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GENETIC STUDIES OF SKIN BARRIER DEFECTS WITH FOCUS ON ATOPIC DERMATITIS

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ABSTRACT

Atopic dermatitis (AD) is a common, complex inflammatory skin disorder where a defect skin barrier is central in the pathogenesis. Mutations in the filaggrin gene cause ichthyosis vulgaris (IV). IV is one of several keratinization disorders named ichthyoses where mutations in skin barrier genes are a common underlying genetic factor. Furthermore, filaggrin mutations are a major risk factor for moderate to severe AD. The aim of the work reported in this thesis is to improve the understanding of the genetic mechanisms of skin barrier defects associated with AD, and to identify whether AD and other common disorders of keratinisation may share genetic susceptibility factors related to skin barrier dysfunction. **Paper I** presents data suggesting that filaggrin mutations may be rare in Ethiopian AD and IV patients, implying other mechanisms should be more important in the pathogenesis of IV and AD in this ethnic group. **Paper II** presents a novel mutation in the steroid sulfatase gene in a patient with clinical signs of common ichthyosis type. In **paper III** association between filaggrin mutations and childhood onset of psoriasis was tested. No association to any prevalent filaggrin mutations was found, and no novel mutations. This indicates that filaggrin loss-of- function variants do not have a strong effect on the onset of psoriasis in childhood.

In **paper IV** it is demonstrated that functional parameters and gene expression in molecular pathways *in vivo* is altered in patients suffering from AD and IV and depend on filaggrin genotype. Patients with filaggrin mutations displayed a severe phenotype with impaired barrier function measured as increased trans-epidermal water loss, and significantly altered pH levels. Furthermore, the numbers of genes with altered expression were significantly higher in patients with low or absent filaggrin expression. These pathways include many genes involved in inflammation, epidermal differentiation, lipid metabolism, cell signalling and adhesion. **Paper V** represents a candidate gene study where expression analysis links the epidermal transglutaminases 1 and 3 to the manifestation of AD and genetic analysis suggests that genetic variation at the transglutaminase 1 locus could be involved in the development of the disease.

The results of the work reported in this thesis provides additional descriptive information and further elucidates the pathogenesis underlying AD and other disorders of keratinization, in particular in relation to filaggrin deficiency. Better understanding of the genetic factors and molecular and functional consequences should hopefully enable future individually designed barrier restoring therapy.

LIST OF PUBLICATIONS

I. **Winge MCG**, Bilcha KD, Lieden A, Shibeshi D, Sandilands A, Wahlgren CF, McLean WH, Nordenskjöld M, Bradley M. Novel filaggrin mutation but no other loss-of-function variants found in Ethiopian patients with atopic dermatitis. Br J Dermatol. 2011 Nov; 165(5):1074-80.

II. **Winge MCG**, Hoppe T, Lieden A, Nordenskjöld M, Vahlquist A, Wahlgren CF, Törmä H, Bradley M, Berne B. Novel point mutation in the STS gene in a patient with X-linked recessive ichthyosis. J Dermatol Sci. 2011 Jul;63(1):62-4.

III. **Winge MCG**, Suneson J, Lysell J, Nikamo P, Liedén A, Nordenskjöld M, Wahlgren CF, Bradley M, Ståhle M. Lack of association between filaggrin gene mutations and onset of psoriasis in childhood. J Eur Acad Dermatol Venereol. 2011 Dec. *In press.*

IV. **Winge MCG**, Hoppe T, Berne B, Vahlquist A, Nordenskjöld M, Bradley M, Törmä H. Filaggrin Genotype Determines Functional and Molecular Alterations in Skin of Patients with Atopic Dermatitis and Ichthyosis Vulgaris. PLoS ONE 2011 Dec;6(12):e28254.

V. Liedén A*, Sääf A*, **Winge MCG**, Kockum I, Ekelund E, Wahlgren CF, Nordenskjöld M, Bradley M. Genetic association and expression analysis of the epidermal transglutaminases in atopic dermatitis. *Submitted for publication.*

CONTENTS

LIST OF ABBREVIATIONS

ABCA12	ATP-binding cassette, sub-family A (ABC1), member 12
aCGH	array-based comparative genomic hybridization
AD	atopic dermatitis
ALOX12B	arachidonate 12-lipoxygenase, 12R type
ALOXE3	arachidonate lipoxygenase 3
cDNA	complementary DNA
CE	cornified envelope
CNV	copy number variant
CYP4F22	cytochrome P450, family 4, subfamily F, polypeptide 22
EDC	epidermal differentiation complex
ERAP1	endoplasmic reticulum amino peptidase 1
FLG	filaggrin
GWAS	genome wide association study
HLA	human leukocyte antigen
HWE	Hardy-Weinberg equilibrium
IHC	immunohistochemistry
IV	ichthyosis vulgaris
KIF	keratin intermediate filament
LCE3B/C	late cornified envelope 3b/3c
LD	linkage disequilibrium
MLPA	multiplex ligation-dependent probe amplification
mRNA	messenger RNA
NIPAL4	NIPA-like domain containing 4
OMIM	online Mendelian inheritance in man
OR	odds ratio
PDT	pedigree disequilibrium test
PPR	pathogen pattern recognition receptor
qPCR	quantitative real time polymerase chain reaction
RANTES	regulated upon activation normal T cell expressed and secreted
SC	stratum corneum
SPR	small proline rich proteins
STS	steroid sulfatase
TEWL	trans epidermal water loss
TGM	transglutaminase
XLI	x-linked recessive ichthyosis

1 INTRODUCTION

1.1 GENES, CHROMOSOMES AND MUTATIONS

Deoxyribonucleic acid (DNA) molecules can be found organized in chromosomes of the nucleus and in the mitochondria of eukaryotic cells. DNA consists of large polymers with a linear backbone of sugar and phosphate residues. The sugar part consists of deoxyribose and additional sugar residues are linked by phosphodiester-bonds. Attached to a carbon atom in each sugar residue is a nitrogen base, consisting of adenine (A), cytosine (C), guanine (G) or thymine (T). A sugar with its attached base is called nucleoside, whereas a nucleotide is a nucleoside with a phosphate group attached. This is the basic repeating unit of a DNA strand. The composition of a ribonucleic acid (RNA) molecule is similar to that of a DNA, but RNA contain ribose sugar residues instead of deoxyribose, and uracil (U) as nitrogen base instead of thymine (T). Whereas RNA molecules exist as single strands, the structure of DNA is a double helix, where two DNA strands are bound together by hydrogen bonds. Hydrogen bonding occurs between opposed bases of the two strands according to the rules of Watson-Crick. A specifically binds to T and C specifically binds to G. As a consequence, the base composition of DNA is not random, the amount of A equals that of T, and the amount of C equals that of G (1). Human genome is the term describing the total genetic information (DNA content) in human cells. The majority of the genome is in the nucleus and a minority in the mitochondrial genome. A gene is a functional nucleotide sequence in a certain position on a chromosome, and the majority encodes a specific product (e.g. a protein). The human genome contains approximately 21000 genes coding for proteins or functional RNA, distributed on 24 chromosomes (22 autosomes and two sex chromosomes, X and Y) (2). Each chromosome contains one single DNA molecule tightly packed by histones and other proteins (Fig 1).

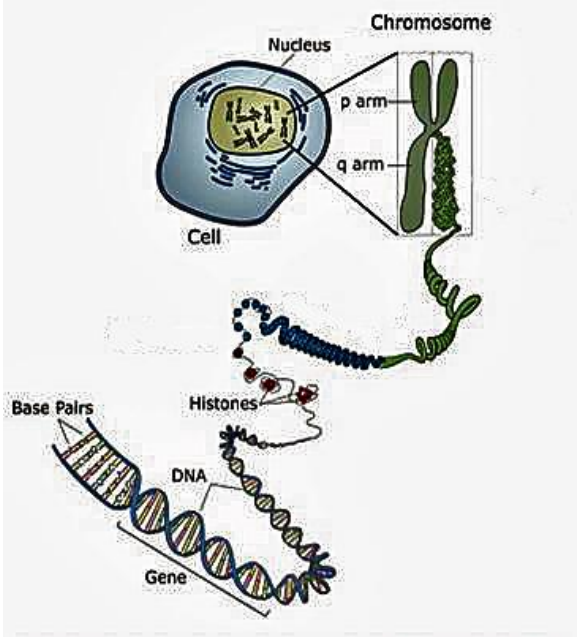

Figure 1: Schematic overview of the structural organization of the human genome. DNA molecules have a linear backbone of sugar phosphate and attached bases consisting of either A, C, G or T. DNA is structured as a double helix condensed around histone proteins forming chromosomes. The genome is contained in 24 chromosomes (22 autosomes and two sex chromosomes, X and Y) and exists in the nucleus in eukaryotic cells. In addition, the mitochondria located in the cytoplasm contain DNA coding for mitochondrial genes. Figure modified from the National Human Genome Research Institute.

The expression of genetic information in eukaryotic cells is largely a one-way system: DNA stored on chromosomes in the cell nuclei is transcribed into synthesis of messenger RNA (mRNA). MRNA in turn is transported into the cytoplasm and translated into protein. This flow of genetic information has been described as the central dogma of molecular biology (3). A set of three nucleotides is called a codon, and encodes one amino acid, which constitutes the basic repeating structure of a protein. Specific start and stop codons encode start and termination of translation into proteins.

Cells in an organism are able to divide and replicate their genetic content. This process is called mitosis. When gametes are formed (sperm and egg cells) a specialized form of cell division occurs called meiosis. Each pair of chromosomes is separated prior to replication. During meiosis exchange of genetic material occur between maternal and paternal chromosomes. This is called crossing-over or recombination and is a principal mechanism for increasing genetic admixture between generations. On the autosomes two copies of each gene are inherited from the parents; a gene may thus exist in two alternative forms (alleles). An individual with two identical alleles at a certain region (locus) is said to be homozygous for that allele, whereas an individual with two different alleles are said to be heterozygous (3).

The human DNA is not static; it is subject to a variety of different types of heritable or acquired changes. Larger scale changes can include loss, gain or rearrangements of parts of or entire chromosomes, whereas smaller scale changes may be grouped into subclasses depending on the outcome on the adjacent DNA sequence. Base substitutions usually include the replacement of a single base. Deletions mean that one or several nucleotides have been lost from the sequence and insertions that of one or more nucleotides has been added to a sequence. Variations in a DNA sequence that are fairly common (at least 1 %) are called polymorphisms (4). Polymorphisms affecting a single nucleotide are commonly referred to as single nucleotide polymorphisms (SNPs).

Changes in base constitution with lower frequency in the populations and that may have a pathogenic effect are called mutations. They can range in size from a single base to a large segment of a chromosome. Gene mutations can either be inherited, or arise *de novo*. Common chromosomal aberrations include insertions, deletions, translocations and inversions. Such mutations are often pathogenic. Mutations in a single gene have varying effects on health, depending on whether they alter function of essential proteins. These types of mutations include missense mutations (DNA base change resulting in an amino-acid substitution), nonsense mutations (DNA base change resulting in a stop-codon and ending translation) and frame-shift mutations (shifts the reading frame for the three bases encoding one amino acid). DNA base changes underling these mutations are frequently caused by insertions, deletions or duplications. Mutations may be "silent" in the sense that there is no known effect for the individual. Only a small percentage of gene mutations cause genetic disorders, most do not affect health. Also, potential pathogenic mutations are often repaired enzymatically prior to protein expression. A very small proportion may in fact have a positive effect and thus driving evolution of the species. This can for instance be achieved by an altered expressed protein that may have improved or changed function that better help the organism to adapt to environmental changes (5).

1.1.1 Genetics of inherited diseases

Inherited genetic diseases can be divided into monogenic and multifactorial (complex) diseases. Monogenic phenotypes or disorders are those whose presence or absence depends on the genotype at a single locus. They follow Mendelian inheritance laws and a phenotype is said to be inherited dominantly if present in a heterozygote carrier of a certain genotype, and recessive if manifesting only in a homozygous individual (3). There is a strong correlation between phenotype and genotype in these disorders and they can often be recognized by the characteristic inheritance pattern they give rise to.

Complex diseases do not follow Mendelian inheritance laws. They are common, and may be both polygenic (multiple susceptibility genes) and/or multifactorial (multiple genes interacting with environmental factors). Polygenic interaction may be explained by an additive or a

multiplicative model. The effect of two or more genes can equal the sum of their independent effect (additive); or the genes can interact in a way that results in a greater risk than that posed by each gene independently (multiplicative) (6). Individual genes behind complex diseases are not likely to be either necessary or sufficient for manifestation of the disease but, rather, a combination of susceptibility genes increases the disease risk. In fact, individuals with the same phenotype may have different combinations of risk-increasing genes and environmental factors. Such genetic variations underlying a similar phenotype are called genetic heterogeneity, and when involving different loci epistasis. Individuals may also have an inherited susceptibility allele without manifesting the disease. This phenomenon is called incomplete penetrance. All of these parameters have to be taken into account when investigating the genetic background underlying complex traits such as atopic dermatitis (7).

1.1.2 Linkage disequilibrium and haplotypes

One powerful approach to identify genetic factors behind complex diseases has been to study association between disease and genetic markers. Linkage disequilibrium (LD) is the non-random association of alleles between genetic loci, which occur when loci are located close on the same chromosome and therefore are not or only rarely separated by crossing over (8). It has been of great significance to understand the patterns of LD for the implementation of candidate gene studies and then genome-wide association studies (GWAS). These rely on the ability of genotyping of markers such as SNPs to trace other genetic variation, associated with underlying diseases (9, 10). The strength of the LD between two markers is influenced by intrinsic factors like recombination and mutation rates, but also extrinsic aspects such as population size, admixture and selection. Nearby alleles on the same chromosome tend to be together as a block. Such a linked block of alleles is called a haplotype. Polymorphisms that uniquely identify distinct haplotypes are called tagging SNP and may hence be used to reduce the number of SNPs analyzed.

1.2 THE SKIN

All organisms have an outer layer that delimits the body and separates it from the environment. In humans this outer layer is the skin and the intestine. The skin is the largest organ of the human body. Main functions include protection against physical damage, defense against biological invasion, regulation of molecular passage and signal transmission (7, 11). This finely tuned balance between protection from harmful pathogens and bidirectional signal exchange is provided by a network of structural, cellular, and molecular elements, collectively referred to as the skin barrier.

1.2.1 Embryological origin and anatomy of the skin

The skin is divided into three principal layers, the epidermis, the dermis and the subcutis. The skin arises from two major embryological elements, the prospective epidermis from the early gastrula, and the prospective mesoderm which is brought into contact with epidermis during gastrulation. The mesoderm provides the dermis and is essential for inducing differentiation of epidermal structures such as hair follicles. The dermis in turn subsequently also forms the subcutis (11).

The major cell in epidermis is the keratinocyte, 95 % of the total. The keratinocyte moves progressively from the epidermal basement membrane towards the skin surface, forming several distinct layers during this process (keratinization), forming stratified squamous epithelium. The epidermis can be divided into the stratum basale, stratum spinosum, stratum granolusum and stratum corneum (SC). Besides keratinocytes also melanocytes, Langerhan's cells and Merkel cells reside in the epidermis. The stratum basale is usually described as one-cell-layer thick (11). In the stratum spinosum the

keratinocytes are enlarged and are interconnected by numerous desomosomal plaques, which interact between keratinocytes to form a stabilizing network of interconnections. Cytoskeleton filaments also attach close to desmosomes to provide stability across the cell layers. The stratum spinosum is succeeded by the stratum granulosum. This layer contains both keratohyalin granules and lamellar bodies. Lamellar bodies discharge lipid components into the intercellular space, which is important for barrier function. The keratohyalin granules contain profilaggrin, contributing to skin barrier homeostasis. Tight junctions are located just below the SC at the level of the stratum granulosum (12) and function as the "gate" for the passage of water, ions, and solutes through the paracellular pathway (13). The outermost layer is the SC where the keratinocytes (now called corneocytes) have lost their nucleus and cytoplasmic organelles. The SC consists consisting of multiple layers of corneocytes. Located within the stratum corneum, the cornified envelope (CE) is an insoluble protein matrix vital for skin-barrier function and integrity. It replaces the plasma membrane of the granular cells during cornification. The SC barrier is maintained by the complex interaction of the CE, intra-cytoplasmic moisturizing factors, and a complex lipid mixture in the extracellular space. A constant, regulated turnover of keratinocytes moves from the stratum basale, being shed in the SC. The total turnover time in the epidermis is thought to take approximately 50-75 days (14). The process of desquamation where the corneocytes are shed involves degradation by proteases of the laminated lipids in the intercellular spaces and loss of desmosomal interconnections (15).

The dermis is a resilient tissue which provides nutrition to the epidermis as well as a supportive function against mechanical injury. The dermis contains few cells, the majority being fibroblasts that secrete dermal constituents. Other cells include mast cells, melanocytes and immunological cells such as macrophages and lymphocytes. The dermis is a matrix where polysaccharides and proteins are linked to produce macromolecules with high water binding capacity. Included among these proteins are collagen, which has great tensile strength, and elastin which provides elasticity. The polysaccharides include glucoseaminoglucans and hyalorinic acid. These have a major role in the supporting matrix of the connective tissue (16).

1.2.2 Epidermal differentiation and the skin barrier

The process of keratinization in the epidermis includes changes in keratins, CE proteins, plasma-membrane glycoproteins, intercellular lipids, desmosomes and other adhesion proteins. The process is thought to be mediated by calcium levels that tightly control differentiation and activation of genes encoding epidermal structural proteins (17). In the basal layers proteins such as keratin 5 and 14 are the main structural proteins. Higher up in the stratum spinosum, desmogleins are expressed together with transglutaminase 1 and 5. In the granular layer proteins such as filaggrin, cornulin, transglutaminase 1 and 3 and keratins 1 and 10 are active; and in the SC involucrin, loricrin, filaggrin, small proline-rich proteins (SPRs) and S100A proteins are abundant. The cells are flattened and their keratin filaments are aligned under the influence of filament aggregating proteins (filaggrins). Filaggrin aggregates the keratinocyte cytoskeleton prior to being crosslinked with other epidermal proteins and lipids by epidermal transglutaminase enzymes, forming the CE barrier (Fig. 1). Subsequently filaggrin degradation products, including urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA) act as natural moisturizing factors (NMF) and play a central role in maintaining hydration of the SC and affect enzyme activity, pH and antimicrobial defense (18, 19).

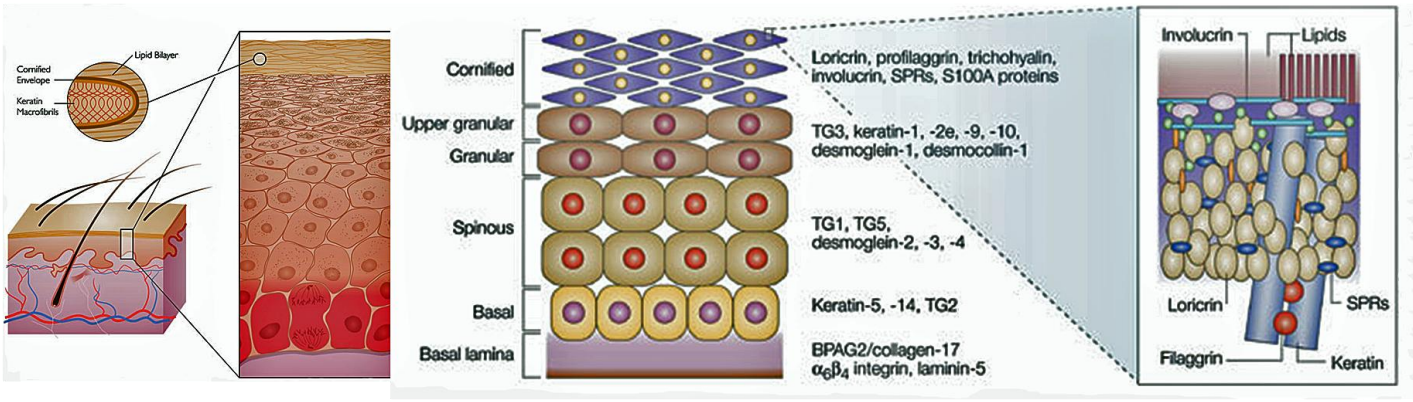

Figure 1: Schematic overview of the structural composition of the skin barrier and gene expression localization in the epidermis. The skin is divided into the epidermis, dermis and the subcutis. The epidermis is the outermost layer and consists of the basal layer, the stratum spinosum, stratum granulosum and the stratum corneum. The process of keratinization takes place in the epidermis as the basal keratinocyte differentiates from the basal layer into the cornified stratum corneum. The cornified envelope within the SC is an insoluble cross linked protein and lipid barrier embedded in a lipid bilayer. Figure modified from Candi 2005 (20) and Segre 2006 (21).

1.2.3 The immune system in the skin

The skin barrier has a mechanical, chemical, and immunologic component. The role of the latter is to elicit a powerful defense reaction in the case of danger and, at the same time, prevent such a reaction against harmless substances. Immune responses originating from the skin are initiated and executed by cells and molecules of both the innate and the adaptive immune system. Innate reactions are the first line of host defense, and are typically rapid, poorly discriminating, and lack memory. Adaptive responses, in contrast, show a high degree of specificity as well as memory but need a prolonged time for its development. As a consequence, innate and adaptive responses are parallel events influencing each other. In fact, the type and magnitude of the innate reactions often determine the quality and quantity of adaptive responses (22).

The main functions of the innate immune system involves induction of immediate responses against potentially harmful microorganisms such as bacteria, fungi and viruses (23). Inflammatory responses by immune cells of the innate system (i.e. granulocytes and macrophages) can be rapidly triggered and followed by activation of dendritic cells and natural killer cells (24, 25). Resident skin cells such as keratinocytes also contribute to the innate immune response by inducing secretion of antimicrobial peptides, and mast-cells may provide strong pro-inflammatory effect when activated (26). The innate system engulfs and destroys pathogens, triggers pro-inflammatory responses and helps present antigen by antigen presenting cells such as dendritic cells; subsequently priming the adaptive immune response (27). The presentation of antigen through an antigen-presenting cell takes place on major histocompability complex (MHC) molecules. In humans, the MHC are called human leukocyte antigen (HLA). Dendritic cells contribute to initiating both primary and secondary adaptive immune responses, and in the skin are subdivided according to immunophenotype into Langerhans cells, inflammatory dendritic epidermal cells and plasmatocytoid dendritic cells (28). This network of dendritic cells is

regarded as a first barrier of the normal skin immune system against environment. It may upon antigen exposure migrate to the draining lymph node for antigen presentation (28). However, the innate system may also discriminate between antigens. This process is mediated by pathogen-associated molecular patterns (PAMPs) capable of showing target specificity towards molecules such as bacteria, viruses and fungi (24). In mammals the receptors handling PAMP recognition are called pattern recognition receptors (PRR). The major PRR of the innate immune system are toll-like receptors, nucleotide oligo domain-like receptors and RIG-I-like receptors. These PRR are germ line encoded and are constitutively expressed by both immune and non-immune cells. Following PAMP recognition, PRR activate signaling pathways that may lead to more antigen-defined innate immune response (27). This response also helps prime the subsequent antigen-specific immune response.

Adaptive immunity includes acquired but time-delayed defense mechanisms against pathogens. Adaptive immune responses are mediated by T and B cells. B cells have specificity for a defined antigen that has been presented by an antigen-presenting cell. Following exposure, clonal expansion of the antigen-specific B cell takes place. These specific B cells can bind and produce antibodies targeting the antigen. Antibodies produced by B cells may also activate mast-cells that upon activation release pro-inflammatory mediators. T cells can have diverse functions, and are activated in the lymph node after antigen exposure. They expand rapidly and secrete cytokines that regulate the following immune response. Depending on the cytokine milieu, the native T cell differentiates to Th1, Th2, Th17 or any other subgroup (including Cd8+ cytotoxic T cells), that in turn secrete specific cytokines. T cells contain highly diverse antigen receptors and are generated by DNA rearrangement events. They can recognize both novel and conserved antigens. This system requires recombination leading to different cell clones capable of specific protein recognition, instead of pattern recognition. This more complex immune response has as a major advantage that it may be built against many structural proteins and is able to improve its reactions with repeated exposure. The major disadvantage following this is a considerable risk of autoimmunity (28).

1.3 ATOPIC DERMATITS

Atopic dermatitis (AD; OMIM# 603165), is an inflammatory skin disorder that affects up to 20 % of children and 3-5% of adults in the western world (29, 30). Diagnosis rests on clinical features and the U.K. working party's criteria are frequently used. They are pruritus, typical distribution, early onset, dry skin and a personal or family history of atopic disease (31). During infancy AD affects mainly the face, scalp and extensor surfaces of the extremities. In older children and in those with persistent AD, lichenification develops and affected sites usually include the flexural folds of the extremities. In adults, chronic hand eczema may be the primary manifestation of AD but eczema in the head and neck region is also common (32). The age-dependent localization of AD lesional areas is still largely unknown although proposed predisposing factors include local thickness of the SC and the variation in exposure to exogenous substances, such as irritants and allergens, together with so far unknown factors (19).

1.3.1 AD nomenclature and atopy

AD is included among the atopic disorders, together with allergic asthma and allergic rhinoconjunctivitis. AD is frequently also referred to as atopic eczema, or eczema according to the World Allergy Organization (33). Atopy is defined as a personal and/or familial propensity to produce IgE antibodies and sensitization in response to environmental proteins (33). Although AD is strongly associated with a tendency to produce IgE antibodies, this is not always the case (34). For instance, the atopy prevalence in AD hospital surveys have been estimated to 47-75 % (34). The name AD is widely used in the publications

underlying the present work, is still the OMIM and medical subject headings (MESH) terms and is therefore used throughout, although the terminology is debated (35). The phenomenon of AD in infants often recurring with other atopic manifestations later in life has been referred to as the atopic march (36). Underlying atopy has been considered to be critical in linking AD, allergic rhinitis and allergic asthma and the concept of the atopic march has been supported by cross-sectional and longitudinal studies (37). Patients with AD may develop a typical sequence of AD and allergic asthma and rhinitis, which develop at certain ages. Some may persist for several years, whereas others may resolve with increasing age (38). The mechanisms of growing out of AD remain largely unknown and could be influenced by both genetic and environmental factors (37). Approximately 70% of patients with AD develop allergic asthma compared with 20-30% of patients with mild AD and approximately 8% in the general population (37). However, the development of atopic diseases is individually influenced by both genetic and environmental factors and they may still (while sharing genetic and environmental risk factors) develop independently from each other.

1.3.2 AD pathogenesis

Central in AD pathogenesis are combinations of acquired and inherited factors thought to alter the epidermal structure. These changes in the physiological skin barrier predispose to increased antigen penetration and are followed by immune activation, which in turn has negative consequences for skin barrier homeostasis (39). In addition to genetic disease mechanisms, environmental and individual trigger factors are important (40).

Environmental trigger factors described for AD include food allergens such as cow's milk and hen's egg in children, and allergens such as house dust mites, pets and pollen in both children and adults (40). In addition, super-infections are common in patients with AD, particularly with *Staphylococcus aureus* that colonize more than 90% of patients. This may aggravate skin inflammation and tissue damage by induction of T cell mediation by an superantigenic effect, by specific IgE immune responses against *Staphylococcus aureus* and toxins and through toll-like receptor-mediated immune reactivity (41). In addition other factors such as physiological conditions of the skin barrier altered by using soap and detergents on the skin are thought to mediate the release of pro-inflammatory cytokines from keratinocytes (19). Also, psychological factors are important, and psychological stress is a significant contributor to the disease course through direct and indirect effects on immune response, cutaneous neuropeptide expression, and skin barrier function by inhibition of epidermal lipid synthesis (42).

Studies have shown that the barrier function in AD is altered. This barrier defect, evident e.g. from studies on transepidermal water loss (TEWL), can be seen in active lesions, where the inflammatory response is likely to play an important role, but also in non-lesional skin areas suggesting that the barrier defect is an underlying factor in AD. The impaired homeostasis of the skin leads to increased TEWL as well as changes in gene expression patterns (43) and enzymatic activity (44). This dysfunction is the result of one or more of several factors, and may in addition to environmental factors include reduced levels of SC lipids (45-47); acquired or genetic defects in key epidermal differentiation proteins such as filaggrin (48-51) or epidermal enzymes (49, 52, 53) .

The immune response in AD skin is characterized by infiltration of T cells and dermal dendritic cells, with distinct subsets of cell types including more increased Th2 lymphocytes than Th1 and Th17, and often overrepresentation of increased IgE and eosinophil numbers in circulation (54-56). The defective epithelial barrier in AD is thought to

lead to penetration by epicutaneous antigens. These antigens encounter antigen-presenting cells such as dendritic cells that induce Th2 cells to produce cytokines such as Il4 and IL13. These cytokines in turn induce an IgE class switching as well as promotes Th2 cell survival. The cytokines produced by the increasing number of Th2 cells skews the production away from Th1 and Th17. Also such cytokines have direct effects on the epidermis, by inhibiting terminal differentiation and production of anti-microbial peptides by keratinocytes, leading to an increasingly disrupted epidermal barrier (50, 58) as well as predisposing to AD associated

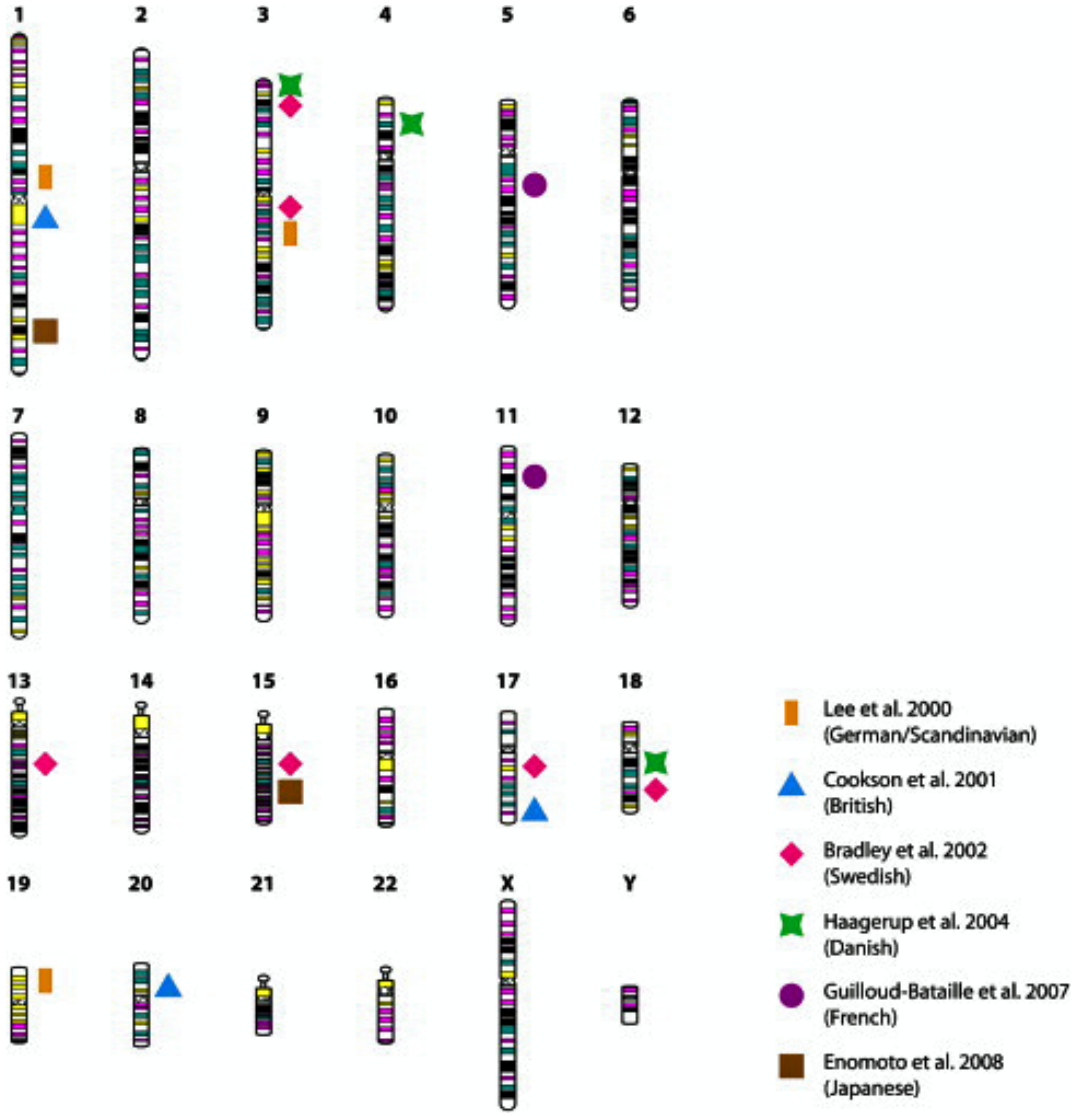

Figure 2: Overview of genome wide linkage studies of AD. 22 autosomal and sex chromosomes represented with candidate regions highlighted. Figure modified from Barnes 2010 (57).

super-infection (59). However, a subset of AD patients have been proposed having a intrinsic type of AD, as opposed to extrinsic AD (60). They do not display increased IgE and eosinophil numbers in circulation, other atopic manifestations, and do not have a distinct Th2 switching. Furthermore, the barrier dysfunction in these patients is thought be minor, and smaller

molecules than protein allergens may be of more importance for the subsequent inflammatory response (61). This terminology is however debated, and there may exist a dynamic relationship between these putative subtypes (62).

1.3.3 Genetics of AD

Pathogenesis is a complex interaction between environmental factors and genetic predisposition. The genetic predisposition has been indicated by high family incidence and concordance of 0.72-0.86 in monozygotic and 0.21-0.23 in dizygotic twins (63, 64).

Previous genome-wide linkage screens of AD (65-70) (Fig. 2) have reported suggested linkage and other chromosomal regions such as on chromosome 14 (71) has been associated to AD. The European AD GWAS published to-date has confirmed 1q21 as a major susceptibility locus (72) as well as an open reading frame on chromosome 11 (*C11orf30*); and recent replication have indicated that the *C11orf30* locus may have an epistatic effect with the strong association to filaggrin (*FLG*) on chromosome 1q21 (73).

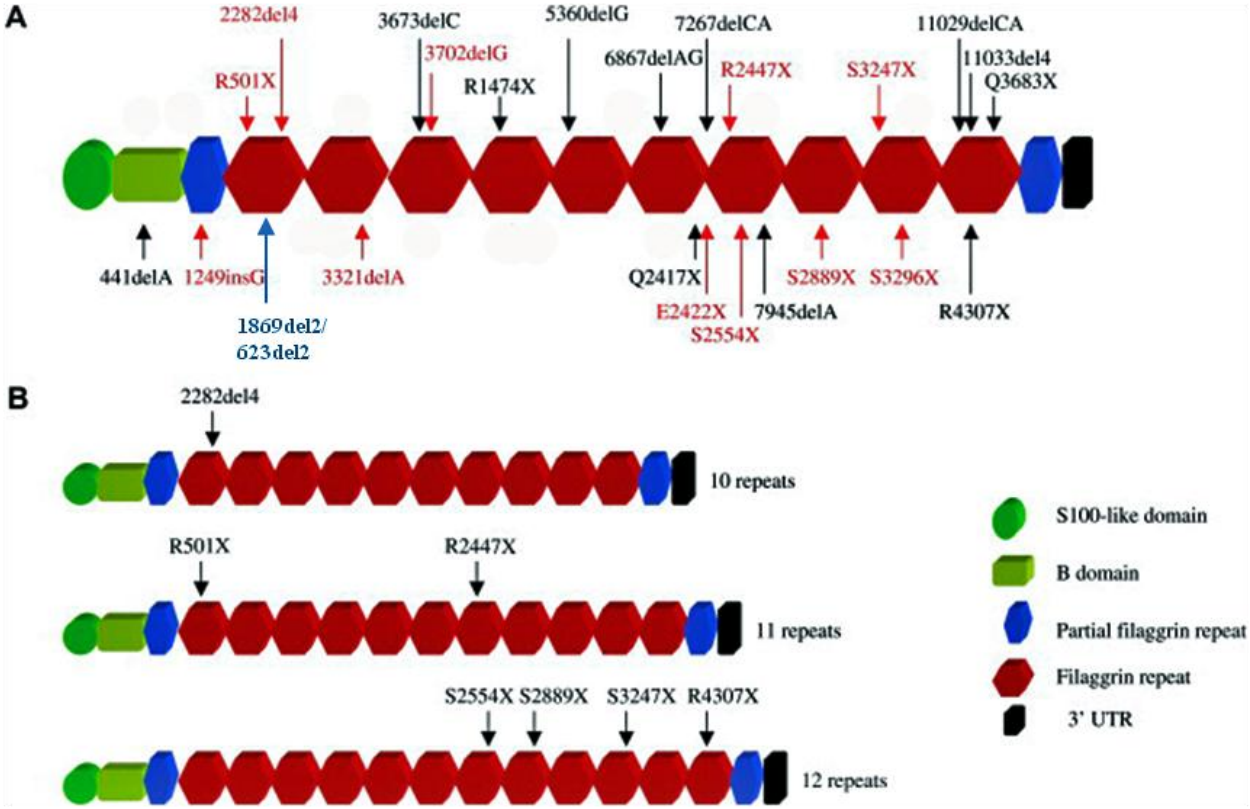

Figure 3: *FLG* organization and location of described mutations. *FLG* is composed of a large transcript encoded by three exons, of which the third encodes the *FLG* repeats. The majority of the *FLG* sequence consists of repeating 35 kiloDalton units separated by a short amino-acid linker peptide (A). Prevalent mutations are marked in red, family specific in black, and the novel mutation 623del2 detected in paper I in blue (here also termed 1869del2). *FLG* has a variable number of repeats, consisting of 10, 11 or 12 units (B). Recurrent mutations can occur in the 10, 11 or 12-repeat allele. Picture modified from O'Regan 2009 (74). UTR= Untranslated region.

The epidermal differentiation complex (*EDC*) on chromosome 1q21 has been mapped in previous genome-wide linkage studies (57) as well as in GWAS (72) as a susceptibility region for both AD and psoriasis (66, 75). The *EDC* contains a cluster of genes of importance for terminal differentiation of keratinocytes and subsequently for skin barrier function and integrity (76). *EDC* proteins share significant sequence similarities, and phylogenetic association suggests that these proteins are derived from a common ancestor, evolving to meet tissue-specific demands (74, 77). In the *EDC* lies the *FLG* gene, encoding filaggrin. In 2006, loss-of-function variants in the *FLG* gene were identified in patients of Scottish, Irish and European-American descent, as the causative genetic factor in the most common form of ichthyosis, ichthyosis vulgaris (IV; Online Mendelian Inheritance in Man #146700) (78). There were indications that AD patients had decreased expression of filaggrin in the skin (79) and there is a clinical overlap between IV and AD. When tested, the *FLG* variants were strongly associated with AD in an Irish AD cohort (48). This is so far the most significant genetic finding associated with AD (51), and it is estimated that 42% of all *FLG* mutations carriers develop AD (80). It has been shown that *FLG* mutations are population-specific and a difference in the spectra of mutations has been described. At present, more than 40 loss-of-function mutations have been reported in European and Asian populations (81).

FLG mutations are the major determinants of the levels of the filaggrin breakdown products contributing to the NMF, such as pyrrolidone carboxylic acid and urocanic acid (19). However, filaggrin deficiency may also lead to a disturbed epithelial differentiation and prone to skin inflammation (80). The integrity of the stratum corneum is maintained primarily by extracellular lipid lamellae (82). Filaggrin deficiency may contribute to defective lipid lamellae through several mechanisms. By impaired keratin intermediate filament (KIF) aggregation in the SC, the maturation and excretion of extracellular lamellar bodies is disturbed (83). Further, tight junctions that seal epidermal cell-cell integrity seem to be reduced in number in filaggrin-deficient individuals, together with a decreased density of corneodesmosin, which is the major component of corneodesmosomes (critical for SC cell-to-cell adhesion) (12, 83).

Filaggrin breakdown products are acidic, and elevated skin-surface pH observed in filaggrin–deficient individuals (84) may be important as the effect of several epidermal serine proteases depends on pH. A more neutral or alkaline pH may activate kallikrein serine proteases with major downstream consequences, including blockade of lamellar-body secretion (85). Activation of serine proteases may also drive Th2-inflammation even in the absence of allergen priming (86). It has also recently been shown that variation in intragenic copy number of *FLG* repeats (Fig. 3) contributes to a dose dependent reduction in AD risk independent of mutations; with an OR of 0.88 for each additional repeat compared to the 10 repeat allele (87).

Experimental evidence for the hypothesis that antigens enter through an impaired epidermal barrier inducing systemic allergen-specific IgE responses is supported in a mice with filaggrin frame shift mutation, analogous to human filaggrin mutation. Epicutaneous application of allergen to these mice resulted in cutaneous inflammatory infiltrates and enhanced cutaneous allergen priming with development of IgE antibody responses (88).

The genetic association with genes involved in the skin barrier homeostasis, such as *FLG* (48) and protease activity (89, 90) and immune response genes demonstrate the molecular background to AD is complex. Various clusters of genes are altered, including inflammatory and epidermal differentiation genes (43). Table 1 lists selected genes that have been associated with AD in at least two studies. These genes include functions in the adaptive and innate

immune response as well as proteins involved in the terminal differentiation (54).

1.4 ICHTHYOSIS

The ichthyoses form a large, clinically and etiologically heterogeneous group of cornification disorders that typically affect all or most of the skin surface (91). There are at least six distinct clinical subtypes that belong to the hereditary non-syndromic ichthyoses; harlequin ichthyosis (OMIM#242500); lamellar ichthyosis (OMIM#242300); congenital icthyosiform erytrodermia (OMIM#242100); epidermolytic (OMIM#113800) and superficial epidermolytic ichthyosis (OMIM#146800); recessive x-linked ichthyosis (XLI; OMIM#242100) and the mildest most common form; IV (92). IV affects 1:250 to 1:400 and 37-50% of these patients also have atopic manifestations (78). Central for the ichthyoses are disturbed pathways related to the intercellular lipid layer, cornified cell envelope formation or function of the keratin network; all leading to a subsequent disturbed skin barrier (92).

1.4.1 Genetics of ichthyosis

The known causative genes underlying common ichthyoses include *ABCA12*, *ALOXE3*, *ALOX12B*, *FLJ39501*, *NIPAL4*, *FLG* and *STS*. *ABCA12* encodes a known keratinocyte lipid transporter associated with lipid transport in lamellar granules, and a loss of *ABCA12* function leads to defective lipid transport in the keratinocytes, resulting in the severe harlequin ichthyosis phenotype. Other causative genes for ichthyoses are transglutaminase 1 (*TGM1*), keratin 1, 10 and 2 and steroid sulfatase (*STS*). *TGM1* encodes an enzyme with a role in cornified cell envelope formation and mutations are causative for lamellar ichthyosis (93). Keratin 1, 10 and 2 encodes proteins involved in the keratin network of suprabasal keratinocytes, and mutations are causative of epidermolytic ichthyosis and superficial epidermolytic ichthyosis, respectively. XLI in turn is caused by partial or complete deletions or inactivating mutations in the *STS* gene leading to deficient STS activity. STS degrades cholesterol sulfate in the intercellular spaces of the SC, and deficiency leads to both malformation of the intercellular lipid layer and a delay in corneodesomsome degradation, resulting in abnormal desquamation (94). Finally, it was found in 2006 that mutations in the *FLG* gene resulting in filaggrin dysfunction are the causative genetic factor for IV (78). Table 1 lists genes associated with distinct subtypes of common ichthyoses.

1.4.2 Ichthyosis and AD

It is well established that IV is commonly associated with atopic manifestations in 37-50% of cases (78), and that the genetic underlying factor causing IV, *FLG* mutations, is strongly associated to AD (48). It has been widely replicated that 20-40% of European and Asian patients with moderate-to-severe AD carry *FLG* mutations. However, all carriers do not manifest AD or IV and roughly 7-10% of the general European population (95) carry at least one *FLG* loss-of-function allele, regardless of symptoms. *FLG* mutations may also modify the course in XLI (96, 97). However, no association has been described regarding atopic manifestations and XLI or other rare forms of ichthyosis, although impairment in pathways important for skin barrier function are, like in AD, evident in these conditions.

Table 1: List of selected replicated genes associated to AD, psoriasis or ichthyosis.

Table modified from Guttman-Yaski et al (122), Akiyama et al (92) and Barnes (57). All gene names are abbreviated followed by reference to initial association study.

Atopic Dermatitis	Chr. region	Psoriasis	Chr. region	Ichthyosis	Chr. region
ADAPTIVE IMMUNE RESPONSE GENES					
Interleukin 4/13 (*IL4/13)* (98, 99)	5q31	Interleukin 4/13 (*IL4/13*) (100)	5q31		
Interleukin 4 RA (*IL4RA*) (101)	5p13	Human leukocyte antigen-C/Psoriasis susceptibility 1 (*HLA-C/PSORS1*) (102, 103)	6P21.3		
Mast cell chymase 1 (*CMA1*) (104)	14q11.2	Interleukin 23R (*IL23R*) (100, 103)	1p31.3		
Interleukin 18 (*IL18*) (105)	11q22.2-q22.3	Interleukin 23A (*IL23A*) (100, 103)	12q13.3		
RANTES (106)	17q11.2-q12	Interleukin 12B (*IL12B*) (107)	5q33.3		
INNATE IMMUNE RESPONSE GENES					
Nuclear-binding oligo-domain 1 (*NOD1*) (108)	7p15-p14	Tumor necrosis factor AIP3 (*TNFAIP3*) (100, 103)	6p23		
Toll-like receptor 2 (*TLR2*) (109)	4q32	Tumor necrosis factor AIP3 interacting protein 1 (*TNIP1*) (100, 103)	5q33.1		
Cluster of differentiation antigen 14 (*CD14*) (110)	5q31.1				
Defensin β1 (*DEFB1*) (111)	8p23.1				
Glutathione S-transferase pi 1 (*GSTP1)* (112)	11q13				
TERMINAL DIFFERENTIATION AND BARRIER GENES					
Serine peptidase inhibitor, Kazal-type 5 (*SPINK-5)* (113)	5q32	Late cornified envelope 3B (*LCE3B*) (114)	1q21.3		
C11orf30 (72)		Late cornified envelope 3C (*LCE3C*) (114)	1q21.3		
Filaggrin (*FLG*) (48)	1q21.3			Filaggrin (*FLG*) (78)	1q21.3
				Transglutaminase 1 (*TGM1*) (93)	14q11.2
KERATIN NETWORK GENES					
				Keratin 1 (*KRT1*) (115)	12q12-13
				Keratin 2 (*KRT2*) (116)	12q11-q13
				Keratin 10 (*KRT10*) (115)	17q21
INTERCELLULAR LIPID ORGANISATION GENES					
				ALOXE3 (117)	17p13.1
				ALOX12B (117)	17p13.1
				ABCA12 (118)	2q34
				CYP4F22 (119)	19p12
				NIPAL4 (120)	5q33
				Steroid sulfatase (*STS*) (121)	Xp22.32

1.5 PSORIASIS

Psoriasis (OMIM#177900) is an heterogeneous disease that affects roughly 2% of the adult population (123) with 0.5 % having onset before 15 years of age (124). It is characterized by inflammation of the skin and sometimes joints (psoriatic arthritis) (125). The disease varies in severity depending on both inheritance and environmental factors. Some patients may manifest mild disease with isolated scaling erythematous plaques on the elbows, knees, or scalp, whereas in others almost the entire cutaneous surface is affected (123). The most common form of psoriasis, plaque psoriasis, occurs in more than 80% of affected patients. Other sub-types include inverse psoriasis and guttate psoriasis (123). Although psoriasis is characterized by proliferation of the epidermis, the immune system has a prominent role in development of this disease, and psoriasis patients have in addition increased risk of acquiring comorbidities such as ischemic heart disease, hypertension, type 2 diabetes mellitus and obesity (125).

1.5.1 Genetics of psoriasis

There is a strong genetic background underlying psoriasis pathogenesis with strong association to pathways involving antigen-presentation such as the *HLA-C* complex and *ERAP1* (103) gene. T cell signaling and NFKB-pathway activation are also associated with manifestation of the disease (126), and a disturbed interplay between T cells is thought to be a key feature underlying the pathogenesis (127). Recently, it has been shown that deletions in genes important for epidermal protein expression are also associated with psoriasis (128). This indicates that like AD, psoriasis display a complex interplay between epidermal barrier function and immunological response. Table 1 lists commonly replicated genes associated with psoriasis.

1.5.2 Psoriasis and AD

AD and psoriasis are two of the most common inflammatory skin disorders and are genetically complex, multifactorial, and do not follow a Mendelian pattern of inheritance (59). Concomitant manifestation of psoriasis and AD is thought to be rare (129). The epidermal differentiation complex (*EDC*) region on chromosome 1q21 has been highlighted as a susceptibility locus for both AD and psoriasis (130). The *EDC* genes encode proteins in the uppermost layers of epidermis vital for keratinocyte differentiation and barrier integrity (131). This locus has previously been identified and named as *PSORS4* (132), and it has been replicated that polymorphisms in the *EDC* genes *LCE3B* and *LCE3C* (late cornified envelope 3B and 3C) influence psoriasis susceptibility (128) although no association has been shown with AD (131). However, altogether abnormalities in epidermal protein expression seem important for the pathogenesis in both AD and psoriasis.

2 AIMS OF THE PRESENT WORK

The overall aim was to improve our understanding of the genetic mechanisms underlying skin barrier defects with the main focus on AD. Specific aims were:

- to further explore the spectrum of *FLG* mutations in different populations as a genetic susceptibility factor underlying AD and IV by studying the role of *FLG* mutations in an Ethiopian case control material.

- to investigate whether mutations in the *FLG* gene may impact childhood onset of psoriasis.

- to study whether the functional and molecular alterations in AD and IV skin are dependent on filaggrin deficiency, and whether *FLG* genotype determines the type of downstream molecular pathways affected.

- to test whether genetic variability at the epidermal transglutaminase gene loci may contribute to AD susceptibility, and if alterations in transglutaminase gene expression is linked to manifestation of the disease.

3 MATERIAL AND METHODS

3.1 CLINICAL MATERIAL

A Swedish AD family material, an Ethiopian AD case control material, a Swedish ichthyosis material and a Swedish psoriasis case control material have been used in the papers included in this work.

An Ethiopian case control material was included in paper I and consists of patients with AD and/or IV (cases; n=110) and subjects without past or present history of AD, dry skin or atopic manifestations (controls; n=103). This was done in collaboration with the Skin Department at Black Lion University Hospital in Addis Ababa, Ethiopia. All patients had been diagnosed with AD by a dermatologist, based on clinical examination and according to the UK Working Party's diagnostic criteria (31). The IV diagnosis was based on clinical examination. Genetic testing was performed of the *STS* gene to exclude X-linked recessive ichthyosis. All individuals included were interviewed using a standardized questionnaire regarding atopic manifestations. Age of onset of AD, food allergy (past or present), urticaria, allergic asthma or rhinoconjunctivitis were all assessed through the questionnaire together with family history of atopy. In addition, there is detailed information available from 53 AD patients regarding associated phenotypes and both total serum IgE-levels as well as allergen-specific serum IgE-levels.

Total serum IgE and allergen-specific serum IgE against *Dermatophagoides pteronyssinus*, hen's egg, and mold mix (Mx2) including *Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Candida albicans*, *Alternaria alternate* and *Helminthosporium halodes* were measured using the ImmunoCAP method (Phadia AB, Uppsala, Sweden). Cutoffs for raised total serum IgE was 22.3 kU/L (9 monts-5 years), 263 kU/L (5-20 years) and 122 kU/L (>20 years). For allergen-specific serum IgE, concentrations below 35 kU/L were considered a negative result. Phenotype data can be found in Table 2.

Table 2: Phenotypes of AD patients, IV patients and healthy controls from an Ethiopian AD case-control material.

Phenotype of Ethiopian AD patients (n=103)	**%**
Female sex	43.6
Age (median; range)	7.0;0.3-34.0
Family history of atopy (asthma, AD or allergic rhinoconjunctivitis)	49.5
Personal history of asthma or allergic rhinoconjunctivitis	27.1
Early age of onset (<2 years)	65
Keratosis pilaris / palmar hyperlinearity	15.5
Xerosis	99
Mild AD (SCORAD <15)	2
Moderate AD (SCORAD 15-40)	65
Severe AD (SCORAD >40)	33
Phenotype of Ethiopian IV patients (n=7)	**%**
Female sex	42.8
Age (median; range)	11.0; 0.7-65.0
U.K. working party's criteria for AD	16.7
Family history of atopy (asthma, AD or allergic rhinoconjunctivitis)	28.6
Personal history of asthma or allergic rhinoconjunctivitis	0
Keratosis pilaris / palmar hyperlinearity	100
Xerosis	100
Phenotype of Ethiopian healthy controls (n=103)	**%**
Female sex	53.4
Age (median; range)	9.0; 0.7-60.0
U.K. working party's criteria for AD criteria	0
Family history of atopy (asthma, AD or allergic rhinoconjunctivitis)	0
Personal history of asthma or allergic rhinoconjunctivitis	0
Keratosis pilaris / palmar hyperlinearity	0
Xerosis	0

Among AD patients where serum was avaliable, 26/53 (49%) showed elevated total serum IgE (median 120 kU/L; inter-quartile range 44–540). Of the allergen-specific serum IgE, 6/53 (11%) patients had elevated levels against hen's egg (all aged 0–5 years), 6/53 (11%) against mould mix and 12/53 (23%) against *Dermatophagoides pteronyssinus.* Patients with severe AD had

significantly higher total serum IgE (p-value= 0.012) and higher allergen-specific serum IgE levels against *Dermatophagoides pteronyssinus* (31%) than patients with mild to moderate AD (20%), although this did not reach statistical significance (p-value= 0.32). Age and gender distribution were similar in the mild to moderate and severe AD subgroups. Allergen-specific serum IgE levels in patients with mild to moderate and severe AD are summarized in Table 3.

Table 3: Total and allergen-specific serum IgE levels for patients with AD.

Total and allergen specific IgE-levels	Mild to moderate AD	Severe AD
Number of individuals	40	13
Total serum IgE (kU/L) (median± SD)	78±810	400±1350
Elevated total serum IgE (>100 kU/L) (%)	41	76.9
Serum egg IgE positive (>0.35 kU/L) (%)	10.3	15.5
Serum *Der p*[1]. IgE positive (>0.35 kU/L) (%)	20.0	30.8
Serum mold mix positive (>0.35 kU/L) (%)	10.0	15.5

[1]*Der p*, *Dermatophagoides pteronyssinus*.

In paper II, a patient with clinical signs of ichthyosis was analyzed. The patient was male and 73 years old. He displayed dry, gray-brown scales especially prominent on the legs and the extensor surfaces of the arms, without flexural involvement or involvement of the soles or palms. He was adopted as a baby, and he has been told that his biological father had dry skin in the flexural areas; otherwise there was no knowledge about any family history of skin manifestations or atopy.

A Swedish psoriasis case control material was used in study III consisting of 241 children with onset of psoriasis below 15 years of age and of 314 healthy controls. They were identified at the Dermatology Department, Karolinska University Hospital, Solna, Sweden, and diagnosed by the same dermatologist. Blood samples were taken and medical history was recorded using a standardized questionnaire. Psoriasis severity was assessed with the Psoriasis Area and Severity Index (PASI) (133) and graded with an arbitrary disease severity score (1-7).

Patients from a Swedish AD family material were analyzed in paper IV and V. They were recruited during 1995-1997 at the Dermatology Departments of the Karolinska Solna and Danderyd Hospital, Sweden. Families with at least two affected siblings were included, resulting in 539 nuclear families from a total of 1753 individuals. All the siblings were diagnosed with AD by the same dermatologist, based on clinical examination and according to the UK Working Party's diagnostic criteria (31). All siblings were interviewed using a standardized questionnaire regarding atopic manifestations. Age of onset of AD, food allergy (past or present), urticaria, allergic asthma or rhinoconjunctivitis were all assessed through the questionnaire together with any family history of atopy. In addition, detailed individual information is available on AD severity (Table 4), associated phenotypes, total serum IgE-levels and allergen-specific serum IgE-levels. Total serum IgE and allergen-specific serum IgE were measured using the Pharmacia CAP System IgE FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala Sweden). Cutoffs for raised total serum IgE was 22.3 kU/L (9 monts-5 years), 263 kU/L (5-20 years) and 122 kU/L (>20 years). Allergen-specific serum IgE antibodies were analyzed against house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), cat, dog, horse, birch, timothy grass, mugwort, olive, *Cladosporium herbarum* and/or *Parietaria judaica* and food mix (including hen's egg white, cow's milk, soya bean, peanut, fish and wheat flour). For phenotype data of included AD siblings see Table 5.

Table 4: Arbitrary assessment of AD severity. Each factor yields a score of 1-2, with a combined maximum score of 5. Presence of AD in one or both sides in bilateral structures was considered as one site.

Factor	Score
Age at onset <2 years	1
Hospitalization for AD	1
Affected sites on examination	
0	0
0 - 3	1
>3	2
Raised total and/or allergen-specific IgE	1
Maximum Score	5

Table 5: Phenotype of included AD siblings from a Swedish AD family material.

Phenotype of Swedish AD siblings (n=1097)	%
Female sex	65
Age (median;range)	29; 4-65
U.K. Working Party's criteria for AD criteria	100
Early onset	78
Elevated total and/or allergen-specific serum IgE	74
Severe atopic dermatitis	15
Personal history of AD and asthma	39
Personal history of AD and rhinoconjunctivitis	66

For paper IV; patients (n=43) with AD (n=35), AD and IV (n=5) and IV (n=3) together with controls (n=15; subjects without past or present history of AD, dry skin or other atopic manifestations) were identified at the Dermatology Outpatient Clinics at Karolinska University Hospital Solna, Sweden; Sophiahemmet Stockholm, Sweden and Uppsala University Hospital, Uppsala, Sweden; or recruited from a Swedish family material with known *FLG* genotype as described previously (134) and used in paper V. All patients were investigated by a dermatologist performing clinical examination and recording medical history with a standardized questionnaire. Inclusion criteria were age between 18-65 years and diagnosed AD and/or IV. Exclusion criteria were pregnancy; other concomitant skin disease; recent UV-treatment; or recent use of topical or systemic glucocorticoids, immunosuppressives or retinoids (< 4 weeks). AD was diagnosed according to the UK Working Party's diagnostic criteria, severity being assessed using the scoring atopic dermatitis index (SCORAD) (135). IV was diagnosed by clinical examination and genetic testing of the *FLG* gene. In male patients with ichthyosis genetic testing of the *STS* gene was performed to rule out XLI. Other atopic manifestations such as allergic asthma and allergic rhinoconjunctivitis were assessed through the questionnaire. Blood samples and punch biopsies were taken from both patients and controls. Two 3 mm punch biopsies were obtained from a non-lesional area on each patient's forearm. TEWL was assessed using a Tewameter TM 300 Multi Probe Adapter (Courage + Khazaka electronic GmbH, Köln, Germany) and skin pH was measured using a skin-pH-Meter PH 905 Multi Probe Adapter (Courage + Khazaka electronic GmbH). TEWL and pH were measured from the forearms of patients and controls. The patients were divided into three groups (AD *FLG* +/+; AD *FLG* +/- and AD/IV *FLG* -/-) depending on genotype of the European *FLG*-mutations R501X, 2282del4, S3247X and R2447X (136). Phenotype characteristics of each patient group can be found in Table 6. The control group contained 43% females and the average age was 52 years (range 24-75 years).

Table 6: Phenotype of included AD/IV patients from a Swedish AD and IV material. The AD FLG +/+ group consisted of 14 patients, the AD FLG +/- of 14 patients and the AD FLG -/- of 15 patients, respectively.

Phenotype of Swedish AD/IV patients	AD FLG +/+ (n= 14)	AD FLG +/- (n=14)	AD FLG -/- (n=15)
Female sex (%)	78.6	64.3	46.7
Age (median; range)	56 (28-78)	54 (28-71)	59 (44-70)
SCORAD[1] (median; range)	7.6 (0-14.7)	15.4 (6.2-25.8)	14.1 (7-44.5)

[1] SCORAD of patients with AD.

3.2 GENOTYPING

Genotyping is the process of determining the genes (genotype) of an individual by examining the individual's DNA sequence on the basis of biological assays. This can be done in several ways. For papers included in this work, DNA sequencing, allelic discrimination and MLPA were used.

3.2.1 DNA sequencing

DNA sequencing is a method to detect sequence alterations and was used in papers I-V. The principle of Sanger sequencing includes denaturing and hybridizing a double stranded amplified PCR product using target primers, DNA polymerase and fluorescence-labeled dideoxynucleotides in addition to the normal nucleotides found in DNA. Dideoxynucleotides contain a hydrogen group on the 3' carbon instead of a hydroxyl group. These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides, as a phosphodiester bond cannot form between the dideoxynucleotide and the next nucleotide to be integrated. This terminates the DNA chain. Size separation of the DNA strands is performed with capillary electrophoresis and fluorescence is detected with a charge-colored device-camera in an automatic DNA sequencer. The differently labeled nucleotides are presented as peaks of different colors in generated chromatograms, and can subsequently be compared to a reference sequence.

3.2.2 Allelic discrimination

Allelic discrimination was used in papers I-V, and is a method that using forward and reverse PCR primers to amplify a region that includes an SNP polymorphic site. Allele discrimination is achieved using fluorescence resonance energy transfer (FRET) combined with one or two allele-specific probes that hybridize to the SNP site. The probes will have a fluorophore linked to their 5' end and a quencher molecule linked to their 3' end. While the probe is intact, the quencher will remain in close proximity to the fluorophore, eliminating the fluorophore's signal. During the PCR amplification step, if the allele-specific probe is perfectly complementary to the SNP allele, it will bind to the target DNA strand and then get degraded by 5'-nuclease activity of the Taq polymerase as it extends the DNA from the PCR primers. The degradation of the probe results in the separation of the fluorophore from the quencher molecule, generating a detectable signal. If the allele-specific probe is not perfectly complementary, it will have a lower melting temperature and not bind as efficiently. This prevents the nuclease from acting on the probe (137).

3.2.3 Multiplex ligand-dependent probe amplification

MLPA (Multiplex Ligation-dependent Probe Amplification) was used in paper I, II and IV and is a multiplex PCR method to detect abnormal copy numbers of genomic DNA or RNA sequences, by distinguishing sequences differing in nucleotides (138). In MLPA the two nucleotide half-probes hybridize with the adjacent target DNA sequence permitting ligation between the half-probes and creating one whole probe. The MLPA half-probes are designed so each ligation product has a unique size between 87 and 130 nucleotides. Ligation products are amplified in a subsequent PCR amplification and by using fluorescently labeled primers the PCR-product can be separated and measured by capillary electrophoresis. Comparison of the relative peak area of each amplification product to a normal control reflects the relative copy number of the target sequence.

3.3 GENE EXPRESSION ANALYSIS

Gene expression is the term used to describe the transcription of information contained within DNA into mRNA molecules that are translated into proteins. Gene expression is a highly complex and tightly regulated process that allows a cell to respond dynamically both to environmental stimuli and to its own changing needs. This mechanism acts as a switch to

control which genes are expressed in a cell and also as a gradient that increases or decreases the level of expression of particular genes as necessary. Prior to determining expression of a gene, most methods require conversion of mRNA into complementary DNA (cDNA).

There are several methods of synthezing cDNA. Briefly, the principle of cDNA synthesis includes generation of complementary DNA from a single strand of mRNA based of the pairing of RNA base pairs (A;U;G;C) to their DNA complements (T;A;C;G). Using mature mRNA; two sets of primers may be used in parallel: a poly-A oligonucleotide primer is hybridized onto the poly-A tail of the mRNA template, and in a separate reaction random hexamer primers may be hybridized, capable of hybridizing anywhere on the RNA. Subsequently, reverse transcriptase is added along with deoxynucleotide trisphosphates. This generates a complementary strand of DNA hybridized to the original mRNA strand. The RNA is digested using an RNAse; leaving a single stranded DNA. A DNA polymerase can subsequently transcribe a complementary sequence, generated a double stranded cDNA with corresponding sequence to the mRNA strand of interest.

3.3.1 Microarray expression analysis

A way to determine at what level a certain gene is expressed is microarray expression analysis. For paper IV, Human Gene 1.0 ST Array GeneChip (Affymetrix Inc, Santa Clara, CA) was used to hybridize cDNA derived from the mRNA of normal and patient samples. Microarrays contain immobilized DNA segments corresponding to regions of the genome, and differently fluorescent labeled test and reference cDNA are competitively hybridized to these segments. The test and reference cDNA are labeled and mixed prior to being hybridized on the existing probes on the array. Subsequently the array is washed before the array is loaded into scanning software measuring the ratio of the generated signal (Command Console Software, Affymetrix Inc).

3.3.2 Real-time quantitative PCR

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (qPCR), was used in papers IV-V. It is a PCR based method used to amplify and simultaneously quantify a targeted DNA molecule. The quantity can be either an absolute number of copies or a relative amount. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene and is the method used for paper IV-V. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards. The general principle of DNA quantification by real-time PCR relies on plotting fluorescence against the number of cycles on a logarithmic scale. A threshold for detection of DNA-based fluorescence is set slightly above background. The number of cycles at which the fluorescence exceeds the threshold is called the cycle threshold, C_t. During the exponential amplification phase, the sequence of the DNA target doubles every cycle; for instance a DNA sample who's C_t reaches the threshold compared to another sample by 3 cycles contained $2^3 = 8$ times more template. To quantify gene expression, the C_t from the gene of interest is divided by C_t from a housekeeping gene in the same sample to normalize for variation in the amount and quality between different samples. This normalization procedure is commonly called the $\Delta\Delta C_t$-method and permits comparison of expression of a gene of interest among different samples. However, for such comparison, expression of the normalizing reference gene needs to be very similar across all the samples. For paper IV the genes *E4A*, *18S*, and *GAPDH* were used as endogenous controls as they all displayed relative stability and low variability across the samples run on the array. For paper V; *18S* was used as endogenous control.

3.3.3 Immunohistochemical analysis

Immunohistochemistry (IHC) was used in papers I and V and refers to the process of detecting antigens (e.g. proteins) in cells of a tissue section using the principle of antibodies binding specifically to antigens in biological tissues. The term IHC is derived from "immuno" in reference to antibodies used in the procedure, and "histo" meaning tissue. IHC is commonly used to understand the distribution and localization of markers and differentially expressed proteins in different tissues. An antibody-antigen interaction can be visualized in a number of ways. Commonly, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore.

3.4 DATA AND STATISTICAL ANALYSIS

Relationships between allele and genotype frequencies in each analyzed SNP were tested in paper III-V in accordance with Hardy and Weinberg. If the observed and predicted frequency differs, a deviation from the Hardy Weinberg equilibrium (HWE) is said to exist. A deviation in HWE may indicate technical problems with the assay. HWE was tested using a *chi-square test* as implemented in the Haploview 4.1 program (139).

In paper I; differences in total serum-IgE and allergen-specific serum IgE-levels were compared between mild to moderate and severe AD using the *Mann-Whitney U test*, and p-values below 0.05 were considered statistically significant. *The Mann-Whitney U* test is a non-parametric statistical hypothesis test for assessing whether one of two samples of independent observations tends to have larger values than the other when a normal distribution cannot be assumed.

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For paper III, the significance of allele frequencies was calculated using *Fisher's exact test* and the significance of difference in disease severity and age of onset was calculated using *student's t-test.* P-values below 0.05 were considered statistically significant. *Fisher's exact test* is a statistical significance test used in the analysis of contingency tables where sample sizes are small. The test is useful for categorical data. A *t-test* is a test commonly applied when a normal distribution is expected. When the value of the scaling term of the normal distribution is unknown, and estimated based on the data the test statistic follows a student's t distribution.

For paper IV a two-way analysis of variance (*ANOVA*) was performed to identify differentially expressed genes between the different experimental groups in the microarray analysis using the Partek Genomics Suite 6.4. *ANOVA* is a generalized t-test useful for comparing distribution of more than two groups. For each comparison between two experimental groups the fold change of every annotated gene, together with their corresponding p-value, was exported to Microsoft Office Excel. For qPCR, the relative mRNA expression and statistical significance were calculated using the REST 2009 software (available at www.qiagen.com) using *Fisher's exact test*. For genes chosen for pathway analysis, significance was corrected with *Bonferroni multiple testing*. *Bonferroni correction* is a method used to counteract the problem of errors generated when doing multiple comparisons. Briefly, it is based on the idea that if "n" hypotheses are tested on a set of data, one way to maintain statistical significance is to estimate that significance is maintained by calculating 1/"n" times what it would be if only one hypothesis were tested. Statistical significance for SCORAD was calculated using s*tudent's t -test*. Gene mapping to pathways was performed using DAVID bioinformatics resources (140) with the KEGG pathway analysis option. Gene pathway analysis was also performed using Ingenuity Pathway Analysis**.**

In paper V the *pedigree disequilibrium test (PDT*) and odds ratio (OR) were performed using the Unphased (3.1.3) program (141). *Transmission disequilibrium test* (*TDT*) is a family-based association test for the presence of genetic linkage between a genetic marker and a trait. *PDT* is a modified test of family linkage that can use all potentially informative data from a pedigree, even from extended pedigrees. OR is a common measurement of effect size. The OR for minor alleles was estimated relative to the major allele and the OR for haplotypes relative to all the other haplotypes in the same block together. For the haplotype analysis the specific test haplotype option was used with a rare haplotype frequency maximum of 2%, a zero haplotype frequency maximum of 1% and the estimate missing genotypes option. Associations with significant p-values were corrected for multiple testing by performing 10000 permutations in the Unphased program. LD between polymorphisms in studied regions was calculated using the Haploview program.

Statistical power for studies III and V was estimated using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/; Harvard Medical School). The power of a statistical test is the probability that the test will reject the null hypothesis when the hypothesis is actually false (false negative). Power analysis can be used to calculate the minimum sample size required to estimate detection on of an effect of a given size. Power analysis can also be used to calculate the minimum effect size that is likely to be detected in a paper using a given sample size.

4 RESULTS AND DISCUSSION

There is thought to be a complex interplay between skin barrier dysfunction and subsequent immunological response in common inflammatory disorders of keratinisation such as AD and psoriasis. The papers in this work have aimed to further delineate the genetic factors underlying barrier dysfunction mainly in AD, but also to identify whether AD and other common disorders of keratinisation may share genetic susceptibility factors.

4.1 FILAGGRIN MUTATION MAPPING IN ETHIOPIAN PATIENTS WITH AD AND IV *(PAPER I)*

Mutations in the *FLG* gene are considered the most significant finding to date underlying the barrier impairment seen in AD. At present, more than 40 loss-of-function mutations have been reported in European and Asian populations, and filaggrin deficiency is considered a strong risk factor for developing AD (81). The global contribution of *FLG* mutations to the development of AD and IV remains to be further elucidated; and the mutation spectrum has not been established in native African populations. In paper I we investigated the role of *FLG* mutations in AD and IV patients from an African population. We fully sequenced the *FLG* gene in 40 individuals and genotyped 103 Ethiopian individuals with AD and/or IV for the prevalent European loss-of-function variants. Neither European nor Asian variants were found. In one patient, a novel *FLG* mutation was discovered (Figure 4). No other mutations were found. In addition, no difference in filaggrin expression was detected in AD or IV skin compared to healthy control skin.

Our results suggest that *FLG* mutations are rare in Ethiopian AD and IV patients compared to other described populations. The prevalence of *FLG* mutations is reportedly as high as 17 – 42 % in European populations with moderate-to-severe AD (142), and 20 - 30 % of AD patients in Asian populations (143). Our result suggests a prevalence of ~2-3% among Ethiopian AD patients. It has been proposed that *FLG* mutations are population specific (144) and a difference in the spectra of mutations has been described. A theory is that the *FLG* mutations occurred after the major separation of the respective populations (145). We wanted to limit the ancestral admixture and focused primarily on one distinct population, where the admixture of other previously-studied populations was expected to be limited. However, extensive genetic diversity is described in the African population, which restricts what generalizations can be made for other African populations than the Ethiopian (146). There is also a risk of underestimating the full spectrum of *FLG* mutations in this population given the sample size of fully sequenced AD and IV patients in the paper.

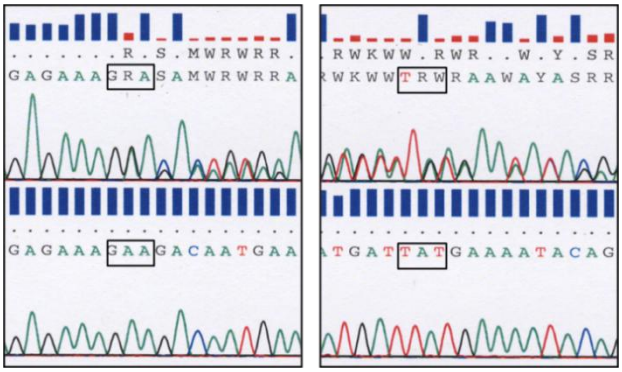

Figure 4: Sequence results displaying *FLG* mutation 623del2. A two base pair deletion at codon 623 (623del2) (top left) leading to a premature stop codon (TGA) at codon 632 (top right). Wild type *FLG* corresponding to codon 623 marked (GAA) (bottom left) and wild type FLG corresponding to codon 632 marked (TAT) (bottom right).

An interesting question arising is why *FLG* mutations are so prevalent in the European population in general, as the overall prevalence regardless of symptoms has been estimated to 9 % (48). A theory is that it could have been of evolutionary value to carry a mutation causing a more permeable skin barrier. Repeated low-level exposure of pathogens to antigen-presenting cells in the skin might increase the immunity against infections during pandemics, e.g. the bubonic plague that wiped out 30-60 % of the entire European population (147). This sort of balanced selection favoring a heterozygous state has been proposed in other conditions such as the underlying sickle-cell anemia mutations seen in malaria-endemic areas (148). Varying susceptibility to malaria between different populations has previously been demonstrated by polymorphisms in several loci affecting immune response pathways (149, 150). This genotypic and phenotypic adaptation may also play a role in other immune-mediated pathways. If the prerequisites for balanced selection favoring a more permeable skin barrier have not been fulfilled in the African population, this could help explain the low prevalence of *FLG* mutations found in Ethiopian AD and IV patients.

Having *FLG* mutations in a setting such as Africa might even be a disadvantage for a single individual, since filaggrin contains numerous histidine residues which during filaggrin degradation serve as substrates for the formation of UCA. UCA has been proposed to act as a UV-absorbing substance in the stratum corneum (151) and filaggrin deficiency has been associated with lower concentrations of UCA in skin cultures (152) and *in vivo* (153). Skin cultures have shown that irradiation of filaggrin-deficient skin results in increased cyclobutane pyrimidine dimers and caspase 3 activation; both suggesting increased DNA damage and apoptosis (152). Thus, individuals with filaggrin-deficient skin might be subject to increased DNA damage in an environment with high UV radiation.

These theories are so far speculations and our findings of a low incidence of *FLG* mutations in the material analyzed might have other explanations. Secondary down regulation of filaggrin in the absence of mutations has been discussed previously (58). However, our IHC analysis does not indicate any apparent down regulation in lesional skin with the phenotype of AD or IV (n=7) compared to healthy control skin (Figure 5). The phenotype is of great importance, and it has been suggested that the absence of both palmar hyperlinearity and keratosis pilaris may give a negative prediction value of *FLG* mutations as high as 92 % (154). The novel mutation 623del2 was detected in an AD patient with palmar hyperlinearity and keratosis pilaris. Had our AD material comprised more patients with palmar hyperlinearity and keratosis pilaris, the frequency of *FLG* mutations might have been higher, although in our present paper 65.7 % of the sequenced AD and IV patients had at least one of these clinical features. It is also premature to rule out mutations in the non-coding part of the *FLG* gene or in the processing of the precursor profilaggrin, as correct processing is important for the functionality of filaggrin (155).

In summary, our result indicates that common European *FLG* loss-of-function variants are rare in Ethiopian AD and IV patients implying that other factors play a more important role for the pathogenesis of IV and AD in this ethnic group.

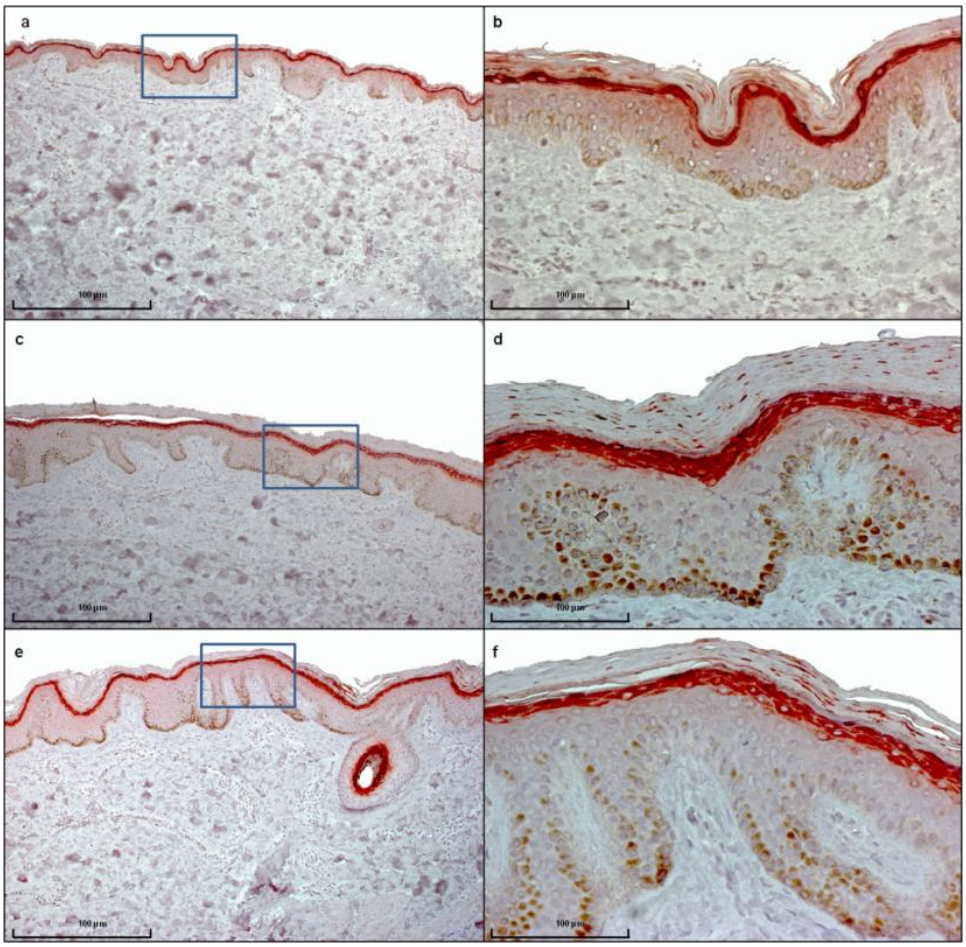

Figure 5: Immunohistochemistry of filaggrin showing expression in Ethiopian healthy control skin, AD skin and IV skin. Staining of filaggrin expression was detectable in the stratum granulosum of the epidermis in representative healthy control skin (a) x100 and (b) corresponding x400 magnification of highlighted area in (a); AD skin (c) x100 and (d) corresponding x400 magnification of highlighted area in (c); and IV skin (e) x100 and (f) corresponding x400 magnification of highlighted area in (e). Hyperplasia of the epidermis is visible in AD (c-d); and IV (e-f) skin compared to control skin (a-b). Scale bars represent 100 µm.

4.2 IDENTIFYING THE UNDERLYING GENETIC FACTOR IN A PATIENT WITH ICHTHYOSIS *(PAPER II)*

We encountered a Swedish patient with typical clinical signs of common ichthyosis of either IV or XLI-type. The patient exhibited dry gray-brown scales especially prominent on the legs and the extensor surfaces of the arms. XLI occurs predominantly in men and may look almost indistinguishable from IV, but skin histology and surface pH differ in the two conditions (156). To determine the exact cause, MLPA was run for the *STS* gene to rule out XLI, and *FLG* genotyping was performed for the *FLG* mutations described in European population. No deletions were found in the coding region of *STS* and no *FLG* mutations were detected. Direct sequencing was subsequently performed for the

STS gene. In exon 3 a non-sense mutation was found using both forward and reverse primer; a one base pair substitution C>T changing arginine (CGA) into a premature stop (TGA) (Figure 6).

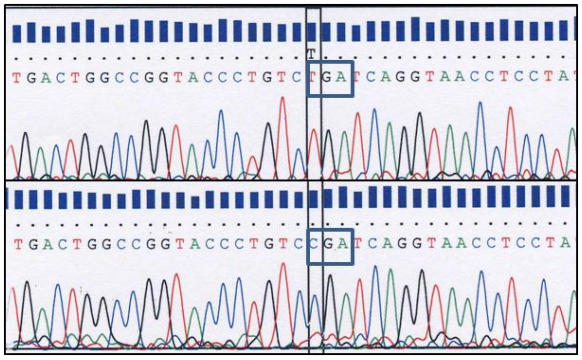

Figure 6: Mutation in the *STS* gene in a patient with ichthyosis. *STS* sequencing results with a one base pair substitution C>T introducing stop (TGA) in exon 3 at amino acid 90 (top) and healthy control showing arginine (CGA) at amino acid 90 (bottom).

The *STS* gene is located on Xp22.3 and encodes a 62kDa polypeptide containing 10 exons (158). In the skin STS is expressed in the epidermis and is thought to have a role in steroid production and lipid metabolism (158, 159). *STS* mutations are heterogeneous and in addition to the more common deletions there are to our knowledge 13 described point mutations (Human Gene Mutations Database, 2010-11-23), 12 in the coding region and one in the non-coding region. Of these, 10 are missense mutations, two represent non-sense and the one in the non-coding region affects a splice junction site (96, 157). The point mutations are all in exons 5 - 10 (Figure 7). Most partial deletions are also in this area, however partial deletions have also been described in exons 1-5 (157). Although the genotypic deficiencies are heterogeneous, they all lead to loss of STS enzyme activity. This leads to defective desquamation through corneocyte retention as well as phase separation in the stratum corneum through excess STS substrate cholesterol sulphate, causing the characteristic phenotype (94). Here we present an additional *STS*

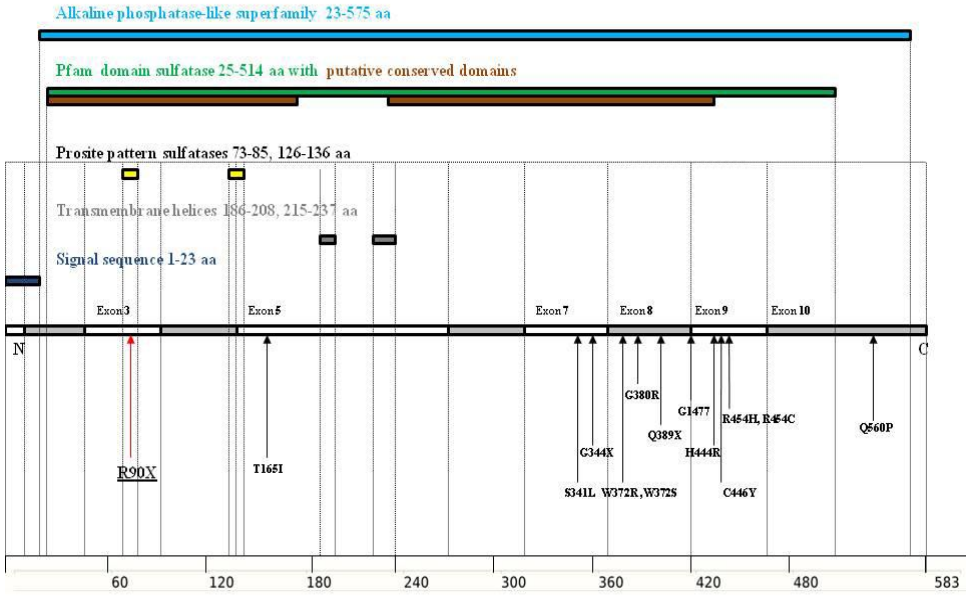

Figure 7: Schematic overview of domains and features of the 583 aa steroid sulfatase protein. Novel point mutation site R90X in exon 3 shown with red arrow, previously described mutation sites shown with black arrow (96, 157).

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mutation, the most 5' point mutation yet described. This emphasizes the value of sequencing the entire gene when the clinical picture match that of XLI but no deletions are found.

4.3 NO ASSOCIATION BETWEEN FILAGGRIN MUTATIONS AND EARLY ONSET OF PSORIASIS *(PAPER III)*

Both psoriasis and AD are considered to exhibit disturbed epidermal barrier function (160). In AD, *FLG* mutations may lead to a defect barrier, allowing for per-cutaneous allergen and irritant priming and subsequent immunological response (88). In psoriasis no association has been found with truncating *FLG* mutations (130, 161) although a disturbed skin barrier is evident (160). *FLG* mutations are associated with an early onset of the disease in AD (162), and have been proposed as a modifying factor in other conditions of keratinization such as XLI (96). We therefore set out to investigate whether *FLG* mutations has any association with early onset of psoriasis.

The *FLG* gene was genotyped in all patients and controls using allelic discrimination (n=555) and sequencing (n=20) (Table 7). We found no association to prevalent *FLG* mutations, and did not detect any novel *FLG* mutations in early onset psoriasis patients. This indicates that *FLG* loss-of-function variants should not modify the age of onset of psoriasis among our patients and that there was no indication of any additional prevalent *FLG* mutation. Nevertheless, as our patients displayed mild-to-moderate psoriasis and were of European descent, the role of *FLG* mutations in severe psoriasis or for psoriasis susceptibility in other populations might be different.

Still, lowered levels of filaggrin expression have been demonstrated in psoriasis (160). In AD skin the presence of Th2-mediated cytokines highly expressed in AD skin such as IL4, IL13 and IL25 down-regulates the expression of filaggrin even in the absence of *FLG* mutations (50, 58). However, the immunological response in psoriasis is thought to be more Th1- and Th17-driven than Th2 (163) and it is possible that reduced levels of filaggrin protein in psoriatic skin is mainly due to disturbed epidermal differentiation, or a consequence of alternative gene regulation; but this has to be further investigated.

Table 7: *FLG* genotyping results in early onset psoriasis patients and controls. Columns R501X, 2282del4, R2447X and S3247X show the presence of four prevalent European *FLG* mutations in early onset psoriasis patients and controls. Rows "AA" corresponds to homozygous wild type for *FLG*; "Aa" to heterozygous for either common *FLG* null alleles and "aa" to homozygous or compound heterozygous for either *FLG* null alleles. Allele frequencies were compared using Fisher's exact test.

	R501X		2282del4		R2447X		S3247X		Combined genotype	
	Controls	Psoriasis	Controls	Psoriasis	Controls	Psoriasis	Controls	Psoriasis	Controls	Psoriasis
AA	311	234	302	238	313	240	312	241	296	230
Aa	3	6	12	3	1	1	2	0	18	10
aa	0	1	0	0	0	0	0	0	0	1
n	314	241	314	241	314	241	314	241	314	241
P-value		0.11		0.11		1.00		0.51		0.57

4.4 FILAGGRIN GENOTYPE DETERMINES MOLECULAR AND FUNCTIONAL ALTERATIONS IN SKIN OF PATIENTS WITH AD AND IV *(PAPER IV)*

Our objective was to study whether the functional and molecular alterations in AD and IV skin depend on filaggrin loss-of-function, and whether *FLG* genotype determines the type of downstream molecular pathway affected.

Patients with AD and/or IV (n=43) and controls (n=15) were recruited from two Swedish outpatient clinics and a Swedish AD family material with known *FLG* genotype. They were clinically examined and their medical history recorded using a standardized questionnaire. Blood samples and punch biopsies were taken from non-lesional skin and TEWL and skin pH was assessed with standard techniques. In addition to *FLG* genotyping, the *STS* gene was analyzed to exclude XLI. Microarrays and qPCR were used to compare differences in gene expression depending on *FLG* genotype.

FLG was down-regulated both by microarray analysis and qPCR in all AD/IV groups compared to the healthy controls (Fig 5). Although there was a gradient of down-regulation depending on *FLG* genotype with the lowest filaggrin expression in patients with *FLG* -/- genotype followed by the *FLG* +/- genotype, the *FLG* +/+ group also displayed down-regulation compared to healthy control skin. Recent studies have shown that pro-inflammatory cytokines may modulate the expression of filaggrin, which might be one of the underlying explanations of our finding (50, 164). Recent studies have shown that pro-inflammatory cytokines may modulate the expression of filaggrin, which might be one of the underlying explanations of our finding (50, 164).

Many of the genes significantly altered in our gene expression analysis map to regions previously linked to AD (57), further highlighting these regions as candidates for AD susceptibility. The distributions of these differentially expressed genes in our study depended on *FLG* genotype, where several clusters were unique for each group, and others overlapped (Fig. 9). Genes from these groups were mapped to significantly altered pathways in each patient group (Table 8). The functional alterations evident from the significantly higher TEWL and pH (Fig. 8) in the filaggrin-deficient groups may influence the number of induced or repressed genes involved in tightly regulated processes such as inflammatory response, following a more permeable barrier, as well as enzymatic activity where the pH level is important (19).

The importance of changes in TEWL and pH has recently been highlighted in filaggrin deficient skin. Reduced levels of filaggrin degradation products are proposed to increase TEWL and pH, decreasing SC hydration and altering enzymatic activity (83, 84). This may account for alterations in corneocyte and lipid organization within the SC (83). Given the frequent phenotypic overlap between dry skin, IV and AD (evident in our *FLG* -/- group as well); it is proposed that these functional alterations are important for the pathogenesis in both IV and AD skin with filaggrin deficiency.

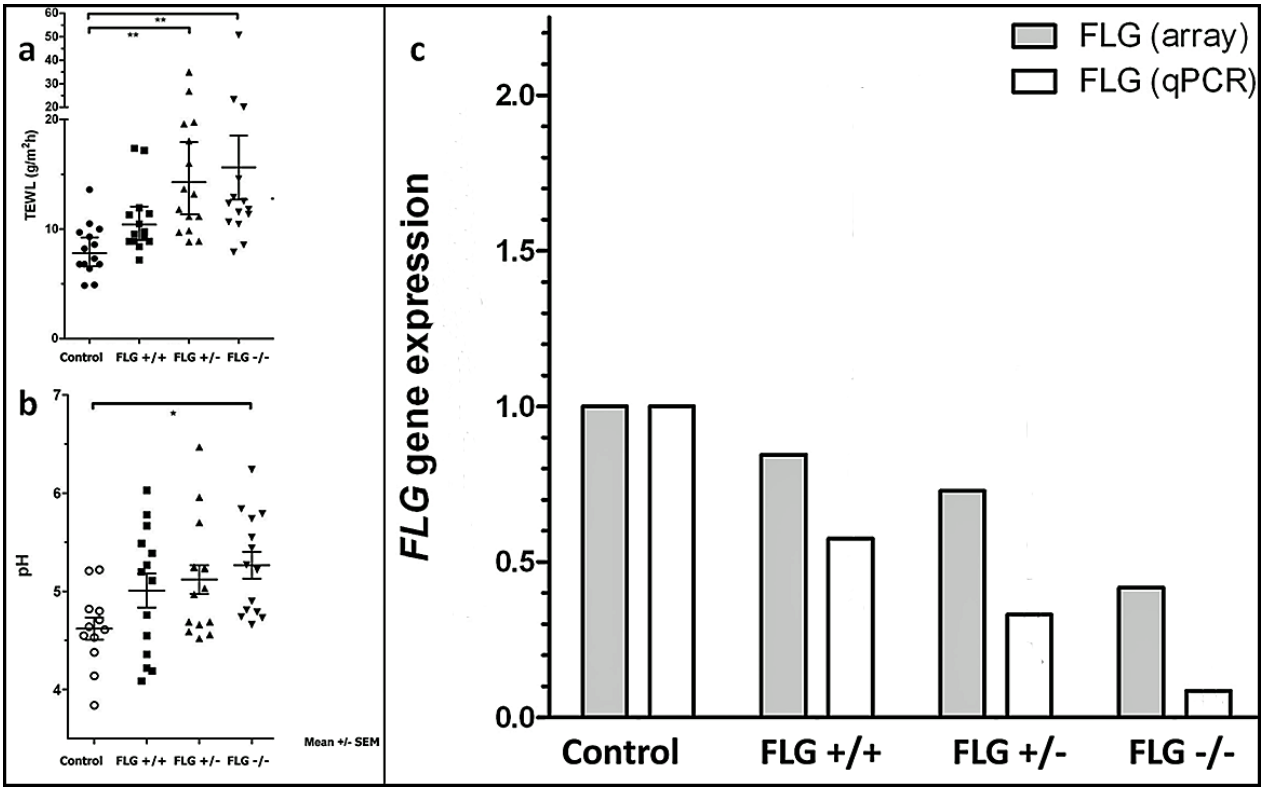

Figure 8: Mean trans-epidermal water loss (TEWL) (a) and pH (b) and decrease in mRNA expression (c) in the AD *FLG* +/+, AD *FLG* +/- and the AD/IV *FLG* -/- group. All groups compared to a healthy control group. Significant changes are denoted with * and ** ($p<0.05$ and $p<0.01$), respectively. All groups had significantly altered filaggrin expression compared to the healthy control group with qPCR; for the *FLG* +/+ p=0.043, the *FLG* +/- p=0.001 and the *FLG* -/- group p=0.001. From the array expression results the *FLG* +/+ group was lower but not significant (p=0.59) whereas the expression was significantly lower in the *FLG* +/- (p=0.04) and *FLG* -/- groups ($p=8x10^{-6}$).

In support of this hypothesis, our AD patients without *FLG* mutations displayed lower functional barrier impairment measured by TEWL, lower pH and significantly lower mean SCORAD than AD patients with *FLG* mutations (The AD *FLG* +/+ had significantly lower SCORAD than the AD *FLG* +/- and the AD patients in the AD/IV *FLG* -/- group (p=0.02)). In addition, the lowest number of significantly altered genes was detected in our AD *FLG* +/+ group. This suggests a correlation between number of affected genes, barrier impairment and disease severity among included AD patients.

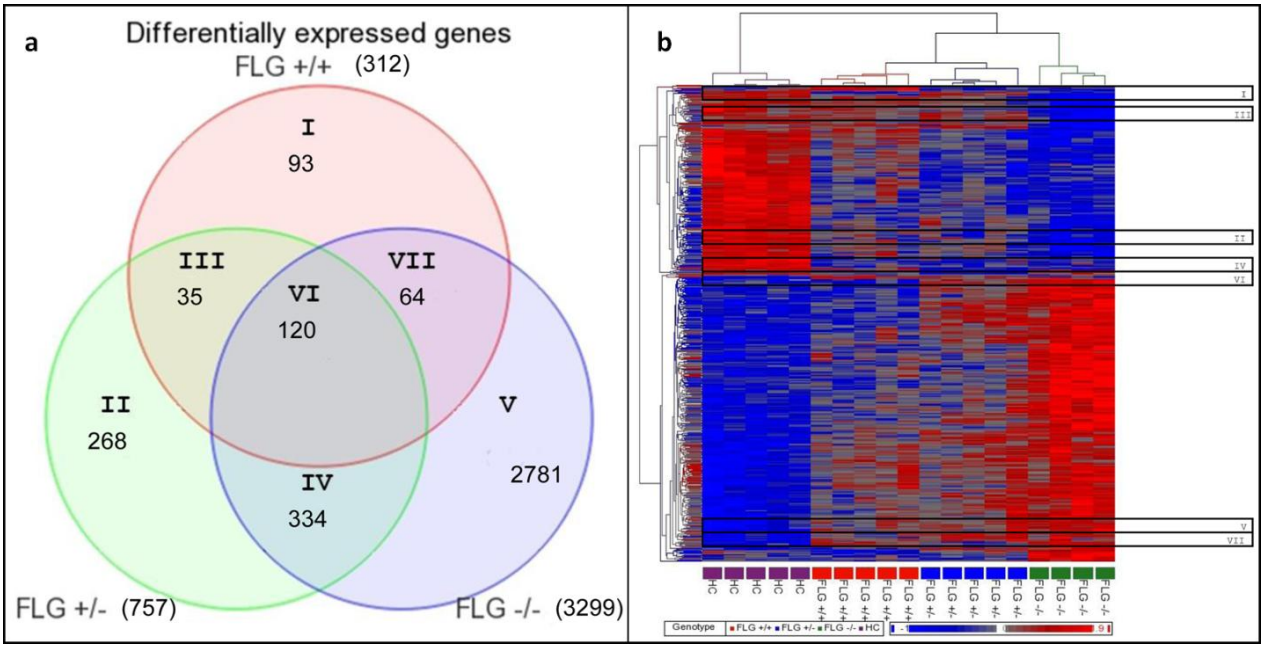

Figure 9: Top overlapping differentially expressed genes in AD skin (a) and heat map of transcriptional levels of genes in AD skin and controls (b). Genes with altered expression in patients with *FLG* wild type genotype *(FLG +/+)*, heterozygote *FLG* mutation genotype (*FLG +/-)*, and homozygote *FLG* mutation genotype (*FLG -/-)*. Clusters containing differentially expressed genes in marked regions I-VII are corresponding regions in a) and b). A list of p-values and fold changes for all differentially expressed genes are described in supplementary Table S1 online for paper IV. Hierarchal clustering analysis was performed in both the gene (row) and experiment (column) dimension. Contrast value for each gene is shown, e.g. the standardized mean difference between the gene's expression in the group versus overall expression.

Of the many genes previously associated to AD (57) several were also dysregulated in our array data, such as serine protease inhibitor kazal-type 5 (*SPINK-5)*, mast cell chymase *(CMA1)* and interleukin 4 *(IL-4)*. Any discrepancies regarding expression of inflammatory mediators commonly found in AD may, at least in part, be due to lower expression of these genes in non-lesional skin. The molecular mechanisms involved in the phenotype of AD following the functional barrier impairment in our material involve altered pathways such as cytoskeleton structure, calcium- and phospatidylinositol signaling and ATP binding cassette (ABC) transport system (Table 8). It has been suggested that filaggrin is of importance for cytoskeleton organization by aggregating KIFs; and that filaggrin deficiency may cause cytoskeleton abnormalities such as perinuclear keratin retraction in granular cells (83). KIF polymerization is actin-dependent (165) and subsequently actin-cytoskeleton aberrations may contribute to the peripheral KIF retraction previously demonstrated in filaggrin-deficient skin (83).

Table 8: Top altered molecular pathways depending on *FLG* genotype. All included molecules were mapped using KEGG Pathway analysis. Presented p-values are uncorrected. Significant p-values after Bonferroni correction are indicated as * (p-value below 0.05) and ** (p-value below 0.005). Candidate genes mapped to each pathway are outlined in the supplementary information online for paper IV.

AD *FLG* +/+	**# genes**	**p-value**
Focal adhesion (hsa04510)	30	$1x10^{-3}$
Regulation of actin cytoskeleton (hsa04810)	29	$6x10^{-3}$
ECM-receptor interaction (hsa04512)	14	$2x10^{-2}$
AD *FLG* +/-	**# genes**	**p-value**
ECM-receptor interaction (hsa04512)	29	$5x10^{-6}$**
Focal adhesion (hsa04510)	52	$9x10^{-6}$**
ABC transporters (hsa02010)	19	$1x10^{-5}$**
Regulation of actin cytoskeleton (hsa04810)	47	$2x10^{-3}$*
Calcium signaling pathway (hsa04020)	37	$1x10^{-2}$
T cell receptor signaling pathway (hsa04660)	23	$4x10^{-2}$
Adherens junction (hsa04520)	17	$7x10^{-2}$
AD/IV *FLG* -/-	**# genes**	**p-value**
Phosphatidylinositol signaling system (hsa04070)	54	$8x10^{-7}$**
Focal adhesion (hsa04510)	118	$8x10^{-7}$**
ECM-receptor interaction (hsa04512)	57	$2x10^{-6}$**
Regulation of actin cytoskeleton (hsa04810)	123	$3x10^{-6}$**
Calcium signaling pathway (hsa04020)	100	$4x10^{-5}$*
T cell receptor signaling pathway (hsa04660)	64	$3x10^{-4}$
ABC transporters (hsa02010)	31	$3x10^{-4}$
Tight junction (hsa04530)	71	$7x10^{-3}$

However, the role of filaggrin in impaired intermediate filament aggregation has been challenged (152) and other factors than filaggrin deficiency may explain the alterations in the pathway for the regulation of the actin cytoskeleton. Our findings support this, as pathways for actin-cytoskeleton regulation were altered in all our patient groups including the group without *FLG* mutations. In addition, several keratins (including keratin 1 and 10) were significantly down-regulated in AD patients both with and without *FLG* mutations. As the actin filament system has been suggested to be involved in KIF transport (165), it is possible that increased actin cytoskeleton regulation is a compensatory mechanism following lower keratin expression. Altogether, our data suggests that both keratin expression and KIF regulation are subject to modulation in AD skin independently of *FLG* mutations.

Filaggrin may be involved in calcium metabolism in the skin (166), and the calcium gradient is important for epidermal differentiation. A loss of this gradient increases keratinocyte proliferation and decreases differentiation (167). Impaired calcium metabolism has been demonstrated in other conditions where the skin barrier is disrupted, such as Hailey-Hailey disease (168) and in psoriatic skin (169). Further, defective lipid transportation and defects in lamellar body extrusion have previously been reported in AD (170, 171) and mutations in this pathway may cause severe ichthyotic conditions such as HI (172). Our filaggrin-deficient groups showed alterations in the pathways for calcium signaling and for ABC transport system, indicating that alterations in these pathways are involved in the pathogenesis of IV and AD with filaggrin deficiency.

In conclusion, we have demonstrated that functional parameters and gene expression in molecular pathways *in vivo* is altered in patients suffering from AD and IV and depend on filaggrin genotype. Patients with *FLG* mutations display a severe phenotype with impaired barrier function measured as increased TEWL, and significantly altered pH levels. Furthermore, the number of genes with altered expression is significantly higher in patients with low or absent filaggrin expression. These pathways include many genes involved in inflammation, epidermal differentiation, lipid metabolism, cell signalling and adhesion.

4.5 GENETIC ASSOCIATION AND EXPRESSION ANALYSIS OF THE EPIDERMAL TRANSGLUTAMINASES IN AD *(PAPER V)*

The epidermal transglutaminases genes *TGM1*, *TGM3* and *TGM5* encode cross-linking enzymes in the epidermis and map in genomic regions that have previously been linked to AD and associated phenotypes. We therefore set out to characterize the expression and test genetic association of the epidermal transglutaminases on the defective skin barrier evident in AD. Genotyping was performed for 47 SNPs in total covering the *TGM1*, *TGM3* and *TGM5* locus in the Swedish family material. Expression analysis of the epidermal transglutaminases was performed with qPCR and IHC.

Results of expression analysis of the *TGM1*, *TGM3 and TGM5* gene using qPCR showed a significantly higher level of *TGM3* mRNA in lesional skin from AD patients than in skin from healthy controls (Fig. 8). IHC analysis indicated an increase of TG1 and TG3 expression in a majority of lesional skin samples, while only some of the non-lesional skin samples appeared to have an increase in these proteins compared to healthy controls. Further, in lesional skin characterized by hyperplasia, TG1 and TG3 expression was found in several of the suprabasal layers, while in skin from healthy individuals the proteins was localized in the outermost granular layer of the epidermis. No apparent differences regarding TG5 expression were found (Fig 8).

Expression data presented in our study show that the expression of the *TGM3* gene is markedly increased in the skin of AD patients compared to the skin of healthy controls, both at transcript level and on protein level (Figure 10). Increased protein expression was also evident for TG1 but without a significant difference at mRNA level.

PDT revealed a significant genetic association for one SNP, rs941505, located in the *TGM1* region. The minor allele was under-transmitted to offspring with AD and allergen-specific IgE (OR=0.60, CI=0.43-0.84). In accordance with this, we also found an association for a haplotype tagged by the minor allele of rs941505 (Table 9). It is possible that individuals carrying the associated *TGM1* haplotype may have a reduced risk of developing a skin barrier dysfunction, thereby reducing the allergen penetrance in the epidermis and the risk of subsequent development of AD. However, alternative explanations are conceivable and further studies are required to fully explain the association.

The MAPPER database (173) positions the *TGM1* SNP rs941505 in an evolutionarily conserved part of the predicted promoter region, 900 bases upstream of the transcription start site. Notably, previous studies have indicated that the first 1.6 kilo bases upstream of the gene are necessary and sufficient for normal tissue- and cell layer-specific expression in transgenic mice (174). Analysis in the RAVEN software (http://www.cisreg.ca/cgi-bin/RAVEN/a) and the MAPPER database suggests that the rs941505 SNP alters putative binding sites of transcription factors overlapping this position.

The *TGM1* gene is positioned in a close (~2.3 kilo bases) head-to-tail arrangement with the Rab geranylgeranyl transferase (*RABGGTA*) gene, and analyzing the LD structure in this region shows that a haplotype block including the associated SNP extends into this gene. The *RABGGTA* gene encodes the α-subunit of the GGTase-II enzyme that attaches geranylgeranyl moieties (acting as membrane anchors) on Rab proteins (175). The Rab proteins are GTPases involved in the regulation of organelle biogenesis and vesicle transport (176).

To date only one genome wide association study (GWAS) exploring AD in the European population has been published (72). This study did not identify the *TGM1* gene region as a susceptibility locus.

Table 9: Results of Pedigree Disequilibrium Test for the inferred *TGM1* haplotypes in the Swedish AD families.

Haplotypes																										AD		AD IgE+4	
rs17102	rs71587	rs27485	rs11264		rs71512		rs27485	rs22733	rs94150	rs72942		rs11627	rs12895	rs37	rs10134	rs22953	rs10136	rs12586											
Block 1							**Block 2**						**Block 3**													**HF[1]**	**OR[2]**	**P**	**OR**	**P**
G	T	C	C	-								-														0.608	1.19 (1.01-1.39)	0.87	1.12 (0.93-1.35)	0.79
A	C	C	A	-								-														0.174	0.55 (0.43-0.70)	0.48	0.95 (0.75-1.20)	0.62
G	C	T	C	-								-														0.145	1.04 (0.84-1.28)	0.22	1.04 (0.82-1.33)	0.24
G	C	C	C	-								-														0.033	0.70 (0.45-1.07)	0.17	0.81 (0.48-1.36)	0.56
				-			C	C	A	A		-														0.570	1.31 (1.13-1.53)	0.08	1.29 (1.09-1.54)	0.04 (NS[3])
				-			C	C	A	G		-														0.213	0.93 (0.79-1.10)	0.42	0.97 (0.78-1.19)	0.76
				-			C	C	**T**	G		-														0.094	0.79 (0.61-1.01)	0.06	**0.56 (0.39-0.79)**	**0.002 (0.03)**
				-			C	T	A	G		-														0.057	0.96 (0.71-1.30)	0.84	1.03 (0.71-1.48)	0.54
				-			T	C	A	A		-														0.055	1.03 (0.75-1.40)	0.88	0.88 (0.60-1.29)	0.89
												-	T	G	T	A	G									0.488	1.16 (1.01-1.34)	0.04 (NS[3])	1.19 (1.01-1.40)	0.04 (NS[3])
												-	C	G	T	G	G									0.198	0.93 (0.77-1.11)	0.40	0.93 (0.74-1.16)	0.51
												-	C	G	T	A	G									0.129	0.90 (0.72-1.12)	0.33	0.92 (0.70-1.78)	0.49
												-	C	G	T	G	A									0.068	0.83 (0.64-1.10)	0.20	0.81 (0.59-1.12)	0.20
												-	C	A	T	G	G									0.058	1.18 (0.85-1.62)	0.68	1.00 (0.66-1.48)	0.81
												-	C	G	G	G	A									0.057	0.84 (0.59-1.18)	0.31	0.77 (0.51-1.17)	0.22

[1]Haplotype Frequency (HF) in the whole material. [2]Odds ratio (OR) of haplotype relative to all the other haplotypes in the same block together, 95% confidence intervals in brackets. [3]Permutated p-values given with brackets, NS = non-significant. Haplotypes with a frequency below 1.5% have been omitted. [4] AD IgE+ subgroup of AD patients with allergen-specific IgE.

Looking at the SNPs present on the arrays used in the GWAS, including a 200 kb region surrounding the associated SNP rs941505, we conclude that none of these are in high LD with rs941505 (based on the HapMap CEU data set). Given the LD and the effect size seen for the AD phenotype we estimate that the statistical power to detect the rs941505 association was below 30% in both discovery sets of the GWAS. This may explain why this study did not identify the *TGM1* region as a susceptibility locus.

In conclusion, expressional analysis links the *TGM1* and *TGM3* gene to the manifestation of AD. Further, the results from the genetic analysis suggest that genetic variation at the *TGM1* locus could be involved in the development of the disease. Independent sets of patients and controls are now needed to confirm the genetic association found in the AD family

material and further studies are needed to identify the actual disease causing variations.

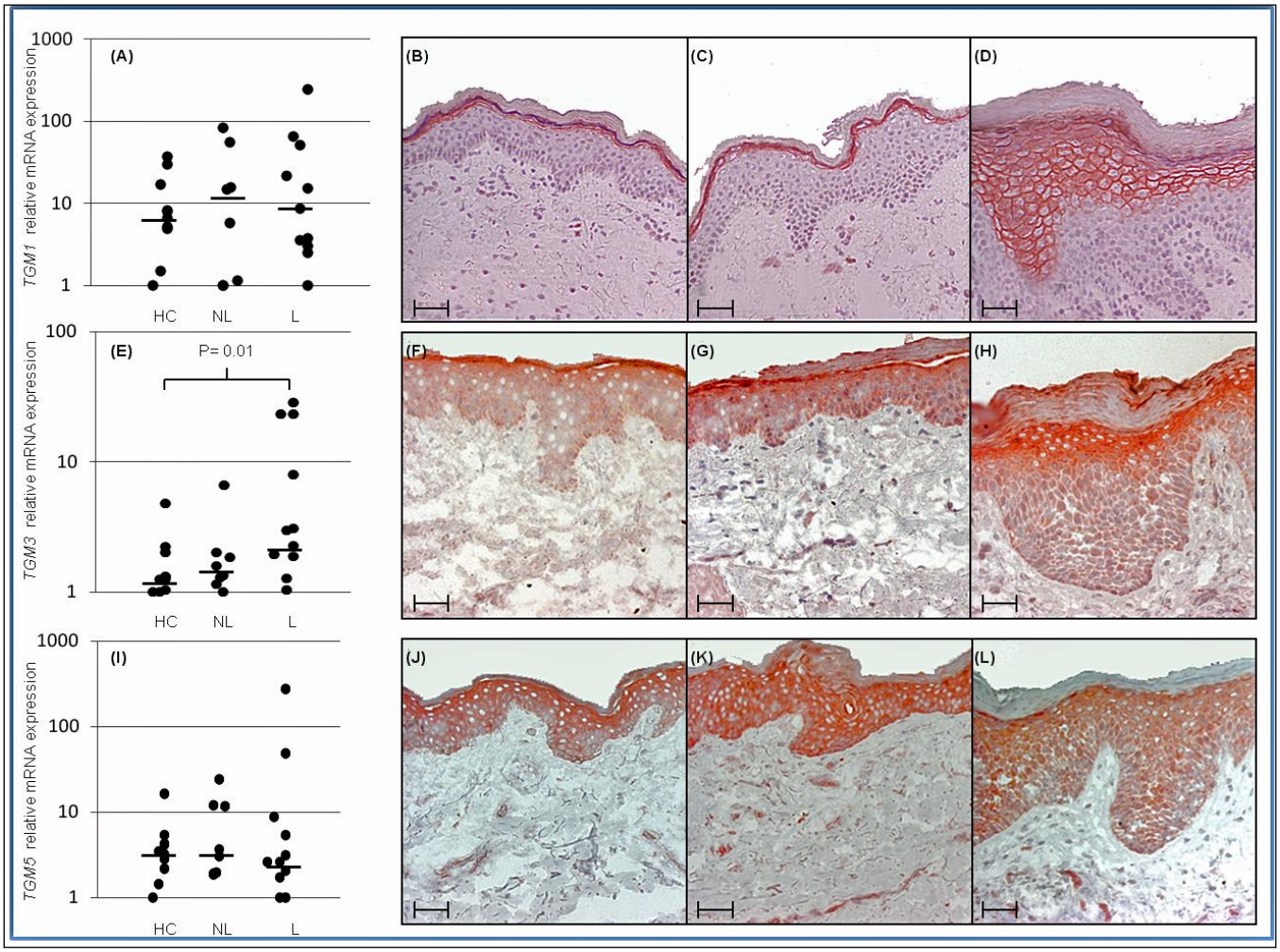

Figure 10: Analysis of the epidermal transglutaminases *TGM1* (A-D), *TGM3* (E-H) and *TGM5* (I-L) mRNA expression in the skin of healthy controls (HC; B;F,J) and the non lesional (NL;C,G;K) and lesional (L; D;H;L) skin of AD patients.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work in this thesis have aimed at further delineating the genetic factors underlying barrier dysfunction mainly in AD, but also at identifying whether AD and other common disorders of keratinisation may share genetic susceptibility factors. AD is a multifactorial, heterogeneous genetic disease resulting from the interaction of genes with environmental factors. The identification of genetic variants such as the *FLG* mutations underlying epidermal barrier dysfunction in patients with AD suggests that, in many cases, a primary breakdown in the skin barrier is the initial event in the development of the disease, followed by a triggered immune response. It has been shown that the spectrum of *FLG* mutations differ between populations. Our results indicate that *FLG* loss-of-function variant are less common in Ethiopian AD and IV patients, and other mechanisms are more important for the pathogenesis of IV and AD in this ethnic group. Such mechanisms could include other environmental or genetic triggers of the immune response and involve other barrier related genes. *FLG* mutations may also exist in the non-coding regions, or there may exist abnormalities in profilaggrin processing. Taking the distinct genotype-phenotype correlation between IV and *FLG* mutations in Europe and Asia into account, genetic associations may be found by analyzing additional IV patients, and test any findings in our AD case control material. Next generation sequencing can be used, by which larger genomic regions can be analyzed and possibly reveal additional genetic associations. It is also possible that IV is less common in the Ethiopian population, which needs to be determined by further epidemiological studies.

We have also genotyped the *FLG* gene in children with psoriasis to determine whether *FLG* mutations could be associated with childhood onset of psoriasis, as shown in AD. No association was found, and no novel mutations were detected, indicating that *FLG* mutations are not a strong modifying factor for the onset of psoriasis. To date, the strongest genetic association with psoriasis is with the *HLA-C* and *ERAP1* immune-regulatory genes, or markers in high LD with these genomic regions. Further sequencing of the *EDC* is needed to determine which other genetic variants than the *LCE3B/LCE3C* polymorphisms are contributors to the *PSORS4* association on chromosome 1q21, and if other epidermal barrier genes outside this locus may predispose to psoriasis susceptibility.

The most likely model for the development of AD is a gene and environmental dosage effect, wherein the combination of genetic and environmental factors determines the likelihood of developing the disease or determining disease severity. Our results have revealed several functional and molecular alterations *in vivo* in patients suffering from AD and IV depending on *FLG* genotype. Disease severity of AD, the gradient of TEWL and pH are increased in patients carrying *FLG* mutations. Furthermore, the number of genes with altered expression was significantly higher in patients with low or absent filaggrin expression. These pathways included many genes involved in inflammation, epidermal differentiation, lipid metabolism, cell signalling and adhesion.

As the epidermal transglutaminases, *TGM1*, *TGM3* and *TGM5* encodes important cross-linking enzymes in the epidermis and map in genomic regions that have previously been linked to AD and associated phenotypes, we characterized the expression and tested whether genetic variation influenced AD susceptibility. Our findings show that expression analysis links the *TGM1* and *TGM3* gene to the manifestation of AD. Increased expression of these enzymes may be a reflection of an altered barrier function and/or ongoing inflammation. Further, genetic analysis suggests that genetic variation at the

TGM1 locus could be involved in the development of the disease. Our results indicate that there is a haplotype, carried by ~19% of the individuals, that protects against the development of AD and allergen specific serum IgE. Independent sets of patients and controls are now needed to confirm the genetic association found in the AD family material and further studies, including sequencing, are needed to identify the actual disease causing variations.

The results of the work reported in this thesis has provided additional descriptive information and further elucidated the pathogenesis underlying AD, in particular in relation to filaggrin deficiency. However, the genetic basis for AD is still largely unknown, and next generation sequencing should provide important clues in the near future. It is possible that *FLG* will be used as a diagnostic tool for individualizing therapy. Studies are currently underway investigating the therapeutic potential of barrier enhancement such as emollients with SC lipids, and there is experimental evidence indicating that the *FLG* gene is amendable to up regulation. For AD, early preventive measures aiming at repairing the barrier might prevent an elicited immune response and subsequent atopic march.

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