.6A, Table A.4**), while males were more likely to develop asthma if they were exposed to antibiotics from birth – 1 year of age (OR = 1.24 per additional course of antibiotics, CI = 1.0 – 1.5; **Figure 2.6B, Table A.4**).

Analysis of antibiotics and respiratory infections separately showed that both males and females receiving antibiotics from 6 months to 1 year of age are at a higher risk of developing preschool-age asthma (OR_{males}= 1.36 per additional course of antibiotics, CI = 1.1 – 1.7 $OR_{females}$ = 1.45 per additional course of antibiotics, CI = 1.0 – 2.1; **Figures 2.7A&B, Table A.4**). However notably, the analysis confirmed that females are at higher risk of preschool-age asthma if they are exposed to antibiotics prenatally (OR_{asthma} = 1.82 per additional course of antibiotics, CI = 1.2 – 2.6; **Figure 2.7A, Table A.4),** while the increased likelihood to develop asthma due to antibiotics given at birth (identified in **Figure 2.5B**) was specific to males (OR = 1.66 per

additional course of antibiotics, CI = 1.2 – 2.3; **Figure 2.7B**, **Table A.4**). Notably, no females received antibiotics at birth, which could potentially skew this finding. However, formal interaction testing between sex and prenatal and birth antibiotics did confirm the female sex-specific association with prenatal antibiotics (OR = 2.07 per additional course of antibiotics, CI = 1.17 – 3.76).

Further, increased likelihood of asthma development associated with respiratory infections from 6-months to 1-year, was also specific to males ($OR_{6mo\text{-}1Y}$ = 1.73, CI = 1.1 – 2.6; **Figure 2.7B**), though this is potentially due to the distribution of respiratory infections over the course of the first year of life for females (females are more likely to develop asthma if they have respiratory infection at any time point in the first year of life, though none of these associations were statistically significant).

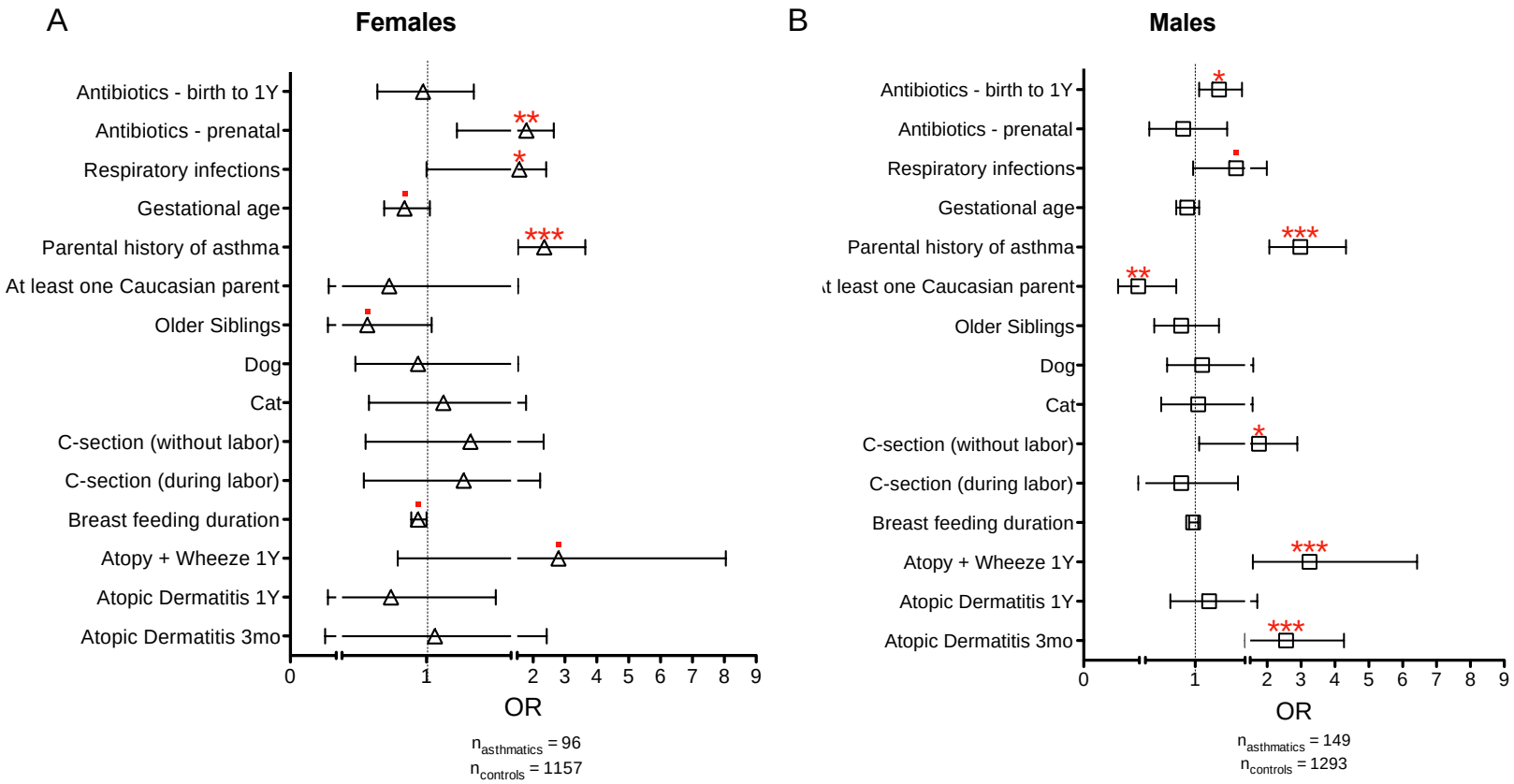

Figure 2.6 Multivariate logistic regression analysis of 3-year asthma stratified by sex

A) Females, **B)** males. Exact ORs, CIs, and p-values reported in **Table A.4**. Triangles (females) and squares (males) denote ORs and horizontal lines denote CIs. The vertical dotted line at 1 highlights CIs that span across 1. CIs that cross 1 are associated with non-significant ORs.

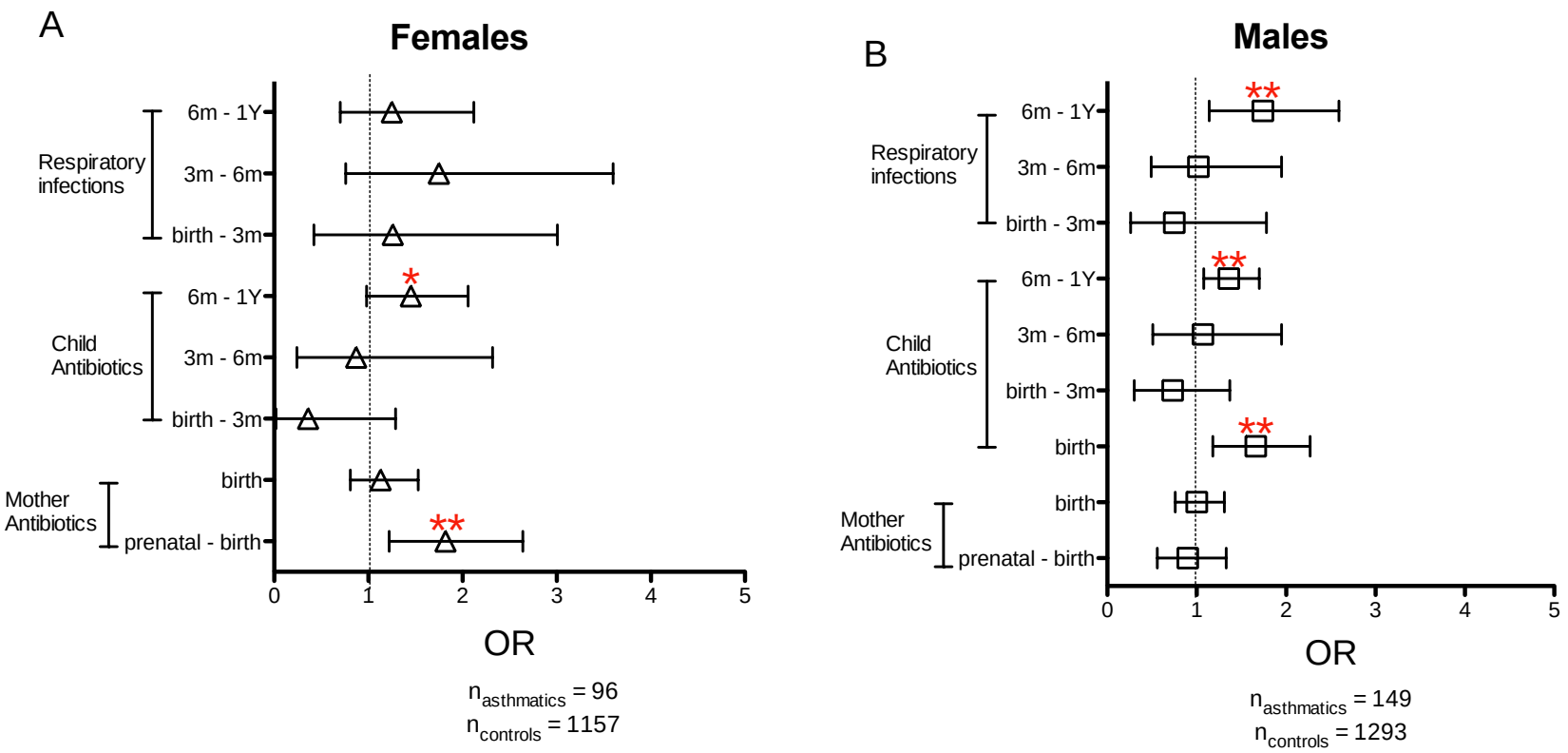

Figure 2.7 Associations of respiratory infections and antibiotics with 3-year asthma stratified by sex

A) Females, **B)** males. Exact ORs, CIs, and p-values reported in **Table A.6**. Triangles (females) and squares (males) denote ORs and horizontal lines denote CIs. The vertical dotted line at 1 highlights CIs that span across 1. CIs that cross 1 are associated with non-significant ORs. There were no 3-year asthmatic females that were prescribed antibiotics at birth – these covariates were removed from these analyses to construct a better logistic regression model.

2.5 Discussion

This study was designed to provide a comprehensive epidemiological analysis of genetic and early life hygiene and microflora hypothesis related factors in the context of the atopic march. The major novel findings from this study are:

a) Identification of hygiene and microflora hypothesis related risk (antibiotics, respiratory infections, and C-section birth without labor and rupture of membranes) and protective factors (older siblings, breast-feeding duration, and increased gestational age) for preschool-age asthma.

b) Increased gestational age, even in the late third trimester, has significant benefits for infant immune health related to asthma and allergies.

c) Identification of sex-specific associations for the timing of antibiotic exposure and development of asthma; with males more likely to develop asthma if they received antibiotics at birth and females more likely if they received antibiotics prenatally. Both males and females were more likely to develop asthma if they received antibiotics from 6 months to 1 year of age.

Respiratory infections, non-Caucasian ethnicity, male sex, and positive parental history of asthma were identified as risk factors for both 1-year atopy and wheezing and preschool-age asthma by 3-years, implicating these particular variables in the progression of the atopic march. With regard to other epidemiological studies, there are discrepancies regarding the roles of early

life factors in increasing or decreasing the likelihood of developing asthma and atopic disease (237). Parental history of asthma is a well-established risk factor for asthma development in children (237). Further, studies have shown that children born <37 weeks gestation are at greater risk of asthma development (237). However, none of the children enrolled in the CHILD study were born before 34 weeks and 4 days gestation, yet we still identified increasing gestational age as a protective factor against preschool-age asthma. Thus, our study suggests that gestational length, even within the late third trimester (34 – 42 weeks), still has significant implications for childhood immune development.

Immune dysregulation is a well-established consequence of preterm birth, however a recent research proposes the microbiota as a potential link, mediating the immune development of the infant *in utero (95, 238)*. Preterm birth has been associated with changes in the placental and vaginal microbiota of pregnant mothers (95, 239, 240). Changes to the placental microbiota or the maternal vaginal microbiota, can result in alterations in intrauterine immune development as well as changes in the colonization of the infant pre- and during birth (90, 91, 110). Though more research is needed to confirm, mechanistically, how late-preterm birth is associated with asthma, these changes in colonization could bias the development of the infant immune system toward a hyper-inflammatory state conferring asthma and allergies.

A number of additional factors associated with the hygiene and microflora hypotheses were identified as protective and risk factors for preschool-age asthma. Breast-feeding was associated with a small decrease in the risk of asthma ($p = 0.06$) and C-section birth without labor was associated with increased risk of developing the disease. Though breast-feeding is often considered beneficial for the immune development of the child, the results regarding its role in asthma and atopic disease are controversial (228, 237, 241, 242). However, though this

finding is not statistically significant (at $p \leq 0.05$), our study does support breast-feeding as a potentially protective factor against asthma development. Notably, C-section birth during labor was not associated with increased risk, which, in line with other epidemiological studies, suggests that membrane rupture is necessary for protection against immune dysregulation (243). Further, both of these variables have known influences on the intestinal microbiota; vaginal birth colonizes the infant through contact with maternal vaginal, fecal, and skin microbiota while breast-feeding promotes microbial colonization through contact with maternal skin and breast-milk microbes and prebiotic substances in human milk (87, 110, 244, 245). More research is needed to determine whether these early life factors drive the development of asthma via alterations to the infant or maternal microbiotas.

In line with Strachan's original hygiene hypothesis, our study also found that having at least one older sibling is a protective factor against preschool-age asthma. Contact with older siblings could mediate the development of immune tolerance through increased microbial exposure, which could increase the diversity of the intestinal microbiota (246, 247). Notably, one recent cross-sectional study showed that the presence of siblings in the household was associated with a specific fecal microbiota taxonomic profile (Bifidobacterium-dominant) (248). However, a recent study conducted in 1-month old infants found that children with older siblings showed an exaggerated Type 1 and Type 17-related immune responses, which may be initiated *in utero*, suggesting that the effect of older siblings on infant immune development could begin prior to exposure to environmental microbes (249). However, the placental, amniotic, and maternal vaginal microbiomes were not analyzed in this study, and it is possible that these bacterial populations play a role in the immune priming of the fetus, as multiple pregnancies could shift

the compositions of these maternal microbial populations and lead to changes in intrauterine immune cell-microbe interactions.

Consistent with previous studies, our study found that males are more prone to develop respiratory infections than females (250). Respiratory pathogens are associated with the induction of Type-1 and Type-17 mediated airway inflammation and viral respiratory infections are associated with increased wheezing in early life, which is a significant risk factor for later asthma development (251, 252). Further, acute Influenza (a common viral infection among the CHILD cohort) can exacerbate allergic responses in OVA-challenged mice, suggesting that early life viral infections can enhance the severity of atopy (253). However it is also possible that children with respiratory infections are being over prescribed antibiotics, which disturb the intestinal and/or airway microbiotas and increase susceptibility to asthma and atopic disease (potentially mediated by an increased severity to respiratory infections in early life or through other microbe-immune cell interfaces) (254, 255). Independent of respiratory infections, antibiotics have been shown to induce lung inflammation in mouse models of experimental asthma (146, 209). Further, treatment with streptomycin perinatally was shown in mice to alter the intestinal microbiota and increase the severity of hypersensitivity pneumonitis (a Th1/Th17 mediated lung disease), highlighting the similarity in effects on immune dysregulation exerted by airway pathogens and early life antibiotic exposure (147). However, we did not address the seasonality of respiratory illnesses in children relative to when the children in this study were born, which could be a potential confounding variable associated with increasing a child's risk of asthma.

The associations with antibiotic exposure were perhaps the most compelling environmental influences on asthma development in this study. First, even in contemporary

medical practice in Canada, 1-in-5 infants are exposed to antibiotics in the first year of life, these rates have not dropped despite many public health initiatives around antibiotic stewardship to decrease antibiotic usage (256). Our results show an increased likelihood in males and females to develop asthma if they received antibiotics between 6-months and 1-year of age. A recent study showed that children exposed to antibiotics from 6-months to 1-year show delayed intestinal microbiota maturation, characterized by shifts in specific bacterial taxa (110). Thus it is possible that antibiotic perturbations to the intestinal microbiota during this time period could stunt the maturation of the infant intestinal microbiota, which could bias the developing infant immune system toward IgE-mediated hypersensitivities. However, further research is needed to determine the role of antibiotics in infant immune development, as it is also possible that the reason children (or mothers, in the case of prenatal antibiotics) are being prescribed antibiotics (i.e. prescription of antibiotics for infections) is the true risk factor for childhood asthma (257).

We also found that antibiotics given at specific time points increased the risk of asthma in a sex-specific manner (prenatal antibiotics were a risk factor for females, while males were more likely to develop asthma if they received antibiotics at birth). Early life antibiotic exposure has been shown to increase fat-mass accumulation and worsen asthma symptoms in mice, which appear to be mediated by antibiotic-induced alterations to the intestinal microbiota (133, 146). Regarding the sex-specific differences, there is evidence that the intestinal microbiome composition modifies sex hormone levels, which can ultimately regulate autoimmune disease progression (as shown using the nonobese diabetic mouse model of type 1 diabetes) (258, 259). In mice, maternal exposure to antibiotics has been shown to increase weight and fat accumulation more profoundly in males, which is also mediated by gut microbial alterations (260). Lastly, farm living has been shown to decrease risk of asthma in a sex-specific manner,

suggesting that sex-specific microbiota mediate these associations with asthma (261). Thus, it is possible that these early life antibiotic exposures do alter the intestinal microbiota in a sex-specific manner, however much more research is needed to determine mechanistically if these sex-specific time-sensitive findings ultimately shift the intestinal microbiota toward one that promotes asthma and atopic disease.

Altogether, our study proposes a number of hygiene-hypothesis related variables as protective and risk factors for asthma and atopic disease development. A particular strength of this study is our focus on the first year of life, as these protective and risk related effects occur prior to the onset of disease. Additionally, this study does not focus on one particular risk or protective factor, but highlights the importance of both genetic and environmental variables in the progression of the atopic march. We also point out the high rate of antibiotic use in Canada and emphasize that antibiotic use has other health related implications aside from promoting antibiotic resistant bacteria.

As with all research, this study has limitations. Though we do not claim to present a complete predictive model of clinical factors associated with childhood asthma, we do not account for all variables (heritable, environmental, and microbiome-related) that are likely contributing to asthma development in Canada (such as, farm or farm-animal exposure, air pollution, parental smoking). The goal of this study was to focus on microbiome-associated factors and thus, it is possible that given the inclusion or exclusion of other variables, that some of these associations with childhood asthma will shift. Further, we report fairly small (yet significant) increases or decreases in the likelihood of developing childhood asthma for many of the microbiome-associated factors. This is consistent with other studies, but it does suggest a potentially bigger role of heritable factors (or factors not included in this analysis) in the

development of asthma and atopic disease (237). Thus, though roles of the microbiome in asthma have been identified, it is possible that children are genetically predisposed to asthma, which alters their microbiome prior to the development of disease, and whether the microbiome mediates the development of asthma due to these factors is still unknown (205, 262, 263).

The environmental exposures discussed in this study suggest a role of the gut microbiota in infant immune development (particularly, a role of the intestinal microbiota implied by the increased risk of asthma associated with oral/intravenous antibiotics). However whether these variables mediate gut microbial changes, which in turn mediate infant immune development is speculative. Large cohort combination microbiome-epidemiological studies are needed to assess gut microbial dysbiosis associated with immune hypersensitivities while adjusting for these factors. Further, statistical methods such as path analysis and structural equation modeling can be applied to large microbiome cohorts to determine if these early life factors drive gut microbial alterations that are associated with asthma and atopic disease. Longitudinal analyses continuing after 3 years of age will also be important to assess the roles of these factors in subjects diagnosed with asthma later in life. Further, similar studies should be conducted in areas where asthma is more or less prevalent, as it is likely that other environmental and genetic exposures have more important roles in other parts of the world.

2.6 Conclusions

In conclusion, this study highlights roles of genetic and environmental factors in the progression of the atopic march. We also highlight hygiene-hypothesis related risk and protective factors, which propose a role of the microbiota in shaping the infant immune system. Of note,

our study found that breast-feeding, increasing gestational age (in the late third trimester), and older siblings were protective against preschool-age asthma, while pre- and post-natal antibiotic exposure and respiratory infections were risk factors for preschool-age asthma. After sex-stratification, antibiotics from 6-months to 1-year were associated with increased likelihood of preschool-age asthma in both males and females. However, females were more likely to develop asthma if they were exposed to antibiotics prenatally, while birth antibiotics were a risk factor for males. Ultimately, the risk factors that we have identified for preschool-age asthma are all modifiable through evidence-based education and policy including decreasing early life antibiotic exposure, encouraging breast feeding and when possible, facilitating vaginal delivery.

Chapter 3: The role of the human intestinal microbiota in an early life atopic disease phenotype

3.1 Synopsis

Asthma is the most prevalent pediatric chronic disease and affects over 300 million people worldwide. Recent evidence in animal models has identified a 'critical window' early in life where perturbations to the gut microbiota (dysbiosis) are most influential in experimental asthma. However, current research has yet to translate these results into humans. We compared the 3-month and 1-year gut microbiota of 319 subjects enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) Study, and show that one-year-old atopic wheezing infants exhibited transient gut microbial dysbiosis during the first 100 days of life. The relative abundance of the bacterial genera *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* were significantly decreased in these children. Further, this decrease in the abundance of specific bacterial taxa was accompanied by shifts in the functional potential of the intestinal microbiota measured by short chain fatty acid analysis. These results enhance the potential for future microbe-based diagnostics and therapies, potentially in the form of probiotics, to prevent the development of asthma and other atopic diseases in children.

3.2 Introduction

Asthma is a chronic inflammatory disease of the airways currently affecting over 300 million people worldwide (1, 264). It is also the most prevalent childhood disease in westernized countries (1, 264) highlighting the marked disparity in prevalence between developed and

developing countries (45). Like other immune-mediated diseases, asthma is multifactorial, caused by both genetic and environmental components. Genome-wide association studies have yielded several gene loci associated with asthma, but these do not explain the largest proportion of asthma heritability (13). Epidemiologic studies have identified a number of early life environmental exposures associated with asthma that may better explain the sharp increase in asthma prevalence over the past few decades. Many of these exposures are associated with early life events known to alter the microbiota, including pre- and perinatal antibiotics, delivery by cesarean section, urban (vs. farm) living, and formula feeding (49, 81, 110, 146, 209).

The Microflora hypothesis suggests perturbations to the intestinal microbiota as the link between these environmental changes and our immune system, and many recent studies have identified the intestinal microbiota as a potential therapeutic target in the prevention of asthma and atopic disease (153, 202, 233, 265). There is now evidence in mice of an early life 'critical window' in which the effects of gut microbial dysbiosis are most influential in human immune development (143, 209, 266). Russell *et al.* showed that perinatal antibiotic treatment of OVA-challenged (asthma-induced) mice exacerbates airway inflammation by increasing serum and surface bound IgE and decreasing T-reg cell accumulation in the colon (209). Research associating these early life gut microbial changes with asthma development, however, has not yet been translated into humans.

Early life gut microbial alterations are not limited to shifts in the prevalence of gut microbes (143, 209) but also include changes in the production of bacterial derived metabolites such as SCFAs (155). The SCFAs, acetate and propionate, were implicated in the reduction of airway cellular infiltration in a mouse model of lung inflammation (155). Shifts in SCFAs have also been associated with the development of food allergies in children (267). Analysis of these

microbial-derived metabolites in infants, could provide a mechanistic link between early life gut microbial dysbiosis and changes in the infant immune system leading to hypersensitivity diseases like asthma.

Designed to elucidate the factors involved in asthma and atopic disease development, the Canadian Healthy Infant Longitudinal Development (CHILD) Study is a multi-centre, longitudinal, prospective, general population birth cohort study following infants from pregnancy until 5-years of age. In the previous chapter, I showed that in the CHILD study, hygiene and microflora hypothesis related factors were associated with increased risk of (antibiotic exposure and respiratory infections) and protection from (duration of breast-feeding, vaginal birth, older siblings, and increased gestational age) 1-year atopy and wheezing and 3-year (preschool-age) asthma. We also identified the 1-year atopic wheezing phenotype as the high-risk phenotype, including subjects most likely to develop preschool-age asthma compared to controls.

Here we provide evidence in a cohort of 319 human subjects enrolled in the CHILD study supporting a link between gut microbial dysbiosis in the first 100 days of life, characterized by reductions in four bacterial genera, *Lachnospira, Veillonella, Faecalibacterium*, and *Rothia* (FLVR), and an increased risk to develop atopy and wheezing by 1 year of age. These reductions were accompanied by a decrease in fecal acetate, revealing a functional role of the intestinal microbiota in atopic disease development in human infants. This comprehensive analysis provides a novel understanding of early life alterations in the intestinal microbiota that precede asthma development in children and highlights an early life 'critical window' in humans during which these gut microbial alterations could be used as diagnostics and therapeutics for asthma and atopic disease.

3.3 Materials and methods

3.3.1 Study design

Details of the CHILD study are described in **Section 2.3.1**. Briefly, the CHILD study is a multi-centre longitudinal, prospective, general population birth cohort study of 3,623 mother-infant pairs recruited at 4 sites across Canada (Vancouver, Edmonton, Manitoba, Toronto) (234, 235). The study follows infants from pregnancy to age 5-years, with clinical assessments by CHILD study clinicians at ages 1, 3, and 5-years. Data up to 3-years of age was used for determining which children were diagnosed with asthma by 3-years of age (n_{asthma} = 19). The University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board approved the research protocols for studies on human samples and each participating parent or legal guardian gave signed informed consent.

3.3.2 Inclusion/Exclusion criteria

This study was based on a nested-case control design to analyze the fecal microbiota of infants enrolled in the CHILD study. Children were selected based on skin prick testing and wheezing data described in Chapter 2, **Section 2.3.2** and availability of biological samples (described below).

Biological samples: Of the subjects with valid skin prick test and wheezing data (**Section 2.3.2**) only subjects with both a 3-month and a 1-year stool sample were considered for inclusion in this analysis. For the control group, subjects from whom additional biological samples were collected by the CHILD study (such as blood or urine) were selected over subjects missing any of these samples.

Of the 3,412 enrolled in the general CHILD Study cohort, 1,427 children had completed the 1-year clinical assessment at the time of selection in September 2012. 163 subjects were excluded due to incomplete skin prick test data or a positive response to glycerin or a negative response to histamine and all other allergens. The remaining 1,264 subjects were grouped into the four clinical phenotypes, atopy + wheeze (AW) (n = 35), atopy only (n = 150), wheeze only (n = 216), and controls (n = 863) and assessed for the availability of a 3-month and a 1-year stool sample [n numbers for children with 3-month and 1-year stool samples available: AW (n = 25), atopy only (n = 112), wheeze only (n = 179), and controls (n = 106)]. Subjects were then excluded from the study if, after preparation and sequencing of the 16S DNA, the sequence results were inadequate (i.e. not enough sequence reads per stool sample) [final n numbers, AW (n = 22), atopy only (n = 87), wheeze only (n = 136), and controls (n = 74)].

The subsets of samples for qPCR analysis included all but one AW sample and 19 (1-year) and 20 (3-months) randomly selected control samples. 13 AW and 13 control samples were submitted for SCFA analysis (**Figure 1.2**). An exact logistic regression model was used to confirm that all subsets used in this study were representative of the larger cohort analyzed (**Tables B.1 – B.3**; $n_{AW} = 22$, $n_{control} = 74$). The number of samples selected depended on the availability of the fecal samples, which tended to be very limited in this study of human infants.

3.3.3 Definitions of clinical variables

Antibiotic exposure birth to 1-year and birth to 3-months: 'At least 1 or more' = received at least one antibiotic, 'none' = did not receive any antibiotics. Reference is 'none'.

Atopic dermatitis or Eczema at 3-months or 1-year: 'yes' = diagnosed with atopic dermatitis (also referred to as eczema is a chronic skin disease characterized by itchy, inflamed skin) at 3-months (reported in 3-month CHILD health questionnaire) or at 1-year (diagnosed by a CHILD clinician at the 1-year clinical assessment or a non-CHILD clinician as reported in one-year CHILD health questionnaire). 'No' = no diagnosis. Reference level is 'no'.

Breast feeding birth to 3-months and birth to 1-year: 'yes' = breast-fed for at least 3-months or at least 1-year (respectively). 'No' = not breast-fed for the entirety of that time (3-months or 1-year, respectively). Reference level is 'no'.

Maternal and paternal history of asthma: Defined as 'yes' or 'no'. Reference level is 'no'.

Mode of birth: Reference level is cesarean section birth.

Sex: Reference level is female.

3.3.4 16S microbial community analysis

3.3.4.1 Fecal DNA extraction and preparation

DNA was extracted from ~50 mg of stool. Samples were mechanically lysed using Mo bio dry bead tubes (Mo Bio Laboratories) and the Fastprep homogenizer (FastPrep Instrument, MP Biochemicals) prior to DNA extraction with the QIAGEN DNA Stool Mini Kit.
All samples were amplified by PCR in triplicate using barcoded primer pairs flanking the V3 region of the 16S gene (**Table 3.1**) as previously described (268). Each 50μL PCR reaction

contained 22µL water, 25µL Top Taq Master Mix, 0.5µL of each forward and reverse barcoded primer, and 2µL template DNA. The PCR program consisted of an initial DNA denaturation step at 95 ^{o}C for (5 min), 25 cycles of DNA denaturation at 95 ^{o}C (1 min), an annealing step at 50 ^{o}C (1min), an elongation step at 72 ^{o}C (1 min), and a final elongation step at 72 ^{o}C (7 min). Controls without template DNA were included to ensure that no contamination occurred. Amplicons were run on a 2% agarose gel to ensure adequate amplification. Amplicons displaying bands at ~160kb were purified using the Illustra GX PCR DNA Purification kit. Purified samples were diluted 1:50 and quantified using PICOGreen (Invitrogen) in the TECAN M200 (excitation at 480ηm and emission at 520ηm).

Table 3.1 16S V3 region primers and barcodes

Forward		Reverse	
Primer ID	**Sequence**	**Primer ID**	**Sequence**
341F/A	CTGATCNNNNCCTACGGGAGGCAGCAG	518R/a	aaccccATTACCGCGGCTGCTGG
341F/B	AGCATCNNNNCCTACGGGAGGCAGCAG	518R/b	ccaacaATTACCGCGGCTGCTGG
341F/C	CGATTANNNNCCTACGGGAGGCAGCAG	518R/c	agttccATTACCGCGGCTGCTGG
341F/D	CATTCANNNNCCTACGGGAGGCAGCAG	518R/d	accggcATTACCGCGGCTGCTGG
341F/E	AAGCTANNNNCCTACGGGAGGCAGCAG	518R/e	caactaATTACCGCGGCTGCTGG
341F/F	GCTGTANNNNCCTACGGGAGGCAGCAG	518R/f	ccacgcATTACCGCGGCTGCTGG
341F/G	ATGGCANNNNCCTACGGGAGGCAGCAG	518R/g	ctatacATTACCGCGGCTGCTGG
341F/H	GCCTAANNNNCCTACGGGAGGCAGCAG	518R/h	tacagcATTACCGCGGCTGCTGG
341F/I	GTAGCCNNNNCCTACGGGAGGCAGCAG	518R/i	atgtcaATTACCGCGGCTGCTGG
341F/J	AAGTGCNNNNCCTACGGGAGGCAGCAG	518R/j	ttaggcATTACCGCGGCTGCTGG
341F/K	ATTATANNNNCCTACGGGAGGCAGCAG	518R/k	ggctacATTACCGCGGCTGCTGG
341F/L	CCAGCANNNNCCTACGGGAGGCAGCAG	518R/l	acgataATTACCGCGGCTGCTGG
341F/M	TGGTCANNNNCCTACGGGAGGCAGCAG	518R/m	ctcagaATTACCGCGGCTGCTGG
341F/N	CCACTCNNNNCCTACGGGAGGCAGCAG	518R/n	ccgtccATTACCGCGGCTGCTGG

Forward		Reverse	
Primer ID	**Sequence**	**Primer ID**	**Sequence**
341F/O	CGCGGCNNNNCCTACGGGAGGCAGCAG	518R/o	tgaccaATTACCGCGGCTGCTGG
341F/P	GAATGANNNNCCTACGGGAGGCAGCAG	518R/p	cttgtaATTACCGCGGCTGCTGG
341F/Q	GCGCCANNNNCCTACGGGAGGCAGCAG	518R/q	aagcgaATTACCGCGGCTGCTGG
341F/R	CTCTACNNNNCCTACGGGAGGCAGCAG	518R/r	tcattcATTACCGCGGCTGCTGG
341F/S	GGTTTCNNNNCCTACGGGAGGCAGCAG	518R/s	tggcgcATTACCGCGGCTGCTGG
341F/T	TAAGGCNNNNCCTACGGGAGGCAGCAG	518R/t	aaggacATTACCGCGGCTGCTGG
341F/U	TCGGGANNNNCCTACGGGAGGCAGCAG	518R/u	atcctaATTACCGCGGCTGCTGG
341F/V	TTCGAANNNNCCTACGGGAGGCAGCAG	518R/v	cactcaATTACCGCGGCTGCTGG
341F/W	GCGGACNNNNCCTACGGGAGGCAGCAG	518R/w	ccgcaaATTACCGCGGCTGCTGG
341F/X	ATTGGCNNNNCCTACGGGAGGCAGCAG	518R/x	gaaaccATTACCGCGGCTGCTGG
341F/Y	TTATTCNNNNCCTACGGGAGGCAGCAG	518R/y	gccttaATTACCGCGGCTGCTGG
341F/Z	TGGAGCNNNNCCTACGGGAGGCAGCAG	518R/z	tcccgaATTACCGCGGCTGCTGG
341F/AA	CTTCGANNNNCCTACGGGAGGCAGCAG	518R/aa	ttcgaaATTACCGCGGCTGCTGG
341F/AB	GGAGAANNNNCCTACGGGAGGCAGCAG	518R/ab	gtccgcATTACCGCGGCTGCTGG
341F/AC	TTTCACNNNNCCTACGGGAGGCAGCAG	518R/ac	aaagcaATTACCGCGGCTGCTGG
341F/AD	TCCGTCNNNNCCTACGGGAGGCAGCAG	518R/ad	agaagaATTACCGCGGCTGCTGG
341F/AE	TGTGCCNNNNCCTACGGGAGGCAGCAG	518R/ae	gaataaATTACCGCGGCTGCTGG
341F/AF	TGCCGANNNNCCTACGGGAGGCAGCAG	518R/af	gctccaATTACCGCGGCTGCTGG
341F/AG	GGCCACNNNNCCTACGGGAGGCAGCAG	518R/ag	ttctccATTACCGCGGCTGCTGG
341F/AH	TATATCNNNNCCTACGGGAGGCAGCAG	518R/ah	gtgaaaATTACCGCGGCTGCTGG
341F/AI	CAGGCCNNNNCCTACGGGAGGCAGCAG	518R/ai	cagatcATTACCGCGGCTGCTGG
341F/AJ	GGTAGANNNNCCTACGGGAGGCAGCAG	518R/aj	aaatgcATTACCGCGGCTGCTGG
341F/AK	CGAAACNNNNCCTACGGGAGGCAGCAG	518R/ak	acaaacATTACCGCGGCTGCTGG

3.3.4.2 Illumina sequencing

Pooled PCR amplicons were diluted to 20ηg/μL and sequenced at the V3 hyper-variable region using Hi-Seq 2000 bidirectional Illumina sequencing and Cluster Kit v4 (Macrogen Inc.).

Library preparation was done using TruSeq DNA Sample Prep V2 Kit (Illumina) with 100ηg of DNA sample and QC library by Bioanalyzer DNA 1000chip (Agilent).

3.3.4.3 Bioinformatics

Samples were pre-processed, denoised, and quality filtered by size using Mothur (269). Representative sequences were clustered into operational taxonomic units (OTUs) using CrunchClust (270) and classified against the Greengenes Database (271) according to 97% similarity. Any OTUs present less than 5 times among all samples were removed from the analysis.

3.3.5 Quantitative Polymerase Chain Reaction:

To validate sequencing results, the abundance of specific fecal bacterial genera was measured in the 16S rDNA V3 amplicons using group-specific 16S rDNA gene primers for the following genera; *Lachnospira*, *Veillonella*, *Rothia*, *Faecalibacterium*, and *Bifidobacterium* (**Table 3.2**). All AW samples and a randomly selected, but representative equal number of control samples were analyzed by qPCR (**Table B.1**). All reactions were carried out in the 7500 Fast Real-Time System (Applied Biosystems) or the ViiA 7 Real-Time PCR System (Life Technologies Inc.). Each 10μL reaction contained 5μL of IQ SYBR green supermix (Bio-Rad, 5uL), 0.1μL of each forward and reverse primer, 0.8μL of nuclease-free water, and 4μL of the V3 amplicon. The qPCR program consisted of an initial step at 95 ^{o}C (15 min), 40 cycles of 15s at 94 ^{o}C, 30s at 60 ^{o}C, and 30s at 72 ^{o}C, and a final cycle of 95 ^{o}C at 15s, 60 ^{o}C at 1 min, 95 ^{o}C at 15s, and 60 ^{o}C at 15s. Per primer set, at least two dilutions were run per sample and all dilutions

were run in duplicate. Samples were normalized according to the ΔC_T method using total 16S rDNA (Bacteria, **Table 3.2**) as the reference gene.

Table 3.2 Taxon-specific qPCR primers

Taxon	Forward	Reverse
Bacteria	ACT CCT ACG GGA GGC AGC AGT	ATT ACC GCG GCT GCT GGC
Bifidobacterium spp.	CTC CTG GAA ACG GGT GGT AAT	ATA GGA CGC GAC CCC ATC CCA
Veillonella spp.	AAG CTA TCA CTG AAG GAG GG	TCC CAA TGT GGC CGT TCA TCC
Rothia spp.	GCC TGG GAA ACT GGG TCT AAT	CAA GCT GAT AGG CCG TGA G
Faecalibacterium spp.	GGA GCG ATC CGC TTT GAG ATG	AAC CTC TCA GTC CGG CTA CCG A
Lachnospira spp.	GCA ACG CGA AGA ACC TTA CC	ACC ACC TGT CAC CGA TGT TC

3.3.6 Short-Chain Fatty Acid Analysis:

Fecal samples were combined with 25% phosphoric acid, vortexed, and centrifuged until a clear supernatant was obtained. Supernatants were submitted for gas chromatography analysis to the Department of Agricultural, Food and Nutritional Science of the University of Alberta. Only 13 AW samples contained enough material for this analysis and 13 additional control samples were randomly selected for this analysis (**Tables B.2 & B.3**). Samples were analyzed as previously described with modifications (272). Briefly, samples were combined with 4-methyl-valeric acid as an internal standard and 0.2 ml was injected into the Bruker Scion 456 gas chromatograph, using a Stabilwax-DA 30m x 0.53mm x 0.5um column (Restek). A standard solution containing acetic acid, proprionic acid, isobutyric acid, butyric acid, isovaleric acid,

valeric acid, and caproic acid, combined with internal standard was injected in every run. The PTV injector and FID detector temperatures were held at 250 °C for the entire run. The oven was started at 80 °C and immediately ramped to 210 °C at 45 °C/min, where it was held for 5.11 mins. Total run time was 8.00 mins. Helium was used at a constant flow of 20.00ml/min. Sample concentrations were normalized to the wet weight of feces.

3.3.7 Statistical Analysis:

3.3.7.1 Regression analyses

An exact logistic regression model based on Markov Chain Monte Carlo (MCMC) sampling was developed and ORs were used to evaluate the risk associated with the AW group according to specific clinical data (273-275). ORs and the adjusted lower and upper 95% confidence intervals were calculated according to the following formula $e^{(\ln(OR))}$ and $e^{(\ln(CI)}$, respectively. ln(CI) is equal to the exact upper and lower confidence intervals (**Table 3.3**). Only subjects for which all the data were available were included in the model ($n_{AW} = 21$, $n_{control} = 74$). This same model was also used to confirm that all subsets of control and AW samples used in this study were representative of the entire cohort (**Tables B.1 – B.3**).

A logistic regression model using the glm2 package in R was used to confirm that this population of subjects was representative of the larger CHILD cohort analyzed in Chapter 2 of this thesis (276). Notably, only 303 subjects from this chapter were included as the remaining 15 subjects had insufficient data to be classified according to an asthma diagnosis at age 3 years or withdrew from the CHILD study before age 3 years (**Table D.1**).

3.3.7.2 Microbial community and SCFA analyses

We assessed fecal microbial diversity and the relative abundance of bacterial taxa using Phyloseq along with additional R-based computational tools in R-studio (R-Studio) (277-283). Principal components analysis (PCA) was conducted using MetaboAnalyst and statistically confirmed by permanova (283-285). The Shannon diversity index was calculated using Phyloseq and statistically confirmed by Mann-Whitney (GraphPad Prism software, version 5c) (283). The 'mt' function in Phylsoeq was used to calculate multi-inference-adjusted p-values to identify differentially abundant OTUs between the 3-month and 1-year samples and among the four phenotypes; AW, atopy only, wheeze only, and controls (283). Differences between the control and AW groups were determined by Mann-Whitney for qPCR. All SCFAs were subject to the Shapiro-Wilk test for normality and differences between control and AW groups were determined by Mann-Whitney. No samples were excluded from statistical tests and the F-test found no significant differences between the variances of the groups. All data points in graphs represent biological replicates. Statistical significance was defined as $p \leq 0.05$.

3.4 Results

3.4.1 Assessment of asthma risk in CHILD study subjects

Using a nested case-control design, we selected 319 1-year-old children from the CHILD study for gut microbiome analysis (see **Section 3.3.2** for inclusion and exclusion criteria based on allergen skin prick testing and wheezing). These study participants were grouped into four clinically-distinct phenotypes based on allergy skin prick testing (i.e. atopy) and clinical wheeze data at 1-year of age: atopy + wheeze (AW, n = 22), atopy only (n = 87), wheeze only (n = 136),

and controls (n = 74) **(**full details regarding the classification of these phenotypes are described in Chapter 2, **Section 2.3.2**; **Figure 3.1).**

Given that the CHILD study is longitudinal, over the course of this study the children in this cohort reached 3-years of age and thus 2 and 3-year clinical data was used to confirm the clinical significance of the 1-year phenotypes; children were assessed by a CHILD clinician at 3-years of age for the diagnosis of asthma. In this cohort (n= 319), the AW group was 21.5 times more likely than the control group [p = 0.002; 95% CI: 2.4 to 196.0], 3.9 times more likely than the atopy only group [p = 0.0429, 95% CI: 1.09 to 14.5], and 5.4 times more likely than the wheeze only group [p = 0.0137, 95% CI: 1.5 to 19.0] to be diagnosed with asthma by 3-years of age. The wheeze only group was 4 times more likely (ns) and the atopy only group was 5.4 times more likely (ns) than the control group to develop asthma by 3-years of age. Together, this diagnosis of asthma at 3-years of age emphasizes the clinical relevance of the AW phenotype at age 1-year among this sub-population of the CHILD study (**Figure 3.1**).

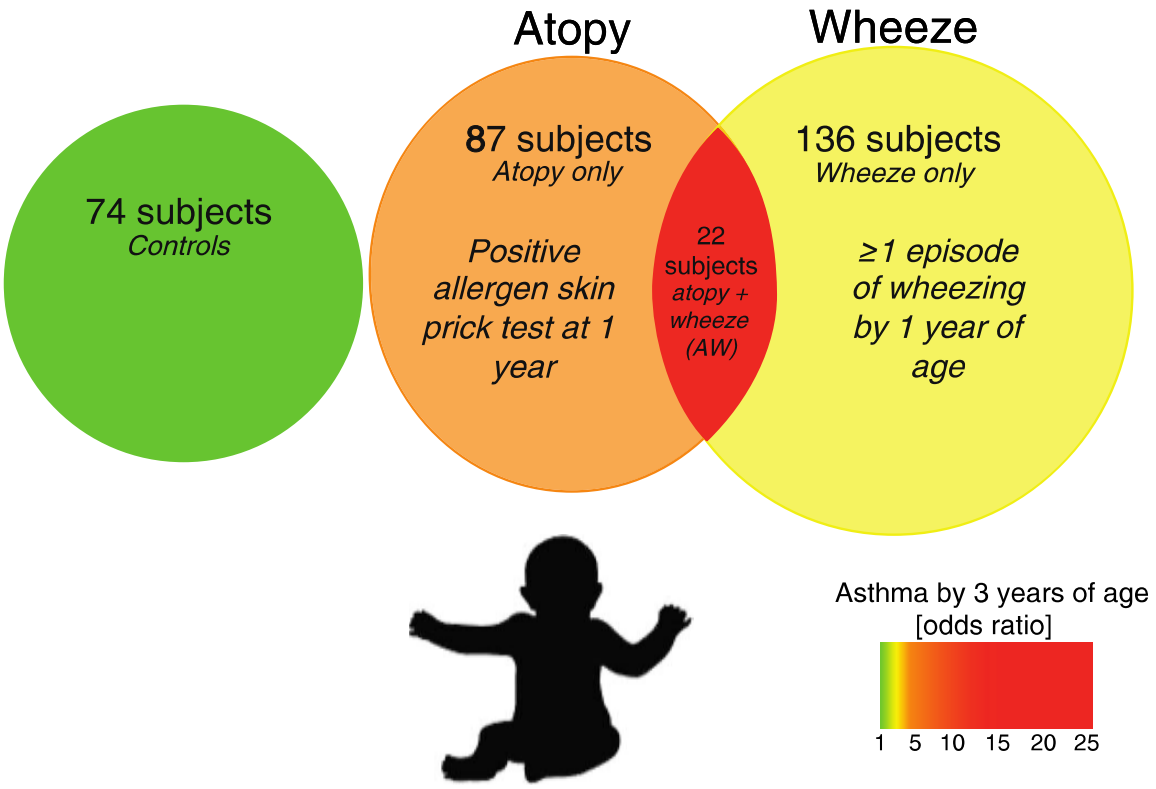

Figure 3.1 Classification of study participants

Distribution of 319 subjects among the four 1-year clinical phenotypes based on skin prick tests and wheeze data at 1-year of age: controls, atopy + wheeze (AW), atopy only, and wheeze only. The odds ratios associating the phenotypes to a 3-year asthma diagnosis are signified as a heat-map color relative to the control group (odds ratios: AW vs controls, 21.5 [p = 0.0022; 95% CI: 2.4 to 196.0]; atopy only vs controls, 5.4 [ns]; wheeze only vs controls, 4.0 [ns], (printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015).

3.4.2 Early life environmental risk factors associated with AW phenotype

We found no significant differences in early life environmental exposures for this sample cohort when compared to the larger cohort analyzed in Chapter 2, suggesting that this sub-population of CHILD subjects is representative of the larger CHILD study cohort (Chapter 2 n = 2,695). However, we did enrich for atopy and wheezing when selecting these subjects for microbiome analysis; and as expected based on the results from Chapter 2 (**Figs. 2.3 & 2.4**), we did find significant differences between the groups according to maternal asthma (OR = 1.5, p = 0.0004, **Table D.1)**.

In line with the results from Chapter 2, exact logistic regression analysis identified antibiotic exposure in the first year of life [OR: 5.6, p = 0.009] and atopic dermatitis at 1-year [OR: 6.4, p = 0.01] as factors that increased a subject's risk of being classified in the AW group compared to controls **(Table 3.3)**. As identified in Chapter 2, cesarean birth and decreased duration of breast-feeding in infancy are also common factors associated with gut microbial dysbiosis, but were not significant factors in this sub-population, likely due to the size of the cohort analyzed (n_{AW} = 22, $n_{controls}$ = 74). However, the association of antibiotic exposure during the first year of life with the AW phenotype supported assessment of the intestinal microbiota composition among infants at high risk of asthma development.

Table 3.3 Early life environmental factors associated with AW phenotype

Variable		Phenotype		OR*	95% CI		P-value
		Atopy + Wheeze (AW)	Controls		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	9 (42%)	12 (16%)	5.6	1.3	81	0.009 **
	None	12 (58%)	62 (84%)				
	Total (100%)	21	74				
Antibiotic Exposure (birth to 3-months of age)	1 or more	0 (0%)	4 (5%)	0.33	-	3.3	0.24
	None	21 (100%)	70 (95%)				
	Total (100%)	21	74				
Atopic dermatitis at 3-months	Yes	5 (24%)	4 (5%)	2.2	0.1	18.2	0.53
	No	16 (76%)	70 (95%)				
	Total (100%)	21	74				
Atopic dermatitis at 1-year	Yes	13 (62%)	18 (24%)	6.4	1.5	67	0.01 **
	No	8 (38%)	56 (76%)				
	Total (100%)	21	74				
Sex	Female	7 (33%)	38 (51%)	0.38	0.07	1.4	0.15
	Male	14 (67%)	36 (49%)				
	Total (100%)	21	74				
Mode of birth	Vaginal	16 (76%)	58 (78%)	0.74	0.15	4.1	1
	Caesarean	5 (24%)	16 (23%)				
	Total (100%)	21	74				
Breast Feeding (birth to 3-months)	Yes	15 (71%)	60 (81%)	0.5	0.07	4.1	0.69
	No	6 (29%)	14 (19%)				
	Total (100%)	21	74				
Breast Feeding (birth to 1-year)	Yes	7 (33%)	24 (32%)	1.2	0.2	6.7	0.73
	No	14 (67%)	50 (68%)				
	Total (100%)	21	74				
Maternal Asthma	Yes	7 (33%)	24 (32%)	1.25	0.1	7.4	1
	No	14 (67%)	50 (68%)				
	Total (100%)	21	74				
Paternal Asthma	Yes	3 (14%)	10 (14%)	1.1	0.1	7.24	1
	No	18 (86%)	64 (86%)				
	Total (100%)	21	74				

*The group listed first for each variable (i.e. 1 or more for antibiotic exposure) is the reference group for interpreting the odds ratio.
(-) A finite lower or upper bound for the confidence interval could not be obtained because the observed value of the sufficient statistic is the maximum possible value.

3.4.3 Gut microbial compositional changes from 3-months to 1-year of age

Consistent with microbiome studies in other cohorts of young children, principal component analysis (PCA) identified age as the main driver of microbial and metabolic shifts in this cohort **(Figure 3.2A)** (86). There was also a significant increase in overall gut bacterial diversity from 3-months to 1-year of age (**Figure 3.2B,** Shannon diversity index, $p \leq 0.01$). Highlighting further the transient compositional state of the infant intestinal microbiota, we identified significant shifts in the abundance of specific bacterial taxa at each time point (**Figure 3.2C, Table 3.4**). By 3-months of age, the infant microbiota is dominated by strict anaerobes (such as members of the *Bifidobacteriaceae* and *Lachnospiraceae* families) and any remnants of facultative anaerobes (Enterobacteriaceae) are completely diminished by 1-year. Ten OTUs exhibiting the largest changes in median abundance from 3-months to 1-year are presented as a heat map **(Figure 3.2D)**. The majority of these taxa increase from 3-months to 1-year, however there is a significant decrease in some taxa, such as *Bifidobacterium longum*, likely due to changes in the infants' diet (transitioning from milk to solid foods).

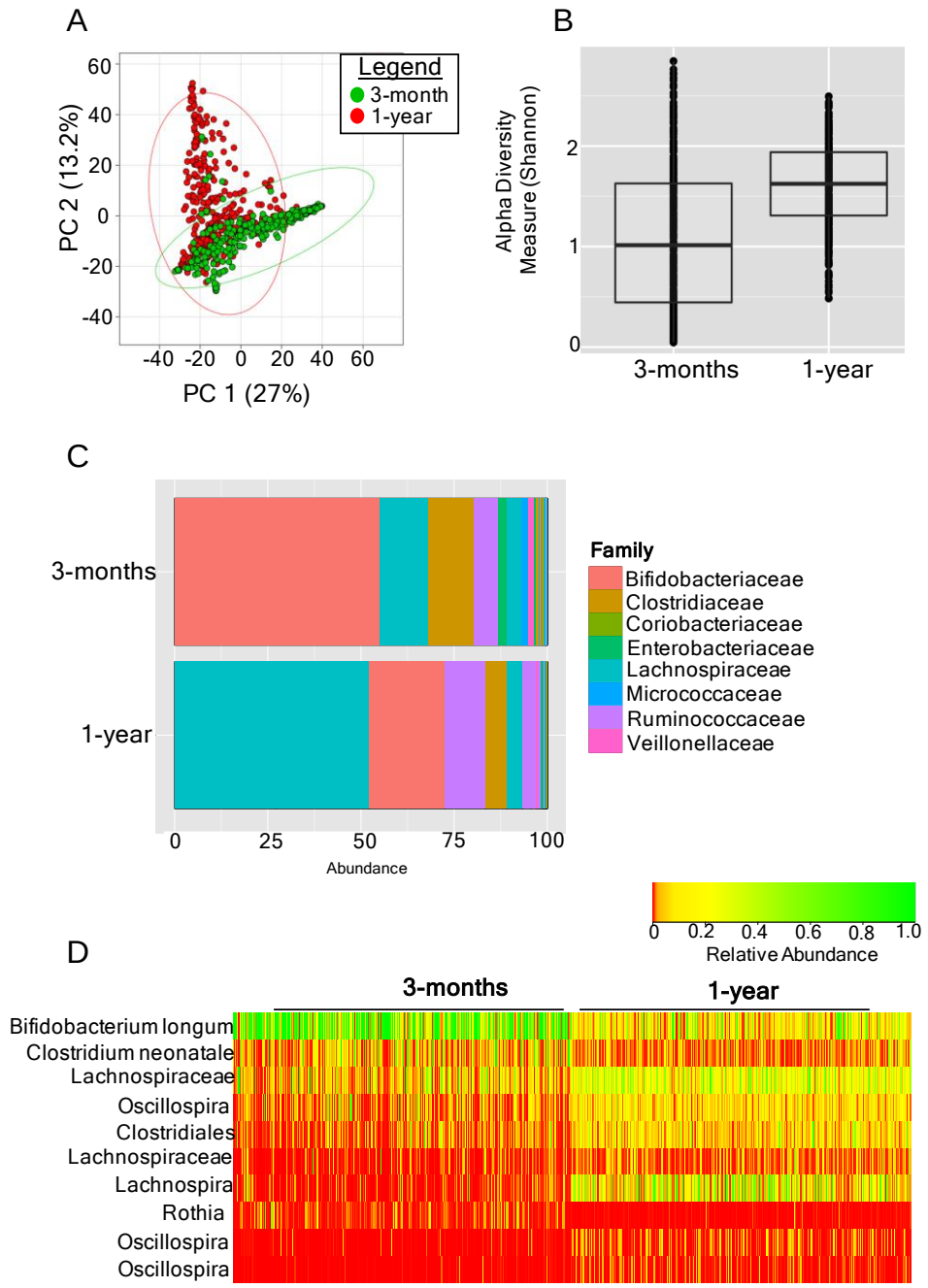

Figure 3.2 Compositional differences between the 3-months and 1-year fecal microbiota

A) Multivariate analysis by PCA of the 3-month and 1-year gut microbiota of 319 children, statistically compared by permanova (p=0.0005). **B)** Alpha diversity was compared at 3-months and 1-year of age using the Shannon Diversity Index, statistically confirmed by Mann Whitney

(p=0.0001, shown as box plots, upper and lower "hinges" correspond to the first and third quartiles (the 25th and 75th percentiles)). **C)** Relative abundances of the top 100 OTUs represented by eight bacterial families. **D)** Heat map displaying the top 10 statistically significant differentially abundant OTUs between 3-months and 1-year of age. Each rectangle is one subject. Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

Table 3.4 Differentially abundant OTUs between 3-month and 1-year fecal microbiota

OTU	Adjusted P value	Median Relative Abundance 3-months	Median Abundance 1-year	Taxonomic Classification
1	0.0019	0.6	0.1	Actinobacteria, Actinobacteria, Bifidobacteriales, Bifidobacteriaceae, *Bifidobacterium, longum*
4	0.0019	0.02	0.01	Firmicutes, Clostridia, Clostridiales, Clostridiaceae, *Clostridium, neonatale*
2	0.0019	0.04	0.2	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
6	0.0019	0.01	0.06	Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Oscillospira*
5	0.0019	0.01	0.05	Firmicutes, Clostridia, Clostridiales
7	0.0289	1.00E-03	5.00E-03	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
3	0.0019	1.00E-03	0.09	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae, *Lachnospira*
20	0.0019	2.00E-03	4.00E-04	Actinobacteria, Actinobacteria, Actinomycetales, Micrococcaceae, *Rothia*
8	0.0019	2.00E-03	1.00E-04	Proteobacteria, Gammaproteobaceria, Enterobacteriales, Enterobacteriaceae
9	0.0087	8.00E-04	6.00E-04	Firmicutes, Clostridia, Clostridiales, Veillonellaceae, *Veillonella*
31	0.0019	2.00E-03	1.00E-04	Bacteria
10	0.0019	6.00E-04	2.00E-04	Proteobacteria, Gammaproteobaceria, Enterobacteriales, Enterobacteriaceae
12	0.0244	2.00E-02	1.80E-04	Firmicutes, Clostridia, Clostridiales, Veillonellaceae, *Veillonella*
53	0.0019	2.00E-04	2.00E-05	Actinobacteria, Coriobacteria, Coriobacteriales, Coriobacteriaceae, *Atopobium*
32	0.0019	2.00E-04	0	Firmicutes, Clostridia, Clostridiales, Clostridiaceae
13	0.0019	0	2.00E-03	Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Oscillospira*
34	0.0019	0	2.00E-04	Bacteria
39	0.0019	2.00E-04	1.70E-04	Firmicutes, Clostridia, Clostridiales,
38	0.0019	8.00E-05	0	Firmicutes, Clostridia, Clostridiales, Clostridiaceae
47	0.0019	4.00E-04	1.00E-04	Bacteria
113	0.0019	2.00E-04	4.00E-05	Actinobacteria, Actinobacteria, Bifidobacteriales, Bifidobacteriaceae, *Bifidobacterium, longum*
15	0.0019	2.00E-05	1.00E-03	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
16	0.0019	0	2.00E-03	Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Faecalibacterium*
48	0.0019	4.20E-05	1.40E-05	Actinobacteria
14	0.0019	0	1.00E-03	Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Oscillospira*
30	0.0019	0	4.00E-05	Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, *Oscillospira*
156	0.0019	8.00E-05	0	Actinobacteria, Actinobacteria, Bifidobacteriales, Bifidobacteriaceae, *Bifidobacterium, longum*
49	0.0019	2.00E-04	1.00E-04	Bacteria
72	0.0019	2.00E-04	0	Proteobacteria, Gammaproteobaceria,
70	0.0019	8.00E-05	0	Bacteria
64	0.0019	9.00E-05	4.00E-05	Bacteria
24	0.0075	0	4.00E-05	Proteobacteria, Alphaproteobacteria, RF32
68	0.0019	4.00E-05	6.00E-05	Bacteria
84	0.0428	4.50E-05	5.00E-05	Bacteria
94	0.0019	3.50E-05	3.60E-05	Bacteria
11	0.0019	0	2.00E-05	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
56	0.0419	4.20E-05	5.00E-05	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
29	0.0019	0	1.00E-04	Firmicutes, Clostridia, Clostridiales
23	0.0019	0	8.00E-05	Firmicutes, Clostridia, Clostridiales
27	0.0019	0	9.00E-05	Firmicutes, Clostridia, Clostridiales
40	0.0019	0	3.00E-04	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
42	0.0019	0	2.00E-04	Firmicutes, Clostridia, Clostridiales, Lachnospiraceae

3.4.4 Alterations in the intestinal microbiota of AW infants

3.4.4.1 16S rRNA sequence analysis

Overall gut community composition did not differ substantially among the clinical phenotypes, as shown by PCA of the 3-month and 1-year samples **(Figs. 3.3A)**. Additionally, although previous studies have shown a decrease in microbial diversity in fecal samples from asthmatic patients, our study did not reveal any significant differences in diversity among the four phenotypes **(Figure 3.3B)** (221)**.**

Nevertheless, a comparison of relative family abundance according to the clinical phenotypes identified differences in the prevalence of some less abundant bacterial taxa (i.e. Micrococcaceae and Veillonellaceae) in the 3-month stool samples **(Figure 3.4A)**, differences which were not present at 1-year **(Figure 3.4B)**. Statistical analysis of the top 100 OTUs yielded no significant differentially abundant OTUs across phenotypes at 3-months or 1-year after correction for multiple comparisons, however qualitative analysis of the relative genera abundance across phenotypes highlights lower abundances of *Lachnospira, Rothia, Veillonella, Faecalibacterium* and a higher abundance of *Oscillospira* and *Epulopiscium* in the AW group at 3-months **(Figure 3.5A,** mt test (ns)**).** In the 1-year fecal microbiota, these genus-specific differences are less apparent, though not completely eradicated (i.e. less *Epulopiscium* and *Dialister* in AW group; **Figure 3.5B**, mt test (ns)).

Comparison of relative taxa abundances among atopic vs. non-atopic children (atopic n = 109, non-atopic n = 210) or wheezing vs. non-wheezing children (wheeze n = 156, non-wheeze n = 163) did not identify any significant (according to the mt test) or observable differences in the 3-month or 1-year gut microbiota, suggesting that these results are not driven by these single factors alone (**Figure 3.6 & 3.7**). This, and the significant likelihood of the AW subjects to be

diagnosed with asthma by 3-years of age over the remaining phenotypes, prompted validation of the 16S rRNA sequence data among the two extreme phenotypes (AW and controls) using genus-specific quantitative PCR (qPCR).

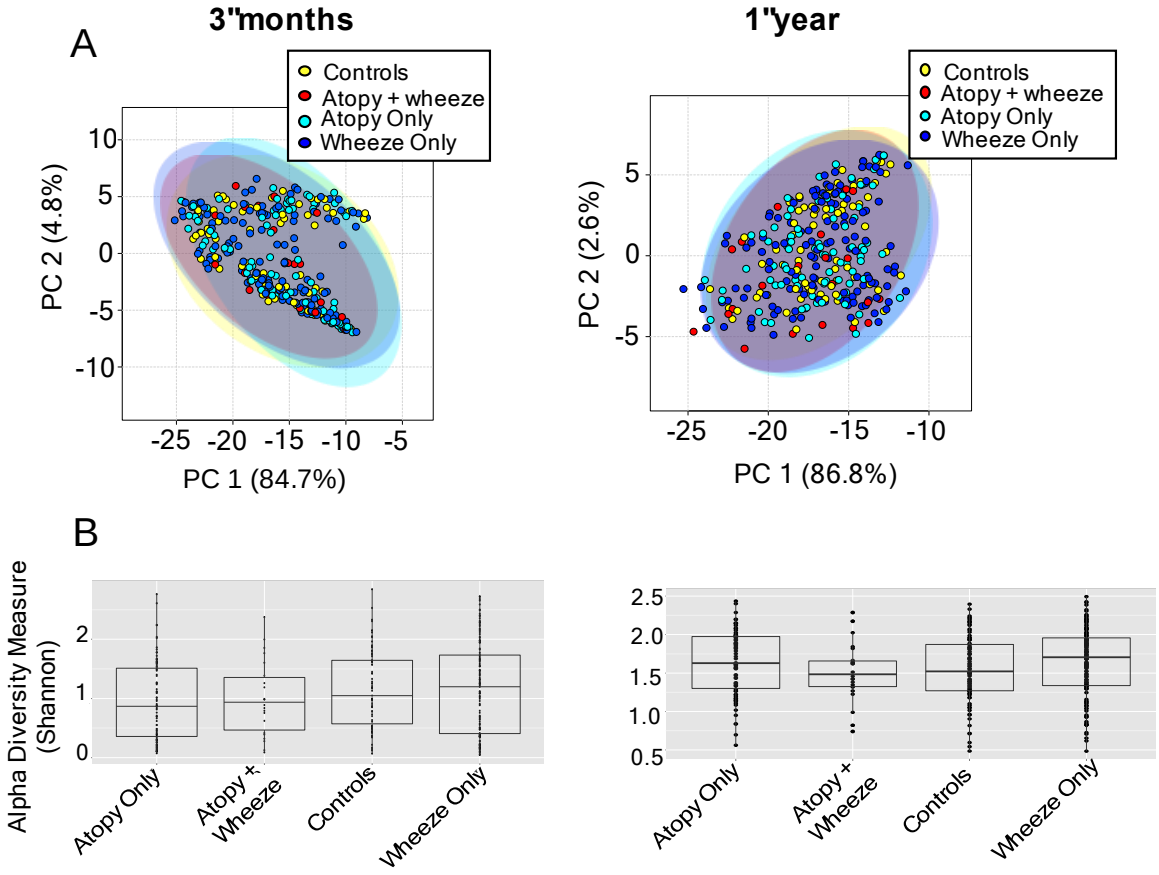

Figure 3.3 PCA and alpha diversity do not show global compositional differences between phenotypes

PCA of the gut microbiota among the four clinical phenotypes at 3-months and 1-year. **B)** Alpha diversity (Shannon Diversity Index) among the four clinical phenotypes at 3-months and 1-year (shown as box plots, upper and lower "hinges" correspond to the first and third quartiles (the

25th and 75th percentiles)). Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

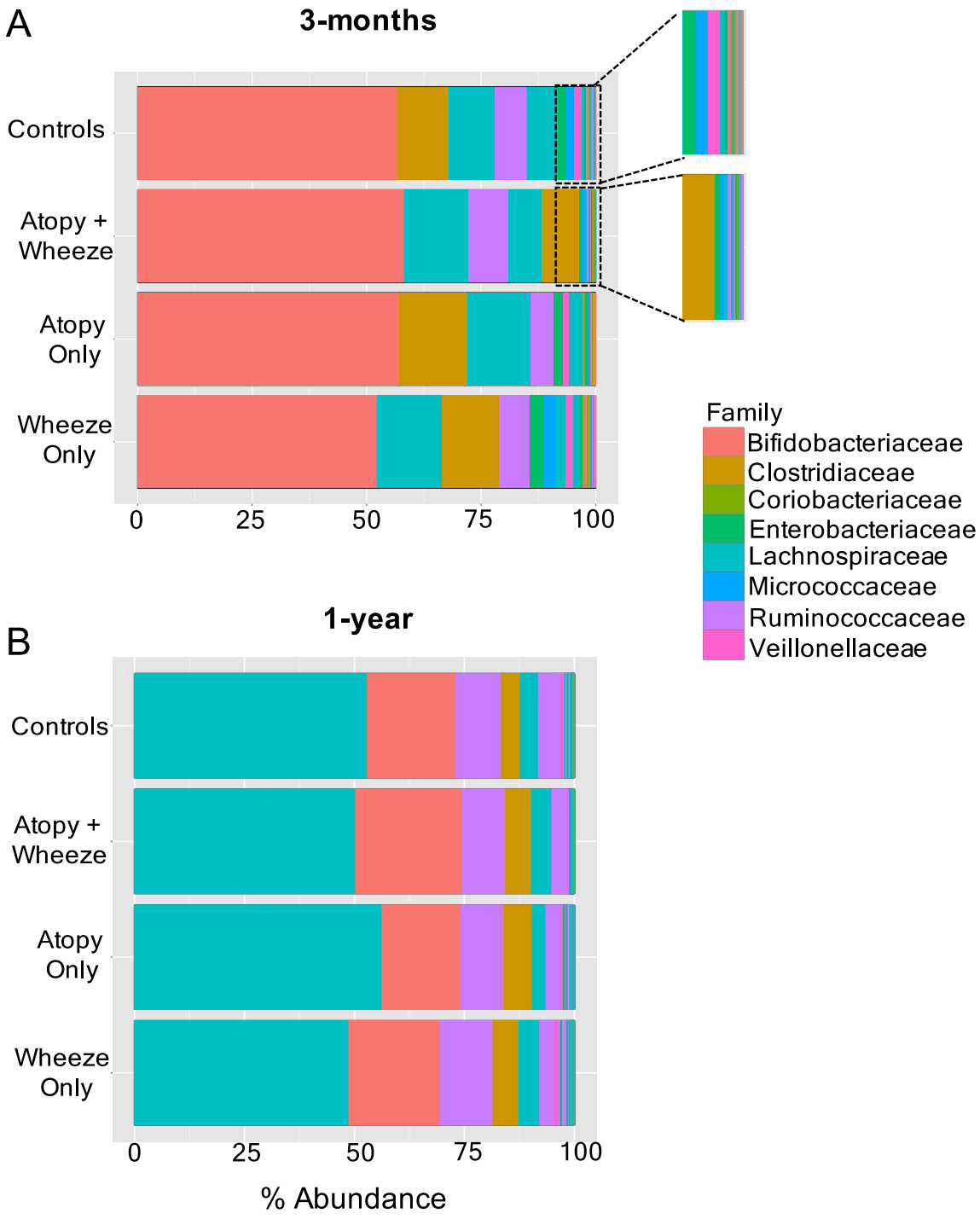

Figure 3.4 Relative family abundance among the four phenotypes

A) Relative abundance of bacterial families within the top 100 OTUs among the four phenotypes at 3-months. **B)** Relative abundances of bacterial families (within the top 100 OTUs) among the

four phenotypes at 1-year. Rectangles represent specific OTUs, which are organized in order of abundance. Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

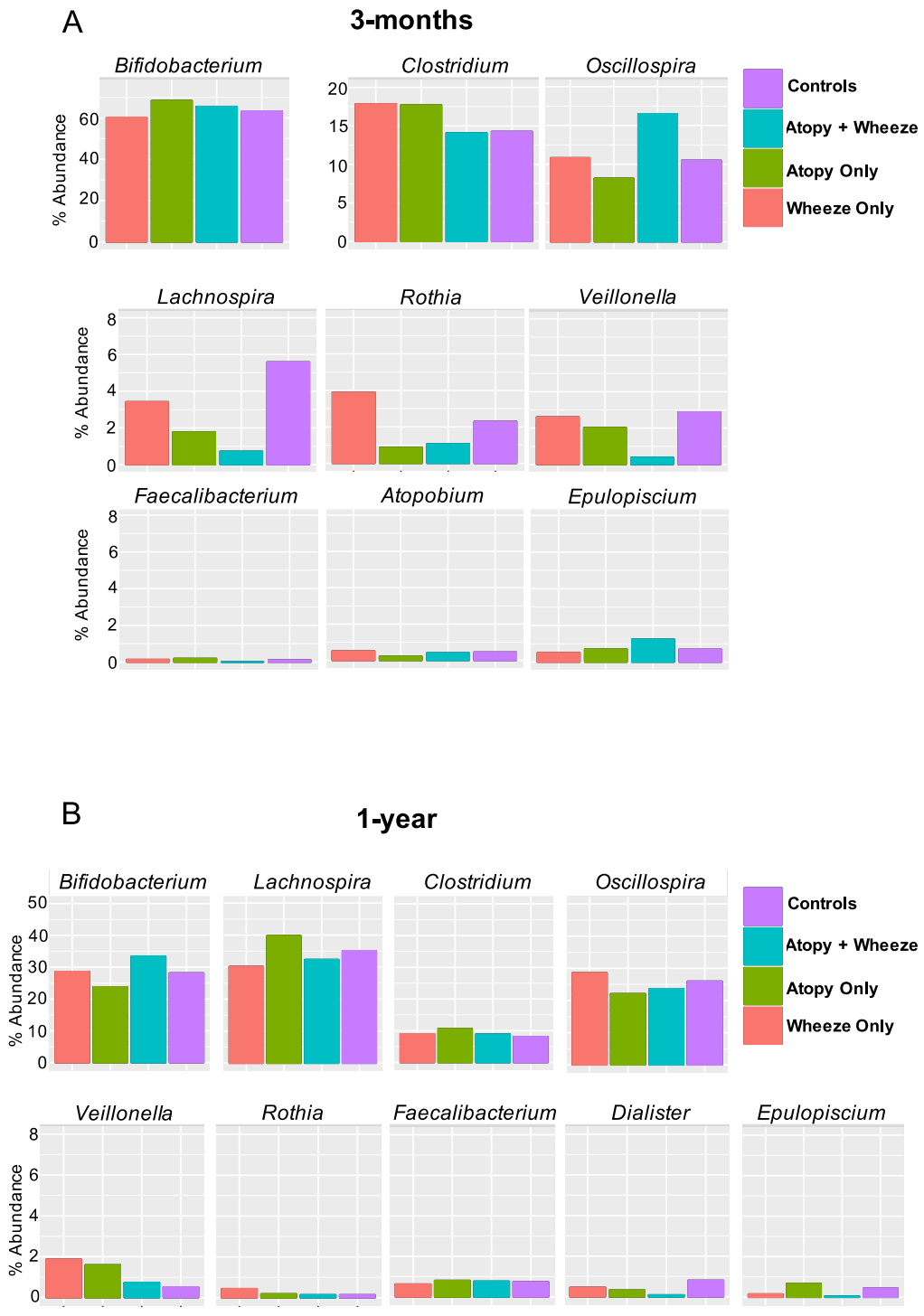

Figure 3.5 Relative genera abundance among the four phenotypes

Relative abundance of bacterial genera within the top 100 OTUs across the four phenotypes at **A)** 3-months and **B)** 1-year.

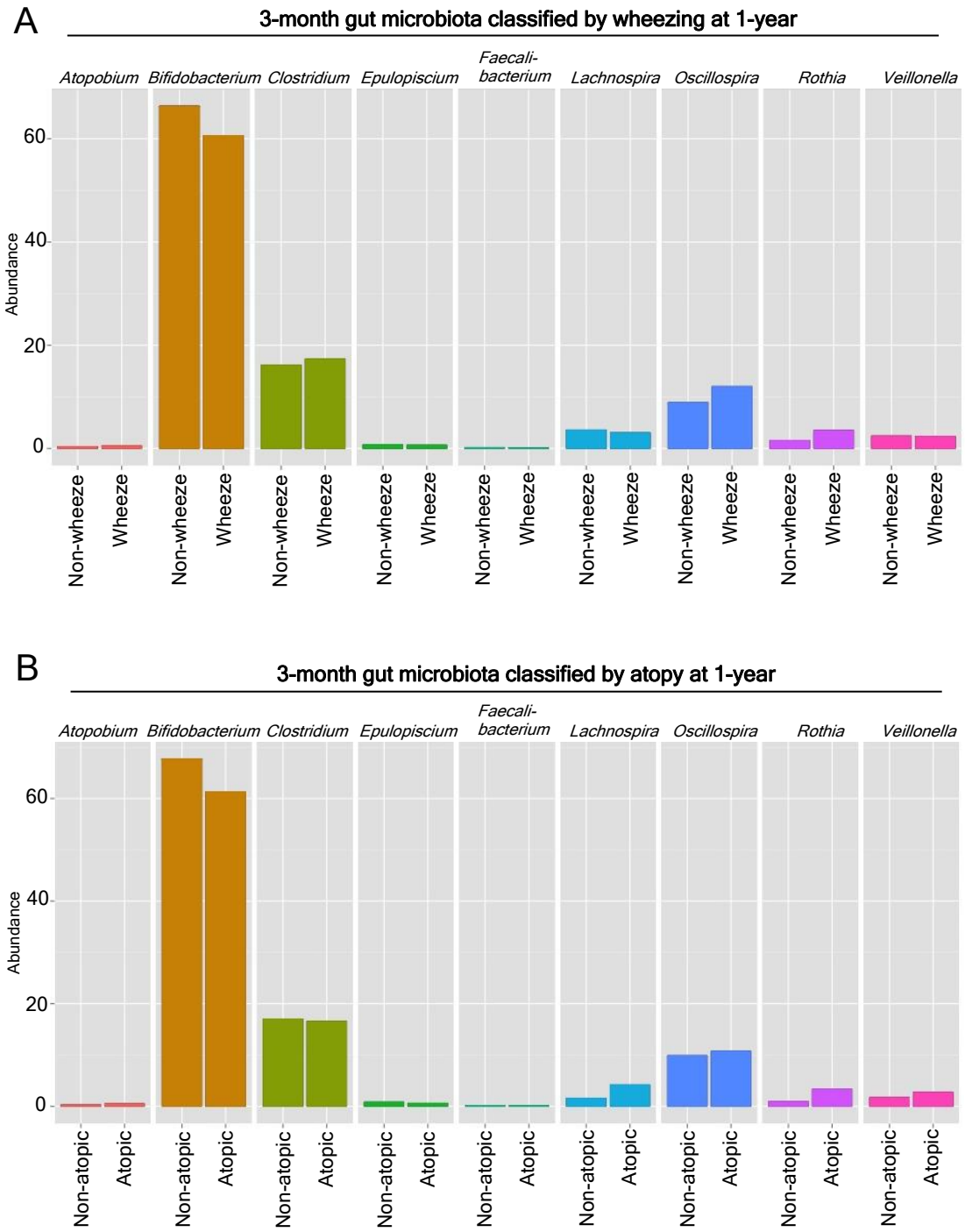

Figure 3.6 3-month fecal microbiota among A) wheeze/non-wheeze or B) atopy/non-atopy

Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

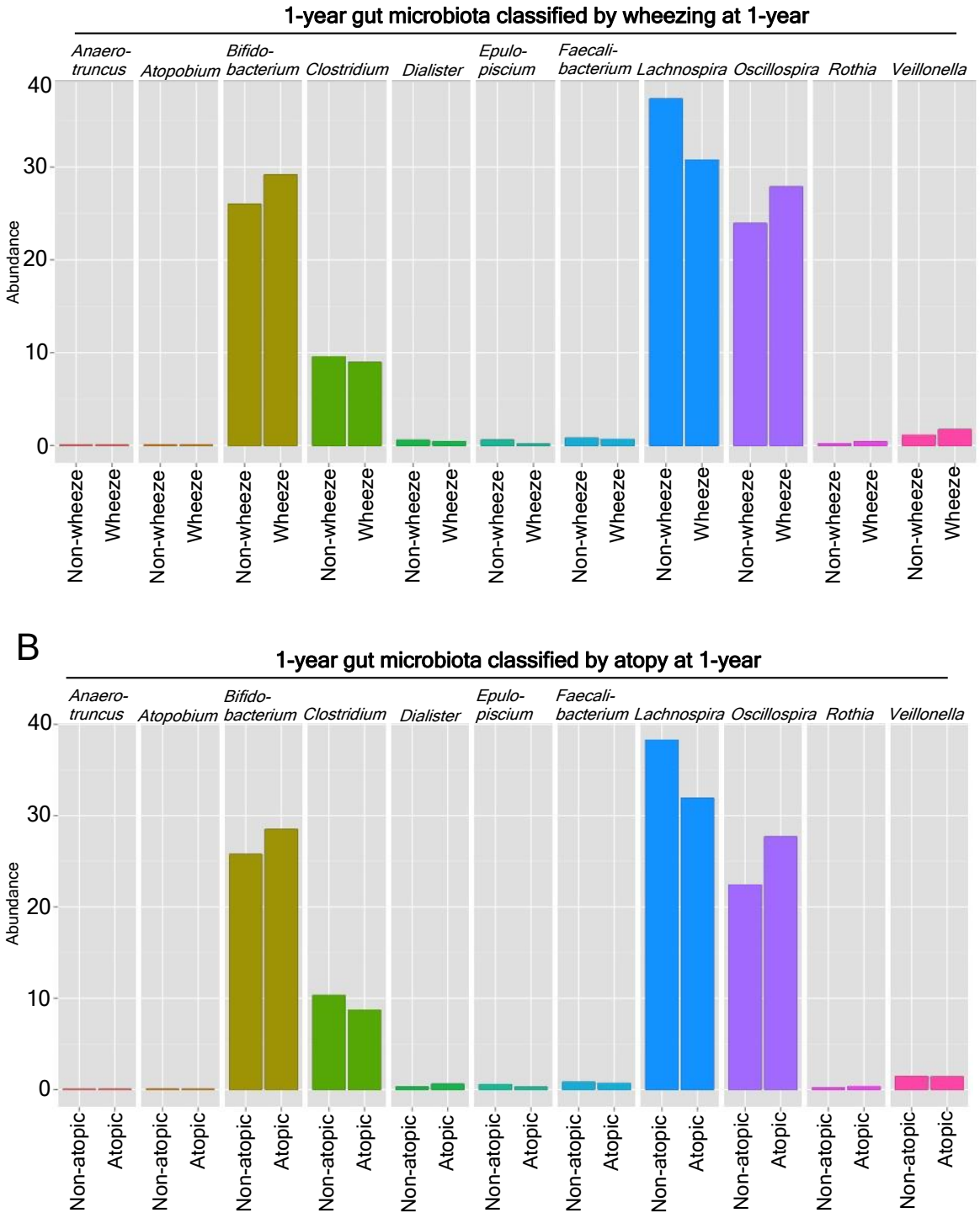

Figure 3.7 1-year fecal microbiota among A) wheeze/non-wheeze or B) atopy/non-atopy

Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

3.4.3.2 qPCR validation of FLVR decrease in AW subjects

Based on the availability of samples, we analyzed a subset of subjects (n_{AW} = 21, $n_{Control\ 3\text{-}months}$ = 20, $n_{Control1\text{-}year}$ = 19), which we established were representative of the original cohort (n_{AW} = 22, $n_{control}$ = 74) based on an exact logistic regression model (**Table B.1**). qPCR confirmed significantly lower abundances of *Veillonella*, *Lachnospira*, *Rothia*, and *Faecalibacterium* in the 3-month AW stool samples compared to controls **(Figure 3.8,** Mann-Whitney P≤0.001**)**. The abundance of *Bifidobacterium,* a taxon with similar relative abundance in AW and controls, was measured to further validate the consistency between the 16S and the qPCR results. We were unable to validate primers for *Epulopiscium*, *Dialister,* and *Oscillospira*, likely due to a combination of the following; the need for higher specificity primers (i.e. potentially down to the species level), the low abundance of these taxa, and other inhibitory effects of the PCR setup.

Consistent with the 16S rRNA sequencing results, these differences were much less apparent in the 1-year stool (*Veillonella* and *Lachnospira* showed less significant differences (P≤0.05) whereas the other 3 genera were not significantly different), suggesting that reductions in these genera in the 3-month microbiota are associated with atopy and wheezing by 1-year of age (**Figure 3.8)**.

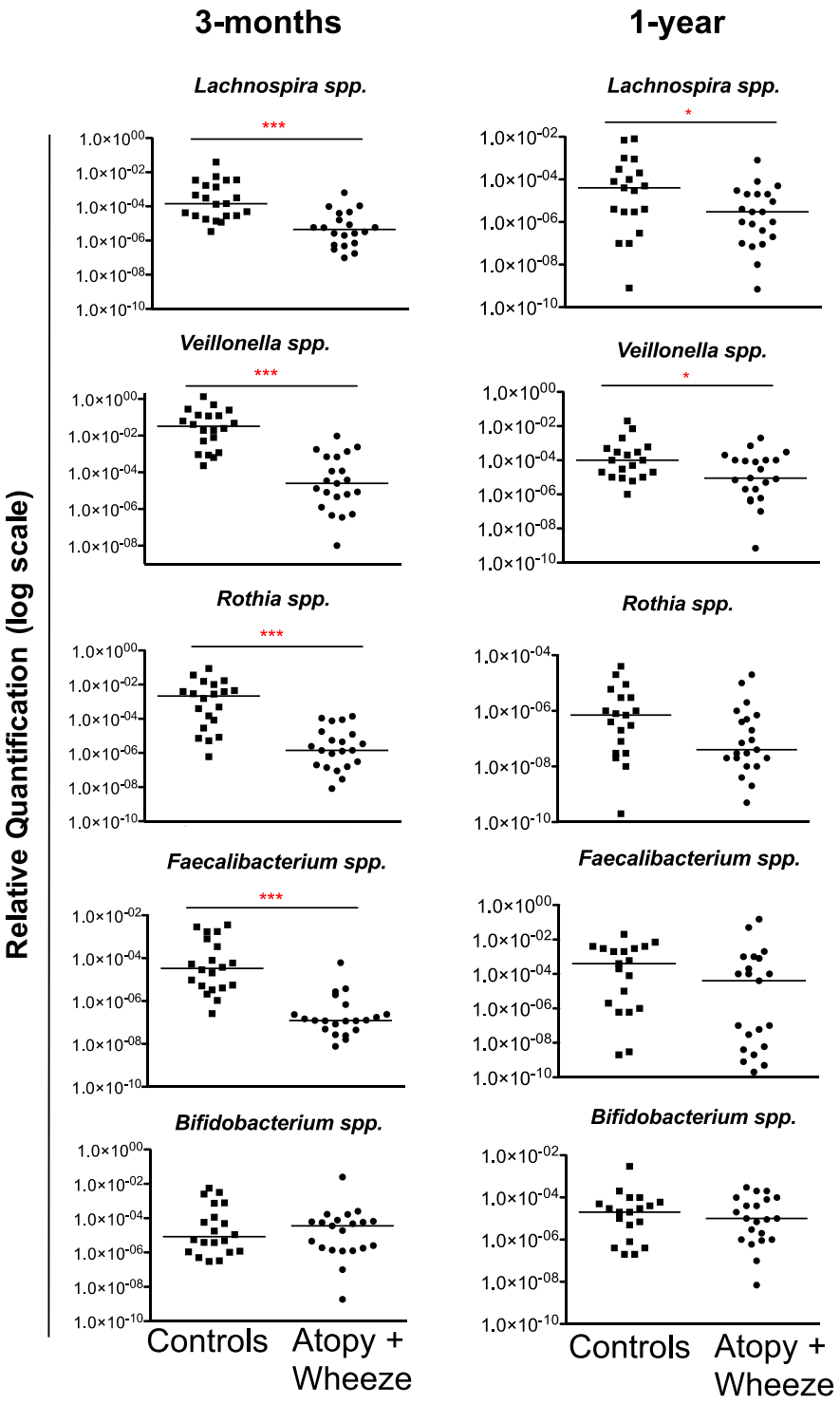

Figure 3.8 qPCR validation of 16S sequencing shows reduction in FLVR taxa among AW subjects

qPCR on selected genera relative to total 16S amplification in all AW fecal samples and a randomly selected subset of control fecal samples at 3-months (n_{CTRL} = 20 n_{AW} = 21) and 1-year

(n_{CTRL} = 19 n_{AW} = 21) [Mann Whitney; *Veillonella* p_{3mo} = 0.0001, p_{1y} = 0.0368; *Lachnospira* p_{3mo} = 0.0002, p_{1y} = 0.029; *Rothia* p_{3mo} = 0.0001, p_{1y} = ns; *Faecalibacterium* p_{3mo} = 0.0001, p_{1y} = ns; *Bifidobacterium* p_{3mo} = ns, p_{1y} = ns]. Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

3.4.5 Reduction in fecal acetate production associated with AW phenotype

The functional implications of the gut community in AW children were further investigated by measuring SCFA levels in feces. An exact logistic regression model was used to confirm that the control and AW subsets used for SCFA analyses were representative of the larger cohort (N_{AW} = 22, $N_{control}$ = 74) (**Tables B.2 – B.3)**. At 3-months of age, fecal samples of AW children had a significantly lower concentration of acetate (**Figure 3.9**) and there were no differences in the remaining SCFAs. A biweight correlation analysis between the top 50 OTUs organized into families and acetate did not show any significant correlations between specific taxa and acetate. This could be because the ability to ferment 6-carbon sugars and produce this SCFA is shared by several bacterial species belonging to phylogenetically distant taxa, or due to a difference in acetate intestinal uptake. Interestingly at one year, all SCFAs were significantly lower in the AW group except acetate, suggesting that a reduction in the concentration of this particular SCFA is specific to the 3-month time point. It is possible that the reductions in the other SCFAs at 1-year are due to shifts in bacterial populations not identified by our analyses. Nevertheless, changes in the concentration of these microbial-derived metabolites serve as a functional marker of gut dysbiosis in early infancy that is linked to asthma risk.

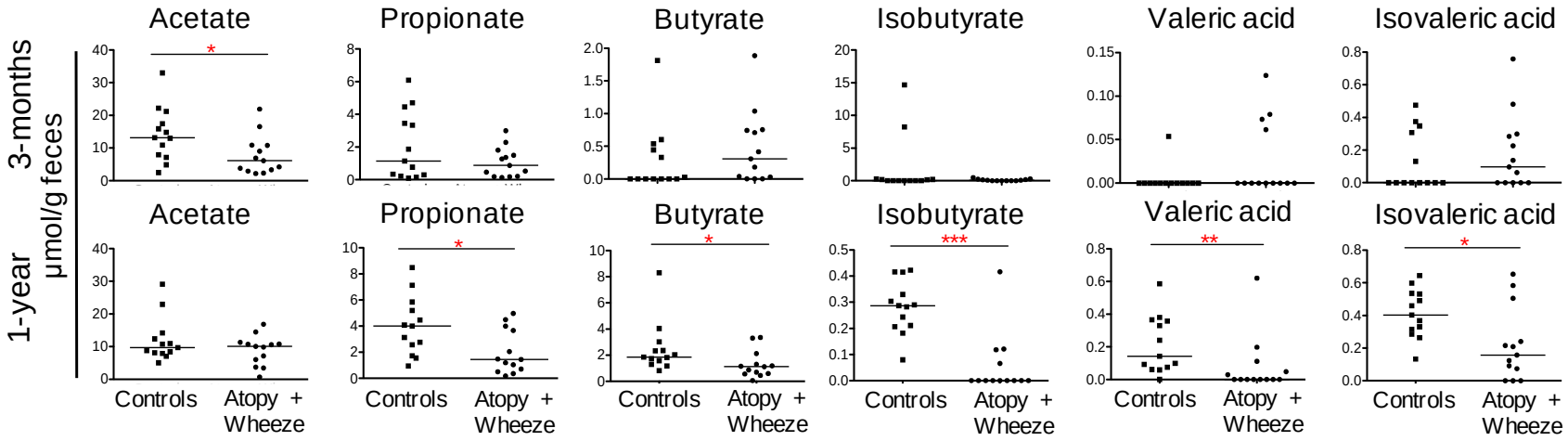

Figure 3.9 Fecal short chain fatty acid production at 3-months and 1-year

Concentration of SCFAs (µmol/g feces) in feces of AW and control samples at 3-months and 1-year of age, measured by gas chromatography and normalized to feces wet weight (n_{AW} = 13, $n_{Control}$ = 13, Mann-Whitney 3-months: acetate p = 0.03, remaining SCFAs are non-significant; Mann-Whitney 1-year: p acetate = ns, p propionate = 0.02, p butyrate = 0.02, p iso-butyrate = 0.0002, p valeric acid = 0.007, p iso-valeric acid = 0.02). Printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al.*, *Sci Trans Med* 2015.

3.5 Discussion

This study was designed to analyze the intestinal microbiome composition and functional potential in 1-year-old atopic wheezing subjects determined to be at high risk of asthma development. The major novel findings include:

a) Establishment of an early life critical window in which gut microbial dysbiosis is most influential in human immune development (specifically in the development of atopy and wheezing).

b) Identification of taxa-specific dysbiosis in the 3-month microbiota among 1-year old atopic wheezing subjects, characterized by reductions in four bacterial taxa (*Faecalibacterium, Lachnospira, Rothia,* and *Veillonella).*

c) A functional connection linking this taxa-specific early life gut microbial dysbiosis was also identified through SCFA analysis, as acetate was the only stool SCFA reduced in AW subjects at 3-months.

These findings indicate that in humans the first 100 days of life represent an early life 'critical window' in which gut microbial dysbiosis is associated with risk of asthma and atopic disease. Compositional shifts characterized by an analysis of overall bacterial diversity and specific bacterial taxa from 3-months to 1- year of age highlight the transient nature of the intestinal microbiota in the first year of infancy. This is consistent with previous studies, highlighting substantial shifts in gut microbial composition throughout the first two years of life

(86, 286). Further, early life gut microbial dysbiosis has been shown to have a much more profound effect on our immune development than disturbances that occur later in life, likely due to the subsequent development of the immune system during infancy (143, 146, 194, 209, 230, 266, 287).

Interestingly, the early life microbial dysbiosis characterized in atopic wheezing children is driven by reductions in four specific bacterial genera, *Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia*, rather than changes in overall community composition or diversity, which have been observed in previous studies (221, 288). Regarding the role of these particular taxa in atopic disease, the abundance of *Faecalibacterium prasnitzii* was reduced in the intestinal microbiota of atopic children aged 4 – 14 years, however this species was actually increased in subjects (children and adults) with atopic dermatitis (223, 289). *Veillonella* is a type of subgingival taxa and was reported higher in abundance in the upper respiratory tracts of adult controls when compared to adult asthmatic and chronic obstructive pulmonary disease (COPD) patients (290). Further, this taxon was also inversely associated with infant wheeze when sequenced from oropharyngeal samples (291). *Rothia* derived from mattress dust samples of children aged 6 – 12 years enrolled in the GABRIEL advanced study using denaturation gradient gel electrophoresis, was negatively associated with allergic rhinoconjunctivitis (292). Additionally, Clostridium clusters VI (includes *Faecalibacterium*) and XVIa (includes *Lachnospira*) have been shown to induce colonic T-regs and reduce OVA-specific serum IgE production in mice, further supporting their role in protecting against asthma and atopic disease (153). Notably, these studies suggest protective effects of all four of these bacterial taxa with regard to allergic disease, aside from (223), which suggests *Faecalibacterium* is promoting atopic dermatitis development. However none of the studies described here assess the intestinal

microbiome in infants. Our study shows decreases in these four taxa in the first 100 days of life, suggesting that therapeutic interventions to address this genus-specific gut microbial dysbiosis and potentially protect against asthma and atopic disease would need to occur very early in life.

Enhanced appreciation of the human microbiome has revealed that a compositional difference in bacterial communities does not necessarily translate to functional differences (293). We addressed this potential disconnect by measuring SCFAs and found a reduction in fecal acetate in the AW children at 3-months of age. At 1-year, all SCFAs were significantly lower in the AW group except for acetate. It is possible that these 1-year SCFA changes could have occurred due to reductions in other bacterial populations not detected by our sequencing technique or due to shifts in taxa we were unable to validate by qPCR (e.g. *Dialister* and *Epulopiscium*). Consequently, these SCFAs could also serve as biomarkers of atopy and wheezing in the first year of infancy.

This chapter features results from a collaborative manuscript published in *Science Translational Medicine* in September 2015. Consequently, I did not include results from portions of the manuscript, which I did not lead (including the PICRUSt analysis, lead by Dr. Dimitriu, and the OVA-challenged mouse model, lead by Dr. Arrieta). This mouse model was used as mechanistic approach to understand how these four bacterial taxa might influence lung inflammation. Adult germ-free mice were inoculated with stool from one AW subject or with the same human inoculum supplemented with live FLVR. The fecal microbiota of the subsequent generation (F1) was analyzed and was clearly distinguishable between mice supplemented with FLVR or not (205). However, regarding the SCFA production among the FLVR supplemented mice, we found an increase in butyrate production after supplementation with FLVR, with no changes in acetate production (205). Additional animal studies have implicated propionate,

acetate, and butyrate in protection against airway inflammation, and this protective effect has been attributed to the stimulation of T-regs and DCs capable of preventing Th2-type immune responses (121, 155, 196, 198). However, supporting the role of acetate as a functional mediator between the intestinal microbiota and atopic disease development in humans, one study obtained serum from pregnant women and identified a negative correlation between maternal acetate levels and percentage of infants requiring two or more general practitioner visits for cough or wheeze (198). Our results in combination with these previous studies suggest that in humans, acetate may be the mediator between early life gut microbial alterations and the development of IgE-mediated diseases, while in mice, these three major SCFAs all exhibit anti-inflammatory effects protective against lung inflammation.

Altogether, this study involves the largest human infant cohort for which gut microbial composition and functional potential has been assessed in the context of atopy, wheezing, and asthma risk. Further, we identified the first 100 days of life as the early life 'critical window' in which microbe-based intervention strategies to protect against childhood atopic disease could be initiated. However this study also has limitations. We did not identify Bacteroidetes spp. as a prominent intestinal phylum in the stool samples at either time point, potentially due to biases associated with the particular hyper-variable region (V3) analyzed (79). However, it is unlikely that this bias would alter our results regarding FLVR, since we were able to validate the 16S sequence results by qPCR and showed that FLVR was able to ameliorate lung inflammation in a mouse model of experimental asthma (205). We were also unable to validate primers for the all the differentially abundant genera identified in the 16S sequencing analysis (*Oscillospira, Epulopiscium, Dialister*), thus it is entirely possible that these technical biases would hide shifts

in additional taxa at both 3-months and 1-year that are associated with atopy and wheezing in 1-year-old children.

Regarding the epidemiological analyses from Chapter 2, this chapter provides evidence of microbial dysbiosis among atopic and wheezing subjects, which are at a high risk of preschool age asthma development. However, the population of AW subjects was too small to elucidate whether these early life hygiene/microflora hypothesis related variables are contributing to this early life gut dysbiosis. Longitudinal exploration in larger-scale human cohorts beginning ideally before 3-months of age will be necessary to confirm the universality of our findings, further elucidate the role of these early life factors in the colonization of the infant gut, and better characterize this early life window.

Additionally, given that 16S rRNA sequencing provides researchers with only a compositional analysis of the microbiota, metagenomic sequencing in a similar cohort would provide a more in depth view of the compositional and functional changes among atopic/asthmatic and healthy children. Using the OVA-challenged mouse model, our group was able to show a potential causal role of the FLVR taxa in conferring protection against lung inflammation in these mice (205). However, future human microbiota colonization experiments in mice, aimed to improve the resemblance to the human infant microbiota, will help to determine the immune mechanisms by which FLVR suppresses allergic lung inflammation. Lastly, it is important to note that the children analyzed in this study were not diagnosed with asthma, but identified as high-risk subjects using early life clinical factors (skin prick testing and wheezing). Future analyses of the intestinal microbiota among preschool and school age asthmatic and healthy children will better determine the role of specific bacterial taxa in protecting and promoting human asthma.

3.6 Conclusions

In conclusion, our data support transient early life gut microbial dysbiosis, characterized by reductions in four bacterial genera (*Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia*), as an indicator of early life atopic disease (atopy and wheezing). We identified a reduction in fecal acetate, providing a mechanistic link between this taxa-specific dysbiosis that will prompt further functional studies to determine the role of SCFAs in asthma and atopic disease pathogenesis in humans. Together, our findings establish an important role for the early life intestinal microbiota in shaping immune system development, and enhance the potential for using rationally designed microbe-based therapies (e.g. pre- and probiotics) to prevent the development of asthma and atopic diseases that begin in childhood.

Chapter 4: Opposing shifts in specific bacterial taxa in early life are associated with increased risk of preschool-age asthma

4.1 Synopsis

Asthma is a chronic disease of the airways affecting one in ten children in Westernized countries. Recently, our group showed that specific bacterial genera in early life are associated with atopy and wheezing in one-year-old children. However, little is known about the link between the early life gut microbiome and the diagnosis of asthma in preschool age children. To determine the role of the gut microbiota in preschool age asthma, children up to 4 years of age enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) study were classified as asthmatic (n = 39) or matched healthy controls (n = 37). 16S rRNA sequencing and quantitative PCR (qPCR) were used to analyze the composition of the 3-month and 1-year gut microbiome of these children. At 3-months the abundance of the genus, *Lachnospira* (L), was decreased (p = 0.008), while the abundance of the species, *Clostridium neonatale* (*C. neonatale*, C), was increased (p = 0.07) in asthmatics. Quartile analysis of stool composition at 3-months revealed a negative association between the ratio of these two bacteria (L/C) and asthma risk by 4-years of age (quartile 1: Odds ratio (OR) = 15, p = 0.02, CI = 1.8 – 124.7; quartile 2: OR = 1.0, ns; quartile 3: OR = 0.37, ns). We conclude that opposing shifts in the relative abundances of *Lachnospira* and *C. neonatale* in the first 3 months of life are associated with preschool age asthma, and that the L/C ratio may serve as a potential early life biomarker to predict asthma development.

4.2 Introduction

Asthma is a multifactorial disease driven by both genetic and environmental factors. While there have been remarkable improvements in the treatment of asthma over the past few decades, there are currently no preventative treatments and asthma remains the most prevalent childhood disease (affecting one-in-ten children) in many countries (1). Multiple lines of evidence suggest that environmental factors contribute to the development of asthma, particularly the geographical disparity in disease prevalence and the observation that asthma rates have increased considerably since the 1980s—all within a single human generation (18). The Microflora Hypothesis suggests that early life perturbations, driven by environmental factors such as antibiotic exposure and mode of birth (vaginal vs. C-section), alter the bacteria populating the intestine (i.e. cause dysbiosis) and disrupt the natural microbiota-immune cell interface critical in promoting immune tolerance (233). Instead this dysbiosis skews the immune system toward immune-mediated and hypersensitivity disorders (294, 295).

The intestinal microbiota has been implicated as a potential therapeutic target for the prevention of IgE-mediated hypersensitivity diseases (146, 205, 221, 222). In the previous chapter, our group associated early life decreases in four bacterial genera, *Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia* (nicknamed FLVR), with atopy and wheezing in 1-year-old children enrolled in the CHILD Study (205). However, further research assessing the role of specific gut bacteria in the development of asthma in preschool age children is necessary before preventative treatments for this burdensome disease can be established.

Here, we describe results assessing the intestinal microbiome composition among children diagnosed with asthma by four years of age and control children with no history of atopy, wheezing, or asthma. We show that opposing shifts in the abundance of two Clostridial

taxa, *Lachnospira* and *C. neonatale*, are associated with the diagnosis of asthma by age four years. We quantify this gut dysbiosis by calculating the ratio of *Lachnospira/C. neonatale* and show an inverse correlation between this ratio in the first three months of life and the odds of developing asthma by four years of age. This ratio, in combination with the individual shifts in these two taxa in the first 100 days of life, may have potential important clinical implications with regard to asthma diagnosis and prevention.

4.3 Materials and methods

4.3.1 CHILD study design and ethics approval

Details of the CHILD Study are described in **Section 2.3.1.**

A parent or legal guardian gave signed informed consent and all research protocols for the following studies in human samples were approved by The University of British Columbia/Children's and Women's Health Centre of British Columbia Research Ethics Board.

4.3.2 Subject Classification

This study is based on a nested case-control design and comprised of subjects enrolled in the CHILD Study that were analyzed in our previous report (205). Only children with complete 3-year data from the CHILD Study were included in this analysis (286 total subjects) and classified as follows. If a subject received a physician diagnosis of asthma by four years of age or was prescribed inhaled asthma medications (inhaled corticosteroids or bronchodilators) from three – four years of age, they were included in the asthmatic group (n = 39). To be classified as

controls (n = 37) subjects were required to be negative for asthma or inhaled medication use, negative for atopy (based on standardized allergen skin prick testing at one- and three-years of age) and negative for wheezing (based on questionnaire analysis repeated 6 times from birth – four years of age combined with clinical assessments at ages 1 and 3 years). See **Section 2.3.1** for details on wheezing and allergen skin prick testing.

4.3.3 Definitions of clinical variables

Antibiotic exposure: Continuous covariate defined by the number of oral and/or intravenous episodes of antibiotics from birth to 3-months or birth to 1-year of age.

Atopic dermatitis or Eczema: 'Yes' = diagnosed with atopic dermatitis (also referred to as eczema is a chronic skin disease characterized by itchy, inflamed skin) at 3-months (reported in 3-month CHILD health questionnaire) or at 1-year (diagnosed by a CHILD clinician at the 1-year clinical assessment or a non-CHILD clinician as reported in one-year CHILD health questionnaire). 'No' = no diagnosis.

Feeding methods: Continuous covariate defined by the duration (in months) a child was breast-fed.

Parental history of asthma: Defined as neither parent having asthma versus at least one parent having asthma. Reference level is neither parent.

Mode of birth: Reference is cesarean section birth.

Sex: Reference is female.

4.3.4 Microbial community analysis

Full details regarding our 16S rDNA extraction, PCR amplification, and bioinformatics have been previously described (see **Section 3.3.4**) (205). Briefly, DNA was extracted from 3-month and 1-year stool samples using Mo-bio dry bead tubes (Mo Bio Laboratories), the Fastprep homogenizer (FastPrep Instrument, MP Biochemicals) or the Disruptor Genie (Scientific Industries, Inc.) and the Qiagen DNA stool mini kit.

DNA samples were amplified by PCR in triplicate using barcoded primer pairs spanning the V3 region of the 16S gene (205, 268). V3 PCR amplicons were sequenced using Hi-Seq 2000 bidirectional Illumina sequencing (Macrogen Inc.). Sequences were quality filtered and denoised using Mothur (269) and clustered into operational taxonomic units (OTUs) using CrunchClust (270). Clusters were classified against the Greengenes Database according to 97% similarity (Levenshtein distance = 5) (271). OTUs with a frequency less than five among all samples were excluded.

4.3.5 qPCR primer design and validation

Sequences for the 16S rRNA genes of the bacterial genera and species of interest and of closely related bacteria were aligned by CLUSTAL-W using MEGA6 alignment explorer and inspected for conserved and variable regions. Based on this analysis, we designed genus-specific primer candidates for *Lachnospira* and *Rothia* and species-specific primer candidates for *C. neonatale*. Primer candidates were assessed for specificity against all bacterial sequences using

Primer-Blast. The primer melting temperature, secondary structure and dimer formation, and G+C content were analyzed using OligoAnalyzer3.1 (Integrated DNA Technologies). Primer pairs meeting all these requirements were validated using the standard curve method in metagenomic DNA extracted from human fecal samples (**Table 4.1**).

4.3.6 Quantitative PCR conditions

Each 10µL reaction contained 5µL of IQ SYBR green supermix (Bio-Rad), 0.1µL of each forward and reverse primer, 0.8µL of nuclease-free water, and 4µLof fecal DNA extract. All reactions were carried out in the ViiA 7 Real-Time PCR System (Life Technologies Inc.) under the following conditions: an initial step at 95 ºC (5 min), 40 cycles of 15s at 94 ºC, 30s at the specific annealing temperature for each primer set (**Table 4.1)**, 30s at 72 ºC (*C. neonatale*, *Veillonella* (205), and Bacteria (296)) or 20s at 72 ºC (*Rothia*, and *Lachnospira*), and a final cycle of 95 ºC at 15s, 60 ºC at 1 min, 95 ºC at 15s, and 60 ºC at 15s. All samples were run in triplicate and normalized according to the ΔC_T method using total 16S rDNA (Bacteria (296), **Table 4.1**) as the reference gene. Samples with Ct values for Bacteria that were two standard deviations higher than the total mean (based on all Bacteria Ct values for 3-months and 1-year), indicating very low baseline levels of 16S DNA, were excluded from the analysis.

Table 4.1 qPCR primer sequences, annealing temperatures, and product sizes

Bacterium	Forward Sequence	Reverse Sequence	Annealing Temperature (°C)	Product Size (bp)
Lachnospira	GTAAAGGGAGTGTAGGTGGCA	AACAGTTTCAATAGCAGTTCCG	58	78
Rothia	AGGCTTGACATATACTGGACCG	CCATGCACCACCTGTATACC	61	72
Veillonella (205)	AAGCTATCACTGAAGGAGGG	TCCCAATGTGGCCGTTCATCC	60	110
Clostridium neonatale	GCTCTGTCTTTAGGGACGAT	CTCAAGTTGAGCCCGAGTAT	54	171
Bacteria (296) (total 16S)	TCCTACGGGAGGCAGCAGT	GGACTACCAGGGTATCTAATCCTGTT	60	442

4.3.7.1 Multivariate logistic regression analysis

Statistical significance was defined as $P \leq 0.05$. Using the glm2 package in R, a logistic regression model was used to evaluate potential associations between the clinical variables and the asthmatic group (**Table 4.2**) (276). Missing data was imputed with the mode of the data set for categorical variables. We report the odds ratio (OR) and the corresponding confidence intervals. ORs above 1 imply an increased likelihood that a child would develop asthma, while ORs below 1 imply a decreased likelihood. This same model was used to confirm that all subsets of 3-month and 1-year asthmatic and control samples used in this study were representative of the larger cohort $n_{Asthmatic} = 39$, $n_{Control} = 37$, **Tables C.1 – C.4**). Additionally this model was used to confirm that the 76 subjects analyzed throughout this chapter were representative of the larger CHILD cohort analyzed in Chapter 2 (**Table D.2**).

4.3.7.2 16S rRNA sequence analysis

The microbial diversity of the fecal microbiota (based on the Shannon alpha diversity index) of asthmatics and controls was analyzed in Phyloseq (283). Deseq2 (297) was used to calculate the multi-inference adjusted p-values (based on false discovery rate, FDR) and log2 fold changes associated with differentially abundant OTUs between asthmatics and controls. Principal components analysis (PCA) was conducted using MetaboAnalyst (284, 285).

This study was based on a nested case-control design to study the intestinal microbiota among asthmatic and control children. La Rosa *et al.* report that power for microbiome analyses is associated with the number of reads per sample (298). *Post-hoc* power analysis of the 3-month 16S data, based on the read counts for the top 46 OTUs identified as differentially abundant by Deseq2, using the HMP R package for hypothesis testing and power calculations, resulted in a power calculation of 0.98; suggesting strong statistical power for the findings we report (298).

4.3.7.3 qPCR analyses

Differences between asthmatics and controls were assessed by the Mann-Whitney test. Differences between atopic, non-atopic asthmatics, and controls were assessed by the Kruskal-Wallis test and subject to the Dunn's multiple comparisons test. All qPCR analyses were carried out using GraphPad Prism version 5c.

4.3.7.4 Calculation of bacterial ratios and quartile analysis

All ratios (**Figures 4.4 & 4.5**) were calculated by dividing the relative quantification (RQ) values (or OTU read counts normalized to relative abundance) at 3-months and 1-year. Quartiles were calculated for the L/C ratio at both time points, *Lachnospira*, and *C. neonatale*

individually at 3-months and 1-year. Quartiles were categorized from low (quartile 1) to high (quartile 4) and were used to calculate ORs to determine if increases or decreases in these bacteria or ratios were associated with preschool age asthma development. ORs above 1 imply an increased likelihood of developing asthma; ORs below 1 imply a decreased likelihood.

4.4 Results

4.4.1 Characterization of the cohort

Of the 319 CHILD Study subjects with microbiome data at 3-months and 1-year of age, 286 subjects (analyzed in the previous two chapters) had reached three years of age at the time this study began in Spring 2015. Of these 286 subjects, 39 were classified as asthmatic based on physician diagnosis or having been prescribed inhaled medications used to treat asthma up to four years of age. To clarify, Chapter 2 describes asthmatic subjects diagnosed or prescribed asthma medications up to age 3, however for this study we used data up to age 4 years to increase the n number of the asthmatic group. For comparison, we identified 37 control subjects who had no evidence of asthma or allergic disease. These control subjects were negative for asthma and also negative for atopy and wheezing from birth to three years of age.

We found no significant differences in early life environmental exposures for this sample cohort when compared to the larger cohort analyzed in Chapter 2, suggesting that this sub-population of CHILD subjects is representative of the larger CHILD study cohort (Chapter 2 n = 2,695). Similar to the representative analysis in Chapter 3, because we did enrich for asthmatic subjects for this chapter, we did find significant differences between the groups according to parental asthma (OR = 2.11, CI = 1.34 – 3.39, p = 0.001, **Table D.2)**.

For this chapter, we did not identify any significant differences between asthmatic and control subjects according to gender, birth mode (vaginal vs. caesarean section), feeding practices (breast fed vs. formula fed), and antibiotic exposure using a multivariate logistic regression model **(Table 4.2)**. However in line with the results from Chapter 2, children diagnosed with atopic dermatitis at 1-year of age or those with parental history of asthma were more likely to develop preschool age asthma (OR 1-year AD: 5.36, CI = 1.06 – 22.87, p = 0.04; OR parental history = 4.52, CI = 1.54 – 13.46, p = 0.006, **Table 4.2**).

Table 4.2 Multivariate logistic regression analysis of key clinical variables

Variable		Phenotype		OR	95% CI		P-value
		Asthmatics	Controls		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	14 (36%)	5 (14%)	2.05	0.85	5.00	0.11
	None	25 (64%)	32 (86%)				
	Total (100%)	39	37				
Antibiotic Exposure (birth to 3-months of age)	1 or more	3 (8%)	2 (5%)	0.29	0.04	2.13	0.22
	None	36 (92%)	35 (95%)				
	Total (100%)	39	37				
AD at 3-months	Yes	7 (18%)	1 (3%)	3.1	0.18	53.0	0.44
	No	32 (82%)	36 (97%)				
	Total (100%)	39	37				
AD at 1-year	Yes	15 (38%)	3 (8%)	5.36	1.06	22.87	**0.04**
	No	24 (62%)	34 (92%)				
	Total (100%)	39	37				

Variable		Phenotype		OR	95% CI		P-value
		Asthmatics	Controls		Lower	Upper	
Sex	Female	18 (46%)	17 (46%)	0.83	0.27	2.48	0.73
	Male	21 (54%)	20 (54%)				
	Total (100%)	39	37				
Mode of birth	Cesarean	8 (21%)	5 (14%)	0.85	0.2	3.63	0.82
	Vaginal	31 (79%)	32 (86%)				
	Total (100%)	39	37				
Breast Feeding	Yes	38 (97%)	34 (92%)	0.97	0.85	1.1	0.69
	No	1 (3%)	3 (8%)				
	Total (100%)	39	37				
Parental Asthma	Neither parent	12 (31%)	26 (70%)	4.52	1.54	13.46	**0.006**
	At least one parent	27 (69%)	11 (30%)				
	Total (100%)	39	37				

Abbreviations: OR = odds ratio, AD = atopic dermatitis, CI = confidence interval.

4.4.2 Microbial community analysis by 16S ribosomal RNA gene amplicon sequencing suggests roles for *Lachnospira* and *Clostridium neonatale*

The global gut microbial community composition in stool samples taken at 3-months or 1-year of age did not differ between asthmatics and controls (as shown by principal components analysis and analysis of microbial diversity at 3-months and 1-year **(Figure 4.1**)**).** Beyond the analysis of global microbial community composition, we used Deseq2 with Benjamini-Hochberg adjustment (for FDR at an alpha threshold of 0.1) to identify differentially abundant OTUs

between asthmatics and controls at 3-months or 1-year; with statistical significance defined as P≤0.05. At 3 months of age, five differentially abundant OTUs were identified (**Figure 4.2A, Table 4.3**). Of note, OTUs 4 (*C. neonatale*, p = 0.076) and 32 (Clostridiaceae, p = 0.005) were increased in the asthmatic group (**Figure 4.2A, Table 4.3**) while OTUs 5 (Clostridiales, p = 0.046) and 3 *(Lachnospira*, p = 0.098) were decreased in asthmatics. At 1 year of age, six differentially abundant OTUs were identified. Of note, three of these OTUs were classified into the family Lachnospiraceae (one was statistically significant; OTU 40, p = 0.032; **Figure 4.2B, Table 4.3**). Additionally, two other FLVR bacteria (*Veillonella* and *Rothia*) were increased in asthmatics at one-year, though only *Rothia* was statistically significant (p = 0.003; **Figure 4.2B, Table 4.3**).

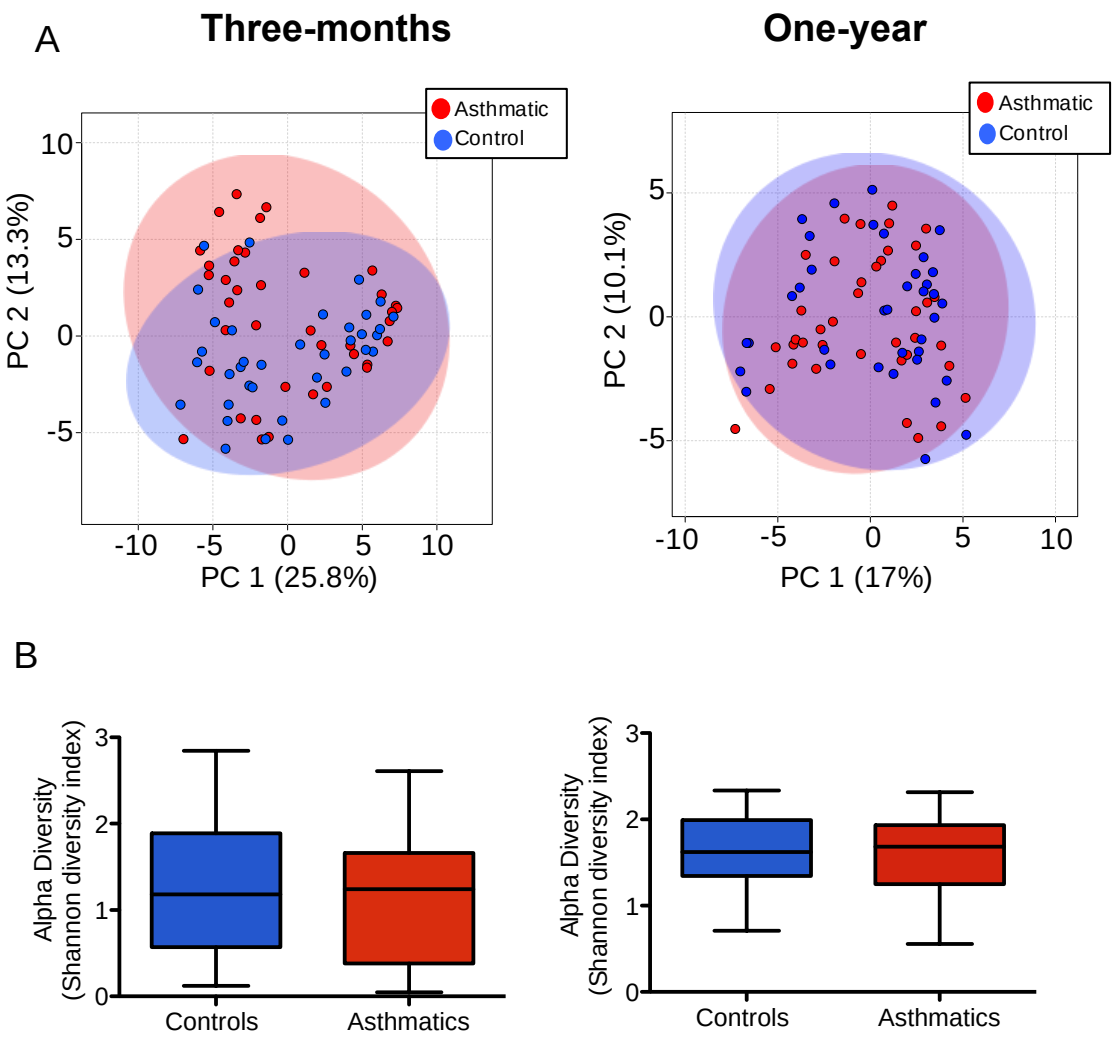

Figure 4.1 Global community analysis of the 3-month and 1-year fecal microbiota among asthmatics and controls

A) Multivariate analysis by PCA among asthmatics and controls at 3-months and 1-year. **B)** Alpha diversity (Shannon diversity index) of the gut microbial community between asthmatics and controls at 3-months and 1-year (shown as box plots, upper and lower hinges correspond to the first and third quartiles (25th and 75th percentiles). Originally published in Stiemsma *et al.* *Clinical Science*. Sep 2016.

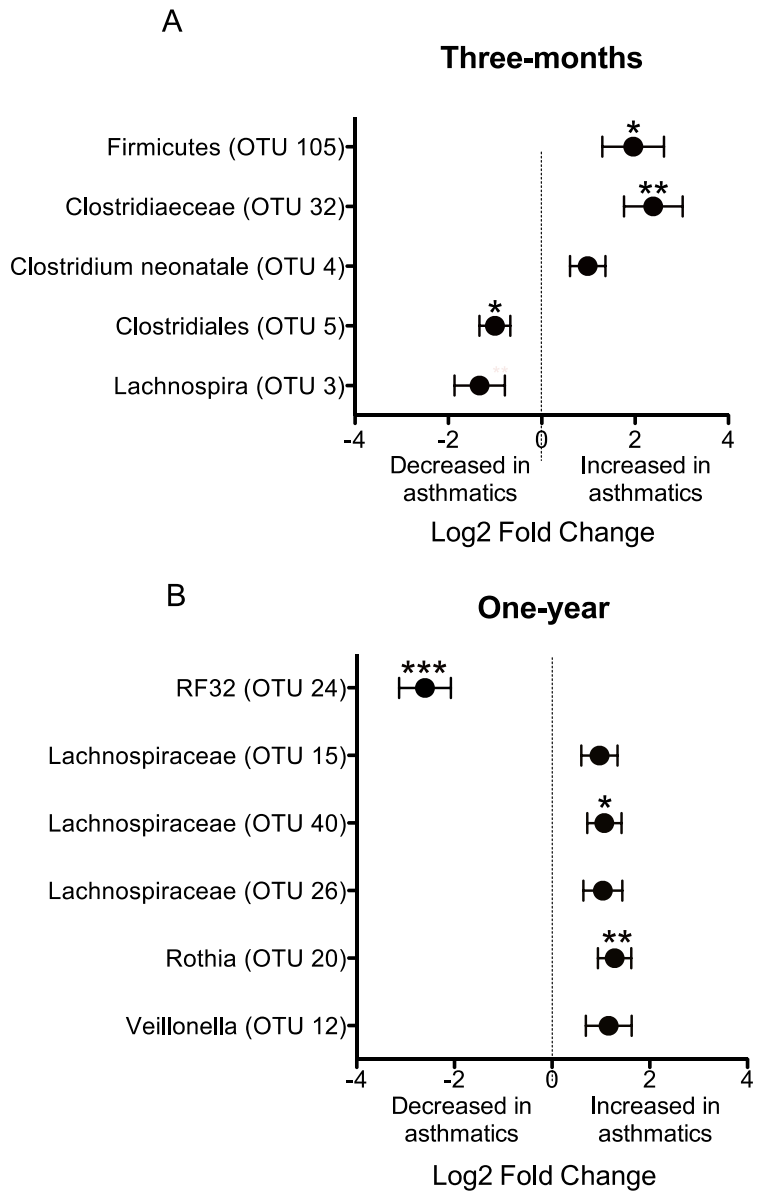

Figure 4.2 Differentially abundant OTUs identified by Deseq2 analysis at A) 3-months and B) 1-year

Each circle represents a specific OTU. An alpha threshold of 0.1 after Benjamini-Hochberg (for FDR) correction was used as a cutoff to identify these OTUs. Significant OTUs are specified as follows; $p < 0.05$ *, $p<0.01$ **, $p<0.001$ ***, [3-months: Clostridiaceae OTU 32 $p = 0.005$; *C. neonatale* OTU 4 $p = 0.076$; Clostridiales OTU 5 $p = 0.035$; *Lachnospira* OTU 3 $p = 0.098$;

Firmicutes OTU 105 p = 0.035; One-year: RF32 OTU 24 p = $3.64e^{-05}$; Lachnospiraceae OTU 15 p = 0.078, OTU 40 p = 0.032, OTU 26 p = 0.078; *Rothia* OTU 20 p = 0.003; *Veillonella* OTU 12 p = 0.098]. N numbers; n asthmatics = 39, n controls = 37. Error bars represent standard error of the log2 fold change. Originally published in Stiemsma *et al. Clinical Science*. Sep 2016.

Table 4.3 Differentially abundant OTUs between asthmatics and controls in the 3-month and 1-year fecal microbiotas after Benjamini-Hochberg adjustment for FDR (alpha = 0.1)

OTU	Mean	log2 fold change (relative to asthmatic group)	Log2 fold change standard error	p-value	p-value adj.	Taxonomic Classification
Three-month OTUs						
4	794.94	0.99	0.38	0.009	0.076	Bacteria, Firmicutes, Clostridia, Clostridiales, Clostridiaceae, *Clostridium*, *neonatale*
5	198.89	-1.00	0.33	0.003	0.035	Bacteria, Firmicutes, Clostridia, Clostridiales
32	63.81	2.39	0.63	0.0002	0.005	Bacteria, Firmicutes, Clostridia, Clostridiales, Clostridiaceae
3	132.53	-1.33	0.54	0.014	0.098	Bacteria, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae, *Lachnospira*
105	11.31	1.96	0.66	0.003	0.035	Bacteria, Firmicutes
One-year OTUs						
20	15.61	1.28	0.34	0.0001	0.003	Bacteria, Actinobacteria, Actinobacteria, Actinomycetales, Micrococcaceae, *Rothia*
24	26.32	-2.6	0.53	$8.09e^{-07}$	$3.64e^{-05}$	Bacteria, Proteobacteria, Alphaproteobacteria, RF32
40	11.39	1.07	0.35	0.002	0.032	Bacteria, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
12	21.66	1.16	0.47	0.012	0.099	Bacteria, Firmicutes, Clostridia, Clostridiales, Veillonellaceae, *Veillonella*
15	54.87	0.97	0.37	0.008	0.078	Bacteria, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae
26	18.68	1.04	0.4	0.009	0.078	Bacteria, Firmicutes, Clostridia, Clostridiales, Lachnospiraceae

4.4.3 Independent validation of 16S ribosomal RNA sequencing

In an effort to identify, more specifically, bacteria that could be used as biomarkers or probiotic treatments for asthma, we chose to validate these sequencing findings only for those OTUs classified down to the genus level (i.e. *C. neonatale*, *Lachnospira*, *Veillonella*, and *Rothia*) using quantitative PCR (qPCR). 16S sequencing uses barcoded primers to amplify a hypervariable region of the 16S gene, while qPCR uses taxon-specific primers for amplification from metagenomic DNA. This makes qPCR an effective validation method for 16S sequencing results. Thus, informed by our findings from 16S sequence analysis (**Figure 4.2, Table 4.3**), we designed and optimized genus-specific primers for the genera, *Lachnospira* and *Rothia,* and species-specific primers for the species, *C. neonatale.* We used previously published primers for *Veillonella (205)* (**Table 4.1**, three-months $n_{asthmatic} = 33$, $n_{control} = 24$; one-year $n_{asthmatic} = 35$, $n_{control} = 28$). Subjects were included in this analysis based on sample availability and these subsets were determined to be representative of the larger cohort using a logistic regression model (**Tables C.1 – C.4**). qPCR identified a significant reduction in the abundance of *Lachnospira* in the 3-month fecal microbiota but not the 1-year fecal microbiota of asthmatics compared to controls (**Figure 4.3A,** Mann-Whitney $p_{3months} = 0.008$). No significant differences in the abundance of *Veillonella* or *Rothia* were observed between asthmatics and controls at 3-months or 1-year (**Figure 4.3C & D**). Further, analysis by qPCR did not confirm a significantly higher abundance of *C. neonatale* in asthmatics at 3-months (**Figure 4.3B**). At 1-year however, qPCR did identify a significantly lower abundance of this taxon in asthmatics (**Figure 4.3B**, Mann-Whitney $p = 0.02$).

Interpreting these results as fold-changes relative to the control group further elucidates these apparent shifts in abundance. According to these qPCR findings, at 3 months asthmatic subjects were colonized with 1/5 less *Lachnospira* and 31 times more *C. neonatale*. While at 1 year, asthmatics were colonized with 16-times more *C. neonatale* and showed no difference in *Lachnospira* colonization. These opposing shifts in *Lachnospira* and *C. neonatale* lead us to hypothesize that a ratio calculation of *Lachnospira/C. neonatale* may be a quantifiable indicator of dysbiosis in asthmatic subjects.

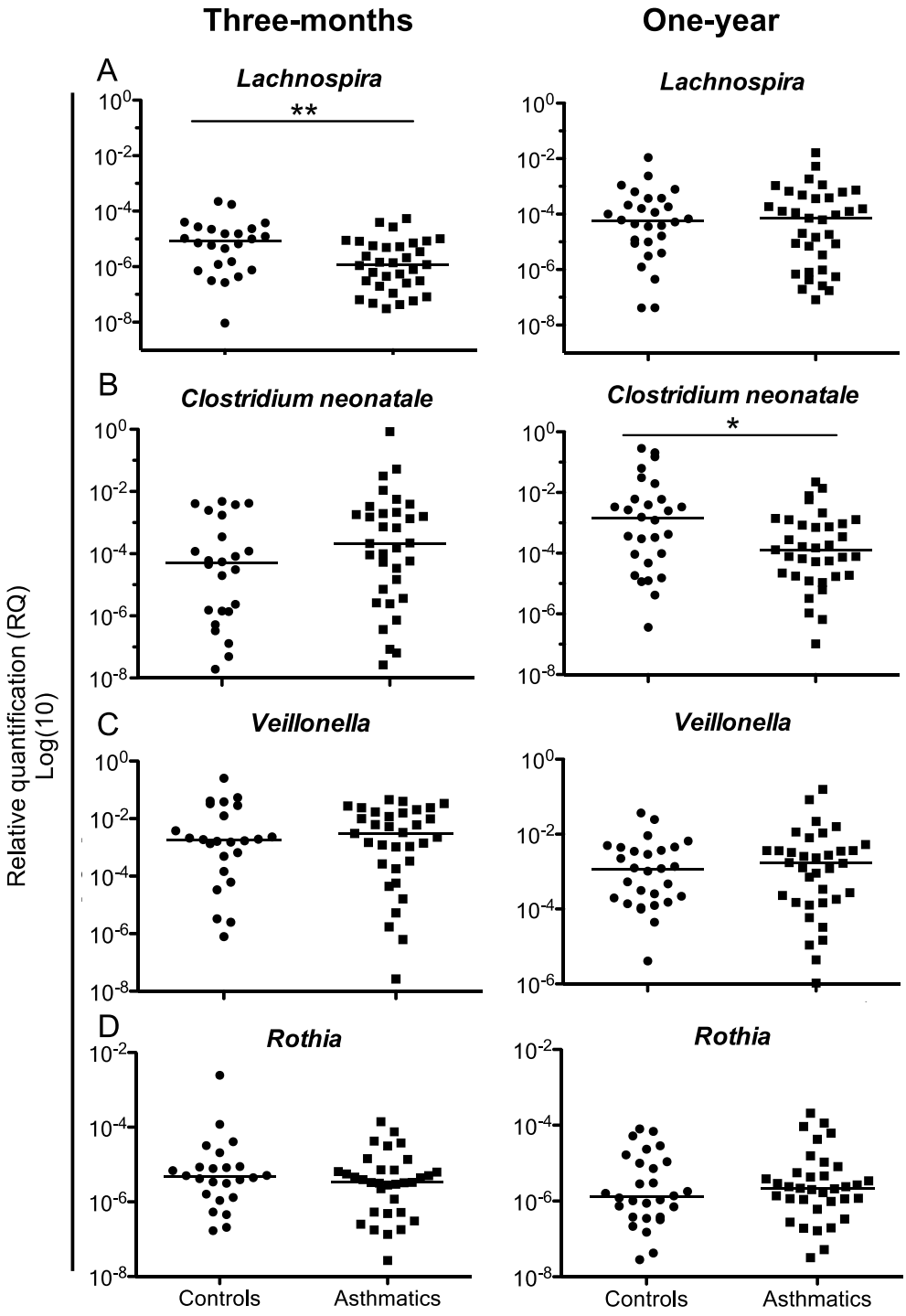

Figure 4.3 qPCR validation of 16S rRNA sequencing in the 3-month and 1-year fecal microbiota

A) qPCR quantification of *Lachnospira*: Mann Whitney: 3-months p = 0.008, 1-year (ns), **B)** qPCR quantification of *C. neonatale*: Mann Whitney: 3-months (ns), 1-year p = 0.02, **C)**

Veillonella (ns), and **D)** *Rothia* (ns) in the 3-month and 1-year gut microbiota. Line represents the median; n_{3mo} Asthmatic = 33, n_{3mo} Control = 24, n_{1Y} Asthmatic = 35, n_{1Y} Control = 28. Star representation; $p \leq 0.05$*, $p \leq 0.01$**. Originally published in Stiemsma *et al. Clinical Science*. Sep 2016.

4.4.4 *Lachnospira/C. neonatale* ratio to quantify dysbiosis

To assess if the relationship between these two bacteria is a quantifiable measure of dysbiosis related to preschool age asthma development, we calculated the ratio of *Lachnospira/C. neonatale* (L/C) for asthmatics and controls based on the relative quantification values from the qPCR analysis. At 3-months, the L/C ratio was significantly lower in asthmatics compared to controls (**Figure 4.4A,** Mann-Whitney p = 0.008). Calculating the ratio of *Lachnospira* to *C. neonatale* using the 16S rRNA read counts normalized to relative abundance confirmed this association (Mann-Whitney p = 0.0001). Interestingly, at 1-year a positive association was observed between the L/C ratio and the asthmatic phenotype (**Figure 4.4B**, Mann-Whitney p = 0.049), though the 16S rRNA read count ratio did not confirm this.

Notably, we did not identify any significant differences between asthmatics and controls after calculating ratios using the RQ values for *Veillonella* and *Rothia* in combination with *Lachnospira* and *C. neonatale* at 3-months (R/C, L/R, V/C, L/V, **Figure 4.5**). At 1-year we did identify significant differences between asthmatics and controls for both the R/C and V/C, suggesting that this decrease is mediated solely by the abundance of *C. neonatale*.

Further, the 3-month qPCR findings (specifically, the decrease in *Lachnospira* and the L/C ratio) are independent of antibiotic exposure, which is commonly associated with

disturbances to the intestinal microbiota (**Figure C.3 & C.4**). Sub-group analyses aimed at parsing out the specificity of these associations with atopic disorders in general did not identify significant differences between atopic and non-atopic asthmatics and the decreases in *Lachnospira* and the L/C ratio remained significant after excluding subjects diagnosed with AD at 3-months or 1-year or with parental history of asthma (**Figs. C.1, C.2, C.5 – C.10**). However, the decrease in *C. neonatale* and the increase in the L/C ratio at 1-year were not independent of these exposures. Thus in aggregate, these specificity analyses suggest that the diagnostic potential for these two particular bacterial taxa alone or as a ratio is greater if analyzed in the first 3-months of life (**Figs. C.1, C.2, C.5 – C.10**).

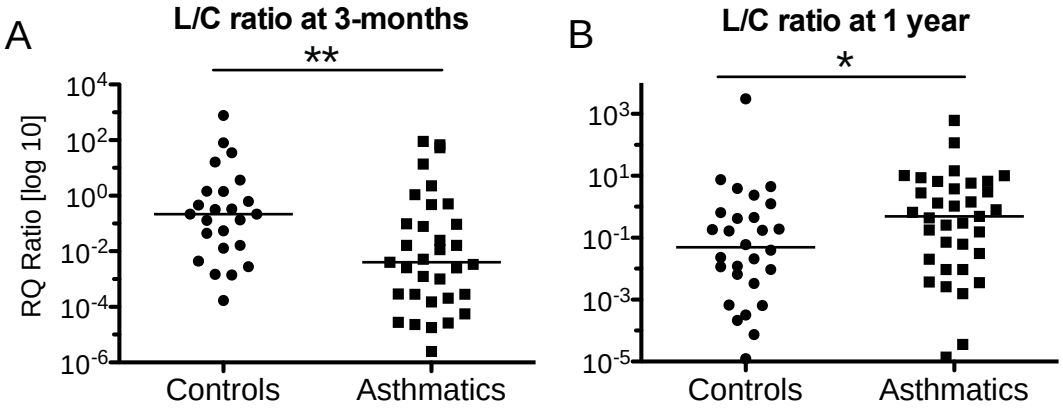

Figure 4.4 Ratio assessment of *Lachnospira* and *C. neonatale*

Ratio of *Lachnospira/C. neonatale* (L/C) relative quantification (RQ) values at **A)** 3-months and **B)** 1-year. Line represents the median; n_{3mo} Asthmatic = 33, n_{3mo} Control = 24; Mann Whitney p = 0.008; n_{1y} Asthmatic = 35, n_{1y} Controls = 28; Mann-Whitney p = 0.048. Stars indicate significant ORs; $p \leq 0.05$*, $p \leq 0.01$**. Originally published in Stiemsma *et al. Clinical Science*. Sep 2016.

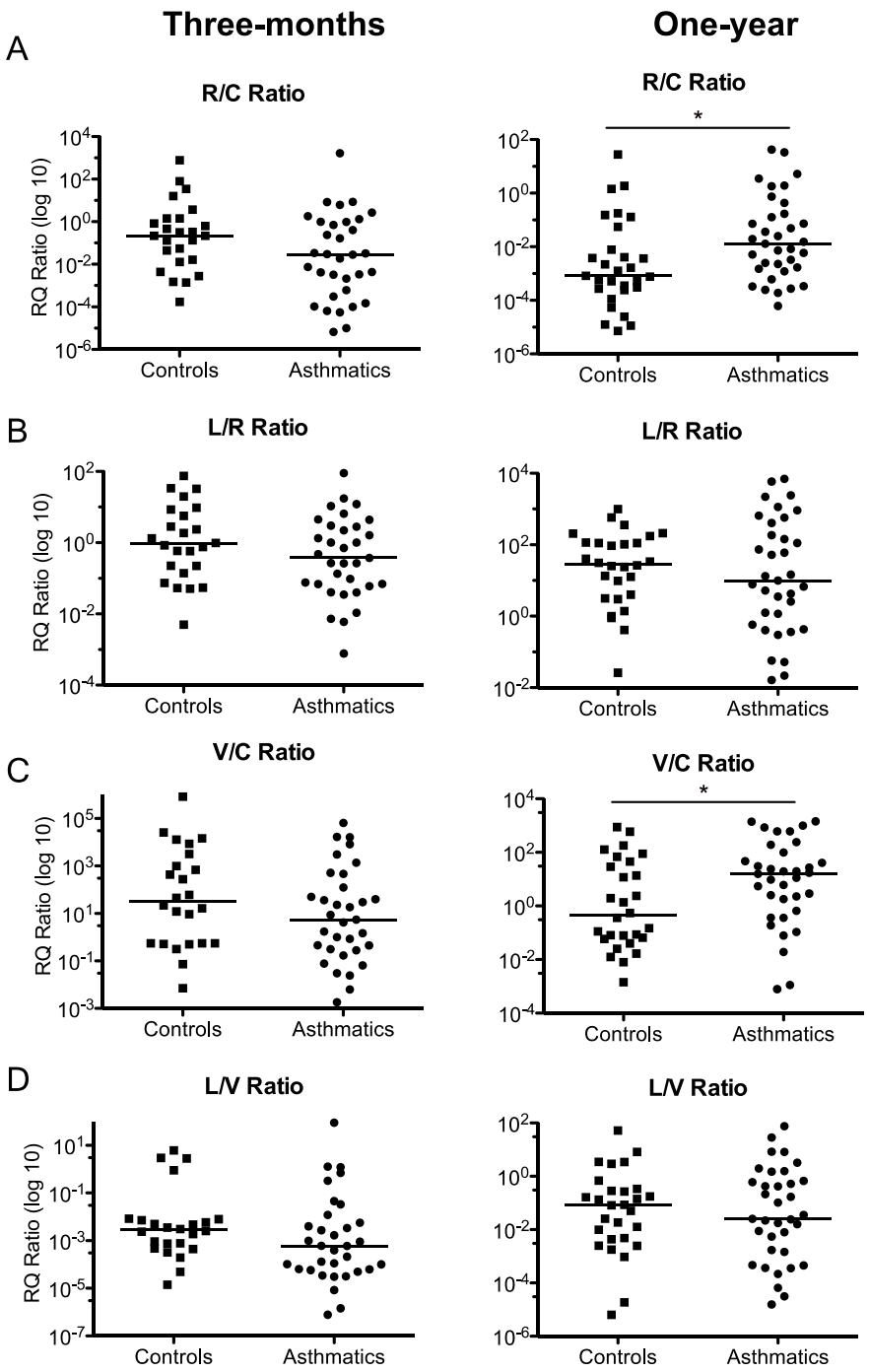

Figure 4.5 Additional ratio analyses

A) *Rothia/C. neonatale* (R/C) [Mann-Whitney p = 0.01], **B)** *Lachnospira/Rothia* (L/R), **C)** *Veillonella/C. neonatale* (V/C) [Mann-Whitney p = 0.03], and **D)** *Lachnospira/Veillonella* (L/V).

Star representation; p ≤0.05 *. Originally published in Stiemsma *et al. Clinical Science*. Sep 2016.

4.4.5 Quartile analysis of the *Lachnospira/C. neonatale* ratio

To assess this ratio at higher fidelity and to determine its potential as a microbe-based diagnostic technique, we analyzed the L/C ratios at 3-months and 1-year as quartiles. Quartiles were determined based on the median and range of the qPCR RQ values and allowed for the categorization of these values into dichotomous variables ranging from the lowest L/C ratios (quartile 1) to the highest L/C ratios (quartile 4). Odds ratios were calculated for each quartile; an odds ratio above 1 is associated with higher odds of developing asthma, while an odds ratio below 1 is associated with lower odds of developing asthma. At 3-months, the odds ratio of being classified into the asthmatic group decreases as the quartiles increase (as the ratio of L/C increases), with a plateau after quartile 3 (OR quartile 1 = 15, p = 0.004, FDR Adjusted p = 0.02; OR quartile 2 = 0.96, ns; OR quartile 3 = 0.37, ns; OR quartile 4 = 0.44, ns), suggesting a protective effect against asthma development associated with increases in the L/C ratio at three-months (**Figure 4.6, Table 4.4)**. At 1-year there were no significant associations, reinforcing the importance of the first 100 days of life as the critical window in which microbial biomarkers for identifying subjects at high risk of asthma are most applicable (**Figure 4.6**, **Table 4.4**).

In addition to the significant associations between the L/C ratio and asthma diagnosis, quartile analysis yielded similar trends when *Lachnospira* and *C. neonatale* were analyzed individually, but similar to the L/C ratio, these trends were only apparent at the 3 month time point (**Figure 4.7, Table 4.4**). Consequently, these results support quantification of microbial

dysbiosis in the first 3-months of life by calculating the ratio of *Lachnospira* to *C. neonatale*, but the individual effects of these two bacterial taxa should also be taken into account.

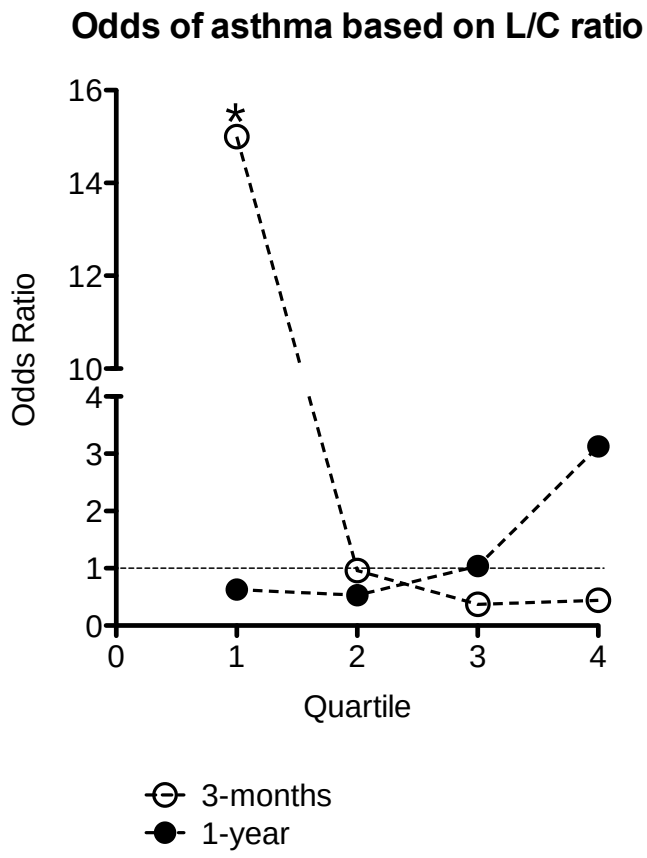

Figure 4.6 Quartile analysis of L/C ratio

Line graph representing the likelihood of asthma diagnosis based on quartile analysis of the L/C ratios at 3-months and 1-year (i.e. quartile 1 = low L/C ratio, quartile 4 = high L/C ratio). Points above the dotted line indicate increased odds of developing asthma; points below the dotted line indicate decreased odds of developing asthma. 95% CIs: 3-months - quartile 1 = 1.8 – 124.7, quartile 2 = 0.3 – 3.25, quartile 3 = 0.11 -1.24, quartile 4 = 0.3 – 1.5; 1 year – quartile 1 = 0.19 –

2.0, quartile 2 = 0.17 – 1.66, quartile 3 = 0.33 – 3.26, quartile 4 = 0.88 – 11.13. Stars indicate significant ORs; p≤0.05*. Originally published in Stiemsma *et al. Clinical Science*. Sep 2016.

Table 4.4 Quartile analysis of L/C ratio, *Lachnospira,* and *C. neonatale*

3-months												
Quartile	L/C Ratio				*Lachnospira*				*Clostridium neonatale*			
	OR	CI	P-value	Adj. P	OR	CI	P-value	Adj. P	OR	CI	P-value	Adj. P
1	**15**	**1.8 - 124.7**	**0.004**	**0.02**	3.5	0.85 - 14.34	0.12	0.24	0.3	0.08 - 1.05	0.07	0.28
2	0.96	0.3 - 3.25	1.0	1.0	1.4	0.41 - 5.0	0.76	0.76	0.93	0.28 - 3.25	1	1
3	0.37	0.11 - 1.24	0.13	0.26	1.65	0.48 - 5.68	0.55	0.73	2.17	0.59 - 8.02	0.35	1
4	0.44	0.13 - 1.5	0.22	0.59	0.12	**0.03 - 0.5**	**0.002**	**0.008**	1.7	0.48 - 5.7	0.55	1
1-year												
Quartile	L/C Ratio				*Lachnospira*				*Clostridium neonatale*			
	OR	CI	P-value	Adj. P	OR	CI	P-value	Adj. P	OR	CI	P-value	Adj. P
1	0.63	0.19 - 2.0	0.55	0.55	1.84	0.55 - 6.2	0.38	0.38	1.27	0.39 - 4.13	0.77	1
2	0.53	0.17 - 1.66	0.38	0.38	0.37	0.12 - 1.2	0.14	0.14	**4.92**	**1.23 - 19.58**	**0.02**	**0.04**
3	1.04	0.33 - 3.26	1	1	1.04	0.33 - 3.26	1	1	0.89	0.28 - 2.85	1	1
4	3.13	0.88 - 11.13	0.09	0.09	1.47	0.46 - 4.69	0.57	0.57	**0.15**	**0.04 - 0.55**	**0.003**	**0.01**

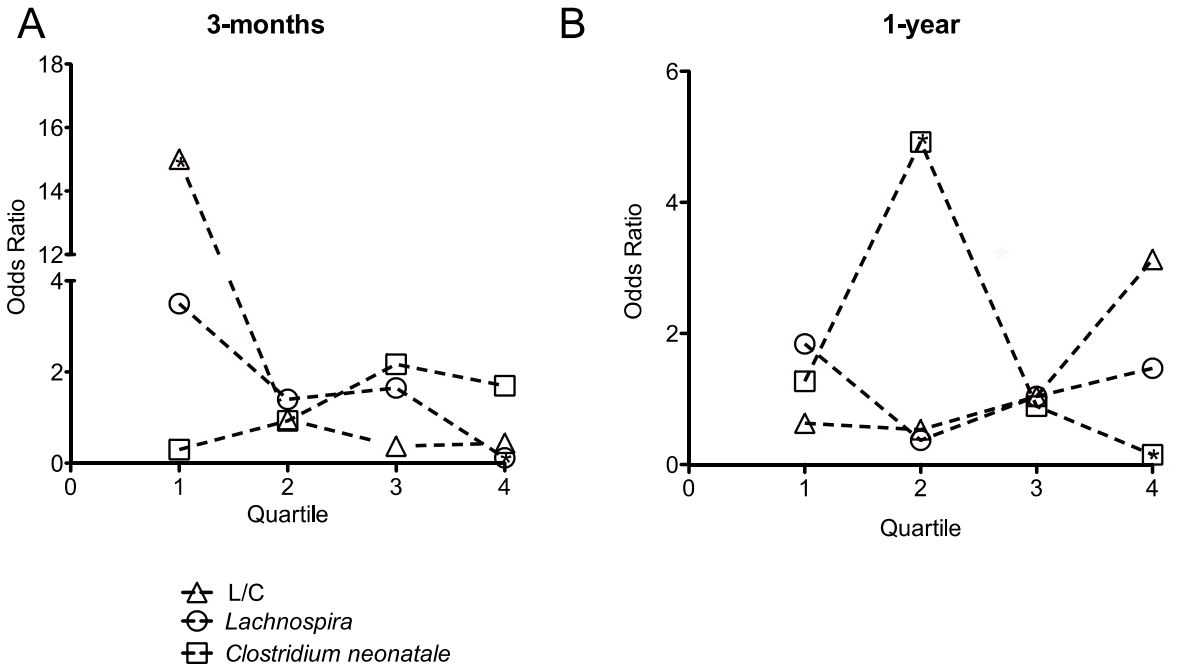

Figure 4.7 Quartile analysis at A) 3-months and B) 1-year for the L/C ratio, *Lachnospira*, and *C. neonatale*

Quartile 1 = low L/C ratio, quartile 4 = high L/C ratio. Star representation; p ≤0.05 *. Originally published in Stiemsma *et al. Clinical Science*. Sep 2016.

4.5 Discussion

This chapter was designed to compare the intestinal microbiota composition among children diagnosed with preschool-age asthma (by age 4-years) or prescribed inhaled asthma medications to a subset of controls negative for atopy, wheezing, and asthma. The major novel findings from this study include:

a) Identification of opposing shifts in the relative abundance of specific bacterial taxa, *Lachnospira* and *C. neonatale*, which are associated with asthma diagnosed by four years of age.

b) Assessment of these bacterial shifts as a ratio (L/C) represents a novel method of quantifying taxa-specific intestinal dysbiosis.

c) Quartile analysis of this ratio and of these two bacterial taxa individually could be used as a microbe-based diagnostic to identify subjects at high risk of developing preschool age asthma.

Through our assessment of the intestinal microbiome among asthmatic and control children, we found evidence of bacterial dysbiosis in the 3-month stool of children diagnosed with asthma at 4 years of age. Specifically, we found a reduction in the abundance of *Lachnospira,* and an increase in the species, *C. neonatale*, in the 3-month fecal microbiota of asthmatic children. These findings extend the previous chapter where we identified four bacterial genera (FLVR) that were less abundant in 3-month stool samples of children identified with atopy and wheezing at age one year by showing that a reduction in *Lachnospira* (one of the FLVR bacteria associated with atopic wheezing children) is a potential indicator of asthma diagnosed in preschool age children. Further, this study supports the first 3 months of life as the early life 'critical window' in which the human immune system is most influenced by changes in gut microbiome composition.

Both *Lachnospira* and *C. neonatale* are intriguing bacteria with biologically compelling links to asthma and allergic disease. Although little is currently known about *C. neonatale*, recent research has implicated this species in neonatal necrotizing enterocolitis and proposes its

classification into the *Clostridium* genus *sensu stricto* (Cluster I) (299). Consistent with our findings, *Clostridium* Cluster I has been positively correlated with atopic dermatitis in humans raising the possibility that this particular Cluster I species may play a role in other atopic disorders (such as asthma) (224). In addition to our previous work identifying a reduction in *Lachnospira* in children at the highest risk of asthma development, Clostridium Cluster XIVa (which includes *Lachnospira*) has been shown to promote colonic regulatory T cell accumulation and lower levels of OVA-specific IgE (153, 205). The individual opposing shifts in the abundance of *Lachnospira* and *C. neonatale* in the first 3 months of life suggest that these specific gut bacterial taxa play a role in protecting against (in the case of *Lachnospira*) or promoting (in the case of *C. neonatale*) the development of a preschool age asthmatic phenotype, in addition to their previously identified roles in other atopic disorders.

These findings are supported by analysis of the L/C ratio, which is significantly lower in asthmatics at 3 months of age. Associative quartile analysis of the L/C ratio with odds of asthma development further supports this association, with the odds of asthma development decreasing as the L/C ratio increases. This ratio was calculated as a quantifiable measure of dysbiosis based on two bacterial taxa, however this does not negate the associations observed with the two bacteria individually (specifically the reduction in *Lachnospira* at 3 months). Quartile analysis of the L/C ratio and *Lachnospira* at 3 months identified children at lower odds of developing asthma (L/C ratio: OR quartile 3 = 0.37, ns OR quartile 4 = 0.44, ns) with the *Lachnospira* analysis identifying children at the lowest odds (OR quartile 4 = 0.12, $p = 0.002$, adj. $p = 0.008$). Only quartile analysis of the L/C ratio, however, identified children with the highest odds of developing preschool age asthma (quartile 1 OR = 15, $p = 0.004$, adj. $p = 0.02$), an important clinical finding with regard to early asthma diagnosis and potential prevention of this disease.

For example, it could be possible to use the L/C ratio as a biomarker for the identification and prediction of subjects with increased potential to develop asthma later in life.

Collectively, these results expand on the current knowledge of the role of the intestinal microbiome in atopic disease, supporting the roles of specific gut bacteria in promoting or protecting against asthma development in children. However the etiology of asthma is complex, as asthma and other atopic disorders are highly intertwined through the 'atopic march' of disease progression in early childhood (12). The qPCR results at 3-months are not influenced by parental history of asthma or atopic dermatitis in the first year of life and we found no significant differences between atopic and non-atopic asthmatics, as highlighted in the sub-group analyses (**Figs. C.1, C.2 & C.5 – C.10**). However our study cohort was enriched for atopic children and the control subset chosen based on the absence of atopic disorders in the first three years of life, making it difficult to determine whether these particular bacteria are specific to asthma or also associated with other preschool age allergic diseases. The epidemiological analysis in Chapter 2 highlights the overlap among childhood atopic diseases (3-year-old asthmatic children were more likely to develop asthma if they were positive for atopy and wheezing at 1-year and atopic dermatitis at 3-months). Thus, it is possible that *Lachnospira* and *C. neonatale* are associated with other preschool-age atopic disorders and it will be important for future studies to determine the diagnostic and probiotic potential of these taxa in atopic diseases in general.

Regarding the role of other early life factors in the development of the intestinal microbiota, logistic regression analysis among this subset of CHILD Study subjects did not identify any hygiene/microflora hypothesis related variables as risk or protective factors for preschool-age asthma. Antibiotic exposure in the first year of life was associated with increased risk of asthma (OR = 2.05, p = 0.11), though this was not a statistically significant association

and does not does not confirm that this taxa-specific dysbiosis is caused by these early life factors. Further, the significant decrease in *Lachnospira* and the L/C ratio remained after sub-group analyses excluding subjects exposed to antibiotics in the first 3-months of life (**Figures. C.3 & C.4**), suggesting that antibiotics during this time period are not contributing to taxon-specific gut microbial dysbiosis associated with asthma development. Notably however, we did not identify antibiotics during this time point as a significant risk factor for 1-year atopy and wheezing or preschool-age asthma in our analyses in Chapter 2. Thus it remains possible that other variables identified in Chapter 2, such as birth mode, duration of breast-feeding, older siblings, gestational age, respiratory infections, and antibiotics given to the infant prenatally or at birth could be influencing the infant microbiota and the subsequent development of asthma in children. In the future, large cohort studies should combine epidemiological and microbiome analysis techniques to elucidate how these early life factors might influence the infant gut microbiome and subsequently, the development of preschool-age asthma.

Additionally, as identified in the previous chapter, this study supports the first 100 days of life as the early life 'critical window' during which changes to the intestinal microbiome are most influential in promoting the development of IgE-mediated hypersensitivities in humans (205). The 3-month findings also possess the greatest diagnostic potential as quartile analysis of the L/C ratio identified children at the highest risk of asthma development and *Lachnospira* analysis identified children at the lowest risk. However future studies should include repeated microbiome analyses beginning before 3 months and continuing up to 1 year of age to more accurately define this early life critical window in humans.

Lastly, this chapter does not provide causative evidence for the role of these bacterial taxa in asthma development, though we did previously demonstrate that *Lachnospira* (along with

the three other FLVR bacteria) ameliorated lung inflammation in an OVA-challenged mouse model (205). Additional translational studies combining human and animal research are necessary to mechanistically define how these bacterial taxa protect against or promote hypersensitivity diseases like asthma.

4.6 Conclusions

In conclusion, this study highlights two Clostridial species with contrasting roles in the development of preschool asthma—*Lachnospira* and *C. neonatale*. Assessment of these bacteria as a ratio (L/C) represents a novel quantification method for measuring taxon-specific gut dysbiosis. Additionally, this study emphasizes the importance of the first 100 days of life as the critical window during which transient gut microbial dysbiosis is associated with immune dysregulation and asthma later in life. Moving forward, this work (along with the results from the previous two chapters) will inform the development of biomarkers to predict risk of asthma and the establishment of rationally designed probiotic regimens to protect children from asthma.

Chapter 5: Conclusion

5.1 Relevance and contribution to the field

5.1.1 Introduction

As suggested by the geographical disparity of asthma and allergies (higher rates observed in Canada and the U.S. versus Asia and Eastern Europe), these hypersensitivity disorders are often viewed as diseases of the Western world (4, 6, 7). Asthma specifically, however, remains the most prevalent childhood disease, affecting approximately one in ten children worldwide (4). Further, asthma is both disruptive to daily living and extremely dangerous if attacks are left untreated, suggesting it as the most burdensome of the atopic diseases (2, 3, 5).

Often IgE-mediated diseases manifest in early childhood and follow an 'atopic march' from early life atopy to asthma development by preschool and school age (12). This immunological heterogeneity, in combination with the genetic and environmental components contributing to asthma development, makes elucidating preventative treatments for asthma challenging. Regarding asthma etiology, the short developmental time frame associated with increased prevalence of IgE-mediated diseases (from the 1980s onward) decreases the likelihood that a changing genetic component is involved (300). Hence, researchers are focusing on the potential roles of environmental factors, such as diet and antibiotic use, in an effort to understand the rise in prevalence of these diseases in the Westernized world (227). An in depth look at the relationship between these 'hygienic' environmental factors and asthma and atopic disease development suggests that lack of exposure to infectious agents in early life may be the culprit for the increase in atopic disease prevalence, a concept most commonly referred to today as the 'hygiene hypothesis' (56).

More recently however, this theory was expanded to include the indigenous microbes resident to the intestine (the microbiota) in the 'microflora hypothesis', which proposes an increase in the prevalence of asthma and allergies due to early life perturbations (i.e. through the use of antibiotics or due to changes in diet), which alter the resident microflora-immune cell interactions necessary for immune tolerance (64, 233). Instead, these disturbances shift the microbiota toward a composition that confers immune dysregulation and hypersensitivities (64, 233).

The work described in this thesis focuses on the role of the intestinal microbiome in human asthma and atopic disease. The novel contributions this thesis makes to human microbiome and asthma epidemiology research are described in the next sections.

5.1.2 Role of early life environmental factors in asthma development

Through an in depth epidemiological analysis in Chapter 2, we identified roles of risk and protective hygiene and microflora hypothesis related factors in the context of the atopic march among a cohort of 2,695 CHILD study subjects. Specifically, we identified pre- and post-natal antibiotic exposure, C-section birth without labor, and respiratory infections in the first year of life as significant risk factors for preschool-age asthma and increased duration of breast-feeding, older siblings, and increasing gestational-age as protective factors against preschool-age asthma. We also highlight time-sensitive sex-specific associations for antibiotic exposure, with males more likely to develop asthma if they received antibiotics at birth and females more likely if they received antibiotics prenatally. Both males and females were more likely to develop asthma if they received antibiotics from 6-months to 1-year of age. Notably, there is evidence of sex-

specific responses to antibiotics, however much more mechanistic research is needed to confirm these sex-specific associations with the development of asthma in humans (260).

Collectively these results suggest that there are environmental risk factors for asthma that are unavoidable. However, it may be possible to protect children from asthma development through conscious changes to the Westernized lifestyle, potentially via decreased over-usage of antibiotics, encouraging breast-feeding and facilitating vaginal delivery, and a general shift in our relationship with microbes, as Chapters 3 and 4 of this thesis further establish the many benefits conferred to humans by bacteria.

5.1.3 Establishment of an early life critical window for microbial intervention in humans

Studies in mouse models of lung inflammation (e.g. OVA, HDM) have identified an early life 'critical window' during which supplementation with specific gut bacteria protects against airway inflammation and antibiotic perturbations to the intestinal microflora enhance hypersensitivity disease (143, 146, 147, 194, 204, 209). However, the work presented in Chapters 3 and 4 of this thesis is the first to identify the first 100 days of life as the critical window for gut microbial intervention in humans.

Though some of the taxa specific changes we observed among AW and asthmatic children were still apparent at 1-year (AW subjects showed a decrease in *Lachnospira* and *Veillonella* and in SCFAs while asthmatics showed a decrease in *C. neonatale* and increase in the L/C ratio at 1-year), the 3-month gut bacterial shifts were more statistically significant and robust against sub-group analyses (**Figures C.1 – C.10**). Further, with regard to the 3-month quartile analyses in Chapter 4, this time point allowed for the identification of children at the highest and lowest odds of developing asthma (lowest L/C quartile OR = 15; highest *Lachnospira* quartile

OR = 0.12) emphasizing this time window as the most clinically relevant in the development of microbe-based diagnostics for asthma and atopic disease.

5.1.4 Specific gut bacterial taxa are associated with asthma and atopic disease in humans

At the time, the few human studies analyzing the intestinal microbiota in asthmatic children identified only global gut microbial differences (i.e. shifts in alpha diversity) in early life (221). However, the work discussed in Chapters 3 and 4 found no differences in bacterial diversity between AWs or asthmatics and controls, but instead characterized shifts in less abundant gut bacterial genera and one bacterial species in asthma and atopic disease (reduced abundances of FLVR genera in 1-year-old AW children in Chapter 3 and contrasting shifts of *Lachnospira* and *C. neonatale* in preschool asthma in Chapter 4).

Clinically, these findings are more relevant than global gut microbial changes, as specific bacterial genera and species are more applicable in the development of probiotic therapies to prevent these diseases. Notably however, in the past year additional bacterial taxa as well as other microbes (e.g. fungi) have been associated with asthma and atopic disease development in children (206, 263). In fact, a recent study published in *Nature Medicine* was able to distinguish asthmatic and atopic children by their neonatal (35 days post birth) intestinal microbiome compositions (263). Children in the highest risk group showed shifts in specific bacterial and fungal taxa, highlighting roles of various gut microbes in human immune development, which are identifiable even earlier than 3-months of age (263). Thus, even now it is becoming more evident that there are likely many other gut microbes associated with asthma and atopic disease development in humans. Additionally, it is likely that the 'critical window' for identifying these gut microbial shifts is even smaller than 3-months of age. However, the work presented in this

thesis was the first to highlight these particular taxa (FLVR and *C. neonatale*) in childhood asthma and atopic disease and will inform the development of novel probiotic strategies and biomarkers to protect children from and predict risk of asthma development.

5.1.5 Specific bacterial taxa and bacterial by-products as candidates for microbe-based therapeutic strategies

The work described in this thesis supports the use of rationally designed microbe-based therapies (pre- and probiotics) initiated in infancy to prevent asthma and atopic disease development in children. Notably, our group further implicated the FLVR taxa in protection against asthma development, as these taxa ameliorated lung inflammation in an OVA-challenged mouse model (205).

Additionally, Chapter 4 highlights the biomarker potential of specific bacterial taxa via quartile analysis of the L/C ratio. We were able to identify children at very high risk of asthma development through this analysis (children in the lowest quartile were 15 times more likely than the other quartiles to develop preschool age asthma), which has significant clinical implications with regard to early diagnosis and prevention of this disease. It may also be possible to use *Lachnospira* specifically as a method of identifying children that are at very low risk of asthma development, as quartile analysis of this taxon identified children at the lowest odds of developing asthma (OR – 0.12). Thus in aggregate, it may be possible to employ these taxa as bacterial biomarkers of asthma and, subsequently, as probiotic treatments to prevent the disease.

Additionally in Chapter 3, we found an inverse association with one bacterial-associated metabolite, the fecal SCFA, acetate. Acetate (among other SCFAs) has been implicated in protection from asthma and allergic diseases in animal models and humans (155, 198). Our

research suggests that this particular SCFA could be used to modulate early life immune development and stimulate proliferation of many beneficial, anti-inflammatory, bacteria (such as *Lachnospira spp.)* that consume acetate, versus introducing probiotic supplements that stimulate the growth of only specific bacterial species. Altogether, the work in this thesis highlights novel and clinically important roles for specific gut bacterial taxa and SCFAs in asthma and other atopic disorders, but the future of this research is ripe with opportunity as there is much more to elucidate before these particular taxa can be employed to protect children from this burdensome disease.

5.2 Future directions

This work has significant implications for human health by enhancing awareness of environmental risks associated with the Westernized lifestyle (e.g. antibiotics, C-section birth) and by establishing further the importance of indigenous gut microbes in the context of asthma and atopic disease. However, there are a number of unanswered questions that will need to be addressed in future studies.

5.2.1 How are these bacterial taxa mediating changes in immune development?

This research identified candidate taxa that may serve as probiotics to prevent asthma and/or as biomarkers to identify high-risk children early in life. The use of human samples is imperative to draw significant conclusions related to human health, however due to challenges in ethics and feasibility, this work is highly correlative and the mechanisms behind these associations can only be inferred. We did establish a causal role of the FLVR taxa in an OVA-

challenged mouse model, however many questions remain and future studies by our group will focus on elucidating these mechanisms using various mouse models of asthma (HDM, OVA, etc.) colonized with a humanized microbiota from older asthmatic and control subjects (3- and 5-year olds) (205).

In conjunction with this mechanistic work, our group is sequencing another 500 – 1000 3-month and 1-year stool samples from the CHILD study to validate these findings and determine if there are any additional taxa that should also be considered. Additionally, targeted-metabolomic and shotgun metagenomic sequencing strategies using stool, urine, and potentially breast-milk samples from these children and their mothers will better characterize the functional roles of these specific taxa in infant immune development. It is also vital that these findings be validated in additional longitudinal cohorts in North America and other parts of the world, as it is possible these results are specific to the Canadian population.

Further, as mentioned in **Section 5.1.4**, it is possible that the most ideal critical window of intervention is even earlier than 100 days post birth. Thus to better elucidate this early life critical window, additional longitudinal cohorts should begin sample collection before 3-months of age and include additional time points up to age 1 year. Additionally, the collection of additional biological samples (namely blood) during the first 3-months of life (though it is not often feasible) would be ideal to determine whether these gut microbial alterations occur prior to immune-dysregulation or vice versa.

5.2.2 Is the intestinal microbiota the mediator between early life environmental exposures and human immune development?

This thesis does provide evidence that known microbiota disturbances (e.g. antibiotics, C-section birth) do increase or decrease a child's risk for asthma development, however there is a major gap in our understanding of whether these environmental factors mediate immune development through perturbations to the intestinal microbiota. It was beyond the scope of this thesis to determine how infants are acquiring or not acquiring the bacterial taxa associated with protection from (FLVR) or risk of (*C. neonatale*) asthma development. Further, the asthmatics and control groups were too small to make any statistically significant claims regarding the effects of environmental factors in shaping the intestinal microbiota of asthmatic subjects. We were able to confirm in our microbiome cohort that antibiotic exposure increased the likelihood of atopy and wheezing (discussed in Chapter 3), but this does not confirm whether antibiotics in the first year of life altered infant immune development through changes to the intestinal microbiota composition. Also notably, antibiotics in the first three months of life were not identified as a significant risk factor for the development of asthma or atopy and wheezing, suggesting that these microbiota alterations are caused by other environmental factors (e.g. mode of birth, gestational age, breast-feeding, etc.) or antibiotic exposure even earlier in life (prenatal or at birth). The additional sequencing of CHILD samples (discussed above) will allow for enhanced analysis of the clinical metadata associated with each subject (i.e. antibiotic exposure, birth mode, breast-feeding) in conjunction with the microbiome sequence data. Machine learning techniques and epidemiological analyses such as path analysis and structural equation modeling can be employed using this larger n number to determine if these early life environmental factors

mediate shifts in childhood immune development through perturbations to the intestinal microbiome composition and functional potential.

Additionally, though this thesis focuses on the intestinal bacterial microbiota, there are many other microbial organisms (fungi and other eukarya, and viruses) that also play key roles in host physiology and immune development (23, 263, 301-303). Additionally, recent research analyzing the lung and airway microbiotas in lung inflammation suggests that these microbial consortiums are equally important in lung diseases, but are often overlooked due to the low biomass of these body sites compared to the human intestine (31, 255, 304). Future studies analyzing the microbiota among asthmatic and atopic versus healthy control infants should also consider the contribution of other microbial organisms and body sites as this would 1) likely provide important insights as to how children are being colonized with the FLVR taxa and 2) identify noteworthy changes in other microbial organisms (in the gut, or more locally in the lung) that will also play important roles in the pathogenesis of this disease.

5.2.3 How might the FLVR taxa be applied to prevent asthma and atopic disease in children?

This work highlights the potential for pro- and pre-biotic therapies to prevent the development of asthma and atopic diseases in children. Ideally, once more research confirming what combinations of the FLVR taxa work best to reduce susceptibility to asthma (through humanized mouse models and additional cohort analyses), these taxa could be given as a probiotic to infants shortly after birth (perhaps through supplemented infant formula). Additionally, supplementation of infants with acetate alone or in combination with the FLVR

taxa could also be a pre- or synbiotic option to protect infants from asthma and atopic disease development. However there are concerns regarding the feasibility and safety of supplementing newborns in a clinical trial with these bacterial taxa. Currently, researchers are working to understand the mechanisms behind the environmental associations discussed in Chapter 2 and whether the potential exists to correct early life disruptions to the microbiome. Dominguez-Bello *et al.* exposed cesarean born infants to vaginal fluids at birth and found that this method partially restored the infant gut microbiota to one comparable to that of vaginally born infants, however the long term consequences of this restoration are not yet known (305). It is also clear that the maternal microbiota plays a prominent role in colonizing the infant (110, 117). Thus, with regard to using the FLVR taxa as protective agents against asthma development, another potential clinical trial might involve probiotic treatment with FLVR to pregnant mothers in an effort to colonize the fetus prenatally, through the birth canal, and/or via contact with maternal skin and milk microbiota during breast-feeding. Similar trials could be conducted with SCFAs or other prebiotic substances in an effort to colonize the infant gut with the FLVR taxa pre- and shortly after birth.

5.2.4 Conclusions

In conclusion, this thesis highlights roles of specific bacterial taxa in the first 100 days of life in an early life atopic disease phenotype (AW) and in children diagnosed with preschool age asthma. We also identified risk and protective hygiene/microflora hypothesis related factors, which could potentially be manipulated in early life to protect children from developing asthma and atopic disease. Ultimately, the novel work presented in this thesis has promising future directions, offering an exciting outlook for future treatment and potential prevention of asthma

and atopic disease in infants. Additionally, this work enhances appreciation of the infant intestinal microbiota, and the many ways in which these indigenous bacteria play vital roles in the development of human immunity.

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Appendices

A. Supplementary information for Chapter 2

Table A.1 Cohort Characteristics

Variable		Included in these analyses	Excluded subjects with either phenotype
Atopy + Wheeze at 1-year	AW	72 (3%)	2 (0.6%)
	Non-AW	2,623 (97%)	226 (65%)
	No phenotype	0 (0%)	119 (34.4%)
	Total (100%)	2,695	347
Asthma at 3-years	Asthma	245 (9%)	13 (4%)
	Non-asthma	2,450 (91%)	106 (31%)
	No phenotype	0 (0%)	228 (65%)
	Total (100%)	2,695	347
Atopic dermatitis at 3-months	Yes	195 (7%)	31 (9%)
	No	2,500 (93%)	316 (91%)
	Total (100%)	2,695	347
Atopic dermatitis at 1-year	Yes	532 (20%)	50 (1%)
	No	2,163 (80%)	27 (86%)
	Total (100%)	2,695	347
Sex	Female	1,253 (46%)	167 (48%)
	Male	1,442 (54%)	180 (52%)
	Total (100%)	2,695	347
Mode of birth	Vaginal	2,030 (75%)	256 (74%)
	C-section with labor	349 (13%)	49 (14%)
	C-section without labor	316 (12%)	42 (12%)
	Total (100%)	2,695	347
Breast-feeding*	0 weeks	94 (6%)	8 (2%)
	Greater than 0 weeks	2,601 (97%)	339 (98%)
	Total (100%)	2,695	347

Variable		Included in these analyses	Excluded subjects with either phenotype
Cat	Yes	660 (24%)	74 (21%)
	No	2,035 (76%)	273 (79%)
	Total (100%)	2,695	347
Dog	Yes	809 (30%)	77 (22%)
	No	1,886 (70%)	270 (78%)
	Total (100%)	2,695	347
Older siblings	At least 1	1,265 (47%)	189 (54%)
	None	1,430 (53%)	158 (46%)
	Total (100%)	2,695	347
Ethnicity	At least 1 Caucasian parent	2,325 (86%)	281 (81%)
	Neither parents Caucasian	370 (14%)	66 (19%)
	Total (100%)	2,695	347
Parental history of asthma	At least 1 parent with history of asthma	884 (33%)	95 (27%)
	Neither parent with history of asthma	1,811 (67%)	252 (73%)
	Total (100%)	2,695	347
Prenatal antibiotics*	Yes	287 (11%)	33 (17%)
	No	2,408 (89%)	287 (83%)
	Total (100%)	2,695	347
Antibiotics birth to 1Y*	Yes	589 (22%)	43 (12%)
	No	2,109 (78%)	304 (88%)
	Total (100%)	2,695	347
Gestational Age*	34 weeks	15 (1%)	0 (0%)
	Greater than 34 weeks	2,680 (99%)	347 (100%)
	Total (100%)	2,695	347

*Continuous covariate

Table A.2 Associations of the four 1-year clinical phenotypes with childhood asthma at 3 years of age

1-year phenotype	3-year asthma	3-year no asthma	Total	OR	95% CI	P-value
AW	21	51	72	6.48	3.78 – 11.13	1.38e-11
Wheeze Only	62	340	402	2.89	2.07 – 4.01	3.79e-10
Atopy Only	49	276	325	2.80	1.95 – 4.01	1.77e-08
Controls	112	1,783	1,896	1	-	-

Table A.3 Associations of clinical variables with 1-year AW, and 3-year asthma

Variable		1-year AW AW = 72, Non-AW = 2,623					3Y Asthma Asthma = 245, Non-asthma = 2,450				
		AW	Non-AW	OR	CI	p-value	Asthma	Non-asthma	OR	CI	p-value
Atopic dermatitis 3m	Yes	15	180	3.49	1.8 – 6.4	9.67e-05	34	161	1.96	1.3 – 3.0	0.002
	%	21	7				14	7			
Atopic dermatitis 1Y	Yes	-	-	-	-	-	66	466	1.04	0.7 – 1.4	0.83
	%	-	-				27	19			
Atopy + wheeze 1Y	Yes	-	-	-	-	-	21	51	2.98	1.7 – 5.2	0.0002
	%	-	-				9	2			
Sex	Male	50	1392	1.78	1.1 – 3.0	0.03	149	1293	1.27	1.0 – 1.7	0.1
	%	69	53				61	53			
Mode of birth	C-section without labor	10	306	1.35	0.6 – 2.7	0.42	39	277	1.51	1.0 – 2.2	0.04
	%	14	12				16	12			
	C-section during labor	11	338	1.07	0.5 – 2.1	0.84	36	813	1.01	0.7 – 1.5	0.95
	%	15	13				15	33			
Breast feeding*	0 months	3	91	1.02	0.96 – 1.08	0.63	9	85	0.97	0.9 – 1.0	0.06
	%	4	3				4	3			
Cat	Yes	17	673	1.1	0.6 – 1.9	0.75	59	601	1.07	0.8 – 1.5	0.66
	%	24	26				24	25			
Dog	Yes	20	789	1.03	0.6 – 1.8	0.9	73	736	1.02	0.8 – 1.4	0.9
	%	28	30				30	30			
Older siblings	At least 1	31	1234	0.83	0.5 – 1.4	0.46	103	1162	0.75	0.6 – 1.0	0.05
	%	43	47				42	47			
Ethnicity	At least one Caucasian parent	56	2269	0.58	0.3 – 1.1	0.09	197	2128	0.6	0.4 – 0.9	0.007
	%	78	87				80	87			
Parental history of asthma	At least one asthmatic parent	30	854	1.71	1.0 – 2.8	0.03	126	758	2.57	2.0 – 3.4	2.69e-11
	%	42	33				51	31			
Prenatal antibiotics*	Yes	11	276	1.32	0.8 – 2.0	0.27	34	253	1.24	0.9 – 1.6	0.14
	%	15	11				14	10			
Antibiotics from birth to 1Y*	Yes	23	563	1.11	0.9 – 1.4	0.37	76	510	1.16	1.0 – 1.3	0.04
	%	32	21				31	21			
Gestational age*	34 weeks	1	14	0.98	0.8 – 1.2	0.83	4	11	0.91	0.8 – 1.0	0.05
	%	1	0.5				2	0.4			

Variable		1-year AW AW = 72, Non-AW = 2,623					3Y Asthma Asthma = 245, Non-asthma = 2,450				
		AW	**Non-AW**	**OR**	**CI**	**p-value**	**Asthma**	**Non-asthma**	**OR**	**CI**	**p-value**
Respiratory infections birth to 1Y	Yes	36	533	3.0	2.0 – 4.4	3.05e-08	70	499	1.46	1.1 – 1.9	0.006
	%	50	20				29	20			

*Continuous variable

Table A.4 Sex stratified associations of clinical variables with 3-year asthma

Variable		Females Asthma = 96, Non-AW = 1,157					Males Asthma = 149, Non-asthma = 1,293				
		Asthma	Non-asthma	OR	CI	p-value	Asthma	Non-asthma	OR	CI	p-value
Atopic dermatitis 3m	Yes	6	68	1.05	0.4 – 2.4	0.92	28	93	2.56	1.5 – 4.3	0.0004
	%	6	6				19	7			
Atopic dermatitis 1Y	Yes	15	184	0.79	0.4 – 1.4	0.44	51	282	1.14	0.8 – 1.7	0.53
	%	16	16				34	22			
Atopy + wheeze 1Y	Yes	5	17	2.8	0.8 – 8.1	0.07	16	34	3.25	1.6 – 6.4	0.0009
	%	5	1				11	3			
Sex	Male	-	-	-	-	-	-	-	-	-	-
	%	-	-				-	-			
Mode of birth	C-section without labor	14	144	1.26	0.6 – 2.3	0.48	25	133	1.76	1.0 – 2.9	0.03
	%	15	12				17	10			
	C-section during labor	14	132	1.22	0.6 – 2.2	0.54	22	181	0.86	0.5 – 1.4	0.57
	%	15	11				15	14			
Breast feeding*	0 months	4	44	0.95	0.9 – 1.0	0.05	5	41	0.98	0.9 – 1.0	0.43
	%	4	4				3	3			
Cat	Yes	26	296	1.1	0.7 – 1.8	0.71	33	305	1.03	0.7 – 1.6	0.9
	%	27	26				22	24			
Dog	Yes	30	352	0.95	0.6 – 1.5	0.84	43	384	1.07	0.7 – 1.6	0.73
	%	31	30				29	30			
Older siblings	At least 1	37	559	0.65	0.4 – 1.0	0.07	66	603	0.86	0.6 – 1.2	0.41
	%	39	48				44	47			
Ethnicity	At least one Caucasian parent	81	1001	0.78	0.4 – 1.5	0.45	116	1127	0.49	0.3 – 0.8	0.004
	%	84	87				78	87			
Parental history of asthma	At least one asthmatic parent	48	360	2.35	1.5 – 3.6	0.0001	78	398	2.98	2.1 – 4.3	6.52e-09
	%	48	31				52	31			
Prenatal antibiotics*	Yes	16	109	1.79	1.2 – 2.7	0.004	18	144	0.88	0.5 – 1.3	0.57
	%	17	9				12	11			
Antibiotics from birth to 1Y*	Yes	25	214	0.98	0.7 – 1.3	0.88	51	296	1.24	1.0 – 1.5	0.01
	%	26	18				34	23			
Gestational age*	34 weeks	0	5	0.87	0.8 – 1.0	0.09	4	6	0.92	0.8 – 1.0	0.19
	%	0	0.4				3	0.5			

Variable		Females Asthma = 96, Non-AW = 1,157					Males Asthma = 149, Non-asthma = 1,293				
		Asthma	**Non-asthma**	**OR**	**CI**	**p-value**	**Asthma**	**Non-asthma**	**OR**	**CI**	**p-value**
Respiratory infections birth to 1Y	Yes	24	226	1.57	1.0 – 2.4	0.04	46	273	1.41	1.0 – 2.0	0.06
	%	25	20				31	21			

*Continuous variable

Table A.5 Associations of antibiotics and respiratory infections with 1-year AW, and 3-year asthma

Variable		1-year AW AW = 72, Non-AW = 2,623					3-year Asthma Asthma = 245, Non-asthma = 2,450				
		AW	Non-AW	OR	CI	p-value	Asthma	Non-asthma	OR	CI	p-value
Prenatal antibiotics	Yes	11	276	1.32ς	0.8 – 2.0ς	0.23ς	34	253	1.3ς	0.9 – 1.6ς	0.1ς
	%	15	11	1.3ϕ	0.8 – 2.0ϕ	0.28ϕ	14	10	1.3ϕ	0.9 – 1.6ϕ	0.12ϕ
Maternal antibiotics at birth	Yes	28	1054	0.89ς	0.6 – 1.3ς	0.54ς	107	975	1.07ς	0.9 – 1.3ς	0.54ς
	%	39	40	0.88ϕ	0.6 – 1.3ϕ	0.52ϕ	44	40	1.07ϕ	0.9 – 1.3ϕ	0.55ϕ
Child antibiotics at birth	Yes	4	77	1.45ς	0.8 – 2.3ς	0.16ς	13	68	1.35ς	1.0 – 1.8ς	0.05ς
	%	6	3	1.52ϕ	0.8 – 2.4ϕ	0.12ϕ	5	3	1.36ϕ	1.0 – 1.8ϕ	0.05ϕ
Child antibiotics birth to 3-months	Yes	1	97	0.32ς	0.02 – 1.2ς	0.23ς	5	93	0.61ς	0.3 – 1.1ς	0.15ς
	%	1	4	0.33ϕ	0.02 – 1.2ϕ	0.24ϕ	2	4	0.64ϕ	0.3 – 1.1ϕ	0.19ϕ
Child antibiotics 3-months to 6-months	Yes	8	102	2.1ς	1.0 – 3.9ς	0.03ς	14	96	1.13ς	0.6 – 1.9ς	0.66ς
	%	11	4	1.54ϕ	0.7 – 3.1ϕ	0.27ϕ	6	4	1.02ϕ	0.6 – 1.7ϕ	0.94ϕ
Child antibiotics 6-months to 1-year	Yes	15	373	1.39ς	1.0 – 1.8ς	0.02ς	59	329	1.46ς	1.2 – 1.8ς	5.83e-05ς
	%	21	14	1.2ϕ	0.9 – 1.6ϕ	0.22ϕ	24	13	1.4ϕ	1.2 – 1.7ϕ	0.0005ϕ
Respiratory infections birth to 3-months	Yes	5	116	1.23ς	0.4 – 2.9ς	0.67ς	10	111	0.82ς	0.4 – 1.5ς	0.56ς
	%	7	4	1.49ϕ	0.5 – 3.6ϕ	0.41ϕ	4	5	0.91ϕ	0.4 – 1.7ϕ	0.79ϕ
Respiratory infections 3-months to 6-months	Yes	11	161	2.33ς	1.13 – 4.4ς	0.01ς	20	152	1.27ς	0.8 – 2.0ς	0.34ς
	%	15	6	2.03ϕ	0.9 – 4.1ϕ	0.06ϕ	8	6	1.3ϕ	0.7 – 2.0ϕ	0.39ϕ
Respiratory infections 6-months to 1-year	Yes	29	414	3.4ς	2.1 – 5.5ς	9.8e-07ς	59	384	1.7ς	1.2 – 2.3ς	0.001ς
	%	40	16	3.09ϕ	1.9 – 5.1ϕ	1.22e-05ϕ	24	16	1.5ϕ	1.1 – 2.1ϕ	0.01ϕ

ς Crude

ϕ Adjusted for respiratory infections (in the case of antibiotics) or antibiotics (in the case of respiratory infections)

All antibiotic variables in this analysis are continuous variables

Table A.6 Sex stratified associations of antibiotics and respiratory infections with 3-year asthma

Variable		Females Asthma = 96, Non-AW = 1,157					Males Asthma = 149, Non-asthma = 1,293				
		Asthma	Non-asthma	OR	CI	p-value	Asthma	Non-asthma	OR	CI	p-value
Prenatal antibiotics	Yes	16	109	1.79ς	1.2 – 2.6ς	0.003ς	18	144	0.91ς	0.6 – 1.3ς	0.67ς
	%	17	9	1.82ϕ	1.2 – 2.6ϕ	0.002ϕ	12	11	0.9ϕ	0.6 – 1.3ϕ	0.62ϕ
Maternal antibiotics at birth	Yes	39	465	1.13ς	0.8 – 1.5ς	0.46ς	68	510	1.0ς	0.8 – 1.3ς	0.98ς
	%	41	40	1.13ϕ	0.8 – 1.5ϕ	0.46ϕ	46	39	1.0ϕ	0.8 – 1.3ϕ	0.99ϕ
Child antibiotics at birth	Yes	0	27	-	-	-	41	13	1.65ς	1.2 – 2.3ς	0.002ς
	%	0	2				28	1	1.66ϕ	1.2 – 2.3ϕ	0.002ϕ
Child antibiotics birth to 3-months	Yes	1	34	0.37ς	0.02 – 1.3ς	0.28ς	4	59	0.67ς	0.3 – 1.3ς	0.29ς
	%	1	3	0.36ϕ	0.02 – 1.3ϕ	0.28ϕ	3	5	0.73ϕ	0.3 – 1.4ϕ	0.4ϕ
Child antibiotics 3-months to 6-months	Yes	4	36	1.09ς	0.3 – 2.7ς	0.87ς	10	60	1.13ς	0.6 – 2.0ς	0.7ς
	%	4	3	1.87ϕ	0.2 – 2.3ϕ	0.8ϕ	7	5	1.07ϕ	0.5 – 2.0ϕ	0.84ϕ
Child antibiotics 6-months to 1-year	Yes	22	146	1.47ς	1.0 – 2.1ς	0.03ς	37	183	1.44ς	0.2 – 1.8ς	0.001ς
	%	23	13	1.45ϕ	1.0 – 2.1ϕ	0.04ϕ	25	14	1.36ϕ	1.1 – 1.7ϕ	0.007ϕ
Respiratory infections birth to 3-months	Yes	5	51	1.1ς	0.06 – 0.1ς	0.85ς	5	60	0.65ς	0.2 – 1.5ς	0.36ς
	%	5	4	1.26ϕ	0.4 – 3.0ϕ	0.64ϕ	3	5	0.75ϕ	0.3 – 1.8ϕ	0.56ϕ
Respiratory infections 3-months to 6-months	Yes	9	67	1.63ς	0.7 – 3.2ς	0.19ς	11	85	1.05ς	0.5 – 1.9ς	0.89ς
	%	9	6	1.75ϕ	0.8 – 3.6ϕ	0.16ϕ	7	7	1.02ϕ	0.5 – 2.0ϕ	0.95ϕ
Respiratory infections 6-months to 1-year	Yes	19	173	1.37ς	0.8 – 2.3ς	0.25ς	40	211	1.9ς	1.3 – 2.8ς	0.001ς
	%	20	15	1.3ϕ	0.7 – 2.1ϕ	0.43ϕ	27	16	1.74ϕ	1.1 – 2.6ϕ	0.008ϕ

ς Crude
ϕ Adjusted for respiratory infections (in the case of antibiotics) or antibiotics (in the case of respiratory infections)
All antibiotic variables in this analysis are continuous variables

Table A.7 Details regarding missing data

Variable	% of missing data
Atopic dermatitis at 3-months	3.0%
Atopic dermatitis at 1-year	0.3%
Breast feeding Duration	0.4%
Birth mode	1.0%
Older Siblings	2.0%
Dog	12.0%
Cat	12.0%
Parental ethnicity	0.04% missing both mom and dad ethnicity
Parental asthma	0.8% missing both parents, 2% missing mom history, 11% missing dad history
Prenatal maternal antibiotics	16.0%
Maternal antibiotics at birth	1.0%
Child antibiotics at birth	10%
Child antibiotics 3-months	4.0%
Child antibiotics 6-months	11.0%
Child antibiotics 6-months to 1-year	13.0%

Table A.8 Spearman correlation analysis for associations of continuous variables with 3-year asthma

Variable	Spearman r	CI	p-value	FDR adjusted p-value
Prenatal antibiotics	0.03	-0.005 – 0.07	0.08	0.14
Maternal antibiotics at birth	0.02	-0.02 – 0.06	0.29	0.31
Child antibiotics at birth	0.04	0.003 – 0.08	0.03	0.05
Child antibiotics birth to 3-months	-0.03	-0.07 – 0.01	0.16	0.23
Child antibiotics 3-months to 6-months	0.03	-0.01 – 0.06	0.18	0.23
Child antibiotics 6-months to 1-year	0.09	0.05 – 0.1	0.0001	0.0005
Child antibiotics birth to 1Y	0.08	0.04 – 0.1	0.0001	0.0005
Breast-feeding	-0.05	-0.09 – -0.01	0.007	0.02
Gestational age	-0.05	-0.08 – -0.006	0.02	0.05

B. Supplementary information for Chapter 3

All supplementary tables are printed with permission from American Association for the Advancement of Science (AAAS), Arrieta*, Stiemsma* *et al., Sci Trans Med* 2015.
Abbreviations: AD = atopic dermatitis, CI = confidence interval.

Table B.1 Exact multivariate logistic regression model of control qPCR subset

Variable		Phenotype		OR*	95% CI		P-value
		Controls (subset)	Controls (whole cohort)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	4 (20%)	12 (16%)	0.95	0.07	7.54	1
	None	16 (80%)	62 (84%)				
	Total (100%)	20	74				
Antibiotic Exposure (birth to 3-months of age)	1 or more	2 (10%)	4 (5%)	2.24	0.09	68.7	1
	None	18 (90%)	70 (95%)				
	Total (100%)	20	74				
AD at 3-months	Yes	4 (20%)	4 (5%)	1.8	0.14	16.44	0.6
	No	16 (80%)	70 (95%)				
	Total (100%)	20	74				
AD at 1-year	Yes	3 (15%)	18 (24%)	0.55	0.06	3.0	0.7
	No	17 (85%)	56 (76%)				
	Total (100%)	20	74				
Sex	Female	10 (50%)	38 (51%)	0.85	0.24	3.06	0.8
	Male	10 (50%)	36 (49%)				
	Total (100%)	20	74				
Mode of birth	Vaginal	13 (65%)	58 (78%)	0.63	0.12	3.3	0.71
	Caesarean	7 (35%)	16 (23%)				
	Total (100%)	20	74				
Breast Feeding (birth to 3-months)	Yes	14 (70%)	60 (81%)	0.44	0.06	5.9	1
	No	6 (30%)	14 (19%)				
	Total (100%)	20	74				

Variable		Phenotype		OR*	95% CI		P-value
		Controls (subset)	Controls (whole cohort)		Lower	Upper	
Breast Feeding (birth to 1-year)	Yes	3 (15%)	25 (34%)	0.84	0.1	64.07	0.26
	No	17 (85%)	49 (66%)				
	Total (100%)	20	74				
Maternal Asthma	Yes	7 (35%)	24 (32%)	1.73	0.45	6.36	0.36
	No	13 (65%)	50 (68%)				
	Total (100%)	20	74				
Paternal Asthma	Yes	1 (5%)	10 (14%)	0.55	0.01	5	1
	No	19 (95%)	64 (86%)				
	Total (100%)	20	74				

*The group listed first for each variable (i.e. 1 or more for antibiotic exposure) is the reference group for interpreting the odds ratio.

Table B.2 Exact multivariate logistic regression model of control SCFA subset

Variable		Phenotype		OR*	95% CI		P-value
		Controls (subset)	Controls (whole cohort)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	3 (24%)	12 (16%)	1.2	0.07	11.8	1
	None	10 (76%)	62 (84%)				
	Total (100%)	13	74				
Antibiotic Exposure (birth to 3-months of age)	1 or more	1 (8%)	4 (5%)	0.96	0.009	41.26	1
	None	12 (92%)	70 (95%)				
	Total (100%)	13	74				
AD at 3-months	Yes	1 (8%)	4 (5%)	1.24	0.02	15.95	1
	No	12 (92%)	70 (95%)				
	Total (100%)	13	74				
AD at 1-year	Yes	2 (15%)	18 (24%)	0.82	0.08	4.48	1
	No	11 (85%)	56 (76%)				
	Total (100%)	13	74				
Sex	Female	6 (54%)	38 (51%)	0.67	0.13	3.00	0.53
	Male	7 (46%)	36 (49%)				
	Total (100%)	13	74				
Mode of birth	Vaginal	8 (62%)	58 (78%)	0.69	0.14	3.9	0.69
	Caesarean	5 (38%)	16 (23%)				
	Total (100%)	13	74				
Breast Feeding (birth to 3-months)	Yes	10 (77%)	60 (81%)	0.68	0.09	5.1	1
	No	3 (23%)	14 (19%)				
	Total (100%)	13	74				
Breast Feeding (birth to 1-year)	Yes	2 (15%)	25 (34%)	0.53	0.05	4.06	0.69
	No	11 (85%)	49 (66%)				
	Total (100%)	13	74				
Maternal Asthma	Yes	5 (38%)	24 (32%)	1.4	0.3	6.36	0.73
	No	8 (62%)	50 (68%)				
	Total (100%)	13	74				

Variable		Phenotype		OR*	95% CI		P-value
		Controls (subset)	Controls (whole cohort)		Lower	Upper	
Paternal Asthma	Yes	1 (8%)	10 (14%)	0.63	0.01	5.81	1
	No	12 (92%)	64 (86%)				
	Total (100%)	13	74				

*The group listed first for each variable (i.e. 1 or more for antibiotic exposure) is the reference group for interpreting the odds ratio.

Table B.3 Exact multivariate logistic regression model of AW SCFA subset

Variable		Phenotype		Odds Ratio*	95% CI		P-value
		AW (subset)	AW (whole cohort)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	5 (38%)	9 (42%)	1.58	0.09	21.76	1
	None	8 (62%)	12 (58%)				
	Total (100%)	13	21				
Antibiotic Exposure (birth to 3-months of age)	1 or more	0 (0%)	0 (0%)	-	-	-	-
	None	13 (100%)	21 (100%)				
	Total (100%)	13	21				
AD at 3-months	Yes	2 (15%)	5 (24%)	1.8	0.01	735.1	1
	No	11 (85%)	16 (76%)				
	Total (100%)	13	21				
AD at 1-year	Yes	9 (69%)	13 (62%)	1.62	0.07	66.69	1
	No	4 (31%)	8 (38%)				
	Total (100%)	13	21				
Sex	Female	6 (54%)	7 (33%)	1.72	0.17	36.6	1
	Male	7 (46%)	14 (67%)				
	Total (100%)	13	21				
Mode of birth	Vaginal	10 (77%)	16 (76%)	1.06	0.04	26.57	1
	Caesarean	3 (23%)	5 (24%)				
	Total (100%)	13	21				
Breast Feeding (birth to 3-months)	Yes	9 (69%)	15 (71%)	0.86	0.05	8.8	1
	No	4 (31%)	6 (29%)				
	Total (100%)	13	21				
Breast Feeding (birth to 1-year)	Yes	3 (23%)	7 (33%)	1.65	0.11	26.31	1
	No	10 (77%)	14 (67%)				
	Total (100%)	13	21				

Variable		Phenotype		Odds Ratio*	95% CI		P-value
		AW (subset)	AW (whole cohort)		Lower	Upper	
Maternal Asthma	Yes	4 (31%)	7 (33%)	2.5	0.12	601.85	1
	No	9 (69%)	14 (67%)				
	Total (100%)	13	21				
Paternal Asthma	Yes	3 (23%)	3 (14%)	1.2	0.07	24.53	1
	No	10 (77%)	18 (86%)				
	Total (100%)	13	21				

*The group listed first for each variable (i.e. 1 or more for antibiotic exposure) is the reference group for interpreting the odds ratio.

*Only subjects for which all the clinical metadata was available could be included in this model.

(-) A finite lower or upper bound for the confidence interval could not be obtained because the observed value of the sufficient statistic is the maximum possible value.

C. Supplementary information for Chapter 4

All supplementary tables and figures were originally published in Stiemsma *et al. Clinical Science*. Sep 2016.
Abbreviations: OR = Odds ratio, AD = atopic dermatitis, CI = confidence interval.
(-) Not able to compute due to distribution of subjects (0 subject in one group).

Table C.1 Multivariate logistic regression table comparing asthmatic 3-month qPCR subset to asthmatic children not qPCRed

Variable		Phenotype		OR	95% CI		P-value
		Asthmatics (qPCRed)	Asthmatics (not qPCRed)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	11 (33%)	3 (50%)	1.39	0.38	5.00	0.62
	None	22 (67%)	3 (50%)				
	Total (100%)	33	6				
Antibiotic Exposure (birth to 3-months of age)	1 or more	2 (6%)	1 (17%)	0.59	0.07	5.05	0.63
	None	31 (94%)	5 (83%)				
	Total (100%)	33	6				
AD at 3-months	Yes	5 (15%)	2 (33%)	0.37	0.02	5.41	0.47
	No	28 (85%)	4 (67%)				
	Total (100%)	33	6				
AD at 1-year	Yes	9 (27%)	6 (100%)	-	-	-	-
	No	24 (73%)	0 (0%)				
	Total (100%)	33	6				
Sex	Female	18 (55%)	0 (0%)	-	-	-	-
	Male	15 (45%)	6 (100%)				
	Total (100%)	33	6				
Mode of birth	Cesarean	5 (15%)	3 (50%)	4.95	0.71	34.81	0.12
	Vaginal	28 (85%)	3 (50%)				
	Total (100%)	33	6				
Breast Feeding	Yes	32 (97%)	6 (100%)	-	-	-	-
	No	1 (3%)	0 (0%)				
	Total (100%)	33	6				

Variable		Phenotype		OR	95% CI		P-value
		Asthmatics (qPCRed)	Asthmatics (not qPCRed)		Lower	Upper	
Parental Asthma	Neither	10 (30%)	2 (33%)	0.75	0.09	6.55	0.8
	At least one parent	23 (70%)	4 (67%)				
	Total (100%)	33	6				

Table C.2 Multivariate logistic regression table comparing asthmatic 1-year qPCR subset to asthmatic children not qPCRed

Variable		Phenotype		OR	95% CI		P-value
		Asthmatics (qPCRed)	Asthmatics (not qPCRed)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	13 (37%)	1 25%)	2.01	0.33	12.43	0.99
	None	22 (63%)	4 (75%)				
	Total (100%)	35	4				
Antibiotic Exposure (birth to 3-months of age)	1 or more	3 (8%)	0 (0%)	-	-	-	-
	None	32 (92%)	4 (100%)				
	Total (100%)	35	4				
AD at 3-months	Yes	7 (20%)	0 (0%)	-	-	-	-
	No	28 (80%)	4 (100%)				
	Total (100%)	35	4				
AD at 1-year	Yes	13 (37%)	2 (50%)	0.74	0.07	7.61	0.8
	No	22 (63%)	2 (50%)				
	Total (100%)	35	4				
Sex	Female	17 (49%)	1 (25%)	0.37	0.03	5.52	0.48
	Male	18 (51%)	3 (75%)				
	Total (100%)	35	4				
Mode of birth	Cesarean	8 (23%)	0 (0%)	-	-	-	-
	Vaginal	27 (77%)	4 (100%)				
	Total (100%)	35	4				
Breast Feeding	Yes	34 (97%)	4 (100%)	-	-	-	-
	No	1 (3%)	0 (0%)				
	Total (100%)	35	4				
Parental Asthma	Neither	11 (31%)	1 (25%)	0.85	0.08	12.31	0.99
	At least one parent	24 (69%)	3 (75%)				
	Total (100%)	35	4				

Table C.3 Multivariate logistic regression table comparing control 3-month qPCR subset to control children not qPCRed

Variable		Phenotype		OR	95% CI		P-value
		Controls (qPCRed)	Controls (not qPCRed)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	4 (17%)	1 (8%)	1.17	0.31	4.44	0.82
	None	20 (83%)	12 (92%)				
	Total (100%)	24	13				
Antibiotic Exposure (birth to 3-months of age)	1 or more	2 (8%)	0 (0%)	-	-	-	-
	None	22 (92%)	13 (100%)				
	Total (100%)	24	13				
AD at 3-months	Yes	0 (0%)	1 (8%)	-	-	-	-
	No	24 (100%)	12 (92%)				
	Total (100%)	24	13				
AD at 1-year	Yes	2 (8%)	1 (8%)	1.66	0.11	26.04	0.72
	No	22 (88%)	12 (92%)				
	Total (100%)	24	13				
Sex	Female	11 (46%)	6 (46%)	0.74	0.15	3.71	0.72
	Male	13 (54%)	7 (54%)				
	Total (100%)	24	13				
Mode of birth	Cesarean	3 (13%)	2 (15%)	1.88	0.21	16.61	0.57
	Vaginal	21 (87%)	11 (85%)				
	Total (100%)	24	13				
Breast Feeding	Yes	21 (87%)	13 (100%)	0.97	0.82	1.15	0.71
	No	3 (13%)	0 (0%)				
	Total (100%)	24	13				
Parental Asthma	Neither	19 (79%)	7 (54%)	0.26	0.05	1.32	0.14
	At least one parent	5 (21%)	6 (46%)				
	Total (100%)	24	13				

Table C.4 Multivariate logistic regression table comparing control one-year qPCR subset to control children not qPCRed

Variable		Phenotype		OR	95% CI		P-value
		Controls (qPCRed)	Controls (not qPCRed)		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	5 (18%)	0 (0%)	-	-	-	-
	None	23 (82%)	9 (100%)				
	Total (100%)	28	9				
Antibiotic Exposure (birth to 3-months of age)	1 or more	2 (7%)	0 (0%)	-	-	-	-
	None	26 (93%)	9 (100%)				
	Total (100%)	28	9				
AD at 3-months	Yes	0 (0%)	1 (11%)	-	-	-	-
	No	28 (100%)	8 (89%)				
	Total (100%)	28	9				
AD at 1-year	Yes	3 (10%)	0 (0%)	-	-	-	-
	No	25 (90%)	9 (100%)				
	Total (100%)	28	9				
Sex	Female	12 (43%)	5 (56%)	1.57	0.3	8.33	0.59
	Male	16 (57%)	4 (44%)				
	Total (100%)	28	9				
Mode of birth	Cesarean	4 (14%)	1 (11%)	0.66	0.05	8.08	0.75
	Vaginal	24 (86%)	8 (89%)				
	Total (100%)	28	9				
Breast Feeding	Yes	26 (93%)	8 (89%)	0.96	0.8	1.16	0.71
	No	2 (7%)	1 (11%)				
	Total (100%)	28	9				
Parental Asthma	Neither	21 (75%)	0.48	-0.74	0.09	2.46	0.38
	At least one parent	7 (25%)	4 (44%)				
	Total (100%)	28	9				

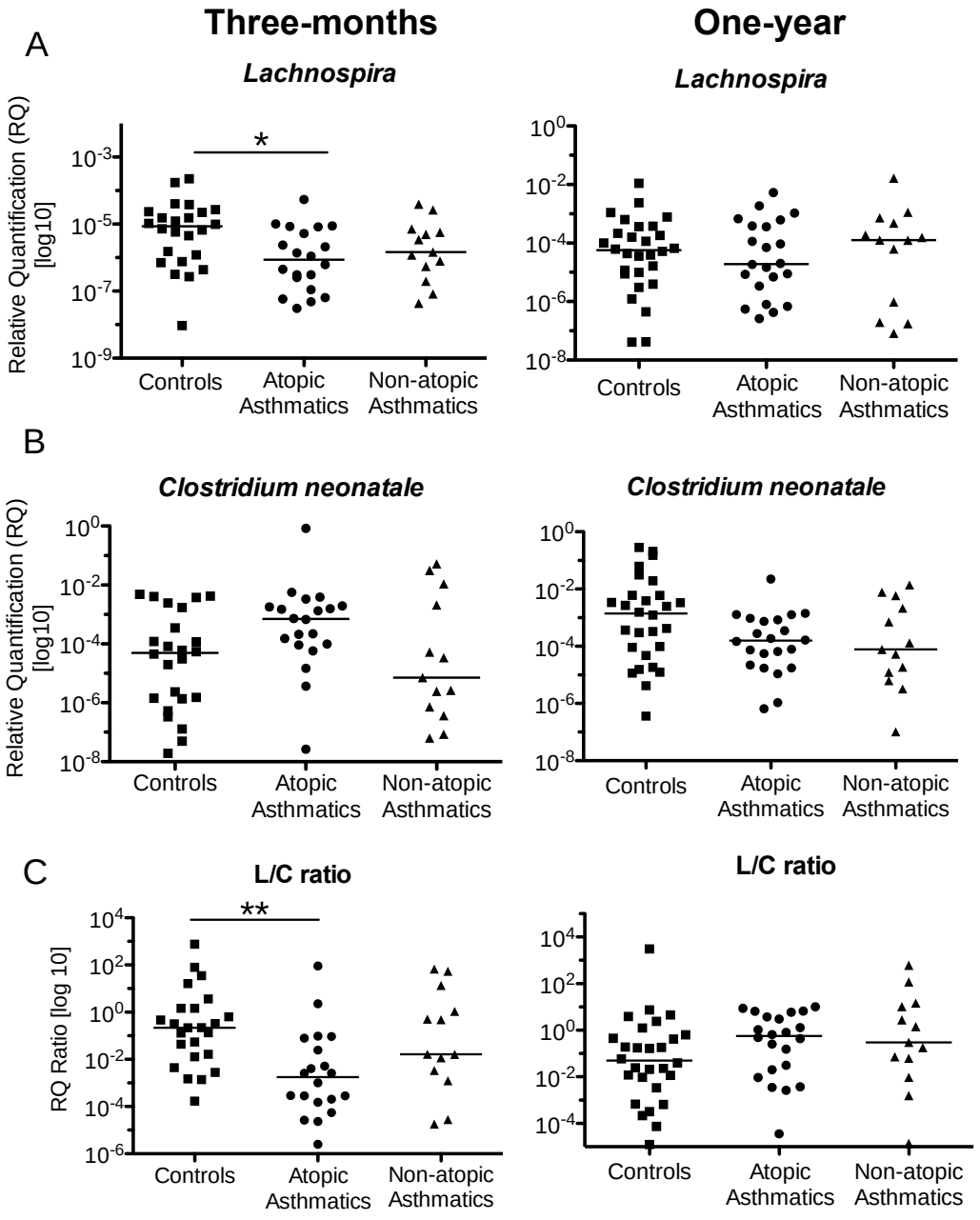

Figure C.1 Sub-group analysis according to atopic status

A) *Lachnospira* [Mann-Whitney p = 0.02], **B)** *C. neonatale*, and C) L/C ratio [Mann-Whitney p = 0.004] at 3-months and 1-year. Star representation; p ≤0.05 *, p ≤ 0.01 **. n_{3mo} Atopic asthmatics = 20, Non-atopic asthmatics = 13, Control = 24, n_{1Y} Atopic asthmatic = 22, Non-atopic asthmatics = 13, Control = 28.

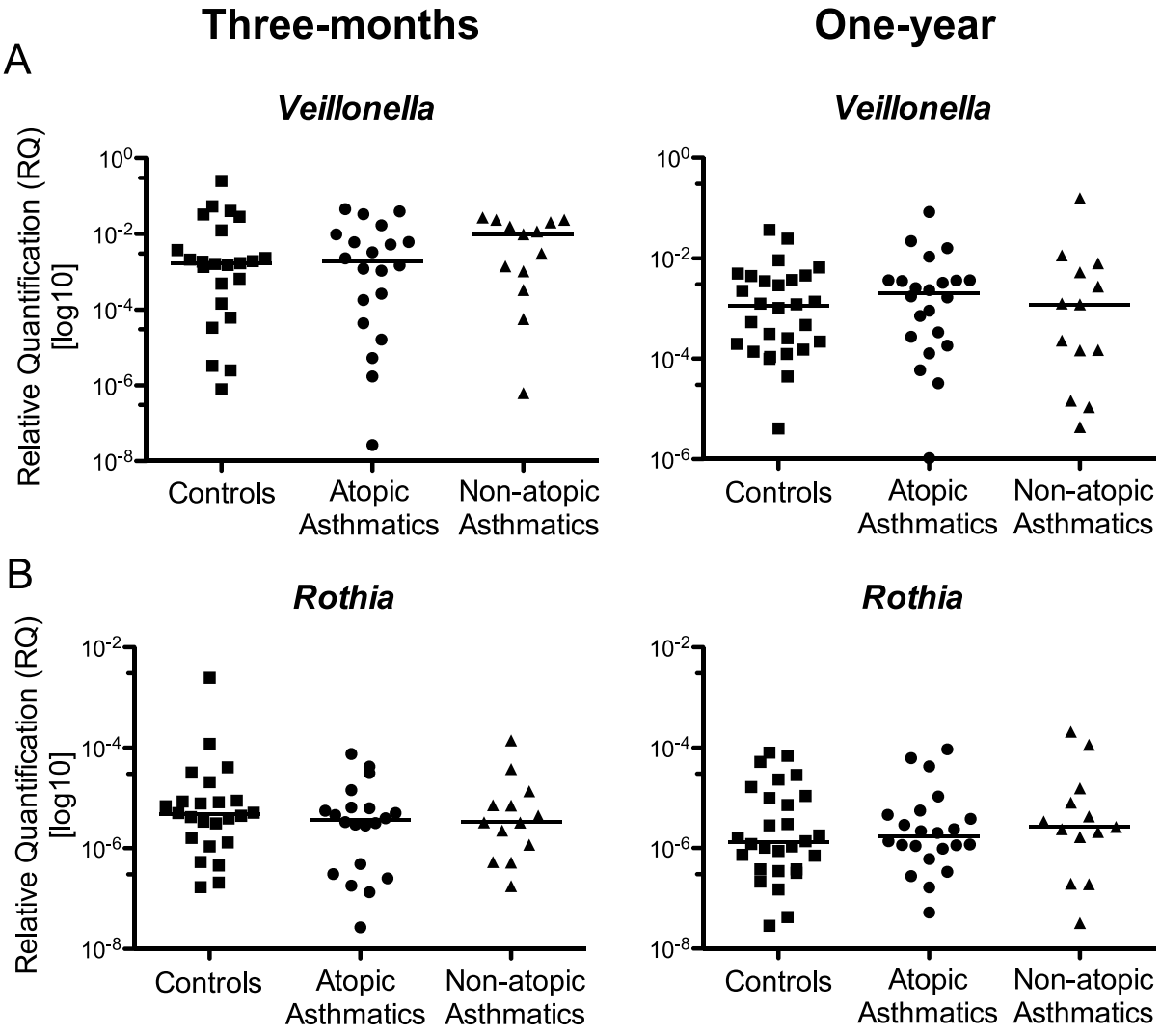

Figure C.2 Sub-group analysis according to atopic status

A) *Veillonella*, **B)** *Rothia* at 3-months and 1-year. n_{3mo} Atopic asthmatics = 20, Non-atopic asthmatics = 13, Control = 24, n_{1Y} Atopic asthmatic = 22, Non-atopic asthmatics = 13, Control = 28.

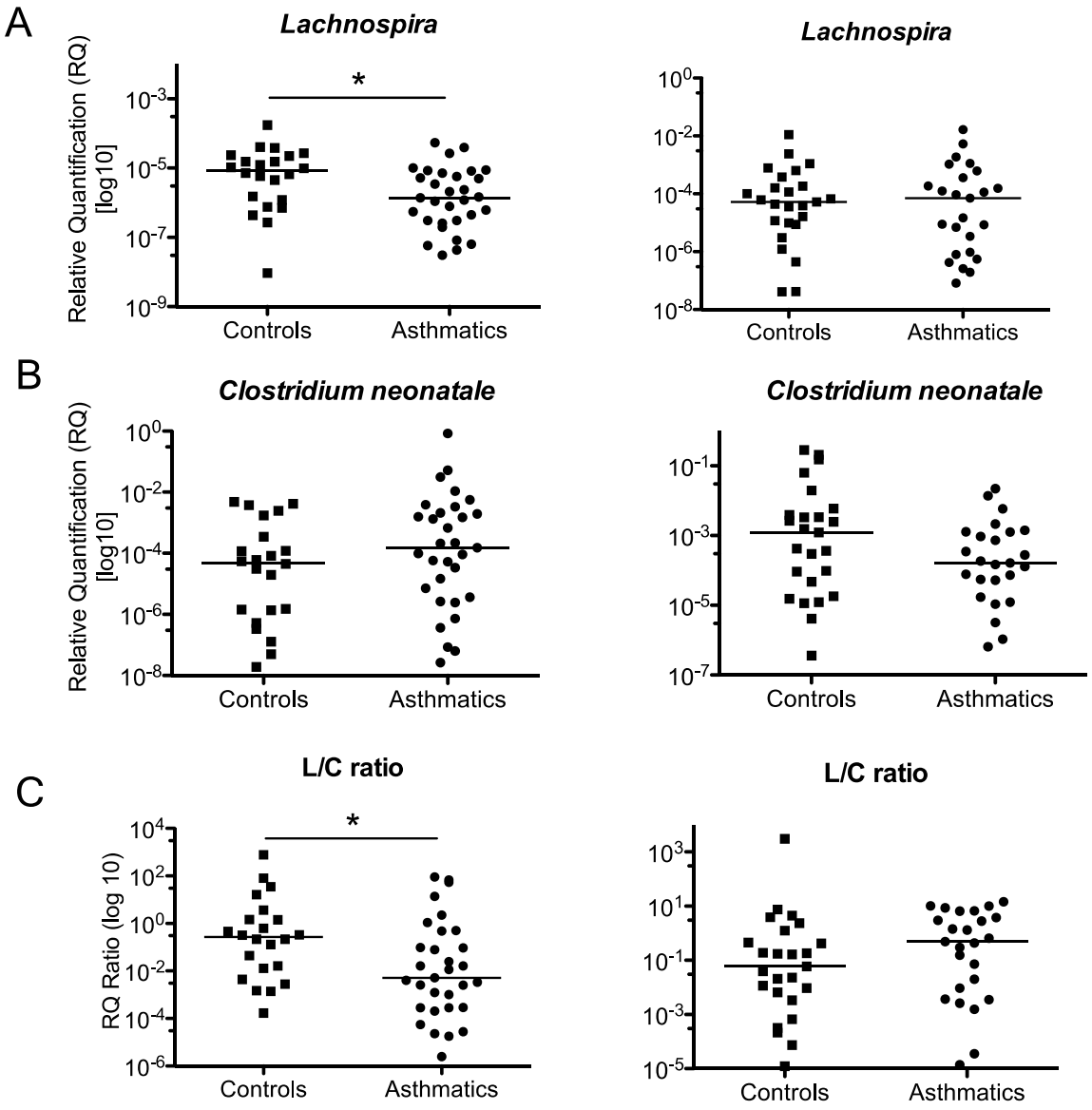

Figure C.3 Sub-group analysis among subjects receiving no antibiotics

A) *Lachnospira* [Mann-Whitney p = 0.02, **B)** *C. neonatale*, **C)** L/C ratio [Mann-Whitney p = 0.02] at 3-months and 1-year. Star representation; p ≤0.05 *. n_{3mo} Asthmatics = 31, Controls = 22, n_{1Y} Asthmatic = 25, Controls = 25.

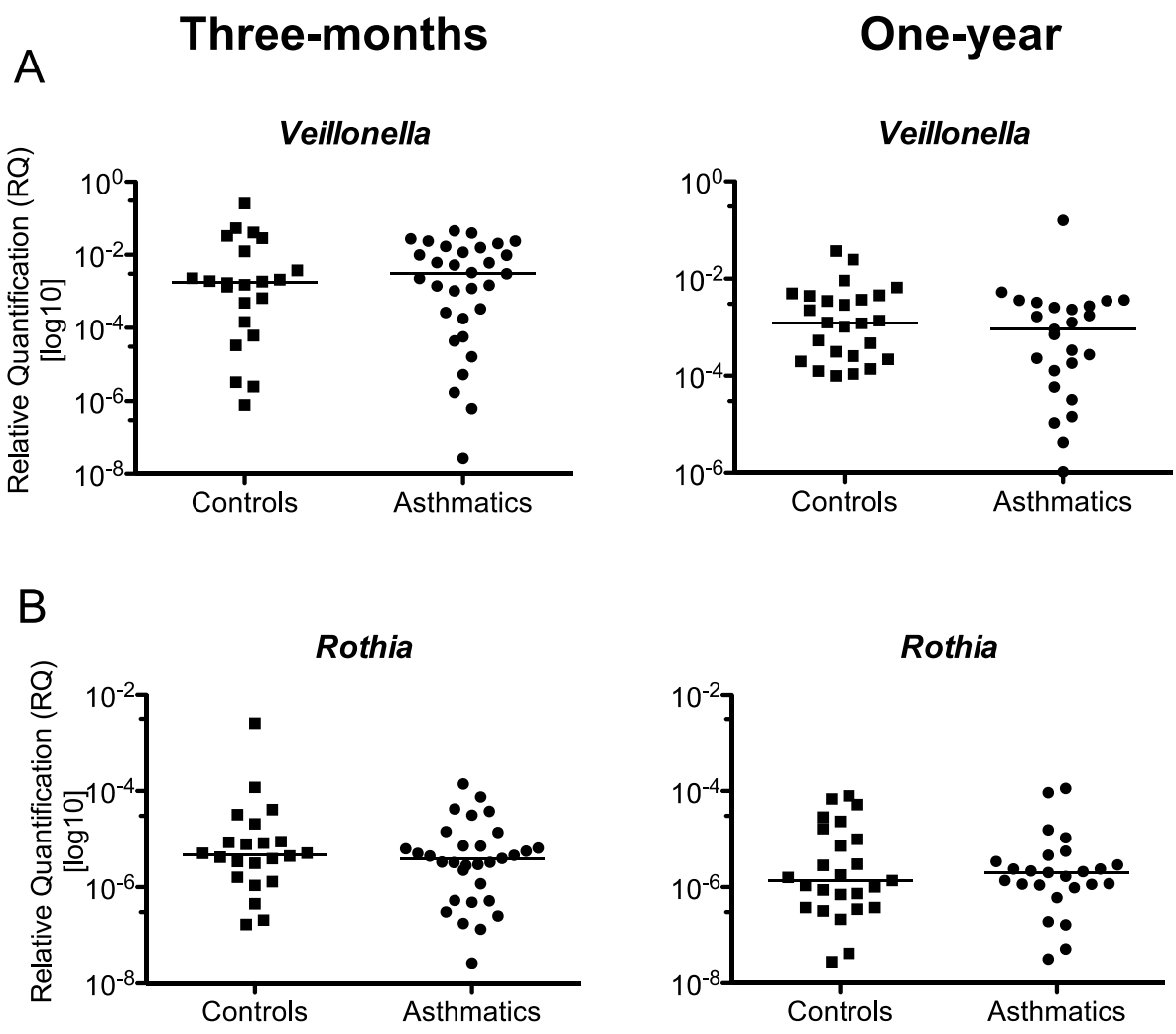

Figure C.4 Sub-group analysis among subjects receiving no antibiotics

A) *Veillonella,* **B)** *Rothia* at 3-months and 1-year. n_{3mo} Asthmatics = 31, Controls = 22, n_{1Y} Asthmatic = 25, Controls = 25.

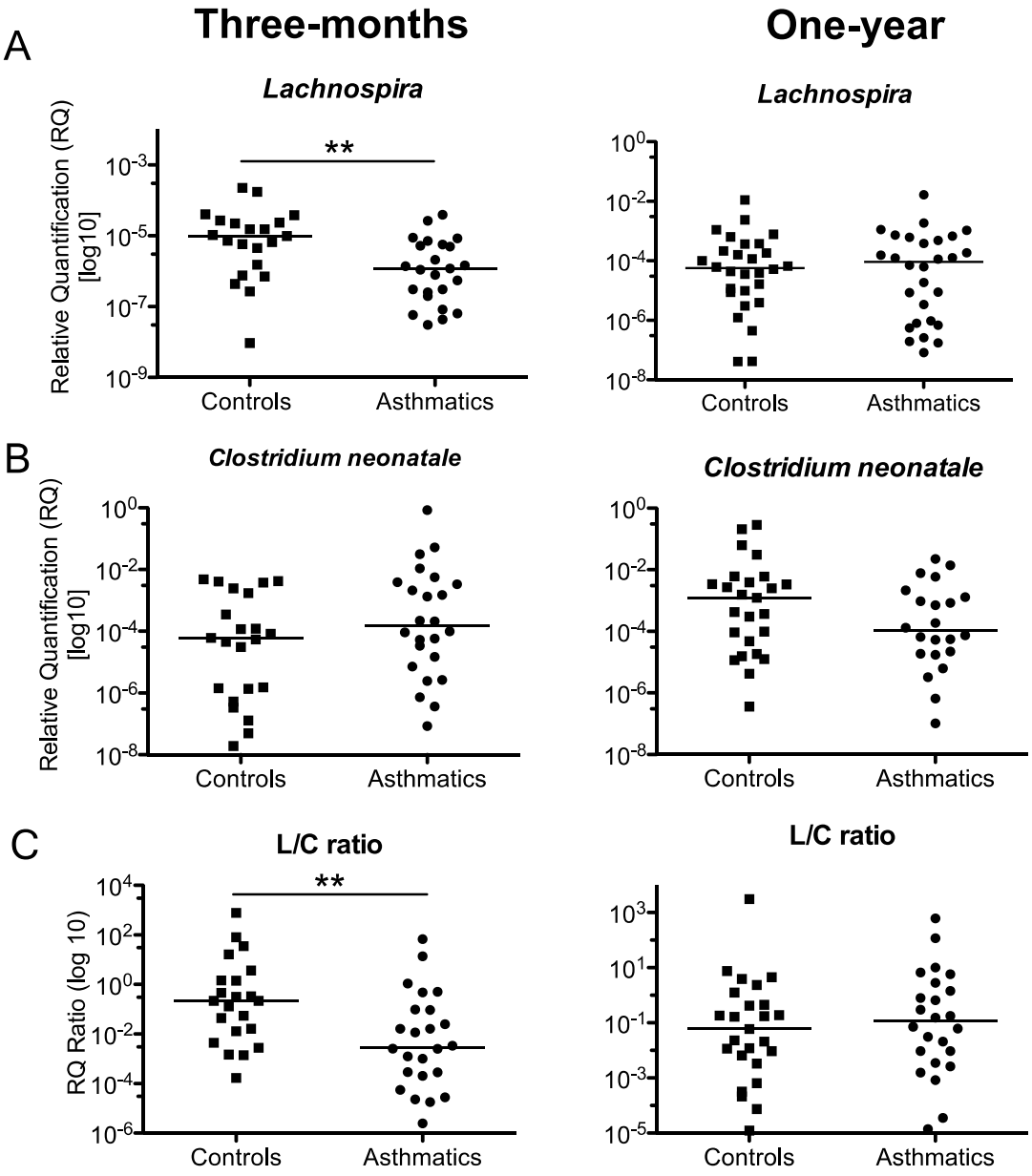

Figure C.5 Sub-group analysis among subjects with no atopic dermatitis at 3-months

A) *Lachnospira* [Mann-Whitney p = 0.01, **B)** *C. neonatale*, **C)** L/C ratio [Mann-Whitney p = 0.004] at 3-months and 1-year. Star representation; p ≤0.05 *, p ≤0.01 **. n_{3mo} Asthmatics = 28, Controls = 24, n_{1Y} Asthmatic = 28, Controls = 28.

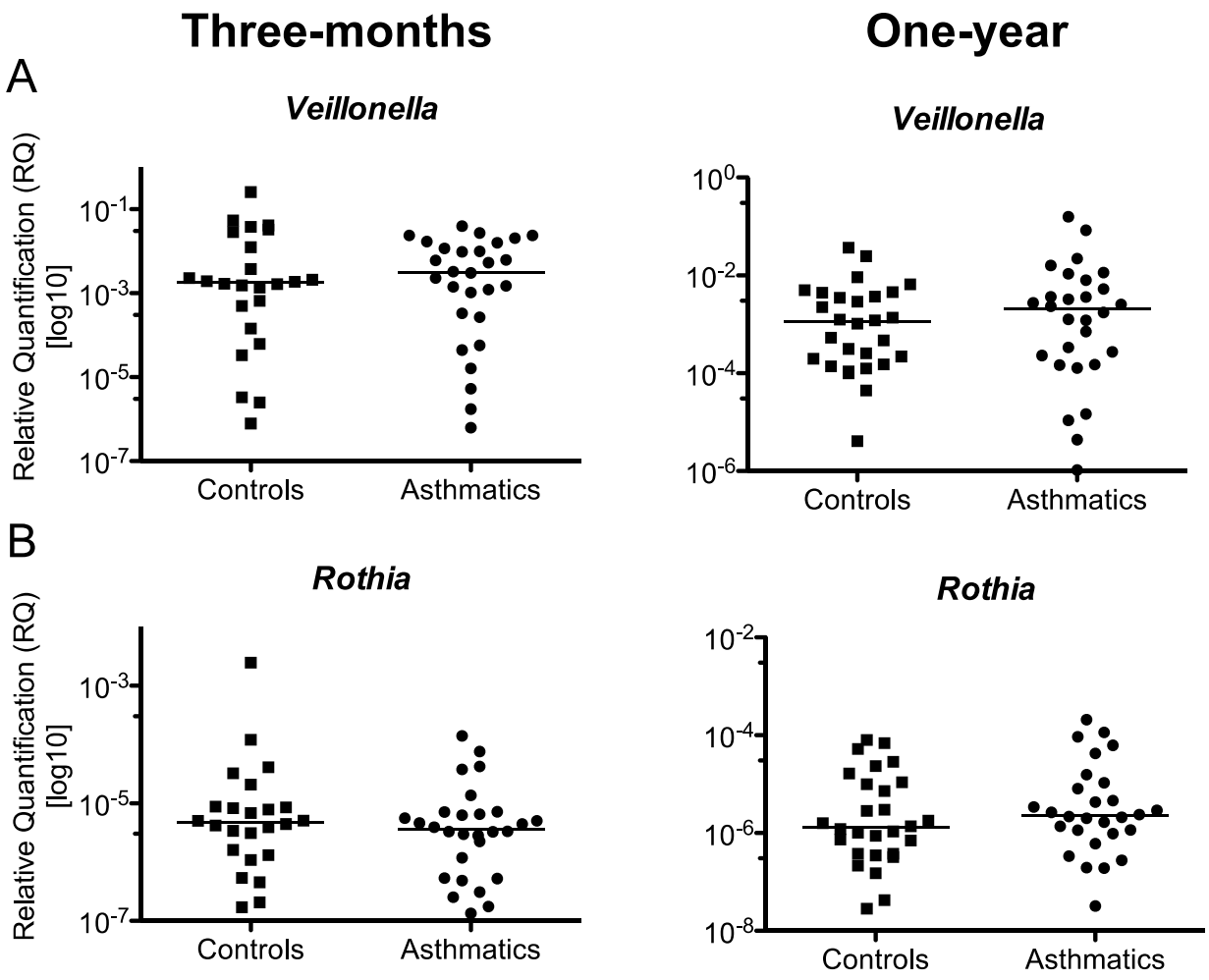

Figure C.6 Sub-group analysis among subjects with no atopic dermatitis at 3-months

A) *Veillonella,* **B)** *Rothia* at 3-months and 1-year. n_{3mo} Asthmatics = 28, Controls = 24, n_{1Y} Asthmatic = 28, Controls = 28.

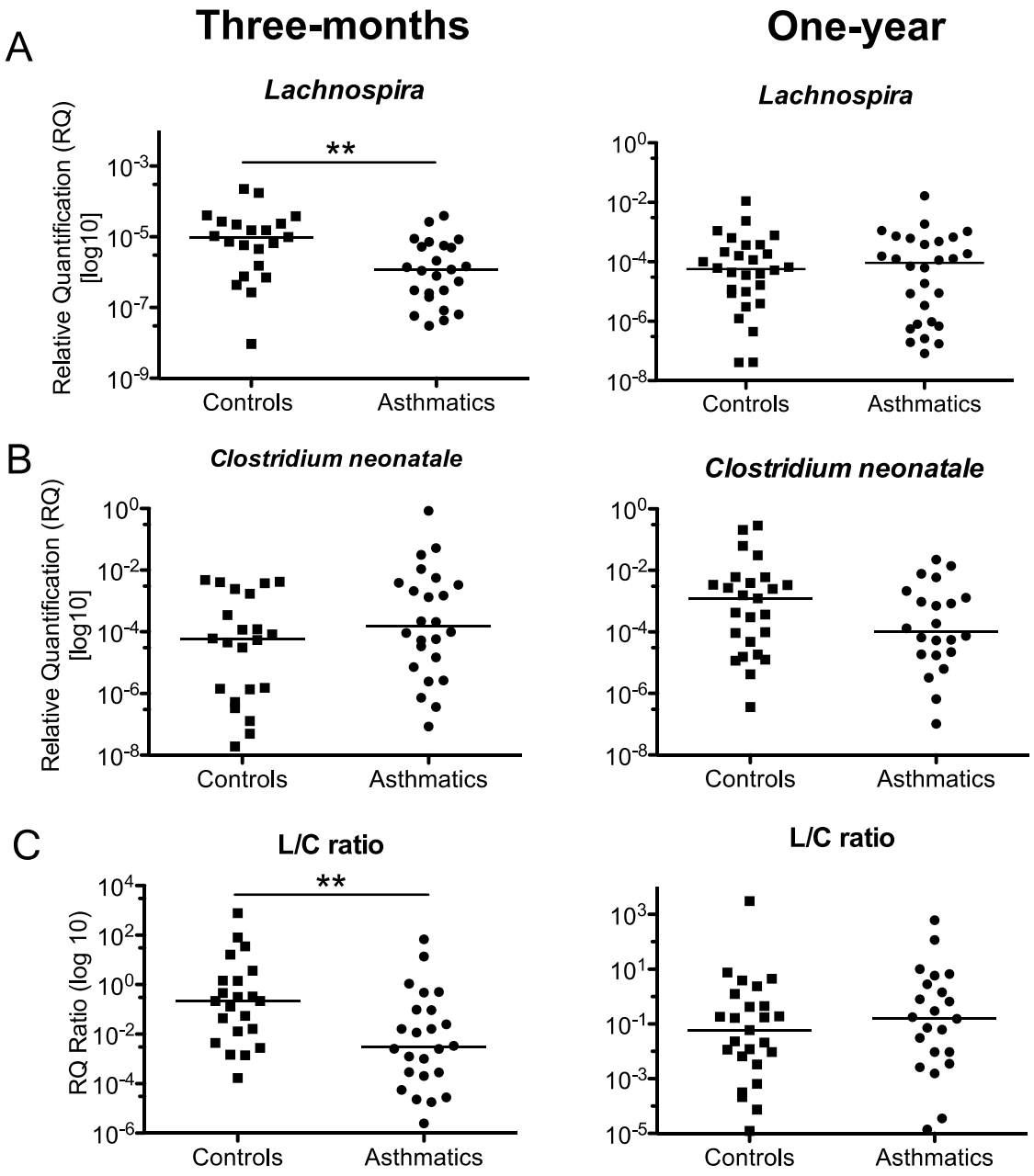

Figure C.7 Sub-group analysis among subjects with no atopic dermatitis at 1-year

A) *Lachnospira* [Mann-Whitney p = 0.005]**, B)** *C. neonatale,* **C)** L/C ratio [Mann-Whitney p = 0.007] at 3-months and 1-year. Star representation; $p \leq 0.05$ *, $p \leq 0.01$ **. n_{3mo} Asthmatics = 24, Controls = 21, n_{1Y} Asthmatic = 22, Controls = 25.

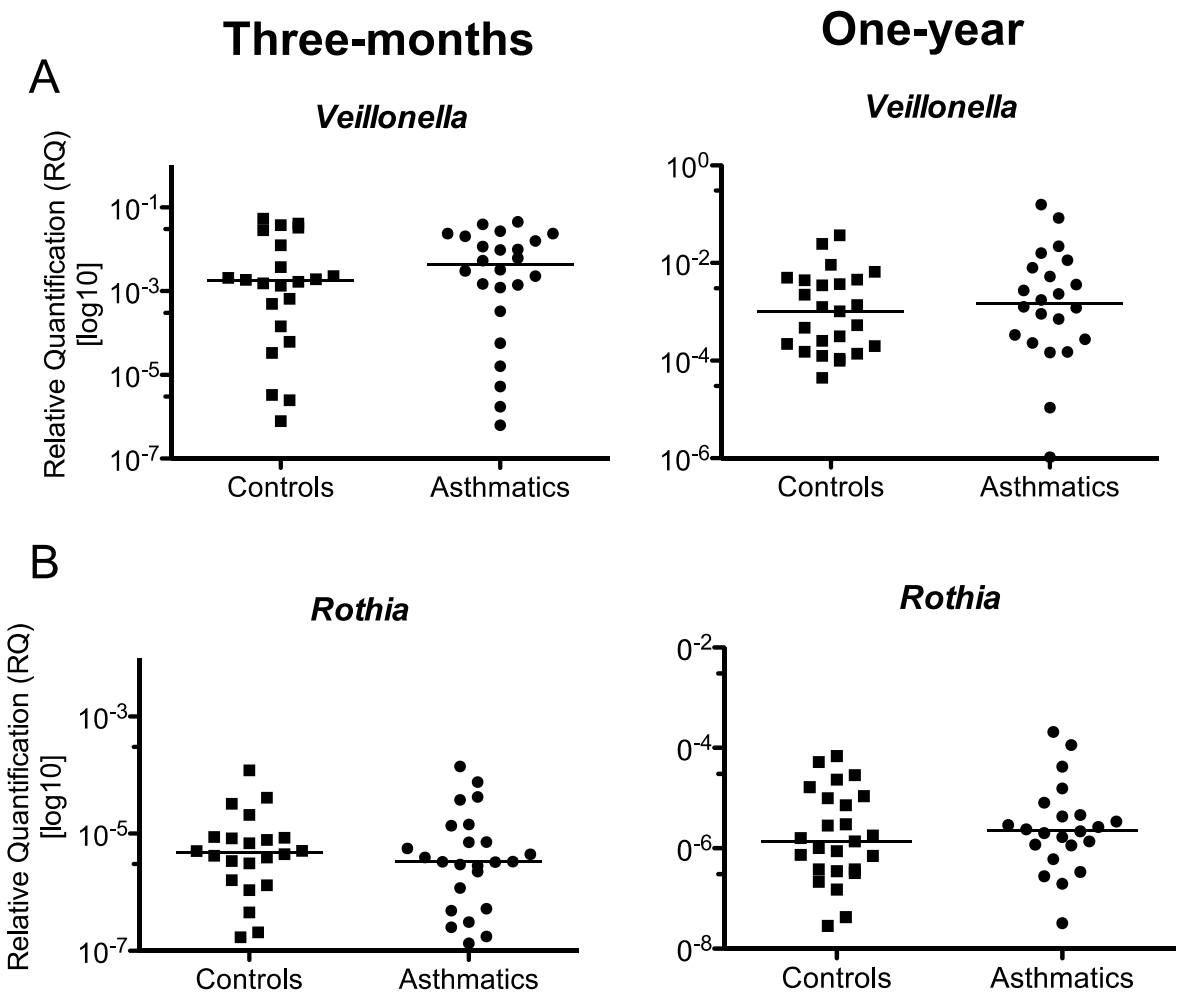

Figure C.8 Sub-group analysis among subjects with no atopic dermatitis at 1-year

A) *Veillonella***, B)** *Rothia* at 3-months and 1-year. n_{3mo} Asthmatics = 24, Controls = 21, n_{1Y} Asthmatic = 22, Controls = 25.

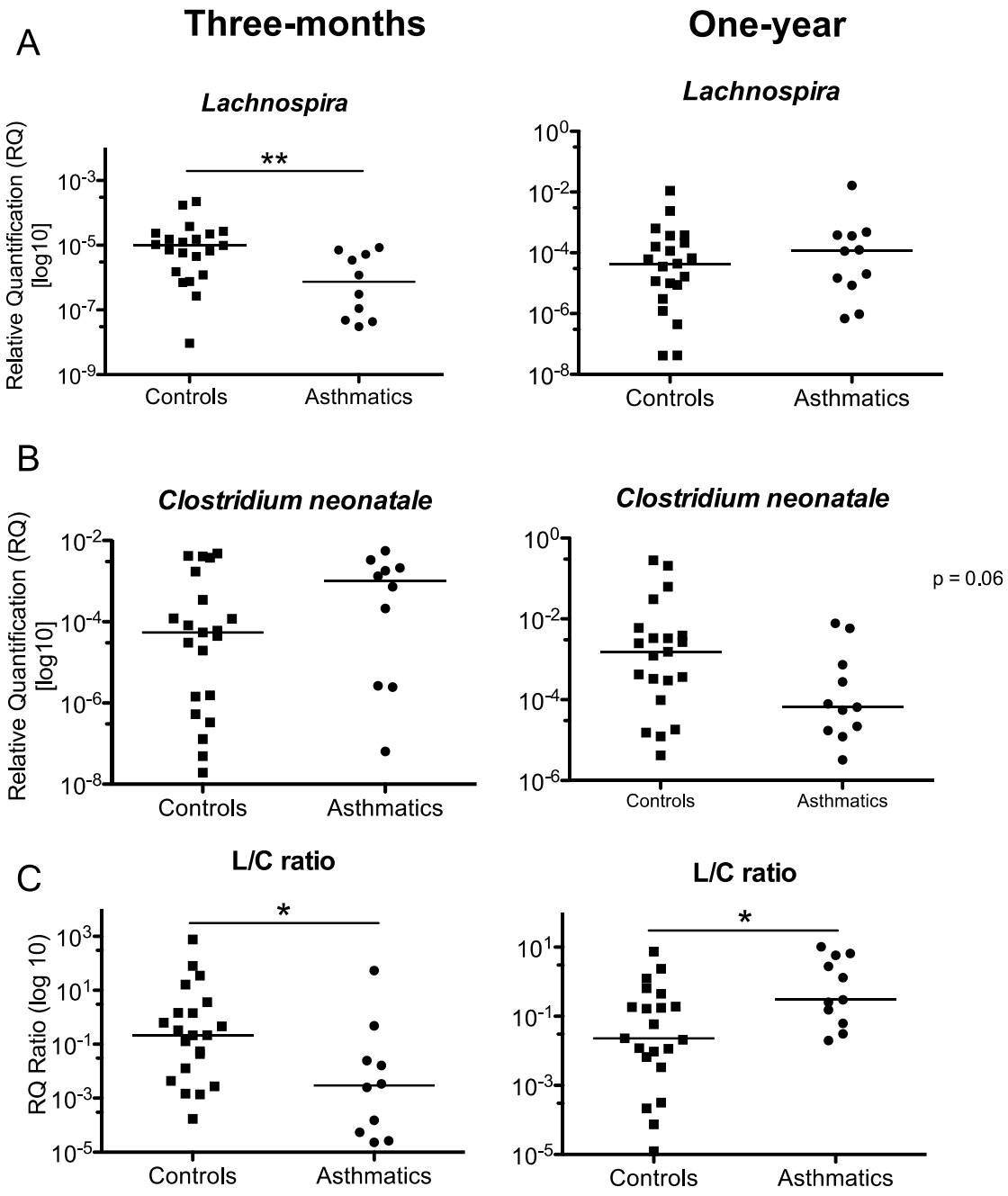

Figure C.9 Sub-group analysis among subjects with no parental history of asthma

A) *Lachnospira* [Mann-Whitney p = 0.008]**, B)** *C. neonatale,* **C)** L/C ratio [Mann-Whitney p_{3mo} = 0.02, p_{1y} = 0.02] at 3-months and 1-year. Star representation; p ≤0.05 *. n_{3mo} Asthmatics = 10, Controls = 21, n_{1Y} Asthmatic = 11, Controls = 21.

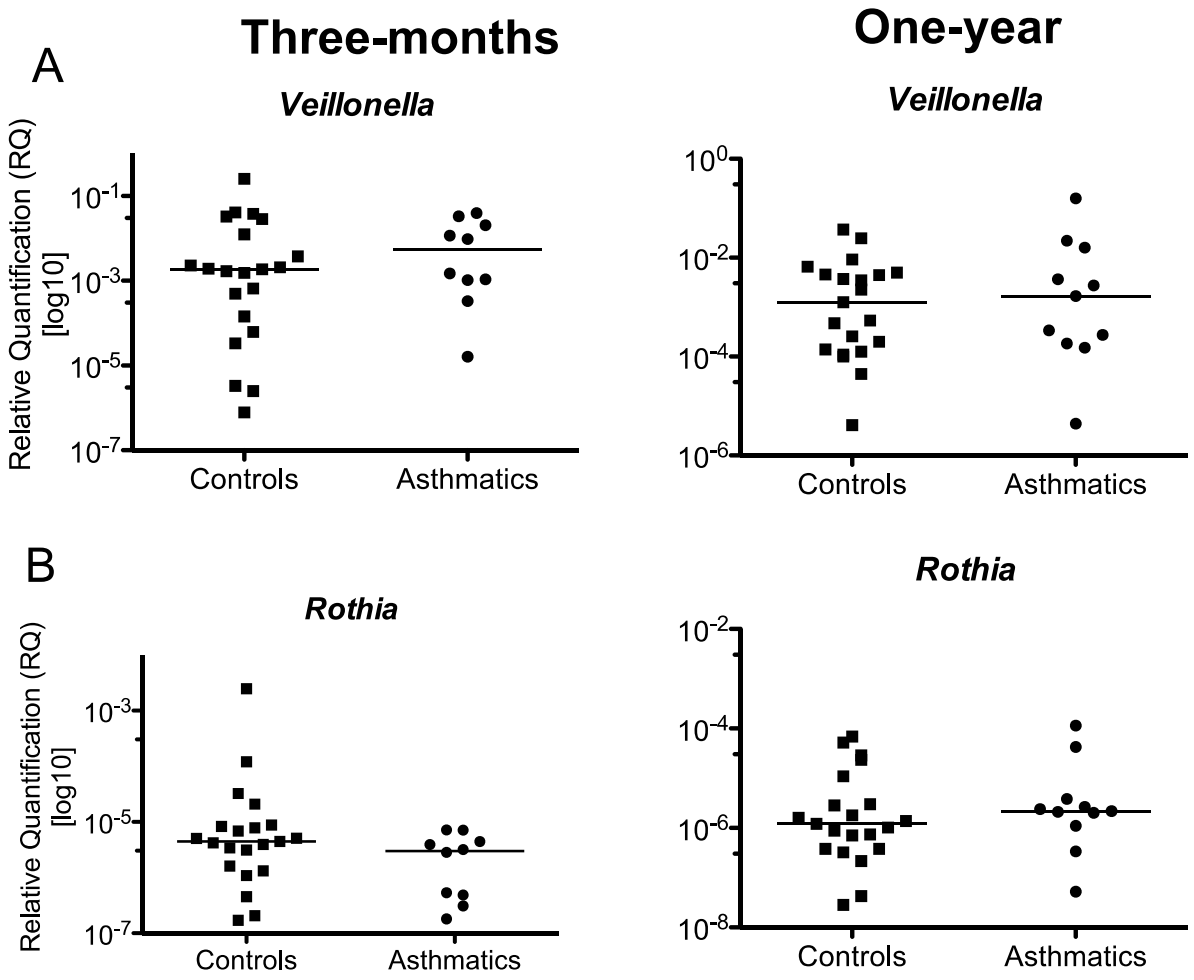

Figure C.10 Sub-group analysis among subjects with no parental history of asthma

A) *Veillonella*, **B)** *Rothia* at 3-months and 1-year. n_{3mo} Asthmatics = 10, Controls = 21, n_{1Y} Asthmatic = 11, Controls = 21.

D. Representative analyses for subsets analyzed in Chapters 3 and 4 relative to cohort analyzed in Chapter 2

Table D.1 Multivariate logistic regression analysis of Chapter 3 subset.

Variable		Phenotype		OR	95% CI		P-value
		Chapter 3	Chapter 2		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	79 (26%)	507 (21%)	1.29	0.95	1.82	0.1
	None	224 (74%)	1885 (79%)				
	Total (100%)	303	2,392				
Antibiotic Exposure (birth to 3-months of age)	1 or more	20 (7%)	149 (6%)	0.82	0.45	1.35	0.46
	None	283 (93%)	2243 (94%)				
	Total (100%)	319	2,392				
AD at 3-months	Yes	28 (9%)	167 (7%)	1.23	0.82	2.01	0.34
	No	275 (91%)	2225 (93%)				
	Total (100%)	303	2,392				
AD at 1-year	Yes	72 (24%)	460 (19%)	1.2	0.9	1.65	0.2
	No	231 (76%)	1932 (81%)				
	Total (100%)	303	2,392				
Sex	Female	125 (41%)	1128 (47%)	1.23	0.96	1.65	0.1
	Male	178 (59%)	1264 (53%)				
	Total (100%)	303	2,392				
Mode of birth	Vaginal	238 (79%)	1792 (75%)	1.26	0.94	1.65	0.12
	Caesarean	65 (21%)	600 (25%)				
	Total (100%)	303	2,392				
Breast-feeding 3-months	Yes	261 (86%)	2082 (87%)	1.01	0.67	1.49	0.97
	No	42 (14%)	310 (13%)				
	Total (100%)	303	2,392				

Variable		Phenotype		OR	95% CI		P-value
		Chapter 3	Chapter 2		Lower	Upper	
Breast-feeding 1-year	Yes	131 (43%)	1113 (47%)	0.88	0.67	1.1	0.34
	No	172 (57%)	1279 (53%)				
	Total (100%)	303	2,392				
Paternal Asthma	Yes	45 (15%)	375 (16%)	0.93	0.67	1.34	0.68
	No	258 (85%)	2017 (84%)				
	Total (100%)	303	2,392				
Maternal Asthma	Yes	83 (27%)	477 (20%)	1.5	1.1	2.01	0.004
	No	220 (73%)	1915 (80%)				
	Total (100%)	303	2,392				
Parental Ethnicity	One Caucasian parent	261 (86%)	2064 (86%)	0.98	0.67	1.35	0.93
	No	42 (14%)	261 (14%)				
	Total (100%)	303	2,392				

Note: Only 303 subjects were included in this model. The remaining 15 subjects were not included in the Chapter 2 analysis because they either withdrew from the study before reaching age 3 years or did not have complete data to classify them with an asthmatic phenotype at age 3 years.

Table D.2 Multivariate logistic regression analysis of Chapter 4 subset.

Variable		Phenotype		OR	95% CI		P-value
		Chapter 4	Chapter 2		Lower	Upper	
Antibiotic Exposure (birth to 1-year of age)	1 or more	19 (25%)	567 (22%)	1.14	0.61	2.01	0.67
	None	57 (75%)	2052 (78%)				
	Total (100%)	76	2,619				
Antibiotic Exposure (birth to 3-months of age)	1 or more	5 (7%)	164 (6%)	0.94	0.33	2.72	0.91
	None	71 (93%)	2455 (94%)				
	Total (100%)	76	2,619				
AD at 3-months	Yes	8 (11%)	187 (7%)	1.47	0.67	3.32	0.33
	No	68 (89%)	2432 (93%)				
	Total (100%)	76	2,619				
AD at 1-year	Yes	18 (24%)	514 (20%)	1.15	0.67	2.01	0.63
	No	58 (76%)	2105 (80%)				
	Total (100%)	76	2,619				
Sex	Female	35 (46%)	1218 (47%)	0.99	0.61	1.64	0.98
	Male	41 (54%)	1401 (53%)				
	Total (100%)	76	2,619				
Mode of birth	Vaginal	63 (83%)	1967 (75%)	1.7	0.92	3.00	0.09
	Caesarean	13 (17%)	652 (25%)				
	Total (100%)	76	2,619				
Breast Feeding	Yes	72 (95%)	2529 (97%)	0.96	0.91	1.02	0.2
	No	4 (5%)	90 (3%)				
	Total (100%)	76	2,619				
Parental Asthma	Yes	38 (50%)	846 (32%)	2.11	1.34	3.39	0.001
	No	38 (50%)	1773 (68%)				
	Total (100%)	76	2,619				

Variable		Phenotype		OR	95% CI		P-value
		Chapter 4	Chapter 2		Lower	Upper	
Parental Ethnicity	One Caucasian Parent	66 (87%)	2259 (86%)	0.96	0.5	2.01	0.92
	No	10 (13%)	360 (14%)				
	Total (100%)	76	2,619				

E. Additional publications not included in Preface

Stiemsma, L.T., Turvey, S.E., and Finlay, B.B. An antibiotic-altered microbiota provides fuel for the enteric foe. *Cell Res*. 2014;24:5-6.

McKinnon ML, Rozmus J, Fung SY, Hirschfeld AF, Del Bel KL, **Thomas L.T.**, Marr N, Martin SD, Marwaha AK, Priatel JJ, Tan R, Senger C, Tsang A, Prendiville J, Junker AK, Seear M, Schultz KR, Sly LM, Holt RA, Patel MS, Friedman JM, Turvey SE. Combined immunodeficiency associated with homozygous MALT1 mutations. *J Allergy Clin Immunol* 2014; 133: 1458-1462, 1462 e1451-1457.

*Published under birth name, Thomas.