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RESEARCH ARTICLE

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ACE2, *TMPRSS2*, and Furin variants and SARS-CoV-2 infection in Madrid, Spain

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Abstract

It has been suggested that some individuals may present genetic susceptibility to SARS-CoV-2 infection, with particular research interest in variants of the *ACE2* and *TMPRSS2* genes, involved in viral penetration into cells, in different populations and geographic regions, although insufficient information is currently available. This study addresses the apparently reasonable hypothesis that variants of these genes may modulate viral infectivity, making some individuals more vulnerable than others. Through whole-exome sequencing, the frequency of exonic variants of the *ACE2*, *TMPRSS2*, and *Furin* genes was analyzed in relation to presence or absence of SARS-CoV-2 infection in a familial multiple sclerosis cohort including 120 individuals from Madrid. The *ACE2* gene showed a low level of polymorphism, and none variant was significantly associated with SARS-CoV-2 infection. These variants have previously been detected in Italy. While *TMPRSS2* is highly polymorphic, the variants found do not coincide with those described in other studies, with the exception of rs75603675, which may be associated with SARS-CoV-2 infection. The synonymous variants rs61735792 and rs61735794 showed a significant association with infection. Despite the limited number of patients with SARS-CoV-2 infection, some variants, especially in *TMPRSS2*, may be associated with COVID-19.

KEYWORDS

ACE2, coronavirus, COVID-19, familial multiple sclerosis, SARS-CoV-2, Spain, *TMPRSS2*, whole-exome sequencing

1 | INTRODUCTION

The spike proteins of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV), NL63-CoV,[1] and SARS-CoV-2 coronaviruses[2] bind to the angiotensin-converting enzyme 2 (ACE2) receptor. It has been suggested that the latter virus, responsible for the coronavirus disease 2019 (COVID-19) epidemic, has greater affinity for ACE2, which may explain the speed with which it spreads.[3] It has also been proposed that ACE2 expression directly correlates with SARS-CoV and SARS-CoV-2 infection,[4] and that mortality in infected patients may be influenced by the level of binding to the receptor.[5] Thus, single-nucleotide polymorphisms of the *ACE2* gene are thought to influence susceptibility to SARS-CoV-2 infection.[6] This should not be surprising, as genetic variants of the receptors targeted by the virus may influence its binding and penetration: this phenomenon was reported with the dipeptidyl peptidase-4 receptor in the case of the MERS-CoV coronavirus.[7]

ACE2 variants may modify the risk of arterial hypertension; therefore, it may be hypothesized that different variants of the gene may present different affinities for the virus.[6,8,9] Arterial hypertension has been associated with variants rs4240157, rs4646155, rs4830542, and rs21068809 in China[10,11]; rs21068809 in India; rs2158083 in

Canada[12]; and rs776995986, rs769062069, rs765152220, and rs750145841 in the Middle East[13]; and the combination of ACE I/D and ACE2 polymorphisms in Brazil.[14] The data reported by Benetti et al[15] in Italy are particularly relevant to the Spanish setting. These findings appear to demonstrate the influence of geographic or ethnic differences in the presence of these variants.[16-18]

Studies into genetic susceptibility to SARS-CoV-2 infection have focused on genetic variants of *ACE2* in different populations; currently, evidence on the subject is scarce and contradictory.[19] The gene encoding transmembrane protease serine 2 (*TMPRSS2*) is another candidate gene studied in relation to COVID-19. *TMPRSS2* expression increases ACE2-mediated invasion of cells by SARS-CoV-2.[20-23] Therefore, the hypothesis that *ACE2* and *TMPRSS2* variants may modulate viral infectivity in humans,[24] making some individuals more vulnerable than others, seems reasonable. Recently, other host factors including Furin, TMPRSS4, and lyosomal cathepsins have been shown to be relevant for SARS-CoV-2 entry into host cells.[25-27] In this study, whole-exome sequencing (WES) is used to analyze variants in *ACE2*, *TMPRSS2*, and *Furin* in a cohort of patients with familial multiple sclerosis (MS) and their relatives.

2 | MATERIAL AND METHODS

2.1 | Sample description

The study cohort comprises 23 families including at least 2 members diagnosed with MS according to the 2010 McDonald criteria[28]; WES was performed in 138 individuals: 52 patients with MS and 86 unaffected family members. Information was gathered on a group of 120 selected individuals through a questionnaire administered to patients enquiring about SARS-CoV-2 infection among their relatives.

2.2 | Definition of COVID-19

One relevant consideration was how infection should be defined, given that, in accordance with the instructions of the Spanish healthcare authorities, biological studies were not routinely used to confirm the diagnosis. Both in patients with MS and in the remaining participants, diagnosis of SARS-CoV-2 infection was established based on the criteria described below, obtained using the questionnaire. An individual was considered to be infected if they met any of the following criteria: (a) compatible symptoms, with positive PCR results obtained during the episode or positive serology results obtained afterwards; (b) episode of at least 7 days' duration of fever associated with at least 2 of the following symptoms: dry cough, diarrhea, pneumonia, and chest pain; (c) episode of at least 7 days' duration of fever and olfactory alterations[29] associated with at least one of the following symptoms: dry cough, diarrhea, pneumonia, and chest pain; (d) episode similar to those described in (b) and (c), of less than 7 days' duration, in an individual living with somebody with PCR confirmation of infection; and (e) episode of symptoms related to the infection, with or without fever, motivating a physician to order self-isolation.

2.3 | Whole-exome sequencing

The WES methodology followed is published elsewhere,[30] but is also included in the Supplemental material. Sequencing information is included in the European Genome-Phenome Archive. Variants were described using the dbSNP database, which includes data on nucleotide and amino acid sequence changes. Data are also provided on minor allele frequency (MAF) and combined annotation-dependent depletion (CADD) score.

2.4 | Statistical analysis

Statistical analysis was performed using SPSS version 20.0 and PLINK. Results from the descriptive analysis of the variants identified are expressed as absolute frequencies and percentages. A Mantel-Haenszel test was performed to determine the association between each variant and COVID-19 infection, because this is a family-based study. A *P*-value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of the study population

Of the total of 138 individuals who underwent sequencing, 7 patients with MS and 11 unaffected individuals were excluded because of several reasons (mainly because some individuals were not born in Spain or were not living in Madrid during the pandemic). The questionnaire identified seven individuals with SARS-CoV-2 infection belonging to a generation that had not undergone sequencing (three children of a patient with MS and four siblings of another patient with MS). Of the total of 120 subjects analyzed, seven cases of SARS-CoV-2 infection (5.83%) were identified, with the remaining 113 (94.2%) not having been infected. The Supplemental material summarises the demographic and clinical characteristics of the 120 individuals studied, grouped according to presence of MS. The genetic variants identified are also included in the Supplementary material. Table 1 shows the frequency of each variant in patients with MS and unaffected individuals with and without SARS-CoV-2 infection.

3.2 | Analysis of the *ACE2* gene

ACE2 polymorphisms were very rare in the cohort. A total of 103 (91.2%) individuals without COVID-19 presented no exonic variants of the gene: 39 patients with MS (90.1%) and 64 unaffected individuals (90.1%). In the group of individuals with SARS-CoV-2 infection, 6 (85.7%) presented no exonic variant.

TABLE 1 Synonymous and non-synonymous *ACE2* and *TMPRSS2* variants identified in a study of 120 individuals

			MS (n = 45)		Unaffected individuals (n = 75)		Total (n = 120)		
Gene	Variant (rs)	Nucleotide change	SARS-CoV-2– (n = 42)	SARS-CoV-2+ (n = 3)	SARS-CoV-2– (n = 71)	SARS-CoV-2+ (n = 4)	SARS-CoV-2 – (n = 113)	SARS-CoV-2+ (n = 7)	*P*-value
ACE2	rs35803318	c.2247G>A	2 (4.8%)	0 (0.0%)	6 (8.5%)	0 (0.0%)	8 (7.1%)	0 (0.0%)	NS
ACE2	rs41303171	c.2158A>G	1 (2.4%)	0 (0.0%)	1 (1.4%)	1 (25.0%)	2 (1.8%)	1 (14.3%)	NS
TMPRSS2	rs17854725	c.879T>C	38 (90.5%)	3 (100.0%)	57 (80.3%)	4 (100.0%)	95 (84.1%)	7 (100.0%)	NS
TMPRSS2	rs61735789	c.651C>T	3 (7.1%)	0 (0.0%)	5 (7.0%)	0 (0.0%)	8 (7.1%)	0 (0.0%)	NS
TMPRSS2	rs75603675	c.23G>T	13 (31.0%)	2 (66.7%)	26 (36.6%)	2 (50.0%)	39 (34.5%)	4 (57.1%)	NS
TMPRSS2	rs2298659	c.888C>T	12 (28.6%)	1 (33.3%)	26 (36.6%)	4 (100.0%)	38 (33.6%)	5 (71.4%)	NS
TMPRSS2	rs12329760	c.589G>A	11 (26.2%)	1 (33.3%)	24 (33.8%)	2 (50.0%)	35 (31.0%)	3 (42.9%)	NS
TMPRSS2	rs3787950	c.336A>G	11 (26.2%)	0 (0.0%)	9 (12.7%)	2 (50.0%)	20 (17.7%)	2 (28.6%)	NS
TMPRSS2	rs61735794	c.1266G>A	1 (2.4%)	0 (0.0%)	1 (1.4%)	2 (50.0%)	2 (1.8%)	2 (28.6%)	**<.0001**
TMPRSS2	rs61735792	c.300C>T	0 (0.0%)	0 (0.0%)	1 (1.4%)	2 (50.0%)	1 (0.9%)	2 (28.6%)	**<.0001**
TMPRSS2	rs142750000	c.1578G>A	1 (2.4%)	0 (0.0%)	3 (4.2%)	0 (0.0%)	4 (3.5%)	0 (0.0%)	NS
TMPRSS2	rs200291871	c.22G>C	0 (0.0%)	0 (0.0%)	2 (2.8%)	0 (0.0%)	2 (1.8%)	0 (0.0%)	NS
TMPRSS2	rs141788162	c.759C>T	1 (2.4%)	0 (0.0%)	2 (2.8%)	0 (0.0%)	3 (2.7%)	0 (0.0%)	NS
Furin	rs6226	c.1851G>C	39 (92.9%)	3 (100.0%)	67 (94.4%)	4 (100.0%)	106 (93.8%)	7 (100.0%)	NS
Furin	rs753334944	c.2334C>T	0 (0.0%)	0 (0.0%)	3 (4.2%)	0 (0.0%)	3 (2.7%)	0 (0.0%)	NS
Furin	rs16944971	c.128C>T	1 (2.4%)	1 (33.3%)	5 (7.0%)	0 (0.0%)	6 (5.3%)	1 (14.3%)	NS
Furin	ND (chr15:91424678)	c.1956_1956delG	1 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.9%)	0 (0.0%)	NS
Furin	rs73489557	c.183C>T	1 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.9%)	0 (0.0%)	NS
Furin	rs6225	c.1392G>T	0 (0.0%)	0 (0.0%)	2 (2.8%)	0 (0.0%)	2 (1.8%)	0 (0.0%)	NS

Note: Absolute frequency and percentage of individuals presenting these variants according to presence of MS and SARS-CoV-2 infection. Mantel-Haenszel test was performed to determine the association between each variants and COVID-19.

Only two exonic *ACE2* variants were detected in the cohort. First, rs35803318 (c.2247G>A), a synonymous variant with a low population frequency (MAF, 0.038) and a CADD score of 4.97, was observed in eight individuals without SARS-CoV-2 infection (7.1%) and in no individual with the infection. The other variant, rs41303171 (c.2158A>G), a missense variant with a CADD score of 15.09 (MAF, 0.016), was observed in two individuals without SARS-CoV-2 infection (1.8%; one with MS and one unaffected individual) and one patient with the infection (14.3%) ($\chi^2 = 0.652$; $P > .05$). These variants were not detected in homozygosis in any individual.

3.3 | Analysis of the *TMPRSS2* gene

This gene showed a higher level of polymorphism, with 109 patients without SARS-CoV-2 infection (96.5%) and 7 with the infection (100.0%) presenting variants. Among patients with MS, 41 (97.6%) individuals without SARS-CoV-2 infection and 3 with the infection (100.0%) presented variants of *TMPRSS2*. In the group of unaffected individuals, 68 (95.8%) without SARS-CoV-2 infection and 4 (100.0%) with the infection presented *TMPRSS2* variants. No difference was observed in distribution between men and women. Eleven variants were detected: three missense (rs75603675, rs12329760, and rs200291871) and eight synonymous variants (rs17854725, rs61735789, rs2298659, rs3787950, rs61735794, rs61735792, rs142750000, and rs141788162) (Table 1). The synonymous variant rs61735792 (c.300C>T; MAF, 0.01; CADD score, 0.216) was observed in individuals unaffected by MS in one family, with 2 (28%) presenting SARS-CoV-2 infection and 1 (0.9%) not infected ($\chi^2 = 14.2$; $P < .001$). The synonymous variant rs2298659 (c.888C>T; MAF, 0.23; CADD score, 0.713) was observed in 38 (33.6%) individuals without SARS-CoV-2 infection and five (71.4%) individuals with the infection (four of whom did not have MS); this difference was not statistically significant ($\chi^2 = 2.4$; $P = .115$). The synonymous variant rs61735794 (c.300C>T; MAF, 0.001; CADD score, 0.216) was present in two individuals without SARS-CoV-2 (1.8%) and two individuals with the infection (28.6%); this difference was statistically significant ($\chi^2 = 14.2$; $P < .001$). No significant differences were found between individuals with and without SARS-CoV-2 infection for the remaining *TMPRSS2* variants. No variant was detected in homozygosis.

3.4 | Analysis of the *furin* gene

Several variants were found in the furin gene in our sample. However, none of them was associated with COVID-19 (Table 1).

4 | DISCUSSION

Previous studies have searched for genetic factors associated with susceptibility to SARS-CoV-2 infection. It has been suggested that the virus is associated with several variants of the *OAS1*, *MX1*, *MBL2*, *CCL2*, *CCL5*, *ASHG*, *IFNgamma*, *CD14*, and *CD209* genes.[31-34] *MBL2* variant rs1800450 may also be associated.[35] It has also been proposed that genetic susceptibility to COVID-19 should be analyzed in association with *ACE2* variants in different populations. ACE2 is the functional receptor that mediates invasion of host cells by SARS-CoV-2. The other candidate gene related to COVID-19 susceptibility is *TMPRSS2*. Expression of the gene increases levels of ACE2-mediated invasion of cells by SARS-CoV-2, as the TMPRSS2 protein acts as a coreceptor.[36] Therefore, *ACE2* and *TMPRSS2* variants may modulate viral infectivity in humans,[37] making some individuals more vulnerable than others; a genetic effect may be involved, according to the results of a recent twin study.[38] In this study, we have used a cohort of families including patients with MS, with the aim to evaluate the association between genetic variants in specific genes and SARS-CoV-2 infection. We have divided Section 4 according to the genes examined.

4.1 | *ACE2* gene

This gene showed a low frequency of polymorphisms and was not associated with infection, which seems to contradict the hypothesis that *ACE2* variants are involved in infectivity, given the high rates of SARS-CoV-2 infection in Madrid. This study detected the missense variant rs41303171, which replaces asparagine with aspartate at position 720; this rare variant, practically absent in Asia and very infrequent in Africa and the Middle East, was also reported by Benetti et al[15] The relevance of this variant is controversial. Codon 720 is located far from the binding site targeted by the viral spike protein,[8] so it is unlikely that the variant could influence infectivity; however, its potential relevance may be related to its proximity to the site of cleavage by *TMPRSS2*.[13] According to Benetti et al,[15] this variant is significantly more frequent in patients than among controls. Similarly, the variant was associated with greater infection rates in the present study, particularly among individuals unaffected by MS; however, the data are insufficient to support this association.

The other *ACE2* variant detected, rs35803318, is synonymous and is considered non-pathogenic, although Ardeshirdavani et al[39] suggest that it is the only variant associated with infection. Conversely, Benetti et al[15] report that this variant was much more frequent among controls than among infected patients; these results are similar to those of the present study, in which this variant was not observed in any patient with SARS-CoV-2 infection. However, insufficient data are available to support a possible protective role.

4.2 | *TMPRSS2* gene

TMPRSS2, which is involved in proteolytic cleavage of ACE2 and the SARS-CoV-2 spike protein, leading to viral penetration into the host cell, is a highly polymorphic gene with numerous variants displaying considerable variations in human population frequency; practically all members of this cohort presented some variant. Several authors have detected variants of this gene that may influence infection risk, including rs977728, rs139010197, rs353163, and rs150048716.[40] Variant rs35074065 causes overexpression of the protease,[41] and may therefore constitute a candidate variant.[42] Variant rs75603675 has also been described as a possible candidate, although its pathogenicity has not been demonstrated. In the cohort studied here, this variant was more frequent among infected individuals, although this difference was not statistically significant. The synonymous variant rs61735789 was also associated with infection, although the difference was not significant. Benetti et al[15] describe 2 haplotypes of this gene that may be related to infection. The first contains at least the variants rs463727, rs34624090, rs55964536, rs734056, rs4290734, rs34783969, rs11702475, rs35899679, and rs35041537, and has been functionally linked to rs8134378. None of these variants were detected in this cohort. The second haplotype contains rs2070788, rs9974589, and rs7364083; none of these variants were detected in the present study. However, the missense variants rs12329760 and rs200291871 did appear in the cohort, but showed no association with infection. The first variant is potentially pathogenic. The synonymous variants rs61735794 and rs61735792 were also detected at significantly different frequencies in the groups of individuals with and without SARS-CoV-2 infection. Synonymous variants are not usually pathogenic, although we may consider that the risk may be not related to the production of a pathological protein, as with missense mutations, but to smaller modifications.

4.3 | *Furin* gene

Furin has been implicated in the SARS-CoV-2 infectivity. S protein is cleaved by TMPSS2 with the collaboration of furin, which has been linked to the entry of the virus in the respiratory tract and also with an increased risk of contagion.[43] To our knowledge, genetic variants of furin and its association with COVID-19 have not been examined. In our study, we did not found association between *Furin* variants and COVID-19.

4.4 | Implications for patients with MS

MS has previously been linked to coronavirus infections[44,45]; therefore, it would be beneficial to establish whether the pandemic has a

specific impact on these patients. Furthermore, polymorphisms of genes related to the renin-angiotensin system, although not *ACE2* specifically, have been studied in these patients.[46] There is also debate around the effect of MS treatment on COVID-19 severity,[47,48] and it has been suggested that some drugs acting on the immune system may protect against the infection.[49-52] While this issue lies beyond the scope of the current study, comparison of variants between patients with MS and unaffected individuals seems not to show that these genes play a pathogenic role.

5 | LIMITATIONS

In this study, WES findings were used to analyze the frequency of *ACE2* and *TMPRSS2* variants in Madrid, at a time of high incidence of COVID-19. The main limitation of the study is the small number of patients in the cohort with SARS-CoV-2 infection, which reduces the statistical value of the results. Similarly, the low sample size limits the possibility to evaluate other aspects of COVID-19 that could have some genetic influence, including the severity of the infection. The low number of cases may be explained by particularly strict adherence to lockdown and safety measures among members of the cohort due to potential risks related to MS; furthermore, the potential protective effect of treatment for MS, which constitutes the basis of the cohort, has also been suggested, despite a majority of participants being unaffected. Beyond these considerations, the fact that the cohort includes patients with MS should not constitute a bias as a high proportion of its members do not have the disease. Another limitation is the lack of confirmation of SARS-CoV-2 infection in many cases: for a considerable period of time during the pandemic, the Spanish healthcare authorities recommended that infected patients be quarantined without PCR confirmation of the infection. Finally, WES only analyses exonic variants; therefore, the study may have failed to detect potentially related intronic variants. Some strengths of the study are that it identified the frequency of variants of these genes in a geographic region where this had not previously been analyzed, and that human subjects were tested, rather than samples from a gene bank, enabling more precise analysis of the results.

6 | CONCLUSIONS

The results show that *ACE2* presents a low level of polymorphism, with only two variants (rs41303171 and rs35803318) being identified, corroborating the findings of Benetti et al[15] While *TMPRSS2* is highly polymorphic, the variants identified are not reported in other studies, with the exception of rs75603675. The synonymous variants rs61735794 and rs61735792 showed a significant association with SARS-CoV-2 infection. Further studies should be performed in different geographic regions, given the ethnic characteristics of these variants. In any case, this study provides data on the region of Madrid. The description of potential genetic variants clinically associated with COVID-19 provides a first step in the knowledge of genetic influences to the infection by SARS-CoV-2, although susceptibility should be proven in experimental studies performed in vitro.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Lead researchers: JAMG, JMG; study design: VP, JAMG, and JMG. Patient assessment: JAMG, PME, JMG, and JPE. Family studies: VP, LTF, LH, and PME. Data coordination: LH and UGP. Database: LTF, LH, and JMG. Data filtering and analysis: LTF and LH. Statistical analysis: JAMG. Analysis of results: all authors. Figures and tables: LTF. Drafting of manuscript: JMG, LTF, LH, and JAMG. Revision of manuscript: all authors.

ETHICS AND CONSENT

This study was approved by the clinical research ethics committee of Hospital Clínico San Carlos (WES and familial multiple sclerosis project [ref. XXXX] and COVID-19 and multiple sclerosis [ref. 20/242-E]). All participants undergoing WES gave written informed consent. Verbal consent was obtained from patients for the collection of data on COVID-19. Data were handled in observance of Spanish legislation on data protection (Organic Law 15/1999 of 13 December). The study complies with the principles of the Declaration of Helsinki ("Recommendations guiding doctors in biomedical research involving human subjects," Helsinki 1964, modified in October 2013).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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