PADI4 Polymorphisms Confer Risk of Anti-CCP-Positive Rheumatoid Arthritis in Synergy With HLA-DRB1*04 and Smoking

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Peptidylarginine deiminases (PADs) catalyze citrullination, a post-translational modification playing a pathogenic role in anti-citrullinated protein antibody (ACPA)-positive rheumatoid arthritis (RA). The interplay between single nucleotide polymorphisms (SNPs) in the PADI genes and known risk factors for ACPA-positive RA, including smoking, HLA-DR4 and -1, and the PTPN22 R620W polymorphism, was investigated. We typed four PADI2 SNPs, four PADI4 SNPs, and the PTPN22 R620W SNP in 445 Danish RA patients and 533 age-matched healthy controls, as well as in 200 North American RA patients and 100 age- and sex-matched controls. The HLA-DRB1 locus was typed in the Danish cohort. Logistic regression analyses, adjusted for age, sex, smoking status, and PTPN22 R620W, revealed increased risk of anti-CCP-positive RA in carriers of rs11203367(T) (OR: 1.22, p=0.03) and reduced risk in carriers of rs2240335(A) in PADI4 (OR: 0.82, p=0.04). rs74058715(T) in PADI4 conferred reduced risk of anti-CCP-negative RA (OR: 0.38, p=0.003). In HLA-DRB1*04-positive individuals, specifically, the risk of anti-CCP-positive RA was increased by carriage of PADI4 rs1748033(T) (OR: 1.54, p=0.007) and decreased by carriage of PADI4 rs74058715(T) (OR: 0.44, p=0.01), and we observed an interaction between these SNPs and HLA-DRB1*04 (p=0.004 and p=0.008, respectively) Thus, PADI4 polymorphisms associate with ACPA-positive RA, particularly in HLA-DRB1*04-positive individuals, and with ACPA-negative RA independently of HLA-DRB1*04.

Keywords: rheumatoid arthritis, peptidylarginine deiminase (PAD), anti-citrullinated proteins antibodies (ACPA), HLA-DRB1*04, single nucleotide polymorphism (SNP)

INTRODUCTION

Peptidylarginine deiminases (PADs) catalyze the post-translational conversion of peptidylarginine to peptidylcitrulline. Citrullination is involved in many physiological functions, including skin keratinization, neuron myelination, and formation of neutrophil extracellular traps (NETs) (1–3) but is also thought play a pathogenic role in anti-citrullinated protein-antibody (ACPA)-positive rheumatoid arthritis (RA) (4). ACPAs, routinely detected using the anti-cyclic citrullinated peptide (anti-CCP) test (5), are often present years before clinically overt disease (6), and anti-CCP-positive RA patients have a worse prognosis with a higher degree of erosive damage than anti-CPP-negative RA patients (7). ACPA-positive RA is strongly associated with HLA molecules containing the so-called shared epitope (SE), which are capable of binding certain citrullinated peptides (8–10). The shared epitope is present in most HLA-DRB1*04 subtypes (HLA-DRB1*0401, HLA-DRB1*0404, HLA-DRB1*0405, and HLA-DRB1*0408 but not in HLA-DR4B1*0402) and HLA-DRB1*01 subtypes (HLA-DRB1*0101 and HLA-DRB1*0102). However, HLA-DRB1*04, which is carried by 67–78% of anti-CCP-positive RA patients (10–12), correlates more strongly with anti-CCP-positive RA than HLA-DRB1*01 (reported in 22–51% of anti-CCP-positive RA patients) (10–12). In addition, the R620W variant of protein tyrosine phosphatase, non-receptor type 22 (PTPN22), confers increased risk of ACPA-positive RA (13, 14), as does smoking (9).

The five existing PAD isoforms in humans are encoded by the PADI1-4 and -6 genes (15). PAD2 and PAD4 are expressed by immune cells (16–18) and are present in the synovium of RA patients, where their expression levels correlate with those of inflammatory markers (18, 19). Some investigators have found that PAD4 generates autoantigens recognized by ACPAs more efficiently than PAD2 (20), while others have shown that the two PAD isoforms are approximately equally efficient (21).

Studies on Asian populations have shown associations between RA and single nucleotide polymorphisms (SNPs) in PADI2 (22, 23) as well as SNPs in PADI4 (22–27). However, the observed associations with PADI2 SNPs in Asian populations have not been replicated in studies on European or North American cohorts, while a few studies have confirmed the association of the PADI4 SNPs with RA in those populations (28–34). Notably, one study reported associations between one SNP in PADI4 (rs2240340) and RA in a North American cohort, alone or when combined with a Swedish cohort, but not in the Swedish cohort alone (31). A haplotype of PADI4 has been reported to confer susceptibility to RA in Asians (24), but not in a British study (30). The haplotype consists of the minor alleles of rs11203366, rs11203367, and rs874881 encoding the amino acid substitutions Gly55Ser, Val82Ala, and Gly112Ala, respectively, and rs1748033 which is a synonymous SNP (**Figure 1**). Besides, the minor allele (A) of the synonymous SNP rs2240035 has been shown to decrease the

risk of RA in Asians and North Americans (26, 27, 35). Together with rs74058715, which is located in the 5' untranslated region (UTR) of PADI4, rs2240035 has been shown to regulate PAD4 expression levels in neutrophils and monocytes (36).

Only few studies on PADI polymorphism in RA have stratified the patients according to HLA types (28, 32, 37, 38) and, generally, studies addressing the influence of HLA types on development of RA have not taken PADI polymorphism into account. We hypothesized that PADI polymorphisms exert their greatest influence in subjects carrying HLA types that bind citrullinated peptides most strongly, i.e., HLA-DRB1*04.

Our aim was to determine if anti-CCP-positive or anti-CCP-negative RA is associated with SNPs in PADI2 or PADI4 in a Danish and a North American cohort of RA patients and healthy controls. Moreover, we aimed at elucidating the interaction of PADI SNPs with HLA-DRB1*04 or HLA-DRB1*01 in predisposing to RA.
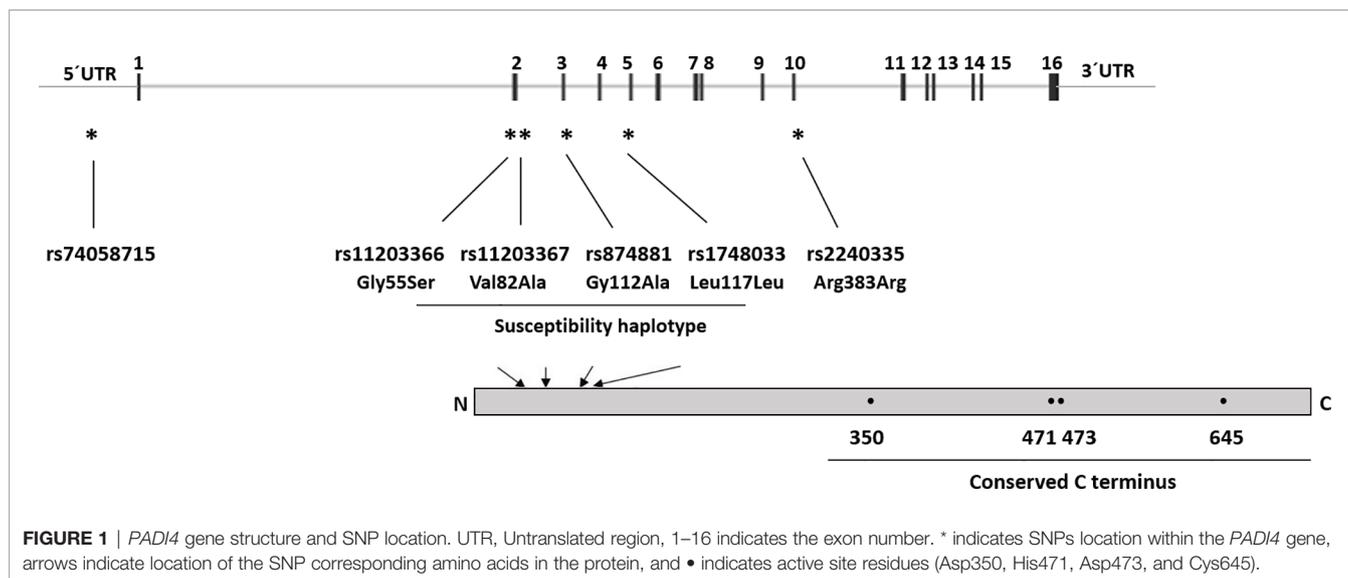
MATERIALS AND METHODS

Patients and Controls

The Danish cohort included 445 RA patients clinically identified at 21 rheumatology departments across Denmark and 533 population controls matched for age (**Table 1**). The cohort has previously been reported on (39). The RA patients had less than 5 years of disease duration and fulfilled the American College of Rheumatology 1987 classification criteria for RA (40). Healthy controls, frequency-matched by birth year, were randomly selected from the Danish population, and blood samples were collected by general practitioners. Written informed consent was obtained from all study subjects, and the study was approved by the Scientific Ethical Committees for Copenhagen and Frederiksberg (KF 01-039/01), the Danish Data Protection Agency (2001–41–0658), and the Institutional Review Board at Statens Serum Institut (21–00050). The North American cohort included 200 RA patients and 100 age- and sex-matched controls without autoimmune disease selected from the University of Wisconsin (UW) Rheumatology Biorepository described in (35, 41) (**Table 1**). The RA patients were initially identified by having at least two outpatient visits with RA-associated ICD codes within 24 months (42), or one visit and a positive anti-CCP test. Diagnosis was confirmed by manual review of rheumatology notes. All subjects gave written informed consent, and the study was approved by the Institutional Review Board of the University of Wisconsin-Madison (#2015-0156).

SNP Selection

SNPs in PADI2 (rs2057094, rs2076616, rs2235912, and rs1005753) and PADI4 (rs74058715, rs11203367, rs1748033, and rs2240335) were selected based on literature search of previously reported associations with RA (22–27, 43). rs11203367 was chosen as a tag-SNP for the two other SNPs encoding amino acid substitutions in the RA susceptibility haplotype (rs11203366 and rs874881), given the strong linkage disequilibrium (LD) between these three SNPs (44). Additionally, as previously shown, rs11203367 is also in strong LD ($r^2 > 0.8$), with the intronic SNPs rs2240340 and rs11203368 (44), two other SNPs associated with RA in previous studies (23, 31);

FIGURE 1 | *PADI4* gene structure and SNP location. UTR, Untranslated region, 1–16 indicates the exon number. * indicates SNPs location within the *PADI4* gene, arrows indicate location of the SNP corresponding amino acids in the protein, and • indicates active site residues (Asp350, His471, Asp473, and Cys645).

therefore these intron located SNPs were excluded from typing. The *PTPN22* missense SNP rs2476601 (R620W) was also included, given the reported association with RA and its known interactions with other risk factors like smoking and carriage of the SE (14). None of the SNPs investigated deviated significantly from Hardy-Weinberg equilibrium, in either cases or controls.

Genotyping

DNA was extracted from whole blood samples from the two cohorts as previously described (35, 39) and stored at −80°C until use. Samples were genotyped for *PADI* SNPs and the rs2476601 SNP in *PTPN22*, encoding R620W, by means of an in-house multiplex SNP assay protocol, as previously described (45). In brief, the method included a polymerase chain reaction (PCR) to amplify the selected SNP sites and an allele-specific primer extension (ASPE) reaction for labeling ASPE-oligonucleotides, followed by hybridization to MagPlex-TAG™ bead sets (Luminex Corporation, Austin, TX, USA) for analysis on the

Luminex platform (Luminex Corporation, Austin, TX, USA). All assay runs included a panel of control samples with known genotypes (Coriell Cell Repository, Camden, NJ, USA) as well as no template negative controls. Low-resolution *HLA-DRB1* typing of the Danish cohort had been performed prior to this study, according to the 11th Histocompatibility Workshop protocol (46).

Statistical Analysis

Hardy-Weinberg equilibrium was calculated with the "hwde"-package in R (R Foundation for Statistical Computing, Vienna, Austria). Associations between SNPs and disease were tested using multiple logistic regressions with adjustment for age, sex, smoking status, and carriage of PTPN22 R620W. P-values and odds ratios (ORs) with 95% confidence intervals (95% CI) were calculated for minor allele counts (trend test). Analyses of the Danish and North American cohorts were performed separately and, in order to increase statistical power, repeated after merging

TABLE 1 | Demographic, clinical, and genetic characteristics of anti-CCP-positive and anti-CCP-negative rheumatoid arthritis (RA) patients and controls.

	Danish cohort (n= 978)			North American cohort (n= 300)		
	Anti-CCP- positive RA (n = 309)	Anti-CCP- negative RA (n = 136)	Controls (n = 533)	Anti-CCP- positive RA (n = 104)	Anti-CCP- negative RA (n = 96)	Controls (n =100)
Age, median (range)	52 (19–68)	53 (21–69)	53 (19–69)	64 (38–87)	63 (34–90)	63 (33–95)
Women, n (%)	209 (68%)	104 (76%)	327 (61%)	72 (69%)	83 (86%)	79 (79%)
Ever smokers, n (%)	226 (73%)	85 (62%)	323 (61%)	57 (55%)	46 (48%)	39 (39%)
Any PTPN22 R620W, n (%)	98 (32%)	33 (24%)	108 (20%)	26 (25%)	28 (29%)	22 (22%)
Rheumatoid factor IgM, n (%)	275 (89%)	40 (29%)	64 (12%)	76 (73%)[‡]	30 (31%)[‡]	NA
Any *HLA-DRB1*04*, n (%)	227 (73%)	50 (37%)	193 (36%)	NA	NA	NA
Any *HLA-DRB1*01*, n (%)	78 (25%)	28 (21%)	102 (19%)	NA	NA	NA
No *HLA-DRB1*04* or *HLA-DRB1*01*, n (%)	47 (15%)	64 (47%)	257 (48%)	NA	NA	NA
Caucasian ethnicity, n (%)	(99%)	(99%)	(99%)	(93%)	(96%)	(94%)

CCP, cyclic citrullinated peptide; RA, rheumatoid arthritis; HLA, human leukocyte antigen; NA, not available.
[‡]info missing for one anti-CCP-positive and seven anti-CCP-negative subjects.

of the two cohorts. *Post-hoc* analyses after stratification for *HLA-DRB1* alleles were performed in the Danish cohort, with further adjustment for the number of *HLA-DRB1*04* or *HLA-DRB1*01* alleles carried. Disease risk due to combined effects of PTPN22 R620W, *HLA-DRB1* alleles, *PADI4* alleles, and smoking was calculated by means of logistic-regression models, with adjustment for age and sex. Gene-gene interaction and gene-exposure interaction were calculated by logistic regression analyses including all single variables and interaction variables in the regression model. A full factorial set of interactions was presented to the model and only conditionally included if they significantly improved the predictive performance of the model. Analyses were performed in R Studio Version 1.0.153 (RStudio Inc., Boston, MA, USA) using R version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria) and in SPSS 25.0 (IBM, New York, USA). The level of statistical significance was set at $p < 0.05$.

RESULTS

The characteristics of the Danish and North American cohorts included in this study are shown in **Table 1**. The North American cohort had a median age of 63 years (range 33–95), which was higher than the 53 years (range 19–69) of the Danish cohort, and it contained more women than the Danish cohort, particularly within the anti-CCP-negative RA and healthy control groups. Anti-CCP-positive RA patients were more often smokers than anti-CCP-negative patients in both cohorts, but the Danish cohort contained more ever smokers than the North American cohort in all three study groups. All analyses were adjusted for age, sex, smoking status, and cohort origin.

Associations Between PADI Polymorphisms and RA

We studied associations of the *PADI* SNPs with anti-CCP-positive and anti-CCP-negative RA separately. Given that carriage of PTPN22 R620W is a known risk factor for anti-CCP-positive RA (14), we adjusted all analyses for this variable

too. To increase statistical power, we merged the two cohorts in these analyses but adjusted the analyses for the origin of the cohort. We found that none of the *PADI2* SNPs included in this study associated with anti-CCP-positive or anti-CCP-negative RA (**Table 2**).

Among the *PADI4* SNPs examined, the minor allele (T) of rs11203367 [the tag-SNP for the previously reported RA susceptibility haplotype (24, 30)], was associated with an increased risk of anti-CCP-positive RA (OR: 1.22, p=0.03) (**Table 2**). The minor allele (A) of rs2240335, on the other hand, appeared to protect against both anti-CCP-positive RA (OR: 0.82, p=0.04) and anti-CCP-negative RA (OR: 0.78, p=0.05). Moreover, the minor allele (T) of rs74058715 appeared to protect against anti-CCP-negative RA (OR: 0.38, p=0.003) (**Table 2**).

The majority of these reported associations was largely driven by the North American cohort, with exception of the association of rs74058715 and anti-CCP-negative RA (**Supplementary Table 1**).

Association of PADI Polymorphisms With RA by HLA-Type

The Danish cohort was HLA-typed, so we reanalyzed their data, taking this variable into account. We chose to examine the influence of *HLA-DRB1*04* and *HLA-DRB1*01* separately, since *HLA-DRB1*04* is more strongly associated with anti-CCP-positive RA than *HLA-DRB1*01* (12) (subjects were grouped as illustrated in **Supplementary Figure 1**; of note, subjects carrying both alleles were included in the analysis of *HLA-DRB1*04*).

Inclusion of HLA-DR type in the analysis did not influence the overall conclusions with respect to SNPs in *PADI2* (data not shown). In *HLA-DRB1*04*-positive subjects, on the other hand, the minor allele (T) of rs74058715 was associated with a significantly reduced risk of anti-CCP-positive RA after adjustment for age, sex, smoking status, PTPN22 R620W, and number of *HLA-DRB1*04* alleles (p=0.01; **Table 3**). This effect can be observed in **Figure 2A**, which shows the cumulative ORs associated with different combinations of four risk factors (*HLA-DRB1*04* alleles, *PADI4* alleles, PTPN22 R620W and smoking) compared to the reference group without any of these risk

TABLE 2 | Associations between selected polymorphisms in the *PADI* genes and anti-CCP-positive or anti-CCP-negative RA.

	Minor allele	Controls (n = 633)			Anti-CCP-positive RA (n = 413)						Anti-CCP-negative RA (n = 232)					
		pp	pq	qq	pp	pq	qq	OR	95% CI	p-value	pp	pq	qq	OR	95% CI	p-value
PADI2																
rs2057094	C	242	289	102	170	175	68	1.00	[0.83;1.19]	0.97	78	111	43	1.14	[0.92;1.44]	0.23
rs2076616	C	312	260	61	213	161	39	0.98	[0.81;1.19]	0.85	101	102	29	1.19	[0.94;1.50]	0.15
rs2235912	C	231	301	101	170	190	53	0.88	[0.73;1.06]	0.17	83	110	39	1.06	[0.84;1.33]	0.62
rs1005753	G	240	303	90	137	220	56	1.12	[0.92;1.35]	0.24	93	107	32	0.97	[0.77;1.22]	0.79
PADI4																
rs74058715	T	553	79	1	369	43	1	0.85	[0.57;1.26]	0.41	220	12	0	**0.38**	**[0.20;0.73]**	**0.003**
rs11203367	T	218	309	106	121	205	87	**1.22**	**[1.02;1.47]**	**0.03**	64	136	32	1.09	[0.86;1.38]	0.47
rs1748033	T	297	281	55	178	186	49	1.18	[0.97;1.44]	0.09	92	119	21	1.16	[0.91;1.48]	0.24
rs2240335	A	267	297	69	199	175	39	**0.82**	**[0.67;0.99]**	**0.04**	108	111	13	**0.78**	**[0.61;1.00]**	**0.05**

CCP, Cyclic Citrullinated Peptide; RA, Rheumatoid Arthritis; p, major allele; q, minor allele; OR, Odds Ratio; 95%CI, 95% confidence interval. Values marked in bold indicate 95% CI excluding 1.00 and p<0.05. Logistic regression with adjustment for age, sex, ever smoking and carriage of PTPN22 R620W.

TABLE 3 | Association of *PADI4* SNPs with RA by HLA-type.

	Controls (n = 533)			Anti-CCP-positive RA (n = 309)						Anti-CCP-negative RA (n = 136)					
	pp	pq	qq	pp	pq	qq	OR	95% CI	p-value	pp	pq	qq	OR	95% CI	p-value
rs74058715															
*HLA-DRB1*04*+[1]	161	32	0	207	20	0	**0.44**	**[0.23;0.84]**	**0.01**	47	3	0	0.31	[0.09;1.08]	0.06
*HLA-DRB1*01*+[2]	75	8	0	29	6	0	2.35	[0.69;8.01]	0.17	21	1	0	0.58	[0.06;5.13]	0.62
Other HLA-types	229	27	1	42	5	0	1.02	[0.37;2.85]	0.96	60	4	0	0.51	[0.17;1.49]	0.22
rs11203367															
*HLA-DRB1*04*+[1]	71	92	30	65	115	47	1.32	[0.99;1.77]	0.06	12	33	5	1.11	[0.67;1.82]	0.69
*HLA-DRB1*01*+[2]	26	40	17	11	20	4	0.70	[0.38;1.30]	0.26	7	14	1	0.72	[0.34;1.52]	0.39
Other HLA-types	87	121	49	17	21	9	1.07	[0.68;1.70]	0.76	18	36	10	1.05	[0.71;1.55]	0.82
rs1748033															
*HLA-DRB1*04*+[1]	105	74	14	94	104	29	**1.54**	**[1.13;2.12]**	**0.007**	20	28	2	1.28	[0.75;2.16]	0.36
*HLA-DRB1*01*+[2]	35	39	9	15	17	3	0.78	[0.40;1.50]	0.46	12	9	1	0.64	[0.29;1.44]	0.29
Other HLA-types	110	124	23	27	15	5	0.85	[0.50;1.43]	0.54	23	35	6	1.20	[0.78;1.84]	0.41
rs2240335															
*HLA-DRB1*04*+[1]	77	96	20	106	98	23	0.81	[0.59;1.11]	0.18	21	26	3	0.91	[0.53;1.54]	0.72
*HLA-DRB1*01*+[2]	37	36	10	20	11	4	0.78	[0.41;1.49]	0.46	6	16	0	1.16	[0.55;2.47]	0.70
Other HLA-types	116	117	24	20	20	7	1.13	[0.69;1.83]	0.63	31	28	5	0.90	[0.58;1.38]	0.63

*CCP, cyclic citrullinated peptide; RA, rheumatoid arthritis; HLA, human leukocyte antigen; p, major allele; q, minor allele; OR, odds ratio; 95% CI, 95% confidence interval. Logistic regression with adjustment for age, sex, ever smoking, carriage of PTPN22 R620W, and carriage of HLA-DRB1*04 or HLA-DRB1*01. Values marked in bold indicate 95% CI excluding 1.00 and p<0.05.*
*[1]Including heterozygotes carrying both HLA-DRB1*04 and HLA-DRB1*01; adjusted for the number of HLA-DRB1*04 alleles.*
*[2]Excluding heterozygotes carrying both HLA-DRB1*04 and HLA-DRB1*04; adjusted for the number of HLA-DRB1*01 alleles.*

factors. Hence, the ORs for *HLA-DRB1*04*-positive subjects who carried the minor allele (light blue bars) were generally lower than the ORs for *HLA-DRB1*04*-positive subjects who did not carry this allele (dark blue bars). By contrast, the minor allele (T) of rs11203367 conferred a borderline significant increase in OR for anti-CCP-positive RA in *HLA-DRB1*04*-positive subjects (p=0.06, **Table 3**), as visualized in **Figure 2B** (light blue bars for carriers *versus* dark blue bars for non-carriers). Notably, rs1748033(T), which belongs to the same RA susceptibility haplotype as rs11203367(T), associated strongly with anti-CCP-positive RA in *HLA-DRB1*04*-positive individuals (p=0.007; **Table 3**), with higher cumulative ORs for carriers of this allele compared to non-carriers (**Figure 2C**, light blue bars *vs* dark blue bars).

No clear pattern was observed for *HLA-DRB1*01*-positive individuals or for subjects who carried neither *HLA-DRB1*04* nor *HLA-DRB1*01* (**Table 3** and gray bars in **Figure 2**). Neither were any associations between SNPs in *PADI* and anti-CCP-negative RA revealed after adjustment for HLA type (**Table 3**).

Gene-Gene and Gene-Exposure Interactions

In view of the associations between SNPs in *PADI4* and anti-CCP-positive RA and the known interaction between the SE and smoking as risk factors for anti-CCP-positive RA, we analyzed the interactions between *PADI4* SNPs and other risk factors, i.e., *HLA-DRB1*04*, *HLA-DRB1*01*, PTPN22 R620W, and smoking on the risk of anti-CCP-positive or anti-CCP-negative RA.

We observed a strong interaction between the major allele (C) of rs74058715, *HLA-DRB1*04* and ever-smoking, that associated with increased risk of anti-CCP-positive RA (p=0.008, **Supplementary Table 2**). Similarly, the minor allele (T) of rs1748033 and *HLA-DRB1*04* interacted synergistically increasing the risk of anti-CCP-positive RA, irrespective of smoking status (p=0.004, **Supplementary Table 2**). Regarding

the risk of anti-CCP-negative RA, we also observed an interaction between the minor allele (T) of rs1748033, *HLA-DRB1*04* and ever-smoking (p=0.05, **Supplementary Table 2**).
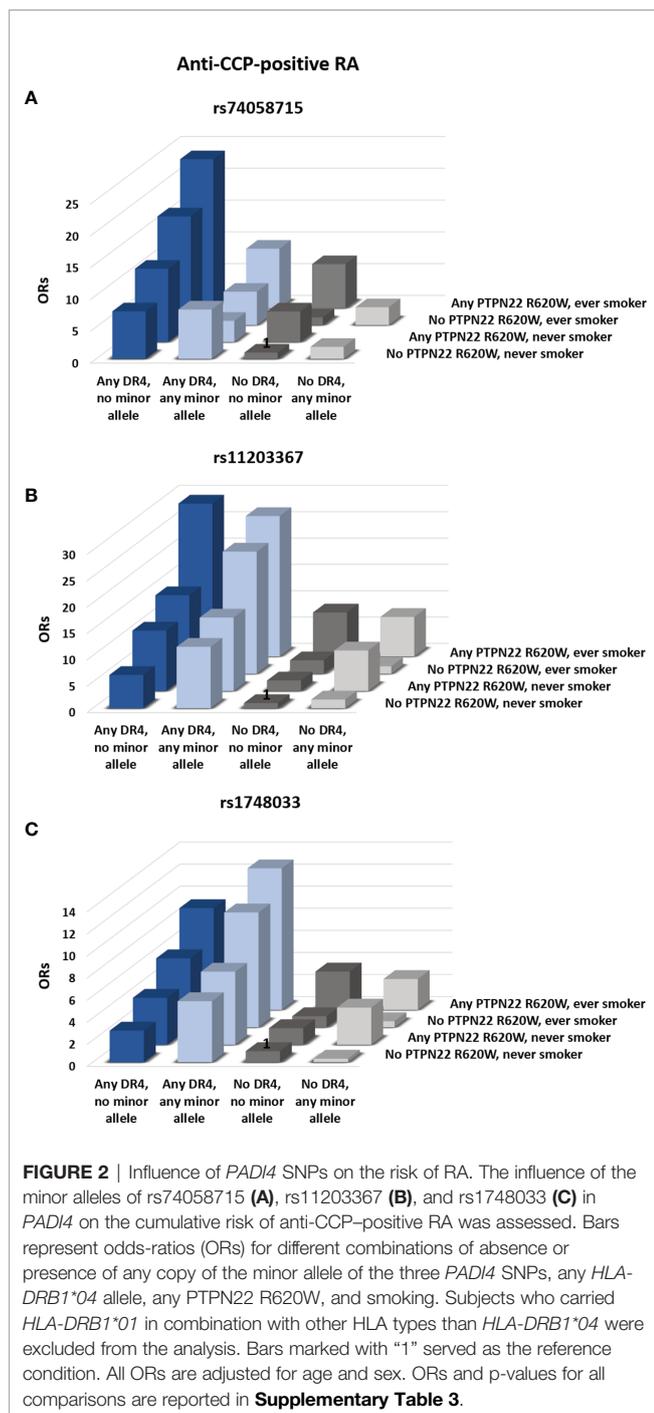
DISCUSSION

PAD2 and PAD4 are thought to be involved in the pathogenesis of ACPA-positive RA by catalyzing the formation of citrullinated autoantigens that are recognized by T cells and targeted by ACPAs (8). However, citrullination by PADs may have other potentially pathogenic roles, including regulation of T-cell responses (47, 48) and induction of NET formation (3, 49). Accordingly, SNPs in *PADI2* and *PADI4* have been shown to associate with RA, mainly in Asian populations (22–27).

This study did not show significant associations between *PADI2* SNPs and RA in the two mainly Caucasian cohorts examined. We cannot rule out, however, that the minor allele (C) of rs2235912 confers resistance to anti-CCP-positive RA, as suggested by a weak tendency in that direction, which is in agreement with findings from a previous meta-analysis (43).

After merging the Danish and North American cohorts and adjusting the model for the origin of the cohorts, we found that the minor allele (T) of rs11203367 in *PADI4* (tag-SNP for a haplotype containing Gly55Ser, Val82Ala, and Gly112Ala) associated with anti-CCP-positive RA with an OR of 1.22. The association was largely driven by the North American cohort, in accordance with a previous study showing association with rs2240340, which is in high LD with the SNP in our study (31). Furthermore, the minor allele (A) of the exon-synonymous SNP rs2240335 was associated with a decreased risk of anti-CCP-positive RA with an OR of 0.82, in agreement with previous results (35).

An especially strong association was observed in *HLA-DRB1*04*-positive subjects in the Danish cohort between the

FIGURE 2 | Influence of *PADI4* SNPs on the risk of RA. The influence of the minor alleles of rs74058715 **(A)**, rs11203367 **(B)**, and rs1748033 **(C)** in *PADI4* on the cumulative risk of anti-CCP–positive RA was assessed. Bars represent odds-ratios (ORs) for different combinations of absence or presence of any copy of the minor allele of the three *PADI4* SNPs, any *HLA-DRB1*04* allele, any PTPN22 R620W, and smoking. Subjects who carried *HLA-DRB1*01* in combination with other HLA types than *HLA-DRB1*04* were excluded from the analysis. Bars marked with "1" served as the reference condition. All ORs are adjusted for age and sex. ORs and p-values for all comparisons are reported in **Supplementary Table 3**.

minor allele (T) of rs1748033 and anti-CCP-positive RA, and a similar tendency was observed for rs11203367, which is also part of RA the susceptibility haplotype. In individuals carrying *HLA-DRB1*04*, the minor (T) allele of rs74058715 was also associated with reduced risk of anti-CCP-positive RA with an OR of 0.44. This SNP is not part of the RA susceptibility haplotype but is associated with decreased expression of PAD4 in neutrophils and monocytes (36). These results suggest that SNPs in *PADI4* alter the cumulative risk of disease conferred by other well-

characterized risk factors (*HLA-DRB1*04*, PTPN22 R620W, and smoking). In accordance, we observed an interaction between the major allele (C) of rs74058715, *HLA-DRB1*04*, and ever-smoking, as well as between the minor allele (T) of rs1748033 and *HLA-DRB1*04* in increasing the risk of anti-CCP-positive RA.

Somewhat unexpectedly, we observed that the minor (T) allele of rs74058715 was associated with reduced risk of anti-CCP-negative RA, and the minor allele (A) of rs2240335 showed a similar tendency. Furthermore, we observed an interaction between the minor allele (T) of rs1748033, *HLA-DRB1*04*, and ever-smoking in conferring risk of anti-CCP-negative RA. Generation of citrullinated autoantigens does not explain a pathogenic role for citrullination in ACPA-negative RA, but *PADI* polymorphisms may be associated with differences in citrullination patterns that may have an adverse influence on the generation of pro-inflammatory mediators (50), regulation of T-cell responses (47, 48), or NET formation (3, 49) potentially leading to tissue damage.

Our study is limited by the relatively low number of subjects included and the lack of HLA-typing of the North American cohort. Furthermore, only low-resolution HLA-typing was performed so the *HLA-DRB1*04*-positive group may have included individuals carrying *HLA-DRB1*0402*, which does not contain the SE and has been associated with a protective effect against RA (51).

It is important to note that although *HLA-DRB1*04* and *HLA-DRB1*01* are often grouped together as SE-containing serotypes, we chose to separate them in our analysis, since the association of *HLA-DRB1*04* with RA is considerably stronger than that of *HLA-DRB1*01* (12). We found no association between SNPs in *PADI* and RA in *HLA-DRB1*01*-positive individuals, and no interaction between *PADI4* SNPs and *HLA-DRB1*01*, which may be due to the low power of the study. On the other hand, cohort stratifications and lack of correction due to the exploratory nature of the study may have led to type I errors. Thus, similar and more robust analyses in larger cohorts are warranted.

Our study suggests that certain SNPs in *PADI4* are risk factors for ACPA-positive RA, especially in *HLA-DRB1*04*-positive individuals. Furthermore, it shows that *PADI4* SNPs may also play a role in ACPA-negative RA.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Scientific Ethical Committees for Copenhagen and Frederiksberg (KF 01-039/01), the Danish Data Protection Agency (2001–41–0658), the Institutional Review Board at Statens Serum Institut (21–00050), and the Institutional

Review Board of the University of Wisconsin–Madison (#2015-0156). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM, CN, DD, MS, MF, PG, SJ, and CE designed the study. LM and PG carried out the experiments. LM, CE, and SJ carried out the statistical analyses. LM, CN, and CE drafted the manuscript. SJ, MF, and MS provided the sample material and the clinical data for the Danish and North American cohorts, respectively. DD, NØ, and MS revised the manuscript critically. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Senshu T, Kan S, Ogawa H, Manabe M, Asaga H. Preferential Deimination of Keratin K1 and Filaggrin During the Terminal Differentiation of Human Epidermis. *Biochem Biophys Res Commun* (1996) 719:712–9. doi: 10.1006/bbrc.1996.1240

2. Falcão AM, Meijer M, Scaglione A, Rinwa P, Agirre E, Liang J, et al. PAD2-Mediated Citrullination Contributes to Efficient Oligodendrocyte Differentiation and Myelination. *Cell Rep* (2019) 27(4):1090–102.e10. doi: 10.1016/j.celrep.2019.03.108

3. Holmes CL, Shim D, Kernien J, Johnson CJ, Nett JE, Shelef MA. Insight Into Neutrophil Extracellular Traps Through Systematic Evaluation of Citrullination and Peptidylarginine Deiminases. *J Immunol Res* (2019) 2019. doi: 10.1155/2019/2160192

4. Schellekens GA, De Jong BAW, Van Den Hoogen FHJ, Van De Putte LBA, Van Venrooij WJ. Citrulline Is an Essential Constituent of Antigenic Determinants Recognized by Rheumatoid Arthritis-Specific Autoantibodies. *J Clin Invest* (1998) 101(1):273–81. doi: 10.1172/JCI1316

5. Schellekens GA, Visser H, De Jong BA, Van Den Hoogen FH, Hazes JM, Breedveld FC, et al. The Diagnostic Properties of Rheumatoid Arthritis Antibodies Recognizing a Cyclic Citrullinated Peptide. *Arthritis Rheum* (2000) 43(1):155–63. doi: 10.1002/1529-0131(200001)43:1<155::AID-ANR20>3.0.CO;2-3

6. Rantapää-Dahlqvist S, De Jong BAW, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies Against Cyclic Citrullinated Peptide and IgA Rheumatoid Factor Predict the Development of Rheumatoid Arthritis. *Arthritis Rheum* (2003) 48(10):2741–9. doi: 10.1002/art.11223

7. van der Helm-van Mil AHM, Verpoort KN, Breedveld FC, Toes REM, Huizinga TWJ. Antibodies to Citrullinated Proteins and Differences in Clinical Progression of Rheumatoid Arthritis. *Arthritis Res Ther* (2005) 7(5):R949-58. doi: 10.1186/ar1767

8. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. Cutting Edge: The Conversion of Arginine to Citrulline Allows for a High-Affinity Peptide Interaction With the Rheumatoid Arthritis-Associated HLA-DRB1*0401 MHC Class II Molecule. *J Immunol* (2003) 171(2):538–41. doi: 10.4049/jimmunol.171.2.538

9. Klareskog L, Stolt P, Lundberg K, Källberg H, Bengtsson C, Grunewald J, et al. A New Model for an Etiology of Rheumatoid Arthritis: Smoking may Trigger HLA-DR (Shared Epitope)-Restricted Immune Reactions to Autoantigens Modified by Citrullination. *Arthritis Rheum* (2006) 54(1):38–46. doi: 10.1002/art.21575

10. Ding B, Padyukov L, Lundström E, Seielstad M, Plenge RM, Oksenberg JR, et al. Different Patterns of Associations With Anti–Citrullinated Protein Antibody–Positive and Anti–Citrullinated Protein Antibody– Negative Rheumatoid Arthritis in the Extended Major Histocompatibility Complex Region. *Arthritis Rheum* (2009) 60(1):30–8. doi: 10.1002/art.24135

11. Pedersen M, Jacobsen S, Garred P, Madsen HO, Klarlund M, Svejgaard A, et al. Strong Combined Gene-Environment Effects in Anti-Cyclic Citrullinated Peptide-Positive Rheumatoid Arthritis: A Nationwide Case-Control Study in Denmark. *Arthritis Rheum* (2007) 56(5):1446–53. doi: 10.1002/art.22597

12. Lundström E, Källberg H, Alfredsson L, Klareskog L, Padyukov L. Gene-Environment Interaction Between the DRB1 Shared Epitope and Smoking in the Risk of Anti-Citrullinated Protein Antibody-Positive Rheumatoid Arthritis: All Alleles are Important. *Arthritis Rheum* (2009) 60(6):1597–603. doi: 10.1002/art.24572

13. Begovich AB, Carlton VEH, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, et al. Report A Missense Single-Nucleotide Polymorphism in a Gene Encoding a Protein Tyrosine Phosphatase (PTPN22) Is Associated With Rheumatoid Arthritis. *Am J Hum Genet* (2004) 75(2):330–7. doi: 10.1086/422827

14. Källberg H, Padyukov L, Plenge RM, Rönnelid J, Gregersen PK, van der Helm-van Mil AHM, et al. Gene-Gene and Gene-Environment Interactions Involving HLA-BRB1, PTPN22, and Smoking in Two Subsets of Rheumatoid Arthritis. *Am J Hum Genet* (2007) 80(5):867–75. doi: 10.1086/516736

15. Vossenaar ER, Zendman AJW, Van Venrooij WJ, Pruijn GJM. PAD, a Growing Family of Citrullinating Enzymes: Genes, Features and Involvement in Disease. *BioEssays* (2003) 25:1106–18. doi: 10.1002/bies.10357

16. Asaga H, Nakashima K, Senshu T, Ishigami A. Immunocytochemical Localization of Peptidylarginine Deiminase in Human Eosinophils and Neutrophils. *J Leuk Biol* (2001) 70(1):46–51.

17. Vossenaar ER, Radstake TRD, van der Heijden A, van Mansum MAM, Dieteren C, de Rooij D-J, et al. Expression and Activity of Citrullinating

Peptidylarginine Deiminase Enzymes in Monocytes and Macrophages. *Ann Rheum Dis* (2004) 63(4):373–81. doi: 10.1136/ard.2003.012211

18. Foulquier C, Sebbag M, Clavel C, Chapuy-Regaud S, Al Badine R, Méchin MC, et al. Peptidyl Arginine Deiminase Type 2 (PAD-2) and PAD-4 But Not PAD-1, PAD-3, and PAD-6 are Expressed in Rheumatoid Arthritis Synovium in Close Association With Tissue Inflammation. *Arthritis Rheum* (2007) 56 (11):3541–53. doi: 10.1002/art.22983

19. Damgaard D, Senolt L, Nielsen MF, Pruijn GJ, Nielsen CH. Demonstration of Extracellular Peptidylarginine Deiminase (PAD) Activity in Synovial Fluid of Patients With Rheumatoid Arthritis Using a Novel Assay for Citrullination of Fibrinogen. *Arthritis Res Ther* (2014) 16(6):498. doi: 10.1186/s13075-014-0498-9

20. Blachère NE, Parveen S, Frank MO, Dill BD, Molina H, Orange DE. High-Titer Rheumatoid Arthritis Antibodies Preferentially Bind Fibrinogen Citrullinated by Peptidylarginine Deiminase 4. *Arthritis Rheumatol* (2017) 69(5):986–95. doi: 10.1002/art.40035

21. Nakayama-Hamada M, Suzuki A, Kubota K, Takazawa T, Ohsaka M, Kawaida R, et al. Comparison of Enzymatic Properties Between Hpadi2 and Hpadi4. *Biochem Biophys Res Commun* (2005) 327(1):192–200. doi: 10.1016/j.bbrc.2004.11.152

22. Too CL, Murad S, Dhaliwal JS, Larsson P, Jiang X, Ding B, et al. Polymorphisms in Peptidylarginine Deiminase Associate With Rheumatoid Arthritis in Diverse Asian Populations: Evidence From MyEIRA Study and Meta-Analysis. *Arthritis Res Ther* (2012) 14(6):R250. doi: 10.1186/ar4093

23. Chang X, Xia Y, Pan J, Meng Q, Zhao Y, Yan X. PADI2 is Significantly Associated With Rheumatoid Arthritis. *PloS One* (2013) 8(12):e81259. doi: 10.1371/journal.pone.0081259

24. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, et al. Functional Haplotypes of PADI4, Encoding Citrullinating Enzyme Peptidylarginine Deiminase 4, are Associated With Rheumatoid Arthritis. *Nat Genet* (2003) 34(4):395–402. doi: 10.1038/ng1206

25. Lee YH, Rho YH, Choi SJ, Ji JD, Song GG. PADI4 Polymorphisms and Rheumatoid Arthritis Susceptibility: A Meta-Analysis. *Rheumatol Int* (2007) 27(9):827–33. doi: 10.1007/s00296-007-0320-y

26. Terao C, Yamada R, Ohmura K, Takahashi M, Kawaguchi T, Kochi Y, et al. The Human AIRE Gene at Chromosome 21q22 is a Genetic Determinant for the Predisposition to Rheumatoid Arthritis in Japanese Population. *Hum Mol Genet* (2011) 20(13):2680–5. doi: 10.1093/hmg/ddr161

27. Freudenberg J, Lee HS, Han BG, Shin HD, Kang YM, Sung YK, et al. Genome-Wide Association Study of Rheumatoid Arthritis in Koreans: Population-Specific Loci as Well as Overlap With European Susceptibility Loci. *Arthritis Rheum* (2011) 63(4):884–93. doi: 10.1002/art.30235

28. Gandjbakhch F, Fajardy I, Ferré B, Dubucquoi S, Flipo RM, Roger N, et al. A Functional Haplotype of PADI4 Gene in Rheumatoid Arthritis: Positive Correlation in a French Population. *J Rheumatol* (2009) 36(5):881–6. doi: 10.3899/jrheum.080398

29. Martinez A, Valdivia A, Pascual-Salcedo D, Lamas JR, Fernandez-Arquero M, Balsa A, et al. PADI4 Polymorphisms Are Not Associated With Rheumatoid Arthritis in the Spanish Population. *Rheumatol (Oxford)* (2005) 44(10):1263–6. doi: 10.1093/rheumatology/kei008

30. Barton A, Bowes J, Eyre S, Spreckley K, Hinks A, John S, et al. A Functional Haplotype of the PADI4 Gene Associated With Rheumatoid Arthritis in a Japanese Population Is Not Associated in a United Kingdom Population. *Arthritis Rheum* (2004) 50(4):1117–21. doi: 10.1002/art.20169

31. Plenge RM, Padyukov L, Remmers EF, Purcell S, Lee AT, Karlson EW, et al. Replication of Putative Candidate-Gene Associations With Rheumatoid Arthritis in >4,000 Samples From North America and Sweden: Association of Susceptibility With PTPN22, CTLA4, and PADI4. *Am J Hum Genet* (2005) 77(6):1044–60. doi: 10.1086/498651

32. Burr ML, Naseem H, Hinks A, Eyre S, Gibbons LJ, Bowes J, et al. PADI4 Genotype Is Not Associated With Rheumatoid Arthritis in a Large UK Caucasian Population. *Ann Rheum Dis* (2010) 69(4):666–70. doi: 10.1136/ard.2009.111294

33. Hoppe B, Häupl T, Gruber R, Kiesewetter H, Burmester GR, Salama A, et al. Detailed Analysis of the Variability of Peptidylarginine Deiminase Type 4 in German Patients With Rheumatoid Arthritis: A Case-Control Study. *Arthritis Res Ther* (2006) 8(2):R34. doi: 10.1186/ar1889

34. Farago B, Talian GC, Maasz A, Magyari L, Horvatovich K, Kovacs B, et al. Prevalence of Functional Haplotypes of the Peptidylarginine Deiminase Citrullinating Enzyme Gene in Patients With Rheumatoid Arthritis: No

Influence of the Presence of Anti-Citrullinated Peptide Antibodies. *Clin Exp Rheumatol* (2007) 25(4):523–8.

35. Mergaert MA, Bawadekar M, Nguyen QT, Massarenti L, Holmes LC, Rebernick R, et al. Reduced Anti-Histone Antibodies and Increased Risk of Rheumatoid Arthritis Associated With a Single Nucleotide Polymorphism in PADI4 in North Americans. *Vol. 20 Int J Mol Sci* (2019) p:3093. doi: 10.3390/ijms20123093

36. Naranbhai V, Fairfax BP, Makino S, Humburg P, Wong D, Ng E, et al. Genomic Modulators of Gene Expression in Human Neutrophils. *Nat Commun* (2015) 6(May):1–13. doi: 10.1038/ncomms8545

37. Bang SY, Han TU, Choi CB, Sung YK, Bae SC, Kang C. Peptidyl Arginine Deiminase Type IV (PADI4) Haplotypes Interact With Shared Epitope Regardless of Anti-Cyclic Citrullinated Peptide Antibody or Erosive Joint Status in Rheumatoid Arthritis: A Case Control Study. *Arthritis Res Ther* (2010) 12(3):R115. doi: 10.1186/ar3051

38. Poór G, Nagy Z, Schmidt Z, Brózik M, Merétey K, Gergely P. Genetic Background of Anticyclic Citrullinated Peptide Autoantibody Production in Hungarian Patients With Rheumatoid Arthritis. *Ann N Y Acad Sci* (2007) 1110:23–32. doi: 10.1196/annals.1423.004

39. Pedersen M, Jacobsen S, Klarlund M, Pedersen BV, Wiik A, Wohlfahrt J, et al. Environmental Risk Factors Differ Between Rheumatoid Arthritis With and Without Auto-Antibodies Against Cyclic Citrullinated Peptides. *Arthritis Res Ther* (2006) 8(4):R133. doi: 10.1186/ar2022

40. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 Revised Criteria for the Classification of Rheumatoid Arthritis. *Arthritis Rheumatol* (1988) 31 (3):315–24. doi: 10.1002/art.1780310302

41. Rebernick R, Fahmy L, Glover C, Bawadekar M, Shim D, Holmes CL, et al. DNA Area and NETosis Analysis (DANA): A High-Throughput Method to Quantify Neutrophil Extracellular Traps in Fluorescent Microscope Images. *Biol Proced Online* (2018) 20(1):1–9. doi: 10.1186/s12575-018-0072-y

42. Katz JN, Barrett J, Liang MH, Bacon AM, Kaplan H, Kieval RI, et al. Sensitivity and Positive Predictive Value of Medicare Part B Physician Claims for Rheumatologic Diagnoses and Procedures. *Arthritis Rheum* (1997) 40 (9):1594–600. doi: 10.1002/art.1780400908

43. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of Rheumatoid Arthritis Contributes to Biology and Drug Discovery. *Nature* (2014) 506(7488):376–81. doi: 10.1038/nature12873

44. Massarenti L, Enevold C, Damgaard D, Ødum N, Nielsen CH, Jacobsen S. Peptidylarginine Deiminase-4 Gene Polymorphisms are Associated With Systemic Lupus Erythematosus and Lupus Nephritis. *Scand J Rheumatol* (2019) 48(2):133–40. doi: 10.1080/03009742.2018.1488273

45. Enevold C, Oturai AB, Sørensen PS, Ryder LP, Koch-Henriksen N, Bendtzen K. Multiple Sclerosis and Polymorphisms of Innate Pattern Recognition Receptors TLR1-10, NOD1-2, DDX58, and IFIH1. *J Neuroimmunol* (2009) 212(1–2):125–31. doi: 10.1016/j.jneuroim.2009.04.008

46. Kimura A, Sasazuki T. Eleventh International Histocompatibility Workshop Reference Protocol for the HLA DNA-Typing Technique, in: Tsuji K, Azaiwa M, Sazasuki T (eds): HLA 1991. Proceedings of the Eleventh International Histocompatibility Workshop and Conference, vol I, Oxford, Oxford Science Publications, (1992) pp. 397-419.

47. Kawalkowska J, Quirke AM, Ghari F, Davis S, Subramanian V, Thompson PR, et al. Abrogation of Collagen-Induced Arthritis by a Peptidyl Arginine Deiminase Inhibitor Is Associated With Modulation of T Cell-Mediated Immune Responses. *Sci Rep* (2016) 6(May):1–12. doi: 10.1038/srep26430

48. Sun B, Chang HH, Salinger A, Tomita B, Bawadekar M, Holmes CL, et al. Reciprocal Regulation of Th2 and Th17 Cells by PAD2-Mediated Citrullination. *JCI Insight* (2019) 4(22):e129687. doi: 10.1172/jci.insight.129687

49. Khandpur R, Carmona-rivera C, Vivekanandan-giri A, Yalavarthi S, Knight JS, Friday S, et al. NETs Are a Source of Citrullinated Autoantigens and Stimulate Inflammatory Responses in Rheumatoid Arthritis. *Sci Transl Med* (2013) 5(178):178ra40. doi: 10.1126/scitranslmed.3005580

50. Sun B, Dwivedi N, Bechtel TJ, Paulsen JL, Muth A, Bawadekar M, et al. Citrullination of NF-kB P65 Promotes Its Nuclear Localization and TLR-Induced Expression of IL-1β and TNF-α. *Sci Immunol* (2017) 2:1–13. doi: 10.1126/sciimmunol.aal3062

51. Hall FC, Weeks DE, Camilleri JP, Williams LA, Amos N, Darke C, et al. Influence of the HLA-DRB1 Locus on Susceptibility and Severity in

Rheumatoid Arthritis. *QJM - Mon J Assoc Physicians* (1996) 89(11):821–9. doi: 10.1093/qjmed/89.11.821

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