Doublesex and mab-3 related transcription factor 1 (*DMRT1*) is a sex-specific genetic determinant of childhood-onset asthma and is expressed in testis and macrophages

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

Objective: We analyzed whether 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 Multicentre Asthma Genetics in Childhood Study (MAGICS)/Phase II International Study of Asthma and Allergies in Childhood (ISAAC II) population on a genome-wide level, and findings

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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 using electrophoretic mobility shift 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 using 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 nonasthmatic control subjects (lowest $P = 5.21 \times 10^{-6}$). Replication of this interaction was successful in 2 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 the 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. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Asthma, genetic association, DMRT1, interaction, rs3812523, sex, single nucleotide polymorphism*

Abbreviations used

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
Ct:	Cycle threshold
DAG:	Dutch Asthma Genome-wide Association Study
DMRT:	Doublesex and mab-3 related transcription factor
EMSA:	Electrophoretic mobility shift assay
Freiburg:	Clinical study population recruited in Freiburg, Germany
ISAAC II:	Phase II International Study of Asthma and Allergies in Childhood
LD:	Linkage disequilibrium
MAF:	Minor allele frequency
MAGICS:	Multicentre Asthma Genetics in Childhood Study
MCP1:	Monocyte chemotactic protein 1
PIAMA:	Prevention and Incidence of Asthma and Mite Allergy
SNP:	Single nucleotide polymorphism
Tomsk:	Clinical study population primarily recruited in Tomsk, Russia

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] whereas remission seems to be more pronounced in boys during puberty.[3] During adolescence and adulthood, more female than male subjects 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, sex-specific differences in social behavior, exposure to environmental disease triggers, and disease awareness can exist.[6,7]

However, sex-specific differences in age of onset and persistence of asthma can 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 to therapeutic intervention. In this study we conducted a genome-wide search for sex-specific associations with childhood asthma, performed replication studies in 7 different study populations, analyzed the functional relevance of associated polymorphisms, and approached the potential role of the associated gene in asthma pathogenesis.

METHODS

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

Interactions between sex and single nucleotide polymorphisms (SNPs) on childhood-onset asthma risk were analyzed on a genome-wide level in the Multicentre Asthma Genetics in Childhood Study (MAGICS)/Phase II International Study of Asthma and Allergies in Childhood (ISAAC II) population (see the Methods section and Table E1 in this article's Online Repository at www.jacionline.org for population details). A total of 1361 subjects (703 asthmatic subjects with 65.3% male subjects and 658 nonasthmatic control subjects with 49.7% male subjects) with chip-based SNP genotypes were available (Sentrix HumanHap300 BeadChip; Illumina, San Diego, Calif).[8] All calculations were carried out with the PLINK software package, version 1.07, by using the following filtering parameters: minor allele frequency (MAF) of 0.05 or greater, SNP genotyping rate of 0.95 or greater, and Hardy-Weinberg disequilibrium P value in the control population of .0001 or greater.[9] 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 7 independent populations with childhood-onset asthma phenotypes (the Avon Longitudinal Study of Parents and Children [ALSPAC]; the clinical study population recruited in Ankara, Turkey [Ankara]; the Children, Allergy, Milieu, Stockholm an Epidemiological Study [BAMSE]; the Dutch Asthma Genome-wide Association Study [DAG]; the clinical study population recruited in Freiburg, Germany [Freiburg]; the Prevention and Incidence of Asthma and Mite Allergy [PIAMA] study; and the clinical study population primarily recruited in Tomsk, Russia [Tomsk]; see the Methods section and Table E1 in this article's Online Repository 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 to determine the extent of sex-specific asthma associations in the doublesex and mab-3 related transcription factor 1 *(DMRT1)* locus. Genetic fine mapping was performed by using a tagging SNP approach based on HapMap data and enriched with SNPs from the 1000 Genomes Project and the SNPper database (see the Methods section in this article's Online Repository for details).[10-12] Polymorphisms not present in the MAGICS/ISAAC II data set of chip- and imputation-based genotypes were genotyped by using 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 by using PLINK. Male-specific asthma associations were investigated for independence 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 means of alignment of amino acid sequences of all human *DMRT* gene family members (Vector NTI Advance 11; Invitrogen, Life Technologies, Carlsbad, Calif). The functional effect of nonsynonymous SNPs was assessed with the PolyPhen-2 software tool.[16] Phylogenetic shadowing between human subjects and mice 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 using electrophoretic mobility shift assays (EMSAs; see the Methods section and Table E6 in this article's Online Repository at www.jacionline.org for details). 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 gels. 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 activity of the putative *DMRT1* promoter were investigated in dual luciferase reporter assays (see the Methods section in this article's Online Repository for details). In short, approximately 2 kb of genomic sequence upstream of *DMRT1* was cloned into the pGL3-Basic vector upstream of the Firefly Luciferase gene (see Fig E2 and Table E7 in this article's Online Repository at www.jacionline.org; 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 a readout of promoter activity. Cotransfection of Renilla Luciferase driven by the 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 the testes only.[19] To identify *DMRT1* expression in further potentially relevant tissues, we conducted quantitative real-time PCR on a cDNA library of human tissues and cell lines (see the Methods section in this article's Online Repository for more details). Expression levels of *18S rRNA* and the Δ cycle threshold (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 tissue. Details on ethical guidelines for sample collection and methods for immunohistochemical and hematoxylin staining can be found in the Methods section in this article's Online Repository.

RESULTS

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 ($P = 3.29 \times 10^{-6}$), which is located 8 kb upstream of the uncharacterized gene *LOC100505817* on chromosome 18 (Fig 1). Almost identical significance levels were observed for rs4500131 and rs2146532 ($P = 5.21 \times 10^{-6}$ and $P = 7.45 \times 10^{-6}$, respectively), which are located in the *DMRT1* gene on chromosome 9p24, and an additional 2 polymorphisms in the *DMRT1* locus (rs3812523 and rs1323271) were among the 100 strongest interaction signals (Fig 1 and see Table E2, *A*, in this article's Online Repository at www.jacionline.org). Other interaction signals were distributed

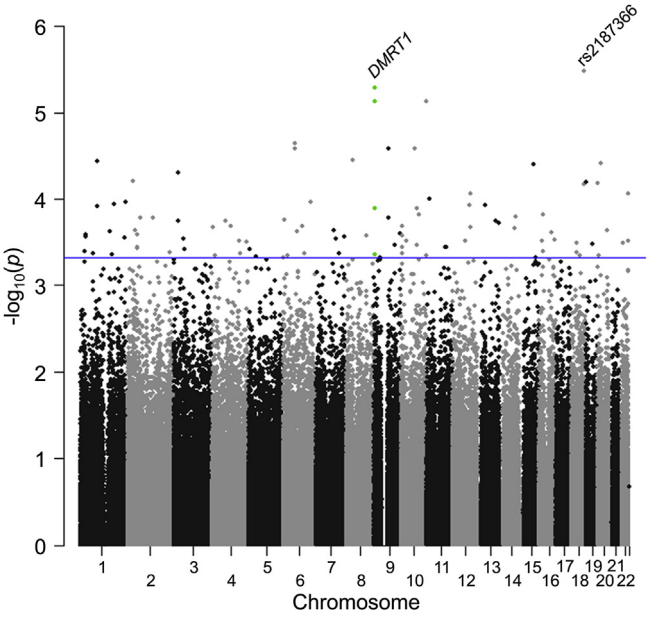

FIG 1. Genome-wide interaction analysis between sex and polymorphisms on asthma risk. The Manhattan plot depicts *P* values of interactions between sex and SNPs on childhood asthma associations in the MAGICS/ISAAC II population. The 100 most significant observations are indicated above the *blue line*, and polymorphism rs2187366 on chromosome 18 obtained the lowest *P* value ($P = 3.29 \times 10^{-6}$). The top 100 interaction signals were distributed over the genome, with not more than 2 signals within 1000 kb (details available from the authors on request), except the *DMRT1* locus on chromosome 9, which contained 4 interaction signals within 5 kb (highlighted in green: rs4500131, $P = 5.21 \times 10^{-6}$; rs2146532, $P = 7.45 \times 10^{-6}$; rs3812523, $P = 1.28 \times 10^{-4}$; rs1323271, $P = 4.37 \times 10^{-4}$).

over the genome, with no more than 2 signals within 1000 kb. Because of the known male-specific biological function of *DMRT1*, this locus was selected for further analyses, despite ranging slightly below a genome-wide significance level at a *P* value of less than 1.6×10^{-7}.[19] Asthma association data for *DMRT1* polymorphisms revealed protective effects for the minor allele in male subjects with odds ratios of less than 1, whereas female subjects displayed no or opposite effects (Fig 2 and see Table E2, *B* and *C*).

Replication analyses of *DMRT1* polymorphisms rs4500131 and rs3812523 ($r^2 = 0.43$ in MAGICS/ISAAC II) were performed in 6 (rs4500131) and 7 (rs3812523) independent populations with childhood-onset asthma. Polymorphisms rs2146532 and rs1323271 were not investigated during replication because of 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 (Fig 2 and see Table E3 in this article's Online Repository at www.jacionline.org). Comparable with the MAGICS/ISAAC II population, the rs4500131 minor allele displayed protective effects in male asthmatic subjects in the clinical Tomsk and DAG cohorts, as well as a trend in the birth cohort PIAMA. For female asthmatic subjects, 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 asthmatic subjects in Tomsk and a trend in Ankara and the PIAMA cohort. As for rs4500131, rs3812523 results for female asthmatic subjects were inhomogeneous, with a

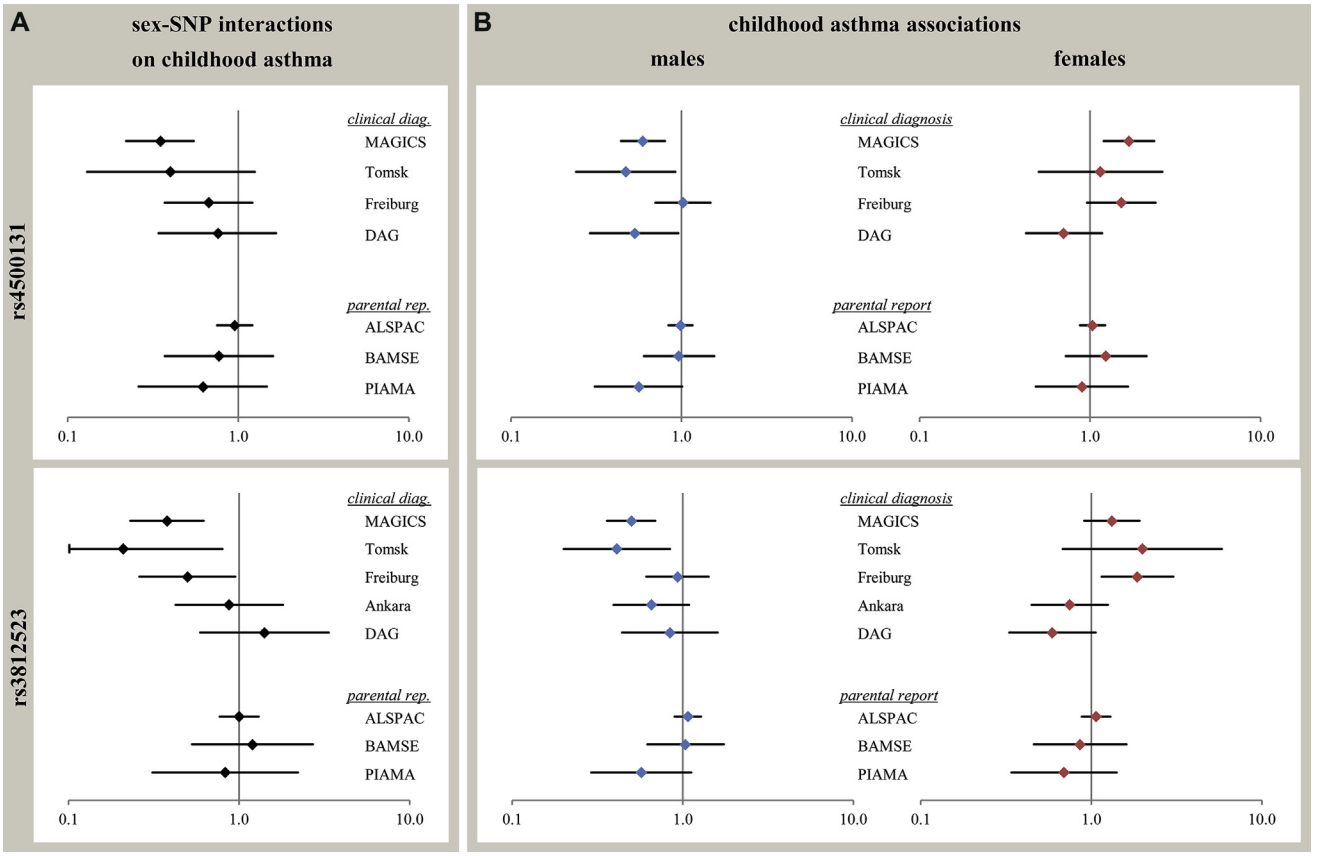

FIG 2. *DMRT1* sex-specific effects on asthma associations. Polymorphisms identified in the MAGICS/ISAAC II discovery population were analyzed in up to 7 independent populations. Results for rs2146532 and rs1323271 are not shown because of high LD with rs4500131. **A,** Sex-SNP interactions on asthma 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 male subjects in Tomsk and DAG and a trend in the PIAMA cohort. Polymorphism rs3812523 replicated this protective effect in male subjects in Tomsk, as well as a trend in Ankara and the PIAMA cohort. Female subjects showed inhomogeneous results, with risk effects in some populations and a trend for protection in other populations.

significant risk effect in Freiburg and a trend for risk in Tomsk, whereas 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; and $r^2 \geq 0.8$). Detailed information on all genotyped and analyzed SNPs is provided in Table E4 in this article's Online Repository at www.jacionline.org. Significant male-specific effects ($P_{male} < .01$) clustered to 23 polymorphisms in the 5′ gene region. These SNPs belonged to 5 different tagging bins, with the strongest signal originating from rs3812523 (Fig 3 and Table I). Male-specific association signals based on imputation for rs7022951, rs2025309, rs4338164, and rs3739583 with asthma and rs7040024 and rs6477321 with eczema were verified by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (eczema data are not shown but are available from the authors on request).

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

All polymorphisms with male-specific asthma associations at a *P* value of less than .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). Comparison between human and mouse sequences identified 5 SNPs to be located in phylogenetically conserved regions (rs7022951, rs7858180, rs3812523, rs3739583, and rs2146532), and allele-specific effects on transcription factor binding were predicted for 4 polymorphisms (rs7022951, rs3812523, rs6477293, and rs2181402; Table I). 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 and see Table E5 in this article's Online Repository at www.jacionline.org; detailed data on *in silico* analyses are available on 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 nonrisk G allele of rs3812523 (conferring a lower risk for asthma

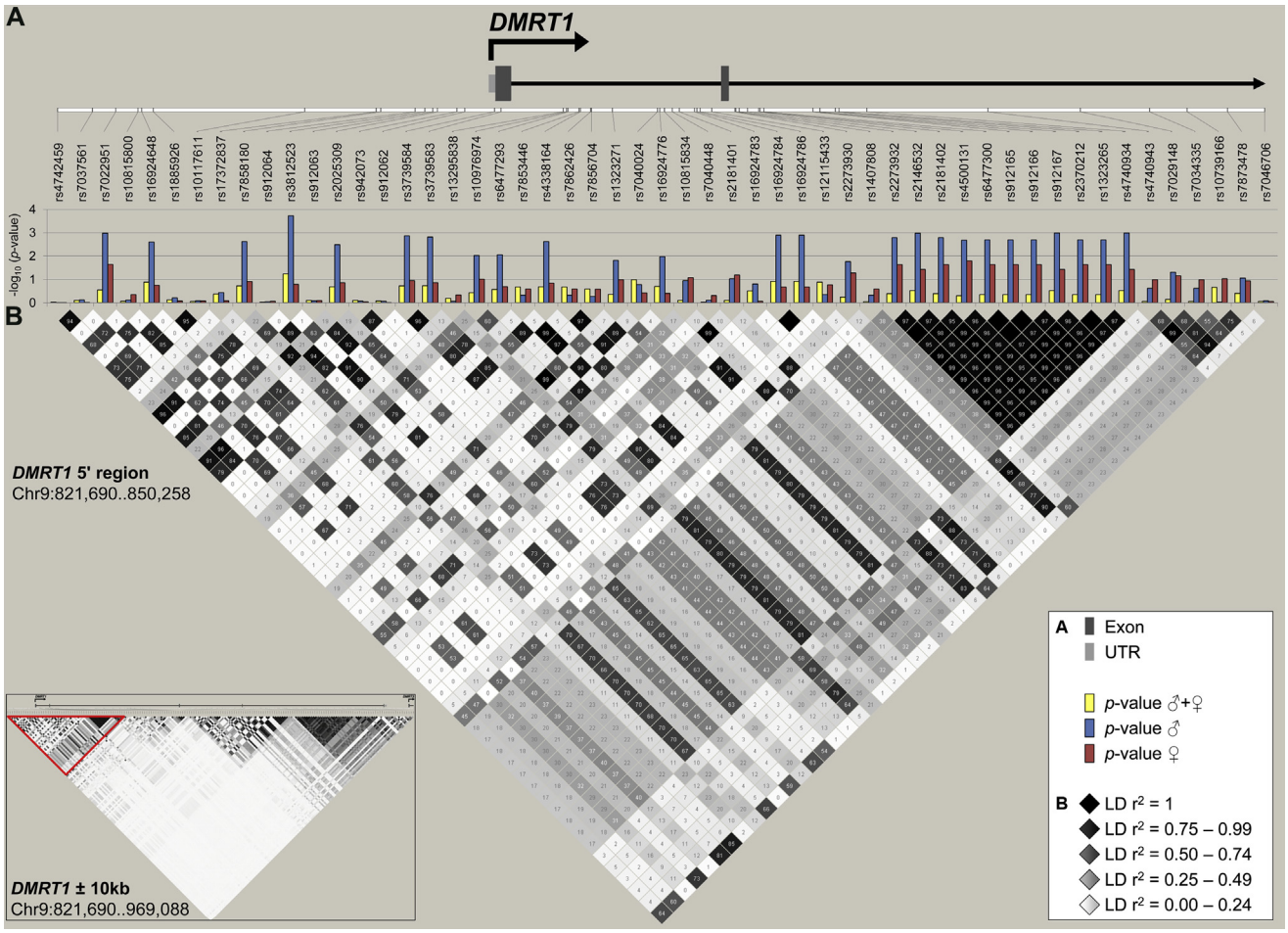

FIG 3. Fine mapping of male-specific asthma associations in *DMRT1*. Genome-wide analysis identified clustering of sex-SNP interactions on asthma risk to the *DMRT1* locus. Subsequently, *DMRT1* was genetically fine mapped to determine sex-specific asthma associations. **A,** Male-specific asthma associations clustered to the 5′ region of *DMRT1*. In female subjects 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 LD structure between polymorphisms was measured by using r^2 analysis.

in male subjects) in nuclear protein extract from HEK-293 cells, which was not present for the risk A allele (Fig 4, complex I, lanes 3-6). Competition experiments demonstrated a high allele specificity of this DNA-protein complex (Fig 4, lanes 7-8). Competition with a high-affinity activator protein 1 (AP-1) consensus site successfully abrogated the DNA-protein complex (Fig 4, lane 9). The dimeric AP-1 transcription factor can be formed by different members of the Jun, Fos, activating transcription factor, and Jun dimerization protein families.[21] Antibody-specific supershift experiments revealed a supershift of the DNA-protein complex after incubation with a c-Jun antibody (Fig 4, lane 11) and negative results for c-Fos (Fig 4, lane 12), activating transcription factor 7 (Fig 4, lane 13), and Jun dimerization protein 2 (data not shown).

A second DNA-protein complex at a lower molecular weight also displayed allele-specific effects (Fig 4, complex II, lanes 3-6). However, for both alleles, the cross-competitions were incomplete (Fig 4, lanes 1 and 8), illustrating that different DNA-protein complexes are overlaid at this height of the EMSA gel. This finding is supported by competition with the AP-1 consensus site (see Fig E1, complex II, lanes 5 and 14 in this article's Online Repository at www.jacionline.org), leading to complete abrogation of this complex only in the nonrisk allele. Antibody-specific supershifts in both the nonrisk and risk alleles confirmed that c-Jun and c-Fos paricipate in this complex (see Fig E1, complex II, lanes 2, 3, 16, and 17). However, even after successful c-Jun and c-Fos supershifts, an unidentified complex remains visible in the risk allele (see Fig E1, complex II, lanes 2 and 3).

Dual luciferase reporter assays were conducted to test whether the allele-specific binding of transcription factors to rs3812523 observed in EMSA has an influence on its activity as a promoter. A strong increase in luciferase signal between the empty pGL3-Basic vector and the 2 pGL3-*DMRT1* vectors demonstrates the functionality of this putative *DMRT1* promoter region (Fig 5). Comparison between the rs3812523 risk and nonrisk alleles displayed a significantly lower expression of the Firefly Luciferase gene under the control of the nonrisk allele in all tested conditions, with the strongest effect in unstimulated HEK-293 cells ($P = .0008$).

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

					Asthma association*			
					Sex-combined analysis		Male-specific analysis	
SNP	Tagging bin	r^2	Localization	MAF	Odds ratio (95 % CI)	*P* value	Odds ratio (95 % CI)	*P* value
rs7022951‡	1		5′ Upstream	0.13	0.88 (0.69-1.12)	.282	**0.58 (0.42-0.80)**	**.0010**
rs16924648‡	2		5′ Upstream	0.12	0.82 (0.63-1.06)	.129	**0.58 (0.41-0.83)**	**.0025**
rs7858180	6	0.93	5′ Upstream	0.19	0.86 (0.68-1.08)	.188	**0.62 (0.46-0.85)**	**.0023**
rs2025309‡	6		5′ Upstream	0.19	0.87 (0.70-1.08)	.206	**0.64 (0.48-0.86)**	**.0032**
rs3812523	8	0.82	5′ Upstream	0.14	0.79 (0.61-1.01)	.058	**0.53 (0.38-0.74)**	**.00019**
rs3739584	8	0.87	Exon 1 5′ UTR	0.13	0.85 (0.67-1.08)	.189	**0.60 (0.43-0.82)**	**.0013**
rs3739583	8	0.85	Exon 1 (S45T)	0.13	0.85 (0.67-1.08)	.185	**0.60 (0.44-0.82)**	**.0015**
rs6477293	8	0.90	Intron 1	0.14	0.87 (0.68-1.11)	.267	**0.65 (0.47-0.90)**	**.0088**
rs4338164‡	8		Intron 1	0.13	0.86 (0.67-1.09)	.206	**0.61 (0.45-0.84)**	**.0023**
rs16924776	8	0.80	Intron 1	0.13	0.85 (0.66-1.09)	.199	0.65 (0.47-0.90)	.010
rs16924784	8	0.85	Intron 1	0.12	0.82 (0.63-1.06)	.120	**0.57 (0.41-0.80)**	**.0013**
rs16924786	8	0.85	Intron 1	0.12	0.82 (0.63-1.06)	.120	**0.57 (0.41-0.80)**	**.0013**
rs10976974	14	0.80	Intron 1	0.22	0.90 (0.72-1.13)	.374	**0.67 (0.50-0.91)**	**.0094**
rs1323271	14	0.81	Intron 1	0.22	0.92 (0.73-1.15)	.438	0.69 (0.51-0.93)	.015
rs2273932	14	0.96	Intron 2	0.23	0.91 (0.73-1.14)	.410	**0.62 (0.46-0.84)**	**.0016**
rs2146532	14	0.99	Intron 2	0.23	0.89 (0.71-1.11)	.296	**0.61 (0.45-0.82)**	**.0010**
rs2181402	14	0.96	Intron 2	0.23	0.91 (0.73-1.14)	.410	**0.62 (0.46-0.84)**	**.0016**
rs4500131‡	14		Intron 2	0.23	0.93 (0.74-1.16)	.497	**0.62 (0.46-0.84)**	**.0021**
rs6477300	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	.443	**0.63 (0.47-0.84)**	**.0020**
rs912165	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	.443	**0.63 (0.47-0.84)**	**.0020**
rs912166	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	.443	**0.63 (0.47-0.84)**	**.0020**
rs912167	14	0.99	Intron 2	0.23	0.89 (0.71-1.11)	.296	**0.61 (0.45-0.82)**	**.0010**
rs2370212	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	.443	**0.63 (0.47-0.84)**	**.0020**
rs1323265	14	0.96	Intron 2	0.23	0.92 (0.74-1.14)	.443	**0.63 (0.47-0.84)**	**.0020**
rs4740934	14	0.99	Intron 2	0.23	0.89 (0.71-1.11)	.296	**0.61 (0.45-0.82)**	**.0010**

+/−, Positive or negative outcome; *NA*, analysis method not applicable; *TF*, transcription factor; *UTR*, untranslated region.

*After fine mapping of *DMRT1*, a total of 23 polymorphisms with male-specific effects on asthma status ($P_{male} < .01$; in boldface) were identified in the MAGICS/ISAAC II data set. Information is given on pairwise LD between SNPs and their tagging SNPs (‡). The SNP localization within the *DMRT1* gene structure and the MAFs in the MAGICS/ISAAC II population are listed.

†*In silico* analyses for functional relevance of SNPs were carried out at 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.

DMRT1 is expressed in testis tissue and macrophages in the lung

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

Immunohistochemistry for DMRT1 presented strong nuclear staining of adult testis Sertoli cells and possibly some germ cells (Fig 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, chronic obstructive pulmonary disease, or idiopathic pulmonary fibrosis presented strong staining for DMRT1 in all alveolar macrophages and also less prominent staining 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. The 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 (see Fig E3 in this article's Online Repository at www.jacionline.org).

DISCUSSION

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 American subjects in which the polymorphism rs2549003, which is located near interferon regulatory factor 1 *(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 ($P = .89$) or asthma association in male subjects ($P_{male} = .39$) 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 male subjects and weaker but opposite effects in female subjects. Replication of rs4500131 and rs3812523 ($r^2 = 0.43$) was successful in some but not all populations. Interestingly, the clinical Tomsk and Freiburg populations

TABLE I. *(Continued)*

Female-specific analysis		*In silico* analyses†				
Odds ratio (95 % CI)	*P* value	Conserved AA sequence (human DMRT family)/ influence on protein function	Conserved genomic region (human and mouse)	Allele-specific effect on TF binding	Conditional association analysis	Strongest association signal
1.54 (1.06-2.23)	.023	NA	+	+	−	−
1.31 (0.88-1.95)	.179	NA	−	−	−	−
1.32 (0.93-1.88)	.124	NA	+	−	−	−
1.30 (0.92-1.82)	.138	NA	−	−	NA	−
1.31 (0.90-1.93)	.162	NA	+	+	+	+
1.36 (0.93-1.97)	.113	NA	−	−	NA	−
1.33 (0.91-1.95)	.137	−/−	+	−	NA	−
1.28 (0.87-1.88)	.203	NA	−	+	NA	−
1.32 (0.91-1.93)	.142	NA	−	−	NA	−
1.19 (0.80-1.76)	.394	NA	NA	NA	NA	NA
1.29 (0.86-1.93)	.211	NA	−	−	NA	−
1.29 (0.86-1.93)	.211	NA	−	−	NA	−
1.34 (0.95-1.90)	.097	NA	−	−	NA	−
1.33 (0.94-1.89)	.103	NA	NA	NA	NA	NA
1.49 (1.06-2.11)	.023	NA	−	−	NA	−
1.44 (1.02-2.03)	.037	NA	+	−	NA	−
1.49 (1.06-2.11)	.023	NA	−	+	NA	−
1.54 (1.08-2.18)	.016	NA	−	−	NA	−
1.49 (1.06-2.11)	.023	NA	−	−	NA	−
1.49 (1.06-2.11)	.023	NA	−	−	NA	−
1.49 (1.06-2.11)	.023	NA	−	−	NA	−
1.44 (1.02-2.03)	.037	NA	−	−	−	−
1.49 (1.06-2.11)	.023	NA	−	−	NA	−
1.49 (1.06-2.11)	.023	NA	−	−	NA	−
1.44 (1.02-2.03)	.037	NA	−	−	−	−

presented the best replication of our findings, whereas the clinically recruited DAG population with an 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 might be less pronounced in a population-based milder asthma phenotype compared with that in populations recruited from tertiary clinical centers. Additionally, opposite effect directions between male and female subjects, as well as ambiguous effects in female subjects 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 male subjects because of the known biological function of DMRT1 in testis tissue, 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, which is located in the putative promoter of *DMRT1*, as the best candidate that can drive the sex-specific asthma association. EMSA displayed allele-specific binding of c-Jun to the nonrisk allele of rs3812523, whereas a second DNA-protein complex containing heterodimeric AP-1 (c-Jun/c-Fos) exhibited quantitative effects in binding affinity between risk and nonrisk alleles. 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 phorbol 12-myristate 13-acetate leads to a relatively lower promoter activity, which is in line with observations of inhibited *DMRT1* expression in primary mouse Sertoli cells after phorbol 12-myristate 13-acetate 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 the cause of childhood asthma between sexes. *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,

Probe	risk allele				nonrisk allele										
Antibody											c-Jun	c-Fos	ATF-7	‡	
Competition	non-risk allele	risk-allele					non-risk allele	risk-allele	AP1	†					
Nuclear extract	HEK P/I	HEK P/I	HEK P/I	HEK unst.	HEK unst.	HEK P/I	HEK P/I	HEK P/I	HEK P/I	HEK P/I	HEK P/I	HEK P/I	HEK P/I	HEK P/I	
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

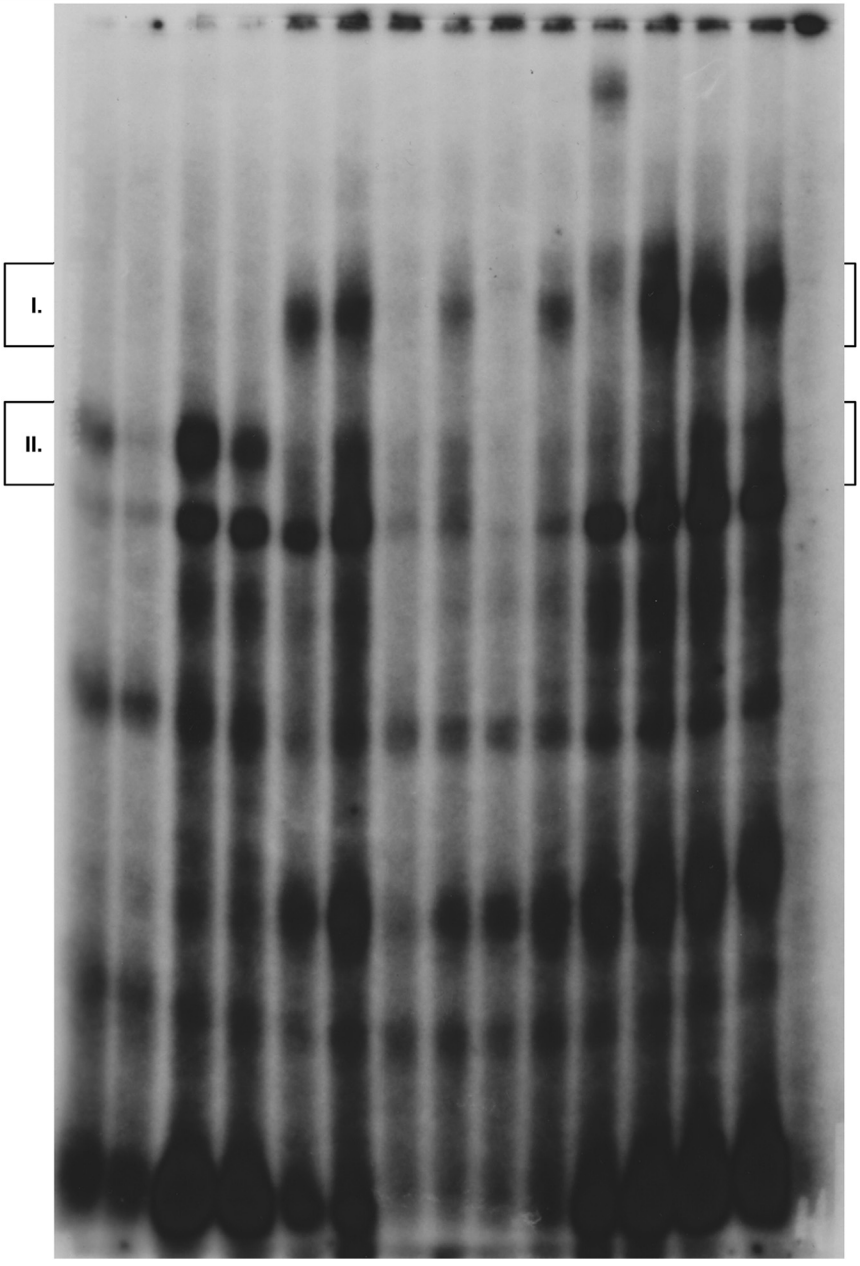

FIG 4. Allele-specific binding of c-Jun to rs3812523. EMSAs were carried out for the risk and nonrisk alleles of rs3812523 by using nuclear protein extracts from HEK-293 cells without stimulation and after stimulation with phorbol 12-myristate 13-acetate–ionomycin (P/I). *Complex I,* Distinct binding of proteins to the nonrisk allele was observed for unstimulated and P/I-stimulated conditions *(lanes 3-6)*. Competition with the risk allele proved specificity of protein binding to the nonrisk allele (*lanes 7* and *8*). Competition with an AP-1 consensus site suggested involvement of this transcription factor *(lane 9)*. Incubation with a c-Jun antibody proved c-Jun binding by means of retardation of the DNA-protein complex *(lane 11)*. Antibody tests for the additional AP-1–associated molecules c-Fos and activating transcription factor 7 *(ATF-7)* showed no effect (*lanes 12* and *13*). *Complex II,* Further allele-specific effects were observed *(lanes 3-6)*, which were shown to involve different overlaying DNA-protein complexes because of incomplete cross-competitions (*lanes 1* and *8*). Further information on the identity of these complexes is given in Fig E1. †Unrelated double-stranded DNA probe. ‡Unrelated antibody.

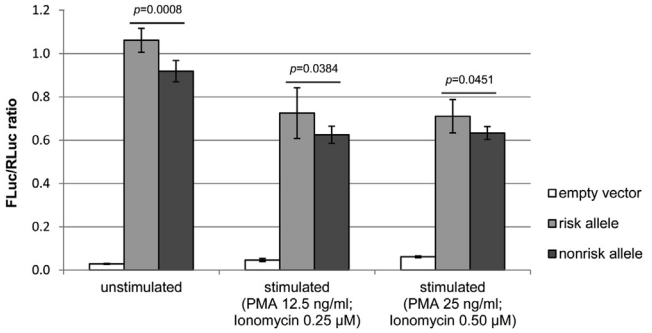

FIG 5. Influence of rs3812523 on promoter activity. Expression of Firefly Luciferase proved the functionality of the genomic sequence upstream of *DMRT1* as a promoter compared with the empty vector. Substitution of the rs3812523 risk allele with the nonrisk allele caused a significant decrease in promoter 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 6 samples. *PMA12.5ng/ml; Ionomycin0.25μM* represents 8 samples. *PMA,* Phorbol 12-myristate 13-acetate.

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 transdifferentiate toward female ovarian granulosa cells.[29] In the same study specific deletion of *DMRT1* in fetal Sertoli cells caused increased estradiol levels and reduced androgen activity. It has been shown that *DMRT1* is continuously expressed in human testis tissue from fetal stages until puberty and thereafter. However, this expression appears to follow a spatiotemporal expression pattern in different testis cell types, whereas in human ovaries *DMRT1* expression is only present in early fetal stages and downregulated in later fetal stages.[26] One could speculate that rs3812523-driven regulation of *DMRT1* expression might influence, to some extent, the timing of testis development and, as a consequence, also the levels and timing of sexual hormone expression. It is an attractive notion that boys carrying the nonrisk 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, whereas immunohistochemistry data revealed low levels of DMRT1 in alveolar macrophages in control lung tissue. In contrast, strong DMRT1 staining was observed in the cytoplasm of alveolar macrophages in patients with interstitial lung disease (desquamative interstitial pneumonia and idiopathic pulmonary fibrosis) and patients with chronic obstructive pulmonary disease, which might indicate a disease-associated function of DMRT1. Bilateral lung explants harvested during lung transplantation from adult asthmatic patients displayed DMRT1 staining similar to that seen in patients with other lung diseases. However, samples derived from adult patients with asthma accompanied by smoking-induced end-stage lung disease (displaying emphysematous changes and anthracosis in addition to the 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 asthma. Unfortunately, childhood asthma samples were not available. The sporadic staining of subsets of macrophages and high endothelial venules in the appendix, thymus, lymph node, and spleen suggests that DMRT1 has a function also in these tissues outside the lung (see Fig E3). 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 that could be causative for the genetic association signals observed in female subjects in our analyses. Furthermore, sex-specific gene regulation has been suggested for DMRT1, with repression of *Stra8* expression in male mice but enhancement of *Stra8* expression in female mice.[30] The underlying mechanism is unknown, but sex-specific gene regulation in human subjects 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, such as the potential of DMRT1 translocalization 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 PBMCs (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 with those in heterozygous carriers ($P = .037$). Moreover, we were able to support this observation with a trend for lower *MCP1* mRNA levels in risk allele carriers ($P = .101$, for details, see the Methods section and Fig E4 in this article's Online Repository at www.jacionline.org).[34] The explorative character of this analysis with a relatively small sample size needs to be acknowledged, as well as the unexpected downregulation 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 are provided in Tables E9 and E10 and Fig E5 in this article's Online Repository at www.jacionline.org) and observed weak but significant associations in male subjects 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 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

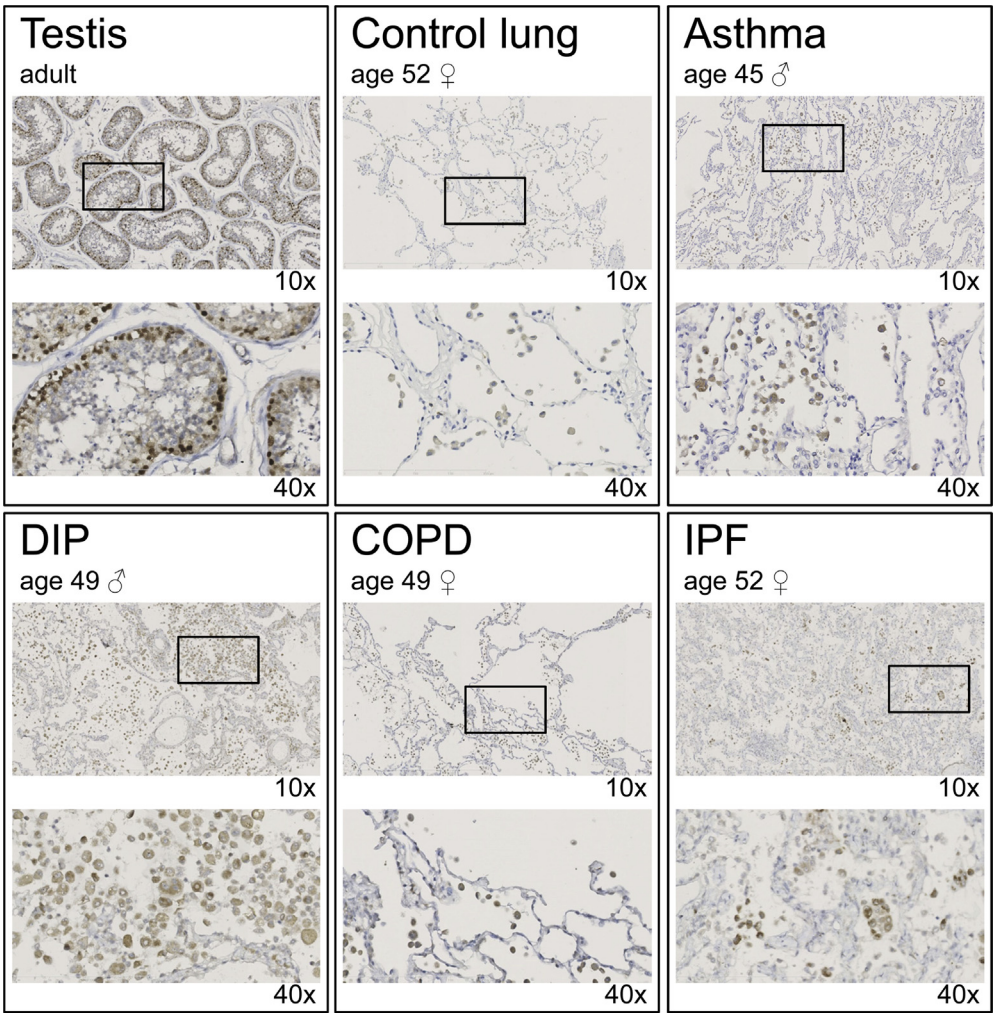

FIG 6. Immunohistochemistry for DMRT1. DMRT1 staining in adult testis tissue was present in nuclei of Sertoli cells and possibly immature germ cells. Lung tissue from patients with normal lung function showed weak DMRT1 staining in alveolar macrophages, whereas tissue from patients with different lung diseases displayed strong DMRT1 staining in all alveolar macrophages and, to a lesser 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); *COPD*, chronic obstructive pulmonary disease; *DIP*, desquamative interstitial pneumonia; *IPF*, idiopathic pulmonary fibrosis.
Tissue samples from lungs of asthmatic subjects 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).

testis development, as well as our demonstration of *DMRT1* expression in alveolar macrophages, opens potential for further functional investigations. The precise analysis of spatiotemporal gene expression of *DMRT1* will have to follow to elucidate whether and how *DMRT1* expression in testis tissue or in cells of the immune system affects asthma pathogenesis in male and female subjects.

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

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