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The Role of Collagen Deposition Biomarkers for Myocardial Interstitial Fibrosis in Atrial Fibrillation Cases

A Thesis

Submitted to the Council of the College of Medicine / University of Kerbala in Partial Fulfillment of the Requirements for the Degree of Master in Clinical Chemistry

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Dedication

- To my dear country
- To my father, may god have mercy on him, the man who taught me how-to live-in dignity and glory.
- To my mother, I cannot find words to express my thanks to her for what she offered me.
- I thank you with all my heart.
- I am grateful to all of you.

Mustafa Hameed Mahdi

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Summary

Myocardial interstitial fibrosis is one of the most common complications of cardiomyopathy because increased collagen deposition alters the interstitial structure of the myocardium. It is also a major cause of cardiac systolic and diastolic dysfunction and changes clinical outcomes in patients with non-ischemic heart disease. As a result, myocardial interstitial fibrosis leads to atrial fibrillation, which is a supraventricular arrhythmia characterized by irregular and ineffective atrial contractions, electrical activation and an absence of the P wave. The symptoms of atrial fibrillation include palpitations, chest pain, dyspnea and syncope. The risk factors reasons are causing myocardial interstitial fibrosis and both of them lead to atrial fibrillation.

Collagen deposition may be a novel pathway marker to distinguish the absence or presence of myocardial interstitial fibrosis in atrial fibrillation patients. This study searched for the relationship between serum levels of PICP, CITP and myocardial interstitial fibrosis in atrial fibrillation patients and the effect of risk factors on the outcomes. Additionally, the efficacy of serum PICP and CITP as biomarkers for diagnosing atrial fibrillation.

The study protocol was approved by the ethical committee at Kerbala University College of Medicine, Al-Zahraa Teaching Hospital / Wasit in the City of Al-Kut. Verbal approval is taken from all patients included in the study before sampling.

The present study involved 120 participants, ranging in age from 22 to 90 years. Sixty of them were healthy and served as a control group and sixty of them had atrial fibrillation and were diagnosed by electrocardiography interpretation, If the electrocardiography does not detect atrial fibrillation despite a strong suspicion, it may be necessary to document the arrhythmia with a Holter monitor. Atrial fibrillation patients are subdivided according to American College of Cardiology, American Heart Association and European Society of Cardiology recommendations into four groups: paroxysmal, persistent, permanent and lone atrial fibrillation. A serum sample from each participants was used to measure the myocardial interstitial fibrosis markers such as PICP and CITP, which were analyzed by enzyme linked immunosorbent assay, while the serum lipid profiles was measured by an Abbott Laboratories device.

The current study revealed that the ratio of females to males was approximately 2:1, and the mean differences of values in the biomarker levels (S.PICP, S.CITP, and

their ratio) based on the echocardiography findings and electrocardiography interpretation in the cases of atrial fibrillation include several biomarkers that are considered statistically significant, which include gender groups, hypertension group, body mass index groups, left atrial diameter, left ventricular diameter, ejection fraction percentage groups and atrial fibrillation groups at $P \leq 0.05$. While lipid profiles, paroxysmal and lone atrial fibrillation at the S.PICP/S.CITP ratio were considered non-significant at $P>0.05$.

According to the receiver operating characteristic curve to determine the efficacy of S.PICP and S.CITP for the detection of myocardial interstitial fibrosis, the most highly specific and sensitive biomarkers for the prognosis of myocardial interstitial fibrosis are S.CITP with a sensitivity of 93.2% and a specificity of 83.3% and has an area under curve of 0.829 ; followed by S.PICP with a sensitivity of 86.7% and a specificity of 81.7% and has an area under curve of 0.782 . While their ratio of S.PICP/S.CITP is not specific for the prognosis of myocardial interstitial fibrosis due to having an area under curve less than 0.5 with a sensitivity of 22% and a specificity of 95% and this is a value rejected in the prediction of myocardial interstitial fibrosis. Additionally, receiver operating characteristic curve for evaluating the effectiveness of left ventricular and left atrial diameter in diagnosing dilation or enlargement, the most specific and sensitive parameter for diagnosing dilation is left ventricular with a sensitivity of 86.7% and a specificity of 81.7% and has an area under curve of 0.683; It is followed by left atrial enlargement with a sensitivity of 78.3%, a specificity of 58.3% and an area under curve of 0.723. Elevated S.PICP and S.CITP levels can be used to indicate myocardial interstitial fibrosis in patients with atrial fibrillation. The S.PICP level with a cutoff value of more than 157 *ng/mL* may be a novel biomarker for evaluating myocardial interstitial fibrosis in patients with atrial fibrillation. The S.CITP level with a cutoff value greater than 165.5 *ng/mL* can be used as an alternative biomarker in patients with atrial fibrillation independent of myocardial interstitial fibrosis. Most atrial fibrillation patients have myocardial interstitial fibrosis and comorbidities such as obesity and hypertension. An high level of serum vitamin C leads to elevated levels of both markers S.PICP and S.CITP for some individuals in the control group, but the increase in levels for both markers S.PICP and S.CITP in the AF group represents myocardial interstitial fibrosis.

List of Contents

List of Abbreviations	
Abbreviations	***Difinition***
AAC	American Association of Colleges
ACC	American College of Cardiology
AF	Atrial Fibrillation
AHA	American Heart Associatio
AMI	Acute Myocardial Infarction
AT-II	Angiotensin-II
AUC	Area Under Curve
AVN	Atrioventricular Node
BBB	Bundle Branch Block
BMI	Body Mass Index
BP	Blood Pressure
bpm	beats per minute
C	Carboxy
CCU	Cardiac Care Unit
CD+	Excessive Collagen Type- I Deposition
CE	Cholesterol Esterase
CITP	Carboxyterminal Telopeptide of Type 1 Collagen
CKD	Chronic kidney disease
CI%	Confidence interval
cm	Centimeters
CO	Cholesterol Oxidase
COL1	Collagen Type I
COL3	Collagen Types III
CTX-1	Carboxy Terminal Telopeptide of Type-1 Collagen
CVD	Cardiovascular Disease
d	Precision
D.W	Distilled Water
DCM	Dilated Cardiomyopathy
DSBMT	2, N, N- bis (4-Sulpho butyl) m-Toluidine Disodium
ECGs	Electrocardiograms
ECM	Extracellular Matrix
EF%	Ejection Fraction Percentage
ELISA	Enzyme Linked Immunosorbent Assay
EP	Electrophysiology
ESC	European Society of Cardiology
FHS	Framingham Heart Study
HCM	Hypertrophic Cardiomyopathy
HDL	High-Density Lipoprotein
HF	Heart Failure
HFpEF	Heart Failure preserved Ejection Fraction
HFrEF	Heart Failure reduced Ejection Fraction
HHD	Hypertensive Heart Disease
HRP	Horseradish Peroxidase

HTN	Hypertension
IHD	Ischemic Heart Disease
IL	Interleukin
IM	Myocardial Infarction
IQR	Inter Quartile Range
KDa	Kilodaltons
Kg	kilograms
LA	Left Atrial
LBB	Left Bundle Branch
LBBB	Left Bundle Branch Block
LDL	Low-Density Lipoprotein Cholesterol
LFT	Liver Function Tests
LSD	Least Significant Differences
LV	Left Ventricular
LVEF%	Left Ventricle Ejection Fraction Percentage
m	Meters
MIF	Myocardial Interstitial Fibrosis
mmHg	Millimertres of mercury
MMPs	Metalloproteinase
MRI	Magnetic Resonance Imaging
N	Amino
n	Sample Size
NPV	Negative Predictive Values
NS	Non-significant
NSR	Normal Sinus Rhythm
O.D.	Optical Density
O.P.	Operation
OR	Odds Ratio
P	Prevalence
PICP	Carboxyterminal Propeptide of Type-1 Procollagen
PIIICP	Carboxyterminal Propeptide of Type- III Procollagen
PIIINP	Aminoterminal Propeptide of Type- III Procollagen
PINP	Aminoterminal Propeptide of Type- I Procollagen
PPV	Positive Predictive Values
QoL	Quality of Life
RBB	Right Bundle Branch
RBBB	Right Bundle Branch Block
RFT	Renal Function Test
ROC	Receiver Operating Characteristic
RV	Right Ventricle
S	Significant
SAN	Sinoatrial Node
Sn	Sensitivity
SND	Sinus Node Dysfunction
Sp	Specificity
SPSS	Statistical Package for The Social Sciences

St.	Standard
TG	Triglyceride
TNF-B	Tumour Necrosis Factor-B
TIMPs	Tissue Inhibitors of Metalloproteinase
USA	United States of America
VC	Vitamin-C
VLDL	Very Low-Density Lipoprotein Cholesterol
WHO	World Health Organization
Z	Statistic for a level of Confidence (1.96 for 95%)

Chapter One

Introduction

and

Literatures Review

1. Introduction and Literatures Review

1.1. Introduction

The heart functions as two consecutive pumps with many electrical and mechanical parts **(Walker & Colledge, 2011)**. The heart weighs 250–390 g in males and 200–275 g in females & is slightly larger than the owner's closed fist, measuring around 12 centimeters in length and 9 centimeters in width **(Jenkins & Tortora, 2016)**.

It is located within the thoracic cavity (chest) behind and to the left of the sternum, the breastbone and the mediastinum (between the lungs) **(Peate & Nalr, 2017)** (Figure 1-1).

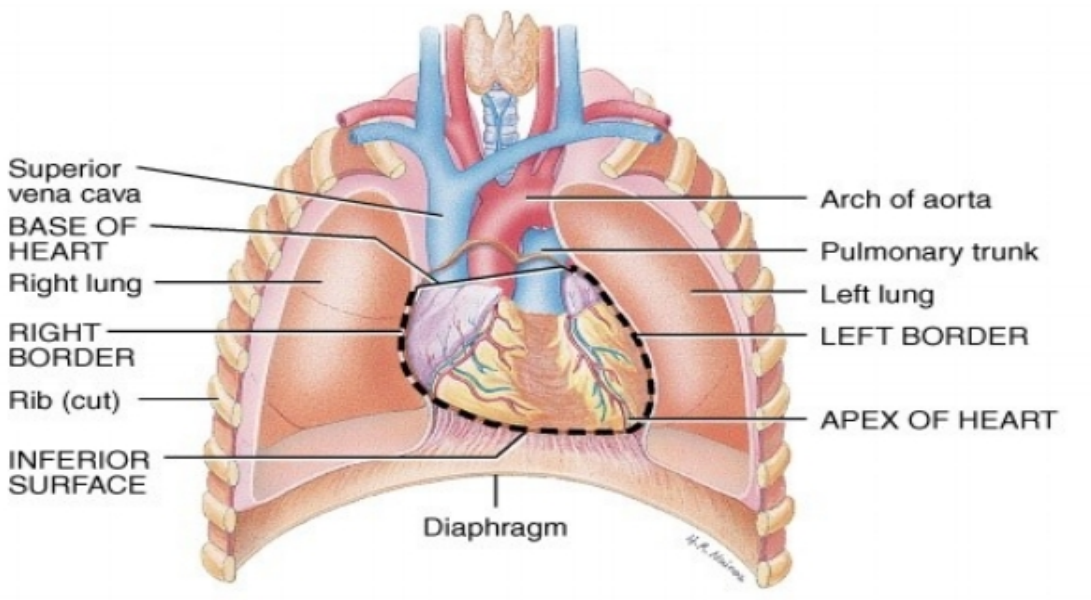


Figure 1-1: Location of the heart (Tortora & Derrickson, 2009)

The heart is split into four chambers by vertical septa: the right and left atria and the right and left ventricles. A pair of atria receive blood and pump it into a pair of ventricles, which then pump blood into the arteries. The right atrium receives relatively low oxygen systemic blood and pumps it into the right ventricle, then pumps it into the pulmonary circuit. The lungs

exchange oxygen and carbon dioxide and oxygen rich blood returns to the left atrium, which pumps blood into the left ventricle, which pumps blood to the rest of the systemic circuit via the aorta **(Snell, 2011)**. (Figure1-2)

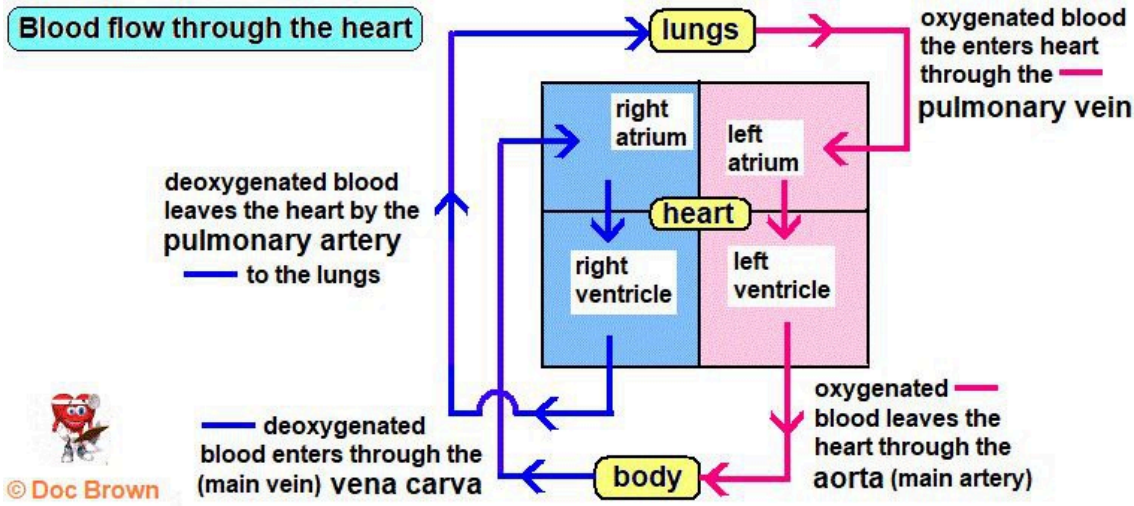


Figure1-2:A Simple illustration of blood flow through the heart (Peate & Nalr, 2017)

There are three basic types of cardiac muscle in the heart: atrial muscle, ventricular muscle & specialized excitatory and conducting muscle fibers. The atrial and ventricular types of muscle contraction occur in the same way that skeletal muscle does but for much longer. However, the specialized excitatory and conductive fibers of the heart contract only weakly because they contain a small number of contractile fibers, instead; they display either spontaneous rhythmical electrical discharge in the form of action potentials or the conduction of the action potentials through the heart, providing an excitatory system that regulates the rhythmical beating of the heart **(Hall, 2015)**.

A resting adult's typical heart rate is between 70-90*bpm*; The rhythmic contractile process occurs spontaneously in the conducting system; The impulse goes to various areas of the heart causing the atria to contract first and simultaneously, followed by the contractions of both ventricles simultaneously; The slight delay in signal transduction from the atria to the

ventricles allows the atria to empty their blood to the ventricles prior to having the ventricles contract. The heart's conducting system comprises specialized cardiac muscle in the sinoatrial node (SAN) (Pacemaker), positioned in the right atrium's upper wall; The atrioventricular node(AVN) is strategically located on the lower part of the atrial septum above the septal attachment of the tricuspid valve. The atrioventricular bundle conducts the cardiac impulse to the ventricles. The atrioventricular node (bundle of His) transmits impulses from the AV node to the Purkinje fibres and separates into branches, one for each ventricle; The right bundle branch (RBB) sends electrical impulses to the right ventricle (RV), whereas the left bundle branch (LBB) sends electrical impulses to the left ventricle (LV) and Purkinje fibres (specialized cardiac muscle fibres that constitute the heart's conducting system and pump out blood) (**Ganesan et al., 2016**).(Figure1-3)

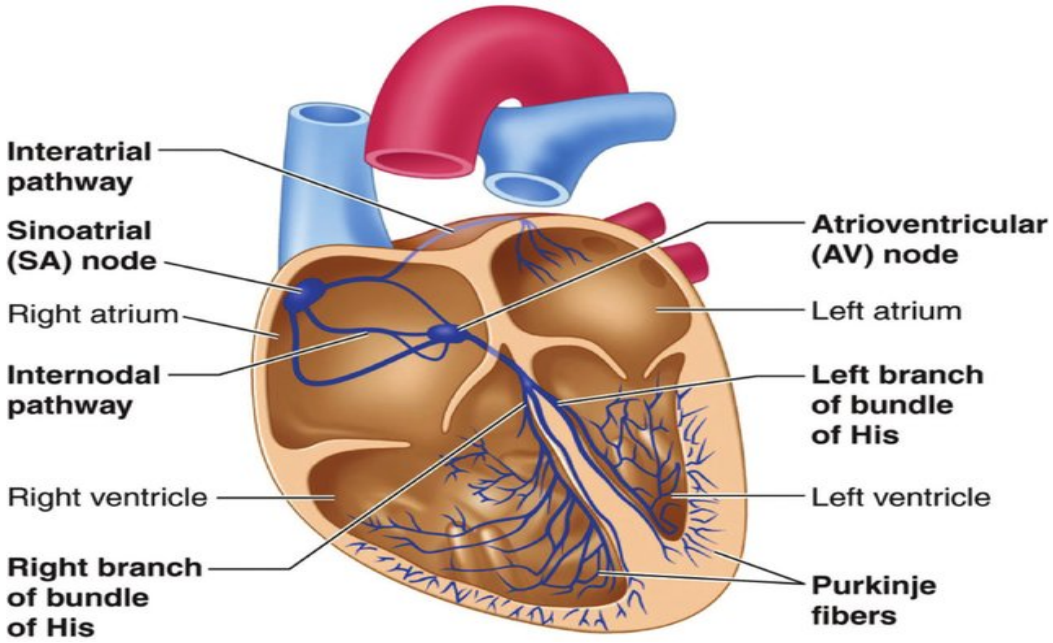


Figure1-3:Heart's electrical conduction system symbolizes the normal sinus rhythm (NSR) route (Ganesan et al., 2016)

Sinus node dysfunction (SND) indicates a wide variety of disorders involving sinus node and atrial impulse generation and propagation, resulting in the inability of (SN) to generate heart rates that meet the body's physiological requirements; Causes of SND can be classified as intrinsic (secondary to a pathological condition involving the sinus node itself such as hypertension, cardiomyopathy, collagen vascular disease and atrial tachyarrhythmias) **(Csepe et al., 2015)** or extrinsic (caused by depression of sinus node function by external factors such as drugs (e.g. beta blockers or autonomic influences) **(Sheldon et al., 2015)**.

The Structural abnormalities (e.g., loss of sinus nodal cells and fibrosis) and modifications in the normal gradient of electrophysiology (EP) properties in the sinus node complex are likely responsible for the slowdown of pacemaker associated with normal ageing and cardiovascular disease (e.g., atrial fibrillation [AF], heart failure [HF]). In addition, autonomic modulation and structural remodelling have the potential to cause not only a decrease in sinus pacemaker activity but also a full blockage of conduction along all nodal conduction channels **(Milanesi,Bucchi & Baruscotti, 2015)**. (See Figure 1-4)

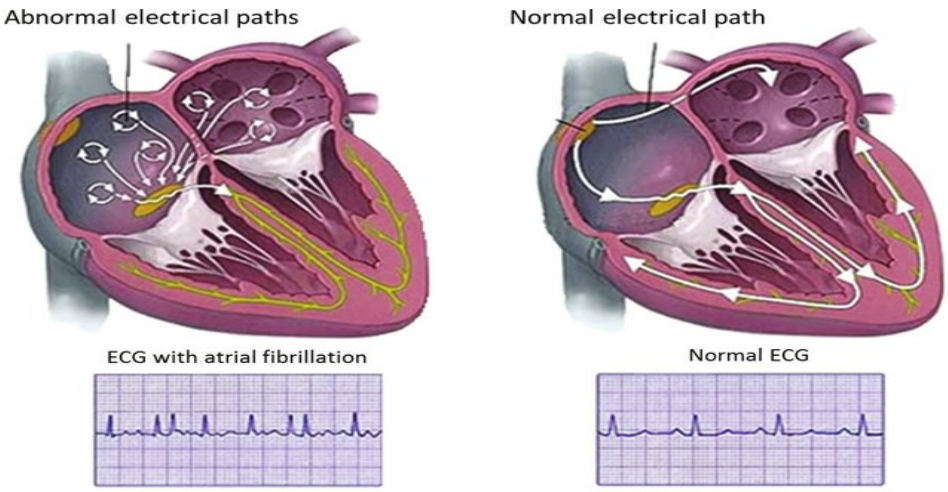


Figure 1-4: Atrial fibrillation (AF) Compared to Normal Sinus Rhythm (NSR) (Ganesan et al., 2016)

Arrhythmias are deviations from the usual heart rate or rhythm. Atrial fibrillation (AF) is an example of an arrhythmia **(NHLBI, 2021)**. AF is a supraventricular tachyarrhythmia distinguished by irregular atrial contractions, electrical activation and ineffective atrial contraction. AF is distinguished by irregular R-R intervals (where atrioventricular conduction is not obstructed), the absence of discernible recurrent P waves and irregular atrial activations **(Hindricks et al., 2021)**.

The prevalence of atrial fibrillation (AF) in the general population of the western world has been estimated to range from 0.5% - 2%. However, the true frequency of AF is likely greater due to the prevalence of asymptomatic (subclinical) AF. In addition, the prevalence of (AF) is steadily rising in the general population largely due to increased longevity and the effectiveness in reducing overall cardiovascular mortality, as well as the increased prevalence of risk factors for AF (such as hypertension [HTN] and obesity). In 2010, the prevalence of AF was expected to range between 2.7 and 6.1 million in the United States. It is predicted to increase to between 5.6 and 12 million by 2050. In Europe, around 8 million people are affected by AF. It is projected to climb to 18 million by 2060 (**Krijthe et al., 2013**).

The clinical risk factors for atrial fibrillation (AF) include being over the age of 65, caucasian, males gender, thyroid dysfunction, having had atrial fibrillation before, high blood pressure, mitral valve disease, heart failure, left ventricular hypertrophy, diastolic dysfunction, left atrial enlargement, stopping beta blocker therapy, being overweight, a low BMI, chronic obstructive pulmonary disease, a long PR interval, diabetes, smoking and drinking alcohol **(Bidar et al., 2014)**. In these instances, AF typically fades with treating the triggering illness.

Symptomatic or asymptomatic atrial fibrillation (AF) might occur even in the same patient. The symptoms of AF vary based on the ventricular

rate, the underlying functional status, the duration of AF, the presence and severity of structural heart disease and the patient's perception **(Garimella et al., 2015)**. Most individuals with AF have palpitations, chest pain, dyspnea, non specific weariness, vertigo and syncope, despite significant interindividual and intraindividual variability **(Dillon & Ghanbari, 2014)**. Although palpitation or the perception of an irregular heartbeat is significant in more than half of patients with atrial fibrillation (especially in those with paroxysmal AF), its connection with confirmed arrhythmia is poor. Significant activity intolerance can be caused by dyspnea and exhaustion.

It is possible to categorize atrial fibrillation (AF). Permanent (AF) refers to AF in which cardioversion has failed or AF has sustained for over a year and further attempts to restore normal sinus rhythm (NSR) have failed. AF that terminates spontaneously within seven days is termed paroxysmal and AF that is present continuously for more than seven days and less than one year is called persistent (**Calkins et al., 2017**). Each of these classifications has ramifications for the underlying mechanisms and the therapeutic response. When a patient has suffered two or more episodes of AF the condition is classed as recurrent.

The phrase "lone" AF has been described inconsistently in the medical literature but it often refers to younger AF patients with no clinical or echocardiographic signs of cardiovascular illness, hypertension (HTN) or diabetes **(January et al., 2014)**.

Electrocardiograms (ECGs) are crucial to cardiac diagnosis and routine care **(Fan et al., 2018)**. ECG monitoring to diagnose atrial fibrillation (AF) **(Attia et al., 2019)**. If an ECG fails to detect AF despite a strong suspicion, a Holter or cardiac event monitor may be necessary to document the AF **(Fuster et al., 2006)**.

Myocardial interstitial fibrosis (MIF) is a major cause of cardiac systolic and diastolic dysfunction and poor clinical outcomes in patients with non ischemic heart disease (IHD). As a result, MIF is a common finding in patients with heart failure (HF) who have non IHD such as diabetic cardiomyopathy or hypertensive heart disease (HTN) (**Shimizu et al., 1993**) non IHD dilated cardiomyopathy (DCM), **(Falcao-Pires et al., 2011)** hypertrophic cardiomyopathy (**Brooks et al., 2003)** and aortic stenosis (**Anderson, Sutton & Lie, 1979**).

This review study will focus on myocardial interstitial fibrosis as determined by the quantity of collagen deposition (captured by S.PICP & S.CITP in serum) that arises as the major alteration in the ventricular extracellular matrix (ECM); it will focus primarily on the importance of MIF in its relationship to AF and how fibrosis interferes with heart function through collagen deposition, It was examined whether the levels of S.CITP and S.PICP obtained from the MIF sample had a prognostic impact on the atrial fibrillation patient.

1.2. Literatures Review

1.2.1. Myocardial Interstitial Fibrosis

1.2.1.1. Definition and Classification

Myocardial Interstitial Fibrosis (MIF) is an increased collagen deposition that alters the myocardial interstitial structure and is quantitatively characterised by an increase in the proportion of total myocardial tissue occupied by collagen fibres as determined by collagen-specific staining of myocardial tissue samples **(Hoyt et al., 1984)**. Therefore, MIF is a common observation in patients with heart failure (HF) who have non ischemic heart illnesses such as diabetic cardiomyopathy, hypertensive cardiac disease **(Van Hoeven & Factor, 1990)**, aortic stenosis (**Shimizu et al., 1993**), hypertrophic cardiomyopathy **(Shirani et al., 2000)** and non ischemic dilated cardiomyopathy (DCM) **(Brooks et al., 2003)**.

The MIF is crucial to myocardial remodelling in patients with HF caused by various non ischemic cardiac diseases. It significantly impacts left ventricular (LV) and systolic and diastolic cardiac dysfunction and causes poor clinical outcomes in non IHD patients **(González et al., 2018)**. (Central Illustration, Fig. 1.5)

Recently, the mechanisms and pathways underlying the disruptions in the turnover of myocardial fibrillar collagen lead to both tissue and organ fibrosis in general **(Weiskirchen, Weiskirchen & Tacke, 2019)**. Moreover, MIF in particular was examined in detail **(Cowling et al., 2019)**. consequently, a quick outline of the essential steps of the fibrotic process is provided in central illustration, Fig. 1.5.

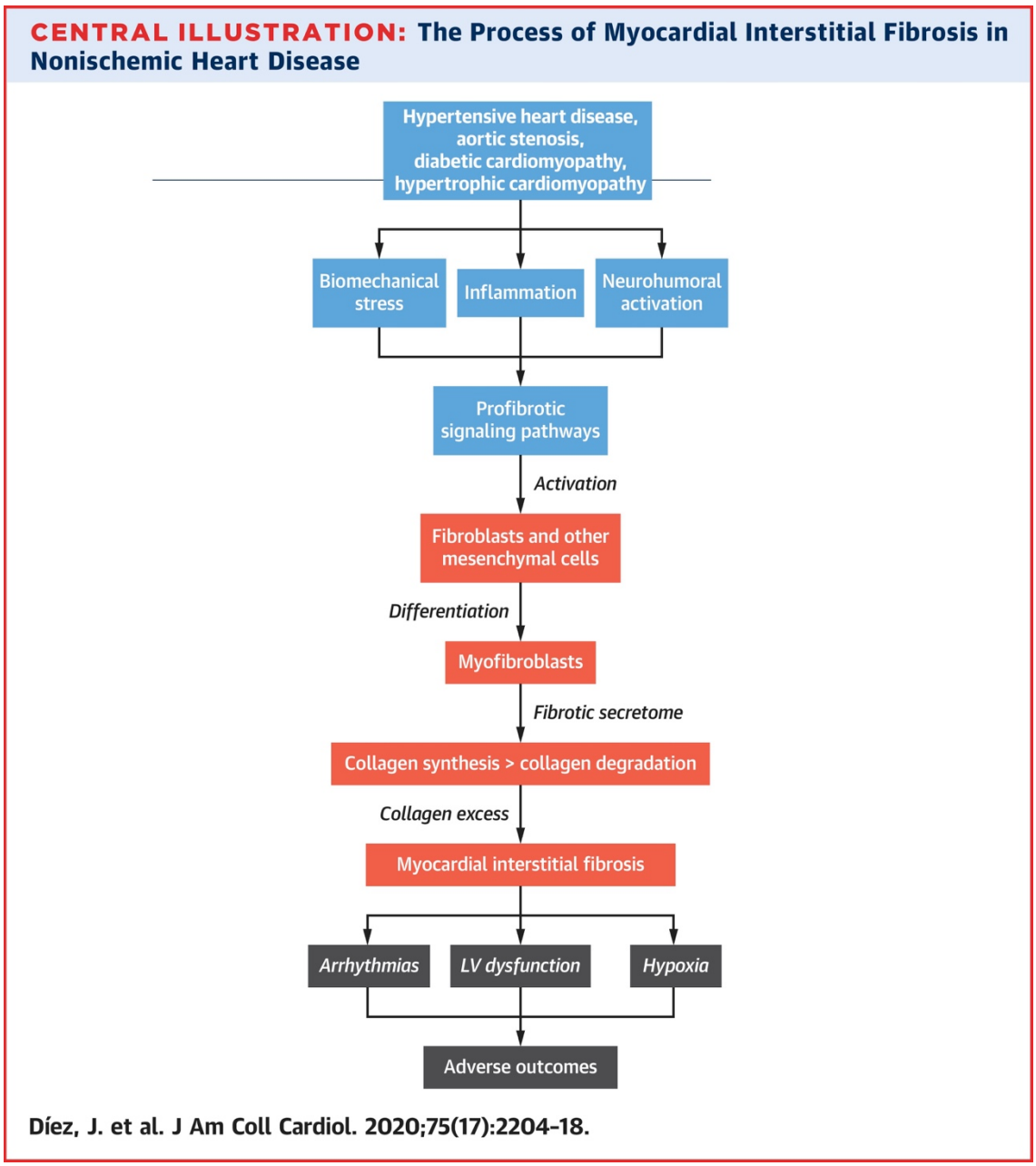


Figure 1-5: Steps of MIF in nonischemic heart disease (Central Illustration) (Díez, González & Kovacic, 2020)

Histopathological categorization distinguishes two forms of fibrosis: (See Figure 1-6)

"Replacement fibrosis" occurs after cardiac injuries such as an acute myocardial infarction (IM), when collagen and the ECM replace necrotic cells **(Burstein & Nattel, 2008)**. It is also referred to as reparative fibrosis.

"Reactive fibrosis" is an inflammatory process (often caused by volume or pressure overload or genetically mediated) that leads to the deposition of fibrous tissue between cells (interstitial) and blood vessels (perivascular) (**Kong, Christia & Frangogiannis, 2014**).

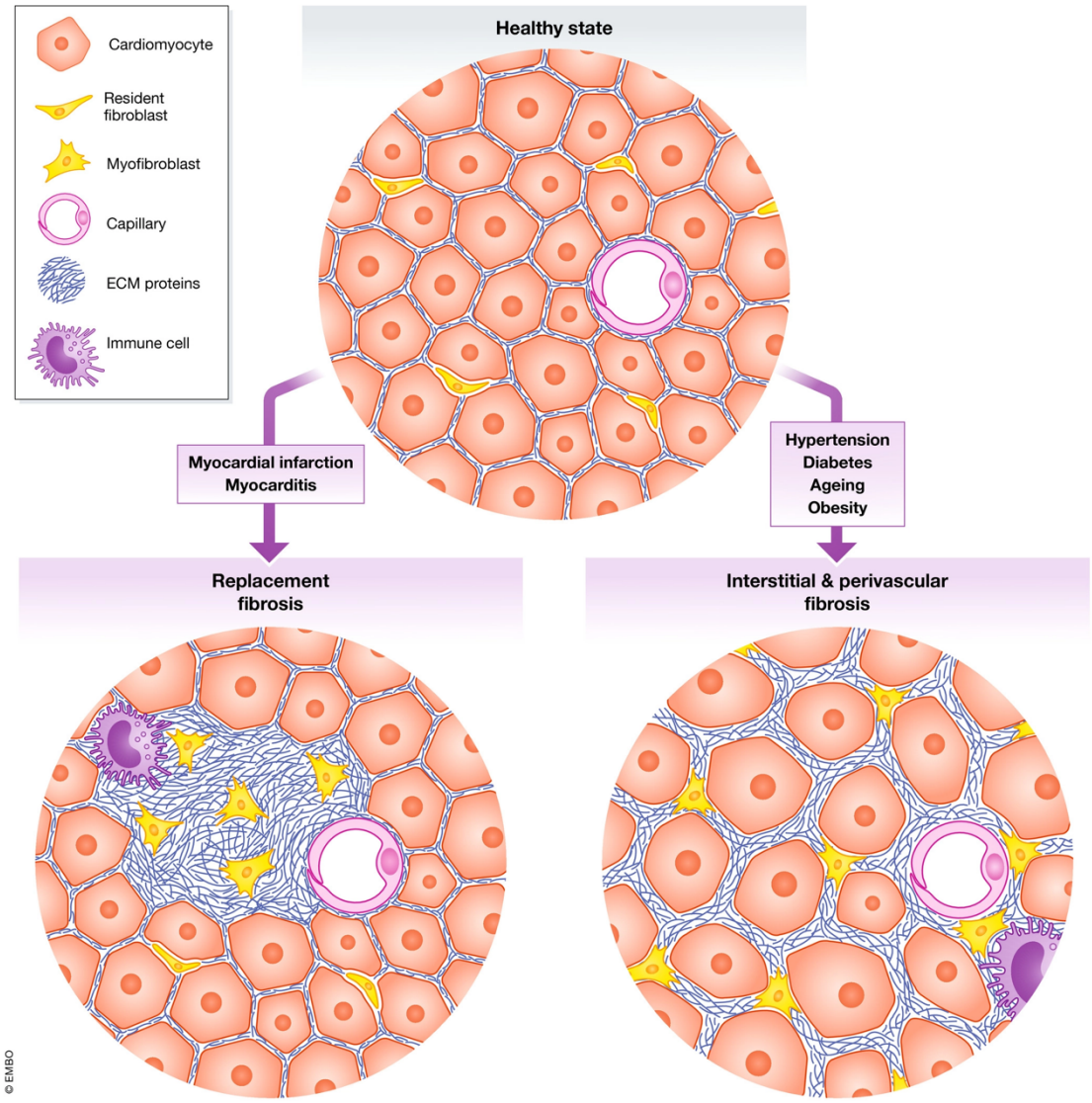


Figure 1-6:Histological distinctions exist among interstitial/perivascular and replacement fibrosis (Sweeney, Corden & Cook, 2020).

Replacement vs. interstitial or perivascular fibrosis illustration depicting the differential spatial deposition of extracellular matrix (ECM) in the two types of cardiac fibrosis as well as changes in cell architecture

cardiomyocyte dilatation, inflammatory cell infiltration and myofibroblast activation. **(Sweeney, Corden & Cook, 2020)**.

Fibrosis can be classified based on the structure, size and organization of the fibrous tissue **(Hansen, Zhao & Fedorov, 2017)**.

- Interstitial fibrosis: hypertrophy and thickening of the extracellular matrix (ECM).
- Diffuse fibrosis: involves areas with a mixture of myocardium & collagen fibres.
- Compact fibrosis: consists of regions of dense collagen devoid of cardiac tissue.
- Patchy fibrosis: consists of myocardial collagen bundles & long strands.

Myocardial fibrosis is typically classified as either replacement or reactive. (See Fig 1-7) During acute and significant tissue damage in each myocardial infarction (MI) or myocarditis, necrotic cardiomyocytes are replaced with reparative fibrosis; different forms of cardiac damage lead to reactive fibrosis with ECM architecture and content varying according to the pathogenic cause of the damage **(Frangogiannis, 2021)**.

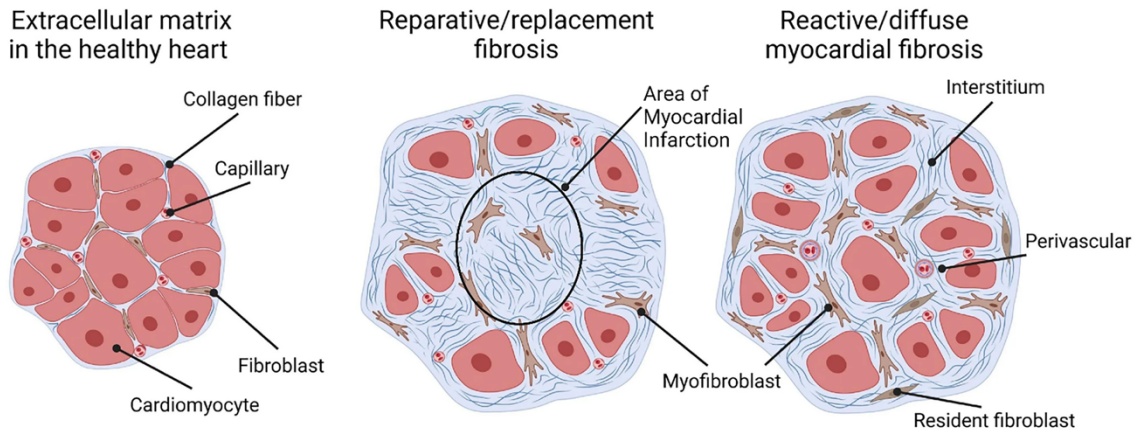


Figure 1-7: Classifications of cardiac fibrosis with permission from Schimmel *et al.* (Schimmel et al., 2022)

1.2.1.2. The Cardiac Extracellular Matrix: Structure and Function

The extracellular matrix (ECM) comprises a fibrillar network, basement membrane, proteoglycans and fibrillar proteins like fibronectin, collagen, elastin, fibrillin and laminin **(Järveläinen et al., 2009**). Collectively, they maintain the integrity of the structure of the surrounding cells, thereby ensuring their stability. In addition, the ECM has been connected to transmit crucial biochemical signals for proper tissue growth; The ECM is present in all tissues, although the distribution of matrix components in every organ is distinct (**Alberts et al., 2008**). For instance, most of the heart's extracellular matrix (ECM) is made up of collagen types I (85%) and types III (11%) **(De Jong et al., 2011)**. The classic conception of the myocardial ECM was that it provided the structural integrity of the heart as an inert mechanical scaffold. Nowadays, it is regarded as a dynamic network with significant metabolic activity and numerous complicated activities including regulating molecular signalling, cell proliferation, differentiation, movement, attraction and protein interactions **(Lukashev & Werb, 1998**). In addition, it modulates myocardial remodelling in both physiological and pathological states. Hence, the ECM plays a crucial role in regulating cardiovascular homeostasis **(Alberts et al., 2008)**. With the accumulation of new knowledge regarding the structure and function of the heart ECM, myocardial fibroblasts have been identified as the principal source of collagen type I (COL1) & collagen types III (COL3) peptides for the myocardium; It can be established that they are the primary cells in the heart that produce collagen **(Kong, Christia & Frangogiannis, 2014)**.

The fibrillar collagen is initially generated as procollagen, which is subsequently divided by specialized proteinase into carboxy (C) and amino (N) terminal propeptides:

- N-terminal polypeptides of COL1 & COL3 (PINP and PIIINP).

- C-terminal polypeptides of COL1 & COL3 (PICP and PIIICP).

Then the substance is released into circulation. Once the propeptides are cleaved, the triple helical chain "will create huge collagen fibrils with other collagen chains" (**Anderson, Sutton & Lie, 1979**).

The collagenases (MMP-1, MMP-8 & MMP-13) hydrolyze these collagen fibrils forming telopeptides and releasing small type I collagen telopeptides (CITP,12-kDa) into the circulation **(Eghbali et al., 1988)**. Non helical derivatives undergo spontaneous denaturation of large peptides **(Lijnen, Petrov & Fagard, 2000)**. The gelatinases MMP-2 & MMP-9 subsequently completely dissolve them into inactive bits. (See Fig. 1-8)

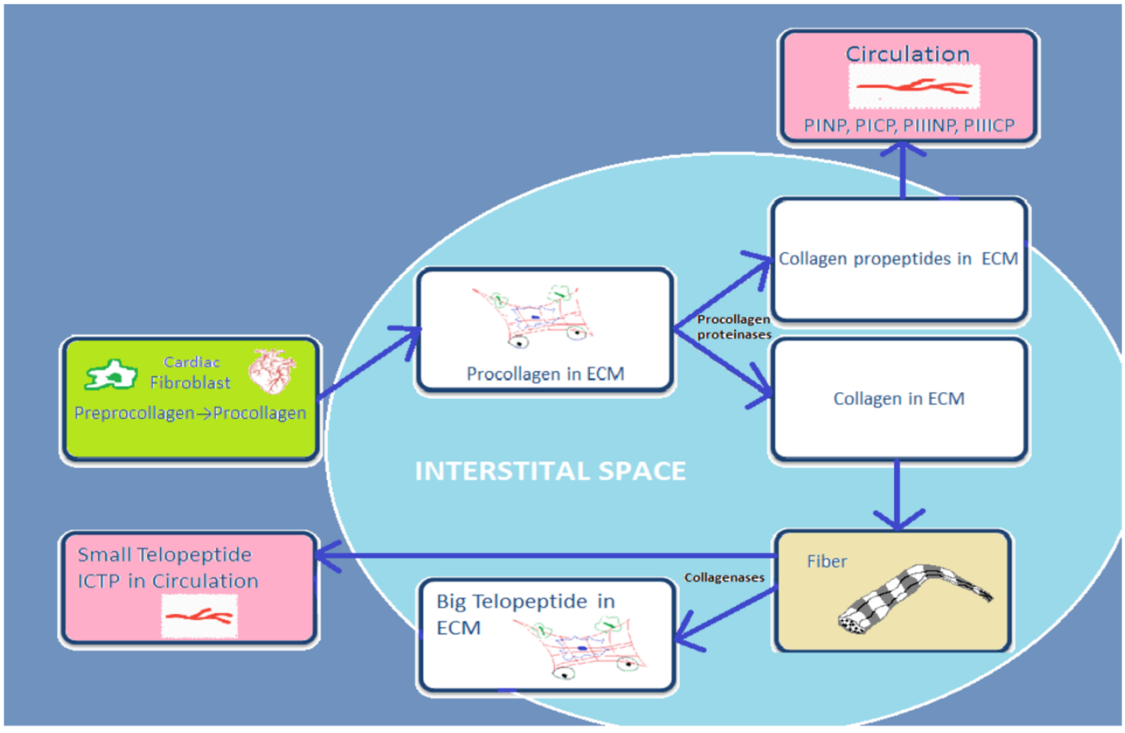


Figure 1-8: A schematic representation of the basic steps in the synthesis & degradation of collagen type-I & type-III (Nikolov & Popovski, 2022)

The extracellular matrix (ECM) plays a crucial role in the contractility of the cardio (**Lukashev & Werb, 1998**). Interstitial fluid and many proteins made by fibroblasts, like collagen & proteoglycans, make up

the ECM or interstitium. Collagen is the primary component of the cardiac extracellular matrix (ECM); Collagen not only keeps the heart's form & size but also gives it tensile strength **(Caulfield & Janicki, 1997)**.

1.2.1.3. The Collagen Turnover

Fibroblasts that have differentiated into myofibroblasts control collagen turnover. (See Figure 1-9) Furthermore, proinflammatory cytokines released by monocytes and macrophages such as tumour necrosis factor-B (TNF-B), interleukin-1& interleukin-6 (IL-1, IL-6) alter the function of fibroblasts & myofibroblasts (**López, González & Díez, 2010)**.

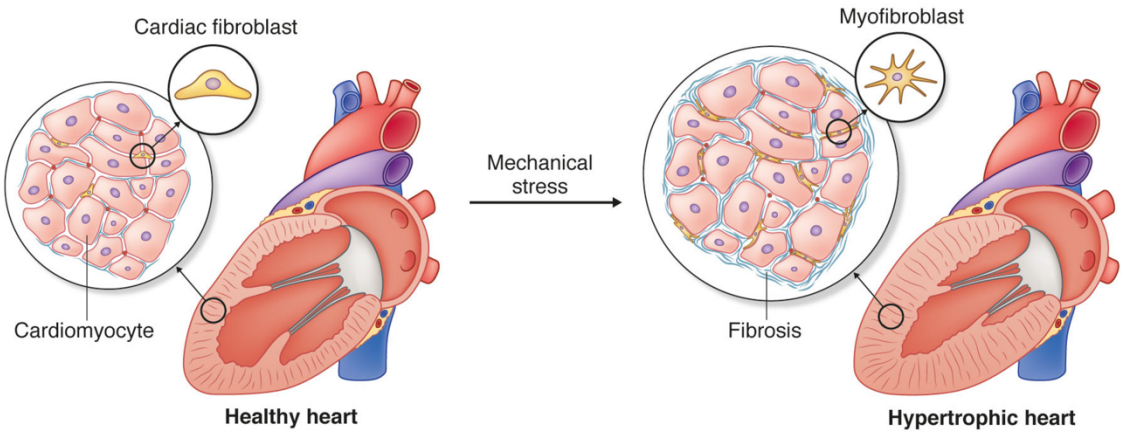


Figure 1-9: Mechanical stress during cardiac remodelling, such as hypertrophic remodelling, causes fibrosis. (Van Putten, Shafieyan & Hinz, 2016)

These cells, in response to the many factors such as mechanical stretch, wall stress, paracrine & autocrine substances created locally like angiotensin-II(AT-II), growth factors like connective tissue growth factor transforming or growth factor-β as well as hormones acquired from the circulation like aldosterone. Furthermore, it includes changes in their rates of migration and proliferation as well as modifications in their capacity to synthesize and secrete fibrillar collagen precursors, "specifically two more abundant subtypes observed in the heart: procollagen types I & types III" in

addition to enzymes that transform procollagen precursors into mature collagen capable of producing fibrils and fibres "like procollagen proteinase and lysyl oxidase" **(López, González & Díez, 2010**).

Fibroblasts produce matrix degrading enzymes such as metalloproteinase commonly known as (MMPs) and their inhibitors, which are regulated by various secreted extracellular protease and produced by fibroblasts, 'the tissue inhibitors of metalloproteinase aka (TIMPs)' **(McKleroy, Lee & Atabai, 2013)**. (See FIG.1-10)

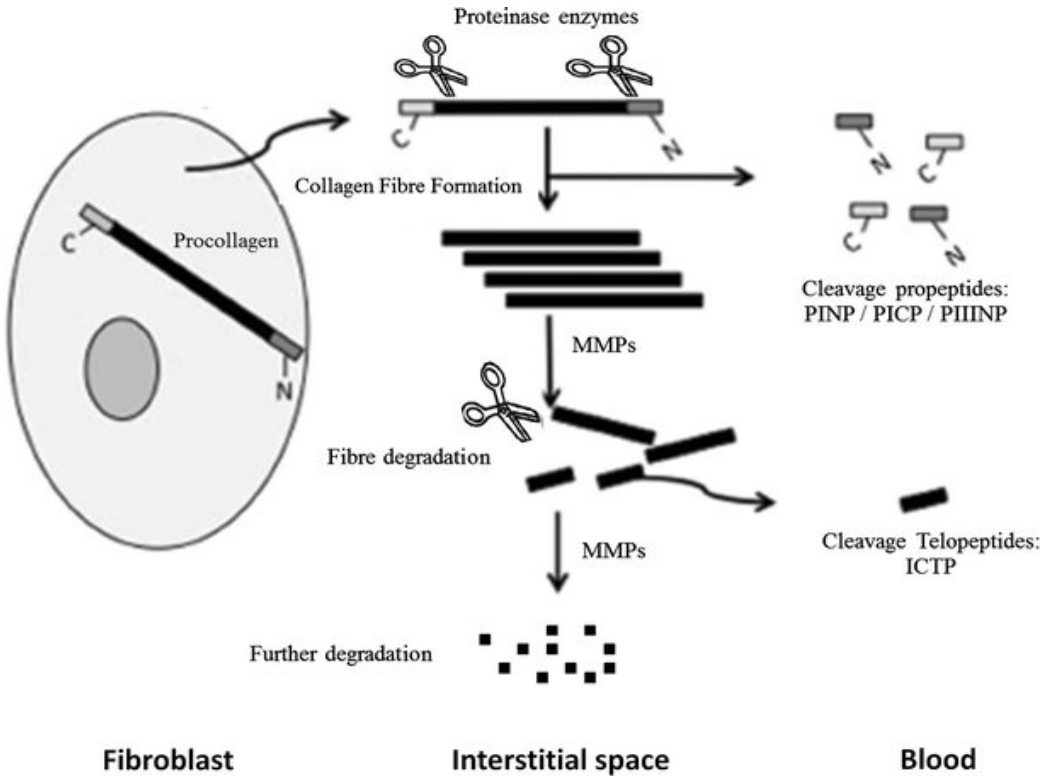


Figure 1-10: A schematic representation of collagen turnover (De Jong et al., 2011)

Heart failure is a cardiovascular disease (CVD) characterized by abnormal collagen turnover **(Moore et al., 2012)**. Myocardial collagen deposition and fibrosis have been associated with elevated serum levels of PICP, PINP, PIIICP, and PIIINP, which indeed reflect an increase in the synthesis of cardiac collagen types I and III. Whereas the elevated serum level of CITP reflect lower collagen degradation in cardiac collagen type I

(Fan et al., 2012). According to these results, heart illness disrupts the equilibrium between the synthesis and degradation of cardiac collagen **(Alla et al., 2006)**.

Cardiac injury is regarded as the initial event in the etiology of myocardial fibrosis according to a widely held belief. Thus, the MMP/TIMP system fails and the degrading activity of MMP-1, -2, -8, -9 &-13 is disrupted **(Gyöngyösi et al., 2017)**. Thus, fibroblasts in the heart become hyperactivated and transdifferentiated into myofibroblasts, which increases the production of collagen types I and III, followed by a decrease in collagen degradation processes and abnormal collagen deposition in the myocardium. Thus, decreased collagen turnover impact the remodelling of cardiac ECM abnormally and COL1/COL3 derived peptides are discharged into the circulation **(Ravassa et al., 2017; Hinderer & Schenke-Layland, 2019**). It can initiate an endless loop of COL1/COL3 over-deposition and subsequent inhibition of degradation. These factors may all contribute to the onset of myocardial fibrosis in heart failure. However, the molecular mechanisms underlying the development and progression of cardiac fibrosis remain obscure **(Hinderer & Schenke-Layland, 2019)**. (See Figure 1-11)

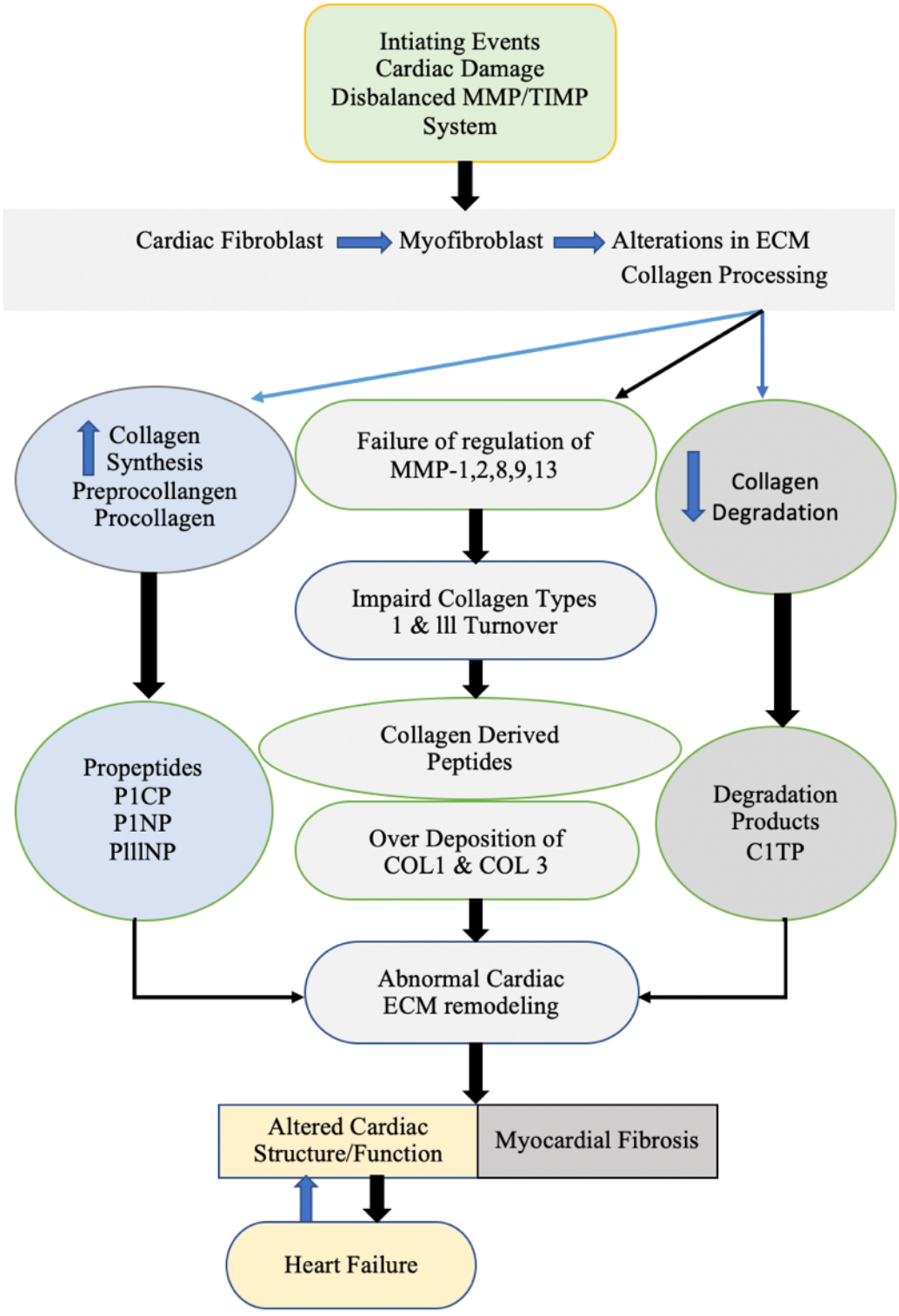


Figure 1-11: A schematic depiction of the collagen I and collagen III turnover impairment steps contributing to myocardial fibrosis in heart failure (López et al., 2021)

1.2.1.4. Myocardial Fibrosis in Non Ischemic Dilated Cardiomyopathy

Non ischemic dilated cardiomyopathy (DCM) is the leading cause of heart failure (HF) and transplantation; A poor prognosis and acute arrhythmias are related to an increase in the amount of replacement fibrosis identified by magnetic resonance imaging (MRI) (**Barison et al., 2015**). In DCM, fibrosis is usually distributed in a patchy or diffuse pattern in the mid wall **(Barison et al., 2015)**.

1.2.1.5. Myocardial Fibrosis and Atrial Fibrillation

Myocardial Fibrosis (MF) is a hallmark of structural remodelling of atrial fibrillation (AF); studies have shown that collagen content in the atrial muscle of patients who have isolated AF is significantly increased compared to the control group of sinus rhythm and rise in collagen can improve heterogeneity of myocardial conduction and heart's electrical instability and facilitate the ability to form re-entry, which increases the risk of AF **(Verheule et al., 2004)**. Additionally, the greater the amount of fibrous tissue in the myocardium lead to a greater the susceptibility to AF and this suggesting that MF may offer the matrix for the onset and maintenance of AF; In the rat model of MF generated by isoproterenol, the susceptibility to AF was increased and this indicates that MF is a major substrate for atrial fibrillation **(Ma et al., 2020)**.

Changes in electrophysiology and structure increase the establishment of focal conduction block and affect potential action duration and spontaneous AF **(Pan et al., 2019)**. MF also has comparable pathophysiological properties. Fibrosis for instance, can delay local atrial conduction and an elevation in conduction heterogeneity resulting in reentrant and localized atrial arrhythmias **(Nattel, Li & Yue, 2000)**. MF enhances the occurrence of AF **(Ahlberg et al., 2018)**. In addition, prolonged

rapid atrial pacing can result in the buildup of ECM protein in atrial muscle; It was also discovered that AF could result in MF **(Slawik et al., 2019)**.

So far, the research shows that MF and AF may affect each other and create a vicious circle; Disorder of collagen organization on one can then split the cardiomyocytes resulting in aberrant electrical conduction between the cardiomyocytes, which offers a substrate for the development of AF **(Xu et al., 2004)**. Nonetheless, there are several divergent viewpoints; Increased MF typically accompanies AF. However, AF including persistent AF can frequently occur without increasing fibrosis **(Pan et al., 2019)**.

1.2.2. The Atrial Fibrillation

1.2.2.1. Definition and Classification

Atrial Fibrillation (AF) is a heart arrhythmia characterized by the irregular and uncontrolled electrical activity of the atria, which interferes with the contractile characteristics of the ventricles, hence diminishing cardiac function and causing pain. It is the most prevalent arrhythmia linked to comorbidities and elevated risk of death **(Beyerbach & Zipes, 2004; Nattel & Opie, 2006**). Patients with atrial fibrillation have a 45-fold greater risk of stroke, a 23-fold elevated risk of heart failure and a 2-fold greater risk of overall mortality **(Wang et al., 2003)**. In addition, uneven RR intervals distinguish AF rhythm and the absence of prominent P waves on the ECG **(Camm et al., 2010; Kirchhof et al., 2016)**.

The following categorization method is recommended for AF management (**Fuster et al., 2006**), according to ACC/AHA/ESC recommendations: **(Hersi & Wyse, 2005)**

- Paroxysmal AF: Episodes that begin and finish spontaneously, typically lasting less than 24 hours but sometimes up to 7 days.
- Persistent AF: Refers to episodes that last longer than seven days but less than a year and necessitate pharmaceutical or electrical treatment to stop.

- Permanent AF: Refers to episodes that have endured for an extended period (more than a year) and have resisted all attempts to stop them.
- Lone AF: Affects individuals younger than 60 years of age without clinical or echocardiographic causes.

1.2.2.2. The Problem of Atrial Fibrillation

Atrial fibrillation (AF) is the most prevalent type of irregular heartbeat and affects 0.7% of people aged 55–59 years and 17.8% of people over the age of 85 years in the European population **(Heeringa et al., 2006)**. It is clinically characterized by a loss of apparent sinoatrial node (SAN) activity, irregular atrial excitation & propagation and an irregularly ventricular beat; Death & morbidity caused by thromboembolism as a result of thrombus formation due to blood stagnation in the atrium (usually the left atrial [LA] accessory) and due to aberrant ventricular rate and rhythm, loss of atrial function, the arrhythmia can cause debilitating discomfort and a decline in quality of life but in some patients, it may be asymptomatic **(Dorian et al., 2000; Van den Berg et al., 2001)**.

1.2.2.3. The Symptoms of Atrial Fibrillation

Atrial fibrillation (AF) symptoms vary a lot from person to person and even over time in the same person. However, the most prevalent symptoms include the following: (**Rienstra et al., 2012; Kirchhof et al., 2016**).

- Palpitations
- Chest pain
- Dyspnea
- Syncope
- Difficulty sleeping
- Vertigo
- Psychological anxiety
- Weariness
- Exercise Intolerance

Not all reported symptoms are related to atrial fibrillation because risk factors and comorbidities of AF may create similar symptoms **(Rienstra et al., 2012**). Asymptomatic AF has the same prognostic impact as symptomatic AF (**Kamel et al., 2009; Dilaveris et al., 2017**).

1.2.2.4. The Epidemiology of Atrial Fibrillation

The prevalence & incidence of atrial fibrillation (AF) is rising worldwide according to the Framingham Heart Study (FHS) & the prevalence of AF has tripled in the past 50 years **(Schnabel et al., 2015)**. According to the global burden of disease research, AF affected approximately 46.3 million people worldwide in 2016 **(Benjamin et al., 2019)**, while in 2004 the lifespan risk of AF was evaluated to be approximately 1 in 4 for white males and females older than 40 (**Lloyd-Jones et al., 2004**).

A decade ago, lifetime risk assessments for white persons reached approximately 1 in 3 and for black persons 1 in 5.8 as for the United States alone, between 3 and 6 million individuals have AF, which is anticipated to rise to (≈6-16 million) by 2050 (**Miyasaka et al., 2006; Mou et al., 2018)**. (See Figure 1.12)

In Europe, the prevalence of AF among adults aged 55 and older was ≈9 million in 2010, projected to reach 14 million by 2060 **(Krijthe et al., 2013; Di Carlo et al., 2019**). By 2050, it is anticipated that at least 72 million Asians will be diagnosed with atrial fibrillation with ≈3 million experiencing AF related strokes **(Chiang, Wang & Lip, 2014)**. In the past decade, knowledge and identification of atrial fibrillation (AF) have increased, which is significant given that around one third of the AF population is asymptomatic (**Dilaveris & Kennedy, 2017)**. Hence, the worldwide AF burden is undoubtedly underestimated. (See Figure 1-12)

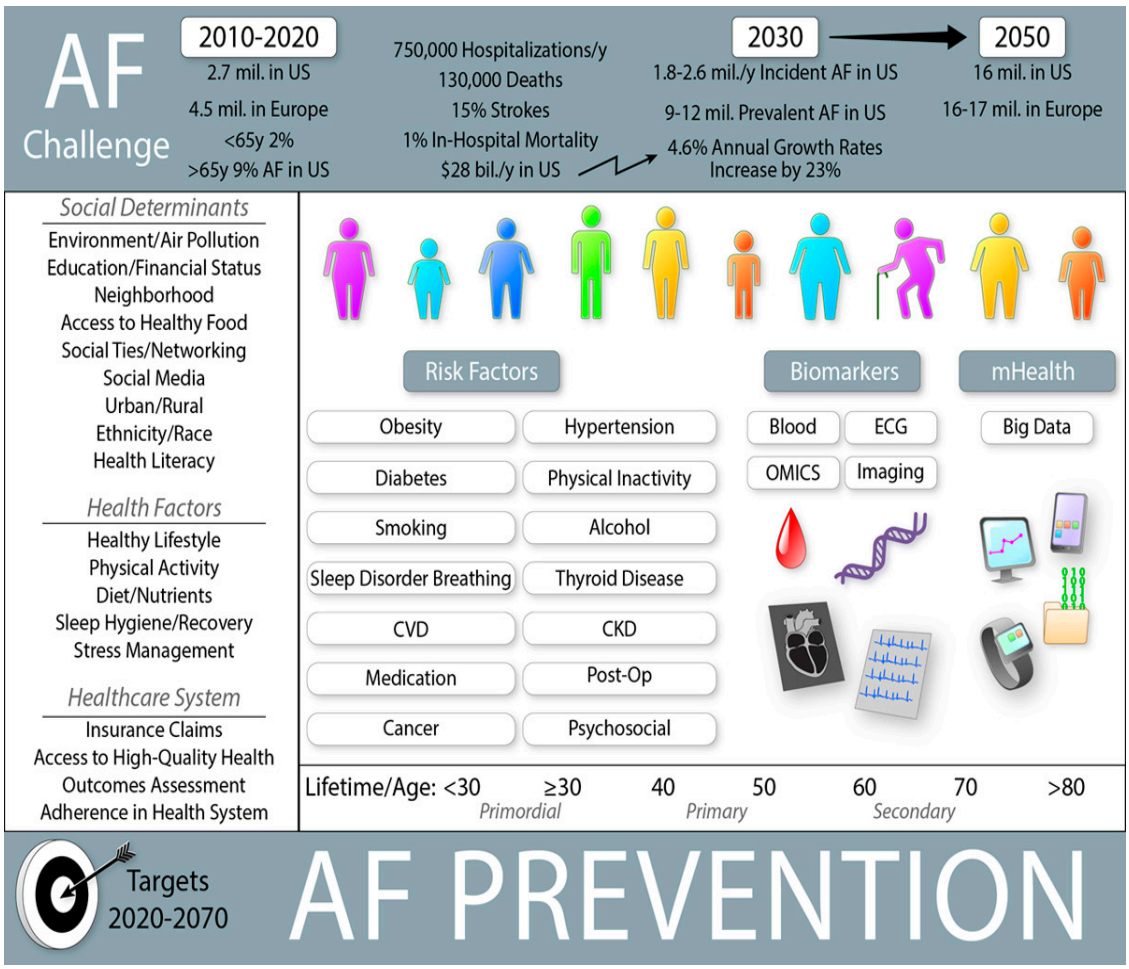


Figure 1-12: Obstacles in the Epidemiology of Atrial Fibrillation (AF), The illustration was created by Dr. Kornej & Smith (2020). (CKD) stands for chronic kidney disease**, (O.P.)** for operation, and **(QoL)** for quality of life.

1.2.2.4. The Rick Factor for Atrial Fibrillation

Several risk factors have been identified as contributors to the onset and development of atrial fibrillation (AF), including non modifiable risk factors such as age and gender and modifiable risk factors such as obesity, hypertension and dyslipidemia **(Heijman et al., 2021; Volgman et al., 2021; Jiang et al., 2022**). In addition to these variables, the improved AF prediction score includes ECG derived PR interval and LV dilatation, Figure 1.13 **(Alonso et al., 2013; Kolek et al., 2016; Calkins et al., 2017)**.

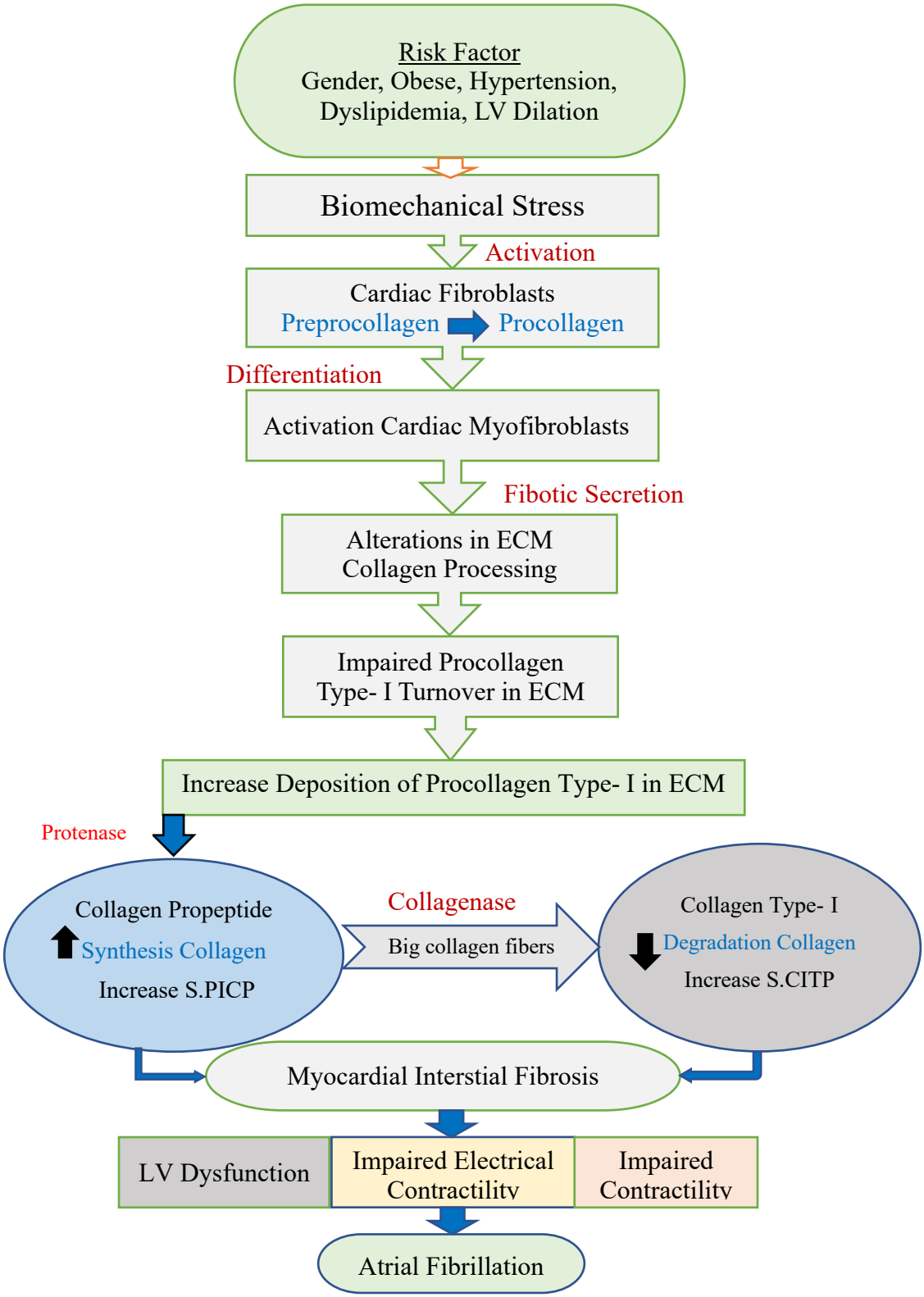


Figure 1-13 Schematic representation of Impaired turnover of type I collagen steps in synthesis and degradation that contribute to myocardial interstitial fibrosis in atrial fibrillation; Researcher Mustafa Hameed created the proposed illustration

1.3. Diagnosis of Atrial Fibrillation

1.3.1. Diagnosis of Atrial Fibrillation by Non Biochemical Tools

1.3.1.1. Electrocardiography

Electrocardiogram (ECG) tracing demonstrating atrial fibrillation (AF) is required to establish the diagnosis of AF. Conventionally, an episode lasting at least 30 seconds is required to diagnose clinical AF **(Steinberg et al., 2018)**.

All patients performed a standard 12-lead ECG. The atrial fibrillation (AF) diagnosis was confirmed using ECGs collected during the event. The absence of P waves, rough or fine fibrillatory waves and irregular RR intervals such as an irregular pattern characterize atrial fibrillation. **(Antman & Eugene, 1997)**. Figure 1-14 depicts well known ECG patterns for diagnosing atrial fibrillation including the absence of P waves and the development of F waves.

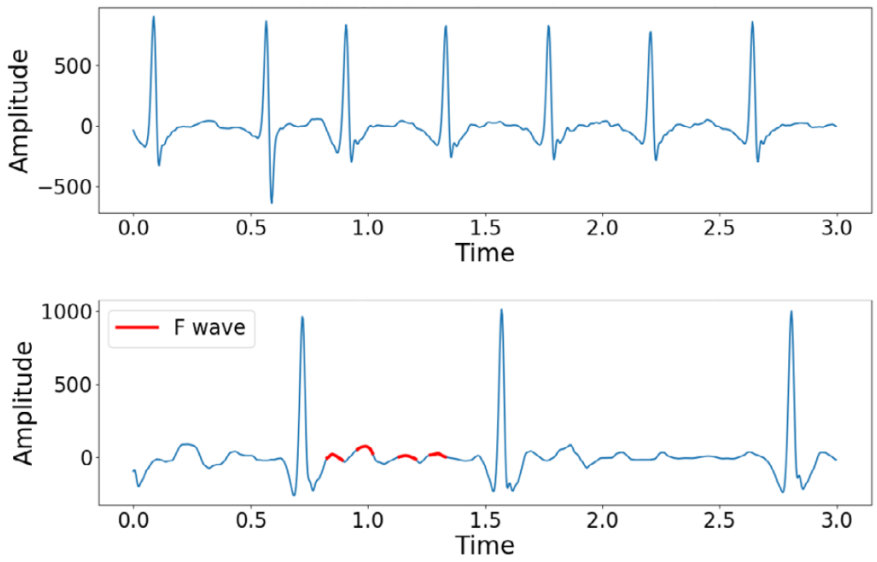


Figure 1-14: The image above exhibits the absence of a P wave, while the one below exhibits the presence of an F wave. (Chen et al., 2018)

Recent research has demonstrated connections between the bundle branch block (BBB) and ECG prognosis for individuals with cardiac disease (**Karaca et al., 2016**). In addition, the left bundle branch block (LBBB) is a

major predisposing factor for systolic cardiac failure and atrial fibrillation if it causes an extension of the QRS duration AF (**Cho et al., 2010**).

LBBB is a change in ventricular conduction that may result in ventricular dyssynchrony and heart failure (HF) **(Kumar, Stevenson & John, 2015**). whereas right BBB (RBBB) like most cases has a pathological etiology but may also be found in normal people **(Fahy et al., 1996)**. It was discovered that left BBB (LBBB) has been related to cardiovascular disease complications in much larger cases than right BBB (RBBB).

1.3.1.2. Echocardiography

Echocardiography is used to look for structural problems in the heart, check how well the heart is working and measure the size of the atrium (**Dillon & Ghanbari, 2014)**. At each beating when the heart contracts, it expels blood from the two pumping chambers known as ventricles, which are then refilled with blood when the heart relaxes (**Dunlay et al., 2012**). Patients were evaluated for LVEF%, left ventricular (LV), and left atrial (LA) size, presence of mitral and aortic regurgitation causes a degree of LV diastolic dysfunction (**Carasso et al., 2005)**.

Ejection fraction (EF) is the quantity of blood pushed out of the ventricle with each heartbeat. Under physiological loading conditions, an LVEF of 55% or more is considered normal, while an EF of less than 50% is considered abnormal. Specifically, EFs between 50 and 55% are regarded as borderline (**Gaasch et al., 2009; Dunlay et al., 2012**). Moreover, valvular, myocardial, pericardial or congenital heart disease is a risk factor for atrial fibrillation. In addition, echocardiography is now a crucial component of the guidelines for treating patients with atrial fibrillation **(Fuster et al. & Managem, 2001)**.

1.3.1.3. Holter Monitor (24-h, 48-h and 72-h)

Some people may not have symptoms, while others may experience a stroke or apparent heart failure or a cardiovascular system collapse. If the ECG does not detect atrial fibrillation despite strong suspicion, it may be necessary to document the arrhythmia using a holter monitor or cardiac event monitor. Extending the recording period from 24 to 72 hours can increase the prevalence of arrhythmias (**Fuster et al., 2006**).

1.3.2. Diagnosis of AF by proposed MIF Biochemical Tests

1.3.2.1. Carboxyterminal Propeptide of Type-I Procollagen (PICP)

1.3.2.1.1. Collagen Synthesis

Collagen type I is a heterotrimeric molecule consisting of two α 1 chains and one α 2 chain. The protein undergoes posttranslational changes throughout its production to generate the procollagen chain; this precursor is released into the extracellular area and cleaved by particular proteinase (**Rodriguez-Pascual & Slatter, 2016**). Carboxyterminal propeptide of type I procollagen (S.PICP; 100 kilodaltons), which is liberated into the circulation and collagen type I generated by cleavage have a 1:1 stoichiometric ratio (**Querejeta et al., 2004)**.

The coronary sinus is where the heart releases S.PICP into the peripheral circulation (**Querejeta et al., 2004)**. Whether plasma PICP and cardiac collagen content have a favourable association remains debatable. According to a cross sectional study plasma PICP concentration in HCM patients were favourably linked with myocardial S.PICP content and myocardial collagen volume fraction (**Yang et al., 2019**). Similarly, Ferreira *et al*. demonstrated that the serum concentration of S.PICP in hypertensive patients prior to medication treatment was significantly higher (**Ferreira et al., 2019**).

1.3.2.1.2. Serum PICP

Under stable production from additional cardiac sources, it has been found that the S.PICP in the peripheral blood of people with hypertensive heart disease (HHD) is mostly from the heart due to a positive serum concentration gradient from the coronary artery sinus to the opposite vein is higher in HHD patients than in people with normal blood pressure; The level of S.PICP in the peripheral blood and coronary arteries are strongly linked to HHD patients **(Querejeta et al., 2004**). S.PICP related to cardiac fibrosis in HHD patients **(Querejeta et al., 2000; Querejeta et al., 2004)**. In addition, it has been shown that PICP concentration quantifies myocardial fibrosis (**Querejeta et al., 2000**), and the stiffness of the LV compartment have been demonstrated to change simultaneously in response to antihypertensive medication in patients with HHD (**López et al., 2004)**. Finally, increased concentrations of PICP detect severe fibrosis and predict HF with preserved ejection fraction in patients with HHD with acceptable sensitivity and specificity **(Querejeta et al., 2000; Martos et al., 2009)**

1.3.2.2. Carboxyterminal Telopeptide of Type I Collagen (CITP)

1.3.2.2.1. Collagen Breakdown

The carboxyterminal telopeptide of collagen type I (S.CITP; 12 kilodaltons) is a crosslinked terminal peptide liberated in a 1:1 stoichiometric ratio after the disintegration of collagen type I fibrils **(Risteli et al., 1993)**. So this allows for the reliable determination of collagen degradation in a cross sectional investigation, S.CITP level were elevated in HCM patients, although S.PICP and S.PINP concentrations were unaffected indicating a shift in the collagen balance towards collagen type I breakdown (**Lombardi et al., 2003)**. So this is a significant finding as collagen deposition is typically responsible for increased cardiac stiffness. However, several additional investigations have demonstrated that the association between

S.CITP and myocardial fibrosis is contested; Serum CITP concentrations were substantially greater in individuals with heart failure (HF) and atrial fibrillation (AF) than in healthy controls (**Morine et al., 2016)**. Nevertheless, Nagao *et al*. showed that serum CITP level in DCM patients were not linked with left ventricular remodelling characteristics or the expression of heart collagen types I and III (**Nagao et al., 2018**).

Moreover, several investigations have verified the prognostic significance of S.CITP; Serum CITP was an independent predictor of cardiovascular death in individuals with acute myocardial infarction (AMI) according to Manhenke *et al*. (**Manhenke et al., 2011**). In this prospective analysis of 233 patients with AMI, 56% attained the composite endpoint of HF symptoms or CV death during the years of followup and plasma CITP was elevated in patients who died from any cause. Similarly, serum CITP is beneficial for identifying cardiac events in patients with heart failure (HF) (**Kitahara et al., 2007**). In conclusion, S.CITP may improve the diagnosis or prognosis of myocardial fibrosis.

1.3.2.2.2. Serum CITP

Even though the pathophysiological significance of measuring isolated CITP has yet to be determined and this peptide can be used with PICP to quantify collagen type I turnover indirectly based on data acquired from hypertensive animals (**Díez et al., 1996)**. it has been suggested that the circulating S.PICP: S.CITP ratio may indicate the degree of coupling among collagen type I synthesis and degradation; Intriguingly, the ratio is correlated with the degree of cardiac fibrosis in patients with HHD (**Díez et al., 2002)**. It indicated that an elevated ratio representing the preponderance of synthesis over degradation may cause fibrosis in these patients.

1.4. The Knowledge Gap

Previous studies showed that the biomarkers (S.CITP, S.PICP) increased in patients with many different heart diseases including ischemic heart disease (IHD) patients (**Manhenke et al., 2011)**, hypertensive heart disease (HHD) patients **(Plaksej et al., 2009)**, hypertrophic cardiomyopathy (HCM) patients with heart failure (HF) (**Ho et al., 2010**), atrial fibrillation (AF) with hyperthyroidism (**Takawale et al., 2022)** and Patients with bone diseases including bone turnover (**Oleiwi, Rasheed & Hussein, 2022**).

A Knowledge gap our current research focused on non ischemic heart disease in order to distinguish itself from previous studies; Patients were diagnosed with echocardiography study and some diagnosed with exploratory catheterization and catheterization results were normal to predict S.CITP and S.PICP are both biomarkers of myocardial interstitial fibrosis (MIF). Patients with MIF cause them cardiac dysfunction, altered electrocardiogram, systolic and diastolic imbalances and changes in the heart muscle structure leading to atrial fibrillation.

The current knowledge aimed at clinical assessment and prognosis by investigating the relationship between S.PICP and S.CITP concentrations in AF patients.

1.5. The Implications and Contribution to Knowledge

Despite the current information on circulating biomarkers of collagen metabolism, several significant limitations weaken their medical utility.

Future research projects continue to be required:

- Determine the connection between the measured amounts of these novel biomarkers, the myocardial collagen network and alterations in heart structure and function.
- Validation of additional information provided by a multiple labelling strategy that combines these peptides with standard biochemical markers (e.g., natriuretic or troponin peptides)
- Assess the effects of these metrics on patient care and outcomes.
- Assess the implementation ability and cost effectiveness of this strategy in the community.
- Identify which treatments have shown a significant response to these novel biomarkers.

1.6. The aims of the study

This study was designed to:

1. Investigate the relationship between serum levels of carboxyterminal propeptide of type-I procollagen, carboxyterminal telopeptide of type-I collagen and myocardial interstitial fibrosis in patients diagnosed with atrial fibrillation.

2. Research the impact of fibrosis repressed myocardial interstitial fibrosis on the outcomes.

3. Study the efficacy of serum levels of carboxyterminal propeptide of type-I procollagen and carboxyterminal telopeptide of type-I collagen as biomarkers in the prognosis of atrial fibrillation.

4. To avoid advanced complications of myocardial interstitial fibrosis.

Chapter Two

Materials and Methods

2- Materials and Methods

2.1. The Study Design and Setting

A case-control study analyzed whether or not there were significant differences between outcomes & look for exposure among cases & controls, as well as was used to determine risk factors that may contribute to atrial fibrillation (AF) cases by comparing them with the control group.

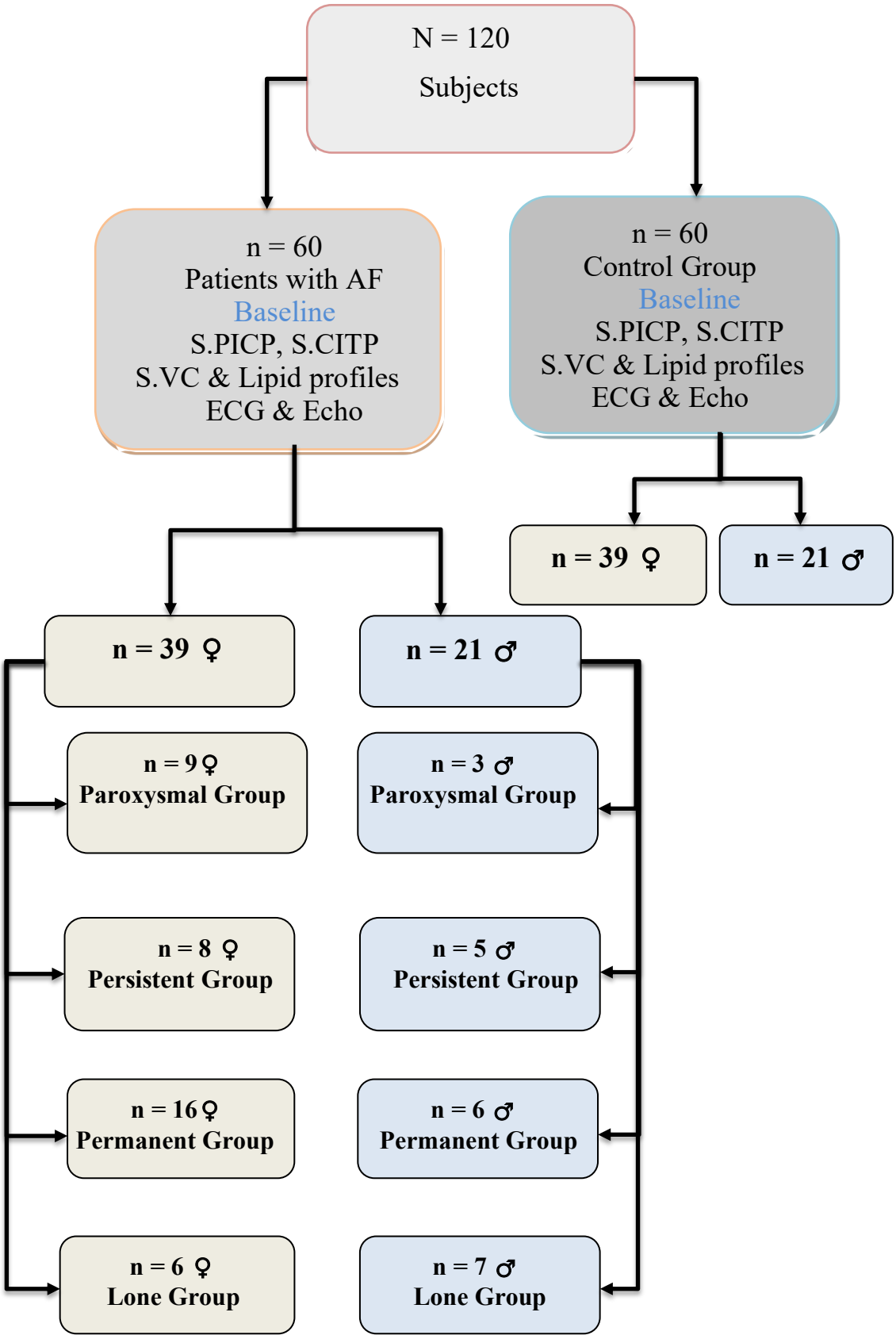
The study was conducted at the Al-Zahraa Teaching Hospital in Al-Kut / Wasit, a teaching hospital with more than 400 beds. This observational study observed patients in a cardiac care unit (CCU), wards, cardiac consultancies, and the emergency department from August 1, 2022, through December 31, 2022. The hospital ethics committee approved the study plan, and all patients or their families were informed of this before taking blood. The patient entered the study when the diagnosis of atrial fibrillation was confirmed.

The study was executed for (120) samples with ages ranging between (22-90 years), (60) of them diagnosed with atrial fibrillation (AF) and (60) as the apparently healthy control group, taking into account the matching between age, race, and gender as the number of patient group (39-females & 21-males) and the number of the control group (39-females & 21-males).

ECG interpretation, Holter monitoring, an echo study and observation of chest pain, palpation, syncope, dyspnea, and risk factors such as gender, hypertension, body mass index and dyslipidemia in diagnosed atrial fibrillation patients.

The biomarker of myocardial interstitial fibrosis (MIF) in AF cases (S.PICP, S.CITP) & comorbidities markers (lipid profiles) & vitamin-C level were tested for all samples.

A case-control study, according to AF divided individuals into 4-groups:



Example of case study atrial fibrillation

A 61-year-old patient was sent to the emergency assessment unit at Al-Zahraa Teaching Hospital / Wasit in the city of Kut with symptoms of palpitations, confusion, and dyspnea. His family's history of discomfort and tachycardia spans five days. In the previous twenty-four hours, he also complained of shortness of breath. He was taken to the hospital by the emergency department after collapsing at home.

Two months earlier, he had suffered identical symptoms but did not seek medical help and appeared to heal rapidly. Following examination and evaluation by the admitting physician, the following data is obtained:

Prior medical background

Before the diagnosis of hypertension five years ago, there was no history of cardiovascular illness. The patient is a heavy smoker (>20 cigarettes per day).

Drug history

No known allergies. The patient was prescribed lisinopril tablets 20 *mg* once daily but was poorly compliant with treatment.

Signs and symptoms on examination

- Blood pressure 100/70 *mmHg*.
- Heart rate 175 *bpm*, irregular.
- Respiratory rate 25 breaths per minute.
- No basal crackles in the lungs.

Diagnosis of Case

Atrial Fibrillation by the ECG interpretation.

Relevant test results include full blood counts, lipid profile tests, thyroid function tests, blood sugar, liver function tests (LFT), electrolytes and renal function tests (RFT). In addition, the echo study and all test results were normal at admission and discharge from the emergency department.

The patient is then transferred to the Cardiac Care Unit (CCU), where he is diagnosed with persistent atrial fibrillation.

Interpretation of case referring to the patient with atrial fibrillation:

- Symptoms such as chest pain, palpitation, dyspnea and syncope.
- Risk factors such as hypertension and heavy smoker.
- Signs such as tachycardia and irregular pulse.
- ECG shows a rapid & irregular QRS rhythm with an absence of P-wave.

2.1.1. The Patients Inclusion Criteria

The sixty of patients with non-ischemic atrial fibrillation were taken and permission was taken from all patients to participate in this study; they underwent the clinical examination steps, which included:

- Vital signs such as blood pressure and heart rate.
- Laboratory investigations such as lipid profiles.
- Height and weight to body mass index (BMI) calculator.

Moreover, family history of AF, age, symptoms, evaluation ECG interpretation, Echo study & normal coronary angiography, diagnosis by a cardiologist as a patient group, AF divided into 4-groups:

- Group A: 12 Patients with Paroxysmal atrial fibrillation.
- Group B: 13 Patients with persistent atrial fibrillation.
- Group C: 22 Patients with permanent atrial fibrillation.
- Group D: 13 Patients with lone atrial fibrillation.

2.1.2. The Patients Exclusion Criteria

This study excluded patients with ischemic heart disease (IHD) and those taking collagen, both healthy and ill.

2.1.3. The Control Criteria

Blood samples were obtained from 60 healthy individuals without heart illness, especially atrial fibrillation (AF) & who do not have a family history of AF. They do not have a risk factor that causes AF, considering the age and gender matching with the patients' group where the number of females was-39 and the number of males was-21; all of them underwent questionnaires, laboratory investigations, ECG interpretation & Echo study.

2.1.4. The Sampling

2.1.4.1. The Sample Size

The study was conducted worldwide and the prevalence was 2% **(Rahman, Kwan & Benjamin, 2016)**. Moreover, recent data indicate that atrial fibrillation affects between 1% and 4% of the populations of Australia, Europe, and the United States **(Zulkifly, Lip & Lane, 2018)**; and from this prevalence a sample size can be calculated **(Daniel & Cross, 2018):**

$n = [Z^2 \times P(1-P)] / d^2$

$n = [(1.96)^2 \times 0.04(1-0.04)] / (0.05)^2$

$n = 60$

Where:

n = Sample Size

Z = Z Statistic for a level of confidence (1.96 for 95% Confidence level)

P = Expected Prevalence or Proportion

d = Precision (d is considered 0.05 to produced good precision & smaller error of estimate).

2.1.4.2. The Ethical Approval

The study protocol was approved by the ethical committee at Kerbala University- College of Medicine, Al-Zahraa Teaching Hospital / Wasit in the City of Al-Kut.

Verbal approval is taken from all patients included in the study before sampling.

2.1.4.3. The Specimen Collection and Storage

The fasting vein samples were collected from all cases; 5 *mL* of blood was drowned using a syringe, put in a tube containing a gel & clot activator & let clot for 2 The patient is then transferred to the Cardiac Care Unit (CCU), where he is diagnosed with persistent atrial fibrillation by the ECG interpretation. 30 minutes at room temperature, and then separated into serum by centrifugation at 3600 *r/min* for 30 minutes.

The serum was divided into 2- eppendorf:

- The first eppendorf is for immediate checking the lipid profiles levels by Abbot-ARCHITECT c4000.
- The second eppendorf & remainder of the first eppendorf is stored in deep freeze at -80 C^0 to check the levels of S.CITP, S.PICP, and Vitamin-C by ELISA.

2.1.5. The Patients Evaluation

The serum from each patient and control was used to measure collagen synthesis and degradation markers (S.PICP, S.CITP,) and lipid profiles (cholesterol, TG, HDL, VLDL, and LDL). Medical and demographic data were collected for each patient through a questionnaire (**Appendix-1**), body mass index (BMI), heart rate, blood pressure (BP), ejection fraction percentage (EF%), left atrial (LA) diameter, and left ventricular (LV) diameter. ECG interpretation was used to diagnose atrial fibrillation (**Appendix-2**).

2.1.5.1. Measurement of The Body Mass Index

The equation determined the body mass index (BMI): a person's weight in kilograms (*Kg*) divided by the square of the person's height in meters (m^2) using appropriate balance as in the equation below:

BMI = Kg/m^2

The body mass index was classified according to the world health organization (WHO) in Table 2.1 **(Seo et al., 2019).**

Table 2.1. BMI Status (Seo et al., 2019)

N	BMI	Unit	Classify by BMI status
1	Below 18.5	Kg/m^2	Underweight
2	18.5-24.9	Kg/m^2	Normal Weight
3	25.0-29.9	Kg/m^2	Pre-obesity
4	30.0-34.9	Kg/m^2	Obesity Class-1
5	35.0-39.9	Kg/m^2	Obesity Class-2
6	Above 40	Kg/m^2	Obesity Class-3

2.1.5.2. Measurement of The Heart Rate

The human heart rate can be classified according to the heart rate per minute, as shown in Table 2.2 (**Yuantoro, Rosmiati & Siradj , 2018)**.

Table 2.2. Classification of Heart Rate (Yuantoro, Rosmiati & Siradj , 2018)

N	Heart Rate	Units	Categories of Heart Rate
1	< 60	*bpm*	Bradycardia
2	60-100	*bpm*	Normal Sinus Rhythm
3	>100	*bpm*	Tachycardia

2.1.5.3. Measurement of The Blood Pressure

Human blood pressure (BP) can be classified according to a report from the American College of Cardiology (ACC) / American Heart Association

(AHA) task force on clinical practice guidelines, as shown in the following table 2.3 **(Whelton, Carey, Mancia, G. et al.,, 2022).**

Table 2.3. Classification of Blood Pressure (Whelton, Carey, Mancia, G. et al.,, 2022)

N	Systolic BP/ Diastolic BP	Units	BP Category
1	$< 120/< 80$	*mm Hg*	Normal Blood Pressure
2	$120 - 129/< 80$	*mm Hg*	Elevated Blood Pressure
3	$130 - 139/80 - 89$	*mm Hg*	Hypertension, stage 1
4	$\geq 140/\geq 90$	*mm Hg*	Hypertension, stage 2

2.1.5.4. Measurement of The Ejection Fraction Percentage

The ejection fraction (EF) is the percentage of blood discharged from the left ventricle during each contraction (heartbeat).

Using echocardiography, patients can be classified according to a performance measure report from the American College of Cardiology (ACC) guideline recommended practice, as shown in Table 2.4 (**ACC, 2019)**.

Table 2.4. Categorization of The Ejection Fraction Percentage (EF%) (ACC, 2019)

N	**Ejection Fraction Percentage (EF%)**	**EF% Category**
1	Greater Than 70%	Hyperdynamic
2	50%-70%	Normal
3	40%-49%	Mild Dysfunction
4	30%-39%	Moderate Dysfunction
5	Less Than 30%	Severe Dysfunction

2.1.5.5. Measurement of The Left Atrial diameter

The left atrial (LA) diameter can be measured in centimeters (*cm*) using echocardiography and can be classified as shown in Table 2.5(**Appendix-3**).

Table 2.5. Categorization of The LA Diameter of Female (Lang et al., 2006)

N	Diameter	units	Category of the LA
1	2.7 - 3.8	*cm*	Normal Range
2	3.9 - 4.2	*cm*	Mild Enlargement
3	4.3 - 4.6	*cm*	Moderate Enlargement
4	≥ 4.7	*cm*	Severe Enlargement

Table 2.5. Categorization of The LA Diameter of male (Lang et al., 2006)

N	Diameter	units	Category of the LA
1	3.0 - 4.0	*cm*	Normal Range
2	4.1- 4.6	*cm*	Mild Enlargement
3	4.7 – 5.2	*cm*	Moderate Enlargement
4	≥ 5.2	*cm*	Severe Enlargement

2.1.5.6. Measurement of The Left Ventricular diameter

The left ventricular (LV) diameter can be measured in centimeters (*cm*) using echocardiography and can be classified according to a report from the American College of Cardiology (ACC), as shown in the following table 2.6 **(Appendix-4)**.

Table 2.6. Categorization of The LV diameter of female (Lang et al., 2005)

N	Diameter	units	Category of the LV
1	3.9 – 5.3	*cm*	Normal Range
2	5.4 – 5.7	*cm*	Mild Dilated
3	5.8 – 6.1	*cm*	Moderate Dilated
4	≥ 6.2	*cm*	Severe Dilated

Table 2.6. Categorization of The LV diameter of male (Lang et al., 2005)

N	Diameter	units	Category of the LV
1	4.2 – 5.9	*cm*	Normal Range
2	6.0- 6.3	*cm*	Mild Dilated
3	6.4 – 6.8	*cm*	Moderate Dilated
4	≥ 6.9	*cm*	Severe Dilated

2.2. The Materials

2.2.1. The Materials & Tools

The materials and tools, along with their suppliers, used in this investigation are listed in Table 2.7.

Table 2.7: The Materials & Tools Used in the Study

N	Name	Company	Country
1	Absorbent filter paper	Cytive	China
2	Balance	Detecto	USA
3	Cotton	Modawa Medical	Iraq
4	Distilled Water		Iraq
5	Eppendorf Tubes (1.5 *mL*)	Kang Gia	China
6	Gel tubes (6*mL*)	EZ Medical lab	China
7	Gloves	MedTech	Malaysia
8	Graduated Cylinder (1500 *mL)*	Eterna Duran	Germany
9	Hard Ice Gels Packs	Hepo International	China
10	Laboratory Stopwatches & Timers	Spectrum	USA
11	Micropipette (10-100 µl)	Slamed®	Germany
12	Pipette Rack Stand	Slamed®	Germany
13	Pipette Tips-100µl (yellow)	Kirgen®	China
14	Pipette(100-1000µl)	Slamed®	Germany
15	Plaster	Life Plus Medical	China
16	Plate Sealer	BT Lab	China

17	Pre-Coated ELISA Plate (96-well)	BT Lab	China
18	Specimen Transport Bag	Hepo International	China
19	Surgical Spirit	Ameya Fze	UAE
20	Syringe (5 *mL/cc*)	Yuandong Medical	China
21	Tourniquet	KaWe	Germany
22	Tube Rack	Slamed®	Germany

2.2.2. The Laboratory Kits

Laboratory kits used in the study were of the highest purity and are listed in Table (2.8) below with their suppliers: -

Table 2.8: The Laboratory kits which are used in this study

N	Name	Company	Country
1	CITP Kit	BT Lab	China
2	Lipid profiles Kits	Abbott Laboratories	USA
3	PICP Kit	BT Lab	China
4	Vitamin-C Kit	BT Lab	China

2.2.3. The Instruments

All the instruments that were used in this study are shown below in Table 2.9.

Table 2.9: The instruments used in this study

N	Name	Company	Country
1	Architect c4000	Abbott Laboratories	USA
2	Centrifuge	Hettich	Germany
3	Deep freezer (-80 c^{o})	ALS	UK

4	Distillateur	Bibby Science	Englan
5	ELISA Dia Reader	Dialab	Austria
6	ELISA Dia Washer	Dialab	Austria
7	Incubator (37 c^{o})	Pasteur	France
8	Refrigerator (-2 to -8 c^{o})	Dairei	Denmark

2.3. The Methods

2.3.1. The Measurement of Serum CITP

❖ **Principle of Assay**

- This sandwich kit is for accurate quantitative human detection C-terminal telopeptide of type-I collagen (also known as CTX-I) in serum.
- This kit is an enzyme-linked immunosorbent assay (ELISA).
- The plate was pre-coated with a human CTX-I antibody.
- CTX-I in the sample was added and bound to antibodies coated on the wells.
- A biotinylated human CTX-I antibody was added and binds to CTX-I in the sample.
- Streptavidin-HRP was subsequently added and bound to the biotinylated CTX-I antibody.
- The plate was placed in the incubator.
- Unbound streptavidin-HRP was removed during the washing step.
- A substrate solution was added and the colour develops proportionally to the amount of human CTX-I.
- An acid-stop solution was added to terminate the process, and the absorbance was measured directly at 450 *nm.*

❖ **Preparation of Reagent**

- All reagents (**Sample & Standard**) were brought to room temperature before use.
- A 120 *µL* of **standard** (1600 *ng/mL*) was reconstituted with 120*µl* of standard diluent to generate an 800 *ng/ml* standard stock solution.

Allow the standard to sit for 15 minutes with gentle agitation prior to making dilutions. Prepare standard points by serially diluting the standard stock solution (800 *ng/ml*) 1:2 with standard diluent to produce 400 *ng/ml*, 200 *ng/ml*, 100 *ng/ml*, and 50 *ng/ml* solutions. Standard diluent serves as the zero standards (0 *ng/ml*) Fig. 2.1. Any remaining solution should be frozen at -20°C and used within one month.

- The dilution of the proposed standard solutions was as follows

800 *ng/ml*	Standard-5	120µl Original Standard+120µl Standard Diluent
400 *ng/ml*	Standard-4	120µl Standard-5 + 120µl Standard Diluent
200 *ng/ml*	Standard-3	120µl Standard-4 + 120µl Standard Diluent
100 *ng/ml*	Standard-2	120µl Standard-3 + 120µl Standard Diluent
50 *ng/ml*	Standard-1	120µl Standard-2 + 120µl Standard Diluent

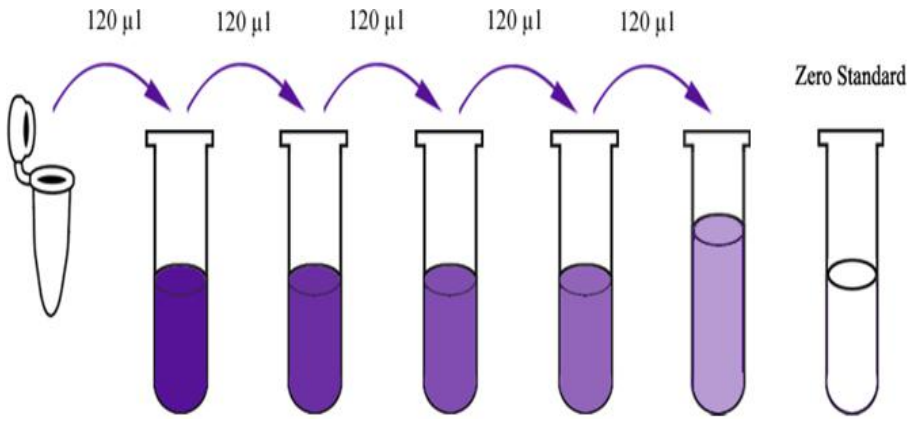


Fig 2.1: Serial dilution of Standard S.CITP

❖ **Wash Buffer**

- A 20 *mL* of buffer wash 25x was added into a graduated cylinder.
- A 480 *mL* of distilled water (D.W) was added into a graduated cylinder to yield 500 *mL* wash buffer dilute.
- A 500 *mL* of wash buffer dilute in a graduated cylinder was added into the washing bottle ELISA, Fig.2.7.

❖ **Procedure of Assay**

- A 50*µl* of the standard (St1– St6) was added to the standard well (A1–F1) only.
- A 40µl of the sample (S1–S90) was added to the sample wells (G1–H12) only.
- A 10*µl* of anti-CTX-1 antibody was added to the sample wells.
- A 50*µl* of streptavidin-HRP was added to the sample & standard wells.
- Wells was a mix.
- The plate was covered with a new sealer.
- The plate was incubated at 37 °C for 60 minutes.
- The sealer was removed from the plate.
- The plate was washed with washing buffer five times and dried on paper towels.
- A 50*µl* of the substrate solution A&B was added into all well.
- A new sealant was used to cover the plate.
- The plate was incubated in the dark at 37 °C for 10 minutes.
- A 50 *µl* of the stop solution was added into each well, and the blue colour changed into yellow immediately.
- The plate was directly added to a microplate reader ELISA & set to 450 *nm*.
- The reading was the absorption (A value) to determine the absorption level of each well, Fig.2.7. **(Appendix-5)**
- The results were printed using a graphic standing curve.

❖ **Calculate of Result**

The concentration of serum CITP, absorption and concentration of standard is evaluated by a standard curve by the ELISA Dia Reader as shown in the table.2.10.

The standard curve is formed by graphing the average A. for every standard on the vertical (Y) line versus the concentration on the horizontal

(X) line and drawing a best-fit curve across the points on the graph, as illustrated in Figure 2.2.

Table 2.10: The standard curve of Human S.CITP

N0. of Standard (St)	Concentration of standard	absorption (A)
St_1	50 *ng/ml*	0.346
St_2	100 *ng/ml*	0.543
St_3	200 *ng/ml*	0.888
St_4	400 *ng/ml*	1.375
St_5	800 *ng/ml*	2.209

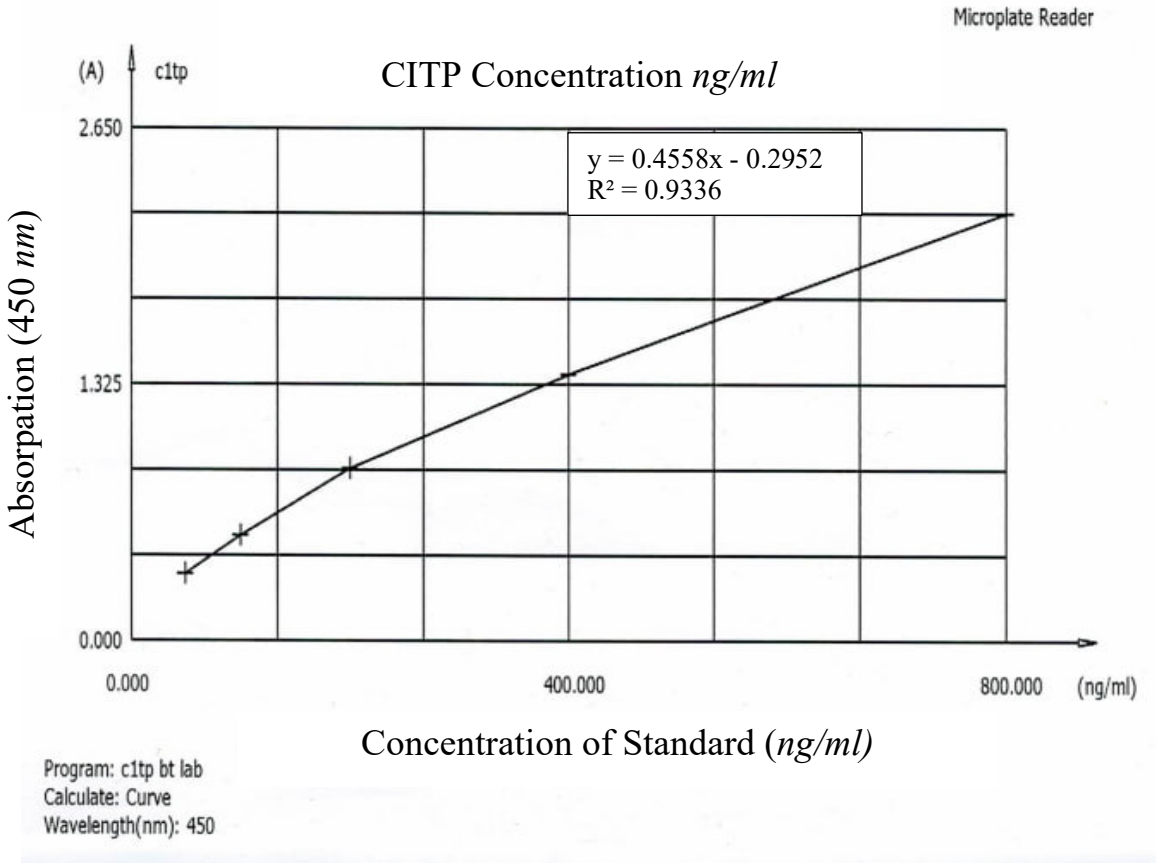


Fig 2.2: The Standard Curve of Human S.CITP

2.3.2. The Measurement of Serum S.PICP

❖ **Principle of Assay**

- This sandwich kit is intended for the precise quantitative human detection of carboxyterminal propeptide of type-1 procollagen (also known as S.PICP) in serum.
- This kit is an enzyme-linked immunosorbent assay (ELISA).
- The plate was pre-coated with a human PICP antibody.
- S.PICP in the sample was added and binds to antibodies coated on the wells.
- A biotinylated human S.PICP Antibody was added and binds to S.PICP in the sample.
- Streptavidin-HRP was added & bound to the biotinylated S.PICP antibody.
- The plate was placed in the incubator at 37c
- Unbound streptavidin-HRP was removed during the washing step.
- A substrate solution was added, and colour developed proportionately to the amount of human S.PICP.
- The reaction is terminated was added an acidic stop solution, and the absorbance was measured directly at 450 *nm*.

❖ **Preparation of Reagent**

- All reagents (**Sample & Standard**) were brought to room temperature before use.
- A 120 μL of **standard** (1600 *ng/mL*) was reconstituted with 120μl of standard diluent to generate an 800 *ng/ml* standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare standard points by serially diluting the standard stock solution (800 *ng/ml*) 1:2 with standard diluent to produce 400 *ng/ml*, 200 *ng/ml*, 100 *ng/ml*, and 50 *ng/ml* solutions. Standard diluent serves as the zero standards (0 *ng/ml*) Fig. 2.3. Any

remaining solution should be frozen at -20°C and used within one month.

- The dilution of the proposed standard solutions was as follows

800 *ng/ml*	Standard-5	120µl Original Standard+120µl Standard Diluent
400 *ng/ml*	Standard-4	120µl Standard-5 + 120µl Standard Diluent
200 *ng/ml*	Standard-3	120µl Standard-4 + 120µl Standard Diluent
100 *ng/ml*	Standard-2	120µl Standard-3 + 120µl Standard Diluent
50 *ng/ml*	Standard-1	120µl Standard-2 + 120µl Standard Diluent

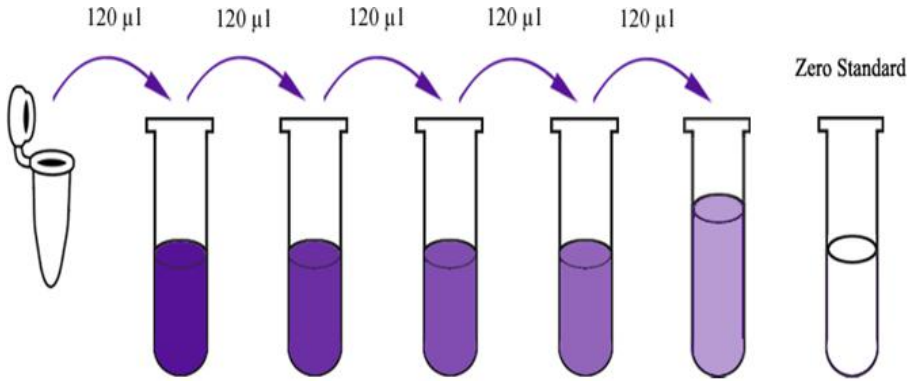


Fig 2.3: Serial dilution of Standard S.PICP

❖ **Wash Buffer**

- A 20 *mL* of wash buffer 25x was added into graduated cylinder.
- A 480 *mL* of distilled water (D.W) was added into graduated cylinder to yield 500 *mL* wash buffer dilute.
- A 500 *mL* of wash buffer dilute in a graduated cylinder was added into the washing bottle ELISA, Fig.2.7.

❖ **Procedure of Assay**

- A 50*µl* of standard (St1– St6) was added to standard well (A1–F1) only.
- A 40*µl* of a sample (S1–S90) was added to sample wells (G1–H12) only.

- A 10μl of anti-S.PICP antibody was added to sample wells.
- A 50μl of streptavidin-HRP was added to sample & standard wells.
- Wells was a mix.
- The plate was covered with a new sealer.
- The plate was incubated at 37 °C for 60 minutes.
- The sealer was removed from the plate.
- The plate was washed with washing buffer five times and dried on paper towels.
- 50μl of the substrate solution A & B was added into each well.
- The plate was covered with a new sealer.
- The plate was incubated in the dark at 37 °C for 10 minutes.
- A 50 μl of the stop solution in each well, the blue colour will change into yellow immediately.
- The plate was directly added to a microplate reader ELISA & set to 450nm.
- The reading was the absorpation (A value) to determine the absorption level of each well, Fig.2.7. **(Appendix-6)**
- The results were printed using a graphic standing curve.

❖ **Calculate of Result**

The concentration of serum S.PICP, absorption and concentration of standard is evaluated by a standard curve by the ELISA Dia Reader as shown in the table.2.11.

The standard curve is calculated by graphing the average A for every standard on the vertical (Y) line versus the concentration on the horizontal (X) line & draw a best-fit curve through the points on the graph, as shown in Fig. 2.4.

Table 2.11: The standard curve of Human S.PICP

N0. of Standard (St)	Concentration of standard	absorption (A)
St_1	50 *ng/ml*	0.333
St_2	100 *ng/ml*	0.492
St_3	200 *ng/ml*	0.816
St_4	400 *ng/ml*	1.286
St_5	800 *ng/ml*	2.304

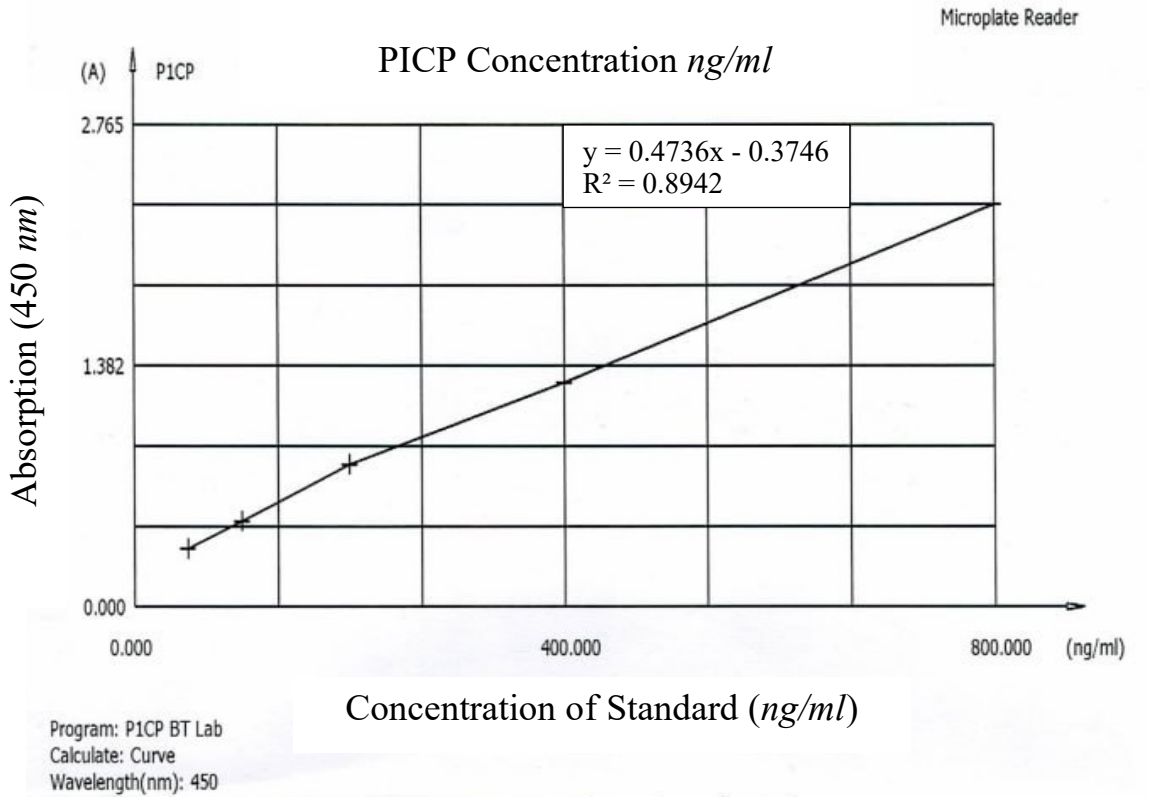


Fig 2.4: The Standard Curve of Human S.PICP

2.3.3. The Measurement of Serum Vitamin-C

❖ **Principle of Assay**

- This sandwich kit is intended for the precise quantitative human serum detection of Vitamin-C (VC).
- This kit is an enzyme-linked immunosorbent assay (ELISA).

- The plate was pre-coated with a human vitamin-C antibody.
- Vitamin C in the sample was added and bound to antibodies coated on the wells.
- Biotinylated human vitamin-C Antibody was added and binds to Vitamin-C in the sample.
- Streptavidin-HRP was added & bound to the biotinylated vitamin-C antibody.
- The plate was placed in the incubator at 37c.
- Unbound streptavidin-HRP was removed during the washing step.
- A substrate solution was added and colour developed in proportion to the amount of human vitamin C.
- The absorbance was measured at 450 *nm* after adding an acidic stop solution to terminate the reaction.

❖ **Preparation of Reagent**

- All reagents (**Sample & Standard**) were brought to room temperature before use.
- A 120*µl* of **standard** (320 *ng/ml*) was reconstituted with 120*µl* of standard diluent to generate a 160 *ng/ml* standard stock solution. Allow the standard to sit for 15 minutes with gentle agitation prior to making dilutions. Prepare standard points by serially diluting the standard stock solution (160 *ng/ml*) 1:2 with standard diluent to produce 80 *ng/ml*, 40 *ng/ml*, 20 *ng/ml* and 10 *ng/ml* solutions. Standard diluent serves as the zero standards (0 *ng/ml*) Fig. 2.5. Any remaining solution should be frozen at -20°C and used within one month.
- The dilution of the proposed standard solutions was as follows:

160 *ng/ml*	Standard-5	120µl Original Standard+120µl Standard Diluent
80 *ng/ml*	Standard-4	120µl Standard-5 + 120µl Standard Diluent
40 *ng/ml*	Standard-3	120µl Standard-4 + 120µl Standard Diluent

20 *ng/ml*	Standard-2	120µl Standard-3 + 120µl Standard Diluent
10 *ng/ml*	Standard-1	120µl Standard-2 + 120µl Standard Diluent

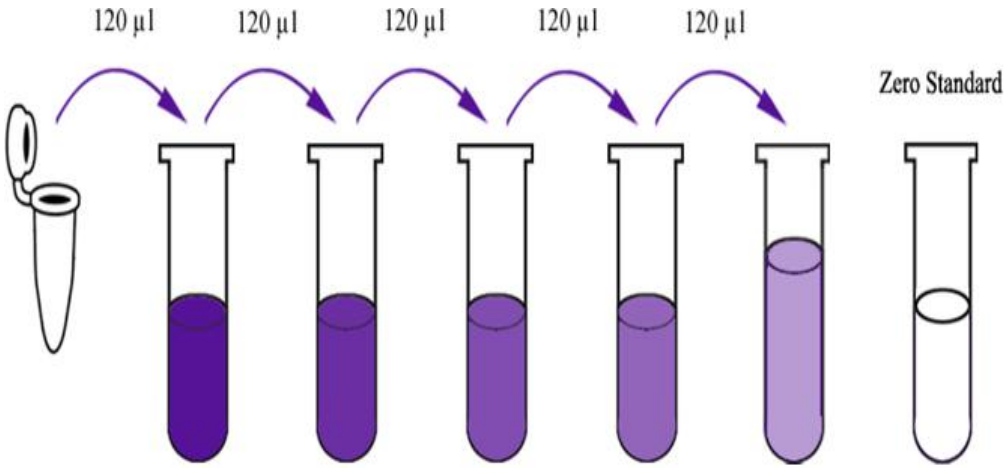


Fig 2.5: Serial dilution of Standard Vitamin C

❖ **Wash Buffer**

- A 20 *mL* of wash buffer 25x was added into graduated cylinder.
- A 480 *mL* of distilled water (D.W) was added into graduated cylinder to yield 500 *mL* wash buffer dilute.
- A 500 *mL* of wash buffer dilute in a graduated cylinder was added into the washing bottle ELISA, Fig.2.7.

❖ **Procedure of Assay**

- A 50*µl* of the standard (St1– St6) was added to standard wells (A1–F1) only.
- A 40*µl* of the sample (S1–S90) was added to sample wells (G1–H12) only.
- A 10*µl* of the anti-vitamin-C antibody was added to sample wells.
- A 50*µl* of the streptavidin-HRP was added to sample & standard wells.
- Wells was a mix.
- The plate was covered with a sealer.

- The plate was incubated at 37 °C for 60 minutes.
- The sealer was removed from the plate.
- Wash the plate with the wash buffer five times & blot the plate onto paper towels.
- 50μl of the substrate solution A&B was added into each well.
- The plate was covered with a new sealer.
- The plate was let to incubate for 10 minutes in the dark at 37 °C.
- 50 μl of the stop solution was added in each well; the blue colour will change into yellow immediately.
- The plate was directly added to a microplate reader ELISA & set to 450nm.
- Read the absorption (A value) to determine the absorption level of each well, Fig.2.7. **(Appendix-7)**
- Print the results with the graphic curve stander.

❖ **Calculate of Result**

The concentration of serum vitamin-C and absorption & concentration of standard is evaluated by a standard curve by the ELISA Dia Reader as shown in the table.2.12.

The standard curve is formed by graphing the average absorption for every standard on the vertical (Y) line versus the concentration on the horizontal (X) line and drawing a best-fit curve across the points on the graph, as depicted in Fig. 2.6.

Table 2.12: The standard curve of Human Vitamin-C

N0. of Standard (St)	Concentration of standard	absorption (A)
St_1	160 *ng/ml*	0.562
St_2	80 *ng/ml*	0.810
St_3	40 *ng/ml*	1.414
St_4	20 *ng/ml*	2.047
St_5	10 *ng/ml*	2.478

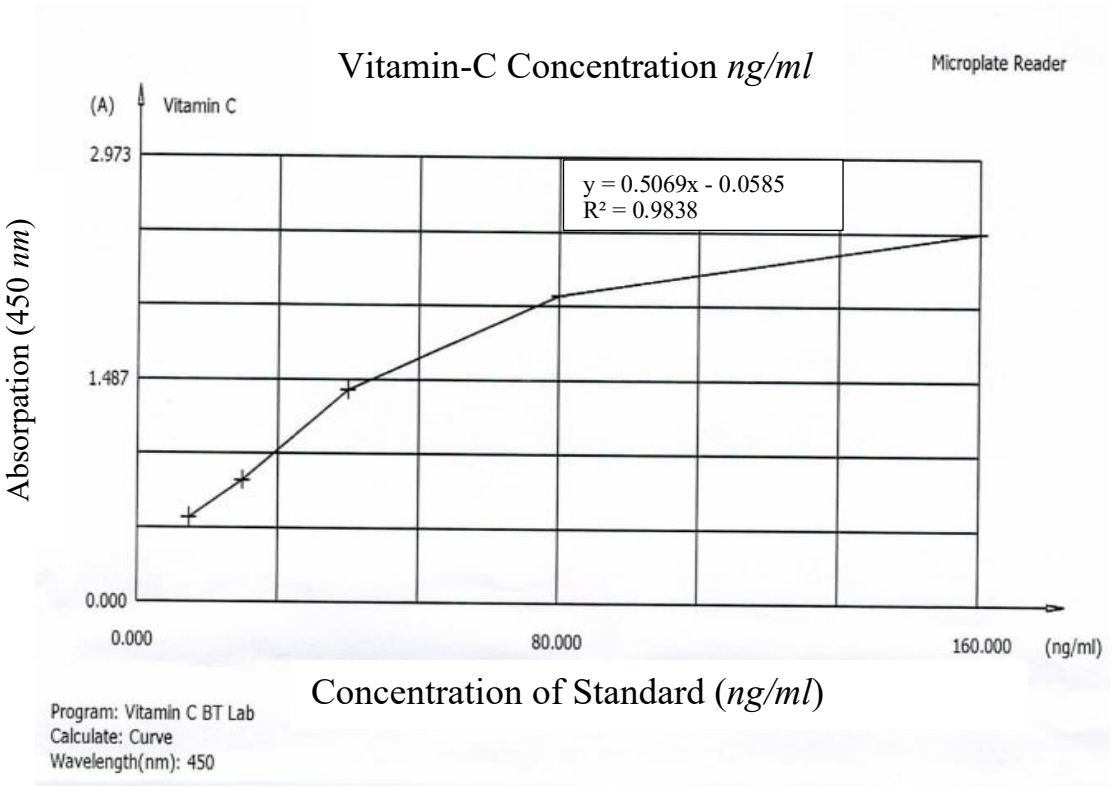


Fig 2.6: The Standard Curve of Human Vitamin-C

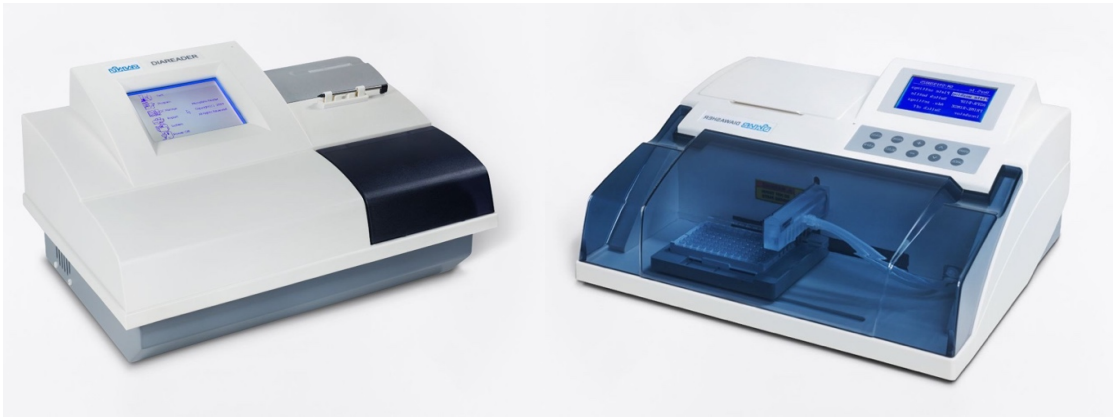


Fig. 2.7: DiaReader and Wash Reader The microtiter plate reader is used for the investigation of CITP, PICP, and Vitamin-C biomarkers (DIALAB GmbH., 2019)

2.3.4. Serum Cholesterol

- **Methodology by Enzymatic**
- **Principle of Assay**
  - The cholesterol assay is used for the quantitation of cholesterol in human serum.

- Cholesterol ester + $H_2O \xrightarrow{Cholestrol\ esterase}$ Cholesterol + fatty acids
- Cholesterol + $O_2 \xrightarrow{Cholesterol\ oxidase}$ Cholest-4-ene-3-one + H_2O_2
- H_2O_2 + HBA+ 4-AAP $\xrightarrow{Peroxidas}$ Chromophore (quinoneimine dye)
- The chromophore (a quinonimine dye) was quantitated at 500 *nm*.

❖ **Normal Value:** in serum according to **(Expert Panel on Detection, 2001)**

- < 200 *mg/dL*---------- > **Desirable**
- 200-239 *mg/dL*---------->**Borderline**
- ≥ 240 *mg/dL*----------> **High**

❖ **Procedure of Assay**

The Architect Abbott laboratories device measures the cholesterol level, Fig.2.8. **(Appendix-8)**

2.3.5. Serum Triglyceride

❖ **Methodology by Enzymatic**

❖ **Principle of Assay**

- The triglyceride (TG) assay is used to quantify TG in human serum.
- Triglycerides $\xrightarrow{Lipase}$ Free Fatty Acids + Glycerol
- Glycerol + ATP $\xrightarrow{glycerol\ kinase}$ Glycerol-3-Phosohate + ADP
- Glycerol-3-Phosohate $\xrightarrow{glycerol\ phosohate\ oxidase}$ DAP + H_2O_2
- H_2O_2 + 4-AAP + 4-CP $\xrightarrow{Peroxidase}$ Red Colored Dye
- The absorbance of this dye is proportional to the triglyceride concentration present in the sample.

❖ **Normal Value:** in serum according to (**Expert Panel on Detection, 2001**)

- < 150 *mg/dL*---------- > **Normal**
- 150-199 *mg/dL*----------> **Borderline High**
- 200-499 *mg/dL*----------> **High**
- ≥ 500 *mg/dL*----------> **Very High**

❖ **Procedure of Assay**

The Architect Abbott laboratories device measures the triglyceride level, Fig.2.8. **(Appendix-9)**

2.3.6. Serum High-Density Lipoprotein (HDL)

❖ **The methodology** by Accelerator Selective Detergent.

❖ **Principle of Assay**

- The ultra high-density lipoprotein (UHDL) assay is used to quantify HDL in human serum.
- The UHDL assay is a homogeneous method for directly measuring HDL cholesterol concentrations in serum.
- The method uses a 2-reagent format & properties of a special detergent.
- This method is based on accelerating the reaction of cholesterol oxidase (CO) with Non-HDL un-esterified cholesterol and dissolving HDL cholesterol selectively using a specific detergent.
- The first reagent, Non-HDL un-esterified cholesterol is subject to an enzyme reaction and a peroxidase reaction consumes the peroxide generated with DSBMT, Yielding a colorless product.
- The second reagent consists of a detergent (capable of solubilizing HDL cholesterol), cholesterol esterase (CE) and a chromogenic coupler to developer for the quantitative determination of HDL cholesterol.

❖ **Normal Value:** in serum according to **(Expert Panel on Detection, 2001)**

- < 40 *mg/dL*----------> **Major Risk Factor For Heart Disease.**
- ≥ 60 *mg/dL*----------> **Negative Risk Factor For Heart Disease.**

❖ **Procedure of Assay**

The Architect Abbott laboratories device measures the HDL cholesterol level, Fig.2.7. **(Appendix-10)**

2.3.7. Serum Very Low-Density Lipoprotein-Cholesterol (VLDL-C)

The Architect Abbott laboratories device calculated the VLDL-C level according to Friedewald Equation **(Friedewald, Levy & Fredrickson, 1972**), Fig.2.8.

VLDL-C = Triglycerides(*mg/dL*) /5

❖ **Normal Value:** Less Than 20 *mg/dL*

2.3.8. Serum Low-Density Lipoprotein-Cholesterol (LDL-C)

The Architect Abbott laboratories device calculated the LDL-C level according to Friedewald Equation **(Friedewald, Levy & Fredrickson, 1972)**, Fig.2.8.

LDL-C= Total Cholesterol(*mg/dL*) **–** (HDL **–** VLDL) *mg/dL*

❖ **Normal Value:** Up to 160 *mg/dL*

Fig. 2.8: Abbot-ARCHITECT c4000 clinical chemistry is used for the investigation of lipid profile samples (Corelaboratory, 2018)

2.4. The Statistical analysis

Information from the questionnaire and all test results from study group samples were entered into a data sheet. The data analysis for this work was generated utilising the social sciences statistical package, version 28.0 (IBM, SPSS, Chicago, Illinois, USA), and the real statistics resource pack software for mac (Release 8.6) of the resource pack for excel 2016, (Copyright, 2013 –2020) (**Zaiontz, 2013**).

Descriptive statistics were applied to each group's data. Values were illustrated by n (%) for categorical; scale variables were presented by mean ± 2 standard deviation for normal data, while for non-normal data, continuous variables were presented by the interquartile range (IQR) and median. The distribution of the data was examined using the Shapiro-Wilk test as a numerical measure of normality. For abnormal distribution, the univariate analysis was performed using an independent Kruskal Wallis test for continuous variables.

Analytical and statistical tests confirmed significant differences in categorical variables among the parameters. Results of all hypothesis test with p-values ≤ 0.05 (two-sided) were considered statistically significant.

The simultaneous confidence level for each confidence interval was calculated using Fisher's least significant difference (LSD) test. This simultaneous confidence level represents the probability that each confidence interval contains the true change. Fisher's LSD test was employed in ANOVA to produce confidence intervals for all pairwise differences between biomarkers and study groups.

The optimal threshold with high specificity and sensitivity for study cases was detected using receiver operating characteristic (ROC) analysis.

ChapterThree

Results

3. Results

This study included 120 participants; the gender distribution was 78 females and 42 males; the ratio of females to males was approximately 2:1. The ages of the participants ranged from 22 to 90 years, and they were divided into two groups: 60 of them were diagnosed with atrial fibrillation (AF), and 60 were used as a healthy control group. Atrial fibrillation group was divided into subgroups, The clinical characteristics of the four groups of atrial fibrillation patients are displayed in the figures below.

Most of patients were within the age group above 60 years, as presented in Fig. 3.1. While the body mass index was distributed based on the BMI status according to the World Health Organization, it was found that most of the patients were classified as pre-obese to obese, as presented in Fig. 3.2.

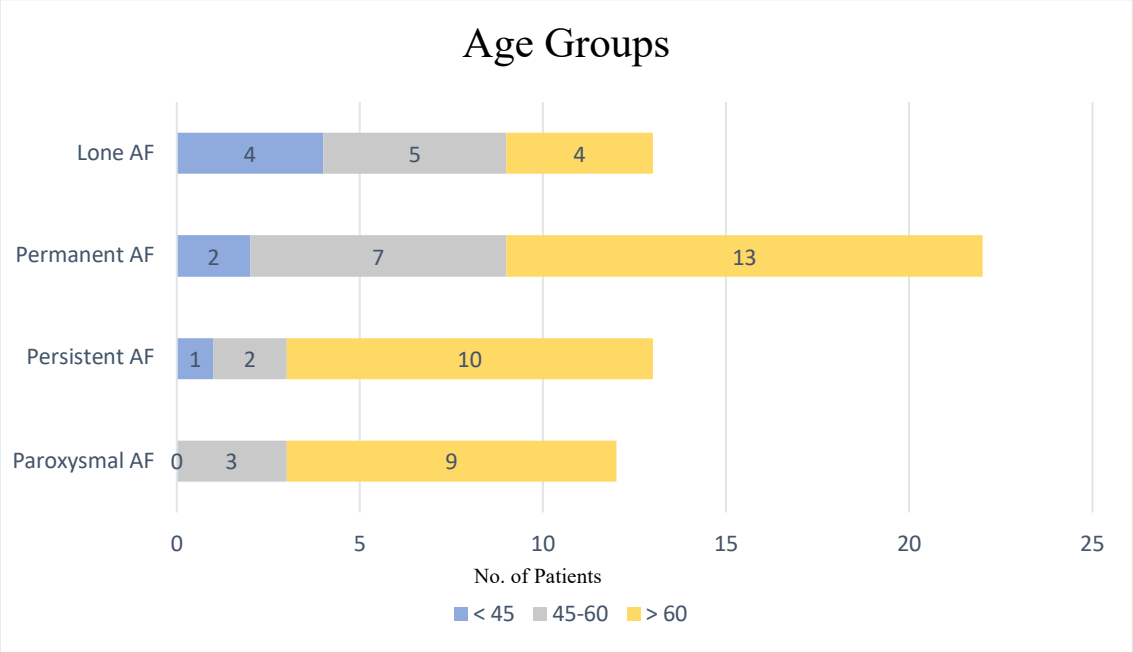


Figure 3.1: Distribution of Age groups among patients with AF

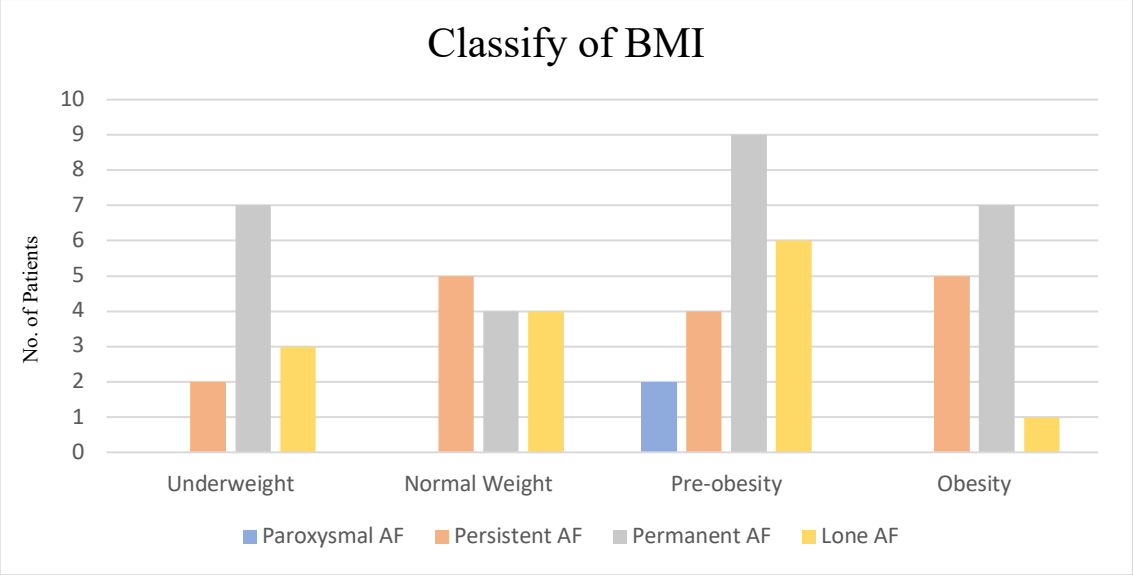


Figure 3.2: Distribution of BMI groups among patients with AF

The blood pressure (BP) was classified according to an American College of Cardiology (ACC) and American Heart Association (AHA) task force on clinical practice guidelines; results indicated that about 29% of the patients were within normal blood pressure, 19% of the patients were within elevated BP, and 52% were within hypertension (HTN) stages 1 and 2, as presented in (Fig. 3.3).

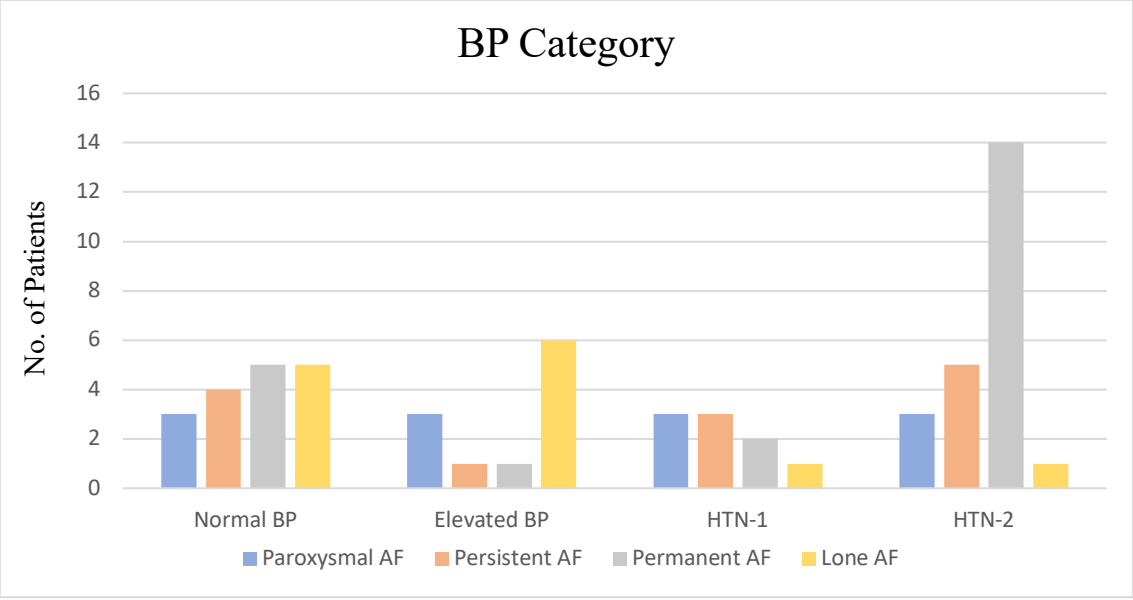


Figure 3.3: Distribution of Blood Pressure among patients with AF

Comorbidities (hypertension and dyslipidemia) were distributed among patients with atrial fibrillation. Results revealed that about 70% of the AF patient groups had hypertension, and 23% were confirmed to have dyslipidemia, as presented in Fig. 3.4.

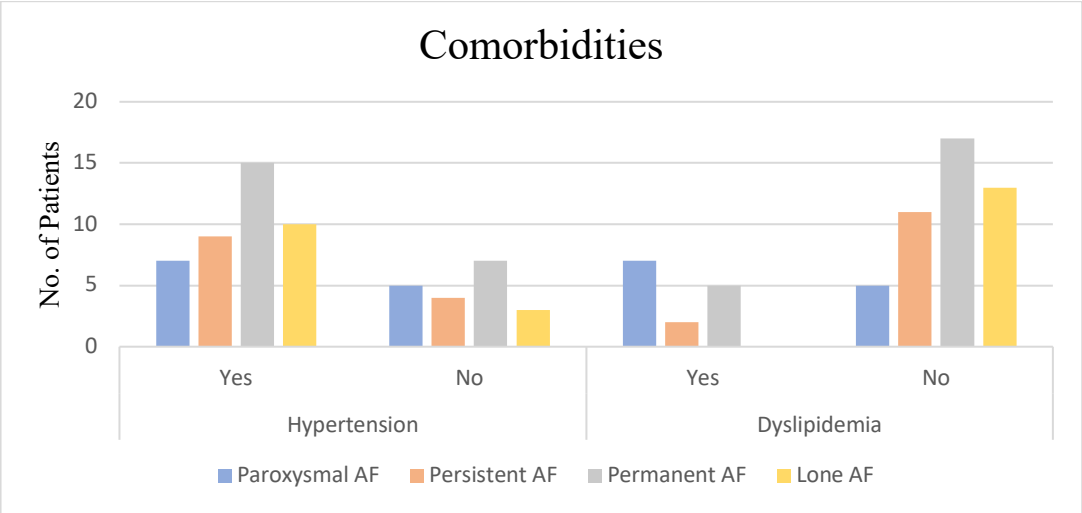


Figure 3.4: Distribution of comorbidities among patients with AF

The groups of patients with atrial fibrillation are listed in Fig. 3.5. Patients were divided into four subgroups based on disease duration. Group A: 12 patients with paroxysmal AF; Group B: 13 patients with persistent AF; Group C: 22 patients with permanent AF; and Group D: 13 patients with lone AF.

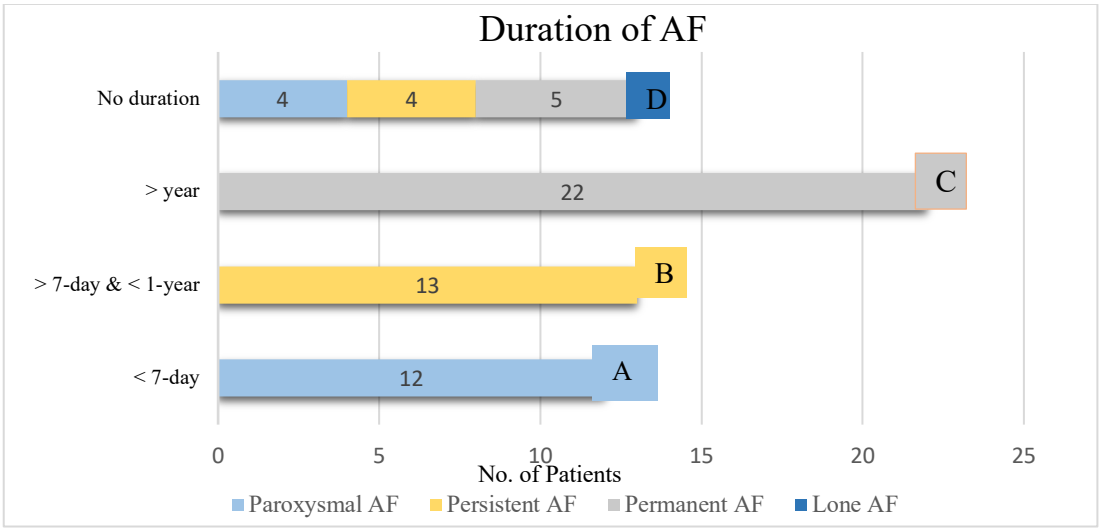


Figure 3.5: Distribution of disease duration among patients with AF

Subsequently, regarding the distribution of symptoms among AF patients, Fig. 3.6 demonstrated that about 57% of the patients had chest pain, 88% presented with palpitations, 80% reported dyspnea and only 23% indicated the presence of syncope.

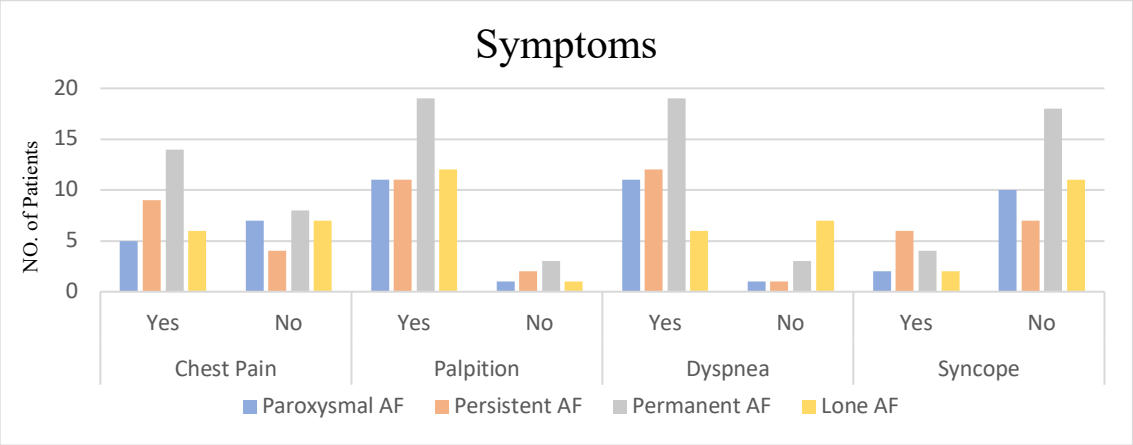


Figure 3.6: Distribution of Symptoms among patients with AF

Bundle branch block (BBB) is a condition in which the heart's electrical signal is blocked or disrupted, resulting in an arrhythmia. It was a diagnosis based on ECG interpretation. Fig. 3.7 shows the distribution of ECG interpretation among AF patients. Results indicated that 13% of the patients had a present LBBB, only 3% had a present RBBB, and 38% had an abnormality in the heart rate, such as tachycardia.

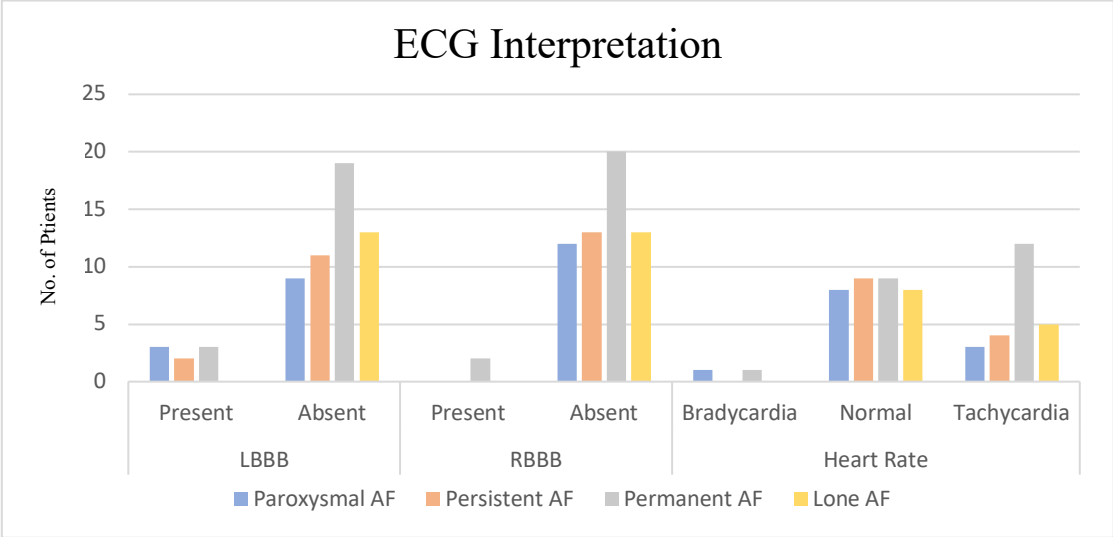


Figure 3.7: Distribution of ECG Interpretation among AF patients

Through an echocardiographic study, abnormalities and dysfunctions in the cardiac structure were selected as clinical confounders. Fig. 3.8 shows how the echocardiography study results are spread among AF patients. Results revealed that 67% were in the normal range and 25% of the patients had mild, 6% moderate, and 2% severe enlargements in LA diameter. While 63% were in the normal range and 27% of the patients had mild, 7% moderate, and 3% severe dilatation in LV diameter. Additionally, 73% were in the normal range and 15% of the patients had mild, 10% moderate, and 2% severe dysfunction in EF%.

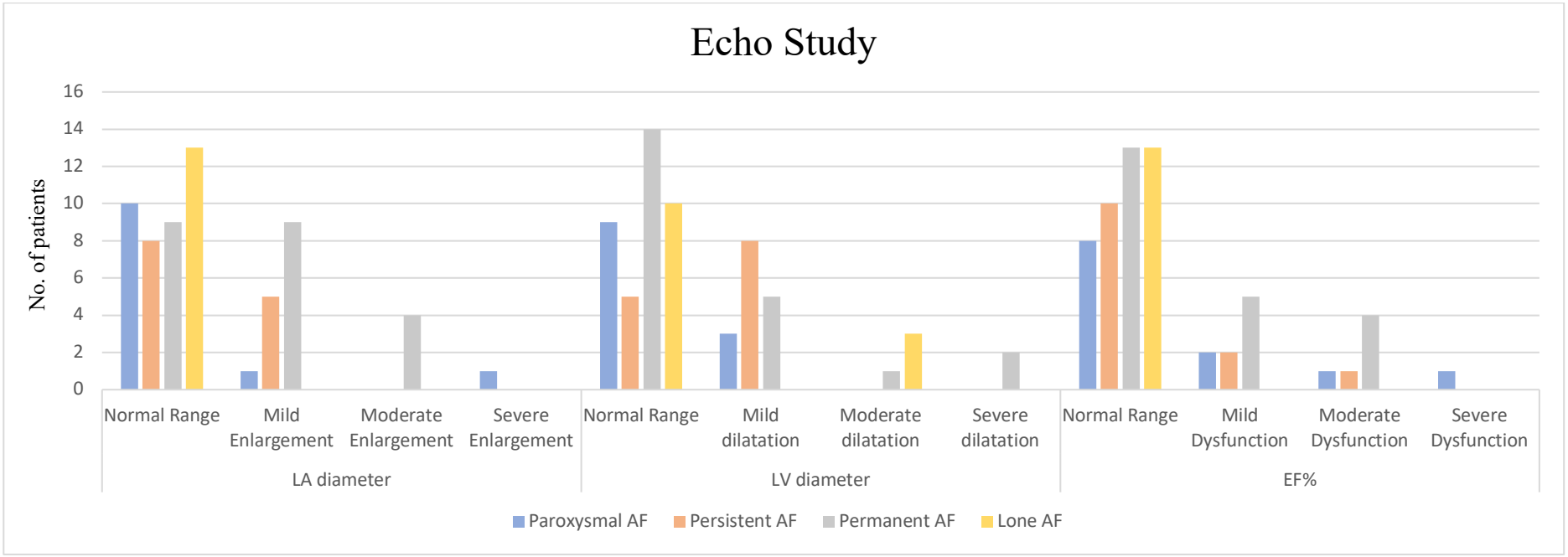


Figure 3.8: Distribution of Echocardiography Study among patients with AF

3.1. The relationship between gender and atrial fibrillation patients

In figure (3.9), the gender distribution in this study was 39 (65% females) and 21 (35% males), and the ratio of females to males was apperoaxmally 2:1.

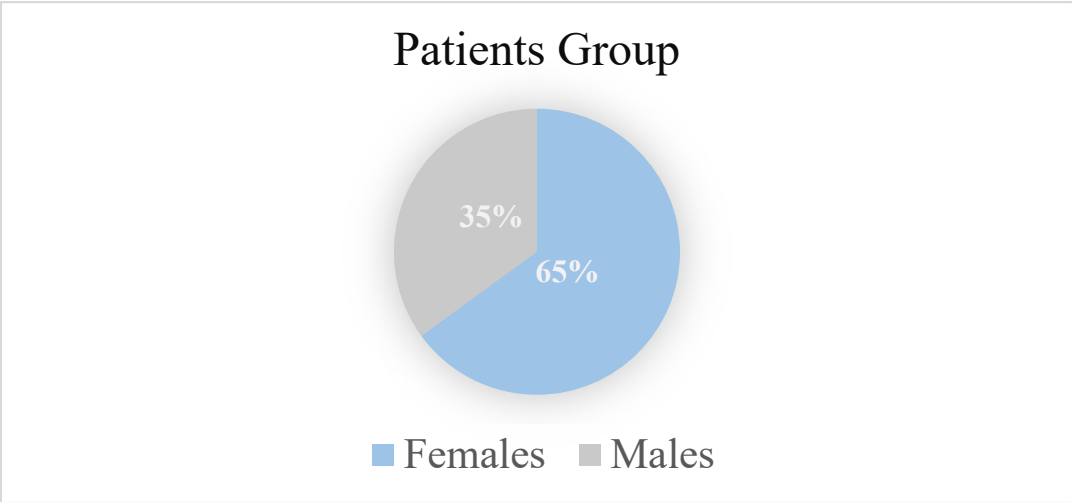


Figure 3.9: Distribution of gander groups among patients with AF

The mean level of S.PICP and S.CIPT based on gender differences was presented in Fig. 3.10. Females group had a higher mean level of both markers than the males group

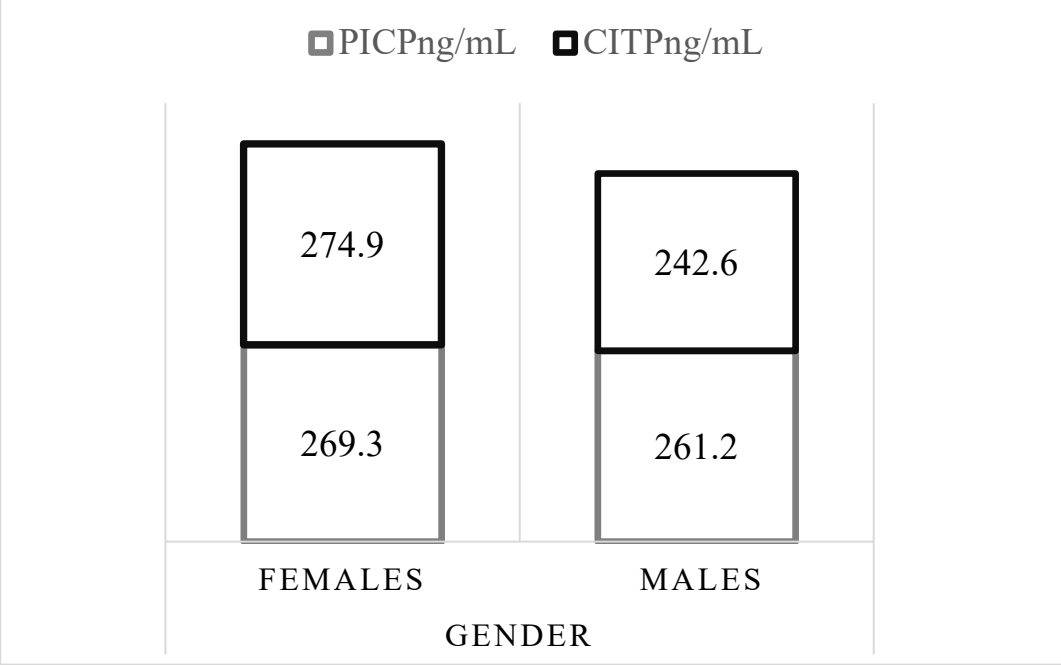


Figure 3.10: Distribution of S.PICP and S.CITP among gender in AF patients

3.2 The mean differences of collagen synthesis and degradation markers in clinical variables

Data from earlier research revealed that both collagen synthesis and degradation markers, S.PICP and S.CIPT are related to myocardial fibrosis and validated by endomyocardial biopsies(**Raafs et al., 2021)**.

Figure 3.11 shows the clinical variables evaluated using the chi-squared /Fisher's exact test and the ANOVA test for the mean values of S.PICP and S.CITP. In terms of BMI differences, the obese group had a small increase in both markers compared to the pre-obesity group.

The blood pressure group with hypertension had a higher mean level of both markers than the elevated blood pressure (BP) group.

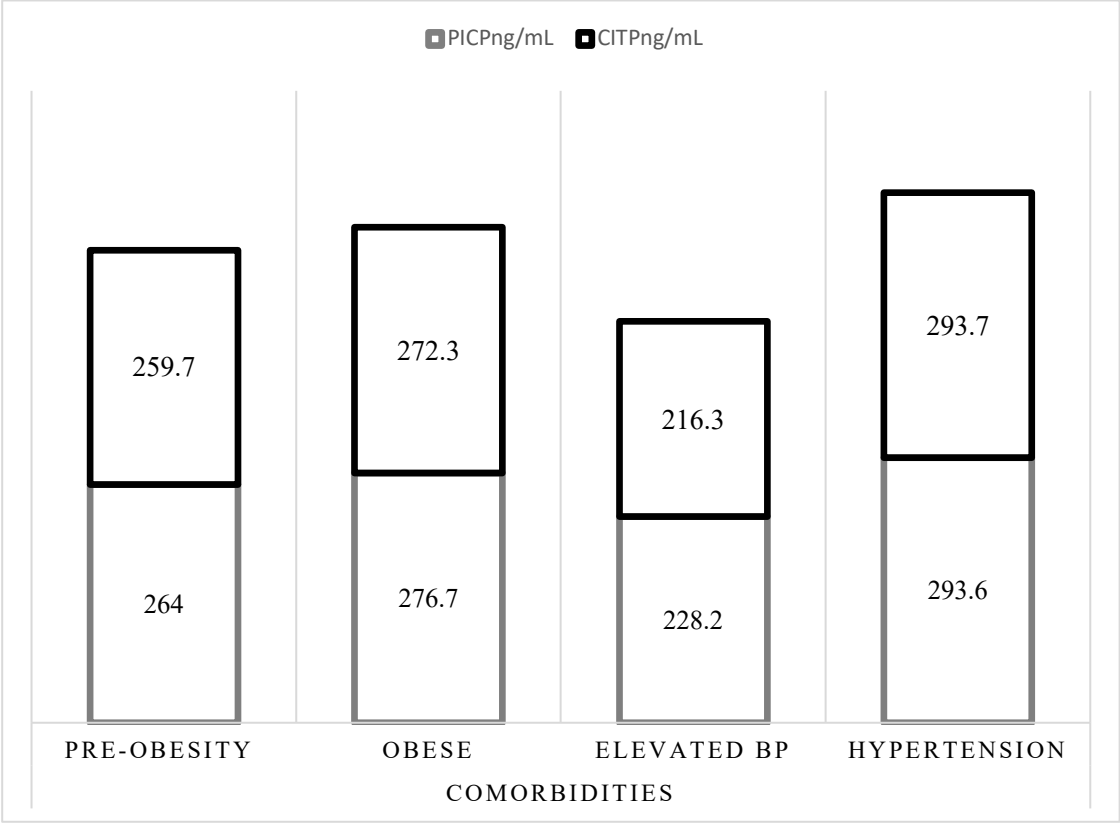


Figure 3.11: Distribution of S.PICP and S.CITP among independent variables in AF patients

3.3. Lipid profiles

Although there was a broad range of variation in the levels of lipid profiles in patient groups, results showed insignificant differences ($P > 0.05$) in cholesterol, TG, HDL, VLDL and LDL in AF patients compared to the control group, Fig.3.12

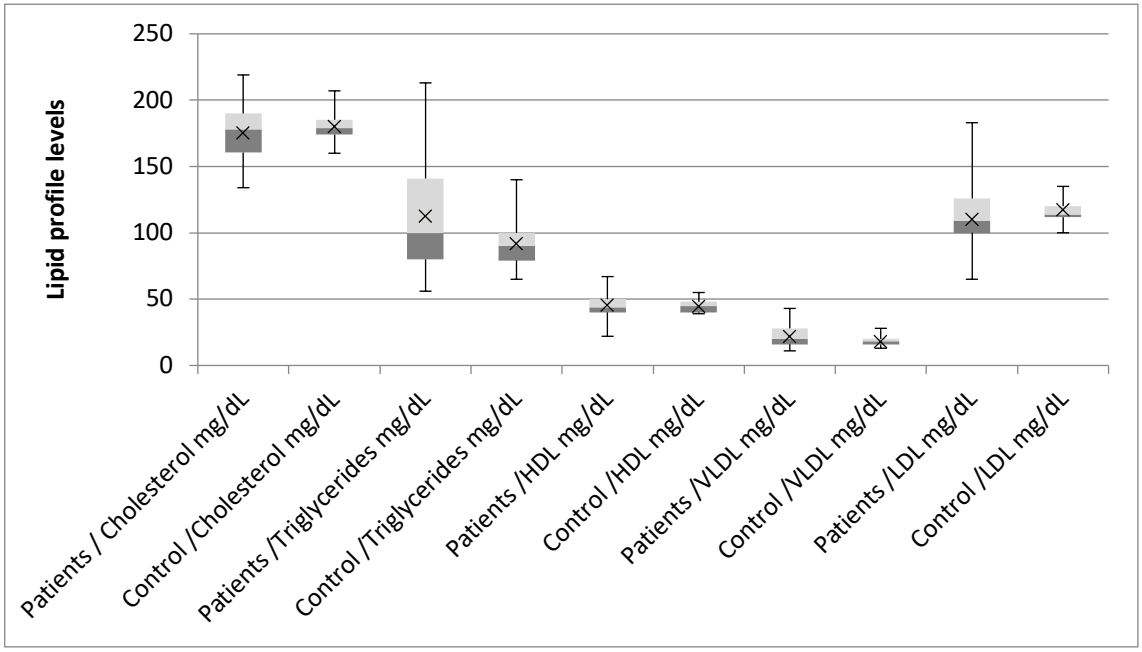


Figure.3.12: Boxplot of the Distribution of Lipid Profile Levels among AF Patient Groups Compared to the Control Group

TG: Triglyceride, **HDL**: High-density lipoprotein; LDL: Low-density lipoprotein; **VLDL**: Very Low-Density Lipoprotein.

- **Normal Range**
  - Cholesterol: 200-239 *mg/dL*
  - TG: < 150 *mg/dL*
  - *HDL: ≥ 60 mg/dL*
  - VLDL: Less Than 20 *mg/dL*
  - LDL: Up to 160 *mg/dL*

3.4. The mean serum vitamin C level between the AF group and the control group

The mean level of serum vitamin C was high in the control group compared to the AF group as in Table 3.1 & Fig. 3.13, and this high level of serum vitamin C leads to elevated levels of both markers S.PICP and S.CITP for some individuals in the control group, but the increase in levels for both markers S.PICP and S.CITP in the AF group represents myocardial interstitial fibrosis (MIF) as in Fig. 3.14.

Table 3.1: The Distribution of the Mean S.VC Level among Groups of AF Patients Compared to the Control Group

Vitamin-C	Mean± SD	P-value
Control Group	58.7±6.26	0.654 [**NS**]
Patient Group	46.9±4.24	

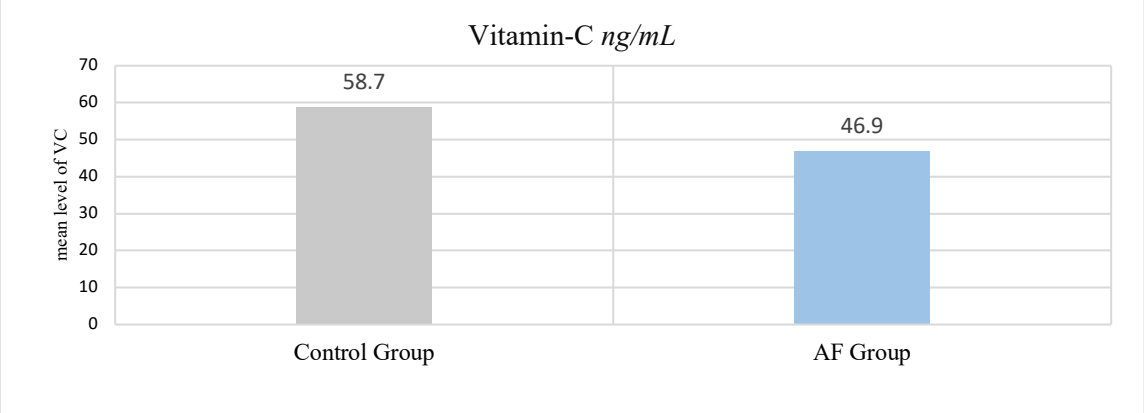


Figure 3.13: The Distribution of the Mean S.VC Level among Groups of AF Patients Compared to the Control Group

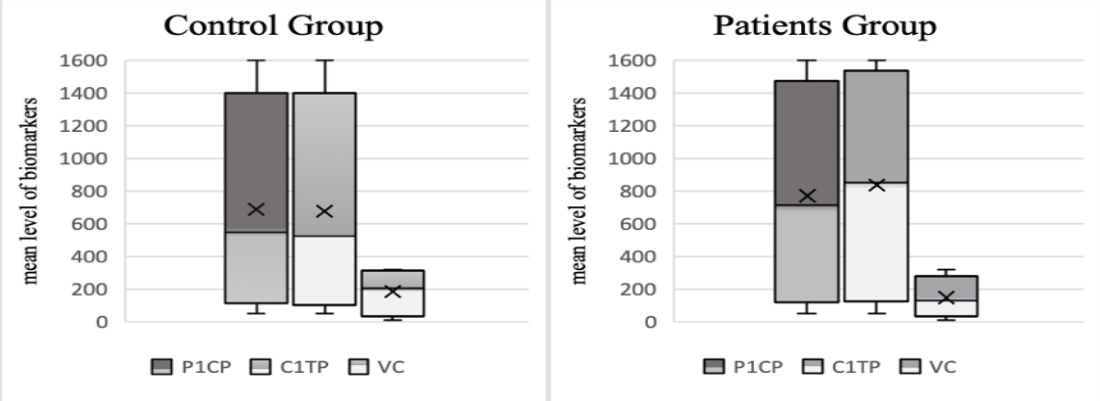


Figure 3.14: Box plot of the distribution of the mean level of biomarkers among the AF patient groups compared to the control group

3.5 Distribution Mean of S.PICP Level and S.CITP Level as Markers of Collagen Deposition

It was reported that high levels of circulating collagen synthesis and degradation might be associated with a higher risk of heart failure (HF) in a certain community. This study would examine the role of collagen turnover and markers of collagen deposition in atrial fibrillation (AF) cases.

Table 3.2 and Figure 3.15 illustrates the mean distribution levels of S.PICP and S.CITP values by T- Test. Both indicators were significantly higher in AF cases than in the healthy control group. The mean levels were 682.5 and 675, respectively.

Table 3.2: the distribution of the mean S.PICP level and S.CITP level among the AF patient groups compared to the control group

Biomerkers		mean±SD	P-Value
PICP	Control Group	682.5±197.28	0.05 [S]
	Patient Group	770±159.39	
CITP	Control Group	675±176.95	0.012 [S]
	Patient Group	837.5±142.03	

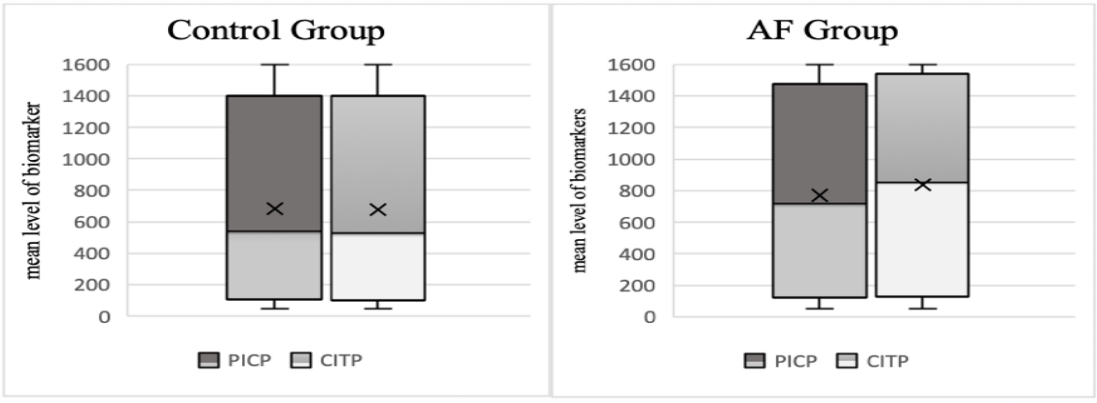


Figure 3.15: Box plot of the distribution of the mean S.PICP level and S.CITP level among the AF patient groups compared to the control group

3.6 Association of proposed biochemical markers with all study groups

Fisher's LSD method calculated confidence intervals for all paired changes between biomarkers and dependent variables while controlling for the individual error rate at the chosen significance level.

This simultaneous confidence level is the chance that the real difference is in all confidence intervals. There were multiple outliers, as

determined by the boxplot; data was normally distributed for each group, as determined by the Shapiro-Wilk test (P>0.05); and variances were homogeneous, as determined by Levene's test of homogeneity of variances (P = 0.353). The data were presented as mean levels in Fig. 3.16.

Compared to the control group, the mean level of S.PICP was significantly higher in all AF groups than in the control group (P≤ 0.05). Similarly, the mean level of S.CITP was significantly higher in all AF groups than in the control group (P≤ 0.05). as shown in Fig. 3.16.

The mean level of the marker of collagen synthesis to collagen degradation ratio was only significant between persistent and permanent AF groups compared to the control group (P≤ 0.05), as shown in Fig. 3.16.

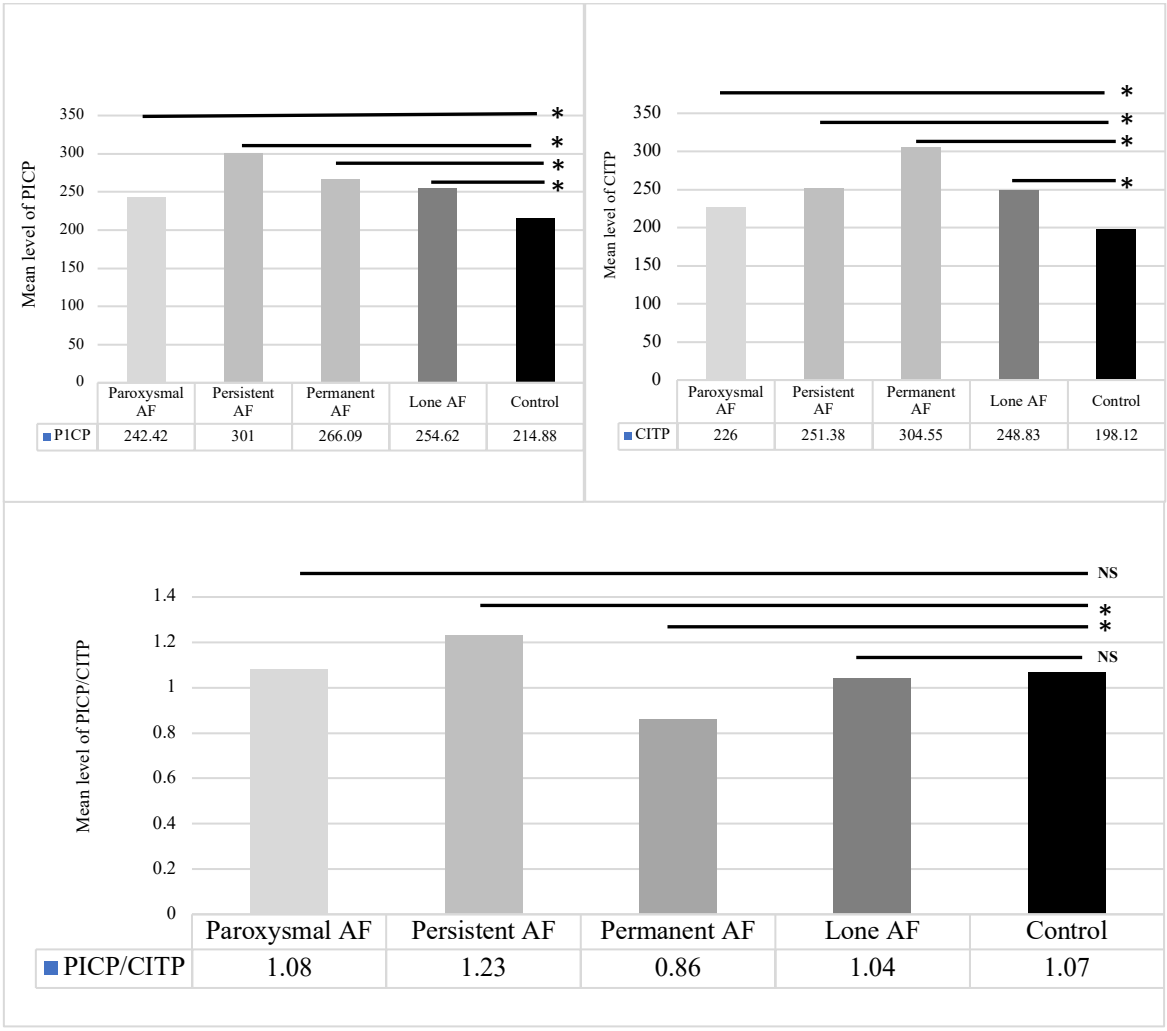


Figure.3.16: Multiple comparisons of dependent variable and least significant difference post hoc test in all groups studied

3.7 The receiver operating curve (ROC) curve of serum PICP and CITP and their ratio for the diagnosis of MIF in AF cases

Table 3.3. shows the AUC & ROC for assessing S.PICP, S.CITP and their ratio concentrations as potential diagnoses markers of MIF in AF cases.

S.PICP, S.CITP and their ratio biomarkers exhibited high diagnostic accuracy for predicting AF.

The S.PICP produced an AUC of 0.829 (0.688–0.876; P<0.001). The best cut-0ff Value of S.PICP for detection of MIF is 157*ng/mL* with sensitivity 86.7%, Specificity 81.7%, PPV 81.25%, NPN 85.71% & accuracy 83.33%.

The S.CITP produced an AUC of 0.782 (0.741**–** 0.917**;** P<0.001). The best cut-0ff Value of S.CITP for early detection of MIF is 165.5 *ng/mL* with sensitivity 93.2%, Specificity 83.3%, PPV 84.62%, NPN 90.91% & accuracy 87.5%.

Ratio S.PICP / S.CITP has an AUC of 0.395 (0.287-0.503**;** P<0.048) with a sensitivity of 22%, Specificity 95%, PPV 81.25%, NPV 54.81% & accuracy 58.33%.

Table 3.3: The ROC curve for the optimal threshold that assesses S.PICP, S.CITP and their ratio for diagnosing MIF

Biomarker	Cutoff Value	AUC	CI (95%)	Sn. %	Sp. %	P-value	PPV %	NPV %	Accuracy %
S.PICP *ng/ mL*	157	0.829	0.688-0.876	86.7	81.7	<0.001[S]	81.25	85.71	83.33
S.CITP *ng/ mL*	165.5	0.782	0.741-0.917	93.2	83.3	<0.001[**S**]	84.62	90.91	87.5
$\frac{\text{S. PICP}}{\text{S. CITP}}$	/	0.395	0.287-0.503	22	95	0.048[**S**]	81.25	54.81	58.33

p<0.05 considered significantly different, [**S**]= Significant, [**NS**]= Non-significant, [**Sn**]= Sensitivity [**Sp**]= Specificity, [**PPV**]= Positive Predictive Values, [**NPV**]= Negative Predictive Values, [**PICP**] = Carboxyterminal Propeptide of Type-1 Procollagen, [**CITP**] = Carboxyterminal Telopeptide of Type 1 Collagen

❖ To determine the efficiency of (S.PICP, S.CITP and Ratio S.PICP / S.CITP) in the detection of MIF, the results indicated that the biomarker with the highest specificity for MIF was S.CITP (sensitivity 93.2%), followed by the most sensitive biomarker for MIF was S.PICP (sensitivity 86.7%), but reflecting the low sensitivity of MIF was the ratio of S.PICP/S.CITP with a sensitivity of 22%.

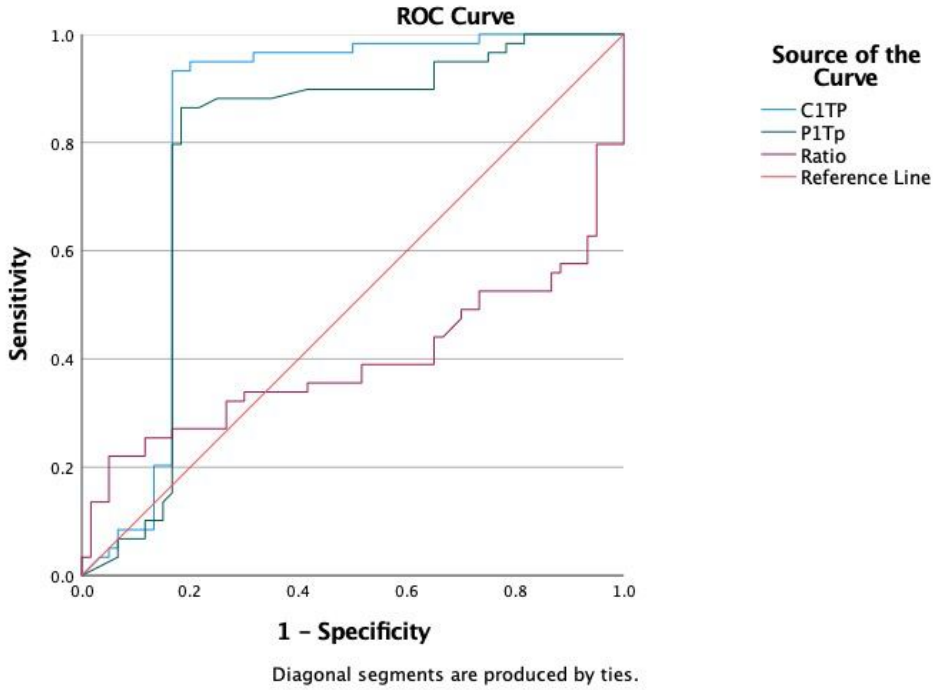


Figure 3.17: ROC curve for optimal diagnostic point analysis for predicting MIF in AF patients using S.PICP, S.CITP and their ratio

❖ Intergroup comparisons of the clinical biomarkers' proposed cut-off values were also performed. Table 3.4 shows the number of patients in each AF group who had or did not have myocardial interstitial fibrosis (MIF) based on cut-off values measured by ROC.

Table 3.4: Distribution of patients in AF groups according to the proposed cut-off value for clinical biomarkers

Biomarker	Cut off Value	Paroxysmal AF, n=12	Persistent AF, n=13	Permanent AF, n=22	Lone AF, n=13	Total	P value
S.PICP *ng/mL*	**>157**	11	11	17	13	**52**	**<0.001[S]**
	<157	1	2	5	0	8	
S.CITP *ng/mL*	**>165.5**	11	13	2	11	**37**	**<0.001[S]**
	<165.5	1	0	20	2	23	
p<0.05 considered significantly different**, [S]=** Significant**, [NS]=** Non-significant							

3.8. Association of Biochemical Markers and Echocardiography Findings with independent risk factors

Multinomial logistic regression was applied to analyse the results; the left ventricular (LV) diameter was measured in centimetres (*cm*) by using echocardiography and classified based on the American College of Cardiology (ACC) (**Lang et al., 2006**); S.PICP, S.CITP levels were evaluated.

Table 3.5 indicates that elevated levels of these markers in individuals with atrial fibrillation represent independent significant risk factors ($P \leq 0.05$) when compared to the control group (OR of S.PICP: 4.431, 54.003; 95% Cl: 0.164-119.982 and 2.645-1102.537). While the left ventricle dilated only mildly group in the OR of the S.PICP level, there were no significant differences ($P > 0.05$) compared to the control group.

There were no significant differences in the OR of the S.CITP level ($P > 0.05$) among AF patients with mild, moderate, and severe left ventricular dilatation compared to the control group.

Table 3.5: The association of the independen variable factor with the odds ratio for left ventricular diameter across all study groups and the total number of subjects (N = 120) was shown as follows:

Clinical Variable	LV diameter Group N= 120	OR (Lower – upper)	P value
PICP *ng/mL*			
	Normal	1[a]	**-**
	Mildly Dilated	1.210(0.117-12.498)	0.103[NS]
	Moderately Dilated	4.431(0.164-119.982)	**0.012[S]**
	Severely Dilated	54.003(2.645-1102.537)	**0.01[S]**
CITP *ng/ mL*			
	Normal	1[a]	**-**
	Mildly Dilated	1.002(0.999-1.004)	0.183[NS]
	Moderately Dilated	1.000(0.993-1.007)	0.08[NS]
	Severely Dilated	1.007(1.001-1.012)	0.536[NS]

- **P≤0.05** Considered Significantly Different, [**S**]= Significant, [**NS**]= Non-Significant
- [**1[a]**] = The reference category is normal range; [**LV**]= Left Ventricular, [**OR**]= Odds Ratio

3.9. The Relationship Between Proposed Biochemical Markers and Echocardiography Findings for Patients with Atrial Fibrillation

The group with mild enlargement in the left atrium (LA) had higher levels of S.PICP and S.CIPT than those with moderate to severe enlargement. In contrast, the group with moderate to severe dilation with a thick wall in the left ventricular (LV) had higher levels of S.PICP and S.CITP than the group with mild dilation with a thick wall.

The groups with moderate to severe ejection fraction dysfunction had higher mean levels of both markers than those with mild dysfunction.

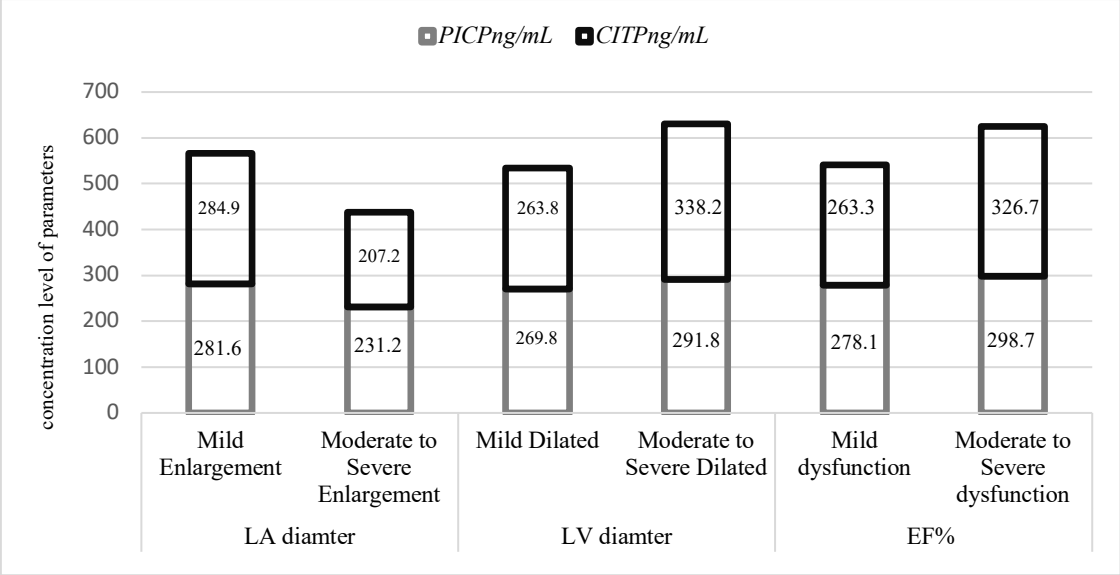


Figure 3.18: The mean differences in the biomarkers (PICP and CITP) levels based on the echocardiography findings in case of atrial fibrillation

3.10. Receiver Operating Curve (ROC) of LA and LV diameter for early-stage diagnosis of enlargement and dilation in MIF with AF cases

- Table 3.6 shows the AUC and ROC for evaluating LA and LV diameter as possible early stage thick wall enlargement and dilation diagnoses in MIF with AF cases.
- The LA diameter produced an AUC of 0.723 (0.633-0.812; P<0.001). The best cut-off Value of LA for early enlargement detection is 3.55*cm* with a sensitivity 78.3%, specificity 58.3%, PPV 62.64%, NPV 89.66% & accuracy 69.17%.
- The LV diameter produced an AUC of 0.683 (0.587-0.778; P<0.001). The best cut-0ff Value of LV for early dilation detection is 4.95*cm* with sensitivity 86.7%, specificity 81.7%, PPV 65.28%, NPV 72.92% & accuracy 68.33%.

Table 3.6: The optimal threshold ROC curve evaluating LA and LV diameters for the diagnosis of early stage enlargement and dilation in MIF with AF cases

Echo Study	Cut off Value	AUC	CI 95%	Sn. %	Sp. %	P-value	PPV %	NPV %	Accuracy %
LA diameter *cm*	3.55	0.723	0.633-0.812	**78.3**	58.3	<0.001[S]	62.64	89.66	69.17%
LV diameter *cm*	4.95	0.683	0.587-0.778	**86.7**	81.7	<0.001[S]	65.28	72.92	68.33%

[**P≤0.05**] considered significantly different, [**S**]= Significant, [**NS**]= Non-Significant, [**Sn**]= Sensitivity, [**Sp**]=Specificity, [**PPV**]= Positive Predictive Values, [**NPV**]= Negative Predictive Values, [**AUC**]= Area Under the Curve, [**LV**]= Left Ventricular, [**LA**]= Left Atrial

- The efficiency of LA and LV diameter in the detection of enlargement and dilation of wall thickness was performed to predict their sensitivity toward MIF in AF cases
- The sensitivity of LV was 86.7%, followed by LA sensitivity of 78.3%, as described in figure.3.20.

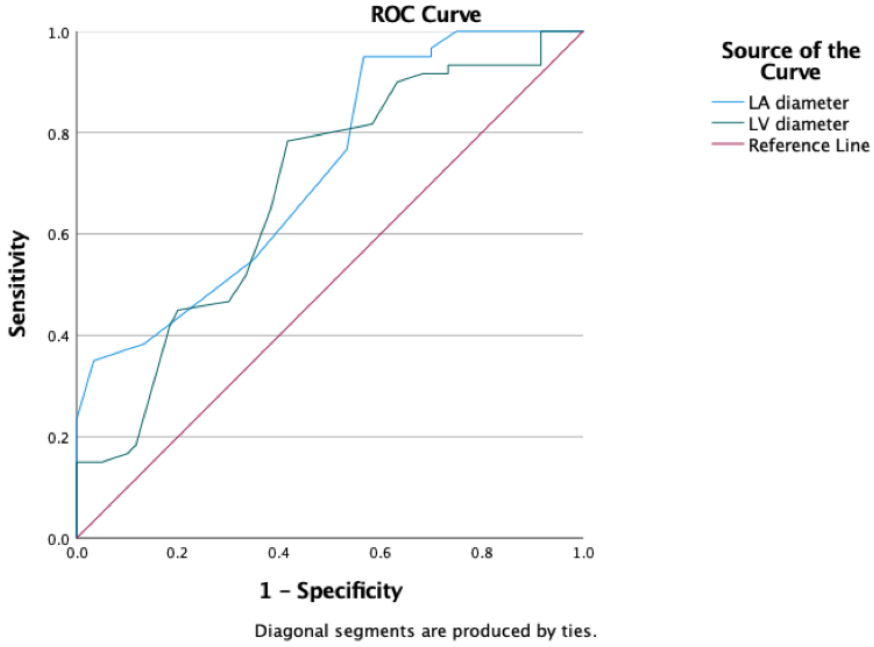


Figure 3.19: ROC curve for optimal diagnostic point analysis for predicting early stage enlargement and dilation in MIF with AF cases using LA and LV diameter

❖ Intergroup comparisons of the echocardiography study parameter cut-off values were performed. Table 3.7 shows the number of patients in each AF group who had or did not have a dilation and wall thickness based on the cutoff values measured by ROC.

Table 3.7: Distribution of Patients in Atrial Fibrillation Groups According to Suggested Cutoff Value for Echocardiography Findings

Echo Study	Cut off Value	AF patients n=60				Total	P-value
		Paroxysmal AF, n=12	Persistent AF, n=13	Permanent AF, n= 22	Lone AF, n= 13		
LA diameter *cm*	>3.55	11	13	20	13	**57**	<0.001[**S**]
	<3.55	1	0	2	0	3	
LV diameter *cm*	>4.95	8	12	16	11	**47**	<0.001[**S**]
	<4.95	4	1	6	2	13	

❖ Table 3.8 compares echo study parameters and clinical biomarkers proposed from the cutoff values. The prognostic cutoff value for types of AF patients is highly significant in all comparisons, and likewise, the intergroup comparison of the sensitivity, specificity and accuracy of both, where it shows:

Table 3.8: The sensitivity, specificity, and accuracy cutoff values were used to compare the proposed clinical biomarkers and the echocardiography study

Groups	Parameters	AUC	CI 95%	Sn. %	Sp. %	P-value	PPV %	NPV %	Accuracy %
Clinical Biomarkers Proposed	S.PICP *ng/mL*	0.782	0.688-0.876	**86.7**	81.7	<0.001[**S**]	81.25	85.71	**83.33%**
	S.CITP *ng/mL*	0.829	0.741-0.917	**93.2**	83.3	<0.001[**S**]	84.62	90.91	**87.5%**
Echo Study	LA diameter *cm*	0.723	0.633-0.812	**78.3**	58.3	<0.001[**S**]	62.64	89.66	**69.17%**
	LV diameter *cm*	0.683	0.587-0.778	**86.7**	81.7	<0.001[**S**]	65.28	72.92	**68.33%**

[**P≤0.05**] considered significantly different, [**S**]= Significant, [**NS**]= Non-Significant, [**Sn**]= Sensitivity, [**Sp**]= Specificity, [**PPV**]= Positive Predictive Values, [**NPV**]= Negative Predictive Values, [**PICP**] = Carboxyterminal Propeptide of Type-1 Procollagen, [**CITP**] = Carboxyterminal Telopeptide of Type 1 Collagen, [**AUC**]= Area Under the Curve, [**LV**]= Left Ventricular, [**LA**]= Left Atrial

Chapter Four

Discussion

4- Discussion

The current study investigated the relationship between excess collagen deposition levels and myocardial interstitial fibrosis (MIF) in atrial fibrillation (AF) patients; some were diagnosed with left atrial (LA) enlargement and others with left ventricular (LV) dilation with wall thickness or ejection fraction percentage (EF%) dysfunction. The results indicate that most patients had mild enlargement in the LA. At the same time, the threshold value of the biomarker depended on excess collagen deposition and was directly related to the intensity of MIF and LA enlargement in AF patients. Likewise, results indicate that most patients had moderate to severe dilation in the LV. At the same time, the threshold value of biomarker depended on excess collagen deposition and was directly related to the intensity of MIF and LV dilation with thickness wall in AF patients. Moreover, the results indicate that most patients had moderate to severe dysfunction in the EF%.

Additionally, the degree of collagen deposition dependence, MIF, LA enlargement, LV dilation with thickness wall and EF% dysfunction was associated with various demographics and comorbidities, such as obesity, hypertension, and most AF groups. Still, it was not associated with dyslipidemia and the S.PICP/S.CITP ratio in both their paroxysmal and lone AF.

4.1. Gender distribution of atrial fibrillation patients

The study showed that the distribution of females was greater than that of males (65%: 35% in Fig. 3.9). The distribution by gender was similar to a study conducted at the Bezmi Alem Vakif University in Turkey on 52 patients with atrial fibrillation, which it was found that the distribution of females to males was 62% to 38% (**Sonmez et al., 2014**). However, unlike a study conducted at the University of Utah in the USA on 908 patients

receiving treatment for atrial fibrillation, it found that the females to males distribution was 34.8%: 65.2% **(Akoum et al., 2018)**.

4.2. Mean Biomarkers among Studied Parameter

In Figure 3.10, results revealed a significant difference in mean levels of S.PICP and S.CITP in patient groups based on gender, as female patients had a higher mean level of each of the biomarkers than male patients. The result agrees with the research defined by Kobayashi *et al*. (2022), which states that patients with elevated serum PICP are only related to gender (higher in women) (**Kobayashi et al., 2022**). This result was not similar to a study conducted in the USA at Loyola University Medical Centre, which showed that serum PICP and CITP in males were elevated on a mean compared to females (**Odeh et al., 2023**).

The reason is that men's risk of developing atrial fibrillation is 1.5–2.0 times higher than women's (**Ko et al., 2016**). However, because women live longer than men and atrial fibrillation increase in prevalence with age, the absolute number of women with atrial fibrillation outnumbers men (**Gillis, 2017**). after 75 years of age about 60% of the people with atrial fibrillation are women (**Volgman et al., 2020)**. Females are associated with a higher incidence of atrial fibrillation mortality worldwide likely due to thromboembolic risk **(Zhang et al., 2021)**.

Figure 3.11 reveals small significant differences between groups of BMI patients. The obese group slightly increased in both biomarkers compared to the preobese group. This finding is consistent with previous reports that found obesity to be associated with higher serum PICP (**Eschalier et al., 2014**). Nevertheless, in contrast to the study in 113 obese patients (body mass index ≥ 30 kg/m^2) without other concomitants, spironolactone (versus placebo) reduced serum PICP; the change in PICP was associated with an improvement in left ventricular diastolic function (**Kosmala et al., 2013**).

The reason for this is that there is a strong association between obesity and atrial fibrillation (**Tedrow et al., 2010**). Obesity increased the risk of atrial fibrillation by 49% in the general population. In addition, obesity can directly affect the atrial myocardium by releasing adipokines, which promote inflammation and fibrosis **(Hatem & Sanders, 2014)**.

Recent research has revealed new insights into the effects of cardiac adipose tissue, such as epicardial fat, which is a powerful predictor of AF and leads to myocardial fatty infiltration and adipokine-induced fibrosis. Weight loss leads to a regression of adiposity related fibrosis, structural abnormalities, conduction abnormalities and reduction in AF burden. As a result, weight loss and risk factor treatment are now established pillars of AF management **(Mahajan & Wong, 2021)**.

In Figure 3.11, the results showed that there were statistically significant differences between the groups of blood pressure (BP); the group with hypertension (HTN) had higher levels of S.PICP and S.CITP than the group with elevated blood pressure and the result is consistent with Ravassa *et al.,* who reported that levels of PICP and the ratio of serum carboxyterminal telopeptide of collagen type I (CITP) to MMP1 corresponding to severe collagen deposition and increased collagen crosslinking respectively, allow the stratification of patients with HF attributable to hypertensive heart disease (**Ravassa et al., 2017)**. Similarly, research defined by Ferreira *et al*. (2019) states that the serum level of PICP were significantly higher in patients with hypertension before drug treatment (**Ferreira et al., 2019)**. Another study reported that increased serum CITP and PICP levels were associated with hypertensive left ventricular fibrosis and diastolic dysfunction (**Lindsay, Maxwell & Dunn, 2002**)**.** However, unlike research defined by Lopez *et al*. (2012), which states that PICP level decreased in patients with hypertensive heart failure who received torasemide treatment **(López et al., 2012**). Another study reported that

reduced plasma CITP level were linked to fibrosis in one study with essential hypertension patients (**Laviades et al., 1998**).

The reason is that hypertension can affect collagen turnover, as it is associated with enlarged heart muscle cells and increased ventricular wall stiffness, which means increased collagen fibres. As a result, patients with latent hypertension show higher levels of COL1 synthesis markers such as PICP (**Nikolov & Popovski, 2022)**. Hypertension (HT) confers the highest population attributable risk among factors leading to atrial fibrillation (AF). Atrial remodelling due to hypertension is progressive but also reversible. However, inhibition of the renin-angiotensin-aldosterone system has shown the greatest promise in improving AF outcomes **(Lau, Shenasa & Shenasa, 2021).** Similarly, prolonged myocardial stress due to hypertension and other risk factors is thought to increase ECM deposition and lead to fibrosis that may compromise myocardial function and impair electrical conduction favouring the advent of arrhythmias and HF; Collagen synthesis is a dynamic process involving metabolically active myofibroblasts **(Condorelli, Jotti & Pagiatakis, 2016**).

The results revealed insignificant differences ($P>0.05$) in cholesterol, TG, HDL, VLDL and LDL in AF group compared to the control group in Fig. 3.12; This result is consistent with a study conducted in the USA at the University of Minnesota, which showed no significant differences in the distribution of lipid profiles between AF patients and a control group (**Duprez et al., 2018**). Similarly, the result was consistent with previous studies that showed no significant differences in lipid profiles between atrial fibrillation patients and the control group (**Barasch et al., 2011)**.

However, the results were inconsistent with the Chinese study conducted at Anzhen Hospital, which showed that blood lipid levels were lower in patients with atrial fibrillation **(Li et al., 2018)**. As well, unlike research defined by Duprez *et al.* (2018), which states that total cholesterol

and HDL cholesterol was lower at a higher S.CITP level **(Duprez et al., 2018)**. The reason is that the predisposition to hypertrophic cardiomyopathy is due to low plasma levels of HDL-C **(Schillaci et al., 2001; Velagaleti et al., 2009)**, which leads to an increased risk of atrial fibrillation due to structural changes in the atria rather than changes in lipid profiles. A recent study showed that patients with atrial fibrillation had lower levels of TC, LDL-C and HDL-C than those who did not develop atrial fibrillation and that low HDL-C levels predicted atrial fibrillation (**Barkas et al., 2017**).

Also, there was a high in mean serum vitamin C (S.VC) in the control group compared to the AF group, as shown in Table 3.1 & Figure 3.13. This high in S.VC leads to an elevation of both markers S.PICP and S.CITP in some individuals of healthy group. However, an increase in levels for both markers S.PICP and S.CITP in the AF group represents myocardial interstitial fibrosis (MIF), as in Fig. 3.14, which agrees with previous studies indicating that vitamin C stimulates hydroxylation, which acts to form collagen fibres by binding together amino acids, such as proline and lysine (**Peterkofsky, 1991; Yamauchi & Sricholpech, 2012**). Fibrillar collagens which comprise at least 90% of the total mass of mammalian collagen are initially synthesized as single strands called procollagens **(Di Lullo et al., 2002**). Procollagens convert to mature collagen fibres by hydroxylated (**Gorres & Raines, 2010**). Under physiological conditions, fibroblasts take up the required proline and lysine to synthesize procollagen, but only a very small amount of procollagen peptides maintain the structure of ECM and most of the procollagen peptides are degraded. Major types of procollagen are implicated in the elevated levels of S.PICP & S.CITP **(Kikuchi, Tanno & Kobayashi, 2012)**.

The reason is that clinical and preclinical data indicated that changes in the intensity of collagen biosynthesis resulted from regulation by ascorbic acid (**Phillips, Tajima & Pinnell, 1992**). Further research found

that S.PICP and S.CITP are involved in myocardial fibrosis (MF). Under pathological conditions, the degradation of type-I collagen decreases and leads to an imbalance of the type-I collagen proportion resulting in myocardial fibrosis **(Li et al., 2022).**

Table 3.2 & Figure 3.15 illustrates the distribution of S.PICP and S.CITP values; both indicators were significantly higher in AF cases compared to the healthy control group. The result is consistent with the research defined by Swartz *et al*. (2012), which states that the S.PICP and S.CITP were elevated in the patient group compared to the control group (**Swartz et al., 2012**). Similarly, the result agrees with a Finlandia study conducted on 70 patients with atrial fibrillation that found the C-terminal telopeptide of collagen type-I (S.CITP) was significantly higher in atrial fibrillation patients than in the control group (**Kallergis et al., 2008**).

Figure 3.16 illustrates that the mean level of S.PICP was significantly higher in all AF groups compared to the control group ($P \leq 0.05$). The finding was consistent with another study that found that the increase in serum PICP was higher in patients with paroxysmal, persistent and permanent atrial fibrillation **(Tziakas et al., 2007; Kallergis et al., 2008**). These results also agree with a study conducted at the University of Navarra Clinic in Spain on 150 patients with atrial fibrillation that found a high serum carboxyterminal propeptide of procollagen type-I (PICP) level that reflects excessive myocardial collagen type-I deposition (CD+) of circulating biomarkers is associated with the prevalence and incidence of atrial fibrillation in heart failure patients and that increased collagen type-I deposition leads to myocardial interstitial fibrosis **(Ravassa et al., 2019)**. This statement is substantiated by Boldt *et al.* (2004) in their report. Similarly, the mean level of S.CITP was significantly higher in all AF groups than in the control group ($P \leq 0.05$), as shown in Fig. 3.16. This finding is consistent with a study conducted in the USA at Loyola University Medical

Center, which showed that circulating collagen proteins S.CITP were significantly elevated in all patients with atrial fibrillation compared to the healthy group (**Odeh et al., 2023)**. Likewise, research defined by Tziakas *et al.* (2007) states that serum CITP level were significantly (P = 0.001) higher in the AF group than in the normal sinus rhythm (SR) group (**Tziakas et al., 2007**).

The present study revealed that the mean level of the marker of collagen synthesis to collagen degradation ratio was only significant between persistent and permanent AF compared to the control ($P \leq 0.05$), as presented in Fig. 3.16, While the mean level of the S.PICP/S.CITP ratio for the paroxysmal and lone atrial fibrillation group was not significantly different from the control group($P>0.05$). Unfortunately, the previous study did not measure markers of the S.PICP/S.CITP ratio may be one reason for the observed differences between types of AF patients.

The reason for this is that only the persistent and permanent atrial fibrillation groups have statistical significance because the disease duration lasts a long time and is linked to other health problems, unlike the cases in the paroxysmal and lone atrial fibrillation groups.

Figure 3.18 shows the parameters of the echocardiogram study that categorized the left atrial (LA) enlargement groups and the mean of the prognostic biomarker of these groups revealed highly significant between groups enlargement differences for LA patients. The group with mild enlargement in the LA had higher levels of S.PICP and S.CITP than the group with moderate to severe enlargement. This result was consistent with the Chinese study performed in the hospital of Nanjing Medical University, which showed that serum PICP had been found to correlate with the presence of left atrial (LA) fibrosis in AF patients (**Zhao et al., 2014**). Additionally, S.CITP is involved in the process of LA fibrosis in AF **(Kallergis et al., 2008)**. The reason is that fibroblasts increase collagen accumulation, which plays a role in atrial electrical activity and structural remodelling (**Gurses et**

al., 2019). Fibrosis has been linked to the pathophysiology of atrial fibrillation with atrial fibrosis causing conduction disturbances and is a central component of atrial remodelling in atrial fibrillation. Cardiac fibroblasts are the cells responsible for fibrosis formation and are activated by risk factors (HTN, HF and obesity). Thus, fibrosis contributes to atrial fibrillation. Managing risk factors and fibrotic conditions is essential to suppress atrial fibrosis and atrial fibrillation risk **(Harada & Nattel, 2021)**.

Figure 3.18 showed that the parameters of the echocardiogram study classified left ventricular dilatation groups with a thick wall and the mean predicted biomarker of these groups revealed highly significant differences between dilatation groups of left ventricular (LV) patients; the group with moderate to severe dilation in the LV with a thick wall had higher levels of S.PICP and S.CITP than the group with mild dilation with a thick wall. The result was consistent with the Chinai study that was performed in Fuwai Hospital (Beijing), which showed that serum S.PICP level was elevated in patients with hypertrophic cardiomyopathy (HCM) (**Yang et al., 2019**). In contrast, a study by Lombardi *et al*. showed that serum CITP level was elevated in patients with HCM (**Lombardi et al., 2003**).

However, it conflicts with a previous study report that found elevated serum marker of collagen metabolism at S.CITP concentrations in peripheral venous blood samples in patients with mild to moderate heart failure due to dilated cardiomyopathy (DCM) and a slight increase in S.PICP level was found. However, the differences in S.PICP were not statistically significant **(Schwartzkopff et al., 2002)**. Similarly, Investigations of S.PICP in patients with HCM and cardiomyopathies show elevated serum PICP in patients with mild to moderately dilated cardiomyopathy **(González et al., 2019).** The reason is that myocardial stiffness or fibrosis is caused by increased collagen deposition, which increases S.CITP level **(Morine et al., 2016)**.

Figure 3.18, the mean of the predicted biomarkers determined by echocardiogram study parameters shows highly significant differences between the ejection fraction (EF%) patient groups; the group with moderate to severe dysfunction in the EF% had higher levels of S.PICP and S.CITP than the group with mild dysfunction. This result was consistent with previous literature findings that serum PICP level were associated with the severity of HFrEF, with mortality in HFpEF and HFrEF **(Löfsjögård et al., 2017).** It conflicts with previously reported data that the values of S.PICP were lower in HFpEF patients (**Ravassa et al., 2018**).

4.3 Odds Ratio between Biochemical Markers and Echocardiography Study

Table 3.5 showed significant independent risk factors for left ventricular ($P \leq 0.05$) in S.PICP level when compared with the control group; the result was consistent with a study conducted in the USA at Texas Tech University on 40 patients with left ventricular (LV) diastolic dysfunction, which showed that serum PICP proved to be a powerful predictor of restrictive like filling as seen in the most severe form of LV in HFpEF and HFrEF **(Martos et al., 2007; Roongsritong et al., 2008)**.

While There were no significant differences ($P > 0.05$) in the OR of S.PICP level between the mild left ventricle dilatation group and the control group. Furthermore, Table 3.3 there were no significant differences in the OR of the S.CITP level ($P > 0.05$) among AF patients with mild, moderate, and severe left ventricular dilatation compared to the control group.This outcome is in line with the study by Lombardi *et al*. (2003), which claims that patients with LV dilated cardiomyopathy did not have significantly different serum S.PICP and S.CITP levels (**Lombardi et al., 2003)**. On the contrary, this finding is inconsistent with previous reports of elevated S.CITP value in patients with mild to moderate cardiomyopathy (**Schwartzkopff et al., 2002**).

4.4 Receiver Operating Characteristic Curve (ROC-Curve) and Area Under Curve Score (AUC-Score)

The current study revealed that measuring serum PICP (at a cut-off value of > 157 *ng/mL*) and CITP (at a cut-off value of > 165.5 *ng/mL*) were the best biomarkers for distinguishing myocardium interstitial fibrosis (MIF) with atrial fibrillation (AF) patients from the healthy group (Table 3.3 and Fig. 3.17). These biomarkers have a higher area under the curve than their ratio of S.PICP/S.CITP. Results contribute to the theory of collagen deposition as a major mechanism involved in MIF with atrial fibrillation patients. These results are consistent with previous studies showing that elevated S.CITP may facilitate the diagnosis or prognosis of myocardial fibrosis **(Ding et al., 2020)**. This result is also consistent with the study conducted at the hospital of Nanjing Medical University in China on 90 patients with atrial fibrillation. It was found that S.PICP is a sensitive biomarker of fibrosis in patients with atrial fibrillation **(Zhao et al., 2014).** Also, this statement is corroborated by López *et al*. (2015) in their report. That is interpreted as serum PICP and CITP being the best biomarkers for differentiating between patients and controls.

Results also confirmed that left ventricular diameter has more sensitivity and specificity than left atrial diameter in early detecting co-extension of myocardial interstitial fibrosis (MIF) in atrial fibrillation patients (Table 3.6 & Fig. 3.19). So, these results made the left ventricular diameter more useful than the left atrial diameter. The left ventricular diameter was more useful in detecting early coextension of myocardial interstitial fibrosis (MIF) in atrial fibrillation patients.

Table 3.8 shows that the clinical biomarkers proposed have higher sensitivity, specificity and accuracy than echocardiography findings in the early detection of MIF in patients with atrial fibrillation. These results are consistent with previous studies showing that an echocardiographic study

does not reliably identify myocardial fibrosis in patients with hypertrophic cardiomyopathy (HCM). Still, it is reliably diagnosed by magnetic resonance imaging (MRI). An echocardiographic study may be useful in preidentifying those who benefit from functional magnetic resonance imaging (fMRI) to confirm the finding of myocardial scarring. MRI: one of its advantages is its high diagnostic capacity, but it is time consuming, expensive, requires intravenous contrast injection and is only sometimes readily available. Therefore, echocardiographic studies cannot substitute for MRI (**Compton et al., 2016)**. That is interpreted as serum PICP and CITP being the best predictive biomarkers of myocardial fibrosis because both have a sensitivity of 86.7%, a specificity of 81.7%, a sensitivity of 93.2%, and a specificity of 83.3%, respectively. These biomarkers are inexpensive and readily available.

There is a cutoff value for the LV to distinguish a left ventricle that showed a normal or abnormal diameter by echo study if the abnormality is classified according to the AAC as mild, moderate and severe. In contrast, S.CITP and S.PICP can improve the accuracy of LV diagnosis in these cases because they reflect myocardial structural fibrosis or LV remodelling rather than dilatation or diameter changes.

Table 3.8 shows the ROC curve that was carried out to assess the diagnostic performance of S.PICP and S.CITP and whether they can be used as novel diagnostic tests compared to echocardiography with almost the same sensitivity and specificity as echocardiography. The reason is that echocardiography remains the primary method for detecting underlying heart diseases such as atrial or ventricular dilatation, heart valve disease or left ventricular systolic dysfunction **(Bax, Marsan and Delgado, 2015)**.

The proposed biomarkers have advantages including a more sensitive, specific and accurate analysis than echocardiographic findings in diagnosing fibrosis because the proposed biomarkers are quantitative

methods that use serum PICP and CITP levels to determine atrial and ventricular fibrosis. Neither echocardiographic study technique can provide information regarding the spatial location of fibrous regions within the atria and ventricles (**Begg et al., 2016**).

These biomarkers are associated with the development of cardiac fibrosis by changing their concentration according to the degree of myocardial interstitial fibrosis. Because of their low molecular weight, most of these biomarkers are produced by cardiac myofibroblasts and accumulate in the interstitial myocardium. If the myocardium is fibrotic due to excess collagen deposition, it will increase biomarker levels and lead to myocardial stiffness or fibrosis, impaired contractility, LV dysfunction and electrical alteration; all of these reasons lead to atrial fibrillation. This subsequently leads to increased levels in the blood and urine as collagen is secreted within a few hours of the occurrence of myocardial interstitial fibrosis even before the onset of atrial fibrillation. Therefore, these biomarkers are promising for detecting myocardial interstitial fibrosis in its earliest stages.

Chapter Five

Conclusions and Recommendations

5.1. Conclusions

1. Serum PICP and CITP levels can indicate myocardial interstitial fibrosis in patients with atrial fibrillation.

2. Fibrosis represented by myocardial interstitial fibrosis and its risk factors significantly impacts serum PICP and CITP levels, while serum lipid profiles alone have no significant impact.

3. Serum PICP level with a cutoff value of more than 157 *ng/mL* may be an excellent biomarker for evaluating myocardial interstitial fibrosis in atrial fibrillation patients, with a sensitivity of 86.7% and a specificity of 81.7%. A serum CITP level with a cutoff value greater than 165.5 *ng/mL* can be used as an alternative biomarker for patients with atrial fibrillation independent of myocardial interstitial fibrosis with a sensitivity of 93.2% and a specificity of 83.3%.

4. The majority of atrial fibrillation patients have myocardial interstitial fibrosis and comorbidities such as obesity and hypertension.

5.2. Recommendations

1. Studying the role of serum PICP and CITP levels in the pathogenesis of atrial fibrillation will be needed to answer whether this role can be translated into therapeutic targets.
2. Study other biomarkers of collagen deposition like PIIICP, PINP, and PIIINP in a large sample size of patients with atrial fibrillation. So, this knowledge gives clinicians the ability to predict who will develop cardiomyopathy early and improve prevention of this devastating disease.

References

References:

Ahlberg, G., Refsgaard, L., Lundegaard, P.R. *et al*., 2018. 'Rare truncating variants in the sarcomeric protein titin associate with familial and early-onset atrial fibrillation'. *Nature communications,* 9(1), p.4316.

Akoum, N., Mahnkopf, C., Kholmovski, E.G. et al., 2018. 'Age and sex differences in atrial fibrosis among patients with atrial fibrillation'. *EP Europace*, 20(7), pp.1086-1092.

Alberts, B., Johnson, A., Walter, P. *et al., 2008.* 'Molecular biology of the cell', 5th edn. *Garland Science. New York.*

Alla, F., Kearney-Schwartz, A., Radauceanu, A. *et al*., 2006. 'Early changes in serum markers of cardiac extra-cellular matrix turnover in patients with uncomplicated hypertension and type II diabetes'. *European journal of heart failure*, 8(2), pp.147-153.

Alonso, A., Krijthe, B.P., Aspelund, T. *et al*., 2013. 'Simple risk model predicts incidence of atrial fibrillation in a racially and geographically diverse population: the CHARGE-AF consortium'. *Journal of the American Heart Association,* 2(2), p. e000102.

Whelton, P.K., Carey, R.M., Mancia, G. et al., 2022. 'Harmonization of the American College of Cardiology/American Heart Association and European Society of Cardiology/European Society of Hypertension blood pressure/hypertension guidelines: comparisons, reflections, and recommendations'. *European Heart Journal*, *43*(35), pp.3302-3311.

American College of Cardiology, 2019. 'Left ventricular ejection fraction LVEF assessment (outpatient setting) '.

Anderson, K.R., Sutton, M.G. and Lie, J.T., 1979. 'Histopathological Types Of Cardiac Fibrosis In Myocardial Disease'. *The Journal of pathology*, 128(2), pp. 79-85.

Andrade, J., Khairy, P., Dobrev, D. and Nattel, S., 2014. 'The clinical profile and pathophysiology of atrial fibrillation: relationships among clinical features, epidemiology, and mechanisms'. *Circulation research*, 114(9), pp.1453-1468.

Antman, EM., & Eugene, B., 1997. 'Text book of cardiovascular medicine. Philadelphia: W.B'. *Saunders Company*; p. 1208.

Attia, Z.I., Noseworthy, P.A., Lopez-Jimenez, F. *et al.* 2019. 'An Artificial Intelligence-enabled ECG Algorithm For The Identification Of Patients With Atrial Fibrillation During Sinus Rhythm: a retrospective analysis of outcome prediction'. *Lancet* 394(10201), pp. 861–867.

Barasch, E., Gottdiener, J.S., Aurigemma, G. *et al*., 2009. 'Association between elevated fibrosis markers and heart failure in the elderly: the cardiovascular health study'. *Circulation: Heart Failure*, 2(4), pp.303-310.

Barasch, E., Gottdiener, J.S., Aurigemma, G. *et al*., 2011. 'The relationship between serum markers of collagen turnover and cardiovascular outcome in the elderly: the Cardiovascular Health Study'. Circulation: Heart Failure, 4(6), pp.733-739.

Barison, A., Grigoratos, C., Todiere, G. *et al*., 2015. 'Myocardial interstitial remodelling in non-ischaemic dilated cardiomyopathy: insights from cardiovascular magnetic resonance'. *Heart failure reviews,* 20, pp.731-749.

Barkas, F., Elisaf, M., Korantzopoulos, P. *et al*., 2017. 'The CHADS2 and CHA2DS2-VASc scores predict atrial fibrillation in dyslipidemic individuals: role of incorporating low high-density lipoprotein cholesterol levels'. International Journal of Cardiology, 241, pp.194-199.

Bax, J.J., Marsan, N.A. and Delgado, V. 2015. 'Non-invasive imaging in atrial fibrillation: focus on prognosis and catheter ablation'. Heart, 101(2), pp.94-100.

Begg, G.A., Holden, A.V., Lip, G.Y. *et al*., 2016. 'Assessment of atrial fibrosis for the rhythm control of atrial fibrillation'. International journal of cardiology, 220, pp.155-161.

Benjamin, E.J., Muntner, P., Alonso, A. *et al*., 2019. 'Heart disease and stroke statistics—2019 update: a report from the American Heart Association'. *Circulation*, 139(10), pp. e56-e528.

Beyerbach, D.M. & Zipes, D.P., 2004. 'Mortality as an endpoint in atrial fibrillation'. *Heart Rhythm,* 1(2), pp.8-19.

Bidar, E., Maesen, B., Nieman, F. *et al*., 2014. 'A prospective Randomized Controlled Trial On The Incidence and Predictors Of late-Phase Postoperative Atrial Fibrillation Up To 30 Days and The Preventive Value Of Biatrial Pacing'. *Heart Rhythm*, 11(7), pp.1156-1162.

Boldt, A., Wetzel, U., Lauschke, J. *et al*., 2004. 'Fibrosis in left atrial tissue of patients with atrial fibrillation with and without underlying mitral valve disease'. Heart, 90(4), pp.400-405.

Brooks, A., Schinde, V., Bateman, A.C. *et al*., 2003. 'Interstitial Fibrosis In The Dilated Non-Ischaemic Myocardium'. *Heart,* 89(10), pp.1255-1256.

Burstein, B. & Nattel, S., 2008. 'Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation'. *Journal of the American College of Cardiology,* 51(8), pp.802-809.

Calkins, H., Hindricks, G., Cappato, R. *et al*., 2017. '2017 HRS/ EHRA /ECAS /APHRS/ SOLAECE Expert Consensus Statement On Catheter and Surgical Ablation Of Atrial Fibrillation'. *Heart Rhythm*.14(10), e275-e444.

Camm, A.J., Kirchhof, P., Lip, G.Y. *et al.* & European Heart Rhythm Association, 2010. 'Guidelines for the management of atrial fibrillation: the Task Force for the Management of Atrial Fibrillation of the European Society of Cardiology (ESC) '. *Eur Heart J,* 31(19), pp.2369-2429.

Carasso, S., Sandach, A., Beinart, R. *et al*., & Echocardiography Working Group of the Israel Heart Society, 2005. 'Usefulness of four echocardiographic risk assessments in predicting 30-day outcome in acute myocardial infarction'. *The American journal of cardiology*, 96(1), pp.25-30.

Caulfield, J.B. & Janicki, J.S., 1997. 'Structure and function of myocardial fibrillar collagen'. *Technology and Health Care,* 5(1-2), pp.95-113.

Chen, Y., Wang, X., Jung, Y. *et al*., 2018.'Classification of short single-lead electrocardiograms (ECGs) for atrial fibrillation detection using piecewise linear spline and XGBoost'. *Physiological measurement*, 39(10), p.104006.

Chiang, C.E., Wang, K.L. & Lip, G.Y., 2014. 'Stroke prevention in atrial fibrillation: an Asian perspective'. *Thrombosis and haemostasis,* 112(05), pp.789-797.

Cho, G.Y., Kim, H.K., Kim, Y.J. *et al*., 2010. 'Electrical and mechanical dyssynchrony for prediction of cardiac events in patients with systolic heart failure'. *Heart,* 96(13), pp.1029-1032.

Compton, G., Nield, L., Dragulescu, A. *et al*., 2016. 'Echocardiography as a screening test for myocardial scarring in children with hypertrophic cardiomyopathy'. *International journal of pediatrics*, 2016.

Condorelli, G., Jotti, G.S. and Pagiatakis, C., 2016. 'Fibroblast senescence as a therapeutic target of myocardial fibrosis: beyond spironolactone? '. Journal of the American College of Cardiology, 67(17), pp.2029-2031.

Corelaboratory, 2018. 'Abbott Architect-c4000, p. 2251'.

Cowling, R.T., Kupsky, D., Kahn, A.M. *et al*., 2019. 'Mechanisms of cardiac collagen deposition in experimental models and human disease'. *Translational Research,* 209, pp.138-155.

Csepe, T.A., Kalyanasundaram, A., Hansen, B.J. *et al*., 2015. 'Fibrosis: a structural modulator of sinoatrial node physiology and dysfunction'. *Frontiers in physiology,* 37(6).

Daniel, W.W. & Cross, C.L., 2018. 'Biostatistics: a foundation for analysis in the health sciences'. *Wiley*.

de Jong, S., van Veen, T.A., de Bakker, J.M. *et al*., (2011). '*Biomarkers of myocardial fibrosis*'. *Journal of cardiovascular pharmacology*, 57(5), pp.522-535.

Di Carlo, A., Bellino, L., Consoli, D. *et al*., 2019. 'National Research Program: Progetto FAI. La Fibrillazione Atriale in Italia. Prevalence of atrial fibrillation in the Italian elderly population and projections from 2020 to 2060 for Italy and the European Union: the FAI Project**'**. *Europace*, 21(10), pp.1468-1475.

Di Lullo, G.A., Sweeney, S.M., Korkko, J. *et al*., 2002. 'Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen'. *Journal of Biological Chemistry*, 277(6), pp.4223-4231.

DIALAB, GmbH., 2019. 'DIAReader ELX8OOG and DIAWasher ELX5O/8'.

Díez, J., González, A. & Kovacic, J.C., 2020. 'Myocardial interstitial fibrosis in nonischemic heart disease, part 3/4: JACC focus seminar'. *Journal of the American College of Cardiology*, 75(17), pp.2204-2218.

Díez, J., Panizo, A., Gil, M.J. *et al*., 1996. 'Serum markers of collagen type I metabolism in spontaneously hypertensive rats: relation to myocardial fibrosis'. *Circulation,* 93(5), pp.1026-1032.

Díez, J., Querejeta, R., López, B. *et al*., 2002. ' Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients**'**. *Circulation,* 105(21), pp.2512-2517.

Dilaveris, P.E. & Kennedy, H.L., 2017. 'Silent atrial fibrillation: epidemiology, diagnosis, and clinical impact'. *Clinical cardiology*, 40(6), pp.413-418.

Dillon, P. & Ghanbari, H., 2014. 'Diagnostic evaluation and follow-up of patients with atrial fibrillation'. *Cardiology Clinics*, 32(4), pp.507-519.

Dorian, P., Jung, W., Newman, D. *et al*., 2000. 'The impairment of health-related quality of life in patients with intermittent atrial fibrillation: implications for the assessment of investigational therapy'. *Journal of the American College of Cardiology*, 36(4), pp.1303-1309.

Dunlay, S.M., Roger, V.L., Weston, S.A. *et al*., 2012. 'Longitudinal changes in ejection fraction in heart failure patients with preserved and reduced ejection fraction'. *Circulation: Heart Failure,* 5(6), pp.720-726.

Duprez, D.A., Heckbert, S.R., Alonso, A. *et al*., 2018. 'Collagen biomarkers and incidence of new onset of atrial fibrillation in subjects with no overt cardiovascular disease at baseline: the multi-ethnic study of atherosclerosis'. Circulation: Arrhythmia and Electrophysiology, 11(10), p. e006557.

Eghbali, M., Czaja, M.J., Zeydel, M. *et al*., 1988. 'Collagen chain mRNAs in isolated heart cells from young and adult rats'. *Journal of molecular and cellular cardiology, 20(3), pp.267-276.*

Eschalier, R., Rossignol, P., Kearney-Schwartz, A. *et al*., 2014. 'Features of cardiac remodelling, associated with blood pressure and fibrosis biomarkers, are frequent in subjects with abdominal obesity'. Hypertension, 63(4), pp.740-746.

Expert Panel on Detection, E., 2001. 'Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III)'. *Jama*, 285(19), pp.2486-2497.

Fahy, G.J., Pinski, S.L., Miller, D.P. *et al*., 1996. 'Natural history of isolated bundle branch block'. *The American journal of cardiology*, 77(14), pp.1185-1190.

Falcao-Pires, I., Hamdani, N., Borbély, A. *et al*., 2011. 'Diabetes Mellitus Worsens Diastolic Left Ventricular Dysfunction In Aortic Stenosis Through

Altered Myocardial Structure and Cardiomyocyte Stiffness'. Circulation, 124(10), pp.1151-1159.

Fan, D., Takawale, A., Lee, J. *et al*., 2012. 'Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease'. *Fibrogenesis &Tissue Repair,* 5-15.

Fan, X., Yao, Q., Cai, Y. *et al*. 2018. 'Multiscaled Fusion Of Deep Convolutional Neural Networks For Screening Atrial Fibrillation From Single Lead Short ECG Recordings'. IEEE J. Biomed. *Health Inform*, 22(6), pp.1744–1753.

Ferreira, J.P., Rossignol, P., Pizard, A. *et al*., 2019. 'Potential spironolactone effects on collagen metabolism biomarkers in patients with uncontrolled blood pressure'. Heart, 105(4), pp.307-314.

Frangogiannis, N.G., 2021. 'Cardiac fibrosis'. *Cardiovascular research, 117(6), pp.1450-1488.*

Friedewald, W.T., Levy, R.I. & Fredrickson, D.S., 1972. ' Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge'. *Clinical chemistry*, 18(6), pp.499-502.

Fuster, V., Ryden, L.E., Asinger, R.W. *et al*., 2001. 'American College of Cardiology/American Heart Association Task Force on Practice Guidelines; European Society of Cardiology Committee for Practice Guidelines and Policy Conferences (Committee to Develop Guidelines for the Management of Patients With Atrial Fibrillation); North American Society of Pacing and Electrophysiology. ACC/AHA/ESC Guidelines for the Management of Patients With Atrial Fibrillation: Executive Summary A Report of the American College of Cardiology/American Heart Association Task' *Circulation,* 104 (no): 2118-2150.

Fuster, V., Rydén, L.E., Cannom, D.S. *et al*. 2006. 'Acc/aha/esc 2006 guidelines for the management of patients with atrial fibrillation: A report of the american college of cardiology/american heart association task force on

practice guidelines and the european society of cardiology committee for practice guidelines (writing committee to revise the 2001 guidelines for the management of patients with atrial fibrillation): Developed in collaboration with the european heart rhythm association and the heart rhythm society'. *Circulation*, 114(7), pp. e257-e354.

Fuster, V., Rydén, L.E., Cannom, D.S. *et al*., (2006). 'ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation: full text: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Revise the 2001 guidelines for the management of patients with atrial fibrillation) developed in collaboration with the European Heart Rhythm Association and the Heart Rhythm Society'. *Europace,* 8(9), pp.651-745.

Gaasch, W.H., Delorey, D.E., Kueffer, F.J. *et al*., 2009. 'Distribution of left ventricular ejection fraction in patients with ischemic and hypertensive heart disease and chronic heart failure'. *The American journal of cardiology*, 104(10), pp.1413-1415.

Ganesan, P., Sterling, M., Ladavich, S. *et al*. 2016. '*Computer-aided clinical decision support systems for atrial fibrillation*'. *In Computer-aided Technologies-Applications in Engineering and Medicine*. Intech Open.

Garimella, R.S., Chung, E.H., Mounsey, J.P. *et al*. 2015. '*Accuracy Of Patient Perception Of Their Prevailing Rhythm: A Comparative Analysis Of Monitor Data and Questionnaire Responses In Patients With Atrial Fibrillation*'. *Heart Rhythm,* 12(4), pp.658-665.

Gillis, A.M., 2017. 'Atrial fibrillation and ventricular arrhythmias: sex differences in electrophysiology, epidemiology, clinical presentation, and clinical outcomes'. *Circulation*, 135(6), pp.593-608.

González, A., López, B., Querejeta, R. *et al*., 2010. 'Filling pressures and collagen metabolism in hypertensive patients with heart failure and normal ejection fraction'. *Hypertension*, 55(6), pp.1418-1424.

González, A., López, B., Ravassa, S. *et al*., 2019. 'The complex dynamics of myocardial interstitial fibrosis in heart failure'. Focus on collagen cross-linking. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1866(9), pp.1421-1432.

González, A., Schelbert, E.B., Díez, J. *et al.*, 2018. '*Myocardial interstitial fibrosis in heart failure: biological and translational perspectives*'**.** *Journal of the American College of Cardiology*, 71(15), pp.1696-1706.

Gorres, K.L. & Raines, R.T., 2010. 'Prolyl 4-hydroxylase'. *Critical reviews in biochemistry and molecular biology*, 45(2), pp.106-124.

Gurses, K.M., Yalcin, M.U., Kocyigit, D. *et al*., 2019. 'Serum galectin-3 level predicts early recurrence following successful direct-current cardioversion in persistent atrial fibrillation patients'. *Türk Kardiyoloji Derneği Arşivi*, *47*(7).

Gyöngyösi, M., Winkler, J., Ramos, I. *et al*., 2017. 'Myocardial fibrosis: biomedical research from bench to bedside'. *European journal of heart failure*,19(2), pp.177-191.

Hall, J. E. (2015). *The Heart',* Hall, J. E.(ed.). '*Pocket Companion to Guyton & Hall Textbook of Medical Physiology*'.13ThEd. USA: patricia tannian. E-Book. *Elsevier Health Sciences*, pp.63-78.

Hansen, B.J., Zhao, J. & Fedorov, V.V., 2017. '*Fibrosis and atrial fibrillation: computerized and optical mapping: a view into the human atria at submillimeter resolution*'. *JACC: Clinical Electrophysiology, 3(6), pp.531-546.*

Harada, M. & Nattel, S., 2021. 'Implications of inflammation and fibrosis in atrial fibrillation pathophysiology'. *Cardiac electrophysiology clinics*, 13(1), pp.25-35.

Hatem, S.N. and Sanders, P., 2014. 'Epicardial adipose tissue and atrial fibrillation'. *Cardiovascular research*, 102(2), pp.205-213.

Heeringa, J., van der Kuip, D.A., Hofman, A. *et al*., 2006. 'Prevalence, incidence and lifetime risk of atrial fibrillation: the Rotterdam study'. *European heart journal*, 27(8), pp.949-953.

Heijman, J., Sutanto, H., Crijns, H.J. *et al*., 2021. 'Computational models of atrial fibrillation: Achievements, challenges, and perspectives for improving clinical care'. *Cardiovascular Research*, 117(7), pp.1682-1699.

Hersi, A.; & Wyse, D.G., 2005. '*Management of atrial fibrillation*'. *Current problems in cardiology,* 30(4), pp.175-233.

Hinderer, S., & Schenke-Layland, K., 2019. 'Cardiac fibrosis–A short review of causes and therapeutic strategies'. *Advanced drug delivery reviews*, 146, pp.77-82.

Hindricks, G., Potpara, T., Dagres, N. *et al*. 2021. '*2020. ESC Guidelines For The Diagnosis and Management Of Atrial Fibrillation Developed in Collaboration With The European Association Of Cardio-Thoracic Surgery (EACTS)* '. *Eur. Heart J*., 42(5): pp. 373-498.

Ho, C.Y., López, B., Coelho-Filho, O.R. *et al*., 2010. 'Myocardial fibrosis as an early manifestation of hypertrophic cardiomyopathy'. *New England Journal of Medicine*, 363(6), pp.552-563.

Hoyt, R.H., Ericksen, E., Collins, S.M. *et al*. 1984. 'Computer-assisted quantitation of myocardial fibrosis in histologic sections'. *Archives of pathology & laboratory medicine, 108(4), pp.280-283.*

January, C.T., Wann, L.S., Alpert, J.S. *et al*. 2014. '2014 AHA/ACC/HRS Guideline For The Management Of Patients With Atrial Fibrillation: A

Report Of The American College Of Cardiology/American Heart Association Task Force on Practice Guidelines and the Heart Rhythm Society'. *Journal of the American College of Cardiology*, 64(21), e1-e76.

Järveläinen, H., Sainio, A., Koulu, M. et al. 2009. 'Extracellular matrix molecules: potential targets in pharmacotherapy'. *Pharmacological reviews, 61(2), pp.198-223.*

Jenkins, G., Tortora, G.J., 2016. '*Anatomy and physiology*'. John Wiley & Sons.

Jiang, Q., Yang, L., Chen, M.L. et al., 2022. 'Lipid Profile and Atrial Fibrillation: Is There Any Link?' *Reviews in Cardiovascular Medicine*, 23(8), p.272.

Kallergis, E.M., Manios, E.G., Kanoupakis, E.M. *et al*., 2008. 'Extracellular matrix alterations in patients with paroxysmal and persistent atrial fibrillation: biochemical assessment of collagen type-I turnover'. *Journal of the American College of Cardiology*, 52(3), pp.211-215.

Kamel, H., Lees, K.R., Lyden, P.D. *et al*. & Virtual International Stroke Trials Archive Investigators., 2009. 'Delayed detection of atrial fibrillation after ischemic stroke'. *Journal of Stroke and Cerebrovascular Diseases*, 18(6), pp.453-457.

Karaca, O., Cakal, B., Omaygenc, M.O.*et al*., 2016. 'Native electrocardiographic QRS duration after cardiac resynchronization therapy: The impact on clinical outcomes and prognosis'. *Journal of Cardiac Failure*, 22(10), pp.772-780.

Kawamura, M., Munetsugu, Y., Kawasaki, S. *et al*., 2012. 'Type III procollagen-N-peptide as a predictor of persistent atrial fibrillation recurrence after cardioversion'. *Europace*, 14(12), pp.1719-1725.

Kirchhof, P., benussi s, Kotecha d, *et al*., 2016 '*esC guidelines for the management of atrial fibrillation developed in collaboration with eACTs*'. *eur heart J.* 37: 2893-962.

Kitahara, T., Takeishi, Y., Arimoto, T. et al., 2007. 'Serum carboxy-terminal telopeptide of type I collagen (ICTP) predicts cardiac events in chronic heart

failure patients with preserved left ventricular systolic function'. *Circulation Journal*, 71(6), pp.929-935.

Ko, D., Rahman, F., Schnabel, R.B. *et al*., 2016. 'Atrial fibrillation in women: epidemiology, pathophysiology, presentation, and prognosis'. *Nature Reviews Cardiology*, 13(6), pp.321-332.

Kobayashi, M., Girerd, N., Ferreira, J.P. *et al*., 2022. 'The association between markers of type I collagen synthesis and echocardiographic response to spironolactone in patients at risk of heart failure: findings from the HOMAGE trial'. *European journal of heart failure*, 24(9), pp.1559-1568.

Kolek, M.J., Graves, A.J., Xu, M. *et al*., 2016. 'Evaluation of a prediction model for the development of atrial fibrillation in a repository of electronic medical records'. *JAMA cardiology*, 1(9), pp.1007-1013.

Kong, P., Christia, P. & Frangogiannis, N.G., 2014. '*The pathogenesis of cardiac fibrosis*'. *Cellular and molecular life sciences*, 71, pp.549-574.

Kornej, J. & Smith, Ben., 2020. 'Epidemiology of atrial fibrillation in the 21st century: novel methods and new insights'. *Circulation research*, 127(1), pp.4-20.

Kosmala, W., Przewlocka-Kosmala, M., Szczepanik-Osadnik, H. *et al*., 2013. 'Fibrosis and cardiac function in obesity: a randomised controlled trial of aldosterone blockade'. *Heart*, 99(5), pp.320-326.

Krijthe, B.P., Kunst, A., Benjamin, E.J. *et al*., 2013. 'Projections on the number of individuals with atrial fibrillation in the European Union, from 2000 to 2060'. *European heart journal*, 34(35), pp.2746-2751.

Kumar, S., Stevenson, W.G. & John, R.M., 2015. 'Arrhythmias in dilated cardiomyopathy'. *Card Electrophysiol Clin* 7: 221–233.

Lang, R.M. et al., 2006. 'Recommendations for chamber quantification'. *European journal of echocardiography*, 7(2), pp.79-108.

Lang, R.M., Bierig, M., Devereux, R.B. *et al*., 2005. 'Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology'. *Journal of the American society of echocardiography*, 18(12), pp.1440-1463.

Lau, D.H., Shenasa, H.A. & Shenasa, M., 2021. 'Hypertension, prehypertension, hypertensive heart disease, and atrial fibrillation'. *Cardiac Electrophysiology Clinics*, 13(1), pp.37-45.

Laviades, C., Varo, N., Fernández, J. *et al*., 1998. 'Abnormalities of the extracellular degradation of collagen type I in essential hypertension'. *Circulation*, 98(6), pp.535-540.

Li, G., Yang, J., Zhang, D. *et al*., 2022. 'Research progress of myocardial fibrosis and atrial fibrillation'. *Frontiers in Cardiovascular Medicine*, 9, p.889706.

Li, X., Gao, L., Wang, Z. *et al*., 2018. 'Lipid profile and incidence of atrial fibrillation: a prospective cohort study in China'. *Clinical cardiology*, 41(3), pp.314-320.

Li, Z.Z., Du, X., Guo, X.Y. *et al*., 2018. 'Association between blood lipid profiles and atrial fibrillation: a case-control study'. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, 24, p.3903.

Lijnen, P.J., Petrov, V.V. & Fagard, R.H., *2000.* '*Induction of cardiac fibrosis by transforming growth factor-β1*'. *Molecular genetics and metabolism, 71(1-2), pp.418-435.*

Lindsay, M.M., Maxwell, P. and Dunn, F.G., 2002. 'TIMP-1: a marker of left ventricular diastolic dysfunction and fibrosis in hypertension'. Hypertension, 40(2), pp.136-141.

Lloyd-Jones, D.M., Wang, T.J., Leip, E.P. *et al*., 2004. 'Lifetime risk for development of atrial fibrillation: the Framingham Heart Study'. *Circulation*, 110(9), pp.1042-1046.

Löfsjögård, J., Kahan, T., Díez, J., López, B. et al., 2017. 'Usefulness of collagen carboxy-terminal propeptide and telopeptide to predict disturbances of long-term mortality in patients≥ 60 years with heart failure and reduced ejection fraction'. *The American Journal of Cardiology*, 119(12), pp.2042-2048.

Lombardi, R., Betocchi, S., Losi, M.A. *et al*., 2003. 'Myocardial collagen turnover in hypertrophic cardiomyopathy'. *Circulation*, 108(12), pp.1455-1460.

López, B., González, A. & Díez, J., 2010. 'Circulating biomarkers of collagen metabolism in cardiac diseases'. *Circulation,*121(14):1645-54.

López, B., González, A., Ravassa, S. *et al*., 2015. 'Circulating biomarkers of myocardial fibrosis: the need for a reappraisal'. *Journal of the American College of Cardiology*, 65(22), pp.2449-2456.

López, B., Querejeta, R., González, A. *et al*., 2004. 'Effects of loop diuretics on myocardial fibrosis and collagen type I turnover in chronic heart failure'. *Journal of the American College of Cardiology,* 43(11), pp.2028-2035.

López, B., Querejeta, R., González, A. *et al*., 2012. 'Collagen cross-linking but not collagen amount associates with elevated filling pressures in hypertensive patients with stage C heart failure: potential role of lysyl oxidase'. Hypertension, 60(3), pp.677-683.

López, B., Ravassa, S., Moreno, M.U. *et al*., 2021. 'Diffuse myocardial fibrosis: mechanisms, diagnosis and therapeutic approaches'. *Nature Reviews Cardiology,* 18(7), pp.479-498.

Lukashev, M.E & Werb, Z., 1998. 'ECM signalling: orchestrating cell behaviour and misbehaviour'. *Trends in cell biology*, 8(11), pp.437-441.

Ma, S., Ma, J., Tu, Q. *et al*., 2020. 'Isoproterenol increases left atrial fibrosis and susceptibility to atrial fibrillation by inducing atrial ischemic infarction in rats'. *Frontiers in Pharmacology,* 11, p.493.

Mahajan, R. & Wong, C.X., 2021. 'Obesity and metabolic syndrome in atrial fibrillation: cardiac and noncardiac adipose tissue in atrial fibrillation'. *Cardiac electrophysiology clinics*, 13(1), pp.77-86.

Manhenke, C., Ørn, S., Squire, I. *et al*., 2011. 'The prognostic value of circulating markers of collagen turnover after acute myocardial infarction'. *International journal of cardiology,* 150(3), pp.277-282.

Martos, R., Baugh, J., Ledwidge, M. *et al*., 2007. 'Diastolic heart failure: evidence of increased myocardial collagen turnover linked to diastolic dysfunction'. *Circulation,* 115(7), pp.888-895.

Martos, R., Baugh, J., Ledwidge, M. *et al*., 2009. 'Diagnosis of heart failure with preserved ejection fraction: improved accuracy with the use of markers of collagen turnover'. *European journal of heart failure*, 11(2), pp.191-197.

McKleroy, W., Lee, T.H. & Atabai, K., 2013. 'Always cleave up your mess: targeting collagen degradation to treat tissue fibrosis'. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 304(11), pp. L709-L721.

Milanesi, R., Bucchi, A. & Baruscotti, M., 2015. 'The Genetic Basis For Inherited Forms Of Sinoatrial Dysfunction and Atrioventricular Node Dysfunction'. *Journal of Interventional Cardiac Electrophysiology*, 43(2), pp. 121-134.

Miyasaka, Y., Barnes, M.E., Gersh, B.J. et al., 2006. 'Secular trends in incidence of atrial fibrillation in Olmsted County, Minnesota, 1980 to 2000, and implications on the projections for future prevalence'. *Circulation*, 114(2), pp.119-125.

Moore, L., Fan, D., Basu, R. *et al*., 2012. 'Tissue inhibitor of metalloproteinases (TIMPs) in heart failure'. *Heart failure reviews, 17, pp.693-706.*

Morine, K.J., Paruchuri, V., Qiao, X. et al., 2016. 'Circulating multimarker profile of patients with symptomatic heart failure supports enhanced fibrotic degradation and decreased angiogenesis'. *Biomarkers,* 21(1), pp.91-97.

Mou, L., Norby, F.L., Chen, L.Y. *et al*., 2018. 'Lifetime risk of atrial fibrillation by race and socioeconomic status: ARIC Study (Atherosclerosis Risk in Communities)'. *Circulation: Arrhythmia and Electrophysiology*, 11(7), p. e006350.

Nagao, K., Inada, T., Tamura, A. *et al*., 2018. 'Circulating markers of collagen types I, III, and IV in patients with dilated cardiomyopathy: relationships with myocardial collagen expression'. *ESC heart failure*, 5(6), pp.1044-1051.

National Heart, Lung & Blood Institute., 2021. '*Arrhythmia*'. Available from: https://www.nhlbi.nih.gov/health-topics/arrhythmia. Accessed [May 21, 2021].

Nattel, S. & Opie, L.H., 2006. '*Controversies in atrial fibrillation*'. *The Lancet,* 367(9506), pp.262-272.

Nattel, S.; Li, D. & Yue, L., 2000. '*Basic mechanisms of atrial fibrillation—very new insights into very old ideas*'. *Annual Review of Physiology,* 62(1), pp.51-77.

Nikolov, A. & Popovski, N., 2022. 'Extracellular matrix in heart disease: Focus on circulating collagen type I and III derived peptides as biomarkers of myocardial fibrosis and their potential in the prognosis of heart failure: A concise review'. *Metabolites,* 12(4), p.297.

Oleiwi, E.A., Rasheed, M.K. & Hussein, K.G., 2022. 'Assessment of Total Procollagen Type 1 Intact N-terminal Propeptide, C-telopeptide of type 1 collagen, Bone Mineral Density and its Relationship to Body Mass Index in Men with Type 2 Diabetes'. *Journal of the Faculty of Medicine*, 64(2).

Pan, Z., Ai, T., Chang, P.C. et al., 2019. '*Atrial fibrillation and electrophysiology in transgenic mice with cardiac-restricted overexpression of FKBP12*'. *American Journal of Physiology-Heart and Circulatory Physiology,* 316(2), pp.H371-H379.

Peate, Ian. & Nalr, Muralitharan. (eds). 2017. '*The cardiac system*'. *In Clare, Carl. (ed.).* '*Fundamentals of Anatomy and Physiology*': *For Nursing and Healthcare Students.* 2nd Ed, UK: John Wiley & Sons, pp:224

Peterkofsky, B., 1991. 'Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy'. *The American journal of clinical nutrition*, 54(6), pp.1135S-1140S.

Phillips, C.L., Tajima, S. & Pinnell, S.R., 1992. 'Ascorbic acid and transforming growth factor-β1 increase collagen biosynthesis via different mechanisms: coordinate regulation of proα1 (I) and proα1 (III) collagens'. *Archives of biochemistry and biophysics*, 295(2), pp.397-403.

Plaksej, R., Kosmala, W., Frantz, S., *et al*., 2009. 'Relation of circulating markers of fibrosis and progression of left and right ventricular dysfunction in hypertensive patients with heart failure'. *Journal of hypertension*, 27(12), pp.2483-2491.

Querejeta, R., López, B., González, A. *et al*., 2004. 'Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis'. *Circulation*, 110(10), pp.1263-1268.

Querejeta, R., Varo, N., López, B. *et al*., 2000. 'Serum carboxy-terminal propeptide of procollagen type I is a marker of myocardial fibrosis in hypertensive heart disease'. *Circulation,* 101(14), pp.1729-1735.

Raafs, A.G., Verdonschot, J.A., Henkens, M.T. *et al*., 2021. 'The combination of carboxy-terminal propeptide of procollagen type I blood levels and late gadolinium enhancement at cardiac magnetic resonance provides additional prognostic information in idiopathic dilated cardiomyopathy–A multilevel

assessment of myocardial fibrosis in dilated cardiomyopathy'. *European journal of heart failure*, 23(6), pp.933-944.

Rahman, F., Kwan, G.F. & Benjamin, E.J., 2016. 'Global epidemiology of atrial fibrillation'. *Nature Reviews Cardiology*, 13(8), pp.501-501.

Ravassa, S., Ballesteros, G., López, B. *et al*., 2019. 'Combination of circulating type I collagen-related biomarkers is associated with atrial fibrillation'. *Journal of the American College of Cardiology*, 73(12), pp.1398-1410.

Ravassa, S., López, B., Querejeta, R. *et al*., 2017. 'Phenotyping of myocardial fibrosis in hypertensive patients with heart failure. Influence on the clinical outcome'. *Journal of hypertension*, 35(4), pp.853-861.

Ravassa, S., Trippel, T., Bach, D. *et al*., 2018. 'Biomarker-based phenotyping of myocardial fibrosis identifies patients with heart failure with preserved ejection fraction resistant to the beneficial effects of spironolactone: results from the Aldo-DHF trial'. *European Journal of heart failure*, 20(9), pp.1290-1299.

Rienstra, M., Lubitz, S.A., Mahida, S. *et al*.,2012. 'Symptoms and functional status of patients with atrial fibrillation: state of the art and future research opportunities'. *Circulation*, 125(23), pp.2933-2943.

Risteli, J., Elomaa, I., Niemi, S. *et al*., 1993. 'Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: a new serum marker of bone collagen degradation'. *Clinical chemistry*, 39(4), pp.635-640.

Rodriguez-Pascual, F. & Slatter, D.A., 2016. 'Collagen cross-linking: insights on the evolution of metazoan extracellular matrix'. *Scientific reports*, 6(1), pp.1-7.

Roongsritong, C., Sadhu, A., Pierce, M. *et al*., 2008. 'Plasma Carboxy-Terminal Peptide of Procollagen Type I Is an Independent Predictor of Diastolic

Function in Patients With Advanced Systolic Heart Failure'. *Congestive Heart Failure*, 14(6), pp.302-306.

Schillaci, G., Vaudo, G., Reboldi, G. *et al*., 2001. 'High-density lipoprotein cholesterol and left ventricular hypertrophy in essential hypertension'. *Journal of hypertension*, 19(12), pp.2265-2270.

Schimmel, K., Ichimura, K., Reddy, S. et al. 2022. 'Cardiac fibrosis in the pressure overloaded left and right ventricle as a therapeutic target'. *Frontiers in cardiovascular medicine, 9, p.886553.*

Schnabel, R.B., Yin, X., Gona, P. *et al*., 2015. '50-year trends in atrial fibrillation prevalence, incidence, risk factors, and mortality in the Framingham Heart Study: a cohort study'. *The Lancet*, 386(9989), pp.154-162.

Schwartzkopff, B., Fassbach, M., Pelzer, B. *et al*., 2002. 'Elevated serum markers of collagen degradation in patients with mild to moderate dilated cardiomyopathy'. *European Journal of Heart Failure*, 4(4), pp.439-444.

Seo, M.H., Lee, W.Y., Kim, S.S. *et al*., 2019. '2018 Korean society for the study of obesity guideline for the management of obesity in Korea'. *Journal of obesity & metabolic syndrome*, 28(1), p.40.

Sheldon, R.S., Grubb II, B.P., Olshansky, B. *et al*., 2015. 'heart rhythm society expert consensus statement on the diagnosis and treatment of postural tachycardia syndrome, inappropriate sinus tachycardia, and vasovagal syncope'. *Heart Rhythm*. 12(6): e41-63.

Shimizu, M., Umeda, K., Sugihara, N. *et al*., 1993. 'Collagen Remodelling In Myocardia Of Patients With Diabetes'. *Journal of clinical pathology*, 46(1), pp. 32-36.

Shirani, J., Pick, R., Roberts, W.C. *et al*. 2000. 'Morphology and significance of the left ventricular collagen network in young patients with hypertrophic cardiomyopathy and sudden cardiac death'. *Journal of the American College of Cardiology*, 35(1), pp.36-44.

Slawik, J., Adrian, L., Hohl, M. *et al*., 2019. 'Irregular pacing of ventricular cardiomyocytes induces pro-fibrotic signalling involving paracrine effects of transforming growth factor beta and connective tissue growth factor'. *European Journal of Heart Failure,* 21(4), pp.482-491.

Snell, R. S., 2011. '*The Thorax: Part II—The Thoracic Cavity*'. In Snell, R. S.(ed.). *Clinical anatomy by regions. 9Th*.New York: Lippincott Williams & Wilkins, pp:82-85.

Sonmez, O., Ertem, F.U., Vatankulu, M.A. *et al*., 2014. 'Novel fibro-inflammation markers in assessing left atrial remodeling in non-valvular atrial fibrillation'. Medical science monitor: *international medical journal of experimental and clinical research*, 20, p.463.

Steinberg, J.S., O'Connell, H., Li, S. *et al*., 2018. 'Thirty-second gold standard definition of atrial fibrillation and its relationship with subsequent arrhythmia patterns: analysis of a large prospective device database'. *Circulation: Arrhythmia and Electrophysiology,* 11(7), p. e006274.

Swartz, M.F., Fink, G.W., Sarwar, M.F. *et al*., 2012. 'Elevated pre-operative serum peptides for collagen I and III synthesis result in post-surgical atrial fibrillation'. Journal of the American College of Cardiology, 60(18), pp.1799-1806.

Sweeney, M., Corden, B. & Cook, S.A., 2020. '*Targeting cardiac fibrosis in heart failure with preserved ejection fraction: mirage or miracle*'*? EMBO molecular medicine,* 12(10), p. e10865.

Takawale, A., Aguilar, M., Bouchrit, Y. *et al*., 2022. 'Mechanisms and Management of Thyroid Disease and Atrial Fibrillation: Impact of Atrial Electrical Remodeling and Cardiac Fibrosis'. *Cells*, 11(24), p.4047.

Tedrow, U.B., Conen, D., Ridker, P.M. *et al*., 2010. 'The long-and short-term impact of elevated body mass index on the risk of new atrial fibrillation: the

WHS (Women's Health Study)'. *Journal of the American College of Cardiology*, 55(21), pp.2319-2327.

Tortora, G.J., & Derrickson, B.H., 2009. '*Principles of Anatomy and Physiology*'. 12th Ed. Hoboken, NJ: John Wiley & Sons.

Tziakas, D.N., Chalikias, G.K., Papanas, N. *et al*., 2007. 'Circulating levels of collagen type I degradation marker depend on the type of atrial fibrillation'. *Europace*, 9(8), pp.589-596.

van den Berg, M.P., Hassink, R.J., Tuinenburg, A.E. *et al*., 2001. 'Quality of life in patients with paroxysmal atrial fibrillation and its predictors: importance of the autonomic nervous system'. *European heart journal*, 22(3), pp.247-253.

Van Hoeven, K.H. & Factor, S.M., 1990. 'A comparison of the pathological spectrum of hypertensive, diabetic, and hypertensive-diabetic heart disease'. *Circulation,* 82(3), pp.848-855.

Van Putten, S., Shafieyan, Y. & Hinz, B., 2016. 'Mechanical control of cardiac myofibroblasts'. *Journal of molecular and cellular cardiology*, 93, pp.133-142.

Velagaleti, R.S., Massaro, J., Vasan, R.S. *et al*., 2009. 'Relations of lipid concentrations to heart failure incidence: the Framingham Heart Study'. Circulation, 120(23), pp.2345-2351.

Verdecchia, P., Reboldi, G., Gattobigio, R. *et al*., 2003. 'Atrial fibrillation in hypertension: predictors and outcome'. *hypertension*, 41(2), pp.218-223.

Verheule, S., Sato, T., Everett IV, T. *et al*., 2004. 'Increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-β1'. *Circulation research,* 94(11), pp.1458-1465.

Volgman, A.S., Benjamin, E.J., Curtis, A.B. et al., 2021. 'Women and atrial fibrillation'. *Journal of Cardiovascular Electrophysiology*, 32(10), pp.2793-2807.

Walker, B. R. & Colledge, N. R. (eds.). 2013. 'Cardiovascular disease'. In Newby, D.E. (eds.). 'Davidson's principles and practice of medicine' .21st Ed, e-book. *Elsevier Health Sciences*, pp:524.

Wang, T.J., Larson, M.G., Levy, D. *et al*., 2003. 'Temporal relations of atrial fibrillation and congestive heart failure and their joint influence on mortality: the Framingham Heart Study'. *Circulation,* 107(23), pp.2920-2925.

Weiskirchen, R., Weiskirchen, S. & Tacke, F., 2019. 'Organ and tissue fibrosis: Molecular signals, cellular mechanisms and translational implications'. *Molecular aspects of medicine,* 65, pp.2-15.

Xu, J., Cui, G., Esmailian, F. *et al*., 2004. 'Atrial extracellular matrix remodeling and the maintenance of atrial fibrillation'. *Circulation,* 109(3), pp.363-368.

Yamauchi, M. & Sricholpech, M., 2012. 'Lysine post-translational modifications of collagen'. *Essays in biochemistry*, 52, pp.113-133.

Yang, C., Qiao, S., Song, Y. *et al*., 2019. 'Procollagen type I carboxy-terminal propeptide (PICP) and MMP-2 are potential biomarkers of myocardial fibrosis in patients with hypertrophic cardiomyopathy'. *Cardiovascular Pathology*, 43, p.107150.

Zaiontz, C., 2013. Real Statistics resource pack. Available: www. real-statistics. com/free-download/real-statistics-resource-pack/. (18 March 2023)

Zhang, J., Johnsen, S.P., Guo, Y. et al., 2021. 'Epidemiology of atrial fibrillation: geographic / ecological risk factors, age, sex, genetics'. *Cardiac electrophysiology clinics*, 13(1), pp.1-23.

Zhao, F., Zhang, S., Chen, Y. *et al*., 2014. 'Increased expression of NF-AT3 and NF-AT4 in the atria correlates with procollagen I carboxyl-terminal peptide

and TGF-β1 levels in serum of patients with atrial fibrillation'. BMC Cardiovascular Disorders, 14, pp.1-12.

Zulkifly, H., Lip, G.Y. and Lane, D.A., 2018. 'Epidemiology of atrial fibrillation'. *International journal of clinical practice*, 72(3), p. e13070.

Appendices

Appendices:

Appendix-1

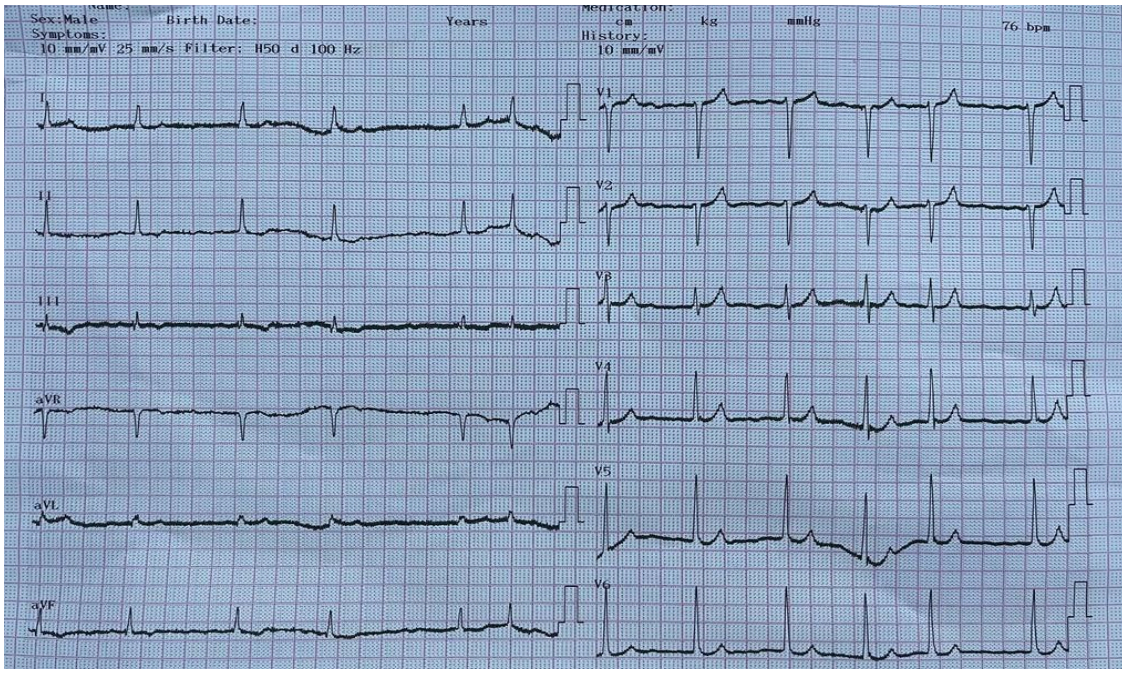
Questionaire

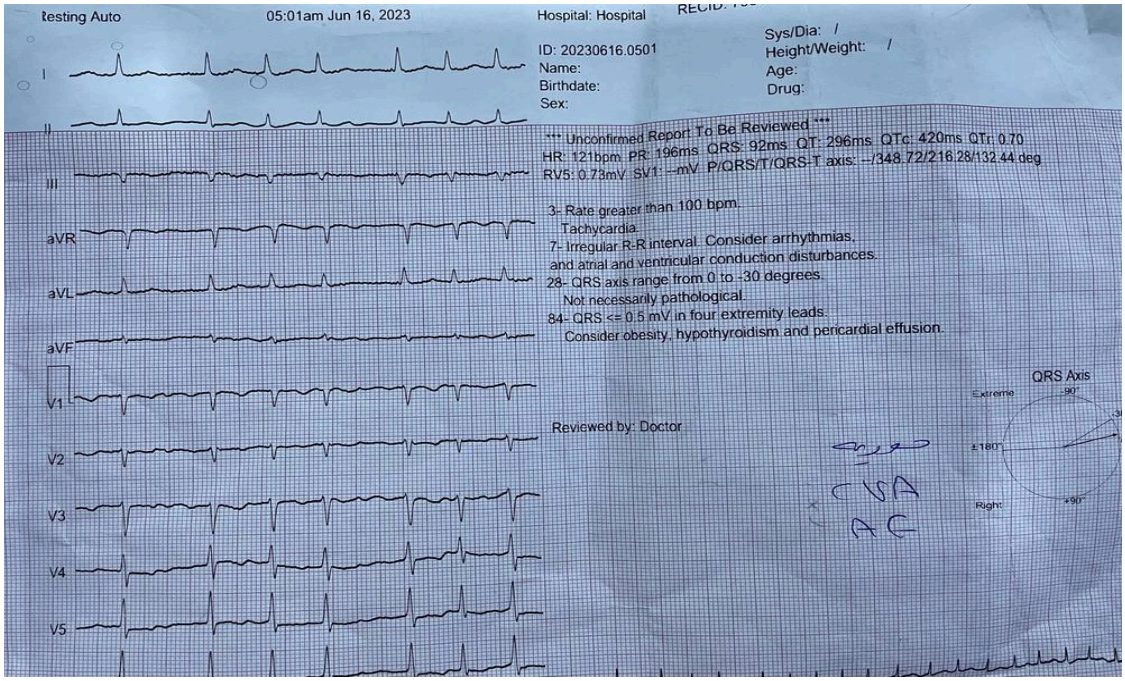
Name Patient:		**Paroxysmal AF**	**Persistent AF**	**Permanent AF**	**Lone AF**
Clinical Variable					
Duration of AF	***< 7-day***				
	>7-day & < 1-year				
	> 1-year				
Age *years*	*< 45*				
	45-60				
	>60				
BMI *Kg/m²*	*Underweight*				
	Normal Weight				
	Pre-obesity				
	Obesity Class-1				
	Obesity Class-2				
	Obesity Class-3				
HR *bpm*	*Bradycardia*				
	Normal				
	Tachycardia				
B.P *mm Hg*	*Normal HTN*				
	Elevated HTN				
	HTN Stage-1				
	HTN Stage-2				
Comorbidities					
HTN	*Yes*				
	NO				
Dyslipidemia	*Yes*				
	NO				
Symptoms					
palpitation	*Yes*				
	NO				
Chest pain	*Yes*				
	NO				

Dyspnea	*Yes*				
	NO				
Syncope	*Yes*				
	NO				
ECG Interpretation					
HR ***bpm***	*Bradycardia*				
	Normal				
	Tachycardia				
LBBB	Absent				
	Present				
RBBB	Absent				
	Present				
Echo Study					
EF%	Hyperdynamic				
	Normal				
	Mild				
	Moderate				
	Severe				
LA diameter	Normal				
	Mild				
	Moderate				
	Severe				
LV diameter	Normal				
	Mild				
	Moderate				
	Severe				

Appendix-2

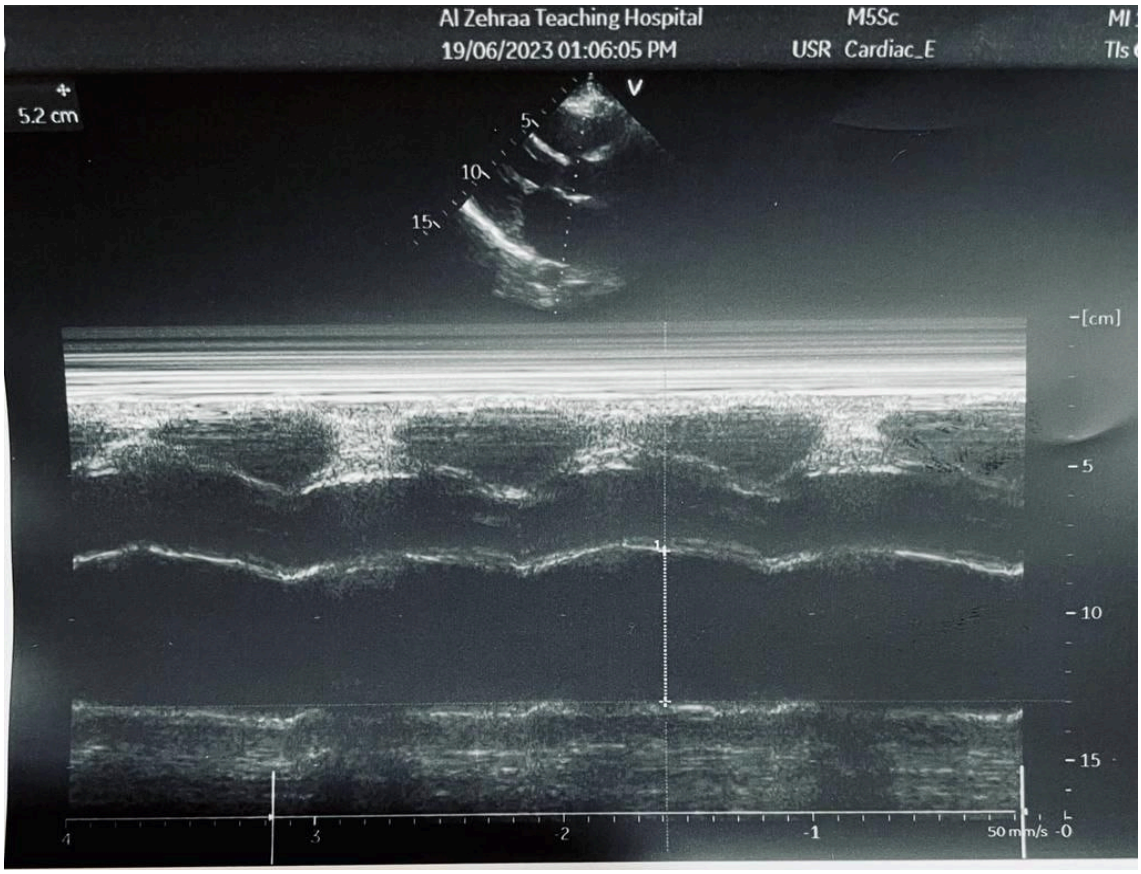
ECG interpretation of atrial fibrillation

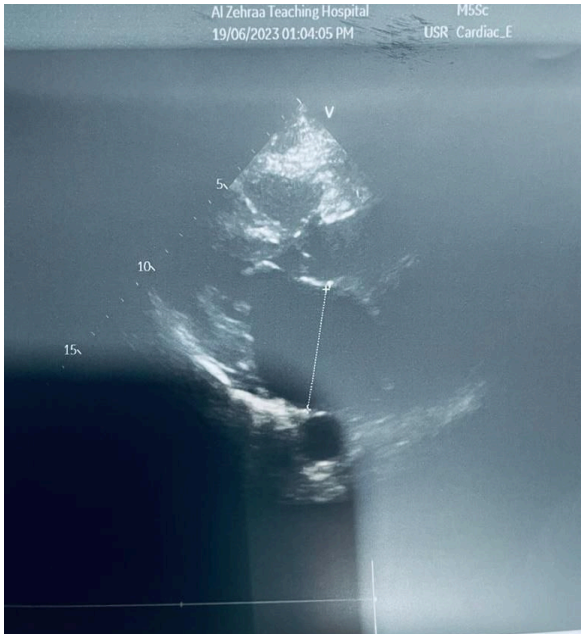


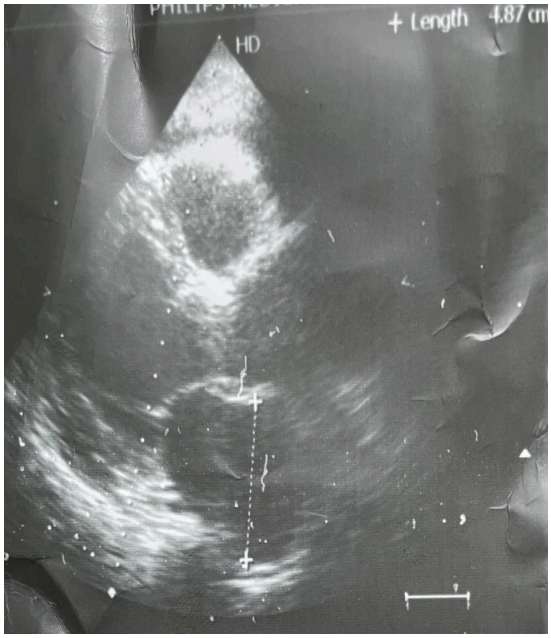


Appendix-3

Echo study of left atrial enlargement

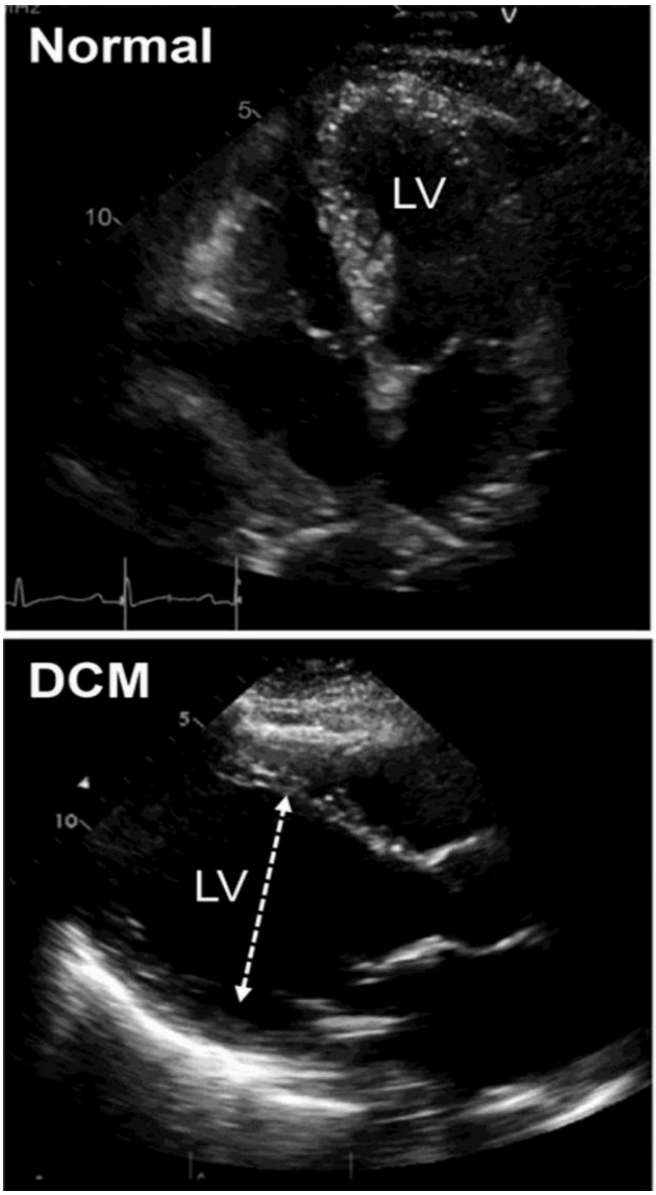






Appendix-4

Echo study of left ventricular dilated



Appendix-5

Human Cross-Linked C-telopeptide of Type 1 Collagen ELISA Kit

User Instruction

Cat.No E1349Hu

Standard Curve Range: 7-1500 *ng/mL*

Sensitivity: 4.21 *ng/mL*

Size: 96 wells / 48 wells

Storage: Store the reagents at 2-8°C. For over 6-month storage refer to the expiration date keep it at -20°C. Avoid repeated thaw cycles. If individual reagents are opened it is recommended that the kit be used within 1 month.

*This product is for research use only, not for use in diagnosis procedures. It's highly recommended to read this instruction entirely before use.

Precision

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

CV (%) = SD/mean x 100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

Intended Use

This sandwich kit is for the accurate quantitative detection of Human Cross-Linked C-telopeptide of Type 1 Collagen (also known as CTX-1) in serum, plasma, cell culture supernates, Ascites, tissue homogenates or other biological fluids.

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human CTX-1 antibody. CTX-1 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human CTX-1 Antibody is added and binds to CTX-1 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated CTX-1 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human CTX-1. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Provided

Components	Quantity (96T)
Standard Solution (1600ng/ml)	0.5ml x1
Pre-coated ELISA	12 * 8 well strips x1 3ml x1
Standard Diluent	6ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution	6ml x1
Substrate Solution	20ml x1
Wash Buffer Concentrate (25x)	1ml x1
Biotinylated Human CTX-1 Antibody	1
User Instruction	2 pics
Plate Sealer	1 pic
Zipper bag	1 pic

Material Required But Not Supplied

- 37°C±0.5°C incubator
- Absorbent paper
- Precision pipettes and disposable pipette tips
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10nm wavelength filter

Precautions

- Prior to use, the kit and sample should be warmed naturally to room temperature 30 minutes.
- This instruction must be strictly followed in the experiment.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remain from deterioration. Cover all reagents when not in use.
- Make sure pipetting order and rate of addition from well-to-well when pipetting reagents.
- Pipette tips and plate sealer in hand should be clean and disposable to avoid cross-contamination.
- Avoid using the reagents from different batches together.
- Substrate solution B is sensitive to light, don't expose substrate solution B to light for a long time.
- Stop solution contains acid. Please wear eye, hand and skin protection when using this material. Avoid contact of skin or mucous membranes with kit reagent.
- The kit should not be used beyond the expiration date.

Specimen Collection

Serum Allow serum to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Plasma Collect plasma using EDTA or heparin as an anticoagulant. After mix 10-20 minutes, centrifuge samples for 20 minutes at 2000-3000 RPM. Collect the supernatant without sediment.

Urine/Ascites/ Cerebrospinal fluid Collect by sterile tube. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Cell culture supernatant Collect by sterile tubes. When detecting secrete components, centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatants. When detecting the components in the cell, use PBS (pH 7.2-7.4) to dilute cell suspension, the cell concentration of approximately 1 million/ml. Damage cells through repeated freeze-thaw cycles to let out the inside components. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Tissue Rinse tissues in ice-cold PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. Mince tissues and homogenize them in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to get thesupernatant.

Note

- Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the

standard curve, users must contact us to determine the optimal sample for their particular experiments.

- Samples to be used within 5 days should be stored at 2-8°C. Samples should be aliquoted or must be stored at -20°C within 1 month or -80°C within 6 months. Avoid repeated freeze thaw cycles.
- Samples should be brought to room temperature before starting the assay.
- Centrifuge to collect sample before use.
- Samples containing NaN3 can't be tested as it inhibits the activity of Horse Radish Peroxidase (HRP).
- Collect the supernatants carefully. When sediments occurred during storage, centrifugation should be performed again.
- Hemolysis can greatly impact the validity of test results. Take care to minimize hemolysis.

*Sample can't be diluted with this kit. Owing to the the material we use to prepare the kit; the sample matrix interference may falsely depress the specificity and accuracy of the assay.

Summary

1. Prepare all reagents, samples and standards.

2. Add sample and ELISA reagent into each well. Incubate for 1 hour at 37°C.

3. Wash the plate 5 times.

4. Add substrate solution A and B. Incubate for 10 minutes at 37°C.

5. Add stop solution and color develops.

6. Read the OD value within 10 minutes.

Appendix-6

Human Carboxyterminal propeptide of type 1 procollagen ELISA Kit User Instruction

Cat.No E1373Hu

Standard Curve Range: 5-1500 *ng/mL*

Sensitivity: 2.26 *ng/mL*

Size: 96 wells

Storage: Store the reagents at 2-8°C. For over 6-month storage refer to the expiration date keep it at -20°C. Avoid repeated thaw cycles. If individual reagents are opened it is recommended that the kit be used within 1 month.

*This product is for research use only, not for use in diagnosis procedures. It's highly recommended to read this instruction entirely before use.

Precision

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

CV (%) = SD/mean x 100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

Intended Use

This sandwich kit is for the accurate quantitative detection of Human Carboxyterminal propeptide of type 1 procollagen (also known as P1CP) in serum, plasma, cell culture supernates, Ascites, tissue homogenates or other biological fluids.

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human P1CP antibody. P1CP present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human P1CP Antibody is added and binds to P1CP in the sample.

Then Streptavidin-HRP is added and binds to the Biotinylated P1CP antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human P1CP. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Provided

Components	Quantity (96T)
Standard Solution (1600ng/ml)	0.5ml x1
Pre-coated ELISA	12 * 8 well strips x1 3ml x1
Standard Diluent	6ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution	6ml x1
Substrate Solution	20ml x1
Wash Buffer Concentrate (25x)	1ml x1
Biotinylated Human P1CP Antibody	1
User Instruction	2 pics
Plate Sealer	1 pic
Zipper bag	1 pic

Material Required But Not Supplied

- 37°C±0.5°C incubator
- Absorbent paper
- Precision pipettes and disposable pipette tips
- Clean tubes
- Deionized or distilled water

- Microplate reader with 450 ± 10nm wavelength filter

Precautions

- Prior to use, the kit and sample should be warmed naturally to room temperature 30 minutes.
- This instruction must be strictly followed in the experiment.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remain from deterioration. Cover all reagents when not in use.
- Make sure pipetting order and rate of addition from well-to-well when pipetting reagents.
- Pipette tips and plate sealer in hand should be clean and disposable to avoid cross-contamination.
- Avoid using the reagents from different batches together.
- Substrate solution B is sensitive to light, don't expose substrate solution B to light for a long time.
- Stop solution contains acid. Please wear eye, hand and skin protection when using this material. Avoid contact of skin or mucous membranes with kit reagent.
- The kit should not be used beyond the expiration date.

Specimen Collection

Serum Allow serum to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Plasma Collect plasma using EDTA or heparin as an anticoagulant. After mix 10-20 minutes, centrifuge samples for 20 minutes at 2000-3000 RPM. Collect the supernatant without sediment.

Urine/Ascites/ Cerebrospinal fluid Collect by sterile tube. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Cell culture supernatant Collect by sterile tubes. When detecting secrete components, centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatants. When detecting the components in the cell, use PBS (pH 7.2-7.4) to dilute cell suspension , the cell concentration of approximately 1 million/ml. Damage cells through repeated freeze-thaw cycles to let out the inside components. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Tissue Rinse tissues in ice-cold PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. Mince tissues and homogenize them in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to get thesupernatant.

Note

- Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the standard curve, users must contact us to determine the optimal sample for their particular experiments.
- Samples to be used within 5 days should be stored at 2-8°C. Samples should be aliquoted or must be stored at -20°C within 1 month or -80°C within 6 months. Avoid repeated freeze thaw cycles.
- Samples should be brought to room temperature before starting the assay.

- Centrifuge to collect sample before use.
- Samples containing NaN3 can't be tested as it inhibits the activity of Horse Radish Peroxidase (HRP).
- Collect the supernatants carefully. When sediments occurred during storage, centrifugation should be performed again.
- Hemolysis can greatly impact the validity of test results. Take care to minimize hemolysis.

*Sample can't be diluted with this kit. Owing to the the material we use to prepare the kit, the sample matrix interference may falsely depress the specificity and accuracy of the assay.

Summary

1. Prepare all reagents, samples and standards.

2. Add sample and ELISA reagent into each well. Incubate for 1 hour at 37°C.

3. Wash the plate 5 times.

4. Add substrate solution A and B. Incubate for 10 minutes at 37°C.

5. Add stop solution and color develops.

6. Read the OD value within 10 minutes.

Appendix-7

Human Vitamin C ELISA Kit
USER INSTRUCTION
Cat.No E1538Hu
Standard Curve Range: 1-300 *ng/ml*
Sensitivity: 0.52 *ng/ml*
Size: 96 wells

Storage: Store the reagents at 2-8°C. For over 6-month storage refer to the expiration date keep it at -20°C. Avoid repeated thaw cycles. If individual reagents are opened it is recommended that the kit be used within 1 month.

*This product is for research use only, not for use in diagnosis procedures. It's highly recommended to read this instruction entirely before use.

Precision

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

CV (%) = SD/mean x 100

Intra-Assay: CV<8%

Inter-Assay: CV<10%

Intended Use

This sandwich kit is for the accurate quantitative detection of Human Vitamin C (also known as VC) in serum, plasma, cell culture supernates, Ascites, tissue homogenates or other biological fluids.

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human VC antibody. VC present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human VC Antibody is added and binds to VC in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated VC antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human VC. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Provided

Components	Quantity (96T)
Standard Solution (320ng/ml)	0.5ml x1
Pre-coated ELISA	12 * 8 well strips x1 3ml x1
Standard Diluent	6ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution	6ml x1
Substrate Solution	20ml x1
Wash Buffer Concentrate (25x)	1ml x1
Biotinylated Human VC Antibody	1
User Instruction	2 pics
Plate Sealer	1 pic
Zipper bag	1 pic

Material Required But Not Supplied

- 37°C±0.5°C incubator
- Absorbent paper
- Precision pipettes and disposable pipette tips
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10nm wavelength filter

Precautions

- Prior to use, the kit and sample should be warmed naturally to room temperature 30 minutes.
- This instruction must be strictly followed in the experiment.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remain from deterioration. Cover all reagents when not in use.
- Make sure pipetting order and rate of addition from well-to-well when pipetting reagents.
- Pipette tips and plate sealer in hand should be clean and disposable to avoid cross-contamination.
- Avoid using the reagents from different batches together.

- Substrate solution B is sensitive to light, don't expose substrate solution B to light for a long time.
- Stop solution contains acid. Please wear eye, hand and skin protection when using this material. Avoid contact of skin or mucous membranes with kit reagent.
- The kit should not be used beyond the expiration date.

Specimen Collection

Serum Allow serum to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Plasma Collect plasma using EDTA or heparin as an anticoagulant. After mix 10-20 minutes, centrifuge samples for 20 minutes at 2000-3000 RPM. Collect the supernatant without sediment.

Urine/Ascites/ Cerebrospinal fluid Collect by sterile tube. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Cell culture supernatant Collect by sterile tubes. When detecting secrete components, centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatants. When detecting the components in the cell, use PBS (pH 7.2-7.4) to dilute cell suspension, the cell concentration of approximately 1 million/ml. Damage cells through repeated freeze-thaw cycles to let out the inside components. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment.

Tissue Rinse tissues in ice-cold PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. Mince tissues and homogenize them in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw

cycles. The homogenates are then centrifuged for 5 minutes at 5000×g to get the supernatant.

Note

- Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the standard curve, users must contact us to determine the optimal sample for their particular experiments.
- Samples to be used within 5 days should be stored at 2-8°C. Samples should be aliquoted or must be stored at -20°C within 1 month or -80°C within 6 months. Avoid repeated freeze thaw cycles.
- Samples should be brought to room temperature before starting the assay.
- Centrifuge to collect sample before use.
- Samples containing NaN3 can't be tested as it inhibits the activity of Horse Radish Peroxidase (HRP).
- Collect the supernatants carefully. When sediments occurred during storage, centrifugation should be performed again.
- Hemolysis can greatly impact the validity of test results. Take care to minimize hemolysis.

*Sample can't be diluted with this kit. Owing to the the material we use to prepare the kit; the sample matrix interference may falsely depress the specificity and accuracy of the assay.

Summary

1. Prepare all reagents, samples and standards.

2. Add sample and ELISA reagent into each well. Incubate for 1 hour at 37°C.

3. Wash the plate 5 times.

4. Add substrate solution A and B. Incubate for 10 minutes at 37°C.

5. Add stop solution and color develops.

6. Read the OD value within 10 minutes.

Appendix-8

Cholesterol

This package insert contains information to run the Cholesterol assay on the ARCHITECT c SystemsTM and the AEROSET System.

Intended Use

The Cholesterol assay is used for the quantitation of cholesterol in human serum or plasma.

Summary and Explanation of The Test

Measurement of serum cholesterol levels can serve as an indicator of liver function, biliary function, intestinal absorption, propensity toward coronary artery disease, and thyroid function. Cholesterol levels are important in the diagnosis and classification of hyperlipoproteinemias. Stress, age, gender, hormonal balance, and pregnancy affect normal cholesterol levels.[1]

The Adult Treatment Panel of the National Cholesterol Education Program (NCEP) recommends that all adults 20 years of age and over should have a fasting lipoprotein profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride) once every five years to screen for coronary heart disease risk.[2]

Principles of Procedures

The use of enzymes to assay cholesterol has been studied by many investigators.[3,4] This reagent is based on the formulation of Allain,

et al.5 and the modification of Roeschlau6 with further improvements to render the reagent stable in solution.

Cholesterol esters are enzymatically hydrolyzed by cholesterol

esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4-aminoantipyrine to form a chromophore (quinoneimine dye) which is quantitated at 500 nm.

Methodology: Enzymatic

Reagents

Reagent Kit

REF 7D62 Cholesterol is supplied as a liquid, ready-to-use, single reagent kit which contains:

R1 10 x 84 mL

Estimated tests per kit: 3,032

Calculation is based on the minimum reagent fill volume per kit.

Reactive Ingredients ---------- > Concentration

Cholesterol Oxidase (Microbial) ---------- > more than 200 *U/L*

Cholesterol Esterase (Microbial)---------- > more than 500 *U/L*

Peroxidase (Horseradish)---------- > more than 300 *U/L*

4-Aminoantipyrine---------- > 0.25 *mmol/L*

HBA---------- > 10 *mmol/L*

The Abbott Clinical Chemistry Cholesterol reagent is certified to be traceable to the National Reference System for Cholesterol, against the Abell-Kendall reference method in a CDC-Certified Cholesterol Reference Method Laboratory Network (CRMLN).

Reagent Handling and Storage

Reagent Handling

Remove air bubbles, if present in the reagent cartridge, with a new applicator stick. Alternatively, allow the reagent to sit at the appropriate storage temperature to allow the bubbles to dissipate. To minimize volume depletion, do not use a transfer pipette to remove the bubbles.

Caution: Reagent bubbles may interfere with proper detection of reagent level in the cartridge, causing insufficient reagent aspiration which could impact results.

Reagent Storage

Unopened reagents are stable until the expiration date when stored at 2 to 8°C.

Reagent stability is 30 days if the reagent is uncapped and onboard.

Sample collection and handling

Suitable Specimens

Serum and plasma are acceptable specimens. The National Cholesterol Education Program (NCEP) recommends using fasting specimens.[2]

• **Serum**: Use serum collected by standard venipuncture techniques into glass or plastic tubes with or without gel barriers. Ensure complete clot formation has taken place prior to centrifugation. Separate serum from red blood cells or gel as soon after collection as possible.

Some specimens, especially those from patients receiving anticoagulant or thrombolytic therapy, may take longer to complete their clotting processes. Fibrin clots may subsequently form in these sera and the clots could cause erroneous test results.

• **Plasma:** Use plasma collected by standard venipuncture techniques into glass or plastic tubes. Acceptable anticoagulants are lithium heparin (with or

without gel barrier) and sodium heparin. Ensure centrifugation is adequate to remove platelets. Separate plasma from red blood cells or gel as soon after collection as possible.

Specimen Storage Serum and plasma

Temperature	**Maximum Storage**
20 to 25°C	> 7 days[5]
2 to 8°C	> 7 days [5,12]
-20°C	> 3 months [5]

Guder et al.[11] suggest storage of frozen specimens at -20°C for no longer than the time interval cited above. However, limitations of laboratory equipment make it necessary in practice for clinical laboratories to establish a range around -20°C for specimen storage. This temperature range may be established from either the freezer manufacturer's specifications or your laboratory standard operating procedure(s) for specimen storage.

NOTE: Stored specimens must be inspected for particulates. If present, mix and centrifuge the specimen to remove particulates prior to testing.

Bibliography

1. Burtis CA, Ashwood ER, editors. Tietz Fundamentals of Clinical Chemistry, 5th ed. Philadelphia, PA: WB Saunders; 2001:480–5.

2. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 2001; 285:2486–97.

3. Flegg HM. An investigation of the determination of serum cholesterol by an enzymatic method. Ann Clin Biochem 1973; 10:79– 84.

4. Richmond W. Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. Clin Chem 1973;19(12):1350–6.

5. Guder WG, Narayanan S, Wisser H, et al. List of analytes—preanalytical variables. Annex In: Samples: From the Patient to the Laboratory. Darmstadt, Germany: GIT Verlag; 1996: Annex 12–3.

6. US Pharmacopeial Convention, Inc. General notices. In: US Pharmacopeia National Formulary, 1995 ed (USP 23/NF 18). Rockville, MD: The US Pharmacopeial Convention, Inc; 1994:11.

Appendix-9

Triglycerides

This package insert contains information to run the Triglyceride assay on the ARCHITECT c SystemsTM and the AEROSET System.

Intended Use

The Triglyceride assay is used for the quantitation of triglyceride in human serum or plasma.

Summary and Explanation of The Test

Triglycerides are a family of lipids absorbed from the diet and produced endogenously from carbohydrates and fatty acids. Measurement of triglyceride is important in the diagnosis and management of hyperlipidemia. These diseases can be genetic or secondary to other disorders including nephrosis, diabetes mellitus, and endocrine disturbances. The National Cholesterol Education Program (NCEP) cites evidence that triglycerides are an independent risk factor for atherosclerosis.[1] Individuals with hypertension, obesity, and/or diabetes are at greater risk than are those without these conditions.[2,3]

The Adult Treatment Panel of the NCEP recommends that all adults 20 years of age and over should have a fasting lipoprotein profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride) once every five years to screen for coronary heart disease risk.[1]

Principles of Procedures

Triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing hydrogen peroxide (H2O2). In a color reaction catalyzed by peroxidase, the H2O2 reacts with 4-aminoantipyrine (4-AAP) and 4-chlorophenol (4-CP) to produce a red colored dye. The absorbance of this dye is proportional to the concentration of triglyceride present in the sample. This analytical methodology is based on the reaction sequence described by Fossati et al.[4] and by McGowan et al.5 In this reagent, 4-chlorophenol is used rather than 2-hydroxy-3,5-dichlor- obenzenesulfonate, used in the Fossati and McGowan studies.

Methodology: Glycerol Phosphate Oxidase

Reagents

Reagent Kit

REF 7D74 Triglyceride is supplied as a liquid, ready-to-use, single reagent kit which contains:

R1 10 x 84 mL

Estimated tests per kit: 3,032

Calculation is based on the minimum reagent fill volume per kit.

Reactive Ingredients	Concentration
ATP	> 2.5 *mmol/L*
Mg2+	> 2.5 *mmol/L*
4-Aminoantipyrine	> 0.4 *mmol/L*
4-Chlorophenol	> 2 *mmol/L*
Peroxidase (Horseradish)	> more than 2,000 *U/L*
GK (Microbial)	> more than 600 *U/L*
GPO (Microbial)	> more than 6,000 *U/L*
Lipoprotein Lipase (Microbial)	> more than 3.000 *U/L*

Reagent Handling and Storage

Reagent Handling

Remove air bubbles, if present in the reagent cartridge, with a new applicator stick. Alternatively, allow the reagent to sit at the appropriate storage temperature to allow the bubbles to dissipate. To minimize volume depletion, do not use a transfer pipette to remove the bubbles.

Caution: Reagent bubbles may interfere with proper detection of reagent level in the cartridge, causing insufficient reagent aspiration which could impact results.

Reagent Storage

Un opened reagents are stable until expiration date when stored at 2 to 8C°.

Reagent stability is 42 days if the reagent is uncapped and onboard.

Sample Collection and Handling

Suitable Specimens

Serum and plasma are acceptable specimens. The National Cholesterol Education Program (NCEP) recommends using fasting specimens.[1]

• **Serum**: Use serum collected by standard venipuncture techniques into glass or plastic tubes with or without gel barriers. Ensure complete clot formation has taken place prior to centrifugation. Separate serum from red blood cells or gel as soon after collection as possible.

Some specimens, especially those from patients receiving anticoagulant or thrombolytic therapy, may take longer to complete their clotting processes. Fibrin clots may subsequently form in these sera and the clots could cause erroneous test results.

• **Plasma**: Use plasma collected by standard venipuncture techniques into glass or plastic tubes. Acceptable anticoagulants are lithium heparin (with or without gel barrier) and sodium heparin. Ensure centrifugation is adequate to remove platelets. Separate plasma from red blood cells or gel as soon after collection as possible.

Sample Collection and Handling

Specimen Storage

Serum and plasma

Temperature Maximum Storage

20 to 25°C----------> 2 days[5]

2 to 8°C---------- > 7 days [5,6]

-20°C---------- >1 year [5]

Guder et al.[10] suggest storage of frozen specimens at -20°C for no longer than the time interval cited above. However, limitations of laboratory equipment make it necessary in practice for clinical laboratories to establish a range around -20°C for specimen storage. This temperature range may be established from either the freezer manufacturer's specifications or your laboratory standard operating procedure(s) for specimen storage.

Note: Stored specimens must be inspected for particulates. If present, mix and centrifuge the specimen to remove particulates prior to testing.

Bibliography

1. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 2001; 285:2486–97.

2. Rubins HB. Triglycerides and coronary heart disease: implications of recent clinical trials. J Cardiovasc Risk 2000;7(5):339–45.

3. Forrester JS. Triglycerides: risk factor or fellow traveler? Curr Opin Cardiol 2001; 16:261–4.

4. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 1982; 28:2077–80.

5. Guder WG, Narayanan S, Wisser H, et al. List of analytes—preanalytical variables. Annex In: Samples: From

the Patient to the Laboratory. Darmstadt, Germany: GIT Verlag; 1996: Annex 22–3.

6. US Pharmacopeial Convention, Inc. General notices. In: US Pharmacopeia National Formulary, 1995 ed (USP 23/NF 18). Rockville, MD: The US Pharmacopeial Convention, Inc; 1994:11.

Appendix-10

Ultra HDL

This package insert contains information to run the Ultra HDL assay on the ARCHITECT c SystemsTM and the AEROSET System.

Intended Use

The Ultra HDL (UHDL) assay is used for the quantitation of high-density lipoprotein (HDL) cholesterol in human serum or plasma.

Summary and Explanation of The Test

Plasma lipoproteins are spherical particles containing varying amounts of cholesterol, triglycerides, phospholipids, and proteins. Phospholipids, free cholesterol, and proteins constitute the outer surface of the lipoprotein particle, while the inner core contains mostly esterified cholesterol and triglyceride. These particles serve to solubilize and transport cholesterol and triglyceride in the bloodstream.

The relative proportions of protein and lipid determine the density of these lipoproteins and provide a basis on which to begin their classification.[1] The classes are: chylomicron, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Numerous clinical studies have shown that the different lipoprotein classes have very distinct and varied effects on coronary heart disease risk.[2]

The principle role of HDL cholesterol in lipid metabolism is the uptake and transport of cholesterol from peripheral tissues to the liver through a process known as reverse cholesterol transport (a proposed cardioprotective mechanism).[3] Low HDL cholesterol levels are strongly associated with an increased risk of coronary heart disease.[4-7]

Hence, the determination of serum HDL cholesterol is a useful tool in identifying high-risk patients. The Adult Treatment Panel of the National

Cholesterol Education Program (NCEP) recommends that in all adults 20 years of age and over, a fasting lipoprotein profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride) should be obtained once every five years to screen for coronary heart disease risk.[8]

Principles of Procedures

The Ultra HDL assay is a homogeneous method for directly measuring HDL cholesterol concentrations in serum or plasma without the need for off-line pretreatment or centrifugation steps.

The method uses a two-reagent format and depends on the properties of a unique detergent. This method is based on accelerating the reaction of cholesterol oxidase (CO) with non-HDL unesterified cholesterol and dissolving HDL cholesterol selectively using a specific detergent. In the first reagent, non-HDL unesterified cholesterol is subject to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction with DSBmT yielding a colorless product. The second reagent consists of a detergent (capable of solubilizing HDL cholesterol), cholesterol esterase (CE), and chromagenic coupler to develop color for the quantitative determination of HDL cholesterol.

Methodology: Accelerator Selective Detergent.

Reagents

Reagent Kit

REF 3K33 Ultra HDL is supplied as a liquid, ready-to-use, two-reagent kit which contains:

R1 4 x 84 *mL*

R2 4 x 32 *mL*

Estimated tests per kit: 1,440

Calculation is based on the minimum reagent fill volume per kit.

Reactive Ingredients **Concentration**

R1 Cholesterol oxidase (E. coli) ---------- > < 1,000 *U/L*

Peroxidase (Horseradish)---------- > < 1,300 *ppg U/L*

N, N-bis (4-sulphobutyl)-m-toluidine-disodium--------- > < 1.0 *mmol/L*

(DSBmT)

Accelerator---------- > < 1.0 *mmol/L*

Ascorbic oxidase (Curcubita sp.) ----------> < 3,000 *U/L*

R2 Cholesterol esterase (Pseudomonas sp.) ---------- > < 1,500 *U/L*

4-Aminoantipyrine ---------- > < 0.1%

Detergent---------- > < 2.0%

The Ultra HDL reagent is certified as traceable to the HDL cholesterol designated comparison method, covering the NCEP medical decision points, by the CDC-Certified Cholesterol Reference Method Laboratory Network (CRMLN).

Reagent Handling and Storage

Reagent Handling

Remove air bubbles, if present in the reagent cartridge, with a new applicator stick. Alternatively, allow the reagent to sit at the appropriate storage temperature to allow the bubbles to dissipate. To minimize volume depletion, do not use a transfer pipette to remove the bubbles.

Caution: Reagent bubbles may interfere with proper detection of reagent level in the cartridge, causing insufficient reagent aspiration which could impact results.

Reagent Storage

• Unopened reagents are stable until the expiration date when stored at 2 to 8°C.

• Do not freeze.

• Protect reagents from direct sunlight.

• Reagent stability is 28 days if the reagent is uncapped and onboard.

Indications of Deterioration

• Quality control results outside of the acceptance criteria defined by your laboratory.

• Presence of turbidity.

Sample Collection and Handling

Suitable Specimens

Serum and plasma are acceptable specimens. The National Cholesterol Education Program (NCEP) recommends using fasting specimens for

a lipoprotein profiles. If the specimen is nonfasting, only the values for total cholesterol and HDL cholesterol are usable.[9]

• **Serum**: Use serum collected by standard venipuncture techniques into glass or plastic tubes with or without gel barriers. Ensure complete clot formation has taken place prior to centrifugation. When processing samples, separate serum from blood cells or gel according to the specimen collection tube manufacturer's instructions.

Some specimens, especially those from patients receiving anticoagulant or thrombolytic therapy, may take longer to complete their clotting processes. Fibrin clots may subsequently form in these sera and the clots could cause erroneous test results.

• **Plasma**: Use plasma collected by standard venipuncture techniques into glass or plastic tubes. Acceptable anticoagulants are sodium heparin, lithium heparin (with or without gel barrier), and spray-dried EDTA. * Ensure centrifugation is adequate to remove platelets. When processing samples, separate plasma from blood cells or

gel according to the specimen collection tube manufacturer's instructions.

***Note**: Lower HDL cholesterol results obtained from EDTA plasma have been attributed to an osmotic dilution effect. The NCEP has suggested multiplying EDTA plasma results by a factor of 1.03 to correct the EDTA result to a serum equivalent value.[10]

Sample Collection and Handling

Specimen Storage

Serum and Plasma

Temperature	**Maximum Storage**
20 to 25°C	> 2 days[5]
2 to 8°C	> 7 days [5,6]
-20°C	>1 year [5]

Guder et al.[10] suggest storage of frozen specimens at -20°C for no longer than the time interval cited above. However, limitations of laboratory equipment make it necessary in practice for clinical laboratories to establish a range around -20°C for specimen storage. This temperature range may be established from either the freezer manufacturer's specifications or your laboratory standard operating procedure(s) for specimen storage.

Note: Stored specimens must be inspected for particulates. If present, mix and centrifuge the specimen to remove particulates prior to testing.

Bibliography

1. Gotto AM. Lipoprotein metabolism and the etiology of hyperlipidemia. Hosp Pract 1988;23(Suppl 1):4–13.

2. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)—Final Report. National

Institutes of Health. National Heart, Lung, and Blood Institute. NIH Publication No. 02-5215. September 2002; I-1–II-22.

3. Badimon JJ, Badimon L, Fuster V. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. J Clin Invest 1990;85(4):1234–41.

4. Castelli WP, Doyle JT, Gordon T, et al. HDL Cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation 1977;55(5):767–72.

5. Gordon T, Castelli WP, Hjortland MC, et al. High-density lipoprotein as a protective factor against coronary heart disease. Am J Med 1977;62(5):707.

6. Williams P, Robinson D, Bailey A. High density lipoprotein and coronary risk factors in normal men. Lancet 1979;1(8107):72–5.

7. Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease; New perspectives based on the Framingham study. Ann Intern Med 1979;90(1):85–91.

8. US Department of Labor, Occupational Safety and Health Administration. 29 CFR Part 1910.1030. Occupational Exposure to Bloodborne Pathogens.

9. Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). JAMA 2001;285(19):2486–97.

10. National Cholesterol Education Program. Recommendations on Lipoprotein Measurement, from the Working Group on Lipoprotein Measurement. National Institutes of Health. National Heart, Lung, and Blood Institute. NIH Publication No. 95-3044. September 1995.

الملخص

التليف الخلالي لعضلة القلب هو واحد من أكثر المضاعفات شيوعاً لاعتلال عضلة القلب لان زيادة ترسيب الكولاجين يغير البنية الخلالية لعضلة القلب. كما إنه سبب رئيسي لخلل القلبي الانقباضي والانبساطي وتغيير النتائج السريرية في المرضى الذين يعانون من آمراض القلب غير التاجية. نتيجة لذلك يؤدي التليف الخلالي لعضلة القلب الى الرجفان الاذيني.

الرجفان الاذيني هو عدم أنتظام ضربات القلب فوق البطين يتميز بانقباضات آذينية غير منتظمة، غير فعالة، تنشيط كهربائي وغياب موجة-P ، تشمل أعراض الرجفان الاذيني الخفقان والم الصدروضيق التنفس والاغماء. أسباب عوامل الخطر تسبب التليف الخلالي لعضلة القلب وكلاهما يؤدي الى الرجفان الاذيني. ترسيب الكولاجين قد يكون علامة مسار جديدة لتميز غياب أو وجود التليف الخلالي لعضلة القلب فى مرضي الرجفان الاذيني.

بحثت هذه الدراسة عن العلاقة بين مستويات الدم ل (PICP, CITP) والتليف الخلالي لعضلة القلب لدى مرضى الرجفان الاذيني وتآثير عوامل الخطر على النتائج. بالاضافة الى ذلك فعالية (S.PICP & S.CITP) كمؤشرات حيوية لتشخيص الرجفان الاذيني.

تمت الموافقة على بروتوكول الدراسة من قبل اللجنة الأخلاقية في كلية الطب جامعة كربلاء، مستشفى الزهراء التعليمي / واسط في مدينة الكوت. تم أخذ الموافقة الشفهية من جميع المرضى المشمولين في الدراسة قبل أخذ العينات.

تضمنت الدراسة الحالية ١٢٠ مشاركاً تتراوح اعمارهم من ٢٢ آلى ٩٠ عاماً. كان ٦٠ منهم بصحة جيدة وكانوا بمثابة مجموعة ضابطة و٦٠ منهم مصاباً بالرجفان الاذيني وتم تشخيصهم بواسطة تفسير تخطيط كهربائية القلب، إذا لم يكشف مخطط كهربائية القلب الرجفان الاذيني على الرغم من الشك القوي فقد يكون من الضروري توثيق عدم انتظام ضربات القلب باستخدام جهاز الهولتر. ينقسم مرضى الرجفان الاذيني وفقاً لتوصيات الكلية الامريكية لامراض القلب/ جمعية القلب الامريكية/ الجمعية الاوربية لامراض القلب الى أربع مجموعات: الرجفان الاذيني الانتيبابي، الرجفان الاذيني المستمر، الرجفان الاذيني الدائم والرجفان الاذيني الوحيد. مصل الدم من كل عينة تم استخدامه لقياس حالات التليف الخلالي لعضلة القلب مثل(PICP & CITP) ، والتي تم تحليلها بواسطة الاليزا بينما تم قياس ملف الدهون بواسطة جهاز أبوت (Abbott).

كشفت الدراسة أن نسبة مرضى الاناث الى الذكور كانت ١:٢، وأن متوسط الفروق للقيم في مستويات المؤشرات الحيوية (S.PICP, S.CITP, and their ratio) بناءً على نتائج تخطيط صدى القلب وتفسير مخطط كهربائية القلب في حالات الرجفان الاذيني تتضمن العديد من المؤشرات الحيوية التي تعتبر ذات دلالة آحصائية والتي تشمل مجموعات الجنس ، مجموعات أرتفاع ضغط الدم ، مجموعات مؤشر كتلة الجسم ، قطر الأذين الأيسر ، قطر البطين الأيسر ، مجموعات النسبة المئوية للكسر

القذفي ومجموعات الرجفان الأذيني عند $P \leq 0.05$. بينما ملف الدهون ، الارتجاف الاذيني الانتيابي والوحيد عند نسبه S.PICP / S.CITP يعتبر غير معنوي عند $P > 0.05$.

وفقاً الى الخاصية العملية للمستقبل لتحديد فعالية S.PICP & S.CITP للكشف عن التليف الخلالي لعضلة القلب ، فإن المؤشرات الحيوية الأكثر تحديداً وحساسية للتنبؤ بالتليف الخلالي لعضلة القلب هي S.CITP بحساسية ٩٣.٢٪ وخصوصية ٨٣.٣٪ ولها مساحة تحت المنحني ٠.٨٢٩ ؛ يليه S.PICP بحساسية ٨٦.٧٪ وخصوصية ٨١.٧٪ ولها مساحة تحت المنحني ٠.٧٨٢. في حين أن نسبتهم S.PICP / S.CITP ليست محددة لتشخيص التليف الخلالي لعضلة القلب بسبب وجود مساحة تحت المنحني أقل من ٠.٥ مع حساسية ٢٢ ٪ وخصوصية ٩٥ ٪ ، وهذه قيمة مرفوضة في التنبؤ بالتليف الخلالي لعضلة القلب. بينما الخاصية العملية للمستقبل لتقييم فعالية قطر البطين الأيسر والأذين الأيسر في تشخيص التمدد أو التوسع، فإن المقياس الأكثر تحديداً وحساسية لتشخيص التمدد هوالبطين الايسر بحساسية ٨٦.٧٪ وخصوصية ٨١.٧٪ ولديه مساحة تحت المنحني ٠.٦٨٣؛ يليه توسع الأذين الأيسر بحساسية ٧٨.٣٪ وخصوصية ٥٨.٣٪ ومساحة تحت المنحني ٠.٧٢٣. يمكن استخدام مستويات S.PICP و S.CITP المرتفعة للإشارة إلى التليف الخلالي لعضلة القلب في مرضى الرجفان الأذيني. قد يكون مستوى S.PICP بقيمة قطع تزيد عن ١٥٧ *نانوغرام/ ملي لتر* علامة بيولوجية جديدة لتقييم التليف الخلالي لعضلة القلب في مرضى الرجفان الأذيني. يمكن أستخدام مستوى S.CITP بقيمة قطع أكبر من ١٦٥.٥ *نانوغرام/ ملي لتر* كمؤشر حيوي بديل في المرضى الذين يعانون من الرجفان الأذيني المستقل عن التليف الخلالي لعضلة القلب. يعاني معظم مرضى الرجفان الأذيني من تليف خلالي في عضلة القلب وأمراض مصاحبة مثل السمنة وأرتفاع ضغط الدم. أرتفاع مستوى مصل فيتامين سي يؤدي الى أرتفاع مستويات كلا العلامتين S.PICP و S.CITP لدى بعض الافراد في المجموعة الاصحاء، لكن الزيادة في مستويات كل من العلامات S.PICP وS.CITP في مجموعة الرجفان الاذيني تمثل التليف الخلالي لعضلة القلب.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء- كلية الطب
فرع الكيمياء السريرية

دورعلامات ترسيب الكولاجين لتليف الخلالي لعضلة القلب في حالات الرجفان الأذيني

رسالة مقدمة الى

جامعة كربلاء - فرع الكيمياء السريرية - مجلس كلية الطب

كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

مصطفى حميد مهدي غافل

بكالوريوس علوم كيمياء / جامعة واسط / ٢٠١٧

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دائرة صحة واسط

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