Grundinformationen

Kurzbeschreibung Ihres Tätigkeitsbereiches

Assistenzarzt in der Infektiologie mit klinischem Schwerpunkt auf der stationären Diagnostik, Therapie und Betreuung von Patienten mit akuten und chronischen Infektionskrankheiten.

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Kurzer Lebenslauf

Nach meiner Ausbildung im Rettungsdienst begann ich mein Studium an der medizinischen Fakultät Pécs und setzte dies an der Universität Duisburg-Essen fort. Während des klinischen Abschnittes begann ich im Rahmen meiner Promotionsarbeit am Institut für Pharmakologie und Pharmakokinetik meine ersten wissenschaftlichen Projekte. Der Hauptfokus meiner Arbeit lag ab 2021 im Rahmen der COVID-19 Pandemie auf einer Dissertation mit dem Einfluss genetischer und klinischer Faktoren auf den Verlauf von COVID-19. Die Ergebnisse dieser Arbeit sind in zwei peer-reviewten Publikationen eingeflossen. Seit Anfang 2024 bin ich als Assistenzarzt in der Klinik für Infektiologie tätig und strebe weitere Forschungsprojekte an.

Allgemeinverständlich formulierte Zusammenfassung ihrer Arbeit:

Diese Arbeit untersucht den Einfluss genetischer und klinischer Marker auf den Verlauf von COVID-19, mit besonderem Fokus auf genetische Varianten im menschlichen Toll-Like-Rezepotor-4-Gen (TLR4-Gen). Analysiert wurde eine Kohorte von 1570 Patienten hinsichtlich demografischer Daten, Vorerkrankungen, Entzündungsparametern sowie dem Genotyp.

Ein zentrales Ergebnis der Untersuchung ist die hohe prognostische Aussagekraft initialer Entzündungsmarker wie Interleukin-6 (IL-6) und Procalcitonin (PCT). Bereits zu Krankheitsbeginn wiesen Patienten, die später schwere oder tödliche Verläufe erlitten, auffällig erhöhte Werte auf – selbst in Fällen mit zunächst mildem klinischem Bild. Diese Marker ermöglichen somit eine frühzeitige Identifikation von Risikopatienten noch vor Auftreten schwerer Symptome.

Zudem zeigt die Analyse, dass Träger des Genotyps von TLR4 ein signifikant geringeres Risiko für schwere Krankheitsverläufe aufweisen. Besonders relevant ist hierbei, dass es sich bei der genetischen Bestimmung des TLR4-Polymorphismus um einen lebenslang stabilen Biomarker handelt, der als dauerhafter, universell einsetzbarer Prädiktor in die Risikobewertung einbezogen werden kann.

Diese Erkenntnisse verdeutlichen die Bedeutung des Zusammenspiels aus genetischer Prädisposition und frühen Entzündungszeichen für die Entwicklung individualisierter Strategien im Umgang mit COVID-19 – insbesondere für die Risikostratifizierung, frühzeitige Intervention und gezielte Therapieplanung.

Zusammenfassende Kurzbeschreibung

Wie beurteilen Sie das Innovationspotential Ihrer Einsendung?

Die vorliegende Arbeit besitzt ein hohes Innovationspotenzial, da sie genetische und klinische Faktoren kombiniert, um den Verlauf von COVID-19 präziser vorhersagen zu können. Besonders neuartig ist die Identifikation des TLR4-Einzelnukleotidpolymorphismus rs4986790 als lebenslang gültigen, stabilen Biomarker, der unabhängig von anderen Risikofaktoren einen prädiktiven Wert für schwere Krankheitsverläufe besitzt.
Darüber hinaus hebt die Studie die große Aussagekraft früh bestimmbarer Entzündungsmarker (IL-6 und PCT) hervor, die bereits zu Beginn der Erkrankung Aufschluss über das spätere Risiko schwerer Verläufe geben, selbst wenn die Patienten zunächst klinisch stabil erscheinen. Insbesondere im Erstkontakt, etwa in der Notaufnahme, erleichtert die frühzeitige Risikobewertung die Entscheidung, ob eine ambulante oder stationäre Behandlung notwendig ist. Damit leistet die Arbeit einen wichtigen Beitrag zur Weiterentwicklung der COVID-19-Versorgung und ist auch auf zukünftige Infektionskrankheiten übertragbar.

Wie beurteilen Sie Ihre Einsendung bezüglich der Nachhaltigkeit?

Die Arbeit leistet einen nachhaltigen Beitrag zur Optimierung von Ressourcen im Gesundheitswesen. Durch die einmalige Bestimmung des genetischen Markers TLR4 rs4986790, der lebenslang gültig ist, können Patienten langfristig besser risikostratifiziert werden. Dies ermöglicht eine gezielte Steuerung von Personal, Bettenkapazitäten und finanziellen Mitteln, insbesondere in Zeiten hoher Belastung des Gesundheitssystems.
Darüber hinaus erleichtert die präzise Vorhersage schwerer Krankheitsverläufe eine effizientere Planung und Verteilung von humanen und finanziellen Krankenhausressourcen. Die Erkenntnisse sind nicht nur für COVID-19 relevant, sondern lassen sich auch auf andere Infektionskrankheiten übertragen, was den nachhaltigen Nutzen der Arbeit zusätzlich unterstreicht.

Beitrag zu einer nachhaltigen Verbesserung der Gesundheit und Lebensqualität

Genetische Prädiktoren wie rs4986790 sind *zeitunabhängige* Biomarker, die nicht von akuten Krankheitsverläufen oder Therapien beeinflusst werden. Sie ermöglichen eine dauerhafte, lebenslange Aussage über das individuelle Risiko. Durch eine gezielte Frühidentifikation von Hochrisikopatient:innen können medizinische Ressourcen effizienter genutzt, Behandlungsstrategien individualisiert und Krankheitsverläufe abgemildert werden. Die Anwendung solcher Marker kann perspektivisch auch zur Impfpriorisierung oder Therapieentscheidung beitragen – mit direktem Einfluss auf die Lebensqualität vulnerabler Gruppen.

Besitzt Ihr Beitrag Standortrelevanz (z.B. für Essen)?

Die Arbeiten wurden am Institut für Pharmakogenetik der Universitätsmedizin Essen durchgeführt, das direkt am Standort Essen sitzt. Deutschlandweit gibt es nur wenige weitere humangenetische Institute und Labore, wie in Berlin, Heidelberg oder Tübingen. Die enge Verzahnung von Forschung und klinischer Praxis am Universitätsklinikum Essen ermöglicht eine effiziente Umsetzung der Ergebnisse in die Patientenversorgung vor Ort. Sie spiegelt die Exzellenz medizinischer Forschung in Essen wider und stärkt die Rolle der Stadt als Wissenschafts- und Gesundheitsstandort. Darüber hinaus unterstreicht sie die Relevanz lokal erhobener Daten für globale Fragestellungen der öffentlichen Gesundheit.

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The single nucleotide polymorphism rs4986790 (c.896A>G) in the gene *TLR4* as a protective factor in corona virus disease 2019 (COVID-19)

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Background and aims: Several factors, such as hypertension and diabetes mellitus, are known to influence the course of coronavirus disease 2019 (COVID-19). However, there is currently little information on genetic markers that influence the severity of COVID-19. In this study, we specifically investigated the single nucleotide polymorphism (SNP) rs4986790 in the *TLR4* gene to identify a universal marker for preclinical prediction of COVID-19 disease progression.

Methods: We analyzed the influence of demographics, pre-existing conditions, inflammatory parameters at the time of hospitalization, and *TLR4* rs4986790 genotype on the outcome of COVID-19 in a comprehensive cohort (N = 1570). We performed multivariable analysis to investigate the impact of each factor.

Results: We confirmed that younger patient age and absence of pre-existing conditions were protective factors against disease progression. Furthermore, when comparing patients with mild SARS-CoV-2 infection with patients who required hospitalization or intensive care or even died due to COVID-19, the AG/GG genotype of *TLR4* rs4986790 was found to be a protective factor against COVID-19 disease progression (OR: 0.51, 95% CI: 0.34 - 0.77, p = 0.001). In addition, we demonstrated that low levels of interleukin-6 (IL-6) and procalcitonin (PCT) had a favorable effect on COVID-19 disease severity. In the subsequent multivariable analysis, we confirmed the absence of cardiovascular disease, low levels of IL-6 and PCT, and *TLR4* rs4986790 AG/GG genotypes as independent predictors of potential hospitalization and reduction of severe or fatal disease course.

Conclusion: In this study, we identified an additional genetic factor that may serve as an invariant predictor of COVID-19 outcome. The *TLR4* rs4986790 AG/GG genotype reduced by half the risk of COVID-19 patients requiring hospitalization, intensive care or to have a fatal outcome. In addition, we were able to confirm the influence of previously known factors such as pre-existing

conditions and inflammatory markers upon the onset of disease on the course of COVID-19. Based on these observations, we hereby provide another prognostic biomarker that could be used in routine diagnostics as a predictive factor for the severity of COVID-19 prior to SARS-CoV-2 infection.

KEYWORDS

SARS-CoV-2, TLR4, COVID-19, polymorphism, rs4986790, disease severity, prognostic marker, IL-6

1 Introduction

Toll-like receptors (TLRs) recognize specific structures called pathogen-associated molecular patterns (PAMPs) and viral proteins on the surface of pathogens such as bacteria and viruses. There exist ten different variants of TLRs, which are located on the cell membrane as well as in endosomes. TLRs are expressed on both adaptive and innate immune cells, including T cells, B cells, dendritic cells, macrophages, and natural killer cells. Each TLR recognize specific structures. TLR3 recognizes double-stranded RNA, TLR4 recognizes lipopolysaccharides, and TLR7/8 recognize single-stranded RNA (1, 2).

Two different signaling pathways are known for TLR4. The first is via myeloid differentiation primary response 88 (MyD88), while the second pathway is via TIR-domain-containing adapter-inducing interferon-β (TRIF). This results in the activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factors (IRFs), leading to the production of type-1 IFN and pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), and tumor necrosis factor-α (TNF-α) (3, 4). The production of pro-inflammatory cytokines and interferons is critical in fighting viral infections. The spike protein of SARS-CoV-2 acts as a ligand for TLR4, which induces a strong protein-protein interaction. This can lead to overactivation of TLR4, resulting in a prolonged or excessive immune response (5, 6).

Excessive levels of cytokines can lead to dysregulation of the immune response, resulting in cytokine storm and cytokine release syndrome. This systemic inflammatory syndrome is characterized by life-threatening levels of circulating cytokines and hyperactivation of immune cells. In the worst case, this exaggerated immune response can lead to multiple organ failure with a fatal outcome of COVID-19 (7). Severe SARS-CoV-2 infection is characterized primarily by elevated levels of IL-6, but also IL-8, IL-10, TNF-α and interferon-γ (IFN-γ). In particular, IL-6 has been associated with a significantly higher mortality and may serve as an indicator for disease prognosis and the severity of in COVID-19 (8).

Receptor activity and sensitivity may be influenced by genetic variation. The single nucleotide polymorphism (SNP) rs4986790 in the *TLR4* gene has been frequently described in the literature (9–12). The SNP is located outside the ligand binding domain of TLR4 and therefore does not affect LPS binding. However, it causes a local conformational change that affects folding, cell surface expression levels, protein stability, and interaction with downstream messenger proteins. This results in a twofold reduction of functional TLR4 (13). In addition to the well-known risk factors for COVID-19, including older age, immunosuppression, chronic pulmonary, hepatic, renal, neurological, cardiovascular disease, and diabetes mellitus, some patients without these conditions also showed severe and fatal outcomes (14). In previous studies, we have shown that genetic factors also influence disease progression. The *ACE2* rs2285666 GG genotype or G-allele were associated with an almost twofold increased risk of infection and a threefold increased risk of a severe or fatal outcome of COVID-19 (15). We also demonstrated that the *GNB3* c.825C>T (rs5443) TT genotype is protective against COVID-19 fatality (16).

Because TLR4 plays a pivotal role in binding to the SARS-CoV-2 spike protein and regulating downstream inflammatory responses (17), we investigated the influence of the SNP rs4986790 (c.896A>G) in the *TLR4* gene and the associated expression of interleukin-6 as an inflammatory mediator in the current study.

2 Methods

2.1 Study participants, recruitment and patient outcome

The study was approved by the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (20-9230-BO) and was conducted in collaboration with the West German Biobank (WBE; 20-WBE-088). Written informed consent was obtained from all patients enrolled in the study.

Enrollment started on March 11, 2020, and ended on May 18, 2021. A total of 1570 SARS-CoV-2 positive patients with at least one positive reverse transcription polymerase chain reaction (RT-PCR) test result were included. Follow-up was completed on June 30, 2021, at which time all patients had been discharged from the hospital or had died during the study. The study included 660 female patients (42.0%) and 910 male patients (58.0%). Age ranged from 18 to 99 years, with a median of 62.0 years. Patients were unvaccinated and did not receive targeted antibody therapy during the observation period.

The categorization was based on ECDC (European Centre for Disease Prevention and Control, 2021) criteria and considered the worst achieved condition after SARS-COV-2 infection. The 'mild' group included outpatients with no or mild symptoms not requiring hospitalization (N = 205, 13.1%). The 'hospitalized' group encompassed all patients who were hospitalized but did not require intensive care at any time (N = 760, 48.4%). To be included in the 'severe' group, patients had to be admitted to an intensive care unit due to SARS-COV-2 infection and/or intubated due to respiratory failure or indirect sequelae of SARS-COV-2 infection (N = 292, 18.6%). The 'fatal' group consisted of patients who died of SARS-COV-2 infection despite receiving medical care after admission (N = 313, 19.9%).

For a comprehensive analysis, we included additional data from the patients' medical history. This included conditions such as hypertension, cardiovascular disease, diabetes mellitus, and laboratory values of interleukin-6 (IL-6) and procalcitonin (PCT).

2.2 Genotyping of *TLR4* rs4986790 (c.896A>G, p.Asp299Gly)

Genomic DNA was extracted from 200 µl EDTA-treated blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed using 2 µl genomic DNA and 30 µl Taq DNA-Polymerase 2x Master Mix Red (Ampliqon, Odense, Denmark) with the following conditions: initial denaturation at 95°C for 5 min; 36 cycles of denaturation at 95° C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s each; final extension at 72°C for 10 min (forward primer: 5′ AGA GGG CCT GTG CAA TTT GA 3' and reverse primer 3′ TCC CAC CTT TGT TGG AAG TGA 5′). PCR products were purified and *TLR4* rs4986790 genotypes were determined by Sanger sequencing.

2.3 Statistical analyses

Correlation of demographics (sex, medical history) and outcome of COVID-19 was calculated using Pearson's chi-squared statistic (χ^2) with Baptista-Pike method for odds ratio (OR) and 95% confidence interval (CI). One-way analysis of variance (ANOVA) was performed using the Kruskal-Wallis test with Dunn's multiple comparison to assess the influence of age, laboratory parameters, and pre-existing conditions on COVID-19 severity. Receiver operating characteristic (ROC) analysis and Youden's J statistic were used to calculate thresholds for laboratory values correlating with severe or fatal disease.

Hardy-Weinberg equilibrium (HWE) was calculated using Pearson's chi-squared goodness-of-fit test (χ^2), and samples were considered to deviate from HWE at a significance level of $p < 0.05$.

The number of patients with the *TLR4* rs4986790 GG genotype was small (N = 7). For this reason, we pooled the patients with *TLR4* rs4986790 AG and GG genotypes into one group.

For genetic association, we calculated OR and 95% CI by Pearson's chi-squared statistic (χ^2) using the Baptista-Pike method for OR and 95% CI, respectively. *P*-values are two-tailed and values < 0.05 were considered significant. Multivariable analysis was used to estimate the independence of age, sex, medical history, laboratory parameters, and *TLR4* rs4986790 genotype by stepwise Cox regression (likelihood ratio test, backwards).

3 Results

From March 11, 2020, to June 30, 2021, 1570 SARS-CoV-2 positive patients were enrolled and evaluated to determine the association between the rs4986790 SNP in the *TLR4* gene and the severity of COVID-19. We also analyzed IL-6 (N = 1060) and PCT (N = 1365) levels in all patients who had a laboratory determination of these parameters at the time of hospital admission. The demographic and clinical characteristics of the patients are summarized in Table 1.

With increasing disease severity the number of male ($p = 0.002$) and older ($p < 0.0001$) patients and the frequency of pre-existing conditions such as arterial hypertension ($p < 0.0001$), other cardiovascular diseases ($p < 0.0001$) and diabetes mellitus ($p = 0.001$) increased significantly.

Using ROC analysis, we estimated a threshold for IL-6 and PCT levels above which the risk of hospitalization and severe or fatal outcome of COVID-19 increased. For IL-6, levels above 18.75 pg/mL blood appear to be critical for a patient to at least be hospitalized or to have a severe or fatal outcome. For PCT, we found that patients with levels above 0.075 ng/mL blood were more likely to be hospitalized or to have a severe or fatal outcome of COVID-19. Patients with a mild or asymptomatic course in the 'mild' group had the lowest IL-6 levels (median = 8.4, N = 93, IQR = 3.7 - 21.2), which significantly increased with disease severity ($p < 0.0001$, Figure 1A), while the highest median IL-6 levels were found in the 'fatal' group (median = 102.0, N = 243, IQR = 38.1 - 226.0). We also observed a significant increase in PCT levels with disease severity ($p < 0.0001$, Figure 1B). Median PCT levels were 10-fold higher in the 'fatal' group (median = 0.36, N = 304, IQR = 0.12 - 1.37) compared to the 'mild' group (median = 0.03, N = 102, IQR = 0.02 - 0.05).

3.1 *TLR4* rs4986790 as a protective factor against severe course of COVID-19

Overall, the observed genotypes for *TLR4* rs4986790 were consistent with HWE in patients with 'mild' ($p = 0.20$), 'hospitalized' ($p = 0.16$), 'severe' ($p = 0.37$), and 'fatal' ($p = 0.17$) SARS-CoV-2 infection. The genotype distribution for all patients according to the severity of SARS-CoV-2 infection is shown in Table 2. Notably, we observed very similar rs4986790 G-allele frequencies (4.0 - 6.0%) in all groups except in patients with 'mild' SARS-CoV-2 infection (8.0%). We assessed, whether carriers of the G-allele or the GG genotype might be better protected against the need for hospitalization or against severe or fatal disease outcome. We found a significant association for protection in rs4986790 AG or GG genotype carriers comparing all patients ('hospitalized', 'severe' and 'fatal') with COVID-19 with those with 'mild' or asymptomatic SARS-CoV-2 infection (OR: 0.51, 95% CI: 0.34 - 0.77; $p = 0.001$, Table 3).

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TABLE 1 Demographics, clinical indications, and outcome of disease in SARS-CoV-2-positive patients.

Characteristics	All patients (N = 1570)	Mild (N = 205)	Hospitalized (N = 760)	Severe (N = 292)	Fatal (N = 313)	*p*-value
Age - years	62.0 (49.0 - 76.0)	47.0 (34.5 - 64.0)	61.0 (48.3 - 76.0)	59.0 (50.0 - 70.0)	71.0 (59.8 - 82.0)	< 0.0001
Male sex	910 (58.0)	107 (52.2)	416 (54.7)	185 (63.4)	202 (64.5)	0.002
Medical History						
Diseases of cardiovascular system[1]	547 (34.8)	11 (5.4)	257 (33.8)	111 (38.0)	168 (53.7)	< 0.0001
Arterial Hypertension	748 (47.6)	29 (14.1)	373 (49.1)	149 (49.1)	197 (62.9)	< 0.0001
Diabetes Mellitus	404 (25.7)	14 (6.8)	214 (28.2)	76 (26.0)	100 (31.9)	0.001
Inflammatory markers						
Interleukin-6 (high) pg/mL[2]	736 (69.4)	24 (25.8)	301 (62.3)	195 (80.9)	216 (88.5)	< 0.0001
Procalcitonin (high) ng/mL[3]	779 (57.1)	16 (15.7)	291 (42.9)	205 (73.3)	267 (87.8)	< 0.0001

Classification according to the ECDC COVID-19 surveillance report. Sex and medical history are expressed as absolute number and percentages. All other values are given as median and interquartile range (IQR). Only data that could be obtained from the patients' medical record were included in the calculation. Both pre-existing conditions and laboratory values for IL-6 and PCT were not fully documented; only values that were collected were included in the calculation. Laboratory values were taken at the time of hospital admission, which in the majority of cases corresponded to the early onset of COVID-19.

[1]e.g. myocardial infarction, coronary heart disease, but not arterial hypertension. [2]Interleukin-6 (high) refers to patients in whom the calculated threshold of 18.75 pg/mL was exceeded. [3]Procalcitonin (high) refers to patients in whom the calculated threshold of 0.075 ng/mL was exceeded. Units: pg/mL = picogram per milliliter, ng/mL = nanogram per milliliter.

In univariate analyses, we also found that age (≤ 62 years, OR: 0.34, 95% CI: 0.24 - 0.47; $p < 0.0001$), absence of pre-existing conditions [cardiovascular disease (OR: 0.16, 95% CI: 0.09 - 0.31; $p < 0.0001$), arterial hypertension (OR: 0.30, 95% CI: 0.20 - 0.46; $p < 0.0001$), diabetes mellitus (OR: 0.34, 95% CI: 0.20 - 0.61; $p < 0.0001$)], low levels of the two selected inflammatory markers IL-6 (OR: 0.13, 95% CI: 0.08 - 0.20; $p < 0.0001$) and PCT (OR: 0.12, 95% CI: 0.07 - 0.21; p < 0.0001) were significant predictors of protection against hospitalization due to SARS-CoV-2 infection or severe or fatal course of COVID-19.

To estimate the independence of the *TLR4* rs4986790 AG or GG genotype as a protective factor compared to the other predictive parameters, namely, age, pre-existing conditions, IL-6 and PCT, we performed multivariable analysis by stepwise Cox regression. We compared the 'mild' group with all others ('hospitalized', 'severe" and 'fatal') to estimate which factors are independent predictors of protection against SARS-CoV-2 infection requiring hospitalization or against COVID-19 severe or fatal outcome. We observed that absence of cardiovascular disease (OR: 0.23, 95% CI: 0.09 - 0.57; $p =$

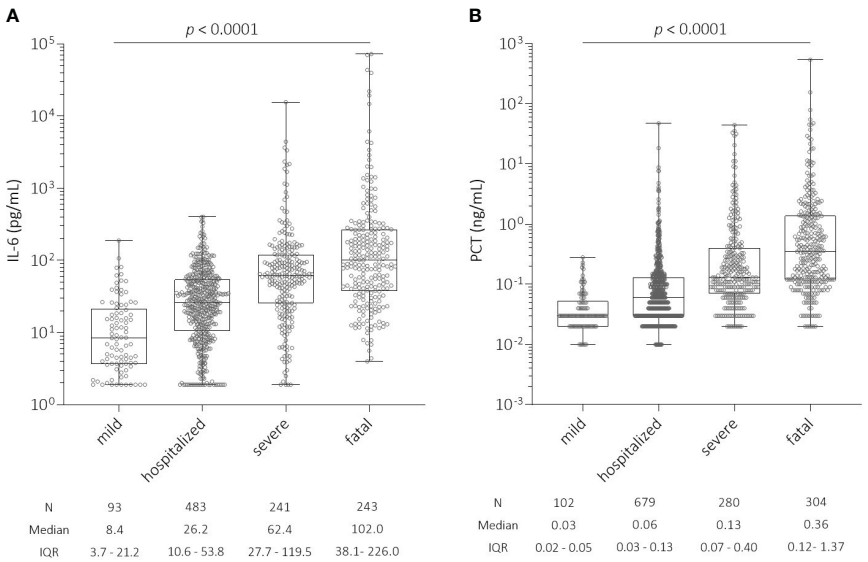

FIGURE 1
(A) Distribution of interleukin-6 (IL-6) levels (pg/mL) at the time of hospital admission among all patients with SARS-CoV-2 infection according to COVID-19 severity. There was a significant increase in IL-6 levels with COVID-19 severity ($p < 0.0001$): 'mild' (N = 93, median = 8.4, IQR = 3.7 - 21.2); 'hospitalized' (N = 483, median = 26.2, IQR = 10.6 - 53.8); 'severe' (N = 241, median = 62.4, IQR = 27.7 - 119.5); and 'fatal' (N = 243, median = 102.0, IQR = 38.1 - 226.0). **(B)** Distribution of procalcitonin (PCT) levels (ng/mL) at the time of hospital admission among all patients with SARS-CoV-2 infection according to COVID-19 severity. There was a significant increase in PCT levels with COVID-19 severity ($p < 0.0001$): 'mild' (N = 102, median = 0.03, IQR = 0.02 - 0.05); 'hospitalized' (N = 679, median = 0.06, IQR = 0.03 - 0.13); 'severe' (N = 280, median = 0.13, IQR = 0.07 - 0.40); and 'fatal' (N = 304, median = 0.36, IQR = 0.12 - 1.37). IL-6, interleukin-6; PCT, procalcitonin; IQR, interquartile range.

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TABLE 2 *TLR4* rs4986790 (c.896A>G) genotype distribution among all patients with SARS-CoV-2 infection according to the severity of COVID-19.

	All patients (N = 1570)	Mild (N = 205)	Hospitalized (N = 760)	Severe (N = 292)	Fatal (N = 313)
TLR4 rs4986790 AA	1410 (89.8 %)	171 (83.4 %)	698 (91.8 %)	258 (88.4 %)	283 (90.4 %)
TLR4 rs4986790 AG	153 (9.7 %)	34 (16.6 %)	59 (7.8 %)	32 (11.0 %)	28 (8.9 %)
TLR4 rs4986790 GG	7 (0.4 %)	0 (0.0 %)	3 (0.4 %)	2 (0.7 %)	2 (0.6 %)
minor allele frequency (G)	0.05	0.08	0.04	0.06	0.05

0.002), low IL-6 (OR: 0.21, 95% CI: 0.12 - 0.36; $p < 0.0001$) and PCT levels (OR: 0.27, 95% CI: 0.14 - 0.52; $p < 0.0001$) and *TLR4* rs4986790 AG or GG genotype (OR: 0.47, 95% CI: 0.23 - 0.96; $p = 0.039$) remained independent protective factors (Table 3).

4 Discussion

Specifically, we observed that the AG or GG genotype of rs4986790 in the *TLR4* gene was associated with protection against hospitalization, intensive care or death from COVID-19. Previously, two other groups investigated the SNP *TLR4* rs4986790 and its association with COVID-19 severity.

In contrast to our observations, Taha *et al.* found that the G-allele of rs4986790 was associated with a significantly higher risk of severe COVID-19 (N = 300, OR = 3.14, 95% CI = 2.02 - 4.88, $p < 0.001$) in a cohort of Egyptian patients (18). The authors also observed a significant increase in IL-6 levels in carriers of the rs4986790 G-allele, which we could not confirm in our study (data not shown).

The completely opposite effect observed in our study, could be due to the different allele distribution in our European (MAF = 0.05) and the Egyptian cohort (MAF = 0.18) in the study by Taha *et al.* On the other hand, we examined a much larger cohort in our study, which could also have led to differences in the observations.

A second study investigated the possible contribution of SNPs in *TLR2* and *TLR4* genes to COVID-19 disease severity and prognosis in an European population (N = 249) consisting mainly of Greek patients with SARS-CoV-2 infection (19). These authors found no significant association of *TLR4* rs4986790 with COVID-19 disease severity, although the minor allele frequency was comparable to that observed in our study (0.04 vs. 0.05). They showed a significantly increased risk of severe COVID-19 in carriers of *TLR2* rs57443708 and/or *TLR4* rs4986791 variants. Although there are discrepancies between our study and the other studies, all analyses show that polymorphisms in the Toll-like receptor gene family appear to have an important influence on the course of COVID-19. The direct functional effects of the SNPs are still largely unclear and need to be evaluated in further studies.

We confirmed that cardiovascular disease, arterial hypertension, and diabetes mellitus are associated with an increased rate of severe progression and fatality in COVID-19 patients, as observed in several other studies. In univariate analysis, we also confirmed the influence of patient age as a prognostic factor (20, 21). Furthermore, we investigated the influence of interleukin-6 and procalcitonin on the course of the disease. Our study showed an association between high levels of both IL-6 and PCT and a severe course or even death in COVID-19 patients at the time of hospital admission. Several other studies have demonstrated that the inflammatory markers IL-6 and PCT are predictors of in-hospital mortality (22–25).

TABLE 3 Protective factors against COVID-19 disease progression comparing patients with mild SARS-CoV-2 infection with patients who required hospitalization or intensive care treatment or had a fatal outcome due to COVID-19.

Factor	Univariate analysis		Multivariable analysis	
	OR [95 % CI]	*p*-value	OR [95 % CI]	*p*-value
Age (≤ 62 years)	0.34 [0.24 - 0.47]	< 0.0001	0.54 [0.30 – 1.00]	0.047
Sex (female)	0.76 [0.57 - 1.03]	0.07	NS	NS
Absence of				
Diseases of the cardiovascular system[1]	0.16 [0.09 - 0.31]	< 0.0001	0.23 [0.09 - 0.57]	0.002
Arterial Hypertension	0.30 [0.20 - 0.46]	< 0.0001	NS	NS
Diabetes mellitus	0.34 [0.20 - 0.61]	< 0.0001	NS	NS
Inflammatory markers				
Interleukin-6 (< 18.75 pg/mL)	0.13 [0.08 - 0.20]	< 0.0001	0.21 [0.12 - 0.36]	< 0.0001
Procalcitonin (< 0.0075 ng/mL)	0.12 [0.07 - 0.21]	< 0.0001	0.27 [0.14 - 0.52]	< 0.0001
***TLR4* rs4986790 AG/GG genotype**	0.51 [0.34 - 0.77]	0.001	0.47 [0.23 - 0.96]	0.039

[1]e.g. myocardial infarction, coronary heart disease, but not arterial hypotension. Units: pg/mL = picogram per milliliter, ng/mL = nanogram per milliliter. OR, odds ratio; CI, confidence interval; NS, not significant in stepwise multivariable analysis.

In this study, only patients who were not vaccinated against COVID-19 were analyzed. The extent to which the prognostic parameters identified in this or other studies play a role in vaccinated patients remains unclear. Given the current evolution of the infection process, with the appearance of more infectious yet potentially harmless variants and thus fewer severe cases, additional factors influencing the course of the disease may need to be identified. In this multivariable analysis, we were able to confirm a younger age, the absence of cardiovascular disease, the G-allele of *TRL4* rs4986790 and low levels of the inflammatory markers IL-6 and PCT upon hospital admission as independent protective factors against hospitalization, intensive care or fatal course of COVID-19. Time-independent factors that can be used to predict the course of COVID-19 are still very rare. For this reason, analysis of host genetics would be an important component that could be easily implemented in routine diagnostics.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (20-9230-BO) and was conducted in collaboration with the West German Biobank (WBE; 20-WBE-088). Written informed consent was obtained from all patients enrolled in the study. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CZ: Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft. KS: Resources, Writing – review & editing. HR: Data curation, Resources, Writing – review & editing. WS: Supervision, Validation, Writing – review & editing. BM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The *GNB3* c.825C>T (rs5443) polymorphism and protection against fatal outcome of corona virus disease 2019 (COVID-19)

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Background and aims: Albeit several factors which influence the outcome of corona virus disease (COVID-19) are already known, genetic markers which may predict the outcome of the disease in hospitalized patients are still very sparse. Thus, in this study, we aimed to analyze whether the single-nucleotide polymorphism (SNP) rs5443 in the gene *GNB3*, which was associated with higher T cell responses in previous studies, might be a suitable biomarker to predict T cell responses and the outcome of COVID-19 in a comprehensive German cohort.

Methods: We analyzed the influence of demographics, pre-existing disorders, laboratory parameters at the time of hospitalization, and *GNB3* rs5443 genotype in a comprehensive cohort (N = 1570) on the outcome of COVID-19. In a sub cohort, we analyzed SARS-CoV-2-specific T cell responses and associated *GNB3* rs5443 genotypes. We investigated the influence of all factors on COVID-19 fatality in multivariable analysis.

Results: We found a younger patient age, normotension or absence of diabetes mellitus or cardiovascular diseases, normal blood cell counts, and low inflammatory markers at hospital admission were protective factors against fatal course of disease. In addition, the rs5443 TT genotype was significantly associated with protection against COVID-19 fatality (OR: 0.60, 95% CI: 0.40–0.92, p = 0.02). We also observed significantly increased SARS-CoV-2-specific T cell responses in rs5443 TT genotype carriers (p = 0.01). Although we observed a significant association of the factors described previously in univariate analysis, only a younger age of the patients, normal blood cell counts, and the *GNB3* rs5443 TT genotype remained independent predictors against COVID-19 fatality in multivariable analysis.

Conclusion: Immutable predictors for COVID-19 fatality are relatively rare. In this study we could show that the TT genotype of the SNP rs5443 in the gene *GNB3* is associated with protection against COVID-19 fatality. It was as well correlated to higher SARS-CoV-2-specific T cell responses, which could result in a milder course of disease in those patients. Based on those observations we hereby provide a further prognostic biomarker, which might be used in routine diagnostics as a predictive factor for COVID-19 mortality already upon hospitalization.

KEYWORDS

GNB3, rs5443, genetic association, T cell response, G protein, COVID-19, SARS-CoV-2, disease severity

Introduction

Heterotrimeric guanine-binding proteins (G proteins) transmit signals from the cell surface, trigger intracellular signal cascades, and involve in a wide variety of physiological processes (Klenke et al., 2011). The gene *GNB3* encodes the G protein subunit β3 and is located on chromosome 12p13.31. The ß-subunits are not only the important regulators of the *a*-subunits of G proteins but also intracellular effectors. The synonymous single-nucleotide polymorphism (SNP) rs5443 (c.825C>T; p.S275=) in the gene *GNB3* is associated with several disorders and affects the pharmacodynamics of many different drugs (Klenke et al., 2011). The T allele of this SNP gives rise to the splice variant Gβ3-s, which lacks 123 nucleotides or 41 amino acids. Aberrant splicing results in a dominant gain of function and G protein activation (Siffert et al., 1998). We could show in previous studies that the rs5443 T allele is associated with increased chemotaxis, migration, and proliferation of B lymphoblasts, neutrophils, and T lymphocytes (Virchow et al., 1998; Virchow et al., 1999; Lindemann et al., 2001; Tummala, 2013). Lindemann et al. (2001) could show that $CD4^+$ T cell counts are increased in individuals carrying the rs5443 T allele. Therefore, it appears that individuals carrying the T allele show an increased function of their cellular immune system.

Adaptive immune responses, especially those of the T cells, are of major importance in SARS-CoV-2 infection. Virus-specific $CD4^+$ and $CD8^+$ T cells produce effector cytokines and exert cytotoxic activity in most patients with SARS-CoV-2 infection, whereas neutralizing antibodies directly interfere with viral entry of host cells (Jung and Shin, 2021). Nevertheless, patients with corona virus disease 2019 (COVID-19) not only show lower proportions of SARS-CoV-2-specific $CD4^+$ or $CD8^+$ T cells but also B cells and NK cells, with increasing disease severity (Huang et al., 2020; Peng et al., 2020; Zeng et al., 2020; Olea et al., 2021). Zeng et al. (2020) observed $CD4^+$ T cell lymphopenia in all severe and fatal cases with SARS-CoV-2 infection in their study. Furthermore, the authors could show that prolonged activation and exhaustion of $CD8^+$ T cells were associated with COVID-19 severity. In single-cell transcriptomic analyses, encompassing over 80,000 virus-reactive $CD8^+$ T single cells, Kusnadi et al. (2021) could show that SARS-CoV-2-reactive $CD8^+$ cells exhibited exhausted phenotypes with a decreased capacity to produce cytokines in severely ill COVID-19 patients.

In light of these observations, we hypothesized that the SNP rs5443 in the gene *GNB3* might influence the T cell response in COVID-19 patients as well and, thereby, the outcome of the disease. To answer this question we analyzed the SNP rs5443 in the gene *GNB3* in a comprehensive retrospective German cohort with SARS-CoV-2 infection and its influence upon T cell response and course of COVID-19.

Methods

Study participants, recruitment, and outcome of the patients

The study was conducted following the approval of the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (20-9230-BO) and in cooperation with the West German Biobank (WBE; 20-WBE-088). Written informed consent was obtained from the study patients.

Enrollment started on 11 March 2020, and ended on 18 May 2021. Altogether, 1,570 SARS-CoV-2-positive patients with at least one positive real-time reverse transcription polymerase chain reaction (RT-PCR) test result were consecutively recruited for the study. Follow-up was completed on 30 June 2021, and at that time all patients either were discharged from the hospital as "cured" or had a fatal outcome of the disease. The clinical outcome was defined as follows according to the criteria of the ECDC (European Center of Disease Prevention and Control, 2021)—"mild': outpatients (N = 205); "hospitalized": inpatients (N = 760); "severe": hospitalized patients admitted to an intensive care unit and/or became dependent on mechanical ventilation (N = 292); "fatal" all cases of COVID-19-related deaths during the hospital stay or within a follow-up of 30 days (N = 313). In contrast to the ECDC classification, where patients counted up to three times, every patient counted only once, according to the worst clinical outcome

observed during the hospital stay in our study. The patients included in this study were of Caucasian origin.

For further statistical analyses, demographic data, medical history, and hematological parameters (erythrocyte, platelet, neutrophil, and lymphocyte counts) at the time of hospital admission were documented for each patient. The medical history included pre-existing disorders of the cardiovascular system (e.g., myocardial infarction, coronary heart disease but not arterial hypertension), arterial hypertension, and diabetes mellitus.

Neutrophil–lymphocyte ratio, platelet–lymphocyte ratio, and systemic immune-inflammation index were calculated as inflammatory markers. The neutrophil-lymphocyte ratio (NLR) is calculated by dividing the number of neutrophils per nanoliter (nl) by the number of lymphocytes per nl from a peripheral blood sample. Similarly, the platelet-lymphocyte ratio (PLR) is calculated, where the number of platelets per nl is divided by the number of lymphocytes per nl in a peripheral blood sample. For the systemic immune-inflammation index (SII), the platelet counts per nl were multiplied by the number of neutrophils per nl and then divided by lymphocyte counts per nl in a peripheral blood sample.

Interferon-γ ELISpot assay

SARS-CoV-2-specific T cell responses were analyzed in 182 randomly selected SARS-CoV-2-positive patients using interferon-γ (IFN-γ) ELISpot assays as previously described (Schwarzkopf et al., 2021). Briefly, ELISpot stripes containing polyvinylidene difluoride (PVDF) membranes (MilliporeSigma™ MultiScreen™ HTS, Fisher Scientific, Schwerte, Germany) were activated with 50 µl of 35% ethanol for 10 s and washed with distilled water. Plates were then coated for 3 hours with 60 µl of monoclonal antibodies against IFN-γ (10 µg/ml of clone 1-D1K, Mabtech, Nacka, Sweden). Thereafter, ELISpot plates were washed and then blocked with 150 µl AIM-V® (Thermo Scientific, Dreireich, Germany). After 30 min at 37°C, AIM-V® was discarded, and duplicates of 250,000 peripheral blood mononuclear cells (PBMC) were grown in the presence or absence of either PepTivator® SARS-CoV-2 protein S1/S2 (600 pmol/ml, Miltenyi Biotec, Bergisch Gladbach, Germany) in 150 µl of AIM-V®. The peptide mix of the S1/S2 protein consists mainly of 15-mer sequences with 11 amino acids overlap, covering the immunodominant sequence domains of the surface glycoprotein of SARS-CoV-2. After 19 h of incubation at 37°C, the ELISpot plates were washed, and captured IFN-γ was detected by incubation for 1 hour with 50 µl of the alkaline phosphatase-conjugated monoclonal antibody against IFN-γ (clone 7-B6-1, Mabtech, Stockholm, Sweden), diluted 1:200 with PBS plus 0.5% bovine serum albumin (BSA). After further washing, 50 µl of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) was added, and purple spots appeared within 7 min. Spot numbers were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). Mean values of duplicate cell cultures were considered. We determined SARS-CoV-2-specific spots by spot increment, defined as stimulated minus non-stimulated values. Stimulated spot numbers > 3-fold higher than negative (unstimulated) controls combined with an increment value of >3 to the antigen were considered positive. Of note, the negative controls reached a mean value of less than one spot.

Genotyping of *GNB3* rs5443 (c.825C>T)

Genomic DNA was extracted from 200 µl EDTA-blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed with 2 µl genomic DNA and 30 µl *Taq* DNA-Polymerase 2x Master Mix Red (Ampliqon, Odense, Denmark), with the following conditions: initial denaturation 94°C for 3 min; 35 cycles with denaturation 94°C for 30 s, annealing at 66°C for 30 s, and elongation 72°C for 30 s each; final elongation 72°C for 10 min (forward primer: 5′ GCT GCC CAG GTC TGA TCC C 3' and reverse primer 3′ TGG GGA GGG TCC TTC CAG C 5′). PCR products were digested with *Bse*DI (Thermo Scientific, Dreireich, Germany), and restriction fragments were analyzed by agarose gel electrophoresis. The various genotype results from restriction fragment length polymorphism (RFLP)-PCR were validated by Sanger sequencing.

Statistical analyses

Correlation of demographics (sex and medical history) and outcome of COVID-19 were calculated using Pearson's chi square (χ^2) statistics using the Baptista–Pike method for the odds ratio (OR) and 95% confidence interval (CI). One-way analysis of variance (ANOVA) was performed using the Kruskal–Wallis test with Dunn's multiple comparison to assess the influence of age, hematological parameters, or inflammatory markers on COVID-19 severity. To calculate thresholds for the laboratory values, which correlate with fatal course of disease receiver operating characteristic (ROC) analysis, Youden`s J statistic was performed.

The number of patients with fatal outcome of disease, for whom IFN-γ ELISpot analyses could be performed, was relatively small. Thus, we defined additional groups to perform statistical analyses to estimate the influence of the T cell response on COVID-19 severity in our cohort. Therefore, patients from the categories "mild" and "hospitalized" were grouped together to the group "moderate," whereas the patients with "severe" and "fatal" COVID-19 were consolidated to the group "serious." The differences in T cell responses as analyzed by IFN-γ ELISpot between patients with "moderate" and "serious" COVID-19 was estimated by Mann–Whitney test.

TABLE 1 Demographics, clinical characteristics, and outcome of the disease in SARS-CoV-2-positive patients. Classification according to the COVID-19 surveillance report of the ECDC: category "mild" is a case that has not been reported as hospitalized or dead. A "severe" case has been admitted to intensive care and/or required mechanical respiratory support. All values are given in medians and interquartile ranges (IQR), except from sex and medical history, which are reported in absolute counts and percentages.

Characteristics	All patients (N = 1570)	Mild (N = 205)	Hospitalized (N = 760)	Severe (N = 292)	Fatal (N = 313)	p-value
Age–years	62.0 (49.0–76.0)	47.0 (34.5–64.0)	62.0 (48.3–76.0)	59.0 (50.0–70.0)	71.0 (59.5–82.0)	$p < 0.0001$
Male sex	910 (58.0)	107 (52.2)	416 (54.7)	185 (63.4)	202 (64.5)	$p = 0.002$
Medical history						
Cardiovascular system[a]	547 (34.8)	11 (5.4)	257 (33.8)	111 (38.0)	168 (53.7)	$p < 0.0001$
Arterial hypertension	748 (47.6)	29 (14.1)	373 (49.1)	149 (51.0)	197 (62.9)	$p < 0.0001$
Diabetes mellitus	404 (25.7)	14 (6.8)	214 (28.2)	76 (26.0)	100 (31.9)	$p = 0.001$
Hematological parameters						
Erythrocytes/nl	4.4 (3.8–4.8)	4.6 (4.2–4.9)	4.4 (4.0–4.9)	4.4 (3.8–4.8)	4.0 (3.4–4.6)	$p < 0.0001$
Platelets/nl	202.0 (156.0–260.0)	204.0 (164.0–270.5)	205.0 (157.0–255.0)	209.0 (169.0–292.0)	189.0 (135.0–242.0)	$p < 0.0001$
Neutrophils/nl	4.9 (3.1–7.5)	3.7 (2.7–5.1)	3.9 (2.6–5.8)	6.3 (4.2–9.3)	7.7 (5.2–11.7)	$p < 0.0001$
Lymphocytes/nl	0.9 (0.7–1.3)	1.1 (0.9–1.5)	1.0 (0.7–1.4)	0.8 (0.6–1.1)	0.7 (0.5–1.1)	$p < 0.0001$
Inflammatory markers						
NLR	5.0 (2.9–9.9)	3.1 (2.1–4.7)	3.8 (2.4–6.2)	7.9 (4.5–13.0)	11.1 (6.0–18.5)	$p < 0.0001$
PLR	217.8 (151.1–326.7)	176.3 (139.1–268.9)	197.7 (140.7–285.5)	269.8 (181.4–418.4)	252.6 (162.9–414.2)	$p < 0.0001$
SII	1031.0 (523.9–2206.0)	717.2 (385.7–1055.0)	769.6 (399.8–1417.0)	1680 (921.9–3466.0)	1917.0 (1010.0–4019.0)	$p < 0.0001$

[a]Cardiovascular system: for example, myocardial infarction, coronary heart disease but not arterial hypertension. Abbreviations: nl = nanoliter; NLR, neutrophil–lymphocyte ratio; PLR, platelet–lymphocyte ratio; SII, systemic immune-inflammation index.

Hardy–Weinberg equilibrium (HWE) was calculated using Pearson's chi square (χ^2) goodness of fit test, and samples were considered as deviant from HWE at a significance level of $p < 0.05$.

For genetic association, we calculated OR and 95% CI by Pearson's chi square (χ^2) statistics using the Baptista–Pike method for OR and 95% CI, respectively. p-values are reported two-sided, and values of <0.05 were considered significant. One-way analysis of variance (ANOVA) was performed using Kruskal–Wallis test with Dunn's multiple comparison test to determine the influence of *GNB3* rs5443 genotype on T cell response as measured by IFN-γ ELISpot assay.

Multivariable analysis was performed to estimate independency of the variables age, sex, medical history, laboratory parameters, and *GNB3* rs5443 genotypes by stepwise Cox regression (likelihood ratio test, backward).

Results

From 11 March 2020 to 30 June 2021, we enrolled and studied 1,570 SARS-CoV-2-positive patients to determine the association of the SNP rs5443 in the gene *GNB3*, with severity of COVID-19. In a sub group of patients (N = 182), who were representative for all severity groups, we additionally analyzed the T cell response to SARS-CoV-2-specific antigens. The demographics and clinical characteristics of the patients are summarized in Table 1. We observed that about 20% of all patients (inpatients and outpatients) and 23% of the hospitalized patients had a fatal outcome of COVID-19. With increasing severity of the disease, we found significantly more elderly and male patients and those who had arterial hypertension, cardiovascular disorders, or diabetes mellitus as pre-existing medical disorders (Table 1). The number of platelets, erythroctytes, and lymphocytes decreased significantly, whereas the neutrophil counts increased with disease severity ($p < 0.0001$, ANOVA). Regarding the inflammatory markers, NLR, PLR, and SII, we observed significantly higher values with increasing severity of COVID-19 as well.

SARS-CoV-2-specific T cell response and *GNB3* rs5443 genotype

In 182 patients, we performed IFN-γ ELISpot assays to determine T cell response to SARS-CoV-2-specific antigens. We were able to analyze patients from all severity groups: "mild" (N = 79); "hospitalized" (N = 82); "severe" (N = 17), and "fatal" (N = 4). The number of patients with fatal outcome of disease, for whom IFN-γ ELISpot analyses could be performed, was relatively small. Thus, we defined additional groups to perform statistical analyses to estimate the influence of the T cell response on COVID-19 severity in

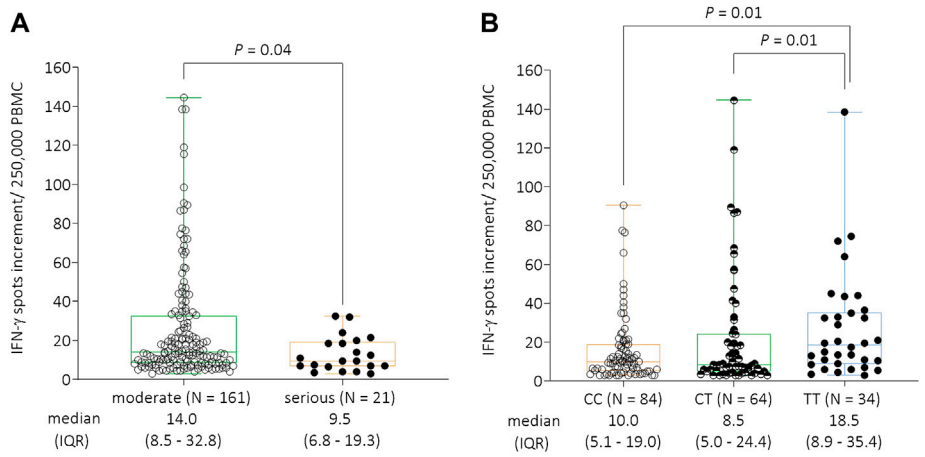

FIGURE 1
(A) IFN-γ ELISpot responses to S1/S2 protein of SARS-CoV-2 per 250,000 peripheral blood mononuclear cells stratified by COVID-19 severity. Due to the low number of cases in the individual groups, patients from the categories "mild" and "hospitalized" were grouped together to the group "moderate," whereas the patients with "severe" and "fatal" COVID-19 were consolidated to the group "serious." There was a significant decline of IFN-γ spots increment comparing the "serious" (N = 21, median = 9.5, and IQR = 6.8–19.3) to the "moderate" (N = 161, median = 13.5, and IQR = 8.0–35.0) groups (p = 0.04). **(B)** IFN-γ ELISpot responses to S1/S2 protein of SARS-CoV-2 per 250,000 peripheral blood mononuclear cells stratified by the *GNB3* rs5443 genotype. Individuals with the TT genotype had significantly higher spots increment (median = 18.5, and IQR = 8.9–35.4) compared to CT (median = 8.5, IQR = 5.0–24.4, and p = 0.01) or CC genotype carriers (median = 10.0, IQR = 5.1–19.0, and p = 0.01). Abbreviations: IFN-γ = interferon gamma; PBMC = peripheral blood mononuclear cells; IQR = interquartile range.

TABLE 2 *GNB3* rs5443 (c.825C>T) genotype distribution among all patients with SARS-CoV-2 infection and subdivided according to the severity of COVID-19.

	All patients (N = 1,570)	Mild (N = 205)	Hospitalized (N = 760)	Severe (N = 292)	Fatal (N = 313)
GNB3 rs5443 CC	700 (44.6)	89 (43.4)	330 (43.4)	121 (41.4)	160 (51.1)
GNB3 rs5443 CT	666 (42.4)	90 (43.9)	324 (42.6)	130 (44.5)	122 (39.0)
GNB3 rs5443 TT	204 (13.0)	26 (12.7)	106 (13.9)	41 (14.0)	31 (9.9)
Minor allele frequency (T)	0.34	0.35	0.35	0.36	0.29

our cohort. Therefore, patients from the categories "mild" and "hospitalized" were grouped together to the group "moderate," whereas the patients with "severe" and "fatal" COVID-19 were consolidated to the group "serious." We observed a significant decline of spots increment in the IFN-γ ELISpot assay comparing the "serious" group (N = 21, median = 9.5, and IQR = 6.8–19.3) to the "moderate" group (N = 161, median = 14.0, IQR = 8.5–32.8, p = 0.04, Figure 1A).

In a next step, we analyzed the influence of *GNB3* rs5443 genotypes on IFN-γ production against SARS-CoV-2-specific antigens. Here, we found a significant increase of IFN-γ spots increment in TT genotype carriers (median = 18.5 and IQR = 8.9–35.4) compared to those with CC genotype (median = 10.0, and IQR = 5.1–19.0) or CT genotype (median = 8.5, and IQR = 5.0–24.4) (both p = 0.01, respectively, Figure 1B).

GNB3 rs5443 as a protective factor against COVID-19 fatality

Overall, the observed genotypes for *GNB3* rs5443 were compatible with HWE in patients with "mild" (p = 0.66), "hospitalized" (p = 0.07), "severe" (p = 0.52), and "fatal" (p = 0.28) SARS-CoV-2 infection. Genotype distributions for all patients and the different groups according to severity of SARS-CoV-2 infection are shown in Table 2. Notably, we observed very similar rs5443 T allele frequencies (35%–36%) in all groups, except from those patients with a "fatal" outcome of COVID-19 (29%). Thus, we estimated, whether T allele or TT genotype carriers might be protected more effectively against fatal outcome of the disease. We found a significant association for protection against COVID-19 fatality in rs5443 TT genotype carriers comparing all patients ("mild," "hospitalized," and "severe") with SARS-CoV-2 infection

TABLE 3 Protective factors against COVID-19 fatality. Abbreviations: nl = nanoliter; NLR = neutrophil-lymphocyte ratio; PLR = platelet-lymphocyte ratio; SII = systemic immune-inflammation index; OR = odds ratio; CI = confidence interval, NS = not significant in stepwise multivariable analysis.

	Univariate analysis		Multivariable analysis	
Factor	**OR (95% CI)**	***p*-value**	**Or ([95% CI)**	***p*-value**
Age (<62 years)	0.35 (0.27–0.45)	<0.0001	0.47 [0.34–0.64)	<0.0001
Sex (female)	0.71 (0.55–0.92)	0.01	NS	NS
Absence of				
Diseases of cardiovascular system	0.41 (0.32–0.53)	<0.0001	NS	NS
Arterial hypertension	0.53 (0.41–0.68)	<0.0001	NS	NS
Diabetes mellitus	0.75 (0.57–0.98)	0.04	NS	NS
Hematological parameters				
Erythrocytes (≥4.0/nl)	0.27 (0.21–0.34)	<0.0001	0.70 (0.52–0.94)	0.02
Platelets (≥133.5/nl)	0.40 (0.29–0.54)	<0.0001	0.42 (0.30–0.60)	<0.0001
Neutrophils (≤6.6/nl)	0.28 (0.21–0.36)	<0.0001	0.32 (0.23–0.45)	<0.0001
Lymphocytes (≥0.9/nl)	0.41 (0.32–0.53)	<0.0001	0.55 (0.41–0.74)	<0.0001
Inflammatory markers				
NLR (<7.3)	0.24 (0.18–0.31)	<0.0001	NS	NS
PLR (<224.6)	0.60 (0.46–0.78)	<0.0001	NS	NS
SII (<1206.4)	0.32 (0.24–0.42)	<0.0001	NS	NS
GNB3 rs5443 TT genotype	0.60 (0.40–0.92)	0.02	0.65 (0.44–0.96)	0.03

and with those who died from COVID-19 (OR: 0.60, 95% CI: 0.40–0.92; $p = 0.02$, Table 3).

Thereupon, we performed multivariable analysis to analyze the independence of the *GNB3* rs5443 TT genotype in comparison to the other predictive parameters: age, pre-existing disorders, hematological parameters, and inflammatory markers. We performed ROC analysis and Youden`s statistic for the numeric variables to estimate a threshold above which the risk for COVID-19 fatality significantly decreased. We found that a younger patient age (<62 years; $p < 0.0001$), erythrocyte (≥4.0/nl; $p = 0.02$), platelet (≥133.5/nl; $p < 0.0001$), neutrophil (<6.6/nl; $p < 0.0001$), and lymphocyte (≥0.9/nl; $p < 0.0001$) counts above these respective thresholds at the time of admission to hospital, and the *GNB3* rs5443 TT genotype ($p = 0.03$) remained independent predictors for protection against COVID-19 fatality (Table 3).

Discussion

Remarkably, we observed that the TT genotype of the SNP rs5443 in the gene *GNB3* was associated with a higher T cell response as estimated by IFN-γ ELISpot assay in our patients. We could not find an association of *GNB3* genotype to lymphocyte or T cell counts. Thus, it seems that the increased T cell response in TT genotype carriers might be related to an increased activation of T cells. Early development of $CD8^+$ T cell responses is correlated to a more effective viral clearance and a mild course of COVID-19. Patients with severe disease display early onset of inflammation as well as delayed and relatively excessive adaptive immune response (Moss, 2022). The SNP rs5443 in the gene *GNB3* was not only correlated to higher T cell responses but also to a significantly reduced risk for COVID-19 fatality in our study in univariate and multivariable analyses.

The underlying mechanism of the influence of *GNB3* genotype on T cell response remains elusive. Juno (2014) could show that *GNB3* TT genotype carriers had a significantly lower *LAG-3* gene expression. The *LAG-3* (lymphocyte activation gene 3) gene is localized on chromosome 12 nearby to *GNB3*, nevertheless there are no SNPS in the gene *LAG-3* in high linkage disequilibrium with rs5443, which could be causative for the different gene expression in *GNB3* TT genotype carriers. LAG-3 was found to be expressed on dysfunctional or exhausted T cells in chronic viral infections and correlated with severity of the infection (Blackburn et al., 2009; Richter et al., 2010). Further studies are needed to analyze whether a reduced *LAG-3* expression is responsible for the T cell activation in *GNB3* TT genotype carriers.

We found that the comorbidities arterial hypertension, other disorders of the cardiovascular system and diabetes mellitus were associated with COVID-19 fatality in univariate analysis. This has already been extensively described in a multitude of studies and meta-analyses (Zhou et al., 2020). A variety of other factors, for example, age, sex, or laboratory parameters, have also been identified to influence the course of COVID-19 (Hobohm et al., 2022). The

infection-fatality ratio of COVID-19 significantly increases through ages 30, 60, and 90 years (COVID-19 Forecasting Team, 2022). Thus, we observed that a younger age (<62 years) was an independent protective factor against COVID-19 fatality in our study as well. Nevertheless, we could not confirm the independent influence of other consistent factors, like sex or pre-existing disorders, in a multivariable analysis.

Normal cell counts of lymphocytes and platelets upon hospital admission are associated with a significantly reduced risk for fatal outcome of COVID-19. Impaired adaptive immune responses as reflected by low counts of white blood cells together with augmented inflammation serve as a good predictor for the course of the disease (Qin et al., 2022). In our study, we noticed impaired white blood cell counts in individuals with severe COVID-19 as well. Several studies could show that the inflammatory markers NLR, PLR, and SII determined upon hospital admission are good predictive markers for in-hospital mortality (Fois et al., 2020; Wang et al., 2021; Sarkar et al., 2022). We also found a significant association for COVID-19 fatality and high NLR, PLR, and SII in the univariate analysis in our study. Nonetheless, those markers did not reach statistical significance in the multivariable analysis. Therefore, it seems even more important to find persistent markers that can predict the course of COVID-19 disease.

Together with a younger patient age, a normal white blood cell count at hospital admission, the *GNB3* rs5443 TT genotype remained an independent protective factor against COVID-19 fatality in our study. Immutable predictors are still relatively rare, thus analyses of genetic host factors might be useful in predicting severity, which could be implemented in routine diagnostics.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials; further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical Faculty of the University of Duisburg-Essen. The patients/participants provided their written informed consent to participate in this study.

Author Contributions

BM: conceptualization, resources, methodology, formal analysis, investigation, supervision, data curation, funding acquisition, project administration, visualization, writing—original draft, and writing—review and editing. KS: resources, methodology, data curation, formal analysis, validation, and writing—review, and editing. CZ, CE, HR, MK, LT, and VR: data curation, resources, and investigation. ML: validation and writing—review and editing. WS and K-HL: conceptualization, validation, supervision, and writing—review, and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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