Interaction of HLA-DRB1 shared epitope and smoking on the development of anti-citrullinated protein antibody positive rheumatoid arthritis in Greek patients

E.N. Mole[1], A.E. Tarassi[2], A.G. Tsirogianni[2], T.E. Athanasiades[2], V.E. Kitsiou[2], D.I. Kouniaki[2], S.V. Gazi[1], P.G. Vlachoyiannopoulos[3]

[1]Department of Rheumatology, KAT General Hospital of Attica, Athens;
[2]Department of Immunology-Histocompatibility, Evangelismos General Hospital, Athens;
[3]Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Greece.

Abstract

Objective

Rheumatoid arthritis (RA) is a complex, multifactorial autoimmune disease, whose aetiopathogenesis involves genetic and environmental factors. The aim of this case-control study is to confirm the impact of interaction of genetic and environmental factors in the pathogenesis of RA in Greek smoker and non-smoker patients.

Methods

We assessed the effects of shared epitope (SE) alleles and smoking on the presence of ACPA autoimmunity in three hundred Greek patients with longstanding RA (150 smokers and 150 non-smokers). Three hundred and forty-six Greek blood donors volunteers and hospital personnel served as controls.

Results

*An increased frequency of HLA-DRB1 *01:01, *10:01, *04:04 and *04:05 alleles, as well as the protective role of *04:03 allele in Greek patients were confirmed during comparison with controls. The presence of any SE influenced the development of RA (OR: 4.37[3.13-6.11], p<0.001). A strong effect for ACPA production was observed in individuals carrying any SE allele (OR: 4.3[2.57-7.22], p<0.001). Single SE carriers in combination with smoking had an increased risk of developing ACPA-positive RA (OR: 6.53[1.47-28.91], p=0.013), which further increased in smokers with a double gene copy (OR: 15.27[1.39-167.52], p=0.026). The strongest interaction, with regard to ACPA-positive RA, was observed in individuals that possessed the HLA-DRB1 *01:01 (OR: 12.55[1.32-119.35], p=0.028) SE allele, whereas the combination of SE genes and smoking did not influence the risk of ACPA-negative RA (OR: 2.01[0.76-5.26], p=0.15).*

Conclusion

We identified that smoking and the presence of SE alleles increased the risk of developing ACPA-positive RA, indicating a strong genetic-environmental correlation that probably triggers the pathogenesis of RA in Greek patients.

Key words

shared epitope, smoking, rheumatoid arthritis, interaction, alleles

Evangelia N. Mole, MD, MSc
Aikaterini E. Tarassi, MD, PhD
Alexandra G. Tsirogianni, MD, PhD
Theophilos E. Athanasiades, PhD
Vasiliki E. Kitsiou, MD, PhD
Diamanto I. Kouniaki, PhD
Sousana V. Gazi, MD, PhD
Panayiotis G. Vlachoyiannopoulos, MD, PhD

Please address correspondence to:
Evangelia N. Mole
2 Nikis Street,
Kifisia 14561, Greece.
E-mail: evamole34@gmail.com

Received on March 31, 2025; accepted in revised form on July 2, 2025.

© Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2025.

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that affects up to 1% of adult population (1) and predominantly involves synovial joints (2), resulting structural damage of cartilage, bone and ligaments (1-3). Subsequently, extra-articular manifestations may occur, affecting significantly morbidity and mortality of patients. The aetiology is not fully understood, however genetic and environmental factors influence the disease pathogenesis (2, 5).

A genetic background has been established in twin and family studies, and RA heritability reached 60% (6). The strongest genetic association is thought to be within the major histocompatibility complex (MHC), also known as the human leukocyte antigen system (HLA), particularly the HLA-DRB1 gene, accounting for 30–50% of the total predisposition (7). The HLA-DR molecules are heterodimers, consisting of an α (DRA) and a β-chain (DRB), the two chains are anchored to the membrane of the antigen-presenting cells and their basic function is presentation of antigenic peptides to T-lymphocytes (8, 9). Multiple HLA-DRB1 alleles encoding the shared epitope (SE) amino acid sequence at positions 70 to 74 in the third hypervariable region of the DRB1 molecule have been associated with a higher risk of RA (10). The SE motif may be directly involved in RA pathogenesis by allowing the presentation of arthritogenic peptides to CD4 T-cells, which further induce production of antibodies against cyclic citrullinated peptides (anti-CCP) by B-cells (6, 11).

Citrullination is a post-transcriptional procedure characterised by conversion of a peptidyl-arginine to peptidyl-citrulline by peptidylarginine deaminase enzyme (PADI) (12). Citrullination imparts neo-antigenic properties to the protein molecule and takes place intracellularly under conditions of cell apoptosis (12).

ACPAs (anti-citrullinated protein antibodies) are detected in the majority of RA patients, and they are highly specific for RA (95%), as 75% of patients with positive antibodies and undifferentiated arthritis will develop RA at least three years later (13). Compared to rheumatoid factor (RF), they have a higher diagnostic value. In addition, seropositive patients experience a more aggressive form of disease, compared to seronegative patients, in terms of disease activity, radiographic damage and progression, while their presence may predict erosive disease (14). The distinction between ACPA-positive and ACPA-negative RA proved particularly useful once the determinant effect of genetic factors (HLA-DRB1-SE) on seropositive RA was recognised (14, 15). A strong association between SE alleles and ACPAs in RA patients has been found, suggesting that DR molecules encoded by SE alleles are involved in the presentation of citrullinated peptides to T-cells (16). A study with human DR4 transgenic mice reported that the presence of citrullinated peptides leads to increased affinity to DR molecules and subsequent activation of CD4 T-cells (16, 17).

Smoking has been recognised as the most important relevant environmental risk factor only for ACPA-positive disease, particularly in individuals carrying one or two copies of HLA-DRB1 SE (14, 18). Numerous studies have highlighted a strong genetic and environmental interplay between smoking and SE alleles in the risk of developing ACPA-positive RA, which increases in proportion to the cumulative exposure to smoking, measured in pack-years (3, 19, 20). This could be explained by the observation of citrullinated proteins in the lungs of smokers, which upon binding to HLA class II molecules, are presented to CD4 T-cells (12). Thus, smoking is associated with an increased risk of developing RA by inducing the production of ACPA antibodies, suggesting mechanisms associated with B-cell activation (6). Recent studies have indicated that smoking not only influences the ACPAs positivity but also correlates with elevated levels of ACPAs and RF (21). However, the precise mechanism by which smoking contributes to ACPA production in patients with different genetic background remains unclear. The reported results are conflicting, possibly due to genetic and environmental heterogeneities among different studied

populations. The genetic background differs between ethnicities. For instance, in Greek RA patients, SE alleles are detected in about half of them and disease seems to be milder (22, 23).

This is a case-control study with prevalent cases in order to confirm the impact of environmental and genetic factors in the pathogenesis of RA in the Greek population. More specifically we aim to assess the association of HLA-DRB1 SE and smoking in the presence or absence of ACPA autoimmunity in Greek RA patients.

Materials and methods

We conducted a case-control study of 300 Greek patients with longstanding RA who were followed up at the Department of Pathophysiology, School of Medicine, University of Athens and Department of Rheumatology of KAT, General Hospital of Attica. All patients met at least four of the American College of Rheumatology criteria for RA (ACR 1987) at the time of enrolment (24). Three hundred and forty-six Greek blood donor volunteers and hospital personnel with known HLA-DRB1 status served as controls, with consideration given to age and gender, after answering questionnaire about tobacco exposure and blood sampling for ACPA determination. Participation was voluntary and written consent obtained from all patients. This study has been approved by the research ethics committee of the National and Kapodistrian University of Athens (protocol code 180 and date of approval 18.09.2019). The data that support the findings of this study will be available in request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

At inclusion, cases and controls were asked to provide blood samples and smoking history was assessed by questionnaires. Current and past smokers were classified as smokers and those who had never smoked were classified as non-smokers. ACPA antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (QUANTA Lite Inova – Diagnostics Inc. San Diego CA 92131). An antibody titre ≥25IU was considered as positive. Genomic DNA was extracted from peripheral venous blood using salting-out procedures (25). The HLA-DRB1 high-resolution typing was performed by molecular techniques: polymerase chain reaction and hybridisation with sequence-specific oligonucleotide probes and/or specific primers (PCR-SSOP, PCR-SSP) (26, 27) in the Department of Immunology - Histocompatibility of the Evangelismos General Hospital of Athens. The HLA-DRB1 *01:01, *01:02, *04:01, *04:02, *04:03, *04:04, *04:05, *04:08, *10:01 were classified as the SE alleles.

Statistics

In a series of multiple logistic regression analyses, odds ratios (ORs) and 95% confidence intervals were calculated, in order to compare the allelic frequency between cases and controls and to clarify the possible effects of SE alleles and smoking on ACPA antibody production. Presence of ACPAs was the dependent variable, non-smokers and non-SE carriers were served as reference groups. In addition, we investigated the interaction between smoking and SE genes with regard to the risk of developing ACPA-positive RA, by performing multiple logistic regression analysis on a multiplicative scale. Analyses were adjusted for age and gender, according to the principle of control selection. *p*-values less than 0.05 were considered as statistically significant. The Statistical Package for the Social Sciences, v. 25 (IBM SPSS statistics) was used.

Table I. Characteristics of the patients and controls.

	RA patients (n=300)	Controls (n=346)
Female (%)	223 (74.3)	260 (75.1)
Male (%)	77 (25.7)	86 (24.9)
Age (mean ± SD) years	65.18 ± 12.05	64.58 ± 11.73
Disease duration (mean ± SD) years	10.23 ± 6.43	-
Current smokers (%)	150 (50)	129 (37.3)
ACPA + (%)	173 (57.7)	106 (30.6)
HLA-DRB1 SE + (%)	194 (64.7)	101 (29.2)

SD: standard deviation

Table II. Frequencies of SE alleles in Greek RA patients and controls.

	RA patients n (%)	Controls n (%)	OR 95% CI, *p*-value
*01:01	67 (22.3)	32 (9.2)	3.24 [1.99-5.25], <0.001
*01:02	15 (5)	11 (3.2)	2.16 [0.93-5.03], 0.07
*04:01	10 (3.3)	7 (2)	2.12 [0.75-5.95], 0.15
*04:02	8 (2.7)	5 (1.4)	2.49 [0.75-8.2], 0.13
*04:03	13 (4.3)	21 (6.1)	0.99 [0.46-2.12], 0.9
*04:04	19 (6.3)	5 (1.4)	5.69 [2.04-15.9], 0.001
*04:05	45 (15)	15 (4.3)	4.56 [2.41-8.61], <0.001
*04:08	3 (1)	0 (0)	8.1 [0.41-158.4], 0.16
*10:01	62 (20.7)	14 (4)	6.47 [3.47-12.06], <0.001
Any SE	194 (64.7)	101 (29.2)	4.37 [3.13-6.11], <0.001
Single SE	141 (47)	83 (24)	4.12 [2.88-5.9], <0.001
Double SE	53 (17.7)	13 (3.8)	9.16 [4.75-17.68], <0.001

Results

Patients' characteristics

The overall study population consisted of 300 Greek RA patients, with mean age 65.18±12.05 years and mean disease duration 10.23±6.43 years, and 346 controls. In total 74.3% of cases were women, 57.7% were ACPA-positive and 64.7% possessed at least one copy of HLA-DRB1 SE allele. More specifically 47% and 17% of cases possessed a single and double copy of SE gene respectively. Among cases 50% were smokers and 50% had never smoked (Table I). Among our study population, only nine cases and five controls were past smokers; these individuals were classified as smokers for the purposes of this analysis.

SE alleles frequencies

An increased frequency of HLA-DRB1 *01:01, *10:01, *04:04, *04:05 alleles,

as well as the protective role of *04:03 alleles in Greek RA patients were confirmed (28, 29). The HLA-DRB1 *01:01 allele was the most common found within the HLA-DR1 group, with allelic frequency of 22.3% among cases *versus* 9.2% of controls (OR: 3.24 [95% CI: 1.99–5.25], $p<0.001$). We identified HLA-DRB1 *10:01 allele in 20.7% of patients *versus* 4% of controls (OR: 6.47 [95% CI: 3.47–12.06], $p<0.001$), HLA-DRB1 *04:04 allele in 6.3% of patients *versus* 1.4% of controls (OR: 5.69 [95% CI: 2.04–15.9], $p=0.001$) and HLA-DRB1 *04:05 allele in 15% among cases *versus* 4.3% among controls (OR: 4.56 [95% CI: 2.41–8.61], $p<0.001$). Additionally, we confirmed the protective role of HLA-DRB1 *04:03 allele in Greek population, with a frequency of 4.3% of patients *versus* 6.1% of controls (OR: 0.9, [95% CI: 0.46–2.12], $p=0.9$) (Table II).

Effects of HLA-DRB1 SE alleles and smoking on RA occurrence

After comparison of patients with controls, we observed that the presence of any HLA-DRB1 SE highly influenced the development of RA (OR: 4.37 [95% CI: 3.13–6.11], $p<0.001$). More specifically, a double gene dose, notably increased the RA occurrence compared to a single gene copy (OR: 9.16 [95% CI: 4.75–17.68], $p<0.001$, OR: 4.12 [95% CI: 2.88–5.9], p<0.001, respectively). Tobacco exposure was also associated with increased RA occurrence (OR: 1.67 [95% CI: 1.17–2.38], $p=0.005$).

Effects of HLA-DRB1 SE alleles on ACPA antibody production

The shared epitope status (single or double gene) influenced the production of ACPA antibodies. A stronger effect was observed in individuals with a double copy of SE (OR: 17.19 [95% CI: 6.17–47.85], $p<0.001$) compared to those with single SE allele (OR: 3.38 [95% CI: 1.97–5.81], $p<0.001$), indicating a gene dosage effect on antibody formation. Among the analysed alleles, *01:01, *04:01, *04:04, *04:05 and *10:01 were significantly associated with production of ACPA antibodies (OR: 5.42 [95% CI: 2.69–10.95], p<0.001, OR: 5.32 [95% CI: 1.04–27.27], $p=0.045$ OR: 9.06 [95% CI: 2.34–35.02], $p=0.001$, OR: 3.61 [95% CI: 1.66–7.85], $p=0.001$ and OR: 6.4 [95% CI: 3.04–13.49], p<0.001) respectively.

Table III. Odds ratio developing ACPA-positive RA in subjects exposed to different combinations of smoking and SE genes.

	ACPA +RA(n) (Ca/Co)	OR 95% CI, *p*-value	ACPA -RA(n) (Ca/Co)	OR 95% CI, *p*-value
Single SE+/Sm+	51/4	6.53 [1.47-28.31], 0.013 *	20/14	2.7 [0.9-7.57], 0.06
Single SE+/Sm-	39/29	2.92 [1.42-6], 0.003	31/36	1.8 [0.8-3.3], 0.07
Single SE-/Sm+	14/16	2.58 [1.02-6.56], 0.04	26/90	0.62 [0.35-1.09],0.09
Single SE-/Sm-	21/48	Ref.	45/96	Ref.
Double SE+/Sm+	37/1	15.27 [1.39-167.52], 0.026 **	2/4	0.6 [0.11-3.27], 0.5
Double SE+/Sm-	11/8	3.71 [1.24-11.09], 0.019	3/0	8.78 [0.5-199], 0.13
Double SE-/Sm+	14/16	2.58 [1.02-6.56], 0.04	26/90	0.62 [0.35-1.09], 0.09
Double SE-/Sm-	21/48	Ref.	45/96	Ref.
*01:01+/Sm+	41/1	12.55 [1.32-119.35], 0.028 ***	6/9	1.13 [0.25-5.14], 0.8
*01:01+/Sm-	12/13	1.15 [0.5-2.76], 0.7	8/9	1.49 [0.55-4.07], 0.42
*01:01-/Sm+	61/20	4.91 [2.45-9.85], <0.001	42/99	0.74 [0.46-1.19], 0.21
*01:01-/Sm-	59/72	Ref.	71/123	Ref.

SE: shared epitope, Sm: smoking, Ca/Co: cases/controls, *$p=0.012$ for interaction, **$p=0.01$ for interaction, ***$p=0.02$ for interaction.

Effects of smoking on ACPA antibody production

Furthermore, we analysed the effect of smoking exposure on ACPAs production and it was determined that smoking conferred to a significant increase in production of auto-antibodies (OR: 2.18 [95% CI: 1.27–3.74], $p=0.004$).

Interaction between smoking and HLA-DRB1 SE alleles in relation to ACPA-positive RA

We aimed to investigate the potential interaction between smoking and SE alleles for ACPA-positive RA by comparing the different combinations of smoking and SE for the presence of ACPA antibodies, in the two subsets of RA (Table III). Non-smokers without the SE allele served as the reference group. Smoking slightly increased the risk for ACPA-positive RA in SE-negative individuals (OR: 2.58 [95% CI: 1.02–6.56], $p=0.04$). Moreover, the presence of a single HLA-DRB1 SE allele in combination with smoking, appeared to increase the risk of developing ACPA-positive RA in Greek patients (OR: 6.53 [95% CI: 1.47–28.91], $p=0.013$, $p=0.012$ for interaction). This risk further increased among smokers who carried double copies of SE (OR: 15.27 [95% CI: 1.39–167.52], $p=0.026$, $p=0.01$ for interaction). This gene-environment interaction was also evident when analysing the combination of tobacco exposure with different subtypes of SE alleles separately. Among the analysed SE alleles, only HLA-DRB1 *01:01 in combination with smoking conferred the highest risk (OR: 12.55 [95% CI: 1.32–119.35], $p=0.028$, $p=0.02$ for interaction). The analysis of *01:01 SE allele resulted quite broad 95% CI, probably due to small sample size of exposed cases among those who had never smoked and did not possess SE alleles. Finally, the combination of SE genes and smoking did not influence the risk of ACPA-negative RA (OR: 2.01 [95% CI: 0.76–5.26], $p=0.15$).

Discussion

Rheumatoid arthritis is a multifactorial disease, influenced by genetic and environmental factors (3). HLA-DRB1 SE alleles, represent the most important RA-associated genetic risk factor (30). The SE not only increases the likelihood of developing RA, but also correlates with earlier disease onset, more severe bone erosions and generation of ACPAs. SE allows the presentation of arthritogenic peptides to CD4 T-cells and further induces ACPAs production by B-cells (31). However, the strength of association between various SE alleles and RA susceptibility varies (30, 32). According to some studies, HLA-DR4 alleles seem to confer higher risk for ACPAs compared with HLA-DR1 and HLA-DR10 alleles (30). This could be explained by the fact that different

alleles encode different amino acid sequence at the position 70-74 of the third hypervariable region (HVR3) of the DB1 molecule, affecting the antigen binding affinity (12). However, Ting *et al*. presented that this different peptide affinity may occur due to β-chain polymorphisms that reside outside the SE motif (33).

On the other hand, the genetic background of RA differs across different regions such as southern and northern Europe, Asia and United States of America (28). For example, RA is less prevalent and less severe in southern than in northern Europe, where the SE alleles are detected in about 75% of RA patients and the disease course tends to be more severe (22, 23). In Greek RA patients, SE alleles are detected in about half and disease seems to be milder (28).

Smoking is the most important and well recognised environmental risk factor for RA, inducing production of ACPAs only in SE carriers (34). In 2007, Klareskog *et al*. studied patients with early RA and by analysing cells from the bronchoalveolar lavage for the presence of citrullinated proteins, identified the strongest genetic-environmental interaction between smoking and SE on the development of ACPA-positive RA, suggesting a new model of aetiology of the disease (34). In particular, the relative risk (RR) for ACPA-positive RA in smokers was 6.5 (95% CI 3.8–11.4) for single and 21 (95% CI 11–40.2) for double SE alleles carriers (34). Unlike the data of the EIRA study, a case – only analysis of 3 North American RA cohorts (NARAC, Inception Cohort and SONORA), verified a weak gene-environment interaction between smoking and SE alleles only in the NARAC cohort, indicating a need for further studies in order to detect other environmental factors that may be associated with citrullination and RA (35). A study that analysed an African-American population with RA failed to show this SE-smoking interaction (36).

In a recent research, incident cases of RA were divided into four subgroups, based on the presence or absence of RF and ACPAs, and different effects of gene-environmental interaction were observed in each group (37). The most dramatic interaction was observed in cases with simultaneous presence of both autoantibodies, suggesting that smoking and SE may play different roles in the pathogenesis of different serologically defined subsets of RA (37). A possible explanation could be that RF generated T-cell independent effects of smoking would enhance class II MCH-dependent T-cell activation against citrullinated proteins at mucosal sites where smoke encounters the immune system (38).

Based on these data, we conducted a case-control study in order to clarify the impact of environmental, life-style and genetic factors on development of RA. The studied population was genetically homogenous, consisting exclusively of Greek patients with RA and two basic conclusions emerged. Firstly, smoking and SE alleles were mainly associated with an increased risk of developing ACPA antibodies. Secondly, we observed a significant genetic-environmental interaction between smoking and SE alleles, particularly in cases of ACPA-positive RA. Additionally, we examined the hypothesis that different SE alleles in association with smoking interacted differently regarding development of ACPA-positive RA.

We observed that 64.7% of patients possessed at least one copy of HLA-DRB1 SE allele, an increased frequency of *01:01, *10:01, *04:04 and *04:05 alleles in Greek RA patients and the protective role of *04:03 alleles in comparison with controls. Presence of a single or double SE allele highly influenced the development of RA (OR: 4.12 and OR: 9.16 respectively). Furthermore, we analysed the impact of SE alleles on ACPAs production. A stronger effect was observed in patients with double SE copy (OR: 17.19) in contrast with individuals with a single SE one (OR: 3.38). Patients carrying *01:01, *04:01, *04:04, *04:05 and *10:01 alleles developed positive ACPAs. On the other hand, smokers were also at significantly increased risk of developing ACPA autoantibodies, an observation that is consistent with previous studies (34, 44). In our study, SE interacted with smoking in the development of ACPA-positive RA. The risk of ACPA-positive RA was strongly influenced by the presence of a single HLA-DRB1 SE (OR: 6.53, p=0.012 for interaction) and further increased in smokers who possessed a double gene copy of SE (OR: 15.27, p=0.01 for interaction). Smoking conferred to a smaller degree in the risk of developing ACPA-positive RA in SE negative patients. Among the analysed alleles, the stronger gene-environmental interaction was observed for the *01:01 allele. The estimated OR of interaction models analyses should be interpreted in the context of small sample sizes that resulted large 95% CI.

Certain SE alleles, such as HLA-DRB1 *04:05 and *10:01 are prevalent in Asia but are less common in the Caucasian populations, in which instead HLA-DRB1 *04:01 and *04:04 are more frequently found (39, 40). A meta-analysis revealed variations in the genetic predisposition to RA across different parts of Europe (28). In certain regions of southern Europe, particularly those outside the Mediterranean basin (*e.g*. northwestern Spain) the genetic susceptibility profile of RA resembles that of northern Europe (28). Even within the Mediterranean Europe region, some heterogeneity across areas, regarding the frequency of SE and the strength of the association with RA susceptibility was observed (28). This could be probably explained by the fact that SE alleles are less prevalent in Greeks than other Mediterranean populations (41). According to Reviron *et al*., in a Latin/Greek population in southern France, DRB1 *01:01 was the most common SE allele (41%) followed by *04:01 (25%) and *04:04 (13%) (42). Similarly, in our studied population, *01:01 allele was also the most prevalent SE allele (22.3%). Contrary to previous studies (41), our research indicated that a significant majority of Greek RA patients (64.7%) possess SE alleles, possibly due to larger sample size of our examined population.

Our study's strengths lie in its inclusion of RA patients and controls from a real-world setting, where controls have been selected after being matched for age and gender. Additional advantages of our research include the genetic homo-

geneity of the studied population, and the equal proportion of smokers and non-smokers, which reduced the risk of bias in assessing environmental effects. Furthermore, past smokers – as was previously mentioned – were classified as smokers, to capture the cumulative lifetime impact of tobacco exposure, which is relevant for understanding gene-environmental interactions in RA. This classification is consistent with prior research, showing that even a history of tobacco exposure, regardless of cessation, can contribute to RA pathogenesis through sustained immune modulation (14).

We collected detailed smoking history, including the onset and duration of smoking relative to disease onset. While the long disease duration at enrolment may introduce some degree of recall bias, our structured data collection was designed to minimise this limitation and improve reliability of smoking exposure assessment. Nonetheless, we acknowledge that recall bias remains a potential source of error.

Several studies across different populations-including Caucasian, Asian and African RA patients-have shown that smoking and/or SE alleles increased the risk of developing ACPA antibodies, suggesting a strong gene-environmental interaction on the disease pathogenesis (18, 31, 35, 43, 44). We demonstrated a significant interaction between smoking and SE alleles in ACPA-positive RA and this might be explained by the observation that MCH class II immunity may be triggered at sites in which smoke interacts with immune system, *i.e.* the lungs and related mucosal tissues of smokers (45, 46).

HLA class II molecules, exhibit a strong association with RA, but this association accounts only for one-third of genetic susceptibility (47). Our study focused on HLA-related factors; however, other genes - such as PTPN22 and PADI4 - and additional environmental exposures (*e.g.* alcohol, coffee, tea, diet, occupational pollutants, and infections) may also play significant roles in RA pathogenesis (2, 35, 48). These factors were not assessed in the current analysis, which represents a limitation. Future work in our cohort will aim to incorporate a broader range of genetic and environmental variables to build a more comprehensive understanding of RA pathogenesis. A recent study comparing Greeks and Polish RA patients, investigated the distribution of gene polymorphisms in relation to the presence of HLA-DRB1 SE alleles. Results suggested that besides HLA-DRB1 alleles, MICA and NKG2D polymorphisms are involved in RA development, with their frequencies differing between the two populations (49). Additionally, a recent case-control study revealed interactions between smoking, alcohol and HLA-DRB1 SE, in ACPA-positive RA, emphasising the need for further research into various environmental and genetic factors for elucidating the aetiology and pathogenesis of RA (50).

Multiple studies undertaken to date have revealed a completely new picture of RA pathogenesis and gave us the knowledge of existing ethnic heterogeneity among different populations. To the best of our knowledge, this is the first study conducted on Greek RA patients to explore the interaction between SE and smoking. We believe it will enhance the understanding of the pathogenesis and clinical presentation of the disease. In summary, our findings suggest that smoking or SE increase the risk of ACPA antibodies in patients with RA, while their combination results in higher risk for ACPA production, suggesting a strong gene-environmental interaction that triggers RA pathogenesis in the Greek RA patients. This interaction seems to apply between smoking and HLA-DRB1 *01:01 allele.

References

1. HARRIS ED Jr: Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med* 1990; 322(18): 1277-89. https://doi.org/10.1056/nejm199005033221805
2. LEE YH, BAE SC, SONG GG: Gene-environmental interaction between smoking and shared epitope on the development of anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis: a meta-analysis. *Int J Rheum Dis* 2014; 17(5): 528-35. https://doi.org/10.1111/1756-185x.12307
3. KLARESKOG L, PADYUKOV L, RÖNNELID J, ALFREDSSON L: Genes, environment and immunity in the development of rheumatoid arthritis. *Curr Opin Immunol* 2006; 18(6): 650-55. https://doi.org/10.1016/j.coi.2006.06.004
4. IMBODEN JB: The immunopathogenesis of rheumatoid arthritis. *Annu Rev Pathol* 2009; 4: 417-34. https://doi.org/10.1146/annurev.pathol.4.110807.092254
5. KLARESKOG L, GREGERSEN PK, HUIZINGA TW: Prevention of autoimmune rheumatic disease: state of the art and future perspectives. *Ann Rheum Dis* 2010; 69(12): 2062-66. https://doi.org/10.1136/ard.2010.142109
6. MACGREGOR AJ, SNIEDER H, RIGBY AS *et al.*: Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum* 2000; 43(1): 30-37. https://doi.org/10.1002/1529-0131(200001)43:1<30::AID-ANR5>3.0.CO;2-B
7. BOWES J, BARTON A: Recent advances in the genetics of RA susceptibility. *Rheumatology* (Oxford) 2008; 47(4): 399-402. https://doi.org/10.1093/rheumatology/ken005
8. MARGULIES DH: How MHC molecules grab citrullinated peptides to foster rheumatoid arthritis. *J Biol Chem* 2018; 293(9): 3252-53. https://doi.org/10.1074/jbc.h118.001829
9. NAGAFUCHI Y, SHODA H, SUMITOMO S *et al.*: Immunophenotyping of rheumatoid arthritis reveals a linkage between HLA-DRB1 genotype, CXCR4 expression on memory CD4(+) T cells, and disease activity. *Sci Rep* 2016; 6: 29338. https://doi.org/10.1038/srep29338
10. MORGAN AW, HAROON-RASHID L, MARTIN SG *et al.*: The shared epitope hypothesis in rheumatoid arthritis: evaluation of alternative classification criteria in a large UK Caucasian cohort. *Arthritis Rheum* 2008; 58(5): 1275-83. https://doi.org/10.1002/art.23432
11. WYSOCKI T, OLESIŃSKA M, PARADOWSKA-GORYCKA A: Current understanding of an emerging role of HLA-DRB1 gene in rheumatoid arthritis-from research to clinical practice. *Cells* 2020; 9(5). https://doi.org/10.3390/cells9051127
12. MAKRYGIANNAKIS D, HERMANSSON M, ULFGREN AK *et al.*: Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Ann Rheum Dis* 2008; 67(10): 1488-92. https://doi.org/10.1136/ard.2007.075192
13. BAX M, VAN HEEMST J, HUIZINGA TW, TOES RE: Genetics of rheumatoid arthritis: what have we learned? *Immunogenetics* 2011; 63(8): 459-66. https://doi.org/10.1007/s00251-011-0528-6
14. KÄLLBERG H, DING B, PADYUKOV L *et al.*: Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke. *Ann Rheum Dis* 2011; 70(3): 508-11. https://doi.org/10.1136/ard.2009.120899
15. PIERINGER H, STUDNICKA-BENKE A: What is causing my arthritis, doctor? A glimpse beyond the usual suspects in the pathogenesis of rheumatoid arthritis. *QJM* 2013; 106(3): 219-28. https://doi.org/10.1093/qjmed/hcs205
16. VAN GAALEN FA, LINN-RASKER SP, VAN VENROOIJ WJ *et al.*: Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. *Arthritis Rheum* 2004; 50(3) 709-15.

https://doi.org/10.1002/art.20044
17. SHIMANE K, KOCHI Y, SUZUKI A *et al*.: An association analysis of HLA-DRB1 with systemic lupus erythematosus and rheumatoid arthritis in a Japanese population: effects of *09:01 allele on disease phenotypes. *Rheumatology* (Oxford) 2013; 52(7): 1172-82. https://doi.org/10.1093/rheumatology/kes427
18. LINN-RASKER SP, VAN DER HELM-VAN MIL AH, VAN GAALEN FA *et al*.: Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. *Ann Rheum Dis* 2006; 65(3): 366-71. https://doi.org/10.1136/ard.2005.041079
19. KARLSON EW, CHANG SC, CUI J *et al*.: Gene-environment interaction between HLA-DRB1 shared epitope and heavy cigarette smoking in predicting incident rheumatoid arthritis. *Ann Rheum Dis* 2010; 69(1): 54-60. https://doi.org/10.1136/ard.2008.102962
20. ISHIKAWA Y, TERAO C: The impact of cigarette smoking on risk of rheumatoid arthritis: a narrative review. *Cells* 2020; 9(2). https://doi.org/10.3390/cells9020475
21. ISHIKAWA Y, IKARI K, HASHIMOTO M *et al*.: Shared epitope defines distinct associations of cigarette smoking with levels of anticitrullinated protein antibody and rheumatoid factor. Ann Rheum Dis 2019; 78(11): 1480-87. https://doi.org/10.1136/annrheumdis-2019-215463
22. DROSOS AA, LANCHBURY JS, PANAYI GS, MOUTSOPOULOS HM: Rheumatoid arthritis in Greek and British patients. A comparative clinical, radiologic, and serologic study. *Arthritis Rheum* 1992; 35(7): 745-48. https://doi.org/10.1002/art.1780350705
23. DROSOS AA, MOUTSOPOULOS HM: Rheumatoid arthritis in Greece: clinical, serological and genetic considerations. *Clin Exp Rheumatol* 1995; 13 (Suppl. 12): S7-12.
24. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31(3): 315-24. https://doi.org/10.1002/art.1780310302
25. MILLER SA, DYKES DD, POLESKY HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16(3): 1215. https://doi.org/10.1093/nar/16.3.1215
26. BUYSE I, DECORTE R, BAENS M *et al*.: Rapid DNA typing of class II HLA antigens using the polymerase chain reaction and reverse dot blot hybridization. *Tissue Antigens* 1993; 41(1): 1-14. https://doi.org/10.1111/j.1399-0039.1993.tb01970.x
27. OLERUP O, ZETTERQUIST H: HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992; 39(5): 225-35. https://doi.org/10.1111/j.1399-0039.1992.tb01940.x
28. IOANNIDIS JP, TARASSI K, PAPADOPOULOS IA *et al*.: Shared epitopes and rheumatoid arthritis: disease associations in Greece and meta-analysis of Mediterranean European populations. *Semin Arthritis Rheum* 2002; 31(6): 361-70. https://doi.org/10.1053/sarh.2002.31725
29. MILICIC A, LEE D, BROWN MA, DARKE C, WORDSWORTH BP: HLA-DR/DQ haplotype in rheumatoid arthritis: novel allelic associations in UK Caucasians. *J Rheumatol* 2002; 29(9): 1821-26. PMID: 12233873
30. VAN DER HELM-VAN MIL AH, VERPOORT KN, LE CESSIE S, HUIZINGA TW, DE VRIES RR, TOES RE: The HLA-DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide. *Arthritis Rheum* 2007; 56(2): 425-32. https://doi.org/10.1002/art.22373
31. BANG SY, LEE KH, CHO SK, LEE HS, LEE KW, BAE SC: Smoking increases rheumatoid arthritis susceptibility in individuals carrying the HLA-DRB1 shared epitope, regardless of rheumatoid factor or anti-cyclic citrullinated peptide antibody status. *Arthritis Rheum* 2010; 62(2): 369-77. https://doi.org/10.1002/art.27272
32. GORMAN JD, LUM RF, CHEN JJ, SUAREZ-ALMAZOR ME, THOMSON G, CRISWELL LA: Impact of shared epitope genotype and ethnicity on erosive disease: a meta-analysis of 3,240 rheumatoid arthritis patients. *Arthritis Rheum* 2004; 50(2): 400-12. https://doi.org/10.1002/art.20006
33. TING YT, PETERSEN J, RAMARATHINAM SH *et al*.: The interplay between citrullination and HLA-DRB1 polymorphism in shaping peptide binding hierarchies in rheumatoid arthritis. *J Biol Chem* 2018; 293(9): 3236-51. https://doi.org/10.1074/jbc.ra117.001013
34. KLARESKOG L, STOLT P, LUNDBERG K *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. https://doi.org/10.1002/art.21575
35. LEE HS, IRIGOYEN P, KERN M *et al*.: Interaction between smoking, the shared epitope, and anti-cyclic citrullinated peptide: a mixed picture in three large North American rheumatoid arthritis cohorts. *Arthritis Rheum* 2007; 56(6): 1745-53. https://doi.org/10.1002/art.22703
36. MIKULS TR, HUGHES LB, WESTFALL AO *et al*.: Cigarette smoking, disease severity and autoantibody expression in African Americans with recent-onset rheumatoid arthritis. *Ann Rheum Dis* 2008; 67(11): 1529-34. https://doi.org/10.1136/ard.2007.082669
37. HEDSTRÖM AK, RÖNNELID J, KLARESKOG L, ALFREDSSON L: Complex relationships of smoking, HLA-DRB1 genes, and serologic profiles in patients with early rheumatoid arthritis: update from a Swedish population-based case-control study. *Arthritis Rheumatol* 2019; 71(9): 1504-11. https://doi.org/10.1002/art.40852
38. JÓNSSON T, THORSTEINSSON J, VALDIMARSSON H: Does smoking stimulate rheumatoid factor production in non-rheumatic individuals? *Apmis* 1998; 106(10): 970-74. https://doi.org/10.1111/j.1699-0463.1998.tb00247.x
39. NEWTON JL, HARNEY SM, WORDSWORTH BP, BROWN MA: A review of the MHC genetics of rheumatoid arthritis. *Genes Immun* 2004; 5(3): 151-57. https://doi.org/10.1038/sj.gene.6364045
40. KOCHI Y, SUZUKI A, YAMADA R, YAMAMOTO K: Genetics of rheumatoid arthritis: underlying evidence of ethnic differences. *J Autoimmun* 2009; 32(3-4): 158-62. https://doi.org/10.1016/j.jaut.2009.02.020
41. BOKI KA, PANAYI GS, VAUGHAN RW, DROSOS AA, MOUTSOPOULOS HM, LANCHBURY JS: HLA class II sequence polymorphisms and susceptibility to rheumatoid arthritis in Greeks. The HLA-DR beta shared-epitope hypothesis accounts for the disease in only a minority of Greek patients. *Arthritis Rheum* 1992; 35(7): 749-55. https://doi.org/10.1002/art.1780350706
42. REVIRON D, PERDRIGER A, TOUSSIROT E *et al*.: Influence of shared epitope-negative HLA-DRB1 alleles on genetic susceptibility to rheumatoid arthritis. *Arthritis Rheum* 2001; 44(3): 535-40. https://doi.org/10.1002/1529-0131(200103)44:3<535::aid-anr101>3.0.co;2-z
43. 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. https://doi.org/10.1002/art.24572
44. MICHOU L, TEIXEIRA VH, PIERLOT C *et al*.: Associations between genetic factors, tobacco smoking and autoantibodies in familial and sporadic rheumatoid arthritis. *Ann Rheum Dis* 2008; 67(4): 466-70. https://doi.org/10.1136/ard.2007.075622
45. KHARLAMOVA N, JIANG X, SHERINA N *et al*.: Antibodies to porphyromonas gingivalis indicate interaction between oral infection, smoking, and risk genes in rheumatoid arthritis etiology. *Arthritis Rheumatol* 2016; 68(3): 604-13. https://doi.org/10.1002/art.39491
46. HOLERS VM, DEMORUELLE MK, KUHN KA *et al*.: Rheumatoid arthritis and the mucosal origins hypothesis: protection turns to destruction. *Nat Rev Rheumatol* 2018; 14(9): 542-57. https://doi.org/10.1038/s41584-018-0070-0
47. DEIGHTON CM, WALKER DJ, GRIFFITHS ID, ROBERTS DF: The contribution of HLA to rheumatoid arthritis. *Clin Genet* 1989; 36(3): 178-82. https://doi.org/10.1111/j.1399-0004.1989.tb03185.x
48. KOKKONEN H, BRINK M, HANSSON M *et al*.: Associations of antibodies against citrullinated peptides with human leukocyte antigen-shared epitope and smoking prior to the development of rheumatoid arthritis. *Arthritis Res Ther* 2015; 17(1): 125. https://doi.org/10.1186/s13075-015-0638-x
49. WIELIŃSKA J, TARASSI K, IWASZKO M *et al*.: Shared epitope and polymorphism of MICA and NKG2D encoding genes in Greek and Polish patients with rheumatoid arthritis. *Cent Eur J Immunol* 2021; 46(1): 92-98. https://doi.org/10.5114/ceji.2021.104425
50. HEDSTRÖM AK, HÖSSJER O, KLARESKOG L, ALFREDSSON L: Interplay between alcohol, smoking and HLA genes in RA aetiology. *RMD Open* 2019; 5(1): e000893. https://doi.org/10.1136/rmdopen-2019-000893