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Identification of a novel genetic locus associated with immune-mediated thrombotic thrombocytopenic purpura

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ABSTRACT

Immune thrombotic thrombocytopenic purpura (iTTP) is an ultra-rare, life-threatening disorder, mediated through severe ADAMTS13 deficiency causing multi-system micro-thrombi formation, and has specific human leukocyte antigen associations. We undertook a large genome-wide association study to investigate additional genetically distinct associations in iTTP. We compared two iTTP patient cohorts with controls, following standardized genome-wide quality control procedures for single-nucleotide polymorphisms and imputed HLA types. Associations were functionally investigated using expression quantitative trait loci (eQTL), and motif binding prediction software. Independent associations consistent with previous findings in iTTP were detected at the HLA locus and in addition a novel association was detected on chromosome 3 (rs9884090, P=5.22x10^{-10}, odds ratio 0.40) in the UK discovery cohort. Meta-analysis, including the French replication cohort, strengthened the associations. The haploblock containing rs9884090 is associated with reduced protein O-glycosyltransferase 1 (POGLUT1) expression (eQTL P<0.05), and functional annotation suggested a potential causative variant (rs71767581). This work implicates POGLUT1 in iTTP pathophysiology and suggests altered post-translational modification of its targets may influence disease susceptibility.

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is an ultra-rare, life-threatening illness, with an annual incidence of approximately 6/million, and with an untreated mortality approaching 90% (10-20% with prompt intervention). It can affect patients of any age, but often affects young adults (30-40 years) and is more common in women.[1] The initial diagnosis of TTP is based on clinical suspicion, but ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) activity <10 IU/dL confirms the diagnosis. Severe deficiency of ADAMTS13 results in failure to cleave ultra-large von Willebrand Factor multimers (UL-VWF), crucial for normal hemostatic function and proteolytic regulation of VWF. ADAMTS13 deficiency in immune TTP (iTTP) is mediated through immunoglobulin G (IgG) autoantibodies.[2,3] The precipitant of the disease in most cases is unclear.[4]

As with many autoimmune diseases, human leukocyte antigen (HLA) type is associated with the risk of developing iTTP, with HLA-DRB1*11, HLA-DQB1*03 and HLADRB3* increasing risk, and HLA-DRB1*04 and HLA-DRB4 (HLA-DR53) being protective in Europeans.[5,6,7] No genetic risk factors outside the HLA genes have previously been shown to be associated with iTTP.

We performed a genome-wide association study (GWAS) in UK and French iTTP cohorts and identified association of alleles both within and beyond the HLA locus.

Methods

Cohorts

As part of the UK TTP registry, patients were consented for DNA analysis (MREC: 08/H0810/54) (see the *Online Supplementary Appendix*). Patients on the UK TTP registry were screened for the clinical diagnosis, and confirmed with an ADAMTS13 level <10 IU/dL at diagnosis (utilizing FRETS methodology)[8] and the presence of an anti-ADAMTS13 autoantibody.[2,3] The French replication cohort TTP samples were obtained from the French Reference Center for TMA (CNR-MAT) and informed consent was obtained from each patient with confirmed iTTP (see above criteria) (Institutional Review Board of Pitié Salpêtrière Hospital; clinicaltrials gov. Identifier: NCT00426686). The European control genotypes were obtained from the Wellcome Trust Case Control Consortium (WTCCC), both the 1958 British Birth Cohort and National Blood Service control samples.[9] In addition, controls were used from the Illumina reference panel[10] and Oxford controls.[11,12]

Genotyping, quality control and imputation

TTP samples were genotyped on the Illumina Human Omni Express single-nucleotide polymorphisms (SNP) chips and controls were genotyped on different SNP chips (see the *Online Supplementary Appendix*). Pre-imputation quality control was performed in all data sets separately, and then in a combined cohort (*Online Supplementary Figure S1*). Quality control (QC) was performed for individuals and SNP. Individuals were selected for further analysis by European ancestry principal component analysis (PCA) (see the *Online Supplementary Figure S2*). Only SNP present in all data sets were subsequently analyzed.

Genome-wide imputation was performed on markers that had passed quality control, and were present in all datasets using Beagle (version 5.0) utilizing the 1.000 Genome Project Phase 3 as a reference panel.[13] In addition to standardized QC, only SNP with a dosage R^2 (DR2) >0.8 were included.

Genome-wide association study and loci characterization

GWAS was performed using SNP & Variation Suite v8, using logistic regression with principal component correction.[14,15] The logistic regression *P*-values, odds ratios (OR) were calculated in addition to λ inflation factors, and QQ plots are shown (*Online Supplementary Figure S3*). A standardized genome wide significance level of $5x10^{-8}$ was applied.[15] For discovery and replication analysis meta-data please contact the authors.

Conditional analyses were undertaken using a full *versus* reduced regression model. Lead SNP at each locus were used as conditional inputs to determine independence, with results plotted using Locus Zoom software.[16]

Imputation of HLA types was performed utilizing SNP2HLA with previously genotyped markers.[17] Imputed HLA types were excluded if the R^2 (confidence) was <0.80. Conditional analyses were subsequently performed as described above.

Expression quantitative trait locus (eQTL) analysis was performed to associate identified SNP with differential gene expression.[18] Additional markers in linkage disequilibrium with the lead SNP at the chromosome 3 locus were identified by LD-link (https://ldlink.nci.nih.gov).[19] Functional annotation of the haploblock was performed using ChipSeq data via the UCSC genome browser (https://genome.ucsc.edu). Binding sites of transcription factors (highlighted through genome annotation) were obtained from FactorBook,[20] and position weight matrix (PWM) binding motifs generated. Binding motifs were generated using Mast-Meme.[21]

Results

Discovery cohort

Following quality control as outlined in the methods (*Online Supplementary Figure S1*) there were 241 TTP cases and 3,200 controls in the UK discovery cohort. Following imputation and quality control 3,649,347 SNP were available for analysis. Association testing was performed using a logistic regression model with PCA correction, and the genomic inflation factor (λ) was 1.0239 (*Online Supplementary Figure S3*).

In the UK discovery cohort two peaks were identified (Figure 1) (*Online Supplementary Figure S4*) (lead SNP are summarized in Table 1). The peak with the strongest association corresponded to the class II HLA region on chromosome 6, with 1,017 SNP reaching genome wide significance. The lead SNP rs28383233 located in the intergenic region between *HLA-DRB1* and *HLA-DQA1* ($P=2.20x10^{-23}$, OR 3.12, 95% Confidence Interval [CI]: 2.49-3.93) (Table 1; Figure 2).

Conditional analysis was performed on rs28383233 and the lead SNP following this was rs1064994 (within *HLA-DQA1*), with a *P*-value of $1.13x10^{-10}$ (OR 2.20, 95% CI: 2.06-3.37). Following conditioning on both rs28383233 and rs1064994 no further markers reached significance within the class II HLA region, indicating that there are two detectable independent genetic associations with iTTP within the HLA region.

HLA imputation was performed on the UK discovery cohort, and following quality control, 95 imputed HLA alleles remained. HLA-DRB1*11:01 was the allele most strongly associated with iTTP, with a *P*-value of $3.25x10^{-17}$ (OR 2.79, 95% CI: 2.23-3.50). Following conditional analysis of HLA-DRB1*1101, no other HLA types reached genome wide significance, but HLA-DQA1*03:01 remained significant (with a HLA-only Bonferroni correction, $P<5.26x10^{-4}$) at $1.49x10^{-6}$ (OR 0.47, 95% CI: 0.33-0.65) suggesting that the protective effect of this allele is independent of HLA-DRB1*11:01.

In addition to the class II HLA peak on chromosome 6, a novel association was observed on chromosome 3. Sixteen markers reached genome wide significance, with the lead SNP, rs9884090(A), having a *P*-value of $5.22x10^{-10}$ (OR 0.40, 95% CI: 0.29-0.56) (Table 1; Figure 3). Upon conditional analysis of the lead SNP no markers reached genome wide significance indicating one detectable signal at this locus. No statistical epistasis was seen between the chromosome 3 and chromosome 6 associations, with each association being independent. Five genes are annotated within this chromosome 3 haploblock: *ARHGAP31, TMEM39A, POGLUT1, TIMMDC1*, and *CD80*.

Replication cohort

Within the French replication cohort there were 112 cases and 2,603 controls following quality control as outlined in the methods (*Online Supplementary Figures S1* and *S2*). 3,649,546 SNP were available for analysis, and asso-

Table 1. Lead single nucleotide polymorphisms identified in the UK discovery cohort.

rsID (position)	Minor allele / Major allele	MAF cases / MAF controls	Logistic regression *P*-value	Odds Ratio (95% CI)
rs9884090 (ch3:119116150)	A/G	0.08/0.19	$P = 5.22x10^{-10}$	0.40 (0.29-0.56)
rs28383233 (ch6:32584153)	G/A	0.64/0.40	$P = 2.20x10^{-23}$	3.12 (2.49-3.93)
rs1064994 (ch6:32611195)	C/T	0.25/0.11	$P = 1.13x10^{-10}$	2.20 (2.06-3.37)

Displayed are Minor/Major Alleles, Minor Allele Frequencies (MAF), logistic regression *P*-value (corrected for principal component analysis stratification), and odds ratio (with 95% Confidence Intervals [CI]). Genomic positions refer to Human Assembly GRCh37/hg19.

ciation testing was performed using a logistic regression model with PCA correction, and λ was 1.0830 (*Online Supplementary Figure S5*).

The association with the lead SNP in the chromosome 3 haploblock, rs9884090(A) was replicated with a *P*-value of 0.001 (OR 0.52), and the two independent lead SNP with the class II HLA peak on chromosome 6 were also replicated (Table 2). The locus zoom plots are shown (*Online Supplementary Figures S6* to *S8*). Imputed HLA type analysis was also consistent with the UK discovery cohort with HLA-DRB1*11:01 and HLA-DQA1*03:01 representing two independent HLA signals.

In addition, a meta-analysis was performed combining the UK and French cohorts (cases 241/112, controls 3,200/2,603 respectively), which demonstrated strengthening of the previously observed signal (rs9884090 P=1.60x10^{-10}, OR 0.47, rs28383233 P=1.22x10^{-42}, OR 3.70, rs1064994 P=5.03x10^{-25}, OR 2.89) (Table 3; *Online Supplementary Figure S9*).

Expression quantitative trait loci and functional DNA analysis

eQTL data from the Genotype Tissue Expression Project and Blood eQTL Browser for the lead SNP at the chromosome 3 locus (rs9884090) demonstrated significant reduction in expression of *POGLUT1* with the protective allele in the majority of tissues tested, including blood cells (P<0.001).[18,22] LD-link identified 20 markers found to be in tight linkage disequilibrium (R2 and D' >0.80) with rs9884090 contained within the chromosomal region (see the *Online Supplementary Table S1*).[19] All markers were functionally annotated with information from the UCSC Genome Browser (Human Assembly GRCh37/hg19)[23,24] (see the *Online Supplementary Table S1*). One variant was particularly noted, rs71767581 (Ch3, 119187422 AC/-del), which is a 2-basepair deletion in the promoter of POGLUT1. This may be functionally important as the haploblock identified is associated with reduced expression in *POGLUT1*. Upon analysis of ChipSeq data in UCSC Genome Browser 14 transcription factors were predicted to bind at this site (see the *Online Supplementary Table S2*), adding further evidence that rs71767581 may be functionally important for POGLUT1 expression.

Discussion

This GWAS, involving two European populations, is the first to be performed in iTTP and shows consistent evidence of association at loci on chromosome 6 and chromosome 3. The associated alleles on chromosome 6 lie within the HLA region and imputation of HLA types and conditional analyses indicated independent association between HLA-DRB1*11:01 (OR 2.79; P=3.25x10^{-17}) and HLA-DQA1*03:01 (OR 0.47; P=1.49x10^{-6}, post conditional analysis), which are consistent, and in linkage with previously published risk and protective associations with iTTP at this locus.[5–7] A recent case-control study comparing frequency of alleles only at immune loci in 190 Italian TTP patients and 1,255 controls identified the HLA variant rs6903608, (in addition to HLA-DQB1*05:03) as conferring a 2.5-fold increase of developing TTP.[25]

Here we also identified a novel association of iTTP with alleles on chromosome 3 tagged by the lead SNP rs9884090. Five genes are located within the associated haploblock: *ARHGAP31, TMEM39A, POGLUT1, TIMMDC1*, and *CD80. ARHGAP31* (rho GTPase activating protein 31) is associated with the autosomal dominant condition Adams-Oliver Syndrome (OMIM 100300).[26] Mutations within *ARHGAP31* have been implicated with abnormal vascular development and VEGF (vascular endothelial growth factor) angiogenesis.[27] Little is understood regarding the function of *TMEM39A* (transmembrane protein 39A). While variants have been implicated in autoimmune disease such as systemic lupus erythematosus[28,29] and multiple sclerosis,[30,31] understanding of

Table 2. French cohort replication of lead single nucleotide polymorphisms identified in the UK discovery cohort.

rsID (position)	Minor Allele / Major Allele	MAF Cases / MAF Controls	Logistic Regression *P*-value	Odds Ratio (95% CI)
rs9884090 (ch3:119116150)	A/G	0.10/0.18	$P = 0.001$	0.52 (0.34-0.81)
rs28383233 (ch6:32584153)	G/A	0.68/0.40	$P = 3.87x10^{-9}$	2.57 (1.87-3.53)
rs1064994 (ch6:32611195)	C/T	0.42/0.11	$P = 5.015x10^{-9}$	2.86 (2.06-3.99)

Displayed are Minor/Major Alleles, Minor Allele Frequencies (MAF), logistic regression *P*-value (corrected for principal component analysis stratification), and odds ratio (with 95% Confidence Intervals [CI]). Genomic positions refer to Human Assembly GRCh37/hg19.

Table 3. Meta-analysis combining UK and French Cohorts, showing lead single nucleotide polymorphisms identified in the UK discover cohort.

rsID (position)	Minor Allele / Major Allele	MAF Cases / MAF Controls	Logistic Regression *P*-value	Odds Ratio (95% CI)
rs9884090 (ch3:119116150)	A/G	0.08/0.19	P =1.60x10-10	0.47 (0.36-0.60)
rs28383233 (ch6:32584153)	G/A	0.64/0.41	P = 1.22x10-42	3.70 (2.81-4.03)
rs1064994 (ch6:32611195)	C/T	0.22/0.11	P = 5.03x10-25	2.89 (2.39-3.49)

Displayed are Minor/Major Alleles, Minor Allele Frequencies (MAF), logistic regression *P*-value (corrected for principal component analysis stratification), and odds ratio (with 95% Confidence Intervals [CI]). Genomic positions refer to Human Assembly GRCh37/hg19.

its function is lacking. *TIMMDC1* is a membrane embedded mitochondrial complex factor, and is associated with mitochondrial disorders.[32] The protein encoded by the CD80 gene functions as a membrane receptor being activated by CTLA-4 or CD28, both of which are T-cell receptors. The downstream mechanisms are T-cell proliferation and cytokine production. CD80 and its receptors have been associated with focal segmental glomerulosclerosis33 and systemic lupus erythematosus.[34,35] *POGLUT1* (protein O-glucosyltransferase 1) is mutated in Dowling-Degos disease-4 (an autosomal dominant genodermatosis with progressive and disfiguring reticulate hyperpigmentation and muscular dystrophy, OMIM 615696) and *POGLUT1* has been shown to catalyse O-glycosylation of epidermal growth factor (EGF)-like repeats.[36,37] EGF-like repeats are well conserved structures, and highly represented with proteins involved in coagulation.[38,39] *In vitro* work has demonstrated POGLUT1 binds and glycosylates specific coagulation factors including factor VII and factor IX.[37,40]

The haploblock identified in this analysis of iTTP (which is tagged by rs9884090(A)) is associated with significantly decreased *POGLUT1* expression by eQTL.[41] Several other genetic variants contained within this haploblock have been associated with other autoimmune diseases, and the majority of these variants have been shown to be in linkage with our lead variant rs9884090 (see the *Online Supplementary Appendix*), supporting the findings described here.[28,29,31,42,43,44] eQTL analysis is a robust tool, that can associate gene expression with specific genetic variants. Our analysis found rs9884090(A) to have a reduced frequency in iTTP, and rs9884090(A) was shown to be associated with significantly decreased *POGLUT1* expression in different eQTL resources.[18,22] In order to locate the underlying genetic variant implicated in this reduced *POGLUT1* expression we used LD-link to identify additional variants, and located a 2-basepair deletion with the *POGLUT1* upstream promoter region that is in tight linkage disequilibrium with the lead associated variant (R2/D'>0.80). As rs9884090(A) confers reduced risk of developing iTTP, we hypothesize that reduced expression of *POGLUT1* leads to altered posttranslational modification (O-glycosylation) of key POGLUT1 targets to reduce the risk of iTTP. The evidence we present supports POGLUT1 as the gene of interest, but we cannot exclude other genes within the associated haploblock. The pathway through which POGLUT1's effects could be mediated remains to be determined. Given there are several reported variants with this haploblock associated with different autoimmune disease, it is likely the downstream functional consequences medicated through POGLUT1 influence immune-regulatory pathways which may generally increase the risk of other autoimmune disease, in addition to iTTP, and may provide insights into potential therapies.[45–56]

In summary, we have identified a novel genetic variant, rs9884090(A), in two independent populations, which is associated with reduced risk of iTTP. Utilizing linkage disequilibrium we have identified a functional variant in tight LD with the lead SNP in the POGLUT1 promoter

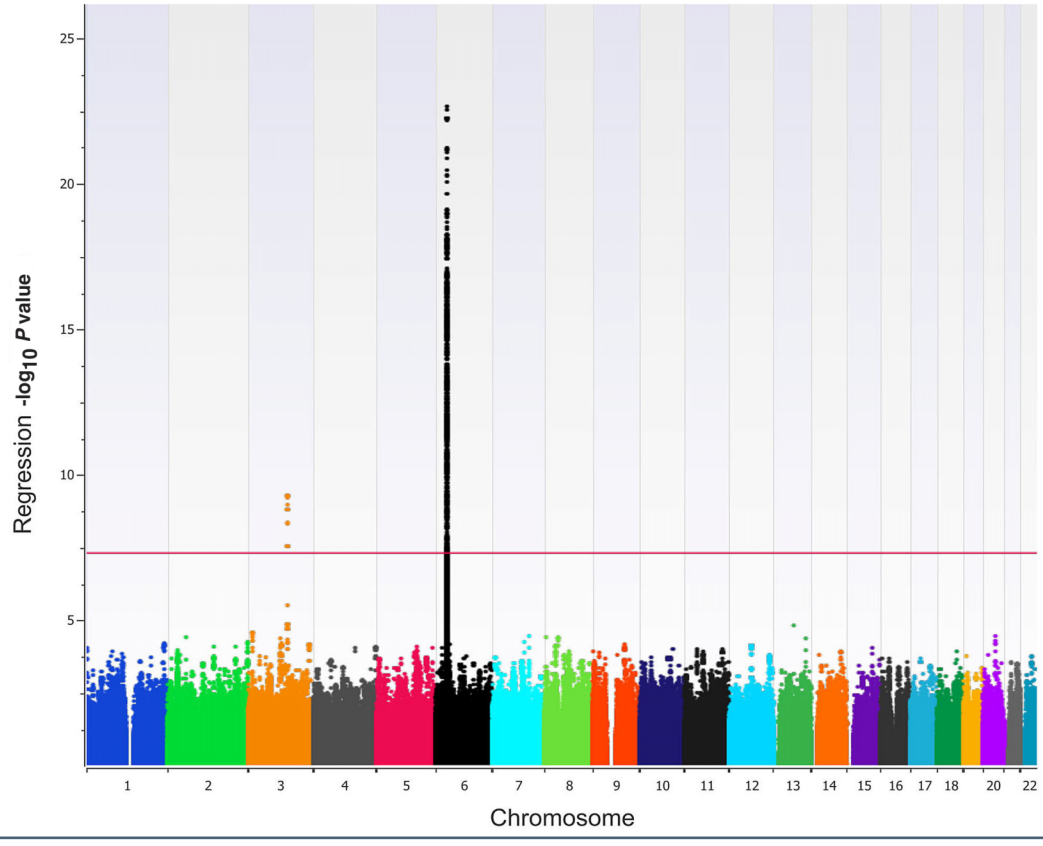

Figure 1. Manhattan plot of genome wide association analysis comparing UK immune thrombotic thrombocytopenic purpura discovery cohort compared with controls. The x-axis shows chromosome location, and the y-axis shows negative logarithmic *P*-values. Standardized genome wide significant $5x10^{-8}$ is depicted by the red line. The human leukocyte antigen peak is visualized on chromosome 6 (black), in addition to the novel chromosome 3 association (orange).

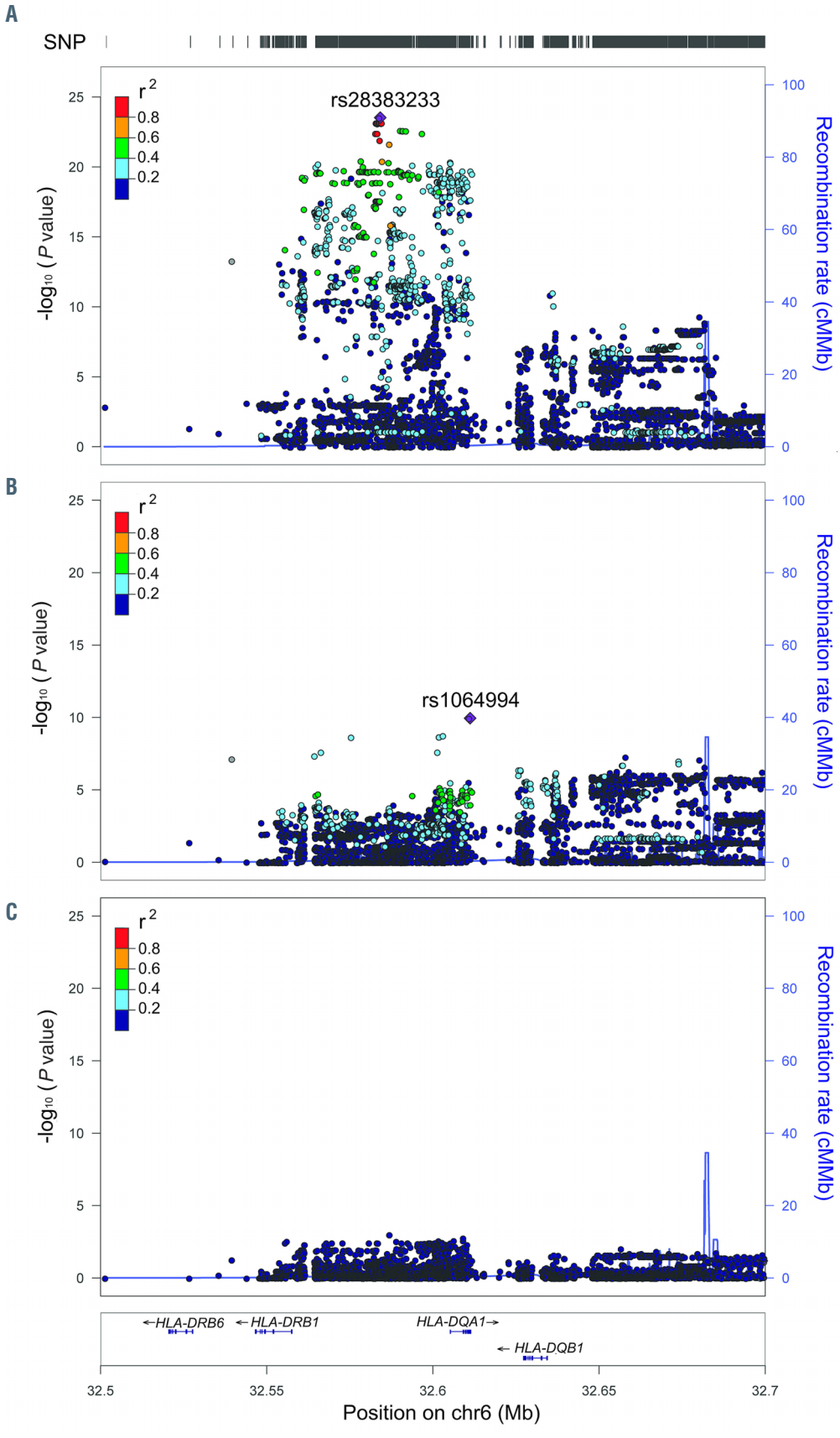

Figure 2. Locus zoom plots of the chromosome 6 peak in the UK discovery cohort. The upper plot (A) shows the unconditioned analysis with the lead single-nucleotide polymorphisms rs28383233, and the middle plot (B) shows analysis conditioned on the lead SNP rs28383233, revealing independent association with rs1064994. The lower plot (C) shows analysis conditioned on both rs28323233 and rs1064994. Genomic positions refer to Human Assembly GRCh37/hg19. chr6: chromosome 6.

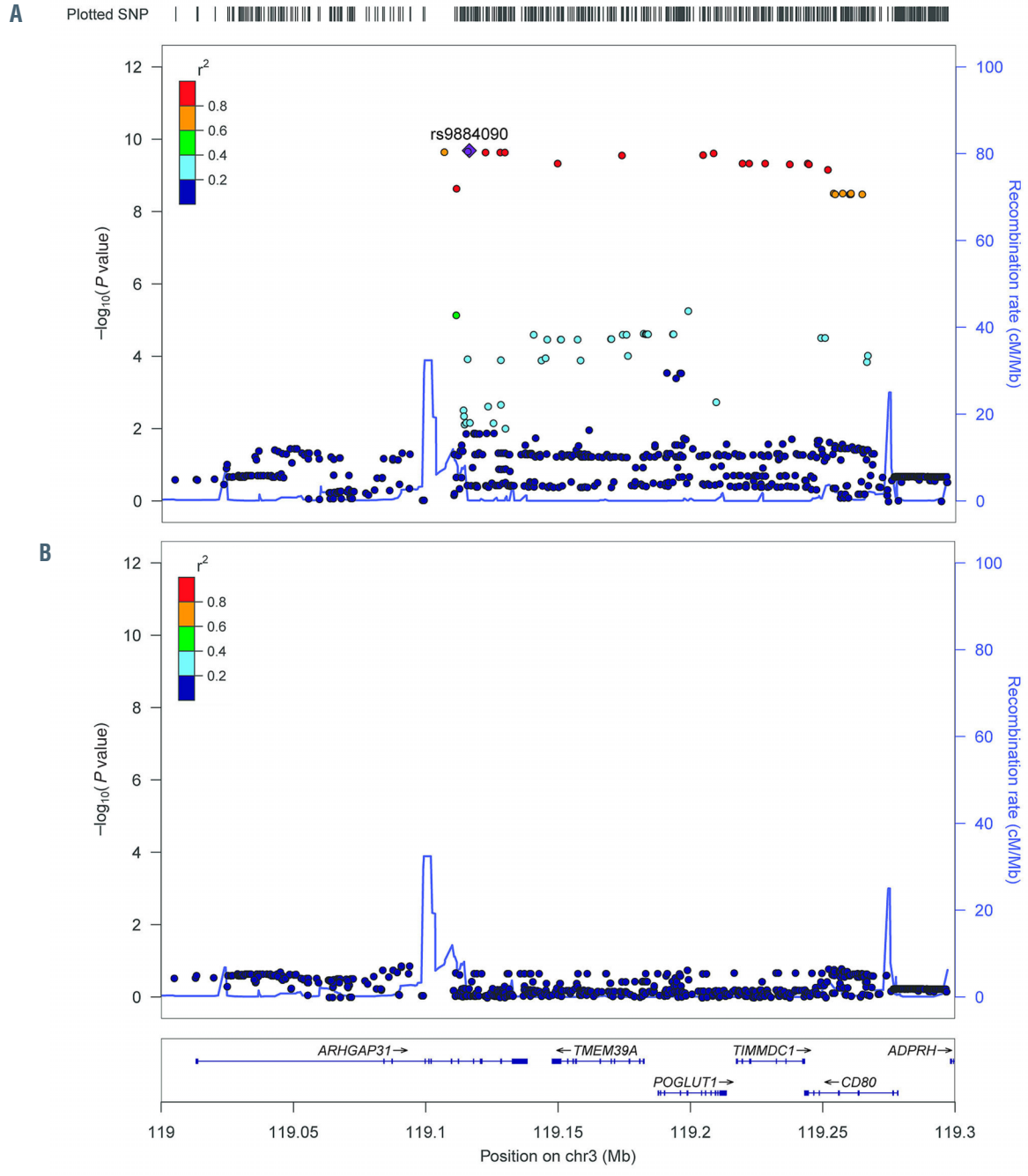

Figure 3. Locus zoom plots of the chromosome 3 peak in the UK discovery cohort. The upper plot (A) shows the unconditioned analysis and the lower plot (B) shows associations of the same markers when conditioned on the lead single-nucleotide polymorphisms (SNP), rs9884090. Genomic positions refer to Human Assembly GRCh37/hg19. chr3: chromosome 3.

site and eQTL demonstrates reduced POGLUT1 expression associated with this variant. We therefore hypothesize this leads to altered O-glycosylation on POGLUT1 targets. Whilst the exact role of POGLUT1 in the pathophysiology of iTTP requires further downstream functional analysis, this work represents an important step forward in our understanding of iTTP.

Disclosures

MJS received research funding from Shire/Takeda; PC sits on the advisory board and received symposia fees from Sanofi, Alexion and Roche, received fees from Octapharma; AV sits on the advisory board of Ablynx/Sanofi, Roche-Chugai, and Shire/Takeda, received course fees and awards from LFB Biomédicaments, Octapharma and CSL-Behring; MT sits on the advisory board of Sanofi; YB sits on the advisory board of Sanofi and Octapharma; PP sits on the advisory board of Sanofi; DPG received honoraria and sits on the advisory board of Alexion; MS consults, received honoraria, sits on the advisory board, received speakers fees from Novarits, received honoraria, sits on the advisory board, received research funding and speakers fees from Shire/Takeda, consults for, received honoraria, sits on the advisory board, received speakers fees from Ablynx/Sanofi and Shire/Takeda, received honoraria, sits on the advisory board and speakers bureau of Alexion, received research funding from Baxalta: All other authors have no conflicts of interest to disclose.

Contributions

MJS designed research, recruited patients, performed research, collected data, analyzed and interpreted data, wrote the manuscript; PC designed research, recruited patients, analyzed and interpreted data, wrote the manuscript; CC, SD, VP and APL performed research, collected data, analyzed and interpreted data, wrote the manuscript; AV designed research, recruited patients, analyzed and interpreted data, wrote the manuscript; MT designed research, recruited patients, analysed and interpreted data, wrote the manuscript; JOC designed research, wrote the manuscript; MH designed research, wrote the manuscript; YB, LG and PP designed research, recruited patients, wrote the manuscript; RK, DPG, HS and MAS designed research, performed research, analyzed and interpreted data, wrote the manuscript.

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