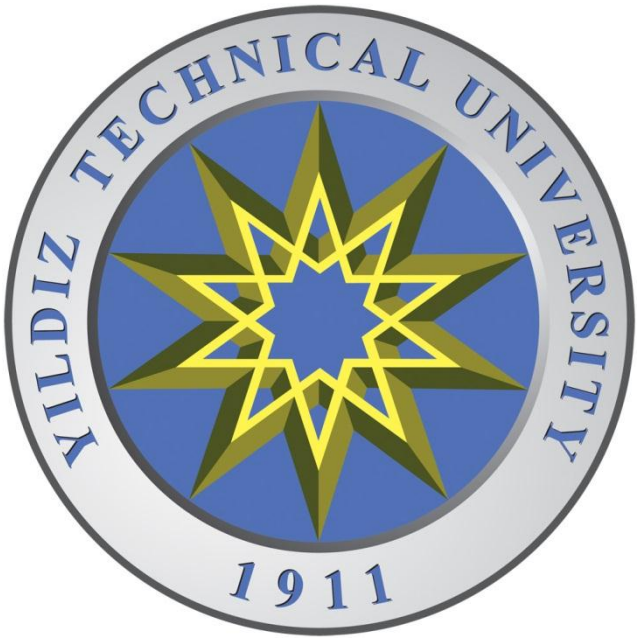

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CLINICAL VALUE OF CIRCULATING MICRORIBONUCLEIC ACIDS MIR-1 AND MIR-21 IN EVALUATING THE DIAGNOSIS OF ACUTE HEART FAILURE IN ASYMPTOMATIC TYPE 2 DIABETIC PATIENTS

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A thesis submitted by Mutaa Abdalmutaleb Abd ALHAYALI in partial fulfillment of the requirements for the degree of **PhD OF SCIENCE** is approved by the committee on 09.09.2019 in Department of Chemistry

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Mutaa Abdalmutaleb Abd ALHAYALI

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LIST OF ABBREVIATIONS

AGE	Advanced glycation end
Bcl-6	B- cell lymphoma 6 protein
CADM1	Cell adhesion molecule 1
CRD	Carbohydrate-recognition domain
CSCs	Cardiac stem cell
DFU	Diabetic foot ulcers
DICER	Endoribonuclease DICER
DR	Diabetic retinopathy
FASL	FAS ligand is type 2 transmembrane protein
GAS5	Growth arrest specific 5
GBM	Glioblastoma
HAND2	Heart and neural crest derivatives expressed protein 2
HCC	Human hepatocellular cancer
HDAC4	Histon deacetylase 4
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HSP60	Heat shock protein 60
HVD	Heart valve disease
IL	Interleukins

INSR	Insulin reseptor
IR	Insulin resistance
KCNJ2	Potassium voltage – gated channel sub family J member
KRLB	Kinase regulation ring bound
lncRNA	Long non coding RNA
MAP k	Mitogen-activated protein kinases
NSCLC	Non – small – cell lung carcinoma
NYHA	New York Heart Association
PDCD4	Programmed cell death 4 protein
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PKCe	Protein kinase C epsilon
PLEKHA1	Pleckstrin homolog domain containing A1
PTB	Phosphor tyrosine-binding
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
qPCR	Quantitative polymerase chain reaction
RAS	Renin angiotensin system
RISC	RNA – induced silencing complex (nuclease activity)
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SCAD	Silent Coronary Artery Disease
SHP2	Serine homology
SPRY1	Sprout protein homolog 1
SRF	Serum receptor factors
STAT3	Signal transducer and activator of transcription 3
TIPE2	Tumor necrosis factor α induced protein 8 like 2
TMEM 49	gene Transmembrane protein 49
UTR	Un-translating region of miRNA

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ABSTRACT

CLINICAL VALUE OF CIRCULATING MICRORIBONUCLEIC ACIDS MIR-1 AND MIR-21 IN EVALUATING THE DIAGNOSIS OF ACUTE HEART FAILURE IN ASYMPTOMATIC TYPE 2 DIABETIC PATIENTS

Mutaa Abdalmutaleb Abd ALHAYALI

Department of Chemistery

PhD Thesis

Adviser: Assoc. Prof. Dr. Volkan SOZER

Co-adviser: Prof. Dr. Hafize UZUN

Objective: To investigate whether the circulating (microRNA-1 and 21) miR-1 and miR-21 expression might be used in the diagnosis of heart failure (HF) and silent coronary artery disease (SD) in asymptomatic type 2 diabetes mellitus (T2DM) patients and to explore the relationship of these miRs with N-terminal pro-brain natriuretic peptide (NT-proBNP) and galectin-3.

Methods: One hundred thirty-five consecutive patients with T2DM and 45 matched control subjects were enrolled in the study. This study consisted of four groups: control group (mean age: 60.23±6.27 years, F/M:23/22), diabetic group (DM) (mean age: 61.50±5.08, F/M:23/22), DM+SCAD group (mean age: 61.61±6.02, F/M:20/25) and DM+ acute HF group (mean age: 62.07±5.26 years, F/M:20/25).

Results: miR-1 was downregulated in the DM, CAD+DM and HF+DM groups by 0.54, 0.54 and 0.12 fold when compared with controls, respectively. miR-1 levels were significantly lower in HF+DM than DM with 0.22 fold changes ($p<0.001$); and in patients with CAD+DM group with 0.22 fold changes ($p<0.001$). Similarly, miR-21 was overexpressed in patients with DM, CAD+DM and HF+DM 1.30, 1.79 and 2.21 fold when compared with controls, respectively. An interesting finding is that miR-21 expression is significantly higher in HF+DM group compared to CAD+DM group.

miR-1 was negatively correlated with NT-proBNP (r=-0.891; p<0.001) and galectin-3 (r=-0.886; p<0.001) in HF+DM group. miR-21 showed strongly positive correlation with (r=0.734; p<0,001) and galectin-3 (r=0.764; p<0.001) in HF+DM group. Conclusion: This results suggest that the circulating decreased miR-1 and increased miR-21 expression is associated with NT-proBNP and galectin-3 levels in acute HF+DM. Especially miR-21 expression might be useful in predicting the onset of acute HF in asymptomatic T2DM patients. miR-21 expression is more valuable than miR-1 in predicting cardiovascular events of acute HF and the combined analysis of miR-21 expression, galectin-3 and NT-proBNP can increase the predictive value of miR-21 expression.

Keywords: Asymptomatic type 2 diabetes mellitus; acute heart failure; silent coronary artery disease; NT-proBNP; galectin-3; miRNA-1; miRNA-21.

ÖZET

Dolaşımdaki mikroribonükleik asitler miR-1 ve miR-21 ' in klinik değerinin Tip 2 diabetik hastalarda akut kalp yetersizliği teşhisindeki önemi

Mutaa Abdalmutaleb Abd ALHAYALI

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Amaç: Tip 2 diabetes mellitus (T2DM) hastalarunda dolaşımdaki (microRNA-1) miR1 and (microRNA-21) miR-21 ekspressiyon kalp yetersizliği ve sessiz koroner arter hastalığı teşhisinde kullanılıp kullanılmayacağının ve mikro RNA lar ile N-terminalpro-brain natriuretic peptide (NT-proBNP) ve galektin-3 ile olan ilgilerinin belirlenmesidiri.

Metodlar: T2DM lu 135 hasta ile 45 kontrol çalışmamıza dahil edildi.Çalışmamızdaki 4 grup: kontrol grubu (ortalama yaş: 60.23±6.27, K/E:23/22), diabetik grup (DM) (ortalama yaş: 61.50±5.08, K/E:23/22), DM+Koroner Arter Hastalığı grup (ortalama yaş:61.61±6.02, K/E:20/25) ve DM+ akut kalp yetmezliği grubu (ortalama yaş: 62.07±5.26,K/E:20/25).

Sonnçlar: Kontrol gruplarıyla mukayesese edildiğinde miR-1, DM, Koroner Arter Hastalığı+DM ve KalpYetmezliği+DM gruplarında 0.54, 0.54 and 0.12 kat azaldı. ($p<0.001$); Bununla beraber miR-1 düzeyi Kalp yetersizliği+DM grubunda, Koroner Arter Hastalığı+DM ve DM gruplarından 0.22 kat düşük bulundu. ($p<0.001$). Benzer şekilde, miR-21 kontrol grubuyla mukayase edildiği zaman DM, Koroner Arter Hastalığı+DM ve Kalp Yetmezliği+DM gruplarında sırasi ile belirtilecek şekilde 1.30, 1.79 ve 2.21 kat arttı ($p<0.001$). İlginç bir bulgudur ki miR-21 ifadesinin Kalp Yetmezliği+DM grubunda Koroner Arter Hastalığı+DM grubuna göre arttığıdır. miR-1 HF+DM grubunda NT-proBNP ve galektin 3 ile negatif korolesyon göstermiştir. Sırası ile ($r=-0.891$; $p<0.001$) ve ($r=-0.886$; $p<0.001$), akut Kalp Yetmezliği+DM.grubunda miR-21 pro BNP ve galaktin 3 ile yüksek pozitif korolasyon gösterdi sırası ile ($r=0.734$; $p<0.001$) ve ($r=0.764$; $p<0.001$).

Tartışma: Bu sonuçlar bize gösterir ki KalpYetmezliği+DM gurubunda azalmış miR-21 ifadesi ve artmış NT-proBNP ve galektin 3 düzeyleri bize; DM hastalarında akut kalp yetersizliğinin başlayabileceğini göstermektedir. Akut kalp yetersizliğinin başlangıcını anlayabilmek için miR-21 ifadesin incelenmesi miR-1 ekspressiyonunu incelenmesinden daha değerlidir ve galektin-3 ve NT-proBNP ile beraber bakıldığında miRNA-21, bize kalp yetersizliğinin başlayabileceğini gösterebileceği anlaşılmaktadır.

Anahtar Kelimeler: Asymptomatik tip 2 diabetes mellitus; akut kalp yetersizliği; sessiz koroner arter hastalık; koroner arter hastalık NT-proBNP; galectin-3; miRNA-1; miRNA-21

CHAPTER 1

INTRODUCTION

1.1 Mini Literature Review

Diabetes is considered as a big problem causing death around the world. Diabetes mellitus type 2 (T2DM) has a negative influence on the prevalence, presentation, severity and prognosis of coronary artery disease (CAD) [1]. T2DM and heart failure (HF) are also common companions in clinical practice. For many years, HF was noted to be a complication of diabetes [2]. Increasing numbers of older patients with diabetes, and their improved survival from cardiovascular events will undoubtedly see a massive increase in patients with both diabetes and HF. Accurate diagnosis of HF is important because the morbidity and mortality of HF is high. A quarter of those with chronic HF have diabetes and over 40% of these patients are hospitalized with worsening HF [1,3].

The natriuretic peptides are a family of ring shaped vasoactive hormones showing considerable sequence homology. There are 4 types of natriuretic peptides A, B, C, D. One of them is N-terminal pro-brain natriuretic peptide (NT-proBNP) which is a prohormone consisting of 76 amino acid. The N-terminal is inactive protein which is splitted from the molecule to formation brain natriuretic peptide (BNP). Both BNP and NT-proBNP are manufactured in response to ventricular stretch and ischemic injury, so their concentrations are important to detection of HF. However, NT-proBNP represents a useful biomarker in the diagnosis and risk stratification of patients with chronic HF because of its more stable form in serum after blood collection. Increment of circulating NT-proBNP levels have used as a biomarker for the apprising of cardiovascular disease [4,5,6,7]. Moreover, NT-proBNP is a potential marker of subclinical atherosclerosis in patients with T2DM [8] and elevated its level in blood is a strong predictor of cardiovascular death [9].

Galectin-3 is a member of the lectin family composed of a protein which is encoded by one gene. One of its features is ability to binding with β-galactosides due to possessing recognition domain of carbohydrate. Galectin-3 contributes in an inflammatory response, repairing tissues addition to demodulation of cardiac ventricular. It is considered a strong biomarker and predictor in HF and recently used to estimation death rates, especially in patients with DM and HF [10,11,12, 13,14].

miRNAs are small non-coding RNAs, with an average 22 nucleotides in a single spiral. Numerous studies have demonstrated that miRNAs can be released into extracellular fluids. miRNAs have hormone-like activities because extracellular miRNAs can be delivered to target cells and they may act as autocrine, paracrine, and/or endocrine regulators to modulate cellular activities [15]. Extracellular miRNAs play a very crucial role in many biological processes such as cancer development, immune system, epithelial-to-mesenchymal transition and fibrosis, in various types of cardiac diseases, and in DM [16]. Dysregulation of miRNA-1 and miRNA-21 was observed in HF [17].

1.2 Objectives of the Thesis

Studies in recent years have shown that the prevalence of heart failure in patient with diabetes is very high, and the prognosis for patients with heart failure is worse in those with diabetes than in those without diabetes [18]. Various epidemiologic data also have shown that pre-diabetes is associated with a high risk of heart failure and suggest an age-adjusted hazard ratio (HR) between 1.2 and 1.7 in different populations of patients with impaired fasting glucose, [19,20] although not confirmed in all studies [21]. Prevalence of pre-diabetes and diabetes is high among patients with heart failure and proves as a relevant predictor of prognosis [18].

miRNAs are small single-strand RNA molecules that influence their target genes at a posttranscriptional level, thereby regulating many biological processes have shown to be involved in regulating beta cell function, insulin response, glucose homeostasis, as well as the pathogenesis of diabetic vascular complications [22,23]. Research in this field has highlighted new mechanistic links between diabetes and cardio vascular disease CVD [24], with many evidences proving the involvement of distinct miRNAs in the pathological steps that lead to atherosclerosis.

Increased incidence of ischemic heart failure (HF) occurs in patients with diabetes mellitus even in the absence of larger infarct size, lower left ventricular ejection fraction (LVEF), or reduced infarct artery patency [25,26].

Therefore, this study aimed to investigate whether the circulating miR-1 and miR-21 expressions might be used in the diagnosis of SCAD and in acute HF with asymptomatic T2DM patients and without diabetes and to explore the relationship of these miRNAs with NT-proBNP and galectin-3.

1.3 Hypothesis

Increased morbidity and mortality associated with ischemic heart failure HF in type 2 diabetic patients requires a deeper understanding of the underpinning pathogenic mechanisms. However, the molecular mechanisms by which diabetes contributes to worsening ischemic HF remain unclear [27]. Patients with T2DM had a poor prognosis due to silent CAD (SCAD) and acute HF, and therefore it is important to define any early predictors of SCAD and acute HF in patients with T2DM. In addition, there is not adequate information in the literature regarding the relationship between SCAD and acute HF patients with T2DM and miR-1 and miR-21 expressions..

CHAPTER 2

LITERATURE REVIEW

2.1 MicroRNAs

MicroRNAs (miRNAs) are a group of short non-coding RNA molecules containing about 21 – 27 nucleotides. They are regulatory molecules of gene expression in eukaryotic species in both normal and pathologic cellular process at the level of post-transcriptional [28,29]. miRNAs are related to the modifying of expressing of gene, by disable or activation transcription via very specific-sequence DNA/RNA binding [30]. In 1993 the first miRNA was explored in Caenorhabditis elegans. The greatest effect of miRNAs have not been recognized until 2000s [31]. About 2000 human miRNAs are recognized by using cloning and sequence analysis. They have an important role in the regulation of target genes. They are binding to complementary regions of messenger transcripts for repressing there translation [32], in 3'-untranslated region (3'-UTR) of mRNA [33].

Mature miRNAs are bound with the 3-UTR of their target region in mRNA and negative regulation of expression in gene occurs via smashing or inhibition translation. Because the ability of new miRNA to regulation the expression of more than one of its target genes, it is considered as a transcriptional factor [34,35,36]. Binding results in mRNA degradation or impaired translation and subsequent decreased protein expression [36].

miRNAs can post transcriptionally silencing protein expression by conjunction with complementary target mRNAs and degrading them, or by malfunctioning translation of mRNAs to proteins [37].

The function of miRNA is regulation of gene expression. It has been predicted that miRNA could regulate about 60% of the whole human genome. miRNAs are abundance present in all human cells. It is thought that each miRNA has more than one of

conserving targets and non-conserving targets. It has been suggested the possibility of each miRNA for regulating an unlimited number of targets [38,39].

miRNAs have a significant presence in differentiation, development of programmed cell death. Addition to the limitation of final phenotype of cancer cells [40], like the let-7 family of miRNAs that target oncogenes leading to inhibition tumor growth [41]. Also they mediate intracellular communication [42], and regulate mRNA which is encoding proteins alter cellular functions. miRNAs involve in stable equilibrium processes in body and pathophysiology demolition in disorders and abnormalities cases [43]. More than one miRNA can be responsible for one gene target. Through regulation of gene expression, miRNAs take part in the regulation of many process in cell, including cholesterol metabolism [44], post-implantation developing [45], synthesis of insulin [46], and hematopoiesis [47]. Modulation of miRNA occurs by exercise [48], chemicals in environment [49] and nutrition factors [50, 51,52,53]. Also miRNAs are regulators in pathogenicity of diseases, as in cardiovascular disease [54]. miRNAs have vital role in animal development by regulating multiple cellular processes including cell fate specification, cell signaling, and tissue morphogenesis [55,56]. In mammals, miRNAs are able to modulate of differentiation hematopoietic lineage. Modern computational speculation of miRNAs target sites explains their participation in an interactive regulatory network of gene [57,58, 59].

Many human diseases, including cancer, may occur due to changes in expression of miRNA [60]. miRNAs may down-regulated during tumor development, resulting in up-regulation of their target genes, so they work to suppress genes of tumor and targeting cell cycle [61]. In similar way, a group of miRNAs are up-regulation through tumorigenesis causative down-regulated of their targets that are genes of suppressing tumor [62].

2.1.1 Biogenesis of miRNAs

The biogenesis of miRNA is a critical process in all organisms because an alteration to any step may cause downstream effects. Transcription a primary miRNAs which may contain single or cluster miRNAs, resulting from transcription genes of miRNA from genome [31]. Primary miRNAs are generated from RNA polymerase II and production from introns and inter-genic regions. These molecules include one or more short sequence that formation structure of thermodynamic stable hairpin [29]. Processing of miRNAs consist of multiple enzyme and proteins like exportin-5 that binding to hairpin

and transfers it outside of nucleus [63]. In cytoplasm, the pre-miRNA reacts the RNase III (DICER) which is binding with its protein (TAR RNA binding protein) to hairpin. The complex of protein –RNA permits DICER to split hairpin to formation 21-23 nucleotides dsRNA which forms mature miRNA and its complement [64,65]. Finally, it is treated with Argonaute protein (AGO2), which is composed of RISC "RNA induced silencing complex". RISC chooses the mature miRNA strand and transfers to the target mRNA where it links with a targeted sequence in the 3′ UTR of the message as in Figure 2.1.

Complex of miRNA: mRNA will contribute to mRNA degradation or translational repression of the message [66]. Dysregulation of components of miRNA –processing lead to disturb the product and appearing disease [67].

miRNAs have function in the pathogenesis and prognosis of disease like neurodegenerative disorder [68], gastrointestinal diseases[69], diabetes[70], cancer and its resistance against chemotherapy [71] and cardiovascular system [72], neurodegeneration, rheumatoid arthritis [73]. Studies show the dysregulation of the miRNA expression in tissue of placenta contributes in pathogenesis of preeclampsia [43,74].

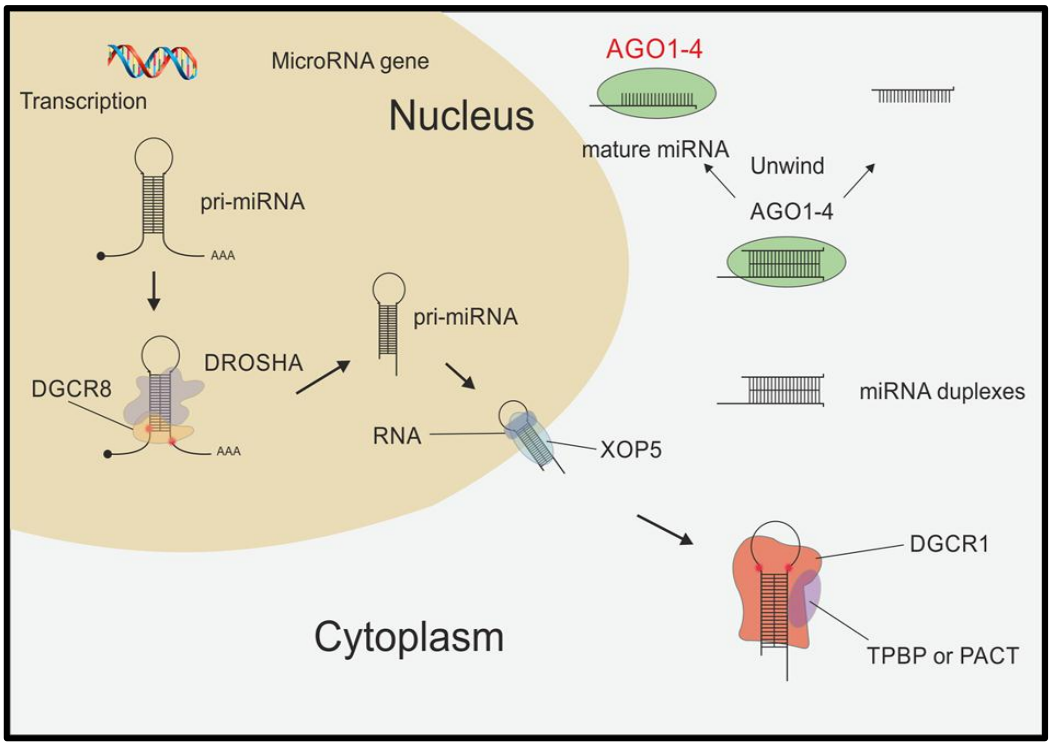

Figure 2.1 miRNA biosynthesis pathway [67]

2.1.2 Biochemistry of miRNAs

miRNAs are more stable compared wto mRNA profiles. Contrasting of mRNAs resides, miRNAs work as an essential regulators of many genes. Therefore, miRNAs give more truly physiological alterations. Circulating miRNAs are more resistance to activity of endogenous ribonuclease. They are certain stable [75], due to existence materials binding with them such as exosomes in blood [76]. miRNAs have been seemed to be stable 10 times more than mRNA, the half‑life of which is ~10 h as in miR‑125b (half‑life, 225 h) [77]. Pure structural features of miRNAs presented in form related with exosomes, and the soluble form with RNA‑binding proteins [78]. Advances in PCR, RT‑qPCR platforms, are confirmed that quantitative analysis of miRNAs from tissue specimens and plasma/serum has more easier and accurate [79].

2.1.3 miRNA's Structure

Production of mature miRNA was reduced affected by lack structural stability of the hairpin that resulting from many variants in the pre-miRNAs [80]. The ability of drosha to recognizing and processing pre-miRNA will be decreased when there was a secondary structure of pre-miRNA far from the standard hairpin. This little variation in sequence leads to strong effects [81]. Through pre-miRNA biogenesis, the situation of split sites of drosha may changes due to stable hairpin structure resulting from mutation in sequence of primary miRNA. Presence of seed regions in mature miRNA belongs to deleting shift the split sites of dicer [82,83]. miRNAs are found also in all fluids in body like serum, plasma, saliva. They are surrounded by membranous vesicles such as micro-particles and exosomes which are protected them from digestion by RNAse [84,85,86,87]. Extracellular miRNAs have vital role in regulation immune response, cellular migration and differentiation and in cell-cell communication [88]. According to above, they are good marker for detection and prediction for different diseases and [89,90,91], metabolic disorders as in case with T2DM [92].

2.1.4 Mechanisms of Action of miRNA

The corresponding among targeted gene (mRNA) and sRNA , miRNA –RISC intercede repression gene can be sectioned in 3 processes : (a) fissure at specific site (b) translational inhibition (c) enhance mRNA degradation (10).

miRNA will cleavage specifically if the mRNA has complementarity to the miRNA or productive translation will be repress. If the mRNA does not have efficient complementarity to be cleaved it has suitable constellation of miRNA complementarity site [93]. The interaction of miRISC – mRNA may executes to some manners of direct and indirect in suppression of translation [83]. Direct modes include: (a) initiation block : translation initiation is inhibited by interfering of miRISC with elF4F- cap and through weaken correlation of "60s subunit" and blocking formation of "80s subunit" of ribosome.

(b) Prohibition of post-initiation: declining ribosomal premature, so "40s /60s" ribosomes were prevented of joining during elongation process [94,95]. Indirect mode occurs by mRNA deadenylation by glycine – tryptophan protein and degradation [96,97]. Subsequent 50 terminal cap was discarded by specific enzyme work to removing the cap [94] as shown in Figure 2.2.

Human miRNAs located among genes coding protein, while one-third of them are located within the introns of mRNA. So they are under the control of the promoter responsible for transcript of primary mRNA. Strong association expressions are noticed between miRNAs and their host genes. That give us evidence that processing of miRNAs and their host genes are from the same primary transcripts [98].

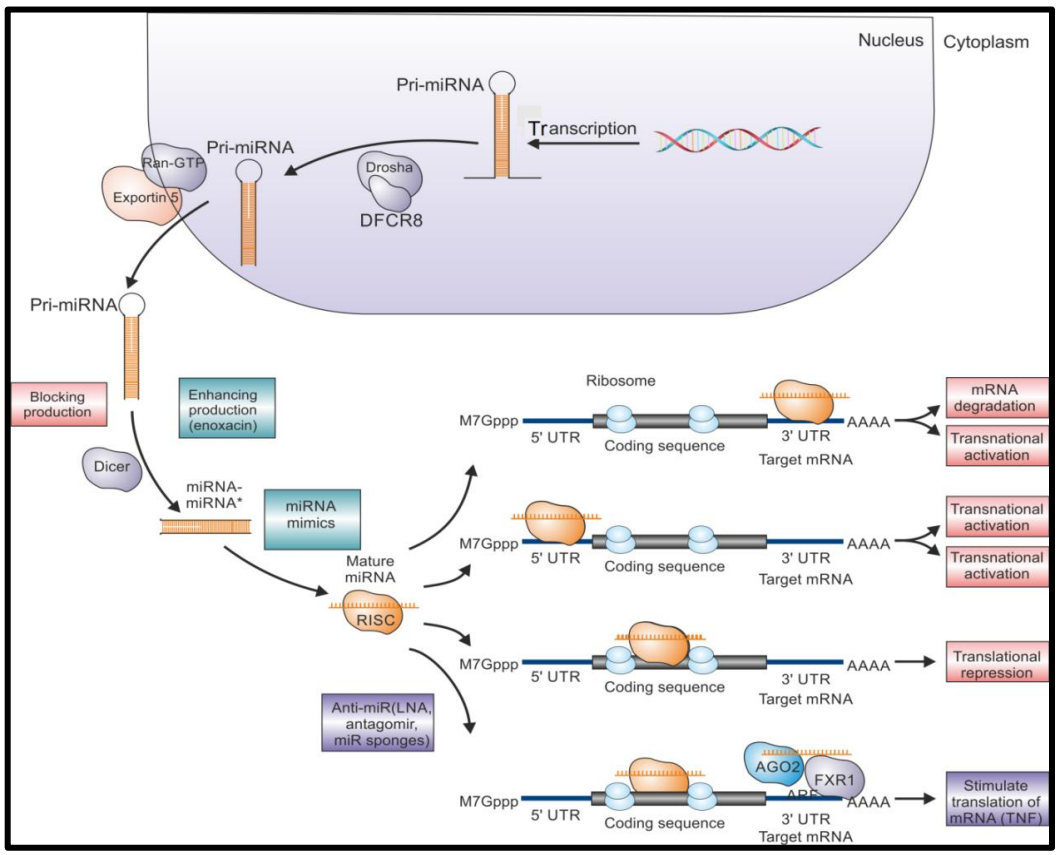

Figure 2.2 Mechanism of action of miRNAs and use of therapeutic agents to block or activate their function [99]

2.1.5 miRNAs As Serum Biomarkers

Serum miRNAs are highly stable. Being resistant to RNase A digestion and other harsh conditions like (low/high pH, boiling, extended storage and freeze/thaw cycles). That makes them ideal for using in the clinical setting as biomarkers. Their expression profiling have demonstrated the presence of different miRNAs expression patterns in pathological condition, colorectal cancer, type 2 diabetes [100], diffuse large B-cell lymphoma [101] and ovarian cancer [102]. As well as physiological conditions such as pregnancy [103]. It can be used miRNAs as markers to follow -up progression of pregnancy and gestational diseases such as preeclampsia [104].

2.2 miR -1

miR-1 is high expression in tissue or cell, but little expression in another cells [105]. It is a critical mediator of cell proliferation and differentiation in cardiac [106,107] and skeletal muscles [108]. In particular, miR-1 is highly enriched in cardiac and skeletal muscle cells as compared to non-striated muscle tissues during animal development and in adults [106,107,108] and has the following sequence:

>hsa-mir-1MI0000651

UGGGAAACAUACUUCUUUAUAUGCCCAUAUGGACCUGCUAAGCUAUGGA
AUGUAAAGAAGUAUGUAUCUCA

It promotes cardiac and skeletal muscle gene expression and muscle differentiation, in part, by repressing the key transcriptional regulators histone deacetylase 4 (HDAC4) [108] or hand2 [109]. It has been shown that tissue-specific expression of miR-1 is dependent on SRF in the heart [109] and Myo D family of transcription factors in skeletal muscle [108,110]. miR-1 is exceedingly abundant in muscle that prevents proliferation of progenitor cells and enhancement myogenesis [108,109,111]. It is expressed in developing skeletal muscle and heart [112, 113, 114].

miR-1 is the first miRNA that has been explored and proved to be a regulator of cardiac development and disease [109,115,116,117]. Cardiac ischemic arrhythmias are developed when miR-1 targets KCNJ2 gene. Some reports confirmed that miR-1 cause severe cardiac infection by influence of the expression of protective proteins in host [115,116]. miR-1 has ability to inhibition cardiac hypertrophy by affecting the growth-related targets, including Ras GTPase-activating protein (RasGAP), cyclin-dependent

kinase9 (Cdk9), fibronectin, and Ras homolog enriched in brain (Rheb) [118]. miR-1 is important for both cardiogenesis, through the regulation of Notch signaling, and skeletal muscle growth during embryonic development of Drosophila. Analysis of the presumptive miR-1 promoter has shown that miR-1 expression is regulated by SRF, MyoD, MEF2, and Twist, all factors known be important in conferring muscle-specific expression [119,120].

miR-1 has direct regulation on protein kinase C epsilon (PKCe) and heat shock protein 60 (HSP60), so this demonstrates that miR-1 is an important factor in cardiac injury [120,121,122]. miR-1 protects heart from hypertrophy and HF by regulation some hypertrophy-related genes like apoptosis regulators, ion channels [123,124,125,126,127]. miR-1 is down-regulated in symptomatic heart failure patients and there was a reduction of its expression in severity of NYHA class [17]. It has a potential therapeutic role against lung cancer [128,129,130]. A study indicates that miR-1 is down-regulation in thyroid carcinogenesis [131]. Expression of miR-1 considers a prognostic marker in patients with breast cancer [132]. miR-1 affects cardiomyocyte growth by negatively regulating expression of calmodulin and calmodulin-dependent nuclear factor in activated T cells (NFAT) signaling, it also targets the 31UTRs of several gene transcripts important in cardiomyocyte growth. These include myocyte enhancer factor 2A (Mef2a) and GATA binding protein 4 (Gata4), and targets key cardiac-specific transcription factors, notch ligand, Delta like 1 (Dll-1), Iroquois-class home domain protein (Irx-5) and heart and neural crest derivatives expressed 2 (Hand2). A further target gene is potassium voltage-gated channel subfamily D member 2 (Kcnd2) [128].

2.2.1 miR-1 Regulates Electrical and Contractile Activity of the Heart

Efficient contraction of heart is the result of highly organized conductive system and effective electromechanical coupling. Electrical stimulation proceeds from apex toward the base in ventricles and repolarize in reverse order [130]. Spatial heterogeneous action-potential duration and conductance guarantee depolarization and repolarization propagate orderly. To ensure the heterogeneity, heart has adopted a spatial specific transcriptional network that tightly regulates expression of ion channel and gap junction. When stimuli reach working cardiomyocyte, it triggers release of calcium which binds to troponin C followed by myosin-actin cross-bridge formation. In this process, myosin light chain kinase (MLCK) potentiates the force and rate of cross-

bridge recruitment in cardiomyocyte and may serve as a major target in regulation of cardiac contraction [132,133].

miR-1 homozygous null mice show prolonged QRS complex, prolonged PR and QT intervals on surface electrocardiograph, indicating defects in atrioventricular (AV) and ventricular conduction. Echocardiography also reveals severely impaired fractional shortening with poor systolic function [134]. miR-1 over expression mice develops frequent atrioventricular block of varying degree as evidenced by prolonged PR interval. The transgenic mice also show impaired contractile and diastolic function that might due to damaged sarcomere assembly [135,136]. Disorder expression of miR-1 results in defects of electrophysiology and contractions in heart causative severe destruction and shortening systolic function which can be discovered by echocardiography [133,134,135,136]. miR-1 over expression appears impairment sarcomere assembly causative obstruction contractile and diastolic function [137,138]. miR-1 and miR-133 are specifically expressed in adult cardiac and skeletal muscle tissues, but not in other tissues tested [139,140].

2.3 miR- 21

miR-21 is aplenty expression in mammalian cells which have up-regulated related to some types of cancer [141], and other diseases in association with regulation of cell proliferation and apoptosis. The coding gene for miR-21 in human is found on chromosome 17q23.2 [142] over lapping with the TMEM 49 gene [143,144] and has the following sequence:

>hsa-mir-21 MI0000077

UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAAC
ACCAGUCGAUGGGCUGUCUGACA

The indication of aberrant expression of miR-21 is came from the profiling of miRNA of human glioblastoma (GBM), brain tumor of glial origin [145]. miR-21 participates in tumors development including cell proliferation, migration and metastasis, by variety mechanisms [146,147]. miR-21 relates to growth of the endocrine pancreas, insulin secretion regulation, and took part in β-cell dysfunction in T2DM [148], glucose homoeostasis, angiogenesis, inflammatory response modulation and complications of micro-and macro-vascular [149]. Suppression expression of mir-21 has an therapeutic effect on diabetic nephropathy [150]. miR-21 is identified a key molecule associated

with a wide range of cancers like in breast cancer which is diagnosing by a raising circulating level of miR-21 [151]. miR-21 over expression was noticed in some solid tumors like colon, breast, gastric and pancreatic cancers [64,152]. Aberrant miR-21 expression has an effect on the growth of HCC (Human hepatocellular cancer) and spread by alteration of phosphatase and tensin homolog (PTEN) expression. The PTEN-dependent pathways included mediating phenotype of cancer cells features like growth, migration and invasion [153]. miR-21 can enhances oncogenesis and progression of various carcinomas through targeted tumor suppressors [154]. miR-21 is up-regulation in leukemic, and its expression rises in patients with chronic lymphocytic leukemia [155]. High level of miR-21 was noticed in cancer cell lines, it represents about 15–25% of other miRNAs [156], pathological stress cells [157,158] and in vascular after balloon operation [159]. Elevated concentration of miR-21 is not a featured for cancer cells but also considers a distinguished feature of pathological cell growth [158]. Also high levels of miR-21 are observed in vascular walls after balloon injury [159]. Low expression miR-21 diminishes formation of neointima in rat artery after a surgery via influencing both apoptosis and proliferation of smooth muscle in vascular [158].

It is clearly that expression of miR-21 is up-regulation in cardiac hypertrophy, promotes cardiac fibrosis and save cardiomyocytes from apoptosis [127]. In cardiac fibrosis, miR-21 has been an effective role [160,161]. mir-21 has ability to impaired cardiac fibroblasts proliferation, using CADM1/STAT3 pathway, targeting CADM1. Serum miR-21 showed potential diagnostic values on heart failure (HF) due to heart valve disease (HVD) [162].

miR-21 is involved in the pathogenesis of proliferative vascular disease such as coronary heart disease [163,164]. It is responsible of cell dysfunction in patients with coronary artery disease by influence of super oxide dismutase expression [165]. miR-21 is important for keep survival of c-kit+ CSCs and allows to proliferation in it [166].

Recent studies indicate to the effect of miR-21 in the immune system function regulation [167]. It keeps non-activated T-Cell and antigen presenting cells in less levels of expression [168]. miR-21 was promoting inflammation and autoimmune disease pathogenesis involving psoriasis, T1DM, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus [64].

miR-21-5p is an “oncomiR”, and non-regulated in cancers [16], it has been classified as an “inflammatory miR”, because its effective role in modifying inflammation and tissue repairing [169,170].

2.3.1 Regulation of miR-21

Detection of miR-21 can be able in mature mast cells, neutrophils, T and B cell. Many extracellular and intracellular signaling molecules participate in regulation of miR-21 expression. It works as a good indicator for activation of immune response, due to ability of IL-4, and lipopolysaccharide to stimulate the miR-21expression in monocytes [171], by activation of transcriptional factors. Positive regulation of miR-21 expression resulting from association among p65 and miR-21 in promoter region [172] inhibits of miR-21 expression occurs when binding transcription repressor Bcl-6 with miR-21 in promoter region [173].

A study indicates that negatively regulated of miR-21 expression related to lncRNA GAS5 "growth arrest specific 5" [174].

2.3.2 Targets of miR-21

Tumor-suppressor genes are the targeting genes of miR-21, such as Akt and p53 activity [175], and transforming growth factor beta [176]. miR-21 has effects in variety biological processes, as cell differentiation, proliferation, and apoptosis [177].

miR-21 enhances immune response, plays great role in autoimmune diseases. Down – regulation of (PDCD4, TIPE2 and FASL) impairs apoptosis of T cells [168,172,178,179]. Down-regulation of miR-21 target (SPRY1) leads to activation of T cells [180], while down-regulation of (PLEKHA1 and CXCR4) causative inhibition of T cell activation [181]. In cardiovascular system (SPRY1, PDCD4 and PTEN) are targeted genes of miR-21 [182].

2.4 Diabetes Mellitus

Diabetes mellitus (DM) is a combination of heterogeneous disorders. DM presents with hyperglycemia and glucose intolerance, as a result of lack of insulin, defective insulin action, or both [183]. Such complications arise due to derangements in the regulatory systems for storage and mobilization of metabolic fuels. These complications include catabolism and anabolism of carbohydrates, lipids and proteins emanating from defective insulin secretion, insulin action, or both [184]. Also DM can be defined as a

group of metabolic disorders characterized by a chronic hyperglycemic condition resulting from defects in insulin secretion, insulin action or both. Permanent neonatal diabetes is caused by glucokinase deficiency, and is an inborn error of the glucose-insulin signaling pathway [185]. Relationship between DM and elevated mortality makes it the direct reason of the most healthy problems and death in world [186, 187]. DM is related with a different implications, like personal, family, social and high cost for the National Health System for each country due to long hospitalization, diagnostic tests [188,189,190,191,192]. The prevalence of DM has been risen at serious rates all over the world [187,193].

Complications of diabetes are vascular originally. In diabetic patients, retinopathy (DR) is one of the most complications which causing a new-onset blindness [194,195,196].

Types of diabetes mellitus is classified according on its etiology and clinical presentation. As such, there are four types of diabetes mellitus: type 1 diabetes, type 2 diabetes, gestational diabetes, and other specific types [183,197].

2.4.1 Type 1 Diabetes Mellitus

T1DM occurs when the β-cells in the pancreas could not able to producing enough amounts of insulin causative elevated blood sugar levels. In many cases, immune system attacks β-cells by mistake, it can considers as "an autoimmune disease" [141].

2.4.2 Type 2 Diabetes Mellitus

T2DM is a disorder in metabolism takes place because of insulin resistance and/or deficiency, it represents 90% of diabetic patients [198,199]. Insulin Resistance (IR) means malfunction in response to insulin in cell and inefficiency of insulin amounts to access glucose balance. IR is a good marker of T2DM [141]. Hyperglycemia causes vascular destruction in T2DM patients [200,201]. Combination of genetic, environmental factors and life style are an essential reasons of T2DM occurrence [202].

Increased weight and obesity epidemic, belong to diet, rareness of practicing sports and activities and interfere with genetic readiness, have an influence of the prevalence of diabetes [200]. Type2 diabetes mellitus (T2DM) was considered a disease of adults, now is widespread in children and teenagers [201]. The increasing incidence of obesity in children and the resultant insulin resistance contributes to the increasing prevalence of T2DM in this population.

2.4.3 Risk Factors for the Incidence of Type2 Diabetes Mellitus

Many factors increase the incidence of T2DM, like gender (female), age (increasing age), diet, lack of sleep, nutritional supplement received by the mother during pregnancy. But the essential factors that play critical role are [199, 203]:

A) Lifestyle: which includes stress, obesity, diet, consumption high quantities of sweets and lack of physical activity

B) Genetic factors: are associated with a family history of DM and found several genes related with the incidence of T2DM.

C) Other medical problems: these include tow essential points first of them use of some medication as diuretics, antihypertensive and antipsychotic treatments. The second suffering from diseases like gestational diabetes, Cushing's syndrome, hyperthyroidism and glucagonomas

2.4.4 Insulin Receptor (INSR)

Insulin is anabolic hormone which enhances of metabolism, energy homeostasis [204]. Insulin signaling has been started by interaction of ligand-receptor. Insulin binding with tyrosine kinase enzyme which represents its receptor involving auto phosphorylation and mediating a steps of phosphorylation reactions, leading to insulin action [205]. It founds in all vertebrate tissues with the highest concentration in the major metabolic organs such as muscle, adipocytes and hepatocytes [206,207]. The receptor consists of 2 subunits of each α and β. The α subunits are localized outside of the cell (extracellular domains) of the receptor complex and bind to insulin. β subunits constitute transmembaranous and intracellular components of the receptor complex. The beta subunits are binding by disulphide linkages. When insulin binding to the alpha subunit, the beta subunit undergoes auto phosphorylation resulting in activation of a cascade of phosphorylation [205]. Decrease in INSR in T2DM patients was confirmed the important role of INSR to maintain insulin sensitivity. In some studies, the augmenting of both miR-195 and miR-15b were confirmed in the animals with obese T2DM and conjugated with down-regulation of INSR. A result of interconnection between these miRNAs and 30-UTR of INSR, a detention of insulin signaling in hepatocytes occurrence [206].

2.4.5 Insulin Signaling

Insulin binding to the α-subunits induces trans-auto phosphorylation of the β-subunits which become activated [208,209]. There are 3 pathways used to activation IR. Phosphatidylinositol 3-kinase (PI3K), Cbl/CAP pathway and mitogen-activated protein kinases (MAP k) [209,210]. The MAP kinase cascade leads to enhanced cell growth, while Cbl/CAP cascade mediates glucose transporting to the plasma membrane [211,212, 213].

Insulin signaling proteins stimulate PI3K cascade to excite insulin metabolic functions [213]. Stimulation of PI-3K creates phosphatidylinositol (3-5) triphosphate, enrolling 3-phosphoinositide– dependent protein kinase-1 and -2 (PDK1 and PDK2) and Akt "which was induced by PDK1 and PDK2 mediated phosphorylation at T308 and S473 respectively" to the plasma membrane [214]. Targets are phosphorylate by Akt, leading to inhibition the synthesis of macromolecules, such "as glycogen synthase kinase-3b and p70S6K" and transcription factor fork head box class O1 (Foxo1). Foxo1 lose its transcriptional activity when phosphorylated by Akt at S253, which influence in regulation of a set of physiological activities such as metabolism, growth of heart muscle and survival [215]. So, phosphorylation of Akt→Foxo1 intermediates the action of insulin and works as an indicator of insulin sensitivity.

Vital molecules in the insulin signaling are Tyrosine-phosphorylated proteins, which are substrates of insulin receptor [214,215,216]. These molecules participate in insulin action through binding of serine homology 2 [217,218,219]. The subunit p85 found in SHP2 and PI3K have a regulatory function in insulin mechanism action [220,221]. Proteins of IRS have the same structure which is featured by existence of an NH2-terminal "pleckstrin homology" (PH) domain close to a phosphor tyrosine-binding (PTB) domain. Subsequent with a different-length of COOH-terminal tail which are phosphorylation sites composed of a number of tyrosine and serine. Elements of cytoskeletal, phospholipids in plasma membrane and protein ligands are interceded the interactions between IR-IRS [222,223]. The domain of kinase regulation ring bound (KRLB) presents just in IRS-2 [224,225], and is in contact with IR in phosphorylation regulatory loop region [211]. Both of IRS-1 and IRS-2 have long tails with 20 phosphorylated Tyr sites [221].

2.4.6 Pathogenesis of Type2 Diabetes Mellitus

The distinguished feature of T2DM is abnormal function of islet cell. Releasing accurate amounts of insulin from β-cell are decreasing compared to elevated level of sugar in blood. Hyperglycemia was quantified according to functional shortage of this islet [226,227,228]. Furthermore, glucagon was produced from α-cells in pancreas motivating production of hepatic glucose. Recently, presence of abnormalities gut hormones as glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic peptide (GIP) are also considered a reason of type 2 diabetes, but it still uncertain [229,230]. Resistance of insulin is the prevalent feature of T2DM, particularity in obesity [231,232]. Impaired insulin secretion and insulin resistance contribute more or less jointly to the development of pathophysiological conditions. Impaired glucose tolerance (IGT) was stimulated through reduction of glucose-responsive early-phase insulin secretion, and insulin secretion reduction after meals causative postprandial hyperglycemia [233,234].

Pathogenesis is supposed existence of genetic defects in regulatory molecules that responsible of glucose metabolism. A study showed that targeted genes responsible of insulin secretion in pancreatic cells and the molecules encompassing mechanism of insulin action have identified as genetic abnormalities that causative pathogenesis as in glucokinase genes, mitochondrial genes, and genes encoding for insulin receptor. Genetic abnormalities in genes -encoding production of insulin, can be considered as a strong causative of pathogenesis. Occurrence of these abnormalities may belong to mutations in the KCNQ1 gene related to insulin secretion [235].

2.4.7 Type2 Diabetes Mellitus and Its Complications

Insulin resistance is an essential reason of T2DM and cardiovascular disease [236]. Serious macrovascular and microvascular complications are resulting from hyperglycemia. Macrovascular complications, cardiovascular deterioration, coronary heart disease and stroke are common in diabetic patients [237]. While, diabetic retinopathy a leading reason of blindness in patients resulting from the microvascular events [238]. Kidneys were also affected by diabetes which causes disruption of glomeruli tubules, obstruction in function of kidney this is called diabetic nephropathy [239]. Nervous system was affected by diabetic, which considers a mean cause of developing chronic diabetic foot ulcers (DFU). The quality persons' lives with diabetes

or one of its related complications was decrease and create many social and economic problems [240,241].

The inflammation status distinctive of T2DM, which causative its severe complications, may be slowed or denied by right nutrition and practice physical activities, regularly. Needing to determined best effective treatment and development of new therapeutic strategies for these complications in patients is still of importance [242,243]. Many studies showed that although using glucose-lowering treatment reduces the risk of occurrence of cardiovascular disease, but the risk of diabetic complications is still unknown due to delayed of interferences are executed after diagnosis of the disease [244,245].

2.5 miRNA and Diabetes Mellitus

2.5.1 miRNAs and Pancreatic β-Cells

miRNAs usually work to target genes responsible of regulation survival and apoptosis of β-cell like Bax and Bcl-2 genes [246]. Concerning with the differentiation of β-cell, the ability of human stem cells to differentiate into cell responsible of producing insulin in vitro gives hope that the regulatory role of miRNAs in this process can be studied. miRNAs have a regulatory roles in β-cell proliferation. For example, miR-375 negatively regulates of Cadm1, the direct target gene, impedes cell growing in various cancer cells lines [247]. On the contrary, miR-181a has positive role due to its reduction action in islets of rats [248]. Expression of miRNAs were estimated, and 4 islet-specific miRNAs (miR-7, miR-375, miR-34a, and miR-146a) give distinctive expressive modes. Many other miRNAs are linked with insulin secretion such as miRNAs (375, 33, 29a, 184, 187, 30a) [249,250], which are regulated by targeting Stx-1a, at-SNARE protein that related with insulin exocytosis [251] .

2.5.2 miRNAs as Type2 Diabetes Mellitus Markers and Its Complications

Pathogenesis of diabetic complications are not easy to follow-up because they are featured by genetic alterations [252]. Using classical parameters to monitoring T2DM progression are not accurately prediction of developing its complications [253,254]. Accordingly, the importance to identify new potential markers have ability to give evidence for patients who are at elevated risk of T2DM-associated complications [255,256].

Using miRNAs as biomarkers for diagnosis diabetes is very useful especially after using microarray technique, and then demonstrate by using qPCR which is giving conceptualize about the ability to determination diagnostic profile of miRNAs types of DM [257,258,259,260,261].

Circulating miRNAs can be used to indicate the occurrence by T2DM or one of its complications, as in using miR-29a/b/c, and miR-192 miR-21 to determine onset of DM and prevent development of DM [262]. miR-21 works to bloke " PTEN", a key modulator in DM [263]. Also using miR-126 as a strong marker for diagnostic Coronary artery disease in plasma of patients of T2DM [264]. In addition, a study showed three serum miRNAs (miR-138, miR376a and miR-15b) as potential biomarkers to distinguish obesity patients from obesity-T2DM and T2DM patients, while it can be used the integration of miR-138 and miR-503 to distinguish between diabetic and obese diabetic patients [265,266]. A levels of miRNAs as (miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486) were reduced in plasma of T2D patients. Other study identified miR-144, miR-146a, miR-150 and miR182 from the blood of T2D patients as the signature miRNAs for prediction of T2DM, while considered miR-126 a distinctive biomarker in the serum of T2DM patients with Coronary artery disease (CAD) because of its own expression modality [266].

miR-21, miR-21-5p, miR-126 and miR126-3p have a significant role in diagnosis of T2DM or its complications because of their expression in the inflammatory response and vascular homeostasis [267]. miR-21 is a strong biomarker in detection of DM due to reducing expression in diabetic wounds [268] and its functions in wound healing [269].

2.6 Heart Failure

Heart failure (HF) is a clinical syndrome cause by structural and functional defects in myocardium resulting in obstruction of ventricular filling or the discharge of blood. The most common cause for HF is reduced left ventricular myocardial function. Heart failure result from pathogenic mechanisms which cause raising hemodynamic overload, immoderate humoral neurons inducement, revising of ventricular, ischemia, untypical myocyte calcium channel, exaggerated or inappropriate of extracellular matrix proliferation and mutations in genes [270]. It can be identified through ideal sings like tiredness, breathlessness and swelling of ankle. That may be accompanied by,

pulmonary splinter, arising pressure of jugular venous and peripheral edema resulting in a reduction of cardiac productivity and elevation intra cardiac pressures through stress, tension and rest. In children, the signs may different according to the age of the child and commonly involve difficulty feeding, sweating, rapid breathing, failing growth [271].

Originally in heart failure, patients have asymptomatic structural or functional distortions in heart" systolic or diastolic left ventricular (LV) dysfunction", then a clinical signs seem to be obvious. Realization these distortions are paramount because their relationship to the serious complications that may be result from it. Identification of these origins are very important and treatment them may reduce death rates in patients [272,273,274].

Symptoms are not enough to distinguish HF and other diseases [275,276]. Heart failure symptoms are resulting from detention of fluids which may be treated readily by using diuretic drugs. Using of displacing of the apical impulse, which may be particular, but is difficult to reveal [274,277,278]. In old, obese people and patients with pulmonary disease symptoms may be so complicated to recognized and explain [279,280,281]. Comparing with elderly patients, heart failure in young patients has another causes, clinical signs and effects [282,283].

2.6.1 Classification of Heart Failure

Heart failure can be divided in to left ventricular, right ventricular or biventricular according to the situation of the deficiency. HF is classified as acute or chronic according to the period onset. Clinically, it is divided into two types depending on its functional condition of heart to: HF with preserved ejection fraction (HFpEF) which is the most prevalent HF phenotype and HF with reduced ejection fraction (HFrEF) [284,285]. According to heart productivity, HF can be divided to high-output failure which means uncommon disorder featured by rising resting cardiac index more than 2.5–4.0 L/min/m², and low-output failure which is more common and featured by insufficient forward cardiac output, predominately through excessing metabolic instance [286,287]. Deliberate oxygen up taking in HF with muscle function deterioration, practicing exercise rehabilitation imitate to be an essential factors in progressing the inflammatory imbalance, sedative high cardiac filling pressure, improving quality of life and reduction death rates related with heart failure [288,289,290].

According to the "American College of Cardiology/American Heart Association" there are four stages for HF [289,290,291]:

A) Elevated risk of HF without symptoms or structural heart infection

B) Found heart disease without HF symptoms.

C) Heart disease with HF symptoms.

D) HF needing for clinical interferences

2.6.2 Risk Factors for Developing Heart Failure

There are many risk factors that lead to damage the cardiac muscle and can be caused heart failure. These involve the following [290]:

- Presences some diseases such as: hypertension, diabetes mellitus, arrhythmia, congenital heart disease, heart valve disease, myocarditis, anemia and thyroid disease.
- Coronary Heart Disease which means decreased quantities of oxygenated blood to heart because of formation plaques on wall of arteries causative HF.
- Other causes such as developing of aging, obese, cancer treatment, drugs abuse, viral infection like HIV virus

2.6.3 Systolic and Diastolic Heart Failure as Distinct Phenotypes

HFpEF and HFrEF may be shared the same clinical phenotype, signs, symptoms, exercise intolerance and mechanisms, but this not means that they are belong to a common pathogenesis, or have the same medical treatment. Actually, it can be distinguished depending on cause and treatment. Studies submit strong evidence that HFpEF and HFrEF represent 2 distinct diseases and they have bimodal distribution not unimodal in patients with HF. Response to therapies, it can be seen that some therapies give benefits when using with HFrEF and failed with HEpEF. Modalities of left ventricular modifying in HFrEF and HFpEF are different. The dilation chamber of left ventricular is an identical featured in HFrEF. Ejection fraction in HErEF is lower because of large size of this chamber, while strok volume is similar to that in healthy persons [284,285].

Regarding to pathogenicity and developing of disease there is a different between HFrEF and HFpEF .The distinct risk factors of HFpEF are age, hypertension and diabetes mellitus, so it can be considered HFpEF as a form of accelerating hypertension.

On the other hand, HFrEF may be advanced acutely responding myocardial loss of function [285,290].

2.6.4 Pathogenesis of Heart Failure in Patients with Diabetes Mellitus

The most prevalent complication of DM is cardiovascular disorder [291]. Both of them sharing the general pathogenic factors. In the diabetic patients heart disorders may evolve to coronary artery disease and high blood pressure. The relationship between heart failure and diabetes mellitus is very strong as confirmed by many epidemiological studies and also supported by proofs from scientific experiments which explained the functional defects cardiac muscle in diabetic patients [292,293]. Occurrence of HF in diabetic patients belongs to many factors such as resistance of insulin, elevated glucose level and up taking some drugs related to DM as insulin, glitazones and sulfonylurea [294,295] as in Figure 2.3.

Some hypotheses like disorders of metabolism, fibrosis of myocardia, destruction calcium balance and other have been given explanations about mechanisms responsible of decreasing myocardial contraction in the DM patients [296].

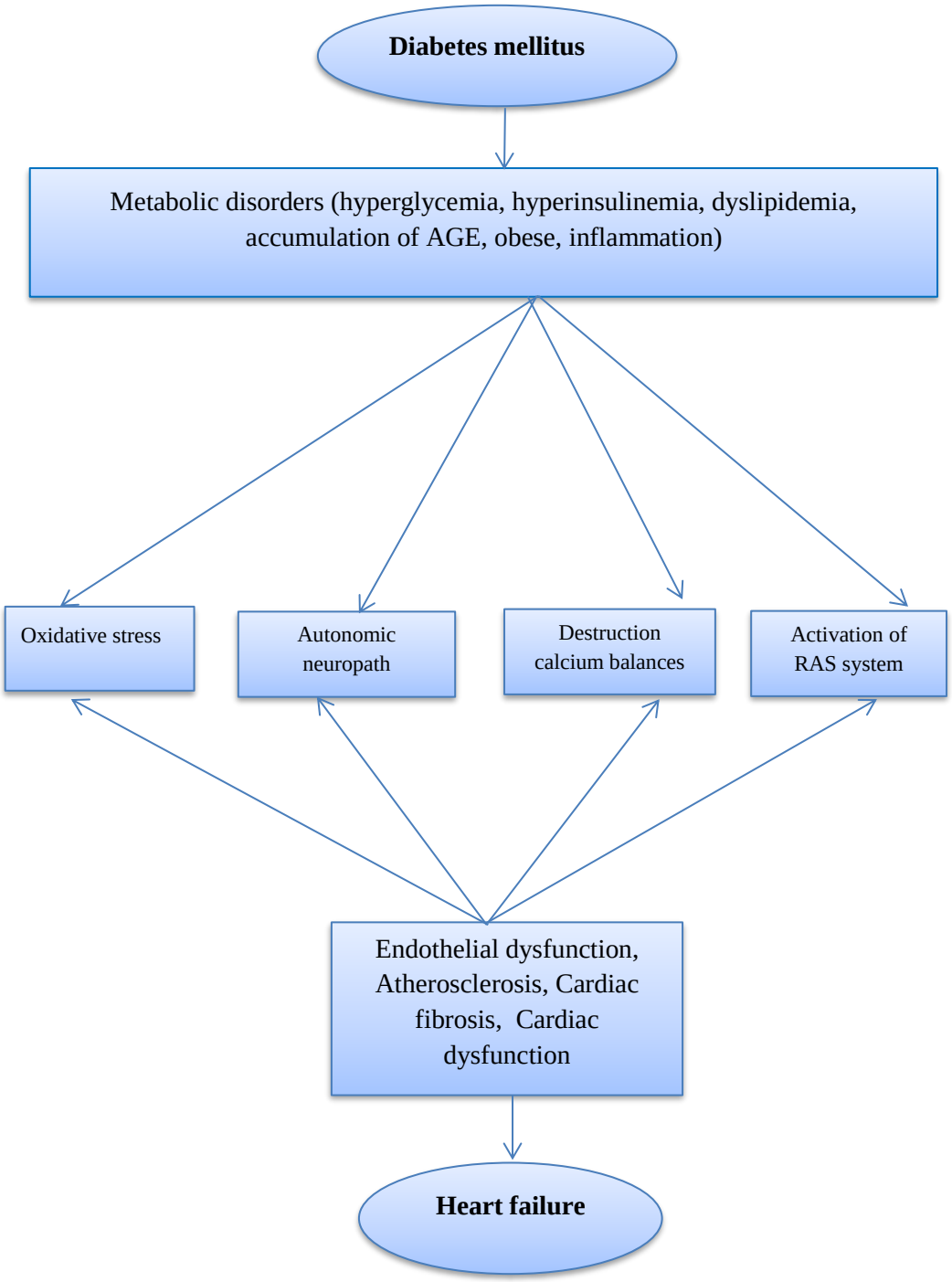

Figure 2.3 Pathophysiology of heart failure in diabetic patients [296]

2.6.5 Frequency of Heart Failure in the Diabetic Patients

The incidence with HF in diabetic patients is elevated in about twice in men and more than five times women compared to healthy people, and the real cause is unknown still now [297]. This combination was distinct of hypertension, age, obesity, coronary artery disease (CAD) and hyperlipidemia [298]. A study showed that using drugs for T2DM would elevate the risk factor of incidence with HF [299,300]. Existence of metabolic disorders, time of undiagnosed DM and duration and need for drugs may expedite developing HF in patients with T2DM. In general, the predominance of DM in the societies ranges between 4% to 6%, and was increasingly in HF cases. Using of pharmaceutical therapies related to T2DM was correlating with a 4.75-fold change excessed HF risk, that means the degree of metabolic troubles, interval of undiagnosed DM, and requirement for pharmalogical therapies, but not the therapies themselves, could be all expedite promoting of HF in T2DM patients. Rates of cardiovascular death in patients with T2DM have been augmented comparison with non-diabetics. Increment incidence with coronary artery disease and stroke were related with a 2–4-fold, and 2–8 fold the risk of HF in diabetic patients. Some studies suggest that using therapeutic strategies for cardiovascular risk factors in patients with T2DM is very substantial in diminution the risk of CVD [301,302,303].

Diabetes also increases the risk of developing heart failure in patients with other causes, as in acute myocardial infarction. It is believed that diabetes promotes the development of myocardial fibrosis and diastolic dysfunction [304,305,306].

2.6.6 Using Functional Alterations in Heart as an Indicator of Heart Failure in Type 2 Diabetes Mellitus Patients

Alterations of heart functions occur in patients with T2DM encompass in weaken diastolic function of the heart, which may outstrip the systolic dysfunction [307]. That was confirmed in diabetic patients distinct of coronary artery disease, hypertension before they were obvious [308]. The left ventricular (LV) ejection time is reduced, but the pre-ejection period and the ratio of pre-ejection period to LV ejection time are increased [309,310,311]. Increased LV wall intensity is noticed. In ejection fraction, LV mass indicator, moreover an age-concerning descend, and an age-concerning augment in diastolic diameter [312,313].

2.6.7 Heart Failure and miRNAs

All vascular diseases may lead to heart failure, which is a status when heart productivity was not match the needs of tissues. The most studies concerning with miRNAs and heart disease, showed that miRNAs embroiled in pathological cases and may also be engaged in heart failure. Particularly, the miRNAs like "miR-21, miR-129 and miR-210" were high expression, while other miRNAs like "miR-30 and miR-182" were low expression [128]. In heart failure, a transition to programmed fetal gene is noticed. These variations to expressed fetal genes may give a share in pathological features noticed in heart failure. Comprehend the expression of miRNA profile in heart failure situation may give us possibility to confirm the selective targets. Growing cardiac muscle was affected by miR-1 which caused negative regulated expression of calmodulin and calmodulin-dependent nuclear factor in activated T cells" (NFAT) signaling. Furthermore, miR-1 has ability to targeting the 3′UTRs genes contribute in growth of cardiomyocyte. miR-1 and miR-30a have a key roles" apoptosis and cardiac hypertrophy" [163]. miR-21 has been targeted genes responsible of apoptosis, hypertrophy and fibrosis of cardiomycytes, while miR-195, miR-499-5p and miR-92a have been targeted genes participating in apoptosis [162,164].

2.7 Galectin-3 (Gal-3)

Galectins are a family of lectins (carbohydrate binding proteins) that have tendencies to β-galactosides. They are involved in many cellular functions as in cell–cell adhesion and apoptosis [314,315]. They are available in immune and epithelial cells of animals [316] and possess one carbohydrate-recognition domain (CRD) at least [317]. There are 17 galectins were identified, most of them have a single CRD-1 while others contain two homologous CRD-2 [318,319]. Currently, 15 members of the galectin family have been described in vertebrates without classical signal sequence, but can function extracellular. According to that, proteins are found in cytoplasm and nucleus in some conditions [320,321].

Gal-3 is one of the most well-studied members of the galectins which was encoded by a single gene, located on chromosome 14, locus "q21–q22" [315,322]. Nuclear expression of Gal-3 is associated with proliferative effects. It has been described that Gal-3 is translocated from the cytosol into the nucleus via a passive and an active pathway [317]. Gal-3 contains several phosphorylation sites and other determinants important for the secretion of Gal-3 [323].

Human Gal-3 is a 31-kDa chimeric protein, has CRD-1 and a non-lectin domain enriched with proline and glycine [318,319]. This unique galectin consists of four structural domains [324,325,326] as in Figure 2.4:

1) NH_2 terminal domain containing a serine phosphorylation site, which has ability to regulation signaling activity in cell.

2) A collagen-α-like sequence is rich in glycine, tyrosine, and proline.

3) COOH terminal domain, which is recognition of β-galactosides

Gal-3 is a monomer in solution and can formation pentamers by means of supple "N-terminal domains" by connected with its saccharide molecules. It is secreted into the extracellular space through non-classic secretion pathway [327], so it is available in cytoplasm, nucleus and cell surface [328,329].

Gal-3 contacts with proteins, carbohydrates, un-glycosylated proteins as receptors on cellular surface and extracellular receptors, which modulate cell-cell adhesion signaling in the extracellular compartment [330,331]. Intracellular Gal-3 has an importance in cell growth, anti-apoptosis signaling, and mRNA interlacing, whereas implication of extracellular Gal-3 was in migration, growth, cell-cell communication, including laminin, fibronectin, and Mac-2 binding protein [332,333,334] as in Figure 2.5. In addition, Gal-3 has also a high binding affinity to advanced glycation end products (AGE) and is considered to be a receptor of AGE. Also influence the activation and regulation of both innate and adaptive immune responses they have been compromised in the pathogenicity of a diversity of diseases, like tumor initiation, advancement, and in migration of malignant tumor [335].

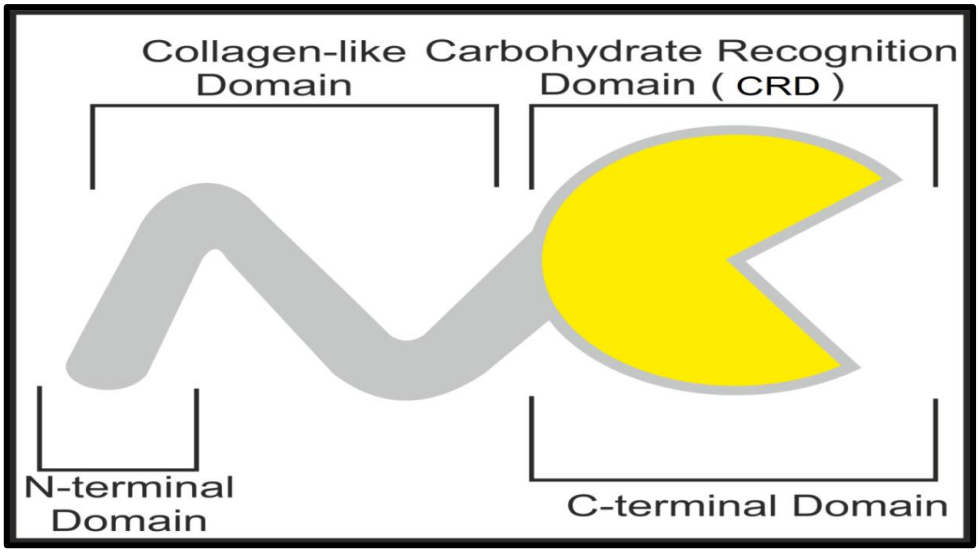

Figure 2.4 Structure of galectin-3[329]

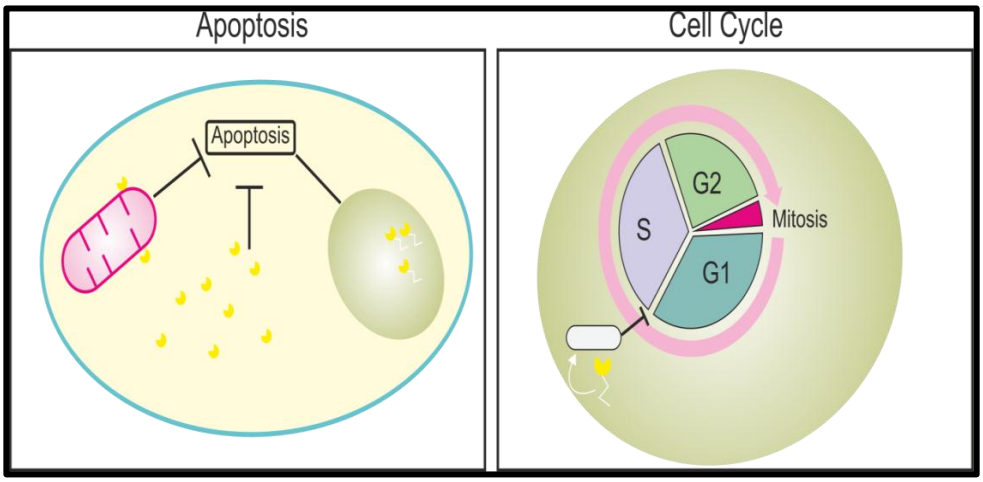

Figure 2.5 Galectin-3 intra cellular functions [329]

2.7.1 Expression of Galectin-3

Stimulation of Gal-3 secretion in macrophage and fibroblasts can be occurred by stress, such as irradiation and heat shock. Expression of Gal-3 has an importance in many physiological and pathological processes, such as inflammation and immune responses, tumor growth and headway, diabetes, moreover to repairing wound. It can be revealed in most proliferative cells as in "tumor cells, eosinophils, neutrophils, macrophages and fibroblasts" [336,337,338,339]. Expression level of Gal-3 can be altered due to external stimulation and environmental conditions [340]. Gal-3 plays a key role in the cardiac reconstructing process by involvement in inflammatory response and homeostasis [341,342]. High expressions of Gal-3 are in tissues as in stomach, colon, spleen, lung, ovary and uterus, while its expressions in kidney, heart, cerebrum, pancreas, and liver are at lower level [343].

2.7.2 Galectin-3 in Type 2 Diabetes Mellitus, Cardiac Remodeling and Heart Failure

Circulating Gal-3 was highest in T2DM patients [344,345,346,347]. It may be a risk factor for vascular complications, such as HF, peripheral artery disease, and other vascular complications [347]. In diabetic patients, Gal-3 concentrations were significantly elevated in subjects with CAD and associated with the formation of plaques [344].

Elevated level of Gal-3 in serum can be used as a potential marker for detection and prediction of HF. Gal-3 was involved in cardiovascular disease as blood pressure, low and high-density lipoprotein, cholesterol, triglyceride, creatinine, urinary albumin excretion rate, pro-BNP and C-reactive protein [348].

Gal-3 is elevated in patients with unstable CAD more than stable counterparts. Thus it may be used as a marker for atherosclerotic plaques [349]. Clinical trials demonstrated that Gal-3 is considered a prognostic marker in heart failure. Gal-3 predictive value was evaluated as a good marker in acute HF [350]. Expression of myocardial Gal-3 was up-regulation in hearts which is developed to HF in animal models. It is useful in a patients with HF to determine their risk and to evaluation this biomarker compared to other conventional risk markers. Gal-3 is a prognostic biomarker for early detection of HF phenotyping, and therapeutic targeting of HFPEF [351,352]. Prognosis utility of Gal-3 in acute heart failure was confirmed by the Food and Drug Administration. It is a

typical marker can be used for early detection of fibrotic cardiac injuries, hypertrophic and risk stratification [353]. Experimental observations found that Gal-3 is increased in decompensated heart failure more than 5-fold compared to compensated hearts [12], and up- regulation in LV dysfunction and not be became shorter to patients with rising AngII signaling [354].

2.8 Natriuretic Peptide

The natriuretic peptide family composed of 4 members ANP, BNP- which they are derived from atria and brain respectively-, CNP and DNP [7]. All of them are belonged to polypeptide precursors and each one contains a ring made of 17 amino acids in animals and 32 amino acids in human as in Figure 2.6. BNP was separated from pig brain in 1988, also it can be found in myocytes of ventricular [355,356].

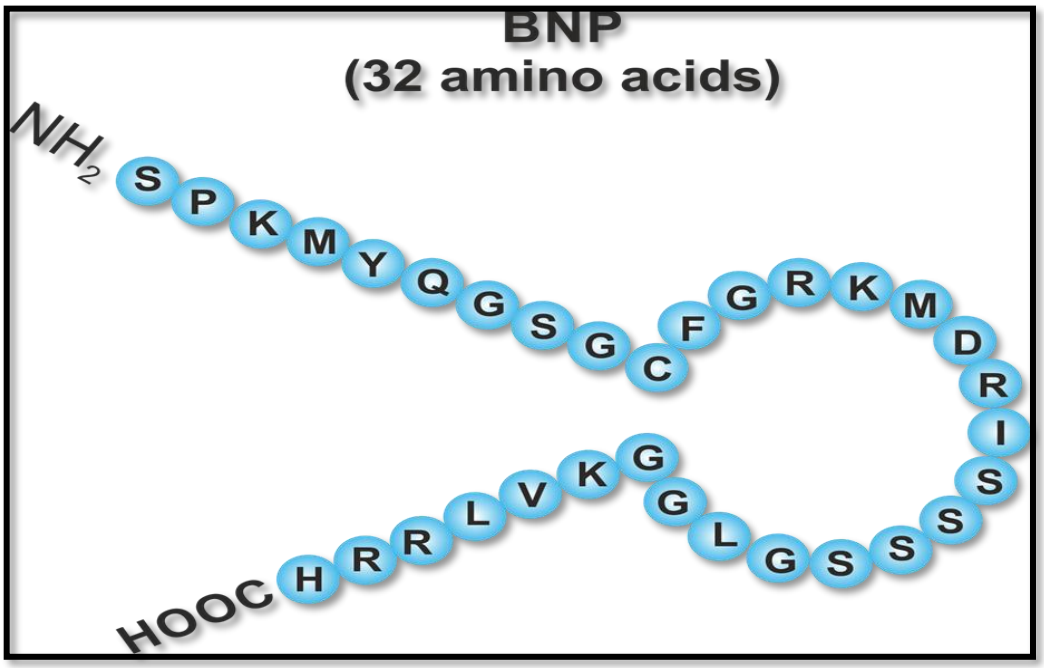

Figure 2.6 Primary structure of human BNP [359]

Ancestor of BNP consists of 134 amino acids. After fissure, prepro-BNP cleaves into two parts a pro-BNP (108 amino acids) and signal peptide (26 amino acids). In turn, pro-BNP breaks up into two molecules active BNP (32-amino acid C-terminal fragment) and the inactive NT-proBNP (76-amino acid N-terminal fragment) [357,358,359]. BNP has a half-life about 20 minutes. BNP and NT-proBNP are similar, just in NT-proBNP longer half-life time which is (72h), while BNP (4h) [359,360].

BNP is stocked in vesicles and released from atria and left ventricular through regulative and constitutive pathways, respectively. Secretion BNP is from the cardiomyocytes of LV, which is dominated at the transcription stage, and the stimulation process takes longer time. BNP is still at high levels for a longer time compared to ANB [361,362,363]. BNP has ability to inhibition fibrotic response that could be influenced on the cardiac fibroblasts through "extracellular signal-related kinase signaling".

Generally, the level of BNP is low in serum of healthy people but in patients it depends on their sex, age and race [364]. Clinically, active form of BNP which is found in plasma and featured by short half-life could be very important in follow up the situation of patients during taking pharmaceutical drugs [365].

2.8.1 Structure of the BNP

The BNP gene is found on the short arm of the human chromosome 1and consists of three exons [366]:

Exon 1: Encoding for a 26-amino acid signal peptide.

Exon 2: Encoding for most of the proBNP sequence.

Exon 3: Encoding for the terminal histidine and the 3´-UTR.

BNP mRNA is translated to 134-amino acid, then removing 26-amino acid signal peptide, the remaining were 108-amino acid proBNP. BNP composed of 2 forms: proBNP-108 is prevalent in ventricular tissue about (60%) and BNP-32 which is prevalent in atria tissue about (60%) [367]. Fission of proBNP into BNP-32 and N-terminal proBNP-76 takes place in the trans-Golgi network by corin convertase enzyme, thereafter into the circulation by a constitutive pathway as in Figure 2.7. Some studies gave an evidences of presence proBNP-108 and augmented proBNP-108/BNP-32 ratio in plasma of patients with severe HF. There was a substitution duplicate for proBNP, which has an ability to retain intron. This transcript contains the first and second exons and the second intron. It encodes and alters BNP [368].

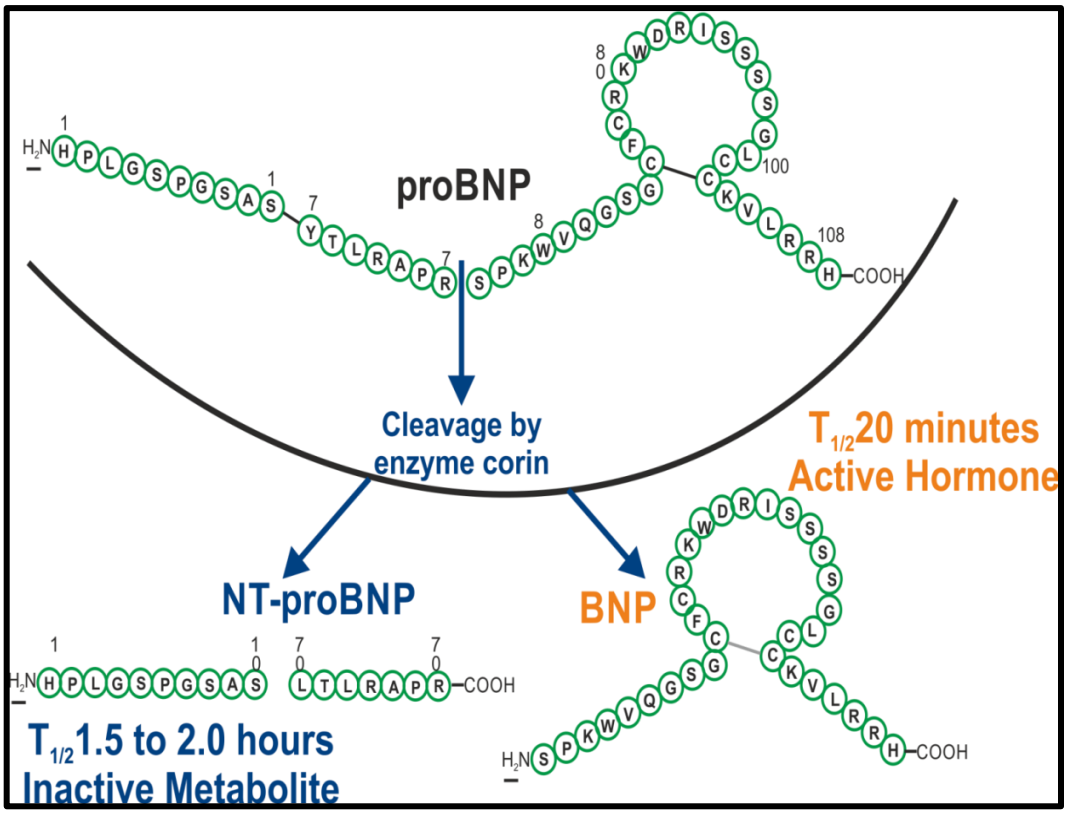

Figure 2.7 Activation of brain natriuretic peptide (BNP) by cleavage from its propeptides [371]

2.8.2 Physiological Effects of Brain Natriuretic Peptides

BNP is a neuro-hormone. It represents an activated form of proBNP, stored as secretory granules in both ventricles and, to a lesser extent, in the atria [290]. It is produced predominantly by cardiac ventricular myocardium, much less by atrial myocardium. Production of BNP is stimulated by increased cardiac wall stress during volume and/or pressure overload. Binding between BNP and its receptor (NPR-A) leads to diuresis, natriuresis [370,371]. High levels of BNP and NTproBNP related to impaired LV ejection fraction [372] and also for detection of asymptomatic LV systolic dysfunction [373].

An elevated in BNP concentration was noticed in chronic heart failure and CAD patients. According to "New York Heart Association" (NYHA) classification, levels of BNP in CAD cases may be more 25 times than its levels in patients without HF. In forms of acute coronary artery disease, the excessive concentration of BNP can be noticed [7,374]. NPs are used to determination patients with HFPEF at highest risk, that due to levels of NPs in patients with HFREF are higher than its levels in HFPEF patients [351]. BNP and NT-proBNP values are very influenced by many factors including renal function, anemia and age [375].

The measurement of BNP concentrations in plasma has an importance in addition to the chest x-ray, Doppler echocardiography and electrocardiogram in diagnosis HF in suspected patients [376]. When physical signs and radiographic results are not clear, NP levels are useful for detection of a new or acutely decompensated HF [377]. In myocardial ischemia, rising concentrations of BNP are also noticed and performed the expansion and adversity of ischemia. Temporary myocardial ischemia may be corresponded to a fast rising in BNP concentration [378].

2.9 High Sensitivity C Reactive Protein (hs-CRP)

C-Reactive protein (CRP) is un-glycosylated member of pentraxin family which due to lectin superfamily [379]. It is a type of α globulin and has a molecular weight about 110,000–140,000 Dalton. CRP consists of 5 subunits, which are assembly as a cyclic shape called pentamer [380]. CRP was discovered firstly through a precipitation reaction among patient's serum and *pneumococcus pneumonia* in 1930. The "polysaccharide fraction C" was extracted from the cell wall of this bacteria, and named C-reactive protein [379]. Each subunit possesses 2 faces, "recognition" face which is

displaying 5 phosphocholin-binding sites and an "effector" face which is comprising complement and Fc-receptor-binding sites. Phosphocholin is an essential molecule of CRP, that exists in lipopolysaccharid of bacteria. CRP has an important role in immune response. It was produced in liver and activated by complement through classical pathway. CRP has ability to motivating the manufacturing cytokines like IL-1 and TNFα [380,381]. Excessively high levels of CRP appear in patients with bacterial infections, autoimmune diseases, and cardiovascular diseases [379,381,382].

Because of lacking CRP its ability to sensitivity through normal extent of inflammatory situation, so using high sensitive CRP (hs-CRP) is very useful to exposing the depressed level inflammation. Immune techniques are used in detecting of CRP and hsCRP such as ELISA, immunonephelometry…etc. [383].

CRP can be used as a prognostic biomarker and predictive risk factor in patients with diabetes mellitus, atherosclerosis and cardiovascular diseases [384]. Highest levels of hsCRP correlate to inflammatory conditions in patients with coronary artery disease and heart failure [380,384,385]. Recent studies have been confirmed that hs-CRP concentrations have a significant biomarker for the whole-body inflammation condition [386]. hs-CRP can be decreased by alteration life pattern and taking pharmaceutical drugs [380].

2.9.1 Correlation between hs-CRP and Coronary Artery Disease

Cardiovascular disease is one of the inflammatory disease in which hs-CRP at a serious high levels [380]. hsCRP considers as an ideal biomarker using for estimating the inflammation in cardiovascular disease [381]. Some studies explain the role of hs-CRP in augmentation the cardiovascular epidemic and death rates [383]. Recent studies gave indicators about the probable linkage between concentration of hs-CRP and coronary artery disease and noticed the excessive high concentration of hs-CRP in patients with CAD [387]. Other study, has used hs-CRP to appreciate the correlation of inflammatory condition with the new occurrence of CAD and pointed out to the ability of using hs-CRP as a predictive risk factor for CAD and systemic inflammation [383]. Rising level of hs- CRP was observed in patients with peripheral arterial disease, stroke and endothelial dysfunction. The hs-CRP also gives an idea about the inflammation condition of arterial wall [380,385].

Actually, remaining hs-CRP at elevated concentrations with obscurity of chronically of intense cases, cardiovascular disease must be taken into account. Interestingly enough, modern studies give evidence show that hs-CRP is, in particular, pertinent in prediction of cardiovascular disease in asymptomatic patients without heart disease. Therefore, hs-CRP can be used in classification patients in the absence of classical cardiovascular risk factors such as smoking and high blood pressure, according on level of hs-CRP, which must be less than '"3.0 mg/L" [380,386].

2.9.2 Correlation of hs-CRP with Insulin Resistance and Metabolic Syndrome

One of the an essential causes of type2 diabetes mellitus is insulin resistance which is involving in the metabolic syndrome, and is featured by abdominal obesity high blood pressure, high glucose level and high-density lipoprotein (HDL) cholesterol. All previous properties, have been confirmed to be related with chronic, can be determined by hs-CRP estimation. Increasing level of hs-CRP points out to substantially relation with T2DM [384]. Excessive concentration of hs-CRP considers a strong predictor and diagnostic biomarker of metabolic disorders and diabetes mellitus even after controlling the traditional factors such as BMI, history of family with DM, and other factors. Level of hs-CRP can be used to indicate to the severity of cardiovascular disease in patients with T2DM [385,386].

Studies showed that using anti-diabetic drugs as metformin supply a low insulin-sensitizing impact and it would make to decrease level of hs-CRP. On the contrary, anti-diabetic drugs with no insulin-sensitizing impact seem to have low or no influence on inflammation processes such as sulfonylureas, which has not ability to lowering CRP levels in patients with T2DM [388].

2.10 Coronary Artery Disease (CAD)

Coronary artery disease (CAD) is a complex chronic inflammatory disease. It is characterized by remodeling and narrowing of the coronary arteries supplying oxygen to the heart. It can has various clinical manifestations, including stable angina, acute coronary syndrome, and sudden cardiac death. It has a complex etiopathogenesis and a multifactorial origin related to environmental factors as diet, smoking, physical activity, and genetic factors [389]. CAD is one of the major causes of death and based on estimates, approaching "1.25 million" persons were infected in the United States per year [390,391]. In recent years, the rate of patients with CAD entered to hospitals is

raised, that gives visualization about elevated incidence and frequency of it [392]. Pattern of life has a significant role in repetition and advancement of common diseases, such as CAD. Difficulty of this disease lies in the patient's ability to change lifestyle and commitment to take medicine away from supervision of doctors [393].

Risk factors of CAD can be divided in to two types [394,395]:

1) **Non-modifiable risk factors:** which cannot be altered and include age, gender, history of the family, and ethnic. Men have a higher risk than women, especially those older than age 45, women older than age 55. Serious risk factors include high levels of serum cholesterol, low-density lipoprotein cholesterol, and triglycerides; lower levels of high-density lipoprotein cholesterol
2) **Modifiable risk factors :** which can be altered by medical and pattern of life interventions and include high blood pressure, hypercholesterolemia, physical inactivity, diabetes mellitus, excessive weight, obese and smoking.

There is a relationship between DM and prevalence with CAD. DM is one of the main risk factors for CAD. Silent CAD is occurring spontaneously generally related to ischemia, which is stimulated by exercise, emotion and reproducible. The standard silent coronary artery disease are [396]:

1. Formerly diagnosis of CAD with no angina, or remaining symptom complex stable for at least 60 days
2. No alteration in recurrence, priod, accelerating cause of relief of angina for at least 60 days
3. No proof for deterioration of cardiac muscle.

CAD is a major cause of death in western and developing countries. This elevating may be due to the rising prevalence of many CAD risk factors like DM [397,398]. The prevalence of DM in patients with CAD is up to 50% in many countries [399].

2.10.1 Pathophysiology of Coronary Heart Disease

Occurrence of CHD belongs to atherosclerosis and its developing is correlated to factors concerning with environment and genetic [390]. Atherosclerosis is featured by gradual packing of "lipids fibrous elements, and inflammatory molecules" in the walls arteries [400]. Oxidized/modified LDL particles are strong ligands that induce the adherence of molecules vascular cell and intercellular molecules at the surface of endothelium, and

encourage monocyte adherence and migration to the "sub-endothelial space". Monocytes are adhesion and immigration to the sub endothelial space and then convert into macrophages in the "intima media", which in turn restrict oxidized LDL through receptors to be foam cells. That would be stimulated cellular and humoral immune response by combination among monocytes, macrophages, T-cell and B-cell in addition to production cytokines and TNFα [390,401]. Developing this process and immigration of smooth muscle cells from the medium layer to intima follows by formation of lesions.

After that smooth muscle cells start to subtract extracellular ligands which are transformed to "fibrous cap". Then lipids would be released and formed "necrotic core". Outcome of this process is formation plaques. There are two types of plaques can be identified depending on equation between formation and degrading of fibrous cap [402,403]:

A) Stable plaques, which are produced by smooth muscle cell from a substance plentiful with collagen type 1and 2, these plaques lead to occurrence of silent coronary artery disease (SCAD)**.**

B) Un stable or vulnerable plaques, which are broken and leading to releasing coagulation proteins causative thrombosis and lead to occurrence of acute coronary syndrome (ACS).

The different clinical manifestations of SCAD are related with various mechanisms involve:

(i) plaque-correlated oculsion of epicardial arteries
(ii) prevalent convulsion of normal or plaque-diseased arteries
(iii) functional disruption of microvascular
(iv) previously acute myocardial necrosis which leads to loss left ventricular its function (ischemic cardiomyopathy)

All mechanisms which are mentioned above may be worked aside or incorporation.

2.10.2 Silent Myocardial Ischemia

Silent myocardial ischemia (SMI) is more predominance in patients with DM compared to the healthy population. Often, there is an appearance of coronary artery disease when diagnosed in diabetic patients in advanced, but delayed diagnosis may be clarified by the subsistence of SMI [311], which is possibly very widespread in patients with DM

due to diabetic neuropathy. In patients with DM, neuropathy can be noticed in only 1 out of 4 of the patients with SMI [312]. The correlation between glucose status and CVD may be dilated to the diabetic threshold and a linkage between them can be exposed in the existence of impaired glucose tolerance and impaired fasting glucose tests [313].

New conception of the pathophysiological principles of myocardial ischemia is based on scientific experiments which point out narrowing in coronary artery determined coronary blood inflow. Myocardial ischemia in SCAD is resulting from a temporary imbalance between blood providing and metabolic requirement. Ischemic results can be predicted by some alterations which include [404,405]:

1) Elevated concentrations of hydrogen and calcium ions in the venous blood that exhaust the ischemic area
2) Presence an indicators of weakness in diastolic and systolic of ventricular accompanied by wall malformations
3) Advanced of ST-T waves alterations
4) Cardiac ischemic pain

2.10.3 Detection of Coronary Artery Disease by Using miRNAs

Coronary artery disease (CAD) considers the mean cause that responsible of elevated levels of death rates in developing countries, in spite of the advance in health care, diagnosis and treatment options. Present day, depending on symptoms, electrocardiogram abnormalities and troponin concentrations in detection of acute coronary syndrome, not enough and need to develop much effective biomarkers and revoke non effective strategies [406]. Using miRNAs in cardiovascular disease are great acceptance as best diagnostic and prognostic biomarkers of "coronary artery disease and acute coronary syndrome".

Modern studies demonstrate the ability of detection of acute myocardial infraction (AMI) is depending on cardiomyocyte-enriched miRNAs [407]. Particularly, these studies pointed to "miR-1, miR-133a, miR-133b, miR-208, and miR-499" were up-regulation in plasma of patients with AMI. Recently, a systematic study suggests that only miR-1, miR-133a, miR-208a/b, and miR-499(a) in plasma and serum are a strong biomarkers for detection of coronary heart disease [408]. Analytic studies estimated the specificity and sensitivity of miR-499 and miR-133a which showed sensitivity and

specificity were [(0.88 ,0.87) and (0.89 , 0.87)], respectively. That makes them a good markers of AMI [407].

A recent study confirmed that circulating miRNAs "miR-132, miR140-3p, and miR-210" can be used as a predictors for cardiovascular disease especially in patients with ACS and SCAD [409]. Several miRNAs such as (miR-133, miR-208a, miR-17-92a, miR14, miR-155), which are responsible of regulation expression in "cardiomyocyte, endothelial cell, vascular smooth cell and inflammatory cell "are associating with CAD, whereas "miR-122 and miR-370" correlating to metabolism of lipid were elevated in patients with severity CAD [410]. Another study demonstrates that miR-126 related with microvesicles, while miR-199a is a good predictor for SCAD patients [411].

2.10.4 Coronary Artery Disease and Type 2 Diabetes Mellitus

In patients with T2DM, CVD is the main reason of death in this population. Between all conditions under the name of cardiovascular diseases, coronary artery disease is the most deadly in diabetic patients. An increasing rates of epidemic and death are in type 2 diabetic patients with CVD compared to non-diabetic persons [412]. Diabetic vascular disease (DV) is in charge of (2-4) - fold increase the prevalence of coronary artery disease (CAD) and stroke, and about (2-8)-fold progress in the risk of HF [413]. Risk factors in type 2 diabetic patients without family history of CAD are similar risk of cardiac proceedings persons with a previous myocardial infarction [414]. Diabetes cases may not necessary be a CVD, so consideration should be given to finding appropriate strategies to prevent diabetic patients from developing CVD [415]. It worth noting the importance of controlling the traditional risk factors in type 2 diabetic patients to decrease the risk of developing coronary artery disease [306]. Furthermore using drugs to controlling of glucose level in T2DM such as "statins and insulin-sensitizer" have a great influence on non- traditional factors [416,417].

There are some essential mechanisms responsible of vascular dysfunction which is the main reason of cardiovascular outcomes in DM. Loss vascular its function associates with adipose tissues, insulin resistance (IR) and alteration in concentrations of different of circulating agents. Oxidative stress (OE) has a significant role in formation of plaques in atherosclerosis, particularly, in DM [418], because its ability to oxidation LDL. Elevated OE in diabetes mellitus due to deficiency of antioxidants elements that may promote increased OE in DM comprise antioxidant deficiencies, high producing of reactive oxygen species and glycation and glycooxilation processes [419].

The association between traditional and nontraditional risk factors is the main cause of increasing prevalence with CAD in patients with T2DM. Correlation between vascular risk and (IR) points out that early appearance of cardiovascular risk before evolving T2DM [420].

Some evidences demonstrate that controlling of blood glucose level, high blood pressure, reducing LDL and loss of weight are the best strategies to decrease risk of cardiovascular in patients with T2DM. Benefits of controlling of cardiovascular are more effective when starts early in conditions with short period of diabetes mellitus and low cardiovascular risk [420,421].

Treatment with DM and decreasing cardiovascular risk factor are an important step to prevent developing events due to strong association between DM and CVD. Furthermore, studies have emphasized that rising ability of coagulation and autonomic neuropathy are predominating appearance in patients with T2DM and may be participate in evolving of CVD, increasing occurrence with myocardial infection and then CHV [421].

CHAPTER 3

MATERIALS AND METHODS

3.1 Subject

The protocol was approved by the local Ethics Comittee of Istanbul Education and Research Hospital (No: 1031, date: 07.07.2017) and was conducted in accordance with Declaration of Helsinki. All subjects were of Turkish descent. All subjects gave their informed consent for inclusion before they participated in the study. Pregnant women, active infection, acute renal failure, hepatic, rheumatic, malignant or endocrine diseases, subarachnoid haemorrhage, chronic lung diseases, acute and chronic pulmonary embolism, and smokers, individuals with a history of chronic alcohol consumption and subjects who were taking certain drugs, such as hepatotoxic drugs (antituberculous, antiepileptic) or oral contraceptive pills were excluded from the study.

All subjects were classified into four different groups.

Control group: A total of 45 healthy subjects who did not have any endocrine, vascular, cardiac or inflammatory diseases were chosen for the control group (mean age: 60.23±6.27 years, F/M:23/22). An oral questionnaire was applied to the subjects and none of our subjects declared that they had a family history of diabetes. They did not have diabetes or glucose intolerance as confirmed by an oral glucose tolerance test (OGTT).

DM group: Patients with T2DM (mean age: 61.50±5.08, F/M:23/22) who were diagnosed according to the American Diabetes Association (ADA) guidelines [422] were included in this study. All of the diabetic patients were being treated for diabetes with insulin (20%) and/or metformin (80%).

DM+CAD group: A total of 45 diabetic patients (mean age: 61.61±6.02, F/M:20/25) with coronary artery disease were enrolled in our study. All of the diabetic patients were being treated for diabetes with insulin (25%) and/or metformin (75%). A total of 86% of diabetic patients in this group had hypertension and they were being treated with beta

blockers (48%), thiazide (28%) and/or ACE inhibitors (14%). Diabetic patients with dyslipidemia (75%) were taking antihyperlipidemic drugs, such as statins. Patients with 2 or 3 vascular occlusion were selected as CAD + DM group.

Acute HF+DM group: A total of 45 patients (mean age: 62.07±5.26 years, F/M:20/25) with acute HF were studied. The diagnostic criteria of acute HF were recommended according to the American Heart Association (AHA) Guidelines. Exclusion criteria: patients with T2DM known HF, chronic obstructive pulmonary disease (COPD), pulmonary artery embolism and/or deep venous thrombosis, or who used to take anticoagulant drugs in the past three months or received blood transfusion recently, patients with a history of malignancy, cognitive dysfunction, mental illness, systemic disease.

3.2 Coronary Angiography

Patients underwent coronary angiography by Seldinger technique. Three-dimensional digital subtraction angiography (3D-DSA) examinations were performed with femoral catheterization with a DSA system (Philips Allura Xper FD20, Netherlands). The coronary angiograms were read by expert cardiologists, the clinical diagnosis, and laboratory results. The cardiologists recorded the location and extent of luminal narrowing for 15 segments of the major coronary arteries [423]. Patients were classified as having CAD if a stenosis of 50% or greater was found in at least one of the segments. Patients without CAD were defined as having less than 50% stenosis in all of the segments. A composite cardiovascular score (0–75) was calculated based on determination of presence of stenosis on a scale of 0–5 of the 15 predetermined coronary artery segments.

3.3 Echocardiography

Each patient underwent a complete transthoracic echocardiographic (TTE) studies with ultrasound systems located in adjacent echocardiography rooms. The device used was the General Electric Vivid S5 (GE Health Medical, Horten, Norway). Left ventricular ejection fraction (LVEF) was calculated according to Simpson’s method [424]. HF was defined according to the clinical criteria of the Framingham Heart Study 6 and by 25% < (LVEF) < 35%, according to bidimensional transthoracic Doppler echocardiography.

3.4 Sample Collection and Measurements

Fasting venous blood samples were drawn between 8 and 10 am after the subjects fasted overnight (10–12 hours). Blood samples were drawn from the brachial veins in brachial fossa and placed into plain tubes (K2-EDTA anticoagulated whole blood) and

anticoagulant free tubes. The samples were centrifuged for 10 minutes at 4000 rpm at 4 °C. Biochemical tests were performed immediately. For the determination of other parameters, serum aliquots were frozen and stored at –80 °C immediately until they were required for further analysis.

3.5 Isolation of miRNAs from Serum:

Serum miRNA was extracted from serum samples using EXTRACTME miRNA KIT (BLIRT, Poland). All isolation protocols were conducted according to manufacturers' instructions, without further modifications.

Isolation protocol according to manufacturers' instructions:

1. Place the serum in a 2 ml tube. Add 400 µl miRNA lysis buffer and 4,4 µl antifoam reagent and vortex for 60 s.
2. Centrifuge for 2 min at maximum speed.
3. Transfer the supernatant into DNA purification column placed in a collection tube. Centrifuge for 30s at ≥ 8000 x g. Keep DNA purification column for further DNA purification. Keep the flow-through.
4. Transfer the flow-through into an sterile, 1.5 ml Eppendorf microcentrifuge tube.
5. Add 0,5 volume of 96-100% ethanol. Mix by pipetting or vortexing for 5 s.
6. Transfer the mixture into an large RNA purification column placed in a collection tube. Centrifuge for 15 s at ≥8000 x g. Keep the flow-through. Keep large RNA purification column for large RNA purification. Minicolumn with large RNA can be stored no longer than 15 min at 4-8°C.
7. Transfer the flow-through into an sterile, 1.5-2 ml Eppendorf microcentrifuge tube.
8. Add 1 volume of 96-100% ethanol. Mix by pipetting or vortexing for 5 s.
9. Transfer 700 µl of the mixture thus obtained into an miRNA purification column placed in a collection tube. Centrifuge for 15s at ≥8000 x g. Discard the flow-through and reuse the column, together with the collection tube.
10. Transfer the remaining mixture into the same miRNA purification column and centrifuge at 15s at ≥8000 x g. Discard the flow-through.
11. Prepare the minicolumns with bound DNA, large RNA and miRNA.
12. Add 700 µl DNA wash buffer on DNA purification column and 700 µl RNA wash buffer on large RNA purification column and centrifuge for 15s at ≥8000 x g.
13. Again apply 500 µl DNA/RNA wash buffer to the each minicolumn and centrifuge at 15s at ≥8000 x g. Discard the flow-through and reuse the collection tube.

14. Again apply 500 µl DNA/RNA wash buffer to the each minicolumn and centrifuge at 30s at ≥8000 x g. Discard the flow-through and reuse the collection tube.

15. Centrifuge for 90s at maximum speed. The wash buffer contains alcohol, which may interfere with some enzymatic reactions and also decrease the elution efficiency. It is therefore vital to remove the alcohol completely from the minicolumn before elution.

16. Discard the collection tube and flow-through and carefully transfer the DNA purification column and large RNA purification column to a sterile, 1.5 ml Eppendorf microcentrifuge tubes and miRNA purification columns to miRNA elution tube.

17. Add 50-100 µl DNA elution buffer, precisely, onto the center of DNA purification column membrane and 30-100 µl RNA elution buffer, precisely, onto the center of large RNA and miRNA purification columns membranes. Other buffer volumes may be used.

18. Centrifuge at ≥8000x g for 1 min.

19. Remove the minicolumns and place the tubes with the eluted large RNA and miRNA in a freezing rack. The isolated RNA and DNA are ready for use in downstream applications or for storage at -80°C.

The concentrations and purities of RNA were estimated using NanoDrop spectrophotometer (ThermoFisher Scientific, USA), and A260/A280 ratio = 1.9 – 2.1 values were considered indicative of relatively pure RNA.

3.6 Synthesis and Amplifing cDNA

To estimate the expression miRNA levels four-step RT-PCR assay was used. Normalization was calculated according to determined miRNA levels

Firstly miRNA samples were transcribed into cDNA using High-Capacity cDNA Reverse Transcriptin (RT) Kit (ThermoFisher Scientific, USA) and RT oligos specific to miRNAs identified in Table 3.1 (SUARGE, Turkey), the cDNA synthesis was performed. Firstly, the 2✕ Reverse Transcription Master Mix was prepared Table 3.2. The 2✕ RT master mix was placed on ice and mix gently. 10 µl of 2✕ RT master mix was pipetted into each well of a 96-well reaction plate or individual tube. 10 µl of RNA sample was pipetted into each well, pipetting up and down two times to mix. the plates was sealed. The plate was briefly centrifuged to spin down the contents and to eliminate any air bubbles. The reaction volume was set to 20 µl and cDNA synthesis was performed by adjusting the the thermal cycler conditions Table 3.

Table 3.1 RT oligos specific to miRNAs

hsa-miR-1 RT	5'GAAAGAAGGCGAGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCG CCTTCTTTCATGGGCAT-3'
hsa-miR-21 RT	5'GAAAGAAGGCGAGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCG CCTTCTTTCTCAACATC-3'
RNU44 RT	5'GAAAGAAGGCGAGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCG CCTTCTTTCAGTCAGTT-3'

These are specific primers for cDNA trancription

hsa-miR-1 Forward	5'-GCAACATACTTCTTTATATGCCCAT-3
hsa-miR-21 Forward	5'-GCGGTAGCTTATCAGACTGATGT-3'
RNU44 Forward	5'-CCTGGATGATGATAAGCAAATG-3'
Universal Reversal	5'-CGAGGAAGAAGACGGAAGAAT-3'

These are specific primers for PCR analaysis

Table 3.2: Materials and volumes required to prepare the 2× RT master mix (per 20-µl reaction)

Components	Volume/Reaction (µl)
	1x Kit without RNase Inhibitor
10× RT Buffer	2.0
25× dNTP Mix (100 mm)	0.8
10× RT specific Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	-
Nuclease-free H_2O	4.2
Total per Reaction	10.0

Table 3.3: The thermal cycler conditions

Step	Temperature °C	Time Sec	Cycle
Activation and denaturation	95	180	35-45 cycles
Denaturation	95	5	
Annealing	60	10	
Extension	72	5-20	
Melt curve analysis	According to the qPCR instrument manual		

Firstly miRNA samples were transcribed into cDNA using the High-Capacity cDNA Reverse Transcription (RT) Kit (ThermoFisher Scientific, USA) and RT oligos specific to miRNAs identified in Table 1 (SUARGE, Turkey), the cDNA synthesis was performed. Then cDNAs amplified and the expression of miR-1 and mir-21 analyzed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using specific AMPLIFYME SYBR Universal Mix (BLIRT, Poland). RNU44 (TaqMan® Small RNA Controls from Applied Biosystems) was used as a small RNA endogenous control.

The relative expression levels of miRNA-1 and miR-21 were calculated using the 2-ΔΔCt method. According to 2-ΔΔCt method the first ΔCT is the difference in threshold cycle between the target and reference genes: ΔCT = CT (a target gene (miR-1 or miR-21))−CT(a reference gene (RNU44)). The ΔΔCT is the difference in ΔCT as described in the above formula between the target and reference samples, which is:

ΔΔCT = ΔCT (patient sample)−ΔCT (control sample) = (CTD − CTB)−(CTC − CTA).

The final result of this method is presented as the fold change of target gene expression in a target sample relative to a reference sample, normalized to a reference gene [1].

3.7 Measurement of Plasma NT-proBNP Concentrations

NT-proBNP levels were analyzed (K2-EDTA anticoagulated whole blood) using time resolved florescence assay on AQT90 FLEX immunoassay analyzer (Radiometer, DK). The results were expressed as pg/mL. Intra- and inter-assay coefficients of variation (CVs) were determined to be 4.1% and 5.2%, respectively.

3.8 Measurement of Plasma Galectin-3 Concentration

Serum galectin-3 levels were also assayed by sandwich ELISA kit (Human galectin-3 kit, Cat. No. E-EL-H1470, Elabscience Biotechnology Co., Wuhan, Hubei, China)). The galectin-3 results were expressed as ng/mL. The lowest level of galectin-3 that can be

detected by this assay was 0.10 ng/mL. Intra and inter-CV were determined to be 6.6% and 7.5%, respectively.

3.8.1 Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human GAL3. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for GAL3 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human GAL3, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human GAL3. The concentration of GAL3 calculated in the samples by comparing the OD of the samples to the standard curve as in Figure 3.1.

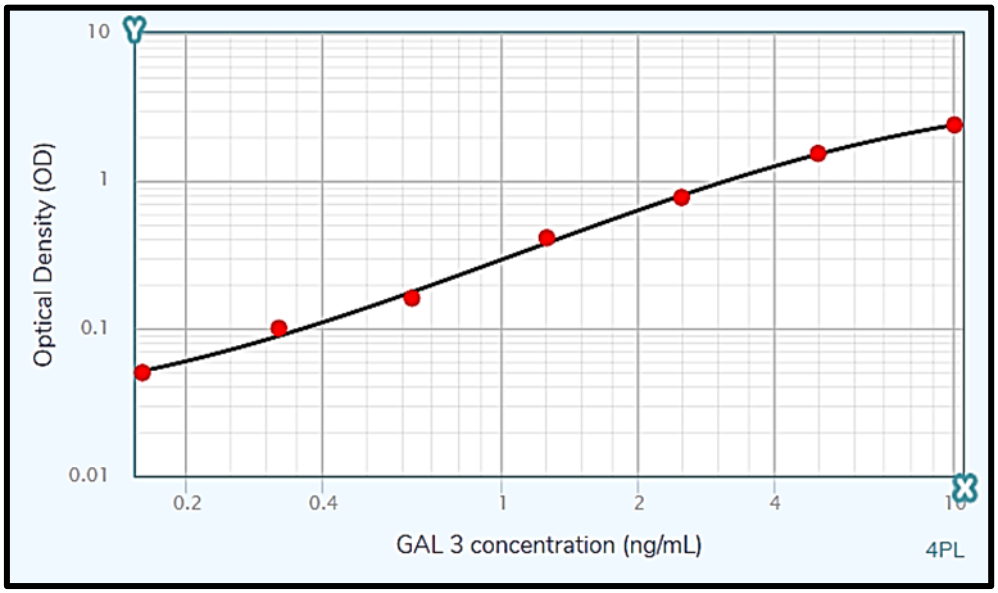

Figure 3.1 Standart curve of galectin-3 (GAL-3)

Biochemical rutin parameters (glucose, total cholesterol, HDL-cholesterol, LDL cholesterol, triglyceride, creatinine, uric acid) were determined using the spectrophotometric methods (Roche Cobas İntegra 400, Roche Diagnostics Ltd.

Germany). HbA1c determination was based on HPLC (Variant Turbo II, Bio-Rad Laboratories, Inc. USA).

3.9 Statistical Analysis

For statistical analyses, SPSS 21.0 was used. Continuous variables were tested for normal distribution by the Shapiro-Wilk test. Results for normally distributed continuous variables are expressed as means ± standard deviations, and we used the unpaired Student's t test to compare mean values. Between-group comparisons of distributions were performed using the Mann-Whitney U test and Wilcoxon's signed-rank sum test. Correlations among continuous variables were assessed using Spearman's rank correlation coefficient [r]. Categorical variables are expressed as numbers (percentages) and were compared using Fisher's exact test. To evaluate the expression levels of miRNAs circulating within the two groups, we decided to use Student's t test for two independent groups. ROC analysis was used to determine the separation power of the parameters. As a result of ROC analysis, cut-off points were determined by using Youden Index. To determine the risk of having the values above the cut-off value, the risk analysis was performed and the OR (odds ratio) values were obtained. The positive predictive values of the combinations according to the cut-off points for the miR-21, NT-proBNP and Galectin-3 parameters were calculated. p values < 0.05 were considered statistically significant.

CHAPTER 4

RESULTS AND DISSCUSION

4.1 Patient's Characteristics

One hundred and thirty five diabetic patients was divided into three subgroups: patients with diabetes mellitus only (DM) (n:45, mean age 61.50 ± 5.08), patients with CAD together with diabetes mellitus (CAD+DM) (n:45, mean age 61.61 ± 6.02) , and patients with heart failure with diabetes (HF+DM) (n:45, mean age 62.07 ± 5.26). There were no statistically significant differences in terms of age and sex between these groups and also between control and these groups (Table 4.1). DBP values of the control individuals were significantly lower than DM, CAD+DM and HF+DM groups (for each $p<0.001$). There was no difference between the other groups in terms of DBP. SBP values of control subjects were significantly lower than DM, CAD+DM, HF+DM groups (for each $p<0.001$). In the DM group, SBP was significantly lower than the CAD+DM group ($p<0.05$). In the CAD+DM group, SBP was significantly lower than the HF+DM group ($p<0.001$), (Table 4.1).

Table 4.1 General characteristics and biochemical parameters of subjects.

	Control group (n=45)	**Diabetes Mellitus (n=45)**	**DM + CAD (n=45)**	**DM + HF (n=45)**
Sex (F/M)	23 (%51)/ 22 (%49)	23 (%51)/ 22 (%49)	20 (%44/ 25 (%56)	20 (%44) /25 (%56)
Age (years)	60.23 ± 6.27	61.50 ± 5.08	61.61 ± 6.02	62.07 ± 5.26
BMI (kg/m^2)	23.33±1.39 a***,b***,c***	29.86±3.18	29.62±2.63	29.21±1.66
DM duration (years)	-	6.83±2.50 b*,c***	7.96±2.73 **a*,**c*	10.50±4.17
DBP (mmHg)	75.32 ± 5.07 a***, b***, c***	83.88 ±6.15	84.93 ± 7.41	84.09 ± 7.80
SBP (mmHg)	113.50 ± 6.22 a***, b***, c***	136.88 ± 5.85 b*	130.71 ± 14.95 c***	141.36 ± 16.40
Glucose (mg/dL)	83.03 ± 9.85 a***,b***,c***	210.03 ± 77.69 b*,c***	164.89 ± 54.90 c***	92.64 ± 4.35
HbA1c	4.74 ± 0.47 a**,b***,c***	10.23 ± 9.67 c*	7.46 ± 1.42 c***	5.63 ± 0.73
Total cholesterol (mg/dL)	169.73 ± 13.90 a***,c***	231.73 ± 60.36 b***,c*	186.39 ± 32.88	198.36 ± 27.81
HDL cholesterol (mg/dL)	51.03 ± 8.87 b***,c***	52.63 ± 14.58 b***,c***	41.39 ± 6.75	40.07 ± 8.49
LDL cholesterol (mg/dL)	93.58 ± 11.43 a***,b***,***c	143.65 ± 41.33 b**,*c	116.00 ± 26.57	119.55 ± 34.19
Triglyceride	99.53 ± 16.24 a***,b***,c***	196.98 ± 135.86	153.39 ± 61.47	149.43 ± 46.30
Creatinine (mg/dL)	0.82 ± 0.16 c***	0.86 ± 0.33 c**	1.17 ± 0.76	1.19 ± 0.49
Uric Acid	6.08 ± 0.88	5.69 ± 1.66	6.30 ± 2.31	6.50 ± 1.33
hsCRP (mg/L)	0.47 ± 0.26 c*	1.79 ± 5.64	1.79 ± 3.19	3.33 ± 5.92
NT-proBNP (pg/mL)	95.10 ± 24.00 a***,**b****,**c***	111.93 ± 25.23 b**,c***	4556.44 ± 6533.47 c*	8764.71 ± 6863.97
Galectin-3 (ng/mL)	4.27 ± 1.13 a***,b***,c***	5.92 ± 1.48 b***,c***	8.50 ± 1.63 c***	10.68 ± 2.81

DM: diabetes mellitus**; DBP:** diastolic blood pressure; **SBP:** systolic blood pressure; **NT-proBNP**: N-terminal pro-brain natriuretic peptide.
a: vs DM, b: vs DM+CAD, c: vs DM+HF
*p<0.05 **p<0.01 ***p<0.001

No correlation was found between the EF with miRNAs and cardiovascular risk factors as lipid parameters, systolic and diastolic pressure, BMI, creatinine, and uric acid. Similarly, there was no association between cardiovascular risk factors (lipid parameters, systolic and diastolic pressure, BMI and miRNAs in CAD+DM patients. It was not shown in the Table 4.1, but there was no correlation between the number of vascular occlusion and cardiovascular risk factors (lipid parameters, systolic and diastolic pressure, BMI) and miRNAs in the CAD+DM group.

4.2 Biochemical Findings and Expression of miRs

Serum NT-proBNP and galectin-3 levels were found to be increased significantly DM, CAD+DM and HF+DM groups with respect to control group, respectively (for NT-proBNP: control vs DM $p<0.001$, control vs CAD+DM $p<0.01$, control vs HF+DM $p<0.001$, DM vs CAD+DM $p<0.01$, DM vs HF+DM $p<0.001$, CAD+DM vs HF+DM $p<0.05$; for Galectin-3 $p<0.001$ for all comparisons) (Table 4.1).

Serum miR-1 levels were significantly lower in patients with DM, CAD+DM and HF+DM groups than in control groups, with 0.54-, 0.54- and 0.12-fold changes, respectively (for each group $p<0.001$). miR-1 levels were significantly lower in patients with HF+DM group than in patients with DM with 0.22-fold changes ($p<0.001$); and in patients with CAD+DM group with 0.22-fold changes ($p<0.001$) (Figure 4.1).

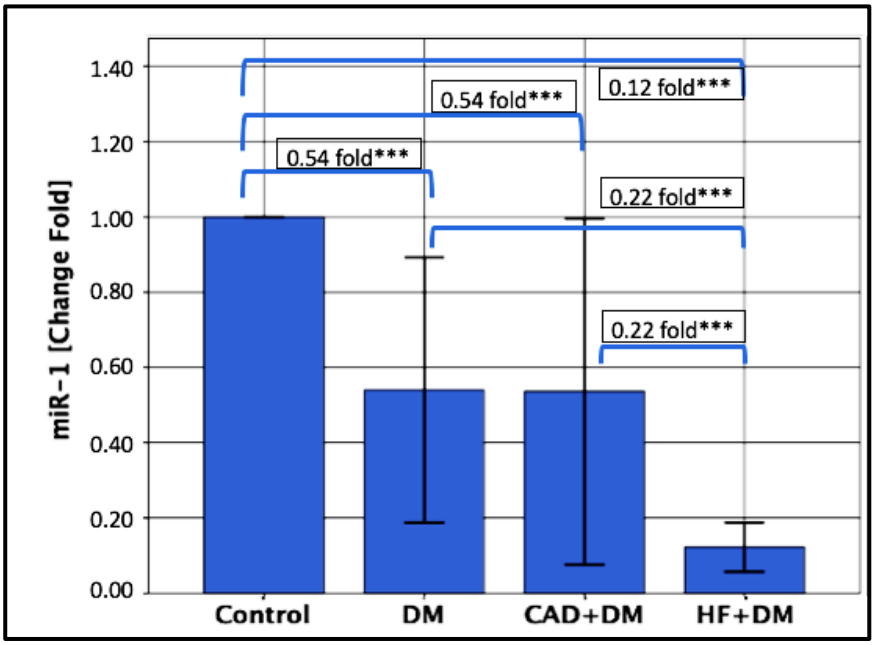

Figure 4.1 Relative expression levels of miR-1**.** miR-1 levels were significantly lower in patients with DM, CAD+DM and HF+DM groups than in control groups, with 0.54-, 0.54- and 0.12-fold changes, respectively. miR-1 levels were significantly lower in patients with HF+DM group than in patients with DM with 0.22-fold changes; and in patients with CAD+DM group with 0.22-fold changes. Data are presented as fold-change derived from mean $2^{-\Delta\Delta CT}$ method. values *p<0.05 **p<0.01 ***p<0.001.

On the contrary, serum miR-21 levels were significantly upregulated in patients with CAD+DM and HF+DM groups than in control groups, with 1.79-, and 2.21-fold changes, respectively (for each group $p<0.001$). miR-21 levels also were significantly higher in patients with HF+DM group than in patients with DM with 1.70-fold changes ($p<0.001$), and in patients with CAD+DM group with 1.24-fold changes ($p<0.01$), in patients with CAD+DM group than in patients with DM with 1.37-fold changes ($p<0.001$) (Figure 4.2).

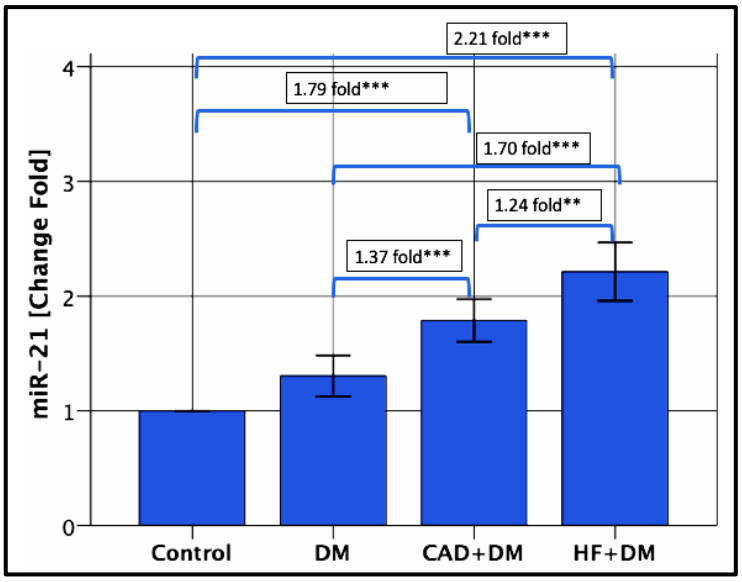

Figure 4.2 Relative expression levels of miR-21**.** miR-21 levels were significantly higher in patients with CAD+DM and HF+DM groups than in control groups, with 1.79-, and 2.21-fold changes, respectively. miR-21 levels were significantly higher in patients with HF+DM group than in patients with DM with 1.70-fold changes, and in patients with CAD+DM group with 1.24-fold changes. In patients with CAD+DM group than in patients with DM with 1.37-fold changes. Data are presented as fold-change derived from mean $2^{-\Delta\Delta CT}$ method. *p<0.05 **p<0.01 ***p<0.001.

Figure 4.3 shows miR-1 correlations with NT-proBNP and galectin-3 in DM, CAD+DM and HF+DM groups. In DM group, miR-1 was found to be positively correlated with NT-proBNP (r=0.419, p<0.01). In other groups, miR-1 was found to be negatively correlated with NT-proBNP (for CAD+DM r=-0.882, p<0.001; for HF+DM r=-0.891, p<0.001) and galectin-3 (for DM r=-0.371, p<0.05; for CAD+DM r=-0.754, p<0.001; for HF+DM r=-0.866, p<0.001).

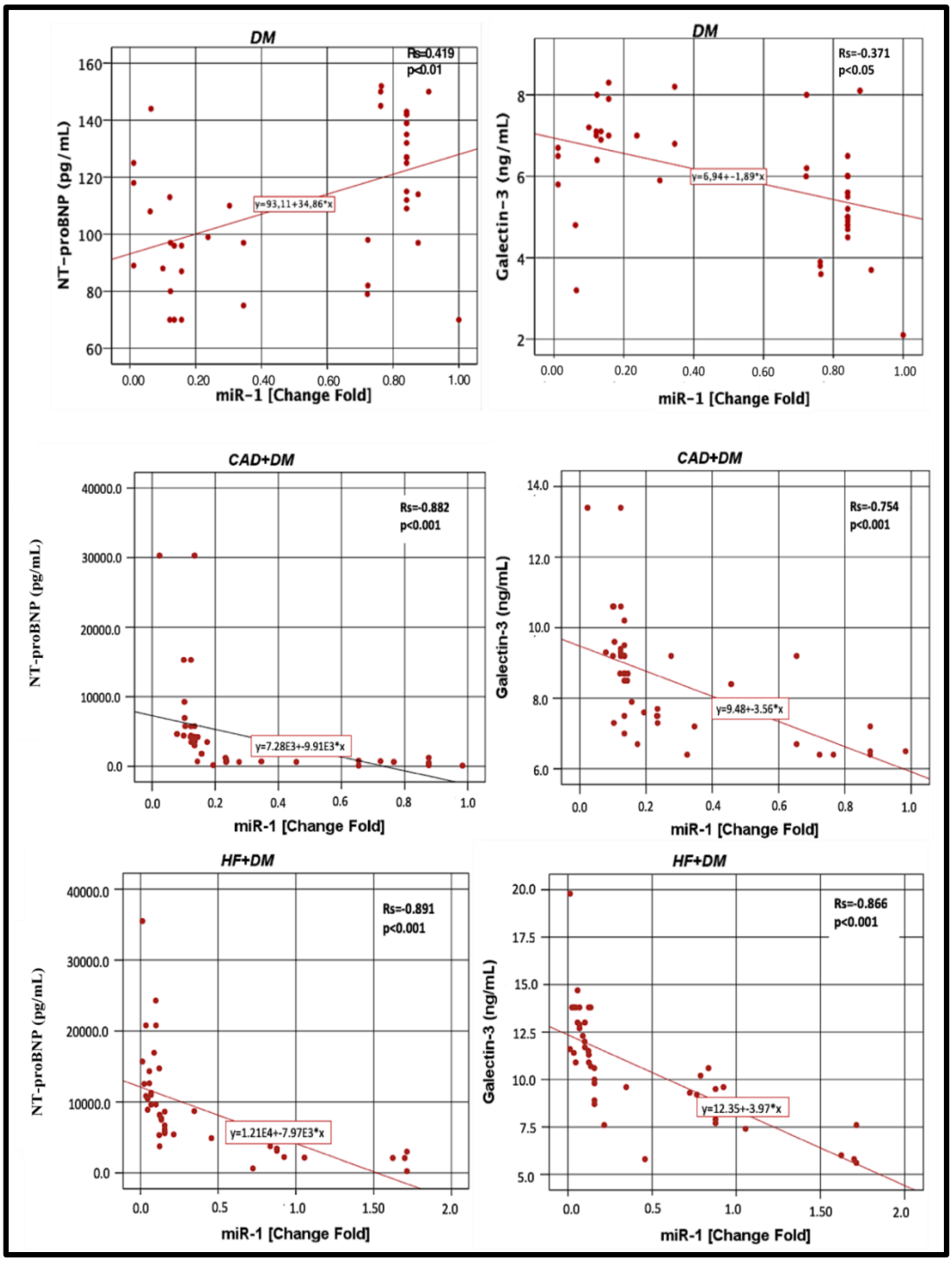

Figure 4. 3 miR-1 correlations with NT-proBNP and galectin-3 in DM, CAD+DM and HF+DM groups. Data are presented as fold-change derived from mean $2^{-\Delta\Delta CT}$ method. Rs: Spearman's rank correlation coefficients (r)

Figure 4.4 shows miR-21 correlations with NT-proBNP and galectin-3 in DM, CAD+DM and HF+DM groups. In contrast to miR-1, in all groups miR-21 was positively correlated with NT-proBNP and (for DM r=0.893, p<0.001; for CAD+DM r=0.898, p<0.001; for HF+DM r=0.734, p<0.001) and galectin-3 (for DM r=0.782, p<0.001; for CAD+DM r=0.773, p<0.001; for HF+DM r=0.764, p<0.001).

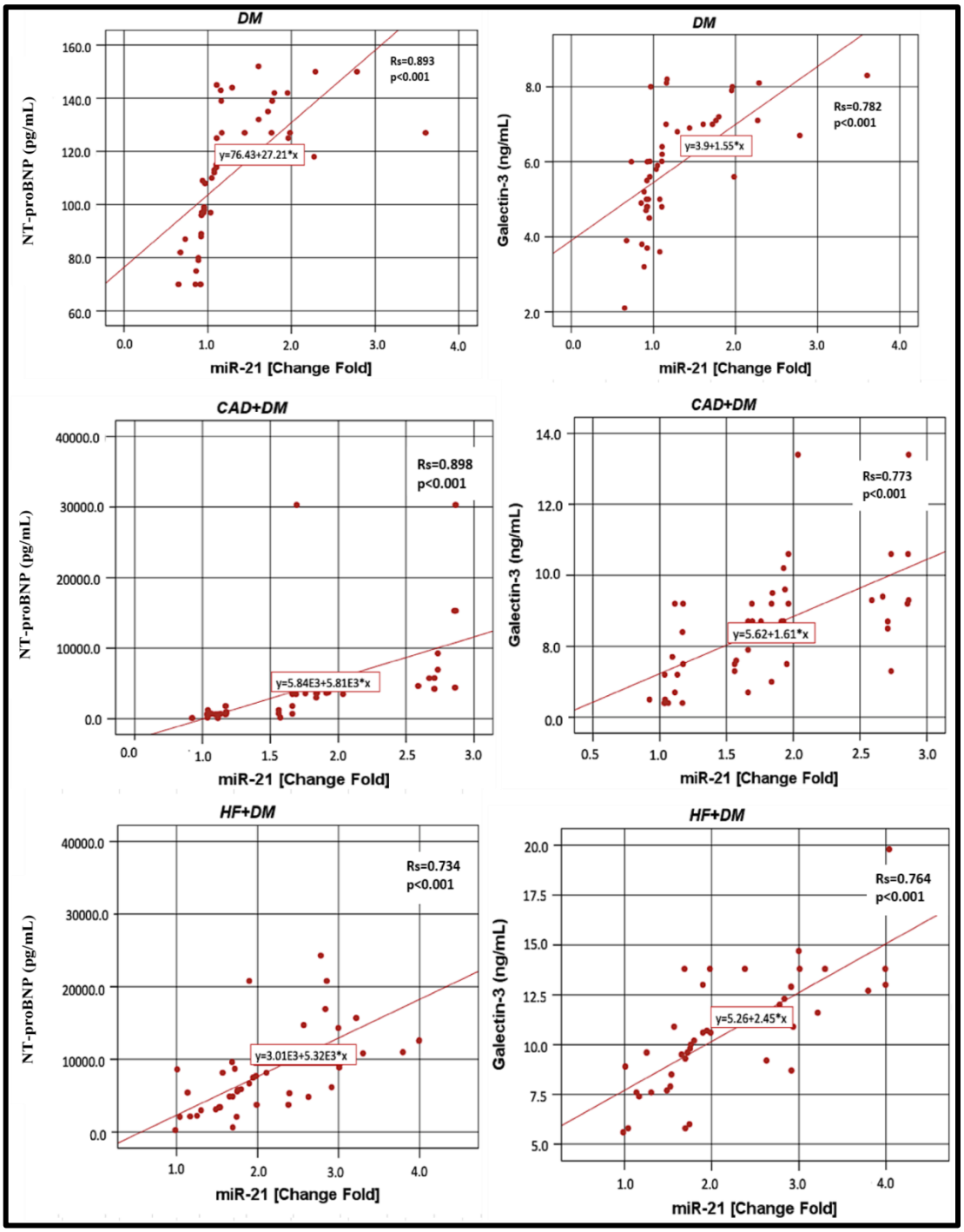

Figure 4. 4 miR-21 correlations with NT-proBNP and galectin-3 in DM, CAD+DM and HF+DM groups. Data are presented as fold-change derived from mean $2^{-\Delta\Delta CT}$ method. Rs: Spearman's rank correlation coefficients (r).

In the second part of the study, ROC curve of serum biomarkers and miRNA fold-change levels were determined for different group combinations and cut-off points were determined (Figure 4.5). According to the results of ROC analysis, miR-21, NT-proBNP and galectin-3 might be a good biomarkers to distinguish HF+DM (Sensitivity: %84.4, Specificity: %71.1, $p<0.001$; Sensitivity: %100, Specificity: %100, $p<0.001$; Sensitivity: %80.0, Specificity: %100, $p<0.001$ respectively for miR-21, NT-proBNP and galectin-3) from DM (Table 4.2). Although it is not as powerful as other parameters, miR-1 levels were found to be statistically significant in differentiating HF+DM patients from DM according to ROC analysis (Sensitivity: %46.7, Specificity: %22.2, $p<0.001$, Table 4.2). Analysis results for other parameters are shown in(Figure 4.5 and Table 4.2).

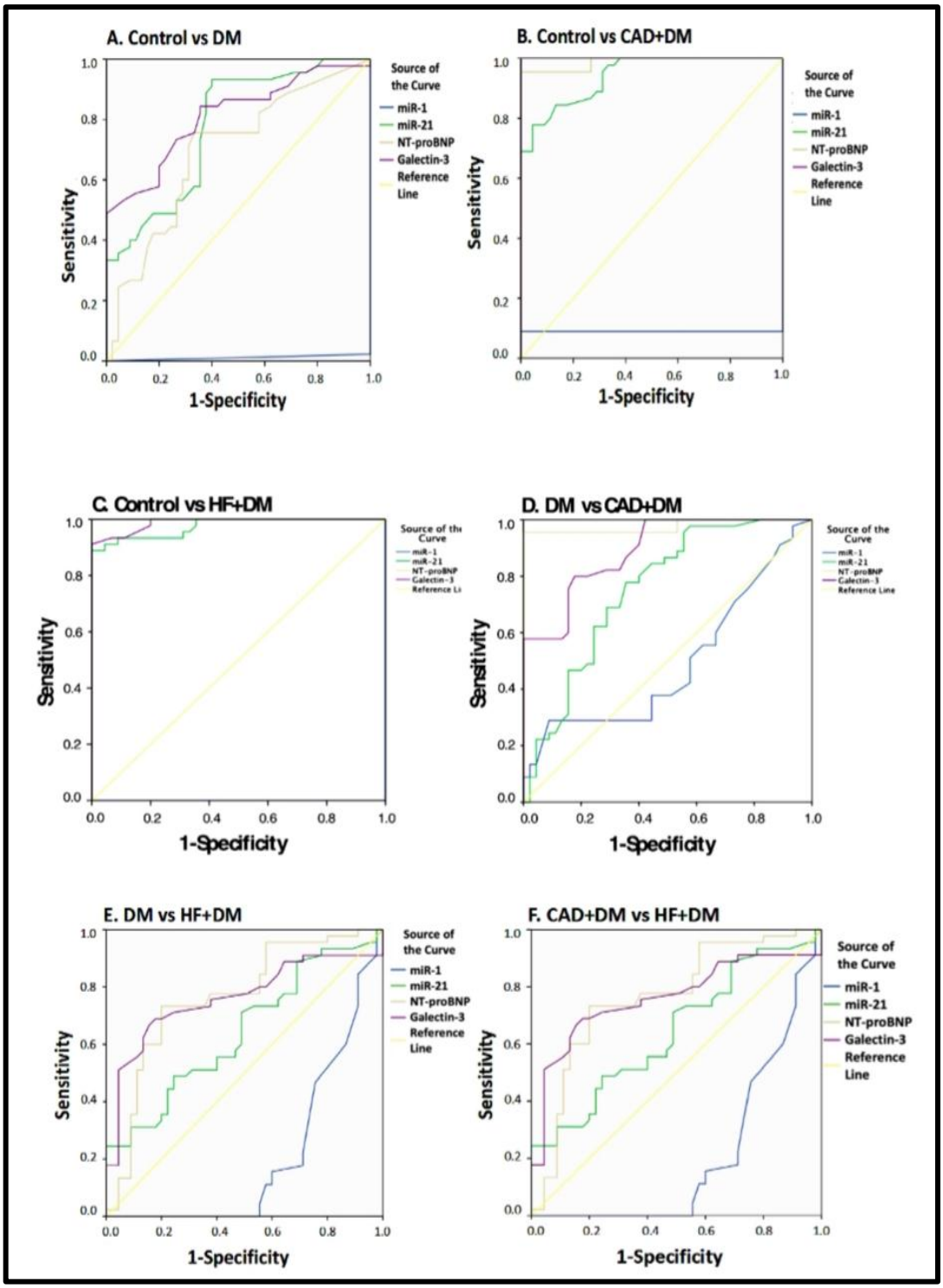

Figure 4. 5 ROC analysis of laboratory findings for different groups: A. Control vs DM groups B. Control vs CAD+DM groups C. Control vs HF+DM groups D. DM vs CAD+DM groups E. DM vs HF+DM groups F. CAD+DM vs HF+DM vs CAD+DM groups.

Table 4.2 ROC analysis of laboratory findings for different groups and risk assesment according to cut-off values

A. Control vs DM						
Variables	**AUC**	**P**	**Sensitivity**	**Specificity**	**Cut-off value**	**OR for cut-off values (95%CI)**
miR-1	0.011	**0.000**	-	-	-	-
miR-21	0.777	**0.000**	0.933	0.600	0.840	**21.000 (5.641-78.173)*** **
NT-proBNP	0.694	**0.002**	0.756	0.667	95.5	**6.182 (2.464-15.512)*** **
Galectin-3	0.812	**0.000**	0.733	0.733	4.95	**7.563 (2.971-19.251)*** **
B. Control vs CAD+DM						
Variables	**AUC**	**P**	**Sensitivity**	**Specificity**	**Cut-off value**	**OR for cut-off values (95%CI)**
miR-1	0.089	**0.000**	-	-	-	-
miR-21	0.944	**0.000**	0.800	0.911	1.130	**38.875 (10.338-124.488)*** **
NT-proBNP	0.988	**0.000**	0.956	1.000	167.5	X
Galectin-3	1.000	**0.000**	1.000	1.000	6.200	X
C. Control vs HF+DM						
Variables	**AUC**	**P**	**Sensitivity**	**Specificity**	**Cut-off value**	**OR for cut-off values (95% CI)**
miR-1	0.000	-	-	-	-	-
miR-21	0.974	**0.000**	0.889	1.000	1.223	X
NT-proBNP	1.000	**0.000**	1.000	1.000	210.500	X
Galectin-3	0.988	**0.000**	0.911	1.000	6.700	X
D. DM vs CAD+DM groups						
Variables	**AUC**	**P**	**Sensitivity**	**Specificity**	**Cut-off value**	**OR for cut-off values (95% CI)**
miR-1	0.492	0.063	-	-	-	Not significant
miR-21	0.755	**0.000**	0.778	0.667	1.168	**7.0 (2.742-17.867)*** **
NT-proBNP	0.976	**0.000**	0.956	1.000	165.5	X
Galectin-3	0.891	**0.000**	0.800	0.822	7.150	**18.5 (6.428-53-245)*** **
E. DM vs HF+DM groups						
Variables	**AUC**	**P**	**Sensitivity**	**Specificity**	**Cut off-Value**	**OR for cut-off values (95%CI)**
miR-1	**0.194**	**0.000**	0.467	0.222	0.125	**1.667 (1.175-2.363)*** **
miR-21	**0.834**	**0.000**	0.844	0.711	1.463	**12.020 (4.320-33.460)*** **
NT-proBNP	**1.000**	**0.000**	1.000	1.000	208.5	X
Galectin-3	**0.933**	**0.000**	0.800	1.000	8.40	X
F. CAD+DM vs HF+DM groups						
Variables	**AUC**	**P**	**Sensitivity**	**Specificity**	**Cut-off value**	**OR for cut-off values (95%CI)**
miR-1	0.207	**0.000**	0.467	0.244	0.125	**1.619 (1.137-2.306)*** **

miR-21	0.640	**0.022**	0.711	0.511	1.695	**2.570 (1.078-6.144)***
NT-proBNP	0.764	**0.000**	0.733	0.800	4747.00	**11.0 (4.108-29.454)*****
Galectin-3	0.761	**0.000**	0.711	0.756	9.250	**7.608 (2.981-19.417)*****

*p<0.05; ***p<0.001; AUC: Area uder curve; OR: Odds Ratio; X: OR could not be calculated because there were no values above or below the cut-off value in the groups.

According to the cut-off values, the risk of the disease was calculated for those who had higher levels than the cut-off values (Table 4.2). When compared to DM, serum miR-1 and miR-21 expression levels were found to be higher than 0.125- and 1.463-fold change, respectively and increased the risk of developing HF+DM by 1.667 fold and 12.020 fold, respectively (Table 4.2). The results of the analysis for other parameters are shown in (Table 4.2).

Compared to the control group; serum galectin-3 levels higher than 4.95 ng/mL were found to increase the risk of developing DM by 7.5 fold. In addition, when DM and CAD+DM groups are compared; serum galectin-3 levels higher than 7.15 ng/mL were shown to increase the risk of CAD+DM by 18.5 fold. And it is interesting that when compared with CAD+DM and HF+DM groups; Galectin-3 levels greater than 9.25 ng/mL were found to increase the risk of HF+DM by 7.6 folds. Serum NT-proBNP levels higher than 95.5 pg/mL increased the risk of developing DM by 6.18 folds (compared to control); and higher than 4747 pg/mL increased the risk of HF+DM (according to CAD+DM) (Table 4.2).

In cases where the value of miR-21 is higher than 1.695 and the NT-proBNP value is higher than 4747 pg/mL, the probability of HF+DM (positive predictive value) was found as 95.2%. In cases where the value of miR-21 was lower than 1.695 and the NT-proBNP value was less than 4747 pg/mL, the probability of being only DM (positive predictive value) was found to be 66.7% (Table 4.3).

Table 4.3 Positive predictive values of combinations based on cut-off for miR-21 and NT-proBNP

	DM		**CAD+DM**		**HF+DM**	
	n	**%**	**n**	**%**	**N**	**%**
miR-21<1.696 and NT-proBNP<4748	34	66.7	15	29.4	2	3.9
miR-21>1.695 and NT-proBNP<4748	11	26.2	21	50.0	10	23.8
miR-21<1.696 and NT-proBNP>4747			8	42.1	11	57.9
miR-21>1.696 and NT-proBNP>4747			1	4.8	20	95.2

In cases where the value of miR-21 was higher than 1.695 and the galectin-3 value was higher than 9.25 ng/mL, the probability of HF+DM (positive predictive value) was found as 73.7%. In cases where the value of miR-21 was less than 1,695 and the galectin-3 value was less than 9.25 ng/mL, the probability of being only DM (positive predictive value) was found to be 51.5% (Table 4.4).

Table 4.4 Positive predictive values of combinations based on cut-off for miR-21 and galectin-3

	DM		CAD+DM		HF+DM	
	n	**%**	**n**	**%**	**n**	**%**
miR-21<1.696 and galectin-3<9.26	34	51.5	22	33.3	10	15.2
miR-21>1.695 and galectin-3<9.26	11	42.3	12	48.0	2	8.0
miR-21<1.696 and galectin-3>9.25			1	25.0	3	75.0
miR-21>1.695 and galectin-3>9.25			10	26.3	28	73.7

In cases where NT-proBNP value was higher than 4747 pg/mL and galectin-3 value was greater than 9.25 ng/mL, the probability of HF+DM (positive predictive value) was found to be 100%. In cases where NT-proBNP value was lower than 4747 pg/mL and galectin-3 value was less than 9.25 ng/mL, the probability of being only DM (positive predictive value) was found as 63.4% (Table 4.5).

Table 4.5 Positive predictive values of combinations based on cut-off for NT-proBNP and Galectin-3

	DM		CAD+DM		HF+DM	
	n	**%**	**n**	**%**	**n**	**%**
NT-proBNP<4748 and galectin-3<9.26	45	63.4	25	35.2	1	1.4
NT-proBNP>4747 and galectin-3<9.26			9	42.9	12	57.1
NT-proBNP<4748 and galectin-3>9.25			11	50.0	11	50.0
NT-proBNP>4747 and galectin-3>9.25					21	100.0

In the cases where the value of miR-21 was higher than 1.695, the NT-proBNP value was 4747 pg/mL and the galectin-3 value was greater than 9.25 ng/mL, the probability of HF+DM (positive predictive value) was found to be 100%. In cases where the value of miR-21 was 1.695, NT-proBNP value was lower than 4747 pg/mL and galectin-3 value was less than 9.25 ng/mL, the probability of being only DM (positive predictive value) was found to be 69.4% (Table 4.6).

Table 4.6 Positive predictive values of combinations based on cut-off for miR-21, NT-proBNP and galectin-3

	DM		CAD+DM		HF+DM	
	n	**%**	**n**	**%**	**n**	**%**
miR-21<1.696 and NT-proBNP<4748 and galectin-3<9.26	34	69.4	14	28.6	1	2.0
miR-21>1.695 and NT-proBNP <4748 and galectin-3<9.26	11	50.0	11	50.0		
miR-21<1.696 and NT-proBNP >4747 and galectin-3<9.26			8	47.1	9	52.9
miR-21>1.696 and NT-proBNP>4747 and galectin-3<9.26			1	33.3	2	66.7
miR-21<1.696 and NT-proBNP<4748 and galectin-3>9.25			1	50.0	1	50.0
miR-21>1.695 and NT-proBNP<4748 and galectin-3>9.25			10	50.0	10	50.0
miR-21<1.696 and NT-proBNP>4747 and galectin-3>9.25					2	100.0
miR-21>1.695 and NT-proBNP>4747 and galectin-3>9.25					18	100.0

4.3 Discussion

In this study, we report that miR-1 and miR-21 expressions were associated with the presence of DM. miR-1 was found to be downregulated, while miR-21 was overexpressed in all patients when compared with the control subjects. We noted several important differences between DM, CAD+DM and HF+DM with respect to miR-1 and miR-21. First, among subjects with DM, miR-1 were the lowest in HF+DM. Second, miR-21 expression is higher in HF+DM group compared to CAD+DM group. Furthermore, miR-1 were negatively correlated with NT-proBNP and galectin-3 levels in CAD+DM group. miR-21 showed stronger positive correlation with NT-proBNP and galectin-3. According to the results of ROC analysis, miR-21 might be a good biomarker to distinguish HF+DM from DM. Our results underscore the importance of decreased miR-1 and increased miR-21 expression as a cardiovascular risk factor and suggest that miR-21 can be used as an early predictor of HF in asymptomatic T2DM patients.

DM is known to be a potent and prevalent risk factor for heart disease. Diabetic cardiomyopathy is an early complication of DM and is revealed with diastolic dysfunction followed by abnormalities in systolic function [425]. Due to New York Heart Association (NYHA) functional class, the mortality risk associated with HF patients is not explained fully with comorbidities (DM, anemia, and renal insufficiency) and treatment strategies [426]. It is not clear if systemic diseases such as DM have effect on the predictive value of biomarkers for HF. In the current study, serum NT-proBNP and galectin-3 levels were the highest in HF+DM. In the study of Ballo et al. [427] the association between NT-proBNP and risk of cardiac events in a population of asymptomatic diabetic patients were enrolled in a primary care setting and NT-proBNP levels added an independent and an incremental prognostic value for the prediction of the clinical outcome. Serum NT-proBNP levels were found to be increased in DM, CAD+DM and HF+DM groups. Furthermore NT-proBNP levels were higher in HF+DM group compared to CAD+DM group. Both the current and the other mentioned study [427,428,429,430] suggest that an elevated plasma NT-proBNP level in asymptomatic patients with DM should alert physicians for an increased risk of the cardiovascular events.

It is interesting that when compared with CAD+DM and HF+DM groups; Galectin-3 levels in CAD+DM was found lower than HF+DM. Tan et al. [431] investigated the

relationship between serum galectin-3 and incident cardiovascular events and all-cause mortality in T2DM patients. According to the results of this study, serum galectin-3 was found related with the adverse outcomes in subjects with or without prevalent CAD independent from the traditional cardiovascular risk factors. The results of galectin-3 in T2DM seems confusing because some studies claim that galectin-3 deficiency is associated with insulin resistance, and galectin-3 elicits a protective effect in T2DM by acting as a receptor for advanced glycation end products (AGEs) [432,433]. However, Li et al. [434] showed that in galectin-3 gene knockout mice which were fed with a high-fat diet, the development of insulin resistance was found to be significantly reduced. Furthermore, the results of this study also provided preliminary evidence that extracellular galectin-3 binds to the insulin receptor directly and attenuates downstream pathways, suggesting galectin-3 to be a novel targetable link between the insulin resistance and T2DM. Holmager et al. [435] suggest that glucose metabolism is associated with circulating galactin-3 in HF, as elevated levels were found in patients with DM and a relation with increasing HbA1c levels was also demonstrated. Both our and the mentioned previous study [431,432,434,435] suggest that there is an urgent need to develop galectin-3 inhibitors that have a high oral bioavailability and a low toxicity profile to combat increased galectin-3 levels which are related with the developing HF in DM.

The circulating miR-1 which have anticardiac hypertrophic effects was found downregulated in all patients groups when compared with the control subjects in the present study. In addition, miR-1 expression was found to be decreased gradually in DM, CAD+DM and HF+DM groups Furthermore miR-1 was negatively correlated with NT-proBNP and galectin-3 levels in CAD+DM and HF+DM and it especially showed the strongest correlation in HF+DM group. Although being not as powerful as the other parameters, still miR-1 levels were found to be statistically significant in differentiating HF+DM patients from DM according to ROC analysis. Similar to our results Sygitowicz et al. [17] demonstrated that miR-1 was significantly downregulated. Downregulation of the expression of miR-1 was correlated with the increase of serum NT-proBNP concentration in patients with symptomatic HF in NYHA class II/III. As no patients with acute myocardial infarction were included in this study, the miR-1 expression pattern was not influenced by the presence of acute ischaemia or myocardial necrosis, which could potentially increase the expression of this type of miR. In the present study, when compared to DM, serum miR-1 expression were found to be higher

than 0.125-fold change and increased the risk of developing HF+DM by 1.667 fold. According to the results of our study, miR-1 expression together with NT-proBNP and galectin-3 levels might be useful in predicting the onset of HF in asymptomatic T2DM patients. There are controversial findings concerning the importance of miR-1 in heart disease. Plasma miR-1 was up-regulated in patients with acute myocardial infarction (AMI)-HF [125,436]. Ai et al. [126] showed that miR-1 levels were significantly higher in the plasma of AMI patients compared with non-AMI subjects and the levels were dropped to normal values on discharge following the medication. Increased circulating miR-1 was not associated with age, gender, blood pressure, DM or the established biomarkers for AMI. Tomaniak et al. [437] found that in symptomatic HF patients with LVH, galectin-3 concentrations and miR-1 expressions were correlated with anatomic changes of the left ventricle. Karakikes et al. [438] restoration of miR-1 gene expression is a strong medicinal planning to invert pressure resulting from cardiac hypertrophy and prohibit maladaptive cardiac reordering. miR-1 was suggested to be a therapeutic potential either via being used to target specific genes, or via becoming a therapeutic target itself [439].

The roles of miR-21 in cardiac diseases are controversial [127,440,441,442,443,444,445,446]. The reason for this is probably because miR-21 plays different roles in different cell types. miR-21 is only present in interstitial cells and correlates with collagen expression in the heart [443]. In the present study, serum miR-21 expression was upregulated in patients in CAD+DM and HF+DM groups and found higher than the control groups, with 1.79-, and 2.21-fold changes, miR-21 levels also were significantly higher in patients with HF+DM group than in patients with DM with 1.70-fold changes. No correlation was found between the EF with miRNAs. But, miR-21 had correlation with NT-proBNP and galectin-3 in DM, CAD+DM and HF+DM groups. Similar to our results, Sygitowicz et al. [17] reported that overexpression of miR-21 was seen in all patients, independent of HF severity and overexpression of miR-21 was correlated significantly with galectin-3 levels. Furthermore, according to the results of ROC analysis, miR-21 might be a good biomarker to distinguish HF+DM from DM. Contrary to our results, Tomaniak et al. [437] found significant down-regulation of miR-21 associated with the increase of LVEDD in symptomatic HF. miR-21 was up regulated in fibroblasts with high glucose treatment and exerted its harmful effects [440]. Dai et al. [447] demonstrated that miR-21 exerted its protective role directly in cardiac myocytes and encouraged further

development of cardiac specific overexpression of miR-21 therapy toward cellular tropism. Circulating some miRs have also been suggested as promising diagnostic biomarkers in patients with T2DM. But, the results of miR-21 in T2DM are controversial [448,449,450, 91]. Other study showed that serum miR-21 level was not associated with T2D, however, its expression was significantly down-regulated in serum of obese diabetic and non-diabetic subjects [448,449]. Zampetaki et al. [91] reported that plasma miR-21 levels were downregulated in T2DM patients when compared to non-diabetic subjects. Similar to our results, an upregulation of circulation miR-21 was reported in subjects with T2D compared to prediabetic subjects [450]. The reason of the discrepancy among different studies remains unclear. A possible explanation regading to the divergence among the studies might be because of the differences in the source of samples (plasma vs serum) or the differences in study populations [448]. When the results of predictive analysis are taken into consideration, we also believe that miR-21, NT-proBNP and galectin-3 as activity panel indicator will be useful especially for HF+DM.

4.4 Conclusion

miR-1 and miR-21 are the main profibrogenic miRs, they are independently associated with the presence and severity (decreased EF) of acute HF in T2DM. miR-1 expression together with NT-proBNP and galectin-3 might be useful in predicting the onset of HF in asymptomatic T2DM patients. miR-21 might be a good biomarker to distinguish HF+DM from DM. These findings led to use miR-1 and miR-21 as the circulating potential biomarkers for therapeutic prospects of HF. The roles of miR-1 and miR-21 in the pathogenesis of symptomatic HF in T2DM need to be determined in large-scale prospective study.

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Papers

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