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DQB1 Locus Alone Explains Most of the Risk and Protection in Narcolepsy with Cataplexy in Europe

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Study Objective: Prior research has identified five common genetic variants associated with narcolepsy with cataplexy in Caucasian patients. To replicate and/or extend these findings, we have tested *HLA-DQB1*, the previously identified 5 variants, and 10 other potential variants in a large European sample of narcolepsy with cataplexy subjects.

Design: Retrospective case-control study.

Setting: A recent study showed that over 76% of significant genome-wide association variants lie within DNase I hypersensitive sites (DHSs). From our previous GWAS, we identified 30 single nucleotide polymorphisms (SNPs) with $P < 10^{-4}$ mapping to DHSs. Ten SNPs tagging these sites, *HLA-DQB1,* and all previously reported SNPs significantly associated with narcolepsy were tested for replication.

Patients and Participants: For GWAS, 1,261 narcolepsy patients and 1,422 HLA-*DQB1*06:02*-matched controls were included. For HLA study, 1,218 patients and 3,541 controls were included.

Measurements and Results: None of the top variants within DHSs were replicated. Out of the five previously reported SNPs, only *rs2858884* within the HLA region ($P < 2x10^{-9}$) and *rs1154155* within the *TRA* locus ($P < 2x10^{-8}$) replicated. *DQB1* typing confirmed that *DQB1*06:02* confers an extraordinary risk (odds ratio 251). Four protective alleles (*DQB1*06:03,* odds ratio 0.17, *DQB1*05:01*, odds ratio 0.56, *DQB1*06:09* odds ratio 0.21, *DQB1*02* odds ratio 0.76) were also identified.

Conclusion: An overwhelming portion of genetic risk for narcolepsy with cataplexy is found at *DQB1* locus. Since *DQB1*06:02* positive subjects are at 251-fold increase in risk for narcolepsy, and all recent cases of narcolepsy after H1N1 vaccination are positive for this allele, *DQB1* genotyping may be relevant to public health policy.

Keywords: Autoimmunity, GWAS, H1N1 vaccination, genetics

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INTRODUCTION

Narcolepsy with cataplexy (hereinafter "narcolepsy") is a disabling and chronic sleep disorder characterized by irresistible sleep episodes and cataplexy (muscle atonia induced by strong emotions).[1,2] The genetics of narcolepsy is complex but includes the strongest association ever reported with a single HLA-*DQB1* allele (*DQB1*06:02*).[3] Because of the discovery of the HLA association, narcolepsy is believed to be an autoimmune disease. The discovery of hypocretin (orexin) deficiency in narcolepsy with cataplexy[4,5] raised the possibility that hypothalamic hypocretin-producing neurons might be the target of an autoimmune attack, motivating the search for potential genetic mechanisms.

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The first genome-wide association study (GWAS) in narcolepsy with European ancestry found a strong association with the T-cell receptor alpha locus (*TRA*), independent of the HLA.[6] Our previous GWAS found a significant protective HLA variant.[7] Another GWAS found evidence for association with *P2RY11*, although with a modest increase in risk (OR = 1.28).[8] More recently, the autoimmune hypothesis was strengthened by finding circulating auto-antibodies against Tribbles homolog 2 (TRIB2), especially close to the disease onset,[9-11] a surge of newly diagnosed cases of narcolepsy in young people following the 2009 H1N1 vaccination,[12-15] and the discovery of associations with common variants in *CTSH* and *TNFSF4*, two other immune-related genes.[16]

Narcolepsy with cataplexy being a rare disease (prevalence = 0.02%-0.05%), studies including larger populations to increase the statistical power to detect small effect size variants are unlikely to become available in the near future. We therefore performed a modified GWAS replication study in 1,261 European narcolepsy patients and 1,422 HLA-*DQB1*06:02*-matched controls.

METHODS

Patients and Controls

Informed consent according to each country's ethics institution was obtained from all subjects, and the experimental protocols were approved by the Lausanne (CHUV) ethical committee.

Samples of patients and controls used for discovery GWAS were described previously[7] and included 562 HLA-*DQB1*06:02* positive narcolepsy with cataplexy patients and 702 origin-matched and HLA-*DQB1*06:02* positive controls of European ancestry. Replication samples included 699 HLA-*DQB1*06:02* positive narcolepsy with cataplexy patients and 720 origin-matched and HLA-*DQB1*06:02* positive healthy controls of European ancestry. All patients were diagnosed according to the International Classification of Sleep Disorders.[17] The origin of patients was: NL: Dutch, DE: German + Austrian, PL: Polish + Slovak, FR: French, SP: Spanish, CH: Swiss, and IT: Italian. Controls were recruited from Dutch, German, Polish, French, Spanish, Swiss, and Italian general populations.

For HLA analysis and estimation of risk for each *DQB1* allele, all centers were required to include any documented *DQB1*06:02* negative patient available. Only *DQB1*06:02* negative patients with low (< 110 pg/mL) or undetectable (< 40 pg/mL) cerebrospinal fluid (CSF) hypocretin-1 were included in the final analysis (N = 20). Hypocretin-1 levels were also available in 294 *DQB1*06:02* positive patients, among whom 252 had undetectable and 31 low levels.[2] In total, *DQB1* typing was available in 1,218 patients and 3,541 country-matched controls (see Table 2).

SNP and HLA Genotyping

SNP genotyping was performed by KBiosciences (UK) using fluorescence-based competitive allele-specific PCR. High-resolution HLA-*DQB1* genotyping was performed either by reverse sequence-specific oligonucleotide (SSO) or direct sequencing methods by each country's accredited HLA laboratories, according to standard protocols.

Exome Sequencing

Genomic DNA was fragmented with a Covaris S2 sonicator (Covaris, Inc.) to obtain DNA fragments with a homogenous size distribution having a maximum at 180-200 base pairs. Preparation of the exome enriched, barcoded sequencing libraries was performed using Agilent SureSelect Human All Exon v3 kit. Libraries were sequenced on Illumina HiSeq 2000, generating 100 bp paired-end reads. Purity filtered reads were aligned to the human genome (assembly hg19).

Data Analysis

SNP analysis was performed by logistic regression. All other individual frequency comparisons for HLA alleles were performed by Mantel-Haenszel test and meta-analyzed by the same procedure, using the meta.MH function in R. In order to account for the number of *DQB1* alleles tested, we applied Bonferroni correction (n = 14); thus nominal P values below $\alpha = 0.0036$ are declared to be significant. To determine which haplotypes confer risk (provide protection) independently of each other, we adapted a step-wise procedure.[18] In each iteration, carriers of the most significantly associated haplotype are removed and the association test (for each remaining haplotype) is repeated for the remaining individuals.

RESULTS

Given that a large number of significant GWAS variants lie within gene regulatory rather than protein coding regions, a recent study found that over 76% of GWAS significant hits map within regulatory regions defined by DNase I hypersensitive sites (DHSs).[19] This suggests that variants within these regulatory regions have a substantially higher probability of showing significant associations. To verify if additional variants associated with narcolepsy can be found, we have mapped all suggestively significant SNPs ($P < 10^{-4}$) from our original GWAS[7] to DHSs and attempted replication of these top hits in an independent European narcolepsy population: 503 SNPs were found with a P value $< 10^{-4}$. Mapping these SNPs to DHSs indicated that only 41 (8.15%) were within these regulatory sites. Among these, 10 mapped within the HLA class II region and 3 within the *TRA* locus, for which significant associations with narcolepsy were already reported. The remaining 28 SNPs could be tagged by 10 SNPs. The two previous significant SNPs, *rs2858884* within the HLA region and *rs1154155* within the *TRA* locus, were also included. Among other significant hits reported recently, *rs7553711* within *TNFSF4* and *rs2305795* within *P2RY11* were assessed in our original GWAS and selected for replication. Because the genotypes for the two others (*rs34593439* and *rs34843303* located in intron 1 of *CTSH*) were not available in our discovery analysis, we selected *rs12148472* ($r^2 = 0.93$ with *rs34593439* and $r^2 = 0.95$ with *rs34843303*) as a proxy for this association. Replication samples included 699 narcolepsy patients and 720 HLA-matched controls.

SNPs *rs2858884* (replication $P = 6.40 \times 10^{-4}$, meta-analysis $P = 1.03 \times 10^{-9}$) within the HLA region and *rs1154155* (replication $P = 2.12 \times 10^{-5}$, meta-analysis $P = 1.56 \times 10^{-8}$) within the *TRA* locus were successfully replicated as expected (Table 1). None of the other SNPs withstood the Bonferroni correction. Nevertheless, *rs2305795* within *P2RY11* had a nominal replication $P = 4.70 \times 10^{-3}$, *rs12148472* within *CTSH* P = 0.017, and *rs7553711* within *TNFSF4* P = 0.022. The last two SNPs were recently reported in an international study in which 584 of our patients were included.[16] Re-analyzing our data without these 584 overlapping patients showed a very similar trend (Table S1).

SNP *rs2858884,* as in our first GWAS, confers protection against narcolepsy (OR = 0.56, 95%CI = 0.47-0.67), even if cases and controls were matched for at least one *DQB1*06:02* allele, strongly suggesting that some *trans DQB1* alleles are protective. HLA alleles frequency distribution shows slight but significant differences among European countries. To find which *trans DQB1* alleles are associated with protection against narcolepsy, we compared patients to healthy controls of the same country of origin. Although *DQB1*06:02* is the most important susceptibility marker in narcolepsy, this association is believed to be neither necessary nor sufficient, because up to 30% of the general population may carry this allele and 5% to 15% of narcolepsy patients may not carry it. We first reassessed the exact number of *DQB1*06:02* negative patients in each country. Table 2 shows the frequency of *DQB1*06:02* positive and negative cases

Table 1—Replication results for SNPs mapping within the DHSs and SNPs already reported as significantly associated with narcolepsy[6-8,16]

rs#	Allele	Chr	Pos	AF_C1:AF_N1	OR1[95%CI]	p1	n1	AF_C2:AF_N2	OR2[95%CI]	p2	n2	OR-Meta[95%CI]	pMeta	nMeta
rs2858884	C	6	32808061	0.219:0.100	0.48[0.37-0.62]	4.65E-08	562:702	0.161:0.114	0.69[0.55-0.85]	6.40E-04	656:697	0.59[0.50-0.62]	1.03E-09	1218:1399
rs1154155	T	14	22072524	0.859:0.801	0.65[0.52-0.81]	1.92E-04	562:702	0.835:0.769	0.66[0.55-0.80]	2.12E-05	665:700	0.66[0.57-0.81]	1.56E-08	1227:1402
rs9291642	T	4	9616373	0.824:0.887	1.77[1.37-2.28]	1.03E-05	562:702	0.858:0.869	1.10[0.88-1.38]	3.92E-01	662:706	1.36[1.15-2.28]	3.63E-04	1224:1408
rs2305795	G	19	10087052	0.480:0.458	0.82[0.63-1.06]	1.31E-01	562:702	0.446:0.390	0.81[0.70-0.94]	4.70E-03	661:701	0.81[0.71-1.06]	1.35E-03	1223:1403
rs4810966	G	20	35703186	0.591:0.502	0.68[0.56-0.81]	2.44E-05	562:702	0.551:0.543	0.97[0.83-1.13]	6.81E-01	665:701	0.84[0.74-0.81]	2.52E-03	1227:1403
rs11085891	G	19	14395367	0.330:0.361	2.53[1.63-3.94]	3.85E-05	562:702	0.375:0.405	1.14[0.97-1.34]	9.94E-02	646:702	1.25[1.08-3.94]	3.22E-03	1208:1404
rs12148472	T	15	77018533	0.868:0.852	0.80[0.60-1.08]	1.41E-01	562:702	0.884:0.851	0.75[0.60-0.94]	1.17E-02	655:696	0.77[0.64-1.08]	3.77E-03	1217:1398
rs7553711	T	1	171398531	0.669:0.631	0.88[0.74-1.05]	1.57E-01	562:702	0.674:0.632	0.83[0.71-0.97]	2.24E-02	662:707	0.85[0.76-1.05]	8.19E-03	1224:1409
rs1338829	G	6	65198664	0.740:0.667	0.68[0.56-0.82]	7.49E-05	562:702	0.691:0.691	1.00[0.85-1.18]	9.87E-01	666:701	0.85[0.75-0.82]	1.08E-02	1228:1403
rs290183	G	11	85061310	0.764:0.828	1.55[1.25-1.92]	7.27E-05	562:702	0.804:0.800	0.98[0.81-1.18]	8.04E-01	664:710	1.19[1.03-1.92]	1.65E-02	1226:1412
rs473267	T	6	83936886	0.204:0.273	1.53[1.25-1.88]	4.57E-05	562:702	0.261:0.255	0.97[0.82-1.15]	7.51E-01	668:710	1.17[1.03-1.88]	1.88E-02	1230:1412
rs213038	C	1	21528791	0.153:0.215	1.63[1.30-2.06]	3.05E-05	562:702	0.188:0.180	0.95[0.78-1.15]	5.92E-01	660:704	1.19[1.02-2.06]	2.31E-02	1222:1406
rs1786783	T	18	33223187	0.787:0.852	1.61[1.28-2.03]	5.31E-05	562:702	0.846:0.834	0.91[0.74-1.12]	3.84E-01	664:707	1.17[1.00-2.03]	4.36E-02	1226:1409
rs2068662	G	12	94892386	0.088:0.042	0.39[0.26-0.58]	4.67E-06	562:702	0.062:0.080	1.30[0.97-1.73]	7.72E-02	669:707	0.87[0.68-0.58]	2.25E-01	1231:1409
rs7222250	T	17	37191995	0.593:0.559	0.40[0.26-0.62]	4.35E-05	562:702	0.555:0.562	1.03[0.88-1.20]	7.23E-01	662:706	0.93[0.80-0.62]	3.18E-01	1224:1408

rs#, SNP number; Allele, Effect allele; Chr, chromosome; Pos, position; AF_C1, allele frequency in controls in the discovery sample; AF_N1, allele frequency in narcoleptics in discovery samples; OR1[95%CI], odds ratio[95% confidence interval] discovery; p1, discovery P-value; n1, number of subjects in discovery (cases, controls); AF_C2, allele frequency in controls in the replication sample; AF_N2, allele frequency in narcoleptics in replication samples; OR2[95%CI], odds ratio[95% confidence interval] replication; p2, replication P-value; n2, number of subjects in replication (cases, controls); pMeta, meta-analysis P-value; nMeta, meta-analysis sample size (cases, controls).

and controls in each selected European country. As opposed to previous reports,[3,20] in our population, fewer than 2% of narcolepsy patients are *DQB1*06:02* negative, confirming the extraordinary association between *DQB1*06:02* and narcolepsy (OR = 251). Although more than 98% of narcolepsy patients are *DQB1*06:02* positive in all European countries, substantial differences exist between different European control populations, with Italy and Spain having many fewer *DQB1*06:02* subjects than the Netherlands or Germany. Based on our observations (Table 2), *DQB1*06:02* has a positive predictive value of 65.68% (95%CI = 63.45%-67.86%) and a negative predictive value of 99.32% (95%CI = 98.95%-99.58%) for narcolepsy.

Given that *DQB1* allele frequencies vary in different populations, we tested if DQB1 phenotypes of the general control populations in Europe indicate protection against narcolepsy. Several differences were found between European countries (e.g., *DQB1*03:01* is significantly protective in French and Italians but not in others), but meta-analysis in all European countries indicated that except for *DQB1*06:02*, all other DQB1 phenotypes had higher frequencies in controls, and all except *DQB1*03:04, 04:02, 05:02*, and *06:04*, were significantly higher (Table 3). This finding confirms that only *DQB1*06:02* (present in 20% of the general European population) confers risk, and the remaining 80% carry a protective *HLA-DQB1* haplotype against narcolepsy. Since 20% of the general European population carry *DQB1*06:02*, the strong susceptibility allele, while only a small fraction (0.02-0.05%) is affected, other protective factors might be involved.

Accordingly, comparison between *DQB1*06:02* positive patients and controls revealed that *rs2858884* confers protection against narcolepsy, suggesting that *DQB1*06:02* positive controls can be protected by a *trans DQB1* allele. We first confirmed that *rs2858884* is strongly associated with several *DQB1* alleles, independent of the case or control status (Table 3). In order to disentangle the effect of protective haplotypes independent of *DQB1*06:02*, we analyzed the data by restricting the comparisons to heterozygous

Table 2—DQB1*0602 association in European countries

Country (case, control)	Case-DQB1+ N (%)	Control-DQB1+ N (%)	OR	P
DE (232, 296)	227 (97.84)	72 (24.3.2)	141.24	9.71E-26
CH (66, 473)	65 (98.48)	102 (21.56)	236.42	7.01E-8
NL (323, 469)	318 (98.45)	114 (24.31)	198.05	3.62E-30
PL (63, 197)	63 (100)	44 (22.33)	438.08	2.65E-09
SP (127, 1,174)	126 (99.21)	170 (14.48)	744.14	5.25E-11
FR (341, 499)	335 (98.24)	94 (18.84)	240.56	1.18R-37
IT (66, 433)	64 (96.97)	30 (6.93)	429.87	3.21E-16
Mantel-Haenszel (meta-analysis)	1,198 (98.36)	626 (17.68)	251.12	1.04E-120

DE, Germany; CH, Switzerland; NL, Netherlands; PL, Poland; SP, Spain; FR, France; IT, Italy; OR, odds ratio.

*DQB1*06:02* cases and controls. We first performed a univariate analysis to estimate the ORs using a general *DQB1*06:02* heterozygous background. A step-wise procedure was then employed, whereby in each step, carriers of the most significantly associated haplotype were removed and the association test (for each haplotype) was repeated for the remaining individuals. This analysis informed us which haplotypes confer protection independently of each other. The most protective *trans* alleles were *DQB1*06:03, DQB1*05:01, DQB1*06:09,* and *DQB1*02* (Table 4). Notably, the extent of their protective effects (ORs) were highly variable across different countries (Cochran's heterogeneity P-values < 10^{-13}). To assess the effect of a second *DQB1*06:02* haplotype in trans, we have reanalyzed the data restricted to *DQB1*06:02* positive individuals (both heterozygous and homozygous). Several differences were found between European countries (e.g., *DQB1*02* is significantly protective in Dutch but not in others), but meta-analysis in all European countries indicated that homozygous *DQB1*06:02* doubled the risk of narcolepsy (Table S2).

Table 3—Mantel-Haenzel odds ratios and *rs2858884* association for DQB1 phenotypes between cases and controls in European countries

DQB1	DE 232-296	CH 66-473	NL 323-469	PL 63-197	SP 127-1,174	FR 341-499	IT 66-433	MH OR	MH P	rs2858884 OR	P
02	0.73	0.54	0.37[d]	0.53	0.58[a]	0.49[c]	0.49	0.51	**1.02E-18**	3.14	1.73E-51
03:01	0.69	0.5	0.88	0.71	0.65	0.59[b]	0.45[a]	0.65	**3.48E-09**	0.31	1.60E-23
03:02	0.23[c]	0.6	0.54[a]	1.41	0.34[a]	0.54	0.78	0.49	**1.52E-10**	0.33	2.89E-09
03:03	0.24[a]	0.34	0.48	0.47	0.39	0.26[a]	0.9	0.37	**2.06E-07**	0.2	8.43E-05
03:04	0.44	1.44	0.99	0.95	0.86	0.48	1.26	0.57	3.28E-01	0	NA
04:02	1.03	0.6	0.93	0.37	0.35	0.8	0.14	0.69	2.55E-02	0.58	2.26E-02
05:01	0.29[c]	0.35	0.24[d]	0.21	0.29[b]	0.33[c]	0.08	0.27	**2.71E-28**	1	9.85E-01
05:02	0.48	0.85	1.21	0.4	1	0.74	1.37	0.85	2.76E-01	0.47	2.16E-03
05:03	0.66	0.41	0.31	0.63	1.35	0.48	0.1	0.5	**2.01E-04**	0.85	4.69E-01
06:01	0.19	0.42	0.42	0.57	0.27	0.06	0.27	0.18	**1.03E-03**	0.76	7.19E-01
06:02	8.07[d]	8.92[d]	7.80[d]	7.45[d]	11.83[d]	9.66[d]	27.08[d]	9.33[d]	**6.23E-268**	0.68	4.85E-03
06:03	0.12[c]	0.3	0.12[c]	0.11	0.05[a]	0.16[c]	0.16	0.13	**2.52E-19**	3.97	5.86E-27
06:04	0.85	0.8	0.81	0.35	0.97	1.11	0.39	0.84	2.38E-01	0.92	6.07E-01
06:09	0.44	3.77	0.59	0.41	0.18	0.07	1.06	0.23	**5.45E-03**	5.59	3.36E-07

[a]P < 0.05; [b]P < 0.01; [c]P < 1E-3; [d]P < 1E-6, P values in bold survive Bonferroni correction. DE, Germany; CH, Switzerland; NL, Netherlands; PL, Poland; SP, Spain; FR, France; IT, Italy; MH, Mantel-Haenzel; OR, odds ratio. Numbers below country codes indicate number of patients-controls.

Table 4—Mantel-Haenzel odds ratios for *trans DQB1* alleles between *DQB1*06:02* heterozygous cases and controls in European countries

								Univariate		Stepwise	
DQB1	DE 176-500	CH 53-102	NL 265-486	PL 59-149	SP 122-170	FR 298-352	IT 56-130	MH OR	MH P	MH OR	MH P
02	1.37	0.97	0.65	0.44	1.45	1.08	0.74	**0.95**	5.96E-01	0.76	**3.51E-03**
03:01	1.29	0.96	1.63	2.07	1.71	1.75	0.96	1.5	8.19E-06	1.06	9.39E-01
03:02	0.59	1.39	1.47	1.96	0.6	0.89	1.94	1.03	8.35E-01	0.74	3.33E-02
03:03	0.51	0.49	1.89	0.66	0.52	0.49	4.59	0.81	3.24E-01	0.63	3.59E-02
03:04	0.88	NA	1.71	0.45	4.01	3.26	NA	1.45	5.99E-01	NA	NA
04:02	2.59	2.79	2.41	11.7	0.56	1.26	0.14	1.63	1.40E-02	1.17	4.39E-01
05:01	0.8	0.66	0.49	0.44	0.7	0.7	0.19	**0.61**	2.21E-04	0.56	**1.43E-05**
05:02	1.44	2.5	1.61	7.13	1.91	1.25	15.03	1.98	3.85E-04	1.60	1.71E-02
05:03	1.53	0.89	0.92	1.53	1.35	0.68	0.21	0.96	8.51E-01	0.71	1.19E-01
06:01	0.29	0.35	8.59	2.29	1.33	0.22	0.44	0.61	3.99E-01	0.44	1.70E-01
06:03	0.12	0.89	0.12	0.27	0.06	0.23	1.12	**0.19**	4.29E-11	0.19	**4.29E-11**
06:04	1.12	2.79	1.79	1.14	1.91	1.5	0.55	1.54	1.51E-02	1.07	7.31E-01
06:09	0.53	0.35	0.21	NA	0.26	0.1	4.59	**0.24**	6.98E-03	0.21	**2.95E-03**

P-values in bold survive Bonferroni correction. DE, Germany; CH, Switzerland; NL, Netherlands; PL, Poland; SP, Spain; FR, France; IT, Italy; MH, Mantel-Haenzel; OR, odds ratio; NA, not applicable. Numbers below country codes indicate number of patients-controls. Multivariate ORs and P-values were calculated in a stepwise fashion, where carriers of the most significant haplotypes are removed in each iteration.[18]

The next question is whether *DQB1*06:02* or another gene in its vicinity is different and unique to narcolepsy. *DQB1*06:02* was partially sequenced in 4 narcolepsy patients and no variation was observed.[21,22] To exclude the possibility that a mutation occurred within this region in narcolepsy patients, we extracted the sequence corresponding to the full length of the HLA class II region from an ongoing exome sequencing project in 10 *DQB1*06:02* homozygous narcolepsy patients. No variant was found in any of the tightly linked *DRB1*15:01*, *DQA1*01:02*, or *DQB1*06:02* genes, confirming that the entire region is identical between narcolepsy patients and *DQB1*06:02* positive controls. Comparison among these 10 patients and the reference sequence indicated that the shared and conserved haplotype includes *DRB5*, *DRB1*, *DQA1*, and *DQB1* genes (150kb, Figure S1).

DISCUSSION

Our GWAS replication confirms the complex HLA-*DQB1* involvement in susceptibility and protection from narcolepsy with cataplexy in Europe. As in most other autoimmune disorders, the overwhelming portion of risk and protection is found within the HLA region, and other common variants found through GWASs have little to negligible contribution. Narcolepsy is a rare disease and underdiagnosed, and large patient populations are not available to gain enough power to detect low effect size genetic variants. Our European

population is one of the largest, and we estimate that nearly 90% of European narcolepsy with cataplexy patients with complete diagnostic work-up and DNA available were included in the present study. Nevertheless, future efforts by including more patients might increase the statistical power to detect additional small effect size variants outside of the HLA region.

Previous studies in European ancestry, Japanese, Korean, and Chinese populations, have identified *DQB1*03:01* as a second susceptibility allele, while *DQB1*05:01*, *06:01*, and *06:03* were found to protect against narcolepsy.[18,23,24] Here, we show that at least two other alleles (*DQB1*02*, and *DQB1*06:09*) also confer protection. The protective effects of *DQB1*05:01*, *06:01*, and *06:03* were interpreted in terms of allele competition.[18] DQ molecules are heterodimers of α- and β-chains, encoded by *DQA1* and *DQB1* loci. The allele competition hypothesis posits that other DQ1 molecules than the susceptibility one (*DQα*01:02/DQβ*06:02*) might compete and thus reduce the availability of susceptibility molecules. However, this hypothesis is not consistent with either an increased risk conferred by *DQB1*03:01* or decreased risk conferred by *DQB1*02* (which heterodimerize with *DQA1*05:01*). Additionally, our finding that *DQB1*06:09* also confers protection while heterodimerizing with the susceptibility *DQA1*01:02*, argues against the allele competition hypothesis. Because of a very tight linkage disequilibrium between *DQA1* and *DQB1*, the respective contribution of each locus is difficult to disentangle. Although *DQA1* typing was not available in our patients and controls, our finding that different *DQB1* alleles in tight linkage with the similar *DQA1* can both predispose and protect against narcolepsy constitutes an additional evidence that *DQB1* more than *DQA1* is the major locus associated with narcolepsy with cataplexy.

Overall, among 20% of the European general population who carry *DQB1*06:02* on one chromosome, on average one-third carries a neutral allele, one-third an additional risk allele, and one-third a protective allele on the other chromosome. Given the ORs in different countries in this study, it is expected that with larger populations, several other alleles with ORs ranging from 0.75 to 0.89, as found here, might turn out to be protective. Also, protective alleles may vary between populations as for instance *DQB1*06:01* is common and strongly protective in Japanese and Koreans,[18,23] while it has a very low frequency in Caucasians. DQ molecules are encoded by *DQA1* and *DQB1* loci, mainly on the same chromosome (*cis*) but also on the opposite chromosome (*trans*). Accordingly, individuals heterozygous for *DQA1*05:01/DQB1*02:01* and *DQA1*03:01/DQB1*03:02* are at 5-fold higher risk for type 1 diabetes than those homozygous for either of the DQ variants,[25] clearly indicating the formation of highly susceptible *trans*-encoded molecules DQ8.5 (*DQA1*05:01/DQB1*03:02*) and DQ2.3 (*DQA1*03:01/DQB1*02:01*). Conversely, in narcolepsy, *DQA1*01:02/DQB1*06:02* and *DQA1*01:03/DQB1*06:03* heterozygotes are rare, probably because trans heterodimers encoded by *DQA1*01:03/DQB1*06:02* or *DQA1*01:02/DQB1*06:03* must be protective (note that such combinations are even found in *cis* in some populations, e.g., *DQA1*01:02/DQB1*06:03* in Slovenia and *DQA1*01:03/DQB1*06:02* in Iran, see http://www.allelefrequencies.net/).

One major difficulty in narrowing down the susceptibility/protective locus within the HLA class II region is the well-documented large conserved extended HLA haplotypes (1 to 4 Mb), including several loci (most important ones being *DRB1*, *DQA1*, and *DQB1*). Nevertheless, comparisons in different ethnic groups strongly suggested that the minimum narcolepsy susceptibility region extends centromeric to *DRB1* locus from *DQA1* to *DQB1*.[24,26,27] This observation is strengthened by the fact that many European ancestry *DRB1*15:01* negative narcolepsy patients still carry *DQA1*01:02-DQB1*06:02* ([27] and personal observation: four patients in the present study). Also, in the present study, *DQB1*06:04* and *DQB1*06:09* that are in almost complete linkage disequilibrium with *DRB1*13:02-DQA1*01:02*, confer opposing susceptibility or protection effects.

For a causal involvement, variants within a locus should result in susceptibility or protection only. In the present study, variants within each *DQB1*02, DQB1*03*, *05*, and *06* allelic group confer either susceptibility or protection, strongly supporting a causal *DQB1* involvement. *DQB1*02* found here as protective, is a common allele (between 17% and 25% carriers in European countries) and is the major susceptibility allele for type 1 diabetes, while *DQB1*06:02* is the major protective allele in this disease. Surprisingly (given the high prevalence of type 1 diabetes), there is not a single narcolepsy patient, so far reported, with type 1 diabetes, indicating an exclusive contribution of *DQB1*06:02* and *DQB1*02* to each condition, suggesting a complex contribution of *DQB1* locus, not only to narcolepsy but also to other HLA-associated disorders. It remains that *DQB1*06:02*, a common variant at the *DQB1* locus, confers an extraordinary risk (251-fold increase) to narcolepsy with cataplexy and almost all other *DQB1* common variants confer risk or protection with effect sizes from 2- to 5-fold. As opposed to all other common variants discovered by GWAS, which have small effects, HLA gene variants represent an exception in all HLA-associated disorders, and deserve renewed interest to understand how a common variant can be causally associated with a disease. In this effort, given its almost complete association with *DQB1*06:02,* narcolepsy with cataplexy is one of the best candidates.

Despite the fact that nearly 100% of narcolepsy with cataplexy patients are *DQB1*06:02* positive, HLA typing is disregarded in the diagnosis of narcolepsy with cataplexy, mainly because 20% of the general population is also positive (low specificity), and because of the cost of high resolution HLA typing. Instead, hypocretin deficiency (hypocretin-1 levels in the CSF < 110 pg/mL), which needs invasive lumbar puncture, is being considered as part of the definition of the condition (although performed in very few doubtful cases). Nevertheless, the almost 100% negative predictive value warrants HLA typing, not only in patients suspected of narcolepsy but especially in "at risk" populations. In this context, all narcolepsy patients, reported to date, who developed the disease after H1N1 vaccination were *DQB1*06:02* positive,[12-15] thus at very high risk.

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