Genetic Susceptibility Factors for Eczema

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"The darkest hour is just before dawn."

(Thomas Fuller)

Für meine Mutter

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Summary

Eczema (atopic dermatitis) is one of the most common chronic inflammatory skin diseases in infants and children with prevalence rates of up to 20%. The disease frequently co-occurs with other atopic disorders such as asthma and rhinitis and is often accompanied by elevated levels of immunoglobulin E (IgE) antibodies and aberrant IgE-mediated responses to otherwise harmless environmental agents.

Eczema is considered a polygenic disease, caused by a complex interplay of various predisposing genes, which additionally interact with environmental, non-genetic components.

This work focuses on the identification of genetic factors contributing to the aetiology of eczema. To this end several candidate gene association studies were performed and the first and to date only genome-wide association studies (GWAS) on eczema as well as on total serum IgE levels were conducted.

This thesis provides the first independent replication study on the filaggrin gene (*FLG*). *FLG* is located in the epidermal differentiation complex (EDC) on chromosome 1q21 and encodes a structural protein with key functions in the formation of the epidermal barrier. In a family-based approach it was clearly shown that the two loss-of-function mutations R501X and 2282del4 in this gene strongly predispose for eczema (Odds Ratio (OR) 2.73; *P*-value 5.1×10^{-8}). Along with a consistent and prominent association between these mutations and eczema subsequent investigations delineated their impact on distinct eczema subtypes as well as on eczema-related traits like asthma and hay fever. Strong associations with allergic sensitization (*P* 2.3×10^{-7}), increased total IgE (*P* 9.8×10^{-8}) and the atopic (OR 3.66; *P* 4.6×10^{-5}), but not the non-atopic form of eczema were observed. Both disease alleles predispose to the early-onset and severe form of eczema (OR 5.21 and 2.65; *P* 2.8×10^{-6} and 0.0043, respectively). It was demonstrated that mutant *FLG* alleles increase the risk for allergic rhinitis (OR 2.64; *P* 2.5×10^{-6}), and patients with *FLG*-related eczema were shown to be at higher risk to develop additionally allergic asthma (OR 3.49; P 1.0×10^{-5}).

Moreover, it was possible to evaluate the relevance of *FLG* risk alleles on the population level in a German cross-sectional study. On the basis of an observed carrier frequency of 7.4% for the four most prevalent mutations the Population Attributable Risk (PAR) was estimated as high as 13.5% and the penetrance reached 38.5%.

The remarkably strong effect of the *FLG* gene on eczema and asthma risk was finally confirmed with the help of a comprehensive meta-analysis based on 24 studies published until 2008. This analysis emphasizes the important role of *FLG* as the first validated and strong genetic risk factor for eczema, and provides a general measure of its impressive effect size. *FLG* mutant alleles were shown to cause a more than threefold increased eczema risk and

clearly convey predisposition to the particular phenotype of asthma occurring in the context of eczema, but apparently not to asthma independent of eczema.

These observations point towards a genetically disturbed epidermal barrier as key event in the pathogenesis of eczema and as risk factor for allergic sensitization and concomitant respiratory disease.

The first GWA study on eczema published in 2009 identified a novel susceptibility locus on chromosome 11q (OR 1.22; P 7.64×10^{-10}), as well as putative additional risk variants (OR 1.20; P 3.52×10^{-5}) apart from *FLG* within the EDC. The associated variant on chromosome 11 is located in an intergenic region between the two plausible candidates *C11orf30* (chromosome 11 open reading frame 30) and *LRRC32* (leucine rich repeat containing 32). Hence the gene affected by the identified risk allele on chromosome 11 is still elusive and subject of current investigations.

With the help of an independent GWAS on total IgE levels it could be shown that variants within the gene encoding the alpha chain of the high affinity receptor for IgE (*FCER1A*) strongly influence serum IgE levels (P 7.08×10^{-19}). Presence of the homozygous state of the rs2427837 minor allele was estimated to decrease serum IgE levels by 35%, and was strongly associated with decreased FCER1A cell surface expression on basophils. Concurrently, the study identified the cytokine cluster on chromosome 5q31 as a second susceptibility locus for altered IgE concentrations (P 6.28×10^{-7} - 4.46×10^{-8}).

Taken together, the discovery and comprehensive description of novel risk variants and potential susceptibility genes for eczema and related phenotypes change and complete our understanding of the complex aetiology of eczema and atopic diseases. Although to date immunological alterations have been the focus of pathophysiological considerations, new concepts recognize the impairment of the skin barrier as well as an abnormal reactivity of the adaptive and innate immune system as important factors.

Eczema causes significant problems in everyday life and has been shown to lead to decreased quality of life, vitality, and mental health of patients, concurrently constituting a high economic burden with direct and indirect costs similar to those of asthma. Hence, the development of new prognostic and therapeutic tools is of essential relevance. Knowledge about the genetic background of eczema translates into a clearer understanding of mechanisms of the disease and ideally into the future development and refinement of diagnostic and therapeutic strategies. The challenge of future studies will be the further dissection of the genetic architecture of eczema and the confirmation of observed relations between variations in the human genome and eczema by functional approaches in order to illuminate the pathophysiological nature of this complex disease.

Zusammenfassung

Das atopische Ekzem („Neurodermitis“, „atopische Dermatitis“) gilt mit einer Prävalenz von bis zu 20% als eine der häufigsten chronisch-entzündliche Hauterkrankung bei Säuglingen und Kindern. Die Krankheit tritt häufig in Kombination mit anderen atopischen Erkrankungen wie Asthma und Rhinitis auf und wird sehr oft von erhöhten Immunglobulin E (IgE) -Spiegeln im Serum und einer veränderten, IgE-vermittelten Immunantwort auf normalerweise harmlose Umweltagentien begleitet.

Das atopische Ekzem wird als polygene Erkrankung verursacht durch ein komplexes Zusammenspiel mehrerer prädisponierender Gene betrachtet, die zusätzlich mit nicht-genetischen Umweltkomponenten interagieren.

Diese Arbeit befasst sich mit der Identifizierung genetischer Faktoren, die ursächlich an der Entstehung des atopischen Ekzems beteiligt sind. Zu diesem Zweck wurden mehrere Kandidatengen-Assoziationsstudien durchgeführt und es erfolgten die ersten und bislang einzigen genomweiten Assoziationsstudien (GWAS) zum atopischen Ekzem sowie zum Serum-IgE-Spiegel.

Diese Doktorarbeit liefert die erste unabhängige Replikationsstudie zum Filaggrin-Gen (*FLG*). *FLG* ist im Epidermalen Differenzierungskomplex (EDC) auf Chromosom 1q21 lokalisiert und kodiert ein Strukturprotein mit Schlüsselfunktion bei der Entwicklung der epidermalen Barriere. In einer Familienstudie wurde klar gezeigt, dass die beiden Nullmutationen R501X und 2282del4 in diesem Gen stark für das atopische Ekzem prädisponieren (Odds Ratio (OR) 2.73; *P*-Wert 5.1×10^{-8}). Zusammen mit einem konsistenten und prominenten Zusammenhang zwischen diesen Mutationen und dem atopischem Ekzem wurde deren Einfluss auf bestimmte Subphänotypen des atopischen Ekzems und verwandte Erkrankungen wie Asthma und Heuschnupfen in weiterführenden Versuchen erfolgreich dargestellt. Signifikante Assoziationen mit allergischer Sensibilisierung (*P* 2.3×10^{-7}), erhöhtem IgE-Spiegel (*P* 9.8×10^{-8}) und der extrinsischen (OR 3.66; *P* 4.6×10^{-5}), nicht aber der intrinsischen Form des atopischen Ekzems wurden beobachtet. Beide Krankheitsallele prädisponieren zu frühem Krankheitsbeginn (<2 Jahre) und schwerer Ausprägung des Ekzems (OR 5.21 bzw. 2.65; *P* 2.8×10^{-6} bzw. 0.0043). Es konnte nachgewiesen werden, dass die mutierten *FLG* Allele das Risiko für eine Erkrankung an allergischer Rhinitis erhöhen (OR 2.64; *P* 2.5×10^{-6}) und Patienten mit *FLG*-bedingtem atopischen Ekzem außerdem ein höheres Risiko tragen, zusätzlich allergisches Asthma zu entwickeln (OR 3.49; *P* 1.0×10^{-5}).

Des Weiteren war es möglich, die Bedeutung der *FLG* Risikoallele auf Populationsebene in einer deutschen Querschnittsstudie aufzuzeigen. Auf Grundlage der beobachteten Trägerfrequenz von 7,4% für die vier häufigsten Mutationen lagen Schätzungen für das Populationsattributable Risiko (PAR) bei 13,5% und die Penetranz erreichte 38,5%.

Die außergewöhnliche Effektgröße dieses Gens in Bezug auf das Erkrankungsrisiko für atopisches Ekzem und Asthma wurde schließlich mit Hilfe einer umfassenden Metaanalyse, die auf 24 bis 2008 publizierten Studien basiert, bestätigt. Diese Analyse verdeutlicht die wichtige Rolle des *FLG*-Gens als erster validierter und starker genetischer Risikofaktor für das atopische Ekzem und liefert eine allgemein gültige Messung seiner beeindruckenden Effektgröße. Die mutierten *FLG*-Allele verursachen ein mehr als dreifach erhöhtes Risiko, an atopischem Ekzem zu erkranken, und sind für die deutliche Prädisposition zum spezifischen Phänotyp von Asthma im Kontext des atopischen Ekzems, nicht aber zu Asthma unabhängig vom atopischen Ekzem verantwortlich.

Diese Beobachtungen deuten auf eine Schlüsselfunktion der genetisch bedingten Störung der epidermalen Barriere in der Pathogenese des atopischen Ekzems und auf ihre Bedeutung als Risikofaktor für allergische Sensibilisierung und begleitendes Asthma hin.

Die erste GWAS zum atopischen Ekzem, die 2009 publiziert wurde, identifizierte einen neuen Suszeptibilitätslocus auf Chromosom 11q (OR 1.22; P 7.64×10^{-10}), sowie mehrere zusätzliche und von *FLG* unabhängige potentielle Risikovarianten im EDC (OR 1.20; P 3.52×10^{-5}). Das durch das identifizierte Risikoallel betroffene Gen auf Chromosom 11 ist noch unbekannt und Gegenstand gegenwärtiger Untersuchungen, da die assoziierte Variante in einer intergenen Region zwischen den beiden plausiblen Kandidaten *C11orf30* (chromosome 11 open reading frame 30) und *LRRC32* (leucine rich repeat containing 32) liegt.

Mit Hilfe einer unabhängigen genomweiten Analyse zum Serum-IgE-Spiegel konnte gezeigt werden, dass Varianten im Gen der α-Kette des hochaffinen IgE-Rezeptors (*FCER1A*) die IgE-Konzentration stark beeinflussen (P 7.08×10^{-19}). Schätzungen zeigten eine Senkung des IgE-Spiegels um 35% bei Vorhandensein des seltenen Allels von rs2427837 im homozygoten Zustand. Derselbe Genotyp war zusätzlich stark mit einer verringerten Expression von FCER1A auf der Oberfläche basophiler Granulozyten assoziiert. Gleichzeitig identifizierte die Studie das Zytokincluster auf Chromosom 5q31 als zweite Suszeptibilitätsregion für einen veränderten IgE-Spiegel (P 6.28×10^{-7} - 4.46×10^{-8}).

Die Entdeckung und Beschreibung neuer Risikovarianten und potentieller Suszeptibilitätsgene für das atopische Ekzem und verwandte Phänotypen verändert und erweitert unser Verständnis der komplexen Ätiologie des atopischen Ekzems und allergischer Erkrankungen. Nachdem bislang immunologische Veränderungen im Zentrum pathophysiologischer Überlegungen standen, sehen neuere Konzepte sowohl die funktionelle Einschränkung der Hautbarriere als auch eine veränderte Reaktionen des adaptiven und angeborenen Immunsystems als bedeutsam an.

Das atopische Ekzem führt zu signifikanten Problemen im Alltag, und betroffene Individuen leiden unter einer einschneidenden Verminderung der Lebensqualität, Vitalität und psychischen Gesundheit. Gleichzeitig stellt die Erkrankung durch direkte und indirekte Kosten,

ähnlich wie Asthma, eine hohe ökonomische Belastung des Gesundheitssystems dar, so dass der Entwicklung neuer prognostischer und therapeutischer Ansätze große Bedeutung zukommt. Das Wissen um die genetischen Hintergründe des atopischen Ekzems führt zu einem besseren Verständnis der Krankheitsmechanismen und damit zur Entwicklung und Verbesserung diagnostischer und therapeutischer Strategien. Die Aufgabe zukünftiger Studien zur weiteren Aufklärung der pathophysiologischen Beschaffenheit dieser komplexen Erkrankung besteht in weiteren Analysen der genetischen Architektur des atopischen Ekzems und der Bestätigung der beobachteten Zusammenhänge zwischen Varianten im menschlichen Genom und der untersuchten Krankheit durch funktionelle Ansätze.

Abbreviations

aa	amino acid
AE	atopic eczema
APC	antigen presenting cell
bp	base pair
C11orf30	chromosome 11 open reading frame 30
CD	Crohn's disease
CD14	monocyte differentiating antigen CD14
Chr	chromosome
CI	confidence interval
CMA1	mast cell chymase
CNV	copy number variation
CRNN	cornulin
DNA	deoxyribonucleic acid
EAACI	European Association of Allergy and Clinical Immunology
EDC	epidermal differentiation complex
EMSA	electrophoretic mobility shift assay
EOMES	eomesodermin
FCER1A	alpha polypeptide of the high affinity receptor I for the Fc fragment of IgE
FLG	filaggrin
FLG2	filaggrin 2
GARP	glycoprotein A repetitions predominant
GINI	German Infant Nutritional Intervention Study
GM-CSF	granulocyte-macrophage-colony-stimulating factor
GUCY2E	guanylat cyclase 2E
GWAS	genome-wide association study
HapMap	haplotype map
HGP	Human Genome Project
HRH4	histamine receptor H4
HRNR	hornerin
IF	intermediate filaments
IgE	immunoglobulin E
IL	interleukin
IL4RA	IL4 receptor alpha-chain
ISAAC	International Study of Asthma and Allergies in Childhood
IV	ichthyosis vulgaris
IVL	involucrin
kD	kilo Dalton
KLK7	kallikrein-related peptidase 7
KORA	Cooperative Health Research in the Region of Augsburg
LCE	late cornified envelope
LCR	locus control region
LD	linkage disequilibrium
LEKTI	lymphoepithelial kazal-type-related inhibitor
LISA	Influences of life-style related factors on the immune system and the development of allergies in childhood
lof	loss-of-function
LOR	loricrin
LRRC32	leucine rich repeat containing 32
LY86	lymphocyte antigen 86
MAF	minor allele frequency
MALDI TOF MS	matrix assisted laser desorption/ionisation time of flight mass spectrometry
Mb	mega base
MEF-2A	myocyte-specific enhancer factor 2A
Mio.	Million
NCBI	National Center for Biotechnology Information
NMF/NMS	natural moisturizing factors/substances
NOD	nucleotide-binding oligomerization domain protein
OR	odds ratio
PAD	pepdidyl-arginin deaminase
PAMP	pathogen-associated molecular pattern
PAR	population attributable risk
PBL	peripheral blood leukocyte
PCA	pyrrolidone carboxylic acid

PRKRIR	protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor)
PRR	pattern recognition receptor
RAD50	DNA repair protein RAD50
RANTES	regulated on activation, normal T-cell expressed and secreted
RFLP	restriction fragment length polymorphism
RHS	RAD50 hypersensitive site
RNA	ribonucleic acid
RPTN	repetin
RT-PCR	real time-polymerase chain reaction
S100A	S100 calcium binding protein A
S. aureus	staphylococcus aureus
SC	stratum corneum
SCCE	stratum corneum chemotryptic enzyme
SCORAD	score of atopic dermatitis
SCTE	stratum corneum tryptic enzyme
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SPINK5	serine protease inhibitor, Kazal type 5
SPR	small proline rich proteins
STAT6	signal transducer and activator of transcription 6
STR	short tandem repeat
TCHH	trichohyalin
TDT	transmission disequilibrium test
TEWL	transepidermal water loss
TGF-beta	transforming growth factor beta
TGM	transglutaminase
T_H1	T-helper cells 1
T_H2	T-helper cells 2
TLR	toll-like receptor
TSLP	thymic stromal lymphopoietin
UCA	urocanic acid
UCSC	University of California Santa Cruz
UK	United Kingdom
UTR	untranslated region
UV	ultraviolet
VNTR	variable number of tandem repeats
WAO	World Allergy Organisation

1 Introduction

1.1 Atopy and atopic diseases

Although the term “atopy” is relatively new, there are several historic documents which refer to “atopic” diseases. Over 2500 years ago, Hippocrates described a condition of an undetermined cause characterized as “itching over [one’s] whole body” (Smith 1994). Likewise, the Emperor Octavianus Augustus was reported to suffer from “extremely itchy skin, seasonal rhinitis and tightness of the chest” by Suetonius (Ring 2005). Formally, the term “atopy” (derived from Greek: “atopos”, “not in the right place”) was introduced 1923 by Coca and Cooke to describe some phenomena of hypersensitiveness in man (Coca and Cooke 1923). They considered ‘‘atopy’’ as a hereditary disorder clinically characterized by asthma or hay fever, which is associated with immediate-type (wheal-and-flare) skin reactions and which is different from ‘‘anaphylaxis’’ as a lack of protection (Portier and Richet 1902), and ‘‘allergy’’ as altered reactivity. Since Prausnitz and Küstner had already demonstrated the passive transfer of immediate hypersensitivity in man by serum (Prausnitz and Küstner 1921), Coca and Grove later designated the causative serum factor as “atopic reagins” (Coca and Grove 1925). In 1933, Wise and Sulzberger included eczema into the group of atopic diseases proposing the term ‘‘atopic dermatitis’’ to denote ‘‘confusing types of localised and generalised lichenification, generalised neurodermatitis or manifestation of atopy” (Wise and Sulzberger 1993). After the identification of “reagins” as immunoglobulin E (IgE) antibodies Pepys defined atopy as inherited tendency to produce increased amounts of IgE against low doses of environmental allergens (Pepys 1994). His definition therefore not implies the presence of clinical symptoms, but describes the patient’s immunologic reaction. Since these years, the definition of “atopy” has been a matter of controversy. Recently a working group of the World Allergy Organization (WAO) proposed the following definition of atopy “[…] a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema (Johansson, Bieber et al. 2004).

1.2 Clinical presentation and diagnosis of eczema

Eczema is a chronic inflammatory skin disease characterized by intense pruritus, dry skin, a relapsing course and typical age-related distribution of skin lesions. In infants, eczematous lesions usually appear on the scalp (milk-crust) and the cheeks, whereas during childhood the flexures, the dorsal parts of the limbs, and the nape are involved. In adolescent and adult patients the head, neck and flexures are often affected. Flares are characterized by oozing

erythematous patches and plaques. Furthermore, lichenification, recurrent superinfections and crusted erosions are common. A lot of patients show characteristic, but not specific atopic stigmata, like hyperlinearity of the palms ("ichthyosis hand"), Dennie-Morgan fold (groove of the lower eyelid), Herthoge's sign (lateral rarefaction of the eyebrow), and white dermographism (Weidinger and Ring 2006).

The majority of patients show an onset of disease in early childhood before the age of five, often referred to as "early-onset eczema" and a spontaneous remission in early adolescence (Williams and Strachan 1998), but the disease also can persist into or start in adulthood (late-onset eczema), making it one of the most common skin disorders throughout all ages.

Eczema frequently co-occurs with other atopic disorders such as asthma, rhinitis and food allergy, and is often marked by elevated levels of serum IgE and an abnormal IgE-mediated response to common allergens (Bieber 2008).

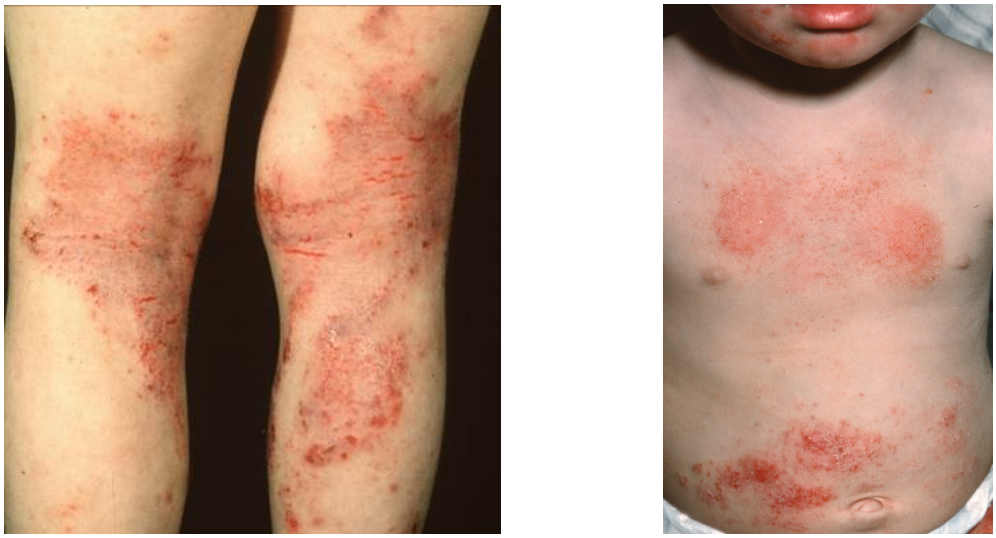

Figure 1: Flexural and abdominal eczema in a child and an infant, respectively. From (Weidinger and Ring 2006).

Thus, eczema shows a wide spectrum of clinical presentations both within and between patients, and it is still unclear whether it represents a single disorder with different clinical manifestations or a group of syndromes with unique or overlapping pathophysiological pathways and a rather uniform clinical presentation. Dermatological examinations of the specific morphology and distribution of skin lesions and further characteristics like the intense pruritus and the chronicity of symptoms are the basis for a medical diagnosis (Weidinger and Ring 2006). In the absence of adequate laboratory tests and specific histological features, numerous lists of diagnostic criteria have been developed in order to establish a definition. Above all, a clear-cut definition of eczema is extremely necessary for the implementation of reliable and reproducible epidemiological, clinical, and genetic research. At present, the United

Kingdom Working Party's diagnostic criteria have been most widely validated and appear to be applicable and repeatable across all ages and many ethnicities (Table 1).
However, as shown in a recent review, the ideal set of diagnostic criteria still has to be established (Brenninkmeijer, Schram et al. 2008).

Mandatory feature

- Itchy skin (within the last year)

In combination with at least three of the following features

- History of flexural dermatitis
- Visible flexural dermatitis
- Age of onset <2 years (for patients >4 years)
- History of atopy
- Xerosis

Table 1: United Kingdom Working Party's Diagnostic Criteria for the Diagnosis of Atopic Dermatitis.

1.3 Aetiology of eczema

Eczema is regarded as a multifactorial disease, which results from complex gene-environment interactions (Cookson 2001; Hoffjan and Epplen 2005). The variability of its clinical presentation between individual patients depends on a variety of different genetic and non-genetic determinants.
Environmental trigger factors such as aeroallergens like dust mite, animal dander, and pollen have been shown to provoke eczema flares and to influence eczema severity (Schafer, Heinrich et al. 1999; Capristo, Romei et al. 2004; Purvis, Thompson et al. 2005) and can provoke exacerbations in adult eczema. Allergic sensitization to food allergens, mainly to cow's milk and hen's egg, which is frequent in infant patients, is also associated with the severity of the disease (Hauk 2008). Further factors are recurrent bacterial, fungal and viral infections. Eczema patients often exhibit extensive colonization of the skin by bacteria and fungi such as Staphylococcus aureus and malassezia. An explanation for this observation might be the deficiency of antimicrobial peptides due to the inflammatory micromilieu in the skin of eczema affected individuals (Ong, Ohtake et al. 2002; Rieg, Steffen et al. 2005; McGirt and Beck 2006; Kisich, Carspecken et al. 2008). Especially persistent colonization with S. aureus is frequent in these individuals and might cause flares (Leung 2003; Elias and Schmuth 2009). Secretion of staphylococcal superantigens (S. aureus enterotoxins) activates macrophages and T cells and the production of enterotoxin-specific IgE antibodies in the host, which correlates with eczema severity (Leung 1993; Bunikowski, Mielke et al. 1999). Stress

(Schmid-Ott, Jaeger et al. 2001), autoreactivity to human proteins (autoantigens) (Natter, Seiberler et al. 1998; Mothes, Niggemann et al. 2005) and other irritant factors like sweat, soap or woolly clothing may also contribute to the pathogenesis of the disease.

Eczema is thought to be part of the syndrome of “atopic diseases”, which are characterized by the production of IgE antibodies to ubiquitous allergens. Immunological abnormalities in eczema patients involve a systemic IgE-mediated T-helper cell 2 (T_H2) dominated immune pattern together with a progressive shift from a T_H2 immune response in acute lesions to a T-helper cell 1 (T_H1) immune response in chronic lesions (Bieber 2008). For the majority of eczema patients IgE mediated allergies play an essential and disease maintaining role (atopic or “extrinsic” eczema). Affected individuals show high total serum IgE levels as well as specific sensitization against food and aeroallergens (Novak, Kruse et al. 2002; Novak and Bieber 2003) and an intranasal, bronchial or epicutaneous application of common aeroallergens can provoke or worsen eczematous skin lesions (Darsow, Vieluf et al. 1995).

Nevertheless, like for asthma bronchiale, there exists a significant amount of eczema patients without any sign of IgE-mediated sensitization. In particular children <2 years and persons with adult-onset eczema are affected by this “intrinsic” or non-atopic form of the disease, and present normal IgE serum levels without any sensitization to food or aero-allergens (Novak and Bieber 2003; Illi, von Mutius et al. 2004). However, children with early-onset non-atopic eczema often become sensitized during the course of the disease, several weeks or month after the first manifestation of skin lesions (Novak and Bieber 2003; Illi, von Mutius et al. 2004). The estimated frequency of the non-atopic form accounts for 16% - 45% of eczema patients (Novak and Bieber 2003). In two multi-centre, multi-national trials wide variation in the pattern of allergic sensitization between different countries was observed, with the highest numbers of sensitized infants in Australia (83%) and the United Kingdom (UK) (79%), a finding that reassembles prevalence for eczema (de Benedictis, Franceschini et al. 2009). There is still an ongoing discussion about the question if IgE is a primary causative factor in asthma and eczema or if it only represents an epiphenomenon of these phenotypes (Williams and Flohr 2006; Flohr, Weiland et al. 2008).

Due to the observation that eczema often precedes the subsequent manifestation of asthma, and since eczema has been shown to be a well-established risk factor for asthma, the concept of a progressive “atopic march” has been developed, i.e. that in susceptible individuals there is a sequential progression from eczema and food allergy to asthma and rhinitis (Wahn and von Mutius 2001; Spergel and Paller 2003; Spergel 2005). While sensitization represents a plausible link between eczema and asthma, recent research indicates that sensitization might as well be a shared epiphenomenon (Novak and Bieber 2003; Illi, von Mutius et al. 2004). Recently provided evidence for lung inflammation caused by epidermal produced thymic stromal lymphopoietin (TSLP) points towards alternative mechanisms apart from allergic

sensitization for the comanifestation of eczema and asthma (He, Oyoshi et al. 2008; Demehri, Morimoto et al. 2009; Zhang, Hener et al. 2009)

The complex pathophysiology of eczema is reflected by the confusing terminology including terms such as “atopic eczema”, “atopic dermatitis”, “childhood eczema”, “atopiform dermatitis” and “flexural dermatitis” frequently used synonymously in the literature. Following the recommendation for a standardized nosology for allergic diseases of the nomenclature committee of the WAO (Johansson, Bieber et al. 2004) and the task-force on nomenclature of the European Academy of Allergy and Clinical Immunology (EAACI) (Johansson, Hourihane et al. 2001), the term “eczema” now includes both subtypes and is used instead of atopic eczema/dermatitis. The term “atopic eczema (AE)” solely designates individuals with eczema and involved IgE (extrinsic form), whereas the rest, formerly known as “intrinsic” form, is referred to as “non-atopic eczema”. However, as pointed out, this division might not adequately reflect the natural history of this disease (Bieber 2008), and it has to be considered that so far most, if not all, studies on the genetics of eczema were performed prior to these suggestions. Additionally, most existing DNA collections have been assembled using older definitions.

To avoid confusion, in this thesis the nomenclature suggested by the WAO is used.

1.4 Epidemiology of eczema

The prevalence of eczema has nearly doubled in the last thirty years, and 15-30% of children and 2-10% of adults are affected nowadays (Williams and Flohr 2006; Stensen, Thomsen et al. 2008). Comprehensive global data on the prevalence of eczema symptoms has been provided by the International Study of Asthma and Allergies in Childhood (ISAAC), a systematic international record of the prevalence of asthma, eczema and rhinitis involving more than 100 countries and 2 Million children. The range of prevalence values for current eczema symptoms in the age groups 6-7 and 13-14 years varied from 0.9% in India and 0.2% in Tibet to 22.5% in Ecuador and 24.6% in Colombia, respectively (Odhiambo, Williams et al. 2009). Overall, in the age group of 6-7 years high prevalence values (≥15%) for current eczema symptoms were commonly found more often in study centres in Oceania (Australia, New Zealand), whereas generally lower values (<5%) were reported for the Indian subcontinent, the Eastern Mediterranean Region and Northern and Eastern Europe. In the age group of 13-14 year old children Africa and Oceania showed overall higher rates for current eczema prevalence in contrast to the Indian subcontinent and participating centres in Northern and Eastern Europe with generally low values. Germany itself ranges in the middle of ranked prevalence rates with values of approximately 8% for current eczema symptoms in both age groups (Odhiambo, Williams et al. 2009).

However, prevalence differed strongly between different nations and even between particular study centres within the same country or geographical region. The observed differences in international and national patterns illustrate the diversity in the global prevalence of this disease and shows that eczema represents a major public health issue not only in industrialized but also in developing countries.

A widely favoured explanation for the rising prevalence is the so-called “hygiene-hypothesis”, which is based on the epidemiological observation of higher prevalence of eczema in urban compared to rural areas. Infections and exposition to microbial antigens during early childhood seem to have a protective influence on the formation of eczema and atopic disease (Strachan 1989; Braun-Fahrlander and Lauener 2003; Zutavern, von Klot et al. 2006) and a relation between the growing prevalence and the higher living standard and improved hygienic conditions resulting in less frequent parasitic infestation is assumed (Schafer, Meyer et al. 2005).

1.5 Evidence for a genetic basis of eczema

There is a longstanding recognition that asthma, eczema, and rhinitis cluster in families (Sneddon 1951; Schaffer 1966), which suggests a strong influence of genetic risk components. Nearly one century ago Cooke and van der Veer already suggested a genetic component after making the observation of an increased incidence for atopic diseases in first degree family members (Cooke and Van der Veer 1916). Parental atopy, in particular eczema, is strongly associated with an increased risk for the offspring to develop the disease (Dold, Wjst et al. 1992; Wadonda-Kabondo, Sterne et al. 2004). The strongest evidence for the importance of genetic factors in atopic disease stems from twin studies. Mono- and dizygotic twins are normally exposed to an identical environmental milieu and life style within their family, thus providing the possibility of measuring genetic influences broadly independent from environmental factors. The concordance rate for eczema among monozygotic twins (0,72-0,86) has been shown to be significantly higher than for dizygotic twins (0,15-0,23), and segregation analyses indicated that genetic components account for more than 70% of the variance in susceptibility to the disease (Larsen, Holm et al. 1986; Schultz Larsen 1993; Thomsen, Ulrik et al. 2006; van Beijsterveldt and Boomsma 2007).

Eczema is considered a poly- or oligogenetic disease, resulting from a complex interplay between various susceptibility loci in the genome. Unlike monogenic disorders, which are caused by mutations in a single gene, eczema seems to arise on the basis of variations in a variety of different genes, which do not follow a Mendelian mode of inheritance (Glazier, Nadeau et al. 2002). Additionally, parent - of - origin effects have been attributed to eczema. Different studies suggested that maternally transmitted alleles are more likely to be associated

with disease in the offspring than inheritance from the father (Walley, Chavanas et al. 2001; Weidinger, Baurecht et al. 2008).

1.6 Methods used in the genetic dissection of eczema

The human DNA sequence between two individuals differs only by approximately 0.1%. These differences are mainly based on four types of polymorphic variants: single nucleotide polymorphisms (SNPs), microsatellites (short tandem repeats, STRs), restriction length fragment polymorphisms (RLFPs) and a variable number of tandem repeat polymorphisms (VNTRs). These polymorphic markers, which not necessarily contribute directly to a certain disease, are commonly used as proxis for susceptibility loci, and have become the genetic tool in the dissection of heritable diseases.
In general, there are two major approaches to identify genetic variants associated with susceptibility to complex traits: linkage analysis and association studies.

1.6.1 Whole-genome linkage studies

Genome-wide linkage analysis is a family-based method. Highly polymorphic genetic markers, usually microsatellites, which are evenly distributed across the genome, are used to analyze co-segregation with the disease in a collection of independent individuals and their parents or within a pedigree of many generations. The most common approach is the linkage analysis of affected sib pairs. To determine the proportion of parental alleles shared at each marker pairs of siblings having a certain disease are genotyped. If there is a significant difference in the ratio of shared marker alleles between affected siblings under the assumption of no linkage, the region surrounding the marker is linked to the disease. One advantage of this method is the independency of any hypothesis, and due to the highly polymorphic nature of microsatellites it requires only a small number of markers. On the other hand it is not possible to identify specific genes as the linked regions typically encompass several mega bases of the genome and might include hundred of different genes. To narrow down the region of peak linkage, follow-up analyses including positional cloning with high-density sets of microsatellite markers and SNPs have to be conducted. Linkage analyses have been very successful in the identification of monogenic disease genes (McKusick 2007), but they have limited power to detect small risk genes for complex traits due to epistasis, incomplete penetrance, polygeneity or phenotypic heterogeneity. None of the genome-wide linkage studies on eczema performed to date has led to the identification of a validated disease gene (see chapter 1.7 Results from whole-genome linkage studies in eczema).

1.6.2 Association studies

With the first publication of the Human Genome Project (HGP) in 2001 (McPherson, Marra et al. 2001), the determination of the sequence of 3 billion base pairs representing the human genome - including newly identified polymorphisms - and their publication in public databases it became possible to analyze markers in distinct genes of interest by genetic association studies. This approach is not, like linkage studies, limited to a family-based study design, but can also analyze the distribution of possible disease markers in unrelated and disease affected individuals compared to healthy control subjects (case-control study design) (Altshuler, Daly et al. 2008). An association between the genotype and the phenotype is assumed if the genetic marker and the disease occur together more often than expected by chance. Association studies provide a more powerful tool for detecting common disease alleles, even if conferring only a modest disease risk (Risch and Merikangas 1996).

The most common form of genetic variations detected by the HGP are SNPs, which represent over 80% of the genetic variation between individuals. A SNP is a site where a single base substitution occurs at a frequency of at least 1% in the population. Approximately one out of every 200 base pairs in the human genome is a SNP. These variants are commonly biallelic and less polymorphic as microsatellites, but they are much more frequent and more easily scored with automatic methods than the latter. Moreover, SNPs often occur in coding (gene) regions of the genome. Terms to classify SNPs due to their genomic position are coding and non-coding, non-synonymous (amino acid change) and synonymous (no amino acid change). The majority of SNPs are non-functional and therefore result in neutral phenotypic outcomes. However, a subset of SNPs can predispose individuals to disease, or influence its severity, progression or individual response to medicine. At the molecular level, these functional SNPs can affect the human phenotype by interfering on both levels of the protein synthesis machinery: non-coding SNPs may disrupt transcription factor binding sites, splice sites and other regulatory elements on the transcriptional level, whereas coding SNPs can cause an amino acid change and alter the functional or structural properties of the translated protein. Together with DNA copy number variations (CNVs), SNPs have become one of the most actively researched areas of genomics in recent years (Feuk, Carson et al. 2006). Until recently, based on technical limitations, association studies have been focused on the analysis of candidate genes, which are selected because of their possible involvement in disease pathogenesis due to their function or because of their location in regions with observed linkage in prior genome-wide linkage scans (positional/functional candidate genes) (Tabor, Risch et al. 2002). However, candidate gene studies are prone to type I (false positive results) and type II errors (false negative results), and an appropriate and stringent study design including independent replication of results is of great importance to achieve solid results. There are

many potential pitfalls in candidate gene association studies such as a lack of power to detect a true association (too small sample size), clinical heterogeneity of the trait in question, which leads to an inhomogeneous study cohort, inappropriate selection of control individuals or the general use of inappropriate statistical models and correction methods for multiple testing (Glazier, Nadeau et al. 2002; Lohmueller, Pearce et al. 2003; Rogers, Raby et al. 2009). Generally, case-control designs are prone to confounding because of population stratification, i.e. ethnic/racial heterogeneity of study subjects (Ober and Hoffjan 2006). Population stratification describes the mixture of two or more subpopulations within a study cohort. If these subpopulations differ in allele frequencies, stratification might lead to false positive results (Figure 2). The Transmission-Disequilibrium-Test (TDT) based on family collections (case-parent trio design) has the advantage of being unaffected by population stratification. Provided that at least one of the parents is heterozygous for a given allele, the TDT analyses if this allele is more often transmitted than untransmitted to the affected offspring (Knapp 1999). Because non-transmitted parental alleles serve as internal controls, reliable ethnic homogeneity is given (Spielman, McGinnis et al. 1993).

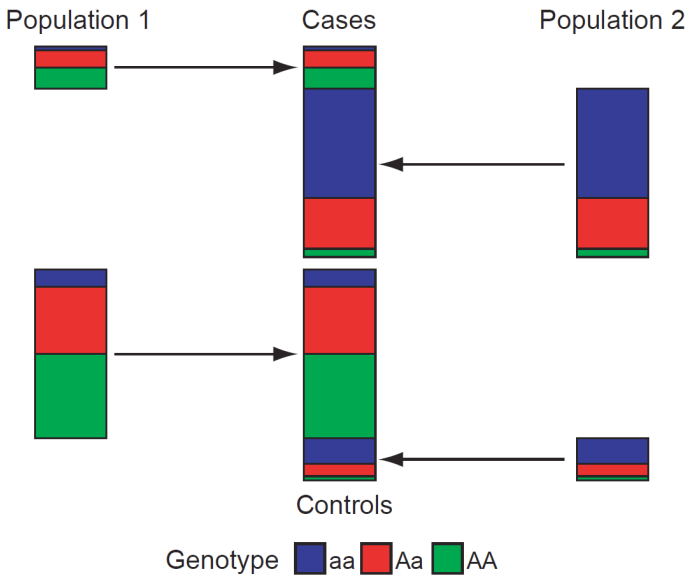

Figure 2: The effect of population stratification at a SNP locus. If the sample group under examination consists of two genetically inhomogeneous subpopulations, allele frequencies differ between cases and controls without true association between genotype and phenotype. In the given example proportion of population 2 is higher within the case group compared to controls, and consequently allele frequency of genotype “aa” is also higher. From (Marchini, Cardon et al. 2004)

Candidate gene association studies have been supplemented lately by genome-wide association approaches. Genotyping-data from the International Haplotype Map (HapMap) Project (International HapMap Consortium 2005), which has been extended quite recently by resequencing-data of the 1000 Genomes Project (http://www.1000genomes.org) together with

the development of chip-based high-throughput genotyping platforms make it now possible to analyze up to 2,5 Million SNPs simultaneously and hypothesis-free. And the next generation of genotyping microarrays has already been announced, allowing the analysis of up to 5 Mio. variants per human sample. As a number of 17.9 Mio. SNPs in the human genome is annotated in public data bases (ensemble release 58, GRCh 37, http://www.ensembl.org/Homo_sapiens/Info/StatsTable), only methodological problems limit the coverage, and there are discussions about the 1000$ genome including genetic variations *in toto* (Bennett, Barnes et al. 2005; Service 2006; von Bubnoff 2008).

One important observation of the HapMap project revealed the genome to be organized in blocks of SNPs in strong linkage disequilibrium (LD) with one another. So far the majority of GWAS are based on a limited set of SNPs, which is supposed to transfer additional information on other common polymorphisms in the human genome through LD-based tagging. LD refers to non-independent inheritance of alleles, e.g. SNPs, at two or more loci, not necessarily located on the same chromosome. This non-random association of different alleles normally located at two different sites nearby each other (Wall and Pritchard 2003) is mainly influenced by the rate of recombination between these two sites, but also by mutation and genetic drift. The degree of LD is normally higher for shorter distances between two alleles, since the distance is directly linked to the probability of recombination events occurring between the two loci.

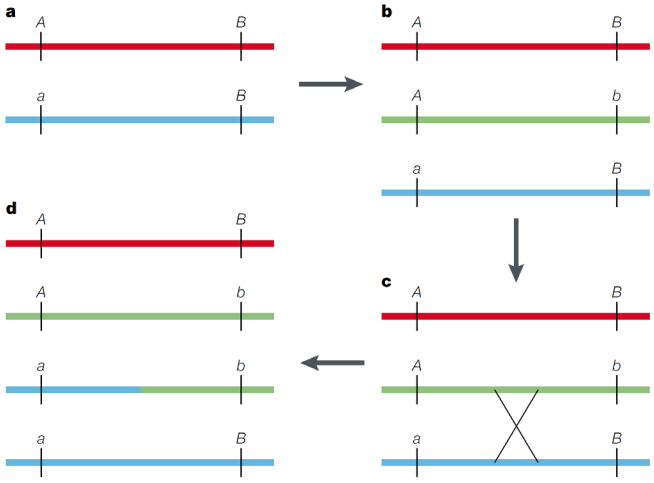

Figure 3: Schematic illustration of formation and erosion of LD structures. (a) shows a polymorphic locus with alleles A and a. (b) With the occurrence of a new SNP (Bb) at a nearby site, the mutant allele b is located on a single chromosome, which additionally bears either allele A or a of the first locus. In the illustrate example the new haplotype Ab (green chromosome) originates. Hence, allele b only occurs in combination with allele A. (c) Due to recombination the fourth possible haplotype is created (d), association between the two loci is disrupted and LD among the markers declines with increasing frequency of the recombinant chromosome in the population. From (Ardlie, Kruglyak et al. 2002)

Common statistical measurements for LD are D' and r^2 ranging between values 0 and 1, with 0 indicating complete equilibrium or independent transmission and 1 complete disequilibrium or dependent transmission. The r^2 measure is called correlation coefficient and depends, in contrast to D' additionally on the frequency of alleles. The LD-structure in a region of interest, e.g. a gene, is essential for genetic association approaches. Two or more SNPs showing a high r^2 value are supposed to provide redundant information in terms of the individual genotype, and allow a maximal coverage of interesting genes with a minimal effort in experimental genotyping.

For the same reason declarations about associations between certain SNPs and certain phenotypes have to be handled with care, as the variant showing association might not be itself the causative variant with functional effect, but only in LD with the latter.

GWAS have already led to the identification of numerous new and robust susceptibility loci for many complex diseases (Frazer, Murray et al. 2009) and represent a very useful tool for the further dissection of complex traits. However, GWAS, like candidate gene association studies, are always dependent on an appropriate study design in terms of study sample size, adequate selection of cases and controls, stringent statistical analysis and reproducibility of results.

1.7 Results from whole-genome linkage studies in eczema

Up to date six genome-wide linkage scans for eczema have been reported pointing towards several possible eczema-linked regions. Major findings were a region on chromosome 3q21 detected in families of mainly German origin (Lee, Wahn et al. 2000) and the loci 1q21 and 17q25 strongly linked to eczema in a British population (Cookson, Ubhi et al. 2001). On chromosome 1q21 resides the EDC and the Keratin-Type I-cluster is located on chromosome 17q25. Both regions harbour gene clusters known to cause genodermatoses through perturbance of epidermal differentiation and function (Irvine and McLean 2003). Further studies in Swedish, Danish and Japanese families on genome wide linkage reported additional findings for 3p24-22, 18q21, 3p26-24 and 15q21 (Bradley, Soderhall et al. 2002; Haagerup, Bjerke et al. 2004; Enomoto, Noguchi et al. 2007). Interestingly, for the majority of regions linkage to psoriasis, a chronic inflammatory disorder of the skin, which is rarely linked with eczema, rather than to allergic asthma has been reported (Cookson, Ubhi et al. 2001).

With the exception of *FLG*, which partly underlies the significant linkage to chromosome 1q21 (Morar, Edster et al. 2007), the responsible disease genes for the remaining loci are still not known.

1.8 Results from candidate gene association studies in eczema

Based on the assumption that the primary defect in eczema is immunological and prior to the discovery of *FLG (*see below), for many years candidate genes involved in immune regulation and signalling have been investigated, such as genes encoding proinflammatory molecules, which are thought to promote T_H2 immune responses, as well as genes that had been associated with asthma and/or atopy.

To date, more than 40 associated candidate genes have been reported in literature (Barnes 2010), but only very few proposed associations could be replicated in at least one independent study, and even for replicated genes at the same time a considerable number of negative reports exists (Table 2).

In contrast to these mainly immunological susceptibility genes only filaggrin, appears to present a strong and consistent eczema risk gene. The first report of association between *FLG* and ichthyosis vulgaris (IV) appeared in 2006 (Smith, Irvine et al. 2006), followed by the identification of several loss-of-function mutations in patients with eczema (Palmer, Irvine et al. 2006). In the meantime an impressive series of more than 20 independent replication studies conducted subsequently after the first replication included in this thesis was performed (Table 2). Scientific evidence for this gene as one of the strongest risk factors for a complex allergic disease is overwhelming. Due to the discovery of *FLG*, susceptibility genes for eczema are now divided into two major functional groups: genes contributing to epidermal or epithelial structures and genes encoding immune regulatory proteins:

1.8.1 Immunoregulatory genes

Genes involved in immune dysregulation commonly fall into the two major groups of antigen presentation and cell-mediated/humoral immune response, and cell signalling/cellular movement. Several candidate genes have been identified in the cytokine cluster on chromosome 5q31-33, which contains a family of functionally related cytokines regulating IgE-synthesis, like interleukin (IL)-3, IL-4, IL-5, IL-13 and the granulocyte-macrophage-colony-stimulating factor (*GM-CSF*). IL-4, IL-5 and IL-13 are produced by T_H2 cells, and the latter two cytokines are known to increase the production of IgE.

One of the most consistent associations has been reported for ***interleukin-13***, where two functional polymorphisms in the promoter region and in exon 4 have been associated with a variety of atopy-related disorders. IL-13 has been shown to be overexpressed in eczema lesions, especially in the non-atopic form (Jeong, Ahn et al. 2003; Tazawa, Sugiura et al. 2004).

Observed increase in the transcriptional activity of ***interleukin-4*** due to a mutation (-590 C/T) in the promoter of this gene seems to predispose for eczema (Kawashima, Noguchi, Arinami, Yamakawa-Kobayashi et al. 1998), too. Association with a gain-of-function mutation in the α-subunit of the ***IL-4 receptor*** (IL4RA) located on chromosome 16q12 additionally could enhance IL-4 mediated effects (Hershey, Friedrich et al. 1997).

Mutations in the promoter region of ***RANTES*** (regulated on activation, normal T-cell expressed and secreted) and association with eczema has been reported by different studies (Nickel, Casolaro et al. 2000; Bai, Tanaka et al. 2005; Tanaka, Roberts et al. 2006). This chemokine is located within the C-C chemokine cluster on chromosome 17q11-12, and belongs to the family of chemotactic cytokines, small signalling proteins regulating leukocyte trafficking at site of inflammation. RANTES has been shown to be overexpressed in lesional skin of eczema patients (Kato, Pawankar et al. 2006) and attracts monocytes, eosinophils, basophils and lymphocytes.

CMA1 encoding the enzyme mast cell chymase, is located on chromosome 14q11.2 and represents the major secreted serine protease of mastcells. Together with histamine and tryptase it seems to play a key role in inflammation, bronchial hyperresponsiveness and tissue remodelling and is therefore an excellent candidate gene not only for atopy but also for eczema. Association between variants in the promoter region of *CMA1* and eczema have been reported in several studies (Mao, Shirakawa et al. 1996; Mao, Shirakawa et al. 1998; Tanaka, Sugiura et al. 1999; Weidinger, Rummler et al. 2005) and an elevated expression level has been noted in lesional and non-lesional skin of affected individuals (Jarvikallio, Naukkarinen et al. 1997; Badertscher, Bronnimann et al. 2005).

NOD1 (nucleotide-binding oligomerization domain protein 1, also designated *CARD4*) is located on chromosome 7p14-15, belongs to the family of so-called pattern-recognition receptors (PRRs) and responds to pathogen-associated molecular patterns (PAMPs) as part of the innate immunity host defence. This receptors are either localized in the cell membrane like e.g. Toll-like receptors (TLRs) or CD14 or cytosolic proteins like NOD1 and NOD2 (Inohara, Ogura et al. 2001; Athman and Philpott 2004). Association with eczema and/or increased total serum IgE levels has been reported for different variants in this gene (Weidinger, Klopp et al. 2005).

1.8.2 Epithelial barrier genes

Another involved gene in the cytokine cluster (5q32), which does not belong to immunological candidates, seems to be ***SPINK5*** (serine peptidase inhibitor, Kazal type 5)***,*** which encodes a putative serine protease inhibitor called LEKTI (or LETK1; Lympho-Epithelial Kazal Type Inhibitor). The protein was shown to be expressed, amongst others, in the epidermis, mucosal

epithelia and organs of the immune system like thymus and lymph nodes (Magert, Kreutzmann et al. 2002). LEKTI appears to be responsible for the inhibition of several serine proteases like Trypsin, Plasmin, Kathepsin G and human neutrophilic elastase, and additional points of attack like allergens with serine protease activity (pollen, components of faeces of house dust mite), microbial proteinases, and mastcell tryptase are under discussion (Walley, Chavanas et al. 2001; Magert, Kreutzmann et al. 2002; Bitoun, Micheloni et al. 2003; Mitsudo, Jayakumar et al. 2003). Most importantly, it is supposed to interact with the stratum corneum chymotryptic enzyme (SCCE) (Komatsu, Takata et al. 2002) and the stratum corneum tryptic enzyme (SCTE) that are involved in desquamation and inflammation. Both enzymes exhibit an increased expression in eczema patients probably pointing out to a shift in the equilibrium between these proteases and their inhibitor LEKTI. Of note, proteolytic activation of profilaggrin, the precursor of filaggrin, by serine proteases is supposed to be regulated by LEKTI. *SPINK5* was identified by Chavanas et al (Chavanas, Bodemer et al. 2000; Chavanas, Garner et al. 2000) as the cause of Netherton syndrome, an autosomal-recessive disease marked by an epidermal barrier disruption and different polymorphisms have been shown to be associated with eczema and asthma in combination with eczema (Walley, Chavanas et al. 2001; Kato, Fukai et al. 2003; Nishio, Noguchi et al. 2003; Kabesch, Carr et al. 2004).

Filaggrin (*FLG*) is located within the EDC on chromosome 1q21.3, a dense cluster of approximately 50 genes mainly encoding proteins involved in the terminal differentiation of the epidermis (Toulza, Mattiuzzo et al. 2007). As already mentioned before, chromosome 1q21 has been shown to be linked to eczema, psoriasis and IV, the latter of which are two common monogenic keratinisation disorders, which are characterized, like eczema, through dry skin and stratum corneum (SC) and permeability barrier abnormalities (Linde 1992; Tabata, Tagami et al. 1998; Walley, Chavanas et al. 2001). *FLG* seems to be the susceptibility gene underlying the linkage signal on chromosome 1q21, and until now, represents the only known responsible disease gene for observed linkage with eczema.

The denomination filaggrin (filament aggregating protein) for a protein isolated from stratum corneum and interacting with intermediate filaments (IF) appeared on the scientific scene in 1981 (Steinert, Cantieri et al. 1981). FLG represents a structural protein with key functions in the formation and maintenance of the cornified envelope, a fact that makes it indispensable for preventing the penetration of environmental agents like microbes or allergens into the organism and, on the other hand, for controlling transepidermal water loss (TEWL) (Candi, Schmidt et al. 2005; Proksch, Brandner et al. 2008). Profilaggrin is expressed as a ~400 kilo Dalton (kD) inactive precursor protein in the stratum granulosum of the epidermis (Figure 4).

It's most important feature, exon 3, is composed of tandem repeats, encoding the functionally active FLG monomers. These FLG repeats are identical in size (324 amino acids (aa)), but show some differences in composition ((Gan, McBride et al. 1990). Three different alleles with

10, 11 or 12 repeats have been shown to exist in the general population (Smith, Irvine et al. 2006). Upon cornification of keratinocytes profilaggrin is proteolytically cleaved into 37 kD filaggrin subunits. These monomers lead to a dense bundling of keratin intermediate filaments, flattening the keratinocytes during their differentiation into corneocytes (Presland, Haydock et al. 1992) (Figure 5). Concurrently, the amino (N)-terminus of the protein is translocated into the cell nucleus, where it supposed to be responsible for apoptotic processes (Ishida-Yamamoto, Takahashi et al. 1998; Pearton, Dale et al. 2002).

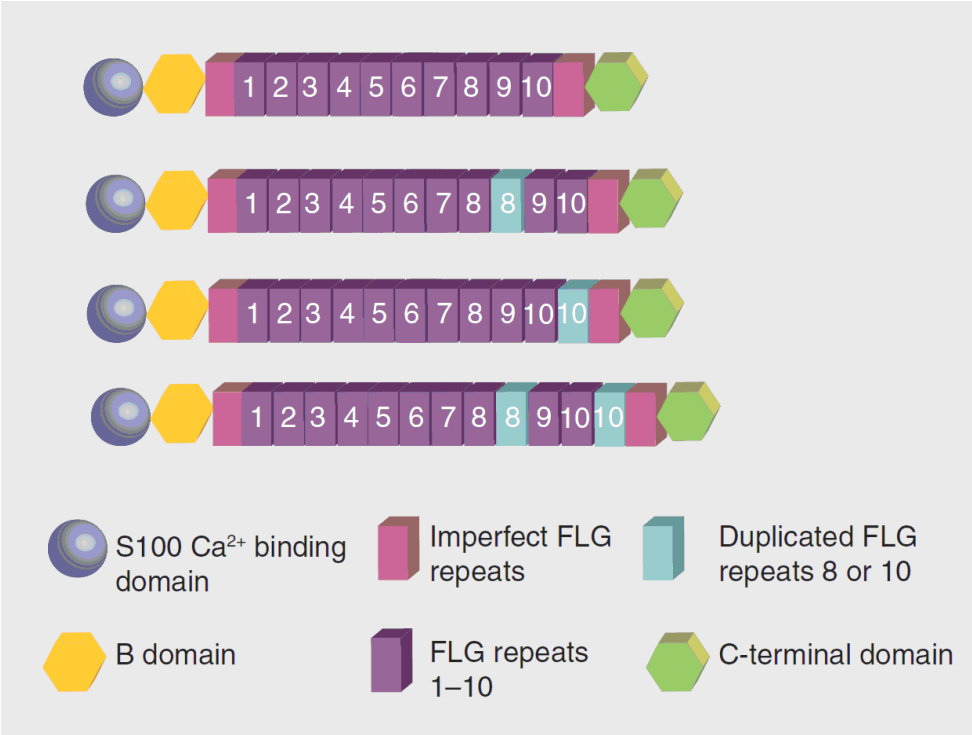

Figure 4: Structure of Profilaggrin: The N-terminus consists of two distinct domains: a conserved S100-like domain and a less conserved cationic B-domain followed by an imperfect FLG-unit and 10-12 almost identical FLG monomers. The C-terminus contains a truncated FLG-unit and a unique peptide of 23 amino acids. There are different size variants of the human *FLG* gene in the population, which have resulted from duplications of repeat 8 and/or repeat 10. It is likely that size variants involving other repeats also exist. A lower number of repeats has been reported to be associated with dry skin (Ginger, Blachford et al. 2005). From (Rodriguez, Illig et al. 2008).

Together with the lipid envelope, the cornified envelope is formed constituting the mortar and brick structure of the epidermis. Additionally, degradation products of filaggrin, free hygroscopic amino acids and their derivates, are thought to serve as natural moisturizers of the skin (NMF, natural moisturizer factors) (reviewed in (Rodriguez, Illig et al. 2008)) and possibly as protection against ultraviolet (UV) radiation (Sandilands, Sutherland et al. 2009).

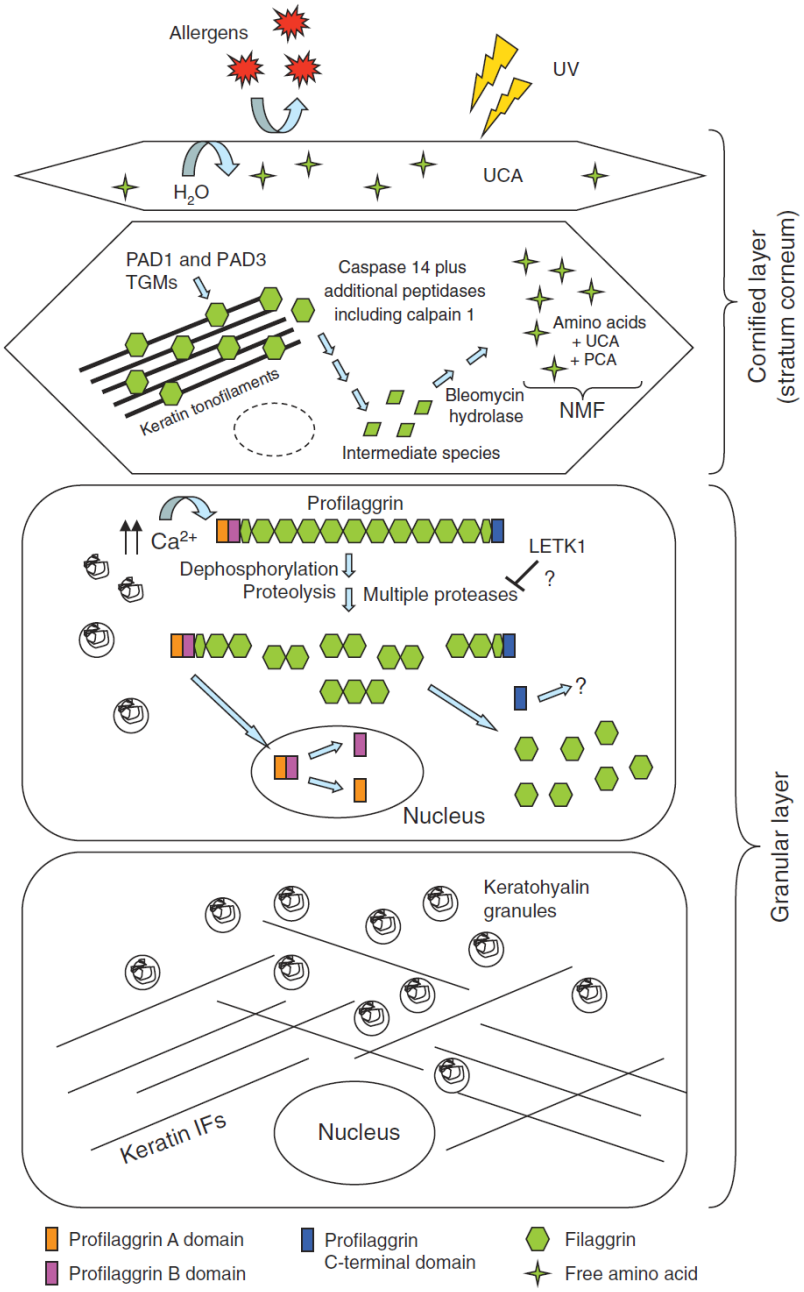

Figure 5: Profilaggrin, the precursor of filaggrin, is expressed in the granular layer of the skin and stored in its insoluble form in keratohyalin granules. Upon an increase of the intracellular Ca^{2+}-level during differentiation of keratinocytes in the stratum granulosum of the epidermis, the granules release the precursor protein, which is subsequently dephosphorylated and proteolytically cleaved into functionally active monomers. The functional FLG subunits lead to an aggregation of keratin intermediate filaments, thereby promoting transformation of keratinocytes into corneocytes, which constitute the stratum corneum. The insoluble keratin matrix is formed by crosslinking of the cytoskeleton by transglutaminases (TGMs). Deimination via pepdidylarginine-deiminases (PADs) and conversion of arginine into citrullin residues promotes further degradation. Subsequent proteolysis by different proteases produces a pool of hygroscopic amino acids and their derivates, like pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA) (from (Sandilands, Sutherland et al. 2009)).

Although more than 20 years ago FLG was suspected for the first time to be involved in different keratinisation disorders like IV, and the finding that its expression is clearly reduced in the epidermis of individuals with this disease (Fleckman, Dale et al. 1985; Sybert, Dale et al. 1985; Fleckman, Holbrook et al. 1987; Nirunsuksiri, Presland et al. 1995; Fleckman and Brumbaugh 2002) in depth analysis of this gene revealed to be quite difficult because of its repetitive structural nature (Sandilands, Terron-Kwiatkowski et al. 2007). In 2006 Irwin McLean and his group finally managed to comprehensively sequence the entire *FLG* gene and to show several mutations to be the cause of IV (Sandilands, O'Regan et al. 2006; Smith, Irvine et al. 2006). Eczema and IV, which is supposed to be the most frequent monogenic skin disorder based on abnormal epidermal differentiation, share a lot of clinical characteristics and are associated with each other (Wells 1966). Subsequently the same group detected significant associations between the same variants and eczema in independent, but relatively small and on the basis of asthma recruited cohorts (Palmer, Irvine et al. 2006).

The great majority of the more than 30 identified variants in the *FLG* gene have a frequency of less than 1% or are family specific/private mutations. The two most prevalent null mutations R501X and 2282del4 show carrier frequencies of <6% in different independent German population-based studies (Novak, Baurecht et al. 2007; Chapter 2.3).

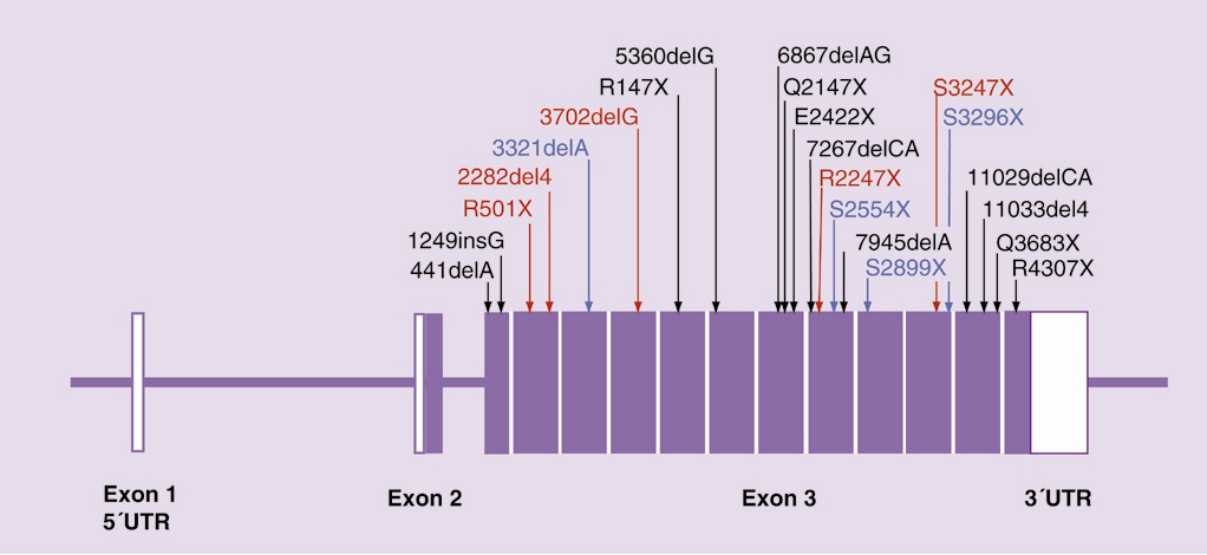

Figure 6: Structure of the human *FLG* gene. Like other members of the S100-'fused type' gene family *FLG* shares a conserved gene structure consisting of three exons. Exon 1 consists of untranslated sequence only, exon 2 contains the start codon and the unusually large exon 3 (12753bp) encodes most of the N-terminal domain and 10-12 filaggrin monomers. Most prevalent mutations are depicted in red, family specific or private mutations in black and variants detected in Asian populations only in blue. Not all mutations identified up to date are included for better overview. From (Rodriguez, Illig et al. 2008).

Interestingly, frequency appears to increase from South to North Europe, which might reflect ethnic differences. In populations from UK and Ireland *FLG* lof-mutations seem to be more prevalent compared to continental Europe (Smith, Irvine et al. 2006; Barker, Palmer et al. 2007; Sandilands, Terron-Kwiatkowski et al. 2007; Brown, Relton et al. 2008; Rice, Patel et al. 2008; Weidinger, Baurecht et al. 2008) but extremely low in an Italian case-control population, which represents the only analysis in South European individuals and furthermore the only negative association study on *FLG* and eczema published so far (Giardina, Paolillo et al. 2008). Until quite recently, none of the reported and associated *FLG* null alleles could be found in public databases (R501X is now included as rs61816761 in the National Center for Biotechnology Information (NCBI), dbSNP build 130, http://www.ncbi.nlm.nih.gov/).

In Asian populations significant associations of eczema and IV with 18 identified *FLG* null alleles have been observed, too, but mutations seem to be highly population specific. Of the 20 described loss-of-function variants found in the European population, the majority with the exception of four observed heterozygous carriers of R501X (Ching, Hon et al. 2009) could not be found in Asian individuals of Japanese, Korean and Singaporean Chinese Populations (Nomura, Sandilands et al. 2007; Enomoto, Hirata et al. 2008; Hamada, Sandilands et al. 2008; Nomura, Akiyama et al. 2008; Hsu, Akiyama et al. 2009) and vice versa. However, this ethnic group seems to have its own exclusive null mutations showing the same strong impact on the development of eczema. All reported variants are frameshift or nonsense mutations leading to a premature translation stop and hence truncation of the profilaggrin molecule. The two most common mutations R501X (a nonsense mutation of the arginine codon 501 to a stop codon) and 2282del4 (a frameshift mutation at position 2282 due to a four base pair (bp) deletion) in the first filaggrin repeat impede any filaggrin synthesis from these alleles. As the 3' gene sequence seems to be important for posttranslational processing into functional filaggrin subunits, even variants located in this region prevent or reduce production of free filaggrin in the stratum corneum (Sandilands, O'Regan et al. 2006; Sandilands, Terron-Kwiatkowski et al. 2007).

In flaky tail (ft/ft) mice, the corresponding mouse model, which resulted from a spontaneous mutation, loss of profilaggrin and filaggrin leads to dry and flaky skin, paw constrictions in the neonatal period and annular tail (Lane 1972; Presland, Boggess et al. 2000). A homozygous frameshift mutation described as a 1bp deletion in the murine *FLG* gene seems to be the reason for insufficient filaggrin production in this mouse model, and can be equated with the null mutations found in human *FLG* (Fallon, Sasaki et al. 2009).

Gene	Location	Phenotype(s)	Number of subjects*	Variant(s)	Association	Reference.
IL13	5q31	childhood AE	52/288 Canadians	rs20541	yes	(He, Chan-Yeung et al. 2003)
				rs1800925	no	
		AE	238/104 Dutch	rs1800925	yes	(Hummelshoj, Bodtger et al. 2003)
		AE	185/102 Japanese	rs20541	yes	(Tsunemi, Saeki et al. 2002)
				rs1800925, rs1881457	no	
		AE	187/98 Germans	rs20541	yes	(Liu, Nickel et al. 2000)
		AE	94/186 Chinese	rs1800925, rs1881457	no	(Chang, Lee et al. 2006)
		childhood AE	178/1358 British	rs1800925, rs2066960, rs1295686, rs20541, rs1295685	no	(Arshad, Karmaus et al. 2008)
IL4	5q31	childhood AE	88 Japanese families	rs2243250	yes	(Kawashima, Noguchi et al. 1998)
		childhood AE	76+25 Australian families	rs2243250, rs2070874, haplotypes	no	(Elliott, Fitzpatrick et al. 2001)
		AE	190/116 Japanese	rs2243250	no	(Tanaka, Sugiura et al. 2001)
		AE severity	406 Swedish families	rs2243250	yes	(Soderhall, Bradley et al. 2002)
		extrinsic AE	60/30 Germans	rs2243250	yes	(Novak, Kruse et al. 2002)
		early-onset AE (<2 years)	52/496 Canadians	rs2243250 (haplotype with IL13 variants))	yes	(He, Chan-Yeung et al. 2003)
		AE	94/186 Chinese	rs2243250, rs2070874	no	(Chang, Lee et al. 2006)
SPINK5	5q32	Childhood AE	148+78 British families	rs2303067	yes	(Walley, Chavanas et al. 2001)
				rs2303063, rs2303064	no	
		AE+asthma	41 Japanese families	rs2303067, rs2303063, rs2303064, rs17860502, rs2303070	yes	(Nishio, Noguchi et al. 2003)
		AE	124/110 Japanese	rs2303067, rs2303063, rs2303061, rs2303062, rs2303066, rs2303068	yes	(Kato, Fukai et al. 2003)
				rs2303063, rs2303065	no	
		AE	308 German families	rs2303067, rs2303063, rs2303064, rs17860502	no	(Folster-Holst, Stoll et al. 2005)
		AE	99/102 French	rs2303067	no	(Hubiche, Ged et al. 2007)
		AE	486 German families	rs2303067	yes	(Weidinger, Baurecht et al. 2008)
		AE	773/3992 Germans		no	
		childhood AE	418/552 Irish/UK			
			1583/7746 UK			
		childhood AE	220/1161 Germans	rs2303067	no	(Kabesch, Carr et al. 2004)
		AE+asthma	78/200 Dutch	rs2303067, rs2303063, rs3756688	no	(Jongepier, Koppelman et al. 2005)
			175 Dutch families			

NOD1 (CARD4)	7p14-15	adult AE	457/1417 Germans	rs2736726, rs2075817, haplotypes	yes	(Weidinger, Klopp et al. 2005)
		AE	189 German families	rs2975632, rs2075822, rs2907749, rs2907748	yes	
		AE	392/297 Germans	haplotypes, SNP-SNP interaction	yes	(Macaluso, Nothnagel et al. 2007)
CMA1	14q11.2	adult AE	100/100 Japanese	rs1800175	yes	(Mao, Shirakawa et al. 1996)
		childhood AE	145/851 Japanese	rs1800175	yes	(Mao, Shirakawa et al. 1998)
		AE	100/101 Japanese	rs1800175	no	(Kawashima, Noguchi et al. 1998)
		intrinsic AE	47/100 Japanese	rs1800175	yes	(Tanaka, Sugiura et al. 1999)
		AE	70/100 Italians	rs1800175	no	(Pascale, Tarani et al. 2001)
		adult AE	242/1875 Germans	rs1800175	yes	(Weidinger, Rummler et al. 2005)
				rs1956923, rs5244, rs5246, rs5247, rs5248, rs5250	no	
IL4RA	16p12-11	AE	101/75 Japanese	rs2057768, rs2107356, rs8060798, rs8060938, rs12927172	yes	(Hosomi, Fukai et al. 2004)
				rs12927543	no	
		adult AE	27/29 Japanese	rs1805011	yes	(Oiso, Fukai et al. 2000)
				rs1805010, rs1805012	no	
		Infantile flexural AE	245/1051 British	rs1801275	yes	(Callard, Hamvas et al. 2002)
		extrinsic AE	302/122 Japanese	rs1805010, rs1805011, rs1801275	no	(Tanaka, Sugiura et al. 2001)
		AE	94/186 Chinese	rs1805011, rs2234898, rs1805012, rs1801275, rs1805015	no	(Chang, Lee et al. 2006)
RANTES	17q11.2	childhood AE	188/98 Germans	rs2107538	yes	(Nickel, Casolaro et al. 2000)
		childhood extrinsic AE	128/303 Hungarian	rs2107538, rs2280788	no	(Kozma, Falus et al. 2002)
		AE	62/14 Japanese	rs2107538	yes	(Bai, Tanaka et al. 2005)
		extrinsic AE	389/177 Japanese	rs2107538, rs2280788	yes	(Tanaka, Roberts et al. 2006)
FLG	1q21.3	AE, extrinsic AE, childhood AE, adult AE, AE+asthma	> 6000/> 27000 > 3000 families Europeans, European-Americans, Japanese, Chinese, Koreans	R501X, 2282del4, etc.	yes	(Palmer, Irvine et al. 2006), > 20 reports reviewed in (Rodriguez, Baurecht et al. 2009)

Table 2: Published candidate genes for which association with eczema (designated AE due to limitation of space) has been reported in at least two independent studies. * Number of cases/controls, cases/cohort or families.

1.9 Aims

The aetiology of eczema is complex, as different genetic as well as environmental factors contribute to the disease. This complexity displays a major challenge to the dissection of underlying mechanisms. Since only a few genes have been identified to influence manifestation of eczema, and as more susceptibility genes are supposed to exist, this work addresses the genetic impact on eczema by identifying and describing novel risk variants and genes for the disease.

As replication studies of reported associations between genetic variants and complex diseases often unmask false positive results, the previously described loss-of-function polymorphisms in the *filaggrin* gene and their association with eczema should be confirmed in a first independent replication study within this thesis. In order to further delineate the role of *FLG* mutations in eczema pathogenesis and subphenotypes (atopic and non-atopic form), intermediate phenotypes like serum IgE levels, and related atopic traits like asthma and rhinitis several association studies including family-based and case-control approaches will be performed. Concurrently its impact in epidemiological terms on eczema and atopic traits in the general population will be determined in a large population-based cross-sectional study population. Refinement of the risk profile of *FLG* null alleles will be achieved by a meta-analysis including the data of 24 association studies on eczema and asthma published until November 2008.

To gain further insights in the genetic architecture of eczema and its endophenotype IgE and to unravel novel susceptibility loci two unbiased genome-wide association studies should be conducted. These hypothesis-free approaches are projected in various large and well phenotyped cohorts with the help of newly developed high throughput SNP genotyping platforms and results will be concurrently validated in different independent replication cohorts.

2 Results

This work includes and is based on the following publications:

I Weidinger S, Illig T, Baurecht HJ, Irvine AD, **Rodríguez E**, Diaz-Lacava A, Klopp N, Wagenpfeil S, Zhao Y, Liao H, Lee SP, Palmer CNA, Jenneck C, Maintz L, Hagemann T, Behrendt H, Ring J, Nothen MM, McLean WHI, Novak N. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. J Allergy Clin Immunol 118(1):214-219, 2006. (Chapter 2.1)

II Weidinger S, **Rodríguez E**, Stahl C, Wagenpfeil S, Klopp N, Illig T, Novak N. Filaggrin mutations strongly predispose to early-onset and extrinsic atopic dermatitis. J Invest Dermatol 127:724-726, 2007. (Chapter 2.2)

III Weidinger S, O'Sullivan M, Illig T, Baurecht HJ, Depner M, **Rodríguez E**, Ruether A, Klopp N, Vogelberg C, Weiland SK, McLean WHI, von Mutius E, Irvine AD, Kabesch M. Filaggrin mutations, atopic eczema, hay fever and asthma in children. J Allergy Clin Immunol 121(5):1203-1209, 2008. (Chapter 2.3)

IV **Rodríguez E**, Baurecht H, Herberich E, Wagenpfeil S, Brown SJ, Cordell HJ, Irvine AD, Weidinger S. Meta-analysis of filaggrin polymorphisms in eczema and asthma: Robust risk factors in atopic disease. J Allergy Clin Immunol, 123(6):1361-70, 2009. (Chapter 2.4)

V Esparza-Gordillo J, Weidinger S, Fölster-Holst R, Bauerfeind A, Ruschendorf F, Patone G, Rohde K, Marenholz I, Schulz F, Kerscher T, Hubner N, Wahn U, Schreiber S, Franke A, Vogler R, Heath S, Baurecht H, Novak N, **Rodríguez E**, Illig T, Lee-Kirsch MA, Ciechanowicz A, Kurek M, Piskackova T, Macek M, Lee YA, Ruether A. A common variant on chromosome 11q13 is associated with atopic dermatitis. Nat Genet, 41(5):596-601, 2009. (Chapter 2.5)

VI Weidinger S, Gieger C, **Rodríguez E**, Baurecht HJ, Mempel M, Klopp N, Gohlke H, Wagenpfeil S, Ollert M, Ring J, Behrendt H, Heinrich J, Novak N, Bieber T, Krämer U, Berdel D, von Berg A, Bauer CP, Herbarth O, Koletzko S, Prokisch H, Mehta D, Meitinger T, Depner M, von Mutius E, Liang L, Moffatt M, Cookson W, Kabesch M, Wichmann HE, Illig T. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. PLoS Genet 4(8):1-9, 2008. (Chapter 2.6)

2.1 Loss of function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitization

Rapid publication

Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease with a strong genetic background. One of the characteristic features of AD and causative factor for the disease is an impaired epidermal skin barrier based on a primary defect of epidermal differentiation.
Objectives: Recently, 2 loss-of-function mutations (R501X and 2282derl4) in the filaggrin gene *(FLG)* that cause ichthyosis vulgaris, one of the most common inherited skin disorders of keratinization, have been reported to be strong predisposing factors for AD.
Methods: We evaluated the association of the loss-of-function mutations R501X and 2282del4 within the *FLG* gene in a large collection of 476 well-characterized white German families with AD by using the transmission-disequilibrium test.
Results: Our family-based approach revealed prominent associations between the 2 loss-of-function *FLG* mutations and AD, as previously observed in a traditional Mendelian linkage analysis and case-control cohort analysis approach. In addition, we observed associations of the *FLG* mutations in particular with the extrinsic subtype of AD, which is characterized by high total serum IgE levels and concomitant allergic sensitizations. Furthermore, *FLG* mutations are significantly associated with palmar hyperlinearity in patients with AD, which represents a shared feature of AD and ichthyosis vulgaris.
Conclusion: Together these data implicate that *FLG* is the first really strong genetic factor identified in a common complex disease.
Clinical implications: These findings underline the crucial role of the skin barrier in preventing allergic sensitization.
(J Allergy Clin Immunol 2006;118:214-9.)

Key words: *Atopic dermatitis, skin barrier, epidermal differentiation complex, polymorphisms, filaggrin*

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease caused by complex interactions between multiple susceptibility genes and environmental factors.[1] Its high familial occurrence and a concordance rate of about 80% among monozygotic twins and 20% among dizygotic twins[2,3] indicate a substantial genetic component. However, the inheritance pattern of atopic diseases, such as asthma, rhinitis, or AD, is complex and does not follow an obvious Mendelian mode.[4] Moreover, the dissection of multifactorial genetic traits like AD is hampered by considerable phenotypic heterogeneity and incomplete penetrance.[5] Nevertheless, in the past decade, significant progress has been made in the field of AD genetics with the identification of numerous loci and candidate genes linked and associated with AD.[6-8] Unfortunately, a high percentage of genetic studies on AD have

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Abbreviations used
AD: Atopic dermatitis
FLG: Filaggrin gene

reported conflicting results, and only very few genetic associations have been successfully replicated in other cohorts. However, replication is fundamental for deciding that an observed association is not due to chance.[9]

In part, this irreproducibility can be attributed to inaccurate phenotyping. Only a clear-cut definition of the phenotype allows the subanalysis of different subtypes of AD, such as extrinsic or intrinsic forms, mild and severe variants, and AD in infancy versus chronic persistent courses or courses with a late-type onset in adulthood, which might all be influenced by different genetic modifications.

While in the past years research on the genetics of AD has focused on candidate genes with effects on innate and adaptive immunity, recently attention has been drawn to the epithelial barrier, and it has been suggested that in AD the epidermal barrier might be impaired by a primary defect of epidermal differentiation.[10] Naturally, the epidermal skin barrier represents an effective protective shield that maintains, to some degree, resistance to environmental agents, such as allergens, microbes, or various irritants. A compromised skin barrier with an enhanced transepidermal water loss and reduced hydration of the skin is a characteristic feature of patients with AD. Further on, a higher pH in the epidermis of patients with AD is suspected to affect the activity of enzymes in the lamellar lipid matrix of the stratum corneum, which are involved in ceramide synthesis and epidermal differentiation.[11]

Supporting this view of a crucial role of the skin barrier in AD, genome-wide linkage analysis has shown a significant linkage signal with AD on chromosome 1q21.[7] This region contains the human epidermal differentiation complex.[12] The epidermal differentiation complex harbors several genes characterized by common structural features, such as a central region of short tandem peptide repeats, that encode proteins involved in the formation of the cornified cell envelope, such as small proline-rich region proteins, S100A proteins, and late envelope proteins, many of which are overexpressed in the skin of patients with AD.[10,13] During the epidermal differentiation process, there is increasing expression of keratin proteins, which form the intermediate filament cytoskeleton on epithelial cells. Similarly, expression of desmosomal proteins increases as keratinocytes differentiate upward and outward in the epidermis, firmly anchoring the keratin network to cell membranes. Filaggrin, which consolidates the keratin filaments into dense bundles at the granular layer–stratum corneum boundary, represents an integral part of the epidermis and is crucial for the development of the cornified envelope to engineer and maintain the barrier function of the uppermost layer of the skin.[14] Very recently, it has been reported that 2 loss-of-function mutations of the filaggrin gene *(FLG)* associated with ichthyosis vulgaris[15] are strong predisposing factors for AD.[16] These *FLG*-null mutations, R501X and 2282del4, led to deficiency of filaggrin peptides in the upper part of the epidermis in homozygote and compound heterozygote patients. In addition to the fact that ichthyosis vulgaris, which is one of the most common inherited skin disorders of keratinization, is frequently associated with AD, patients with AD share some clinical features with patients with ichthyosis vulgaris, such as dry scaly skin, keratosis pilaris, and palmar hyperlinearity, which, because of their relevance in AD, have been incorporated into the basic diagnostic criteria for AD.[17] The observed association of *FLG* mutations with AD support the view that these mutations contribute to the impaired skin barrier in patients with AD and therefore represent an important factor determining the severity and course of AD. To confirm these recent results, we aimed to replicate these associations in a large collection of 476 white German families with AD and to identify subtypes of AD in which these mutations might be of major relevance.

METHODS

Study population

For family-based association analysis, we used 476 complete white German parent-offspring trios for AD who had been recruited in Munich and Bonn, Germany. All subjects had completed a standardized questionnaire, which, next to demographic data, included the basic allergy questions of the European Community Respiratory Health Survey.[18] Both total and specific IgE antibodies against common environmental allergens (grass, birch pollen, cat, and house dust mite) were measured with the help of an enzyme immunoassay (CAP-FEIA, Pharmacia, Uppsala, Sweden or Immulite and DPC Biermann, Bad Nauheim, Germany). Specific sensitization was defined to be present if at least one of the specific IgE antibodies was positive (CAP RAST class $\geq$1, corresponding to $\geq$0.35 kU/L). Subjects were classified as having asthma or allergic rhinoconjunctivitis when they reported a physician's diagnosis of asthma or rhinoconjunctivitis, and AD was diagnosed on the basis of a skin examination by experienced dermatologists using the UK diagnostic criteria for AD.[19] In addition, stigmata of atopic constitution were evaluated.[20] Extrinsic AD was defined as AD with concomitant sensitization and IgE levels of 150 kU/L or greater.[21] IgE levels were considered increased at values of greater than 100 kU/L. All study methods followed the Declaration of Helsinki protocols and were approved by the ethics committee of the "Bayerische Landesärtzekammer" Munich and the ethics committee of the University of Bonn. Written informed consent was obtained from all participants before the beginning of the study.

Genotyping

Genomic DNA was extracted from leukocytes with a commercial DNA isolation kit (Gentra Systems, Minneapolis, Minn), according to the manufacturer's recommendation. Genotyping for R501X was performed with the MassARRAY system (Sequenom, San Diego, Calif), as described recently.[22] Briefly, genomic DNA was amplified by means of PCR with HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). The genotyping assay was carried out by using 5 ng of genomic DNA. PCR primers were used at 167 nM final concentrations for a PCR volume of 6 μL. The PCR condition was 95°C

TABLE I. PCR primers used for genotyping of *FLG* variants

SNP ID	Direction	PCR primer	Extension primer
R501X	Forward	ACGTTGGATGCTGGAGGAAGACAAGGATCG	ATGCCTGGAGCTGTCTC
	Reverse	ACGTTGGATGATGGTGTCCTGACCCTCTTG	
2282del4	DEL4.F2	TCCCGCCACCAG CTCC	
	DEL4.F1	6-FAM-GTGGCTCTGCTGATGGTGA	

Genotyping for R501X was performed with the MassARRAY system (Sequenom, San Diego, Calif), and 2282del4 mutation was typed by using fluorescent PCR, as described previously.[14,21]

for 15 minutes for hot start, followed by denaturing at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute for 45 cycles, and finally incubation at 72°C for 10 minutes. PCR products first were treated with shrimp alkaline phosphatase (Amersham, Freiburg, Germany) for 20 minutes at 37°C to remove excess deoxyribonucleoside triphosphates and afterward for 10 minutes at 85°C to inactivate shrimp alkaline phosphatase. Thermo Sequenase (Amersham, Freiburg, Germany) was used for the base extension reactions. Extension primers were used at a final concentration of 5.4 μM in 10-μL reactions (Table I).[14,21] The base extension reaction condition was 94°C for 2 minutes, followed by 94°C for 5 seconds, 52°C for 5 seconds, and 72°C for 5 seconds for 40 cycles. All reactions (PCR amplification and base extension) were carried out in a Tetrad PCR thermal cycler (MJ Research, Waltham, Mass). The final base extension products were treated with SpectroCLEAN resin (Sequenom, San Diego, Calif) to remove salts in the reaction buffer. This step was carried out with a Multimek 96-channel autopipette (Beckman Coulter, Fullerton, Calif), and 16 μL of resin-water suspension was added to each base extension reaction, making the total volume 26 μL. After a quick centrifugation (2000 rpm for 3 minutes) in an Eppendorf Centrifuge 5810, 10 nL of reaction solution was dispensed onto a 384 format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint nanodispenser (Sequenom). A modified Bruker Biflex matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Sequenom) was used for data acquisitions from the SpectroCHIP. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom).

The 2282del4 mutation was typed by sizing a fluorescently labeled PCR fragment on an Applied Biosystems 3100 or 3730 DNA sequencer, as described previously.[15,22]

Ten-microliter PCR reactions were carried out with primers DEL4.F2 and DEL4.R1 in AmpliTaq Gold buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems), 10 nmol of each deoxyribonucleoside triphosphate, and 1 unit of AmpliTaq Gold DNA polymerase. Reactions were amplified as follows: 94°C (12 minutes) for 1 cycle; 94°C (15 seconds), 58°C (30 seconds), and 72°C (45 seconds) for 30 cycles; and 72°C (5 minutes) for 1 cycle. Fragments were diluted 1:60 and sized against ROX-500 size markers, according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 199 bp, and the 2282del4 allele was 195 bp. All primers are shown in Table I.

Statistical analysis

Descriptive statistics for quantitative and qualitative values were calculated with SAS version 9.1 (SAS Institute, Cary, NC) and are given as means ± SD and relative frequencies or absolute numbers, respectively. Hardy-Weinberg equilibrium was tested by using the χ^2 goodness-of-fit test. Evidence of associations with the binary traits AD, asthma, total serum IgE level (cutoff median), and allergic sensitization was evaluated by using the classical transmission-disequilibrium test implemented in Haploview 3.2. Odds ratios measuring how much more often the variant is transmitted and the wild-type allele is nontransmitted as opposed to the variant being nontransmitted and the wild-type allele being transmitted were calculated by using SAS version 9.1. An additional case-control analysis for parents with AD versus parents without AD from the study trios was calculated by using multiple logistic regression analyses with AD as dependent and sex and mutant allele (yes/no) for either polymorphism as independent variables.

Any *P* values given are 2-sided, subject to a global significance level of 5%, and not adjusted for multiple comparisons. However, most association results remained significant after applying the Bonferroni correction for multiple testing (Bonferroni-corrected significance level: 0.05/21 = 0.0024). Power analyses for transmission-disequilibrium tests concerning AD were calculated with nQuery Advisor version 5.0 by using the McNemar test of equality of paired proportions with a 5% two-sided significance level and the following values as observed in the family data: (1) difference in proportions of carrier allele transmitted and nontransmitted (7%) and (2) proportion of discordant transmissions (ie, the number of transmissions of carrier allele and nontransmission of the wild-type allele or vice versa) related to the total number of parents in the family dataset (16%).

TABLE II. Phenotype characteristics of all individuals (parents and affected offspring) included in analyses

	Trios (n = 476)	
	Parents	Offspring
Male sex (%)	476 (50.0)	196 (41.1)
Mean ± SD age, y	54.71 ± 10.27	22.12 ± 10.76
Median IgE, kU/L	42.0	253.0
AD (%)	90 (9.6)*	476 (100)
Extrinsic AD (%)	59 (6.4)*	397 (83.4)*
Intrinsic AD (%)	15 (1.6)*	79 (16.6)*
Early-onset AD, <2 y (%)	—	285 (62.0)*
Asthma (%)	94 (11.3)*	146 (31.6)*
Rhinitis (%)	278 (33.3)*	269 (58.2)*
Sensitization (%)	278 (33.3)*	352 (79.8)*
Palmar hyperlinearity (%)	ND	113 (47.7)*

ND, Not done.
*Relative proportions are calculated without considering missing values.

RESULTS

The clinical characteristics of the study population are outlined in Table II. The average genotyping success rate for both polymorphisms was 97.8%. Analysis of Mendelian inheritance within the families revealed no major errors. The 2282del4 variant was present in 15.76% of offspring, and the frequency of carriers of R501X was 8.8%, with a combined carrier frequency of 22.75%

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TABLE III. Frequency of *FLG*-null alleles in parents and offspring

	R501X		2282del4		Combined genotype	
Genotype	Parents	Children	Parents	Children	Parents	Children
AA	877 (94.30%)	424 (91.18%)	848 (89.08%)	401 (84.24%)	781 (83.98%)	360 (77.25%)
Aa	53 (5.70%)	41 (8.82%)	103 (10.82%)	72 (15.13%)	145 (15.59%)	96 (20.60%)
Aa	—	—	1 (0.11%)	3 (0.63%)	4 (0.43%)	10 (2.15%)

AA, Wild-type/wild-type *FLG* genotype for R501X and 2282del4 variants; *Aa*, heterozygous genotype for either R501X or 2282del4; *aa*, homozygous R501X or 2282del4 genotype or compound heterozygous genotype.

TABLE IV. Association analysis of *FLG*-null alleles in the trio cohort

	R501X		2282del4		Combined genotype Aa	
Trait	T:U	*P* value	T:U	*P* value	T:U	*P* value
AD	42:11	2.2×10^{-5}	73:30	2.2×10^{-5}	101:37	5.1×10^{-8}
Allergic sensitization	36:05	1.3×10^{-6}	56:25	6.1×10^{-4}	79:26	2.3×10^{-7}
Total IgE level	30:03	2.6×10^{-6}	53:20	.0001	69:19	9.8×10^{-8}
Asthma	16:03	.003*	26:09	.004*	34:10	.0003
Extrinsic AD	40:07	1.5×10^{-6}	62:27	.0002	88:30	9.3×10^{-8}
Intrinsic AD	04:02	NS	11:03	.03*	13:07	NS
Palmar hyperlinearity	16:01	3.0×10^{-4}	24:05	.0004	35:06	5.9×10^{-6}

Combined genotype Aa, Heterozygous genotype for either R501X or 2282del4; *T:U*, transmitted:untransmitted; *NS*, not significant.
*Not significant after Bonferroni correction for multiple testing.

(Table III). In our cohort we did not observe any homozygotes for R501X, but we did observe 3 homozygotes for 2282del4 and 10 compound heterozygotes. Two of the individuals heterozygous for 2282del4 and 6 of the individuals heterozygous for both polymorphisms exhibited asthma in addition to AD, and all compound heterozygotes were characterized by increased total IgE levels and allergic sensitization.

Results of association analysis are summarized in Table IV. Both *FLG* gene variants were significantly overtransmitted to AD-affected offsprings. The strongest association was seen for the combined Aa genotype (presence of either R501X or 2282del4 variant, $P = 5.1 \times 10^{-8}$), pointing to a dominant risk for AD conferred by both alleles. Corresponding odds ratios were 3.64, 2.43, and 2.73 for R501X, 2282del4, and the combined genotype, respectively. Power calculations showed that with our cohort of 476 families, a significance level of .05, and a rate of discordant pairs of 0.156, a difference in proportions of 0.042 for the combined genotype can be detected with a statistical power of 90%. Additional analysis of affected versus unaffected parents confirmed a strong association of both mutations with AD ($P < .0001$ for combined genotype, Table V).

Although a number of phenotypic and genetic studies suggested parent-of-origin effects, in particular maternal effects in AD,[23] we did not observe such effects for R501X and 2282del4 by using a previously described analysis method.[24]

Both *FLG* variants also showed significant association with total serum IgE levels ($P = 9.3 \times 10^{-8}$ for combined genotype), with geometric means for serum IgE of 177.7 kU/L in wild-type/wild-type versus 298.9 kU/L in carriers of either allele, and with allergic sensitization ($P = 2.3 \times 10^{-7}$ for combined genotype). Further on, consistent with the observation of Palmer et al[16] that these *FLG*-null alleles are important risk factors for asthma in association with AD, both variants showed significant association with asthma as a secondary trait ($P = .0003$ for combined genotype). With 270 (274 and 288, respectively) families with offspring also with asthma successfully genotyped for the combined genotype (R501X and 2282del4, respectively), the observed statistical power to detect a difference in transmission and nontransmission proportions of the carrier allele of 9% (4% and 6%, respectively; as observed in our data) when the proportion of discordant transmission is 16% (7% and 12%, respectively; as observed in our data) was 97% (79% and 84%, respectively).

Along with these observations, a highly significant transmission distortion was seen for the extrinsic variant of AD ($P = 9.3 \times 10^{-8}$ for combined genotype). In contrast, no association was seen with intrinsic AD. In our cohort of families with AD, these *FLG* variants were revealed to be also associated with the presence of palmar

TABLE V. Case-control analysis of parents with AD versus parents without AD from the study trios, with logistic regression analyses controlled for sex showing the increased risk of the mutant allele for AD

SNP	Variable	*P* value	OR (95% CI)
2282del4	Mutant allele	.0013	2.5 (1.4-4.3)
R501X	Mutant allele	<.0001	4.1 (2.2-7.9)
Combined genotype	Mutant allele	<.0001	3.3 (2.1-5.4)

SNP, Single nucleotide polymorphism; *OR*, odds ratio.

hyperlinearity in patients with AD ($P = 5.4 \times 10^{-6}$ for combined genotype), which is considered a strong sign for atopic constitution.[20] No correlation between the presence of *FLG*-null mutations and an early onset of AD was observed.

DISCUSSION

Genetic factors appear to be important in the multifactorial pathogenesis of AD, and in recent years there have been many reports of ''significant'' associations.[4] However, most initial findings were not replicated by subsequent association studies, which is of utmost importance to demonstrate a true relationship between a genetic marker and a disease.[25] Notably, the associations between the 2 loss-of-function *FLG* mutations and AD observed by Palmer et al,[16] using traditional Mendelian linkage analysis and case-control cohort analysis approaches, are revealed to be as prominent in our family-based analysis. These observations were strengthened by an additional analysis of unaffected versus affected parents, which confirmed the association of the *FLG* mutations with AD. Thus our results add value by reinforcing the role of *FLG* mutations in AD, which are the first really strong genetic factors identified in this common complex disease. In essence these mutations act in a low-penetrance Mendelian fashion, showing codominant transmission in families.

In addition, our results further support the hypothesis that an impaired skin barrier is an important prerequisite for subsequent allergic sensitization and avails the manifestation of additional allergic respiratory diseases in patients with AD. Functional clues for a crucial role of the skin barrier in the development of sensitizations have already been provided in the past with the help of tape-stripping experiments in mice, which showed that epicutaneous sensitization through barrier-disrupted skin enhances T_H2 cytokine expression within the skin.[26-28] In line with these findings are observations in children with AD who were exposed to topical emollients containing peanut protein. These children showed a significantly increased risk of peanut sensitization,[29] indicating a close relation between the integrity of the skin barrier and the development of skin-mediated sensitizations.

It might be speculated whether disturbance of the epidermal barrier caused by *FLG* variants facilitates allergic sensitization and predisposes to the manifestation of respiratory atopic disease, which classifies the extrinsic variant of AD. The fact that we observed strong associations with allergic sensitization, increased total IgE levels, and extrinsic AD, but not with the intrinsic variant of AD, points to a stringent correlation between FLG status and allergic sensitization. Some of our individuals with intrinsic AD did carry the *FLG* mutations; however, the overall number of intrinsic AD cases was rather low. Further investigations in additional cohorts are necessary to confirm these intriguing preliminary observations.

Although the epidermal barrier matures rapidly after birth, percutaneous absorption of topically applied substances is higher in early infancy.[30] Combined with a markedly higher surface area/weight ratio in infants, a genetically determined dysfunction of the epidermal skin barrier was expected to be of higher relevance in children with a manifestation of AD, in particular during the first 2 years of life. However, the presence of *FLG*-null mutations was not correlated with an early onset of AD in our study cohort.

As mentioned above, the 2 *FLG*-null mutations investigated in this study have been identified originally as a cause of ichthyosis vulgaris, a keratinization disorder that exhibits strong association with AD.[31] In our cohort of families with AD, these *FLG* variants were revealed to be also associated with the presence of palmar hyperlinearity in patients with AD ($P = 5.4 \times 10^{-6}$ for combined genotype), which is characteristic for ichthyosis vulgaris but also considered a strong sign for atopic constitution,[20] thereby providing a further genetic link between these 2 diseases.

Taken together, in view of the data presented here, a genetically determined disruption of the epidermal skin barrier should be regarded as a key event in the pathogenesis of AD and as a considerable risk factor for the development of subsequent sensitizations and respiratory diseases in a subgroup of patients, which might pave the way for a severe and long-lasting atopic career.[32]

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2.2 Filaggrin mutations strongly predispose to early-onset and extrinsic atopic dermatitis

Filaggrin Mutations Strongly Predispose to Early-Onset and Extrinsic Atopic Dermatitis

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TO THE EDITOR

Atopic dermatitis (AD) arises from the interaction between strong genetic and environmental factors (Morar *et al.*, 2006). As it commonly occurs in concert with asthma and/or hay fever, and the majority of patients exhibits elevated serum IgE levels and/or sensitizations against aero- and food allergens, most pathophysiological concepts are dominated by atopic mechanisms and regard IgE-mediated sensitization as central cornerstone in the development of AD (Akdis *et al.*, 2006). However, there is accumulating evidence from epidemiological studies, suggesting that sensitization is not essential for the development of eczema and that atopy may be secondary rather than causative (Williams and Flohr, 2006). In addition, genome-wide screens and candidate gene studies have shown only partial overlap of AD with other atopic phenotypes, but a closer relationship with other epithelial diseases such as psoriasis (Cookson, 2004). Thus, attention has been drawn to the impaired epidermal barrier function, which is a hallmark feature of AD. In this context, the epidermal differentiation complex on chromosome 1q21, for which linkage to both AD and psoriasis has been reported, is of particular interest, as it harbors a number of genes and gene families expressed in the terminally differentiating epithelium (Cookson *et al.*, 2001). Recently, it was demonstrated that two functional mutations within the epidermal differentiation complex gene filaggrin (*FLG*), underlie ichthyosis vulgaris (Smith *et al.*, 2006) and are strong risk factors for AD and asthma in the context of AD in European populations (Palmer *et al.*, 2006). In a

Abbreviations: AD, atopic dermatitis; FLG, filaggrin

Table 1. Frequency of *FLG* null alleles in cases (AD) and controls

	R501X		2282del4		Combined genotype	
Genotype	Cases	Controls	Cases	Controls	Cases	Controls
AA	250 (91.9%)	245 (97.6%)	227 (84.1%)	241 (96.4%)	213 (78.9%)	234 (94.0%)
Aa	22 (8.1%)	6 (2.39%)	43 (15.9%)	9 (3.6%)	50 (18.5%)	15 (6.0%)
aa					7 (2.6%)	

Abbreviations: AD, atopic dermatitis; *FLG*, filaggrin.
''AA'' refers to wild-type/wild-type *FLG* genotype for R501X and 2282del4 variants. ''Aa'' refers to heterozygous genotype for either R501X or 2282del4 and ''aa'' refers to homozygous R501X or 2282del4 genotype or compound heterozygous genotype.

Table 2. Results of the logistic regression models adjusted for age and sex using backward variable selection

	R501X			2282del4			Combined genotype Aa		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value	OR	95% CI	*P*-value
AD	3.51	(1.39; 8.84)	0.0079	4.98	(2.37; 10.48)	2.3×10^{-5}	3.53	(1.92; 6.48)	4.9×10^{-5}
Extrinsic AD	4.02	(1.58; 10.26)	0.0035	5.00	(2.34; 10.69)	3.3×10^{-6}	3.66	(1.96; 6.83)	4.6×10^{-5}
AD+asthma	5.07	(1.73; 14.81)	0.0030	7.49	(3.18; 17.63)	4.0×10^{-6}	5.69	(2.76; 11.73)	2.5×10^{-6}
AD+rhinitis	4.10	(1.55; 10.87)	0.0045	5.83	(2.68; 12.68)	8.9×10^{-6}	4.04	(2.11; 7.72)	2.4×10^{-5}
Total IgE			NS	3.29	(1.56; 6.97)	0.0018	2.14	(1.14; 4.00)	0.0178
Early-onset AD	5.66	(2.05; 15.62)	0.0008	7.18	(3.17; 16.25)	2.3×10^{-6}	5.21	(2.61; 10.40)	2.8×10^{-6}
SCORAD (objective components, median >31)			NS	3.16	(1.49; 6.72)	0.0027	2.65	(1.36; 5.17)	0.0043

Abbreviations: AD, atopic dermatitis; CI, confidence interval; NS, nonsignificant; OR, odds ratio.

subsequent family-based association study, we could show that these mutations particularly predispose to the extrinsic subtype of AD, but not intrinsic AD, suggesting that the skin barrier defect in AD is a key early event that precedes the development of allergic sensitization and respiratory atopy (Weidinger *et al.*, 2006). To replicate these associations, we investigated an independent collection of 274 unrelated adult AD cases (average age 35.9±10.8 years; 61.2% female subjects and 38.8% male subjects) and 252 population-based controls (average age 39.4±16.1 years; 61.5% female subjects and 38.5% male subjects). All patients were recruited in the dermatologic outpatient departments of the University of Bonn and the Technical University Munich, Germany. AD was diagnosed on the basis of a skin examination by experienced dermatologists using the UK diagnostic criteria for AD. The severity of eczema was assessed using the SCORAD (scoring atopic dermatitis index). Subjects were classified as having asthma or allergic rhinitis when they reported a physician's diagnosis of asthma or rhinoconjunctivitis. Hypernormal control individuals without AD or allergic diseases and IgE levels <100 kU/l were selected from the KORA S4 (Cooperative Health Research in the Region of Augsburg Survey 4: 1999–2001) population-based cross-sectional study, which was carried out 1999–2001 in the city and region of Augsburg, South Germany (Weidinger *et al.*, 2005). Total as well as specific IgE antibodies against common environmental allergens were measured with the help of an enzyme-immuno assay (CAP-FEIA, Pharmacia, Uppsala, Sweden). Specific sensitization was defined to be present if at least one of the specific IgE antibodies was positive (CAP-RAST class ⩾1, corresponding to ⩾0.35 kU/l). A raised total serum IgE was taken to be greater than 100 kU/l. Extrinsic AD was defined as AD with concomitant sensitization and/or IgE levels ⩾100 kU/l. Severity of AD was regarded as binary trait using the median value of the objective SCORAD components (31 points) as cutoff.

All study methods were approved by the ethics committee of the ''Bayerische Landesärtzekammer'' Munich and the ethics committee of the University of Bonn and a written and informed consent that complies with all the Declaration of Helsinki Principles was obtained from the participants before the beginning of the study.

Out of the 274 AD cases, 71.7% were extrinsic, 62.6% had allergic rhinitis, 34.8% suffered from asthma, and 41.6% showed an early onset of the disease (<2 years of age). The mean total IgE of AD cases was 1,024 kU/l.

Genotyping for R501X and 2282del4 was performed using the MassARRAY system (Sequenom, San Diego, CA) as described previously (Weidinger *et al.*, 2005). To test for associations with binary traits, logistic regression models were applied with age, sex, and genotype as independent variables. In addition, associations with

IgE levels were evaluated using a linear regression model on log-transformed IgE values with the same covariates. For either regression analysis backward variable selection was applied. Results are given for the estimates of genotype effects after variable selection. The *P*-values are two-sided and subject to a significance level of 0.05. The issue of multiple testing does not affect the overall results and interpretation. Therefore *P*-values were not adjusted.

In our cohort, we did not observe any homozygotes for either mutation, but seven compound heterozygotes. The *FLG* variants were greatly over-represented in AD cases with a combined carrier frequency of 57 (21.1%) *vs* 15 (6.0%) in controls (odds ratio 3.53, 95% confidence interval 1.92–6.48, $P=4.9\times10^{-5}$, Tables 1 and 2). The lower than expected carrier frequency for heterozygote *FLG* status in controls (6 *vs* 10% for other comparable populations) is probably due to the fact that our control population had been screened so as to not have AD, allergic diseases or raised IgE levels and therefore represents a hypernormal type of control population.

The combined carrier frequency among patients with extrinsic AD was 24.6%. The highest carrier frequency was seen in patients with early onset of disease (33 out of 111, 29.7%). In addition, logistic regression analysis indicated that the 2282del4 mutation was associated with a more severe phenotype (odds ratio 3.16, 95% confidence interval 1.49–6.72, $P=0.0027$). The lack of association with severity for R501X may be due to the age at which SCORAD was determined and due to the fact that the SCORAD is a ''snap shot'' variable that does not reflect the overall disease activity. Both *FLG* variants showed significant associations with total serum IgE levels with geometric means for serum IgE (kU/l) 135.6 in wild type/wild type *versus* 317.4 in carriers of at least one mutation ($P=0.010$). This association is confirmed by linear regression using log-transformed values of observed IgE levels as quantitative dependent variable ($P=0.011$). In addition, both asthma and rhinitis in the context of AD were significantly associated ($P=2.5\times10^{-6}$ and $P=2.4\times10^{-5}$ for combined genotype). Consistent with previous findings (Weidinger *et al.*, 2006), no association was seen with the intrinsic subtype of AD. Results of association analysis are summarized in Table 2.

Our present findings confirm that *FLG* mutations are strong risk factors for AD, in particular for the extrinsic subtype of AD, and with concomitant respiratory allergic diseases. Our results further indicate that these variations predispose to an early onset and persistent course of the disease. It seems conceivable that a genetically determined primary epidermal barrier disruption is the key-event in AD that predisposes to secondary allergic manifestations by causing a reduced resistance to environmental agents such as allergens, irritants, and microbes. It will be highly interesting to further evaluate the epidermal differentiation complex for additional candidate genes modulating the skin barrier function.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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2.3 Filaggrin mutations, atopic eczema, hay fever and asthma in children

Atopic dermatitis and skin disease

Filaggrin mutations, atopic eczema, hay fever, and asthma in children

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Background: Mutations in the filaggrin gene (*FLG*) have been shown to play a significant role in ichthyosis vulgaris and eczema, 2 common chronic skin diseases. However, their role in the development of other atopic diseases such as asthma and rhinitis has not yet been clarified in large population-based studies.

Objectives: To study the effect of *FLG* mutations at the population level and their effect on other atopic phenotypes.

Methods: Association analysis of the 2 common *FLG*-null mutations R501X and 2282del4 and 3 recently identified rare *FLG* variants (R2447X, S3247X, 3702delG) was performed on our cross-sectional population of German children (n = 3099) recruited as part of the International Study of Asthma and Allergies in Childhood II in Munich (n = 1159) and Dresden (n = 1940).

Results: *FLG* variants increased the risk for eczema more than 3-fold (odds ratio [OR], 3.12; 95% CI, 2.33-4.173; $P = 2.5 \times 10^{-14}$; population-attributable risk, 13.5%). Independent of eczema, *FLG* mutations conferred a substantial risk for allergic rhinitis (OR, 2.64; 95% CI, 1.76-4.00; $P = 2.5 \times 10^{-6}$; population-attributable risk, 10.8%). Nasal biopsies demonstrated strong filaggrin expression in the cornified epithelium of the nasal vestibular lining, but not the transitional and respiratory nasal epithelia. In contrast, the association with asthma (OR, 1.79; 95% CI, 1.19-2.68; $P = .0048$) was restricted to asthma occurring in the context of eczema, and there was a strong association with the complex phenotype eczema plus asthma (OR, 3.49; 95% CI, 2.00-6.08; $P = 1.0 \times 10^{-5}$).

Conclusion: Our results suggest that *FLG* mutations are key organ specific factors predominantly affecting the development of eczema and confer significant risks of allergic sensitization and allergic rhinitis as well as asthma in the context of eczema.

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Key words: *Atopic eczema, rhinitis, asthma, filaggrin, children*

Recently a breakthrough in the genetics of atopic eczema was achieved by the identification of 2 mutations (R501X and 2282del4) within the filaggrin gene (*FLG*).[1,2] Both mutations lead to a premature translation stop of the filaggrin protein, resulting in a filaggrin deficiency. Further rare *FLG* mutations reported in Irish subjects have also been shown to be functional null alleles.[3]

A substantial proportion of the white population may have a primary barrier dysfunction caused by a genetic filaggrin deficiency and thereby be prone to developing inflammatory skin diseases.[4] Although *FLG* mutations cause ichthyosis vulgaris, one of the most common inherited cutaneous keratinization disorders, they also represent a major predisposing factor for atopic eczema.[1,2] Independent replication studies have provided unequivocal evidence for the exceptionally strong and consistent effect of *FLG* null alleles on white populations, predisposing particularly to an early-onset, severe, and persistent course of eczema.[5]

These initial family-based and case-control studies were all centered on the effect of *FLG* in patients with eczema, whereas so far, the incidence of *FLG* null alleles in the general population and their role in atopic diseases other than eczema has not been determined sufficiently. The expression of filaggrin in the skin and other keratinizing epithelia such as the oral cavity and the conjunctiva[6-8] may suggest that organs in addition to the skin could be affected by *FLG* mutations.

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Abbreviations used
EDC: Epidermal differentiation complex
FLG: Filaggrin gene
HWE: Hardy-Weinberg equilibrium
ISAAC II: International Study of Asthma and Allergies in Childhood, phase II
OR: Odds ratio
PAR: Population-attributable risk
SPT: Skin prick test

We genotyped 3099 German children from the population-based cross-sectional International Study of Asthma and Atopy in Childhood, phase II (ISAAC II) for 5 *FLG* variants previously described to reach a minor allele frequency of at least 0.01 in a study of Irish subjects[3]: the originally identified mutations in *FLG* repeat 1, R501X and 2282del4, as well as the more 3′ mutations R2447X, S3247X, and 3702delG. The role of *FLG* mutations in the manifestation of a range of atopic diseases such as eczema, asthma, and rhinitis was studied. Furthermore, the probability that a person carrying these mutations will develop an atopic disease (penetrance) and the proportion of cases in the population attributable to the mutant *FLG* alleles (population-attributable risk PAR) were determined.

METHODS

Nomenclature

The terminology for atopic diseases is confusing, and terms such as *atopic eczema*, *atopic dermatitis*, *childhood eczema*, *atopiform dermatitis*, and *flexural dermatitis* are frequently used synonymously in the literature. In an attempt to standardize the nosology for allergic diseases, the nomenclature committee of the World Allergy Organization recently published its recommendation for naming allergic diseases such as asthma, eczema, and rhinitis.[9] In this article, we try to follow these recommendations, although this study was performed before the World Allergy Organization suggestions.

Study population and phenotyping

Between 1995 and 1996, a cross-sectional study was performed in Munich and Dresden, Germany,[10] as part of ISAAC II to assess the prevalence of asthma and allergies in all schoolchildren attending 4th class in both cities (age 9-11 years) using standardized and validated study tools.[10-13] Within the study population of 5629 children, all children of German origin with DNA available from Munich (n = 1159) and Dresden (n = 1940) were included in this analysis (n = 3099).

Skin tests and serum measurements for total and specific IgE were performed according to standardized procedures as previously described.[10] Skin prick tests (SPTs) to 6 common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass, and tree pollen, all from ALK-Scherax, Wedel, Germany) were performed. A child was considered atopic if a wheal reaction ≥3 mm occurred to 1 or more allergens after subtraction of the negative control. Specific IgE antibodies against a panel of aeroallergens (Sx1 CAP from Phadia, Freiburg, Germany) and food allergens (FX5 CAP; Phadia) were measured in a range between 0.35 and 100 IU/mL.

Children whose parents reported a physician's diagnosis of endogenous or atopic dermatitis were classified as having eczema. Children whose parents reported a physician's diagnosis of asthma (at least once) or of spastic bronchitis or recurrent asthmatic bronchitis (at least twice) in a self-administered questionnaire were classified as having asthma. The definition of allergic rhinitis was based on the parent's information of a doctor's diagnosis of hay fever in combination with a positive SPT against at least 1 of the allergens tested.

TABLE I. Descriptive characterization of the ISAAC II study population comparing genotyped individuals included in this analysis (N = 3099)

	No.*	Percent
Male sex	1561/3099	50.4
Age (y), mean (SD)	9.6 (0.6)	
Eczema	540/2994	18.0
Atopic eczema	193/2901	6.7
Asthma	272/3054	8.9
Atopic asthma	124/2906	4.3
Allergic rhinitis	214/2938	7.3
Sensitization	777/3002	25.9
Current wheeze	256/3052	8.4
Currently visible eczema	169/3098	5.5
Current allergic rhinitis symptoms	441/3037	14.5

*Number affected/number with data available.

Eczema and asthma were divided into atopic and nonatopic on the basis of positive SPTs against at least 1 of the allergens tested. *Current allergic rhinitis symptoms* was defined as the parent's report of an itchy runny nose and sneezing in the last 12 months in the absence of a cold.

The following subphenotypes of eczema were analyzed: *visible eczema*, defined according to standardized and validated criteria at inspection determined by a trained pediatrician[14]; and *current eczema symptoms*, defined as parent's report of the presence of an itchy rash in the last 12 months that had affected the skin creases.

Written informed consent was obtained from all parents of children included in the study. All study methods were approved by ethics committees of the medical faculty of the University of Münster (ISAAC phase II data center) and the ethics committee of the Bavarian medical council (center for genetic analysis).

Genotyping

Genomic DNA was extracted from whole blood by a standard salting out method.[15] To increase DNA yield, random DNA preamplification with GenomiPhi (Amersham Biosciences, Freiberg, Germany) was performed. DNA samples were genotyped for R501X, 2282del4, and 3702delG using matrix-assisted laser desorption/ionization time-of flight mass spectrometry (www.sequenom.com). PCR assays and associated extension reactions were designed using the SpectroDESIGNER software (Sequenom, San Diego, Calif). All amplification and extension reaction conditions have been previously described.[16] Primer extension products were analyzed by a MassARRAY mass spectrometer (Sequenom; www.bdal.de), and resulting mass spectra were analyzed using the SpectroTYPER RT 2.0 software (Sequenom). Primers for genotyping were ACGTTGGATGCTGGAGGAAGACAAGGA TCG, ACGTTGGATGATGGTGTCCTGA CCCTCTTG, and ATGCCTGG AGCTGTCTC (extension primer) for R501X; ACGTTGGATGTCTTGG TGGCTCTGCTGATG, ACGTTGGATGGGGACATTCAGAA GACTCAG, and GACTCAGACACACAGT (extension primer) for 2282del4; and ACGTT GGATGTGTGAGTGTCTAGAGCTGTC, ACGTTGGATGACAAACAATC AGG AGACGGC, and GCTCCAGGCACTCAGGT (extension primer) for the 3702delG variant. Genotyping of R2447X and S3247X was performed by using the TaqMan allelic discrimination method (Applied Biosystems, Foster City, Calif), and the 3702delG genotyping was repeated using this method as also reported by Sandilands et al.[3] To control for errors and technical problems in genotyping, derived genotype frequencies were compared with the expected allelic population equilibrium based on the Hardy-Weinberg equilibrium (HWE) test.

Histology

Normal nasal tissue in 4-μm sections was obtained at excision of an unrelated dermal-based mesenchymal lesion from an otherwise healthy patient.

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Multiple sections for immunostaining were taken at regular intervals along a continuum from the free alar margin through transitional epithelium and nasal respiratory epithelium. Immunohistochemical staining was performed by using the Biogenesis antifilaggrin antibody (Biogenesis, Poole, UK) (catalog no. 4480-0905) after automated antigen retrieval by heat on the Benchmark automated stainer (Ventana Medical Systems, Tucson, Ariz).

Statistical analysis

Descriptive statistics for quantitative values are given as means (±SDs) and for qualitative values by absolute and relative frequencies. To test the fit of genotype frequencies with the HWE, a χ^2 goodness-of-fit test was used, implemented in PROC ALLELE (SAS statistical software package 9.1; SAS Institute, Cary, NC). Association of binary traits with genetic variations and thus estimation of the odds ratios (ORs) with 95% CIs was evaluated by logistic regression models using SAS 9.1. Models were adjusted for sex, and best parameter sets to fit the model were found by using backward selection methods. In a secondary approach, based on the known genetic effect of *FLG* mutations, eczema was specified as an additional covariate to evaluate genetic effects on binary traits independently of eczema. Values for total serum IgE levels were first log-transformed, and linear regression models were applied. Skin test results were analyzed by using logistic models. The population attributable risk (PAR) was calculated as follows: PAR = $[Prev_E (OR-1)]/[1+Prev_E (OR-1)]$, where $Prev_E$ is the prevalence of exposure (proportion of *FLG* mutation carriers in the population) and OR is the estimated OR from multiple regression models.[17]

RESULTS

The clinical characteristics of the study population are outlined in Table I. For the current study, 5 *FLG* mutations previously identified to result in a deficiency of filaggrin expression were genotyped in 3099 German children. The genotyping success rate was 97.3% for 2282del4, 98.9% for R501X, 97.7%% for R2447X, 96.7% for S3247X, and 97.0% for 3702delG. Carriers of 3702delG could be identified with neither matrix-assisted laser desorption/ionization time-of flight nor TaqMan genotyping in our population. Observed genotype frequencies did not deviate significantly from expected frequencies under the HWE assumption.

In this cross-sectional German population, the 2282del4 variant was present in 4.6% of children, and the frequency of carriers of R501X was 1.9%, with 0.1% of children carrying both mutations concomitantly, resulting in a combined carrier frequency of 6.4%. R2447X was present in 0.7% and S3247X in 0.2% of children. The combined carrier frequency of R501X, 2282del4, R2447X, and S3247X was 7.4% (Tables II and III).

Table IV summarizes the results of association analysis. *FLG* variants were strongly overrepresented in eczema cases (OR, 3.12; 95% CI, 2.33-4.17; $P = 2.5 \times 10^{-14}$) for combined genotype). In addition, in the total population we observed a significantly increased risk of rhinitis (OR, 2.32; 95% CI, 1.59-3.36; $P = 1.0 \times 10^{-5}$) and asthma (OR, 1.79; 95% CI, 1.19-2.68; $P = .0048$). Associations became even stronger when restricting the analysis to the allergic subphenotypes atopic eczema (OR, 4.57; 95% CI, 3.08-6.74; $P = 3.2 \times 10^{-14}$) and allergic rhinitis (OR, 2.64; 95% CI, 1.76-3.96; $P = 2.5 \times 10^{-6}$). Independent analysis of the Munich (n = 1159) and Dresden (n = 1940) populations showed associations with rhinitis and allergic rhinitis in both cohorts (see this article's Table E1 in the Online Repository at www.jacionline.org). Accordingly, *FLG* alleles conferred an increased risk for sensitization as measured by skin test (OR, 1.61; 95% CI, 1.20-2.17; $P = .0017$), which was strongest in individuals

TABLE II. Frequency of *FLG* null alleles R501X, 2282del4, R2447X, and S3247X* in children affected by eczema or allergic rhinitis and unaffected children†‡

	R501x				2282del4				R2447X				S3247X			
	Eczema		Allergic rhinitis		Eczema		Allergic rhinitis		Eczema		Allergic rhinitis		Eczema		Allergic rhinitis	
	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected	Affected	Unaffected
AA	511 (94.6%)	2395 (97.6%)	200 (93.5%)	2650 (97.3%)	479 (88.7%)	2294 (93.5%)	190 (88.8%)	2537 (93.1%)	519 (96.1%)	2386 (97.2%)	207 (96.7%)	2645 (97.1%)	525 (97.2%)	2362 (96.3%)	207 (96.7%)	2627 (96.4%)
Aa	25 (4.6%)	32 (1.3%)	11 (5.1%)	48 (1.8%)	52 (9.6%)	86 (3.5%)	21 (9.8%)	31 (4.0%)	10 (1.9%)	10 (0.4%)	1 (0.5%)	19 (0.7%)	—	6 (0.2%)	—	5 (0.2%)
Aa	—	—	—	—	—	1 (0.04%)	—	1 (0.004%)	—	—	—	—	—	—	—	1 (0.04%)

For combined genotypes of R501X, 2282del4, R2447X, and S3247X variants, the minor allele label *a* refers to the dominant mutant alleles.
*No carriers of 3702G observed.
†Unaffected children are defined as individuals without the investigated trait.
‡Percentages are given with respect to 3099 children included in the study. Individuals with missing genotype or phenotype information are not explicitly shown in the table.

TABLE III. Frequency of combined common *FLG* null alleles in children affected by eczema or allergic rhinitis and unaffected children*†

	Combined genotype (R501X, 2282del4, R2447X, S3247X)			
	Eczema		Allergic rhinitis	
	Affected	Unaffected	Affected	Unaffected
AA	437 (80.9%)	2206 (89.9%)	175 (81.8%)	2420 (88.8%)
Aa	83 (15.4%)	134 (5.5%)	33 (15.4%)	178 (6.5%)
aa	2 (0.4%)	2 (0.08%)	—	4 (0.2%)

For combined genotypes of R501X, 2282del4, R2447X, and S3247X variants, the minor allele label *a* refers to the dominant mutant alleles.
*Unaffected children are defined as individuals without the investigated trait.
†Percentages are given with respect to 3099 children included in the study. Individuals with missing genotype or phenotype information are not explicitly shown in the table.

TABLE IV. ORs and 95% CIs for associations between *FLG* mutations and selected atopic phenotypes based on a logistic regression models adjusted for sex by using backward variable selection (OR calculated for heterozygote vs wild-type)

	Combined genotype (R501X, 2282del4, R2447X, S3247X)			
Phenotype	OR	95% CI		*P* value
Eczema	3.115	2.326	4.173	2.5×10^{-14}
Atopic eczema	4.556	3.080	6.740	3.2×10^{-14}
Currently visible eczema	2.981	1.954	4.548	4.0×10^{-7}
Asthma	1.790	1.194	2.684	.0048
Atopic asthma	1.777	0.994	3.175	.0524
Current wheeze	1.752	1.156	2.655	.0082
Allergic rhinitis	2.640	1.762	3.955	2.5×10^{-6}
Current allergic rhinitis symptoms	1.686	1.200	2.371	.0026
Sensitization (skin test reactivity)	1.609	1.195	2.167	.0017
Sensitization adjusted for eczema	1.460	1.073	1.986	.0160
Allergic rhinitis adjusted for eczema	2.245	1.480	3.406	.0001
Allergic rhinitis adjusted for atopic eczema	1.925	1.214	3.053	.0053
Asthma adjusted for eczema	1.515	0.999	2.299	NS
Asthma adjusted for atopic eczema	1.375	0.851	2.222	NS
Atopic asthma adjusted for eczema	1.379	0.757	2.514	NS
Atopic asthma adjusted for atopic eczema	1.111	0.586	2.107	NS

TABLE V. Association of FLG mutations with selected atopic phenotypes in the presence and absence of eczema

	Allergic rhinitis				Asthma				Atopic asthma			
	OR	95% CI		*P* value	OR	95% CI		*P* value	OR	95% CI		*P* value
Eczema (n = 540)	2.001	1.078	3.176	.0280	1.518	0.831	2.773	NS	1.646	0.766	3.539	NS
No eczema (n = 2454)	2.471	1.415	4.316	.0015	1.525	0.855	2.721	NS	1.046	0.374	2.927	NS

with eczema (OR, 2.04; 95% CI, 1.25-3.33; $P = .0043$). Similar trends were observed for specific serum IgE levels (data not shown). The association with atopic asthma *per se* was only borderline significant (OR, 1.78; 95% CI, 0.99-3.18; $P = .0524$), whereas the complex asthma plus eczema phenotype was strongly associated (OR, 3.49; 95% CI, 2.00-6.08; $P = 1.0 \times 10^{-5}$). After adjusting for eczema or atopic eczema, associations with asthma and atopic asthma still showed a positive trend, but the association was now no longer significant. In contrast, the association with allergic rhinitis (OR, 2.25; 95% CI, 1.48-3.41; $P = 1.0 \times 10^{-4}$; and OR, 1.93; 95% CI, 1.21-3.05; $P = .0053$) remained significant (Table IV). Also, an analysis stratified for eczema showed associations with rhinitis but not with asthma (Table V). However, the association with current allergic rhinitis symptoms was clearly weaker after adjustment for eczema (OR, 1.40; 95% CI, 0.99–2.00; $P = .061$). Additional analyses of subphenotypes showed strong associations of asthma plus rhinitis (OR, 2.63; 95% CI, 1.21-5.70) and rhinitis but no asthma (OR, 2.54; 95% CI, 1.62-4.00), whereas the association of asthma but no rhinitis was only borderline significant (OR, 1.53; 95% CI, 0.941-2.495).

Immunohistochemical staining of skin in multiple, serially taken sections in the nasal vestibule up to the transitional epithelium consistently showed a typical granular pattern of filaggrin staining in the granular layer and a more diffuse pattern in the stratum corneum, consistent with normally differentiated hair-bearing skin. No filaggrin expression was seen in the transitional mucosa or respiratory mucosa (Fig 1).

For eczema, the penetrance of *FLG* null alleles was 38.5%, and the PAR, which indicates the proportion of eczema cases in the population attributable to the mutant *FLG* alleles, was 13.5% on the basis of the observed carrier frequency of mutant *FLG* alleles of 7.4% in the population. For allergic rhinitis, the PAR was 10.8%, and 8.4% when adjusted for eczema, whereas the PAR for allergic rhinitis in combination with eczema was as high as 20.1% and for asthma in combination with eczema was 15.6% (Table VI).

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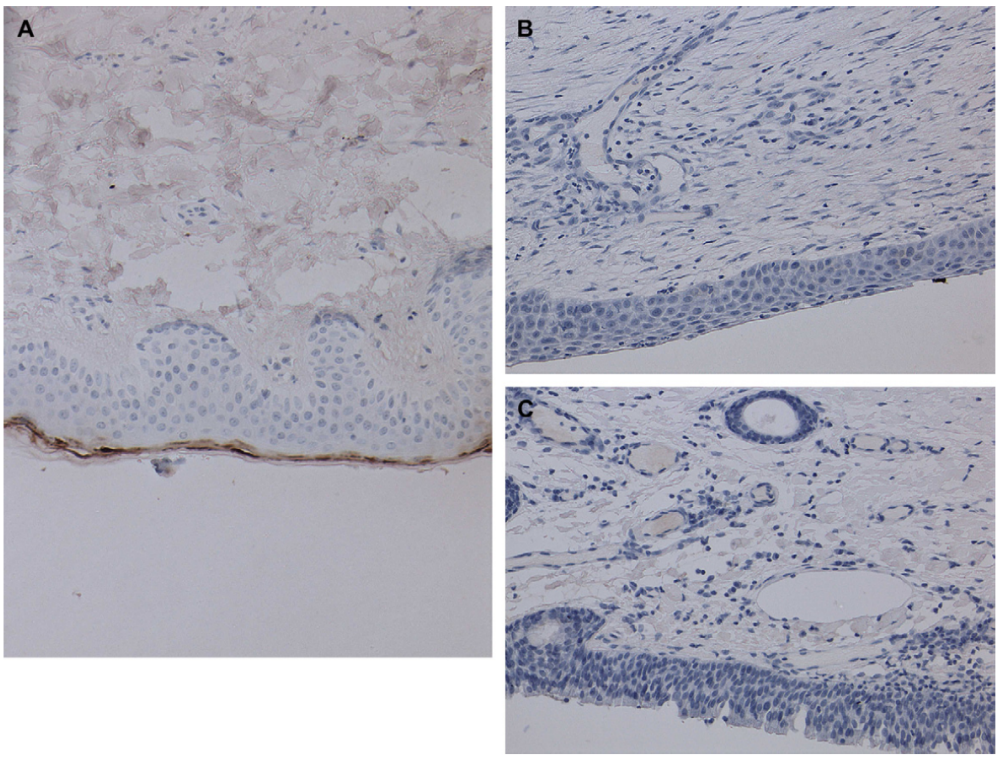

FIG 1. Nasal filaggrin expression. Sections of vestibular skin **(A)**, transitional epithelium **(B)**, and respiratory mucosa **(C)**. In the vestibular skin, staining is identical to the pattern observed in normal hair-bearing skin with strong granular staining in the granular layer and more diffuse staining of the stratum corneum. In transitional and respiratory nasal epithelia, no typical filaggrin staining is demonstrable. There is minimal edge artefact that is not significant. All original magnifications ×100.

DISCUSSION

This is a large-scale population-based study dissecting the influence of *FLG* mutations on eczema and a range of atopic diseases such as asthma and allergic rhinitis in the general population. In addition to exceptionally strong and consistent effects of *FLG* mutations on eczema and atopic eczema, associations with allergic sensitization and allergic rhinitis, independently of eczema, were demonstrated. In contrast, and in line with previous studies,[2,18,19] an association with asthma was noted, but there was no direct effect of *FLG* mutations on asthma or atopic asthma in the absence of eczema. However, as also observed in the large longitudinal population-based Avon Longitudinal Study of Parents and Children, there was a strong association with the complex asthma plus eczema phenotype with an OR of 3.49 in ISAAC II (OR for asthma plus eczema, 3.42 in ALSPAC).[20]

On the basis of these results, *FLG* must be considered to be a major gene in the development of eczema, atopic sensitization, and allergic rhinitis, with a significant effect in asthma that is restricted to patients with a history of eczema.

Filaggrin is a key protein in the formation of the outermost keratin layer of the skin,[21] with additional properties contributing to the hydration of the stratum corneum.[22] Thus, a genetically determined filaggrin deficiency was first considered and confirmed to be involved in ichthyosis vulgaris, a common disease leading to an excessive scaling of the skin, and in eczema.[1] Concerning clinically manifest eczema, *FLG*-null alleles show an incomplete penetrance of 38.5%. Recently we demonstrated a strong association of *FLG* mutations with dry skin as an intermediate trait.[23] Thus, it seems plausible that *FLG* mutations primarily cause a dry or defective skin barrier which, when driven by additional genetic and environmental factors, results in eczema in a large proportion of carriers.

Independently from eczema, *FLG* mutations showed a significant association with allergic sensitization and allergic rhinitis in our population. The association with allergic rhinitis was also independent from the presence (OR, 2.63; 95% CI, 1.21-5.70) or absence (OR, 2.54; 95% CI, 1.62-4.00) of concomitant asthma, and could be observed in both subpopulations when analyzed independently (data not shown). In contrast, the contribution of *FLG* mutations to the complex etiology of asthma *per se* seems to be limited to those with previous eczema.

Our results support recent epidemiologic data indicating an early comanifestation of atopic eczema and asthma rather than a progressive atopic march[24] and a stronger overlap between eczema and allergic rhinitis.[25,26] However, in contrast with allergic rhinitis, the observed risk for the trait current allergic rhinitis symptoms (reflecting the 12-month period prevalence of symptoms before recruitment) was only borderline significant after adjustment for eczema. This observation is in line with findings from the ALSPAC cohort, in which, in contrast with our study,

TABLE VI. PAR estimates and 95% CIs and penetrance (with exact *P* value) caused by *FLG* mutations R501X, 2282del4, R2447X, and S3247X (based on the observed carrier frequency for the combined genotype of 7.4%)

	PAR	95% CI		Penetrance
Eczema	13.5%	8.9%	19.0%	38.5% (1.3×10^{-13})
Allergic rhinitis	10.8%	5.3%	17.9%	15.4% (3.1×10^{-5})
Eczema + allergic rhinitis	20.1%	9.9%	33.4%	8.1% (9.7×10^{-6})
Eczema + asthma	15.6%	6.9%	27.3%	8.3% (3.2×10^{-5})

FLG associations with allergic rhinitis were limited to those with previous eczema.[20] In this context, it has to be considered that our study is a cross-sectional study that documents the precise and detailed phenotypes present in the study population at the time point when they are examined (in this case, 9-11 years of age) and records details of previous illnesses (physician-diagnosed eczema ever) as recalled by the parents at the time of interview. Thus, there is a possibility of recall bias against previous eczema, and it cannot be ruled out that the effect of filaggrin mutations on allergic rhinitis observed here in 2 independent populations was entirely independent of previous eczema and that *FLG* mutations exert an effect on rhinitis as a result of previous eczema, however mild. Carefully phenotyped studies will be needed to clarify these open questions.

Filaggrin is expressed in other keratinizing epithelia—for example, in the oral cavity[7] and the ocular conjunctiva.[6] So far, filaggrin has not been detected in the human bronchial epithelium.[8,27] Here, we demonstrate that filaggrin is expressed in the anterior vestibulum of the nose, but not in transitional and respiratory nasal epithelia. Thus, it seems unlikely that *FLG* mutations exert organ-specific and local effects in the upper airways. Indeed, the mechanisms through which *FLG* mutations contribute to airway disease are not understood yet. Percutaneous priming[28] and secondary immunologic effects from the induction of T_H2 cytokines in epithelia[29] are interesting hypotheses that have been put forward, but need further investigation.

FLG mutations are strongly overrepresented in children with atopic eczema or allergic rhinitis as indicated in Tables II and III. Because of the low frequencies of minor alleles of 2.40% for 2282del4 and 0.96% for R501X, individuals homozygote for the mutations are rarely found, even after genotyping 3099 individuals. Interestingly, 2 out of 3 individuals carrying 2 mutations have eczema, whereas none of them seems to be affected by rhinitis at the age of 9 to 11 years, when the survey was conducted, demonstrating that genotype-phenotype correlation is not completely linear and the penetrance of the *FLG* effect is incomplete. Although these preliminary results are intriguing, caution should be used when drawing conclusions based on the very small numbers of homozygotes seen here.

However, with the estimated penetrance of 38.5% and a PAR of 13.5% for eczema and 10.8% for allergic rhinitis as well as 20.1% and 15.6% for the complex phenotypes eczema plus rhinitis and eczema plus asthma, respectively, the effect of these single gene variants is extremely strong. The PAR for eczema plus asthma is very consistent with the ALSPAC cohort, which estimated this to be 15.6%.[20] Although previously only rough estimates of PAR based on the 2 most common variants existed,[18] this is the first assessment of the PAR truly based on data from the general population and including less frequent variants. Interestingly, both the originally identified null mutations (R501X and 2282del4) and the less common variants R2447X, S3247X, and 3702delG appear to be significantly more prevalent in United Kingdom and Irish populations (compared with continental Europe),[1,3,30] which might reflect ethnic differences. Furthermore, the genetic architecture of *FLG*-related eczema mutations seems to consist of a mixture of prevalent *FLG* mutations common to different populations and several infrequent, family-specific, and private mutations.[31]

The *FLG* gene is located within the epidermal differentiation complex (EDC) on chromosome 1q21. The EDC is a dense cluster of genes involved in the terminal differentiation of the epidermis and formation of the stratum corneum.[32] Because the EDC comprises further genes and gene families important for the differentiation of the human epidermis, it is conceivable that additional genetic variants in other genes of the EDC may additionally contribute to the risk to develop eczema.[33] Whether any of these genes may also be involved in the development of airway disease remains to be seen.

Our results may help us understand better the genetic susceptibility of complex diseases. The strong impact of *FLG* mutations with a rather low frequency on eczema and allergic rhinitis challenges the common disease-common variants hypothesis suggesting that such common diseases as asthma, eczema, and rhinitis are predominantly caused by mutations highly frequent in the population.[34,35] The fact that infrequent mutations may be associated with common diseases needs consideration, because a new generation of genome-wide association studies is emerging, predominantly based on frequent haplotype-tagging polymorphisms. Using these approaches, *FLG* effects would have been missed.

In addition, the "1 airway, 1 disease" hypothesis suggesting that the nose and the lung share the same mucosa and that therefore the same mechanisms lead to the development of rhinitis and asthma, might be an oversimplification.[36,37]

Now that *FLG* mutations have been established as a major cause of atopic eczema, their newly discovered role in the development of allergic rhinitis needs to be further elucidated, including replication of associations and systematic expression studies in the upper and lower airways. Additional large-scale and longitudinal studies will be needed to give definite answers about how *FLG* mutations may affect the development of asthma. Furthermore, ethnic differences in allele frequencies across Europe and the world need to be studied.

Assessing the effects of *FLG* deficiency has been the first step toward the understanding of a major disease mechanism in the development of eczema, asthma, and allergic rhinitis. Reconstitution of filaggrin function or compensation of filaggrin deficiency may be next steps building the groundwork for new strategies in prevention and treatment of these common diseases.

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Council (reference no. G0700314), and donations from anonymous families affected by eczema in the Tayside region of Scotland.

Clinical implications: *FLG* mutations predispose to eczema and are also risk factors for allergic sensitization and allergic rhinitis. Patients with filaggrin-related eczema are at increased risk of developing asthma.

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TABLE E1. Association of *filaggrin* mutations with allergic rhinitis in ISAAC II

	Combined genotype (R501X, 2282del4, R2447X, S3247X)			
Phenotype	**OR**	**95% CI**		***P* value**
Physician-diagnosed allergic rhinitis (Munich and Dresden combined)	2.320	1.598	3.368	1.0×10
Physician-diagnosed allergic rhinitis Munich	3.603	2.012	6.453	1.6×10^{-5}
Physician-diagnosed allergic rhinitis Dresden	1.765	1.079	2.888	.0238
Allergic rhinitis (physician's diagnosis and SPT reaction to inhalant allergen)	2.640	1.762	3.955	2.5×10^{-6}
Allergic rhinitis Munich	4.065	2.199	7.514	7.7×10^{-6}
Allergic rhinitis Dresden	1.975	1.145	3.407	.0144
Current allergic rhinitis symptoms (last 12 mo)	1.686	1.200	2.371	.0026
Current allergic rhinitis symptoms Munich	1.850	1.094	3.128	.0217
Current allergic rhinitis symptoms Dresden	1.571	1.002	2.463	.0491

Combined and independent analyses of filaggrin associations with physician-diagnosed allergic rhinitis, without/in combination with allergic sensitization to inhalant allergens and symptoms of allergic rhinitis within the last 12 months (current symptoms) in the Munich (n = 1159) and Dresden (n = 1940) samples.

2.4 Meta-analysis of filaggrin polymorphisms in eczema and asthma: Robust risk factors in atopic disease

Meta-analysis of filaggrin polymorphisms in eczema and asthma: Robust risk factors in atopic disease

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Background: The discovery of filaggrin *(FLG)* null mutations as a major risk factor for eczema represents a milestone toward the understanding of an important mechanism in this complex disease. However, published studies demonstrate differences concerning design and effect size, and conflicting results for asthma have been reported.

Objectives: We sought to provide a more accurate estimate of *FLG* effect sizes and to better refine *FLG* risk profiles within the broad and inclusive eczema diagnosis. We also sought to provide a more detailed and conclusive estimate of the risk for asthma associated with *FLG* null alleles.

Methods: We performed a meta-analysis of 24 studies on *FLG* mutations and eczema involving 5,791 cases, 26,454 control subjects, and 1,951 families as well as 17 studies on asthma involving 3,138 cases, 17,164 control subjects, and 1,511 offspring.

Results: Both case-control and family studies showed strong associations with eczema. Case-control studies were heterogeneous, whereas family studies yielded more homogeneous results. Combined analysis showed that *FLG* haploinsufficiency strongly increases the eczema risk (odds ratio [OR], 3.12; 95% CI, 2.57-3.79) and is associated with more severe and dermatologist-diagnosed disease. *FLG* mutations are also significantly associated with asthma (OR, 1.48; 95% CI, 1.32-1.66). However, although strong effects for the compound phenotype asthma plus eczema (OR, 3.29; 95% CI, 2.84-3.82) were observed, there appears to be no association with asthma in the absence of eczema.

Conclusions: This meta-analysis summarizes the strong evidence for a high eczema risk conferred by *FLG* mutations and refines their risk profiles, suggesting an association with more severe and secondary care disease. *FLG* mutations are also a robust risk factor for asthma and might help define the asthma endophenotype linked with eczema. (J Allergy Clin Immunol 2009;123:1361-70.)

Key words: *Atopic eczema, atopic dermatitis, eczema, asthma, filaggrin, meta-analysis*

(Atopic) eczema (atopic dermatitis) is the most common chronic inflammatory skin disease and affects up to 30% of children and 10% of adults in developed societies. A considerable percentage of patients with eczema (especially those with moderate or severe disease in secondary care settings) exhibit increased total IgE levels and specific sensitizations to environmental allergens, and eczema often predates the development of allergic airway disease.[1]

Atopic disorders are strongly genetic. Based on the assumption that these diseases are primarily immunologic in etiopathogenesis, for many years genetic research has focused on genes associated with immunologic abnormalities.[2] The recent identification of mutations in the gene encoding the stratum corneum structural protein filaggrin (FLG) as a remarkably strong and widely replicated risk factor for eczema,[3,4] however, has led to a paradigm shift and has placed an inherited disturbance of the epidermal barrier into the center of pathogenetic concepts.[1]

To date, 2 common *FLG* variants (R501X and 2282del4) with an estimated combined allele frequency of approximately 6%, as well as 18 less common variants, all in trans with R501X and 2282del4, have been identified in individuals of European descent. An additional 17 mutations appear to be restricted to individuals of Asian descent. Each of these reported variants lead to nonsense mutations, such as frameshifts or premature stop codons, and either prevent or severely diminish the production of free FLG in the epidermis.[4]

Although *FLG* mutations have been consistently associated with eczema, their role in predisposition to asthma remains a matter of debate.[5,6] In a meta-analysis of 9 studies that had been conducted until March 2007, we observed an overall odds ratio (OR) of 4.09 from eczema case-control studies and an OR of 2.10 from eczema family studies, with substantial heterogeneity among the

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Abbreviations used
FLG: Filaggrin
OR: Odds ratio
PAR: Population-attributable risk

studies.[7] Since then, a large number of additional studies have been published, including investigations on large population-based cohorts and in subjects primarily ascertained through a diagnosis of asthma. To assess the range of *FLG* risk on eczema and to provide a more accurate estimate of the effect size, we report the results of an updated meta-analysis based on 24 studies involving 5,791 eczema cases and 26,454 control subjects, as well as 1,951 eczema families with not less than 3,110 affected offspring. To improve appropriate interpretation, we also assessed heterogeneity and clustered studies according to their study characteristics in subanalyses. Furthermore, we tried to better define the contribution of *FLG* variants to asthma in separate analyses of 3,138 patients with asthma and 17,164 control subjects, as well as 4 family studies including 1,511 affected offspring.

METHODS

Data extraction and abstraction

The extant nosology of atopic disease is confusing, and terms such as *eczema*, *atopic eczema*, and *atopic dermatitis* are frequently used interchangeably in the literature. Recently, a World Allergy Organization report suggested the use of *eczema* as preferable to *atopic dermatitis*.[8] Following this definition, the term *eczema* includes the disease formerly called *atopic eczema* or *atopic dermatitis*, as well as *nonatopic eczema*, whereas the term *atopic eczema* is reserved for those patients with eczema and evidence of IgE involvement. It has to be considered that thus far most studies on *FLG* were performed with DNA collections that had been assembled by using older definitions without distinction between atopic and nonatopic eczema. Analyses presented in this article generally comply with the World Allergy Organization recommendations.

Four researchers (ER, EH, HB, and SW) independently identified published studies and abstracts using the search terms "atopic eczema," "atopic dermatitis," "eczema," "asthma," "FLG," "filaggrin," "R501X," and "2282del4" and their combinations in the databases MEDLINE (National Library of Medicine, Bethesda, Md, 1966-2008) and the ISI Web of Knowledge–Web of Science from the first reported association[9] up to now (April 2006- January 2009). The highest number of items was obtained by using a combination of the terms "eczema/asthma" and "filaggrin," which resulted in 35 studies retrieved from PubMed and 54 from the ISI Web of Knowledge (date of research: November 5, 2008). Different combinations of the remaining search terms did not produce any new results. Double hits, review articles, and publications without data on the effect of the genetic variations on eczema risk or asthma risk or those only providing data in Asian populations were excluded. This resulted in a final list of 26 articles. Two more publications were excluded[10,11] because they did not present original data or estimates of the ORs (including 95% CIs) of family, case-control, or population-based studies on *FLG* and eczema or *FLG* and asthma. The reference lists of all retrieved articles were also reviewed to identify publications on the same topics, which did not reveal additional studies.

The following information was abstracted from each study: study design, country of participants, definition and numbers of cases and control subjects, severity of eczema in cases, frequency of genotypes, and consistency of genotype frequencies with Hardy-Weinberg equilibrium. All studies taken into account presented preferably estimated ORs uncorrected for potential confounders or provided complete contingency tables. To broaden the analysis, we supplemented these studies with adjusted ORs if unadjusted estimates were not provided. We communicated with the primary investigators to obtain clarifications and additional data from their studies when these were not clear in the original publications.

Statistical analysis

In addition to single-mutation analysis of R501X and 2282del4 and additional less common variants in some studies,[12-15] association analysis was also performed on a combined genotype because the variants are in trans and are considered to have biologically equivalent effects.[12] Individuals carrying 1 mutated and 1 wild-type allele were coded as heterozygous and individuals heterozygous for 2 polymorphisms or homozygous for 1 polymorphism were coded as homozygous for the combined genotype.

Initially, we accounted for different study types and evaluated an overall estimate for case-control and family studies separately. For pooling both study types, we used the allelic inheritance model. Following the method of Risch and Merikangas[16] and defining the 2 alleles as A and B, with B being the mutant variant, we let the risk for individuals of genotype AB be θ times the risk for individuals with genotype AA, a genotype relative risk of θ. To facilitate grouped analysis of several family and case-control studies, we assumed a multiplicative relation for 2 B alleles, and thus the genotype relative risk for genotype BB is θ^2. Hence for studies that present a 3 $\times$ 2 contingency table for the 3 possible genotypes for cases and control subjects, we coded the respective SNP covariates as 0 for AA, 1 for AB, and 2 for BB and applied a logistic model for estimating the OR and its respective 95% CI. Because in the study by Giardina et al[17] no mutation carrier (AB genotype and BB genotype) was detected in the control group, the estimation would have generated inappropriate values. Therefore we calculated ORs and 95% CIs from the 2 $\times$ 2 table with 0.5 added to each cell.[18]

Studies that only provided estimates for ORs and 95% CIs were reviewed for the genetic model, and if the allelic model was not used, we asked the authors to provide estimates using an allelic model or supply the 3 $\times$ 2 contingency table.

In family studies the probability of a heterozygote parent to transmit the possible high-risk B allele is as follows: $P = \theta/(1+\theta)$.[16] Thus $1-P = 1/(1+\theta)$ is the inverse probability, and therefore $P/(1-P) = \theta$, which equals the ratio of transmitted B and untransmitted A (T) to untransmitted B and transmitted A (U) alleles. This can be interpreted as an approximate OR as follows[19]: $OR = \frac{T}{U}$.

T can be considered a binomial variable if conditioned on the sum of the numbers of "discordant" pairs (T + U).[19] Thus with the Δ method, we derived a symmetric confidence interval for log(OR) in family studies.

In case-control and family studies we estimated the allelic risk θ, interpreting this as an OR. Hence it is possible to combine both study types to estimate an overall OR when using logistic regression with appropriate coding of genotype variables[20] or by converting the table of genotype counts to allele counts[21] in case-control studies. In support of this methodological analysis, Knapp et al[22] showed that a haplotype relative risk estimate obtained from nuclear families with a single affected child is not larger than an increased relative risk estimate from case-control studies, assuming positive linkage disequilibrium between the marker and disease locus.

For combining the study results, we estimated the overall effect of *FLG* null alleles with a random-effects model.[23] The test of homogeneity was performed according to the method of Cooper and Hedges.[24] When the symmetry assumption held, funnel plots were constructed and the test for asymmetry by Egger et al[25] was applied to detect a potential publication bias.

The main results of meta-analyses are visualized graphically in forest and funnel plots. Forest plots show the estimated ORs and 95% CIs for risk of atopic eczema and asthma imparted by *FLG* variations for individual studies, group analyses, and combined analyses of all groups. Funnel plots might give a hint as to potential publication bias. If no publication bias exists, then the results of the individual studies are distributed symmetrically and funnel shaped around the overall estimate.

For all analyses, we used an Excel to R Interface for meta-analysis,[26] which uses the base, stats, and meta packages in R 2.6.0.[27]

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TABLE I. Association studies of *FLG* polymorphisms and eczema that met the inclusion criteria

	CC									FAM								
	R501X			2282del4			Combined			R501X			2282del4			Combined		
Study	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
Marenholz et al, 2006[35]	6.42	2.55	19.63	2.44	1.14	5.45	3.62	2.02	6.82	1.73	1.15	2.59	2.13	1.60	2.83	2.13	1.68	2.71
Palmer et al, 2006[9]	9.22	4.17	21.24	15.77	5.37	58.07	11.56	5.73	24.74									
Palmer et al, 2006[9]	–	–	–	–	–	–	2.64	1.92	3.63									
Palmer et al, 2006[9]	–	–	–	–	–	–	2.27	1.23	4.37									
Ruether et al 2006,[36]	3.39	1.75	6.58	NA	NA	NA	NA	NA	NA	2.17	1.33	3.56	–	–	–	–	–	–
Weidinger et al 2006,[28]†	4.10	2.20	7.90	2.80	1.60	4.70	3.60	2.30	5.70	3.82*	1.97*	7.42*	2.43*	1.59*	3.72*	2.73*	1.87*	3.98*
Weidinger et al 2007,[31]	3.59	1.52	9.89	5.07	2.53	11.33	4.33*	2.43*	7.70*									
Barker et al 2007,[33]‡	5.09	3.51	7.39	7.27	4.59	11.51	6.29	4.62	8.63									
Betz et al 2007,[37]	–	–	–	–	–	–	2.14	1.24	3.66									
Hubiche et al 2007,[38]†	3.35	1.39	8.62	9.14	3.10	39.48	5.59	2.81	12.06									
Lerbaek et al 2007,[34]§	3.39	0.69	13.16	2.61	0.55	9.49	2.84	0.99	7.47									
Lerbaek et al 2007,[34]	2.14	0.45	7.82	2.14	0.45	7.82	2.32	0.77	6.29									
Morar et al 2007,[29]‖	2.55	1.50	4.34	1.93	1.23	3.03	2.03	1.46	2.81	2.55	1.68	3.86	2.38	1.54	3.67	1.72	1.32	2.24
Novak et al 2008,[39]†	6.25	1.55	25.22	4.86	1.77	13.33	6.78	2.76	16.64									
Rogers et al, 2007[6]	3.09	1.41	7.04	2.02	1.09	3.75	2.38	1.48	3.90	1.38	0.55	3.42	3.75	1.24	11.30	2.40	1.15	5.02
Sandilands et al, 2007[12]	14.05	8.18	25.09	8.78	4.97	15.99	8.18¶	5.69¶	11.97¶									
Stemmler et al, 2007[40]	1.23	0.65	2.27	1.93	1.29	2.89	3.82	2.46	6.09									
Brown et al, 2008[15]	5.55	3.74	8.34	4.13	2.67	6.40	5.61¶	4.08¶	7.82¶									
Brown et al, 2008[14]	1.58	0.87	2.78	2.02	1.08	3.69	1.75¶	1.19¶	2.56¶									
Ekelund et al, 2008[41]										2.68	1.34	5.33	1.85	1.24	2.76	1.81	1.31	2.50
Giardina et al, 2008[17]	5.96#	0.28#	125.03#	1.78	0.29	13.65	–	–	–									
Henderson et al, 2008[32]	2.50	1.91	3.27	2.43	1.87	3.14	2.48*	2.06*	2.99*									
Rice et al, 2008[42]†	2.08	1.29	3.36	1.30	0.74	2.26	1.69	1.14	2.49									
Weidinger et al, 2008[13]†	3.66	2.13	6.22	2.78	1.94	3.95	3.05**	2.29**	4.04**									
Weidinger et al, 2008[30]	–	–	–	–	–	–	4.27	3.28	5.56	3.42	1.80	6.50	2.32	1.52	3.54	2.75	1.91	3.95
Weidinger et al, 2008[30]	–	–	–	–	–	–	5.47	3.71	8.08									
Weidinger et al, 2008[30]	–	–	–	–	–	–	2.27	1.90	2.70									
Pooled estimate	3.54	2.62	4.80	2.97	2.31	3.83	3.39	2.73	4.23	2.23	1.80	2.76	2.18	1.82	2.61	2.03	1.77	2.34
Pooled estimate, both study types	3.14	2.45	4.03	2.78	2.28	3.39	3.12	2.57	3.79									

CC, Case-control studies; *FAM*, family studies; *NA*, not available.
*Not considered for meta-analysis because the cohort was included in Weidinger et al.[30]
†Recalculated from original data.
‡Not considered for meta-analysis because the cohort was included in Brown et al.[15]
§Comparison with Copenhagen Prospective Study on Asthma in Childhood control subjects was not considered for meta-analysis because the control subjects were used in Palmer et al.[9]
‖The case-control approach was not included for pooled estimates, both study types, because cases and control subjects resemble family study offspring.
¶Combined genotype for mutations R501X, 2282del4, R2447X, S3247X, and 3702delG.
#No carriers of R501X were detected in the control group, and ORs were calculated with 2 × 2 contingency tables according to the method of Walter et al.[18]
**Combined genotype for mutations R501X, 2282del4, R2447X and S3247X.

RESULTS

A total of 89 publications, including published abstracts (35 from PubMed and 54 from the ISI Web of Knowledge) were identified, of which 24 met the inclusion criteria for the current meta-analyses on eczema, asthma, or both. Tables I* and II† list all case-control and family-based studies included and show the estimated ORs and 95% CIs for both R501X and 2282del4 and the combined genotype, if provided.

The 2 family panels in Morar et al[29] were combined into 1 panel, and the 2 individual panels are not displayed separately.

The family cohort in Weidinger et al[28] was enlarged with additional families and reanalyzed in Weidinger et al.[30] Therefore the former was excluded from the meta-analysis to prevent duplication. The same applies to the case-control study in Weidinger et al,[31] which was reanalyzed in Weidinger et al[30] for the combined genotype.

For the study by Sandilands et al,[12] the Irish case series analyzed in the original report on *FLG* and atopic eczema by Palmer et al[9] was enlarged with an additional 136 cases. Because the control populations in both studies have very different sample sizes (n = 186 vs 736), we considered both studies independent and assessed their influence in a sensitivity analysis. The same case series enlarged by an additional 230 cases was included in the study by Weidinger et al,[30] but the sample size of the control cohort differs largely, and thus these cohorts were also considered independent.

The Avon Longitudinal Study of Parents and Children was analyzed twice for the effect of *FLG* mutations on eczema risk,[30,32] and hence the study by Henderson et al[32] was not considered in the meta-analysis of the combined genotype and eczema.

The case cohort used by Barker et al[33] was extended and reanalyzed by Brown et al,[15] and therefore the former was dropped from the meta-analysis.

*See references 6, 9, 12–15, 17, and 28–42.
†See references 6, 9, 13, 14, 29, 31, 35, 37, 38, and 40–43.

TABLE II. Association studies of *FLG* polymorphisms and asthma that met the inclusion criteria

	Asthma			Asthma with eczema			Asthma without eczema		
Study	OR	95% CI		OR	95% CI		OR	95% CI	
Marenholz et al, 2006[35]*	3.55	1.87	7.02	5.84	2.69	13.12	2.47	1.01	5.71
Marenholz et al, 2006[35]†				2.79	1.65	4.71			
Palmer et al, 2006[9]				12.94	5.05	35.93			
Palmer et al, 2006[9]	1.73	1.31	2.30	2.64	1.92	3.63	0.97	0.64	1.44
Palmer et al, 2006[9]				2.32	0.75	6.27			
Weidinger et al, 2006[28]				3.40	1.68	6.88			
Betz et al, 2007[37]				4.95	2.29	10.36			
Hubiche et al, 2007[38]‡				5.73	1.88	16.78			
Morar et al, 2007[29]	1.52	1.13	2.05				1.28	0.61	2.48
Rogers et al, 2007[6]	1.52	0.93	2.47	2.40	1.15	5.02	1.00	0.51	1.96
Weidinger et al, 2007[31]§				6.26	3.12	12.57			
Basu et al, 2008[43]‡	1.61	1.08	2.40						
Brown et al, 2008[14],**	1.23	0.82	1.81	2.16	1.34	3.40			
Ekelund et al, 2008[41]				3.58	1.99	6.42			
Henderson et al, 2008[32]	1.28	0.99	1.63	3.38	2.36	4.74	0.78	0.42	1.32
Rice et al, 2008[42]	1.19	0.90	1.57						
Weidinger et al, 2008[13]§,***	1.79	1.22	2.63	3.49	2.00	6.08	1.45	0.83	2.56
Pooled estimate	1.48	1.32	1.66	3.29	2.84	3.82	1.11	0.88	1.41

*Multicenter Allergy Study cohort.
†Genetic Studies in Nuclear Families with Atopic Dermatitis cohort.
‡ORs for the dominant model.
§Recalculated from original data.
**Combined genotype for mutations R501X, 2282del4, R2447X, S3247X, and 3702delG.
***Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

It appears that the studies by Palmer et al[9] and Lerbaek et al[34] used the same control subjects from the Copenhagen Prospective Study on Asthma in Childhood cohort. Because Lerbaek et al[34] also compared the cases derived from the Danish Twin Register with unrelated healthy control subjects from the same register, we included this setting in the meta-analyses.

Eczema

The initial aim of this study was to provide a general and overall measure of effect size based on all available association studies on *FLG* and eczema. However, because the study characteristics varied widely (eg, in terms of definitions of eczema, study size and power, source of control subjects, case-control ratio, and phenotyping procedures), we also carried out a sensitivity analysis by grouping the studies based on their characteristics and a meta-analysis that involved only the most robust studies according to generally accepted guidelines (see Table E1 in this article's Online Repository at www.jacionline.org).[44,45]

Case-control studies

A total of 24 case-control studies on *FLG* and eczema were identified and provided data on the *FLG* combined genotype. Twenty of these studies also provided data for R501X, and 19 provided data for 2282del4, respectively. For the combined genotype, we estimated an overall allelic risk of 3.39 (95% CI, 2.73-4.23) by applying a random-effects model. ORs in the individual studies ranged from 1.69 to 11.56 (Table I and Fig 1), reflecting the large heterogeneity of 83.3%. There was no significant deviation from the symmetry assumption ($P = .2247$), and thus with additional visual inspection, there is no hint of publication bias (Fig 1). For R501X, an OR of 3.54 (95% CI, 2.62-4.80) was calculated in a random-effects model, whereas the OR for 2282del4 was 2.97 (95% CI, 2.31-3.83). For both mutations, no evidence of publication bias was detected by means of formal testing when the symmetry assumption held ($P = .422$ and $P = .194$, respectively; Fig 1).

Family studies

The 6 family studies showed a more homogeneous range of ORs (ie, from 1.72 to 2.75 for the combined genotype; Table I). This suggests a fixed-effects model because the heterogeneity measure shows 0% for both polymorphisms and 20.5% for the combined genotype. The overall OR was estimated 2.03 (95% CI, 1.77-2.34) for the combined genotype, 2.23 (95% CI, 1.80-2.76) for R501X, and 2.18 (95% CI, 1.82-2.61) for 2282del4. No significant deviation from the symmetry assumption could be detected, indicating no publication bias.

Combined analysis of all studies

Because of the interdependence, the case-control study from Morar et al[29] was not included. When combining family-based and case-control studies, we did not detect statistically significant asymmetry for the combined genotype ($P = .1316$), R501X ($P = .371$), and 2282del4 ($P = .078$), although by trend, family studies are more homogeneous and show lower ORs than case-control studies (Fig 1). In a random effects-model we estimated an overall OR of 3.12 (95% CI, 2.57-3.79) for the combined genotype, 3.14 (95% CI, 2.45-4.03) for R501X, and 2.78 (95% CI, 2.28–3.39) for 2282del4, respectively. Studies demonstrated considerable variation in frequency of mutations, in particular in cases and offspring, with carrier frequencies ranging from 0.144 to 0.558 (see Table E2 in this article's Online Repository at

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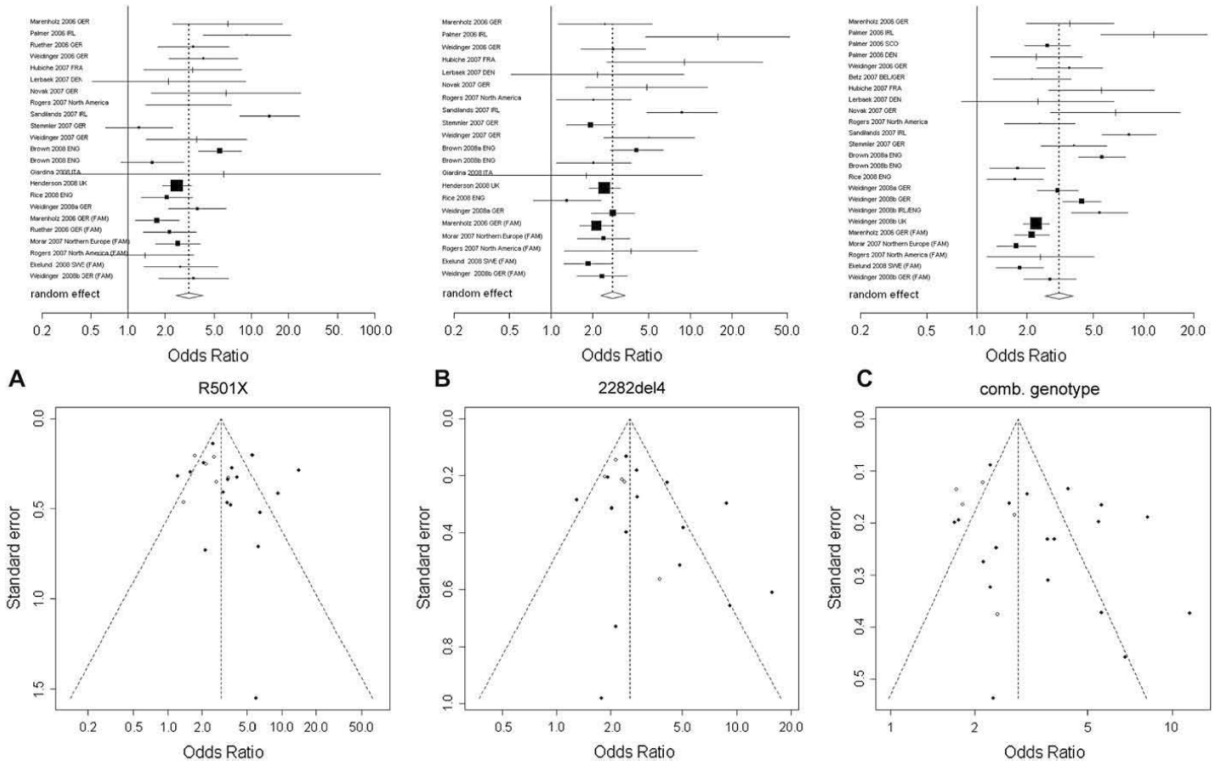

FIG 1. Association of *FLG* mutations R501X and 2282del4 and the combined genotype with increased eczema risk. Forest plots show the ORs and respective 95% CIs for the different studies included in the meta-analysis. Funnel plots show the ORs and SEs for the association. **A**, R501X. **B**, 2282del4. **C**, Combined genotype. Family studies are indicated by *FAM* in the forest plots and by *open circles* in the funnel plots.

www.jacionline.org). Based on the information given in the publications, cases and control subjects were reasonably similar in ancestry. None of the studies adjusted for population stratification (eg, by using non–disease markers).

Grouped analysis based on study characteristics

To account for differences in terms of study design, we performed a sensitivity analysis by grouping the studies based on their characteristics as far as reported in the publications or by means of direct correspondence with the investigators. For all groups, we calculated an overall estimate and restricted analysis to the combined genotype. The studies used in the sensitivity analysis and the grouping criteria are outlined in Table III.‡

Of the 25 studies included in this meta-analysis, 4 were population-based studies (overall OR for eczema, 2.19), 5 were family-based studies (OR, 2.04), and 16 were case-control studies. Five of the 16 case-control studies used population-based control subjects (OR, 4.92), whereas 7 studies used healthy outpatients as control subjects (OR, 2.77), and 4 studies involved other control subjects, such as asthmatic patients without eczema (OR, 5.09). The definition and diagnosis of eczema also differed between the studies. In 12 studies eczema was diagnosed by dermatologists by using validated criteria, such as the UK Working Party criteria or those of Hanifin and Rajka. The overall OR for eczema from these studies was 4.24 (95% CI, 3.09-5.81). In 5 studies eczema was physician diagnosed (overall OR, 2.07; 95% CI, 1.73-2.48), whereas in 4 studies cases were ascertained on the basis of a reported physician's diagnosis in the past (overall OR, 2.33; 95% CI, 1.83-2.97), and in 4 studies other criteria were applied (overall OR, 2.46; 95% CI, 1.97-3.10). Subanalysis of studies on the basis of case/control subject ratio showed overall estimates of 3.52 (<1:4), 3.25 (1:4-1:2), and 3.48 (>1:2), respectively. According to the number of cases examined, we estimated overall ORs of 2.79 (95% CI, 2.02-3.84) for studies involving more than 500 cases, 2.49 (95% CI, 1.92-3.23) for studies involving 200 to 500 cases, and 3.87 (95% CI, 2.70-5.54) for studies involving less than 200 cases. Finally, studies were grouped according to the severity of eczema in cases based on information given in the publications or provided by the investigators through direct correspondence. Studies on cases with predominantly moderate-to-severe eczema yielded a significantly higher OR of 5.16 (95% CI, 3.92-6.80) in contrast to ORs of 2.70 (95% CI, 2.01-3.64) and 2.11 (95% CI, 1.88-2.38) for studies on predominantly mild-to-moderate eczema cases and studies on inhomogeneous cases or cases with unknown severity, respectively. All results are presented in Fig 2.

‡See references 6, 9, 12–15, 28–30, 35, and 37–42.

TABLE III. Association studies of the *FLG* combined genotype and eczema used in the sensitivity analysis

Study	OR	95% CI		Design	Diagnosis	No. of cases	No. of control subjects	Total no.	Case-control ratio	Eczema severity
Marenholz et al, 2006[35]	3.62	2.02	6.82	3	2	189	321	510	>0.5	B
Palmer et al, 2006[9]	11.56	5.73	24.74	4	1	52	186	238	0.25-0.5	A
Palmer et al, 2006[9]	2.64	1.92	3.63	2	4	279	1008	1287	0.25-0.5	C
Palmer et al, 2006[9]	2.27	1.23	4.37	3	2	142	190	332	>0.5	C
Weidinger et al, 2006[28]	3.60	2.30	5.70	3	1	90	862	952	<0.25	C
Betz et al, 2007[37]	2.14	1.24	3.66	4	4	145	473	618	0.25-0.5	C
Hubiche et al, 2007[38]	5.59	2.81	12.06	4	1	99	102	201	>0.5	A
Lerbaek et al, 2007[34]	2.32	0.77	6.29	3	3	26	157	183	<0.25	C
Morar et al, 2007[29]	2.03	1.46	2.81	3	2	657	333	990	>0.5	C
Rogers et al, 2007[6]	2.38	1.48	3.90	3	4	185	461	646	0.25-0.5	C
Sandilands et al, 2007[12]	8.18	5.69	11.97	2	1	188	736	924	0.25-0.5	A
Stemmler et al, 2007[40]	3.82	2.46	6.09	3	1	378	700	1078	>0.5	A
Brown et al, 2008[15]	5.61	4.08	7.82	2	1	186	1035	1221	<0.25	A
Brown et al, 2008[14]	1.75	1.19	2.56	1	1	195	610	805	0.25-0.5	B
Novak et al, 2007[39]	6.78	2.76	16.64	2	1	56	1446	1502	<0.25	B
Rice et al, 2008[42]	1.69	1.14	2.49	1	3	267	5022	5289	<0.25	C
Weidinger et al, 2008[13]	3.05	2.29	4.04	1	3	540	2559	3099	<0.25	B
Weidinger et al, 2008[30]	4.27	3.28	5.56	2	1	773	3992	4765	<0.25	A
Weidinger et al, 2008[30]	5.47	3.71	8.08	4	1	418	552	970	>0.5	A
Weidinger et al, 2008[30]	2.27	1.90	2.70	1	3	1583	6063	7646	0.25-0.5	B
Marenholz et al, 2006[35]	2.13	1.68	2.71	0	2	490		490		C
Morar et al, 2007[29]	1.72	1.32	2.24	0	2	426		426		C
Rogers et al, 2007[6]	2.40	1.15	5.02	0	4	NA		NA		C
Ekelund et al, 2008[41]	1.81	1.31	2.50	0	1	406		406		C
Weidinger et al, 2008[30]	2.75	1.91	3.95	0	1	486		486		A

Design: *0*, family-based study; *1*, population-based (cross-sectional) study; *2*, case-control study with population control subjects; *3*, case-control study with outpatient control subjects with known eczema status; *4*, other. Diagnosis: *1*, dermatologist's diagnosis according to validated criteria; *2*, physician's diagnosis according to validated criteria; *3*, patient-reported physician's diagnosis; *4*, other. Eczema severity: *A*, predominantly moderate to severe; *B*, predominantly mild to moderate; *C*, not reported or inhomogeneous. *NA*, Not available.

In addition, we carried out a meta-analysis on the most robust studies only according to generally accepted standard criteria (ie, after exclusion of studies that appeared to violate predefined criteria, such as Hardy-Weinberg equilibrium).[44,45]

Asthma

To further clarify the inconclusive association between *FLG* and asthma, we conducted a meta-analysis of 9 studies that analyzed the trait asthma, 6 studies that analyzed the trait asthma without eczema, and 14 studies that analyzed asthma plus eczema. For all subphenotypes, we calculated an overall estimate and restricted the analyses to the combined *FLG* genotype. Studies showed considerable differences concerning the definition of asthma (eg, physician's diagnosis, self-report, or both) that were not always specified in detail.

For asthma analyzed independently from eczema status, we estimated an overall OR of 1.48 (95% CI, 1.32-1.66) by using a fixed-effects model because of an observed heterogeneity of 40.9% ($P = .095$). The test of asymmetry did not indicate any publication bias ($P = .066$). However, visually, the funnel plot shows some weak asymmetry caused by the study by Marenholz et al,[35] which might have overestimated the effect.

A stronger effect was observed for the subphenotype asthma plus eczema, with an overall OR of 3.29 (95% CI, 2.84-3.82) in a fixed-effects model (heterogeneity of 39.5%, $P = .064$). There does not appear to be an obvious publication bias toward positive findings, but both the funnel plot and the Egger test ($P = .039$) indicated that the effect for asthma plus eczema might have been overestimated in the small-sized Irish cohort within the initial study by Palmer et al.[9]

In contrast, 5 of 6 somewhat homogeneous studies found no significant association, with asthma without eczema, yielding an overall OR estimate of 1.11 (95% CI, 0.88-1.41) by applying a fixed-effects model (test of heterogeneity, $P = .284$). There was no indication toward publication bias when the symmetry assumption held ($P = .193$). All results are presented in Table II and Fig 3.

DISCUSSION

This meta-analysis of genetic association studies on the most common *FLG* variants in European populations involved 5,791 eczema cases and 26,454 control subjects, as well as 1,951 families. In addition, 3,138 asthma cases, 17,164 control subjects, and 4 family studies with 1,511 asthma-affected offspring were analyzed. This analysis provides the most comprehensive assessment thus far of the relevance of common *FLG* polymorphisms to eczema and represents the first meta-analysis on their effect on asthma. In a previous meta-analysis that involved less than half the number of individuals of the present study, we found a relative risk for eczema of about 3.58 under a dominant genetic model. The same model needs to be fitted to each dataset to combine case-control and family studies into one analysis. It is possible to fit a dominant model to family data by using conditional logistic regression,[20,46] but this would require individual-level family genotype data, which are not available. Thus to enhance direct comparability between case-control and family-based studies, we used regression methods to estimate the multiplicative allelic

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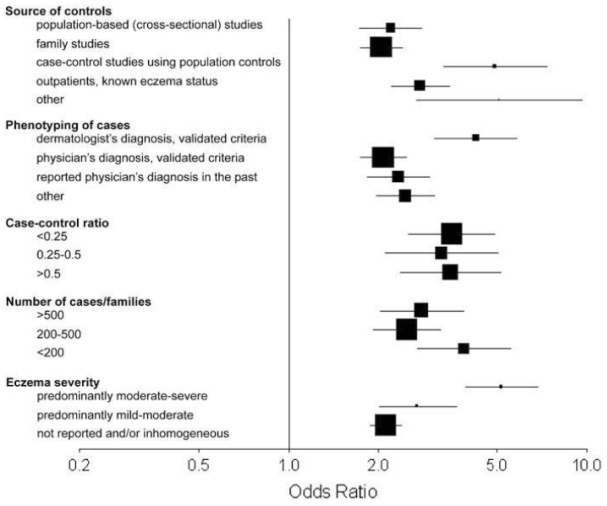

FIG 2. Association of *FLG* polymorphisms (combined genotype) with increased eczema risk grouped by study characteristics.

relative risk in both case-control and family studies.[21] However, results do not differ significantly from those obtained through a dominant model of analysis (combining the heterozygote with the homozygote mutants to compare carriers of ≥1 *FLG* null mutations with wild-type individuals, see Tables E3 and E6).

Our current analysis demonstrates that across all studies there is a high risk conferred by both null polymorphisms, with an estimated overall OR of 3.14, 2.78, and 3.12 for R501X, 2282del4, and the combined genotype, respectively, without formal evidence of publication bias. However, it has to be considered that visual inspection of funnel plots is not completely reliable and that statistical tests that are used to quantify the asymmetry have various limitations.[47] Furthermore, there appear to be large differences in allele frequencies across Europe, depending on the countries in which the participants live and their ethnicity. It has to be noted that although R501X and 2282del4 are common in populations of European ancestry, they are absent in Asian populations, in which a different spectrum of *FLG* variants has been identified. To date, nothing is known about the role of *FLG* mutations in other ethnic groups. Because none of the studies published thus far have adjusted for population stratification, inflated false-positive rates cannot be completely ruled out. However, based on the information given in the publications and provided by the authors through correspondence, cases and control subjects of the single studies did not differ significantly with respect to their ethnic background. In addition, results from case-control studies are strongly supported by family-based studies, which are robust to stratification, and do not differ significantly from those obtained from the most robust case-control studies (see Table E1 in this article's Online Repository at www.jacionline.org). Thus it appears unlikely that population stratification had a significant influence on the outcomes. The OR for eczema is higher for R501X than for 2282del4 in this meta-analysis, suggesting a possible differential risk effect with mutation site; however, there are insufficient large studies on the other uncommon recurrent null alleles to explore this possibility further. Interestingly, the effect of *FLG* on eczema in population-based cross-sectional studies, in which we assume no population stratification, is nearly the same as in family studies, further strengthening our findings and conclusions to the general population (Fig 2, Table I, and see Table E4 in this article's Online Repository at www.jacionline.org).

Although environmental factors are undoubtedly important for the development of eczema in susceptible individuals, it has been estimated in twin studies that almost 80% of the predisposition to eczema is due to genetic factors.[48-51] Eczema and atopic diseases do not follow a simple Mendelian mode of inheritance but represent complex traits with several genes that might be relevant. Thus far, *FLG* has shown the strongest effect, and the results from this meta-analysis clearly illustrate that the magnitude of its effect on the risk of eczema is higher than that of any other confirmed candidate gene for atopic diseases and one of the largest ever shown in the genetics of complex diseases.

Although an impressive number of studies in various cohorts have demonstrated a strong effect of *FLG* variants on eczema risk, with only 2 negative reports (in studies with methodological weaknesses), our overall results showed considerable between-study heterogeneity. This could be due to bias, chance, or real differences in populations or in interactions with other risk factors. Thus attention should be given to the characteristics of the individual studies. Although eczema cases were apparently selected according to appropriate criteria, it has to be considered that eczema is quite a heterogeneous disease, with considerable variability in age of onset, presentation, severity, distribution, and course. Furthermore, there were large differences in terms of study size and power and control selection. Studies also differed in the extent of testing to exclude control subjects with eczema, and in many of them, screening and ascertainment of control subjects were not described in detail. Moreover, none of the studies performed to date adjusted for population stratification.

However, the considerably extended data available at the time of this study allowed better refinement of *FLG* risk profiles within the broad and inclusive diagnosis of eczema. Interestingly, we detected a clear signal that the strongest associations for *FLG* within eczema are in dermatologist-diagnosed cases and severe-to-moderate cases (OR of 4.24 [95% CI, 3.09-5.81] and OR of 5.16 [95% CI, 3.92-6.80], respectively). Because of incomplete data in many of the

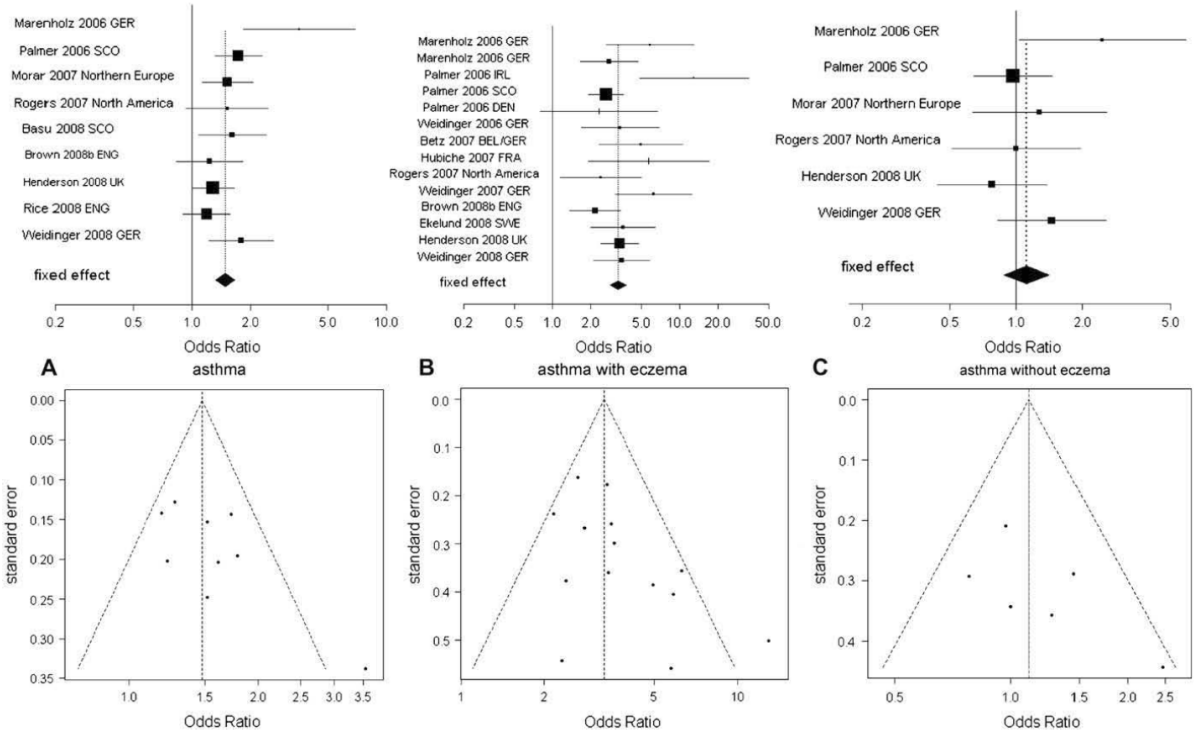

FIG 3. Association of *FLG* polymorphisms (combined genotype) with increased risk to several asthma definitions: **A**, Asthma. **B**, Asthma with eczema. **C**, Asthma without eczema. Forest plots show the ORs and respective 95% CIs for the different studies included in the meta-analysis. Funnel plots show the ORs and SEs for the association.

published studies, we were unable to properly analyze differential effects on atopic versus nonatopic eczema, as has been suggested previously.[13,28,30,31,52] Although the data are not sufficiently robust to ascertain cases as atopic versus nonatopic eczema in all cohorts, in those in which such a clear differentiation was possible, a differential effect is seen, with a stronger OR for atopic eczema (2.86) versus nonatopic eczema (2.47; see Table E5 in this article's Online Repository at www.jacionline.org).

Whereas the association of *FLG* alleles with more severe and dermatologist-diagnosed disease appears real and robust, the mechanisms that underlie this observation remain unclear. One possibility is that in addition to being a susceptibility factor for initiation of eczema, within established eczema, *FLG* null alleles function as a severity factor and amplify disease. Although speculative, this is consistent with previous longitudinal population data that suggest a persistence effect on eczema duration conferred by *FLG* null alleles.[32] More severe, more persistent, or both types of disease might also have an effect on asthma development.

Although the strong effect of a genetically determined *FLG* deficiency on eczema risk is undoubted and consistent with its expression profile and putative functions, a role for *FLG* in asthma has been hitherto less clear. Several studies have suggested that *FLG* mutations also confer an increased risk for the development of asthma. However, there was a large degree of heterogeneity between these studies (eg, in definition of asthma and sample sizes) that might have confounded the analysis. In addition, most of the studies investigated subjects with asthma ascertained through a diagnosis of eczema, whereas only a few examined asthma independently from eczema status, either in population-based cohorts or cohorts ascertained on the basis of asthma (Table II). Meta-analysis of the latter studies showed that *FLG* null alleles are significantly associated with asthma *sensu latu*, with a notable OR of 1.5. This compares quite strongly with other asthma susceptibility genes.[53,54] In particular, the association with asthma and eczema is strongest for the complex phenotype of eczema plus asthma (OR, 3.29). Further analysis based on the available data indicates that the association with asthma appears to be limited to asthma with coexpression of eczema, whereas *FLG* mutations appear to not independently affect the risk for asthma. This observation is supported by results from recent large-scale and robust studies.[6,13,32] Thus it seems conceivable that *FLG* deficiency might predispose to the particular asthma phenotype occurring in the context of eczema in contrast to the form of asthma not linked with eczema and that eczema is on the mechanistic pathway to conferral of risk.

The mechanism of the asthma risk associated with *FLG* null alleles is not yet fully understood. FLG is expressed in the skin[9] and in the outer layers of the oral and nasal mucosae[13,55] but not in the respiratory epithelium of the nose or the lower airways.[13,56] Therefore it has been suggested that *FLG*-associated asthma is

mediated through percutaneous priming[57] and/or secondary, possibly systemic, immunologic mechanisms stimulated through the impaired skin barrier. Interestingly, recent large-scale studies have shown that the risk of asthma (and rhinitis) is not restricted to those with allergic sensitization,[13,32] suggesting that the mechanistic association of eczema and later asthma cannot be simplistically reduced to excess allergen sensitization and IgE production caused by epithelial barrier failure. Clearly, further epidemiologic and mechanistic studies are needed to explore the link between *FLG* deficiency and airway disease.

In conclusion, we report here a comprehensive analysis encompassing all qualifying studies on *FLG* mutations and eczema and asthma published to January 2009. *FLG* null alleles are seen to confer a high risk of eczema, with a greater effect on moderate-to-severe disease and dermatologist-diagnosed disease and a trend toward a stronger effect with atopic or extrinsic disease. Additionally, the overall risk of asthma conferred by *FLG* null alleles is shown to be approximately 1.5 but importantly only in asthma in the context of eczema, suggesting that the complex eczema plus asthma phenotype is a distinct endophenotype of both asthma and eczema. These data suggest that *FLG*-related eczema might have a distinct phenotype and disease trajectory within the current broad definitions of eczema.

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Key messages

- **The effect of *FLG* on eczema risk is higher than that of any other confirmed candidate gene for atopic diseases and one of the largest ever shown in the genetics of complex diseases.**
- **The strongest associations for *FLG* within eczema are in dermatologist-diagnosed cases and moderate-to-severe cases.**
- ***FLG* deficiency predisposes to the particular asthma phenotype occurring in the context of eczema in contrast to the form of asthma not linked with eczema.**

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METHODS

In addition to the allelic inheritance model, a dominant inheritance model was applied for case-control studies, and results of both models were compared. In Table E3 all case-control studies for *FLG* and eczema are displayed, whereas in Table E6 all case-control studies on *FLG* and asthma are shown.

If available, allele and carrier frequencies for case-control studies were calculated, and pooling was done according to the method proposed by Thakkinstian et al.[E1] Furthermore, where appropriate, Hardy-Weinberg equilibrium for each study's control group was assessed by use of the χ^2 test.

The most robust association studies according to generally accepted criteria[E2,E3] were the subject of a separate meta-analysis, which was carried out as described in the Methods section. The following studies were included: Marenholz et al[E4] (case-control and family study), Brown et al,[E5] Weidinger et al,[E6] Weidinger et al[E7] (all 3 case-control studies and the family study), Ekelund et al,[E8] and Morar et al[E9] (family study). Because of interdependence with 1 or more of these studies, the following studies had to be excluded: Weidinger et al,[E10] Morar et al[E9] (case-control study), and Henderson et al.[E11]

For the population-based (cross-sectional) studies, the population-attributable risk (PAR) for eczema was calculated as follows: $PAR=[Prev E\,(OR-1)]/[1+Prev E\,(OR-1)]$, where *PrevE* is the pooled prevalence of exposure (proportion of *FLG* mutation carriers in the population), as determined by using the method of Thakkinstian et al,[E1] and *OR* is the pooled OR from the meta-analysis.[E12]

RESULTS

For eczema, the overall estimates obtained by using a dominant inheritance model did not differ significantly from those obtained by using the allelic inheritance model, whereas for asthma, the effects were slightly different. The lower bound of the 95% CI is higher in the dominant models compared with that in the allelic models. In summary, both inheritance models show consistent results (Table E3, Table E6, and Table I).

Meta-analyses on the most robust association studies on *FLG* and eczema yielded an overall OR of 2.96 (95% CI, 2.29–3.82; Table E1). Meta-analyses of the population-based studies showed a pooled OR of 2.19 (95% CI, 1.72–2.80), with a pooled carrier frequency (combined genotype) of 0.093 (95% CI, 0.051-0.135) and an overall PAR of 10.0% (Table E4).

For atopic eczema, higher ORs were observed compared with those for eczema in general; however, the CIs overlap, and the estimates for atopic eczema are within the CI for eczema (Table E5).

There are only 2 studies that provide information on atopic asthma. For Henderson et al,[E11] we calculated an OR of 2.26 (95% CI, 1.32-3.65), and for Weidinger et al,[E6] an OR of 1.78 (95% CI, 0.99-3.18) was observed. Although for Henderson et al[E11] this estimate is higher than that for asthma, for Weidinger et al,[E6] it is nearly identical.

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TABLE E1. Most robust association studies of *FLG* polymorphisms and eczema

	CC									FAM								
	R501X			2282del4			Combined			R501X			2282del4			Combined		
Study	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
Marenholz et al, 2006[E4]	6.42	2.55	19.63	2.44	1.14	5.45	3.62	2.02	6.82	1.73	1.15	2.59	2.13	1.60	2.83	2.13	1.68	2.71
Weidinger et al, 2007[E10]	3.59	1.52	9.89	5.07	2.53	11.33	4.33*	2.43	7.70									
Morar et al, 2007[E9]†	2.55	1.50	4.34	1.93	1.23	3.03	2.03	1.46	2.81	2.55	1.68	3.86	2.38	1.54	3.67	1.72	1.32	2.24
Brown et al, 2008[E5]	5.55	3.74	8.34	4.13	2.67	6.40	5.61‡	4.08	7.82									
Ekelund et al, 2008[E8]										2.68	1.34	5.33	1.85	1.24	2.76	1.81	1.31	2.50
Henderson et al, 2008[E11]	2.50	1.91	3.27	2.43	1.87	3.14	2.48*	2.06	2.99									
Weidinger et al, 2008[E6]	3.66	2.13	6.22	2.78	1.94	3.95	3.05§	2.29	4.04									
Weidinger et al, 2008[E7]	–	–	–	–	–	–	4.27	3.28	5.56	3.42	1.80	6.50	2.32	1.52	3.54	2.75	1.91	3.95
Weidinger et al, 2008[E7]	–	–	–	–	–	–	5.47	3.71	8.08									
Weidinger et al, 2008[E7]	–	–	–	–	–	–	2.27	1.90	2.70									
Pooled estimate + 95% CI	3.57	2.52	5.05	2.81	2.20	3.60	3.48	2.54	4.76	2.32	1.81	2.97	2.14	1.78	2.58	2.02	1.75	2.33
Pooled estimate, both study types	3.10	2.35	4.10	2.54	2.14	3.00	2.96	2.29	3.82									

CC, Case-control studies; *FAM*, family studies.

*Not considered for meta-analysis because the cohort was included in Weidinger et al.[7]

†This case-control study was not included in the pooled estimate of both study types because the cohort was included in the family study.

‡Combined genotype for mutations R501X, 2282del4, R447X, S3247X, and 3702delG.

§Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

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TABLE E2. Allele frequencies, Hardy-Weinberg equilibrium, carrier frequencies, and ethnic origin of subjects in case-control studies for *FLG* polymorphisms and atopic eczema

	Allele frequencies		HWE control subjects	Carrier frequencies		Ethnicity cases/control subjects
Study	Cases	Control subjects	*P* value	Cases	Control subjects	
Marenholz et al, 2006[E4]	0.094	0.025	0.643	0.167	0.051	Ger
Palmer et al, 2006[E14]	0.337	0.043	0.540	0.558	0.086	Ire/Ire
Palmer et al, 2006[E14]	0.127	0.049	0.083	0.229	0.093	Sco/Sco
Palmer et al, 2006[E14]	0.095	0.042	0.233	0.176	0.079	Den
Ruether et al, 2006[E15]	NA	NA	NA	NA	NA	Ger/Ger
Weidinger et al, 2006[E16]				0.367	0.138	Ger/Ger
Weidinger et al, 2007[E10]*	0.119	0.030	0.624	0.211	0.06	Ger/Ger
Barker et al, 2007[E27]†	0.273	0.046	0.248	0.423	0.088	GB/GB
Betz et al, 2007[E17]	0.083	0.040	0.376	0.152	0.08	Ger, Net/central Eur‡
Hubiche et al, 2007[E18]	0.177	0.035	0.647	0.313	0.071	Fra/Fra
Lerbaek et al, 2007[E19]§	0.115	0.042	0.235	0.231	0.079	Den/Den
Lerbaek et al, 2007[E19]	0.115	0.057	0.446	0.231	0.115	Den
Morar et al, 2007[E9]	NA	NA	NA	0.267	0.141	Mixed (white subjects, Asian subjects)
Novak et al, 2007[E20]	0.172	0.043	0.745	0.344	0.085	Ger/Ger
Rogers et al, 2007[E21]	0.092	0.035	0.442	0.144	0.071	North American (white non-Hispanic subjects)
Sandilands et al, 2007[E22]	0.287	0.039	0.918	0.452	0.076	Ire/Ire
Stemmler et al, 2007[E23]	0.087	0.022	0.207	0.158	0.043	Ger/Ger
Brown et al, 2008[E5]‖	0.299	0.059	0.696	0.457	0.115	Eng/Eng
Brown et al, 2008[E24]‖	0.113	0.065	0.302	0.184	0.129	Eng
Giardina et al, 2008[E25]	NA	NA	NA	NA	NA	Ita/Ita
Henderson et al, 2008[E11]*	0.076	0.032	0.040	0.148	0.064	Eng
Rice et al, 2008[E13]	NA	NA	NA	NA	NA	Eng
Weidinger et al, 2008[E6]¶	0.083	0.029	0.981	0.163	0.058	Ger
Weidinger et al, 2008[E7]	0.123	0.037	0.920	0.221	0.073	Ger/Ger
Weidinger et al, 2008[E7]	0.254	0.049	0.227	0.418	0.098	Eng, Ire/Ire
Weidinger et al, 2008[E7]	0.076	0.036	0.097	0.147	0.072	Eng
Pooled estimate	0.113 (0.079-0.146)	0.037 (0.022-0.052)	–	0.216 (0.174-0.259)	0.075 (0.054-0.096)	

Hardy-Weinberg equilibrium *P* values were calculated for control subjects.
HWE, Hardy-Weinberg equilibrium; *Ger*, Germany; *Ire*, Ireland; *Sco*, Scotland; *Den*, Denmark; *GB*, Great Britain; *Net*, Netherlands; *Eur*, Europe; *Fra*, France; *Eng*, England; *Ita*, Italy; *NA*, Not available.
*Not considered for pooling because the cohort was included in Weidinger et al.[E7]
†Not considered for pooling because the cohort was included in Brown et al.[E5]
‡No details available.
§Comparison with Copenhagen Prospective Study on Asthma in Childhood control subjects was not considered for pooling because control subjects were used in Palmer et al.[E14]
‖Combined genotype for mutations R501X, 2282del4, R447X, S3247X, and 3702delG.
¶Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

TABLE E3. Case-control studies on *FLG* polymorphisms and eczema that met the inclusion criteria by using a dominant genetic model

	R501X			2282del4			Combined		
Study	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Marenholz et al, 2006[E4]	6.65	2.43	18.22	2.42	1.09	5.38	3.73	1.98	7.02
Palmer et al, 2006[E14]	9.22	4.11	20.70	16.76	5.23	53.74	13.40	6.33	28.36
Palmer et al, 2006[E14]	–	–	–	–	–	–	2.89	2.04	4.11
Palmer et al, 2006[E14]	–	–	–	–	–	–	2.49	1.26	4.93
Ruether et al, 2006[E15]	3.59	1.80	7.05	7.10†	3.41†	14.78†	NA	NA	NA
Weidinger et al, 2006[E16]	4.10	2.20	7.90	2.50	1.40	4.30	3.30	2.10	5.40
Weidinger et al, 2007[E10]	3.59	1.43	9.01	5.07	2.42	10.64	–	–	–
Betz et al, 2007[E17]	–	–	–	–	–	–	2.05	1.16	3.62
Hubiche et al, 2007[E18]*	3.35	1.39	8.62	9.14	3.10	39.48	5.59	2.81	12.06
Lerbaek et al, 2007[E19]	2.14	0.54	8.51	2.14	0.54	8.51	2.32	0.82	6.53
Morar et al, 2007[E9]*	2.55	1.50	4.34	1.93	1.23	3.03	2.03	1.46	2.81
Novak et al, 2007[E20]†	6.25	1.55	25.22	4.86	1.77	13.33	6.78	2.76	16.64
Rogers et al, 2007[E21]	3.10	1.36	7.07	1.85	0.93	3.68	2.20	1.26	3.84
Sandilands et al, 2007[E22]	14.05	8.04	24.53	8.94	4.99	16.01	10.02	6.74	14.89
Stemmler et al, 2007[E23]	1.31	0.65	2.62	1.93	1.25	2.96	4.18	2.60	6.73
Brown et al, 2008[E5]**	6.19	4.03	9.51	4.15	2.62	6.56	6.46	4.47	9.32
Brown et al, 2008[E24]**	1.51	0.83	2.75	2.02	1.09	3.72	1.53	0.99	2.37
Giardina et al, 2008[E25]	5.96	0.28	125.04	1.78	0.29	10.79	–	–	–
Henderson et al, 2008[E11]	2.50	1.91	3.27	2.42	1.86	3.14	–	–	–
Rice et al, 2008[E13]*	2.08	1.29	3.36	1.30	0.74	2.26	1.69	1.14	2.49
Weidinger et al, 2008[E6]***	3.66	2.15	6.23	2.86	2.00	4.09	3.16	2.36	4.22
Weidinger et al, 2008[E7]	–	–	–	–	–	–	4.56	3.44	6.05
Weidinger et al, 2008[E7]	–	–	–	–	–	–	5.88	3.85	8.99
Weidinger et al, 2008[E7]	–	–	–	–	–	–	2.23	1.87	2.67
Pooled estimate + 95% CI	3.93	2.73	5.66	3.18	2.41	4.19	3.75	2.87	4.90
Pooled estimate + 95% CI, allelic model	3.84	2.68	5.51	3.18	2.44	4.14	3.59	2.84	4.54

NA, Not available.
*Estimates are calculated with an allelic model by using logistic regression.
†Not used for comparison of the dominant and allelic inheritance models because the allelic model was not applicable.
**Combined genotype for mutations R501X, 2282del4, R2447X, S3247X, and 3702delG.
***Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

TABLE E4. Association studies of *FLG* polymorphisms and eczema in population-based (cross-sectional) cohorts

	Combined genotype				
Study	**OR**	**95% CI**		**PAR**	**Carrier frequencies**
Brown et al, 2008[E24]	1.75*	1.19	2.56	9.6%	0.142
Henderson et al, 2008[E11]†	2.48	2.06	2.99	11.4%	0.087
Rice et al, 2008[E13]‡	1.69	1.14	2.49		
Weidinger et al, 2008[E6]	3.05§	2.29	4.04	13.6%	0.077
Weidinger et al, 2008[E7]	2.27	1.90	2.70	12.1%	0.087
Pooled estimate	2.19	1.72	2.80	10.0%	0.093

*Combined genotype for mutations R501X, 2282del4, R447X, S3247X, and 3702delG.
†Not included in the meta-analysis because it was included in Weidinger et al.[E7]
‡Not included in meta-analysis because the carrier frequency is not given.
§Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

TABLE E5. Association studies of *FLG* polymorphisms and atopic eczema

	R501X			2282del4			Combined		
Study	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Marenholz et al, 2006[E4]							3.75	1.98	7.40
Morar et al, 2007[E9]							2.28	1.46	3.56
Rogers et al, 2007[E21]							2.40	1.47	3.94
Weidinger et al, 2007[E10]	4.02	1.58	10.26	5.00	2.34	10.69	4.02	1.58	10.26
Henderson et al, 2008[E11]							3.19	2.25	4.45
Weidinger et al, 2008[E6]**							4.56	3.08	6.74
Marenholz et al, 2006[E4] (FAM)	2.09	1.26	3.48	2.38	1.70	3.33	2.49	1.86	3.34
Weidinger et al, 2006[E16] (FAM)	5.71	2.56	12.76	2.30	1.46	3.61	2.93	1.94	4.44
Ekelund et al, 2008[E8] (FAM)	4.33	1.88	9.94	2.22	1.41	3.47	2.21	1.50	3.25
Pooled estimate + 95% CI, atopic eczema	3.18	2.23	4.53	2.47	1.98	3.08	2.86	2.49	3.28
Pooled estimate + 95% CI, eczema	2.47	1.69	3.60	2.32	1.74	3.09	2.47	2.14	2.86

FAM, Family study.
**Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

TABLE E6. Case-control studies of *FLG* polymorphisms and asthma that met the inclusion criteria by using a dominant genetic model

	Allelic model									Dominant model								
	Asthma			Asthma with eczema			Asthma without eczema			Asthma			Asthma with eczema			Asthma without eczema		
Study	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Marenholz et al, 2006[E4]	3.55	1.87	7.02	5.84	2.69	13.12	2.47	1.01	5.71	3.61	1.79	4.85	2.22	10.62	14.89	2.47	1.05	5.81
Palmer et al, 2006[E14]				12.94	5.05	35.93							14.17	5.19	38.7			
Palmer et al, 2006[E14]	1.73	1.31	2.30	2.64	1.92	3.63	0.97	0.64	1.44	1.81	1.34	2.46	2.89	2.04	4.11	1.03	0.67	1.57
Palmer et al, 2006[E14]				2.32	0.75	6.27							2.78	0.92	8.42			
Betz et al, 2007[E17]				4.95	2.29	10.36												
Hubiche et al, 2007[E18]*				5.73	1.88	16.78							5.73	1.88	16.78			
Morar et al, 2007[E9]	1.27*	1.02*	1.58*				1.28	0.61	2.48	1.27*	1.02*	1.58*				1.48	0.69	3.14
Weidinger et al, 2007[E10]				6.26	3.12	12.57							6.62	3.25	13.47			
Basu et al, 2008[E26]*	1.61	1.08	2.40							1.61	1.08	2.40						
Brown et al, 2008[E24]**	1.23	0.82	1.81	2.16	1.34	3.40				1.13	0.72	1.77	1.97	1.13	3.45			
Henderson et al, 2008[E11]	1.28	0.99	1.63	3.38	2.36	4.74	0.78	0.42	1.32	1.56	1.17	2.09	3.47	2.42	4.96	0.78	0.44	1.39
Rice et al, 2008[E13]*	1.19	0.90	1.57							1.19	0.90	1.57						
Weidinger et al, 2008[E6]***	1.79	1.22	2.63	3.49	2.10	5.80	1.45	0.83	2.56	1.82	1.22	2.71	3.64	2.11	6.26	1.50	0.84	2.67
Pooled estimate + 95% CI	1.63	1.25	2.12	3.75	2.79	5.04	1.13	0.88	1.46	1.68	1.42	1.99	3.93	2.89	5.36	1.19	0.92	1.55

*Not used for comparison of the dominant and allelic inheritance models because neither the allelic model nor the dominant model was applicable.
**Combined genotype for mutations R501X, 2282del4, R2447X, S3247X, and 3702delG.
***Combined genotype for mutations R501X, 2282del4, R2447X, and S3247X.

2.5 A common variant on chromosome 11q13 is associated with atopic dermatitis

LETTERS

nature genetics

A common variant on chromosome 11q13 is associated with atopic dermatitis

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We conducted a genome-wide association study in 939 individuals with atopic dermatitis and 975 controls as well as 270 complete nuclear families with two affected siblings. SNPs consistently associated with atopic dermatitis in both discovery sets were then investigated in two additional independent replication sets totalling 2,637 cases and 3,957 controls. Highly significant association was found with allele A of rs7927894 on chromosome 11q13.5, located 38 kb downstream of *C11orf30* ($P_{combined} = 7.6 \times 10^{-10}$). Approximately 13% of individuals of European origin are homozygous for rs7927894[A], and their risk of developing atopic dermatitis is 1.47 times that of noncarriers.

Atopic dermatitis (MIM603165), or eczema, is a chronic inflammatory skin disorder and a major manifestation of allergic disease. In industrialized countries, the prevalence of atopic dermatitis is approximately 15%, and there has been a steady increase over the past decades[1]. Genetic and environmental factors interact to determine disease susceptibility[2], and family and twin studies indicate that the genetic contribution is substantial[3]. The molecular mechanisms underlying eczema are not fully understood, although skin barrier defects as well as systemic and cutaneous immune dysfunction in response to allergens or bacterial products are thought to have an important role[4].

To identify genetic variants contributing to atopic dermatitis, we conducted a genome-wide association (GWA) study in two German study populations. We genotyped 939 atopic dermatitis cases and 975 controls (set 1) as well as an independent set of 270 complete nuclear families comprising 1,097 individuals (set 2) on Affymetrix Human mapping 500K and 5.0 arrays (**Fig. 1** and **Supplementary Methods** online). A total of 342,303 markers fulfilled quality control criteria and were analyzed for association with atopic dermatitis. Individuals showing evidence of non-European admixture were excluded, and no additional evidence of genetic stratification was found by principal component analysis with Eigenstrat[5]. Comparison of the observed and expected distribution showed a moderate inflation of the test statistic in set 1 (Cochran-Armitage trend test; inflation factor $\lambda = 1.14$; **Supplementary Fig. 1a** online), which was corrected for by the genomic control method.

No single SNP achieved genome-wide significance after correction for the number of test performed ($P < 1.46 \times 10^{-7}$) in either set 1 or 2 (**Supplementary Fig. 1b**). We then focused on those genomic regions consistently associated with atopic dermatitis in both discovery sets. We required the primary associations to be significant at $P < 0.005$ in either set 1 or 2, and replicated with the same risk allele at $P < 0.05$ in the alternative discovery set (**Fig. 1**). When multiple markers in the same region fulfilled these criteria, we selected the SNP yielding the strongest association. We selected and genotyped 54 SNPs in an independent sample of 1,363 German atopic dermatitis cases and 2,739 German controls (set 3; **Supplementary Table 1** online). We expected to observe less than three association results at a nominal P value of <0.05 under the null hypothesis of no association

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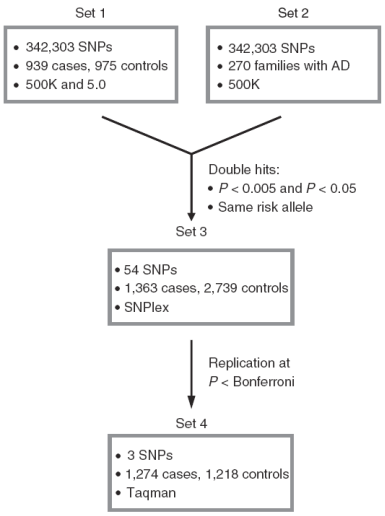

Figure 1 Study design. AD, atopic dermatitis.

(54 × 0.05 = 2.7); instead, we found nine SNPs associated with atopic dermatitis at this threshold, which is more than three times the number of positive results expected to occur by chance. Moreover, three SNPs located on chromosomes 1q21, 9p21 and 11q13 were significantly associated with atopic dermatitis in set 3 after Bonferroni correction for the 54 tests performed (corrected threshold = 0.05/54 = 0.00093).

Results on 1q21 will be shown below. Markers on 9p21 and 11q13 were tested for association in an additional replication set of 1,274 cases and 1,218 controls of European ancestry (set 4). The association finding on chromosome 9p21 was not replicated in set 4 (rs7024096; **Supplementary Table 2** online). However, a strong association with atopic dermatitis, reaching genome-wide significance in the overall analysis of the discovery and follow-up groups, was observed for the A allele of rs7927894 on chromosome 11q13.5 (combined OR = 1.22, 95% CI = 1.15–1.30, $P = 7.6 \times 10^{-10}$; **Table 1**). We computed the genotype-specific OR for rs7927894 in order to investigate the mode of inheritance more carefully. Results from all groups combined demonstrated that association of rs7927894 with atopic dermatitis did not deviate from the additive model ($P = 0.37$). Compared to noncarriers, heterozygous and homozygous carriers of the risk allele had ORs of 1.16 (95% CI = 1.02–1.32) and 1.47 (95% CI = 1.29–1.68), respectively. Considering the average frequency of the risk allele in all control populations (0.363; **Table 1**), individuals homozygous for rs7927894 (AA) represent approximately 13% of the population.

Notably, the same atopic dermatitis risk allele reported here (rs7927894 allele A) has recently been identified as a susceptibility factor for Crohn's disease[6]. Crohn's disease is a complex chronic inflammatory bowel disorder sharing many pathophysiological characteristics with atopic dermatitis, such as recurrent inflammation of the epithelial barrier, defective cutaneous or mucosal barrier function, and deficient innate immune responses against bacterial infections[7]. Present data suggest that rs7927894[A] confers susceptibility to atopic dermatitis and Crohn's disease jointly, which may contribute to the higher incidence of atopic dermatitis observed among individuals with Crohn's disease[8,9].

rs7927894 is located in an intergenic region 38 kb downstream of *C11orf30* (chromosome 11 open reading frame 30) and 68 kb upstream of *LRRC32* (leucine rich repeat containing 32). To identify a potential link to atopic dermatitis, we performed RT-PCR analysis, which showed that both *C11orf30* and *LRRC32* are ubiquitously expressed, including in tissues relevant to atopic dermatitis such as skin and peripheral blood mononuclear cells (**Supplementary Fig. 2** online). Additionally, we assessed a possible regulatory role of rs7927894 on *C11orf30* or *LRRC32* gene expression in the publicly available dataset generated by Dixon *et al.*[10], who conducted a genome-wide association study for global mRNA expression in lymphoblastoid cell lines from asthmatic children. There was no evidence for a *cis*-regulatory effect of rs7927894. However, the possibility of a regulatory effect in another tissue or physiological state cannot be ruled out.

Linkage disequilibrium (LD) analysis in HapMap showed that rs7927894 is located in a 200-kb LD block containing *C11orf30* (**Fig. 2**), suggesting that the functional variant responsible for the observed association may be located within the *C11orf30* region. However, the possibility remains that rs7927894 may be in LD with a functional variant not genotyped in the HapMap project and located outside this LD block. *C11orf30* encodes the nuclear protein EMSY, which binds and inactivates the cancer susceptibility gene *BRCA2*. EMSY associates with chromatin regulators HP1β and BS69, and localizes to sites of repair following DNA damage. It has been implicated in chromatin modification, DNA repair and transcriptional regulation. An increase in *C11orf30* copy number has been reported in epithelium-derived cancer of the breast and ovary[11]. The potential involvement of *C11orf30* in multiple inflammatory and

Table 1 Association of rs7927894[A] on 11q13.5 with atopic dermatitis

	Number		RAF or T:U			
Study population	Cases	Controls	Cases	Controls	OR (95% CI)	*P* value
Discovery groups (GWA)						
Set 1[a]	939	975	0.409	0.372	1.18 (1.03–1.33)	0.027
Set 2	529 (270 families)		294:226		1.30 (1.09-1.55)	0.001
Follow-up groups						
Set 3[a]	1,363	2,739	0.407	0.363	1.21 (1.10–1.33)	2.46×10^{-4}
Set 4	1,274	1,218	0.415	0.357	1.29 (1.15–1.45)	1.94×10^{-5}
GWA[b]						3.83×10^{-4}
Follow-up groups[b]	2,637	3,957	0.411	0.361	1.24 (1.15–1.33)	9.62×10^{-8}
All combined[bc]	4,105	4,932	0.411	0.363	1.22 (1.15–1.30)	7.64×10^{-10}

All *P* values are two-sided. Shown are the number of cases and controls in each data set with the corresponding risk allele frequency (RAF). The number of individuals in set 2 refers to the number of atopic dermatitis cases (529) and the number of families (270). Odds ratios (OR) were calculated by logistic regression analysis and *P* values with the Cochran-Armitage trend test. For set 2, the number of transmissions versus nontransmissions are shown (T:U). OR in set 2 represents the ratio of transmitted/untransmitted alleles.
[a]Results for set 1 and set 3 were adjusted by the genomic control method ($\lambda = 1.14$, **Supplementary Fig. 1**). [b]For the combined analysis, the OR and *P* values were calculated using the inverse variance[28] and Fisher's method[27], respectively. [c]Results from the family-based set 2 were not included in the calculation of allele frequencies and OR for the combined population.

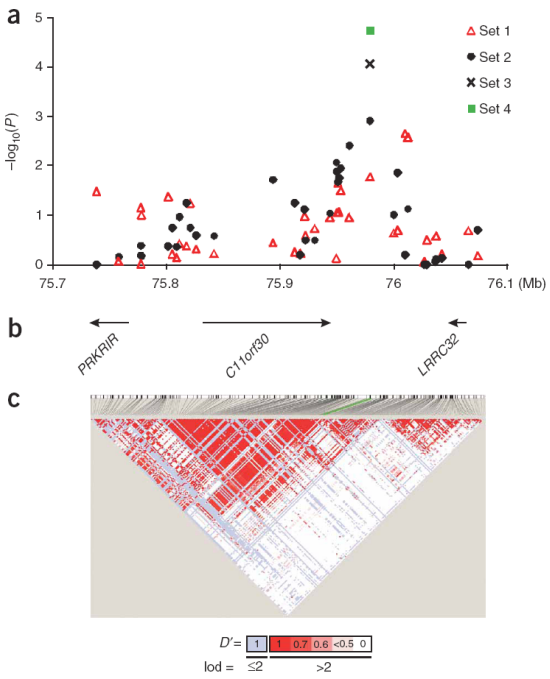

Figure 2 Association results and LD structure in the atopic dermatitis–associated region on 11q13.5. (**a**) Association results for all GWA markers tested in the region. Physical positions are based on NCBI build 36. (**b**) Genes in the region from the UniGene database. (**c**) LD in the CEU HapMap population. Disequilibrium coefficient values for Hapmap Phase II data (v. 22) were generated with Haploview. The lead SNP is indicated by a green line.

malignant epithelial diseases (atopic dermatitis, Crohn's disease and adenocarcinoma) strongly suggests a role for *C11orf30* in epithelial immunity, growth and/or differentiation.

An additional association signal was observed in the epidermal differentiation complex (EDC) on chromosome 1q21, which contains a number of genes that are required for epidermal structure and function[12]. Genotyping of rs6661961 in set 3 confirmed the association reaching genome-wide significance ($P_{\text{combined set 1,2,3}} = 1.2 \times 10^{-9}$; **Table 2**). Data from both discovery sets revealed the presence of multiple disease-associated markers spanning 600 kb within the EDC (**Fig. 3**). Because of the proximity of the well-established atopic dermatitis susceptibility gene *FLG* (filaggrin)[13], we evaluated whether the observed association was due to LD with *FLG* mutations or to additional genetic factors. We genotyped all four *FLG* mutations previously reported in the German population (R501X, 2282del4, R2447X and S3247X)[14] in all study samples. We observed markedly high LD between the *FLG* mutations and rs6661961, which is located 156 kb away (**Supplementary Fig. 3** online). Exclusion of *FLG* mutation carriers abolished the observed association ($P > 0.1$; **Table 2**). This result indicates that the association signals in the EDC were due to *FLG* mutations, reinforcing their role in atopic dermatitis and supporting the robustness of the study. It has been shown that the spectrum of *FLG* mutations varies among human populations, with the more prevalent European mutations (R501X and 2282del4) absent in African and Asian populations[13,15]. This suggests that the mutational events occurred after the separation of the respective human populations. The high LD observed between *FLG* mutations and SNPs located as far as 500 kb away (**Supplementary Fig. 3**) supports this hypothesis and allowed us to the detect *FLG* in our scan.

Notably, a set of markers surrounding the *HRNR* (hornerin) gene, located 78 kb away from *FLG*, showed a strong association with atopic dermatitis after exclusion of *FLG* mutation carriers in both discovery sets (**Fig. 3** and **Table 2**). Marker rs877776 yielded the highest association ($P_{\text{set1}} = 0.0003$, $P_{\text{set2}} = 0.005$; **Table 2**) and was then genotyped in set 3 and set 4. Again, risk allele C of rs877776 showed an increased frequency in cases compared to controls after exclusion of *FLG* mutation carriers, although the trend was not significant ($P_{\text{replication sets 3, 4}} = 0.104$). Altogether, these data are consistent with that of a previous report[16] and suggest that additional atopic dermatitis risk factors exist in the EDC apart from prevalent *FLG* mutations (rs877776, $P_{\text{combined}} = 3.5 \times 10^{-5}$). rs877776 is in LD with a number of candidates involved in skin barrier function (**Fig. 3**), including the genes encoding hornerin, filaggrin, filaggrin2, cornulin and late cornified envelope protein 5A, suggesting that the remaining atopic dermatitis risk factor may be located within or between any of these genes. Finally, no evidence for genetic interaction was observed after testing all possible combinations between the *FLG* mutations, rs877776, and the lead SNP on chromosome 11 (rs7927894).

Six additional loci were nominally replicated at $P < 0.05$ in set 3 but were not significant after multiple-testing correction and thus

Table 2 Association results for the lead SNPs in the EDC before and after stratification for *FLG* mutations

		Full sample		Non-*FLG* mutation carriers	
Marker		rs6661961	rs877776	rs6661961	rs877776
Minor allele		A	C	A	C
GWA set 1	Cases	0.498	0.169	0.408	0.191
	Controls	0.430	0.137	0.409	0.139
	OR	1.31	1.28	0.99	1.46
	P	1.2×10^{-4}	0.01	0.939	2.8×10^{-4}
GWA set 2	T:U	281:217	164:122	150:134	120:75
	OR	1.30	1.34	1.12	1.60
	P	0.003	0.02	0.86	0.005
Follow-up set 3	Cases	0.484	0.156	0.406	0.175
	Controls	0.432	0.158	0.414	0.163
	OR	1.23	0.98	0.97	1.10
	P	2.9×10^{-5}	0.82	0.565	0.19
Follow-up set 4	Cases		0.163		0.181
	Controls		0.159		0.164
	OR		1.02		1.13
	P		0.77		0.13
All combined[a]	OR		1.06		1.20
	P		0.027		3.52×10^{-5}

Shown are the corresponding allelic frequencies of the minor allele and the number of transmitted versus untransmitted alleles for set 2. Non-*FLG* mutation carriers refers to the individuals remaining after exclusion of all individuals or families carrying any of the four genotyped mutations (665 cases, 873 controls in set 1; 161 families in set 2; 984 cases, 2,436 controls in set 3; 980 cases, 1,102 controls in set 4). Note that individuals or families with missing *FLG* mutation genotypes were also excluded.
[a]Results from the family-based set 2 were not included in the calculation of allele frequencies and OR for the combined population.

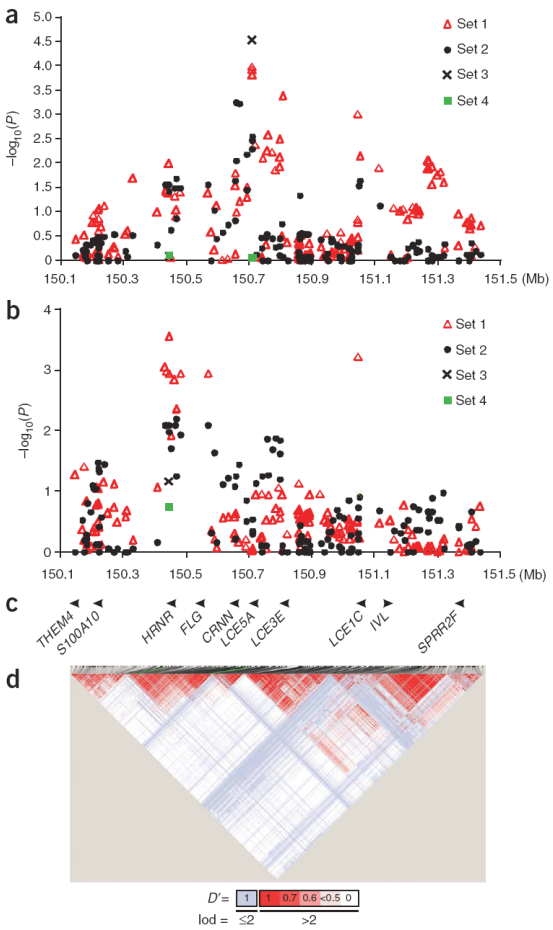

Figure 3 Association results and LD structure in the epidermal differentiation complex (EDC) on 1q21. (**a**) Association results for all GWA markers tested in the region. (**b**) Association results after exclusion of all individuals or families carrying any of the four tested *FLG* mutations. (**c**) Genes in the region from the UniGene database. Owing to the size of the genomic region shown (1.4 Mb) and the high number of genes contained, only selected genes are shown as a reference. (**d**) LD in the CEU HapMap population. Disequilibrium coefficient values for HapMap Phase II data (v. 22) were generated with Haploview. Markers rs877776 and rs6661961 are indicated by a green line.

were not genotyped in set 4 (**Supplementary Table 3** online). Some of these loci affect immune processes related to atopic dermatitis pathogenesis, such as the differentiation of type 2 T helper cells, mast cells, eosinophil and dendritic cell chemotaxis, and pruritus (*HRH4*)[17], Toll-like receptor-mediated responses to bacteria (*LY86*, also called *MD-1*)[18], and $CD8^+$ T cell function (*EOMES*)[19]. Power calculations indicate that our discovery sets had >84% power to detect variants with a relative risk of 1.5 and a minor allele frequency of 20% at $P < 0.005$. The power drops to 12% for a relative risk of 1.2 and the same allele frequency (**Supplementary Methods**). These data suggest that additional low-risk factors remain to be discovered for atopic dermatitis, and bigger samples sizes will be required to identify and replicate them.

Here we present the first genome-wide association study for atopic dermatitis. We identified a common sequence variant on chromosome 11q13.5 associated with atopic dermatitis in all four study groups including more than 4,100 atopic dermatitis cases. Moreover, we provide a list of additional candidate genes, including a new susceptibility locus within the EDC. Further replication in additional cohorts, fine mapping and functional studies will be required to gain a better understanding of the physiological mechanisms underlying this common allergic disorder.

METHODS

Study participants. We evaluated four independent study groups (**Supplementary Methods**). All cases and controls were of European origin. The institutional ethics review boards of the participating centers approved the study protocol, and informed consent was obtained from all probands or their legal guardians. In all four study groups, the physician's diagnosis of eczema was made according to standard criteria in the presence of a chronic or chronically relapsing pruritic dermatitis with the typical morphology and distribution[20,21].

All samples in sets 1, 2 and 3 were German. Set 1 included 939 atopic dermatitis cases and 975 controls; set 3 consisted of 1,363 atopic dermatitis cases and 2,739 controls. All atopic dermatitis cases from sets 1 and 3 were obtained from German university hospitals (Charité Universitätsmedizin Berlin, University of Kiel, Technical University Munich, and University of Bonn), and were of self-reported German ancestry. All German controls in sets 1 and 3 were derived from the previously described population-based Popgen Biobank[22].

Samples in set 2 were recruited at Charité Universitätsmedizin Berlin and consisted of 270 complete nuclear families comprising 1,097 individuals and including 529 children with atopic dermatitis. Families were recruited through two affected siblings with an age of onset below two years of age and moderate-to-severe disease expression.

Set 4 consisted of 1,274 atopic dermatitis cases and 1,218 geographically matched controls from Germany, the Czech Republic and Poland. German atopic dermatitis cases ($n = 456$) were recruited at Charité Universitätsmedizin Berlin. All 360 Czech and 458 Polish atopic dermatitis cases had a positive family history of atopy and were recruited for the EPAAC (Early Prevention of Asthma in Atopic Children, $n = 795$) and ETAC (Early Treatment of the Atopic Child, $n = 23$) trials, two similar randomized, double-blind, placebo-controlled studies on the efficacy of levocetirizine or cetirizine, respectively, in the prevention of asthma[23,24]. The Czech, Polish and German controls were obtained from blood donation programs in Prague, Szczecin and Dresden, respectively. A summary of the study groups is included in **Supplementary Methods**.

Genotyping and quality control of the data. Genotyping for the GWA study was done with Affymetrix 500K and 5.0 Arrays. According to the manufacturer's recommendations, we discarded samples with call rates <93% or 86% after preliminary quality control calling with the Dynamic Model algorithm for 500K and 5.0 arrays, respectively. Samples showing evidence of cryptic relatedness to other study participants or non-European ancestry were excluded. A summary of excluded samples is presented in **Supplementary Methods**. Principal components analysis with Eigenstrat[5] revealed no evidence of population stratification.

We excluded SNPs that had low genotyping rate (<95%), deviated from Hardy-Weinberg equilibrium ($P_{HWE} < 0.0005$ in controls), showed excess heterozygosity (>0.55) or were rare (minor allele frequency (MAF) <1%). The genotype concordance rate between the 500K and 5.0 arrays was evaluated by genotyping 82 samples with both platforms. A group of 8,753 markers with more than two discordant genotypes in this set of 82 genotype pairs were excluded from further analysis, yielding a final genotype concordance rate of 99.9%. Additional quality control criteria are described in **Supplementary Methods**.

All replication genotyping in sets 3 and 4 was done with ligation-based SNPlex or Taqman following the assay manual (see **Supplementary Methods** for additional details).

Statistical analysis. SNP association with atopic dermatitis in sets 1, 3 and 4 was analyzed using the Cochran-Armitage test (ATT) with additive trend as implemented in PLINK[25]. In discovery set 1, we observed a moderate variance inflation ($\lambda = 1.14$) in the distribution of the test statistic (median χ^2_{1df} of the Cochran-Armitage trend test) when compared to the median of the expected distribution under the null hypothesis of no association (**Supplementary Fig. 1a**). After genomic control correction, no evidence of inflation remained in set 1, suggesting that no systematic bias exists in the dataset. To address potential genetic stratification in set 3, we adjusted the *P* values in set 3 with the variance inflation ($\lambda = 1.14$) observed in set 1 because both populations originated from the same study centers in Germany. Additionally, we used available genome-wide Illumina HapMap300 SNP genotypes[26] from control individuals originating from Germany, the Czech Republic and Poland to estimate the expected variance inflation (λ) in set 4. No evidence of variance inflation was observed in this analysis, and thus, no lambda correction was applied to the set 4 results (see **Supplementary Methods** for detailed description). Family-based association analysis in set 2 was done using the transmission disequilibrium test (TDT) implemented in PLINK[25]. An empirical *P* value obtained with a permutation procedure was used to account for the non-independence of the two affected sibs included for each family. Odds ratios (OR) for sets 1, 3 and 4 are based on logistic regression without adjustment for covariates and represent the risk for atopic dermatitis associated with each copy of the risk allele. ORs for set 2 were calculated with PLINK and indicate the ratio of transmissions versus nontransmissions for the risk allele. Combined *P* values were obtained using Fisher's method[27]. ORs were combined by the weighted average of study-specific estimates of the ORs using inverse variance weights[28]. After combining the results from sets 1, 3 and 4, the multiplicative model provided an adequate fit for rs7927894 on chromosome 11. The risk predicted by the genotype-specific model was not significantly different from that predicted by the log-additive (or multiplicative) genetic model ($P = 0.37$), obtained with a likelihood-ratio test. We conducted logistic regression analyses to examine multiplicative gene–gene interaction between the *FLG* mutations and the markers rs7927894 (chromosome 11) and rs877776 (chromosome 1). Regarding the *FLG* genotypes, individuals were coded as noncarriers of any mutations (code 0), as heterozygote carriers (code 1) or as homozygote/composite heterozygote mutation carriers (code 2). For rs877776 and rs7927894, individuals were coded according to the number of minor alleles carried (0, 1 or 2).

Power calculations in case-control analysis were performed with the Genetic Power Calculation software[29], and a previously described method[30] was used to compute power for family-based set 2 (see **Supplementary Methods**).

Tissue expression pattern. We examined the tissue-specific expression of *C11orf30, LRRC32* and *GAPDH* using oligo(dT)-primed cDNA of 17 different human tissues. cDNA samples of 16 tissues were from the human MTC Panels I and II, which are standardized for the expression of *GAPDH* (BD Biosciences). In addition, human skin RNA from a healthy donor was extracted using Trizol (Invitrogen), treated with DNAse and transcribed into cDNA using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics). The *GAPDH* PCR was carried out using the primers provided with the MTC panels, and the *C11orf30* and *LRRC32* PCRs were done using specific primers listed in **Supplementary Table 4** online.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

J.E.G., Y.A.L., S.W., A.R. and N.H. designed the study. Y.A.L. directed the study. Y.A.L., J.E.G., S.W., R.F.-H., U.W., S.S., N.N., M.L.-K., A.C., M.K., T.P. and M.M. provided the case and control samples. J.E.G., S.W., A.R., G.P., A.F., R.V., H.B., T.I. and Y.A.L. contributed to biobanking and management of clinical data. J.E.G., G.P., I.M., F.S. and T.K. performed 500K array and Taqman genotyping. A.R. did the SNPlex genotyping; E.R. contributed to DNA extraction, biobanking and *FLG* genotyping of Munich samples. A.B., F.R., K.R., J.E.G., N.H. and Y.A.L. conducted all statistical analyses and interpreted the data. S.H. estimated population stratification in set 4. J.E.G. and Y.A.L. wrote the manuscript.

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2.6 Genome-wide scan on total serum IgE levels identifies FCER1A as a novel susceptibility locus

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Genome-Wide Scan on Total Serum IgE Levels Identifies *FCER1A* as Novel Susceptibility Locus

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Abstract

High levels of serum IgE are considered markers of parasite and helminth exposure. In addition, they are associated with allergic disorders, play a key role in anti-tumoral defence, and are crucial mediators of autoimmune diseases. Total IgE is a strongly heritable trait. In a genome-wide association study (GWAS), we tested 353,569 SNPs for association with serum IgE levels in 1,530 individuals from the population-based KORA S3/F3 study. Replication was performed in four independent population-based study samples (total n = 9,769 individuals). Functional variants in the gene encoding the alpha chain of the high affinity receptor for IgE (*FCER1A*) on chromosome 1q23 (rs2251746 and rs2427837) were strongly associated with total IgE levels in all cohorts with *P* values of 1.85×10^{-20} and 7.08×10^{-19} in a combined analysis, and in a post-hoc analysis showed additional associations with allergic sensitization ($P=7.78\times10^{-4}$ and $P=1.95\times10^{-3}$). The "top" SNP significantly influenced the cell surface expression of FCER1A on basophils, and genome-wide expression profiles indicated an interesting novel regulatory mechanism of FCER1A expression via GATA-2. Polymorphisms within the *RAD50* gene on chromosome 5q31 were consistently associated with IgE levels (*P* values 6.28×10^{-7}–4.46×10^{-8}) and increased the risk for atopic eczema and asthma. Furthermore, *STAT6* was confirmed as susceptibility locus modulating IgE levels. In this first GWAS on total IgE *FCER1A* was identified and replicated as new susceptibility locus at which common genetic variation influences serum IgE levels. In addition, variants within the *RAD50* gene might represent additional factors within cytokine gene cluster on chromosome 5q31, emphasizing the need for further investigations in this intriguing region. Our data furthermore confirm association of *STAT6* variation with serum IgE levels.

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Author Summary

High levels of serum IgE are considered markers of parasite and helminth exposure. In addition, they are associated with allergic disorders, play a key role in anti-tumoral defence, and are crucial mediators of autoimmune diseases. There is strong evidence that the regulation of serum IgE levels is under a strong genetic control. However, despite numerous loci and candidate genes linked and associated with atopy-related traits, very few have been associated consistently with total IgE. This study describes the first large-scale, genome-wide scan on total IgE. By examining >11,000 German individuals from four independent population-based cohorts, we show that functional variants in the gene encoding the alpha chain of the high affinity receptor for IgE (*FCER1A*) on chromosome 1q23 are strongly associated with total IgE levels. In addition, our data confirm association of *STAT6* variation with serum IgE levels, and suggest that variants within the *RAD50* gene might represent additional factors within cytokine gene cluster on chromosome 5q31, emphasizing the need for further investigations in this intriguing region.

Introduction

High levels of IgE have been considered for many years as markers of parasite and helminth exposure to which they confer resistance [1]. In Western lifestyle countries with less contact, however, elevated IgE levels are associated with allergic disorders [2]. Only recently, it has been established that IgE antibodies also play a key role in anti-tumoral defence [3] and are crucial mediators of autoimmune diseases [4], thus challenging the traditional Th1/Th2 dogma.

High total serum IgE levels are closely correlated with the clinical expression and severity of asthma and allergy [5,6]. The regulation of serum IgE production is largely influenced by familial determinants, and both pedigree- and twin-based studies provided evidence of a strong genetic contribution to the variability of total IgE levels [7,8]. Genetic susceptibility of IgE-responsiveness is likely to be caused by a pattern of polymorphisms in multiple genes regulating immunologic responses[9], but so far only very few loci could be established consistently and robustly, most notable *FCER1B*, *IL-13* and *STAT6* [10,11].

Family and case-control studies indicated that total serum IgE levels are largely determined by genetic factors that are independent of specific IgE responses and that total serum IgE levels are under stronger genetic control than atopic disease [8,12,13,14]. An understanding of the genetic mechanisms regulating total serum IgE levels might also aid in the dissection of the genetic basis of atopic diseases. In an attempt to identify novel genetic variants that affect total IgE levels, we conducted a genome-wide association study (GWAS) in 1,530 German adults and replicated the top signals in altogether 9,769 samples of four independent study populations.

Results

Genome-wide Association Scan

For the GWAS 1,530 individuals from the population-based KORA S3/F3 500 K study with available total IgE levels were typed with the Affymetrix 500 K Array Set. For statistical analysis, we selected SNPs by including only high-quality genotypes to reduce the number of false positive signals. A total of 353,569 SNPs passed all quality control measures and were tested for associations with IgE levels. Figure 1 summarizes the results of the KORA S3/F3 500 K analysis. No single SNPs reached genome-wide significance, but the scan pointed to the gene encoding the alpha chain of the high affinity receptor for IgE (*FCER1A*) on chromosome 1 (Figure 1A). Particularly the quantile-quantile-plot of the *P* values illustrates observed significant associations beyond those expected by chance (Figure 1B).

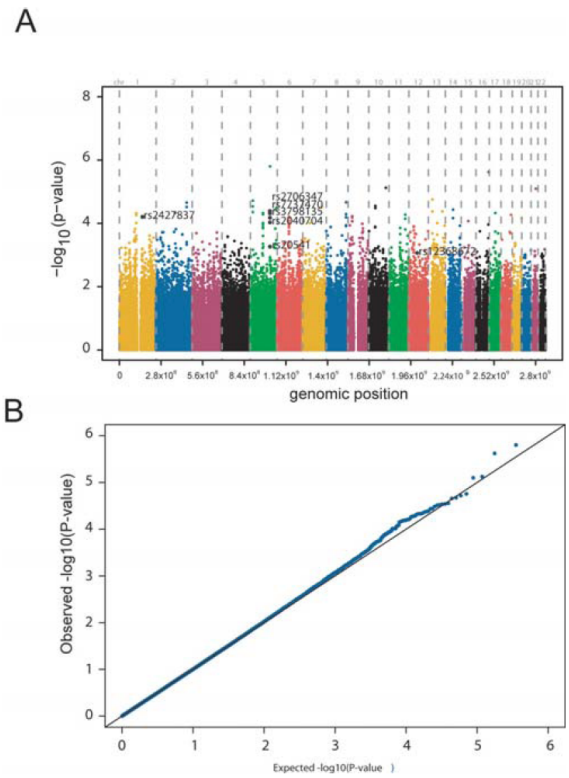

Figure 1. Results of the KORA S3/F3 500 K analysis. a) Genome-wide association study of chromosomal loci for IgE levels: the analysis is based on a population-based sample of 1530 persons. The x-axis represents the genomic position of 353,569 SNPs, and the y-axis shows −log10 (*P* value). b) Quantile-quantile plot of *P* values: Each black dot represents an observed statistic (defined as the −log10(*P* value)) versus the corresponding expected statistic. The line corresponds to the null distribution.
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Replication and Fine-Mapping

For replication in the independent population-based KORA S4 cohort (N = 3,890), we used the following inclusion criteria: (i) $P<10^{-4}$ in the genome wide analysis (39 SNPs, 35 expected); (ii) $P<10^{-3}$ with at least one neighboring SNPs (±100 kb) with $P<10^{-3}$ (45 SNPs). The specific results for all SNPs in the GWAS and KORA S4 are given in supplementary table S3. Six SNPs were significantly associated with total IgE levels in KORA S4 with *P* values ranging from 2.47×10^{-4} to 3.23×10^{-9} (given a Bonferroni-corrected significance level of 5.10×10^{-4}). The strongest associations were observed for rs2427837 ($P=3.23\times10^{-9}$), which is located in the 5′ region of *FCER1A*, and rs12368672 ($P=2.03\times10^{-6}$), which is located in the 5′ region of *STAT6*. In addition, all 4 *RAD50* SNPs which had been selected in the GWAS could be replicated.

Effect estimates of the SNPs in *FCER1A* and *STAT6* were only slightly lower compared to those in the KORA S3/F3 500 K

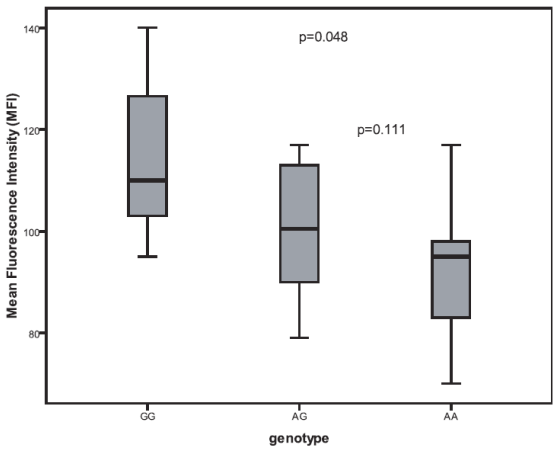

Figure 2. Expression of the FCER1 alpha chain on IgE-stripped basophils. PBMCs were isolated from individuals displaying high sIgE levels and FCER1 alpha chain expression was measured after stripping IgE from its receptor by lactic acid buffer incubation by FACS. Results are expressed as mean fluorescence intensity for FCER1A in the basophile gate. Significance was calculated using the Student's-t-test.
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sample whereas clearly lower effects were observed for the SNPs in *RAD50*. The rare allele "G" of the top ranked SNP rs2427837 in *FCER1A* had an estimated effect per copy of −0.212 based on the logarithm of total IgE. This translates into an estimated decrease of 19.1% in total serum IgE level for the heterozygote genotype and 34.6% for the rare homozygote genotype, which was significantly associated with an increased FCER1A expression on IgE-stripped basophils (Figure 2).

The estimated effect of the *STAT6* SNP rs12368672 was 0.156 resulting in an increase of total IgE of 16.9% and 36.6% for the heterozygote and rare homozygote genotype, respectively. The most significant SNP in the *RAD50* gene (rs2706347) had an effect estimate of 0.143 ($P = 2.26 \times 10^{-4}$) with an associated increase in total IgE of 15.4% and 33.1%. Altogether the variance of total IgE level explained by genotypes of the three replicated regions was about 1.9%.

To fine-map the regions of strong association in greater detail, we selected additional SNPs covering the *FCER1A* and *RAD50* gene region based on HapMap data from individuals of European ancestry. In addition, two previously described promoter SNPs of *FCER1A* (rs2251746, rs2427827) [15,16], as well as 2 SNPs in the *RAD50* hypersensitive site 7 (RHS7) in intron 24 (rs2240032, rs2214370)[17] were included. In total, 14 SNPs were genotyped in KORA S4. We found the strongest association in the proximal promoter region of the *FCER1A* gene, at rs2251746, which was in strong LD ($r^2 = 0.96$) with rs2427837 (Table 1 and Figure 3). The contribution of the two alleles of rs2251746 in homozygotes and heterozygotes is given in Figure S1. Their effect is observed across the full range of IgE values. The strongest observed association of SNP rs2251746 and the distribution of the SNPs in the region are shown in Figure 3A. None of the *RAD50* SNPs in the fine-mapping showed distinctly stronger association with total IgE (Figure 3B). We additionally sequenced all *FCER1A* exons with adjacent intronic sequences in 48 male and 48 female samples selected equally from the extremes of the serum IgE distribution in 3,890 individuals from the KORA S4 cohort. We identified two new mutations, each present in one individual only, and concurrently confirmed three SNPs already annotated in public databases (dbSNP) with validated minor allele frequencies in Europeans. None of the novel mutations were predicted to have functional consequences (for details see Text S1 and Tables S5 and S6). Haplotype analysis for the *FCER1A* gene showed lower total IgE levels with effect estimates ranging from −0.18 to −0.32 for a haplotype described by the rare "G" allele of rs2427837 and the rare "C" allele of rs2251746 (haplotype frequency 26.4%) in comparison to all other common haplotypes carrying both major alleles (Table S7).

For further replication of the KORA S4 results in the population-based children cohorts GINI (n = 1,839), LISA (n = 1,042) and ISAAC (n = 2,998) the top 6 SNPs: rs2251746, rs2427837, rs2040704, rs2706347, rs3798135, rs7737470 and rs12368672 were tested for association with total serum IgE levels. In GINI, all SNPs except rs12368672 yielded significant *P* values ranging from 0.029 to 8.14×10^{-6}. After correction for multiple testing SNP rs2706347 is slightly above the significance level. In LISA, the two *FCER1A* polymorphisms rs2251746 and rs2427837 were strongly associated ($P = 4.18 \times 10^{-5}$ and 6.58×10^{-5}), while the *RAD50* SNPs showed consistent trends, but no statistical significance. In ISAAC, the effect estimates of the two *FCER1A* SNPs were distinctly smaller than in the other replication samples but in the same direction and significantly associated with *P* values of 2.11×10^{-4} for rs2251746 and of 4.27×10^{-4} for rs2427837. The *RAD50* SNPs showed effect estimates in concordance with the other replication samples but were only borderline significant. Additional analysis of markers in the *RAD50-IL13* region in a subset of 526 children from the ISAAC replication cohort (for details see Table S9) indicated presence of one linkage disequilibrium (LD) block, which encompasses the entire *RAD50* gene and extends into the promoter region of the *IL13* gene, whereas rs20541 showed low levels of LD with *RAD50* variants ($r2 < 0.3$) (Figure S2)

In the combined analysis of all replication samples both selected *FCER1A* SNPs ($P = 1.85 \times 10^{-20}$ and 7.08×10^{-19} for rs2251746 and rs2427837, respectively) and *RAD50* SNPs ($P = 6.28 \times 10^{-7}$–4.46×10^{-8}) were significantly associated with IgE levels. Effect estimates were consistent throughout all replication cohorts.

Association Analysis with Dichotomous Traits

In a *post hoc* analysis of the KORA S4 and ISAAC replication cohorts, *FCER1A* polymorphisms rs2251746 and rs2427837 showed association with allergic sensitization ($P = 7.78 \times 10^{-4}$ and 1.95×10^{-3} in KORA, $P = 0.025$ and 0.032 in ISAAC), while there were no significant associations for the dichotomous traits asthma, rhinitis and atopic eczema (AE). However, the number of cases for these traits was relatively low. We therefore additionally typed a cohort of 562 parent-offspring trios for AE from Germany and a population of 638 asthma cases and 633 controls from UK. In these cohorts we observed weak associations of *RAD50* variants with eczema ($P = 0.007$–0.01) and with asthma ($P = 0.017$–0.002, Table S8).

Discussion

In this large-scale population-based GWAS with follow-up investigations in 9,769 individuals from 4 independent population-based study samples we show that functional variants of the gene encoding the alpha chain of the high affinity receptor for IgE (*FCER1A*) are of major importance for the regulation of IgE levels.

The high affinity receptor for IgE represents the central receptor of IgE-induced type I hypersensitivity reactions such as the liberation of vasoactive mediators including serotonin and

Table 1. Association between total IgE and selected SNPs in the GWAS sample and in the four replication samples.

		GWAS KORA S3/F3			Replication KORA S4			Replication GINI			Replication LISA			Replication ISAAC			Combined		
		n = 1,530			n = 3,890			n = 1,839			n = 1,042			n = 2,998			n = 9,769		
Gene	SNP	Est.	P value	Est. %	Est.	P value	Est. %	Est.	P value	Est. %	Est.	P value	Est. %	Est.	P value	Est. %	Est.	P value	Est. %
FCER1A	rs2511211				−0.206	9.28E-08	−18.59												
FCER1A	rs10489854				0.153	2.85E-02	16.52												
FCER1A	rs2494262				0.122	1.67E-04	12.99												
FCER1A	rs2427837	−0.235	6.19E-05	−20.94	−0.212	3.23E-09	−19.12	−0.219	2.51E-05	−19.64	−0.280	6.58E-05	−24.56	−0.145	4.27E-04	−13.53	−0.202	7.08E-19	−18.27
FCER1A	rs12565775				0.119	2.45E-02	12.56												
FCER1A	rs2427824				0.082	2.52E-02	8.49												
FCER1A	rs3845625				0.085	5.09E-02	8.82												
FCER1A	rs2427827				0.120	2.45E-04	12.72												
FCER1A	rs2251746				−0.227	6.07E-10	−20.29	−0.236	8.14E-06	−20.99	−0.290	4.18E-05	−25.17	−0.153	2.11E-04	−14.16	−0.213	1.85E-20	−19.21
RAD50	rs2069812				−0.052	1.42E-01	−4.98												
RAD50	rs2706347	0.236	4.05E-05	26.62	0.143	2.26E-04	15.43	0.122	2.91E-02	13.02	0.118	1.01E-01	12.56	0.095	2.70E-02	9.96	0.120	6.28E-07	12.80
RAD50	rs6884762				0.034	7.22E-01	3.46												
RAD50	rs17772565				−0.096	2.27E-01	−9.17												
RAD50	rs17772583				−0.058	1.24E-01	−5.62												
RAD50	rs3798135	0.227	6.58E-05	25.48	0.142	2.32E-04	15.20	0.173	2.00E-03	18.91	0.107	1.37E-01	11.26	0.101	1.75E-02	10.64	0.129	6.69E-08	13.82
RAD50	rs2040704	0.221	9.25E-05	24.73	0.140	2.47E-04	14.97	0.158	4.40E-03	17.14	0.111	1.21E-01	11.73	0.112	8.22E-03	11.83	0.130	4.46E-08	13.90
RAD50	rs7737470	0.231	4.81E-05	25.99	0.142	2.27E-04	15.28	0.163	3.70E-03	17.70	0.100	1.64E-01	10.55	0.087	4.13E-02	9.12	0.123	3.35E-07	13.07
RAD50	rs2240032				0.137	4.01E-04	14.67												
RAD50	rs2214370				0.136	5.95E-01	14.54												
STAT6	rs12368672	0.167	8.52E-04	18.18	0.156	2.03E-06	16.93	0.016	7.34E-01	1.65	0.075	2.44E-01	7.78				0.108	1.52E-05	11.44

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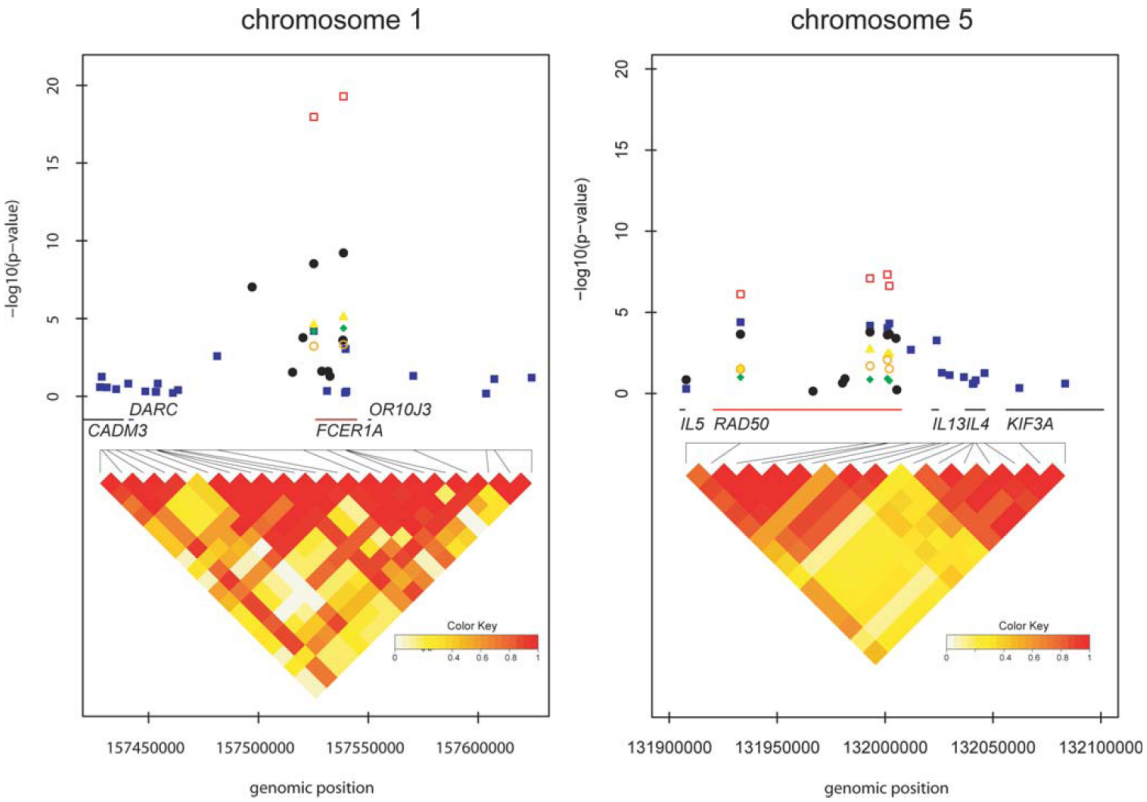

Figure 3. P value and pairwise linkage disequilibrium diagram of the region on chromosome 1q23, area of *FCER1A* (panel A), and chromosome 5q31, area of *RAD50* (panel B). Pairwise LD, measured as D′, was calculated from KORA S3/F3 500 K. Shading represents the magnitude of pairwise LD with a white to red gradient reflecting lower to higher D′ values. Gene regions are indicated by colored bars. *P* value diagram: The x-axis represents the genomic position. The y-axis shows −log10 (*P* values) of KORA S3/F3 500 K (blue), KORA S4 (black), GINI (yellow), LISA (green), ISAAC (orange), combined replication samples (red).
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histamine, but also for the induction of profound immune responses through the activation of NFkappa B and downstream genes [18]. It is usually expressed as a $\alpha\beta\gamma_2$ complex on mast cells and basophils, but additionally as a $\alpha\gamma_2$ complex on antigen-presenting cells (APCs) as shown for dendritic cells and monocytes [18]. Interestingly, in APCs, IgE-recognition of allergens also leads to facilitated allergen uptake via FCER1 and thereby contributes to a preferential activation of Th2-subsets of T-cells. Its expression is substantially influenced by the binding of IgE to either form of the receptor as bound IgE apparently protects the receptor from degradation and thus enhances surface expression without *de novo* protein synthesis. Of note, binding of IgE in the two different complexes only uses the alpha subunit of the receptor lacking contact sites with the beta or gamma subunits. Consequently, the expression level of the alpha subunit is crucial for IgE levels on immune cells [18].

Previous studies suggested linkage of atopy to the gene encoding the β chain of the high-affinity IgE receptor (*FCRER1B*) [19]. FCER1B plays a critical role in regulating the cellular response to IgE and antigen through its capacity to amplify FCER1 signalling and regulate cell-surface expression [18], and there have been several studies which reported an association of *FCER1B* variants and atopy-related traits but conflicting results for total IgE [20,21,22,23,24,25,26,27,28]. In a more recent study, no association between *FCER1B* tagSNPs and IgE levels was observed [22]. The 500 k random SNP array contained only one SNP within as well as 31 SNPs within a 100-kb region around this gene, which were not significantly associated with total IgE. However, we cannot rule out that we missed relevant variants in this gene.

In the present study we identified *FCER1A* as susceptibility locus in a genome-wide association scan and replicated association of the *FCER1A* polymorphism rs2427837 with serum IgE levels in a total of 9,769 individuals from 4 independent population-based cohorts with a combined *P* value of 7.08×10^{-19}. This SNP is in complete LD with the *FCER1A* polymorphism rs2251746, for which we observed a combined *P* value of 1.85×10^{-20}.

Besides the continuous cycling of the IgE receptor subunits from intracellular storage pools to the surface, there is also a substantial expression of the alpha subunit after stimulation with IL-4 which requires *de novo* protein synthesis [18]. This induction is stimulated by the transcription factor GATA-1, which has a binding site in the putative promoter region of the *FCER1A* gene. Notably, in a previous study with Japanese individuals it could be shown that the minor allele of the polymorphism rs2251746 is associated with higher FCER1A expression through enhanced GATA-1 binding [15]. In line with this we observed an increased cell surface expression of FCER1A on IgE-stripped basophils from individuals homozygous for the "G" allele at rs2427837 (Figure 2). Analysis of

the correlation of FCER1A expression with IgE levels in 320 KORA samples where whole genome blood expression profiles were available revealed no significant effect. However, FCER1A expression showed a significant dependency on IL-4 ($P = 0.0087$) and GATA-1 expression ($P = 1.4 \times 10^{-4}$), confirming the known stimulation pathway. Interestingly, we found a highly significant dependency of FCER1A expression on GATA-2 transcript levels ($p = 7.8 \times 10^{-27}$). While whole blood expression levels could easily obscure the situation in basophils, this finding might indicate a novel regulatory mechanisms of FCER1A expression via GATA-2 [18].

The large (>50 kb) *RAD50* gene, which encodes an ubiquitously expressed DNA repair protein, is located within the Th2-cytokine locus on chromosome 5q31, which has been linked with total IgE [29]. It contains multiple conserved non-coding sequences with presumed regulatory function [30]. Remarkably, evidence has been provided for the presence of a locus control region (LCR) within a 25 kb segment of the 3′ region of this gene, which plays an important role in the regulation of Th2 cytokine gene transcription [31]. The core of this LCR is constituted by four *RAD50* hypersensitive sites (RHS) in intron 21 (RHS4-6) and 24 (RHS7) [17,32,33]. The finding of an association between *RAD50* variants and IgE levels is new and biologically compelling. However, it has to be considered that so far *RAD50* has not emerged as candidate, but that several known candidate genes for atopy-related traits map to this region with strong linkage disequlibrium, especially *IL13*, which is one of the strongest and widely replicated candidate genes [10,11]. Notably, two functional *IL13* polymorphisms, *IL13*-1112CT (rs1800925) in the promoter region and *IL13*+2044GA (*IL13* Arg130Gln, rs20541) in Exon 4, have been shown to be associated with a range of atopy-related disorders. *IL13*+2044GA (rs20541) did not pass our selection criteria, and *IL13*-1112CT (rs1800925) is not contained in the Affymetrix 500 K Array Set. Additional analysis of markers in this region including these two SNPs showed one LD block encompassing the entire *RAD50* gene and extending into the *IL13* promoter region, whereas rs20541 showed low levels of LD with *RAD50* SNPs (Figure S2). Thus, we cannot reliably differentiate the specific source of the signal between *RAD50* and *IL13* in our data. Functional studies are needed to assess whether *RAD50* is a true causal gene and to identify the causal genetic variants modulating IgE levels in this region.

The identification and positive replication of the *STAT6* locus, which is located in one of the most frequently identified genomic regions linked to atopy-related phenotypes [34], serves as positive control for the experiment. Our results confirm previous candidate studies which showed that genetic variants in the gene encoding STAT6, a key regulatory element of the TH2 immune response, contribute to the regulation of total serum IgE [35,36].

Other previously reported candidate genes for total IgE showed no or only weak signals in our genome-wide scan (Tables S10 and S11). However, it has to be considered that there are only very few genes that have been associated in the first place to IgE such as *STAT6*, whereas most reported candidate genes for total IgE were investigated in asthma or eczema cohorts [10,11]. In addition, there have been queries with regard to replication for many of the genes reported. Thus, our data obtained in a population-based and ethnically homogeneous sample (South German Caucasians) are not readily comparable with previous candidate gene studies. Furthermore some previously implicated variants were covered insufficiently by the 500 k random SNP array (Table S10).

In summary, in this first GWAS on total IgE *FCER1A* was identified and replicated as new susceptibility locus at which common genetic variation influences serum IgE levels. In addition, our data suggest that variants within the *RAD50* gene might represent additional factors within cytokine gene cluster on chromosome 5q31, emphasizing the need for further investigations in this intriguing region.

Methods

Subjects and Study Design

A detailed description of the GWAS population and the replication samples is given in Text S1 and Table S1. In all studies informed consent has been given, and all studies have been approved by the local ethical committees. The participants were of European origin.

KORA S3/F3 500 K and Replication Sample KORA S4

The study population for the GWAS (KORA S3/F3 500 K) and the first replication cohort were recruited from the KORA S3 and S4 surveys. Both are independent population-based samples from the general population living in the region of Augsburg, Southern Germany, and were examined in 1994/95 (KORA S3) and 1999/2001 (KORA S4). The standardized examinations applied in both surveys have been described in detail elsewhere [37]. In the KORA S3 study 4,856 subjects (participation rate 75%), and in KORA S4 in total 4,261 subjects have been examined (participation rate 67%). 3,006 subjects participated in a follow-up examination of S3 in 2004/05 (KORA F3). For KORA S3/F3 500 K we selected 1,644 subjects of these participants in the age range 25 to 69 years including 1,530 individuals with total IgE level available. From KORA S4, DNA samples from 3,890 individuals with total IgE level were available. Total and specific IgE antibodies to aeroallergens (S×1) were measured using RAST FEIA CAP system (Pharmacia, Freiburg, Germany). Specific sensitization was defined as specific IgE levels ≥0.35KU/l (CAP class >= 1).

GINI and LISA Replication Samples

GINI (German Infant Nutritional Intervention Program) and LISA (Influences of lifestyle-related factors on the immune system and the development of allergies in childhood study) are two ongoing population-based birth cohorts conducted in Germany. A detailed description of screening and recruitment has been provided elsewhere [38]. Briefly, the GINI birth cohort comprises 5,991 newborns, who were recruited between January 1996 and June 1998 in 16 maternity wards in Wesel and Munich, Germany. Children with a positive medical history of atopic disease were invited to a randomized clinical trial with hydrolyzed formulae [39]. The LISA birth cohort study includes 3,097 neonates who were recruited between December 1997 and January 1999 in Munich, Leipzig and Wesel, Germany. Blood samples were collected from 1,962 (51%) and 1,193(50%) children from the GINI and LISA study, respectively, at age 6. Total IgE was determined by standardized methods with CAP-RAST FEIA (Pharmacia Diagnostics, Freiburg, Germany).

ISAAC Replication Sample

Between 1995 and 1996, a cross sectional study was performed in Munich and in Dresden, Germany as part of the International Study of Asthma and Allergy in Childhood phase II (ISAAC II) to assess the prevalence of asthma and allergies in all schoolchildren attending 4th class in both cities (age 9 to 11 years) [40]. Serum measurements for total and specific IgE were performed according to standardized procedures as previously described [40]. Allergic sensitization was defined as positive prick test reaction to at least one out of six common aeroallergens. Within the study population

of 5,629 children, all children of German origin with DNA and total IgE level available were included in this analysis (n = 2,998).

KORA S3/F3 500 K Genotyping and Quality Control

Genotyping for KORA S3/F3 500 K was performed using Affymetrix Gene Chip Human Mapping 500 K Array Set consisting of two chips (Sty I and Nsp I). Genomic DNA was hybridized in accordance with the manufacturer's standard recommendations. Genotypes were determined using BRLMM clustering algorithm. We performed filtering of both conspicuous individuals and single nucleotide polymorphisms (SNPs) to ensure robustness of association analysis. Details on quality criteria are described in Text S1 and Table S2.

SNP Selection for Replication and Fine-Mapping

The power of the replication was estimated for a difference in log total IgE per allele of 0.2 and a nominal significance level of 0.05. The power to detect a true association was above 85% in KORA S4, GINI and ISAAC; whereas in LISA it was about 55%. No single SNPs in the GWAS reached genome-wide significance using a Bonferroni threshold of 1.4×10^{-7}. To fine map the replicated loci in KORA S4 we selected tagging SNPs and used the pairwise tagging algorithm ($r^2>0.8$) implemented in HAPLOVIEW 3.3 (HapMap data release #22, March 2007, on NCBI B36 assembly, dbSNP b126) and additionally selected putative functional SNPs in *FCER1A* and *RAD50*.

SNP Genotyping and Quality Control in the Replication Samples

In all replication samples genotyping of SNPs was realized with the iPLEX (Sequenom San Diego, CA, USA) method by means of matrix assisted laser desorption ionisation-time of flight mass spectrometry method (MALDI-TOF MS, Mass Arraay, Sequenom, San Diego, CA, USA) according to the manufacturers instructions. In KORA S4 for 7 of 84 replicated SNPs a deviation from Hardy-Weinberg-Equilibrium was observed (P value<0.01). In LISA, GINI and ISAAC all replicated SNPs were in HWE. Details on genotyping are described in Text S1 and Table S4.

Mutational Analysis by Cycle Sequencing

FCER1A exons were amplified with intronic primers (Tables S5 and S6) and were directly sequenced using a BigDye Cycle sequencing kit (Applied Biosystems). Genomic DNA (~30 ng) was subjected to PCR amplification carried out in a 15 µl volume containing 1× PCR Master Mix (Promega), 0.25 µM of each forward and reverse primer under the following cycle conditions: initial step at 95°C for 5 min, for 30 cycles at 95°C for 30 s, 58°C (exon 1 62°C) for 30 s, and 72°C for 30 s; and final extension at 72°C for 5 min.

Statistical Analysis of Genetic Effects

In the KORA S3/F3 500 K sample possible population substructures were analyzed (Text S1). Additive genetic models assuming a trend per copy of the minor allele were used to specify the dependency of logarithmic values of total IgE levels on genotype categories. The result is a multiplicative model on the original scale of total IgE with effects interpreted in percental changes. All models were adjusted for gender and in the adult cohorts we adjusted additionally for age. We used a linear regression algorithm implemented in the statistical analysis system R (http://www.r-project.org/) and SAS (Version 9.1.). To select significant SNPs in the genome-wide screening and the replications we used conservative Bonferroni thresholds which corresponded to a nominal level of 0.05. Haplotype reconstruction and haplotype association analysis was performed in the KORA S4 replication sample using the R-library *HaploStats* that allows including all common haplotypes in the linear regression and incorporating age and gender as covariates. The most common haplotype served as reference. Details on haplotype analysis are described in Text S1.

Gene Expression Analysis

Peripheral blood (2.5 ml) was drawn from individuals participating in the KORA study under fasting conditions. The blood samples were collected between 10–12am directly in PAXgene (TM) Blood RNA tubes (PreAnalytiX). The RNA extraction was performed using the PAXgene Blood RNA Kit (Qiagen). RNA and cRNA quality control was carried out using the Bioanalyzer (Agilent) and quantification was done using Ribogreen (Invitrogen). 300–500 ng of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). 1,500 ng of cRNA was hybridized to the Illumina Human-6 v2 Expression BeadChip. Washing steps were carried out in accordance with the Illumina technical note # 11226030 Rev. B. The raw data were exported from the Illumina "Beadstudio" Software to R. The data were converted into logarithmic scores and normalized using the LOWESS method [41]. The association between *FCER1A* gene expression (independent variable) and IgE level (dependent variable) was computed using the linear regression model adjusted for gender.

Supporting Information

Figure S1 Box plot comparing the total IgE levels for the genotypes at rs2251746. The x axis represents the three genotype groups: TT (major homozygote), CT (heterozygote) and CC (minor homozygote). The y axis is the total IgE level on a logarithmic scale. Plot was created in R using the box plot function from the graphics package.
Found at: doi:10.1371/journal.pgen.1000166.s001 (0.38 MB TIF)

Figure S2 Patterns of pairwise LD between the SNPs at the RAD50-IL13 locus.
Found at: doi:10.1371/journal.pgen.1000166.s002 (0.03 MB TIF)

Table S1 Description of study populations.
Found at: doi:10.1371/journal.pgen.1000166.s003 (0.05 MB DOC)

Table S2 KORA S3/F3 500K SNP exclusion. Detailed breakdown of SNPs that were monomorphic or did not pass quality control and therefore did not enter analysis.
Found at: doi:10.1371/journal.pgen.1000166.s004 (0.04 MB DOC)

Table S3 Details on the association analysis of SNPs selected for replication (additive model).
Found at: doi:10.1371/journal.pgen.1000166.s005 (0.25 MB DOC)

Table S4 Genotyping details on replication and fine-mapping stages.
Found at: doi:10.1371/journal.pgen.1000166.s006 (0.15 MB DOC)

Table S5 Primers used to amplify the exons of *FCER1A*.
Found at: doi:10.1371/journal.pgen.1000166.s007 (0.04 MB DOC)

Table S6 Mutational analysis of *FCER1A* exons.
Found at: doi:10.1371/journal.pgen.1000166.s008 (0.04 MB DOC)

Table S7 Associations between *FCERA1* haplotypes and IgE levels in KORA S4. Results correspond to the single SNP analyses where presence of A (rs2427837) and C (rs2251746) alleles at respective positions were strongly associated.
Found at: doi:10.1371/journal.pgen.1000166.s009 (0.05 MB DOC)

Table S8 Association analysis of FCERA1 and RAD50 variants with AE in 562 German AE trios and with asthma in 638 UK asthma cases and 633 controls.
Found at: doi:10.1371/journal.pgen.1000166.s010 (0.06 MB DOC)

Table S9 Extended SNP analysis in the RAD50-IL13 region in a subset of 526 children from the ISAAC replication cohort and association with total IgE levels.
Found at: doi:10.1371/journal.pgen.1000166.s011 (0.05 MB DOC)

Table S10 Genes that have been associated with total IgE ordered by their chromosomal position.
Found at: doi:10.1371/journal.pgen.1000166.s012 (0.16 MB DOC)

Table S11 Affymetrix SNPs in selected candidate genes for total IgE, which yielded a nominal p-value <0.05 in the GWAS. Genes are ordered by their chromosomal position.
Found at: doi:10.1371/journal.pgen.1000166.s013 (0.14 MB DOC)

Text S1 Supplementary information.
Found at: doi:10.1371/journal.pgen.1000166.s014 (0.10 MB DOC)

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Author Contributions

Conceived and designed the experiments: S Weidinger, N Klopp, T Meitinger, HE Wichmann, T Illing. Performed the experiments: E Rodriguez, M Mempel, N Klopp, H Prokisch, D Mehta. Analyzed the data: S Weidinger, C Gieger, H Baurecht, H Gohlke, S Wagenpfeil, M Depner, L Liang, T Illing. Contributed reagents/materials/analysis tools: S Weidinger, H Gohlke, M Ollert, J Ring, H Behrendt, J Heinrich, N Novak, T Bieber, U Krämer, D Berdel, A von Berg, CP Bauer, O Herbarth, S Koletzko, T Meitinger, E von Mutius, MF Moffatt, W Cookson, M Kabesch, HE Wichmann. Wrote the paper: S Weidinger, C Gieger, MF Moffatt, M Kabesch, T Illing.

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2.7 Summary of results

In the following the main findings of each study are shortly summarized:

I. Two recently discovered risk alleles for eczema in the filaggrin gene on chromosome 1q21.3, R501X and 2282del4, were independently replicated for the first time and additionally analyzed regarding their relevance for different subtypes of eczema and related allergic and intermediate phenotypes. Both variants were significantly overtransmitted from parents to affected offspring in a set of 476 German families. The combined genotype Aa (heterozygous carriers of at least one mutant allele) showed strong association with eczema (OR 2.73; *P* $5.1x10^{-8}$), total serum IgE levels (*P* $9,8x10^{-8}$), allergic sensitization (*P* $2.3x10^{-7}$), asthma a secondary trait (*P* 0.0003), atopic eczema (*P* $9.3x10^{-8}$), and the atopy stigmata palmar hyperlinearity (*P* $5.9x10^{-6}$). No association could be seen for the non atopic and early onset forms of eczema and analysis of a possible parent of origin effect was negative (chapter 2.1).

II. For verification of association results *FLG* variants R501X and 2282del4 were analyzed in a second study sample consisting of 272 independent eczema cases and 252 population-based controls from the cross sectional KORA S4 study (Cooperative Health Research in the Region of Augsburg, Survey 4). All previous results could be replicated and additional subphenotypes were shown to be strongly influenced by *FLG* loss-of-function mutations.

Confirmation of associated traits for the combined genotype included eczema (OR 3.53 [95% Confidence Interval (CI) 1.92-6.48]; *P* $4.9x10^{-5}$), atopic eczema (OR 3.66 [95%CI 1.96-6.83]; *P* $4.6x10^{-5}$), asthma in combination with eczema (OR 5.69 [95%CI 2.76-11.73]; *P* $2.5x10^{-6}$), and total serum IgE (OR 2.14 [95%CI 1.14-4.00]; *P* 0.017). Analysis of additional phenotypes revealed significant association with allergic rhinitis in the context of eczema (OR 4.04 [95%CI 2.11-7.72]; *P* $2.4x10^{-5}$), the early onset form of eczema (OR 5.21 [95%CI 2.61-10.40]; *P* $2.8x10^{-6}$) and the more severe and persistent type of eczema as measured with the “Score of Atopic Dermatitis” (SCORAD) (OR 2.65 [95%CI 1.36-5.17]; *P* 0.0043). Again, there was no association observed with the intrinsic form of eczema (chapter 2.2).

III. In order to investigate distribution and impact of *FLG* variants in the general population and to uncover their role in other atopic phenotypes like asthma and rhinitis the five most prevalent variants R501X, 2282del4, R2447X, S3247X and 3702delG were studied in a large cross-sectional study population, consisting of 3099 children recruited in Munich and Dresden as part of the ISAAC II study. Carrier frequencies on population level were 4.6% for 2282del4, 1.9% for R501X, 0.7% for R2447X and 0.2% for S3247X yielding

a combined carrier frequency of 7.4%. No carriers of the 3702delG mutation could be identified.

As expected, the analyzed variants (combined genotype) were strongly overrepresented in eczema affected individuals and increased disease risk more than threefold (OR 3.12 [95%CI 2.33-4.17]; *P* $2.5x10^{-14}$). Accordingly, *FLG* alleles conferred an increased risk for sensitization (OR 1.61 [95%CI 1.20-2.17], *P* 0.0017). Penetrance of *FLG* null alleles reached 38.5%, indicating the proportion of individuals bearing at least one mutant *FLG* allele concurrently presenting the clinical disease phenotype. The proportion of eczema affected individuals in the general population explained by the presence of one or more *FLG* risk variants, commonly described as Population Attributable Risk, was 13.5%. Additionally, significant association with allergic rhinitis independently from eczema status was observed (OR 2.64 [95%CI 1.76-4.00]; *P* $2.5x10^{-6}$), whereas association with asthma appeared only to be significant in combination with eczema (OR 3.49 [95%CI 2.00-6.08]; *P* $1.0x10^{-5}$). Of note, histological analysis of the nasal epithelium showed strong expression of FLG in the vestibular lining, but lack of expression in the transitional epithelium and in the respiratory mucosa (chapter 2.3).

IV. The *FLG* gene and its polymorphisms represent one of the most widely replicated risk factor in the area of complex diseases. However, results of the numerous studies differ largely concerning effect size, and there exists a conflict of statements regarding *FLG* null mutations and their possible influence on asthma. The conducted meta-analysis represents the most comprehensive evaluation of the effect of *FLG* loss-of-function mutations and the first meta-analysis on asthma so far.

A total of 24 studies on *FLG* mutations and eczema comprising nearly 5,800 cases, > 26,000 controls and nearly 2,000 families were included in this analysis. For asthma, there were 17 studies with > 3,000 cases, > 17,000 controls and > 1,500 affected family offspring available. In the combined analysis of the two most common variants R501X and 2282del4 in all family and case-control studies a more than threefold increased risk for eczema was confirmed (OR 3.12 [95%CI 2.57-3.79]). In the case of asthma, it seems to be indeed exclusively the combined asthma + eczema phenotype (OR 3.29, [95%CI 2.84-3.82]) that is genetically determined by *FLG* null alleles, but not asthma without eczema. An evaluation of all included studies concerning study design (e.g. population size, determination of disease status etc.) revealed strongest association for affected individuals with a dermatologist's diagnose and the more severe form of eczema (chapter 2.4).

V. In the first and to date only GWAS on eczema with more than 307000 SNP markers in >3500 cases, >4900 controls and 270 nuclear families (parent-child trios), a so far unknown susceptibility locus on chromosome 11q13.5 could be identified. The minor allele

A of rs7927894 showed a highly significant association ($OR_{combined}$ 1.22 [95%CI 1.15-1.30]; $P_{combined}$ $7.6x10^{-10}$) with the disease in all four independent study populations. The associated SNP rs7927894 is located in an intergenic region on chromosome 11, 38kb downstream of the gene *C11orf30* encoding the EMSY protein and 68kb upstream of *LRRC32* encoding the GARP protein (glycoprotein A repetitions predominant), two pathophysiologically very interesting and hitherto disregarded candidates for eczema. There was no evidence for any regulatory influence of rs7927894 on mRNA expression of both genes in a public available dataset of a GWAS for global mRNA expression in lymphoblastoid cell lines from asthmatic children (Dixon, Liang et al. 2007) and the causal variant(s) are still to be determined.

The observation of an additional association signal located in the EDC around the hornerin (*HRNR*) gene (lead SNP rs877776, $P_{combined}$ $3.5x10^{-5}$) after exclusion of individuals carrying any of the five most prevalent *FLG* mutations points strongly towards a second risk factor in this intriguing region apart from *FLG* and represents a novel susceptibility locus for eczema in the EDC (chapter 2.5).

VI. Analysis of human serum IgE concentrations in a genome-wide scan consisting of more than 353000 SNP markers and more than 11.000 individuals from 4 independent population based cohorts revealed that variants in the α chain of the high affinity receptor for IgE strongly impact basal IgE regulation. Two polymorphisms (rs2251746 and rs2427837) in this gene were strongly associated across all cohorts with combined P-values of $1.85x10^{-20}$ and $7.78x10^{-19}$, respectively. The estimated effect of SNP rs2427837 can be translated in a decrease of approximately 19.1% of IgE level in heterozygous carriers and 34.6% for homozygous carriers of the rare A allele. Additional functional studies showed a significant decrease of FCER1A cell surface expression on basophils for the AA genotype of rs2427837. This observation is in line with a previous Japanese study, which had demonstrated that polymorphism rs2251746, which is in complete LD with rs2427837, alters expression of FCER1A (Hasegawa, Nishiyama et al. 2003). A post-hoc analysis of these two SNPs for different atopic traits demonstrated additional associations with allergic sensitization (*P* $7.78x10^{-4}$ and $1.95x10^{-3}$ for rs2251746 and rs2427837, respectively). Supplemental exon sequencing of *FCER1A* in 48 male and 48 female individuals equally selected from the extremes of serum IgE distribution in KORA S4 did not reveal any new prevalent mutation in this gene.

Apart from *FCER1A*, several variants located within the *IL13-RAD50* locus with consistent effect estimates and combined P-values of $6.28x10^{-7}$ to $4.46x10^{-8}$ showed associations with IgE levels. The most significant SNP rs2706347 was associated with an estimated increase of 15.4% and 33.1% of total IgE for heterozygous/homozygous carriers of the minor allele, respectively. These four associated polymorphisms in the *RAD50* gene were

additionally observed to be risk factors for eczema and asthma in subsequent analyses in a cohort of 562 German eczema families and an asthma case-control study including 638 asthmatics and the same number of controls from UK (chapter 2.6).

3 Discussion

The skin represents a highly effective physical and biomechanical as well as immunological barrier. The immunological skin barrier relies on innate immune receptors and components of the adaptive immune system, whereas the physical barrier is mainly provided by the stratum corneum, a dense layer of protein-rich cells within an intercellular matrix of unpolar lipids. Efficient function of the skin barrier critically depends on proper formation and composition of the SC and it is increasingly recognized that deficiency of the epidermal barrier might be largely due to genetic defects in SC proteins or lipids. With the discovery of loss-of-function mutations in the key SC protein filaggrin as causative for ichthyosis vulgaris and strong risk factors for eczema, first evidence for the involvement of defective structural epidermal proteins due to genetic alterations in skin barrier disruption has been established.

Filaggrin: Evidence for a crucial role of skin barrier dysfunction in eczema

Within the scope of this thesis, three independent association studies on common *FLG* mutations were carried out to validate and further dissect the role of this gene in eczema pathogenesis. By examining a collection of 476 German parent-affected offspring trios the first independent confirmation of the association between common *FLG* loss-of-function mutations (R501X and 2282del4) and eczema could be provided. In addition, it could be shown that these variants are also associated with the presence of palmar hyperlinearity, a characteristic feature for IV called “ichthyosis-hand”, which is also considered a strong sign for atopic constitution and often observed in eczema patients (Przybilla, Ring et al. 1991). In a subsequent case-control and a population-based approach *FLG* null mutations were consistently associated with total and specific serum IgE levels. Accordingly, in all three studies a particularly strong association was observed for the atopic subtype of the disease (eczema with allergic sensitization). It was demonstrated that *FLG* variants also strongly predispose to a subtype of asthma that occurs in the context of eczema. Moreover, FLG deficiency revealed to be a risk factor for the early-onset and severe and persistent subtypes of eczema. In the large-scale population-based study in German children the mutant *FLG* alleles showed an incomplete penetrance of 38%. The population attributable risk reached 13.5%, which means that more than 13% of eczema cases in the population can be attributed to FLG deficiency.

The comprehensive summary of these results as well as those of numerous other studies in the performed meta-analysis provides strong evidence for a genetically determined FLG deficiency as major risk factor for the development of eczema. Careful evaluation of the risk estimates conferred by mutant *FLG* alleles in the different studies, which partially showed large divergences, revealed a more than threefold increase in eczema susceptibility and a

more than threefold elevated risk for the compound phenotype asthma + eczema. Taken together, these observations provide evidence for the hypothesis that the impaired aggregation of filaments in the skin caused by lack of filaggrin leads to an impaired barrier, which might facilitate the penetrance of environmental agents such as allergens. This consequently might promote inflammation and allergic sensitization, and the development of the atopic form of eczema (Fig. 7), eventually followed by asthma and rhinitis. Filaggrin is not expressed in the human bronchial mucosa (Ying, Meng et al. 2006). However, the disruption of the epidermal barrier due to an inherited filaggrin deficiency might allow the entry of allergens and initiation of a systemic T_H2 response affecting organs like the lung (Hudson 2006). The hypothesis that allergic sensitization might happen in the skin is supported by the observation that an exposure to topical emollients containing peanut proteins can lead to peanut allergy in children (Lack, Fox et al. 2003), and that epicutaneous exposure to peanut allergen causes allergic sensitization in mice (Strid and Strobel 2005). Hence, skin-mediated sensitization seems to depend critically on the integrity of the skin barrier and even includes possible sensitization against food allergens.

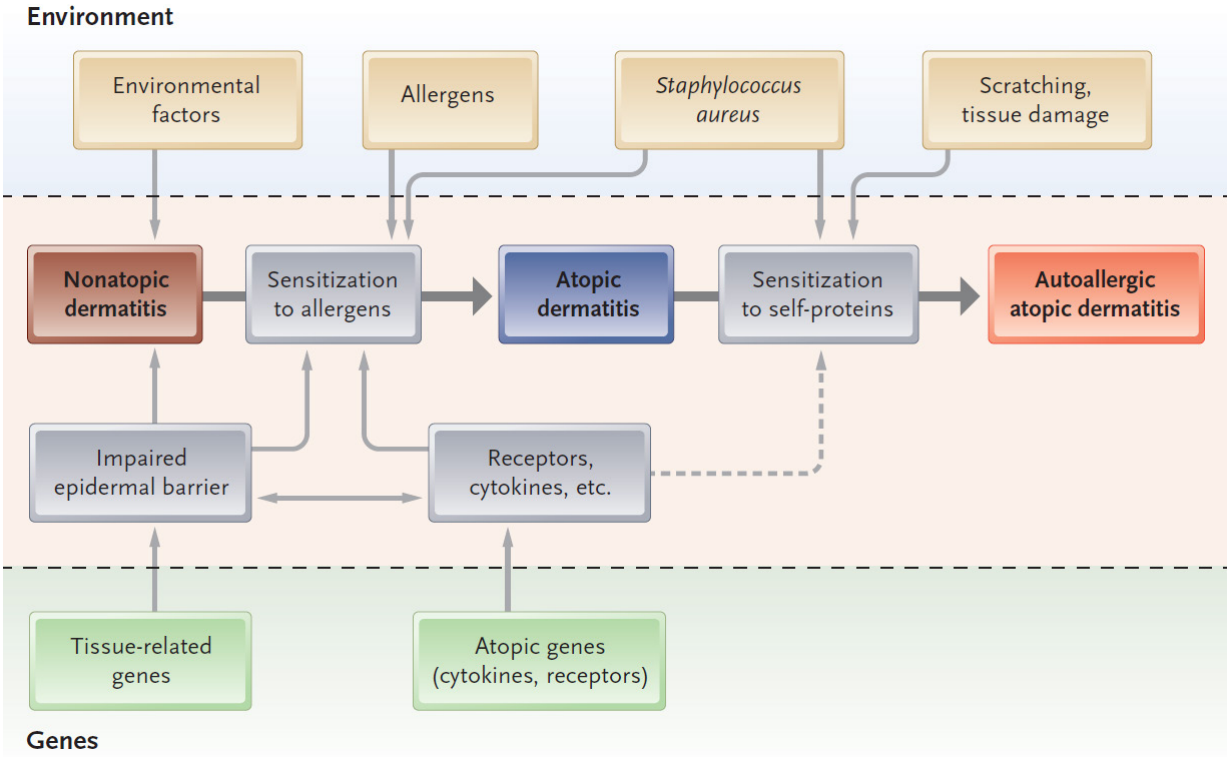

Figure 7: Hypothesis concerning the natural history of eczema. Environmental factors and a genetically determined epidermal barrier dysfunction cause non-atopic eczema as the first manifestation of the disease. Subsequent IgE-mediated sensitization due to a genetically determined immune response dysfunction occurs, additionally triggered by S. aureus infections. Finally, patients may become sensitized to self-proteins because of scratching and the release of structural proteins from damaged tissue. From (Bieber 2008)

This work strongly supports the change in concepts for eczema pathogenesis indicating that a genetically derived defect in the epidermal barrier plays a pivotal role in the development of this disease, probably followed by IgE-dependent sensitization and the subsequent onset of respiratory diseases (Hudson 2006).

However, around 60% of individuals affected by eczema do not carry any of the known mutations in the *FLG* gene, but there are first hints indicating that FLG expression in the skin might be modified by additional genetic and environmental factors. Recently, a decreased expression of filaggrin in acute skin lesions of eczema patients without *FLG* mutations was observed and attributed to the T_H2 cytokine milieu, since it could be shown that the expression of FLG is downregulated by IL-4 and IL-13 (Howell, Kim et al. 2007). Thus, T_H2 cytokines not only represent critical mediators of the allergic immune response, but additionally seem to contribute to the skin barrier disruption in eczema patients. Another possible explanation for reduced levels of the FLG protein in the epidermis of eczema patients displaying the *FLG* wildtype might be an alteration in profilaggrin processing. FLG is primarily expressed and stored as a non-functional precursor in the stratum granulosum, and accurate activation by posttranslational modification is of high importance. The protease SCCE is supposed to be responsible for proteolytic cleavage of profilaggrin (Resing, Thulin et al. 1995) and seems to be regulated by LEKTI, a serine protease inhibitor. A genetic association study performed by our group (Weidinger, Baurecht et al. 2008) on *KLK7* (kallikrein-related peptidase 7), the gene encoding SCCE, as well as *SPINK5*, which encodes LEKTI, did not confirm the association between KLK7 and eczema, as reported in previous studies (Vasilopoulos, Cork et al. 2004) but revealed a polymorphism in SPINK5 to confer increased risk of eczema when maternally inherited. Analysis of a possible interaction between *FLG*, *SPINK5* and *KLK7* in the same study did not show any significant epistatic effects. Therefore, besides a direct genetic influence on expression due to mutations in the *FLG* gene itself, filaggrin insufficiency might be indirectly caused by acquired and/or inherited factors influencing its expression and/or posttranslational processing.

A variety of studies points towards a genetically determined FLG deficiency as risk factor for other epidermal disorders. Apart from (atopic) eczema, our study in a population-based cohort of German adults revealed an additional association of *FLG* mutations with allergic contact sensitization against certain allergens as well as with allergic contact dermatitis (Novak, Baurecht et al. 2007). These observations are in line with studies provided by other groups, which reported associations with chronic-irritant hand-eczema and severe courses of alopecia areata and X-chromosomal ichthyosis (de Jongh, Khrenova et al. 2008), whereas *FLG* variants seem to be no risk factor for psoriasis vulgaris. Although a reduced expression of FLG has been observed in psoriatic lesions (Watanabe, Wagatsuma et al. 1991;

Huffmeier, Traupe et al. 2007), an association between *FLG* variants and psoriasis could not be demonstrated (Weichenthal, Ruether et al. 2007; Zhao, Terron-Kwiatkowski et al. 2007).
The functional consequences and exact phenotypic characteristics caused by *FLG* mutations are not completely understood and the hypothesis of a "dry or defective barrier" in *FLG*-mutation carriers remains to be proven (Irvine 2007). There still remains a need to dissect the possible impact of *FLG* mutations in other allergic diseases, such as asthma, rhinitis, sensitization and allergy to food and aeroallergens, and a variety of other diseases related to skin barrier disruption like psoriasis.
Additional studies in carefully phenotyped, large scale and cross sectional longitudinal study populations are necessary to generate a clearer picture of mutant *FLG* and its influences on allergic diseases, certain subtypes of allergic phenotypes or secondary traits like serum IgE levels. Another helpful measure for detection of further genetic factors would be the subdivision of eczema cases in *FLG*-associated and *FLG*-independent disease, which might additionally facilitate delineation of environmental modifiers.
An alternative approach to gain more insights into particular effects and phenotypic consequences of FLG deficiency might be the use of animal models e.g. a mouse-model, which provides the possibility of analysing various generations due to short life span and fast reproduction of these animals. Breeding of isogenic strains reduces the genetic heterogeneity between subjects, and controlled test settings allow examinations of gene-environment interactions, e.g. reaction to allergen exposure. To date no feasible animal model exists that covers all the different aspects of eczema. Only the flaky tail (*ft*) mouse seems to hold the possibility to serve as comprehensive model for eczema pathogenesis and the role of *FLG*. However, the eczema-related phenotype characterized by inflammatory infiltrates in the epidermis and allergen-specific antibodies in this model only occurs in homozygous individuals, but lung inflammation or airway hyperresponsiveness could not be detected in percutaneously sensitized *ft/ft* mice (Fallon, Sasaki et al. 2009). These observations are contrary to data retrieved from human populations where heterozygosity of *FLG* mutations is sufficient to increase the risk for eczema and eczema-associated asthma. *ft* mice additionally fitted with deficiencies in IgE synthesis or other important T_H2 promoting molecules will help to dissect the role of immune response, whereas exposition of *ft* mice to environmental factors might throw light on exogenous influences and their interaction with FLG deficiency.
Taken together, *FLG* and its different null mutations appear to be the only strong and consistent eczema risk factor across different populations and in multiple large independent studies and their discovery represents a milestone in the area of atopic disease research.
The assessment of phenotypic effects of FLG deficiency represents the first step towards the understanding of an important disease mechanism in the development of eczema, allergic

rhinitis and asthma in the context of eczema. Although the functional consequences of FLG deficiency are still not fully understood, the recent findings on this gene already led to a change in the concept of eczema aetiology. The identification of several loss-of-function mutations in this gene opens up the possibility of reclassification of atopic diseases. The frequency of *FLG* variants in patients with eczema, which adds up to 40%, might be considered for a novel molecular eczema classification, based on underlying genetic defects rather than on clinical symptoms. A next step may be the development of methods for the restoration of epidermal skin barrier by means of superficially applicable crèmes and emollients containing substitutes for natural moisturizing substances (NMS), if not even for the filaggrin peptides itself. Reconstitution of FLG function could be achieved by the use of substances, which allow a read-through of DNA-polymerases over the disease causing null mutations, thereby clearing the lack of filaggrin and restoring functional skin barrier without any restriction. With the development of screening method using *FLG* mutations or deficiency as early biomarker, infants under high risk could be determined and measures for avoiding contact with allergens could be taken. With one or more of these actions the key event of sensitisation, probably leading to the subsequent development and progression of atopic disease like eczema or asthma may be prevented in childhood. Early disease management could improve the outcome and quality of life of patients with eczema.

Genome-wide association study on eczema: identification of a novel susceptibility locus

GWAS are hypothesis-free approaches and very useful tools in the identification of disease-associated genes, especially without prior implication in pathogenic concepts. Likewise, the GWA study included in this thesis revealed a yet uncharacterized locus to be significantly associated with eczema. The study design of two different German discovery sets used in this approach consisted of a case-control and a family-based study population in order to exclude possible population stratification. The case-control set was additionally tested for possible population stratification by principal component analysis with Eigenstrat (Price, Patterson et al. 2006), which did not show any evidence for genetic stratification. Variants with the same risk allele consistently associated with eczema in both sets with $p<0.005$ in one population and $p<0.05$ in the other population were selected for replication in an independent and large cohort of German ancestry. Markers showing significant association in this replication cohort after Bonferroni correction were additionally tested in a second large replication set of European cases and controls. The study revealed an interesting novel susceptibility locus on chromosome 11q13.5, a second association signal independently from *FLG* on chromosome 1q21.3 within the EDC, as well as a number of nominally associated variants within or nearby plausible candidate genes. The latter six loci did not reach the

significance threshold in the first replication set after correction for multiple testing but included promising candidates like *HRH4* (histamine receptor H4), *LY86* (lymphocyte antigen 86) and *EOMES* (eomesodermin). All these genes are known to be involved in immunological pathways like the differentiation of T_H2 cells, mast cells, and eosinophils (*HRH4*) (Thurmond, Gelfand et al. 2008), Toll-like receptor mediated response to bacterial infections (*LY86* or *MD-1*) (Nagai, Shimazu et al. 2002) and CD8+ T-cell function (*EOMES*) (Pearce, Mullen et al. 2003). As the calculated power for the detection of risk variants conferring a relative risk of 1.5 with a minor allele frequency of 20% is only about 12% for the performed analysis, it is very probable that additional low risk variants were missed due to false negative results. Additional genome-wide association or candidate gene approaches providing bigger sample sizes will be needed for follow-up and positive confirmation concerning these loci.

The gene or gene product affected by the rs7927894 risk allele on chromosome 11 still remains to be identified and functionally characterized; the SNP rs7927894 is located in an intergenic region 38 kb downstream of *C11orf30* and 68 kb upstream of *LRRC32*. *C11orf30* codes for the EMSY protein, which has been implicated in DNA repair, chromatin regulation and transcriptional regulation. It has been reported to bind to the breast cancer susceptibility protein BRCA2 and to be involved in epithelial-derived cancer of the breast and the ovaries (Hughes-Davies, Huntsman et al. 2003), and might play a role in epithelial differentiation. GARP, which is encoded by the second nearby gene *LRRC32*, has been shown to be a cell surface molecule expressed on regulatory T-cells, which binds TGF-beta (transforming growth factor beta) (Wang, Wan et al. 2008). Therefore it also represents a plausible candidate for eczema susceptibility, given the important role of T-cell-mediated inflammation.

RNA-expression profiling in different tissues showed ubiquitous expression of both genes, even in eczema relevant tissues like the skin. Although the associated variant is located in an LD block, which extends into C11orf30, it cannot reveal the associated gene, as correlated variants are located at both sides of the SNP. Moreover, rs7927894 or more likely a causative variant in LD with this SNP may be located in a non-coding regulatory sequence with effects on expression of *LRRC32* of even more distant genes others than *C11orf30* and *LRRC32*. Disruption of non-coding long-range regulatory elements, in many cases acting over distances spanning various intermediate genes, is an emerging disease mechanism (Kleinjan and van Heyningen 2005).

Interestingly, association of the rs7927894 allele A has recently also been shown in a GWAS for Crohn's disease (CD) (Barrett, Hansoul et al. 2008), another complex disorder, which is characterized by a chronic and recurrent inflammation of the gastrointestinal tract. CD and eczema are known to show some pathophysiological similarities like inflammation and impairment of the epithelial barrier and restrictions of the innate immune system (Schreiber,

Rosenstiel et al. 2005). SNP rs7927894 seems to convey susceptibility to both diseases, which might contribute to the high incidence of eczema observed in CD patients (Pugh, Rhodes et al. 1979). Thus, genetic factors beyond skin-specific physical barrier genes may have an effect on susceptibility to epithelial inflammation with similar function in different inflammatory diseases affecting both the skin and the gastrointestinal tract. Although highly speculative, it is tempting to suggest that the 11q13.5 variant may also contribute to a T_H1-dominant immune profile as seen in CD as well as in chronic atopic eczema lesions rather than to the T_H2-dominant lesions of acute atopic eczema.

In the meantime, association of rs7927894 [A] has been replicated in an independent Irish study, which confirmed allele frequency of the risk variant and showed a similar OR (O'Regan, Campbell et al. 2010).

The results obtained from the GWAS require further investigations on the functional consequences on the biomolecular level caused by rs7927894 or any functional variant in LD with this SNP. LD structure retrieved from HapMap Phase 1 & 2-full data set (http://hapmap.ncbi.nlm.nih.gov/index.html.en) shows correlation of rs7927894 with a variety of variants on both sides of the SNP. Hence LD data alone does not reveal, which of the two genes is of prior interest for functional analysis. To give a short outline, polymorphisms in LD (D'>0.8) in a 750kb surrounding region of rs7927894 are located mostly in the intergenic region between *C11orf30* and *LRRC32*. Several SNPs can be found in the 3' region of *LRRC32* or in *C11orf30* itself, and some correlated variants are located in the guanylat cyclase 2E pseudogene (*GUCY2E*) downstream of *LRRC32* or in the intergenic region between *C11orf30* and the upstream protein kinase encoding gene *PRKRIR* (protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor)).

In order to draw first conclusions on a possible functional involvement of rs7927894 neighbouring genes, potential differences in mRNA expression for the two candidate genes *C11orf30* and *LRRC32* in the large and well-characterised KORA research platform and genome-wide expression data will be analysed.

Considering the possible regulatory role of the SNP containing intergenic region, an intensely public database research for the rs7927894 surrounding region regarding conservation, regulatory potential and predicted enhancer regions using the University of California Santa Cruz (UCSC) genome browser (http://www.genome.ucsc.edu/), Vista Genome browser (http://pipeline.lbl.gov/cgi-bin/gateway2), and Vista Enhancer browser (http://enhancer.lbl.gov/) was conducted. A conserved sequence with high regulatory potential and different conserved transcription factor binding sites in a 1kb region (Chr11:75,977,000-75,978,000) upstream of rs7927894 was observed. Several SNPs are located in this region and might display LD with the original SNP associated in the GWAS. Unfortunately LD calculation is not possible, because none of these polymorphisms has been

genotyped in the course of the Hap Map project. Therefore a reasonable experiment would be an enhancer analysis by transfection of enhancer minimal promoter constructs into standard cell lines and measurement of enhancer activity by luciferase reporter gene assay. Identification of possible DNA interacting proteins could be examined via electrophoretic mobility shift assays (EMSAs), DNA-affinity purification and mass-spectrometry.

Bioinformatic results obtained from the before mentioned preliminary public database research additionally revealed another very interesting region. A polymorphism (rs1044265) located in the 3' untranslated region (UTR) of *C11orf30* near several microRNA regulatory sites and within a MEF-2A (myocyte-specific enhancer factor 2A) binding site seems to be in strong LD (D'=0.83) with rs7927894. MicroRNAs are supposed to regulate ~30% of all human genes via binding to 3'UTR mRNA sequences, and several polymorphisms in these regions have been shown to exert effect on gene expression (Lau, Lim et al. 2001; Lee and Ambros 2001; Sevignani, Calin et al. 2006; Yanaihara, Caplen et al. 2006). Hence, rs1044265 may interfere with miRNA function leading to differential gene expression of *C11orf30*. To test this hypothesis miRNA regulation assays and mRNA stability assays are planned.

The second significant association signal identified in this GWAS belongs to SNP rs877776 located within the hornerin gene (*HRNR*) in the EDC on chromosome 1q21.3. The risk allele could only be detected after stratification for *FLG* lof-mutations, i.e. statistical analysis after exclusion of individuals carrying any of the known *FLG* variants. This observation reflects the basic need for determination of -at least- the two most common *FLG* mutations R501X and 2282del4 in study populations used in future GWAS on eczema or in candidate gene association studies including loci within the EDC. The possibility of an associated SNP being only in LD with any of the *FLG* variants require stratification in order to detect true association apart from *FLG*. Before stratification for mutant *FLG* carriers the performed GWAS showed a significant association signal of SNP rs6661961 ($P \leq 3x10^{-3}$), which is located in the EDC 156kb downstream of *FLG*. Association with this variant was observed in both discovery populations and replicated in a follow-up study sample. Subsequent LD analysis revealed high LD between this marker and the *FLG* mutations, and stratification for *FLG* mutation carriers abolished the observed association ($P > 0.5$). However, these results serve as a positive control for this association study, as they reconfirm association with *FLG* and thereby robustness of the study. Stratification is all the more important as the EDC still seems to harbour further genes involved in eczema development. A variety of candidates with important function in the development of the epidermal barrier are located in this region, each of which might cause defects in the formation of the cornified envelope. One or more independent risk genes might exist within the EDC that similar to *FLG* but independently of it cause skin barrier dysfunction with the effect of facilitated allergen penetration and systemic

allergic reactions. Risk alleles in *FLG*-unstratified eczema populations might be underrepresented due to 20-40% of eczema patients carrying one or more *FLG* mutation. False negative results could occur because of dilution of novel risk gene effects by *FLG*, whereas the statistical power to detect a true association with further independent risk gene in the EDC increases after exclusion of patients with eczema caused by *FLG* variants.

However, it has to be mentioned that SNP rs877776 could not be replicated within the same Irish study, which successfully reproduced the observed association for rs7927894 (O'Regan, Campbell et al. 2010). Although the examined eczema cases belong to the discovery cohort for *FLG* mutations and are very well characterized for its rare and prevalent loss-of-function variants (Palmer, Irvine et al. 2006) stratification for *FLG* variants did not reveal any association within the EDC. It might be speculated that the Irish study including 500 cases and 1000 controls did not have enough power to detect effects of the *HRNR* variant due to a relatively low allele frequency of ~16% and a small effect size (OR 1.20). As *FLG* mutations are known to be population specific, rs877776 might also be in LD with a yet unidentified *FLG* variant unique to continental Europeans, which does not appear in the Irish study cohort used for replication analysis.

The performed GWAS suggests that *FLG* might not be the only risk gene within the EDC responsible for linkage and association signals. Additionally, a variety of different inflammatory diseases has been linked to the EDC, like psoriasis, asthma, systemic lupus erythematosus (SLE) and rheumatoid arthritis (Willis-Owen, Morar et al. 2007). For most of the latter diseases, no susceptibility gene has been identified within this region so far. However, a recent GWAS on psoriasis assumed the presence of a susceptibility gene in the EDC (Liu, Helms et al. 2008), the PSORS4 locus on 1q21 has been repeatedly confirmed in independent patient cohorts (Capon, Semprini et al. 2001; Giardina, Sinibaldi et al. 2006), and a copy number variation within the late cornified envelope (LCE) genes has been shown to be associated with increased risk for psoriasis (de Cid, Riveira-Munoz et al. 2009; Huffmeier, Bergboer et al. 2010). Hence, the EDC still represents a very promising region in search of further risk genes, not only for eczema, but although for other inflammatory barrier diseases, and its comprehensive analysis is of high importance.

Therefore we have started an in-depth analysis of genetic variants within the EDC in order to discover novel mutations, confirm annotated SNPs and detect rare variants and unravel their role in eczema and psoriasis. The spectrum of polymorphisms in the EDC including the region between the *S100A10* and *S100A1* (S100 calcium binding protein A) genes from position 148700000 to 150500000 on chromosome 1q21.3 will be explored by massive parallel sequencing with next generation sequencing (Illumina Genome Analyzer or Solexa). SNPs and novel mutations in 61 genes of the EDC will be systematically screened by

resequencing this 2megabases (Mb) chromosomal region (Figure 8) in at least 100 eczema/psoriasis cases and 100 healthy controls.

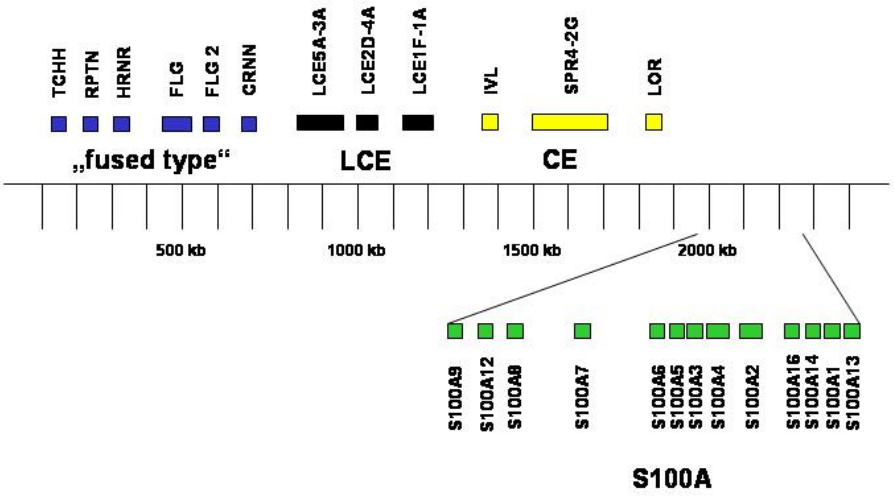

Figure 8: Schematic physical map of human chromosome 1q21.3 representing the different gene families. The EDC includes a region of 1.62 Mb on chromosome 1q21.3 and constitutes a dense cluster of approximately 50 genes involved in the final differentiation of the epidermis. These genes can be subdivided into functionally, structurally and evolutionary related families: the small proline rich proteins (*SPR*s), involucrin (*IVL*) and loricrin (*LOR*), the *S100A* family, the late cornified envelope proteins (*LCE*s) and a few single copy genes of the “fused type” family including filaggrin (*FLG*), filaggrin2 (*FLG2*), hornerin (*HRNR*), cornulin (*CRNN*), repetin (*RPTN*) and trichohyalin (*TCHH*). The fused type family is characterised by a large repeat domain of several protein motifs arranged in tandem (reviewed in ((Rodriguez, Illig et al. 2008)).

Subsequently to in depth sequencing and the discovery of novel variants, an association analysis of selected polymorphisms in sufficiently powered case-control-cohorts for eczema and psoriasis will be necessary, and associated variants in putative disease genes will have to be confirmed in additional and independent study populations. Moreover, validated risk genes will be functionally evaluated by *in vitro*-analysis of tissue-specific and cell-type specific expression patterns during differentiation using real time-PCR (RT- PCR) and *in situ* hybridization.

Limitations of this method are not only its time- and cost expenditure –although next generation sequencing methods already provide a much better time- and cost effectiveness compared to traditional sequencing methods - which restrict the projected approach to a relatively small set of samples to be sequenced. But it’s also hampered by the already mentioned structural nature of a variety of genes like *FLG* and the fused type family in

general, with it's commonly highly repetitive genomic sequence, and the bioinformatic challenge to handle and comprehensively analyze the huge amount of generated data.

Likewise to the fast developing field of new GWAS techniques, the term "next-generation" sequencing already seems to be antiquated, and the new slogan of "Third generation" sequencing has been coined. New methods promise to increase speed of data generation while costs are concurrently decreasing (Check Hayden 2009; Eid, Fehr et al. 2009). However, still we have not reached the 1000$ genome widely thought to be necessary for broad medical use in diagnosis and individualized medical treatment (Mardis 2006).

The strong impact of *FLG* mutations, which have a rather low individual frequency, on eczema and allergic rhinitis challenges the popular hypothesis "common disease-common variant", which proposes that the genetic variations underlying common disorders such as allergic diseases must be alleles, which are themselves very common in the general population (Lander 1996; Chakravarti 1999). Up to now it has provided the scientific paradigm for genome-wide association studies conducted on common diseases (Wang, Barratt et al. 2005). As coverage of all genomic variants within a genome-wide association approach is still not possible, SNP selection for these arrays is generally based on an even physical distribution of markers throughout the genome with regard to genomic LD architecture in order to capture redundant information about as many SNPs as possible, or concentrates on coding regions and recombinant hotspots. However, this strategy does not necessarily hold for all genetic factors involved in polygenic diseases (Pritchard 2001; Pritchard and Cox 2002), and analysis of *FLG* loss-of-function mutations highlights its limitations. Rare variants with minor allele frequencies of < 10% like the *FLG* polymorphisms, which only reach a frequency of ~10% when combined, are generally not included in purchasable standardized genome-wide chip applications and might therefore slip through scientists fingers.

GWA approaches offer the big advantage of hypothesis free search for disease genes that is not hampered by certain disease concepts and restricted to selected candidate genes. GWAS have already proofed to be useful and reliable tools for the identification of new and robust susceptibility loci for different complex diseases (Frazer, Murray et al. 2009). For e.g. Crohn's disease, genome-wide approaches already revealed an impressive number of new risk genes, which, however, only explained a small proportion of the total variance in disease risk (Barrett, Hansoul et al. 2008). This unexpected case of "missing heritability" observed in genome-wide analyses already represents a dictum in the scientific community (Maher 2008). Due to the fact that genome-wide arrays still do not cover variants with a minor allele frequency (MAF) < 10%, rare variants with stronger effects are missed. Many scientists strongly support the concept that few rare variants displaying relatively strong effects as well as numerous common variants with a rather low impact on the phenotype play crucial roles

in the aetiology of complex diseases (Maher 2008; Schork, Murray et al. 2009). Only a combination of both candidate gene association studies and GWAS will give a complete picture of the disease underlying mechanisms. However, as already mentioned, different sequencing projects like the 1000 genome project provide genome-wide platforms with a increasing number of new common and -most importantly- rare variants with minor allele frequencies below 10%. Genome-wide coverage of these SNP arrays will increase significantly during the next years, although whole genome sequencing in large cohorts seems to be the still unfeasible gold-standard for a real genome wide analysis in order to dissect the majority of genetic factors, which underlie complex diseases.

GWAS on total IgE: insights into the genetic architecture of atopic disease risk

In order to identify novel susceptibility genes influencing total IgE levels as a crucial outcome of allergic reactions, a GWAS in five independent, large-scale, and population-based German study samples was performed. In contrast to the often used case-control study design, the population-based approach allows estimation of genetic impact across the full range of IgE levels as observed in the common population, including individuals that are genetically predisposed but clinically unaffected. As none of the tested SNPs reached genome-wide significance ($P<1.4x10^{-7}$) in the KORA S3/F3 discovery set, variants showing P-values of $<10^{-4}$ and/or $P<10^{-3}$ with at least one neighbouring SNP reaching the same P-value were selected for genotyping in the first replication set (KORA S4). Two variants within the gene for the high affinity IgE receptor α chain (FCER1A) were shown to be significantly associated with total IgE, as well as four SNPs in the RAD50 gene, which is located within the T_H2 cytokine-cluster on chromosome 5q31. All SNPs were subsequently validated in three additional children cohorts (LISA (Influences of life-style related factors on the immune system and the development of allergies in childhood), GINI (German Infant Nutritional Intervention Study), ISAAC). Furthermore, tagging SNPs covering the associated loci as well as functionally interesting literature SNPs were selected for finemapping. Association of IgE concentrations with a variant in the signal transducer and activator of transcription 6 gene (*STAT6*), a key regulatory element of the T_H2 immune response, which has been shown to influence regulation of total serum IgE in previous studies (Schedel, Carr et al. 2004; Weidinger, Klopp et al. 2004), was reconfirmed.

The two associated *FCER1A* polymorphism rs2251746 and rs2427837 were consistently associated with alterations in IgE levels across all study populations. The estimated effect of SNP rs2427837 can be translated in a decrease of approximately 19.1% of the IgE level in heterozygous carriers and 34.6% in homozygous carriers of the rare A allele. The most significant SNP in the second associated loci on chromosome 5q31 showed an estimated effect of 15.4% and 33.1% increase in total IgE levels for heterozygous and homozygous

carriers of the risk allele, respectively. Effect estimates for the *STAT6* variant accounted for an increase of 16.9% for the rare heterozygous, and 36.6% for the rare homozygous genotype in total serum IgE concentrations. The variance in total IgE levels explained by the risk alleles of the three detected susceptibility regions accounts for about 1.9%.

Up to date, only very few loci have been found to be consistently associated with serum IgE levels, like for example *IL-13* and *STAT6* (Ober and Hoffjan 2006; Vercelli 2008). Previous candidate gene studies focused exclusively on the β-subunit of the high affinity IgE receptor (*FCER1B*), which is known to play an essential role in the regulation of FCER1 cell surface expression and intracellular signalling (Kraft, Wessendorf et al. 1998; Donnadieu, Jouvin et al. 2000), reported association of variants within this gene and atopy-related traits, but conflicting results for IgE levels (Shirakawa, Li et al. 1994; Shirakawa, Mao et al. 1996; Ulbrecht, Eisenhut et al. 1997; Palmer, Rye et al. 1999; Hizawa, Yamaguchi et al. 2000; Hizawa, Yamaguchi et al. 2001; Traherne, Hill et al. 2003; Hoffjan, Ostrovnaja et al. 2004; Maier, Howson et al. 2006). In this GWAS no evidence for association of *FCER1B* with total IgE levels could be observed, which, however, might be due to low coverage of the *FCER1B* gene on the used Affymetrix 500K array chip.

Instead, the first genome-wide screen on total IgE identified the α chain of the high affinity IgE receptor as a novel susceptibility locus. Additionally performed functional studies on the two SNPs that are located in the putative promoter region of *FCER1A* showed a significant decrease of FCER1A cell surface expression on basophils for the AA genotype of rs2427837 in accordance to a previous Japanese study which demonstrated that polymorphism rs2251746, which is in complete LD with rs2427837, influences expression of FCER1A (Hasegawa, Nishiyama et al. 2003). Interestingly, only the α-subunit of the IgE receptor binds to the IgE-antigen complex, whereas the β and γ subunit lack physical contact. Further examinations revealed weak effects of both SNPs on allergic sensitization, whereas no association with eczema or asthma per se in independent case-control cohorts could be observed. In a recent study in two German birth cohorts rs2251746 was consistently associated with total serum IgE levels from birth up to the age of six years, exerting an allele-dose effect. Again, no significant associations with asthma or eczema or interaction with different environmental exposures could be observed (Chen, Weidinger et al. 2009), indicating a role in basal IgE regulation.

These observations add a new aspect to reports from different functional studies, which propose crucial regulation of cell surface expression of FCER1 through its ligand IgE. Correlation between the concentration of serum IgE and the amount of IgE bound to the basophils has been reported, and monomeric IgE has been shown to increase FCER1 expression on the cell surface (Malveaux, Conroy et al. 1978; Furuichi, Rivera et al. 1985; Quarto, Kinet et al. 1985). It is supposed that IgE stabilizes the receptor complex on the cell

surface and restricts its internalization and degradation while maintaining basal synthesis, whereas there is no evidence for IgE-mediated increase in transcription or translation of FCER1. However, the exact regulatory pathway by which FCER1α expression might be linked to alterations in IgE serum levels remains unknown.

Although the 5q31 region has already been linked to total IgE (Marsh, Neely et al. 1994) *RAD50* has never been recognized as candidate prior to this GWAS. The gene encodes a DNA repair protein that is ubiquitously expressed, and therefore lacks any obvious biological connection to the analyzed trait. However, it contains a locus control region (LCR), which is supposed to regulate expression of the neighbouring T_H2 cytokines (Loots, Locksley et al. 2000). Hence, associated polymorphisms in *RAD50* rather influence T_H2 cytokine gene transcription than RAD50 regulation itself. The LCR is constituted by four RAD50 DNase I hypersensitive sites (RHS) in intron 21 (RHS4-6) and intron 24 (RHS7) (Fields, Lee et al. 2004; Lee and Rao 2004; Lee, Spilianakis et al. 2005). RHS7 has been shown to be rapidly demethylated in murine cell lines of stimulated T_H2-cells (Kim, Fields et al. 2007), an observation that might connect demethylating events in RHS7 with LCR activation under T_H2 conditions. Epigenetics is increasingly recognized as an important factor in complex diseases like eczema, and it provides a link between genetics and environment. Cells are enabled to change their behaviour in response to internal or external environmental influences. The best-studied epigenetic modification of DNA in mammals is methylation of cytosine in CpG dinucleotides (Bird 2002). We concurrently conduct a study analyzing the quantitative methylation status of CpGs within *RAD50* which might reveal epigenetic regulatory pathways influencing cytokine expression and T_H2 immune reaction in atopic individuals. Analysis is carried out using MassCLEAVE™ biochemistry and quantitative high-throughput mass spectrometry (Sequenom® MassARRAY® EpiTYPER system). Regions to be analyzed have been selected regarding presence of CpGs and CpG islands, conservation, regulatory potential and predicted enhancer regions using the UCSC genome browser (http://www.genome.ucsc.edu/), Vista Genome browser (http://pipeline.lbl.gov/cgi-bin/gateway2), and Vista Enhancer browser (http://enhancer.lbl.gov/). To detect candidate RAD50 sites differentially methylated in allergic individuals, in a first step 48 *RAD50* amplicons covering the regions of high interest will be screened in peripheral blood DNA from eczema patients (n=20) and sex- and age-matched healthy controls (n=20) from our own disease collections. As methylation patterns are supposed to be highly tissue-specific, DNA from different cell types has been collected. The projected analysis includes peripheral blood samples, as well as DNA extracted from keratinocytes, T-cells and B-cells. In parallel, transcriptional expression of IL-4, Il-5 and Il-13 in peripheral blood leukocytes (PBL) will be assessed by RT-PCR to seek for correlations with methylation profiles.

Interestingly, further analysis of the *RAD50* SNPs in independent case-control cohorts showed additional association with eczema and asthma, and in a very recently conducted independent GWAS the *RAD50-IL-13* locus was strongly associated with asthma (Li, Howard et al. 2010), further supporting the role of variations within this locus for allergic diseases.
Although *RAD50* represents a new and biologically very interesting candidate due to its supposed regulatory function on T_H2 cytokine expression, it has to be considered that several atopy-related susceptibility genes are located within the same cluster. *IL-13* represents one of this consistently associated genes (Ober and Hoffjan 2006; Vercelli 2008) and very recently, a functional variant in a DNase I hypersensitive site within the neighbouring *IL-13* gene has been shown to be strongly associated with IgE levels (Kiesler, Shakya et al. 2009). Analysis of LD structure in the associated region revealed a LD block including the entire *RAD50* gene and the *IL-13* promoter region, and it is not possible to exclude the possibility of associated SNPs being in LD with functional variants within the *IL-13* gene. Hence, genetic variations within the *RAD50-IL13* locus seem to be of importance for several atopy-related phenotypes, but final designation to one (ore more) specific susceptibility gene(s) is prevented by the complex LD-architecture characterizing this region, and functional studies are needed to dissect the underlying source of genetic association. Generally, elevated IgE levels are supposed to confer resistance against parasite infections, but they are strongly associated with allergic disorders in industrialized countries with less exposure to e.g. helminths. Recently it has been shown that an elevated IgE immune response is also involved in anti-tumoral defence (Gould, Mackay et al. 1999) and autoimmune diseases (Dimson, Giudice et al. 2003). Variability of IgE production has been shown to be largely influenced by genetic factors in pedigree and twin studies (Jacobsen, Herskind et al. 2001; Strachan, Wong et al. 2001). The decision for serum IgE levels as analyzed trait in this GWAS was based on its assumed role as endophenotype in atopic disorders like eczema. IgE largely fulfils the main criteria for such an endophenotype: It represents a precisely measurable and objective trait with sufficient heritability, which is feasibly linked to atopic eczema. An understanding of the genetic mechanisms regulating total serum IgE levels might also aid in the dissection of the genetic basis of atopic diseases. However, different independent GWAS on asthma and eczema only evidenced a small degree of overlap between identified susceptibility loci for atopic traits and total IgE, and disease-specific factors seem to be of high importance. Our knowledge about interrelation between the phenotypically very heterogeneous atopic diseases and the intermediate trait IgE is still very limited. Although the identified susceptibility genes seem to be important for the atopic state per se rather than a specific atopic disease, genetic research on endophenotypes will contribute to the clarification of allergic disease aetiology.

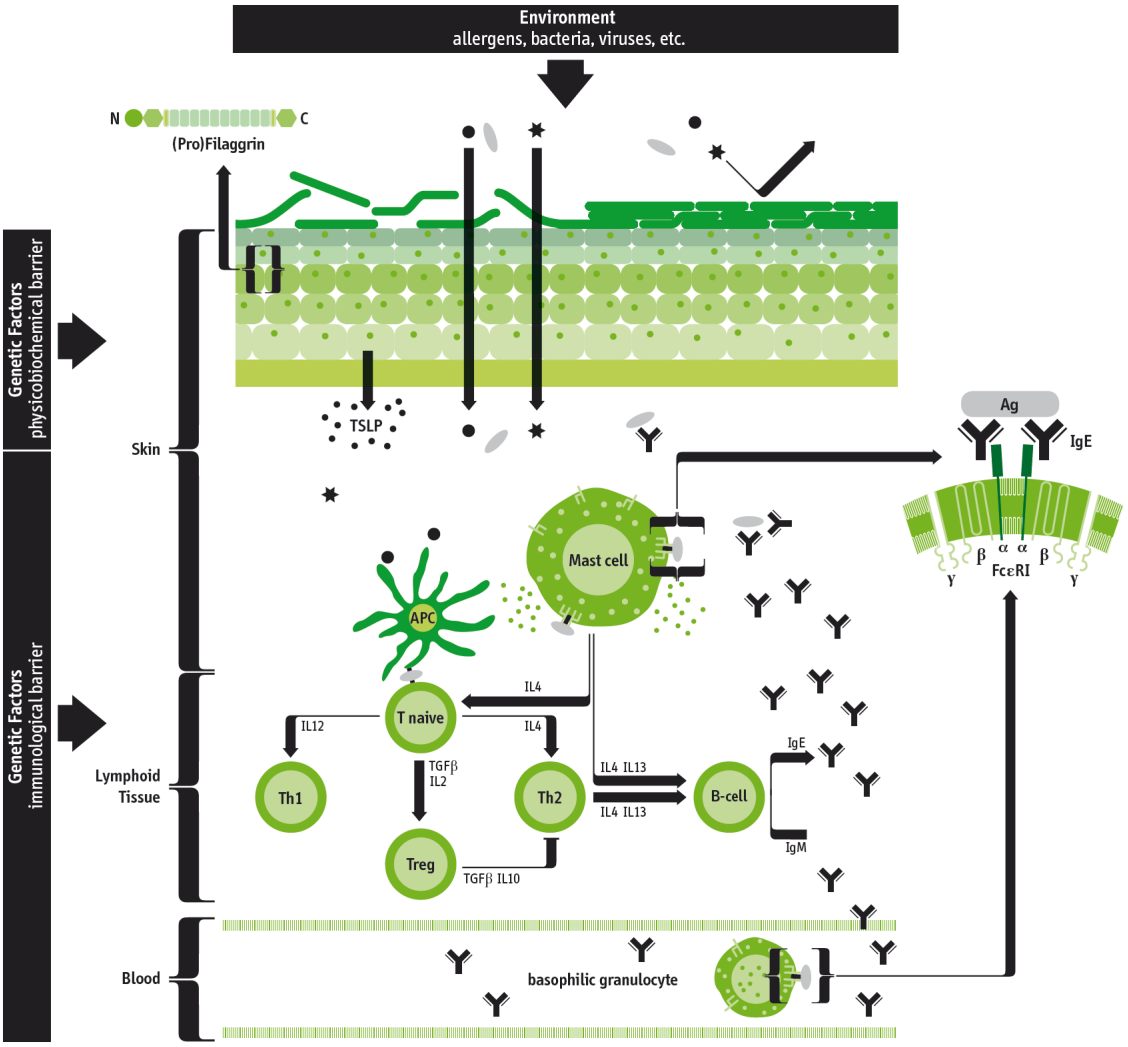

Figure 9: Schematic presentation of the interplay between barrier disruption and immune dysfunction in eczema aetiology highlighting genetically caused FLG deficiency and a mutant high affinity IgE receptor.

4 Concluding remarks

It is widely recognized that atopic diseases such as eczema arise on the interplay of a strong genetic predisposition and environmental factors. New insights into the genetic architecture of eczema and atopy have considerably influenced our understanding of disease mechanism. Until recently common pathophysiological concepts for eczema focused on immunological mechanisms and postulated a central role of allergic sensitization and atopy. With the discovery of *FLG* mutations eczema and atopy are no longer considered as an exclusively immunological dysfunction, and the focus has shifted to the disruption of epithelial barrier integrity. *FLG* also challenges the "common disease-common variant" hypothesis and strengthens the idea of few rare variants conferring strong risk, which account not alone for the genetic aetiology of complex diseases but together with multiple common alleles conferring low risk.

However, new risk genes described in initial association studies are often not reproducible and fail to replicate. To clarify the role of these possible susceptibility genes and in order to identify additional novel risk variants large-scale and well designed whole-genome association studies are needed. New technologies allowing the hypothesis-free search for new susceptibility loci already revealed various robustly associated risk genes for complex diseases, and technical limitations concerning whole genome coverage might be overcome within the next years. While awaiting the promised 1000$ genome which will enable researchers to analyze yet unknown rare variants, different consortia started meta-analysis approaches unifying the data from different large GWAS in order to uncover missing heritability in complex disease, as huge sample numbers provide the power to detect common genetic variants with modest or small effects.

As not only technologies in the field of genomics advance very rapidly, but also new high-throughput methods appear in proteomics and transcriptomics and in the relatively new fields of metabolomics and epigenomics, researchers of this different areas close ranks. The term of system biology tries to meet the complete picture in human disease research, and holds the promise of identification of new and easily applicable diagnostic tools as well as implementation of individualized therapies carefully adapted to the patient's personal background.

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Education and professional experience

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09/1995-01/1998	Apprenticeship as biological laboratory assistant at the Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
05/1998	Diploma studies in biology at the Ludwig-Maximilians-Universität (LMU), Munich, Germany
08/2001-09/2002	Two semesters abroad at the Universidad de Concepción (UDEC), Concepción, Chile, by means of a scholarship of the German Academic Exchange Service (DAAD)
02/2003-09/2003	Zoological practical course at the Universidad de Concepción by means of a DAAD scholarship „Morphological and allozymic differences between two sintopyc species of the *Liolaemus monticola* group from National Park Laguna del Laja (Chile)“
05/2004-03/2005	Diploma thesis at the Helmholtz Zentrum München, Department of Molecular Radiation Biology, advised by PD Dr. med. Horst Zitzelsberger Topic: „Characterisation of markerchromosomes in radiation transformed human breast cell lines“
01/2006 to present	Doctoral candidate advised by PD Dr. med. Stephan Weidinger at the Department of Dermatology and Allergy, Technische Universität München (TUM), Munich, Germany, in cooperation with the Department of Epidemiology (Prof. Dr. Dr. H.-Erich Wichmann), Helmholtz Zentrum München, Neuherberg, Germany

Additional qualifications

08/2004	Fluorescence Activated Cell Sorting (FACS)/Microdissection-Workshop at the Molecular Cytogenetics Laboratory, Veterinary School, University of Cambridge, and the Cancer Cell Unit, Hutchinson/MRC Research Centre, Cambridge, England
08/2006	Advanced training course for statistics and SPSS for physicians at the Institute for Medical Statistics and Epidemiology (IMSE), Technische Universität München, Munich, Germany
03/2007	Basic Methylation Analysis Course, Applied Biosystems (ABI), Darmstadt, Germany
07/2007	18th International Summer School of Epidemiology, The International Centre for Advanced Studies in Health Sciences and Services (ICAS) of the Faculty of Medicine, Ulm University, Ulm, Germany in cooperation with the Akademie für Wissenschaft, Wirtschaft und Technik an der Universität Ulm e.V., Ulm, Germany
09/2009	The Baltic Summer School, Christian-Albrechts-Universität, Kiel, Germany, Founded by the medical faculties of the Universities of Kopenhagen, Kiel and Lund
05/2010	LMU EXTRA Seminar “Personalführung”, Ludwig-Maximilians-Universität, Munich, Germany

Grants and awards

07/2007	Poster presentation at the „Inflammatory Barrier Disease Meeting/Genetic exploration leads to novel therapies“, International Symposium, University Hospital Kiel, Kiel, Germany. Abstract title: Filaggrin mutations are strong risk factors for early-onset and severe atopic dermatitis with allergic sensitizations. Receipt of a travel grant by the University Hospital Kiel
09/2007	Poster presentation at the „37th Annual European Society for Dermatological Research (ESDR) Meeting“, Zurich, Switzerland. Abstract title: Do tagging SNPs evidence the strong association between two functional DNA variants in the Filaggrin gene and atopic dermatitis? Receipt of a travel grant by the ESDR
05/2008	Poster presentation at the „International Investigative Dermatology (IID) Meeting 2008“, Kyoto, Japan. Abstract title: Analysis of gene-gene interaction within the filaggrin pathway. Receipt of a travel grant by the ESDR
06/2008	Oral presentation at the „XXVII Congress of the European Academy of Allergology and Clinical Immunology (EAACI)”, Barcelona, Spain. Abstract title: Filaggrin mutations, atopic eczema, hay fever, and asthma in children. Receipt of a travel grant by the Deutsche Forschungsgemeinschaft (DFG) and the Oral Abstract Session prize awarded by the EAACI
09/2009	Attendance of and poster presentation at the “Baltic Summer School”, Christian-Albrechts-Universität, Kiel, Germany. Receipt of a travel stipend by the Christian-Albrechts-Universität
06/2009	Oral and Poster presentation at the “XXVIII Congress of the European Academy of Allergy and Clinical Immunology (EAACI)”, Warsaw, Poland. Abstract title: Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. Receipt of a travel grant by the EAACI
10/2009	Poster presentation at the “59th Annual Meeting of the American Society of Human Genetics (ASHG)”, Honolulu, Hawaii. Abstract title: Meta analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. Receipt of a travel grant by the German Academic Exchange Service (DAAD)

12/2009 Poster presentation at the “World Allergy Congress 2009 (WAC)”; Buenos Aires, Argentina. Abstract title: Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. Receipt of a travel grant by the World Allergy Organisation (WAO)

Publication list

Weidinger S, Illig T, Baurecht HJ, Irvine AD, **Rodríguez E**, Diaz-Lacava A, Klopp N, Wagenpfeil S, Zhao Y, Liao H, Lee SP, Palmer CNA, Jenneck C, Maintz L, Hagemann T, Behrendt H, Ring J, Nothen MM, McLean WHI, Novak N. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. J Allergy Clin Immunol 118(1):214-219, 2006.

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E. Rodríguez was involved in designing the study, was responsible for biobanking and preparation of samples for analysis, performed all experimental parts of the study, and contributed to the writing of the manuscript.

II Weidinger S, **Rodríguez E**, Stahl C, Wagenpfeil S, Klopp N, Illig T, Novak N. Filaggrin mutations strongly predispose to early-onset and extrinsic atopic dermatitis. J Invest Dermatol 127:724-726, 2007. (Chapter 2.2)

E. Rodríguez was involved in designing the study, was responsible for biobanking and preparation of samples for analysis, performed all experimental parts of the study, and contributed to the writing of the manuscript.

III Weidinger S, O'Sullivan M, Illig T, Baurecht HJ, Depner M, **Rodríguez E**, Ruether A, Klopp N, Vogelberg C, Weiland SK, McLean WHI, von Mutius E, Irvine AD, Kabesch M. Filaggrin mutations, atopic eczema, hay fever and asthma in children. J Allergy Clin Immunol 121(5):1203-1209, 2008. (Chapter 2.3)

E. Rodríguez performed the following experimental parts of the study: assay design, genotyping and quality control for all analysis performed with MALDI-TOF MS. In addition, she contributed to the writing of the manuscript.

IV **Rodríguez E***, Baurecht H*, Herberich E, Wagenpfeil S, Brown SJ, Cordell HJ, Irvine AD, Weidinger S. Meta-analysis of filaggrin polymorphisms in eczema and asthma: Robust risk factors in atopic disease. J Allergy Clin Immunol, 123(6):1361-70, 2009. (Chapter 2.4)

E. Rodríguez conceived and designed the study, performed data extraction from public data bases and data abstraction from published manuscripts, participated in statistical analysis and wrote the manuscript.

* authors contributed equally

V Esparza-Gordillo J, Weidinger S, Fölster-Holst R, Bauerfeind A, Ruschendorf F, Patone G, Rohde K, Marenholz I, Schulz F, Kerscher T, Hubner N, Wahn U, Schreiber S, Franke A, Vogler R, Heath S, Baurecht H, Novak N, **Rodríguez E**, Illig T, Lee-Kirsch MA, Ciechanowicz A, Kurek M, Piskackova T, Macek M, Lee YA, Ruether A. A common

variant on chromosome 11q13 is associated with atopic dermatitis. Nat Genet, 41(5):596-601, 2009. (Chapter 2.5)

E. Rodríguez was responsible for sample preparation and biobanking, as well as assay design and *FLG* genotyping of the analyzed eczema case population from the Universities of Bonn and Munich.

VI Weidinger S, Gieger C, **Rodríguez E**, Baurecht HJ, Mempel M, Klopp N, Gohlke H, Wagenpfeil S, Ollert M, Ring J, Behrendt H, Heinrich J, Novak N, Bieber T, Krämer U, Berdel D, von Berg A, Bauer CP, Herbarth O, Koletzko S, Prokisch H, Mehta D, Meitinger T, Depner M, von Mutius E, Liang L, Moffatt M, Cookson W, Kabesch M, Wichmann HE, Illig T. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. PLoS Genet 4(8):1-9, 2008. (Chapter 2.6)

E. Rodríguez was involved in designing the study, performed main experimental parts of the study (genotyping and quality control of initial screen, SNP selection and genotyping for replication and finemapping, mutational analysis) and contributed to the writing of the manuscript.

PD Dr. med. Stephan Weidinger

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Munich, 20th of July 2010

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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*) Nichtzutreffendes streichen

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