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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.
Eugénie Mutez, MD, 23 Kathrin Brockmann, MD, 4, 5
Angela B. Deutschländer, MD, 24, 26
Georges M. Hadjigeorgiou, MD, 27
Ethimos Dardiotis, MD, 27 Leonidas Stefanis, MD, PhD, 29, 30
Athina Maria Simitsi, MD, PhD, 29
Enza Maria Valente, MD, PhD, 31, 32
Simona Petrucci, MD, PhD, 33, 34 Stefano Duga, PhD, 35, 36
Letizia Straniiero, PhD, 35 Anna Zecchinelli, MD, 37
Gianni Pezzoli, MD, 38 Laura Brighina, MD, PhD, 39, 40
Carlo Ferrarese, MD, PhD, 39, 40 Grazia Annesi, PhD, 41
Andrea Quattrone, MD, 42 Monica Gagliardi, PhD, 43
Hirotaka Matsuo, MD, PhD, 44 Akiko Nakayama, PhD, 44
Nobutaka Hattori, MD, PhD, 45 Kenia Nishioka, MD, PhD, 45
Sun Ju Chung, MD, PhD, 46 Yun Joong Kim, MD, PhD, 47
Pierre Kolber, MD, 48
Bart P.C. van de Warrenburg, MD, PhD, 49
Bastiaan R. Bloem, MD, PhD, 49 Jan Aasly, MD, 50
Mathias Toft, MD, PhD, 51 Lasse Pilhstram, MD, PhD, 51
Leonor Correia Guedes, MD, PhD, 52, 53
Joaquin J. Ferreira, MD, PhD, 52, 54
Soraya Bardien, PhD, 55, 56 Jonathan Carr, PhD, 56
Eduardo Tolosa, MD, PhD, 57, 58 Mario Ezquerra, PhD, 59, 60
Pau Pastor, MD, PhD, 60, 61 Monica Diaz-Fairen, MSc, 60, 61
Karim Wirdefeldt, MD, PhD, 62, 63 Nancy L. Pedersen, PhD, 63
Caroline Ran, PhD, 64 Andrea C. Belin, PhD, 64
Andreas Puschmann, MD, PhD, 65
Emil Ygland Rödström, MD, 65 Carl E. Clarke, MD, 66
Karen E. Morrison, MD, 67 Manuela Tan, PhD, 68
Dimitri Krainc, MD, PhD, 69, 70, 71, 72 Lena F. Burdulla, PhD, 73 Matt J. Farrer, PhD, 73
Reijo Krüger, MD, 6, 68, 74, 75
Thomas Gasser, MD, 4, 5
Manu Sharma, PhD, 75 Nicolas Vincie, PhD, 76
Alexis Elbaz, MD, PhD, 77 on behalf of the Comprehensive Unbiased Risk Factor Assessment for Genetics and Environment in Parkinson’s Disease (Cope-PD) Consortium

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ABSTRACT: Background: Two studies that examined the interaction between HLA-DRB1 and smoking in Parkinson’s disease (PD) yielded findings in opposite directions.

Objective: To perform a large-scale independent replication of the HLA-DRB1 × smoking interaction.

Methods: We genotyped 182 single nucleotide polymorphism (SNPs) associated with smoking initiation in 12,424 cases and 9,480 controls to perform a Mendelian randomization (MR) analysis in strata defined by HLA-DRB1.

Results: At the amino acid level, a valine at position 11 (V11) in HLA-DRB1 displayed the strongest association with PD. MR showed an inverse association between genetically predicted smoking initiation and PD only in absence of V11 (odds ratio, 0.74, 95% confidence interval, 0.59-0.93, PInteraction = 0.028). In silico predictions of the influence of V11 and smoking-induced modifications of α-synuclein on binding affinity showed findings consistent with this interaction pattern.

Conclusions: Despite being one of the most robust findings in PD research, the mechanisms underlying the inverse association between smoking and PD remain unknown. Our findings may help better understand this association. © 2022 The Authors. Movement Disorders published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: Parkinson’s disease; smoking; gene-environment interaction; HLA

Genome-wide association studies (GWAS) in Parkinson’s disease (PD) identified an association with the human leukocyte antigen (HLA) region, in particular with HLA-DRB1. Hollenbach et al1 reported an inverse association of PD with the shared epitope (SE), a combination of amino acids (AA) coded by HLA-DRB1, only in the presence of a valine at position 11 (V11). The strongest association in a cross-ethnic GWAS meta-analysis was an inverse association with a histidine at position 13 (H13) in HLA-DRB1, strongly correlated with V11.5 The latest study, with some overlap with the previous two, highlighted three AA (V11, H13, and H33) encoded by HLA-DRB1 inversely associated with PD.3

Following studies showing interactions between smoking and HLA-DRB1 in other conditions,4,6 Chuang et al7 genotyped one single nucleotide polymorphism (SNP) in the HLA-DRB1 region whose minor G allele is inversely associated with PD (2056 cases, 2723 controls) and reported a significant positive interaction between self-reported smoking and rs660895-G: the inverse association between smoking and PD was stronger in carriers of the AA genotype compared to G-allele carriers.7 Based on a smaller selected sample (837 cases, 918 controls), the study that identified an inverse association of the SE and V11...
combination (SE+V11+) with PD also showed an interaction with smoking, but in the opposite direction: the inverse association between smoking and PD was restricted to SE+V11+ carriers. The authors hypothesized that post-translational modifications of α-synuclein induced by smoking (citrullination/homocitrullination) explained this interaction.

We performed a large-scale independent replication of the HLA-DRB1 × smoking interaction by performing a Mendelian randomization (MR) analysis using smoking predisposing genes as instrumental variables in strata defined by HLA-DRB1.

**Subjects and Methods**

**Courage-PD**

The Comprehensive Unbiased Risk Factor Assessment for Genetics and Environment in Parkinson’s Disease (Courage-PD) consortium pooled individual-level data from 35 studies and used the Neurochip array to genotype participants (Supplementary Appendix S1). Analyses are based on 26 studies with at least 50 cases or controls of European descent (12,424 cases, 9,480 controls); participants’ characteristics are shown in Supplementary Table S1. Additional methods on genotyping and imputation of HLA alleles/haplotypes/AA are available as Supplementary Appendix S1. All studies were approved by local ethical committees following procedures of each country.

**Smoking Initiation: Two-Sample Mendelian Randomization**

Because self-reported smoking was not available in most studies, we used SNPs associated with smoking initiation to perform two-sample MR. Summary statistics for the association between SNPs and smoking initiation (182 SNPs independently associated at \( P < 5 \times 10^{-8} \)) came from the GWAS and Sequencing Consortium of Alcohol and Nicotine use (n = 1,232,091, European descent) (Supplementary Appendix S1), and those for associations with PD came from Courage-PD (Supplementary Table S2).

**In Silico Prediction of Binding Affinity of HLA-DRB1 Alleles to α-Synuclein**

We assessed the binding affinity (nM) of HLA-DRB1 alleles to α-synuclein derived peptides using NetMHCIIpan 4.0 and predicted whether peptides are strong, weak, or non-binders. After targeting 607 four-digit HLA-DRB1 alleles, we restricted our analyses to 34 alleles observed in Courage-PD. Of 126 α-synuclein derived peptides, we retained 96 peptides with lysine residues that can be homocitrullinated to examine the role of smoking-related post-translational modifications. We also performed analyses restricted to a single peptide (Tyrosine 39, Y39) that induces T cell responses in PD patients and was previously used for binding affinity predictions.

**Statistical Analyses**

We used SAS 9.4 (SAS Institute Inc, Cary, NC, USA), STATA16 (StataCorp LP, College Station, TX, USA), and R packages HIBAC and TwoSampleMR (R Foundation for Statistical Computing, Vienna, Austria).

**Interaction between Genetically Predicted Smoking Initiation and HLA-DRB1**

To perform an independent replication of the HLA-DRB1 × smoking interaction, we excluded the French study that contributed to identify the interaction between smoking and rs660895 in PD.

We used the random-effects inverse-variance weighted (IVW) approach to perform MR analyses for genetically predicted smoking initiation in two strata defined by the presence of V11 encoded by HLA-DRB1 alleles (Supplementary Appendix S1). We compared the two MR estimates using the statistic \( \beta_2 - \beta_1 \)/\( \sqrt{SE(\beta_2)^2 + SE(\beta_1)^2} \), where \( \beta_1 \) and \( \beta_2 \) are MR estimates in the two strata with variances \( SE(\beta_1)^2 \) and \( SE(\beta_2)^2 \); this difference represents the interaction between smoking and HLA-DRB1 and follows a normal distribution. In sensitivity analyses, we used other MR approaches that are less powerful, but more robust to pleiotropy (weighted median-method and model-based, MR-PRESSO, MR-Lasso); we also performed analyses after excluding 31 pleotropic SNPs associated with alcohol drinking and/or body mass index (Supplementary Appendix S1).

As secondary analyses, we ran MR analyses stratified by rs660895 and HLA-DRB1*04, which are both inversely associated with PD and in linkage disequilibrium with V11. Analyses stratified by rs660895 have the advantage that they did not involve HLA imputation and are, therefore, based on a larger number of cases and controls than analyses that required HLA imputation.

**In Silico Prediction of Binding Affinity**

To examine the influence of V11 encoded by HLA-DRB1 alleles and homocitrullination (HC) of α-synuclein derived peptides on binding affinity, we described binding affinity for the four groups defined by the combination of V11 and HC. All \( 2 \times 2 \) differences were tested using the Wilcoxon non-parametric test corrected for multiple comparisons. We compared the percentage of binding
| HLA-DRB1 | 0 allele or AA residue | 1/2 alleles or AA residues | Interaction OR (95% CI) | P |
| --- | --- | --- | --- | --- |
| | OR per 1-SD increase in the prevalence of ever smoking (95% CI) | P | P-het. | OR per 1-SD increase in the prevalence of ever smoking (95% CI) | P | P-het. | OR per 1-SD increase in the prevalence of ever smoking (95% CI) | P |
| Valine 11<sup>b</sup> | 6383 controls, 8812 cases | 2212 controls, 2531 cases | 0.74 (0.59–0.93) 9.2 × 10<sup>−3</sup> 0.73 | 1.25 (0.83–1.87) 0.29 0.40 | 1.68 (1.06–2.68) 0.0 |
| Inverse variance weighted | | | | | | | | |
| Weighted median | 0.75 (0.53–1.07) 0.11 | 1.14 (0.61–2.15) 0.68 | 1.52 (0.75–3.11) 0.26 |
| Weighted mode | 0.63 (0.30–1.31) 0.22 | 1.72 (0.38–7.82) 0.48 | 2.74 (0.51–14.77) 0.24 |
| MR-Lasso | No invalid SNP (λ = 0.20) | 1.30 (0.87–1.96) 0.20<sup>e</sup> | 1.76 (1.10–2.81) 0.020 |
| MR-PRESSO | | 0.59 | 0.47 |
| rs660895-G<sup>d</sup> | 6498 controls, 8903 cases | 2982 controls, 3521 cases | 0.73 (0.59–0.91) 4.8 × 10<sup>−3</sup> 0.84 | 1.33 (0.95–1.87) 0.10 0.41 | 1.83 (1.22–2.74) 3.5 × 10<sup>−3</sup> |
| Inverse variance weighted | | | | | | | | |
| Weighted median | 0.72 (0.52–1.00) 0.05 | 1.04 (0.62–1.73) 0.89 | 1.45 (0.78–2.66) 0.24 |
| Weighted mode | 0.68 (0.31–1.48) 0.34 | 0.99 (0.23–4.26) 0.99 | 1.46 (0.30–7.08) 0.66 |
| MR-Lasso | No invalid SNP (λ = 0.19) | 1.25 (0.89–1.75) 0.20<sup>e</sup> | 1.71 (1.14–2.56) 9.1 × 10<sup>−3</sup> |
| MR-PRESSO | | 0.83 | 0.40 |
| HLA-DRB1*04<sup>b</sup> | 6563 controls, 9014 cases | 2032 controls, 2329 cases | 0.73 (0.59–0.92) 6.8 × 10<sup>−3</sup> 0.77 | 1.29 (0.83–2.00) 0.26 0.47 | 1.75 (1.07–2.87) 0.03 |
| Inverse variance weighted | | | | | | | | |
| Weighted median | 0.70 (0.50–0.97) 0.03 | 1.16 (0.59–2.29) 0.66 | 1.67 (0.81–3.46) 0.18 |
| Weighted mode | 0.67 (0.30–1.48) 0.32 | 1.51 (0.34–6.66) 0.59 | 2.26 (0.38–13.39) 0.34 |
| MR-Lasso | No invalid SNP (λ = 0.20) | 1.18 (0.76–1.83) 0.46<sup>f</sup> | 1.61 (0.98–2.64) 0.06 |
| MR-PRESSO | | 0.67 | 0.57 |

Valine 11 amino acids and HLA-DRB1*04 alleles were determined using imputation of HLA alleles and amino acids based on SNPs from the HLA region.

The interaction OR represents the OR in carriers of 1/2 alleles or AA residues divided by the OR in carriers of 0 allele or AA residue.

Total number: 8995 controls, 11,343 cases.

Number of invalid SNPs = 4; λ = 0.17.

Total number: 9480 controls, 12,424 cases.

Number of invalid SNPs = 4; λ = 0.19.

Number of invalid SNPs = 11; λ = 0.19.

SNPs, single nucleotide polymorphism; PD, Parkinson’s disease; OR, odds ratio; CI, confidence interval; AA, amino acid; P-het., P for heterogeneity; λ, tuning parameter for MR-Lasso.
FIG. 1. Forest plot of the association between genetically predicted smoking initiation (inverse variance weighted estimate) and Parkinson’s disease stratified by HLA-DRB1.

| HLA-DRB1 | Group | OR Smoking (95% CI) | P-value | P-Interaction |
|----------|-------|---------------------|---------|--------------|
| **All SNPs (182 SNPs)** | | | | |
| Valine 11 | 0 residue | 0.74 (0.59-0.93) | .0092 | - |
| | 1 or 2 residues | 1.25 (0.83-1.87) | .29 | .03 |
| rs660895 | AA | 0.73 (0.59-0.91) | .0048 | - |
| | AG or GG | 1.33 (0.95-1.87) | .10 | .0035 |
| HLA-DRB1*04 | 0 allele | 0.73 (0.59-0.92) | .0068 | - |
| | 1 or 2 alleles | 1.29 (0.83-2.00) | .26 | .03 |
| **No pleiotropic SNPs (151 SNPs)** | | | | |
| Valine 11 | 0 residue | 0.73 (0.56-0.94) | .01 | - |
| | 1 or 2 residues | 1.42 (0.90-2.25) | .13 | .01 |
| rs660895 | AA | 0.72 (0.56-0.92) | .01 | - |
| | AG or GG | 1.46 (0.99-2.14) | .06 | .003 |
| HLA-DRB1*04 | 0 allele | 0.73 (0.57-0.94) | .01 | - |
| | 1 or 2 alleles | 1.41 (0.86-2.31) | .17 | .02 |

FIG. 2. Prediction of binding affinity (nM) according to the presence of a valine at position 11 (V11) coded by HLA-DRB1 alleles and homocitrullination (HC) of α-synuclein derived peptides. *P values for the comparison versus the reference group (V11⁻HC⁻).
Data Availability

Results

Supplementary Table S3 shows 19 SNPs from the HLA region associated with PD after accounting for multiple comparisons, of which 17 were located near HLA-DRB1 (including rs660895); none of them was associated with smoking initiation ($P > 0.05$). Among 64 alleles of HLA class 2 genes (HLA-DPB1, HLA-DQA1, HLA-DQB1, and HLA-DRB1), five were significantly and inversely associated with PD (HLA-DQA1*03:01, HLA-DQA1*03:03, HLA-DQB1*03:02, HLA-DRB1*04:01, and HLA-DRB1*04:04) (Supplementary Table S4). The odds ratio (OR) for the association of all HLA-DRB1*04 alleles combined with PD was 0.84 (95% confidence interval [CI], 0.78–0.91; $P = 3.9 \times 10^{-6}$). Associations between DRB1 $\sim$ HLA-DQB1 haplotypes and PD are shown in Supplementary Table S4.

Among 131 AA encoded by HLA-DRB1 and 116 by HLA-DQB1, 11 AA were associated (9 inversely, 2 positively) with PD and were all encoded by HLA-DRB1 (Supplementary Table S5). Two AA, V11, and S37, remained significantly associated with PD after a backward stepwise selection procedure, with a stronger association for V11 (OR, 0.85; 95% CI, 0.79–0.92; $P = 2.2 \times 10^{-5}$) than S37 (OR, 1.07; 95% CI, 1.00–1.14; $P = 0.040$). The association of H13 and H33 with PD was explained by V11 (Supplementary Table S6). We found no significant interaction between SE and V11 ($P = 0.29$); only V11 remained associated with PD (OR, 0.81; 95% CI, 0.74–0.89; $P < 10^{-3}$) when both were included in the model (Supplementary Table S7).

The overall association between genetically predicted smoking initiation and PD was of 0.86 (95% CI, 0.73–1.05; $P = 0.10$) without evidence of heterogeneity between SNPs ($P = 0.40$). Compared with 26% (n = 2212) of the controls, 22% (n = 2531) of the cases carried at least one V11 residue. Genetically predicted smoking initiation was inversely associated with PD in the absence of V11 ($OR_{IVW}$, 0.74; 95%, 0.59–0.93; $P = 0.0092$), but not in its presence ($OR_{IVW}$, 1.25; 95% CI, 0.83–1.87; $P = 0.29$), with a positive and significant interaction ($P = 0.03$) (Table 1, Fig. 1). There was no significant heterogeneity across SNPs and MR-Presso did not detect pleiotropy (all $P > 0.10$). Results of pleiotropy-robust approaches were consistent with the IVW method, although CIs were generally larger. Similar conclusions were reached after excluding 31 pleiotropic SNPs (Fig. 1, Supplementary Table S8). Results were similar in analyses stratified by rs660895 or HLA-DRB1*04.

Compared to V11−HC−, V11+HC− and V11−HC + were both associated with decreased binding affinity, with a stronger effect of HC+ than V11+ (Fig. 2, Supplementary Table S9). Alternatively, in the presence of HC+, V11+ increased binding affinity (all peptides) or had no effect (Y39); HC+ had no effect on binding affinity in the presence of V11+. Analyses of binding and non-binding peptides paralleled these results (Supplementary Table S10).

Discussion

We replicate an interaction between HLA-DRB1 and smoking,7 according to which the inverse association between smoking and PD is only present in participants without protective HLA-DRB1 AA/alleles. In silico predictions of binding affinity are consistent with an interaction between V11 and post-translational smoking-induced modifications of α-synuclein derived peptides.

Recent MR studies showed an inverse association between genetically predicted smoking and PD.15–18 These findings are in favor of a causal role of smoking in PD, but the underlying mechanisms remain unknown and gene–environment interactions analyses may contribute to their understanding. The interaction pattern we found is similar to the interaction between self-reported smoking and rs660895 reported by Chuang et al.7 Our study represents a fully independent replication using a different approach to define smoking (MR) and SNP-based imputation of HLA amino acids that allowed us to examine this interaction at the AA level. Therefore, our findings contradict those from Hollenbach et al19 who reported an interaction in the opposite direction based on a selected sample of smaller size.

Lower binding affinity for α-synuclein derived peptides is associated with a weaker immune response that may explain decreased PD risk.19 Our binding affinity analyses are consistent with the interaction pattern we identified. Although V11 and HC both decreased binding affinity for α-synuclein derived peptides in the absence of each other, consistent with the inverse association of V11 and smoking with PD, there was a positive interaction between V11 and HC, whereby both V11 and HC had a weaker or no effect in the presence of each other;
this pattern is consistent with the lack of association between smoking and PD in V11 carriers that we found.

We used MR to define genetically predicted smoking initiation, rather than self-reported smoking; MR has the advantage that, provided that a set of assumptions are met, smoking-PD association estimates are less likely to be biased by confounding or reverse causation than association estimates based on self-reported smoking.8 Another strength of our study compared to Chuang et al7 is that rather than using a single SNP, we used genome-wide data to impute AA encoded by HLA-DRB1. Finally, using an independent dataset, we report similar associations with HLA alleles and AA as previous studies.2,3 One limitation of our HLA-DRB1 × smoking interaction analyses is that the approach we used allowed us to estimate the association between smoking initiation and PD stratified by HLA-DRB1, but did not allow us to estimate the association between HLA-DRB1 and PD stratified by smoking.

Despite being one of the most robust findings in PD, the mechanisms underlying its inverse association with smoking remain unknown. This work represents the first example of large-scale replication of a gene–environment interaction in PD, and allows proposing a biological mechanism to explain the inverse smoking-PD association, in the context of a larger body of work on the relationship between the immune system and PD.19

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Data Availability Statement
Results can be reproduced using the Supplementary material

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Supporting Data
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