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SERUM LEVELS OF CARDIOVASCULAR BIOMARKERS AMONG CHRONIC KIDNEY DISEASE PATIENTS WITH/WITHOUT HEPATITIS C VIRUS INFECTION

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

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HELYAH KANAAN MOHAMMED MOHAMMED

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By Helyah Kanaan Mohammed MOHAMMED

August 2023

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ABSTRACT

SERUM LEVELS OF CARDIOVASCULAR BIOMARKERS AMONG CHRONIC KIDNEY DISEASE PATIENTS WITH/WITHOUT HEPATITIS C VIRUS INFECTION

Helyah Kanaan Mohammed MOHAMMED

Master of Science in Biology

Advisor: Assoc. Prof. Dr. Emin BOZKURT

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August 2023

This case-control study aimed to determine the effect of infection with hepatitis C virus as a risk factor associated with increased incidence of cardiovascular diseases among chronic kidney disease patients. In order to achieve this goal, a total of 180 Iraqi persons (107 males and 73 females; mean age was 51 years) attending both the Center of Renal Disease and Transplantation and the Gastrointestinal Tract Teaching Hospital at Baghdad Medical City Complex were enrolled in the current study. Of them, 130 patients (74 males and 56 females) were infected with the hepatitis C virus and the other 50 persons were controls (33 males and 17 females) clear of any viral existence. Serum specimens were collected from all the participants during the period from June 2022 to the end of August 2022, and data were collected via direct interviews with the participants by using a special questionnaire form. The enzyme-linked immunosorbent assay technique was used to detect the serum levels of the following cardiac dysfunction biomarkers: N-Terminal-pro-brain natriuretic peptide, Interleukin-6, and cardiac Troponin T among all the participants. Data analysis shows that the highest number of participants (55) was recorded in the sixth decade (age 50-59 years). The numbers of males and females were 38 and 17, respectively. Moreover, about 58%, 78%, and 97% of the total number of participants were found to be diabetic, hypertensive, and hypoalbuminemic, respectively. Regarding the cardiac dysfunction biomarkers, data analysis revealed that there were highly statistically significant differences in the mean levels of all the above-mentioned biomarkers in the patients' group in comparison to the control group ($P \leq 0.001$). The mean

of interleukin-6 for the patients and controls were 44 pg/mL and 22 pg/mL, respectively. The mean of cardiac troponin T for the patients and controls were 3 pg/mL and 2 pg/mL, respectively and the mean of N-Terminal-pro-brain natriuretic peptide for the patients and controls were 88 pg/mL and 45 pg/mL, respectively. For the sex and age groups of the participants, two statistically significant differences were demonstrated. The first was between interleukin-6 and the sex of the patients with HCV infection. The mean of interleukin-6 in males was 35 pg/mL, while the mean was 42 pg/mL for the females (P=0.026). The second statistical difference was seen between the age of the patients and their hypertension status. The mean age of hypertensive patients was 53 years, while the mean age was 43 years for the non-hypertensive (P≤0.001). From the above findings, we can conclude that having an infection with the hepatitis C virus may increase the risk of cardiovascular diseases and related conditions among chronic kidney disease patients. The sex and age of the patients play an important predisposing factor.

2023, 39 pages

Keywords: Kidney disease, HCV, Interleukin-6, N-Terminal-pro-brain, Troponin-T

ÖZET

HEPATİT C VİRÜSÜ ENFEKSİYONU OLAN/OLMAYAN KRONİK BÖBREK HASTALIĞI HASTALARINDA KARDİYOVASKÜLER BİYOMARKERLERİN SERUM DÜZEYLERİ

Helyah Kanaan Mohammed MOHAMMED
Biyoloji, Yüksek Lisans
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Eş Danışman: Dr. Öğr. Üyesi Basim Mohammad IBRAHİM
Ağustos 2023

Bu çalışmada, kronik böbrek hastalığı olan hastalarda kardiyovasküler hastalık insidansının artmasıyla ilişkili bir risk faktörü olarak kabul edilen hepatit C virüsü enfeksiyonunun etkisinin belirlenmesi amaçlanmıştır. Bu çalışmanın amacına ulaşmak için Bağdat Tıp Şehri Kompleksi'nde Böbrek Hastalıkları ve Transplantasyon Merkezi ve Gastroenteroloji Eğitim Hastanesi'nde yatan toplam 180 Iraklı (107 erkek ve 73 kadın; ortalama yaş 51) alınmıştır. Numuneler arasında 130 hasta (74 erkek ve 56 kadın) hepatit C virüsü ile enfekte olmuştur ve diğer 50 kontrol hastası (33 erkek ve 17 kadın) herhangi bir viral varlık göstermemiştir. Haziran 2022'den Ağustos 2022'nin sonuna kadar tüm katılımcılardan serum örnekleri toplanmıştır. Veriler, özel bir anket formu kullanılarak katılımcılarla doğrudan görüşmeler yoluyla toplanmıştır. Enzime bağlı immünosorbent tahlil teknolojisi, aşağıdaki kardiyak işlev bozukluğu biyobelirteçlerinin serum seviyelerini tespit etmek için kullanılmıştır: Tüm katılımcılar arasında N-terminal-pro-beyin natriüretik peptid, interlökin-6 ve kardiyak troponin T. Veri analizi, en yüksek katılımcı sayısının (55) 50'li yaşlarda (50-59 yaş) kaydedildiğini göstermektedir. Erkek ve kadın sayısı sırasıyla 38 ve 17 idi. Ayrıca, toplam katılımcı sayısının sırasıyla yaklaşık %58, %78 ve %97'sinin diyabetik, hipertansif ve hipoalbuminemik olduğu bulunmuştur. Kardiyak disfonksiyon biyobelirteçleri ile ilgili olarak, veri analizi, hasta grubunda yukarıda belirtilen tüm biyobelirteçlerin ortalama düzeylerinde kontrol grubuyla karşılaştırıldığında oldukça istatistiksel olarak anlamlı farklılıklar olduğunu ortaya koydu ($P \leqslant 0.001$). Hastalar ve kontroller için interlökin-6 ortalaması sırasıyla 44 pg/mL ve 22

pg/mL idi. Hastalar ve kontroller için kardiyak troponin T ortalaması sırasıyla 3 pg/mL ve 2 pg/mL idi, ve hastalar ve kontroller için N-Terminal-pro-beyin natriüretik peptidinin ortalaması sırasıyla 88 pg/mL ve 45 pg/mL idi. Katılımcıların cinsiyet ve yaş grupları için istatistiksel olarak anlamlı iki farklılık gösterildi. Birincisi, interlökin-6 ile HCV enfeksiyonu olan hastaların cinsiyeti arasındaydı. İnterlökin-6'nın erkeklerde ortalaması 35 pg/mL iken kadınlarda 42 pg/mL idi (P=0.026). İkinci istatistiksel fark, hastaların yaşı ile hipertansif durumları arasında gözlendi. Hipertansif hastaların ortalama yaşı 53 iken, hipertansif olmayanların ortalama yaşı 43 idi (P⩽0.001). Yukarıdaki bulgulardan, hepatit C virüsü enfeksiyonunun kronik böbrek hastalığı hastalarında kardiyovasküler hastalık ve ilişkili durumlar riskini artırabileceği sonucuna varabiliriz. Hastaların cinsiyeti ve yaşı önemli bir predispozan faktördür.

2023, 39 sayfa

Anahtar Kelimeler: Böbrek hastalığı, HCV, İnterlökin-6, N-Terminal-pro-beyin, Troponin-T

PREFACE AND ACKNOWLEDGEMENTS

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Helyah Kanaan Mohammed MOHAMMED
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CONTENTS

LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Celsius Degree
dL	Deciliter
g	Gram
kDa	Kilodalton
≤	Less than or Equal
L	Liter
µL	Microliter
mL	Milliliter
mmol	Millimole
Ng	Nanogram
nm	Nanometer
%	Percentage
pg	Picogram
±	Plus and minus

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ALP	Alkaline phosphatase
ATPase	Adenosine triphosphatase
AUC	Area under the curve
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
CDC	Center of disease control and prevention
CHC	Chronic hepatitis C
CKD	Chronic kidney disease
CMIA	Chemiluminescent microparticle immunoassay
CPRR	Cellular pathogen recognition receptors
CRP	C-Reactive Protein
CTL	Cytotoxic T-lymphocytes
CVD	Cardiovascular diseases
DAA	Direct-acting antivirals
DM	Diabetes mellitus
ECG	Echocardiography
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal disease
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCO_3	Bicarbonate
HCV	Hepatitis C virus
HD	Haemodialysis
HIV	Human Immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
hs-CRP	High-sensitivity C-reactive protein
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
IR	Insulin resistance
IRF-3	Interferon regulatory factor-3
ISG	Interferon stimulated gene
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function associated antigen-1
MC	Mixed cryoglobulinaemia
MHC	Major histocompatibility complex

MI	Myocardial infarction
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NM	Nanometer
NOSA	Non-organ specific autoantibodies
NT-pro-BNP	N-Terminal-pro-Brain natriuretic peptide
OD	Optical density
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffer saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
RF	Rheumatoid factor
RIBA	Recombinant immunoblot assay
RLU	Relative light unit
RPM	Round per minute
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SD	Standart deviation
SAD	Systemic autoimmune diseases
SPSS	Statistical Package for the Social Sciences
Th cells	Helper T-cells
TnC, T, I	Troponin C, T, I
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
WHO	World health organization

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Hepatitis C virus (HCV) is the primary causative agent of viral hepatitis, which is an illness that primarily affects the liver and may eventually result in hepatocellular carcinoma (HCC). Transfusion of blood and blood products represents the most common source of transmission (Naghavi 2019). Chronic kidney illness encompasses a broad spectrum of kidney abnormalities, from relatively little damage to total failure of renal function. The inability of the kidneys to function normally over an extended period is known as chronic kidney disease (CKD). It manifests insidiously and may vary from asymptomatic to so severe that renal function is lost without warning (Zuwala-Jagiello *et al.* 2015). Patients with CKD are at a higher risk for several complications, including cardiovascular disease (CVD). Some examples of diseases falling under this umbrella include heart attacks and strokes (Al-Sultani *et al.* 2016). In nephrology outpatient clinics and wards, the prevalence of HCV infection is elevated, and the reasons behind this elevation include higher prevalence in the general population, absence of standard precautions for infection, effective vaccination, inadequate disinfection of medical devices, and the spread of infection from patient to another (Abbas *et al.* 2020). Chronic HCV infection is associated with CVDs, and this might be linked to the fact that HCV infection induced chronic inflammation, direct endothelial invasion, dysfunction, and changes in the serum levels of heart dysfunction biomarkers (Babiker *et al.* 2017). Cardiac dysfunction biomarkers include interleukin-6 (IL-6), N-terminal-pro-brain natriuretic peptide (NT-pro-BNP), and troponin T (TnT) (Adukauskiene *et al.* 2016, Zhang *et al.* 2017). The primary objectives of the present investigation were to:

1. The following indicators will be measured to ascertain the potential involvement of HCV infection as an additional risk factor for CVDs among CKD patients: Triggering IL-6, NT-pro-BNP, and TnT.
2. Find out the correlation between cardiac dysfunction markers and the serum level of albumin.
3. Determine the possible association between age groups, sex, and chronic diseases (diabetes mellitus (DM) and hypertension) among the patients with the tested parameters.

2 LITERATURE REVIEW

2.1 Hepatitis C Virus

HCV was discovered in 1989 and thought to be the only member of the *Hepacivirus* genus until 2011. The body of the virus is small; consists of a lipid membrane envelope, in addition to an enveloped virion containing a genome of single-stranded positive-sense RNA (Figure 2.1) (Younossi *et al.* 2016).

A micro-RNA found only in the liver (miR-122) promotes HCV replication by enhancing HCV-mRNA synthesis (Dawany 2010).

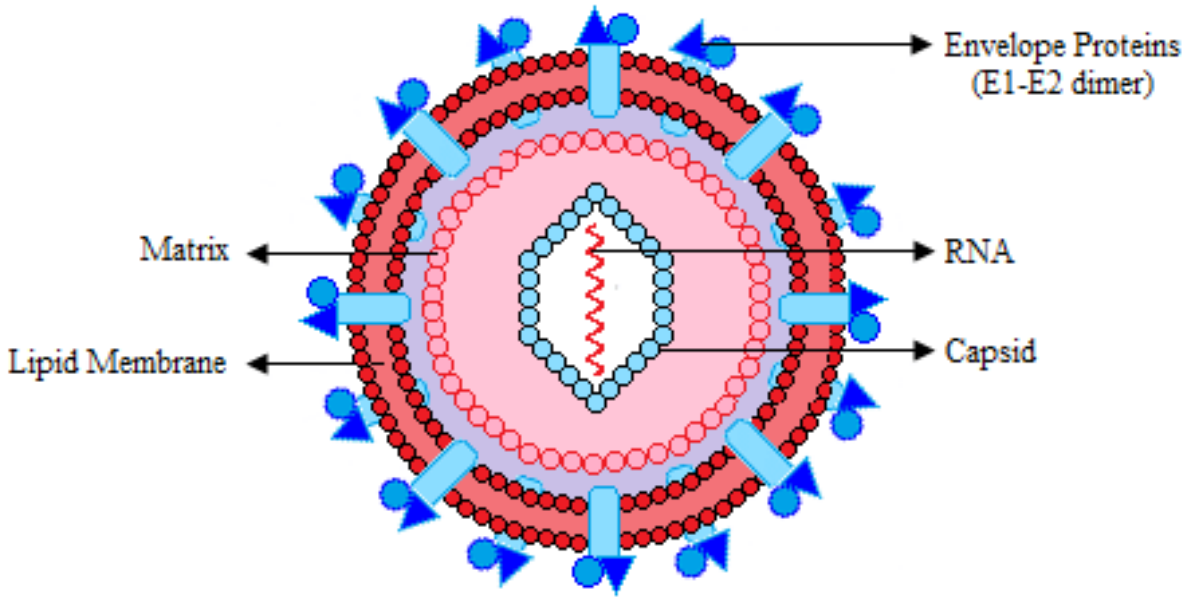

Figure 2.1 Structure of the HCV

2.2 Epidemiology of HCV Infection

There are an estimated 71 million persons infected with HCV across the globe, and nearly 400,000 people have lost their lives to HCV-related liver illnesses (Figure 2.2). Recent worldwide estimates show that the viremic prevalence of HCV infection is less than 1.0% in most affluent nations, even though the quality of epidemiologic data and prevalence estimations varies substantially between countries and within regions (WHO 2022).

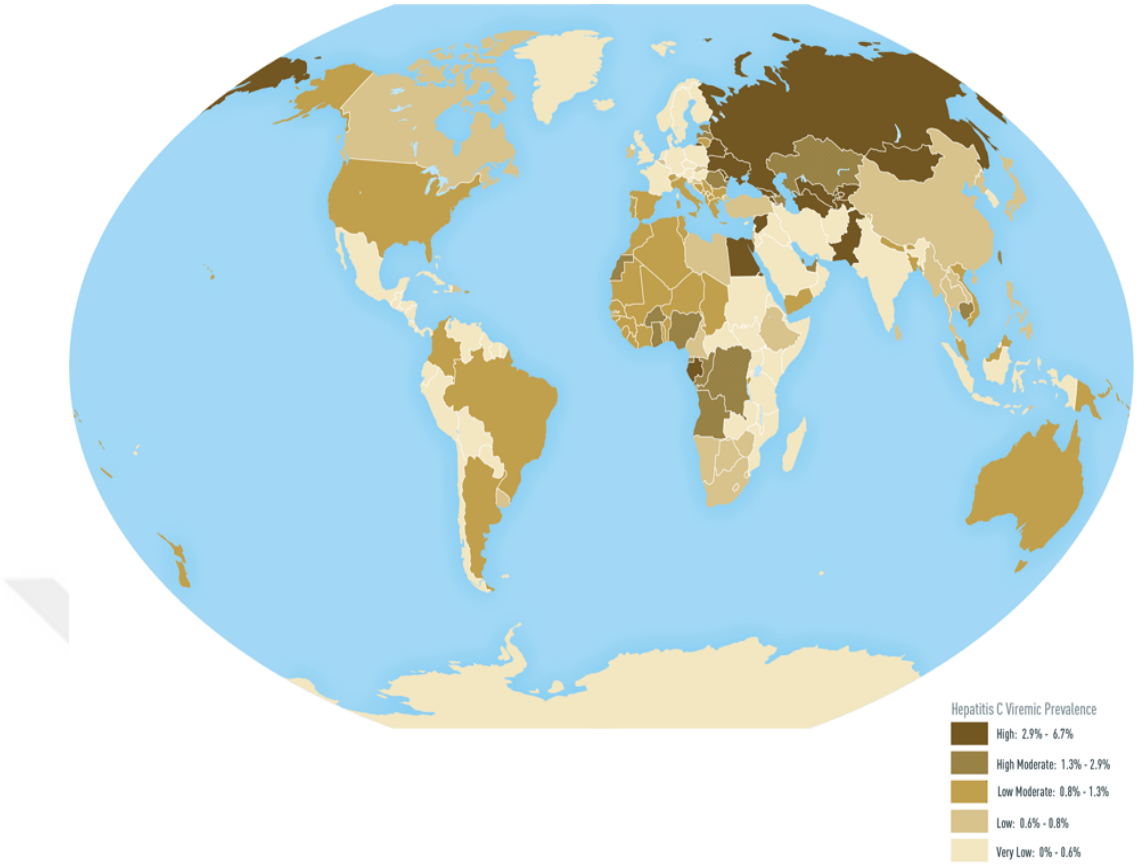

Figure 2.2 Prevalence of HCV infection

2.3 Mode of Transmission

The infection was shown to be transmitted via many routes. The sexual route appears as an important risk factor that rises over time among multiple sexual partners, men who have sex with men, during menstruation, and anal and/or traumatic sex (Hegazy 2016) Perinatal mode of transmission (mother-child) was found to be high as 3-5% of born infants to infected mothers with HCV have acquired anti-HCV antibodies via a trans-placenta route (Indolfi *et al.* 2013). One major risk factor for contracting HCV is receiving a blood or blood product transfusion. According to the WHO, 39 nations do not do regular screening of blood transfusions for blood-borne viruses (Al-Khazraji *et al.* 2019). An emerging epidemic of HCV among injection drug users has been demonstrated, particularly in developed countries, and this might be related to the heavy existence of HCV in spoons and rinsing liquids utilized (Saito and Ueno 2013). Nosocomial

transmission is considered to be high as patients and/or healthcare providers could carry the infection to each other and vice versa (Li and Lo 2015).

2.4 HCV and The Immune System

Antiviral immunity is induced in the liver, virus-specific T-cells are recruited, and infected hepatocytes are eliminated during the early stages of infection when the majority of innate immunity (natural killer cells) is activated. This elimination is done through two pathways; direct (via cytolytic techniques) and indirect (via the production of cytokines). The secreted cytokines prevent the replication of the virus by protecting uninfected cells from being infected (Yamane *et al.* 2013).

The major histocompatibility complex molecules convey viral molecules from the endoplasmic reticulum to the cell surface, where they are identified by T-cells after HCV has infiltrated and replicated inside hepatocytes (Zeisel *et al.* 2013). Development of infected individuals to chronic stage suggests the failure of immunity in eradicating the infection, and this may be related to the emergence of escape-variants that resulted from viral mutations; minimized production of antiviral cytokines and antagonistic peptides (Neumann-Haefelin and Thimme 2013). Figure 2.3 summarizes the immune response to viral hepatitis C.

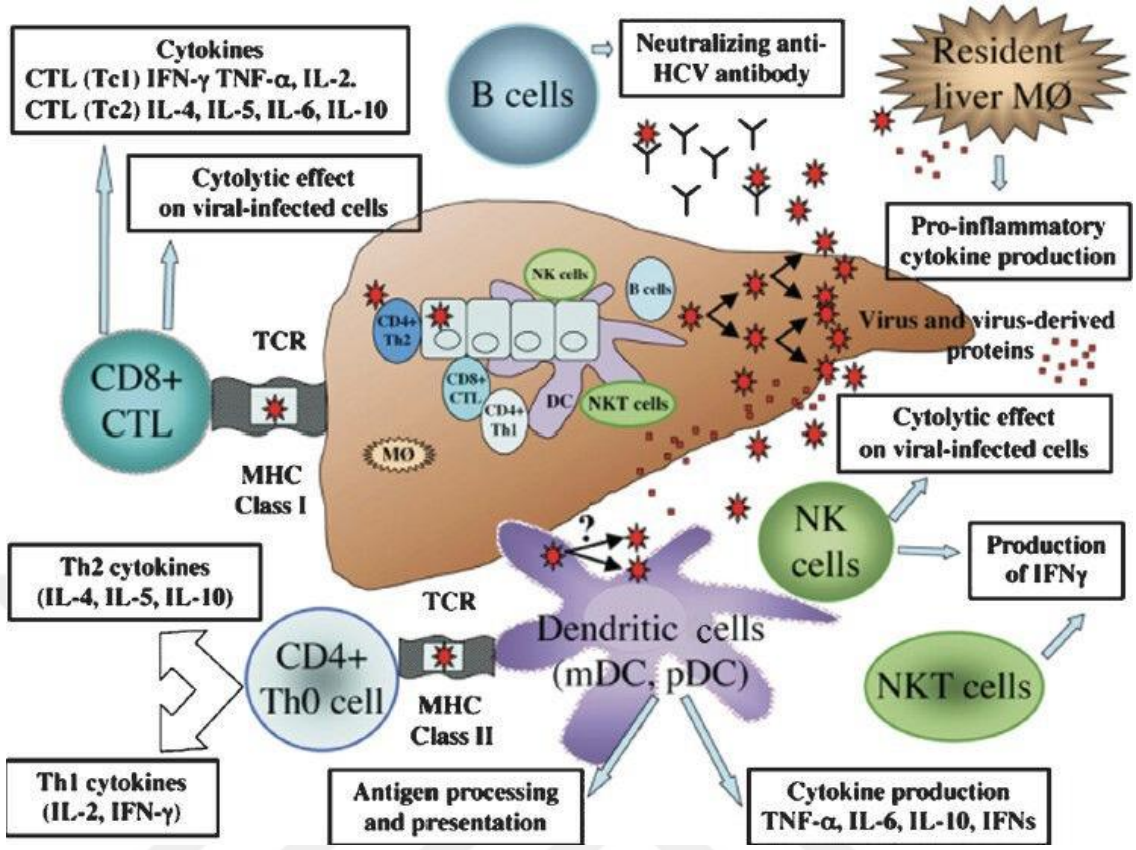

Figure 2.3 Immune response in viral hepatitis C

2.5 Pathogenesis of HCV

HCV causes polyclonal B-cell proliferation and autoreactive T-cell production through HCV lymph-otropism, molecular-mimicry, and bystander activation. Lymphomas arise as a result of further genetic changes. Figure 2.4 displays the biological and natural mechanisms involved in the progression of HCV-induced HCC (Hoshida *et al.* 2014).

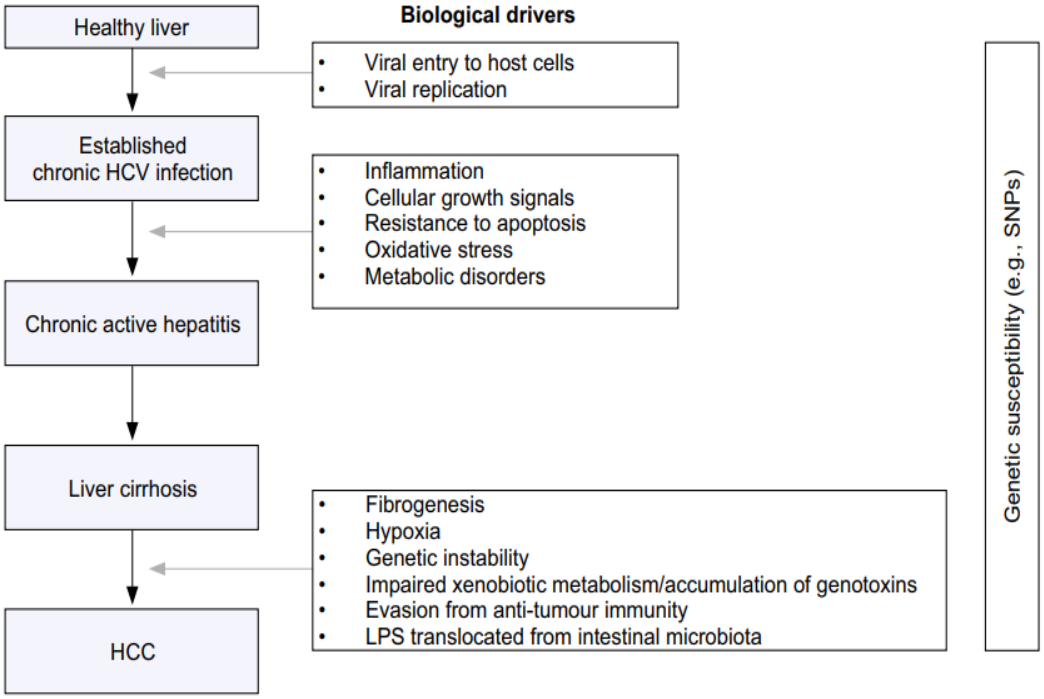

Figure 2.4 Natural history and biological processes in HCV

2.6 Biomarkers of Cardiac Dysfunction

2.6.1 Cardiac Troponin T

Protein is found in the actin filaments. In 1964 it was reported that this protein was a regulator of muscle contraction. It is made up of 2 subunits; troponin C and troponin T has a molecular weight is 37 kDa and it helps in binding the troponin complex to tropomyosin and activating actinomyosin ATPase activity (Katrukha 2013).

Because most patients with renal failure also suffer from heart failure, leading to an elevated serum level of troponin even in the absence of clinical myocardial infarction, the validity of cardiac troponin T in the management of acute coronary artery diseases among CKD patients is a matter of debate (Chew *et al.* 2014). Another reason might be existing which is represented by the accumulation of immuno-reactive cardiac troponin fragments that are associated with the abnormal catabolism of troponin and/or the ESRD-associated skeletal myopathy (Bedimo and Abodunde 2016).

2.6.2 N-Terminal pro-brain natriuretic peptide

A hormone that helps both arteries and veins retain fluid and increase their volume in response to variations in internal heart pressure. This process, however, results in the activation of natriuretic peptide precursor B gene transcription to produce pro-brain natriuretic peptide (Guo *et al.* 2014). Corin and furin help in splitting the resulting pro-BNP to release an active hormone BNP in addition to an inactive amino-terminal fragment NT-pro-BNP in the blood. Because a high level of this marker indicates that the heart is having trouble pumping enough blood to the body, it is used to identify and assess heart failure (Maisel *et al.* 2018).

2.6.3 Interleukin 6

Interleukin-6 (IL-6) is a hormone-like pro-inflammatory cytokine that aids in promoting B-cell differentiation, T-cells activation, CRP hepatic production, and acute phase response regulation (Rose-John 2020). Multiple mechanisms govern IL-6 production, with TNF-α, IL-1β, adipokines, prostaglandins, and toll-like receptors serving as major inducers of IL-6 (Ali Deeb *et al.* 2019). It has been demonstrated that levels of IL-6 are found to be elevated during infections and other medical conditions, including diabetes, kidney disease, and CVDs, and the inability to activate it was associated with recurrent/persistent microbial infections (Kreiner *et al.* 2017).

2.7 Laboratory Diagnosis

The diagnosis of HCV infection depends on both physical examination and laboratory investigation, and it is considered to be very important and vital through its role in differentiating acute from chronic stages, determining the extent of liver damage, and guiding treatment lines (Sagnelli *et al.* 2018). Laboratory detection procedures begin with the diagnosis of anti-HCV antibodies in the blood by the use of enzyme immunoassay or recombinant immunoblot assay (Kim *et al.* 2015). Although these methods are efficient and have reliable diagnostic outcomes, they cannot differentiate between infection stages. This may be related to the fact that anti-HCV specific IgM is variable in acute infectious diseases and identified at increased levels in HCV chronic patients (Ilyas and Ahmad 2014).

Viral RNA represents an initial marker of primary infection before the existence of anti-HCV by many weeks. The using of real-time PCR and multiplex advanced PCR facilitated the diagnosis and monitoring of HCV infection within a short duration (Gupta *et al.* 2014). The screening flowchart of HCV infection is shown in Figure 2.5.

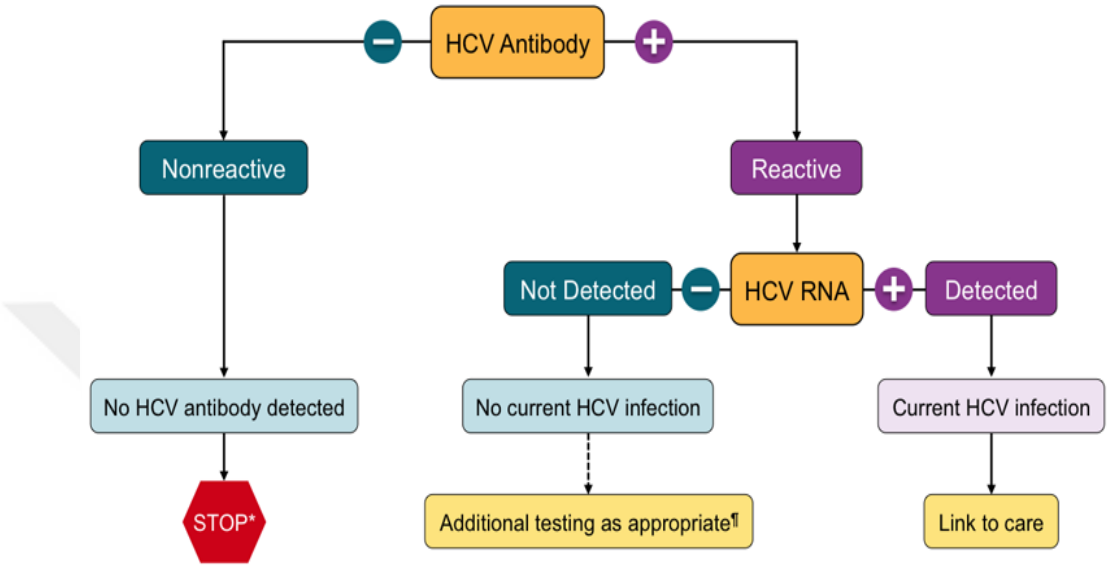

Figure 2.5 The proper order of HCV tests to detect active infection

3. MATERIALS AND METHODS

3.1 Study Design, Location, and Timing

Between June 2022 and August 2022, case-control research was conducted at the Renal Disease and Transplantation Center and the Gastrointestinal Tract Teaching Hospital at the Baghdad Medical City Complex. Data were collected using a medical questionnaire designed for this work and filled via direct interviews with all the participants.

3.2 Subjects

In this research, 180 patients with CKD were recruited and split into two groups:

- **Group 1:** Consist of 130 HCV-infected CKD patients; 74 men and 56 women. The ages of participants varied from 24 to 74.
- **Group 2:** Involve 50 CKD patients who were virus-free (control group); there were 33 men and 17 women in this group. They were between the ages of 22 and 75. Figure 3.1 is a flowchart depicting the research methodology.

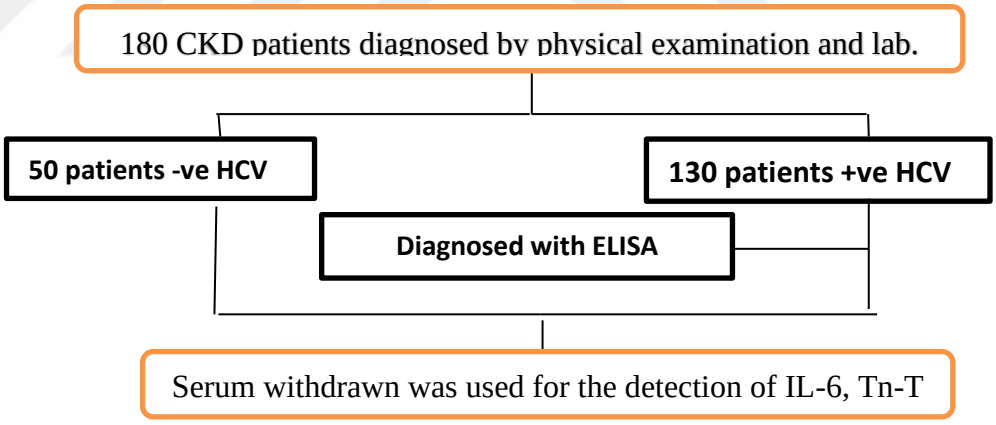

Figure 3.1 Flow chart of the study design

3.3 Criteria

3.3.1 Inclusion criteria

Patients who were diagnosed with CKD and HCV infection had no other coexisting medical issues.

3.3.2 Exclusion Criteria

Antiviral-treated HCV patients and those suffering from other infections or disorders.

3.4 Ethical Considerations

The names of the participants were not included in the questionnaire as they were requested, and consent had been taken before they answered the questionnaire. Ethical approval was obtained according to memorandum No 21005 on 26.05.2022.

3.5 MATERIALS

3.5.1 Instruments

Table 3.1 lists all the research tools that were used in this investigation.

Table 3.1 Instruments used in the study

Instrument	Company	Country
Disposable 5 mL syringes	Jiangsu Kangyou	China
Disposable pipette tips	Boenmed	China
Eppendorf tubes	Boenmed	China
Gel separation tubes	Boenmed	China

3.5.2 EQUIPMENT

The equipment listed in Table 3.2 was used in this study.

Table 3.2 Equipment used in the study

Equipment	Company	Country
ARCHITECT plus (Abbott i2000SR)	Abbott	USA
Centrifuge	Genex	USA
Micropipette 10-1000 µL	Dragon Lab.	China
Microplate reader	BioTek	USA

3.5.3 KITS

The kits listed in Table 3.2 was used in this study.

Table 3.3 Kits used in the current study

Name of kit	Company	Country
ELISA Human IL-6	MyBioSource	USA
ELISA Human TroponinT	Cusabio	USA
ELISA Human NT-pro-BNP	MyBioSource	USA

3.6 METHODS

3.6.1 Collection of blood specimens and data

All patients were subjected to a questionnaire and withdrawn 5 mL blood samples. The questionnaire involves socio-demographic data (age, sex) as well as other information including duration of infection, duration of HD, DM, and hypertension.

3.6.2 Interleukin-6

3.6.2.1 Test principle

This assay determines the quantitative presence of IL-6 in the serum of patients by the using ELISA technique. The microtitration plate was coated with specific antibodies, standards, and specimens were added to each well. A conjugated horseradish peroxidase (HRP) specific antibody was then added, followed by the addition of a substrate solution and color develops in proportion to the amount of IL-6 bounded. The color development is stopped and the optical density (OD) was measured at 450 nm.

3.6.2.2 Reagents preparation

1. Reagents were brought to room temp. before using it for 30 minutes.
2. A fresh standard was prepared for the assay.
3. After diluting 1 mL of wash solution (20x) with 19 mL of distilled water to make 20 mL of washing buffer, we obtained 1 mL of wash solution (1x).

3.6.2.3 Assay procedure

1. Each well received 50 µL of the standard and specimen.
2. Each well, save the blank well, had 100 µL of HRP reagent injected into it.
3. After covering the plate, we left it in the 37°C incubator for an hour.
4. Three changes of washing buffer were used, each time leaving the wells undisturbed for one minute.
5. After adding 50 µL of solution A and solution B to each well and gently mixing them, they were incubated at 37°C for 15 minutes in the dark.

6. After adding 50 µL of stop solution to each well, we lightly tapped the plate to ensure mixing and measured the OD at 450 nm after 15 minutes.

3.6.2.4 Standard Curve of IL-6

Figure 3.2 displays a curve of IL-6 that confirms the standard curve generated by graphing absorbance values versus specimen concentration.

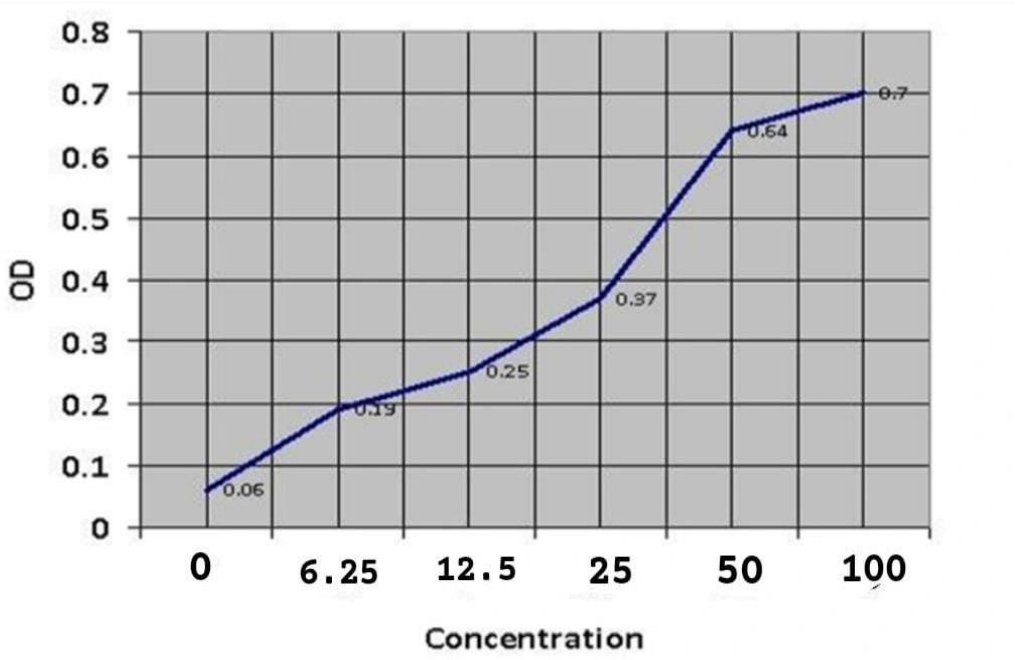

Figure 3.2 Standard curve of IL-6

3.6.3 Cardiac troponin T

3.6.3.1 Test principle

This assay detected the quantitative existence of TnT by ELISA technique. The microtitration plate was coated with specific antibodies, standards, and specimens were added to each well. A conjugated HRP-specific antibody was then added, followed by the addition of a substrate solution and color develops in proportion to the amount of TnT bounded. The color development is stopped and the OD was measured.

3.6.3.2 Reagents preparation

1. All reagents were brought to room temperature before using for 30 minutes.
2. A fresh standard was prepared for the assay.
3. Biotin-antibody (1x) was prepared by the dilution of 10 µL biotin antibody with 990 µL HRP diluent, representing 100-fold dilution as recommended.
4. Conjugated HRP (1x) was prepared by mixing 10 µL HRP avidin with 990 µL of HRP diluent, representing 100-fold dilution as recommended.
5. To make 500 mL washing buffer (1x), 20 mL wash buffer (25x) was combined with distilled water.
6. Reconstitution with 1 mL of sample diluent yielded a 3000 pg/mL stock solution, which was used as the standard. Wait 15 minutes before touching.
7. A concentration of 3000 pg/mL was used for the undiluted standard, while a concentration of 0 pg/mL was used for the sample diluent.

3.6.3.3 Assay procedure

1. We added 100 µL of standard and specimen to each well and let the mixtures incubate at 37^{o}C.
2. Each well was loaded with 100 µL of biotin-antibody (1x) and incubated for 1 hour.
3. Two washes of 200 µL of washing buffer were performed in each well, and each well was let to drain for 1 minute.

4. Each well was given 100 µL of 1x conjugated HRP and incubated for 1 hour at 37°C.
5. As in Step 3, the washing was done again.
6. Each well had 90 µL of a substrate solution added to it, and then it was incubated at 37°C, out of the light, for 15-30 minutes.
7. Within 5 minutes, the OD was measured at 450 nm after adding 50 µL of stop solution to each well.

3.6.3.4 Results calculation

A standard curve was drawn by plotting the absorbance values against the concentration, which was verified by the curve shown in Figure 3.3.

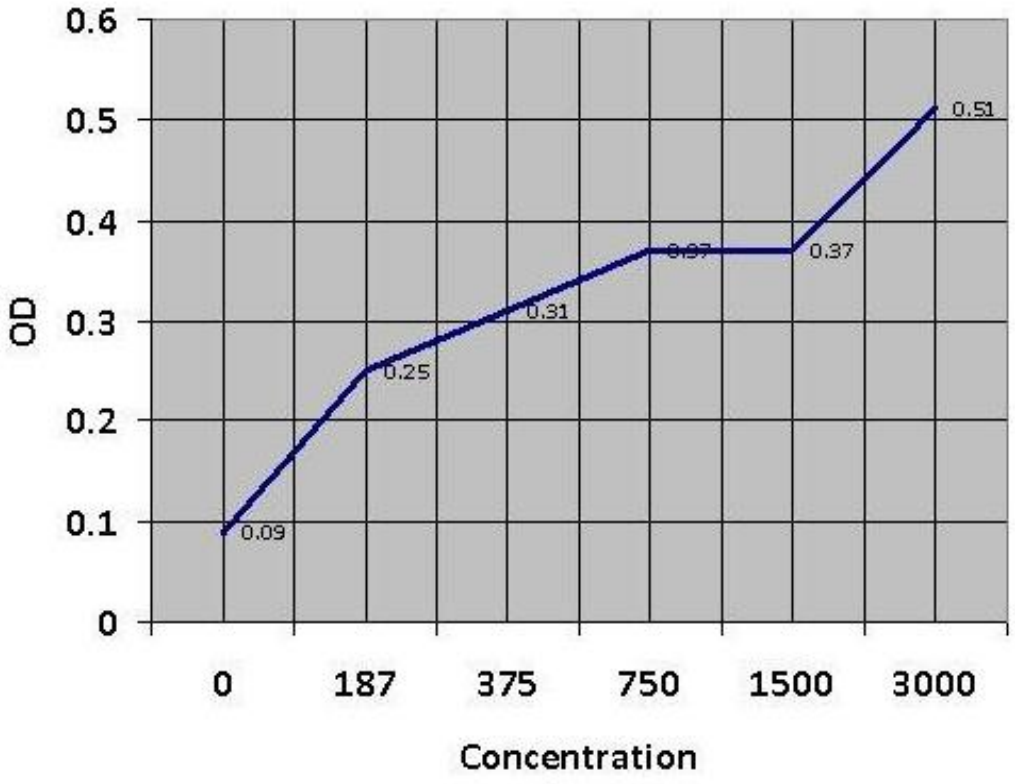

Figure 3.3 Standard curve of TnT

3.6.4 N-Terminal-pro-brain natriuretic peptide

3.6.4.1 Test principle

This quantitative assay determines the existence of NT-pro-BNP in the serum by using the ELISA technique. The wells of the microtitration plate was coated with specific antibodies for the target. Standards, specimens, and conjugated HRP were pipetted into each well and any NT-pro-BNP present was bounded. After washing away any remaining contaminants, a substrate solution was applied, and the presence of NT-pro-BNP was detected by the resulting color. The OD was taken at the end of the coloring process.

3.6.4.2 Reagents preparation

1. Reagents were brought to room temperature before using for 30 minutes.
2. A fresh standard was prepared for the assay.
3. The washing solution (20x) was diluted with distilled water.

3.6.4.3 Assay procedure

1. Each standard well-received 50 µL of the standard.
2. Each well received 10 µL of testing samples and 40 µL of specimen diluent.
3. After incubating at 37^{o}C for an hour, 100 µL of HRP conjugate was added to each well.
4. After standing for one minute, each well was washed twice with 400 µL of washing buffer.
5. After adding 50 µL of solution A and solution B to each well, gently mixing, and incubating at 37°C for 15 minutes, the results were read.
6. After 15 minutes, the OD was measured at 450 nm after adding 50 µL of stop solution to each well.

3.6.4.4 Results calculation

A standard curve was drawn by plotting the absorbance values against the concentration, which was verified by the curve shown in Figure 3.4.

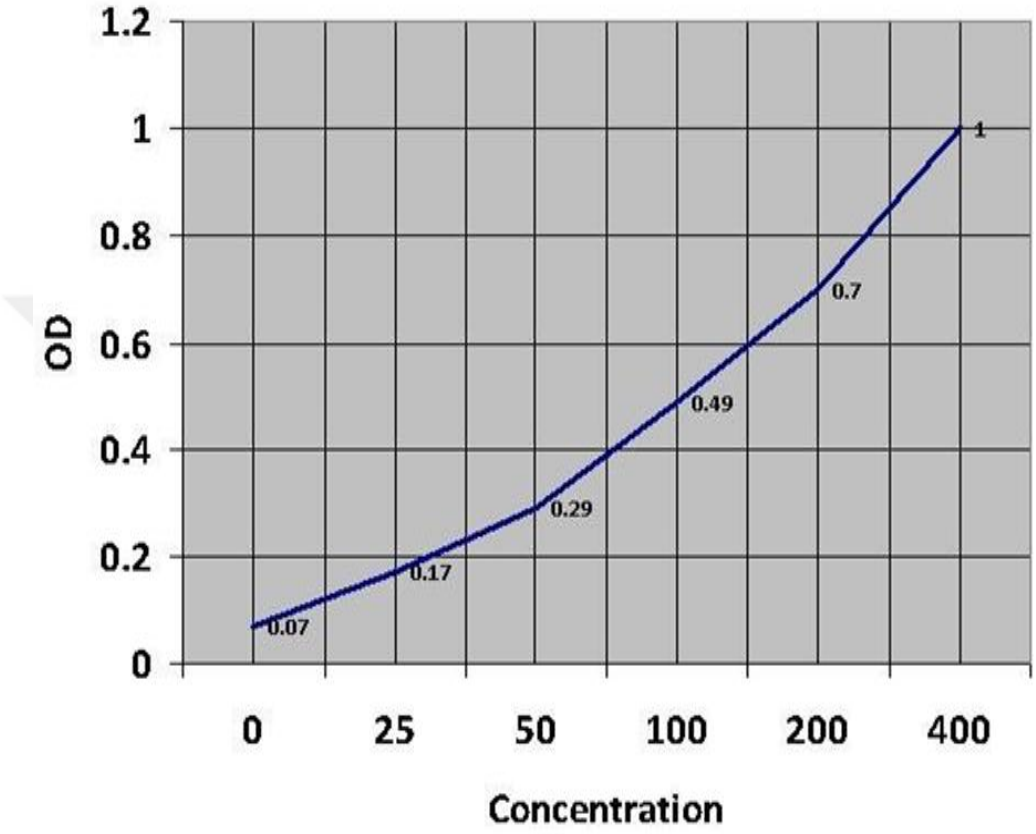

Figure3.4 Standard curve of NT-pro-BNP

3.6.5 Albumin

Data for serum levels of albumin was collected for all the participants from their profiles. The method used for the detection was a fully automated device (Cobas c 111, Roche, Germany).

3.7 Statistical Analysis

After analysis and calculation in SPSS version 25, Microsoft Excel 2017, and JASP, results were laid up in tables and figures. Statistical significance was defined as a P value of less than ≤0.05. Nominal and ordinal variables are displayed as categories, whereas

continuous variables are given as means and standard deviations. An Independent sample T-test was used to assess the significance of the difference between the means of the outcome variable (continuous) among the categories of the predictor variable (categorical). An assumption of equal variances was made for the t-test. The significance of the relationship between the different qualitative variables was evaluated using the chi-square test and the Fisher exact test for qualitative categorical and nominal data.

4. RESULTS AND DISCUSSIONS

4.1 Demographic Parameters

The mean age of participants was 51.58±12.37 years. The number of males was 107 (59%) with a mean age of 52.09±11.57 years, and the number of females was 73 (41%) with a mean age of 50.87±13.46 years. The participants were divided into two groups; 130 (72%) were CKD patients infected with HCV and 50 (28%) CKD patients clear of any viral existence (control group). Data analysis demonstrated that the highest number of patients was recorded in the age group 50-59 years. The results are shown in Tables 4.1, 4.2, and 4.3.

Table 4.1 Distribution of participants according to age groups and sex

Age groups (years)	Total number	Number of males (%)	Number of females (%)
20-29	10	5 (50%)	5 (50%)
30-39	21	13 (62)	8 (38)
40-49	40	25 (63)	15 (37)
50-59	**55**	**38 (69)**	**17 (31)**
60-69	42	18 (43)	24 (57)
70-79	12	8 (67)	4 (33)
Total	**180**	**107 (59%)**	**73 (41%)**

Table 4.2 Distribution of patients according to age groups and sex

Age groups (years)	Total number	Number of males (%)	Number of females (%)
20-29	5	1 (0.76)	4 (3.07)
30-39	16	9 (6.9)	7 (5.38)
40-49	31	18 (13.8)	13 (10)
50-59	**34**	**23 (17.6)**	**11 (8.4)**
60-69	33	15 (11.5)	18 (13.8)
70-79	11	8 (6.1)	3 (2.3)
Total	**130**	**74 (56.36)**	**56 (43.01)**

Table 4.3 Distribution of controls according to age groups and sex

Age groups (years)	Total number	Number of males (%)	Number of females (%)
20-29	5	4 (8)	1 (2)
30-39	5	4 (8)	1 (2)
40-49	9	7 (14)	2 (4)
50-59	**21**	**15 (30)**	**6 (12)**
60-69	9	3 (6)	6 (12)
70-79	1	0 (0)	1 (2)
Total	**50**	**33 (66)**	**17 (34)**

The current case-control study involves a total number of 180 CKD. The highest number of them was recorded in the sixth decade (age 50-59 years). The mean age of the participants was 51.58 years. These findings, however, were in agreement with recent studies done in Iraq (Salman *et al.* 2021, Jouda *et al.* 2023). People begin to show increased vulnerability to age-related illnesses and associated problems beginning in the sixth decade, also called the age of biological decline (Franceschi *et al.* 2018). Moreover, aging was linked to the accumulation of various cellular/molecular damage over time, leading to a gradual decline in physico-mental capacity and making people more susceptible to a wide range of illnesses and health problems (WHO 2022).

The peak age for HCV infection seems to be between 50 and 59 years old, with the frequency seeming to be greater in males than in women. Such prevalence rates were also demonstrated in other studies in males (19.1%) than females (12.7%) (Ilyas and Ahmad 2014). It's possible that variations in research design, patient groups, and test types all

contribute to result inconsistencies. Both infection incidence and treatment efficacy were shown to be affected by sex (Baden *et al.* 2014). However, these results don't square with what we found and with the evidence that female HCV clearance rates are much higher than male rates and that chronic HCV infection progresses more slowly in females than in males (Bakr *et al.* 2006).

4.2 Existence of Comorbidities

Regarding the presence of certain conditions such as DM, hypertension, and hypoalbuminemia, data analysis shows that 58% of the patients have DM, 78% were hypertensive and 97% were hypoalbuminemia. The results are shown in Figure 4.1.

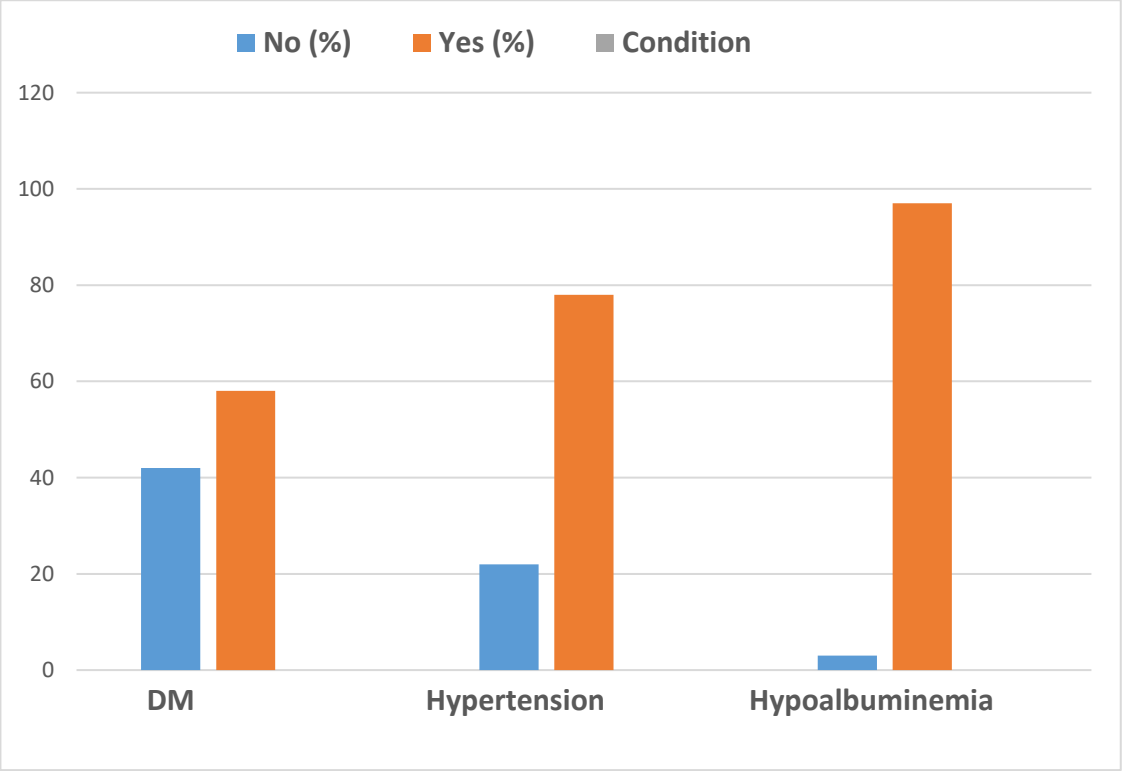

Figure 4.1 Distribution of participants according to the existence of chronic diseases

Hypertension and DM are considered important risk factors that participated in the progression to a CKD condition. Seventy percent to eighty percent of hemodialysis patients have hypertension (blood pressure > 140/90 mm Hg), and only a few of those patients have it under control (Bucharles *et al.* 2018). The current study investigates the

existence of DM, hypertension, and hypoalbuminemia among CKD patients in the presence and/or absence of HCV infection. Data demonstrate that 58% of the patients have DM, 78% were hypertensive with a statistically significant difference in the mean age of hypertensive versus non-hypertensive patients and 97% were hypoalbuminemia. These results were in agreement with the outcomes of other studies demonstrating that these conditions were frequently detected among ESRD patients (Kalantar-Zadeh *et al.* 2021)

The above-mentioned findings, however, disagreed with the outcomes of other studies. Both type 1 and type 2 diabetes had a substantial influence on the development of chronic kidney disease, with the former carrying a 12.9-fold increased risk. Long-term diabetics (>15 years) were 29.8 times more likely to have chronic kidney disease. Having hypertension increased the chance of chronic kidney disease by 3.5 times, and having it for more than 15 years increased the risk by 12.9 times (Khaleel *et al.* 2019) exhibiting that there was a significant difference in the existence of DM and hypertension among HCV patients with/without CKDs. The expression of HCV core proteins and inflammatory cytokines both induce hepatic insulin resistance by altering signaling along the insulin receptor substrate-1 pathway, but these results may not be comparable for a variety of reasons. These may include differences in study design, patient populations, geographic locations, conditions related to these diseases, assay types, and more. However, this, in combination with other variables like food and obesity, might lead to the manifestation of a diabetic phenotype. Hyperglycemia and decreased insulin production occur when insulin resistance progresses to a point where the β-cell can no longer cope (Naing *et al.* 2012, Hum and Jou 2018). Studies done between 2014 and 2018 demonstrated the role of DM as a predisposing factor for CKD and outlined many factors contributing to this condition, including:

1. Patients with ESRD have lower capillary blood glucose levels.
2. Patients with CKD were more likely to have malnutrition and protein-energy wasting, which increased their vulnerability to hypoglycemia.
3. Kidney failure slowed the breakdown and clearance of exogenous and endogenous insulin, increasing its half-life.

4. Renal gluconeogenesis may be diminished by impairments in nephron bulk and function (Rhee *et al.* 2014, Sudha *et al.* 2017, Krishnasamy and Abell 2018).

Concerning hypertension, data show that there was a statistically significant difference in the mean age of hypertensive versus non-hypertensive patients, with a prevalence rate greater than 75%. A study done in 2017 exhibited that there was a relationship between DM and hypertension among HCV patients, who were more aware of their hypertension than non-infected subjects (Awadallah *et al.* 2017). Possible causes include variations in patient populations and regions, as well as variations in factors such as volume overload, sodium retention, arterial stiffness, renin-angiotensin-aldosterone system activation, sleep apnea, sympathetic nervous system activation, and the use of recombinant EPO. The major pathogenic mechanisms of hypertension in this group seem to be all of these variables (Agarwal *et al.* 2014, Khaleel *et al.* 2019).

The use of recombinant human EPO or eprex, however, was also shown to be related to hypertension. Reduction in endogenous EPO was a major cause of renal anemia in CKD patients on HD (Mimura *et al.* 2015). Eprex is a growth factor prescribed for the treatment of symptomatic anemia associated with chronic renal failure in dialysis patients (Terada *et al.* 2021). As a result of remarkably low levels of hemoglobin, the doctors advised patients to continue using EPO to maintain hemoglobin values within the desired concentration range (10-12 g/dL; 6.2-7.5 mmol/L) (Tsubakihara *et al.* 2010, Masakane *et al.* 2015, Liu *et al.* 2015). Since eprex has been linked to an increased risk of hypertensive crisis with encephalopathy and seizures, it should be taken with care in the context of untreated, inadequately treated, or poorly managed hypertension (Mastromarino *et al.* 2011, Kleijn *et al.* 2013), therefore, it is occasionally required to add or increase anti-hypertensive therapy, and if blood pressure cannot be managed, the treatment should be stopped (Nagai *et al.* 2016).

Albumin is a protein made by the liver; serving as liver-working and body oncotic-pressure indicators, preventing fluids from leaking out of blood vessels into the surrounding tissues (Kooman and Sande 2019). Results analysis revealed that more than 95% of the participants have hypoalbuminemia states with a statistically significant among patients versus the controls. Similar results were also demonstrated in other

studies. The mean serum albumin level was 3.6±0.3 g/dL (Yamashita *et al.* 2011). About 60% of hemodialysis patients had albumin values of 4.0 g/dL due to the development of chronic renal disease kidney failure and the commencement of maintenance hemodialysis (Kalantar-Zadeh *et al.* 2021). Documenting a significant reduction in albumin serum levels among CKD patients in general and HCV-infected patients in particular. Patients with HCV, ESRD, or advanced cancer may have experienced fluid accumulation or edema due to hypoalbuminemia, which can be explained by the role serum albumin plays, such as maintaining pH and normal microvascular permeability, mediating coagulation, and possessing antioxidant properties. This led to an increase in inflammatory mediators and more severe liver disease (Hussein 2012, Kalantar-Zadeh *et al.* 2021). Another explanation might be linked to the fact that poor nutritional intake among CKD patients resulted in the diminution of albumin synthesis, which was responsible for hypoalbuminemia among those patients (Al-Rubaie and Hasan 2014, Khaleel *et al.* 2019). Another possible interpretation is that patients with liver disease demonstrate low albumin, which can cause bilateral edema, ascites, or pleural effusion, leading to an elevation in inflammatory mediators (Ijaz *et al.* 2011, Kalantar-Zadeh *et al.* 2021).

4.3 Association Between Studied Biomarkers and the Sex of Participants

There was a statistically significant difference between the mean level of IL-6 only and the patient's sex; the mean level among females was 42.47±20.11 pg/mL, whereas among males was 35.46±21.05 pg/mL ($P \leq 0.05$). For cTnT and NT-pro-BNP biomarkers, however, data revealed that there was no statistically significant difference in the means versus the sex of the participants. The results are shown in Table 4.4.

Table 4.4 Relationship between the studied biomarkers and the sex of the patients

Variables	Sex				P value
	Females (107)		Males (73)		
	Mean	SD	Mean	SD	
IL-6 levels (pg/mL)	42.47	20.11	35.46	21.05	**0.026***
cTnT level (ng/L)	3.23	1.80	3.42	1.99	**0.508**
NT-pro-BNP level (pg/mL)	77.59	35.56	75.61	40.33	**0.734**
*** Statistically significant difference, calculated using independent samples T-test**					

Data analysis exhibited that there was a statistically significant difference in the mean level of IL-6 only with the patient's sex. The mean level among females was 42.47±20.11 pg/mL, whereas among males the mean level was 35.46±21.05 pg/mL ($P \leq 0.05$). Such relationships were also demonstrated in studies done in Italy, the USA, and Egypt (O'Connor *et al.* 2007). The results of this study were as follows. Women showed greater IL-6 production as compared to men, supporting our first hypothesis [12.3±6.1 vs. 6.9±4.4; $F(1,26)=7.16$, $P \leq 0.01$]. Patients' sex and baseline IL-6 blood levels were shown to be significant and independent prognostic indicators linked with increased risk of severe outcomes, and so might be used to improve treatment. Gender variations in the biological pathways that contribute to proinflammatory cytokine activity underlie the observed sex inequalities in the incidence of inflammatory illnesses. In addition, therapies that focus on autonomic processes may represent novel approaches to limiting IL-6 production, which may have effects on the risk of inflammatory diseases in women (O'Connor *et al.* 2007).

4.4 Association Between Age of Participants and Infection With HCV

The mean age of patients infected with HCV in this study was 52.59±12.21 years, whereas the mean age of those who are not infected (control group) was 48.94±12.53 years. There was a 3.65-year age gap between the groups, but no statistically significant difference was found. The results are shown in Table 4.5.

Table 4.5 Relationship between the age group of participants and infection with HCV

Variable	HCV infection status				P value
	Positive (130)		Negative (50)		
	Mean	SD	Mean	SD	
Age (years)	52.59	12.21	48.94	12.53	0.076

4.5 Association Between Age of Participants and Hypertension Status

Regarding the correlation between the age groups of participants and the presence of hypertension, it has been shown that the mean age of the hypertensive patients was 53.79±11.22 years, while for the non-hypertensive patients was 43.85±13.25 years. A difference of 9.94 years was found to be statistically significant (P≤0.001). The results are shown in Table 4.6.

Table 4.6 Association between the age of participants and hypertension status

Variable	Hypertension				P value
	Present (140)		Not present (40)		
	Mean	SD	Mean	SD	
Age (years)	53.79	11.22	43.85	13.25	**≤0.001***
*** Statistically significant difference, calculated using independent samples T-test**					

4.6 Association Between the Studied Biomarkers and HCV Infection

The mean level of IL-6 among HCV patients was 44.31±20.43 pg/mL, while the mean level among the control group was 22.96±12.69 pg/mL. IL-6 levels in HCV patients were significantly higher than in the control group (P≤0.001). It is a proinflammatory cytokine that contributes to B- and T-cell differentiation/activation (Neuman *et al.* 2012). Elevated levels of IL-6 among HCV-infected patients were also demonstrated in other studies (Khan *et al.* 2011) The results of Khan *et al.* (2011) were as follows. Where the level of IL-6 for the control group was 13.69±1.25 and the level of IL for HCV patients was

51.68±2.36, meaning that the level of IL increases in HCV patients, which is consistent with our study. The expression of IL-6 is regulated in many ways, and one of its most important activators is TNF-α (Ali Deeb *et al.* 2019). Elevated serum levels of TNF-α have been demonstrated recently in patients with CKD on HD infected with HCV (Salman *et al.* 2021), and this was found to have resulted directly in an increase in the serum levels of IL-6. This result, however, agrees with the fact that elevated levels of IL-6 have been seen during microbial infections and other medical conditions, including kidney disease, atherosclerosis, ischemic stroke, heart failure, and myocardial infarction (Kreiner *et al.* 2017, Su *et al.* 2021). As a result of oxidative stress, chronic inflammation, and fluid overload, CKD patients have been shown to have elevated blood IL-6 levels. By exacerbating renal damage and setting the stage for CKD problems, IL-6 hastens the disease's development (Costa-Pereira 2014). By suppressing endothelial nitric oxide synthase and adiponectin expression, IL-6 is the initiator of endothelial damage, and recombinant IL-6 injection has been shown to worsen atherosclerosis (Zhuang *et al.* 2015). These results indicate that IL-6 may play a role in explaining why CKD patients have a higher risk of cardiovascular disease (Jasiewicz *et al.* 2015, Velásquez *et al.* 2015).

Regarding the second biomarker cTnT, the mean level among HCV patients was 3.72±2.03 ng/L, whereas the mean level among the control group was 2.36±1.08 ng/L. cTnT levels in HCV patients were significantly higher than in the control group ($P \leq 0.001$). Troponin is a special protein that affects actinomyosin by inhibiting its ATPase activity. It is composed of two types; skeletal and cardiac (Masia *et al.* 2011). In the present investigation, blood levels of the cardiac dysfunction biomarker cTnT were evaluated, and a highly statistically significant difference was detected between the patient and control groups. Consistent with findings from previous Asian research, these findings (Matsumori *et al.* 2006). Where the results of Matsumori *et al.* (2006) showed that 17 of 56 patients (30%) had increased levels of circulating cardiac troponin (cTn) I, and that 28 of 59 patients (48%) with HCV antibodies had detectable levels of cTnT, indicating the presence of persistent myocardial damage. Both kinds of cardiac troponin may be very sensitive and specific prognostic indicators acting as a predictor of unfavorable cardiac outcomes, particularly in patients with CKDs, and it has been shown that HCV infection may be a major predisposing factor for myocarditis (Ingec *et al.* 2014, Badiou *et al.* 2016). Patients with CKD receiving hemodialyses treatment showed no

statistically significant change in blood cTnT levels, according to Castini *et al.* (2017). Heart failure appears to be common among patients with renal failure, and is associated with elevated serum levels of troponin without clinical myocardial infarction; however, there may be several confounding factors that account for these divergent findings, such as the health of the participants, the location of the studies, the study designs, the assays employed, and other conditions related to this infection (Chew *et al.* 2014).

The mean level of the third biomarker (NT-pro-BNP) among HCV patients was 88.31±37.40 pg/mL, while the mean level among the control group was 45.56±18.33 pg/mL. NT-pro-BNP levels in HCV patients were significantly higher than in the control group (P≤0.001). The results were shown in Table 4.7. It is a hormone synthesized in the heart and secreted by the ventricles as a reaction to cardiac wall distension and stretching (Yancy *et al.* 2013). The findings so far are consistent with those of previous research conducted in Japan (Okada *et al.* 2013, Sato 2013). Okada *et al.* (2013) compared to controls (mean 39.8±24.4 pg/mL; median 35.8 pg/mL; range 7.0-108.0), NT-proBNP concentrations in HCV patients were substantially higher (P≤0.05) (mean 71.6±79.1 pg/mL; median 46.0 pg/mL, range 5.0-400.0). 20% of HCV patients and 6% of controls exhibited elevated NT-proBNP. In Egypt Raouf *et al.* (2020) compared to healthy controls (mean: 36.1±28.1 pg/mL), patients with CHC had a substantially higher NT-pro-BNP concentration (mean: 120.6±72.6 pg/mL; P=0.001). 80% of patients with CHC had elevated NT-pro-BNP (>65 pg/mL; the cut-off value of NT-pro-BNP), and 29.1% of patients with CHC had elevated NT-pro-BNP (>125 pg/mL; the suggested cut-off value for heart failure). Chronic HCV infection was independently associated with elevated NT-pro-BNP levels, according to multivariate analyses. And in Iraq, Salman *et al.* (2021) measured NTpro-BNP and cTn-I biomarker concentrations. The cardiac biomarkers assessed in the patient group were significantly different from those in the control group. The average levels of the NTproBNP biomarker were 49.46±19.47 in the control group and 86.95±43.1 in the patient group, while the average levels of the cTn-I biomarker were 278.91±326.8 in the control group and 527.29±445.5 in the patients. Evidence suggests that HCV infection may have contributed to the development of ESRD and myocarditis in some patients by replicating the virus in their hearts, leading to high blood pressure, right ventricular heart failure, and an increase in NT-pro-BNP production (Horii *et al.* 2013, Okada *et al.* 2013, Franceschi *et al.* 2018). According to research conducted in

Egypt in 2011, elevated levels of NT-pro-BNP were closely connected to changes in ECG results in individuals with HCV. As a result, it has been suggested that this biomarker be measured during the patients' subsequent monitoring (Saleh *et al.* 2011).

Table 4.7 Serum levels of the studied biomarkers levels among the studied groups

Variables	**HCV infection status**				**P value**
	Positive (130)		**Negative (50)**		
	Mean	**SD**	**Mean**	**SD**	
IL-6 levels (pg/mL)	44.31	20.42	22.96	12.69	< **0.001***
cTnT levels (ng/L)	3.72	2.03	2.36	1.08	< **0.001***
NT-pro-BNP levels (pg/mL)	88.31	37.40	45.56	18.33	< **0.001***
*** Statistically significant difference, calculated using independent samples T-test**					

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The levels of IL-6, cTnT, and NT-pro-BNP cardiac dysfunction biomarkers were significantly highly elevated in the serum of CKD patients with HCV infection in comparison with the control group ($P \leq 0.001$). These findings suggest that having an HCV infection can be an additional predisposing factor for CVDs among CKD patients.
2. The highest number of CKD patients was recorded among patients in the sixth decade.
3. The majority of patients have HCV infection.
4. Almost all patients were hypoalbuminemia with a prevalence rate of more than 95%.
5. Hypertension and DM appear as common chronic diseases among the participants with prevalence rates of 78% and 58%, respectively.
6. The level of IL-6 was higher in patients compared to the control group.

5.2 Recommendations

1. Design a study determining the effect of the studied biomarkers among patients with HBV infection and compare the outcomes with the results obtained from the current study.
2. Conduct specific studies focusing on the role of CVDs among CKD patients free from any viral infection.
3. Perform epidemiological studies to investigate how CKD patients can get a viral infection.

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