Genetic Investigations of Allergic Disease – Especially Allergic Rhinitis

PhD thesis

Lisbeth Venø Kruse

Department of Human Genetics
Faculty of Health Sciences
University of Aarhus
and
Department of paediatrics
Region hospital Viborg
2011

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Preface and acknowledgements

This thesis is based on work carried out from 2007 to 2011 during my years as a clinical assistant at Department of Paediatrics, Region Hospital Viborg, and PhD student at Department of Human Genetics, Aarhus Faculty of Health Sciences, Aarhus University. The work was mostly done at Department of Human Genetics.

During the making of this thesis, I received help and encouragement from many people, and for this, I would like to express my gratitude to the following individuals:

My main supervisor *Anders Børglum* for sharing his comprehensive knowledge of genetics with me. I would also like to thank him for his amazing ability to always meet yet another question with a smile. He has contributed significantly to this thesis.

Annette Haagerup, my clinical supervisor, for introducing me to science, for her help in starting up my project and getting it funded, and for the good advice and help along the way.

My supervisor *Lars G. Hansen* for his willingness to help me whenever needed. His direct insight into the clinical world of paediatrics and allergology has been of great value to me. I am also grateful that he never forgot that I was a member of his staff when the ward got together for social events, even though he rarely saw me in Viborg on a work day!

Ronald Dahl for his competent guidance, kind help in recruiting patients, and his willingness to supervise my project.

Mette Nyegaard for her friendship and her professional help and support, even when that required helping me with software problems late in the evening.

Birgit Hansen for her meticulous work in the laboratory and her good company on our long drives around Jutland collecting blood samples and meeting families involved in the project.

Steffen Møller-Larsen for his skilled contribution to the project and for sharing his great insight into the mysterious world of computer software and script formation.

Anne Hedemand for help and support along the way and for her skillful work in the laboratory.

Ulla Christensen for her guidance into the puzzling world of basis research and for showing sympathy when things were difficult.

Olav Bennedbæk, Per-Henrik Kaad, Jørgen Mølgaard Henriksen, Maurits Dirdal and Gurli wolf for their great help in the search for project families.

Ditte Demontis, Jakob Grove, Jane Christensen and the rest of my colleagues at the *Department of Human Genetics* and *Department of Clinical Genetics* for the good advice, good laughs, and good working atmosphere.

A big thanks to all the rhinitis families for taking the time and effort to participate in this project.

Finally, a sincere thanks to my dear family and friends. I am grateful for your encouragement and support, especially during the periods when my workload was intense. In particular, I want to thank my beloved husband *Anders* for his patience and support all of the way.

This project has been funded by Region Hospital Viborg, Health Research Fund of Central Denmark Region, Viborg Amts Sundhedsvidenskabelige forskningsudvalg, Sygeforsikringen "danmark"s healthfund, Toyota Foundation Denmark, The A.P. Møller Foundation for the Advancement of Medical Science, and Else and Helene Alstrups Fund.

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I. Lisbeth Venø Kruse, Ulla Christensen, Annette Haagerup, Lars Gudmund Hansen, Steffen Møller Larsen, Mette Deleuran, Dirk Goossens, Jurgen Del-Favero, Stine Krog Venø, Mette Nyegaard and Anders Dupont Børglum: A genome-wide search for linkage to allergic rhinitis in Danish sib-pair families. Submitted.

II. Lisbeth Venø Kruse, Ulla Christensen, Annette Haagerup, Lars Gudmund Hansen, Steffen Møller Larsen, Mette Deleuran, Dirk Goossens, Jurgen Del-Favero, Stine Krog Venø, Mette Nyegaard and Anders Dupont Børglum: A genome-wide search for linkage to atopy and asthma in Danish sib-pair families. Submitted.

III. Lisbeth Venø Kruse, Steffen Møller Larsen, Ulla Christensen, Annette Haagerup, Mette Deleuran, Lars Gudmund Hansen, Stine KrogVenø, Mette Nyegaard and Anders Dupont Børglum: Filaggrin mutations in a Danish hay fever cohort confers susceptibility to atopic dermatitis but not to allergic rhinitis.

Abbreviations

ADAM33	A desintegrin and metalloproteinase domain 33
AC	Allergic conjunctivitis
AD	Atopic dermatitis
ALOX5AP	Arachidonate-5-lipoxygenase-activating protin
AR	Allergic rhinitis
AS	Allergic sensitization
Cc	Case control study
CC16	Clara cell secretory protein 16
CCR	Chemokine receptor
CD14	Monocyte differentiating antigen 14/ Cluster of differentiation 14
CHI3L1	Chitinase 3-like 1 gene
CLC	Charcot-Leyden crystal protein
cM	CentiMorgan
COX	Cyclooxygenase
CTLA	Cytotoxic T lymphocyte-associated
CXCL	Chemokine
CYSLTR	Cysteinyl leukotriene receptor
DAF	Decay-accelerating factor
DENNDIB	DENN/MADD domain containing 1B
DEFB	B-defensin
DPP10	Dipeptidyl peptidase 10
ECP	Eosinophil cationic protein
END1	Endothelin1
Ec	Eczema
Fam	Family study
FcγRIIIa	Fcγ receptor group IIIa
FcεR1α	α-subunit for high-affinity receptor for IgE
FcεR-β	β-subunit for high-affinity receptor for IgE
FcγRIIIa	Fcγ receptor group IIIa
FcεR1α	α-subunit for high-affinity receptor for IgE

FcεR-β	β-subunit for high-affinity receptor for IgE
FLG	Filaggrin
FOJX1	Forkhead-box J1
FOXP	Forkhead BOX P3
GATA	GATA-binding protein
GPRA	G protein-coupled receptor for asthma susceptibility
GSTP1	Glutathione S-transferase P1
GWAS	Genome wide association study
HC	Hay fever combined (AR and / or AC)
HNMT	Histamin N-Metyltransferase
IAR	Intermittent allergic rhinitis
IBD	Identical by decent
IFN	Type I inteferon
IFNGR	Interferon gamma receptor
IgE	Immunoglobulin E
IgE-AR	IgE associated allergic rhinitis
IgE-AD	IgE associated atopic dermatitis
IgE-Asthma	IgE associated asthma
IgE≥100/150	total IgE≥ 150 kU/l for 6 -14 years old, ≥ 100 kU/l for the remaining
IL	Interleukin
IL4RA	Interleukin 4 receptor
IL13RA1	IL-13 receptor α 1 subunit
IL18RAP	IL-18 receptor accessory protein
IL2RB	Interleukin 2 receptor beta
IL1RN	Interleukin 1 receptor antagonist
LELP	Late cornified envelope-like proline-rich
Imp	Imprinting
IRF	Interferon regulatory factor
LTA4H	Leukotrine A4 hydrolase
ITK	IL-2-inducible T-cell kinase
IL1RN	Interleukin 1 receptor antagonist

LOD	The common logarithm of the odds ratio
MAVS	Mitrocholdrial Antiviral Signaling
MOD score	Genehunter MOD generated maximization of LOD score
NOD1	nucleotide-binding oligomerization domain protein 1
NOS	Nitric oxide synthase
NPL	Nonparametric LOD score
OR	Odds ratio
ORMDL3	ORM1-like protein 3
PAR	Persistent allergic rhinitis
PHF	PHD finger protein
PLAU	Urokinase-type plasminogen activator
Pos. sIgE	Positive specific IgE antibody for at least one of the tested allergens
PRR	Prevalence rate ratio.
PTGS	Prostaglandin-endoperoxide synthase
SART-1	Squamous cell carcinoma antigen recognized by T cells 1
Sensitivity	Proportion of subjects with allergy who test positive
Sib	Sibling
sIgE	Specific immunoglobulin E
Specificity	Proportion of subjects without allergy who test negative
SNP	Single nucleotide polymorphism
SPP1	Secreted phosphoprotein 1
SPT	Skin prick testing
STAT	Signal transducer and activator of transcription
T-IgE	Total immunoglobulin E in serum
TLR	Toll-like receptor
TIM	T-cell immunoglobulin domain and mucin domain
UGB	Uteroglobin
VDR	Vitamin D receptor
WT	Wild type

Introduction

" Is asthma hereditary?
I think there can be no doubt that it is"

Henry Hyde Salter. 1860;
"On Asthma: Its Pathology and Treatment"

It has been suspected for centuries that the atopic diseases, allergic rhinitis (AR), asthma, and atopic dermatitis (AD), have a strong heritable component. This suspicion has repeatedly been found to be true [1]. Nevertheless, pinpointing the actual genes behind this group of diseases has proven difficult. This difficulty arises because the atopic diseases mainly fall into the category of complex disorders that does not display classical Mendelian forms of inheritance. There are a few known monogenic diseases with atopic phenotypes [2, 3], but most of the atopic diseases are characterised by being polygenic and multifactorial. Thus, multiple genes interact with each other and/or with environmental factors to cause atopic diseases and form the actual phenotype. Finding all of these genes, especially those with small effects, has proven to be quite a challenge!

All complex diseases are to some extent heterogeneous, and the heterogeneity can be seen in many different domains. Among these are age of onset, severity, and response to treatment. The natural history of allergic rhinitis (AR) is no exception to this. AR can for example be a persistent disease, lasting more than 4 weeks a year and more than 4 days a week, or an intermittent disease with a shorter duration. Different phenotypes and clinical manifestations are likely to work by different pathways and different genes. These differences obviously have implications for genetic research in this area [4]. An often used method to reduce genetic heterogeneity and thereby increase the power of an analysis is to dissect complex traits into the underlying more distinct traits (intermediate phenotypes) – traits such as total or specific IgE [5-7].

Genome-wide linkage study, candidate gene association studies, and genome-wide association studies are some of the tools used to achieve knowledge about the genetic architecture underlying the complex phenotypes. Each of these techniques is described later in this thesis.

To date, more than a thousand studies of the genetics behind allergic diseases have been published. Each of them helps us understand a little bit more about the complex world of allergy genes.
The amount of research done in this field reflects the growing burden that atopic diseases lay on patients and societies around the world. The risk of developing atopy and AR has increased over the last few decades [8-10] even though there are strong signs that the increase has reached a plateau in many Western countries [11-13]. In Denmark, the lifetime prevalence of AR is 15.7%, and for asthma and AD, it is 6.9% and 21.3%, respectively [14].

The three articles in this thesis lay further pieces to the puzzle, with the aim that in a near future, we will see a greater portion of, if not the whole, picture behind the genetic structure underlying allergic diseases.

English summary

Allergic rhinitis (AR) is a complex disorder with a multifactorial, polygenic aetiology. Twin studies have found the genetic contribution to be substantial. However, genetic research on the disease is sparse. AR is mostly considered a phenotypic subgroup in studies of other atopic disorders, an approach that increases the risk of selection bias.

The aim of this study was to identify chromosomal regions (loci) likely to harbour genes influencing the development of AR and related allergic disorders and to investigate a possible association of the filaggrin gene (*FLG)* with AR.

We collected and clinically characterised 127 nuclear families with at least two siblings suffering from AR, including 529 individuals (274 children and 255 parents).
A whole genome linkage scan was performed on this sample using 429 microsatellite markers distributed throughout the genome. For parts of the analysis, we combined the sample with two previously collected Danish sib-pair family samples, reaching a total of 357 families comprising 1,508 individuals, of whom 190 families (812 individuals) segregated AR. The computer programmes Genehunter NPL as well as Genehunter MOD and Genehunter Imprinting were used for obtaining non-parametric and parametric linkage results, respectively.

Furthermore, a candidate gene association study of *FLG* and AR, asthma, atopic dermatitis and allergic sensitization was undertaken. This study examined four *FLG* single nucleotide polymorphisms (SNPs) previously associated with atopic dermatitis. The program FBAT 2.0.3 was used for the family based association analysis.

The main results of the genome wide linkage scan of AR were the following:

- Suggestive evidence of linkage to hay fever combined (i.e. AR and/or allergic conjunctivitis) at 1p13, 1q31, 2q23 and 20p12 with 1p13 showing close to genome wide significance.
- Suggestive evidence of linkage to AR at 1p13, 1q31, 2q14-q21, 2q23, 12q13 and 20p12.
- Suggestive evidence of linkage to IgE associated AR at 1p13, 1q32 and 20p12.
- Indication of linkage (NPL$\geq$ 2) to hay fever combined at 1q21, 4q11-q12, Xp21 and Xp11.
- Indication of linkage (NPL$\geq$ 2) to AR at 1q21, 4q11-q12, 14q12, 22q12-q13 and Xp22-p21.
- Indication of linkage (NPL$\geq$ 2) to IgE associated AR at 14q12 and 20p13.
- Likely maternal imprinting for AR at 2q23.3 (MOD without imprinting 1.74, MOD with imprinting / p(wt,wt),p(wt,d), p(d,wt), p(d,d) = 3.26 / 0.045, 0.25, 0.045, 0.28).
- Possible maternal imprinting at 3q28 (MOD without imprinting 1.52, MOD with imprinting / p(wt,wt),p(wt,d), p(d,wt), p(d,d) = 2.84 / 0.02, 0.71, 0.02, 0.71).
- Indication of linkage to persistent allergic rhinitis at chromosome 21q22 (NPL= 2.03, P value= 0.004, MOD score without / with imprinting = 2.35 /2.33).

The main results of the genome wide linkage scan of atopy and asthma were the following:

- Suggestive evidence of linkage to asthma at 2p13 and 20p12 with 2p13 strongly approaching genome wide significance.
- Suggestive evidence of linkage specific IgE at 1p13 and 20p13.
- Suggestive evidence of linkage to total IgE at 6p24 and 11q12-q13.
- Indication of linkage (NPL$\geq$ 2) to specific IgE at 10q26, 11q22, 14q11, 20p13, 22q12-q13 and Xp22.
- Possible maternal imprinting for specific IgE at 3q22 and 20p12 and for total IgE at 11q22.

The main results of the *FLG* association study were the following:

- Confirmation of association with atopic dermatitis.
- No significant association with AR, asthma or allergic sensitization when the influence of co-existing atopic dermatitis was removed.

In conclusion, we found association of *FLG* with atopic dermatitis but not with AR. We collected an AR family sample and found suggestive linkage of AR to several chromosome regions as well as suggestive linkage to several regions for atopy and asthma.

Danish summary

Allergisk rhinitis (AR) er en kompleks sygdom med multifaktoriel og polygen ætiologi. Tvilling studier har fastslået, at genetikkens bidrag til udviklingen af AR er betydelig. Alligevel er genetisk forskning i området sparsomt. AR behandles oftest som en fænotypisk undergruppe i studier der behandler andre allergiske lidelser. Denne tilgang til forskning i AR genetik øger risikoen for selektions bias.

Målet for dette studie er at identificerer kromosomale regioner, der kan indeholde gener bag AR og andre allergiske lidelser, samt at belyse en mulig association mellem filaggrin genet (*FLG*) og AR.

Vi opsporede, indsamlede og fænotype bestemte en gruppe af 127 danske familier som omfattede 529 individer (274 børn og 255 forældre).
En scanning af hele genomet blev udført med brug af 429 mikrosatellit markører fordelt jævnt over genomet. I dele af analyserne kombinerede vi denne gruppe med to tidligere indsamlede danske familie grupper og opnåede derved total 357 projekt familier omfattende 1,508 individer af hvilke 190 familier (812 individer) segregerede AR. Computer programmerne Genehunter NPL samt Genehunter MOD og Genehunter Imprinting blev brugt for at opnå henholdsvis ikke-parametriske og parametriske analyse resultater.

Vi udførte endvidere et kandidat gen studie af *FLG* og AR, astma, atopisk dermatitis samt allergisk sensibilitet. Dette studie undersøgte fire *FLG* single nucleotide polymorphisms (SNPs) der tidligere er associeret med atopisk dermatitis. Til den familie baserede familie analyse benyttedes programmet FBAT 2.0.3.

Resultaterne af hel genom scanningen for AR var som følgende:

- Sandsynlig kobling til ”høfeber kombineret ” (i.e. AR og/eller allergisk konjunktivitis) ved , 1q31, 2q23 og 20p12 med 1p13 tæt på signifikant evidens for kobling.
- Sandsynlig kobling til AR ved 1p13, 1q31, 2q14-q21, 2q23, 12q13 og 20p12.
- Sandsynlig kobling til IgE associeret AR ved 1p13, 1q32 og 20p12.
- Indikation for kobling (NPL≥ 2) til høfeber kombineret ved 1q21, 4q11-q12, Xp21 og Xp11.
- Indikation for kobling (NPL≥ 2) til AR ved 1q21, 4q11-q12, 14q12, 22q12-q13 og Xp22-p21.
- Indikation for kobling (NPL≥ 2) til IgE associeret AR ved 14q12 og 20p13.
- Sandsynlig maternel imprinting for AR ved 2q23.3 (MOD uden imprinting 1.74, MOD med imprinting / p(wt,wt),p(wt,d), p(d,wt), p(d,d) = 3.26 / 0.045, 0.25, 0.045, 0.28).
- Mulig maternel imprinting for AR ved 3q28 (MOD uden imprinting 1.52, MOD med imprinting / p(wt,wt),p(wt,d), p(d,wt), p(d,d) = 2.84 / 0.02, 0.71, 0.02, 0.71).
- Indikation for kobling til persisterende allergisk rhinitis ved 21q22 (NPL= 2.03, P value= 0.004, MOD score without / with imprinting = 2.35 /2.33).

Resultaterne af hel genom scanningen for atopi og astma var som følgende:

- Sandsynlig kobling til astma ved 2p13 og 20p12 med 2p13 nærmende sig signifikant evidens for kobling.
- Sandsynlig kobling til specifik IgE ved 1p13 og 20p13.
- Sandsynlig kobling til total IgE ved 6p24 og 11q12-q13
- Indikation for kobling (NPL≥ 2) til specifik IgE ved 10q26, 11q22, 14q11, 20p13, 22q12-q13 og Xp22.

- Mulig maternel imprinting for specifik IgE ved 3q22 og 20p12 samt for total IgE ved 11q22.

Resultaterne af *FLG* associations studiet var som følgende:

- Bekræftelse af associationen med atopisk dermatitis.
- Ingen association med AR, astma eller allergisk sensitivitet når påvirkningen af samtidig atopisk dermatitis var fjernet.

Konklusion: Vi fandt association mellem *FLG* og atopisk dermatitis men ikke mellem *FLG* og AR. Vi samlede en AR familie gruppe og fandt adskillige regioner med sandsynlig kobling til AR samt atopi og astma.

Allergy phenotypes

The main focus of this thesis is on atopy and allergic rhinitis, which will be described below in detail. Atopic dermatitis and asthma will also be briefly discussed.

Atopy

Atopy is clinically defined as a person who has a personal and/or familial tendency to become sensitised and produce Immunoglobulin E (IgE) antibodies in response to ordinary allergen exposure. Consequently, atopic individuals can develop IgE-mediated allergic diseases, including rhinitis and asthma. IgE antibodies mediate a hypersensitivity reaction (such as allergic rhinitis) by binding to a high-affinity receptor on the surface of mast cells and basophils, which subsequently activates these cells. The term "atopy" is used to specifically describe IgE sensitisation, which can be assessed by quantification of the IgE antibody level (total IgE antibody or allergen-specific IgE antibody) or a positive skin prick test [15].

Complex diseases are heterogeneous due to differences in the age of onset, the disease severity, the response to treatment and the natural history of the disease. Different phenotypes and clinical manifestations are likely to work by different pathways and different genes[4]. Genetic studies often attempt to reduce the genetic heterogeneity and thereby increase the analytical power; typically, researchers dissect complex traits into the underlying more distinct traits (intermediate phenotypes), such as total IgE or specific IgE [5-7].

Total IgE

The IgE level varies widely with age in normal healthy subjects (Table 1) (http://www.lab.dk

/show.asp?kode=NPU02482 and https://www.sundhed.dk /Artikel.aspx?id=13231.1) and is associated with numerous factors, such as race, sex, smoking habits and parasitic disease [16, 17]. Thus, this large natural range makes it less useful for detecting whether a person is atopic.

The Total IgE level is neither sensitive nor specific for allergic rhinitis (AR) and is therefore not routinely indicated in AR diagnosis [18]. However, a total IgE level greater than 100 before the age of 6 is risk factor for developing AR [19]. The total IgE level is found to be a predictor for the asthma and atopic dermatitis phenotypes [20, 21]. Twin-based studies have suggested that the heritability of the total IgE level is between 60 and 81% [5, 22, 23].

Table 1: Reference ranges for the serum total IgE level in healthy subjects.

Age	Upper limit of the reference range
newborn	1 KU/l
14 days - 1 year	15 KU/l
1-6 years	100 KU/l
6-14 years	150 KU/l
> 14 years	100 KU/l

Adapted from: https://www.sundhed.dk/artikel.aspx?id=13231.1 and http://www.lab.dk/show.asp?kode=NPU02482

Allergen-specific IgE and Skin prick test

Allergies can be antibody or cell mediated, although a majority of patients with allergic symptoms have IgE isotype antibody-mediated allergies. These individuals produce IgE antibodies to a specific allergen (IgE-mediated allergy) [15].

Method of measurement:

Skin prick testing (SPT).

In the skin prick test, a few drops of the purified allergen are gently pricked on to the skin surface, typically on the forearm. If the patient has been sensitised to the applied allergen, a wheal will develop. A positive SPT is defined as a wheal size of $\geq$ 3 mm larger than the negative control. However, certain drugs, such as antihistamines and tricyclic anti-depressants, can affect the SPT results. Additionally, skin prick testing is not recommended if the test area has eczema [1].

Allergen-specific serum IgE.

The Specific IgE level was measured with an ImmunoCAP assay (Phadia Aps, Allerød, Denmark). The ImmunoCAP Phadiatop® is a combined test that detects IgE antibodies against the most common inhalant allergens. Studies have found that the results from Phadiatop® testing agree with the clinical classification of atopic and non-atopic persons in 91.4% of cases. The clinical sensitivity and specificity of Phadiatop® are 93% and 89%, respectively [24]. Once an individual is diagnosed as atopic, individual allergens are tested with the ImmunoCAP Specific IgE, which is an in vitro test that measures the serum concentration of the allergen-specific IgE antibody. The sensitivity and specificity of this test compared to clinical diagnosis are 89% and 91%, respectively [25]. Unlike the SPT, specific IgE measurements are not influenced by drugs or skin disease [1].

Prevalence of positive SPT and elevated S-IgE.

Research over the past several decades indicates that the prevalence of atopy and atopic disease has significantly increased [9, 10]. However, there have been several studies indicating that the prevalence has reached a plateau or has even decreased in areas of the western world during the 1990s [12, 13, 26].

Currently, the frequency of sensitisation measured by SPT and/or S-IgE in children with no clinically diagnosed allergic disease is 17%. For children with allergic rhinitis (AR), atopic dermatitis, and asthma, the frequency is 55%, 47%, and 56%-61%, respectively [27, 28]. For 42% of children with AR and 44% of children with asthma, the diseases are associated with sensitisation. Sensitisation cannot be linked to the worsening of eczema in children with atopic dermatitis [27].

Comparison of test methods.

There is a strong correlation between S-IgE and SPT. A German twin study found that the S-IgE and SPT results are substantially cross-trait twin correlated (0.98), mainly due to shared genetic factors [5]. A comparison of the ImmunoCAP Specific IgE (CAP) and SPT results for 53 inhalant allergens in patients with chronic rhinitis indicated that the tests agreed in 80.6% of the patients, with more $SPT^{+}S\text{-}IgE^{-}$ (11.7%) than $S\text{-}IgE^{+}SPT^{-}$ (7.7%) [29].

Heredity of positive SPT and elevated S-IgE.

The most important single factor statistically associated with the development of allergic disease is whether other family members are affected. A newborn child with no atopic parents has approximately a 5% to 15% risk of developing allergic disease. If one parent is atopic, the risk increases to 20% to 40%, and the risk further increases to 40% to 60% for infants with two atopic parents. Interestingly, the risk with two atopic parents further increases to 50% to 80% if the parents share the same allergic manifestation [30, 31].

Twin-based studies have shown that the estimated heritability of a positive SPT and an elevated serum S-IgE are approximately 0.56 to 0.68 and 0.60 to 0.78, respectively [5, 22, 32, 33]. Interestingly, monozygotic twins often vary in their expression of atopy, which indicates that epigenetic and environmental factors, rather than genetic factors, determine the pattern of

sensitisation. However, the genetic influence is most strongly associated with the age of onset, and to some extent, the type of associated allergic diseases in individuals with a genetic predisposition to atopy [22, 34].

Clinical relevance.

If a patient presents clinical signs of an allergic disease, testing for specific IgE can determine whether the symptoms indeed are caused by allergies. It can also help identify the allergen that is causing the hypersensitivity, which would allow for patient to avoid this allergen and/or initiate allergen immunotherapy [1].

Allergic rhinitis

Definition and diagnosis.

The typical symptoms for allergic rhinitis (AR) are anterior or posterior rhinorrhoea, nasal blockage, sneezing or itching of the nose due to inflammation of the nasal mucosa [16, 18, 19]. According to some definitions, these symptoms must occur for two or more consecutive days and for more than one hour a majority of the days for the symptoms to be pathological. These symptoms can be caused by an IgE or non-IgE mediated (i.e., cell-mediated) reaction in the nose after allergen exposure [35]. AR is often accompanied by allergic conjunctivitis (itch and redness of the eye) [19]. AR can be subdivided into “intermittent” or “persistent” disease (Table 2), and the disease severity can be classified as “mild” or “moderate-severe” [1].

Basic mechanisms of allergic rhinitis.

Allergens can have an enzymatic proteolytic activity and can directly activate cells and induce inflammation, independently of IgE (i.e., a **non-IgE mediated reaction)** [36, 37]. However, most patients with allergic rhinitis have IgE-mediated allergies (IgE-associated allergic rhinitis).

In **IgE-mediated AR,** the antigen penetrates the epithelium and is subsequently presented to a T-lymphocyte by an antigen presenting cell, thereby initiating IgE antibody production. IgE antibodies are produced and bind to receptors on mast cells and other inflammatory cells. When the IgE eventually binds to the allergen on the surface of the inflammatory cell, the cell releases inflammatory mediators that initiate a number of physiological pathways that result in allergy symptoms [1, 38].

Table 2 Allergic rhinitis classification according to the ARIA guidelines [1].

1: **Intermittent allergic rhinitis** (IAR) The patient has symptoms for less than 4 days a week or less than 4 weeks a year
2: **Persistent allergic rhinitis** (PAR) The patient has symptoms for more than 4 days a week and more than 4 weeks a year
A: **Mild allergic rhinitis symptoms:** • Normal sleep • No impairment of daily activities, leisure and/or sports • No impairment of school or work • No troublesome symptoms
B: **Moderate-severe allergic rhinitis symptoms**: • Sleep disturbance • And/or impairment of daily activities, leisure and/or sports • And/or impairment of school or work • And/or troublesome symptoms

Heredity of allergic rhinitis

Twin-based studies have estimated that the AR heritability is between 71% and 96% [39-41]. If a child's father or mother has AR, the child's risk for developing AR increases with a prevalence rate ratio (PRR) of 2.7 (95% CI, 1.8 to 3.9) and 2.2 (95% CI, 1.5 to 3.2), respectively. This risk again

rises to a PRR of 4.5 (95% CI, 3.3 to 6.1) if both parents have AR[42]. Another study indicated that the risk of developing AR is 13% if none of the parents are affected and 40% if one or both parents have AR [43].

The prevalence of allergic rhinitis

Hay fever is a surprisingly modern disease; a few rare descriptions can be traced back to Islamic texts from the 9th century, as well as a few European texts from the 16th century [44]. However, modern hay fever literature is commonly believed to start with John Bostock's article from 1819 describing his own hay fever symptoms [45]. It took Bostock 9 years to collect 28 more cases for a second article [46] because at that time, hay fever was an "uncommon condition" [47]. The prevalence has since increased, and Charles Blackley (1873) began his book on hay fever with the statement: "It would seem that hay-fever has, of late years, been considerably on the increase" [48]. In 1907 the *General Practitioner* journal wrote the following when reviewing William Lloyd's book *Hay Fever, Hay Asthma, Its Causes, Diagnosis and Treatment* [49]: "From our point of view this book is important, firstly, because it treats of a very common complaint" [44]. Thus, hay fever became a common disease during the 19th century. This rise in prevalence continued into the 20th century, and today, 10% to 30% of adults and up to 40% of children suffer from this condition [8, 19, 28, 50]. However, the increase in the prevalence of AR and other allergic disease seems to have reached a plateau during the 1990s, especially in children [12, 13, 26]. There is still an increase in the number of allergy cases in populations as a whole as the "allergic generations" become older. Moreover, the number of people with allergies varies significantly between countries [50]. The prevalence of AR in Danish surveys varies between 14 and 22% [14, 51, 52]. AR is often seen in combination with other allergic diseases, such as atopic dermatitis and asthma [16, 53-55].

One may ask what has caused this increase in allergic rhinitis and other allergic diseases? Because the time span for the increase has been fairly short, changes in the gene pool are therefore not a realistic explanation. Several studies have attempted to answer this question by looking at known risk factors for AR and identifying how they may have changed over time. The actual reason behind the increase is likely to be a combination of the different influences, some of which are described below.

Risk factors and their possible influence on the increase in AR

- The most important single factor associated with the development AR is a **family history of allergy**, especially allergic rhinitis [39-42, 56, 57]. However, this cannot explain the increase in the AR prevalence rate.
- Exposure to **allergens** [1, 58-60]. Currently, the pollen season is approximately three weeks longer for birch and five to six weeks longer for alder and hazel compared to 30 years ago [61]. This results in a significant increase in the total pollen exposure and may thereby contribute to the increase in allergic sensitisation and allergic disease. A further lengthening of the pollen season and an increase in new types of pollen are expected to continue with global warming.
- **Allergic sensitisation.** A positive SPT or S-IgE is a significant risk factor for developing hay fever [28, 62]. Several studies have reported an increase in sensitisation as measured by the SPT or the serum S-IgE [9, 10, 63-65].
- **Urban** living in combination with a "**Western lifestyle**" increases the risk for AR compared to **rural** living. Maybe as a result of changes in diet, exposure to traffic-related particles and parasitic infections, and changes in income and educational level [66, 67]. Thus, increasing urbanisation worldwide may influence the prevalence of allergies.

- **Traffic-related air pollution** is associated with the development of allergies [68-70]. The burden of traffic has significantly increased this last century.
- High **social status** and elevated **educational level** of the patient increases the risk of atopy and AR. This is not a new observation. Blackley wrote in his book in 1873 that hay fever seemed to be confine to the educated classes [1, 44, 71].
- The risk of developing AR increases with **maternal age** [8]. The average age for a first-time mother in Denmark has increased from 23.1 years in 1960 to 29 years in 2008 (http://www.statistikbanken.dk/FOD11).
- **Maternal smoking** during pregnancy and after the child's birth has been shown to increase the risk of sensitisation and AR in several studies [57, 59], whereas other studies have found no association between maternal smoking and AR [72]. The number of Danish women who smoke has decreased from 47% in 1970 to 25% in 2006 [51], which could be related to the stabilisation of the AR prevalence in children. (1970 was the first year "Danmarks statistik" started to register smoking habits among the Danes.)
- Feeding infants with **formula** instead of breast feeding increases the risk of AR. Interestingly, formula feeding was very popular until the 19th century, which coincides with an increase in the prevalence of allergic disease [44, 73, 74].
- A person with **eczema** has an increased risk of developing AR [59].
- Low **indoor air quality** with regard to dampness and ventilation, which can result in mould growth and an elevated number of house dust mites [1, 58, 59, 75].
- A **high birth weight** ($\geq$ 3000 g) increases the risk for developing AR compared to a low birth weight (< 2000 g) [76].
- **A high gestational age** (Children born after week 40 of gestation) is associated with an increased risk for AR compared to babies born before 33 weeks of gestation [76]. However,

a second study concluded that a **low gestational age** (born at least 3 weeks preterm) is a risk factor for AR [57].

- **The male gender** is an independent risk for developing atopy and AR [57].

Protective influences on allergic rhinitis

- A **high number of siblings** appears to be inversely correlated with the risk of atopy and AR [59, 71, 77]. The fertility in Denmark has fallen from 2.6 children per woman in 1966 to 1.8 children per woman in 2005; currently, few women bear more than 2 children [78].
- There is increasing evidence that **living on a farm**, both organic and conventional, protects against AR and asthma [79-83]. One study, however, observed that this protective effect only applies to children of farmers born after 1971 [84].
- Several studies have indicated that **breast-feeding** is associated with a protective effect against atopy and AR [68, 73, 77], whereas other studies have shown that breast-feeding increases the risk of developing allergies [85, 86]. There may be a bias in that mothers who know they have a family history of allergies are more prone to breast-feed their children.
- Chronic infection with **parasitic worms and microbes** is associated with a protective effect against the development of allergic disease. It has been hypothesised that better treatment and health care in all parts of the world have decreased exposure to gut-dwelling microbes and helminths, resulting in abnormal immunoregulation (i.e., an imbalance) between the Th1 and Th2 immune response [87, 88].

Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory disease of the skin. Although termed atopic, the proportion of AD patients that have IgE-mediated sensitivity varies considerably (from 7% to 78%

between studies) [89]. Atopic dermatitis diagnosis is based on the criteria published by Hanifin and Rajka [90] or the diagnostic criteria later developed by British dermatologists (box 1) [91].

A three-fold increase in the cumulative incidence rate of AD among newborn to 7-year-old children was reported in Denmark, from 3% in 1960–1964 to 10% in 1970–1974 [92]. However, several studies indicate that this increase has levelled off during the 1990s [13, 93]. The prevalence of AD among 12-16-year-old children was 21.3% in 2000. Twin-based studies estimated an AD heritability between 86% and 88% [39, 92]. Parental eczema is strongly associated with childhood AD; the odds ratio (OR) is 1.69 (95% confidence interval, 1.47 to 1.95) for maternal eczema alone, 1.74 (1.44 to 2.09) for paternal eczema alone and 2.72 (2.09 to 3.53) for eczema in both parents [94]. Another study reported that AD in children had a PRR of 1.9 (95% CI, 0.3 to 11.8) and 1.5 (95% CI, 0.4 to 5.5) when only the father or only the mother was affected, respectively, and a PRR of 2.3 (95% CI, 0.4 to 13.7) when both parents were affected [42].

BOX 1: The British dermatologist diagnostic guidelines for the diagnosis of atopic dermatitis [91]

AD diagnosis requires:

An itchy skin condition (or parental report of scratching)

Plus three or more of the following:

1. A history of involvement of the skin creases, such as the folds of the elbows, behind the knees, the front of the ankles, or around the neck (incl. cheeks in children under the age of ten)
2. A personal history of asthma or hay fever (or history of atopic disease in a first-degree relative in children under the age of four)
3. A history of a general dry skin in the late year
4. Visible flexural eczema (or eczema involving the cheeks/forehead and under the limbs in children under the age of four)
5. An onset of symptoms before the age of two (not used if the child is under four)

Asthma

Asthma is a chronic inflammatory disease of the upper airways and lungs that is characterised by recurring symptoms that vary in severity and frequency. Symptoms include wheezing, cough, chest tightness and shortness of breath and occur as a result of airflow obstruction, bronchial inflammation, and hyper responsiveness [95].

Asthma frequently coexists with AR, and AR is often a precursor of bronchial asthma in children [1, 55].

Similar to other allergic diseases, the prevalence of asthma has increased worldwide over the past several decades [96]. This increase has stabilised, and a recent Danish study on the prevalence of physician-diagnosed asthma among unselected Danish schoolchildren age 6 to 17 showed no increase in prevalence over 10 years, with a 4.0% prevalence (95% confidence interval (CI) 2.7–5.3%) in 1990–1991 and 3.6% prevalence (95% CI 2.4–4.8%) in 2001 [97].

Twin-based studies have estimated that the heritability for asthma is between 61% and 82% [39, 98, 99]. Variation in the age at asthma onset is mainly due to environmental factors and accounts for 66% of cases compared to genetic factors [98]. A child's risk of developing asthma is 22% to 29% if neither of the parents have asthma and 47% to 55% if one or both parents have asthma [43].

The phenotypes investigated in this thesis

In this thesis, the genetics behind the following phenotypes were investigated.

Allergic rhinitis. The AR phenotype was studied using three approaches. The first two are broad definitions based purely on a clinical symptom-based diagnosis. The first of these approaches is named "Hay fever combined" (HC), and this group contains subjects who present clinical

symptoms of allergic rhinitis and/or allergic conjunctivitis; the second broad group contains subjects who have clinical symptoms of allergic rhinitis (AR). Finally, the third group contains a more defined phenotype (IgE associated AR), where the subjects have AR in combination with a positive specific IgE (defined as a positive specific IgE antibody for at least one of the tested allergens). The defined phenotype of this third group may decrease the genetic heterogeneity and thereby increase the analytical power of the study.

Total IgE. The total IgE was considered elevated if $\geq$ 150 kU/l for children between 6 and 14 and $\geq$ 100 kU/l for the remaining participants.

Allergen-specific serum IgE. The specific IgEs were tested for reactivity with twelve allergens: mite (*D. pternyssinus* and *D. farinae*), cat, horse, dog, timothy grass, birch, mugwort and mould (*Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum*). A Specific IgE $\geq$ 0.35 kU/l was considered elevated.

Asthma. The asthma phenotype was studied using two approaches. The clinical diagnose was based on answers given in a standardised questionnaire recording symptoms, duration and treatment. In addition the phenotype was studied in combination with a positive specific IgE (defined as a positive specific IgE antibody for at least one of the tested allergens). No further clinical testing was performed.

Atopic dermatitis. AD was diagnosed using the British dermatologist diagnostic guidelines for the diagnosis of atopic dermatitis [91] evaluated trough answers given in a standardised questionnaire recording symptoms, duration and treatment. In addition the phenotype was studied in combination with a positive specific IgE (defined as a positive specific IgE antibody for at least one of the tested allergens.

How to analyse complex genetic traits

Molecular genetics is a relatively new area of research. Since the discovery of deoxyribonucleic acid (DNA) in 1944, the discovery of the components that comprise genes, and the realisation in 1959 that there are 46 human chromosomes, the field of genetic research has increasingly expanded [100]. The genetic basis of a thousand single-gene disorders has been identified. In contrast, mapping the genetics underlying complex disorders has proven quite bothersome. Three basic study designs are widely used in the pursuit of new discoveries in this field.

Genome-wide linkage study / Positional cloning by linkage

In linkage analysis, marker polymorphisms are scattered throughout the genome (i.e., g*enome-wide linkage study* or *regional linkage analysis* if only parts of the genome is studied).The current standard aims at including a highly informative marker for every 10 centiMorgan (cM) or a large number of biallelic markers (typically SNP arrays) [101]. These markers are used to determine co-segregation of a disease trait and a marker (or a set of markers) in families affected by the disease of interest [100, 102, 103]. If a region contains a higher than expected number of shared alleles, it is said to be linked to the disease trait and may therefore harbour genes that contribute to the phenotype in question (susceptibility genes). These genes can then become candidates for further analysis [103].

This study design allows for the identification of new regions harbouring genes not previously suspected to have an influence on the disease in question. Genome-wide linkage studies are effective in detecting rare disease alleles with relatively large effects; however, these studies are not so effective at finding genes with modest effects size [103].

Candidate gene association studies

In a candidate gene study, a gene or set of genes suspected to play a role in the disease of interest is selected. The selection is based on knowledge of the protein product or suspected role of the gene in the present pathophysiology. The suspicion may be based on involvement of the gene in other diseases with phenotypic overlap, findings in animal models, or the location of the gene in a region earlier found to be linked to the disease, in which case it is referred to as a positional candidate gene [103, 104]. The incidence of transmission of variants in a selected candidate gene is then compared between affected individuals (cases) and controls. In the case of family-based association studies, one might say that the cases and controls are the alleles transmitted to the affected offspring and the untransmitted alleles among informative (i.e., heterozygote) parents, respectively.

The main limitation of this study design is that only genes with some prior evidence of involvement in the disease are selected for examination.

Genome-wide association study

With the characterisation of millions of single nucleotide polymorphisms (SNPs) in the human genome, the advances in SNP genotyping technology, and the development of statistical software and analysis methods, genome-wide association studies (GWAS) have become possible. In GWAS, hundreds of thousands to more than one million SNPs distributed throughout the genome are examined in each subject [102-105], which enables a hypothesis-independent association study, making it possible to identify genetic variants in novel pathways influencing the investigated trait. To date, hundreds of new loci have been found to influence complex disorders. There is, however, only a very limited number of studies that focus on atopic disorders, and so far, no studies

concerning allergic rhinitis have been published (http://www.genome.gov/GWAStudies/). GWAS will be described in more detail in the chapter "Summary of GWA studies for allergic rhinitis and atopy" later in this thesis.

There are limitations to the GWA study. One is that the current genotype arrays in GWAS have limited power in capturing low frequency variants (i.e., variants with allele frequencies of less than 5%). Another challenge of the GWAS is the difficulty in collecting a big enough sample size that will give statistically significant results [105].

Applied statistical methods in this thesis

Linkage analysis

Genetic linkage describes the tendency of alleles from different loci to be inherited together because the loci are located close to each other on a chromosome. How closely they are located and to which degree they are linked can be measured by the number of crossovers between the homologous chromosomes occurring between the two loci during meiosis (i.e., recombination fraction, θ). If a recombination fraction of 50% ($\theta = 0,5$) is observed, the distance between the loci is said to be (at least) 50 centiMorgan (cM), and no linkage is present between the loci. In the case of complete linkage, no recombination is observed ($\theta = 0$). With a recombination fraction under 50% ($\theta < 0,5$), the two loci are said to be genetically linked.

The purpose of a linkage analysis is to estimate the recombination fraction (θ) and to clarify whether the derivation from 50% recombination is statistically significant if θ is < 0,5.

This is clarified through the calculation of a LOD score (referred to as *the common logarithm of the odds ratio*) [106]. The LOD score represents the $\log_{10}$ of the likelihood of obtaining the observed results under the assumption of linkage divided with the likelihood of the results under the assumption of no linkage:

$$LOD = Z(\theta) = \log_{10} [L (\theta) / L (\theta = 0,5)].$$

A LOD $\geq$ 3 is generally referred to as significant linkage in the analysis of simple monogenic disorders [101, 107]. It is however more difficult to set a significance level to LOD scores when analysing complex diseases. Different proposals have been made addressing this issue (Table 3).

Table 3: Proposed significance levels of LOD scores and p values in genome scans.

Thomson et al. [108]		**Lander et al.** [109]			**Haines et al**. [110]		
Level of significance	**Nominal p-value**	**Level of significance**	**Nominal p-value**	**LOD scores**	**Level of significance**	**Nominal p-value**	**LOD scores**
Week	0.05	Suggestive	7×10^{-4}	2.2	Interesting	3×10^{-2}	1.0
Moderate	0.01	Significant	2×10^{-5}	3.6	Very interesting	9×10^{-4}	2.0
Strong	0.001	Highly significant	3×10^{-7}	5.4	Provisional linkage	3×10^{-5}	3.0
					confirmed linkage	8×10^{-7}	4.0

Modified from Haines,1998 [110]

MOD-score and Imprinting analysis

The classical LOD score method is a parametric analysis requiring trait model parameters to be specified, such as penetrances and disease allele frequency. These parameters are difficult to provide when it comes to complex disorders because the disease model parameters are usually unknown prior to the analysis. A way to overcome this problem is by using a MOD-score analysis in which the parametric LOD score is maximised with respect to the trait model parameters. We used GENEHUNTER MODSCORE version 3.0 and extended the analysis with GENEHUNTER IMPRINTING version 1.3 (http://www.staff.uni-marburg.de/~strauchk/software.html) in which the parent-of-origin effect (imprinting effect) is taken in to consideration with different penetrances for heterozygote individuals, depending on whether the disease causing allele is transmitted by the father or the mother.

Regarding significance levels, a MOD score of three shall be "deflated" by approximately 0.3 to one units to be comparable with LOD scores due to the multiple testing of the maximisation process [111, 112].

Nonparametric linkage analysis

Another way to address the problem of the unknown disease model parameters in the analysis of complex disorders is by performing a nonparametric linkage analysis (NPL) that requires no

assumption about the mode of inheritance of the trait studied. This “model-free” method is based on identifying genomic regions in which patterns of allele sharing among family members correspond to the distribution of the investigated phenotype. We addressed the “model-free” method by the affected sib-pair method using GENEHUNTER-NPL version 2.0 [113] for the autosomal chromosomes. NPL analysis of the X chromosome was done using GENEHUNTER-IMPRINTING version 1.3 [102]. In an affected sib-pair analysis, DNA from families with at least two children with the phenotype in question is collected. The analysis then focuses on the number of parental alleles shared by the affected siblings, namely, the number of alleles shared identical by decent (IBD). In the case of heterozygote parents, the siblings can by chance share 0, 1 or 2 alleles, and this occurrence is expected to happen in 25% (no alleles shared), 50% (one allele shared) or 25% (2 alleles shared) of cases. If the siblings share a particular allele more frequently than expected by chance, it is suspected that a linkage exists between that allele and the phenotype the siblings have in common.

Empiric p value

To evaluate the genome wide level of significance in a given sample, a computer simulation of the sample markers with a given phenotype is commonly performed. This simulation is done by performing a series of LOD or MOD score calculations on the background of genetic marker genotypes generated under the assumption of free recombination between the phenotype and the marker locus (i.e., under the assumption of no linkage to the phenotype). Typically around 5000-10000 calculations are performed to find the empiric p value associated with the observed result. The empiric p-value describes how often it could be expected to obtain a result equal to or greater than the observed under the assumption of no linkage [110]. We calculated the empiric P values for the parametric MOD scores using the simulation features incorporated in Genehunter MOD and

Genehunter-Imprinting [113, 114]. A total of 10000 simulations were performed for each region. Based on the regional empiric p value and the size of the region tested a genome wide empiric p value was calculated (GW empiric p value) indicating the likelihood of obtaining results that were as good as or better than the observed (under the assumption of no linkage) in a whole genome scan.

Genome-wide significant linkage is obtained when the observed (or a higher) score is seen in less than 1 out of 20 simulated genome scans (genome-wide empiric p value < 0.05), and suggestive evidence of linkage is obtained when the observed (or a higher) score is seen once (or fewer times) per simulated genome scan. [109].

Association analysis of a candidate gene

To identify genetic variants associated with the phenotypes of this thesis, we performed a genetic association analysis. The association between the filaggrin gene and allergic rhinitis (AR) was chosen for investigation based on the gene's involvement in atopic dermatitis, another disease associated with AR, and on publications that suggest a connection between AR and filaggrin. The collected family samples allow for performing a family-based association study that uses the untransmitted alleles as internal controls and alleles transmitted to the affected offspring (from the informative (i.e., heterozygous) parents) as cases. An advantage of this approach is that any potential bias from population stratification is avoided [115, 116].

The SNP data was found at the HapMap project (http://www.hapmap.org/). The program FBAT version 2.0.3c was used to perform the family-based association analysis of single SNPs and haplotypes [115-117].

Summary of genome wide linkage scans of allergic rhinitis and atopy

To date only four genome wide scans of the phenotype AR have been published [118-121] reporting evidence of linkage (LOD scores $\geq$ 1 and / or p-value $\leq$ 0.003) on chromosome 1q, 2q, 3q, 4q, 5q, 6p, 6q, 9q,11p, 12p, 12q, 17q, 18q, 22q and Xp (table 4). Of these, only linkage to regions 3p13 and 6p24-p23 have been replicated in a second study [118, 121]. This low number of replications may partly be due to the fact that only a few linkage scans have been conducted in the area of AR.

Table 4: Genome wide linkage scans conducted in allergic rhinitis (AR) or atopy 1996-2010

Ref Year	Sample	Phenotypes	Susceptibility regions
[122] 1996	Australia: 80 families. 44 sib pair siblings with atopy.	sIgE	13q14.2-3
[123] 1999	German /Swedish: 97 families. 86% /73% with SPT / sIgE.	T-IgE sIgE	2p21, 2q32 6p22-p21, 9q31 2p21, 6p21, 9q31
[124] 2000	Netherland: 200 families	T-IgE	5q23-q31, 7q21, 12q23-q24
[125] 2000	French: 107 families	T-IgE SPT	12q21.33 7q11.23, 17q12
[118] 2001	Denmark: 100 families. 33AR families.	AR	4q24-q27 2q12-q33, 3q13, 4p15-q12, 5q13 -q15, 6p24-p23, 12p13, 22q13, y Xp21
[126] 2001	Nederland: 200 families	T-IgE/ sIgE/ SPT	1p31, 2q24-q32, 3q29, 5q23-q31, 6p21, 7q11-q22, 12q23-q24,13q12-q13/ 3q25-q26, 7q11-q22,11q22, 17q25, 18p11, 22q11 / 5p15, 8p23, 10q21-q22, 11q22, 12p13, 13q14, 17q21, 17q25, 22q11
[119] 2002	Japan: 48 families	AR T-IgE sIgE	1q36.2, 4q13.3, 9q34.3 3p24, 5q33, 12p13, 12q24 4p16, 11q14, 16p12
[127] 2002	Denmark: 100 families. 48 atopy families	T-IgE sIgE	3q21-22, 5q31-35, 6p24-p22,11q13, 22q13 1p34, 3q13-22, 4q32, 5q31-35,6p24-p22, 16p11, 22q13, Xp11, Xq21
[128] 2004	German / British / Portuguese: 37 / 19 / 26 families with atopy	SPT	(Strongest) 3q21.3 (Weaker) 2p12, 16q21
[129] 2004	USA: 287 families. 68.5% with pos sIgE	SPT	5q31, 8q12, 11q23, 12p13, 14q32, 20p13-p12.1
[130] 2004	France: 228 families	T-IgE/ SPT/	8p22, 12p13 / 5p15, 13q34, 17q22-24
[131] 2004	USA: 199 families 74% atopic children	[A]Atopy	3q21

(Table 4 continued)

[120] 2005	France: 295 families. 185 AR families.	AR	2q32, 3p24-p14, 9p22, 9q22-q34
[132] 2005	Germany: 201 families	T-IgE	1p36,1q24.3, 7p14.1, 7q11, 7q21, 11p15.5,11q25
		sIgE	2p21, 4q35, 5p13
		SPT	1q23, 3p14
[121] 2005	Sweden: 250 AR families	AR	3q13, 4q34-35, 6p22-24, 9p11-q12, 9q33.2-34.3, 17q11.2, 18q12
[133] 2005	Australian: 202 families. 73% atopic.	SPT	2q35, 3q12, 6p21, 17q21, 20q13
		T-IgE	1q24-q25, 10q22
[134] 2007	Denmark/USA/Europe: 250/ 76/364 families. 641/ 438 families has recorded SPT /total IgE	T-IgE	1q23.1, 5q33, 8q21, 10q22
		SPT	1q23, 3q22-q23, 5q31, 8q21

[A] Atopy involves positive SPT, positive specific IgE and / or elevated total IgE. Only positions with LOD-scores $\geq$ 1 and / or p-value $\leq$ 0.003 are shown [110].
The table is based on literature search using PubMed and the following search string ((Allergic rhinitis OR asthma OR atopy OR IgE OR Immunoglobulin E OR SPT OR skin prick test OR RAST) AND (genome OR linkage OR genome scan OR genome- wide OR scan OR LOD)) NOT (GWAS OR Candidate gene OR polymorphisms). Going back to 1996.

The phenotype atopy has been studied and reported 13 times, often in connection with genome wide linkage studies of asthma [122, 124-135].

The definition of atopy vary between studies. Some define atopy as a positive serum IgE (sIgE) and/or a positive skin prick test (SPT). Some include an elevated total IgE (T-IgE) in the definition of atopy. For the phenotype atopy 19 regions with possible linkage have been reported (Table 4). The regions most likely to harbour true susceptibility genes for atopy are probably the regions 2p22-p21, 6p21 and 12p13 and the regions 5q31 and 7q11-q22 as these regions have shown evidence of linkage in independent studies four and five times, respectively [119, 123-127, 129, 130, 132-134].

An important aspect of genetic studies is there replication by different investigators in different populations. As illustrated in table 4 this replication in later studies is only seldom achieved. Some initial findings will never be replicated. This may be explained by differences in study design and phenotype assessment as well as power limitations and variations in statistical methods [136].

Summary of candidate gene studies in allergic rhinitis and atopy

Candidate gene association studies have revealed several potential candidate genes for allergic rhinitis and atopy. Atopy is most often investigated in association with another phenotype, such as asthma. Table 5 shows 31 genes found to be associated with allergic rhinitis.

Table 5: Studies in which a significant association to allergic rhinitis has been described.

Genes	Year (reference)	Population	Phenotype	Chromosome[A]	Candidate genes
Chemokines and receptors	2004 [137]	Japanese	AR	4q21	CXCL9, XCL10, CXCL11
	2004 [138]	Korean	AR	17q11.2-q12	RANTES
	2005 [139]	Korean	AR	17q11.2	Eotaxin-3
	2007 [140]	Japanese	AR	3p21.3	CCR2, CCR3
Eosinophils	2003 [141]	Japanese	AR	17q23	EPO
	2004 [142]	Japanese	AR	---	---
	2009 [143]	Czech	AR	---	---
	2010 [144]	Korean	AR	14q11.2	ECP (RNase3)
Interleukins	2003 [145]	German	AR	11q22	IL18
and receptors	2003 [146]	Finnish	AR	2q14	IL1
	2004 [147]	German	AR	16p12.1-11.2	IL4RA
	2006 [25]	Korean and Chinese	AR	1p36.11	IL28RA
	2006 [148]	Korean	AR	5q31	IL13
	2006 [149]	Korean	AR	11q22	IL18
	2007 [150]	Czech	AR	---	---
	2008 [151]	Japanese	AR	9p24.1	IL33
	2009 [152]	13 countries	AR	SPT, sIgE	IL13
	2010 [153]	Dutch	AR	5q31	---
Toll-like	2008 [154]	Canadien	AR	9q32-q33	TLR4
receptor	2009 [155]	Korea	AR	4q32	TLR2
Monocyte diffe-	2001 [156]	Nederlands	AR	5q31.1	CD14
rentiating antigen	2010 [157]	Chinese	AR	---	---
Filaggrin	2006 [158]	European	AR+ec.	1q21	FLG
	2007 [159]	German	AR+ec.	---	---
	2008 [160]	Swedish	AR+ec.	---	---
	2008 [161]	German	AR	---	---
	2009 [162]	Nederlands	AR+ec.	---	---
	2010 [163]	Danish	AR	---	---

(Table 5 continued)

Other genes	2004 [164]	Japanese	AR	20p13	ADAM33
	2004 [165]	Korean	AR	5q33.2	TIM-3
	2006 [166]	Turkish	AR	5q31.1	C4
	2006 [167]	Korean	AR	17q22	FOXJ1
	2007 [168]	British	AR	10p15	GATA3
	2008 [169]	Turkish	AR	1q21	FcγRIIIa
	2009 [170]	Jap nese	AR	1q32	DAF
	2009 [171]	Swedish	AR	19q13.1	CLC

[A] positions stated in the articles or found at OMIM (http://www.ncbi.nlm.nih.gov/omim). Significant association: Nominal p value ≤ 0.05
The table is based on literature search using PubMed and the following search string ((Allergic rhinitis OR asthma OR atopy OR IgE OR Immunoglobulin E OR SPT OR skin prick test OR RAST) AND (Candidate gene OR association OR polymorphisms OR mutation)) NOT (Genome wide association study OR GWAS).
CXCL: chemokine, *RANTES*: 'Regulated upon Activation, Normally T-Expressed, and presumably Secreted, *CCR*: chemokine receptor, *EPO*: Eosinophil peroxidise. *ECP*: eosinophil cationic protein, IL: interleukin, *IL4RA*: IL 4 receptor alpha chain, *TLR*: Toll-like receptor, *CD14*: Cluster of differentiation 14, *FLG*: filaggrin, *ADAM*: A desintegrin and metalloproteinase domain, *TIM*: T-cell immunoglobulin domain and mucin domain, *LTC4S*: leukotriene C4 synthase, FOXJ1: Forkhead-box J1, *GATA*: GATA-binding protein, *FcγRIIIa*: Fcγ receptor group IIIa, *DAF*: Decay-accelerating factor, *CLC*: Charcot-Leyden crystal protein, ec.: eczema
Submitted as supplementary table to paper I.

As in linkage studies, replication by different investigators in different populations is desirable. However, the associations to AR have rarely been unambiguously replicated in a number of populations (Table 5). To date the best replicated AR genes are the Interleukin 18 (*IL18)* gene and the Eosinophil peroxidise *(EPO*) gene having been replicated in three independent populations each [141-143, 145, 149, 150]. The Filaggrin (*FLG*) genes association to AR has also been replicated in different studies and different populations but only in the context of AD [158-160, 162]. A possible reason for this low replication rate may be found in differences in study design including phenotype assessment as well as variations in statistical methods and power limitations [136]. Table 6 shows candidate genes associated with atopy.

Table 6: Studies in which a significant association to atopy has been described.

Genes	Year reference	Population	Phenotype	Chromosome[B]	Candidate genes
Chemokines	2003 [172]	Korean	T-IgE	17q21.1–q21	Eotaxin1
and receptors	2005 [173]	Grampian	SPT	17q11.2-q12	RANTES
	2005 [174]	British	sIgE	---	---
Interleukins	2002 [175]	Finnish	SPT	2q14	IL1A
and receptors	2002 [176]	British	T-IgE	16p12.1-11.2	IL4RA
	2003 [141]	Japanese	sIgE	---	---
	2003 [177]	Danish	SPT / sIgE	5q31	IL13
	2003 [178]	German	T-IgE	---	---
	2003 [179]	Chinese	T-IgE	---	---
	2003 [145]	German	sIgE	11q22	IL18
	2003 [180]	Finnish	SPT	2q14	IL1A, IL1B, IL1RN
	2004 [181]	German	sIgE	7p21, 4q26-q27, 6p12- p11, 5q31, 11q22	IL6, IL2 IL4R, IL13, IL18
	2004 [147]	German	T-IgE	16p12.1-11.2	IL4RA
	2004 [182]	Finnish	SPT	2q14, 16p12.1-11.2	IL1A, IL4RA
	2006 [183]	Finnish	SPT	2q14	IL1A
	2008 [184]	Dutch	T-IgE / sIgE	5q31	IL13
	2008 [185]	Dutch	SPT, T-IgE	2q12	IL18R1, IL18RAP
	2009 [152]	13 countries	SPT, T-IgE	5q31	IL13
			SPT, sIgE	6p12- p11	IL4R
	2009 [186]	British	atopy[A]	5q31	IL13
	2010 [153]	Dutch	sIgE	---	---
	2010 [187]	Swiss	SPT, T-IgE	5q31.1	IL4
Monocyte diffe-	2001 [156]	Nederland	sIgE, T-IgE	5q31.1	CD14
rentiating antigen	2003 [188]	Chinese	T-IgE	---	---
	2004 [189]	Australian	SPT	---	---
	2005 [190]	German	sIgE, T-IgE	---	---
	2005 [191]	Taiwanese	T-IgE	---	---
	2007 [192]	Danish	SPT	---	---
	2008 [184]	Dutch	T-IgE / sIgE	---	---
Filaggrin	2006 [158]	German	sIgE+ec.	1q21	FLG
	2006 [193]	German	sIgE, T-IgE	---	---
	2007 [159]	German	T-IgE	---	---
	2008 [160]	Swedish	sIgE	---	---
	2008 [161]	German	SPT	---	---

(Table 6 continued)

IgE-receptor	1993 [194]	British	atopy[A]	11q13	FCεR-β
	2009 [195]	Korean	SPT	11q13	FCεR-β
	2010 [196]	Japanese	T-IgE	1q24	FCεR1α
Toll-like receptor	2004 [197]	British	sIgE / SPT	9q32-q33	TLR4
	2008 [198]	Danish	sIgE	Xp22	TLR7, TLR8
	2008 [154]	Canadian	SPT	9q32-q33	TLR4
	2009 [152]	13 countries	SPT, sIgE	---	---
Major histocom-	2007 [199]	Norwegian	sIgE	6q21.3	HLA class II
patibility complex	2008 [200]	Iranian	T-IgE	6p21.3,	---
Chitinases	2009 [201]	Danish	sIgE	1q32.1	CHI3L1
enzymes	2009 [202]	Korean	SPT	---	---
Cyclooxygenase	2005 [203]	Australian	SPT	9q32	COX1 (PTGS1)
	2007 [204]	China	sIgE	1q25.2 -q25.3	COX2 (PTGS2)
Cysteinyl	2003 [205]	Tristan da Cunha	SPT	13q14	CysLTR2
leukotriene	2006 [206]	British	sIgE	Xq13-q21	CYSLTR1
and receptors	2009 [207]	British	atopy[A]	---	---
Other genes	2002 [208]	Dutch	T-IgE	2q33	CTLA4
	2002 [209]	British	SPT	Chr.11	SART-1
	2003 [210]	Australian	T-IgE	13q14	PHF11
	2003 [211]	Japanese	sIgE	6q23-q24	IFNGR1
	2004 [212]	Canadian	SPT	12q13–23	VDR
	2004 [213]	German	T-IgE	12q13	STAT6
	2004 [214]	European	T-IgE	2q33	CTLA4
	2004 [215]	Czech	T-IgE	12q14–24.2	NOS1
	2005 [216]	Polish	T-IgE	5q32-q34	β2-ADR
	2005 [217]	Finnish	atopy[A]	10p15	GATA3
	2005 [218]	American	SPT	5q33	TIM1, TIM3, ITK
	2006 [219]	Chinese	T-IgE	8p23.1	DEFB1
	2006 [220]	Japanese	T-IgE	4q21-q25	SPP1
	2006 [221]	Hutterites	sIgE	9p21	IFN
	2007 [222]	Danish	sIgE	3q21	CD86
	2007 [223]	Canadian	SPT	10q24	PLAU
	2007 [224]	Indian	T-IgE	1q21	LELP1
	2008 [225]	German	T-IgE, sIgE	5q31.1	IRF-1

(Table 6 continued)

2008 [226]	British	atopy[A]	13q12, 12q22	ALOX5AP, LTA4H
2009 [143]	Czech	T-IgE	17q23	EPO
2009 [170]	Japanese	atopy[A]	1q32	DAF
2009 [227]	Chinese	sIgE/ T-IgE	17q21.1	ORMDL3
2010 [228]	Dutch	sIgE (girls)	Xp11.23	FOXP3

[A] Atopy involves pos. skin prick test (SPT), pos. serum IgE (sIgE) and / or elevated total IgE (T-IgE)
[B] positions state in the articles or found at OMIM (http://www.ncbi.nlm.nih.gov/omim).
Significant association: Nominal p value ≤ 0.05

The table is based on literature search using PubMed and the following search string ((atopy OR IgE OR Immunoglobulin E OR SPT OR skin prick test OR RAST) AND (Candidate gene OR association OR polymorphisms OR mutation)) NOT (Genome wide association study OR GWAS).

IL: interleukin, IL1RN: IL 1 receptor antagonist, IL13RA1: IL-13 receptor α 1 subunit, IL18RAP: IL 18 receptor accessory protein. IL18R1: IL18 receptor, CD14: Cluster of differentiation 14, FCεR-β: β-subunit for high-affinity receptor for IgE, FCεR1α: α-subunit for high-affinity receptor for IgE CHI3L1: chitinase 3-like 1 gene, PTGS: Prostaglandin-endoperoxide synthase, COX: Cyclooxygenase, CYSLTR: Cysteinyl leukotriene receptor, CTLA: Cytotoxic T lymphocyte-associated , SART-1: Squamous cell carcinoma antigen recognized by T cells 1, PHF: PHD finger protein, IFNGR: Interferon gamma receptor, VDR: vitamin D receptor STAT: Signal transducer and activator of transcription, NOS: nitric oxide synthase, ITK: IL-2–inducible T-cell kinase, TIM: T-cell immunoglobulin domain and mucin domain DEFB: b-defensin, SPP1: Secreted phosphoprotein 1, IFN: type I interferon, PLAU: urokinase-type plasminogen activator, LELP: late cornified envelope-like proline-rich, IRF: Interferon regulatory factor, ALOX5AP: Arachidonate-5-lipoxygenase-activating protein, LTA4H: Leukotrine A4 hydrolase, DAF: Decay-accelerating factor, ORMDL3: ORM1-like Protein 3, FOXP3: Forkhead Box P3, ec: eczema.

Summary of candidate gene studies on filaggrin mutations and atopic diseases

Genome-wide screens have shown significant linkage of a variety of disorders to chromosome 1q21. Among these are atopic dermatitis, psoriasis, and ichthyosis vulgaris, along with a number of autoimmune diseases [229]. The filaggrin gene, FLG, is located at chromosome 1q21. The exploration of FLG in connection with the atopic diseases has been met with great interest. The association of atopic dermatitis (AD) with FLG, and the association between FLG and atopy are well established (table 7). Establishing whether there are associations between FLG and allergic rhinitis (AR) and asthma is more difficult. When looking at the candidate gene studies performed on FLG and atopic diseases (table 7), it seems that the phenotypes AR and asthma are only significantly associated with FLG when in context with AD.

Table 7: Candidate gene studies on Filaggrin mutations and atopic diseases.

Phenotype	Year reference	Population	Investigated mutations	Sample size (cases/controls, families/offspring, or cases only)	Significant association yes/no	Ref
AD	Palmer et al. 2006	Irish Danish	A	cc 52/189 cc 142/190	y	[230]
	Weidinger et al. 2006	German	A	fam 476/476	y	[193]
	Marenholz et al. 2006	European German	A	fam 490/903 cc 180/314	y	[158]
	Ruether et al. 2006	German	R501X A	fam 338/338 cc 272/276	y	[231]
	Weidinger et al. 2007	German	A	cc 274/252	y	[159]
	Morar et al. 2007	European	A	fam 657/990	y	[232]
	Stemmler et al. 2007	German	A	cc 378/433	y	[233]
	Sandilands et al.2007	Irish	F	cc 188/736	y	[234]
	Nomura et al. 2007	Japanese	B	cc 143/156	y	[235]
	Rogers at al. 2007	American	A	Fam 460/646	y	[236]
	Hubiche et al. 2007	French	A	cc 99/102	y	[237]
	Nomura et al. 2007	Japanese	D	cc 102/133	y	[238]

(Table 7 continued)

	Howell et al. 2007	American	A	cc 30/39	y	[239]
	Lerbaek et al. 2007	Denmark	A	cc 183/189	n	[240]
	Giardina et al. 2008	Italian	A	cc 178/210	n	[241]
	Ekelund et al. 2008	Swedish	A	fam 406	y	[160]
	Weidinger et al. 2008	German	F	cc 540/2454	y	[161]
	Brown et al. 2008	British	G	cc 186/1035	y	[242]
	Brown et al. 2008	British	F	cc 195/599	y	[243]
	Enomoto et al. 2008	Japanese	B	cc 376/923 fam 105	y	[244]
	Nomura et al. 2008	Japanese	D	cc 102/133	y	[238]
	Henderson et al. 2008	British	A	cc 1445/3810	y	[245]
	Schuttelaar et al. 2009	Nederland	C	cc 934/663	y	[162]
	O´Regan et al. 2009	Irish	E	cc 511/1000	y	[246]
	Bisgaard et al. 2009	Danish British	A	cases 379 cases 503	y	[247]
	Brown et al. 2009	British	G	cases 792	y	[248]
	Greisenegger et al. 2009	Australian German	E	cc 274/402 cc188/402	y	[249]
	Müller et al. 2009	ETAC	A	fam 244/496	y	[250]
	Nemoto-Hasebe et al. 2009	Japanese	p.Lys421X	cc 137/134	y	[251]
	Aslam et al. 2010	European	A	cc 32/30	y	[252]
	Flohr et al. 2010	British	E	cases 88	y	[253]
AD without atopy	Weidinger et al. 2006	German	A	fam 79/79	n	[193]
	Marenholz et al. 2006	German European	A	cc 63/314 fam 490/903	y	[158]
AD with atopy	Weidinger et al. 2006	German	A	fam 397/397	y	[193]
	Weidinger et al. 2007	German	A	cc 196/252	y	[159]
	Morar et al. 2007	European	A	not available	y	[232]
	Marenholz et al. 2006	German European	A	Cc 117/314 fam 490/903	y	[158]
	Ekelund et al. 2008	Swedish	A	fam 406	y	[160]
	Weidinger et al. 2008	German	E	cc 540/2454	y	[161]
	Henderson et al. 2008	British	A	cc 193/4571	y	[245]
Increased AD severity	Weidinger et al. 2007	German	2282del4	cc 274/252	y	[159]
	Ekelund et al. 2008	Swedish	A	fam 406	y	[160]
	Brown et al. 2009	British	G	cases 792	y	[248]
	Ching et al. 2009	Chinese	R501X	cc 174/191	n	[254]
	Flohr et al. 2010	British	E	cases 88	y	[253]
Early AD onset	Weidinger et al. 2006	German	A	Fam 285/285	n	[193]
	Barker et al. 2007	British	A	cc 163/1463	y	[255]
	Weidinger et al. 2007	German	A	cc 111/252	y	[159]

(Table 7 continued)

	Stemmler et al. 2007	German	A	cc 210/433	y	[233]
	Brown et al. 2008	British	G	cc 186/1035	y	[242]
	Enomoto et al. 2008	Japanese	B	cc 376/923 fam 105	y	[244]
	Bisgaard et al. 2009	Danish	A	cases 379	y	[247]
	Greisenegger et al. 2009	Australian German	E	cc 274/402 cc188/402	y	[249]
	Flohr et al. 2010	British	E	cases 88	y	[253]
Persistent AD	Brown et al. 2008	British	G	cc 186/1035	y	[242]
Hyperlinear palms, AD	Weidinger et al. 2006	German	A	113 case	y	[193]
	Brown et al. 2008	British	E	cc 195/599	y	[243]
	Brown et al. 2009	British	G	cases 792	y	[248]
Flexura eczema, AD	De Jongh et al. 2008	German	A	cc 296/no info.	y	[256]
	Brown et al. 2009	British	G	cases 792	y	[248]
Increased T-IgE in AD	Weidinger et al. 2006	German	A	fam 476	y	[193]
	Weidinger et al. 2007	German	A	cc 196/252	y	[159]
AR	Chawes et al. 2010	Danish	A	cc 38/185 (33% with AD)	y	[163]
AR with AD	Marenholz et al. 2006	German European	A	cc 44/314 fam 248	y	[158]
	Weidinger et al. 2007	German	A	cc 172/252	y	[159]
	Ekelund et al. 2008	Swedish	A	fam 406	y	[160]
	Weidinger et al. 2008	German	F	cc 124/2782	y	[161]
	Schuttelaar et al. 2009	Nederland	C	cc 934/663	y	[162]
AR without AD	Weidinger et al. 2008	German	F	cc 124/2782	y	[161]
	Brown et al. 2008	British	F	cc 152/599	n	[243]
	Henderson et al. 2008	British	A	cc 779/3849	n	[245]
	Aslam et al. 2010	European	A	cc 26/25	n	[252]
AR without atopy	Chawes et al. 2010	Danish	A	cc 66/185	n	[163]
Asthma with AD	Palmer et al. 2006	Scottish	A	cc 604/1008	y	[230]
	Weidinger et al. 2006	German	A	fam 476/146	y	[193]
	Marenholz et al. 2006	German European	A	cc 40/314 fam 220	y	[158]
	Weidinger et al. 2007	German	A	cc 95/252	y	[159]
	Rogers at al. 2007	American	A	Fam 460/646	y	[236]
	Ekelund et al. 2008	Swedish	A	fam 406	y	[160]
	Weidinger et al. 2008	German	F	cc 272 /2782	y	[161]
	Brown et al. 2008	British	F	cc 83/599	y	[243]
	Henderson et al. 2008	British	A	cc 447/4808	y	[245]
	Schuttelaar et al. 2009	Nederland	C	cc 934/663	y	[162]
	Müller et al. 2009	ETAC	A	fam 244/496	y	[250]
	Osawa et al. 2010	Japanese	H	cc 172/134 cases 137	y	[257]

(Table 7 continued)

Asthma without AD	Palmer et al. 2006	Scottish	A	cc 604/1008	n	[230]
	Palmer et al.2007	Scottish	A	Cases 405	n	[258]
	Rogers at al. 2007	American	A	Fam 460/646	n	[236]
	Weidinger et al. 2008	German	F	cc 272/2782	n	[161]
	Brown et al. 2008	British	F	cc 167/599	n	[243]
	Henderson et al. 2008	British	A	cc 447/4808	n	[245]
	Schuttelaar et al. 2009	Nederland	C	cc 934/663	n	[162]
	Aslam et al. 2010	European	A	cc 26/28	n	[252]
	Osawa et al. 2010	Japanese	H	cc 172/134 cases 137	n	[257]
Asthma severity	Palmer et al.2007	Scottish	A	cases 874	y	[258]
Asthma exacerbations	Basu et al.2008	Scottish	A	cases 1135	y	[259]
Atopy	Weidinger et al. 2006	German	A	fam 278/352	y	[193]
	Weidinger et al. 2007	German	A	cc 196/252	y	[159]
	Weidinger et al. 2008	German	F	cc 777/2225	y	[161]
	Ekelund et al. 2008	Swedish	A	fam 406	y	[160]
	Enomoto et al. 2008	Japanese	B	cc 376/923 fam 105	y	[244]
	De Jongh et al. 2008	German	A	cc 296/no info.	y	[256]
	Schuttelaar et al. 2009	Nederland	C	cc 934/663	y	[162]
	Greisenegger et al. 2009	Australian German	E	cc 274/402 cc188/402	y	[249]

Modified from Rodriguez et al. (2008) [260]and extended up to date (2010). Fam: family study. Cc: case-control study. ETAC: Treatment of the Atopic Child trial, Caucasian. Atopy: involves pos. skin prick test (SPT), pos. serum IgE (sIgE) and / or elevated total IgE (T-IgE). *A mutations:* R501X, 2282del4. *B mutations*: S2554X, 3321delA. *C mutations*: R501X, 2282del4, R2447X. *D mutations*: S2554X, S2889X, S3269X, 3321delA. *E mutations*: R501X, 2282del4, R2447X, S3247X. *F mutations:* R501X, 2282del4, R2447X, S3247X, 3702delG. *G mutations:* R501X, 2282del4, R2447X, S3247X, 3702delG, 3673delC. H mutations: R501X, 3321delA, S1695S, Q1701X, S2554X, S2889X, S3296X, K4022X

Summary of GWA studies of allergic rhinitis and atopy

Genome-wide association studies (GWAS) are growing in popularity. The characterisation of millions of single nucleotide repeat polymorphisms (SNPs) in the human genome and the availability of these in public databases, the advances in SNP genotyping technology, and the development of statistical software and analysis methods have made this new field of research possible. To date, hundreds of new loci have been found in the field of complex disorders. However, regarding atopic disorders, the number of published GWA studies is limited.

Figure 1

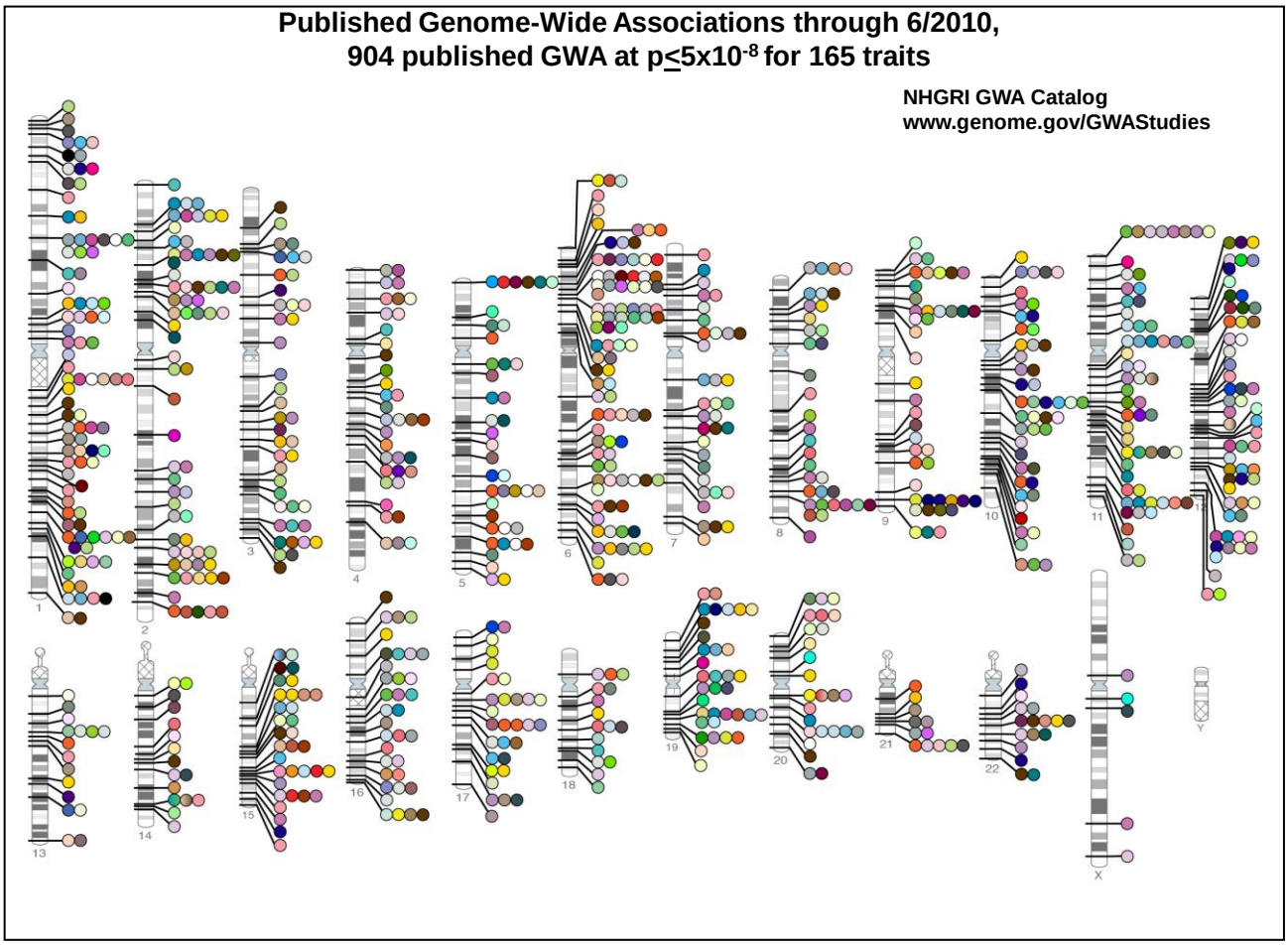


Figure 1 continued

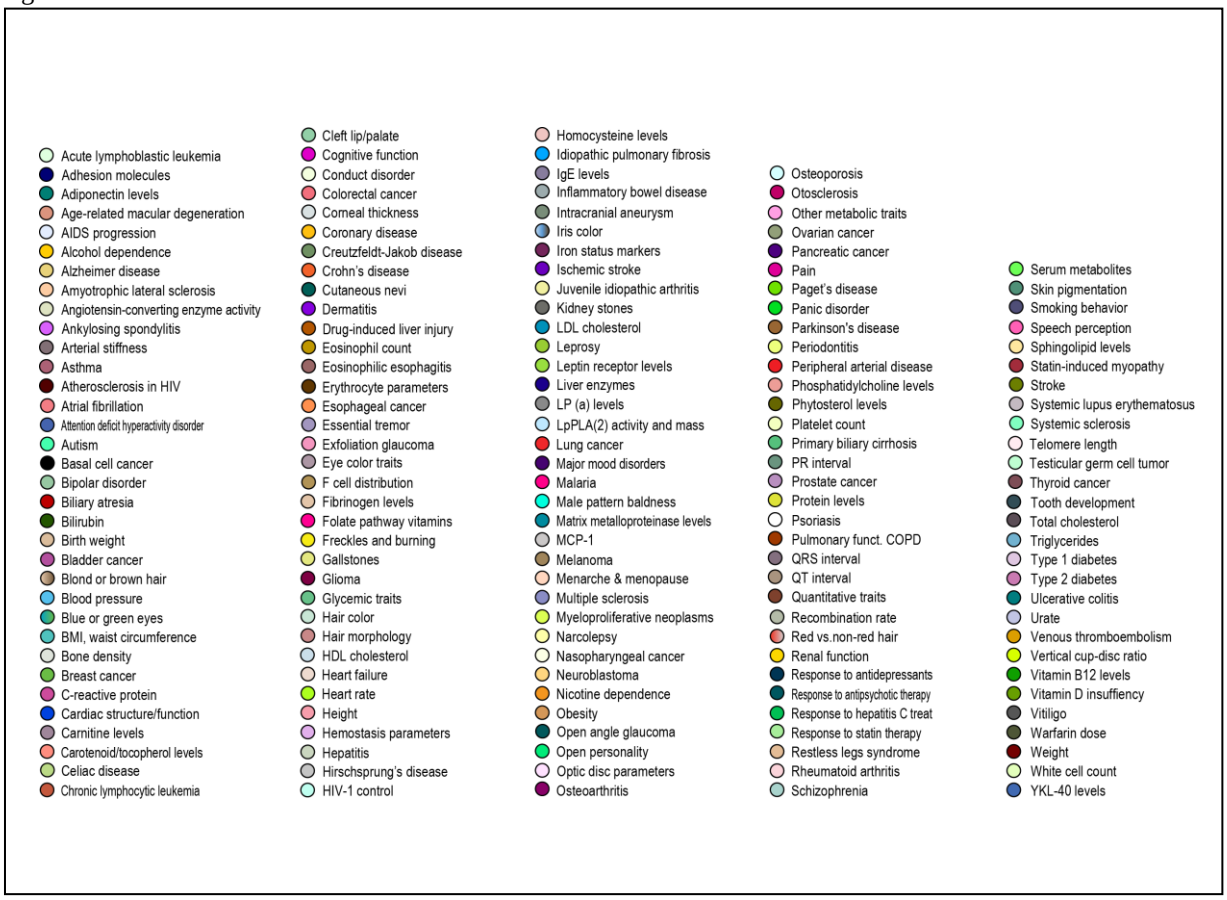


Figure illustrating the investigated trait (color coded) and chromosomal position of findings in published GWA studies of 2010 (http://www.genome.gov/GWAStudies/.)

When conducting a search in the Catalog of Published Genome-Wide Association Studies under National Human Genome Research Institute (http://www.genome.gov/GWAStudies/#1), there were 12 GWA studies concerning asthma (of which only 8 reached the genome wide significance level of $P \leq 5x10^{-8}$) reporting 20 different genes [261-272], one study concerning atopic dermatitis reporting two genes [273], and one study investigating genes behind specific serum IgE levels reporting two genes [274]. Two GWA studies of atopy have been recently published [275, 276]. The 2010 study by Wan et al. revealed no genome wide significant association. Castro-Giner et al. found a significant association of atopy to the Homo sapiens protein kinase-like protein (*SGK493*) located at 2p21 [276]. So far, GWA studies concerning allergic rhinitis or rhinitis in general have not been

published. GWAS has till now only described a small fraction of the heritability behind the atopic disorders. This may be due to the fact that GWAS have limited power in capturing low frequency variants (i.e., variants with allele frequencies of less than 5%)[105]. Findings from conducted Linkage scans may help improving the success of GWAS by pointing to possible susceptibility regions worthy of greater attention in the GWA study.

The aim of the present study

The aim of this thesis was to identify the chromosomal regions (loci) that likely harbour the genes influencing the development of AR and related allergic disorders and to investigate a possible association of the filaggrin gene (*FLG)* with AR. This study was conducted through collection and linkage scanning of a novel AR family sample and association analysis of four *FLG* single nucleotide polymorphisms (SNPs) previously associated with atopic dermatitis.

The rhinitis sample

Recruitment of families

We collected a sample of 127 Danish nuclear families with at least two siblings with AR reaching a total of 286 children and 254 parents. The recruitment of these families was performed through the paediatric departments in Viborg, Aarhus, Hjoerring, Herning and Aalborg as well as the Department of Respiratory Diseases, Aarhus University Hospital and one private paediatric clinic in Aalborg.

Initially, the journals of patients diagnosed with AR at the listed hospital departments and clinics were studied, and the patients with siblings were then contacted by letter. The letter informed the patients about the study and invited the patient and his/her family to return a reply sheet if they had at least two children with AR and wanted to join the project. Approximately 600 letters were sent to possible project families resulting in contact with 132 possible project families of which 127 met the selection criteria.

Questionnaire

All members of the project families filled out a standardised questionnaire recording the symptoms, duration and treatment of rhino-conjunctivitis, AD and asthma as well as information of known or suspected allergens behind the atopic diseases.

Clinical examination

All families were interviewed and the siblings clinically examined by the same doctor (Lisbeth V. Kruse). The interview was based on the questionnaires, looking for possible misunderstandings or skipped questions. Atopic disease was diagnosed according to standard criteria [19]. The diagnoses were evaluated by a second doctor (Annette Haagerup) who assessed the questionnaires without seeing the individuals. A few patients were hereafter excluded. The families were grouped according to phenotypes being present in at least two and as many as four of the siblings (Table 8).

Table 8: Phenotypic features in the rhinitis sample

	Sib-pair families	Parents	Offspring all /with the phenotype [A]
Total	127	254	286
Hay fever combined (HC)	124	248	279 / 267
IgE associated HC[B]	100	200	223 / 209
Allergic rhinitis (AR)	119	238	272 / 258
IgE associated AR[B]	96	200	213 / 200
Rhinoconjunktivitis (RC)	105	210	232 / 224
AR and asthma	35	70	81 / 73
AD	33	66	84 / 76
IgE associated AD[B]	25	50	67 / 57
Asthma	38	80	268 / 258
IgE-Asthma[B]	34	68	79 / 73
Pos. serum IgE	103	204	235 / 219
Total IgE>100/150 KU/l	53	106	142 / 118

[A] All the children in the families / Only the children with the present phenotype in the families.
[B] The present phenotype and a positive specific IgE antibody for at least one of the tested allergens.

The children with allergic rhinitis were further subdivided according to the ARIA guidelines (Table 2, page 27) and were grouped into persistent allergic rhinitis (PAR), intermittent allergic rhinitis (IAR) and children with mild and moderate-severe symptoms (Table 9). The mean age among the children was 16 years, and 52% were male.

Table 9: ARIA subdivisions of children in the rhinitis sample [1].

	Duration	**Severity**		**Total**
		Mild	**Moderate-severe**	
Children with AR	PAR	42	39	81 (31%)
	IAR	26	156	182 (69%)
Children with IgE associated AR*	PAR	37	30	67 (29%)
	IAR	21	143	164 (71%)

* AR *and* a positive specific IgE antibody for at least one of the tested allergens.

Blood was collected from all 540 participants for DNA analysis and for serum measurements of total IgE and allergen-specific IgE antibody (ImmunoCAP system. Phadia Aps, Allerød, Denmark). All families were Caucasian and all were of Danish origin except one father from Greece and one family from Poland; therefore, the sample was of high genetic homogeneity and well suited for genetic studies.

PAPER I:

A genome-wide search for linkage to allergic rhinitis in Danish sib-pair families.

Kruse L.V [1,2], Christensen U [1,3], Haagerup A [1,3], Deleuran M [4], Hansen L.G [2], Møller-Larsen S [1], Venø S.K [1], Goossens D [5,6], Del-Favero J [5,6], Nyegaard M [1,7], Børglum A.D [1]

1 Department of Human Genetics, Aarhus Faculty of Health Sciences, Aarhus University, Aarhus, Denmark

2 Department of Paediatrics, Region Hospital Viborg, Denmark

3 Department of Paediatrics, Aarhus University Hospital, Skejby, Denmark

4 Department of Dermatology, Aarhus University Hospital, Aarhus, Denmark

5 Applied Molecular Genomics Group, Department of Molecular Genetics, VIB, Belgium

6 University of Antwerp (UA), Antwerp, Belgium

7 Department of Haematology, Aalborg Hospital, Aalborg Denmark

Abstract

Allergic rhinitis is a complex disorder with a polygenic, multifactorial etiology. Twin studies have found the genetic contribution to be substantial. However, the focus on genetic research of allergic rhinitis is sparse and only four genome-wide linkage scans have been reported concerning allergic rhinitis. Only one of these specifically collected for the rhinitis phenotype. We collected and clinically characterised a sample consisting of 127 Danish nuclear families with at least two siblings suffering from allergic rhinitis or allergic conjunctivitis including 540 individuals (286 children and 254 parents). A whole genome linkage scan using 429 microsatellite markers was performed on both this sample and an earlier collected sample consisting of 130 with atopic dermatitis and other atopic disorders. A third sib-pair family sample, which was previously collected and genotyped, was added to the analysis increasing the total sample size to 357 families consisting of 1,508 individuals. A total of 190 families (812 individuals) segregated allergic rhinitis. The linkage

analysis software Genehunter NPL, Genehunter MOD, and Genehunter Imprinting were used to obtain non-parametric and parametric linkage results.

We obtained genome wide suggestive evidence for three loci earlier reported (2q14-q23, 2q23 and 12p13) and three novel susceptibility regions (1p13, 1q31-q32 and 20p12) as well as indication of linkage to loci at 1q21, 4q11-q12, 14q11-q12, 20p13,22q12-q13, Xp11 and Xp22-p21. High MOD scores were obtained at 1p13 and 20p12 (MOD without / with imprinting = 4.20 / 4.30 and 3.66 / 4.25, respectively), approaching genome wide significance. Likely maternal imprinting was observed at 2q23, and possible maternal imprinting at 3q28.

Introduction

Allergic rhinitis (AR) is a growing problem worldwide. Today 10% to 30% of adults and up to 40% of children suffer from this condition [8, 19, 28, 50]. However, the rise in prevalence appears to have reached a plateau in many western countries since the 1990s [12, 13, 26]. The typical symptoms are drip, block, and itch of the nose along with sneezing due to inflammation of the nasal mucosa often accompanied by allergic conjunctivitis (itch and redness of the eye) [19]. Allergic rhinitis is often mistakenly considered a disease of little importance. It is however a frequent cause of morbidity, medical treatment costs, lost work days, and reduced school performance [19, 277]. It often significantly affects a patient's quality of life and it is at times accompanied by symptoms such as headache, weakness, malaise and fatigue [19]. Mood changes, depression, and anxiety are also described in relation to AR [16].

Allergic diseases are complex multi-factorial and polygenic disorders. This combination of environmental risk factors and multiple susceptibility genes complicates the elucidation of the underlying genetic architecture. However, the genetic contribution to AR has been shown to be substantial. Twin studies have shown that the estimated heritability for AR is approximately 71% -

96% [39-41]. Considerable research has focused on the genetic basis of asthma and to some extend atopic dermatitis while AR has been somewhat neglected. Only four whole genome scans of the phenotype AR have been reported [118-121] and only three regional linkage analyses of parts of the genome have been reported [135, 278, 279]. More research has been performed in the field of candidate gene association analysis and more than 30 genes showing association with AR have been reported (Table 5, page 44). However, the associations to AR have rarely been unambiguously replicated in a number of populations (Table 5, page 44). To date the best replicated AR genes are the gene of interleukin 18 and the Eosinophil peroxidise gene having been replicated in three independent populations each [141-143, 145, 149, 150]. The Filaggrin (*FLG*) genes association to AR has been replicated in four different studies and different populations but only in the context of AD [158-160, 162]. Currently, no genome wide association studies concerning AR have been published (http://www.genome.gov/GWAStudies /#1). In this study, we performed a whole genome linkage scan of two samples: One sample containing 127 Danish nuclear families, each with at least two siblings suffering from AR or allergic conjunctivitis, and a second sample consisting of 130 Danish nuclear families with atopic dermatitis and other atopic disorders[280]. The two samples were combined with a third sib pair atopy family sample previously collected and genotyped [127] reaching a total of 357 families consisting of 1,508 individuals of whom 190 families (812 individuals) segregated AR.

Method

Subjects

Three family samples (A, B and C) were included in the study. Sample A consisted of 127 Danish nuclear families with a total of 286 children and 254 parents. The recruitment of these families was performed through the paediatric departments in Viborg, Aarhus, Hjoerring, Herning and Aalborg,

as well as the Department of Respiratory Diseases, Aarhus University Hospital and one private paediatric clinic in Aalborg. All individuals were clinically examined and tested through a questionnaire by the same doctor. The questionnaire contained questions about symptoms, duration and treatment of rhino-conjunctivitis, atopic dermatitis and asthma as well as questions about known or suspected allergens behind the atopic diseases. Atopic disease was diagnosed according to standard criteria [19]. The diagnoses were evaluated by a second doctor who assessed the questionnaires without seeing the individuals.

The families were grouped according to phenotypes being present in at least two and as many as four of the siblings (Table I.1). The children with allergic rhinitis were further subdivided according to the ARIA guidelines and were grouped into persistent allergic rhinitis (PAR), intermittent allergic rhinitis (IAR) and children with mild and moderate-severe symptoms (Table I.1). In IAR, the patient has symptoms for less than 4 days or less than 4 weeks a year. PAR patients have symptoms for more than 4 days a week and more than 4 weeks a year. The differentiation of the severity of AR as “mild” or “moderate-severe” disease is based on the absence of all, or the presence of at least one, of the following 4 symptom categories: (i) Sleep disturbance (ii) Impairment of daily activities, leisure and/or sports (iii) Impairment of school or work and (iiii) troublesome symptoms [1].

The mean age among the children was 16 years, and 52% were male. Blood was collected from all participants for DNA analysis and for serum measurements of total IgE and allergen-specific IgE antibody (ImmunoCAP system. Phadia Aps, Allerød, Denmark).

Samples B and C were comprised of two earlier collected Danish sibling-pair family samples, and they were included in the genotyping and/or linkage analyses. Sample B [280] contained 130 Danish nuclear families with at least two full siblings with AD. The mean age among the children was 12.5 years, and 48% were male. Sample C [127] contained 100 Danish nuclear families

enrolled in the Danish Allergy Project. Inclusion criteria were at least two full siblings with a doctor diagnosed atopy such as asthma, rhinitis or atopic dermatitis. The mean age among the children was 14.5 years, and 49% were male. Combined samples B and C accounted for a total of 460 parents and 519 children (Table I.1). Thus, a total of 425 children with AR and/ or allergic conjunctivitis (AC) and 394 parents from 197 Danish nuclear families were analysed in this study. Sample A is a novel sample, and sample B has not been reported previously in respect to allergic rhinitis. However, Sample C has been reported previously [118, 278].

The Danish scientific ethical system approved the protocol. Furthermore, all participants and/or their parents signed informed consent forms.

Phenotype definition

The AR phenotype was studied using three approaches. The first two are broad definitions based purely on a clinical symptom-based diagnosis. The first of these approaches is named "Hay fever combined" (HC), and this group contains subjects who present clinical symptoms of AR and/or AC; the second broad group contains subjects who have clinical symptoms of AR. The third group contains a more defined phenotype (IgE associated AR), where the subjects have AR in combination with a positive specific IgE (defined as a positive specific IgE antibody for at least one of the tested allergens). The defined phenotype of this third group may decrease the genetic heterogeneity and thereby increase the statistical power of the study. The allergen-specific serum IgEs were tested for reactivity with twelve allergens. These allergens were mite (*D. pternyssinus* and *D. farinae*), cat, horse, dog, timothy grass, birch, mugwort and mould (*Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum*). A Specific IgE $\geq$ 0.35 kU/l was considered elevated.

Genotyping

Samples A and B were genotyped at the University of Antwerp, Belgium, using 429 microsatellite markers (415 autosomal and 14 X-linked markers) covering the whole genome. This approach resulted in approximately 465,000 genotypes. The mean intermarker distance was 8.26 centiMorgan (cM), and the range was 0 - 17.6 cM. The markers showed an average heterozygosity of 73%. Sample C was previously genotyped at the Department of Human Genetics, Aarhus, Denmark, using 446 microsatellite markers (432 autosomal and 14 X-linked markers) [280]. Furthermore, sample B was previously genotyped in three candidate regions at the Department of Human Genetics and used a total of 91 markers located on chromosome 3, 4, and 18. In both cases an ABI-310 sequencer from Applied Biosystems was used. These genotypes were also included for analysis to increase the statistical power. All the genotypes were uploaded to the database system BCSNP (BCPlatforms, Finland). Mendelian inconsistencies in the pedigree set were identified and removed using the computer programs Merlin [281] and PedCheck [282]. When inconsistencies were found, the genotypes from the problematic marker were removed for the entire nuclear family.

Statistical analyses

Linkage analyses were performed by the Affected Sib-Pair Method [107]. Multipoint non-parametric linkage analyses, which require no specification of the underlying genetic model for the trait being investigated, were performed on the autosomal chromosomes using the software program Genehunter NPL v. 2.0. Non-parametric analysis of the X chromosome was performed using Genehunter-Imprinting v. 1.3 [113]. On chromosomes where a NPL-score $\geq$ 1,5 was obtained parametric analysis was performed using Genehunter MOD and Genehunter-Imprinting [114]. In both types of analyses, the settings "Modcal: global" and "MOD-score routine: standard" were used. Genehunter MOD maximises the LOD score with respect to different disease-model

parameters. Using Genehunter Imprinting, the "parent-of-origin effect" (imprinting effect) is taken into consideration with different penetrances for heterozygous individuals, depending on whether the disease causing allele is transmitted by the father or the mother. A Genehunter Imprinting MOD-score may give two different heterozygote penetrances. For this score to indicate true imprinting, the following three rules must be considered: (1) A large difference between P(wt/d) and P(d/wt) gives a greater chance of true imprinting. A difference above 0.2 is recommended [114]. (2) The difference between imprinting and non-imprinting MOD scores must be high and preferably exceed 1.5 [283]. (3) The imprinting MOD must be high enough to at least indicate evidence of linkage in the region. When it comes to significance levels, a MOD score shall be "deflated" with approximately 0.3 to one units to be comparable to LOD scores due to the multiple testing of the maximisation process [111, 112].

To evaluate the genome-wide level of significance in our samples, the empiric P values were calculated for the parametric MOD scores using the simulation features incorporated in Genehunter MOD and Genehunter-Imprinting [113, 114]. A total of 10000 simulations were performed for each chromosome/region. Based on the regional empiric p value and the size of the region tested a genome wide empiric p value was calculated indicating the likelihood of obtaining scores that were as high as or higher than the observed (under the assumption of no linkage) in a whole genome scan. A region is suggestive of linkage when the observed result is found to occur by chance less than once in a whole genome scan representing a nominal p value of $7x10^{-4}$. If the observed result is found to occur by chance less 0.05 times in a genome scan, representing a nominal p value of $2x10^{-5}$, the region is significant of linkage [109]. MOD score calculations on the X chromosome was not possible using Genehunter MOD or Genehunter Imprinting.

Table I.1: Phenotypic features in sample A and sample B+C and subdivision of sample A according to ARIA guidelines [1].

Descriptive Statistics

Panel A: Phenotypic features in sample A and sample B+C

Sample	Phenotype	Sib pair families	Parents	Offspring all /with the phenotype [A]
A				
	Total	127	254	286
	Hay fever combined (HC)	124	248	279 / 267
	Allergic rhinitis (AR)	119	238	272 / 258
	IgE associated AR[B]	100	200	213 / 200
B+C				
	Total	230	460	519
	Hay fever combined (HC)	73	146	169 / 158
	Allergic rhinitis (AR)	71	142	167 / 156
	IgE associated AR[B]	65	130	151 / 141

Panel B: Children subdivided according to ARIA guidelines

Sample		Duration	Severity		Total
A			**mild**	**moderate-severe**	
	Children with AR	**PAR**	42	39	**81 (31 %)**
		IAR	26	156	**182 (69%)**
	Children with IgE-AR [B]	**PAR**	37	30	**67 (29%)**
		IAR	21	143	**164 (71%)**

[A] All the children in the families / Only the children with the present phenotype in the families.
[B] AR *and* a positive specific IgE antibody for at least one of the tested allergens (IgE-AR).

Results

The results of the parametric and nonparametric analyses are summarised in Table I.2. The NPL-scores of chromosomes with NPL-scores ≥ 2 in at least one of the three rhinitis phenotypes are illustrated in Figures I.1 and I.2.

In the parametric analysis a number loci reached suggestive evidence of linkage based on empirical data (simulations) with one region at 1p13 showing close to genome wide significance (MOD score 4.20; empiric P_{region} 0.0003; empiric P_{genome} 0.065) for HC in the combined sample.
Besides 1p13 the following chromosomes obtained suggestive evidence of linkage to HC based on the MOD simulation: 1q31(Imprinting MOD 3.38; empiric P_{region}=0.007), 2q23 (imprinting MOD 3.00; empiric P_{region}=0.076), 20p12 (MOD scores without/with imprinting 3.52/3.81; corresponding empiric P_{region}= 0.0025 and 0.0033, respectively),

For the AR phenotype, suggestive evidence of linkage was found for the chromosomes 1p13 (Non-imprinting MOD 2.97; empiric P_{region}=0.0055), 1q31 (Imprinting MOD 3.10; empiric P_{region}=0.054), 2q14-q21(Imprinting MOD 3.33; empiric P_{region}=0.0045), 2q23 (Imprinting MOD 3.26; empiric P_{region}=0.0077), 12p13(Imprinting MOD 3.10; empiric P_{region}=0.038) and 20p12 (MOD scores without/with imprinting 3.66/4.25; corresponding empiric P_{region}= 0.0039 and 0.0036, respectively),

The phenotype IgE-AR revieled suggestive evidence of linkage at chromosome 1p13 (MOD scores without/with imprinting 4.01/4.01; empiric P_{region}= 0.0006 and 0.0008, respectively), 1q32 (MOD scores without/with imprinting 3.16/3.28; empiric P_{region}= 0.003 and 0.007, respectively) and 20p12 (Imprinting MOD 3.30; empiric P_{region}=0.0.018).

In the non-parametric analysis, only chromosome 20p12 reached the level of suggestive linkage as defined by Lander et al. [109]. This was obtained for the combined sample for the phenotypes HC and AR (NPL 2.76; P= $3.8x10^{-4}$ and NPL 2.76; P=$3.7x10^{-4}$, respectively).

Several loci reached or exceeded NPL 2.00 with nominal p values less than 0.01, thereby showing "interesting" evidence of linkage according to Haines et al. [110](Table I.2). These loci were chromosomes 1q21, 4q11-q12, 14q12, 20p13, 22q12-q13, Xp22, Xp21.1 and Xp11.

When performing an NPL analysis on the ARIA sub-phenotypes of AR children in sample A (Table I.1), the sub-sample with mild AR symptoms (N=68) and the IAR sub-sample (N=182) yielded low NPL scores < 1. The sub-sample of children with moderate-serve AR symptoms (N=195) resulted in NPL-scores < 2. Only when analysing AR children with PAR (N = 81) were NPL-scores > 2.0

obtained at chromosome 21q22.3 (NPL= 2.03, P value= 0.004, MOD score without / with imprinting = 2.35 /2.33).

Table I.3 summarises the MOD scores and penetrances for loci showing signs of imprinting. When investigating possible regions with imprinting (Table I.3), most of the loci have scores that differ far less than the recommended 1.5 between the imprinting and the non-imprinting scores. Furthermore, the difference between P(wt/d) and P(d/wt) are mostly well below 0.2. However, at locus 2q23.3 in the AR phenotype, a distance of 1.52 between the imprinting and the non-imprinting score and a P(wt/d) > P(d/wt) of 0.205 was found. At locus 3q28 in the IgE-AR phenotype, a fairly large difference between P(wt/d) > P(d/wt) of 0.69 is found along with an imprinting MOD of 2.84 and a difference between the imprinting and non-imprinting MOD of 1.32.

Table I.2: Result of nonparametric analyses (NPL) and of parametric analyses using Genehunter MOD and Genehunter imprinting (MOD score)

Locus	Marker	Marker map positions (cM)	Sample	HC NPL-score	HC MOD score Without imprinting	HC MOD score With imprinting	AR NPL-score	AR MOD score Without imprinting	AR MOD score With imprinting	IgE-AR NPL-score	IgE-AR MOD score Without imprinting	IgE-AR MOD score With imprinting
1p13.2	**D1S187**	145.45	**A**	-	-	-	1.67	2.13	2.53	1.62	2.56	2.63
-	-	-	**A+B+C**	2.16	**4.20**	**4.30**	2.08	**2.97**	3.15	1.92	**4.01**	**4.01**
1q21.1	**D1S442**	154.74	**A**	1.71	-	-	1.93	-	-	1.84	-	-
-	-	-	**A+B+C**	2.27	-	-	2.18	-	-	-	-	-
1q31.2	D1S3468	205.4	**A+B+C**	1.62	2.63	**3.38**	1.68	2.45	**3.10**	-	-	-
1q32.1	D1S1647	215.99	**A**	-	-	-	-	-	-	2.04	**3.16**	**3.28**
2q14.3-q21.1	D2S2215-D2S2256	134.45-141.62	**A+B+C**	1.81	2.15	2.91	1.81	2.64	**3.33**	-	-	-
2q23.3	D2S2241	156.92	**A**	2.59	2.43	**3.00**	2.34	-	**3.26**	-	-	-
-	-	-	**A+B+C**	2.00	-	-	2.01	-	-	-	-	-
3q28	ATA57D10	206.43	**A**	1.68	-	-	1.92	-	-	1.63	-	2.84
-	-	-	**A+B+C**	1.81	-	-	1.70	-	-	-	-	-
4q11-q12	D4S3255-GATA61B02	61.42-63.58	**A**	1.89	-	-	1.57	-	-	-	-	-
-	-	-	**A+B+C**	2.04	-	-	2.03	-	-	1.88	-	2.05
4q35.1	D4S408	195.06	**A**	-	-	-	-	2.21	2.21	-	-	-
8q22.2	D8S506	110.2	**A**	1.78	-	-	-	-	-	-	-	-
-	-	-	**A+B+C**	1.83	-	-	-	-	-	-	-	-
10q26.12	D10S1230	142.78	**A**	-	-	-	-	-	-	1.63	-	2.53
10q26.13	D10S2322	149.25	**A**	1.72	2.43	2.05	1.50	-	-	-	-	-
11q12.3-q13.2	D11S1883-D11S987	65.05-67.48	**A+B+C**	-	-	-	-	-	-	1,52	-	-
11q22.3	D11S1394	97.92	**A**	1.83	-	-	-	-	-	1.91	-	-
-	-	-	**A+B+C**	-	-	-	-	-	-	1.66	-	-
11q24.1	D11S4464	123	**A+B+C**	1.80	-	-	1.80	-	-	-	-	-
12p13	D12S372-D12S358	6.42-26.23	**A+B+C**	1.55	2.62	2.46	1.55	2.62	**3.10**	-	-	-
13q34	D13S285	110.55	**A+B+C**	1.89	-	-	1.82	-	-	1.74	2.42	2.40
14q12-q11.2	D14S297-D14S741	31.75-36.76	**A**	1.95	-	-	2.17	-	-	2.22	-	-
15q26.3	D15S966	112.58	**A**	1.68	-	-	1.86	-	2.20	1.94	-	-
16q24.1	GATA86C08	120.59	**A**	1.82	-	-	1.99	2.86	2.94	-	-	-
20p13	D20S199	6.25	**A**	-	-	-	-	-	-	2.06	-	-
20p12.3	GATA72E11	21.15	**A**	2.85	2.26	2.38	2.29	-	-	-	-	-
20p12.1	D20S163	31.43	**A+B+C**	**2.76**	**3.52**	**3.81**	**2.76**	**3.66**	**4.25**	2.37	2.79	**3.30**
21q22.2-q22.3	D21S1893-D21S1890	43.67-52.5	**A**	1.57	2.14	2.08	1.66	2.66	2.78	-	-	-
22q12.3-q13.1	D22S1045-D22S685	32.5-42.81	**A**	-	-	-	1.56	-	-	1.82	-	-
-	-	-	**A+B+C**	-	-	-	2.02	2.40	2.34	1.98	2.17	2.16
22q13.32	D22S1161	59.5	**A**	1.88	-	-	-	-	-	-	-	-
Xp22.32	DXS6807	13.50	**A**	-	-	-	2.23	NA	NA	1.92	NA	NA
Xp22.11	DXS989-ATA28C05	29.76	**A+B+C**	-	-	-	-	-	-	1.79	NA	NA
Xp21.1	DXS9907	33.54	**A+B+C**	2.00	NA	NA	2.00	NA	NA	-	-	-
Xp11.3	DXS1003	47.08	**A**	2.24	NA	NA	-	-	-	-	-	-

Only NPL-scores $\geq$1.5 and MOD scores $\geq$ 2 are listed. Bold writing indicates suggestive evidence of linkage based on simulation for the parametric results (MOD scores) and based on the Lander and Kruglyak [109] criteria for the nonparametric results. NA: not applicable.

Table I.3: Mod scores and penetrances in loci showing signs of imprinting.

Phenotype	With imp.			Penetrance				
Locus	yes/no	Sample	MOD score	p(wt/wt)	p(wt/d)	p(Het)	p(d/wt)	p(d/d)
HC								
1p13.2	n	A+B+C	4.20	0.00		0.07		0.27
-	y	-	4.30	0.00	0.07		0.10	0.33
1q31.2	n	A+B+C	2.63	0.008		0.008		0.14
	y	-	3.38	0.045	0.045		0.19	0.50
2q14-q21	n	A+B+C	2.15	0.00		0.003		0.03
	y	-	2.91	0.00	0.015		0.00	0.05
2q23.3	n	A	2.43	0.00		0.25		0.93
-	y	-	3.00	0.00	0.39		0.17	0.95
20p12.3	n	A	2.26	0.05		0.17		0.73
-	y	-	2.38	0.08	0.30		0.27	1.00
20p12.1	n	A+B+C	3.52	0.025		0.1		0.61
-	y	-	3.81	0.025	0.12		0.07	0.59
AR								
1p13.2	n	A	2.13	0.00		0.07		0.15
-	y	-	2.53	0.00	0.05		0.11	0.11
1p13.2	n	A+B+C	2.97	0.02		0.16		0.54
-	y	-	3.15	0.02	0.12		0.17	0.49
1q31.2	n	A+B+C	2.45	0.008		0.008		0.12
-	y	-	3.10	0.05	0.05		0.17	0.46
2q14-q21	n	A+B+C	2.64	0.00		0.025		0.13
	y	-	3.33	0.00	0.10		0.00	0.22
2q23.3	n	A	1.74	0.01		0.01		0.007
	y	-	3.26	0.045	0.25		0.045	0.28
12q13	n	A+B+C	2.62	0.07		0.07		1.00
-	y	-	3.10	0.18	0.18		0.35	1.00
16q24.1	n	A	2.86	0.08		0.62		0.62
-	y	-	2.94	0.07	0.52		0.62	0.62
20p12.1	n	A+B+C	3.66	0.03		0.10		0.57
-	y	-	4.25	0.04	0.15		0.07	0.73
IgE-AR								
1p13.2	n	A	2.56	0.00		0.32		0.89
-	y	-	2.63	0.00	0.30		0.37	0.90
1q32.1	n	A	3.16	0.01		0.72		0.97
-	y	-	3.28	0.00	0.62		0.75	0.95
3q28	n	A	1.52	0.09		0.28		1.00
-	y	-	2.84	0.02	0.71		0.02	0.71
10q26.12	n	A	1.96	0.15		0.38		1.00
-	y	-	2.53	0.16	0.46		0.86	1.00
20p12.1	n	A+B+C	2.79	0.05		0.17		1.00
-	y	-	3.30	0.04	0.19		0.09	0.54

Only loci with MOD score $\geq$ 2.5 and with results indication imprinting (i.e., MOD with imprinting leads to a disease model with p(wt/d) $\neq$ p(d/wt) and MOD score with imprinting > MOD score without imprinting) are listed. “Imp” indicates imprinting.

Penetrances (p) at which the highest MOD score is obtained. "Wt" indicates Wild type (a normal allele), "d" indicates a disease allele. The first and the last value (wt/wt) and (d/d) indicate the probability for a person to be affected if he or she is homozygote with two normal or two abnormal alleles. The second value (Wt/d) / (Het) is the probability of a person who is heterozygous to be affected. The paternal alleles are listed first for the heterozygous population (seen in MOD score with imprinting).

Discussion

The present genome scanning of three Danish family samples revealed a number of loci showing evidence of linkage to allergic rhinitis. Chromosomes 1p13, 1q31-q32, 2q14-q21, 2q23, 12p13 and 20p12 reached suggestive evidence of linkage with 1p13 approaching genome wide significance. NPL above two was obtained at 1q21, 4q11-q12, 14q11-q12, 20p13, 22q12-q13, Xp22-p21 and Xp11. High MOD scores were obtained in several of these regions as well (table I.2) with the highest MOD scores found at 1p13 and 20p12.

Chromosome 1p13 has not been reported earlier in connection with AR nor atopy. The locus has previously shown suggestive linkage to asthma [284]. Possible candidate genes are the GSTM1 (glutathione S-transferase M1) gene [285] and CHIA (Acidic Mammalian Chitinase) located at 1p12-p13 is earlier associated with asthma [286]. Chromosome 20p12 is also a novel finding in regard to AR, however, it is earlier found to be linked to atopy and asthma [127, 284]. No obvious candidate gene exists in the region.

Three of the regions with suggestive linkage to AR in our study have been reported previously (2q14-q21, 2q23 and 12p13) [118] as well as two of the regions with weaker linkage to AR (22q12-q13 and Xp21). These regions were identified from a linkage scan performed by Haagerup et al. The study sample from Haagerup at al. is included in our analysis as sample C. Only linkage obtained at 2q23 is based solely on sample A, the remaining loci are found when analysing the combined phenotype. Thus, these regions do not represent an independent linkage replication, although the evidence for linkage has increased by increasing the sample size. Region Xp11 and 14q12 have been reported earlier in association with allergic sensitisation and asthma, respectively [127, 129, 284].

AR is most often studied as a subgroup of another allergic disease and seldom is allergic rhinitis the main focus of the study. We focused on AR when collecting our sample A, hoping thereby to eliminate a possible selection bias. Linkage analysis of AR is problematic due to the heterogeneity seen in the phenotypic expression of the disease and the genetics underlying the disease. By using three phenotypic approaches to AR including the most defined IgE-AR, we hope to minimise the heterogeneity without missing linkage signals due to smaller sample sizes. In an attempt to narrow the phenotype, we also divided sample A according to the ARIA guidelines into PAR and IAR as well as into "mild" and "moderate-severe" based on symptom severity.

Only four whole genome scans of the phenotype AR [118-121] and three regional linkage analysis of parts of the genome [135, 278, 279] have been reported (Table I.4). For all published AR genome-wide scans (except the study by Yokouchi et al.) [119], the samples were originally collected focusing on other atopic diseases than AR. The AR phenotype was then analysed as a phenotypic subgroup thereby potentially increasing the selection bias and genetic heterogeneity between the studies. Our study is one of the first to focus primarily on the AR phenotype. When comparing AR linked regions in our sample to published independent studies, only sparse overlap in the AR regions are found.

This result may be explained by the difference in study design and phenotype assessment. The phenotype in one study was based solely on answers from a standardised questionnaire [120] while other studies have based the phenotype on doctor confirmed diagnoses [118, 119, 121]. Furthermore, one study examined the phenotype AR [120] while the three other genome-wide studies investigated the defined phenotype IgE-AR [118, 119, 121]. Additionally, power limitations and variations in statistical methods may have interacted.

Our results showed an absolute absence of a signal in the region of 3q13 for AR. This region showed a highly significant linkage in a fine scale linkage study by Brasch-Andersen et al. who also included the sample we have included as sample C [279]. A possible explanation may be that the subjects in this study almost all had asthma as well as AR. In our study only 29% and 42% of the AR sib-pairs in samples A and B, respectively, had co-existing asthma.

A candidate gene on chromosome 1q21 is the Filaggrin gene (*FLG*). Several studies have found an association between *FLG* and atopic dermatitis and atopy as well as AR and asthma when co-excising with atopic dermatitis [260]. DENN/MADD domain containing 1B (*DENND1B*) gene located at locus 1q31.3 is found to associate with asthma [267].A candidate gene at 1q32 is the chitinase 3-like 1 (*CHI3L1*) gene which has been associated with allergic sensibility [201, 202]. Also at 1q32 we find the Decay-accelerating factor (*DAF*) gene earlier found to associate to atopy [170] and the interleukin 10 (*IL10*) gene previously associated with asthma [285]. At 2q14 the Interleukin 1 receptor antagonist (*IL1RN*) gene [175, 180] previously associated with allergic sensibility and the dipeptidyl peptidase 10 (*DPP10*) gene previously associated with asthma [287], resides. At 20p13, a disintegrin and metalloproteinase domain 33 (*ADAM33*) gene has previously been associated with asthma and allergic rhinitis (AR) [164, 285, 288]. At 20p13 the Mitochondrial Antiviral Signaling Protein (*MAVS*) gene found by GWA study to associate to asthma is located [266]. Interleukin 2 receptor beta (*IL2RB*) located at 22q12.3 is earlier found to associate to asthma [264].Toll-like receptor 7 and 8 (*TLR 7* and *TLR8*) located at Xp22 confer susceptibility to several allergic diseases and among these are AR and allergic sensibility [198]. At Xp11 the Forkhead BOX P3 (*FOXP3*) gene is located [228], it is found to associate with allergic sensibility. We are not aware of any previous reported candidate genes in the chromosome regions 14q12, 20p12 or Xp21. However, because it has been shown that the actual disease gene can reside as far as 20 cM away

from the markers with maximum LOD scores [289], the NPL score at Xp22 and Xp21 may actually be addressing the same gene, as may 20p12 and 20p13. Furthermore, a possible candidate gene for 2q23 and 2q21 may be the Histamin N-Metyltransferase (*HNMT)* gene located at 2q22, which has previously been found to be associated with asthma [290].

Inactivation of either the parental or maternal copy of a gene is known as imprinting. Imprinting may be due to either DNA methylation or changes in the chromatin structure. The obtained imprinting MOD-score of 3.26 at locus 2q23.3 supports the presences of imprinting. At locus 3q28 in the IgE-AR phenotype, only a moderate difference of 1.32 was found between the MOD-score with and without imprinting. The MOD score obtained with imprinting is high, suggesting a possible maternal imprinting may exist (Table I.3). We are not aware of any previous allergy-related linkage studies reporting imprinting in these regions.

In conclusion, the genome-wide search by the affected sib-pair method identified several regions that may contain a gene involved in the pathogenesis of allergic rhinitis. Among the regions suggestive of linkage to AR three (2q14-q23, 2q23and 12p13) were reported earlier, as were the weaker associated regions 22q13 and Xp21 [118]; with only 2q23 representing an independent sample because of sample overlap for the remaining findings.

The novel AR regions identified were 1p13 and 20p12 (suggestive of linkage) and 1q21, 14q12, Xp11 and, Xp22 (NPL $\geq$2). MOD-scores above four with and without imprinting strongly support the association of regions 1p13 and 20p12. Likely maternal imprinting was found at chromosome 2q23.3, and possibly maternal imprinting at chromosome 3q28. Analysis of the AR sub-phenotypes PAR, IAR, “mild symptoms” and, “moderate-severe symptoms” [1] resulted in a possible linkage of PAR to chromosome 21q22.

These results may help to identify genes that contribute to AR in the future. Identification of a risk gene for AR or other atopic diseases may increase our knowledge of the pathophysiology of these complex diseases and pave the way for more precise disease-classification and diagnosis, as well as facilitate more personalised treatments.

Table I.4: Results of published genome scans and fine scale mapping conducted in allergic rhinitis (AR)

Ref (year) Sample	No. of markers (scan regions)	Chromosome position(s) with linkage												
		1	2	3	4	5	6	9	11	12	17	18	22	X
[135] (1999) Barbados. 528 subjects.82 % AR	72 (Chr.12q)	---	---	---	---	---	---	---	---	**q21.1**	---	---	---	---
[118] (2001) Denmark: 33AR families.	446 (total)	---	q12-q33	q13	**q24-q27** p15-q12	q13-q15	p24-p23	---	---	p13	---	---	q13	p21
[119] (2002) Japan: 48 AR families	400 (total)	q36.2	---	---	**q13.3**	---	---	q34.3	---	---	---	---	---	---
[278] (2003) Denmark: 30AR families.	97 (8 regions[A])	---	---	---	q32.2	---	---	---	---	---	---	---	---	---
[120] (2005) France: 185 AR families.	396 (total)	---	**q32**	p24-p14	---	---	---	p22	---	---	---	---	---	---
		---	---	---	---	---	---	q22-q34	---	---	---	---	---	---
[121] (2005) Sweden: 250 AR families	367 (total)	---	---	**q13**	q34-q35	---	p24-p22	p11-q12	---	---	q11.2	q12	---	---
		---	---	---	---	---	---	q33-34	---	---	---	---	---	---
[279](2006) Denmark: 125 AR families	28 (3p14.2–q21.2)	---	---	**q13.31**	---	---	---	---	---	---	---	---	---	---

[A] 3p, 3q, 4p, 4q, 5q, 6p, 9p, 12q, 18q, Xp

Table of references [118-121, 135, 278, 279]Only positions with LOD-scores $\geq$ 1 and / or p-value $\leq$ 0.003 are shown (i.e., significance level of interest according to Haines et al. [110]). Positions with LOD-scores > 2.2, P<0.0007 and / or MOD> 3 are written in bold (i.e., suggestive of linkage according to Lander et al.[109])

Acknowledgments

We thank the patients and their families for participating in the project. The helpful and supportive staffs at the outpatient allergy clinics in Viborg, Aarhus, Hjoerring, Herning and Aalborg and the laboratory technicians in Hjoerring and Aalborg are greatly appreciated. The project was funded by Region Hospital Viborg, the Health Research Fund of Central Denmark Region, the Viborg country health-science research committee, Sygeforsikringen "Danmark"s healthfund, the Toyota Foundation of Denmark, The A.P. Møller Foundation for the Advancement of Medical Science, the Else and Helene Alstrups Fund, The Lundbeck Foundation, and the Aarhus Faculty of Health Sciences, Aarhus University.

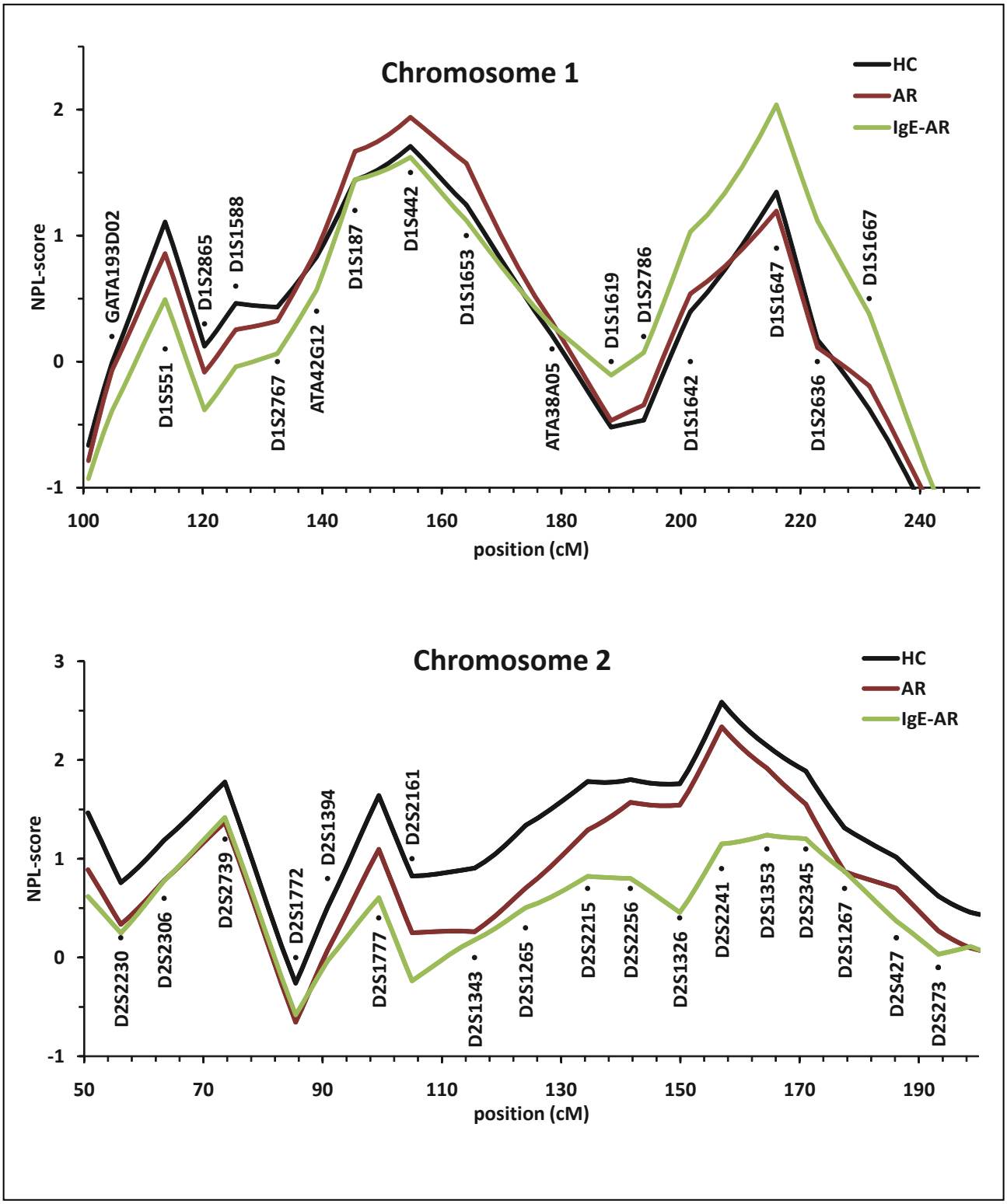
Conflict of interest

None.

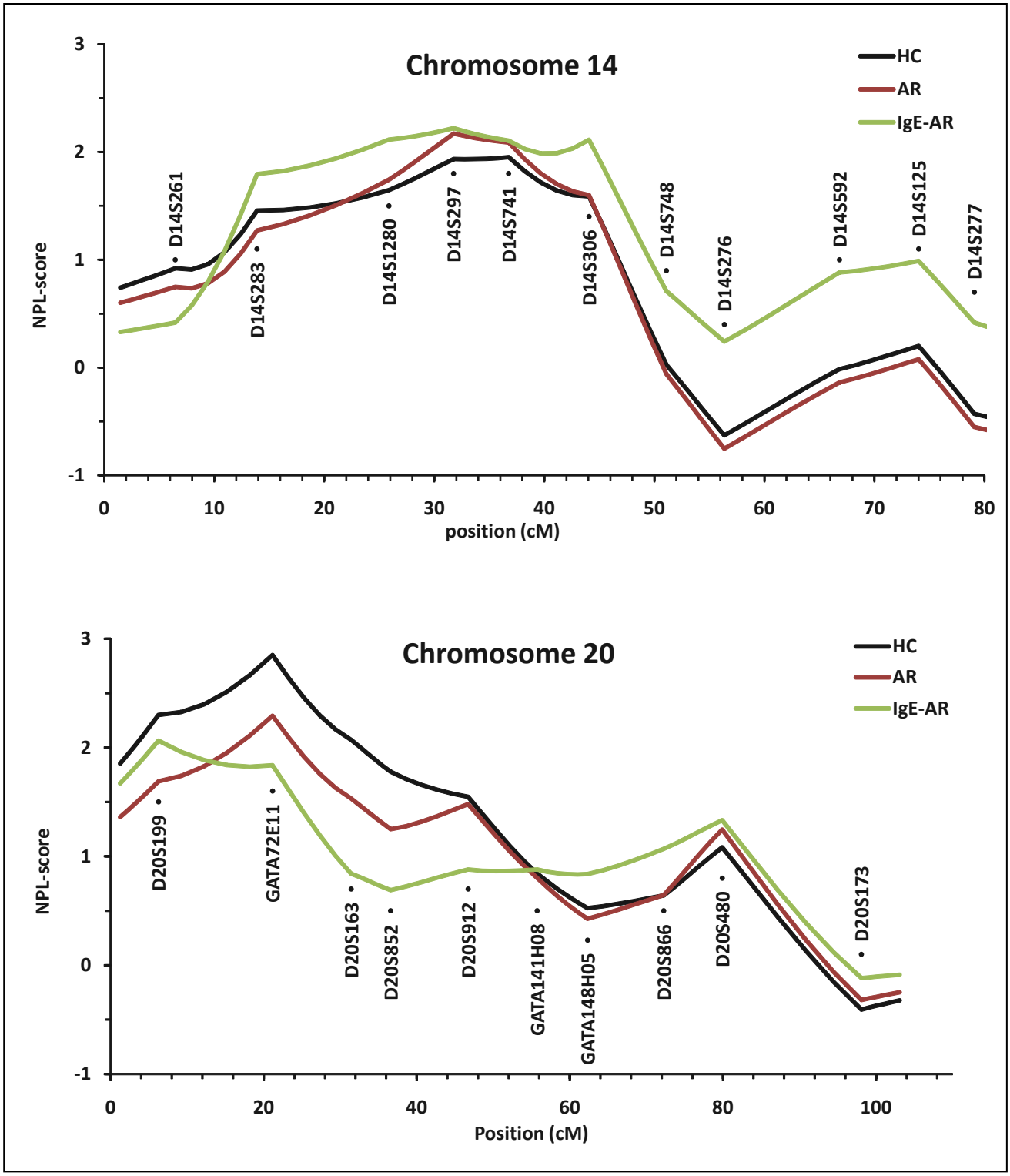
Ethics approval

The Danish scientific ethics system approved the protocol (journal no. 196/3581) and all participants and/or their parents signed informed consent forms.

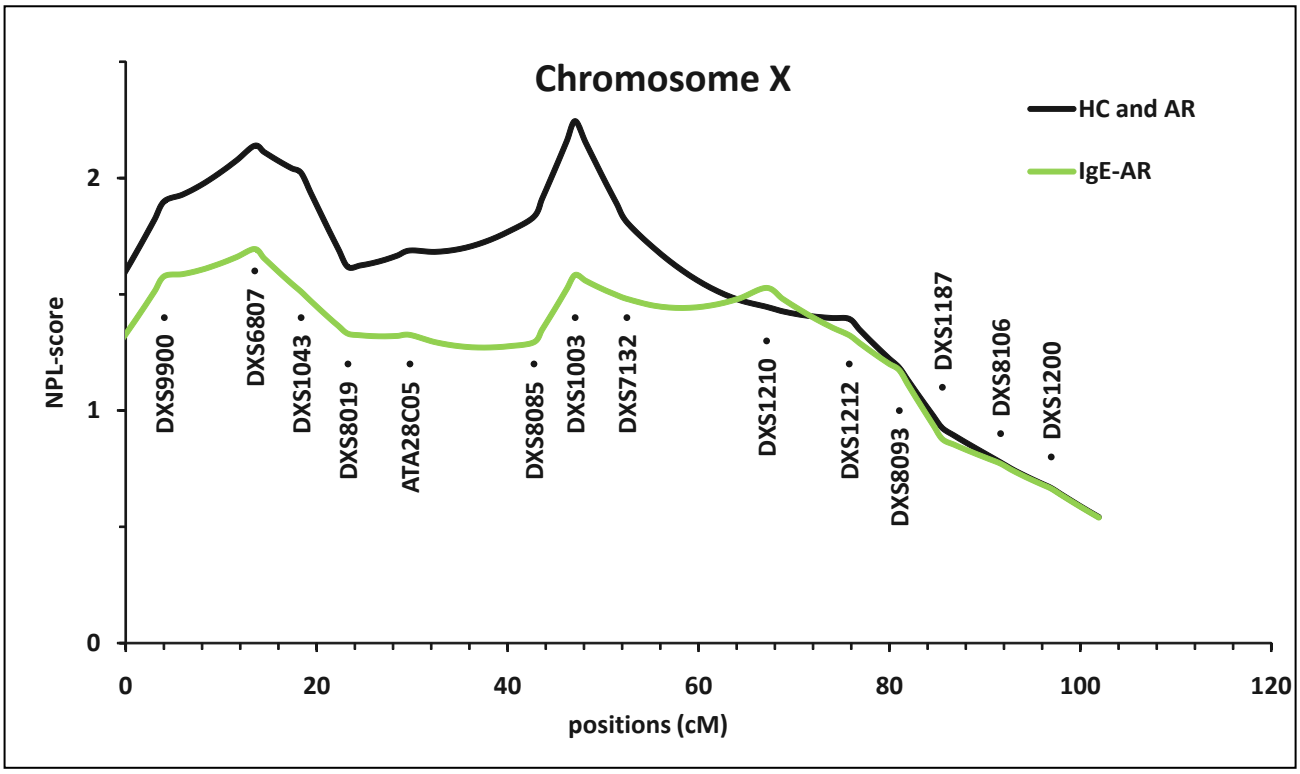
Figure I.1: NPL-scores (Z-scores) Sample A only. Chromosomes with NPL-score ≥2 for one of the three phenotypes are shown.



(Figure I.1continued)

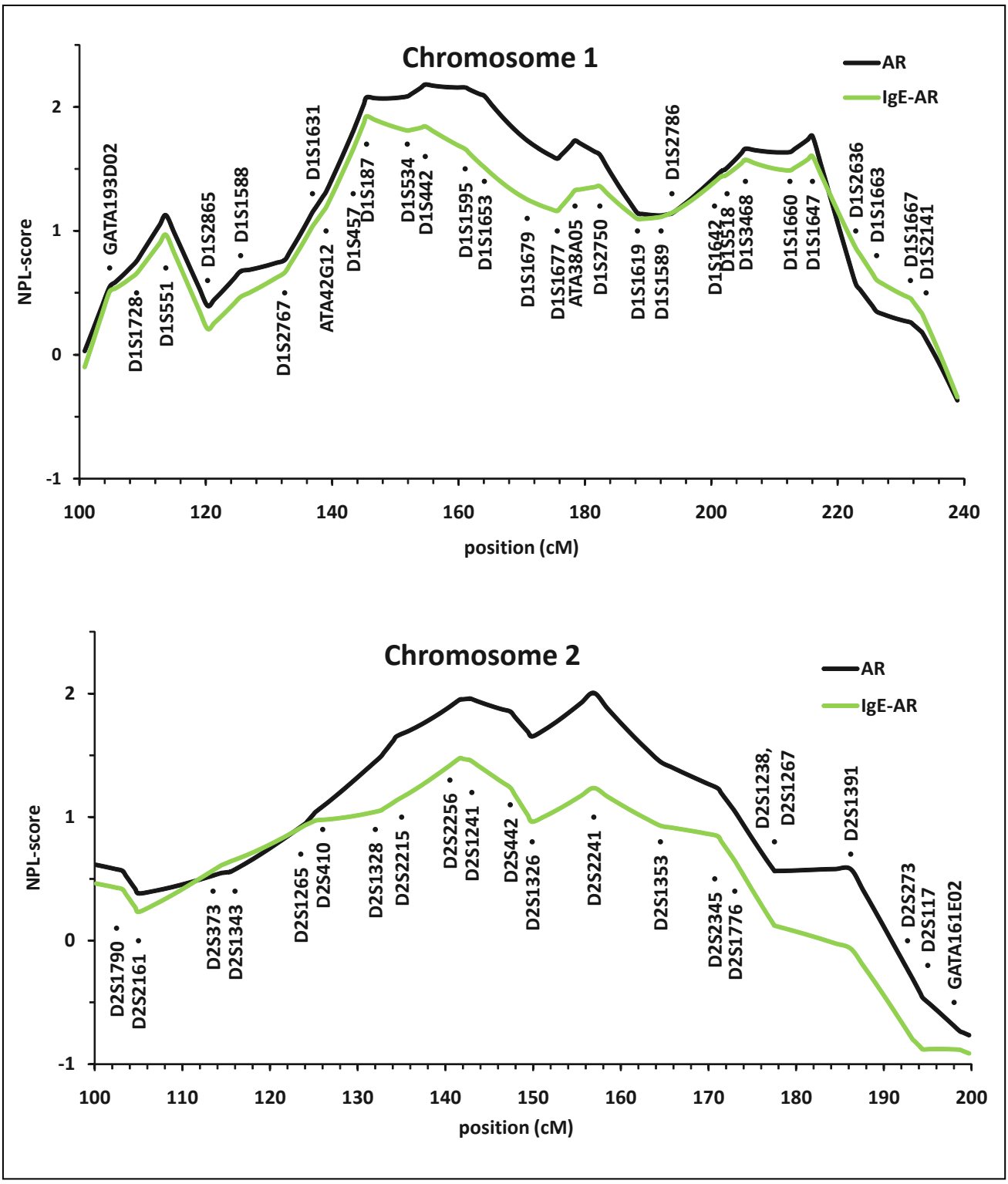


(Figure I.1 continued)

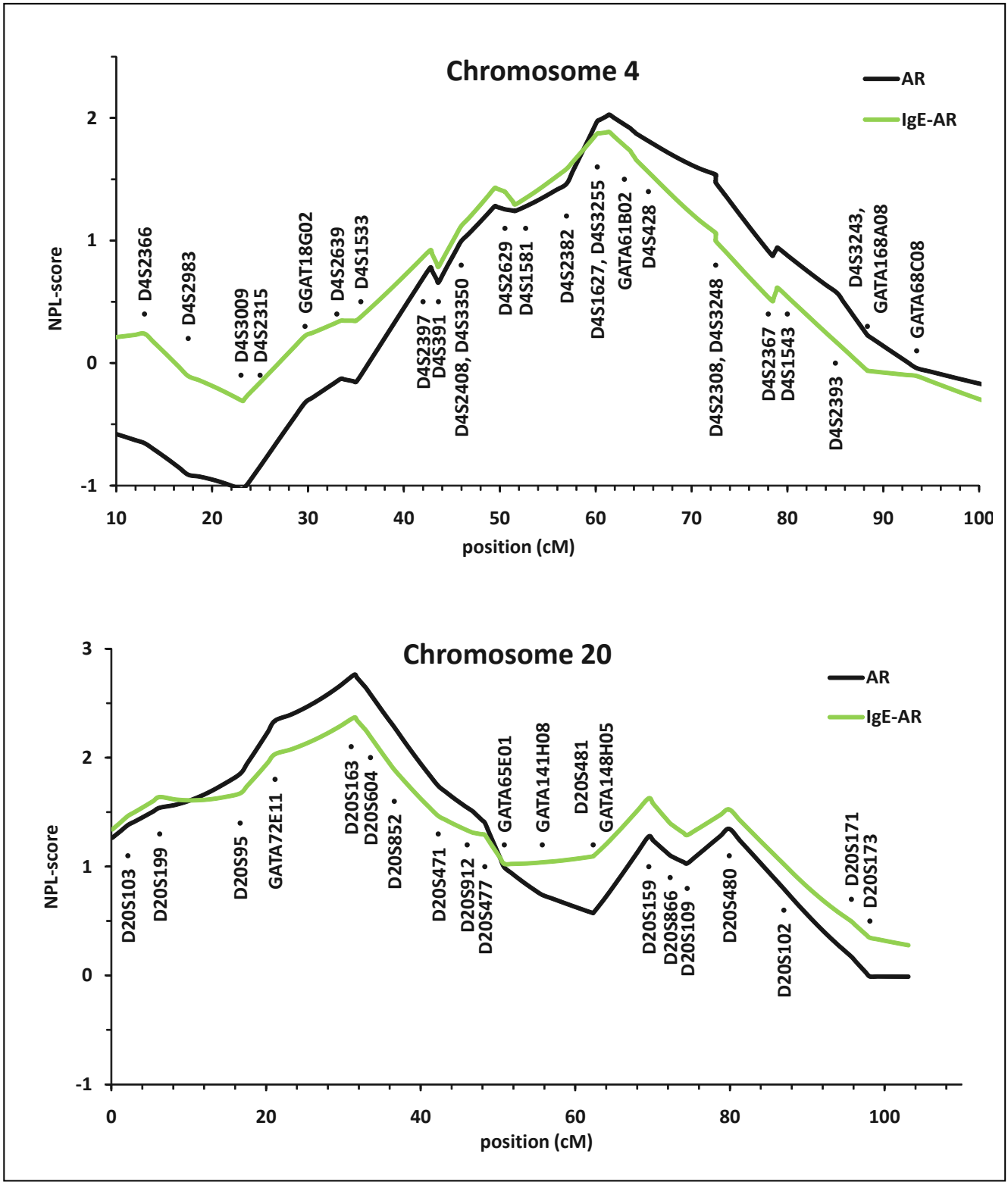


HC: Hay fever combined, AR: Allergic rhinitis, IgE-AR: IgE associated AR (see "Phenotype definition" under "Method" for more details). For the X chromosome, curves created for the HC and AR phenotypes were coincident and the two phenotypes are therefore combined in one curve.

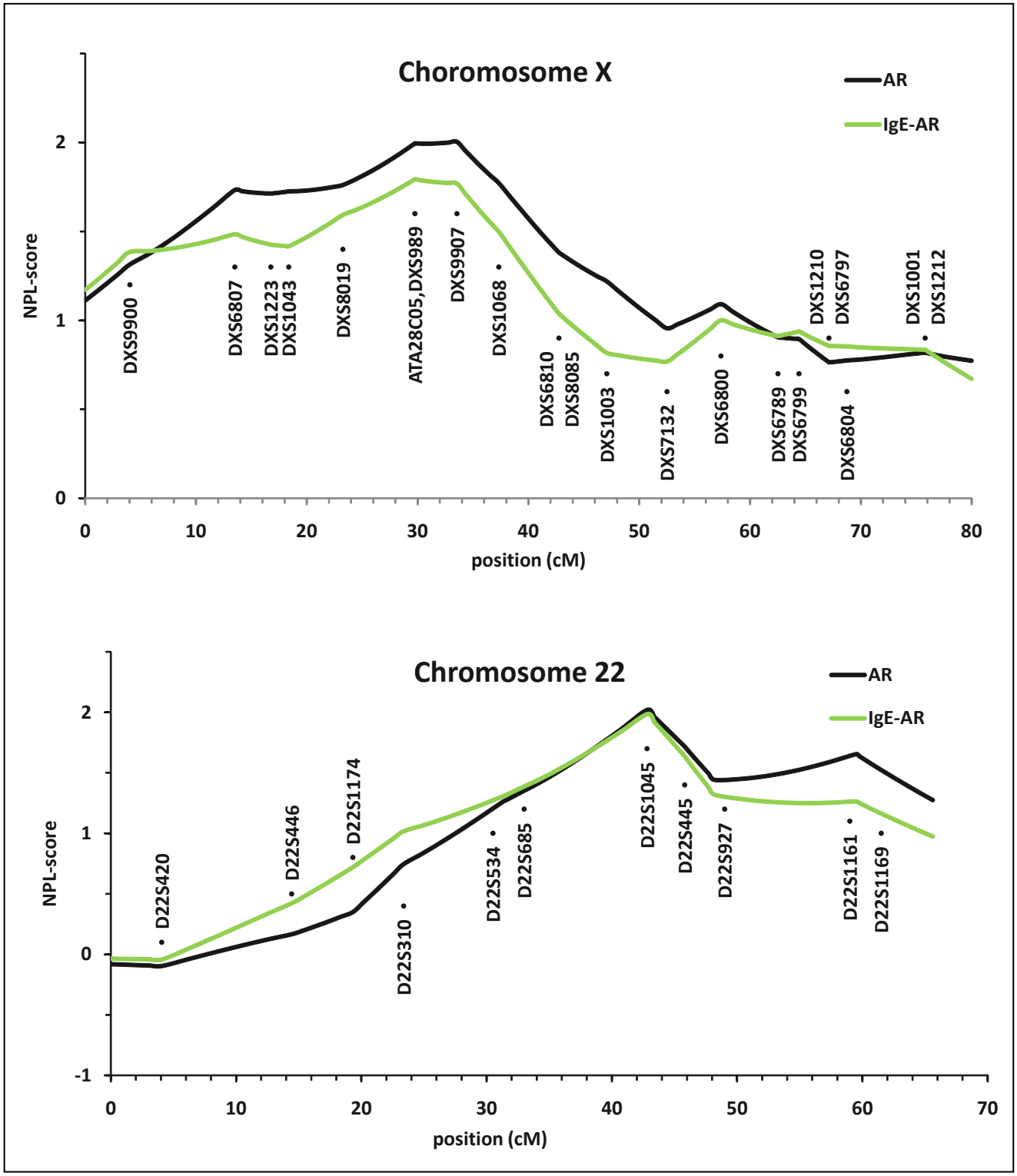
Figure I.2: NPL-scores (Z-scores) for the combined sample (sample A, B and C). Chromosomes with NPL-score ≥2 for one of the phenotypes are shown.



(Figure I.2 continued)



(Figure I.2 continued)



When all three samples are combined the results of the phenotypes HC and AR are almost identical. HC: Hay fever combined, AR: Allergic rhinitis, IgE-AR: IgE associated AR (see "Phenotype definition" under "Method" for more details)

PAPER II:

A genome-wide search for linkage to atopy and asthma in Danish sib-pair families.

Kruse L.V [1,2], Christensen U [1,3], Haagerup A [1,3], Deleuran M [4], Hansen L.G [2], Møller-Larsen S [1], Venø S.K [1], Goossens D [5,6], Del-Favero J [5,6], Nyegaard M [1,7], Børglum A.D [1]

1 Department of Human Genetics, Aarhus Faculty of Health Sciences, Aarhus University, Aarhus, Denmark
2 Department of Paediatrics, Region Hospital Viborg, Denmark
3 Department of Paediatrics, Aarhus University Hospital, Skejby, Denmark
4 Department of Dermatology, Aarhus University Hospital, Aarhus, Denmark
5 Applied Molecular Genomics Group, Department of Molecular Genetics, VIB, Belgium
6 University of Antwerp (UA), Antwerp, Belgium
7 Department of Haematology, Aalborg Hospital, Aalborg Denmark

Abstract

Atopic diseases are complex disorders that are multifactorial and polygenic. Twin studies have found a substantial genetic contribution. We aim to investigate possible linkage to total IgE and allergen specific IgE as well as asthma. We collected and clinically characterised a Danish sample consisting of 127 nuclear families including 540 individuals (286 children and 254 parents). On this sample and a sample previously collected comprising 130 atopic dermatitis families, a whole genome linkage scan was performed using 429 microsatellite markers. A third sib-pair family sample previously collected and genotyped was added to the analysis, reaching a total of 357 families comprising 1,508 individuals, of whom 237, 125 and 92 families had at least two sibs with allergic sensitisation, elevated total IgE or asthma, respectively.

The computer programmes Genehunter NPL as well as Genehunter MOD and Genehunter Imprinting were used to obtain non-parametric and parametric linkage results, respectively.

We found genome-wide suggestive linkage to asthma at 2p13 and 20p12, with 2p13 strongly approaching genome wide significance. Both regions known from previously studies. Evidence of suggestive linkage to specific IgE was obtained at an established locus (20p12) and a novel region (1p13). Linkage to total IgE was confirmed at 6p24 and 11q12-q13. NPL above two was obtained in the allergen specific IgE phenotype at chromosomes 10q26, 11q22, 14q11, 20p13, 22q12-q13 and Xp22. Possible maternal imprinting was seen at 11q22 for the total IgE phenotype and at 3q22 and 20p12 for allergen specific IgE phenotype.

Introduction

The prevalence of atopy and atopic diseases such as asthma has, over the last decades, increased significantly [9, 10]. Studies conducted during the 1990s indicated that the prevalence had reached a plateau, or even decreased, but only in areas of the western world [12, 13, 26].

The amount of research done in the field of atopic diseases reflects the growing burden these disorders lay on patients and societies around the world. Thus, several studies have recently been conducted that investigated the genetic architecture underlying allergies. To date, more than one thousand candidate gene and linkage studies have been published in this field [103]. Replicating the results of earlier studies has, however, proven troublesome. Many explanations for these inconsistencies between results have been offered. Some point to the lack of statistical power and poor study design; others address problems in standardising diagnostic processes for complex disorders, due to the heterogeneous nature of such conditions. The heterogeneity can be seen in many different domains: age of onset, severity of symptoms, response to treatment and the natural history of the disease. Individual phenotypes and clinical manifestations are likely to work by different pathways and genes [4], complicating the elucidation of the genetic architecture underlying the atopic diseases. A method often used to reduce genetic heterogeneity, and thereby

increase the power of an analysis, is the dissection of complex traits into more distinct traits (intermediate phenotypes), such as atopy characterised specifically by total IgE (T-IgE), specific IgE (sIgE) and/or results of a skin prick test (SPT) [5].

The focus of this study was to perform a linkage analysis on the intermediate phenotypes T-IgE and sIgE and, at the same time, investigate possible regions with linkage to asthma in our sample. Twin-based studies have revealed an estimated heritability of T-IgE and sIgE to be between 60% and 81% and 60% and 78%, respectively [5, 22, 23, 32], and the heritability of asthma to be between 61% and 81% [39, 98, 99], confirm the importance and relevance of genetic research.

Method

Subjects

We have included 127 Danish nuclear families in the study (Sample A). In total, 286 children and 254 parents were recruited through the paediatric departments in Viborg, Aarhus, Hjoerring, Herning and Aalborg, the Department of Respiratory Diseases of Aarhus University Hospital and one private paediatric clinic in Aalborg. All persons were clinically examined by the same doctor and completed identical questionnaires. The questionnaire contained questions about symptoms, duration and treatment of rhino-conjunctivitis, atopic dermatitis and asthma, as well as questions about known or suspected allergens responsible for the atopic diseases. Atopic disease was diagnosed according to standard criteria [19]. The diagnoses were evaluated by a second doctor by assessing the questionnaires alone.

The families were sub grouped according to whether the phenotypes were present in at least two, and as many as four, of the siblings (Table II.1). Mean age among the offspring was 16 years; 52% were male.

Blood was drawn from all participants for DNA analysis and for serum measurements of total IgE and allergen-specific IgE antibody (ImmunoCAP system, Phadia Aps, Allerød, Denmark). Two previously collected Danish sib-pair family samples (Samples B and C) were included in the genotyping and/or analyses. Sample B [280] contained 130 Danish nuclear families with at least two full sibs with AD. Mean age among the children was 12.5 years; 48% were male. Sample C [127] contained 100 Danish nuclear families enrolled in the Danish allergy project. Inclusion criteria were at least two full sibs with doctor diagnosed atopy, i.e., asthma, rhinitis or atopic dermatitis. Mean age among the offspring was 14.5 years; 49% were male. Together, these two samples (sample B and C) included 460 parents and 519 children. In total, data from 357 families comprising 1,519 individuals were gathered, in which 213, 130 and 97 families had at least two sibs with allergic sensitisation, elevated total IgE or asthma, respectively (Table II.1). Sample A is a novel sample, whereas Sample B had been previously collected, but not analysed with respect to asthma or atopy. Sample C has already been reported [127, 278]. The Danish scientific ethical system approved the protocol, and all participants and/or their parents signed informed consent forms.

Phenotype definition

T-IgE varies widely depending on age (https://www.sundhed.dk/artikel.aspx?id=13231.1); therefore, a threshold was established to better distinguish changes. For children between 6 and 14 years of age, T-IgE was considered elevated when equal to or above 150 kU/l and for the remaining participants, 100 kU/l. Levels of sIgE were determined for twelve different allergens: Mite (D.pternyssinus and D.farinae), cat, horse, dog, timothy grass, birch, mugwort and mould (Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum and Penicillium chrysogenum). Antibodies to sIgE were considered elevated when $\geq$ 0.35 KU/l. A person was

classified sIgE-positive when sIgE was elevated against at least one of the tested allergens. IgE-associated allergic diseases were defined by the present phenotype combined with sIgE-positivity antibody.

Genotyping

Samples A and B were genotyped at the University of Antwerp, Belgium using 429 microsatellite markers (415 autosomal and 14 X-linked markers) covering the whole genome and resulting in approximately 465,000 genotypes. The mean intermarker distance was 8.26 centiMorgan (cM; range: 0 - 17.64 cM), and the markers showed an average heterozygosity of 73%. Sample C was previously genotyped at the Department of Human Genetics, Aarhus, Denmark using 446 microsatellite markers (432 autosomal and 14 X-linked markers) [280]. Also, at the Department of Human Genetics, Sample B was previously genotyped in three candidate regions using a total of 91 markers using an ABI-310 Sequencer from Applied Biosystems. These genotypes were included in the analyses to increase statistical power. All genotypes were uploaded to BCSNP (BCPlatforms, Finland). Mendelian inconsistencies in the pedigree set were identified and removed using the computer programmes Merlin [281] and PedCheck [282]. In case of inconsistencies, genotypes from the problematic marker were removed for the entire nuclear family.

Statistical analyses

Multipoint parametric linkage analyses were performed via the Affected Sib-Pair Method [107]. Multipoint non-parametric linkage analyses, which required no specification of the underlying genetic model for the trait being investigated, were performed on the autosomal chromosomes using the software program Genehunter NPL v. 2.0. Non-parametric analysis of the X chromosome was performed using Genehunter Imprinting v. 1.3 [113]. On chromosomes where a NPL-score greater

than or equal to 1.5 was obtained, parametric analyses were performed using Genehunter MOD and Genehunter-Imprinting [114]. In both types of analyses, the following settings were used: "Modcal: global" and "MOD score routine: standard". Genehunter MOD maximises the LOD score with respect to different disease-model parameters. Genehunter Imprinting accounts for the "parent-of-origin effect" (imprinting effect) by incorporating different penetrances for heterozygous individuals, depending on whether the disease-causing allele is transmitted by the father or the mother. When calculating a Genehunter Imprinting MOD score, two different heterozygote penetrances may be found. For this to indicate true imprinting, the following three rules can be applied: (I) A large difference between P(wt/d) and P(d/wt) gives a greater chance of true imprinting (a different above 0.2 is recommended [114]); (II) The difference between imprinting and non-imprinting MOD scores must preferably exceed 1.5 [283]; and (III) The imprinting MOD must be high enough to at least indicate evidence of linkage in the region. When it comes to significance levels, Weeks et al. [111] and Hodge et al. [112] have found that a MOD-score of 3 shall be "deflated", with approximately 0.3 to 1 units within the upper boundary being rather conservative in order to be comparable to LOD score.

To evaluate the genome-wide level of significance in our samples, the empiric P values were calculated for the parametric MOD scores using the simulation features incorporated in Genehunter MOD and Genehunter-Imprinting [113, 114]. A total of 10000 simulations were performed for each chromosome/region. Based on the regional empiric p value and the size of the region tested a genome wide empiric p value was calculated indicating the likelihood of obtaining scores that were as high as or higher than the observed (under the assumption of no linkage) in a whole genome scan. A region is suggestive of linkage when the observed result is found to occur by chance less than once in a whole genome scan representing a nominal p value of $7x10^{-4}$. If the observed result is found to occur by chance less 0.05 times in a genome scan, representing a nominal p value of

$2x10^{-5}$, the region is significant of linkage [109]. MOD score calculations on the X chromosome was not possible using Genehunter MOD or Genehunter Imprinting.

Table II.1: Phenotypic features in Sample A and Sample B+C.

	Sib pair	Parents	Offspring
Sample A	families		all /with the phenotype [A]
Total	127	254	286
Pos. serum IgE	103	204	235 / 219
Total IgE>100/150 KU/l	53	106	142 / 118
Asthma	38	80	88 / 82
IgE-Asthma[B]	34	68	76 / 70
Sample B+C			
Total	230	460	519
Pos. serum IgE	110	220	251 / 238
Total IgE>100/150 KU/l	77	154	181 / 165
Asthma	59	118	140 / 126
IgE-Asthma[B]	54	108	124 / 117

[A] All the children in the families / Only the children with the present phenotype in the families.
[B] IgE associated asthma, i.e., asthma *and* a positive specific IgE antibody for at least one of the tested allergens.

Results

The results of nonparametric and parametric analyses are summarised in Table II.2. Regions found to be suggestive of linkage by simulation are written in bold. Figures II.1, II.2 and II.3 show graphs illustrating NPL scores for all three investigated phenotypes on chromosomes where a NPL greater than or equal to 2 is obtained for at least one of the phenotypes.

In the parametric analysis a number of loci reached suggestive evidence of linkage based on empirical data (simulations) with one region strongly approaching genome wide significance linkage to asthma at 2p13 (MOD 4.23; empiric P_{region} =0.0059, empiric P_{genome}=0.07). Chromosome 20p12 obtained suggestive evidence of linkage to asthma based on the MOD simulations (Sample A MOD score without/with imprinting 3.05/3.74; corresponding empiric P_{region}=0.018 and 0.011, respectively. Sample AB MOD without imprinting 3.18; empiric P_{region}=0.013) (Table II.2).

For the phenotype sIgE, simulations revealed suggestive evidence of linkage at chromosomes 1p13 (Sample AB MOD score without/with imprinting 2.94/2.97; corresponding empiric P_{region}=0.0022 and 0.0045, respectively. Sample ABC MOD score without/with imprinting 3.09/3.19; corresponding empiric P_{region}=0.0023 and 0.0034, respectively) and 20p12 (Sample AB MOD score without imprinting 2.81; empiric P_{region}=0.018. Sample ABC MOD score with imprinting 3.42; empiric P_{region}=0.014) (Table II.2).

Analysis and subsequent MOD simulations of the phenotype T-IgE revealed the following two loci to be suggestive of linkage: 6p24 (MOD score without/with imprinting 3.00/3.13; corresponding empiric P_{region}=0.03 and 0.047) and 11q12-q13 (MOD score without/with imprinting 3.11/3.26; corresponding empiric P_{region}=0.018 and 0.034) (Table II.2).

Several loci in the sIgE phenotype reached or exceeded NPL 2.00 with nominal p values less than 0.01, thereby showing "interesting" evidence of linkage according to Haines et al. [110](Table II.2). These loci were chromosomes 10q26, 11q22, 14q11, 20p13, 22q12-q13 and Xp22.

When calculating MOD scores without and with imprinting, we found that all loci had less than the recommended 1.5 between the observed imprinting and non-imprinting scores, and the difference between P(wt/d) and P(d/wt) was mostly well below 0.2 (Table II.2). However, for the T-IgE phenotype at chromosome 11q22, we found P(wt/d) > P(d/wt) with an interval of 0.5. The difference between imprinting and non-imprinting MOD scores was 0.92, and the MOD score with imprinting obtained at this locus reached 3.10. In analysing the sIgE phenotype, chromosome 3q22 with a imprinting MOD of 2.96 revealed a difference between the imprinting and non-imprinting score of 1.44 and P(wt/d) > P(d/wt) with an interval of 0.56. Chromosome 20p12 in the sIgE

phenotype reached an imprinting MOD of 3.42 and disclosed a difference between MOD with and without imprinting of 0.97 and a P(wt/d) > P(d/wt) interval of 0.18.

Table II.2: Results of nonparametric analyses (NPL) and of parametric analyses using Genehunter MOD and Genehunter Imprinting (MOD-score) on total IgE, specific IgE and asthma phenotypes.

Phenotype	Locus	Marker	Marker map Positions (cM)	Sample	NPL-score	P Value	MOD score Without imprinting	MOD score With imprinting / p(wt,wt); p(wt/d); p(d/wt); p(d/d)
T-IgE	**2p23.1**	D2S352	50.65	**A**	1.81	0.003	2.39	2.99 (0.00, 0.12, 0.03, 0.44)
---	**3q22.1**	D3S1541	146.60	**AB**	1.59	0.004	1.74	2.50 (0.00, 0.02, 0.003, 0.06)
---	**3q24**	D3S1744	161.04	**ABC**	1.53	0.01	2.20	2.86 (0.00, 0.32, 0.65, 0.74)
---	**6p24.3**	D6S277	14.61	**ABC**	1.62	0.007	**3.00**	**3.13** (0.00, 0.20, 0.27, 0.71)
---	**11q12.3-q13.2**	D11S1883-D11S987	65.05-67.48	**ABC**	1.84	0.003	**3.11**	**3.26** (0.07, 0.43, 0.54, 1)
---	**11q22.3**	D11S1394	97.92	**A**	1.65	0.007	2.18	3.10 (0.05, 0.54, 0.05, 0.54)
---	**16p12.1**	D16S3145	52.26	**A**	1.56	0.01		
sIgE	**1p13.2**	D1S187	145.45	**AB**	2.06	*0.0048*	**2.94**	**2.97** (0.00, 0.13, 0.11, 0.31)
---	---	---	---	**ABC**	2.06	0.006	**3.09**	**3.19** (0.03, 0.1, 0.14, 0.54)
---	**1q32.1**	D1S1647	215.99	**A**	1.72	0.03		
---	**1q21.1**	D1S442	154.74	**A**	1.63		2.45	2.52 (0.00, 0.61, 0.71, 1.0)
---	**2q16.3**	D2S2739	73.61	**A**	1.89	0.02		
---	**2q23.3**	D2S2241	156.92	**AB**	1.59	0.02		
---	**3p26**	D3S1297-D3S3050	8.31-14.46	**AB**	1.70	0.02		
---	---	---	---	**ABC**	1.5	0.04	2.36	2.75 (0.01, 0.05, 0.02, 1)
---	**3q22.1**	D3S1541	146.60	**ABC**	1.91	0.007		2.96 (0.06, 0.7, 0.14, 0.7)
---	**4q11-q12**	D4S325	61.42	**ABC**	1.84	0.014		
---	**5p15.2**	D5S1991	26.73	**ABC**	1.70	0,029		
---	**6q14**	D6S1031-D6S1270	88.63-92.9	**ABC**	1.62	0.026		2.13 (0.03, 0.09, 0.17, 0.17)
---	**8q22.2**	D8S506	110.2	**AB**	1.71	0.02		
---	---	---	---	**A**	1.69	0.03	2.54	2.54 (0.05, 0.05, 0.05, 0.55)
---	10q26.12	D10S1230	142.78	**AB**	2.01	0.006	2.45	2.72 (0.14, 0.48, 0.76, 0.76)
---	---	---	---	**A**	1.91	0.02		2.03 (0.04, 0.1, 0.16, 0.79)
---	**11q22.3**	D11S1394	97.92	**A**	2.13	0.01		
---	---	---	---	**AB**	1.55	0.02		
---	---	---	---	**ABC**	2.00	0.008		2.00 (0.03, 0.14, 0.17, 0.17)
---	**13q14.11**	D13S1247	38.96	**A**	1.58	0.04		
---	**13q33.3**	D13S173	93.52	**ABC**	1.70	0.02	2.05	1.79 (0.00, 0.09, 0.1, 0.1)
---	**14q11.2**	D14S283	13.89	**A**	2.04	0.02		
---	**14q22-q23**	D14S276-D14S125	56.36-74.01	**AB**	1.52	0.02	2.19	2.21 (0.015, 0.05, 0.02, 1.0)
---	**15q26.3**	D15S966	112.58	**A**	1.96	0.02		
---	**15q26.3**	D15S966	112.58	**AB**	1.52	0.02		
---	**20p13**	D20S199	6.25	**A**	2.55	0.003		
---	**20p12**	GATA72E11-D20S163	21.15-31.43	**AB**	2.38	0.001	**2.81**	2.81 (0.015, 0.015, 0.015, 0.1)
---	---	---	---	**ABC**	2.22	0.004	2.45	**3.42** (0.05, 0.24, 0.06, 0.24)
---	**21q21.1**	D21S1432	2.99	**AB**	1.77	0.01		
---	**22q12.3-q13.1**	D22S685-D22S1045	32.5-42.81	**A**	1.57	0.04		
---	---	---	---	**AB**	1.72	0.02		
---	---	---	---	**ABC**	2.27	0.003	2.29	2.34 (0.05, 0.14, 0.17, 0.4)
---	**Xp22.32**	DXS6807	13.50	**A**	1.91	0.02		
---	**Xp22.11**	DXS989-ATA28C05	29.76	**AB**	1.80	0.01		
---	---	---	---	**ABC**	2.2	0.004		

(Table II.2 continued)

Asthma	**2p13.2**	D2S1394	90.82	**A**	1.54	0.003	**4.23**	**4.36** (0.01, 0.1, 0.01, 0.75)
---	**3p26.2-p26.1**	D3S1560	18.97	**A**	1.57	0.004		
---	**7p14.3**	D7S617	41.69	**A**	1.50	0.002		
---	**11q22.3**	D11S1394	97.92	**ABC**	1.63	0.003	2.03	2.11 (0.01, 0.07, 0.08, 0.09)
---	**20p12**	GATA72E11-D20S163	21.15	**A**	1.84	0.0009	**3.05**	**3.74** (0.01, 0.01, 0.1, 0.85)
---	---	---	---	**AB**	1.73	0.0004	**3.18**	3.18 (0.008, 0.09, 0.09, 0.09)
---	---	---	---	**ABC**	1.62	0.003		

Only NPL-scores $\geq$1.5 and MOD scores $\geq$ 2 are listed. NPL-scores $\geq$ 2.2 and MOD scores $\geq$ 2.5 are written in bold. Asterisk (*) indicates suggestive of linkage based on simulation. Sample AB: Samples A and B combined. Sample ABC: sample A, B and C combined. Penetrances (p) at which the highest MOD with imprinting scores are obtained are listed. "Wt" indicates Wild type (a normal allele), while "d" indicates a disease allele. The first and the last value (wt/wt) and (d/d) indicates the probability for a person to be affected if he ore she is homozygous with two normal or two abnormal alleles. The middle values (Wt/d)/(d/Wt) are the probabilities of affectation for a person who is heterozygous with the paternal allele listed first [114].

Discussion

The present study revealed several loci showing suggestive evidence of linkage to the three phenotypes investigated. Suggestive evidence of linkage was obtained at 2p13 and 20p12 for the asthma phenotype with 2p13 strongly approaching genome wide significance.

For the T-IgE phenotype suggestive evidence of linkage was obtained at 6p24 and 11q12-q13 and at 1p13 and 20p12 for the sIgE phenotype. NPL above two was found in the sIgE phenotype at chromosomes 10q26, 11q22, 14q11, 20p13, 22q12-q13 and Xp22. High MOD scores were obtained in many of the same regions with the highest MOD scores seen for the asthma phenotype at 2p13.

Results of published genome-wide scans conducted in atopy are shown in Table II.3.

Chromosome 2p13 has in a meta-analysis of genome-wide linkage studies of asthma meet suggestive linkage criteria [284].We know of no candidate genes in the area. Beside 2p13 we found region 20p12 to be suggestive of linkage to asthma. 20p12 has been linked to asthma or bronchial hyper-responsiveness (BHR) in earlier studies [284, 291, 292]. BHR is a condition often analysed as an intermediate phenotype in connection with asthma. BHR is always present in asthma;

however, a person can have BHR without having asthma, as seen in patients suffering from chronic obstructive pulmonary disease.

As for the regions suggestive of linkage at T-IgE, both chromosomes 6p24 and 11q13 have been found earlier in connection with a study conducted by Haagerup et al., using the same sample represented in our study as Sample C. Thus, these regions do not represent an independent linkage replication, although the evidence for linkage has increased by increasing the sample size [127]. Chromosome 6p24 is also previously associated with asthma [285].

As for the regions we have found to be suggestive of linkage to sIgE (1p13 and 20p13) region 20p13-p12 has in previous linkage studies been associated with allergic sensitisation (Table II.3)[129] and both regions have earlier shown suggestive linkage to asthma [284].Of the regions with week linkage to sIgE three have been previously reported (11q22, 20p13 and 22q12-q13) [126, 127, 129]. Of these, 22q13 was described by Haagerup et al. and is therefore not representative of an independent replication due to sample overlap. However, the evidence for linkage has increased because of the greater sample size [127].

The following 11 regions have, in earlier publications, been linked to asthma or BHR as significant or suggestive of linkage without detection in our study: 1p31.1-p13.3, 2p14-q22.1, 4p14-q13.3, 5q31-q33, 6p22.3-p21.1, 7q34-qter, 10p14-q11.21, 11q24.1-qter, 12pter-p11.21, 14q32.12-qter and 17pter-q24 [284].

Among possible candidate genes in the studied regions, this study highlights are the following: The endothelin1 (*END1*) gene located at 6p24 is earlier associated with asthma [285]. Located at 11q13

is the β-subunit for high-affinity receptor for IgE (*FCεR-β*) gene, found to associate with atopy [194, 195]. At 11q12 the Clara cell secretory protein (*CC16*) gene is by several studies found to increase the risk for developing asthma [285, 293] as is the glutathione S-transferase P1 (*GSTP1*) gene [285]. The Squamous cell carcinoma antigen recognized by T cells 1 (*SART-1*) gene at 11q22 associate with SPT, but not with asthma [209]. At 20p13, a disintegrin and metalloproteinase domain 33 (*ADAM33*) gene has previously been associated with asthma and allergic rhinitis (AR) [164, 288]. Toll-like receptors 7 and 8 (*TLR7, TLR8*) located at Xp22, confer susceptibility to several allergic diseases, among these, AR and allergic sensibility [198]. We are not aware of any candidate genes in the 22q12-q13 region.

Inactivation of the parental or maternal copy of a gene by either methylation of the DNA or changes in the chromatin structure is known as imprinting. At chromosome 11q22, for the T-IgE phenotype the obtained MOD-score with imprinting of 3.10 support the presence of maternal imprinting despite the moderate difference between the imprinting and non-imprinting score of 0.92. A more pronounced MOD score difference of 1.44 was seen in analysing the sIgE phenotype on chromosome 3q22. Although the region only obtained a modest imprinting MOD of 2.96 it may point to maternal imprinting. At chromosome 20p12, the MOD difference was only 0.97 and the interval between P(wt/d) > P(d/wt) only 0.18. Still, combined with the high imprinting MOD of 3.42 maternal imprinting may be present. We are not aware of any earlier linkage studies concerning allergies to have reported imprinting at these regions.

In conclusion, the genome-wide search by the affected sib-pair method identified several regions that may contain a gene involved in the pathogenesis of atopy and asthma. We found evidence of suggestive linkage to asthma at 2p13 and 20p12, with 2p13 strongly approaching genome wide

significance. Both regions known from previously studies [284, 285]. Genome-wide suggestive linkage to specific IgE was obtained at an established locus (20p12) and a novel region (1p13). Linkage to total IgE was confirmed at 6p24 and 11q12-q13, however, not represent an independent linkage replication due to sample overlap [127]. NPL above two was obtained in the specific IgE phenotype at chromosomes 10q26, 11q22, 14q11, 20p13, 22q12-q13 and Xp22. High MOD scores were obtained in many of the same regions with the highest MOD scores seen for the asthma phenotype at 2p13. Possible maternal imprinting was seen at 11q22 for the T-IgE phenotype and at 3q22 and 20p12 for sIgE.

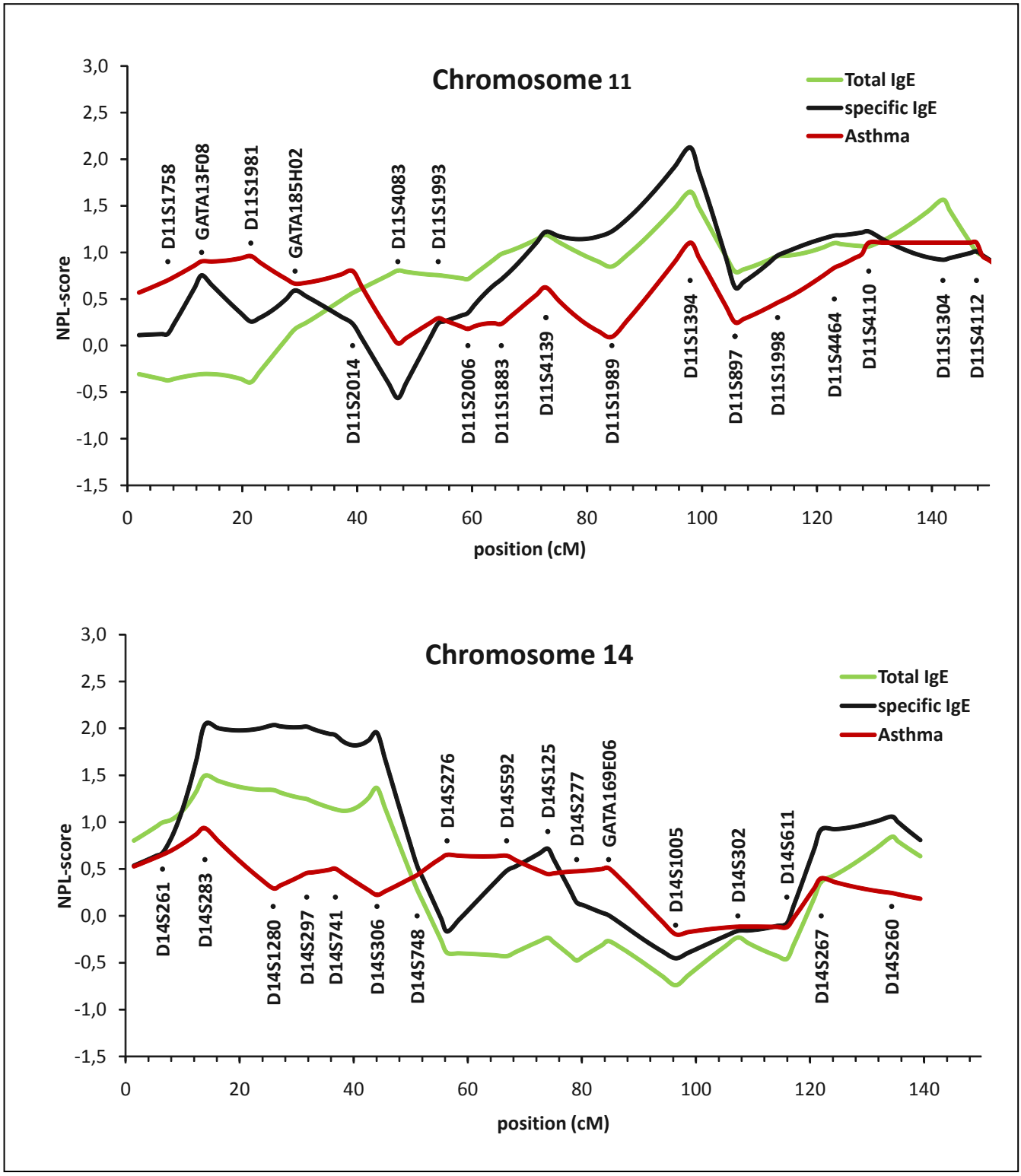
The ability to diagnose, classify and treat atopic diseases is currently met by numerous limitations. We do not fully know why some people become atopic and others do not. If we can unravel the genetics underlying these diseases, we may gain a wealth of knowledge regarding the pathophysiology, thereby clearing the way for more precise disease classification and diagnosis. Further, new findings will facilitate new and possibly even personalised treatments for atopic patients.

Table II.3: Results of published genome scans conducted in atopy.

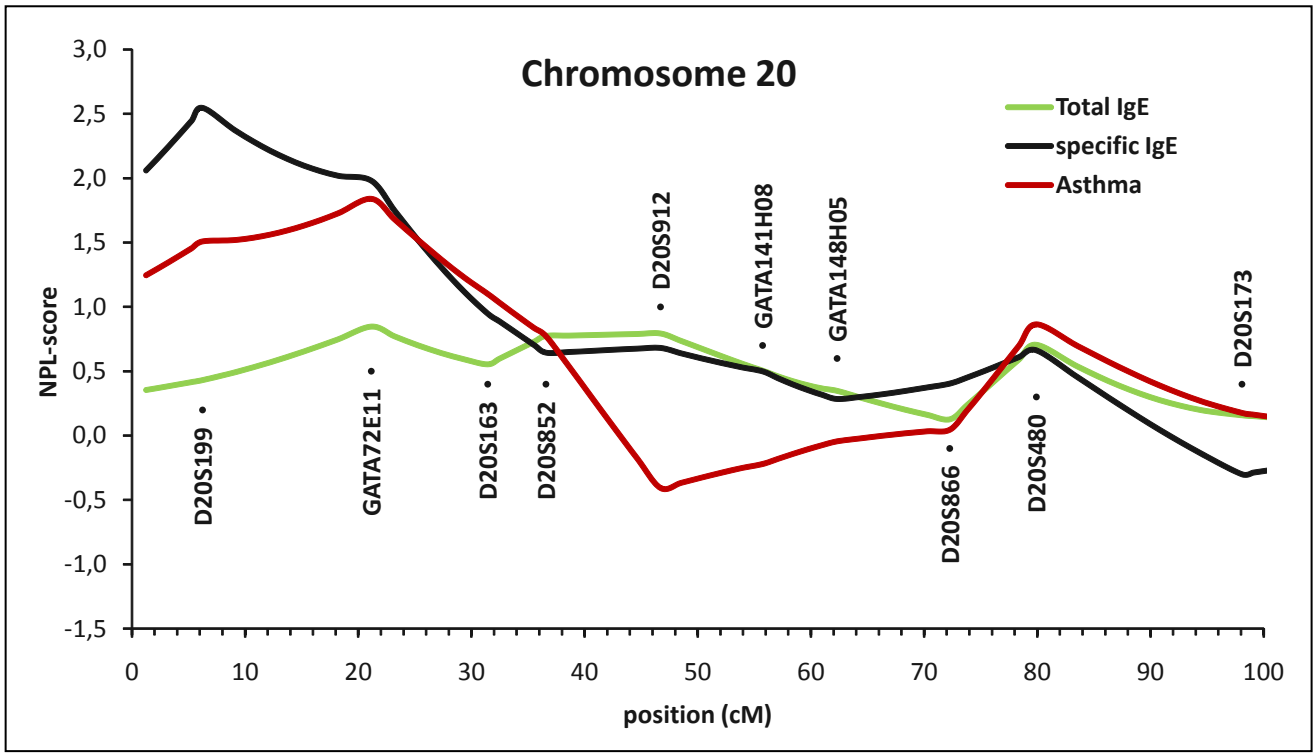
Ref. year	Phenotype	Chromosome position(s) with linkage																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	20	22	X
[122] 1996	sIgE	---	---	---	---	---	---	---	---	---	---	---	---	q14	---	---	---	---	---	---	---
[123] 1999	T-IgE	---	p21,q32	---	---	---	p22-p21	---	---	q31	---	---	---	---	---	---	---	---	---	---	---
	sIgE	---	p21	---	---	---	p21	---	---	q31	---	---	---	---	---	---	---	---	---	---	---
[124] 2000	T-IgE	---	---	---	---	**q23-q31**	---	**q21**	---	---	---	---	**q23-q24**	---	---	---	---	---	---	---	---
[125] 2000	SPT	---	---	---	---	---	---	q11.23	---	---	---	---	---	---	---	---	q12	---	---	---	---
	T-IgE	---	---	---	---	---	---	---	---	---	---	---	q21	---	---	---	---	---	---	---	---
[126] 2001	T-IgE	p31	q24-q32	**q29**	---	**q23-q31**	p21	**q11-q22**	---	---	---	---	**q23-q24**	**q12-q13**	---	---	---	---	---	---	---
	sIgE	---	---	q25-q26	---	---	---	q11-q22	---	---		q22	---	---	---	---	q25	p11	---	q11	---
	SPT	---	---	---	---	p15	---	---	p23	---	q21-q22	q22	p13	q14	---	---	q21,q25	---	---	q11	---
[127] 2002	T-IgE	---	---	**q22**	q32	**q31-q35**	**p24**-p22	---	---	---	---	q13	---	---	---	---	---	---	---	q13	---
	sIgE	p34		q13-q22	q32	q31-q35	**p24**-p22	---	---	---	---	---	---	---	---	p11	---	---	---	q13	p11, q21
[119] 2002	T-IgE	---	---	p24	---	q33	---	---	---	---	---	---	p13, q24	---	---	---	---	---	---	---	---
	sIgE	---	---	---	p16	---	---	---	---	---	---	q14	---	---	---	p12	---	---	---	---	---
[128] 2004	sIgE	---	**p12**	**q21**	---	---	---	---	---	---	---	---	---	---	---	**q21**	---	---	---	---	---
[129] 2004	SPT	---	---	---	---	q31	---	---	q12	---	---	q23	p13	---	q32	---	---	---	p13-p12	---	---
[130] 2004	T-IgE	---	---	---	---	---	---	---	p22	---	---	---	**p13**	---	---	---	---	---	---	---	---
	SPT	---	---	---	---	p15	---	---	---	---	---	---	---	q34	---	---	q22-24	---	---	---	---
[131] 2004	T-IgE or sIgE	---	---	**q21**	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
[132] 2005	T-IgE	**p36, q24-31**	---	---	---	---	---	**p14, q11,q21**	---	---	---	p15.5 q25	---	---	---	---	---	---	---	---	---
	sIgE	---	p21	---	q35	p13	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	SPT	q23	---	p14	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
[133] 2005	T-IgE	**q24-q25**	---	---	---	---		---	---	---	**q22**	---	---	---	---	---	---	---	---	---	---
	SPT	---	**q35**	**q12**	---	---	**p21**	---	---	---	---	---	---	---	---	---	**q21**	---	**q13**	---	---
[134] 2007	T-IgE	**q23**	---	---	---	q33	---	---	**q21**	---	q22	---	---	---	---	---	---	---	---	---	---
	SPT	q23	---	q22-q23	---	q31	---	---	q21	---	---	---	---	---	---	---	---	---	---	---	---

Table of references [119, 122-134]. Only positions with LOD-scores $\geq$ 1 and / or p-values $\leq$ 0.03 are shown (i.e., significance level of interest according to Haines et al.)[110]. Positions with LOD-scores $\geq$ 2.2, P $\leq$ 0.0007 (i.e., suggestive of linkage according to Lander et al.) [109] and / or MOD > 3 are written in bold.

Figure II.1: NPL-scores (Z-scores) of Sample A alone. Chromosomes with a NPL-score ≥ 2 for one of the phenotypes are shown.

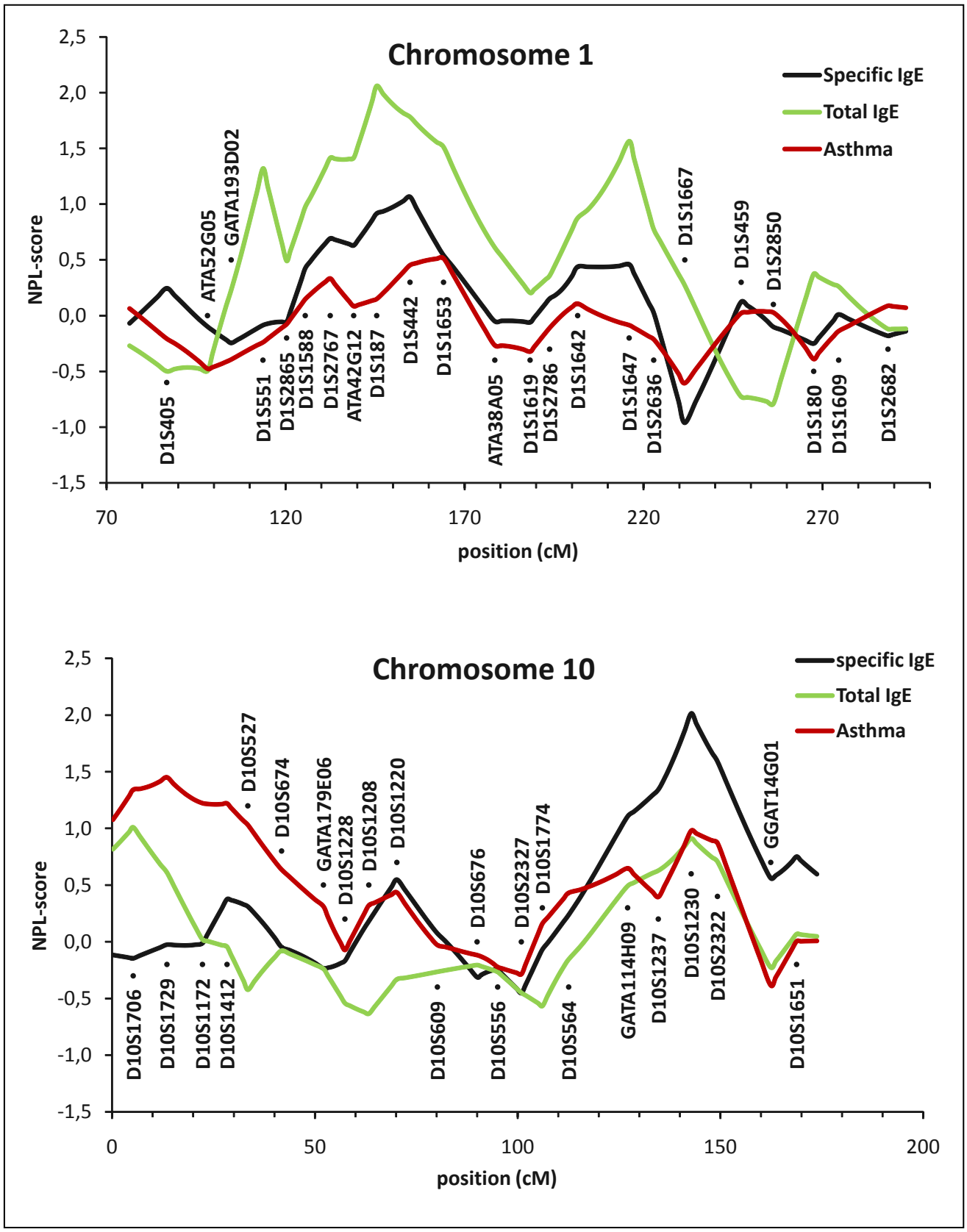


(Figure II.1 continued)

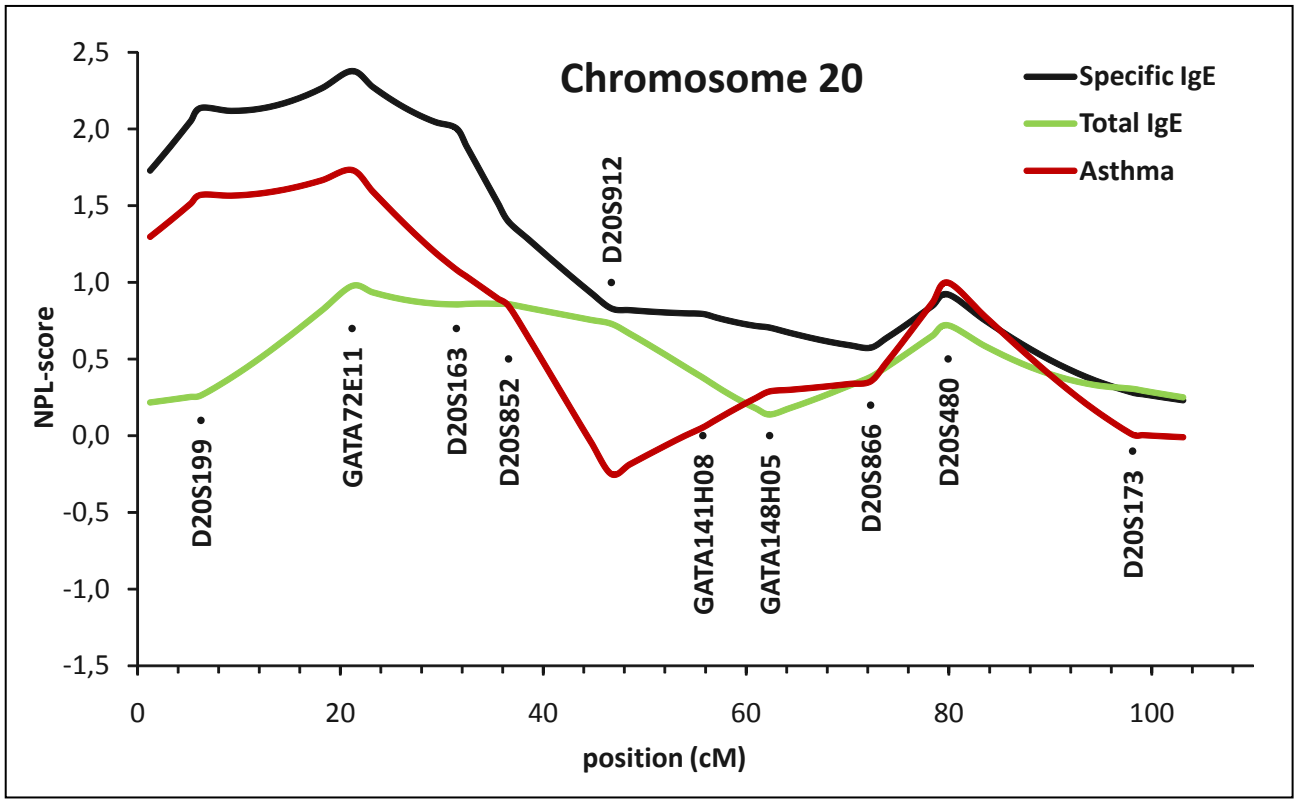


Total IgE: elevated total IgE, specific IgE: elevated specific IgE for at least one of the tested allergens (see "Phenotype definition" under "Method" for more details).

Figure II.2: NPL-scores (Z-scores) for Sample AB. Chromosomes with a NPL-score ≥ 2 for one of the phenotypes are shown.

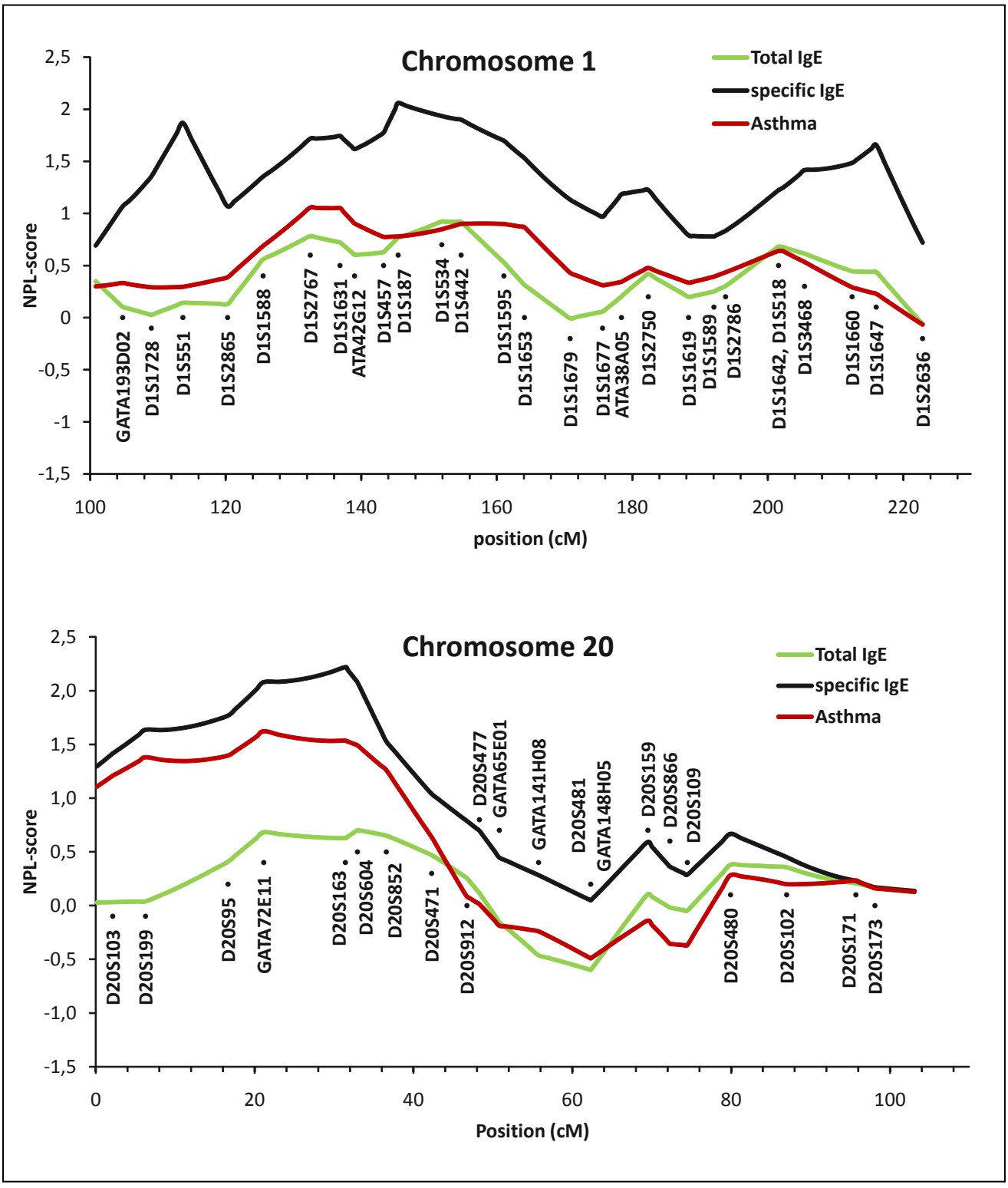


(Figure II.2 continued)

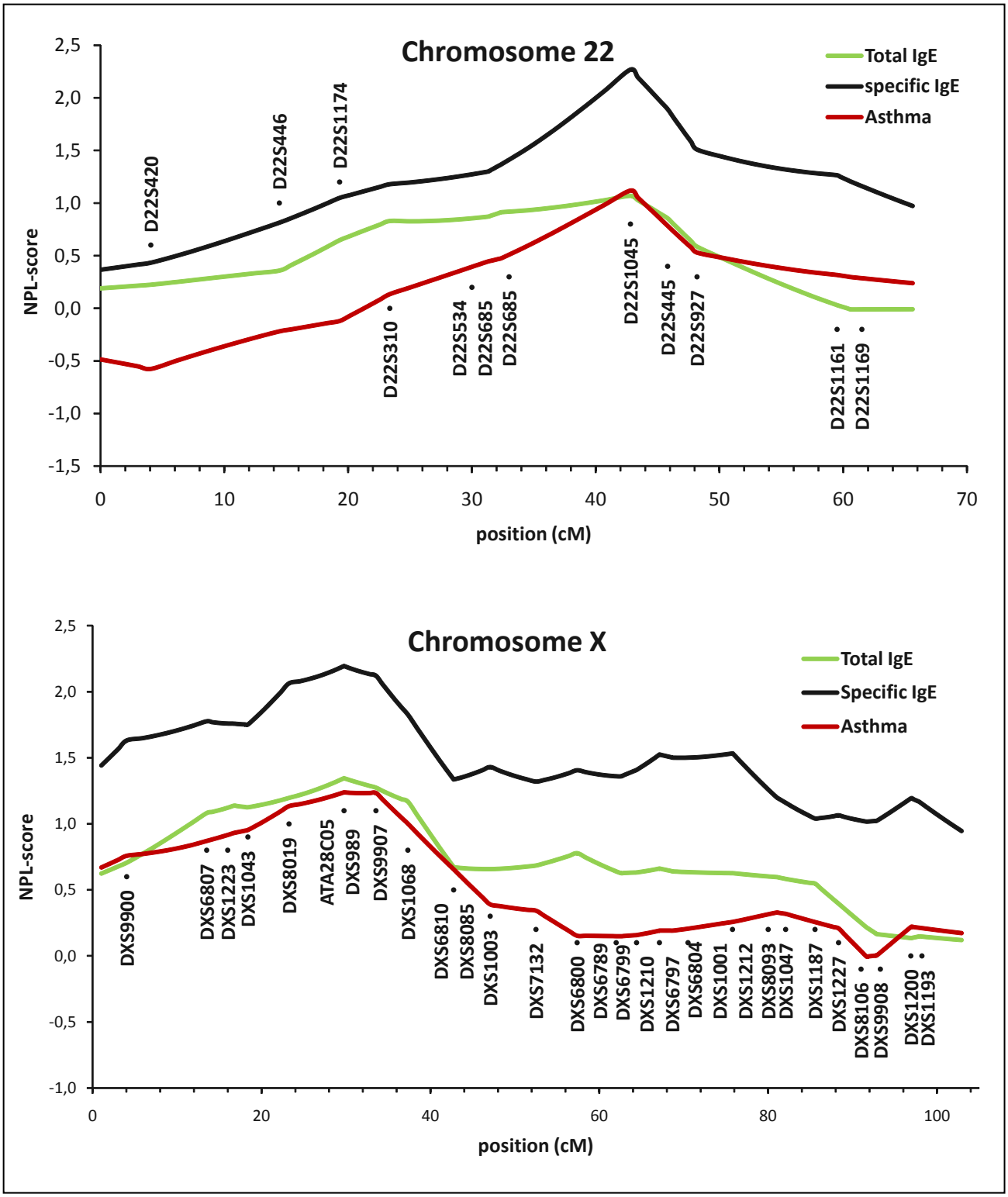


Total IgE: elevated total IgE, specific IgE: elevated specific IgE for at least one of the tested allergens (see "Phenotype definition" under "Method" for more details).

Figure II.3: NPL-scores (Z-scores) for Sample ABC. Chromosomes with a NPL-score ≥ 2 for one of the phenotypes are shown.



(Figure II.3 continued)



Total IgE: elevated total IgE, specific IgE: elevated specific IgE for at least one of the tested allergens (see "Phenotype definition" under "Method" for more details).

Acknowledgments

We thank patients and their families for their willingness to participate in the project. The continuously helpful and supportive staff at the outpatient allergy clinics in Viborg, Aarhus, Hjoerring, Herning and Aalborg and the laboratory technicians in Hjoerring and Aalborg are all greatly appreciated. The project has been funded by Regions Hospital Viborg, Health Research Fund of the Central Denmark Region, Viborg country health-science research committee, Sygeforsikringen "Danmark"s healthfund, Toyota Foundation Denmark, the A.P. Møller Foundation for the Advancement of Medical Science, Else and Helene Alstrups Fund, the Lundbeck Foundation, and Aarhus Faculty of Health Sciences, Aarhus University.

Conflict of interest

None.

Ethics approval

The Danish scientific ethics system approved the protocol (journal no. 196/3581) and all participants and/or their parents signed informed consent forms.

PAPER III:

Filaggrin mutations in a Danish hay fever cohort confers susceptibility to atopic dermatitis but not to allergic rhinitis.

Kruse L.V [1,2], Møller-Larsen S [1], Christensen U [1,3], Haagerup A [1,3], Deleuran M [4], Hansen L.G [2], Venø S.K [1], Nyegaard M [1,5], Børglum A.D [1]

1 Department of Human Genetics, Aarhus Faculty of Health Sciences, Aarhus University, Aarhus, Denmark

2 Department of Paediatrics, Region Hospital Viborg, Denmark

3 Department of Paediatrics, Aarhus University Hospital, Skejby, Denmark

4 Department of Dermatology, Aarhus University Hospital, Aarhus, Denmark

5 Department of Haematology, Aalborg Hospital, Aalborg, Denmark

Abstract

Background: Several studies have found an association between the filaggrin (*FLG*) gene and atopic dermatitis (AD) as well as atopy. A possible association between *FLG* and other atopic conditions such as allergic rhinitis (AR), asthma and elevated total IgE (T-IgE) without the presence of coexisting AD has still not been fully investigated. Therefore, we further explored the possible connection between *FLG* and AR, asthma and T-IgE with and without the influence of AD in the samples as well as the association between *FLG* and AD and allergic sensitisation.

Methods: We collected and clinically characterised a Danish sample consisting of 127 nuclear families with at least two siblings suffering from AR or allergic conjunctivitis, including 540 individuals (286 children and 254 parents). We combined this sample with a previously collected sample comprised of 132 Danish nuclear families collected with the focus of AD among the children reaching a total of 259 families comprising 1,099 individuals of whom 157 families segregated AR (341 AR children). Family based association analysis of single SNPs and haplotypes was performed using the software FBAT V2.0.3.

Result: Two SNPs out of four (2282del4 and R501X) in the *FLG* gene were significantly associated with AD. No association to AR, asthma or allergic sensitisations was found without coexisting AD. *Conclusion:* Susceptibility of *FLG* with AD is affirmed. No association between *FLG* and AR or asthma was found when the influence of co-existing AD is removed.

Introduction

Approximately one thousand studies concerning genes associated with allergic diseases have been published over the last decades [294]. The genes identified that contribute to allergic disease can be placed into four broad groups. The first group contains genes involved with sensing the environment. The genes in the second group are responsible for maintaining the integrity of the epithelial barrier; therefore, they take part in primary defence. The third group of genes regulate the atopic immune response. Finally, the fourth group contains genes involved in tissue response to chronic inflammation [104]. Filaggrin falls into group two and is a part of the epidermal differentiation complex (EDC), which is a cluster of genes on chromosome 1q21 involved in terminal differentiation of the human epidermis [295]. Single nucleotide polymorphisms (SNPs) in the filaggrin (*FLA*) gene have been strongly associated with atopic dermatitis and allergic sensitisation [260]. Interestingly, *FL*G mutations have also been associated with asthma and allergic rhinitis (AR), primarily in children with coexisting atopic dermatitis (Table III.1). The filaggrin protein (FLG) is expressed in the lower part of the stratum corneum of the skin and in the vestibulum of the nose [296, 297]. FLG along with other proteins in the EDC form aggregates at the cellular cytoskeleton, and a protein layer develops that is important for binding lipids and regulating skin permeability to water and external particles, such as allergens [296, 298]. Filaggrin is not expressed in the mucosa of the nose or the lungs; however, it is not clear if a direct connection exists between filaggrin loss of function and allergies of the airways [297, 298]. Nevertheless, it is

possible that loss-of-function mutations in FLG predisposes people to asthma and AR through an increased risk of sensitisation after allergen contact with the skin.

Table III.1: Candidate gene studies on Filaggrin mutations and allergic rhinitis.

Phenotype	Reference Year	Population	Investigated SNPs	Sample size	Significant association yes/no	Ref
AR	Chawes et al. 2010	Danish	A	38 cases, 185 controls (33% with AD)	y	[163]
AR with AD	Marenholz et al. 2006	German European	A	44 cases, 314 controls 248 families	y	[158]
	Weidinger et al. 2007	German	A	167cases, 252 controls	y	[159]
	Ekelund et al. 2008	Swedish	A	406 families	y	[160]
	Weidinger et al. 2008	German	C	124 cases, 2782 controls	y	[161]
	Schuttelaar et al. 2009	Netherlands	B	934 cases, 663 controls	y	[162]
AR without AD	Weidinger et al. 2008	German	C	124 cases, 2782 controls	y	[161]
	Brown et al. 2008	British	C	152 cases, 599 controls	n	[243]
	Henderson et al. 2008	British	A	779 cases, 3849 controls	n	[245]
	Aslam et al. 2010	European	A	26 cases, 25 controls	n	[252]
AR without atopy	Chawes et al. 2010	Danish	A	66 cases, 185 controls	n	[163]

A SNPs: R501X, 2282del4. *B SNPs*: R501X, 2282del4, R2447X. *C SNPs:* R501X, 2282del4, R2447X, S3247X, 3702delG

Methods

Subjects

We included 127 Danish nuclear families with at least two siblings with AR or allergic conjunctivitis in the study (Sample A) containing in total 286 children and 254 parents. This study was conducted through the paediatric departments in Viborg, Aarhus, Hjoerring, Herning and Aalborg, Department of Respiratory Diseases, Aarhus University Hospital and one private paediatric clinic in Aalborg. All participants were clinically examined and questionnaire tested by the same doctor. The questionnaire contained questions regarding symptoms, duration and treatment of allergic rhinitis, allergic conjunctivitis, atopic dermatitis and asthma. Atopic disease was diagnosed according to standard criteria [19](Table III.2). The diagnoses were evaluated by a second doctor who assessed the questionnaires alone. Individuals with uncertain diagnoses were not

included in the analysis. The mean age among the children was 16 years, and 52% were male. Blood was drawn from all participants for DNA analysis and for serum measurements of total IgE and allergen-specific IgE antibody to 12 common allergens (Phadia Aps, Allerød, Denmark).
An earlier collected Danish sib-pair family sample (sample B) was included for genotyping and linkage analyses. Sample B [280] contained 132 Danish nuclear families with at least two full sibs with AD (Table III.2). The mean age among the children was 12.5 years, and 48% were male. Thus, in total, 581 children (382 with AR and 398 with AD) and 518 parents from 259 Danish nuclear families were analysed in the present study. All participants gave informed consent prior to inclusion in the study.

Phenotype definition

Total IgE (T-IgE) varied widely with age (https://www.sundhed.dk/artikel.aspx?id=13231.1). T-IgE was considered elevated if $\geq$ 150 kU/l for children between 6 and 14 and $\geq$ 100 kU/l for the remaining participants. The specific IgEs (sIgE) were tested for reactivity with twelve allergens: mite (*D. pternyssinus* and *D. farinae*), cat, horse, dog, timothy grass, birch, mugwort and mold (*Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum* and *Penicillium chrysogenum*). A specific IgE $\geq$ 0.35 kU/l was considered elevated.
An individual was classified as "positive specific IgE" when the sIgE was elevated against at least one of the tested allergens. Atopic disease was diagnosed according to standard criteria [19]. IgE associated allergic diseases was defined as the present phenotype combined with a positive sIgE antibody for at least one of the tested allergens.

Table III.2: Distribution of families according to the number of genotyped sibs available for linkage analyses

Phenotypes	**No. Of sibs**				**Total no.**
Sample A	**1**	**2**	**3**	**4**	**of families**
Hay fever combined (HC)[A]	3	106	16	2	127
Allergic rhinitis (AR)	7	105	12	2	126
IgE associated AR[B]	24	87	9	0	120
Atopic dermatitis (AD)	44	27	5	1	77
IgE associated AD[B]	47	22	3	0	72
AR without AD	47	52	3	0	102
AR with coexisting AD	51	22	2	0	75
Asthma	53	35	3	0	91
IgE associated Asthma[B]	51	32	2	0	85
Asthma without AD	44	10	1	0	55
Pos. specific IgE (sIgE)	18	94	11	0	123
Elevated total IgE (T-IgE)	45	48	5	0	98
Sample B					
Hay fever combined (HC)[A]	45	34	5	1	85
Allergic rhinitis (AR)	42	32	5	1	80
IgE associated AR[B]	43	28	3	1	75
Atopic dermatitis (AD)	3	111	16	2	132
IgE associated AD[B]	50	49	7	2	108
Asthma	40	23	5	1	69
IgE associated Asthma[B]	38	21	2	1	62
Pos. specific IgE (sIgE)	46	52	9	1	108
Elevated total IgE (T-IgE)	38	32	4	1	75

[A] The phenotype *and* a positive specific IgE antibody for at least one of the tested allergens.

[B] Hay fever combined (HC) contains subjects with clinical symptoms of AR and / or allergic conjunctivitis

Genotyping

The selection of SNPs was based on findings in earlier studies (Table III.1). Genotyping was performed using the Sequenom MassARRAY Genotyping system (Sequenom, San Diego, CA). Primers for PCR and extension probes were designed using the MassARRAY Assay Design 3.1 software (Sequenom). Multiplex PCR was performed in 5 µl reactions containing 10 ng of genomic DNA, 1.25x PCR buffer (Qiagen, Valencia, CA), 0.5 mM dNTPs (Roche, Geneva, Switzerland), 100 nM of each primer (Metabion, Martinsried, Germany) and 0.5U Taq polymerase (Qiagen), using the standard cycling conditions described by Sequenom. The PCR products were treated with arctic shrimp alkaline phosphatase (SAP), and the probe extension reaction (iPLEX) was performed in accordance with its standard protocol (Sequenom). The iPLEX reactions were desalted using resin and spotted on a SpectroCHIP (Sequenom) using a nanodispenser. The samples were analysed

using a Bruker matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometer (Sequenom), and the genotypes were determined using the MassARRAY Type 4.0 software (Sequenom). The primer sequences used were as follows: 5'-ACGTTGGATGATGGTGTCCTGACCCTCTTG, 5'-ACGTTGGATGGAGGAAGACAAGG ATCGCAC and 5'-GCCTGGAGCTGTCTC (extension primer) for R501X (rs61816761); 5'-AC GTTGGATGGTGAGGGACATTCAGAAGAC, 5'-ACGTTGGATGTTGGTGGCTCTGCTGAT GGT and 5'-CTACGACTCAGACACACAGT (extension primer) for 2282del4 (no rs number exists); 5'-CGTTGGATGCATGAATGGTGTCCTGACCC, 5'-ACGTTGGATGAAGACAAG GATCCCACCACA and 5'-GCCTGGAGCTGTCTC (extension primer) for R2447X (no rs number exists); and 5'-ACGTTGGATGTGTCTTCGTGATGGGACCTG, 5'-ACGTTGGATGTGCTTCC AGAAACCATCGTG and 5'-GGTGGCTGGAGCCGTGCCTT (extension primer) for S3247X (no rs number exists). Testing for Mendelian inheritance utilised the FBAT V.2.0.3 programme [115-117]. In case of inconsistencies, all genotypes for the problematic SNP were removed within the nuclear family.

Statistical analysis

Family based association analysis of single SNPs and haplotypes was performed using the software programme FBAT V2.0.3 [115-117]. The data were analysed using an additive inheritance model. This model corresponds to the original TDT test and has been shown to perform well in several situations where the true inheritance model is unknown [115]. In addition, due to a substantial gain in power (results section), we also analysed using a recessive model. Power calculations were performed using PBAT v. 3.61 [299, 300], and the calculations were conducted on the combined allele, which combines the status of the common loss of function mutations (i.e., a compound heterozygote is treated as a homozygote) assuming that they have the same effect on the phenotype.

The allele frequency of the common allele was calculated using data from Thyssen et al. [301]. We used the FBATs ability to test different null hypotheses, namely "no linkage and no association" as well as "no association in the presence of linkage". In calculating the empiric p value for the latter hypothesis, one avoids the cofounding that occurs with more than one affected child per pedigree [302, 303], which would result in an inflation of the p values. The calculations were based only on the informative families. For a family to be informative, the parents must be heterozygous for the allele in question, which makes it possible to determine which parent contributed which allele to the child.

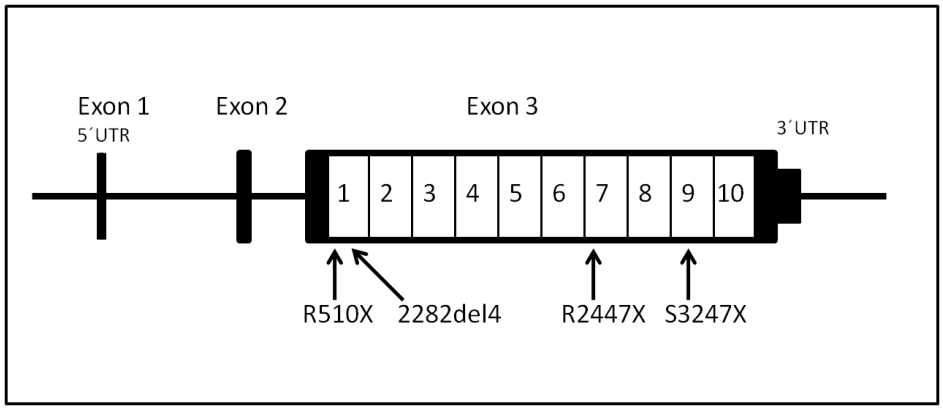


Figure III.1: Structure of the filaggrin (FLG) gene (please note, that the drawing is only a schematic). FLG is made of three exons. Exon 1 and exon 2 are quite short (15 bp and 159 bp long, respectively) and contain only the 5` untranslated region sequence (5ÚTR) and the translation initiation cordon, respectively. The coding sequence for almost all EDC genes is contained in the terminal exon 3. Exon 3 (12-14 kb) consists of 10 to 12 filaggrin repeats (in some individuals the 8th and/or the 10th filaggrin repeat is/are duplicated) and the N-terminal domain. The location of the four mutations genotyped in this article are shown [251, 295, 304].

Results

Four SNPs within the *FLG* gene were genotyped (Figure III.1). R2447X and S3247X were not polymorphic in our population; therefore, we only further analysed 2282del4 and R501X.

All families in sample B had AD or coexisting AD. Overall, 59% of the individuals in sample A had coexisting AD (Table III.3). The results of the association analysis of SNP 2282del4 and R501X analysed individually and combined can be seen in Table III.4.

Table III.3: Sample A

Fraction of coexisting AD in AR families	
Family description	**Coexisting AD percent / N**
Families with 1 AR sibling	N=7
Coexisting AD in 1 sibling	57% / 4
Families with 2 AR siblings	N=105
Coexisting AD in 1 sibling	40% / 42
Coexisting AD in 2 siblings	17.1% / 18
Families with 3 AR siblings	N=12
Coexisting AD in 1 sibling	41.7% / 5
Coexisting AD in 2 siblings	16.7% / 2
Coexisting AD in 3 siblings	16.7% / 2
Families with 4 AR siblings	N=2
Coexisting AD in 2 siblings	100% / 2

The analysis of SNP 2282del4 revealed that in sample A and B combined (Sample AB), a statistical significant association was found for all the investigated phenotypes except AR and IgE associated AR (IgE-AR), with the strongest association to the phenotypes AD and IgE associated AD (IgE-AD, empiric p values of 3.9×10^{-4} and 3.7×10^{-3}, respectively) (Table III.4). When analysing sample B alone, all phenotypes reached a level of significance (empiric p value ≤ 0.05). In sample A, all of

the obtained empiric p values were above 0.05; however, only IgE associated AD in sample A reached a statistical significant nominal p value of 0.029 (Table III.4).

When analysing SNP R501X alone, the tendency of the 2282del4 was repeated with statistically significant empiric p-values obtained in sample B and sample AB, with the highest score found in sample AB in association with the phenotypes AD and IgE-AD (empiric p values of $9.7x10^{-4}$ and $4.5x10^{-3}$, respectively) (Table III.4).

Analysing 2282del 4 and R501X in combination demonstrated that sample A reached statistically significant p values with phenotypes AD and IgE-AD alone (empiric p values of 0.028 and 0.04, respectively). Sample B showed a statistically significant association with all the investigated phenotypes. In sample AB, statistically significant p values were obtained in all the investigated phenotypes but IgE associated AR (IgE-AR) (Table III.4).

In the power calculations, using a significance level of 0.05, a combined allele frequency of 0.0417, and a disease prevalence of 0.2, we were able to sustain a power above 0.8 for a population attributable risk as low as 0.075 for the additive model and 0.016 for the recessive model in the smallest sample. In the combined samples, the corresponding numbers were 0.052 for the additive model and 0.013 for the recessive model.

Discussion

We genotyped four *FLG* SNPs in a large combined Danish sib-pair sample and found statistical significant association with the 2282del and R501X with most of the investigated phenotypes, specifically AD and IgE-AD.

When minimising the influence of co-existing AD, no association with AR, asthma, atopy or T-IgE was found, indicating that the association is driven mainly by AD (Table III.4).

We collected a relatively large sample consisting of families with at least two children with AR (sample A). In diagnosing the participants, we were quite meticulous as described under "methods". By refining the AR phenotype to the defined IgE-AR, we hoped to further minimise the heterogeneity. This important aspect in study design provides the opportunity to determine whether *FLG* confers AR risk independent of AD. Another strength of our study is the homogeneity among the Danish population with the majority being white Caucasians.

Two of the four tested genetic variations were polymorphic in our population. The R501X and 2282del4 FLG mutations are quite common in the continental European population with a minor allele frequency of approximately 3.5% and 3.8%, respectively [305]. In most parts of Europe, the carrier frequency of R501X and 2282del4 in subjects with AD is estimated to be 9% and 14.7%, respectively. A remarkably higher carrier frequency is observed in the United Kingdom and Ireland, reaching 38.5% for R501X and 26.9% for 2282del4, which may reflect ethnic differences [305].

To our knowledge, no study exists describing the carrier frequencies of all four SNPs in a Danish population, but a recent German study by Weidinger et al. found that the carrier frequency of R501X was 1.9% among the children in the study and 4.6% for the 2281del4 mutation. The more

rare FLG mutations R2447X and S3247X were present in 0.7% and 0.2% of the children, respectively. The SNP 3702delG was also genotyped, but the carrier frequency could not be subsequently identified in the study population [161]. If the same relationship between the *FLG* mutation frequencies is present in Denmark, it would explain why the SNP 2282del4 and R501X are polymorphic in our population and R2447X and 3702delG are not.

Our finding of a convincing association of *FLG* with atopic dermatitis has been described earlier in several studies [260]. Surprisingly, we found the association to be strongest in the mixed AD group in all three sample settings and smaller in the atopic AD group both when testing the 2282del4 and R501X SNPs alone and combined. Other studies have found the association between *FLG* and AD to be increased in the atopic subgroup of AD [159, 160, 193, 232].

FLG was only associated with AR in our study in the presence of a high share of coexisting AD as seen in sample B. The association faded below the level of significance when samples A and B were combined for all settings except when analysing SNP 2282del4 and R501X combined (empiric p value=$8.5x10^{-3}$). When analysing sample A alone, no association was observed (p values > 0.1), with sample A being the sample with the lowest share of coexisting AD.

Similar tendencies were registered when analysing the phenotypes of asthma, IgE-asthma, T-IgE and sIgE where an association to *FLG* was present only in the context of a high share of coexisting AD (Table III.4).

Five published studies have shown an association of *FLG* and AR with coexisting AD. In only two of nine earlier studies, the association between *FLG* and AR remained after adjusting for AD [161, 163] (Table III.1). Three studies showed no association of *FLG* and AR without coexisting AD [243, 245, 252]. Thus, this area in *FLG* research has contradicting results. Several reasons for the

low reproducibility between the association studies concerning *FLG* and AR can be considered. First, the method of diagnosing the AR patients varies widely between studies. For example, some phenotypes were based on the diagnosis of doctors after personal contact with the participants of the study [160, 163]. Three studies have based the diagnosis solely on answers to standardised questionnaires [162, 243, 245]. The design of these questionnaires are also quite different (namely "Has your child ever had hay fever" [162], "In the past 12 months, has your child had a problem with sneezing, runny or blocked nose when he/she did not have a cold or the flu" [245] and, "Has your child ever suffered from hay fever? By hay fever we mean bouts of sneezing with a runny nose or itchy eyes in the summer.") [243]. Some diagnosed the participants as having AR when the parents reported that a physician had diagnosed the child with AR [158, 159, 161]. In one study, the actual method of diagnosing AR was not clear [252]. In all but one study [161], the AR diagnosis was independent of specific IgE testing or a skin prick test. Clearly, this inconsistency in defining AR may result in a genetic heterogeneity between studies. Finally, low statistical power and variations in the statistical methods used, as well as differences in the underlying population genetics may explain the divergent results.

Inadequate statistical power from small sample size and low minor allele frequencies can cause type II errors (false-negative results) in studies of genetic association. We can not rule out that our material is underpowered because the effect a variant has on a disease is also important. The population attributable risk for AD for the loss of function mutations has been estimated to 13.5 % [249]. It is likely that the effect on asthma and AR is smaller. However, our power calculations showed that in the recessive analysis of the combined samples, we would be able to sustain a power above 0.8 even if the effect was as low as a tenth of this.

The p values reported in this study are not corrected for multiple testing because the majority of tests were not independent, being carried out on the same set of subjects and investigating related

phenotypes. However, Bonferroni correction of the p values would still leave significant association between the investigated SNPs and AD and IgE-AD in sample B and AB and an association to asthma, IgE-asthma, T-IgE and sIgE in connection with coexisting AD.

We note that our conclusions of no association between *FLG* and AR after adjusting for AD differs markedly from the findings of Chawes et al. who found an association between IgE associated AR and *FLG* in children by the age of 7 years (OR, 3.3; 95% CI, 1.3-8.3; p=0.01) [163] and Weidinger et al. who showed a significant association of *FLG* and AR also after stratifying for eczema (OR, 2.5; 95% CI, 1.4-4.3; p= 0.0015) [161](Table III.1).

In summary, our findings confer the important role of *FLG* in the development of AD. As earlier reported, our analysis showed an association of *FLG* to the phenotypes of asthma, IgE associated asthma, total IgE and specific IgE in connection with coexisting AD and a weak association between *FLG* and AR and IgE-AR again in connection with coexisting AD. No association was seen between *FLG* and AR or asthma when testing a sample with only a few AD patients, suggesting no genetic association of *FLG* and AR in the absence of AD.

Great attention in defining the diagnosis should be taken in future work with candidate genes involved in the pathogenesis of AR, which will hopefully increase the reproducibility of the research.

Acknowledgments

We thank the patients and their families for participating in the project. The helping and supporting staff at the outpatient allergy clinics in Viborg, Aarhus, Hjoerring, Herning and Aalborg, and the laboratory technicians in Hjoerring and Aalborg are greatly acknowledged.

Funding

This project has been funded by the Region Hospital Viborg, the Health Research Fund of Central Denmark Region, the Viborg country health-science research committee, Sygeforsikringen "Danmark"s healthfund, the Toyota Foundation Denmark, The A.P. Møller Foundation for the Advancement of Medical Science, the Else and Helene Alstrups Fund, The Lundbeck Foundation, the Aarhus Faculty of Health Sciences at Aarhus University and the Villum Kann Rasmussen Foundation.

Conflict of interest

None.

Ethics approval

The Danish scientific ethical system approved the protocol (journal no. 196/3581) and all participants and/or their parents signed informed consent forms.

Table III.4

Family based association study of Filaggrin mutation

Phenotypes	AD	IgE-AD[A]	HC	AR	IgE-AR[A]	Asthma	IgE-Asthma[A]	T-IgE	sIgE
Panel A: association of SNP 2282del4									
Sample A									
No. of inf. fam.	11	12	10	11	10	11	12	11	11
Empiric p value[B]	0.108	0.061	0.739	0.869	0.631	0.705	0.479	0.371	0.655
Sample B									
No. of inf. fam.	20	18	17	17	15	16	14	14	18
Empiric p value[B]	**$1.5x10^{-3}$**	**0.024**	**0.027**	**0.027**	0.053	**$7.8x10^{-3}$**	**0.035**	**0.043**	**0.024**
Sample AB									
No. of inf. fam.	32	31	27	28	25	28	27	25	29
Empiric p value[B]	**$3.9x10^{-4}$**	**$3.7x10^{-3}$**	0.051	0.067	0.081	**0.018**	**0.039**	**0.028**	**0.048**
Panel B: association of SNP R501X									
Sample A									
No. of inf. fam.	8	8	7	8	7	7	7	8	5
Empiric p value[B]	0.109	0.366	0.394	0.532	0.782	0.059	0.059	0.467	0.763
Sample B									
No. of inf. fam.	17	22	16	17	17	15	12	14	22
Empiric p value[B]	**$3.9x10^{-3}$**	**$6.6x10^{-3}$**	0.087	0.063	0.077	**0.023**	**0.029**	**0.028**	**$6.6x10^{-3}$**
Sample AB									
No. of inf. fam.	25	30	23	25	24	22	19	22	27
Empiric p value[B]	**$9.7x10^{-4}$**	**$4.5x10^{-3}$**	0.061	0.066	0.101	**$4.6x10^{-3}$**	**$5.2x10^{-3}$**	0.166	**$9.8x10^{-3}$**
Panel C: association of SNP 2282del4 and R501X combined									
Sample A									
No. of inf. fam.	18	20	16	18	17	17	19	19	16
Empiric p value[B]	**0.028**	**0.040**	0.439	0.611	0.579	0.250	0.150	0.869	0.593
Sample B									
No. of inf. fam.	29	28	25	26	24	21	19	20	28
Empiric p value[B]	**$2.6x10^{-5}$**	**$6.7x10^{-4}$**	**$3.9x10^{-3}$**	**$2.9x10^{-3}$**	**$7.9x10^{-3}$**	**$2.5x10^{-3}$**	**$7.3x10^{-3}$**	**$2.8x10^{-3}$**	**$6.7x10^{-4}$**
Sample AB									
No. of inf. fam.	47	48	41	44	41	38	38	39	44
Empiric p value[B]	**$2.2x10^{-6}$**	**$7.1x10^{-5}$**	**$6.2x10^{-3}$**	**$8.5x10^{-3}$**	0.017	**$1.4x10^{-3}$**	**$2.4x10^{-3}$**	**$9.5x10^{-3}$**	**$2.0x10^{-3}$**

[A] The phenotype *and* a positive specific IgE antibody for at least one of the tested allergens. [B] P value based on the null hypotheses "no association in the presence of linkage".
AD: atopic dermatitis, IgE-AD: IgE associated AD, HC: Hay fever combined (Allergic rhinitis and/or atopic conjunctivitis), AR: allergic rhinitis, IgE-AR: IgE associated AR, IgE-Asthma: IgE associated asthma, T-IgE: elevated total IgE, sIgE: elevated allergen specific IgE for at least one of the tested allergens. Empiric p values ≤ 0.05 are written in bold. Sample AB: Samples A and B combined.

General discussion and future perspectives

Results

Genome wide linkage analysis of allergic rhinitis.

In summary, we obtained genome wide suggestive linkage to AR at 1p13, 2q14-q23, 2q23, 12p13 and 20p12 with 1p13 showing close to genome wide significance. Indication of linkage was seen to loci 1q21, 14q12, 22q13; Xp11 and Xp22-p21 (NPL $\geq$2). MOD-scores above four with and without imprinting strongly support the association of regions 1p13 and 20p12. Likely maternal imprinting was found at chromosome 2q23.3, and possibly maternal imprinting at chromosome 3q28. Analysis of the AR sub-phenotypes PAR, IAR, "mild symptoms" and, "moderate-severe symptoms" resulted in a possible linkage of PAR to chromosome 21q22.

Genome wide linkage analysis of atopy and asthma.

We found evidence of suggestive linkage to asthma at 2p13 and 20p12, with 2p13 strongly approaching genome wide significance. Genome-wide suggestive linkage to specific IgE was obtained at 1p13 and 20p12. Linkage to total IgE was confirmed at 6p24 and 11q12-q13. NPL above two was obtained in the specific IgE phenotype at chromosomes 10q26, 11q22, 14q11, 20p13, 22q12-q13 and Xp22. High MOD scores were obtained in many of the same regions with the highest MOD scores seen for the asthma phenotype at 2p13. Possible maternal imprinting was seen at 11q22 for the T-IgE phenotype and at 3q22 and 20p12 for sIgE.

Family based association analysis of filaggrin gene to allergic rhinitis and other atopic diseases.

We investigated the possible association of mutations in the *FLG* gene with AR and found no

association when the influence of co-existing atopic dermatitis (AD) was removed. Susceptibility to AD conveyed by *FLG* mutations was affirmed.

Discussion

To date, considerable research effort has focused on the genetic basis of asthma and, to some extent, AD. However, AR has been somewhat neglected. This atopic phenotype/disease is vastly overlooked, possibly as a consequence of the many patients with very mild symptoms. However, AR ranges in severity from mild to seriously debilitating and is a condition with tremendous financial impact on societies around the world, mainly due to absences at work and school, loss of productivity and treatment costs [306, 307].

When AR is specifically studied, it is, as described in paper I, often as a subgroup of a previously collected sample focussing on another atopic phenotype (i.e. an asthma sample), possibly resulting in selection bias.

The linkage analyses of our novel AR sample supported the previous reported linkage to AR of regions 2q23, Xp21 and Xp22 by Haagerup et al. [118]. No other published studies share our findings. An obvious reason for this would be the very scarce number of publications regarding genome scans and AR, and a likely possibility that would account for the deviations among reported studies could be the variations in study design, especially in defining the phenotype investigated.

A more clearly defined phenotype is one of the most essential components in improving a genetic study. When selection criteria vary between studies, reproducibility is weakened. This aspect proves a challenge when it comes to complex, yet common, diseases, such as the allergic diseases. The description of a person with AR may differ widely, depending on whether it is asked of a non-

professional, a general practitioner or a specialist in allergic diseases. To confirm that all subjects in a sample actually present the symptoms of AR, it is important to examine and interview each person and preferably combine the diagnosis with defined test measurements, such as specific IgE or results from a skin prick test. It is not adequate to simply base the diagnosis on information gathered from a questionnaire, from questions such as "Has your child ever suffered from allergic rhinitis?" In addition, most patients with allergies have more than one concurrent allergic disease. When searching for genes underlying AR, one must be aware of other allergic diseases among the test subjects that may influence the findings (i.e. asthma). It may not be possible to eliminate these concurrent diseases in the test group, but they must be considered in the interpretation of test results.

The substantial heterogeneity in the presentations of allergic diseases, with respect to age of onset, severity and response to treatment, among others, also poses a challenge in genetic research. These differences in clinical manifestations are likely to work via different pathways and involve different genes, potentially reducing the power of the genetic study. One way of making the sample more homogeneous is by narrowing the phenotype. However, narrowing the phenotype results in smaller sample sizes, which also tend to reduce the power of the genetic study. We investigated more narrowly defined AR phenotypes by combining the clinical AR diagnose with a positive sIgE (the IgE-AR phenotype) and also by dividing the AR phenotype according to the ARIA guideline [1]. The result of the ARIA subdivision was fairly small sample sizes and none of these sub-phenotypes obtained results significant or suggestive of linkage based on empirical data (simulations). However, for the sub-phenotype PAR, the NPL score exceeded two at chromosome 21q22 thereby attracting attention. The region 21q22 has not been found in earlier studies and with the limited number of children in this group (N=81) possible type I error (false positive) should be considered.

The suggestive evidence of linkage to asthma obtained at 2p13 and 20p12 has in previous studies been associated with asthma or BHR. This increases the likelihood that these regions indeed may harbour true susceptibility genes for asthma. However, genetic studies on asthma are complicated by differences in defining the asthma phenotype. In our study, the phenotype was defined solely by answers given in a questionnaire. Some studies involve tests in defining the phenotype, e.g. peak flow measurements or reversibility-test [133] and other studies look at asthma exacerbation [259]. Investigating the more biologically defined intermediate phenotypes, T-IgE and sIgE [5-7], was an attempt to reduce the genetic heterogeneity and thereby increase the power of the analysis.

The two regions showing suggestive linkage to T-IgE (6p24 and 11q12-q13) has been reported in a previous study by Haagerup et al [127]. This study investigated one of the samples included in our study (Sample C). Thus, the present findings do not represent an independent replication, although the evidence of linkage was increased by adding the new samples (A and B). Chromosome 6p24 is also previously associated with asthma [285].

As for the two regions showing suggestive linkage to sIgE (1p13 and 20p13), region 20p13-p12 has in previous linkage studies been associated with allergic sensitisation (Table II.3, page 95)[129] and both regions have earlier shown suggestive linkage to asthma [284]. Of the regions with week linkage to sIgE three have been previously reported (11q22, 20p13 and 22q12-q13) [126, 127, 129]. Of these, 22q13 was described by Haagerup et al. [127] and is therefore not an independent replication due to sample overlap. However, the evidence for linkage has increased because of the greater sample size [127]. The sharing of genetic susceptibility regions between the atopy markers and asthma confirms these markers as being intermediate phenotypes of asthma, underlining the importance of atopic pathophysiology in the clinical expression of asthma.

When we investigated the possible association of *FLG* and AR, we found a field represented by contradictory results in the published literature (see Table 7, page 49). Analysing these publications, we found quite large differences in how the AR phenotype had been defined. As mentioned, this provides a major potential for selection bias and consequently minimises the comparability between studies. Having a novel sample meticulously collected with the main focus on AR gave us a unique opportunity to assist in clarifying this area of *FLG* research. Our finding of no association between *FLG* and AR was supported by the fact that we found, as hypothesised, an association between *FLG* and AD in all three sample settings (Sample A alone, Sample B alone and sample A and B combined).

Future perspectives.

AR could clearly benefit from more attention by the field of genetics. In particular, we recommended a focused look at the identified candidate loci at 1p13, 1q21, 2q23, 20p12, Xp11, Xp21 and Xp22. Likewise, in asthma and atopy the most interesting candidate loci emerging from the present study include: 2p13 and 20p12 for asthma, 1p13 and 20p12 for sIgE and 6p24 and 11q12-q13 for T-IgE. Future genetic studies aiming at identifying the specific susceptibility genes and variants located in these loci include fine scale mapping, candidate gene association analysis, and prioritization of the loci in GWAS data [266, 274, 308-310]. The results of the present thesis contribute significantly to a strong basis for this endeavour.

If we can unravel the genetics behind the allergic diseases, we may gain knowledge of the underlying pathogenesis, which in turn can clear the way for improvements in several ways:

- **More precise disease-classification and diagnosis**. An example of this would be subclassing AD patients into those with and without *FLG* mutations. A sub classification as such could help predict the prognosis of the patient.
- **Specify the impact of environmental risks on the individual patient**. An example of this is given by Bisgaard et al. who published a study in 2009 looking at the consequence of having a cat or a dog, if one has a *FLG* mutation. He found that cat ownership increased the effect of a *FLG* mutation on the development of eczema and sensitisation, whereas dog ownership had no consequence for the patients [162].
- **New treatments.** In addition to easing symptoms, the aim of research should strive toward curing the disease. Identification of novel susceptibility genes may represent new drug targets, enabling a causally directed treatment.
- **Personalised medicine.** New treatments should account for the individual patient's genetic constitution. Today, some patients do not benefit from certain treatments, whereas others do. In the future, we may be able to determine the best working treatment for the individual patient upon diagnosis, thereby minimising the discomfort for the patient and increasing the speed of recovery.

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