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Genetic and Environmental Influence on Asthma and Related Phenotypes

The Effect of Profession and Inflammatory Cytokine Genes *IL1A*, *IL1B* and *IL10*

■

ACADEMIC DISSERTATION

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To my family

Abstract

Background: Various environmental and hereditary factors affect asthma risk. The immunological defence against antigens is initiated by antigen presenting cells, which induce a primary inflammatory reaction. Interleukin-1 (IL-1) and IL-10 are among the key mediators of this reaction and polymorphism in their genes can contribute to the development of appropriate or undesirable (asthmatic or allergic) inflammation.

Aims: To assess the effect of environmental factors on asthma risk by comparing the asthma incidence in different occupational groups; to investigate the effect of polymorphism in genes *IL1A*, *IL1B* and *IL10* on asthma and related phenotypes; to ascertain whether sensitisation to common allergens differs between adult asthmatics and controls.

Subjects and methods: The grant for special reimbursement for anti-asthmatic medication from the Social Insurance Institution of Finland was used as asthma criterion. Case ascertainment was evaluated from the case records of 205 asthmatics. The entire 25- to 59-year-old employed population of Finland was followed 1986-1998 to estimate the effect of work-related factors on asthma incidence. Genetic studies were made in a population-based sample of 245 asthmatics and 405 matched controls. The data from allergy testing, spirometry and other laboratory measurements were obtained for all subjects. The base exchange polymorphisms *IL1A*(+4845G>T), *IL1B*(-511C>T), *IL10*(-819C>T), *IL10*(-1082G>A) and *IL10*(-592C>A) were analysed using standard methods.

Results: There were 49575 new cases of asthma during the follow-up. The overall attributable fraction of work-related exposure was 29% for men and 17% for women. The incidence of asthma was higher in women (2.47/1000/year) than in men (1.65/1000/year). The age-adjusted risk was increased especially in agricultural and industrial occupations. *IL1B* gene was associated with asthma in men, heterozygotes running a lower risk (OR 0.5). *IL1A* was associated with atopy in controls and with nasal polyposis in asthmatics. The functioning of the *IL10* haplotypes, which are involved in the regulation of IL-10 levels, were found to differ between asthmatics and controls. *IL10* seems to participate in the regulation of eosinophil counts and IgE levels without effect on asthma susceptibility. Sensitisation to some of the allergens studied (e.g. animals, tree and grass pollens) was more common among asthmatics than controls.

Conclusions: Occupational factors are implicated in asthma in adults much more than previously assumed. The genes coding for inflammatory cytokines IL-1 and IL-10 seem to be associated with severity of asthma. *IL1A* is associated with atopy and nasal polyposis. Further, *IL1B* is associated with asthma in men.

Lyhennelmä

Tausta: Useiden ympäristötekijöiden ja perinnöllisyyden tiedetään vaikuttavan astmariskiin. Elimistön antigeenia esittelevät solut aikaansaavat ensivaiheen tulehdusreaktion, joka aloittaa puolustautumisen altistetta vastaan. Keskeisiä välittäjäaineita tämän tulehdusreaktion säätelyssä ovat interleukiini-1 (IL-1) ja IL-10, joiden geenien monimuotoisuus voi vaikuttaa siihen, kehittyykö ihmiselle altistukseen liittyen tarkoituksenmukainen vai ei-toivottu (astmaattinen tai allerginen) tulehdus.

Tavoitteet: Tutkimuksen tarkoituksena oli arvioida ympäristötekijöiden vaikutusta aikuisten astmariskiin vertaamalla sairauden ilmaantuvuutta eri ammattiryhmissä, tutkia geenien *IL1A*, *IL1B* ja *IL10* monimuotoisuuden vaikutuksia astmaan ja siihen läheisesti liittyviin tekijöihin sekä selvittää eri allergeeneille herkistymisen määrä astmaatikoilla ja vertailuhenkilöillä aikuisväestössä.

Aineisto ja menetelmät: Astmakriteerinä käytettiin Kelan myöntämää astmalääkkeiden erityiskorvausoikeutta. Kriteerin luotettavuus arvioitiin 205 henkilön lääkärinlausunnoista ja sairauskertomusmerkinnöistä. Työympäristön vaikutusta astmariskiin tutkittiin seuraamalla rekisteritietojen perusteella koko suomalaista työssä käyvää (25-59v.) väestöä 1986-1998. Perinnöllisten tekijöiden vaikutusta selvitettiin kliinisesti tutkittujen 245 astmaatikon ja 405 kaltaistetun vertailuhenkilön väestöpohjaisessa aineistossa. Tutkituille tehtiin allergiatesti ja spirometria sekä tutkittiin verinäytteitä. Genotyypitykset kohdista *IL1A*(+4845G>T), *IL1B*(-511C>T), *IL10*(-819C>T), *IL10*(-1082G>A) ja *IL10*(-592C>A) tehtiin aikaisemmin kuvattuja menetelmiä käyttäen.

Tulokset: Seurantatutkimuksen aikana todettiin 49575 uutta astmatapausta, joista työympäristöön liittyvien tekijöiden arvioitiin aiheuttaneen 29% miehillä ja 17% naisilla. Sairauden ilmaantuvuus oli selvästi suurempi naisilla (2,47/1000/vuosi) kuin miehillä (1,65/1000/vuosi). Astmariski oli suurin maatalous- ja teollisuusammateissa. Tutkituista geeneistä *IL1B* näyttää olevan yhteydessä astmariskiin. Heterotsygoottisilla miehillä oli puolet (OR 0,50) pienempi astmariski kuin homotsygoottisilla. *IL1A* geenin puolestaan havaittiin olevan yhteydessä atopiaan vertailuhenkilöillä, kun taas astmaatikoilla se oli yhteydessä sairautta vaikeuttavaan nenäpolypoosiin. IL-10 tuotantoon vaikuttavia *IL10* geenin haplotyyppejä tutkittaessa havaittiin niiden toimivan eri tavalla astmaatikoilla ja vertailuhenkilöillä. *IL10* näyttää osaltaan säätelevän eosinofiilisten valkosolujen määrää ja immunoglobuliini E:n pitoisuutta, mutta sillä ei näyttäisi olevan selvää vaikutusta astmariskiin. Astmaatikot ovat herkistyneet vertailuhenkilöitä useammin vain osalle tutkituista allergeeneista (esim. eläimille, puiden ja heinien siitepölylle).

Johtopäätökset: Työympäristöön liittyvät tekijät selittävät paljon suuremman osan uusista astmatapauksista kuin aikaisemmin on arvioitu. Tulehdusta säätelevien välittäjäaineiden IL-1 ja IL-10 geenit näyttävät vaikuttavan astman vaikeusasteeseen. *IL1A* geeni on yhteydessä atopiaan ja nenäpolypoosiin ja miehillä *IL1B* geeni astmariskiin.

Contents

Abbreviations

AAF	Adult Asthma in Finland study
BAL	bronchoalveolar lavage
BHR	bronchial hyperresponsiveness
CI	confidence interval
Con A	concanavalin A
COPD	chronic obstructive pulmonary disease
DNA	deoxyribonucleic acid
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA	human leukocyte antigen
ICE	interleukin-1 converting enzyme
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
IU/L	international units per litre
LPS	lipopolysaccharide
MFHS	Mini-Finland Health Survey
NK	natural killer cell
NP	nasal polyposis
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RR	risk ratio
SCF	stem cell factor
SII	Social Insurance Institution
SLE	systemic lupus erythematosus
SNDA	subset of newly diagnosed asthmatics
SNP	single nucleotide polymorphism
TGF	tumour growth factor
T_H	T helper cell
TNF	tumour necrosis factor

List of original communications

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals.

I Karjalainen A, Kurppa K, Martikainen R, Klaukka T, Karjalainen J. Work is related to a substantial portion of adult-onset asthma incidence in the Finnish population. *Am J Respir Crit Care Med* 2001;164(4):565-8

II Karjalainen J, Nieminen MM, Aromaa A, Klaukka T, Hurme M. The interleukin-1β genotype carries asthma susceptibility only in males. *J Allergy Clin Immunol* 2002;109(3):514-6

III Karjalainen J, Hulkkonen J, Pessi T, Huhtala H, Nieminen MM, Aromaa A, Klaukka T, Hurme M. The *IL1A* genotype associates with atopy in non-asthmatic adults. *J Allergy Clin Immunol* 2002; 110(3):429-34

IV Karjalainen J, Joki-Erkkilä VP, Hulkkonen J, Pessi T, Nieminen MM, Aromaa A, Klaukka T, Hurme M. The IL1A genotype is associated with nasal polyposis in asthmatic adults. *Allergy* 2003; 58(5):393-396

V Karjalainen J, Hulkkonen J, Nieminen MM, Huhtala H, Aromaa A, Klaukka T, Hurme M. IL-10 gene promoter region polymorphism is associated with eosinophil count and circulating IgE in adult asthma. *Clin Exp Allergy* 2003; 33:78-83

I Introduction

Asthma is one of the most common chronic diseases in both children and adults. Clearly, asthma exacts a heavy toll in both its costs to society and its affect on the individual. Despite progress in treatment of the disease, the expenses it incurs have risen in recent years, the overall annual costs in Finland being estimated at 420 million euros at the start of the 1990s. This included direct costs resulting from medical care and changes in working and living conditions, indirect costs relating to loss of production, and costs resulting from disabilities (Haahtela and Laitinen 1996). Although mortality and total number of days in hospital due to asthma have been decreasing in Finland (Haahtela et al. 2001) the disease is a source of great concern to the sufferer. Adults with asthma are troubled not only by the symptoms themselves, but by limitation of daily activities (occupational, social and physical), sleep impairment and emotional problems such as anxiety and frustration (Juniper 1997).

Both genetic and environmental factors contribute to the inception and evolution of asthma and atopy. To comprehend the pathogenetic mechanisms underlying these disorders, it is essential to identify factors which initiate, intensify and modulate the inflammatory response of the airway and to determine how these immunological and biological processes produce the characteristic airway abnormalities (Busse and Lemanske 2001). Multiple chromosomal regions and polymorphisms of several candidate genes have already been linked to or associated with asthma and atopy (Hakonarson and Wjst 2001). An understanding of the molecular genetic events contributing to the development of allergies and asthma will greatly increase our knowledge of the pathogenesis of these diseases (Borish 1999).

The generally accepted conception is that environmental factors are important for the development of asthma, but the individual must be genetically predisposed

to respond to environmental influences (Los et al. 1999). In this thesis, the environmental influence on asthma incidence is estimated by comparing the overall asthma incidence in different occupations. Further, the roles of inflammatory cytokine genes *IL1A*, *IL1B* and *IL10* in asthma and its associated phenotypes are studied by the means of candidate gene approach. All the studies have been carried out in population-based samples of Finnish adults.

II Review of the literature

Definitions and phenotyping

Asthma

Definition

Asthma is a complex syndrome with a wide range of clinical phenotypes in both adults and children. Its major characteristics include a variable degree of airflow obstruction, bronchial hyperresponsiveness, and airway inflammation (Busse and Lemanske 2001). The definition of asthma has changed during recent decades with accumulating understanding of the immunological mechanisms underlying the disorder. Until the 1980s its basic characteristics were considered to be bronchospasm, oedema and hypersecretion, while the more recent definition by The Global Strategy for Asthma Management and Prevention Report states that "Asthma is a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway

responsiveness to a variety of stimuli". The role of airway inflammation is thus highlighted (NHLBI/WHO 1995).

Several guidelines have been developed for the management of asthma (NHLBI/WHO 1995, NHLBI 1997, The Finnish Society of Respiratory Medicine and The Finnish Paediatric Society 2000). Although there are differences between these guidelines, the treatment of inflammation is the basis of asthma medication strategy in all of them. However, these same recommendations still emphasise lung function measurements for the diagnosis of asthma. It is possible, or even probable, that in future the characterisation of airway inflammation will also be included.

Asthma case ascertainment

Various methods have been used to define asthma in epidemiological studies. No generally applicable golden standard has been presented which would apply in both group-level epidemiology and individual-level clinical practice (Toelle et al. 1992). Most epidemiological studies have used symptom questionnaires and history of physician-diagnosed asthma alone or together with observed bronchial hyperresponsiveness (BHR). Given the variable course of asthma and the different demands imposed by study design it is obvious that no single definition of asthma will be applicable to all studies (Pekkanen and Pearce 1999).

The Finnish national health insurance scheme, which includes a drug reimbursement system, covers the entire population. The Social Insurance Institution (SII) of Finland maintains a register which provides population-level information on the occurrence of asthma. Individuals with clinically well-established persistent asthma are eligible for special reimbursement for medication and are therefore registered. Granted reimbursement has previously been used in Finnish scientific studies on the epidemiology of asthma (Reijula et al. 1996, Haahtela and Klaukka 1998). The Special Reimbursement Register is based on diagnoses made by specialists. Both clinical and physiological criteria for persistent asthma are applied. Since the level of special reimbursement for

anti-asthmatic medication costs is relatively high (75% of total costs), it is likely that more or less all those who fulfil the criteria are registered.

Related phenotypes

The lack of a defined and specific asthma phenotype has been considered a major hurdle in reliably detecting asthma-associated genes (Sandford et al. 1996). Consequently, much emphasis has been placed on surrogate markers of the disease, especially measures of bronchial hyperresponsiveness and atopy, although these are not specific to asthma (Holgate 1997). Moreover, genetic studies on atopy have varied greatly in the approach adopted to determine the atopic state, and these differences have contributed to the difficulties in developing a unified view of the subject (Hopkin 1995).

A high serum immunoglobulin E (IgE) level is one of the most frequently applied surrogate markers in asthma research. Ever since its discovery IgE has been connected with asthma (Johansson 1967). Burrows and his group has studied IgE in a population cohort from Arizona. They found that age, gender and smoking significantly affect serum IgE levels (Barbee et al. 1981b). Whether the subject is allergic or not the age- and gender-standardised IgE levels are associated with asthma (Burrows et al. 1989, Beeh et al. 2000). Moreover, in a prospective setting the age- and gender-standardised IgE levels have been identified as independent risk factors for asthma in both young adults and the elderly (Burrows et al. 1991, Dodge et al. 1994). In a longitudinal study IgE levels were seen to remain relatively unchanged after 35 years of age, while in younger age groups a slight decreasing tendency was found (Barbee et al. 1987).

Most allergic and nonallergic asthmatics have bronchial eosinophilia and there is a significant association between eosinophil activation and asthma severity. There is evidence that the number of eosinophils is increased in asthma in consequence of reduced apoptosis (Bousquet et al. 2000). Peripheral blood eosinophilia is also frequently found in asthma and correlations have been observed between eosinophil levels and severity of symptoms, degree of airflow limitation and bronchial hyperresponsiveness (Horn et al. 1975, Bousquet et al.

1990, Ulrik 1995). Ideally, eosinophils should be measured from the airways, but peripheral blood measurements are often used in asthma surveys as being less time- and labour-consuming than alternative methods (Jarjour et al. 1998, Paggiaro et al. 2002).

Challenge tests are also used in the characterisation of asthmatic subjects. Bronchial hyperresponsiveness is defined as increased responsiveness or reactivity of the bronchi to various physical, chemical and pharmacological stimuli, manifested as airway narrowing (Banik and Holgate 1998). Tests for bronchial hyperresponsiveness, usually histamine or methacholine challenges, are used in clinical practice both as a part of asthma diagnosis and in follow-up of the efficacy of treatment (Cockcroft et al. 1977, Britton et al. 1986, Nieminen 1992, Sont et al. 1996). Some researchers have proposed that in epidemiological studies asthma should be defined on the basis of asthma symptoms together with bronchial hyperresponsiveness (Toelle et al. 1992).

Atopy

The concept of atopy began to emerge with the publication in 1916 of an article on immediate whealing reactions to common allergens in patients suffering from common allergic diseases. The authors concluded forcefully that "such individuals as a group possess a very peculiar capacity to become sensitive in a natural way to certain proteins to which their environment and habits of life frequently expose them" (Cooke and van der Veer 1916). Atopy as a term was first introduced and defined seven years later by Coca and Cooke (1923). At first the new term was widely adopted, but due to problems related to the complexity of the definition, usage of the term decreased during the 1930s and 1940s (Pepys 1994).

The finding of IgE (Ishizaka et al. 1966, Johansson 1967) prepared the theoretical basis for a more simple definition of atopy. In 1975 the following proposal was made: "that form of immunological reactivity of the subject in which reaginic antibody, now identifiable as IgE antibody, is readily produced in response to ordinary exposure to common allergens of the subjects' environment"

(Pepys 1975). This does not imply the presence of symptoms; it is simply a description of the immunologic reactivity of the subject (Pepys 1994). The presence of specific IgE is usually studied by either skin prick test (Dreborg and Frew 1993) or serum assay (Matricardi et al. 1994).

Recently the European Academy of Allergology and Clinical Immunology published a position statement on the revised nomenclature for allergy. The following proposal for a definition of atopy was offered: "Atopy is a personal or familial tendency to produce IgE antibodies in response to low dose of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis." (Johansson et al. 2001). In this present work atopy is defined on the basis of skin prick test. The choice was made in order to keep the contents of the study uniform with the original contributions (especially *Study III*).

Nasal polyposis

Nasal polyposis (NP) is a chronic inflammatory disease of the nasal mucosa which often coexists with asthma (Mygind 1990, Kramer and Rasp 1999). Especially the triad of NP, aspirin intolerance and asthma has long been recognised as a distinct phenotype of asthma (Widal et al. 1922). NP is clinically characterised by oedematous masses in the nasal and paranasal cavities, causing symptoms such as nasal obstruction, anosmia, sneezing, rhinorrhea and itching. Since this disorder is seen to cluster in families, a genetic predisposition has been suggested (Greisner and Settipane 1996). In a recent population-based study the overall prevalence of NP in Finland was found to be 4.3%. The prevalence was 16.5% in subjects with physician-diagnosed asthma and 3.7% in subjects without (Hedman et al. 1999).

Genetics of asthma and atopy

Genetic component in the development of the disease

Allergic disorders have long been known to cluster in families (Cooke and van der Veer 1916). Preliminary evidence for a genetic effect on atopy and asthma has been obtained from twin studies. When aggregation of asthma cases in the Finnish adult twin cohort was studied, it was found that the concordance was higher among monozygotic than dizygotic twins. This led to an estimate of heritability (that proportion of aetiology attributable to genetic factors) of 36% (Nieminen et al. 1991). In the case of young twin cohorts even higher estimates (60 – 87%) for asthma heritability have been reported (Duffy et al. 1990, Harris et al. 1997, Laitinen et al. 1998). Among the phenotypes associated with asthma, the total serum IgE level evinces marked heritability in different populations (Hopp et al. 1984, Hanson et al. 1991), whereas the specificity of the IgE response is mainly determined by environmental factors (Hanson et al. 1991, Tovey et al. 1998).

Furthermore, a 3- to 5-fold genetic effect has also been demonstrated by comparing the relative risk of asthma in the siblings of the proband to the relative risk in the general population (Sandford et al. 1996, Laitinen et al. 1998, Barnes and Marsh 1998).

Despite substantial research efforts, the specific genes contributing to the development of asthma and atopy have not yet been established. Genetic studies on complex diseases such as asthma pose considerable challenges by reason of phenocopies (identical phenotypes can be attributable to different constellations of genes), the large number of genes involved, the effects of any particular gene being possibly fairly modest, and the unknown model of inheritance. Furthermore, the alleles which influence these phenotypes are not necessarily fully penetrant, which means that a proportion of the carriers of a susceptibility allele will not express the phenotype (Ober and Moffatt 2000). Although the absence of major breakthroughs in the search for a genetic basis of allergic diseases has raised scepticism among some scientists, the obvious benefits of the

ongoing research are recognised by the majority. The clinical benefits of studies seeking to identify genes associated with a complex disease such as asthma include the possibility of genetic screening, improved disease classification, and an increased understanding of underlying disease mechanisms (Shapiro and Owen 2002).

Identification of the contributing genes

Two techniques have been used to identify variants behind the observed genetic effect. The genome-wide search followed by positional cloning is a potentially powerful approach which could ultimately identify novel genes influencing the development of asthma and its related phenotypes. This approach to gene identification is particularly appealing in that it does not require a prior hypothesis regarding the role of specific genes or pathways which influence these phenotypes (Ober and Moffatt 2000). An example of this was recently obtained. Before a study identifying ADAM-33 as a positional candidate for asthma (Van Eerdewegh et al. 2002), there was little evidence suggestive of a role for metalloproteinases in asthma pathogenesis.

Several genome-wide screens for asthma and its associated phenotypes have been carried out. The linkage results of all 21 genome scans so far published in this field are available in the Asthma & Allergy Gene Database (http://cooke.gsf.de/) (Wjst and Immervoll 1998). A summary of positive linkage results is presented here in Figure 1. However, this presentation shoud be seen as a rough estimate, as there are serious shortcomings in some of the included studies in relation to phenotyping and sampling. Some of the higher-quality studies have proceeded to positional cloning. These efforts are ongoing with at least four to five different chromosomes.

In contrast to positional cloning, the candidate-gene approach is based on existing knowledge on the pathophysiology of the disease studied, the basic idea being to study genes which are likely to play a role in the condition. *Studies II-V* in the present series utilised the candidate-gene approach. To be precise, they are association studies with candidate genes. The main interest was to establish whether biologically plausible polymorphisms are associated with the phenotypes

in question. By definition, a polymorphism is a variation in the DNA sequence which has an allele frequency of at least 1% in a population (Cavalli-Sforza and Bodmer 1971). There are several types of polymorphism in the genome: so-called SNPs, repeat polymorphisms, insertions, and deletions. Most of the DNA sequence variation in the human genome is in the form of SNPs (Sachidanandam et al. 2001). An analogy can be drawn between epidemiology and candidate-gene studies (Tabor et al. 2002), for which reason this field of research has also been called molecular epidemiology.

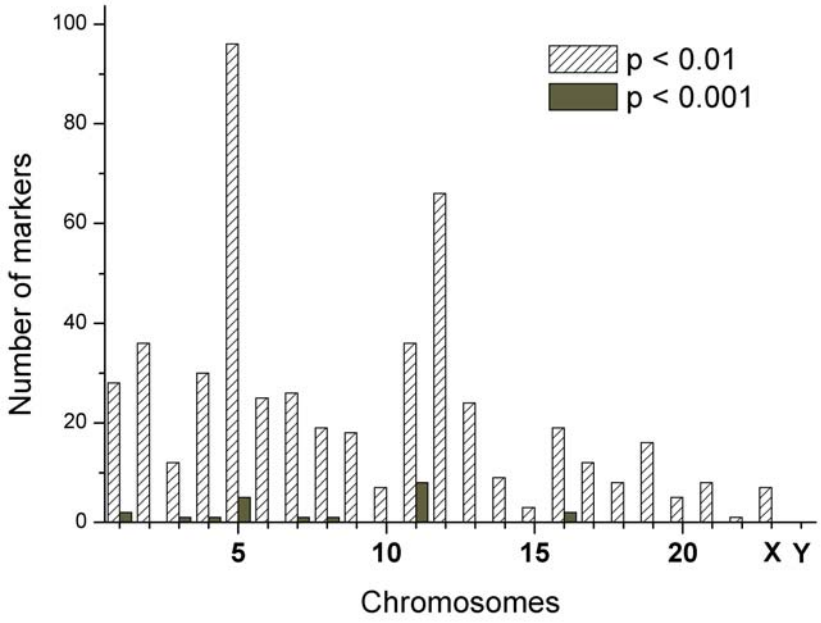

Figure 1. Linkage hits for asthma or allergy

Asthma & Allergy Gene Database cooke.gsf.de 17/02/03. Results from genome scans using asthma or associated phenotype as the trait showing a number of markers which could be assigned to the Marshfield map of 12/00. Data from (Daniels et al. 1996, Zamel et al. 1996, CSGA 1997, Ober et al. 1998, Wjst et al. 1999, Dizier et al. 2000, Yokouchi et al. 2000, Xu et al. 2000, Ober et al. 2000, Lee et al. 2000, Laitinen et al. 2001, Cookson et al. 2001, Haagerup et al. 2001, Xu et al. 2001a, Xu et al. 2001b, Van Eerdewegh et al. 2002, Bradley et al. 2002, Joost et al. 2002, Silverman et al. 2002, Hakonarson et al. 2002)

Inflammatory cytokines in asthma and atopy

Cytokines

Cytokines are signalling proteins in cell-cell communication. They usually have an effect on closely adjacent cells and therefore function in paracrine fashion. Moreover, they often act at a distance (endocrine function) or have an effect on the cell of origin (autocrine function). Cytokines act on target cells to give impulse to a wide array of cellular functions including activation, proliferation, chemotaxis, immunomodulation, relase of other cytokines or mediators, growth and cell differentation, and apoptosis (Chung and Barnes 1999). More than 200 cytokines have been identified, and these are generally divided into the subgroups interleukins, growth factors, chemokines, interferons and colony-stimulating factors. Traditionally cytokines have also been divided by their inflammatory activity into pro-inflammatory (e.g. IL-1, IL-6, TNF-α, TGF-β) and anti-inflammatory (e.g. IL-1Ra, IL-10) subgroups (Callard et al. 1999).

It is important to understand that individual cytokines are always parts of a complex network. Each cytokine has many overlapping functions, each function being potentially mediated by more than one cytokine (Chung and Barnes 1999). Existing knowledge of cytokines and their interactions is based on simplistic models. In future, the major challenge will be to understand biology at the system level. Recent progress in molecular biology and the computational sciences has made possible a new multidisciplinary field of research called systems biology (Kitano 2002). However, for the time being all new data on cytokines must be placed in the context of a network whose functioning is not yet fully understood.

Cytokine gene polymorphisms

It has now been demonstrated that several of the cytokine genes are polymorphic (Haukim et al. 2002). The pathologies of many diseases are influenced by the profiles of cytokine production. Interindividual differences in cytokine profiles

appear to be due, at least in part, to allelic polymorphism within regulatory or coding regions of cytokine gene. The influence of cytokine gene polymorphisms on gene expression and disease has been addressed at two levels of research: studies using *in vitro* or *in vivo* gene expression, and genetic studies involving disease association. Few studies have thus far integrated both of these approaches (Bidwell et al. 1999). Several disease associations with cytokine gene polymorphisms have already been reported (Haukim et al. 2002). For example, the polymorphisms of IL-1 and IL-10 are associated with various diseases of autoimmune or inflammatory nature (Hurme et al. 1998).

Interleukin-1 cytokine family

Interleukin-1 (IL-1) represents a group of proteins which are closely involved in the enhancement of inflammation and host defence. IL-1 was originally discovered independently in several institutes in the late 1970s (Tocci and Schmidt 1997). It in fact consists in a family of three proteins, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra), this latter being an antagonist which has no agonist activity compared with the first two family members (Dinarello 1996). The balance between agonists and antagonists in the IL-1 system is likely to have profound effects on the pathogenesis of inflammatory diseases (Gershenwald et al. 1990).

One of the names formerly used, endogenous pyrogen, reflects the fact that the objective for the first studies concerning IL-1 was to find the factors causing fever. Subsequently other names were given to this small group of proteins on the basis of their discrete biological activities (Tocci and Schmidt 1997). In addition to fever, IL-1 family cytokines have a number of important biologic effects which are summarised in Table 1. The principal cellular sources of IL-1α and IL-1β are monocytes, specialised tissue macrophages such as alveolar and synovial macrophages, Langerhans cells, chondrocytes, endothelial cells, mast cells and fibroblasts (Tocci and Schmidt 1997).

IL-1 is a highly active cytokine which stimulates the production of prostaglandins and nitric oxide, both of which are highly inflammatory. In

addition, IL-1 induces the synthesis of chemokines, small proteins facilitating the entry of inflammatory cells into tissues (Dinarello 2000). Most of the biological effects of IL-1α and IL-1β result from their ability to modulate gene expression in target cells (Tocci and Schmidt 1997). IL-1α remains primarily cell-associated and is found largely in the cytosol and on the plasma membrane of cells, whereas IL-1β is the main secreted form of IL-1 (Dinarello 1994).

Systemic effects	Fever, shock, hypotension, stimulation of HPA axis, neutrophilia, hypoferraemia, hyperlipidaemia, hypoalbuminaemia
Local effects	Angiogenesis, fibrosis, neutrophil influx, chemokine induction
Immunological effects	Enhanced T and B cell stimulation, enhanced lymphokine synthesis, enhanced antibody production (adjuvant effect)
Inflammatory effects	Mediates shock, arthritis, colitis, insulitis and thyroiditis in inflammatory disease; increases adhesion molecules on endothelium; enhances release of arachidonate products, prostanoids, and eicosanoids

Table 1. IL-1 biological effects
Adapted from Rosenwasser (1998)

IL-1α

The synthesised 31kDa precursor of IL-1α remains in the cytosol until it is myristoylated and translocated to the cell membrane. Approximately 10 to 15 % of the IL-1α is myristoylated (Kurt-Jones et al. 1985, Stevenson et al. 1993). IL-

1α may be found in the circulation as it is released from dying cells, or alternatively after proteolytic cleavage of myristoylated IL-1α by calpainin in the process in which the 17 kDa form of IL-1α is formed (Dinarello 1996). Intracellular proIL-1α acts as an autocrine growth factor. It regulates normal cellular differentiation, particularly in epithelial and ectodermal cells. In the case of keratinocytes, constitutive production of large amounts of proIL-1α is found in healthy human skin (Hauser et al. 1986). In the murine T_{H2} cell line, IL-1α was proposed as an essential autocrine and paracrine growth factor using an antisense IL-1α oliconucleotide and anti- IL-1α antibodies (Zubiaga et al. 1991).

*IL-1*β

IL-1β is also primarily synthesised as an immature 31 kDa protein called pro-IL-1β, whose biological activity is marginal. This pro-IL-1β remains cytosolic until converted to mature protein (17.5 kDa) by a proteolytic cleavage with interleukin-1 converting enzyme (ICE) (Dinarello 1996). Overexpression of ICE activity has been found to potentiate Fas-mediated cell death, and inhibition of ICE activity seems to prevent this mode of apoptosis (Los et al. 1995).

IL-1Ra

IL-1Ra is a naturally occurring cytokine whose only function is to prevent a biological response to IL-1 (Dinarello 2000). IL-1Ra is present in secreted (sIL-1Ra) and two intracellular (icIL-1Ra I and icIL-1Ra II) forms, all having similar physiological activities. The antagonist activity of IL-1Ra is based on its capability to bind to the same receptor (with almost the same affinity) as IL-1α and IL-1β, thus interrupting the onset of signal transduction. Inflammatory autoimmune diseases develop spontaneously in IL-1Ra knockout mice (Horai et al. 2000, Nicklin et al. 2000). This is in line with observations in IL-10 knockout mice (Kuhn et al. 1993) and demonstrates the importance of down-regulation of IL-1-mediated inflammation in maintaining normal homeostasis.

IL-1 receptors

Type I IL-1 receptors (IL-1RI) have an extensive cytoplasmic domain and, following ligand binding, transduce the biological effects attributed to IL-1 (Sims et al. 1993). Despite the near-equal affinities of IL-1 and IL-1Ra for IL-1RI, a 10-100 fold molar excess of IL-1Ra is needed to inhibit IL-1 activity (Arend et al. 1990). The best explanation for the potency of IL-1-induced signalling is postreceptor amplification through multiple phosphorylations of protein kinases (Dinarello 1996). The type II receptor (IL-1RII), has an antagonistic role in binding IL-1 protein without inducing signal transduction events. Also soluble IL-1RII (and to a lesser extent soluble IL-1RI) work antagonistically by binding IL-1β in a liquid phase (Colotta et al. 1994, Dinarello 1996).

IL-1 gene complex

IL-1α, IL-1β and IL-1Ra are encoded by separate genes, designated *IL1A*, *IL1B* and *IL1RN*, respectively. All three genes are clustered on the long arm of chromosome 2q13-q21 which spans more than 430 kilobases (Nicklin et al. 1994). In addition the *IL1* gene cluster contains genes coding for several novel cytokines designated *IL1F5 – IL1F10* (Dunn et al. 2001, Figure 2). All *IL1* family genes are polymorphic. Two of these sites of polymorphism, *IL1A* (+4845) and *IL1B* (-511) studied in this thesis, are presented in Figure 2. Several of the *IL1* family polymorphisms have been shown to be associated with either susceptibility to or severity of inflammatory conditions and diseases (Haukim et al. 2002). The alleles in the *IL1* family are inherited in linkage disequilibrium (Cox et al. 1998). This constitutes the allelic polymorphism from conserved haplotypes which can be involved in the development of immune diseases. This result has also been confirmed in a Finnish population (Pessi et al. 2003).

The single G to T base exchange polymorphism in exon five at +4845 of the *IL1A* gene results in an amino acid substitution of alanine to serine (van den Velden and Reitsma 1993). The biological significance of this polymorphism has not yet been conclusively established. *In vitro* findings have demonstrated that *IL1A*2* (SNP –889, which is >99% in linkage disequilibrium with SNP +4845)

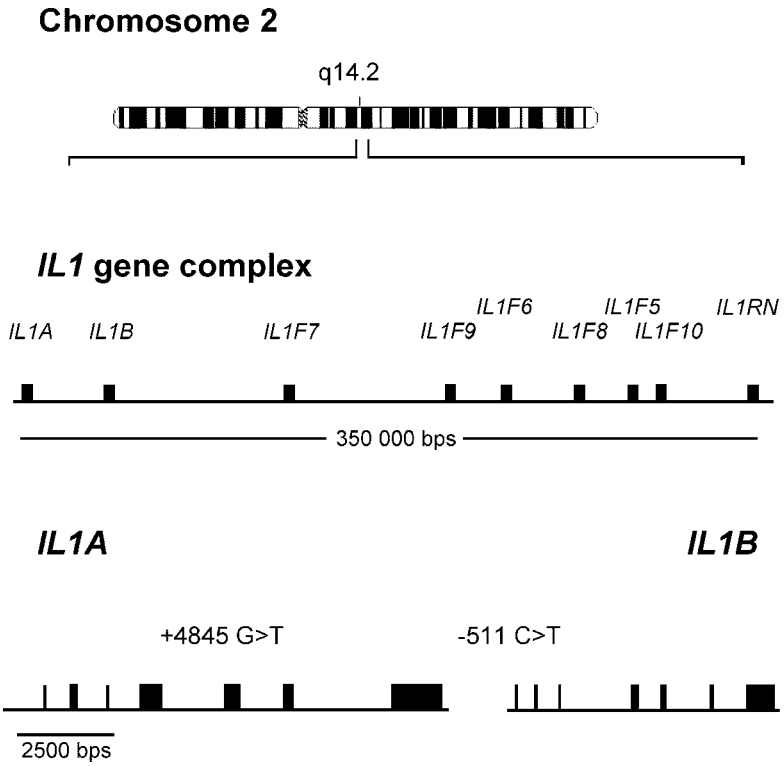

Figure 2. *IL1* gene cluster on chromosome 2

Top: The *IL1* gene cluster is located in 2q13-21. *Middle*: The IL1 family genes (boxes) are within 350 kilobase range. *Bottom*: The sites of *IL1A*(+4845G>T) and *IL1B*(-511C>T) are marked in gene models showing exons (boxes) and introns.

increases the production of IL-1β in healthy subjects (Hulkkonen et al. 2000), whereas *IL1RN**2 has been associated with both high (Santtila et al. 1998) and low (Vamvakopoulos et al. 2002) IL-1β release. The polymorphism can change the amino acid sequence of the protein conformation, this resulting in a difference in biological functions, receptor affinity or half-life of this protein. *IL1A* +4845G>T has been associated with chronic polyarthritis (Jouvenne et al. 1999), and periodontal disease (Cullinan et al. 2001). Moreover, carriers of the minor *IL1A**4845T evinced higher C-reactive protein levels than non-carriers when patients undergoing coronary angiography were studied (Berger et al. 2002).

IL1B –511C>T was first described by di Giovine and associates (1992). This polymorphism has been found to be associated with inflammatory bowel disease (Nemetz et al. 1999), schizophrenia (Katila et al. 1999), and Parkinson's disease (Schulte et al. 2002). Its biological significance is not precisely known, but it has been found to participate in regulating plasma IL-1β levels in healthy individuals (Hulkkonen et al. 2000).

IL-1 in asthma and atopy

IL-1β is an important growth factor for T_{H2} cells in response to antigen-primed antigen presenting cells but not for T_{H1} cells (Greenbaum et al. 1988). Synergistic effects between IL-1 and IL-6 have been reported for the activation of T cells (Sironi et al. 1989, Elias et al. 1989).

Patients with symptomatic asthma show increased levels of IL-1β in BAL fluid compared with patients with asymptomatic asthma (Broide et al. 1992). This difference was also found when asthmatics were compared with healthy volunteers, and furthermore, asthmatics had an increase in IL-1β-specific mRNA transcripts in BAL fluid macrophages (Borish et al. 1992). Increased expression of IL-1β in the asthmatic airway epithelium has been reported, together with an increased number of macrophages expressing IL-1β (Sousa et al. 1996). Moreover, treatment of asthma with corticosteroids inhibits IL-1β expression in the airway wall epithelium without affecting IL-1ra expression (Sousa et al. 1997).

In atopic dermatitis and in atopy in general, IL-1 has been shown to be part of both the early and the late phase response within the skin (Rosenwasser 1998). IL-1α alpha mediates inflammatory reactions in the skin and upregulates the expression of other pro-inflammatory cytokines. It also regulates its receptor antagonist primarily at transcriptional level (La and Fischer 2001). On the other hand, IL-1β is locally upregulated in the early phases of skin sensitisation to contact allergen (Kermani et al. 2000).

Interleukin-10

Interleukin-10 (IL-10) was first described as Cytokine Synthesis Inhibitory Factor in mice (Fiorentino et al. 1989). The main function of this pleiotropic mediator appears to be to limit and ultimately terminate inflammatory responses (Moore et al. 2001). IL-10 is usually considered to have a role in the downregulation of cell-mediated and cytotoxic inflammatory responses, being thus a potent anti-inflammatory mediator. The natural cellular sources of IL-10 in man are T_{H1} and T_{H2} CD4+ cells, activated monocytes, and B cells (especially CD5+ or EBV infected B cells) (Powrie et al. 1997).

The anti-inflammatory properties of IL-10 are partly mediated by its ability to downregulate HLA II expression and antigen presentation of monocyte-macrophage lineage cells (Bogdan et al. 1991, de Waal Malefyt et al. 1991, Powrie et al. 1997). Interestingly, IL-10 has closely related homologs in several virus genomes of which the homology to the Ebstein Barr virus was first found (Moore et al. 1990). This shows that IL-10 has an important role in regulating immune and inflammatory responses (Moore et al. 2001). In addition, IL-10 inhibits the proliferation and cytokine production of T cells responding to antigen as well as IFN-γ production by NK cells (Powrie et al. 1997). IL-10 also has a number of proinflammatory and haematological functions. It promotes B cell activation and differentiation and induces immunoglobulin synthesis and autoantibody production (Rousset et al. 1992, Llorente et al. 1995).

Levels of circulating IL-10 are elevated in SLE, rheumatoid arthritis and Sjögren's syndrome (Llorente et al. 1994), which would imply that this cytokine is involved in the pathogenesis of autoimmune diseases. Moreover, blockade of circulating IL-10 with neutralising antibodies in SLE-prone mice delays the onset of the disease and increases survival, suggesting that IL-10 promotes the development of SLE in the mouse (Ishida et al. 1994). Studies on gene knock-out mice suggest that IL-10 has a protective function against antigen-driven inflammatory responses (Rennick et al. 1997); IL-10 -/- mice developed chronic enterocolitis with marked mononuclear cell infiltration and high IL-1, IL-6, TNF-α and nitric oxide levels in colonic lesions (Kuhn et al. 1993, Berg et al. 1996). A protective role of IL-10 has also been observed in experimental endotoxin shock,

as the lethal dose of endotoxin was 40 times lower in IL-10 -/- mice compared to control mice (Berg et al. 1995).

IL-10 receptor

The IL-10 receptor is composed of at least two subunits, the ligand binding IL-10R1 and an accessory subunit IL-10R2 (Moore et al. 2001). The IL-10 receptor is mainly detected on haematopoietic cells (Liu et al. 1994). The human IL-10 receptor gene maps to a gene-rich area at chromosome 11q23.3 (Taniyama et al. 1995).

IL10 gene

The *IL10* gene is located at chromosome 1q31-q32 (Kim et al. 1992, Eskdale et al. 1997). The human IL-10 gene is composed of five exons (Powrie et al. 1997). It seems that IL-10 production is controlled mainly at transcriptional level and the genetic polymorphism of the 5' flanking region may partly explain this variation (Turner et al. 1997, Eskdale et al. 1998). Twin and family studies have suggested that approximately 75 % of the variation in IL-10 production is genetically determined (Westendorp et al. 1997).

Three single base polymorphisms located at positions -1082G>A, -819C>T, and -592C>A form combinations in which only three haplotypes (GCC, ACC and ATA) have been described in Caucasian populations (Turner et al. 1997, Hulkkonen et al. 2001). These haplotypes seem to be of functional relevance, since they have been linked to IL-10 production. The *IL10* GCC haplotype been found to be associated with high IL-10 production in Con A stimulated peripheral blood mononuclear cell cultures (Turner et al. 1997) and in patients suffering from Sjögren's syndrome (Hulkkonen et al. 2001). The ATA haplotype has been associated with lower transcriptional activity than the GCC haplotype and the ATA/ATA genotype with lower IL-10 production in LPS-stimulated whole blood cultures than the other genotypes (Crawley et al. 1999). An Australian research group confirmed these *in vitro* results in a small number (n=10) of healthy subjects (Edwards-Smith et al. 1999). Furthermore, they

divided the *IL10* haplotype combinations into high producers (GCC/GCC), intermediate producers (GCC/ACC, GCC/ATA) and low producers (ATA/ATA, ACC/ATA, ACC/ACC).

The above-mentioned promoter region haplotypes have been found to be related to the severity of inflammatory diseases. During the last few years, the ATA haplotype has been associated with severe asthma (Lim et al. 1998), rheumatoid arthritis (Hajeer et al. 1998), and juvenile rheumatoid arthritis (Crawley et al. 1999). Moreover, these haplotypes have been held to be of therapeutic significance in chronic hepatitis C, as patients having the GCC ("high-producer") haplotype showed poor response to IFN-α therapy (Edwards-Smith et al. 1999). These results suggest that an individual clinical strategy could be applicable for patients depending on their *IL10* genetics. The published functional and association studies on *IL10* promoter region haplotypes are summarised in Table 2.

IL10 haplotypes	Functional observations and disease associations	Reference
GCC	High IL-10 production in stimulated PBMCs	(Turner et al. 1997)
	High IL-10 levels in Sjögren's syndrome patients	(Hulkkonen et al. 2001)
	Poor response to IFN-a therapy in patients having hepatitis C	(Edwards-Smith et al. 1999)
	More common in SLE patients with renal involvement	(Lazarus et al. 1997)
ACC	Low or intermediate production of IL-10	(Edwards-Smith et al. 1999)
ATA	Lower transcriptional activity than GCC and association with juvenile rheumatoid arthritis	(Crawley et al. 1999)
	Protects against early EBV infection	(Helminen et al. 2001)
	Association with susceptibility to herpes zoster	(Haanpää et al. 2002)
	Association with severe asthma	(Lim et al. 1998)
	Association with rheumatoid arthritis	(Hajeer et al. 1998)
	More common in SLE patients with neuropsychiatric manifestations	(Rood et al. 1999)

Table 2. Overview of previous *IL10* haplotype findings

IL-10 in asthma

IL-10 has unique functional effects, a number of which are relevant to asthma. It inhibits the production of several proinflammatory cytokines such as IL-1b, TNF-a, and GM-CSF, as well as chemokines (Barnes 2001). The absence of IL-10 (e.g. in IL-10 gene knockout mice) results in severe allergen-induced airway inflammation with exaggerated production of IL-4, IL-5 and IFN-c compared with that in wild-type mice (Umetsu and DeKruyff 1999). This is concordant with observations in humans. Significantly less IL-10 is found in the lungs of patients with asthma (Borish et al. 1996, John et al. 1998). *IL10* has been considered as a candidate gene in asthma. The allele A at -592 (located in some studies at -571) has been associated with increased total IgE levels in asthmatic subjects (Hobbs et al. 1998) and the promoter region haplotype has been associated with severe asthma (Lim et al. 1998).

IL-10 in atopy

Since IL-10 was found to inhibit survival of and cytokine production by eosinophils stimulated with LPS, a role of IL-10 in modulating allergic responses has been noted (Takanaski et al. 1994). It has since been shown that IL-10 could also inhibit production of cytokines such as TNF and IL-6 by stimulated mast cells (Arock et al. 1996, Marshall et al. 1996). These in vitro findings were in line with findings in previously sensitised mice where a single intranasal dose of IL-10 concurrent with antigen challenge specifically inhibited airway neutrophilia, eosinophilia and TNF related to antigen challenge (Zuany-Amorim et al. 1995). The influences of IL-10 on allergic inflammation are summarised in Table 3.

Therapeutic use of IL-10

The idea of using IL-10 as a treatment in inflammatory diseases has emerged due to its diverse anti-inflammatory characteristics. IL-10 administration to humans

even at high doses (up to 100 μg/kg) is quite safe and has been shown to suppress the production of proinflammatory cytokines such as IL-1, IL-6 and TNF-α after LPS stimulation in whole blood or PBMC cultures (Chernoff et al. 1995, Huhn et al. 1996). Some experience has already been gained in humans. Recombinant IL-10 has proved to be effective in the control of inflammatory bowel disease (van Deventer et al. 1997). In the case of allergic diseases, animal studies have demonstrated that IL-10 can inhibit allergic airway inflammation (Zuany-Amorim et al. 1995). Based on these findings IL-10 is now considered as a potential therapeutic agent in many inflammatory diseases (Barnes 2001). Moreover, fascinating new perspectives recently emerged when an international group of scientists showed that it is possible to generate autogenous populations of IL-10-producing regulatory T cells *in vitro* and to successfully restore them to the site of inflammation (Barrat et al. 2002).

Target	Effect
IgE production	Inhibition when present at time of allergen processing
	Stimulation of committed B lymphocytes
Mast cells	Inhibition of cytokine production
	No influence on histamine release
	Cofactor for mast cell proliferation (murine)
Eosinophils	Inhibition of eosinophilopoesis and production of antiapoptotic cytokines
	Inhibition of cytokine production
Neutrophils	Inhibition of cytokine production
T helper lymphocytes	Inhibition of allergen-processing and presentation to T helper lymphocytes and subsequent T helper activation
	Direct inhibition of TH2 lymphocyte production of IL-5

Table 3. Influences of IL-10 on allergic inflammation
Adapted from Borish (1998)

Other inflammatory cytokines in asthma and atopy

The role of other inflammatory cytokines in asthma and atopy is not studied in this thesis. This is not to say that they do not contribute to these disorders. Other pro-inflammatory cytokines include TNF, IL-6, IL-11, GM-CSF, and SCF. Other anti-inflammatory cytokines include INF-γ, IL-12, and IL-18.

The principal source of TNF-α is the macrophage and its secretion is upregulated by other cytokines such as IL-1, GM-CSF and INF-γ (Chung and Barnes 1999). The effects of TNF-α are very similar to those of IL-1β, as there is close interaction in the signal transduction pathway of these two cytokines (Eder 1997). TNF-α may thus have an important effect on asthmatic inflammation (Kips et al. 1993). The role of IL-6 and IL-11 in asthma and atopy remains unclear. Increased release of IL-6 from alveolar macrophages has been found in asthmatic patients after allergen challenge and increased basal release compared with non-asthmatic subjects (Gosset et al. 1991, Broide et al. 1992). However, these findings may be attributable to IL-1 (Dinarello 1996). There is little information concerning SCF in allergic disorders, but GM-CSF has been shown to be increasingly expressed in the airway epithelium of asthmatic patients (Sousa et al. 1993). GM-CSF accounts for the increased eosinophil survival activity of BAL fluid and is involved in chronic eosinophilia and airway remodelling in asthma (Park et al. 1998).

IFN-γ has unique functional effects, a number of which are relevant to asthma. IFN-γ is produced by T_{H1} cells and it exerts an inhibitory effect on T_{H2} cells (Romagnani 1990). It also inhibits antigen-induced eosinophil recruitment and IL-4 induced IgE synthesis by B cells (Nakajima et al. 1993, Chung and Barnes 1999). However, it also has pro-inflammatory effects, as it increases the production of cytokines such as IL-1 and TNF-α, and the expression of adhesion molecules in the airway wall (Look et al. 1992). IL-12 mediates its anti-inflammatory effect mainly by IFN-γ (Bruselle et al. 1997). Interestingly, allergen immunotherapy results in an increase in IL-12 expression (Hamid et al. 1997).

III Aims of the study

1. To assess the reliability of register data on granted special reimbursement for anti-asthmatic medication by SII as an asthma case ascertainment instrument.

2. To estimate the effect of work-related environmental factors on asthma risk by comparing the asthma incidence in different occupational groups.

3. To establish whether sensitisation to common allergens differs between adult asthmatics and controls.

4. To analyse the role of *IL1A* +4845G>T and *IL1B* –511C>T polymorphism in the susceptibility to asthma and their associations with disease-related phenotypes.

5. To investigate the promoter region haplotypes of the *IL10* gene (formed of three SNPs: -1082G>A, -819C>T, and -592C>A) in asthma in order to determine whether they predispose patients to the disease or whether they are associated with disease characteristics.

IV Subjects and methods

Study populations

The data in this study were obtained from three different study populations. All samples were population-based. In all of them the asthmatic subjects were entitled to special reimbursement for asthma medication from the Social Insurance Institution of Finland. The special reimbursement right is granted if the criteria for persistent asthma are fulfilled as certified by a specialist (The SII criteria are given in Table 4). In addition, in *Study I* patients with recognised occupational asthma were also regarded as asthma cases. Their medication is compensated from the Statutory Accident Insurance and registered by the Finnish Register of Occupational Diseases. The diagnosis of occupational asthma is made by a chest physician and evidence of a causal link between a specific workplace exposure and the disease is required.

Study population for assessment of asthma diagnoses (Study I)

The studied asthmatic subjects were extracted from the study population of the Finnish Health Care Survey 1995/96 (Arinen et al. 1998), where the target cohort was the household population resident in Finland at that time. All institutionalised persons (about 63 000) were excluded, and the final target population thus comprised around five million persons. The design consisted in stratified one-stage cluster sampling in which households formed the clusters. In the survey sample of 12 936 persons 205 individuals were identified who had received reimbursement rights to asthma medication in 1986-98 and were aged 25-59 at that time.

Study population for asthma incidence study (Study I)

The study involved a follow-up of the Finnish population employed during the period 1986-98. Technically it consisted of three cohorts from consecutive national population censuses. All employed Finns without pre-existing asthma and aged between 25 and 59 years on 31 December 1985, 1990 or 1995 were classified according to their occupation and were followed for asthma during the years 1986-90, 1991-95 or 1996-98, respectively. There were 0.99, 1.01 and 0.88 million employed men and 0.90, 0.95 and 0.83 employed women under follow-up in the 1985, 1990 and 1995 cohorts, respectively. For each follow-up period the cases and person-years were calculated according to the occupation the person was engaged in at the start of the follow-up.

A typical history, clinical features and course of asthma must be documented in the medical certificate and the following physiological criteria are applied:

At least one of criteria 1 to 3

1. A variation of $\geq$ 20% in diurnal PEF recording (reference to maximal value)
2. An increase of $\geq$ 15% in PEF or FEV_1 with β_2-agonist
3. A decrease of $\geq$ 15% in PEF or FEV_1 in exercise testing

And

4. A continuing regular use of asthma medication which has lasted for six months at the time of the decision

Table 4. The diagnostic criteria for asthma for granted special medication reimbursement from the Social Insurance Institution.

PEF = Peak expiratory flow rate. In addition to the percentage limits given, an absolute limit value of $\geq$ 50 l/min is required. FEV_1 = Forced expiratory volume in one second. In addition to the percentage limits given, an absolute limit value of $\geq$ 0.15 l is required.

Study population for the genetic studies (Studies II-V)

This study population included all subjects who participated in the clinical phase of the Adult Asthma in Finland (AAF) project. All had responded in the AAF postal questionnaire phase, in which the study population consisted of two different population-based samples. One sample was based on the cohort of the Mini-Finland Health Survey (MFHS) and the other was drawn from the registers of the Social Insurance Institution (sample of newly diagnosed asthmatics). Figure 3 summarises how the final study population was formed. The ethnic origin of the cohort was uniform.

The Mini-Finland Health Survey subset: In the MFHS (Aromaa et al. 1989) the sampling method used was two-stage stratified cluster design. Forty strata were formed out of clusters of one or more neighbouring municipalities which were as similar as possible with regard to the stratum variables. One stratum was then picked at random to represent each stratum. In the second stage, the persons to be studied were drawn at random from the Social Insurance Institution population register so that the whole sample became self-weighting. The sample represented all Finns aged 30 or over. Basic health examination data were obtained on 7217 persons (90% of the whole sample).

Those who were selected as asthma cases in the AAF study either had asthma when the Mini-Finland Health Survey field work was done or had developed new asthma during the fifteen-year follow-up period according to the data that they have been granted reimbursement for prescriptions of anti-asthmatic agents. Two controls not suffering from asthma or chronic obstructive pulmonary disease were initially selected for each subject from the MFHS population. No other exclusion criteria were used for controls. Cases and controls were matched for age, gender and area of residence.

Subjects who responded to the AAF postal questionnaire were invited to participate in the clinical phase. Altogether 404 out of 472 invited subjects from the MFHS population attended the clinical phase. One control was excluded due to insufficient blood sample.

Subset of newly diagnosed asthmatics and their controls: To obtain quantitatively better data for the cross-sectional analysis, a sample of newly

diagnosed asthmatics (SNDA) and controls for them was drawn from the Social Insurance Institution registers. The cases comprised a random sample of 30 - 65 year old asthmatics to whom entitlement was granted during 1996. Two controls (matched for age, gender and area of residence) were initially drawn for each subject from the population register. The sample size in the AAF postal questionnaire phase was 1400 asthmatics and 2800 controls. From among those who responded to the postal questionnaire 107 asthmatics and their 141 controls were invited to attend the clinical phase of AAF to increase the statistical power of the genotype analyses. One asthmatic was excluded due to insufficient blood sample.

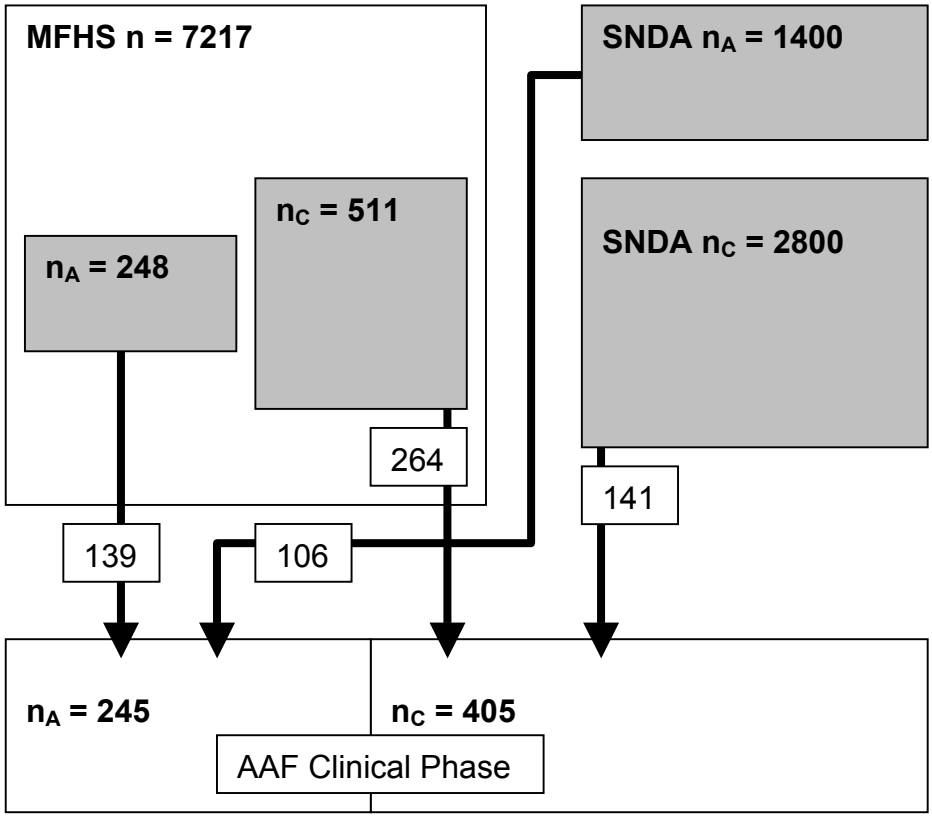

Figure 3. Formation of AAF clinical phase study population.

Grey boxes represent subsets of the AAF postal questionnaire study. n_A = number of asthmatics, n_C = number of controls

Analysis of medical certificates and hospital records

The reliability of register data on the right to special reimbursement for anti-asthmatic medication was assessed by analysing data from the SII medical certificates and, if these were incomplete, data from hospital records (with the patient's permission). All information on disease symptoms, clinical and laboratory findings and smoking history was recorded and then compared with SII criteria for special reimbursement at the individual level.

Allergy testing

Skin prick tests were performed by specially trained nurses with a panel of 22 common allergen extracts (ALK A/S, Copenhagen, Denmark). The allergens used were birch, alder, timothy grass, meadow foxtail, mugwort, dog, cat, horse, cow dander, Dermatophagoides farinae, Dermatophagoides pteronyssinus, Acarus siro, Tyrophagus putrescentiae, Lepidoglyphus destructor, Alternaria alternata, Cladosporium herbarum, Aspergillus fumigatus, oats, barley, barley flour, wheat flour, and rye flour. The test sites were placed on the volar side of the forearm. A test was considered positive when the mean diameter of the weal was at least 3 mm larger than that of the negative control. The patient was considered prick test-positive if at least one allergen gave a positive result.

Laboratory analyses

All analyses were made in the Centre for Laboratory Medicine, Tampere University Hospital. Total serum immunoglobulin E (IgE) determinations were carried out by the immunoluminometric method (Ciba Corning Diagnostics, Halsted, U.K.) and eosinophil counting using Technicon H3 analysers (Bayer Diagnostica, Tarrytown, NY, USA).

Lung function measurements

Lung function was measured with the Vitalograph® (Vitalograph Ltd., Buckingham, U.K.) spirometer, and the results were corrected for BTPS (body temperature and pressure, saturated with water vapour). Results of two technically faultless measurements were recorded, and the highest readings for FEV_1 and FVC were taken as the final results, whether obtained at the same measurement or not. The only reason for rejecting a measurement was a technically incomplete or otherwise unsuccessful blow. Calibration was performed according to manufacturer's instructions. Finnish reference values (Viljanen et al. 1982) were used when converting results to per cent predicted form.

Genotyping methods

IL1A (+4845G>T) and IL1B (–511C>T)

G-to-T base exchange at position +4845 of the *IL1A* gene was analysed by the modified method of van den Velden (van den Velden and Reitsma 1993). PCR primers 5'-ATG GTT TTA GAA ATC ATC AAG CCT AGG GCA- 3' and 5'-AAT GAA AGG AGG GGA GGA TGA CAG AAA TGT- 3' amplified exon 5 but introduced a mutation (T-G) in the primer, which facilitates analysis of this polymorphism by *Sat I* (MBI Fermentas, Vilna, Lithuania) digestion.

The region containing the AvaI polymorphic site at position –511 (C-to-T base exhange) of *IL1B* was amplified by PCR (di Giovine et al. 1992). The oligonucleotides 5'-TGGCATTGATCTGGTTCATC-3' and 5'-GTTTAGGAATCTTCCCACTT-3' were used as primers. Fragments were analysed by electrophoresis on 9% PAGE and stained with ethidium bromide.

IL10 (-819C>T), IL10 (-1082G>A) and IL10 (-592C>A)

The C/T base exchange polymorphism at –819 of the IL-10 gene was analysed by PCR, RFLP and gel electrophoresis as previously described (Mok et al. 1998). The –1082 G/A and –592 C/A polymorphisms of the IL-10 gene were analysed by PCR and RFLP and gel electrophoresis (Edwards-Smith et al. 1999).

Ethics

Approval for the AAF study was obtained from the ethical committee at Tampere University Hospital. All subjects gave informed consent to participate. In *Study I* the study protocol was approved by the ethical committees of the participating institutions. In assessment of the reliability of the asthma diagnoses (*Study I*) written permission was obtained from subjects to explore medical case records (if their medical certificate was incomplete).

Statistical methods

Statistical calculations were carried out on SPSS (ver. 10.1, SPSS Inc., Chicago, Illinois) and Statistica software (ver. Win.5.1D, StatSoft Inc, Tulsa, OK, USA). Both parametric and non-parametric tests were used. Findings were considered statistically significant at $p < 0.05$. Genotype frequencies were compared using chi-square test with appropriate degrees of freedom. Bonferroni correction and Yates correction were applied when appropriate. The tests also included Mann-Whitney U test, Kruskall-Wallis test and two-way ANOVA (the post-hoc comparisons were carried out by the LSD test of means). Further, logistic regression analysis was used in *Study III*.

Odds ratios (OR) with 95% confidence interval (CI) were calculated by CIA software (ver. 1.1, copyrighted by MJ Gardner and the British Medical Journal, 1989), Hardy-Weinberg equations were calculated by Arlequin (Schneider et al. 2000) and power calculations in *Study V* by PS software (Dupont and Plummer,

Jr. 1997). The population attributable fraction (*Study I*) was estimated according to the formulas given for adjusted analysis (Rothman and Greenland 1997).

V Results

Assessment of the reliability of asthma diagnoses

A complete medical certificate or the case history from the hospital records was available for all those identified 205 cases of the representative population sample which belonged to our study population (*Study I*). The SII diagnostic criteria (Table 4) were fulfilled in 183 (89%) of the cases and in another 20 cases (10%) the diagnosis of asthma had been made by a chest physician on the basis of other clinical data (most often a history of typical asthma symptoms together with observed bronchial hyperresponsiveness). In addition, there were two cases of post-tuberculotic respiratory problems in which asthma was clearly not present. Of the 203 patients with asthma, 20 also had another chronic pulmonary disease (18 patients had COPD and two had chronic symptomatic bronchiectasis).

The effect of work-related exposures on overall asthma incidence

There were 49 575 incident cases of asthma during the follow-up, of which 2464 (5,0%) were cases of recognised occupational asthma. The incidence of asthma was 1.65/1000/year for men and 2.47/1000/year for women. The age-adjusted risk was increased especially in agricultural, mining and manufacturing occupations and service work. When non-administrative work (exposed) as a whole was compared with administrative work (unexposed) in the age-adjusted model, the relative risk was 1.45 (1.37-1.53) in men and 1.27 (1.23-1.31) in women. These risk estimates did not change when adjustment for follow-up period was included in the model. Based on the above risk estimates and the exposure prevalences

among cases, the overall attributable fraction of exposure was 29% (95% CI 25-33%) for men and 17% (95% CI 15-19%) for women.

In men the age-adjusted relative risk was significantly increased in the major occupational groups of agriculture (RR 2.12, 95%CI 1.99-2.26), mining (1.95, 1.58-2.40), manufacturing (1.56, 1.47-1.65), service (1.53, 1.42-1.66), transport and communications (1.31, 1.22-1.40) and sales work (1.14, 1.05-1.23). In women a significant increase was found in the major occupational groups of agriculture (1.84, 1.76-1.92), service (1.41, 1.35-1.46), manufacturing (1.33, 1.27-1.39), transport and communications (1.22, 1.13-1.31) and sales work (1.13, 1.08-1.18) and in the group comprising technical, physical science, social science, humanistic and artistic work (including medical and nursing work; 1.06, 1.03-1.10).

Atopy in adult asthmatics and controls

Prevalences of positive reactions to specific allergens and differences between asthmatics and controls are given in Figure 4. A positive finding in the skin test was more common among female (62.0%) than male asthmatics (47.3%, $p = 0.025$; χ^2 test df = 1). In the control group the percentage of skin test-positive subjects was similar in both genders, 38.9 in females and 37.3 in males ($p = 0.756$; χ^2 test df = 1). There were no differences between the genders in the number of positive reactions in the skin test in either of the groups.

Serum IgE was significantly higher in asthmatics than in controls ($p < 0.001$; Mann-Whitney-U test); in asthmatics the median IgE was 43 IU/L (quartile range 15-144), in controls 20 IU/L (quartile range 8-66). The IgE level was significantly associated with skin test results in both groups. In quartiles by IgE a positive reaction in the skin test was observed in 37.1, 47.5, 67.8 and 73.8% ($p < .001$; χ^2 test df = 3) in asthmatics and correspondingly 17.1, 31.6, 42.6 and 63.3 ($p < 0.001$; χ^2 test df = 3) in controls. Increasing age was also significantly associated with fewer positive skin test results in both groups ($p < .001$; χ^2 test df = 4).

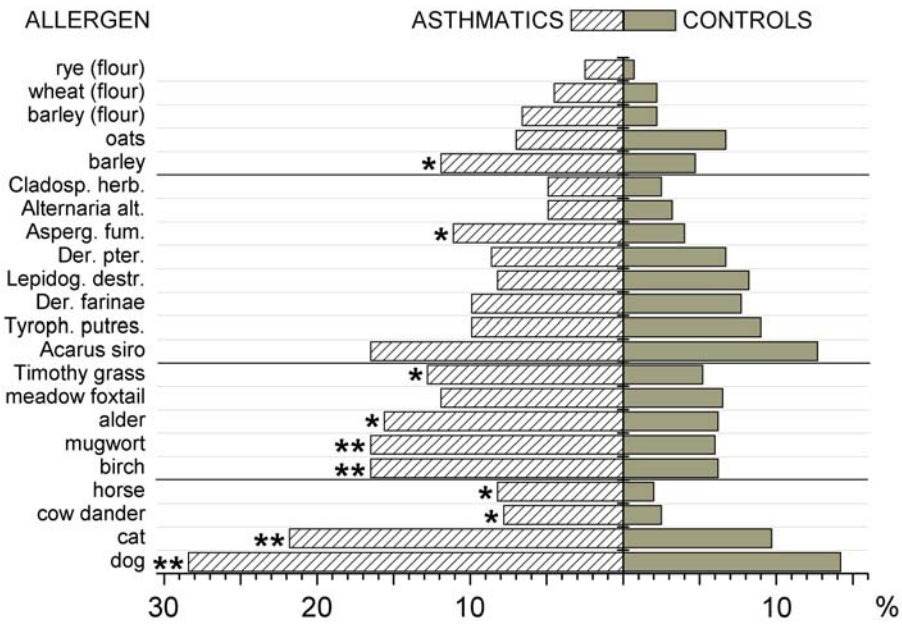

Figure 4. Positive reactions to specific allergens in skin prick test in asthmatics and controls

* p < 0.05 and ** p < 0.01 for difference between the groups.

IL1 genotype and asthma susceptibility

Asthmatics and controls evinced similar *IL1A* +4845G>T and *IL1B* –511C>T genotype and allele frequencies in the study population (Table 5). However, in males the *IL1B* –511C>T genotype distribution differed significantly between asthmatics and controls (p=0.031; χ^2 test df = 2), this due mainly to the decreased number of the C.T heterozygotes in the patient group (p=0.011; χ^2 test df = 1). Consequently, heterozygocity must be seen to exert a significant protective effect (OR 0.50, 95%CI 0.30 - 0.86). To establish whether the effect of the *IL1B* (-511) genotypes is stronger in an asthma subgroup, the patients and controls were divided into groups using the serum IgE level as criterion. The genotype distribution between cases and controls was significantly different only in males with a lower IgE level (serum IgE < 100 IU/ml). The odds ratio of the C.T heterozygotes was further decreased (0.37, 0.19 - 0.71) and that of the pooled homozygotes increased (2.70, 1.40 - 5.19).

	IL1A			*IL1B*		
	Asthmatics (n = 241) %	Controls (n = 397) %	P value	Asthmatics (n = 243) %	Controls (n = 400) %	P value
Genotype						
1.1	44.4	46.5		41.6	34.3	
1.2	47.3	41.8	.222[1]	42.4	50.8	.105[1]
2.2	8.2	11.8		16.0	15.0	
Allele frequency						
allele 1	68	67	.769[2]	62.8	59.6	.265[2]
allele 2	32	33		37.2	40.4	

Table 5. The *IL1A* +4845G>T and *IL1B* –511C>T genotype and allele frequencies in all asthma patients and controls

IL1A +4845 (allele 1 = G, allele 2 = T) and *IL1B* -511 (allele 1 = C, allele 2 = T).

P value calculated by chi-square test [1]in 3x2 table and [2]in 2x2 table.

Nasal polyposis in asthmatics and IL1 genotypes

The prevalence of NP in asthmatics was 14.3%. As expected, NP was associated with severity of asthma. Patients with NP had more nightly symptoms and used inhaled steroids more often than those without NP. In addition, there was a greater need for periodical oral steroid within the last 12 months in the NP group. However, no clear association was found with lung function, atopy or blood eosinophil count.

The distribution of the *IL1A* +4845G>T genotype differed significantly between asthmatics with and without NP (p=0.005; χ^2 test df = 2). This was due mainly to the increased number of allele G homozygotes among subjects with nasal polyposis. The odds ratio for G.G genotype was 2.73 (95%CI 1.40 – 5.32). Moreover, when surgical removal of nasal polyps was used as an additional criterion for NP the odds ratio for subjects with the G.G genotype was further

increased to 3.51 (1.47 – 8.38). For *IL1B* –511C>T, the genotype distribution was similar in patients with and without NP.

IL1 genotype and atopy

In controls, the *IL1A* +4845G>T genotype distribution was significantly different between skin test-positive and skin test-negative individuals (p= 0.006; χ^2 test df = 2). This was caused by an increase in the frequency of the minor allele (allele T) in the skin test-negative population (Table 6). The protective effect of *IL1A**T was somewhat stronger in males. For allele T carriers the odds ratio was 0.58 (0.38 - 0.88) in males and 0.72 (0.52 - 0.99) in females. There was no significant association between the studied *IL1A* genotype and skin test results in asthmatics (Table 6).

	Asthmatics			Controls		
	SPT - (n = 103) %	SPT + (n = 137) %	P value	SPT - (n = 245) %	SPT + (n = 150) %	P value
Genotype						
G.G	43.3	45.3		40.1	56.7	
G.T	48.1	46.7	.950[1]	47.0	33.3	.006[1]
T.T	8.7	8.0		13.0	10.0	
Allele frequency						
allele G	67	69	.761[2]	64	73	.004[2]
allele T	33	31		36	27	
Carriers of allele T						
carrier	55		.759[2]	60	43	.001[2]

Table 6. The *IL1A* (+4845) genotypes in skin test-negative (SPT -) and -positive (SPT +) subjects

IL1A genotyping was successful in 241/245 asthma patients and in 397/405 controls. P value calculated by chi-square test [1]in 3x2 table and [2]in 2x2 table.

Carriers and non-carriers of allele T did not differ in relation to skin weal size caused by a positive control substance, nor did they differ in reactivity to negative control substance. When specific allergens were analysed separately, carriers of allele T had fewer positive reactions to all but two allergens (birch and *Aspergillus fumigatus*). In all quartiles by serum IgE, the proportion of *IL1A* allele T carriers was higher in skin test-negative subjects, but the finding was statistically significant only in the subgroup of highest IgE values (IgE ≥ 67 IU/l: in skin test-negatives 66.7% vs. 38.3% in positives, p = 0.007; χ^2 test df = 1).

To ascertain whether *IL1A* (+4845) allele T carrier status has an independent and significant role, all factors here observed to have an effect on skin test results were entered into a logistic regression model. In this model the allele G homozygous controls had an almost two-fold higher risk of positive skin test result compared to carriers of allele T. However, serum IgE quartiles and age (as continuous variable) were the most significant predictors of skin test outcome. Gender had no independent role in the model (Figure 5).

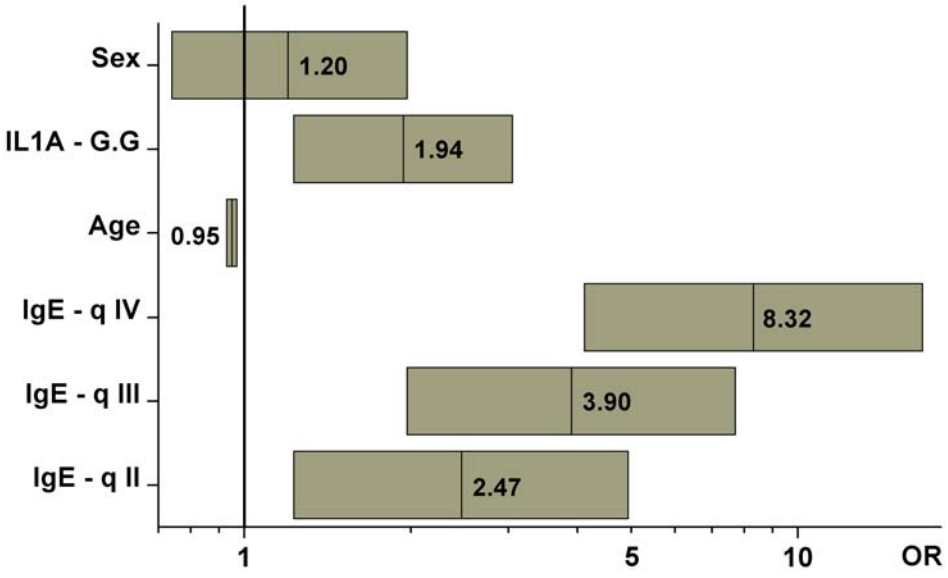

Figure 5. Odds ratio and 95% confidence intervals (boxes) for positive skin test for studied factors in logistic regression model

P value for individual parameters in the model: IgE < 0.001, Age < 0.001, IL1A genotype 0.004, Sex 0.456. Hosmer and Lemeshow goodness-of-fit test, P = 0.327 (df = 8).

IL10 effects on asthma susceptibility and lung function

Individual IL-10 genotypes, haplotype carrier rate and haplotype carrier frequencies were similar in healthy controls and in patients with asthma. This finding proved consistent when the genders were analysed separately. Thus, the IL10 haplotypes had no effect on asthma susceptibility, neither were there any significant differences in lung function (as measured with FEV_1 % predicted) between IL-10 haplotypes determined in the asthma and control groups. However, smoking male controls carrying the GCC haplotype had impaired lung function when compared with non-carriers of the haplotype ($p < 0.05$; Mann-Whitney-U test).

IL10 haplotypes in relation to eosinophil counts and serum IgE levels

Asthmatics with the ATA/ATA genotype had higher eosinophil counts than those with other genotypes (Figure 1, p=0.0267; Kruskall-Wallis test). A converse trend of association was noted in controls (p=0.0983; Kruskall-Wallis test). No differences were found in eosinophil counts when asthmatic subjects and controls were categorised on the basis of GCC, ACC or ATA haplotype carrier status.

The male asthmatics with ATA haplotype had 2.8-fold higher IgE levels than ATA-negative male asthmatics (Figure 6, in two-way ANOVA p=0.0112, 1 df), and smoking had no effect. Moreover, no combined effect of smoking and ATA was found. An opposite association was found in ATA-positive control males, who had 1.9-fold lower IgE levels than their ATA-negative counterparts (Figure 6, in two-way ANOVA p=0.0420, 1df) and no effect of smoking or combined effect was found. In females ATA carrier state had no effect on IgE. Smoking, however, had an independent effect (p=0.0065 for asthmatics and p=0.0257 for controls, respectively).

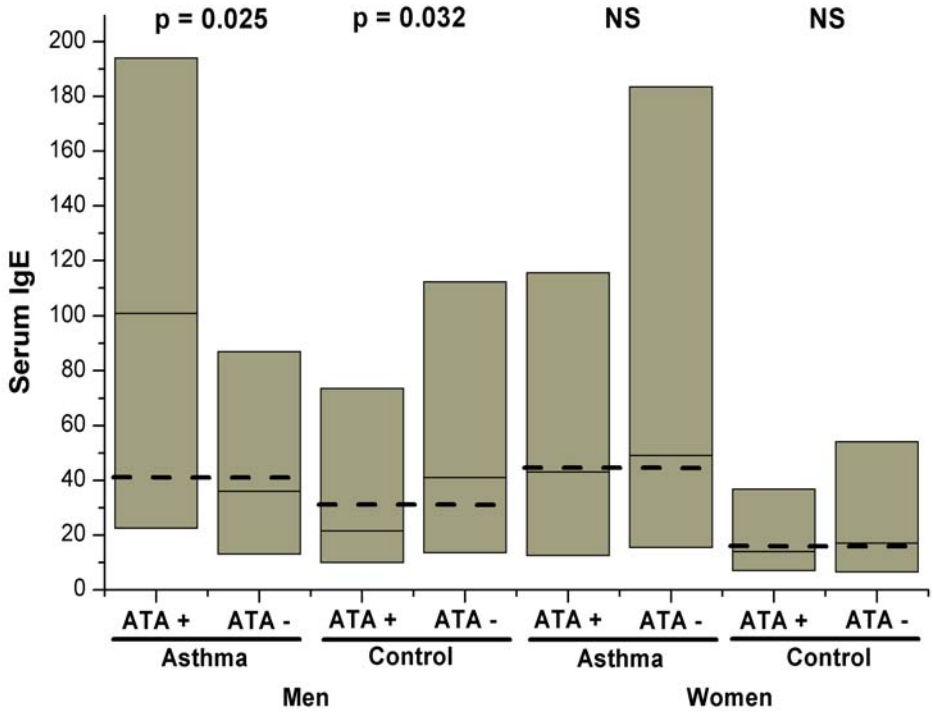

Figure 6. Serum IgE in subjects categorised by ATA haplotype carrier state, disease status and gender.

Values are median (horizontal lines) and quartile range (grey boxes), given in IU/L. Dashed horizontal lines indicate overall median values in corresponding groups

VI Discussion

Case ascertainment

The availability and quality of register-based data differs between countries. Some notable register-based studies in the field of obstructive lung diseases in different parts of the world have recently been published (Sin and Tu 2001, Soriano et al. 2002). Register-based studies have their limitations, but they are not subject to the problems related to representative sampling and the non-response frequently encountered in population-based questionnaire studies. The national Special Reimbursement Register is based on diagnoses made mainly by chest physicians. Clinical and physiological criteria for persistent asthma are applied. Examination of the medical certificates and hospital records indicated that a clinical diagnosis of asthma had been recorded in 99% of cases. This is similar to the 97% reported in an earlier regional study (Kauppi et al. 1998). The prevalence of asthma according to the criteria used in the Finnish adult population is 3.3% (National Agency for Medicines and Kela 1998).

Examination of the asthma diagnoses indicated that the positive predictive value of our definition is very high as compared to the definitions of asthma used in most epidemiological studies (Pekkanen and Pearce 1999). By definition, positive predictive value in this case is the proportion of true asthmatics among all those who test positive according to the case ascertainment instrument used (i.e. register data on special reimbursement right). On the other hand the sensitivity of this criterion is lower than for most other definitions of asthma, as this category comprises only persistent cases.

Since the level of special reimbursement for anti-asthmatic medication costs is relatively high (75% of total costs), it is likely that almost all those who fulfil the

criteria are registered. The only major exception concerns patients with recognised occupational asthma who receive full compensation for medication from the Statutory Accident Insurance. These cases are registered in the Finnish Register of Occupational Diseases and are thus also available for epidemiological studies. In our series (*Study I*) occupational asthma accounted for only 5% of all new cases of persistent asthma (in the age-group 25 - 64 years).

In Germany it has been reported that a significant proportion of subjects having COPD were identified as asthmatics when data on anti-asthmatic medication from a prescription database were used as asthma criterion (Himmel et al. 2001). The register data we applied succeeded much better in this setting. Overlapping of asthma and COPD was found in 9.8% of cases. This is well in line with the previous 10% estimate of the amount of overlapping made by Barnes (2000).

Due to the good positive predictive value of granted special reimbursement right for anti asthmatic medication our approach can be considered suitable when relative risks of persistent asthma are studied. Moreover, for the same reason entitled reimbursement would also seem a practicable selection criterion for asthmatics in population-based case-control studies in Finland, as for example *studies II, III and V* here. Positive predictive value is much more important than the proportion of possible false negatives in the study population in a study where the relative risk associated with a certain genotype is studied (Brenner and Gefeller 1993). The occurrence of false negatives in the sample would only increase the support for a null hypothesis ("no association") and thus weaken the support for the study hypothesis.

Work-related incidence

The total work-related excess of adult-onset asthma incidence was much higher than previously estimated (Blanc and Toren 1999). The attributable fraction of occupational factors was 29% for men and 17% for women. The fact that only 5% of adult-onset asthma was diagnosed as occupational asthma explains why previous estimates have been lower. These figures were based on occupational

asthma as defined by the national compensation scheme. This type of data has acknowledged weaknesses in coverage and case ascertainment (Meredith and Nordman 1996).

The work-related excess of asthma was most clearly seen in agricultural, manufacturing and service work. This is in line with the findings in a recent multinational cross-sectional study which reported an attributable fraction of 5-10% for occupation in a population aged 20-44 years (Kogevinas et al. 1999). Gender also has a significant impact on asthma morbidity. Among adults, both the prevalence and the incidence of asthma are higher in females (De Marco et al. 2000).

Exposure to workplace air pollution occurs more often in occupations associated with a low socio-economic status. Such an association could prove confounding due to life-style factors and possibly result in overestimation of the work-related risk. A separate analysis using lower-level administrative workers as a reference group did not however indicate such a detriment to work-related risk estimates. Even though a substantial effect on asthma incidence was found we may still have underestimated the risk and the attributable fraction because a complete lack of harmful occupational exposure in our reference occupation is not tenable (e.g. exposure to paper dust, emissions from office equipment or other impurities due to poor ventilation). Moreover, change of work due to respiratory symptoms is frequent prior to the diagnosis of asthma and may also have biased the risk estimates towards unity (Kogevinas et al. 1996).

Atopy

Atopic findings in Finnish adults have recently been studied in two population based samples (Kilpeläinen 2001, Vartiainen et al. 2002). The prevalence of positive findings to individual allergens in the skin test in our control group was comparable to those reported from North Karelia (Vartiainen et al. 2002). A clear difference (2-fold higher prevalence in North Karelia) was seen only with birch and Timothy grass allergens. The sample in that study was slightly younger (25 – 55 years) and included asthmatics, which can explain the difference.

A decline in skin test reactivity in older age groups has previously been observed (Barbee et al. 1981a). This could be due either to a true decline in immunological reactivity or a decrease in the ability of the skin to react to immunological challenges (Baldacci et al. 2001). Gilchrest and associates showed that the skin of elderly people is hypocellular and hypovascular as compared to that of young adults, and quantification of mast cells and venular cross-sections revealed approximately 50% and 35% reductions, respectively (Gilchrest et al. 1982). However, these aging changes in skin reactivity do not necessarily reflect milder overall allergic activity. In a long-term follow-up study of patients with allergic rhinitis, no association between symptoms and skin test reactivity was observed (Simola et al. 1999).

Atopy was significantly more common among asthmatics. Ten of the 22 allergens tested were significantly more often positive in asthmatics than in population based controls. These ten included all the four animal allergens tested (dog, cat, horse and cow dander) and the two most common Finnish tree pollen allergens (birch and alder). These all are well known for their capacity to cause asthmatic symptoms together with mugwort, Timothy grass and *Aspergillus*. However, it was somewhat surprising that the reaction to barley was also significantly more common in asthmatics. Although in many countries the house dust mite is the most important indoor allergen (Sporik et al. 1990), in Finnish studies the role of this agent has proved to be small (Timonen et al. 1995, Remes and Korppi 1996). This is assumed to be the consequence of the cold and dry climate in Finland during winter.

IL1 gene complex and asthma susceptibility

Several regions in the human genome have been linked to asthma or associated phenotypes in more than one genome-wide screen. One of these regions is located in chromosome 2q (Daniels et al. 1996, Ober et al. 1998, Wjst et al. 1999). The *IL1* gene family is located in this region and has therefore been considered a promising candidate locus (Los et al. 1999, Hakonarson and Wjst 2001).

Although it has not been the major cytokine of interest among asthma researchers, IL-1 has been suspected to be involved in asthma pathogenesis (Busse and Lemanske 2001). IL-1β is an important pro-inflammatory cytokine and the polymorphisms of this gene have been shown to be associated with a number of immune diseases (Hurme et al. 1998).

We found that the *IL1B* gene carries asthma susceptibility, but only in males. In the case of the *IL1A* genotype no contribution to asthma susceptibility was observed. There are clear gender-dependent differences in the body's defence mechanisms in response to invading microbes and likewise in the prevalences of inflammatory and autoimmune diseases (Lahita 2000). Since it is highly unlikely that the frequencies of the alleles of the cytokine genes vary between males and females, one might argue that the functions of a given allele are gender-dependent. This could be manifested e.g. in different allelic associations in some diseases. Interestingly, the regulation of IL-1 secretion has been found to be fundamentally different in women compared to that in men (Lynch et al. 1994). Our findings may therefore partly explain why females run a higher risk of developing new asthma after puberty (De Marco et al. 2000).

The effect of homozygocity/heterozygocity on biological mechanisms, e.g. IL-1β protein production, is unknown. It has however recently been observed that *IL1B* (–511C>T) heterozygocity has a protective effect against serious manifestations of meningococcal disease (Read et al. 2000). Such a heterozygote advantage may thus be valid in several diseases of inflammatory or infectious nature. The findings described here can make a significant contribution to the total asthma prevalence due to the high frequency of the functional genotypes (i.e. 50%/50% *IL1B* homozygote/heterozygote ratio in the population).

It should however be borne in mind that a negative association has also been published (Unoki et al. 2000). This kind of inconsistency in association findings is not unique when using the candidate gene approach (Albuquerque et al. 1998). The reasons for this may lie in genetic heterogeneity, differences in case ascertainment and environmental differences between populations (Los et al. 1999). In the case of *IL1B*, for example, the prevalences of polymorphisms are

dramatically lower in Chinese subjects than those reported for Europeans (Armitage et al. 2000).

Nasal polyposis

IL-1 seems to have an important role in the pathogenesis of NP. The development of NP has been held to be the result of stimulation of the epithelium by aerodynamic changes, allowing irritants to metabolically or physically alter or injure the surface epithelium (Bernstein 2001). The second phase would thereafter be related to activity of tumour necrosis factor-α and IL-1β. The expression of these two cytokines has been detected on both RNA and protein level in the epithelium and endothelium of the nasal polyps (Bernstein 2001). Their most important consists in enhancing the expression of adhesion molecules on endothelial cells. This results in an increased transendothelial migration of eosinophils into the tissues (Kramer and Rasp 1999), which has been regarded as the third phase in the development of NP (Bernstein 2001).

Interestingly, we found here no association between *IL1B* –511C>T polymorphism and NP, whereas in the case of *IL1A* +4845G>T polymorphism an association was seen. The biological significance of the *IL1A* exon 5 polymorphism has not been conclusively established. The single G to T base exchange polymorphism in exon five at +4845 of the *IL1A* gene causes an amino acid substitution of alanine to serine (van den Velden and Reitsma 1993). The change in the amino acid sequence of IL-1α may alter protein conformation, resulting in a difference in biological functions, receptor affinity or half-life of this protein. It is also possible that the +4845 allele associations described here are mediated by other adjacent genes linked to the +4845 site, or by a combined effect of the +4845 alleles and the other alleles embedded in the *IL1* gene complex.

NP and asthma appear to have a cumulative negative effect on the quality of life (Radenne et al. 1999). In our study population NP was associated with increased asthma symptoms and usage of anti-inflammatory medication. It is

therefore plausibile to consider asthma with NP as a clinically important subgroup of asthmatics. However, it should be kept in mind that aspirin hypersensitivity and chronic rhinosinusitis are also common disorders which overlap significantly with NP (Mygind et al. 2000, ten Brinke et al. 2002). Especially the triad of NP, aspirin intolerance and asthma has long been recognised as a distinct phenotype of asthma (Widal et al. 1922).

IL1A and atopy

IL-1α mediates inflammatory reactions in the skin and upregulates the expression of other pro-inflammatory cytokines. It also regulates its receptor antagonist primarily at transcriptional level (La and Fischer 2001). In atopic dermatitis and in atopy in general, IL-1 has been shown to be part of both the early and the late phase response within the skin (Rosenwasser 1998). For example, IL-1β is locally upregulated in the early phases of skin sensitisation to contact allergen (Kermani et al. 2000) and there are significant inter-individual differences in dendritic cell IL-1β mRNA production *in vitro* (Pichowski et al. 2001).

Our results suggest that IL-1α is involved in the regulation of skin inflammation triggered primarily via IgE-mediated mechanisms, carriers of allele T at this locus having a lower inflammatory rate. The findings described here may thus explain some of the inconsistencies between skin test results and other clinical data. We also found an association between the *IL1A* genotype and a history of allergic skin symptoms in the control group. However, in controls serum IgE levels and skin test results were not consistent with the symptoms revealed. It is thus not certain that these symptoms were of truly atopic nature. Some individuals exhibit a heightened skin sensitivity which is often related to a high incidence of adverse reactions to cosmetics and toiletries. Self-perceived sensitive skin is equally common in atopics and non-atopics (Willis et al. 2001). It may be that our result for the *IL1A* genotype and a history of allergic skin symptoms may actually indicate an association with sensitive skin. On the other hand Reilly and co-workers found no difference in skin blister fluid IL-1α levels between subjects with sensitive skin and controls (Reilly et al. 2000).

We consider our control group to represent the general population reasonably well. The only exclusion criterion for controls was a diagnosis of asthma or chronic obstructive pulmonary disease. The prevalence of asthma according to the criteria used in the Finnish adult population is 3.3% (National Agency for Medicines and Kela 1998) and thus the effect of asthmatics on skin prick test results in the population is of minor significance. It is therefore our opinion that allele T carriers in the general population have a reduced risk of positive skin test reactions. In asthmatics factors involved in the disease process seem to override the effect of allele T carrier status on skin test results.

IL10 haplotypes and asthma

Our finding is well in line with the results of Lim and coworkers (1998), who observed that the low IL-10-producing haplotype was more common in patients with severe asthma; however, no clear effect on asthma susceptibility was noted.

Lung function measurements, mainly by spirometry, are of vital importance when diagnosing asthma and assessing its severity. Forced expiratory volume in one second (FEV_1) presented as per cent of predicted is a reliable measure of airway obstruction. We found no previous studies reporting an association (or lack of association) between *IL10* and lung function. This could have been expected on the basis of previously reported findings (Lim et al. 1998). Moreover, Mäkelä and associates demonstrated in mice that IL-10 has an important role in airway hyperresponsiveness (Mäkelä et al. 2000). We observed no clear connection between *IL10* haplotypes and airway obstruction in asthmatics. Regarding smoking status our study population was fairly heterogeneous, but the above result held good when smoking status was taken into account.

One interesting association was seen in male smokers, which could possibly play a role in chronic obstructive pulmonary disease. There is some evidence that polymorphisms of cytokine genes exert an effect on the pathogenesis of airway obstruction. Recently *IL1* haplotypes have been associated with lung function decline in healthy smokers (Joos et al. 2001) and non-smoking asthmatics (Karjalainen et al. 2002). Taking into account that IL-10 is usually considered an

anti-inflammatory cytokine, it was interesting that smoking male controls with the high IL-10-producing GCC haplotype had impaired lung function.

Most allergic and nonallergic asthmatics have bronchial eosinophilia and there is a significant association between eosinophil activation and asthma severity (Bousquet et al. 2000). The number of eosinophils is increased in asthma in consequence of reduced apoptosis (Bousquet et al. 2000). Besides several other anti-inflammatory effects IL-10 is also known to induce eosinophil cell death (Takanaski et al. 1994). In a recently published study Immervoll and coworkers found an association of eosinophil cell count with the polymorphic site *IL10* -592 in asthma family material (Immervoll et al. 2001). The ATA/ATA genotype has previously been linked to lower IL-10 production than other genotypes (Crawley et al. 1999, Hulkkonen et al. 2001). It is possible that the high eosinophil counts observed here in ATA/ATA genotypic asthmatics are an end result of genetically determined low IL-10 production capacity and diminished IL-10-mediated eosinophil cell apoptosis.

As these effects were not seen in healthy control subjects, the results suggest that *IL10* genotype effects are enhanced whenever there is disease stress which drives towards eosinophilia. This kind of disease-specific association of *IL10* genotypes seems to be typical likewise for circulating IL-10, as *IL10* polymorphism primarily regulates IL-10 levels in patients with primary Sjögren's syndrome but not in healthy control subjects (Hulkkonen et al. 2001). It would thus appear that the basal and induced *IL10* effects are differently regulated.

Independent of allergic status, elevated serum IgE is a risk factor for asthma (Burrows et al. 1989, Beeh et al. 2000). Humbert and coworkers have shown that there is also similar cytokine (Humbert et al. 1996a) and high-affinity IgE receptor (Humbert et al. 1996b) expression in bronchial biopsies in both allergic and intrinsic asthmatics. Moreover, IgE plays a critical role in the development of allergen-specific bronchial hyperresponsiveness in asthmatics (Rabe 1998). A role of IgE in asthma is now further evidenced by the promising results reported with the new anti-IgE monoclonal antibody treatment (Busse 2001).

IL-10 is known to inhibit IL-4-induced IgE synthesis by PBMC (Punnonen et al. 1993, Jeannin et al. 1998). On the other hand, IL-10 has been shown to increase IgE production in B cells which are already IgE-switched (Jeannin et al.

1998). Hobbs and coworkers have previously studied the *IL10* -571 polymorphic site in asthmatic subjects (here -592) (Hobbs et al. 1998). They showed an association with allele A at this site and high IgE levels in asthmatics. Our finding concerning the association of the ATA haplotype with high IgE in asthmatic is consistent with their data.

We consider that the previously reported effect of the ATA haplotype on asthma severity (Lim et al. 1998) may be transmitted by eosinophils and IgE. Although asthmatic subjects with the rare ATA/ATA genotype were distinct in respect of their eosinophil counts, we observed no distinct associations with the ATA/ATA genotype and clinical phenotype in these patients. This may be explained by the fact that the number of cases with the ATA/ATA genotype was somewhat small for reliable frequency analysis. It is also likely that due to the subtle nature of allelic effects larger materials and longitudinal studies are needed to find this kind of phenotypic differences.

Evaluation of the methods used and statistical significance

Finnish registers provide an exceptional opportunity to do epidemiological research. Our study (*Study I*) is the first report on occupation-related asthma incidence where all employed citizens in a country have been followed. Obvious strengths in our study are large sample size and good positive predictive value of case ascertainment criteria. Lack of individual-level data (e.g. smoking and family history) is the most evident disadvantage of the study design adopted.

Studies II, III and *V* were carried out in a case-control setting. They thus have their highest statistical power in relation to the persistent asthma phenotype. Ideally, every phenotype should be studied in its own case-control sample, but this would not be cost effective. Since we were concerned to study genetic associations between several polymorphisms and several clinical phenotypes, we faced the problem of multiple comparisons. The p-values for genetic associations were not adjusted for multiple comparisons (i.e. Bonferroni procedure). There are several statistical points which suggest that the Bonferroni procedure should not be used in our setting: 1) The use of Bonferroni correction is not statistically appropriate in the presence of statistical dependence. There is clearly a

dependence between the studied phenotypes as well as between different IL-10 haplotypes. 2) In frequency analyses the overall frequencies of the groups were compared using omnibus (overall effect) statistics with n x 2 chi-square testing standardised for n-1 degrees for freedom (where n is the number of cells, i.e. number of genotypes or haplotypes in a contingency table). Adjustment for the number of degrees of freedom itself is a corrective procedure for multiple testing. 3) The effect of IL-10 genotypes and haplotypes on the IgE and eosinophil phenotypes were compared by non-parametric statistics using Kruskall-Wallis ANOVA. The Kruskall-Wallis test automatically adjusts the significance level for the number of terms (i.e. takes account of the number of degrees of freedom), which itself, as explained above, is a corrective measure for multiple testing. 4) Most importantly, the use of the Bonferroni procedure is in dispute in the literature. As Bonferroni correction reduces the probability of type I error by reducing the alpha level, it similarly increases the chance of type II (false negative) error, thus increasing the probability of accidentally losing the true positive finding.

One possible way to deal with true statistical significance in association studies is to use simulations (Laitinen et al. 1997, Laitinen et al. 2000). These mathematically advanced tests are considered to be more accurate than the traditional tests, but due to their complexity they are challenging to both researchers and readers. Utilisation of simulations could have improved our analyses.

Studies II-V were made using a candidate gene-based association analysis. Many researchers have considered this a relevant method in studying complex genetic traits, but considerable criticism has also been raised by others due to the insufficient quality of the published reports (NatureGenetics 1999, Laitinen et al. 2000). Association studies can have greater statistical power than linkage studies in detecting several genes of small effect (Risch and Merikangas 1996, Tabor et al. 2002), but they have been criticised by reason of the non-replication of results. It should always be kept in mind that the main objective in association studies is to generate hypotheses and to identify alleles which might have a role in the disease process. Functional studies and independent replication of results are as important here as they are in any field of medical research.

According the editorial published in the leading journal of genetic research, high-quality association studies should ideally have certain properties: large sample sizes and small p values; they should report associations which make biological sense and alleles which affect the gene product in a physiologically meaningful way. In addition, they should contain an initial study as well as independent replication; the association should be observed in both family-based and population-based studies and the odds ratio and/or attributable risk should be high (NatureGenetics 1999). Quite similar issues were emphasised in a recent opinion article (Tabor et al. 2002).

Considering how these goals or recommendations were attained in *Studies II-V*, some observations may be made. We used geographically matched controls to minimise the effect of genetic variation within the Finnish population (Kittles et al. 1998). The studies were made in a population-based sample and therefore there should be no significant bias related to referral pattern of cases. The sample size was larger than in most published cytokine gene association studies (Haukim et al. 2002). The genotypes we studied have been shown to affect protein structure (*IL1A* +4845G>T) or regulation of production (*IL1B* –511C>T and *IL10* promoter region haplotypes). The role of the studied cytokines in asthma and atopy has been widely recognised. Moreover, subgroup analyses were undertaken only in cases where there was a biological basis for doing so. In addition, the phenotype of the controls was carefully studied. However, our findings were not tested in independent samples and there are not yet sufficient data on the functional changes these studied polymorphisms cause. These shortcomings are evident and they must be seen as a future task for our research group.

VII Conclusions

Special reimbursement for anti-asthmatic medication granted by the Social Insurance Institution is a reliable asthma case ascertainment instrument in adults in Finland. It can be considered suitable for Finnish register-based studies on the relative risks of persistent asthma and as a practicable selection criterion for asthmatics in population-based case-control studies. However, other means of case identification should be considered in settings where the sensitivity of the instrument used is considered critical.

Occupational factors are involved in asthma in adults much more than has hitherto been assumed. For all occupational factors the attributable fraction estimate is 17% in women and 29% in men. Occupational asthma accounted for only 5% of all cases of asthma, which is clearly less than the attributable fraction estimate. In other words the major part of the work-related excess may have not been the target for any preventive actions, which have mainly been guided by surveillance data concerning recognised occupational asthma. Whichever the mechanism, the adverse public health impact of occupational factors in adult-onset asthma and the potential for prevention seem much greater than previously assumed.

Atopy is closely related to asthma in Finnish adults. A significant difference between asthmatics and controls was found in the prevalence of sensitisation to animal allergens, birch, alder, mugwort, Timothy grass, *Aspergillus* and barley. Age and serum IgE levels were closely related to positive findings in skin prick testing.

The incidence of asthma is significantly higher in adult women than in men. The genetic background of asthma was found to differ between adult men and women.

In the case of men an effect of the *IL1B* -511 locus was seen, while in females it had no effect. Nonetheless, the findings described here can make a significant contribution to the total asthma prevalence due to the high frequency of the functional genotypes (i.e. 50%/50% IL-1β homozygote / heterozygote ratio in the population).

IL1A +4845 G>T single nucleotide polymorphism was found to be associated with atopy in our control population. *IL1A* gene is involved in the regulation of skin inflammation triggered primarily via IgE-mediated mechanisms, non-carriers of allele T at locus +4845 having a reduced risk of positive skin test reactions. This finding may explain some of the inconsistencies between skin test results and other clinical data. Moreover, this encourages us to explore further the relation of IL-1 gene family and atopy.

The *IL1A* gene was associated with nasal polyposis among asthmatic patients. This is of interest since clinically patients with nasal polyposis are considered a distinct subgroup of asthma. Our findings indicate that *IL1A* can play a role as an inflammatory modulator in the pathogenesis of this disease. To our knowledge this was the first study to produce evidence of a specific gene locus associated with nasal polyposis.

The *IL10* promoter region haplotypes (formed of three SNPs: -1082G>A, -819C>T, and -592C>A) do not carry asthma susceptibility, nor have they any significant effect on lung function in asthmatics. Our results suggest that the eosinophil counts and serum IgE are differently regulated by the IL-10 genotype in asthmatic and in normal subjects. When combined with findings from other studies these results indicate that the IL-10 genotype becomes important in cases in which disease stress affects the immune system.

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