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RESEARCH ARTICLE

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FURIN gene variants (rs6224/rs4702) as potential markers of death and cardiovascular traits in severe COVID-19

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Abstract

Furin is a protease that plays a key role in the infection cycle of SARS-CoV-2 by cleaving the viral proteins during the virus particle assembly. In addition, Furin regulates several physiological processes related to cardio-metabolic traits. DNA variants in the *FURIN* gene are candidates to regulate the risk of developing these traits as well as the susceptibility to severe COVID-19. We genotyped two functional *FURIN* variants (rs6224/rs4702) in 428 COVID-19 patients in the intensive care unit. The association with death ($N = 106$) and hypertension, diabetes, and hyperlipidaemia was statistically evaluated. The risk of death was associated with age, hypertension, and hypercholesterolemia. The two *FURIN* alleles linked to higher expression (rs6224 T and rs4702 A) were significantly increased in the death cases (odds ratio= 1.40 and 1.43). Homozygosis for the two high expression genotypes (rs6224 TT and rs4702 AA) and for the T-A haplotype was associated with an increased risk of hypercholesterolemia. In the multiple logistic regression both, hypercholesterolemia and the TT + AA genotype were significantly associated with death. In conclusion, besides its association with hypercholesterolemia, *FURIN* variants might be independent risk factors for the risk of death among COVID-19 patients.

KEYWORDS

COVID-19, FURIN, gene polymorphism, hypercholesterolemia, SARS-CoV-2

1 | INTRODUCTION

The coronavirus SARS-CoV-2 responsible for the COVID-19 pandemic enters the human cells through the binding of the viral spike (S) protein to the membrane angiotensin-converting enzyme-2 (ACE-2).[1] The S-proteinis a trimeric transmembrane glycoprotein with two main external domains: S1, which contains the receptor-binding domain, and S2 which contains the membrane fusion domain.[2–4] The native S protein is subjected to activation by protease-mediated cleavage at two distinct sites. The cell-surface serine protease TMPRSS2 is essential for the S-proteolysis and enables endosome-independent virus entry by a route that avoids antiviral IFITM-proteins.[1,5] While in the SARS-CoV-1 that causes the SARS both incisions occur after the virus has bound to the receptor, the SARS-CoV-2 contains a polybasic cleavage site (PRRAR) that is cut by the furin protease during the viral assembly in the infected cell.[6] This first

cut preactivates the viral particles and might explain the higher infective capacity of SARS-CoV-2 compared to other viruses that require two protease-cuts. Interestingly, some of the SARS-CoV-2 variants of concern could enhance the cleavage by furin, likely increasing the infection capacity.[7–9]

Furin is a membrane-bound protease of the subtilisin-like proprotein convertase family whose natural cellular function is to process precursor proteins but is also utilized by a number of pathogens to process their envelope proteins.[10–14] The furin polybasic insertion endows SARS-CoV-2 with a selective advantage.[15] In ferrets the lack of the S1/S2 furin site shed to lower titres among infected animals, that were unable to transmit the virus to cohoused animals.[16]

COVID-19 symptoms range from none or mild symptoms to severe pneumonia with admission to the intensive care unit (ICU), and this heterogeneity might be partly explained by host genetic variants that influence vulnerability to infection or modulate the immunological responses. The *FURIN* gene is a candidate to regulate the risk of COVID-19 severity. A single-nucleotide polymorphism (SNP, rs4702 A/G) located in the 3′-untranslated region of the gene influences alveolar and neuron infection by SARS-CoV-2 in vitro.[17] *FURIN* expression would be down-regulated by the rs4702 G allele, which is located in the binding site of a micro RNA (miR-338-3p).[17,18]

FURIN variants associated with differences in gene expression could modify the individual´s susceptibility to developing severe COVID-19 pneumonia with an increased risk of death. Our aim was to determine the association between two common *FURIN* variants and adverse outcomes in COVID-19 patients.

2 | PATIENTS AND METHODS

We obtained the demographic and clinical data of 428 patients who required admission in the ICU due to COVID-19 (mean age: 64, range: 24–95). All the participants were of European ancestry and from the region of Asturias (Northern Spain, total population of 1 million). The study was approved by the Ethics Committee of Principado de Asturias (Oviedo, Spain). All the patients or their next of kin gave their consent to participate in the study.

These patients were hospitalized between March (2020) and February (2021), a period in which three pandemic waves took place in our community. They were followed till disease remission with hospital discharge or death.

The hypercholesterolemia, diabetes, and hypertension status were obtained from the medical records. We measured the D-Dimer and IL6 blood levels at ICU admission. Based on previous reports values <2000 ng/ml (D-Dimer) or <15 pg/ml (IL6) were considered the predictors of severity.[19,20]

The *FURIN* rs6224 T/G and rs4702 A/G were determined by real-time PCR with Taqman assays (Thermo Fisher; Figure S). The rs6224 SNP was genotyped instead of rs17514846 A/C because the two were in almost complete linkage disequilibrium ($D´ = 0.98$; $r^2 = 0.94$) and there was no custom Taqman assay for rs17514846.

TABLE 1 Main values in the COVID-19 ICU patients ($N = 428$)

	Deceased $N = 106$	Survivors $N = 322$	p value
Male	76 (72%)	236 (74%)	0.71
Female	30 (28%)	86 (26%)	
Age mean, SD	71 ± 10	62 ± 12	$\mathbf{4 \times 10^{-13}}$
Age IQ range	55–71	66–78	
BMI, range	21–51	19–52	0.32
BMI >30	50%	53%	0.29
Diabetes	27 (26%)	75 (23%)	0.66
Type 2	21	68	
Type 1	0	2	
Unconfirmed	4	5	
Hypercholesterol	62 (59%)	147 (46%)	**0.02**
Hypertension	69 (65%)	166 (52%)	**0.02**
IL6[a] median	91	40	
pg/ml (IQ range)	50–131	12–127	
≤15 pg/ml	6 (6%)	63 (28%)	
15–70 pg/ml	32 (32%)	80 (36%)	
>70 pg/ml	62 (63%)	80 (36%)	$\mathbf{6 \times 10^{-6}}$
D-Dimer[b] ng/ml	1496	976	
Median (IQ range)	(936–2546)	(579–1594)	
<500 ng/ml	11 (12%)	44 (19%)	
501–2000 ng/ml	48 (52%)	133 (60%)	
>2000 ng/ml	33 (36%)	46 (21%)	**0.01**

Abbreviations: BMI, body mass index; ICU, intensive care unit.
[a]Measured in 323 patients.
[b]Measured in 315 patients.

The rs17514846 A/C (intron 1 of *FURIN* gene) was associated with differential expression, with cultured vascular endothelial cells with AA genotype showing higher *FURIN* expression than CC cells.[21] As a quality control of the method we confirmed the genotypes of several individuals by Sanger sequencing of PCR-fragments (Figures S).

The statistical analysis was performed with the R-free software (www.r-project.org). The logistic regression (linear generalized model) was used to compare mean values and frequencies between the groups.

3 | RESULTS

3.1 | *FURIN* variants and mortality

We determined the association between the study variables and death in the COVID-19 patients (Table 1). The next variables were

significantly associated with death in the univariate logistic regression: age, hypertension, hypercholesterolemia, IL6 >70 pg/ml, and D-Dimer >2000 ng/ml. The two FURIN SNPs did not deviate from the Hardy-Weinberg equilibrium. Alleles rs6224 T and rs4702 A (linked to increased *FURIN* expression) were associated with a higher risk of death (Table 2). In the multivariate logistic regression only age, IL6 >70, and the rs4702 AA genotype remained significant predictors of death among the COVID-19 patients (Table 3).

TABLE 2 Genotype and allele frequencies in the death and survivors

	Death *N* = 106	Survivors *N* = 322	*p* value	OR (95% CI)
Rs6224 T/G				
GG	28 (26%)	116 (36%)		
GT	51 (48%)	147 (46%)	0.07	1.57 (0.96–2.56)
TT	27 (26%)	59 (18%)		
T-frequency	0.50	0.41	0.03	1.40 (1.03–1.92)
Rs4702 A/G				
AA	42 (40%)	84 (26%)		
AG	44 (41%)	164 (51%)		
GG	20 (19%)	74 (23%)	0.01	1.86 (1.17–2.95)
A-frequency	0.60	0.52	0.03	1.43 (1.04–2.03)

Note: The *p* and OR values for the rs6224 (T allele and GT + TT genotypes) and rs4702 (A allele and AA genotype) are also shown.

Abbreviations: CI, confidence interval; OR, odds ratio.

3.2 | Haplotypes

The two *FURIN* SNPs were in linkage disequilibrium with two major haplotypes, T-A (higher expression) and G-A (Figure S). We found a significantly higher frequency of the rs6224 T-rs4702 A haplotype among deceased patients ($p = 0.04$, odds ratio [OR] = 1.41, 95% confidence interval = 1.01–1.95) (Figure 1). This suggested an increased risk of death among patients with the high expression haplotype. In agreement, double homozygotes TT + AA were significantly increased in the deceased patients (38% vs. 25%; $p = 0.015$) with OR = 1.83 (1.11–2.98).

3.3 | FURIN and hypercholesterolemia

\We compared the allele, genotype, and haplotype frequencies between the patients according to hypertension, diabetes, blood lipid, IL6, and D-Dimer levels (Tables S). The two SNPs were associated with hypercholesterolemia, with the two high expression genotypes (rs6224 TT and rs4702 AA) showing a higher risk. Homozygotes for the high expression T-A haplotype were significantly increased among the hypercholesterolemics (Table S). To determine whether the association between the *FURIN* variants and death might be explained by the hypercholesterolemia status we performed a multiple logistic regression. Both, hypercholesterolemia and the TT + AA genotype were significantly associated with death, with OR = 1.74 (1.01–2.85) for the genotype combination. This suggested that besides its association with hypercholesterolemia, *FURIN* variants might be independent risk factors for the risk of death among COVID-19 patients.

TABLE 3 Statistical differences between the deceased and survivors

	Univariate		Multivariate	
	p value	OR (95% CI)	*p* value	OR (95% CI)
Age	1×10^{-10}	1.09 (1.06–1.12)	7×10^{-4}	1.05 (1.02–1.09)
Male	0.71	0.91 (0.56–1.50)	0.21	
D-Dimer >2000 ng/ml	0.01	2.15 (1.26–3.61)	0.18	
IL6 >70 pg/ml	8×10^{-6}	3.04 (1.87–5.01)	1×10^{-3}	2.61 (1.47–4.71)
Hypertension	0.02	1.74 (1.11–2.77)	0.31	
Hyperchol	0.02	1.68 (1.08–2.63)	0.18	
Diabetes	0.66	1.12 (0.67–1.85)	0.37	
Rs6224 TT + GT	0.07	1.57 (0.97–2.59)	0.54	
Rs4702 AA	0.01	1.86 (1.17–2.95)	0.04	1.94 (1.01–3.71)

Note: Variables not significant in the multivariate logistic regression were correlated with advanced age.

The next variables were correlated with age:

D-Dimer >2000 ng/ml, $p = 0.0004$.

IL6 >70 pg/ml, $p = 0.01$.

Hypercholesterolemia, $p = 0.001$.

Diabetes, $p = 0.001$.

Hypertension, $p = 6 \times 10^{-7}$.

Abbreviations: CI, confidence interval; OR, odds ratio.

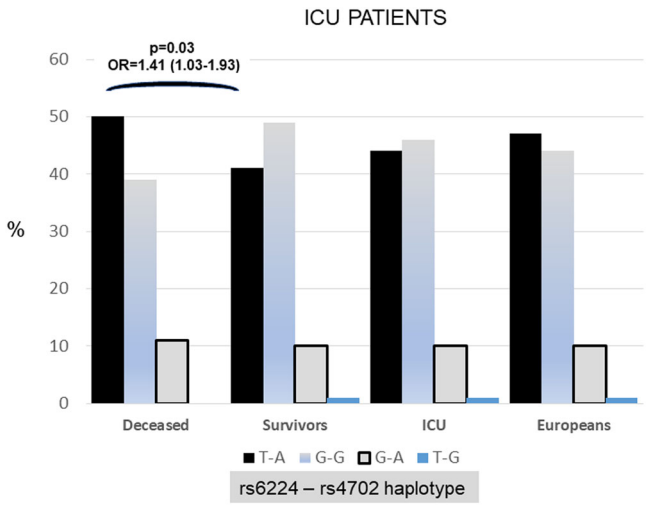

FIGURE 1 Haplotype frequencies in the different groups. rs6224 T-rs4702 A was significantly increased in the deceased ICU patients. ICU, intensive care unit

4 | DISCUSSION

The involvement of *FURIN* gene variants in COVID-19 has been hypothesized by some authors. An exome sequencing of a limited number of Multiple Sclerosis patients ($n = 120$) from Spain identified several coding variants without statistical significance between those positive and negative for SARS-CoV-2 infection.[22] This study was limited by the only analysis of the *FURIN* coding exons and did not compare the COVID-19 clinical forms. There a two main reasons why *FURIN* gene variants might contribute to the COVID-19 outcomes. First, as cellular protease furin plays an important role in SARS-CoV-2 biology due to its pivotal role in the processing of the Spike protein during the virus-particle assembly. The presence of the furin-site facilitates viral replication and enhanced infectivity. The mechanisms that enhanced the furin activation of Spike have been related to increased syncytia formation in pneumocytes.[23,24] SARS-CoV-2 induces the formation of multinucleated syncytia through the interaction of the S protein on the surface with the ACE-2 on neighboring cells. These syncytia may contribute to viral dissemination, immune evasion, and inflammatory response, and have been related to the lymphopenia that characterizes severe COVID-19.[25]

As protease furin plays a role in the activation of proteins involved in multiple cellular pathways, including some that contribute to cardiometabolic traits. A cross-sectional analysis of 2312 participants (mean age: 53 years) found that individuals with the lowest serum furin had average systolic, diastolic, and mean arterial blood pressures higher than the corresponding pressures in individuals with the highest serum quartile.[26] Furin cleaves the pro-renin receptor PRR to generate soluble sPRR. The PRR is a regulator of the renin-angiotensin system (RAS), and the sPRR is a candidate biomarker that reflects the status of the tissue RAS and the risk for cardiovascular traits such as hypertension.[27] Therefore, the role of furin in the regulation of RAS function might explain the association between *FURIN* variants and hypertension, a major determinant of COVID-19 severity. In this regard, the rs4702 GG genotype (linked to lower *FURIN* expression) has been associated with an increased risk of hypertension.[26–30]

Furin is a key regulator of the PCSK9 expression, a protein that regulates the number of LDL receptors and the level of blood lipids.[31,32] Higher circulating furin has been associated with increased LDL-cholesterol in plasma.[33] Furin might be also associated with COVID-19 severity through platelet activation and the risk of thrombotic events. Patients with coronary artery disease and SARS-CoV-2 positive displayed a significantly enhanced platelet activation.[34] Moreover, furin plasma levels were significantly increased in patients with a poor clinical prognosis.

The association between *FURIN* variants and the risk for viral infections has been reported.[35–37] In the SARS-CoV-2 infection these variants could explain the heterogeneous response, with individuals remaining asymptomatic while others develop severe pneumonia with an increased risk of death. Variants that increase the furin expression could be associated with worse outcomes (Figure 2). Our data were in agreement with this hypothesis because the rs6224 T and rs4702 A (high expression alleles) were significantly increased in the deceased patients. Furin has been associated with the risk of developing cardiovascular traits, such as hypertension, hypercholesterolemia, and diabetes, which are risk factors for COVID-19 severity and increased mortality. In our study, the two *FURIN* high-expression genotypes were associated with a significantly higher risk of hypercholesterolemia. Blood furin concentration has been associated with LDL cholesterol.[31–33] The association between circulating furin and lipid values could be mediated by its capacity to regulate the expression and activity of pcsk9, a protein that competes with the LDL-cholesterol particles by binding to the LDL-receptor.[38,39] Also, furin converts the propeptide form of Bmp1 into an active form that cleaves the LDL-R making it unable to bind the LDL particles.[40] Thus, the effect of *FURIN* variants on the risk of death might be secondary to hypercholesterolemia, which was significantly associated with death. However, the fact that the risk genotypes were associated with death among the COVID-19 patients independently of hypercholesterolemia suggested that these *FURIN* gene variants might contribute to disease severity by themselves.

5 | LIMITATIONS

Our study has several limitations, mainly the reduced sample size. Additional studies with larger cohorts from different populations are necessary to confirm the association with COVID-19 severity. Also, we studied patients with the most severe disease manifestation represented by the ICU patients that are at increased risk of death. Patients who did not require ICU admission, or who showed symptoms that did not require hospitalization were not studied and no conclusion about the effect of the *FURIN* variants on these manifestations can be concluded. The studied variants might regulate the risk of hypercholesterolemia and COVID-19 severity through a

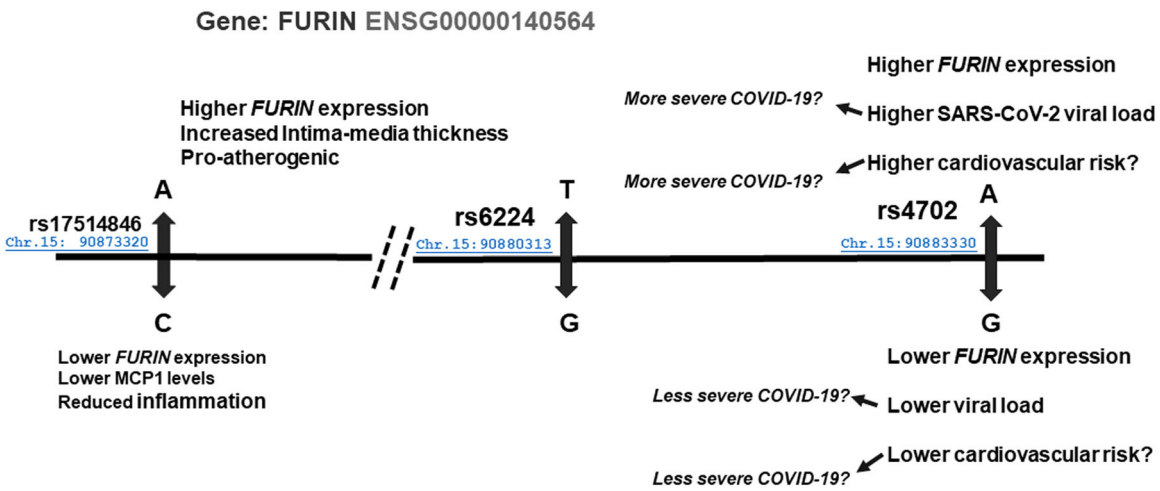

FIGURE 2 Proposed mechanism for the association between FURIN gene variants and increased risk of COVID-19 severity. Variants linked to increased furin expression might enhance SARS-CoV-2 infectivity by facilitating the viral particles' assembly in the host cell. In addition, these FURIN variants would increase the risk of developing cardiovascular traits, such as hypercholesterolemia. These variants have been also associated with increased vascular inflammation, a hallmark of atherosclerosis. The FURIN gene maps to chromosome 15, and the position of the SNPs relative to the coordinate sequence (Ensembl reference gene) is indicated. The two genotyped variants were located in intron 13 (rs6224) and the 3´-UTR (rs4702). SNP, single-nucleotide polymorphism; UTR, untranslated region

differential expression of the protein, but the level of blood circulating furin was not measured in our patients.

6 | CONCLUSIONS

The *FURIN* high expression alleles rs6224 T and rs4702 A were associated with an increased risk of death in COVID-19. These variants were also associated with hyperlipaemia, a risk factor for disease severity and mortality. However, both *FURIN* variants were independently associated with death suggesting that furin is a risk factor for COVID-19 severity beyond its role in cholesterol hemostasis.

Our work was based on a limited number of patients and requires validation in larger cohorts from different regions.

AUTHOR CONTRIBUTIONS

Lead researchers: Eliecer Coto, Guillermo M. Albaiceta; *Study design*: Eliecer Coto, Guillermo M. Albaiceta, Juan Gómez; *Patient assessment and data acquisition*: Guillermo M. Albaiceta, Laura Amado-Rodríguez, Marta García-Clemente, Elías Cuesta-LlavonaL, Santiago Melón, Marta E. Alvarez-Argüelles, José A. Boga, Susana Rojo-Alba, Victoria Alvarez, Sergio Pérez-Oliveira, Juan Gómez; *Database*: Eliecer Coto, Guillermo M. Albaiceta, Laura Amado-Rodríguez, Elías Cuesta-Llavona; *Genotyping*: Eliecer Coto, Elías Cuesta-LlavonaL, Daniel Vázquez-Coto, Belén Alonso, Sara Iglesias; *Data filtering and analysis*: Eliecer Coto; *Statistical analysis*: Eliecer Coto, Daniel Vázquez-Coto; *Analysis of results*: Eliecer Coto; *Writing the manuscript*: Eliecer Coto; *Revision of manuscript*: all the authors.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. An excel file with the raw data would be available for meta-analysis research.

ETHICS STATEMENT

This study was approved by the clinical research ethics committee of Hospital Universitario Central Asturias (HUCA). All the participants or they next of keen gave written or verbal consent. Data were handled in observance of Spanish legislation on data protection. The study complies with the principles of the Declaration of Helsinki ("Recommendations guiding doctors in biomedical research involving human subjects").

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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