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Incidence of cereblon protein in intensive care patients: a cross-sectional study

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Abstract. – OBJECTIVE: Sepsis remains a common cause of death. The cereblon (CRBN) protein, which is involved in important cellular processes, plays a role in sepsis. This cross-sectional study aimed to show the CRBN protein expression and its effects on patients in the intensive care unit.

PATIENTS AND METHODS: Samples were taken by deep tracheal aspiration from patients. The presence of CRBN was pathologically investigated using immunohistochemical tests and polyclonal antibodies against CRBN. The relationship between gender, sepsis, steroid, survival and the presence of CRBN was examined.

RESULTS: Respiratory and neurologic diseases were the most common reasons for admission. Acinetobacter was the most frequent microorganism. In patients with more than normal inflammatory cells, a negative correlation was observed between CRBN expression and leukocyte rate (p=0.031). In patients with CRBN, there was no correlation between steroid and mortality, APACHE/Glasgow score, hospital stay length, and ICU.

CONCLUSIONS: Although the prognosis for sepsis is better in CRBN-deficiency animals, the presence of CRBN in humans does not affect it. In our study, CRBN decreased as inflammatory cells increased in the patient's aspiration material. The response to steroids, an immunomodulator, did not change with the presence of the immunomodulator target molecule, CRBN. Therefore, using immunomodulators in the treatment of sepsis should be reconsidered.

Key Words:
Cereblon, CRBN, Critical care, Sepsis, Mortality.

Introduction

Sepsis is still a significant cause of morbidity and mortality[1]. The annual death rate is around 5.3 million[2]. It involves multiple aspects of the interaction between the infecting pathogens and the host. The host response depends on several factors and is vital in mortality[3].

Alveolar macrophages are the primary phagocytes of the body's first line of defense against harmful microbes, called the innate immune system. They absorb microorganisms and destroy them. They also produce cytokines that attract other immune cells to the lungs. Macrophages are important for modulating the antibacterial function of neutrophils. A study showed[4] that macrophages are essential for modulating the antibacterial function of neutrophils and play an essential role in sepsis.

The cereblon (CRBN) protein is involved in critical cellular processes. Multiple disorders (multiple myeloma, acute lung injury, e.g.) are associated with CRBN dysregulation[5-7]. In the heart, liver, and skeletal muscle, disrupting CRBN has increased Adenosine monophosphate (AMP)-activated protein kinase (AMPK) activity[8-10]. Thus, it slows down the inflammatory process.

CRBN is the primary target of immunomodulatory drugs, especially thalidomide derivatives. Its degradation of transcriptional proteins leads to multiple myeloma (MM) cell progression, reducing survival[11]. Thalidomide and a derivative drug called lenalidomide significantly extend patients' survival with MM[12].

CRBN has also been shown to play a role in sepsis. CRBN was protective against organ injury by decreasing inflammation in a septic animal model[13]. It is also a profibrotic regulator and might be a potential target for treating lung fibrosis[14].

Studies[11,12] have been conducted on the serological change of CRBN protein, especially in MM

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cells. However, in the English language literature, there is no immunohistochemical study of the localization and presence of CRBN expression in inflammatory cells in non-neoplastic processes. Our primary aim in this study was to demonstrate the presence of CRBN in patients with sepsis and its effect on mortality. Our second goal was to show whether the effect of immunomodulatory drugs in patients with sepsis changes with the presence of CRBN.

Patients and Methods

Patient Selection

After obtaining the Ethics Committee permissions (İzmir Democracy University Ethics Committee permission No: 2020/21-02), (Clinical trials number: NCT05083520, https://beta.clinicaltrials.gov/study/NCT05083520), the patients hospitalized in the ICU were examined. The work has been carried out in accordance with the Code of Ethics of the World Medical Association. The inclusion and exclusion criteria were determined taking into consideration the COVID-19 pandemic that started in December 2019. COVID-19 patients were excluded because, at the time, the relationship between the CRBN protein and COVID-19 was unknown.

Inclusion criteria were: age ≥18, negative COVID-19 Polymerase Chain Reaction (PCR) test, being intubated, getting permission from the patient's relatives to be included in the study, and having enough secretion to count cells by aspiration through the endotracheal tube.

The exclusion criteria were: age <18, positive COVID-19 PCR test, having sepsis cases other than pulmonary sepsis, not getting consent from patients' relatives, not having enough secretion to count cells by aspiration through the endotracheal tube, and not having enough cells in the secretion.

A total of 116 patients were followed up between September 1st, 2020, and September 1st, 2021. Forty-six patients with COVID-19 were not included in the study. Seventy patients proven non-COVID-19 with negative COVID-19 PCR tests were eligible for inclusion in the study. Four of these patients could not be included in the study because their relatives did not consent. Eight patients could not be included because sufficient pulmonary secretion material was not obtained in the aspiration performed during the intubated follow-up period. The tracheal aspiration procedure was applied to the remaining fifty-eight intubated patients. However, at the stage of microscopic examination, two patients were excluded from the study because there were no cells in the material to be examined. The study continued with 56 patients (Figure 1). Informed consent was obtained from all individual participants included in the study. The patient samples were taken and numbered solely by the intensive care physicians responsible for data collection.

Tracheal Aspiration Procedure

The tracheal aspiration procedure was performed in the first 12 hours after the patient's intubation. The patients in the semi-fowler position were oxygenated with 100% O_2 for two minutes before tracheal aspiration. Following aseptic rules, aspiration was performed through the endotracheal tube or tracheostomy cannula. Paul's Tracheal Culture Bottle was used to store secretions. The patients were ventilated with 100% O_2 for two minutes.

Storage of Samples

An equal volume of pre-prepared fixatives (a mixture of the same volume of 95% ethyl alcohol and 10% formalin) was added to the patient's secretion.

Making Cell Blocks

For preparing cell blocks, each specimen with fixative was centrifuged for 10 minutes at 2,000 rpm. The supernatant was eliminated. The cell button was resuspended in the same fixative and centrifuged at 3,000 rpm for 10 minutes. For 4-6 hours, the tube was set aside. After discarding the supernatant, the cell button was gently removed and placed in a labeled tissue cassette. The specimen was processed and implanted. Tissue sections with a thickness of 3-4 μm were cut from the cell blocks. For morphological examination, sections were stained with Hematoxylin and Eosin. Only modest numbers of lymphocytes, neutrophils, and other inflammatory cells are found in bronchial aspiration (BAL) fluid from healthy, non-smoking adults without lung disease. Alveolar macrophages make up to 90% of the cell population in the lungs. The specimens were considered adequate or unsatisfactory in this study according to specific criteria, namely insufficiency of alveolar macrophages (i.e., less than 10 alveolar macrophages/high-power field), excess of airway-derived epithelial cells (i.e., more than the presence of alveolar macrophages), a mucopurulent exudate, degeneration changes, or laboratory processing artifacts. Specimens found

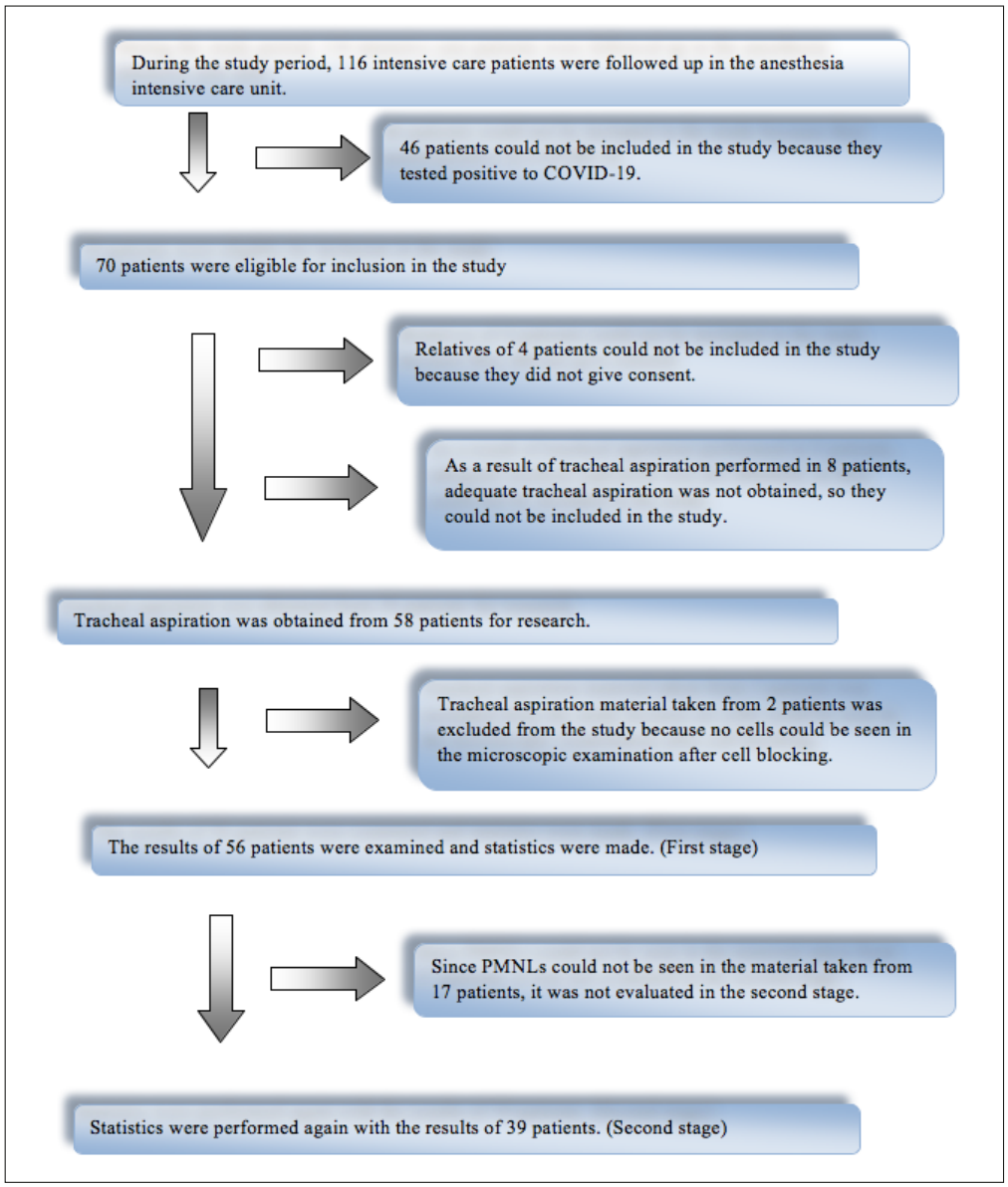

Figure 1. Flow diagram of the study.

insufficient for evaluation and/or unsatisfactory were excluded from the study[15].

Immunohistochemical (IHC) Tests

The streptavidin-biotin peroxidase method was used (Invitrogen, Carlsbad, CA, USA). Serial 4-µm sections, obtained from paraffin blocks, were treated with a microwave with a heat-induced epitope retrieval procedure. Slides were left for 20 minutes in 10 mM/L citrate buffer at pH 6.0, cooled at room temperature for 20 minutes, and blocked to retrieve endogenous peroxidase and biotin. Polyclonal antibody against CRBN (Invitrogen, PA5-38037, 1/200 dilution) was used. A brown granular or diffuse cytoplasmic and/or nuclear staining for CRBN within inflammatory cells was considered positive. The slides were reviewed by a pathologist who was blinded to patients' clinical symptoms. Staining patterns were categorized based on intensity and location.

A single pathologist took part in the study to standardize the evaluation. Our pathology professor has 35 years of professional experience. Cytoplasmic CRBN expression was examined microscopically and recorded as present/absent.

Statistical Analysis

Since there are no clinical studies on the effects of CRBN on sepsis, a power analysis was performed with preclinical studies. Yang et al[7] attempted to explore the effects of CRBN on the progression of acute lung injury (ALI) in mice. In the power analysis based on the number of CRBN knockdown mice, the number of patients required for the study to be 80% power was determined as 32. The power analysis program (G*Power 3.1.9.2, Düsseldorf, Germany) was used. Therefore, the research sample will consist of at least 45 patients diagnosed with sepsis and hospitalized in the intensive care unit. All statistical analyses were performed using SPSS (version 25.0, IBM Corp., Armonk, NY, USA). The quantitative data were recorded as mean values ± standard deviation (SD) and analyzed using the Student's *t*-test. Tukey's post hoc test was used to validate ANOVA for comparing measurement data among groups. In cases where the necessary conditions for parametric analysis were not met, Mann-Whitney U was used to compare the quantitative data of the two groups. The Chi-square or Fisher's exact test was used to compare categorical variables recorded as percentages. Differences were considered significant when the *p*-value was lower than 0.05.

Results

Considering only patients whose immunohistochemical expression of CRBN could be evaluated, the study population consisted of 56 patients, 32 males (57.1%) and 24 (42.9%) females. The mean age of the patients was 70.13±13. The average hospitalization period was 30.04±17.5 days, and the mean follow-up time in the intensive care unit was 27.5±18.3 days.

The patients in our study were categorized into specific diagnostic groups, as illustrated in Figure 2. Respiratory (COPD, pneumonia, pulmonary cancer, etc.) and neurologic diseases (cerebrovascular occlusion, hemorrhage, etc.) were the most common reasons for admission (43,1% and 22.4%). The sum of the cumulative percentage of the diagnostic groups is shown with the red line. Accordingly, most patients had respiratory and neurologic diseases (65.5%).

Forty-two of 56 patients had one or more concomitant chronic diseases. While 36 (64.29%) of our patients had sepsis at the sampling time, 20 (35.71%) did not. The most frequently grown microorganism was Acinetobacter, encountered in 18 patients.

While complete/partial cure to treatment was obtained in 9 (16.1%) patients, 47 (83.9%) patients died. Different microbiological agents were grown in the cultures of 33 (58.9%) patients in the microbiological examination of bronchoalveolar fluid. According to the antibiograms or with prophylactic aims, 51 (91.1%) patients used different types of antibiotics.

In all patients, examination of immunohistochemically stained cell block materials revealed cytoplasmic or nuclear expression of CRBN in macrophages or polymorphonuclear leucocytes (PMNLs) (Figure 3).

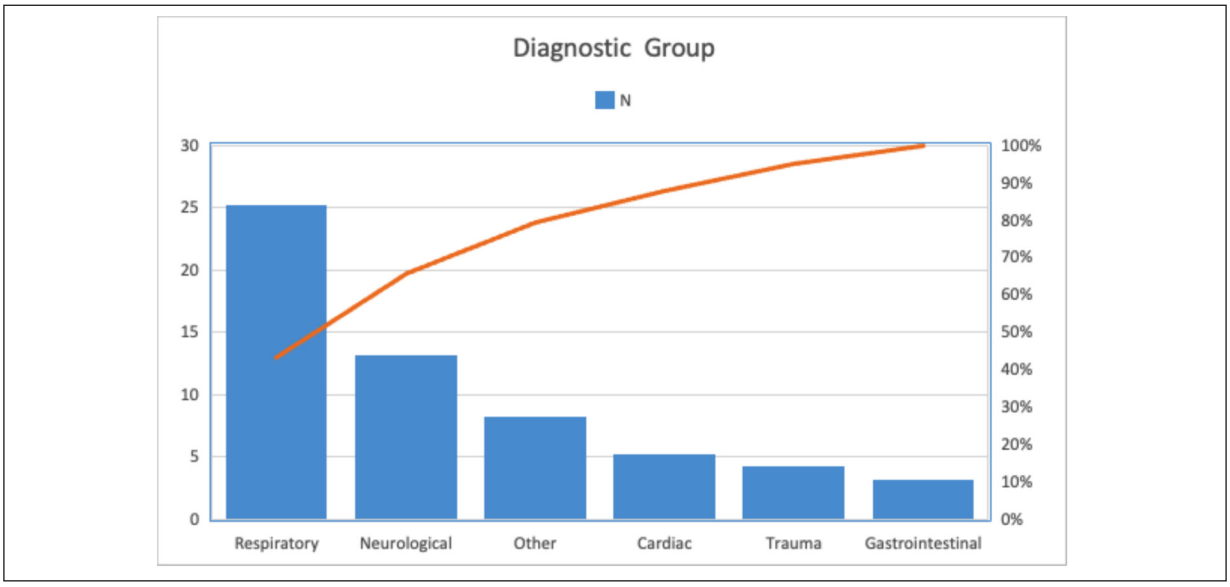

Figure 2. The diagnostic groups of patients.

Cereblon protein was detected in macrophages or neutrophils in 24 patients, but it was not detected in the other 32 patients. No statistically significant relationship was detected between the presence of CRBN expression and gender (p-value=0.876), the presence of sepsis (p-value=0.376), the use of steroids (p-value=0.322), and survival (p-value=0.598) (Table I).

Although PMNLs were present in some patients, no CRBN expression was determined in PMNLs. PMNL cells were seen in trace amounts in some patients. More than normal PMNL cells (>3% PMNL cells) were detected in the bronchial aspiration fluid (BAL) of only 39 patients. When these patients with more than normal PMNL cells were examined, CRBN expression and different clinicopathological parameters were re-evaluated. Of these 39 patients, 23 were males (59%), and 16 were females (41%). The mean age of these patients was 69.1±14 years (range 25-91 years). In these 39 cases, no statistically significant relationship was detected between the presence of CRBN expression and gender (p=0.894), the presence of sepsis (p-value=0.584), the use of steroids (p-value=0.297), and survival (p-value=0.465). On the contrary, there was a negative correlation between CRBN expression and PMNLs rate (p-value=0.031) by Chi-square analysis (Table I). Cereblon protein incidence decreased when the number of PMNLs in the tracheal aspiration material of the patients increased (Figure 4).

When these 39 patients were examined, there was no difference between CRBN expressions with age (p=0.478), Glasgow score (p=0.647), Apache score (p=0.478), and hospitalization time (p=0.627).

We investigated whether steroid, an immunomodulator, makes a difference in patients with CRBN. First, 24 patients with CRBN expression among all patients (56) were examined. The effect of steroid treatment on survival was measured. No difference was detected between the Apache Score, Glasgow Score, length of hospital and ICU stay (Table II). Afterwards, 39 patients with PMNLs in tracheal aspiration material were analyzed. Among 19 patients with CRBN expression, the effect of steroid treatment on mortality, intensive care score, and length of stay was evaluated. No significant difference was found.

Discussion

In our research, with regards to our first goal, "to demonstrate the presence of CRBN in patients with sepsis and its effect on mortality", we saw that CRBN synthesis in sepsis patients did not change mortality, intensive care scores (such as Apache, Glasgow) or length of hospital stay. When our second goal, "to show whether the effect of immunomodulatory drugs in patients with sepsis changes with the presence of CRBN", was examined, it was observed that using steroids

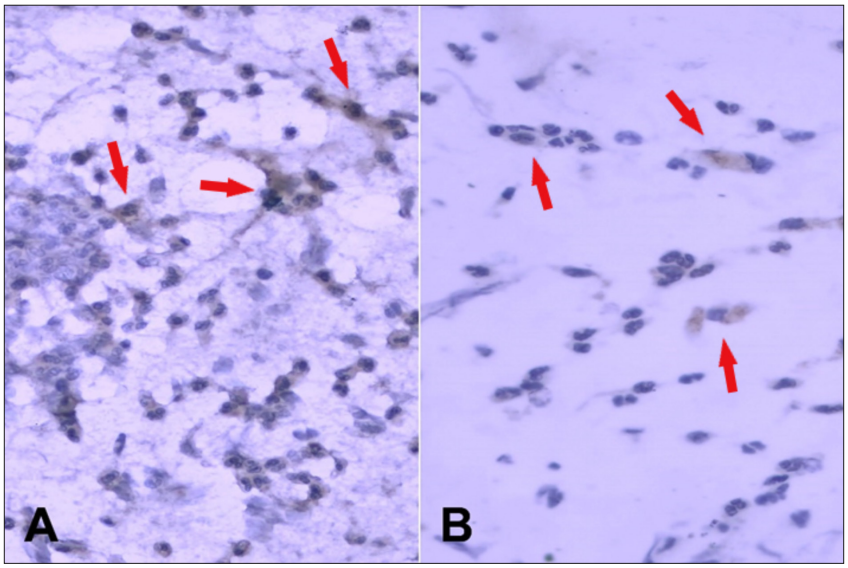

Figure 3. Histopathological evaluation reveals the differences of the CRBN expressions according to the cell types: (**A**) note the presence of CRBN expressions in both macrophages and neutrophils (DAB x 400), (**B**) note the presence of CRBN expressions in only macrophages (DAB x 400).

Table I. Relationship between the presence of CRBN and gender, sepsis, use of steroid, and survival.

Relationship between presence of CRBN and gender, sepsis, use of steroid, and survival in all patients					
		Number of patients without CRBN	Number of patients with CRBN	Total	*p*
Gender	Male	18	14	32	0.876
	Female	14	10	24	
	Total	32	24	56	
Sepsis	Absence	13	7	20	0.376
	Present	19	17	36	
	Total	32	24	56	
Use of steroid	No	20	18	38	0.322
	Yes	12	6	18	
	Total	32	24	56	
Survival	Safe	5	4	9	0.598
	Ex	27	20	47	
	Total	32	24	56	
Relationship between presence of CRBN and gender, sepsis, use of steroid, and survival in patients with high PMNL cells					
		Number of patients without CRBN	Number of patients with CRBN	Total	*p*
Gender	Male	12	11	23	0.894
	Female	8	8	16	
	Total	20	19	39	
Sepsis	Absence	8	6	14	0.584
	Present	12	13	25	
	Total	20	19	39	
Use of Steroid	No	11	13	24	0.297
	Yes	9	6	15	
	Total	20	19	39	
Survival	Safe	3	4	7	0.465
	Ex	17	15	32	
	Total	20	19	39	
Rate of PMNLs among inflammatory cells	≥51%	11	4	15	0.031*
	<50%	9	15	24	
	Total	20	19	39	

*$p<0.05$.

(an immunomodulatory drug) for all patients and patients with high CRBN synthesis, did not change mortality, intensive care scores (such as Apache, Glasgow) or length of hospital stay. In addition, we found a decrease in the neutrophil count when there was an increase in the presence of CRBN protein.

In a sepsis study conducted by Gil et al[13], the role of CRBN in polymicrobial sepsis induced by cecal ligation and puncture (CLP) was investigated. CRBN-deficient (KO) mice were used for the study[13]. Resistance to polymicrobial sepsis has been demonstrated in CRBN-deficient mice. Survival 6 days after CLP was significantly higher in KO mice (50%) compared to wild-type (WT) controls (0%). In KO mice, peripheral blood bacterial load is lower; lung damage is more minimal. Activation of AMPK and heme oxygenase-1 (HO-1) in peritoneal macrophages from WT mice was found lower. AMPK has been shown to protect against organ injury by suppressing inflammation. This study demonstrated that CRBN expression plays an attractive role in CLP-induced sepsis and peritoneal macrophage response. This creates a new approach to sepsis. In our study, a relationship between the presence of CRBN in bronchial secretion and survival could not be established. However, as PMNLs increased in the aspiration material, CRBN expression decreased. In cases with a nearly fibrinopurulent exudate and deletion PMNLs, cellular CRBN expression is lost, which means that CRBN cannot have a protective effect against organ damage.

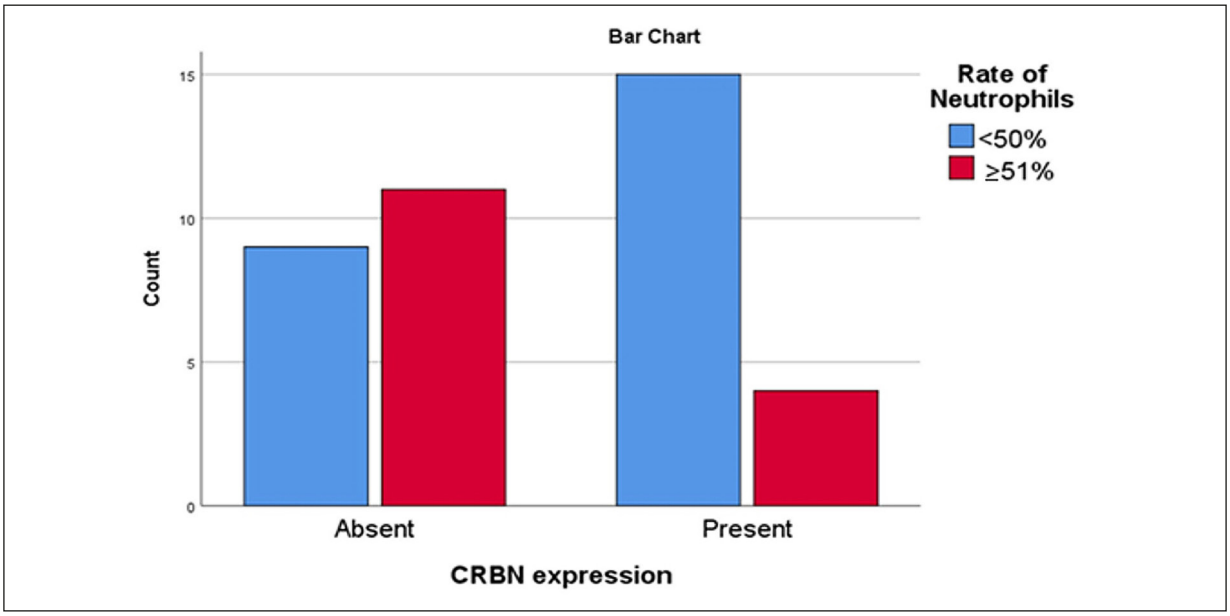

Figure 4. Negative correlation between the presence of CRBN expression and PMNLs rate (p-value=0.031) by Chi-square analysis.

Table II. The effect of steroid treatment on mortality and intensive care parameters.

The effect of steroid treatment on mortality and intensive care parameters in all patients (24) having CRBN					
	Number of patients who died	**Apache score**	**Glasgow score**	**Length of hospital stay (days)**	**Length of stay in the ICU (days)**
Treated with steroids (n: 6)	4	24.5+6.4	10.6+3.6	30.3+14.3	24+14
Did not treated with steroids (n: 18)	16	26.3±7.8	7.1+2.7	30.9+16.5	29.9+17.3
p	0.251	0.974	0.066	0.494	0.581
The effect of steroid treatment on mortality and intensive care parameters in patients (19) with high PMNLs and CRBN in tracheal aspiration					
	Number of patients who died	**Apache score**	**Glasgow score**	**Length of hospital stay (days)**	**Length of stay in the ICU (days)**
Treated with steroids (n: 6)	4	24.5+6.4	10.6+3.6	30.3+14.3	24+14
Did not treated with steroids (n: 13)	11	25.7+8.5	7.1+2.7	30.8+18.9	30.6+18.9
p	0.373	0.898	0.087	0.701	0.639

The AMPK, negatively controlled by cereblon, has previously been linked to pulmonary fibrosis. Kang et al[14] interpreted the role of CRBN in bleomycin (BLM)-induced pulmonary fibrosis in mice. In CRBN knockout (KO) mice, BLM-induced fibrosis was significantly reduced. According to this study, CRBN is a profibrotic regulator and might be used as a potential target to treat lung fibrosis.

A study examining the mechanism of acute kidney injury (AKI) in sepsis investigated the role of CRBN. Sepsis was induced by applying lipopolysaccharide (LPS) on human kidney 2 (HK2) cells. Circ_0114428 and CRBN levels were higher in septic AKI blood samples and LPS-induced HK2 cells. LPS-induced apoptosis, inflammation, oxidative stress, and ER stress were rescued

by CRBN overexpression. The Circ_0114428 knockdown attenuated LPS-induced HK2 cell injury. CRBN expression was significantly raised in serum from septic AKI patients. It was suggested that CRBN played a staminal role in kidney damage due to sepsis, in which circ_0114428 might be related to its function16. However, in our study, the presence of cereblon suppressed the increase in PMNLs. This may indicate that inflammation is suppressed. For a reason whose mechanism we do not know, we found that the presence of cereblon and the presence of inflammatory cells showed a negative correlation. In contrast to the protective effect in KO mice without cereblon in the study of Gil et al[13], we saw fibrinopurulent exudate and abundant PMNL cells in patients without cereblon in our study.

CRBN has a role in chronic inflammation-related conditions and regulates the inflammatory response. CRBN plays a nonenzymatic role in inflammation, leading to suppression of NF-kB activation and increased pro-inflammatory cytokine levels[17].

A study[18] investigating other mechanisms revealed that the transcriptional activity of the activator protein 1 (AP-1) complex is decreased, and CRBN reduced the mRNA expression and the protein levels of several pro-inflammatory cytokines. The researchers introduced a new molecular mechanism by which CRBN adjusts the inflammatory response and apoptosis. CRBN promotes or inhibits the ubiquitination of two critical molecules at different levels of the inflammatory cascade. So, the inflammatory response is suppressed. LPS is an inflammatory stimulus and can also provoke macrophages' apoptosis. Therefore, modulating the AP-1 signaling pathway can be a promising therapeutic strategy for treating inflammation-associated diseases.

It is known that CRBN is synthesized in the human retinal cell. In an experimental study, the effect of the absence of CRBN on the condition of retinitis was examined. In that study, retinitis was induced in human retinal cells by LPS. IL-6 and MCP-1 proteins are increased mediators in retinitis. IL-6 and MCP-1 protein synthesis is decreased in CRBN knockdown (KD) retinal cells19.

In an animal study[13], CRBN reduced the inflammatory response. However, in our study, the inflammatory cells decreased as the amount of CRBN in the lung secretion increased. This made us think that the inflammatory response to CRBN might be altered in genetically engineered CRBN KD animals. Other mechanisms may be at play.

CRBN is used as a target port to determine the treatment of inflammatory events and neoplastic diseases. Immunomodulatory drugs (IMiDs) are a class of compounds that can be used to attenuate the inflammatory response. IMiDs such as thalidomide and its structural analogs (lenalidomide, pomalidomide) are also used in cancer therapy, for example, multiple myeloma (MM), and myelodysplastic syndrome (MDS)[20,21].

CRBN has been identified as a mutual direct and major target of IMiDs[22]. A novel CRBN modulator, CC-885, has just been discovered. CC-885, unlike other IMiDs, has a strong anti-solid tumor effect[23]. CC-885 was found to increase the antitumor activity of Volasertib, a drug used in non-small-cell lung cancer (NSCLC). CC-885 works by selectively promoting CRBN, increasing the sensitivity of NSCLC to volasertib. It can be used in combination therapy to treat lung cancer[24].

The relationship between neoplastic diseases and CRBN has been extensively investigated in MM. It has been suggested that MM patients with high CRBN expression are sensitive to IMiD treatment and show a good clinical course. Decreased CRBN protein levels have also been reported to be specifically associated with the development of lenalidomide resistance during treatment in 77% of lenalidomide-resistant MM patients[25-27].

Western blot, immunoprecipitation, and immunohistochemistry were used to assess CRBN expression in some investigations[28,29]. Chang et al[30] used the full-length human CRBN protein as the antigen to create CRBN-specific monoclonal antibodies (mAbs). These mAbs are extremely specific. A commercial antibody developed for research purposes was used in our study, and there was no problem with negative-positive controls.

Although CRBN increased the response in cancer treatment in some studies[25-27], our study found no statistically significant relationship between CRBN expression and clinicopathological and prognostic parameters. This may be because we found lower CRBN synthesis in patients with an increased inflammatory response. We could not observe a difference in mortality since this decrease did not contribute to the effect of IMiDs and other drugs used. This situation caused us to question the use of IMiDs like steroids in patients with severe inflammation in BAL. If CRBN, the primary target of IMiDs, is low in patients, we should not use IMiDs such as steroids and should not take the risk of side effects. In addition, there is a tendency to initiate IMiDs according to the presence of CRBN in cancer patients. However, in non-neoplastic inflammatory events, there is

no practice to start treatment based on the presence of CRBN. More comprehensive studies are needed to enlighten these topics.

Since our study coincided with the pandemic period, our intensive care unit served as a corona intensive care unit during the peak periods of the epidemic. This caused us difficulty in finding non-COVID-19 cases. In addition, in this challenging period, when anesthesiologists' workload increased, patient follow-up for scientific research became difficult. Temporary assignments, difficulties in supplying materials, loss of motivation of staff, approaching every patient as a possible corona case, and cautious approach to procedures such as tracheal aspiration that cause an increase in the number of droplets were some of these difficulties.

Conclusions

This study was conducted in non-COVID-19 patients. Due to the pandemic, our intensive care unit served as COVID-19 intensive care for some periods, which caused the prolongation of the case coll ection process for the research. In this study, some of the patients were in the "sepsis" stage, some in the "severe sepsis" stage, and some in the "septic shock" stage. In future studies, examining patients with sepsis of the same severity is recommended. Although there are many publications[5,8,13] on cereblon, there are no similar studies in humans. Therefore, the hypothesis was based only on animal or cell studies. It would be helpful if cereblon could also be investigated in the blood. However, our hospital did not have the necessary materials. In our study, immunohistochemical cytoplasmic cereblon expression was categorized as present/absent by an experienced pathologist. It is recommended to perform further studies in which digital images are obtained by virtual microscopy and quantitatively graded according to the intensity of staining.

CRBN expression is not a biomarker to be used to treat IMiDs. In particular, cells expressing high amounts of CRBN are resistant to proteasome inhibitor-induced death in MM investigations, indicating that CRBN is important in chemotherapeutic treatment-induced cell death. However, the situation may not be similar in sepsis. The non-specificity of the market's most widely used polyclonal CRBN antibodies requires a reproducibly accurate method of detecting cereblon protein in inflammatory cells. In addition to developing new monoclonal CRBN antibodies and optimizing the immunohistochemical staining method, further studies are needed to determine the most appropriate treatment strategy for CRBN expression.

Availability of Data and Materials

The data supporting this article is available from the corresponding author on reasonable request.

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Conflict of Interest

The authors of this study declare that they have no competing interests.

Informed Consent

Informed consent was obtained from all patients.

Authors' Contributions

Conceptualization and design: PA; collection of the data: KA, DAY, CE; pathologic examination: GD; performing the statistical analysis: HG, GD; drafting and writing: PA, KK; designed the figure: ZS; editing: PA, GD, ZS. All authors read and approved the final version of the manuscript.

Ethics Approval

The study has complied with the principles outlined in the Declaration of Helsinki, and the Izmir Democracy University Ethics Committee approved the study protocol (2020/21-02).

Confidentiality of Data

The authors state that all data gathered in this research were only used solely for research and were kept confidential.

Clinical Trials Number

NCT05083520, https://beta.clinicaltrials.gov/study/NCT05083520.

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