Article Type: Original Article: Epidemiology and Genetics

Childhood asthma is associated with mutations and gene expression differences of *ORMDL* genes which can interact

Short title: *ORMDLs* and childhood asthma

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12652

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ABSTRACT

Background: Genome-wide association studies identified *ORMDL3* as a plausible asthma candidate gene. ORMDL proteins regulate sphingolipid metabolism and ceramide homeostasis, participate in lymphocyte activation and eosinophil recruitment. Strong sequence homology between the three *ORMDL* genes and ORMDL protein conservation amongst different species suggest that they may have shared functions. We hypothesized that if single nucleotide polymorphisms (SNPs) in *ORMDL3* alter its gene expression and play a role in asthma, variants in *ORMDL1* and *ORMDL2* might also be associated with asthma.

Methods: Asthma associations of 44 genotyped SNPs were determined in at least 1,303 subjects (651 asthmatics). *ORMDL*s expression was evaluated in peripheral blood mononuclear cells (PBMC) from 55 subjects (8 asthmatics) before and after allergen stimulation, and in blood (n=60, 5 asthmatics). Allele-specific *cis*-effects on *ORMDLs* expression were assessed. Interactions between human ORMDL proteins were determined in living cells.

Results: Sixteen SNPs in all three *ORMDL*s were associated with asthma (14 in *ORMDL3*). Baseline expression of *ORMDL1* (p=1.7*10^{-6}) and *ORMDL2* (p=4.9*10^{-5}) was significantly higher in PBMC from asthmatics, while induction of *ORMDL*s upon stimulation was stronger in non-asthmatics. Disease-

associated alleles (rs8079416, rs4795405, rs3902920) alter *ORMDL3* expression. ORMDL proteins formed homo- and heterooligomers and displayed similar patterns of interaction with SERCA2 and SPT1.

Conclusions: Polymorphisms in *ORMDL* genes are associated with asthma. Asthmatics exhibit increased *ORMDL* levels, suggesting that *ORMDL*s contribute to asthma. Formation of heterooligomers and similar interaction patterns with proteins involved in calcium homeostasis and sphingolipid metabolism could indicate shared biological roles of ORMDLs, influencing airway remodeling and hyperresponsiveness.

Keywords: SNP; childhood asthma; PBMC; BRET; protein-protein interaction

ABBREVIATIONS

BIP	Endoplasmic reticulum luminal Ca^{2+}-binding protein grp78
BRET	Bioluminescence energy transfer
Ca^{2+}	Calcium
CI	Confidence interval
Derp1	*Dermatophagoides pteronyssinus* (house dust mite)
EXACT	EXpression Analysis CohorT
eQTL	Expression quantitative trait locus
GWAS	Genome-wide association study
ISAAC II	International Study of Asthma and Allergies in Childhood, phase II

LD	Linkage disequilibrium
LpA	lipid A
MAF	Minor allele frequency
MAGICS	Multicenter Asthma Genetics in Childhood Study
MALDI-TOF-MS	Matrix-Assisted-Laser-Desorption/Ionization-Time-Of-Flight-Mass-Spectrometry
MCAD	Mitochondrial medium-chain acyl-CoA dehydrogenase
OR	Odds ratio
ORMDL (*ORMDL*)	ORM1-like protein (gene)
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
Ppg	Peptidoglycan
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RFC	Relative fold change
Rluc	Renilla luciferase
SERCA2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
SNP	Single nucleotide polymorphism
SPT1	Serine palmitoyltransferase 1
UTR	Untranslated region

YFP	Yellow fluorescent protein
18S rRNA	*eukaryotic 18S ribosomal RNA*

INTRODUCTION (301)

Chromosome 17q21 has been the first (1) and most widely replicated asthma susceptibility locus discovered by genome-wide association studies (GWAS) (2-4). However, none of the genes within the locus had previously been related to the disease. Single nucleotide polymorphisms (SNPs) from 17q21 showing highly significant associations with childhood asthma correlated with the expression of *ORMDL3* transcripts, suggesting *ORMDL3* to be a plausible asthma-candidate gene in the locus (1). Later, allele-specific gene expression was also observed for other genes in chromosome 17q21 (5, 6), questioning the role of *ORMDL3* in asthma.

ORMDL3 belongs to a gene family including also *ORMDL1* (chromosome 2q32) and *ORMDL2* (chromosome 12q13.2) (7). ORMDLs are transmembrane proteins expressed in the endoplasmic reticulum (7, 8), which act as negative regulators in the sphingolipid metabolism (9) and in the maintenance of ceramide homeostasis of mammalian cells (10). In addition, ORMDL3 participates in the intracellular calcium turnover, cellular stress responses (11), lymphocyte activation (12) and eosinophil recruitment (13). Strong sequence homology of paralogs and orthologs in the *ORMDL* gene and ORMDL protein family has been observed. Protein conservation between human ORMDLs exceeds 80% (7), suggesting that ORMDL family members may have shared biological functions. We hypothesized that if SNPs in 17q21 change the function and/or expression of *ORMDL3* and contribute to asthma development, also genetic variants within the other two gene family members,

ORMDL1 and *ORMDL2*, may be associated with childhood asthma. If this were the case, the role of *ORMDL3* as an asthma candidate gene within the 17q21 locus would be strengthened.

To investigate that hypothesis, we performed fine mapping of the three genetic regions harboring *ORMDL*s, utilizing HapMap (14), 1000 Genomes (15) and our own re-sequencing data, analyzed *ORMDL1*, *-2* and *-3* expression patterns in non-asthmatic and asthmatic individuals and determined functional interactions of all three ORMDL proteins.

METHODS

Detailed descriptions of the methods and study cohorts are provided in the Online Supporting information.

Genotyping, SNP selection and genetic association analyses

As previously described in detail (16, 17), DNA from 1,422 children with German and Austrian genetic background (728 asthmatics) was genotyped by Illumina Sentrix HumanHap300 BeadChip (Illumina, Inc., San Diego, USA). The majority of the asthmatic children (*n*=655) were derived from the Multicenter Asthma Genetics in Childhood Study (MAGICS) (1, 18) where asthma was diagnosed by pediatric pulmonologists. Unaffected individuals (*n*=694) and additional 73 asthmatics were obtained randomly from the large (*n*=5,629) cross-sectional International Study of Asthma and Allergy in Childhood, phase II (ISAAC II) (1, 19) (physician's diagnosis of asthma and/ or recurrent spastic or asthmatic bronchitis reported by their parents). These two cohorts were then investigated in a case-reference design. The very similar study protocols of MAGICS and ISAAC II were approved by the respective ethics committees and written informed consent was obtained from parents of participants.

Polymorphisms in *ORMDL1* (Chr. 2: 190,632,838..190,656,054), *ORMDL2* (Chr. 12: 56,205,389.. 56,218,425) and *ORMDL3* (Chr. 17: 38,069,949..38,092,713) and their closest genomic vicinity were selected for genotyping from: (I) chip-genotyping (1, 18); (II) additional selection from HapMap (14) (*ORMDL1* and *ORMDL2*); (III) fine mapping and functional investigation of the 17q21 locus and *ORMDL3*; (IV) 1000 Genomes Pilot phase data (15); (V) additional mutational screening (direct sequencing in at least 40 subjects) on *ORMDL2* (see Fig. S2, S3 and S4, Online Supporting information). Genotyping data required to conduct association analyses were obtained also *de novo* by matrix-assisted-laser-desorption/ionization-time-of-flight-mass-spectrometry (MALDI-TOF-MS) (20) resulting in a maximal dataset of 1,446 individuals (763 asthmatics), while data from chip genotyping was available 1,303 (651 asthmatics) individuals only (Table S1).

Real-time quantitative PCR gene expression analysis

Peripheral blood mononuclear cells (PBMC) from non-asthmatic adult subjects (*n*=47, asthma status was determined based on self-reported questionnaire information) recruited in the EXpression Analysis CohorT (EXACT, *n*=113 individuals) and atopic asthmatics (physician's diagnosis of asthma and atopy status) from the ZAP II study (*n*=8) were isolated and processed as previously reported (5, 21). Subjects were assigned as non-asthmatics if they had no asthma. PBMC were stimulated for 48 hours with phytohemagglutinin (PHA, 5 µg/ mL), *D. pteronyssinus* (Derp1, 30 µg/ mL), lipid A (LpA, 0.1 µg/ mL) or peptidoglycan (Ppg, 10 µg/ mL). Total RNA isolated from PBMC and whole blood was converted to cDNA and expression levels of the targeted genes were investigated by quantitative real-time PCR (qRT-PCR) using *18S rRNA* as an endogenous control (see Tables S6 and S7 in the Online Supporting information). Data analyses were conducted based on the comparative delta-delta C_t ($\Delta\Delta C_t$) method and relative fold changes (RFC) were determined (5, 22). Allele-specific effects of rs5742940 (*ORMDL1*), rs7954619 (*ORMDL2*), and rs8079416, rs4795405, rs12603332,

rs3902920 (*ORMDL3* and its close genomic vicinity) on the respective *ORMDL* expression were investigated in non-asthmatic subjects (*n*=47).

Statistical analyses

Linkage disequilibrium (LD) calculations were conducted by Haploview v.4.2 software (23). All pairwise LD values correspond to the square of the correlation coefficient (r^2). Associations of SNPs with asthma and disease subphenotypes were modelled by logistic regression and reported as odds ratios (ORs) with 95% confidence intervals (95% CI). Gene-by-gene interactions between the *ORMDL*s were evaluated by application of previously developed risk score model (24, 25) using logistic regression. The variant with the strongest asthma association from each *ORMDL* gene was subjected to analysis (i.e. rs5742940, rs7954619 and rs8079416, respectively). A risk value of one (presence of genetic risk) or zero (absence) was assigned to the respective risk alleles which were then summed up and subjects with one, two or three risk alleles were grouped together and compared to the reference population with a risk score of zero. Dominant model was applied for *ORMDL1* and *-3* and recessive – in case of *ORMDL2*.

Calculations were conducted using Plink software package 1.07 (26) (http://pngu.mgh.harvard.edu/~purcell/plink). Gene expression data were analyzed using R software (27).

Physical interaction analyses of ORMDL family proteins

Physical interactions between ORMDL proteins in living COS-7 cells were determined using bioluminescence resonance energy transfer (BRET) where Renilla luciferase (Rluc) served as donor and yellow fluorescent protein (YFP) as the acceptor of energy transfer (28). BRET experiments were performed as previously described (29). Interactions were classified as positive when the BRET ratio was above a method-specific threshold of 0.094.

RESULTS

ORMDL family genes are associated with asthma

As SNPs in *ORMDL3* are associated with asthma, we systematically investigated variants in all three *ORMDL* genes for their effect on the disease. For this purpose we performed a mutation screening in all three *ORMDL*s, applying sequencing as well as bioinformatics tools. Genotypes of 44 polymorphisms with a minor allele frequency (MAF) ≥0.01 were obtained from 1,446 asthmatic and non-asthmatic children. This included 16 SNPs in *ORMDL1*, 9 in *ORMDL2* and 19 in *ORMDL3* (Fig. 1, Table 1, and Table S2 in the Online Supporting information). Genotyping data from our cohort were subjected to LD-based (r^2>0.9) tagging. Polymorphisms in *ORMDL1*, *ORMDL2* and *ORMDL3* and their genomic vicinities formed 11, 8 and 15 tagging bins, respectively.

SNPs in *ORMDL* genes were analyzed for their association with asthma and its atopic and non-atopic subphenotypes (Table 1, Table S2). Polymorphism rs5742940 located in the putative promoter of *ORMDL1* was associated with asthma [p=0.009, OR (95% CI) = 2.31 (1.23-4.33)]. When subphenotypes of asthma were analyzed (Table S2), this variant was associated with atopic asthma [p=0.008, OR (95% CI) = 3.11 (1.34-7.20)] but not with non-atopic asthma (Table S2). Polymorphism rs7954619 just upstream of the putative *ORMDL2* promoter region demonstrated association with asthma [p=0.017, OR (95% CI) = 0.68 (0.49-0.93)]. As expected, SNPs in *ORMDL3* and its closest genomic surrounding on the 17q21 locus showed multiple highly significant associations with asthma (Table 1-C). In brief, 14 SNPs in 10 tagging bins were associated with asthma. Polymorphisms rs8079416 (tagging bin 15) and rs4065275 (tagging bin 1) were only in moderate pairwise LD (r^2 = 0.52, which was the highest r^2 detectable between the two bins) (Fig. 1-C), suggesting that the association signals were at least partly independent between these two tagging bins. The p-values and ORs observed for the remaining significantly associated SNPs corresponded to LD distance between particular SNP and the polymorphisms from bins 1 and 2 (highest r^2 between them = 0.50)

and bins 1 and 5 (highest r^2 = 0.80) or both tagging bins 1 and 15 (bins 9, 10 and 13) (Table 1-C, Fig. 1-C). The highest LD between: (1) bins 1 and 9 was r^2 = 0.73; (2) bins 1 and 10 was r^2 = 0.49; and (3) bins 1 and 13 was r^2 = 0.41, which suggests that these association signals are not completely dependent as well. Polymorphisms from bins 6, 7 and 8 were only in a weak LD with bins 1 and/or 15, thus it is more difficult to relate their associations to tagging bins 1 or 15, the major sources of the association signal in our data (Table 1-C, Fig. 1-C). When conditional analyses were performed, distinct asthma association signals from bins 1, 5 and 15 (data not shown) remained significant. However, reduced effect sizes were observed. Thus, each of these bins explains additional part of the association signal. Disease associations of variants within bins 9, 10 and 13 seem also not entirely related to the strong signal from bin1.

ORMDL expression levels differ between non-asthmatics and asthmatics

When *ORMDL1-3* expression in PBMC was compared between non-asthmatics (*n*=47) and allergic asthmatics (*n*=8), higher *ORMDL*s levels were observed in asthmatics. The baseline expression measured in unstimulated PBMC between both groups differed for *ORMDL1* (p=1.7*10^{-6}), and *ORMDL2* (p=4.9*10^{-5}) but was not significant for *ORMDL3* (Fig. 2). Differences in whole blood (Fig. S5, Supporting information) between asthmatics and non-asthmatics were significant for *ORMDL3* (p=0.005) but not for *ORMDL1* and *ORMDL2* (see Fig. S5, Supporting information).

Stimulation with all allergens (except for Derp1 and LpA in case of *ORMDL3)* led to substantially increased *ORMDL1, -2* and *-3* levels in PBMC of non-asthmatic subjects and this effect was the strongest for *ORMDL2* (Fig. 3 and see Fig. S6). After PHA stimulation *ORMDL2* displayed almost 8-fold elevated mRNA levels compared to unstimulated PBMC, while for the rest of the stimuli RFC was approximately 4-fold (Fig. 3 and Fig. S6). In asthmatics, *ORMDL2* was increased about 4-fold (PHA), 3-

and 2-fold (LpA), respectively (Fig. S6). After accounting for the baseline differences in unstimulated PBMC between healthy and diseased subjects, we found that the constitutive *ORMDL1*, *-2* and *-3* expression in asthmatics is much higher (Fig. 3-A, -B and -C). In other words, the affected individuals have already "pre-induced" *ORMDL* levels and in case of *ORMDL2* after PHA application the RFC in asthmatics was 12 times higher compared with the unstimulated PBMC from non-asthmatics. Significant induction after stimulation in asthmatics was observed for *ORMDL2* and *ORMDL3* (except for PHA and LpA) but not for *ORMDL1* (Fig. S6).

Allele-specific ORMDL3 variants alter ORMDL3 expression

Six polymorphisms (rs5742940, rs7954619, rs8079416, rs4795405, rs12603332 and rs3902920) were subjected to analysis aiming to dissect whether the gene expression of each *ORMDL* is affected by the presence of certain asthma-associated alleles. The top associated SNPs from *ORMDL1* (rs5742940) and *ORMDL2* (rs7954619), as well as four from the *ORMDL3* region (representing the three highly associated tagging bins): 1 (2 SNPs), 5 and 15), were analyzed in a cohort of adult non-asthmatics (n=47). An effect was observed for three of the investigated frequent 17q21 SNPs (Table 2 and Table S3), two of which (rs8079416 and rs4795405) are *ORMDL3* variants not being in high pairwise LD (r^2=0.43). The risk "C" allele of rs8079416 (tagging bin 15) was associated with increased *ORMDL3* expression not only after stimulation with Derp1 (p=0.004), LpA (p=0.038) and Ppg (p=0.021) but in the unstimulated samples (p=0.015) as well. The non-risk "T" allele of rs4795405 (tagging bin 5) was associated with significant reduction of *ORMDL3* expression after stimulation and this effect was detected for all stimuli (except Ppg) with lowest p=0.01 for Derp1. Similarly, the non-risk "T" allele of rs3902920 (tagging bin 1) exhibited associations with decreased *ORMDL3* levels upon stimulation with Derp1 (p=0.046). Only a borderline association with reduction of the *ORMDL3* gene expression was observed for rs12603332 after Derp1 exposure (p=0.060). Significantly increased *ORMDL1* expression was observed after Derp1 (p=0.035) and Ppg stimulation (p=0.011)

when the risk "A" allele of rs5742940 was present. However, such effects were not detectable for the *ORMDL2* polymorphism rs7954619 (Table 2 and Table S3).

Gene-by-gene interaction effects of the ORMDL genes on asthma

When combined effects of risk alleles in the three *ORMDL* genes were investigated, we found multiplicative increases of risk ratios when two [p=5.58*10^{-4}, OR (95% CI) = 4.13 (1.85-9.26)] or all three risk alleles [p=1.74*10^{-4}, OR (95% CI) = 8.81 (2.75-25.09)] were present. Apparently, the accumulation of risk alleles leads to a synergistic effect on asthma (Table 3).

Physical protein-protein interactions of human ORMDLs

Using co-immunoprecipitation and BRET we determined whether ORMDL1, -2 and -3 interact physically with each other. All expression constructs of ORMDL proteins were located in the endoplasmic reticulum in living cultured fibroblasts (COS-7 cells). We observed homomeric oligomers of ORMDL1, ORMDL2, and ORMDL3 (Fig. 4-A), as well as heteromeric (Fig. 4-B) interactions between all three combinations of ORMDL proteins. To characterize distinct ORMDL protein complexes, we performed BRET saturation experiments (30, 31) allowing determination of relative binding affinities ($BRET_{50}$) (Fig. 4-A and -B and see Table S9, Online Supporting information). Of note, homooligomers of ORMDL1 assembled with a 2-fold higher apparent affinity than ORMDL2 and ORMDL3 homooligomers. Moreover, for both ORMDL3/ORMDL2 and ORMDL2/ORMDL1 the apparent affinity to form heterooligomers was higher than observed for ORMDL2 or ORMDL3 homooligomers, respectively.

We also compared the interaction patterns of all three ORMDLs with sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) and endoplasmic reticulum membrane based serine palmitoyltransferase 1 (SPT1) (11) as a functional link between them had been suggested (9, 11). Indeed, ORMDL1, ORMDL2, and ORMDL3 physically interacted with SERCA2 and SPT1 (Fig. 4-C). There was no significant difference in the signal intensity among the members of ORMDL protein family.

DISCUSSION

Chromosome 17q21 is the most widely replicated asthma susceptibility locus to date. However, extensive LD spreads among a number of asthma-associated SNPs within 17q21 genes. Thus, the source of the association signal has not been determined yet. Our first expression studies pointed towards *ORMDL3* as the most plausible asthma candidate gene from the 17q21 locus (1). Moreover, the ORMDL family members demonstrate high sequence homology, which suggests that they might share similar biological roles. This is the first study systematically investigating genetic variability in *ORMDL1*, *-2* and *-3* genes in the context of asthma. Our in-depth analyses revealed one asthma-associated variant in *ORMDL1* (rs5742940) and one in *ORMDL2* (rs7954619) and this findings have not been reported previously.

Within *ORMDL3* however, there were 14 disease-associated polymorphisms of which 9 (covering 5 tagging bins) were associated with $p \leq 10^{-5}$ or lower, extending our previous findings from the original GWAS (1). Of these, eight polymorphisms have not been studied for associations with asthma previously.

When we analyzed the association with the subphenotypes atopic and non-atopic asthma, we found that variants in the three *ORMDL* genes are associated with asthma in general. Association with asthma related to atopy status was detectable for rs5742940 (*ORMDL1*). However, the results of our subphenotype analyses have to be interpreted with caution due to the small number of subjects with non-atopic asthma in this cohort.

Polymorphisms in *ORMDL1* and *ORMDL2* demonstrated weaker disease associations compared to *ORMDL3* SNPs. Of note, most of the asthma-associated *ORMDL3* polymorphisms are common variants with MAF often above 0.30 and those in *ORMDL1* and *ORMDL2* loci have considerably lower MAF. Thus, this might be one reason that we detected associations with smaller magnitude.

We investigated whether distinct expression patterns of the *ORMDL* family genes exist in non-asthmatic and asthmatic subjects in response to adaptive and innate immunity stimuli. We compared mRNA levels in PBMC from atopic asthmatics to non-diseased controls. Indeed, all three *ORMDL* genes exhibit higher expression in asthmatics at baseline reaching significance for *ORMDL1* and *ORMDL2* in PBMC and only in whole blood for *ORMDL3*. This observation may be explained by the fact that PBMC fraction includes cell populations that might have important impact on the asthma phenotype, such as lymphocytes and macrophages. Whole blood specimens contain other cells potentially relevant in asthma pathogenesis such as neutrophils, basophil granulocytes and eosinophils which also contribute to certain asthma phenotypes (32-36). Therefore, the differences determined at baseline level (unstimulated PBMC) between asthmatics and non-asthmatics for *ORMDL1* and *ORMDL2* and in whole blood samples for *ORMDL3* might have a different functional origin, i.e. depending on the specific cell type investigated. In a recent study it has been shown that mice overexpress *ORMDL3* in the eosinophils recruited to their airways and that in fact *ORMDL3*

regulates eosinophil trafficking (13). Another study provided data in this respect not only for *ORMDL3*, but also for *ORMDL1* and *ORMDL2* (37). *ORMDL3* was strongly induced in bronchial epithelium, lung macrophages and lung eosinophils after allergen challenge (37). In the same study *ORMDL3* was significantly higher expressed in bone marrow derived eosinophils compared to the other two *ORMDL* genes (37). In contrast, *ORMDL2* was predominantly expressed in peripheral blood neutrophils (37).

Of note, asthmatics in our gene expression cohort were all allergic to grass pollen, and thus, they were already "sensitised" even when we studied unstimulated PBMC. Nevertheless, we compared *ORMDL*s expression in non-asthmatics and asthmatics after allergen stimulation. Significant induction of the three genes was observed being the strongest for *ORMDL2* while it appeared to be rather similar between *ORMDL1* and *ORMDL3*. Gene expression had the same direction (increased) in both groups but the degree of inducibility upon stimulation was higher in non-asthmatic subjects. In asthmatics it seemed that *ORMDL* genes appeared to be already "pre-induced". While the presence of allergic inflammation in our cohort of diseased subjects might lead to increased *ORMDL* levels, the local airway inflammation in asthmatics exists independently of atopy. Asthma-associated SNPs within the *ORMDL* genes (except the rare rs5742940 in *ORMDL1*) displayed associations with asthma in general and not with atopic asthma alone. Thus, it is rather likely that the baseline differences in *ORMDL1-3* expression between healthy and diseased subjects are driven by the asthma status rather than by atopy (38).

These data demonstrated that all three *ORMDL* genes exhibit highly elevated expression in atopic asthmatics compared with non-asthmatics supporting the involvement of all three genes in the development of asthma. Based on our association data we analyzed allele-specific effects of

rs5742940 (*ORMDL1*), rs7954619 (*ORMDL2*), rs8079416 (*ORMDL3*, bin 15), rs4795405 (*ORMDL3,* tagging bin 5), rs12603332 (*ORMDL3*, bin 1) and rs3902920 (*ORMDL3* region, bin 1) on the expression of *ORMDL* genes in the group of non-asthmatic subjects. *Cis*-effects of SNPs on *ORMDL3* expression were observed for three out of four analyzed 17q21 SNPs within this region. To our knowledge this is the first report providing data for the SNPs in *ORMDL1* and *ORMDL2*, as well as for rs3902920 in *ORMDL3*. The pairwise LD between rs8079416 and rs4795405 is weak (r^2=0.43) and it is rather likely that each of them exhibits at least partly independent functional effect. Despite the high LD (r^2=0.79 and r^2=0.74) observed between rs4795405 and the two SNPs (rs12603332 and rs3902920, respectively) from tagging bin 1, it is possible that rs4795405 has its own functional impact on *ORMDL3* expression that is not completely dependent on the influence of the SNPs from tagging bin 1 (data not shown). Their functional aspects are studied in detail elsewhere (Schedel et al., JACI 2015, *in press*).

Although we could detect that *ORMDL1* expression is influenced by the presence of the rs5742940 risk "A" allele, we did not observe the same for rs7954619 on *ORMDL2* expression in our relatively small cohort. This may be due to the small sample size and subsequently insufficient power because of the low allele frequency of this SNP. It has to be acknowledged that here we present data not corrected for multiple testing, as this is not a hypothesis free approach and non-independent analyses have been performed. Significant results exceed those expected by chance but further replication in independent cohorts should follow. Thus, we cannot exclude the potential effects of these rare *ORMDL1* and *-2* polymorphisms. In fact, both SNPs are located just upstream in the putative promoter regions and *in silico* analyses predicted changes in transcription factor binding (see Table S10, Online Supporting information) potentially affecting *ORMDL1* and *ORMDL2* promoter activities. Moreover, when we queried currently available eQTL (Expression quantitative trait loci) databases (see Table S11), we found that rs7954619 (*ORMDL2*) and the *ORMDL3* variants rs8079416,

rs4795405 and rs12603332 influence the respective *ORMDL* gene expression in cell types and tissues involved in the mechanisms of asthma inflammation such as lymphocytes, lymphoblastoid cells and lung tissue. However, no information is yet available for rs5742940 (*ORMDL1*).

Looking from the other perspective, we tried to identify all currently known SNPs that influence *ORMDL1* expression. An extensive eQTL query (all databases are described in Table S11, p. 44 Supporting information) revealed 346 variants that we further checked for their asthma associations in the GABRIEL dataset (3). Data for 67 SNPs was available and for three of them modest disease associations were observed: rs6761221 (p=0.035), rs785567 (p=0.036) and rs7603201 (p=0.044). However, the number of associated variants expected by chance would be three (or even less because some of the 67 SNPs will be in high LD with each other). Thus, seems that common variants regulating *ORMDL1* expression do not exhibit strong associations with asthma in the large independent GABRIEL study.

Nevertheless, considering that our study is focused only on the three *ORMDL* genes and their closest genomic vicinity, we cannot exclude the possibility for existence of variants in long distance acting regulatory elements that could specifically affect *ORMDL*s expression levels depending on disease status.

In this study we identified significant gene-by-gene interactions between SNPs in *ORMDL* family members in a risk score model. Combinations of polymorphisms in two or all three genes show multiplicative rather than additive effects. Furthermore, we demonstrated that all ORMDL proteins are capable of forming homooligomers as well as heterooligomers with any of the other two family members. These findings are in accordance with data from a very recent study (39) focused on the role of ORMDL proteins in the *de novo* ceramide synthesis where it has been also shown that the three ORMDLs interact with each other. As *ORMDL* genes were found to be expressed concomitantly in various tissues (7), ORMDL proteins may exert their function as monomers, dimers or oligomers in

different combinations. However, the dissection of their functional quaternary structure requires further research. Protein oligomerization facilitates the constitution of larger complexes with higher stability as well as with expanded conformational complexity and this allows the formation of additional sites for specific protein interactions (40). We demonstrated that all ORMDL proteins physically interacted with SERCA2 and with SPT1, which are involved in the calcium homeostasis (SERCA2) and sphingolipid metabolism (SPT1). Since ORM1 and ORM2 proteins (in yeast) and ORMDL3 (in mice) are potential regulators of these processes, allele-specific and allergen-dependent expression of *ORMDL*s may have a downstream impact on pathways associated with ORMDL-binding proteins. Our findings are in line with recent studies demonstrating that *ORM1* and *ORM2* overexpression in yeast leads to decreased sphingolipid synthesis (9) and that the three ORMDL proteins participate in the regulation of ceramide production in mammalian cells (10). Taken together, our results support the initial hypothesis of a shared biological role of the ORMDL family proteins. Moreover, altered SERCA2 expression contributes to airway remodeling (41) and disturbed sphingolipid synthesis in the respiratory tract of mice provokes airway hyperresponsiveness (42) - both considered as major hallmarks of asthma.

In summary, our data demonstrated that all three *ORMDL* genes are associated with childhood asthma. Together with our findings from the differential *ORMDL* expression patterns in non-asthmatics vs. diseased subjects, gene-by-gene interactions and concomitant biological functions of the ORMDLs, this work suggests that *ORMDL* genes play a role in disease development. Possibly, other genes in 17q21 may additionally contribute to the asthma association signal but SNPs changing *ORMDL3* function seem to significantly influence asthma susceptibility. Further studies are needed to dissect whether allergens have the ability to trigger *ORMDL*s (over)expression in lung-specific cell types and if the ORMDL proteins contribute to the local lung inflammation in asthma.

ACKNOWLEDGMENTS

We thank all participants in MAGIC, ISAAC II, EXACT and ZAP II studies. We are grateful to Bernd Genser for the statistical support and to Michaela Kolletzki and Linda Güralay for their contribution in the preparation of LD and gene structures schemes. We thank Hannover Biomedical Research School (HBRS) and the PhD program "Molecular Medicine" for supporting Antoaneta A. Toncheva with a PhD fellowship.

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Sequencing and genotyping experiments: DPP, NK, AF and TI.

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Protein interaction experiments: SWG, JMK, ACM.

Statistical analysis and data interpretation: MK, SM, VDG, AAT, DPP and MS.

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FUNDING

This work was funded by the German Research Council (DFG) by grant SFB587, project B16, Project B8 (principal investigator Jens M. Hohlfeld) and the German ministry of education and research (BMBF) as part of the national genome research network (NGFN), with grant NGFN 01GS0810, a

Marie Curie research grant 236137 to MS, and the Bavarian Genome Research Network (BayGene) and LMU*excellent* grant 42595-6 to SWG and ACM.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no competing financial or personal interests.

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TABLES

Table 1. Asthma associations of 44 SNPs within the *ORMDL* genes in the combined MAGICS/ISAAC II case-reference study cohort (*n*=1,446, 763 asthmatics)

SNP	Tagging bin	Minor allele	MAF		Source	Asthma (combined)		Number of subjects analyzed	
			cases	controls		OR (95% CI)	*p*-value	cases	controls
A. *ORMDL1*/ chromosome 2q32									
rs10197331	1	A	0.277	0.279	1K/ MALDI	0.99 (0.84-1.17)	0.932	734	653
rs6942	1	C	0.276	0.287	HM/ MALDI	0.95 (0.81-1.12)	0.557	740	621
rs7591929	1	G	0.276	0.283	CHIP	0.96 (0.81-1.14)	0.680	651	652
rs1899025	1	C	0.277	0.282	CHIP	0.97 (0.82-1.15)	0.746	651	652
rs12990184	2	C	0.023	0.031	HM/ MALDI	0.74 (0.46-1.18)	0.206	744	622
rs77607351	3	C	0.055	0.047	1K/ MALDI	1.16 (0.83-1.63)	0.384	743	676
rs5742959	3	T	0.059	0.048	1K/ MALDI	1.25 (0.89-1.75)	0.195	702	649
rs76405401	4	T	0.015	0.014	1K/ MALDI	1.10 (0.59-2.07)	0.764	741	666
rs75410971	5	G	0.008	0.015	1K/ MALDI	0.53 (0.25-1.11)	0.093	696	641
rs17199067	6	A	0.037	0.033	1K/ MALDI	1.11 (0.74-1.67)	0.599	698	642
rs17271246	7	C	0.146	0.140	1K/ MALDI	1.05 (0.85-1.30)	0.660	735	662
rs5742926	7	T	0.158	0.145	HM/ MALDI	1.10 (0.89-1.35)	0.374	748	623
rs2352709	8	A	0.073	0.068	HM/ MALDI	1.08 (0.80-1.44)	0.620	743	620
rs5742933	9	C	0.203	0.220	1K/ MALDI	0.91 (0.76-1.09)	0.304	699	647
rs5742940	10	A	0.024	0.011	1K/ MALDI	**2.31 (1.23-4.33)**	**0.009**	739	664
rs116275586	11	A	0.034	0.038	1K/ MALDI	0.90 (0.60-1.34)	0.604	746	675
B. *ORMDL2*/ chromosome 12q13.2									
rs2887998	1	A	0.060	0.078	1K/ MALDI	0.76 (0.56-1.02)	0.070	698	636
rs7954619	1	G	0.052	0.075	HM/ MALDI	**0.68 (0.49-0.93)**	**0.017**	710	617
rs10083186	2	A	0.049	0.054	1K/ MALDI	0.90 (0.64-1.27)	0.558	742	667
rs117960147	3	C	0.025	0.030	1K/ MALDI	0.81 (0.51-1.30)	0.387	723	645
rs3759101	4	T	0.038	0.048	HM/ MALDI	0.78 (0.54-1.14)	0.197	748	624
rs56108400	5	T	0.225	0.240	1K/ MALDI	0.92 (0.77-1.10)	0.343	737	673

rs12308277	6	T	0.008	0.016	HM/ MALDI	0.49 (0.24-1.02)	0.056	747	625
rs7316533	7	C	0.055	0.060	1K/ MALDI	0.90 (0.65-1.24)	0.529	739	672
rs75446180	8	C	0.030	0.027	1K/ MALDI	1.11 (0.71-1.73)	0.643	741	674
C. *ORMDL3*/ chromosome 17q21									
rs7216389	1	C	0.425	0.475	CHIP	**0.67 (0.58-0.79)**	**$4.88*10^{-7}$**	651	652
rs3902920*	1	T	0.415	0.482	1K/ MALDI	**0.66 (0.57-0.77)**	**$6.61*10^{-8}$**	734	664
rs4065275	1	A	0.419	0.482	HM/ MALDI	**0.67 (0.58-0.78)**	**$3.36*10^{-7}$**	751	622
rs12603332	1	T	0.412	0.480	HM/ MALDI	**0.66 (0.56-0.76)**	**$6.70*10^{-8}$**	724	608
rs1031459*	2	C	0.305	0.355	1K/ MALDI	**0.79 (0.67-0.93)**	**0.004**	730	653
rs3169572	3	T	0.037	0.052	HM/ MALDI	0.70 (0.48-1.01)	0.055	750	582
rs7942*	4	C	0.017	0.019	1K/ MALDI	0.87 (0.49-1.51)	0.613	747	676
rs8076131	5	G	0.391	0.477	HM/ MALDI	**0.72 (0.62-0.83)**	**$1.14*10^{-5}$**	745	641
rs4795405	5	T	0.376	0.469	CHIP	**0.69 (0.59-0.81)**	**$3.31*10^{-6}$**	651	651
rs17608925	6	C	0.077	0.110	HM/ MALDI	**0.68 (0.53-0.89)**	**0.004**	747	581
rs3744246	7	T	0.163	0.197	HM/ MALDI	**0.80 (0.67-0.97)**	**0.024**	756	669
rs4795402	8	A	0.196	0.242	HM/ MALDI	**0.77 (0.64-0.93)**	**0.007**	695	568
rs4794820	9	A	0.367	0.458	1K/ MALDI	**0.69 (0.59-0.80)**	**$1.35*10^{-6}$**	738	669
rs56199421*	10	C	0.378	0.459	1K/ MALDI	**0.71 (0.61-0.83)**	**$1.26*10^{-5}$**	740	663
rs73985231*	11	C	0.058	0.075	1K/ MALDI	0.77 (0.57-1.03)	0.076	749	673
rs8065244*	12	G	0.092	0.114	1K/ MALDI	0.79 (0.62-1.01)	0.059	742	673
rs7207600*	13	G	0.323	0.377	1K/ MALDI	**0.78 (0.67-0.92)**	**0.002**	740	668
rs76285844*	14	C	0.099	0.097	1K/ MALDI	1.01 (0.79-1.30)	0.926	741	677
rs8079416	15	C	0.472	0.422	CHIP	**1.55 (1.32-1.81)**	**$6.08*10^{-8}$**	651	652

Abbreviations: SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; 1K – 1000 Genomes; HM – HapMap database; Chip – Illumina BeadChip genotyping; MALDI - MALDI-TOF-MS genotyping.

Number of individuals with non-missing phenotype involved in the respective calculations for the different rounds of genotyping: Asthma - MALDI-TOF-MS (763 cases/ 683 controls); CHIP (651 cases/ 652 controls). Data has not been corrected for multiple testing.

Novel data we report here for: 14 *ORMDL1* polymorphisms; 9 *ORMDL2* polymorphisms; and for 8 *ORMDL3* polymorphisms (indicated with *).

Table 2. Allele-specific effects of asthma-associated polymorphisms on *ORMDL* genes expression in PBMC (*n*=47, adult non-asthmatics)

SNP/ *Cis*-effects on gene	Allele*	MAF†	*p*-value Unstimulated/ Expression (dCt)	*p*-value PHA/ Expression (dCt)	*p*-value Derp1/ Expression (dCt)	*p*-value LpA/ Expression (dCt)	*p*-value Ppg/ Expression (dCt)
rs5742940/ *ORMDL1*	Risk (A)	0.02	0.057 -	0.236 -	**0.035** increased	0.414 -	**0.011** increased
rs7954619‡/ *ORMDL2*	Non-risk (G)	0.09	0.374 -	0.949 -	0.390 -	0.991 -	0.331 -
rs8079416/ *ORMDL3*	Risk (C)	0.40	**0.015** increased	0.234 -	**0.004** increased	**0.038** increased	**0.021** increased
rs4795405/ *ORMDL3*	Non-risk (T)	0.48	0.169 -	**0.038** decreased	**0.010** decreased	**0.028** decreased	0.062 -
rs12603332‡/ *ORMDL3*	Non-risk (T)	0.46	0.118 -	0.177 -	0.060 -	0.247 -	0.187 -
rs3902920/ *ORMDL3*	Non-risk (T)	0.47	0.092 -	0.126 -	**0.046** decreased	0.263 -	0.168 -

Abbreviations: PBMC - peripheral blood mononuclear cells; SNP - Single nucleotide polymorphism; MAF - Minor allele frequency; PHA – Phytohemagglutinin; Derp1 - *Dermatophagoides pteronyssinus* (house dust mite); LpA - lipid A; Ppg – Peptidoglycan.

Significant results ($p < 0.05$) are indicated in bold.

*As "risk" and "non-risk" allele in terms of asthma status, we refer to the effects observed in our genetic asthma association results (Table 1, main text).

[†]MAF in this table is determined according to the analyzed here subset (*n*=47) from the EXACT cohort.

[‡]rs7954619 and rs12603332 were successfully genotyped in 46 adult non-asthmatics.

Table 3. Chance of having asthma with regard to risk score based on the number of risk genotypes

Risk score	*n*	OR (95% CI)	*p*-value
0	34	-	-
1 vs 0	378	2.36 (1.04-5.35)	**0.039**
2 vs 0	718	4.13 (1.85-9.26)	**$5.58*10^{-4}$**
3 vs 0	32	8.81 (2.75-25.09)	**$1.74*10^{-4}$**

The statistical analysis was conducted using logistic regression. Significant *p*-values ($p < 0.05$) are indicated in bold. *n* - number of subjects; OR – odds ratio; CI - confidence interval. Risk score = 0 – neither of the risk alleles is present. Risk score = 1 – only one risk allele is present. Risk score = 2 – two risk alleles are present. Risk score = 3 – all three risk alleles are present.

FIGURE LEGENDS

Figure 1. Linkage disequilibrium (LD) between genotyped polymorphisms in *ORMDL1* (A), *ORMDL2* (B) and *ORMDL3* (C) loci

Schematic representation of *ORMDL1* (A), *ORMDL2* (B) and *ORMDL3* (C) gene regions. Chromosomal positions are provided according to NCBI Genome Build 37.3. Only genotyped in this study single nucleotide polymorphisms (SNPs) are depicted. Identification (rs) numbers of the SNPs are given vertically. Tagging bins are numbered horizontally, in brackets. Asthma-associated SNPs are underlined. Dark grey blocks in the gene structures indicate exons and light grey blocks indicate 5' and 3' untranslated regions (UTRs).

Figure 2. Expression of *ORMDL* genes in PBMC from non-asthmatic (*n*=47) and asthmatic (*n*=8) adults

Differential gene expression (mRNA) levels for each of the three *ORMDL* genes were assessed using the delta C_t (ΔC_t) values, where $\Delta Ct = C_{t(target\ gene)} - C_{t(18S\ rRNA)}$ for each sample, respectively. To better indicate that lower ΔC_t values correspond to higher gene expression levels, the Y-axis of each graphic has been reversed. Significant *p*-values ($p < 0.05$) are indicated in bold. PBMC - Peripheral blood mononuclear cells. Data are given as means ± standard deviation (SD).

Figure 3. Induction of *ORMDL* genes mRNA expression in PBMC within non-asthmatic (*n*=47) and asthmatic adults (*n*=8) after stimulation

*Relative fold changes (RFC) in *ORMDL1* (A), *ORMDL2* (B) and *ORMDL3* (C) expression after stimulation for non-asthmatics (light grey bars) and asthmatics (dark grey bars) were compared with the unstimulated samples from non-asthmatics (RFC = 1) as a baseline. For the asthmatics' cohort

RFC for each targeted gene was set to a fixed value (3.194 for *ORMDL1*, 3.056 for *ORMDL2,* and 1.527 for *ORMDL3*) that represents the expression differences (in RFC) at baseline level between non-asthmatics and asthmatics. PBMC - Peripheral blood mononuclear cells; PHA – phytohemagglutinin; Derp1 - *Dermatophagoides pteronyssinus* (house dust mite); LpA - lipid A; Ppg – peptidoglycan. Data are given as means ± standard deviation (SD).

Figure 4. Oligomerization and interaction patterns of ORMDL proteins

Homomeric (A) and heteromeric (B) interactions of ORMDL1, ORMDL2, and ORMDL3 analyzed by co-immunoprecipitation (CoIP, left panels) or bioluminescence resonance energy transfer (BRET, middle panels) and BRET saturation experiments (right panels). For CoIP experiments ORMDL proteins carrying C-terminal HA-tag or V5-tag were co-expressed in COS-7 cells, precipitated using anti-HA (immunoprecipitation - IP) and detected by anti-V5 (immunoblot - IB). Cytosolic phenylalanine hydroxylase served as a control (see Fig. S7 in the Online Supporting information). For BRET experiments N-terminal fusion proteins of ORMDL and Renilla luciferase (donor) or yellow fluorescent protein (acceptor) were co-expressed in COS-7 cells and the rate of energy transfer (BRET ratio) was determined by luminescence detection. In BRET saturation experiments the ratio of ORMDL acceptor fusion proteins to ORMDL donor fusion proteins was varied yielding a hyperbolic saturation of BRET ratios for positive interactions of ORMDL proteins, whereas the co-expression of ORMDL1 and mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD) resulted in linear regression indicative for a negative interaction. For BRET experiments, data are given as means ± SD of *n*=4 replicates, black dotted lines indicate the threshold for positive interactions of 0.094. (C) BRET experiments testing interaction of ORMDL1, ORMDL2, and ORMDL3 with SERCA2 or SPT1. ORMDL donor fusion proteins were co-expressed with SERCA2 or SPT1 acceptor fusion proteins in COS-7 cells and the BRET ratio was determined. Endoplasmic reticulum luminal Ca^{2+}-binding protein grp78 (BIP) and mitochondrial MCAD served as controls. Data are given as means ± SD of *n*=3 replicates.