M. Sc. Thesis

by

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A Thesis Submitted to the

Graduate School of Health Sciences

in

Partial Fullfilment of the Requirements for

the Degree of

Master of Science

in

Medical Microbiology

Koc University

August 29, 2014

Koc University

Graduate School of Health Sciences

This is to certify that I have examined this copy of a master's

thesis by

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and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

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THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF CRIMEAN-CONGO HEMORRHAGIC FEVER (CCHF)

ABSTRACT

Background:

Crimean-Congo hemorrhagic fever (CCHF) is caused by infection with a tick-borne virus *(Nairovirus)* in the family *Bunyaviridae*. The disease was described in Asia, Africa and Europe. Pathogenesis of the CCHF is poorly understood, but the mechanism is similar to the other viral hemorrhagic fevers and the dramatic outcome of the disease cannot be directly associated with the virus induced host factors or the cytokine storm caused by the viremia. In this study, we aimed to show the role of cytokines in the pathogenesis of the CCHF by studying the serum samples at the different stages of the disease.

Methods:

A total of 48 laboratory confirmed CCHF patients, who were hospitalized in Ankara Numune Training and Research Hospital were included to the study. Disease severity of the patients was evaluated at the time of the admisson, according to the severity scoring index (SSI). Twelve (25%) of the patients were in mild group (SSI 0-2), 27 (54%) in moderate group (SSI 3-9), and 9 (21%) patients in severe group (SSI > 9 or fatal cases). Disease course was divided into four groups by starting from onset of symptoms; 0-3 days (stage I), 4-7 days (stage II), 8-11 days (stage III), and 12-15 days (stage IV). Sera from all the patients were obtained on admission. Then, consequent serum samples of the selected patients in different severity groups were examined for determination of cytokine concentrations in different stages of the disease. Approximately 3-4 serum samples from each patient, total number of 112 serum samples were analyzed. All the samples were tested for interleukin (IL)-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), interferon gamma (IFN-γ) monocyte chemoattractive protein (MCP-1), macrophage inflammatory protein (MIP-1β), and TNF-α by using the Bio-Plex Pro^{TM} human cytokine 17-plex immunoassay system (Bio-rad Laboratories, USA)

Results:

The mean age of all the patients was 51 and there was no difference between the severity groups in terms of age distribution. Approximately half of the patients were male in all severity groups. Fever and headache were the most common symptoms. Disorders related with hemorrhage such as petechia (p=0.03), ecchymosis (p=0.04), melena (p=0.001) were more common in severe cases when compared with other groups. On admission, IL-13 concentration was found to be higher in severe patients (p=0.02). IL-10, TNF-α and IL-6 levels did not show any significant increase (p>0.05). MIP-1β concentrations were found to be significantly higher in mild cases than moderate or severe cases (p=0.02). Also, IL-7 concentrations were detected significantly higher in mild cases that in moderate and severe cases (p=0.04). MIP-1β concentrations in the disease stage of II (p=0.009) and III (p=0.034) were detected significantly higher in mild cases than moderate and severe cases. In the stage III of the disease, in severe cases, we observed significant increase of IL-10 levels than mild and moderate cases (p=0.04). Also, IL-7 and IL-13 were found be higher in severe cases in stages III and II respectively (p=0.02, p=0.04).

Conclusion:

The reports of the previous studies were inconsistent in terms of cytokine levels and disease severity. Because, previous studies did not account the time point of obtained serum sample. By this study we performed an analysis by considering the time point of serum sample, and adjusted the data according to the days from onset of symptoms. MIP-1β could be considered a good prognosis factor in the disease pathogenesis.

KIRIM-KONGO KANAMALI ATEŞİ (KKKA) HASTALIĞININ PATOGENEZİNDE SİTOKİNLERİN ROLÜ

ÖZETÇE

Geçmiş:

Kırım-Kongo Kanamalı Ateşi (KKKA) keneler tarafından taşınan, *Bunyaviridae* ailesine bağlı *Nairovirus* cinsinden bir virüsün neden olduğu infeksiyondur. Asya, Afrika ve Avrupa'da tanımlanmış olan bu hastalığın patogenezi tam olarak anlaşılamamakla birlikte, diğer viral kanamalı ateşlerin mekanizmalarıyla benzerlik gösterir. Hastalığın semptomları, virüsün indüklediği konak faktörleri ya da vireminin sebep olduğu sitokin fırtınası ile direkt olarak ilişkilendirilememiştir. Bu çalışmada, hastalığın farklı dönemlerinde alınan serumların analizi ile sitokinlerin KKKA hastalığının patogenezindeki rolünün gösterilmesi hedeflenmiştir.

Yöntemler:

Ankara Numune Eğitim ve Araştırma Hastanesi'ne başvuran, laboratuvar testleri ile hastalığı doğrulanmış 48 KKKA hastası çalışmaya alınmıştır. Hastalığın derecesi, hastaların hastaneye yatış anında ciddiyet skor indeksi (CSİ)' ne göre belirlenmiştir. On iki (25%) hasta hafif (CSİ 0-2), 27 (54%) hasta orta (CSİ 3-9) ve 9 (21%) hasta ciddi (CSİ > 9 ya da ex vakalar) olarak değerlendirilmiştir. Hastalığın seyri, hastalık başlangıcından itibaren 0-3 gün (dönem I), 4-7 gün (dönem II), 8-11 gün (dönem III) ve 12-15 gün (dönem IV) olmak üzere dörde ayrılmıştır. Tüm hastalardan hastaneye ilk yatış günlerinde serum örneği alınmıştır. Çalışmada, hastalık şiddeti farklı olarak seçilmiş hastalardan alınmış ardışık serum örnekleri ile çalışılarak, hastalığın farklı dönemlerindeki sitokin konsantrasyonlarının analizi yapılmıştır. Her hastadan yaklaşık 3-4 örnek olacak şekilde toplam 112 tane serum örneği çalışmaya dahil edilmiştir. Tüm serum örnekleri interlökin (IL)-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, granülosit koloni stimülan faktör (G-CSF), granülosit makrofaj koloni stimülan faktör (GM-CSF), interferon gama (IFN-γ), monosit kemotaktik protein (MCP-1), makrofaj inflamatuvar protein (MIP-1β) ve tümör nekroz faktorü (TNF-α) için Bio-Plex Pro™ immunoassay sistemi ile analiz edilmiştir. (Bio-Rad, Amerika)

Bulgular:

Çalışmaya alınan hastaların yaş ortalaması 51 olup farklı hastalık şiddetlerinden oluşan gruplarda yaş dağılımı bakımından bir fark yoktur. Tüm hastalık gruplarının yaklaşık yarısı erkek hastalardan oluşmaktadır. Ateş ve baş ağrısı tüm gruplarda ortak semptomlar olarak saptanmıştır. Petesi ($p=0.03$), ekimoz ($p=0.04$) ve melena ($p=0.001$) gibi kanama ilişkili problemler ağır hastalarda diğer gruplardaki hastalara göre daha çok gözlemlenmiştir. Ağır hastalarda IL-13 konsantrasyonları hastaneye ilk yatış günlerinde daha yüksek görülmüştür ($p=0.02$). IL-10, TNF-α ve IL-6 seviyelerinde önemli bir artış gözlemlenmemiştir ($p>0.05$). Hastalığın hafif seyrettiği hastalarda MIP-1β konsantrasyonu orta ve ağır hastalardan önemli derecede yüksek bulunmuştur ($p=0.02$). Aynı hasta grubunda IL-7 konsantrasyonları da benzer sonuçlar göstermiştir ($p=0.04$). Hastalığın II. ve III. dönemlerinde MIP-1β konsantrasyonları, hastalığın hafif seyrettiği hastalarda ağır ve orta olanlara göre önemli derecede yüksek bulunmuştur ($p=0.009$ ve $p=0.034$). Ağır hastalarda hastalığın III. döneminde IL-10 seviyesinde orta ve ağır hastalara göre önemli yükselme saptanmıştır ($p=0.04$). Ayrıca IL-7 ve IL-13 seviyeleri de sırasıyla III. ve II. dönemlerde ağır hastalarda önemli derecede yüksek bulunmuştur ($p=0.02$, $p=0.04$).

Sonuç:

Önceki çalışmalar serum örneklerinin toplanma zamanlarının göz ardı edilmesi sebebiyle sitokin seviyeleri ve hastalık seyri açısından çelişkili sonuçlar içermektedir. Bu çalışmada, serum örneklerinin toplanma zamanları dikkate alınmış ve bulgular hastalık semptomlarının gözlenmeye başlama süresi ile ilişkilendirilmiştir. MIP-1β sitokininin, KKKA hastalığı için iyi bir prognoz faktörü olarak değerlendirilebileceği sonucuna varılmıştır.

ACKNOWLEDGEMENTS

I would like to thank;

Assoc. Prof. Füsun Can, for being the advisor that almost all graduate students can only dream of. She always stood by me not only as an advisor but a friend that you can confide in, an elder that you can consult, but above all, a mother that always cares and thinks only about the health and happiness. Everyday, she proved me again and again, being the salt of the earth, not the academic knowledge alone takes you one step furter.

Prof. Dr. Önder Ergönül, for his self-sacrifice in everything about me. Day by day, as I see his work pace, his determination and mostly, his happiness and hope in life remindes me that being a student of his can be one of the best things that can happen to a student.

Prof. Dr. Mahinur Akkaya, for supporting me since my undergraduate times about everything from my studies to world view, sharing (or actually passing) her passion of being happy despite everyting in the world and trusting me.

My loving mum and dad, for supporting, trusting and believing me in everything that I do from the start; not only about my academic plans but also everything I do in my life. They raised me with such a love, harmony and trust, now I know I can overcome anything that comes to my way, be strong, be healthy and mostly, happy at the same time. I want to thank them for just being who they are.

Nazlı Ataç for being the impossible personality in the world. I could never believe someone can be that modest, helpful, supportive and loving. Sharing someone's happiness or sadness is one thing; but living this happiness or sadness for someone else is another. I would like to thank her for lifting the weight of the world for me.

To the most important two people in my life…who made me who I am, who were and will always be with me at any condition.

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NOMENCLATURE

AST: Aspartate transferase

ALT: Alanine transferase

CCHF: Crimean-Congo hemorrhagic fever

CCHFV: Crimean-Congo hemorrhagic fever virus

CPK: Creatinine phosphokinase

DIC: Disseminated intravascular coagulation

DUGV: Dugbe virus

ELISA: Enzyme linked immunosorbent assay

FBG: Fibrinogen

FFP: Fresh Frozen Plasma

G-CSF: Granulocyte colony stimulating factor

GM-CSF: Granulocyte-monocyte colony stimulating factor

HCW: Health Care Workers

HF: Hemorrhagic fever

HFRS: Hemorrhagic fever with renal syndrome

HFV: Hemorrhagic fever virus

HAZV: Hazara virus

IFN-γ: Interferon gamma

IL: Interneukin

IgG: Immunoglobulin G

IgM: Immunoglobulin M

LDH: Lactate dehydrogenase

L: Large

M: Medium

MIP: Macrophage inflammatory protein

MCP: Monocyte chemoattractive protein

NK: Natural killer

NP: Nucleoprotein

OTP: Ovarian tumor protease

PLT: Platelets

PT: Prothrombin time

aPTT: Activated partial thromboplastin time

RdRp: RNA dependent RNA polymerase

RT-PCR: Reverse transcriptase polymerase chain reaction

RVF: Rift Valley Virus

S: Small

SA-PE: streptavidin-phycoerythin

sTNFR: Soluble Tumor Necrosis Factor Receptor

VHF: Viral hemorrhagic fever

1. Introduction

1.1 Viral Hemorrhagic Fevers (VHF)

The bleeding throughout the body, accompanied with fever is a syndrome called viral hemorrhagic fever (VHF). Patients with VHF display malaise and prostration, increase in vascular permeability and abnormal coagulation pattern in the body. Vascular permeability increase due to the damage in vascular endothelium results in bleeding which the volume of blood loss is an indicator of the disease severity in organs. The symptoms caused are the result of the malfunctioning of the innate immune system response of the host but the replication of the viruses of the hemorrhagic fevers can directly be the cause of the pathological symptoms of the VHF. Rapid infection and deterioration of the dendritic cells, instant and cryptic death of lymphocytes and the release of cytokines from the infected cells collectively cause alterations in the permeability and the function of the vascular system, inducing disorders in coagulation pattern leading to the substantial symptoms of VHF (1) .

The causative agents of HF are, although diverse, mostly single-stranded RNA viruses. These viruses can cause infection in human system by contact trough infected animals or the vectors themselves which are usually arthropods. Although the geographical area is highly restricted, HF viruses can cause serious infections as nowadays the progress of jet travelling together with the increasing population, increases the incidence of contacting the infection agents. Furthermore, sporadic and unexpected outbreaks mostly seen in areas where logistic barriers and cultural restrictions prevent organized investigations (2).

VHFs gained public attention as they can easily spread among patients and health-care staff with a high morbidity and mortality rate, and also with the news media. However, the knowledge obtained related with the VHFs is still slow due to the restrictions of safe working environment. Furthermore, these agents can easily reach high concentrations in cell culture which is another reason for them to be assessed as a potential threat. The infectivity, high morbidity and mortality rates and the characteristics of the VHFs caught public and expert attention in the area of infectious diseases (1).

1.1.1 History and epidemiology

Members of *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae* families are the HF viruses. (Table1.1) Viruses belonging to these families have different transmission patterns geographical distributions but the reservoirs or the arthropod vectors for them play a significant role in determining the geographical area. Also, the reservoir for *Filoviridae* family is still unknown. Ecology and the characteristics of the families also play role in the transmission of the HF viruses in natural course and nosocomial transmission. Reservoirs for the HF viruses are mostly rural therefore patient's history, his/her living place is one of the key points in diagnosis. Transmission between human is possible but nosocomial transmission, usually by the contact with infected body fluids or blood, is also as common as human to human transmission. Transmission of the HF viruses through air is unlikely but still the possibility cannot be completely ruled out (1).

Table 1.1 Hemorragic fever viruses.(1)

Family	**Virus**	**Disease**	**Distribution**	**Source**	**Incubation (days)**
Arenaviridae					
Arenavirus	Lassa	Lassa fever	West Africa	Rodent	5-16
	Junin	Argantine HF	South America	Rodent	7-14
	Machupo	Bolivian HF	South America	Rodent	9-15
	Sabia	Brazilian HF	South America	Rodent	7-14
	Guanarito	Venezuelan HF	South America	Rodent	7-14
	Whitewater Arroyo	Unnamed HF	North America	Rodent	Unknown
Bunyaviridae					
Nairovirus	Crimean-Congo HF	Crimean-Congo HF	Africa, Central Asia, Eastern Europe, Middle East	Tick	3-12
Phlebovirus	Rift Valley Fever	Rift Valley Fever	Africa, Saudi Arabia, Yemen	Mosquito	2-6
Hantavirus	Agents of	HFRS	Asia, Balkans,	Rodent	9-35

	HFRS		Europe*		
Filoviridae					
Ebola Virus**	Ebola	Ebola HF	Africa	Unknown	2-21
Marburg virus	Marburg	Marburg HF	Africa	Unknown	2-14
Flaviviridae					
Flavivirus	Dengue	Dengue	Asia, Africa, Pacific, Americas	Mosquito	Unknown
	Yellow fever	Yellow fever	Africa, Tropical Americas	Mosquito	3-6
	Omsk HF	Omsk HF	Central Asia	Tick	2-9
	Kyasanur forest disease	Kyasanur forest disease	India	Tick	2-9

* Agents of Hanta Pulmonary Syndrome were isolated in North America.

** There are four species of Ebola: Zaire, Sudan, Reston, Ivory Coast

1.1.1.1 Bunyaviridae

Bunyaviridae family is composed of five genera: *Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus* and *Tospovirus*. Among these five genera three of them which are *Phlebovirus, Nairovirus* and *Hantavirus* cause hemorrhagic fever. Transmission routes of these genera are through arthropod vectors such as mosquitos, ticks and phlebotomine flies. However, airborne transmission is also present in some cases (3, 4)

a. Phlebovirus

Rift Valley Fever (RVF) virus is a Phlebovirus causing RVF, described in 1931 in Kenya among livestock as an enzootic hepatitis (5). It quickly infects human and livestock through mosquitos as vectors. It can spread quickly through contact with blood or body fluids

of the infected animal or human. Especially slaughterhouses pose highly risk as contact with contaminated meat is easy. Airborne exposure is another risk factor for laboratory workers. In 2000 RVF affected humans and livestock outside Africa for the first time, spreading through Saudi Arabia and Yemen (6).

b. Hantaviruses

There are about 20 different types exist in Hantavirus genus but 9 of them which are Hantaan, Seoul, Puumala, Dobrava, Sin Nombre, New York, Black Creek Canal, Andes, and Bayou pose the highest clinical risks among the others. Each one of them have their own unique vector, geographic distribution and symptoms caused in an individual. Hantaviruses display different characteristics than the other viruses that belong to *Bunyaviridae* family as their vector is not an arthropod but rodent. Direct contact with infected rodents or excretes of them drives most of the infections in human. Human to human and airborne transmission was found after the hantavirus pulmonary syndrome (HPS) outbreak in southwest Argentina (7). Hantavirus infections started to be called as Korean HF during the Korean War after it was discovered in Manchuria along the Amur River(8). The virus causing Korean HF with renal syndrome (HFRS) is called Hanteen, striped field mouse (*Apodemus agrarius)* as a reservoir, was described in 1977 and in Korea, Japan, and China, is still present. There are other hantaviruses which are responsible for the HFRS such as Seoul, Puumala, Sin Nombre viruses and HPS such as Black Creek Canal, New York, Andes, and Bayou; latter found after the 1993 epidemic in Four Corners, United States (9, 10). All of these viruses cause different forms of HFRS or HPS and symptoms caused by the infection are accompanied with cardiopulmonary malfunctions and hemorrhage (10).

c. Nairoviruses

7 species among Nairovirus genus composed of tick-borne viruses, defined as human or animal pathogens. Molecular characterization of the species is present; therefore relationship analysis could be made referring to that information. For Crimean-Congo Hemorrhagic Fever virus (CCHFV), Hazara virus (HAZV) and Dugbe virus (DUGV) full sequence information is available and for some other members of Nairovirus genus, partial

sequence information is present. DUGV is one of the most well characterized viruses in the genus and with the surveillance studies found to be endemic to Africa. DUGV causes thrombocytopenia with mild febrile illness in humans, transmitted through ticks to vertebrates (11).

CCHFV is the most well characterized virus in Nairovirus genus and is a zoonotic disease infecting vertebrates. It can be transmitted by the ticks, contact with infected animals, blood, body fluids and by aerosol. Although *Hyalomma marginatum* species stands out, CCHFV can be transmitted through at least 29 different species. CCHF is first identified in 1969, years after the outbreak of 1944-1945 in Crimea and a similar one in Belgian Congo in 1956 (12).

1.2 Crimean-Congo hemorrhagic fever (CCHF)

Crimean-Congo hemorrhagic fever (CCHF) is the most widespread tick-borne viral infection affecting humans, can be seen in a widespread area starting from China extending through southern Asia, Middle East, southern Europe to Africa.

Crimean-Congo hemorrhagic fever virus (CCHFV) is the agent responsible for the infection. It infects domestic mammals and human through several genera belonging to ixodid (hard) ticks. Ticks pass the virus through domestic and wild mammals and induce a transient viremia without these mammals showing any symptoms of illness. Tick bites, contact with blood or body fluids of an infected animal or a patient with CCHF. Hyalomma species are primarily responsible for infecting human (13).

CCHF is first recognized in 1944 in former Soviet Union and emerged in the nearby geographic locations after years such as Bulgaria and South Africa. Confirmed cases of CCHF increased after 2000, reported in Turkey, Irani India, Greece, Republic of Georgia and Balkans for the first time. Also, same species of ticks were also discovered in deers in Spain. Around 6300 cases confirmed in 10 years after it was first seen in Turkey in 2002. Increase of incidents can also be seen in Iran after its first recognition in1999 (14). Figure 1.a and 1.b shows the reported and published cases per year.

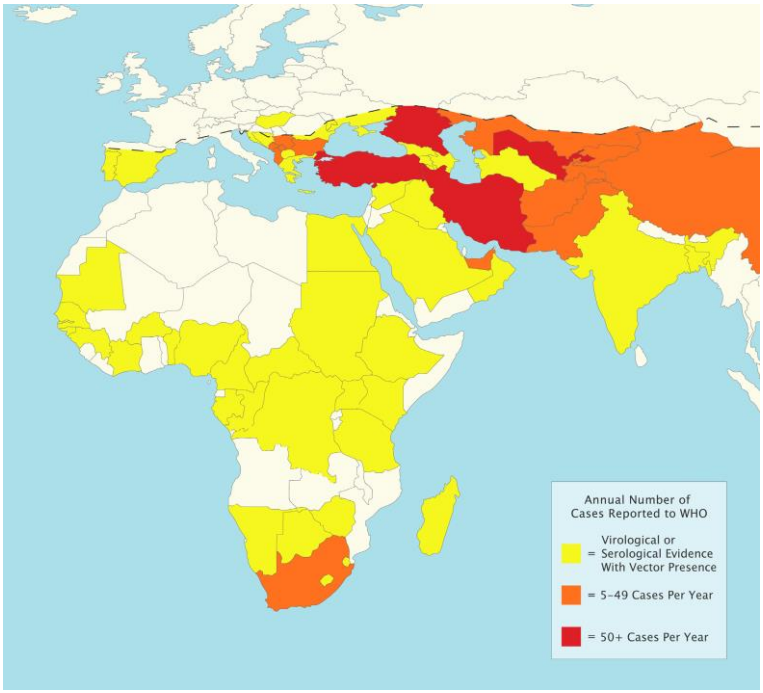

Figure 1.a. Distribution of reported CCHF cases per year. (13)

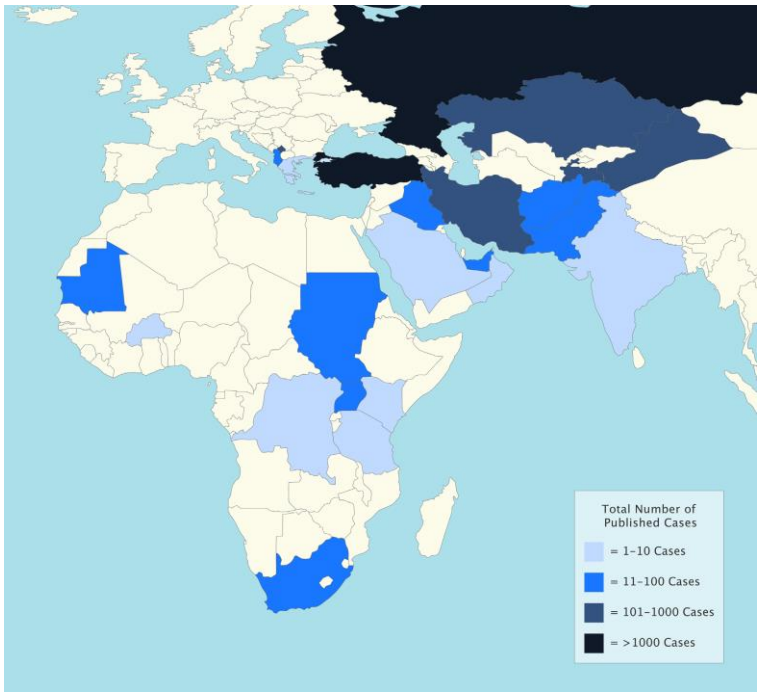

Figure 1.b. Total number of published CCHF cases.(13)

1.2.1 The causative agent of CCHF

1.2.1.1 Classification

Members of the Nairovirus genus are distinguished from one another with their L (large) segments. CCHFV and HAZV form the CCHF serogroup among the other seven serogroups of the genus. HAZV, discovered in Pakistan from the ricks on rodents, is not known to cause disease in humans (3, 15).

1.2.1.2. Virion structure, genome organization and encoded proteins

The virion responsible for causing CCHF is spherical and about 80-100nm in diameter, covered with a lipid envelope. The lipid envelope is composed of G_N and G_c glycoproteins associated with binding of the virion to receptors on cells. Antibodies neutralizing the G_N and G_c glycoproteins are synthesized while infecting the cell.

CCHF virion has tripartite genome which are small (S), medium (M) and large (L), covered by nucleoprotein (NP). For transcription and replication of the genetic material, RNA dependent RNA polymerase (RdRp) is present. Figure 1.2 shows the schematic representation of the CCHFV.

In all species belong to nairovirus genus; terminal complementary sequences which are 50-UCUCAAAGA and 30-AGAGUUUCU are conserved. Formation of rigid panhandle structures and non-covalently closed circular RNA molecules are resultant from intra-strand base-pairing between the terminal nucleotides. Terminal base-pairing exposes the functional promoter regions for the interaction of RdRp as deduced from the other bunyaviruses (16, 17).

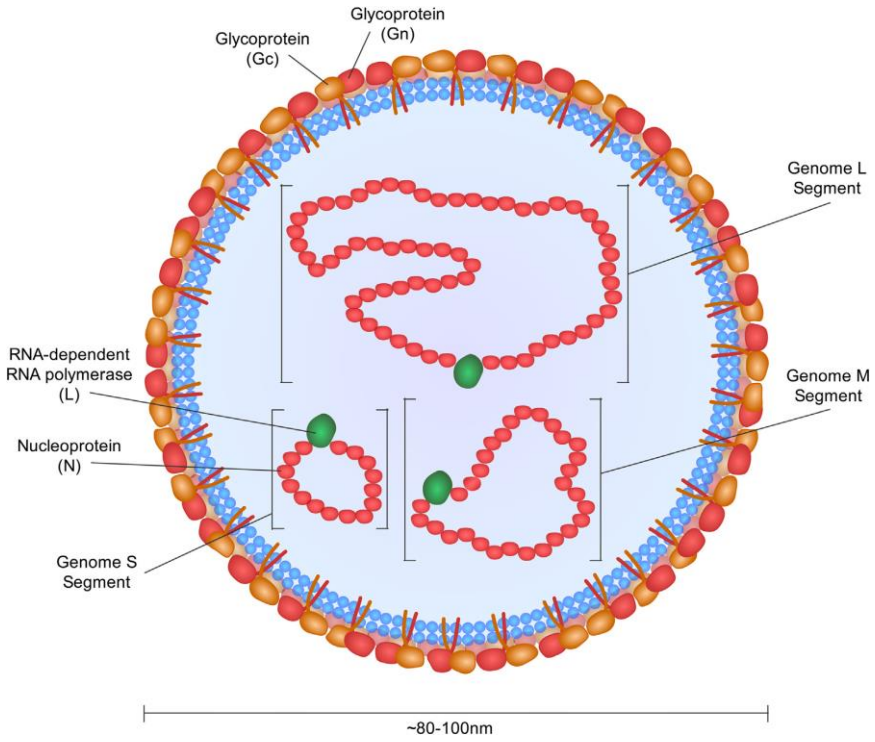

Figure 1.2. Schematic representation of the CCHFV.(13)

a. The S segment

Nucleoprotein (NP) of the CCHF virion is encoded by the S segment. S segment is composed of a large globular domain consisting N- and C- terminals of the polypeptide and a bulged part that contains a conserved caspase-3 cleavage site (18, 19). RNA binding is associated with the globular domain of the NP (20). Head-to-tail interactions of the protein, producing a helical structure, are due to the oligomerization of the NP in order to encapsulate the virion RNA. Due to the inhibition effect on the enhanced RNA polymerase activity, cleavage is thought to have a regulatory affect as NP is cleaved in apoptotic cells in the late infection phase. However, role of the caspase-3 cleavage site is unknown in viral replication (18). NP of the CCHFV together with the NPs of other RNA viruses shows that structurally they are closest to the arenavirus, Lassa. Also, for detection of virus specific immunoglubulin G (IgG)'s can be detected with enzyme-linked immunosorbent assay (ELISA) with NPs expressed in bacteria (21).

b. The M segment

Two type I transmembrane proteins, G_N and G_C, are made up after cotranslational cleavage and post-translational processing starting from endoplasmic reticulum (ER) and end in Golgi body to form the only polyprotein that is encoded by the segment M of the CCHF virion (22, 23).

This polyprotein is firstly cleaved into into 140 kDa PreGN and 85 kDa PreG_C segments by a host signal peptidase. Host proteases SKI-1 and furin further cleaves the PreG_N segment from conserved recognition sites to produce 58 kDa G_N and small protein fragments. One of these small protein segments is a nonstructural protein, NSm, also found in other viruses that belong to bunyaviridae family (22, 24). PreG_N and PreG_C accumulate in the Golgi apparatus if the cells do not have SKI-1 protease and virus paritcles secreted by the cell do not contain surface glycoproteins (25). For the last modifications of G_N and G_C, N-linked glycosylation is also needed (26). For the correct folding to be achieved in G_C, G_N functions like a chaperon (27).

High numbers of cysteine residues are present in glycoproteins of CCHFVs, ensures that there exist a lot of disulfide bonds, producing a complex structure. Mucin-like extensions of the G_N N-terminus produces the extensive O-glycosylation potential (23, 28, 29).
Also, binding to viral DNA is possible with the cytoplasmic tail of the GN as it has two zinc fingers (30).

c. The L segment

Nairoviruses, including CCHFV, has an L segment that is approximately twice the size of the other viruses in Bunyaviridae family. L segment has only one open reading frame, composed of approximately 12,000 nucleotides. It encodes an acid polyprotein that contains about 4000 amino acids, involving an ovarian tumor protease (OTU) domain near the N-terminus. Segments that are homologous to the viral topoisomerase, zinc finger and leucine zipper motifs follow the array (31).
Near the C-terminus, catalytic domain of RdRp is found. High similarity of the RdRp catalytic domain is found between Dugbe virus and CCHFV, which is another member of the Nairovirus genus. L polyprotein is thought to be autocatalytically cleaved by the OTU cysteine protease that is present near the N terminus (32). Though, no clear effect on the

replication is observed when the OTU cysteine protease is deleted from the system. Removing the ubiquitin part from cellular proteins is thought to be the only role of OTU protease system that may be seen as a key to understand host-cell antiviral mechanisms (33) Figure 1.3 summarizes the segments and the cleavage sites of the CCHFV genome.

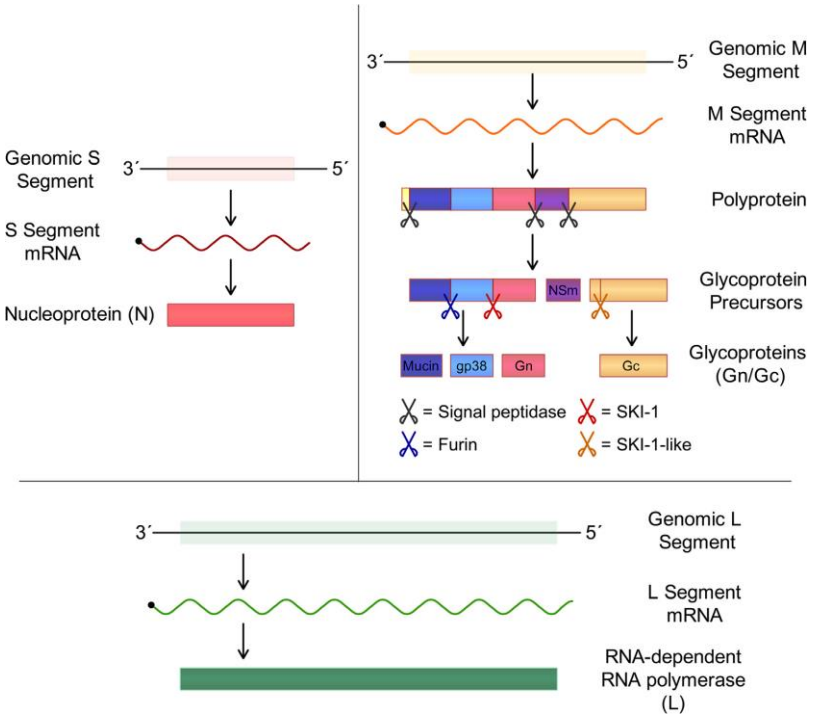

Figure 1.3. Segments and cleavage sites of the CCHFV genome.(13)

1.2.1.3. Replication cycle

Although there is no strict cellular receptor found for CCHF, Gc, which is one of the two soluble ectodomains, is associated with the binding of target cells. Nucleolin, which is a host molecule, is found to be playing a key role for cell entry (34).

Entry route of the virus to the cell is via clathrin-dependent endocytosis (35). After virus enters the cell, the first thing is the synthesis of the primer-intermediates to synthesize the negative strand so that the complementary positive strand can be synthesized. There are models suggested about the synthesis of positive-sense antigenomes and negative-sense progeny genomes without the involvement of primers (36).

Internalization process, congregation of the molecules synthesized for virion in the host and emergence of the virion are dependent on the microtubules of the host cell (37). The synthesis of the glycoproteins, G_N and G_C, the surface glycoproteins of the virions is a

multistep convoluted process, involving the synthesis of precursors PreGN and PreGC in the ER. They are then transported to the Golgi body in heterodimer form and then further cleaved, glycosylated, folded and then integrated into the membrane of the virions. When new, mature virus particles are ready, they bud out of the Golgi body (27). Figure 1.4 summarizes the replication cycle of CCHFV.

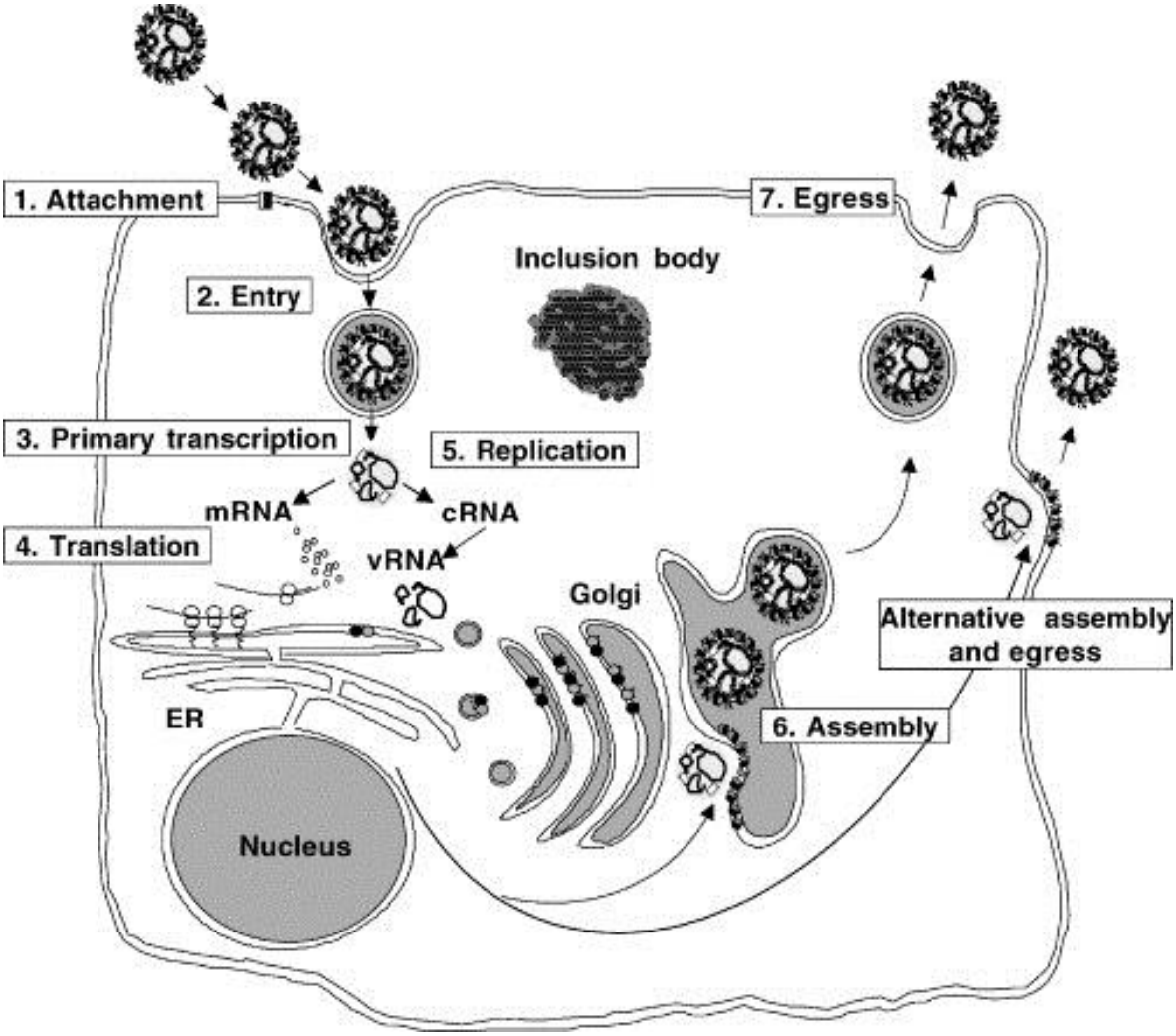

Figure 1.4. Replication cycle of CCHFV.(38)

1.2.2. Maintenance and transmission of CCHFV

The causative agent of the CCHF is decided to be circulating among ticks and different kinds of small and large mammals. Studies conducted on both field-collected ticks and in experimental setups for the vector competence in laboratories suggested that the perpetuation of the vector is conferred to ticks. On the other hand, vertebrates infected with CCHFV do not show symptoms of the illness but experience a transient viremia. Detection of the mammalian host for the CCHFV is restricted to the detection of the specific antibodies that belong to virus through the random collection of sera from livestock or wild animals. Transmission route of the virus is also detected through the antibody detection from the patients, history of contact with ticks, body fluids or the patients themselves or through the detection of the specific antibodies of the CCHFV from the areas of endemic populations (13).

1.2.3. Tick vectors of CCHFV

In the order of Parasitiforms, ticks are arachnids that belong to three different families; Ixodidae, Argasidae and Nuttalliellidae. Family Nuttalliellidae consists only of one species, *Nuttalliella namaqua*. The family Ixodidae consists of members having hard, rigid scutum while the family Argasidae has members with soft body. Diet of the ticks is through draining blood, called hematophagy, from a vertebrate, once in each of their instar, the developmental stage, for their metamorphosis starting from larval stage through nymph to completely mature adult tick. Adult female ticks are also in need of taking an extra blood meal to accumulate nutrients in order to produce eggs. Ticks have hypostomes, specialized for the hematophagy. The tick saliva containing anticoagulation factors and immunomodulatory proteins thought to expedite the infection of the virus carried through ticks and induce a specific immune system response so that the host would not further be invaded (4, 39). Transmission route of CCHFV is through transstadial, transovarial or veneral transmission among the ticks that belong to the Ixodidae family. After their transmission, during summer and spring seasons, viruses begin replication when their reservoir, tick, transmits it through the mammalian host while obtaining the blood needed for the metamorphosis, maturation or egg production.

After the transmission of the virus to the mammalian host, a transient viremia is observed but the tick remains as the natural reservoir for the virus as they remain infected for

several years, therefore they can transmit the virus to more hosts during their blood meals in years.

Among mammalians, humans are not the source of the infection; they are infected only accidentally, therefore they are the final hosts for the virus. One of the most important research areas of the CCHFV maintenance is to detect the possible competent vectors for the ticks which may render a lifelong infection and which the agent can effectively be transmitted from adult females to their eggs and by copulation, adult males to adult females. The research to find other tick species is restricted by the fact that the detection of viral antigens, identifying genomic sequences or the infective virus identified from a wild-caught tick can give false positive results as a blood meal from a CCHFV infected vertebrate host can be supplied to any arthropod. Therefore, the capability of the maintenance of the virus through vertical and venereal transmission is ignored. Only if the transovarial and venereal transmission of the virus is achieved by a vector, then the vector can be counted as a competent one.

It should be kept in mind while analyzing the maintenance of the CCHFV that some tick species are able to serve as a reservoir for the CCHFV and transmit it through the mammals, but do not serve as a source to infect humans. Therefore, effect of the other species in maintaining the virus cannot only be referred to human infections as humans are most likely to be infected by Hyalomma species when they enter an enzootic focus. Identification of candidate vectors for the maintenance of the CCHFV can be initiated from tick surveys gathered from wild and domestic animals but further vector competence studies should also be conducted for confirmation (13).

1.2.4. Transmission Routes of CCHFV

1.2.4.1 Vertical transmission

The ability of a tick vector to support the replication of the virus in the tissues as it passes through its maturation steps from larval stage to nymph and to fully mature adult stage, so that virus can transmit through eggs; and also be transmitted through adult males to adult females through copulation (40-44). After the CCHFV transferred to its competent vector, it starts replicating in the lining midgut lining and spread through other tissues and reaches high

concentrations especially in salivary glands and reproductive organs (45). Even if the concentration of the virus is low in the tissues, as female ticks can lay thousands of eggs, transovarial transmission can result in a large population of infected ticks (46).

1.2.4.2 Horizontal transmission

Spring and summer months constitute the season which ticks transmit CCHFV to the mammalian hosts as it is the period for larvae and nymph stages to support their growth and metamorphosis; and egg production period for adult females to be supported with the blood meals. Replication of the virus starts immediately in a host after its transmission during a blood meal of the vector and virus starts spreading through the tissues and bloodstream. Therefore, the host can serve as an infection source for more virus- naïve ticks. A tick may stay on the host for several weeks inducing the possibility of infection of the host from the infected tick or vice versa, from an infected host to the tick it's feeding on. Nonetheless, not all mammals can be infected with CCHFV or even if they do, do not maintain virus concentrations high enough to infect ticks (43, 47). Furthermore, in order to initiate the infection from the midgut lining, ingested amount of the virus can also be an important factor as not all the quantities are sufficient enough. The co-feeding mechanism (48-50) a second way of transmission, in which the tick saliva containing the virus spreads to adjacent ticks ingesting their blood meal, emerged through evolution. Co-feeding is believed to be enhanced by the substances present in the saliva (51). This transmission type is also referred as a nonviremic transmission as it is not necessary for the host animal to be infected beforehand. Transovarial transmisson of the virus is augmented by direct disclosure to adult females. After a tick finishes its blood meal for its growth and maturation, it may stay on the host until its prospective instar, drop off or molt and then look for a new host for the new blood meal. Transmisson routes of CCHF by ticks are summarized in Figure 1.4. Some members like Hyalomma marginatum, of the Hyalomma genus can be a host for two ticks in larvae and nymph stages that choose rodents, hares, ground-feeding birds for their hosts during feeding and molting; after they reach full maturity, they obtain their blood meals from large mammals such as sheep and cattles. There are also ticks that are called as "three-host" which they leave the host in each of their molting stage (13).

Circulation of the CCHFV is highly associated with the different cycles of tick-host interactions. There are different species of ticks serving as a reservoir which are called

"ambush" or "hunting" ticks depending on their pattern of encountering their host. Hyalomma species fall into "hunting" ticks category as they may go to elevations up to 400m in order to find hosts (13).

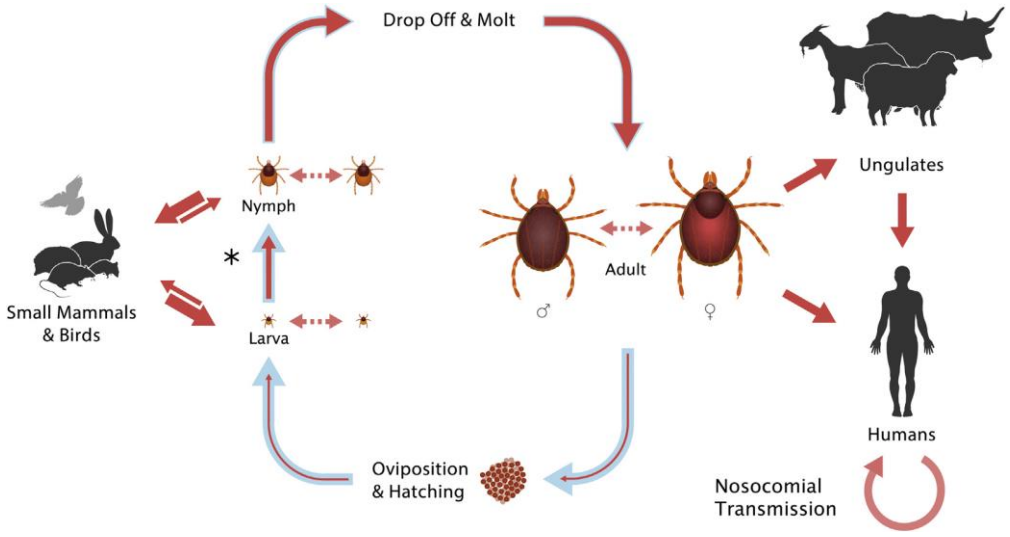

Figure 1.5. Transmission routes of CCHF.(13)

1.2.5 Transmission of CCHFV to humans

The infection of the humans by the CCHFV is usually by being bitten [figure!!!]by an infected tick in a rural area. Incidences occur in spring and summer months as the adult stages of the ticks of Hyalomma species must ingest their blood meals for their maturation. However, incidences can be seen in warm winters, when infected ticks survive (52). Without direct exposure to ticks, humans can be infected through the direct contact with blood or body fluids of an infected animal. Regarding this, abattoir workers are a high risk group for the infection. Human to human transmission of the infection is also possible if there is a contact with the body fluids or blood of an infected person within 7-10 days after the infection. Nonetheless, standard barriers within the healthcare facilities are an acceptable method of protection (53, 54).

1.2.6 Clinical and laboratory features of CCHF

1.2.6.1 Clinical features

CCHFV infections are usually mild cases but can be severe hemorrhagic cases with nonspecific febrile illness. In 1944, during the outbreak in Crimea, symptoms of the patients admitted to the hospital were reported as sudden fever, headaches, muscular pain, vomiting, hyperemia of face and oropharynx, hemorrhagic rash, ecchymoses and also bleeding from several locations such as nasopharynx and gastrointestinal tract (55). Cases reported from the other outbreaks during history included similar findings (56, 57).

Humans are the only host for the CCHFV that exhibit the symptoms explained above, other than newborn mice. Other vertebrate hosts do not show hemorrhage or other symptoms associated with CCHFV infection; severe hemorrhage is the result of the infection if the host is human (58).

Progress of the CCHF is divided into four phases indicated as incubation period, prehemorrhagic, hemorrhagic and convalescent periods (58) as summarized in Figure 1.5.

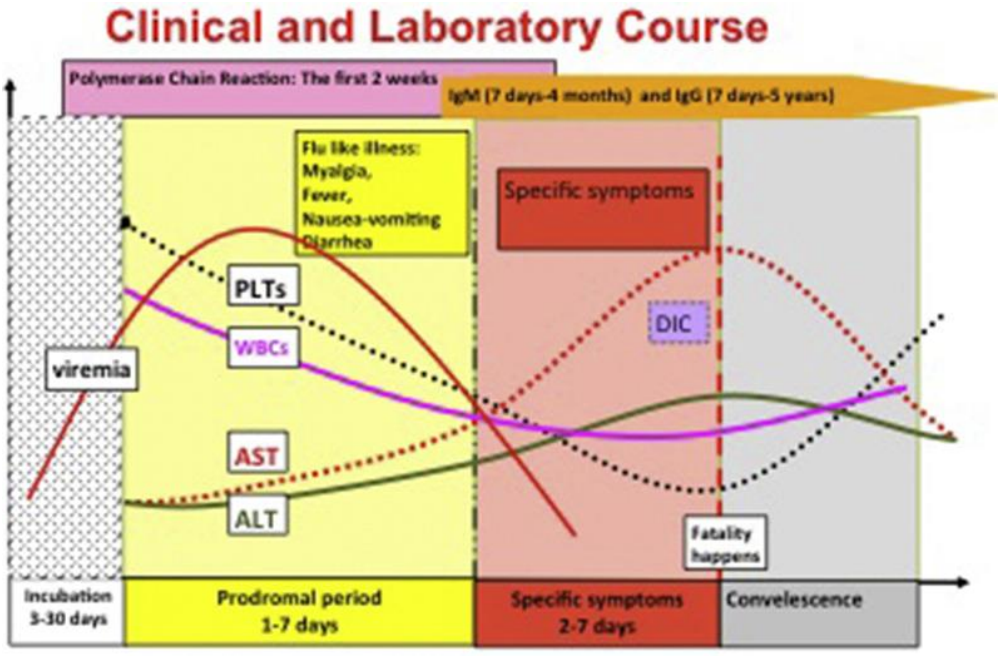

Figure 1.6. Clinical and laboratory course of CCHF. (59)

- Incubation period:

  Time interval of the incubation period is highly variable depending on the route of the virus entrance into patient's bloodstream. Incubation period after the bite of an infected tick is about 1-5 days, whereas, if the virus is acquired through the contact with infected tissue or body fluids, it is about 5-7, up to 13 days (57, 60). Furthermore, it is hypothesized that the length of the incubation period is also affected by the changes in the host and phenotypical properties of the CCHFV, which is associated with the virulence and viral load; in turn, affecting the outcome of the disease (61).

- Prehemorrhagic period:

  Headache, dizziness, vomiting, appetite loss, lethargy, photophobia, back and abdominal pain, several other nonspecific symptoms mark the prehemorrhagic period of the CCHF, accompanied with fever which is around 39-41°C. Fever inclination can prolong up to 5-12 days or can show a biphasic course (13, 38).

- Hemorrhagic period:

  On the 3-5 days of the disease course, hemorrhagic phase prevails (57). Petechial rash of the skin, conjunctiva and mucous membranes which ends up in cutaneous ecchymoses, mostly in extremities, are the first symptoms marking the period. Melena, hematemesis and epitaxis are common and bleeding in gastrointestinal tract and urinary tract is seen. In some cases cerebral hemorrhage, bleeding from vagina and abdominal musculature is reported (56). Hepatomegaly and splenomegaly are also seen frequently in this period.

- Convalescence:

  Full recovery can extend a year if a patient survives CCHF (57). Weakness, hair and appetite loss, sensory impairments, polyneuritis, liver and kidney problems and several other health problems are reported after recovery (56). Recent studies in Turkey and Iran do not report similar findings (62, 63) and reoccurrence is not seen.

In some cases, neuropsychiatric problems including abrupt changes in mood, confusion, aggression and violent behavior are reported (56, 64). Bradycardia and cardiovascular changes may also be observed, however in the 1994-95 outbreak in United Arab Emirates, no vascular problems reported while 8 out of 11 (72,7%) cases died (65).

1.2.6.2 Pathogenesis

Nonstructural proteins with molecular weights changing from 8 to 40000d are called cytokines. There is no strict three dimensional structure or motifs to define cytokines, but they are classified according to their biological activities. During innate and adaptive immunological responses, cytokines, which are proteins, are secreted, acting as inflammatory mediators or modulatory molecules during the course of hemorrhagic fevers (66). In both disease onset and homeostatic regulation, cytokines are involved in several processes such as coagulation and fibrinolysis activators (67). Mostly, they are associated with the host response mechanisms against infections, causing striking changes when they are involved in homeostatic mechanisms (68).

Cytokines regulate the actions of the host such as infection, immune responses, inflammation and trauma. Some cytokines are classified as proinflammatory cytokines, promoting inflammation and some of the others induce healing and classified as anti-inflammatory. The classification is related with the synthesis of small mediator molecules up-regulating during the inflammation (68). The genes related to proinflammatory cytokines induce the factors for platelet-activation factors, prostanoids and nitric oxide (NO). Some other group of genes induces the leukocyte passage between circulation and the tissues. Some other interleukines are classified as chemokines such as IL-8, causing the acitvation of the neutrophils for degranulation and cause tissue damage. Also, IL-1β and TNF- α induce molecules related with endothelial adhesion and important for leukocyte adhesion on the surface of the endothelials before passing through the tissues. Altogether, inflammation is mediated by cytokines by a cascade of different molecules and is not a natural process in healthy individuals. Endotoxins and inflammatory products induce the cascade in an individual and IL-1β, TNF- α and in some cases IFN-γ particularly affects the activation of the genes; IL-1β and TNF- α acting synergistically. T-cells, IL-1β and TNF- α which are activated by and infection or a toxin, starts the inflammatory mediator cascade by targeting the endothelium. The suppression of the cascade is achieved by the anti-inflammatory

cytokines IL-4, IL-10 and IL-13; blocking the cytokines, chemokines and adhesion factors. IL-7 also contributes the immune response by stimulating the proliferation of all cells in the lymphoid lineage (B cells, T cells and natural killer (NK) cells). There is always a balance between the two classes of cytokines, determining the outcome of the disease.

Chemokines are all small heparin-binding proteins that constitute a large family of peptides (60-100 amino acids) structurally related to cytokines, whose main function is to regulate cell trafficking. Monocyte chemoattractant protein-1 (MCP-1/CCL2), and MIP-1β are the members of chemokine family. MCP-1 regulates migration and infiltration of monocytes/macrophages.

MIP-1β activates human granulocytes (neutrophils, eosinophils and basophils) which can lead to acute neutrophilic inflammation. They also induce the synthesis and release of other proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and TNF-α from fibroblasts and macrophages (69). IL-8 is another known chemokine and inflammatory cytokine (70). The cytokines associated with CCHF infection is shown in Figure 1.7.

In this study, we aimed to correlate the role of cytokines in the pathogenesis of the CCHF by analyzing the serum samples from the patients at different stages of the disease by catching the cytokine storm.

Although the network and multiple regulatory pathways of the cytokines are complex and not completely understood, they are thought to be one of the key factors in the classification of the disease severity and prognosis. In the disease pathogenesis, endothelial cells, viral load and coagulation cascades are very important. For CCHF, disease targets the blood and endothelium. While viremia occurs in the first two stages of the disease, improper activation of the T helper 1 (CD4 Th1) lymphocytes produce TNF-α and IFN-γ, which in turn induce macrophages to induce over secretion of IL-1β and IL-6, which are involved in several processes such as coagulation and fibrinolysis activation. Production of vasodilator substances, coagulant protein activation and platelet aggregation damages the endothelium while it can also be infected directly by virus itself. Activation of various substances and/or viremia collectively causes disseminated intravascular coagulation DIC and multiorgan failure (57, 63, 71) .

In 1944 Crimean outbreak, the diagnosis for the disease was clearly associated with the vascular dysfunction, causing hemorrhage and leakage of plasma into the interstitial space (55). Since these days, coagulation abnormalities during the early stages of the disease are

seen and DIC is observed in severe cases of CCHF patients (56, 72). However the cause of vascular dysfunction is due to viremia of endothelium or as an indirect cause, circulating proinflammatory mediators, is still unclear. The only report which correlates the CCHFV to be the direct cause of vascular dysfunction was a 1997 report on 12 autopsies, describing viral RNA detection and the antigens in mononuclear phagocytes (72). However, it should be noted that the vascular permeability cannot be directly associated with the presence of the virus in endothelial cells at time of death as virus can be seen right after the initiation of the disease. On the other hand, procoagulant state and hemorrhage can be associated with acute inflammatory response, resembling the septic shock produced by gram-negative bacterial infection (73).

Studies with Ebola HF, which has many common features with CCHF, point out that vascular dysfunction results from host-induced mechanisms, such as the release of proinflammatory cytokines, platelet aggregation and degranulation, leukocyte adhesion and intrinsic coagulation cascade activation (73, 74).

Many clinical features are common with CCHF and other viral hemorrhagic fevers. After the end of the incubation period, fever, headache, muscle ache, nausea and some other nonspecific symptoms are seen. Outstandingly, forms of hemorrhage such as petechiae, eccymoses, melena and hematemesis are seen in CCHF than other viral hemorrhagic fevers. In severe cases, situation turns quickly into DIC, bleeding and shock (75).

The first effects of infection seem to be the erythrocyte leakage and plasma leakage from the circulatory system to tissues. The damage to the endothelial tissues restricts the platelet aggregation triggering the coagulation cascade, triggering the clotting factor deficiencies leading to hemorrhages. (76). For CCHFV, it is believed that the leakage causing the hemorrhage is resultant from the damage to the tight junctions generating the endothelial barriers. However, it could not directly be associated with the virus-induced host factors, but epithelial cell line modal of CCHFV do not cause damage to the tight junctions or induce necrosis or apoptosis of the cells, suggesting that the hemorrhage could be the result of high levels of proinflammatory cytokines, which is also associated with other hemorrhagic fever viruses such as Ebola or Dengue (77).

As most of the hemorrhagic fevers show similar clinical features and pathogenesis pathways, a similar cytokine storm model of Dengue fever can be suggested for CCHF as seen in Figure 1.6.

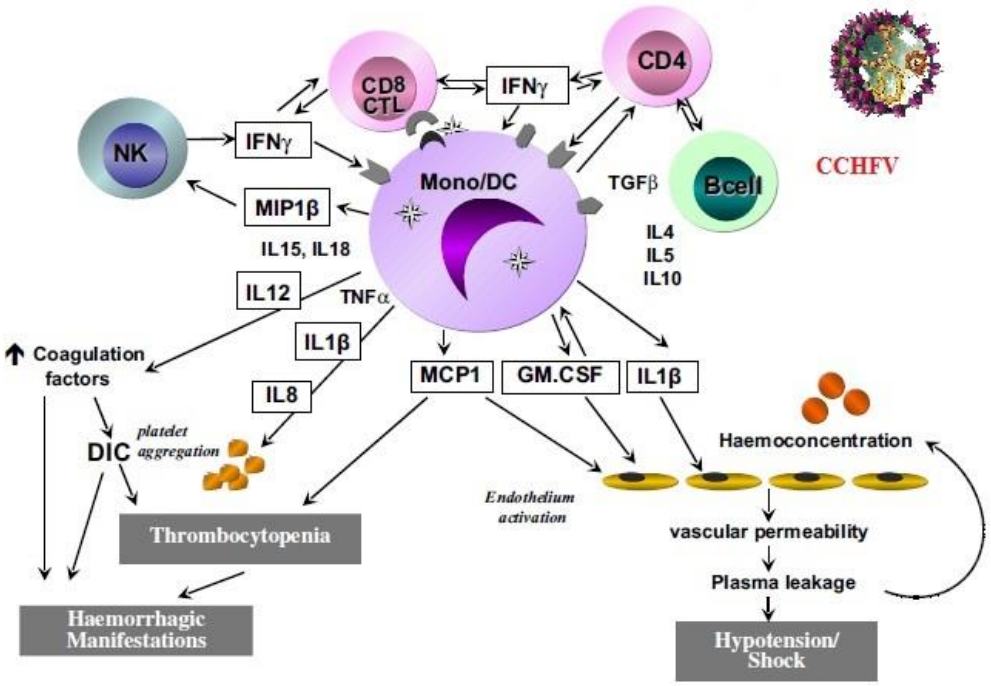

Figure 1.7. Pathogenesis of CCHFV.(78)

Several other studies indicate that viral replication in antigen presenting cells; cytokine liberation and circulation, and T cell activation are likely not to be a linear process. A combination of positive/negative feedbacks, viral clearance and pathological changes collectively direct to vascular dysfunction, impaired permability, therfore circulatory collapse, eventually leading to disease outcome (78).

After the disease onset, increase in aspartate and alanine aminotransferase (AST and ALT) in the serum, increase or decrease in total white blood cell and lymphocyte count in relation with sample collection time, decrease in platelet count and fibrinogen level and prolonged prothrombin and partial prothromboplastin times (PT and aPTT) indicates the presence of the virus in the bloodstream (56).

After hemorrhage and shock, viremia declines and in the patients who survive an immunoglobulin M (IgM) response is seen, which is evaluated as an early diagnostic marker. Also, destruction of erythrocytes, platalets and some leukocytes, which is called

hemophagocytosis, is seen in some cases, which is believed to be resultant from the monocyte activation by the proinflammatory cytokines (71, 79, 80).

Levels of proinflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF-α), in the plasma of the patients are present and significantly higher in fatal cases than the nonfatal ones (81). Several other studies indicate the importance of the proinflammatory cytokines. High TNF-α levels were correlated with severe cases and increment IL-6 was correlated with severe and mild cases in a study conducted with a study population of mild and severe cases in Turkey (76). Also, both TNF-α and IL-6 levels were found to be high in fatal cases compared to the nonfatal ones.

High levels of IFN-γ, TNF-α and IL-10 are associated with fatal cases in a study in Kosovo (2); and high levels of TNF-α, soluble tumor necrosis factor receptor (sTNF-R), IL-6 and IL-10 were associated with a fatal Albanian patient (82).

Also, IL-12 is an important cytokine in Th1 immune response activation and IL-10 is the negative regulator of this process, making IL-10/IL-12 ratio an important indicator of immune status regarding the anti-inflammatory and proinflammatory states (83). Other studies conducted also show that high levels of proinflammatory cytokines (IFN-γ and TNF-α) and antiinflammatory cytokines (IL-6 and IL-10) suggest that they play important roles in the pathogenesis of CCHF and other hemorrhagic fevers such as Lassa and Ebola (2).

Effect of the cytokines IL-4, IL-6, IL-10, MCP-1 and MIP-1β were also studied intensively in dengue virus patients and a significant increase in circulating levels during febrile phase is observed. IL-7 is also found to be increased during febrile phase and it is also significant while compared to defervesence. Levels of IL-1β, IL-13 and IFN- γ are found to be increased compared to control samples in defervesence; however, no significant change was observed in the levels of IL-5, IL-12, and IL-17 between healthy individuals and patients diagnosed with dengue (78).

1.2.7 Diagnosis CCHF

Early diagnosis of the CCHF is very important not only for the outcome of the patient, but also to prevent nosocomial infections and further transmission of the illness (38). If there is a tick bite history, a travel history to an endemic region or exposure to blood or tissues of livestock or human patients and the early symptoms of the disease such as fever, myalgia and diarrhea is observed with the other non-specific symptoms in a person, together with the

symptoms pointing out the presence of vascular leakage and endothelial damage, CCHF should be suspected. If the initial laboratory tests suggests leucopenia, thrombocytopenia and an elevation is observed in serum AST and ALT levels, CCHF suspicion is highly strengthened (13). Differential diagnosis should include other etiologies depending on the origin of the patient and risks of potential exposure (84).

Diagnosis of the CCHF is performed by viral RNA genome by reverse transcriptase polymerase chain reaction (RT-PCR) and/or specific IgM antibody detection in human serum or blood by ELISA (84). The detection of the infectious virus is evaluated as the "gold standard" of the diagnosis, however it is mostly impossible as working with CCHFV requires Biosafety Level 4 containment, which is only available in South Africa among CCHF endemic countries. Therefore, the standard diagnostic test for CCHF is considered to be RT-PCR. During the first 7-10 days of the disease, patients are viremic; therefore virus-specific IgM can be detected by the end of the first week of the disease, followed by the appearance of IgG after a short time period. However, acute patients may fail to develop an antibody response, and IgM may not be detected at the time of testing, making RT-PCR the most reliable test during a CCHF case. If the detection of viral load can be achieved, it will also provide information to the clinican about disease severity and likelihood of death (38).

IgM antibodies diminish in several months after the illness, but the presence of IgG antibodies for longer time periods enables retrospective diagnosis for CCHF (13).

1.2.8 Treatment and prevention of CCHF

1.2.8.1 Treatment

CCHF infections usually do not require hospitalization as they usually progress as a non-specific febrile illness in mild cases. However, for the cases developing hemorrhage and hypotension, the essential part of the case management in CCHF is the supportive therapy (13). By monitoring the bleeding status, thrombocyte solutions and fresh frozen plasma (FFP) are given to the patients. The volume replacement requirement is due to the increased vascular permeability due to the decrease in blood pressure and diminished organ perfusion; FFP and platelet provision is required in the cases of impairments in coagulation. Furthermore, blood transfusion is the requirement in the extreme hemorrhage cases (13).

The only antiviral drug that has been used in the therapy of the viral hemorrhagic fever syndromes including CCHF and LF. The viruses that belong to *Bunyaviridae* family are

generally sensitive to ribavirin (85) and there are *in vitro* studies showing the ribavirin affectivity against CCHFV (86-88). Ribavirin was found to be beneficial in the early stages of the disease in clinical practice (80, 89-91) and placed in the World Health Organization's 15th Model List of Essential Medicines to be used against CCHFV.

During 1944-1945 Crimean outbreak, anti-CCHF immune globulin prepared from the plasma taken from the surviving patients of CCHF, was recommended by Chumakov at that time, but no significant evidence was found that is effective after the applications in Soviet Union (92). On the other hand, anti-CCHF immune globulin therapy is still in use in Bulgaria (93-95).

CCHFV replication is inhibited by Type I IFN in vitro, (96-98) but the only clinical use was in 1985 Tygerberg outbreak in South Africa, where it was applied on 6 patients and no beneficial effect was observed (12).

1.2.8.2 Prevention

Bunyaviridae family is highly infectious after the contact with blood or budy fluids of an infected patient. In the case of a suspected person, infection control professionals should directly be informed as well as the health department of area. Clinical laboratories and public health authorities should also be informed (84).

The way to prevent the infection is not to be exposed to the the CCHFV. To fulfill the purpose, people who are evaluated in the high risk population such as slaughterhouse workers, veterinarians, sheep herders or health care workers (HCW) should avoid the contact with ticks, infected animals, blood or body fluids of the infected animal or humans. Wearing gloves and trying to minimize the direct contact of the skin with the infected animals, patients, blood or body fluilds are effective and practical ways of this prevention. For HCWs, barrier nursing methods should be used.

Tick control is not a practical way for disease prevention as *Hyalomma* species are common in worldwide but treatments with acaricides are effective in endemic areas.

There were some vaccine production trials in history resulting in a detectable antibody production in the persons taking the vaccine, however as the population that is under the risk of the exposure to a CCHFV, a modern, large-scale vaccine production seems unlikely (13).

2. Material and methods

2.1 Study population

A total of 48 patients were included the study. Patients in the study population were admitted to the Infectious Diseases and Clinical Microbiology Clinic of Ankara Numune Education and Research Hospital between 2007 and 2009 with CCHF diagnosis. Serum specimens of acute period were collected and sent to the national reference laboratory of Turkey, Refik Saydam Hygiene Center for confirmation. Cases were confirmed by ELISA for detection of IgM postivity and/or polymerase chain reaction (PCR). After the admission to the hospital, biochemical laboratory parameters and complete blood counts were measured daily. Daily serum samples were collected for cytokine studies and stored -20 °C until analysis day. One serum sample which was taken on the day of admission was studied for each patient and additionally, 12 mild, 27 moderate and 9 severe cases were selected for detailed analysis. Consequent serum samples of the selected patients were categorized into 4 stages of the acute phase of the disease. Stage I describes disease days 0-3; stage II 4-7, stage III 8-11 and stage IV 12-15. The serum samples of each patient that are available in each stage of the disease were selected for further cytokine determination. Approximately 3-4 serum samples from each patient and a total of 98 serum samples were analyzed in the study. The study was approved by the ethical board with the approval number 2014.088.IRB2.025.

Disease severity of the patients was evaluated on admission depending on the severity scoring index (SSI). 5 parameters which are platelet count (0–3 points), activated partial thromboplastin time (aPTT, 0–3 points), fibrinogen (0–3 points), bleeding (0–3 points), and somnolence (0 or 1 points; Table 2.1) included for the calculation. (62)

Table 2.1 Severity scoring index (SSI) for CCHF. (99)

SSI parameter	**Score**
Platelet count, ($x10^3$ platelets/mm^3)	
>150	0
150-50	1
49-20	2
< 20	3
aPTT, sec	
≤34	0
35-45	1
46-59	2
>60	3
Fibrinogen level, mg/dL	
≥180	0
179-160	1
159-120	2
<120	3
Bleeding	
No	0
Petechia	1
Ecchymosis	2
Bleeding	3
Somnolence	
No	0
Yes	1

2.2 Cytokine assays

With the patient serums collected from Infectious Diseases and Clinical Microbiology Clinic of Ankara Numune Education and Research Hospital between 2007 and 2009 with CCHF, 17-plex cytokine assay were studied. Cytokines (IL)-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte

colony stimulating factor (GM-CSF), interferon gamma (IFN-γ) monocyte chemoattractive protein (MCP-1), macrophage inflammatory protein (MIP-1β), and tumor necrosis factor (TNF-α) were studied.

Bio-Rad Pro Human Cytokine assays depend on biometric capture sandwich immunoassay basis. For the cytokine measurement, system includes beads which are fluorescently dyed and adjoint with monoclonal antibodies for target proteins. Protocol is based on incubation of antibody coupled beads with the standards, samples and controls; then biotinylated detection antibodies. There are washing steps for the unbound molecules between the incubation steps, with the Bio-Plex Pro wash station. Streptavidin-phycoerythrin (SA-PE) mixture is used as a reporter after the washing step of the unbound antibodies. After the removal of excess SA-PE with another washing step period, the assay plate is run through Bio-Plex MAGPIX to obtain concentration titres, which is achieved by the measurment of fluorescent intensity of the beads and bound SA-PE complex. Bio-Plex Manager™ is used for data collection. (Figure 2.1)

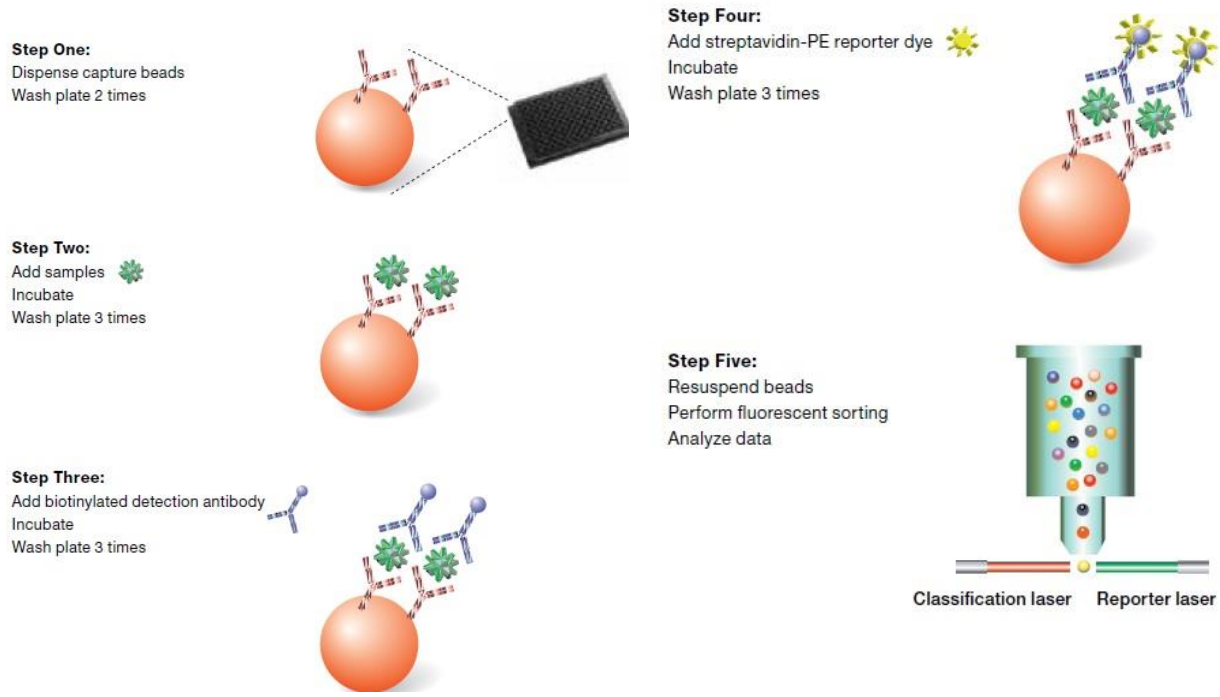

Figure 2.1 Workflow of sandwich-based Bio-Plex® assay (Bio-rad Laboratories, USA)

2.2.1 Preparation of the standards

500µL standard diluent is added into the standard vial and vortexed for 5 secs. Then, standard vial is incubated in ice for 30 mins. While standard solution is in incubation, 9 tubes (from S1 to S8 for standards and B for blank) are labelled. After the incubation, 72µL standard diluent is added into S1 tube and for the rest of the labelled tubes, 150 µL standard diluent is added. 128µL from the standard solution is added into the S1 tube and rest of the standards are serially diluted in 1:4 ration by taking 50µL from the S1 tube and adding it to S2; repeating it until the 8th tube, vortexing each tube for 5 secs. No dilutions performed in "blank" tube. Standards and blank is kept in ice until they are added to the plate. Preparation of the standards should be about the same time with the end of the sample preparation and should not be kept too long before their transfer to the plate containing the beads.

2.2.2 Preparation of the bead mixture

5175µL assay buffer is mixed with 575µL 10X bead mixture in a 15mL falcon tube. Tube is gently vortexed and kept in aluminum foil. 50µL bead solution is added to the each well in the plate.

2.2.3 Preparation of the antibody solution

2700µL detection antibody diluent is mixed with 300µL detection antibody in a 15mL falcon tube. Tube is gently vortexed and kept in aluminum foil. Gently vortexed before adding to the wells. Preparation of the antibody solutions should be about the same time with the last washing step after the first incubation (30mins) period of the samples and should not be kept too long before the transfer to the plate conatining the beads.

2.2.4 Preparation of the streptavidin-phycoerythin (SA-PE) solution

5940µL assay buffer is mixed with 60µL 100X SA-PE in a 15mL falcon tube. Tube is gently vortexed and kept in aluminum foil. Gently vortexed before adding to the wells. Preparation of the SA-PE should be about the same time with the last washing step after the second incubation period (30mins) of the samples and should not be kept too long before the transfer to the plate conatining the beads.

2.2.5 Preparation of samples

A flat-bottom 96-well plate is used for sample preparation. 150µL sample diluent is added to each well to be used except the first two columns and the first two cells of the 3rd and 4th column as they will be used for standards and the blank. 50µL patient serum is added into each well. Therefore 1:4 dilution for the serums is achieved. After 1:4 dilution of the samples in a flat-bottom 96-well plate, 100µL wash buffer is added onto each well that contains 50µL bead solution. Plate is secured on the magnetic washer and kept 1 mins. Without removing the plate from the magnetic washer, wash buffer is eliminated by turning the plate upside down. This wash step is repeated for two times. After washing the beads, samples diluted in another flat-bottom 96-well plate are transferred to the plate containing beads. Then, standards which have been kept in ice are added into the cells A1-A2 to H1-H8. Blank is added into A3 and A4. Plate is then sealed, covered with aluminum foil and incubated 30mins on shaking incubator at lowest speed. After the incubation, washing step is repeated for 3 times with 100µL wash buffer for each well in each washing step. Then 25µL antibody is pipetted to each well, plate is sealed, covered with aluminum foil and incubated 30mins on s shaking incubator at lowest speed. After the incubation, washing step is repeated for 3 times. Then 50µL streptavidin-phycoerythin (SA-PE) fluorescent reporter dye is pipetted to each well, plate is sealed, covered with aluminum foil and incubated 10mins on s shaking incubator at lowest speed. After the incubation, washing step is repeated for 3 times with 100µL wash buffer for each well in each washing step. Then 125µL assay buffer is pipetted to each well and cytokine concentration titres are read in Bio-Plex MAGPIX.

2.3 Statistical Analysis

Stata version 11 was used for statistical analysis of the concentrations of the serum samples included in the study.

3. Results

Study population includes 48 patients; 12(25%) in mild group with severity scores 0-2, 27(54%) in moderate group with severity scores 3-9 and 9(21%) with severity scores higher than 9 and/or ex patients. (Figure 3.1)

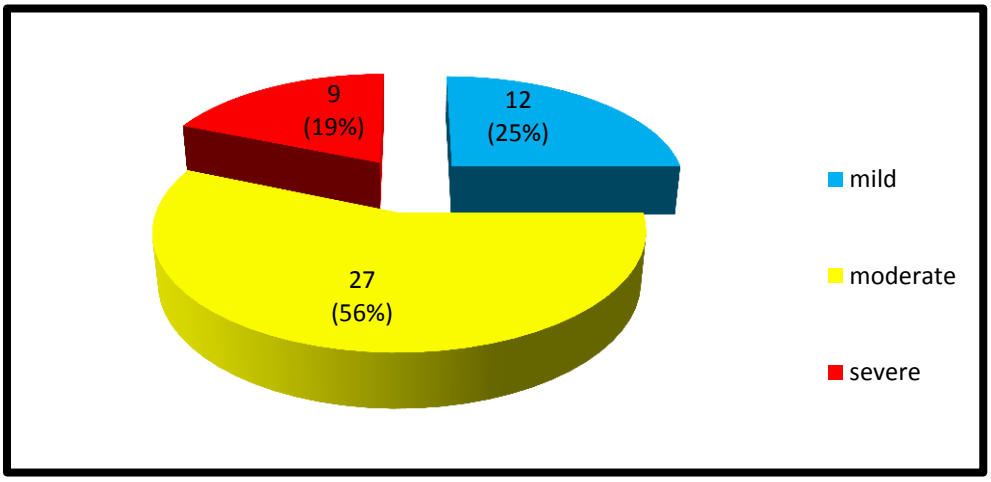

Figure 3.1. Distribution of the study population.

The mean age of all the patients was 50 and there was no difference between the severity groups in terms of age distribution. Approximately half of the patients were male in all severity groups. (Table 3.1)

Table 3.1 Demographic characteristics of the patients in different severty groups.

Characteristic	All patients (N=48)	Mild (n=12)	Moderate (n=27)	Severe (n=9)
Age, mean (SD)	50(16)	57(15)	51(15)	47(17)
Male gender	50.00	50.00	60.00	40.00

Tick bite history of the patients was positive in 66.67% in all groups. Other ways of transmission could not be found. Time onset of the symptoms was similar in all severity groups.

All the patients were diagnosed by PCR or by IgM antibody positivity. PCR testing were performed on 40/48 patients and 39 of 40 (97.50%) were positive. IgM antibody was searched among 12/48 patients and found to be positive in 11 out of 12(91.67%). Figure 3.2 summarizes the clinical findings of the patients with CCHF.

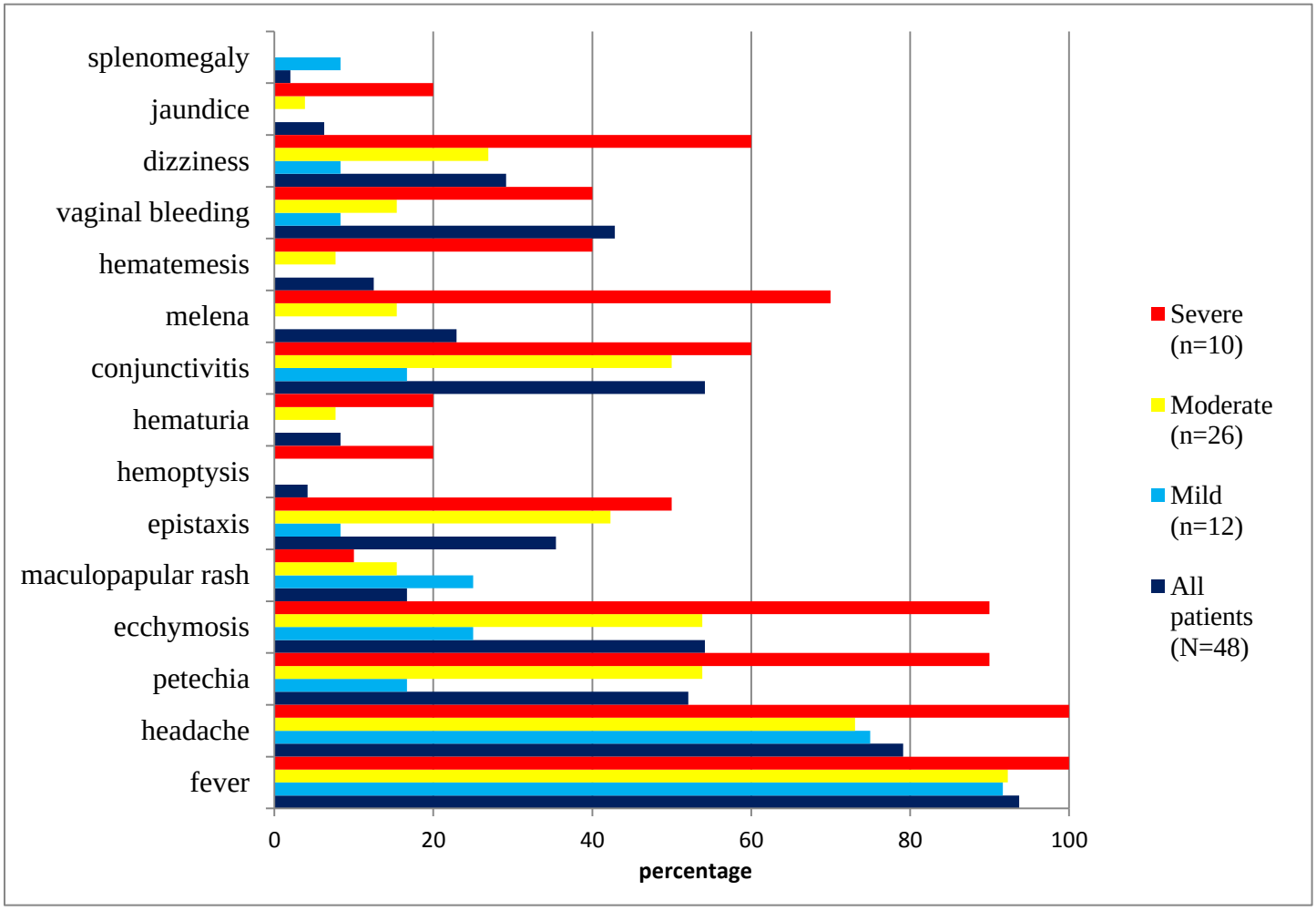

Figure 3.2 Clinical findings of CCHF patients in different severity groups.

According to clinical findings, fever and headache were the most common symptoms of the patients in all severity groups. Disorders related with hemorrhage such as petechia (p=0.03), ecchymosis (p=0.04), melena (p=0.001) were more common in severe cases when compared with other groups.(figure 3.2)

All of the patients had leukopenia, thrombocytopenia, and elevated aspartate transferase (AST), alanine transferase (ALT), lactic dehydrogenase (LDH), and creatinine phosphokinase (CPK) levels. AST (p=0.001), ALT (p= 0.003), and LDH (p=0.000) levels were significantly higher; prothrombin time (pt) (p=0.000) and activated partial thromboplastin time (aPTT) (p=0.000) were significantly longer; and fibrinogen (FBG) (p=0.000) levels were significantly lower in the patients with severe CCHF, compared with those in the patients with mild and moderate groups (Table 3.2). Platelet counts (plt) were low and creatin phosphate kinase (CPK) levels were high in all groups. (Figure 3.3)

Table 3.2. Pathological laboratory findings for 48 patients with mild, moderate or severe CCHF.

Characteristic	**All patients (N=48)**	**Mild (n=12)**	**Moderate (n=27)**	**Severe (n=9)**
Highest AST (U/L)	(25-2404)/360,5	(25-368)/98	(64-2404)/360,5	(363-2053)/953
Highest ALT (U/L)	(15-1037)/169	(15-292)/67,5	(34-1037)/183,5	(158-1015)/451
Lowest platelets($x10^3$/mL)	(4-130)/17	(29-130)/62	(4-114)/15	(8-34)/11,5
Lowest WBCs($x10^3/mm^3$)	(900-10500)/1650	(1100-2700)/1850	(900-2600)/1450	(1300-10500)/1850
Highest LDH (U/L)	(149-7559)/776,5	(149-767)/295	(269-4596)/860	(901-7559)/2591,5
Highest CPK (U/L)	(53-10177)/655,5	(68-1694)/234,5	(53-4010)/744,5	(261-10177)/929,5
Lowest PT(sec)	(11-32)/13	(11-16)/12	(11-23)/13,5	(13-32)/20,5
Lowest aPTT(sec)	(26-152)/42	(25-41)/32	(27-78)/44,5	(33-152)/96,5
Lowest FBG (g/L)	(64-382)/224,5	(218-382)/241	(64-349)/209,5	(75-272)125,5

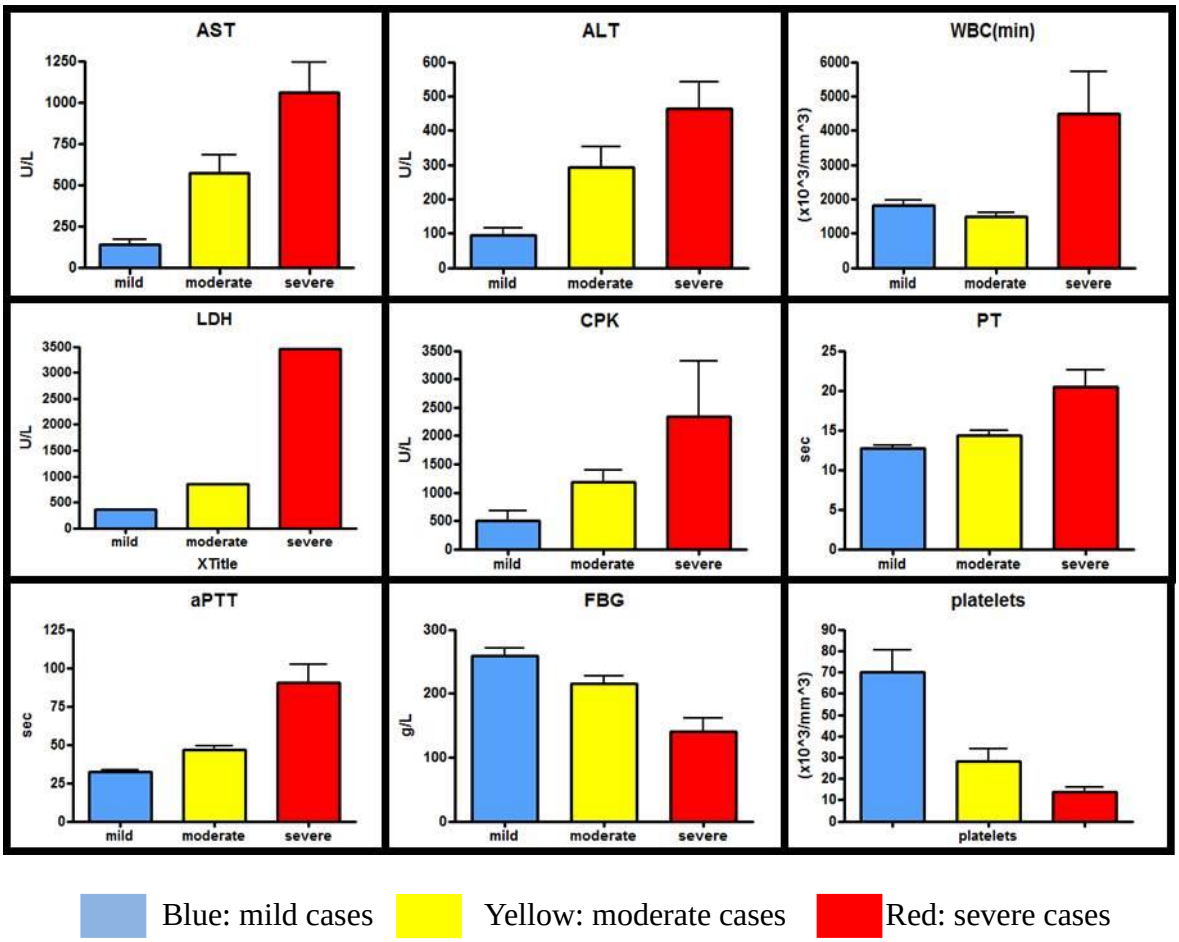

Figure 3.3. Laboratory findings of the patients in different severity groups.

We studied the cytokine profile of the patients with CCHF in order to compare mild, moderate and severe cases during the acute phase of the disease. Table 3.4 shows data of plasma cytokine contents of the patients, which were sorted in four groups according to their reported function.

Table 3.3 Cytokine levels of the serum samples of the patiens with different severity groups.

cytokines		Disease severity groups		
		Mild (range)/median	Moderate (range)/median	Severe (range)/median
inflammatory cytokines	IL-1β	(0.04-64.60)/0.61	(0.09-73.36)/1.44	(0.28-1.81)/0.47
	TNF-α	(0.37-17.25)/3.91	(0.11-42.14)/3.40	(0.31-4.75)/1.09
	IFN-γ	(8.66-454.29)/42.19	(6.41-3922.14)/37.04	(12.67-48.20)/44.24
	IL-12	(0.04-205.38)/2.61	(0.14-97.69)/2.71	(0.14-16.95)/0.61
humoral cytokines	IL-4	(0.04-0-76)/0.59	(0.02-3.67)/0.58	(0.03-0.78)/0.59
	IL-5	(0.19-2.05)/0.26	(0.18-6.77)/0.19	(0.19-1.61)/0.23
	IL-6	(0.14-949.57)/7.30	(0.80-451.78)/10.80	(0.49-101.85)/18.41
	IL-10	(2.84-162.11)/24.19	(1.18-146.56)/19.79	(17.91-112.36)/47.33
	IL-13	(0.04-4.74)/0.29	(0.02-34.01)/1.22	(0.06-14.12)/0.78
growth & cellular cytokines	IL-2	(0.05-46.28)/1.17	(0.08-31.41)/1.16	(0.17-28.83)/0.63
	IL-7	(0.60-165.91)/2.10	(0.07-32.01)/1.30	(0.03-9.68)/0.47
	IL-17A	(0.86-1.92)/1.46	(0.70-10.27)/1.04	(0.70-1.809/1.29
	G-CSF	(0.23-16.25)/1.51	(0.15-40.40)/2.98	(0.10-8.47)/2.68
	GM-CSF	(0.21-78.99)/16.95	(0.53-236.23)/35.52	(10.10-90.26)/25.20
chemokines	IL-8	(2.20-619.24)/17.11	(2.00-111.49)/12.30	(5.63-61.99)/13.53
	MCP-1	(20.35-1131.33)/74.72	(3.32-1558.48)/97.01	(5.62-286.65)/44.23
	MIP-1β	(36.09-1869.77)/194.51	(3.38-2091.55)/58.29	(25.95-1923.95)/45.40

We found that IFN-γ, IL-6, GM-CSF, IL-8, MCP-1 and MIP-1β levels were elevated during acute phase of the disease. The ranges and median values are indicated in Table 3.3.

We observed that cytokine concentrations of MIP-1β, which is classified under chemokines, were significantly increased during mild CCHF infection when compared with moderate and severe CCHF cases. ($p=0.02$) Also, cytokine concentrations of IL-7 were significantly increased during mild CCHF infection when compared with moderate and severe CCHF cases. ($p<0.04$)

None of the inflammatory cytokines showed significant increase in different severity groups. Among humoral cytokines, IL-13 significantly increased in severe and moderate cases. (p= 0.02)

Ranges of four groups of cytokines and their median values are given in table 3.3 and figure 3.4.

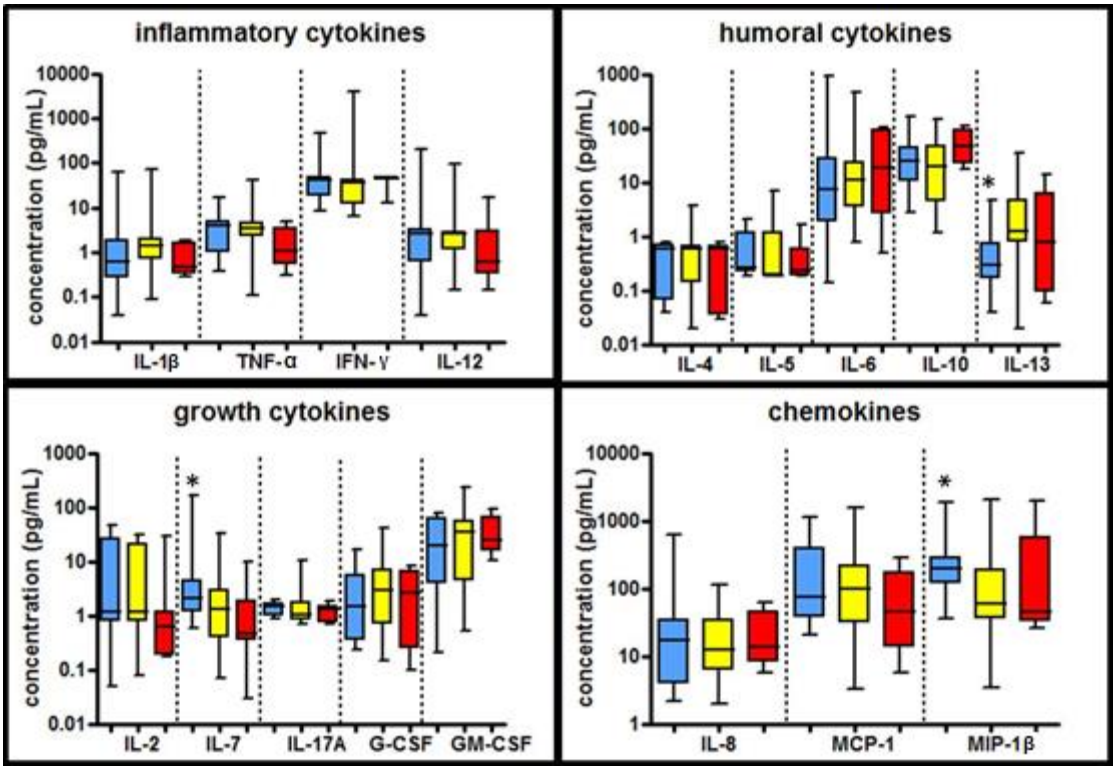

Figure 3.4. Ranges of four cytokine groups and their median values.

Table 3.4. Ranges and median values of each cytokine in different stages of the disease.

cytokines	mild			
	I	II	III	IV
IL-1β	(0.04-2.43)/1.24	(0.20-64.60)/0.61	(0.07-900.00)/2.30	(0.68-3.90)/1.84
IL-2	(0.05-46.28)/23.17	(0.62-70.21)/1.66	(0.03-40.94)/1.51	(0.40-21.63)/0.83
IL-4	(0.04-0.30)/0.17	(0.02-0.76)/0.37	(0.02-2.10)/0.33	(0.04-0.78)/0.62
IL-5	(0.27-2.05)/1.16	(0.19-2.05)/0.24	(0.19-1.15)/0.24	(0.19-1.15)/0.21
IL-6	(9.05-110.14)/59.60	(0.14-949.57)/7.30	(0.98-243.64)/61.58	(0.99-78.59)/1.35
IL-7	(1.83-91.03)/46.43	(0.20-189.83)/1.30	(0.29-69.65)/1.47	(1.21-6.69)/4.69
IL-8	(18.80-33.39)/26.10	(2.20-619.24)/21.40	(3.45-173.93)/84.39	(12.41-86.96)/16.17
IL-10	(20.98-110.62)/65.80	(1.00-293.02)/16.57	(0.66-79.70)/1.51	(0.91-80.47)/7.28
IL-12	(1.37-205.38)/103.38	(0.04-318.52)/2.63	(0.14-163.91)/2.65	(0.34-3.68)/1.61
IL-13	(0.32-4.74)/2.53	(0.04-2.95)/0.23	(0.06-1.19)/0.37	(0.04-1.13)/0.49
IL-17A	(0.97-1.57)/1.27	(0.48-1.92)/1.29	(0.00-20.42)/1.51	(0.90-1.96)/1.11

G-CSF	(0.32-6.89)/3.61	(0.12-16.25)/1.51	(0.12-198.36)/2.06	(0.12-4.55)/0.65
GM-CSF	(15.38-77.46)/46.42	(0.21-311.95)/28.19	(4.67-385.60)/37.74	(2.58-81.28)/2.58
IFN-γ	(35.46-168.11)/101.79	(8.66-576.28)/42.96	(8.82-1511.99)/46.58	(10.74-43.73)/38.91
MCP-1	(95.91-878.91)/478.43	(20.35-1131.33)/53.85	(5.81-700.00)/51.26	(14.24-71.08)/35.27
MIP-1β	(181.97-1869.77)/1025.87	(36.09-2233.75)/210.30	(29.39-1728.39)/727.10	(36.25-832.47)/97.90
TNF-α	(0.43-5.64)/3.04	(0.35-17.25)/4.55	(0.59-92.22)/4.14	(1.76-4.75)/3.26
	moderate			
	I	II	III	IV
IL-1β	(0.37-2.01)/1.13	(0.006-73.36)/1.37	(0.21-60.34)/0.58	(0.62-30.26)/0.62
IL-2	(0.46-22.14)/0.91	(0.03-31.41)/0.91	(0.18-31.92)/9.35	(0.13-30.89)/2.71
IL-4	(0.04-1.05)/0.53	(0.02-3.67)/0.57	(0.03-0.81)/0.48	(0.00-1.37)/0.57
IL-5	(0.19-2.05)/0.20	(0.18-6.77)/0.21	(0.19-1.61)/0.21	(0.19-2.27)/0.20
IL-6	(6.49-78.56)/12.00	(0.80-26.08)/8.28	(0.09-322.23)/4.45	(0.01-451.78)/6.80
IL-7	(0.46-32.01)/5.54	(0.07-5.54)/0.54	(0.03-2.96)/0.68	(0.07-5.26)/1.78
IL-8	(5.13-44.53)/12.49	(2.00-622.89)/14.21	(2.49-189.10)/9.84	(2.64-573.89)/55.37
IL-10	(1.64-146.56)/55.55	(1.18-137.17)/12.09	(0.89-91.61)/8.43	(1.00-28.90)/5.20
IL-12	(0.46-5.39)/2.81	(0.14-97.69)/2.55	(0.04-9.67)/1.82	(0.04-4.68)/2.56
IL-13	(0.33-29.39)/1.23	(0.02-34.10)/1.09	(0.02-7.49)/0.31	(0.06-1.79)/0.38
IL-17A	(0.86-10.27)/1.32	(0.70-1.88)/1.49	(0.26-1.96)/1.55	(0.07-2.31)/1.61
G-CSF	(0.23-9.04)/2.98	(0.15-40.40)/1.45	(0.12-59.04)/4.63	(0.12-85.09)/4.00
GM-CSF	(4.88-236.23)/76.16	(0.53-81.12)/7.01	(1.57-138.40)/8.21	(0.62-135.50)/5.24
IFN-γ	(12.03-285.83)/37.56	(0.41-3922.14)/36.27	(6.09-477.56)/26.10	(6.25-288.08)/13.39
MCP-1	(53.98-232.30)/91.80	(3.32-1558.48)/124.73	(3.57-598.14)/22.38	(6.08-996.92)/26.38
MIP-1β	(51.34-1078.31)/122.66	(7.93-540.97)/70.41	(3.38-458.45)/42.59	(22.95-2091.55)/95.53
TNF-α	(0.11-9.37)/3.13	(0.11-11.81)/3.26	(0.33-11.34)/3.75	(0.33-54.25)/4.45
	severe			
	I	II	III	IV
IL-1β	(0.28-1.81)/0.35	(0.24-1.74)/0.38	(0.31-1.94)/1.54	(0.37-1.81)/1.09
IL-2	(0.19-1.14)/0.63	(0.17-28.83)/0.41	(0.17-23.68)/1.11	(0.03-1.07)/0.55
IL-4	(0.03-0.65)/0.04	(0.03-0.78)/0.05	(0.03-0.76)/0.57	(0.63-0.78)/0.71
IL-5	(0.23-1.61)/0.23	(0.19-0.24)/0.22	(0.19-0.92)/0.21	(0.19-0.19)/0.19
IL-6	(1.69-93.60)/87.96	(0.49-101.85)/4.83	(0.66-106.34)/11.33	(5.66-18.32)/11.99
IL-7	(0.47-9.68)/0.47	(0.29-1.69)/0.45	(0.03-2.10)/1.26	(2.37-3.78)/3.08
IL-8	(6.79-61.99)/39.78	(5.63-47.57)/9.40	(9.34-42.78)/13.53	(4.70-90.57)/25.35
IL-10	(17.91-112.36)/87.20	(7.65-93.32)/17.00	(9.32-71.28)/25.57	(4.43-8.65)/6.54
IL-12	(0.34-3.14)/0.45	(0.14-16.95)/0.33	(0.31-7.73)/2.71	(2.74-13.84)/8.29
IL-13	(0.11-14.12)/0.71	(0.07-7.94)/0.76	(0.06-22.64)/2.75	(0.05-1.09)/0.57
IL-17A	(0.74-1.45)/1.32	(0.79-1.80)/1.33	(0.70-1.51)/0.77	(0.75-1.92)/0.93
G-CSF	(0.10-8.38)/0.12	(0.07-8.47)/3.53	(0.38-110.53)/2.35	(0.24-4.95)/2.60
GM-CSF	(14.86-48.71)/21.13	(25.20-90.26)/41.54	(6.94-73.50)/38.91	(40.96-80.82)/60.89
IFN-γ	(44.24-48.20)/45.45	(12.67-48.89)/32.38	(12.51-47.37)/45.28	(12.19-40.64)/26.41
MCP-1	(5.62-286.65)/161.48	(15.43-62.38)/41.34	(5.25-181.86)/47.88	(56.96-67.26)/62.11

MIP-1β	(26.87-1073.15)/42.95	(20.06-65.45)/44.87	(25.95-1923.95)/36.21	(42.41-77.52)/59.97
TNF-α	(0.31-3.53)/0.54	(0.35-4.75)/0.48	(0.54-4.35)/2.11	(3.26-4.75)/4.01

Figures 3.5-3.21 show the cytokine profiles of the patients' serum samples at the different stages of the acute disease.

As indicated in Figure 3.20, MIP-1β concentrations were both high in mild cases at the stages II (p=0.009) and III (p=0.03).

IL-7 concentrations were also higher in mild cases at disease stage III. (p=0.02) (Figure 3.10)

IL-13 concentration was found to be increased significantly in severe cases in stage II (p=0.02) and IL-10 concentrations were also high in stage III (p= 0.04) for severe cases, although no significant difference was detected in the first serum samples. (Figure 3.14, Figure 3.12)

There were no significant difference observed between the concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-17A, G-CSF, GM-CSF, MCP-1,IFN-γ and TNF-α cytokines in any disease stage.

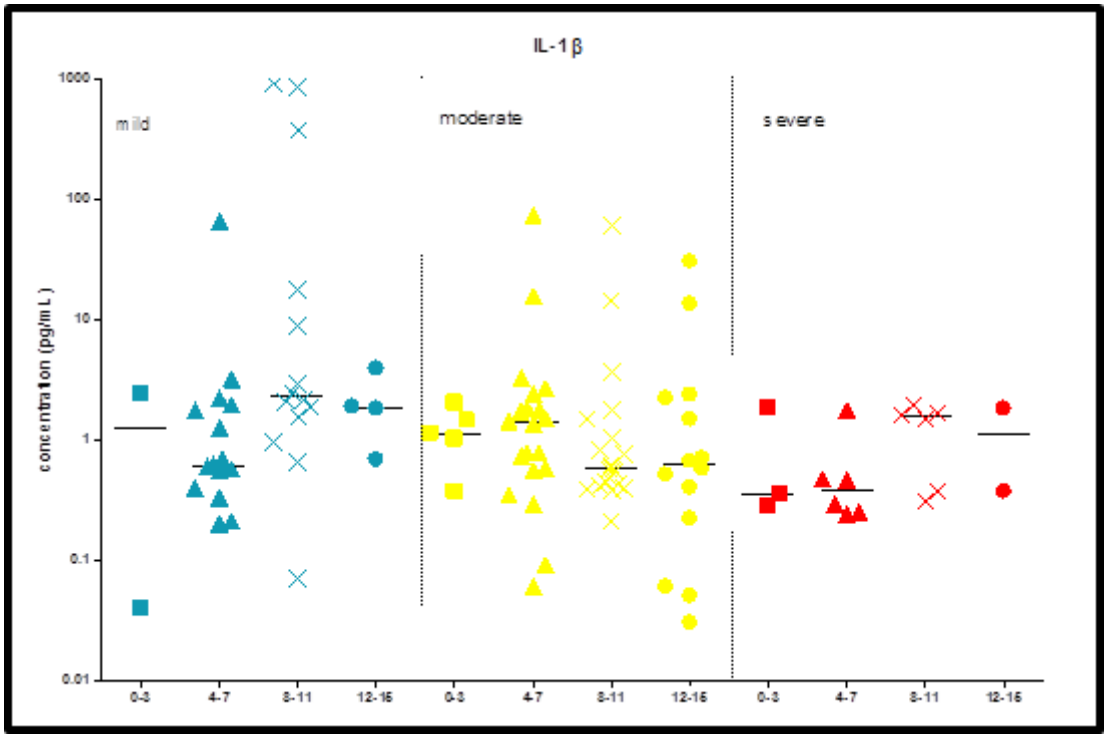

Figure 3.5. IL-1β plot for all severity groups in different stages.

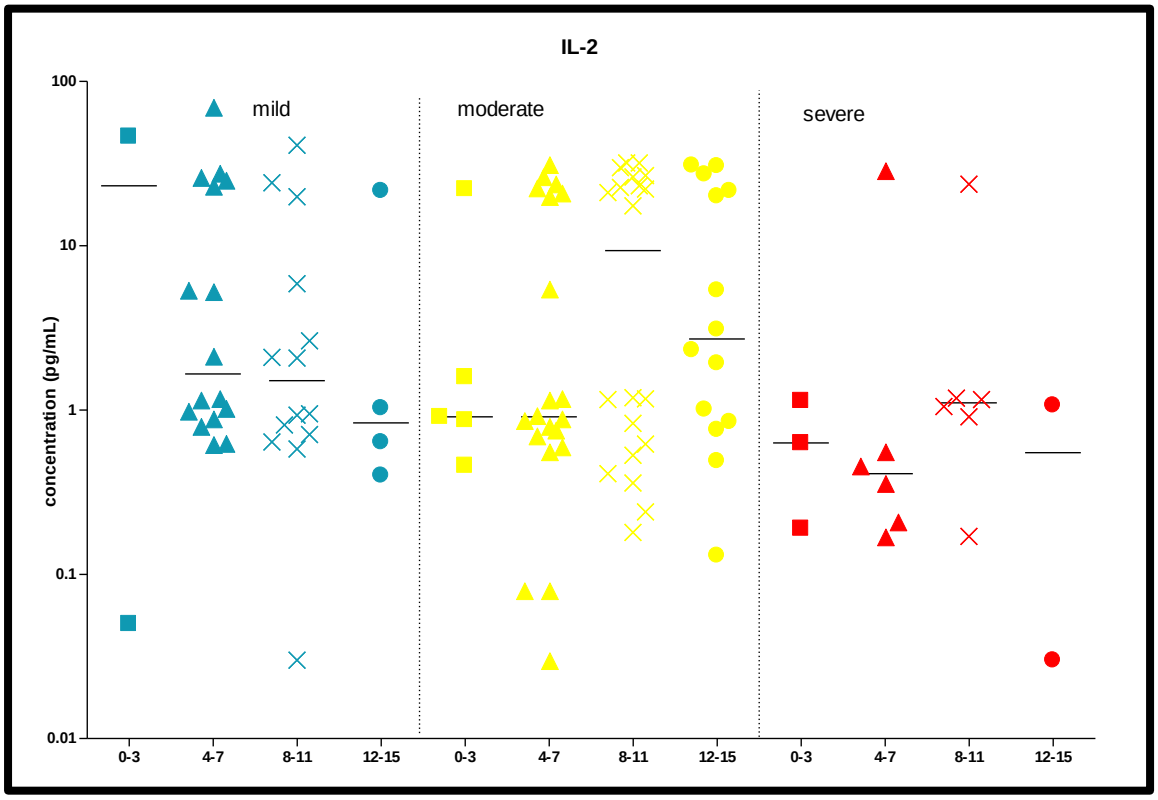

Figure 3.6. IL-2 plot for all severity groups in different stages.

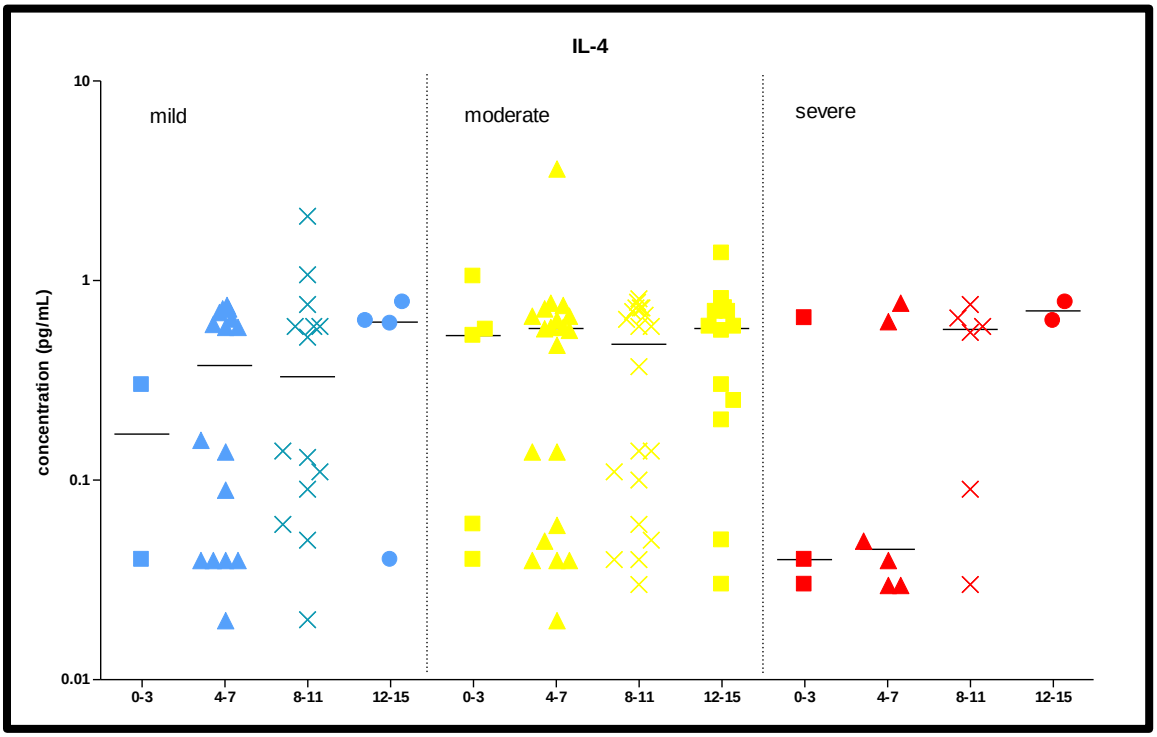

Figure 3.7. IL-4 plot for all severity groups in different stages.

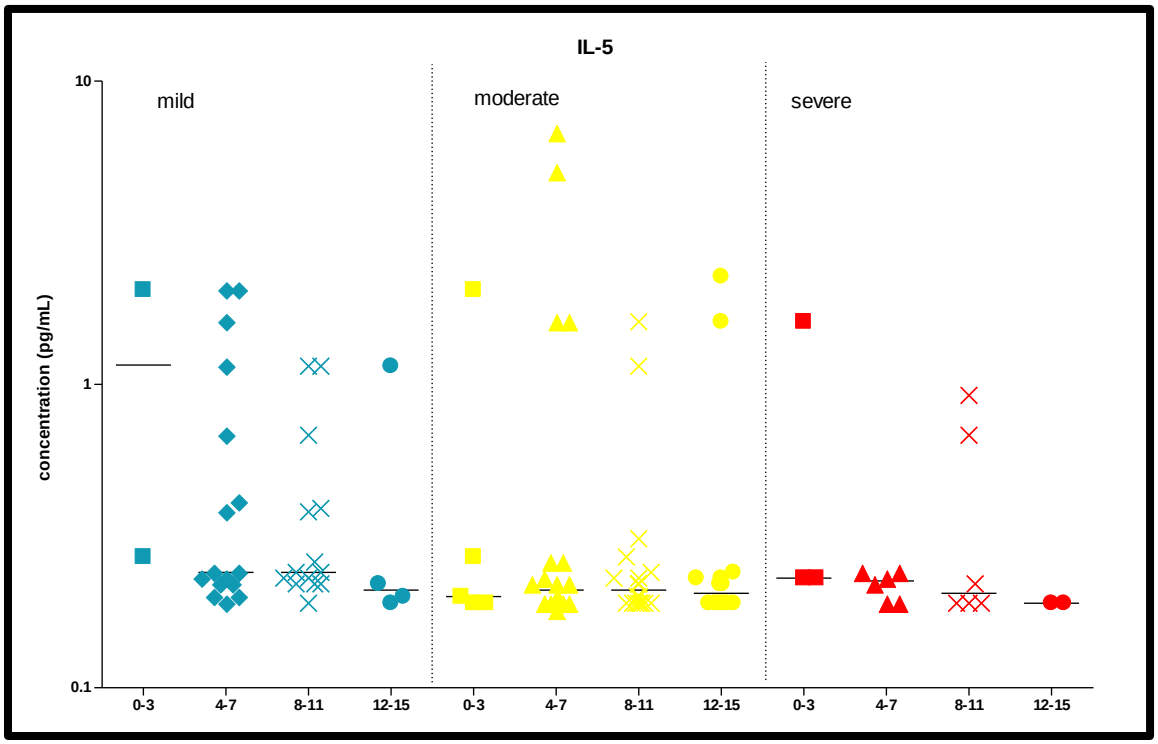

Figure 3.8. IL-5 plot for all severity groups in different stages.

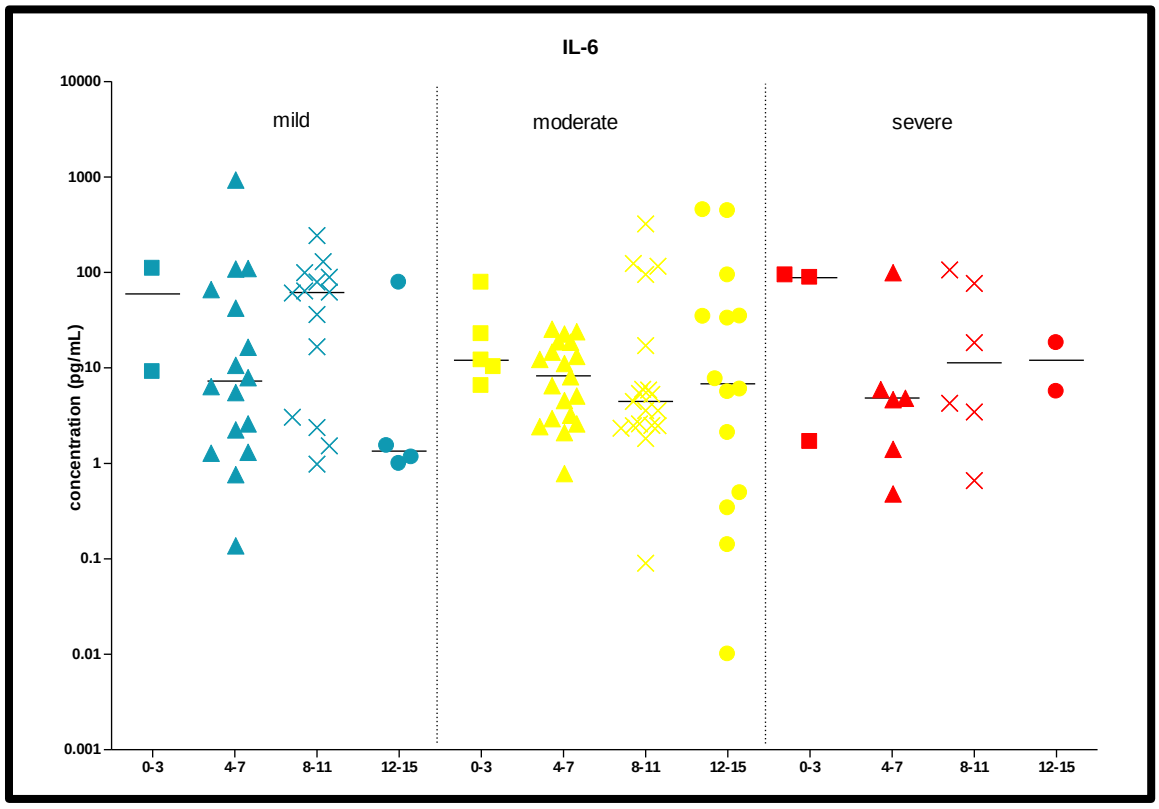

Figure 3.9. IL-6 plot for all severity groups in different stages.

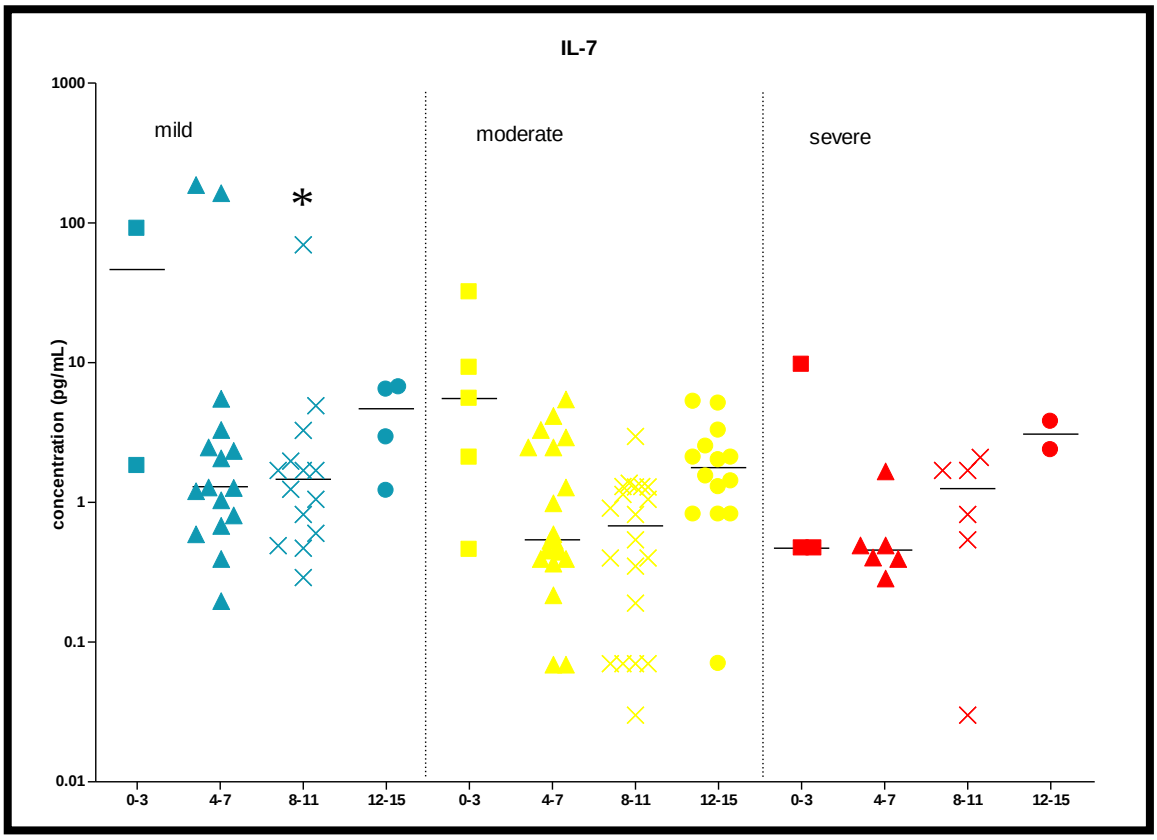

Figure 3.10. IL-7 plot for all severity groups in different stages.

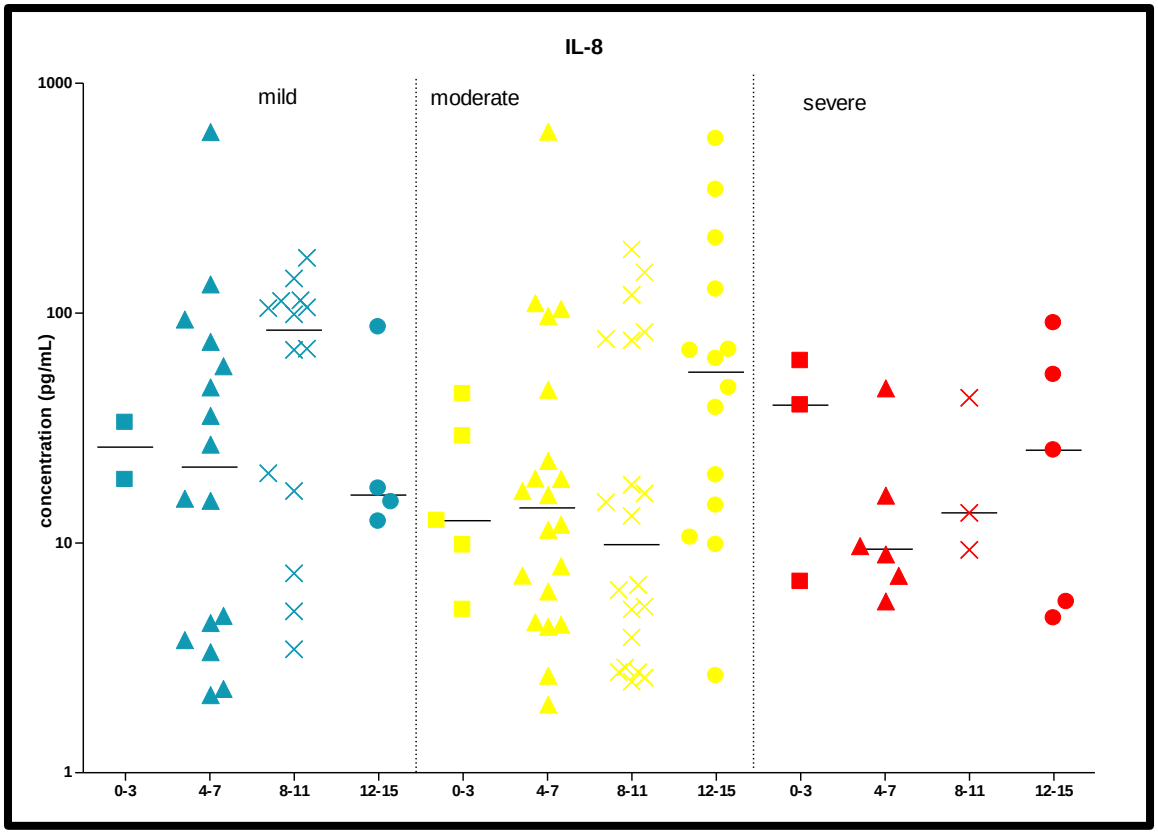

Figure 3.11. IL-8 plot for all severity groups in different stages.

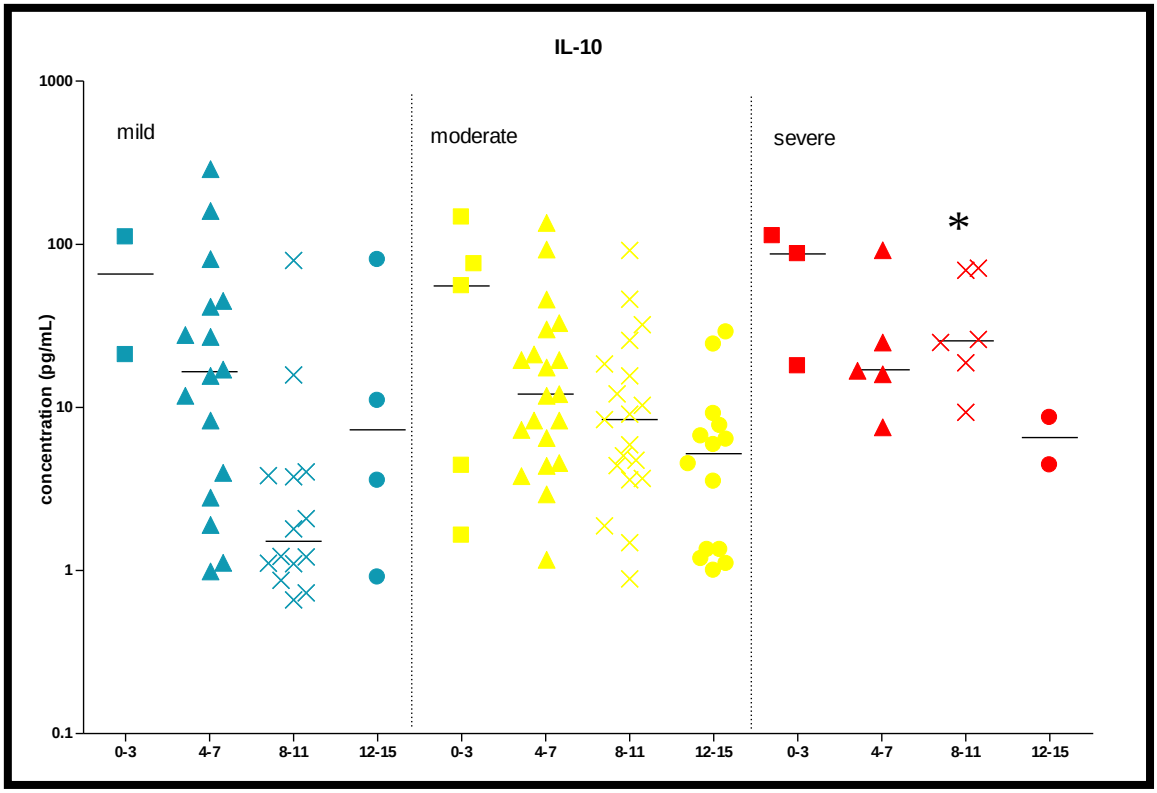

Figure 3.12. IL-10 plot for all severity groups in different stages.

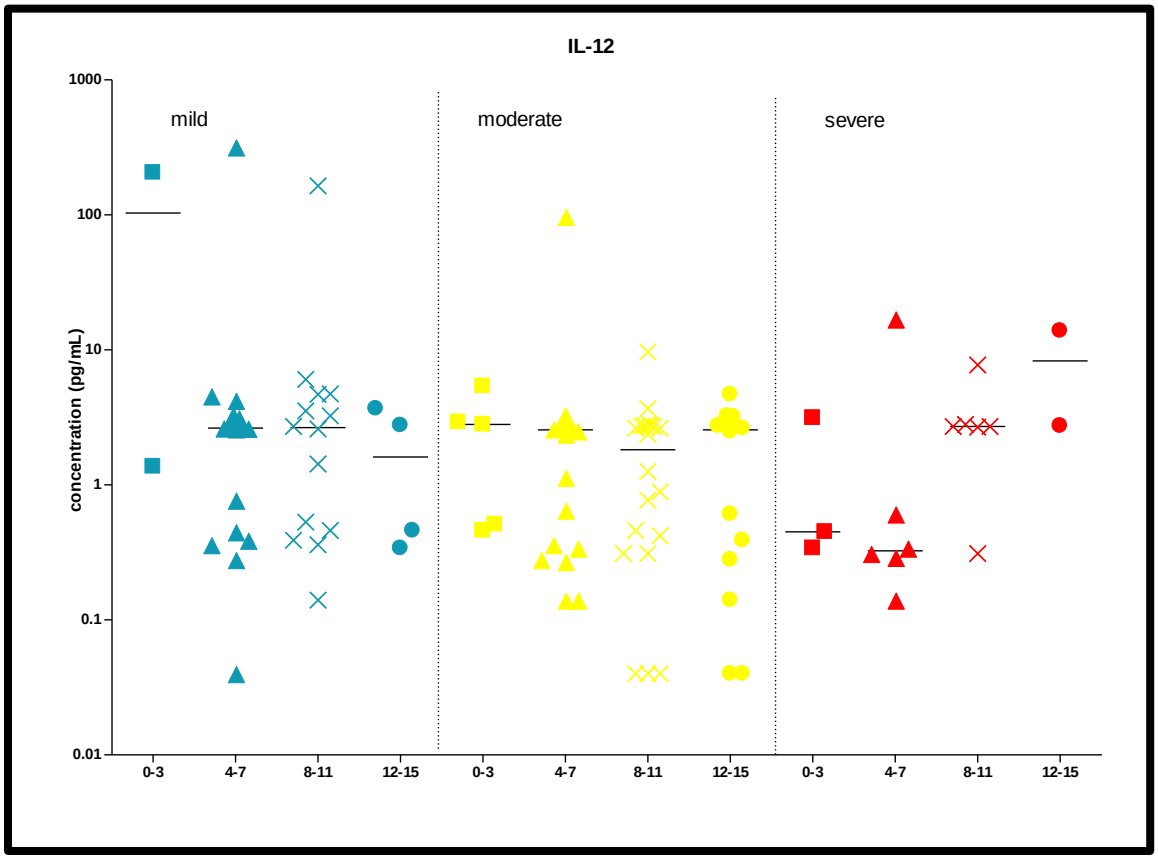

Figure 3.13. IL-12 plot for all severity groups in different stages.

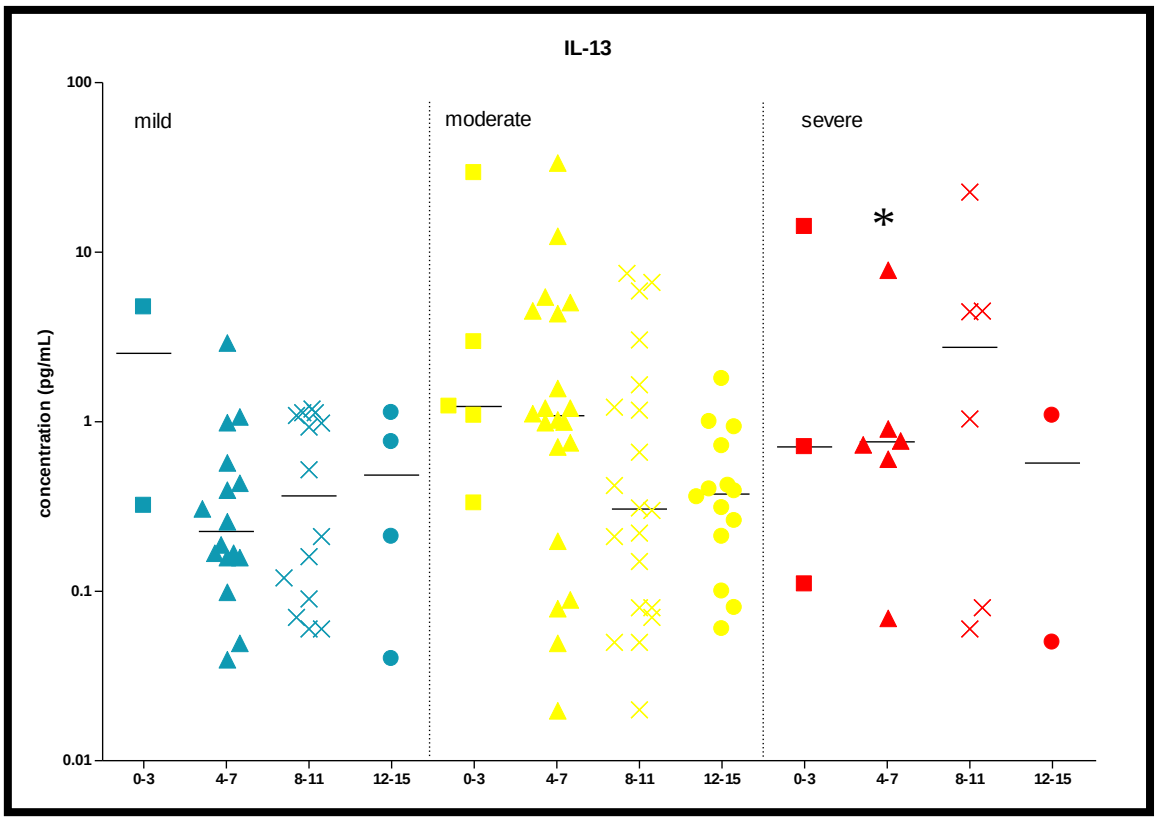

Figure 3.14. IL-13 plot for all severity groups in different stages.

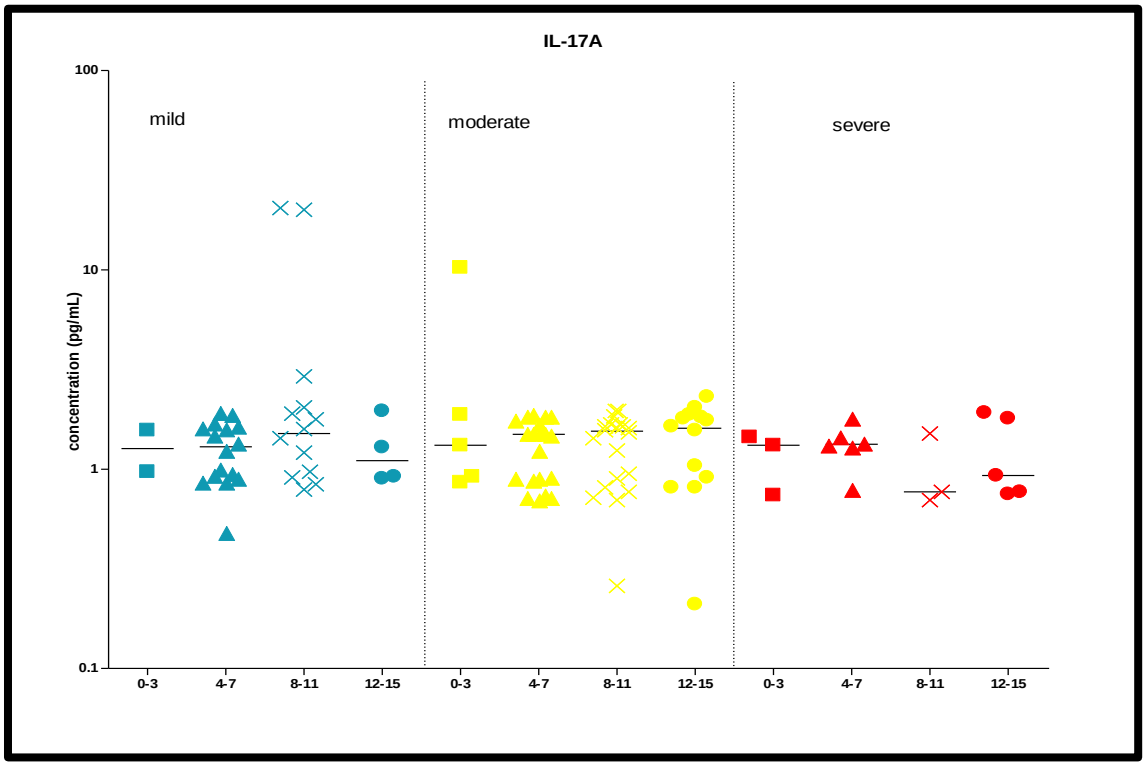

Figure 3.15. IL-17A plot for all severity groups in different stages.

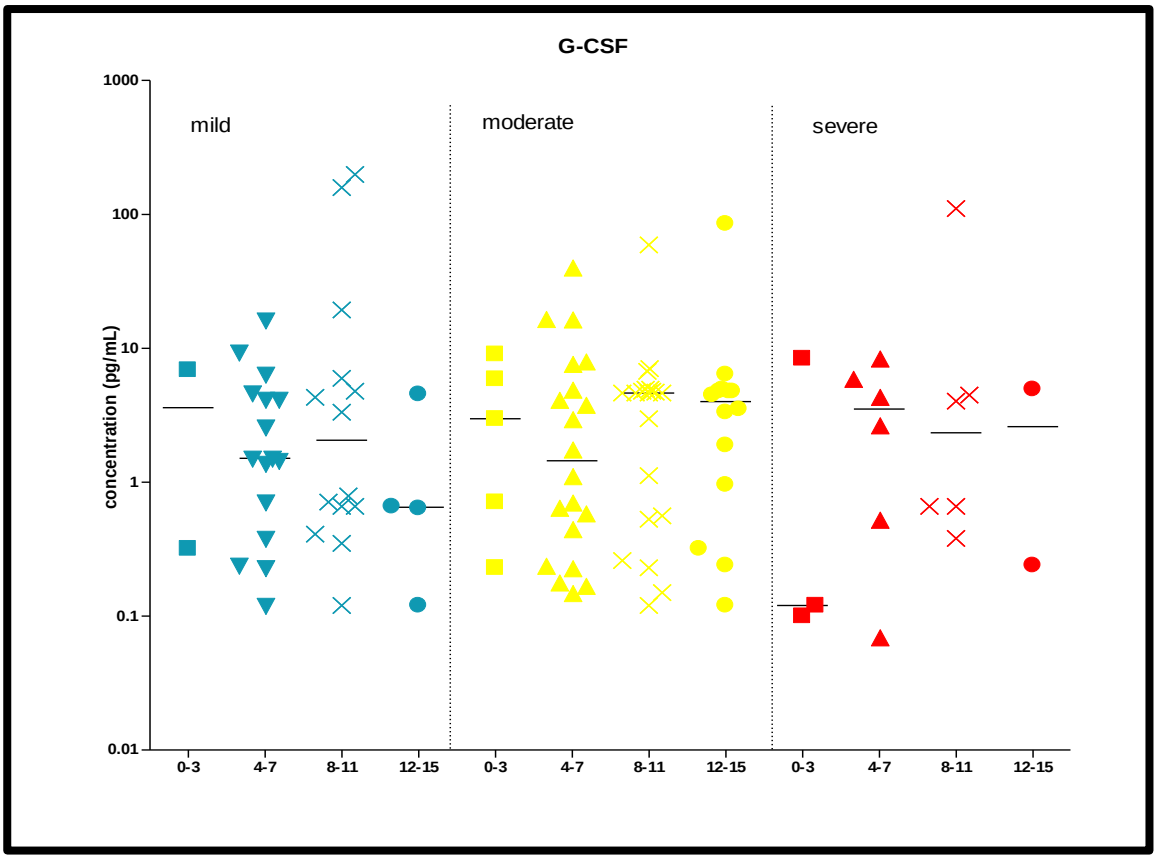

Figure 3.16. G-CSF plot for all severity groups in different stages.

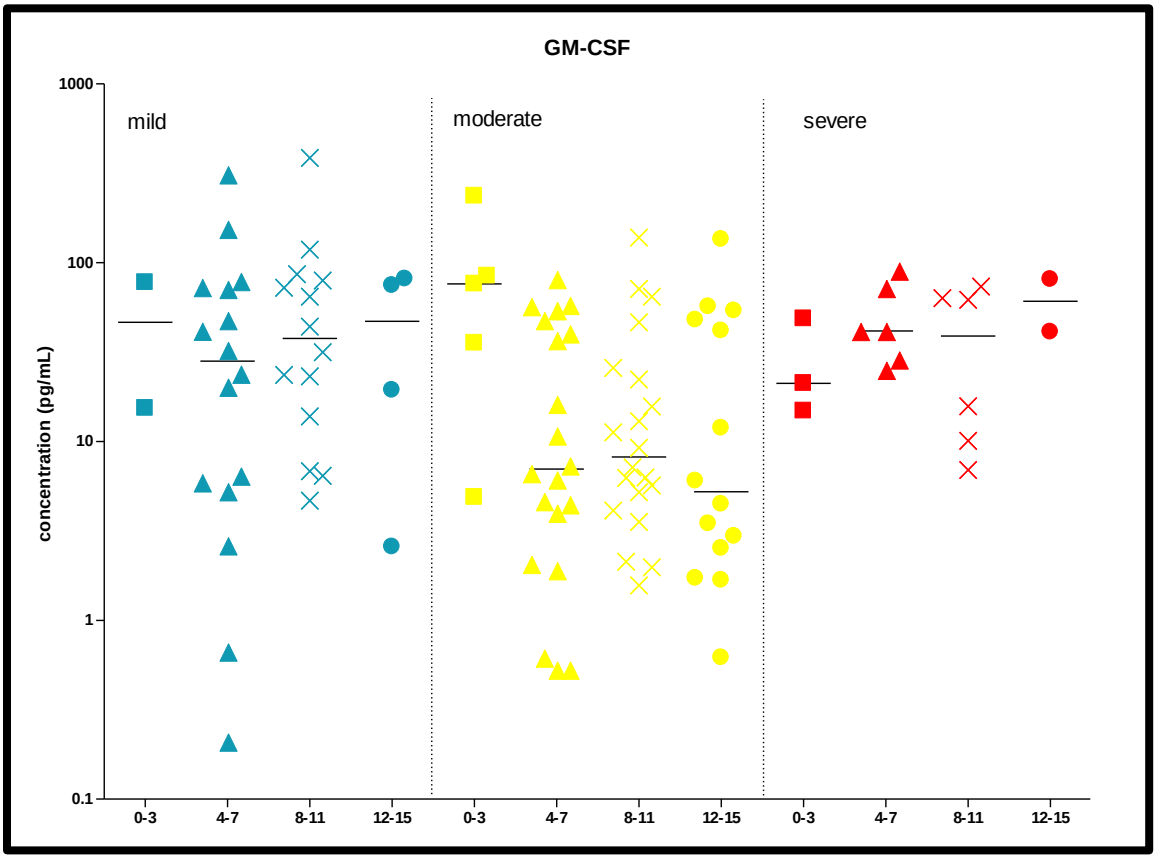

Figure 3.17. GM-CSF plot for all severity groups in different stages.

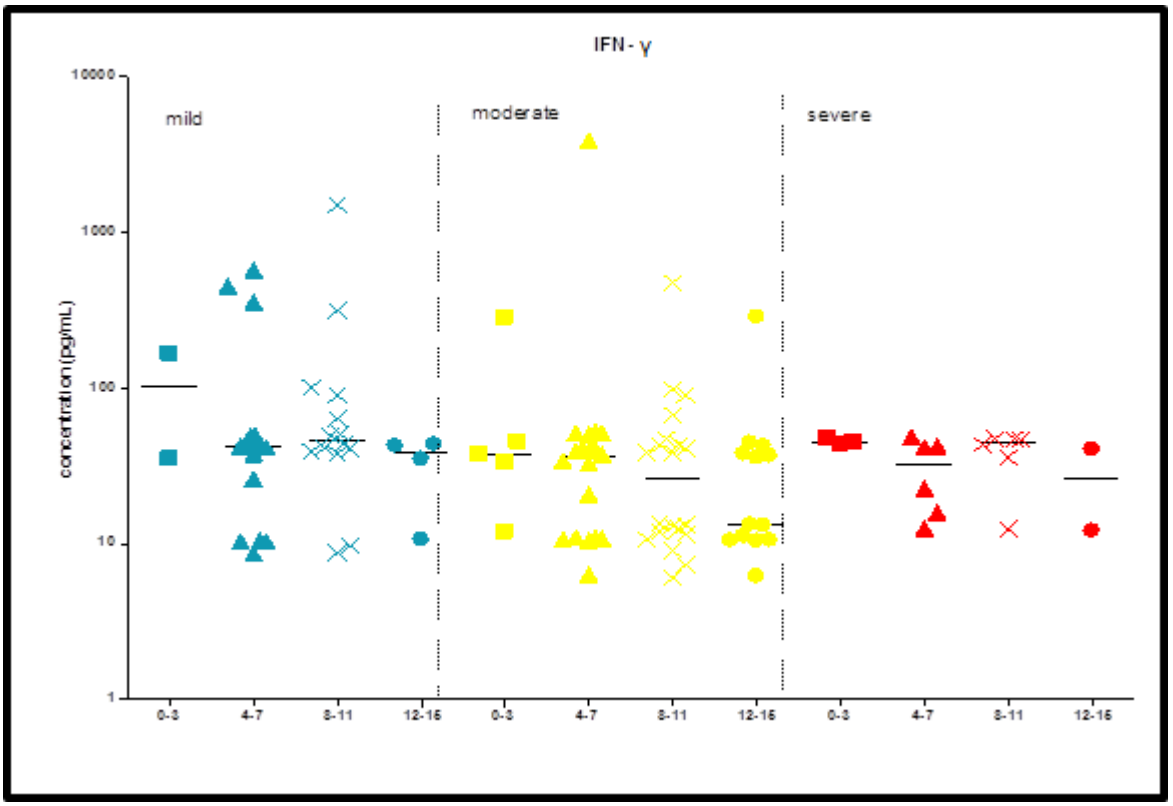

Figure 3.18. IFN-γ plot for all severity groups in different stages.

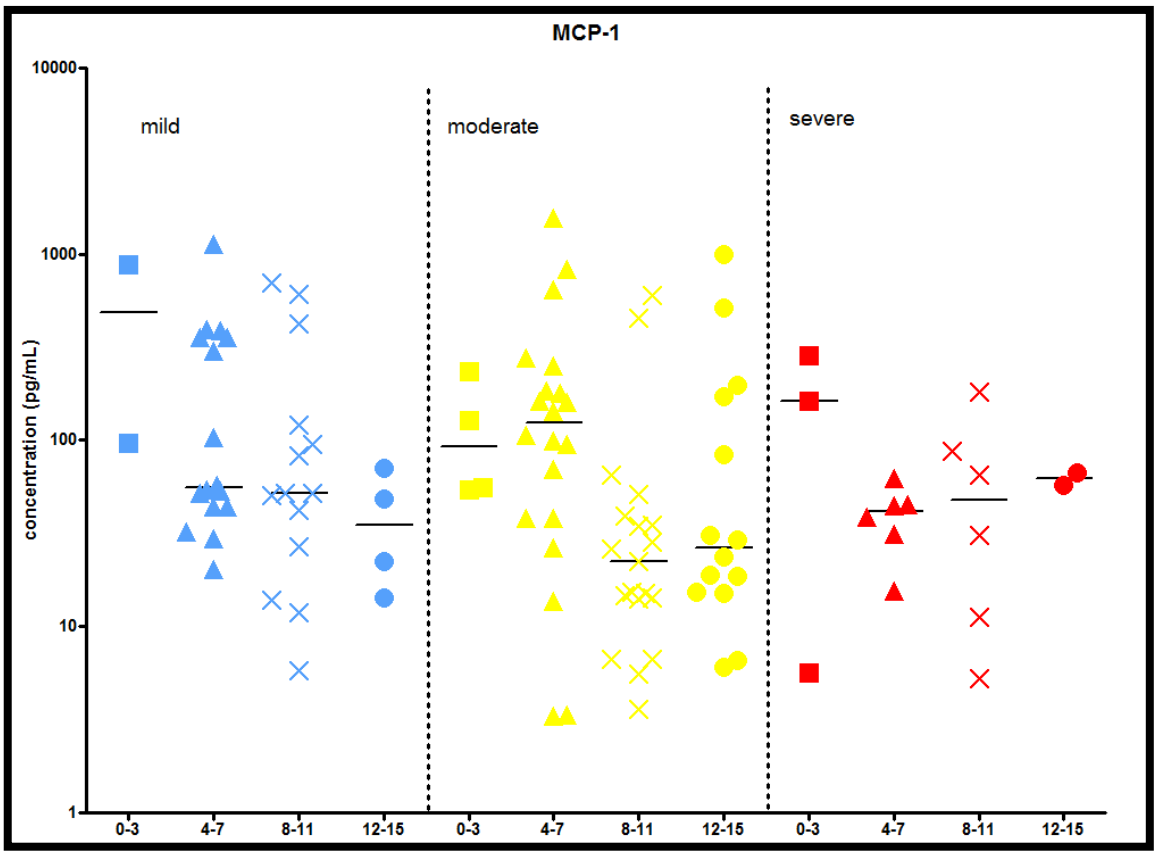

Figure 3.19. MCP-1 plot for all severity groups in different stages.

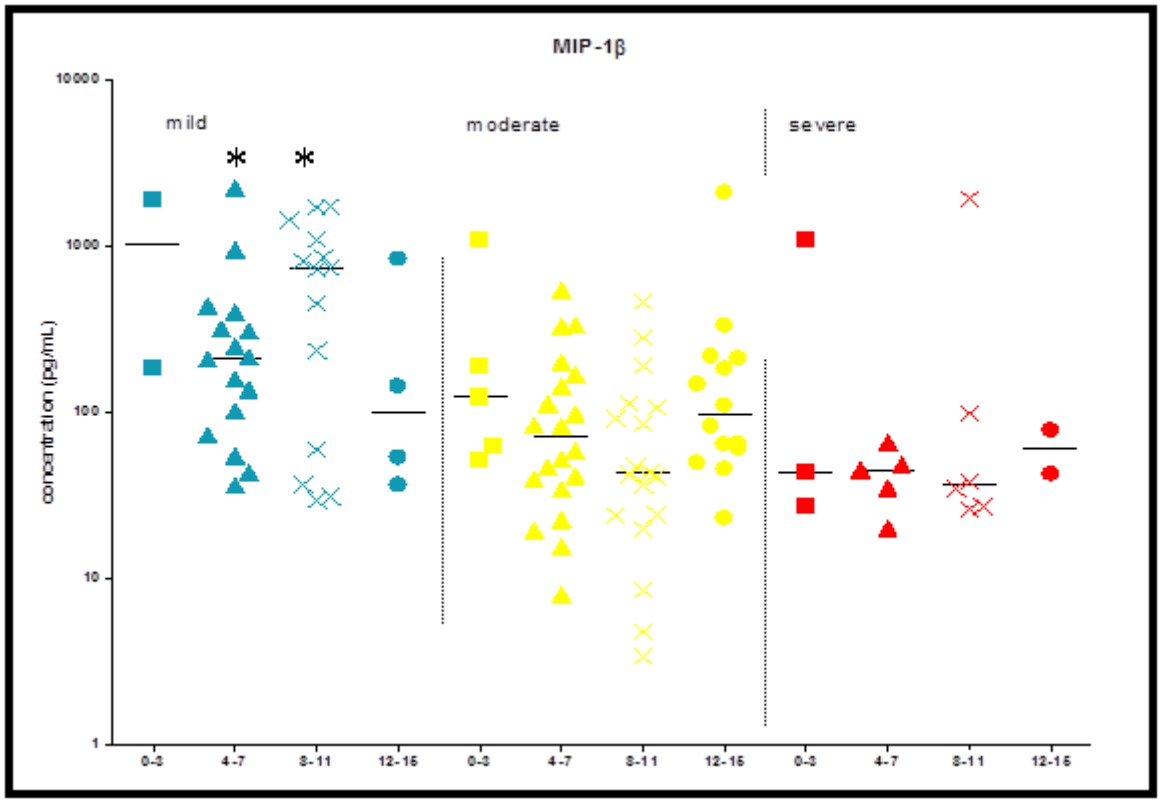

Figure 3.20. MIP-1β plot for all severity groups in different stages.

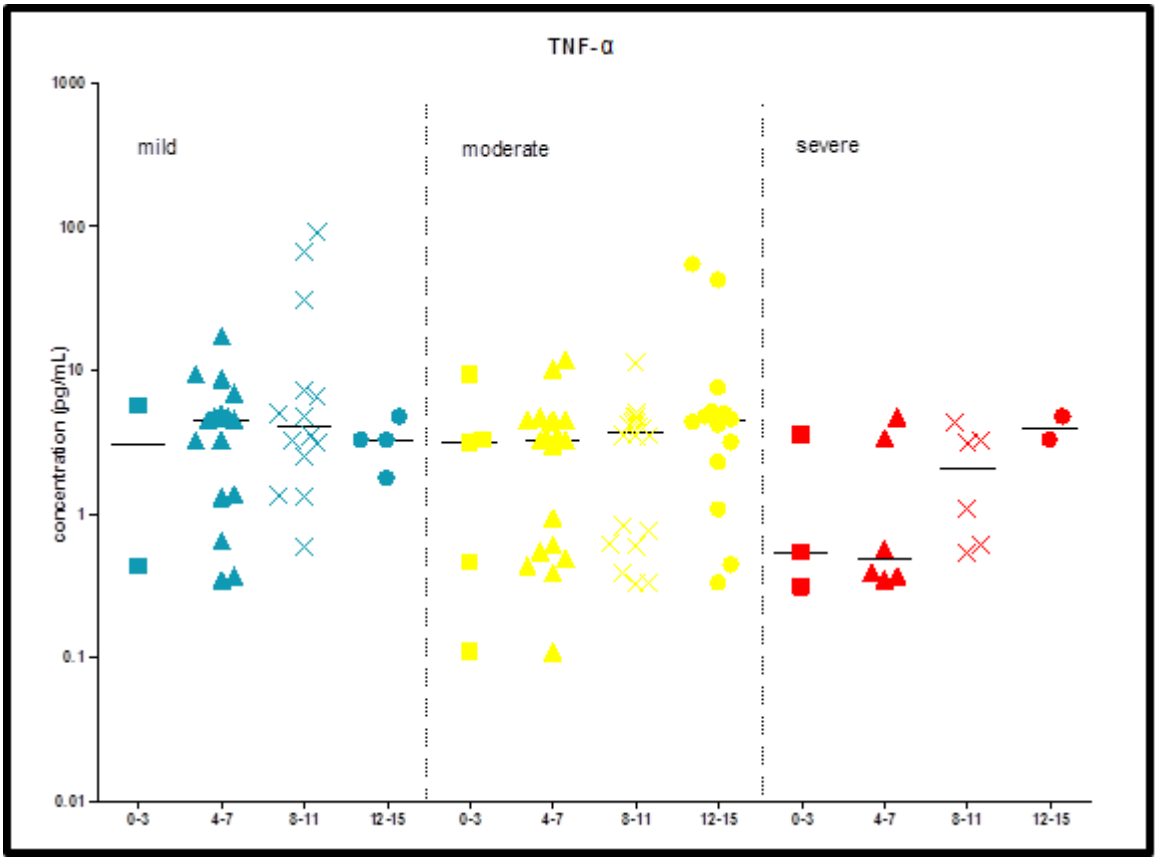

Figure 3.21. TNF-α plot for all severity groups in different stages.

4. Discussion

Pathogenesis of CCHF is yet not clearly understood. Like other hemorrhagic fever virus, CCHFV targets the cells that induce antiviral immune response, most likely leading to overwhelming virus particles and vascular dysregulation (100).

One of the main characteristics of the viral hemorrhagic fevers is the impairment of vascular tissue leading to changes in vascular permeability and hemorrhage. However, the endothelial damage is most likely is not due to the direct effect of virus replication but because of the various host induced immune mechanisms such as cytokine storm (74). IL-1β, TNF-α, IL-6 have been shown to be major triggers and regulators of inflammatory responses to microbial pathogens, inducing cytokine release, endothelial cell activation, acute phase protein synthesis and fever. MCP-1, MIP-1β have a role in recruitment of the cells to the site of infection and also enhance the capacity of these cells to adhere to the vascular endothelium (101).

Some of the recent studies about cytokines in CCHF patients showed that cytokines may play a role in the pathogenesis of the disease (76). In a study in Turkish patients, TNF-α, IL-6, IL-1β have been reported to be associated with disease severity. Serum concentrations of proinflammatory cytokines IL-6 and TNF- α were significantly higher in fatal patients than patiens with nonfatal CCHF, although there was no significant difference in the levels of IL-10 between the two groups (76). On the contrary, Saksida et al showed that significant increase in the levels of IL-10 and TNF-α in fatal CCHF cases than moderate and severe cases. Increased levels of IL-10 and poor outcome had also been reported in patients with dengue fever and ebola (101). In addition, CCHF infected dentritic cells had been shown to release IL-10 and TNF-α in vitro (102).

In our study, the serum samples that were taken on the day of admission, IL-13 concentration was observed higher in severe patients. IL-10, TNF-α and IL-6 levels did not show any significant increase. Our results were in accordance with the recent studies in Turkey (103, 104). In a study that evaluates cytokines as a Th1/Th2 marker in the CCHF; IL-10 levels were found to be significantly higher in severe patients groups. Whereas, IL-6 and TNF-α levels did not show any difference (104). In another study among children and adult cases IL-2, IL-5, IL-9, IL-12 and IL-13 levels were higher in fatal cases (103). No difference

were detected in TNF-α, IFN-γ, IL-1β, IL-17A, IL-22, IL-10, IL-6 and IL-4 in the patients who died and those who survived. They concluded that milder clinical course in children with CCHF cannot be explained with cytokine network alone.

By this study, we describe the role of MIP-1β cytokine in the pathogenesis of the CCHF for the first time and MIP-1β concentrations were detected significantly higher in mild cases that in moderate and severe cases. MIP-1β is produced not only by human monocytes and dendritic cells induced by different stimuli but also by activated natural killer (NK) cells and lymphocytes. MIP-1β is chemoattractive and activating factor for NK cells, therefore it is effective in the virus clearance mechanisms as they have antiviral cytokine production and cytotoxic activity against infected cells. (78). That finding suggests that MIP-1β can be a good prognostic factor for CCHF infections. Also, IL-7 is an inducer of MIP-1β during the immune response and is known to be released during febrile phase of the disease. In this study, IL-7 concentrations were found to be significantly higher in mild cases than moderate and severe cases.

There is detection bias in the studies performed for cytokine responses of the immune system. Cytokines may produce transient peaks and cannot be detected at the time point of the obtained serum samples. In other words, the major concern is great variability in cytokine levels during disease course. Accordingly, there are inconsistent findings and their comments in cytokine research literature. In order to overcome this obstacle we adjusted the time point for cytokines according to time (days) from the onset of the symptoms. This is one of the strongest features of this study.

Sample size is another limitation in cytokine literature. Since we categorized the patients according to their days of the onset of symptoms, in some groups we suffered from low sample size. On the other hand, our sample size was satisfactory in comparison with other similar reports.

In this study, consequent serum samples from the patients were studied. Our earliest samples from the patients were at the day 2, latest ones were from day 14. We think that in this time period, testing of the cytokines in the serums 3 days apart could catch the cytokine storm peaks. In the stage III of the disease, in severe cases, we observed significant increase of IL-10 levels, altough we could not detect it at the first serum samples. These findings

support our hypothesis that cytokine studies should be conducted with the serum samples that are arranged according to the stages of the disease.

In conclusion, this study showed that IL-7, IL-13 and MIP-1β are increased in CCHF patients. IL-7, MIP-1β are shown to be increased in mild patients whereas, IL-13 is shown to be increased in severe cases. This is the first report of MIP-1β cytokine role in CCHF patogenesis. Because of the inconsistent findings and disagreement related with the cytokines, to evaluate the association of the cytokines with disease pathogenesis, serum samples from the patients should be selected in accordance with the different stages of the disease.

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