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Permalink

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Journal

Human Molecular Genetics, 23(22)

ISSN

0964-6906

Authors

Kuiper, Jonas JW
Van Setten, Jessica
Ripke, Stephan
[et al.](#)

Publication Date

2014-11-15

DOI

10.1093/hmg/ddu307

Peer reviewed

A genome-wide association study identifies a functional *ERAP2* haplotype associated with birdshot chorioretinopathy

Jonas J.W. Kuiper^{1,2,†}, Jessica Van Setten^{3,†}, Stephan Ripke^{6,7}, Ruben Van 'T Slot³, Flip Mulder³, Tom Missotten⁸, G. Seerp Baarsma⁸, Laurent C. Francioli³, Sara L. Pulit³, Carolien G.F. De Kovel³, Ninette Ten Dam-Van Loon¹, Anneke I. Den Hollander^{9,10}, Paulien Huis in het Veld⁹, Carel B. Hoyng⁹, Miguel Cordero-Coma¹¹, Javier Martín¹², Victor Llorenç¹³, Bharti Arya¹⁴, Dhanes Thomas¹⁴, Steven C. Bakker⁴, Roel A. Ophoff^{4,15,16}, Aniki Rothova^{1,17}, Paul I.W. De Bakker^{3,5,‡}, Tuna Mutis^{2,‡} and Bobby P.C. Koeleman^{3,‡,*}

¹Department of Ophthalmology, ²Department of Clinical Chemistry and Hematology, ³Department of Medical Genetics, ⁴Department of Psychiatry, Rudolph Magnus Institute of Neuroscience, ⁵Department of Epidemiology, University Medical Center Utrecht, Utrecht, The Netherlands, ⁶Program in Medical and Population Genetics, Broad Institute of Harvard and MIT, Cambridge, MA, USA, ⁷Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA, ⁸The Rotterdam Eye Hospital, Rotterdam, The Netherlands, ⁹Department of Ophthalmology and ¹⁰Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, ¹¹Unidad de Uveitis. Servicio de Oftalmología, Hospital Universitario de León, León, Spain, ¹²Instituto de Parasitología y Biomedicina López-Neyra, IPBLN, CSIC, Granada, Spain, ¹³Institut Clinic d'Oftalmologia (ICOF), Hospital Clinic de Barcelona, Barcelona, Spain, ¹⁴Moorfields Eye Hospital NHS Foundation Trust, London, UK, ¹⁵Center for Neurobehavioral Genetics, Semel Institute for Neuroscience & Human Behavior, ¹⁶Department of Human Genetics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA and ¹⁷Erasmus University Medical Center, Rotterdam, The Netherlands

Received February 21, 2014; Revised June 5, 2014; Accepted June 12, 2014

Birdshot chorioretinopathy (BSCR) is a rare form of autoimmune uveitis that can lead to severe visual impairment. Intriguingly, >95% of cases carry the HLA-A29 allele, which defines the strongest documented HLA association for a human disease. We have conducted a genome-wide association study in 96 Dutch and 27 Spanish cases, and 398 unrelated Dutch and 380 Spanish controls. Fine-mapping the primary MHC association through high-resolution imputation at classical HLA loci, identified HLA-A*29:02 as the principal MHC association (odds ratio (OR) = 157.5, 95% CI 91.6–272.6, $P = 6.6 \times 10^{-74}$). We also identified two novel susceptibility loci at 5q15 near *ERAP2* (rs7705093; OR = 2.3, 95% CI 1.7–3.1, for the T allele, $P = 8.6 \times 10^{-8}$) and at 14q32.31 in the *TECPR2* gene (rs150571175; OR = 6.1, 95% CI 3.2–11.7, for the A allele, $P = 3.2 \times 10^{-8}$). The association near *ERAP2* was confirmed in an independent British case–control samples (combined meta-analysis $P = 1.7 \times 10^{-9}$). Functional analyses revealed that the risk allele of the polymorphism near *ERAP2* is strongly associated with high mRNA and protein expression of *ERAP2* in B cells. This study further defined an extremely strong MHC risk component in BSCR, and detected evidence for a novel disease mechanism that affects peptide processing in the endoplasmic reticulum.

*To whom correspondence should be addressed at: Lundlaan 6, 3584 CG Utrecht, The Netherlands. Tel: +31 887568116; Fax: +31 887567984; Email: B.P.C.Koeleman@umcutrecht.nl

[†]These authors equally contributed to this work.

[‡]These authors jointly directed this work.

INTRODUCTION

Birdshot chorioretinopathy (BSCR; MIM605808) is a rare ocular disorder that has a very strong HLA association, with >95% of cases carrying the HLA-A29 allele (1–3). BSCR manifests as a severe progressive intraocular inflammation of the posterior eye segment, typically leading to extensive retinal atrophy and blindness (4,5). The disease is most often observed in middle-aged and elderly individuals of European descent and has a slight female predominance (3,6). Current treatment strategies include various immunosuppressive medications but fail to prevent or cease the retinal atrophy. HLA-A29 is relatively common in European populations, but only a tiny subset of HLA-A29-positive individuals develops BSCR (6). It is therefore assumed that other genes than *HLA-A* and additional exogenous factors are involved in the development of the disease.

RESULTS

To identify BSCR susceptibility genes and to fine-map the association within the MHC region, we conducted a genome-wide association study in 96 Dutch and 27 Spanish BSCR cases and 398 unaffected Dutch and 380 Spanish controls from European ancestry. After quality control and genome-wide SNP imputation, we analyzed a total of 9,932,851 SNPs in 117 cases and 693 controls (Materials and Methods; Supplementary Material, Fig. S1). We did not see evidence for population stratification ($\lambda_{GC} = 0.96$; Supplementary Material, Fig. S2).

The strongest association signal was located within the HLA class I region for rs142115394 ($P = 6.3 \times 10^{-17}$). We next imputed and tested classical alleles and amino acid polymorphisms in *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DPA1*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1* and *HLA-DRB1* following a recently described procedure (7). The strongest association of all variants tested mapped to the classical HLA-A*29:02 allele ($P = 6.6 \times 10^{-74}$) (Fig. 1 and Table 1; Supplementary Material, Table S1). We found HLA-A*29:01 to be nominally associated ($P = 0.02$) but not significant once the A*29:02 effect was accounted for

($P = 0.093$), indicating that the classical A29 effect can be primarily attributed to the A*29:02 allele. No polymorphic amino acid position could explain the data better than A*29:02 (Supplementary Material, Table S2). Controlling for the HLA-A*29:02 effect, no other classical *HLA-A* allele was significantly associated (best adjusted $P = 3.0 \times 10^{-4}$ for HLA-A*30:01).

Outside the MHC region, a novel association was found at 5q15 for rs7705093 ($P = 8.6 \times 10^{-8}$) within the *LNPEP* gene encoding *leucyl/cystinyl aminopeptidase* (Table 1). This association was further strengthened in 30 British cases and 2793 control samples (combined meta-analysis $P = 1.7 \times 10^{-9}$). Due to linkage disequilibrium (LD), several other SNPs across the locus also showed strong association (Supplementary Material, Fig. S3). This locus includes the three members of the M1 family of aminopeptidases; *ERAP1*, *ERAP2* and the *LNPEP* gene (8). *ERAP1* and *ERAP2* encode *endoplasmic reticulum aminopeptidase 1* and *2*, respectively, and are both involved in antigen processing and presentation by HLA class I molecules to T cells (8–10). The *LNPEP* gene encodes *leucyl/cystinyl aminopeptidase* that specifically functions in peptide trimming for cross-presentation in dendritic cells (11).

To explore the biological relevance of the associated variation in the 5q15 locus, we first investigated whether the associated SNPs also influence expression levels of these three genes using the expression quantitative trait loci (eQTL) database GeneVar (12). The strongest QTL for *ERAP2* expression in LCL cells ($P = 2.0 \times 10^{-114}$ from GeneVar) is observed for rs10044354 ($P = 1.2 \times 10^{-7}$ for association with BSCR), which is almost in perfect LD with the lead SNP rs7705093 ($r^2 = 0.98$ in the controls; Supplementary Material, Fig. S4). These SNPs are statistically indistinguishable with respect to BSCR risk ($P > 0.2$). The rs10044354 (and SNPs in LD with rs10044354) appeared to have no significant impact on *LNPEP* or *ERAP1* expression ($P > 0.2$ from GeneVar).

Subsequently, we tested if this variant is also correlated to protein levels of *ERAP2* in B-cell lines from individuals from the Centre d'Etude du Polymorphisme Humain (CEPH) panel (13) and five BSCR patients. Indeed, homozygous carriers for

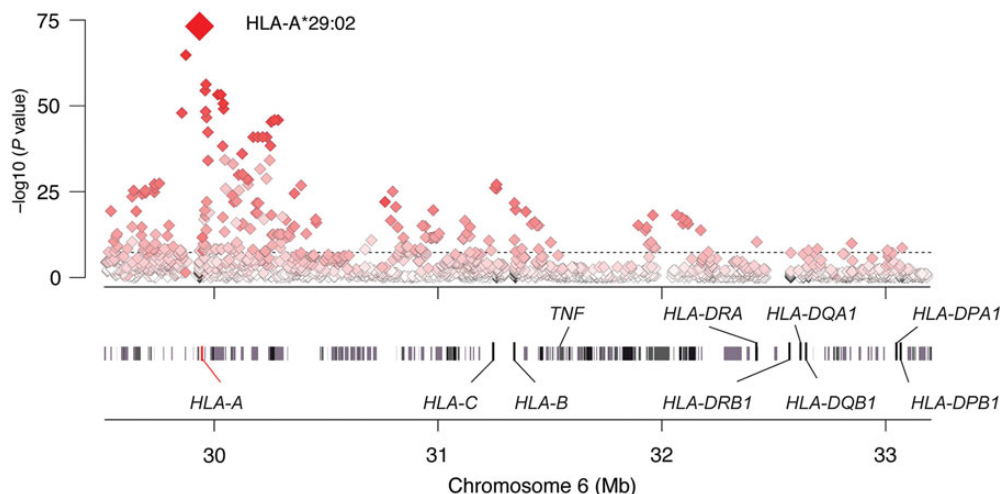


Figure 1. Association tests within the MHC region to birdshot chorioretinopathy. The strongest MHC signal mapped to HLA-A*29:02 allele. The shading depicts the strength of the correlation (r^2) between HLA-A*29:02 (red diamond) and the SNPs tested in the region. Gene positions are obtained from the human genome build 37 (GRCh37/hg19).

Table 1. HLA-A29:02 and newly associated loci identified in this study

Region	Dutch cohort			Spanish cohort			Mega-analysis			British cohort (replication)			Meta-analysis				
	Allele	Case MAF	Control MAF	OR (95% CI)	P	Case MAF	Control MAF	OR (95% CI)	P	Case MAF	Control MAF	OR (95% CI)	P	Case MAF	Control MAF	OR (95% CI)	P
HLA	HLA-A29:02	0.422	0.026	303.0 (124.4, 845.6)	2.0×10^{-63}	0.548	0.084	25.4 (8.9, 94.9)	9.3×10^{-12}	157.5 (91.6, 272.6)	6.6×10^{-74}	2.0 (1.1, 3.6)	0.011	2.2 (1.7–2.9)	1.7 $\times 10^{-9}$	4.6 (2.6–8.3)*	$1.6 \times 10^{-7*}$
ERAP2-LNPEP	rs7705093-T	0.618	0.405	2.6 (1.8, 3.7)	2.7×10^{-7}	0.554	0.399	2.0 (1.0, 3.7)	3.8×10^{-2}	2.3 (1.7, 3.1)	8.6×10^{-8}	0.621	0.442	2.0 (1.1, 3.6)	0.011	2.2 (1.7–2.9)	1.7×10^{-9}
TECPR2	rs150571175-A	0.075	0.017	5.9 (2.5, 14.2)	6.4×10^{-5}	0.183	0.041	6.0 (2.2, 16.5)	6.0×10^{-4}	6.1 (3.2, 11.7)	3.2×10^{-8}	0.033*	0.036*	1.0 (0.2–1.4)*	1*	4.6 (2.6–8.3)*	$1.6 \times 10^{-7*}$

*This SNP failed genotyping by Sanger sequencing, so replication genotyping for this locus was at rs7159505, which had $r^2 = 0.83$ as calculated from Go NL.

the rs10044354 C allele showed little or no ERAP2 protein expression in a quantitative western blot analysis, whereas hetero- or homozygous carriers of the rs10044354T risk allele showed higher protein levels of ERAP2 ($P = 0.009$) in cases and controls (Fig. 2). In contrast, we did not observe an effect of this SNP on LNPEP expression, consistent with the mRNA findings from GeneVar (Fig. 2, bottom).

Another novel association was detected at 14q32.31 in the *TECPR2* gene (rs150571175, $P = 3.2 \times 10^{-8}$) (Table 1; Supplementary Material, Fig. S3). However, this association could not be confirmed in the British cohort ($P = 1.0$; combined meta-analysis $P = 1.6 \times 10^{-7}$). The protein encoded by *TECPR2* (tectonin beta-propeller repeat-containing 2) interacts with Atg8 orthologs and functions in autophagosome accumulation for autophagy (14).

Because BSCR is considered an autoimmune disease and shares signatures of T helper 17-cell responses with other autoimmune disorders (15–19), we hypothesized that known risk loci for other autoimmune diseases may also be associated with BSCR. Therefore, we tested 289 bona fide non-HLA SNP associations in autoimmune disorders (Supplementary Material, Table S3). For all BSCR Dutch and Spanish cases and controls, we computed a weighted genetic risk score based on the observed number of risk alleles and the known effect sizes for each autoimmune disease separately. We performed a similar test for SNPs associated to either height or LDL levels as two control traits. After correction for multiple testing, we obtained no significant association between increased risk scores and BSCR risk for any of the autoimmune diseases tested, except for wGRS Psoriasis (higher for BSCR) and LDL (lower in BSCR) (Supplementary Material, Table S4). However, these associations were only nominally significant in the Dutch cohort but not in the Spanish cohort. Although this would suggest that BSCR shares no additional susceptibility loci with other autoimmune diseases, it is possible that the sample size and power was simply too low to detect such an overlap.

DISCUSSION

To date, the association of HLA-A29 with BSCR is the strongest association described between an HLA class I allele and human disease (2,6). Although this association has been known for over three decades (1), lack of understanding of the contribution of HLA-A29 to the pathogenesis of BSCR, impeded it to become an absolute criterion for diagnosis (22). HLA-A29 can be subdivided in at least 17 subtypes (23), but most common in Caucasians are the subtypes HLA-A*29:01 and HLA-A*29:02 that have both been most associated with BSCR (24). However, the imputation analyses of the HLA region revealed that the nominal association of HLA-A*29:01 disappeared once the A*29:02 effect was accounted for, indicating that the classical A29 effect in BSCR can be primarily attributed to the A*29:02 allele and comes with unusually large effect size in terms of explained variance (Nagelkerke's index = r^2 [controlled for top 4 PCs and cohorts] = 0.595). However, since HLA-A29 is relatively common in European populations (7%) and very few HLA-A29-positive individuals develop BSCR (6), other additional genetic or exogenous factors are required for the development of the disease. Here, we provide evidence for an

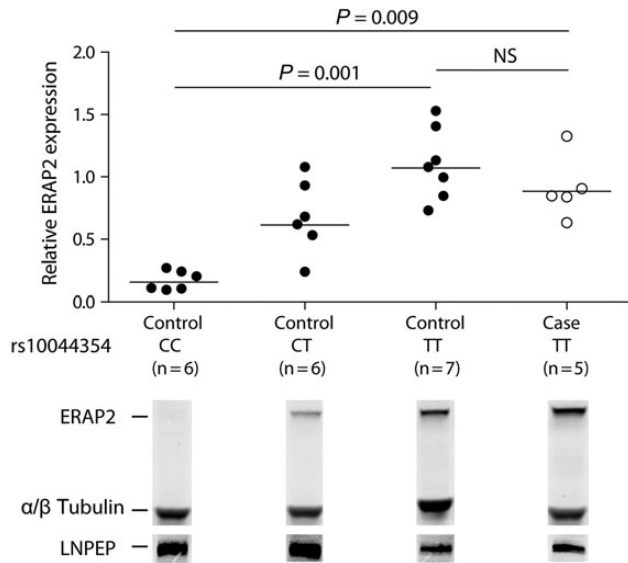


Figure 2. Expression of ERAP2 and LNPEP in CEPH control and BSCR according to rs10044354 genotype. B-cell lines from individuals from the CEPH panel (13) and five BSCR patients were lysed and ERAP2 and LNPEP expression was assessed after SDS-PAGE and western blotting with antibodies to ERAP2 and LNPEP. The endogenous levels of both α - and β -tubulin total protein were analyzed as a loading control. The horizontal lines indicate the means. Kruskal-Wallis test with Dunn's multiple-comparison *post hoc* test was used to assess differences in the levels of ERAP2 between B-cell line groups according to rs10044354 genotype.

association with *ERAP2* and speculate about a possible association of *TECPR2*.

The *ERAP2* gene is located on chromosome 5 and is positioned between the closely related *ERAP1* and *LNPEP* genes (8,9). The *ERAP2* gene encodes for an aminopeptidase located in the endoplasmic reticulum (ER) that is involved in the final processing and presentation of antigenic precursors, together with the closely related and better characterized *ERAP1* (10). In fact, *ERAP2* and *ERAP1* were shown to form a heterodimer to combine their restricted sequence specificities for peptide trimming (10). Notably, some antigenic peptides, including the immunodominant epitope derived from HIV gag protein, have been shown to be solely dependent upon *ERAP2* trimming (25).

Recent genome-wide association studies have implicated a role for *ERAP1* and *ERAP2* in several HLA-associated autoimmune diseases such as psoriasis (26), ankylosing spondylitis (20,21) and Crohn's disease (27), or juvenile idiopathic arthritis (28), suggesting an important functional role for these genes in the immunopathology of autoimmune diseases. Of note, all these *ERAP1*- or *ERAP2*-associated diseases share a major HLA class I association that contrasts the major HLA class II association found in many other autoimmune diseases not associated to *ERAP1* or *ERAP2*. In this respect, our study substantiates an emerging concept as we also found a strong link between *ERAP2* and BSCR, an autoimmune disease affecting the eye that manifests almost exclusively in HLA-A29-positive Caucasians.

Similar to HLA genes, *ERAP2* has been shown to undergo balancing selection, which maintains two main haplotypes A and B, with highly differential protein expression due to alternative mRNA splicing (29). We discovered that the SNPs associated

with BSCR at 5q15 also serve as tags for these haplotypes, with high expression levels of *ERAP2* in BSCR patients and no or low expression in homozygous negative healthy individuals. Based on our results, and given the fact that *ERAP2* has nonoverlapping trimming capacities (10) with *ERAP1*, it is likely that *ERAP2* has a specific contribution to the immunopathology of BSCR, by differential generation or destruction of immunodominant epitopes capable of binding to HLA-A29. Modulation of the antigen processing and presentation pathway by targeting *ERAP2* in BSCR is particularly of interest since, in contrast to *ERAP1*, a rather high frequency of *ERAP2*-deficient individuals is maintained in the population without any clear adverse effects on their immunity and healthy (29). However, the efficacy of selective inhibition of *ERAP2* in BSCR, while remaining *ERAP1* function, remains to be clarified and involves the use of novel inhibitors that targets this family of pleiotropic aminopeptidases (30).

We found a significant association for *TECPR2* in the Dutch and Spanish cohorts, but these findings could not be replicated in British patients. *TECPR2* encodes for *TECPR2* (*tectonin beta-propeller repeat-containing 2*) that interacts with Atg8 orthologs and is a positive regulator of autophagy and functions in autophagosome accumulation (14). A mutation in the *TECPR2* gene was recently implicated in hereditary spastic paraparesis (31). The eye maintains an unconventional form of autophagy that is crucial for the function and health of the photoreceptors in the retina (32,33). Interestingly, interfering with autophagy by rapamycin, a potent inducer of autophagy, can paradoxically reduce or exacerbate uveitis (34,35). Moreover, autophagy-related genes have also been associated with other autoimmune diseases, including SLE and Crohn's disease (36,37). Further investigation of the association of the *TECPR2* gene in other BSCR populations is necessary to clarify its possible contribution to the pathogenesis of BSCR.

In conclusion, we identified HLA-A*29:02 as the principal MHC association and report variants near the *ERAP2* gene at 5q15 that predispose to BSCR. Expression studies indicate that the disease-associated variants at 5q15 correlate to mRNA and protein expression levels of *ERAP2*, suggesting that differential peptide processing and antigen presentation to T cells is an essential disease mechanism for BSCR.

MATERIALS AND METHODS

Subject collection

A total of 96 unrelated Dutch BSCR cases, 27 Spanish BSCR cases and 398 unrelated Dutch healthy controls and 380 Spanish controls, all from European ancestry, are enrolled in this study. BSCR diagnosis was based on criteria established by international consensus (22). Dutch cases were recruited at the Department of Ophthalmology at the University Medical Center Utrecht, the Eye Hospital Rotterdam and Radboud University Nijmegen Medical Center, the Netherlands. These three institutes represent the largest registry of BSCR patients in the Netherlands; we estimate that >90% of all BSCR cases in the Netherlands are included in this cohort. Spanish cases were recruited at the department of ophthalmology at the Hospital Clinico San Carlos, Madrid, Hospital Clinico San Cecilio, Granada, Hospital de Cruces, Bilbao, Hospital Clinic Barcelona, Hospital Carlos Haya, Malaga and Hospital Universitario, Leon.

Dutch controls were collected at UMC Utrecht and Spanish controls were recruited from the National bloodbank. For replication, we included an additional 30 UK BSCR cases recruited at the department of ophthalmology at the Moorfields Eye Hospital NHS Foundation Trust, London. As controls, we used genotype data from the UKBS and 58BC controls, which are part of the WTCCC study (38). The UKBS cohort contained 1397 control individuals selected from a UK sample of blood donors, and the 58BC contained 1396 control individuals from the 1958 British Birth Cohort (39). This study was performed in compliance with the guidelines of the Declaration of Helsinki and has the approval of the local Institutional Review Boards. Study details were explained to all cases and controls, and the collection of blood was carried out after obtaining written informed consent from each participant.

Genotyping, quality control and association analysis

All Dutch and Spanish samples were genotyped using the Illumina Human OmniExpress BeadChip, which contains 730 525 SNPs. Data for the British 1958 Birth Cohort and UKBS were obtained from the European Genome-Phenome Archive at <https://www.ebi.ac.uk/ega/>. These individuals were genotyped using the Affymetrix GeneChip 500K platform and the Illumina ImmunoChip platform. We used PLINK (40) to clean the genotype data using standard quality control parameters: we removed SNPs with low frequency (<0.02), out of Hardy–Weinberg equilibrium ($P < 10^{-6}$), or with excess missingness ($>5\%$). We also checked for relatedness and inconsistent gender. Principal components analysis was performed to detect population stratification. Overall, we excluded two Dutch and four Spanish cases, and four Dutch and 85 Spanish controls, and kept a total of 393 831 SNPs for subsequent analyses. Imputation of untyped SNPs was performed using Minimac (41). As reference panel, we used the 998 phased haplotypes from the Genome of the Netherlands Project release 4 encompassing 19 763 454 SNPs (42). We included the top four principal components as covariates in logistic regression models using PLINK (version 1.08) and R (version 2.11.1). SNPs with MAF $<1\%$ or with imputation quality (info score) <0.01 were excluded from analyses. Genome-wide distribution of the test statistic indicated no evidence for population stratification (QQ plots Supplementary Material, Fig. S2). As previously described (7), we imputed classical alleles and amino acid polymorphisms in *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DPA1*, *HLA-DPBI*, *HLA-DQA1*, *HLA-DQB1* and *HLA-DRB1* at a four-digit resolution, as well as an additional 3,117 SNPs across the MHC, using a large reference panel of 2,767 individuals of European descent. We imputed cases and controls together. For the association analyses in the MHC, we assessed the significance of the improvement in fit by calculating the deviance (defined as $-2 \times$ the log likelihood), which follows a χ^2 distribution.

Protein expression assays

We assessed ERAP2 and LNPEP protein levels in EBV transformed B cells (EBV-LCL), from 19 individuals from the CEPH panel and EBV-LCLs generated from peripheral blood mononuclear cells of five BSCR patients, by western blotting (13). From the CEPH panel, we selected six homozygotes for the C allele, six

heterozygotes and seven homozygotes for the T allele (NA07022, NA07029, NA07056, NA07345, NA07357, NA10838, NA10846, NA10854, NA10863, NA11839, NA12003, NA12056, NA12144, NA12145, NA12239, NA12249, NA12812, NA12815 and NA12872). Protein extracts (20 $\mu\text{g}/\text{lane}$) from EBV-LCL cells were separated on a 4–12% NuPageBis–Tris gel (Invitrogen) and transferred to a PVDF membrane. Proteins were detected using a 1:2500 dilution of primary antibody [goat anti-ERAP2 polyclonal antibody (AF3830, R&D Systems), mouse anti-ERAP2 polyclonal (ab69037, Abcam), sheep anti-LNPEP polyclonal antibody (AF6386, R&D Systems) and anti- α/β -tubulin monoclonal prepared in Rabbit (#2148, Cell Signaling)]. Anti- and anti-rabbit secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes; 1:2500) and anti-goat secondary antibody conjugated to IRDye800 (Li-Cor; 1:2500) were used to probe primary antibodies. Protein bands were detected and quantified by western blotting with the Odyssey system (Li-Cor). The ratio of the intensity of the ERAP2- α/β -tubulin band of CC-homozygotes to CT-heterozygotes and TT-homozygotes was calculated using ImageJ.

Transcriptional analysis

eQTL analysis was performed based on data available from the Sanger Institute GENEVAR project for lymphoblastoid-cell lines from 726 HapMap 3.0 CEU individuals and three tissue types (LCL cell type was used in the analysis) collected from 856 healthy female twins of the MuTHER resource (12,43). Differences in the levels of ERAP2 in B-cell line between groups according to rs10044354 genotype were analyzed using non-parametric one-way ANOVA; Kruskal–Wallis test with Dunn's multiple-comparison *post hoc* test in GraphPad Prism 5 software. Statistical significance was accepted at a significance level of $P < 0.05$.

Genetic risk score

To test the cumulative effect of validated SNPs associated with other autoimmune diseases (ankylosing spondylitis, rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, vitiligo, type 1 diabetes and multiple sclerosis) (20,26,44–49), we computed a weighted genetic risk score in all cases and controls of our study. We summed the estimated dosages of the known risk alleles (for a given autoimmune disease) weighted by the natural log of the published odds ratios (Supplementary Material, Table S3). We tested the genetic risk scores for each disease as a continuous variable for association to BSCR status using logistic regression, including the top four principal components as covariates. We corrected for multiple testing of 10 traits in total (including height (50–52) and LDL (53,54) as a negative control).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank all participants involved in this study, including the Birdshot Uveitis Society (<http://birdshot.org.uk/>), The

Moorfield Biobank (UK) and Spanish Birdshot Group (GENUVE). This study was supported by the combined grants from the Dr F.P. Fischer Stichting, Amersfoort; the Algemene Nederlandse Vereniging Ter Voorkoming Van Blindheid, Doorn; the Landelijke Stichting Voor Blinden en Slechtzienden, Utrecht, the Stichting Nederlands Oogheelkundig Onderzoek (SNOO), Rotterdam and the Blindenpenning Stichting, Amsterdam, the Netherlands.

Conflict of Interest statement. None declared.

FUNDING

Genotyping of Dutch control sample was supported by NIH/NIMH MH090553 to R.A.O. P.I.W.d.B. is a recipient of a VIDI Award from the Netherlands Organization for Scientific Research (N.W.O. project number 016.126.354).

WEB RESOURCES

Summary statistics of all SNPs included in this study are available for download through: <http://cmm.umcutrecht.nl/publications/koелеman/birdshot/>; Beagle Genetic Analysis Software, <http://faculty.washington.edu/browning/beagle/beagle.html> PLINK statistical software, <http://pngu.mgh.harvard.edu/~purcell/plink/>; R project, <http://www.r-project.org/>; ImageJ, <http://rsbweb.nih.gov/ij/index.html>; GeneVar, <http://sanger.ac.uk/resources/software/genevar/>; The 1000 Genomes Project, <http://www.1000genomes.org/>; LocusZoom, <http://csg.sph.umich.edu/locuszoom/>; SNP2HLA imputation pipeline, <http://www.broadinstitute.org/mpg/snp2hla/>; Ensemble Genome Browser, <http://www.ensembl.org/>.

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