

1 **Analysis of the individual and aggregate genetic contributions of previously**  
2 **identified *SPINK5*, *KLK7* and *FLG* polymorphisms to eczema risk**

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55 **ABSTRACT**

56 **Background**

57 Polymorphisms in the serine protease inhibitor gene *SPINK5* and the serine protease  
58 *KLK7* appear to confer risk to eczema in some cohorts but these findings have not  
59 been widely replicated. These genes encode proteins thought to be involved in  
60 regulation of post-translation processing of filaggrin, the strongest identified genetic risk  
61 factor for eczema to date.

62 **Objectives**

63 To clarify the individual risk of eczema conferred by the *SPINK5* polymorphism-  
64 rs2303067 (Lys420Ser) and a previously described insertion in the 3'UTR of *KLK7* and  
65 to examine potential epistatic effects between these variants and *FLG* mutations.

66 **Methods**

67 Initially we examined the effects of these polymorphisms and *FLG* in 486 unrelated  
68 cases from a German family-based study, an additional 287 German cases, and 418  
69 unrelated Irish/English eczema cases (n for 3 genes studied = 1191 vs. 4544 controls).  
70 We then additionally studied the *SPINK5* polymorphism and *FLG* mutations in 1583  
71 eczema patients from the ALSPAC cohort (n for 2 genes studied = 2774 vs. 10607  
72 controls).

73 **Results**

74 No association was seen with the *SPINK5* or *KLK7* variants in the case-control  
75 analysis; however, a weaker effect was observed for the *SPINK5* variant with maternal  
76 transmission in the family-based study. No interactions were seen between the  
77 polymorphisms in *KLK7*, *SPINK5* and *FLG*.

78 **Conclusion**

79 The *SPINK5* 420LysSer mutation confers a risk of eczema when maternally inherited,  
80 but is not a major eczema risk factor. The *KLK7* insertion appears to confer no risk of

81 eczema. We found no interaction between the *SPINK5* risk allele or the putative *KLK7*  
82 risk allele and *FLG* mutations.

83 **Abstract word count: 254**

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#### 85 **Key Messages**

- 86 • The *SPINK5* Lys420Ser polymorphism confers a risk of eczema when maternally  
87 inherited, but is not a major genetic contributor to eczema risk
- 88 • A previously reported association of a *KLK7* insertion and eczema could not be  
89 confirmed
- 90 • There is no evidence for epistatic effects between *KLK7* or *SPINK5* variants and  
91 *FLG* mutations

92

#### 93 **Capsule Summary**

94 Previously reported polymorphisms in *SPINK5*, *KLK7* and *FLG* were studied in 2774  
95 eczema cases and 10607 controls. No association with eczema was seen with the  
96 *KLK7* insertion, a weak maternal effect was seen with the *SPINK5* Lys420Ser  
97 polymorphism; neither polymorphism had an epistatic effect with *FLG*.

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101 **Key words:** Eczema, atopy, skin barrier, stratum corneum, epistasis

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104 **Abbreviations:**

105	AIC	Akaike Information Criterion
106	ALSPAC	Avon Longitudinal Study of Parents and Children
107	CI	Confidence Interval
108	FLG	Filaggrin
109	IC	Logistic regression model with an interaction score
110	<i>KLK7</i>	Kallikrein-related peptidase 7
111	KORA	Co-operative Health Research in the Region of Augsburg
112	LEKTI	Lympho-epithelial Kazal type inhibitor
113	LRM	Logistic regression model with product interaction terms
114	MAF	Minor Allele Frequency
115	MNM	Multinomial regression model
116	OR	Odds Ratio
117	RF	Random forest
118	SE	Standard error
119	SNP	Single nucleotide polymorphism
120	SPINK5	Serine peptidase inhibitor, Kazal type 5
121	SSCE	Stratum corneum chymotryptic enzyme
122	TDT	Transmission disequilibrium test

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142 region of Scotland.

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145 **CONFLICT OF INTEREST STATEMENT**

146 WHIM holds patents related to diagnosis and therapeutic correction of the filaggrin  
147 gene. All other authors declare that they have no competing financial or other interests.

148

149 **Introduction**

150 Eczema is a common chronic inflammatory skin disease with a complex, multifactorial  
151 aetiology and a strong genetic component<sup>1, 2</sup>. Like other complex diseases eczema is  
152 hypothesized to be determined by many genetic factors interacting with environmental  
153 components<sup>2</sup>. In the most commonly accepted paradigm for complex diseases, single  
154 genetic factors are considered to contribute only a modest amount to the total variation  
155 in the trait, but are likely to exert additive or synergistic effects known as epistatic  
156 interactions<sup>3</sup>.

157 The identification of two common (R501X and 2282del4) and several rare mutations  
158 within the *filaggrin* (*FLG*) gene causing a deficiency of this key protein involved in skin  
159 barrier function delineated a major genetic risk for eczema<sup>4-6</sup>. Subsequently an  
160 impressive series of replication studies<sup>6-21</sup> confirmed that these polymorphisms confer  
161 an exceptionally strong risk for eczema and subsequent allergen sensitization and that  
162 *FLG* is one of the strongest known genes for complex diseases in general<sup>22-25</sup>.

163 These observations suggest that the breakdown of the epidermal barrier represents  
164 one of the primary events in the development of eczema. This breach might then allow  
165 increased penetration of antigens, allergens and irritants from the environment and  
166 thereby predispose to allergic sensitization and aberrant responses to microbial  
167 infection<sup>26-28</sup>.

168 Filaggrin is initially synthesized as biologically inactive profilaggrin, which is expressed  
169 as a highly phosphorylated insoluble protein in the granular layer of the epidermis.  
170 During the transition from granular cells to flattened squames, profilaggrin is processed  
171 to biologically active filaggrin monomers by several dephosphorylation and proteolytic  
172 steps<sup>29, 30</sup>, the impairment of which might also impair skin barrier function. One of the  
173 proteases that has been suggested to be implicated in profilaggrin processing is the  
174 stratum corneum chymotryptic enzyme (SSCE)<sup>31, 32</sup>, which is possibly regulated by the

175 serine protease inhibitor LETKI, encoded by *SPINK5*<sup>32-34</sup>. Interestingly, an insertion in  
176 the 3' untranslated region of the *kallikrein 7* gene (*KLK7*) encoding SCCE<sup>35</sup> has been  
177 reported to be associated with eczema. Early genome wide linkage analysis of eczema  
178 family studies suggested a potential locus on 5q31 and, after identification of 6 common  
179 polymorphisms in *SPINK5*, the variant Lys420Ser, was associated with eczema in a  
180 cohort of British children,<sup>36</sup> this association has been replicated in 2 small Japanese  
181 studies,<sup>37, 38</sup> but other studies have failed to replicate this association.{REFS HERE}  
182 However, whereas *FLG* has been firmly established as a major gene for eczema, the  
183 reported effects of *KLK7* and *SPINK5* variants are rather weak and so far lack robust  
184 confirmation in replication cohort studies. Therefore, the aim of the present study was  
185 to address the existing literature and to clarify the role of these previously reported  
186 polymorphisms in *SPINK5* or *KLK7* in eczema. In addition, given their potential effects  
187 on post translational modification of filaggrin, we also sought to examine gene-gene  
188 interactions between *FLG*, *KLK7* and *SPINK5*.

189

## 190 **Methods:**

### 191 **Study populations**

192 *SPINK5*, *KLK7* and *FLG* variants were typed in a cohort of 486 German parent-  
193 offspring trios for eczema, a collection of 418 English and Irish eczema cases, and 552  
194 Irish blood donor controls, an additional series of 287 eczema cases from Germany and  
195 the population-based cross-sectional KORA S4 cohort (n=3992). In addition, the  
196 population-based ALSPAC cohort (n=7646) was typed for the Lys420Ser *SPINK5*  
197 polymorphism and the two most common *FLG* mutations R501X and 2282del4. Finally,  
198 to increase power, we performed a pooled analysis on all available data from all  
199 cohorts (for details on study populations see supplementary table 1).

200 The study designs have been described in detail elsewhere<sup>9, 18, 39</sup>. Briefly, KORA S4  
201 represents a sample of the general adult population of German nationality in the region  
202 of Augsburg recruited from October 1999 to April 2001. The survey comprised 4261  
203 unrelated men and women between 25 and 74 years of age. All subjects had to  
204 complete a standardised questionnaire, that, in addition to demographic data included  
205 the basic allergy questions of the European Community Respiratory Health Survey  
206 (ECRHS) on respiratory health<sup>40</sup>. All individuals received a skin examination by  
207 experienced senior dermatologists, who had been additionally trained before the start  
208 of the study, according to the criteria of Hanifin & Rajka<sup>41</sup> and the UK diagnostic criteria  
209 for eczema<sup>42</sup>.

210 All German eczema cases were unrelated and of white origin with eczema diagnosed  
211 on the basis of a skin examination by experienced dermatologists using the UK  
212 diagnostic criteria<sup>42</sup>. In the family collection, 10.4% of the parents suffered from eczema  
213 (9.3% affected fathers and 11.1% affected mothers).

214 Eczema cases from Ireland were recruited through attendance at a hospital-based  
215 clinic in Our Lady's Children's Hospital Crumlin and the diagnosis was made according  
216 to the UK diagnostic guidelines by an experienced paediatric dermatologist (ADI, GO'R  
217 or RW). The English eczema cohort was recruited from hospital-based clinics in  
218 London and Newcastle and has been described previously<sup>11</sup>. A summary of the  
219 demographics for all eczema study populations examined is presented in table E1 in  
220 the online repository.

221 The Avon Longitudinal Study of Parents and Children (ALSPAC) is a longitudinal,  
222 population-based birth cohort study that recruited 14541 unrelated pregnant women  
223 resident in Avon, UK with expected dates of delivery between 1<sup>st</sup> April 1991 and 31<sup>st</sup>  
224 December 1992. There were 14,062 liveborn children. The study protocol has been  
225 described previously<sup>43, 44</sup> and further details are on the ALSPAC website:



226 <http://www.alspac.bris.ac.uk>. At 6, 18, 30 and 42 months of age the mothers were  
227 asked whether their child had skin rashes in the joints or creases of the body. As in  
228 previous studies, we defined individuals with eczema as those with reports of flexural  
229 dermatitis at 2 time points between 6 and 42 months<sup>25, 43, 44</sup>

230 All study methods were approved by the relevant local authorities and a written and  
231 informed consent that complies with all the Declaration of Helsinki Principles was  
232 obtained from all participants.

233

### 234 **Genotyping:**

235 Genotyping in German samples was performed using the MassARRAY system  
236 (Sequenom, San Diego, USA) as described previously<sup>9</sup>. Genotyping calls were made  
237 in real time with MASSARRAY RT software (Sequenom). Primers as well as allele  
238 frequencies in the population-based KORA S4 cohort (n=4198) are shown in the online  
239 repository table E2.

240 The Lys420Ser polymorphism in *SPINK5* (rs2303067) was typed in the Irish and  
241 English eczema cohorts and controls using a predesigned SNP Taqman Genotyping  
242 Assay from Applied Biosystems (product C\_2000249\_10) and run on a 7900HT Fast  
243 Real-Time PCR system using the manufacturers recommended protocol. The 4bp  
244 insertion in the 3'UTR of *KLK7* was typed in Irish and English eczema cohorts and  
245 controls by sizing of fluorescently labelled PCR products on an Applied Biosystems  
246 3130xl Genetic Analyser. 10ml PCR reactions were performed using 25ng of genomic  
247 DNA with 400nM forward primer (5' gtt tct tca agt gtg caa gtt cac caa 3') and 400nM  
248 FAM-labelled reverse primer (5' gat tgg ttt atc aac agg gc 3') in AmpliTaq Gold Buffer  
249 containing 1.5mM MgCl<sub>2</sub>, 10nmol of each dNTP, 4% v/v DMSO and 0.25U AmpliTaq  
250 Gold polymerase (Applied Biosystems). PCR reactions were amplified using an

251 annealing temperature of 58°C. Diluted PCR products were sized against ROX-500  
252 size standards (Applied Biosystems). Allele sizes were 201bp and 205bp (insertion).  
253 For the ALSPAC cohort the 2 commonest *FLG* mutations were typed as previously  
254 described<sup>25</sup> and the *SPINK5* Lys420Ser polymorphism was typed by Taqman assay.  
255 As we discovered three negative associations for *KLK7* in the eczema cohorts we did  
256 not perform *KLK7* analysis in this very large population cohort.

257

### 258 **Statistical analyses:**

259 Descriptive statistics for quantitative and qualitative values are given by mean ±  
260 standard deviation (SD) and relative frequencies or absolute numbers, respectively.  
261 Deviation from Hardy-Weinberg equilibrium was tested in parents for the family-  
262 analyses and in controls for the case-control analyses. In the family-setting we  
263 analysed association of single SNPs with eczema using the classical transmission  
264 disequilibrium test (TDT). Parent-of-origin effects were investigated with the method  
265 proposed by Weinberg<sup>45</sup>.

266 Case-control analyses for single SNPs was performed using logistic regression models  
267 adjusted for age and gender. In order not to constrain the analyses to a specific genetic  
268 model we modelled the three categorical genotypes by two dummy variables.

269

270 Gene-gene interaction analyses was performed after excluding individuals with two  
271 mutant *FLG* alleles since these individuals do not express the filaggrin protein and  
272 therefore a biological interaction with post-translational modifications by SSCE and  
273 *LEKTI* is not plausible.

274 In order to estimate interaction effects between the single polymorphisms in *FLG*,  
275 *SSCE* and *LEKTI*, four different approaches were carried out. In any of these  
276 approaches we adjusted the models for the common covariates age and gender. Firstly,

277 interaction was evaluated using the logistic regression model with product interaction  
278 terms (LRM). The Akaike Information Criterion (AIC) was used to select the appropriate  
279 model<sup>46</sup> Secondly, we defined an interaction score (IC) which counts the number of  
280 copies of the potentially disease-associated alleles<sup>47</sup>, which we used as a covariate in  
281 the logistic model. Thirdly, we modelled maternal effects observed for *SPINK5* using  
282 affected offspring from families only and estimated ORs for two different affection  
283 status.. compared with controls in a multinomial regression model (MRM), which was  
284 carried out with BayesX 1.50<sup>48</sup>. An elaborate description of the statistical methods are  
285 given in the online repository.

286 For any of these regression approaches the quantitative covariate age was modelled  
287 non-parametrically in a general additive model framework (GAM)

288 Finally, for further exploration, variable importance measures were computed by means  
289 of the random forest method. Random forests provide variable importance measures  
290 that can be employed to detect variables relevant for predicting the response. The most  
291 commonly used variable importance measure is the permutation importance<sup>49</sup>.

292 High positive values of the importance measure obtained indicate a high variable  
293 importance. Small positive or negative values indicate that a variable is irrelevant for  
294 predicting the response.

295 Additionally, we pooled all three study cohorts to increase the power of detecting any  
296 single SNP and interaction effect. As British and German populations might be slightly  
297 different with regard to ethnicity<sup>17</sup> we accounted for a population effect in every analysis  
298 approach by introducing a binary independent variable which codes 1="British/Irish  
299 origin" and 0="German origin". Thus we corrected the estimated genetic effect for  
300 potential population differences.

301 Power calculations<sup>50</sup> for the pooled single SNP analyses were performed with  
302 nQuery7.0 assuming a dominant model.

303 All statistical analyses were carried out with R 2.6.0<sup>51</sup>, unless otherwise stated.

304 **Results:**

305 **Single gene analyses**

306 First we examined the effect of the individual polymorphisms in predisposition to  
307 eczema in our samples. Allele frequencies are presented in supplementary table 1. The  
308 *SPINK5* and *KLK7* polymorphisms showed comparable allele frequencies across all  
309 study populations. *FLG* polymorphism results have been published for the German  
310 family study previously<sup>9, 52</sup>, although for this study further families and cases were  
311 tested to increase statistical power, in particular when looking for interaction between  
312 alleles. In the German family cohort, *FLG* polymorphisms greatly increased the risk for  
313 eczema (OR=2.75, 95%CI=1.93-3.98,  $p=1.8 \times 10^{-8}$ ), whereas the *SPINK5* polymorphism  
314 rs2303067 showed only a slight over-transmission to eczema-affected offspring  
315 (OR=1.25, 95%CI=1.04-1.50,  $p=0.018$ ). No associations were seen for the *KLK7* 3'UTR  
316 insertion (Table 1). In this family cohort, the power to detect a proportion of 1.2 between  
317 transmission of the risk allele vs. non transmission (which corresponds to a difference  
318 in the proportions of 5% given the observed number of discordant pairs) was 35% for  
319 the *SPINK5* polymorphism and 44% for the *KLK7* insertion. The power to detect a  
320 difference in the proportion of 9% for *SPINK5* and of 8% for *KLK7* was 80%.

321 Since parent-of-origin effects have been reported for *SPINK5* variants<sup>36</sup>, we also tested  
322 for differences between maternal and paternal allele sharing and we confirmed a  
323 stronger association for the maternally inherited rs23030067 A-allele and a relative risk  
324 of transmission of maternal alleles compared to paternal alleles of 2.18 (95% CI 1.38-  
325 3.43,  $p=0.0008$ ). The observed power to detect parent-of-origin effects as described in  
326 <sup>45</sup> was greater than 90%. In the pooled German case-control cohort the presence of at  
327 least one *FLG* variant greatly increased the risk for eczema (OR=4.56, 95%CI=3.44-  
328 6.05,  $p=5.9 \times 10^{-26}$ ). In contrast, neither the *SPINK5* nor the *KLK7* variant were  
329 associated with eczema (Table 2).

330 In the Irish case-control cohort comparable results could be seen: presence of a *FLG*  
331 null allele increased the risk for eczema about 5.88 fold (95%CI=3.85-8.99,  $p=2.8 \times 10^{-16}$ ).  
332 No association between *SPINK5* or *KLK7* and eczema was detected (Table 2).

333 The analyses of ALSPAC also showed a strong *FLG* effect (OR=2.23, 95%CI=1.87-  
334 2.67,  $1.2 \times 10^{-18}$ ). Consistent with our other cohorts, no association between the  
335 Lys420Ser *SPINK5* polymorphism and eczema was found (Table 2).

336 For the pooled study in all models a population effect was estimated as a confounder  
337 with  $p$ -values  $< 10^{-4}$ . An exceptionally strong *FLG* effect on eczema was seen for the  
338 pooled analyses (OR=3.36, 95%CI=2.97-3.79,  $p=1.3 \times 10^{-84}$ ) with a power of >99% for  
339 the observed proportion of *FLG* variants of 0.21 in the cases. No association was  
340 observed for the *SPINK5* variant. The power to detect an increased risk in OR=1.2 for  
341 carriers of the *SPINK5* rare allele compared to non-carriers with an observed proportion  
342 of 0.73 in the cases was 95% assuming a dominant genetic model. For *KLK7*, we had  
343 78% power to detect an OR of 1.2 in the pooled analyses with an observed MAF in the  
344 cases of 0.56.

345

#### 346 **Gene-gene interaction analyses:**

347 Gene-gene interactions were examined in all three cohorts separately and together in a  
348 pooled analysis.

349 The LRM approach in the German case-control cohort was the best fitting model on the  
350 basis of minimization of AIC with main effects of *FLG* and rs2303067 (*SPINK5*) and  
351 their product interaction terms. In this model only *FLG* showed a significant genetic  
352 effect. In the Irish case-control cohort only *FLG* along with the covariates age and  
353 gender remained in the model according to the AIC (Table 3).

354 In the IC approach, in addition to one mutant *FLG* allele the A allele of rs2303067  
355 (*SPINK5*) as well as the *KLK7* insertion were defined as “risk” variants. By deriving a

356 score from the numbers of variant copies in the German case-control cohort we  
357 observed a tendency for an increasing risk with the number of risk alleles an individual  
358 carried. The fall in OR in the last category can probably be attributed to the low number  
359 of observations, as reflected by the wide CI. Interestingly, the effect size increases with  
360 the number of risk alleles probably because of the increased chance of risk *FLG* alleles  
361 in these cells.

362 For the Irish/British case-control cohort similar results were observed, but the OR in the  
363 four variant group was more than twice as high as in the German case-control analyses.  
364 Using the MNM approach, we tried to account for the maternal parent-of-origin effect  
365 reported by Walley et al. 2001<sup>36</sup>. In our family collection we observed a tendency for an  
366 increasing risk of development of eczema caused by *FLG* mutations (regardless of  
367 inheritance of these *FLG* mutations), if the *SPINK5* SNP rs2303067 was inherited from  
368 the mother. There was an increased risk of eczema compared with paternal inheritance.  
369 However, the null hypothesis for equal *FLG* risk in both response categories could not  
370 be rejected.

371 In the RF approach (Table 4), in the German sample only age and *FLG* status showed  
372 positive variable importance values. The average out-of-bag prediction accuracy was  
373 between 87.7% for the random forests and 88.3% for bagging. However, this is due to  
374 the fact that the average specificity was close to 100%, while the sensitivity was around  
375 43% in the sample with approximately 21% cases. In the Irish case-control cohort only  
376 age and to some extent *FLG* and gender were suitable for predicting eczema. The  
377 average out-of-bag prediction accuracy was between 83.2% for the random forests and  
378 84.4% for bagging.

379 The pooled analyses revealed consistent results to the previously estimated effects. In  
380 all models we estimated a population effect as a confounder with  $p$ -values  $< 10^{-4}$ .

## 381 Discussion

382 This large-scale study examined variants in three candidate genes, which have  
383 previously been reported to be associated with eczema. All genes encode proteins that  
384 are involved in the highly organized process of epidermal differentiation and are  
385 important for the maintenance of the skin barrier function. In addition, due to their  
386 biological interactions we hypothesized that there might also be gene-gene-interactions.  
387 Filaggrin is a key protein for the development of the cornified envelope and the process  
388 of cornification<sup>53</sup>. Two common null mutations in the *FLG* gene (R501X, 2282del4) have  
389 been firmly established as strong risk factors for eczema<sup>22, 23</sup>. *KLK7* encodes the  
390 protease SSCE, which has been suggested to be involved in the complex proteolytic  
391 processing of filaggrin. An insertion in the 3'UTR of the *KLK7* gene possibly influencing  
392 SSCE activity has been reported to be associated with eczema in a UK case-control  
393 study, but this association has not been replicated so far<sup>14, 35</sup>. *SPINK5* is the gene  
394 defective in Netherton syndrome and encodes the serine proteinase inhibitor LEKTI,  
395 which has been implicated in the regulation of SSCE activity. An association of a  
396 *SPINK5* SNP with eczema has previously been reported<sup>36</sup>, but was not confirmed in a  
397 recent study<sup>54</sup>.

398 Using a large cohort of German families and an Irish/English case-control series as well  
399 as a pooled and enlarged German case-control collection and the longitudinal ALSPAC  
400 cohort, we first examined the individual SNPs. Results from these analyses suggest  
401 that, of the tested polymorphisms, only the *FLG* mutations represent important and  
402 replicable genetic determinants for eczema, whereas the *SPINK5* variant Lys420Ser  
403 appears to have a weaker effect, and only when maternally inherited, and the *KLK7*  
404 insertion does not exert an effect. However, our data does not preclude that there are  
405 other variants in *SPINK5* and/or *KLK7* of importance for eczema.

406



407 In a second step we aimed at elucidating potential gene-gene-interaction. Using several  
408 statistical approaches we found no evidence for an interaction between variants in  
409 these three genes. The fact that the random forests permutation importance is  
410 essentially zero for the *SPINK5* and *KLK7* variants confirms that these variants are not  
411 relevant for predicting eczema, neither individually nor in interactions with each other,  
412 as interactions would be captured by the random forest variable importance<sup>55</sup>. Since  
413 there was a considerable difference in age between cases and controls, we considered  
414 age as a covariate in all analyses and modelled it non-parametrically. However, this  
415 leads to little variability in the response data explained by *FLG* mutations in the RF  
416 approach.

417 It is widely hypothesized that complex human diseases such as eczema result from an  
418 unknown number of genetic factors, each of which influences susceptibility through  
419 interactions with other genes and with environmental factors<sup>56, 57</sup>. With whole-genome  
420 association studies with hundreds of thousands of measured genetic variations  
421 emerging, analyses of the complex molecular interactions on the DNA level is of utmost  
422 importance and it will be necessary to develop innovative statistical methods. For  
423 eczema, this is the first study that directly addresses this issue by exploring the effect of  
424 potential interactions among genes encoding proteins in the filaggrin expression and  
425 processing pathways. Using diverse and complementary statistical approaches in this  
426 large sample we did not find evidence for epistatic effects between *FLG* and *KLK7*  
427 variants that significantly predict eczema risk. Thus, while our data underlines the  
428 exceptional importance of filaggrin deficiency for eczema risk, it does not support the  
429 hypothesis that its effect is dependent on or modified by *KLK7*. The results of the  
430 pooled analyses and the family analyses give a hint that *SPINK5* may be a potential  
431 player (when maternally inherited) within the filaggrin cascade but this association

432 requires further exploration. However, it cannot be excluded that acquired alterations in  
433 filaggrin processing or variations in other genes in the same pathway such as *KLK5*  
434 might contribute to eczema susceptibility. Functional studies are needed to explore the  
435 individual roles of products of genes within the filaggrin pathway and their biologic  
436 interactions, and future large-scale studies using powerful statistical methods will aid in  
437 elucidating the relationship between combinations of polymorphisms for eczema  
438 susceptibility.



439 **Table 1: ORs and 95% CIs for associations between polymorphisms and eczema in the**  
 440 **family-based analysis. T, transmitted; U, untransmitted; comb., combined.**  
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<b>German families (A)</b>	<b>Gene</b>	<b>Polymorphism</b>	<b>T:U</b>	<b>OR</b>	<b>95% CI</b>	<b>P-value</b>
	<i>FLG</i>	r501x	41: 12	3.42	1.84 6.70	1.2 x10 <sup>-4</sup>
		2282del4	72: 31	2.32	1.54 3.57	8.1 x10 <sup>-5</sup>
		comb. genotype	110: 40	2.75	1.93 3.98	1.8 x10 <sup>-8</sup>
	<i>SPINK5</i>	rs2303067	259: 207	1.25	1.04 1.50	0.01815
	<i>KLK7</i>	AACC ins	183: 177	1.03	0.84 1.27	0.79215

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444 **Table 2: Associations between polymorphisms and eczema in the case-control**  
 445 **approaches. All logistic regression models are adjusted for age and gender. Every block**  
 446 **refers to a single model where only estimates of the genetic variables are displayed. No**  
 447 **specific genetic model was assumed and estimates are given for every genotype**  
 448 **compared to the wildtype. comb., combined; het, heterozygous; hom, homozygous.**  
 449 **Populations as designated in table 1 are indicated in brackets.**  
 450

	Gene	Polymorphism	OR	95% CI		P-value
<b>German (A,B,E)</b>  cases: 773 controls: 3992 total: 4765	FLG	comb. genotype (het. vs. wt)	4.15	3.10	5.56	1.3x10 <sup>-21</sup>
		comb. genotype (hom. vs. wt)	23.11	7.23	73.89	1.2x10 <sup>-7</sup>
	SPINK5	rs2303067(het. vs. wt)	1.14	0.87	1.49	0.34285
		rs2303067(hom. vs. wt)	1.22	0.89	1.67	0.21252
	KLK7	AACC ins (het. vs. wt)	1.11	0.89	1.40	0.35472
AACC ins (hom. vs. wt)		0.95	0.64	1.42	0.81033	
<b>Irish/English (C,F)</b>  cases: 418 controls: 552 total: 970	FLG	comb. genotype (het. vs. wt)	4.34	2.77	6.79	1.3x10 <sup>-10</sup>
		comb. genotype (hom. vs. wt)	2.6x10 <sup>56</sup>	0	∞	1.0
	SPINK5	rs2303067(het. vs. wt)	0.78	0.52	1.18	0.23945
		rs2303067(hom. vs. wt)	1.15	0.71	1.87	0.57398
	KLK7	(het. vs. wt)	1.29	0.89	1.86	0.17756
(hom. vs. wt)		0.91	0.47	1.76	0.76988	
<b>ALSPAC (D,G)</b>  cases: 1583 controls: 6063 total: 7646	FLG	comb. genotype (het. vs. wt)	2.17	1.81	2.60	3.5x10 <sup>-17</sup>
		comb. genotype (hom. vs. wt)	3.3x10 <sup>6</sup>	0	∞	0.94524
	SPINK5	rs2303067(het. vs. wt)	0.96	0.84	1.09	0.52176
		rs2303067(hom. vs. wt)	1.14	0.98	1.34	0.09156
<b>Pooled* (A,B,C,D,E,F,G)</b>  cases: 2774 controls: 10607 total: 13381	FLG	comb. genotype (het. vs. wt)	3.04	2.68	3.44	8.5x10 <sup>-68</sup>
		comb. genotype (hom. vs. wt)	49.38	19.72	123.61	8.2x10 <sup>-17</sup>
	SPINK5	rs2303067(het. vs. wt)	0.97	0.87	1.07	0.50850
		rs2303067(hom. vs. wt)	1.13	1.00	1.27	0.04545

451 \* for the pooled analyses we estimated in all models a population effect as confounder with p-values < 10<sup>-4</sup>

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**Table 3: Associations between polymorphisms or gene-gene interaction terms and eczema. All models are adjusted for age and gender. Every block refers to a single model. LRM, logistic regression model with product interaction terms; IC, logistic regression model with interaction score; MNM, multinomial logistic model considering maternal inheritance. Populations as designated in table 1 are indicated in brackets.**

German						
	gene-gene interaction term	OR	95% CI		P-value	
LRM (A,B,E)	FLG comb. genotype (het. vs. wt.)	6.11	4.09	9.12	3.3x10 <sup>-10</sup>	
	rs2303067 (het. vs. wt.)	1.28	0.87	1.90	0.11862	
	rs2303067 (hom. vs. wt.)	1.28	0.83	1.97	0.18638	
	FLG comb. genotype (het.) rs2303067 (het.)	0.62	0.34	1.11	0.17805	
	FLG comb. genotype (het.) rs2303067 (hom.)	0.61	0.06	6.20	0.23915	
IC (A,B,E)	<i>interaction score</i> *	1 vs. 0	1.08	0.72	1.61	0.71881
		2 vs. 0	1.18	0.80	1.76	0.39974
		3 vs. 0	1.67	1.08	2.57	0.01982
		4 vs. 0	2.75	1.53	4.92	0.00067
		5 vs. 0	1.45	0.14	14.70	0.75107
MNM (A,E)	Multinomial logit trait	covariates	OR	95% CI		P-value
			0: controls	1:gender f vs. m	1.69	1.12
	1: eczema & SPINK5 (not maternal)	1: FLG comb. genotype	5.22	3.12	8.73	4.9x10 <sup>-7</sup>
		2:gender f vs. m	1.32	0.87	2.00	0.18653
	2: eczema & SPINK5 (maternal)	2: FLG comb. genotype	6.16	3.70	10.26	1.1x10 <sup>-7</sup>
test $\beta_{1.combGeno}=\beta_{2.combGeno}$					0.80071	
Irish						
	gene-gene interaction term	OR	95% CI		P-value	
LRM (C,F)	FLG comb. genotype (het. vs. wt.)	4.34	2.77	6.79	1.3 x 10 <sup>-10</sup>	
IC (C,F)	<i>interaction score</i> *	1 vs. 0	1.32	0.64	2.75	0.45470
		2 vs. 0	1.18	0.56	2.46	0.66276
		3 vs. 0	1.96	0.92	4.20	0.08175
		4 vs. 0	5.17	1.88	14.24	0.00148
		5 vs. 0	0.01	~0	2.6x10 <sup>10</sup>	0.74599
ALSPAC						
	gene-gene interaction term	OR	95% CI		P-value	
LRM (D,G)	FLG comb. genotype (het. vs. wt.)	2.26	1.63	3.14	1.2 x 10 <sup>-6</sup>	
	rs2303067 (het. vs. wt.)	1.02	0.87	1.19	0.83390	
	rs2303067 (hom. vs. wt.)	1.18	0.98	1.41	0.07389	
	FLG comb. genotype (het.) rs2303067 (het.)	0.90	0.59	1.38	0.63859	
	FLG comb. genotype (het.) rs2303067 (hom.)	1.04	0.62	1.74	0.87865	
IC (D,G)	<i>interaction score</i> *	1 vs. 0	1.06	0.92	1.23	0.39907
		2 vs. 0	1.29	1.09	1.51	0.00245
		3 vs. 0	2.72	1.84	4.02	5.4 x 10 <sup>-7</sup>
pooled**						
	gene-gene interaction term	OR	95% CI		P-value	
LRM (A,B,C,D ,E,F,G)	FLG comb. genotype (het. vs. wt.)	3.43	2.71	4.35	1.7 x 10 <sup>-24</sup>	
	rs2303067 (het. vs. wt.)	1.03	0.92	1.16	0.58801	
	rs2303067 (hom. vs. wt.)	1.15	1.00	1.32	0.04914	
	FLG comb. genotype (het.) rs2303067 (het.)	0.81	0.60	1.09	0.16864	
	FLG comb. genotype (het.) rs2303067 (hom.)	0.96	0.67	1.37	0.82037	
IC (A,B,C,D ,E,F,G)	<i>interaction score</i> *	1 vs. 0	1.10	0.99	1.23	0.08116
		2 vs. 0	1.37	1.21	1.55	6.5 x 10 <sup>-7</sup>
		3 vs. 0	3.66	2.83	4.75	1.2 x 10 <sup>-22</sup>

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\*score: number of copies of the "risk" alleles in FLG comb. genotype, SPINK5 rs2303067 and KLK7, for ALSPAC and pooled analysis the score is reduced to the number of copies of FLG and SPINK5 alleles  
\*\*for the pooled analyses in all models we estimated a population effect as confounder with p-values<10

461 **Table 4: Results for random forests with 2 randomly pre-selected variables in each split.**  
 462 **The average permutation importance +/- 2 standard errors of the mean over 100**  
 463 **iterations are displayed for each variable. Results for random forests with 5 randomly**  
 464 **preselected variables, i.e. for bagging, were almost identical. High positive values**  
 465 **indicate a high variable importance. Small positive or negative values indicate that a**  
 466 **variable is irrelevant for predicting the response. Populations as designated in table 1**  
 467 **are indicated in brackets.**  
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RF	German ( <i>A,B,E</i> )		Irish ( <i>C,F</i> )		ALSPAC ( <i>D,G</i> )		Pooled ( <i>A,B,C,D,E,F,G</i> )	
	mean - 2 se	mean - 2 se	mean - 2 se	mean + 2 se	mean - 2 se	mean + 2 se	mean - 2 se	mean + 2 se
age	0.12179	0.12241	0.19797	0.19891	n.a.	n.a.	n.a.	n.a.
gender	-0.00037	-0.00032	0.01075	0.01106	-0.00004	-0.00003	-0.00022	-0.00021
<i>FLG</i> comb. genotype	0.00465	0.00473	0.05204	0.05253	0.00001	0.00002	0.00468	0.00474
<i>SPINK5</i>	-0.00021	-0.00016	0.00040	0.00059	-0.00003	-0.00002	-0.00024	-0.00022
<i>KLK7</i>	-0.00015	-0.00011	-0.00112	-0.00097	n.a.	n.a.	n.a.	n.a.
population	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-0.00008	-0.00006

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472 **Figure 1:**  
473 **Residual plot of age after fitting a general linear model for individual SNP**  
474 **analyses. The anscombe residuals show a functional form and do not spread**  
475 **randomly. Hence a general additive model is fitted for the data.**

476

477 **Figure 2:**  
478 **Residual plot of age after fitting a general linear model for SNP-SNP interaction**  
479 **analyses. The anscombe residuals show a functional form and do not spread**  
480 **randomly. Hence a general additive model is fitted for the data. The upper row**  
481 **reflects anscombe residuals of the German case-control analyses; the lower row**  
482 **shows the anscombe residuals for the same models in the Irish case-control**  
483 **cohort.**

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486 **References:**

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- 488 1. van Beijsterveldt CE, Boomsma DI. Genetics of parentally reported asthma, eczema  
489 and rhinitis in 5-yr-old twins. *Eur Respir J* 2007; 29:516-21.
- 490 2. Morar N, Willis-Owen SA, Moffatt MF, Cookson WO. The genetics of atopic  
491 dermatitis. *J Allergy Clin Immunol* 2006; 118:24-34; quiz 5-6.
- 492 3. Glazier AM, Nadeau JH, Aitman TJ. Finding genes that underlie complex traits.  
493 *Science* 2002; 298:2345-9.
- 494 4. Smith FJ, Irvine AD, Terron-Kwiatkowski A, Sandilands A, Campbell LE, Zhao Y, et  
495 al. Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris.  
496 *Nat Genet* 2006; 38:337-42.
- 497 5. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al.  
498 Common loss-of-function variants of the epidermal barrier protein filaggrin are a  
499 major predisposing factor for atopic dermatitis. *Nat Genet* 2006; 38:441-6.
- 500 6. Sandilands A, O'Regan GM, Liao H, Zhao Y, Terron-Kwiatkowski A, Watson RM, et  
501 al. Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis  
502 vulgaris and predispose individuals to atopic dermatitis. *J Invest Dermatol* 2006;  
503 126:1770-5.
- 504 7. Ruether A, Stoll M, Schwarz T, Schreiber S, Folster-Holst R. Filaggrin loss-of-  
505 function variant contributes to atopic dermatitis risk in the population of Northern  
506 Germany. *Br J Dermatol* 2006; 155:1093-4.
- 507 8. Marenholz I, Nickel R, Ruschendorf F, Schulz F, Esparza-Gordillo J, Kerscher T, et al.  
508 Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic  
509 march. *J Allergy Clin Immunol* 2006; 118:866-71.
- 510 9. Weidinger S, Illig T, Baurecht H, Irvine AD, Rodriguez E, Diaz-Lacava A, et al. Loss-  
511 of-function variations within the filaggrin gene predispose for atopic dermatitis with  
512 allergic sensitizations. *J Allergy Clin Immunol* 2006; 118:214-9.
- 513 10. Stemmler S, Parwez Q, Petrasch-Parwez E, Epplen JT, Hoffjan S. Two Common  
514 Loss-of-Function Mutations within the Filaggrin Gene Predispose for Early Onset of  
515 Atopic Dermatitis. *J Invest Dermatol* 2006.
- 516 11. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null Mutations in the  
517 Filaggrin Gene (FLG) Determine Major Susceptibility to Early-Onset Atopic  
518 Dermatitis that Persists into Adulthood. *J Invest Dermatol* 2006.
- 519 12. Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson  
520 RM, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent  
521 and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 2007; 39:650-  
522 4.
- 523 13. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null mutations in the  
524 filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis  
525 that persists into adulthood. *J Invest Dermatol* 2007; 127:564-7.
- 526 14. Hubiche T, Ged C, Benard A, Leaute-Labreze C, McElreavey K, de Verneuil H, et al.  
527 Analysis of SPINK 5, KLK 7 and FLG genotypes in a French atopic dermatitis cohort.  
528 *Acta Derm Venereol* 2007; 87:499-505.
- 529 15. Morar N, Cookson WO, Harper JI, Moffatt MF. Filaggrin mutations in children with  
530 severe atopic dermatitis. *J Invest Dermatol* 2007; 127:1667-72.
- 531 16. Nomura T, Sandilands A, Akiyama M, Liao H, Evans AT, Sakai K, et al. Unique  
532 mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic  
533 dermatitis. *J Allergy Clin Immunol* 2007; 119:434-40.
- 534 17. Rogers AJ, Celedon JC, Lasky-Su JA, Weiss ST, Raby BA. Filaggrin mutations  
535 confer susceptibility to atopic dermatitis but not to asthma. *J Allergy Clin Immunol*  
536 2007; 120:1332-7.

- 537 18. Weidinger S, Rodriguez E, Stahl C, Wagenpfeil S, Klopp N, Illig T, et al. Filaggrin  
538 mutations strongly predispose to early-onset and extrinsic atopic dermatitis. *J Invest*  
539 *Dermatol* 2007; 127:724-6.
- 540 19. Nomura T, Akiyama M, Sandilands A, Nemoto-Hasebe I, Sakai K, Nagasaki A, et al.  
541 Specific Filaggrin Mutations Cause Ichthyosis Vulgaris and Are Significantly  
542 Associated with Atopic Dermatitis in Japan. *J Invest Dermatol* 2008.
- 543 20. Ekelund E, Lieden A, Link J, Lee SP, D'Amato M, Palmer CN, et al. Loss-of-function  
544 variants of the filaggrin gene are associated with atopic eczema and associated  
545 phenotypes in Swedish families. *Acta Derm Venereol* 2008; 88:15-9.
- 546 21. Brown SJ, Relton CL, Liao H, Zhao Y, Sandilands A, Wilson IJ, et al. Filaggrin null  
547 mutations and childhood atopic eczema: a population-based case-control study. *J*  
548 *Allergy Clin Immunol* 2008; 121:940-46 e3.
- 549 22. Baurecht H, Irvine AD, Novak N, Illig T, Buhler B, Ring J, et al. Towards a major risk  
550 factor for atopic eczema: meta-analysis of filaggrin mutation data. *J Allergy Clin*  
551 *Immunol* 2007; in press.
- 552 23. Rodriguez E, Illig T, Weidinger S. Filaggrin loss-of-function mutations and  
553 association with allergic diseases. *Pharmacogenomics* 2008; 9:399-413.
- 554 24. Weidinger S, O'Sullivan M, Illig T, Baurecht H, Depner M, Rodriguez E, et al.  
555 Filaggrin mutations, atopic eczema, hay fever, and asthma in children. *J Allergy Clin*  
556 *Immunol* 2008.
- 557 25. Henderson J, Northstone K, Lee SP, Liao H, Zhao Y, Pembrey M, et al. The burden of  
558 disease associated with filaggrin mutations: a population-based, longitudinal birth  
559 cohort study. *J Allergy Clin Immunol* 2008; 121:872-7 e9.
- 560 26. Hudson TJ. Skin barrier function and allergic risk. *Nat Genet* 2006; 38:399-400.
- 561 27. McLean WH, Hull PR. Breach delivery: increased solute uptake points to a defective  
562 skin barrier in atopic dermatitis. *J Invest Dermatol* 2007; 127:8-10.
- 563 28. Sicherer SH, Leung DY. Advances in allergic skin disease, anaphylaxis, and  
564 hypersensitivity reactions to foods, drugs, and insects. *J Allergy Clin Immunol* 2007;  
565 119:1462-9.
- 566 29. Resing KA, Walsh KA, Dale BA. Identification of two intermediates during  
567 processing of profilaggrin to filaggrin in neonatal mouse epidermis. *J Cell Biol* 1984;  
568 99:1372-8.
- 569 30. Resing KA, Walsh KA, Haugen-Scofield J, Dale BA. Identification of proteolytic  
570 cleavage sites in the conversion of profilaggrin to filaggrin in mammalian epidermis. *J*  
571 *Biol Chem* 1989; 264:1837-45.
- 572 31. Resing KA, Thulin C, Whiting K, al-Alawi N, Mostad S. Characterization of  
573 profilaggrin endoproteinase 1. A regulated cytoplasmic endoproteinase of epidermis. *J*  
574 *Biol Chem* 1995; 270:28193-8.
- 575 32. Descargues P, Deraison C, Bonnart C, Kreft M, Kishibe M, Ishida-Yamamoto A, et al.  
576 Spink5-deficient mice mimic Netherton syndrome through degradation of desmoglein  
577 1 by epidermal protease hyperactivity. *Nat Genet* 2005; 37:56-65.
- 578 33. Komatsu N, Takata M, Otsuki N, Ohka R, Amano O, Takehara K, et al. Elevated  
579 stratum corneum hydrolytic activity in Netherton syndrome suggests an inhibitory  
580 regulation of desquamation by SPINK5-derived peptides. *J Invest Dermatol* 2002;  
581 118:436-43.
- 582 34. Bitoun E, Micheloni A, Lamant L, Bonnart C, Tartaglia-Polcini A, Cobbold C, et al.  
583 LEKTI proteolytic processing in human primary keratinocytes, tissue distribution and  
584 defective expression in Netherton syndrome. *Hum Mol Genet* 2003; 12:2417-30.
- 585 35. Vasilopoulos Y, Cork MJ, Murphy R, Williams HC, Robinson DA, Duff GW, et al.  
586 Genetic association between an AACC insertion in the 3'UTR of the stratum corneum  
587 chymotryptic enzyme gene and atopic dermatitis. *J Invest Dermatol* 2004; 123:62-6.

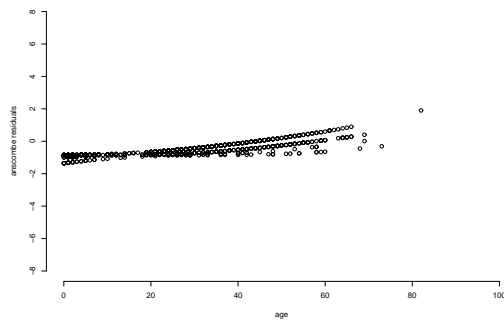
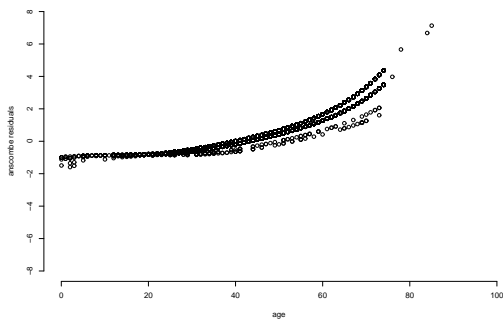
- 588 36. Walley AJ, Chavanas S, Moffatt MF, Esnouf RM, Ubhi B, Lawrence R, et al. Gene  
589 polymorphism in Netherton and common atopic disease. *Nat Genet* 2001; 29:175-8.
- 590 37. Kato A, Fukai K, Oiso N, Hosomi N, Murakami T, Ishii M. Association of SPINK5  
591 gene polymorphisms with atopic dermatitis in the Japanese population. *Br J Dermatol*  
592 2003; 148:665-9.
- 593 38. Nishio Y, Noguchi E, Shibasaki M, Kamioka M, Ichikawa E, Ichikawa K, et al.  
594 Association between polymorphisms in the SPINK5 gene and atopic dermatitis in the  
595 Japanese. *Genes Immun* 2003; 4:515-7.
- 596 39. Rathmann W, Haastert B, Icks A, Lowel H, Meisinger C, Holle R, et al. High  
597 prevalence of undiagnosed diabetes mellitus in Southern Germany: target populations  
598 for efficient screening. The KORA survey 2000. *Diabetologia* 2003; 46:182-9.
- 599 40. Burney PG, Luczynska C, Chinn S, Jarvis D. The European Community Respiratory  
600 Health Survey. *Eur Respir J* 1994; 7:954-60.
- 601 41. Hanifin JM, Rajka G. Diagnostic features of atopic eczema. *Acta Derm Venereol*  
602 1980;44-7.
- 603 42. Williams HC, Burney PG, Hay RJ, Archer CB, Shipley MJ, Hunter JJ, et al. The U.K.  
604 Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum  
605 set of discriminators for atopic dermatitis. *Br J Dermatol* 1994; 131:383-96.
- 606 43. Golding J, Pembrey M, Jones R. ALSPAC--the Avon Longitudinal Study of Parents  
607 and Children. I. Study methodology. *Paediatr Perinat Epidemiol* 2001; 15:74-87.
- 608 44. Jones RW, Ring S, Tyfield L, Hamvas R, Simmons H, Pembrey M, et al. A new  
609 human genetic resource: a DNA bank established as part of the Avon longitudinal  
610 study of pregnancy and childhood (ALSPAC). *Eur J Hum Genet* 2000; 8:653-60.
- 611 45. Weinberg CR. Methods for detection of parent-of-origin effects in genetic studies of  
612 case-parents triads. *Am J Hum Genet* 1999; 65:229-35.
- 613 46. Foster MR. Key concepts in model selection: Performance and generalizability. *J*  
614 *Math Psychol* 2000; 44:205-31.
- 615 47. Weedon MN, McCarthy MI, Hitman G, Walker M, Groves CJ, Zeggini E, et al.  
616 Combining information from common type 2 diabetes risk polymorphisms improves  
617 disease prediction. *PLoS Med* 2006; 3:e374.
- 618 48. Brezger A, Kneib T, Lang S. BayesX - Software for Bayesian Inference in Structured  
619 Additive Regression Models. Munich, 2007.
- 620 49. Strobl C, Boulesteix AL, Zeileis A, Hothorn T. Bias in random forest variable  
621 importance measures: illustrations, sources and a solution. *BMC Bioinformatics* 2007;  
622 8:25.
- 623 50. Fleiss JL, Tytun A, Ury HK. A Simple Approximation for Calculating Sample Sizes  
624 for Comparing Independent Proportions. *Biometrics* 1980; 36:343-6.
- 625 51. Team RDC. R: A Language and Environment for Statistical Computing. Vienna: R  
626 Foundation for Statistical Computing, 2007.
- 627 52. Weidinger S, Rodriguez E, Stahl C, Wagenpfeil S, Klopp N, Illig T, et al. Filaggrin  
628 mutations strongly predispose to early-onset and extrinsic atopic dermatitis *J Invest*  
629 *Dermatol* 2006; 126:in press.
- 630 53. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the  
631 skin. *Nat Rev Mol Cell Biol* 2005; 6:328-40.
- 632 54. Folster-Holst R, Stoll M, Koch WA, Hampe J, Christophers E, Schreiber S. Lack of  
633 association of SPINK5 polymorphisms with nonsyndromic atopic dermatitis in the  
634 population of Northern Germany. *Br J Dermatol* 2005; 152:1365-7.
- 635 55. Lunetta KL, Hayward LB, Segal J, Van Eerdewegh P. Screening large-scale  
636 association study data: exploiting interactions using random forests. *BMC Genet*  
637 2004; 5:32.

- 638 56. Moore JH. The ubiquitous nature of epistasis in determining susceptibility to common  
639 human diseases. *Hum Hered* 2003; 56:73-82.  
640 57. Moore JH. A global view of epistasis. *Nat Genet* 2005; 37:13-4.  
641  
642

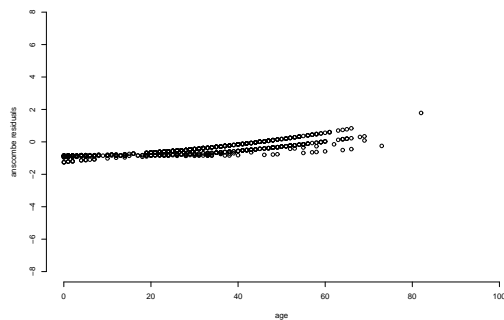
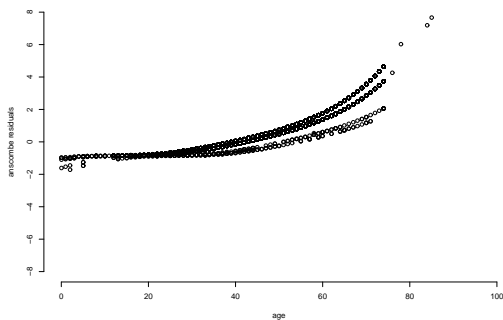
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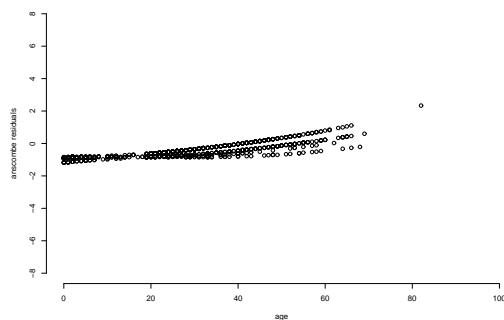
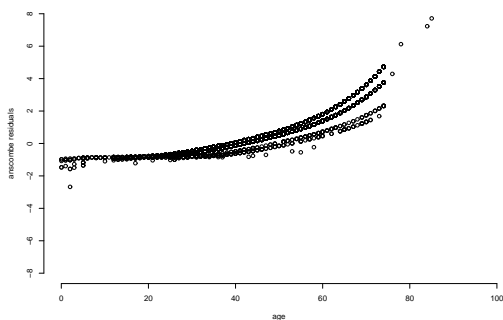
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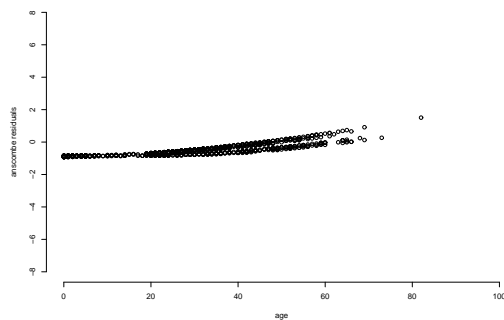
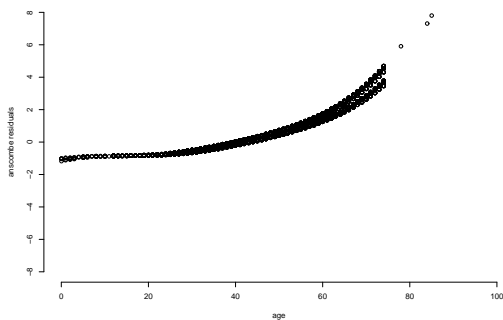
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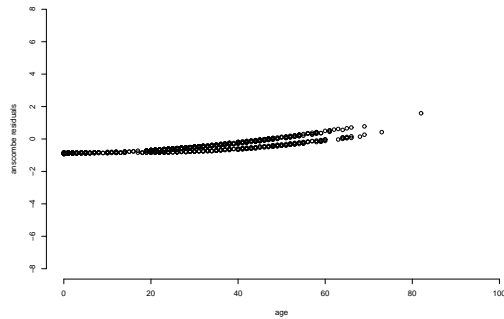
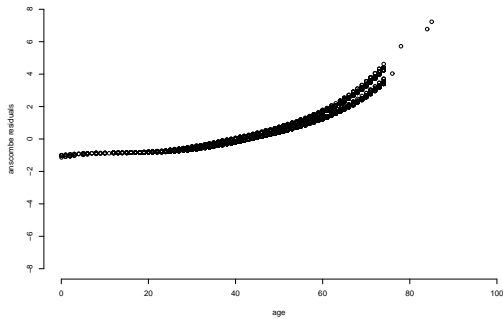
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comb.Geno



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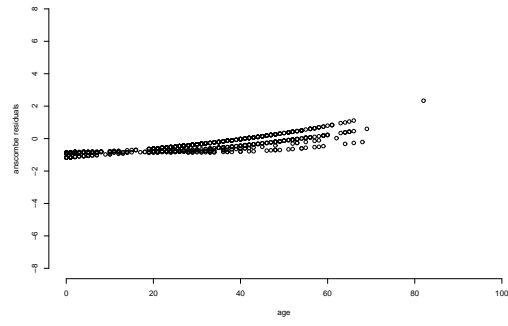
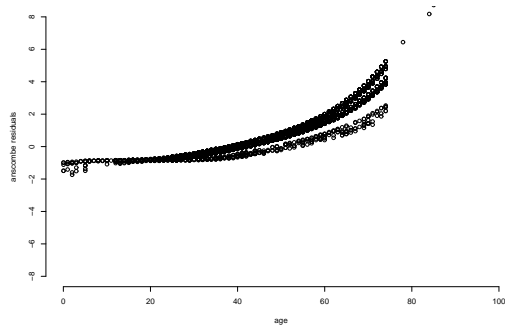
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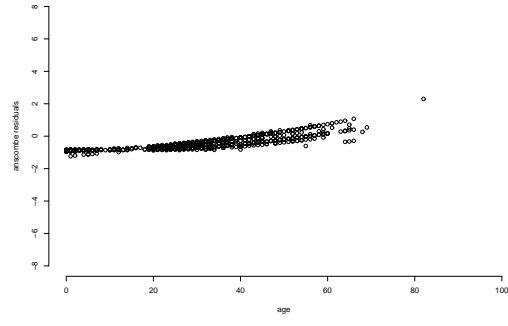
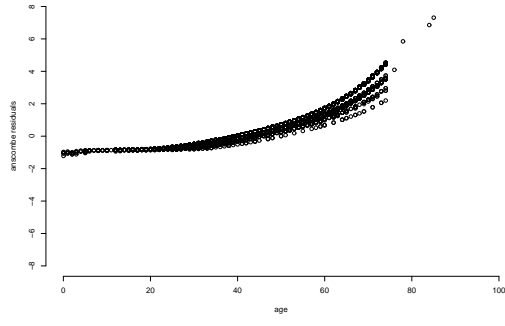
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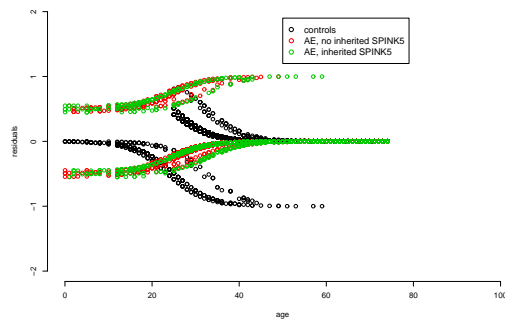
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MNM



## Online Repository:

### Analysis of the individual and aggregate genetic contributions of previously identified *SPINK5*, *KLK7* and *FLG* polymorphisms to eczema risk

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## METHODS

### Statistical methods:

Descriptive statistics for quantitative and qualitative values are given by mean  $\pm$  standard deviation (SD) and relative frequencies or absolute numbers, respectively. Deviation from Hardy-Weinberg equilibrium was tested in parents for the family-analyses and in controls for the case-control analyses. In the family-setting we analysed association of single SNPs with eczema using the classical transmission disequilibrium test (TDT). Parent-of-origin effects were investigated with the method proposed by Weinberg<sup>1</sup>.

Case-control analyses for single SNPs was performed using logistic regression models adjusted for age and gender. In order not to constrain the analyses to a specific genetic model we modelled the three categorical genotypes by two dummy variables. Thus we estimated separate effects for heterozygotes and homozygotes as compared to wildtype. As the English and Irish case series are ethnically related, were shown to have highly similar genotypes for *FLG*<sup>2, 3</sup>, and here had almost identical MAFs for *KLK7* and *SPINK5* polymorphisms (supplementary table 3), we analysed these cases together.

Gene-gene interaction analyses was performed after excluding individuals with two mutant *FLG* alleles since these individuals do not express the filaggrin protein and therefore a biological interaction with post-translational modifications by SSCE and LEKTI is not plausible.

In order to estimate interaction effects between the single polymorphisms in *FLG*, *SSCE* and *LEKTI*, four different approaches were carried out. In any of these approaches we adjusted the models for the common covariates age and gender. Firstly, interaction was evaluated using the logistic regression model with product



interaction terms (LRM). We started with a sparse model of the known covariates age, gender and *FLG*. Incrementally we extended the model with additional SNPs in *SPINK5* and *KLK7* and SNP-SNP-product-interaction terms. The Akaike Information Criterion (AIC) was used to select the appropriate model<sup>4</sup>.

Secondly, we defined an interaction score (IC) which counts the number of copies of the potentially disease-associated alleles<sup>5</sup>, which we used as a covariate in the logistic model. Thirdly, we modelled maternal effects observed for *SPINK5* using affected offspring from families only. We constructed a three-categorical trait: unaffected controls, affected offspring with no mutant allele inherited from the mother, affected offspring with a mutant allele inherited from the mother. We then estimated ORs for both affected status compared with controls in a multinomial regression model (MRM).

For any of these regression approaches the quantitative covariate age was modelled non-parametrically in a general additive model framework (GAM) due to the functional structure of anscombe residuals after applying analyses in the general linear model framework (GLM). For the multinomial model we used a REML-approach<sup>6</sup> implemented in BayesX 1.507<sup>7</sup>.

Finally, for further exploration, variable importance measures were computed by means of the random forest method. Random forests, and the related method bagging, are an ensemble method where a set of classification or regression trees is aggregated for prediction<sup>8, 9</sup>. Random forests provide variable importance measures that can be employed to detect variables relevant for predicting the response. The most commonly used variable importance measure is the permutation importance<sup>10</sup>. For variable selection purposes the advantage of the random forest permutation accuracy importance measure as compared to univariate screening methods is that it covers the impact of each predictor variable individually as well as in multivariate

interactions with other predictor variables. For example, Lunetta et al.<sup>11</sup> demonstrated that genetic markers relevant in interactions with other markers or environmental variables can be detected more efficiently by means of random forests than by means of univariate screening methods like Fisher's exact test<sup>11</sup>. Here the random forest implementation *cforest* from the package *party*<sup>12, 13</sup> in the R system for statistical computing<sup>14</sup> is used, because it guarantees unbiased variable selection for predictor variables of different scales of measurement<sup>10</sup>. Predictor variables considered here were age, gender and respective SNP variables.

To assess the stability of the results 100 random forests with 500 trees each were fitted with the configuration guaranteeing unbiased variable selection suggested by Strobl et al.<sup>10</sup>. The random forests were built with either 2 randomly pre-selected variables in each split (argument *mtry*=2) or 3 randomly pre-selected variables in each split (*mtry*=3) for comparison. The latter approach is equivalent to bagging, which is contained in random forests as the special case where the number of randomly pre-selected variables is equal to the number of available variables. High positive values of the importance measure obtained indicate a high variable importance. Small positive or negative values indicate that a variable is irrelevant for predicting the response.

Additionally, we pooled all three study cohorts to increase the power of detecting any single SNP and interaction effect. As British and German populations might be slightly different with regard to ethnicity<sup>15</sup> we accounted for a population effect in every analysis approach by introducing a binary variable which codes 1="British/Irish origin" and 0="German origin". Thus we corrected the estimated genetic effect for potential population differences. Power calculations<sup>16</sup> for the pooled single SNP analyses were performed with nQuery7.0 assuming a dominant model.

All statistical analyses were carried out with R 2.6.0<sup>14</sup>, unless otherwise stated.

**Table E1: Descriptive characterization of cases and control populations. N.a., not available**

	n	Mean age in yrs. (std)	Male gender	Mean IgE (std)	Country	Notation
German offspring	486	22.04 (10.64)	198 (40.7%)	990.4 (2472.7)	Germany	A
German cases	287	35.55 (16.15)	112 (39.0%)	1442.2 (2380.6)	Germany	B
Irish/English cases	418	19.42 (18.43)	199 (51.4%)	3008.0 (6170.0)	Ireland	C
ALSPAC cases*	1583	3.5	849 (53.6%)	286.0 (539.0)	England	D
Sum cases	2774					
KORA S4	3992	49.51 (13.90)	1971 (49.9%)	114.2 (1535.5)	Germany	E
Irish controls	552	35.71 (12.27)	170 (30.8%)	n.a.	Ireland	F
ALSPAC controls	6063	3.5*	3135 (51.7%)	200.4 (462.7)	England	G
Sum controls	10607					

\* Eczema status determined in all children at 42 months in ALSPAC cohort

**Table E2: Genotyping details and minor allele frequencies in the KORA S4 population-based cohort. MAF, minor allele frequency; DIR, direction**

SNP ID	MAF	DIR	PCR Primer	Extension Primer
R501X	0.013	fwd rev	ACGTTGGATGCTGGAGGAAGACAAGGATCG ACGTTGGATGATGGTGTCTGACCCTCTTG	ATGCCTGGAGCTGTCTC
2282del4	0.025	fwd rev	ACGTTGGATGTTGGTGGCTCTGCTGATGGT ACGTTGGATGGTGAGGGACATTCAGAAGAC	GAAGACTCAGACACACAGT
rs2303067	0.479	fwd rev	ACGTTGGATGCCATCCTTTTTAGCCAAGC ACGTTGGATGCCTCAAAGGAAGCTGTACTC	GATTGTCTTTTGTCTTCTTGATT
AACC ins	0.312	fwd rev	ACGTTGGATGTGATTGGTTTATCAACAGG ACGTTGGATGGACGCCGATGACCTATGAAG	TTTCCTCAAAGATATATTTAAACC

## References:

1. Weinberg CR. Methods for detection of parent-of-origin effects in genetic studies of case-parents triads. *Am J Hum Genet* 1999; 65:229-35.
2. Sandilands A, O'Regan GM, Liao H, Zhao Y, Terron-Kwiatkowski A, Watson RM, et al. Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis vulgaris and predispose individuals to atopic dermatitis. *J Invest Dermatol* 2006; 126:1770-5.
3. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null Mutations in the Filaggrin Gene (FLG) Determine Major Susceptibility to Early-Onset Atopic Dermatitis that Persists into Adulthood. *J Invest Dermatol* 2006.
4. Foster MR. Key concepts in model selection: Performance and generalizability. *J Math Psychol* 2000; 44:205-31.
5. Weedon MN, McCarthy MI, Hitman G, Walker M, Groves CJ, Zeggini E, et al. Combining information from common type 2 diabetes risk polymorphisms improves disease prediction. *PLoS Med* 2006; 3:e374.
6. Kneib T, Baumgartner B, Steiner WJ. Semiparametric Multinomial Logit Models for Analysing Consumer Choice Behaviour. *ASTA Advances in Statistical Analysis* 2007; 91:225-44.
7. Brezger A, Kneib T, Lang S. *BayesX - Software for Bayesian Inference in Structured Additive Regression Models*. Munich, 2007.
8. Breiman L. Arcing classifiers. *The Annals of Statistics* 1998; 26:801-49.
9. Breiman L. Random forests. *Machine Learning* 2001; 45:5-32.
10. Strobl C, Boulesteix AL, Zeileis A, Hothorn T. Bias in random forest variable importance measures: illustrations, sources and a solution. *BMC Bioinformatics* 2007; 8:25.
11. Lunetta KL, Hayward LB, Segal J, Van Eerdewegh P. Screening large-scale association study data: exploiting interactions using random forests. *BMC Genet* 2004; 5:32.
12. Hothorn T, Hornik K, Zeileis A. Unbiased recursive partitioning: a conditional inference framework. *Journal of Computational and Graphical Statistics* 2006; 15:651-74.
13. Hothorn T, Hornik K, Zeileis A. *Party: A laboratory for recursive part(y)itioningR* package version 0.9-0. 2006.
14. Team RDC. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing, 2007.
15. Rogers AJ, Celedon JC, Lasky-Su JA, Weiss ST, Raby BA. Filaggrin mutations confer susceptibility to atopic dermatitis but not to asthma. *J Allergy Clin Immunol* 2007; 120:1332-7.
16. Fleiss JL, Tytun A, Ury HK. A Simple Approximation for Calculating Sample Sizes for Comparing Independent Proportions. *Biometrics* 1980; 36:343-6.