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Genome-Wide Association Study of Hepatitis C Virus- and Cryoglobulin-Related Vasculitis

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Abstract

Objectives—The host genetic basis of mixed cryoglobulin vasculitis is not well understood and has not been studied in large cohorts. A genome-wide association study was conducted among 356

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Authorship and Conflict of Interest Statements

ALZ, PC, MV, MC, AM, EC, LG, BT, VP, LBD, SIK, MPB, GML, AYK, LA, DLT collected data. DLT, GLW, PD, RL designed the research analysis and performed the statistical analysis. RL and DT designed and performed the targeted genotyping. DLT, GLW, ALZ, LG, and PD wrote the manuscript. All authors interpreted the data and revised the manuscript.

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HCV RNA positive individuals with cryoglobulin-related vasculitis and 447 ethnically-matched, HCV RNA positive controls.

Methods—All cases had both serum cryoglobulins as well as a vasculitis syndrome. A total of 899,641 markers from the Illumina HumanOmni1-Quad chip were analyzed using logistic regression adjusted for sex, as well as genetically-determined ancestry. Replication of select single nucleotide polymorphisms (SNPs) was conducted using 91 cases and 180 controls, adjusting for sex and country of origin.

Results—The most significant associations were identified on chromosome 6 near the *NOTCH4* and *MHC class II* genes. A genome-wide significant association was detected on chromosome 6 at SNP rs9461776 (OR= 2.16, $p=1.16E-07$) between *HLA-DRB1* and *DQA1*: this association was further replicated in additional independent samples (meta-analysis $p=7.1\times 10^{-9}$).

Conclusions—A genome-wide significant association with cryoglobulin related vasculitis was identified with SNPs near *NOTCH4* and *MHC Class II* genes. The two regions are correlated and it is difficult to disentangle which gene is responsible for the association with MC vasculitis in this extended MHC region.

Keywords

Genome-Wide Association Study; Cryoglobulinemia; Human Leukocyte Antigen; SNP

Introduction

More than 185 million persons have been exposed to hepatitis C virus (HCV) and an estimated 130 million have chronic infection.¹ Although the main target of this virus is the hepatocyte, HCV also appears to infect lymphocytes.² Patients with chronic HCV infection typically produce anti-HCV IgG and can develop not only hepatic disease but also extrahepatic conditions amongst which the most frequent is mixed cryoglobulinemia (MC).^{3, 4} MC vasculitis is both an autoimmune and B-lymphoproliferative disorder, clinically benign, but evolving in 8–10% of cases into a frank non-Hodgkin's lymphoma (NHL).⁵ Cryoglobulins are called “mixed” because they are composed of both IgGs and IgMs, which can be partially monoclonal (type II MC) or totally polyclonal (type III MC) and have rheumatoid factor (RF) activity.^{6–8} The prefix “cryo” refers to the property that these immune complexes precipitate at temperatures below 37° C and dissolve with rewarming.⁹

Approximately half of persons with chronic HCV infection have serum cryoglobulins, but only ~5% develop a clinically evident MC vasculitis syndrome.^{10, 11} This HCV-related, cryoglobulinemic vasculitis most commonly manifests in the skin (palpable purpura, 80%), joints (70%), peripheral nerves (60%), and kidney (immune complex nephritis, 20%).¹¹ Due to the wide range of symptoms, MC vasculitis subjects are often clinically referred to different specialties, thus the actual prevalence may be underestimated.¹⁰

The clinical vasculitis syndrome can be improved or resolved by therapy directed at HCV infection and/or the B cell proliferation (e.g., with rituximab), inflammation (e.g., with corticosteroids), or serum cryoglobulins (e.g., with plasmapheresis).^{12–17} While, the

pathogenesis of the MC vasculitis syndrome is still unclear accumulating evidence points to a role for host genetics. There are no consistent differences in the infecting viruses of persons with chronic infection with or without the vasculitis syndrome.^{4, 18} There are marked ethnic and regional differences in the prevalence of MC vasculitis with the highest prevalence in persons of Mediterranean descent.^{10, 19–21} Pozzato and colleagues found only Mediterranean descent explained the much higher prevalence of MC vasculitis in Italy compared to Japan.²² In addition, several studies have identified associations between MC vasculitis and particular HLA alleles.^{23–27} The purpose of this investigation was to perform a genome-wide, multi-center study to investigate the host genetic basis for MC vasculitis in persons with chronic HCV infection.

Results

We identified 356 individuals with cryoglobulin-related vasculitis and chronic hepatitis c virus (HCV) infection. A total of 447 controls with chronic HCV infection but with no cryoglobulin-related vasculitis were selected from a previously published GWAS of spontaneous clearance versus chronic infection of HCV, or were specifically screened for this study.²⁸ After quality control measures, 899,641 single nucleotide polymorphisms (SNPs) were compared between cases and controls.

The most significant association was located on chromosome 6p21.32 (Figure 1, Table 1). A SNP, rs2071286, located within an intronic region of *NOTCH4* ($P=6.2\times 10^{-9}$) conferred 2.15 times the odds of having cryoglobulin-related vasculitis within chronically infected patients for each risk allele. An additional SNP within *NOTCH4* at rs2071279 ($P=1.6\times 10^{-7}$) had a similar effect size with each risk allele (T) resulting in 1.90 times the odds of disease. To confirm these findings, replication was attempted for both of these SNPs, however rs2071286 did not reach significance ($P=0.13$) and rs2071279 failed in production. Additionally, the next most significant *NOTCH4* findings (rs9267820 and rs9267833) also did not meet the replication threshold ($P>0.01$) (Table 2).

The second most significant association was found nearly 400 kilobases away within the Major Histocompatibility Complex (MHC) between *HLA-DRB1* and *HLA-DQA1* at SNP rs9461776 ($P=1.2\times 10^{-7}$). Each additional copy of the risk allele (G) was associated with 2.16 times the odds of cryoglobulin-related vasculitis. This SNP was significantly replicated in an independent sample of cases and controls ($P=0.01$). When the discovery and replication stages were combined within a meta-analysis, rs9461776 had a p-value of 7.1×10^{-9} (OR=2.02, $I^2=0$). Imputation of additional SNPs in both the *NOTCH4* and *MHC* regions areas did not yield more significant signals than the actual genotyped SNPs, which may reflect the strong linkage disequilibrium (LD) in the region.

The LD structure around the top *NOTCH4* association (Figure 2) suggests that there are two distinct blocks of LD defining *NOTCH4* and the MHC region. However, rs2071286 and rs2071279 in *NOTCH4* are in strong LD ($D' = 0.98$, Figure 2) and both are in LD with the HLA class II SNP rs9461776 ($D' = 0.71$ and 0.73 , respectively) despite low r^2 values likely due to differences in minor allele frequencies (Figure 2). To determine if the two regions might be statistically representing the same underlying association, we performed a

conditional analysis. The associations in this region were conditioned on the *NOTCH4* SNP, rs2071286, and the other associations in this region were attenuated to $P < 10^{-4}$. (Figure 3) When the associations were conditioned on the top HLA Class II SNP, rs9461776, the other associations in the region were also attenuated to $P < 10^{-4}$. These results do not clarify if the underlying causal allele in this region is attributable to *NOTCH4* or to the HLA Class II alleles.

Discussion

In this investigation we found strong evidence of a host genetic basis for MC vasculitis focused in and around the *MHC* class II and *NOTCH4* genes. Although there have been reported associations of MC vasculitis with the Class II MHC region the findings have been inconsistent. Cacoub and colleagues found an association of the HLA class II allele *DRB1*11* with HCV-related cryoglobulinemia, although Amoroso and colleagues failed to find a significant association with HLA DR or DQ loci.^{26, 27} In a separate study by De Re and colleagues, *DR5* and *DQ3* alleles were associated with HCV-related cryoglobulinemic vasculitis.²⁹ In a study of 25 HCV positive MC vasculitis patients and 407 controls, Lenzi and coworkers suggested an HLA-B8-DR3 haplotype associated with susceptibility to MC vasculitis, partially confirmed by a Chinese report.^{23, 24} The complexity of the MHC region, the ethnic differences in populations and the smaller sample sizes in the earlier studies may also contribute to the differences in association results. In this genome wide association study of European individuals we do identify a significant association in the extended HLA Class II region that is independent of the recently associated HLA Class II HCV viral clearance region.²⁸

However, the nearby *NOTCH4* gene also shows association with MC vasculitis in the current study. The Notch proteins are a family of transmembrane proteins (Notch 1–4) that serve as receptors for a signal transduction pathway. The Notch genes are involved with promotion and inhibition of cell differentiation and gene expression in a positive feedback loop. Genome-wide associations have shown significant associations of *NOTCH4* with systemic sclerosis, myeloperoxidase levels, age related macular degeneration, asthma and schizophrenia.^{30, 31} Additionally independently of HLA genes, *NOTCH4* polymorphisms have been associated with autoimmune disorders.^{32, 33} The importance of Notch signaling in T cell development makes it a viable candidate gene for MC vasculitis. However, the SNPs that mark *NOTCH4* and *MHC class II* are too strongly correlated to discriminate the degree to which each region contributes to the association, and there is no tractable experimental model of MC vasculitis that can be used to dissect the association further.

In summary, based on our GWAS data based on a large sample of MC vasculitis patients and controls identified two significant regions with implicated polymorphisms: *MHC Class II* and *NOTCH4*. However, we do not have evidence to indicate the causal variants for these associations. Each of these genes may play a role but further investigation is needed to determine the biologic link to this syndrome.

Materials and Methods

Subjects

The case participants in both the discovery and replication stages were enrolled in 9 different studies using study-specific case definitions (Supplementary methods and Supplementary Table 1). A total of 1,074 individuals were identified from the following studies: Toulouse Cohort, BAHSTION (Boston Acute HCV Study: Transmission, Immunity and Outcomes Network), Charles, RomeCryo, Mangia, REVELL (Correlates of Resolved Versus Low-Level Viremic Hepatitis C Infection in Blood Donors), Cacoub (Paris VIREP-C Study), United Kingdom Drug Use cohort and MASVE (Center for Systemic Manifestations of Hepatitis Viruses) (see Supplemental Methods). Controls were individuals with chronic HCV infection from a previously published GWAS.²⁸ The research was approved by the relevant institutional review boards for each study.

Genotyping

In the initial analysis, 3,454 individuals were genotyped on the Illumina Human Omni-Quad array at the Center for Inherited Disease Research (CIDR). Samples included both cases and controls that were recruited specifically for MC vasculitis (N=497), as well as a previously published GWAS for spontaneous HCV resolution versus chronic infection (N=2,957). A total of 1,134,514 SNPs were released with genotype and intensity data. Standard quality control measures were performed for both the individual and SNP. Individuals were excluded if the percentage of missing data was >2% and/or individuals were more related than expected (1st degree relatives). Markers were excluded if the percentage of missing genotypes was > 5%, the minor allele frequency (MAF) was < 1% or there were strong deviations from Hardy-Weinberg equilibrium ($p > 10^{-5}$). Principal components analysis (PCA) was used to determine genetic ancestry using 41,871 autosomal independent SNPs, excluding regions known to be associated with ethnicity, such as the extended *HLA* region and the *lactase* gene. Only individuals that clustered with European ancestry were included in further analyses (N=983). Within individuals of European-descent, a gradient was apparent from the United Kingdom to Italy, with the French participants clustering in the middle (Supplemental Figure 1A and 1B). This is consistent with prior studies showing a North-South ancestral cline in Europe.³⁴ The association between the principal components (PCs) and outcome was statistically significant for only the first two PCs ($P < 0.05$), therefore both PCs were included in all genome-wide analyses. The inflation factor (λ) after adjusting for the first two PCs and sex was 0.97, suggesting there was no genomic inflation due to confounding. The final genome-wide data set included 983 individuals (356 cases and 627 controls) at 899,641 locations across the genome.

Statistical Analysis

The initial analysis was the discovery phase and included cryoglobulinemia-associated vasculitis cases (n=356) and the HCV GWAS controls (n=447) with similar ancestral clustering (PCA determined) and chronic HCV infection from 8 different studies (Toulouse Cohort, BAHSTION, RomeCryo, Mangia, REVELL, Cacoub (Paris VIREP-C Study), the UK Drug Cohort, and MASVE). The controls consisted of individuals with chronic HCV infection from the HCV GWAS (N=355), as well as a set of controls recruited specifically

for this study (N=92). These latter were consecutively recruited patients with chronic HCV infection showing complete absence of features of mixed cryoglobulinemic vasculitis or other autoimmune and/or lymphoproliferative disorders (i.e, purpura, arthralgia, asthenia, polyneuropathy, nephritis, sicca syndrome, cryoglobulinemia, rheumatoid factor activity, complement consumption, autoantibodies, monoclonality) confirmed during at least two years of follow-up in two yearly evaluations.

All tests of association were conducted in the statistical software, Plink.³⁵ GWAS was performed using logistic regression, under an additive genetic model adjusting for the first two PCs and sex. A significance threshold of $P < 5 \times 10^{-7}$ was used to assess genome-wide significance.³⁶ The $-\log_{10}$ P -values from each SNP's logistic regression was plotted to generate a Manhattan plot (Figure 1). A conditional analysis was conducted using the top-associated SNPs (rs2071286, rs9461776) by adding the SNP as a covariate to the logistic regression and evaluating the association.

Imputation

SNP Imputation was conducted to increase the density of untyped markers around significant and suggestive association signals using the statistical program IMPUTE2³⁷ using a multi-ethnic reference panel from four continents (Africa, North and South America, Asia, and Europe) as part of the 1000 Genomes Project.³⁸ The imputed genotypes were analyzed using logistic regression under an additive model adjusting for the first two principal components and sex. An Expectation-Maximization (EM) algorithm was used to incorporate the probabilities of each genotype in the logistic regression.

Replication

To confirm the novel findings from the discovery phase of the GWAS, a replication study was performed using convenience samples for the cases and a subset of controls with chronic HCV from the previously published GWAS. Controls were selected to be matched 2:1 with the cases based on their study's country-of-origin. (Table 1) SNPs were genotyped that had reached either genome-wide significance ($P < 5 \times 10^{-7}$) or were SNPs in linkage disequilibrium with the genome-wide significant SNPs and had suggestive P -values ($P < 5 \times 10^{-6}$). A total of 4 SNPs were analyzed for replication in 92 cases and 179 controls from 7 study sites including one new site not represented in the discovery stage (Toulouse Cohort, BAHSTION, Charles, RomeCryo, Mangia, Cacoub (Paris VIREP-C Study), and MASVE). Using the standard Qiagen spin protocol, DNA for genotyping was extracted from stored cells using the QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD). Genotyping for single nucleotide polymorphisms (SNPs) was performed with TaqMan SNP genotyping assays (Life Technologies, Carlsbad, CA), which uses unique primers and probes sets to target each polymorphism. Taqman's standard protocol for 5.0ul reactions was utilized. The Roche LightCycler 480 Real-Time System (Roche Applied Science, Indianapolis, IN) was used to amplify the DNA and measure the fluorescence from each reaction to determine the genotype using Roche's Endpoint Genotyping Software.

Tests of association were conducted in Plink using logistic regression, adjusted for study country-of-origin and sex. A conventional P -value threshold was used for replication

($P < 0.01$). A meta-analysis was conducted between the discovery and replication associations using a fixed-effects model in META^{39, 40} and evaluated for heterogeneity (I^2). Linkage disequilibrium was estimated in Haploview.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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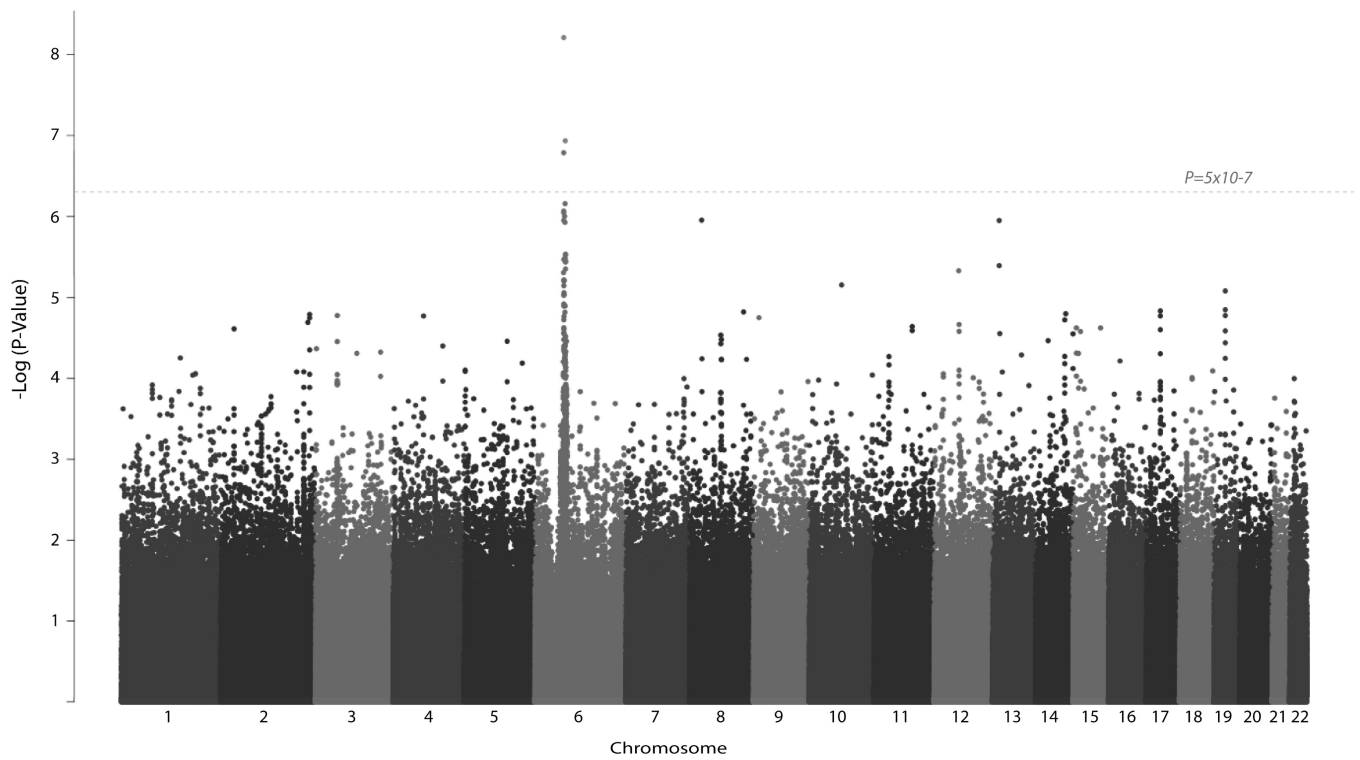


Figure 1. Manhattan Plot of GWAS Results. Significance is indicated by the $-\log$ transformation of the P-value on the y-axis. (e.g. P-value=0.001 denoted as 3) Associations are organized by chromosome on the x-axis. Genome-wide significance is indicated by the dashed line ($P < 5 \times 10^{-7}$).

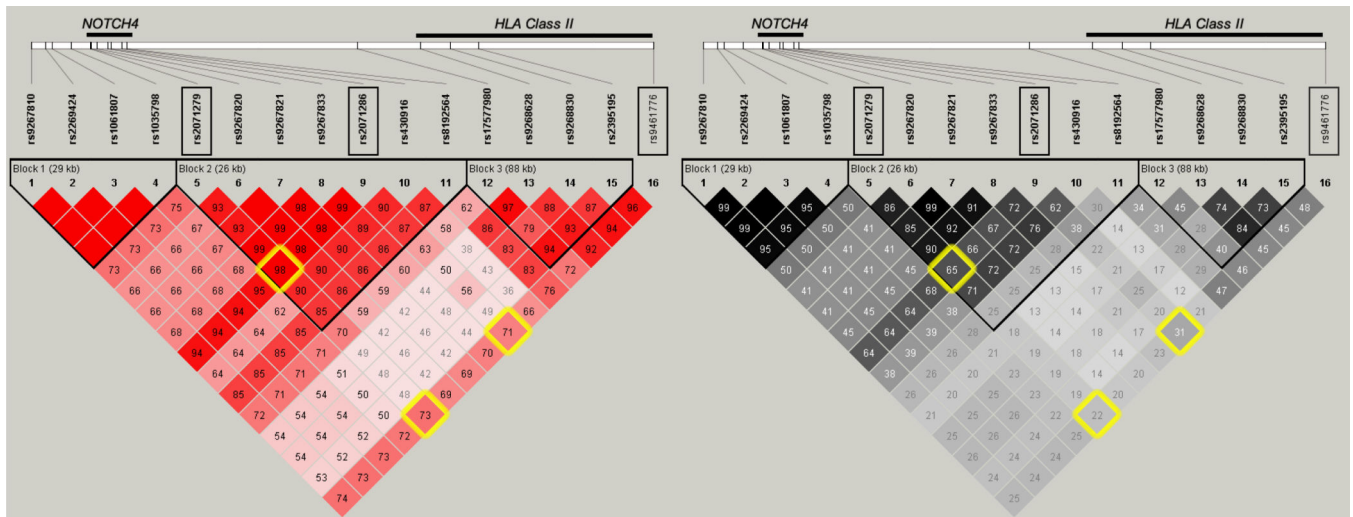


Figure 2. Linkage disequilibrium for SNPs with $P < 10^{-5}$ in terms of D' (red) and r^2 (grey). The top SNPs' pairwise linkage disequilibrium measures are highlighted in yellow. Of note is the HLA Class II SNP that is in long-range LD with the NOTCH4 SNPs to the left. Genes are depicted at the top and the corresponding SNPs are below in the LD plot. Black Triangles depict blocks of LD.

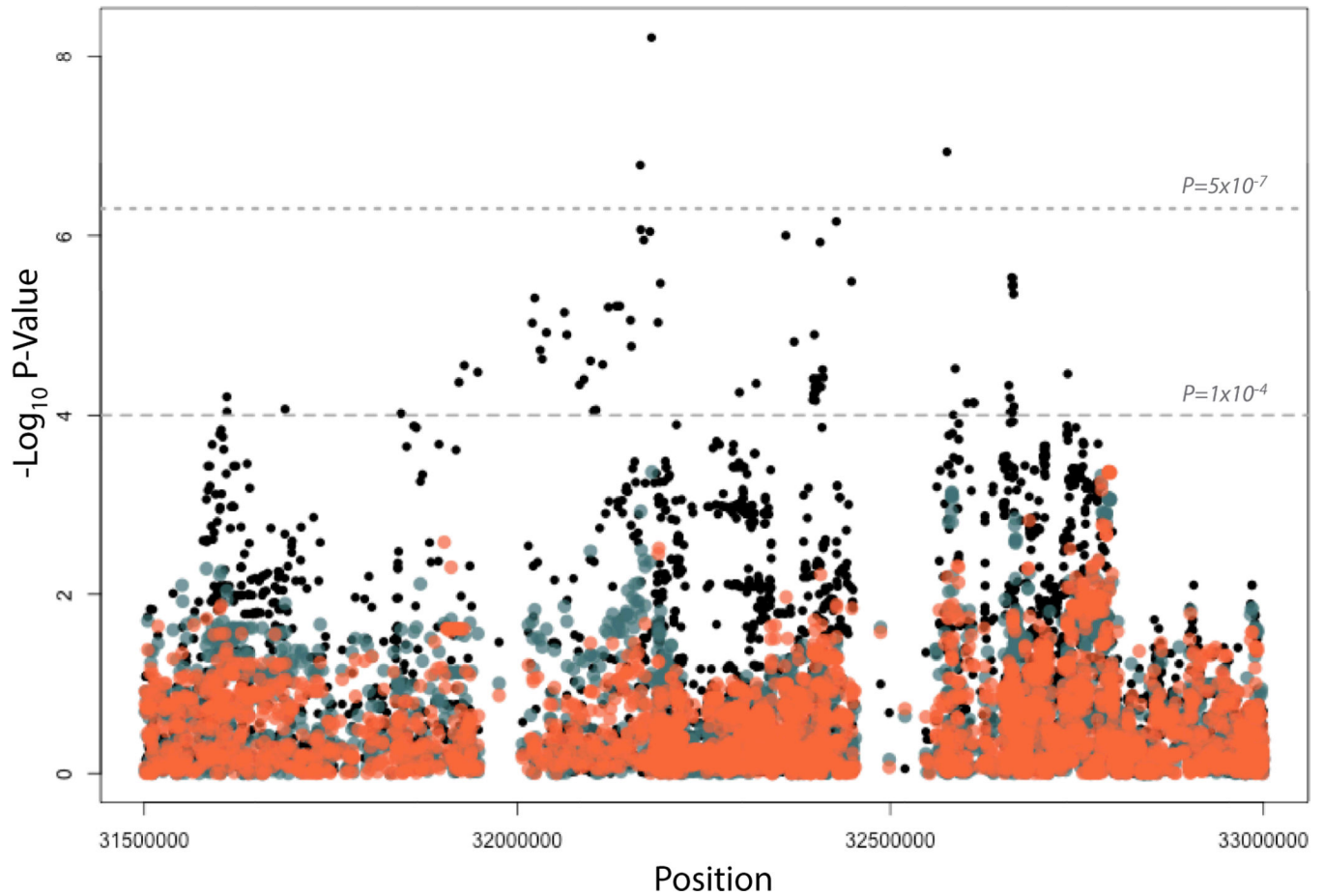


Figure 3. Conditional Associations in NOTCH4 and HLA Class II Region

The original associations are shown in black, with the associations conditioning on the top NOTCH4 SNP shown in orange. The associations conditioning on the top HLA Class II SNP is shown in blue. Genome-wide significance is indicated with the gray dashed line ($P=5\times 10^{-7}$). A threshold of 10^{-4} is shown with the lower dashed line

Table 1

Study demographics for the discovery and replication populations by case and control status.

	Discovery Samples			Replication Samples			Everyone		
	Control	Case	Total	Control	Case	Total	Control	Case	Total
% Female (N)	26.4 (118)	66.9 (238)	54.6 (438)	45.0 (81)	69.2 (63)	53.1 (144)	31.7 (199)	67.3 (301)	46.6 (500)
<i>Toulouse Cohort (FR)</i>	1	0	1	29	0	29	30	0	30
<i>BAHSTION (US)</i>	78	0	78	37	0	37	115	0	115
<i>Charles (US)</i>	0	0	0	0	20	20	0	20	20
<i>RomeCryo (IT)</i>	0	74	74	2	16	18	2	90	92
Study (country)	35	0	35	0	0	0	35	0	35
<i>Mangia (IT)</i>	114	25	139	111	24	135	225	49	274
<i>REVELL (US)</i>	127	0	127	0	0	0	127	0	127
<i>Cacoub (FR)</i>	0	72	72	0	15	15	0	87	87
<i>MASVE (IT)</i>	92	185	277	0	17	17	92	202	294
Total	447	356	803	179	92	271	626	448	1074

Table 2

Most significant associations from the Discovery GWAS of MC Vasculitis

Chr	SNP	MAF	OR	P-value	P _{Rep}	P _{Meta}	Gene
6	rs2071286	0.29	2.15	6.2E-09	0.18	1.7E-08	<i>NOTCH4</i>
6	rs9461776*	0.21	2.16	1.2E-07	0.01*	7.1E-09	<i>HLA-DRB1/DQA1</i>
6	rs2071279	0.38	1.90	1.6E-07	NA	NA	<i>NOTCH4</i>
6	rs9267820	0.37	1.83	8.6E-07	0.12	5.9E-07	<i>NOTCH4</i>
6	rs9267833	0.36	1.83	9.0E-07	0.12	6.0E-07	<i>NOTCH4</i>