Prenatal and early childhood antibiotic exposure and the risk of neurodevelopmental disorders

by

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Background: Early life changes in microbiota composition as a result of antibiotic exposure potentially impair brain development and increase the risk of neurodevelopmental disorders. In this project, I examined the association of antibiotic exposure during the first year of life and prenatal antibiotic exposure with the risk of autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD).

Methods: A population-based cohort study was conducted which included children born in Manitoba, Canada between the fiscal years 1998 and 2016/2017 and utilized administrative databases in Manitoba. Exposure was defined as having filled one or more antibiotic prescriptions during the first year of life or pregnancy. The outcome was ASD or ADHD diagnosis identified in hospital abstracts, physician visits, drug dispensations or educational special needs funding data. Cox proportional hazards regression models were used to estimate the risk of ASD or ADHD in the entire population and a sibling cohort. The risk of ADHD was additionally estimated in a high dimensional propensity score (HDPS)-matched cohort.

Results: In a cohort of 214 834 children, antibiotic use in the first year of life was associated with a small reduction in ASD risk (Hazard ratio [HR] 0.91, 95% CI 0.84-0.99) but this was not observed in the sibling cohort (HR 1.03, 95% CI 0.86-1.23). Prenatal antibiotic exposure was associated with a small increase in ASD risk in the overall cohort (HR 1.10, 95% CI 1.01-1.19) and in the sibling cohort (HR 1.08, 95% CI 0.90-1.30).

In a cohort of 187 605 children, antibiotic exposure in the first year of life was not associated with ADHD risk in the HDPS-matched cohort (HR 1.02, 95% CI 0.97-1.08) and in the

sibling cohort (HR 0.96, 95% CI 0.89-1.03). Prenatal antibiotic exposure was associated with increased ADHD risk in the HDPS-matched cohort (HR 1.22, 95% CI 1.17-1.27) but the risk was attenuated in the sibling cohort (HR 1.06, 95% CI 0.99 - 1.13).

Conclusions: No association was observed between antibiotic exposure in the first year of life and ASD or ADHD risk. In addition, no clinically-significant association was observed between prenatal antibiotic exposure and ASD or ADHD risk.

ACKNOWLEDGMENTS

I would like to express my gratitude to my advisors: Dr. I fan Kuo and Dr. Silvia Alessi-Severini for the great mentorship, outstanding guidance, kindness, and encouragement. I deeply appreciate giving me the liberty to follow my passion and the numerous opportunities they facilitated to help building my skills as an independent researcher and teacher. It was a privilege working with both of you. I am truly grateful to my advisory committee members: Dr. Salaheddin Mahmud and Dr. Marni Brownell for their invaluable input and guidance in shaping the research project and for the countless manuscript edits.

I would like to thank the staff at the Manitoba Centre for Health Policy. Specifically, Charles Burchill, Heather Prior and Matt Dahl for their guidance in working with administrative databases and SAS coding. I would also like to thank Kristine Kroeker from the George & Fay Yee Centre for Healthcare Innovation for sharing her statistical expertise.

I would like to thank my family and friends in Jordan for their support and understanding when I missed their special days and occasions. I am thankful to Donica Janzen, Wajd Alkabbani, Kevin Friesen and all the students in the Pharmacoepidemiology student group (PEG) for their support, input and the endless practices. Donica and Wajd, thank you for making conference travels much more enjoyable. Kevin, thank you for making SAS coding more fun than it really is.

I am grateful to have received the University of Manitoba Graduate Fellowship and the Evelyn Shapiro Award for Health Services Research, which helped funding this project. I am thankful to the College of Pharmacy at the University of Manitoba for the continuous support throughout my Ph.D. program.

I acknowledge the Manitoba Centre for Health Policy for use of data contained in the Manitoba Population Research Data Repository under project #H2016:244 (HIPC# 2016/2017 – 11). I thank Manitoba Health, Healthy Living and Senior, Winnipeg Regional Health Authority, Manitoba Education and Training, Manitoba Department of Families and Healthy Child Manitoba for the contribution of data used in this study. The results and conclusions are those of the authors and no official endorsement by the Manitoba Centre for Health Policy, Manitoba Health, Healthy Living and Senior or other data providers is intended or should be inferred.

DEDICATION

In memory of my father who encouraged me to pursue graduate studies but did not live to see it happen.

To my husband and best friend Sultan for his endless love, for inspiring me to chase my dream and for constantly being by my side.

To my son Rayan, my ultimate source of pride and the joy of my life.

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LIST OF ABBREVIATIONS

AAP: The American Academy of Pediatrics

ADD: Attention deficit disorder

ADHD: Attention-deficit/hyperactivity disorder

ASD: Autism spectrum disorder

ATC: Anatomical Therapeutic Classification

CADDRA: The Canadian ADHD Resource Alliance

CI: Confidence interval

CNS: The central nervous system

DPIN: Drug Program Information Network

DSM: The Diagnostic and Statistical Manual of Mental Disorders

DSM-5: The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

FDA: The Food and Drug Administration

GIT: The gastrointestinal tract

HDPS: High Dimensional Propensity Scores

HR: Hazard ratio

ICD: The International Classification of Disease coding system

ICD-10: The 10th revision of the International Classification of Disease coding system

ICD-9-CM: The 9th revision - Clinical Modifications of the International Classification of Disease coding system

ID: Intellectual disability

IQR: Interquartile range

NASS: National Autism Spectrum Disorder Surveillance System

NDD: Neurodevelopmental disorders

NSCH: National Survey of Children's Health

OR: Odds ratio

PDD: Pervasive developmental disorder

PHIN: Scrambled Personal Health Identification Number

SAMIN: The Social Allowances Management Information Network

SD: Standard deviation

SEFI: The Socio-Economic Factor Index

SES: Socioeconomic status

UK: The United Kingdom

US: The United States

YLDs: Years lived with disability

PREFACE

This thesis is written in a grouped manuscript style and includes work that has been published or submitted for publication in peer-reviewed journals. The manuscripts which are included in the thesis were conceptualized and executed by Amani Hamad in collaboration with her thesis advisors and advisory committee: Dr. I fan Kuo, Dr. Silvia Alessi-Severini, Dr. Salaheddin Mahmud and Dr. Marni Brownell. Amani Hamad is the guarantor and takes full responsibility for the integrity of the manuscripts and accuracy of the analysis and interpretation. All authors on the manuscripts contributed to study concept, design and results interpretation. All authors revised and approved the manuscripts for publication.

The thesis includes six chapters: an introductory chapter, four manuscripts which are published or submitted for publication in peer-reviewed journals, and a concluding chapter. The manuscripts generated from this thesis are:

- Chapter 2: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Early Childhood Antibiotics Use and Autism Spectrum Disorders: A Population-Based Cohort Study. *Int J Epidemiol* 2018; 47 (5):1497-1506
- Chapter 3: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Prenatal Antibiotics Exposure and the Risk of Autism Spectrum Disorders: A Population-Based Cohort Study. *PLOS ONE* 2019; 14: e0221921
- Chapter 4: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Antibiotic Exposure in the First Year of Life and the Risk of Attention-Deficit/Hyperactivity Disorder: a Population-Based Cohort Study. *Am J Epidemiol* 2019; 188: 1923–1931

- Chapter 5: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Prenatal Antibiotic Exposure and the Risk of Attention Deficit/Hyperactivity Disorder: A Population-Based Cohort Study. *CMAJ (submitted)* 2019

Chapter 1: Introduction and Rationale

1.1. Background

Neurodevelopmental disorders (NDD) are defined by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) as a group of conditions characterized by developmental deficits resulting in impairments of functioning, which manifest early in development.[1] Autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) are the most commonly diagnosed NDD.[2–4]

ASD is a disorder of significant burden with 62 million people affected and a prevalence of 0.83% worldwide.[5] A prevalence of 0.67% was reported in the United States (US) in 2000, which increased to 1.7% in 2014.[6] In several regions of Canada, a prevalence of 0.46 - 0.53% was reported in 2003, which increased to 0.83 - 1.09% in 2008.[7] The worldwide ADHD prevalence is estimated at 5.3 to 7.1% in children and adolescents.[8,9] In Canada, national-level data on ADHD prevalence are not yet available. In the province of Manitoba, ADHD prevalence in children aged 6 to 19 years was 5.5% between 2005 and 2009, which increased to 6.8% between 2009 and 2013.[10]

The exact etiology of NDD is not well-established but an interaction of genetics and environmental factors is proposed.[11–15] A considerable amount of literature examined prenatal and postnatal environmental factors and reported an association of several factors with ASD and ADHD.[16–26] However, it remains unclear how these environmental factors may play a role in the development of these disorders, if any. Recent research proposed microbiota as a contributor to the etiology of NDD as it has been shown that children with these disorders have an abnormal microbiota composition, *dysbiosis*.[27–35] Early life changes in microbiota

composition, which can be induced by exposure to antibiotics, disrupts the gut-brain axis and potentially impairs brain development and increases the risk of NDD.[34,36–42] This project aimed to examine the association between antibiotic exposure in early life and the risk of ASD and ADHD.

1.2. Disease definitions

1.2.1. ASD

Autism was first described by Leo Kanner in 1943 when he reported the condition in 11 children and defined it as the "innate inability to form normal emotional contacts with others".[43] Up until the 1970s, the condition was considered a type of childhood schizophrenia. In the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM) published in 1980, autism was categorized as a distinct disorder. The diagnostic criteria were detailed in the revised form of DSM-3 to include 8 to 16 criteria among areas of communication, social and restricted interest or activities and to include a new category "Pervasive Developmental Disorder, Not Otherwise Specified". In the 1994 fourth edition of DSM and subsequent revision in 2000, autism disorder was classified as a type of pervasive developmental disorder (PDD), along with other distinct disorders including Asperger's disorder, childhood disintegrative disorder, and Rett's syndrome.

In 2013, the fifth edition of DSM (DSM-5) was published and the term ASD was coined to collectively describe the disorders previously classified under PDD, except for Rett's syndrome, under a uniform set of diagnostic criteria. As a result, ASD is presently defined as a neurodevelopmental disorder affecting social communication and social interaction, with restricted repetitive patterns of behaviour or activities.[1] Under DSM-5, two diagnostic criteria

aimed to describe the symptoms of ASD "Persistent impairment in reciprocal social communication and social interaction" plus "restricted, repetitive patterns of behavior".[1] The goal of transitioning to DSM-5 diagnostic criteria for ASD was driven by the inconsistency in applying DSM-4 criteria in different practice settings.[1] A large validation study reported a sensitivity of 91% for ASD diagnosis in children previously diagnosed with PDD, indicating that most children diagnosed with PDD under DSM-4 criteria fulfill ASD diagnostic criteria under DSM-5.[44]

According to the diagnostic criteria in DSM-5, an ASD diagnosis is feasible at the age of 12 to 24 months. However, symptoms can be recognized earlier than 12 months or later than 24 months depending on the severity of the condition.[1] The American Academy of Pediatrics (AAP) recommends screening for ASD in children as young as 18 months since communication and language development delays in addition to mild motor delays are usually detectable at this age.[45–47] Several screening tools were developed to identify ASD in younger children.[48–51] The "Modified Checklist for Autism in Toddlers, Revised with Follow-Up" is among the most commonly used tools and has been shown to be valid in children as young as 18 months of age.[48]

1.2.2. ADHD

ADHD is a neurodevelopmental disorder characterized by a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development.[1] ADHD was first described in 1902 by Sir George Still, a British pediatrician who defined the condition as "an abnormal defect of moral control in children".[52] In 1952, with the introduction of the first edition of DSM, it was referred to as "minimal brain dysfunction". In 1968, in the second edition

of DSM, it was referred to as “hyperkinetic reaction of childhood”, which excludes inattention as a diagnostic criterion. It was not until 1980 with DSM-3 that inattention was included and the term “attention deficit disorder (ADD), with or without hyperactivity” was used. In DSM-3 revision, the term changed into ADHD with diagnostic criteria including several symptoms within either inattention or hyperactivity. In 1994, DSM-4 introduced ADHD subtypes: ADHD predominantly inattentive, ADHD predominantly hyperactive-impulsive or ADHD combined type. In 2013, diagnostic criteria under DSM-5 were broadened to include adolescents and adults.[1]

AAP recommends screening for ADHD in children as young as 4 years of age.[53] This recommendation is based on a review of 9 studies reporting evidence of reliability and validity of ADHD diagnostic criteria in children aged 4 to 5 years.[54] The most recent edition of the practice guideline by the Canadian ADHD Resource Alliance (CADDRA) acknowledged that ADHD can be diagnosed in preschoolers and cited AAP recommendation on screening for ADHD as early as age 4.[55] The Canadian Pediatric Society did not recommend a minimum age of diagnosis for ADHD in their 2018 position statement on ADHD diagnosis.[56]

1.3. Epidemiology

1.3.1. ASD

Among all mental disorders, ASD is the leading cause of disabilities in children younger than 5 years and is the fourth leading cause of disabilities in children 5 to 14 years of age.[57] The disorder accounts for over 9 million years lived with disability (YLDs) worldwide.[58] In 2016, there were 62 million ASD-diagnosed cases worldwide, with a prevalence of 0.83% across all age groups.[5] ASD prevalence in 8-year-old children in the US was 1.7% in 2014, an increase from

0.67% in 2000.[6] In all data years, males were four times more likely to be diagnosed with ASD than females.[6]

In Canada, the Public Health Agency of Canada's National Autism Spectrum Disorder Surveillance System (NASS) reported a combined ASD prevalence of 1.52% in children aged 5 to 17 years based on 2015 data from six provinces and one territory.[59] In the province of Manitoba, which was not included in NASS findings, ASD prevalence was estimated to be 0.49% in children aged 5 to 9 years between 1996 and 2000. This estimate increased to 0.88% between 2001 and 2005 and 1.25% between 2009 and 2014.[60,61] ASD prevalence in younger Manitoba children aged 1 to 5 years increased from 0.46% in 2004 to 0.97% in 2015.[62]

Less is known about the worldwide incidence of ASD. A Danish study estimated ASD incidence in children and adults at 4 cases per 10 000 person-years in 2010.[63] The highest incidence rate was reported in children 4 to 13 years of age with an incidence of 17-18 cases per 10 000 person-years. In the United Kingdom (UK), ASD incidence rate in 8-years-old children was 10 cases per 10 000 in boys and 2 cases per 10 000 in girls in 2010.[64] In the Canadian province of Alberta, an ASD incidence of 14 cases per 10 000 person-years was reported in 2008 among 3-year-old children.[65] In the Avalon Peninsula of the Canadian province of Newfoundland and Labrador, ASD incidence in children aged 1 to 14 years was 10 cases per 10 000 in 2006 and 17 cases per 10 000 in 2010.[66] In Manitoba, ASD incidence in children aged 1 to 5 years increased from 16 cases per 10 000 person-years in 2004 to 39 cases per 10 000 person-years in 2015.[62]

The observed increase in ASD prevalence and incidence over the past years might be partially attributed to changes in diagnostic criteria, increased public and healthcare professional awareness and methodological differences in epidemiological studies.[67–70] The

higher prevalence of ASD in males was consistently shown in previous studies.[71–74] Several mechanisms were suggested to explain the sex bias in ASD prevalence. The first mechanism is the difference in ASD clinical phenotype between males and females. Symptoms of aggressive and repetitive behaviour are more commonly observed in male ASD patients.[72,75] On the other hand, females tend to have more internalizing symptoms including depression and anxiety, which are harder to identify,[76] and may have contributed to underreporting of ASD diagnoses among females. Another mechanism suggests the male sex hormone, testosterone, as a contributor to the higher prevalence of ASD observed in males.[71,77] Sex chromosomal genes have been suggested as another mechanism for ASD male bias. It was proposed that ASD may be an X-linked disorder but familial ASD transmission patterns did not support this theory.[71]

1.3.2. ADHD

The worldwide burden of ADHD is significant. Around 500 000 YLDs are attributable to ADHD.[78] In males aged 10 to 14 years, ADHD is the 34th highest contributor to YLDs, which is higher than diabetes and intellectual disability.[78] A significant proportion of children with ADHD continue to meet the diagnostic criteria or have ADHD symptoms in adulthood and live with long term adverse health and social outcomes.[79–82] Adult ADHD patients were reported to have higher rates of motor vehicle collisions, criminal offences, comorbid depression and anxiety, drug abuse and work absenteeism in addition to having less work productivity and marriage instability. [83–87]

The worldwide ADHD prevalence ranges from 5.3 to 7.1% in children and adolescents.[8,9] According to the US National Survey of Children's Health (NSCH), parent-reported ADHD prevalence in children aged 4 to 17 years was 8.8% in 2011.[88] Among those, 69% were taking

ADHD medications. In Canada, national-level data on ADHD prevalence are not yet available. In Manitoba, the 5-year ADHD prevalence was 5.5% between 2005 and 2009, which increased to 6.8% between 2009 and 2013.[10] Less is known about the incidence of ADHD in the general population. A Danish registry study estimated an ADHD incidence in children and adults at 9 cases per 10 000 person-years in 2010.[89] In Sweden, the annual incidence of ADHD increased from 4 to 9 cases per 10 000 persons between 2007 and 2011.[90]

Similar to ASD, a higher prevalence of ADHD in males was observed in many studies.[8,91,92] Several mechanisms have been suggested to explain this male bias in the development of ADHD. One mechanism suggests the difference in ADHD symptoms in males and females. Male patients present more frequently with symptoms of inattention and aggressive behaviour compared to females, which makes it easier to identify and diagnose the disorder in males.[93] Another mechanism involves the difference in the threshold of polygenic ADHD risk score, an aggregate of alleles indexing genetic risk for ADHD. It is suggested that a higher threshold of genetic risk is required in females to manifest ADHD symptoms. This theory is supported by the observation of higher number of ADHD symptoms in siblings of females with ADHD than siblings of males with ADHD.[94]

1.4. Treatment

ASD is a chronic condition without a known cure. However, behavioural and pharmacological treatments are offered to improve functionality. Behavioural therapy is the mainstay of treatment in ASD patients. Psychotropic medications including antipsychotics and antidepressants, can be used to control the symptoms.[95–97] Among those medications, only aripiprazole and risperidone have a US Food and Drug Administration (FDA) labelled indication

for autism-related irritability. Currently, none of these medications is approved by Health Canada for use in children with ASD.

As with ASD, ADHD is a chronic condition with no curative therapy. However, pharmacological treatments are available and recommended in certain patients to manage the core symptoms of ADHD, usually in combination with behavioural therapy. CNS stimulants, such as amphetamine and methylphenidate, are considered the first-line treatment for ADHD in most adults and children older than 6 years.[53,55,98] Atomoxetine and guanfacine are considered second-line treatments due to lower treatment response rates.[55,98] They have minimal CNS stimulant properties and thus have lower abuse potential.[53,55,98] CNS stimulants, atomoxetine, and guanfacine are all approved by the US-FDA and Health Canada for ADHD in adults and children older than 6 years.

1.5. Co-occurrence

Co-occurrence is common among NDD; 59% to 78% of patients with ASD meet the diagnostic criteria for ADHD.[99–101] Although DSM-4 recommended ASD diagnosis as an exclusion criterion for ADHD diagnosis, the most recent edition (DSM-5) acknowledged the fact that these two disorders could overlap. It is currently recommended that if a patient meets the diagnostic criteria for both ASD and ADHD, the patient should receive both diagnoses.[1] Children with both ASD and ADHD diagnoses usually present with more severe symptoms and greater functioning impairment.[102]

1.6. Etiology

1.6.1. Genetic factors

Genetic factors have been proven to have a major role in ASD etiology based on twin studies, family studies and studies of molecular genetics. A population-based observational study of twins investigated the role of genetics and shared environmental factors in the etiology of ASD.[11] The study included 192 twin pairs with at least one twin within each pair having ASD. Probandwise concordance was reported, an estimate of the proportion of twins who have a condition who also have an affected twin. Probandwise concordance was higher in monozygotic compared to dizygotic twins: 77% (95% confidence interval [CI], 65-86%) versus 31% (95% CI, 16-46%), respectively in males and 50% (95% CI, 16-84%) versus 36% (95% CI, 11-60%), respectively in females. Higher probandwise concordance among monozygotic twins who share 100% of their genetic material compared to dizygotic twins who only share 50% supports a genetic contribution to ASD etiology. However, the same study found only a moderate genetic heritability of 38% (95% CI, 14%-67%) and a significant contribution from shared environmental factors, which accounted for 58% (95% CI, 30%-80%) of variance in ASD liability. A population-based cohort study of 13 164 children with ASD reported a relative recurrence risk of 6.9 (95% CI, 6.1-7.8) in full siblings, 2.4 (95% CI, 1.4-4.1) in maternal half-siblings and 1.5 (95% CI, 0.7-3.4) in paternal half-siblings.[103] The higher recurrence risk in full siblings who share 50% of their genetic material compared to half-siblings who only share 25% indicates a genetic contribution to ASD etiology. Moreover, the higher relative risk in maternal half-siblings who share the intrauterine environment compared to paternal half-siblings indicates a role of environmental factors.

To date, three single gene conditions have been identified for association with ASD: fragile X syndrome, methyl-CPG-binding protein 2 spectrum disorders, and phosphatase and

tensin homolog related conditions.[104] AAP and the American Academy of Neurology recommend testing for Fragile X in ASD patients who meet defined criteria.[105,106] However, studies have reported a small ASD diagnostic yield with fragile X testing.[107–109] The identified ASD susceptibility genes account collectively for only 10 to 20% of the cases which suggests a contribution of environmental factors.[12]

Genetic factors play a vital role in the etiology of ADHD with a heritability of 76%.[110] In a population-based study of 1452 pairs of female twins, ADHD concordance was significantly higher in monozygotic twins compared to dizygotic twins in all comorbid ADHD classes (odds ratio [OR] from 2.5, 95% CI 1.9-3.5 to 19.4, 95% CI 6.3-60.0).[111] Genetic contribution was also observed in family studies. A study of 894 children with ADHD and their 1135 siblings reported a relative recurrence risk of 9, 95% CI 7.7-10.4 in the siblings.[112] In an adoption study examining the rates of ADHD in parents of 126 children diagnosed with the disorder, 6% of adoptive parents versus 18% of biological parents had ADHD. A control group of biological parents of non-ADHD children had an ADHD risk of 3%.[113] Many single genes have been identified for an association with ADHD including DAT1, DRD4, DRD5, 5HTT, HTR1B, CDH13, PRKG1, CAMK1D, ITGAE, and ITGA11. However, no single gene accounts for the high heritability of the disorder.[114,115] Genome-wide association studies showed that third of ADHD heritability is a result of polygenic effects with many variants having small individual effects.[14]

1.6.2. Environmental factors

Literature to date examined many prenatal and postnatal environmental factors as predictors of ASD [16–19,22,23]. A meta-analysis of forty studies examined over 60 perinatal and neonatal risk factors and found an association with abnormal presentation, fetal distress, birth injury or

trauma, multiple birth, maternal hemorrhage, summer birth, low birth weight, congenital malformation, low 5-minute Apgar score, feeding difficulties, meconium aspiration, neonatal anemia, ABO or Rh incompatibility, and hyperbilirubinemia.[17] The same research group published a previous meta-analysis examining 50 prenatal risk factors and found an association with advanced maternal age, prenatal medication use, bleeding, having a mother born abroad and prenatal infections.[16] A subsequent case-control study of 286 children with autism found an association with maternal passive smoking, family history of psychiatric disorders, neonatal jaundice, preterm delivery, advanced maternal age and threatened abortion.[18] In a more recent meta-analysis, birth complications, maternal obesity, maternal diabetes, and caesarian section were found to be associated with ASD risk.[116]

Few studies investigated the association of prenatal medication exposure and ASD risk. Antidepressants were studied in association with NDD because of their effects on serotoninergic pathways involved in the etiology of these disorders.[117–121] A recent meta-analysis of 6 population-based studies suggested an increased risk of ASD in children exposed to antidepressants prenatally (OR 1.81, 95% CI 1.49-2.20).[122] When the analysis was adjusted for maternal psychiatric illness, a reduction in the risk estimate was observed but remained significant (OR 1.52, 95% CI 1.09-2.12). Supplementation of folic acid in the 4 weeks prior to conception until 8 weeks after conception was found to be associated with lower rates of autism (OR 0.61, 95% CI 0.41-0.90).[123] In a population-based cohort study, out of 260 children who were exposed to anticonvulsants prenatally, 4.6% met ASD diagnostic criteria.[124] Sodium valproate was the agent most commonly associated with ASD risk which is, as suggested by the authors, mediated through an epigenetic mechanism by inhibiting histone deacetylase protein

involved in the DNA methylation process. So far, the available evidence does not implicate any of the reported environmental factors in ASD etiology due to methodological limitations, such as confounding bias, and inconsistencies between studies.

Similar to ASD, a combination of genetic and environmental factors is implicated in the etiology of ADHD.[14,15] A population-based case-control study investigating prenatal and postnatal risk factors of ADHD in 604 children, reported an association of low paternal education, prenatal smoking exposure, prenatal illicit drug use and maternal depression with higher ADHD symptom scores.[24] Another case-control study of 50 ADHD children and their matched controls reported an association between birth complications, parental psychiatric disorder, early childhood trauma and non-maternal child care and ADHD risk.[25] In contrast, prolonged breastfeeding, which was defined as breastfeeding for more than 3 months, was associated with a lower risk of ADHD.[25] Other studies reported an association of ADHD with low birth weight, preterm delivery, low 5-minute Apgar score, neonatal jaundice, childhood asthma, maternal epilepsy, psychological stress during pregnancy and heavy maternal smoking.[125–132,26]

A limited number of studies investigated the association between prenatal medication exposure and ADHD risk. A case-control study of 7874 children reported an association of ADHD with prenatal antidepressant exposure after controlling for maternal major depression (OR 1.81, 95% CI 1.22-2.70).[133] A more recent population-based cohort study also reported an increased ADHD risk with prenatal exposure to antidepressants but with a smaller effect size (Hazard ratio [HR] 1.39, 95% CI 1.07-1.82).[134] Another population-based cohort study investigated prenatal acetaminophen exposure in 64 322 infants and found an association of acetaminophen exposure with hyperkinetic diagnosis (HR 1.37, 95% CI 1.19-1.59) and with receiving ADHD

treatment (HR 1.29, 95% CI 1.15-1.44).[135] The potential mechanism of this association is the endocrine disrupting properties of acetaminophen that may alter maternal sex and thyroid hormones which are necessary for fetus brain development.[135] This study had the strengths of the prospective design, the large sample size, and the use of database-recorded ADHD diagnosis. However, misclassification is a potential limitation since the exposure and the timing of the exposure were collected by telephone interviews and are susceptible to recall bias. Confounding by indication is another limitation. The authors accounted for fever, infections, and inflammatory conditions; however, acetaminophen could have been used for other conditions such as pain management. The association of prenatal acetaminophen exposure and ADHD risk was observed in two additional studies, despite having similar study limitations.[136,137] As with ASD, the available evidence is not enough to implicate any of these associated factors in ADHD etiology.

1.6.3. Emerging theory: microbiota involvement

1.6.3.1. Microbiota colonization

Human bodies host a large and diverse collection of microorganisms accounting for half of the total human cell count.[138] The majority of these microorganisms, which are collectively called microbiota, resides in the gastrointestinal tract (GIT).[139] Until recently, the fetus gut was believed to be germ-free and that microbiota colonization starts during delivery. However, studies demonstrated colonization of the placenta, amniotic fluid and infant meconium, which suggests in-utero microbiota transfer.[140–143] In a recent study, antibiotic-altered microbial community administered to germ-free pregnant mice transmitted to the offspring and was maintained for at least 21 weeks after birth.[144]

Microbiota colonization continues during delivery and is influenced by the mode of delivery. Infants born vaginally acquire microbiota resembling vaginal bacterial community while those delivered by caesarian section acquire microbiota resembling skin surface bacterial community.[145,146] The main differences observed in caesarean delivered infants are the lower content of *Bifidobacteria* and *Bacteroides fragilis,* and the higher content of *Clostridium difficile* and *Escherichia coli.*[147] Preterm infants were found to have a significantly higher content of *Clostridium difficile* than full-term infants.[147] Colonization continues during early infancy and is influenced by environmental factors such as breastfeeding, which is associated with a higher content of *Bifidobacterium* and a lower content of *Clostridium difficile*, *Bacteroides fragilis* and *Escherichia coli* compared to formula feeding.[147–149] Hospitalization and the use of prebiotics, probiotics, and antibiotics during the pre, peri or postnatal periods is associated with a higher content of Proteobacteria, Firmicutes, Enterobacteriaceae, *Staphylococcus*, *Propionibacterium,* and *Corynebacterium*.[147–149]

Recent evidence suggests that antibiotic administration induces long-term changes to microbiota composition. In one study, data on perinatal antibiotic exposure of 184 Manitoban infants were collected and gut microbiota content at one year of age was determined by analyzing fecal samples.[150] It was found that maternal exposure to antibiotics prior to or shortly after delivery was associated with a significant increase in *Clostridium* content in infants after one year of delivery, which can either be a result of a placental transfer or breastfeeding transfer. Other effects included a lower content of the Bacteroidetes phylum and a higher content of the Proteobacteria phylum and *Akkermansia* genus.[150] Another study examined gut microbiota of three subjects after receiving two courses of the antibiotic ciprofloxacin.[151]

Significant changes in microbiota composition occurred as soon as 3 to 4 days after the initiation of each course. By the end of the study period of 10 months, the composition was not completely back to its initial state. A single clindamycin dose administered to mice resulted in profound changes in microbiota composition for at least four weeks which included a loss of 90% of the microbial taxa of the cecum.[152] Additional human and animal studies showed that prenatal antibiotic exposure induced profound and persistent changes in microbiota composition in the offspring and caused several immunological alterations.[153–155]

1.6.3.2. Microbiota association with NDD

Recently, more research has been conducted to identify microbiota composition and explore its relationship with health and disease states of the host. Animal studies provided preliminary evidence for an association between microbiota and several local and systemic diseases including gastrointestinal, psychiatric, metabolic, autoimmune, neurological and neurodevelopmental disorders.[156–163]

Microbiota has a major role in the communication between the GIT and the central nervous system (CNS), a relation referred to as the microbiota-gut-brain axis.[36,37,164,165] Several mechanisms have been suggested to explain microbial role in this communication pathway, which included endocrine, nervous, and immune mechanisms.[166] Some microbial metabolites affect the brain through an immune or sympathetic nervous system activation.[36,37] For example, microbiota-induced immune system activation produces cytokines that can affect brain function.[167] Additionally, short chain fatty acids, which are common microbial metabolites, influence the function and differentiation of immune system cells.[168] Kynurenic acid, a metabolic product of some bacteria, is part of the metabolic pathway of tryptophan, which is a

precursor for the neurotransmitter serotonin known to be involved in the pathophysiology of psychiatric disorders.[37,169,170] Microbiota, directly or through metabolites, can also induce vagal nerve activation which modulates mood and stress.[37,164] In addition, microbiota regulates the release of gut peptides, which communicate with the brain via a hormonal pathway.[37] Microbiota interacts with the host endocrine signaling and is a critical component for the development of the hypothalamic pituitary adrenal system postnatally.[165] Moreover, microbiota mediate the production of neurotransmitters including gamma aminobutyric acid (GABA), the major inhibitory neurotransmitter of the CNS, which is involved in many pathways and functions in the body.[36,171]

The hypothesis of microbiota involvement in NDD etiology was first suggested based on the frequent GIT symptoms observed in children with ASD.[172,27,173–175] A population-based case-control study examined GIT symptoms in 960 children with ASD, typical development or with developmental delay.[172] Children with ASD were more likely to develop GIT symptoms including abdominal pain, gaseousness, diarrhea, constipation or pain on stooling (OR 7.92, 95% CI 4.89-12.85). In addition, children with frequent GIT symptoms had worse ASD symptom scores, suggesting that dysbiosis contributes to the etiology of the disorder.[31,172] Many studies examined gut microbiota in children with ASD and found that anaerobic bacteria of the genus *Clostridium* are present in significant amounts in comparison to the control group.[27,28,30,176] Antibiotic use is a major risk factor for developing an infection with the *Clostridium species, Clostridium difficile, which is* known to produce neurotoxins that inhibit neurotransmitter release in nerve cells.[177,178] Other studies additionally reported significantly higher content of *Bacteroidetes* and *Lactobacillus* but a lower content of *Bifidobacterium* in children with

autism.[31–33] Lower content of *Faecalibacterium* and a higher content of *Bifidobacterium* were also observed in children with ADHD.[34,35] Early life dysbiosis is believed to affect neurodevelopment and produce long term effects on behaviour and mental health.[36,37,42,161]

Interventions that change microbiota composition have been utilized to modulate brain activity and affect mood and behaviour. In a randomized controlled trial of 40 healthy adult participants, a four-week intake of probiotics versus placebo significantly reduced cognitive reactivity to sad mood by reducing rumination and aggressive thoughts.[179] In another study, a four-week intake of fermented milk with probiotic by 12 healthy women affected brain activity in areas that control the processing of emotion and sensation.[180] In a preliminary study, 75 infants were randomized to receive either a probiotic (*Lactobacillus rhamnosus*) or placebo during the first 6 months of their lives.[181] A follow-up at 13 years of age revealed a lower risk of ADHD and Asperger's disease in infants who received probiotics. No specific pattern of change in microbiota composition was found except for a lower amount of *Bifidobacterium* at 6 months of age in children later diagnosed with ADHD or Asperger's disease. In an open-label study of 18 children with ASD, a fecal microbiota transplant for 7 to 8 weeks was associated with a significant improvement in GIT symptoms and behavioural ASD symptoms.[182] In another open-label trial, an oral dose of vancomycin was given to 11 children with autism for 8 weeks and was followed by a probiotic mixture for another 4 weeks.[183] Improvement in both behavioural and communication symptoms was achieved but was not maintained at follow up. These findings appear to provide some evidence of gut microbiota involvement in NDD etiology and insight into potential treatment modalities.

1.6.3.3. Association of antibiotics and NDD

The current knowledge of microbiota role as a determinant of neurodevelopment and the influence of antibiotics in inducing dysbiosis raises a question on the association between antibiotic exposure in early life and the development of NDD. An exploratory population-based cohort study investigated the association of self-reported maternal infections, febrile episodes, and prenatal antibiotic use with the risk for ASD and infantile autism.[184] The authors found that in a cohort of 96 736 children, no association was observed between maternal infection or febrile episodes and the risk of ASD. However, there was a non-statistically significant trend toward an increased ASD risk with prenatal exposure to antibiotics (HR 1.20, 95% CI 1.00-1.40). This study presents many strengths, including the prospective design, the large sample size, the standard definition of disease outcomes and the numerous covariates included in the analysis. However, the main concern in this study design is the potential of exposure misclassification which was retrospectively self-reported by telephone interviews. Additionally, because of the exploratory nature of the study, it was not specifically designed to examine antibiotics exposure and ASD risk. A recent population-based study using Danish registries followed 780 547 children and found an association of ASD with a diagnosis of otitis media (HR 1.83, 95% CI 1.71-1.95) and with a broad-spectrum antibiotic dispensation (HR 1.29, 95% CI 1.17-1.43).[185] The association of otitis media and ASD did not change after adjusting for broad-spectrum antibiotic dispensation. Furthermore, additional exposure to a broad-spectrum antibiotic in those diagnosed with otitis media had no significant effect on the risk estimate (HR 1.77, 95% CI 1.36-2.30) which suggests that the observed risk with antibiotic exposure is potentially confounded by the indication. The risk of ASD did not increase with higher number of antibiotic dispensations which indicates a

lack of dose-response effect. This was a large cohort study with a long follow-up period which included several important confounders; sex, age, maternal and paternal age at birth, gestational age, parity, parental psychiatric disorders, and previous hospitalizations. However, the study lacked information on many other important confounders such as socioeconomic status, prenatal smoking and alcohol intake, birth complications, mode of delivery and breastfeeding. Genetics and shared environmental factors were also not accounted for. In addition, the study did not have a specific exposure window and included the first antibiotic dispensation which could have occurred anytime during study period. Finally, the study did not examine the risk associated with different antibiotic classes.

A birth cohort study investigated the association of ADHD symptoms with medications used during pregnancy in 871 women.[136] Mothers were interviewed just after birth and were asked yes or no questions on the use of acetaminophen, aspirin, antacids, and antibiotics. ADHD symptoms were measured using a questionnaire completed by the parent when the child was 7 and 11 years of age and by the child at age 11. There was a trend toward higher scores of ADHD symptoms in children who received antibiotics, but it was not found to be statistically significant (mean score difference 0.40, 95% CI –0.50-1.30 and 0.80, 95% CI –0.10-1.70, in child and parent-scoring, respectively). This study has the strengths of the prospective design, the relatively large sample size, and the inclusion of numerous covariates in the analysis. However, a few limitations are present. Misclassification of the exposure is a concern since it was self-reported retrospectively by the mothers just after birth. Moreover, exposure data were collected in a simple yes or no format which lacks information about the type of antibiotic and the number of antibiotic courses received. In addition, outcome data were based on ADHD symptoms

reported by the parents and children rather than a standard diagnostic definition. Finally, restricting the study population to children of women with European ethnicity limits the generalizability of study findings. In a recent population-based cohort study of 671 592 children, antibiotic use in the first two years of life was not found to be associated with ADHD risk in between-within sibling survival models (HR 0.98, 95% CI 0.90-1.07 for penicillin and 0.99, 95% CI 0.92-1.06 for broader spectrum antibiotics).[186] This was a well-designed study with several strengths. The study had a large sample size, identified the exposure and outcome in administrative databases which avoids recall bias, included numerous potential confounders in the analyses, and conducted a sibling-controlled design to minimize unmeasured confounding. The study; however, did not capture the information required to examine a dose-response effect such as the number of antibiotic courses or cumulative duration. Additionally, as the study population was restricted to native Danish children, findings are not necessarily generalizable to other children.

1.7. Utilization of antibiotics in early life

Among all prescription medications, antibiotics are the most frequently dispensed to children accounting for 24% to 27% of all prescriptions;[187] 39.7% of infants below the age of one received at least one antibiotic.[188] In 2010, amoxicillin followed by azithromycin were the most frequently dispensed antibiotics in infants and children.[187] Over-prescription and inappropriate antibiotic prescription were observed in many studies.[187–190] For example, in 23% of pediatric ambulatory visits antibiotics, 50% of which were broad-spectrum antibiotics, were inappropriately dispensed for a respiratory condition without a valid indication.[189] Among

Canadian preschool children, 49% received an antibiotic for upper respiratory tract infection, 32% for serous otitis media, 44% for acute laryngitis or tracheitis and 24% for influenza.[190]

Antibiotics are also commonly prescribed to pregnant women. Up to 33% of pregnant women received antibiotics.[191–193] A large proportion of these antibiotics were prescribed without a valid indication.[191] Of women who received antibiotics during pregnancy, 6.4% received a harmful or a contraindicated antibiotic including tetracyclines, sulfonamides, and quinolones, and 76% received an antibiotic that is not a first line agent for their condition.[192,193]

1.8. Rationale and research objectives

1.8.1. Rationale

Microbiota emerged as a potential contributor to NDD etiology through its role in the microbiota-gut-brain axis. Therefore, early life dysbiosis can impair neurodevelopment and have long term adverse effects on behaviour and mental health. Antibiotic exposure induces long-term dysbiosis, which is proposed as a potential contributor in the etiology of NDD. This project aimed to investigate the association between early life antibiotic exposure and the risk of NDD. Antibiotics are commonly prescribed to children and pregnant women. Over-prescription of antibiotics remains an existing problem as observed in many studies. Accordingly, identifying an association between antibiotic exposure and the risk of NDD, if any, is an important public health concern. Findings from this research provide insight into the possible etiology of NDD which can inform disease prevention strategies.

1.8.2. Objectives

The aim of this thesis is to investigate the association between antibiotic exposure early in life and the risk of NDD, with the following specific objectives addressed in subsequent chapters:

1. To examine the association between antibiotic exposure in the first year of life and ASD risk *(Chapter 2).*
2. To examine the association between prenatal antibiotic exposure and ASD risk *(Chapter 3)*.
3. To examine the association between antibiotic exposure in the first year of life and ADHD risk *(Chapter 4)*.
4. To examine the association between prenatal antibiotic exposure and ADHD risk *(Chapter 5)*.

Chapter 2: Early Childhood Antibiotics Use and Autism Spectrum Disorders: A Population-Based Cohort Study

2.1. Overview

This manuscript is the first of two manuscripts that examine the association of early life antibiotic exposure and ASD risk. This chapter addresses the first project objective, which assesses the association using the first year of life as the exposure period. The manuscript is based on a population-based cohort study of children born in Manitoba between the fiscal years 1998 and 2016 and utilized administrative health data from the Manitoba Population Research Data Repository. Cox proportional hazards regression models were used to estimate the risk of developing ASD in the overall population and in a cohort of exposure-discordant siblings. Study findings suggested no association between antibiotic exposure in the first year of life and ASD risk.

Citation: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Early Childhood Antibiotics Use and Autism Spectrum Disorders: A Population-Based Cohort Study. *Int J Epidemiol* 2018; 47 (5):1497-1506

2.2. Abstract

Background: Changes in microbiota composition as a result of antibiotics use in early life has been proposed as a possible contributor in the etiology of autism spectrum disorders (ASD). We aimed to examine the association between early life antibiotic exposure and risk of ASD.

Methods: This was a population-based cohort study which included all live births in Manitoba, Canada between April 1, 1998 and March 31, 2016. We utilized administrative health data from the Manitoba Population Research Data Repository. Exposure was defined as having filled one or more antibiotic prescription during the first year of life. The main outcome was ASD diagnosis. Cox proportional hazards regression models were used to estimate the risk of developing ASD in the overall population and in a sibling cohort.

Results: Of all subjects in the cohort (n=214 834), 94 024 (43.8%) filled an antibiotic prescription during the first year of life. During follow-up, 2965 children received an ASD diagnosis. Compared to children who did not use antibiotics during the first year of life, those who received antibiotics had a reduced risk of ASD (adjusted HR 0.91, 95% CI 0.84-0.99). Number of treatment courses and cumulative duration of antibiotic exposure were not associated with ASD. In the sibling-controlled analysis, early life antibiotic exposure was not associated with ASD (adjusted HR 1.03, 95% CI 0.86-1.23).

Conclusions: Our findings suggested no clinically significant association between early life antibiotics exposure and risk of ASD and should provide assurance to concerned prescribers and parents.

2.3. Introduction

Autism spectrum disorders (ASD) are among the most commonly diagnosed neurodevelopmental disorders with 52 million cases worldwide.[1–3] They represent a group of disorders characterized by impairment in social communication and interaction with repetitive patterns of behavior.[4] The global prevalence of ASD has been increasing over the years, yet the exact etiology remains unclear.[2,5–7] A complex genetics model is proposed to explain the etiology of ASD where an interaction of several gene variants along with environmental factors is required for the disorder to manifest.[8,9] A substantial amount of literature has examined prenatal and postnatal environmental factors as predictors for ASD [10–16] ; however, causal relationships remain inconclusive. As such, it remains unclear how these environmental factors may play a role in the development of ASD, if any.

Recent research has pointed to a potential role of human microbiota in the etiology of neurodevelopmental disorders. Microbiota, a diverse collection of microorganisms in the human body, has a major role in the communication between the gastrointestinal tract and the central nervous system, a relation referred to as the microbiota-gut-brain axis.[17–19] It has been shown that children with ASD presenting with frequent gastrointestinal symptoms have worse ASD symptom scores, which can be attributed to changes in gastrointestinal microbiota.[20] Moreover, many studies examining gastrointestinal microbiota in children with ASD reported significant differences in microbiota composition compared to control groups.[21–24] In a recent exploratory study, extended-duration microbiota transfer therapy to subjects with ASD resulted in improving gastrointestinal and behavioral symptoms of ASD.[25]

Consistent with this theory, it has been hypothesized that early life changes to gut microbiota composition, potentially induced by antibiotics exposure, may impair the gut-brain axis and, as a result, increase the risk of ASD.[18,17,26–29] In this study, we aimed to examine the association between antibiotic use during the first year of life and the risk of ASD.

2.4. Methods

2.4.1. Design and subjects

We conducted a population-based cohort study utilizing the Manitoba Population Research Data Repository, which provides a comprehensive collection of administrative, registry, survey, and other data on all Manitoba residents and is housed at the Manitoba Centre for Health Policy (MCHP). The health system in the province of Manitoba is universal and publicly funded, hence, any encounter with the health system or drug dispensation is captured in the Repository. All patient records in the Repository are de-identified, and linkage among different databases was achieved through scrambled Personal Health Identification Numbers (PHIN).

The cohort included all births identified in the Manitoba Health Insurance Registry between April 1, 1998 and March 31, 2016. The birthdate was assigned as the index date for cohort entry. A minimum of 18 months of valid Manitoba health registration was required for children to be included in the cohort and we excluded subjects whose mothers had less than two years of valid Manitoba health registration prior to index date. Children were followed until the earliest of a diagnosis of ASD, migration out of province, age of 18 years, death or end of study period (March 31, 2016).

Other data sources of the study included the Drug Program Information Network (DPIN), In-hospital Pharmaceuticals, Hospital Abstracts, physician claims from the Medical Services database, the Manitoba Education and Training Special Needs Funding data, the Hospital Newborn to Mother Link Registry, BabyFirst - Families First Screen and the Social Allowances Management Information Network (SAMIN) (see Supplementary Table 1 for description of data sources). The study was approved by the University of Manitoba Health Research Ethics Board and the Health Information Privacy Committee of Manitoba Health, Seniors and Active Living.

2.4.2. Exposure

The primary measure of exposure was defined as having filled one or more antibiotic prescriptions during the first year of life as recorded in the DPIN, which captures all prescription drug dispensation outside the hospital setting. We further analyzed the exposure based on the number of antibiotic courses received, cumulative duration within the first year of life and the class of antibiotic (see Supplementary Table 2 for antibiotics classification and ATC codes).

2.4.3. Outcome

The primary outcome was ASD diagnosis identified between the age of 18 months and the end of follow-up period, using claims from the hospital discharge abstracts, medical services (physician claims) and the Manitoba Education and Training Special Needs Funding data. In Canada, the 9th and 10th revisions of the International Classification of Disease (ICD) coding system are currently used to report health services in physician claims (outpatient) and hospital discharge abstracts (inpatient admissions), respectively.

We defined ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (*DSM-5*), which includes childhood autism, atypical autism, Asperger's disorder,

childhood disintegrative disorder, other pervasive developmental disorders and pervasive developmental disorders not otherwise specified.[4] As established by previous research, [13,30–32] ASD was defined as one or more hospitalizations with an ASD code (ICD-9 299.0, 299.1, 299.8 or 299.9, or ICD-10 F84.0, F84.1, F84.3, F84.5, F84.8 or F84.9), one or more physician visit with ICD-9 code of 299 or presence of an "ASD" identifier in the Manitoba Education and Training Special Needs Funding data. This definition was based on a previous validated algorithm with a positive predictive value of 88%, [33] with the addition of educational data as a source to identify ASD.

2.4.4. Covariates

Sociodemographic characteristics, potential confounders and previously reported ASD predictors were included as covariates.[10–16,28,34–36] Maternal covariates included region of residence (urban or rural) and socioeconomic status (SES), prenatal smoking, prenatal alcohol or drug use, prenatal infections, medical conditions of interest, and prenatal use of antidepressants (see Supplementary Table 3 and Table 4 for list of medications included as covariates and diagnostic criteria for maternal and childhood medical conditions). Since previous literature reported an increased risk of ASD at maternal ages above 30 years,[10] we included mothers' age at delivery with three categories: less than 30, 30 to 39, and 40 years or greater. Mothers' physician visits in the year prior to pregnancy were also included to control for differences in healthcare access. Child covariates included sex, size for gestational age, mode of delivery, birth complications, breastfeeding initiation, multiple births, birth order (first born or subsequent), and medical conditions of interest. Season of birth and year of birth were also included to adjust for detection bias. In addition, we included childhood infections to account for potential bias due to confounding by indication.

2.4.5. Statistical analysis

Multivariable Cox proportional hazards regression model was used to examine the association between antibiotic exposure and ASD diagnosis, adjusting for significant confounders and covariates. Subjects were right censored at migration out of province, death, age of 18 years or end of study period if they did not receive an ASD diagnosis. We excluded subjects with missing data on any of the relevant covariates from the analysis. Hazard ratios were estimated to compare risk of ASD among those exposed to antibiotics and those who were not exposed. Interactions of covariates with antibiotic exposure were explored. Correlation matrix and chi-square tests were conducted to examine multicollinearity among covariates. Proportional hazards assumptions were tested by examining the correlation between follow-up time and Schoenfeld residuals of exposure.

The main analysis included history of antibiotic exposure in the first year of life as the predictor and was stratified based on region and sex as potential effect modifiers. We conducted secondary analyses to examine the association based on the number of antibiotic courses, antibiotic classes and cumulative duration within the first year of life.

Multiple sensitivity analyses were conducted. First, we restricted the cohort to children with diagnosed infection in the first year of life to account for potential confounding by indication. Second, to address potential misclassification of the outcome, we applied a stricter diagnostic algorithm to identify ASD by requiring one hospitalization, two physician claims within three years or one physician claim plus educational special needs funding for ASD within three years. Additional sensitivity analyses were also done by modifying the minimum age for ASD diagnosis to one and two years old, altering the antibiotics exposure window to six and 18

months of life, including in-hospital antibiotic use in identifying exposure, and examining the risk of ASD co-occurring with attention deficit hyperactivity disorder (ADHD) or intellectual disability (ID).

To account for the likelihood of unmeasured confounding due to familial and genetic factors, we conducted a sibling-controlled analysis by limiting the cohort to subjects who have at least one maternal sibling discordant in antibiotic exposure. Using multivariable Cox proportional hazards regression models, the sibling cohort was stratified by their mothers to examine the association between antibiotic exposure and ASD diagnoses, adjusted for the same covariates used in the main model except for healthcare access and maternal medical conditions, which we assumed to be static across pregnancies. The statistical software SAS® 9.4 (SAS Institute; Cary, NC) was used for all data analyses.

2.5. Results

2.5.1. Description of study population

There was a total of 261 655 births in Manitoba between 1998 and 2016. Of those, 214 834 children met the criteria for cohort inclusion (Figure 2.1). The cohort was well balanced in sex (51.3% males) and geographical area (54.4% were living in urban regions). Other baseline characteristics are presented in Table 1. Of all subjects, 43.8% received at least one antibiotic course during the first year of life. Among those, 75.8% received one or two courses, 56.1% received antibiotics for less than or equal to 2 weeks and 58.6% received a penicillin antibiotic (see Supplementary Table 5 for description of antibiotics use). Comparing baseline characteristics between the two exposure groups showed a lack of balance in several variables such as sex, socioeconomic status, prenatal smoking and childhood medical conditions. Prenatal

smoking, alcohol and drug use were excluded from the main model due to large percentage of missing data (Table 1) but were subsequently examined in a sensitivity analysis. Subjects were followed for a total of 1 943 612 person-years with a median of 8.6 person-years (IQR 4.8-13.2). During follow-up, 2965 children received an ASD diagnosis at a mean age of 5.48 years (SD 3.24). The crude incidences for ASD diagnosis were 1.46 per 1000 person-years and 1.59 per 1000 person-years in children exposed and unexposed to antibiotics in early life, respectively.

2.5.2. Cox regression model for overall cohort

In the main analysis, antibiotic exposure was not found to be associated with ASD diagnosis (HR 0.93, 95% CI 0.87-1.00). After adjusting for covariates, the risk estimates varied very little. Although statistical significance was found (HR 0.91, 95% CI 0.84-0.99), we were unable to conclusively reject the null given the confidence interval limits' proximity to 1.00. Stratifying the analysis by sex and region also resulted in a statistically significant association in males (adjusted HR 0.91, 95% CI 0.83-1.00), and with those residing in urban regions (adjusted HR 0.85, 95% CI 0.77-0.94). In secondary analyses, number of antibiotic courses and cumulative duration were not associated with ASD (Table 2).

2.5.3. Sensitivity analyses

No major shift in risk estimates was observed in the planned sensitivity analyses. The association between early childhood antibiotics exposure and ASD was consistent across the different sensitivity analyses with changes in exposure, outcome definitions and other parameters (Figure 2.2).

2.5.4. Sibling cohort analysis

The sibling cohort included 80 225 subjects with 57 063 sibling pairs discordant in exposure status (Figure 2.1). In this cohort, 1012 subjects developed ASD during a median follow up of 9.1 (IQR 5.6-12.9) years. For baseline characteristics of the sibling cohort, see Supplementary Table 6. Early life antibiotic exposure was not associated with ASD in the sibling-controlled analysis (adjusted HR 1.03, 95% CI 0.86-1.23). No substantial variation in the risk association was observed in all secondary analyses and all estimates remained non-significant (Table 3).

2.6. Discussion

The main analysis of this large population-based cohort study consisting of all births occurring in Manitoba over an 18-year period showed a trend towards reduced risk of ASD in infants exposed to antibiotics in their first year of lives compared to those not exposed. This marginal association was observed in males, and in those residing in urban areas when stratified by sex and region. In secondary analyses, risk reduction appeared to be significant in those exposed to macrolides and penicillins. Number of antibiotic courses or cumulative duration on antibiotics was not associated with ASD risk. Several sensitivity analyses modifying various exposure and outcome parameters affirmed the robustness of the risk estimates in the main model. Despite the statistically significant findings in the main models, we do not believe the observed association was clinically meaningful.

There were a few causes for concern with the findings from our main model. While the risk estimate was robust, the confidence interval approximated null. There was also an absence of a dose response effect when antibiotic exposure was stratified by cumulative duration or number of antibiotic courses, which casted further uncertainty on the observed association between early life antibiotic exposure and ASD. The most critical issue affecting the validity of

the findings from the primary analyses, however, was the fact that the main model could not account for confounding due to environmental, genetic and other familial or social factors known to be major contributors in the ASD disease model. To address these outstanding confounders, we explored the association in subsequent analyses using a sibling cohort.

In the analyses based on a sibling-controlled design, antibiotics use in the first year of life was not associated with risk of developing ASD in both unadjusted and adjusted models. The associations remained non-significant after stratifying by sex and region, and when exposure was examined by the number of courses, cumulative duration, and antibiotic class. The discrepancy in results between the models based on the overall birth cohort and the sibling cohort suggested high susceptibility of the former to systematic confounding by familial factors that cannot be identified or measured in administrative databases. This led us to conclude that the marginal association between early life antibiotic exposure and ASD observed in the main model was unlikely to be meaningful, and that based on the sibling-controlled design, antibiotic exposure in infant years did not appear to be associated with ASD.

Antibiotics are the most frequently prescribed medications for children, where over-prescription and inappropriate use are often observed. [37–40] Accordingly, identifying antibiotics' association with ASD, if any, would be a public health interest. Previous research suggested a role for microbiota in the development of ASD through disruption of gut-brain axis. [20–24,29] One research group examined gut microbiota of infants exposed to antibiotics perinatally and found significant changes in both the diversity and quantities of microbiota composition at one year of age. [27] Hence, it has since been stipulated that early life antibiotics exposure could induce long-term changes to microbiota composition. Previous observational studies reported an increased

risk of several childhood diseases, including asthma, inflammatory bowel disease, idiopathic juvenile arthritis, obesity and eczema, [41–51] with early life antibiotic induced microbiota changes as the proposed etiology. On the contrary, we did not observe this expected increase in ASD rates in children exposed to antibiotics in their first years of life. This could be attributed to the magnitude of microbiota changes being too small to impair neurodevelopment, or that a specific profile of microbiota changes is responsible for neurodevelopmental disorders.

Our study has several strengths. We utilized 18 years of population-based data, allowing for a large sample size and long follow-up period. In addition, we examined a comprehensive set of potential confounders and ASD predictors for inclusion in the model. Most importantly, we conducted a sibling-controlled analysis to account for unmeasured confounding due to genetics and shared environmental factors. Since we did not exclude any children on basis other than Manitoba Health Insurance registration, the results are likely generalizable.

We identified a few potential limitations in our study. First, drug dispensation may not accurately reflect actual drug use, potentially resulting in misclassification of the exposure, which could bias the results towards the null. We also did not consider antibiotic exposure in the hospital setting due to poor database quality. We did, however, include in-hospital pharmaceutical data in our sensitivity analysis and the risk estimates were similar. Second, while the utilized ASD identification algorithm was adopted from previous research, it has not been independently validated. A non-differential misclassification of the outcome is therefore possible, which could bias the results toward the null. We explored this prospect in a sensitivity analysis using a stricter algorithm to identify ASD and the risk estimates remained consistent. Also, it was difficult to estimate the appropriate exposure time window between antibiotic use

and ASD diagnosis because little is known about the underlying mechanism and latency period for which impairment in neurodevelopment occur. In subsequent sensitivity analyses, we varied antibiotic exposure to within the first 6 and 18 months of life, and the risk estimates were not sensitive to changes in exposure time window. Finally, despite all our efforts to control for confounding by including multiple relevant covariates in the model and by utilizing a sibling cohort, there was still a potential for unmeasured confounding from covariates that could not be obtained from the Repository. For example, we were not able to fully control for both maternal and paternal genetic contribution as our sibling cohort used maternal siblings only. Also, we could not examine other factors that may impact microbiota composition, such as diet, use of probiotics, metabolic changes etc. The complexity of gut microbiota and consequences following any changes make the association with childhood diseases a challenging one to investigate and warrants further studies to examine such risk on both biological and population levels.

2.7. Conclusion

Our results suggest that antibiotics use during the first year of life is not associated with development of ASD. Lack of dose response and most importantly, lack of association in the sibling-controlled analysis confirms main analysis findings. We also found that the risk of ASD is highly susceptible to confounding by unmeasured shared familial factors. A sibling-controlled analysis limited such unmeasured confounding to a certain extent and should be considered in all future observational studies examining risk of ASD.

2.8. Acknowledgment

We would like to thank Charles Burchill and Heather Prior from the Manitoba Centre for Health policy for their valuable support. The authors acknowledge the Manitoba Centre for Health Policy for use of data contained in the Manitoba Population Research Data Repository under project #H2016:244 (HIPC# 2016/2017 – 11). The results and conclusions are those of the authors and no official endorsement by the Manitoba Centre for Health Policy, Manitoba Health, Healthy Living and Senior or other data providers is intended or should be inferred. Data used in this study are from the Manitoba Population Research Data Repository housed at the Manitoba Centre for Health Policy, University of Manitoba and were derived from data provided by Manitoba Health Healthy Living and Senior, Winnipeg Regional Health Authority, Manitoba Department of Families, Healthy Child Manitoba and Manitoba Education and Training.

2.9. Figures and tables

Table 2.1. Characteristics of study cohort: overall and by antibiotics exposure status

Characteristic	Number (%)		
	All subjects N=214 834	Antibiotic use during the first year of life	
		No N= 120 810	Yes N= 94 024
Male	110 107 (51.3)	59 240 (49.0)	50 867 (54.1)
Urban region	116 865 (54.4)	64 806 (53.6)	52 059 (55.4)
Socioeconomic status (SES)[a]:			
High	21 212 (9.9)	13 286 (11.0)	7926 (8.4)
Middle	77 708 (36.2)	45 578 (37.7)	32 130 (34.2)
Low-mid	67 059 (31.2)	35 857 (29.7)	31 202 (33.2)
Low	48 855 (22.7)	26 089 (21.6)	22 766 (24.2)
Receipt of income assistance [b]	37 158 (17.3)	14 058 (11.6)	23 100 (24.6)
Maternal age at delivery (years):			
< 30	128 229 (59.7)	68 793 (56.9)	59 436 (63.2)
30-39	81 963 (38.2)	49 101 (40.6)	32 862 (35.0)
>= 40	4642 (2.2)	2916 (2.4)	1726 (1.8)
Breastfeeding initiation [c]	172 952 (80.8)	99 269 (82.5)	73 683 (78.7)
Multiple birth [d]	5383 (2.5)	3202 (2.7)	2181 (2.3)
Caesarian section	43 782 (20.4)	24 751 (20.5)	19 031 (20.2)
Birth complications	21 022 (9.8)	11 493 (9.5)	9529 (10.1)
First born child	80 758 (37.6)	49 211 (40.7)	31 547 (33.6)
Small for gestational age [e]	16 507 (7.7)	9501 (7.9)	7006 (7.5)
Prenatal alcohol/drug use [f]	12 959 (12.3)	6407 (11.4)	6552 (13.4)
Prenatal smoking [g]	20 844 (19.4)	9199 (16.0)	11 645 (23.4)
Childhood medical conditions:			
Infections:			
None	67 430 (31.4)	59 635 (49.4)	7795 (8.3)
Mild-moderate [h]	134 794 (62.7)	56 960 (47.2)	77 834 (82.8)
Severe [i]	12 610 (5.9)	4215 (3.5)	8395 (8.9)
Epilepsy	1056 (0.5)	424 (0.4)	632 (0.7)
Neonatal jaundice	20 517 (9.6)	10 956 (9.1)	9561 (10.2)
Other developmental disabilities	859 (0.4)	355 (0.3)	504 (0.5)
Asthma	26 641 (12.4)	5276 (4.4)	21 365 (22.7)
Maternal medical conditions:			
Mood and anxiety disorders	15 885 (7.4)	7528 (6.2)	8357 (8.9)
Schizophrenia	196 (0.1)	111 (0.1)	85 (0.1)
Diabetes	6085 (2.8)	3372 (2.8)	2713 (2.9)
Prenatal infections	67 559 (31.5)	31 506 (26.1)	36 053 (38.3)
Prenatal antidepressants exposure	5759 (2.7)	2900 (2.4)	2859 (3.0)
Year of birth:			
1998-2001	47 107 (21.9)	20 593 (17.1)	26 514 (28.2)
2002-2005	48 596 (22.6)	25 260 (20.9)	23 336 (24.8)
2006-2009	53 052 (24.7)	31 640 (26.2)	21 412 (22.8)
2010-2014	66 079 (30.8)	43 317 (35.9)	22 762 (24.2)
Season of birth:			
Winter	49 039 (22.8)	27 677 (22.9)	21 362 (22.7)
spring	56 517 (26.3)	30 493 (25.2)	26 024 (27.7)

Characteristic	Number (%)		
	All subjects N=214 834	Antibiotic use during the first year of life	
		No N= 120 810	Yes N= 94 024
Summer	58 987 (27.5)	33 331 (27.6)	25 656 (27.3)
Fall	50 291 (23.4)	29 309 (24.3)	20 982 (22.3)

Percentages are calculated based on non-missing data

[a] Based on the Socioeconomic factor index, a neighborhood level measure based on Canada census, which was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Missing data for 886 (0.4%) subjects

[d] Defined as the number of births following a multiple gestation pregnancy

[e] Defined as having birth weight below the 10^{th} percentile for the gestational age and sex. Missing data for 502 (0.2%) subjects

[f] Missing data for 109 491 (51.0%) subjects

[g] Missing data for 107 550 (50.1%) subjects

[h] Defined as having an infection code in physician claims only

[i] Defined as having a hospitalization with an infection code

Table 2.2. Association between antibiotics use and risk of ASD in the overall cohort

Variable	Person-years	Number of events	HR (95% CI)	
			Unadjusted	Adjusted [a]
Main analysis				
History of antibiotics use	1 943 612	2965	0.93 (0.87 – 1.00)	0.91 (0.84 – 0.99)
Stratified by sex:				
Male	991 939	2401	0.86 (0.79 – 0.93)	0.91 (0.83 – 1.00)
Female	951 673	564	0.97 (0.82 – 1.14)	0.92 (0.76 – 1.12)
Stratified by region:				
Rural	887 497	953	1.10 (0.97 – 1.25)	1.01 (0.88 – 1.17)
Urban	1 056 105	2012	0.89 (0.82 – 0.97)	0.85 (0.77 – 0.94)
Secondary analyses				
Antibiotic class				
None	1 013 186	1610	1.00 [Reference]	1.00 [Reference]
Penicillin	358 792	1131	0.93 (0.86 – 1.01)	0.92 (0.84 – 1.00)
Macrolides and related antibiotics	254 769	366	0.92 (0.82 – 1.03)	0.87 (0.77 – 0.99)
Non-penicillin β-lactams	201 080	315	1.00 (0.88 – 1.12)	0.93 (0.82 – 1.05)
Others	115 785	156	0.89 (0.76 – 1.05)	0.92 (0.77 – 1.09)
Number of antibiotic courses				
0	1 013 186	1610	1.00 [Reference]	1.00 [Reference]
1	453 484	657	0.94 (0.86 – 1.03)	0.92 (0.83 – 1.01)
2	229 957	329	0.94 (0.83 – 1.06)	0.89 (0.78 – 1.01)
3	120 114	179	0.98 (0.84 – 1.15)	0.93 (0.79 – 1.09)
>= 4	126 871	190	0.99 (0.86 – 1.16)	0.90 (0.76 – 1.06)
Cumulative antibiotic duration (days)				
0	1 013 186	1610	1.00 [Reference]	1.00 [Reference]
1-7	173 235	243	0.89 (0.78 – 1.02)	0.91 (0.79 – 1.05)
8 – 14	320 640	464	0.92 (0.83 – 1.02)	0.91 (0.82 – 1.01)
15-21	177 468	269	0.98 (0.86 – 1.11)	0.95 (0.82 – 1.08)
>21	259 083	379	0.95 (0.85 – 1.06)	0.88 (0.78 – 1.00)

[a] Adjusted for sex, region, healthcare access, SES, maternal age at delivery, maternal medical conditions (mood and anxiety disorders, schizophrenia and diabetes, prenatal infections), prenatal antidepressants use, size for gestational age, childhood medical conditions (epilepsy, infections, neonatal jaundice, asthma and a diagnosis with other developmental disability disorder), birth complications, mode of delivery, multiple birth, breastfeeding initiation, year of birth, season of birth, and birth order

Table 2.3. Association between antibiotics use and risk of ASD in siblings' cohort

Variable	Person-years	Number of events	HR (95% CI)	
			Unadjusted	Adjusted [a]
Main analysis				
History of antibiotics use	746 135	1012	1.03 (0.89 – 1.19)	1.03 (0.86 – 1.23)
Stratified by sex:				
Male	380 952	817	0.99 (0.80 – 1.23)	0.98 (0.76 – 1.26)
Female	365 184	195	0.85 (0.54 – 1.33)	0.76 (0.41 – 1.39)
Stratified by region:				
Rural	393 205	395	1.14 (0.89 – 1.45)	1.10 (0.80 – 1.50)
Urban	352 930	617	1.03 (0.85 – 1.24)	1.00 (0.79 – 1.28)
Secondary analyses				
Antibiotic class				
None	368 556	477	1.00 [Reference]	1.00 [Reference]
Penicillin antibiotics	306 227	435	1.11 (0.95 – 1.29)	1.11 (0.93 – 1.34)
Macrolides and related antibiotics	94 429	134	1.13 (0.89 – 1.42)	1.13 (0.87 – 1.46)
Non-penicillin β-lactams	78 132	130	1.31 (1.04 – 1.66)	1.18 (0.91 – 1.53)
Others	37 229	56	1.14 (0.81 – 1.59)	1.22 (0.85 – 1.75)
Number of antibiotic courses				
0	368 556	477	1.00 [Reference]	1.00 [Reference]
1	210 538	282	0.97 (0.81 – 1.16)	0.98 (0.79 – 1.21)
2	89 899	131	1.07 (0.82 – 1.39)	1.09 (0.80 – 1.47)
3	41 010	69	1.42 (0.97 – 2.08)	1.46 (0.94 – 2.26)
>= 4	36 132	53	0.99 (0.65 – 1.50)	0.87 (0.54 – 1.40)
Cumulative antibiotic duration (days)				
0	368 556	477	1.00 [Reference]	1.00 [Reference]
1-7	85 069	112	0.93 (0.71 – 1.22)	1.00 (0.73 – 1.37)
8 – 14	142 259	190	0.98 (0.79 – 1.21)	0.95 (0.74 – 1.21)
15-21	68 965	112	1.28 (0.95 – 1.72)	1.30 (0.91 – 1.84)
>21	81 286	121	1.08 (0.81 – 1.43)	1.05 (0.75 – 1.48)

[a] Adjusted for sex, region, SES, maternal age at delivery, prenatal infections, prenatal antidepressants use, size for gestational age, childhood medical conditions (epilepsy, infections, neonatal jaundice, asthma and a diagnosis with other developmental disability disorder), birth complications, mode of delivery, multiple birth, breastfeeding initiation, year of birth, season of birth, and birth order

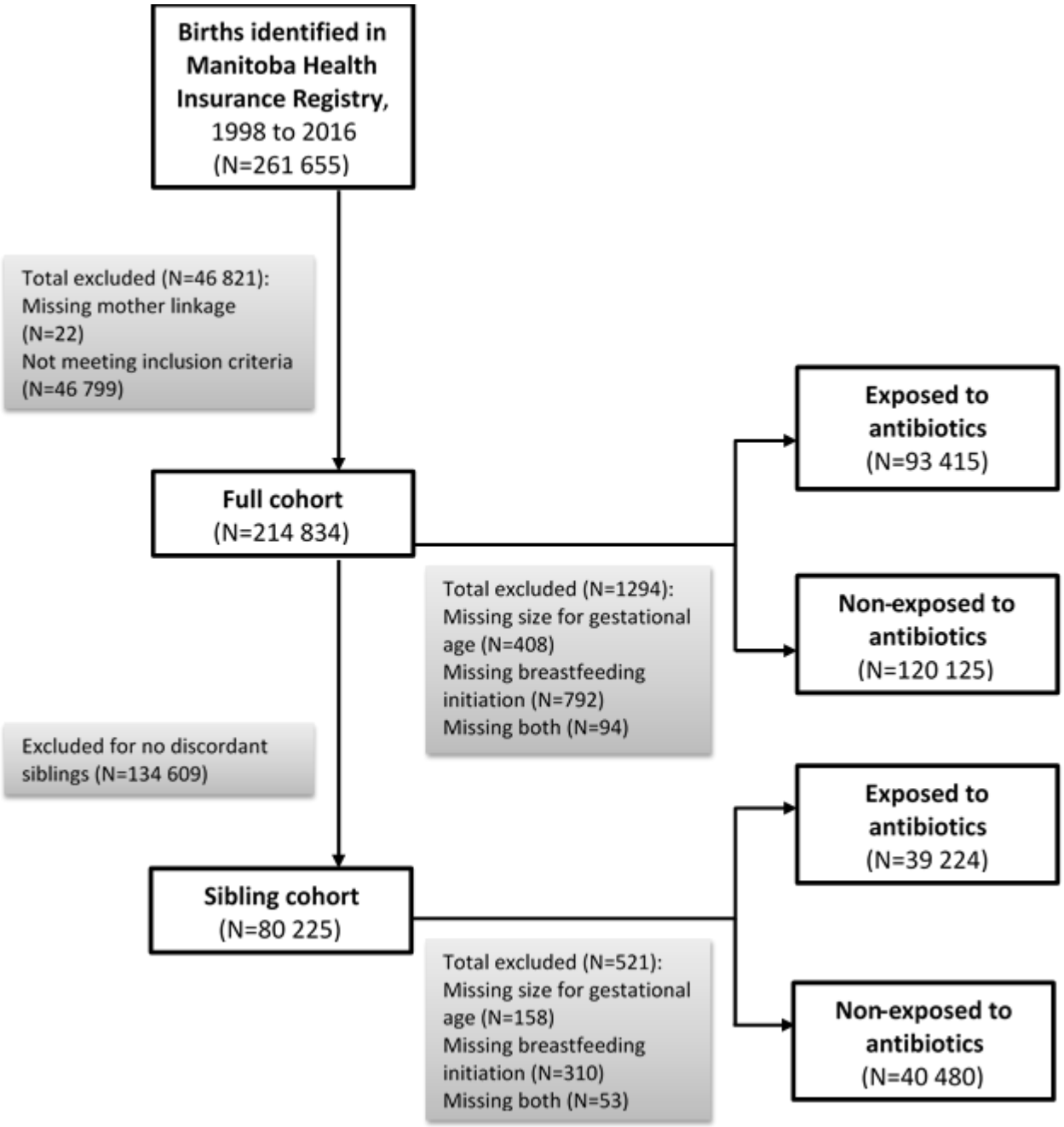

Figure 2.1. Study populations: overall and sibling cohort

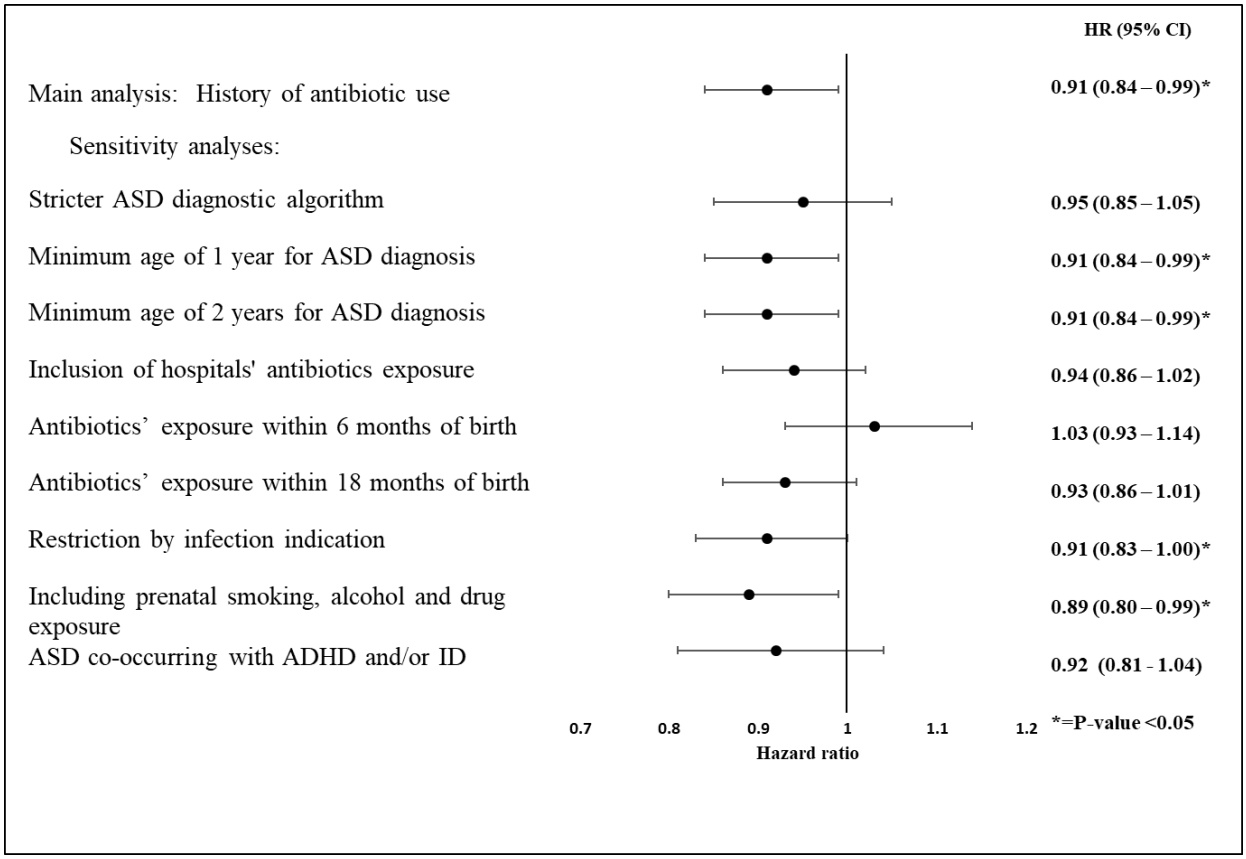

Figure 2.2. Forest plot with sensitivity analyses risk estimates

Supplementary Table 2.1. Description of data sources

Database	Description	Data years
Drug Program Information Network (DPIN)	An electronic drug system that captures all prescriptions dispensed from community pharmacies to residents and is maintained by Manitoba Health, Seniors and Active Living. It includes prescription information such as generic name, Anatomical Therapeutic Chemical (ATC) code, date of dispensation, strength and days supplied.	1995/96 - 2015/16
In-hospital Pharmaceuticals	An inpatient pharmacy system that provides dispensing information on pharmaceutical use at three Winnipeg hospitals: Health Sciences Centre, Concordia Hospital and Riverview Health Centre.	1999-2012
Hospital discharge abstracts	Includes records of all patients' hospital admissions with summaries for demographic data such as gender and postal code, and clinical data including up to 25 diagnosis codes and 20 procedure codes.	1970/71 - 2015/16
Medical Services database	Physician claims in the Medical Services database include records of claims for any physician visits in offices, hospitals and outpatient departments in Manitoba in addition to some information on Manitobans' physician visits outside the province. Claims are submitted by physicians electronically for service reimbursement by Manitoba Health, Seniors and Active Living.	1970/71 - 2015/16
The Manitoba Education Special Needs data file	Education data includes education records on special school funding received for students with special needs, including those with ASD.	1995/96 - 2015/16
The Hospital Newborn to Mother Link	Serves to match the baby's birth hospital record with the mother's obstetrical delivery record and contains basic demographic and hospital data on newborns and their mothers for in-hospital births.	1984/85 - 2015/16
BabyFirst - Families First Screen	Collected by Public Health Nurses on nearly all families with newborns in Manitoba and maintained by Heathy Child Manitoba. Contains records of newborns identifying biological, social, and demographic risk factors including prenatal smoking, alcohol and drug use.	2000 – 2013
The Social Allowances Management Information Network	Provides information concerning employment and income assistance received by Manitoba residents.	1995/96 - 2014/15
Canada Census	A population survey with aggregate demographic information for all persons within a dissemination area in Canada and is conducted by Statistics Canada every five years.	1971 – 2016

Supplementary Table 2.2. Antibiotic classification

Class	Generic name	Anatomical Therapeutic Chemical
Penicillin antibiotics	Amoxicillin, ampicillin, bacampicillin, penicillin, piperacillin, pivampicillin, pivmecillinam, cloxacillin, floxacillin, nafcillin, ticarcillin, tazobactam	J01C
Non-penicillin β-lactams	Cefazolin, cefepime, cefixime, cefotaxime, cefotetan, cefoxitin, cefpodoxime, cefprozil, ceftazidime, ceftizoxime, ceftobiprole, ceftriaxone, cefuroxime, cephalexin, cephalothin, aztreonam, cefaclor, cefadroxil, cefamandole, doripenem, ertapenem, meropenem	J01D
Macrolides and related antibiotics	Dalfopristin, telithromycin, azithromycin, clarithromycin, clindamycin, erythromycin, lincomycin	J01F, J01R
Other antibiotics	Minocycline, doxycycline, demeclocycline, rolitetracycline, tetracycline, ciprofloxacin, gatifloxacin, nalidixic acid, gemifloxacin, grepafloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin, trovafloxacin, methenamine, fosfomycin, tedizolid, nitrofurantoin, metronidazole, linezolid, fusidate, fusidic acid, bacitracin, vancomycin, colistin, polymyxin B, telavancin, tobramycin, gentamicin, daptomycin, amikacin, spectinomycin, neomycin, netilmicin, spiramycin, streptomycin, chloramphenicol, trimethoprim, sulfamethoxazole, sulfadiazine, sulfapyridine, sulfisoxazole	J01A, J01B, J01G, J01E, J01M, J01X

Supplementary Table 2.3. Prenatal antidepressants list [a]

Class	Generic name	Anatomical Therapeutic Chemical
Antidepressants	Desipramine, imipramine, clomipramine , trimipramine, amitriptyline, nortriptyline, protriptyline, doxepin, amoxapine, amineptine, maprotiline, fluoxetine, citalopram, paroxetine, sertraline, fluvoxamine, escitalopram, phenelzine, tranylcypromine, moclobemide, tryptophan, trazodone, nefazodone, mirtazapine, bupropion, venlafaxine, milnaacipran, duloxetine, desvenlafaxine, vilazodone, vortioxetine.	N06A

[a]: Two prescription fills during pregnancy was required

Supplementary Table 2.4. Diagnostic criteria of medical conditions

Covariate	Period	Diagnostic Criteria
Maternal medical conditions:		
Mood and anxiety disorders	Within 3 years before index date	✓ One or more hospitalizations with a diagnosis for depressive disorder, affective psychoses, neurotic depression or adjustment reaction: ICD-9-CM codes 296.1-296.8, 300.4, 309 or 311; ICD-10-CA codes F31, F32, F33, F34.1, F38.0, F38.1, F41.2, F43.1, F43.2, F43.8, F53.0, F93.0 or with a diagnosis for an anxiety state, phobic disorders or obsessive-compulsive disorders: ICD-9-CM codes 300.0, 300.2, 300.3, 300.7; ICD-10-CA codes F40, F41.0, F41.1, F41.3, F41.8, F41.9, F42, F45.2, OR ✓ One or more hospitalizations with a diagnosis for anxiety disorders: ICD-9-CM code 300; ICD-10-CA codes F32, F34.1, F40, F41, F42, F44, F45.0, F45.1, F45.2, F48, F68.0, or F99 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ One or more physician visits with a diagnosis for depressive disorder or affective psychoses: ICD-9-CM codes 296, 311, OR ✓ One or more physician visits with a diagnosis for anxiety disorders: ICD-9-CM code 300 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ Three or more physician visits with a diagnosis for anxiety disorders or adjustment reaction: ICD-9-CM code 300, 309.
Schizophrenia	Within 3 years before index date	✓ One or more hospitalization with a diagnosis for schizophrenia: ICD-9-CM code 295; ICD-10-CA codes F20, F21, F23.2, F25, OR ✓ One or more physician visits with a diagnosis for schizophrenia: ICD-9-CM code 295.
Diabetes mellitus	Within 1 year before index date	✓ One or more hospitalizations with a diabetes diagnosis: ICD-9-CM code 250, 648.0, 648.8; ICD-10-CA codes E10-E14, O24, OR ✓ Two or more physician visits with a diabetes diagnosis: ICD-9-CM code 250, OR ✓ One or more prescriptions for a diabetes medication (ATC: A10A, A10B).
Prenatal infection	During pregnancy	A hospitalization or physician visit with any of the following codes: Eye and ear infections: ICD-9-CM codes 370*, 372*, 380*, 381*, 382; ICD-10-CA codes H10, H13.1, H16, H19.1, H19.2, H60, H62, H65, H66, H67 Upper respiratory tract infection: ICD-9-CM codes 460–465; ICD-10-CA codes J00-J06 Lower respiratory tract infection: ICD-9-CM codes 466, 481–488; ICD-10-CA codes J09- J18, J20-J22 Genitourinary system infection: ICD-9-CM codes 590, 595, 597, 599*, 601, 604, 614-617, 771*; ICD-10-CA codes N10-N12, N30, N33.0, N33.8, N37.0, N39.0, N41, N45, N70-N77 Central nervous system infections: ICD-9-CM codes 320, 321, 323, 324, 728*; ICD-10-CA codes G00-G02, G04-G07

Covariate	Period	Diagnostic Criteria
Prenatal infection	During pregnancy	Skin infections: ICD-9-CM codes 680-686; ICD-10-CA codes L00- L08, M72.6 Cardiovascular system infections: ICD-9-CM codes 391, 420, 421, 422, 424*; ICD-10-CA codes I01, I30, I32.0, I32.1, I33, I38, I39.8, I40, I41.0, I41.1, I41.2 Musculoskeletal system infections: ICD-9-CM codes 711, 730; ICD-10-CA codes M00- M01, M03, M46.2, M86 Bacteremia/Septicemia: ICD-9-CM codes 771*, 790*, 995*; ICD-10-CA codes P36, A22.7, A26.7, A02.1, A32.7, A02.1, A32.7, A40, A41, A42.7, B37.7 Gastrointestinal system infections: ICD-9-CM codes 535, 540, 541, 542, 566, 567, 530*, 572*, 575*; ICD-10-CA codes K20, K29.0, K29.1, K29.8, K35, K61, K65, K67, K81.0 Other Parasitic and infectious diseases: ICD-9-CM codes 001-139; ICD-10-CA codes A00-B99
Childhood medical conditions:		
Birth complications	At birth	Include vacuum, forceps or breech procedures: ICD-9-CM procedure codes 72, 73.3, 763.2, 652.2, 669.6; CCI codes 5MD53, 5MD54, 5MD55, 5MD56, shoulder dystocia: 660.4, placental abruption: 641.2, cord prolapse: 663, uterine rupture: 665.1 or other perinatal complications: 760-779.
Epilepsy	Within the first year of life	✓ One or more hospitalization for seizure disorder: ICD-9-CM codes 345, 649.4; ICD-10-CA codes G40, G41, OR ✓ One or more physician visit for seizure disorder: ICD-9-CM code 345, OR ✓ One or more prescription for an anticonvulsant medication (ATC: N03A)
Asthma	Within the first year of life	✓ One or more hospitalizations with a diagnosis of asthma: ICD-9-CM codes 493; ICD-10-CA code J45, OR ✓ One or more physician visits with a diagnosis of asthma: ICD-9-CM codes 493, OR ✓ One or more prescriptions for asthma medication (ATC: R03, R06AX17)
Other developmental disabilities	Within the first year of life	✓ One or more hospitalizations with diagnoses for intellectual disabilities, Down's syndrome, autosomal deletion syndromes, Prader-Willi syndrome, other specified congenital anomalies, or fetal alcohol syndrome: ICD-9-CM codes 317, 318, 319, 758.0- 758.3, 759.8, 760.71; ICD-10-CA: F70, F71, F72, F73, F78, F79, P04.3, Q86, Q87, Q89.8, Q90, Q91, Q93, Q99.2. ✓ One or more physician visit with diagnoses for intellectual disabilities: ICD-9-CM: 317, 318, 319
Neonatal jaundice	Within the first 4 weeks of life	✓ One or more hospitalizations with diagnoses for Jaundice: ICD-9-CM code 774; ICD–10–CA codes P58, P59, OR ✓ One or more physician visit with diagnoses for Jaundice: ICD-9-CM code 774
Infection	Within the first year of life	Similar to maternal definition

Supplementary Table 2.5. Description of antibiotics use

	Number (%)
Antibiotic class	
Penicillin antibiotics	77 984 (58.6)
Macrolides and related antibiotics	24 807 (18.7)
Non-penicillin β-lactams	20 680 (15.6)
Others	9527 (7.2)
Total	132 998
Number of courses	
1	48 440 (51.5)
2	22 804 (24.3)
3	11 419 (12.1)
>=4	11 361 (12.1)
Total	94 024
Cumulative duration (days)	
1-7	19 325 (20.6)
8-14	33 418 (35.5)
15-21	17 523 (18.6)
>=22	23 758 (25.3)
Total	94 024

Supplementary Table 2.6. Characteristics of sibling cohort

Characteristic	Number (%)		
	All subjects N=80 225	Antibiotic use during the first year of life	
		No N= 40 734	Yes N= 39 491
Male	41 168 (51.3)	19 560 (48.0)	21 608 (54.7)
Urban region	37 694 (47.0)	18 407 (45.2)	19 287 (48.8)
Socioeconomic status (SES)[b]:			
High	6584 (8.2)	3355 (8.2)	3229 (8.2)
Middle	26 669 (33.2)	13 582 (33.3)	13 087 (33.1)
Low-mid	24 458 (30.5)	12 147 (29.8)	12 311 (31.2)
Low	22 514 (28.1)	11 650 (28.6)	10 864 (27.5)
Receipt of income assistance [c]	15 991 (19.9)	6868 (16.9)	9123 (23.1)
Mothers age at delivery:			
< 30	52 145(65.0)	26 005 (63.8)	26 140 (66.2)
30-39	26 975 (33.6)	14 126 (34.7)	12 849 (32.5)
>= 40	1105 (1.4)	603 (1.5)	502 (1.3)
Breastfeeding initiation [d]	62 012 (77.7)	31 402 (77.4)	30 610 (77.9)
Multiple birth [e]	2392 (3.0)	1251 (3.1)	1141 (2.9)
Caesarian section	14 188 (17.7)	7187 (17.6)	7001 (17.7)
Birth complications	5435 (6.8)	2695 (6.6)	2740 (6.9)
First born child	22 575 (28.1)	11 150 (27.4)	11 425 (28.9)
Small for gestational age [f]	5643 (7.1)	2906 (7.2)	2737 (7.0)
Prenatal alcohol/drug use [g]	5236 (12.6)	2413 (11.9)	2823 (13.4)
Prenatal smoking [h]	8678 (20.6)	3965 (19.2)	4713 (21.9)
Childhood medical conditions:			
Infections:			
None	21 560 (26.9)	17 480 (42.9)	4080 (10.3)
Mild-moderate [i]	52 648 (65.6)	21 292 (52.3)	31 356 (79.4)
Severe [j]	6017 (7.5)	1962 (4.8)	4055 (10.3)
Epilepsy	419 (0.5)	155 (0.4)	264 (0.7)
Neonatal jaundice	7517 (9.4)	3774 (9.3)	3743 (9.5)
Other developmental disabilities	363 (0.5)	132 (0.3)	231 (0.6)
Asthma	10 680 (13.3)	2370 (5.8)	8310 (21.0)
Maternal medical conditions:			
Mood and anxiety disorders	5879 (7.3)	2826 (6.9)	3053 (7.7)
Schizophrenia	71 (0.1)	38 (0.1)	33 (0.1)
Diabetes	2309 (2.9)	1221 (3.0)	1088 (2.8)
Prenatal infections	25 833 (32.2)	12 099 (29.7)	13 734 (34.8)
Prenatal antidepressants exposure	2119 (2.6)	1036 (2.5)	1083 (2.7)
Year of birth:			
1998-2001	15 098 (18.8)	6856 (16.8)	8242 (20.9)
2002-2005	20 751 (25.9)	10 205 (25.1)	10 546 (26.7)
2006-2009	22 749 (28.4)	11 965 (29.4)	10 784 (27.3)
2010-2014	21 627 (27.0)	11 708 (28.7)	9919 (25.1)
Season of birth:			
Winter	18 655 (23.3)	9488 (23.3)	9167 (23.2)
Spring	20 888 (26.0)	10 110 (24.8)	10 778 (27.3)
Summer	21 838 (27.2)	11 134 (27.3)	10 704 (27.1)
Fall	18 844 (23.5)	10 002 (24.6)	8842 (22.4)

Characteristic	Number (%)		
	All subjects N=80 225	Antibiotic use during the first year of life	
		No N= 40 734	Yes N= 39 491

Percentages are calculated based on non-missing data

[a] Based on the Socioeconomic factor index, a neighborhood level measure based on Canada census, which was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Missing data for 363 (0.5%) subjects

[d] Defined as the number of births following a multiple gestation pregnancy

[e] Defined as having birth weight below the 10^{th} percentile for the gestational age and sex. Missing data for 211 (0.3%) subjects

[f] Missing data for 38 816 (48.4%) subjects

[g] Missing data for 38 034 (47.4%) subjects

[h] Defined as having an infection code in physician claims only

[i] Defined as having a hospitalization with an infection code

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Chapter 3: Prenatal Antibiotics Exposure and the Risk of Autism Spectrum Disorders: A Population-Based Cohort Study

3.1. Overview

This manuscript is the second of two manuscripts that examine the association of early life antibiotic exposure and ASD risk. This chapter addresses the second project objective, which assesses the association of antibiotics exposure during pregnancy and the risk of developing ASD. The manuscript is based on a population-based cohort study of children born in Manitoba between the fiscal years 1998 and 2016 and utilized administrative health data from the Manitoba Population Research Data Repository. Cox proportional hazards regression models were used to estimate the risk of developing ASD in the overall population and in a cohort of exposure-discordant siblings. Study findings suggested a small and clinically non-significant increase in ASD risk in children exposed to antibiotics prenatally.

Citation: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Prenatal Antibiotics Exposure and the Risk of Autism Spectrum Disorders: A Population-Based Cohort Study. *PLOS ONE* 2019; 14: e0221921

3.2. Abstract

Background: Prenatal antibiotic exposure induces changes in infants' gut microbiota composition and is suggested as a possible contributor in the development of autism spectrum disorders (ASD). In this study, we examined the association between prenatal antibiotic exposure and the risk of ASD.

Methods: This was a population-based cohort study utilizing the Manitoba Population Research Data Repository. The cohort included 214 834 children born in Manitoba, Canada between April 1, 1998 and March 31, 2016. Exposure was defined as having filled one or more antibiotic prescription during pregnancy. The outcome was autism spectrum disorder diagnosis. Multivariable Cox proportional hazards regression was used to estimate the risk of developing ASD in the overall cohort and in a sibling cohort.

Results: Of all subjects, 80 750 (37.6%) were exposed to antibiotics prenatally. During follow-up, 2965 children received an ASD diagnosis. Compared to children who were not exposed to antibiotics prenatally, those who were exposed had a higher risk of ASD: (adjusted HR 1.10, 95% CI 1.01-1.19). The association was observed in those exposed to antibiotics in the second or third trimester (HR 1.11, 95% CI 1.01-1.23 and 1.17, 95% CI 1.06-1.30, respectively). In the siblings' cohort, ASD risk estimate remained unchanged (adjusted HR 1.08, 95% CI 0.90-1.30, although it was not statistically significant.

Conclusions: Prenatal antibiotic exposure is associated with a small increase in the risk of ASD. Given the potential of residual confounding beyond what it was controlled through our study

design and because of possible confounding by indication, such a small risk increase in the population is not expected to be clinically significant.

3.3. Introduction

Autism spectrum disorders (ASD) are characterized by impairment in social communication and interaction with repetitive patterns of behavior.[1] The burden of ASD is significant with 62 million cases worldwide.[2] Genetics are primary contributors to the development of ASD; however, the increasing prevalence of ASD suggests a role of environmental factors.[3–7]

Abnormal composition of microbiota, the community of microorganisms residing in the human body, has been observed in children with ASD and is proposed as a contributor to ASD development.[8–11] Recent research have shown that the fetal gut is not germ free and suggests maternal microbiota transfer before birth.[12,13] Antibiotic-altered microbiota administered to pregnant mice demonstrated transmission of the same alteration of microorganisms to the offspring. Despite absence of direct exposure to antibiotics, the offspring maintained this microbial composition for at least 21 weeks and were found to be at higher risk of developing colitis.[14] In another study, treating pregnant mice with antibiotics directly resulted in persistent reduction in offspring gut microbiota diversity and in immunological alterations.[15] Antibiotic-induced changes in fetal microbiota composition can disrupt the gut-brain axis, potentially impairing neurodevelopment and increasing the risk of ASD.[8,9,16]

Although previous observational studies reported several prenatal and postnatal environmental factors as predictors of ASD.[17–19] potential of confounding and other study flaws limited the clinical applicability of these associations. In this study, we aimed to examine the

association between prenatal antibiotic exposure and the risk of ASD. Antibiotics over-prescription and inappropriate use are often observed during pregnancy.[20–22] Given the high frequency of antibiotic prescribing in this population.[20,23] identifying an association between antibiotic use and ASD, if any, would be of public health interest.

3.4. Methods

3.4.1. Design and subjects

This was a population-based cohort study utilizing administrative health data from the Manitoba Population Research Data Repository housed at the Manitoba Centre for Health Policy. The Repository is a collection of administrative, registry, survey and other data that come from different provincial government departments such as health, education and justice. Under a universal, provincial health delivery system, the Repository captures all encounters by all residents with the healthcare system including physician visits and drug dispensation, collected for claim purposes. Patient records in the Repository are de-identified. Scrambled Personal Health Identification Numbers (PHIN) are used for linkage among different databases.

The cohort consisted of all live births identified in the Manitoba Health Insurance Registry between April 1, 1998 and March 31, 2016. Children were required to have continuous enrollment with Manitoba Health for at least 18 months after their birthdate, which was also the cohort index date, to ensure that they have met the minimum age of ASD diagnosis.[24] In addition, to obtain data on maternal covariates, the mothers were required to have at least two years of Manitoba Health enrollment prior to index date. Children were followed until a diagnosis of ASD, migration out of province, 18th birthday, death or end of study period (March

31, 2016), whichever occurred first. Subjects with missing data on any of the relevant covariates were excluded from the cohort. To account for unmeasured familial environmental and genetic confounders that are shared between siblings, we identified a cohort of maternal siblings who have different exposure status to prenatal antibiotics, i.e., one or more of the siblings were exposed to antibiotics prenatally and one or more were not.

Other data sources of the study included the Drug Program Information Network (DPIN), In-hospital Pharmaceuticals, Hospital Abstracts, physician claims from the Medical Services database, the Manitoba Education and Training Special Needs Funding data, the Hospital Newborn to Mother Link Registry, BabyFirst - Families First Screen and the Social Allowances Management Information Network (SAMIN) (Supplementary Table 1). The study was approved by the University of Manitoba Health Research Ethics Board and the Health Information Privacy Committee of Manitoba Health, Seniors and Active Living.

3.4.2. Exposure

The exposure was identified in DPIN, a record of outpatient drug dispensations, and was defined as having filled one or more antibiotic prescriptions during pregnancy (Supplementary Table 2). The first day of pregnancy was estimated as the difference between birth date and gestational age. Gestational age is approximated from the first date of women's last menstrual period and is identified in the Hospital Newborn to Mother Link Registry. In secondary analyses, the exposure was examined based on the pregnancy trimester, number of antibiotic courses received, cumulative duration and class of antibiotic. Grouping of these variables was based on their frequency in the cohort.

3.4.3. Outcome

The primary outcome was ASD diagnosis after 18 months of age and included childhood autism, atypical autism, Asperger's disorder, childhood disintegrative disorder, other pervasive developmental disorders and pervasive developmental disorders not otherwise specified.[1]

The 9th and 10th revisions of the International Classification of Disease (ICD) coding system were used to identify ASD diagnosis. ASD was defined as one or more hospitalization with an ASD code (ICD-9 299.0, 299.1, 299.8 or 299.9, or ICD-10 F84.0, F84.1, F84.3, F84.5, F84.8 or F84.9), one or more physician visit with ASD code (ICD-9 code of 299) or presence of an "ASD" identifier in the Manitoba Education and Training Special Needs Funding data.[7,25,26] A validation study reported a positive predictive value of 88% using one or more hospitalizations or physician visits.[27] Including educational data as a source to identify ASD is expected to increase the sensitivity.[28]

3.4.4. Covariates

The models were adjusted for region of residence (urban or rural), socioeconomic status (SES), mothers' age at delivery (less than 30, 30 to 39, and 40 years or greater), prenatal use of medications and maternal medical conditions of interest (Supplementary Tables 3 and 4). SES was measured using the Socio-Economic Factor Index (SEFI), an area level measure derived from Census data. In addition, receiving income assistance was explored as a proxy of individual level SES. Data on prenatal smoking, alcohol or drug use were obtained from the BabyFirst - Families First Screen. A large amount of missing data was observed for these variables; accordingly, they were not included in the main analysis but were explored in a sensitivity analysis restricted to children who had data on these variables. Number of mothers' physician visits in the year prior

to pregnancy was included as a measure of healthcare access. Indication of the dispensed antibiotic is not reliably captured in the administrative databases; hence, we were not able to account for the specific indication for which the antibiotics were prescribed. The models were also adjusted for child covariates including sex, size for gestational age, mode of delivery, birth complications, breast feeding initiation, multiple births, birth order (first born or subsequent), season of birth, year of birth, early childhood antibiotics use and medical conditions of interest (Supplementary Table 4).

3.4.5. Statistical analysis

Multivariable Cox proportional hazards regression was used to examine the association between antibiotic exposure and ASD diagnosis. To account for correlation among siblings, regression models were stratified by the mothers to examine this association within the sibling cohort. The analysis was stratified by sex and region to examine potential effect modification. Multicollinearity among covariates and interactions with antibiotic exposure were explored. Proportional hazards assumption was tested by examining the correlation between follow-up time and Schoenfeld residuals of the independent variables.

In the planned sensitivity analyses, we restricted the cohort to children whose mothers had an ICD code for infection during pregnancy. We applied a stricter ASD identification algorithm, which required one hospitalization or two physician claims within three years or one physician claim plus educational special needs funding for ASD within three years. We varied the minimum age for ASD diagnosis to one and two years old, we included inpatient antibiotic use in identifying the exposure and we included prenatal smoking, alcohol or drug use. In addition, we conducted two negative-control analyses by examining maternal antibiotic exposure in the year

before pregnancy and the year after birth. The statistical software SAS® 9.4 (SAS Institute; Cary, NC) was used for all data analyses.

3.5. Results

3.5.1. Description of study population

A total of 214 834 children met the inclusion criteria (Figure 3.1). About 51% were males and 54.4% resided in an urban region (Table 1); 37.6% of the children were exposed to antibiotics prenatally. The majority were exposed to one antibiotic course (62.8%) or were exposed for less than or equal to two weeks (74.1%). 54.6% were exposed to a penicillin antibiotic (Supplementary Table 5).

During a follow-up of 1 943 612 person-years with a median of 8.6 person-years (Interquartile range 4.8-13.2), 2965 children received an ASD diagnosis. The crude incidence rates for ASD diagnosis were 1.62 per 1000 person-years and 1.47 per 1000 person-years in children exposed and unexposed to antibiotics prenatally, respectively.

The sibling cohort included 75 896 subjects, with 53 840 exposure discordant pairs (Figure 3.1). In this cohort, 977 subjects developed ASD during a median follow up of 9.0 person-years (Interquartile range 5.5-12.9). Baseline characteristics of the sibling cohort are further described in Supplementary Table 6.

3.5.2. Cox regression models

Prenatal antibiotic exposure was associated with a small increase in ASD risk (HR 1.11, 95% CI 1.03-1.19). After adjusting for covariates (Table 2), the risk estimates remained unchanged (HR 1.10, 95% CI 1.01-1.19). An interaction between antibiotics use and region was statistically

significant (p-value = 0.02). The association with ASD risk was statistically significant in children residing in rural regions (HR 1.25, 95% CI 1.08-1.44), but not in those residing in urban regions (HR 1.02, 95% CI 0.92-1.13). In secondary analyses, statistically significant association was observed for those exposed to antibiotics in the second or third trimester (HR 1.11, 95% CI 1.01-1.23) and 1.17, 95% CI 1.06-1.30], respectively) or those exposed to penicillins or another β-lactam antibiotics (HR 1.13, 95% CI 1.04-1.24 and 1.18, 95% CI 1.03-1.37), respectively). Analysis based on cumulative duration of antibiotic exposure showed a dose response effect, with the highest risk observed in those exposed to antibiotics for longer than 14 days (HR 1.15, 95% CI 1.01-1.30). In the sibling cohort, prenatal antibiotic exposure was associated with a small, non-statistically significant increase in the risk of ASD (HR 1.08, 95% CI 0.90-1.30). No substantial variation in the risk association was observed in all secondary analyses (Table 3).

3.5.3. Sensitivity analyses

The association between prenatal antibiotic exposure and ASD was consistent across the different sensitivity analyses (Figure 3.2). The risk ranged from HR 1.04, 95% CI 0.91-1.19 when restricting the cohort to those who had an infection diagnostic code during pregnancy to HR 1.11, 95% CI 1.02-1.20 when including inpatient antibiotic use. Maternal antibiotic exposure in the year before pregnancy and the year after birth were not found to be associated with ASD risk in the child (HR 0.98, 95% CI 0.91-1.06 and 1.03, 95% CI 0.94-1.12, respectively).

3.6. Discussion

Findings from this large population-based cohort study showed a 10% increase in the risk of ASD in children exposed to antibiotics prenatally compared to those who were not exposed. This

association was dependent on region and was only observed in those residing in rural regions. The increased risk was shown in those exposed to penicillins and other β-lactams and in those exposed to antibiotics in the second or third trimester. The highest risk was observed in those exposed to antibiotics for longer than two weeks or who received 3 or more antibiotic courses. The lack of association in the two negative controls provides confidence that the findings are reliable.

Since the main model could not account for all confounding sources, we explored the association using a sibling cohort design to address environmental, genetic, and other familial or social factors. In the analysis of the sibling cohort, the risk of ASD with prenatal antibiotic exposure did not change significantly, except the association was no longer statistically significant. This could be explained by the smaller sample size of the sibling cohort. This led us to conclude that prenatal antibiotic exposure appears to be associated with a small increase in ASD risk. Nevertheless, we believe the observed risk is too small to be clinically meaningful and may have been influenced by residual confounding from variables that could not be identified in the Repository, not shared by sibling pairs or not recorded correctly.

Study findings are consistent with a previous exploratory population-based cohort study conducted by Atladóttir HO et al in Denmark.[29] The study investigated the association of self-reported maternal infections, febrile episodes and prenatal antibiotics use with the risk for ASD. There was no association of maternal infection or febrile episodes with the risk for ASD. However, there was an increased risk of ASD with prenatal use of antibiotics (HR 1.20, 95% CI 1.00-1.40). Due to the exploratory nature of this study, and the potential misclassification of the self-reported exposure data, the findings needed to be replicated in a large study designed to

address this research question using reliable exposure data sources. Even though the study by Atladóttir HO et al. did not find an association between prenatal infection and ASD risk, other studies reported increased ASD risk with multiple prenatal infections or infections requiring hospitalization.[30,31] Accordingly, confounding by indication is a concern and may have influenced the findings of the current study.

Our study has several strengths including the large sample size, long follow-up period, the use of administrative databases to identify the exposure and the outcome, including many potential confounders and the sibling-controlled design that minimized confounding by unmeasured familial factors. Despite the mentioned strengths, few limitations need to be considered. Exposure misclassification is a concern since drug dispensation does not necessarily indicate drug use. In addition, inpatient antibiotic dispensations were not included in the main analysis due to the limited data years and geographic coverage of the inpatient dispensation database. However, the observed association did not change significantly in the sensitivity analysis that included the subset of data available on inpatient dispensations. Outcome misclassification is another concern given that the utilized ASD identification algorithm has not been independently validated, yet using a stricter ASD identification algorithm did not change the risk estimate. The potential for unmeasured confounding from variables that are not shared by sibling pairs is a potential limitation. For example, we could only identify maternal siblings due to the lack of reliable linked data identifying the fathers. Hence, the sibling cohort included both full and half siblings, which is not ideal to account for confounding by genetic factors. Confounding by indication is another limitation and may have influenced study findings. Future studies are needed to explore other methods to control for confounding in examining similar

associations. In addition, studies are recommended to investigate antibiotic-induced microbial dysbiosis and microbiota involvement in neurodevelopmental disorders at the biological level to shed light on the etiology of these disorders and inform disease prevention.

3.7. Conclusion

Our results suggest that prenatal antibiotic exposure is associated with a small, albeit clinically non-significant increase in the risk of ASD which may have been influenced by unmeasured confounding.

3.8. Acknowledgment

We would like to thank Charles Burchill and Heather Prior from the Manitoba Centre for Health policy for their valuable support. The authors acknowledge the data providers and the Manitoba Centre for Health Policy for use of data contained in the Manitoba Population Research Data Repository under project #H2016:244 (HIPC# 2016/2017 – 11). The results and conclusions are those of the authors and no official endorsement by the Manitoba Centre for Health Policy, Manitoba Health, Healthy Living and Senior or other data providers is intended or should be inferred. Data used in this study are from the Manitoba Population Research Data Repository housed at the Manitoba Centre for Health Policy, University of Manitoba and were derived from data provided by Manitoba Health Healthy Living and Senior, Winnipeg Regional Health Authority, Manitoba Department of Families, Healthy Child Manitoba and Manitoba Education and Training.

3.9. Figures and tables

Table 3.1. Characteristics of study cohort: overall and by antibiotic exposure status

Characteristic	Number (%)		
	All subjects N=214 834	Antibiotic use during pregnancy	
		NO N= 134 084	YES N= 80 750
Male	110 107 (51.3)	68 691 (51.2)	41 416 (51.3)
Urban region	116 865 (54.4)	74 887 (55.9)	41 978 (52.0)
Socioeconomic status (SES)[a]:			
High	21 212 (9.9)	14 912 (11.1)	6300 (7.8)
Middle	77 708 (36.2)	50 861 (37.9)	26 847 (33.3)
Low-mid	67 059 (31.2)	40 410 (30.1)	26 649 (33.0)
Low	48 855 (22.7)	27 901 (20.8)	20 954 (26.0)
Receipt of income assistance [b]	37 158 (17.3)	17 058 (12.7)	20 100 (24.9)
Mothers age at delivery (years):			
< 30	128 229 (59.7)	76 411 (57.0)	51 818 (64.2)
30-39	81 963 (38.2)	54 602 (40.7)	27 361 (33.9)
>= 40	4642 (2.2)	3071 (2.3)	1571 (2.0)
Breastfeeding initiation [c]	172 952 (80.8)	110 425 (82.7)	62 527 (77.7)
Multiple birth [d]	5383 (2.5)	3246 (2.4)	2137 (2.7)
Caesarian section delivery	43 782 (20.4)	26 810 (20.0)	16 972 (21.0)
Birth complications	21 022 (9.8)	13 092 (9.8)	7930 (9.8)
First born child	80 758 (37.6)	52 334 (39.0)	28 424 (35.2)
Small for gestational age [e]	16 507 (7.7)	10 445 (7.8)	6062 (7.5)
Prenatal alcohol/drug use [f]	12 959 (12.3)	7265 (10.9)	5694 (14.7)
Prenatal smoking [g]	20 844 (19.4)	10 687 (15.8)	10 157 (25.7)
Childhood medical conditions:			
Infections:	67 430 (31.4)	48 169 (35.9)	19 261 (23.9)
None	134 794 (62.7)	78 881 (58.8)	55 913 (69.2)
Mild-moderate [h]	12 610 (5.9)	7034 (5.3)	5576 (6.9)
Severe [i]	1056 (0.5)	589 (0.4)	467 (0.6)
Epilepsy	20 517 (9.6)	11 934 (8.9)	8583 (10.6)
Neonatal jaundice	859 (0.4)	489 (0.4)	370 (0.5)
Other developmental disabilities	26 641 (12.4)	13 522 (10.1)	13 119 (16.3)
Early life antibiotic exposure [j]	94 024 (43.8)	51 524 (38.4)	42 500 (52.6)
Maternal medical conditions:			
Mood and anxiety disorders	15 885 (7.4)	7493 (5.6)	8392 (10.4)
Schizophrenia	196 (0.1)	87 (0.1)	109 (0.1)
Diabetes	6085 (2.8)	3268 (2.4)	2817 (3.5)
Infections	67 559 (31.5)	22 214 (16.6)	45 345 (56.2)
Prenatal medications use:			
Antidepressants	5759 (2.7)	2773 (2.1)	2986 (3.7)
Antipsychotics	613 (0.3)	236 (0.2)	377 (0.5)
Anticonvulsants	1282 (0.6)	552 (0.4)	730 (0.9)
Cardiovascular medications	3370 (1.6)	1887 (1.4)	1483 (1.8)
Year of birth:			
1998-2001	47 107 (21.9)	28 817 (21.5)	18 290 (22.7)
2002-2005	48 596 (22.6)	30 789 (23.0)	17 807 (22.1)
2006-2009	53 052 (24.7)	33 269 (24.8)	19 783 (24.5)

Characteristic	Number (%)		
	All subjects N=214 834	**Antibiotic use during pregnancy**	
		NO N= 134 084	**YES N= 80 750**
2010-2014	66 079 (30.8)	41 209 (30.7)	24 870 (30.8)
Season of birth:			
Winter	49 039 (22.8)	30 678 (22.9)	18 361 (22.7)
Spring	56 517 (26.3)	34 935 (26.1)	21 582 (26.7)
Summer	58 987 (27.5)	36 831 (27.5)	22 156 (27.4)
Fall	50 291 (23.4)	31 640 (23.6)	18 651 (23.1)

Percentages are calculated based on non-missing data

[a] The Socio-Economic Factor Index (SEFI) was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Missing data for 886 (0.4%) subjects

[d] Defined as the number of births following a multiple gestation pregnancy

[e] Defined as having birth weight below the 10th percentile for the gestational age and sex. Missing data for 502 (0.2%) subjects

[f] Missing data for 109 491 (51.0%) subjects

[g] Missing data for 107 550 (50.1%) subjects

[h] Defined as having an infection code in physician claims only

[i] Defined as having a hospitalization with an infection code

[j] Defined as filling one or more antibiotic prescription during the first year of life

Table 3.2. Association between antibiotic exposure and risk of ASD in the overall cohort

Variable	Person-years	Number of events	HR (95% CI)	
			Unadjusted	Adjusted [a]
Main analysis				
Prenatal antibiotic exposure	1 943 612	2965	1.11 (1.03 - 1.19)	1.10 (1.01 - 1.19)
Stratified by sex:				
Male	991 939	2401	1.11 (1.02 - 1.20)	1.12 (1.02 - 1.22)
Female	951 673	564	1.10 (0.93 - 1.30)	1.01 (0.84 - 1.23)
Stratified by region:				
Rural	887 497	953	1.28 (1.13 - 1.46)	1.25 (1.08 - 1.44)
Urban	1 056 105	2012	1.06 (0.97 - 1.17)	1.02 (0.92 - 1.13)
Exposure by trimester: [b]				
None	1 211 385	1778	1.00 (Reference]	1.00 [Reference]
First	330 263	527	1.09 (0.99 - 1.20)	1.04 (0.94 - 1.16)
Second	372 136	620	1.14 (1.04 - 1.25)	1.11 (1.01 - 1.23)
Third	331 159	549	1.13 (1.03 - 1.25)	1.17 (1.06 - 1.30)
Secondary analyses				
Antibiotic class				
None	1 211 385	1778	1.00 [Reference]	1.00 [Reference]
Penicillin antibiotics	494 406	813	1.12 (1.03 - 1.22)	1.13 (1.04 - 1.24)
Non-penicillin β-lactams	132 014	233	1.19 (1.04 - 1.36)	1.18 (1.03 - 1.37)
Macrolides and related antibiotics	135 917	222	1.11 (0.97 - 1.28)	1.04 (0.90 - 1.20)
Others	194 826	302	1.06 (0.94 - 1.20)	1.01 (0.98 - 1.15)
Number of antibiotic courses				
0	1 211 385	1778	1.00 [Reference]	1.00 [Reference]
1	458 378	739	1.10 (1.10 - 1.20)	1.10 (1.00 - 1.20)
2	166 695	266	1.09 (0.96 - 1.24)	1.07 (0.93 - 1.22)
>=3	107 154	182	1.16 (1.00 - 1.35)	1.16 (0.98 - 1.37)
Cumulative antibiotic duration (days)				
0	1 211 385	1778	1.00 [Reference]	1.00 [Reference]
1 – 7	300 554	472	1.07 (0.96 - 1.18)	1.07 (0.96 - 1.19)
8 – 14	237 233	384	1.11 (0.99 - 1.24)	1.10 (0.98 - 1.24)
>14	194 441	331	1.17 (1.04 - 1.31)	1.15 (1.01 - 1.30)

[a] Adjusted for sex, region, SES, maternal age at delivery, maternal medical conditions (mood and anxiety disorders, schizophrenia, DM, prenatal infections) prenatal antidepressants use, size for gestational age, childhood medical conditions (epilepsy, infections, neonatal jaundice, asthma and a diagnosis with other developmental disability disorder), antibiotics use in the first year of life, birth complications, mode of delivery, multiple birth, breastfeeding initiation, year of birth, season of birth, and birth order, health care access

[b] First trimester = 0 to 13 weeks of pregnancy, second trimester = 14 to 27 weeks, third trimester = 28 weeks until date of birth

Table 3.3. Association between antibiotic exposure and risk of ASD in the sibling cohort

Variable	Person-years	Number of events	HR (95% CI)	
			Unadjusted	Adjusted [a]
Main analysis				
Prenatal antibiotics exposure	700 610	977	1.05 (0.90 -1.22)	1.08 (0.90 - 1.30)
Stratified by sex:				
Male	357 076	786	1.21 (0.97 - 1.52)	1.14 (0.89 - 1.47)
Female	343 535	191	1.03 (0.64 - 1.64)	1.10 (0.57 - 2.11)
Stratified by region:				
Rural	361 486	378	1.12 (0.87 - 1.45)	1.19 (0.85 - 1.67)
Urban	339 124	599	1.07 (0.88 - 1.30)	1.07 (0.84 - 1.37)
Exposure by trimester: [b]				
None	368 871	501	1.00 [Reference]	1.00 [Reference]
First	141 496	190	1.15 (0.94 - 1.41)	1.25 (1.00 - 1.58)
Second	159 418	249	1.13 (0.94 - 1.36)	1.16 (0.94 - 1.44)
Third	143 039	207	1.21 (0.99 - 1.47)	1.25 (1.00 - 1.57)
Secondary analyses				
Antibiotic class				
None	368 871	501	1.00 [Reference]	1.00 [Reference]
Penicillin antibiotics	219 436	327	1.11 (0.94 - 1.32)	1.18 (0.97 - 1.45)
Macrolides and related antibiotics	59 611	93	1.27 (0.96 - 1.68)	1.24 (0.91 - 1.68)
Non-penicillin β-lactams	57 224	82	1.27 (0.95 - 1.71)	1.29 (0.94 - 1.77)
Others	80 109	101	0.96 (0.74 - 1.26)	1.01 (0.75 - 1.36)
Number of antibiotic courses				
0	368 871	501	1.00 [Reference]	1.00 [Reference]
1	226 323	311	0.99 (0.83 - 1.18)	1.04 (0.85 - 1.28)
2	70 245	115	1.14 (0.86 - 1.51)	1.17 (0.84 - 1.62)
>= 3	35 171	50	1.33 (0.87 - 2.03)	1.20 (0.73 - 1.96)
Cumulative antibiotic duration (days)				
0	368 871	501	1.00 [Reference]	1.00 [Reference]
1-7	148 816	200	0.97 (0.78 - 1.19)	1.04 (0.81 - 1.32)
8-14	111 409	165	1.03 (0.82 - 1.30)	1.06 (0.81 - 1.38)
>14	71 514	111	1.29 (0.96 - 1.72)	1.26 (0.89 - 1.79)

[a] Adjusted for sex, region, SES, maternal age at delivery, prenatal infections, prenatal antidepressants use, size for gestational age, childhood medical conditions [epilepsy, infections, neonatal jaundice, asthma and a diagnosis with other developmental disability disorder), antibiotics use in the first year of life, birth complications, mode of delivery, multiple birth, breastfeeding initiation, year of birth, season of birth, and birth order

[b] First trimester = 0 to 13 weeks of pregnancy, second = 13 to 27 weeks, third = 28 weeks until date of birth

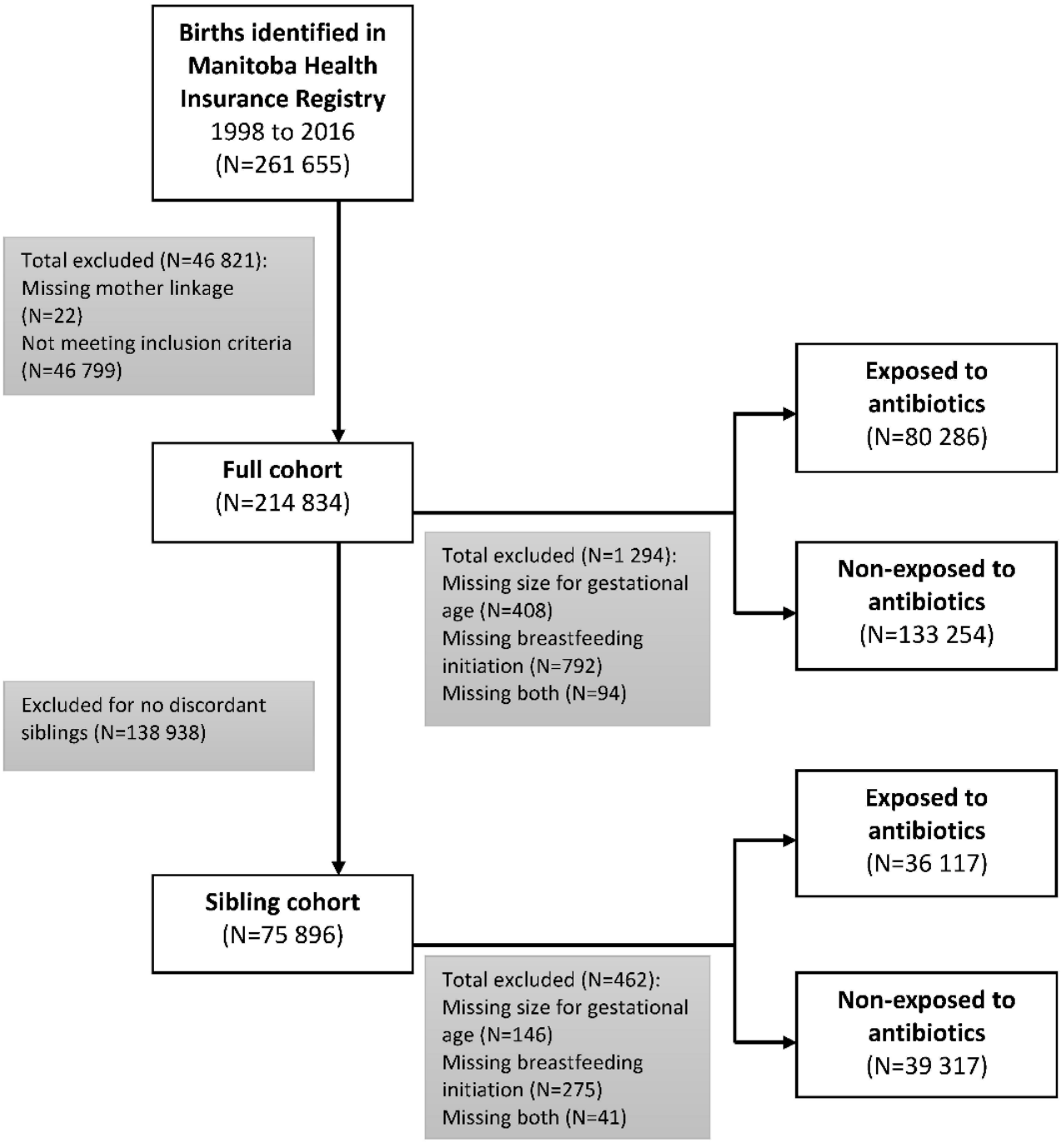

Figure 3.1. Study populations: overall and sibling cohort

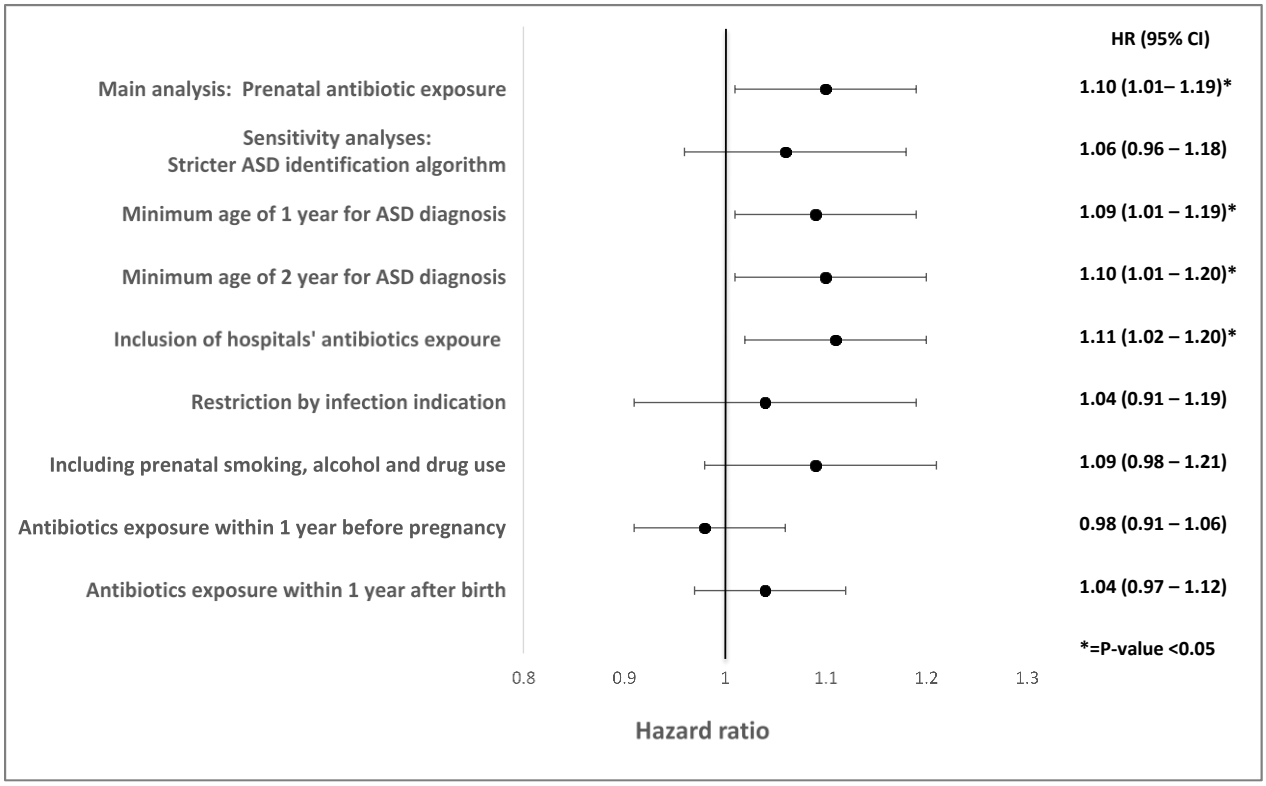

Figure 3.2. Forest plot with sensitivity analyses risk estimates

Supplementary Table 3.1. Description and years of data sources

Database	Description	Data years
Drug Program Information Network (DPIN)	An electronic drug system that captures all prescriptions dispensed from community pharmacies to residents and is maintained by Manitoba Health, Seniors and Active Living. It includes prescription information such as generic name, Anatomical Therapeutic Chemical (ATC) code, date of dispensation, strength and days supplied.	1995/96 - 2015/16
In-hospital Pharmaceuticals	An inpatient pharmacy system that provides dispensing information on pharmaceutical use at three Winnipeg hospitals: Health Sciences Centre, Concordia Hospital and Riverview Health Centre.	1999-2012
Hospital discharge abstracts	Includes records of all patients' hospital admissions with summaries for demographic data such as gender and postal code, and clinical data including up to 25 diagnosis codes and 20 procedure codes.	1970/71 - 2015/16
Medical Services database	Physician claims in the Medical Services database include records of claims for any physician visits in offices, hospitals and outpatient departments in Manitoba in addition to some information on Manitobans' physician visits outside the province. Claims are submitted by physicians electronically for service reimbursement by Manitoba Health, Seniors and Active Living.	1970/71 - 2015/16
The Manitoba Education and Training Special Needs funding data file	Includes education records on special school funding received for students with special needs, including those with ASD.	1995/96 - 2015/16
The Hospital Newborn to Mother Link	Serves to match the baby's birth hospital record with the mother's obstetrical delivery record and contains basic demographic and hospital data on newborns and their mothers for in-hospital births.	1984/85 - 2015/16
BabyFirst - Families First Screen	Collected by Public Health Nurses on nearly all families with newborns in Manitoba and maintained by Heathy Child Manitoba. Contains records of newborns identifying biological, social, and demographic risk factors including prenatal smoking, alcohol and drug use.	2000 – 2013
The Social Allowances Management Information Network	Provides information concerning employment and income assistance received by Manitoba residents.	1995/96 - 2014/15
Canada Census	A population survey with aggregate demographic information for all persons within a dissemination area in Canada and is conducted by Statistics Canada every five years.	1971 – 2016

Supplementary Table 3.2. Antibiotics classification according to Anatomical Therapeutic Chemical (ATC)

Class	Generic name	ATC
Penicillin antibiotics	Amoxicillin, ampicillin, bacampicillin, penicillin, piperacillin, pivampicillin, pivmecillinam, cloxacillin, floxacillin, nafcillin, ticarcillin, tazobactam	J01C
Non-penicillin β-lactams	Cefazolin, cefepime, cefixime, cefotaxime, cefotetan, cefoxitin, cefpodoxime, cefprozil, ceftazidime, ceftizoxime, ceftobiprole, ceftriaxone, cefuroxime, cephalexin, cephalothin, aztreonam, cefaclor, cefadroxil, cefamandole, doripenem, ertapenem, meropenem	J01D
Macrolides and related antibiotics	Dalfopristin, telithromycin, azithromycin, clarithromycin, clindamycin, erythromycin, lincomycin	J01F, J01R
Other antibiotics	Minocycline, doxycycline, demeclocycline, rolitetracycline, tetracycline, ciprofloxacin, gatifloxacin, nalidixic acid, gemifloxacin, grepafloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin, trovafloxacin, methenamine, fosfomycin, tedizolid, nitrofurantoin, metronidazole, linezolid, fusidate, fusidic acid, bacitracin, vancomycin, colistin, polymyxin B, telavancin, tobramycin, gentamicin, daptomycin, amikacin, spectinomycin, neomycin, netilmicin, spiramycin, streptomycin, chloramphenicol, trimethoprim, sulfamethoxazole, sulfadiazine, sulfapyridine, sulfisoxazole	J01A, J01B, J01G, J01E, J01M, J01X

Supplementary Table 3.3. Prenatal medications explored as covariates [a]

Class	Generic name	ATC
Antidepressants	Desipramine, imipramine, clomipramine, trimipramine, amitriptyline, nortriptyline, protriptyline, doxepin, amoxapine, amineptine, maprotiline, fluoxetine, citalopram, paroxetine, sertraline, fluvoxamine, escitalopram, phenelzine, tranylcypromine, moclobemide, tryptophan, trazodone, nefazodone, mirtazapine, bupropion, venlafaxine, milnaacipran, duloxetine, desvenlafaxine, vilazodone, vortioxetine.	N06A
Cardiovascular medications	Antihypertensive agents: reserpine, methyldopa, clonidine, guanfacine, prazosin, doxazosin, guanethidine, diazoxide, hydralazine, minoxidil, nitroprusside, bosentan, ambrisentan, macitentan, riociguat. Peripheral vasodilators: phentolamine, nicotinic acid, pentoxifylline, ergoloid mesylates, cyclandelate. Beta blockers: oxprenolol, pindolol, propranolol, timolol, sotalol, nadolol, metoprolol, atenolol, acebutolol, bisoprolol, esmolol, nebivolol, labetalol, carvedilol. Calcium channel blockers: amlodipine, felodipine, nicardipine, nifedipine, nimodipine, clevidipine, verapamil, diltiazem. Agents acting on the renin-angiotensin system: captopril, enalapril, lisinopril, perindopril, ramipril, quinapril, benazepril, cilazapril, fosinopril, trandolapril, losartan, eprosartan, valsartan, irbesartan, candesartan, telmisartan, olmesartan, azilsartan, aliskiren.	C02 C04 C07 C08 C09
Antipsychotics	Chlorpromazine, promazine, fluphenazine, perphenazine, prochlorperazine, trifluoperazine, thioproperazine, perazine, periciazine, thioridazine, mesoridazine, pipotiazine, haloperidol, droperidol, ziprasidone, lurasidone, flupentixol, clopenthixol, zuclopenthixol, fluspirilene, pimozide, loxapine, clozapine, olanzapine, quetiapine, asenapine, lithium, risperidone, aripiprazole, paliperidone, brexpiprazole.	N05A
Anticonvulsants	Phenobarbital, primidone, phenytoin, fosphenytoin, ethosuximide, clonazepam, carbamazepine, oxcarbazepine, rufinamide, eslicarbazepine, valproic acid, vigabatrin, lamotrigine, topiramate, gabapentin, levetiracetam, pregabalin, stiripentol, lacosamide, perampanel, brivaracetam, beclamide.	N03A

[a]: Two prescription fills during pregnancy was required

Supplementary Table 3.4. Identification algorithm of medical conditions

Covariate	Period	Diagnostic Criteria
Maternal medical conditions:		
Mood and anxiety disorders	Within 3 years before index date	✓ One or more hospitalizations with a diagnosis for depressive disorder, affective psychoses, neurotic depression or adjustment reaction: ICD-9-CM codes 296.1-296.8, 300.4, 309 or 311; ICD-10-CA codes F31, F32, F33, F34.1, F38.0, F38.1, F41.2, F43.1, F43.2, F43.8, F53.0, F93.0 or with a diagnosis for an anxiety state, phobic disorders or obsessive-compulsive disorders: ICD-9-CM codes 300.0, 300.2, 300.3, 300.7; ICD-10-CA codes F40, F41.0, F41.1, F41.3, F41.8, F41.9, F42, F45.2, OR ✓ One or more hospitalizations with a diagnosis for anxiety disorders: ICD-9-CM code 300; ICD-10-CA codes F32, F34.1, F40, F41, F42, F44, F45.0, F45.1, F45.2, F48, F68.0, or F99 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ One or more physician visits with a diagnosis for depressive disorder or affective psychoses: ICD-9-CM codes 296, 311, OR ✓ One or more physician visits with a diagnosis for anxiety disorders: ICD-9-CM code 300 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ Three or more physician visits with a diagnosis for anxiety disorders or adjustment reaction: ICD-9-CM code 300, 309.
Schizophrenia	Within 3 years before index date	✓ One or more hospitalization with a diagnosis for schizophrenia: ICD-9-CM code 295; ICD-10-CA codes F20, F21, F23.2, F25, OR ✓ One or more physician visits with a diagnosis for schizophrenia: ICD-9-CM code 295.
Diabetes mellitus	Within 1 year before index date	✓ One or more hospitalizations with a diabetes diagnosis: ICD-9-CM code 250, 648.0, 648.8; ICD-10-CA codes E10-E14, O24, OR ✓ Two or more physician visits with a diabetes diagnosis: ICD-9-CM code 250, OR ✓ One or more prescriptions for a diabetes medication (ATC: A10A, A10B).
Prenatal infection	During pregnancy	A hospitalization or physician visit with any of the following codes: ✓ Eye and ear infections: ICD-9-CM codes 370, 372, 380, 381, 382; ICD-10-CA codes H10, H13.1, H16, H19.1, H19.2, H60, H62, H65, H66, H67. ✓ Upper respiratory tract infection: ICD-9-CM codes 460–465; ICD-10-CA codes J00-J06. ✓ Lower respiratory tract infection: ICD-9-CM codes 466, 481–488; ICD-10-CA codes J09- J18, J20-J22. ✓ Genitourinary system infection: ICD-9-CM codes 590, 595, 597, 599, 601, 604, 614-617, 771; ICD-10-CA codes N10-N12, N30, N33.0, N33.8, N37.0, N39.0, N41, N45, N70-N77. ✓ Central nervous system infections: ICD-9-CM codes 320, 321, 323, 324, 728; ICD-10-CA codes G00-G02, G04-G07

Covariate	Period	Diagnostic Criteria
Prenatal infection, continued	During pregnancy	✓ Skin infections: ICD-9-CM codes 680-686; ICD-10-CA codes L00- L08, M72.6. ✓ Cardiovascular system infections: ICD-9-CM codes 391, 420, 421, 422, 424; ICD-10-CA codes I01, I30, I32.0, I32.1, I33, I38, I39.8, I40, I41.0, I41.1, I41.2. ✓ Musculoskeletal system infections: ICD-9-CM codes 711, 730; ICD-10-CA codes M00- M01, M03, M46.2, M86. ✓ Bacteremia/Septicemia: ICD-9-CM codes 771, 790, 995; ICD-10-CA codes P36, A22.7, A26.7, A02.1, A32.7, A02.1, A32.7, A40, A41, A42.7, B37.7. ✓ Gastrointestinal system infections: ICD-9-CM codes 535, 540, 541, 542, 566, 567, 530, 572, 575; ICD-10-CA codes K20, K29.0, K29.1, K29.8, K35, K61, K65, K67, K81.0. ✓ Other Parasitic and infectious diseases: ICD-9-CM codes 001-139; ICD-10-CA codes A00-B99.
Childhood medical conditions:		
Birth complications	At birth	Include vacuum, forceps or breech procedures: ICD-9-CM procedure codes 72, 73.3, 763.2, 652.2, 669.6; CCI codes 5MD53, 5MD54, 5MD55, 5MD56, shoulder dystocia: 660.4, placental abruption: 641.2, cord prolapse: 663, uterine rupture: 665.1 or other perinatal complications: 760-779.
Epilepsy	Within the first year of life	✓ One or more hospitalization for seizure disorder: ICD-9-CM codes 345, 649.4; ICD-10-CA codes G40, G41, OR ✓ One or more physician visit for seizure disorder: ICD-9-CM code 345, OR ✓ One or more prescription for an anticonvulsant medication (ATC: N03A)
Asthma	Within the first year of life	✓ One or more hospitalizations with a diagnosis of asthma: ICD-9-CM codes 493; ICD-10-CA code J45, OR ✓ One or more physician visits with a diagnosis of asthma: ICD-9-CM codes 493, OR ✓ One or more prescriptions for asthma medication (ATC: R03, R06AX17)
Other developmental disabilities	Within the first year of life	✓ One or more hospitalizations with diagnoses for intellectual disabilities, Down's syndrome, autosomal deletion syndromes, Prader-Willi syndrome, other specified congenital anomalies, or fetal alcohol syndrome: ICD-9-CM codes 317, 318, 319, 758.0- 758.3, 759.8, 760.71; ICD-10-CA: F70, F71, F72, F73, F78, F79, P04.3, Q86, Q87, Q89.8, Q90, Q91, Q93, Q99.2. ✓ One or more physician visit with diagnoses for intellectual disabilities: ICD-9-CM: 317, 318, 319
Neonatal jaundice	Within the first 4 weeks of life	✓ One or more hospitalizations with diagnoses for Jaundice: ICD-9-CM code 774; ICD–10–CA codes P58, P59, OR ✓ One or more physician visit with diagnoses for Jaundice: ICD-9-CM code 774
Infection	Within the first year of life	Similar to maternal definition

Supplementary Table 3.5. Description of antibiotic use

	Number (%)
Antibiotic class	
Penicillin antibiotics	53 232 (50.2)
Non-penicillin β-lactams	16 321 (15.4)
Macrolides and related antibiotics	14 799 (14.0)
Others	21 687 (20.5)
Total	106 039
Trimester [a]	
1	36 563 (32.1)
2	41 368 (36.3)
3	35 907 (31.5)
Total	113 838
Number of courses	
1	50 685 (62.8)
2	18 427 (22.8)
>=3	11 638 (14.4)
Total	80 750
Cumulative duration (days)	
1-7	34 213 (42.4)
8-14	25 593 (31.7)
>14	20 944 (25.9)
Total	80 750
[a] First trimester = 0 to 13 weeks of pregnancy, second trimester = 14 to 27 weeks, third trimester = 28 weeks until date of birth	

Supplementary Table 3.6. Characteristics of sibling cohort: overall and by antibiotics exposure status

Characteristic	**Number (%)**		
	All subjects N=75 896	**Antibiotic use during pregnancy**	
		No N= 39 577	**Yes N= 36 319**
Male	38 788 (51.1)	20 186 (51.0)	18 602 (51.2)
Urban region	36 648 (48.3)	18 688 (47.2)	17 960 (49.5)
Socioeconomic status (SES)[a]:			
High	5913 (7.8)	3051 (7.7)	2862 (7.9)
Middle	24 463 (32.2)	12 849 (32.5)	11 614 (32.0)
Low-mid	23 604 (31.1)	12 160 (30.7)	11 444 (31.5)
Low	21 916 (28.9)	11 517 (29.1)	10 399 (28.6)
Receipt of income assistance [b]	16 779 (22.1)	7953 (20.1)	8826 (24.3)
Mothers age at delivery:			
< 30	50 132(66.1)	26 242 (66.3)	23 890 (65.8)
30-39	24 780 (32.7)	12 839 (32.4)	11 941 (32.9)
>= 40	984 (1.3)	496 (1.3)	488 (1.3)
Breastfeeding initiation [c]	58 576 (77.5)	30 635 (77.8)	27 941 (77.2)
Multiple birth [d]	1548 (2.0)	747 (1.9)	801 (2.2)
Caesarian section	13 178 (17.4)	6701 (16.9)	6477 (17.8)
Birth complications	5582 (7.4)	2905 (7.3)	2677 (7.4)
First born child	21 037 (27.7)	11 419 (28.9)	9618 (26.5)
Small for gestational age [e]	5141 (6.8)	2682 (6.8)	2459 (6.8)
Prenatal alcohol/drug use [f]	5279 (13.5)	2679 (13.1)	2600 (13.9)
Prenatal smoking [g]	8881 (22.3)	4322 (20.8)	4559 (23.9)
Childhood medical conditions:			
Infections:	21 731 (28.6)	12 184 (30.8)	9547 (26.3)
None	48 736 (64.2)	24 541 (62.0)	24 195 (66.6)
Mild-moderate [h]	5429 (7.2)	2852 (7.2)	2577 (7.1)
Severe [i]	387 (0.5)	204 (0.5)	183 (0.5)
Epilepsy	7004 (9.2)	3615 (9.1)	3389 (9.3)
Neonatal jaundice	330 (0.4)	159 (0.4)	171 (0.5)
Other developmental disabilities	10 817 (14.3)	5232 (13.2)	5585 (15.4)
Asthma	21 731 (28.6)	12 184 (30.8)	9547 (26.3)
Early life antibiotics exposure [j]	36 013 (47.5)	17 680 (44.7)	18 333 (50.5)
Maternal medical conditions:			
Mood and anxiety disorders	5779 (7.6)	2603 (6.6)	3176 (8.7)
Schizophrenia	73 (0.1)	37 (0.1)	36 (0.1)
Diabetes	2163 (2.9)	1066 (2.7)	1097 (3.0)
Prenatal infections	26 693 (35.2)	7663 (19.4)	19 030 (52.4)
Prenatal medications use:			
Antidepressants	2101 (2.8)	969 (2.5)	1132 (3.1)
Antipsychotics	228 (0.3)	101 (0.3)	127 (0.4)
Anticonvulsants	493 (0.7)	209 (0.5)	284 (0.8)
Cardiovascular medications	1001 (1.3)	487 (1.2)	514 (1.4)
Year of birth:			
1998-2001	14 145 (18.6)	7329 (18.5)	6816 (18.8)
2002-2005	19 390 (25.6)	10 472 (26.5)	8918 (24.6)
2006-2009	21 426 (28.2)	11 285 (28.5)	10 141 (27.9)

Characteristic	Number (%)		
	All subjects N=75 896	Antibiotic use during pregnancy	
		No N= 39 577	Yes N= 36 319
2010-2014	20 935 (27.6)	10 491 (26.5)	10 444 (28.8)
Season of birth:			
Winter	17 661 (23.3)	9258 (23.4)	8403 (23.1)
Spring	19 755 (26.0)	10 127 (25.6)	9628 (26.5)
Summer	20 598 (27.1)	10 727 (27.1)	9871 (27.2)
Fall	17 882 (23.6)	9465 (23.9)	8417 (23.2)

Percentages are calculated based on non-missing data

[a] Socio-Economic Factor Index (SEFI) was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Missing data for 316 (0.4%) subjects

[d] Defined as the number of births following a multiple gestation pregnancy

[e] Defined as having birth weight below the 10th percentile for the gestational age and sex. Missing data for 187 (0.2%) subjects

[f] Missing data for 36 810 (48.5%) subjects

[g] Missing data for 36 018 (47.5%) subjects

[h] Defined as having an infection code in physician claims only

[i] Defined as having a hospitalization with an infection code

[j] Defined as filling one or more antibiotic prescription during the first year of life identified in DPIN

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Chapter 4: Antibiotic Exposure in the First Year of Life and the Risk of Attention-Deficit/Hyperactivity Disorder: a Population-Based Cohort Study

4.1. Overview

This manuscript is the first of two manuscripts aiming to examine the association of early life antibiotic exposure and ADHD risk. This chapter addresses the third project objective, which assesses the association using the first year of life as the exposure period. The manuscript is based on a population-based cohort study of children born in Manitoba between the fiscal years 1998 and 2017 and utilized administrative health data from the Manitoba Population Research Data Repository. Cox proportional hazards regression models were used to estimate the risk of developing ADHD in a high dimensional propensity scores-matched cohort and in a cohort of exposure-discordant siblings. The high dimensional propensity scores-matched cohort was added to the protocol of objectives 3 and 4 as an additional method to account for unmeasured confounding and to compliment the results of the sibling-design. Study findings suggested no association between antibiotic exposure in the first year of life and ADHD risk.

Citation: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Antibiotic Exposure in the First Year of Life and the Risk of Attention-Deficit/Hyperactivity Disorder: a Population-Based Cohort Study. *Am J Epidemiol* 2019; 188: 1923–1931.

4.2. Abstract

Background: Early childhood antibiotic exposure induces changes in gut microbiota reportedly associated with the development of Attention-Deficit/Hyperactivity Disorder (ADHD). We conducted a study to examine the association between antibiotic use in the first year of life and ADHD risk.

Methods: We conducted a population-based cohort study, which included children born in the Manitoba, Canada between 1998 and 2017. Exposure was defined as having filled one or more antibiotic prescriptions during the first year of life. ADHD diagnosis was identified in hospital abstracts, physician visits or drug dispensations. Risk of developing ADHD was estimated using Cox proportional hazards regression in a high dimensional propensity score-matched cohort (n= 69 738) and a sibling cohort (n= 67 671).

Results: ADHD risk was not associated with antibiotic exposure in the matched-cohort (HR 1.02, 95% CI 0.97-1.08 and in the sibling cohort (HR 0.96, 95% CI 0.89-1.03). In secondary analyses of the matched cohort, ADHD risk increase was observed in those exposed to 4 or more antibiotic courses or a duration longer than 3 weeks. These associations were not observed in the sibling cohort.

Conclusions: Antibiotic exposure in the first year of life does not pose an ADHD risk on a population level.

4.3. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is the most common neurodevelopmental disorder and is associated with significant burden worldwide.[1,2] More than half of the children

with ADHD continue to have the disorder in adulthood and live with many long-term adverse health and social outcomes.[3–6]

The worldwide ADHD prevalence in children was estimated at 7.2%.[7] In the Canadian province of Manitoba, an annual age-standardized prevalence of 1.5% was reported for ADHD in 1999, which increased to 2.8% in 2012.[8] Similar trends were reported in several other Canadian provinces.[8] Genetic factors have been demonstrated to play a role in ADHD etiology with a hereditability of 70-80%.[9–14] Additionally, several environmental factors have shown possible association with ADHD risk, including low level of paternal education, low maternal age at delivery, prenatal smoking, prenatal illicit drug use, birth complications, low birth weight, preterm delivery, neonatal jaundice, childhood asthma, maternal depression, prenatal antidepressants and acetaminophen exposure.[15–27]

Gut microbiota plays an important role in the communication between the gut and the brain, via the gut-brain axis.[28–30] Early life changes in microbiota composition result in different brain developmental trajectories, potentially contributing to disorders such as ADHD.[29–31] Recent research has shown that ADHD subjects have different microbiota composition compared to non-ADHD subjects.[32] A small randomized study reported a lower risk of ADHD in infants who received a probiotic in the first 6 months of life, suggesting that microbiota does play a role in the development of ADHD.[33] It has been demonstrated that exposure to antibiotics in early life disrupts the equilibrium of gut microbiota and could potentially contribute to the development of ADHD.[30,31,34] In this study, we examined the association between antibiotic use in the first year of life and the risk of ADHD.

4.4. Methods

4.4.1. Design and subjects

This was a population-based cohort study that utilized data from the Manitoba Population Research Data Repository. The Repository databases capture encounters with the healthcare system and drug dispensations for Manitoba residents through a universal and publicly funded provincial health system. Linkage among different databases was achieved through scrambled Personal Health Identification Numbers. The study was approved by the University of Manitoba Health Research Ethics Board and the Health Information Privacy Committee.

We identified a birth cohort of all children registered in the Manitoba Health Insurance Registry who were born between April 1, 1998 and March 31, 2017 with the birthdate as the assigned index date for cohort entry. To meet the recommendations of the American Academy of Pediatrics on screening for ADHD at 4 years of age [35], we required a minimum of 4 years of valid Manitoba health registration since birth for the children to be included in the cohort. We also required a minimum of 2 years of health registration for the mothers prior to child's birthdate to capture relevant covariates. Children were followed until diagnosis of ADHD, migration out of province, 18th birthdate, death or end of study period (March 31, 2017), whichever occurred earlier.

Other data sources used in the study included the Drug Program Information Network, In-hospital Pharmaceuticals, Hospital Abstracts, physician claims from the Medical Services database, the Hospital Newborn to Mother Link Registry, BabyFirst - Families First Screen and the Social Allowances Management Information Network (Supplementary Table 1). Currently in

Manitoba, the 9th - Clinical Modifications (ICD-9-CM) and the 10th (ICD-10) revisions of the International Classification of Disease coding system are used in physician claims (outpatient) and hospital discharge abstracts (inpatient admissions), respectively.

To account for unmeasured confounding due to genetic and other shared familial factors, we analyzed the association between antibiotic exposure and ADHD risk in a separate sibling cohort, consisting of children from the overall cohort with at least one maternal sibling discordant in antibiotic exposure.

4.4.2. Exposure

Exposure to antibiotics was identified in the Drug Program Information Network, which captures all prescription drug dispensation through community pharmacies. Early childhood antibiotics use was defined as having filled one or more antibiotic prescriptions during the first year of life. Additionally, we analyzed the exposure based on the number of antibiotic courses received, cumulative duration within the first year of life and the class of antibiotic (Supplementary Table 2).

4.4.3. Outcome

The primary outcome was ADHD diagnosis identified between the age of 4 years and the end of follow-up period. An established ADHD identification algorithm was utilized, which defines ADHD diagnosis as one or more hospitalizations with a diagnosis of hyperkinetic syndrome (ICD-9-CM code 314 or ICD-10 code F90); or one or more physician visits with a diagnosis of hyperkinetic syndrome (ICD-9-CM code 314); or two or more prescriptions for ADHD drugs (Anatomical Therapeutic Chemical code N06BA) within a year without a diagnosis of conduct

disorder (ICD-9-CM code 312 or ICD-10 codes F63, F91, F92); or disturbance of emotions (ICD-9-CM code 313 or ICD-10 codes F93, F94); or cataplexy/narcolepsy (ICD-9-CM code 347 or ICD-10 code G47.4).[36–38]

4.4.4. Covariates

High Dimensional Propensity Scores (HDPS) were estimated using 10 dimensions from hospital abstracts, physician visits and the Drug Program Information Network. Mothers' hospital medical diagnoses and procedures (2 dimensions) and outpatient medical diagnoses and tariff codes (2 dimensions) were identified during the year prior to birthdate. Prenatal drug dispensations excluding antibiotics (1 dimension) were identified during the 9 months prior to birthdate. Children's hospital medical diagnoses and procedures (2 dimensions), outpatient medical diagnoses and tariff codes (2 dimensions) and drug dispensations excluding antibiotics (1 dimension) were identified during the year following birthdate. Within each dimension, the top 200 prevalent variables were identified and were ranked based on their frequency into once, sporadic and frequent. A total of 500 variables were selected from the candidate variables. The selection was based on the amount of imbalance of the variable between the two exposure groups and the association between the variable and the outcome of interest.

In addition to the 500 variables, a set of sociodemographic characteristics and other clinically important variables were identified and included in the HDPS estimation.[17,20,21,23,26,27,39,40] The pre-identified variables included sex, region of residence (urban or rural), mothers' age at delivery, the Socio-Economic Factor Index, which is an area-level measure of socioeconomic status derived from Census data [41], receiving income assistance, which was used as a proxy of individual level socio-economic status, season of birth

and year of birth. We also included preterm delivery, mode of delivery (vaginal or caesarian section), breastfeeding initiation, birth complications, size for gestational age and birth order (firstborn or subsequent), which were all derived from hospital birth records. We excluded children with missing values on any of these variables (Figure 4.1). Number of mothers' physician visits in the year prior to pregnancy was included to control for differences in healthcare access. Prenatal smoking, alcohol and drug use were explored for inclusion and were obtained from the BabyFirst - Families First Screen (Supplementary Table 1). A large amount of missing data was observed for these variables; accordingly, they were included in a sensitivity analysis in a subset of the cohort who had these variables available.

4.4.5. Statistical analysis

Antibiotic-exposed and unexposed children were matched by a one-to-one nearest neighbor greedy match on sex, birthdate (within 365 days) and propensity scores within a caliper of 0.05. Balance across the exposure groups was examined by calculating the standardized difference for the pre-identified variables in addition to clinically important maternal and childhood medical conditions (Supplementary Table 3).[22,24,42,43] Within the matched cohort, a Cox proportional hazards regression model, stratified by the matched pair, was used to compare the risk of ADHD among those exposed and unexposed to antibiotics. For secondary analyses, based on the number of antibiotic courses, cumulative duration and the class of antibiotic, Bonferroni-adjusted confidence intervals and alpha level were estimated to correct for inflation due to multiple comparisons. Proportional hazards assumption was tested using Kolmogorov-type supremum test and a time interaction was included when the assumption was violated.

Within the sibling cohort, a multivariable Cox proportional hazards regression model was used and was stratified by the mothers to account for correlation among sibling pairs. The model was adjusted for sex, mothers' age at delivery (less than 30, 30 to 39, and 40 years or greater), size for gestational age, preterm delivery, mode of delivery (vaginal or caesarian-section), breastfeeding initiation, birth order (firstborn or subsequent), year of birth, birth complications, prenatal infections, prenatal antidepressants exposure in addition to relevant childhood medical conditions (infection, epilepsy, asthma, neonatal jaundice and a diagnosis with other developmental disabilities). The statistical software SAS® 9.4 (SAS Institute; Cary, NC) was used for all data analyses.

4.4.6. Sensitivity analyses

Multiple sensitivity analyses were planned and conducted in matched cohorts. First, to address potential misclassification of the outcome, we applied a stricter identification algorithm requiring one hospitalization, two physician claims within three years, or two or more prescriptions for ADHD drugs in a year plus one physician claim within three years. Second, a minimum age of 6 years was required to identify ADHD based on the guideline recommendation by the Canadian ADHD Resource Alliance.[44] In another sensitivity analysis, for those who received an ADHD diagnosis after the age of 4, we stopped follow up at the first date of ADHD diagnosis even if it occurred before the age of 4. Additional sensitivity analyses were done by modifying antibiotic exposure window to six months and two years of life, starting follow up at the time of exposure for the exposed and their matched subjects, including in-hospital antibiotic use and adjusting for prenatal smoking, alcohol and drug use in a subset of the cohort who had these variables available.

4.5. Results

4.5.1. Description of study population

There was a total of 289 449 births in Manitoba between the fiscal years 1998 and 2017. Of those, 187 605 children met the inclusion criteria (Figure 4.1); 51.2% of the children included in the cohort were males, and 54.2% were living in urban regions; 84 424 (45.0%) children received at least one antibiotic dispensation during the first year of life. Among those, 75.1% received one or two courses, 55.2% received antibiotics for less than or equal to 2 weeks, and 58.2% received a penicillin antibiotic (Supplementary Table 4). Several baseline characteristics were imbalanced between the two exposure groups such as receipt of income assistance, prenatal smoking, and some childhood medical conditions (Tables 1 and 2). Children were followed for a total of 1 990 452 person-years with a median of 10.1 person-years (interquartile range 6.8, 14.3). During follow-up, 16 290 (8.7%) children received an ADHD diagnosis at a mean age (standard deviation) of 8.05 (2.88) years. The crude incidences for ADHD diagnosis were 9.8 cases per 1000 person-years and 6.7 cases per 1000 person-years in children exposed and unexposed to antibiotics in the first year of life, respectively.

4.5.2. Description of the matched cohort

Of the study cohort, 69 738 children were matched on HDPS, sex, and birthdate. Baseline characteristics were well balanced between the two exposure groups with standardized differences of less than 0.1 (Tables 1 and 2). Children in the matched cohort were followed for a total of 740 715 person-years with a median of 10.1 person-years (interquartile range 6.9, 14.3). During follow-up, 6087 (8.7%) children received an ADHD diagnosis at a mean age (standard deviation) of 8.0 (2.87) years. The crude incidences for ADHD diagnosis were 8.2 cases per 1000

person-years and 8.2 cases per 1000 person-years in children exposed and unexposed to antibiotics in the first year of life, respectively.

4.5.3. Cox regression models in the overall and matched cohorts

In the overall cohort, antibiotic exposure was associated with ADHD diagnosis (unadjusted HR 1.62, 95% CI 1.48-1.78). In the matched cohort, ADHD risk was not found to be associated with antibiotic exposure (HR 1.02, 95% CI 0.97-1.08). However, in secondary analyses, antibiotic exposure was found to be associated with ADHD diagnosis in those receiving at least 4 antibiotic courses or for a duration longer than 3 weeks (HR 1.57, 95% CI 1.23-2.00 and HR 1.38, 95% CI 1.17-1.64, respectively). Examining the association based on antibiotic class showed an increased ADHD risk with all antibiotic classes but was highest with non-penicillin β-lactams (Anatomical Therapeutic Chemical J01D) with HR 1.23, 95% CI 1.07-1.42 (Table 3).

4.5.4. Sensitivity analyses

No major shifts in risk estimates were observed in most of the planned sensitivity analyses (Figure 4.2). However, ADHD risk estimates appeared to be sensitive to changing the exposure-time window of antibiotics, with HR 1.08, 95% CI 1.03-1.14 and HR 1.22, 95% CI 1.16-1.28) when exposure was varied to the first 6 months or 2 years of life.

4.5.5. Sibling-controlled analyses

The sibling cohort included 67 671 children and 46 967 exposure discordant pairs (Figure 4.1). Baseline characteristics of the sibling cohort are described in Supplementary Table 5. Of this cohort, 33 374 (49.3%) children were exposed to antibiotics in the first year of life. Description of antibiotics use in the sibling cohort is summarized in Supplementary Table 4. During a follow

up of 729 540 person-years, 6021 children developed ADHD. The crude incidences for ADHD diagnosis were 8.8 cases per 1000 person-years and 7.7 cases per 1000 person-years in children exposed and unexposed to antibiotics in the first year of life, respectively. Early childhood antibiotic exposure was not associated with the risk of ADHD in the sibling cohort (adjusted HR 0.96, 95% CI 0.89-1.03). No substantial variation in the risk association was observed in all secondary analyses (Table 3). Despite a slight imprecision, a small increase in ADHD risk was observed with exposure to non-penicillin beta-lactams (HR = 1.14, 95% CI: 1.00, 1.31).

4.6. Discussion

This was a population-based cohort study consisting of all births occurring in Manitoba that examined the association between early childhood antibiotic exposure and the risk of ADHD. In the unadjusted model based on the overall population, antibiotic exposure in the first year of life was associated with ADHD risk. In the HDPS-matched cohort, no increased risk of ADHD was observed in children exposed to antibiotics in their first year of life. In secondary analyses of the matched cohort, ADHD risk increase was observed in those exposed to 4 or more antibiotic courses or for a duration longer than 3 weeks. Stratifying the analysis based on antibiotic class resulted in a risk increase in all antibiotic classes. Several sensitivity analyses that modified various exposure and outcome parameters affirmed the robustness of the risk estimates in the main model. However, the association appeared to be susceptible to changes in antibiotic exposure window.

Since the main model could not account for all confounding sources, we explored the association using exposure-discordant sibling cohort design to address environmental, genetic and other shared familial factors. Within the sibling cohort, antibiotic use in the first year of life

was not associated with the risk of developing ADHD, which confirms findings from the main analysis of the HDPS-matched cohort. No association was observed in all secondary analyses based on the number of antibiotic courses, cumulative duration or class of antibiotic with the exception of a small increase in ADHD risk with exposure to non-penicillin β-lactams.

The disagreement in secondary analyses findings between the models based on the HDPS-matched cohort and the sibling cohort suggested susceptibility of the former to confounding by genetics and other shared familial factors that cannot be identified in administrative databases. This indicates that the association between early life antibiotic exposure and ADHD observed in the secondary analyses of the matched cohort was likely confounded by unobserved factors. This led us to conclude that antibiotic exposure in the first year of life is not associated with ADHD risk.

Our study has several strengths. We utilized 19 years of population-based data, which allowed for a large sample size and a long follow-up period. Thus, the results are generalizable to all children residing in Manitoba. Additionally, we used an established algorithm to identify ADHD from administrative databases. Most importantly, we used a HDPS-matching and a sibling-controlled design to minimize confounding. The Manitoba Repository provides a rich source of information to estimate HDPS and to make the exposure groups similar in regard to baseline characteristics, which reduces the sources of unmeasured confounding. The Repository also allows linking of siblings through the mother's scrambled Personal Health Identification Number enabling a sibling-controlled design, which reduces confounding due to genetics or shared environmental factors.

Despite the mentioned strengths, a few limitations were identified. First, antibiotic dispensation was used as a proxy for actual use potentially resulting in misclassification of the exposure. We also did not include hospital-antibiotic exposure in the main analysis due to concerns about the quality of the available data, which is limited in data years and in geographic coverage. We did, however, include in-hospital dispensations in one sensitivity analysis and the risk estimates remained robust. Second, non-differential misclassification of the outcome is a potential limitation since the utilized ADHD identification algorithm has not been independently validated. However, in a sensitivity analysis, we used a stricter algorithm to identify ADHD and the risk estimates were similar. Additionally, although we attempted to control for confounding by HDPS-matching and by utilizing a sibling cohort, there was still a potential for unmeasured confounding from variables that could not be obtained from the Repository and are not shared by sibling pairs. For example, the sibling cohort does not control for all genetic confounding since it included both half-siblings and full siblings, which accounts for 25% to 50%, at most, of the genetic component of the disorder. Finally, little is known about the consequences of antibiotic-induced microbiota changes; accordingly, the exposure time-window for an association with ADHD is unknown. In two sensitivity analyses, exposure within the first 6 months or 2 years of life appeared to have an effect on changing the ADHD risk estimates. This finding warrants future studies examining ADHD risk with antibiotic exposure at other stages of childhood.

4.7. Conclusion

Our findings suggest that antibiotic use during the first year of life is not associated with the development of ADHD. The association observed in secondary analyses of the HDPS-matched

cohort appears to be overestimated by shared familial factors. Given that the exposure time-window for the association between antibiotic-induced microbiota changes with ADHD is not well defined, future studies are warranted to examine antibiotic exposure at other stages of childhood. Furthermore, the risk of childhood diseases resulting from antibiotic-induced microbiota changes is yet to be substantiated and would benefit from a further examination at the biological level.

4.8. Acknowledgment

We thank Heather Prior and Matthew Dahl from the Manitoba Centre for Health policy for their valuable support. The authors acknowledge the Manitoba Centre for Health Policy for use of data contained in the Manitoba Population Research Data Repository under project #H2016:244 (HIPC# 2016/2017 – 11). The results and conclusions are those of the authors and no official endorsement by the Manitoba Centre for Health Policy, Manitoba Health, Healthy Living and Senior or other data providers is intended or should be inferred. Data used in this study are from the Manitoba Population Research Data Repository housed at the Manitoba Centre for Health Policy, University of Manitoba and were derived from data provided by Manitoba Health Healthy Living and Senior, Winnipeg Regional Health Authority, Manitoba Department of Families and Healthy Child Manitoba.

4.9. Figures and tables

Table 4.1. Sociodemographic and birth characteristics of the overall and matched cohorts

Characteristic	Overall cohort N=187 605			Matched cohort N = 69 738		
	Number (%)		Standardized difference	Number (%)		Standardized difference
	Antibiotic exposed N=84 424	Unexposed N=103 181		Antibiotic exposed N=34 869	Unexposed N=34 869	
Male	45 578 (54.0)	50 488 (48.9)	0.101	18 152 (52.1)	18 152 (52.1)	0.000
Urban region	46 632 (55.2)	55 091 (53.4)	0.037	18 966 (54.4)	19 046 (54.6)	0.005
Socioeconomic status (SES) [a]			0.137			0.086
High	6994 (8.3)	11 219 (10.9)		3060 (8.8)	3972 (11.4)	
Middle	28 669 (34.0)	38 463 (37.3)		12 719 (36.3)	12 716 (36.5)	
Low-mid	28 100 (33.3)	30 510 (29.6)		11 681 (33.5)	10 301 (29.5)	
Low	20 661 (24.5)	22 989 (22.3)		7409 (21.3)	7880 (22.6)	
Receiving income assistance [b]	23 870 (28.3)	15 285 (14.8)	0.332	7550 (21.7)	6954 (19.9)	0.042
Maternal age at delivery [c]	27.8 (5.9)	28.7 (5.9)	0.15	28.2 (5.8)	28.3 (5.9)	0.020
Breastfeeding initiation [d]	65 962 (78.5)	84 208 (81.9)	0.087	28 035 (80.4)	28 314 (81.2)	0.020
Preterm delivery [e]	6295 (7.5)	7422 (7.2)	0.010	2616 (7.5)	2570 (7.4)	0.005
Caesarian delivery	16 918 (20.0)	20 949 (20.3)	0.007	7023 (20.1)	7161 (20.5)	0.010
Firstborn child	28 493 (33.8)	42 098 (40.8)	0.146	12 819 (36.8)	12 896 (37.0)	0.005
Small for gestational age [f]	6286 (7.5)	8033 (7.8)	0.013	2605 (7.5)	2684 (7.7)	0.009
Birth complications	8804 (10.4)	10 089 (9.8)	0.022	3552 (10.2)	3480 (10.0)	0.007

Values are numbers (percentages) unless stated otherwise. Percentages are calculated based on non-missing data

[a] Based on the socioeconomic factor index, a neighborhood level measure based on Canada census, was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Values are expressed in mean (Standard deviation) in years

[d] Missing data for 789 children (0.4%) of the overall cohort

[e] Defined as gestational age less than 37 weeks. Missing data for 193 children (0.1%) of the overall cohort

[f] Defined as having birth weight below the 10th percentile for the gestational age and sex. Missing data for 264 children (0.1%) of the overall cohort

Table 4.2. Medical conditions and prenatal exposures of the overall and matched cohorts

Characteristic	Overall cohort N=187 605			Matched cohort N = 69 738		
	Number (%)		Standardized difference	Number (%)		Standardized difference
	Antibiotic exposed N=84 424	Unexposed N=103 181		Antibiotic exposed N=34 869	Unexposed N=34 869	
Maternal medical conditions and prenatal exposures						
Mood and anxiety disorders	17 408 (20.6)	16 176 (15.7)	0.129	6422 (18.4)	6248 (17.9)	0.013
Schizophrenia	159 (0.2)	159 (0.2)	0.008	57 (0.2)	68 (0.2)	0.007
Asthma	7628 (9.0)	6328 (6.1)	0.110	2742 (7.9)	2712 (7.8)	0.003
Developmental disabilities	2137 (2.5)	1886 (1.8)	0.048	797 (2.3)	763 (2.2)	0.007
Prenatal infections	32 549 (38.6)	27 191 (26.4)	0.264	11 643 (33.4)	11 386 (32.7)	0.016
Diabetes Mellitus	2813 (3.3)	3500 (3.4)	0.003	1109 (3.2)	1174 (3.4)	0.010
Prenatal smoking [a]	13 012 (23.2)	11 085 (15.8)	0.189	4897 (20.0)	4360 (18.7)	0.032
Prenatal alcohol/drug use [b]	7758 (14.1)	8307 (12.0)	0.062	3126 (13.0)	2910 (12.7)	0.009
Childhood medical conditions						
Asthma	19 337 (22.9)	4647 (4.5)	0.555	4717 (13.5)	3772 (10.8)	0.083
Epilepsy	553 (0.7)	366 (0.4)	0.042	156 (0.5)	194 (0.6)	0.015
Neonatal jaundice	8551 (10.1)	9329 (9.0)	0.037	3295 (9.5)	3505 (10.0)	0.020
Infections	77 906 (92.3)	53 343 (51.7)	1.013	28 990 (83.1)	27 845 (79.9)	0.085
Other developmental disabilities	384 (0.5)	250 (0.2)	0.036	135 (0.4)	102 (0.3)	0.016

Values are numbers (percentages) unless stated otherwise. Percentages are calculated based on non-missing data
[a] Missing data for 61 390 (32.7%) children in the overall cohort and for 21 996 (31.5%) children in the matched cohort
[b] Missing data for 63 238 (33.7%) children in the overall cohort and for 22 752 (32.6%) children in the matched cohort

Table 4.3. Association between antibiotic use in the first year of life and the risk of attention deficit/hyperactivity disorder

Variable	Matched cohort N = 69 738		Sibling cohort N = 67 671		
	Number of events	**HR (95% CI)**	**Number of events**	**HR (95% CI)**	**Adjusted [a] HR (95% CI)**
Main analysis					
Antibiotic exposure	6087	1.02 (0.97-1.08)	6021	1.03 (0.97-1.10)	0.96 (0.89-1.03)
Secondary analyses [b]					
Antibiotic class [c]					
None	3042	1.00 [Reference]	2783	1.00 [Reference]	1.00 [Reference]
Penicillin antibiotics	2432	1.11 (1.03-1.19)	2697	1.11 (1.03-1.21)	1.03 (0.94-1.14)
Macrolides and related antibiotics	730	1.18 (1.04-1.34)	903	1.18 (1.05-1.32)	1.08 (0.95-1.23)
Non-penicillin β-lactams	567	1.23 (1.07-1.42)	723	1.26 (1.11-1.43)	1.14 (1.00-1.31)
Others	305	1.22 (1.01-1.48)	377	1.19 (1.01-1.41)	1.12 (0.94-1.33)
Number of antibiotic courses					
0	3042	1.00 [Reference]	2783	1.00 [Reference]	1.00 [Reference]
1	1914	0.94 (0.87-1.02)	1565	0.98 (0.89-1.09)	0.94 (0.84-1.05)
2	621	1.09 (0.94-1.27)	792	1.02 (0.89-1.18)	0.93 (0.80-1.09)
3	202	1.09 (0.83-1.43)	430	1.20 (0.99-1.44)	1.10 (0.90-1.34)
>= 4	308	1.57 (1.23-2.00)	451	1.10 (0.92-1.33)	0.97 (0.79-1.19)
Cumulative antibiotic duration (days)					
0	3042	1.00 [Reference]	2783	1.00 [Reference]	1.00 [Reference]
1-7	733	0.92 (0.81-1.06)	622	0.98 (0.84-1.14)	0.95 (0.80-1.11)
8 – 14	1261	0.94 (0.85-1.04)	1072	0.99 (0.88-1.11)	0.92 (0.81-1.05)
15-21	470	1.10 (0.92-1.32)	611	1.04 (0.89-1.21)	0.96 (0.81-1.14)
>21	581	1.38 (1.17-1.64)	933	1.13 (0.99-1.30)	1.03 (0.88-1.20)

[a] Adjusted for sex, breastfeeding initiation, mode of delivery, preterm delivery, size for gestational age, mothers' age at delivery, birth complications, birth order, year of birth, childhood medical conditions (asthma, epilepsy, infections, neonatal jaundice and a diagnosis with other developmental disorder), prenatal infections and prenatal antidepressants exposure.

[b] Bonferroni adjusted alpha = 0.0125

[c] A child can be exposed to multiple classes and therefore a member of multiple categories

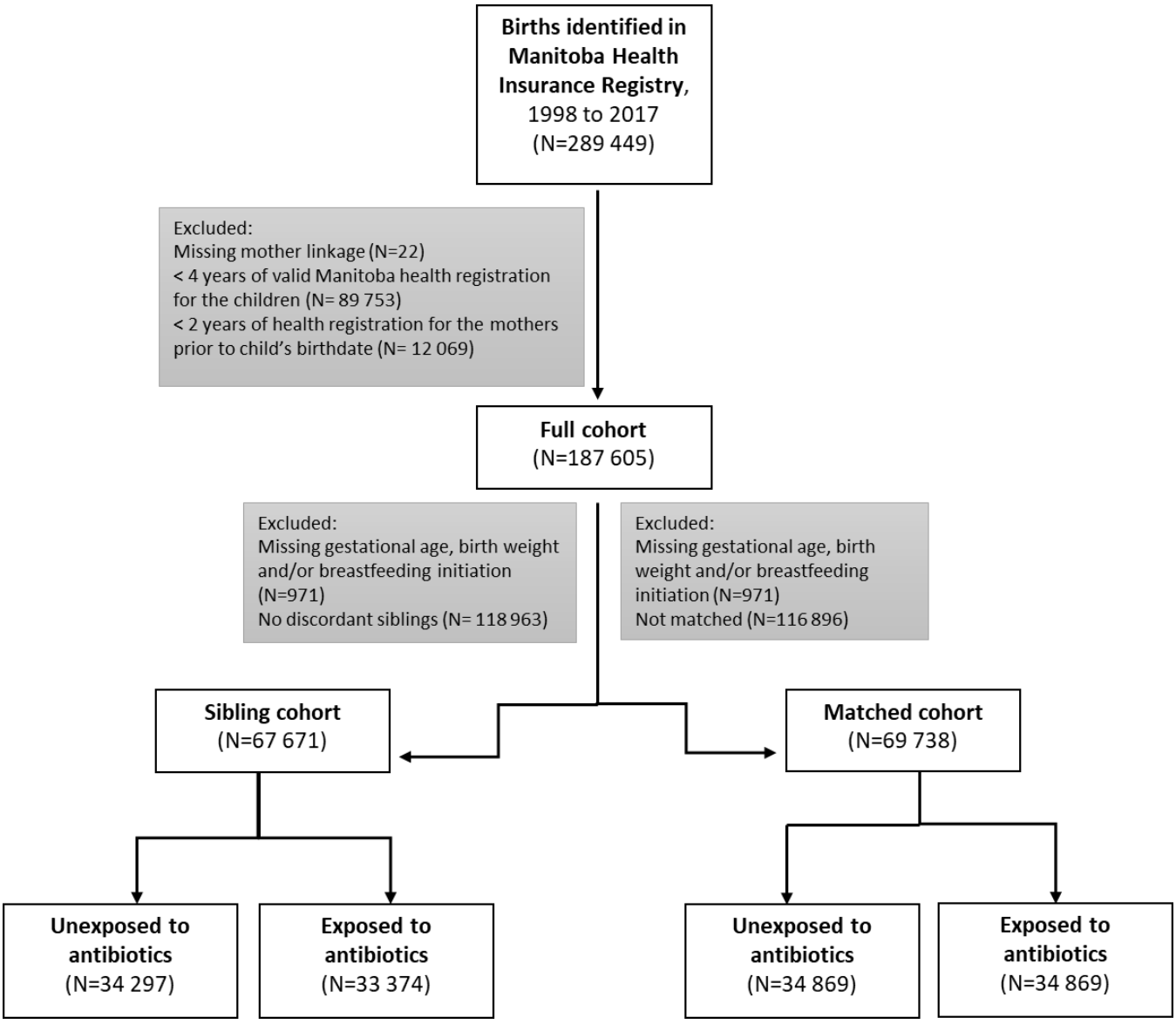

Figure 4.1. Study populations: overall, matched and sibling cohort

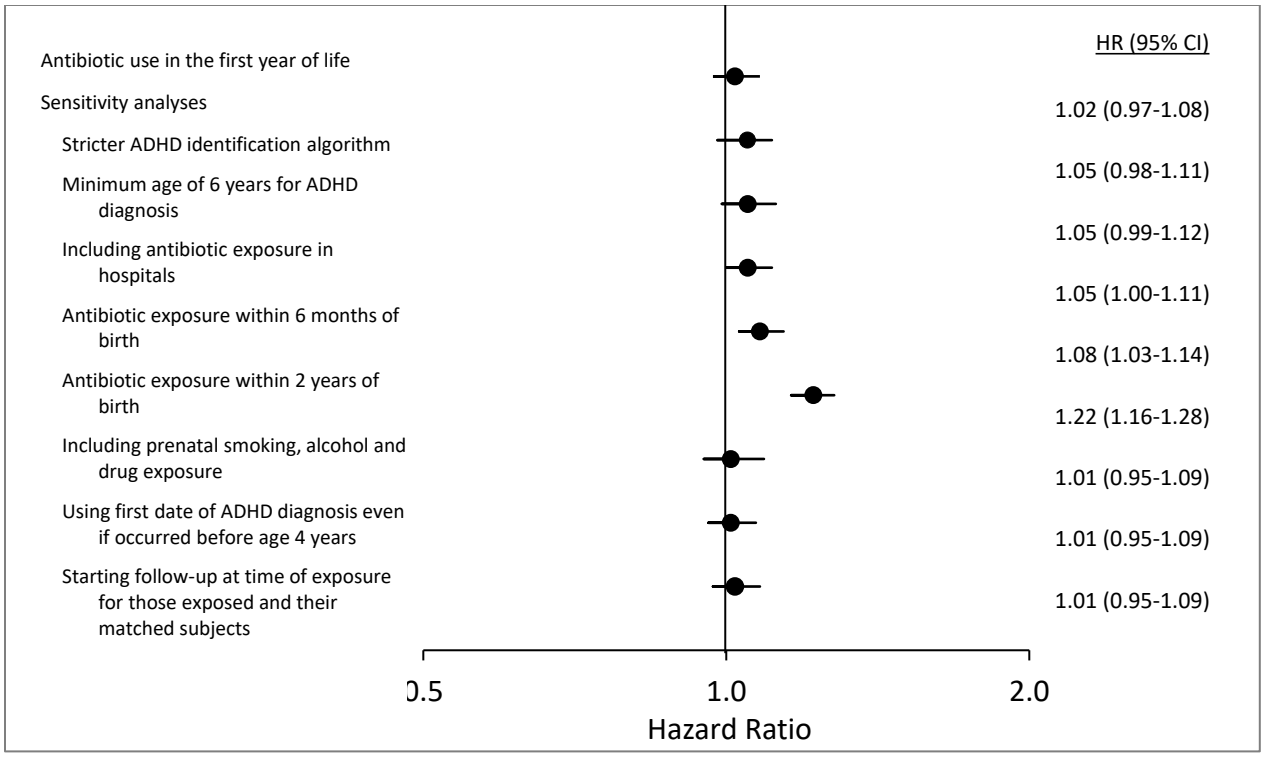

Figure 4.2. Risk estimates for the planned sensitivity analyses

Supplementary Table 4.1. Description of data sources

Database	Description	Data years
The Manitoba Health Insurance Registry	A population-based registry that collects demographic and insurance coverage information of Manitoba residents and is maintained by Manitoba Health, Seniors and Active Living.	1970/71 – 2016/17
Drug Program Information Network	An electronic drug system that captures all prescriptions dispensed from community pharmacies to residents and is maintained by Manitoba Health, Seniors and Active Living. It includes prescription information such as generic name, Anatomical Therapeutic Chemical code, date of dispensation, strength and days supplied.	1995/96 – 2016/17
In-hospital Pharmaceuticals	An inpatient pharmacy system that provides dispensing information on pharmaceutical use at three Winnipeg hospitals: Health Sciences Centre, Concordia Hospital and Riverview Health Centre.	1999 – 2012
Hospital discharge abstracts	Includes records of all patients' hospital admissions with summaries for demographic data such as gender and postal code, and clinical data including up to 25 diagnosis codes and 20 procedure codes.	1970/71 – 2016/17
Medical Services database	Physician claims in the Medical Services database include records of claims for any physician visits in offices, hospitals and outpatient departments in Manitoba in addition to some information on Manitobans' physician visits outside the province. Claims are submitted by physicians electronically for service reimbursement by Manitoba Health, Seniors and Active Living.	1970/71 – 2016/17
The Hospital Newborn to Mother Link	Serves to match the baby's birth hospital record with the mother's obstetrical delivery record and contains basic demographic and hospital data on newborns and their mothers for in-hospital births.	1984/85 – 2016/17
BabyFirst - Families First Screen	Collected by Public Health Nurses on nearly all families with newborns in Manitoba and maintained by Heathy Child Manitoba. Contains records of newborns identifying biological, social, and demographic risk factors including prenatal smoking, alcohol and drug use.	2000 – 2016
The Social Allowances Management Information Network	Provides information concerning employment and income assistance received by Manitoba residents.	1995/96 – 2016/17
Canada Census	A population survey with aggregate demographic information for all persons within a dissemination area in Canada and is conducted by Statistics Canada every five years.	1971 – 2016

Supplementary Table 4.2. Antibiotics classification

Class	Generic name	Anatomical Therapeutic Chemical
Penicillin antibiotics	Amoxicillin, ampicillin, bacampicillin, penicillin, piperacillin, pivampicillin, pivmecillinam, cloxacillin, floxacillin, nafcillin, ticarcillin, tazobactam	J01C
Non-penicillin β-lactams	Cefazolin, cefepime, cefixime, cefotaxime, cefotetan, cefoxitin, cefpodoxime, cefprozil, ceftazidime, ceftizoxime, ceftobiprole, ceftriaxone, cefuroxime, cephalexin, cephalothin, aztreonam, cefaclor, cefadroxil, cefamandole, doripenem, ertapenem, meropenem	J01D
Macrolides and related antibiotics	Dalfopristin, telithromycin, azithromycin, clarithromycin, clindamycin, erythromycin, lincomycin	J01F, J01R
Other antibiotics	Minocycline, doxycycline, demeclocycline, rolitetracycline, tetracycline, ciprofloxacin, gatifloxacin, nalidixic acid, gemifloxacin, grepafloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin, trovafloxacin, methenamine, fosfomycin, tedizolid, nitrofurantoin, metronidazole, linezolid, fusidate, fusidic acid, bacitracin, vancomycin, colistin, polymyxin B, telavancin, tobramycin, gentamicin, daptomycin, amikacin, spectinomycin, neomycin, netilmicin, spiramycin, streptomycin, chloramphenicol, trimethoprim, sulfamethoxazole, sulfadiazine, sulfapyridine, sulfisoxazole	J01A, J01B, J01G, J01E, J01M, J01X

Supplementary Table 4.3. Identification algorithms of medical conditions explored for balance between exposure groups

Covariate	Period	Diagnostic Criteria
Maternal medical conditions		
Mood and anxiety disorders	Within 3 years before index date	✓ One or more hospitalizations with a diagnosis for depressive disorder, affective psychoses, neurotic depression or adjustment reaction: ICD-9-CM codes 296.1-296.8, 300.4, 309 or 311; ICD-10-CA codes F31, F32, F33, F34.1, F38.0, F38.1, F41.2, F43.1, F43.2, F43.8, F53.0, F93.0 or with a diagnosis for an anxiety state, phobic disorders or obsessive-compulsive disorders: ICD-9-CM codes 300.0, 300.2, 300.3, 300.7; ICD-10-CA codes F40, F41.0, F41.1, F41.3, F41.8, F41.9, F42, F45.2, OR ✓ One or more hospitalizations with a diagnosis for anxiety disorders: ICD-9-CM code 300; ICD-10-CA codes F32, F34.1, F40, F41, F42, F44, F45.0, F45.1, F45.2, F48, F68.0, or F99 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ One or more physician visits with a diagnosis for depressive disorder or affective psychoses: ICD-9-CM codes 296, 311, OR ✓ One or more physician visits with a diagnosis for anxiety disorders: ICD-9-CM code 300 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ Three or more physician visits with a diagnosis for anxiety disorders or adjustment reaction: ICD-9-CM code 300, 309.
Schizophrenia	Within 3 years before index date	✓ One or more hospitalization with a diagnosis for schizophrenia: ICD-9-CM code 295; ICD-10-CA codes F20, F21, F23.2, F25, OR ✓ One or more physician visits with a diagnosis for schizophrenia: ICD-9-CM code 295.
Developmental disability	Any time before index date	✓ One or more hospitalizations with diagnoses for autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD) or intellectual disabilities: ICD-9-CM codes 299, 314, 317, 318, 319; ICD-10-CA: F70, F71, F72, F73, F78, F79, F84, F90. ✓ One or more physician visit with diagnoses for ASD, ADHD or intellectual disabilities: ICD-9-CM: 299, 314, 317, 318, 319
Prenatal infection	During pregnancy	A hospitalization or physician visit with any of the following codes: ✓ Eye and ear infections: ICD-9-CM codes 370*, 372*, 380*, 381*, 382; ICD-10-CA codes H10, H13.1, H16, H19.1, H19.2, H60, H62, H65, H66, H67 ✓ Upper respiratory tract infection: ICD-9-CM codes 460–465; ICD-10-CA codes J00-J06 ✓ Lower respiratory tract infection: ICD-9-CM codes 466, 481–488; ICD-10-CA codes J09- J18, J20-J22 ✓ Genitourinary system infection: ICD-9-CM codes 590, 595, 597, 599*, 601, 604, 614-617, 771*; ICD-10-CA codes N10-N12, N30, N33.0, N33.8, N37.0, N39.0, N41, N45, N70-N77 ✓ Central nervous system infections: ICD-9-CM codes 320, 321, 323, 324, 728*; ICD-10-CA codes G00-G02, G04-G07 ✓ Skin infections: ICD-9-CM codes 680-686; ICD-10-CA codes L00- L08, M72.6 ✓

Covariate	Period	Diagnostic Criteria
Prenatal infection, continued	During pregnancy	✓ Cardiovascular system infections: ICD-9-CM codes 391, 420, 421, 422, 424*; ICD-10-CA codes I01, I30, I32.0, I32.1, I33, I38, I39.8, I40, I41.0, I41.1, I41.2 ✓ Musculoskeletal system infections: ICD-9-CM codes 711, 730; ICD-10-CA codes M00- M01, M03, M46.2, M86 ✓ Bacteremia/Septicemia: ICD-9-CM codes 771*, 790*, 995*; ICD-10-CA codes P36, A22.7, A26.7, A02.1, A32.7, A02.1, A32.7, A40, A41, A42.7, B37.7 ✓ Gastrointestinal system infections: ICD-9-CM codes 535, 540, 541, 542, 566, 567, 530*, 572*, 575*; ICD-10-CA codes K20, K29.0, K29.1, K29.8, K35, K61, K65, K67, K81.0 ✓ Other Parasitic and infectious diseases: ICD-9-CM codes 001-139; ICD-10-CA codes A00-B99
Diabetes mellitus	Within 1 year before index date	✓ One or more hospitalizations with a diabetes diagnosis: ICD-9-CM code 250, 648.0, 648.8; ICD-10-CA codes E10-E14, O24, OR ✓ Two or more physician visits with a diabetes diagnosis: ICD-9-CM code 250, OR ✓ One or more prescriptions for a diabetes medication (ATC: A10A, A10B).
Asthma	Within 1 year before index date	✓ One or more hospitalizations with a diagnosis of asthma: ICD-9-CM codes 493; ICD-10-CA code J45, OR ✓ One or more physician visits with a diagnosis of asthma: ICD-9-CM codes 493, OR ✓ One or more prescriptions for asthma medication (ATC: R03, R06AX17)
Childhood medical conditions:		
Birth complications	At birth	Include vacuum, forceps or breech procedures: ICD-9-CM procedure codes 72, 73.3, 763.2, 652.2, 669.6; CCI codes 5MD53, 5MD54, 5MD55, 5MD56, shoulder dystocia: 660.4, placental abruption: 641.2, cord prolapse: 663, uterine rupture: 665.1 or other perinatal complications: 760-779.
Epilepsy	Within the first year of life	✓ One or more hospitalization for seizure disorder: ICD-9-CM codes 345, 649.4; ICD-10-CA codes G40, G41, OR ✓ One or more physician visits for seizure disorder: ICD-9-CM code 345, OR ✓ One or more prescription for an anticonvulsant medication (ATC: N03A)
Asthma	Within the first year of life	✓ One or more hospitalizations with a diagnosis of asthma: ICD-9-CM codes 493; ICD-10-CA code J45, OR ✓ One or more physician visits with a diagnosis of asthma: ICD-9-CM codes 493, OR ✓ One or more prescriptions for asthma medication (ATC: R03, R06AX17)
Other developmental disabilities	Within the first year of life	✓ One or more hospitalizations with diagnoses for ASD, intellectual disabilities, Down's syndrome, autosomal deletion syndromes, Prader-Willi syndrome, other specified congenital anomalies, or fetal alcohol syndrome: ICD-9-CM codes 299, 317, 318, 319, 758.0- 758.3, 759.8, 760.71; ICD-10-CA: F70, F71, F72, F73, F78, F79, F84, P04.3, Q86, Q87, Q89.8, Q90, Q91, Q93, Q99.2. ✓ One or more physician visit with diagnoses for ASD or intellectual disabilities: ICD-9-CM: 299, 317, 318, 319
Neonatal jaundice	Within the first 4 weeks of life	✓ One or more hospitalizations with diagnoses for Jaundice: ICD-9-CM code 774; ICD–10–CA codes P58, P59, OR ✓ One or more physician visit with diagnoses for Jaundice: ICD-9-CM code 774
Infection	Within the first year of life	Similar to maternal definition

Supplementary Table 4.4. Description of Antibiotic Use in the Overall, Matched and Sibling Cohorts

	Number (%)		
	Overall cohort N = 187 605	**Matched cohort N = 69 738**	**Sibling cohort N = 67 671**
Antibiotic class			
Penicillin antibiotics	70 028 (58.2)	26 875 (62.9)	26 905 (59.8)
Macrolides and related antibiotics	22 766 (18.9)	7130 (16.7)	8244 (18.3)
Non-penicillin β-lactams	18 607 (15.5)	6101 (14.3)	7001 (15.6)
Others	9000 (7.5)	2628 (6.1)	2871 (6.4)
Total	120 401 (100)	42 734 (100)	45 021 (100)
Number of courses			
1	42 761 (50.7)	24 714 (70.9)	19 172 (57.4)
2	20 603 (24.4)	6560 (18.8)	7820 (23.4)
3	10 454 (12.4)	1913 (5.5)	3449 (10.3)
>=4	10 606 (12.6)	1682 (4.8)	2933 (8.8)
Total	84 424 (100)	34 869 (100)	33 374 (100)
Cumulative duration (days)			
1-7	16 777 (19.87)	10 145 (29.1)	7819 (23.4)
8-14	29 790 (35.29)	15 766 (45.2)	12 852 (38.5)
15-21	15 834 (18.76)	4852 (13.9)	5987 (17.9)
>21	22 023 (26.09)	4106 (11.8)	6716 (20.1)
Total	84 424 (100)	34 869 (100)	33 374 (100)

Supplementary Table 4.5. Baseline Characteristics of the Sibling Cohort

Characteristic	**Number (%)**		
	All subjects N=67 671	**Antibiotic exposure in the first year of life**	
		No N= 34 297	**Yes N=33 374**
Male	34 612 (51.2)	16 416 (47.9)	18 196 (54.5)
Urban region	31 923 (47.2)	15 622 (45.6)	16 301 (48.8)
Socioeconomic status (SES) [a]			
High	5529 (8.2)	2803 (8.2)	2726 (8.2)
Middle	22 561 (33.3)	11 487 (33.5)	11 074 (33.2)
Low-mid	20 460 (30.2)	10 142 (29.6)	10 318 (30.9)
Low	19 121 (28.3)	9865 (28.8)	9256 (27.7)
Mothers age at delivery [b]	27.62 (5.62)	27.84 (5.57)	27.39 (5.66)
Breastfeeding initiation	52 473 (77.5)	26 491 (77.2)	25 982 (77.9)
Preterm delivery [c]	5036 (7.4)	2514 (7.3)	2522 (7.6)
Small for gestational age [d]	4749 (7.0)	2443 (7.1)	2306 (6.9)
Caesarian section	11 892 (17.6)	6019 (17.6)	5873 (17.6)
Birth complications	5237 (7.7)	2611 (7.6)	2626 (7.9)
First born child	19 156 (28.3)	9452 (27.6)	9704 (29.1)
Prenatal alcohol/drug use [e]	5495 (12.5)	2634 (12.0)	2861 (12.9)
Prenatal smoking [f]	9181 (20.5)	4439 (19.9)	4742 (21.1)
Childhood medical conditions:			
Infections	50 144 (74.1)	20 046 (58.4)	30 098 (90.2)
Epilepsy	339 (0.5)	128 (0.4)	211 (0.6)
Neonatal jaundice	6313 (9.3)	3153 (9.2)	3160 (9.5)
Other developmental disabilities	267 (0.4)	94 (0.3)	173 (0.5)
Asthma	9049 (13.4)	2033 (5.9)	7016 (21.0)
Prenatal infections	21 877 (32.3)	10 256 (29.9)	11 621 (34.8)
Prenatal antidepressants exposure	1631 (2.4)	784 (2.3)	847 (2.5)
Year of birth:			
1998-2001	14 248 (21.1)	6523 (19.0)	7725 (23.2)
2002-2005	19 569 (28.9)	9684 (28.2)	9885 (29.6)
2006-2009	20 923 (30.9)	11 052 (32.2)	9885 (29.6)
2010-2013	12 923 (19.1)	7038 (20.5)	5885 (17.6)

Values are numbers (percentages) unless stated otherwise. Percentages are calculated based on non-missing data

[a] Based on the socioeconomic factor index, a neighborhood level measure based on Canada census, was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Values expressed in mean (Standard deviation) in years

[c] Defined as gestation age less than 37 weeks

[d] Defined as having birth weight below the 10th percentile for the gestational age and sex.

[e] Missing data for 23 638 (34.9%) subjects

[f] Missing data for 22 891 (33.8%) subjects

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Chapter 5: Prenatal Antibiotic Exposure and the Risk of Attention Deficit/Hyperactivity Disorder: A Population-Based Cohort Study

5.1. Overview

This manuscript is the second of two manuscripts that examine the association of early life antibiotic exposure and ADHD risk. This chapter addresses the fourth project objective, which assesses the association of antibiotics exposure during pregnancy and the risk of developing ADHD. The manuscript is based on a population-based cohort study of children born in Manitoba between the fiscal years 1998 and 2017 and utilized administrative health data from the Manitoba Population Research Data Repository. Cox proportional hazards regression models were used to estimate the risk of developing ADHD in the overall population, a high dimensional propensity scores-matched cohort and in a cohort of exposure-discordant siblings. The high dimensional propensity scores-matched cohort was added to the protocol of objectives 3 and 4 as an additional method to account for unmeasured confounding and to compliment the results of the sibling-design. Study findings suggested no clinically significant association between prenatal antibiotic exposure and ADHD risk.

Citation: Hamad AF, Alessi-Severini S, Mahmud S, Brownell M and Kuo I. Prenatal Antibiotic Exposure and the Risk of Attention Deficit/Hyperactivity Disorder: A Population-Based Cohort Study. *CMAJ (submitted)* 2019

5.2. Abstract

Background: Dysbiosis induced by prenatal antibiotic exposure is a potential contributor to the development of attention-deficit/hyperactivity disorder (ADHD). We examined the association between prenatal antibiotic exposure and ADHD risk.

Methods: This was a population-based cohort study of children born in Manitoba, Canada between 1998 and 2017 and their mothers. Exposure was defined as having filled one or more antibiotic prescriptions during pregnancy. The outcome was ADHD diagnosis in offspring identified in hospitalizations, physician visits or drug dispensations. Risk of ADHD was estimated using Cox proportional hazards regression in the entire cohort, a high dimensional propensity scores (HDPS)-matched cohort, and an exposure-discordant sibling cohort.

Results: The cohort included 187 605 children; 16 290 (8.7%) children received an ADHD diagnosis. Prenatal antibiotic exposure was associated with increased ADHD risk (HR 1.22, 95% CI 1.18-1.26). In the HDPS-matched cohort of 129 674 children, prenatal antibiotic exposure was associated with ADHD risk (HR 1.22, 95% CI 1.17-1.27). The highest risk was observed in those receiving 3 or more antibiotic courses (HR 1.26, 95% CI 1.14-1.39) and for a duration longer than 2 weeks (HR 1.29, 95% CI 1.20-1.39). In the sibling cohort of 64 019 children, prenatal antibiotic exposure was not associated with ADHD risk (HR 1.06, 95% CI 0.99 - 1.13).

Conclusion: Prenatal antibiotic exposure is not associated with a clinically significant increase in ADHD risk. The risk increase observed in the overall and HDPS-matched cohorts is likely overestimated by unmeasured confounding which was minimized in the sibling cohort.

5.3. Introduction

Attention deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder with a significant burden.[1,2] The disorder is associated with many long term adverse health and social outcomes such as comorbid depression and anxiety, drug abuse, motor vehicle collisions, criminal offences, and work absenteeism.[3–6] The worldwide ADHD prevalence is estimated at 5.3 to 7.1% in children and adolescents.[7,8] While no national data are available on ADHD prevalence in Canada, ADHD prevalence in children aged 6 to 19 years was 5.5% between 2005 to 2009 in the province of Manitoba, and increased to 6.8% between 2009 and 2013.[9] Genetic factors are significant contributors to the etiology of ADHD with a hereditability of about 74%.[10,11] In addition, environmental factors such as maternal age at delivery, prenatal smoking and illicit drug use, maternal depression and prenatal exposure to antidepressant medications have also been suggested to be associated with ADHD risk.[12–16]

Recent studies have shown that subjects with ADHD have abnormal microbiota composition,[17,18] but the exact mechanism by which microbiota is involved in the etiology of the disorder is unknown. It is proposed that microbiota dysbiosis disrupts the communication between the gut and the brain, via the gut-brain axis, potentially contributing to the development of neurodevelopmental disorders.[19–24] Given the recent evidence on microbiota transfer in utero and on antibiotic-induced fetal dysbiosis, [25–28] we aimed to examine the association between prenatal antibiotic exposure and the risk of ADHD.

5.4. Methods

5.4.1. Design, Study Setting and Participants

We conducted a population-based cohort study of children born between 1998 and 2017 in the province of Manitoba – Canada. Data were extracted from the Manitoba Population Research Data Repository, a collection of administrative, registry, survey and other types of data housed at the Manitoba Centre for Health Policy. The universal and publicly funded provincial health system allows for all encounters with the health system and drug dispensations for Manitoba residents to be captured in the Repository. All patient records in the Repository are de-identified, but linkable at the person level using a scrambled identifier.

All children registered in the Manitoba Health Insurance Registry who were born between April 1, 1998 and March 31, 2017 were identified. A minimum of 4 years of valid Manitoba health registration was required for the children to be included in the cohort. This was required to meet the minimum age of 4 years for an ADHD diagnosis as recommended by the American Academy of Pediatrics.[29] To capture maternal baseline characteristics, we required a minimum of 2 years of health registration for the mothers prior to child's birthdate. Children were followed from birthdate, which determined the index date, until the earliest of a diagnosis of ADHD, migration out of province, age of 18 years, death or end of study period (March 31, 2017).

We additionally identified a cohort of children who had at least one maternal sibling discordant in prenatal antibiotic exposure. We examined the association between prenatal antibiotic exposure and ADHD risk in this sibling cohort to reduce unmeasured confounding due to genetic, and shared environmental and social factors.

Other data sources used in the study included outpatient prescription-medication dispensations, in-hospital dispensations, physician outpatient claims, hospitalization records, birth records, a screen of newborn risk factors in addition to employment and income assistance database (Supplementary Table 1). The study was approved by the University of Manitoba Health Research Ethics Board and the Health Information Privacy Committee of Manitoba Health, Seniors and Active Living.

5.4.2. Exposure

Prenatal exposure to antibiotics was defined as having filled one or more outpatient antibiotic prescriptions during pregnancy. Birth date and gestational age, which was based on the first date of women's last menstrual period, were identified in birth records and were used to estimate conception date. In secondary analyses, we examined the association between prenatal antibiotic exposure and ADHD risk based on pregnancy trimester, number of antibiotic courses, cumulative duration of use and antibiotic class (Supplementary Table 2).

5.4.3. Outcome

The main outcome measure was ADHD diagnosis at a minimum age of 4 years. The International Classification of Disease (ICD) coding system in the 9th - Clinical Modifications (ICD-9-CM) and the 10th (ICD-10) revisions were used to identify ADHD diagnosis in physician outpatient claims and hospitalization records, respectively. The Anatomical Therapeutic Chemical (ATC) Drug Classification System was used to identify ADHD drugs in prescription dispensations. A standard identification algorithm was utilized which defines ADHD diagnosis as one or more hospitalizations with a diagnosis of hyperkinetic syndrome (ICD-9-CM code 314 or ICD-10 code

F90); or one or more physician visits with a diagnosis of hyperkinetic syndrome (ICD-9-CM code 314); or two or more prescriptions for ADHD drugs (ATC code N06BA) within a year without a diagnosis of conduct disorder (ICD-9-CM code 312 or ICD-10 codes F63, F91, F92); or disturbance of emotions (ICD-9-CM code 313 or ICD-10 codes F93, F94); or cataplexy/narcolepsy (ICD-9-CM code 347 or ICD-10 code G47.4).[9,30–33]

5.4.4. Covariates

Sociodemographic characteristics and other clinically important variables were explored for inclusion in the analyses.[12,34,35] Models based on the overall cohort were adjusted for sex, mother's age at delivery (less than 30, 30 to 39, and 40 years or greater), region of residence (urban or rural), receipt of income assistance, year of birth, birth order (firstborn or subsequent), mother's medical conditions (mood and anxiety disorder, developmental disability and asthma) prenatal antidepressant exposure and the number of mother's physician visits in the year prior to conception as a measure of healthcare use. Models based on the sibling cohort were adjusted for sex, mothers' age at delivery, birth order, year of birth, and prenatal antidepressant exposure.

High Dimensional Propensity Scores (HDPS) were estimated using 5 dimensions and were used to match children who were exposed to antibiotics to those who were not. Mothers' hospital medical diagnoses and procedures (2 dimensions), outpatient medical diagnoses and tariff codes (2 dimensions) and drug dispensations (1 dimension) were identified during the year prior to the estimated conception date. The 200 most prevalent variables were identified in each dimension and were ranked based on their frequency into once, sporadic, and frequent; 500 variables were selected for inclusion in HDPS estimation in addition to sex, region of

residence, Socio-Economic Factor Index, a measure of socioeconomic status derived from Census data and is based on area of residence,[36] receiving income assistance, mother's age at delivery, birth order, season of birth, year of birth, and the number of mother's physician visits in the year prior to pregnancy. Prenatal smoking, alcohol and drug use, which were obtained from a screen of newborn risk factors, were included in a sensitivity analysis of a subset of the cohort that had these variables available.

5.4.5. Statistical analysis

In both the overall and the sibling cohort, multivariable Cox proportional hazards regression models were used to compare ADHD risk among those exposed and unexposed to antibiotics prenatally. The model was stratified by the mothers in the sibling cohort.

Unadjusted Cox proportional hazards regression models, stratified by the matched pair, were used to compare ADHD risk in the HDPS-matched cohort. Children who were exposed to antibiotics prenatally were matched to those who were not exposed in a one-to-one nearest neighbor greedy match on sex, birthdate (within 365 days) and HDPS within a caliper of 0.05. Balance across the two exposure groups was examined by calculating the standardized differences for sociodemographic characteristics, birth characteristics, and relevant maternal medical conditions at baseline (Supplementary Table 3).

In the secondary analyses, indicator variables were used for each of the categories and were mutually adjusted for in the models. Proportional hazards assumption was tested by examining the correlation between various forms of follow-up time and Schoenfeld residuals of

the independent variables. The statistical software SAS® 9.4 (SAS Institute; Cary, NC) was used for all data analyses.

5.4.6. ***Sensitivity and negative-control analyses***

We conducted several sensitivity analyses in the overall cohort. First, we applied a stricter identification algorithm requiring one hospitalization, two physician claims within three years or two or more prescriptions for ADHD drugs in a year plus one physician claim within three years. Second, we required a minimum age of 6 years to identify ADHD diagnosis based on recommendations by the Canadian ADHD Resource Alliance.[37] Third, we included in-hospital antibiotic dispensation in exposure definition, which was not included in the main analysis due to the limited data years and geographic coverage of the in-hospital dispensation dataset. Other sensitivity analyses were conducted by including prenatal smoking, alcohol and drug use in HDPS estimation, and restricting the cohort to firstborn children. We also examined maternal antibiotic exposure in the year before conception and the year after birth as negative controls.

5.5. Results

5.5.1. Description of study population

Out of 289 449 births in Manitoba between the fiscal years 1998 and 2017, 187 605 children met the inclusion criteria (Figure 5.1). The cohort was well balanced with 51.2% males, and 54.2% residing in urban regions; 70 554 (37.6%) children were exposed to antibiotics prenatally; 62.7% of the exposed received only one antibiotic course, 73.9 % received antibiotics for a cumulative duration of less than or equal to 2 weeks and 50.7% received a penicillin antibiotic only (Supplementary Table 4). Children were followed for a total of 1 990 452 person-years with

a median of 10.1 person-years (Interquartile range [IQR) 6.8 – 14.3). During follow-up, 16 290 (8.7%) children received an ADHD diagnosis at a median age of 7.5 years (IQR 6.0 – 9.5). The crude incidences for ADHD diagnosis were 10.3 cases per 1000 person-years and 6.9 cases per 1000 person-years in children exposed and unexposed to antibiotics prenatally, respectively.

A total of 129 674 children were matched on HDPS, sex, and birthdate. Baseline characteristics were well balanced between the two exposure groups with standardized differences of less than 0.1 for all the examined variables except for maternal asthma with a standardized difference of 0.15 (Table 1). Children were followed for a total of 1 374 484 person-years with a median of 10.0 person-years (IQR 6.8 – 14.4). During follow-up, 12 099 (9.3%) children received an ADHD diagnosis. The crude incidences for ADHD diagnosis were 9.6 cases per 1000 person-years and 8.0 cases per 1000 person-years in children exposed and unexposed to antibiotics prenatally, respectively.

The sibling cohort included 64 019 children and 44 491 exposure-discordant pairs (Figure 5.1). Baseline characteristics and description of antibiotic exposure in the sibling cohort are described in Supplementary Tables 5 and 4, respectively. During a follow up of 687 350 person-years, 6025 (9.4%) children received an ADHD diagnosis. The crude incidences for ADHD diagnosis were 9.5 cases per 1000 person-years and 8.1 cases per 1000 person-years in children exposed and unexposed to antibiotics prenatally, respectively.

5.5.2. Main analysis

Prenatal antibiotic exposure was associated with ADHD risk in the overall cohort (HR 1.22, 95% CI 1.18 – 1.26). The highest risk was observed in those receiving 3 or more antibiotic courses or

for a duration longer than 2 weeks (HR 1.39, 95% CI 1.31-1.47 and HR 1.33, 95% CI 1.27-1.39, respectively). The association did not change significantly in the matched cohort (HR 1.22, 95% CI 1.17-1.27). The highest risk in the matched cohort was observed in those receiving 3 or more antibiotic courses (HR 1.26, 95% CI 1.14-1.39), received antibiotics for a duration longer than 2 weeks (HR 1.29, 95% CI 1.20-1.39) or children who were exposed to macrolides (HR 1.35, 95% CI 1.23-1.48). No significant variation in the risk was observed with exposure in different pregnancy trimesters (Table 2). In the sibling cohort, prenatal antibiotic exposure was not associated with the risk of ADHD (HR 1.06, 95% CI 0.99-1.13). No significant variation in the risk was observed in the secondary analyses (Table 3).

5.5.3. ***Sensitivity and negative-control analyses***

No major changes in the risk estimates were found in all planned sensitivity analyses (Figure 5.2). Maternal exposure to antibiotics in the year before conception and in the year after childbirth were found to be associated with ADHD risk in the child (HR 1.18, 95% CI 1.15-1.22 and 1.23, 95% CI 1.19-1.27, respectively). Contrary to the analysis in the overall cohort, maternal exposure to antibiotics in the year before conception and after childbirth was not associated with the ADHD risk in the sibling cohort (HR 1.04, 95% CI 0.97-1.11 and 0.99, 95% CI 0.92-1.06, respectively).

5.6. Discussion

We found an increased ADHD risk in children exposed to antibiotics prenatally. The highest risk was observed in those exposed to 3 or more antibiotic courses or for a duration longer than 2 weeks. The risk of ADHD did not change significantly after matching children on HDPS, sex, and

birthdate. In the sibling cohort, prenatal antibiotic exposure was not associated with the risk of developing ADHD. Several sensitivity analyses confirmed that the association is not sensitive to changes in exposure, outcome or covariate definitions. An association was observed in both planned negative controls in the overall cohort but not in the sibling cohort.

The risk of ADHD was comparable in the overall cohort and the HDPS-matched cohort, which indicates that HDPS matching did not add much to controlling confounding beyond what was provided by the covariates included in the multivariable model. Instead, the risk of ADHD faded in the sibling cohort, suggesting that the risk observed in the overall and HDPS-matched cohorts may have been overestimated as a result of unmeasured confounding by genetics and other shared factors.

The association between maternal exposure in the year before conception and the year after childbirth with ADHD risk in the child indicates that the observed association between prenatal antibiotic exposure and ADHD might have been confounded by unobserved factors. The fact that this association was observed in the overall cohort but not in the sibling cohort suggests that the analysis conducted in the sibling cohort was less sensitive to unmeasured confounding. This is not unexpected given the significant contribution of genetics in the etiology of ADHD, [10,11] which is partially adjusted for in the sibling-controlled analysis. In light of this observation, we believe the sibling cohort captures more accurately the risk of prenatal antibiotic exposure on ADHD development, which was not found to be significant. Future studies are warranted to examine other potential dysbiosis-inducing factors in association with ADHD risk. Moreover, the role of microbiota in the development of ADHD is yet to be investigated at the biological and population level.

Our study has several strengths. The Manitoba Population Research Data Repository contains a large collection of data sources and provides comprehensive data on important characteristics and health system encounters of the entire Manitoba population. This allowed for a population-based study with a large sample size and a long follow-up period. Therefore, study findings are likely generalizable to all Manitoban children. We used a standard algorithm to identify ADHD from administrative databases, which eliminates any reporting bias. In addition, and to minimize the potential for confounding, we conducted HDPS-matching and a sibling-controlled design.

Despite the strengths mentioned above, it is important to acknowledge study limitations. Although we used an ADHD identification algorithm that was adopted from previous research, it has not been validated. Misclassification of the outcome is therefore a potential limitation. To address this issue, we conducted a sensitivity analysis with a stricter identification algorithm and study findings did not change. Misclassification of the exposure is another potential limitation since exposure was defined based on dispensation rather than actual use and it did not include in-hospital use for the reasons discussed in the methods section. In a sensitivity analysis, we included data available on in-hospital antibiotic dispensation and the risk estimate remained unchanged. Another potential limitation is unmeasured confounding by variables which are unavailable in the Repository and are not shared by sibling pairs. Unmeasured confounding in the overall and the HDPS-matched cohorts may have overestimated the risk estimates. However, the sibling-controlled design likely avoided confounding by genetics and shared familial factors to some extent and, therefore, it is recommended for future studies examining ADHD risk.

5.7. Conclusion

Study findings suggest that prenatal antibiotic exposure is not associated with a clinically significant increase in the risk of developing ADHD.

5.8. Acknowledgment

We would like to thank Charles Burchill, Matt Dahl and Heather Prior from the Manitoba Centre for Health policy for their valuable support.

We acknowledge the Manitoba Centre for Health Policy for use of data contained in the Manitoba Population Research Data Repository under project #H2016:244 (HIPC# 2016/2017 – 11). The results and conclusions are those of the authors and no official endorsement by the Manitoba Centre for Health Policy, Manitoba Health, Healthy Living and Senior or other data providers is intended or should be inferred. Data used in this study are from the Manitoba Population Research Data Repository housed at the Manitoba Centre for Health Policy, University of Manitoba and were derived from data provided by Manitoba Health, Healthy Living and Senior, Winnipeg Regional Health Authority, Manitoba Department of Families and Healthy Child Manitoba.

5.9. Figures and tables

Table 5.1. Baseline and birth characteristics of the study cohort: overall and matched

Characteristic	Overall cohort N=187 605			Matched cohort N = 129 674		
	Number (%)		Standardized difference	Number (%)		Standardized difference
	Unexposed N=117 051	Exposed N=70 554		Unexposed N=64 837	Exposed N=64 837	
Male	59 894 (51.2)	36 172 (51.3)	0.002	33 358 (51.5)	33 358 (51.5)	0.000
Urban region	65 305 (55.8)	36 418 (51.6)	0.084	33 417 (51.5)	33 488 (51.7)	0.002
Socioeconomic status (SES)[a]:			0.174			0.066
High	12 873 (11.0)	5340 (7.6)		5995 (9.3)	5223 (8.1)	
Middle	44 027 (37.6)	23 105 (32.8)		23 084 (35.6)	22 065 (34.0)	
Low-mid	35 291 (30.2)	23 319 (33.1)		20 448 (31.5)	21 425 (33.0)	
Low	24 860 (21.2)	18 790 (26.6)		15 310 (23.6)	16 124 (24.9)	
Receiving income assistance [b]	18 503 (15.8)	20 652 (29.3)	0.326	15 681 (24.2)	16 717 (25.8)	0.037
Maternal age at delivery [c]	28.7 (5.8)	27.6 (5.9)	0.185	27.8 (5.8)	27.7 (5.9)	0.008
Breastfeeding initiation [d]	95 919 (82.3)	54 251 (77.2)	0.127	51 865 (80.4)	50 709 (78.5)	0.045
Preterm delivery [e]	8139 (7.0)	5578 (7.9)	0.036	4694 (7.3)	4962 (7.7)	0.016
Caesarian delivery	23 213 (19.8)	14 654 (20.8)	0.023	12 837 (19.8)	13 571 (20.9)	0.028
Firstborn child	45 645 (39.0)	24 946 (35.4)	0.075	24 272 (37.4)	23 731 (36.6)	0.017
Small for gestational age [f]	9046 (7.7)	5273 (7.5)	0.010	4943 (7.6)	4795 (7.4)	0.009
Birth complications [g]	11 743 (10.0)	7150 (10.1)	0.003	6356 (9.8)	6623 (10.2)	0.014
Prenatal smoking [h]	12 296 (15.4)	11 801 (25.3)	0.247	8518 (19.7)	10 131 (23.4)	0.089
Prenatal alcohol/drug use [i]	8948 (11.4)	7117 (15.5)	0.121	5719 (13.4)	6270 (14.7)	0.037
Maternal medical condition:						
Mood and anxiety disorders	17 082 (14.6)	16 502 (23.4)	0.226	11 930 (18.4)	13 567 (20.9)	0.064
Asthma	5557 (4.8)	8399 (11.9)	0.261	4083 (6.3)	6854 (10.8)	0.154
Developmental disabilities	1994 (1.7)	2029 (2.9)	0.078	1382 (2.1)	1689 (2.6)	0.031

Characteristic	Overall cohort N=187 605			Matched cohort N = 129 674		
	Number (%)		Standardized difference	Number (%)		Standardized difference
	Unexposed N=117 051	Exposed N=70 554		Unexposed N=64 837	Exposed N=64 837	

Percentages are calculated based on non-missing data

[a] Based on the Socioeconomic factor index, a neighborhood level measure based on Canada census, was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Mean age in years (Standard deviation)

[d] Missing data for 789 children (0.4%) of the overall cohort and 554 (0.4%) children in the matched cohort

[e] Defined as gestational age less than 37 weeks. Missing data for 193 children (0.1%) of the overall cohort and 133 (0.1%) children in the matched cohort

[f] Defined as having birth weight below the 10th percentile for the gestational age and sex. Missing data for 264 children (0.1%) of the overall cohort and 186 (0.1%) children in the matched cohort

[g] Include vacuum, forceps or breech procedures: ICD-9-CM procedure codes 72, 73.3, 763.2, 652.2, 669.6; CCI codes 5MD53, 5MD54, 5MD55, 5MD56, shoulder dystocia: 660.4, placental abruption: 641.2, cord prolapse: 663, uterine rupture: 665.1 or other perinatal complications: 760-779

[h] Missing data for 61 390 (32.7%) children in the overall cohort and 43 092 (33.2%) in the matched cohort

[i] Missing data for 63 238 (33.7%) children in the overall cohort and 44 351 (34.2%) in the matched cohort

Table 5.2. Association between antibiotic exposure and risk of ADHD: overall and matched cohort

Variable	Overall cohort N = 187 605			HDPS-matched cohort N = 129 674		
	Number of events	Person-years	Adjusted [a] HR (95% CI)	Number of events	Person-years	Unadjusted HR (95% CI)
Main analysis						
Prenatal antibiotic exposure						
Unexposed	8649	1 247 467	1.00 [Reference]	5500	690 186	1.00 [Reference]
Exposed	7641	742 985	1.22 (1.18 – 1.26)	6599	684 298	1.22 (1.17 – 1.27)
Secondary analyses						
Exposure by trimester [b]						
None	8649	1 247 467	1.00 [Reference]	5500	690 186	1.00 [Reference]
First	3722	333 818	1.13 (1.09 – 1.17)	3094	298 488	1.13 (1.06 – 1.20)
Second	3919	352 091	1.17 (1.13 – 1.22)	3270	317 862	1.21 (1.14 – 1.28)
Third	3256	314 554	1.12 (1.07 – 1.16)	2752	286 035	1.08 (1.01 – 1.15)
Antibiotic class						
None	8649	1 247 467	1.00 [Reference]	5500	690 186	1.00 [Reference]
Penicillin antibiotics	5150	500 082	1.15 (1.12 – 1.19)	4419	458 112	1.13 (1.08 – 1.19)
Non-penicillin β-lactams	1489	133 529	1.18 (1.12 – 1.25)	1220	118 134	1.14 (1.04 – 1.25)
Macrolides and related antibiotics	1675	131 987	1.17 (1.11 – 1.24)	1336	116 915	1.35 (1.23 – 1.48)
Others	2176	194 694	1.09 (1.04 – 1.14)	1806	174 912	1.08 (1.00 – 1.17)
Number of antibiotic courses						
0	8649	1 247 467	1.00 [Reference]	5500	690 186	1.00 [Reference]
1	4317	467 362	1.16 (1.12 – 1.20)	3938	444 343	1.20 (1.14 – 1.26)
2	1898	168 403	1.26 (1.20 – 1.33)	1623	153 050	1.25 (1.15 – 1.35)
>= 3	1426	107 221	1.39 (1.31 – 1.47)	1038	86 904	1.26 (1.14 – 1.39)
Cumulative antibiotic duration (days)						
0	8649	1 247 467	1.00 [Reference]	5500	690 186	1.00 [Reference]
1-7	2780	306 543	1.16 (1.11 – 1.21)	2534	291 069	1.18 (1.11 – 1.25)
8 – 14	2412	241 468	1.20 (1.14 – 1.25)	2143	226 189	1.21 (1.13 – 1.30)
>14	2449	194 975	1.33 (1.27 – 1.39)	1922	167 040	1.29 (1.20 – 1.39)

[a] Adjusted for sex, mother's age at delivery, region of residence, receipt of income assistance, year of birth, birth order, mother's medical conditions, prenatal antidepressant exposure and number of mother's physician visits in the year prior to pregnancy.

[b] First trimester = 0 to 13 weeks of pregnancy, second trimester = 14 to 27 weeks, third trimester = 28 weeks until date of birth

Table 5.3. Association between antibiotic exposure and risk of ADHD in the sibling cohort

Variable	Sibling cohort N = 64 019			
	Number of events	Person-years	Unadjusted HR (95% CI)	Adjusted [a] HR (95% CI)
Main analysis				
Prenatal antibiotics exposure				
Unexposed	2913	360 947	1.00 [Reference]	1.00 [Reference]
Exposed	3112	326 402	1.07 (1.01 – 1.14)	1.06 (0.99 – 1.13)
Secondary analyses				
Exposure by trimester [b]				
None	2913	360 947	1.00 [Reference]	1.00 [Reference]
First	1446	138 371	1.02 (0.94 – 1.11)	1.03 (0.94 – 1.13)
Second	1506	145 603	1.13 (1.04 – 1.23)	1.10 (1.00 – 1.20)
Third	1258	131 952	1.02 (0.93 – 1.11)	1.02 (0.93 – 1.12)
Antibiotic class				
None	2913	360 947	1.00 [Reference]	1.00 [Reference]
Penicillin antibiotics	2063	215 600	1.01 (0.94 – 1.09)	1.03 (0.95 – 1.11)
Non-penicillin β-lactams	582	55 855	1.18 (1.04 – 1.34)	1.09 (0.96 – 1.25)
Macrolides and related antibiotics	700	55 595	1.12 (0.99 – 1.26)	1.07 (0.95 – 1.21)
Others	784	77 095	1.01 (0.90 – 1.13)	1.01 (0.89 – 1.13)
Number of antibiotic courses				
0	2913	360 947	1.00 [Reference]	1.00 [Reference]
1	1921	223 769	1.05 (0.98 – 1.13)	1.04 (0.96 -1.12)
2	750	68 775	1.10 (0.98 – 1.28)	1.11 (0.99 -1.25)
>= 3	441	33 859	1.12 (0.97 – 1.30)	1.10 (0.95 -1.28)
Cumulative antibiotic duration (days)				
0	2913	360 947	1.00 [Reference]	1.00 [Reference]
1-7	1233	146 636	1.06 (0.97 – 1.16)	1.04 (0.95 – 1.15)
8 – 14	1039	110 020	1.04 (0.94 – 1.14)	1.03 (0.94 – 1.14)
>14	840	69 746	1.14 (1.02 – 1.28)	1.13 (1.01 – 1.27)

[a] Adjusted for sex, mothers' age at delivery, birth order, year of birth and prenatal antidepressants exposure.
[b] First trimester = 0 to 13 weeks of pregnancy, second trimester = 14 to 27 weeks, third trimester = 28 weeks until date of birth

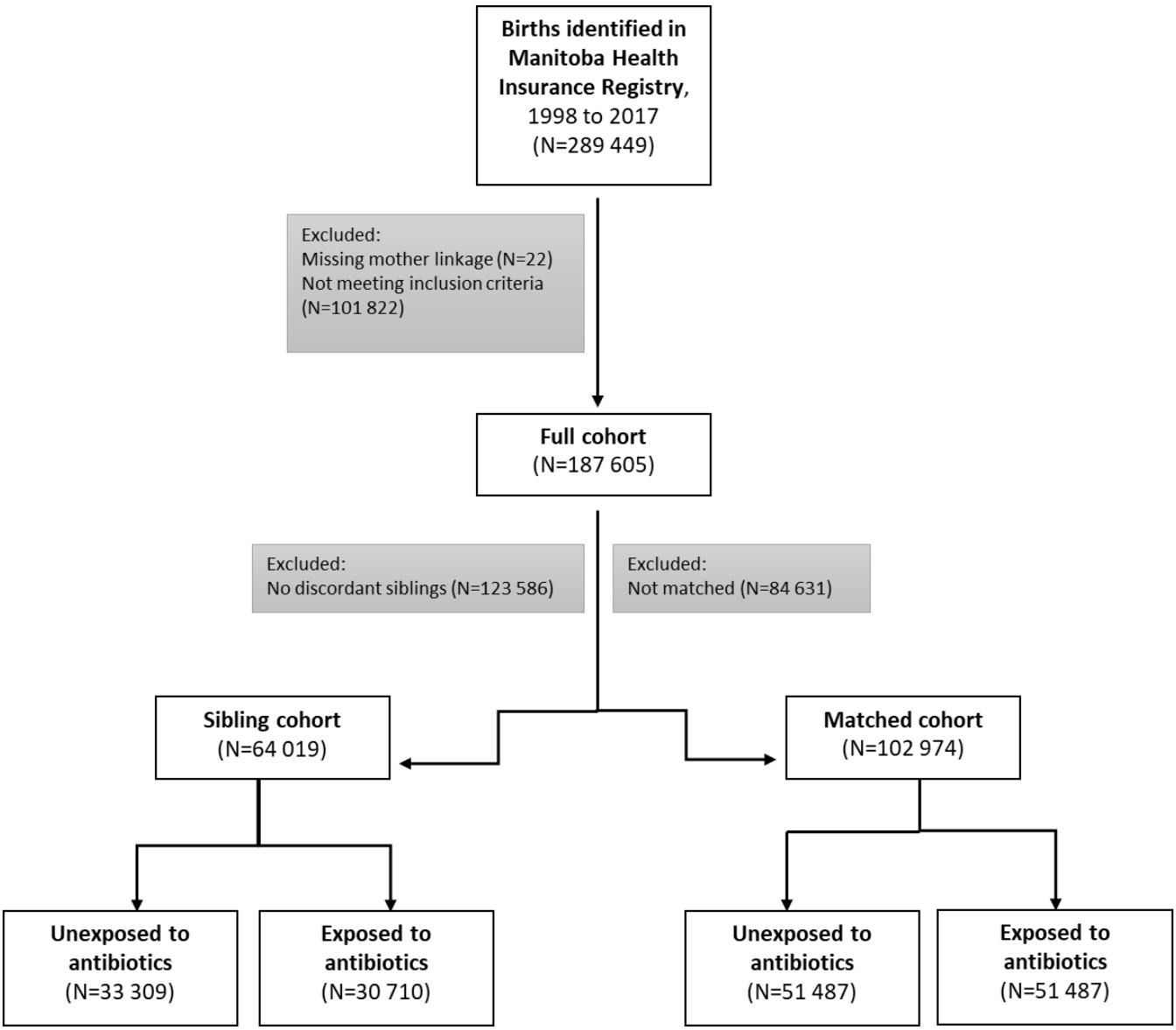

Figure 5.1. Study populations: overall, matched and sibling cohorts

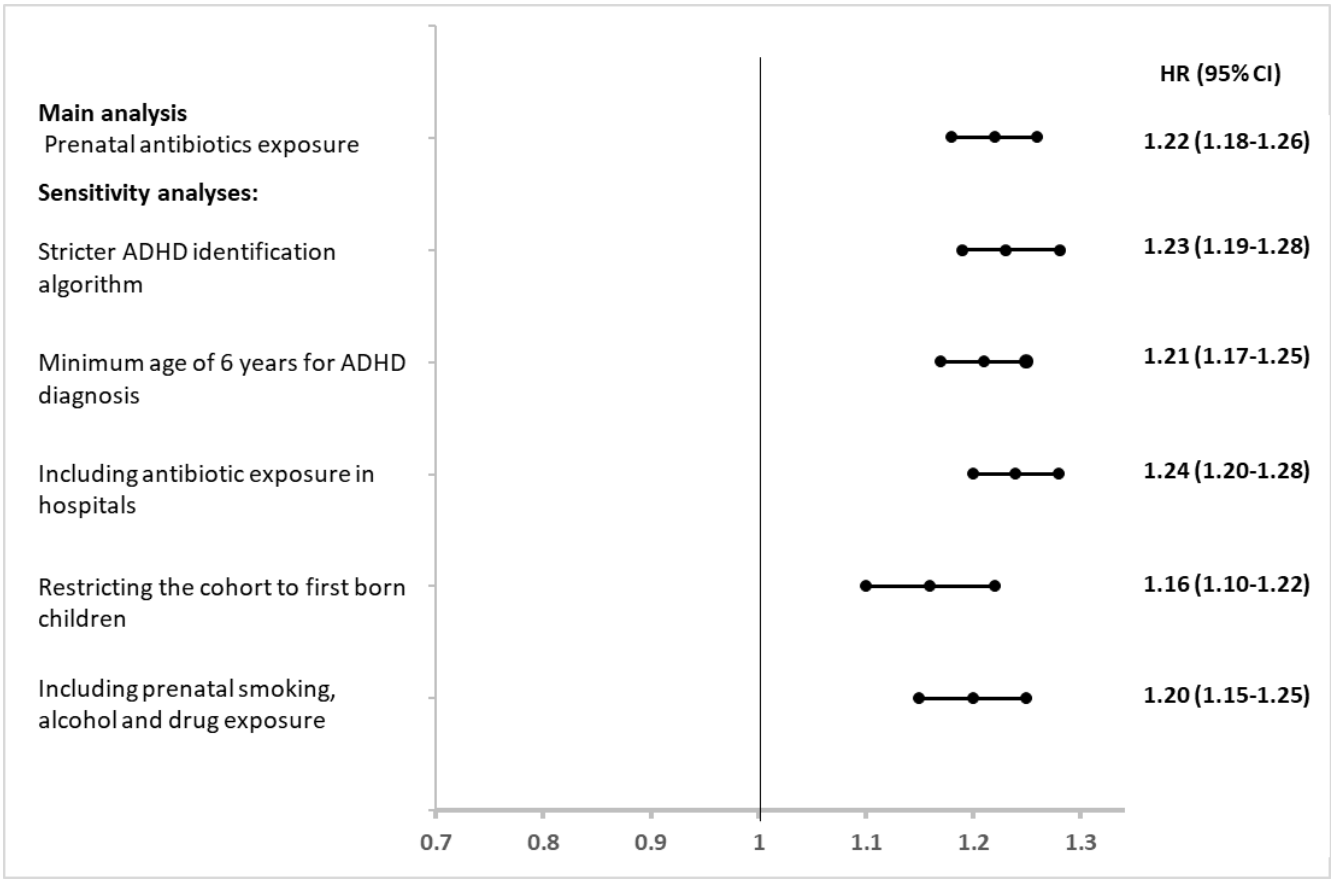

Figure 5.2. Risk estimates from sensitivity analyses in the overall cohort

Supplementary Table 5.1. Description of data sources

Database	Description	Data years
The Manitoba Health Insurance Registry	A population-based registry that collects demographic and insurance coverage information of Manitoba residents and is maintained by Manitoba Health, Seniors and Active Living.	1970/71 – 2016/17
Drug Program Information Network	An electronic drug system that captures all prescriptions dispensed from community pharmacies to residents and is maintained by Manitoba Health, Seniors and Active Living. It includes prescription information such as generic name, Anatomical Therapeutic Chemical code, date of dispensation, strength and days supplied.	1995/96 – 2016/17
In-hospital Pharmaceuticals	An inpatient pharmacy system that provides dispensing information on pharmaceutical use at three Winnipeg hospitals: Health Sciences Centre, Concordia Hospital and Riverview Health Centre.	1999 – 2012
Hospital discharge abstracts	Includes records of all patients' hospital admissions with summaries for demographic data such as gender and postal code, and clinical data including up to 25 diagnosis codes and 20 procedure codes.	1970/71 – 2016/17
Medical Services database	Physician claims in the Medical Services database include records of claims for any physician visits in offices, hospitals and outpatient departments in Manitoba in addition to some information on Manitobans' physician visits outside the province. Claims are submitted by physicians electronically for service reimbursement by Manitoba Health, Seniors and Active Living.	1970/71 – 2016/17
The Hospital Newborn to Mother Link	Serves to match the baby's birth hospital record with the mother's obstetrical delivery record and contains basic demographic and hospital data on newborns and their mothers for in-hospital births.	1984/85 – 2016/17
BabyFirst - Families First Screen	Collected by Public Health Nurses on nearly all families with newborns in Manitoba and maintained by Heathy Child Manitoba. Contains records of newborns identifying biological, social, and demographic risk factors including prenatal smoking, alcohol and drug use.	2000 – 2016
The Social Allowances Management Information Network	Provides information concerning employment and income assistance received by Manitoba residents.	1995/96 – 2016/17
Canada Census	A population survey with aggregate demographic information for all persons within a dissemination area in Canada and is conducted by Statistics Canada every five years.	1971 – 2016

Supplementary Table 5.2. Antibiotics classification

Class	Generic name	Anatomical Therapeutic Chemical
Penicillin antibiotics	Amoxicillin, ampicillin, bacampicillin, penicillin, piperacillin, pivampicillin, pivmecillinam, cloxacillin, floxacillin, nafcillin, ticarcillin, tazobactam	J01C
Non-penicillin β-lactams	Cefazolin, cefepime, cefixime, cefotaxime, cefotetan, cefoxitin, cefpodoxime, cefprozil, ceftazidime, ceftizoxime, ceftobiprole, ceftriaxone, cefuroxime, cephalexin, cephalothin, aztreonam, cefaclor, cefadroxil, cefamandole, doripenem, ertapenem, meropenem	J01D
Macrolides and related antibiotics	Dalfopristin, telithromycin, azithromycin, clarithromycin, clindamycin, erythromycin, lincomycin	J01F, J01R
Other antibiotics	Minocycline, doxycycline, demeclocycline, rolitetracycline, tetracycline, ciprofloxacin, gatifloxacin, nalidixic acid, gemifloxacin, grepafloxacin, levofloxacin, moxifloxacin, norfloxacin, ofloxacin, trovafloxacin, methenamine, fosfomycin, tedizolid, nitrofurantoin, metronidazole, linezolid, fusidate, fusidic acid, bacitracin, vancomycin, colistin, polymyxin B, telavancin, tobramycin, gentamicin, daptomycin, amikacin, spectinomycin, neomycin, netilmicin, spiramycin, streptomycin, chloramphenicol, trimethoprim, sulfamethoxazole, sulfadiazine, sulfapyridine, sulfisoxazole	J01A, J01B, J01G, J01E, J01M, J01X

Supplementary Table 5.3. Identification algorithms of maternal medical conditions

Covariate	Period	Diagnostic Criteria
Mood and anxiety disorders	Within 3 years before index date	✓ One or more hospitalizations with a diagnosis for depressive disorder, affective psychoses, neurotic depression or adjustment reaction: ICD-9-CM codes 296.1-296.8, 300.4, 309 or 311; ICD-10-CA codes F31, F32, F33, F34.1, F38.0, F38.1, F41.2, F43.1, F43.2, F43.8, F53.0, F93.0 or with a diagnosis for an anxiety state, phobic disorders or obsessive-compulsive disorders: ICD-9-CM codes 300.0, 300.2, 300.3, 300.7; ICD-10-CA codes F40, F41.0, F41.1, F41.3, F41.8, F41.9, F42, F45.2, OR ✓ One or more hospitalizations with a diagnosis for anxiety disorders: ICD-9-CM code 300; ICD-10-CA codes F32, F34.1, F40, F41, F42, F44, F45.0, F45.1, F45.2, F48, F68.0, or F99 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ One or more physician visits with a diagnosis for depressive disorder or affective psychoses: ICD-9-CM codes 296, 311, OR ✓ One or more physician visits with a diagnosis for anxiety disorders: ICD-9-CM code 300 AND one or more prescriptions for an antidepressant or mood stabilizer, including medications with the ATC codes N05AN01, N05BA, N06A, OR ✓ Three or more physician visits with a diagnosis for anxiety disorders or adjustment reaction: ICD-9-CM code 300, 309.
Developmental disability	Any time before index date	✓ One or more hospitalizations with diagnoses for autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD) or intellectual disabilities: ICD-9-CM codes 299, 314, 317, 318, 319; ICD-10-CA: F70, F71, F72, F73, F78, F79, F84, F90. ✓ One or more physician visit with diagnoses for ASD, ADHD or intellectual disabilities: ICD-9-CM: 299, 314, 317, 318, 319
Asthma	Within 1 year before index date	✓ One or more hospitalizations with a diagnosis of asthma: ICD-9-CM codes 493; ICD-10-CA code J45, OR ✓ One or more physician visits with a diagnosis of asthma: ICD-9-CM codes 493, OR ✓ One or more prescriptions for asthma medication (ATC: R03, R06AX17)

Supplementary Table 5.4. Description of prenatal antibiotic exposure: overall, matched cohort and sibling cohort

	Number (%)		
	Overall cohort N = 187 605	**Matched cohort N = 129 674**	**Sibling cohort N = 64 019**
Antibiotic class			
Penicillin antibiotics	46 936 (50.7)	42 883 (51.4)	20 171 (52.1)
Macrolides and related antibiotics	13 100 (14.2)	11 554 (13.8)	5557 (14.4)
Non-penicillin β-lactams	13 764 (14.9)	12 181 (14.6)	5687 (14.7)
Others	18 806 (20.3)	16 851 (20.2)	7301 (18.9)
Total	92 606	83 469	38 716
Exposure by trimester [a]			
First	31 923 (33.5)	28 476 (33.2)	13 097 (33.3)
Second	33 768 (35.5)	30 419 (35.5)	13 865 (35.3)
Third	29 540 (31.0)	26 788 (31.3)	12 320 (31.4)
Total	95 231	85 683	39 282
Number of courses			
1	44 223 (62.7)	41 986 (64.8)	20 951 (68.2)
2	16 086 (22.8)	14 562 (22.5)	6504 (21.2)
>=3	10 245 (14.5)	8289 (12.8)	3255 (10,6)
Total	70 554	64 837	30 710
Cumulative duration (days)			
1-7	29 551 (41.9)	28 030 (43.2)	13 860 (45.1)
8-14	22 572 (32.0)	21 085 (32.5)	10 211 (33.3)
>14	18 431 (26.1)	15 722 (24.2)	6639 (21.6)
Total	70 554	64 837	30 710

[a] First trimester = 0 to 13 weeks of pregnancy, second trimester = 14 to 27 weeks, third trimester = 28 weeks until date of birth

Supplementary Table 5.5. Baseline characteristics of the sibling cohort

Characteristic	Number (%)		
	All subjects N=64 019	Prenatal antibiotic exposure	
		No N= 33 309	Yes N=30 710
Male	32 662 (51.0)	16 972 (51.0)	15 690 (51.1)
Urban region	30 884 (48.2)	15 745 (47.3)	15 139 (49.3)
Socioeconomic status (SES)[a]:			
High	4920 (7.7)	2539 (7.6)	2381 (7.8)
Middle	20 473 (32.0)	10 715 (32.2)	9758 (31.8)
Low-mid	19 859 (31.0)	10 220 (30.7)	9639 (31.4)
Low	18 767 (29.3)	9835 (29.5)	8932 (29.1)
Received income assistance [b]	16 803 (26.3)	8101 (24.3)	8702 (28.3)
Mothers age at delivery [c]	27.4 (5.6)	27.4 (5.6)	27.4 (5.6)
Breastfeeding initiation [d]	49 222 (77.2)	25 664 (77.4)	23 558 (77.0)
Preterm delivery [e]	4506 (7.1)	2330 (7.0)	2176 (7.1)
Small for gestational age [f]	4324 (6.8)	2241 (6.7)	2083 (6.8)
Caesarian section	1192 (17.3)	5660 (17.0)	5432 (17.7)
Birth complications	4845 (7.6)	2512 (7.5)	2333 (7.6)
Firstborn child	17 744 (27.7)	9638 (28.9)	8106 (26.4)
Prenatal alcohol/drug use [g]	5625 (13.4)	2783 (12.9)	2842 (14.0)
Prenatal smoking [h]	9470 (22.2)	4542 (20.7)	4928 (23.8)
Maternal medical conditions:			
Mood and anxiety disorders	11 806 (18.4)	5648 (17.0)	6158 (20.1)
Asthma	4881 (7.6)	1882 (5.7)	2999 (9.8)
Developmental disabilities	1309 (2.0)	646 (1.9)	663 (2.2)

Percentages are calculated based on non-missing data

[a] Based on the Socioeconomic factor index, a neighborhood level measure based on Canada census, which was categorized with cut off points within one standard deviation from the mean into high, middle, low middle and low SES

[b] Defined as receiving income assistance for at least two months within 1 year before to 18 months after index date

[c] Mean (Standard deviation)

[d] Missing for 270 (0.4%) subjects

[e] Defined as gestation age less than 37 weeks, missing for 77 (0.1%) subjects

[f] Defined as having birth weight below the 10th percentile for the gestational age and sex. Missing for 106 (0.2%) subjects

[g] Missing data for 22 172 (34.6%) subjects

[h] Missing data for 21 385 (33.4%) subjects

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Chapter 6: Conclusions and Future Directions

In this population-based study of all Manitoba births between the fiscal years 1998 and 2016/2017, exposure to antibiotics in the first year of life was not found to be associated with increased ASD risk in the children. Similarly, exposure to antibiotics in the first year of life was not found to be associated with increased ADHD risk. Prenatal exposure to antibiotics was associated with a small, albeit clinically non-significant increase in the risk of ASD in offspring. Prenatal exposure to antibiotics was also not associated with a clinically significant increase in the risk of ADHD.

The potential association between antibiotic exposure in early life and the risk of NDD is based on the role microbiota plays in brain development and functioning and on antibiotics-induced dysbiosis.[30,34,36–42,150–155] Children with NDD have been shown to have abnormal microbiota composition.[27–35] Some of the main alterations include a higher content of *Clostridium, Bacteroidetes, Faecalibacterium,* and *Lactobacillus* and a lower content of *Bifidobacterium* which is generally known as protective bacteria.[27,28,30–35,176,194] The causes of these microbiota changes are unknown but could have been induced by antibiotic exposure in early life. Antibiotics are the most frequently prescribed medications for children and are commonly prescribed to pregnant women, where over-prescription and inappropriate use are often observed.[187,188,190–193] Accordingly, identifying antibiotics' association with NDD, if any, would be of public health interest.

By altering gut microbiota composition, antibiotics allow the overgrowth of pathogenic bacteria and influence many processes in the body including the development of the immune system, energy extraction from food, lipid and carbohydrate metabolism, hormonal regulation and neurodevelopment.[36,37,41,195–198] These consequences of antibiotic-induced dysbiosis are the bases for numerous studies investigating an association between antibiotic exposure in early life and childhood diseases.[199–216] Despite some inconsistencies in the available literature examining the association of antibiotic exposure and childhood diseases, several meta-analyses showed an association is present with asthma, inflammatory bowel disease, obesity, and eczema.[199,212–216] A meta-analysis of 22 observational studies examining prenatal and early childhood antibiotic exposure reported a significant association with asthma with OR 1.52, 95% CI 1.30-1.77 for early childhood exposure and OR 1.24, 95% CI 1.02-1.50 for prenatal exposure.[199] A meta-analysis of 11 observational studies reported an increased risk of inflammatory bowel disease with a previous antibiotic exposure (OR 1.57, 95% CI 1.27-1.94).[216] Two meta-analyses reported a small increase in the risk of obesity in children exposed to antibiotics in the first 2 years of life with OR ranging from 1.05, 95% CI 1.00-1.11 to 1.11, 95% CI 1.02-1.20.[214,215] A population-based case-control study found that exposure to antibiotics in the two and a half years prior to diagnosis with juvenile idiopathic arthritis was associated with a higher risk of the disease (OR 2.1, 95% CI 1.2-3.5).[208] Another population-based case-control study reported an increased risk of juvenile idiopathic arthritis in children exposed to antibiotics in the first 2 years of life (OR 1.4, 95% CI 1.2-1.6).[217] A recent meta-analysis of 22 observational studies found that antibiotic exposure in the first 2 years of life is associated with an increased risk of eczema (OR 1.26, 95% CI 1.15-

1.37).[213] The effect size of the association of antibiotics with childhood diseases was small in many cases and could have been overestimated by unmeasured confounding.

In this study, the expected increase in ASD and ADHD rates in children exposed to antibiotics in their first year of life was not observed. A clinically-significant increase in ASD and ADHD rates in children exposed to antibiotics prenatally was not observed as well. This could be attributed to the magnitude of antibiotic-induced dysbiosis being too small to impair neurodevelopment, the duration of dysbiosis being too short to result in long-term effects on neurodevelopment or that a specific profile of dysbiosis is responsible for NDD and was not identified in this thesis project. Study findings align with another population-based study by Axelsson et al which used a sibling design and reported no association between antibiotic exposure in the first two years of life and ADHD risk.[186] On the other hand, a population-based study by Wimberley et al reported an increased ASD risk in children exposed to broad-spectrum antibiotics.[185] This risk is potentially confounded by the indication because of the observed increase in ASD risk in children exposed to otitis media regardless of antibiotic use, and the lack of additional risk in children with otitis media who were also exposed to antibiotics.

To minimize the potential of confounding in this study, HDPS-matching (chapters 4 and 5) and a sibling-controlled design (chapters 2 to 5) were additionally conducted. The HDPS-matching was used to identify confounders or proxies of confounders available in administrative databases and then balance the two exposure groups according to the confounders. The sibling-controlled design was used to reduce unmeasured confounding due to genetic and shared

environmental and social factors. The sibling-controlled analysis was found to be less sensitive to unmeasured confounding than the analysis based on the entire cohort or the HDPS-matched cohort. This is potentially attributed to the adjustment of genetic confounding in the sibling-controlled analysis given the significant contribution of genetics in the etiology of ADHD and ASD.[11,12,15,110] This can also be explained by the stronger collective confounding of unmeasured genetic and shared environmental factors compared to the factors identifiable in administrative databases. Therefore, the sibling-controlled analysis seems to capture more accurately the risk of NDD and is recommended in future studies examining NDD risk.

The role of microbiota in the development of NDD is yet to be investigated at the biological and population level. In addition, factors contributing to dysbiosis in children with these disorders need to be investigated in future studies. Addressing these research questions would provide insight into the etiology of these disorders and inform strategies for disease prevention.

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Appendix A: Sample sizes for the sensitivity analyses of chapters 2 to 5

Sensitivity analysis	**Sample size**
Chapter 2: Early Childhood Antibiotics Use and Autism Spectrum Disorders: A Population-Based Cohort Study	
Stricter ASD identification algorithm	213 539
Minimum age of 1 year for ASD diagnosis	213 539
Minimum age of 2 years for ASD diagnosis	213 539
Inclusion of hospitals' antibiotics exposure	213 539
Antibiotics' exposure within 6 months of birth	213 539
Antibiotics' exposure within 18 months of birth	213 539
Restriction by infection indication	146 498
Including prenatal smoking, alcohol and drug exposure	103 718
ASD co-occurring with ADHD and/or ID	213 539
Chapter 3: Prenatal Antibiotics Exposure and the Risk of Autism Spectrum Disorders: A Population-Based Cohort Study	
Stricter ASD identification algorithm	213 540
Minimum age of 1 year for ASD diagnosis	213 540
Minimum age of 2 year for ASD diagnosis	213 540
Inclusion of antibiotics exposure from in-hospital pharmaceuticals database	213 540
Restriction by infection indication	67 187
Including prenatal smoking, alcohol and drug use	103 719
Antibiotics exposure within 1 year before pregnancy	213 540
Antibiotics exposure within 1 year after birth	213 540
Chapter 4: Antibiotic Exposure in the First Year of Life and the Risk of Attention-Deficit/Hyperactivity Disorder: a Population-Based Cohort Study	
Stricter ADHD identification algorithm	70 096
Minimum age of 6 years for ADHD diagnosis	61 846
Including antibiotic exposure in hospitals	71 646
Antibiotic exposure within 6 months of birth	58 622

Antibiotic exposure within 2 years of birth	102 728
Including prenatal smoking, alcohol and drug exposure	43 744
Using first date of ADHD diagnosis even if occurred before age 4	69 738
Start follow up at time of exposure for those exposed and their matched subjects	69 738
Chapter 5: Prenatal Antibiotic Exposure and the Risk of Attention Deficit/ Hyperactivity Disorder: A Population-Based Cohort Study	
Stricter ADHD identification algorithm	187 605
Minimum age of 6 years for ADHD diagnosis	166 582
Including antibiotic exposure in hospitals	187 605
Restricting the cohort to first born children	70 591
Including prenatal smoking, alcohol and drug exposure	122 566
Antibiotics exposure within 1 year before conception	187 605
Antibiotics exposure within 1 year after birth	187 605

Appendix B: Health Research Ethics Board approval

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada, R3E 0W3
Telephone : 204-789-3255
Fax: 204-789-3414

UNIVERSITY OF MANITOBA | Research Ethics - Bannatyne
Office of the Vice-President (Research and International)

HEALTH RESEARCH ETHICS BOARD (HREB)
CERTIFICATE OF FINAL APPROVAL FOR NEW STUDIES
Delegated Review

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Amani Hamad	U of M and MCHP/College of Pharmacy	HS19851 (H2016:244)
APPROVAL DATE: June 9, 2016	**EXPIRY DATE:** June 9, 2017	
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable): Dr. I. fan Kuo		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
N/A	Prenatal and Early Childhood Antibiotics Exposure and Risk for Neurodevelopmental Disorders
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: University of Manitoba Start Up Funds	

Submission Date of Investigator Documents:	HREB Receipt Date of Documents:
June 6, 2016	June 6, 2016

THE FOLLOWING ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol: Proposal		June 6, 2016
Consent and Assent Form(s):		
Other: Data Extraction Form (Appendix A)- undated		submitted June 6, 2016

CERTIFICATION
The above named research study/project has been reviewed in a ***delegated manner*** by the University of Manitoba (UM) Health Research Board (HREB) and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM HREB.

HREB ATTESTATION
The University of Manitoba (UM) Research Board (HREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the HREB complies with the membership requirements for Research Ethics Boards defined in Division 5

of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. The study is acceptable on scientific and ethical grounds for the ethics of human use only. ***For logistics of performing the study, approval must be sought from the relevant institution(s).***
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. **This approval is valid until the expiry date noted on this certificate of approval. A Bannatyne Campus Annual Study Status Report** must be submitted to the HREB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the HREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form.**
6. Adverse events and unanticipated problems must be reported to the HREB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM HREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report**.

Sincerely,

[redacted]

Chair, Health Research Ethics Board
Bannatyne Campus

Please quote the above Human Ethics Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255/ **Fax**: (204) 789-3414

Appendix C: Health Information Privacy Committee approval

Manitoba
Health, Healthy Living and Seniors
Health Information Privacy Committee
4040-300 Carlton Street, Winnipeg, Manitoba, Canada R3B 3M9
T 204-786-7204 F 204-944-1911
www.manitoba.ca

September 7, 2016

Amani Hamad
College of Pharmacy, University of Manitoba
750 McDermot Ave
Winnipeg, MB R3E 0T5
hamada@myumanitoba.ca

HIPC No. 2016/2017 – 11
File number to be quoted on correspondence

Dear Amani Hamad,

Re: Prenatal and Early Childhood Antibiotics Exposure and Risk for Neurodevelopment Disorders

The Health Information Privacy Committee has considered and ***approved*** your request for access to data for the purposes of the above named project.

Any significant changes to the proposed study design should be reported to the Chair/HIPC for consideration in advance of their implementation. Also, please be reminded that any manuscripts and presentation materials resulting from this study must be submitted to Manitoba Health, Healthy Living and Seniors for review. Specifically, manuscripts must be submitted *at least **30** calendar days* prior to the intended publication and presentation materials must be submitted *at least **10** calendar days* prior to the presentation.

Researcher Agreement will need to be completed before work on this project can commence. This will be initiated by **MCHP**. If you have any questions or concerns, please do not hesitate to contact [redacted] Committee Coordinator at [redacted]

Yours truly,

[redacted]
Chair, Health Information Privacy Committee

c.c. [redacted]

Manitoba
spirited energy

Appendix D: Winnipeg Regional Health Authority approval

Winnipeg Regional Health Authority | Office régional de la santé de Winnipeg
Caring for Health | À l'écoute de notre santé

4th Floor – 753 McDermot Ave.
Winnipeg, Manitoba
R3E 0T6 CANADA
TEL: 204.594.5321
FAX: 204.789.3956
www.wrha.mb.ca

CHI Evaluation Platform

July 20, 2016

Amani Hamad
Faculty of Health Sciences, College of Pharmacy
University of Manitoba
750 McDermot Avenue
Winnipeg, MB R3E 0T5

Dear Amani:

Re: "Prenatal and Early Childhood Antibiotics Exposure and the Risk for Neurodevelopmental Disorders"

We are pleased to inform you that your request for the above-named study that was submitted in July 2016 has been approved by the Winnipeg Regional Health Authority (WRHA) Research Access and Approval Committee.

Specifically,

I approve the request the access and use of the WRHA's In-Hospital Pharmaceuticals data for the above-named project.

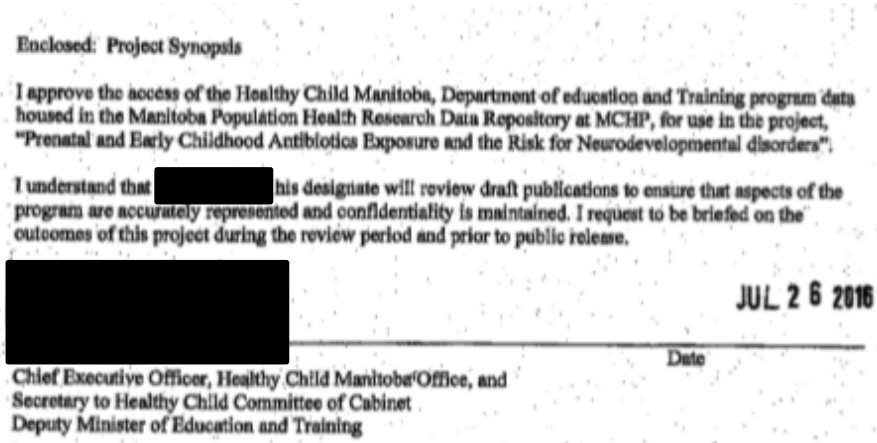
We extend best wishes for successful completion of your study.

Yours sincerely,

[redacted]
Director, Evaluation Platform, Centre for Healthcare Innovation
WRHA Research Access and Approval Committee
Winnipeg Regional Health Authority

cc: [redacted] Chief Privacy Officer, WRHA
ior Executive Director, Division of Quality and System Performance,
Executive Director, Information Management & Analytics, Manitoba
nd Seniors
ordinator
sitory Access Coordinator
P Research Program Coordinator

Appendix E: Healthy Child Manitoba approval



Enclosed: Project Synopsis

I approve the access of the Healthy Child Manitoba, Department of education and Training program data housed in the Manitoba Population Health Research Data Repository at MCHP, for use in the project, "Prenatal and Early Childhood Antibiotics Exposure and the Risk for Neurodevelopmental disorders".

I understand that [redacted] his designate will review draft publications to ensure that aspects of the program are accurately represented and confidentiality is maintained. I request to be briefed on the outcomes of this project during the review period and prior to public release.

[redacted]

JUL 2 6 2016

Date

Chief Executive Officer, Healthy Child Manitoba Office, and
Secretary to Healthy Child Committee of Cabinet
Deputy Minister of Education and Training

Appendix F: Department of Families approval

[redacted]

From: [redacted]
Sent: Friday, July 22, 2016 8:41 AM
To: [redacted]
Cc: +WPG1038 - P&P-ED (FAM)
Subject: FW: MCHP Data Access Request FAMLSP16-00115

Hello [redacted]

Please see Deputy Minister [redacted] approval below for this data access request.

Thank you,

[redacted]

Legislation and Strategic Policy Branch (Department of Families)

[redacted]

From: [redacted]
Sent: 2016-Jul-22 8:36 AM
To: [redacted]
Subject: RE: MCHP Data Access Request FAMLSP16-00115

[redacted] has reviewed/approved

Appendix G: Manitoba Education and Training approval

Manitoba

Education and Training

School Programs Division
Robert Fletcher Building
307–1181 Portage Avenue, Winnipeg, Manitoba, Canada R3G 0T3
T 204-945-7935 F 204-945-8303
www.edu.gov.mb.ca/k12

July 28, 2016

Amani Hamad
Manitoba Centre for Health Policy
Max Rady College of Medicine
408-727 McDermot Avenue
Winnipeg, Manitoba R3E 3P5

Dear Amani Hamad:

Re: Prenatal and Early Childhood Antibiotics and Risk for Neurodevelopmental Disorders

We have reviewed your proposal and approve the access and use of the Department of Education and Training data located in the Population Health Research Data Repository at MCHP for the project entitled, *Prenatal and Early Childhood Antibiotics and Risk for Neurodevelopmental Disorders*.

Sincerely,

[redacted]
Assistant Deputy Minister

[redacted] Deputy Minister