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EARLY LIFE ORIGINS OF ASTHMA
GENETIC AND ENVIRONMENTAL FACTORS IN TWIN AND KIN

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Karolinska Institutet

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EARLY LIFE ORIGINS OF ASTHMA
Genetic and environmental factors in twin and kin

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W*e don’t choose our parents or our siblings. Still, what they share with us in terms of genes and environment will affect us throughout life. So it matters what family we are born into, but only to a certain extent. We are still individuals with the possibility to be totally unique.*

To my family

ABSTRACT

During the last decades there has been an upsurge of studies investigating how early life exposures may affect subsequent health outcomes in childhood. For instance, low birth weight and exposure to antibiotics during fetal or early life have been suggested to increase the risk of childhood asthma. Twin- and sibling comparisons can help to account for confounding factors shared within families and to shed light on potential causal pathways.

Thus, in **Study I**, we aimed to investigate if low birth weight, as a proxy for fetal growth restriction in twins, was associated with the development of asthma in a cohort of 10 918 Swedish twins aged 9 or 12 years. We found a significant association which was thought to be explained by unique individual factors and not due to familial confounding or gestational age. It can be speculated that restricted fetal growth affects lung development in utero which influences the risk of developing asthma in childhood.

In **Study II,** we aimed to further understand the adverse effects of restricted fetal growth, by specifically investigating its association with childhood lung function in a cohort of 576 twins aged 9-14 years at invitation to the study. We found a significant association between fetal growth restriction and reduced forced expiratory volume in the first second, which could be explained by unique factors of each twin. Similar effects were found in non-asthmatic individuals, whereas other factors may be of importance for the association between fetal growth and lung function in individuals with asthma.

To be able to study asthma in register-based studies, a valid measure of the disease is needed. In **Study III**, medical records for roughly 1700 individuals, corresponding to prescription dates of asthma medications or to discharge dates accompanying asthma diagnoses and identified from population-based drug- and patient registers, were collected from health care units and evaluated against pre-defined criteria of asthma. We found a high positive predictive value for asthma medication as a proxy for asthma in older children and the majority of children with an asthma diagnosis in the patient register fulfilled pre-defined criteria of asthma.

In **Study IV**, the previously suggested association between antibiotic exposure in fetal and early life and childhood asthma (based on the validated outcome measure from *Study III*) was investigated in a cohort of 493 785 children. We found an association between antibiotic exposure both in fetal and early life and asthma. Yet, sibling control analyses suggested that the associations were due to shared factors within families, and confounding by indication or reverse causation due to respiratory infections.

In conclusion, shared genetic and environmental factors contributed to the association between antibiotics and asthma, but not between fetal growth and asthma and lung function, respectively. Genetically informed designs to control for familial confounding are useful tools to help provide a clearer understanding of the etiology of asthma. In addition, asthma identified from population-based registers can be used as a validated outcome measure and contribute towards future studies on asthma using register-based data.

SVENSK SAMMANFATTNING

Under de senaste decennierna har ett stort fokus lagts på att försöka förstå hur faktorer under fosterlivet eller tidiga barnår påverkar individens hälsa senare i livet. Det har exempelvis föreslagits att låg födelsevikt, exponering för antibiotika under fostertiden eller att barnet själv har blivit behandlat med antibiotika tidigt i livet leder till en ökad risk för astma senare i barndomen. Tvilling- och syskonstudier kan bidra till ökad förståelse om sambanden är kausala eller om de är orsakade av genetik- och miljöfaktorer som delas inom familjer.

I **studie I** undersökte vi sambandet mellan låg födelsevikt och astma i barndomen. Studien omfattade 10 918 tvillingar, 9 eller 12 år gamla, och våra resultat tyder på att låg födelsevikt ökar risken för astma oavsett om barnet föds för tidigt eller i fullgången tid, samt oberoende av delade genetik- och miljöfaktorer. Resultaten skulle kunna tolkas som att hämmad fostertillväxt påverkar fostrets lungutveckling och därmed risken för astma.

I **studie II** studerade vi därför om fostertillväxt påverkar lungfunktionen i barndomen och om ett eventuellt samband påverkas av astma. Våra resultat, baserade på 576 tvillingar i åldrarna 9-14 år, tyder på att hämmad fostertillväxt leder till försämrad lungfunktion. Sambandet kunde inte förklaras av för tidig födelse eller delade genetik- och miljöfaktorer, och sågs även hos barn utan astma. Det förefaller dock som att andra faktorer verkar påverka sambandet mellan fostertillväxt och lungfunktion hos dem som har astma.

I Sverige finns det flera nationella hälsoregister. För att kunna studera astma i register-baserade studier behövs ett bra utfallsmått. I **studie III** validerade vi således mått på astma i journaler för ca 1700 individer från primär- och specialistsjukvården, identifierade från hälsoregistren. Våra resultat tyder på att astmaläkemedel rapporterade i Läkemedelsregistret kan användas som ett precist mått på astma hos barn, och att majoriteten av barn som har fått en astmadiagnos i specialistsjukvården uppfyllde förbestämda kriterier för en astmadiagnos.

I **studie IV** undersökte vi det tidigare föreslagna sambandet mellan antibiotika under fostertiden eller tidigt i livet och astma i barndomen i en syskonstudie, där astma identifierats från hälsoregistren (baserat på studie III). Studien omfattande nästan en halv miljon barn och våra resultat tyder på att antibiotika i sig inte orsakar astma utan att sambandet orsakas av delade faktorer inom familjer samt luftvägsinfektioner.

Sammanfattningsvis talar våra resultat för att delade genetik- och miljöfaktorer bidrog till sambandet mellan antibiotika och astma, men inte till sambandet mellan hämmad fostertillväxt och astma respektive lungfunktion. Vi har också visat att astma identifierat från hälsoregister kan fungera som ett bra mått på astma, och kan användas i framtida studier. Slutligen, tvilling- och syskonstudier i kombination med information från populations-baserade register kan bidra till ökad förståelse av tidiga orsaker till astma hos barn.

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(not included in thesis)

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II. Tedner SG, Örtqvist AK, Almqvist C. Fetal growth and risk of childhood asthma and allergic disease. Clin Exp Allergy. 2012;42(10):1430-47.

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LIST OF ABBREVIATIONS

ATC	Anatomical Therapeutic Chemical
ATS/ERS	American Thoracic Society/European Respiratory Society
BPD	Bronchopulmonary Dysplasia
β2	Beta-2 adrenergic receptor
CATSS	Child and Adolescent Twin Study in Sweden
DAG	Directed Acyclic Graph
DZ	Dizygotic
FEV_1	Forced Expiratory Volume in first second
FVC	Forced Vital Capacity
ICD 10	International Classification of Disease, version 10
ICS	Inhaled Corticosteroids
ISAAC	International Study of Asthma and Allergies in Childhood
LISA	Longitudinal Integration database for Health Insurance and Labor Market Studies
LLN	Lower Limits of Normal
LTRA	Leukotriene Receptor Antagonist
MBR	Medical Birth Register
MGR	Multi-Generation Register
MZ	Monozygotic
NBHW	National Board of Health and Welfare
NPR	National Patient Register
PEF	Peak Expiratory Flow
PIN	Personal Identification Number
PPV	Positive Predictive Value
RDS	Respiratory Distress Syndrome
SD	Standard Deviation
SGA	Small for Gestational Age
SPDR	Swedish Prescribed Drug Register
STOPPA	Swedish Twin study On Prediction and Prevention of Asthma
STR	Swedish Twin Register
Th	T-helper cell
TPR	Total Population Register

1 INTRODUCTION

1.1 ASTHMA

"Every breath counts", the slogan of the European Respiratory Society

1.1.1 History

People have suffered from asthma for millennia. The term asthma originates from the Greek word aazein which means to pant or exhale with open mouth (1). First descriptions of asthma symptoms in writing are found in the Illiad of Homer (700 BC) and were first used as a medical term by the schools of Hippocrates (460-357 BC), even though asthma then was more looked upon as a symptom related to any type of dyspnea and not as clinical entity in itself. Five hundred years later, Aretaeus the Cappadocian, and much later Floyer in his Treaties of Asthma (in 1698), described asthma largely matching the clinical descriptions used today (2).

1.1.2 Prevalence

Asthma is a common chronic disease among children worldwide (3) and approximately one in ten children in Sweden has asthma (4, 5) (*Figure 1*). It is a disease afflicting all socioeconomic classes; however, low socioeconomic status has been reported to increase the disease risk (6). While mortality and hospitalization rates of asthma have decreased in the

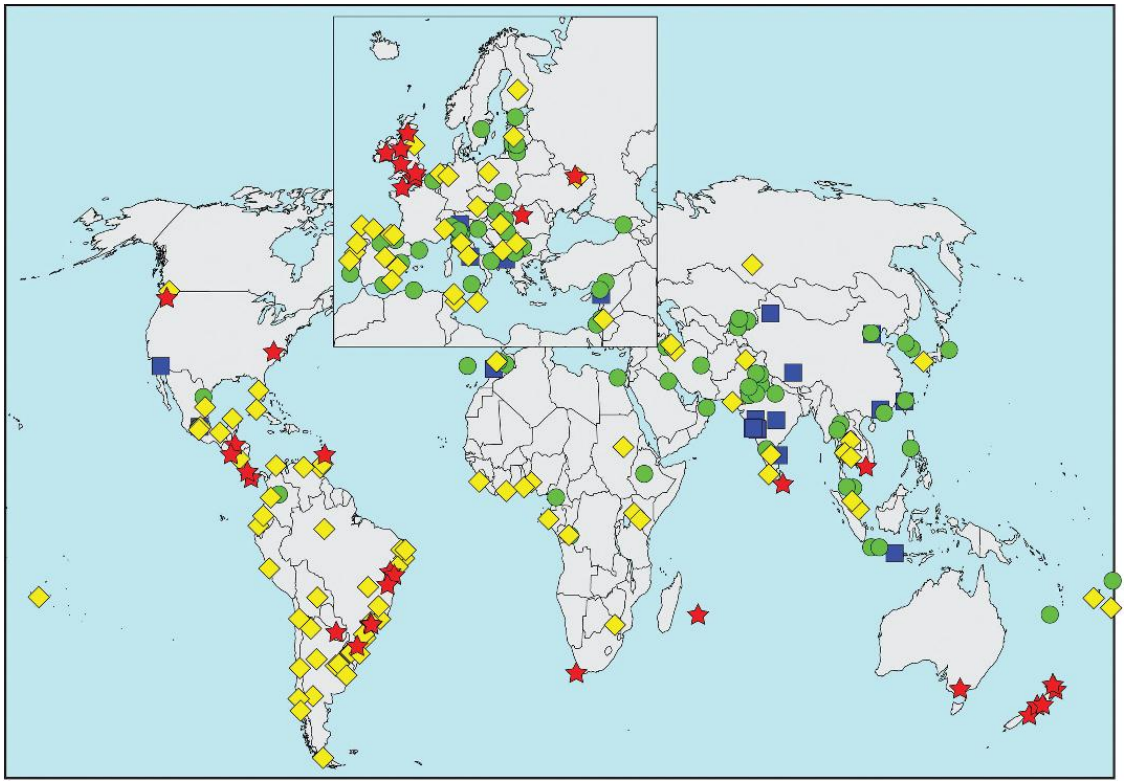


Figure 1. Prevalence of current wheeze according to a written questionnaire in 13–14 year olds from the Phase Three of the International Study of Asthma and Allergies in Childhood. The symbols indicate prevalence values of <5% (blue square), 5 to<10% (green circle), 10 to <20% (yellow diamond) and >20% (red star). *From Lai CK, et al. Thorax. 2009;64(6):476-83. Reproduced with permission from BMJ Publishing Group Ltd.*

last decades (7, 8) the prevalence of the disease has increased all around the world (9). Although some recent studies have suggested that the increase in asthma prevalence seem to have reached a plateau (10-12), the direct and indirect costs for asthma are, and will continue to be, high (13).

1.1.3 Characteristics

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by a history of recurrent episodes of wheezing, breathlessness, cough, and tightness in the chest, which reverse spontaneously or after use of medication (14, 15). The childhood asthma disease spectrum is widely recognized and it has been suggested that asthma is not just one disease, but many united by some common clinical features such as intermittent wheezing and reversible airway obstruction (15).

The term 'phenotype' can be used to describe any observable properties or traits of an organism, and is supposed to arise from the interaction of the individual's genes with the environment. Examples of different phenotypes of asthma are atopic and non-atopic asthma, different wheezing phenotypes (transient, intermediate-onset, late-onset and persistent), and obese non-eosinophilic asthma (15-19). The disease severity differs from child to child, and the disability associated with asthma can differ with the level of treatment and disease control (13, 14, 20).

There are three characteristic pathophysiological changes seen in the asthma patient (14, 15); 1) the presence of airway inflammation, where more than 100 different mediators (chemokines, cytokines, histamine etc.) have been identified; 2) structural changes often described as airway remodeling with increased smooth muscles, mucus hyper secretion and narrowing of the airways, due to contraction of smooth muscle and local edema, and; 3) airway hyper responsiveness, due to stimuli of free nerve ends that are uncovered when the epithelial in the airways is damaged.

1.1.4 Clinical diagnosis

The majority of Swedish children receive their asthma diagnosis in the primary health care clinics or at specialist pediatric outpatient clinics. A diagnosis is based on a thorough patient history regarding current symptoms, family history of asthma and allergic disease, home environment, potential triggers, and previous medical history, together with a clinical examination including measures of lung function and allergy tests.

Dynamic spirometry and lung function

Lung function is one important determinant of asthma, and can be evaluated through dynamic spirometry from school-age and onwards (21), whereas other techniques may be used in younger children (22). Spirometry is a standardized test in terms of performance, methodology and equipment specifications (21). The procedure is highly dependent on the individual's cooperation, and imprecision in the lung function measurement may arise when

competent guidance and support from trained personnel is lacking. A reversibility test with inhalation of a short-acting bronchodilator may identify obstructive airways (21).

The procedure creates a flow-volume loop, where at the start of the test, both flow and volume are equal to zero (*Figure 2*). When the patient exhales forcibly, the curve rapidly mounts to a peak, which is referred to as the peak expiratory flow (PEF). After the PEF, the curve descends and the flow decreases as more air is expired. A normal flow-volume loop will descend in a fairly straight line from the top until reaching the bottom, where the forced vital capacity (FVC) is measured. The forced expiratory volume in the first second (FEV_1) is the volume of air which can be forcibly exhaled from the lungs during the first second of a FVC procedure.

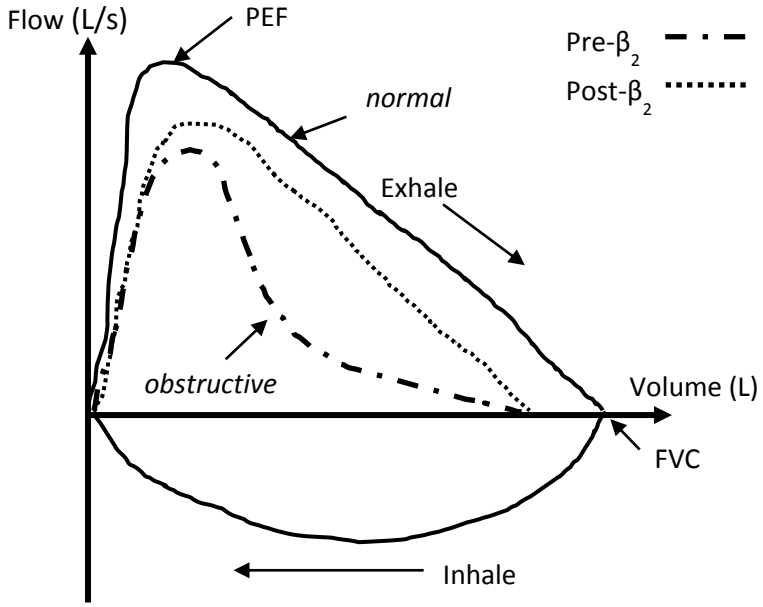


Figure 2. Spirogram with a normal flow-volume loop and a concave flow-volume loop before bronchodilator (pre-β_2), indicating airflow limitation, and after bronchodilator (post-β_2).

Characteristics of the spirogram in individuals with obstruction of the airways, such as individuals with asthma, include a concave flow-volume loop, a lower FEV_1 to FVC ratio indicating obstruction, and lower levels of FEV_1 before bronchodilator use in relation to reference values taking age, sex, height and ethnicity into account (*Figure 2*) (14, 23). A clinically relevant threshold is commonly defined as <80% of the predicted FEV_1 value or as <LLN (lower limits of normal) in z-scores, which is usually <-1.64 standard deviations from the average (23). In addition, ≥12% reversibility, assessed as percent change in FEV_1 from pre to post inhalation of a bronchodilator, is in line with a diagnosis of asthma. However, individuals with lower levels of reversibility (~10%) may have some relief from symptoms with improved treatment. It should also be noted that lung function measurements can be normal in asthmatic individuals with well-controlled asthma (14). In cases when the spirometry is normal, but the patient history still suggests asthma, other diagnostic tools may be used (14, 24).

1.1.5 Treatment

The treatment of asthma is both pharmacological and non-pharmacological (which involves environmental interventions such as elimination of allergens or other triggers). Pharmacological treatments include inhaled β2-agonists, inhaled corticosteroids (ICS) and leukotriene receptor antagonists (LTRA) (*Figure 3*)(15).

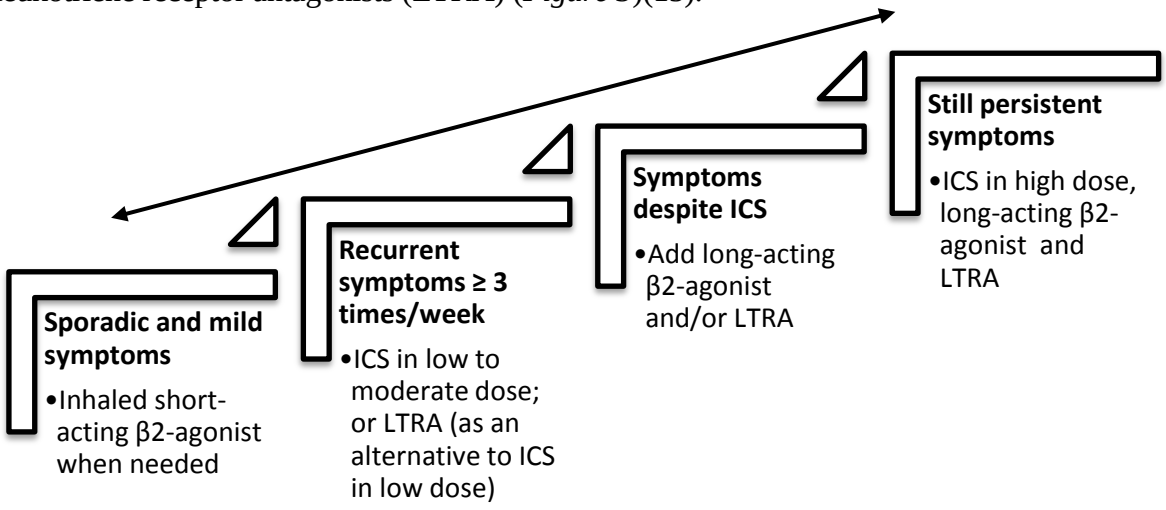


Figure 3. Pharmacological treatment of asthma in children ≥6 years of age, based on treatment algorithm recommended by the Swedish Pediatric Society's section of Allergy. Short-acting β2-agonists should be used at all levels if the child has symptoms. The arrow indicates the importance of increasing treatment when needed or reducing treatment when possible. For exact dosages and treatment algorithm in younger children, see www.barnallergisektionen.se or www.fass.se

1.1.6 Measures of asthma in observational studies

Owing to the variable nature of asthma symptoms both between and within individuals, clinical assessment is regarded the best method to identify asthma (25). However, medical examinations are costly and other methods such as child and parental answers to standardized questionnaires, *e.g.* those developed in the International Study of Asthma and Allergies in Childhood (ISAAC) (26), have often been used and proved valid in measuring prevalence of asthma symptoms (27, 28).

Sweden has a long tradition of register-based research (29). Using register-based data is one way to make use of already collected longitudinal information for a large number of individuals. Using such data limits costs, time spent on collection, and also avoids potential recall bias. As the data are population-based, the findings are also generalizable, at least within the specific country and time period. As asthma in Sweden is often diagnosed in the primary health care clinics, the diagnosis will not be recorded in the patient register as it only includes information from in- and outpatient hospital clinics (30). Instead, a validated measure, based on filled prescriptions of asthma medication, may function as proxy for the disease.

1.2 EARLY LIFE ORIGINS OF ASTHMA

Today, we know that asthma is a multifactorial disease where environmental and genetic factors interplay (31). Numerous environmental factors have been reported to increase the risk of asthma such as viral infections, pre- and postnatal exposure to tobacco smoke, air pollution and low socio-economic status. Others have suggested that children growing up on farms or have older siblings may have lower risk of asthma. In addition, numerous genetic variants have been associated with asthma. While these factors are important, they lie outside the scope of this thesis. The focus of this thesis has been to further study fetal growth and exposure to antibiotics in fetal and early life as potential risk factors for asthma. No specific genetic variants are addressed in this thesis. Instead, genetic factors that are shared within twin pairs and siblings in families are taken into account in the analyses as potential familial confounders for the associations between fetal growth/antibiotics and asthma/lung function.

1.2.1 Fetal growth

"And the day came when the risk to remain tight in a bud was more painful than the risk it took to blossom" Anaïs Nin[*]

Pregnancy duration and antenatal care in Sweden

In Sweden, most pregnant women attend an antenatal care clinic from approximately week 10 of pregnancy up until delivery, with more frequent visits closer to the expected due date. Duration of pregnancy can be estimated according to Naegele's rule resulting in a pregnancy duration of approximately 280 days (40 weeks) (32). The fetus' gestational age, can also be estimated by ultrasound, which has been routinely offered to all pregnant women in Sweden around week 16-21, since the 1990's and accepted by almost all women (33). A term birth takes place between week 37 and 42 of gestation. Delivery before week 37 is thus defined as preterm and after week 42 post-term. The pregnancy duration is furthermore divided into three trimesters; 1st (day 1-91), 2nd (day 92-189) and 3rd (day 190 to delivery).

Birth weight, fetal growth restriction and small for gestational age

Birth weight is the product of both gestational age and fetal growth. Normal birth weight for a term infant in Sweden is between 2700 and 4400 grams (34, 35) and low birth weight is defined as a weight below 2500 grams (36). Historically, birth weight has frequently been used by many countries as an indicator for fetal growth, since it is a practical and inexpensive measurement (36). However, birth weight is a single-point measurement and does not take gestational age into account.

At the antenatal care clinics, rough estimates of fetal growth is measured throughout the pregnancy usually based on maternal weight gain, symphysis-fundus height measurements and complemented by ultrasound measurements if any deviations from the expected growth curve occur (37, 38). 'Fetal growth restriction' implies that fetal growth has been restricted by

[*]Author disputed; however, the quote is frequently attributed to Anaïs Nin, but without a specific source to her work.

factors affecting the intrauterine environment or the delivery of nutrients to the fetus, which prevents the fetus to reach its full growth potential (37). A surrogate, but not strictly synonymous, term that is usually used instead, is 'small for gestational age' (SGA) which, by definition, implies that the birth weight falls below the 10th percentile (39) or <2 SD below the mean weight for sex-specific gestational age (35). However, it should be recognized that SGA infants may either be pathologically or constitutionally small.

The determinants of fetal growth are a fetus with a certain growth potential, a space to grow in and supply line to enable it. The mother influences the potential for growth both by providing genes and intrauterine environment (which in turn will be a result of both her genes and environment). Determinants of fetal growth can be divided into genetic, demographic, placental, environmental and medical factors, where the most common causes for restricted fetal growth in the western world are placental insufficiency and smoking (*Figure 4*) (37, 40-42).

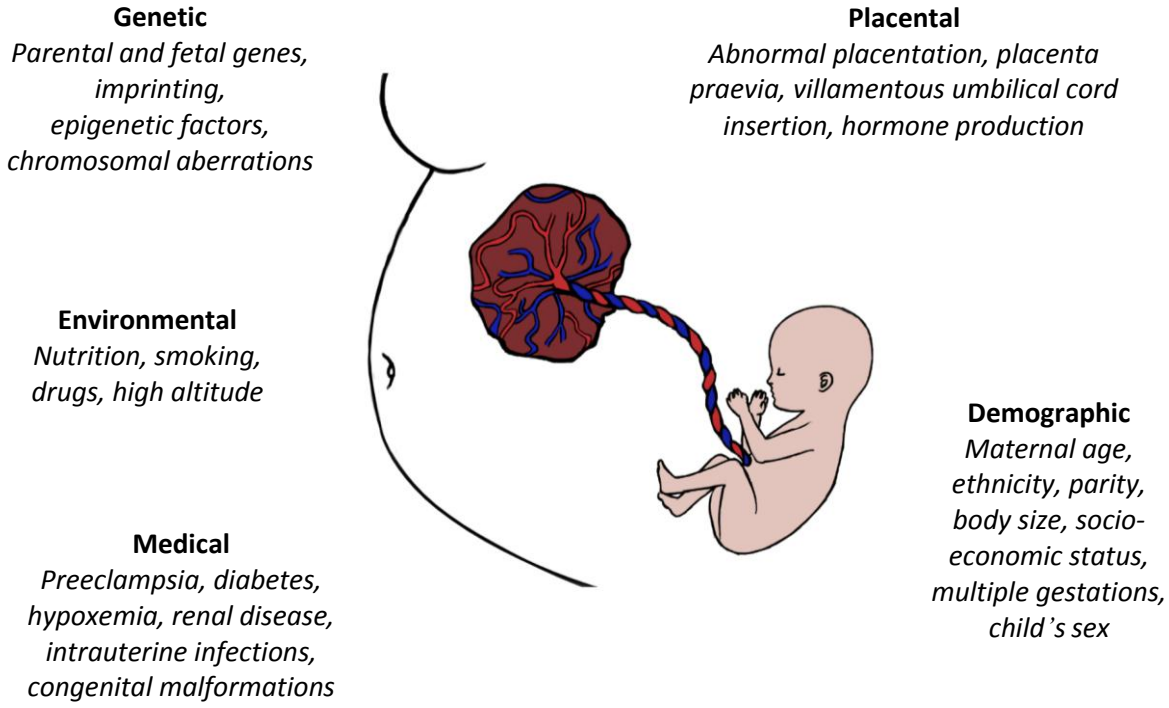


Figure 4. Determinants of fetal growth. *Figure modified from Tedner SG et al. Clin Exp Allergy 2012;42(10):1430-47. Reproduced with permission from John Wiley and Sons.*

Fetal growth and respiratory health

Appropriate fetal growth is important for health in both the short and the long term perspective (37, 43, 44). The first researchers to propose that early life deprivation could predispose later health, more specifically the risk of coronary heart disease, in what has become known as the 'fetal origin hypothesis', were David Barker and Clive Osmond in 1986 (45, 46), building on findings from others (47-49). The hypothesis has been elaborated and refined several times and evolved into the Developmental Origins of Health and Disease (DOHaD) hypothesis, and suggests that the fetus makes physiological adaptations in response

to under-nutrition in utero to prepare itself for postnatal conditions. These adaptations may be beneficial in utero, but may be unfavorable if the postnatal environment has been wrongly 'predicted'(50). There is increasing epidemiological evidence that respiratory health is similarly modified through perinatal experiences, as proposed in the Developmental Origins of Respiratory Health and Disease (DORHaD) concept (44, 51).

Overview of lung development

The development of the lung is initiated around 4 weeks of gestation (embryonic phase), as a derivate of the foregut and by the sixth week of gestation, the two lungs can be distinguished as separate structures (52). During the next stage (pseudo glandular phase), all conducting airways and blood vessels are formed and epithelial cells appear, followed by growth and maturation of the peripheral airways, and differentiation of epithelial cells to form type 1 and type 2 alveolar cells (canicular phase). Alveoli start to appear in week 29 (saccular-alveolar phase) and continuously increase in number throughout the pregnancy. By 40 weeks of gestation, approximately 100-150 million alveoli have formed. Although numbers of alveoli are thought to be complete by 2–4 years of age, alveoli might have the capability to multiply beyond this age (52, 53). After birth, lung size increases proportionally with age, height, sex and ethnicity (23). Maximum lung volumes are reached around the age of 22 years in men and slightly earlier in women, after which point FEV_1 and FVC decline with age, even in healthy individuals due to gradual loss of elasticity (52).

Birth characteristics and asthma and lung function

Previous studies investigating birth characteristics such as birth weight, gestational age and fetal growth and asthma and lung function have found conflicting results. In studies linking birth weight with asthma some have suggested an increased risk of asthma with low birth weight (54-57), or an increased risk with high birth weight (58), while still others have found no association (59-62). Furthermore, while some have found an association between gestational age and asthma (54, 63, 64), others have not (56, 61, 62). Studies that have investigated the association between fetal growth and asthma are more sparse, potentially due to difficulties in properly assessing fetal growth. However, studies using SGA as marker of fetal growth have also found inconsistent results where some have indicated an increased risk of asthma (64, 65), and others have found no association (54, 62), or even an inverse association with a decreased risk of asthma (66).

Previous studies have also reported that being born preterm (67-69) and with low birth weight (70-72) is associated with lung function deficits in child-and adulthood, whereas others have found no association (73, 74). As for asthma, the association between fetal growth and lung function has been less well studied. However, some studies have found an association between SGA and impaired lung function (75, 76).

Potential pathways

Figure 5 illustrates potential pathways of the association between fetal growth and asthma and lung function. For instance, genetic factors play an important role in fetal growth (77-80), in asthma and lung function (31, 81-86). However, it remains unclear how genetic determinants for fetal growth and asthma and/or lung function are related, and whether reported findings on *e.g.* epigenetic modifications are causal or merely a response to an adverse intra- or post-uterine environment. Environmental factors in utero and/or in postnatal life that have been investigated as potentially important for fetal growth, asthma and/or lung function, include maternal smoking during pregnancy (87), vitamin D (52, 88, 89), air pollution (90-92), viral infections (93) and early allergen sensitization (94). It also seems possible that postnatal growth and obesity may be of significance for the development of asthma (95, 96) and lung function (76, 97).

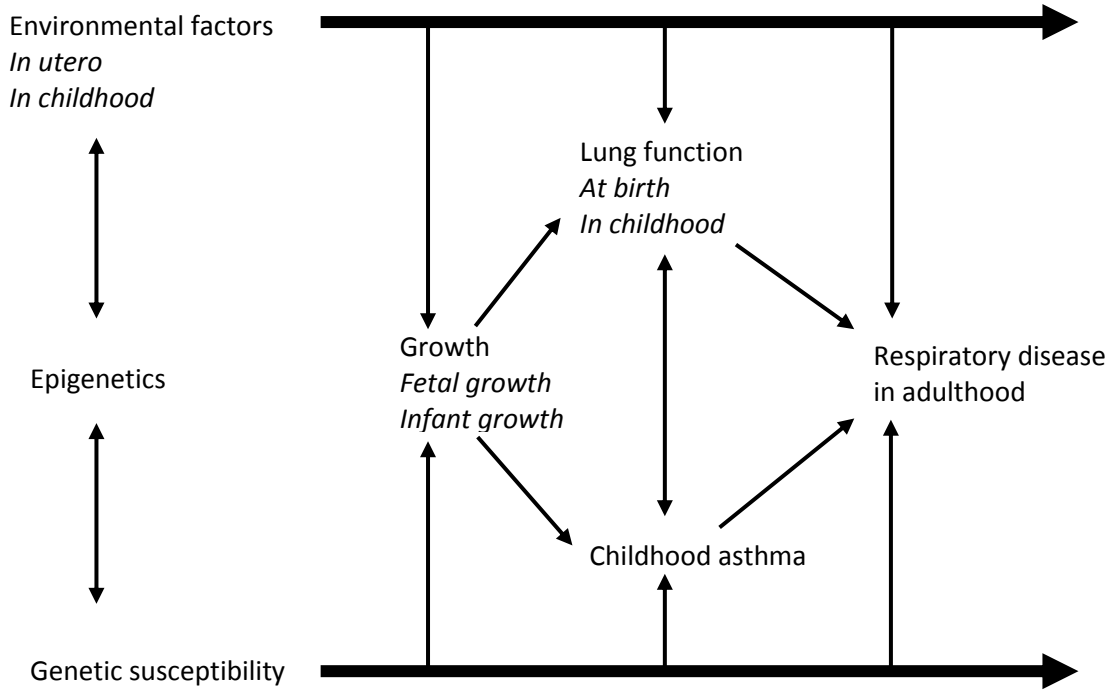


Figure 5. Potential pathways leading from adverse fetal and childhood exposures to growth adaptations and respiratory health outcomes.

Major concerns regarding previous literature lie in the fact that birth weight, gestational age, and fetal growth are strongly correlated, and that it has been difficult to disentangle effects from these exposures. Although studies have controlled for confounders, residual confounding from factors that are difficult to measure, such as genetic susceptibility, diet during pregnancy, and socio-economic status, may still be a problem and may lead to spurious conclusions. Here, studies of twins may function as a tool to disentangle true effects from genetic and environmental confounding. In addition, studies of twins may allow investigating fetal growth independently of gestational age, due to the fact that twins are born at the same gestational age. Thus, a difference in birth weight within a twin pair may be regarded as difference in fetal growth.

1.2.2 Antibiotics

"One sometimes finds what one is not looking for" Sir Alexander Fleming

The discovery

The introduction of antimicrobial agents into general clinical practice denotes one of the milestones of medical advances in modern medicine. In 1928, Alexander Fleming became famous after discovering a mold identified as Penicillium notatum, in a Petri dish seeded with the bacteria Staphylococcus aureus, which actively killed the bacteria (98). After the discovery of penicillin, and during the last half of the 20th century, a number of new antimicrobials came into clinical use, giving clinicians a variety of choices when treating many types of infectious diseases that had previously been deadly or caused major adverse health effects (99).

Prescription patterns

Figure 6 illustrates the consumption of antibiotics in outpatient care in different European countries (100). The first choice of antibiotics for all European countries is penicillin, but the proportion of different types of penicillin varies (101). While the consumption of antibiotics has slowly decreased in Sweden during the last decade, the consumption of antibiotics globally has increased (102).

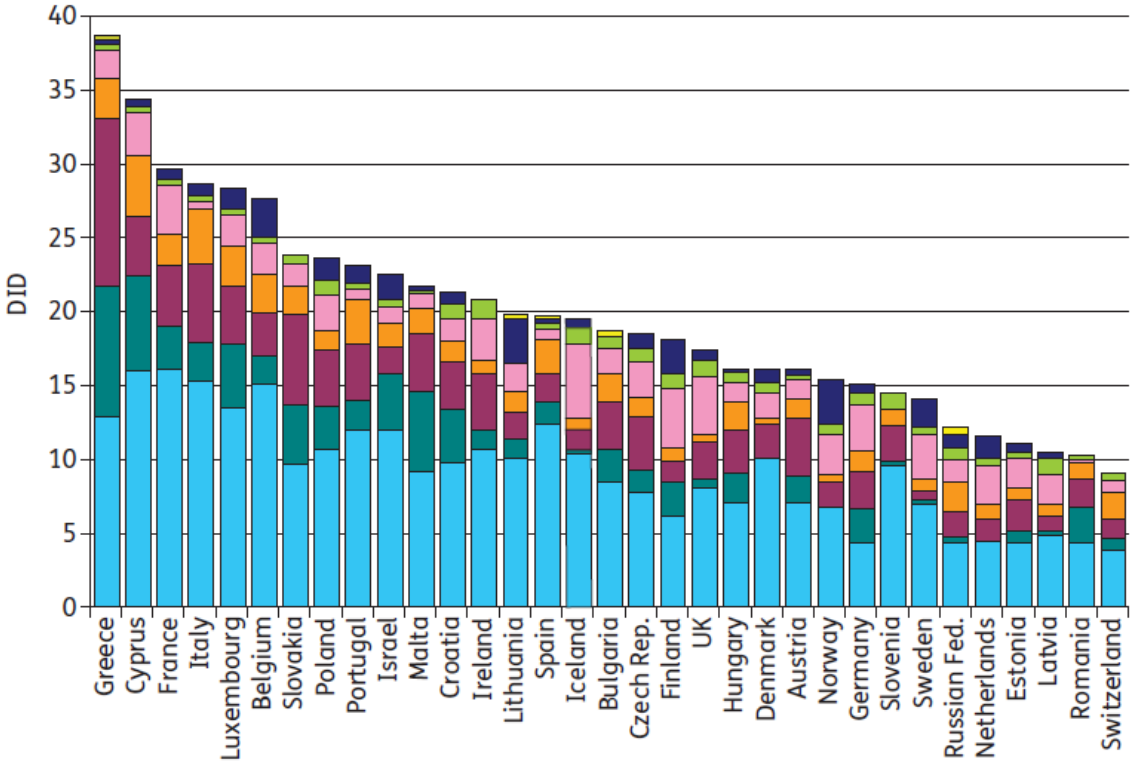


Figure 6. Total outpatient antibiotic use in 33 European countries in 2009. DID – defined daily doses per 1000 inhabitants per day; Light blue – Penicillins; dark green – Cephalosporins; purple – Macrolides; orange – Quinolones; pink – Tetracyclines; light green – sulphonamides; dark blue: urinary antiseptics; yellow – others. *From Adriaenssens N, et al. J Antimicrob Chemother. 2011;66 Suppl 6:vi3-12. Reproduced with permission from Oxford University Press.*

Notably, the effectiveness of antibiotics against bacterial infections has been reduced as bacteria have developed ways of staying alive and becoming resistant to antibiotics (103). As the majority of antibiotic prescriptions are issued in outpatient care, a reduction in these prescriptions would have a large impact on the total use of antibiotics (101, 104). Acute respiratory infections are commonly treated with antibiotics in outpatient care (105), and children contribute to a key part of the prescriptions filled (106). Yet, acute respiratory infections in children are most often viral or of non-severe bacterial etiology (105), and the effectiveness of antibiotics in these cases is limited.

Antibiotics and asthma

The increasing use of antibiotics (100) has co-occurred with the rising prevalence of childhood asthma (9). This has led to a surge of observational studies investigating the potential harmful role of exposure to antibiotics in fetal and early life and subsequent development of childhood wheeze and asthma (107, 108). In 1998, Farooqi and Hopkin suggested that exposure to antibiotics in early childhood increases the risk of asthma (109). Not long after, Benn and colleagues reported that exposure to antibiotics in fetal life increases the risk of subsequent childhood asthma (110). So far, the majority of studies investigating the association between antibiotic exposure and asthma have found an increased risk of asthma (107-117); however, evidence for a causal association is lacking.

Potential mechanisms

The 'hygiene hypothesis', proposed by Strachan in 1989 suggests that the lack of microbial exposure, as a result of very hygienic conditions in early life, may have an impact on the balance of the immune system, leading to the development of allergic diseases (118). With the identification of T-helper cells type 1 (Th1) and type 2 (Th2), the theory became plausible as some environmental factors seemed to be able to induce an allergy-protective immune response by Th1 cells (119). Based on the hygiene hypothesis, it is rational to propose that the increase of early life exposure to antibiotics reduces impact of microorganisms and subsequently promotes allergic immune responses. For instance, antibiotics have been suggested to induce a Th2-skewed response, driving the immune system towards an allergic pathway (120).

In 2005, Mairi Noverr and Gary Huffgnale presented an alternative interpretation of the data supporting the 'hygiene hypothesis', namely the 'microflora hypothesis', which proposed that alterations in the gastrointestinal microbiota due to antibiotics may disturb mechanisms of immunological tolerance in the mucosa, leading to an increased risk of allergic airway disease in genetically susceptible individuals (121).

Different possible biological mechanisms have been suggested for the association between fetal exposure to antibiotics and childhood asthma. It has been hypothesized that maternal antibiotic treatment could activate a disease process in perinatal life in susceptible individuals, or that maternal antibiotics may alter the microflora colonization patterns in neonatal life, or that inflammation due to a maternal infection could modulate the fetus'

developing lungs, leading to subsequent asthma (60, 122-124). However, clear confirmed biological explanations are lacking.

Despite these biologically plausible links between antibiotic use and asthma, it has also been suggested that antibiotics may not cause asthma at all, but that the observed associations have been due to confounding by indication or reverse causation. This could be the case because of respiratory infections and diagnostic uncertainty in the nature of wheeze in young children (125) or attributable to genetic predisposition to both infection and asthma (122, 126). The association could also be due to residual confounding (127). Studies of siblings may function as useful tool to disentangle potential causal effects due to antibiotics from effects due to genetic and environmental confounding, as siblings may be differently exposed to antibiotics, but at the same time share genes, early life environment and parental factors (127).

1.3 TWIN AND KIN

Twins have stimulated myths and stories for centuries, from Greek mythology to Shakespeare's "Comedy of errors". Sir Francis Galton noted in 1875 that the study of twins may allow *"to weigh in just scales the respective effects of nature and nurture"* (128) and twins have continued to intrigue and inspire scientists, owing to the possibility to partition the observed variance of a trait into genetic and environmental variation. As an extension of twin studies, methods comparing full- and half siblings within families, cousins, or offspring of twins or siblings, have been developed (127). In this thesis, we take advantage of the unique features of twins and siblings to further assess causality of previously suggested associations.

In Sweden, about 110,000 children are born each year and each woman gives birth to approximately two children, meaning that most children will have a sibling at one point. Approximately 3% of the births are multiple births, the majority of which are twin births. Twinning occurs as a result of the implantation and maintenance of two embryos and can happen spontaneously in two ways; either through an early splitting of one fertilized egg (zygote) or through separate fertilization of two eggs (129). Approximately one-third of all twins come from one fertilized zygote and are accordingly called monozygotic (MZ), whereas the remaining two-thirds stem from two zygotes and are thus called dizygotic (DZ). During the last decades, the rate of twinning has increased around the world, which is thought to be due to increasing maternal age at first child and assisted reproductive therapies (130).

Differences in fetal growth

Compared to singletons, twins will need to share both the space and supply line from the mother in utero, where the foundation for this sharing depends on the placentation (129). An embryo is enclosed in the inner amnion membrane, and the outer chorion, which is the connection to the maternal circulation via the placenta. Embryos that are separate, as is the case for singletons and DZ twins, will develop their own membranes and are able to form individual placentas (although some of the DZ placentas will fuse together). MZ twins may also have their own placenta if the division of the fertilized egg occurs before the formation of the chorion. If the division of the egg happens later, the MZ twins will have their own

amnion membrane, but there will only be one chorion and thus one placenta to be shared by the twins. In rare cases, the MZ twins will share both amnion and chorion (129).

Up until the 3rd trimester, the prenatal growth of twins is commonly described to be similar to that of singletons (131). From then on it has been suggested that the twin growth velocity is down-regulated as a response to the environment when the combined size of the fetuses goes beyond a certain threshold (132). In a cohort of twins, fetal genes, maternal factors and placental function are expected to influence the variation in fetal growth. Zygosity and chorionicity further influence fetal growth, which probably is a reflection of the importance of placentation and the location of the chord insertion (129). Compared to singletons, the unique constraints on twins, due to their sharing of space and supply line, likely overshadow most other influences on growth. The variation in growth between unrelated twins is likely influenced by the same determinants as described for singletons, *i.e.* the individual growth potential and supply line supporting it.

Shared genes within families – genetically informed samples

A fertilized human egg should contain a nucleus with our genome separated into 46 chromosomes divided into 23 pairs, where one chromosome in each pair comes from the mother and the other from the father. Since we get half of our chromosomes from our mothers and half from our fathers, singleton full siblings will thus share, on average, 50% of their segregating genes, as will DZ twins that stem from two separately fertilized eggs. Half siblings will consequently share approximately 25% of their segregating genes. On the other hand, MZ twins that stem from one fertilized egg will share 100% of their genes. While the genetic setup is expected to be the same for MZ twins, epigenetic modulations of the DNA (potentially influencing how genes are expressed) are unique for the individual, thus MZ twins also differ to some extent (133). As a result of the genetic similarities and dissimilarities between twins and siblings it is possible to partition the observed variance of a trait into genetic and environmental variation.

Unique and shared environment

In twin and siblings studies, the environmental variance of a trait is commonly divided into unique and shared environment. The shared environmental factors are factors common to a twin pair or siblings in a family, such as socioeconomic status, home environment, upbringing, and health seeking behavior. These are factors that make twins in a twin pair or siblings in a family more similar. The unique factors are those that each individual experiences throughout life, such as exposure to certain medications and treatment procedures, infections or other medical conditions, personal tobacco usage, physical activity and diet. A difference in shared intrauterine environment for MZ and DZ twins is largely ascribed to the MZ twins who share a placenta, which have a greater potential for inter-fetal exchange (through vascular anastomoses).

1.4 CAUSAL INFERENCE IN OBSERVTIONAL STUDIES

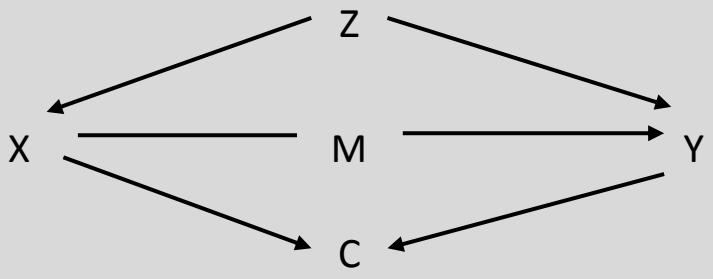
Causal inference is the procedure used to help draw conclusions whether an occurrence of an effect is caused by an exposure or intervention or only associated with it (134). By randomizing individuals to different interventions in an experimental study, the investigator aims to control all parameters of the study, including the timing of the exposure in relation to the outcome. This means that one can, with rather high certainty, assume that any observed association between the exposure and the outcome was caused by the intervention. When randomized controlled trials are not possible due to economical, practical or ethical reasons, observational epidemiological studies can be used to investigate associations between exposures and outcomes, *i.e.* to identify potential risk or protective factors for a disease. However, due to the fact that practically all environmental exposures are non-randomly assigned in the population (for instance, some people are more prone to perform regular physical activity whereas some are more prone to smoke) one has to ask if associations seen in an observational study are causal or induced by problems inherent to the study design. When assessing causality in an epidemiological study, Directed Acyclic Graphs (DAGs) may be used (*Box 1*) (135).

Box 1. A note on DAGs

The DAG can be applied to various analyses, for example, a study of the association between an exposure (X) and an outcome (Y). A directed arrow between these two variables indicate that X may cause Y, but the arrow does not say anything about whether the association is positive or negative, or about strength of the association.

A known and measured factor that is a common cause to the exposure and the outcome is defined as a confounder (denoted Z). A factor that lies in the causal pathway between the exposure and the outcome is defined as a mediator (denoted M). There are almost always mediators on the causal pathway, but this does not indicate that the exposure is not causal.

When the exposure and the outcome are common causes for a third factor (a common effect), this factor is called a collider (denoted C). Pathways through colliders are closed, unless the collider is adjusted for which will then open the path and potentially cause spurious associations.



1.5 INTERNAL VALIDITY

An epidemiological study can be viewed as an exercise in measurement, where the goal is to obtain an accurate result, with as little error as possible. Validity distresses how closely we

measure what we intend to measure. Bias, a term used to describe any type of systematic error in a study, can infiltrate epidemiological studies from numerous directions and threaten the internal validity of the study (134). Efforts to avoid systematic errors include appropriate study design, data collection and analysis. In this thesis, bias will be classified in three broad categories; confounding, selection, and information bias.

1.5.1 Confounding

Mere associations do not mean causality; thus, even though a specific exposure and a specific outcome may be associated, the exposure might not cause the outcome. Confounding of an association is defined as when there is a common cause of exposure and outcome and can be thought of as a mixing of effects (134). For a factor to be a confounder, it cannot be an effect of the exposure, thus acting as a mediator in the causal pathway. Confounding can cause bias in either direction, *i.e.* an over- or underestimation of the effect. In some cases, the bias from confounding can be strong enough to reverse the supposed direction of an effect (136).

*Figure 7*and *8* display two examples of confounding. In the first example, a measure of the confounder maternal smoking can be relatively easily identified, for instance in the Medical Birth Register or through questionnaires (even though the validity of the variable has to be taken into consideration). In the second example, information on the confounder respiratory infections could potentially be found in medical records or collected through parental questionnaires, or identified by using different groups of antibiotics as a proxy measurement for the type of infection that is assumed to be treated.

Figure 7. The exposure birth weight, the outcome asthma and the confounder maternal smoking during pregnancy.

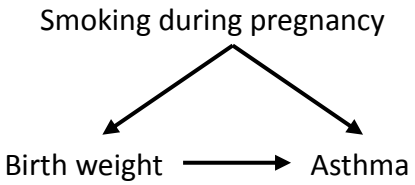
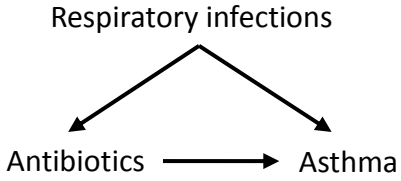


Figure 8. The exposure antibiotics, the outcome asthma and the confounder respiratory infections. This type of confounding is called *confounding by indication*.



If confounding is not controlled adequately in the study design by *e.g.* randomization or matching, it is possible to adjust the association for this variable, thereby closing the pathway from the exposure to the outcome through the confounder. There are standard methods for this, such as stratification which means estimating the association separately for different levels of the confounder. One can also use regression model adjustments which are used to find estimates averaged over the different levels of the confounder. As the layers of confounding are left behind, one approaches deeper causal understanding of the underlying biology.

Another source of confounding comes from variables that are shared within families, including genetic and environmental factors, from now on denoted as *familial confounding*. Two examples of familial confounding in this thesis are displayed in *Figure 9 and 10.*

Figure 9. The exposure birth weight, the outcome asthma and the confounder diet during pregnancy – environmental confounding.

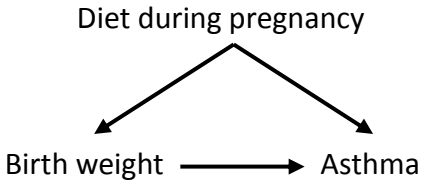
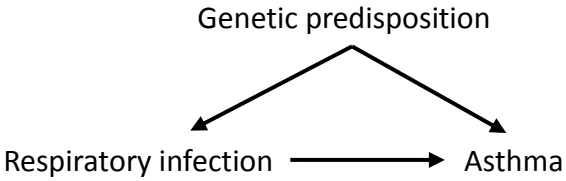


Figure 10. The exposure respiratory infection, the outcome asthma and the confounder genetic predisposition – genetic confounding.



In these examples, diet patterns and genetic predisposition are variables that are difficult to measure with high validity in observational studies, and are therefore often left out in analyses and referred to as *residual* or *unmeasured confounding.* Studies that ignore these types of unmeasured genetic and environmental familial confounders may therefore produce biased inference (127).

1.5.2 Selection bias

The term "selection bias" is commonly applied to biases resulting from inappropriate selection of controls in case-control studies, from differential loss-to-follow up in longitudinal studies, volunteer bias, healthy-worker bias, and nonresponse bias (134). The source of those biases arise from conditioning on a common effect (*Figure 11*) (137). Non-random selection in itself does not automatically result in selection bias, but for selection bias to arise, the intended exposure as well as outcome of the study must be associated with the common effect, *e.g.* participation status (137).

Figure 11. An association between birth weight and asthma is introduced by conditioning on participation in the study if this is an effect of a cause of birth weight through socio-economic status, and of a cause of asthma through family history of asthma.

1.5.3 Information bias

When the exposure, outcome or other variables in the model, are not measured perfectly, information bias (misclassification) may occur (134). If the misclassification of the exposure is random with respect to any other variable in the model, or vice versa, the misclassification is referred to as non-differential. Non-differential misclassification of the exposure is

regarded to lead to an attenuation of the true effect. Non-differential misclassification of the outcome will lead to larger variability in the data. If misclassification between comparison groups differs systematically (differential misclassification), the bias can go in either direction.

A specific type of information bias is the issue of *reverse causality*, which represents the chicken and egg dilemma. When an exposure is time-varying, or when the onset of a disease (the outcome) is difficult to measure, it can be difficult to ascertain if the exposure preceded the outcome or if the outcome actually came first. *Figure 12* illustrates how the outcome of asthma (Y) precedes the exposure of antibiotics (X). For instance, early symptoms of asthma (Y*) such as wheezing, may be mistaken for a respiratory infection, leading to antibiotic treatment. When the symptoms don't disappear with antibiotic treatment, the subject might seek health care again and then given a diagnosis of asthma (Y).

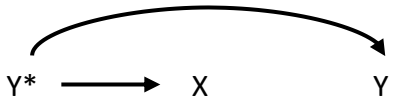


Figure 12. DAG illustrating the issue of reverse causality, where early symptoms of asthma precede antibiotic treatment.

Another common type of information bias is *recall bias*, a type of differential misclassification, which occurs when a subject is asked about the exposure after the disease has occurred, as in case-control studies or retrospective cohort studies. For instance, it has been suggested that acetaminophen use in early childhood is associated with an increased risk of asthma (138). However, it has been questioned whether this is a causal association or not, as *e.g.* parents to children with asthma may be more or less likely to report early use of acetaminophen compared to parents to children without asthma, which could then induce a spurious association. Recall bias is distinct from the more general problem of remembering and reporting events, which happens to all people and tends to be a non-differential misclassification.

1.6 EXTERNAL VALIDITY

Systematic errors determine the internal validity, and as the "internal" indicates, this concerns only the subjects studied. If the subjects under study are representative of the population we want to make inference about, the target population, then findings may be generalizable to this population as well. This is referred to as external validity or generalizability. The issue of generalizability of findings in twins compared to singletons and families with siblings compared to families with only one child will be discussed later.

2 AIMS

Much focus has been put into the idea of how our future may be programmed in utero or early in childhood. For instance, low birth weight and exposure to antibiotics in fetal life and early childhood have been suggested to increase the risk of asthma or lung function impairment in childhood. Twin- and sibling comparisons can help account for confounding factors shared within families and shed light on potential causal pathways. Furthermore, to be able to study asthma in register-based research, a valid measure of the disease is needed.

Specifically, we aimed to:

- investigate if low birth weight, as a proxy for fetal growth restriction in twins, was associated with an increased risk of asthma in childhood, independent of gestational age and familial factors (**Study I**)

- examine if fetal growth restriction was associated with impaired lung function in childhood, independent of gestational age and familial factors, and whether the potential association differed in individuals with and without asthma (**Study II**)

- assess whether asthma medication reported in the Swedish Prescribed Drug Register could function as a proxy for asthma and evaluate if asthma diagnoses in the National Patient Register fulfilled predefined criteria of asthma **(Study III)**

- study if antibiotic exposure in fetal life and early childhood was associatied with subsequent childhood asthma, independent of familial factors **(Study IV)**

3 MATERIAL AND METHODS

3.1 AT A GLANCE

	Population	Material	Method
I	10 918 twins, 9- or 12 years old, included in CATSS	*Exposure*: Birth weight, gestational age and fetal growth from the MBR *Outcome*: 'Asthma ever' from parental interview in CATSS *Confounders* from CATSS and MBR	Generalized estimating equations with the logit link and exchangeable covariance structure within pairs for the full cohort analyses (between-family) and conditional logistic regression for the within-twin pair analyses
II	576 twins, 9-14 years old, included in STOPPA	*Exposure*: Birth weight, gestational age and fetal growth from MBR *Outcome*: Lung function measures from dynamic spirometry *Effect modifier*: Current asthma from parental questionnaire in STOPPA *Confounders* from MBR and parental questionnaire in STOPPA	Linear regression with robust sandwich estimator for the full cohort analyses (between-family) and linear regression with fixed effects estimator for the within-twin pair analyses
III	Samples of individuals with a history of asthma* medication or diagnosis aged 0-17 years (study base, N=81 580)	Asthma medication (ATC: R03) from the SPDR and asthma diagnoses (ICD 10: J45) from the NPR, and medical records for 1710 individuals from 1117 health care units around Sweden	Descriptive statistics in terms of period prevalence, incidence, positive predictive value, and proportions
IV	493 785 children born to women pregnant between July 2005 and December 2010, followed from start of pregnancy to school-age	*Exposure*: Systemic antibiotics (ATC: J01) from SPDR *Outcome*: Asthma on the basis of an asthma diagnosis in the NPR (ICD 10: J45) in combination with asthma medication (ATC: R03) in the SPDR. This outcome was validated in study III. *Confounders* from LISA, MBR and NPR	Cox proportional hazard model with robust sandwich estimator for the full cohort analyses (between-family) and stratified Cox proportional hazard model for the within-sibling analyses

* Please note that study III also includes a validation of asthma in adults and of eczema medication as a proxy for eczema in childhood. However, as the focus of this thesis has purposely been on childhood asthma, these parts of the study are only mentioned briefly in the method section and further information can be found in the manuscript.

3.2 PERSONAL IDENTIFICATION NUMBER

Sweden has a long history of holding registers and producing population statistics. In 1686, the Swedish church began to register parish members into local registers (church books), which enabled the church and the Swedish state to keep a population census, enroll individuals to the army, and for tax issues (139).

In 1947, personal records including information on date and place of birth, sex, and address were established for all individuals who were registered in a local parish register and each individual was assigned a personal identification number (PIN). In 1991, the responsibility of the local parish registers was moved to the local tax offices, and from then onwards the responsibility of the PIN was taken care of by the National Tax Office.

When a child is born in Sweden, the obstetric department, the responsible midwife (for births outside the hospital), or the parents (if no midwife is present at the delivery), are obliged to report the birth to the National Tax Board within one month after the delivery. Historically, the newborn child has been assigned a temporary number at the delivery ward and then the PIN was sent to the home within a few days. However, since a few years back, newborns are now given the PIN directly in the delivery ward through an automatic linkage between the National Tax Board and Obstetrix, which is the electronic health record system used by more than 90% of the maternity and delivery units in Sweden.

The PIN consists of a six-digit birth date and a four-digit identification number. It is a unique identifier and the basis for all public administration in Sweden. Thus, all administrative and other registers are indexed by this number. This enables unambiguous linkage between national registers and other study specific or clinical data.

Immigrants who become permanent residents or intend to stay in Sweden for at least one year, also receive a personal identification number. Immigrants, who do no not fulfill this condition, but who use the Swedish social security system are assigned a coordination number by the National Tax Board. Individuals with coordination numbers are not included in national demographic or health registers. Hence, residents with coordination numbers will not be sampled in national register-based studies in Sweden.

3.3 DATA SOURCES

3.3.1 Registers

All four studies in this thesis take advantage of Swedish national population-based registers. National registers containing primarily demographic information are kept by Statistic Sweden, whereas the population-based national health registers are kept by the National Board of Health and Welfare (NBHW). The Swedish Twin Register (STR) is hosted by the Department of Medical Epidemiology and Biostatistics at Karolinska Institutet.

Total population register

The Total Population Register (TPR) was established by Statistics Sweden in 1968, when large sections of the local population registers were computerized (140). Information on births, deaths, place of residence, civil status, migration, relations and citizenship are reported from the local tax offices to the National Tax Board, with a daily notification to the TPR.

Multi-Generation Register

The Multi-Generation Register (MGR) is part of the TPR and links all Swedish residents to their parents, allowing for identification of family constellations, including identification of full- and half-siblings and cousins (141). The register was established in the early 1990s' and includes all individuals, so-called *index persons*, who have been registered in Sweden some since 1961. The register contains information on the index person's personal identification number, and the personal identification numbers of biological or adoptive parents (*Figure 12*). Linkage between index persons and parents is possible if the parents were alive and a resident of Sweden when the personal identification number was introduced in 1947 or thereafter. Immigrated index persons are only linked to their parents if they immigrated with their parents before age 18. The register was used in *Study IV* to identify siblings.

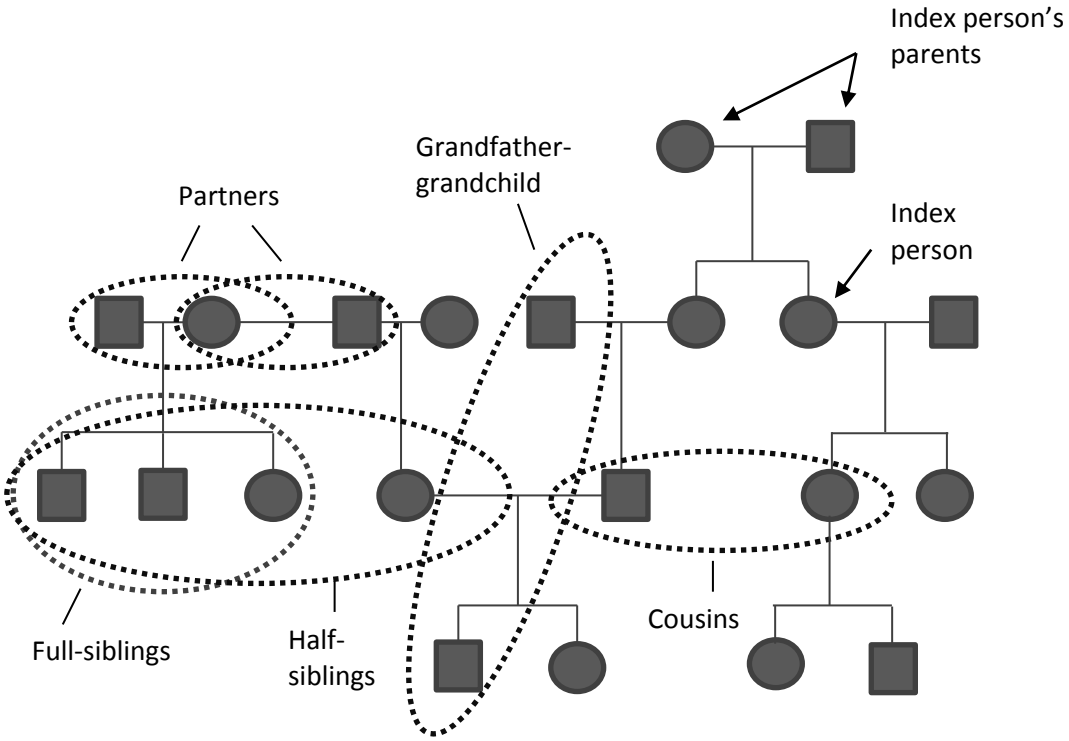


Figure 12. Graphic presentation of four generations in the Multi-Generation Register, where circles represents females and squares represents males. Different family constellations that can be identified from the register are suggested.

Longitudinal Integration database for Health Insurance and Labor Market Studies

The Longitudinal Integration database for Health Insurance and Labor Market Studies (LISA), held by Statistic Sweden, includes information on employment, disposable income, education, and area of residence among other data for all individuals aged 16 years or older and registered in Sweden. The database presently holds annual registers since year 1990 (142). Information from the register was used in *Study IV*, where the highest level of education for either parent was identified.

Medical Birth Register

Since 1973, all pregnancies resulting in a delivery have been reported by the delivering unit to the Medical Birth Register (MBR), kept by the NBHW. The information in the register is derived from four medical records; two records from the antenatal care of the mother, the delivery record and the record of the newborn infant examination. Records for a small percentage of all infants (approximately 2%) are missing completely; for others, the information is incomplete for example, due to missing data from antenatal-care clinics or pediatric wards (143). By linkage to registers provided by Statistics Sweden, information on the mother's country of birth and education at the time of pregnancy are added to the MBR. Information regarding exposures and potential confounders was collected from the register and used in *Study I, II and IV*.

National Patient Register

The National Patient Register (NPR) was established in 1964 by the NBHW and is based on hospital discharge records (144). The register includes information on dates of admission and discharge, type of hospital department, whether the hospital visit was scheduled or not and the cause of hospitalization. The cause of hospitalization is further divided into primary and secondary diagnoses, coded at the time of discharge according to the current version of the Swedish translation of the International Classification of Disease (ICD) as determined by the WHO. The diagnosis codes are entered more or less free hand by the attending physician into the register, meaning that data withdrawals from the register by researchers may include spelling mistakes and data irregularities. Nevertheless, it has been estimated that more than 98% of the hospital visits have been reported correctly (145).

From the time of its establishment, the register has expanded from only including data from six counties (out of 21), to covering approximately 85% of the discharges in 1983, to having complete national coverage from January 1987 and onwards. In 2001, outpatient visits from hospital-based clinics began to be reported as well and today, approximately 80% of the outpatient visits are recorded. However, data from primary health care clinics are not included in the NPR. Information from the register was used in *Study II, III and IV*.

Prescribed Drug Register

In July 2005, the Swedish Prescribed Drug Register (SPDR), the newest population-based health register held by the NBHW, was established. The register contains data on all

dispensed prescribed medications from outpatient care and in the primary health care for all Swedish residents (146). The register includes information on the dispensed item including dosage, expenditure and reimbursement, age, sex and place of residence of the patient, prescription and dispensing date, a prescriber code and the prescriber's profession. All drugs are classified according to the Anatomical Therapeutic Chemical (ATC) classification system (147). However, the register does not include data on over-the-counter (OTC) drugs, drugs used in hospitals or drugs that were prescribed but not dispensed. Furthermore, the indication for the drug is only available if noted by the physician on the prescription and then only available in free text. Information from the register was used primarily in *Study III* and *IV*.

3.3.2 Twin cohorts

The STR was established in the late 1950s' with the intention to study the health effects of smoking and alcohol, with the capacity to control for genetic liability for disease (148). The register includes information on twins born in Sweden since 1886, which at present is more than 95,000 twin pairs (148, 149). The twins under study in this thesis were born from 1992 and onwards and are included in the Child and Adolescent Twin Study in Sweden (150) and the Swedish Twin study On Prediction and Prevention of Asthma (151).

The Child and Adolescent Twin Study in Sweden

In *Study I*, the study population was derived from The Child and Adolescent Twin Study in Sweden (CATSS) which is an ongoing longitudinal twin study targeting all twins born in Sweden since July 1992. Since 2004, the STR has systematically approached parents of 9-year old twins with an invitation to participate in a telephone interview regarding their children's somatic and mental health and social environment. During the first three years of the study, twins aged 12 years (born 1992-1994) were also included. By March 2014, parental interviews concerning ~ 23 900 twins have been completed, with an overall response rate of 70% (149). Analyses of the differences between non-responders and responders, based on a merge of data from approximately 11,000 twins from CATSS, Statistics Sweden, and NBHW, have shown that non-responders more often belong to a low socio-economic stratum and have more neuropsychiatric diagnoses such as attention-deficit hyperactivity disorder and autism spectrum disorder (150).

Zygosity in CATSS is determined by DNA (in saliva) or by using answers to questions about similarity such as "Are your twins like two peas in a pod, or more like siblings in general?", which have shown an accuracy of around 95% (148). Building on the recruitment of CATSS, several follow-ups and extensions have been initiated, such as a follow-up at age 15 and 18, and other cohorts with the aims to study more disease specific questions (149).

The Swedish Twin study On Prediction and Prevention of Asthma

One of the extension studies from CATSS is the Swedish Twin study On Prediction and Prevention of Asthma (STOPPA) (151). STOPPA was initiated in May 2011 and completed in June 2014. Based on questions on asthma and wheezing (26) included in the CATSS interview, an algorithm was created to identify twins discordant and concordant for a history of asthma/wheezing. MZ and DZ same-sexed twins aged 9-14 years old at the invitation, were invited with their parents to a clinical examination. The twins were examined in collaboration with pediatric allergy and asthma clinics in Gothenburg, Linköping, Karlstad, Umeå, Växjö, Lund, and at Lomma Primary Health Care Centre. In Stockholm, the twins were examined at test centers at Karolinska Institutet (*Figure 13*). At the examination the twins and parents answered a questionnaire each, and the twins were thereafter invited to measure their weight and height, their lung function by dynamic spirometry and fractional exhaled nitric oxide, as well as give blood-, saliva-, urine- and fecal samples. In total, 752 twins were included in STOPPA, with a response rate of approximately 52% from the first contact by a research nurse over telephone.

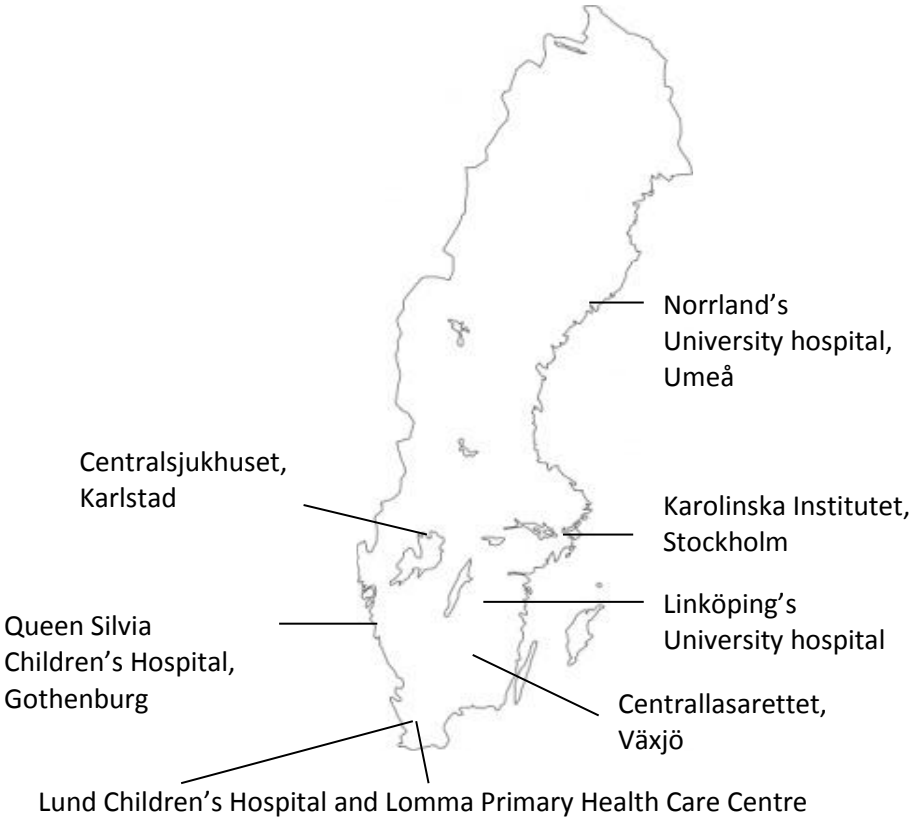


Figure 13. The location of the clinical examinations in STOPPA.

In *Study II*, objective measures of lung function from spirometry were used. The individual was asked to take the deepest breath possible and then exhale into a sensor as hard as possible, for as long as possible. The procedure was repeated at least three times before and after inhalation of a short-acting bronchodilator. The procedure and evaluation of spirograms were performed in accordance with guidelines by the American Thoracic Society/European

Respiratory Society (ATS/ERS) recommendations (21). The all-age reference values were used to calculate z-scores and lower limits of normal (23).

3.3.3 Medical records

Medical records are mainly an important tool for health care personnel to secure patient safety and all health care, public or private, that is provided is required by law to be documented in the medical record. Nowadays, the majority of the medical records are electronic and the patients' medical history is stored in databases based on their PIN. In addition to being a tool in the clinicians' daily practice, the medical record may also be used for evaluation of health care units, in legal contexts, as reference material, and for quality control in a research setting. In *Study III,* patient medical records were identified based on the PIN in combination with the name of the health care unit, and used to validate information on asthma medication in the SPDR and diagnosis in the NPR.

In short, for the validation of asthma medication as a proxy for asthma, individuals fulfilling criteria for asthma medication in the SPDR (Figure 1 in paper III) without a diagnosis of asthma in the NPR were identified, and medical records for a random sample of these individuals were requested and reviewed (*Figure 14*). For the assessment of asthma diagnoses in the NPR, individuals with a diagnosis in the register, independent of their history of asthma medication, were identified and medical records for a random sample of these individuals were requested and reviewed.

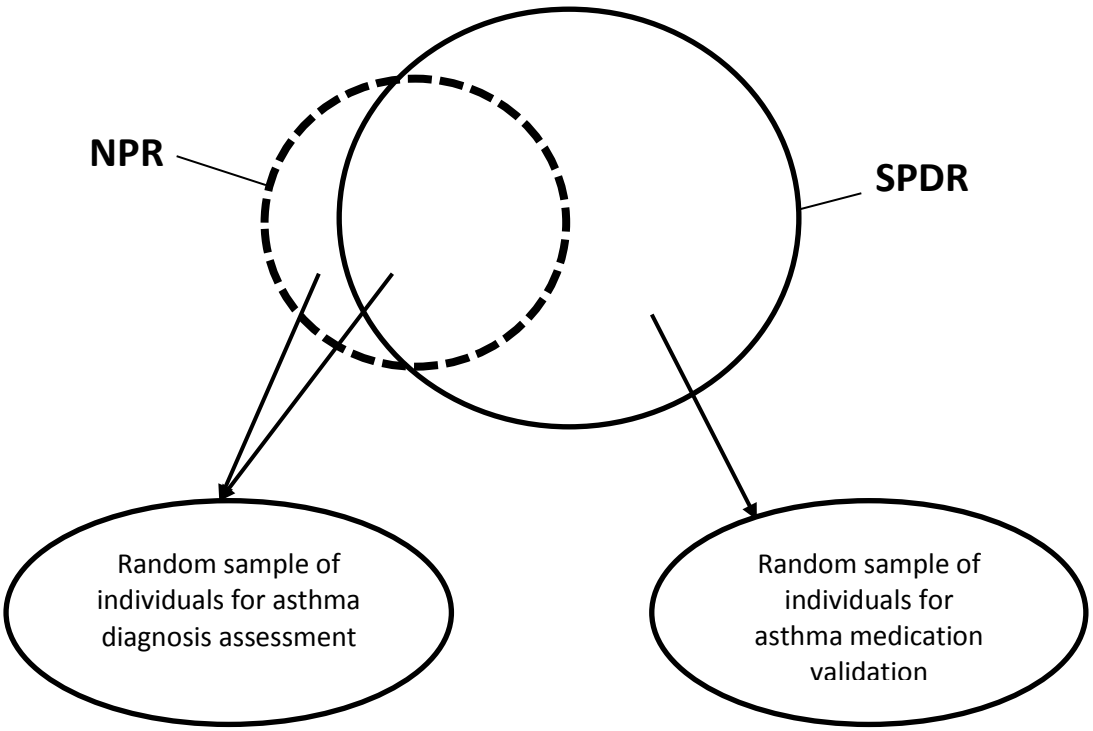


Figure 14. Linkage between the Swedish Prescribed Drug Register (SPDR) and National Patient Register (NPR), to identify individuals to be included in the validation of asthma medication as a proxy for asthma, and assessment of asthma diagnoses in the NPR.

3.4 BETWEEN- AND WITHIN-FAMILY DESIGN

As has been previously discussed, an association between an exposure and an outcome does not automatically imply causality. Other factors may influence the association and these factors can only be adjusted for if they are known and measured. Unmeasured and non-measurable variables may induce a spurious *i.e.* non-causal association between two variables, and lead to incorrect interpretation and conclusions.

The first step is to acknowledge that confounding might be present, and the second step is to adjust for the confounders, which can be problematic when dealing with unmeasured confounders. When the confounder is shared within a twin pair or within siblings in a family (*familial confounding*), as in the case with genetic alleles or certain behaviors, a possibility for better inference arises (127).

In a traditional between-family analysis, individuals from different families are compared (*upper panel*, *Figure 15*). In a within-family analysis, individuals (twins/siblings) are compared with individuals from the same family, making it possible to study the association between an exposure and an outcome, while adjusting for familial factors (*lower panel*, *Figure 15*).

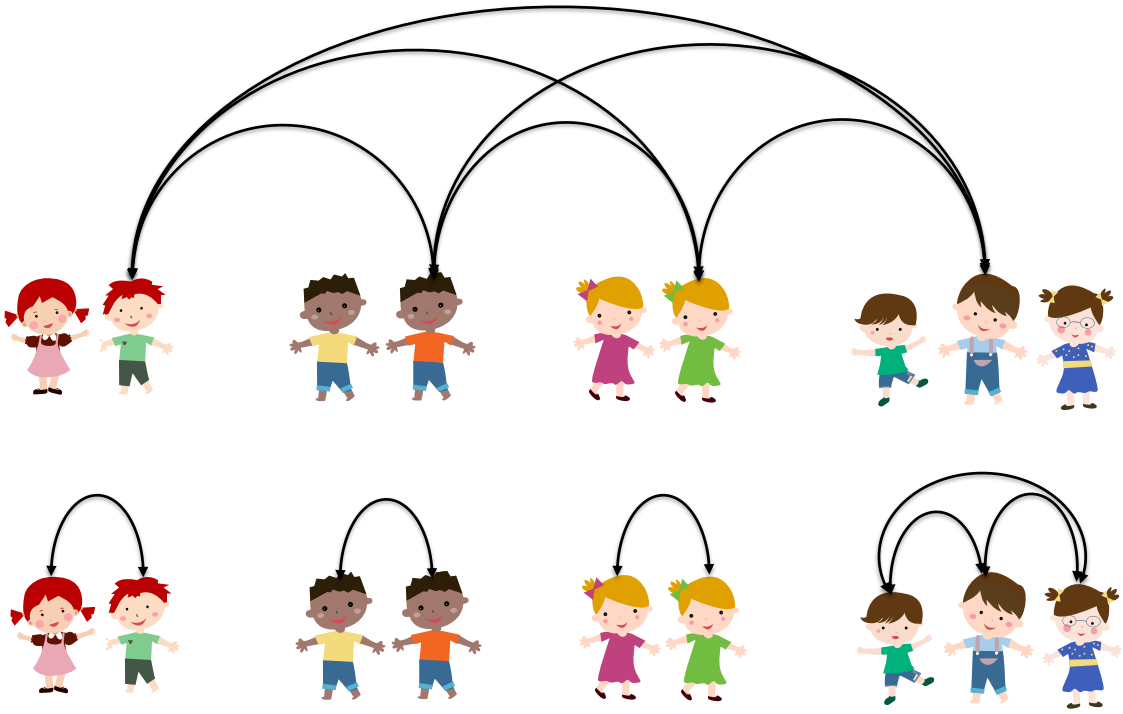


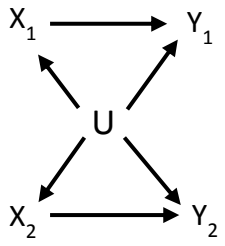
Figure 15. Between-family (upper panel) and within-family (lower panel) analysis.

Adjusting for familial factors is possible due to the fact that MZ twins share all of their genes, while DZ twins and full siblings share on average half of their segregating genes. Furthermore, twins and siblings share parental factors and intrauterine exposure to some extent. If associations seen in a cohort of twins/siblings remain when non-affected twins/ siblings are used as controls to the affected individual, then factors unique to the individual are assumed to be involved in the underlying causal pathway. In contrast, if the association is

attenuated or disappears, then factors common to the twins/siblings are involved. Thus, within-twin or within-family studies are based on individuals who are not randomly assigned to a setting, but instead take advantage of 'natural experiments' based on family constellations. Comparisons of findings within MZ versus DZ twin pairs can further provide insight into the role of genetic factors. If there is no difference in the strength of the association between the exposure and outcome between MZ and DZ twins of same sex, then the association is thought to be independent of genetic factors.

In *Study I, II and IV* the study participants are first regarded as any other cohort population, and associations are investigated between individuals independent of their family constellation. Analyses are controlled for known and measured confounders by including them in the models. Secondly, to gain more insight about the role of early life risk factors for subsequent childhood asthma, we take advantage of each individual's family constellation to investigate family-adjusted estimates: within-twin pairs and within-sibling group estimates.

Figure 16 illustrates a simple situation of familial confounding, where sub-index 1 represents twin/sibling 1 and sub-index 2 represents twin/sibling 2. In this situation, estimations of the association between X and Y, without adjustment for the unknown confounder U, would produce a biased estimate. If U is an unmeasured or non-measureable shared familial confounder, within-family adjustments may be used.



3.5 STATISTICAL ANALYSES

Random error

Random error is the variability in data that we cannot explain (134). Random error may stem from random biological processes, but could also stem from sampling. The random variability will in turn affect the precision of the sample estimation. Statistical analyses of epidemiological data attempt to assess variability in the data in an effort to distinguish chance findings from true results that might be replicated upon repetition of the work. Any study with a study population smaller than infinitely large would only give an estimation of the true value. The random error can be reduced by increasing the study population size.

Handling of clustered data

While the degree of similarity in twins and siblings tend to be the objective in within-twin or within-sibling studies, it is also a feature that calls for special care in the methods used for statistical inference. In most statistical analyses, one fundamental assumption is that the observations in a model are independent from each other. Assuming the opposite, when observations are in fact positively correlated as with twins and siblings, can lead to overestimation of the statistical information of the data with too narrow confidence intervals or too low P-values (152). In the statistical methods used for *Study I, II and IV*, the size of the

correlations between individuals (twins and siblings) are not of interest on their own, but rather viewed as nuisance factors that need to be accounted for.

3.5.1 Study I & II

As a first step in *Study I* (CATSS), the association between the exposure variables birth weight, gestational age, and birth weight for gestational age as a measure of fetal growth (by using SD scores (SDS) for birth weight by gestational week and sex) and the outcome variable 'asthma ever' (yes/no) was investigated in the full cohort of twins. The proc genmod procedure (which applies generalized estimating equations (GEE)) in SAS was implemented with the logit link and the exchangeable covariance structure. GEE is one method to handle clustered data and was therefore used to account for dependences within-twin pairs. The exposures were both assessed as categorical and continuous variables.

Firstly in *Study II* (STOPPA), the associations between the same exposure variables as in *Study I* and continuous measures of lung function (FEV_1, FVC, FEV_1/FVC and post bronchodilator change in FEV_1) were investigated with linear regression and the sandwich estimator of standard errors to account for clustering within-twin pairs. We also investigated whether the exposure variables influenced asthma, which subsequently influenced lung function (asthma acting as a mediator), or if the observed effect of the exposures on lung function differed in those with and without asthma (asthma acting as an effect modifier), by including asthma as a covariate (test of mediation) and as an interaction term with the exposure variables (test of effect modification), in the full cohort analyses.

In a second step of *Study I and II*, within-twin pair analyses were performed, using conditional logistic regression in *Study I* and linear regression with the fixed-effects estimator in *Study II*. In the sample produced by twin pair matching, the regression analyses estimates the effect (change in log-odds of asthma or change in z-score in lung function) of the difference between two observations in the strata. Thus, a continuous measure of birth weight will estimate the linear effect of the within-twin pair difference in birth weight. In *Study I*, the odds ratio were reported in relation to 1000 g negative deviance in birth weight from the twin pair mean, while in *Study II*, the coefficient corresponded to a 500 g negative deviance from the twin pair mean.

For more information on specific confounders and effect modifiers included in the models, please see papers I and II.

3.5.2 Study III

In *Study III*, the period prevalence of asthma medication (and/or eczema medications) was estimated as the proportion of individuals in the Swedish population for whom medication was dispensed during the year 2008. The nominator (number of drug users in 2008) was retrieved from the SPDR and the denominator (number of individuals within the age span during 2008) from Statistics Sweden.

Incidence estimates were based on all new users of asthma medication between 2008 and 2009 and estimated person-time was based on population data from Statistics Sweden. The period of 2008-2009 was based on the waiting-time distribution. The waiting-time distribution, is a frequency distribution of first time occurrences of medication use within a time window, and essentially addresses when users of a specific medication will first appear inside a time window, when all information outside the defined time window is disregarded (153). For medication intended for chronic treatment, current users will generally be captured in the beginning of the time window. The distribution graph will eventually be dominated by new (incident) users after a certain period of time, depending on the type of medication and the expected time interval between dispensed prescriptions (Figure 2 in paper III). Except for information on incidence, seasonality of dispensed medication can also be assessed.

Positive predictive value (PPV) was used to validate asthma medication as a proxy for asthmatic disease (*Box 2*). PPV was estimated as the proportion of children defined as having asthma according to a gold standard who also fulfilled asthma medication criteria (numerator), to all children fulfilling the asthma medication criteria (denominator). For information on specific medication criteria in different age groups, please see Figure 1 paper III.

Box 2. Validation measures

		Gold standard		
		Yes	No	PPV=*a/(a+b)*
Test outcome	Yes	*a (true positive)*	*b (false positive)*	*Sensitivity=a/(a+c)*
	No	*c (false negative)*	*d (true negative)*	*Specificity=d/(b+d)*

Our gold standard for asthma was either having a record of an asthma diagnosis (ICD 10: J45) in the medical record, or fulfilling pre-defined criteria of asthma suggested by the Swedish Pediatric Society's section for Allergy (*Box 3*) (154).

Box 3. Asthma according to Swedish Pediatric Society's section for Allergy

- ≥ 3 obstructive periods before 2 years of age and/or;
- ≥ 1 obstructive period after 2 years of age and/or;
- ≥ 1 obstructive period independent of age when the child has ≥1 of the following: eczema, allergy, parents and/or siblings with asthma or no improvement between periods of respiratory tract infections.

Children under two years of age with ≤ 2 asthma-like symptoms during respiratory tract infections and without symptoms between infections are defined as suffering from obstructive bronchitis.

Asthma diagnoses reported in the NPR were evaluated by calculating the proportion of individuals with an asthma diagnosis in the register that also were defined as having asthma according to criteria by Swedish Pediatric Society.

A brief note: eczema medication as a proxy for eczema in children was also evaluated in *Study III*. The original plan was to use validated standardized criteria as the gold standard for eczema (155), but unfortunately the information in the medical records was too limited, thus leaving us with information only on given diagnoses in the medical record. Firstly, each medical record was reviewed to see if the child had been given the umbrella diagnosis of unspecific dermatitis, and secondly a specific diagnosis of dermatitis, namely eczema (L20), seborrhoeic dermatitis (L21) or contact (allergic (L23); non-allergic (L24)) dermatitis (L25).

3.5.3 Study IV

In *Study IV*, a Cox proportional hazard model, with the age of the child as the analysis time scale and the date of birth as time of origin, was used to investigate the association between exposure to antibiotics in fetal life and early childhood and asthma. The robust sandwich estimator was used to take clustering within the cohort into account.

A stratified Cox proportional hazard model was used to investigate the association between antibiotics in fetal life and childhood and asthma, among siblings within families. In this model, each family has its own baseline hazard rate reflecting the family's shared factors. The exposure comparisons (antibiotics vs. no antibiotics), are thus made within the family. The model takes differences in follow-up time into account. Hence, only sibling pairs discordant for antibiotics as well as asthma were informative for the analyses, *i.e.* contributed with information to the estimates. Indeed, to be informative, the sibling without asthma should have at least as long follow-up time as the sibling with asthma.

To study the possibility of confounding by indication and reverse causation from respiratory infections, all analyses were performed with the exposure of antibiotics divided into three groups (117, 125): "any antibiotics", "airway antibiotics", and "urinary tract or skin and soft tissue antibiotics" as proxies for the indications of the treatments. We also included information on assumed exposure to "any antibiotics" within hospitals based on information on diagnoses reported in the NPR that we have previously shown to have a high correlation with antibiotic treatment (156).

For details regarding confounders included in the models and information on sub- and sensitivity analyses, please see paper IV.

4 ETHICAL CONSIDERATIONS

Ethics is about structuring, stimulating and maintaining a consciousness and a discussion on how to act. The applications of the Ethical Review Act (2003:460) that are important for studies included in this thesis are those addressing the handling of sensitive personal data, physical interventions of study participants, and collection of biological samples. As an underlying rule in the Ethical Review Act, all individuals that are involved in research shall, with few exceptions, be informed about the research, their participation in it, and have the option to participate or not. Consent to participate is only valid after the study subject has been properly informed about the study. If the study participant is a child, then it becomes more difficult to obtain adequate informed consent, as their ability to judge risk and assess consequences is limited and they can more easily be influenced by others. In the Ethical Review Act (18 §), children 15 years and older should be properly informed and give their own consent to participation. In younger children, the legal guardians of the child should be informed and give consent. However, the child should also be informed in a manner considered appropriate for their age group, and if the child opposes to the study, then the parents' consent is not valid.

In *Study I*, the children were 9 and 12 years of age, and the parents were thus informed and given the option to participate in the CATSS study including linkage to the national registers. In *Study II*, both children and parents were informed and asked for consent. All collected data (questionnaire, samples, objective measures) in STOPPA have been de-identified and the PIN replaced with a study number. The children and parents only consented to participate in STOPPA. In both CATSS and STOPPA, withdrawal from the studies is possible at any point in time.

As previously described, informed consent applies to all research involving humans, with few exceptions. These exceptions include register-based studies, where data that have already been collected are used. In *Study III*, no informed consent was collected from the patient for whom we collected medical records. Instead, each head of the prescribing/treating health care unit was asked for permission, in accordance with the ethical permission of the study. All medical records were de-identified and replaced with a study number before the medical records were reviewed. To perform the study a large number of medical records had to be collected and we assessed that the benefit of informing the individual patient was smaller than the possible anxiety a request would involve. Furthermore, as asthma and eczema are common diseases in childhood often treated in the primary health care clinics, we evaluated the intrusion of integrity to be relative small.

In *Study IV*, anonymized data for approximately half a million individuals were requested from the population-based demographic and health registers, and participation consent was not requested. However, as the study examined a common exposure and outcome, the risk of identifying specific individuals within the cohort was regarded as small, as well as the risk of intrusion of the personal integrity.

5 MAIN RESULTS AND DISCUSSION

5.1 STUDY I & II

We found that the risk of childhood asthma increased with reduced birth weight, gestational age and fetal growth, in the full cohort of *Study I*. Similarly, we found that FEV_1 decreased with reduced birth weight, gestational age and fetal growth, although not significantly, in the full cohort of *Study II*. We also found that post bronchodilator change in FEV_1 increased with decreasing gestational age. No significant associations were found for FVC and FEV_1/FVC.

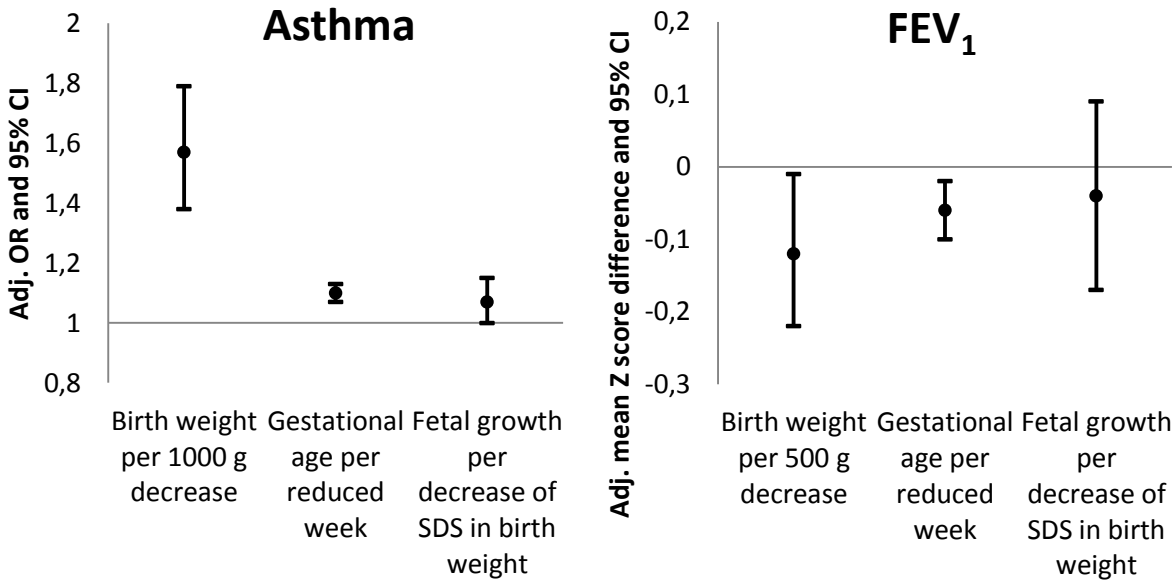


Figure 17. Asthma (left panel, *Study I*) and FEV_1 (right panel, *Study II*) in relation to birth weight, gestational age and fetal growth in full cohort analyses.

In the within-twin pair analyses, the association between birth weight, as a proxy for fetal growth in twins, and asthma (*Study I)* and FEV_1 (*Study II)*, remained.

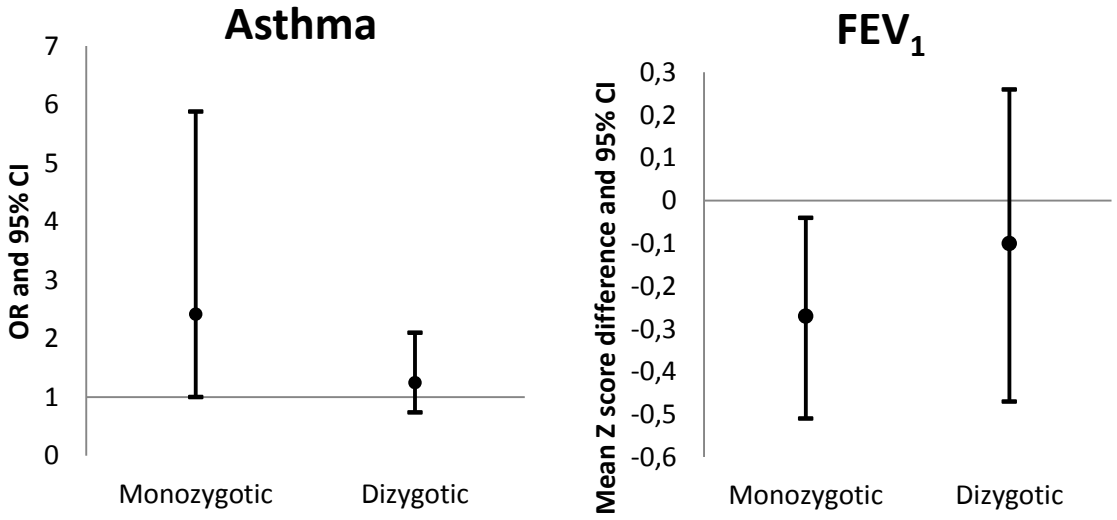


Figure 18. Asthma (left panel, *Study I*) and FEV_1 (right panel, *Study II*) in relation to fetal growth within-twin pairs.

Furthermore, there was no significant difference in strength of the associations between MZ and DZ twins in *Study I* or in *Study II*, and no significant differences were found between preterm and term twins.

In the full cohort analyses in *Study II*, we further tested whether birth weight, gestational age, and/or fetal growth influenced asthma which subsequently influenced lung function (mediation), or if the observed effect of the exposures on lung function differed in those with and without asthma (effect modification).

We did not find any evidence for asthma being a mediator between the birth characteristics and lung function; however, while similar results were seen for individuals without asthma, an opposite pattern was found in those with asthma where FEV_1 increased with decreasing birth weight and gestational age (*p* for interaction ≤ 0.05).

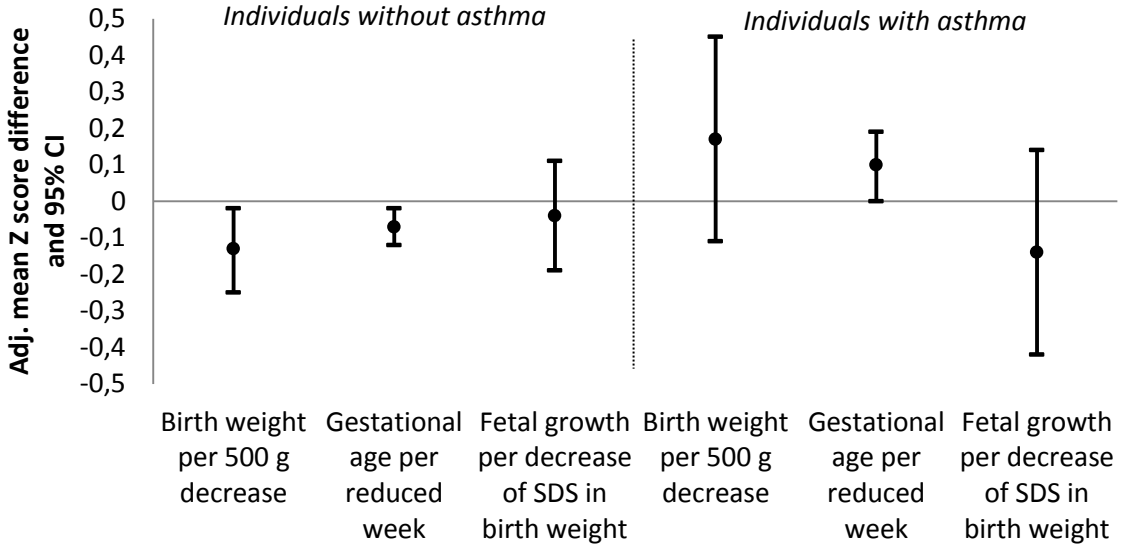


Figure 19. Lung function in relation to birth weight, gestational age and fetal growth, in individuals with (right panel) and without (left panel) asthma.

Please see paper II for further results regarding other lung function measurements, and sensitivity analysis excluding individuals with a history of respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD).

5.2 STUDY III

The period prevalence of any asthma medication in the year 2008 was 7.5% in pre-school children (0-4.5 years) and 6.4% in school-age children (>4.5-17 years). *Figure 20* illustrates the prevalence of filled prescriptions of different asthma medications during the same year. The incidence of pre-school asthma medication users was 3.70/100 person-years (95% CI 3.66-3.74) and 2.12/100 person-years (95% CI 2.10-2.14) in school-age children.

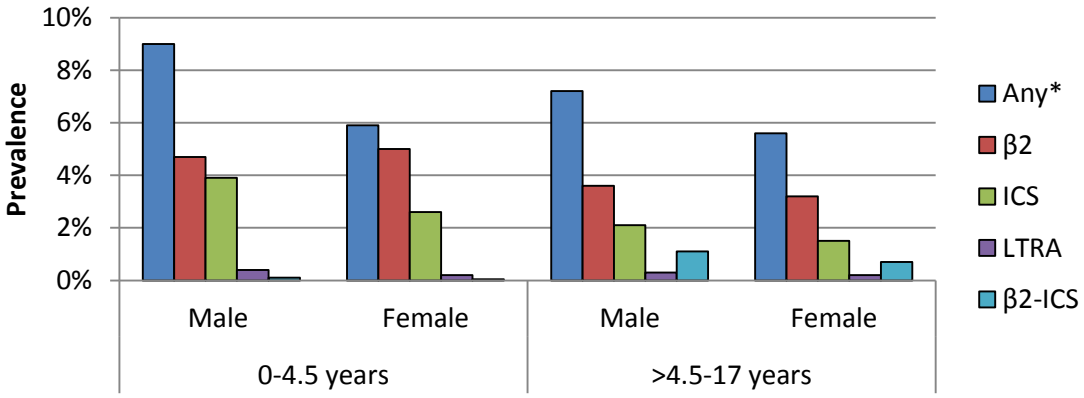


Figure 20. Prevalence of filled prescriptions of asthma medication during the year 2008. *Any of the following: β2 - inhaled β2-agonists; ICS - inhaled corticosteroids; LTRA - leukotriene receptor antagonists; β2-ICS - fixed combinations of β2-agonists and corticosteroids.

When using an asthma diagnosis (J45) recorded in the medical record as gold standard, pre-school children had a PPV of 68% for asthma medication as a proxy for asthma (*Figure 21*). Corresponding PPV in school-age children was 89%. The remaining 32% (pre-school) and 11% (school-age) corresponded to acute bronchitis/bronchiolitis, chronic bronchitis and upper respiratory tract infections. When using asthma criteria by the Swedish Pediatric Society as gold standard, pre-school children had a PPV of 75% for asthma medication as a proxy for asthma, whereas school-age children had a PPV of 94%. In the assessment of asthma diagnoses in the NPR, 78% of the pre-school children and 99% of school-age children fulfilled the asthma criteria by the Swedish Pediatric Society.

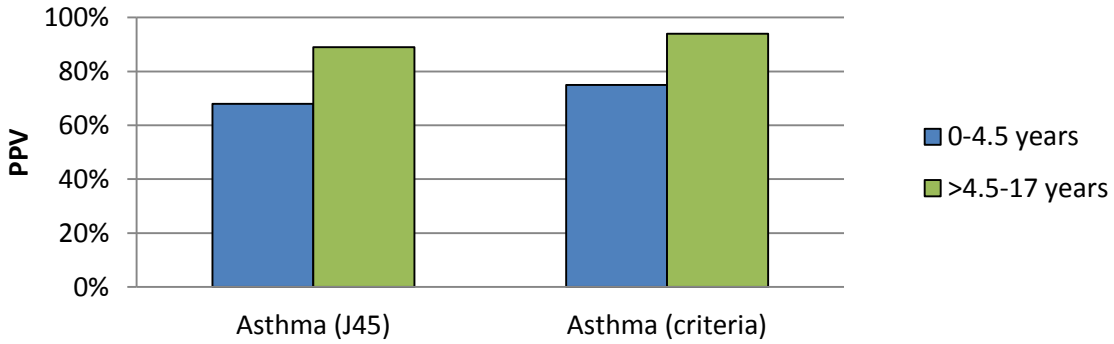


Figure 21. PPV for asthma medication as a proxy for asthma diagnosis in children, using either asthma diagnosis or asthma defined from criteria by the Swedish Pediatric Society, as gold standard.

Please see paper III for results regarding eczema and asthma in adults.

5.3 STUDY IV

We found significant associations between exposure to all groups of antibiotics in fetal life and childhood asthma in analyses of the full cohort of all children. In the within-sibling analyses, the associations disappeared.

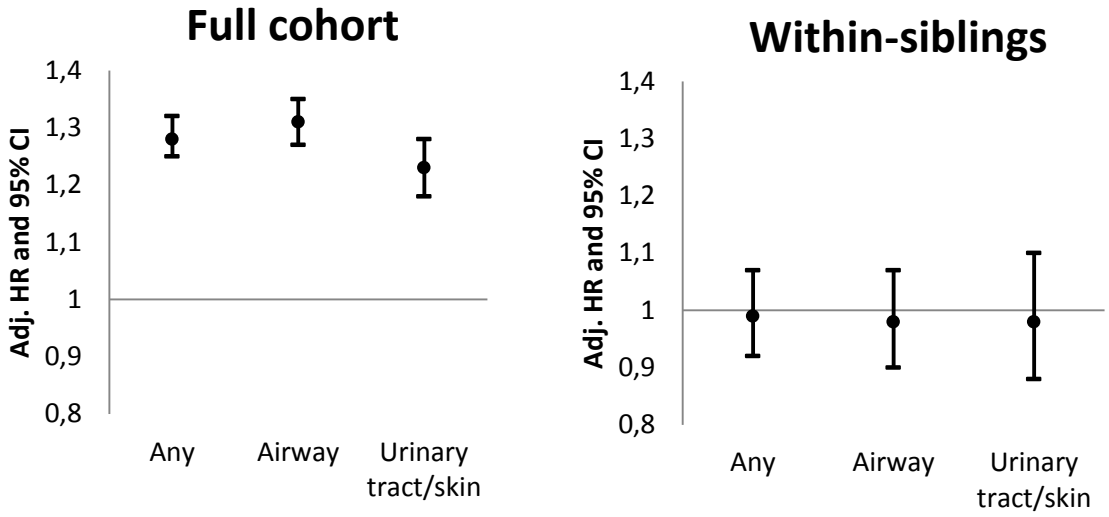


Figure 22. Full cohort analyses (left panel) and within-sibling analyses (right panel) of antibiotic exposure in fetal life and subsequent asthma.

We also found significant associations between any, airway and urinary tract/skin antibiotics in childhood and asthma in the full cohort analyses of all children. The highest risk of asthma was found in children exposed to airway antibiotics during the first six months in life.

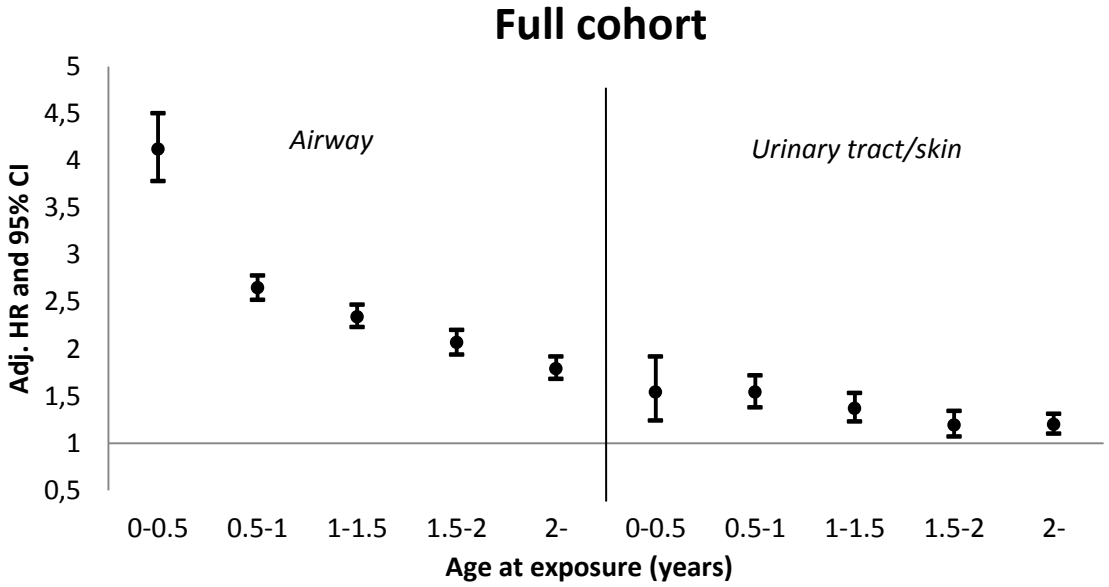


Figure 23. Full cohort analyses of airway (left panel) and urinary tract/skin (right panel) antibiotic exposure in early childhood and subsequent asthma.

In the within-sibling analyses, the association between urinary tract/skin antibiotics and asthma disappeared, and decreased markedly for airway antibiotics.

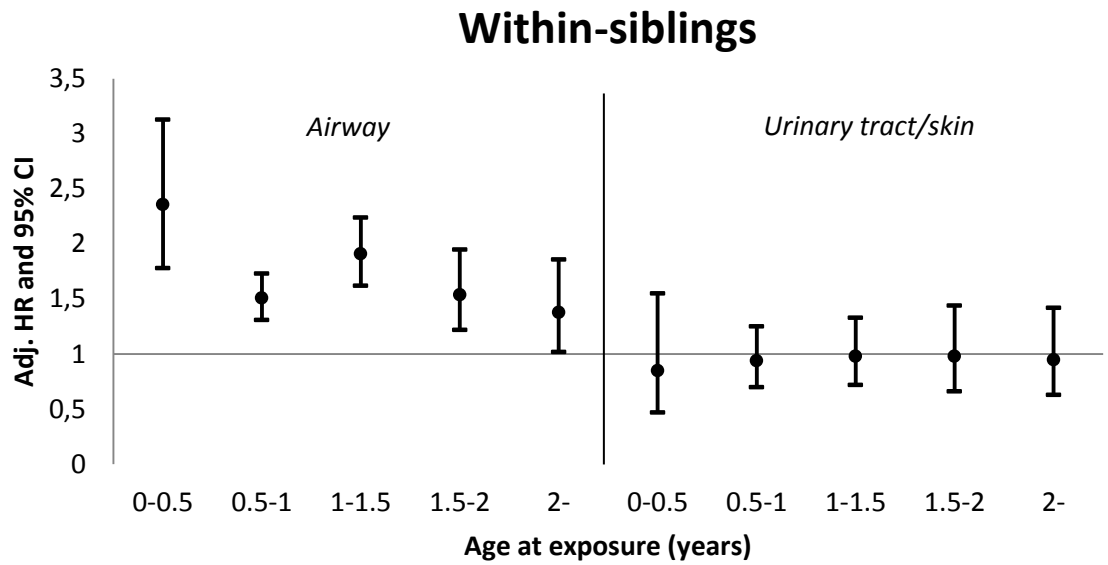


Figure 24. Within-sibling analyses of airway (left panel) and urinary tract/skin (right panel) antibiotic exposure in early childhood and subsequent asthma.

In full cohort analyses, using antibiotics during the first year of life as exposure and incident asthma from two years of age as the outcome, significant associations were found for all three groups of antibiotics. In the within-sibling analyses, the association disappeared for all groups of antibiotics.

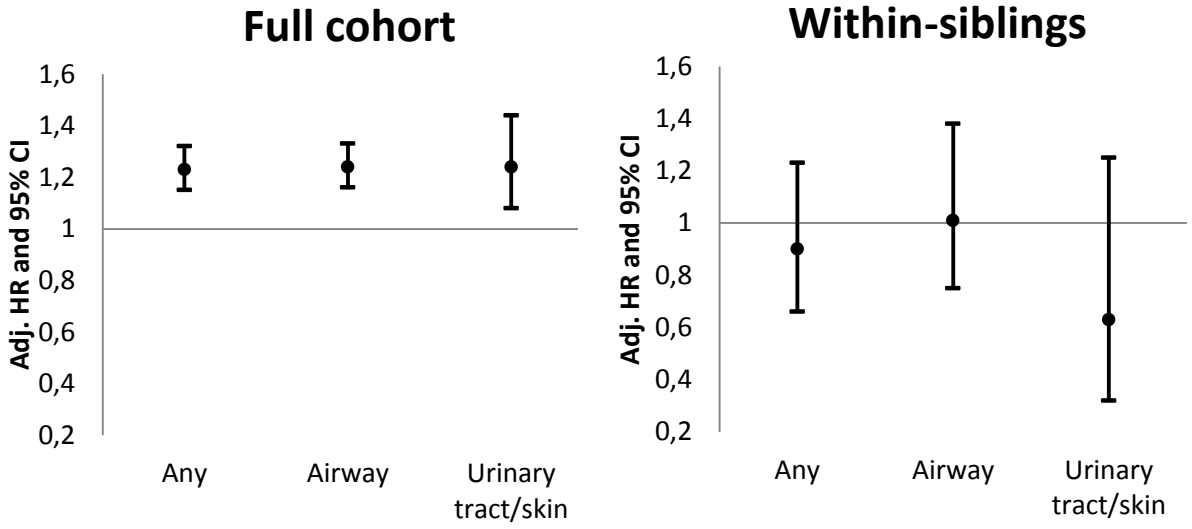


Figure 25. Full cohort analyses (left panel) and within-sibling analyses (right panel) of antibiotic exposure during the first year of life and incident asthma from two years of age.

Please see paper IV for further results from sub- and sensitivity analyses.

5.4 INTERNAL VALIDITY

5.4.1 Confounding

The major advantages with within-family based designs, such as full-sibling comparisons and twin-comparisons, include the possibility to account for all genetic and environmental selection factors that makes the siblings/twins similar (127). For twins, the comparison also accounts for all confounds associated with age, shared prenatal factors that make the twins similar and factors correlated with sex in MZ twins and DZ same-sexed twins. However, sibling and twin comparisons also have limitations; they do not rule out factors that make the siblings/twins dissimilar (non-shared factors).

5.4.2 Selection of participants

The study subjects in this thesis are all members of cohort samples from some theoretical source population. Subjects that meet a set of inclusion criteria (*e.g.* inclusion in a register, birth year, participation in data collection, etc.) are selected and followed for a fixed period of time (*Study I and II*). Subject can also be followed prospectively and individuals may be censored due to migration or death, as is the case in *Study IV*.

Other than fulfilling features that define the source population in *Study I* (such as being a twin at 9 or 12 years of age living in Sweden with Swedish-speaking parents), the twins of CATSS were characterized by their parents willingness to participate in the study. Participation can be associated with socio-economic status (157) or family history of disease (158). Any greater influence from potential unmeasured common causes of participation (such as socio-economic status or family history of asthma) on the association between birth weight and asthma (as illustrated in *figure 11, page 15*) is, however, considered unlikely especially with respect to within-twin pair birth weight. In addition, a response rate of almost 80% should diminish concerns about strong volunteer selection.

In *Study II*, a second level of criteria for participation from the CATSS study was included, namely agreeing to a clinical examination. The overall response rate was 52% in the study, and somewhat higher in MZ twins compared to DZ twins (57% and 47% respectively). The group of twins where no one in the pair had a history of asthma or wheeze, independent of zygosity, had the highest participation rate. The clinical examinations were performed at eight pediatric clinics or test centers around Sweden, and the twins and families were reimbursed for travel and living expenses. Thus, individuals should not have been self-excluded due to economic reasons, although other reasons for being unable to travel may have affected the participation for some families. Generally, representativeness is most likely only a concern if one believes that the association between fetal growth and lung function would be due to a different etiology in those who did not participate (159).

In *Study III*, random samples of individuals were selected from the source population, thus reducing risk of self-selection, or selection due to prescribing patterns or socio-economic status. However, the head of each health care unit decided whether or not to send the medical

records for each patient. Nevertheless, we did not find any regional differences in the proportion of received medical records.

In *Study IV*, the study population was restricted to children born to women living in Sweden during their whole pregnancy. Follow-up was censored at the study outcome of asthma, emigration, death or end of study period, whichever occurred first. Less than 1% of the children emigrated and 0.3% were lost to follow-up due to death. Thus, selection due to loss-of-follow up is considered unlikely.

5.4.3 Measurement error

Asthma

In *Study I*, the main outcome of asthma was defined based on an affirmative answer to "Has your child ever had asthma?", which is a question from the ISAAC questionnaire (26). Mistaking other respiratory noises as asthma symptoms may lead to over-reporting (160), therefore misclassification of the outcome has to be considered. In a Swedish validation study of the ISAAC questionnaire, parental reported prevalence was significantly higher compared to information based on medical records (161). Nonetheless, we obtained similar results using secondary outcomes of at least one asthma attack during the past 12 months or a doctor's diagnosis of asthma. In addition, the potential misclassification is most likely non-differential with regard to the exposure.

In *Study II*, in analyses of asthma as a mediator or effect modifier, current asthma was defined as affirmative answers to both "Does your child have asthma?" and "Has your child had wheezing or whistling in the chest in the last 12 months?". For this study we also had information on prescribed asthma medications in the SPDR and asthma diagnoses in the NPR, and used the validated asthma outcome measurement from *Study III* to confirm 90% of the parentally reported asthma cases.

In *Study IV* asthma was defined on the basis of *Study III*. As the majority of our study population in *Study IV* was of pre-school age, there was a concern that only using asthma medication as a proxy as the outcome, would not be satisfactory, since 25% were classified as having other asthma-like diagnosis. Thus, we chose to define asthma as a diagnosis in the NPR and asthma medication in the SPDR according to the criteria in *Study III*. This stricter definition reduces the number of cases, as the majority of children receive an asthma diagnosis in the primary health care clinic, which does not report to the NPR. This means that some of those that are classified as discordant sibling pairs in the analyses may actually be concordant. On the other hand, sensitivity analyses using asthma medication and diagnosis as separate outcomes, gave similar results and did not affect the study conclusion.

Lung function

In *Study II*, lung function was measured with dynamic spirometry according to ATS/ERS guidelines by trained personnel blinded for the exposure and asthma status (21). Accordingly,

any imprecision in the lung function measurement due to low cooperation or weak technique is most likely non-differential with respect to the exposure variables. It could be discussed whether post bronchodilator change in FEV_1 reflects a true effect of the bronchodilator or if it reflects an improvement in technique. We found no indication that the technique improved significantly from pre to post bronchodilator assessment, as approximately 87% of the twins had reproducible measures of FEV_1 before bronchodilator, while 88% had reproducible measures afterwards.

Birth characteristics

The foundation of the association between fetal growth and asthma or lung function mainly relies on proxies of growth at birth. As discussed in the introduction, both proxy measures of fetal growth and fetal growth *per se* are determined by many factors. Unrelated individuals may differ in these measures due to differences in gestational age, genetics, environment, and maternal constitution. When comparisons are made within-twin pairs all factors shared by the twins are held constant, thus growth discordance in twins is mainly a result of unequal share of supply and difference in genetic factors in DZ twins.

The potential influence from cross-misclassification needs to be considered in within-twin pair comparison of exposures, such as birth weight. Assigning a twin characteristic of its co-twin will distort any true association and lead spurious associations. In *Study I and II*, efforts were made to minimize cross-misclassification, by comparing information on birth weight and birth order from the MBR with parental-reported information.

Antibiotics

In *Study IV*, antibiotic exposure was defined as having filled a prescription of antibiotics, which is not equal to adherence to treatment (162), and as a result we may have overestimated the actual number of exposed. Furthermore, we did not have any information on the actual indication for the antibiotics, but instead divided them into three groups based on treatment guidelines and expertise knowledge. Our main purpose was to keep the group of urinary tract/skin antibiotics "as clean as possible" and making sure that antibiotics which may be prescribed for both urinary tract/skin infections and for airway infections (*e.g.* cephalosporins), were excluded from the urinary tract/skin antibiotic group. By doing this, we aimed to avoid misclassification of this group of antibiotics to further assess confounding from respiratory infections.

5.5 EXTERNAL VALIDITY

Generalizability *(i.e. external validity*) of twin studies to the general population is regularly questioned. A potential threat to representativeness could be if twins were to be different from singletons with respect to underlying biological mechanisms for the phenotype under study. In regards to the fetal programming theory, it has been suggested that the growth restraint that twins experience compared to singletons, would put twins at a different risk of subsequent disease. Studies investigating the risk of asthma in twins compared to singletons,

have reported inconsistent results, with some reporting a decreased risk of asthma in twins compared to singletons (163, 164) whereas others report no significant differences (165, 166). Preliminary results from our research group report an increased risk of childhood asthma in pre-school twins compared to singletons, however, this increased risk was mediated by gestational age and birth weight. Adjustment for these factors leads to a significantly decreased risk of childhood asthma in twins. Thus, the general growth constraint of twinning (because of shared space and supply line) may not influence subsequent asthma, whereas factors that makes twins experience different growth from their co-twin could. Several of these factors would be fetal growth determinants common also for non-twins (167).

Generalizability to non-sibling populations from studies investigating antibiotics and asthma using sibling control design can be discussed. For instance, it has been reported that older siblings might be protective to younger siblings developing asthma (168), therefore birth order can be suspected to influence our results. We found a significant interaction between birth order and exposure for any and airway antibiotics with a slightly higher hazard ratio of asthma in first-born children. Yet, the hazard ratios for first-borns, non-first-borns, and the whole cohort independent of birth order, were very similar. Thus, it is not likely that the difference in effect based on birth order has any clinical implications. In addition, there was no significant interaction between birth order and urinary tract/skin antibiotics. It is therefore unlikely that the lack of an association in the sibling analyses for urinary tract/skin antibiotics is due to families with one child. Instead, we believe that our result imply that the association between antibiotics in childhood and asthma is confounded by familial factors, by indication or reverse causation because of respiratory infections.

6 CONCLUSIONS AND IMPLICATIONS

Decreasing birth weight was associated with asthma and decreased FEV_1 in childhood, respectively, in full cohort analyses and in within-twin pair analyses. This indicates that fetal growth affects future development of asthma and lung function, independent of shared environmental factors and gestational age. Similar findings were found in MZ and DZ twins and in preterm and term infants, implying that the associations also were independent of shared genetic factors and that prematurity cannot explain the association between fetal growth and asthma and lung function (**Study I** and **II**).

Analyses of differential effects in individuals with and without asthma for the association between fetal growth and lung function showed similar results for non-asthmatics but not for asthmatics, proposing that other factors may be of importance in individuals with asthma (**Study II**).

A high PPV was found for asthma medication as a proxy for asthma in school-age children, while a somewhat lower PPV was found for pre-school children. A large proportion of both pre-school and school-age children with reported asthma diagnoses in the NPR fulfilled pre-defined criteria of asthma. Thus, asthma medication may function as a valid measure of asthma in itself in children of school-age and older, while asthma medication in combination with an asthma diagnosis may be used in future studies of pre-school children in order to avoid misclassification from obstructive bronchitis (**Study III**).

Exposure to antibiotics during fetal life was associated with an increased risk of asthma in full cohort analyses, but not in analyses using siblings without asthma as controls to siblings with asthma. This indicates that shared genetic and environmental factors confound the association between fetal antibiotic exposure and asthma (**Study IV**).

Exposure to antibiotics in early childhood was associated with an increased risk of asthma in full cohort analyses. In analyses using sibling controls, the association disappeared for urinary tract/skin antibiotics and decreased markedly for airway antibiotics, implying that shared genetic and environmental factors confound the association between early life antibiotic exposure and asthma. The remaining effect of airway antibiotics may be caused by confounding by indication or reverse causation, which is not accounted for in the sibling analyses (**Study IV**).

7 FUTURE PERSPECTIVE

Pregnancy is evidently an immensely important time for future health, but whether insults, such as impaired fetal growth, that disturb normal lung development can manifest as asthma is most likely only one part of this emerging story. Though we believe within-twin pair comparisons can provide valuable indications about the role of fetal growth on subsequent health, the design is associated with challenges. Future studies in regards to the role of fetal environment including the nature of twins' shared environment in utero and the reasons for fetal growth discordance in twins may increase our understanding whether results from twin studies are generalizable to the general population.

As MZ twins share all their genes and intrauterine environment, dissimilarities between the individuals in a MZ twin pair could be an effect of environmental factors or genetic variation that arises in utero after the splitting of the fertilized egg or after birth. As we and others have reported that fetal growth, independently of shared genetic and environmental factors, affects asthma (57, 169, 170) and measures of lung function, it can be speculated that genetic variants such as epigenetic changes, may be involved in the underlying causal pathway. In addition, longitudinal studies have suggested that lung function impairment at birth predicts later respiratory morbidity such as asthma (171, 172). Hence it is possible that individuals with lung function deficits at birth due to *e.g.* fetal growth restriction may be differently susceptible for postnatal exposures and further adverse effects of lung function and development of asthma, compared to infants with appropriate fetal growth. Thus, an improved understanding on to what level environmentally induced epigenetic changes could affect future health, or be inherited, is warranted.

While our results indicate that antibiotics do not seem to cause childhood asthma, this does not justify increased use of antibiotics. On the contrary, bearing in mind the threat of antibiotic resistance, the need of antibiotics should be re-considered one more time before prescribing it to a child with a respiratory infection. Yet, whether our findings of a non-causal association between antibiotics in fetal and early life and childhood asthma are the truth or not, is likely a debate that will continue. Whereas a randomized controlled trial seems unfeasible in regards to ethics, choice of comparator treatment, and low contamination between treatment arms, longitudinal studies with the possibility to take familial confounding into account have been one way to help us further understand the association between antibiotics and asthma. Nevertheless, confirmation of our results in other populations is of interest. In order to perform these types of studies a large population with known family constellations is needed.

Sweden and the other Nordic countries are commonly described as gold mines for researchers with regards to our population-based registers as they can be used to study risk and protective factors for outcomes in individuals and families followed throughout life. Together, the Nordic countries' registers cover over 25 million inhabitants (29) and the similarities between the countries' health- and welfare system and our personal identification numbers, create a good platform for collaborations. Nonetheless, easily accessible collaborations are

hindered by practical, legal, and ethical questions that need to be solved. While validation studies are time-consuming and may not be regarded as 'exciting research', the data should be validated in each country and to the situation for which they are supposed to be used, since there may still be differences between the countries.

So far, none of the Nordic countries have access to a population-based register with information from the primary health care clinics. Thus, other sources have to be used to identify individuals with asthma in register-based studies, and asthma medication and diagnosis from the drug and patient registers seem to be well-suited for this purpose (55, 173-175). However, we have limited possibilities to investigate different phenotypes of asthma in the Swedish registers, which could be of importance as it has been reported that antibiotic exposure in childhood is associated with an increased risk of atopic asthma, but not with non-atopic asthma (115). Moreover, a more well-structured reporting on indications of treatment in the SPDR would facilitate studies on potential consequences of treatments, avoid misclassification of exposures, and take confounding by indication further into account. In addition, a population-based register on drug treatments within hospitals would lead to a better coverage of medication use in the population. It would also make it possible to investigate the individual risk of in-patient treatments.

While this thesis provides some clues about the role of fetal growth and antibiotics and the risk of childhood asthma and lung function impairment, the degree to which disease risk is established during fetal and postnatal life, the essential mechanisms, and susceptible developmental windows are yet to be determined. Efforts should be made to follow individuals from the time before pregnancy to adulthood with valid data in family designs.

8 ERRATA

No thesis is perfect and that includes this one, and even before having it printed, I can report on two errors:

- *Paper I*
  There is an error in Table 1 for the variable maternal BMI, where the number and percentage of individuals with asthma for each category should be BMI <18.5: 15 (6.9%); 18.5-24.9: 774 (13.2%); 25.0-29.9: 303 (14.2%); ≥30.0: 126 (5.9%); Missing: 283 (14.2%)
- *Paper IV*
  A proof change was taken incorrectly. In the second paragraph under the statistical analysis section of the methods, the second sentence should have read: ”For the controls we used all full siblings (excluding 9896 children for whom the father’s identity was unknown) who did not have the outcome of interest yet and who were still in the study at the age at which the index child developed asthma.” The word ”age” is missing in the published article.

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