INFLAMMATION AND "STRESS" IN ASTHMA AND ALLERGY; ARE OXIDATIVE STATE AND CORTISOL LEVELS INVOLVED?

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1 PREFACE

1.1 Acknowledgements

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1.2 Selected abbreviations

aOR: adjusted Odds Ratio

Aspirin: Acetylsalicylic acid

BHR: Bronchial hyperresponsiveness

COX-2: Cyclooxygenase-2

CRP: C-reactive protein

EAACI: European Academy of Allergology and Clinical Immunology

EBC: Exhaled Breath Condensate

ECA: Environment and Childhood Asthma

ECP: Eosinophil Cationic Protein

FeNO: Fractional exhaled Nitric Oxide

GA^2LEN: Global Allergy and Asthma European Network

GINA: Global Initiative for Asthma

FEF_{50}: Forced Expiratory Flow at 50% of the vital capacity

HPA: Hypothalamic-pituitary-adrenal

Hs-CRP: High sensitivity CRP

ICS: Inhaled corticosteroids

IgE: Immunoglobulin E

ISAAC: International Study group of Asthma and Allergies in Childhood

MDI: Metered dose inhaler

$MEF_{50:}$ Mid-expiratory flow

NO: nitric oxide

OR: Odds Ratio

PGE2: Prostaglandin E2

ROS: reactive oxygen species

SARI: Selenium Asthma Research Integration

SPT: Skin prick test

Th: T-helper

T_{reg}: T regulatory

1.3 Summary

There is evidence that allergic diseases, such as asthma and allergic rhinitis are mediated by oxidative stress, possibly involving declining lung antioxidant defences related to increased oxidant airway damage. Several vitamins have anti-oxidant effects including vitamin C, vitamin E, the retinols, vitamin A, α- and β- carotene, and the dietary intake of these antioxidants have been linked to the presence and severity of asthma. Non-dietary hydrophilic endogenous serum antioxidants such as albumin and uric acid have a potential to protect tissues against oxidant injury and are substantial contributors to the total serum antioxidant status. Transferrin may have antioxidant effects inhibiting iron-mediated oxidation through the capacity for binding free iron, whereas serum ferritin as a biochemical marker of body iron stores is the preferred marker for iron-related oxidative stress. Increased use of paracetamol and concurrently decreased use of acetylsalicylic acid (aspirin) during the last thirty years has been hypothesised as one of many explanations for the rise in asthma prevalence as detoxification of paracetamol may deplete stores of the antioxidant glutathione. The model for understanding associations between asthma and psychological "stress" is based upon the assumption that "stress" operates by altering the magnitude of airway inflammatory responses to irritants, allergens, and infections in individuals with asthma. Stress in chronic disease is thought to induce an attenuated responsiveness of the hypothalamic-pituitary-adrenal (HPA)-axis with a lower secretion of cortisol and thereby an increased production of proinflammatory cytokines typically counter-regulated by cortisol.

The overall aim of the study was to investigate the role of oxidative stress and cortisol levels in allergic disease.

The present study is based on two different asthma study populations; the GA^{2}LEN (Global Allergy and Asthma European Network) multi-centre Selenium Asthma Research Integration (SARI) study, using data from all European centres and individual data from the Norwegian SARI study and the Environment and Childhood Asthma (ECA) birth cohort study from Oslo. Asthma and allergy outcomes were assessed through questionnaires and clinical investigations, including lung function testing, allergological investigations, measurement of fractional exhaled Nitric Oxide (FeNO) and bronchial hyperresponsiveness.

In the SARI study plasma selenium levels were found to vary throughout the European population, being lowest in the Norwegian subjects, but with no overall significant association with asthma. Reduced levels of the major serum antioxidant albumin were found in Norwegian children with asthma and reduced albumin was associated with increased FeNO, a marker of allergic inflammation in asthma. In poorly controlled asthma we found reduced levels of plasma Vitamin E and serum transferrin. Co-morbidity of asthma and allergic rhinitis was associated with reduced serum albumin levels and increased serum levels of the oxidant ferritin.

In the ECA study maternal intake of paracetamol during pregnancy was associated with current allergic rhinitis at 10 years in boys and girls. A history of asthma, but not current asthma at 10 years was significantly associated with paracetamol exposure between 0-6 months of age boys and girls even in relation to contemporaneous airway infections. A gender effect was observed, revealing that allergic sensitization was associated with infant paracetamol use in female offspring, also after adjusting for concomitant airway infections.
In the SARI study children with asthma on moderate or high doses of inhaled corticosteroids had reduced salivary cortisol, but co-morbidity of asthma and rhinitis was also associated with

reduced cortisol levels. Furthermore, in children with a co-morbidity of asthma and allergic rhinitis on no or insubstantial anti-inflammatory treatment salivary cortisol levels were associated with reduced albumin and elevated high sensitivity CRP (Hs-CRP) levels.

Overall the present thesis suggests a significant association between oxidative stress and allergic diseases, although a relation between selenium and asthma was not established. The pre-oxidant paracetamol emerges as an independent risk factor for allergic diseases. Reduced levels of the stress hormone cortisol in saliva were associated with allergic disease. Although there was no overall association between oxidative stress and salivary cortisol levels, asthmatic children with rhinitis on no or low doses of Inhaled corticosteroids (ICS) appeared to constitute a specific subtype of allergic disease in which morning cortisol levels were associated with reduced albumin and increased Hs-CRP levels.

1.4 List of papers

Paper #1

Burney P, Potts J, Makowska J, Kowalski M, Phillips J, Gnatiuc L, Shaheen S, Joos G, Van Cauwenberge P, van Zele T, Verbruggen K, van Durme Y, Derudder I, Wohrl S, Godnic-Cvar J, Salameh B, Skadhauge L, Thomsen G, Zuberbier T, Bergmann KC, Heinzerling L, Renz H, Al-Fakhri N, Kosche B, Hildenberg A, Papadopoulos NG, Xepapadaki P, Zannikos K, Gjomarkaj M, Bruno A, Pace E, Bonini S, Bresciani M, Gramiccioni C, Fokkens W, Weersink EJ, Carlsen KH, **Bakkeheim E**, Loureiro C, Villanueva CM, Sanjuas C, Zock JP, Lundback B, Janson C. A case-control study of the relation between plasma selenium and asthma in European populations: a GAL2EN project. Allergy 2008 Jul; 63:865-71.

Paper #2

Bakkeheim E., Mowinckel P., Carlsen KH., Burney P, Lødrup Carlsen KC. Altered oxidative state in schoolchildren with asthma. Pediatric Allergy and Immunology 2010: Jul 13. [Epub ahead of print]

Paper #3

Bakkeheim E., Mowinckel P., Carlsen KH., Håland G., Lødrup Carlsen KC. Paracetamol in early infancy; the risk of childhood allergy and asthma. Acta Paediatrica 2010: Jul 8. [Epub ahead of print]

Paper #4

Bakkeheim E., Mowinckel P., Carlsen KH., Burney P, Lødrup Carlsen KC. Reduced basal salivary cortisol in children with asthma and allergic rhinitis. Acta Paediatrica 2009: Nov 13. [Epub ahead of print]

2 GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

2.1 Asthma and allergy definitions

Asthma.

Asthma is a complex disease with airways inflammation associated with poorly understood genetic and environmental factors. Asthma symptoms are largely characterized by recurrent episodes of wheezing, shortness of breath, chest tightness and coughing. The present scientific approach in the definition of this disorder has given rise to varying definitions;

The Global Initiative for Asthma (GINA) revised guidelines from 2006 (update 2009) gives an operational description of asthma as:

> *a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable airflow obstruction that is often reversible either spontaneously or with treatment (1).*

The British guideline on the management of asthma from the British Thoracic Society has a more observational assessment when describing the asthma disease:

> *The diagnosis of asthma is a clinical one; there is no standardised definition of the type, severity or frequency of symptoms, nor of the findings on investigation. The absence of a gold standard definition means that it is not possible to make clear evidence based recommendations on how to make a diagnosis of asthma. Central to all definitions is the presence of symptoms (more than one of wheeze, breathlessness,*

chest tightness or cough) and of variable airflow obstruction. More recent descriptions of asthma in children and in adults have included airway hyper-responsiveness and airway inflammation as components of the disease. How these features relate to each other, how they are best measured and how they contribute to the clinical manifestations of asthma, remains unclear (2) .

The phenotypes of childhood asthma have evolved partly in order to elucidate the natural history of asthma (3;4) and the risk of asthma after early life wheezing (5), but also to identify specific distinct characteristics of the heterogeneous asthma disease (6). A remaining challenge in early childhood, where bronchial obstruction is a common phenomenon, is the identification of persistent asthma in contrast to transient disease (7;8). Moreover, there is an obvious need to facilitate research within early life events that can improve lung growth in the damaged lung and prevent damage to the potentially healthy in early life and over the life course (7)

Allergy.

A hypersensitivity response is accomplished by unexpected reactions in the skin and mucosal surfaces. Thus the European Academy of Allergology and Clinical Immunology (EAACI) nomenclature task force proposed the following definition to hypersensitivity:

Hypersensitivity causes objectively reproducible symptoms or signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects (8)

Allergy can be antibody- or cell-mediated, and in most patients the antibody typically responsible for an allergic reaction belongs to the immunoglobulin E (IgE) isotype and these patients may be said to suffer from IgE-mediated allergy. The EAACI *nomenclature* task force came up with this definition of allergy:

> *Allergy is a hypersensitivity reaction initiated by immunologic mechanisms (8).*

Although the term allergy has been used synonymously with IgE-mediated disorders, the present definition also includes non-IgE-mediated allergy which will not be further considered in the present thesis.

The term "atopy" (from the Greek *atopos,* meaning out of place) is often used to describe IgE-mediated diseases. Atopic subjects have a hereditary predisposition to produce IgE antibodies against common environmental allergens and have one or more atopic diseases (i.e. allergic rhinitis, asthma and atopic eczema) (9) . The EAACI nomenclature task force proposed the following definition of atopy:

> *Atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis (8).*

The terms atopy and atopic should be reserved to describe this clinical trait and predisposition, and not be used to describe diseases. The first manifestations of atopy in a child are often ‘‘allergic’’ symptoms, such as abdominal pain and vomiting/diarrhea,

symptoms of bronchial obstruction, and skin rashes/angioedema, with the subsequent detection of specific IgE antibody some time later in many cases (8).

Allergic rhinitis.

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced after allergen exposure by an IgE-mediated inflammation of the nasal membranes. The EAACI nomenclature task force employs this definition for allergic rhinitis:

> *The symptoms resulting from an immunologically mediated hypersensitivity reaction in the nose should be called allergic rhinitis (8).*

The WHO document ‘‘Allergic Rhinitis and its Impact on Asthma’’ (10) recommends that the old terms ‘‘seasonal’’ and ‘‘perennial’’, which are not useful where climatic seasons are perennial, should be replaced by the terms intermittent allergic rhinitis and persistent allergic rhinitis, respectively. However, in describing symptoms during the pollen season in a case of pollen induced allergic rhinitis, the old term ‘‘seasonal allergic rhinitis” is valid (8).

2.2 Epidemiology of asthma and allergic disease

The increase in asthma prevalence over the past decades (11) has been called an asthma epidemic and the prevalence of allergic rhinitis and allergic dermatitis has also increased considerably in the industrialized world (12). The presence of allergic sensitization does not at all times involve allergic symptoms and both asthma and allergic rhinitis may occur without detection of IgE-mediated antibodies (10). The term “allergic diseases” typically include

asthma, allergic rhinitis and allergic dermatitis, as well as anaphylaxis, however, the present thesis will be limited to a focus on asthma and allergic rhinitis only.

Allergic disease thus represent a major and increasing health problem in developed countries (13), with asthma as the third leading cause of hospitalisation among children under 18 years of age in the United States, exceeded only by pneumonia and injuries (14). The prevalence of current asthma in Danish school children aged 7-17 years increased from 5.3% in 1986 to 11.7% in 2001 (15). The International Study group of Asthma and Allergies in Childhood (ISAAC) phase III study showed rising prevalence of asthma for children aged 6-7 years and 13-14 years in many centres, but there was an absence of increase in prevalence of asthma symptoms for the older age-groups in centres with existing high prevalence pointing to the development of an upper limit plateau (16). In the Environment and Childhood Asthma (ECA) birth-cohort study from our research group of children born 1992-93 in Oslo, Norway it was demonstrated that every fifth child at 10 years age had a history of asthma (17), a figure likely to reflect the situation in Norway in general with similar results among school children found during the first part of the 1990s with asthma prevalence in Northern Norway of 12.3% (18) and in Mid-Norway of 10.2% (19), but lower in West Norway (5.4%) (20).

The prevalence of allergic rhinitis at 16 years of age in cohorts of British children born in 1958 and 1970 increased from 12% in the earlier cohort to 23% and in the later cohort (14), however in Australia between 1992 and 2002 the prevalence of hay fever and allergic sensitization remained unchanged (21).

2.3 Co-morbidity of asthma and allergic rhinitis

There is compelling evidence of a close relationship between the upper and lower airways in asthma and allergic rhinitis (22). Upper airway disease with nasal blockage, mouth breathing, day time sleepiness and hearing impairment is more prevalent in asthmatic children aged 2-5 years compared with healthy controls (23). In the ECA study, having current symptoms of rhinitis was closely associated with conjunctivitis, asthma, allergic dermatitis and BHR in childhood, with conjunctivitis and BHR being strongly linked to rhinitis with concomitant allergic sensitization (24) (Figure 1).

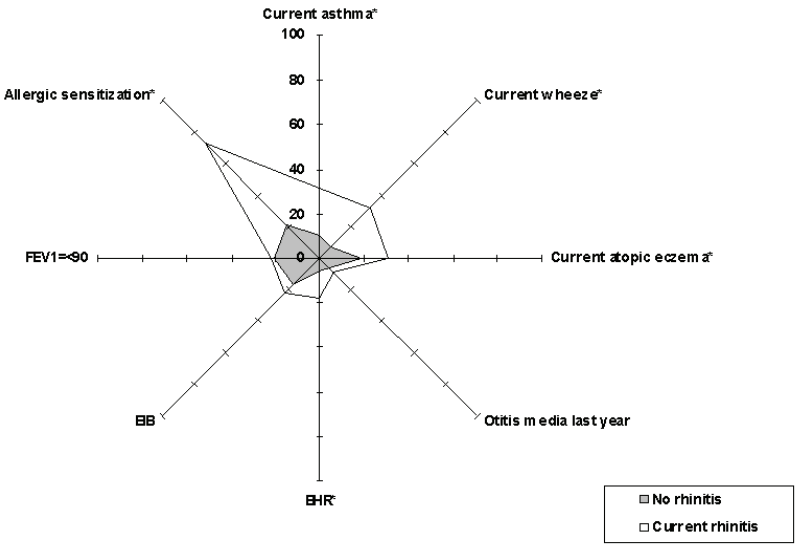


Figure 1: *Radarplot of distribution of co-morbidities and clinical characteristics in children without (n=761) and with (n=254) current rhinitis. BHR are presented as PD20≤ 1 µmol methacholine. The scales are in per cent, (* = p< 0.001, or otherwise no significant difference between the groups). With permission from Bertelsen RJ (The indoor environment and childhood allergic disease; the importance of allergens and microbial components, doctoral thesis 2010, University of Oslo, Faculty of Medicine).*

Allergic rhinitis is present in the majority of patients with asthma, and a significant minority of patients with rhinitis have concomitant asthma (25). Asthma and allergy are closely associated in children and allergic sensitisation is associated with the degree of exposure to allergens (26). Allergen exposure can increase airway responsiveness in non-asthmatic subjects with allergic rhinitis and is associated with an increase in markers of lower airway inflammation, particularly with indoor allergens. Furthermore, nasal allergen provocation can induce bronchial inflammation and vice versa, suggesting close interrelations between upper and lower airways (27).

2.4 Development of allergic diseases and airways inflammation

Risk factors for chronic respiratory disease can be operative years in advance of disease onset and the development of asthma with or without allergic sensitization and other chronic lung diseases progresses with different pace and in diverse inflammatory conditions (28). From a number of previous studies it is known that the naive Th (T-helper) 0 lymphocyte develops either into Th1 cells as part of the cellular defence against bacteria or viruses or into Th2 cells which are liable for the production of IgE and subsequent allergic diseases (29;30). The currently widely accepted model of asthma development is a disease of Th2-mediated chronic airway inflammation, in which asthma is a consequence of chronic inflammation of the airways, engaged basically by Th2 lymphocytes reacting to inhaled allergens and antigens (31). The original classification of Th1 and Th2 pathways has recently been expanded to include additional effector Th cell subsets including Th9, Th17 and T regulatory (T_{reg}) cells (32). The parallel increase of both Th2- associated allergic diseases and Th1-associated autoimmune diseases has put forward the hypothesis that both allergic and autoimmune diseases might result from a functional or quantitative deficiency of T_{reg} cells that control Th1 and Th2 immune responses (33). In this context allergic disease can be the consequence of an

inappropriate balance between allergen activation of T_{reg} cells and effector Th2 cells, and this imbalance could result from a deficiency in suppression by T_{reg} cells or strong activation signals could overcome such regulation (36).

The complex and multiple factors influencing and interacting in the development and expression of allergic diseases may be divided into environmental factors or host factors (1). The numerous environmental factors include allergens, infections, tobacco smoke, air pollution, chemical irritants, diet/lifestyle and stress and the host factors are genetic (genes predisposing to allergic sensitization and/or airway hyperresponsiveness), obesity and gender (1;13).

The extent of this thesis does not give room for a comprehensive presentation of obesity, air pollutants, smoking and chemical irritants as common risk factors for asthma or allergic disease. Likewise the genetic basis in asthma and allergy is beyond the scope of this thesis.

Environmental factors.

Asthma involving allergic sensitization develops as a result of processes in early life, involving the augmentation of a systemic susceptibility to IgE-sensitization and a local susceptibility to airway inflammation with following injure to lung tissues and altered respiratory function (34). One of the major risk factors for asthma is sensitization to allergens resulting from a failure to generate protective immunologic tolerance (35). This tolerance process is orchestrated by airway mucosal dendritic cells and normally results in programming of T_{reg} cells, which inhibit activation of the Th2 memory cells that, among other activities, drive IgE production with subsequent IgE-mediated tissue damage (35). Birth-cohort studies have shown that early sensitization to house dust mite allergens, cat dander and

dog dander are independent risk factors for asthma in children (36;37). However the relationship between allergen exposure and sensitization in children is not straightforward. It depends on the allergen, the dose, the time of exposure, the child's age and the genetics (38;39).

Although early development of allergic disease is a strong predictor of later asthma (40), environmental factors other than allergen exposure are also considerable in the early life origins of allergic disease. Exposure to infections early in life influences the development of a child's immune system along a "non-allergic" Th1-cells pathway, leading to a reduced risk of asthma and other allergic diseases. The proposition of an association between infections and allergic disease has evolved in various ways exploring the role of overt viral and bacterial infections, the significance of environmental exposure to microbial compounds, and their effect on underlying responses of innate and adaptive immunity (41). However, higher numbers of respiratory infections in the first year of life has been found to be a predictor of asthma onset by age 3.3 (46). Infections with rhinovirus during the first 3 years of life have recently been expressed to increase the risk of developing asthma by 6 years 26-fold, compared with 3 fold for allergen sensitization (47).

Paralleling the asthma epidemic, a decreased intake of dietary substances that contribute to antioxidant defence is observed, possibly contributing to the population increase of allergy and asthma (42). On the other hand a large German birth-cohort study found no evidence supporting a delayed introduction of solid diet beyond 4 or 6 months for the prevention of asthma, allergic rhinitis, and food or inhalant sensitization at the age of 6 years (43). Furthermore the American Academy of Pediatrics stated in their guidelines that there is no convincing evidence that maternal manipulation of diet during pregnancy or lactation, use of

soy products, or infant dietary restrictions beyond 4-6 months has any effect on the development of allergic disease (44). However, regular fish consumption before one year of age appears to be associated with a reduced risk of allergic disease and sensitization to food and inhalant allergens during the first four years of life (45).

A possible relationship between outdoor pollution and the development of allergic disease the findings of many studies are weak or contradictory (15;55), but ambient air pollution particles assist Th2 skewing of the immune response by exerting oxidant stress effects on the immune system, including modification of dendritic cell function (46). A Candian population-based study which examined early-life (*in utero* and during the first year of life) exposure to traffic-related air pollution found an association between elevated early-life exposure to traffic-related air pollution and a higher risk of asthma in preschool-aged children (47), but in 9- to 10-year-old children in Oslo no positive associations of long-term traffic-related exposures with asthma onset or with current respiratory symptoms were found (48). Exposures to tobacco smoke both in prenatal life and after birth are associated with measurable harmful effects including a greater risk of developing asthma-like symptoms (1;49), and reduced lung function is found in newborn babies of smoking mothers (50;51). Evidence of increased risk of allergies following maternal or environmental smoking is however less clear (52). Any further in depth discussion on this is outside the scope of the present thesis.

Host factors.

The search for specific genes involved in susceptibility to atopy (immunoglobulin E responses to allergens) or asthma continues, but it is unlikely that a complex and heterogeneous disease such as asthma will have only one or a small number of genes associated with disease expression (53). A large number of genes have been associated with asthma, (54;55).

Recently an association was demonstrated between a specific TBX21 haplotype and allergic asthma in children, implicating that T-bet genes might be helpful in identifying individuals at risk for allergic asthma (56). Epigenetic regulation may in part mediate the complex gene-by-environment interactions that can lead to asthma, and the variable natural history of asthma may be a result of epigenetic changes, such as DNA methylation, covalent histone modifications, microRNA changes, and chromatin alterations, after early or later environmental exposures (57;58).

Gender difference in allergic disease prevalence is well documented, but mechanisms for the differences are not known. Male sex is a risk factor for asthma in children and prior to puberty the prevalence of asthma may be nearly twice as great in boys as in girls (1), however, the switch to a female preponderance through puberty and in adults is well recognised (59).

Host-environment interactions.

In a group of genetically at-risk asthma children it has been shown that the risk of asthma at 6 years was related to both biological (total IgE at six months age) and psychosocial variables (parenting difficulties at 3 weeks age), supporting that asthma starts early in life, with the developing immune system interacting with environmental influences (60). Psychological stress may be an additional environmental factor that augments oxidative toxicity and increases airway inflammation and psychological stress has pro-oxidant properties that augment oxidative processes and increases the likelihood of oxidative stress–induced pathology (61). Although, asthma is clearly associated with a systemic predisposition for allergic Th2 cell cytokine responses, independent local immune events seem to be responsible for the development of allergic airway inflammation, and epithelial respiratory cells are now also recognized as key producers of cytokines, chemokines and growth factors in the airways

(62). Increased oxidative stress has been directly linked to oxidation of proteins DNA and lipids, causing direct lung injury or inducing a variety of cellular responses through the generation of secondary metabolic reactive oxygen species with subsequent effect on remodelling of extra-cellular matrix, mitochondrial respiration, cell proliferation and protective mechanisms in the lungs, such as the surfactant and the antiprotease screens (63). Effective repair mechanisms and immune modulation may also be targets of oxidative stress and oxidative stress is also thought to be a central event in inflammatory responses through the activation of transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), and thus through signal transduction and gene expression of pro-inflammatory mediators (64).

Lifetime course of asthma

Asthma may appear the first time at any moment during life, although typically begins in early life, with sub-optimal foetal growth, maternal micronutrient deficiencies, or smoking during pregnancy being associated with impaired lung function and later asthma (65;66). Asthmatic children may experience repeated remission and relapse (72;73), with a previous notion that a high proportion of children "grew out" of the disease or changed the phenotypic expression of their disease (38;67). Throughout adolescence, major changes occur in the natural history of asthma, including gender reversal with a developing female predominance (68) and remission in about 50% of asthmatics but the disease may return later in adulthood in those supposed to have become healthy (69). Recent cohort-studies have demonstrated that not only chronic childhood asthma continues until adulthood, but also that asthma with onset in early adulthood has its origins in early childhood (70-72). Features estimating persistence of asthma consist of early-onset persistent wheezing, severity of disease, reduced lung function and BHR, sensitization to multiple allergens and allergic co-morbidity (72).

Teenagers who are asymptomatic could actually have pathological signs of sub-clinical asthma, persistent BHR, airway inflammation and thickening of the reticular layer of the airways basement membrane (73-75). On the basis of these large numbers of converging observations, current hypotheses focus on epithelial barrier function and it has been suggested that in asthma a structurally and functionally defective lower airways epithelium underlies abnormal responses to the inhaled environment leading to enhanced signalling between the airway epithelium and the Epithelial–Mesenchymal Trophic Unit (EMTU) and immune cells (76). The above reflections have been listed out in a recent review, pointing out that the natural history of asthma is one of remission and relapse, thus making it important to consider the disease over the life course (76) as illustrated in figure 2.

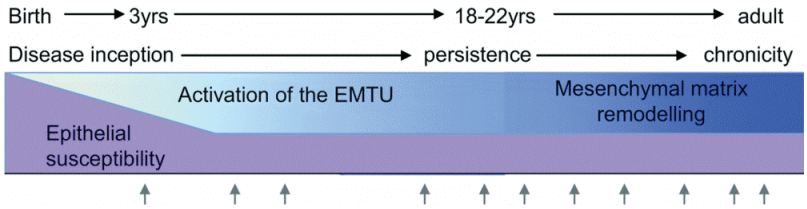


Figure 2: *Schematic representation of asthma from infancy into adulthood. The arrows indicate exacerbations of asthma. EMTU = Epithelial–Mesenchymal Trophic Unit. (From: Holgate ST, Clinical Science (2010) 118, (439–450) "open access")*

"Window of opportunity"

There is growing evidence to support that early deviations from the normal developmental pattern enhance risks for disease later in life with the recognition of a unique "window of opportunity" to permit or prevent the emergence of childhood and adult diseases (77). Foetal or neonatal programming is the phenomenon encompassing deviations from the normal developmental pattern and figure 3 illustrates the complexity of different cumulative interactive mechanisms, including oxidative stress, that are involved in foetal-neonatal programming (78).

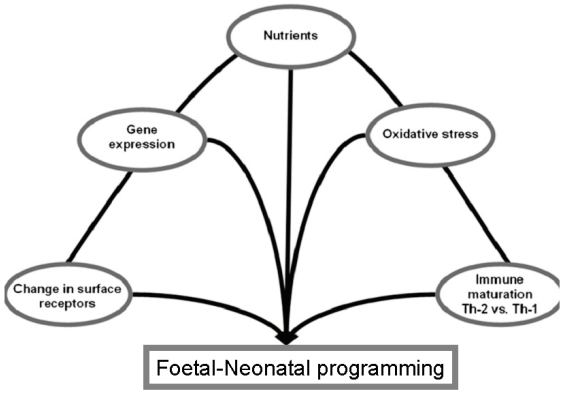
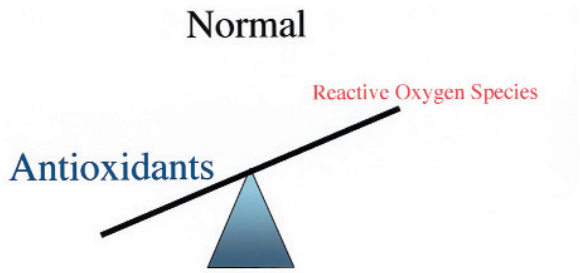


Figure 3: *Five cumulative interactive mechanisms under the umbrella of foetal-neonatal programming explain adulthood consequences. (Modified figure derived from the original by; Nesterenko TH et al., Amer J Perinatol 2009; 26: 191-198.)*

The initiation and development of allergic diseases are characterized by ongoing inflammation and several studies have shown that reactive oxygen species (ROS) play a key role in initiation as well as amplification of inflammation in asthmatic airways (79). ROS are oxygen ions [singlet oxygen, superoxide ($O_2^{\cdot-}$)] or oxygen-containing radicals [hydroxyl, $OH^{\cdot-}$], and their reaction products [e.g. hydrogen peroxide (H_2O_2)] are increasingly recognized as signalling intermediates in their own right that can contribute to developmentally relevant adaptive or maladaptive molecular responses in early life (80). ROS generation through endogenous mechanisms or exogenous environmental exposure is critical to the inflammatory response through perpetuation and amplification of pro-inflammatory signalling pathways (64). If the amount of antioxidants are reduced or the number of reactive oxygen species is increased, the balance of antioxidants and reactive oxygen species may be angled in the direction of oxidative stress as illustrated in figure 4 (81).

A)



B)

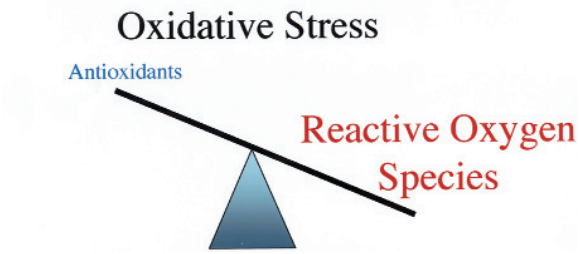


Figure 4: *An imbalance between reactive oxygen species and antioxidants can lead to elevated stress.* ***A,*** *Normally, there are sufficient antioxidants in the respiratory tract such that the production of a small amount of reactive oxygen species is inconsequential.* ***B,*** *If either antioxidants are diminished or production of reactive oxygen species is amplified, the balance of antioxidants and reactive oxygen species is tipped towards oxidative stress. (Reprinted from:Bowler RP; JACI 2002; 110:349-356 , with permission from Elsevier).*

Children who will go on to develop asthma are more likely to show acquired, persistent deficits in lung function if the first symptoms of the disease occur during the first 3 years of life (82). Structural and functional studies of children with asthma symptoms at different ages have provided strong support for the existence of a "window of opportunity" for the establishment of deficits in lung function and airway remodelling in asthma before the age of three (90;91), a time when intervention may modify the natural history of asthma. The impaired balance of Th1 and Th2 cells in the Th2 direction would increase the risk of allergy, especially in the vulnerable phase after birth, when the immune system is immature and inflammatory processes may advance (27).

Markers of airway inflammation

The diagnosis of asthma and of other components of this disease is facilitated through objective measurements (83). Presentation of asthma may vary between individuals, and no test definitely confirms or rules out a diagnosis of asthma. The measurement of bronchial hyperresponsiveness (BHR), fractional exhaled nitric oxide (FeNO) and markers of inflammation in exhaled breath condensate (EBC) or blood may contribute to the understanding of the underlying mechanisms in asthma.

The measurement of BHR has been used in animal models and in humans to study the mechanisms of allergen-induced airway inflammation and the associated physiological changes, as well in the development of new drugs for asthma (84). BHR is used to aid a diagnosis in asthma, although studies up to now have failed to demonstrate any clear association between BHR and counts of inflammatory cells from airway fluids or tissues (94). Although measurements of BHR are proposed to be more useful in diagnosing allergic than non-allergic asthma, there are important differences in diagnostic sensitivity between boys and girls with a sensitivity of 71% and specificity of 69% at PC20 ≤ 4.0 mg/ml in girls whether sensitized to allergens or not, but of no help in the diagnosis of asthma in non-sensitized boys (85). Exercise induced asthma consists of bronchial obstruction occurring immediately, or soon after, physical exercise as a result of increased respiratory water and heat loss due to increased ventilation during exercise, with the subsequent release of mediators and stimulation of airways receptors (86). Exercise-induced bronchoconstriction is consequently often used as a measure of indirect BHR and employed in epidemiological studies (87;88).

FeNO measurements are currently used in daily clinical practise, with FeNO being a marker of airway inflammation and FeNO may have the ability of being a marker also of airway oxidative stress (89). Recent studies report that high FeNO levels support the diagnosis of asthma (90;91), but a study from our own group (90) demonstrates that FeNO is mostly related to allergic sensitisation with a high specificity (0.97), but a low sensitivity (0.41). FeNO is significantly increased in acute asthma or steroid-dependent acute asthma (92) or when the maintenance therapies of steroids are reduced, but FeNO normalises rapidly in patients with stable asthma after steroid treatment (93). Yet, different biomarkers in EBC may turn out to be of an additional value to FeNO in diagnosing asthma, although further exploration of such measures is warranted to define their value for diagnosing and characterising the disease, establishing level of asthma control as well as severity in childhood (94). The serum level of eosinophil cationic protein (ECP) has been used as a clinical marker for disease activity in asthma, and serum ECP levels reflect the intensity of eosinophilic airway inflammation (95). There is no consistent correlation between serum ECP levels and clinical phenotypes, but a relatively high inter-individual variation (95), whereas it is unlikely to be useful for establishing the diagnosis of asthma in an individual patient (96). Measurement of C-reactive protein (CRP) is commonly used to control for infectious disease (97), but high sensitivity CRP (Hs-CRP) is also proposed a surrogate marker of airway-inflammation in asthma (98) and serum Hs-CRP-levels are related to the state of exacerbation and allergic inflammation in asthma (99).

2.5 Nutrients and oxidative state in allergic disease

Diet and nutrients

It has been hypothesized that the recent increase in the prevalence of asthma may, in part, be a consequence of changing diet (100). Today's diet foods are processed in factories, stored and transported over great distances. This is in contrast to the diet of past generations containing foods produced and marketed locally and eaten fresh or fermented (100). According to the National Food Survey of 2005 (101), the UK intake of green vegetables, apples and saturated fats (butter) has decreased, and consumption of fruit juices, low fat spreads and vegetable oils has increased.

The role of dietary factors in asthma is unclear (102). Epidemiological studies in adults and children have demonstrated a relationship between dietary antioxidants and lipids with parameters of allergic diseases (103). Reduced lung function is reported in children with inadequate dietary antioxidant vitamin intake (104). Other studies suggest that early introduction of daily fresh fruit or vegetables may decrease the risk of childhood asthma (116;117). Daily fresh fruit consumption appears to have a beneficial effect on asthma symptoms and lung function in school children in some studies (105;106), however providing free fruit at school for one year does not have any immediate effect on prevalence or severity of asthma in young children (107).

The suggestion that antioxidant supplements can prevent chronic diseases has not been proved or consistently supported by the findings of published intervention trials (108). The evidence from prospective studies and randomized clinical trials is neither consistent nor conclusive (109). Notwithstanding some promising hypotheses and findings, concluding evidence about

the role of specific nutrients, food types, or dietary patterns in the development of asthma has not yet been demonstrated (110).

Oxidative stress

The respiratory airways and the lungs have an antioxidant system protecting against exposure to noxious oxidants (111), and there is evidence that allergic diseases, such as asthma, rhinitis, and allergic dermatitis, are mediated by oxidative stress (81), possibly involving declining lung antioxidant defences related to increased oxidant airway damage (42). Excessive ROS production in asthma leads to alteration in key enzymatic as well as nonenzymatic antioxidants such as glutathione, vitamins C and E, beta-carotene, uric acid, thioredoxin, superoxide dismutases, catalase, and glutathione peroxidases leading to oxidant-antioxidant imbalance in airways with subsequent emerge of chronic inflammation (112). The generation of oxygen free radicals by activated inflammatory cells produces many of the pathophysiologic oxidative changes associated with chronic obstructive airways disease (108). The oxidative changes include damaging biomolecules and cell components, triggering the activation of specific signalling pathways, creating toxic products, altering gene expression and enzyme activity, and disrupting normal repair mechanisms (113). It is suggested that specific inflammatory abnormalities exist in the airways of subjects with mild-to-moderate persistent asthma, in which an inflammatory state is often associated with increased generation of reactive oxygen species and the damaging effects of free radicals (114). The role of oxidative stress in allergic rhinitis is not well studied, but is likely to be similar to that of asthma (81).

Dietary antioxidants

Dietary antioxidants may be particularly important during childhood with growing and developing airways (100). A sub-optimal antioxidant status during the first 3 years of life ("window of opportunity") may result in oxidative airway damage with potentially long-term effects on airway calibre, airway compliance or both. Reduced dietary intake of vitamins, selenium, flavonoids, and fruits by mothers during pregnancy and by young children has the potential to predispose to asthma development, not only by affecting airway growth but also by promoting Th2-differentiation and allergic sensitization (111;128). The reduced antioxidant status during foetal and the first 3 years of life may increase the likelihood for initial critical encounters between cells involved in allergic immune responses and allergens that result potent influences on Th-cell polarization in the Th2 direction (103;115).

Dietary studies suggest associations between oxidative stress, bronchial inflammation, development of asthmatic symptoms, and reduced cellular functions (114;116). Several vitamins have anti-oxidant effects including vitamin C, vitamin E, the retinols, vitamin A, α- and β- carotene (117). The dietary intake of these antioxidants have been linked to the presence and severity of asthma (114) and an inverse relationship between intake of fruit, juice, vegetables and the vitamins A, C and E and lung function has been observed in children (118).

Selenium (Greek σελήνη *selene* meaning "Moon") was discovered in 1817 by Jöns Jakob Berzelius, who found the element associated with tellurium (named for the Earth) (119). As selenium is necessary for the adequate function of the enzyme glutathione peroxidase, it is an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defence systems, and immune function (120;121). In diseases

involving oxidative stress and inflammation higher selenium status has been associated with better outcome (122), but it has also been suggested that selenium intake and allergic airway inflammation are not related in a simple dose-response manner (123). The role of selenium in asthma has been studied extensively, and serum selenium levels have been shown to be lower in asthmatic patients than in controls in several studies (124-126). One study showed that a lower intake of selenium was associated with asthma (140), albeit two of the recent randomised trials failed to show a benefit from selenium supplementation in asthmatic adults (141;142).

Endogenous hydrophilic antioxidants

Non-dietary hydrophilic endogenous serum antioxidants such as albumin and uric acid have a potential to protect tissues against oxidant injury (118), and albumin and uric acid are substantial contributors to the total serum antioxidant status with as much as 28% and 19.3%, respectively, in available assays (127). Bilirubin acts as an important co-antioxidant for Vitamin E, inhibiting plasma and low-density lipoprotein lipid peroxidation. High serum bilirubin was associated with relief of asthma symptoms in adults (128;129).

Free iron can act as an oxidant and catalyze generation of highly reactive hydroxyl radicals with reports of positive associations between childhood asthma and iron levels (126). In this perspective transferrin may have antioxidant effects inhibiting iron-mediated oxidation through the capacity for binding free iron (130), whereas serum ferritin as a biochemical marker of body iron stores, is the preferred marker for iron-related oxidative stress in adults according to the Third National Health and Nutrition Examination Survey from the USA (131).

2.6 Paracetamol and development of allergic disease

The increasing use of paracetamol rather than acetylsalicylic acid (aspirin) during the last thirty years is suggested to be one of many explanations for the increase in asthma prevalence (132). Detoxification of paracetamol may deplete stores of glutathione, which is one of the major antioxidants present in the lung (133), and a reduced source of glutathione may lead to increased oxidative damage to the epithelium and following increased frequency and severity of asthma attacks in susceptible individuals (134). Furthermore, when glutathione levels are low, defective processing of disulfide bonds that are key in antigen presentation has been hypothesized (151;152). Another mechanism for the association between paracetamol and allergic disease is that aspirin, but not paracetamol, inhibits cyclooxygenase-2 (COX-2) activity (132). During common respiratory viral infections, which are well known risk factors for asthma, prostaglandin E2 (PGE2) is produced through the actions of COX-2. As PGE2 promotes Th2 and inhibits Th1 type cytokine generation, the decreased use of aspirin may be a factor in facilitating allergic sensitization and asthma by augmenting the relative Th1/Th2 cytokine imbalance in genetically predisposed children (132). Figure 5 gives an overview of the proposed association between paracetamol effects and asthma development.

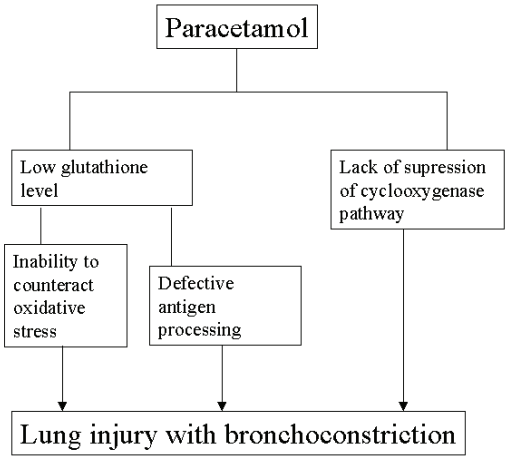


Figure 5:*Proposed mechanisms for paracetamol-induced lung injury*

In British adults frequent paracetamol use, but not the use of aspirin was associated with asthma morbidity and allergic rhinitis (135). Therapeutic doses of paracetamol have since been shown to reduce anti-oxidant capacity in the blood (133), and the association has been confirmed in a large survey in the USA (136). A randomised trial in the USA of paracetamol and ibuprofen use for paediatric febrile illness found increased risk of asthma morbidity in the paracetamol group compared to the ibuprofen group (137).

Use of therapeutic doses of paracetamol during pregnancy and during the early months of life may play a role in the development of allergic and non-allergic asthma in children, and exposure for paracetamol during pregnancy has been associated with allergic asthma in children aged 3-10 years (138;139). Use of paracetamol between birth and 6 months of age, and between 4 and 6 months of age was found to be associated with non-allergic asthma (140). These observational studies with reports on the association between use of in early life and later childhood asthma have controlled for several confounding factors, but the question

whether paracetamol use is merely a marker of contemporary airway infections has not been completely resolved.

The present thesis will contemplate the oxidative balance in allergic diseases in relation to the pre-oxidant paracetamol, the marker of oxidative stress; ferritin and the antioxidants albumin, bilirubin, selenium, transferrin, uric acid and vitamin E (figure 6).

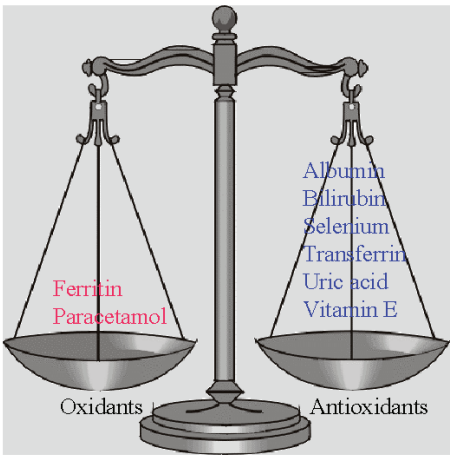


Figure 6: *Oxidative balance illustrated by the "weighed up" oxidants and antioxidants in the present survey.*

2.7 "Stress" and cortisol levels in asthma and allergic rhinitis

Not only oxidative stress, but also psychological stress is known to worsen the course of asthma, although the underlying mechanisms are poorly understood. This problem is especially difficult since stress elicits secretion of cortisol, a hormone that reduces airway inflammation (141). Abnormal neural function contributes to the pathogenesis of airway disease and in addition to affecting airway physiology, the nerves produce and release inflammatory mediators, contributing to the recruitment of activated inflammatory cells which in turn affect the function of airway nerves and release of neurotransmitters (142). The model for understanding associations between asthma and stress is based upon the assumption that psychological stress operates by altering the magnitude of airway inflammatory responses to irritants, allergens, and infections in individuals with asthma. The biological pathways for how stress amplifies the immune response to asthma triggers include the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic-adrenal-medullary axis, and the sympathetic and parasympathetic arms of the autonomic nervous system (143).

Interactions between the nervous system and the immune system include release of several biological compounds such as glucocorticosteroids, catecholamines, cytokines and neuropeptides (144). Glucocorticosteroids and catecholamines as end-products of the stress system may together with histamine have effects on Th-cells and influence the onset and/or course of inflammatory or allergic disease (145). Stress in chronic disease is thought to induce an attenuated responsiveness of the HPA-axis with a lower secretion of cortisol and thereby an increased production of cytokines typically counter-regulated by cortisol (146;147). These patterns suggest that strained parent-child relations as well as perhaps stress in general, bring about adverse outcomes in asthma by diminishing cortisol's ability to regulate cytokine activity and subsequent airway inflammation with ameliorated asthma symptoms (141).

Newborn babies of atopic parents were found to have a lower morning surge of cortisol and decreased diurnal variation of cortisol (148), which may affect the development of allergy in early life through interactions with inflammatory mediators. It has also been proposed that lung function varies with plasma cortisol levels; the number of circulating inflammatory cells varies with plasma cortisol levels; and low levels of endogenous cortisol may be associated with risk for asthma (149). Supporting this theory it has been shown that treatment with inhaled corticosteroids may increase the numbers and restore impaired function of pulmonary T_{reg} cells in asthmatic subjects (33)

Inhaled corticosteroids (ICS) are the first-line therapy for persistent asthma in children. However, individual responses and sensitivity to steroids are recognized, both in terms of effect and side effects. Although probably mostly related to dose, HPA-axis suppression is reported in children using ICS (150-152). Anti-inflammatory treatment with ICS has the potential to augment adrenal function in asthmatics with subnormal adrenal responses at baseline (153). We do presently not know whether an attenuated response of the HPA-axis in childhood allergic disease is a consequence of inherently lower cortisol production in certain subgroups, or due to stress factors or ongoing chronic allergic inflammatory processes.

3 AIMS OF THE STUDY (Research Questions 1-5)

The overall aim of the study was to investigate the role of oxidative stress and cortisol levels in allergic disease.

The specific Research Questions (RQ) were;

RQ1. Is the dietary antioxidant Selenium reduced in asthma?

RQ2. Are allergic diseases in children associated with markers of oxidative stress?

RQ3. Are markers of oxidative stress associated with inflammation in allergic disease?

RQ4. What is the role of the pre-oxidant paracetamol in the risk and expression of allergic disease?

RQ5. Are levels of the stress hormone cortisol in saliva associated with allergic diseases in children?

4 SUBJECTS AND METHODS

4.1 Designs

The present thesis is based on two different asthma study populations; the GA^{2}LEN (Global Allergy and Asthma European Network) multi-centre Selenium Asthma Research Integration (SARI) study, using data from all European centres and individual data from the Norwegian SARI study (Oslo), and the Environment and Childhood Asthma (ECA) birth cohort study from Oslo.

4.1.1 *SARI study.*

4.1.1.1 The European SARI multi-centre study

is a cross-sectional case-control study comprising a population of children and adults across Europe to assess if plasma selenium level is associated with asthma? The study comprised two steps. First, a population-based survey was conducted, through a simple screening questionnaire sent by post. Adult cases were aged 20 to 45 years, living in a defined area and had both a self-reported diagnosis of asthma and either wheezing, or shortness of breath, or waking at night with breathlessness in the previous 12 months (154). Adult controls lived in the same area, were aged 20 to 45 years old and had neither a diagnosis of asthma nor any of the three symptoms. Two centres (Athens and Oslo) sampled paediatric populations and these were recruited in the same way as the adults, but in the age group 7-14 years.

The subjects with asthma as well as controls without asthma were identified and eligible for recruitment into the second step; a clinical investigation. However, some centres were unable to identify the minimum required number of asthmatic subjects from the surveys, in this circumstance further cases could be recruited from clinics providing that they met the criteria and were not treated in the clinic for other non-allergic conditions.

4.1.1.2 The Norwegian SARI study

The Norwegian study population within the SARI study is a cross-sectional case-control study of 102 children, 50 with current asthma and 52 non-asthmatic controls recruited as described for the multi-centre study. 110 children agreed to participate in the study, but eight children with a history of asthma but no current asthma symptoms were excluded from the analyses. 42 cases and 51 controls from this population were included in the multi-centre study based on the information from the screening questionnaire. In the Norwegian SARI study we used doctors diagnosis of asthma and not parental report of asthma diagnosis as inclusion criteria, hence including more cases than in the multi-centre study. Furthermore one control subject had checked for asthma diagnosis in the questionnaire, which was verified to be wrong, and thus analysed as a control.

4.1.2 *'Environment and Childhood Asthma' (ECA) study.*

The present thesis used data from several follow-up investigations in the prospective birth cohort 'Environment and Childhood Asthma' (ECA) study in Oslo, described in detail previously (155).

Inclusion at birth

In brief all healthy term children with a birth weight of at least 2000 g living in the township of Oslo during 15 months from January 1st 1992 were invited provided sufficient language skills were present among the parents with 3754 children included.

0-2 years

The initial ECA study concerned the first 2 years of life with half-yearly questionnaires from birth, lung function measurements at birth in 802 children, and a nested case-control study at

2 years including children with physician confirmed recurrent bronchial obstruction (*n* = 306) and age-matched controls (*n* = 306) of whom 562 underwent detailed examinations during the first two years (155).

10-year follow up

The 10-year follow-up study (2001–2004) with detailed relevant investigations included 1019/1215 (84%) of the children with lung function measurements at birth and/or a clinical 2-year investigation. An overview of the ECA study is given in figure 6.

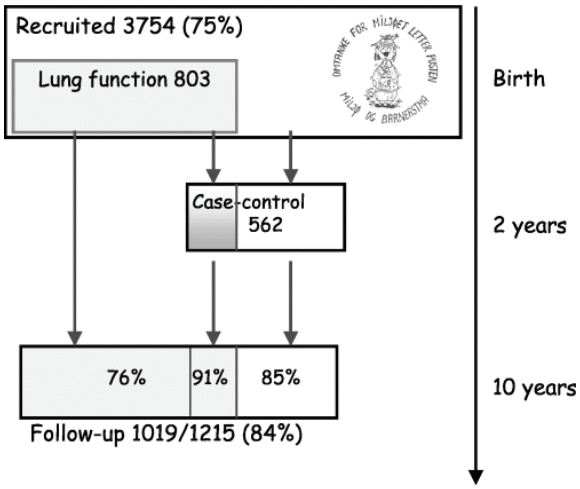


Figure 6*: Illustration of the Environment and Childhood Asthma Study design, with the number of children included in the 10-yr follow-up. The 1215 children who were invited to the follow-up were all traceable children with either lung-function measured at birth (n = 802) and/or included in the case–control group at 2 yr (n = 562). Only outcomes from the 10-yr follow-up were used in the present thesis. From: Carlsen KC et al; Allergy 2006 Apr;61(4):454-60 with permission from John Wiley & Sons.*

4.2 Populations

4.2.1 *The SARI European multi-centre study*

In the multi-centre SARI study 569 subjects with asthma and 576 controls without asthma were included, in which 991 adults (20-45 years of age) were enrolled in 12 centres and 154 children (7-14 years) in two centres, Oslo and Athens. The main characteristics of the study subjects are presented in table 1.

Table 1: Numbers and characteristics of cases and controls recruited by centre – gender, age, smoking, supplement use in the SARI multi-centre study.

Centre	Numbers		% female		Mean age (years)		% current smokers		% supplement users		% atopic	
	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case
Ghent	59	76	56	61	32	29	17	27	28	25	40	85
Vienna	46	12	35	31	35	33	48	31	20	8	47	92
Odense	41	50	69	63	34	32	17	27	44	47	28	77
Berlin	33	18	27	38	39	38	46	54	22	38	19	81
Palermo	53	41	56	52	34	33	25	26	11	14	46	73
Rome	21	28	72	86	35	34	24	25	10	14	24	89
Amsterdam	38	38	59	78	35	33	17	13	22	35	44	90
Lodz	44	52	45	44	31	29	16	33	9	25	34	82
Coimbra	46	51	51	65	33	32	34	9	11	11	22	78
Barcelona	44	44	58	58	34	33	30	31	10	2	26	80
Stockholm	17	38	59	61	39	38	29	18	18	24	35	92
London	51	50	57	70	38	38	17	32	38	30	49	72
Athens	32	29	44	34	10	10	-	-	6	10	41	62
Oslo	51	42	50	47	9	9	-	-	56	51	25	60
Overall	576	569	52	58	35	33	26	26	23	25	35	79

From: Burney P. et al, Allergy 2008; 63:865-71, with permission from John Wiley & Sons

For analysis the subjects were divided into two groups by severity, intermittent and mild persistent disease (night time symptoms twice or less per month, trouble breathing less than once a day, FEV1 (at least 80% predicted and using at most a medium dose of inhaled steroid) and moderate or severe persistent disease who had more than these symptoms.

4.2.2 *The Norwegian SARI study*

Mean age of the children was 9.65 years (range 7-12 years), without significant age difference between the 50 children with asthma and 52 non-asthmatic controls. Allergic rhinitis was significantly more often found in children with asthma compared to controls ($p<0.0001$). The clinical characteristics of the study subjects are presented in table 2.

Table 2: Clinical characteristics of the study subjects in the Norwegian SARI study.

	Asthma	Controls
n	50	52
Boys n (%)	28 (56)	26(50)
Age, yr(Mean (SD))	9.6 (1.5)	9.7 (1.2)
SPT any positive n (%)	24 (48)	7 (13)
Allergic rhinitis (%)	19 (38)	4 (8)

4.2.3 *The ECA study*

The 1019 children were similar to the non-included birth-cohort regarding gender, maternal smoking and paracetamol use in pregnancy and between 0 to 6 months age of the child. Due to lack of data three subjects could not be classified in relation to asthma, thus analyses were done in 1016 (99.7 %) of the study subjects. We found no significant difference between boys and girls regarding paracetamol use, maternal smoking, airway infections, parental asthma or parental allergy (table 3).

Table 3: Clinical data and paracetamol use of the 1016 children in the prospective birth-cohort study, also stratified by boys and girls.

	All children n=1016 N (%)	Boys n=548 N (%)	Girls n=468 N (%)
Paracetamol (yes)			
1.trimester	31 (3)	15 (3)	16 (3)
2. + 3. trimester	32 (3)	15 (3)	17(4)
0-6 months of age	83 (8)	42 (8)	41 (9)
Daily tobacco smoking (yes)			
Mothers in pregnancy	173 (17)	77 (14)	96 (21)
Parents 0-6 months of age	345 (34)	177 (32)	168 (36)
Presence of upper airway infection before six months age (yes)	105 (10)	59 (11)	46 (10)
Presence of lower airway infection before six months age (yes)	87 (9)	55 (10)	32 (7)
Parental asthma (yes)	183 (18)	97 (18)	86 (18)
Parental allergic rhinitis (yes)	488 (48)	270 (49)	218 (47)

4.3 Methods

4.3.1 *Questionnaires*

SARI study

Central training was given to staff in each centre prior to involvement with the main data collection. Instruction included adherence to standard protocols for administering questionnaires, undertaking skin prick tests and spirometry and handling specimens. Questionnaires used mostly questions that had been used in other surveys and the questionnaires were forward and back translated, compared and reconciled. Socio-economic status was assessed from current or last occupation (of father in the case of children) and classified as professional, managerial, skilled, semi-skilled, unskilled or student. Smoking status was classified as never smoked and exposed to less than one hour environmental tobacco smoke per day, never smoked and exposed to at least one hour of environmental tobacco smoke per day, ex-smokers and current smokers.

ECA study

Birth:

The questionnaire at birth included detailed questions related to family health, pregnancy complications, medication use (what and when) as well as environmental and socio-economic aspects.

Six months:

At six months parents recorded specifically all health events (what, how often and age), medication use and contact with health care. Infectious diseases were listed including common upper and lower airway infections as well as other common febrile conditions. Medication use was listed in relation to possible asthma-related treatment, and given with open questions for listing of antibiotics and any other medication. Ascertained for negative answers was done answering no to any medications in the first six months of life.

Two years:

The questionnaire at two years included among the records of child health, medication and diseases, a specific question of paracetamol use, which revealed that less than 1 % of the subjects who had reported paracetamol use at 6 months reported no paracetamol use ever at 2 years. By summarizing the half-yearly reports of medication use from pregnancy until 2 years age we found that the under-reporting of paracetamol exposure compared to the reported use with specific questions for paracetamol at the two year investigation was limited to 20% or less.

10-year follow-up:

A detailed parental structured interview included central ISAAC questions related to airways symptoms of the child was performed (17), with detailed questions regarding environmental exposure, life-style, eczema and allergic rhinitis.

4.3.2 *Clinical and laboratory investigations*

The SARI study and the 10-year follow-up in the ECA study.

In the SARI study spirometry was performed by the EasyOne spirometer according to the ERS/ATS guidelines (156), however, using the reference values of Zapletal (157) for children (up to 18 years). The study subjects withheld short-acting beta 2-agonist and long acting beta2-agonists for at least 6 and 12 hours, respectively, prior to investigation. Examinations were performed at least one week after any respiratory infection.

In the ECA study lung-function at 10 years (reported only in relation to BHR measurement) was measured by forced expiratory flow volumes according to the ERS/ATS guidelines (156) using a SensorMedics VMax 20c (SensorMedics Diagnostics, Yorba Linda, CA, USA). BHR was measured by methacholine bronchial provocation with the Spira nebuliser (Spira Respiratory Care Centre Ltd, Hämeenlinna, Finland) consistent with international guidelines (158). We reported the inhaled dose causing 20% reduction in FEV_1 (PD_{20}), with a maximum inhaled cumulated dose of metacholine of 22.4 μmol.

A standardized exercise test (88) was in the ECA study performed on a second test-day (within one week) by a 6-8 min treadmill run, of which the last 4 min at 95% of maximal heart rate with a 5% incline of the treadmill. An exercise test was regarded as positive with a fall in FEV_1 ≥10% of baseline FEV_1 within 3-20 min after termination of running

In the Norwegian SARI study and in the ECA study FeNO was measured by Ecomedis CLD 88, before spirometry tests: NO-free air was inhaled to near total lung capacity, followed immediately by full exhalation at a constant flow of 50 ml/sec, and the expiratory pressure was maintained between 5-20 mmHg to close the soft palate. FeNO was recorded as mean value from three (alternatively two in a few patients) successive reproducible plateaus according the ERS/ATS-guidelines (159).

In the SARI study skin prick tests (SPT) were undertaken for a common European panel (160) using ALK-Abello reagents against timothy pollen, cat dander, Dermatophagoides farinae, olive, birch, parietaria, alternaria, histamine (10mg/ml) and diluent controls. The widest diameter and its perpendicular diameter were measured, with the mean of these diameters registered. The SPTs in the ECA study were also performed ALK-Abello (161) reagents with histamine as positive and saline as negative controls. Allergens used were: house dust mite (*Dermatophagoides pteronnysinus* and *Dermatophagoides farinae*), German cockroach, dog, cat, rabbit dander, birch, timothy (grass), mugwort, moulds (*Cladosporium herbarium* and *Alternaria alternata*), egg white, milk, peanut and codfish. A positive SPT was defined as the wheal being equal to or more than 3 mm above the diluent control for any of the tested allergens.

Serum was analysed for total and specific immunoglobulin E (IgE) and (s-IgE), respectively, using the UniCAP fluoroenzyme immunoassay according to the manufacturer's instructions (Pharmacia Upjohn, Uppsala, Sweden). For s-IgE common inhalant and food allergens were used as listed above.

In the SARI study vacutainer plastic blood collection tubes and 7 ml gel separator tubes with heparin were used to collect plasma for selenium analyses. Samples were centrifuged at 1100-1300 g for 10 minutes within 2 hours of collection and the plasma was stored at -20^{o}C. All specimens from all centres were shipped to Germany and analysed in one laboratory (Marburg, Germany) by atomic absorption spectroscopy using a Perkin Elmer 4110 ZL Zeeman Corrected Graphite Furnace Atomic Absorption Spectrometer (162). Plasma vitamin E (α-tocopherol) was analysed at A/S Vitas, Oslo by an HPLC system as described by Richenheimer et al except that the fluorescence detector with excitation and emission wavelength was set to 295 and 330 nm, respectively (163). Serum concentrations of albumin, uric acid, transferrin and bilirubin were measured with the Cobas Integra 800 (Roche Diagnostics AS, Basel, Switzerland); ferritin was measured with Centaur (Bayer Diagnostica AS, Leverkusen, Germany), high sensitivity CRP (hs-CRP) was analysed by the immunoturbidimetric method Cobas Integra 800 (Roche Diagnostics AS, Basel, Switzerland) at Oslo University Hospital, Ullevål, Department of Clinical Chemistry respectively.

Saliva was in the Norwegian SARI study collected in Salivette® (Sarstedt, Germany) cotton tubes. The timing of the sampling was post-awakening between 07-09 AM and pre-bedtime between 21-23 PM on the day before the clinical visit. The cotton tubes were placed in a plastic tube after sampling and brought with the study subjects to our clinic, where the tubes were centrifuged at 2000g for 15 minutes and the saliva frozen at – 70 ° C until analysed. Salivary cortisol was analysed with Radioimmunoassay at Oslo University Hospital, Aker, Hormone laboratory within 2-11 months after sampling. A standard commercial Coat-A-Count kit from Diagnostic Products Corporation, Los Angeles, CA, USA was used, all samples were analysed in duplicate and the mean value registered.

4.3.3 *Clinical outcomes*

4.3.3.1 Asthma

In the SARI multi-centre study the following definition was used for cases with asthma: Self-reported (eventually parental reported) diagnosis of asthma and either wheezing, or shortness of breath, or waking at night with breathlessness in the previous 12 months. Controls had neither a diagnosis of asthma nor any of the three above listed symptoms.

Secondarily the subjects were divided into two groups by severity, intermittent and mild persistent disease (night time symptoms twice or less per month, trouble breathing less than once a day, FEV1 (at least 80% predicted and using at most a medium dose of inhaled steroid)) and moderate or severe persistent disease that had more than these symptoms.

In the Norwegian study population within the SARI study the main outcome was *current asthma:* defined as having doctor's diagnosis of asthma and reported wheeze in the previous 12 months prior to study investigation.

Secondary asthma outcomes were:

asthma with FeNO ≥ 20 ppb, the cut-off value for FeNO corresponding to the upper reference limit for healthy children (91).

asthma control (the last year): defined according to the GINA guidelines (1) as controlled versus partly controlled or uncontrolled. Partly controlled or uncontrolled asthma was classified in children reporting any of the following: daytime symptoms more than twice a week, any limitations of activities, any nocturnal symptoms, need for reliever treatment more than twice a week, one or more asthma exacerbation per

year or FEV_1 percent predicted < 80%. The other asthma children were registered as having controlled asthma.

Controls were children without current asthma and *Healthy* children were defined as having neither current asthma nor allergic rhinitis.

In the ECA study a *History of asthma* at 10 years was defined as at least two of three criteria fulfilled by the time of investigation:

Dyspnoea, chest tightness and/or wheezing.

Doctor's diagnosis of asthma.

Use of asthma medication (ß-2 agonist, sodium chromoglycate, inhaled or systemic corticosteroids, leukotriene antagonists and/or aminophylline).

Current asthma at 10 years was defined as having a history of asthma as defined above, plus symptoms and/or medication within the last year and/or a positive exercise test (17).
Current wheeze at 10 years was defined as wheezing, tightness or whistling in the chest in the past 12 months.

4.3.3.2 Allergic sensitization

In the SARI study the presence of *Allergic sensitization* was reported as any positive SPT as defined above. In the ECA-study *Allergic sensitization* was defined as a positive SPT to at least one allergen and/or any s- IgE $\geq$ 0.35 kU/L.

4.3.3.3 Allergic rhinitis

In the Norwegian SARI study *Allergic rhinitis* was defined as doctor's diagnosis of allergic rhinitis and nasal allergic symptoms within the last 12 months prior to study investigation. In the ECA-study *Current allergic rhinitis was* defined as doctor's diagnosis of allergic rhinitis, nasal allergic symptoms within the last 12 months prior to study investigation and allergic sensitization at the time of investigation.

4.3.3.4 Exhaled nitric oxide and bronchial hyperresponsiveness

In the Norwegian SARI study we used *FeNO ≥ 20 ppb* as the outcome due to the available literature at the time of data analyses with cut-off value for FeNO at 20 ppb corresponding to the upper reference limit for healthy children (91). The cut-off level for FeNO of 20 was obtained based upon a previous study from Israel (91), and appeared to be an appropriate marker of inflammation in asthma, as only one control subject (with allergic rhinitis) had levels above this cut-off. In the ECA study a recent publication from our group demonstrated that *FeN0 ≥ 16.7 ppb* was the cut-off value for FeNO corresponding to the upper reference limit for healthy children in this population at 10 years (90).

BHR was only assessed in the ECA study and with *Mild to moderate bronchial hyperresponsiveness defined* as PD_{20} >1 and ≤ 8µmol metacholine and *Severe bronchial hyperresponsiveness* defined as PD_{20} ≤ 1µmol metacholine.

4.4 Statistical analyses

Demographic results are given as mean or median values with standard deviation (SD), 95% confidence interval (95%CI) or per cent when appropriate. Groups were compared using unpaired t-test for continuous variables when normally distributed, otherwise by non-parametric tests (Mann-Whitney). Multiple group comparisons with salivary cortisol as

explanatory variable employed Kruskal-Wallis test following Bonferroni-Holm post hoc test for pair wise multiple comparisons. Otherwise multiple group comparisons made use of one way Analysis of Variance (ANOVA) for continuous variables followed by Tukey's (normal distribution) and Dunnet's (when not normally distributed) post hoc tests. Pearson's chi-square tests were employed for categorical variables. Multiple logistic regression techniques using were used to estimate associations between outcomes and the explanatory variables. The procedure used for the multivariate regression models was Hosmer backward elimination starting with all the candidate variables in the model. At each step, the least significant variable was removed. This process continued until only significant variables or confounders remained in the model. Furthermore, all candidate variables were tested for confounding. In the final model interactions and model assumptions were explored (164). The model assumptions were tested out using Hosmer's and Lemeschow's goodness of fit test. Robust linear regression was employed for associations between salivary cortisol as dependent variable and outcomes. Analyses were performed using Statistical Package for Social Sciences 15.0 (SPSS inc. Chicago, Ill). All p-values (two-sided) equal to or below 0.05 were considered significant.

4.5 Ethical considerations

Local ethical committee approval was given in each centre for the SARI multi-centre study and for both the SARI study and the ECA study approval was obtained from the Regional Medical Ethical committee and the Norwegian Data Inspectorate. The studies were registered in the Norwegian Bio Bank Registry. Each participant was provided with an information sheet explaining the study and parents signed a consent form prior to study participation.

5 RESULTS

5.1 Plasma levels of the dietary antioxidant selenium in asthma

Mean plasma selenium concentrations among the controls varied throughout Europe, ranging in adults from 116.3 μg/L in Palermo to 67.7 μg/L in Vienna and from 80.3 μg/L in the children in Athens to 56.1 μg/L among the children in Oslo (table 4).

Table 4: Plasma selenium concentrations (μg/L) by centre

Centre	Control (Mean, (SD) (Range) (n)	Case (Mean, (SD) (Range) (n)	Difference in Selenium concentration (μg/L)
Ghent	82.7 (16.0) (53.7-124.8) 59	85.9 (17.5) (60.0-131.9) 76	-3.12 (p=0.29)
Vienna	67.7 (13.1) (35.5-112.1) 46	66.7 (10.2) (56.1-89.2) 12	0.96 (p=0.81)
Odense	76.4 (11.5) (60.0-99.5) 41	79.2 (11.3) (56.1-101.9) 50	-2.79 (p=0.25)
Berlin	82.4 (15.2) (60.8-114.5) 33	87.0 (9.7) (70.3-105.1) 18	-4.67 (p=0.24)
Palermo	116.3 (17.5) (82.1-148.4) 53	113.0 (19.8) (72.6-153.2) 41	3.21 (p=0.41)
Rome	88.6 (13.0) (64.7-112.1) 21	88.5 (11.3) (69.5-115.3) 28	0.10 (p=0.98)
Amsterdam	82.0 (11.9) (64.0-120.8) 38	87.7 (15.8) (58.4-138.2) 38	-5.69 (p=0.08)
Lodz	79.2 (14.8) (44.2-113.7) 44	69.5 (15.1) (34.0-110.5) 52	9.67 (p=0.002)
Coimbra	95.7 (11.0) (68.7-114.5) 46	94.5 (13.9) (54.5-124.8) 51	1.26 (p=0.63)
Barcelona	102.1 (13.4) (79.0-135.0) 44	100.0 (16.1) (74.2-138.2) 44	2.19 (p=0.49)
Stockholm	79.9 (8.5) (63.2-101.0) 17	79.5 (11.6) (52.9-99.5) 38	0.39 (p=0.90)
London	99.0 (17.8) (62.4-142.1) 51	97.2 (19.9) (48.2-151.6) 50	1.79 (p=0.63)
Athens	80.3 (13.1) (56.1-120.0) 30	82.7 (10.9) (56.1-109.8) 27	-2.37 (p=0.46)
Oslo	56.1 (9.6) (37.9-86.1) 51	56.4 (9.4) (39.5-75.0) 42	-0.34 (p=0.87)
OVERALL	85.4 (21.0) (35.5-148.4) 574	85.7 (20.3) (34.0-153.2) 567	-0.30 (p=0.81)

From: Burney P. et al, Allergy 2008; 63:865-71, with permission from John Wiley & Sons

Random effects meta-analysis of the results from the centres showed no overall association between asthma and plasma selenium (Odds ratio (OR)/10μg/L increase in plasma selenium:

1.04 (95% confidence interval: 0.89 , 1.21), though there was a significantly protective effect in Lodz (0.48 (0.29 , 0.78)) and a marginally significant adverse effect in Amsterdam (1.68 (0.98 , 2.90)) and Ghent (1.35 (1.03 , 1.77)) (Figure 7).

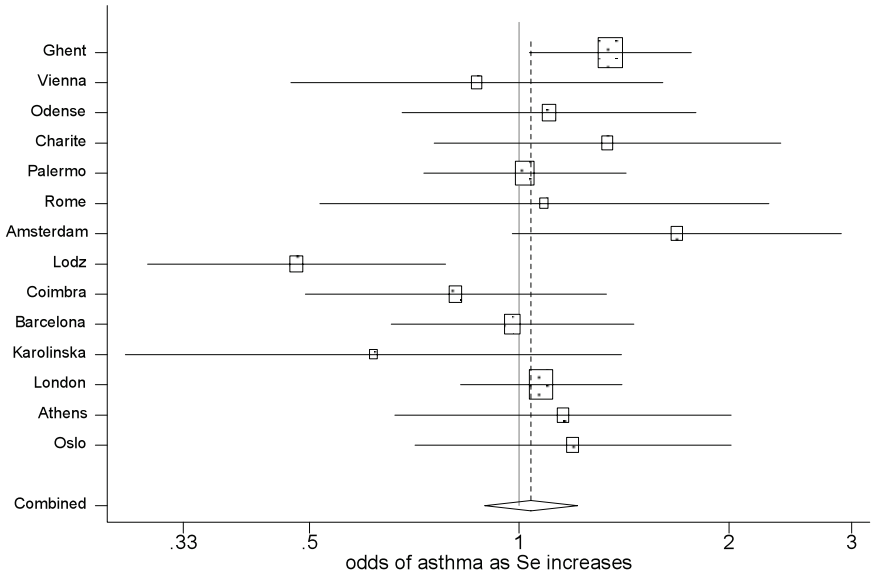


Figure 7: *Graph showing meta-analysis of odds of asthma as plasma selenium increases linearly per 10 μg/L. Effects are adjusted for gender, age, smoking, socio-economic status, supplement use, paracetamol use and body mass index. (From: Burney P. et al, Allergy 2008; 63:865-71, with permission from John Wiley & Sons)*

Comparing moderate and severe persistent asthma with intermittent and mild persistent asthma, selenium was associated with less severe asthma (OR: 0.37 per 10 μg/L increase in selenium (95%CI: 0.15 , 0.88)) in Lodz. The effect went the other way in Coimbra 2.00 (0.99 , 4.01) and overall the effect was minimal 0.95 (0.68 , 1.32) with significant heterogeneity between the centres (Q= 20.908 on 11 degrees of freedom (p= 0.034)). Examination of the characteristics of the centres did not give a clear idea of any systematic difference that might explain the differences between the centres.

There was no evidence of any interactions between a positive SPT or gender and selenium. In the Norwegian Sari study there were no associations between selenium and outcomes of allergic diseases and the analyses did not reveal any interaction with the other antioxidants, ferritin or hs-CRP.

5.2 Markers of oxidative state in allergic diseases

Oxidative state in allergic diseases was assessed in relation to the serum oxidant ferritin and the antioxidants albumin, bilirubin, selenium, transferrin, uric acid and vitamin E.

Asthma

In the Norwegian SARI study current asthma was inversely associated with albumin (g/l) adjusted odds ratio (aOR) (0.81 (0.66 , 0.99)p=0.048) compared to healthy children in a backward stepwise logistic regression model adjusting for age and gender. The serum or plasma levels given in table 5 demonstrate that except for albumin, there were no significant differences between asthma and controls for any analysed antioxidants or for the oxidant ferritin. We also controlled for possible ongoing infection through measuring hs-CRP in serum and the serum levels were not significantly different in asthma compared to controls.

Table 5: Levels of antioxidants, ferritin and hs-CRP in current asthma and controls.

	Current asthma (n=50)	Controls (n=52)	p-values
Selenium µmol/l mean (95%CI)	0.70 (0.67 , 0.74)	0.71 (0.68 , 0.74)	0.72
Vitamin E µmol/l mean (95%CI)	23 (22 , 25)	23 (22 , 24)	0.63
Ferritin µg/l (95%CI) mean (95%CI)	32.5 (26.9 , 38.2)	29.7 (26.6, 32.8)	0.38
Transferrin g/l mean (95%CI)	3.00 (2.91 , 3.10)	2.94 (2.86 , 3.02)	0.26
Albumin g/l mean (95%CI)	46.6 (46.0 , 47.2)	47.4 (46.8 , 47.9)	0.05
Bilirubin µmol/l median (95%CI)	7.0 (5.7 , 7.4)	6.8 (6.9 , 7.9)	0.63†
Uric acid µmol/l median (95%CI)	224 (205 , 245)	216 (196 , 238)	0.30†
Hs-CRP mg/l median (95%CI)	3.6 (3.4 , 3.9)	3.4 (3.1 , 3.6)	0.10†

P-values given by Students t-test or by †Mann-Whitney-test. (From: Bakkeheim E. et al, Pediatr Allergy and Immunol 2010 [Epub ahead of print] with permission from John Wiley & Sons).

Poorly controlled asthma (n= 24) was associated with lower vitamin E levels (µmol/l) (aOR 0.79 (0.65 , 0.95)p=0.02), lower transferrin levels (per 0.1 g/l) aOR (0.72 (0.57 , 0.92)p<0.01) and higher albumin levels aOR (1.53 (1.03 , 2.28)p=0.04) compared to the 26 subjects with controlled asthma (after adjusting for age and gender).

Allergic rhinitis

Children with both current asthma and allergic rhinitis (n=19) had significantly reduced albumin (aOR=0.70 (0.50, 0.99) p=0.04) and higher ferritin levels (mg/l) (aOR=1.04 (1.00 , 1.09) p=0.03) compared with the 48 healthy children.

5.3 Oxidative state and the association with inflammatory markers in allergic disease

Using FeNO levels ≥20 ppb (vs < 20 ppb) identified asthmatic children in all but 1 control child who was subsequently excluded from the remaining 51 controls in the following analyses. Current asthmatics with high FeNO ≥ 20 (ppb) had the lowest albumin levels (mean difference -1.6 g/l);(p=0.02) compared to controls (Figure 8)

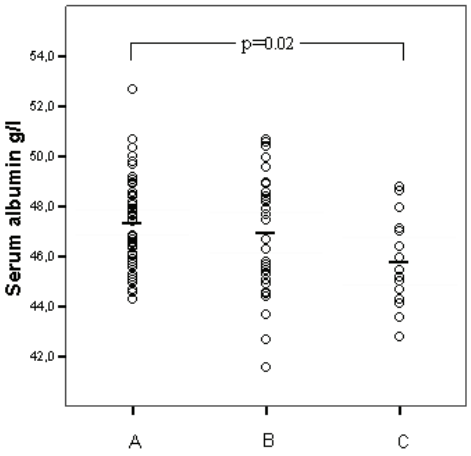


Figure 8: *Levels of serum albumin in controls with FeNO < 20.0 (ppb)(n=51) (A), asthma with FeNO < 20.0 (n=34)(B) and asthma with FeNO ≥ 20.0 (n=16) (C). Bold horizontal lines indicate mean values. Only significant p-values are shown. (From: Bakkeheim E. et al, Pediatr Allergy and Immunol 2010 [Epub ahead of print] with permission from John Wiley & Sons.*

Slightly increased levels of Hs-CRP (within the normal range of CRP) was found in asthmatic children with FeNO ≥ 20 ppb (median difference 0.5 mg/l);(p=0.02) compared to controls with FeNO < 20. In the multivariate analyses (adjusted for age and gender) a high FeNO ≥ 20 (ppb) was associated only with reduced albumin (g/l) aOR (0.60 (0.40 , 0.89)p=0.012) compared to controls with FeNO < 20.

5.4 Paracetamol

Asthma

Maternal use of paracetamol was reported by 31 and 32 women in the in the first and second-third trimester, respectively and never in 972 women in the ECA study. 83 children (42 boys and 41 girls) received at least one dose of paracetamol until six months age (see table 3). Paracetamol use during pregnancy was not associated with a history of asthma at 10 years, but use between 0-6 months of age tended OR (95%CI) (1.53 (0.94 , 2.43)p=0.070) to increase

the risk of a history of asthma. The multivariate regression analyses demonstrated that paracetamol exposure between 0-6 months age significantly increased the risk of a history of asthma after adjusting for gender and the other significant confounders (table 6).

Table 6: Adjusted odds ratios* (aOR) of having a history of asthma at 10 years age by intake of paracetamol between 0-6 months age.

	adjusted Odds Ratios	95 % CI
Gender	**0.60**	(0.45 , 0.80)
Paracetamol 0-6 months	**1.68**	(1.04 , 2.72)
Current allergic rhinitis	**2.64**	(1.88 , 3.71)
Parental asthma	**2.36**	(1.67 , 3.22)

**Significant Odds Ratios in bold.*
The following variables were included in the stepwise backward logistic regression analyses: gender (eventually stratified by boy/girl), paracetamol use in pregnancy (1. or 2.+3. trimester), paracetamol use between 0-6 months age, current asthma at 10 years, current allergic rhinitis at 10 years, allergic sensitization at 10 years, history of asthma at 10 years, current wheeze at 10 years, maternal smoking in pregnancy, mother's education level at birth of child, father's education level at birth of child, family income at the time of birth of child, parental asthma, parental allergy and maternal health in pregnancy. From: Bakkeheim E. et al, Acta Paediatrica 2010 [Epub ahead of print] with permission from John Wiley & Sons)

Gender was a clinically relevant robust confounder and stratified by gender paracetamol intake between 0-6 months was significantly associated with a history of asthma at 10 years in girls, but not in boys OR 2.20 (1.13 , 4.30) and 1.16 (0.61 , 2.23) respectively. Although parental asthma was a significant confounder, the adjusted OR for paracetamol use between 0-6 months remained almost unchanged 2.24 (1.13 , 4.14) for the risk of a history of asthma in girls.

Paracetamol exposure during pregnancy or before six months of age was not associated with current asthma or current wheeze.

Allergic rhinitis

The risk of current allergic rhinitis was in the ECA study increased by maternal intake of paracetamol in the first trimester. This association was robust, remained significant (table 7a) and was only to a minor degree influenced by (table 7 b) adjustment for the significant confounders.

Table 7:
a) The crude risk of current allergic rhinitis at 10 years by each of the 5 remaining significant covariates in logistic regression.

	Univariate Odds Ratios (95%CI)
Paracetamol (no/yes) 1.trimester	2.30 (1.06 , 4.97)
Father's education level† at birth of child	0.88 (0.78 , 0.99)
Parental allergy (no/yes)	3.01 (2.12 , 4.26)
Current asthma (no/yes)	5.91 (4.06, 8.61
Current wheeze (no/yes)	7.11 (4.82, 10.5)

b) The adjusted risk of current allergic rhinitis by paracetamol use in the first trimester of pregnancy also showing the effect of adjusting for significant covariates.

	Adjusted for:	Multivariate Odds Ratios (95%CI)
Paracetamol (no/yes) 1.trimester	Gender	2.37 (1.09 , 5.15)
Paracetamol (no/yes) 1.trimester	Gender, father's education level at birth of child†	2.76 (1.24 , 6.10)
Paracetamol (no/yes) 1.trimester	Gender, father's education level at birth of child†, parental allergy	2.42 (1.08 , 5.45)
Paracetamol (no/yes) 1.trimester	Gender, father's education level at birth of child†, parental allergy, current asthma	2.60 (1.16 , 6.06)
Paracetamol (no/yes) 1.trimester	Gender, father's education level at birth of child†, parental allergy, current asthma, current wheeze	2.42 (1.01 , 5.79)

† Education level gradually listed from 1-6 (1=lowest education level, 6 = university degree).

Allergic sensitization

There was no overall association between paracetamol use in pregnancy or use between 0-6 months and allergic sensitization at 10 years in the multivariate regression analyses. However, in girls, but not boys, paracetamol used in the first six months of age increased the risk of allergic sensitization (odds ratio (OR) 2.20 (1.15 , 4.22). Although current wheeze and

parental allergy were significant confounders, the adjusted OR for paracetamol remained unchanged 2.23 (1.15, 4.33) for the risk of allergic sensitization in girls.

Inflammatory markers

In the ECA study maternal use of paracetamol in the second and/or third trimester of pregnancy significantly increased the risk of high FeNO ≥ 16.7 (ppb) levels in the offspring at 10 years with OR 3.87 (1.31 , 11.37). This association remained significant in the multivariate analyses (table 8).

Table 8: Adjusted odds ratios* (aOR) of having a FeNO ≥ 16.7 (ppb) at 10 years age by maternal intake of paracetamol in the 2.+ 3. trimester.

	adjusted Odds Ratios	95 % CI
Gender	0.95	(0.53 , 1.89)
Paracetamol 2. +3. trimester	**4.83**	(1.44, 16.17)
Allergic sensitization	**5.38**	2.83 , 10.25)
History of asthma	**2.41**	(1.26 , 4.60)
Current wheeze	**2.96**	(1.50 , 5.85)
Mother's education level	**1.36**	(1.05 , 1.75)

**Significant Odds Ratios in bold.*
The following variables were included in the stepwise backward logistic regression analyses: gender (eventually stratified by boy/girl), paracetamol use in pregnancy (1. or 2.+3. trimester), paracetamol use between 0-6 months age, current asthma at 10 years, current allergic rhinitis at 10 years, allergic sensitization at 10 years, history of asthma at 10 years, current wheeze at 10 years, maternal smoking in pregnancy, mother's education level at birth of child, father's education level at birth of child, family income at the time of birth of child, parental asthma, parental allergy and maternal health in pregnancy. From: Bakkeheim E. et al, Acta Paediatrica 2010 [Epub ahead of print] with permission from John Wiley & Sons)

Paracetamol intake by the mother in the first trimester increased the risk of severe BHR at 10 years (OR 2.63 (1.05 , 6.58)) but this association was no longer significant after adjustment in multivariate analyses. However, daughters of mothers who used paracetamol in the first trimester or in the second and/or third trimester of pregnancy had increased risk of severe

BHR with unadjusted OR 6.10 (1.83 , 20.4) and 4.04 (1.08 , 15.13) respectively. In multivariate analyses, only the OR for paracetamol use in the first trimester remained significant 5.48 (1.37 , 21.82) (after adjustment for the significant confounders current wheeze and allergic sensitization .

In forward regression analyses the risk of having current asthma, a history of asthma or current wheeze at 10 years after paracetamol exposure before six months of age were not significantly influenced by reported upper and lower airway infections between 0-6 months of age.

5.5 Basal salivary cortisol levels and the association with allergic diseases in children

In the Norwegian SARI study morning salivary cortisol median (95%CI) nmol/l was lower in asthmatics (8.7 (7.1 , 9.7)) compared to controls 10.4 ((9.6 , 11.8)p=0.006). Evening salivary cortisol levels in children with asthma (0.8 (0.7 , 0.9)) were also significantly lower than in controls (1.0 (0.8 , 1.1)p=0.012). In multiple logistic regression analyses including age and gender, current asthma was not significantly associated with morning salivary cortisol or evening salivary cortisol compared to healthy children. Further regression analyses demonstrated that asthmatics using moderate or high doses of inhaled corticosteroids (ICS) had reduced morning salivary cortisol adjusted (for age and gender) odds ratio (aOR) (95%CI) (0.54 (0.37 , 0.80)p=0.002) nmol/l and reduced evening cortisol (0.09 (0.01 , 0.6)p=0.02) compared to healthy children. Children with asthma as well as rhinitis on no or low doses of ICS had reduced morning cortisol aOR (0.73 (0.56 , 0.96)p=0.02) compared to healthy children. Linear regression analyses demonstrated that the use of ICS was significantly associated with reduced morning (p=0.003) and evening (p=0.04) cortisol levels

after adjustment for age and gender. When excluding children with moderate to high doses of ICS from the analyses, there was no significant influence of low doses of ICS on morning or evening cortisol levels. A significant association was found between morning cortisol levels and allergic rhinitis (p=0.03).

Figure 9 demonstrates the crude salivary morning cortisol levels in healthy controls and asthmatics grouped by rhinitis state and use of ICS.

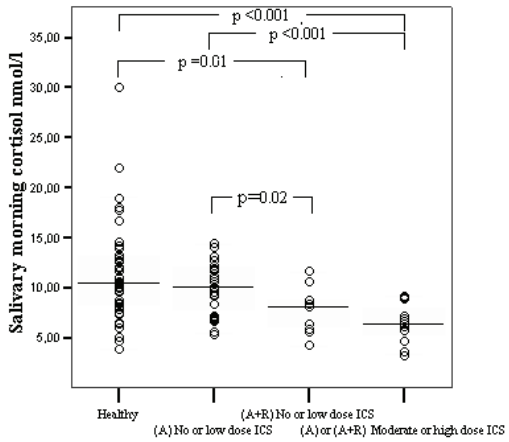


Figure 9: *Levels of salivary morning cortisol in healthy (n=48), asthma only (A) on no or low dose ICS (n=27), asthma and rhinitis (A+R) on no or low dose ICS (n=11) and (A) or (A+R) children on moderate ICS dose (n=12). Horizontal bold lines indicate median values. Only significant p-values are shown. (From: Bakkeheim E. et al, Acta Paediatrica 2009 [Epub ahead of print] with permission from John Wiley & Sons)*

Salivary cortisol levels and the association with oxidative state

There was no overall association in a linear regression analysis between salivary morning cortisol and each of the following explanatory variables after adjustment for age and gender; dietary antioxidants Vitamin E and Selenium, the non-dietary antioxidants; albumin, uric acid, bilirubin and transferrin, the oxidant ferritin and Hs-CRP. However, among the 11 asthmatic

11 asthmatic children with rhinitis using no or low doses of ICS, salivary morning cortisol levels were associated with reduced albumin levels *beta* (95%CI) (–0.72 (-1.43 , -0.16)p=0.046) and increased Hs-CRP levels (2.62 (0.89 , 4.35)p=0.009) after adjustment for age and gender. In the multivariate linear regression analyses, we found that only Hs-CRP remained significantly (2.08 (0.51 , 3.65)p=0.018) associated with morning cortisol, and not albumin (–0.46 (-0.98 , 0.06)p=0.078) when Hs-CRP and albumin were analysed in the same model (unpublished data).

6 GENERAL DISCUSSION

6.1 Main findings of the present study

The present thesis draws attention to the associations between different aspects of oxidative stress, together with glucocorticosteroids as end products of the biological stress system, and outcome measures in allergic disease.

The role of selenium in asthma

The results of the selenium levels in our European population, reported from the SARI multi-centre study, are complementary to the conclusions of two of the latest randomised trials, which failed to show a benefit from supplementation of the diet of asthmatic adults with selenium (165;166). These in contrast to a recent study from Denmark reporting serum selenium concentrations inversely associated with asthma (183). The SARI-study is a large European study and taken together these results suggest that selenium, at levels reported in this study, is unlikely to play any significant role in the rise in asthma prevalence. This does not, however, exclude the possibility that it is involved in specific sub-groups of patients or under specific conditions. It also does not imply that the low levels of selenium in Europe are a matter for complacency. The plasma selenium levels among subjects without asthma were very variable and tended to be highest in the southern centres. In this perspective it is noticeable that the Norwegian children in the present study were found to have the lowest levels of plasma selenium throughout Europe.

In the USA, where levels are generally much higher than in Europe, a significant protective effect of selenium in other diseases, such as cancer development was seen in randomised trials in participants specifically selected from low-selenium regions of the United States (167-170). Furthermore, a role for oxidant stress has been suggested in the adverse effects on the

skeleton of smoking, and in the USA a reduced dietary intake of selenium substantially increased the risk of hip fracture in current smokers (171), whereas this protective effect by selenium intake not was seen in Sweden (172).

Oxidative state in allergic diseases

Antioxidants

The relationship between the serum antioxidant albumin and allergic diseases is unclear and investigated in few studies. The reduced albumin levels in the present study of children with asthma and allergic rhinitis are supported by the results from Japanese school children and Turkish adults with asthma (173;174). In Australian adults albumin was lower in severe asthma and albumin correlated positively with lung function (118). Albumin, the most abundant circulating protein in plasma, exerts important antioxidant activities and may contribute up to 28% of total anti-oxidant status (127). The albumin molecule acts through its multiple-binding sites and free radical-trapping properties. In physiological or pathological conditions, albumin function associated with changes in the oxidative balance, the albumin structure, and its beneficial antioxidant properties can be altered. In general, albumin constitutes the major plasma protein target of oxidant stress (175;176).

Bilirubin was not significantly related to asthma or allergic rhinitis in the present study, but bilirubin levels of the children were within the normal range. Transient relief of severe asthma symptoms during hyperbilirubinemia has been reported (129), but in our study there was no relationship between bilirubin levels and asthma control. The analyses did not confirm any correlation or interaction with other antioxidants or with ferritin as oxidant in contrast to previous in vitro studies of bilirubin acting as a co-antioxidant with vitamin E and albumin (144;193).

The presently observed association between reduced levels of the antioxidant transferrin in partly controlled or uncontrolled asthma compared to controlled asthma has not been frequently described, and a previous report of the association between transferrin and asthma observed no difference in transferrin levels between adult asthmatics and healthy controls (177). Transferrin has the ability to inhibit lipid peroxidation in the lung epithelial lining fluid (178), contributing to the serum antioxidant capacity in patients with asthma (179), but our finding is uncertain. The negative correlation between transferrin and ferritin, however, was expected due to their inversely related effects in regulating body iron stores (180).

The increased uric acid levels found in asthma with allergic rhinitis in the Norwegian SARI study may reflect an upregulation in order to compensate for lower levels of other antioxidants or increased levels of oxidants (127). The non-dietary hydrophilic endogenous serum antioxidant uric acid has a potential to protect tissues against oxidant injury (118), and uric acid contributes to the total serum antioxidant status with as much 19.3% (127). However, a recently published study found no association between allergic disease and plasma levels of uric acid among more than 1000 young adults in Chile (198).

The reduced levels of vitamin E in partly controlled or uncontrolled asthma compared to controlled asthma in the Norwegian SARI study may reflect lower antioxidative ability in less controlled asthma, in line with reports of increased oxidative stress in acute exacerbations of asthma (181). Reduced Vitamin E levels were previously found in Turkish children with mild to moderate asthma (182). In Australian adults vitamin E was reduced in severe asthma, as well as in mild to moderate asthma compared to healthy subjects respectively (118). A recent systematic review and meta-analysis of the association between antioxidant vitamins and

asthma outcome measures found that Vitamin E intake was associated with the severity of asthma (183). However, a randomized trial of dietary supplementation with vitamin E showed no benefit to current standard treatment in English adults with mild to moderate asthma (184). A plausible biological support for the observation is that vitamin E is a fat-soluble antioxidant with a defence role against oxidant-induced membrane injury (103) and that this defence may be altered during asthma exacerbations.

The oxidant ferritin

The regression analyses demonstrated an inverse relation between the antioxidant albumin and the oxidant ferritin in children with asthma and allergic rhinitis, which may express an oxidative imbalance or even oxidative stress in these subjects, with the increased ferritin levels possibly involved in the allergic inflammation. There is scarce literature support for our findings. In Turkish adults there was no association asthma and ferritin (126;174), however, ferritin correlated negatively with plasma vitamin antioxidants in South-Korean adults (131). In iron overload, albumin with the supportive effect of bilirubin has the potential of offering anti-oxidant protection (176). On one hand, there is little doubt that iron causes oxidative stress, but on the other, it is far from clear whether oxidative stress, so generated, leads to poor clinical outcomes (185). A recent study concluded that lung injury after ozone exposure is iron dependent (186). One of the few other studies on this topic suggests that increased iron and decreased selenium concentrations in patients with childhood asthma may be responsible for the oxidant/antioxidant imbalance (126).

Markers of oxidative stress and the association with inflammation in allergic diseases

The albumin levels in the Norwegian SARI study were lowest in children with increased levels of FeNO, implicating an inverse association between albumin levels and inflammatory

activity. FeNO as an objective measure of inflammation in asthma was more strongly associated to reduced albumin than the presence of allergic rhinitis. The reduced albumin levels in asthma, with increased FeNO or with co-morbidity of allergic rhinitis, connect albumin levels with the allergic inflammatory state of these children. Hs-CRP, the proposed surrogate marker of airway-inflammation in asthma (98) showed slightly increased levels of Hs-CRP (within the normal range of CRP) in asthmatic children with high FeNO, emphasizing an increased intensity of allergic inflammation in these subjects. These levels of hs-CRP were unlikely to reflect ongoing infection in these subjects (97).

The role of the pre-oxidant paracetamol in the risk and expression of allergic disease

In the ECA study paracetamol use before six months age was significantly associated with a history of asthma at 10 years after adjusting for relevant confounders. This is in line with studies from Singapore (140), New Zeeland (187) and the ISAAC phase III study (138) for childhood asthma and current wheeze after paracetamol intake in the first year of life. The increased risk of a history of asthma in the present study supports the assumption of a relationship between paracetamol exposure in early life and later childhood asthma, but in this population the more strict definition of current asthma is more likely to reflect the clinical disease state of the children (17). The significant association between paracetamol exposure in infancy and a history of asthma, but not current asthma, may question a causal versus casual relationship, as the former outcome may reflect early wheezing and reverse causation. Although relatively few children in the present study were exposed to paracetamol between 0-6 months, it has been shown that the incidence of wheezing declines with age, and that the associations between risk factors for allergic disease and clinical outcomes are likely to be strongest early in life (67). Paracetamol use was in the present study not associated with

current wheeze at 10 years. Current wheeze as outcome at 10 years had little relation to other objective measures in the ECA study (data not shown).

Maternal intake of paracetamol in the first trimester of pregnancy increased the risk of current allergic rhinitis at 10 years. Furthermore, the risk of allergic sensitization at 10 years in girls was increased after paracetamol intake before six months of age. These findings are consistent with reports from Ethiopia (188), the association between reported paracetamol use and eczema and hay fever in New Zealand children (187) and increased risk of allergic rhinitis in Mexican children respectively (189). Paracetamol for fever in the first year of life increased the risk of allergic rhinitis and eczema in the children in the ISAAC phase three study, and a possible biological explanation is a modification in Th1/Th2 balance towards a disposition for allergic diseases after early exposure for paracetamol (138).

In the ECA study an elevated FeNO was associated with paracetamol use in the second or third trimester and daughters of women using paracetamol during pregnancy were at increased risk of severe BHR at 10 years age. A few recent reports observed increased risk of wheeze, asthma and allergic disease after paracetamol intake in pregnancy (140;190;191), and in line with our 10-year-old children, increased BHR was observed in Danish neonates after maternal intake of paracetamol during the third trimester (192). We are not aware of previous reports of associations between FeNO, indicating an inflammatory process, and paracetamol intake during early childhood. Prospective studies have demonstrated that frequent use of paracetamol in pregnancy may increase the risk of wheezing in the offspring (211;212) and the increased risk of an elevated FeNO and severe BHR after paracetamol exposure in pregnancy indicates that intrauterine inflammatory processes may be altered due to biological effects of paracetamol.

Our results indicate an increased susceptibility in the female gender for the development of allergic disease after paracetamol exposure in early life. This has not previously been reported. Gender and age-dependent differences have been reported for toxicity of paracetamol in rats (193), and there are reports of impaired activity of the glucuronidation pathway for paracetamol in women compared to men (194;195). This is particularly of interest in view of the more frequent allergic disease in pre-pubertal boys compared to a female dominance post-puberty (196).

The analyses in the present study could separate the effects of the infections itself and the use of paracetamol, and as paracetamol in childhood mainly is used for febrile infections, the consistency of the odds ratios after adjustment for upper and lower airway infections gives further evidence in favour of paracetamol as an independent risk factor for allergic diseases.

Levels of the stress hormone cortisol in saliva and the association with allergic diseases

In the logistic regression model adjusting for age and gender, having both asthma and allergic rhinitis was associated with reduced basal salivary morning cortisol compared with healthy children. Several studies of serum cortisol in allergic disease have been published, showing lower spot cortisol among policemen (n=270) with asthma (197) and lower cortisol at midnight in 7-16 year old children with allergic asthma (198). On the other hand a recent study reported similar basal salivary cortisol levels in 47 asthmatic children and adolescents (without information on allergic state) and 45 controls (199). Also adolescents from Colorado aged 12-19 years with allergic diseases (mainly allergic rhinitis) had similar basal salivary cortisol levels and diurnal patterns to controls, in contrast to a significantly lower cortisol response to laboratory-induced stress (200). Among German children exposed to recurrent

maternal distress, an elevation in cortisol levels occurred in response to an acute stressor when there was no accompanying diagnosis of asthma, whereas, in comparison, children with asthma exhibited lower cortisol levels; especially in allergic and bronchial hyperresponsive asthma (201). A study of neonates with disposition to allergic disease showed altered responsiveness of the HPA-axis to stress, which may increase the vulnerability to developing manifestations of allergic disease in later life (202). However, children with low adrenal reserve may also improve their adrenal responses as a result of long-term treatment with ICS (203). A prospective 12-month study of a cohort of 41 pre-adolescent asthmatic children that were placed on long-term treatment with ICS demonstrated that those 10 % with a low adrenal reserve before starting ICS, as well as more than half of the remaining cohort, showed improved adrenal responses while receiving long-term ICS (224).

The significant association between allergic rhinitis and reduced morning cortisol in the linear regression analyses and the differences between cortisol levels in current asthma children with and without allergic rhinitis, all on no or low doses of ICS, suggest an additive effect of having both conditions. Consequently, the lower basal morning cortisol levels in children with co- morbidity of asthma and rhinitis may be associated with the additive effect of having both diseases on reduced secretion of cortisol at the adrenal gland.

The lowest salivary cortisol levels were present in asthmatic children using moderate to high ICS doses, emphasising a dose-dependent adrenal suppression in children on moderate to high doses of ICS. However, children with low adrenal reserve may also improve their adrenal responses as a result of long-term treatment with ICS (203) and the present study along with

earlier studies (204) emphasize the importance of documenting the use of exogen corticosteroids when employing salivary cortisol measurements.

Salivary cortisol and oxidative state

Although not described in the included papers of the present thesis, we demonstrated (non-published data) in the Norwegian SARI study that children with both asthma and allergic rhinitis using no or insubstantial anti-inflammatory treatment appeared to have an association between reduced cortisol levels and reduced albumin or elevated Hs-CRP. Previous studies suggest that low levels of endogenous cortisol may be associated with asthma (167;221), and the children with co-morbidity of asthma and rhinitis in our study may represent a phenotype with inherently lower cortisol production.

There are relatively few available studies with respect to the association between cortisol levels and oxidative state. However it is known that chronic stress can affect human health through a myriad of behavioural and biochemical pathways, for instance a co-elevation of cortisol and insulin, and suppression of certain anabolic hormones leading to an environment of systemic inflammation and oxidative stress (205). Chronic stress and chronic inflammation is frequently observed in allergic disease, and the chronic inflammation may be associated with a lower response of the HPA-axis as different pro-inflammatory cytokines inhibit the ACTH-induced production of cortisol (206;207). In a recent study of adult patients with chronic heart failure it was demonstrated that serum cortisol levels were a complementary and incremental cardiac event risk predictor in combination with Brain Natriuretic Peptide and that cardiac event prediction based on cortisol levels was influenced by oxidative stress (208).

6.2 Strengths and limitations

Strengths

This large multinational European SARI case-control design is an appropriate method when investigating relatively frequent and long-lasting diseases such as asthma and allergy. A major strength was that the cases were drawn from the same populations as the controls. In the Norwegian SARI study the asthma diagnosis is likely to be correct, supported by the reduced lung function and increased FeNO, as well as more frequent allergic sensitisation in current asthma compared to control.

An additional strength in the SARI multi-centre study was that plasma selenium measurements were all made in one single laboratory accredited according to ISO/IEC 17025 standards. The analyses of Vitamin E, endogenous hydrophilic antioxidants ferritin and Hs-CRP were common analyses done in batches, with standardised methodology. We chose to perform separate analyses of antioxidants and ferritin rather than in a compiled kit of plasma reactive oxidants, as it strengthens the understanding of the analyses and biological associations. The concentration of cortisol in saliva, a relatively novel measurement, reflects the unbound fraction of circulating cortisol, and has in adults with cortisol excess a diagnostic value similar to that of serum cortisol (209). The use of salivary cortisol measurements in the present study mitigates the potential increase in cortisol levels by stress and anxiety experienced by blood sampling.

The ECA study is a prospective birth-cohort-study running since 1992 including detailed questionnaires every six months until two years of age and follow up at 10 years age. The design was chosen in order to investigate asthma and allergic disease factors associated with asthma development incorporating environmental exposure. The longitudinal design

decreases the risk of recall bias and misclassification emphasizing an increased level of evidence (210). We used a strict definition of asthma, which has been found to correlate with objective measures and genetic markers of allergic disease (56). A major strength of the ECA study was the detailed recording of respiratory and other infections in the first six months of life, including any medication uses during pregnancy and the first half of infancy.

Limitations

In the cross-sectional case-control design of the SARI study the objectively measured variables for exposure are especially suitable for evaluating potential associations. However, the questionnaire information related to various exposures portrays increased vulnerability to bias and the influence from non-recognized factors and confounders. In this context we cannot conclude on causality, but the discriminatory value of our findings would have to be further assessed in other population studies (211). Hence effect associations within different populations associated with both plasma selenium and asthma could act as a confounder and could produce either a spurious positive or negative effect or a spurious lack of association. For this reason we have in the SARI multi-centre study controlled for area of residence, gender, age, smoking, socio-economic status, body mass index and use of supplements, and these factors had relatively little effect on the estimated effects and both the negative and the positive effects associated with raised plasma selenium levels increased after this adjustment. This argues against confounding by at least these variables. The data do, however, suggest variation in the association between the sites, which may be important. Heterogeneity between the centres could be due to chance, to negative or positive confounding in some of the areas or to effect modification. The possibility that the variability in the findings between the centres is due to chance cannot be discounted. The heterogeneity evident in the data is of only marginal significance ($p=0.088$ in the most powerful of the analyses).

A limitation of the Norwegian SARI study was that only four of the controls had allergic rhinitis, and this group was too small for relevant statistical analyses of associations between control children with allergic rhinitis and levels of antioxidants, the oxidant ferritin, Hs-CRP or cortisol levels. Furthermore, basal cortisol measurements are usually considered inferior to dynamic testing (203). It has though been demonstrated that salivary morning cortisol in adult asthma patients receiving ICS has potential as a screening method for detecting pronounced HPA- axis suppression (212). Cortisol sampling was done during one day only, but there is notable intra-individual variability in salivary cortisol levels (213), which is not so apparent when collection is timed to awakening (204). A cross-sectional study like the Norwegian SARI study does not provide information on the response of adrenal function to ICS treatment. However, with so many steroid naïve, measuring basal salivary cortisol reflects the level of secretion at the adrenal gland, not the rest of the HPA-axis or the responsiveness upon stimulation.

A shortcoming of the ECA study was that we could not, for the entire cohort report the number of doses paracetamol taken by the pregnant mothers or given to the children, and were subsequently not able to analyse dose-response associations. The use of open questions for paracetamol use in epidemiological studies may be associated with under-reporting of the exposure in contrast to the use of specific questions. By summarizing the half-yearly reports of medication use from pregnancy until 2 years age we controlled for eventually under-reporting of paracetamol exposure and we ascertained for negative answers by reports of the parents answering no to any medications in the first six months of life.

6.3 Clinical implications and future perspectives

The heterogeneity of allergic disease with multiple early life risk factors pointing to different phenotypic expressions of asthma and allergy highlights the importance of exploring the underlying basal mechanisms.

The present study does not support an important role for selenium in allergic disease. The SARI multi-centre study, a large European study, demonstrated no association between plasma selenium levels and asthma. Although there are strong theoretical grounds for believing that selenium levels might influence asthma, the present study does not warrant a need for further studies exploring this hypothesis.

In physiological or pathological conditions, function associated with changes in the oxidative balance, the albumin structure, and its beneficial antioxidant properties can be altered. In general, albumin constitutes the major plasma protein target of oxidant stress (175;176). The most evident finding in the Norwegian SARI study of oxidative state was the reduced albumin levels in asthma with increased FeNO or with co-morbidity of allergic rhinitis. The system of oxidants and antioxidants in mammals accentuate towards a balance, and in allergic disease in humans one may hypothesize that this homeostasis may be disordered. Oxidant-antioxidant imbalance leads to pathophysiological effects associated with asthma such as vascular permeability, mucus hypersecretion, smooth muscle contraction, and epithelial shedding (112). The underlying markers of inflammation in allergic diseases are associated with markers of oxidative stress in the present study, supporting a biological plausibility for the association between altered oxidative state and allergic inflammation.

Epidemiological data support the scientific evidence of oxidant-antioxidant imbalance in asthmatics, and the reduced levels of Vitamin E and transferrin in poorly controlled asthma may further assist this position. We will need to explore further the association between reduced asthma control and oxidative state, as it appears likely that those who are temporarily deficient of antioxidants will be those who may benefit at most of antioxidant supplementation. This may make it necessary to combine the research and the clinical investigations in the laboratories and outpatient clinics with further exploration in the emergency rooms, identifying those patients with severe or uncontrolled asthma and with possibly distorted oxidative and inflammatory state. Although intervention studies with antioxidant supplements have not been convincing, the supplementation of antioxidants to boost the endogenous antioxidants or scavenge excessive oxidant production could be employed to diminish the inflammatory response in susceptible individuals with asthma by restoring oxidant-antioxidant balance.

The positive association between ferritin levels and asthma with allergic rhinitis indicates an increased inflammatory level in these subjects and probably increased oxidative stress. Iron supplements in premature infants and older infants and toddlers have been proposed to induce oxidative stress, but recent studies have failed to show any adverse health effects after high-dose oral iron supplementation (235;236). This does not imply a more liberal indication for iron supplements, and this topic deserves a larger focus than has been the case to date, with the vast use of iron supplements through early childhood in premature as well as term infants. The association between iron-related oxidative stress and allergic disease should be further assessed in population studies.

Uric acid levels were within the normal range in asthma and controls. The increased uric acid levels found in asthma with allergic rhinitis were not confirmed in the regression analyses. An upregulation of uric acid in subjects with allergic inflammatory disease would have to be further explored in other studies.

The lack of association between the normal levels of bilirubin and allergic disease in the present study does not highlight a role for bilirubin in asthma or allergic rhinitis. However, the few reports of relief of asthma symptoms during acute hyperbilirubinemia and the theoretical biological mechanisms presented previously in the thesis permit the elaboration of further surveys on this issue.

Our finding of increased Hs-CRP levels in asthmatics with elevated FeNO gives a further indication of the inflammatory disorder in these children. In a previous study, levels of Hs-CRP in exhaled breath condensate seem to be correlated with those measured in serum (214), and this association may in the future provide another useful diagnostic tool for detecting and monitoring low-grade inflammation of patients with asthma near to the affected organ; namely the lung. A major challenge is, however, to identify appropriate markers or mediators of inflammation that are useful in diagnosis and understanding underlying mechanisms (215).

The problem of ingestion of pro-oxidative compounds is related to the issues of temporal low antioxidant status. The inflammatory and oxidative state of the airway epithelium and the underlying mesenchyme may be crucial in translating the allergic phenotype into the lower airways, with temporal development of different asthma sub-phenotypes with conflicting responses to asthma treatment and natural history of disease (76). The finding of increased risk of allergic diseases in female offspring exposed to paracetamol in pregnancy or the first

six months of life suggests that gender differences in susceptibility to triggers of asthma and allergy may diverse the risk of allergic development after ingestion of paracetamol. The current evidence points to the necessity of rising parents' awareness that early use of paracetamol in infancy may contribute to the risk of asthma development. Thus; a restrictive use of paracetamol should be advocated. The present results, endorsing paracetamol use in early infancy as an independent risk factor for allergic disease, underline this position. However, to estimate the exact effect of paracetamol on outcomes in allergic disease, only a population-based randomised trial of adequate power and duration to examine the incidence of childhood asthma with paracetamol compared with ibuprofen (eventually aspirin) would bring us closer to a definite answer to the question of causality.

The reduced salivary cortisol levels in asthma with allergic rhinitis may be related to allergic disease or the ongoing chronic allergic inflammatory process. Glucocorticosteroids are the only drugs available today with the ability to block several of the inflammatory and pro-inflammatory stages in allergic disease including asthma. The clinical effects of ICS have been demonstrated in numerous of studies in children with asthma, with effects containing improved lung function, reduced number of exacerbations, enhanced symptom control and less reactive airways, but there are also many non-responders, particularly in young age (216;217). Most studies of adrenal function in asthmatic children have focused on the risk of suppression of the HPA-axis in children treated with ICS, but a recent review of the literature states that the use of ICS in children leads to minimal risk of clinically relevant adverse effects on growth, bone density or cortisol levels (151). In this perspective our study and the present literature support the measurement of basal salivary morning cortisol as convenient in monitoring treatment with inhaled corticosteroids in children. There is potential for the identification of adrenal insufficiency when using salivary cortisol measurement with

Synacthen stimulation (218) and it is expected that the measurement of salivary cortisol will become routine in the evaluation of patients with disorders of the HPA-axis (219). With respect to the role of cortisol levels in allergic disease, the initiation of a birth-cohort study assessing cortisol levels at birth, with further repeated measurements of cortisol levels until manifestation of asthma and allergy symptoms in later childhood, would make it possible to shed light on the pertinent question; do inherently lower cortisol levels increase the risk of developing asthma or allergy? Such a study would also act in response to the uncertainty of whether cortisol levels change as the disease develops, particularly in relation to the initial levels measured from birth and onwards. The evening salivary cortisol levels in the present study were low and relatively close to the detectable limit. This, together with small differences between outcomes in evening levels, impair the clinical relevance of measuring basal evening cortisol in asthma children.

7 MAIN CONCLUSIONS

Based on the findings in the current thesis in relation to previous knowledge and discussion we are able to conclude to the research questions as follows:

There was no overall association between plasma selenium levels and asthma in the investigated European population. Selenium is unlikely to play a major role in the asthma epidemic over the recent decades.

Reduced levels of the major serum antioxidant albumin were found in children with asthma. Co-morbidity of asthma and allergic rhinitis was associated with reduced albumin levels and increased levels of the oxidant ferritin. Reduced levels of the antioxidants vitamin E and transferrin were found in poorly controlled asthma. Allergic diseases were associated with markers of oxidative stress.

Reduced levels of the serum antioxidant albumin were associated with high FeNO, a marker of allergic inflammation in asthma. Hs-CRP, a surrogate marker of airway-inflammation in asthma was increased in asthmatic children with high FeNO. Markers of oxidative stress were associated with inflammation in allergic diseases.

We found no association between current symptoms of asthma or wheeze at 10 years and early paracetamol intake. A history of asthma and current allergic rhinitis were significantly associated with early paracetamol exposure in boys and girls. However a gender effect was observed revealing that allergic sensitization was associated with paracetamol use in early infancy in female offspring. The presence of concomitant airway infections in early life did

not alter the results. Paracetamol use in pregnancy or early infancy was an independent risk factor for allergic disease.

Asthmatic children with rhinitis on no or low doses of inhaled corticosteroids had reduced salivary morning cortisol levels. Levels of the stress hormone cortisol measured in saliva are associated with allergic diseases in children.

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