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GENETIC STUDIES OF CANDIDATE GENES IN ECZEMA

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To my mother

ABSTRACT

Eczema is a common inflammatory skin disorder characterised by itching and relapsing eczematous lesions. It usually presents in early childhood and affects about 10-20% of all children. The background is multifactorial, with both environmental risk factors and hereditary factors contributing to the development of the disease. The aim with this thesis was to identify susceptibility genes that contribute to eczema development.

In a systematic analysis of global gene-expression patterns in eczema skin, we found the *SOCS3* gene to be significantly more highly expressed than in skin from healthy controls and immunohistochemical analysis confirmed an elevation of the SOCS3 protein. Furthermore, we found a genetic association between eczema and a haplotype in the *SOCS3* gene in two independent groups of patients ($p<0.02$ and $p<0.03$). These results strongly suggest that *SOCS3*, located in a chromosomal region previously linked to the disease (17q25), is a susceptibility gene for eczema.

Recent studies have identified two loss-of-function variants, R501X and 2282del4, in the filaggrin (*FLG*) gene as predisposing factors for eczema. To determine the frequency of these variants and test for association we analysed transmission in 406 multiplex eczema families. In accordance with previous studies we found association between the filaggrin gene variants and atopic eczema ($p<1\times10^{-7}$). The highest odds ratio for the combined allele was found for the subgroup with a severe eczema phenotype (OR 4.73 (1.98–11.29), $p<4\times10^{-8}$). Association was also found with raised total serum IgE, allergic asthma, and allergic rhinoconjunctivitis occurring in the context of eczema. Our results support an important role for the filaggrin gene variants R501X and 2282del4 in the development and severity of atopic eczema.

In order to identify new molecular disease determinants of eczema, we analysed differentially expressed genes in a mouse model with an eczema-like phenotype. *CRNN* was identified and altered gene expression was confirmed with Real Time PCR. The *CRNN* gene was then further investigated by genetic association analysis. We found association with atopic eczema, but the observed association is likely to be explained by linkage disequilibrium between the *CRNN* gene and the *FLG* 2282del4 mutation. Therefore, the role of *CRNN* in eczema needs to be further evaluated.

Lack of genetic association between eczema and the asthma susceptibility gene *NPSR1* was found when analysing seven polymorphisms in the gene in five European patient materials. Expression of NPSR1 in the epidermis showed no apparent difference between eczema patients and healthy controls. In addition there was no association with asthma, elevated IgE, or atopic sensitisation in the context of eczema.

In summary, the *SOCS3* gene has been identified as a potential novel susceptibility gene for eczema. Furthermore, the *FLG* gene has been confirmed as an important susceptibility gene for eczema and a marker of disease severity. Finally, the *CRNN* gene has been identified as a differentially expressed gene in eczema skin.

LIST OF PUBLICATIONS

I. Ekelund E, Sääf A, Tengvall-Linder M, Melen E, Link J, Barker J, Reynolds NJ, Meggitt SJ, Kere J, Wahlgren C-F, Pershagen G, Wickman M, Nordenskjöld M, Kockum I, and Bradley M
Elevated Expression and Genetic Association Links the *SOCS3* Gene to Atopic Dermatitis
Am J Hum Genet 2006;78(6):1060-5

II. Ekelund E*, Liedén A*, Link J, Lee SP, D'Amato M, Palmer CNA, Kockum I, and Bradley M
Loss-of-function Variants of the Filaggrin Gene are Associated with Atopic Eczema and Associated Phenotypes in Swedish Families
Acta Derm Venereol 2008;88:15–19 *Authors contributed equally

III. Liedén A, Ekelund E, Kuo I-C, Kockum I, Huang C-H, Mallbris L, Lee SP, Seng LK, Chin GY, Wahlgren C-F, Palmer CNA, Björkstén B, Ståhle M, Nordenskjöld M, Bradley M, Chua KY, and D'Amato M
Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema
Submitted

IV. Ekelund E, Bradley M, Weidinger S, Jovanovic DL, Johansson C, Wahlgren C-F, Lindgren CM, Jakob T, Illig T, von Mutius E, Braun-Fahrländer C, Doekes G, Riedler J, Scheynius A, Pershagen G, Nordenskjöld M, Kockum I, and Kere J
Lack of association between Neuropeptide S Receptor 1 (*NPSR1*) and eczema in five European populations
Submitted

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LIST OF ABBREVIATIONS

APT	Atopy patch test
BAMSE	Barn allergi miljö Stockholm epidemiologi
cDNA	Complementary DNA
cM	centimorgan
CI	Confidence interval
CRNN	Cornulin
DNA	Deoxyribonucleic acid
DZ	Dizygotic twin
EDC	Epidermal differentiation complex
FLG	Filaggrin
HWE	Hardy-Weinberg equilibrium
IDEC	Inflammatory dendritic epidermal cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KORA	Cooperative health research in the Augsburg region
LC	Langerhans cell
LD	Linkage disequilibrium
LOD	Logarithm of the odds
mRNA	Messenger ribonucleic acid
MZ	Monozygotic twin
NPSR1	Neuropeptide S receptor 1 gene
OR	Odds ratio
PARSIFAL	Prevention of allergy - risk factors for sensitisation in children related to farming and anthroposophic lifestyle
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDT	Pedigree disequilibrium test
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
TDT	Transmission disequilibrium test
Th	T-helper
UTR	Untranslated region

INTRODUCTION

GENETICS

Basic genetics

The human genome is the term used to describe the total genetic information in human cells. The genetic information is stored in macromolecules of deoxyribonucleic acid (DNA), which mostly specify synthesis of proteins. DNA molecules are large polymers with a backbone of sugar and phosphate and attached nitrogenous bases (adenine, cytosine, guanine, and thymine). A sugar with an attached base is a nucleoside, and a nucleoside with a phosphate group is a nucleotide, which is the basic repeat unit of a DNA strand (Strachan and Read 2004). The majority of the human genome is located in the nucleus of cells and contains approximately 30 000 genes and several other functional elements that are important for the function of cells. The genes and their sequence of DNA bases represent a code for synthesizing proteins. The fundamental unit of this genetic code is termed a codon, which consists of three nucleotides and codes for one unit of protein, an amino acid.

Two DNA strands are organized into a double helix where the strands are bound to each other in an antiparallel way (Watson and Crick 1953). By this organisation the DNA is stabilized and replication can occur simultaneously on each strand. In humans, the DNA helix is organized in 23 pairs of chromosomes, with many hundreds of genes on each chromosome (Figure 1). We have two copies of each autosome, one inherited from our father and one from our mother. A person having two identical gene copies (alleles) in a region (locus) on the chromosome are said to be homozygous, whereas a person with two different alleles are said to be heterozygous.

Figure 1. A chromosome pair, with one chromosomes DNA sequence magnified.

Cells in an organism have the ability to divide and make exact copies of themselves and their genetic content. This process is called mitosis. When gametes (sperm and egg cells) are formed there is a specialized form of cell division occurring called meiosis. It involves separation of the chromosomes in each pair, but before that also an exchange of genetic material between the two homologous chromosomes in a pair (Figure 2). This process of genetic recombination helps preserve genetic variability within a species.

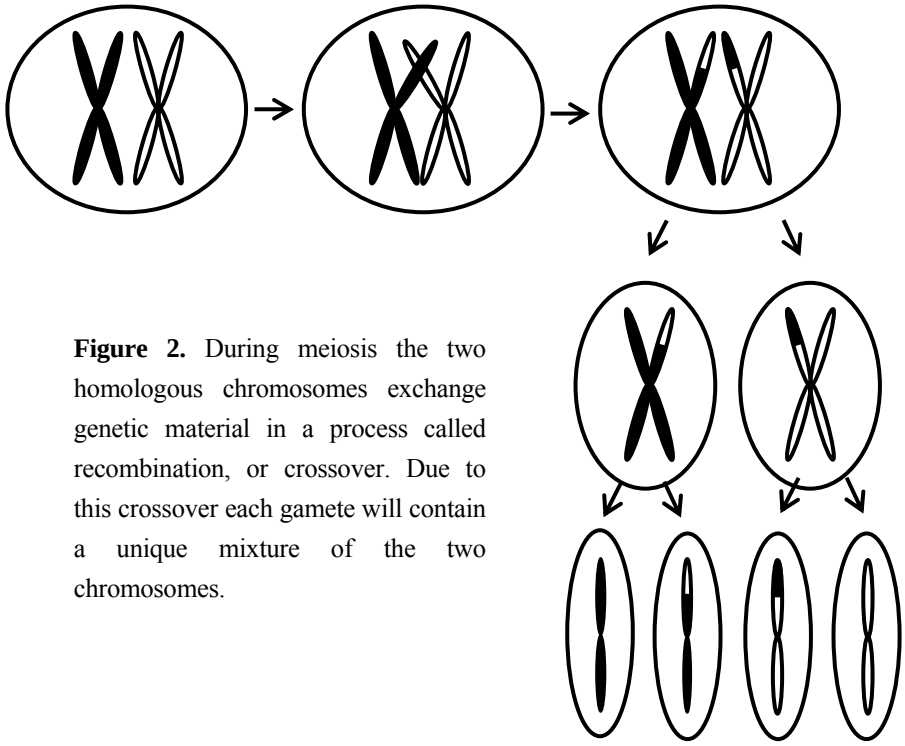


Figure 2. During meiosis the two homologous chromosomes exchange genetic material in a process called recombination, or crossover. Due to this crossover each gamete will contain a unique mixture of the two chromosomes.

Variations in the DNA sequence

Changes in the DNA sequence may lead to disruption of gene function, which can cause disease. These genetic changes include single base substitutions, deletions and insertions of different sizes, and chromosome translocations and inversions. Even in healthy individuals the DNA sequence is not totally identical, although very similar. There are common variants in the DNA sequence among people, approximately one every 1 200 nucleotides. These variants are called polymorphisms if the less common allele occur in more than 1% of the individuals in any population (Brookes 1999). One common form of polymorphism is called single nucleotide polymorphism (SNP), where only one nucleotide is changed in the sequence (Figure 3). Usually the sequence varies between two possible nucleotides in a SNP position. It is estimated that the

human genome contain at least 10 million common SNPs (Frazer, Ballinger et al. 2007). A SNP may be located within a gene and then it may result in an altered protein. However, the vast majority of SNPs are silent that we do not know of any functional consequence of.

Figure 3. Illustration of a single nucleotide polymorphism (SNP) in the DNA sequence in three individuals.

Linkage disequilibrium and haplotypes

Polymorphisms in close proximity tend to be inherited together. This means that individuals who carry a particular SNP allele at one site often carry specific alleles at other nearby SNP locations. This correlation along a chromosome is known as linkage disequilibrium (LD). LD exists because of shared ancestry of the chromosomes we carry today. When a new variant arises through a mutation in the DNA sequence it is located on a chromosome that has a unique combination of older polymorphisms surrounding it. LD is usually measured pair-wise between SNPs, either by using r^2 or D′. Both measures range from 0 (no disequilibrium) to 1 (complete disequilibrium). The measure r^2 represent a statistical correlation between two SNP sites, whereas D′ less than 1 indicates that recombination has occurred between the sites (Wall and Pritchard 2003). As expected, LD is inversely related to the distance between the markers, but LD is also variable in different genomic regions and between populations. As mentioned above, nearby alleles on the same chromosome tend to be transmitted together as a block. Such a linked block is known as a haplotype. The length of these haplotypes vary in the genome and one hypothesis is that the genome is divided into regions with high LD separated by recombination hotspots with low LD (Wall and Pritchard 2003). Polymorphisms that uniquely identify haplotypes are called tag SNPs and are often used in genetic studies since they reduce the number of SNPs that need to be analysed.

Genetics of diseases

Monogenic diseases

In 1865, the Austrian monk Gregor Mendel (1822-1884) published his findings of characteristic inheritance in the pea plant. He also postulated two laws of inheritance long before DNA was discovered. Mendel's first law of segregation states that during gamete formation each member of an allelic pair separates from the other member to form the genetic constitution of the gametes. This means that parents transmit to an offspring one randomly chosen allele of the two present at each locus. Mendel's second law of independent assortment predicts that during gamete formation alleles at one locus segregate independently of alleles at other loci (Connor and Ferguson-Smith 1984). Mendelian phenotypes or disorders are those whose presence or absence depends on the genotype at a single locus. There is a strong correlation between genotype and phenotype in these disorders. They can be recognized by the characteristic pedigree pattern they give rise to. A phenotype is said to be inherited dominantly if present in a heterozygous carrier, and recessively if manifest only in a homozygous individual (Strachan and Read 2004).

Complex diseases

So called complex diseases do not follow the Mendelian inheritance laws. They are common disorders that are polygenic (multiple genes) and/or multifactorial (multiple genes interacting with environmental and lifestyle factors). Assuming a threshold model, the disease manifests in those who have a liability beyond a set value (Haines and Pericak-Vance 1998) (Figure 4).

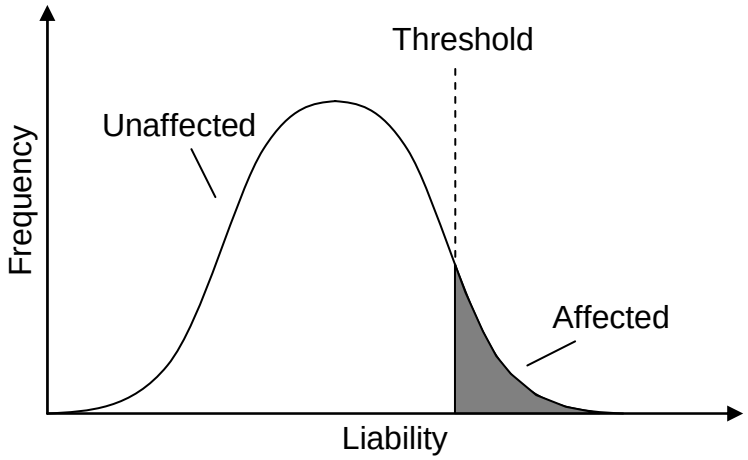


Figure 4. The multifactorial threshold model. Modified from (Haines and Pericak-Vance 1998).

Interaction of multiple genes is usually explained by either an additive or a multiplicative genetic model. The effect of two or more genes can equal the sum of their independent effect (additive) or the genes can interact in such a way that results in an even greater risk than generated independently by each gene (multiplicative) (Haines and Pericak-Vance 1998). It is likely that individual genes behind a complex disease are neither necessary nor sufficient for disease development, but it seems that a combination of susceptibility genes increases the liability to disease. It is also likely that individuals with the same phenotype have different combinations of risk-increasing genes and environmental factors involved. Since variations in any of several different genes may result in very similar phenotypes (genetic heterogeneity) and the different loci may interact with each other (epistasis), the complexity is increased. Some individuals that inherit the susceptibility allele do not manifest the disease (incomplete penetrance), whereas others develop the disease due to environmental factors independent of the susceptibility allele (phenocopies). The individual genetic factors that contribute to complex diseases are thought to be of low effect size, with genotype relative risk of 1.2-2.0 which is the same as 20-100% increase in risk for carriers of the risk genotype (Morar, Willis-Owen et al. 2006).

Finding susceptibility genes

If susceptibility genes for complex diseases are identified it will increase the knowledge about pathophysiology of diseases. Identifying possible drug targets may also lead to the development of improved treatment. To offer specific preventive lifestyle advice on the basis of genotype can also be a possibility if susceptibility genes and their interaction with environmental factors are made clear. Different approaches can be used in the search for novel susceptibility genes in complex diseases. Some of the methods are listed below.

Linkage analysis

Linkage analysis is a method where localization of the disease causing variant is done by analysing the pattern of inheritance in families. Individuals are genotyped for markers spaced evenly across the genome, usually every 10 centimorgan (cM). The aim is to locate a disease gene by finding markers that co-segregate with the disease more often than expected by random segregation. The closer the marker and the disease gene are located, the more likely it is that they will be inherited together, since it is then less

likely that they will be separated by recombination. Classical linkage analysis is referred to as parametric or model-based analysis, since the parameters for the genetic model has to be provided. The parameters include mode of inheritance, gene penetrance estimates, and allele frequencies. The probability of linkage is given as the logarithm of the odds (LOD) score, where the odds of linkage represent the ratio between two hypotheses: the alternative hypothesis that the loci are linked and the null hypothesis where there is no linkage between the loci. LOD scores can be summed up from a set of families used and evidence of linkage is found if the maximum LOD score exceeds a predefined threshold. Parametric linkage analysis has successfully localized and identified more than 1 000 genes causing monogenic diseases showing a Mendelian inheritance pattern.

When analysing linkage in a complex disease, a non-parametric or model-free linkage analysis is preferable since the mode of inheritance is not known. One widely used method is the affected sib-pair analysis. Using this method one needs to genotype families with at least two affected siblings and identify excess of sharing of alleles identical-by-descent. The expected sharing of alleles identical-by-descent for siblings is 25% for not sharing any, 50% for sharing one, and 25% for sharing both alleles. If there is excess sharing of alleles this will lead to an increase in LOD score.

Different significance levels for a LOD score has been proposed depending on the method used (Lander and Kruglyak 1995; Nyholt 2000). Therefore, significance levels of linkage analyses are best presented with a corresponding p-value as well as LOD score values. For non-parametric sib-pair analysis the term *suggestive linkage* is used for a LOD score of 2.2 ($p \leq 0.00074$) and a LOD score ≥ 3.6 is regarded as genome-wide *significant* ($p \leq 0.000022$). This means that evidence of suggestive linkage will occur randomly one time in a genome-wide linkage analysis and that evidence of significant linkage will occur by chance once in every 20 genome-wide linkage scan.

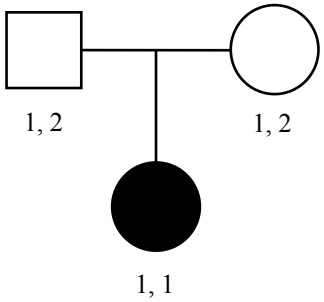
Association studies

Association analysis is usually based on pre-existing knowledge of the function of genes (candidate gene approach), but can also be applied in genome-wide association studies. In the candidate gene approach, you start with a gene that you think may play a role in disease pathogenesis. With association analysis you look for a statistical

association between a genotype and a phenotype. In other words, you test whether a specific allele is more common among the affected than among the unaffected individuals. If you find allelic association to the phenotype this can be explained either by direct biological action of the allele or by linkage disequilibrium with a nearby located susceptibility gene.

Association analysis can be performed in case-control studies where you compare the frequency of the allele among patients and unrelated healthy individuals in a χ^2 test. The control individuals should be matched for ethnicity and other factors such as age and gender. This is important because of the risk of population stratification (multiple population subtypes) which can lead to spurious associations.

Association analysis can also be performed in family materials with affected individuals. In this setting there is no concern that population stratification will lead to spurious associations (Haines and Pericak-Vance 1998). The most commonly used family-based method is the transmission disequilibrium test (TDT) where you include an affected child and its two parents (Spielman, McGinnis et al. 1993). Parents that are heterozygous at a marker locus are considered and you observe the frequency with which the two alleles are transmitted to the affected offspring. Deviation from the expected equal frequency transmission of the two alleles is tested in a standard χ^2 test. The TDT test statistics are $\chi^2 = (a-b)^2/(a+b)$ where **a** is the number of times the first allele is transmitted to affected offspring and **b** the number of times the other allele is transmitted. The null hypothesis is that the two alleles (a, b) are transmitted equally (a=b), which means that the disease locus and the marker are not associated (Figure 5).



OBSERVED		NON-TRANSMITTED ALLELE	
		Allele 1	Allele 2
TRANSMITTED ALLELE	Allele 1	0	**2**
	Allele 2	0	0

EXPECTED		NON-TRANSMITTED ALLELE	
		Allele 1	Allele 2
TRANSMITTED ALLELE	Allele 1	0	**1**
	Allele 2	**1**	0

Figure 5. TDT scoring in a family. In this example the $\chi^2 = (2-0)^2/(2+0)=2$

TDT approach also tests for linkage in the presence of association. When dealing with late onset diseases where parental DNA is hard to come by one may use a modified version of TDT called Sib-TDT, where the frequencies of the marker allele is compared between affected and unaffected siblings. Compared to TDT, sib-TDT is has less power to detect association.

In order to make the method valid when there are multiple affected offspring TDT has been modified (Martin, Kaplan et al. 1997). But an even further refinement to the method is the pedigree disequilibrium test (PDT) (Martin, Monks et al. 2000). This method makes it possible to include extended families and test for association.

Gene expression analysis

Another approach to gain knowledge about complex diseases is to study differential expression of genes. Changes in gene expression can be detected by microarray technologies, which harbour a high capacity to monitor the expression of many genes simultaneously and thus provide information about disease pathology and important mechanisms of the involved pathways. By using the microarray technology, transcriptional levels of thousands of different gene sequences can be monitored in one experiment. Different approaches can be applied to gene expression analyses. The gene expression in cells or whole tissues can be compared between patients and healthy controls. The gene expression can also be analysed in different stages of disease. Microarray studies can be designed as large-scale analysis, where the whole genome is represented on the array, or as analysis of a subset of genes. The microarray technology has been improved during the last years. Not only has the number of genes that simultaneously can be monitored in one experiment increased, but today microarray technology is a useful tool to detect post-transcriptional regulation mechanisms such as alternative splicing of genes and their products.

Animal models

When studying genetics in complex diseases, animal models have certain advantages. Environmental exposures can be controlled and the genetic background can be homogeneous through inbreeding. It is possible to study spontaneously developed phenotypes but also induced phenotypes. However, an animal model of disease will never be exactly the same as the human disease leading to difficulties in interpretation

of results. The development of transgenic mouse models also has the potential to be informative about different candidate genes and the development of complex diseases.

ECZEMA

Clinical signs

Eczema, also referred to as atopic dermatitis, is a common inflammatory skin disease. Symptoms include itch and chronic relapsing eczematous lesions at typical locations. It usually present in early years, about 70% below the age of 2 years (Williams and Wüthrich 2000) and 90% below age of 4 years (Ring, Przybilla et al. 2006).

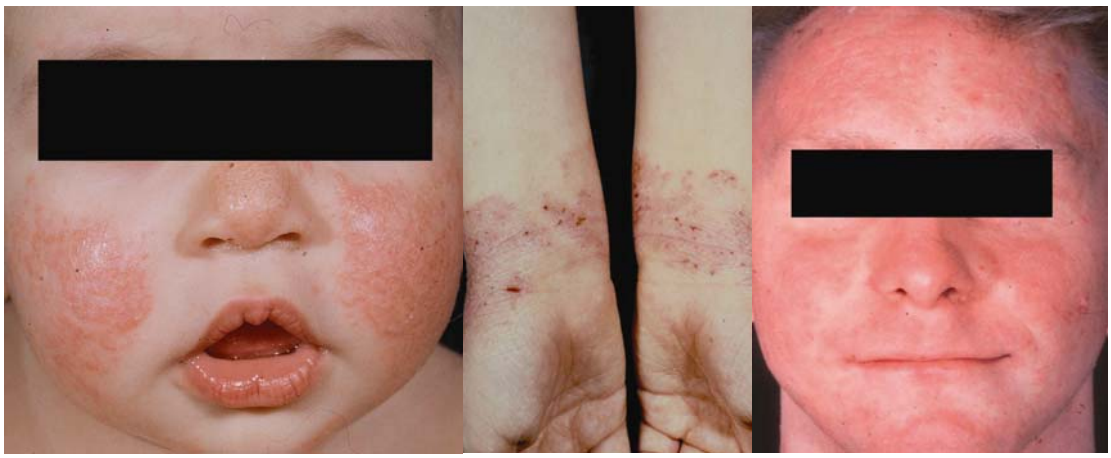


Figure 6. Typical appearance and location of eczematous lesions in infancy, childhood, and adulthood.

In infancy the lesions are typically localized symmetrically on extensor sides of arms and legs, and in the face (Ring, Przybilla et al. 2006). During childhood the lesions are commonly located symmetrically on flexural sides. The main clinical picture in adolescent and adulthood is eczema in the head and neck region, and hand eczema (Figure 6). It has been reported that eczema spontaneously clears in about 50 to 70% of patients in a ten-year period when onset is in childhood. But the problem with these studies is that eczema can appear after a long period of clearance and the recurrence rate is directly proportional to the frequency of follow-up. The natural history of eczema is not fully understood and some argue that one can never grow out of eczema. The tendency to react with eczema might always be present, even if some individuals are apparently free from disease during adolescent (Williams and Wüthrich 2000). Quality of life is greatly reduced for children and adults with eczema. For instance the itching leads to sleep disorders for over 60% of the children with eczema (Lewis-Jones 2006). Sleep deprivation consequently leads to impaired functioning for the whole

family at school and work. The reduced quality of life caused by childhood eczema has been shown to be greater or equal to other common childhood diseases such as asthma or diabetes (Beattie and Lewis-Jones 2006).

Diagnostic criteria

There is no biochemical marker that can serve as a diagnostic tool for eczema. The diagnosis is solely dependent on clinical symptoms and signs. These symptoms and signs have been organized into diagnostic criteria by different clinicians. The classic criteria proposed by Hanifin and Rajka in 1980 is based on the presence of at least three out of four major, and three out of 23 additional minor criteria (Hanifin 1980). The four major criteria are: pruritus, typical morphology and distribution of skin lesions, chronic or relapsing course, and personal or family history of atopy. The UK Working Party Diagnostic Criteria from 1994 define eczema as an itchy condition, plus three or more of the following: history of involvement of the skin creases, personal history of allergic asthma or allergic rhinoconjunctivitis, history of dry skin in the past year, visible flexural eczema, and onset during the first 2 years of life (Williams, Burney et al. 1994(I); Williams, Burney et al. 1994(II); Williams, Burney et al. 1994(III)).

Prevalence

The prevalence of eczema and other allergic diseases has increased markedly during the last decades (Schultz Larsen and Hanifin 1992; Schultz Larsen, Diepgen et al. 1996). Between years 1979 and 1991 the prevalence of eczema among school children in Sweden was reported to have been more than doubled (Åberg, Hesselmar et al. 1995). The prevalence of eczema is now reported to be one of the highest in the world, 22% among 7-year olds and 13% among 13-year olds (Asher, Montefort et al. 2006).

Atopy and nomenclature

Eczema is considered to be one of the so called atopic disorders. The concept of *atopy* was originally introduced in 1923 by Coca and Cooke as meaning *strange disease* or *not in the right place*. The term *atopy* has since then been used meaning different things (Williams 2000). In 2003, the World Allergy Organization, proposed a revised nomenclature of the term *atopy* which defines it as "*a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a*

consequence these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema" (Johansson, Bieber et al. 2004). Although eczema is strongly associated with a tendency to produce IgE antibodies there is a debate whether this is central to eczema development or whether this is an epiphenomenon of disease activity (Flohr, Johansson et al. 2004). Not all patients with eczema are atopic; up to two thirds have no measurable allergen-specific IgE antibodies. The proportion of eczema with sensitization is higher in a hospital setting compared to in the community, which may reflect differences in disease severity (Flohr, Johansson et al. 2004). Consequently there have been efforts done in dividing eczema into different subgroups, such as an intrinsic and extrinsic form based on the presence or absence of reactivity to allergens (Williams 2000). The World Allergy Organization has also recently revised the nomenclature of eczema in the same manner as with the term atopy. They propose the use of *atopic eczema* to mean eczema in a person with the atopic constitution. This should be determined by measuring IgE levels in the patient. *Non-atopic eczema* is subsequently present in a patient with eczema without allergic sensitization. The term *eczema* should be used as an umbrella term as long as the immunological mechanism of eczema is unclear (Johansson, Bieber et al. 2004). An illustration of this revised nomenclature is shown in Figure 7. However, this new terminology has not yet been widely accepted.

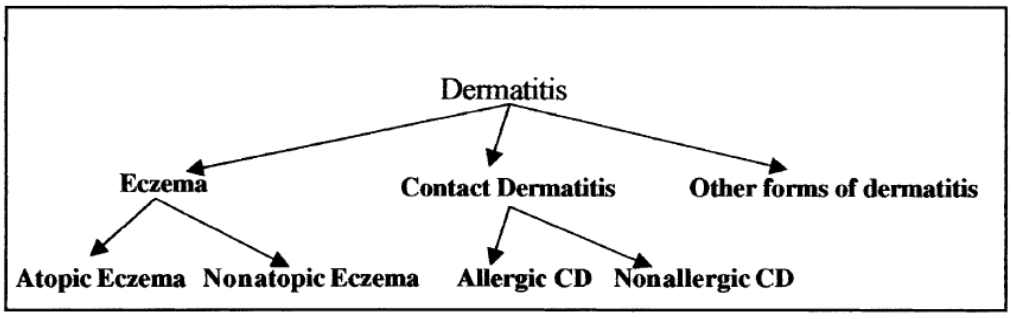


Figure 7. Revised nomenclature on inflammatory skin disorders (Johansson et al. 2004)

Associated atopic manifestations

There are among patients with eczema often co-morbidity with other atopic disorders such as allergic asthma and allergic rhinoconjunctivitis. It has been argued that *the atopic march* is the natural history of atopic manifestations. The advocators of the atopic march theory claims that the clinical signs of eczema is the start of a march that follows through the development of allergic asthma and allergic rhinoconjunctivitis (Spergel and Paller 2003). The theory is based on comparisons of prevalence figures of

the diseases in different age groups. When looking more closely on the relationship between eczema and asthma development, studies have shown that wheezing in early years and a specific sensitization pattern are predictors of asthma development (Illi, von Mutius et al. 2004). This finding speaks in favour of early co-manifestation of two atopic disorders rather than an increased risk of developing allergic asthma if affected by early eczema (Williams and Flohr 2006).

Environmental factors

The rapid increase in prevalence suggests that environmental factors, which are important for the development of eczema, have changed rather than genes. In particular environmental factors linked to the *Western lifestyle* seem important, since there is a large variation worldwide in the prevalence of allergic diseases with a north-south gradient, where the lowest prevalence is reported close to the equator (Flohr, Pascoe et al. 2005; Asher, Montefort et al. 2006). Eczema is also more common in urban than in rural communities and migrant studies have demonstrated that immigrants take on the risk of the community when they move to more westernized countries. The risk of developing eczema is also found to be higher for children growing up in smaller families and in families of higher socioeconomic class (Flohr, Pascoe et al. 2005).

The *hygiene hypothesis* was formulated in the late 1980s, and is based on the finding that there is a decreased risk of developing eczema with an increase in number of siblings (Strachan 1989). It was suggested that this is due to increased exposure to infections that protects the younger siblings against eczema. A recent systematic review of the epidemiological evidence behind this hypothesis (Flohr, Pascoe et al. 2005) finds that there is currently no clear epidemiologic evidence to suggest that exposure to a specific infection reduces the risk of childhood eczema. In fact, they found that some childhood infections, such as measles, are associated with an increased risk of eczema development. Furthermore, no clear evidence was found that routine childhood vaccinations are increasing the risk of developing eczema. But the review found studies that provided evidence of a positive association between the use of antibiotics and an increased risk of eczema. Some studies suggest that probiotics (Lactobacillus GG) can both reduce eczema severity (Flohr, Pascoe et al. 2005) and reduce the risk of developing eczema with 50% (Kalliomaki, Salminen et al. 2001; Kalliomaki, Salminen et al. 2003). These epidemiological findings may help sort out what factors in the

anthroposophic lifestyle that contributes to the lower risk of eczema found in children with anthroposophic background in Sweden (Alm, Swartz et al. 1999). The anthroposophic lifestyle has several characteristics including a restrictive use of antibiotics and vaccinations, but they also consume fermented vegetables rich in Lactobacillus.

Other aspects of hygiene could also be important environmental factors, e.g. extensive washing and the use of soap and detergents. In a large birth cohort from Britain, the question was raised whether the general hygiene such as frequency of washing affects the risk of eczema. In this study they found a hygiene score to be associated with an increased risk of eczema, especially severe eczema (Flohr, Pascoe et al. 2005). Not only has the frequency of washing increased during the last decades, the use of soap and detergents has also increased, which may affect the skin barrier (Cork, Robinson et al. 2006). This may play a pivotal role for the increasing eczema prevalence.

Other environmental factors have also been suggested for possible effects on eczema development, such as climate factors, air-pollution, smoking, and dietary factors (Asher, Montefort et al. 2006). Although environmental factors clearly play an important role in the development of eczema, it is of great importance to explore the genetic factors that contribute to disease development. If such genetic factors are identified the understanding of pathophysiological mechanisms will improve. This may in turn give indications also on the interplay with environmental factors.

Pathophysiology of eczema

The pathophysiology of eczema is a product of complex interactions between defects in the epidermal skin barrier function and deficiencies in the innate and the adaptive immune responses.

Skin barrier

The skin acts as a barrier in many ways. It protects the body against water loss, chemical and physical insult, and protects us from microorganisms. The epidermis, which is the uppermost part of the skin function as a barrier against the environment by means of several layers of corneocytes. Corneocytes are flattened dead keratinocytes that have proliferated from the basal layers of the epidermis and been terminally

differentiated in the outermost part (cornified layer). The corneocytes are locked together by corneodesmosomes that provide strength and structural integrity to the cornified layer. The barrier is constantly regenerated by terminally differentiating keratinocytes in a process known as cornification or keratinization. Desquamation is the process by which the epidermis is maintained at a constant thickness. In the desquamation process, corneocytes that are shed from the surface are replaced from underneath by keratinocytes undergoing terminal differentiation. The desquamation process is facilitated by degrading proteases that are regulated by protease inhibitors (Candi, Schmidt et al. 2005; Cork, Robinson et al. 2006).

In eczema, the skin barrier is dysfunctional in different aspects. The skin is dry and shows increased transepidermal water loss, as well as reduced content of skin lipids. In patients with eczema the pH is also higher than in healthy controls, which leads to increased protease activity in the skin (Candi, Schmidt et al. 2005; Cork, Robinson et al. 2006; Maintz and Novak 2007). As discussed later, structural skin barrier proteins also seem to be affected and to play a role in eczema pathophysiology.

Immune system

Innate immunity is the first line of defence against microbes and responds rapidly to invasion. Important mechanisms in innate immunity are the toll-like receptors on cell surfaces that recognize molecular pattern of microbes and trigger the immune system. It has been speculated whether deficiencies in these pattern recognition receptors contribute to the imbalanced immune response seen in eczema (Maintz and Novak 2007). Another mechanism by which an impaired innate immune response is thought to be involved in the pathogenesis of eczema is by deficient production of antibacterial peptides. Skin in eczema patients show deficient production of β defensin (HRD-2) and LL-37 compared to psoriasis skin and this may increase the vulnerability for patients with eczema to be colonised by *S. aureus* (Ong, Ohtake et al. 2002; Nomura, Goleva et al. 2003).

Alterations in the adaptive immune response are also part of the eczema pathophysiology (Figure 8). Keratinocytes from eczema patients have shown to produce increased amounts of pro-inflammatory cytokines, such as GM-CSF, TNF-α, IL1, and IL18. The cytokine production may be induced by mechanical trauma caused

by scratching of the skin (Homey, Steinhoff et al. 2006), or by microbial and antigenic invasion (Maintz and Novak 2007). The pro-inflammatory cytokines in turn induce the production of chemokines, which attracts T-cells to the skin.

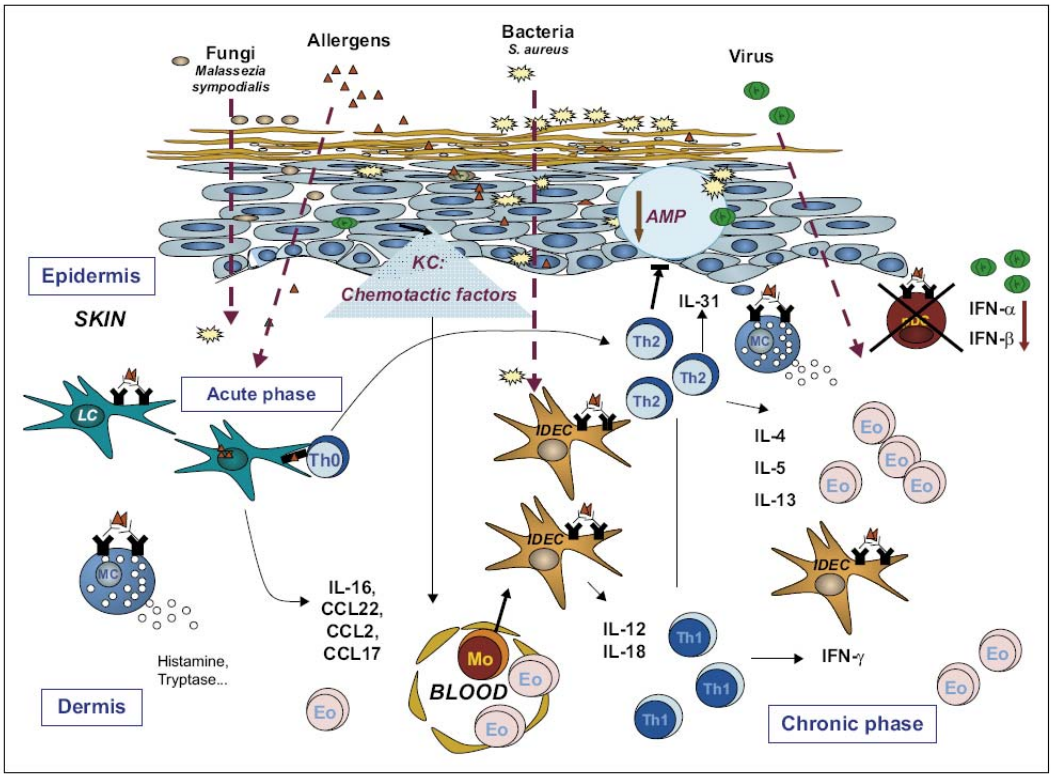


Figure 8. Schematic overview of some of the factors involved in eczema pathophysiology. Abbreviations: AMP = antimicrobial peptides; CCL = chemotactic cytokine ligand; Eo = eosinophil; IDEC = inflammatory dendritic epidermal cells; IL = interleukin; IFN = interferon; KC = keratinocyte; LC = Langerhans cells; MC = mast cells; Mo = monocyte. (Maintz and Novak 2007)

The migration of T-cells to the inflamed skin plays an essential role in the development of eczema. Eczema can be divided into an acute phase, where the number of T-helper type 2 (Th2) cells expressing IL-4 and IL-13 are increased compared to in normal skin. IL-4 mediates the immunoglobulin (Ig) isotype switch in B-cells that leads to production of IgE (Homey, Steinhoff et al. 2006). A chronic eczematous lesion is more Th1-dominated and the cytokines expressed are for instance IFN-γ and IL-12 (Leung, Boguniewicz et al. 2004; Maintz and Novak 2007). T-regulatory cells are another subtype of T-cells that regulate the balance between Th1- and Th2-cells and modified function of T-regulatory cells seems to be associated with eczema (Verhagen, Akdis et al. 2006). Dendritic cells are antigen presenting cells that are shown to be of importance for the pathophysiology of eczema. In eczema skin, high amounts of Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC) have been shown, both which

express the high affinity receptor for IgE. LC has an important role in initiating the Th2 immune response by antigen-presentation to T-cells, but also in recruiting IDEC into the skin that by the release of IL-12 and IL-18 is contributing to the switch towards a Th1-dominated immune response (Novak and Bieber 2005). Other cell types than mentioned above are also thought to have a role in eczema pathophysiology, e.g. mast cells, eosinophils, and plasmacytoid dendritic cells (Maintz and Novak 2007).

Trigger factors

There are several factors that can act as triggers for the eczema reaction, such as allergens, microorganisms, stress and irritants. Food allergens can in up to 40% of children with moderate to severe eczema act as trigger factors (Leung and Bieber 2003). Food allergen-specific T-cells have been cloned from the skin lesions of patients with eczema, indicating that food can contribute to immune response (Akdis, Akdis et al. 2006). Also by inhaling aeroallergens such as house dust mite, or by applying them on the skin, eczematous lesions may be triggered (Leung and Bieber 2003). Another factor that exacerbates eczema lesions is the bacteria *Staphylococcus aureus* that are found in increased numbers in over 90% of eczema patients (Baker 2006). There are several reasons for this exacerbating effect of *S. aureus*, for instance the bacteria is secreting super-antigenic exotoxins that stimulate T-cells and macrophages (Leung, Harbeck et al. 1993). Furthermore, microbes (e.g. *S. aureus* and house dust mite) are known to produce proteins that through protease activity damage the skin barrier and can trigger eczema (Cork, Robinson et al. 2006). Irritants such as wool and detergents can also trigger eczematous lesions (Akdis, Akdis et al. 2006; Cork, Robinson et al. 2006).

GENETICS OF ECZEMA

Heritability of eczema

Familial aggregation

Familial aggregation is the clustering of affected individuals in families. This can be due both to shared genes and shared environment. The risk of developing eczema if neither of the parents has eczema is 10-15%. If only one parent has eczema the risk is 25-30%, and if both parents have the disease 50-75% of their offspring will develop eczema (Schultz Larsen 1991). Furthermore, it is more likely to develop the same atopic manifestation as the parents. Children whose parents suffer from eczema have a

higher risk of developing eczema than children with parents suffering from allergic asthma or allergic rhinoconjunctivitis (Dold, Wjst et al. 1992). This may indicate that there are separate and/or additional genes for eczema and the other atopic manifestations.

A measure of familial aggregation is λ_s, which is the ratio of the risk for a sibling to be affected compared to the population risk. A higher value of λ_s is consistent with a greater contribution of genetics in the development of the trait. For a purely genetic disease the λ_s value can be as high as 500 (a recessive trait such as cystic fibrosis) or 5 000 (a dominant disorder such as Huntington disease), but for a complex trait the estimated λ_s is much lower (Haines and Pericak-Vance 1998). For eczema the λ_s is usually estimated to about 2-3.

Twin studies

Another way of estimating the genetic component versus the environmental component is to study twins. Monozygotic twins (MZ) are genetically identical, whereas dizygotic twins (DZ) share on average one-half of their genes. If both twins in a pair are affected by the same disease they are said to be concordant. Comparing the concordance rates among MZ and DZ gives you an idea of the genetic contribution to the disease. A higher concordance for MZ than DZ twins is indicative of the involvement of genetic factors in the susceptibility. In two Danish studies of twin pairs with eczema a concordance rate of 0.72-0.86 in MZ twins and a concordance rate of 0.21-0.23 in DZ twin pairs was shown (Larsen, Holm et al. 1986; Schultz Larsen 1993).

Previous genetics studies in eczema

Linkage studies

Genome-wide linkage screens in eczema have so far been published in five different studies (Lee, Wahn et al. 2000; Cookson, Ubhi et al. 2001; Bradley, Söderhäll et al. 2002; Haagerup, Bjerke et al. 2004; Enomoto, Noguchi et al. 2007). At least suggestive linkage has been reported for a number of different chromosomal regions, such as: 1q21, 3p26-24, 3p24-22, 3q14, 3q21, 4p15-14, 13q14, 15q14-15, 15q21, 17q21, 17q25, 18q11-12, 18q21, and 20p. Overall, there is no substantial overlap between the peaks (Table 1), but simulations have shown that the linkage peak can vary up to 30 cM from

the disease locus (Roberts, MacLean et al. 1999) making overlap likely between some of the peaks.

Table 1. Results of genome-wide linkage analyses in eczema.

Study	No of families	1q	3p	3q	4p	13q	15q	17q	18q	20p
Lee et al. 2000	199			3q21						
Cookson et al. 2001	148	1q21						17q25		20p
Bradley et al. 2002	109		3p24-22	3q14		13q14	15q14-15	17q21	18q21	
Haagerup et al. 2004	100		3p26-24		4p15-14				18q11-12	
Enomoto et al. 2007	77						15q21			

Some overlaps do also occur between eczema and other inflammatory diseases, such as psoriasis and asthma. The peaks on chromosome 1q21, 3q21, and 17q25 overlap with psoriasis peaks (Bowcock and Cookson 2004) and the peaks on 1q21, 3q21, 13q, and 18q11-12 overlap with asthma peaks (Morar, Willis-Owen et al. 2006; Willis-Owen, Morar et al. 2007). The 17q25 peak also overlaps with linkage peaks in two other skin disorders, seborrhea-like dermatitis and epidermodysplasia verruciformis (Willis-Owen, Morar et al. 2007). Overlapping peaks with both asthma and other skin disorders is likely to reflect the presence of specific genes for skin inflammation as well as specific genes for atopy. Generally, finding overlaps between eczema and other diseases may reflect pleiotropism (the ability of a single gene factor to moderate multiple phenotypes), different susceptibility genes located in the same gene cluster, or pure chance (Willis-Owen, Morar et al. 2007).

Candidate gene studies

Several candidate genes for eczema have been proposed in the literature. They have been selected as possible candidate genes to study because of gene function or involvement in pathways of known importance in eczema pathophysiology. Often in combination with being located in chromosomal regions linked to eczema. Some candidate genes in eczema are listed in Table 2, showing relatively few replications of positive findings. I will below briefly discuss some of the candidate genes that are the most replicated and widely accepted.

Table 2. Candidate gene studies in eczema. Modified from (Hoffjan and Epplen 2005; Morar, Willis-Owen et al. 2006).

GENE	GENE NAME	REGION	STUDY REFERENCES
FLG	Filaggrin	1q21	(Marenholz, Nickel et al. 2006; Palmer, Irvine et al. 2006; Ruether, Stoll et al. 2006; Weidinger, Illig et al. 2006; Barker, Palmer et al. 2007; Morar, Cookson et al. 2007; Nomura, Sandilands et al. 2007; Stemmler, Parwez et al. 2007; Weidinger, Rodriguez et al. 2007)
CTLA4	Cytotoxic T lymphocyte-associated 4	2q33	(Jones, Wu et al. 2006)
CSTA	Cystatin A	3q21	(Vasilopoulos, Cork et al. 2007)
COL29A1	Collagen XXIX alpha 1	3q22.1	(Söderhäll, Marenholz et al. 2007)
IL-2	Interleukin 2	4q27	(Christensen, Haagerup et al. 2006)
TLR2	Toll-like receptor 2	4q32	(Ahmad-Nejad, Mrabet-Dahbi et al. 2004)
IRF2	Interferon regulatory factor 2	4q35.1	(Nishio, Noguchi et al. 2001)
CD14	Monocyte differention antigen CD14	5q31.3	(Lange, Heinzmann et al. 2005)
IL-5	Interleukin 5	5q31	(Yamamoto, Sugiura et al. 2003)
GM-CSF	Granulocyte-macrophage colony-stimulating factor	5q31.3	(Rafatpanah, Bennett et al. 2003)
SPINK 5	Serine protease inhibitor, Kazal type 5	5q31-33	(Walley, Chavanas et al. 2001; Kato, Fukai et al. 2003; Nishio, Noguchi et al. 2003; Kabesch, Carr et al. 2004; Kusunoki, Okafuji et al. 2005)
IL-13	Interleukin 13	5q31	(Liu, Nickel et al. 2000; Tsunemi, Saeki et al. 2002; He, Chan-Yeung et al. 2003; Hummelshøj, Bodtger et al. 2003)
IL-4	Interleukin 4	5q31	(Kawashima, Noguchi et al. 1998; Novak, Kruse et al. 2002)
IL-12B	Interleukin 12B	5q31-33	(Tsunemi, Saeki et al. 2002)
TIM1	T-cell immunoglobin domain and mucin domain protein 1	5q33	(Chae, Song et al. 2003)
NOD1	Caspase recruitment domain-containing protein 4 (*CARD4*)	7p15-14	(Weidinger, Klopp et al. 2005)
FCER1B	Fc-epsilon receptor I beta-chain	11q13	(Cox, Moffatt et al. 1998; Söderhäll, Bradley et al. 2001)
GSTP1	Glutathione s-transferase pi	11q13	(Safronova, Vavilin et al. 2003; Vavilin, Safronova et al. 2003)
IL-18	Interleukin 18	11q22	(Novak, Kruse et al. 2005)
PHF11	PHD finger protein 11	13q14	(Jang, Stewart et al. 2005)
CMA1	Mast cell chymase 1	14q11	(Mao, Shirakawa et al. 1996; Mao, Shirakawa et al. 1998; Tanaka, Sugiura et al. 1999; Iwanaga, McEuen

			et al. 2004; Weidinger, Rummler et al. 2005)
IL4R	IL-4 receptor alfa chain	16p12-11	(Hershey, Friedrich et al. 1997; Oiso, Fukai et al. 2000; Callard, Hamvas et al. 2002; Novak, Kruse et al. 2002; Hosomi, Fukai et al. 2004)
NOD2	Caspase recruitment domain-containing protein 15 (*CARD15*)	16q21	(Kabesch, Peters et al. 2003)
CCL5	Regulated on activation, normally T cell expressed and secreted (*RANTES*)	17q11-12	(Nickel, Casolaro et al. 2000; Tanaka, Roberts et al. 2006)
CCL11	Eosinophil chemotactic protein (*Eotaxin*)	17q21	(Tsunemi, Saeki et al. 2002)
IL12RB1	Interleukin-12 receptor, beta-1	19p13.1	(Takahashi, Akahoshi et al. 2005)
TGFB1	Transforming growth factor, beta-1	19q13.1	(Arkwright, Chase et al. 2001)
SCCE	Stratum corneum chymotryptic enzyme (*KLK7*)	19q13.3	(Vasilopoulos, Cork et al. 2004)
GSTT1	Glutathione s-transferase theta-1	22q11.2	(Vavilin, Safronova et al. 2003)

Reports of lack of association with eczema exist for some candidate genes but are not listed here.

Filaggrin (FLG) gene

The *FLG* gene located on chromosome 1q21 is the most recently identified and so far strongest candidate gene for eczema. Null mutations in the gene encoding profilaggrin was originally found to cause the keratinizing disorder ichthyosis vulgaris (Smith, Irvine et al. 2006) and soon after these null mutations where shown to be a major predisposing factor for eczema (Palmer, Irvine et al. 2006). The carrier frequency of the two loss-of-function variants, R501X and 2282del4, is estimated to be about 9% in European populations. Several replications of the association with these variants in European populations have been published to date (Baurecht, Irvine et al. 2007), and unique variants in Japanese eczema patients have been found to be associated with the disease (Nomura, Sandilands et al. 2007). Except in Asian population, no loss-of-function variants have so far been identified in non-European populations.

Serine protease inhibitor, Kazal type 5 (SPINK 5) gene

Polymorphisms in the gene *SPINK5* causes the rare recessive disorder Netherton syndrome. *SPINK5* (Chavanas, Bodemer et al. 2000) has also been associated with eczema in several patient materials (Walley, Chavanas et al. 2001; Kato, Fukai et al. 2003; Nishio, Noguchi et al. 2003; Kabesch, Carr et al. 2004; Kusunoki, Okafuji et al. 2005). *SPINK5* encodes a serine protease inhibitor, which is thought to be important for skin barrier function (i.e. involved in the desquamation process in stratum corneum) (Cork, Robinson et al. 2006).

Interleukin 13 (IL13) gene

Interleukin 13, a cytokine expressed by Th2 cells is thought to play an important role in the pathogenesis of eczema (Leung, Boguniewicz et al. 2004). The gene encoding IL13 is located in the cytokine gene cluster on chromosome 5q31. In addition to being associated with eczema in several studies (Liu, Nickel et al. 2000; Tsunemi, Saeki et al. 2002; He, Chan-Yeung et al. 2003; Hummelshøj, Bodtger et al. 2003), the IL13 gene has also been associated with atopic manifestations in general (Hoffjan and Epplen 2005).

Mast cell chymase 1 (CMA1) gene

Mast cell chymase 1 is a chymotrypsin-like serine protease primarily stored in secretory granules in mast cells. The gene coding for mast cell chymase (*CMA1*), is located in a region previously linked to eczema, 14q11. Several studies have reported association of polymorphisms in the *CMA1* gene with eczema but not with other atopic manifestations (Mao, Shirakawa et al. 1996; Mao, Shirakawa et al. 1998; Tanaka, Sugiura et al. 1999; Iwanaga, McEuen et al. 2004; Weidinger, Rummler et al. 2005). The most pronounced effect have been observed in individuals at the low end of the total serum IgE spectrum (Mao, Shirakawa et al. 1998) indicating an association to non-atopic eczema. But there are studies that do not find any association to *CMA1* (Kawashima, Noguchi et al. 1998; Pascale, Tarani et al. 2001; Söderhäll, Bradley et al. 2001).

Interleukin 4 receptor alpha (IL4RA) gene

The receptor for IL4 and IL13, share a common α-chain encoded by *IL4RA* gene. Several amino acid changing polymorphisms have been identified and some polymorphisms associate with eczema (Hershey, Friedrich et al. 1997; Oiso, Fukai et al. 2000; Callard, Hamvas et al. 2002; Novak, Kruse et al. 2002; Hosomi, Fukai et al. 2004), others with other atopic manifestations (Hoffjan and Epplen 2005). There are also reports that do not find *IL4RA* to be associated with eczema (Tanaka, Sugiura et al. 2001; Söderhäll, Bradley et al. 2002). This makes the contribution of IL4RA to eczema development somewhat unclear.

Gene expression studies

At least two large-scale microarray analyses has to date been preformed on skin from eczema patients (Sugiura, Ebise et al. 2005; Olsson, Broberg et al. 2006). In a large-

scale DNA microarray study, where expression in eczema skin and normal control skin was analysed, Sugiura, Ebise et al. 2005 report ten genes that showed at least a five-fold difference in expression. Four out of the 10 genes showing the largest changes in expression in eczema skin are genes located in the epidermal differentiation complex on 1q21 (S100 calcium-binding protein A8 and S100 calcium-binding protein A7 were up-regulated, whereas loricrin and filaggrin were down-regulated). In order to identify genes that may contribute to transepidermal water loss in eczema, Olsson, Broberg et al. 2006 analysed gene expression in skin from eczema patients and healthy controls using large-scale DNA microarray. In this publication they only report one gene aquaporin 3 (AQP3) to be up-regulated in eczema skin as compared to in healthy skin. Furthermore in a microarray study of a subset of genes, Tenascin-C was found to be five times more up-regulated in lesional eczema skin as compared with in non-lesional or healthy control skin (Ogawa, Ito et al. 2005).

Microarray studies have also compared gene expression profiles in eczema with gene expression profiles in psoriasis. Either as large-scale analysis (Nomura, Gao et al. 2003) or in a subset of genes involved in innate immunity and host defence (Nomura, Goleva et al. 2003; Hijnen, Nijhuis et al. 2005). In large-scale analysis by Nomura and Gao et al, genes that showed at least five-fold increase in expression in eczema skin as compared to psoriasis skin were Nel-like2; the CC chemokines CCL-18, CCL-27, and CCL-13 (that are known to attract Th2-cells and eosinophils), and Tenascin-C (Nomura, Gao et al. 2003). Regarding the innate immune response, lower expression of the antibacterial peptides: HBD-2, iNOS, and IL-8 was found in eczema skin (Nomura, Goleva et al. 2003). These results were confirmed two years later by Hijnen et al. who reported the expression level of antibacterial proteins to be higher in psoriasis skin compared with in eczema skin (Hijnen, Nijhuis et al. 2005).

Animal models in eczema

Dogs and horses, and possibly also cats, develop eczema spontaneously. The prevalence of eczema is reported to be up to 10% among dogs (Marsella and Olivry 2003). As mice have a shorter life span and are easier to breed the use of mouse models for eczema can be very useful and several models for eczema exists. For instance, the NC/Nga mouse spontaneously develops skin lesions that are very similar to human eczema, if they are raised under conventional conditions. If raised in pathogen-free

environment the skin lesions do not occur (Tanaka and Matsuda 2006). Another mouse model is the NOA mouse (Naruto Research Institute Otsuka Atrichia) characterized by ulcerative skin lesions, accumulation of mast cells, and increased serum IgE levels (Natori, Tamari et al. 1999).

AIMS OF THE STUDY

The general aim of the thesis was to identify susceptibility genes for eczema.

The specific aims of the thesis were:

I. To analyse whether the candidate gene, *SOCS3*, selected based on results from a previous linkage scan and a gene expression study in eczema, is associated with the disease and thereby could be a susceptibility gene for eczema.

II. To determine the frequency of the recently identified loss-of-function variants in *FLG*, an important component of the skin barrier, in a Swedish eczema family material and test the association with eczema and associated phenotypes.

III. To analyse if *CRNN*, a potential susceptibility gene identified through gene expression studies in a mouse model, is associated with eczema in a family based association study.

IV. To evaluate if the *NPSR1* gene, a susceptibility gene for asthma and elevated serum IgE, could be a susceptibility gene also for eczema.

MATERIALS AND METHODS

PATIENTS

Papers I-IV

Swedish family material

The patient material used in these studies (**Papers I**, **II**, **III**, and **IV**) is a subset of a larger material collected for genetic analyses during 1995-1997 in the Stockholm area (Bradley, Kockum et al. 2000). Families with at least two siblings affected by eczema were recruited through the patient registers of the Department of Dermatology at Karolinska University Hospital (Solna), and Danderyd Hospital. Families with a child affected by eczema were identified and contacted. Patients were included in the study if the had at least one affected sibling and were over 4 years of age. The families were interviewed and parents were included in the study regardless of their atopic status. All the information about the families and their different aspects of eczema and different atopic phenotypes has been gathered in a database.

Clinical examination

The siblings were examined by the same dermatologist and included as affected by eczema if they fulfilled the UK Working Party's Diagnostic Criteria (Williams, Burney et al. 1994, I-III). The parents were not clinically examined but answered a questionnaire based on the UK Working Party's Diagnostic Criteria for eczema.

Atopic manifestations

All siblings were interviewed in a standardized manner covering different aspects of atopy and eczema. The interview included information about the eczema (age of onset, hospitalization, medication, duration, severity), past or present food allergy, urticaria, allergic asthma, and allergic rhinoconjunctivitis. Atopic manifestations among parents, grandparents, non-participating siblings, spouses and children to the affected siblings were recorded.

IgE quantification

IgE antibodies were quantified in all affected siblings. The total serum IgE was determined using the Pharmacia CAP System IgE FEIA (Phadia AB, Uppsala,

Sweden). The age-specific cut-offs were; 22.3 kU/L (9 months–5 years), 263 kU/L (5-20 years) and 122 kU/L (>20 years).

Allergen-specific IgE antibodies against Phadiatop®, a mixture of inhalant allergens, were analysed with the Pharmacia CAP System Phadiatop®FEIA. The inhalant allergens were: Dermatophagoides pteronyssinus, Dermatophagoides farinae, cat, dog, horse, birch, timothy grass, mugwort, olive, Cladosporium herbarum, and Parietaria judaica. Phadiatop® was recorded as either positive or negative.

Allergen-specific IgE antibodies against a mixture of food allergens (fx5) were analysed with the Pharmacia CAP RAST®FEIA. The food allergens were: hen's egg white, cow's milk, soya bean, peanut, fish, and wheat flour. The RAST results were divided into six classes, where a concentration <0.35kU/L represented a negative result (class 0).

Severity scoring

An arbitrary score for the severity of eczema was obtained using the classifications shown in Table 3.

Table 3. Severity score of eczema

Factor		Score
Age at onset <2 years		1
Hospitalization for eczema		1
Number of sites* manifesting eczema at examination:		
	0	0
	1-3	1
	>3	2
Raised total and/or allergen-specific IgE		1
Maximum score		5

* Presence of eczema in one or both sides in bilateral structures was considered as one site

Characteristics of the patient material

Of the 1 097 affected siblings, 667 were females and 430 male, and the median age at examination was 29 years. A majority had an onset before age of two years (78%), and 74% had elevated total and/or allergen-specific serum IgE-levels. Seventy-two percent of the siblings affected by eczema also suffered from asthma and/or allergic rhinoconjunctivitis (Figure 9) (Bradley, Kockum et al. 2000).

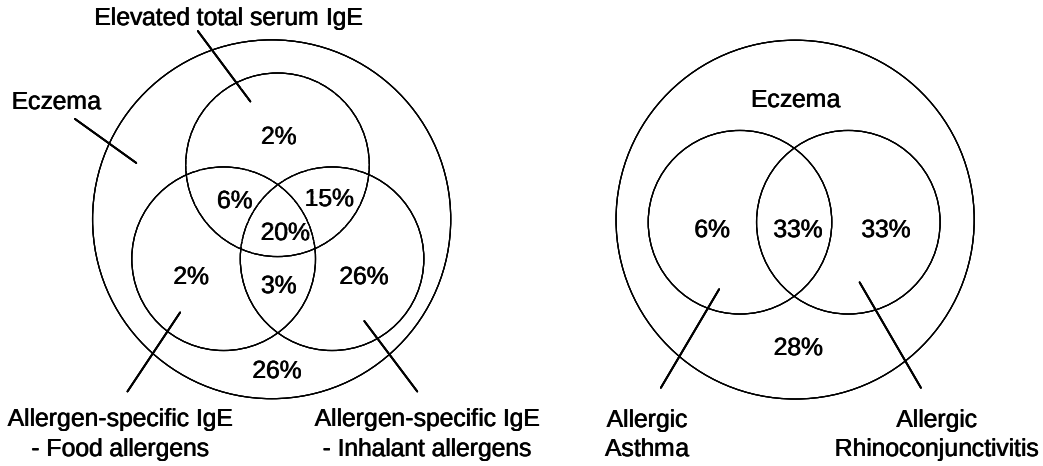


Figure 9. Prevalence of associated phenotypes in the 1 097 siblings affected by eczema.

The presented phenotypic characteristics of the patient material are a description of the material as a whole; the genotyping was performed on DNA from 1514 individuals from 406 families.

Additional patients in Paper I

In **Paper I**, we used two independent patient materials to confirm the genetic association found.

Swedish case control material

We performed genetic analysis in a nested case-control sample consisting of 555 children up to four years of age from a population-based birth cohort abbreviated BAMSE (Böhme, Lannero et al. 2002; Wickman, Kull et al. 2002; Melen, Gullsten et al. 2004). The children with reported symptoms of dry skin in combination with itchy rash for at least two weeks with typical localization, and/or a doctors' diagnosis of eczema were classified as having eczema (Böhme, Lannero et al. 2002). Allergen-specific serum IgE antibodies to inhalant and food allergens were measured in all children. Children that reported no eczema, no asthma or allergic rhinoconjunctivitis, and had no allergen-specific IgE were used as healthy controls. Mean age was 4.3 years. Of the 555 individuals, 328 were affected by eczema and 227 were controls.

British case control material

We also performed genetic analysis in a case-control study from the United Kingdom, comprising 187 adult eczema patients and 230 age and sex matched controls (Veal, Reynolds et al. 2005). All patients were diagnosed by a dermatologist using standard

clinical criteria of Hanifin and Rajka (Hanifin 1992). The mean age was 45 years. Total serum IgE levels were raised in 127 of the eczema patients and 164 had other atopic manifestations. The controls were not suffering from eczema, but were not checked for IgE levels or other atopic manifestations.

Two sets of individuals were also used in **Paper I**, for gene expression and immunohistochemistry analyses.

Patients for gene expression analysis

Nine adult patients with eczema were collected at the Dermatology Unit of Karolinska University Hospital (Solna). From each individual two punch biopsies were taken; one from lesional skin and one from non-involved phosphate-buffered-saline (PBS)-patch-tested skin on the back. The patients were diagnosed with eczema using the UK Working Party's Diagnostic Criteria (Williams, Burney et al. 1994, I). They had been without treatment for the last two months and were considered by a dermatologist to be moderately to severely affected by eczema. As controls we used four healthy control individuals from the Stockholm area. Two punch biopsies were taken from the back; one from normal skin and one from PBS-patch-tested skin. All patients had allergen-specific IgE antibodies but none of the controls. Each punch biopsy provided 10-30 micrograms of RNA. Total RNA was extracted from each skin biopsy using Trizol (Invitrogen, Carlsbad, CA, USA), and total RNA was linearly amplified according to the Ambion MessageAmp procedure.

Patients for immunohistochemical analysis

Applying the same diagnostic criteria, fifteen eczema patients were also collected for the immunohistochemical analysis. Two punch biopsies from the trunk were taken from each individual; one from lesional skin and one from non-involved skin. Eight adult healthy controls from the Stockholm area were also recruited, and biopsies were taken from normal skin.

Additional patients in Paper IV

In **Paper IV**, four European eczema patient materials were also used for the genetic analyses and a set of individuals for immunohistochemical analysis.

PARSIFAL

From the cross-sectional study PARSIFAL (Prevention of Allergy - Risk factors for Sensitisation In children related to Farming and Anthroposophic Lifestyle) samples from 3113 school-children aged 5-13 years from five Western European countries were included (Melen, Bruce et al. 2005). The study was designed to investigate the role of different lifestyles and environmental exposures in farm children, Steiner-school-children and two corresponding reference groups, to identify protective factors against the development of asthma and allergic disorders. Information on environmental exposures and health endpoints were reported by the parents of the children. Children ever diagnosed with asthma, or obstructive bronchitis more than once, were considered to have asthma. Children diagnosed with hay fever and who ever had had symptoms of hay fever, were considered to have rhinoconjunctivitis. The subjects were classified as having eczema when reporting ever having an intermittent itchy rash lasting at least 6 months and having a doctors' diagnose of atopic eczema. 218 children were affected by eczema in this patient material. The study design is described in detail elsewhere (Alfven, Braun-Fahrlander et al. 2006).

German family material

From Germany we used 224 families of Caucasian origin (complete trios and extended families) that had been recruited between January 2001 and December 2003 through an offspring with eczema (689 individuals) (Weidinger, Klopp et al. 2004). The affected offspring were classified as having eczema (n=302) on the basis of clinical examination by dermatologists applying the UK Working Party's Diagnostic Criteria (Williams, Burney et al. 1994, I). The subjects were classified as having allergic asthma or allergic rhinoconjunctivitis when they reported a physician's diagnosis.

KORA C and S4

The KORA (Cooperative Health Research in the Augsburg Region) S3 and S4 surveys are large population-based cross-sectional studies on adults performed from 1994 to 1995 and from 1999 to 2001 in the city and region of Augsburg, South Germany. KORA C represents an enriched sample of 1502 subjects out of the 4178 KORA S3 individuals, who had valid allergen-specific serum IgE results. Subjects were selected, stratified by age and sex, to provide 50% with and 50% without allergen-specific IgE to at least one of the allergens tested and furthermore stratified, so that within these groups

50% had reported symptoms of atopic diseases such as rhinoconjunctivitis, asthma, or eczema. DNA was available from 1420 individuals and 30 of them suffered from eczema. From KORA S4 (n=4261), 227 individuals with eczema and 227 matched controls were selected. The subjects were classified as having asthma, rhinoconjunctivitis or eczema when they reported a physician's diagnosis of asthma, rhinoconjunctivitis or eczema. The sampling frames and study designs have been described previously (Illig, Bongardt et al. 2003; Weidinger, Klopp et al. 2004).

In all four additional populations in **Paper IV**, allergen-specific IgE antibodies against a mixture of inhalant allergens were measured using the same method as described for the family material from Sweden. Additionally in the PARSIFAL study, antibodies against a mix of six common food allergens were measured using the same method as in the family material from Sweden. Total serum IgE was measured in all subjects in the different cohorts (except in the PARSIFAL study) again using the same method as in the family material from Sweden.

Patients for immunohistochemical analysis

Six adult eczema patients with positive atopy patch test (APT) reactivity to house dust mites, *Dermatophagoides pteronyssinus*, were included (two males, four females, age 37 to 65). Four had elevated total serum IgE levels (498-13900 kU/L) and three of these had allergen-specific serum IgE to *D. pteronyssinus* (ImmunoCAP, Phadia AB). Skin biopsies were taken from the APT sites with *D. pteronyssinus* at 6, 24, 48, and 72 h after application and at 72 h after application with the vehicle alone and were snap-frozen (Holm, Matuseviciene et al. 2004). In addition, skin biopsies were obtained from 8 healthy donors.

Phenotypes analysed

The affected individuals have been divided into different subgroups according to their different phenotypes. In the different studies we have analysed the following phenotypes:

In **Paper I**, we analysed the phenotype eczema.

In **Paper II**, we analysed eight phenotypes; eczema, atopic eczema, non-atopic eczema, severe eczema (≥4 in severity score), early onset of eczema (before the age of 2 years), eczema concomitant with raised total serum IgE, allergic asthma concomitant with eczema, and allergic rhinoconjunctivitis concomitant with eczema.

In **Paper III**, the phenotypes eczema, atopic eczema, and non-atopic eczema were analysed.

In **Paper IV**, we analysed the phenotype eczema in all five patient materials. Since PARSIFAL and KORA C participants were not primarily recruited as eczema patients we excluded them when we analysed four associated phenotypes; asthma concomitant with eczema, rhinoconjunctivitis concomitant with eczema, eczema with concomitant atopic sensitization (atopic eczema), and eczema concomitant with elevated total serum IgE. Individuals with total serum IgE levels above the 66th percentile in each population were defined as having an elevated level.

METHODS

Genotyping and SNP selection

In **Paper I**, the SNP selection was based on SNP information from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP). SNPs that previously had been validated repeatedly and had a minor allele frequency over 5% were chosen with priority. In **Paper III**, the SNP selection was based on HapMap genotype data (Frazer, Ballinger et al. 2007) downloaded from the HapMap project website (http://www.hapmap.org) and tag SNPs were selected using the Tagger implementation in the HAPLOVIEW program (Barrett, Fry et al. 2005). A minor allele frequency of 5% was used as cut-off together with a LOD threshold of 3.0 for multi-marker test and an r^2 threshold of 0.8 between SNPs. In **Papers II** and **IV**, the variants and SNPs were chosen based upon their significant associations in previous studies.

Genomic DNA was extracted from venous blood using standard phenol-chloroform procedures. In **Papers I-III**, the genotyping of most of the DNA samples were performed with TaqMan® SNP Genotyping Assays (Applied Biosystems [ABI]). This method is based on allelic discrimination of the wild type allele and the variant allele by

two MGB-probes. Each of the two probes is labelled with a different fluorescent dye, FAM or VIC. Primers and probes were obtained from ABI. The PCRs were carried out in 384-well plates with 5 ng of genomic DNA, 5 µl of a reaction mix containing the specific TaqMan assay solution and 1X TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). Amplification was performed following the Taqman Universal PCR protocol (95°C for 10 min, followed by 40 cycles at 92°C for 15 sec and 60°C for 1 min). Allelic discrimination was performed with the ABI PRISM® 7900HT Sequence Detection System and the SDS 2.2.1 program (Applied Biosystems).

In **Paper II,** genotyping and allelic discrimination of the 2282del4 variant was performed by Colin Palmer's lab in Dundee, Scotland, using a TaqMan-based assay as described more thoroughly in (Sandilands, Terron-Kwiatkowski et al. 2007).

In **Paper IV**, the DNA samples were genotyped using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (SEQUENOM Inc., San Diego, California). This method is based on an allele-specific extension reaction that allows for the differentiation of alleles. Because of different mass of the incorporated nucleotides the extension products can easily be differentiated in a mass spectrum. The genotyping was performed at either the Department of Biosciences at Novum, Karolinska Institutet, Huddinge or at the Department of Dermatology and Allergy, Technical University Munich, Germany as described (Melen, Bruce et al. 2005; Weidinger, Klopp et al. 2005). Briefly, at the Department of Biosciences PCR assays and associated extension reactions were designed using the SpectroDESIGNER software (Sequenom Inc., San Diego, California) and primers were obtained from Metabion GmbH (Planegg-Martinsried, Germany). All amplification reactions were run in the same conditions in a total volume of 5 ml with 2.5 ng of genomic DNA, 1 pmol of each amplification primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$ and 0.2 U of HotStarTaq DNA Polymerase (Qiagen). Reactions were heated at 95°C for 15 min, subjected to 45 cycles of amplification (20 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C) before a final extension of 7 min at 72°C. Extension reactions were conducted in a total volume of 9 ml using 5 pmol of allele-specific extension primer and the Mass EXTEND Reagents Kit before being cleaned using SpectroCLEANER (Sequenom Inc., San Diego, California) on a MULTIMEK 96 automated 96-channels robot

(Beckman Coulter, Fullerton, California). Clean primer extension products were loaded onto a 384-elements chip with a nanoliter pipetting system (SpectroCHIP, SpectroJet, Sequenom) and analysed by a MassARRAY mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting mass spectra were analysed for peak identification using the SpectroTYPER RT 2.0 software (Sequenom).

Quality assessment

As genotyping errors can affect the ability to detect association and also give rise to false positive results, the data was evaluated in different ways prior to association analyses.

One advantage with genotyping family materials is that one has the possibility to use the genotype data and find inconsistencies in Mendelian inheritance, for example too many alleles in the siblings or alleles not derived from the parents. Families were evaluated for inheritance inconsistencies for all markers using the software zGENSTAT 1.128 (H. Zazzi, unpublished). All genotypes that showed inconsistencies were re-analysed and either corrected or removed.

The relationship between allele frequency and the predicted genotype frequencies in a random sample in the population was originally described by Hardy and Weinberg (Strachan and Read 2004). If the observed genotype frequency differs from the expected, deviation from Hardy-Weinberg Equilibrium (HWE) exist. Deviation may indicate non-specific amplification of the assay or genotyping errors such as missed amplification of one of the alleles or inaccurate calling of alleles (Hosking, Lumsden et al. 2004). In zGENSTAT, the expected number of homozygotes is calculated based on the estimated allele frequencies. Using a χ^2 test, the observed number of homozygotes is compared with the expected. A marker showing significant deviation ($p<0.001$) from the expected number of homozygotes was regarded as being in Hardy-Weinberg disequilibrium, and the marker was therefore disregarded. If disequilibrium was observed only in a subset of the samples, this subset was excluded from further analyses.

Mouse model for eczema

In **Paper III**, we used a mouse model to screen for genes with altered transcript levels in the skin after induction of an eczema-like phenotype. The eczema-like phenotype was induced by epicutaneous patching with 50 μg recombinant Der p2 repeatedly on six to eight week old female BALB/c J mice from the Jackson Laboratory (Bar Habor, Maine). Immunohistochemistry on skin biopsies from sensitized mice collected on day 50 showed eczema-like features such as: hyperplasia of the epidermal and dermal layer, hyperkeratosis and spongiosis, with infiltration of inflammatory cells (mainly lymphocytes and eosinophils). The mice sensitized with allergen produced Der p2-specific IgE, IgG1 and IgG2a progressively during the protocol period which was not seen in control mice. T-cells from the mice sensitized with allergen showed cytokine profiles consistent with Th2 immune response (increased IL-4, IL-5, IL-13, IL-9, and IL-10).

Gene expression

cDNA microarray analysis

In **Paper I,** microarray analysis was performed using human cDNA microarrays containing 41 792 sequences (that represent approximately 24 500 unique genes) manufactured by the Stanford Microarray Facility (http://www.microarray.org/sfgf). Fluorescently labelled cDNA prepared from amplified RNA was hybridized to the array in a two-colour comparative format, with eczema patient or healthy control samples labelled with Cy-5, and a reference pool of human mRNAs (Stratagene) derived from ten cell lines labelled with Cy-3. Array images were scanned by using an Axon Scanner 4000B (Axon Instruments, Union City, CA), and data were analysed by using GenePix 3.0 (Axon Instruments, Union City, CA). Data were normalized and retrieved as the $\log_2$ ratio of fluorescence intensities of the sample (Cy5) and the reference (Cy3).

Quantitative real time PCR

Regular polymerase chain reaction (PCR) is not quantitative as the accumulated PCR products are measured after termination of the reaction when most products have reached a plateau level. Real Time PCR on the other hand is a quantitative method which measures the amount of fluorescent PCR product continuously throughout the reaction. For the Real Time PCR experiments in **Paper III**, 1 μg of total RNA from

samples was reversely transcribed into cDNA with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) using an oligo(dT)$_{12-18}$ in the reaction mixture. Primer Express™ 2.0 software from Applied Biosystems (Foster City, California) was used to design specific primers. All primers were designed to overlap an exon-exon junction to avoid amplification of genomic DNA. Each primer pair was tested to produce only one product by a dissociation test (melting temperature of the double stranded product) performed in each run. The product of each designed primer pair was run on a gel to confirm the correct size. Furthermore, the PCR efficiency for all products was tested in a dilution experiment to confirm that the efficiency was independent of template concentration in the range found in the analysed samples. Reactions were carried out in triplicate for each gene in an ABI Prism 7500SDS, using SYBR Green (a dye that binds double stranded DNA) according to manufacturer's instructions (Applied Biosystems). After normalization with the endogenous housekeeping gene Hypoxantine phosphate ribosyl transferase, relative gene expression levels were determined by the comparative C_T method.

Immunohistochemistry

Immunohistochemistry detects cell-specific expression of protein. Staining was performed on snap frozen acetone-fixed, 6-µm-thick, vertical cryostat. The method used was the avidin-biotin complex (ABC) method (Hsu, Raine et al. 1981; Hsu, Raine et al. 1981) (**Papers I** and **IV**) (Vector Laboratories Inc. Burlingame, CA). In this method a secondary rabbit anti-mouse antibody labelled with biotin is attached to the primary antibody. When adding a preformed avidin-biotin-enzyme complex, each secondary antibody is surrounded by enzyme that catalyzes a staining reaction. In the last step of the procedure, the tissue antigen is localized by incubation with a substrate (3-amino-9-ethylcarbazole) for the enzyme. The sections were treated with H_2O_2 to block endogenous peroxidase activity, and non-specific binding of the antibodies was reduced by incubating the sections with normal goat serum (Dako Cytomation A/S, Glostrup, Denmark) and avidin/biotin blocking kit (Vector Laboratories Inc.) prior to staining.

In **Paper I**, a mouse monoclonal antibody against human SOCS-3, dilution 1/250, purchased from Assay Designs (Ann Arbor, MI, USA) was added to incubate with the specimens for 30 minutes. A biotinylated secondary rabbit anti-mouse antibody,

dilution 1/200 (Vector Laboratories) was also added. Counterstaining was made with Mayer's haematoxylin and as a negative control, irrelevant mouse IgG1 was used.

In **Paper IV**, affinity purified rabbit antibodies directed against NPSR1 isoforms A and B respectively, was obtained (GeneOS Oy, Helsinki, Finland), and used at an IgG concentration of 5μg/mL. Pre-immune rabbit serum and irrelevant primary antibody were used as negative controls. Positive staining with the rabbit antibodies directed against NPSR1 was blocked by pre-incubation of the antibodies with their corresponding peptide overnight, before it was added to the skin sections.

Statistical analysis

When testing for allelic association in **Paper I** the statistical software UNPHASED (version 2.403) (Dudbridge 2003) was used. The method used in the analyses of the family material is a PDT test (Pdtphase command), whereas in the two case-control materials an ordinary χ^2 test was used (Cocaphase command). The same software was used to evaluate the LD structure in the region. Since this software does not report transmission frequencies the transmission frequencies were estimated with the program PDT (version 4.0) for alleles and the TRANSMIT program (version 2.5.2) (Clayton 1999) for haplotypes. A non-parametric linkage analysis were also performed using ALLEGRO program (Gudbjartsson, Jonasson et al. 2000), with exponential model with power weighting of families. Power calculation for case control studies were performed using the PS software, version 2.1.31. PS calculates the expected power for a given number of cases and controls assuming certain values of genotype/allele frequencies among controls, OR for the genotype/allele and $\alpha <$ the predetermined cut-off for significance (Dupont 1997). Correction for testing multiple markers was done by the method of Bonferroni (where the p-value is corrected by multiplying with the number of tests performed) either by the number of tested SNPs (n = 3) or the number of tested haplotypes (haplotypes with frequency >5%, n = 4). In **Paper I**, we performed a meta-analysis of the two case control studies in order to evaluate heterogeneity in the two study populations and increase the power. The meta-analysis was performed according to the method described by Woolf (Woolf 1955).

In **Paper II**, the PDT method as implemented in UNPHASED version 3.0.7 was used. The following analysis options were applied; full model option, rare frequency

threshold of 3%, uncertain and missing genotypes option, and model odds ratio in parents. Odds ratio (OR) for alleles was estimated relative to the most common haplotype. P-values were not corrected for multiple comparisons.

In **Paper III**, expression data were compared by Mann-Whitney test except for the comparison between lesional and non-lesional skin from the same patient where the Wilcoxon test was used. The TRANSMIT program (version 2.5.4) was used to test for genetic association. This program performs a transmission disequilibrium test that correctly uses information from families with more than one affected individual. Calculation of pair-wise linkage disequilibrium between SNPs was performed in the software HAPLOVIEW (Barrett, Fry et al. 2005). P-values were not corrected for multiple comparisons in this study.

Using the UNPHASED (3.0.10) program in **Paper IV** allowed for analyses of association in family-based materials and case-control materials combined (Dudbridge 2006). This was done in addition to the analyses performed in the patient materials separately. The following analysis options were applied: full model option, rare frequency threshold of 3%, uncertain haplotypes option for the joint analysis and the three case-control studies, and uncertain and missing genotypes option for the two family materials. With individual haplotype option in the haplotype analyses, a score test of the difference in risk between haplotypes and all the others pooled together was performed, although the OR is shown relative to a reference haplotype (the most frequent haplotype, H1). In the analyses of associated phenotypes in eczema context the zero frequency threshold were set to 0.01, whereas in all the other analyses the default value was used. Power analysis was performed using the GENETIC POWER CALCULATOR (Purcell, Cherny et al. 2003) with model parameters set to calculate the power to detect a dominant effect. Significant p-values were corrected with permutation test generated by the UNPHASED program by randomizing the transmission status of the parental haplotypes.

RESULTS AND DISCUSSION

SOCS3, A CANDIDATE GENE FOR ECZEMA

*Elevated Expression and Genetic Association Links the SOCS3 Gene to Atopic Dermatitis (**Paper I**)*

To search systematically for gene expression patterns associated with eczema, we adopted a genome-wide approach using human cDNA microarrays from skin biopsies from eczema patients and healthy controls. In order to link the differential expression of Suppressor of Cytokine Signaling 3 (*SOCS3)* gene to changes in its protein expression, we used immunohistochemistry on biopsy specimens from a different set of eczema patients and controls. Furthermore, we investigated genetic association between the *SOCS3* gene and eczema in three independent patient materials. We used a set of 406 Swedish families, a nested case-control sample from a population-based birth cohort of Swedish children (n=555) and a case-control study with adult eczema patients from the UK (n=417).

When analysing the microarray data, we found approximately 5 000 genes consistently differentially expressed between skin from eczema patients and control individuals (Sääf et al. manuscript in preparation). Among all the differentially expressed genes, we focused specifically on genes located within chromosomal regions previously linked to eczema. The *SOCS3* gene located in chromosome region 17q25, was found to be significantly more highly expressed in skin from eczema patients as compared to healthy individuals ($p<0.03$, corrected for multiple testing) (Table 1 and Figure 1 in Paper I).

We also found a higher expression of SOCS3 protein in dendritic cells and an increased number of SOCS3 positive cells in epidermis from lesional eczema skin (median 5.6 cells/mm, range 1.1-10.5) compared to non-lesional eczema skin (median 1.1 cells/mm, range 0-5.6, $p<0.05$) and normal skin (median 0.8 cells/mm, range 0-5.9, $p<0.0008$) (Figure 2 in Paper I).

Two SNPs in the *SOCS3* gene were significantly associated with the eczema phenotype (rs12952093, p<0.03 and rs4969170, p<0.02). All the analysed SNPs fell into two haplotype blocks. We then identified three haplotype-tagging SNPs as representative of these haplotype blocks: rs12952093 and rs4969170 in block 1, and rs4969168 in block 2. Two of them were in the promoter region of the gene (rs12952093 and rs4969170) and the third was in 3′UTR (rs4969168). One haplotype from the first block was positively associated with eczema (A-A p<0.02) and one was negatively associated (C-G p<0.04) (Table 4).

Table 4. Genetic Association of *SOCS3* with eczema in Swedish Family Material

Allele or Haplotype				No. of	Transmission Frequency	
rs12952093	*rs4969170*	*rs4969168*	Frequency	Transmissions	(%)	*P*
A			0.31	286	55	0.03
	A		0.37	351	54	0.02
		G	0.83	761	50	NS
C	G		0.61	1140	49	0.04
A	A		0.32	582	53*	0.02
C	G	G	0.64	1118	49	NS
A	A	G	0.16	389	54*	NS

* These transmission frequencies are now more accurately calculated than in the original article.

When finding association to polymorphisms in *SOCS3*, we wanted to determine whether this association could explain the previously found linkage peak on 17q25. The *SOCS3* gene was found to be linked to eczema in these families (LOD=1.63, p<0.04). However, the linkage didn't reach the previously shown suggestive linkage level ($p<7.4x10^{-4}$) in chromosomal region 17q25, indicating that *SOCS3* is not the only gene contributing to the broad peak in the region.

To confirm the genetic association we analysed the three haplotype-tagging SNPs in two independent sets of patients and controls: one nested case-control sample consisting of 555 Swedish children, and one case-control study comprising 187 adult eczema patients and 230 age and sex matched controls from the UK. When analysing the Swedish case-control samples, we found a highly significant genetic association between eczema and both the positively associated haplotype (p<0.03) and the negatively associated haplotype (p<0.02) (Table 5). However, we could not confirm these associations in the UK study although the power was over 80% to detect an

association to the haplotypes, assuming the same haplotype frequencies as observed in the Swedish case-control study. But most likely there is still not power enough in this patient material to detect an association, since a meta-analysis performed in the two case-control sets showed that the risk haplotype was significantly associated ($p<0.02$, with combined OR 1.37 (1.07-1.77) with 95%CI) and a test for heterogeneity between the studies was not significant.

Table 5. Genetic Association of *SOCS3* with eczema in a Swedish nested Case Control Study

Allele or Haplotype			Frequency	Frequency		
rs12952093	*rs4969170*	*rs4969168*	in Controls	in Patients	OR (95% CI)	*P*
A			0.26	0.33	1.45 (1.10-1.92)	0.03
	A		0.31	0.41	1.51 (1.16-1.97)	0.009
		G	0.85	0.84	0.90 (0.62-1.31)	NS
C	G		0.68	0.58	0.67 (0.51-0.87)	0.02
A	A		0.24*	0.33*	1.50 (1.12-2.00)	0.03
C	G	G	0.69	0.58	0.63 (0.48-0.82)	0.004
A	A	G	0.15	0.23	1.72 (1.22-2.43)	0.01

* The original article had these frequencies erroneously switched.

The consistently elevated expression of SOCS3 in skin of eczema patients, together with evidence for genetic association in two independent patient materials, makes *SOCS3* a strong candidate gene for eczema susceptibility. This evidence is reinforced by the potential pathophysiological link between the activity of SOCS3 and the pathogenesis of eczema. SOCS3 is one of eight members of the intracellular protein family, suppressors of cytokine signaling (SOCS). The SOCS proteins regulate cytokine signaling in a negative-feedback loop (Alexander 2002). SOCS3 is generally not highly expressed, but expression is induced by a variety of cytokines (Alexander and Hilton 2004). SOCS3 is thought to bind to the activation loop of Janus kinases, inhibiting kinase activity and thereby suppressing further cytokine signaling. The fact that *SOCS3* could play a role in the pathogenesis of eczema is shown by Seki, Inouse et al. 2003 who found SOCS3 to be expressed predominantly in Th2-cells where it has an important role in regulating the onset and maintenance of Th2-mediated responses. They also found that SOCS3 expression correlated with severity of asthma and eczema, as well as with high levels of serum IgE. Eczema patients show significantly increased SOCS3 expression in peripheral T-cells. Transgenic mice that over-express SOCS3 have an increased production of Th2-associated cytokines and transgenic mice with a

reduction of SOCS3 expression showed suppressed Th2-development. Therefore, SOCS3 seems to be part of the regulation of Th2-responses in eczema. However, the presence or absence of eczema-like features in the *SOCS3* transgenic mice and expression of SOCS3 protein in cell types other than T-cells was not reported (Seki, Inoue et al. 2003).

To conclude Paper I, we found that *SOCS3* is consistently more highly expressed in skin from patients with eczema than in normal control skin and specific haplotypes of the *SOCS3* gene are significantly associated with the disease. We suggest that *SOCS3* may play an important role in eczema pathogenesis. Despite the connection between *SOCS3* and eczema, it is still unclear how the polymorphisms in the *SOCS3* gene affect the development of eczema, but there may be a direct effect on transcription, since two of the associated SNPs are located in predicted transcription factor binding sites.

After the publication of these results, we wanted to gain more information about how the genetic association between *SOCS3* and eczema could influence the eczema development or pathogenesis. We aimed at sequencing the full sequence of the *SOCS3* region (14 kB) in a set of patients with the different haplotypes represented in order to identify novel SNPs that could be even more associated with the disease than those previously genotyped. The LD pattern in the region indicates that there could be parts of the *SOCS3* region that is not fully tagged by our initial analysis of SNPs. These analyses have not yet been completed. In parallel, we are focusing on the 3′UTR region where a possible binding site for the microRNA miR-203 resides (Sonkoly, Wei et al. 2007). A polymorphism at that binding site could have vast impact on the regulation of SOCS3 expression.

FILAGGRIN, A SKIN BARRIER GENE LINKED TO ECZEMA

*Loss-of-function Variants of the Filaggrin Gene are Associated with Atopic Eczema and Associated Phenotypes in Swedish Families (**Paper II**)*

Recently published studies on the EDC member *FLG* have identified two common loss-of-function variants, R501X and 2282del4, to be causative factors in ichthyosis vulgaris and major predisposing factors in the development of eczema (Palmer, Irvine et al. 2006; Smith, Irvine et al. 2006). In order to analyse the frequency of the R510X and 2282del4 variants in the Filaggrin gene (*FLG*) and their association with eczema and associated phenotypes, we used PDT analysis in a Swedish family material consisting of 406 multiplex families.

Both R501X and 2282del4 were over-transmitted to eczema-affected offspring ($p=1.5\times10^{-5}$ and $p=2.9\times10^{-5}$) and also the combined allele was over-transmitted ($p=1.3\times10^{-6}$) (Table 6). Dividing the siblings into atopic and non-atopic eczema subgroups, the atopic group showed an OR of 2.21 (1.50-3.25) for the combined allele ($p=9.5\times10^{-8}$). The *FLG* variants were even more over-transmitted to affected siblings in the severe eczema phenotype (severity scoring ≥4), where the combined allele showed an OR of 4.73 (1.98-11.29) with $p=3.6\times10^{-8}$. In the non-atopic eczema group, there was no significant association with either of the two *FLG* variants.

Table 6. Results of PDT for Filaggrin variants in the Swedish eczema families

TRAIT	R501X		2282DEL4		COMBINED	
	OR (95%CI)	*P*	**OR** (95%CI)	*P*	**OR** (95%CI)	*P*
ECZEMA	**2.68** (1.34-5.33)	1.5×10^{-5}	**1.85** (1.24-2.76)	2.9×10^{-5}	**1.81** (1.31-2.50)	1.3×10^{-6}
ATOPIC ECZEMA	**4.33** (1.88-9.94)	2.6×10^{-7}	**2.22** (1.41-3.47)	4.1×10^{-6}	**2.21** (1.50-3.25)	9.5×10^{-8}
NON-ATOPIC ECZEMA	**2.07** (0.78-5.47)	NS	**0.99** (0.54-1.80)	NS	**1.07** (0.62-1.84)	NS
EARLY ONSET	**4.37** (1.79-10.64)	2.8×10^{-7}	**2.14** (1.41-3.23)	4.2×10^{-5}	**2.09** (1.46-2.97)	1.2×10^{-6}
SEVERE ECZEMA	**5.29** (3.32-8.84)	1.3×10^{-12}	**6.34** (1.90-21.2)	1.5×10^{-7}	**4.73** (1.98-11.29)	3.6×10^{-8}
ALLERGIC ASTHMA	**3.94** (1.12-13.87)	8.4×10^{-6}	**3.85** (1.97-7.51)	2.4×10^{-7}	**3.58** (1.99-6.42)	6.5×10^{-9}
ALLERGIC RHINITIS	**5.07** (1.47-17.46)	2.7×10^{-12}	**1.97** (1.28-3.03)	7.8×10^{-5}	**2.03** (1.39-2.97)	7.0×10^{-7}
RAISED TOTAL SERUM IGE	**4.33** (1.26-14.81)	8.8×10^{-6}	**1.85** (1.14-2.98)	0.0066	**1.93** (1.24-2.99)	0.00054

OR: Odds ratio of minor allele relative to major allele, NS: non-significant, CI: confidence interval.
OR for R501X in the severe eczema phenotype is calculated in a χ^2 test.

In addition to the analysis of association with eczema, we analysed association to other atopic phenotypes in the eczema families. Association was found for raised total serum IgE, allergic asthma and allergic rhinoconjunctivitis, with the most significant association found for the R501X variant and allergic rhinoconjunctivitis, $p=2.7\times10^{-12}$, OR 5.07 (1.47-17.46) (Table 6).

The effect of *FLG* null status on the severity of the eczema and associated phenotypes was further illustrated when we correlated the genotype of the affected siblings with the frequency of the phenotypes. For this analysis, one sibling from each family was randomly selected and only individuals with full genotype information were used. The frequency of the genotypes were, wild-type=313, either heterozygote=67 and homozygotes=6 (or compound heterozygotes). As shown in Figure 10, there was an increase in the frequency of the eczema-associated phenotypes in the heterozygotes compared to wild-type carriers, and in the homozygotes compared to the heterozygotes.

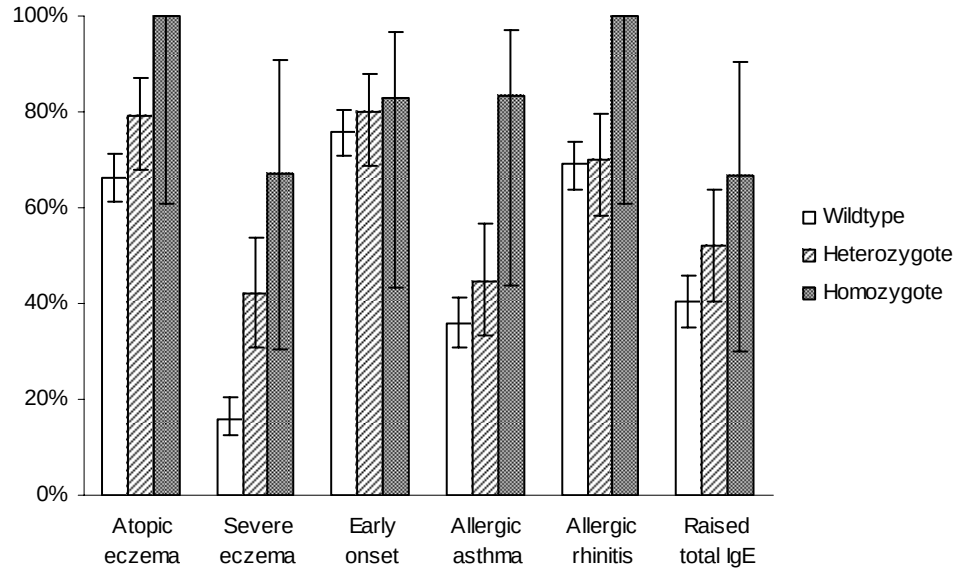


Figure 10. Correlation of FLG genotype and phenotype frequencies in eczema-affected siblings. There was a significantly higher frequency of the severe eczema phenotype among heterozygous carriers of a *FLG* null allele compared to among wt/wt ($p<1.4 \times 10^{-6}$). The 95% confidence interval is shown by error bars.

When analysing the subgroup with the severe eczema phenotype, 43.4% of the individuals were carriers of an *FLG* null allele. As illustrated (Figure 10), 67% of the homozygote carriers and 42% of the heterozygote carriers belonged to the severe eczema phenotype. Only 16% of wild-type carriers of *FLG* belonged to this phenotype

group. Our results may therefore support the hypothesis that individuals with eczema who carry *FLG* null alleles would be more likely to suffer from a persistent severe disease. In our material, 78% of the patients had early onset (≤2 years of age), but the effect of carrying an *FLG* null allele seems only marginal on early-onset (Figure 10).

The association with eczema and associated phenotypes has, since the original publication by Palmer, Irvine et al. 2006, been replicated and extended in a number of populations in Western Europe (Baurecht, Irvine et al. 2007). In the first analysis of *FLG* variants in a Swedish population, we present data that confirm the importance of *FLG* variants as susceptibility factors for eczema and eczema-associated phenotypes. In accordance with other family-based studies we found an over-transmission of the FLG variants in our material (Marenholz, Nickel et al. 2006; Weidinger, Illig et al. 2006; Morar, Cookson et al. 2007; Weidinger, Rodriguez et al. 2007). There was a strong association with eczema, but as reported in some previous studies, we found that the association was mainly to the atopic subgroup of eczema (Weidinger, Illig et al. 2006; Morar, Cookson et al. 2007; Weidinger, Rodriguez et al. 2007). In fact the non-atopic eczema phenotype was not associated to *FLG* in our study even if the power to detect a high-risk allele with an OR of 2.0 was over 95% in our family material, assuming the allele frequency to be 0.086 for carrying a *FLG* null allele. The highest odds ratio was found in the severe eczema phenotype group and more than 40% of individuals with severe eczema carried an *FLG* null allele. Our results therefore support the hypothesis that individuals with eczema who carry *FLG* null alleles would be more likely to suffer from a severe disease. Several studies have indicated that *FLG* variants may be susceptibility factors for asthma (Marenholz, Nickel et al. 2006; Palmer, Irvine et al. 2006; Weidinger, Illig et al. 2006; Palmer, Ismail et al. 2007; Weidinger, Rodriguez et al. 2007), rhinoconjunctivitis (Marenholz, Nickel et al. 2006; Weidinger, Rodriguez et al. 2007) and raised total IgE (Weidinger, Illig et al. 2006; Weidinger, Rodriguez et al. 2007) in eczema patients. A similar pattern of association with allergic asthma, allergic rhinoconjunctivitis, and raised total IgE was also seen in our material. However, most studies have shown that there is no association with either asthma or rhinoconjunctivitis when eczema-affected individuals are excluded from the study populations (Marenholz, Nickel et al. 2006; Palmer, Irvine et al. 2006; Morar, Cookson et al. 2007; Rogers, Celedon et al. 2007). However, association with asthma severity independent of

eczema status has been found (Palmer, Ismail et al. 2007), so the matter of Filaggrin's association with atopy is far from solved.

To summarize the knowledge about the common variants in the Filaggrin gene, it seems that *FLG* variants are strongly associated with eczema. In a recent meta-analysis, the estimated effect size of *FLG* variants corresponds to an OR of 4.0 and 2.0 respectively for case control studies and family-based association studies (Baurecht, Irvine et al. 2007). The association is particularly strong with severe eczema and/or with atopic eczema. The interpretation of this could be that the skin barrier (i.e. the function of the filaggrin protein) is crucial for maintaining the skin free of eczematous reactions. As mention above, we found no association with non-atopic eczema. Intuitively one might think that non-atopic eczema would also be associated with variants in a gene coding for an important component of the skin barrier. The lack of association with non-atopic eczema could be explained by the importance of the *FLG* gene for the integrity of the skin barrier. Carrying a dysfunctional *FLG* gene may lead to atopic eczema because a disrupted skin barrier will increase the epidermal allergen transfer and eventually cause allergic sensitization.

To conclude our findings in Paper II, we found a strong association of loss-of-function variants in *FLG* with eczema. This was especially seen in patients with a severe phenotype and/or with atopic eczema. This supports the importance of the *FLG* gene variants R501X and 2282del4 in the development and severity of atopic eczema.

CORNULIN – A NOVEL SKIN BARRIER GENE?

*Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema (**Paper III**)*

The aim of Paper III, was to identify new molecular disease determinants of eczema and test for genetic association with possible candidate genes. For this purpose an eczema-like phenotype was induced in mice by epicutaneous patching with a major allergen from the house dust mite *Dermatophagoides Pteronyssinus* (Figure 1 and 2a-b, in Paper III). In the mouse model, differentially expressed sequences was identified through the Subtractive Suppression Hybridization method (von Stein 2001). 163 genes were identified and from this data set we confirmed differential expression of genes by Real Time PCR. Most striking was the down-regulation of the *CRNN* gene in Der p2-patched animals compared to controls, p=0.004 (Figure 11).

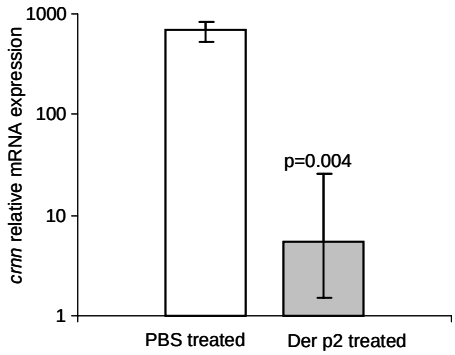


Figure 11. mRNA expression of the crnn gene analysed by Real Time PCR in Der p2-patched skin compared to PBS controls. The data are presented as median values and the 1st and 3rd quartile are shown by error bars.

The transcriptional level of *CRNN* was analysed in skin biopsies from 15 eczema patients (both non-lesional and lesional areas) and 9 healthy controls. *CRNN* mRNA expression was reduced in the eczematous skin, with the majority of patients showing a two-fold reduction or more in lesional areas compared to non-lesional (p=0.009). There was also a significantly lower expression in lesional skin compared to the skin from healthy individuals (Figure 3 in Paper III). Given this finding and the location of *CRNN* within the epidermal differentiation complex, we considered *CRNN* as a candidate gene for eczema, and tested for genetic association.

Six haplotype-tagging SNPs in the gene region were genotyped. A TDT analysis revealed the polymorphism rs941934 to be significantly associated with atopic eczema

and the minor allele to be over-transmitted in this phenotype (p=0.006) (Table 1 in Paper III). An analysis of LD in the region revealed the SNPs to be contained within a single LD block, giving rise to five haplotypes with a frequency over 2% (table 7). One of these haplotypes, CCATAT, tagged by the SNP rs941934, was again associated with atopic eczema, and over-transmitted in this group of patients (p=0.004).

Table 7. Association of *CRNN* haplotypes with Eczema, Atopic eczema and Non-atopic eczema

HAPLOTYPES						ECZEMA				ATOPIC ECZEMA			NON-ATOPIC ECZEMA		
rs10494275	rs4845766	rs941934	rs4240877	rs1923492	rs1923493	HF	N	%	*P*	N	%	*P*	N	%	*P*
T	C	G	C	G	G	0.067	131	50.9	0.74	89	53.2	0.35	40	45.6	0.36
C	A	G	C	G	T	0.634	1144	49.8	0.65	712	49.3	0.35	422	50.7	0.43
C	C	G	T	A	T	0.076	130	47.9	0.43	84	47.2	0.41	45	49.1	0.85
C	C	A	T	A	T	0.137	266	53.6	0.04	170	56.7	0.004	91	48.1	0.51
C	C	G	C	G	G	0.086	146	47.2	0.25	88	44.3	0.07	56	52.4	0.56

Haplotype Frequency (HF) in the whole material, Number of transmissions (N), Transmission frequency (%)

While this article was being put together, two variants in the filaggrin gene was identified as major susceptibility factors for eczema (Palmer, Irvine et al. 2006). Given the relatively short distance (~100 kilobases) between the polymorphism rs941934 in *CRNN* and the two variants R510X and 2282del4 in *FLG*, we decided to investigate the linkage disequilibrium structure between these genes in our genotype data.

Analysis of LD revealed a D′ value of 0.86 and an r^2 value of 0.26 between rs941934 and the 2282del4 variant in the *FLG* gene. Including the *FLG* variants in the haplotype analysis showed that the 2282del4 splits the associated *CRNN* haplotype into two different haplotypes (Table 8). Only the haplotype carrying the 2282del4 variant was still significantly associated with eczema (p=0.002) and atopic eczema (p=0.0002). As expected the haplotype tagged by the *FLG* R501X variant was also significantly associated with eczema and atopic eczema (Table 8).

Table 8. Association of CRNN-FLG haplotypes with Eczema, Atopic eczema and Non-atopic eczema

HAPLOTYPES								ECZEMA				ATOPIC ECZEMA			NON-ATOPIC ECZEMA		
rs10494275	rs4845766	rs941934	rs4240877	rs1923492	rs1923493	2282_del4	R501X	HF	N	%	*P*	N	%	*P*	N	%	*P*
T	C	G	C	G	G	WT	C	0.067	122	52.1	0.47	85	54.0	0.27	36	47.8	0.67
C	A	G	C	G	T	WT	C	0.606	980	49.1	0.17	623	48.4	0.06	350	50.6	0.58
C	A	G	C	G	T	WT	T	0.022	54	72.4	4.8×10^{-6}	40	76.8	1.1×10^{-5}	14	61.9	0.19
C	C	G	T	A	T	WT	C	0.077	121	48.7	0.65	79	47.9	0.56	41	50.1	0.99
C	C	A	T	A	T	WT	C	0.086	132	47.5	0.34	75	48.3	0.62	54	45.5	0.27
C	C	A	T	A	T	Del	C	0.046	96	61.3	0.002	74	66.6	0.0002	21	48.6	0.85
C	C	G	C	G	G	WT	C	0.084	129	46.2	0.15	79	43.4	0.05	47	51.5	0.75

Haplotype Frequency (HF) in the whole material, Number of transmissions (N), Transmission frequency (%)
Wild type (WT), FLG variant 2282del4 (Del)

Cornulin is a recently identified member of the fused gene family, located on chromosome 1q21. The protein has a Ca^{2+}-binding N-terminal domain and show structural similarities with both precursor proteins of the cornified envelope (such as repetin, profilaggrin, and trichohyalin) and S100 proteins. Cornulin expression is mainly localized to the granular layer of epidermis and cornulin is said to be a marker of late epidermal differentiation (Contzler, Favre et al. 2005). It has been suggested that cornulin is up-regulated during, and might play a role in, stress response in squameous epithelium (Darragh, Hunter et al. 2006). Eczematous skin is characterized by increased keratinocyte proliferation, impaired differentiation and increased apoptosis of keratinocytes (Trautmann, Akdis et al. 2000; Proksch, Folster-Holst et al. 2006). The reduced expression of *CRNN* found in our study could therefore be a part of the pathogenetic mechanism behind the impaired differentiation and barrier dysfunction seen in eczema. Reduced transcript levels of other cornified envelope genes, e.g. *FLG* and *LOR*, has also been found in eczema and factors regulating their expression are therefore interesting candidates in eczema pathogenesis (Sugiura, Ebise et al. 2005).

To summarize Paper III, we have identified *CRNN* as a new differentially expressed gene in eczema, and shown that genetic variation at the *CRNN* gene locus is associated

with eczema susceptibility. Although the observed association between eczema and polymorphisms at the *CRNN* locus is likely to be explained by LD between the *CRNN* gene and the *FLG* 2282del4 mutation, the reduced levels of the *CRNN* transcript may be part of disease pathology.

THE ASTHMA GENE NPSR1 IS NOT ASSOCIATED WITH ECZEMA

*Lack of association between Neuropeptide S Receptor 1 (NPSR1) and Eczema in five European populations (**Paper IV**)*

In year 2004, the *NPSR1* gene (also known as *GPRA*) was identified as a susceptibility gene for asthma and elevated serum IgE levels (Laitinen, Polvi et al. 2004). The aim of Paper IV was to search for a potential association between the Neuropeptide S Receptor 1 (*NPSR1*) gene and eczema in a large patient material. PDT analysis was performed in two family materials and association analysis was performed in three case-control materials. All patient materials originated from Europe and seven haplotype-tagging SNPs in the *NPSR1* gene were genotyped in 6275 individuals (1848 eczema patients). In order to analyse the expression pattern of the two isoforms A and B of NPSR1 in the skin, immunohistochemistry was performed. We also wanted to test for association with associated phenotypes such as asthma, rhinoconjunctivitis, atopic sensitization, and elevated total serum IgE levels, in the context of eczema.

We found no association with eczema for any of the seven SNPs in any of the five different populations or in a joint analysis with all materials pooled together. There was also lack of association with any of the seven common haplotypes of *NPSR1* in the different patient materials, also when pooling the materials into a joint analysis (Table 9).

Table 9. Lack of association of *NPSR1* haplotypes with eczema in all five materials

HAPLOTYPE	CASE	CONTROL	CASE FREQUENCY	CONTROL FREQUENCY	OR	95% CI	P-VALUE
H1	549	1287	0.325	0.317	1.0		ns
H2	370	871	0.219	0.215	1.05	0.89-1.23	ns
H3/H6	415	982	0.246	0.242	0.99	0.85-1.16	ns
H4	123	314	0.073	0.077	0.95	0.76-1.20	ns
H5	96	224	0.057	0.055	0.91	0.71-1.17	ns
H7	95	255	0.056	0.063	0.87	0.67-1.13	ns

In both eczema patients and healthy controls, we found a strong epidermal expression of NPSR1 isoform A, although weaker in the basal proliferating keratinocytes. NPSR1 isoform B showed a weak but homogenous epidermal expression in all individuals

(Figure I in Paper IV). None of the isoforms showed differences in epidermal NPSR1 expression between eczema patients and healthy controls.

In the analyses of atopic phenotypes in the context of eczema, we found rhinoconjunctivitis to be significantly associated with the minor allele of polymorphism rs324396 in *NPSR1* (OR 1.25 (1.06-1.47, $p<0.03$ corrected after 1000 permutations). No significant association was found with *NPSR1* and any of the other phenotypes, even if the power to detect such an association was over 80%.

Asthma and eczema can both be manifestations of atopic diseases and the diseases share some pathogenic and immunological features, such as the tendency to develop allergen-specific IgE against common allergens in our environment. The association of *NPSR1* with asthma and raised IgE originally described by Laitinen, Polvi et al. 2004 has been replicated in several studies (Kormann, Carr et al. 2005; Melen, Bruce et al. 2005; Feng, Hong et al. 2006). Genetic association has also been found to other allergic phenotypes, such as atopic sensitization and allergic rhinoconjunctivitis (Melen, Bruce et al. 2005). Recently, inflammatory bowel disease has also been shown to associate with one haplotype in *NPSR1* (H2) and with the functional SNP (rs324981, Asn107Ile) (D'Amato, Bruce et al. 2007). The NPSR1 protein is found to be expressed in the bronchus, the gut, and in the skin (Laitinen, Polvi et al. 2004; Vendelin, Pulkkinen et al. 2005). In Paper IV, we wanted to elucidate whether any of the SNPs in the *NPSR1* gene also were associated with eczema, and if the NPSR1 protein expression was altered in eczema skin as compared to healthy skin. We found no association between eczema and any of the analysed SNPs in *NPSR1* gene or the common haplotypes. Our results suggest that genetic variants in *NPSR1* do not influence the susceptibility for eczema, thus confirming the results found by a German study that found no association of polymorphism rs232922 with eczema (Söderhäll, Marenholz et al. 2005), and a group from UK who reported lack of association with any of the haplotypes H1-H7 in NPSR1 and adult eczema (Veal, Reynolds et al. 2005). Considering the number of eczema patients (1848) and the estimated power of 93%, it is likely a true association with eczema would have been found assuming that the impact of *NPSR1* gene variants would be of a similar magnitude in eczema and asthma.

In conclusion, we found a lack of genetic association of seven *NPSR1* polymorphisms with eczema in five European eczema materials. In addition, the expression pattern of NPSR1 isoforms in the epidermis of healthy individuals and eczema patients do not differ. Taken together, these findings suggest that *NPSR1* gene is not a susceptibility gene for eczema.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The overall aim of this thesis has been to identify susceptibility genes for eczema. In the past years it has become more and more evident that the pathophysiology of eczema is based both on defects in skin barrier function and immunological responses. In the work presented here we found genetic association between eczema and the *SOCS3* gene, the *FLG* gene, and partly also the *CRNN* gene, which is well in line with the dual contribution of both the immune system and the skin barrier. To gain more knowledge of the interplay between these, it is important to study gene-gene interactions and also gene-environmental interactions. Studies that further analyse genes in the epidermal differentiation complex and the interactions between genes involved in skin barrier function are most likely to be presented in the future. Understanding the function and role of different gene products in the development of eczema would be facilitated with the use of animal models. In animal models it is possible to study interactions between genes and environment since both the genetics and environmental exposures can be controlled. The gene-environment interaction can also be studied in prospective birth cohorts that gather information on environmental exposures, such as the BAMSE cohort.

It would be of particular interest to study regulation of the *SOCS3* gene and elucidate in what way the polymorphisms located distally in the promoter region may affect the expression. This can be done in different ways, either by cloning the regulatory sequence of *SOCS3* into a vector upstream of a reporter gene, or indirectly by correlating the genotype of different individuals and their expression of SOCS3. To identify enhancers and transcription factors involved in the expression of SOCS3 would also be of great interest.

Regarding the association of atopic diseases and eczema, it has been suggested in recent literature that there are at least some predisposing genes and genetic loci behind eczema that are specific to eczema (Morar, Willis-Owen et al. 2006). But it is still of importance to determine whether the atopic diseases share some genetic predisposition, or whether they are merely co-manifested due to shared risk factors in the environment (Williams and Flohr 2006). In our study of *NPSR1*, first identified as an asthma

susceptibility gene, we found no evidence of a genetic association with eczema, or with asthma in the context of eczema. If this reflects different subgroups of asthma depending on the context in which the disease develops is not yet known. Furthermore, it would be interesting to find out whether the NPSR1 protein takes part in eczema pathophysiology even if not genetically associated.

It is my hope that better understanding of the genetics behind eczema eventually will contribute to improved therapeutic strategies in eczema, and to a possibility to offer specific preventive lifestyle advice on the basis of genotype in susceptibility genes. Hopefully in the end, this will lead to a decrease in eczema prevalence and an increase in quality of life for affected individuals.

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