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Original Article

DMRT1 is a sex-specific genetic determinant of childhood onset asthma and is expressed in testis and macrophages

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ABSTRACT (239 words)

Background:

Asthma is a disease affecting more boys than girls during childhood and more women than men in adulthood. The mechanisms behind these sex-specific differences are not yet understood.

Objective:

We analyzed if and how genetic factors contribute to sex-specific predisposition to childhood onset asthma.

Methods:

Interactions between sex and polymorphisms on childhood asthma risk were evaluated in the MAGICS/ISAAC II population on a genome-wide level and findings were validated in independent populations. Genetic fine mapping of sex-specific asthma association signals was performed and putatively causal polymorphisms were characterized *in vitro* by EMSA and luciferase activity assays. Gene and protein expression of the identified gene *doublesex and mab-3 related transcription factor 1* (*DMRT1*) were measured in different human tissues by quantitative Real-Time PCR and immunohistochemistry.

Results:

Polymorphisms in the testis-associated gene *DMRT1* displayed interactions with sex on asthma status in a population of primarily clinically defined asthmatic children and non-asthmatic controls (lowest $p=5.21 \times 10^{-6}$). Replication of this interaction was successful in two childhood populations clinically assessed for asthma but showed heterogeneous results in other population based samples. Polymorphism rs3812523 located in the putative *DMRT1* promoter was associated with allele-specific changes in transcription factor binding and promoter activity *in vitro*. DMRT1 expression was observed not only in testis but also in lung macrophages.

Conclusion:

DMRT1 might influence sex-specific patterns of childhood asthma and its expression in testis tissue and lung macrophages suggests a potential involvement in hormone or immune cell regulation.

CLINICAL IMPLICATIONS

Sex-specific genetic associations identified *DMRT1* as a candidate for sex-specific effects in childhood asthma. Expression of DMRT1 in testis and alveolar macrophages suggests its involvement in hormone or immune cell regulation.

CAPSULE SUMMARY

The clinical presentation of asthma differs considerably between boys and girls. Distinct genetic predisposition seems to be present and *DMRT1* is a plausible candidate for sex-specific effects in childhood asthma.

KEY WORDS

asthma; genetic association; DMRT1; interaction; rs3812523; sex; SNP

ABBREVIATIONS

ALSPAC	Avon Longitudinal Study of Parents And Children
Ankara	Clinical study population recruited in Ankara, Turkey
AP-1	Activator Protein-1
BAMSE	Children, Allergy, Milieu, Stockholm an Epidemiological Study
bp	Base pair
CI	Confidence interval
CEU	Utah residents with Northern and Western European ancestry from the CEPH collection
COPD	Chronic obstructive pulmonary disease
DAG	Dutch asthma GWA study
DIP	Desquamative interstitial pneumonia
DLR assay	Dual Luciferase Reporter assay
DMRT	Doublesex and mab-3 related transcription factor
ds	Double-stranded
EMSA	Electrophoretic mobility shift assay
Freiburg	Clinical study population recruited in Freiburg, Germany
GABRIEL	A Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community
GR	Glucocorticoid receptor
GWA	Genome-wide association
HEK-293	Human embryonic kidney 293 cell line
HWE	Hardy–Weinberg equilibrium
IPF	Idiopathic pulmonary fibrosis
ISAAC II	International Study of Asthma and Allergies in Childhood, phase II
Jurkat	Cell line established from T cell leukemia
kb	Kilobase pair
LD	Linkage disequilibrium
MAF	Minor allele frequency
MAGICS	Multicentre Asthma Genetics In Childhood Study
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Mb	Megabase pair
MCP1	Monocyte chemotactic protein-1

OR	Odds ratio
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PIAMA	Prevention and Incidence of Asthma and Mite Allergy
qRT-PCR	Quantitative Real-Time polymerase chain reaction
rs	Reference SNP
SNP	Single-nucleotide polymorphism
Tomsk	Clinical study population primarily recruited in Tomsk, Russia

INTRODUCTION (195 words)

During childhood, asthma prevalence is higher in boys than in girls with a ratio of up to 2:1.[1] However, female sex is a risk factor for the persistence of asthma symptoms into adulthood,[2] while remission seems to be more pronounced in boys during puberty.[3] During adolescence and adulthood, more females than males acquire asthma[4] resulting in a female predominance in asthma prevalence among adults.[5] Changes in physiology and the hormonal milieu during puberty are suggested mechanisms for sex-specific disease susceptibility.[6] Furthermore, gender-specific differences in social behavior, exposure to environmental disease triggers and disease awareness may exist.[6,7]

However, sex-specific differences in age of onset and persistence of asthma may also result from distinct dissimilarities in genetic susceptibility and in mechanisms of disease development between girls and boys. Identifying the genetic basis of sex-differences in asthma onset and its course is urgently needed to develop accurate prognostic markers and a more personalized approach towards therapeutic intervention. In this study we conducted a genome-wide search for sex-specific associations with childhood asthma, performed replication studies in seven different study populations, analyzed functional relevance of associated polymorphisms and approached the potential role of the associated gene in asthma pathogenesis.

METHODS (701 words)

Genome-wide study of sex-SNP interactions on childhood onset asthma risk

Interactions between sex and single nucleotide polymorphisms (SNP) on childhood onset asthma risk were analyzed on a genome-wide level in the MAGICS/ISAAC II population (see Online Repository methods and Table E1 for population details). A total of 1,361 subjects (703 asthmatics with 65.3% males and 658 non-asthmatic controls with 49.7% males) with chip based SNP genotypes were available (Sentrix HumanHap300 BeadChip, Illumina Inc., San Diego, USA).[8] All calculations were carried out with the PLINK software package version 1.07 using the following filtering parameters: minor allele frequency (MAF) ≥0.05; SNP genotyping rate ≥0.95; Hardy-Weinberg disequilibrium *p*-value in the control population ≥0.0001.[9] A logistic regression was used to model dominant SNP effects on asthma status in the complete MAGICS/ISAAC II data set as well as in male and female subsets. For the interaction analysis an additional sex-SNP interaction term was introduced in the regression model.

Replication of selected interaction signals was performed in seven independent populations with childhood onset asthma phenotypes (ALSPAC, Ankara, BAMSE, DAG, Freiburg, PIAMA, Tomsk; see Online Repository methods and Table E1 for population details).

Genetic fine mapping of the *DMRT1* locus

Fine mapping and linkage disequilibrium (LD) analyses were carried out in the MAGICS/ISAAC II data set in order to determine the extent of sex-specific asthma associations in the *DMRT1* locus. Genetic fine mapping was performed using a tagging SNP approach based on HapMap data and enriched by SNPs from the 1,000 genomes project and the SNPper database (details in the Online Repository).[10-12] Polymorphisms not present in the MAGICS/ISAAC II data set of chip and imputation based genotypes were genotyped by mass spectrometry as described previously.[13,14] LD structure and tagging bins of *DMRT1* in the

MAGICS/ISAAC II population were calculated with Haploview software (*DMRT1* ±10 kb; MAF ≥ 0.05; pairwise LD threshold $r^2 \geq 0.8$).[15] Dominant SNP effects on asthma status were determined for both sexes using PLINK. Male-specific asthma associations were investigated for independency in a set of conditional analyses, in which the strongest associated SNP from every tagging bin was individually used as a covariate in logistic regression.

In silico analysis of putative SNP function

Localization of SNPs to conserved coding regions of *DMRT1* was determined by alignment of amino acid sequences of all human *DMRT* gene family members (Vector NTI Advance™ 11). The functional effect of nonsynonymous SNPs was assessed with the PolyPhen-2 software tool.[16] Phylogenetic shadowing between human and mouse was investigated with the Vista Genome Browser (settings: *DMRT1* ±10 kb flanking sequences; 70 % sequence conservation in window size 50 bp).[17] Allele-specific changes in transcription factor binding were predicted with AliBaba2.1.[18]

Electrophoretic mobility shift assay

Allele-specific differences in DNA-protein interaction were investigated by electrophoretic mobility shift assays (EMSA; details in the Online Repository). Briefly, isotope-labeled DNA probes carrying the SNP allele of interest were incubated with nuclear protein extracts from HEK-293 cells and resulting DNA-protein complexes were resolved on gel. Competition experiments with unlabeled probes or addition of specific antibodies were used to identify DNA-protein complexes.

Dual Luciferase Reporter Assay

Allele-specific effects on the activity of the putative *DMRT1* promoter were investigated in dual luciferase reporter (DLR) assays (details in the Online Repository). In short, approximately 2 kb

of genomic sequence upstream of *DMRT1* were cloned into the pGL3-Basic vector upstream of the *Firefly luciferase* gene (Figure E2 in the Online Repository; Promega, Mannheim, Germany). Vectors carrying the SNP allele of interest were used for transfection of HEK-293 cells and Firefly Luciferase activity was used as readout of promoter activity. Co-transfection of *Renilla luciferase* driven by thymidine kinase promoter was used for normalization of the Firefly Luciferase signal.

DMRT1 **expression in human tissues**

Current literature describes considerable expression of *DMRT1* in testis only.[19] To identify *DMRT1* expression in further potentially relevant tissues, we conducted quantitative Real-Time PCR (qRT-PCR) on a cDNA library of human tissues and cell lines (details in the Online Repository). Expression levels of *18S rRNA* and the delta-Ct method (Ct_{DMRT1}-$Ct_{18SrRNA}$) were used to calculate relative gene expression.[20]

DMRT1 immunohistochemistry was performed on human tissue sections from testis and lung. Details on ethical guidelines for sample collection and methods for immunohistochemical and hematoxylin staining can be found in the Online Repository.

RESULTS (1202 words)

Genome-wide analysis of sex-specific effects on asthma risk and *DMRT1* fine mapping

The strongest sex-SNP interaction on asthma status was observed for rs2187366 (p=3.29x10^{-6}), located 8 kb upstream of the uncharacterized gene *LOC100505817* on chromosome 18 (Figure 1). Almost identical significance levels were observed for rs4500131 and rs2146532 (p=5.21x10^{-6} and p=7.45x10^{-6}, respectively), located in the *DMRT1* gene on chromosome 9p24, and an additional two polymorphisms in the *DMRT1* locus (rs3812523 and rs1323271) were among the 100 strongest interaction signals (Figure 1 and Table E2-a in the Online Repository). Other interaction signals were distributed over the genome with no more than two signals within 1,000 kb. Due to the known male-specific biological function of DMRT1 this locus was selected for further analyses, despite ranging slightly below a genome-wide significance level of p<1.6x10^{-7}.[19] Asthma association data for *DMRT1* polymorphisms revealed protective effects for the minor allele in males with odds ratios (OR) < 1, while females displayed none or opposite effects (Figure 2 and Table E2-b,c).

Replication analyses of *DMRT1* polymorphisms rs4500131 and rs3812523 (r^2 = 0.43 in MAGICS/ISAAC II) were performed in six, respectively seven, independent populations with childhood onset asthma. Polymorphisms rs2146532 and rs1323271 were not investigated during replication due to their high LD with rs4500131 (r^2 = 0.98 and r^2 = 0.80, respectively). Polymorphism rs4500131 exhibited a trend for interaction in clinical studies from Tomsk and Freiburg (Figure 2 and Table E3). Comparable to the MAGICS/ISAAC II population, the rs4500131 minor allele displayed protective effects in male asthmatics in the clinical studies Tomsk and DAG as well as a trend in the birth cohort PIAMA. For female asthmatics a trend for replication of risk effects was observed in Freiburg whereas DAG displayed a protective trend. For rs3812523 significant replications of the sex interaction were observed in Tomsk and Freiburg, as well as a significant protective effect in male asthmatics in Tomsk and a trend in

Ankara and PIAMA. Like for rs4500131, rs3812523 results for female asthmatics were inhomogeneous with a significant risk effect in Freiburg and a trend for risk in Tomsk while Ankara and DAG displayed trends for protective effects.

Genetic fine mapping of the 147 kb *DMRT1* locus in the MAGICS/ISAAC II population identified 182 SNPs in 58 tagging bins (*DMRT1* ±10 kb; MAF ≥ 0.05; $r^2 \geq 0.8$). Detailed information on all genotyped and analyzed SNPs is provided in Table E4 in the Online Repository. Significant male-specific effects ($p_{male} < 0.01$) clustered to 23 polymorphisms in the 5' gene region. These SNPs belonged to five different tagging bins with the strongest signal originating from rs3812523 (Figure 3 and Table I-a). Male-specific association signals based on imputation for rs7022951, rs2025309, rs4338164 and rs3739583 with asthma and rs7040024 and rs6477321 with eczema were verified by MALDI-TOF MS (eczema data not shown and available from the authors upon request).

In silico and conditional analyses identify rs3812523 as a candidate for functional investigation

All polymorphisms with male-specific asthma associations with $p < 0.01$ were analyzed *in silico*. Alignment of the human DMRT protein family revealed high similarity in the amino acid sequence in the DM domain for DNA binding. Polymorphism rs3739583 lies outside of this domain and the amino acid exchange from serine to threonine was predicted to be benign (Table I-b). Comparison between human and mouse sequences identified five SNPs to be located in phylogenetically conserved regions (rs7022951, rs7858180, rs3812523, rs3739583, rs2146532) and allele-specific effects on transcription factor binding were predicted for four polymorphisms (rs7022951, rs3812523, rs6477293, rs2181402; Table I-b). Conditional analysis using the strongest associated SNP from every tagging bin with male-specific associations as a covariate revealed that associations of the remaining 22 SNPs are dependent on rs3812523 (Table I-b and

Table E5 in the Online Repository; detailed data on *in silico* analyses is available upon request from the authors). In conclusion, *in silico* and conditional analyses suggested rs3812523 to be the driving force for the male-specific asthma association.

rs3812523 demonstrates allele-specific effects on transcription factor binding and promoter activity *in vitro*

EMSA identified a specific DNA-protein complex for the non-risk 'G' allele of rs3812523 (conferring a lower risk for asthma in males) in nuclear protein extract from HEK-293 cells, which was not present for the risk 'A' allele (Figure 4, complex I; lanes 3-6). Competition experiments demonstrated a high allele-specificity of this DNA-protein complex (Figure 4, lanes 7-8). Competition with a high affinity AP-1 consensus site successfully abrogated the DNA-protein complex (lane 9). The dimeric AP-1 transcription factor can be formed by different members of the Jun, Fos, ATF and JDP protein families.[21] Antibody-specific supershift experiments revealed a supershift of the DNA-protein complex after incubation with a c-Jun antibody (lane 11) and negative results for c-Fos (lane 12), ATF-7 (lane 13) and JDP2 (data not shown).

A second DNA-protein complex at a lower molecular weight also displayed allele-specific effects (complex II; lanes 3-6). However, for both alleles the cross-competitions were incomplete (lanes 1 and 8) illustrating that different DNA-protein complexes are overlaying at this height of the EMSA gel. This finding is supported by competition with the AP-1 consensus site (Figure E1 in the Online Repository; complex II; lanes 5 and 14) leading to complete abrogation of this complex only in the non-risk allele. Again, c-Jun and additionally c-Fos have a role in the non-risk allele and also in the risk allele due to antibody-specific supershifts (Figure E1; complex II; lanes 2,3,16,17). However, even after successful c-Jun and c-Fos supershifts, an unidentified complex remains visible in the risk-allele (Figure E1; complex II; lanes 2 and 3).

DLR assays were conducted to test if the allele-specific binding of transcription factors to rs3812523 observed in EMSA has an influence on the activity as a promoter. A strong increase in Luciferase signal between the empty pGL3-Basic vector and the two pGL3-*DMRT1* vectors demonstrate the functionality of this putative *DMRT1* promoter region (Figure 5). Comparison between the rs3812523 risk and non-risk alleles displayed a significantly lower expression of the *Firefly luciferase* gene under the control of the non-risk allele in all tested conditions with the strongest effect in unstimulated HEK-293 cells (p=0.0008).

DMRT1 is expressed in testis and macrophages in the lung

We screened a cDNA library of human tissue samples and human cell lines for *DMRT1* expression (Table E8 in the Online Repository). As expected, testis displayed strong *DMRT1* expression but lower mRNA levels were also observed in immunologically relevant cell lines (Jurkat and YT) and in HEK-293 cells.

Immunohistochemistry for DMRT1 presented strong nuclear staining of adult testis Sertoli cells and possibly some germ cells (Figure 6).[19] Lung tissue from patients with normal lung function displayed weak DMRT1 staining in alveolar macrophages. Furthermore, lung tissue from adults with asthma, desquamative interstitial pneumonia (DIP), chronic obstructive pulmonary disease (COPD) or idiopathic pulmonary fibrosis (IPF) presented a strong staining of DMRT1 in all alveolar macrophages and less prominent also in interstitial macrophages. In contrast to staining in testis tissue, DMRT1 appears to primarily locate to the cytoplasm of macrophages in the lung. There was no staining of granulocytes, lymphocytes or plasma cells. Appendix, thymus, lymph node, and spleen indicated only staining in a subset of macrophages, mainly in the germinal center (so-called tingible body macrophages) and vascular structures, morphologically high-endothelial venules (Figure E3).

DISCUSSION (1421 words)

Our genome-wide analysis for interactions between sex and SNP effects on childhood asthma risk identified a number of strong interactions close to the genome-wide significance level set for simple association studies. Our analysis did not replicate sex-SNP interaction results from a previous study on asthma in European Americans where polymorphism rs2549003, located near *IRF1* on chromosome 5q31, displayed a significant sex-SNP interaction on asthma with male-specific effects.[22] In our data set, no trend for interaction (p=0.89) or asthma association in males (p_{male}=0.39) could be observed for rs2548997, which was described to be in complete LD with rs2549003 in the same study.

Polymorphisms in *DMRT1* displayed strong asthma associations in males and weaker but opposite effects in females. Replication of rs4500131 and rs3812523 (r^2 = 0.43) was successful in some, but not in all populations. Interestingly, the clinical populations Tomsk and Freiburg presented the best replication of our findings, while the clinically recruited DAG population with algorithm based asthma diagnosis replicated part of the observations. Effects in birth cohorts with parental report of doctor's asthma diagnosis were weaker or not present (ALSPAC, BAMSE and PIAMA). This might indicate that sex-specific effects of DMRT1 on asthma pathogenesis may be less pronounced in a population based milder asthma phenotype compared to populations recruited from tertiary clinical centers. Additionally, opposite effect directions between males and females as well as ambiguous effects in females from different studies could indicate that DMRT1 function in asthma pathogenesis might be modulated by yet unknown population-specific environmental factors.[23]

Our fine mapping focused on males due to the known biological function of DMRT1 in testis and this approach identified that asthma associations are defined to 23 polymorphisms in the 5'-region of *DMRT1*. Conditional analysis and *in silico* prediction suggested rs3812523, located in

the putative promoter of *DMRT1*, as the best candidate that may drive the sex-specific asthma association. EMSA displayed allele-specific binding of c-Jun to the non-risk allele of rs3812523, while a second DNA-protein complex, containing heterodimeric AP-1 (c-Jun/c-Fos), exhibited quantitative effects in binding affinity between risk and non-risk allele. Additionally, the risk allele specifically binds another yet unidentified transcription factor. In line with these observations, our promoter activity assays also demonstrated significant allele-specific effects for rs3812523. Interestingly, stimulation with PMA leads to a relatively lower promoter activity, which is in line with observations of inhibited *DMRT1* expression in primary mice Sertoli cells after PMA stimulation.[24] These *in vitro* analyses allow us to assume that also the *in vivo* expression of *DMRT1* might be influenced by rs3812523, which could contribute to the functional relevance of the association with asthma.

With *DMRT1* we propose a potential factor for genetically driven differences in childhood asthma etiology between sexes. The *doublesex and male abnormal 3 related transcription factor 1* (*DMRT1*) belongs to a family of transcriptional regulators containing the DM domain DNA binding motif, which was first identified in a comparison of the *Drosophila melanogaster* gene *doublesex* and the *Caenorhabditis elegans* gene *male abnormal 3*.[25] This DM domain can be found in a wide spectrum of animal taxa and its function in sexual differentiation has been reviewed in detail by Matson and Zarkower.[19] For mammals Matson and Zarkower describe exclusive expression of *DMRT1* in the gonads, which becomes male-specific during fetal testes differentiation. Human adult testes express *DMRT1* in Sertoli cells and spermatogonia, but not in most other testicular cells.[26] Genetic studies linked human *DMRT1* with testicular germ cell cancer and XY gonadal disorders of sexual development.[27,28] Deletion of *DMRT1* in male adult mice caused testicular Sertoli cells to trans-differentiate towards female ovarian granulosa cells.[29] In the same study, specific deletion of *DMRT1* in fetal Sertoli cells caused elevated oestradiol levels and reduced androgen activity. It has been shown that *DMRT1* is continuously

expressed in human testis from fetal stages until puberty and thereafter. However, this expression appears to follow a spatio-temporal expression pattern in different testis cell types, whereas in human ovaries *DMRT1* expression is only present in early fetal stages and down-regulated thereafter in later fetal stages.[26] One could speculate that rs3812523 driven regulation of *DMRT1* expression might to some extent influence the timing of testis development and as a consequence also levels and timing of sexual hormone expression. It is an attractive notion that boys carrying the non-risk allele benefit from an effect that might normally not occur until puberty when "outgrowing" of asthma symptoms is frequently observed.[3]

However, it is possible that *DMRT1* exerts its influence on asthma from another tissue than testis. Our cDNA screen detected *DMRT1* expression in lymphoid cell lines while immunohistochemistry data revealed low levels of DMRT1 in alveolar macrophages in control lung. In contrast to this, strong DMRT1 staining was observed in the cytoplasm of alveolar macrophages in patients with interstitial lung disease (DIP and IPF) and patients with chronic obstructive pulmonary disease (COPD), which might indicate a disease associated function of DMRT1. Bilateral lung explants harvested during lung transplantation from adult asthmatics displayed DMRT1 staining similar to that in other lung diseases. However, asthma samples derived from adult patients with asthma accompanied by smoking-induced end-stage lung disease (displaying emphysematous changes and anthracosis besides histologic findings typically associated with asthma, such as chronic inflammation of the small airways, hypertrophy of bronchial smooth muscles and mucous plugs) present a considerably different phenotype than childhood asthmatics. Unfortunately, childhood asthma samples were not available. The sporadic staining of subsets of macrophages and high endothelial venules in appendix, thymus, lymph node, and spleen suggests that DMRT1 has a function also in these tissues outside the lung (Figure E3 in the Online Repository). Importantly, also female patients displayed positive staining of macrophages and for now and in contrast to male testis tissue this cell type appears to

be the only site of DMRT1 expression which could be causative for the genetic association signals observed in females in our analyses. Furthermore, sex-specific gene regulation has been suggested for DMRT1 with repression of *Stra8* in male but enhancing *Stra8* expression in female mice.[30] The underlying mechanism is unknown but sex-specific gene regulation in humans by DMRT1 might explain the opposing effect directions of asthma associations observed between sexes in our study.

Localization of DMRT1 to the cytoplasm of macrophages might indicate inactivation of its transcriptional function. Considering the regulatory function of alveolar macrophages in inflammatory responses of the lung,[31] detailed studies on DMRT1 function in these cells need to follow, e.g. the potential of DMRT1 trans-localization to the nucleus upon macrophage activation. To date, little is known on the nuclear import of human DMRT1, but an importin-β1-dependent mechanism has been suggested and the potential role of importins in the regulation of allergic immune responses has recently been reviewed.[32,33] Interestingly, in an explorative study we observed significant correlations between rs3812523 and cytokines secreted from cultured peripheral blood mononuclear cells (PBMC; n=61 adults). Among others, protein levels of monocyte chemotactic protein-1 (MCP1), which is also produced by macrophages, were significantly lower in homozygous carriers of the rs3812523 risk allele compared to heterozygous carriers (p=0.037). Moreover, we were able to support this observation with a trend for lower *MCP1* mRNA levels in risk allele carriers (p=0.101; details in the Online Repository methods and Figure E4).[34] The explorative character of this analysis with relatively small sample size needs to be acknowledged as well as the unexpected down-regulation of the proinflammatory cytokine MCP1 in carriers of the risk allele for asthma, but this effect might serve as a first indication for transcriptional regulation of DMRT1 in immune cells, which warrants further investigation.

Finally, we also conducted sex-specific analyses for asthma associations in the MAGICS/ISAAC II population for all known human *DMRT* genes (details in the Online Repository; Table E10; Figure E5) and observed weak but significant associations in males in *DMRT2* and *DMRTB1*. This identification of further sex-specific associations within the *DMRT* gene family supports our results for *DMRT1* and suggests a possible role of further DM domain genes in childhood asthma. Additionally, in our sex-combined analysis we observed an asthma association of *DMRTA1* which is a gene recently also identified in a genetic study on atopic dermatitis.[35]

In conclusion, we present *DMRT1* as a novel candidate to explain sex-specific asthma effects during childhood. Its role in testis development as well as also our demonstration of *DMRT1* expression in alveolar macrophages opens potential for further functional investigations. The precise analysis of spatio-temporal gene expression of *DMRT1* will have to follow in order to elucidate if and how *DMRT1* expression in testis or in cells of the immune system affects asthma pathogenesis in males and females.

AUTHORS CONTRIBUTIONS

Maximilian Schieck contributed to study design, statistical analyses, replication data interpretation, cDNA library preparation and immunohistochemistry interpretation, performed genome-wide interaction analysis, *DMRT1* fine mapping, *in silico* analyses, *DMRT* gene family analysis, EMSA, *DMRT1* qRT-PCR, BioPlex measurements and construct preparation for DLR assays, drafted the first version of the manuscript and wrote the final version of the manuscript; **Jan P. Schouten** performed statistical analyses during genome-wide interaction and replication phase and contributed to data interpretation and manuscript preparation; **Sven Michel** performed statistical analyses during *DMRT1* fine mapping and contributed to genotyping and data interpretation; **Kathrin Suttner** contributed to study design, EMSA experiments and construct preparation for DLR assays; **Antoaneta A. Toncheva** contributed to cDNA library preparation and performed *MCP1* qRT-PCR; **Vincent Gaertner** contributed to statistical analyses during *DMRT1* replication analyses; **Simone Lipinski** performed DLR assays; **Thomas Illig, Andre Franke** performed MALDI-TOF MS genotyping; **Michael Klintschar** provided human tissue samples for cDNA library; **Omer Kalayci, Umit M Sahiner, Esra Birben, Erik Melén, Göran Pershagen, Maxim B. Freidin, Ludmila M. Ogorodova, Raquel Granell, John Henderson, Bert Brunekreef, Henriëtte A. Smit, Dirkje Postma and Gerard Koppelman** contributed to data collection of the replication cohorts**; Christian Vogelberg, Andrea von Berg, Albrecht Bufe, Andrea Heinzmann, Otto Laub, Ernst Rietschel, Burkhard Simma, Thomas Frischer and Jon Genuneit** contributed to MAGICS data collection; **Danny Jonigk** provided asthma lung tissue samples; **Judith M. Vonk** contributed to statistical analyses during replication phase; **Wim Timens** provided lung and other tissue specimen and performed immunohistochemistry; **H. Marike Boezen** designed and contributed to statistical analyses during genome-wide interaction and replication phase and to data interpretation and manuscript preparation; **Michael Kabesch** developed the study design, participated in data collection, analyses and interpretation, and wrote the final version of the manuscript. All authors checked, contributed to and approved the manuscript.

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Table I. Male-specific asthma associations in the *DMRT1* locus and *in silico* analyses for SNP function

a. After fine mapping of *DMRT1* a total of 23 polymorphisms with male-specific effects on asthma status ($p_{male} < 0.01$) were identified in the MAGICS/ISAAC II data set. Information is given on pairwise LD between SNPs and their tagging SNP (*). The SNP localization within the *DMRT1* gene structure and the MAF in the MAGICS/ISAAC II population are listed. **b**. *In silico* analyses for functional relevance of SNPs were carried out on the protein and DNA level, but also statistical effects were considered to identify a candidate SNP for functional *in vitro* analysis. Polymorphism rs3812523 in the putative promoter of *DMRT1* was identified as a target for functional *in vitro* analysis.

na: analysis method not applicable; +/-: positive or negative outcome

a.					**Asthma association**						**b.** *In silico* **analyses**				
					Sex-combined analysis		**Male-specific analysis**		**Female-specific analysis**		**Conserved AA sequence** (human DMRT family) / **influence on protein function**	**Conserved genomic region** (Human & Mouse)	**Allele-specific effect on TF binding**	**Conditional association analysis**	**Strongest association signal**
SNP	**Tagging bin**	**r^2**	**Localization**	**MAF**	**Odds Ratio (95 % CI)**	***p*-value**	**Odds Ratio (95 % CI)**	***p*-value**	**Odds Ratio (95 % CI)**	***p*-value**					
rs7022951*	1		5' upstream	0.13	0.88 (0.69-1.12)	0.282	**0.58 (0.42-0.80)**	**0.0010**	1.54 (1.06-2.23)	0.023	na	+	+	-	-
rs16924648*	2		5' upstream	0.12	0.82 (0.63-1.06)	0.129	**0.58 (0.41-0.83)**	**0.0025**	1.31 (0.88-1.95)	0.179	na	-	-	-	-
rs7858180	6	0.93	5' upstream	0.19	0.86 (0.68-1.08)	0.188	**0.62 (0.46-0.85)**	**0.0023**	1.32 (0.93-1.88)	0.124	na	+	-	-	-
rs2025309*	6		5' upstream	0.19	0.87 (0.70-1.08)	0.206	**0.64 (0.48-0.86)**	**0.0032**	1.30 (0.92-1.82)	0.138	na	-	-	na	-
rs3812523	8	0.82	5' upstream	0.14	0.79 (0.61-1.01)	0.058	**0.53 (0.38-0.74)**	**0.00019**	1.31 (0.90-1.93)	0.162	na	+	+	+	+
rs3739584	8	0.87	Exon 1 5' UTR	0.13	0.85 (0.67-1.08)	0.189	**0.60 (0.43-0.82)**	**0.0013**	1.36 (0.93-1.97)	0.113	na	-	-	na	-
rs3739583	8	0.85	Exon 1 (S45T)	0.13	0.85 (0.67-1.08)	0.185	**0.60 (0.44-0.82)**	**0.0015**	1.33 (0.91-1.95)	0.137	**-/-**	+	-	na	-
rs6477293	8	0.90	Intron 1	0.14	0.87 (0.68-1.11)	0.267	**0.65 (0.47-0.90)**	**0.0088**	1.28 (0.87-1.88)	0.203	na	-	+	na	-
rs4338164*	8		Intron 1	0.13	0.86 (0.67-1.09)	0.206	**0.61 (0.45-0.84)**	**0.0023**	1.32 (0.91-1.93)	0.142	na	-	-	na	-
rs16924776	8	0.80	Intron 1	0.13	0.85 (0.66-1.09)	0.199	0.65 (0.47-0.90)	0.010	1.19 (0.80-1.76)	0.394	na	na	na	na	Na
rs16924784	8	0.85	Intron 1	0.12	0.82 (0.63-1.06)	0.120	**0.57 (0.41-0.80)**	**0.0013**	1.29 (0.86-1.93)	0.211	na	-	-	na	-
rs16924786	8	0.85	Intron 1	0.12	0.82 (0.63-1.06)	0.120	**0.57 (0.41-0.80)**	**0.0013**	1.29 (0.86-1.93)	0.211	na	-	-	na	-
rs10976974	14	0.80	Intron 1	0.22	0.90 (0.72-1.13)	0.374	**0.67 (0.50-0.91)**	**0.0094**	1.34 (0.95-1.90)	0.097	na	-	-	na	-
rs1323271	14	0.81	Intron 1	0.22	0.92 (0.73-1.15)	0.438	0.69 (0.51-0.93)	0.015	1.33 (0.94-1.89)	0.103	na	na	na	na	Na
rs2273932	14	0.96	Intron 2	0.23	0.91 (0.73-1.14)	0.410	**0.62 (0.46-0.84)**	**0.0016**	1.49 (1.06-2.11)	0.023	na	-	-	na	-
rs2146532	14	0.99	Intron 2	0.23	0.89 (0.71-1.11)	0.296	**0.61 (0.45-0.82)**	**0.0010**	1.44 (1.02-2.03)	0.037	na	+	-	na	-
rs2181402	14	0.96	Intron 2	0.23	0.91 (0.73-1.14)	0.410	**0.62 (0.46-0.84)**	**0.0016**	1.49 (1.06-2.11)	0.023	na	-	+	na	-
rs4500131*	14		Intron 2	0.23	0.93 (0.74-1.16)	0.497	**0.62 (0.46-0.84)**	**0.0021**	1.54 (1.08-2.18)	0.016	na	-	-	na	-
rs6477300	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	0.443	**0.63 (0.47-0.84)**	**0.0020**	1.49 (1.06-2.11)	0.023	na	-	-	na	-
rs912165	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	0.443	**0.63 (0.47-0.84)**	**0.0020**	1.49 (1.06-2.11)	0.023	na	-	-	na	-
rs912166	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	0.443	**0.63 (0.47-0.84)**	**0.0020**	1.49 (1.06-2.11)	0.023	na	-	-	na	-
rs912167	14	0.99	Intron 2	0.23	0.89 (0.71-1.11)	0.296	**0.61 (0.45-0.82)**	**0.0010**	1.44 (1.02-2.03)	0.037	na	-	-	-	-
rs2370212	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	0.443	**0.63 (0.47-0.84)**	**0.0020**	1.49 (1.06-2.11)	0.023	na	-	-	na	-
rs1323265	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	0.443	**0.63 (0.47-0.84)**	**0.0020**	1.49 (1.06-2.11)	0.023	na	-	-	na	-
rs4740934	14	0.99	Intron 2	0.23	0.89 (0.71-1.11)	0.296	**0.61 (0.45-0.82)**	**0.0010**	1.44 (1.02-2.03)	0.037	na	-	-	-	-

Figure 1. Genome-wide interaction analysis between sex and polymorphisms on asthma risk
The Manhattan plot depicts *p*-values of interactions between sex and SNPs on childhood asthma associations in the MAGICS/ISAAC II population. The 100 most significant observations are indicated above the blue line and polymorphism rs2187366 on chr. 18 obtained the lowest *p*-value ($p=3.29x10^{-6}$). Top 100 interaction signals were distributed over the genome with not more than two signals within 1,000 kb (details available from the authors upon request), except the *DMRT1* locus on chr. 9, which contained four interaction signals within 5 kb (highlighted in green: rs4500131 $p=5.21x10^{-6}$; rs2146532 $p=7.45x10^{-6}$; rs3812523 $p=1.28x10^{-4}$; rs1323271 $p=4.37x10^{-4}$).

Figure 2. *DMRT1* sex-specific effects on asthma associations
Polymorphisms identified in the MAGICS/ISAAC II discovery population were analyzed in up to seven independent populations. Results for rs2146532 and rs1323271 are not shown due to high LD with rs4500131. **a. Sex-SNP interactions on asthma association.** Polymorphism rs4500131 displayed a trend for interaction in Tomsk, whereas replication of interaction was observed for rs3812523 in Tomsk and Freiburg**. b. Sex-specific asthma associations.** The minor allele of rs4500131 displayed protective effects in males in Tomsk and DAG and a trend in PIAMA. Polymorphism rs3812523 replicated this protective effect in males in Tomsk, as well as a trend in Ankara and PIAMA. Females showed inhomogeneous results with risk effects in some populations and a trend for protection in other populations.

Figure 3. Fine mapping of male-specific asthma associations in *DMRT1*
Genome-wide analysis identified clustering of sex-SNP interactions on asthma risk to the *DMRT1* locus. Subsequently, *DMRT1* was genetically fine mapped to determine sex-specific asthma associations. **a.** Male-specific asthma associations clustered to the 5' region of *DMRT1*. In females weaker but opposite effects were observed (see Table I for effect directions). The localization of all analyzed SNPs within this area is depicted. Colored bars represent *p*-values ($-\log_{10}$) for sex-combined (yellow), male-specific (blue) and female-specific (red) analyses for asthma associations in the MAGICS/ISAAC II data set. **b**. The Linkage Disequilibrium (LD) structure between polymorphisms was measured by r^2.

Figure 4. Allele-specific binding of c-Jun to rs3812523
Electrophoretic mobility shift assays (EMSA) were carried out for the risk and non-risk alleles of rs3812523 with nuclear protein extracts from HEK-293 cells without stimulation (unst.) and after stimulation with PMA-Ionomycin (P/I). **Complex I**: Distinct binding of proteins to the non-risk allele was observed for unstimulated and P/I-stimulated conditions (lanes 3-6). Competition with the risk-allele proofed specificity of protein binding to the non-risk allele (lanes 7 & 8). Competition with an AP-1 consensus site suggested involvement of this transcription factor (lane 9). Incubation with a c-Jun antibody proofed c-Jun binding by retardation of the DNA-protein complex (lane 11). Antibody tests for additional AP-1 associated molecules c-Fos and ATF-7 showed no effect (lanes12 and 13). **Complex II**: Further allele-specific effects were observed (lanes 3-6) which were shown to involve different overlaying DNA-protein complexes due to incomplete cross-competitions (lanes 1 and 8). Further information on the identity of these complexes is given in Figure E1 in the Online Repository.
† unrelated ds DNA probe
‡ unrelated antibody

Figure 5. Influence of rs3812523 on promoter activity
Expression of *Firefly luciferase* proofed functionality of the genomic sequence upstream of *DMRT1* as a promoter compared to the empty vector. Substitution of the rs3812523 risk allele with the non-risk allele caused a significant decline in promoter activity in all tested conditions.
Firefly luciferase expression was normalized to expression of *Renilla luciferase* in the same sample (FLuc/RLuc ratio). The conditions "unstimulated" and "PMA25ng/ml; Ionomycin0.5µM" represent six samples. "PMA12.5ng/ml; Ionomycin0.25µM" represents eight samples.

Figure 6. Immunohistochemistry for DMRT1

DMRT1 staining in adult testis tissue was present in nuclei of Sertoli cells and possibly immature germ cells. Lung tissue from patients with normal lung function showed weak DMRT1 staining in alveolar macrophages, whereas tissue from patients with different lung diseases displayed strong DMRT1 staining in all alveolar macrophages and to a lower extent also in interstitial macrophages. Staining in the lung was compatible with localization of DMRT1 to the cytosolic compartment. No staining was observed in granulocytes, lymphocytes or plasma cells.

Asthma: asthma bronchiale (adult patient with accompanying emphysematous changes)
DIP: desquamative interstitial pneumonia
COPD: chronic obstructive pulmonary disease
IPF: idiopathic pulmonary fibrosis

Tissue samples from asthmatic lung were kindly provided by the Institute of Pathology (Hannover Medical School, Germany). All other tissue samples were kindly provided by the Department of Pathology and Medical Biology (University of Groningen, The Netherlands)