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Common *Toll-like receptor 7* variants define disease risk and phenotypes in juvenile-onset systemic lupus erythematosus

Yves Renaudineau [a,b,2], Francesca Sposito [c,2], Valentina Natoli [c,d], Amandine Charras [c], Jenny Hawkes [c], Joni Roachdown [c], Mathieu Fusaro [a,b], Eve MD. Smith [c,e], Michael W. Beresford [c,e], Christian M. Hedrich [c,e,*], on behalf of theUK jSLE Cohort Study [1]

[a] *Immunology Department Laboratory, Referral Medical Biology Laboratory, Institut Fédératif de Biologie, Toulouse University Hospital Center, France*
[b] *INFINITy, Toulouse Institute for Infectious and Inflammatory Diseases, INSERM, U1291, CNRS U5051, University Toulouse III, Toulouse, France*
[c] *Department of Women's & Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool, UK*
[d] *Università Degli Studi di Genova, Dipartimento di Neuroscienze, Riabilitazione, Oftalmologia, Genetica e Scienze Materno-infantili, DINOGMI, Genoa, Italy*
[e] *Department of Rheumatology, Alder Hey Children's NHS Foundation Trust, Liverpool, UK*

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ABSTRACT

Toll-like receptor *(TLR)7* contributes to type I interferon (IFN) expression in systemic lupus erythematosus (SLE). This study investigated genetic variability of *TLR7* in 319 juvenile-onset (j)SLE patients from the UK. New generation sequencing was used to associate "common" *TLR7* variants with demographic and clinical features. Three jSLE-associated variants with *in silico* predicted impact on gene function presented minor allele frequencies ≥5 %: rs2302267/n.-20T > G (*TLR7* promoter); rs179008/p.Gln11Leu (missense variant with predicted loss-of-function); and rs3853839/c.*881C > G (*TLR7* 3′UTR). The risk to develop jSLE was increased in African/Caribbean girls carrying rs3853839 GC/GG (OR: 1.8; 95 %-CI: 1.2–2.9), while the risk associated with this variant was reduced in European girls (OR: 0.5; 95 %-CI: 0.4–0.7). At inclusion, rs3853839 minor G allele carrier status associated with activity in the mucocutaneous BILAG domain ($p = 0.004$), "older" age at diagnosis ($p = 0.003$, Asian), C3 consumption ($p = 0.015$, boys), and higher anti-dsDNA antibody levels ($p = 0.015$, African/Caribbean). The negative linkage disequilibrium between rs179008 (T-C/TT) and rs3853839 (CC) associated with increased global disease activity (pBILAG-2004), and activity in the constitutional and musculoskeletal pBILAG domains. Functionally, rs2302267/n.-20T > G, may protect from leukopenia through reduced *TLR7* promoter activity, while rs3853839/c.*881C > G-3′UTR increases TLR7 mRNA stability contributing to increased gene expression. In conclusion, common *TLR7* variants may influence jSLE risk and organ involvement in an ancestry-specific manner. Observations argue for genetic risk stratification and future consideration of gene variants affecting *TLR7* to guide personalized treatment and care strategies.

1. Introduction

Systemic lupus erythematosus (SLE) is a severe autoimmune/inflammatory disease that can affect any organ of the human body [1]. Approximately 15–20 % of SLE patients develop disease during childhood or adolescence and are therefore classified as having juvenile-onset SLE (jSLE) [2,3]. When compared to patients with adult-onset disease, jSLE patients exhibit more variable clinical presentations, higher disease activity and more organ damage already at diagnosis with a higher need for immunomodulating treatments [4,5].

In females, puberty represents a critical event for the development of SLE. Notably, in children <7 years of age, sex distribution is almost equal but changes to a 9–10:1 imbalance towards female sex after puberty. Factors favoring female sex in jSLE are manifold and include hormonal factors (estrogens) and the duplication of the X chromosome that contains SLE-associated genes [6]. Indeed, the number of X chromosomes associates with SLE risk. Incidences of SLE that range between 0.2 and 0.5/100,000 individuals/year in men (46, XY) and women with

* Corresponding author. Department of Women's & Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool, UK
E-mail address: christian.hedrich@liverpool.ac.uk (C.M. Hedrich).
[1] UK jSLE Cohort Study Group.
[2] Equal contribution.

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Turner's syndrome (45, X0), 2–15/100,000 individuals/year in euploid women (46, XX), and further 10-15-fold increased risk among individuals with polysomy X (e.g., 47, XXX; 48, XXXX, etc.) [7].

Among the immune-related genes on the X chromosome (15 % of the 1146 X genes), *Toll-like receptor (TLR)7* encodes for the innate pattern recognition receptor TLR7 that is centrally involved in the activation of type I interferons (IFN). Notably, *TLR7* and Xp22.2, a genomic region distal to *TLR7*, have been identified as risk loci for SLE. Indeed, TLR7 dysregulation in SLE results from several molecular disturbances, including (but may not be limited to) i) defective X chromosome inactivation (XCI) and resulting bi-allelic gene expression in women [8], ii) *TLR7* gene duplication (e.g., the *Yaa* Y-linked autoimmune accelerator gene) [9], iii) common gene variants affecting TLR7 expression [10–12], iv) type I IFN gene:gene interrelations, referred to as epistasis, involving *TLR7* gene polymorphisms [13], and/or v) single nucleotide polymorphisms (SNPs) affecting transcript stability [14]. Recently, ultra-rare gene variants affecting *TLR7* [15,16] or *UNC93B1* [17,18], a scaffolding protein involved in subcellular trafficking and functional control of TLR7, have been linked with TLR7 gain-of-function resulting in SLE or SLE/like disease [16] further underscoring the importance of a tight control of TLR7 signaling for immune homeostasis.

This project delivered a detailed genotype/haplotype analysis of the sex-chromosome-associated *TLR7* locus, linking common variants with a minor allele frequency (MAF) > 5 % among jSLE patients with clinical and biological phenotypes as well as functional impact.

2. Material and methods

2.1. Study cohort

Genomic DNA samples, isolated from whole blood, were selected from 319 jSLE patients enrolled in the UK J-SLE Cohort Study (http://www.liv.ac.uk/ukjsle), with disease-onset before their 18th birthday, and who fulfilled ≥4 American College of Rheumatology (ACR) 1997 classification criteria for SLE [19]. This cohort excluded the twelve patients previously classified as having monosomic "genetic" SLE from the bioinformatical and statistical analysis [5,20]. The following datasets were collected at inclusion and during follow-up (i) demographic information (age, sex, ethnic background); (ii) global disease activity scores, including the pediatric British Isles Lupus Assessment Grade 2004 (pBILAG-2004) and the SLE Disease Activity Index 2000 (SLEDAI-2K) [19,20]; (iii) presence and/or severity of disease at the organ level (numeric version of pBILAG-2004: A = 12, B = 8, C = 1, and D/E = 0) [21]; (iv) SLE-associated laboratory markers, including anti-double-stranded (ds)DNA, anti-Smith (Sm), anti-ribonucleoprotein (RNP), anti-Sjögren's-syndrome-related antigen A (SSA)/Ro and SSB/La autoantibodies (Abs), and complement C3 and C4 fractions as previously described [22–25]. Furthermore, during follow-up, the following datapoints were collected: (v) highest disease activity score (SLEDAI, and pBILAG-2004), organ domain evolution ("major improvement", "minor improvement"/"stability", and "deterioration" as suggested previously [20]), flares per year, and treatment intensity groups. Patients were categorised in the "intensive treatment" group if, throughout the disease course, they received either cyclophosphamide, rituximab or belimumab, and/or two or more conventional disease-modifying anti-rheumatic drugs (DMARDs), excluding hydroxychloroquine, simultaneously for ≥2 consecutive visits, and in the "non-intensive treatment" group, when a maximum of one conventional DMARD, including hydroxychloroquine, was used at a time. In the analysis, organ/system involvement was deemed to be present when a pBILAG-2004 score equal to A, B, or C was reported. The study received ethical approval from the National Research Ethics Service Northwest (REC 06/Q1502/77). The study was conducted in accordance with the declaration of Helsinki, and all participants and/or their legal guardians gave written informed patient assent/consent and parental consent.

2.2. Sequencing and bioinformatic analysis

Sequence capture probes (NimbleGen/Roche) were designed to target exonic regions and exon:intron junctions of pre-selected genes, as well as defined regions around previosyly reported SLE-associated non-coring variants (SNPs) as described previously [20]. Among the genes sequences in total was *TLR7*. Sequencing libraries were prepared from genomic DNA, hybridized to the probes and then sequenced with 150bp paired-end reads using Illumina MiSeq technology (Illumina). Demultiplexing, adaptor and quality trimming (Cutadapt v1.2.1, Sickle v1.2) of reads was performed [26,27]. Polymerase Chain Reaction (PCR) duplicates were identified and excluded from the dataset using Picard [28]. Sequencing data were aligned to the human reference genome (hg38) using bwa [29] and variants were subsequently detected with Genome Analysis Toolkit (GATK) Software [29,30].

The publicly available Ensembl Variant Effect Predictor tool (https://www.ensembl.org/) using *in silico* SnpEFF, *Sorting Intolerant From Tolerant* (SIFT) and *Combined Annotation Dependent Depletion* (CADD) scores were used to predict functional consequences of variants. The *Genotype-Tissue Expression* website (GTEx, https://gtexportal.org/) was used to test methylation (m) and expression (e) quantitative trait loci (QTL) association at *TLR7*, and clinical associations were investigated using Pubmed, and ClinVar (July 2023) as described previously [31–33].

To determine minor allele frequencies (MAF) within the jSLE patient cohort, we adopted an allele coding strategy by considering hemizygous men (0 null, 2 hemizygous for the minor allele) equivalent to homozygous females (0 null, 1 heterozygous, 2 homozygous) as proposed previously [34]. Allele frequencies were extracted from three large-scale international genome projects that consider racial origin: namely *Allele Frequency Aggregator* (ALFA) release 2 project from the *database of Genotypes and Phenotypes* (dbGaP), 1000 genomes project and the *genome Aggregation Database* (gnomAD) (https://www.ncbi.nlm.nih.gov/snp). Haplotype analyses and linkage disequilibrium (LD) parameters (D', rho, and *p* value) were investigated by using SNPstats (http://bioinfo.iconcologia.net/SNPstats) [35].

2.3. Prediction of functional impact and laboratory assays

Transcription factor:DNA interactions **–** To map SNPs against their exact genomic location, previously reported regulatory elements and/or cross-species conserved sequences that may be of regulatory impact [36], we performed a web-based alignment of mammalian *TLR7* genes using the University of California, Santa Cruz Genome Browser (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&position=chr1%3A206940947-206945839).

Transcription factor binding elements affected by the enhancer polymorphism rs2302267 (n.-20T > G) were predicted using USCS Genome Browser (GRCh37/hg19; https://genome-euro.ucsc.edu). The 50bp-spanning flaking sequence of rs2302267 (n.-20T > G) was used to identify disruption of transcription factor motifs applying RStudio environment and packages TxDb.Hsapiens.UCSC.hg19.knownGene, BSgenome.Hsapiens.UCSC.hg19, PWMEnrich, PWMEnrich.Hsapiens.background, GenomicFeatures, and Biostrings.

Forced gene expression **–** To test the functional impact of the "common" 3′UTR variant rs3853839 (MAF >5 %), empty pcDNA3.1 plasmids or expression plasmids carrying the wild-type *TLR7* cDNA plus the 3′UTR sequence or the *TLR7* cDNA plus the variant 3′UTR sequence were introduced into HEK293 T cells (ATCC) using Lipofectamine 2000. After over-night incubation, some cells were stimulated with the TLR7/8 agonist resiquimod (R848) as described previously [37]. Cells were harvested after 18h and lysed to isolate RNA and generate cDNA (kit) for qRT-PCR analysis of IFN associated gene expression ("IFN signature"). The following genes were tested: IFIT1 (forward 5′-TGAGCCTCCT TGGGTTCGTCTAC-3′, reverse 5′-CTCAAAGTCAGCAGCCAGTCTCAG-3′); IFI27 (forward 5′-CTTCACTGCGGCGGGAATC-3′, reverse 5′-CCAG

GATGAACTTGGTCAATCC-3′); IFI44L (forward 5′-AGCCGTCAGGGATG TACTATAAC-3′, reverse 5′-AGGGAATCATTTGGCTCTGTAGA-3′); ISG15 (forward 5′-CACAGCCATGGGCTGGGACCTG, reverse 5′-GCACGCCATC TTCTGGGTGA-3′); RSAD2 (forward 5′-GCATCGTGAGCAATGGA-3′ reverse 5′-CCACGGCCAATAAGGAC-3′); SIGLEC1 (forward 5′-GGCTGT TACGATGGTTTATGATGT-3′, reverse 5′-AATCAAAGGCATCATTTTAG GGATA-3′). IFN signatures were calculated using the methods previously suggested by Rice et al. [38].

RNA stability assays – To test effects of the *TLR7* 3′UTR variant rs3853839 on mRNA stability, HEK293 T cells (ATCC) were transfected with empty plasmids, expression plasmids carrying wild-type or variant *TLR7* rs3853839 (GenScript) using Lipofectamine 2000 as above. Cells were inculcated overnight; some cells were treated with vehicle controls (DMSO) or the transcriptional inhibitor 5,6-dichloro-l-@-ribo-furanosyl benzimidazole (DRB) (20μg/mL,Merck) [39,40]. TLR7 (or IFN, as indicated) expression was quantified using RT-PCR.

Luciferase reporter assays and transcription factor recruitment– To investigate effects of rs2302267 (n.-20T > G) on *TLR7* promoter activity, we generated luciferase reporter assays (pGL3.1 vectors) including wild-type or variant core promoter promoter sequences (covering positions −220 to +18 from the transcriptional start site (Fig. 7B)). HEK293T cells were transfected with the indicated plasmids, using Luciferase 2000 reagent. Five hours after transfection, cells were collected and lysed, and luciferase activity was quantified using the Promega Dual Luciferase Assay System (Promega).

Based on *in silico* prediction of transcription factor binding motifs affected by TLR7 rs2302267 (see above; USCS Genome Browser), we investigated transcription factor recruitment to the region under resting conditions (with vehicle control dimethyl sulfoxide (DMSO)) and after stimulation with phorbol 12-myristate 13-acetate (PMA) as. Anti-LEF1 (Cell Signaling Technology, cat. 76010S), anti-TCF7L1 (Invitrogen, cat. PA5-40327), and normal rabbit IgG were obtained (Invitrogen, MAGnify™ Chromatin Immunoprecipitation System, cat. 492024). Reporter ChIP off the luciferase reporter constructs transfected into HEK293 T cells was carried out according to the manufacturer's instructions (Invitrogen, Life Technologies) and as reported previously [41]. ChIP-DNA was subject to real-time qPCR.

2.4. Statistical analysis

Quantitative data are presented as median ± interquartile [IQ] and were compared using Mann-Whitney test for pairwise comparisons or multiple Kruskal-Wallis with post-hoc Dunn's test for multiple group comparisons. Fisher's exact test was selected for qualitative data, and Odds Ratios (OR) with 95 % Confidence Intervals (95 % CI) were calculated where appropriate. Statistical significance was assessed with two-tailed p values, and assumed with $p < 0.05$. When justified, the p value threshold was adjusted for multiple comparisons using post-hoc False Discovery Rates (FDR). Analyses were completed using PRISM 9.5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Demographic and clinical information

A total of 319 jSLE patients were enrolled in this study, including 51 boys and 268 girls (Table 1). The median age at diagnosis was 12.8 years [IQ: 10.3–14.5]; median age at inclusion in the jSLE study cohort was 13.0 years [IQ: 11.0–15.0]. Most patients were of European (45.8 %), followed by Asian (34.5 %), and black African/Caribbean (16.9 %) ancestry. At first visit/inclusion, patients predominantly exhibited hematological (62.9 %), mucocutaneous (58.8 %), musculoskeletal (55.3 %), constitutional (40.6 %), and renal (39.0 %) involvement.

When comparing boys and girls, except for ancestry ($p < 10^{-4}$), comparable profiles were reported regarding age at diagnosis, pBILAG-2004 and SLEDAI-2K global scores, pBILAG-2004 organ domains (qualitatively and quantitatively), as well as autoantibody profiles including IgG anti-dsDNA Abs, C3/C4 complement fractions, and white blood cell (WBC) counts.

Table 1
Demographic and clinical information.

	All (n = 319)	Males (n = 51)	Females (n = 268)	*p* value (M vs F, FDR: p < 0.005)
Age at diagnosis	12.8 [10.3–14.5]	12.2 [9.8–14.3]	12.8 [10.5–14.5]	0.19
Age at 1st visit	13.0 [11.0–15.0]	13.0 [10.0–14.5]	13.0 [11.0–15.0]	0.07
African/Asian/ European/ Unknown	54 (16.9 %)/110 (34.4 %)/146 (45.7 %)/9 (2.8 %)	6 (11.7)/25 (49 %)/18 (35.3 %)/2 (3.9 %)	48 (17.9 %)/85 (31.7 %)/128 (47.8 %)/7 (2.6 %)	***<10⁻⁴****
pBILAG-2004	7.0 [2.0–15.0]	9.5 [3.0–15.0]	7.0 [2.0–15.0]	0.46
SLEDAI-2K	8.0 [4.0–16.0]	7.0 [2.0–16.0]	9.0 [4.0–16.0]	0.26
Constitutional, % and pBILAG score	129/318 (40.6 %) 0 [0–8]	21/50 (42 %) 0 [0–8]	108/268 (40.3 %) 0 [0–8]	0.87* 0.86
Mucocutaneous, % and score	187/318 (58.8 %) 1 [0–8]	29/50 (58 %) 1 [0–8]	158/268 (59.0 %) 1 [0–8]	0.99* 0.49
Musculoskeletal, % and score	176/318 (55.3 %) 1 [0–8]	20/50 (40.0 %) 0 [0–3]	156/268 (58.2 %) 1 [0–8]	***0.02**** 0.67
Hematological, % and score	200/318 (62.9 %) 1 [0–8]	34/50 (68 %) 1 [0–1]	166/268 (61.9 %) 1 [0–8]	0.52* 0.85
Renal, % and score	124/318 (39.0 %) 0 [0–8]	21/50 (42 %) 0 [0–8]	103/268 (38.4 %) 0 [0–8]	0.63* 0.61
Neurological, % and score	31/318 (9.7 %) 0 [0-0]	6/50 (12 %) 0 [0-0]	25/268 (9.3 %) 0 [0-0]	0.60* 0.56
Cardiorespiratory, % and score	40/318 (12.6 %) 0 [0-0]	4/50 (8 %) 0 [0-0]	36/268 (13.4 %) 0 [0-0]	0.35* 0.28
Gastrointestinal, % and score	23/318 (7.2 %) 0 [0-0]	3/50 (6 %) 0 [0-0]	20/268 (7.5 %) 0 [0-0]	0.99* 0.73
Ophthalmic, % and score	5/318 (1.6 %) 0 [0-0]	2/50 (4 %) 0 [0-0]	3/268 (1.1 %) 0 [0-0]	0.17* 0.17
dsDNA (%) and level (IU/mL)	180/296 (60.8 %) 36 [7–181]	22/47 (46.8 %) 17 [2–109]	158/249 (63.5 %) 38 [9–192]	0.03* 0.10
C3 low (%) and level (g/L)	125/310 (40.3 %) 0.9 [0.6–1.2]	19/50 (38 %) 1.0 [0.6–1.3]	106/260 (40.8 %) 0.9 [0.6–1.2]	0.75* 0.35
C4 low (%) and level (g/L)	174/311 (55.9 %) 0.12 [0.07–0.20]	26/49 (53.1 %) 0.14 [0.07–0.27]	148/262 (56.5 %) 0.12 [0.07–0.20]	0.75* 0.22
White blood cell count (10^9/L)	5.7 [4.1–8.0]	6.1 [4.5–9.5]	5.6 [4.0–7.8]	0.18

Statistical analysis: * Fisher's exact test, if not specified non-parametric Mann-Whitney test.

3.2. TLR7 variants, frequency, and predicted functional impact

Fifty-seven individual *TLR7* gene variants were identified in 319 patients from the UK jSLE cohort affecting the genomic region between positions X:12,867,038 to X:12,890,367 (23.3 kb, GRCh38) (Fig. 1). Gene variants were *in silico* predicted to be: i) of "high impact" (n = 2), ii) of "low impact" (synonymous, n = 10); iii) tolerated/benign missense variants (n = 15); or iv) "modifier" variants when present upstream of

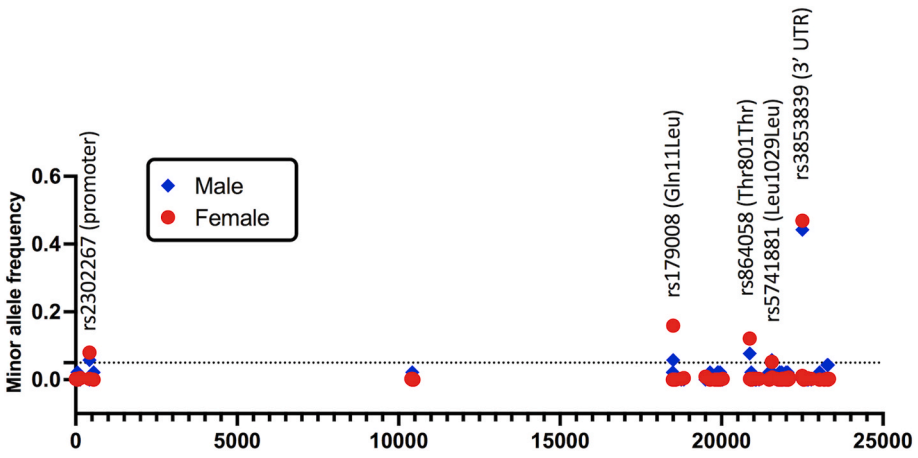


Fig. 1. Genetic variations at *TLR7* in patients with jSLE. Single Nucleotide Polymorphisms (SNPs) with MAF ≥5 % (dotted line). Results are presented stratified by patients' sexes (blue: boys; red: girls). Abbreviations and symbols: Gln: glutamine; Leu: leucine; Thr: threonine; Ser: serine; Glu: glutamic acid; 3′UTR: 3′ untranslated region. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

TLR7 exon 1 (n = 6), in *TLR7* introns (n = 6), or in the 3' untranslated region/UTR (n = 30).

To determine MAFs of *TLR7* variants across the jSLE cohort, hemizygous boys (0 null, 2 hemizygous for the minor allele) were considered equivalent to homozygous girls (0 null, 1 heterozygous, 2 homozygous). Accordingly, MAF ≥5 % delivered 5 candidates: rs2302267 (*TLR7* promoter, n.-20T > G), rs179008 (missense variant, c.32A > T, Gln11Leu), rs3853839 (*TLR7* 3′UTR variant, c.*881C > G), and two synonymous variants: rs864058 (c.2403G > A, Thr801Thr) and rs5741881 (c.3087A > G, Leu1029Leu) that were not further considered.

Next, the *genotype-tissue-expression* (GTEx) database was accessed to test the impact of common SNPs (MAF >5 %) on *TLR7* expression (Table 2). Results suggested that rs2302267 controls *TLR7* promoter DNA methylation status (mQTL), and *TLR7* expression (eQTL) when rs179008 and rs3853839 are also present. Further literature searches retrieved that both rs179008 and rs3853839 have previously been associated with SLE in selected ancestral groups [10,12,42]. Both variants influence IFN expression either by controlling TLR7 protein half-life (rs179008; minor T allele associates with reduced TLR7 half-life and lower IFN expression) [8], or through preventing miRNA-directed TLR7 degradation (rs3853839; minor G allele increases TLR7 mRNA stability) [14]. Because of their ability to interfere with TLR7 and/or IFN signaling, rs2302267, rs179008, and rs3853839 were further studied.

3.3. *TLR7* variants and risk of developing SLE

Because the clinical significance of rs179008 and rs385839 in SLE varies in relation to ancestry [12,42,43], we compared variant frequency according to ancestral background and sexes among selected jSLE patients. Notably, rs3853839 presented a strong bias in both boys ($p = 0.001$) and girls ($p < 10^{-6}$), with an enrichment of the rs3853839 in Asian patients as compared to White Europeans (Fig. 2A). Enrichment of rs179008 among European patients was recorded in both girls ($p = 3 \times 10^{-4}$, alleles TT + TA vs AA) and boys ($p = 0.03$). Notably, rs2302267 distribution was independent of ancestry.

Next, to compare MAFs for rs2302267, rs179008, and rs3853839 between jSLE patients and controls, three international genome projects that consider ancestry information were selected, namely *ALFA*, the *1000 genomes project*, and *GnomAD* (Fig. 2B). Data from South Asian controls were selected instead of "all Asians" based on the predominance of this group among Asian jSLE patients in the UK JSLE cohort (62.9 %) [44,45]. Notably, in the *GnomAD* project database, results for South Asian individuals were only available for rs179008. Minor allele frequencies of *TLR7* SNPs between the three genome projects were similar after considering ancestry (not significant, 2 × 3 Fisher's exact test), allowing us to combine data from the three-genome projects to conduct comparisons with jSLE patients in boys and girls.

Table 2
TLR7 common variants identified across 328 jSLE patients from the UK jSLE cohort Study.

Variants	rs2302267	rs179008	rs3853839
Position (GRCh38)	X: 12,867,459	X: 12,885,540	X: 12,887,911
HGVS_C	n.-20T > G	c.32A > T	c.*881C > G
HGVS_P	−20T > G	Gln11Leu	*881C > G
Gene Location	Promoter	Exon 3/3	3′UTR
Predicted consequence	–	Tolerated (SIFT), benign (CADD)	–
Consequence	Regulatory promoter variant (hypomorphic variant)	Protein half-life reduced in women carrying the minor T allele [8]	miRNA-758, -3148 modulator [14]
QTL (GTEx)	mQTL, eQTL	eQTL	eQTL
Association with SLE	–	Africans & Mexicans [10,42]	East Asians [12]
Females: homo/ heterozygous/ no (% carriers)	6/25/237 (11.6 %)	4/72/192 (28.3 %)	51/103/114 (57.5 %)
Males: hemizygous/no (%)	3/47 (5.9 %)	3/47 (5.9 %)	22/29 (43.1 %)
Healthy population frequency	ancestry bias (1–11 %)	ancestry bias (0–21 %)	ancestry bias (16–85 %)

TLR7: Toll-like receptor 7; jSLE: juvenile Systemic Lupus Erythematosus; UK, United Kingdom; Ser: Serine; Glu: Glutamic acid; GRCh38: Genome Reference Consortium human genome 38; X: chromosome X; HGVS: Human Genome Variation Society; T: Threonine; G: Glycine; A: Alanine; C: Cysteine; Gln: Glutamine; Leu: Leucine; 3′UTR: 3′ Untranslated Region; SnpEFF: Single Nucleotide Polymorphism Effect; SIFT: Sorting Intolerant From Tolerant; CADD: Combined Annotation Dependent Depletion; QTL: Quantitative Trait Locus; GTEx: Genotype-Tissue Expression; mQTL: methylation Quantitative Trait Locus; eQTL: expression Quantitative Trait Locus; no: number.

Comparing MAFs between female jSLE patients and combined control data retrieved increased risk for the development of jSLE associated with rs3853839 in girls of African/Caribbean ancestry (OR: 1.8, 95 % CI: 1.2–2.9, $p = 0.008$), no difference in girls of Asian ancestry, and protective effects in European girls (OR: 0.5, 95 % CI: 0.4–0.7, $p = 3 \times 10^{-6}$) (Fig. 2C). Conversely, no differences were identified between boys with jSLE across ethnic groups (Fig. 2D), which may be the result of the small sample size (n = 51) and/or to a sex bias as reported with rs3853839 between boys and girls of African/Caribbean ancestry (OR: infinity, 95 % CI: infinity-0.76, $p = 0.03$) (Fig. 2E).

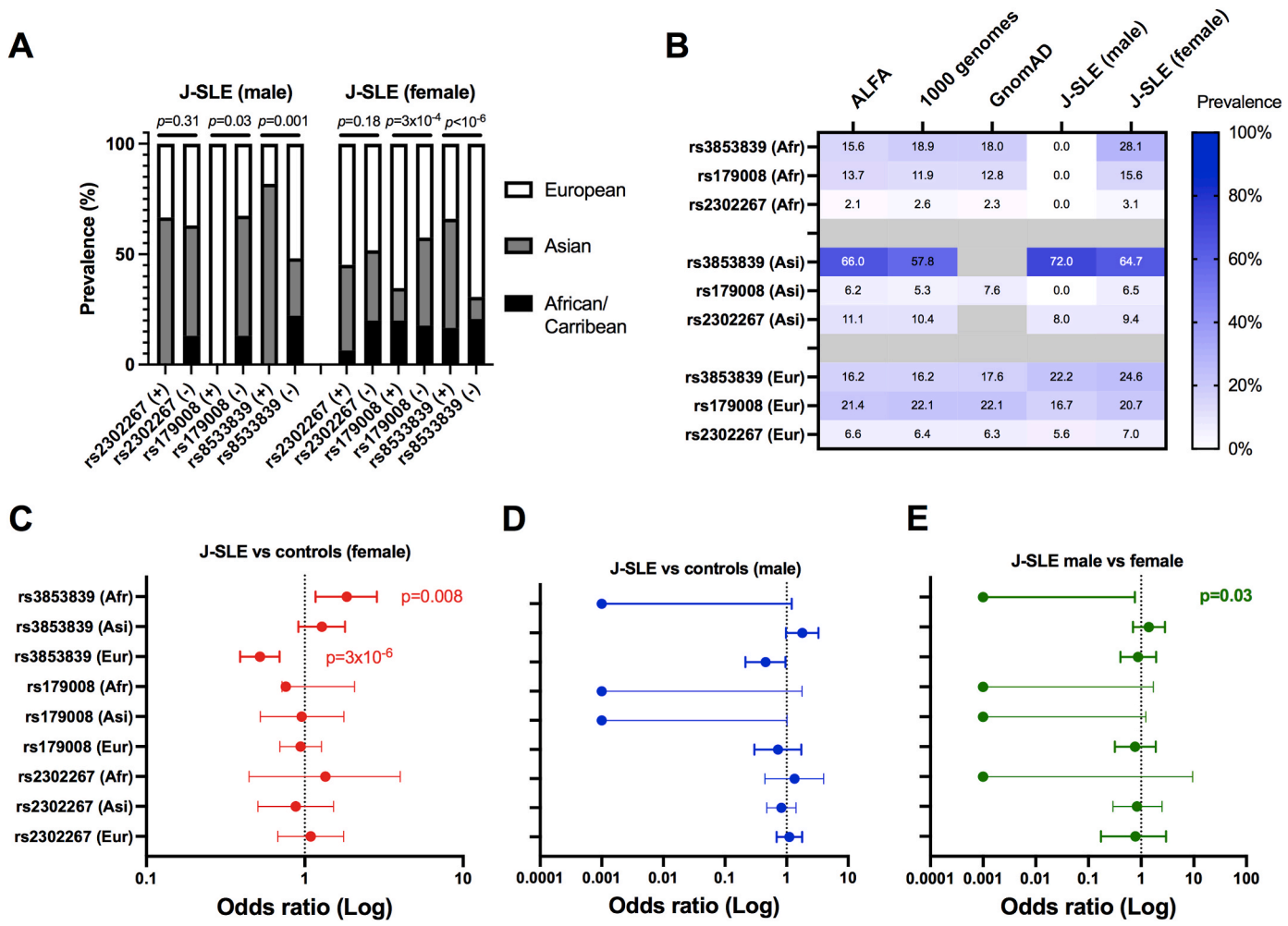


Fig. 2. ***TLR7*** **allelic distribution in jSLE patients stratified by sex (male/female) and ancestry. A and B)** Allelic frequency in jSLE (male/female) according to ancestry as compared to publicly available allele frequency data (ALFA, the 1000 genomes project, and gnomAD). **C-E)** Odds ratios (with 95 % confidence intervals) comparing female jSLE patients *versus* controls (ALFA+1000 genomes + GnomAD projects) (**C**); Odds ratios comparing male jSLE patients *versus* controls (**D**); Odds ratios comparing jSLE males *versus* females (**E**). P values are displayed when significant. Abbreviations and symbols: Afr: African/Caribbean ancestry; Asi: Asian ancestry; Eur: European ancestry; (+): jSLE hetero/homozygous minor allele carrier; (−): jSLE homozygous major allele carrier.

3.4. TLR7 variants and associated clinical characteristics

To test associations between the three selected *TLR7* variants and clinical characteristics at inclusion and during follow-up, jSLE patients were analyzed according to ancestry and sex. Nine associations remained after adjustment for multiple comparisons, predominantly at baseline.

Findings at baseline included associations of the rs3853839 minor G alleles with (i) mucocutaneous involvement (p = 0.004 in all jSLE patients, p = 0.015 in jSLE females); (ii) "older" age at diagnosis among jSLE patients of Asian ancestry (11.4 years [8.5–12.9] *versus* 13.3 years [11.3–14.8], p = 0.003); (iii) C3 complement consumption among boys with jSLE (p = 0.015); and (iv) higher anti-dsDNA antibody levels among jSLE patients of African/Caribbean ancestry (anti-dsDNA Abs: 25 UI/mL [10–97] *versus* 113 UI/ml [36−300] in G allele carriers, p = 0.01) (Fig. 3). The rs2302267 minor G allele was positively associated with higher white cell counts among boys with jSLE (p = 0.02). Presence of the rs179008 minor T allele was associated with elevated ESR among jSLE patients of European ancestry.

During follow-up, "deterioration" in the mucocutaneous BILAG domain was reported in all jSLE patients carrying the rs2302267 minor G allele (p = 0.007), while "deterioration" in the constitutional BILAG domain was recorded in Asian patients carryign the rs179008 minor T allele (p = 0.002). Notably, no associations were reported relating to the number of flare events per year, and/or therapy strategy chosen.

3.5. Haplotype structure and clinical risk in jSLE

Lastly, the *SNPStats* web tool (https://snpstats.net/start.html) was accessed to test whether rs2302267, rs179008 and rs3853839 were in linkage disequilibrium. The rs179008 and rs3853839 polymorphisms were in negative linkage disequilibrium (D' = 0.77, r = −0.25 and $p = 3 \times 10^{-10}$). Conversely, rs230267 was neither linked with rs179008 (p = 0.17) nor with rs3853839 (p = 0.6) (Fig. 4).

The resulting haplotype including the minor rs179008 T allele and the major rs3853839 C allele was identified in 6 European jSLE patients including 3 boys (T-C) and 3 girls (TT-CC). According to the recessive/dominant model, a negative impact on SLE may be suspected for this non-random haplotype (Fig. 5). At diagnosis, the rs179008/rs3853839 T-C/TT-CC haplotype block associated with high disease activity (median pBILAG-2004 16.5 [13.8–20.5] in TT-CC vs 7.0 [2.0–15.0]; p = 0.01) and elevated white blood cell counts (median WBC 10.1 [7.2–14.8] $x10^9$/L in TT-CC vs 5.7 [4.0–8.1] $x10^9$/L; p = 0.01). Notably, constitutional and musculoskeletal involvement (p = 0.03 and p = 0.04, respectively) did not retain statistical significance after correction for multiple comparisons ($0.01 < p \leq 0.05$).

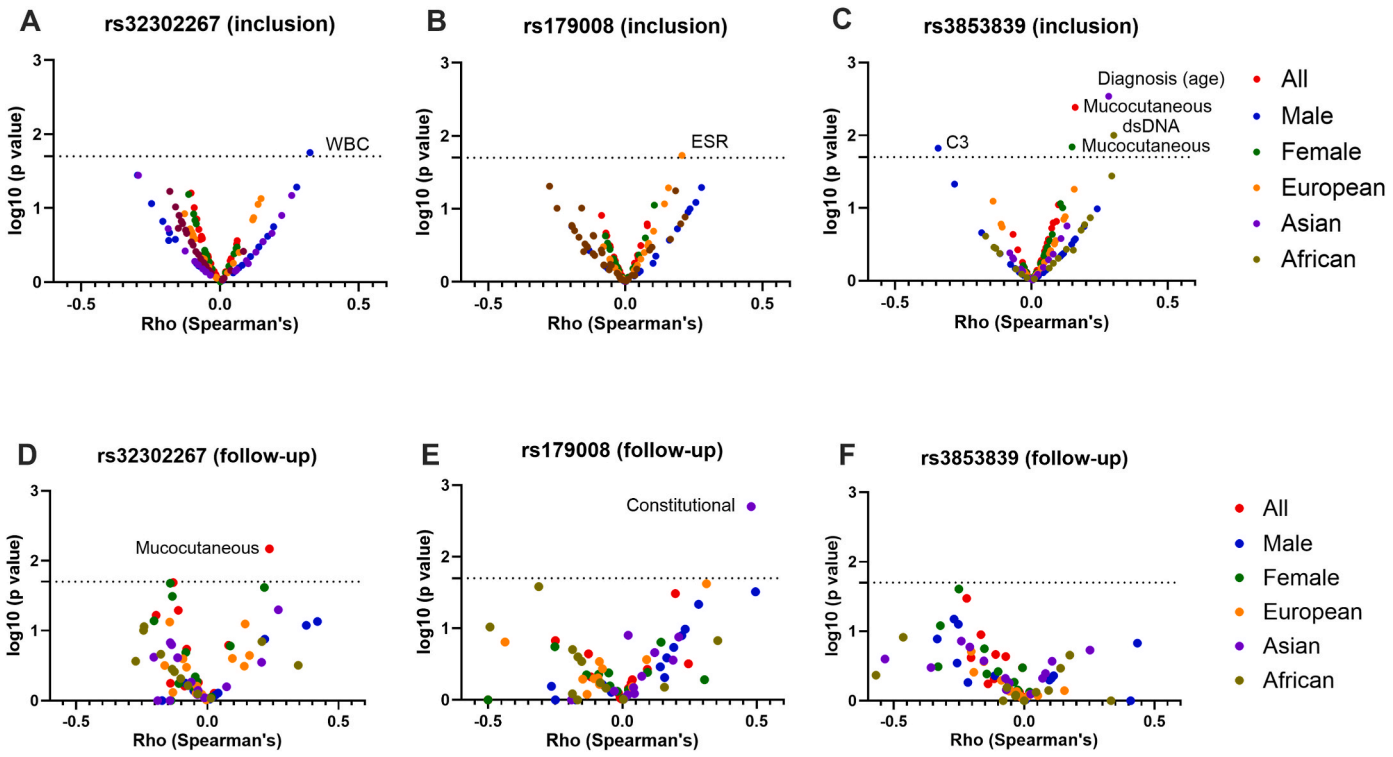
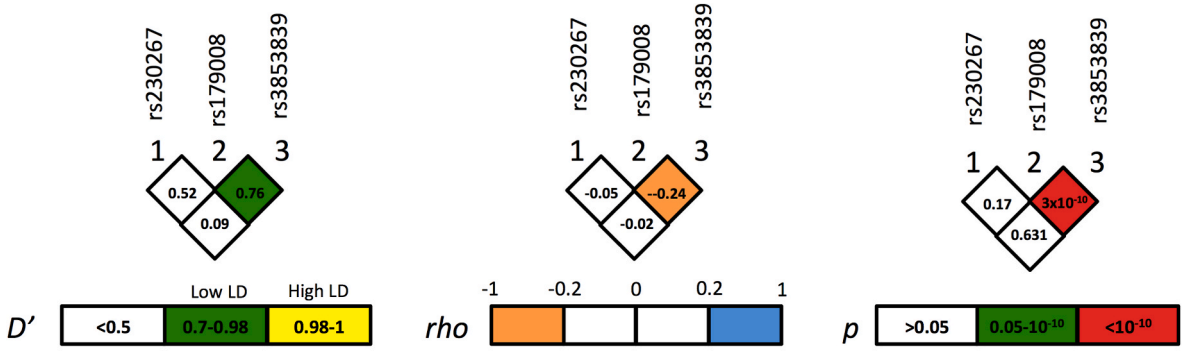


Fig. 3. ***TLR7*** **variants associate with clinical features in jSLE at baseline and during follow-up. A-F)** Three main *TLR7* variants associate with clinical characteristics according to ancestry and sex. **A and D)** rs2302267 allele carriers. **B and E)** rs179008 allele carriers. **C and F)** rs3853839 allele carriers. Spearman's tests with minor allele numbers: 0, 1, and 2. Volcano plots are depicting correlation Rho values and absolute log10 p-values with significant dot lines fixed at $p < 0.02$ after adjustment for multiple comparisons. DsDNA: double-stranded DNA antibody titres; ESR: erythrocyte sedimentation rate; WBC: white blood count.



	rs3853839			
rs230267		CC	GC	GG
	TT	125	95	65
	TG	14	5	6
	GG	4	3	2

	rs3853839			
rs179008		CC	GC	GG
	AA	95	76	69
	TA	42	26	4
	TT	6	1	0

Fig. 4. ***TLR7*** **variants are in linkage disequilibrium**. D′ values from LD analysis (https://snpstats.net/), rho values and p values are displayed as indicated. Allele distribution between rs3853839 and rs230267 (left) or between rs3853839 and rs179008 (right).

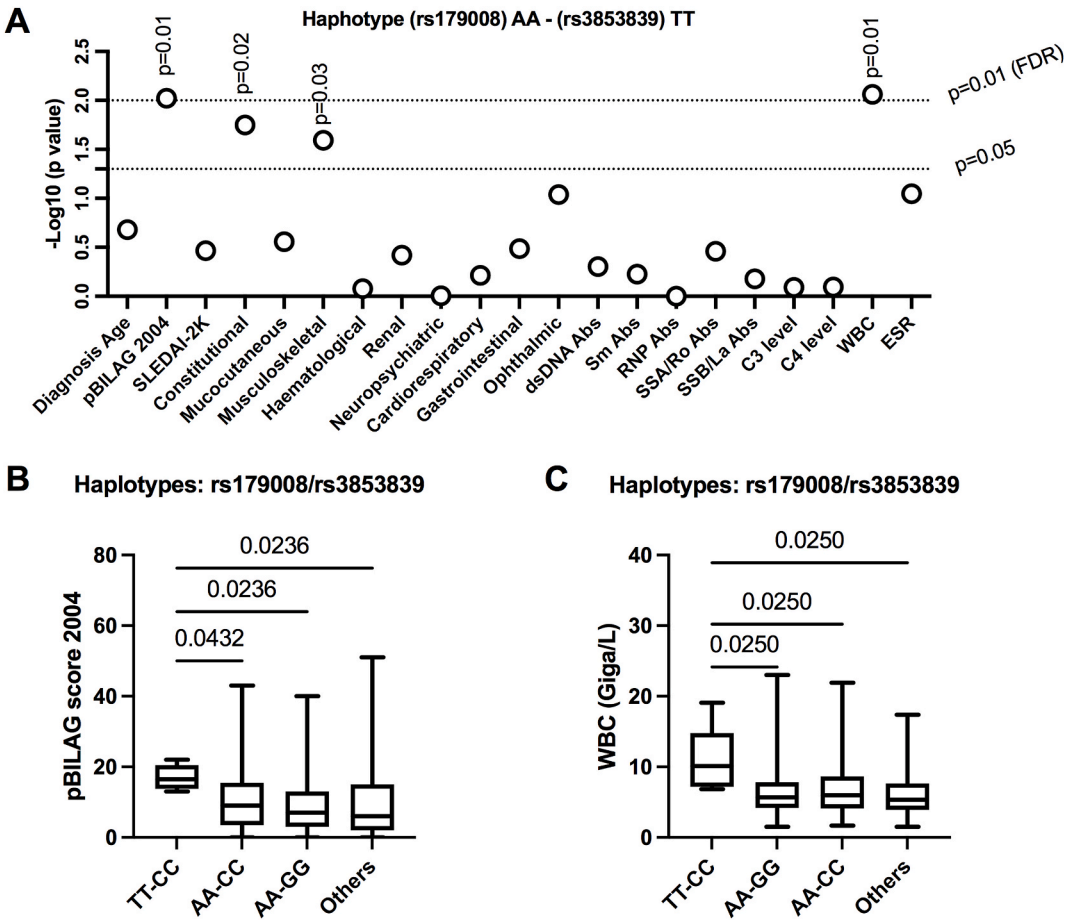


Fig. 5. Associations between clinical features and *TLR7* haplotypes in jSLE. A) Clinical characteristics of patients carrying the T-C/TT-CC haplotype between the T allele at rs179008 and the C allele at rs3853839. P values are indicated when significant and false discovery rates (FDR) were used to correct for multiple comparisons allowing to consider p values with a low discriminating efficacy ($0.01<p \leq 0.05$, gray circle) and p values with a high discriminating efficacy ($p < 0.01$, black circle) for Mann-Whitney tests. **B)** Pediatric British Isles Lupus Assessment Grade 2004 (pBILAG-2004) score according to the rs179008/rs3853839 haplotype. **C)** Peripheral white blood cell count (WBC).

3.6. Functional studies

Two common *TLR7* variants (MAF >5 %) were selected for functional testing, i) rs3853839 (*TLR7* c.*881C > G affecting the 3′UTR), and ii) the promoter variant rs2302267 (n.-20T > G).

The *TLR7* gene consists of 3 exons with high cross-species conservation (Fig. 6A). The 1st exon is a pseudo-exon as it is not translated (5′ untranslated region; 5′ UTR), and only a section of exon 2 is. Thus, the proximal promoter region is complex and incudes regulatory elements in the 1st intron (between exons 1 and 2). In addition to the 5′ UTR, *TLR7* also has a 3' UTR that has previously been suggested to govern microRNA mediated regulation of mRNA stability [14]. Both common jSLE-associated *TLR7* variants identified here map to these complex regulatory regions.

The rs3853839 minor G allele affecting the *TLR7* 3′UTR (c.*881C > G) had previously been linked with SLE in East Asian population [12,14]. However, functional testing has been limited to preliminary luciferase assays that suggested increased mRNA stability [14]. Thus, we generated *TLR7* expression plasmids comprising the *TLR7* cDNA sequence including the major (C) or minor (G) allele. Plasmids were transfected into HEK293 T cells, some cells were stimulated the TLR7/8 agonist resiquimod (R848) as indicated to test TLR7 functionality, and TLR7 and IFN expression were measured. Under resting conditions, cells transfected with expression plasmids carrying the rs3853839 minor G allele (c.*881G) exhibited increased TLR7 (Fig. 6B) and type I IFN signature gene (Fig. 6C) expression when compared to cells expressing the major allele (c.*881C). Because 3′UTRs are frequently involved in fine-tuning of gene expression through the regulation of RNA degradation by micro-RNAs [46], we tested RNA stability of wild-type and variant TLR7 applying 5,6-dichlorobenzimidazole (DRB) assays [39,40]. Inhibition of RNA polymerases with DRB revealed increased mRNA stability of transcripts carrying the rs3853839 minor G over the major C allele (Fig. 6D and E).

Lastly, we investigated how the rs2302267 (n.-20T > G) promoter variant may affect TLR7 expression (Fig. 7A). We generated luciferase promoter plasmids (pGL3 backbone) carrying the wild-type or variant (rs2302267) TLR7 promoter sequence (Fig. 7B). Luciferase reporter assays in HEK 293T cells identified increased activity associated with wild-type (n.-20T) when compared to variant (n.-20G) promoter sequences (Fig. 7C). Bioinformatic analysis of transcription factor:DNA interactions predicted rs2302267 to disrupt a highly conserved transcription binding element for lymphoid enhanced binding factor 1 (LEF1), transcription factor 7 like proteins TCF7L1 and TCF7L2, mitochondrial ribosomal protein 1 (MRPL1), and ventral anterior homeobox 2 (VAX2) (Fig. 7D). Finally, to test effects of rs2302267 on transcription factor recruitment, we performed reporter ChIP assays [41] that confirmed increased recruitment of the transcription factor TCF7L1 (formerly TCF3) [47] (but not the also tested LEF1) to promoter constructs carrying the minor G allele (rs2302267) when compared to constructs including the wild-type major T allele ($p = 0.05$) (Fig. 7E).

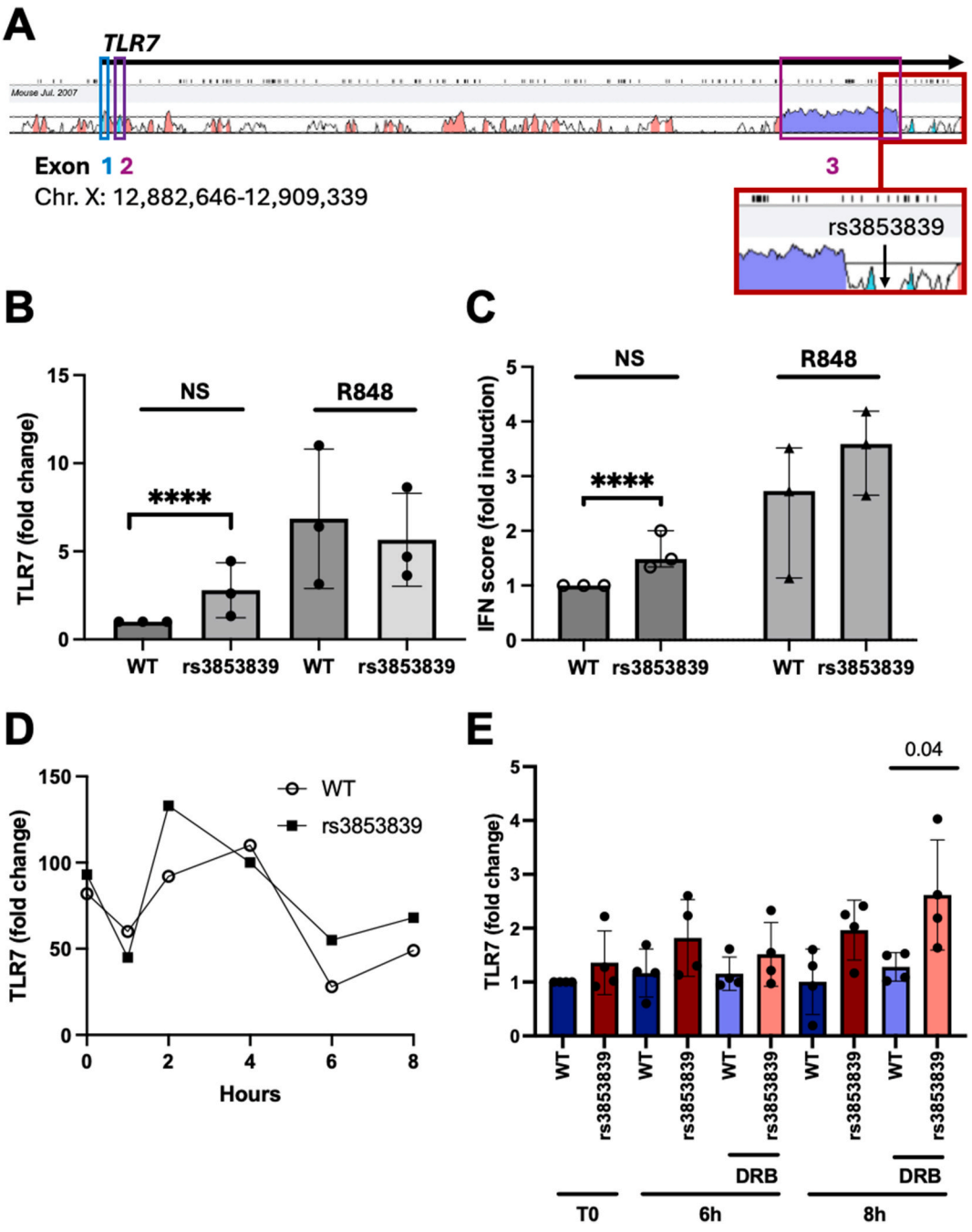


Fig. 6. ***TLR7*** **rs3853839 increases mRNA stability and IFN expression. A)** Alignment of the human and mouse *TLR7* genes to identify sequence conservation across exons, non-coding and regulatory elements. Exons are displayed in purple, conserved non-coding elements in pink, and untranslated regions in turquoise. The *TLR7* gene consists of 3 exons with high cross-species conservation. The 1st exon represents an untranslated pseudo-exon (5′ untranslated region/UTR; turquoise). Exons 2 and 3 are protein coding (purple). In addition to the 5′ UTR, *TLR7* also has a 3′ UTR. The rs3853839 single-nucleotide polymorphism (SNP) localises to the 3′UTR. **B)** mRNA expression of wild-type (WT) and variant TLR7 under resting (NS) conditions and in response to stimulation with the TLR7/8 agonist resiquimod (ST), were tested ($n = 3$; $p < 0.001$). **C)** Type I IFN signature in HEK293T cells expressing WT or variant TLR7 under resting (NS) conditions and in response to stimulation with resiquimod (ST) ($n = 3$; $p < 0.001$). **D, E)** mRNA stability in HEK293T cells forced to express wild-type (WT) or variant TLR7. Transcription was inhibited with DRB, 2h after transfection to, assess relative TLR7 mRNA stability over time (single experiment) (D) one representative experiment; E) $n = 4$; $p = 0.04$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Through its involvement in the sensing and removal of immune complexes and its associated role in controlling IFN expression, the endosomal TLR7 plays a critical role during innate immune responses against single-stranded (ss)RNA viruses [43]. Dysregulation of TLR7 responses has been linked with autoimmune/inflammatory diseases, including SLE [48].

The important role of TLR7 in SLE has recently been underscored by the identification of ultra-rare SLE-causing variants in the *TLR7* gene. The p.Tyr264His *TLR7 de novo* gain-of-function variant, first identified in a 7-year-old jSLE patient, causes severe SLE phenotypes through increased survival of B lymphocytes, the accumulation of atypical $CD11c^+$ B cells, and spontaneous formation of extrafollicular germinal

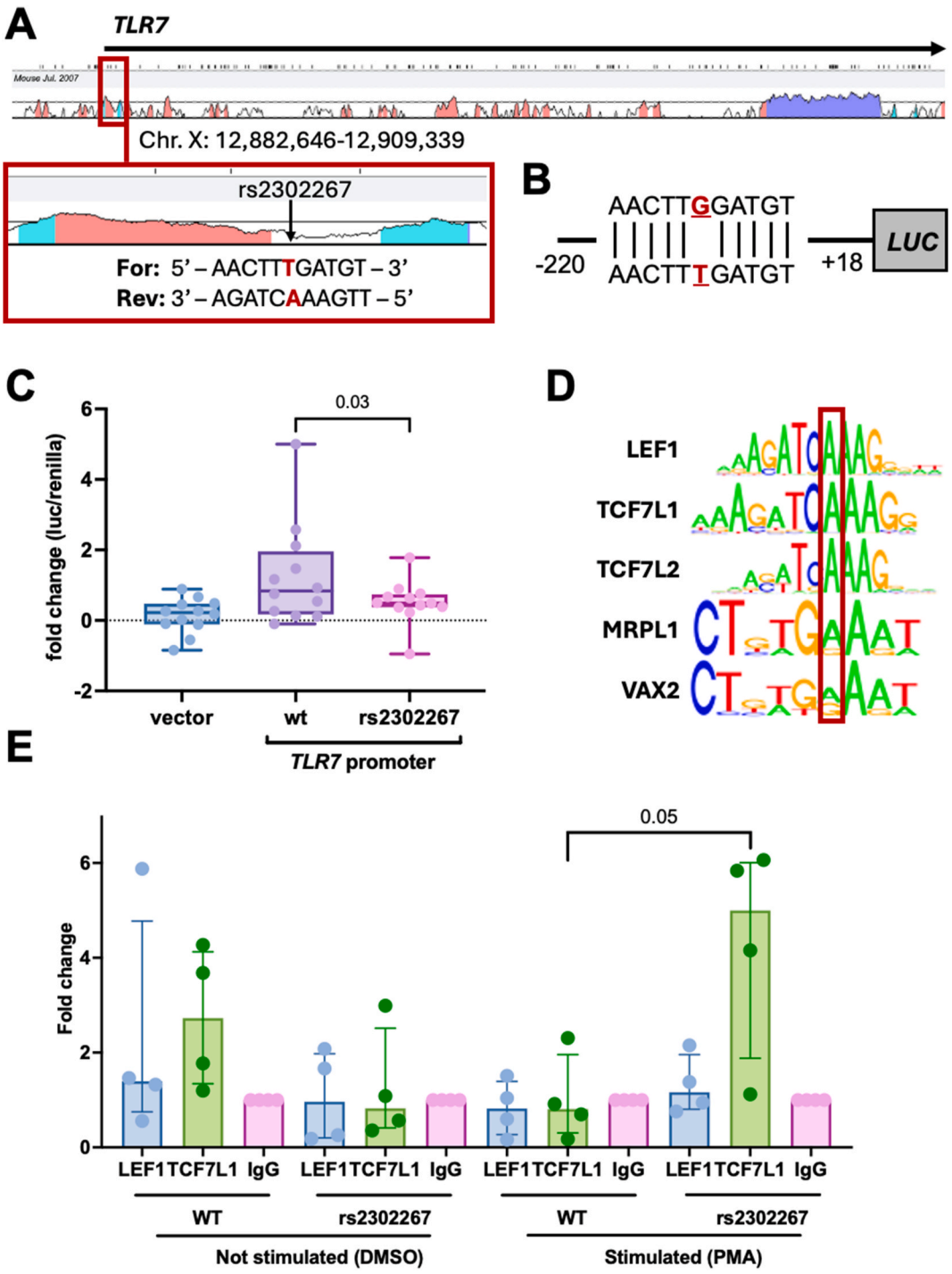


Fig. 7. ***TLR7*** **rs2302267 (n.-20T > G) increases** ***TLR7*** **promoter activity. A)** Alignment of the human and mouse *TLR7* genes to identify sequence conservation across exons, non-coding and regulatory elements. Exons are displayed in purple, conserved non-coding elements in pink, and untranslated regions in turquoise. The rs2302267 single-nucleotide polymorphism (SNP) localises to the 1st intron/proximal promoter. **B)** Luciferase reporter assays were generated (pGL3 backbone) including the TLR7 core promoter sequence (covering the proximal 220 base pairs). To test effects of rs2302267 on promoter activity, the proximal 200 base pair spanning wild-type (flanking sequence with major T allele; bottom panel) or variant (flanking sequence with minor G allele; upperpanel) promoter sequence was introduced. **C)** « Spontaneous » luciferase activity in cell transfected with « empty » pGL3 vectors and such carrying the wild-type (WT) or variant (rs2302267) TLR7 core promoter sequence (n = 10; p = 0.04). **D)** *In silico* analyses identified the region spanning rs2302267 as an consensus transcription factor binding element. **E)** Chromatin immunoprecipitation assays in cells transfected wild-type or variant TLR7 promoter construct (from B, C) identified differential recruitment of the TCF7L1/TCF3 transcription factor to the construct carrying the rs2302267 variant (n = 3; p = 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

center B cells [15]. The same study reported additional large effect size variants affecting *TLR7* (Arg28Gly and Leu507*) in two SLE patients.

This study provides further evidence for the key role of *TLR7* in jSLE development and associated differential disease presentations. The SLE-associated rs179008 minor T allele identified here has been reported to dampen IFN responses in dendritic cells from individuals of European ancestry [8]. Here, we further provide arguments to consider associations with inflammation (elevated ESR) in European jSLE patients and "deterioration" over time in the constitutional pBILAG domain among Asian jSLE patients. This may result from a more potent inflammatory cytokine response mediated by, for example, a compensatory TLR8 response [49]. It furthermore represents a risk factor for infections with RNA viruses, including Zika virus [50], severe acute respiratory syndrome coronavirus 2 (SARS-Cov2) [51], dengue virus [52,53], and human immunodeficiency virus (HIV) [54]. The constantly growing body of evidence linking *TLR7* variants with increased susceptibility and severity of viral infections alongside the impact of TLR7 dysregulation in SLE patients, supports the previously proposed role of virus infections as a trigger of SLE onset and flares, which may be amplified in genetically predisposed individuals [55–57]. However, in contrast to viral infections, the exact contribution of *TLR7* rs179008 to SLE remains controversial and is associated with select populations, such as patients from Brazil and Mexico (single-center studies), and Africa (meta--analysis) [10,42,58]. Conversely, no rs179008 associations were identified in European and Asian populations [59,60]. Seemingly conflicting data may be related to: (i) sex bias [8]; (ii) ethnic bias introduced by control cohorts (rs179008 TC/TT frequencies range from 21.4 to 22.1 % in Europeans, 11.9–13.7 % in African/Caribbean individuals, and 5.3–7.6 % in South Asian individuals as reported here); and (iii) linkage disequilibrium between *TLR7* variants. Notably, the presence of linkage disequilibrium is supported by this study, identifying the T-C/TT-CC haplotype in 6 European jSLE patients that was associated with increased disease activity (pBILAG-2004 but not SLEDAI-2K that is less sensitive to changes in disease activity and inflammation), and a slight increase in leukocyte numbers.

The presence of the rs179008/rs3853839 T-C/TT-CC haplotype in European jSLE patients provides a likely explanation for the "paradoxical" observation that the rs3853839 minor allele represents a risk for jSLE in European girls while protecting African/Caribbean girls (Fig. 2C). Further experiments are required to investigate combined effects of the T-C/TT-CC haplotype on IFN and pro-inflammatory cytokine expression. Indeed, according to previous reports [61], both rs179008-TT and rs3853839-CC genotypes are individually associated with "low" expression of IFN in healthy women, while rs3853839-CC associates with increased IL-6 expression. Impact of the T-C/TT-CC haplotype on interferon-γ may be also suspected, because SLE patients expressing blocking antibodies against the type II IFN-γ exhibit increased disease activity, "high" anti-dsDNA Ab titers, and IFN-related gene expression [62–65]. On the other hand, the presence of antibodies against IFNs and the use of inactivating monoclonal antibodies targeting IFN or IL-6 receptors are effective in the treatment of SLE [66–69].

In line with results from this study, carriers of the rs3853839 GC/GG allele express >2.7-fold increased levels of IFNs and exhibit pronounced IFN signatures when compared to controls, which is a predisposing factor for SLE in select populations from Asia [11,14,70], Europe [71], and Africa/the Caribbeans [72]. Another study in East Asian populations (Chinese and Japanese) reported >2.5-fold increased IFN expression in individuals with the rs3853839 minor G allele which was more pronounced in men [12]. The relatively small overall sample size, especially considering male patients, may explain why the here presented study did not identify associations between rs3853839 in boys across ancestries. Based on functional data from this study and previous reports using a luciferase model [14], variable IFN expression associated with the rs3853839 minor G allele is likely the result of miR-3148-mediated differences in *TLR7* mRNA stability between the major C and the minor G allele. Across studies, the rs3853839 minor G allele associated with disease activity or clinical phenotypes, which was confirmed here, including associations at inclusion with oral ulcers and neurologic involvement [73]. Moreover, this study delivered associations with mucocutaneous involvement in all jSLE patients, C3 complement consumption among boys with jSLE, and elevated anti-dsDNA titres in jSLE patients of African/Caribbean ancestry.

Lastly, the rs2302267 promoter variant (*TLR7* n.-20T > G), present in 11.6 % of female and 5.9 % of male jSLE patients of this cohort, associated with increased leukocyte counts and more "deterioration" in the mucocutaneous pBILAG domain when compared to the remaining cohort. This may be explained by the hypomorphic nature of rs2302267 that negatively affects promoter activity in luciferase experiments through reduced transcription factor recruitment. Notably, after stimulation with PMA, rs2302267 associated with increased recruitment of the transcriptional repressor TCF7L1 (formerly TCF3) [47]. Because TLR7 signaling enhances IFN expression [74,75], and IFN centrally contributes to lymphopenia in SLE or during virus infections [76,77], rs2302267 may be a protective factor reducing haematological involvement in jSLE. Indeed, loss-of-function affecting TCF7L1/TCF3 has previously been linked with immunodeficiency, mild cytopenia and SLE-like clinical pictures [78,79].

While presenting the largest available dataset on *TLR7* variants in jSLE patients, accessing a large national cohort from the UK, this study is limited by a relatively small sample size, especially considering male patients and "minority" ancestries in the UK. Furthermore, functional studies in primary human immune cells from patients were not possible because biosamples were not available.

5. Conclusions

This study linked jSLE susceptibility and phenotypes with "common" *TLR7* variants. The rs179008 variant was previously reported to reduce TLR7 protein half-life, subsequently impairing IFN expression in women carrying the minor T allele. The common rs3853839 G allele increases TLR7 mRNA stability, thereby affecting disease risk in African/Caribbean girls. In European jSLE patients, the rs179008/rs3853839 (TT-CC) haplotype, in both boys and girls, associates with "high" disease activity. The rs2302267 (n.-20T > G) variant, through reduced *TLR7* promoter activity, may protect from lymphopenia. While observations require confirmation in larger independent cohorts, they suggest a key role for TLR7 in SLE pathophysiology and disease expression. Based on these observations and lessons from monogenic SLE patients with gene variants affecting *TLR7* and its regulation [15–18], molecular phenotyping including TLR7 locus analysis may allow for future risk stratification and individualized treatment and care.

CRediT authorship contribution statement

Yves Renaudineau: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesca Sposito:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Valentina Natoli:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Amandine Charras:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Jenny Hawkes:** Writing – review & editing, Investigation, Data curation. **Joni Roachdown:** Writing – review & editing, Investigation, Data curation. **Mathieu Fusaro:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Eve MD. Smith:** Writing – review & editing, Data curation. **Michael W. Beresford:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Christian M. Hedrich:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration,

Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Disclosures

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Data availability

Data will be made available on request.

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