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## IMPACT OF ATOPIC DERMATITIS AND LOSS-OF-FUNCTION MUTATIONS IN THE FILAGGRIN GENE ON THE DEVELOPMENT OF OCCUPATIONAL IRRITANT CONTACT DERMATITIS

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### Abstract

**Background**—Atopic dermatitis (AD) and loss-of-function mutations in the filaggrin gene (*FLG*) are both associated with chronic irritant contact dermatitis (ICD). As *FLG* mutations also are a major risk factor for AD, it is not clear whether *FLG* mutations are an independent risk factor for ICD or whether the risk is mediated by AD.

**Objectives**—To investigate the relative contribution and interaction of *FLG* mutations and AD in German patients with occupational ICD and controls (vocational school apprentices).

**Methods**—A total of 634 patients and 393 controls were genotyped for R501X, 2282del4, R2447X and S3247X. Current or past flexural eczema was used as an indicator of AD.

**Results**—*FLG* mutations were found in 15.9% of the patients with ICD and 8.3% of the controls, with a crude odds ratio (OR) of 2.09 [95% confidence interval (CI) 1.33 – 3.28] for the combined genotype. The adjusted OR for *FLG* mutations, corrected for AD, was 1.62 (95% CI 1.01 – 2.58). Subjects with AD were at approximately three times higher risk to develop ICD (OR= 2.89; 95% CI 2.09 – 3.99). There was no evidence of an interaction between these two risk factors.

**Conclusions**—Our results indicate that both *FLG* mutations and AD increase the risk of ICD. Individuals with concurrent *FLG* mutations and AD are at the highest risk of developing ICD.

### INTRODUCTION

Contact dermatitis (CD) is one of the most common occupational diseases in industrialized countries. In European surveys among workers and apprentices in ‘high-risk’ occupations, such as hairdressing, healthcare and metalworking, the 1-year prevalence varied between 20% and 30%, and mild skin symptoms were present in up to 50% of the workers or apprentices<sup>1-7</sup>. CD is commonly divided into allergic CD (ACD) and irritant CD (ICD), of which ICD is the more common in occupational settings<sup>8</sup>. ICD is caused by repeated exposure to irritants, for example soaps, detergents, disinfectants and water (‘wet work’)<sup>9</sup>. In addition to environmental factors, it is assumed that the risk of developing ICD is influenced by endogenous factors, of which atopic dermatitis (AD) is the most important. It has been estimated that AD increases the risk of developing ICD by a factor of between two and

four<sup>10;11</sup>. In Germany and the Netherlands, a history of AD is used to identify susceptible individuals in prevention programs in high risk occupations<sup>12;13</sup>. However, the mechanisms by which AD influences the development of ICD are still largely unknown. Patients with AD have an impaired epidermal barrier function, even in uninvolved skin<sup>14;15</sup>. One possible factor contributing to an impaired skin barrier function in AD is a decreased level of the epidermal protein filaggrin, caused by loss-of function mutations in the filaggrin (*FLG*) gene. Filaggrin is important for the structure, function and hydration of the stratum corneum<sup>16</sup>, which is the principal barrier of the skin. Reduced levels of filaggrin may lead to increased penetration of irritants and allergens through the skin, and subsequent inflammation<sup>17-20</sup>. *FLG* loss-of-function mutations are a major risk factor for AD; approximately 20-30% of patients with AD carry a *FLG* mutation<sup>21-30</sup>. However, skin barrier is also reduced in some patients with AD who do not carry *FLG* mutations<sup>15</sup>, and the majority of heterozygous *FLG* carriers never develop AD<sup>31</sup>.

So far it is unclear whether *FLG* mutations are an independent risk factor for ICD – e.g. due to a deficient skin barrier or an altered inflammatory status – or if they work through AD. It is also not clear how these mutations interact with the inflammatory processes that are characteristic of AD.

We previously studied the prevalence of the R501X and 2282del4 *FLG* mutations in 296 patients with ICD and a control group of 217 vocational school apprentices. We showed that *FLG* mutations doubled the risk for occupational ICD<sup>32</sup>. Here we present a continuation of that study, increasing the number of patients (n=634) and controls (n= 393). In addition to R501X and 2282del4, we genotyped our samples for two less common null mutations: R2447X and S3247X. Together these four different mutations constitute to more than 90% of the *FLG* mutations found in European populations<sup>33</sup>. The aim of the present study was to investigate the relative contribution and interaction of *FLG* mutations and AD to the risk of acquiring ICD.

## METHODS

### Study population

Ethical approval was obtained from the ethics committee of the University of Osnabrück. Patients were recruited from a specialized clinic for treatment of occupational skin diseases, following a joint study protocol<sup>34;35</sup>. Between 2005 and 2011, all consecutive patients who presented with chronic CD of the hands for at least 3 months (either present at the time of examination or medically verified in the past), were of European descent, were at least 18 years of age and did not suffer from further chronic inflammatory diseases (e.g. rheumatoid arthritis, Crohn disease, systemic lupus erythematosus or psoriasis), as assessed by anamnesis, were asked to provide a DNA sample obtained by a buccal swab. A total of 634 patients fulfilling these inclusion criteria and having a primary diagnosis of ICD according to the dermatologists were included in the present study. For each patient, a full medical and dermatological history was taken, including information about sex, age, diagnosis, age of onset of CD and history of flexural eczema. The diagnosis of ICD was based on a patient's history, exposure to irritants, clinical distribution, presence of skin lesions and exclusion of other dermatologic entities, and patients having no clinically relevant type-IV-sensitization. Patients were patch tested to an extended range of allergens, including standardized and customized substances. All patients were tested at least with the European standard tray, and tests were conducted and read according to international guidelines.<sup>36</sup> Controls were recruited from vocational schools training students in high-risk occupations for hand eczema, e.g. hairdressing, nursing, metalworking, food and catering, or floristry. Of the 500 trainees asked to participate, 477 agreed. Of these, 84 were excluded because they were not of European descent or because they suffered from chronic inflammatory disease (e.g.

rheumatoid arthritis or psoriasis). The remaining 393 trainees were in their second or third year of schooling. Following written informed consent, the controls were asked to complete a questionnaire including information about sex, age, and medical history, particularly with regard to the skin and to atopic symptoms (flexural eczema, rhinitis and asthma). Additionally, a subset of 245 students underwent a brief examination by an experienced dermatologist, who assessed present flexural eczema, anamnesis of childhood eczema and family history of rhinoconjunctivitis, allergic asthma and AD. Current or past flexural eczema in the patients, or self-reported flexural eczema in the controls was used as an indicator of AD.

### Filaggrin genotyping

DNA material was obtained from buccal mucosa cells with buccal swabs (GenetiLab Diagnostic & Research, Pordenone, Italy). For each subject, two swabs were obtained and 2 ml lysis buffer (Puregene<sup>®</sup> Cell Lysis Solution, Gentra Systems, Minneapolis, MN, USA) was added to each swab to disrupt the cells and stabilize the DNA. Extraction and genotyping for R501X, R2447X and S3247X was performed by KBioscience (<http://www.kbioscience.co.uk>). Genotyping was performed using the KASP single nucleotide polymorphism genotyping system (KBioscience), a homogeneous fluorescent resonance energy transfer-based system, coupled with competitive allele specific polymerase chain reaction (PCR). Blind duplicates and Hardy-Weinberg equilibrium tests were used as quality control tests.

R501X was genotyped by using the primer pair 5'-GAATGCCTGGAGCTGTCTCG-3' (C-allele) and 5'-CTGAATGCCTGGAGCTGTCTCA-3' (T-allele) with the common allele primer 5'-GCACTGGAGGAAGACAAGGATCG-3'. R2447X was genotyped by using the primer pair 5'-GAGTGCCTGGAGCTGTCTCG-3' (C-allele) and 5'-GAGTGCCTGGAGCTGTCTCA-3' (T-allele) with the common allele primer 5'-GAGGAAGACAAGGATCCCACCACA-3'. S3247X was genotyped by using the primer pair 5'-GTGTCTGGAGCCGTGCCTTG-3' (C-allele) and 5'-GGTGTCTGGAGCCGTGCCTTT-3' (A-allele) with the common primer 5'-CTTCCAGAAACCATCGTGGATCTGT-3'.

Genotyping for 2282del4 was performed by sizing a fluorescently labeled PCR fragment on an Applied Biosystems 3100 or 3730 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.) as described previously<sup>26;32</sup>.

### Statistical analysis

The observed genotype frequencies were compared with the expected Hardy-Weinberg distribution by  $\chi^2$ -test using an online calculator<sup>37</sup>. Differences in median age of onset of ICD among the patients were assessed with the Mann-Whitney U-test. To estimate the risk of disease conferred by a particular genotype, we calculated the odds ratios (ORs) with 95% confidence intervals (CIs) using  $\chi^2$ -tests comparing the heterozygous and homozygous variant allele genotypes with the wildtype genotype. The effect of *FLG* loss-of-function mutations, AD and possible interaction effects were analysed using logistic regression with backwards selection of variables. The statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, U.S.A.).

## RESULTS

The demographic characteristics of patients and controls are shown in Table 1. The median ages of the patients and controls were 43 and 19 years, respectively. The median age at onset of ICD among the patients was 32 years, except for hairdressers and beauticians, who

developed ICD on average at 19 years of age. The age at onset of ICD was significantly lower in patients with AD than in patients without AD (median age 25 vs. 37 years;  $p < 0.0001$ ). *FLG* loss-of-function mutations did not influence the age of onset of ICD in the patients (data not shown).

The genotype distributions of the 2282del4, R501X, R2447X and S3247X polymorphisms observed in patients and controls did not deviate significantly from the Hardy-Weinberg equilibrium. *FLG* loss-of-function mutation prevalence and allele frequencies for patients and controls are displayed in Table 2. *FLG* loss-of-function mutations were significantly more prevalent in patients with ICD compared with controls, with a crude OR of 2.09 (95% CI 1.33 – 3.28) for the combined carrier allele.

Table 3 shows the prevalence of *FLG* mutations and AD in patients and controls. A history of flexural eczema, used as an indicator of AD, was about twice as common among patients with ICD compared with controls (41.1% vs. 18.7%, respectively). Of the 245 controls who underwent a brief dermatological examination, 40 (16.3%) reported present or past flexural eczema in the questionnaire. Five of them revealed flexural eczema on the day of examination and another 33 were diagnosed with childhood flexural eczema according to their past medical history. *FLG* mutations were present in 13.6% of controls with a history of AD, compared with 7.4% of controls without a history of AD. Among patients, the carrier frequencies of *FLG* mutations were 22.6% and 10.7% in patients with or without a history of AD, respectively. Approximately 70% of the controls with *FLG* mutations – i.e. 6% of the total control population – had no history of AD.

Logistic regression analysis revealed that both *FLG* mutations and AD were significant risk factors for ICD; the effect of AD (OR 2.89) exceeded that of *FLG* (OR 1.61; Table 4). There was no significant interaction effect between *FLG* mutations and AD ( $p = 0.67$ ).

## DISCUSSION

In this study we confirmed the association between *FLG* loss-of-function mutations and the risk of developing ICD that we reported in our previous pilot study, which included only two *FLG* mutations, R501X and 2282del4, and was carried out on a smaller sample<sup>32</sup>. The crude OR for the combined mutant allele based on four polymorphisms (R501X, 2282del4, R2447X and S3247X) was 2.09 (95% CI 1.33 – 3.28), which is comparable with the OR of 1.91 found in our earlier investigation. Here, we show for the first time a significant association of ICD with *FLG* loss-of-function mutations, even if the analysis is adjusted for AD (OR 1.61; 95% CI 1.01 – 2.58). A history of AD increased the risk to develop ICD approximately threefold (OR 2.89; 95% CI 2.08 – 4.03). Thus, according to the regression model, concomitant presence of AD and *FLG* mutations would result in a 4.7-fold increased risk. We found *FLG* loss-of-function mutations in 15.9% of the ICD patients and in 8.3% of the controls. The *FLG* carrier frequency of 8.3% in our control group is in agreement with a general prevalence of 7-10% in European populations<sup>16</sup>. Among cases with current or past AD, the prevalence of *FLG* loss-of-function mutations was 22.6%, which is in line with previously reported carrier frequencies of 21% for the four most common mutations in German adult patients with AD<sup>23;30</sup>. Theoretically, some selection bias could have occurred if more susceptible apprentices (e.g. with a history of AD) had chosen to avoid high risk occupations. However, as genotype distribution and prevalence of flexural eczema were similar to those reported in studies among the German general population, and dropout between the first and second year had been negligible, preselection of our control population was unlikely<sup>38</sup>.

On the other hand, some recall bias could have occurred, as in our control group a history of flexural eczema, as a proxy for AD, was assessed by self-administered questionnaires. As in the patient group a history of flexural eczema was assessed by a standardized interview, we also performed examination by a dermatologist in a subset of controls. Self-reported history of flexural eczema correlated well with the dermatologist's conducted anamnesis. Furthermore, the prevalence of flexural eczema reported by our control subjects (19%) was in agreement with an earlier reported lifetime prevalence among German adolescent populations of 14 – 25%<sup>39;40</sup>.

Another possible source of bias might be the age difference between controls and patients (median ages 19 and 43 years, respectively). Thus, we may have missed some cases of adult-onset AD in our control group. Epidemiologic data on late-onset AD are scarce, but some reports indicate that the proportion of patients with disease starting in adulthood is approximately 5%<sup>40-42</sup>. Therefore, we performed a second analysis with adjusted prevalence of AD in the controls ( $\chi^2$ -test with Mantel Haenzel correction). Adjustment for age did not change the outcomes of the analysis.

To date, most studies investigating polymorphisms in *FLG* have focused on possible associations with AD, and only a few studies have addressed CD. In a 2009 pilot study Molin *et al.*<sup>43</sup> investigated two *FLG* loss-of-function mutations in 122 German nonatopic patients with different subtypes of chronic hand eczema (atopic hand eczema cases were excluded), and compared them to 95 control individuals of unknown origin. Marginally significant associations with *FLG* were reported for a subgroup of patients diagnosed with a combination of ICD and ACD, but not in the subgroup with ICD alone. However, several limitations limit the informative value of this study, such as the small sample size and the choice of the control population. In 2010, Thyssen *et al.*<sup>44</sup> performed a cross-sectional study genotyping R501X and 2282del4 in 3335 adults recruited from a random sample (n = 7931) of the Danish general population. The participants were patch tested and filled in a questionnaire addressing the presence of AD and hand eczema – including ICD, ACD, and atopic hand eczema – during the previous 12 months. *FLG* loss-of-function mutations were over-represented in cases of hand eczema in subjects with AD (OR 2.98; 95% CI: 1.27 – 7.01), but not in subjects without AD (OR 0.82; 95% CI 0.41-1.67). The combined presence of AD and *FLG* loss-of-function mutation status yielded an OR for hand eczema of 3.23 (95% CI 1.51 – 6.91).

The increased susceptibility to ICD in carriers of *FLG* mutations might at least partly be explained by barrier dysfunction, as demonstrated in patients with ichthyosis vulgaris without concomitant AD<sup>17</sup>, in *FLG*<sup>-/-</sup> mice<sup>45</sup> and in infants with and without eczema<sup>46</sup>. Recently, we reported that patients with AD with *FLG* mutations had elevated levels of pro-inflammatory IL-1 cytokines<sup>47</sup>, which might influence inflammatory response after exposure to irritating chemicals. A reduced threshold to inflammation from topically applied irritants has been shown in filaggrin-deficient ('flaky tail') mice<sup>19</sup>.

On the other hand, patients with AD without *FLG* mutations also showed a deficient skin barrier and reduced expression of filaggrin break-down products<sup>15;31;46;48</sup>. Furthermore, filaggrin expression can also be reduced by *FLG* intragenic copy number variations<sup>49</sup>, through downregulation by inflammatory cytokines<sup>33;50</sup> or by modulation of enzymatic processes<sup>16</sup>. The fact that in the present study AD had a stronger effect than *FLG* loss-of-function mutations indicates that other factors, e.g. immunological processes, may play a role in individual susceptibility to ICD next to an impaired skin barrier.

However, it has to be stressed that exposure to skin-irritating factors remains the major causative factor for ICD, and intrinsic factors such as AD and *FLG* mutations only modify

the risk. Excessive environmental exposure to irritants and/or allergens, not only in the workplace but also at home (e.g. nickel), may even conceal the role of genetic susceptibility in epidemiological studies. Unfortunately, the design of our case-control study did not allow for including exposure as a risk factor for ICD. To gain more insight in the complex interplay between *FLG* loss-of-function mutations, atopic predisposition and exposure, a prospective cohort design would be preferable.

In summary, our results indicate that both *FLG* loss-of-function mutations and AD significantly increase the risk of ICD, with respective ORs of 1.61 and 2.89. Individuals with both *FLG* mutations and AD have an approximately four- to fivefold increased risk of developing ICD.

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**Table 1**

Demographic characteristics of patients and controls

		<b>Patients<sup>a</sup></b>	<b>Controls<sup>a</sup></b>
Total N		634	393
Sex, n (%)			
	Males	236 (38)	149 (38)
	Females	392 (62)	244 (62)
Age (years), median (25 - 75%)		43 (31 - 51)	19 (18 - 22)
Occupational Sector, n (%)			
	Hairdressing / Beauty	75 (12)	48 (12)
	Healthcare	252 (40)	95 (24)
	Cleaning	28 (5)	-
	Metalwork / Mechanics	122 (20)	128 (33)
	Construction	30 (5)	-
	Food and Catering	44 (7)	18 (5)
	Floristry and Gardening	14 (2)	-
	Other	64 (10)	100 (26)
Age at onset of irritant contact dermatitis (years), median (25 - 75%)		32 (22 - 43)	-

<sup>a</sup> Subgroup totals may not add up to the total N due to missing data.

**Table 2**

Genotype and allele frequencies among patients (N = 634) and controls (N = 393)

Polymorphism	Genotype	Patients n (%) <sup>a</sup>	Controls n (%) <sup>a</sup>	Odds ratio (95% confidence interval)
R501X	AA	587 (94.5)	350 (97.5)	
	Aa	34 (5.5)	9 (2.5)	
	aa	0 (0.0)	0 (0.0)	
	Wild-type allele	1208 (97.3)	709 (98.7)	1.00
	Mutation allele	34 (2.7)	9 (1.3)	2.25 (1.07 – 4.75)*
2282del4	AA	567 (90.6)	350 (95.1)	
	Aa	58 (9.3)	18 (4.9)	
	aa	1 (0.2)	0 (0.0)	
	Wild-type allele	1192 (95.2)	718 (97.6)	1.00
	Mutation allele	60 (4.8)	18 (2.4)	2.02 (1.17 – 3.49)*
R2447X	AA	599 (99.2)	345 (99.4)	
	Aa	5 (0.8)	2 (0.6)	
	aa	0 (0.0)	0 (0.0)	
	Wild-type allele	1203 (99.6)	692 (99.7)	1.00
	Mutation allele	5 (0.4)	2 (0.3)	1.44 (0.28 – 7.46)
S3247X	AA	608 (99.2)	357 (99.7)	
	Aa	5 (0.8)	1 (0.3)	
	aa	0 (0.0)	0 (0.0)	
	Wild-type allele	1221 (99.6)	715 (99.9)	1.00
	Mutation allele	5 (0.4)	1 (0.1)	2.94 (0.34 – 25.23)
Combined	AA	499 (84.1)	299 (91.7)	
	Aa	87 (14.7)	27 (8.3)	
	aa <sup>b</sup>	7 (1.2)	0 (0.0)	
	Wild-type allele	1085 (91.5)	625 (95.9)	1.00
	Mutation allele	101 (8.5)	27 (4.1)	2.09 (1.33 – 3.28)*

<sup>a</sup>Total number of subjects may differ between polymorphisms due to genotyping failures<sup>b</sup>Homozygous or compound heterozygous\* Significant at  $p < 0.05$

**Table 3**

Prevalence of atopic dermatitis (AD) and filaggrin gene (*FLG*) loss-of-function mutations in patients and controls

		Controls			Patients		
		<i>FLG</i> loss-of-function mutation			<i>FLG</i> loss-of-function mutation		
		No	Yes	Total	No	Yes	Total
History of AD <sup>a</sup>	No	237 (75.2%)	19 (6.0%)	256 (81.3%)	301 (52.6%)	36 (6.3%)	337 (58.9%)
	Yes	51 (16.2%)	8 (2.5%)	59 (18.7%)	182 (31.8%)	53 (9.3%)	235 (41.1%)
	Total	288 (91.4%)	27 (8.6%)	315 (100%)	483 (84.4%)	89 (15.6%)	572 (100%)

<sup>a</sup> AD was defined by current or past flexural eczema

**Table 4**

Logistic regression model for the increased risk of developing irritant contact dermatitis due to atopic dermatitis (AD) and filaggrin gene (*FLG*) loss-of-function mutations

	Total N <sup>b</sup>	AD <sup>a</sup>		FLG loss-of-function mutation	
		No n (%)	Yes n (%)	No n (%)	Yes n (%)
Controls	393	308 (81.3%)	71 (18.7%)	299 (91.7%)	27 (8.3%)
Patients	634	361 (59.2%)	249 (40.8%)	499 (84.1%)	94 (15.9%)
Adjusted odds ratio (95% confidence interval) <sup>c</sup>		2.89 (2.08 – 4.03)*		1.61 (1.01 – 2.58)*	

<sup>a</sup> AD was defined by current or past flexural eczema

<sup>b</sup> Subgroup totals may not add up to the total N due to genotyping failures and/or missing data on flexural eczema

<sup>c</sup> Adjusted for AD and *FLG* mutations, respectively

\* Significant at  $p < 0.05$