

DNA methylation within melatonin receptor 1A (*MTNR1A*) mediates paternally transmitted genetic variant effect on asthma plus rhinitis

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Background: Asthma and allergic rhinitis (AR) are common allergic comorbidities with a strong genetic component in which epigenetic mechanisms might be involved.

Objective: We aimed to identify novel risk loci for asthma and AR while accounting for parent-of-origin effect.

Methods: We performed a series of genetic analyses, taking into account the parent-of-origin effect in families ascertained through asthma: (1) genome-wide linkage scan of asthma and

AR in 615 European families, (2) association analysis with 1233 single nucleotide polymorphisms (SNPs) covering the significant linkage region in 162 French Epidemiological Study on the Genetics and Environment of Asthma families with replication in 154 Canadian Saguenay-Lac-Saint-Jean asthma study families, and (3) association analysis of disease and significant SNPs with DNA methylation (DNAm) at CpG sites in 40 Saguenay-Lac-Saint-Jean asthma study families.

Results: We detected a significant paternal linkage of the 4q35 region to asthma and allergic rhinitis comorbidity (AAR; $P = 7.2 \times 10^{-5}$). Association analysis in this region showed strong evidence for the effect of the paternally inherited G allele of rs10009104 on AAR ($P = 1.1 \times 10^{-5}$, reaching the multiple-testing corrected threshold). This paternally inherited allele was also significantly associated with DNAm levels at the cg02303933 site ($P = 1.7 \times 10^{-4}$). Differential DNAm at this site was found to mediate the identified SNP-AAR association.

Conclusion: By integrating genetic and epigenetic data, we identified that a differentially methylated CpG site within the melatonin receptor 1A (*MTNR1A*) gene mediates the effect of a paternally transmitted genetic variant on the comorbidity of asthma and AR. This study provides a novel insight into the role of epigenetic mechanisms in patients with allergic respiratory diseases. (J Allergy Clin Immunol 2016;■■■■:■■■■-■■■■.)

Key words: Linkage analysis, positional cloning, asthma, allergic rhinitis, parent-of-origin effect, DNA methylation, genetic association study

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The prevalence of allergy-related diseases, such as asthma and rhinitis, has reached epidemic proportions in high-income countries and represents a global health problem for all ages and all ethnic groups. Asthma and allergic rhinitis (AR) affect 5% to 16% and 10% to 25% of persons worldwide, respectively.^{1,2} Asthma and AR frequently coexist in the same subjects.³ Indeed, more than 60% of patients with asthma have concomitant AR, whereas 10% to 40% of patients with AR have asthma comorbidity,⁴ suggesting that these diseases might share a common cause, overlapping pathogenic pathways, or both.

The risk of asthma and AR has a strong genetic component, with estimated heritability ranging from 35% to 75%.² Genome-wide association studies (GWASs) have been successful in identifying loci associated with asthma and AR, with a number of loci

Abbreviations used

AAR:	Asthma and allergic rhinitis comorbidity
AR:	Allergic rhinitis
CIT:	Causal inference test
CNG:	Centre National de Génotypage
CTCF:	CCCTC-binding factor
DNAm:	DNA methylation
EGEA:	Epidemiological Study on the Genetics and Environment of Asthma
GWAS:	Genome-wide association study
MCPPAT:	Monte Carlo Pedigree Parental Asymmetry Test
MTNR1A:	Melatonin receptor 1A
PO-LRT:	Parent-of-origin likelihood ratio test
POO:	Parent of origin
QC:	Quality control
SLSJ:	Saguenay-Lac-Saint-Jean asthma study
SNP:	Single nucleotide polymorphism

shared by both diseases.^{5,6} A few genetic studies have examined asthma and AR comorbidities and led to the identification of new loci that were not detected when they were analyzed separately; these include *ZBTB10*, *CLEC16A*, and *NFIA* genes.^{7,8}

Despite the success of GWASs for discovering common risk alleles associated with asthma, AR, or both, the genetic factors identified to date account only for a small part of the genetic component of these diseases.¹ Sources of this hidden heritability might include epigenetic mechanisms, such as DNA methylation (DNAm). Epigenetic modifications are heritable changes influencing gene expression without alteration of the underlying DNA sequence. Several lines of evidence support a role for epigenetics in asthma and allergy, which are immune-mediated diseases characterized by a T_H2 -type immune response.⁹ Epigenetic mechanisms are known to play a role in T-cell differentiation and regulation.¹⁰ Moreover, environmental factors associated with asthma and allergy have been reported to induce epigenetic changes in experimental settings.¹⁰ The importance of epigenetics in allergy-related diseases has been recently evidenced in human subjects by using an epigenome-wide association study of total IgE levels, where DNAm at 3 CpG sites was found to account for 13% of IgE variation, which is 10-fold higher than that accounted for by GWAS single nucleotide polymorphisms (SNPs).¹¹ DNAm can cause partial or complete silencing of 1 parental allele (imprinting) at a specific locus and can lead to an effect of that polymorphism on disease in offspring that differs according to the parental origin (paternal or maternal) of the allele (parent-of-origin [POO] effect).

The goal of the present study was to identify novel risk loci for allergic diseases defined by asthma, AR, and asthma and allergic rhinitis comorbidity (AAR) through a positional cloning approach taking into account the POO effect and to determine whether the effect of associated SNPs is mediated by DNAm. For that purpose, we developed a 3-step analysis strategy in family samples ascertained through asthma: (1) genome-wide linkage scan of asthma, AR, and AAR in 3 European family data sets; (2) association analysis of SNPs covering the significant linkage region in the French Epidemiological Study on the Genetics and Environment of Asthma (EGEA) discovery data set with validation in a French Canadian data set; and (3) association analyses of DNAm levels with both genetic variants and disease, followed by the causal inference test (CIT).

METHODS**Analysis strategy and family samples**

The study design is described in Fig E1 in this article's Online Repository at www.jacionline.org. The first step consisted of a genome-wide linkage analysis of allergic diseases conducted in 615 families from 3 independent European family samples: EGEA,¹² the Medical Research Council Asthma UK National family collection,¹³ and Italian families.¹⁴ All families were ascertained through at least 1 asthmatic proband recruited in chest clinics, as previously described.¹²⁻¹⁴ Asthma was based on a physician's diagnosis and international standardized questionnaires. AR was defined by the report of at least 1 of the following symptoms: sneezing or runny nose to hay/flowers, animals, or dust. AAR was defined as the coexistence of the 2 diseases (asthma and AR) in the same subject. Details on study characteristics and phenotype definitions are provided in the **Methods** section and Table E1 in this article's Online Repository at www.jacionline.org.

In the second step the region of linkage found significantly linked to AAR in the presence of the POO effect was explored for association in the EGEA study. This study was the only one of the 3 data sets used for linkage analysis that had genotyped SNPs in both parents and offspring, thus allowing testing for the POO effect. After applying stringent quality control (QC) criteria, there were 531 family members belonging to 162 families with at least 1 offspring with AAR and 2 genotyped parents (see the **Methods** section in this article's Online Repository for details). The EGEA top associated SNPs were then tested for validation in an independent family study, the Saguenay-Lac-Saint-Jean asthma study (SLSJ), which included 253 French Canadian multigenerational families ascertained through 2 probands with asthma.¹⁵ Asthma definition in probands was based on at least 2 of the following criteria: (1) a minimum of 3 clinic visits for acute asthma within 1 year, (2) 2 or more asthma-related hospital admissions within 1 year, or (3) steroid dependency. In relatives asthma was defined by (1) a history of asthma (validated by a physician) or (2) asthma-related symptoms and bronchial hyperresponsiveness (see Table E1). The definition of AR was similar to the one used in the EGEA study (see Table E1). After QC of the genotypic data, the SLSJ analysis sample included 486 family members from 154 families with at least 1 genotyped offspring with AAR and 2 genotyped parents (see the **Methods** section in this article's Online Repository for details).

In the third step we investigated whether the significant POO effects of SNPs on AAR were driven by differential DNAm levels measured in 40 SLSJ families by using CIT.¹⁶

For all independent studies, ethical approval was obtained from the appropriate institutional ethic committees, and all subjects provided written informed consent.

Genotyping

The 3 data sets used for linkage analysis had been genotyped by using the same panel of 396 microsatellites at the Centre National de Génotypage (CNG, Evry, France) as part of our linkage analysis program.¹⁷ After QC of the genotypic data, the analysis sample included a total of 2702 subjects belonging to 615 families with at least 2 genotyped sibs.

The discovery and replication association analyses were based on SNP data generated in the EGEA and SLSJ data sets by using the Illumina 610K array (Illumina, San Diego, Calif) at CNG as part of the European GABRIEL asthma consortium.¹⁸ The same QC criteria were applied to both data sets, as described elsewhere.¹⁹ The region analyzed for association included a total of 1233 genotyped SNPs covering the 6-Mb linkage region (95% CI defined by a 1.5-LOD decrease from the linkage peak).

Methylation levels at CpG sites were measured by using DNA from peripheral blood leukocytes in a subset of SLSJ subjects ($n = 167$) by using the Infinium HumanMethylation450 BeadChip at Genome Quebec Innovation Centre (Montreal, Quebec, Canada).¹¹ After QC, a total of 159 subjects belonging to 40 nuclear SLSJ families were available for DNAm association analysis (see the **Methods** section in this article's Online Repository for details). Differential white cell counts (eosinophils, neutrophils, basophils, monocytes, and lymphocytes) were measured by using an automated counter in SLSJ data.

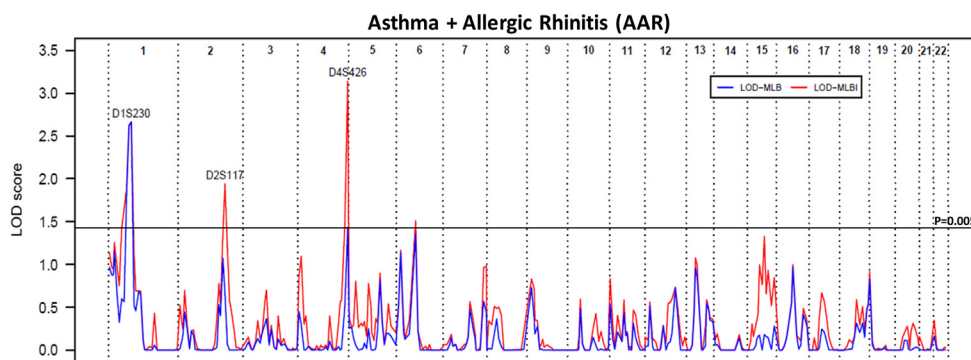


FIG 1. Genome-wide linkage scan conducted for AAR in European families with or without taking into account the POO effect (LOD maximum likelihood binomial method extended for imprinting [LOD-MLBI] or LOD maximum likelihood binomial method [LOD-MLB] curves, respectively). Genetic distance is plotted on the x-axis, and LOD score is plotted on the y-axis.

Statistical analyses

The genome-wide multipoint linkage analysis for the 3 disease entities (asthma, AR, and AAR) used a model-free approach, the maximum likelihood binomial method that can be applied to the whole sibships, and was extended to account for the POO effect (maximum likelihood binomial method extended for imprinting).²⁰ Linkage was tested with and without the POO effect by using likelihood ratio tests (see the [Methods](#) section in this article's Online Repository for details).

Association of the significant linkage region detected for AAR in the presence of the POO effect was investigated by using the Monte Carlo Pedigree Parental Asymmetry Test (MCPPAT) screening method applied to all SNPs covering this region. This method tests for the asymmetry of transmission of the risk allele between the father and mother to heterozygous affected offspring (see the [Methods](#) section in this article's Online Repository for details).²¹ Association results reaching a critical threshold of 0.001 were validated by using a second method, the parent-of-origin likelihood ratio test (PO-LRT).²² In addition to testing for the POO effect, this method allows distinguishing the POO effect from the maternal genotype effect on disease in affected offspring (see the [Methods](#) section in this article's Online Repository for details). It also provides estimates of these different effects, whereas the MCPPAT does not. PO-LRT was only applied to MCPPAT-associated SNPs to reduce the multiple testing issue because it requires performing multiple model comparisons (POO effect only, maternal genotype effect only, or both POO and maternal genotype effects). SNPs detected in EGEA families at a P value of .001 or less with both the MCPPAT and PO-LRT methods were followed up for replication in the SLSJ families and then analyzed in the pooled EGEA and SLSJ samples by using PO-LRT. Significance of the POO effect was finally assessed by using a multiple testing corrected threshold that takes into account linkage disequilibrium between SNPs, as proposed by Li and Ji.²³

To determine whether the significant POO effect of SNPs on disease was driven by differential DNAm of paternal and maternal chromosomes, we carried out association analysis with DNAm levels at CpG sites lying close to the SNPs. We tested for association of both paternally and maternally inherited alleles of AAR-associated SNPs and DNAm levels using a linear regression model adjusted for white cell type proportions (eosinophils, neutrophils, basophils, monocytes, and lymphocytes) and applied to offspring of SLSJ families (see the [Methods](#) section in this article's Online Repository for details). The DNAm levels at CpG sites that were found to be associated with AAR-associated SNPs were subsequently tested for association with disease (AAR) in offspring by using a logistic regression model. In all regression models we used a robust sandwich estimation of variance to take into account familial dependencies. At each step, a Bonferroni correction was applied to correct for the total number of tested CpG sites.

Finally, we carried out a CIT for DNAm levels associated with both SNPs and disease to establish the epigenetic mediation of the POO effect of SNP-AAR association. This approach is based on a series of conditional association analyses described in the [Methods](#) section in this article's Online Repository.¹⁶

If DNAm is a mediator of the relationship between SNP and AAR, the association between SNP and AAR is expected to decrease or even disappear when adjusting for DNAm levels in the regression model while the association between DNAm levels and disease remains significant.

RESULTS

Genome-wide linkage screen

The genome-wide linkage screen conducted for asthma, AR, and AAR in 1472 genotyped siblings (see [Tables E2 and E3](#) in this article's Online Repository at www.jacionline.org) showed significant evidence for linkage of the 4q35 region to AAR when taking into account the POO effect ([Fig 1](#)). The LOD score reached 3.14 ($P = 7.2 \times 10^{-5}$) at D4S426 when alleles were transmitted from the father, whereas there was only modest evidence for linkage when the POO effect was ignored (LOD, 1.44; $P = .005$). No significant linkage with any other phenotype was detected (see [Fig E2](#) and [Table E4](#) in this article's Online Repository at www.jacionline.org).

Association analysis with SNPs taking into account the POO effect

Of 1233 SNPs in the 4q35 linkage region, 5 ($r^2 < 0.8$) showed an association with AAR at a P value of .001 or less by using MCPPAT in 162 French EGEA families (see [Table E5](#) in this article's Online Repository at www.jacionline.org), and 2 of them were confirmed at the same level by using PO-LRT under the best-fitting model of paternally transmitted risk allele effect ([Table I](#) and see [Table E6](#) in this article's Online Repository at www.jacionline.org).²² Among these SNPs taken forward for replication in SLSJ families (see [Table E5](#)), rs10009104 showed nominally significant association with AAR ($P = .02$) under the same paternal-origin effect model. The EGEA and SLSJ pooled analysis improved evidence for this association ($P = 1.1 \times 10^{-5}$) that achieved the multiple testing corrected threshold of 7.2×10^{-5} ([Table I](#)). The ratio of effect for the paternally versus maternally inherited rs10009104-G allele (allelic frequency, 0.72) was 1.96 (95% CI, 1.43-2.70).

Association analysis with DNAm levels at CpG sites taking into account the POO effect

To assess whether association of the paternally inherited rs10009104-G allele with AAR was driven by differential

TABLE I. Association of 4q35 SNPs with asthma and AR in EGEA and SLSJ families by using the PO-LRT²²

Marker	Position (bp)*	Nearest gene or pseudogene (kb distance)	Baseline/ effect allele	Effect allele frequency	EGEA, 162 families		SLSJ, 154 families		Pooled EGEA+SLSJ, 316 families	
					I _p † (95% CI)	P value	I _p † (95% CI)	P value	I _p † (95% CI)	P value
rs10009104	187,490,834	<i>MTNRIA</i> (14)	A/G	0.72	2.13 (1.37-3.23)	.0002	1.75 (1.10-2.86)	.02	1.96 (1.43-2.70)	$1.1 \times 10^{-5} \ddagger$
rs7687115	188,608,857	<i>LOC105377603</i>	G/A	0.56	2.70 (1.69-4.17)	$2.7 \times 10^{-6} \ddagger$	0.87 (0.56-1.33)	.51	—	—

These 2 SNPs are those passing a *P* value of .001 or less in EGEA families and taken forward replication in SLSJ families (see the [Methods](#) section for more details).

*SNP positions from GRCh37.1 assembly.

†I_p indicates the imprinting parameter reflecting a differential transmission of the risk allele to the affected child from fathers versus mothers. I_p is equal to 1 if there is no POO effect.

‡Significant *P* values after correction for multiple testing ($P \leq 7.2 \times 10^{-5}$).

DNAm of parental chromosomes, we tested for association between rs10009104 and DNAm levels at 24 CpG sites located in a 50-kb region around rs10009104 in 40 SLSJ families (159 subjects, see [Table E7](#) in this article's Online Repository at www.jacionline.org). The paternally inherited rs10009104-G allele was nominally associated with DNAm levels at 2 CpG sites, 1 of which met the significance threshold after Bonferroni correction (cg02303933: $P = 1.7 \times 10^{-4}$, [Table II](#)). This CpG site was also associated with AAR ($P = .006$, [Table II](#)). These results were not influenced by adjustment on white cell-type proportions.

We used the CIT to assess whether DNAm levels at cg02303933 mediate the effect of the paternally inherited rs10009104-G allele on AAR ([Fig 2](#)). In a regression model that included the effects of both DNAm and SNP on disease (AAR), we found that methylation levels at cg02303933 remained associated with AAR ($P = .03$), whereas the paternally inherited G allele of rs10009104 did not ($P = .28$). This demonstrates that the paternally inherited rs10009104-G allele effect on AAR is mediated by DNAm variations at this site. Moreover, we estimated that the variance in AAR risk accounted for by the cg02303933 CpG site was 3 times higher than that explained by the rs10009104 SNP.

Using the UCSC genome browser (<http://genome.ucsc.edu/>), we found that rs10009104 is located at 14 kb from the melatonin receptor 1A (*MTNRIA*) gene near a recombination hotspot and is in strong to moderate linkage disequilibrium with 6 SNPs upstream of *MTNRIA* or within *MTNRIA* intron 1 ($D' = 0.9$, $0.60 \leq r^2 \leq 0.73$; see [Fig E3](#) in this article's Online Repository at www.jacionline.org). cg02303933 is located in that intron in the north shore of a CpG island and at a 15-kb distance from rs10009104. This CpG site lies in overlapping functional elements, including DNase I hypersensitivity clusters and CCCTC-binding factor (CTCF)-binding site (see [Fig E4](#) in this article's Online Repository at www.jacionline.org).

DISCUSSION

The present study is the first to investigate genetic variants involved in the comorbidity of asthma and AR while accounting for the POO effect in both linkage and association analyses and seeking for methylation quantitative trait loci. Through a positional cloning approach, we identified a paternally transmitted genetic variant (rs10009104-G allele) associated with AAR in the 4q35 linkage region. We demonstrated that the effect of this paternally inherited variant on AAR was mediated by DNAm variation within the *MTNRIA* gene.

Evidence for association between rs10009104 and AAR rests on the results obtained from the large EGEA discovery family sample and validated in French Canadian families, an independent data set that was not part of the previous linkage study, plus strong improvement of results reaching statistical significance when combining these 2 samples. Validation of this result was also obtained in EGEA through the use of different statistical methods, which provided concordant findings. Our results were thus strengthened by validation at both the population and statistical methodology levels. Moreover, stringent thresholds were used at all steps to correct for multiple testing.

The rs10009104 SNP associated with AAR is located near the linkage peak (1.6 Mb from D4S426) detected in the pooled analysis of 3 European data sets. We checked that this SNP accounted for most of the linkage signal (see the [Methods](#) and [Results](#) sections in this article's Online Repository at www.jacionline.org for details). Previous linkage screens have detected the 4q35 region linked to asthma, AR, bronchial hyperresponsiveness, and atopy-related phenotypes in different populations.²⁴⁻²⁶ A maternal linkage between D4S426 and both atopy and total serum IgE levels has been reported in families of European ancestry.²⁶ Although this reported POO effect differs from that found in the present study, it should be noted that it was related to different phenotypes and that the published linkage study was not followed up by association analysis.²⁶ Nevertheless, the previous and current findings support the presence of the POO effect and potentially epigenetic mechanisms in the 4q35 region.

Existing methodologies investigating the POO effect require genotypic data of high quality in all family members (offspring and both parents). To obtain a denser map of a surrounding region of rs10009104 and encompassing *MTNRIA*, we repeated association analyses (PO-LRT) using HapMap2 imputed SNPs (best guess genotype) with stringent postimputation QC criteria ($r^2 \geq 0.9$). These analyses supported our original finding with additional signals located in the upstream region of *MTNRIA* and in intron 1 of that gene ($P < 3 \times 10^{-3}$), with the best signal still being detected at rs10009104 (results not shown). The single GWAS conducted thus far for AAR, as well as the GWASs for asthma, AR, and allergy and asthma-related phenotypes, did not report any association with 4q35 genetic variants.^{6,7} However, none of these studies had taken into account the POO effect.

Association analysis of rs10009104 with DNAm levels at CpG sites in the 4q35 region showed significant association with cg02303933. This CpG site lies within a CTCF-binding site. CTCF has a central role in chromatin organization and gene expression, and its implication in imprinting is well established.²⁷ Displaying properties of an insulator, CTCF mediates interactions between distant DNA sequences among which regulatory

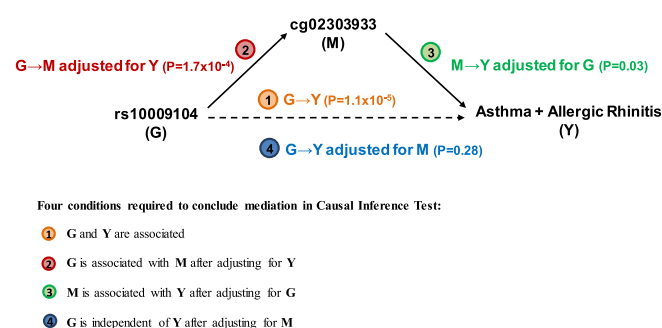
TABLE II. Association of DNAm levels at CpG sites of the 4q35 region with the rs10009104-G allele and AAR in offspring of 40 SLSJ families under a POO effect model

Illumina ID probes	Position (build 37.1)	Gene	Association between DNAm at CpG site and rs10009104-G allele							Association between DNAm at CpG site and AAR, <i>P</i> value	
			β_{wPat}	SD β_{wPat}	P_{op}	β_{wMat}	SD β_{wMat}	P_{om}	P_{diff}		
cg02303933	187,475,669	<i>MTNR1A</i>	1.18	0.31	$1.7 \times 10^{-4*}$	0.01	0.52	.98	.04	.006*	
cg02756176	187,491,362	—	1.34	0.64	.04	1.04	0.53	.05	.69	.06	

These 2 CpG sites are those nominally associated with the paternally inherited rs10009104-G allele ($P_{op} \leq .05$).

P_{diff} , Statistical significance of the difference between β_{wPat} and β_{wMat} assessed by using bootstrapping; P_{op} and P_{om} , statistical significances of the regression coefficients β_{wPat} and β_{wMat} assessed by using bootstrapping ($n = 10,000$ iterations); β_{wPat} and β_{wMat} , regression coefficients of the estimates of paternal or maternal within-family components of rs10009104 genotype score with G allele as the effect allele (see the [Methods](#) section in this article's Online Repository for more details).

*Significant association after Bonferroni correction.

**FIG 2.** Establishing the epigenetic mediation of genetic risk: CIT applied with the paternally inherited rs10009104-G allele as the causal factor (G), DNAm levels at cg02303933 as a potential mediator (M), and asthma plus rhinitis as the outcome (Y).

sequences acting as enhancer and promoter. CTCF plays multiple roles in hematopoietic cell lineages, regulating early thymocyte development,²⁸ cell differentiation, and cytokine production in helper T cells.²⁹ Methylation at this site might disrupt the fixation of CTCF and could impair its regulatory function. Nominal significant association of rs10009104 was also found with DNAm levels at cg02756176. Interestingly, the same association has been reported in adipose tissues.³⁰ In addition, a part of the 4q35 linkage region spanning 4 kb, from 187.488 Mb to 187.492 Mb, and encompassing rs10009104 was described as an allele-specific methylation region in human embryos.³¹ These reports strengthen the evidence for a broad differentially methylated region in 4q35.

The disease-associated CpG site (cg02303933) is located in intron 1 of the *MTNR1A* gene, which encodes a high-affinity G protein-coupled receptor for melatonin. *MTNR1A* is an integral membrane protein that responds to a variety of extracellular stimuli and modulates several signal transduction pathways, among which are G_i protein-adenylyl cyclase (cyclic AMP) and extracellular signal-regulated kinase 1/2, 2 pathways involved in inflammation regulation.³² *MTNR1A* is expressed in numerous organs and cell types, including the lung and B and T cells. *MTNR1A* appears to be a good candidate for AAR based on several lines of evidence. Indeed, *MTNR1A* participates in the action of melatonin, which can have an important immunomodulatory effect in patients with allergic diseases, including antioxidant effects, and an action on T_H2 signaling pathways. Notably, in experimental models melatonin administration regulates smooth muscle tone, attenuates airway inflammation and lung mucus production, and reduces proinflammatory cytokine levels in human bronchial

epithelial cell lines.^{33,34} Both melatonin secretion and *MTNR1A* expression are regulated in a circadian manner.³² Interestingly, a circadian variation in asthma and AR symptoms is well described, with worsening symptoms overnight.³⁵ Moreover, the circadian cycle is known to be regulated by epigenetic mechanisms.³⁶

Although the present linkage analysis included 3 different data sets totaling 615 families and the association study was performed in 2 data sets (for discovery and validation) totaling 316 families, additional replication of our findings might be desirable. However, access to asthma family data sets with comparable study design, phenotype definitions, and genotyping is limited, especially in the GWAS era. We did not find any 4q35 genetic variant associated with the expression (expression quantitative trait locus) of *MTNR1A* in blood and lung tissue (see the [Results](#) section in this article's Online Repository for details). It should be noted that current expression quantitative trait locus databases report data without distinguishing the parental origin of the effect allele. We examined DNAm, one of the epigenetic marks implicated in gene expression regulation, to explain the detected POO effect in the 4q35 region. However, other epigenetic modifications could be investigated, such as histone modifications.¹⁰ Moreover, increase of CpG site density with a sequencing method might reveal additional CpG sites associated with AAR. Further investigations of the 4q35 locus, such as biological experiments, will have the potential to validate methylation findings and improve our understanding of the genetic factors and epigenetic mechanisms underlying the risk of asthma plus rhinitis.

In conclusion, we identified that differential DNAm within *MTNR1A* mediates the effect of a paternally inherited genetic variant on the co-occurrence of asthma and AR. The present study highlights the importance of integrating genetic and epigenetic data in family-based studies to uncover such imprinting mechanisms and therefore to identify new genes in complex diseases.

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Key messages

- A differential DNA-methylated CpG site within *MTNRI*A mediates the effect of a paternally inherited genetic variant on the co-occurrence of asthma and AR.
- The variance in asthma and AR risk accounted for by this methylated CpG site was 3 times higher than that explained by the detected genetic variant.
- Examination of both DNAm profiles and SNP data bring better insight into epigenetic mechanisms, mediating the associations between genetic variants and complex diseases.

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1 **Online Repository Materials**

2 **DNA methylation within *MTNR1A* mediates paternally transmitted genetic variant**
3 **effect on asthma-plus-rhinitis**

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METHODS

Study populations

French EGEA study

The EGEA study is a 20-year longitudinal survey which combines a case-control study and a family study of asthmatic cases (<https://egeanet.vjf.inserm.fr>). The protocol and descriptive characteristics have been described elsewhere.¹ Briefly, probands (388 asthmatic patients) were recruited from six chest clinics in five French cities between 1991 and 1995 (EGEA1) and their 1,244 family members were included, either by including the proband's parents and siblings, or by including the proband's spouse and children. In addition, 415 population-based controls were recruited. Inclusion criteria for asthmatic probands were positive answers to four standardized questions (1/ Have you ever had attacks of breathlessness at rest with wheezing? 2/ Have you ever had an asthma attack? If yes 2a/ was this diagnosis confirmed by a physician? 2b/ Have you had an asthma attack in the last 12 months?). The asthmatic probands and controls were between 7 and 70 years old at time of ascertainment. All probands and their two parents were of European ancestry and were born in France. All subjects responded to a questionnaire based on international standardized tools to diagnose asthma and to determine respiratory and allergic symptoms, treatments, and environmental exposures.¹ In probands' relatives, asthma was defined as a positive answer to "Have you ever had attacks of breathlessness at rest with wheezing?" and/or "Have you ever had asthma attacks?". Ethical approval was obtained from the relevant institutional review board committees (Cochin Port-Royal Hospital and Necker-Enfants Malades Hospital, Paris). Written informed consent was signed by all adult participants and by kin or guardians of the minors/children. Data were collected through face-to-face interviews and examination included extensive phenotypic characterization (detailed clinical data based on standardized questionnaires, skin prick tests, lung function tests, bronchial responsiveness, blood samples, white blood cell counts, total

IgE), data on risk factors (environmental exposures, diet, physical activity, hormone-related events) and drug consumption.

The present study used family data at EGEA1 only. First, linkage analyses were performed on 295 families with at least two genotyped offspring (726 siblings) after QC of the genotypic data.² Then, association analyses taking into account parent-of-origin effect were conducted on families with at least one genotyped and affected offspring and whose both parents were genotyped.

UK family collection (MRCA)

The Medical Research Council (MRC) UK National family collection included 207 nuclear families, recruited through at least one proband with severe (Step 3) childhood onset asthma. Children and their parents were administered a standard questionnaire (based on the American Thoracic Society and International Study of Asthma and Allergies in Childhood (ISAAC) questionnaires³) by a nurse practitioner or a doctor. Asthma was defined as a positive response to the question: “Has your doctor ever told you that you have asthma?” Proband had Step 3 asthma or worse according to the British Thoracic Society guidelines (high-dose inhaled steroids, or low-dose inhaled steroids and a long-acting β -agonist).⁴ Siblings were included regardless of asthma status. After QC of the genotypic data, linkage analyses were performed on 161 MRCA families with at least two genotyped and affected offspring (359 siblings).

Italian family collection

The Italian asthma sample has been described previously.⁵ Briefly, all families were ascertained through an allergic asthmatic child and recruited at the Pediatric Clinic of the University of Verona and at the Bolzano Hospital, both located in north-eastern Italy, between

1995 and 1998. The individuals were characterized for clinical asthma through physician diagnosis according to the American Thoracic Society criteria,³ and including a positive response to a validated respiratory questionnaire.⁶ Subjects underwent a clinical evaluation including a methacholine challenge test. Total serum IgE levels were determined, and skin prick tests to a panel of common allergens were performed. The protocol was approved by the local ethical committee and informed consent was obtained in the collections of blood and clinical information for all the participants. After QC of the genotypic data, 170 Italian families with at least one genotyped and affected sibpair were included in the genome-wide linkage screen (387 siblings).

SLSJ

The Saguenay-Lac-Saint-Jean (SLSJ) asthma study consists of French-Canadian families ascertained through two asthmatic probands. Probands were included in the study if they fulfilled at least two of the following criteria: 1) a minimum of three clinic visits for acute asthma within one year; 2) two or more asthma-related hospital admissions within one year; or 3) steroid dependency, as defined by either six months of oral, or one year of inhaled corticosteroid use. Individuals from 253 independent families were included in the study. Families were included in the study if at least one parent was available for phenotypic assessment, at least one parent was unaffected, and all four grandparents were of French-Canadian origin. Family members were considered asthmatics if both a self-reported history of asthma and a history of physician-diagnosed asthma were available, or by clinical evaluation following a methacholine provocation test.⁷ The SLSJ local ethics committee approved the study, and all subjects gave informed consent.

DNA methylation at CpG sites in white blood cells of individuals of SLSJ families

DNA methylation levels at CpG sites were measured in peripheral blood leukocytes from a subset of SLSJ subjects (N=167) using Infinium HumanMethylation450 BeadChip at Genome Quebec Innovation Centre (Montreal, Canada).⁸ Details on DNA methylation measurement and quality control steps are described in Liang *et al.*⁸

For the present study, we focused on DNA methylation levels at CpG sites located in a 50kb region around rs10009104 for which we observed parent-of-origin effect on asthma-plus-rhinitis risk. Methylation values were standardized and normalized before analysis.

Statistical methods

Step1: Genome-wide linkage screen

Linkage analyses were performed by a model-free approach, the Maximum Likelihood Binomial (MLB) method which is likelihood-based and can be applied to the whole sibship of affected subjects.⁹ In brief, the principle of this method is based on the binomial distribution of the number of affected sibs receiving a given parental allele from heterozygous parents. The probability α for an affected sib to receive from his/her parent the marker allele transmitted with the disease allele, is equal to 0.5 under the null hypothesis of no linkage whereas α is greater than 0.5 under the hypothesis of linkage. Test for linkage is performed using a likelihood ratio test (LRT) statistic, $LRT=2\ln[L(\alpha)/L(\alpha=0.5)]$ with the LRT being asymptotically distributed as a mixture distribution of $0.5 \chi^2_{0df}$ and $0.5 \chi^2_{1df}$.

This method has been extended to account for parent-of-origin (POO) effects, specifically genomic imprinting.¹⁰ The MLB-I method relies on the dissociation of the central parameter α into two parameters, α_p and α_m , which characterize paternal and maternal transmissions, respectively.

Depending on the equality of the two parameters and the departure of at least one of them from 0.5, five models can be considered: M1: no linkage and no imprinting ($\alpha_m=\alpha_p=0.5$), M2:

linkage and no imprinting ($\alpha_m = \alpha_p > 0.5$), M3: linkage and complete maternal imprinting ($\alpha_m = 0.5, \alpha_p > 0.5$), M4: linkage and complete paternal imprinting ($\alpha_m > 0.5, \alpha_p = 0.5$), M5: linkage and imprinting ($\alpha_m > 0.5, \alpha_p > 0.5, \alpha_m \neq \alpha_p$). To test genomic imprinting hypothesis, we tested successively the following models: 1) M1 *versus* M5 (test of linkage taking into account imprinting), 2) M5 *versus* M3 (test of complete maternal imprinting) and 3) M5 *versus* M4 (test of complete paternal imprinting). The first test follows a mixture of $\frac{1}{4} \chi^2_{0df} + \frac{1}{2} \chi^2_{1df} + \frac{1}{4} \chi^2_{2df}$ and the two others tests follow a mixture of $\frac{1}{2} \chi^2_{0df} + \frac{1}{2} \chi^2_{1df}$.

The sibship distribution of families included in linkage analyses is shown in Table E3.

Step 2: Association analysis with SNPs taking into account parent-of-origin effect

We investigated the association between asthma-plus-rhinitis and SNPs belonging to 4q35 region using two approaches taking into account parent-of-origin effect: MCPPAT and PO-LRT.

These methods are based on case-parents trios that are genotyped and can be categorized based on the number of copies of a particular allele (0, 1 or 2 copies of the allele of interest) carried by the mother, the father, and the child (denoted “M”, “F”, and “C”, respectively). A case-parents triad could have 15 possible familial genotype combinations. The two methods consider the mating types in which the mother and the father carry different number of copy of the risk allele, with further stratification on the number of inherited copies of this allele by the child.

Monte Carlo Pedigree-PAT approach (MCPPAT)

The Parental-Asymmetry Test (PAT)¹¹ is based on case-parents trios and looks for asymmetry of risk allele transmission to heterozygous affected offspring.

If we note N_{MFC} the number of families that fall into genotype category MFC, this test is based on the binomial probability 0.5 distribution of the count $Y = N_{211} + N_{201} + N_{101}$ (where the mother has transmitted the risk allele to the child) from the total $Y + X$, with $X = N_{121} + N_{021} + N_{011}$ (where the father has transmitted the risk allele to the child). The test statistics measures the asymmetry in risk allele transmission between mothers and fathers to affected offspring and follows a Mc Nemar χ^2 distribution with 1 df, under the null hypothesis that there is no parent-of-origin effect. This test assumes that maternal genotype has no direct effect on the risk of disease in the child.

We used the Monte Carlo Pedigree-PAT approach, an extension of the PAT method that can be applied to nuclear families with multiple affected offspring and with some missing genotypes.^{12, 13} MCPPAT was performed using the software package MC-PDT (<http://www.stat.osu.edu/statgen/SOFTWARE/MC-PDT/>) implemented in R.

Parent-of-origin likelihood ratio test (PO-LRT)

The approach based on parent-of-origin likelihood ratio test (PO-LRT) enables the identification of both imprinting (that leads to POO) and maternal genotype effects influencing disease risk in the child as maternal genotype effect can mimic POO effect.¹¹

This model considers 1) maternally mediated in utero effects (maternal genotypic effects on the phenotype of the foetus), modelled by two parameters: S_1 and S_2 , and 2) a separate term for imprinting (I_m). Here, S_1 and S_2 correspond to the maternally mediated relative risks for a mother with one copy or two copies of the risk allele of a given variant, respectively, compared to a mother with no copy of this allele. The imprinting parameter reflects a differential transmission of alleles to affected child from mothers (M) *versus* fathers (F). The parameter I_m will be 1 if there is no parent-of-origin effect; I_m will be > 1 if the maternally derived copy of the allele is associated with a higher risk of disease than the paternally

183 inherited copy and will be < 1 if the paternally inherited copy of the allele is associated with a
 184 higher risk of disease than the maternally inherited copy.

185 The model expresses the probability that the mother has transmitted the risk allele to the
 186 affected child rather than the father ($M > F$) as a function of three parameters: I_m , S_1 and S_2 .
 187 When the affected child has inherited at least one copy of the risk allele, $M > F$ corresponds to
 188 maternal transmission whereas $M < F$ corresponds to paternal transmission. The
 189 corresponding ratio, that is, $\Pr[M > F]/\Pr[M < F]$, takes a simple linear form under a
 190 logarithmic transformation with three parameters (α , β and γ). The parameter α accounts for
 191 parent-of-origin effect ($e(\alpha) = I_m$) whereas the parameters β and γ accounts for maternal
 192 effects ($e(\beta) = S_2$ and $e(\gamma) = S_1$). Testing for parent-of-origin effect (M1) consists in testing
 193 that $\alpha = 0$ by means of a LRT and follows a χ^2 distribution with 1 df. Testing for maternal
 194 effects (M2) consists in testing that $\beta = \gamma = 0$ by means of a LRT and follows a χ^2 distribution
 195 with 2 df. Finally we can assume that both effects (maternal and POO) could exist (M3) by
 196 testing that $\alpha = \beta = \gamma = 0$ by means of a LRT and follows a χ^2 distribution with 3 df. We can
 197 also compare M3 model to the two previous ones (M1 and M2) and in this case the test
 198 follows a χ^2 distribution with 2 or 1 df respectively.

199 Note that according to the results of our genome-wide linkage study, we were looking for a
 200 risk allele transmitted by the father to affected offspring. Thus, we decided to present the
 201 imprinting parameter I_p , the probability that the father has transmitted the risk allele to the
 202 affected child rather than the mother ($F > M$) that corresponds to the inverse of I_m : $I_p = 1/I_m$.

203 We extended the PO-LRT originally proposed for trios (affected offspring and both parents)
 204 to two and three sibs to deal with our data. Our implementation in R is available on request.

205 We checked for transmission-ratio distortion or departure from Mendelian transmission
 206 proportion in unaffected families using PO-LRT, and we found no evidence for parent-of-
 207 origin effect for any tested SNPs.

208

209 **Homogeneity test**

210 We investigated whether the association between the replicated SNP and asthma plus allergic
211 rhinitis comorbidity was specific to this combined phenotype or common to asthma and
212 allergic rhinitis, by testing for heterogeneity of association according to disease status defined
213 by the presence of the two diseases “asthma-plus-rhinitis” *versus* the presence of one disease
214 “asthma only or AR only”.

215

216 **Identification of polymorphism explaining the linkage signal**

217 We investigated to what extent the SNP reaching the significance level of association by PO-
218 LRT pooled analysis of discovery and replication studies accounted for the linkage signal. For
219 that purpose, we tested for linkage of asthma-plus-rhinitis to the microsatellite markers used
220 in the linkage scan using the MLBI method stratified according to the genotypes at the
221 replicated SNP. The empirical significance of this linkage signal was assessed by a
222 resampling without replacement procedure (10,000 random selections of a given number of
223 families).

224

225 ***Step 3: Association analysis with DNA methylation levels at CpG sites taking into account*** 226 ***parent-of-origin effect***

227 **SNP-DNA_m levels at CpG sites association test while accounting for parent-of-origin** 228 **effect**

229 To test for association of both paternally and maternally inherited AAR-associated SNPs and
230 DNA_m levels at CpG sites in SLSJ families, we applied a linear regression model to
231 offspring. We used the genotypes and phenotypes from the offspring as well as the genotypes
232 of their parents. For each individual of family j (father, mother and offspring i), a genotype

score (g_{ip} , g_{im} and g_{ij}) corresponding to the number of copies of the allele of interest (0, 1, 2) at a given SNP is derived from the observed genotype. We decomposed g_{ij} , the genotype score of the offspring i of family j , into between-family (b_i) and within-family (w_{ij}) components.¹⁴ The between-family component (b_i) is the expected genotype of the offspring i and corresponds to the probability that this offspring receives the allele of interest conditionally on his/her parent genotypes: $b_i = \frac{g_{ip} + g_{im}}{2}$. The within-family component (w_{ij}) is equal to the difference between g_{ij} and b_i . In the context of parent-of-origin effect model, b_i and w_{ij} are decomposed in two parameters according to the maternally ($b_{\text{Mat}i}$ and $w_{\text{Mat}ij}$) and paternally ($b_{\text{Pat}i}$ and $w_{\text{Pat}ij}$) origin of the allele of interest.

The model testing the association of DNAm levels at CpG sites and both paternally and maternally inherited allele at a given SNP can be written as followed:

$$\mu_{ij} = \mu + \beta_{b\text{Mat}} b_{\text{Mat}i} + \beta_{w\text{Mat}} w_{\text{Mat}ij} + \beta_{b\text{Pat}} b_{\text{Pat}i} + \beta_{w\text{Pat}} w_{\text{Pat}ij}$$

In this model, the variation in the quantitative trait (DNAm levels) is related to $w_{\text{Mat}ij}$ and $w_{\text{Pat}ij}$. We implemented this approach in STATA®. All analyses were adjusted for asthma-plus-rhinitis, with a robust sandwich estimation of the variance to take into account familial dependencies.¹⁵ We also included in the model the white cell type proportions (eosinophils, basophils, neutrophils, monocytes and lymphocytes).

We tested 1) for association of paternally ($\beta_{w\text{Pat}} \neq 0$) and maternally ($\beta_{w\text{Mat}} \neq 0$) inherited alleles of AAR-associated SNPs and DNAm levels and 2) for significant difference between the paternal and maternal associations ($\beta_{w\text{Pat}} \neq \beta_{w\text{Mat}}$) when one parameter in test 1 was significant. Statistical significance of the regression coefficients $\beta_{w\text{Pat}}$ and $\beta_{w\text{Mat}}$, and of their difference was assessed using bootstrap (N=10,000 iterations).

Causal inference test (CIT)

We applied the causal inference test to establish the epigenetic mediation of parent-of-origin effect of SNP-asthma-plus-rhinitis association. The CIT performs statistical tests for four conditions, all of which must be met to conclude that methylation mediation is occurring. This approach is based on a series of conditional association analyses^{16, 17} for the evaluation of the following effects: 1) SNP on asthma-plus-rhinitis risk while accounting for parent-of-origin effect, 2) SNP on DNA methylation levels adjusted for asthma-plus-rhinitis while accounting for parent-of-origin effect, 3) DNA methylation levels on asthma-plus-rhinitis risk adjusted on SNP allelic origin, and 4) SNP allelic origin on asthma-plus-rhinitis risk adjusted for methylation. If DNA methylation is a mediator of the relationship between SNP and asthma-plus-rhinitis, the association between SNP and asthma-plus-rhinitis is expected to drop or even to disappear when adjusting for DNAm levels in the regression model while the association between DNAm levels and disease remains significant. All analyses were performed while accounting for POO effect.

eQTL analysis and functional annotations

We queried whether significant SNPs (or their proxies) were expression quantitative trait loci (eQTLs). We used existing eQTL databases in multiple tissues¹⁸⁻²¹ for populations of European ancestry.

Functional annotations of significant SNPs (or their proxies) and CpG sites were obtained using UCSC (<http://genome.ucsc.edu/>).²²

RESULTS

The association of rs10009104 was found to be stronger for the comorbidity AAR than the single morbidities “asthma alone” or “AR alone” (P for test of homogeneity equal to 0.03, see table E8).

We investigated whether the detected association with rs10009104, lying at 1.6 Mb from the linkage peak, accounted for the linkage in the 4q35 region. We used rs10009104 that has shown the strongest association signal after replication in the pool EGEA and SLSJ analysis to identify 66 families from the original linkage in which at least one copy of the disease-associated allele (G nucleotide) had been transmitted to affected offspring. Nonparametric linkage analysis (MLB-I) in the 66 associated families yielded significant evidence for linkage (LOD-MLBI= 2.80), demonstrating that the linkage signal was attributable to these families. The significance of this linkage signal was assessed by performing MLB-I linkage analysis in 10,000 random selections of 66 families. An empirical significance level was calculated as the proportion of replicates for which the maximum LOD-MLBI was equal or greater than that obtained in the actual analysis. The probability of obtaining a LOD-MLBI of 2.80 by chance in a random selection of 66 families was estimated by 10,000 simulations to be 0.03.

FIGURE LEGENDS

Figure E1. Study design and work flow diagram for the positional cloning study of 4q35 region for the asthma and allergic rhinitis comorbidity (AAR) while accounting for parent-of-origin effect (POO).

Figure E2. Genome-wide linkage scan conducted in European families for asthma and allergic rhinitis with or without taking into account parent-of-origin effect (LOD-MLBI or LOD-MLB curves respectively). For each phenotype, genetic distance is plotted on the x-axis and LOD score on the y-axis.

Figure E3. Linkage Disequilibrium (LD) plot of *MTNR1A* region using Hapmap2 CEU reference sample. Values and colours reflect r^2 and D' respectively.

Figure E4: Functional annotation in *MTNR1A* gene region (chr4: 187,475,215–187,491,897; hg19 assembly) using UCSC Genome Browser.

Selected tracks include histone modifications marks (HMM), DNaseI hypersensitivity sites, transcription factor binding sites and chromatin state segmentation. DNaseI hypersensitivity sites and transcription factor binding sites are identified by gray boxes. Chromatin states are grouped and colored to highlight predicted functional elements (light red: weak promoter, purple: inactive/poised promoter, yellow: weak/poised enhancer, blue: insulator, gray: polycomb repressed). Selected cell lines are H1 human embryonic stem cells (H1-hESC), lymphoblastoid cell lines (GM12878) and normal human lung fibroblasts (NHLF). For clarifications, see the UCSC genome database website (<http://genome.ucsc.edu/>). Positions of the cg02303933 CpG site, lying in *MTNR1A* intron 1, and the rs10009104 SNP (located at a 15kb distance), are indicated by blue arrows.

320 Table E1. Phenotype definitions and genotyping information of linkage and association samples

Study	Country	Phenotype definition		Genome-wide linkage screen	Association
		Asthma	Allergic Rhinitis	Genotyping platform No microsatellites (autosomal) after QC	Genotyping platform
EGEA	France	Being a proband or having answered positively to at least one of the two questions (1/ Have you ever had attacks of breathlessness at rest with wheezing?, 2/ Have you ever had an asthma attack?); plus the presence of bronchial-hyper responsiveness or hospitalization for asthma in life or asthma therapy for linkage analysis	At least one of the following symptoms: sneezing or runny nose to hay/flowers or animals or dust	Marker Set MD 10 378 microsatellites	Illumina 610K in parents & offspring
MRCA	UK	“Has your doctor ever told you that you have asthma?” and probands had a Step 3 or worse according to the British Thoracic Society guidelines (high-dose inhaled steroids, or low-dose inhaled steroids and a long-acting β -agonist) ⁴	Positive answer to the question “Has your child ever had a problem with sneezing or a runny or blocked nose, when he/she did not have a cold or the ‘flu?’”	Marker Set MD 10 373 microsatellites	Illumina 317K in offspring only
Verona	Italy	Physician diagnosis according to the definition of the American Thoracic Society ³ . All patients defined as asthmatics had recurrent symptoms, clinical improvement from inhaled steroids, and reversibility of the bronchial obstruction after administration of b2-agonists with an increase in FEV1 greater than 12.	Presence of at least one of the following symptoms: sneezing or runny nose to hay/flower or animals or dust (questionnaire)	Marker Set MD 10 377 microsatellites	-
SLSJ	Canada	Probands: two of the following criteria: 1) a minimum of three clinic visits for acute asthma within one year; or 2) ≥ 2 asthma-related hospital admissions within one year; or 3) steroid dependency, as defined by either six months of oral, or one year of inhaled corticosteroid use. Relatives: 1) history of asthma (validated by a physician), or (2) asthma-related symptoms and bronchial-hyper responsiveness	At least one of the following symptoms: sneezing or runny nose to hay/flowers or animals or dust	-	Illumina 610K in parents & offspring Infinium HumanMethylation450 BeadChip in a subset of individuals

321

Table E2. Phenotypic characteristics of 1,472 genotyped siblings in 615 families of European ancestry included in the genome-wide linkage study

	All offspring N=1,472	EGEA N=726	MRCA N=359	Verona N=387
Sex, n (% males)	798 (54.2)	386 (53.2)	194 (54.0)	218 (56.3)
Age, mean (SD)	14.5 (9.2)	16.1 (7.7)	11.5 (4.8)	14.4 (13.2)
Asthma, n (%)	810 (55.0)	380 (52.3)	239 (66.6)	191 (49.3)
Atopy (SPT+), n (%)	886 (60.2)	478 (65.8)	143 (39.8)	265(68.5)
Allergic Rhinitis, n (%)	725 (49.2)	320 (44.1)	194 (54.0)	211 (54.5)
Asthma + Allergic Rhinitis, n (%)	539 (36.6)	230 (31.7)	159 (44.3)	150 (38.7)

Table E3. Distribution of families according to the number of genotyped sibs available for linkage analyses of asthma, allergic rhinitis, and “asthma + allergic rhinitis” comorbidity

Phenotypes	No. of sibs			Total no. of Families / Offspring
	2	3	4	
Asthma	204	25	2	231 / 491
Allergic Rhinitis	184	18	6	208 / 446
Asthma + Allergic Rhinitis	103	5	1	109 / 225

Table E4. Microsatellites markers linked to asthma, allergic rhinitis, or asthma and allergic rhinitis comorbidity (AAR) in 615 European families while accounting for parent-of-origin effect

Chr.*	Marker	Positions (Mb) (build 37.1)	Phenotype	Linkage analysis accounting for parent-of-origin (POO) effect			Standard linkage analysis		Best model
				LOD-MLBI	P-value LI / LMI or LPI [†]	Linkage parameters [‡] α_p and α_m under LI model	LOD-MLB	P-value	
1	D1S2797	46.93	Asthma	2.39	0.001 / -	$\alpha_p = 0.70, \alpha_m = 0.69$	2.38	4.6×10^{-4}	No POO effect
			AAR	1.90	0.005 / -	$\alpha_p = 0.74, \alpha_m = 0.70$	1.83	0.002	No POO effect
1	D1S2890	57.87	Asthma	1.93	0.004 / -	$\alpha_p = 0.69, \alpha_m = 0.69$	1.93	0.001	No POO effect
			AAR	2.63	8.4×10^{-4} / -	$\alpha_p = 0.75, \alpha_m = 0.74$	2.62	2.6×10^{-4}	No POO effect
1	D1S230	60.71	AAR	2.67	7.7×10^{-4} / -	$\alpha_p = 0.75, \alpha_m = 0.74$	2.66	2.3×10^{-4}	No POO effect
2	D2S117	195.61	AAR	1.94	0.004 / 0.001	$\alpha_p = 0.77, \alpha_m = 0.50$	0.66	0.04	Paternal linkage
4	D4S426	189.10	AAR	3.14	2.5×10^{-4} / 7.2×10^{-5}	$\alpha_p = 0.81, \alpha_m = 0.50$	1.44	0.005	Paternal linkage
17	D17S944	61.43	Asthma	2.13	0.003 / 8.7×10^{-4}	$\alpha_p = 0.53, \alpha_m = 0.73$	1.03	0.01	Maternal linkage

Results shown are those passing $P \leq 0.005$ using multipoint LOD-MLBI scores

*Chr.: chromosome

[†]The MLB-LI test follows a mixture of $\frac{1}{4} \chi^2_{0df} + \frac{1}{2} \chi^2_{1df} + \frac{1}{4} \chi^2_{2df}$ when both α_p and α_m parameters (paternal and maternal) are estimated (test of linkage taking into account POO effect) and a mixture of $\frac{1}{2} \chi^2_{0df} + \frac{1}{2} \chi^2_{1df}$ when a single parameter α_m (or α_p) is estimated in the context of maternal imprinting (LMI) with α_m fixed to be equal to 0.5 (or in the context of paternal imprinting (LPI) with α_p fixed equal to 0.5).

[‡]Linkage parameters α_p and α_m were computed under the model of linkage taking into account POO effect.

Table E5. Characteristics of individuals belonging to 162 EGEA families and 154 SLSJ families included in SNP association analysis with asthma and allergic rhinitis comorbidity while accounting for parent-of-origin effect

Characteristics	EGEA				SLSJ			
	Father (N=162)	Mother (N=162)	Affected Sibs		Father (N=154)	Mother (N=154)	Affected Sibs	
			< 16 yrs (N=120)	≥ 16 yrs (N=87)			< 16 yrs (N=77)	≥ 16 yrs (N=101)
Age in years, mean ± SD	46.9 (7.9)	45.1 (7.5)	11.5 (2.3)	23.2 (5.3)	47 (12.7)	48 (11.9)	10.9 (3.1)	27.3 (9.1)
Sex, men (%)	-	-	78 (65)	39 (44.8)	-	-	44 (57.1)	35 (34.6)
Asthma, n (%)	36 (22.2)	34 (21.0)	-	-	42 (27.3)	60 (39.0)	-	-
Asthma Age of onset, mean ± SD	16.5 (14.7)	20.2 (17.6)	4.4 (3.2)	10.8 (6.6)	21.4 (20.0)	27.1 (17.7)	3.6 (3.2)	11.9 (10.8)
Asthma Age of onset < 4yrs, n (%)	11 (6.8)	8 (4.9)	68 (56.7)	15 (17.2)	9 (21.9)	4 (6.8)	50 (71.4)	32 (31.7)
Allergic Rhinitis, n (%)	66 (40.7)	70 (43.2)	-	-	51 (33.1)	63 (40.9)	-	-
Asthma+Allergic rhinitis, n (%)	25 (15.4)	26 (16)	-	-	29 (18.8)	37 (24.0)	-	-
SPT ⁺ , n (%)	83 (51.2)	63 (38.9)	103 (85.8)	77 (88.0)	79 (51.3)	78 (50.6)	62 (80.5)	90 (89.1)
ETS [†] exposure in early life, n (%)	114 (70.4)	114 (70.4)	47 (39.2)	54 (62.1)	-	-	33 (47.1)	53 (64.6)
BHR [‡] PD20 ≤ 4 mg methacholine, n (%)	18 (17.1)	52 (44.8)	60 (92.3)	39 (82.9)	24 (19.2)	39 (31.2)	36 (72.0)	71 (77.2)
%FEV ₁ [#] , mean ± SD	101.2 (18.4)	108.7 (16.8)	92.7 (14.6)	97.9 (13.6)	91.7 (18.7)	96.9 (19.9)	88.5 (13.5)	93.1 (16.2)
Log10 EOS ^{**} (cells/mm ³), mean ± SD	2.2 (0.2)	2.1 (0.3)	2.7 (0.3)	2.4 (0.3)	2.2 (4.2)	1.8 (4.0)	4.1 (4.3)	3.4 (3.9)
Log10 IgE ^{††} (IU/ml), mean ± SD	1.8 (0.7)	1.6 (0.7)	2.6 (0.5)	2.4 (0.5)	2.1 (0.7)	1.9 (0.6)	2.4 (0.6)	2.5 (0.7)

These 316 families included at least one genotyped sib suffering of asthma and allergic rhinitis, and two genotyped parents.

* SPT: positive Skin Prick Test response to at least one of 11 or 24 aero-allergens in EGEA and SLSJ respectively

† ETS: Environmental Tobacco Smoke exposure in early life

‡ BHR: Bronchial Hyperresponsiveness

% FEV₁: percentage of predicted forced expiratory volume in 1 second based on age, height and gender

** EOS: blood eosinophil counts

349 ^{††} IgE: total serum Immunoglobulin E levels

350 Table E6. SNPs in 4q35 region showing association with asthma and allergic rhinitis comorbidity in 162 EGEA families under a parent-of-origin
351 effect model

352

Marker	Position (Mb)*	Nearest gene (kb distance)	Baseline/ Effect alleles	Effect allele Frequency	MCPPAT [†] P-value	PO-LRT [†] P-value	PO-LRT Best-fitting Model [‡]
rs7667992	186.663	<i>SORBS2</i>	G/T	0.62	0.001	0.004	Maternal
rs2375801	187.472	<i>MTNR1A</i>	C/T	0.68	0.001	0.007	Imprinting
rs10009104	187.491	<i>MTNR1A</i> (14)	A/G	0.72	0.0006	0.0002	Imprinting
rs6812381	188.493	<i>LOC100506272</i>	A/G	0.58	0.001	0.002	Imprinting
rs6826655 [#]	188.608	<i>LOC105377603</i>	T/G	0.56	1.3x10^{-5**}	3.1x10^{-6**}	Imprinting
rs7687115 [#]	188.609	<i>LOC105377603</i>	G/A	0.56	1.3x10^{-5**}	2.7x10^{-6**}	Imprinting

353 The six SNPs showed are those passing $P \leq 0.001$ with MCPPAT[†] method in EGEA discovery families that are taken forward validation with
354 PO-LRT[†] method

355 *SNP positions from GRCh37.1 assembly

356 [†]MCPPAT refers to Monte-Carlo Pedigree Parental Asymmetry Test and PO-LRT refers to Parent-of-Origin Likelihood Ratio Test

357 [‡]Imprinting refers to a parent-of-origin best-fitting model; Maternal refers to a maternal genotype effect best-fitting model

358 [#]SNPs in complete LD ($D'=1$ and $r^2=1$)

359 ^{**}indicate P-values significant after correction for multiple testing ($P \leq 7.2 \times 10^{-5}$)

360 Table E7. Characteristics of 40 SLSJ complete nuclear families (159 subjects) used to test association between paternally inherited rs10009104-G
 361 allele and DNA methylation levels at CpG sites measured in white blood cells

362

Characteristics	Father (N=40)	Mother (N=40)	Sibs (N=79)
Age in years, mean $\pm$ SD	45.0 (8.4)	42.8 (7.6)	15.6 (8.5)
Sex, men (%)	-	-	42 (53.2)
Asthma, n (%)	14 (35.0)	16 (40.0)	41 (51.9)
Allergic Rhinitis, n (%)	19 (50.0)	22 (55.0)	41 (52.6)
Asthma+Allergic rhinitis, n (%)	8 (20.0)	11 (27.5)	26 (32.9)
No Asthma & No allergic rhinitis, n (%)	14 (35.0)	13 (32.5)	23 (29.1)
SPT ⁺ , n (%)	29 (72.5)	21 (53.9)	58 (73.4)
ETS [†] exposure in early life, n (%)	-	-	28 (35.4)

363

364 * SPT+: positive Skin Prick Test response to at least one aero-allergen

365 [†] ETS: Environmental Tobacco Smoke exposure in early life

366 Table E8. PO-LRT* association results and homogeneity tests for paternally inherited rs10009104-G allele[†] with asthma only and allergic rhinitis
 367 (AR) only in pooled EGEA and SLSJ families
 368

Phenotype	Pooled EGEA and SLSJ families		
	N _{fam}	I _p [‡] (95% CI)	P-value
AAR [#]	316	1.96 [1.43–2.70]	1.1x10^{-5†}
Asthma only	196	1.39 [0.95–2.04]	0.08
AR only	115	2.08 [1.10–3.85]	0.03
Asthma only or AR only	290	1.52 [1.10–2.08]	0.01
Homogeneity test		Chi2	P-value
AAR versus Asthma only or AR only		4.83	0.03

369

370 *PO-LRT refers to Parent-of-Origin Likelihood Ratio Test

371 [†]Significant SNP associated with AAR in the EGEA and SLSJ pooled analysis after correction for multiple testing ($P \leq 7.2 \times 10^{-5}$)

372 [‡]I_p refers to the PO-LRT imprinting parameter reflecting a differential transmission of alleles to affected child from fathers *versus* mothers.

373 [#]AAR refers to asthma and allergic rhinitis comorbidity.

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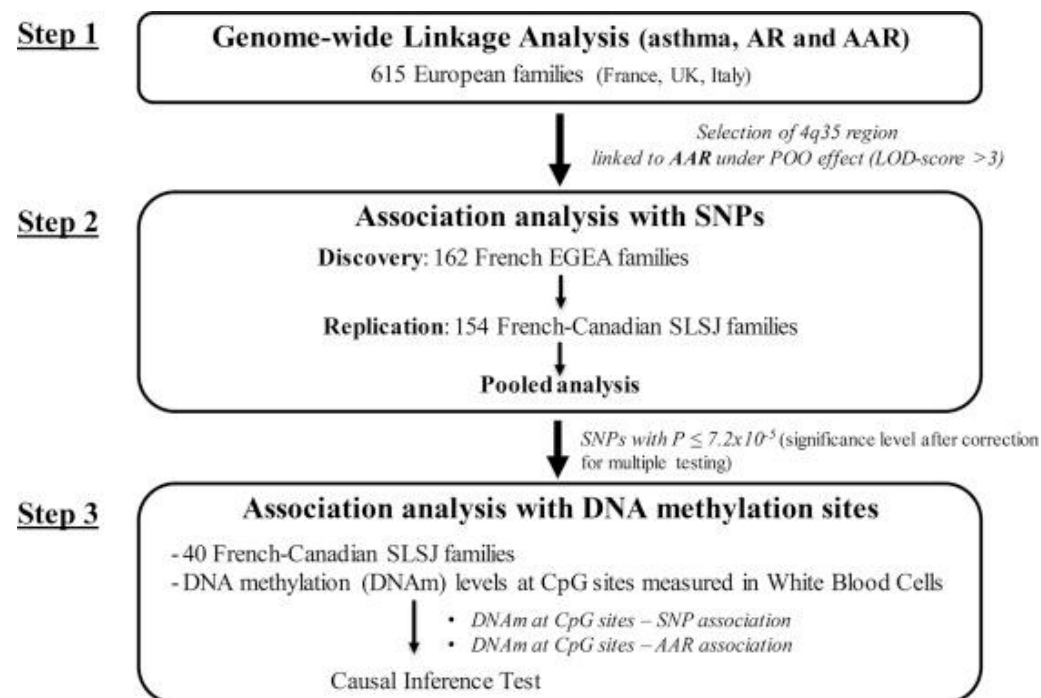


Fig E1.

Chloé Sarnowski, Catherine Laprise, Giovanni Malerba, Miriam F. Moffatt, Marie-Hélène Dizier, Andréanne Morin, Quentin B. Vincent, Klaus Rohde, Jorge Esparza-Gordillo, Patricia Margaritte-Jeannin, Liming Liang, Young-Ae Lee, Jean Bousquet, Valérie Siroux, Pier Franco Pignatti, William O. Cookson, Mark Lathrop, Tomi Pastinen, Florence Demeais, Emmanuelle Bouzigon, **DNA methylation within melatonin receptor 1A (MTNR1A) mediates paternally transmitted genetic variant effect on asthma plus rhinitis**

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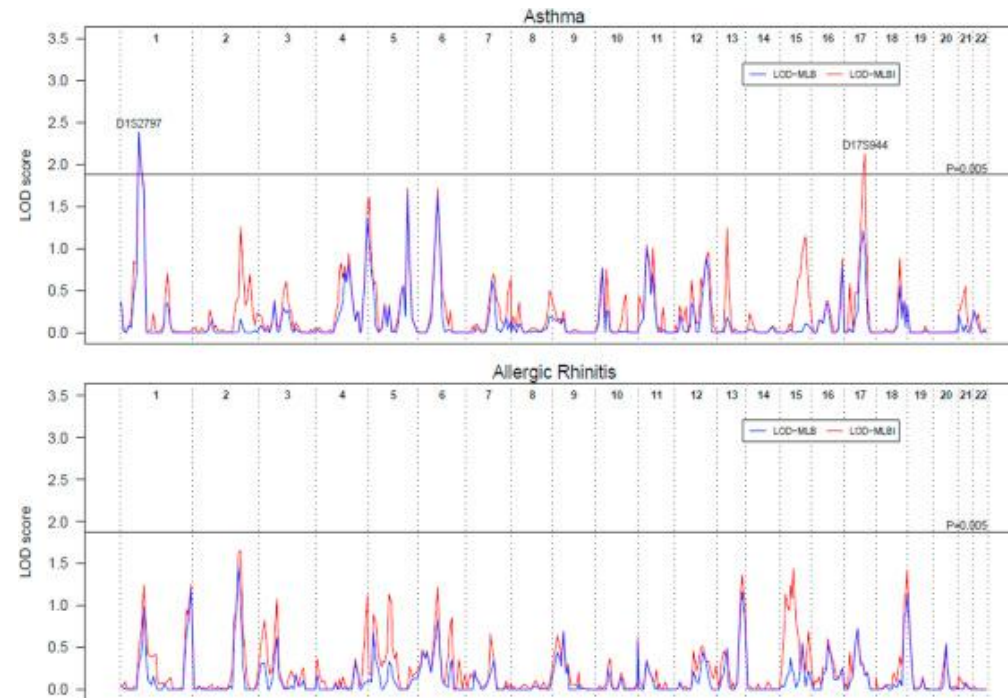


Fig E2.

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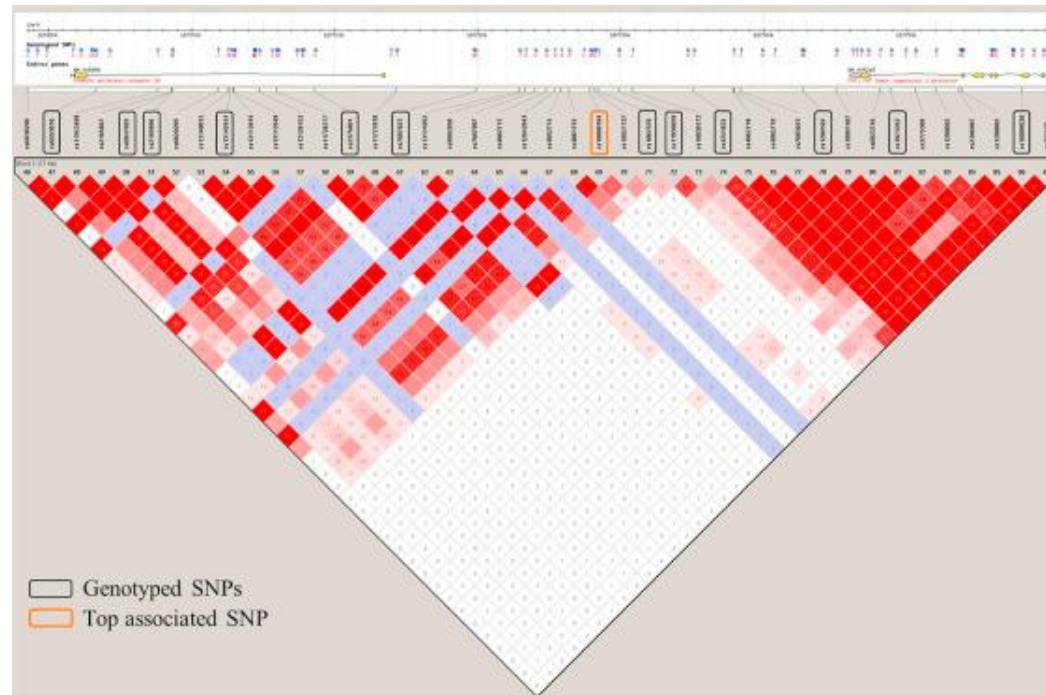


Fig E3.

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Fig E4.

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