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## Research article

# Genetic variants in the NF- $\kappa$ B signaling pathway (*NFKB1*, *NFKBIA*, *NFKBIZ*) and risk of critical outcome among COVID-19 patients

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## ABSTRACT

The NF- $\kappa$ B signaling pathway is a key regulator of inflammation in the response to SARS-CoV-2 infection. This pathway has been implicated in the hyperinflammatory state that characterizes the severe forms of COVID-19. The genetic variation of the NF- $\kappa$ B components might thus explain the predisposition to critical outcomes of this viral disease. We aimed to study the role of the common *NFKB1* rs28362491, *NFKBIA* rs696 and *NFKBIZ* rs3217713 variants in the risk of developing severe COVID-19 with ICU admission. A total of 470 Spanish patients requiring respiratory support in the ICU were studied (99 deceased and 371 survivors). Compared to healthy population controls (N = 300), the *NFKBIA* rs696 GG genotype was increased in the patients (p = 0.045; OR = 1.37). The *NFKBIZ* rs3217713 insertion homozygosity was associated with a significant risk of death (p = 0.02; OR = 1.76) and was also related to increased D-dimer values (p = 0.0078, OR = 1.96). This gene has been implicated in sepsis in mice and rats. Moreover, we found a trend toward lower expression of the *NFKBIZ* transcript in total blood from II patients.

In conclusion, variants in the NF- $\kappa$ B genes might be associated with the risk of developing severe COVID-19, with a significant effect of the *NFKBIZ* gene on mortality. Our results were based on a limited number of patients and require validation in larger cohorts from other populations.

## 1. Introduction

COVID-19 is the disease caused by the SARS-CoV-2, a respiratory virus of the *Betacoronavirus* genre [1]. The clinical manifestations of this disease range from asymptomatic forms that do not require hospitalization to severe illness with ICU admission and high risk of mortality [2]. Many of COVID-19 severe complications are explained by an exacerbated inflammatory response (cytokine storm) that leads to acute lung injury and acute respiratory distress syndrome, and to multiple organ dysfunction at a systemic level, resembling a septic process [3–6]. In addition to well recognized risk factors such as advanced age, male sex and hypertension, the genetic background of the host has been widely associated with the risk of developing severe COVID-19.

The genes that encode the components of the NF- $\kappa$ B pathway are strong candidates to serve as modulators of the outcome.

The NF- $\kappa$ B signaling pathway plays a key role in the promotion of the innate immune response against pathogens [7]. SARS-CoV-2 triggers the NF- $\kappa$ B activation through several mechanisms [8–10]. The result of the activation is the translocation of the NF- $\kappa$ B transcription factor to the nucleus, where it promotes the expression of multiple genes that mediate inflammation. In predisposed people, overstimulation and positive feedback loops can lead to a NF- $\kappa$ B pathway hyperactivation, which in turn promotes an exacerbated inflammatory response mediated by high levels of cytokines [8,11]. Among others, NF- $\kappa$ B induces the synthesis of IL-6, a cytokine with a leading role in inflammation and a strong link to the severity of COVID-19 [12]. IL-

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6 and other cytokines interplay with the coagulation pathways increasing the risk of thromboembolic events and mortality [13]. The NF- $\kappa$ B signaling pathway might also explain the increased risk of adverse outcome among patients with cardiovascular traits such as hypertension and hyperlipidemia [14,15].

The NF- $\kappa$ B signaling depends tightly on the functionality of its components, both activators and inhibitors. Several polymorphisms in the genes encoding components of this pathway can predispose to an altered activation of NF- $\kappa$ B and have been associated with the risk of developing several inflammation-mediated diseases [16–19]. We hypothesized that common NF- $\kappa$ B variants might play a role in the risk of developing severe COVID-19. We addressed this issue by studying candidate gene variants in *NFKB1*, *NFKBIA* and *NFKBIZ*.

## 2. Patients and methods

### 2.1. Study subjects

This study was approved by the Regional Ethical Committee, and all the participants (or their next of kin) gave their informed consent to participate in a research project about the genetic basis of COVID-19. Our study involved 470 COVID-19 patients (as confirmed by a positive result in a RT-qPCR testing in nasopharyngeal exudate) admitted to the Intensive Care Unit (ICU) at Hospital Universitario Central de Asturias. All of them were of European ancestry from the region of Asturias (Northern Spain, with a total population of about 1 million people), and were recruited from January 2020 to May 2021 when three pandemic waves occurred.

All the analytical values were measured at ICU admission. IL-6 > 70 pg/mL and D-dimer > 2,000 ng/mL were considered as the cutoff values based on previous reports stating their relation to higher risk of death in COVID-19 [16–21]. Pre-existing hypertension, hyperlipidemia and diabetes were obtained from the medical records.

We also studied 300 controls from the general population of Asturias prior to the SARS-CoV-2 pandemic. They matched the patients on age and ethnicity, and were studied with the only purpose of determining the genotype and allele frequencies in the general population in the context of a case-control study.

### 2.2. DNA isolation and NF- $\kappa$ B variants genotyping

The DNA of all the participants was obtained from 1 mL of blood in EDTA extraction tubes, and isolated with the Benchtop automated DNA extraction system (Promega Biotech Ibérica S.L, Promega Corporation). The DNA was stored at  $-20^{\circ}\text{C}$ .

We studied the *NFKB1* rs28362491, *NFKBIA* rs696 and *NFKBIZ* rs3217713 variants. The three were chosen based on their reported functional effects on the NF- $\kappa$ B signaling and their previous association to immune-mediated and inflammatory diseases [22,23].

*NFKB1* rs28362491 and *NFKBIA* rs696 were genotyped by allelic discrimination assays with TaqMan MGB probes purchased from Thermo Fisher Scientific (assays id. C\_61632788\_30 for rs28362491 and id. C\_145669\_30 for rs696). DNAs were amplified in 96 well qPCRs plates following the instructions of the manufacturer, using a 7500 Fast Real-Time PCR System (Applied Biosystems). Briefly, approximately 30 ng of each DNA was amplified in a final volume of 10  $\mu\text{L}$  with 0.2  $\mu\text{L}$  of the probe and 1x TaqMan Genotyping master mix (Thermo Fisher Scientific), in 40 cycles of 15 s at  $95^{\circ}\text{C}$  and 60 s at  $60^{\circ}\text{C}$ .

The *NFKBIZ* rs3217713 was genotyped by simple PCR followed by 5% agarose gel electrophoresis, with visualization on a transilluminator and discrimination between the two alleles based on their length (Supplementary Fig. 1) [23]. Briefly, the DNAs (100 ng each) were amplified in 96 plates in a volume of 20  $\mu\text{L}$  with a mix containing 0.5 U of Taq Polymerase (EURx Molecular Biology Products), standard

1x polymerase mix and 10 pmol of each primer, in 30 cycles of  $95^{\circ}\text{C}$ –30 s,  $62^{\circ}\text{C}$  1 min,  $72^{\circ}\text{C}$ –30 s. The accuracy of the genotyping method was previously validated by Sanger sequencing PCR fragments from the three genotypes [23].

### 2.3. *NFKBIZ* transcript levels in total blood

The *NFKBIZ* transcript levels (cDNA) were determined from total blood leukocytes of 52 patients. The blood from these patients was collected at hospital admission in 5 mL tubes contained RNA-later for RNA stability (Tempus™ Blood RNA Tubes, Thermo Fisher Scientific). Initially, the RNA was isolated from 1 mL of total blood of 60 patients with a commercial kit following the manufacturer instructions (GeneMatrix Universal Blood RNA Purification Kit, EURx Molecular Biology Products). The RNA was retro-transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Thermo Fisher Scientific). Each cDNA was amplified with a B-actin (*ACTB*) TaqMan Expression Assay and samples that gave a cycle threshold (Ct) > 30 were discarded due to low performance that suggested RNA degradation. A total of 52 samples met the quality criteria, and each cDNA was amplified in triplicate with *NFKBIZ* and *ACTB* TaqMan expression assays (Thermo Fisher Scientific, assays id hs00944739\_g1 for *NFKBIZ* and Hs99999903\_m1 for *ACTB*) in a real-time PCR equipment (ABI 7500, Thermo Fisher Scientific). The mean Ct of the triplicates was used as the value for transcript abundance (Supplementary Fig. 2, Supplementary Table 2). For each sample, the ratio of the *NFKBIZ*/*ACTB* Ct values indicated the normalized *NFKBIZ* expression, with lower ratios (lower *NFKBIZ* Ct values) corresponding to increased transcript levels.

### 2.4. Statistical analysis

All the anthropometric, analytical and genetic values were collected in an Excel file (available as requested to the corresponding author). The statistical analyses were performed in R (<https://www.r-project.org>) using the RStudio software (<https://www.rstudio.com>). Chi-squared tests were done to compare genotype and allele frequencies between groups. Odds Ratio (OR) values and their 95% confidence intervals (95% CI) were determined by logistic regression (generalized linear model). A  $p < 0.05$  was taken as the cutoff for significance.

## 3. Results

Table 1 summarizes the main values in the study cohort. COVID-19 mortality was significantly associated with older age ( $p = 2.05 \times 10^{-11}$ ) and hypertension ( $p = 0.02$ ). There was also a trend towards an association to mortality in hyperlipidemia. These findings kept in line with the previous characterization of these variables as main risk factors of COVID-19 severity. These variables were correlated with age (Table 2). An IL-6 > 70 pg/mL at ICU admission was also more common among deceased patients. A D-dimer > 2,000 ng/mL at ICU admission was not a significant predictor of mortality in our patients.

Genotype frequencies in patients and population controls are presented in Table 3. No deviation from the Hardy-Weinberg equilibrium was observed in patients and controls. The allele frequencies in the controls were in line with the reported among Europeans. The *NFKBIA* rs696 GG genotype was significantly increased in the ICU patients compared to controls ( $p = 0.045$ ; OR = 1.37, 95% CI = 1.00–1.87). No significant difference was observed in patients vs. controls for the *NFKB1* and *NFKBIZ* variants.

We compared the genotype frequencies between survivors and non-survivors and found a significant association of the *NFKBIZ* rs3217713 II genotype with mortality ( $p = 0.02$ ; OR = 1.76, 95% CI = 1.08–2.86) (Table 3). We found that this genotype was increased in patients

**Table 1**

Main values in the severe COVID-19 patients, showing p-values and Odds Ratios (OR) with 95% confidence intervals (95% CI) in the univariate logistic regression.

	Death N = 99	Survivors N = 371	p-value	OR (95% CI)
Male	71 (72 %)	270 (73 %)	0.83	0.95 (0.58–1.57)
Age (mean ± SD)	70.26 ± 10.23	61.59 ± 12.42	<b>2.05 × 10<sup>-11</sup></b>	1.08 (1.06–1.11)
Hypertension	64 (65 %)	192 (52 %)	<b>0.02</b>	1.70 (1.08–2.72)
Hyperlipidemia	52 (56 %)	164 (45 %)	0.06	1.54 (0.97–2.44)
Diabetes	21 (23 %)	83 (23 %)	0.95	0.98 (0.56–1.67)
IL-6 > 70 pg/mL <sup>1</sup>	42 (55 %)	122 (36 %)	<b>0.002</b>	2.14 (1.30–3.55)
D-dimer > 2000 ng/mL <sup>2</sup>	24 (26 %)	77 (24 %)	0.82	1.06 (0.62–1.79)

<sup>1</sup> IL-6 was determined in 77 deceased patients and 340 survivors.

<sup>2</sup> D-dimer was determined in 94 deceased patients and 316 survivors.

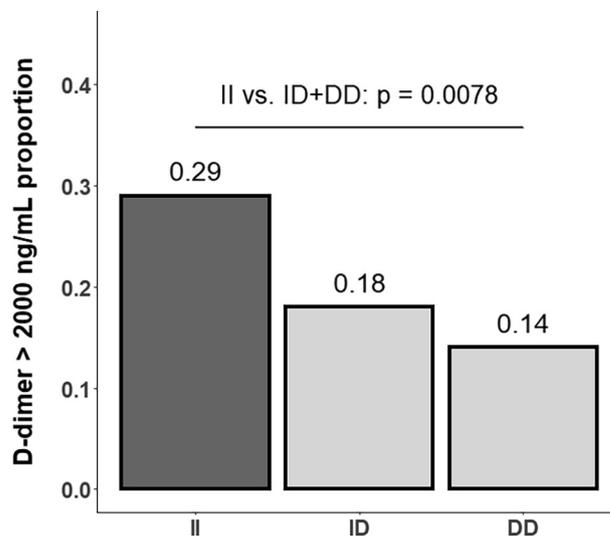
**Table 2**

Multiple logistic regression values for the ICU deceased patients vs. survivors.

	p-value	OR	95% CI
<i>NFKB1Z</i> II	<b>0.04</b>	1.89	1.65–3.53
Age	<b>10<sup>-6</sup></b>	1.07	1.04–1.10
Male	0.70	0.89	0.48–1.68
Hypertension	0.51	0.83	0.47–1.56
Hyperlipidemia	0.74	0.91	0.51–1.60
IL-6 > 70 pg/mL	<b>0.01</b>	2.14	1.24–3.73
D-dimer > 2000 ng/mL	0.51	0.81	0.41–1.52

with D-dimer > 2,000 ng/mL (p = 0.0078; OR = 1.96, 95% CI = 1.20–3.28) (Fig. 1, Supplementary Table 1C).

To explore the potential effect of the rs3217713 variant, we measured the expression of *NFKB1Z* in total blood leukocytes from 52 patients (DD = 3, ID = 18, II = 31) (Supplementary Table 2). The mean values of the *NFKB1Z/ACTB* Ct ratios were 1.267 for DD/ID and 1.320 for II, showing a trend (p = 0.052) toward lower expression (higher Ct ratios) among the rs3217713 II patients.



**Fig. 1.** Genotype frequencies related to levels of D-dimer above 2000 ng/mL among the ICU patients. Raw data presented in Supplementary Table 1C.

**Table 3**

Genotype and allele frequencies for *NFKB1* rs28362491 (indel), *NFKB1A* rs696 (c.\*126G > A) and *NFKB1Z* rs3217713 (indel) in the COVID-19 ICU patients (deceased and survivors) and controls. European MAFs (1000 Genomes Project): *NFKB1* rs28362491 D = 0.40; *NFKB1A* rs696 A = 0.39; *NFKB1Z* rs3217713 D = 0.23.

SNP	Group	N	Genotype freq. (%)			p-value; OR (95% CI)	Allele freq. (%)		p-value; OR (95% CI)
<b><i>NFKB1</i> rs28362491</b>	Deceased	99	II 36 (0.36)	ID 47 (0.47)	DD 16 (0.16)	ID + DD vs. II: 0.69; 1.10 (0.69–1.74)	I 119 (0.60)	D 79 (0.40)	D vs. I: 0.83;1.03 (0.75–1.43)
	Survivors	371	143 (0.39)	166 (0.45)	62 (0.17)		452 (0.61)	290 (0.39)	
	ICU	470	179 (0.38)	213 (0.45)	78 (0.17)	DD vs II + ID: 0.64;1.10 (0.74–1.63)	571 (0.61)	369 (0.39)	D vs. I: 0.92;1.01 (0.82–1.25)
	Controls	300	112 (0.37)	142 (0.47)	46 (0.15)		366 (0.61)	234 (0.39)	
<b><i>NFKB1A</i> rs696</b>	Deceased	99	GG 42 (0.42)	GA 40 (0.40)	AA 17 (0.17)	GG vs. GA + AA: 0.21;1.33 (0.85–2.10)	G 124 (0.63)	A 74 (0.37)	G vs. A: 0.38;1.16 (0.84–1.60)
	Survivors	371	132 (0.36)	175 (0.47)	64 (0.17)		439 (0.59)	303 (0.41)	
	ICU	470	174 (0.37)	215 (0.46)	81 (0.17)	GG vs. GA + AA: <b>0.045</b> ; 1.37 (1.00–1.87)	563 (0.60)	377 (0.40)	G vs. A: <b>0.0498</b> ;1.23 (1.00–1.51)
	Controls	300	90 (0.30)	149 (0.50)	61 (0.20)		329 (0.55)	271 (0.45)	
<b><i>NFKB1Z</i> rs3217713</b>	Deceased	99	II 71 (0.72)	ID 25 (0.25)	DD 3 (0.03)	II vs. ID + DD: <b>0.02</b> ;1.76 (1.08–2.86)	I 167 (0.84)	D 31 (0.16)	I vs. D: <b>0.025</b> ;1.61 (1.06–2.45)
	Survivors	371	219 (0.59)	133 (0.36)	19 (0.05)		571 (0.77)	171 (0.23)	
	ICU	470	290 (0.62)	158 (0.34)	22 (0.05)	ID + DD vs. II: 0.28; 1.18 (0.87–1.61)	738 (0.79)	202 (0.21)	I vs. D: 0.30;1.14 (0.88–1.49)
	Controls	288	189 (0.66)	87 (0.30)	12 (0.04)		465 (0.81)	111 (0.19)	

#### 4. Discussion

The main finding of our study was the association between the *NFKB1Z* rs3217713 II genotype and a higher risk of death among severe ICU COVID-19 patients. The functional effect of this variant is unknown, but one study previously associated rs645781—a polymorphism in strong linkage disequilibrium with rs3217713—to higher risk of invasive pneumococcal disease [24,25]. The *NFKB1Z* gene has been found deregulated in mouse and rat models of sepsis [26,27]. Moreover, the I $\kappa$ B $\zeta$  protein that *NFKB1Z* codes for regulates human monocyte pro-inflammatory responses induced by *Streptococcus pneumoniae* [28]. Of note, a single-cell deep-immune profiling of bronchoalveolar lavage samples from patients with critical COVID-19 in comparison to non-COVID-19 pneumonia and normal lung identified *NFKB1Z* as a gene upregulated in this disease, along with other NF- $\kappa$ B genes such as *NFKB1A* [29,30]. The overactivation of *NFKB1Z* in response to SARS-CoV-2 was in agreement with the regulatory effect of this gene in response to viral infection, activating leukocyte subsets (i.e., Th17) and promoting the expression of pro-inflammatory mediators [31–33]. Miyake et al. reported that *Nfkbiz*<sup>-/-</sup> mice had an impaired production of IFN- $\gamma$  and a decline in natural killer cells (NKs) activity, making them highly susceptible to mouse cytomegalovirus infection [32]. These results pointed to I $\kappa$ B $\zeta$  as essential for the activation of NK cells and antiviral host defense responses.

In addition, *NFKB1Z* rs3217713 II was associated to D-dimer levels above 2,000 ng/mL, suggesting a relation to thrombotic events. NF- $\kappa$ B has been considered a major pathway leading to platelet activation in response to the SARS-CoV-2 infection. This pathway is induced both by a direct interaction with the virus and by an interplay with inflammation, with TLR2 and TLR4 being important upstream inducers [34]. Interestingly, these receptors are also stimulated by lipopolysaccharide and other bacterial cell wall components, and they have been found to play a key role in sepsis, making a connection to I $\kappa$ B $\zeta$ 's involvement in pro-thrombotic pathways [27,35]. The *NFKB1Z* rs3217713 II might thus be associated with enhanced activity of the TLR2/4-NF- $\kappa$ B axis, favoring platelet activation and explaining our observations [34]. Interestingly, the connection between endothelial-specific NF- $\kappa$ B and D-dimer levels was also demonstrated in a mouse transgenic model subjected to septic stimuli [36,37]. In these studies, the blockade of endothelial NF- $\kappa$ B restored plasma fibrinogen levels and reduced plasma D-dimer levels after lipopolysaccharide injection, which caused a procoagulant response defined by marked increase of plasma coagulation factors, decreased fibrinogen and increased D-dimer in plasma, and widespread tissue fibrin deposition. In line with this study, we found a lower expression of *NFKB1Z* in total leukocytes of II patients, that could be associated to a lessened capacity of NF- $\kappa$ B inhibition among II compared to D-carriers, potentially leading to the elevated D-dimer levels found in the former group. However, this observation was limited by the small number of transcripts analyzed (N = 52) and by the fact that it was based on total blood leukocytes. Cell-specific (such as immune subpopulations, endothelia, or lung) studies are thus of utmost relevance.

We also found a higher frequency of *NFKB1A* rs696 GG genotype among the patients in comparison to controls. The *NFKB1A* gene encodes I $\kappa$ B $\alpha$ , a key inhibitor of the NF- $\kappa$ B signaling that acts by blocking the translocation of the RelA/p50 active dimers to the nucleus [7]. The rs696 variant has been related to changes in the *NFKB1A* function because it might affect a miRNA binding site. The G allele has been linked to decreased *NFKB1A* mRNA stability *in vitro* and lower inhibitory activity [16–18]. A lesser inhibition of NF- $\kappa$ B led by rs696 G could promote the proinflammatory signaling of the pathway, thereby increasing the risk of developing COVID-19 complications and ICU admission. Although these functional data support a role for the genetic variant in severe COVID-19, our study compared ICU patients with population controls, and we cannot exclude that the *NFKB1A* vari-

ant was a marker for wide COVID-19 manifestations besides the ones found in severe outcomes with ICU-support. To address this issue, individuals covering the whole spectrum of the disease should be genotyped.

The *NFKB1Z* indel was previously associated with the risk of symptomatic coronary artery disease angiographically confirmed [22]. This effect was not determined in our work because of the lack of available data, but might be worth of consideration for further studies dealing with the long-term effect of COVID-19. In this regard, the NF- $\kappa$ B variants might contribute to define the risk of future cardiac events in patients with previous critical COVID-19.

Finally, we acknowledge that our study presents some limitations. It was based on a limited sample size, and the results should be validated in larger cohorts from different populations, particularly to shed light on the putative role of *NFKB1Z* in the increased mortality. Also, we used D-dimer values as an indicator of coagulation disorders, but a direct proof of these conditions would require imaging techniques not performed routinely on our patients. Elucidating the relationship between *NFKB1Z* and thrombotic disorders that course with high D-dimer levels would also be valuable. Our results are biologically plausible given the pivotal role of the NF- $\kappa$ B pathway in the COVID-19 pathophysiology, but further studies would be of utmost interest to define the functional links with disease risk and mortality in order to contribute to the management of this disease.

#### 5. Conclusions

The *NFKB1Z* rs3217713 insertion allele was a risk factor for mortality among COVID-19 ICU patients, and it was also related to high D-dimer levels. This gene was previously associated with the inflammatory status in critical COVID-19, which would explain the association between its functional variants and severe disease outcomes. Studies from other populations are necessary to confirm our results, as well as functional studies to link the gene variants to COVID-19 pathology.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Author contributions

Lead researchers: EC, GMA; study design: EC, GMA, JG; patient assessment and data acquisition: GMA, LAR, ECLL, JGO; database: EC, GMA, LAR, ECL; genotyping: EC, DGC, DM, DVC; data filtering and analysis: EC, DGC; statistical analysis: EC, DGC; analysis of results: EC, DGC; writing the manuscript: EC, DGC; revision of manuscript: all the authors.

#### Ethics and consent

This study was approved by the clinical research ethics committee of Hospital Universitario Central Asturias (HUCA). All the participants or their next of kin 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 (“Recom-

mentations guiding doctors in biomedical research involving human subjects”).

### 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.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.humimm.2022.06.002>.

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