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Original Article

The effect of inflammatory cytokines and the level of vitamin D on prognosis in Crimean-Congo hemorrhagic fever

Emine Parlak[1], Ayşe Ertürk[2], Yasemin Çağ[3], Engin Sebin[4], Musa Gümüşdere[4]

[1]Department of Infectious Diseases and Clinical Microbiology, Atatürk University Faculty of Medicine, Erzurum, Turkey; [2]Department of Infectious Diseases and Clinical Microbiology, Recep Tayyip Erdoğan University Faculty of Medicine, Rize, Turkey; [3]Department of Infectious Diseases and Clinical Microbiology, Lütfi Kırdar Training and Research Hospital, İstanbul, Turkey; [4]Department of Medical Biochemistry, Faculty of Medicine, Atatürk University, Erzurum, Turkey

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Abstract: Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne viral disease. Its pathogenesis basically involves endothelial damage. The aim of this study was to determine serum IL2, IL6, IL 10 and 25 OH Vitamin D levels in patients with CCHF and also to reveal their role in the clinical course and prognosis of the disease. Diagnosis of CCHF was confirmed using the positive polymerase chain reaction (PCR) test and/or positive IgM antibody by enzyme-linked immunosorbent assay (ELISA). Serum IL-2, IL-6, IL-10 and total 25 OH Vitamin D levels were also measured using ELISA. Eighty CCHF patients and 110 healthy controls were enrolled. IL2, IL6 and IL10 levels were significantly higher in the patient group. IL 6 and IL 10 levels were significantly higher in the fatal group. There was a positive correlation between Vitamin D and AST ($r=0.402$; $P<0.001$), and another positive correlation between IL-6 and CK ($r=0.714$; $P<0.001$). High IL6 and L10 levels are a significant indicator of fatality. Cytokines are only one of the factors responsible for mortality. We conclude that the pathogenesis of the disease can be better understood by elucidating the complicated cytokine network.

Keywords: Crimean-Congo hemorrhagic fever, cytokine, fatality, vitamin D Introduction

Introduction

Crimean-Congo Hemorrhagic Fever is the most widespread zoonotic infectious disease caused by ticks [1, 2]. The agent is a Nairovirus type virus from the family Bunyaviridae. The disease is endemic or sporadic in Asia, Eastern Europe, the Middle East and Africa [1, 3]. CCHF usually involves a tick bite or contact with an infected animal, infected human blood, infected organs or nosocomial transmission [1]. The most common symptoms in clinical practice are sudden fever, headache, lethargy, myalgia and dizziness [4]. Laboratory diagnosis and virus isolation include serological tests, such as the ELISA, which determines IgM and IgG, and molecular-based tests, such as reverse transcription-polymerase chain reaction (RT-PCR), which determines the viral genome [1, 2, 5]. Mortality varies depending on region and mode of transmission, and is reported at between 2.8% and 70% [6]. The fatality level in Turkey according to Ministry of Health figures is 5% [7].

Endothelial injury lies at the basis of pathogenesis of CCHF [1]. This can happen indirectly, with the virus or host factors triggered by the virus leading to endothelial activation and dysfunction, and/or through direct viral infection [8]. Microvascular injury and hemostasis compromise may occur. Uncontrolled cytokine release, the 'cytokine storm', has an important procoagulant effect [9]. Recent studies have shown that pro- and anti-inflammatory cytokines play an important role in the pathogenesis of CCHF [10, 11]. Cytokine levels (IL2, IL6, IL10, IL12, tumour necrosis factor-alpha (TNF-α) and interferon alpha) in patients with CCHF have been investigated in several studies. Increases in cytokine levels have been found to be directly correlated with increase viral load, increased mortality and severity of disease [4,

10-13]. Endothelial damage characteristically contributes to eruptions and hemostatic defects [14]. Significantly higher IL-6 and TNF-α levels have been reported in non-fatal cases compared to fatal cases [10, 11]. Cytokines are one of the factors responsible for mortality [1, 7, 10-12, 14, 15].

Vitamin D is essential for bone growth and for calcium and phosphorus metabolism [16]. It also acts as a hormone, playing a key role in preventing some cancers, autoimmune diseases and some infectious diseases, such as tuberculosis. Vitamin D deficiency is a major health problem in society [17]. The vitamin is an important component of the innate immune system [16]. Active Vitamin D permits the release of antimicrobial peptides (cathelicidin, defensin) from monocytes, neutrophils and epithelial cells. Recent studies have reported a correlation between Vitamin D deficiency and an increased incidence of some infectious diseases [18]. Vitamin D deficiency has been associated with decreased muscle strength, colon cancer, cardiovascular diseases, autoimmune diseases, rheumatoid arthritis and systemic lupus erythematosus [17]. The effects of Vitamin D on clinical outcome and prognosis in CCHF have not previously been investigated. The purpose of this study is to measure cytokine and Vitamin D levels and to investigate their effect on disease severity and pathogenesis.

Material and methods

Patients and methods

Patients with confirmed diagnosis were included. Eighty adult patients hospitalized and treated with a diagnosis of CCHF at the Atatürk University Infectious Diseases Clinic, Turkey between April 2012 and September 2013 were enrolled. Patients were classified according to defined criteria of severity [8, 19]. Diagnosis of CCHF was confirmed by positive RT-PCR test and/or positive IgM antibody by enzyme linked immunosorbent assay (ELISA). The research is a prospective case control study. A healthy control group was constituted from 100 individuals selected from male and female volunteers with the same characteristics. Control group serum levels were measured only once. University ethical committee approval was granted for the study. Consent was received from all patients and controls. Patients' clinical and laboratory characteristics were recorded prospectively onto forms. Patients and healthy controls were non-smokers, and did not consume alcohol or use drugs. Subjects with additional disease (heart disease, renal failure, malignity) were excluded. Two blood samples were collected in order to obtain serum. These were centrifuged for 5 min at 2000 rpm after standing for 30 min, and the serum obtained was placed into Eppendorf tubes. Patient and healthy control group serum were stored at -70°C. The other tube was sent to the Erzurum Regional Institute of Hygiene for diagnostic confirmation.

Biochemical and hematological parameters

Serum AST, ALT, creatine phosphokinase (CK), and lactate dehydrogenase (LDH) levels were measured using original kits (Roche Diagnostics, Mannheim, Germany). Biochemical measurements were determined by standard laboratory methods. Hemogram parameters were determined the Beckman Coulter LH 780 (Beckman Coulter Ireland Inc. Mervue, Galway, Ireland) device in the laboratory. Hemogram parameters standard biochemical techniques were studied in kits (LH 780, USA). Prothrombin time (PT-INR) and activated partial thromboplastin time (aPTT) were analyzed in the ACL Top 700® (Instrumentation Laboratory, Bedford, MA, USA).

Serum IL-2, IL-6, IL-10 and vitamin D level measurement

Blood samples were taken from all the participants. Each collected blood sample was immediately centrifuged for 10 min at 4000 rpm and +4°C. Resulting sera were aliquoted into Eppendorf tubes. Tubes were kept at -70°C in deep freeze until they were analyzed. Serum IL-2, IL-6, IL-10 and total 25-hydroxyvitamin D (25-OH Vitamin D) (D2 and D3) levels were measured using ELISA methods according to the manufacturer's instructions (DiaSource Inc., Nivelle, Belgium). Detection limits of IL-2, IL-6, IL-10 and 25-OH Vitamin D measurements were 0.05 U/mL, 2 pg/mL, 1.6 pg/mL and 1.5 ng/mL respectively. The Results were below detection limits.

Statistical analysis

All data were recorded onto IBM SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) for statistical analysis. Pearson's chi square test

Table 1. Comparison between non-fatal and fatal CCHF patients laboratory values

	Fatal (mean ± SD)	Nonfatal (mean ± SD)	P
WBC (×10^9/L)	5400.00±5703.069	2446.67±1734.650	0.004
Platelets (×10^9/L)	29400.00±15339.492	57226.67±42275.024	0.009
AST (IU/L)	1850.80±1776.804	301.19±307.945	0.001
ALT (IU/L)	872.20±898.221	206.52±391.489	0.001
CK (IU/L)	2402.00±3015.181	711.04±844.807	0.001
LDH (IU/L)	5585.20±7473.921	779.45±1160.250	0.000
PT (sec)	13.1800±3.75659	11.6080±1.98588	0.111
aPTT (sec)	45.3400±14.22913	36.1507±8.37932	0.026
INR	1.3740±0.35620	1.0872±0.19576	0.004

CCHF: Crimean-Congo hemorrhagic fever; WBC: white blood cell counts; PT: prothrombin time; aPTT: activated partial thromboplastin time; INR: international normalized ratio.

Table 2. Comparison of severe and mild/moderate cases laboratory values

	Mild/moderate cases	Severe cases	P
WBC (×10^9/L)	2223.21±1066.002	3583.33±3612.017	0.012
Platelet (×10^9/L)	69696.43±41509.657	22333.33±13779.842	0.001
AST	194.02±184.610	874.08±961.371	0.001
ALT	122.55±123.173	541.13±747.984	0.001
CK	601.14±761.468	1319.75±1642.054	0.05
LDH	476.29±203.467	2488.04±3962.020	0.001
PT	11.2929±1.63883	12.6708±2.79945	0.007
PTT	34.0036±5.49489	43.0750±12.08363	0.001
İNR	1.0588±017154	1.2133±0.27258	0.003

was used to compare categoric variables. Significance of constant variables was analyzed using the t test. Student's t test, Fischer's exact test, forward stepwise analysis and correlation analysis were used at statistical analysis.

Results

Eighty confirmed cases of CCHF and 110 healthy controls were enrolled in the study. Mean age of patients was 49.5±17.1, and 44.8±16.1 in the control group. Mean age was 66.60±12.75 in the non-surviving patients and 48.35±16.85 in the surviving patients. The non-surviving patients were of more advanced age (P=0.02).

Length of hospitalization was 10.29±7.89 days in the severe cases and 6.77±2.04 days in the mild-moderate cases (P=0.041). No significant difference was determined between the patient and control groups in terms of age or gender. Males constituted 52.6% of the patients and females 47.4%; 37.5% of patients were housewives, 28.8% were engaged in animal husbandry and 25% in agriculture. Contact with ticks was present in 65%. Based on severity criteria, 24 (30%) patients were in the severe group and 56 (70%) in the mild-moderate group. Seventy-five (93.75%) patients were discharged in a healthy condition, and 5 (6.25%) died. The most common presentation symptoms were lethargy (98.8%), lack of appetite (91.3%), fever (87.5%) and widespread body pain (82.5%). Physical examination findings included hepatomegaly (63.8%), splenomegaly (46.3%) and hyperemia (42.5%). Hemorrhage was seen in 25% of patients from different regions.

Laboratory parameters at comparison of the fatal and nonfatal patients are shown in **Table 1**. Laboratory parameters at comparison of the severe and mild-moderate groups are shown in **Table 2**. Patient and control group IL2, IL6, IL10 and Vitamin D levels are shown in **Table 3**. IL2, IL6 and IL10 values were significantly higher in the patient group compared to the control group. Severe and mild/moderate cases IL2, IL6, IL10 and Vitamin D levels are shown in **Table 4**. IL10 levels were significantly higher in the severe group than in the mild-moderate group. IL6 and IL10 levels were significantly higher in the fatal group compared to the surviving group. IL10 was significantly higher in patients receiving blood products, while no difference was determined between IL2, IL6 or Vitamin D levels. The results of the correlation analysis performed for each result are shown in **Table 5**. A comparison of significant variables affecting disease severity is shown in **Table 6**.

Mean serum cytokine concentrations were determined in fatal, nonfatal and total cases

Table 3. Patient and control group IL2, IL6, IL10 and Vitamin D levels

Tests	Patients	Control group	*P* value
IL2 U/ml	1.36±0.28	1.17±0.39	0.000
IL6 pg/ml	172.26±619.60	24.1336±43.04965	0.013
IL10 pg/ml	89.62±152.39	2.19±2.78	0.000
Vit D ng/ml	25.74±26.62	19.78±21.45	0.09

IL2; 0.05 U/ml, IL6; 2 pg/ml, IL10; 1.6 pg/ml, Vit D; 1.5 ng/ml sınır değerleri.

Table 4. Severe and mild/moderate cases IL2, IL6, IL10 and Vitamin D levels

Tests	Severe cases	Mild/moderate cases	*P* value
IL2	1.38±0.44	1.36±0.18	0.286
IL6	349.02±961.81	96.52±380.91	0.009
IL10	152.87±219.57	62.52±103.44	0.01
Vit D	30.45±30.25	23.72±24.92	0.158

Table 5. Fatal and nonfatal group IL2, IL6, IL10 and Vitamin D levels

Tests	Fatal	Nonfatal	*P* value
IL2	1.31±0.16	1.36±0.29	0.68
IL6	1259.07±1969.49	99.81±339.18	0.001
IL10	244.12±305.26	79.32±134.29	0.01
Vit D	45.77±51.21	24.40±24.20	0.08

Table 6. Comparison of significant variables affecting disease severity

	P	Odds ratio	95% confidence interval
Hemorrhage	0.002	7.958	2.12-29.78
Altered consciousness	0.015	0.098	0.01-0.64
Hepatomegaly	0.034	5.396	1.13-25.59

(**Figure 1**). There was a positive correlation between IL-6 and CK (r=0.714; P<0.001) **Figure 2** and Vitamin D and AST (r=0.402; P<0.001) **Figure 3**. IL6 was positively correlated with AST, ALT, CK, WBC and LDH. IL 10 was negatively correlated with platelet count (r=0.285; P=0.01) and positively correlated with CK (r=0.256; P=0.02).

Binary logistic regression analysis was performed to determine the factors affecting severity of disease. Categoric variables identified as significant at two-way comparisons (hepatomegaly, splenomegaly, cough, altered consciousness and bleeding) were included in the model. Forward stepwise analysis was performed. Sensitivity of the model was 62.5%, specificity 91.1% and general predictive level 82.5%. The Nagelkerke R square value was 0.474. Of the variables examined, the presence of bleeding increased disease severity 7.9-fold (P=0.002) at a 95% confidence interval [2.12-29.78]. PCR and/or ELISA tests for CCHF were positive in all patients.

Discussion

Crieman-Congo hemorrhagic fever is a tick borne zoonotic infection characterized by fever, trombocytopenia and hemorrhage [19]. The pathogenesis of CCHF is still unclear [15]. The principle targets in CCHFV are mononuclear cells, hepatocytes and the endothelium [1, 7]. The most important stage in the pathogenesis of CCHF is the involvement of the endothelium and endothelial damage has been shown to develop under the effect of inflammatory factors released against the virus, rather than from a direct effect of the virus [1, 4, 9, 10, 20]. Viral spread leads to inflammation, particularly in mononuclear cells and neutrophils in tissue and organs. Systemic inflammatory response syndrome (SIRS) may develop with the activation of macrophages and endothelial cells [10]. Shock, intraabdominal hemorrhage, cerebral hemorrhage, severe anemia, dehydration, myocardial infarct, pulmonary edema and pleural effusion are seen in patients that die from the disease [21].

Endothelial damage leads to hemostatic deficiency by activating the intrinsic coagulation cascade through thrombocyte adhesion, aggregation and degranulation. This results in intravascular coagulation (DIC) and widespread hemorrhage. DIC is a condition in CCHF resulting from excess consumption in plasma of coagulation factors [22].

Virus-related hemophagocytic lenfohistiositozis is frequently seen in CCHF [1]. Dilber et al. [3] determined findings compatible with hemophagocytosis in approximately 30% out of 21 pediatric patients. One study from Turkey determined reactive hemophagocytosis and histio-

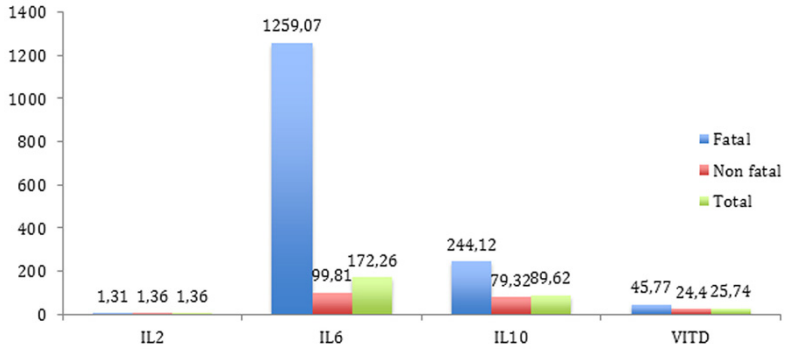

Figure 1. Mean serum cytokine concentrations in fatal, nonfatal and total cases.

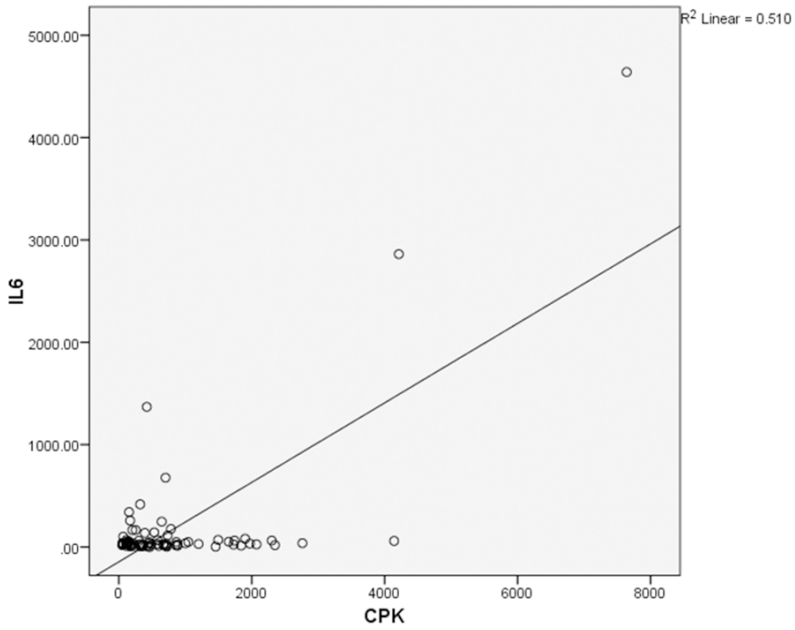

Figure 2. Correlation between IL-6 and creatine phosphokinase. r=0.714; P<0.001.

cytosis proliferation in 7 (50%) out of 14 patients [23]. These studies suggest that hemophagocytosis may play a role in cytopenia observed during CCHF infection.

The most important factor in recovery from CCHF is the immune system. Weak or no antibody response, high viral load titers in circulation and elevated serum cytokine levels are present in fatal cases. A correlation has been shown between antibody response and survival. Inflammatory mediators play an important role in fatal cases [24].

Hemorrhage, the major symptom, indicates increased vascular permeability and endothelial dysfunction [9]. Proinflammatory cytokine induction, endothelial injury, leukocyte adhesion, platelet aggregation and degranulation can be activated with the intrinsic coagulation cascade.

IL2 receptor (IL2 r) can be used as a marker of T lymphocyte activation, because it is released by activated T lymphocytes. Very high levels of IL2 r have been determined in chronic active hepatitis B and acute liver failure. Increased IL2 r levels are regarded as an indicator of probable disease severity in hepatitis C. One study comparing cases of severe and non-severe CCHF with each other and with controls reported significant elevation of IL2 and endothelin-1. These parameters were suggested to be correlated with severity of disease and prognosis in children [26]. Although IL2 levels were higher in patients, no significant elevation was determined. This may be because soluble IL2 r was not investigated in this study. Temporary inhibition of proinflammatory cytokines and activation of anti-inflammatory cytokines can be seen in fatal cases of CCHF.

High serums TNF-α levels in particular have been reported to be associated with severe disease in yellow fever [25]. TNF-α elevation occurs in severe cases and IL6 elevation in severe and mild cases. Papa et al. reported that TNF-α levels were correlated with severity of disease, and that IL6 levels were significant in severe and mild cases. IL6 and TNF-α levels were elevated in the single fatal case in the series [11]. One study involving 3 fatal and 27 non-fatal cases reported higher IL6 and TNF-α levels in the fatal group. Increased IL6 and

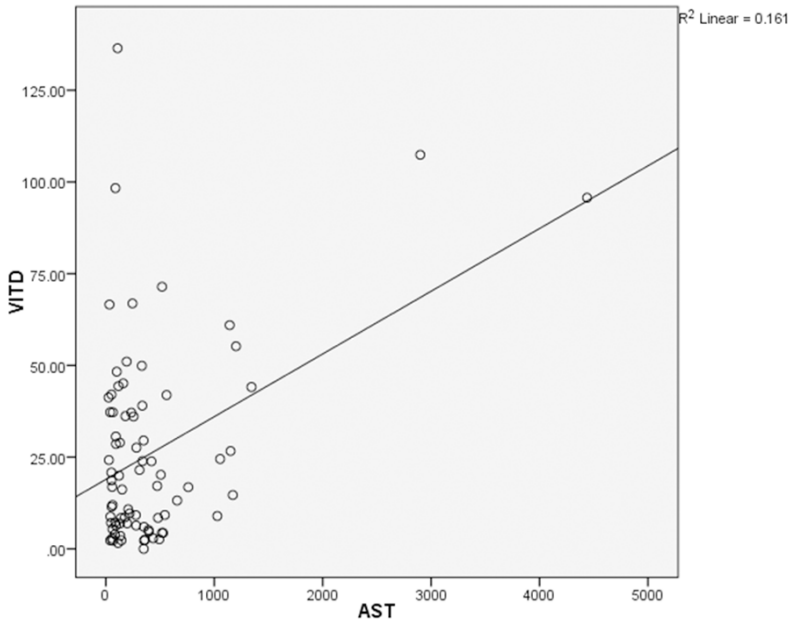

Figure 3. Correlation between vitamin D and aspartate aminotransferase. r=0.402; P<0.001.

TNF-α levels exhibited a parallel effect with DIC score [10]. IL6 and TNF-α levels rose significantly in the fatal group [10, 11]. Kaya et al. associated a high viral load, absence of CCHF specific antibody and IL 6 and TNF-α elevation with fatal outcome [7]. However, some studies have reported no difference in terms of IL 6 levels between fatal and non-fatal cases [14, 15]. Sancakdar et al. [9] reported that IL6 and TNF-α levels did not differ significantly between severe and non-severe groups in child patients, but that severe group values were significantly higher than those in healthy controls. In our study, we determined significantly elevated IL 6 levels in patients and in fatal cases. We think that IL6 response, which was significantly elevated in patients and fatal cases, can play a significant role in determining the severity of CCHF infection.

Humoral immunity (Th2) deriving from IL10 activation is more important for endothelial injury in the pathogenesis of CCHF [9, 14]. IL10 activity is known to have a negative effect in this process, in which IL12 plays a significant role in Th1 immune response activation. Watson et al. determined a correlation between the IL12/IL10 ratio and viral load [20]. IL10 was not correlated with mortality in a study from Turkey. However, a negative correlation was determined with DIC score [10]. Saksida et al. [14] associated high levels of IL10, INF gamma and TNF-α with poor prognosis. A significant positive correlation was reported between cytokine levels and viral load. Elevated IL-10, TNF-α and interferon-gamma levels were shown in fatal cases [14]. Statistically significantly higher levels of cytokines such as IL10, IL12 and TNF-α have been reported in non-surviving patients compared to surviving patients [27]. IL 10 levels were higher in the fatal group in another study from Albania [11]. In another study, IL10 levels were significantly higher in the severe group compared to the non-severe group, and significantly higher in the non-severe group compared to the controls [9]. In a study of 54 pediatric cases of CCHF, Sancakdar et al. [9] compared severe and non-severe goups with healthy controls and reported significantly IL10 and endothelin 1 in the severe group. A study involving 87 patients infected with the Ebola virus reported IL10 elevation in fatal cases [28]. In our study, too, elevated levels of IL10 clearly play a role in determining severity of disease in the pathogenesis of CCHF.

Ertürk et al. [29] showed high neutrophil gelatinase-associated lipocalin serum levels in CCHF. This molecule has been shown to be a good prognostic factor in adult patients. Increases in sVCAM-1 and ICAM-1 have been identified as a significant marker of CCHF infection [4]. Insufficient antibody response has been reported in patients dying due to CCHF. Inflammatory mediators play a significant role in fatal cases [30].

Vitamin D plays an immunomodulator role in the innate and adaptive immune systems [17, 18]. Low Vitamin D levels have been shown to be associated with upper respiratory tract and enteric infections, pneumonia, otitis media, Clostridium infections, vaginosis, urinary tract infections, sepsis, influenza, Dengue fever, hepatitis B, hepatitis C and HIV infections [16]. The fight against some infections may be asso-

ciated with suppression of proinflammatory cytokines (IL12, TNF-α and interferon-gamma). Vitamin D causes a decrease in some cytokines while suppressing mononuclear cell and T lymphocyte cell proliferation [17]. The immunoregulatory and gene expression-regulating effects in pathogenesis are known in Dengue virus infection. The relation of Vitamin D with severity of Dengue fever has been investigated by altering TNF-α and IL10 levels. Vitamin D prevents Dengue fever growth by causing changes through cytokines [31]. The question of future support therapy may arise in patients with low Vitamin D levels [32, 33]. No difference was determined in our study between fatal-nonfatal cases and patients and the control group. This was attributed to the fact that the groups were not distributed homogenously and that the Vitamin D deficiency is a common occurrence in people dwelling in this region.

Ozsürekçi et al. [15] reported no difference in terms of cytokines between children and adults in the context of disease severity. They concluded that a mild course in children cannot be explained in terms of cytokines, but may be associated with the immune system. Early interferon synthesis was found to be responsible for host protection, while later interferon response was associated with a severe course [15, 34]. However, in their study of 25 children Tezer et al. [35] reported that low cytokine levels were good prognostic criteria for a good outcome. Severity/fatality criteria differ between adults and children [9]. There may be differences in terms of genetic variation, immunological response and time of cytokine response.

The limitations of this study are: that the three apoptotic pathways were not analyzed, viral load was not investigated, the low numbers of patients and fatal cases included variation in numbers of days of hospitalization, and that cytokines were not investigated based on stages of the disease.

In conclusion, cytokines play a key role in the pathogenesis of CCHF and disease progression. Cytokine circulation may be affected by genetic differences among patients and viral load. These results suggest that IL2, IL6 and IL10 levels may be a good predictor of disease prognosis. Cytokines also play the main role in fatality in CCHF. When all variables (cellular immunity and humoral immunity markers, apoptotic pathways and clinical parameters) are analyzed together, it may be possible to understand why the disease follows a severe course in some individuals and a mild or moderate course in others. The most effective approach will be the entry into use of a safe and effective vaccine.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Emine Parlak, Department of Infectious Diseases and Clinical Microbiology, School of Medicine, Atatürk University, Erzurum, Turkey. Tel: +90 4422317428; Fax: +90 4423160777; E-mail: eparlak1@yahoo.com

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