ROLE OF EPICARDIAL ADIPOSE TISSUE SECRETED INTERLEUKIN-34 IN FIBROBLAST TO MYOFIBROBLAST TRANSDIFFERENTIATION AND ATRIAL FIBROSIS

Divya Jyothi Madipally

July 2021

Director of Dissertation: Dr. Jacques Robidoux

Major Department: Pharmacology and Toxicology

The adiposity of the heart has been proven to be significantly associated with cardiac dysfunction. Chronic low-grade inflammation of the adipose tissue and cardiac fibrosis are common complications in type 2 diabetes mellitus (T2DM). Characterized by excessive deposition of extracellular matrix (ECM) proteins like collagen types I and III, and transdifferentiation of cardiac fibroblasts to myofibroblasts, cardiac fibrosis is majorly involved in the etiology of cardiac remodeling in T2DM. These fibrogenic actions are suggested to be mediated by the inappropriate release of adipocytokines, triggering a local inflammatory state in the heart. The aim of this study was to measure the cytokines secreted by the EAT and evaluate their correlation with atrial fibrosis in patients with T2DM. Sixty-four patients undergoing cardiac surgery were enrolled in this study. Masson's trichrome staining was performed on frozen sections of right atrial appendage (RAA) to quantify myocardial fibrosis. EAT medium was used for measuring 49 cytokines using a combination of multiplex ELISAs. Primary cardiac fibroblasts were incubated with or without Interleukin-34 (IL-34), Transforming Growth Factor-β (TGF-β), and with or without TGF-β receptor (TGF-βR) and CSF1- receptor (CSF1-R) inhibitors. Immunofluorescent staining for α-

Smooth Muscle Actin (α-SMA) was performed to evaluate fibroblast to myofibroblast transdifferentiation, and western blotting analysis of collagen types-I and III was performed to evaluate fibrogenesis. Histology revealed increased myocardial fibrosis in the RAA of patients with T2DM. Myocardial fibrosis also correlated with the plasma glycated hemoglobin A1c (HbA1c) levels. Of the cytokines secreted by EAT explants 11 correlated with myocardial fibrosis, and 3 correlated with the presence of T2DM. Among these cytokines, IL-34 and soluble IL-6 receptor β were correlating with the RAA fibrosis. Furthermore, IL-34 promoted fibrogenesis in cardiac fibroblasts. Interestingly, neither the inhibition of TGF-βR involved in most fibrotic models nor CSF1-R, the principal receptor for IL-34, attenuated IL-34 induced fibroblast differentiation. In conclusion, elevated levels of IL-34 in the EAT of patients with T2DM correlated with atrial fibrosis and promoted fibroblast to myofibroblast transdifferentiation independent of TGFβ-R and CSF1-R.

ROLE OF EPICARDIAL ADIPOSE TISSUE SECRETED INTERLEUKIN-34 IN FIBROBLAST TO MYOFIBROBLAST TRANSDIFFERENTIATION AND ATRIAL FIBROSIS

A Dissertation Presented to The Faculty of the Department of Pharmacology and Toxicology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor in Philosophy in Pharmacology and Toxicology

By

Divya Jyothi Madipally

July 2021

ROLE OF EPICARDIAL ADIPOSE TISSUE SECRETED INTERLEUKIN-34 IN FIBROBLAST TO MYOFIBROBLAST TRANSDIFFERENTIATION AND ATRIAL FIBROSIS

By

Divya Jyothi Madipally

APPROVED BY:

Director of Dissertation _____
Jacques Robidoux, PhD

Committee Member _____
Ethan J Anderson, PhD

Committee Member _____
Kymberly Gowdy, PhD

Committee Member _____
Srinivas Sriramula, PhD

Committee Member _____
Lisandra E. de Castro Brás, PhD

Chair of the Department/Committee Member _____
David Taylor, PhD

Dean of the Graduate School _____
Paul J. Gemperline, PhD

DEDICATION

To my uncle, Dr. Janna R Pathi

This is your PhD too.

ACKNOWLEDGEMENT

First and foremost, I thank God for all that He has brought me through, for giving me the strength, joy, peace and for all His love and provisions.

To my mentor Dr. Jacques Robidoux thank you for your mentorship and guidance. It has truly been a privilege to work with you, and I cannot thank enough for your constant effort in making me an independent scientist. It all goes back to 2017, I'm grateful to you for choosing me as PhD student and for believing in me, I have learnt how to enjoy science from you. I thank all my research committee members Dr. David Taylor, Dr. Srinivas Sriramula, Dr. Kymberly Gowdy, Dr. Lisandra E. de Castro Brás, Dr. Ethan J Anderson, and research collaborators Dr. Alwair and Dr. Kiser. And a special thanks to Dr. Paul Gemperline for his encouragement and support for the graduate students at East Carolina University.

I thank all the staff members and my fellow graduate students, in the Pharmacology and Toxicology Department at East Carolina University for their support and guidance. I acknowledge Dr. Margaret Nelson, Dr. Jared Shine, Dr. Dan Landin, Barbara Davis, Bin Lui, and my colleagues for making science and work more fun.

I express my heartfelt gratitude to my parents, siblings, and my two lovely nieces Vaishali and Vedanshi for their constant love and support. Finally, my dearest uncle Dr. Janna R Pathi, to whom I owe this PhD, and everything I will contribute to the society as a scientist for the rest of my life – I say thank you for your unconditional love, support, and confidence in me to become a successful scientist.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Name
α-SMA	Alpha smooth muscle actin
ANOVA	Analysis of variance
AF	Atrial fibrillation
BMI	Body mass index
BSA	Bovine serum albumin
CVDs	Cardiovascular diseases
CSF-1	Colony stimulating factor – 1
CABG	Coronary artery bypass surgery
CRP	C-reactive protein
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme linked immunosorbent assay
EAT	Epicardial adipose tissue
ECM	Extracellular matrix
IL-1β	Interleukin - 1β
IL-34	Interleukin - 34
IL-6	Interleukin – 6
ICAM	Intracellular adhesion molecule
MMPs	Matrix metalloproteinases
MAPKs	Mitogen activated protein kinases
MCP-1	Monocyte chemoattractant protein – 1
NDM	Non-diabetic
NF-ΚB	Nuclear factor kappa B
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
POAF	Post-operative AF
RAA	Right atrial appendage

RT	Room temperature
SAA	Serum amyloid
SD	Standard deviation
TGF-βR	TGF - β receptor
TIMPs	Tissue inhibitor of metalloproteinases
TGF-β	Transforming growth factor – β
TNF-α	Tumor necrosis factor alpha
T2DM	Type 2 diabetes mellitus
VCAM	Vascular cell adhesion molecule
EDTA	2,2',2",2"'-(ethane-1,2 diyldinitrilo) tetraacetic acid
DAPI	4'6'- diaminido-2-phenylindole

CHAPTER ONE

INTRODUCTION

1.0 Cardiac fibrosis

Nearly all the cardiovascular diseases (CVDs) are associated with cardiac fibrosis [1]. Fibrosis is mainly characterized by the accumulation of fibroblasts, and an increased deposition of extracellular matrix (ECM) proteins like collagen types I and III, leading to hypertrophy and altered function [2]. Cardiac fibrosis is manifested in two forms, reactive interstitial fibrosis, and replacement fibrosis [3]. Reactive interstitial fibrosis refers to the expansion of the cardiac interstitial space due to excessive deposition of ECM without significant loss of cardiomyocytes [4]. Whereas replacement fibrosis occurs as a response to a cardiac injury where cardiac tissue that is damaged or dead is replaced with collagen-based scarring [5].

Reactive interstitial fibrosis is an adaptive response that aims to preserve the cardiac functional capacity, but progresses to replacement fibrosis, resulting in cardiac hypertrophy and necrosis [6]. Several studies in both human and animal models explored the type and extent of cardiomyocyte death related hypertrophy and necrosis [6-11]. Studies in conditions like myocardial infarction show that there is an initial inflammatory reaction followed by cardiomyocyte death, resulting in replacement fibrosis [12]. Pathological conditions like diabetic cardiomyopathy (DCM), hypertension (HTN), and type 2 diabetes mellitus (T2DM) are known to induce chronic interstitial and perivascular deposition of collagen eventually leading to fibrotic scarring [13-15]. Although the underlying pathophysiological conditions leading to fibrotic

remodeling differ in patients with various CVDs, the cellular effectors involved in fibrotic remodeling are common, with the involvement of similar networks of molecular signals [16].

1.1 Cardiac fibroblasts and myofibroblasts

In the heart, the myofibroblasts are the principal producers of the ECM and contribute significantly to cardiac fibrosis [17]. However, the source of these myofibroblasts in not completely known and remains an area of active research [18, 19]. Myofibroblasts are thought to be derived via the activation of the resident fibroblasts [19]. However, this view is being challenged by the evidence that there is a phenotypic heterogenicity among the fibroblasts [20], not only between organs, but also within the same organ in physiological and pathological conditions [21, 22].

What are cardiac fibroblasts? Fibroblasts are the largest cell population of the myocardium [23]. These are cells with mesenchymal origin that produce a variety of matrix proteins and biochemical mediators such as growth factors and proteases [24]. Morphologically, fibroblasts are flat spindle shaped cells with more than one processes originating from their cell body. Locally in the cardiac tissue, fibroblasts are the only cell types that are not associated with a base membrane [25]. Cardiac fibroblasts are an essential cell type in the heart that are responsible for the homeostasis of the ECM. These are pleiomorphic cells whose expression and arrangement of the contractile proteins is mainly dependent on several factors in their microenvironment like the presence of cytokines, and chemokines [22]. Upon injury, these cells transform to myofibroblast phenotype and contribute to fibrosis. The distinction between the fibroblasts and myofibroblasts is commonly based on their ability to express contractile proteins such as alpha smooth muscle actin (α-SMA), that are principally involved in the transition to the myofibroblast phenotype [26]. This transition of fibroblasts to myofibroblasts progressively leads to heart failure [8].

1.2 Extracellular matrix (ECM)

The ECM is a very dynamic non-cellular three-dimensional network that is present in all tissue types and plays a crucial role in the physiology and pathology of the heart [27]. The ECM does not simply function as a mechanical support system to preserve tissue integrity, but also serves as a signaling hub that is involved in signaling cascades critical for cell function, and act as a reservoir of growth factors that can be released during an injury to regulate cell behavior and activate a repair program inside the myocardium [28]. A healthy cardiac ECM is comprised of an interstitial component that surrounds all the myocardial cells and forms a structural scaffold, and a pericellular component that is in close proximity to certain cell types.

The basement membrane of the ECM is comprised of collagens, laminins, and other proteoglycans that separate cardiomyocytes from the surrounding interstitial matrix [29]. In mammalian hearts the ECM is organized in three interconnected levels: the epimysium encasing the entire organ, the perimysium defining the major myofiber bundles, and the endomysium that surrounds the individual cardiomyocytes [30]. Additionally, the intramyocardial ECM network that includes the endomysial and perimysial layers, contains significant amounts of matrix proteins that are present in the adventitia of the myocardial arteries and arterioles [30, 31].

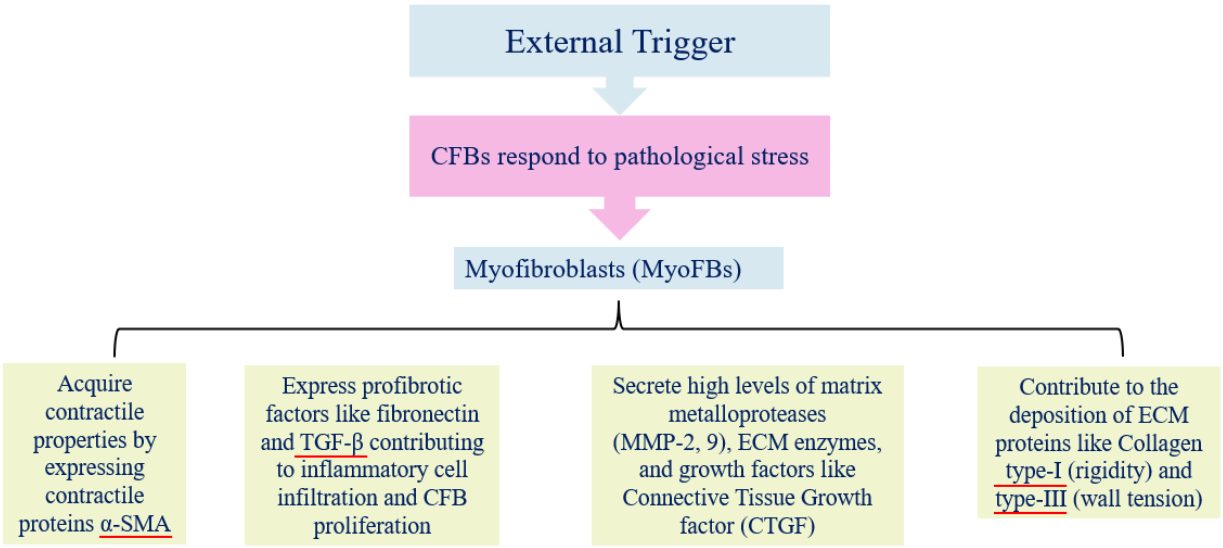
1.3 Collagen types I and III

The crosslinking and remodeling of the ECM occur as a result of tissue-injury repair in the heart and are both associated with cardiac fibrosis [32]. Earlier studies have shown that the composition of a healthy cardiac ECM predominantly consists of collagen types-I and III as the major structural components inside the interstitial matrix [33]. Type-I collagen typically forms thick-rod like fibers

in the epimysium and the perimysium, it is also the structural component of the cardiac interstitium [34]. Type-III collagen is known to form a fine network of fibers that are more commonly found in the endomysium [35].

Studies have shown that the relative amounts of collagen type-I versus collagen type-III fibers regulates the myocardial compliance in cardiac remodeling. Collagen type-I forms thicker and stiffer fibers, whereas the finer reticular collagen type-III fibers are known to be more compliant and increase the elasticity of the ECM. Additionally, alteration in the ratio of collagen types I and III is attributed to cardiac fibrosis. This ratio is dependent on the underlying cause of the fibrosis. Studies in experimental MI models have shown an increased expression of collagen type-I [36-38], while the experimental models of ischemic cardiomyopathy showed an increase in collagen type-III [35]. Some studies also reported that the accumulation of both collagen types I and III followed by a maturation process leads to a fibrotic scar formation [39]. Therefore, the collagen types I and III serve as good parameters to understand the biology and underlying mechanisms that are involved in cardiac fibrosis (Figure-1).

Figure 1: Markers of cardiac fibrosis in pathological conditions



2.0 Inflammation

2.1 Role of inflammation in cardiovascular diseases:

Inflammation and its failure to resolve are considered central in the development and worsening of several CVDs [40]. Followed by all the observations regarding the role of these inflammatory processes, our knowledge about their crucial role in the pathology of cardiac fibrosis is expanding [41]. For example, elevated levels of inflammatory markers like C-reactive protein (CRP) and serum amyloid A (SAA) have been shown to be predictive of future cardiovascular events in several clinical settings [42-45].

Although the importance of inflammation in CVDs is expanding rapidly, one of the major challenges has been that inflammation is more often used to cover a wide variety of processes and observations. For instance, 'inflammation' can include observing non-specific changes in acute-phase proteins (such as CRP ad SAA) produced in the liver, remotely from the site of pathology, but also encompasses a nuanced understanding of local molecular and cellular processes in the heart or the vessel wall that is intimately involved in the disease causation.

Most of these biomarkers currently used to measure the inflammatory state in cardiac fibrosis are somewhat non-specific (for example, CRP, TNF-α, and IL-6). They reflect the downstream consequences of inflammation, while lacking information related to the site specific to activation and do not differentiate the functionally important activation pathways. Therefore, the challenge is in identifying inflammatory markers that are specific to the pathology and progression of cardiac fibrosis. Hence, identifying the local source and effects of these inflammatory mediators, and their related signaling pathways will provide a better understanding

of these inflammatory cytokines and may provide supporting evidence for the development of novel therapeutic strategies.

2.2 Pro- and anti- inflammatory cytokines in cardiac fibrosis

2.2.1 Transforming growth factor – beta (TGF-β)

Several studies have proven that TGF-β is a known fibrogenic growth factor involved in the initiation and exacerbation of fibrosis [46]. Significant experimental evidence supports the concept that TGF-β mediated tissue fibrosis has direct implication on fibroblasts [47]. In mouse models of fibrosis, fibroblast specific deletion of TGF-β receptor (TGF-βR) markedly attenuated the fibrotic response [48, 49]. TGF-β stimulation promotes the transdifferentiation of fibroblasts to myofibroblasts [50-53]. In addition to its role in myofibroblast transdifferentiation, TGF-β exerts ECM remodeling, by promoting increased production of collagen types I and III [54-56].

There are three different isoforms of TGF-β in mammals which include TGF-β1, TGF-β2, and TGF-β3, all encoded by three different genes [57]. Of these isoforms, the most predominant one to be involved in cardiac fibrosis is TGF-β1. Previous studies in humans and experimental animal models have shown that increased TGF-β1 is associated with worsening of cardiac fibrosis in heart failure [58-60]. Increased TGF-β1 binds to TGF-βRI (also called ALK5), and TGF-βRII to activate smad 2/3 pathway, thus promoting cardiac fibrosis [61, 62]

2.2.2 Tumor necrosis factor-alpha (TNF-α)

TNF-α is an acute phase reactive protein and is an inflammatory cytokine with pleiotropic biological effects. In the circulatory system, TNF-α is identified in several myocardial diseases such as acute coronary syndrome [63], acute MI [64], myocarditis [63, 65], dilated cardiomyopathy

and congestive heart failure (CHF) [66-68]. The role of TNF-α in cardiac fibrosis was confirmed by a study reporting that TNF-α global knockout mice showed attenuated inflammatory response and cardiac fibrosis induced by pressure overload [69].

2.2.3 Interleukin 1β (IL-1β)

Studies have shown controversial role of IL-1β in cardiac fibrosis. IL-1β could promote the migration of rat cardiac fibroblasts [70]. Inconsistent with this study, IL-1β inhibited myofibroblast transdifferentiation that was induced by TGF-β [71]. This observation was further supported by an in vivo study using mice with IL-1β deficiency which showed reduced cardiac dysfunction but exacerbated cardiac fibrosis [9]. However, other studies have shown an improved left ventricular function in patients with systemic inflammatory conditions like rheumatoid arthritis followed by IL-1β inhibition [72]. Together, these findings show an important role of IL-1β in cardiac fibrosis.

2.2.4 Interleukin-34 (IL-34)

IL-34 is a novel cytokine that was identified in 2008 as a ligand of colony stimulating factor receptor-1 (CSFR-1) [73]. IL-34 is known to bind to the extracellular domains of the CSFR-1, to the receptor-type protein-tyrosine phosphatase-zeta (PTP-ζ) [74], and to the chondroitin sulfate chains of syndecan-1 [75, 76]. Although it is a recently discovered cytokine, IL-34 has been identified as a multifunctional cytokine that regulates a variety of cellular responses including cell adhesion, differentiation, proliferation, metabolism, angiogenesis, inflammation, and immune responses. Based on the pathological condition involved, increased expression of IL-34 is shown to be associated with several inflammatory cascades, or conversely, mediate immune tolerance [73, 77, 78]. The immunoregulatory properties of IL-34 appear to be significant in several diseases.

Therefore, investigating the role of IL-34 in the immune microenvironment will be of great importance for a comprehensive understanding of its pathophysiological implications.

The expression of IL-34 can be detected at mRNA and protein levels in various types of cells [73]. However, the regulation of IL-34 secretion may depend on the cell type. In the extracellular biofluids IL-34 can be detected at low concentrations in serum/plasma, cerebral spinal fluid, synovial fluid, and saliva. In the nervous system IL-34 exhibits distinct developmental brain expression patterns and regulates the neuronal progenitor cell maintenance and maturation [79]. In contrast, in Langerhans cells microglia develop independently of IL-34 during embryogenesis but rely on it for their maintenance in specific regions of the adult brain [80]. Similarly, microglia rely on IL-34 for survival and homeostasis in specific regions of the brain. Collectively the physiological importance of IL-34 expression in these conditions remains to be explored in future works.

Among the receptors involved in IL-34 cell signaling, the Colony Stimulating Factor 1 – Receptor (CSF-1R) was widely explored [81-84]. Lin et al. first identified that IL-34 is the second ligand for CSF1-R through comprehensive proteomic analysis [78]. Despite lacking similarity with CSF-1 or other proteins, IL-34 is shown to tightly bind to CSF1-R and promote the differentiation, proliferation and survival of monocytes, macrophages, and osteoclasts similar to CSF-1 [85, 86]. Actions of IL-34 are implicated to be complex by the discovery of two other distinct receptors: the PTP-ζ, and Syndecan-1, suggesting additional roles for IL-34, compared to CSF-1. Thus, exploring the role of IL-34 and the downstream signaling receptors in CF might provide the potential for novel therapeutic targets.

3.0 Epicardial adipose tissue (EAT)

3.1 Role of EAT in cardiovascular diseases:

Interest in organ specific adiposity is growing rapidly, as a substantial accumulation of scientific-based evidence suggests that the anatomic location of the adipose tissue is an important contributor to the pathophysiology of several CVDs [87-91]. Epicardial adipose tissue (EAT) is a visceral fat tissue surrounding the heart and has gained interest over the past decade with the focus on investigating the cardiometabolic risk factors associated with it [92, 93]. EAT has drawn so much attention due to its close proximity to the myocardium, and its peculiar metabolic and clinic measurability.

EAT is located between the myocardium and the visceral pericardium (Figure-2) [94]. EAT and the pericardial adipose tissue (PAT) differ in their vascularization, EAT is supplied by the branches of the coronary arteries whereas PAT is vascularized from non-coronary sources [95]. Since there is no muscle fascia or barrier that divides EAT and the myocardium these two tissue layers in the heart share the same microcirculation. Because of its anatomical proximity to the heart, and the absence of fascial boundaries, EAT may interact locally with the myocardium through paracrine and vasocrine secretion of several pro- and anti- inflammatory adipocytokines [96, 97].

Figure-2: Epicardial adipose tissue

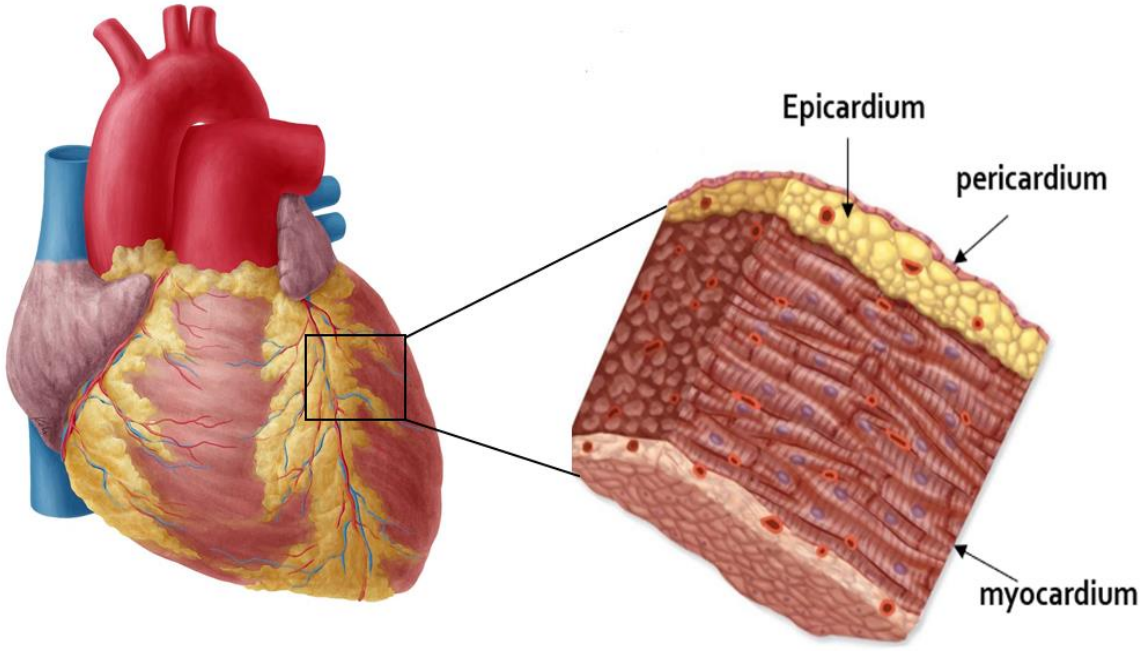


Figure-2: Epicardial adipose tissue as a visceral adipose tissue located between the pericardium and the myocardium, with very close proximity to the myocardium (Adapted from [98]).

3.2 EAT between physiology and pathophysiology

EAT is not merely a lipid-storage tissue of the heart but is also actively involved in regulating energy homeostasis locally in the heart [91]. It is considered as a metabolically active organ that serves as a source of several bioactive molecules [99]. There are two major mechanisms of interaction between the myocardium and the epicardium that have been mentioned earlier as the paracrine and the vasocrine pathways [100]. The paracrine release of the cytokines from the periadventitial epicardium could traverse the coronary wall in an outside-to-inside manner. Given the dense inflammatory infiltrate within the human EAT and its complex cellularity, it is possible to postulate that these cytokines are secreted by various cells in the EAT [101, 102]. Earlier studies have shown that EAT plays a significant role in the inflammatory process within the atherosclerotic plaque. A substantial amount of inflammatory infiltrate has been found in the EAT obtained during coronary artery bypass surgery (CABG) of subjects with severe coronary artery disease (CAD) [103-105]. Several inflammatory adipocytokines with atherogenic properties have been found to be secreted by the EAT through paracrine and vasocrine signaling pathways. [106-108]. Further, the expression and secretion of these inflammatory cytokines is higher in human EAT than in the subcutaneous fat of obese patients with CAD [109].

3.3 EAT transduces inflammation to the myocardium

Collectively all the aforementioned studies suggest that EAT can act as a transducer to mediate the effects of chronic low-grade systemic inflammation to the myocardium in a manner similar to its ability to adversely influence coronary arteries and atherogenesis. First, evidence suggests a correlation between the EAT thickness and the extent of cardiac inflammation in obesity [110], T2DM, and with cardiac fibrosis in patients with heart failure (HF) [111-113]. Second, evidence

points towards a strong association between the accumulation and inflammation of EAT, and the development of cardiac arrythmias [114-116]. It is possible that this interplay is most likely mediated by EAT causing fibrosis of the adjacent myocardium. This can be attributed to the structural and functional abnormalities of the EAT near the foci of the myocardium.

Third, the accumulation of the EAT is closely associated with an impaired myocardial microcirculation, cardiac diastolic filling abnormalities, increased vascular stiffness, and left atrial dilatation [117-120]. Fourth, patients with chronic low-grade inflammation not only accumulate EAT, but also accelerates the progression of atherosclerosis that is presented with microcirculatory derangements leading to fibrosis and HF [121-124]. Together these studies suggest a strong correlation between the chronic inflammation associated release of proinflammatory cytokines by the EAT and cardiac fibrosis (Figure-3).

Figure-3: Epicardial adipose tissue in inflammation and cardiac fibrosis

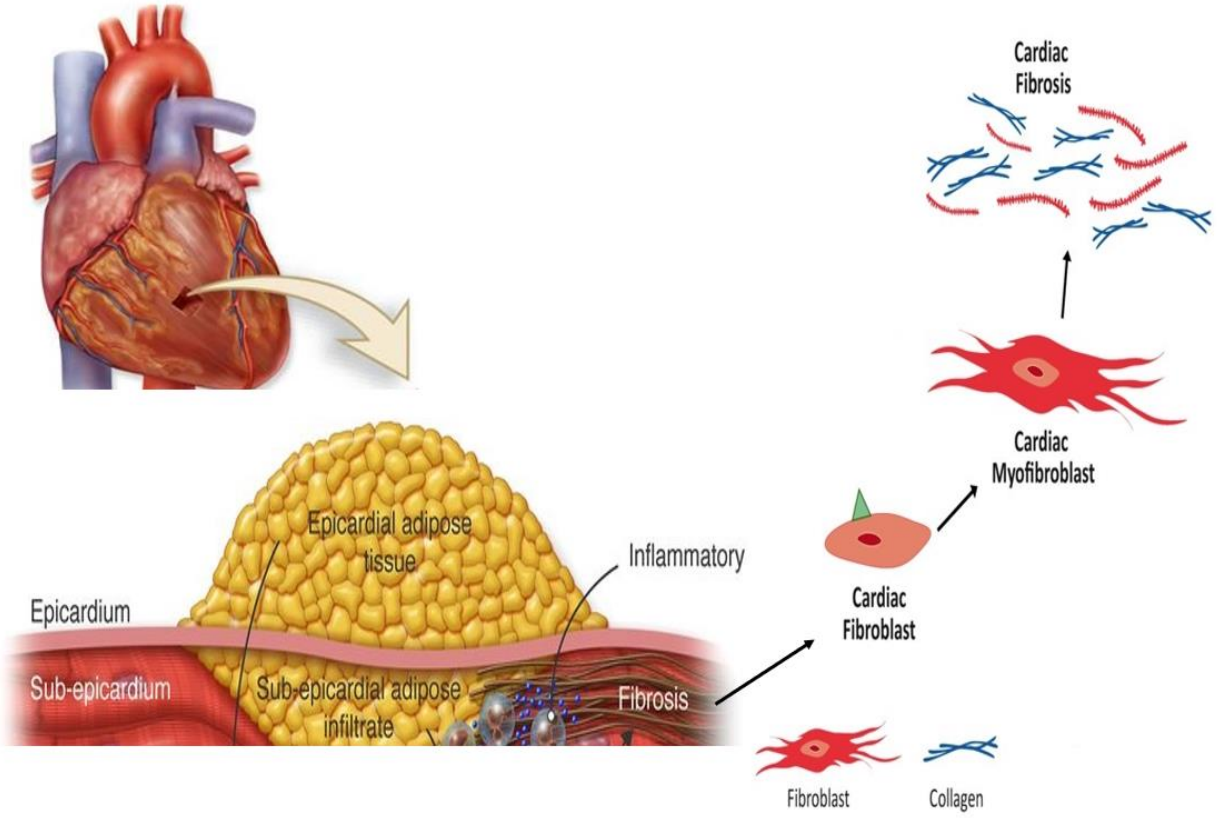


Figure 3: Increased levels of EAT in pathological conditions leading to the release of inflammatory cytokines leads to the transdifferentiation of cardiac fibroblasts to myofibroblasts and promotes cardiac fibrosis (Adapted from [2]).

4.0 Type 2 diabetes mellitus

4.1 Type 2 diabetes mellitus associated inflammation:

T2DM is associated with a decrease in insulin-mediated glucose uptake referred to as 'insulin resistance', initially the pancreatic islet cells compensate for the insulin resistance by enhancing the cell production and insulin secretory activity. Gradually, as the functional capacity of these islet β-cells reaches a threshold in compensating the extent of insulin resistance, there is an insulin secretory defect that ultimately develops as T2DM. This leads to the development of several long-term consequences involving microvascular and macrovascular changes. Studies have shown that these cellular changes may induce specific inflammatory responses creating a chronic low-grade inflammatory environment or exacerbate an existing inflammatory condition [125-128].

There are various cytokines and chemokines that are elevated in T2DM [129]. Cross-sectional and prospective studies have explored elevated levels of C-reactive protein (CRP), fibrinogen, plasminogen activating factor inhibitor [129-131]. Experimental animal models have shown that the production of tumor necrosis factor (TNF) by cells in the adipose tissue of obese rodents show early evidence of tissue inflammation in the pathogenesis of T2DM [131-133]. Serum levels of cytokines such as CRP have been widely explored as markers of inflammation and mediators of atherosclerosis in patients with T2DM [134]. Other cytokines like interleukin-6 are also shown to predict macrovascular events and mortality in T2DM patients with baseline CVD risk factors, when compared to CRP or fibrinogen levels [135, 136]. Adiponectin, which is predominantly synthesized in the adipose tissue is shown to have substantial anti-inflammatory properties by modulating dyslipidemia, and mechanisms associated with atherosclerotic risk in T2DM [137-140]. Studies like these have shown the significant role of inflammation in T2DM,

and the contribution of several pro- and anti-inflammatory mediators that are potentially involved in increasing the T2DM patient risk for CVDs.

4.2 Type 2 diabetes mellitus in cardiovascular disease

T2DM is an established risk factor for CVD. Several studies have shown that people with T2DM carry a 2-6-fold increased risk of CVD related morbidity and mortality compared to NDM [141, 142]. Vascular changes associated with T2DM are known to increase the risk of CAD and stroke by 2-4-fold, which further progress to HF [141]. Majority of the vascular changes in T2DM are related to the vascular adiposity, insulin resistance, and changes in the levels of circulating or highly expressed inflammatory factors [143, 144].

Consistent evidence proves the complex interaction of several risk factors such as the dyslipidemia, insulin resistance, and inflammation that can increase the risk of CVD in T2DM subjects. Major advances have been made in exploring the influence of these non-traditional risk factors for CVD in T2DM. However, currently we do not have any strong evidence showing that regular monitoring of these risk factors in T2DM can serve as better diagnostic tools. Therefore, further research focusing on the factors that lead to the vascular changes locally in the heart might be required to understand the internal impact of T2DM on CVDs.

4.3 Type 2 diabetes mellitus and cardiac fibrosis:

Siperstein et.al (1968) were the first to document that ECM alterations and muscle capillary basement membrane thickening occur in major target organs involved in diabetes [145]. These morphological and biochemical disturbances of the ECM are related to the internal organ

dysfunction [146]. As discussed earlier, ECM comprises a network of collagens, elastin's, structural glycoproteins, integrins, and several cytokines and chemokines that not only provide the mechanical support for the cells, but they also mediate complex cell-cell interactions in the vascular tissue. Myocardial tissue biopsies obtained from diabetic subjects showed increased collagen type-III in comparison to the non-diabetics [147]

Song et.al in 2006 showed that in animal models of T2DM increased intimal proliferation, thickness, and ECM deposition occur in the mesenteric arteries and aorta, increasing the risk of atherosclerosis and CAD [127]. This is mainly mediated by the cardiac remodeling and hypertrophy associated with increased expression of several cytokines, chemokines and other markers of the vascular smooth muscle cells in the myocardium [37, 38].

Expression of factors involved in cardiac fibrosis such as fibronectin vary in their proportion in the myocardium of T2DM subjects [148]. Proteoglycans [PGs] such as versican, decorin, and biglycan are shown to be involved in the pathogenesis of diabetic nephropathy [149]. The above- mentioned PGs are known to accumulate in atherosclerotic lesions, there by leading to ECM turnover events associated with cardiac fibrosis, such as migration and proliferation of CFBs, lipid metabolism and increased fatty acid circulation [150]. Collectively, these findings suggest the importance of exploring the source and the role of the cardiac fibrosis mediators involved in the ECM turnover in T2DM.

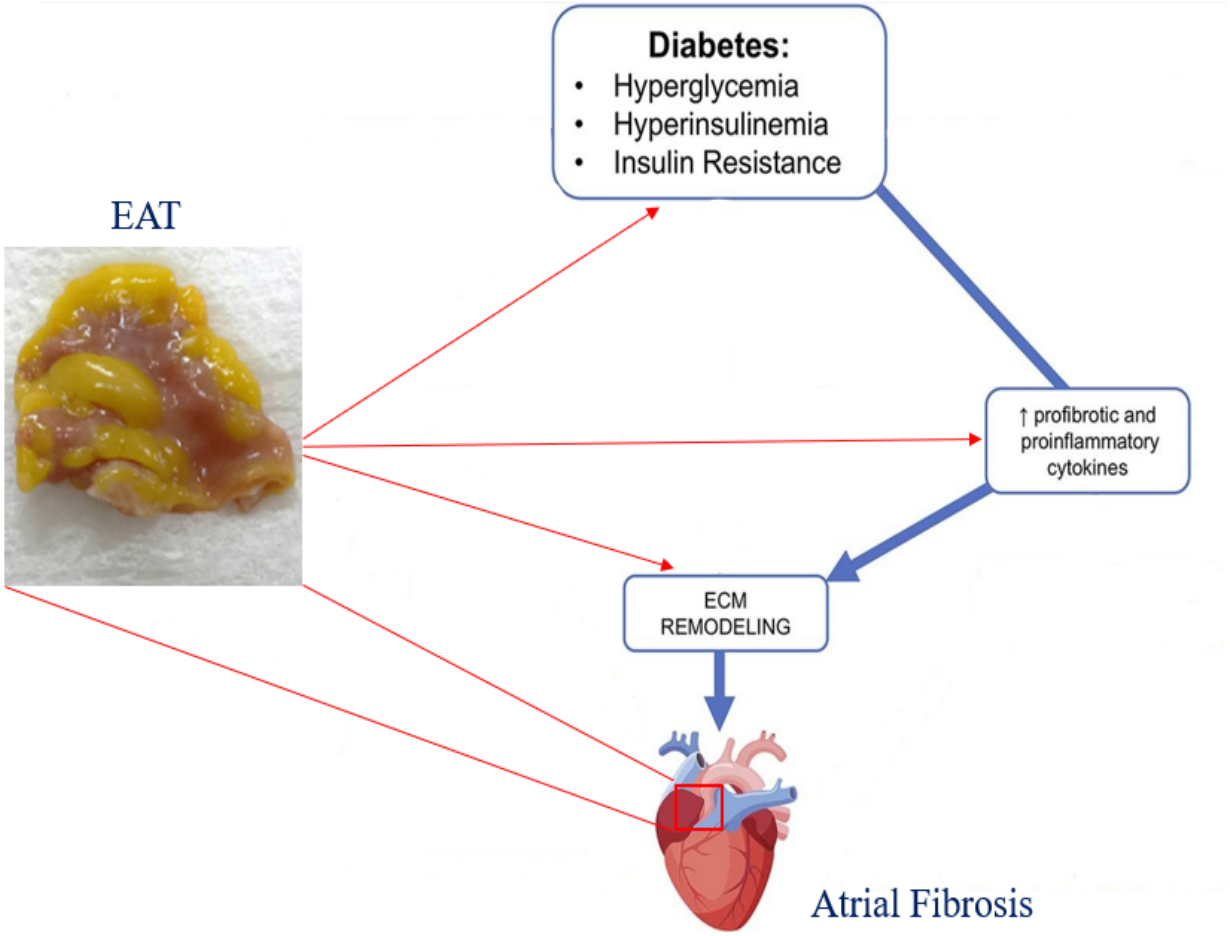
4.4 Type 2 diabetes mellitus and epicardial adipose tissue:

As discussed earlier, EAT is a visceral adipose tissue that can secrete pro-and anti-inflammatory cytokines, and chemokines locally in the heart via paracrine or vasocrine pathways to regulate the myocardial and coronary artery function, and lipid and energy homeostasis [94, 151, 152]. EAT has the ability to release and uptake free fatty acids and mediate low-glucose utilization, which is actively involved in metabolic syndrome and CAD [153]. Recent studies have shown that EAT is associated with fasting glucose levels, insulin resistance, and adiponectin in T2DM subjects, further an increase in EAT was observed in patients with T2DM [154, 155].

Compared to non-diabetic counterparts T2DM subjects have a significantly increased EAT, with clinical consequences of CVD risk [151, 156]. Chun et.al showed a significant correlation between left ventricular EAT thickness and the prevalence of T2DM in Korean men [157]. All these studies collectively suggest the importance of EAT and its paracrine, and vasocrine mediated inflammatory effects in T2DM.

Owing to the location of EAT, studying T2DM induced inflammatory cytokines/chemokines in the EAT that have the potential to promote fibrogenesis, may provide novel mechanisms of EAT-T2DM induced cardiac fibrosis, and help in developing new therapeutic targets. Thus, the **central hypothesis of this study is that 'Adipocytokines and chemokines are secreted by the EAT of T2DM, and a subset of these factors correlate with atrial fibrosis and are involved in the activation of CFBs and MyoFBs, thereby promoting cardiac fibrosis'**. (Figure-4)

Figure 4: Central hypothesis



CHAPTER TWO

MATERIALS AND METHODS

Study design

Between September 2017 and November 2019, approximately 112 patients who underwent coronary artery bypass grafting (CABG) surgery were enrolled in the study. Forty-eight patients were excluded because of insufficient adipose tissue biopsy samples, higher BMI, or above the age of 75 years, with the study enrolment criteria summarized in Table 1. Demographic and clinical characteristics of these 64 (n=32 T2DM; n=32 NDM) patients are summarized in Table 2. The study was approved by the Institutional Review Board (IRB) of East Carolina University (UMCIRB09-0669), and all patients have provided a written informed consent.

Blood collection

Blood was drawn from the patients an hour prior to the surgery BD Vacutainer blood collection tubes with buffered sodium citrate as anticoagulant. For plasma, the blood tubes were centrifuged at 800 rpm for 10 minutes and stored in aliquots at -80°C. Red blood fraction (RBF) and the plasma rich protein (PRP) were collected by centrifuging further at 400 rpm for 10 minutes and stored in aliquots at -80°C [158].

Table-1: Patient inclusion and exclusion criteria for study enrolment

Study Enrolment Criteria

Inclusion criteria	Exclusion criteria
• Age group ~ 50-75 years old	• Heart Failure
• CABG, Aortic Valve Replacement, or both	• Previous CABG
• BMI: 25-29.9 kg/m2	• Known Malignancy
	• Sepsis
	• Mitral Valve surgery
	• Hepatitis B/C
	• Obese patients (BMI ≥ 30.0 kg/m2)

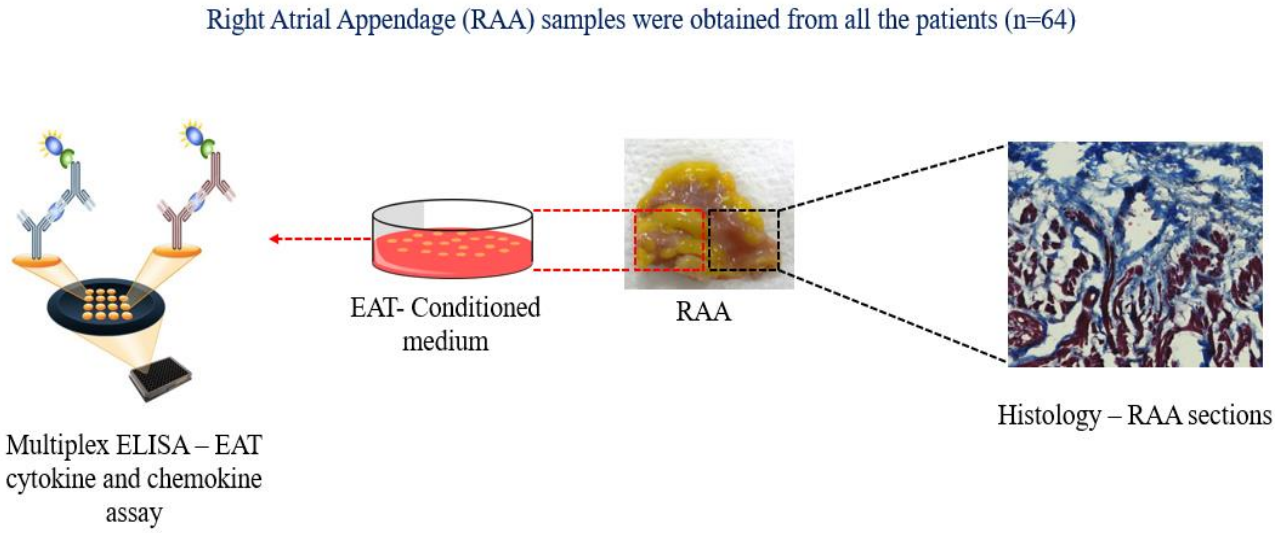
Tissue collection and culture

Tissue biopsy samples were obtained before the initiation of the cardiopulmonary bypass. EAT was extracted from the Right Atrial Appendage (RAA) obtained from the patients. The specimens were rinsed in phosphate buffer saline (PBS) and divided into 3 portions. One portion was fixed in 4% Paraformaldehyde (PFA), embedded in OCT compound, and stored in -80°C for histology [159]. After removing the visible blood vessels, the second portion was frozen immediately in liquid nitrogen and stored in -80°C for immunoblotting assays. The third portion was weighed, cut into small pieces ($\approx 1mm^3$), and transferred into a cell culture plate. According to the tissue weight ($\sim$20mg) media (0.01% BSA, 1% pen-strep, 0.5% fungizone in 500 ml DMEM) was added and incubated at 37°C in a CO2 incubator [158, 159]. After 24 hours, the conditioned media (CM) were collected and centrifuged for 2 minutes. The supernatants were then filtered in centrifugal filter units (Millipore durapore PVDF 5.0um) and stored in -80°C for measurement of secreted inflammatory mediators using multiplex ELISA (Figure-5) [159].

Multiplex enzyme linked immunoassay (ELISA)

Inflammatory mediators released by the EAT in the CM were measured using Bio-Rad ELISA kits (Bio-Plex Pro human cytokine assay) according to the manufacturer recommended procedure. The CM of EAT was resubstituted in 0.1 % BSA media for the ELISA analysis according to the protocol. The Bio-Plex Pro Human 37-plex, 27-plex, 40-plex, and a 3-plex were used for measuring the EAT-secretome. Samples were then normalized, and protein concentrations were obtained by the BCA assay kit (Thermo-scientific, USA).

Figure 5: Experimental design for histology and multiplex ELISA

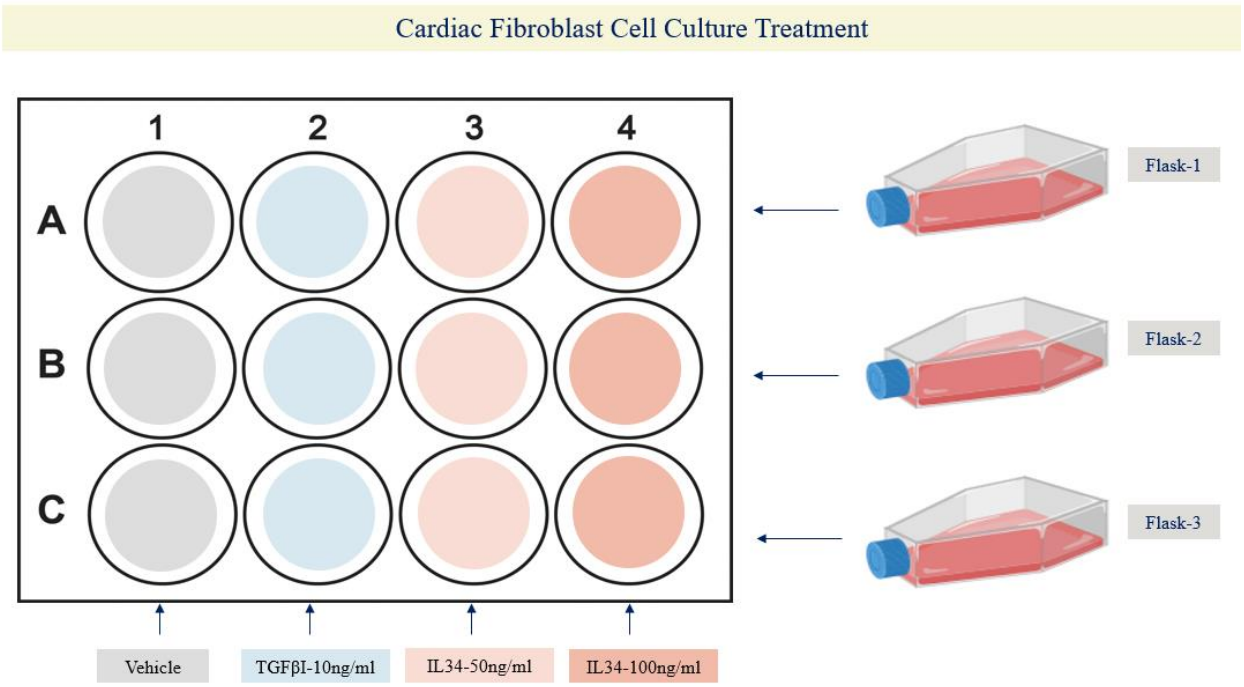


Cell culture

The tissue culture flasks and plates were pre-coated with gelatin (G1393-100ml, Sigma). Using aseptic technique under a laminar flow hood, 3ml/flask (T-75), and 250µl/well (12-well plate) of 0.2% gelatin solution in sterilized water was added to the cell culture surface area. The cell culture flasks and plates were gently rocked to coat the surface, followed by which they were dried in the laminar hood for 2 hours [160].

Human Cardiac Fibroblasts from healthy subjects were obtained from Promocell and maintained in Fibroblasts media and cultured according to the manufacturer's instructions. Three different batches of cells between passages 4 and 6 were studied independently under similar conditions [161]. Cells were seeded in 12-well plates (5×10^4 per well) and stimulated with TGF-β1 (10ng/ml, R&D systems) as positive control [162], and IL-34 (50ng/ml & 100ng/ml, PeproTech) treatment for 72 hours , and collected at 0h, 24h, 48h, and 72h for immunofluorescent staining (Figure-6) [163].

Figure 6: Experimental design for in vitro cardiac fibroblast culture and treatment



TGF-β-receptor inhibition

In most fibrotic organs the fibroblasts express TGF-β that mainly signals through the ALK5-type I TGF-β receptor pathway [164]. According to the manufacturers protocol, in human cardiac fibroblasts RepSox is shown to inhibit ATP binding to ALK5 with IC_{50} of 23nM. Therefore, based on previous studies and the manufacturers protocol, for the experimental design of the current study we used 230nM, RepSox-TGFβR-1/ALK5 (MedChem Express, USA) as the TGFβ-1 receptor inhibitor [165, 166].

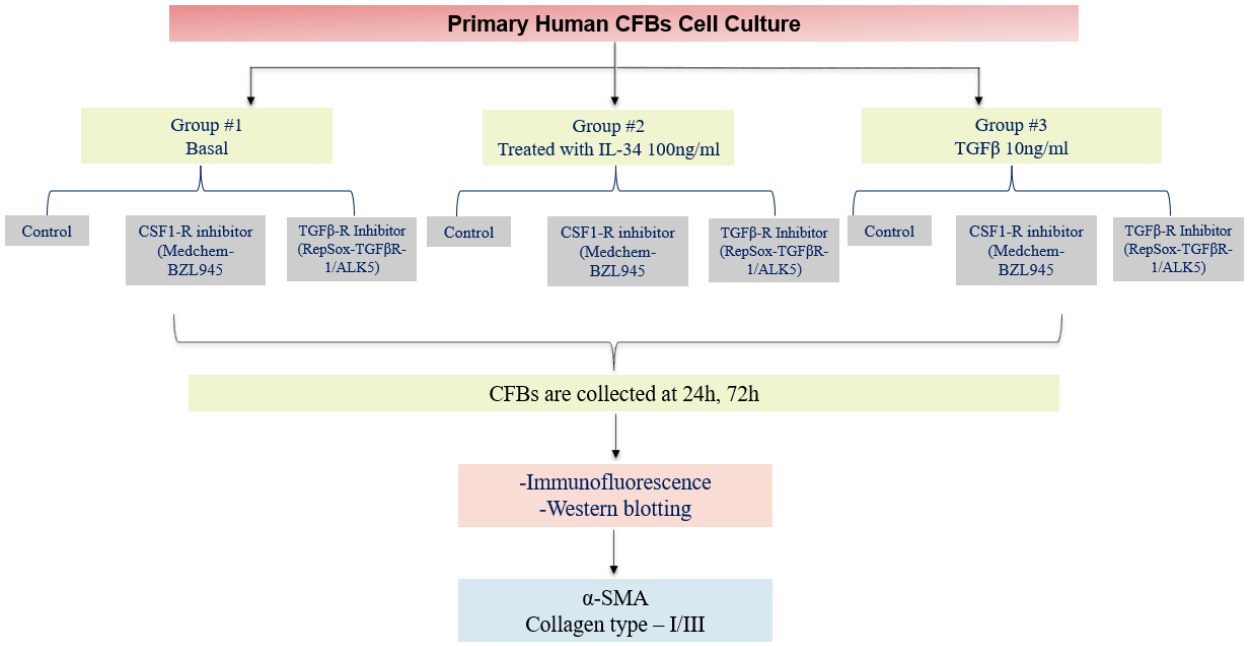
Human fibroblasts (Promocell, USA) were cultured cell passages between 4-6 were seeded in 12-well plates (5 x 10^4 per well) and pre-treated for 1 hour with TGFβ-Receptor Inhibitor (230nM,RepSox-TGFβR-1/ALK5, MedChem Express, USA) [167]. Followed by the receptor inhibition, the cells were stimulated similar to our previous treatment groups with TGF-β1 (10ng/ml, R&D systems) [162], and IL-34 (50ng/ml & 100ng/ml, PeproTech) for 72h, and collected at 0h, 24h, and 72h (Figure 7)[163].

CSF1-Receptor inhibitor treatment

Studies have revealed that CSF-1R binds both IL-34 and CSF-1, but through distinct surfaces resulting in unique receptor conformations and downstream signaling events. Activation of CSF-1R signaling was thought to be mediated only by the homodimeric growth factor CSF-1. However, Li et al. identified IL-34 as a tissue-specific ligand binding to CSF-1R. Considering the central role of CSF1-R in IL-34 signaling, according to previous studies and the manufacturers protocol we have used 670nM CSF1-R inhibitor to investigate the effect of CSF1-R inhibition on IL-34 induced fibrogenesis [168-170].

Three different batches of cardiac fibroblasts between passages 4 and 6 were cultured independently under similar conditions. Cells were seeded in 12-well plates (5×10^4 per well) and pre-treated with CSF-1R inhibitor (670nM) for 1 hour. Followed by which the cells were stimulated with TGF-β1 (10ng/ml, R&D systems) as positive control, and IL-34 (100ng/ml, PeproTech) treatment for 72 hours, and collected at 0h, 24h, and 72h for immunofluorescent staining(Figure 7).

Figure 7: TGFβ- and CSF1- receptor inhibitor treatment groups



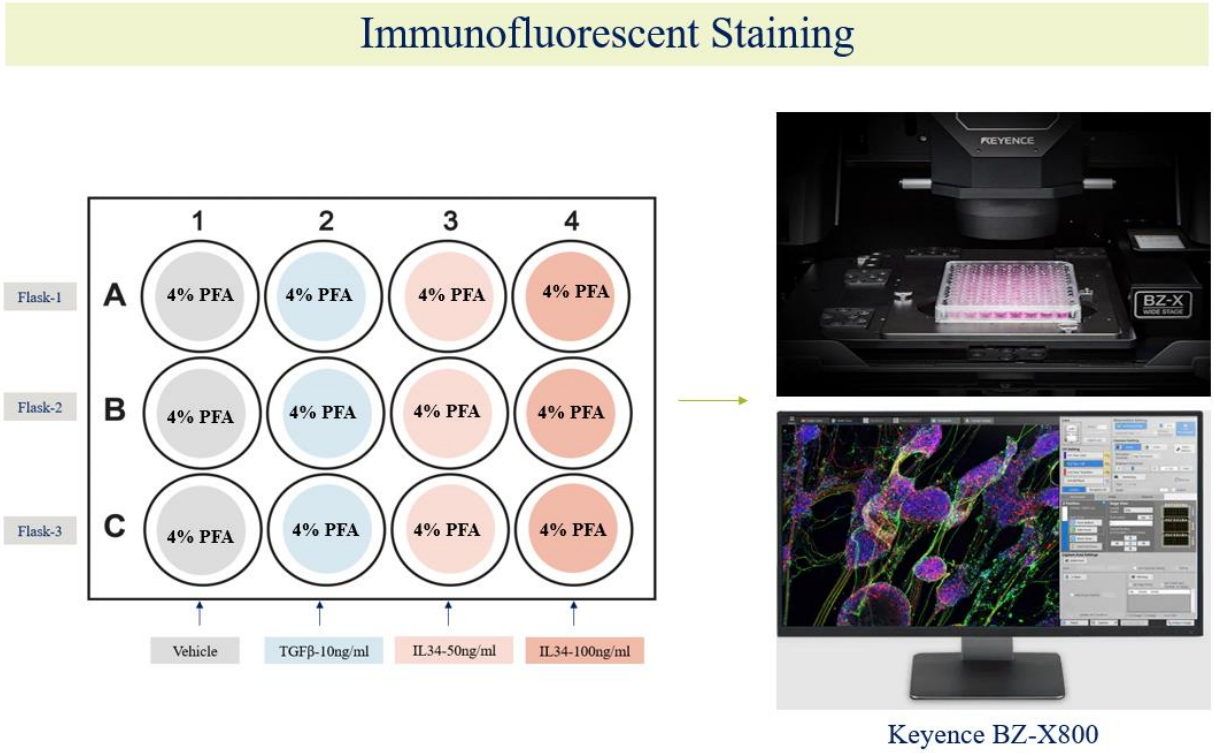
Immunofluorescent staining

Cardiac fibroblasts after treatments were collected at different time points. The culture medium was removed, and the cells were gently rinsed with 500µl/well of PBS (Phosphate buffer Saline). The cells were then fixed in 4% paraformaldehyde for 20minutes. After permeabilization with 0.25% Triton X-100 for 30 minutes at room temperature (RT), the cells were blocked with 5% donkey serum in 0.1% PBST (500µl/well) for 1h at RT [161]. Subsequently, the cells were incubated with primary antibodies against α-SMA (1:200; ab5694, Abcam) for 2 hours at RT [171]. Then, the cells were incubated with secondary antibody (anti-IgG, alexa-488, 1:1000) for 1h at RT [171]. Finally, the cells were counterstained with 4', 6' – diamidino-2-phenylindole (DAPI) (1:10000, D9542-10mg, Sigma) before capturing images by using a fluorescence microscope (Keyence BZ-X800) [Figure-8].

Western blotting

Protein levels were detected by western blot analysis. Briefly, the cardiac fibroblast cells after treatment with TGF-β(10ng/ml), IL-34-50ng/ml, and IL-34-100ng/ml were collected at 48h, and 72h time points. Cell lysates were separated by gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes and blocked with 2.5% nonfat milk. The membranes were then incubated with the following optimally diluted primary antibodies – Collagen Type-1 (1:2000, 14695-1-AP, Proteintech USA); Collagen Type-III (1:1000, 22734-1-AP, Proteintech USA); GAPDH (1:1000, ZG003, Invitrogen, USA) for 1.5hours at RT. Appropriate secondary antibodies were sequentially added. Protein expression was assessed with the Licor Image studio lite 5.2 imaging system.

Figure 8: Experimental design for immunofluorescent staining



Plasma collection

Blood was drawn from the patients an hour prior to the surgery in BD Vacutainer blood collection tubes with buffered sodium citrate as anticoagulant. For plasma, the blood tubes were centrifuged at 800 rpm for 10 minutes and stored in aliquots at -80°C. Plasma, Red blood fraction (RBF), and the plasma rich protein (PRP) were collected by centrifuging further at 400 rpm for 10 minutes and stored in aliquots at -80°C.

Human plasma IL-34 direct ELISA

Plasma levels of IL-34 were measured in the plasma extracted from the patient blood samples using Human IL-34 AccuSignal ELISA kit (K0A0826; Rockland, USA). Briefly, the plasma samples were diluted in the sample diluent buffer (1:2 dilution), standards, antibody detection diluents, and buffers were prepared, and the assay was performed according to the manufacturers protocol.

Statistical analysis

All the continuous variables are presented as mean $\pm$ SD. Data was analyzed using GraphPad prism-8 software, determined by linear regression, unpaired t-test, and one-way ANOVA followed by Tukey & Dunnett multiple comparisons. The student t-test was used for evaluation of a single variable among two groups, and one-way ANOVA with multiple comparisons was used to compare various treatment groups.

CHAPTER THREE

Interleukin-34 Secreted by Epicardial Adipose Tissue Correlates with Atrial Fibrosis and Elevated in Patients with Type-2-Diabetes Mellitus: IL-34 Promotes Transdifferentiation of Myofibroblasts independently of the TGF-β Receptor

Divya Jyothi Madipally[1], Sky Reece[1], Shahab Akhter[2], Hazaim Alwair[2], Joani Zary[3], Bin Luo[1], Srinivas Sriramula[1], Ethan J Anderson[4], Jacques Robidoux[1,5]

Affiliations

[1]Department of Pharmacology and Toxicology, East Carolina University, Greenville, NC, USA

[2]Department of Cardiovascular Sciences, East Carolina University, Greenville, NC, USA

[3]Department of Anatomy and Physiology, East Carolina University, Greenville, NC, USA

[4]Department of Pharmaceutical Sciences and Experimental Therapeutics, University of Iowa, Iowa city, IA, USA

[5]East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC, USA

ABSTRACT

Epicardial adipose tissue (EAT) and subepicardial fibrosis often extend to myocardial fibrosis. While it is known that EAT secretes inflammatory factors and that inflammation has been suggested as an independent risk factor for myocardial fibrosis, the nature of the mediators causing myocardial fibrosis in patients with type 2 diabetes Mellitus (T2DM) is unknown. The aim of this study was to assess whether cytokines differentially secreted by EAT isolated from patients with or without T2DM correlates with atrial fibrosis and promotes myofibroblast differentiation. Right atrial appendages (RAA) were obtained from 64 patients, 32 with and 32 without T2DM undergoing cardiac surgery. Of the 49 cytokines quantified by ELISA, 11 correlated with the extent of fibrosis of the RAA measured by Masson's trichrome staining. Of all the secreted factors, IL-34 and sIL-6Rβ were also elevated in the medium exposed to EAT from patients with T2DM. Finally, IL-34 increased the expression of α-smooth muscle actin independently of the transforming growth factor-β receptor as well as the production of collagen I and III. In conclusion, this study provides the first evidence that the EAT of patients with T2DM secretes IL-34, which in turn correlates with atrial fibrosis and can promote fibroblast to myofibroblast transdifferentiation.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is an established risk factor for cardiovascular diseases [172]. One of the hallmarks of these diseases, irrespective of their etiologies is the presence myocardial fibrosis which is characterized by an expansion of the myocardial interstitium due to exaggerated deposition of extracellular matrix [173]. In that respect not only the presence of T2DM is an independent risk factor for myocardial fibrosis, but many studies have suggested that the presence of T2DM exacerbates the fibrosis associated with other etiologies including hypertension, atherosclerosis, or coronary artery diseases [174]. In these diseases, myocardial fibrosis has been proposed to be of pathophysiological significance because of it interferes with both the electrical and mechanical dysfunctions of the heart [175, 176]. Admittedly most of what we know about myocardial fibrosis relates to ventricular fibrosis, however the right atrial appendages (RAA) of patients with T2DM also display significant levels of stiffness and myocardial fibrosis [177].

Many cell types have been proposed to participate in myocardial fibrosis, however it is beyond reasonable doubt that resident cardiac fibroblasts, the most abundant cell type in the heart, are important players [173]. Moreover, it has been shown that in most fibrotic diseases and fibrosis models that fibroblasts are activated into matrix-secreting and matrix remodeling myofibroblast in a disease- or insult-specific fashion [173, 178]. Another characteristic of these cells, also the reason for their name, is that they display many properties of smooth muscle cells including the expression of α-smooth muscle actin (α-SMA) which gives them contractile properties [179].

In most tissues including the heart, transforming growth factor-β (TGF-β) supper family are considered at the center stage of fibrosis whether as the primary mediator or downstream of other

profibrotic factors [180]. The effects of TGF-βs are pleiotropic but their abilities to promote the expression of collagens and inhibit interstitial collagenase are crucial in fibrosis [181, 182]. As eluded above, many other factors have been shown to be able to promote fibrosis in vivo or in vitro including anti-inflammatory and proinflammatory cytokines [183, 184].

Recently, a role for the epicardial adipose tissue (EAT) in atrial fibrosis has been proposed [185, 186]. This attractive hypothesis is supported by several line of evidence. First, EAT expansion of the tissue correlates with the development of atrial fibrosis [185]. Second, there is no barrier separating the epicardium and the myocardium, allowing it, or the fibrosis within, to invade the myocardium [186]. Third, the absence of barrier and the shared microvasculature should also allow EAT secreted factors to act in an endocrine / paracrine fashion on the myocardium. Comparing the 181 EAT secretory products from 11 patients without (NDM) or with T2DM it was found that 6 factors including the TGF-β family member activin A was elevated in the conditioned-medium from the patients with T2DM [187]. Another study also found Activin A to be secreted by the human EAT explants, but in addition that it was able to promote atrial fibrosis in an organ-culture model of rat atria [185]. Whether TGF-β or any other profibrotic factor are involved in cardiac fibrosis or T2DM-associated fibrosis remains unknown. Thus, the aim of this study was to assess whether cytokines differentially secreted by EAT isolated from patients with or without T2DM correlates with atrial fibrosis and promotes myofibroblast differentiation.

MATERIALS AND METHODS

Study design

Between September 2017 and November 2019, approximately 112 patients who underwent coronary artery bypass grafting (CABG) surgery were enrolled in the study. Forty-eight patients were excluded because of insufficient adipose tissue biopsy samples, higher BMI, or above the age of 75 years. Demographic and clinical characteristics of these 64 (n=32 T2DM; n=32 NDM) patients are summarized in Table 1. The study was approved by the Institutional Review Board (IRB) of East Carolina University (UMCIRB09-0669), and all patients have provided a written informed consent.

Blood collection

Blood was drawn from the patients an hour prior to the surgery BD Vacutainer blood collection tubes with buffered sodium citrate as anticoagulant. For plasma, the blood tubes were centrifuged at 800 rpm for 10 minutes and stored in aliquots at -80°C. Red blood fraction (RBF) and the plasma rich protein (PRP) were collected by centrifuging further at 400 rpm for 10 minutes and stored in aliquots at -80°C [158].

Tissue collection and culture

Tissue biopsy samples were obtained before the initiation of the cardiopulmonary bypass. EAT was extracted from the Right Atrial Appendage (RAA) obtained from the patients. The specimens were rinsed in phosphate buffer saline (PBS) and divided into 3 portions. One portion was fixed in 4% Paraformaldehyde (PFA), embedded in OCT compound, and stored in -80°C for histology

[159]. After removing the visible blood vessels, the second portion was frozen immediately in liquid nitrogen and stored in -80°C for immunoblotting assays. The third portion was weighed, cut into small pieces ($\approx 1mm^3$), and transferred into a cell culture plate. According to the tissue weight ($\sim$20mg) media (0.01% BSA, 1% pen-strep, 0.5% fungizone in 500 ml DMEM) was added and incubated at 37°C in a CO2 incubator [158, 159]. After 24 hours, the conditioned media (CM) were collected and centrifuged for 2 minutes. The supernatants were then filtered in centrifugal filter units (Millipore durapore PVDF 5.0um) and stored in -80°C for measurement of secreted inflammatory mediators using multiplex ELISA [159].

Multiplex enzyme linked immunoassay (ELISA)

Inflammatory mediators released by the EAT in the CM were measured using Bio-Rad ELISA kits (Bio-Plex Pro human cytokine assay) according to the manufacturer recommended procedure. The CM of EAT was resubstituted in 0.1 % BSA media for the ELISA analysis according to the protocol. The Bio-Plex Pro Human 37-plex, 27-plex, 40-plex, and a 3-plex were used for measuring the EAT-secretome. Samples were then normalized, and protein concentrations were obtained by the BCA assay kit (Thermo-scientific, USA).

Cell culture

The tissue culture flasks and plates were pre-coated with gelatin (G1393-100ml, Sigma). Using aseptic technique under a laminar flow hood, 3ml/flask (T-75), and 250µl/well (12-well plate) of 0.2% gelatin solution in sterilized water was added to the cell culture surface area. The cell culture flasks and plates were gently rocked to coat the surface, followed by which they were dried in the laminar hood for 2 hours [160].

Human Cardiac Fibroblasts from healthy subjects were obtained from Promocell and maintained in Fibroblasts media and cultured according to the manufacturer's instructions. Three different batches of cells between passages 4 and 6 were studied independently under similar conditions [161]. Cells were seeded in 12-well plates (5×10^4 per well) and stimulated with TGF-β1 (10ng/ml, R&D systems) as positive control [162], and IL-34 (50ng/ml & 100ng/ml, PeproTech) treatment for 72 hours , and collected at 0h, 24h, 48h, and 72h for immunofluorescent staining [163].

TGF-β-receptor inhibition

Human cardiac fibroblasts (Promocell, USA) cultured cell passages between 4-6 were seeded in 12-well plates (5×10^4 per well) and pre-treated for 1 hour with TGFβ-Receptor Inhibitor (230nM,RepSox-TGFβR-1/ALK5, MedChem Express, USA) [167]. Followed by the receptor inhibition, the cells were stimulated similar to our previous treatment groups with TGF-β1 (10ng/ml, R&D systems) [162], and IL-34 (50ng/ml & 100ng/ml, PeproTech) for 72h, and collected at 0h, 24h, and 72h [163].

Immunofluorescent staining

Cardiac fibroblasts after treatments were collected at different time points. The culture medium was removed, and the cells were gently rinsed with 500µl/well of PBS (Phosphate buffer Saline). The cells were then fixed in 4% paraformaldehyde for 20minutes. After permeabilization with 0.25% Triton X-100 for 30 minutes at room temperature (RT), the cells were blocked with 5% donkey serum in 0.1% PBST (500µl/well) for 1h at RT [161]. Subsequently, the cells were incubated with primary antibodies against α-SMA (1:200; ab5694, Abcam) for 2 hours at RT

[171]. Then, the cells were incubated with secondary antibody (anti-IgG, alexa-488, 1:1000) for 1h at RT [171]. Finally, the cells were counterstained with 4', 6' – diamidino-2-phenylindole (DAPI) (1:10000, D9542-10mg, Sigma) before capturing images by using a fluorescence microscope (Keyence BZ-X800).

Western blotting

Protein levels were detected by western blot analysis. Briefly, the cardiac fibroblast cells after treatment with TGF-β(10ng/ml), IL-34-50ng/ml, and IL-34-100ng/ml were collected at 48h, and 72h time points. Cell lysates were separated by gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes and blocked with 2.5% nonfat milk. The membranes were then incubated with the following optimally diluted primary antibodies – Collagen Type-1 (1:2000, 14695-1-AP, Proteintech USA); Collagen Type-III (1:1000, 22734-1-AP, Proteintech USA); GAPDH (1:1000, ZG003, Invitrogen, USA) for 1.5hours at RT. Appropriate secondary antibodies were sequentially added. Protein expression was assessed with the Licor Image studio lite 5.2 imaging system.

Statistical analysis

All the continuous variables are presented as mean $\pm$ SD. Data was analyzed using GraphPad prism-8 software, determined by linear regression, unpaired t-test, and one-way ANOVA followed by Tukey & Dunnett multiple comparisons. The student t-test was used for evaluation of a single variable among two groups, and one-way ANOVA with multiple comparisons was used to compare various treatment groups.

RESULTS

Patient characteristics

A total of 64 patients were enrolled in the study, all patients were advanced middle ages (62.9 ± 1.2 years). Table-2: 50% of the patients were T2DM (n=32) with the mean age of 63.37 ± 6.18, and average BMI (kg/m2) of 27.14 ± 3. According to the risk factors mentioned in Table-2, 72% of T2DM patients had dyslipidemia, 90.6% were hypertensive, and 37.05% were smoking. The patient medication during the surgery were as following – 75% of the T2DM patients were on β-blockers, 46.87% on ACEIs/ARBs, 31% were on diuretics, 50% were on CCBs, 69% were on statins, 25% on nitrates, and 96.87% were on metformin. Average EF% was 52 ± 11. Up to 81.75% of the patients underwent CABG surgery, 3.21% underwent aortic valve replacement, and 15.04% underwent both CABG and valve replacement. A total of 31.25% of patients had prior MI, 3 % had a history of AF, and 9.57% patients developed POAF.

Atrial fibrosis in patients with T2DM

Figure 9: Despite all the patients being advanced middle ages (62.9 ± 1.2), Masson's trichrome staining comparing atrial fibrosis in the RAA revealed that the accumulation of collagen-stained for blue in and around the EAT was more marked in patients with T2DM compared to NDM (Figures a-d). Subsequently, the extent of atrial fibrosis (total collagen) was more marked in the RAA of patients with T2DM ($p<0.001$, Figure-e), and correlates with the HbA1c levels ($p<0.05$, Figure-f).

Correlation between atrial fibrosis and EAT-cytokines

Figure 10: A total of 49 cytokines were quantified in the EAT-CM using multiplex ELISA, and a linear aggression analysis was performed correlating the extent of atrial fibrosis in the RAA with the levels of cytokines in the EAT (Figure-a). Of the analyte quantified a total of 11 cytokines including IL-34 (***p<0.0001, Figure-b), sIL-6Rβ (*p<0.0181, Figure-c), IL-27 (***p<0.0001, Figure-d), IFN-R (***p<0.0001, Figure-e), IL-12(***p<0.0001), IL-20, IL-22, IL-29, TNFSF-13B, and Osteocalcin (Table-3) positively correlated with RAA fibrosis.

Comparison of the cytokines secreted by EAT of T2DM vs NDM

Figure 11: We compared the 49 cytokines secreted by the EAT among the T2DM vs NDM patients (Figure-a). Of these, 3 cytokines IL-34 (Figure-b), sIL-6Rβ (Figure-c), and IL-11 (Figure-d) were significantly higher in the EAT patients with T2DM. Further, IL-34 and sIL-6Rβ were both elevated in T2DM-EAT and correlated with atrial fibrosis in the RAA (Figure-b,c).

IL-34 induced transdifferentiation of cardiac fibroblasts to myofibroblasts

Figure 12: In comparison to the control groups, cardiac fibroblasts treated with TGF-β1 (10ng/ml) as positive control had a significant (****p<0.0001, Figures b-d) increase in the expression of α-SMA after 24h (12.01-fold); 48h (10.03-fold); and 72h (8.16-fold). As anticipated, cells treated with IL-34 (50ng/ml) had significant (****p<0.0001) increase in the expression of α-SMA after 24h (12.2-fold); 48h (11.46-fold); and 72h (9.41-fold) indicating increased transdifferentiation of fibroblasts to myofibroblasts similar to our positive control (TGF-β1). Fibroblasts treated with IL-34 (100ng/ml) had slightly higher expression of α-SMA (****p<0.0001, Figures b-d) at 24h (13.2-

fold); 48h (11.75-fold); and 72h (10.7-fold) when compared to control, TGF-β1 and IL-34 (50ng/ml).

IL-34 induced expression of collagen types-I and III

Figure 13: In comparison to the control groups, cardiac fibroblasts treated with TGF-β1 (10ng/ml) as positive control had a significant (****p<0.0001, Figures b-d) increase in the expression of both Collagen types- I and III after 48h and 72h. Further, cells treated with IL-34 both 50ng/ml and 100ng/ml had a significant(****p<0.0001, Figures b-d)) increase in the levels of Collagen types – I and III similar to the TGF-β positive control group at both 48h and 72h.

Effect of TGF-βR inhibition on IL-34 induced transdifferentiation of cardiac fibroblasts to myofibroblasts

Figure 14: Similar to our previous results, both TGF-β (10ng/ml) and IL-34(100ng/ml) treatment groups had a significant (****p<0.0001) increase in the MyoFBs/Nuclei ratio after 24h (Figure-c); and 72h(Figure-d). However, inhibition of TGFβR-inhibitor had a significant decrease in the MyoFBs/Nuclei ratio (***P<0.0001) at 24h(Figure-b), and 72h(Figure-d) in the TGFβ(10ng/ml) treated group in comparison to the control, and TGFβ-R inhibitor groups. Interestingly, inhibition of TGFβ-receptor did not have any effect on IL-34-100ng/ml treatment induced differentiation of MyoFBs at both 24(Figure-e), and 72h(Figure-f). These results suggest that IL-34 might be inducing fibrosis in cardiac fibroblasts in a TGF-β receptor independent pathway.

DISCUSSION:

Knowledge of the molecular pathways involved in the pathophysiological processes leading to atrial fibrosis in patients with type 2 diabetes (T2DM) is critical to permit the establishment of a framework for a specific and efficacious therapeutic approach. In the current study we identified eleven cytokines that correlate with atrial fibrosis and three with an increased secretion from the EAT of patients with T2DM. Of these cytokines, we substantiate that IL-34 can promote the transdifferentiation of cardiac fibroblasts into myofibroblasts that express α-SMA and secrete collagen types I and III.

In 2015, Venteclef et al. were the first to demonstrate that a product of the EAT secretome, activin A is able to promote fibrosis [185] To our knowledge, our study is the first study to extend the short list of EAT profibrotic factors by adding IL-34, a relatively novel cytokine identified in 2008 as a ligand for the colony stimulating factor receptor (CSFR)-1 [188]. IL-34 is somewhat promiscuous as it also binds to two other receptors, receptor-type protein-tyrosine phosphatase-zeta [74], and chondroitin sulfate chains of syndecan-1 [75]. With respect to fibrosis the tissular expression of IL-34 has been shown to correlate with liver fibrosis in patients with non-alcoholic fatty liver disease [189]. In another study it was shown that in patients with systemic sclerosis, elevated serum levels of IL-34 correlated with the presence of lung fibrosis [190].

Interestingly serum levels of IL-34 have been associated with the severity cardiac dysfunction in coronary artery disease as well as heart failure [191] [78]. These two studies did not evaluate whether plasma IL-34 correlated with the extent of ventricular fibrosis but considering

the roles of fibrosis in these diseases and the extent of EAT distribution on the ventricles we can safely speculate that our observation could hold true in the ventricles as well.

Our results also demonstrate that IL-34 mediated transdifferentiation of cardiac fibroblasts to myofibroblasts is independent of the TGF-βR inhibition. In our study both IL-34 and TGF-β promoted the expression of α-SMA but only TGF-β effects were inhibited by TGFβ-R inhibition. This adds IL-34 to the short list of TGF-β-independent profibrotic factors. However, because of IL-34 promiscuity for multiple receptors the characterization of the molecular mechanisms including cellular signaling that is involved in IL-34-induced activation of fibroblast is an ongoing effort beyond the scope of this paper.

Our study is the first to establish that sIL-6Rβ is elevated in the EAT of patients with T2DM and correlated with atrial fibrosis. There are two soluble IL-6 receptor isoforms, sIL-6R α and sIL-6R β [192]. Ancey et al. showed that IL-6 treatments reduce collagen content in adult cardiac fibroblasts by binding to the membrane form of the IL-6 receptor and the signal transducer glycoprotein 130 [193]. While on the contrary, co-culturing of sIL-6R with IL-6 produced a 4-fold increase in the collagen content at through what is called the trans-signaling [193].

In a study with similar power as ours (n of 54 vs n of 62) Abe et al. evaluated the EAT content of 27 factors and showed that 16 cytokines quantified from EAT homogenates correlated with the severity of myocardial fibrosis evaluated by collagen quantification from left atrial appendage (LAA) [159]. Although all the factors measured in that study were also measured in our study, only one correlation, IL-12, could be replicated. Different anatomical sampling (LAA vs RAA), the quantification of tissue content vs explant secretion, and histological vs

immunological evaluation of fibrosis can explain these differences. Nevertheless, IL-12 secretion was not increased in EAT explants from patients with T2DM and there is no evidence so far that IL-12 promotes fibroblast activation.

Beside IL-12, we found 8 other factors (IL-20, IL-22, IL-27, IL-29, IFN-α2, soluble IFN-R, osteocalcin, and TNFSF-13B) that correlated with atrial fibrosis, but the secretion was not increased in the EAT explants of patient with T2DM. To our knowledge this is the first report of a correlation between the secretion of any of these factors and atrial fibrosis.

IL-27, a member of the IL-12 family, was shown to be highly expressed in fibrotic tissues, but is antifibrotic. As IL-27 was shown to inhibit lung fibrosis and TGF-β-induced fibroblast activation in a model of pulmonary fibrosis [194]. In addition, deletion of its receptor, IL-27R α, was shown to prevent renal fibrosis in a model of post renal kidney injury [195]. Therefore, although this is to be tested experimentally, IL-27 is somewhat unlikely to be profibrotic.

IL-20 and IL-22 and the more distantly related IL-29 are all members of the IL-10 family [196]. Recently, Tsai et al. demonstrated that anti-IL-20 antibody protects against ischemia/reperfusion induced myocardial remodeling in rats [197]. Yongxin et al [198] demonstrated that IL-22 levels are elevated in the atrium and plasma of patients with atrial fibrillation, and increased collagen synthesis in TGF-β1 treated cardiac fibroblasts. There are no reports showing a direct role for IL-29 in any type of fibrosis or fibroblast activation, but the fact that it binds to the IL-10R2, and IL-10 was shown to be involved in cardiac fibrosis in two mouse models of heart failure. Thus, a role for IL-29 can be reasonably hypothesized in mediating cardiac fibrosis.

Further, our study also showed a positive correlation between cytokines like IFN-α, IFN-R, osteocalcin, and TNFSF-13b secreted by the EAT and atrial fibrosis. Han et al. showed that IFN-α knockout mice treated with Ang-II showed a significant reduction in the levels of α-SMA in cardiac fibroblasts [199]. Desentis et al. showed that Osteocalcin has a protective role in diabetic pathogenesis [200]. TNFSF13b, also known as the B-cell activating factor (BAFF), belongs to the TNF ligand family [201]. Recently, Yarchoan et al. in 2020 showed a strong association between TNFSF-13b and autoimmunity [202]. Collectively, with the current study serving as supporting evidence, further research is required to explore the possible role of these cytokines in the pathogenesis of cardiac fibrosis.

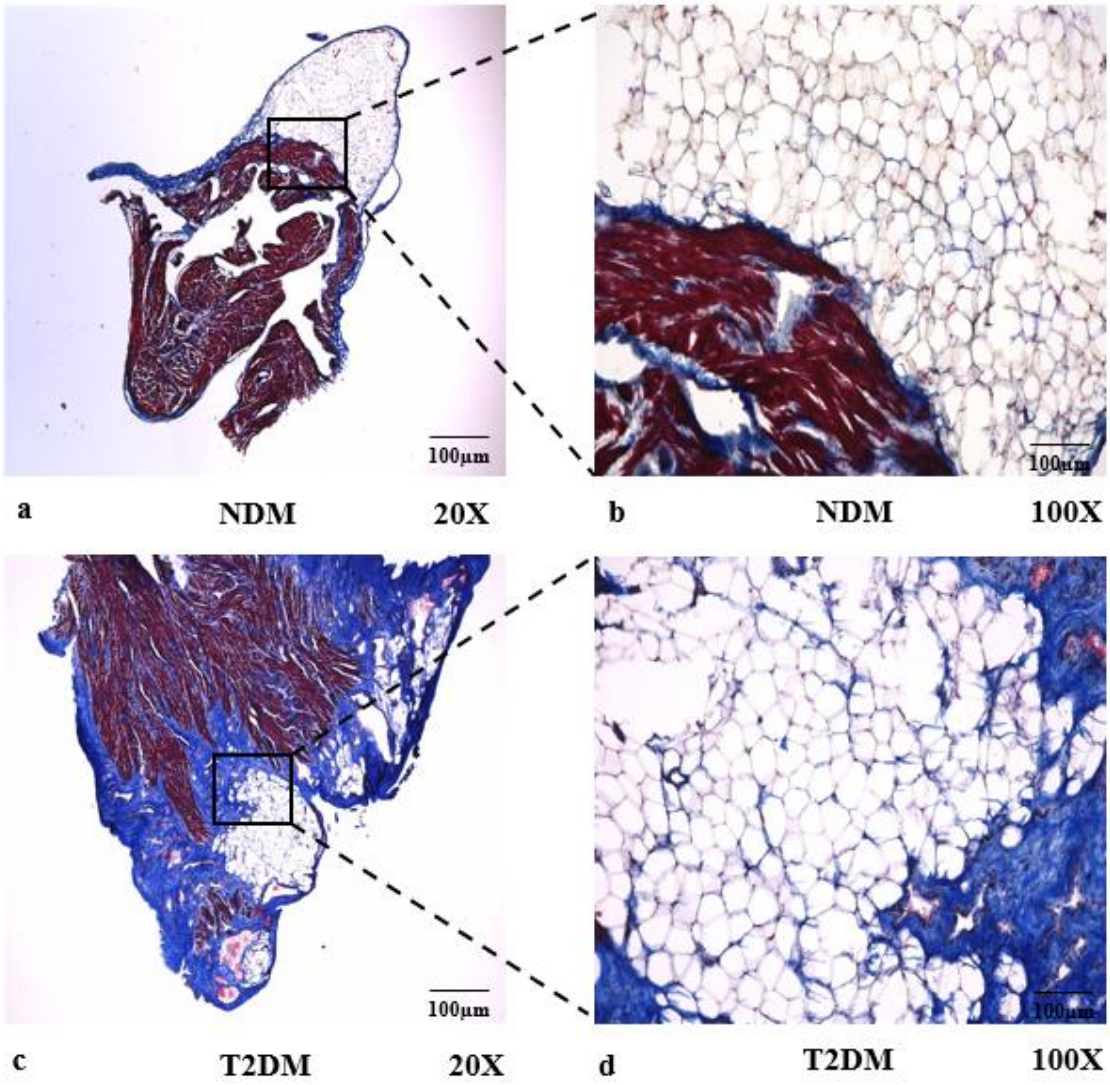
In conclusion, our study revealed that several cytokines released by the EAT had a strong correlation with the extent of fibrosis in the RAA. Of these cytokines, IL-34 and sIL-6Rβ were highly secreted by the EAT of patients with T2DM and correlated with atrial fibrosis. Further IL-34 was able to independently induce the transdifferentiation of cardiac fibroblasts to myofibroblasts invitro, and inhibition of TGF-βR did not have a significant effect on this. Therefore, we characterized the role of IL-34 as a potential candidate for its pathophysiological basis in atrial fibrosis. Further, we also found other cytokines including IL-22, IFN-α2, IFN-R, IL-12, IL-20, IL-29, TNFSF-13β, and osteocalcin that significantly correlated with atrial fibrosis. However, as discussed, further research is required to completely understand the role of these cytokines in T2DM and cardiac fibrosis.

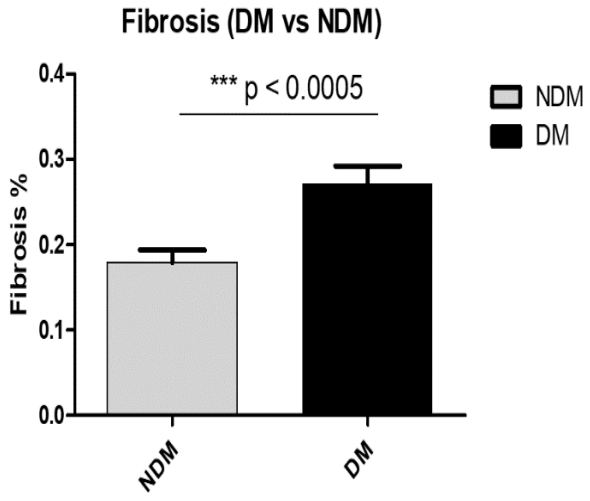
FIGURES

Table-2: Patient demographics

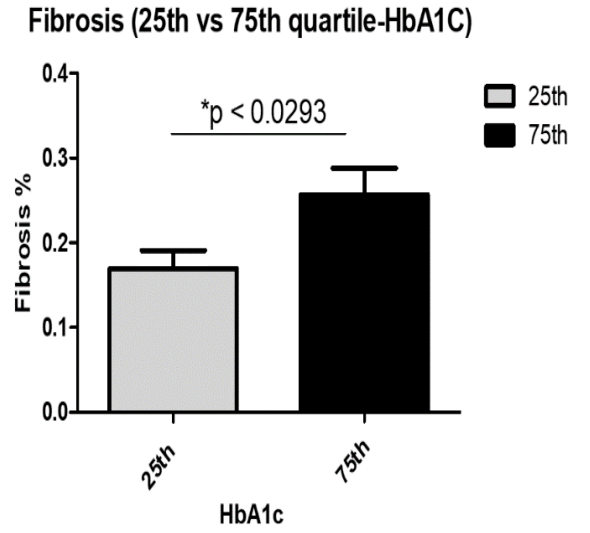
Table-1 Patient Demographics	NDM (n=32)	T2DM (n=32)	P-value
Age (y), (mean +/- SD)	60.75 ±10	63.37 ± 6.18	
Sex %			
* Male	90.63%	87.05%	0.89463
* Female	9.38%	12.50%	0.70546
BMI, Kg/m2 (mean +/- SD)	28.15 ± 3	27.14 ± 3	
Risk Factors %			
* Diabetes	0%	100%	0.0001
* Dyslipidemia	37.50%	72%	0.06298
* Hypertension	68.75%	90.60%	0.32699
* Smoking	46.25%	37.05%	0.5637
Medications, %			
* β-Blockers	78.12%	75%	0.8864
* ACE1/ARBs	9.38%	46.87%	0.00468
* Diuretics	19%	31%	0.31731
* CCBs	18.75%	50%	0.03301
* Statins	38%	69%	0.08635
* Nitrates	28.12%	25%	0.80837
* Metformin	16%	96.87%	0.0001
EF, % (mean +/- SD)	50 ± 12	52 ± 11	
Surgical Procedure			
* CABG	80.75%	81.75%	0.88864
* Valve Replacement	3.05%	3.21%	0.5637
* Combined (CABG+Valve)	16.20%	15.04%	0.76302
Prior MI, %	25%	31.25%	0.63735
History of AF	6%	3%	0.5637
POAF	9.04%	9.57%	0.70546

Figure 9: Comparison of the extent of atrial fibrosis in the RAA of NDM patients with T2DM patients




Fibrosis (DM vs NDM)
*** p < 0.0005
NDM
DM
Fibrosis %
0.4
0.3
0.2
0.1
0.0
NDM
DM

e


Fibrosis (25th vs 75th quartile-HbA1C)
*p < 0.0293
25th
75th
Fibrosis %
0.4
0.3
0.2
0.1
0.0
25th
75th
HbA1c

f

Figure-9: Representative images of Masson's trichrome staining of the RAA comparing the extent of collagen deposited in and around the EAT of NDM patients (Figure-a) with the EAT of patients with T2DM (Figure-c). As shown in the microscopic images, histological evidence revealed that the accumulation of collagen-stained blue indicative of ECM-collagen mediated fibrosis was more marked in the RAA of T2DM (Figure-d) when compared to the NDM (Figure-b). Further, quantification graphs show that the % of atrial fibrosis was significantly higher in the RAA of T2DM in comparison to the NDM (***$P<0.0005$, Figure-e), and positively correlated with the levels of HbA1c in T2DM (*$p<0.0293$, Figure-f).

Figure-10: Correlation between the extent of atrial fibrosis in the RAA and the levels of the cytokines secreted by EAT

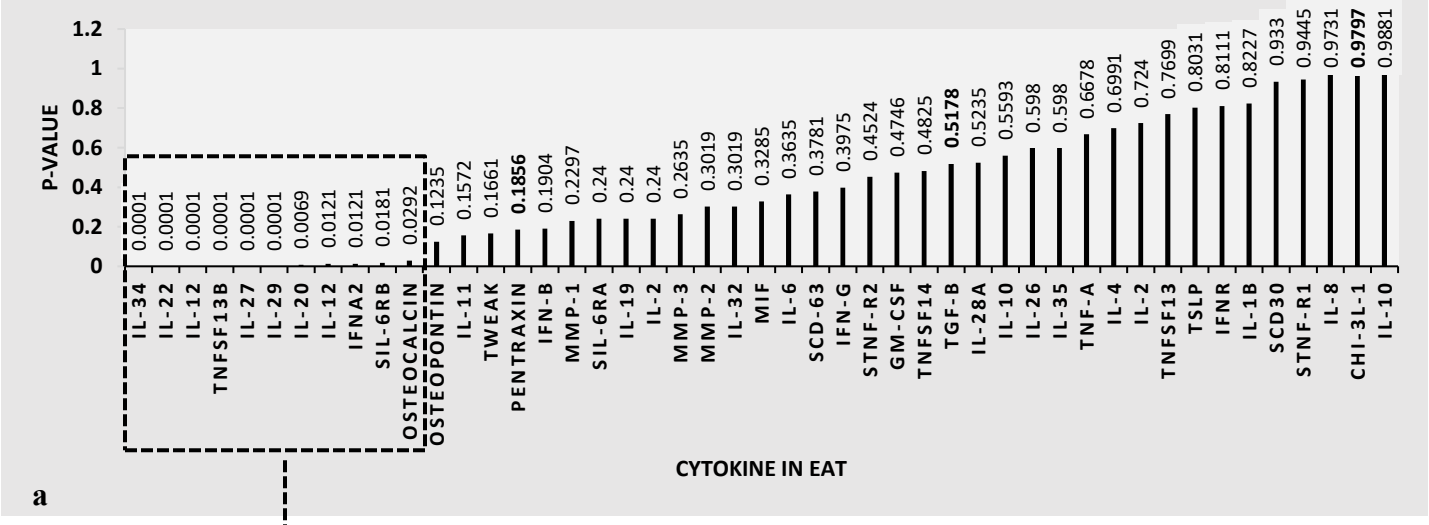
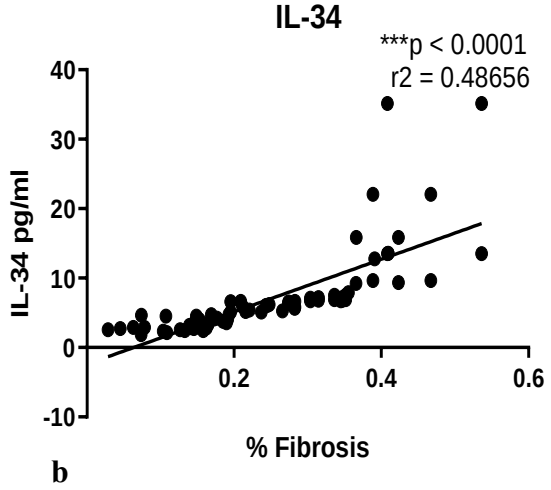
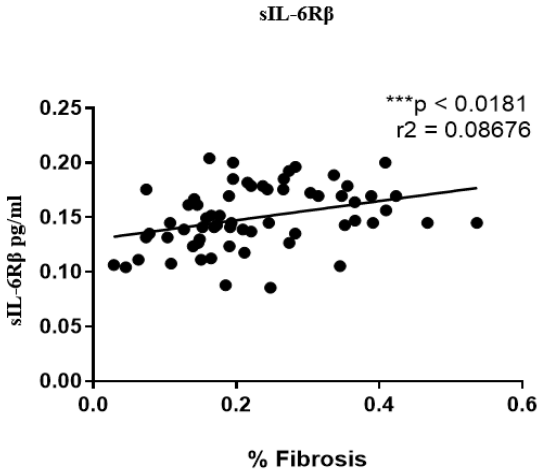


Table-3: Correlation between total collagen in RAA and cytokines in EAT		
Variable	**r2**	**P-value**
IL-27	0.7593	< 0.0001
sIL-6rb	0.08676	0.0181
IL-34	0.4865	< 0.0001
IL-22	0.4774	< 0.0001
IFN-A2	0.1121	0.0121
IFN-R	0.7593	< 0.0001
IL-12	0.4774	< 0.0001
IL-20	0.1121	0.0069
IL-29	0.7593	< 0.0001
TNFSF-13B	0.4774	< 0.0001
Osteocalcin	0.07442	0.0292
IL = Interleukin; s IL = soluble Interleukin; IFN-A2 = Interferon alpha-2; TNFSF = Tumor necrosis factor soluble factor.		

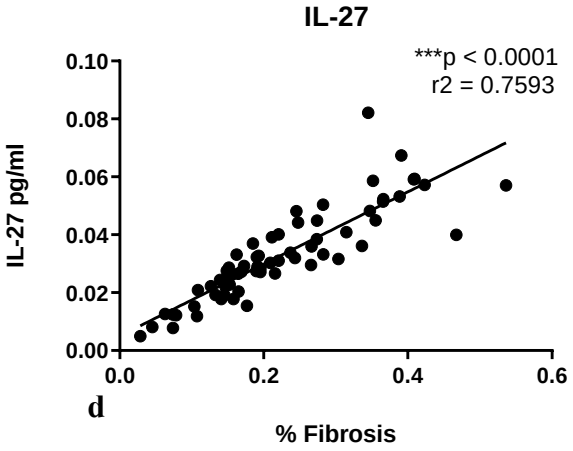
Quantification graphs comparing the extent of atrial fibrosis in the RAA with the levels of cytokines secreted by the EAT



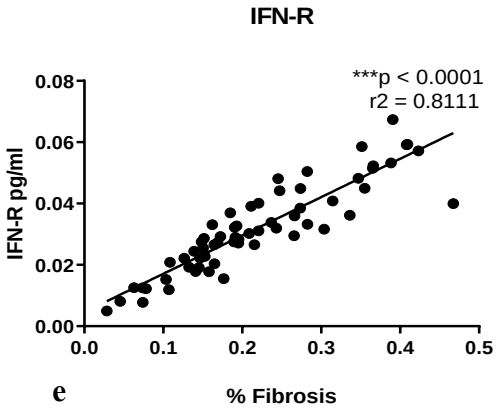
b



c



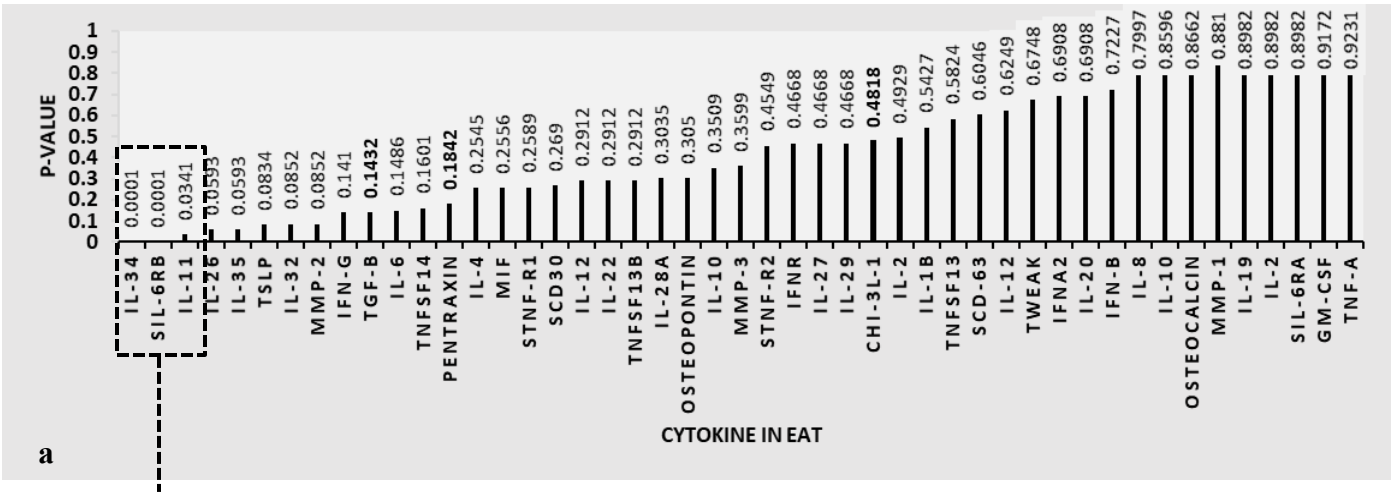
d

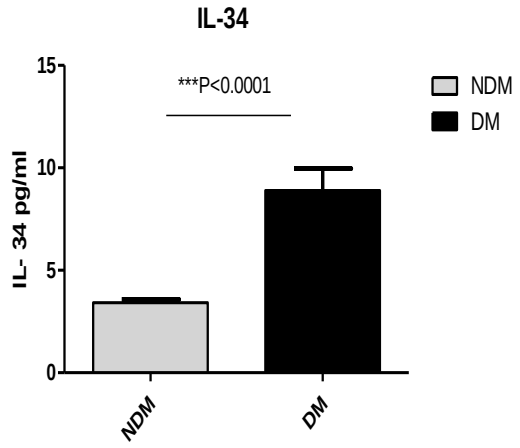


e

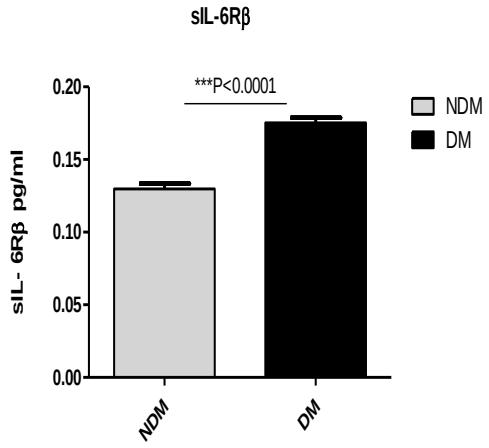
Figure-10: Representative graph showing 49 EAT-cytokines with the p-values of their correlation with atrial fibrosis (Figure-a). Table-2 are the results of the 11-cytokines that correlated with the atrial fibrosis quantified with the total collagen in the RAA samples. Linear regression analysis showing a significant positive correlation between the percentage of atrial fibrosis on X-axis related to the level of IL-34 (***$p<0.0001$, Figure-b) in EAT on Y-axis. Similarly, significant positive correlation was observed between the atrial fibrosis % and the levels of sIL-6RB (**$p<0.0181$, Figure-c), IL-27 (***$p < 0.0001$, Figure-d), and IFN-R (***$p<0.000$, Figure-e).

Figure 11: Comparison of the levels of cytokines secreted by the EAT among NDM vs T2DM

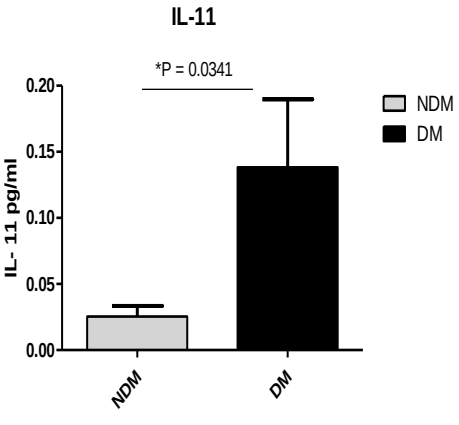




b



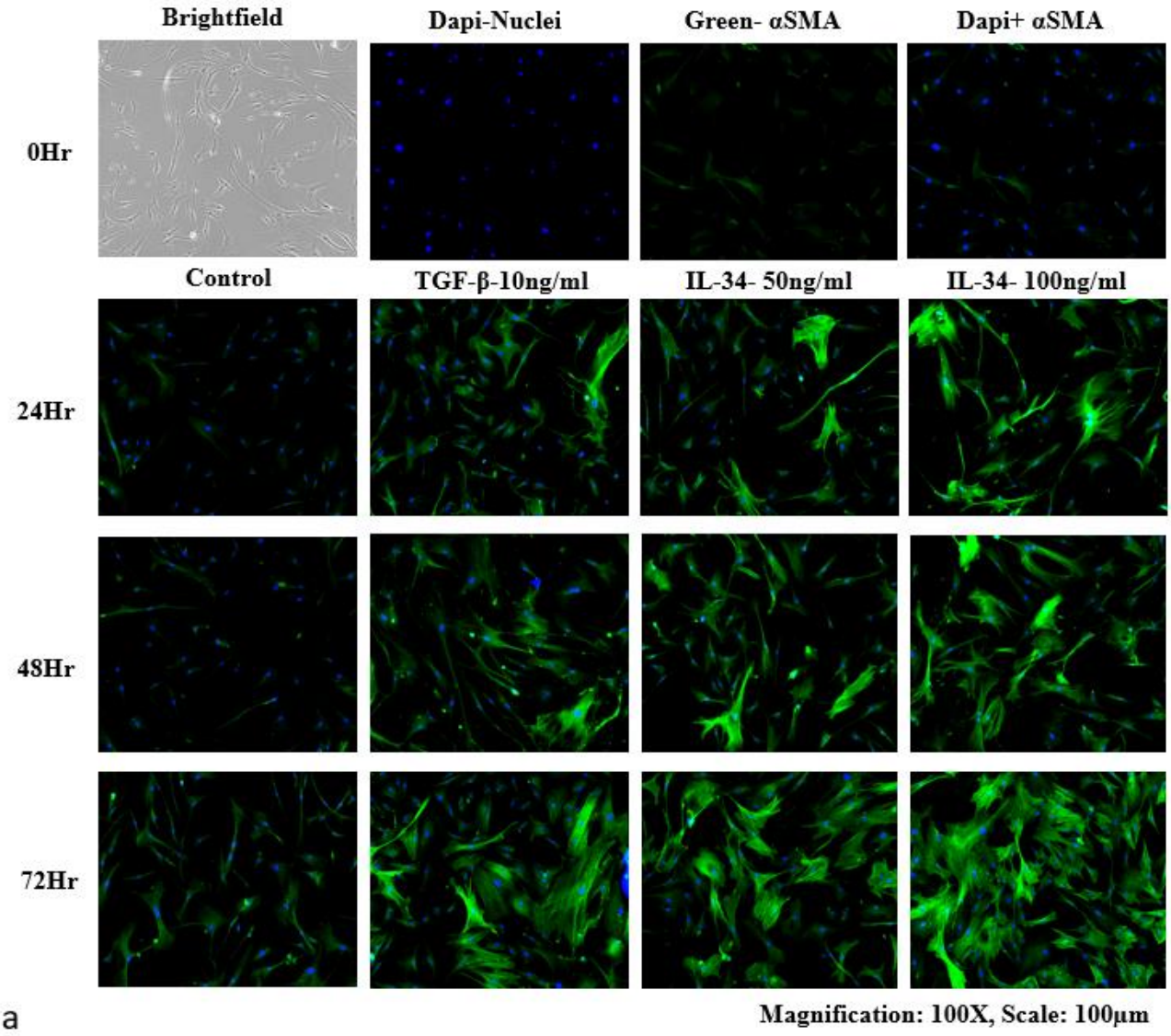
c

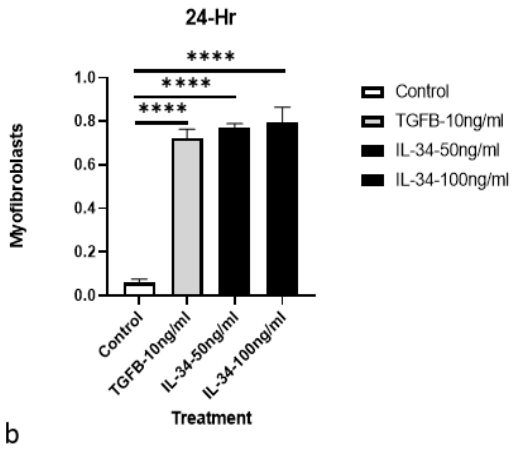


d

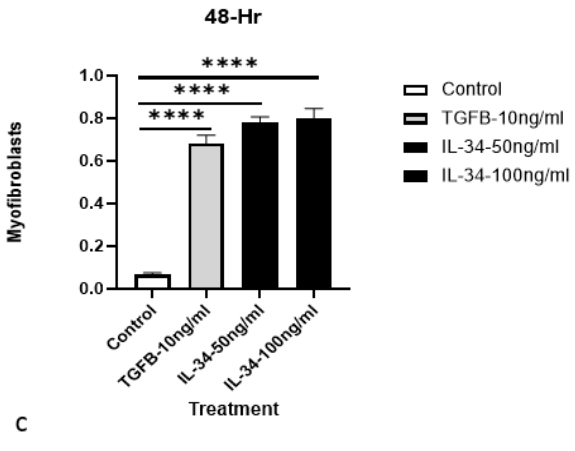
Figure-11: Representative graph showing 49 EAT-cytokines compared in patients with T2DM and NDM with the cytokine on the X-axis and p-values on the Y-axis (Figure-a). Graphs below are a comparison of the levels of EAT-cytokines on Y-axis in T2DM (n=32), and NDM (n=32). IL-34 (***p<0.0001, Figure-b), sIL – 6Rβ (***p<0.0001, Figure-c), and IL-11 (*p = 0.0341, Figure-d) were significantly higher in the EAT of patients with T2DM compared to the NDM.

Figure 12: Immunofluorescent staining measuring the expression of α-SMA in cardiac fibroblasts in IL-34 induced transdifferentiation of cardiac fibroblasts to myofibroblasts

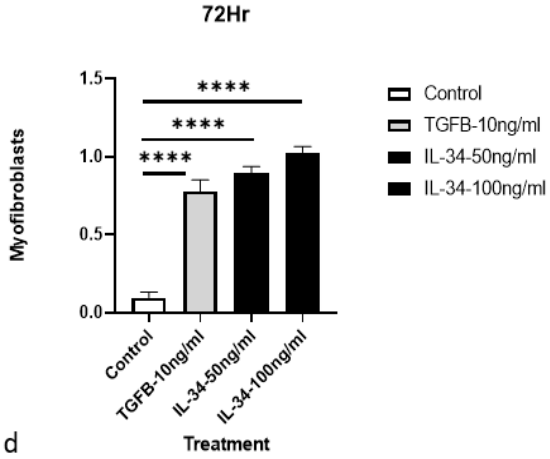



24-Hr
Myofibroblasts
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
Control
TGFB-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
Control
TGFB-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
Treatment

b


48-Hr
Myofibroblasts
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
Control
TGFB-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
Control
TGFB-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
Treatment

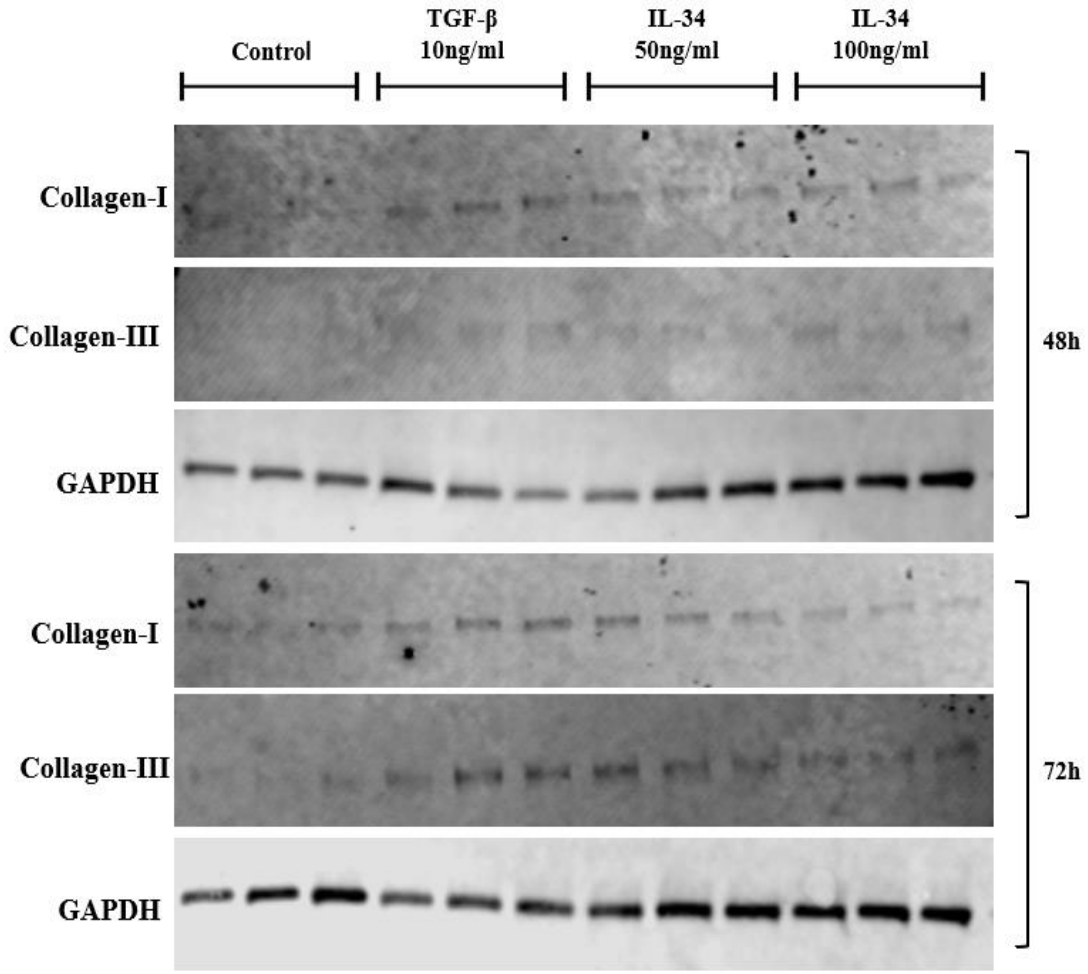
c


72Hr
Myofibroblasts
1.5
1.0
0.5
0.0
****
****
****
Control
TGFB-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
Control
TGFB-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
Treatment

d

Figure 12: Representative micrographs showing fibroblasts at 100x magnification, scale bar: 100μm of brightfield, stained blue for nuclei with Dapi, green for α-SMA, and the overlay of Dapi + α-SMA at 0Hr. Immunofluorescence staining was used to measure the expression of α-SMA in the TGF-β1-10ng/ml, IL-34-50ng/ml, and IL-34-100ng/ml treated fibroblasts in comparison to the control at 24h, 48h, and 72h (Figure-a). Representative graphs showing the comparison of control and treatment groups at 24h (Figure-b), 48h (Figure-c), and 72h (Figure-d) - had a significant increase in the average number of myofibroblasts after TGF-β1 (****$p<0.0001$), IL-34-50ng/ml (****$p<0.0001$), and IL-34-100ng/ml (****$p<0.0001$) treatment.

Figure 13: Western blot analysis measuring the expression of collagen types I and III in cardiac fibroblasts in IL-34 induced fibrogenesis at 48Hr and 72Hr time points



a

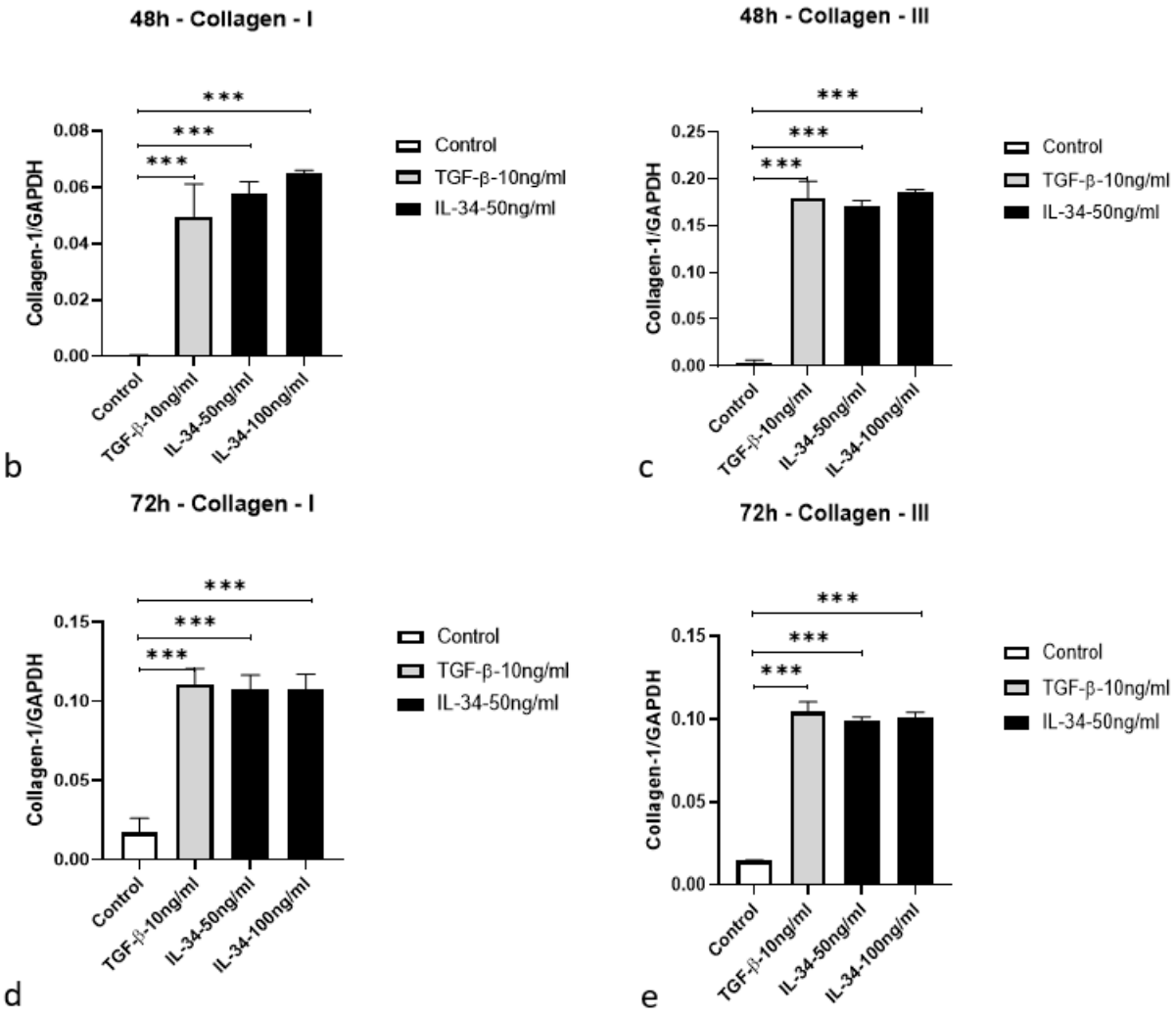
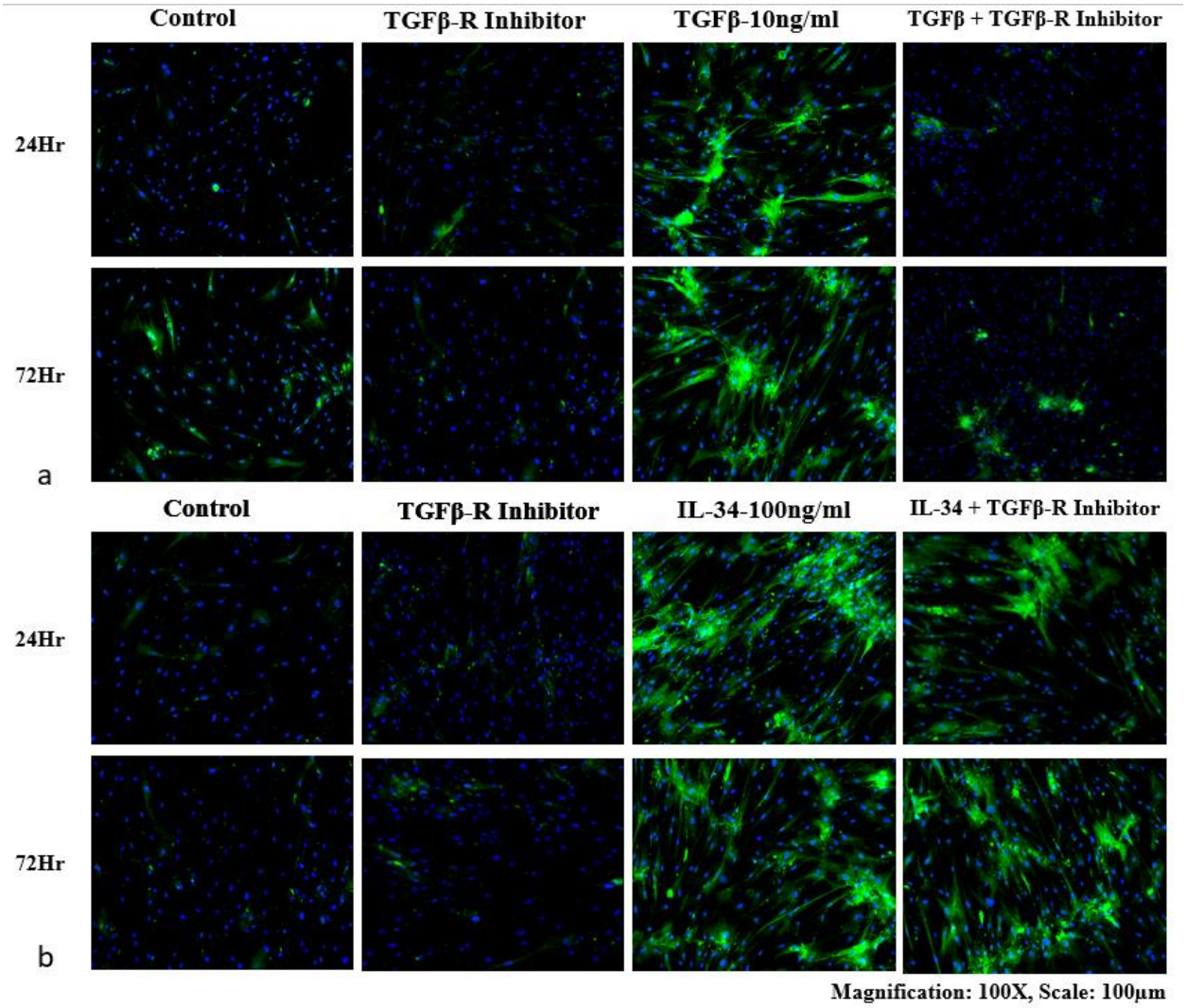
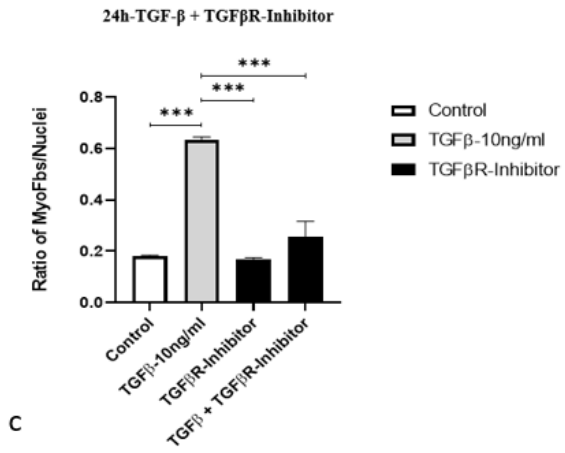

48h - Collagen - I
Collagen-1/GAPDH
0.08
0.06
0.04
0.02
0.00
***
***
***
Control
TGF-β-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
b
48h - Collagen - III
Collagen-1/GAPDH
0.25
0.20
0.15
0.10
0.05
0.00
***
***
***
Control
TGF-β-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
c
72h - Collagen - I
Collagen-1/GAPDH
0.15
0.10
0.05
0.00
***
***
***
Control
TGF-β-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
d
72h - Collagen - III
Collagen-1/GAPDH
0.15
0.10
0.05
0.00
***
***
***
Control
TGF-β-10ng/ml
IL-34-50ng/ml
IL-34-100ng/ml
e

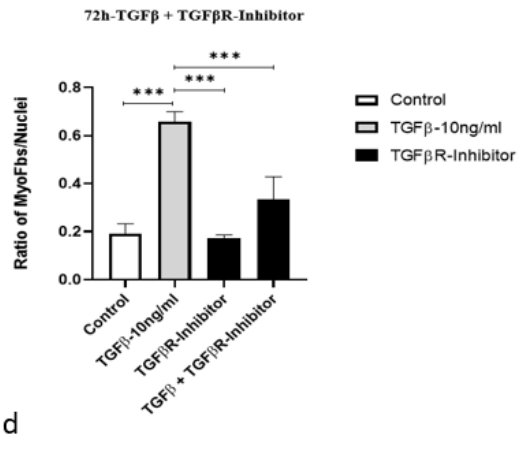
Figure-13: Western blot comparing the screening of protein expression of collagen type-I and type-III among the control, TGF-β1 (10ng/ml), IL-34 (50ng/ml), and IL-34 (100ng/ml) treatments groups at 48hr and 72 hr (Figure-a). Quantitative graphs showing a significant increase in the expression of collagen types I and III among the control and treatment groups at both 48hr and 72hr time points (****$p<0.0001$, Figures: d-e).

Figure-14: Effect of TGF-β receptor inhibition on TGF-β and IL-34 induced transdifferentiation of cardiac fibroblasts to myofibroblasts at 24Hr and 72Hr time points

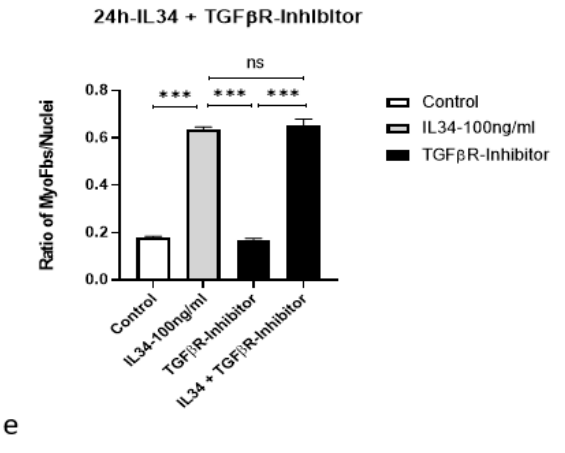



24h-TGF-β + TGFβR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
***
***
***
Control
TGFβ-10ng/ml
TGFβR-Inhibitor
Control
TGFβ-10ng/ml
TGFβR-Inhibitor
TGFβ + TGFβR-Inhibitor

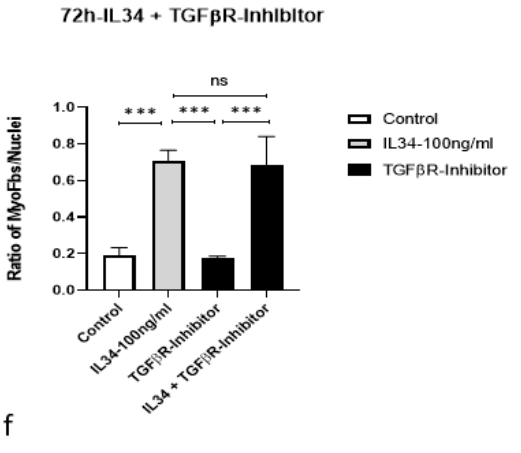
c


72h-TGFβ + TGFβR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
***
***
***
Control
TGFβ-10ng/ml
TGFβR-Inhibitor
Control
TGFβ-10ng/ml
TGFβR-Inhibitor
TGFβ + TGFβR-Inhibitor

d


24h-IL34 + TGFβR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
***
***
***
ns
Control
IL34-100ng/ml
TGFβR-Inhibitor
Control
IL34-100ng/ml
TGFβR-Inhibitor
IL34 + TGFβR-Inhibitor

e


72h-IL34 + TGFβR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
1.0
***
***
***
ns
Control
IL34-100ng/ml
TGFβR-Inhibitor
Control
IL34-100ng/ml
TGFβR-Inhibitor
IL34 + TGFβR-Inhibitor

f

Figure 14: Microscopic images showing fibroblasts at 100x magnification, Scale bars: 100µm. Immunofluorescent staining was used to measure the expression of α-SMA expression in the control, TGF-β1-10ng/ml, TGFβ-R Inhibitor-230nM, and TGFβ+TGFβ-R Inhibitor at 24h and 72h (Figure-a). And α-SMA expression in the control, IL-34-100ng/ml, TGFβ-R Inhibitor-230nM, and IL34+TGFβ-R Inhibitor at 24h and 72h (Figure-b). Representative graphs comparing the ratio of Myofibroblasts (MyoFBs)/Nuclei among the control and treatment groups show a significant increase in the MyoFBs/Nuclei ratio in TGF-β-10ng/ml treatment group, which was significantly reduced in the TGFβ-R inhibitor group, and TGFβ +TGFβ-R Inhibitor group at 24Hr and 72Hr (***$p<0.0001$, Figure-c,d). Further, TGFβ-R inhibitor had no significant reduction on IL-34-100ng/ml induced fibrosis at 24Hr and 72Hr (Figure- e,f).

CHAPTER FOUR

Role of CSF-1 receptor in IL-34 induced cardiac fibrosis

CSF-1R is a type III high-affinity receptor tyrosine kinase expressed on myeloid progenitors for their development, survival, and proliferation. Activation of CSF-1R signaling was thought to be mediated only by the homodimeric growth factor CSF-1. However, Li et al. identified IL-34 as a tissue-specific ligand binding to CSF-1R [78]. When transgenically expressed in CSF1op/op mice, IL-34 rescued all defects resulted from CSF-1 deficiency. Further, Nakamichi et al identified pivotal roles of age-increased IL-34 in the development and maintenance of osteoclast precursors reservoir in the spleen and their transfer to bone in CSF1op/op mice [203]. Studies have then revealed that CSF-1R binds both IL-34 and CSF-1, but through distinct surfaces resulting in unique receptor conformations and downstream signaling events. Through the current study we wanted to investigate the role of CSF-1R in IL-34 induced trans-differentiation of cardiac fibroblasts to Myofibroblasts.

Materials and methods

Cell culture

The tissue culture flasks and 12-well plates were pre-coated with gelatin (G1393-100ml, Sigma). Using aseptic technique under a laminar flow hood, 3ml/flask (T-75), and 250ul/well (12-well plate) of 0.2% gelatin solution in sterilized water was added to the cell culture surface area. The cell culture flasks and plates were gently rocked to coat the surface, followed by which they were dried in the laminar hood for 2 hours. Human Cardiac Fibroblasts from healthy subjects were obtained from Promocell and maintained in Fibroblast media and cultured according to the

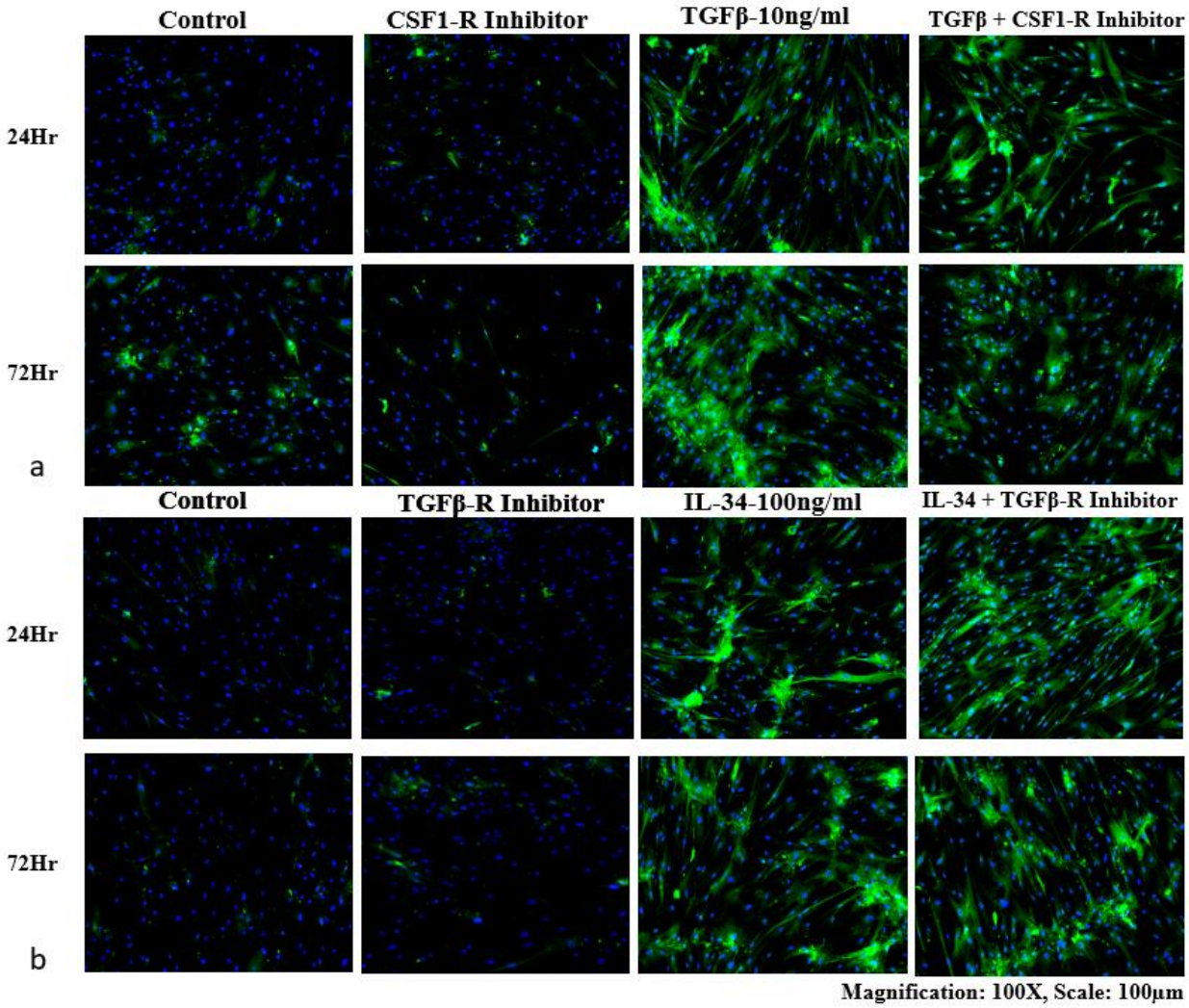
manufacturer's instructions. Three different batches of cells between passages 4 and 6 were studied independently under similar conditions. Cells were seeded in 12-well plates (5×10^4 per well) and pre-treated with CSF-1R inhibitor (670nM) for 1 hour. Followed by which the cells were stimulated with TGF-β1 (10ng/ml, R&D systems) as positive control, and IL-34 (100ng/ml, PeproTech) treatment for 72 hours, and collected at 0h, 24h, and 72h for immunofluorescent staining.

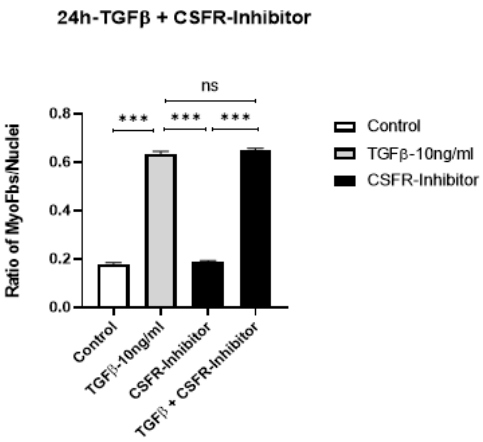
Immunofluorescent staining

Cardiac fibroblasts after treatment were collected at 0h, 24h, and 72h. the culture medium was removed, and the cells were gently rinsed with 500µl/well of PBS (Phosphate buffer Saline). The cells were then fixed in 4% paraformaldehyde for 20minutes. After permeabilization with 0.25% Triton X-100 for 30 minutes at room temperature (RT), the cells were blocked with 5% donkey serum in 0.1% PBST (500µl/well) for 1h at RT. Subsequently, the cells were incubated with primary antibodies against α-SMA (1:200; ab5694, Abcam) for 2 hours at RT. Then, the cells were incubated with secondary antibody (anti-IgG, alexa-488, 1:1000) for 1h at RT. Finally, the cells were counterstained with 4', 6' – diamidino-2-phenylindole (DAPI) (1:10000, D9542-10mg, Sigma) before capturing images by using a fluorescence microscope (Keyence BZ-X800).

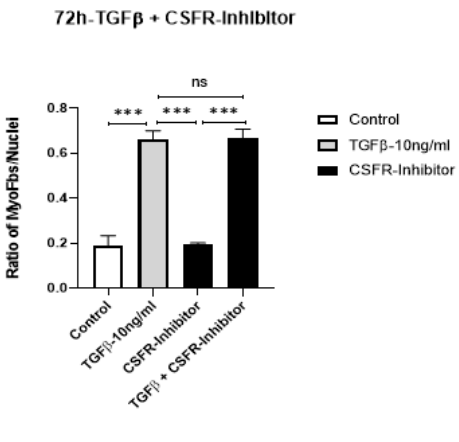
RESULTS

Figure15: Effect of CSF1-receptor inhibition on TGF-β and IL-34 induced transdifferentiation of cardiac fibroblasts to myofibroblasts at 24Hr and 72Hr time points

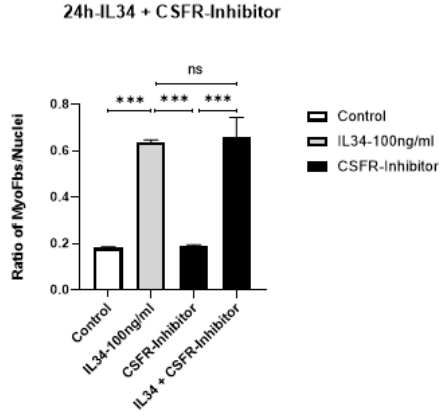



24h-TGFβ + CSFR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
ns
***
***
***
Control
TGFβ-10ng/ml
CSFR-Inhibitor
Control
TGFβ-10ng/ml
CSFR-Inhibitor
TGFβ + CSFR-Inhibitor

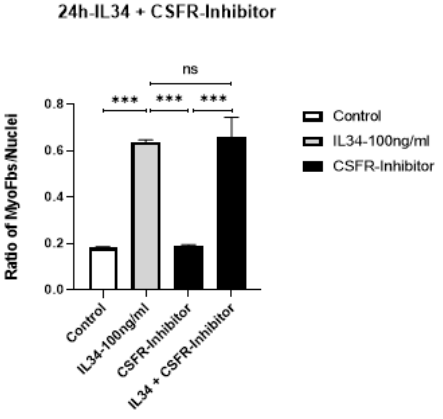
c


72h-TGFβ + CSFR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
ns
***
***
***
Control
TGFβ-10ng/ml
CSFR-Inhibitor
Control
TGFβ-10ng/ml
CSFR-Inhibitor
TGFβ + CSFR-Inhibitor

d


24h-IL34 + CSFR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
ns
***
***
***
Control
IL34-100ng/ml
CSFR-Inhibitor
Control
IL34-100ng/ml
CSFR-Inhibitor
IL34 + CSFR-Inhibitor

e


24h-IL34 + CSFR-Inhibitor
Ratio of MyoFbs/Nuclei
0.0
0.2
0.4
0.6
0.8
ns
***
***
***
Control
IL34-100ng/ml
CSFR-Inhibitor
Control
IL34-100ng/ml
CSFR-Inhibitor
IL34 + CSFR-Inhibitor

f

Figure 15: Microscopic images showing fibroblasts at 100x magnification, Scale bars: 100µm. Immunofluorescent staining was used to measure the expression of α-SMA expression in the control, TGF-β1-10ng/ml, CSF1-R Inhibitor-670nM, and TGFβ+CSF1-R Inhibitor at 24h and 72h (Figure-a). And α-SMA expression in the control, IL-34-100ng/ml, CSF1-R Inhibitor-670nM, and IL34+CSF1-R Inhibitor at 24h and 72h (Figure-b). Representative graphs comparing the ratio of Myofibroblasts (MyoFBs)/Nuclei among the control and treatment groups show a significant increase in the MyoFBs/Nuclei ratio in TGF-β-10ng/ml and IL-34-100ng/ml treatment groups, which was not significantly reduced in the TGFβ +CSF1-R Inhibitor groups (Figure-c,d), and the IL34 + CSF1R inhibitor groups at 24Hr and 72Hr (Figure-e,f).

CHAPTER FIVE

Differential levels of IL-34 in plasma of patients with T2DM

Clinical studies over the past decade have been exploring the role of IL-34 as a potential inflammatory biomarker for the predicting CVD risk in T2DM [204, 205]. Katarzyna et al. in a clinical study showed that serum IL-34 levels were significantly higher in patients with vascular diabetic complications [204]. Several other studies showed that serum IL-34 levels significantly and positively correlated with insulin resistance-related metabolic parameters [73, 80]. Further, IL-34 has been shown to augment fat accumulation and inhibit the stimulatory effects of insulin on glucose transport [205].

Eun-ji et al demonstrated that IL-34 is expressed in human adipose tissue, and that the circulating concentration of IL-34 is associated with insulin resistance [205]. IL-34 is shown to orchestrate immune responses in systemic inflammatory conditions like rheumatoid arthritis [80, 206], and inflammatory bowel disease [207, 208]. Through this study we want to determine if IL-34 is differentially secreted in plasma of patients with T2DM, and its possible correlation with atrial fibrosis.

Materials and methods

Study design

Between September 2017 and November 2019, approximately 112 patients who underwent coronary artery bypass grafting (CABG) surgery were enrolled in the study. Forty-eight patients were excluded because of insufficient adipose tissue biopsy samples, higher BMI, or above the

age of 75 years. Plasma samples from a total of n=40 patients with T2DM (n=20), and NDM (n=20) were collected, and used for measuring the IL-34 levels. The study was approved by the Institutional Review Board (IRB) of East Carolina University (UMCIRB09-0669), and all patients have provided a written informed consent.

Blood collection

Blood was drawn from the patients an hour prior to the surgery in BD Vacutainer blood collection tubes with buffered sodium citrate as anticoagulant. For plasma, the blood tubes were centrifuged at 800 rpm for 10 minutes and stored in aliquots at -80°C. Plasma, Red blood fraction (RBF), and the plasma rich protein (PRP) were collected by centrifuging further at 400 rpm for 10 minutes and stored in aliquots at -80°C.

Human plasma IL-34 direct ELISA

Plasma levels of IL-34 were measured in the plasma extracted from the patient blood samples using Human IL-34 AccuSignal ELISA kit (K0A0826; Rockland, USA). Briefly, the plasma samples were diluted in the sample diluent buffer (1:2 dilution), standards, antibody detection diluents, and buffers were prepared, and the assay was performed according to the manufacturers protocol.

Statistical analysis

All the continuous variables are presented as mean $\pm$ SD. Data was analyzed using GraphPad prism-8 software, determined by unpaired student t-test, and linear regression. The student t-test was used for evaluation of a single variable among two groups.

RESULTS

Figure 16: Comparison of the plasma levels of IL-34 in NDM vs T2DM patients, and correlation with the extent of atrial fibrosis in the RAA

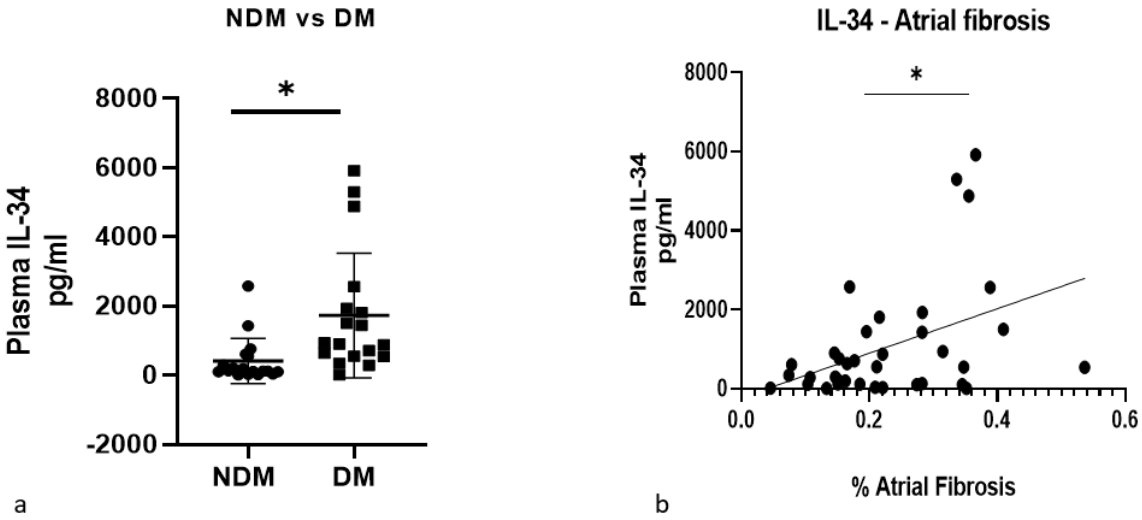


Figure-16: Quantification graphs show plasma IL-34 levels to be significantly high in the T2DM (n=20) group compared to the NDM(n=20) [**p<0.0024, Figure-a]. Further, a simple linear regression analysis was performed correlating the extent of fibrosis in the RAA with the plasma IL-34 levels. And the extent of fibrosis in the patients positively correlated with the plasma IL-34 levels (*p<0.0112, Figure-b).

FINAL DISCUSSION

EAT has been suggested as a source of resident fibroblasts that can be activated by inflammatory stimuli and migrate to the myocardium as myofibroblasts. Adipose tissues are complex organs involved in the production of several inflammatory mediators [209-211]. Several lines of evidence suggest that EAT participates in the inflammatory processes within the myocardium including a correlation between EAT thickness and the degree of cardiac inflammation [211, 212]. Patients with T2DM not only accumulate EAT but have been shown to present with myocardial fibrosis [213, 214]. Collectively, these studies suggest that cytokines released by EAT participate in myocardial fibrosis.

Therefore, the first aim of this study was to measure the secretion of various cytokines released by the EAT explants and determine whether EAT of patients with T2DM have a higher secretion rates of these cytokines, and if there is a possible correlation between these cytokines with the extent of atrial fibrosis observed in patients with T2DM. Using EAT conditioned medium, we measured a total of 49 cytokines, and of the analyte quantified 11 cytokines secreted by the EAT correlated with atrial fibrosis, and 3 cytokines were elevated in T2DM. Of these, IL-34 was both highly expressed in the EAT of T2DM and correlated with atrial fibrosis. These findings suggest that cytokines like IL-34 could possibly be the candidates for the pathophysiological basis of atrial fibrosis in patients with T2DM.

Further, the second aim of this study was to determine the potential of IL-34 to promote fibrogenesis. To the best of our knowledge, this is the first study demonstrating that IL-34 treatment can cause human cardiac fibroblasts to transform to myofibroblasts. The cells treated with IL-34 contained multiple strands of highly organized α – SMA expression in the cytoskeleton.

Although both TGF-β1, and IL-34 treatment groups increased the myofibroblast differentiation, IL-34 treatment group specifically had more marked expression of α – SMA. Further, IL-34 also had a significant increase in the protein expression of both collagen types I and III indicating ECM degradation.

In recent years, significant progress has been made in understanding the receptors and the downstream molecular mechanisms involved in promoting cardiac fibroblast induced fibrosis. Among these, TGF-β (I/II) receptors and its downstream canonical and non-canonical signaling pathways play a key role in cardiac fibrosis and is supported by ample evidence [215-217]. Similar to previous studies, our results suggest that TGFβ-R inhibition significantly reduced the myofibroblast differentiation in the TGF-β treated cardiac fibroblasts. Interestingly, TGFβ-R inhibition did not have any significant effect on the IL-34 induced differentiation of CFBs to myofibroblasts, suggesting that IL-34 might be mediating fibrosis in a TGFβ-R independent pathway.

Lin.et.al identified that IL-34 is a tissue-specific ligand binding to CSF-1R. Furthermore, several studies have shown that CSF-1R binds both IL-34 and CSF-1 through distinct surfaces that result in forming unique receptor conformations and downstream signaling events. In the present study, we wanted to investigate the role of CSF-1R in IL-34 induced transdifferentiation of cardiac fibroblasts to myofibroblasts in spite of the CSF-1R inhibition, suggesting that CSF-1R may not be involved in IL-34 induced cardiac fibrosis.

However, in the current study a positive control for the CSF-1R inhibitor treatment was not included, therefore, further studies are required with a CSF-1 treatment group with the CSF-

1R inhibitor to confirm the effect of the CSF1-R inhibitor treatment. Together, the findings in our study show that neither TGFβ-1R nor CSF-1R are centrally involved in IL-34 induced transdifferentiation of cardiac fibroblasts to Myofibroblasts. Therefore, further studies are required to investigate the role of the newly discovered receptors that IL-34 is shown to bind and mediate it effects through including Syndecan-1 [75-77, 81], and PTP-ζ [74, 81, 85] receptors.

Some studies showed an association between serum IL-34 levels and T2DM. Katarzyna et al. in a clinical study showed that serum IL-34 levels were significantly higher in patients with vascular diabetic complications. In our study we found that IL-34 is differentially secreted in plasma of patients with T2DM, and significantly correlated with atrial fibrosis. Although our study is the first to show a positive correlation between plasma IL-34 and fibrosis in the RAA, further research is required to completely understand the role/contribution of plasma IL-34 in the EAT-cytokine-mediated cardiac fibrosis and T2DM.

sIL-6Rβ

Beside the membrane-bound IL-6R form for IL-6 signaling, Muller-Newen et al. in 1996 was the first to show that there are two soluble receptor isoforms of IL-6 that are generated during post-transcriptional or translational processes [192]. Unlike the soluble receptors of cytokines such as IL-1 or TNF-α which inhibit the effects of their ligands [218, 219], the IL-6/sIL-6R complex is shown to promote IL-6 expression [220]. Therefore, the population of potential IL-6 expressing cells is strongly increased by the presence of the sIL-6R isoforms [221, 222].

Siwik et.al found that incubation of rat cardiac fibroblasts with IL-6 alone reduced collagen content in the media by 11% compared to the TNF-α and IL-1β treatment groups [223]. Study by

Ancey et al. on adult cardiac fibroblasts showed that IL-6 treatment somewhat reduced collagen content. Interestingly, Ancey et.al also found that co-culturing of sIL-6R with IL-6 produced a 4-fold increase in the collagen content at the highest concentration of sIL-6R [193]. Similar to these findings, our study found a strong correlation between the levels of sIL-6Rβ and atrial fibrosis in patients with T2DM. Further research is required to explore and validate the role of IL-6/sIL-6Rβ in T2DM and cardiac fibrosis.

Interleukin-22

Studies by Yongxin et al [198] demonstrated that IL-22 levels are elevated in the atrium and plasma of patients with atrial fibrillation, and increased collagen synthesis in TGF – β1 treated cardiac fibroblasts via the JNK pathway. IL-22 is a multifunctional cytokine involved in a variety of biological processes, including inflammatory reactions, oxidative stress, apoptosis, autophagy, cell migration, and endothelial dysfunction [224-228]. Previous studies have revealed that IL-22 regulates the progression of a variety of CVDs, including hypertension, atherosclerosis, and viral myocarditis [228-230]. Although these studies have explored the role of IL-22 in several invitro animal, and prospective observational studies in humans, to the best of our knowledge, our study was the first to elucidate the levels of IL-22 in the RAA of patients with T2DM, and its correlation with atrial fibrosis. However, further research is required to investigate the specific mechanisms involved in IL-22 mediated cardiac fibrosis.

Interleukin-27

IL-27, one of the cytokines in the IL-12 family, is considered to have both pro- and anti-inflammatory properties [231]. Interestingly, the role of IL-27 in promoting or suppressing

inflammation varies among diseases [232]. Kotaro et al. suggested that plasma levels of IL-27 were high in patients with CAD, but not in acute coronary syndrome [233]. IL-27 has also been shown to have pathogenic role in T2DM, Wang et al [234] detected high levels of IL-27 in diabetic mice, and that blockade of IL-27 significantly delayed the onset of diabetic splenocyte transferred diabetes. These results demonstrate a possible pathogenic role of IL-27 in T2DM. Considering the diverse role of IL-27 in various diseases, it would be interesting to further elucidate the specific role of IL-27 in promoting myocardial fibrosis.

Interferon alpha (IFN-α) and interferon receptor (IFN-R)

Interferons (IFNs) are a family of cytokines that cause a myriad of pathological responses [235-237]. Along with immune cells, the interferons are also secreted by other cell types such as cardiac fibroblasts [238, 239]. Several studies on interferons such as IFN-γ and its receptor IFN-R have explored their role in cardiac fibrosis [240]. Han et al. showed that IFN-γ knockout mice treated with Ang-II showed a significant reduction in the levels of α-SMA in cardiac fibroblasts [199]. Similarly, mice null for IFN-R had a decrease in cardiac hypertrophy and fibrosis, and a reduction in the infiltration of macrophages and T-cells [241]. Similar to these studies, our study showed a significant correlation between IFN-α, and IFN-R and atrial fibrosis. Because extensive data have demonstrated a critical role of inflammation in the fibrotic response, further research is needed for better understanding the role in of IFN-α and IFN-R in the pathogenesis of cardiac fibrosis in T2DM.

Interleukin -12

Similar to the interferon family, IL-12 is a heterodimeric cytokine with differential roles [242, 243]. Produced primarily by antigen-presenting cells like macrophages and dendritic cells [244, 245]. The primary effect of IL-12 is to stimulate differentiation of Th1 cells and is a key regulator of M1-phenotypoe macrophages mediating pro-inflammatory response in certain infectious diseases [246-248]. Fairweather et al. showed that deficiency of IL-12 protects against coxsackievirus B3-induced myocarditis by increasing macrophage and neutrophil infiltration [249]. Further exploration of the role of IL-12 in T2DM induced cardiac fibrosis is needed considering its strong correlation with atrial fibrosis.

Interleukin – 20

IL-20 is a member of the IL-10 family of cytokines and was first discovered in 2001 [250]. It acts on multiple cell types by activating a heterodimeric receptor complex IL-20R/IL-22R1 [251]. Studies indicate that the interaction of IL-20 with these receptors might have proinflammatory effects in chronic inflammatory diseases like T2DM, rheumatoid arthritis, and osteoporosis [252-255]. Recently, Tsai et al in 2021 demonstrated that anti-IL-20 antibody protects against ischemia/reperfusion-impaired myocardial function and cardiac remodeling in rats [197]. Considering the fact that IL-20 strongly correlated with atrial fibrosis in our study, a future direction would be to look into the role of IL-20 in promoting cardiac fibrosis.

Interleukin-29

IL-29, a member of the type 3 interferon family, plays a critical role in host-defense against viral infections [256-259]. Tian et al. in 2020 explored the role of IL-29 in the pathogenesis of obesity-induced inflammation and insulin resistance [260], showing that inhibition of IL-29 could reduce inflammatory cytokine production in macrophage-adipocyte coculture system mimicking an obese and T2DM microenvironment [260]. Our results provide the supporting evidence of IL-29 correlation with atrial fibrosis, thus exploration of its proinflammatory role in T2DM induced cardiac fibrosis is needed.

Osteocalcin

Our study found a significant correlation between osteocalcin and atrial fibrosis. Osteocalcin is one of the major non-collagenous proteins of the bone matrix. It is mainly produced by osteoblasts and is generally regarded as a marker of bone formation [261-264]. Desentis et al. showed that Osteocalcin has a protective in diabetic pathogenesis [200]. And another study demonstrated a strong correlation between hyperglycemia and total osteocalcin levels in T2DM patients [265]. However, we did not see a significant increase in the osteocalcin levels in the EAT of T2DM patients compared to the NDM, and this might possibly be attributed to the fact that these patients are under diabetic management.

Tumor necrosis superfamily – 13b (TNFSF-13b)

TNFSF13b, also known as the B-cell activating factor (BAFF), belongs to the TNF ligand family [201]. Recently, Yarchoan et al. in 2020 showed a strong association between TNFSF-13b and

autoimmunity [202]. However, TNFSF-13b has relatively been less explored for its association with T2DM, and cardiac remodeling. Therefore, with the current study serving as a supporting evidence, further research exploring the possible role of TNFSF13b in cardiac fibrosis is needed.

PERSPECTIVE

Despite the significant amount of research now addressing cardiac fibrosis, the understanding of its pathogenesis, clinical implications, and management remains limited. In terms of the pathophysiology of the cardiac fibrosis, more governing factors should be explored to improve diagnostic methods and identify therapeutic targets. Our knowledge of the role of T2DM-EAT in promoting cardiac fibrosis is somewhat limited. This is because changes in the EAT of human heart cannot be reproduced in most animal models. Currently, there is no specific medical therapy directly targeting cardiac fibrosis. Many of the clinical experiments have been devoted to the known target sites of myocardial fibrosis for the discovery of new medical interventions. Therefore, the rationale for this project was to identify EAT-derived factors in T2DM that correlate with cardiac fibrosis.

Our study revealed that several cytokines released by the EAT had a strong correlation with the extent of fibrosis in the RAA. IL-34 was able to induce fibrogenesis independently of TGF-β- and CSF1-receptors in vitro. Further, we also found that other cytokines including IL-22, IFN-α2, IFN-R, IL-12, IL-20, IL-29, TNFSF-13β, and osteocalcin that significantly correlated with atrial fibrosis. Using the current study as supporting evidence, further research investigating the role of these cytokines in T2DM-cardiac fibrosis may provide the scientific framework for the development of new therapeutic strategies for cardiac fibrosis

REFERENCES

[1] J.G. Travers, F.A. Kamal, J. Robbins, K.E. Yutzey and B.C. Blaxall, "'Cardiac Fibrosis: The Fibroblast Awakens," *Circ.Res.*, vol. 118, no. 6, March 18, pp. 1021-1040.

[2] S. Hinderer and K. Schenke-Layland, "'Cardiac fibrosis - A short review of causes and therapeutic strategies," *Adv.Drug Deliv.Rev.*, vol. 146, June 01, pp. 77-82.

[3] S. Park, N.B. Nguyen, A. Pezhouman and R. Ardehali, "'Cardiac fibrosis: potential therapeutic targets," *Transl.Res.*, vol. 209, July 01, pp. 121-137.

[4] V. Talman and H. Ruskoaho, "'Cardiac fibrosis in myocardial infarction-from repair and remodeling to regeneration," *Cell Tissue Res.*, vol. 365, no. 3, September 01, pp. 563-581.

[5] C.W.L. Chin, R.J. Everett, J. Kwiecinski, A.T. Vesey, E. Yeung, G. Esson, W. Jenkins, M. Koo, S. Mirsadraee, A.C. White, A.G. Japp, S.K. Prasad, S. Semple, D.E. Newby and M.R. Dweck, "'Myocardial Fibrosis and Cardiac Decompensation in Aortic Stenosis," *JACC Cardiovasc.Imaging*, vol. 10, no. 11, November 01, pp. 1320-1333.

[6] M. Picard, K.J. Wright, D. Ritchie, M.M. Thomas and R.T. Hepple, "'Mitochondrial function in permeabilized cardiomyocytes is largely preserved in the senescent rat myocardium," *PLoS One*, vol. 7, no. 8, pp. e43003.

[7] L. van Heerebeek, N. Hamdani, M.L. Handoko, I. Falcao-Pires, R.J. Musters, K. Kupreishvili, A.J. Ijsselmuiden, C.G. Schalkwijk, J.G. Bronzwaer, M. Diamant, A. Borbely, J. van der Velden, G.J. Stienen, G.J. Laarman, H.W. Niessen and W.J. Paulus, "'Diastolic stiffness of the failing diabetic heart: importance of fibrosis, advanced glycation end products, and myocyte resting tension," *Circulation*, vol. 117, no. 1, January 01, pp. 43-51.

[8] A.M. Segura, O.H. Frazier and L.M. Buja, "'Fibrosis and heart failure," *Heart Fail.Rev.*, vol. 19, no. 2, March 01, pp. 173-185.

[9] S. Honsho, S. Nishikawa, K. Amano, K. Zen, Y. Adachi, E. Kishita, A. Matsui, A. Katsume, S. Yamaguchi, K. Nishikawa, K. Isoda, D.W. Riches, S. Matoba, M. Okigaki and H. Matsubara, "'Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways," *Circ.Res.*, vol. 105, no. 11, November 20, pp. 1149-1158.

[10] R.A. Norris, T.K. Borg, J.T. Butcher, T.A. Baudino, I. Banerjee and R.R. Markwald, "'Neonatal and adult cardiovascular pathophysiological remodeling and repair: developmental role of periostin," *Ann.N.Y.Acad.Sci.*, vol. 1123, March 01, pp. 30-40.

[11] S. Fredj, J. Bescond, C. Louault and D. Potreau, "'Interactions between cardiac cells enhance cardiomyocyte hypertrophy and increase fibroblast proliferation," *J.Cell.Physiol.*, vol. 202, no. 3, March 01, pp. 891-899.

[12] V. Talman and H. Ruskoaho, "'Cardiac fibrosis in myocardial infarction-from repair and remodeling to regeneration," *Cell Tissue Res.*, vol. 365, no. 3, September 01, pp. 563-581.

[13] G. Jia, M.A. Hill and J.R. Sowers, "'Diabetic Cardiomyopathy: An Update of Mechanisms Contributing to This Clinical Entity," *Circ.Res.*, vol. 122, no. 4, February 16, pp. 624-638.

[14] A. Harvey, A.C. Montezano, R.A. Lopes, F. Rios and R.M. Touyz, "'Vascular Fibrosis in Aging and Hypertension: Molecular Mechanisms and Clinical Implications," *Can.J.Cardiol.*, vol. 32, no. 5, May 01, pp. 659-668.

[15] I. Russo and N.G. Frangogiannis, "'Diabetes-associated cardiac fibrosis: Cellular effectors, molecular mechanisms and therapeutic opportunities," *J.Mol.Cell.Cardiol.*, vol. 90, January 01, pp. 84-93.

[16] T.A. Wynn, "'Cellular and molecular mechanisms of fibrosis," *J.Pathol.*, vol. 214, no. 2, January 01, pp. 199-210.

[17] K.T. Weber, Y. Sun, S.K. Bhattacharya, R.A. Ahokas and I.C. Gerling, "'Myofibroblast-mediated mechanisms of pathological remodelling of the heart," *Nat.Rev.Cardiol.*, vol. 10, no. 1, January 01, pp. 15-26.

[18] X. Sun, B. Nkennor, O. Mastikhina, K. Soon and S.S. Nunes, "'Endothelium-mediated contributions to fibrosis," *Semin.Cell Dev.Biol.*, vol. 101, May 01, pp. 78-86.

[19] N.G. Frangogiannis, "'Cardiac fibrosis," *Cardiovasc.Res.*, vol. 117, no. 6, May 25, pp. 1450-1488.

[20] S.R. Ali, S. Ranjbarvaziri, M. Talkhabi, P. Zhao, A. Subat, A. Hojjat, P. Kamran, A.M. Muller, K.S. Volz, Z. Tang, K. Red-Horse and R. Ardehali, "'Developmental heterogeneity of cardiac fibroblasts does not predict pathological proliferation and activation," *Circ.Res.*, vol. 115, no. 7, September 12, pp. 625-635.

[21] G. Krenning, E.M. Zeisberg and R. Kalluri, "'The origin of fibroblasts and mechanism of cardiac fibrosis," *J.Cell.Physiol.*, vol. 225, no. 3, November 01, pp. 631-637.

[22] C.A. Souders, S.L. Bowers and T.A. Baudino, "'Cardiac fibroblast: the renaissance cell," *Circ.Res.*, vol. 105, no. 12, December 04, pp. 1164-1176.

[23] T. Moore-Morris, N. Guimaraes-Camboa, I. Banerjee, A.C. Zambon, T. Kisseleva, A. Velayoudon, W.B. Stallcup, Y. Gu, N.D. Dalton, M. Cedenilla, R. Gomez-Amaro, B. Zhou, D.A. Brenner, K.L. Peterson, J. Chen and S.M. Evans, "'Resident fibroblast lineages mediate pressure overload-induced cardiac fibrosis," *J.Clin.Invest.*, vol. 124, no. 7, July 01, pp. 2921-2934.

[24] M.D. Tallquist and J.D. Molkentin, "'Redefining the identity of cardiac fibroblasts," *Nat.Rev.Cardiol.*, vol. 14, no. 8, August 01, pp. 484-491.

[25] K.M. Fries, T. Blieden, R.J. Looney, G.D. Sempowski, M.R. Silvera, R.A. Willis and R.P. Phipps, "'Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis," *Clin.Immunol.Immunopathol.*, vol. 72, no. 3, September 01, pp. 283-292.

[26] O. Kanisicak, H. Khalil, M.J. Ivey, J. Karch, B.D. Maliken, R.N. Correll, M.J. Brody, S.C. J Lin, B.J. Aronow, M.D. Tallquist and J.D. Molkentin, "'Genetic lineage tracing defines myofibroblast origin and function in the injured heart," *Nat.Commun.*, vol. 7, July 22, pp. 12260.

[27] A.D. Theocharis, S.S. Skandalis, C. Gialeli and N.K. Karamanos, "'Extracellular matrix structure," *Adv.Drug Deliv.Rev.*, vol. 97, February 01, pp. 4-27.

[28] R.O. Hynes, "'The extracellular matrix: not just pretty fibrils," *Science*, vol. 326, no. 5957, November 27, pp. 1216-1219.

[29] C. Bonnans, J. Chou and Z. Werb, "'Remodelling the extracellular matrix in development and disease," *Nat.Rev.Mol.Cell Biol.*, vol. 15, no. 12, December 01, pp. 786-801.

[30] N. Bildyug, "'Extracellular Matrix in Regulation of Contractile System in Cardiomyocytes," *Int.J.Mol.Sci.*, vol. 20, no. 20, October 11, pp. 10.3390/ijms20205054.

[31] O.V. Gritsenko, G.A. Chumakova, I.V. Shevlyakov and N.G. Veselovskaya, "'Extracellular matrix of the heart and its changes in myocardial fibrosis," *Kardiologiia*, vol. 60, no. 6, July 07, pp. 773.

[32] M. Shimizu, K. Umeda, N. Sugihara, H. Yoshio, H. Ino, R. Takeda, Y. Okada and I. Nakanishi, "'Collagen remodelling in myocardia of patients with diabetes," *J.Clin.Pathol.*, vol. 46, no. 1, January 01, pp. 32-36.

[33] M.A. Rahman, "'Collagen of Extracellular Matrix from Marine Invertebrates and Its Medical Applications," *Mar.Drugs*, vol. 17, no. 2, February 14, pp. 10.3390/md17020118.

[34] D. Mukherjee and S. Sen, "'Alteration of cardiac collagen phenotypes in hypertensive hypertrophy: role of blood pressure," *J.Mol.Cell.Cardiol.*, vol. 25, no. 2, February 01, pp. 185-196.

[35] A. Uchinaka, M. Yoshida, K. Tanaka, Y. Hamada, S. Mori, Y. Maeno, S. Miyagawa, Y. Sawa, K. Nagata, H. Yamamoto and N. Kawaguchi, "'Overexpression of collagen type III in injured myocardium prevents cardiac systolic dysfunction by changing the balance of collagen distribution," *J.Thorac.Cardiovasc.Surg.*, vol. 156, no. 1, July 01, pp. 217-226.e3.

[36] E. Hookana, M.J. Junttila, K.S. Kaikkonen, K. Porvari, H. Kaija, J. Risteli, M.L. Kortelainen and H.V. Huikuri, "'Increased type I collagen synthesis in victims of sudden cardiac death due to idiopathic myocardial fibrosis," *Ann.Med.*, vol. 46, no. 5, August 01, pp. 318-323.

[37] C.A. Meschiari, O.K. Ero, H. Pan, T. Finkel and M.L. Lindsey, '"The impact of aging on cardiac extracellular matrix," *Geroscience*, vol. 39, no. 1, February 01, pp. 7-18.

[38] C.A. Meschiari, O.K. Ero, H. Pan, T. Finkel and M.L. Lindsey, '"The impact of aging on cardiac extracellular matrix," *Geroscience*, vol. 39, no. 1, February 01, pp. 7-18.

[39] D.J. Crossman, X. Shen, M. Jullig, M. Munro, Y. Hou, M. Middleditch, D. Shrestha, A. Li, S. Lal, C.G. Dos Remedios, D. Baddeley, P.N. Ruygrok and C. Soeller, '"Increased collagen within the transverse tubules in human heart failure," *Cardiovasc.Res.*, vol. 113, no. 8, July 01, pp. 879-891.

[40] E. Golia, G. Limongelli, F. Natale, F. Fimiani, V. Maddaloni, I. Pariggiano, R. Bianchi, M. Crisci, L. D'Acierno, R. Giordano, G. Di Palma, M. Conte, P. Golino, M.G. Russo, R. Calabro and P. Calabro, '"Inflammation and cardiovascular disease: from pathogenesis to therapeutic target," *Curr.Atheroscler.Rep.*, vol. 16, no. 9, September 01, pp. 435-z.

[41] P. Soysal, F. Arik, L. Smith, S.E. Jackson and A.T. Isik, '"Inflammation, Frailty and Cardiovascular Disease," *Adv.Exp.Med.Biol.*, vol. 1216, pp. 55-64.

[42] B.D. Johnson, K.E. Kip, O.C. Marroquin, P.M. Ridker, S.F. Kelsey, L.J. Shaw, C.J. Pepine, B. Sharaf, C.N. Bairey Merz, G. Sopko, M.B. Olson, S.E. Reis and L. National Heart, '"Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE)," *Circulation*, vol. 109, no. 6, February 17, pp. 726-732.

[43] M.B. Schulze, E.B. Rimm, T. Li, N. Rifai, M.J. Stampfer and F.B. Hu, '"C-reactive protein and incident cardiovascular events among men with diabetes," *Diabetes Care*, vol. 27, no. 4, April 01, pp. 889-894.

[44] P.M. Ridker, C.H. Hennekens, J.E. Buring and N. Rifai, '"C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women," *N.Engl.J.Med.*, vol. 342, no. 12, March 23, pp. 836-843.

[45] J. Schweizer, A. Bustamante, V. Lapierre-Fetaud, J. Faura, N. Scherrer, L. Azurmendi Gil, F. Fluri, V. Schutz, A. Luft, S. Boned, J.C. Sanchez, J. Montaner and M. Katan, '"SAA (Serum Amyloid A): A Novel Predictor of Stroke-Associated Infections," *Stroke*, vol. 51, no. 12, December 01, pp. 3523-3530.

[46] A. Biernacka, M. Dobaczewski and N.G. Frangogiannis, '"TGF-beta signaling in fibrosis," *Growth Factors*, vol. 29, no. 5, October 01, pp. 196-202.

[47] N. Frangogiannis, '"Transforming growth factor-beta in tissue fibrosis," *J.Exp.Med.*, vol. 217, no. 3, February 13, pp. e20190103.

[48] H. Khalil, O. Kanisicak, V. Prasad, R.N. Correll, X. Fu, T. Schips, R.J. Vagnozzi, R. Liu, T. Huynh, S.J. Lee, J. Karch and J.D. Molkentin, "'Fibroblast-specific TGF-beta-Smad2/3 signaling underlies cardiac fibrosis," *J.Clin.Invest.*, vol. 127, no. 10, October 02, pp. 3770-3783.

[49] Y. Wei, T.J. Kim, D.H. Peng, D. Duan, D.L. Gibbons, M. Yamauchi, J.R. Jackson, C.J. Le Saux, C. Calhoun, J. Peters, R. Derynck, B.J. Backes and H.A. Chapman, "'Fibroblast-specific inhibition of TGF-beta1 signaling attenuates lung and tumor fibrosis," *J.Clin.Invest.*, vol. 127, no. 10, October 02, pp. 3675-3688.

[50] G. Gabbiani, "'The role of contractile proteins in wound healing and fibrocontractive diseases," *Methods Achiev.Exp.Pathol.*, vol. 9, pp. 187-206.

[51] B. Hinz, "'Myofibroblasts," *Exp.Eye Res.*, vol. 142, January 01, pp. 56-70.

[52] K. Falholt, R. Cutfield, R. Alejandro, A. Volund, L.G. Heding and D.H. Mintz, "'Influence of portal delivery of insulin on intracellular glucose and lipid metabolism," *Metabolism*, vol. 40, no. 2, February 01, pp. 122-126.

[53] A.V. Shinde, C. Humeres and N.G. Frangogiannis, "'The role of alpha-smooth muscle actin in fibroblast-mediated matrix contraction and remodeling," *Biochim.Biophys.Acta Mol.Basis Dis.*, vol. 1863, no. 1, January 01, pp. 298-309.

[54] R.J. McAnulty, J.S. Campa, A.D. Cambrey and G.J. Laurent, "'The effect of transforming growth factor beta on rates of procollagen synthesis and degradation in vitro," *Biochim.Biophys.Acta*, vol. 1091, no. 2, January 31, pp. 231-235.

[55] R. Raghow, A.E. Postlethwaite, J. Keski-Oja, H.L. Moses and A.H. Kang, "'Transforming growth factor-beta increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts," *J.Clin.Invest.*, vol. 79, no. 4, April 01, pp. 1285-1288.

[56] A.M. Boak, R. Roy, J. Berk, L. Taylor, P. Polgar, R.H. Goldstein and H.M. Kagan, "'Regulation of lysyl oxidase expression in lung fibroblasts by transforming growth factor-beta 1 and prostaglandin E2," *Am.J.Respir.Cell Mol.Biol.*, vol. 11, no. 6, December 01, pp. 751-755.

[57] M. Okubo, R. Chiba, T. Karakida, H. Yamazaki, R. Yamamoto, S. Kobayashi, T. Niwa, H.C. Margolis, T. Nagano, Y. Yamakoshi and K. Gomi, "'Potential function of TGF-beta isoforms in maturation-stage ameloblasts," *J.Oral Biosci.*, vol. 61, no. 1, March 01, pp. 43-54.

[58] M. Lu, Q. Qin, J. Yao, L. Sun and X. Qin, "'Induction of LOX by TGF-beta1/Smad/AP-1 signaling aggravates rat myocardial fibrosis and heart failure," *IUBMB Life*, vol. 71, no. 11, November 01, pp. 1729-1739.

[59] M. Behnes, U. Hoffmann, S. Lang, C. Weiss, P. Ahmad-Nejad, M. Neumaier, M. Borggrefe and M. Brueckmann, "'Transforming growth factor beta 1 (TGF-beta 1) in atrial fibrillation and acute congestive heart failure," *Clin.Res.Cardiol.*, vol. 100, no. 4, April 01, pp. 335-342.

[60] M. Ma, K. Watanabe, M.I. Wahed, M. Inoue, T. Sekiguchi, T. Kouda, Y. Ohta, M. Nakazawa, Y. Yoshida, T. Yamamoto, H. Hanawa, M. Kodama, K. Fuse and Y. Aizawa, "'Inhibition of progression of heart failure and expression of TGF-beta 1 mRNA in rats with heart failure by the ACE inhibitor quinapril," *J.Cardiovasc.Pharmacol.*, vol. 38 Suppl 1, October 01, pp. 51.

[61] M. Lu, Q. Qin, J. Yao, L. Sun and X. Qin, "'Induction of LOX by TGF-beta1/Smad/AP-1 signaling aggravates rat myocardial fibrosis and heart failure," *IUBMB Life*, vol. 71, no. 11, November 01, pp. 1729-1739.

[62] S. Schafer, S. Viswanathan, A.A. Widjaja, W.W. Lim, A. Moreno-Moral, D.M. DeLaughter, B. Ng, G. Patone, K. Chow, E. Khin, J. Tan, S.P. Chothani, L. Ye, O.J.L. Rackham, N.S.J. Ko, N.E. Sahib, C.J. Pua, N.T.G. Zhen, C. Xie, M. Wang, H. Maatz, S. Lim, K. Saar, S. Blachut, E. Petretto, S. Schmidt, T. Putoczki, N. Guimaraes-Camboa, H. Wakimoto, S. van Heesch, K. Sigmundsson, S.L. Lim, J.L. Soon, V.T.T. Chao, Y.L. Chua, T.E. Tan, S.M. Evans, Y.J. Loh, M.H. Jamal, K.K. Ong, K.C. Chua, B.H. Ong, M.J. Chakaramakkil, J.G. Seidman, C.E. Seidman, N. Hubner, K.Y.K. Sin and S.A. Cook, "'IL-11 is a crucial determinant of cardiovascular fibrosis," *Nature*, vol. 552, no. 7683, December 07, pp. 110-115.

[63] M. Sun, M. Chen, F. Dawood, U. Zurawska, J.Y. Li, T. Parker, Z. Kassiri, L.A. Kirshenbaum, M. Arnold, R. Khokha and P.P. Liu, "'Tumor necrosis factor-alpha mediates cardiac remodeling and ventricular dysfunction after pressure overload state," *Circulation*, vol. 115, no. 11, March 20, pp. 1398-1407.

[64] M. Sun, F. Dawood, W.H. Wen, M. Chen, I. Dixon, L.A. Kirshenbaum and P.P. Liu, "'Excessive tumor necrosis factor activation after infarction contributes to susceptibility of myocardial rupture and left ventricular dysfunction," *Circulation*, vol. 110, no. 20, November 16, pp. 3221-3228.

[65] H. Wada, K. Saito, T. Kanda, I. Kobayashi, H. Fujii, S. Fujigaki, N. Maekawa, H. Takatsu, H. Fujiwara, K. Sekikawa and M. Seishima, "'Tumor necrosis factor-alpha (TNF-alpha) plays a protective role in acute viralmyocarditis in mice: A study using mice lacking TNF-alpha," *Circulation*, vol. 103, no. 5, February 06, pp. 743-749.

[66] T. Kubota, C.F. McTiernan, C.S. Frye, S.E. Slawson, B.H. Lemster, A.P. Koretsky, A.J. Demetris and A.M. Feldman, "'Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha," *Circ.Res.*, vol. 81, no. 4, October 01, pp. 627-635.

[67] K. Venkatachalam, B. Venkatesan, A.J. Valente, P.C. Melby, S. Nandish, J.E. Reusch, R.A. Clark and B. Chandrasekar, "'WISP1, a pro-mitogenic, pro-survival factor, mediates tumor necrosis factor-alpha (TNF-alpha)-stimulated cardiac fibroblast proliferation but inhibits TNF-alpha-induced cardiomyocyte death," *J.Biol.Chem.*, vol. 284, no. 21, May 22, pp. 14414-14427.

[68] M. Valgimigli, C. Ceconi, P. Malagutti, E. Merli, O. Soukhomovskaia, G. Francolini, G. Cicchitelli, A. Olivares, G. Parrinello, G. Percoco, G. Guardigli, D. Mele, R. Pirani and R. Ferrari, "'Tumor necrosis factor-alpha receptor 1 is a major predictor of mortality and new-onset heart failure in patients with acute myocardial infarction: the Cytokine-Activation and Long-Term Prognosis in Myocardial Infarction (C-ALPHA) study," *Circulation*, vol. 111, no. 7, February 22, pp. 863-870.

[69] K. Miao, L. Zhou, H. Ba, C. Li, H. Gu, B. Yin, J. Wang, X.P. Yang, Z. Li and D.W. Wang, "'Transmembrane tumor necrosis factor alpha attenuates pressure-overload cardiac hypertrophy via tumor necrosis factor receptor 2," *PLoS Biol.*, vol. 18, no. 12, December 03, pp. e3000967.

[70] L.M. Biasucci, G. Liuzzo, G. Fantuzzi, G. Caligiuri, A.G. Rebuzzi, F. Ginnetti, C.A. Dinarello and A. Maseri, "'Increasing levels of interleukin (IL)-1Ra and IL-6 during the first 2 days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events," *Circulation*, vol. 99, no. 16, April 27, pp. 2079-2084.

[71] H. Bronnum, T. Eskildsen, D.C. Andersen, M. Schneider and S.P. Sheikh, "'IL-1beta suppresses TGF-beta-mediated myofibroblast differentiation in cardiac fibroblasts," *Growth Factors*, vol. 31, no. 3, June 01, pp. 81-89.

[72] I. Ikonomidis, J.P. Lekakis, M. Nikolaou, I. Paraskevaidis, I. Andreadou, T. Kaplanoglou, P. Katsimbri, G. Skarantavos, P.N. Soucacos and D.T. Kremastinos, "'Inhibition of interleukin-1 by anakinra improves vascular and left ventricular function in patients with rheumatoid arthritis," *Circulation*, vol. 117, no. 20, May 20, pp. 2662-2669.

[73] M. Baghdadi, H. Endo, Y. Tanaka, H. Wada and K.I. Seino, "'Interleukin 34, from pathogenesis to clinical applications," *Cytokine*, vol. 99, November 01, pp. 139-147.

[74] S. Nandi, M. Cioce, Y.G. Yeung, E. Nieves, L. Tesfa, H. Lin, A.W. Hsu, R. Halenbeck, H.Y. Cheng, S. Gokhan, M.F. Mehler and E.R. Stanley, "'Receptor-type protein-tyrosine phosphatase zeta is a functional receptor for interleukin-34," *J.Biol.Chem.*, vol. 288, no. 30, July 26, pp. 21972-21986.

[75] A.I. Segaliny, R. Brion, E. Mortier, M. Maillasson, M. Cherel, Y. Jacques, B. Le Goff and D. Heymann, "'Syndecan-1 regulates the biological activities of interleukin-34," *Biochim.Biophys.Acta*, vol. 1853, no. 5, May 01, pp. 1010-1021.

[76] A.I. Segaliny, R. Brion, E. Mortier, M. Maillasson, M. Cherel, Y. Jacques, B. Le Goff and D. Heymann, "'Syndecan-1 regulates the biological activities of interleukin-34," *Biochim.Biophys.Acta*, vol. 1853, no. 5, May 01, pp. 1010-1021.

[77] K. Van Raemdonck, S. Umar, K. Palasiewicz, M.V. Volin, H.A. Elshabrawy, B. Romay, C. Tetali, A. Ahmed, M.A. Amin, R.K. Zomorrodi, N. Sweiss and S. Shahrara, "'IL-34 reprograms glycolytic and osteoclastic RA macrophages via Syndecan-1 and M-CSFR," *Arthritis Rheumatol.*, May 13.

[78] Z. Li, D. Jin, Y. Wu, K. Zhang, P. Hu, X. Cao and Z. Chen, "'Increased serum interleukin-34 in patients with coronary artery disease," *J.Int.Med.Res.*, vol. 40, no. 5, pp. 1866-1870.

[79] S. Nandi, S. Gokhan, X.M. Dai, S. Wei, G. Enikolopov, H. Lin, M.F. Mehler and E.R. Stanley, "'The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate neural progenitor cell maintenance and maturation," *Dev.Biol.*, vol. 367, no. 2, July 15, pp. 100-113.

[80] S.J. Moon, Y.S. Hong, J.H. Ju, S.K. Kwok, S.H. Park and J.K. Min, "'Increased levels of interleukin 34 in serum and synovial fluid are associated with rheumatoid factor and anticyclic citrullinated peptide antibody titers in patients with rheumatoid arthritis," *J.Rheumatol.*, vol. 40, no. 11, November 01, pp. 1842-1849.

[81] C. Guillonneau, S. Bezie and I. Anegon, "'Immunoregulatory properties of the cytokine IL-34," *Cell Mol.Life Sci.*, vol. 74, no. 14, July 01, pp. 2569-2586.

[82] V. Chitu, S. Gokhan, S. Nandi, M.F. Mehler and E.R. Stanley, "'Emerging Roles for CSF-1 Receptor and its Ligands in the Nervous System," *Trends Neurosci.*, vol. 39, no. 6, June 01, pp. 378-393.

[83] S. Boulakirba, A. Pfeifer, R. Mhaidly, S. Obba, M. Goulard, T. Schmitt, P. Chaintreuil, A. Calleja, N. Furstoss, F. Orange, S. Lacas-Gervais, L. Boyer, S. Marchetti, E. Verhoeyen, F. Luciano, G. Robert, P. Auberger and A. Jacquel, "'IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential," *Sci.Rep.*, vol. 8, no. 1, January 10, pp. 256-4.

[84] E.L. Masteller and B.R. Wong, "'Targeting IL-34 in chronic inflammation," *Drug Discov.Today*, vol. 19, no. 8, August 01, pp. 1212-1216.

[85] C. Guillonneau, S. Bezie and I. Anegon, "'Immunoregulatory properties of the cytokine IL-34," *Cell Mol.Life Sci.*, vol. 74, no. 14, July 01, pp. 2569-2586.

[86] M. Baud'huin, R. Renault, C. Charrier, A. Riet, A. Moreau, R. Brion, F. Gouin, L. Duplomb and D. Heymann, "'Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis," *J.Pathol.*, vol. 221, no. 1, May 01, pp. 77-86.

[87] L. Scheja and J. Heeren, "'The endocrine function of adipose tissues in health and cardiometabolic disease," *Nat.Rev.Endocrinol.*, vol. 15, no. 9, September 01, pp. 507-524.

[88] T. Becher, S. Palanisamy, D.J. Kramer, M. Eljalby, S.J. Marx, A.G. Wibmer, S.D. Butler, C.S. Jiang, R. Vaughan, H. Schoder, A. Mark and P. Cohen, "'Brown adipose tissue is associated with cardiometabolic health," *Nat.Med.*, vol. 27, no. 1, January 01, pp. 58-65.

[89] S. Bini, L. D'Erasmo, A. Di Costanzo, I. Minicocci, V. Pecce and M. Arca, "'The Interplay between Angiopoietin-Like Proteins and Adipose Tissue: Another Piece of the Relationship

between Adiposopathy and Cardiometabolic Diseases?" *Int.J.Mol.Sci.*, vol. 22, no. 2, January 13, pp. 10.3390/ijms22020742.

[90] J.A. Victorio and A.P. Davel, "'Perivascular Adipose Tissue Oxidative Stress on the Pathophysiology of Cardiometabolic Diseases," *Curr.Hypertens.Rev.*, vol. 16, no. 3, pp. 192-200.

[91] D.F. Sun, N. Kangaharan, B. Costello, S.J. Nicholls, C.A. Emdin, R. Tse, C. Gallagher, A. Kaur, K.C. Roberts-Thomson, R. Mahajan, D.H. Lau, P. Sanders and C.X. Wong, "'Epicardial and subcutaneous adipose tissue in Indigenous and non-Indigenous individuals: Implications for cardiometabolic diseases," *Obes.Res.Clin.Pract.*, vol. 14, no. 1, February 01, pp. 99-102.

[92] I.J. Neeland, R. Ross, J.P. Despres, Y. Matsuzawa, S. Yamashita, I. Shai, J. Seidell, P. Magni, R.D. Santos, B. Arsenault, A. Cuevas, F.B. Hu, B. Griffin, A. Zambon, P. Barter, J.C. Fruchart, R.H. Eckel, International Atherosclerosis Society and International Chair on Cardiometabolic Risk Working Group on Visceral Obesity, "'Visceral and ectopic fat, atherosclerosis, and cardiometabolic disease: a position statement," *Lancet Diabetes Endocrinol.*, vol. 7, no. 9, September 01, pp. 715-725.

[93] A.C. Villasante Fricke and G. Iacobellis, "'Epicardial Adipose Tissue: Clinical Biomarker of Cardio-Metabolic Risk," *Int.J.Mol.Sci.*, vol. 20, no. 23, November 28, pp. 10.3390/ijms20235989.

[94] C.K. Wu, H.Y. Tsai, M.M. Su, Y.F. Wu, J.J. Hwang, J.L. Lin, L.Y. Lin and J.J. Chen, "'Evolutional change in epicardial fat and its correlation with myocardial diffuse fibrosis in heart failure patients," *J.Clin.Lipidol.*, vol. 11, no. 6, December 01, pp. 1421-1431.

[95] R. Rafeh, A. Viveiros, G.Y. Oudit and A.F. El-Yazbi, "'Targeting perivascular and epicardial adipose tissue inflammation: therapeutic opportunities for cardiovascular disease," *Clin.Sci.(Lond)*, vol. 134, no. 7, April 17, pp. 827-851.

[96] T. Mazurek, L. Zhang, A. Zalewski, J.D. Mannion, J.T. Diehl, H. Arafat, L. Sarov-Blat, S. O'Brien, E.A. Keiper, A.G. Johnson, J. Martin, B.J. Goldstein and Y. Shi, "'Human epicardial adipose tissue is a source of inflammatory mediators," *Circulation*, vol. 108, no. 20, November 18, pp. 2460-2466.

[97] G. Iacobellis, "'Local and systemic effects of the multifaceted epicardial adipose tissue depot," *Nat.Rev.Endocrinol.*, vol. 11, no. 6, June 01, pp. 363-371.

[98] M. Zhou, H. Wang, J. Chen and L. Zhao, "'Epicardial adipose tissue and atrial fibrillation: Possible mechanisms, potential therapies, and future directions," *Pacing Clin.Electrophysiol.*, vol. 43, no. 1, January 01, pp. 133-145.

[99] G. Berg, V. Miksztowicz, C. Morales and M. Barchuk, "'Epicardial Adipose Tissue in Cardiovascular Disease," *Adv.Exp.Med.Biol.*, vol. 1127, pp. 131-143.

[100] Y. Song, F. Song, C. Wu, Y.X. Hong and G. Li, "'The roles of epicardial adipose tissue in heart failure," *Heart Fail.Rev.*, June 29.

[101] G. Iacobellis, "'Epicardial adipose tissue in endocrine and metabolic diseases," *Endocrine*, vol. 46, no. 1, May 01, pp. 8-15.

[102] L. Mach, H. Bedanova, M. Soucek, M. Karpisek, T. Konecny, P. Nemec and M. Orban, "'Impact of cardiopulmonary bypass surgery on cytokines in epicardial adipose tissue: comparison with subcutaneous fat," *Perfusion*, vol. 32, no. 4, May 01, pp. 279-284.

[103] V.B. Patel, S. Shah, S. Verma and G.Y. Oudit, "'Epicardial adipose tissue as a metabolic transducer: role in heart failure and coronary artery disease," *Heart Fail.Rev.*, vol. 22, no. 6, November 01, pp. 889-902.

[104] T. Kataoka, K. Harada, A. Tanaka, T. Onishi, S. Matsunaga, H. Funakubo, K. Harada, T. Nagao, N. Shinoda, N. Marui, K. Niwa, H. Tashiro, Y. Hitora, K. Furusawa, H. Ishii, T. Amano and T. Murohara, "'Relationship between epicardial adipose tissue volume and coronary artery spasm," *Int.J.Cardiol.*, vol. 324, February 01, pp. 8-12.

[105] G. Conceicao, D. Martins, I. M Miranda, A.F. Leite-Moreira, R. Vitorino and I. Falcao-Pires, "'Unraveling the Role of Epicardial Adipose Tissue in Coronary Artery Disease: Partners in Crime?" *Int.J.Mol.Sci.*, vol. 21, no. 22, November 23, pp. 10.3390/ijms21228866.

[106] A.A. Keresztesi, G. Asofie, M.A. Simion and H. Jung, "'Correlation between epicardial adipose tissue thickness and the degree of coronary artery atherosclerosis," *Turk.J.Med.Sci.*, vol. 48, no. 1, February 23, pp. 40-45.

[107] T. Mazurek, L. Zhang, A. Zalewski, J.D. Mannion, J.T. Diehl, H. Arafat, L. Sarov-Blat, S. O'Brien, E.A. Keiper, A.G. Johnson, J. Martin, B.J. Goldstein and Y. Shi, "'Human epicardial adipose tissue is a source of inflammatory mediators," *Circulation*, vol. 108, no. 20, November 18, pp. 2460-2466.

[108] L. Cerit, "'Epicardial adipose tissue and cardiometabolic risk," *Clin.Nutr.*, vol. 36, no. 5, October 01, pp. 1452.

[109] S. Eiras, E. Teijeira-Fernandez, L.G. Shamagian, A.L. Fernandez, A. Vazquez-Boquete and J.R. Gonzalez-Juanatey, "'Extension of coronary artery disease is associated with increased IL-6 and decreased adiponectin gene expression in epicardial adipose tissue," *Cytokine*, vol. 43, no. 2, August 01, pp. 174-180.

[110] M. Packer, "'Epicardial Adipose Tissue May Mediate Deleterious Effects of Obesity and Inflammation on the Myocardium," *J.Am.Coll.Cardiol.*, vol. 71, no. 20, May 22, pp. 2360-2372.

[111] N. Gonzalez, Z. Moreno-Villegas, A. Gonzalez-Bris, J. Egido and O. Lorenzo, "'Regulation of visceral and epicardial adipose tissue for preventing cardiovascular injuries associated to obesity and diabetes," *Cardiovasc.Diabetol.*, vol. 16, no. 1, April 04, pp. 44-4.

[112] R.H. Christensen, B.J. von Scholten, C.S. Hansen, M.T. Jensen, T. Vilsboll, P. Rossing and P.G. Jorgensen, "'Epicardial adipose tissue predicts incident cardiovascular disease and mortality in patients with type 2 diabetes," *Cardiovasc.Diabetol.*, vol. 18, no. 1, August 30, pp. 114-y.

[113] R.H. Christensen, B.J. von Scholten, L.L. Lehrskov, P. Rossing and P.G. Jorgensen, "'Epicardial adipose tissue: an emerging biomarker of cardiovascular complications in type 2 diabetes?" *Ther.Adv.Endocrinol.Metab.*, vol. 11, May 25, pp. 2042018820928824.

[114] C.J. Nalliah, J.R. Bell, A.J.A. Raaijmakers, H.M. Waddell, S.P. Wells, G.B. Bernasochi, M.K. Montgomery, S. Binny, T. Watts, S.B. Joshi, E. Lui, C.B. Sim, M. Larobina, M. O'Keefe, J. Goldblatt, A. Royse, G. Lee, E.R. Porrello, M.J. Watt, P.M. Kistler, P. Sanders, L.M.D. Delbridge and J.M. Kalman, "'Epicardial Adipose Tissue Accumulation Confers Atrial Conduction Abnormality," *J.Am.Coll.Cardiol.*, vol. 76, no. 10, September 08, pp. 1197-1211.

[115] D.H. Lau, D. Linz and P. Sanders, "'New Findings in Atrial Fibrillation Mechanisms," *Card.Electrophysiol.Clin.*, vol. 11, no. 4, December 01, pp. 563-571.

[116] M. Packer, "'Disease-treatment interactions in the management of patients with obesity and diabetes who have atrial fibrillation: the potential mediating influence of epicardial adipose tissue," *Cardiovasc.Diabetol.*, vol. 18, no. 1, September 24, pp. 121-9.

[117] M. Packer, "'Epicardial Adipose Tissue May Mediate Deleterious Effects of Obesity and Inflammation on the Myocardium," *J.Am.Coll.Cardiol.*, vol. 71, no. 20, May 22, pp. 2360-2372.

[118] M. Zhou, H. Wang, J. Chen and L. Zhao, "'Epicardial adipose tissue and atrial fibrillation: Possible mechanisms, potential therapies, and future directions," *Pacing Clin.Electrophysiol.*, vol. 43, no. 1, January 01, pp. 133-145.

[119] G. Iacobellis, "'Local and systemic effects of the multifaceted epicardial adipose tissue depot," *Nat.Rev.Endocrinol.*, vol. 11, no. 6, June 01, pp. 363-371.

[120] O. Gruzdeva, E. Uchasova, Y. Dyleva, D. Borodkina, O. Akbasheva, E. Belik, V. Karetnikova, N. Brel, A. Kokov, V. Kashtalap and O. Barbarash, "'Relationships between epicardial adipose tissue thickness and adipo-fibrokine indicator profiles post-myocardial infarction," *Cardiovasc.Diabetol.*, vol. 17, no. 1, March 16, pp. 40-y.

[121] C. Colom, D. Vilades, M. Perez-Cuellar, R. Leta, A. Rivas-Urbina, G. Carreras, J. Ordonez-Llanos, A. Perez and J.L. Sanchez-Quesada, "'Associations between epicardial adipose tissue, subclinical atherosclerosis and high-density lipoprotein composition in type 1 diabetes," *Cardiovasc.Diabetol.*, vol. 17, no. 1, December 07, pp. 156-9.

[122] N. Ei Ei Khaing, T.E. Shyong, J. Lee, C.Y. Soekojo, A. Ng and R.M. Van Dam, '"Epicardial and visceral adipose tissue in relation to subclinical atherosclerosis in a Chinese population," *PLoS One*, vol. 13, no. 4, April 25, pp. e0196328.

[123] N.R. Aeddula, W. Cheungpasitporn, C. Thongprayoon and S. Pathireddy, '"Epicardial Adipose Tissue and Renal Disease," *J.Clin.Med.*, vol. 8, no. 3, March 02, pp. 10.3390/jcm8030299.

[124] I.J. Neeland, R. Ross, J.P. Despres, Y. Matsuzawa, S. Yamashita, I. Shai, J. Seidell, P. Magni, R.D. Santos, B. Arsenault, A. Cuevas, F.B. Hu, B. Griffin, A. Zambon, P. Barter, J.C. Fruchart, R.H. Eckel, International Atherosclerosis Society and International Chair on Cardiometabolic Risk Working Group on Visceral Obesity, '"Visceral and ectopic fat, atherosclerosis, and cardiometabolic disease: a position statement," *Lancet Diabetes Endocrinol.*, vol. 7, no. 9, September 01, pp. 715-725.

[125] A.B. Goldfine and S.E. Shoelson, '"Therapeutic approaches targeting inflammation for diabetes and associated cardiovascular risk," *J.Clin.Invest.*, vol. 127, no. 1, January 03, pp. 83-93.

[126] G.S. Hotamisligil, '"Inflammation and metabolic disorders," *Nature*, vol. 444, no. 7121, December 14, pp. 860-867.

[127] W. Song and A. Ergul, '"Type-2 diabetes-induced changes in vascular extracellular matrix gene expression: relation to vessel size," *Cardiovasc.Diabetol.*, vol. 5, February 17, pp. 3-3.

[128] G. Lowe, M. Woodward, G. Hillis, A. Rumley, Q. Li, S. Harrap, M. Marre, P. Hamet, A. Patel, N. Poulter and J. Chalmers, '"Circulating inflammatory markers and the risk of vascular complications and mortality in people with type 2 diabetes and cardiovascular disease or risk factors: the ADVANCE study," *Diabetes*, vol. 63, no. 3, March 01, pp. 1115-1123.

[129] A. Brahimaj, S. Ligthart, M. Ghanbari, M.A. Ikram, A. Hofman, O.H. Franco, M. Kavousi and A. Dehghan, '"Novel inflammatory markers for incident pre-diabetes and type 2 diabetes: the Rotterdam Study," *Eur.J.Epidemiol.*, vol. 32, no. 3, March 01, pp. 217-226.

[130] L. Pretorius, G.J.A. Thomson, R.C.M. Adams, T.A. Nell, W.A. Laubscher and E. Pretorius, '"Platelet activity and hypercoagulation in type 2 diabetes," *Cardiovasc.Diabetol.*, vol. 17, no. 1, November 02, pp. 141-z.

[131] H.C. Karantonis, E. Fragopoulou, S. Antonopoulou, J. Rementzis, C. Phenekos and C.A. Demopoulos, '"Effect of fast-food Mediterranean-type diet on type 2 diabetics and healthy human subjects' platelet aggregation," *Diabetes Res.Clin.Pract.*, vol. 72, no. 1, April 01, pp. 33-41.

[132] K. Karstoft and B.K. Pedersen, '"Exercise and type 2 diabetes: focus on metabolism and inflammation," *Immunol.Cell Biol.*, vol. 94, no. 2, February 01, pp. 146-150.

[133] C. Liu, X. Feng, Q. Li, Y. Wang, Q. Li and M. Hua, "'Adiponectin, TNF-alpha and inflammatory cytokines and risk of type 2 diabetes: A systematic review and meta-analysis," *Cytokine*, vol. 86, October 01, pp. 100-109.

[134] M.N. Atalar, S. Abusoglu, A. Unlu, O. Tok, S.H. Ipekci, S. Baldane and L. Kebapcilar, "'Assessment of serum galectin-3, methylated arginine and Hs-CRP levels in type 2 diabetes and prediabetes," *Life Sci.*, vol. 231, August 15, pp. 116577.

[135] M. Akbari and V. Hassan-Zadeh, "'IL-6 signalling pathways and the development of type 2 diabetes," *Inflammopharmacology*, vol. 26, no. 3, June 01, pp. 685-698.

[136] K. Rehman, M.S.H. Akash, A. Liaqat, S. Kamal, M.I. Qadir and A. Rasul, "'Role of Interleukin-6 in Development of Insulin Resistance and Type 2 Diabetes Mellitus," *Crit.Rev.Eukaryot.Gene Expr.*, vol. 27, no. 3, pp. 229-236.

[137] G.H. Schernthaner and H. Stangl, "'Reduced adiponectin receptor signalling accelerates atherosclerosis and may worsen the outcome in type 2 diabetes mellitus - another one of those missing links?" *Atherosclerosis*, vol. 229, no. 1, July 01, pp. 30-31.

[138] B.B. Duncan, M.I. Schmidt, J.S. Pankow, H. Bang, D. Couper, C.M. Ballantyne, R.C. Hoogeveen and G. Heiss, "'Adiponectin and the development of type 2 diabetes: the atherosclerosis risk in communities study," *Diabetes*, vol. 53, no. 9, September 01, pp. 2473-2478.

[139] A.E. Achari and S.K. Jain, "'Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction," *Int.J.Mol.Sci.*, vol. 18, no. 6, June 21, pp. 10.3390/ijms18061321.

[140] N. Katsiki, C. Mantzoros and D.P. Mikhailidis, "'Adiponectin, lipids and atherosclerosis," *Curr.Opin.Lipidol.*, vol. 28, no. 4, August 01, pp. 347-354.

[141] R.J. Henning, "'Type-2 diabetes mellitus and cardiovascular disease," *Future Cardiol.*, vol. 14, no. 6, November 01, pp. 491-509.

[142] A. Pandey, S. Chawla and P. Guchhait, "'Type-2 diabetes: Current understanding and future perspectives," *IUBMB Life*, vol. 67, no. 7, July 01, pp. 506-513.

[143] S. Carbone, J.H. O'Keefe and C.J. Lavie, "'SGLT2 Inhibition, Visceral Adiposity, Weight, and Type 2 Diabetes Mellitus," *Obesity (Silver Spring)*, vol. 28, no. 7, July 01, pp. 1173.

[144] P. Lopez-Jaramillo, D. Gomez-Arbelaez, J. Lopez-Lopez, C. Lopez-Lopez, J. Martinez-Ortega, A. Gomez-Rodriguez and S. Triana-Cubillos, "'The role of leptin/adiponectin ratio in metabolic syndrome and diabetes," *Horm.Mol.Biol.Clin.Investig.*, vol. 18, no. 1, April 01, pp. 37-45.

[145] M.D. Siperstein, R.H. Unger and L.L. Madison, "'Studies of muscle capillary basement membranes in normal subjects, diabetic, and prediabetic patients," *J.Clin.Invest.*, vol. 47, no. 9, September 01, pp. 1973-1999.

[146] K.R. Feingold, W.S. Browner and M.D. Siperstein, "'Prospective studies of muscle capillary basement membrane width in prediabetics," *J.Clin.Endocrinol.Metab.*, vol. 69, no. 4, October 01, pp. 784-789.

[147] J.H. Liu, Y. Chen, Z. Zhen, L.M. Ho, A. Tsang, M. Yuen, K. Lam, H.F. Tse and K.H. Yiu, "'Relationship of biomarkers of extracellular matrix with myocardial function in Type 2 diabetes mellitus," *Biomark Med.*, vol. 11, no. 7, July 01, pp. 569-578.

[148] M. Konieczynska, A.H. Bryk, K.P. Malinowski, K. Draga and A. Undas, "'Interplay between elevated cellular fibronectin and plasma fibrin clot properties in type 2 diabetes," *Thromb.Haemost.*, vol. 117, no. 9, August 30, pp. 1671-1678.

[149] M.R. Hayden, J.R. Sowers and S.C. Tyagi, "'The central role of vascular extracellular matrix and basement membrane remodeling in metabolic syndrome and type 2 diabetes: the matrix preloaded," *Cardiovasc.Diabetol.*, vol. 4, June 28, pp. 9-9.

[150] X. Wang, Y. Lu, Y. Xie, J. Shen and M. Xiang, "'Emerging roles of proteoglycans in cardiac remodeling," *Int.J.Cardiol.*, vol. 278, March 01, pp. 192-198.

[151] C. Bambace, A. Sepe, E. Zoico, M. Telesca, D. Olioso, S. Venturi, A. Rossi, F. Corzato, S. Faccioli, L. Cominacini, F. Santini and M. Zamboni, "'Inflammatory profile in subcutaneous and epicardial adipose tissue in men with and without diabetes," *Heart Vessels*, vol. 29, no. 1, January 01, pp. 42-48.

[152] Y.H. Lai, C.H. Yun, F.S. Yang, C.C. Liu, Y.J. Wu, J.Y. Kuo, H.I. Yeh, T.Y. Lin, H.G. Bezerra, S.C. Shih, C.H. Tsai and C.L. Hung, "'Epicardial adipose tissue relating to anthropometrics, metabolic derangements and fatty liver disease independently contributes to serum high-sensitivity C-reactive protein beyond body fat composition: a study validated with computed tomography," *J.Am.Soc.Echocardiogr.*, vol. 25, no. 2, February 01, pp. 234-241.

[153] D.M. Ouwens, H. Sell, S. Greulich and J. Eckel, "'The role of epicardial and perivascular adipose tissue in the pathophysiology of cardiovascular disease," *J.Cell.Mol.Med.*, vol. 14, no. 9, September 01, pp. 2223-2234.

[154] U. Smith and B.B. Kahn, "'Adipose tissue regulates insulin sensitivity: role of adipogenesis, de novo lipogenesis and novel lipids," *J.Intern.Med.*, vol. 280, no. 5, November 01, pp. 465-475.

[155] T. Watanabe, K. Watanabe-Kominato, Y. Takahashi, M. Kojima and R. Watanabe, "'Adipose Tissue-Derived Omentin-1 Function and Regulation," *Compr.Physiol.*, vol. 7, no. 3, June 18, pp. 765-781.

[156] D.K. Song, Y.S. Hong, H. Lee, J.Y. Oh, Y.A. Sung and Y. Kim, "'Increased Epicardial Adipose Tissue Thickness in Type 2 Diabetes Mellitus and Obesity," *Diabetes Metab.J.*, vol. 39, no. 5, October 01, pp. 405-413.

[157] H. Chun, E. Suh, A.R. Byun, H.R. Park and K.W. Shim, "'Epicardial fat thickness is associated to type 2 diabetes mellitus in Korean men: a cross-sectional study," *Cardiovasc.Diabetol.*, vol. 14, May 03, pp. 46-7.

[158] T. Mazurek, L. Zhang, A. Zalewski, J.D. Mannion, J.T. Diehl, H. Arafat, L. Sarov-Blat, S. O'Brien, E.A. Keiper, A.G. Johnson, J. Martin, B.J. Goldstein and Y. Shi, "'Human epicardial adipose tissue is a source of inflammatory mediators," *Circulation*, vol. 108, no. 20, November 18, pp. 2460-2466.

[159] I. Abe, Y. Teshima, H. Kondo, H. Kaku, S. Kira, Y. Ikebe, S. Saito, A. Fukui, T. Shinohara, K. Yufu, M. Nakagawa, N. Hijiya, M. Moriyama, T. Shimada, S. Miyamoto and N. Takahashi, "'Association of fibrotic remodeling and cytokines/chemokines content in epicardial adipose tissue with atrial myocardial fibrosis in patients with atrial fibrillation," *Heart Rhythm*, June 13.

[160] E. Bassat, Y.E. Mutlak, A. Genzelinakh, I.Y. Shadrin, K. Baruch Umansky, O. Yifa, D. Kain, D. Rajchman, J. Leach, D. Riabov Bassat, Y. Udi, R. Sarig, I. Sagi, J.F. Martin, N. Bursac, S. Cohen and E. Tzahor, "'The extracellular matrix protein agrin promotes heart regeneration in mice," *Nature*, vol. 547, no. 7662, July 13, pp. 179-184.

[161] H. Tao, P. Shi, H.Y. Xuan and X.S. Ding, "'DNA methyltransferase-1 inactivation of androgen receptor axis triggers homocysteine induced cardiac fibroblast autophagy in diabetic cardiac fibrosis," *Arch.Biochem.Biophys.*, vol. 692, October 15, pp. 108521.

[162] N. Cho, S.E. Razipour and M.L. McCain, "'Featured Article: TGF-beta1 dominates extracellular matrix rigidity for inducing differentiation of human cardiac fibroblasts to myofibroblasts," *Exp.Biol.Med.(Maywood)*, vol. 243, no. 7, April 01, pp. 601-612.

[163] K. Lin, J. Ma, Y. Peng, M. Sun, K. Xu, R. Wu and J. Lin, "'Autocrine Production of Interleukin-34 Promotes the Development of Endometriosis through CSF1R/JAK3/STAT6 signaling," *Sci.Rep.*, vol. 9, no. 1, November 14, pp. 16781-1.

[164] I. Gueorguieva, A.L. Cleverly, A. Stauber, N. Sada Pillay, J.A. Rodon, C.P. Miles, J.M. Yingling and M.M. Lahn, "'Defining a therapeutic window for the novel TGF-beta inhibitor LY2157299 monohydrate based on a pharmacokinetic/pharmacodynamic model," *Br.J.Clin.Pharmacol.*, vol. 77, no. 5, May 01, pp. 796-807.

[165] J.K. Ichida, J. Blanchard, K. Lam, E.Y. Son, J.E. Chung, D. Egli, K.M. Loh, A.C. Carter, F.P. Di Giorgio, K. Koszka, D. Huangfu, H. Akutsu, D.R. Liu, L.L. Rubin and K. Eggan, "'A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog," *Cell.Stem Cell.*, vol. 5, no. 5, November 06, pp. 491-503.

[166] J.K. Ichida, J. Blanchard, K. Lam, E.Y. Son, J.E. Chung, D. Egli, K.M. Loh, A.C. Carter, F.P. Di Giorgio, K. Koszka, D. Huangfu, H. Akutsu, D.R. Liu, L.L. Rubin and K. Eggan, "'A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog," *Cell.Stem Cell.*, vol. 5, no. 5, November 06, pp. 491-503.

[167] F. Gellibert, J. Woolven, M.H. Fouchet, N. Mathews, H. Goodland, V. Lovegrove, A. Laroze, V.L. Nguyen, S. Sautet, R. Wang, C. Janson, W. Smith, G. Krysa, V. Boullay, A.C. De Gouville, S. Huet and D. Hartley, "'Identification of 1,5-naphthyridine derivatives as a novel series of potent and selective TGF-beta type I receptor inhibitors," *J.Med.Chem.*, vol. 47, no. 18, August 26, pp. 4494-4506.

[168] Z. Xu, Y. Rao, Y. Huang, T. Zhou, R. Feng, S. Xiong, T.F. Yuan, S. Qin, Y. Lu, X. Zhou, X. Li, B. Qin, Y. Mao and B. Peng, "'Efficient Strategies for Microglia Replacement in the Central Nervous System," *Cell.Rep.*, vol. 32, no. 6, August 11, pp. 108041.

[169] S. Lee, X.Q. Shi, A. Fan, B. West and J. Zhang, "'Targeting macrophage and microglia activation with colony stimulating factor 1 receptor inhibitor is an effective strategy to treat injury-triggered neuropathic pain," *Mol.Pain*, vol. 14, December 01, pp. 1744806918764979.

[170] E. Spangenberg, P.L. Severson, L.A. Hohsfield, J. Crapser, J. Zhang, E.A. Burton, Y. Zhang, W. Spevak, J. Lin, N.Y. Phan, G. Habets, A. Rymar, G. Tsang, J. Walters, M. Nespi, P. Singh, S. Broome, P. Ibrahim, C. Zhang, G. Bollag, B.L. West and K.N. Green, "'Sustained microglial depletion with CSF1R inhibitor impairs parenchymal plaque development in an Alzheimer's disease model," *Nat.Commun.*, vol. 10, no. 1, August 21, pp. 3758-z.

[171] B. Lloyd-Lewis, F.M. Davis, O.B. Harris, J.R. Hitchcock, F.C. Lourenco, M. Pasche and C.J. Watson, "'Imaging the mammary gland and mammary tumours in 3D: optical tissue clearing and immunofluorescence methods," *Breast Cancer Res.*, vol. 18, no. 1, December 13, pp. 127-9.

[172] W.B. Kannel and D.L. McGee, "'Diabetes and cardiovascular disease. The Framingham study," *JAMA*, vol. 241, no. 19, May 11, pp. 2035-2038.

[173] N.G. Frangogiannis, "'Cardiac fibrosis: Cell biological mechanisms, molecular pathways and therapeutic opportunities," *Mol.Aspects Med.*, vol. 65, February 01, pp. 70-99.

[174] I. Russo and N.G. Frangogiannis, "'Diabetes-associated cardiac fibrosis: Cellular effectors, molecular mechanisms and therapeutic opportunities," *J.Mol.Cell.Cardiol.*, vol. 90, January 01, pp. 84-93.

[175] S. Nattel, Burstein U - Dobrev, Dobromir and D. Dobrev, "'Atrial remodeling and atrial fibrillation: mechanisms and implications," *Circ.Arrhythm Electrophysiol.*, vol. 1, no. 1, April 01, pp. 62-73.

[176] B. Burstein and S. Nattel, "'Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation," *J.Am.Coll.Cardiol.*, vol. 51, no. 8, February 26, pp. 802-809.

[177] R.R. Lamberts, S.J. Lingam, H.Y. Wang, I.A. Bollen, G. Hughes, I.F. Galvin, R.W. Bunton, A. Bahn, R. Katare, J.C. Baldi, M.J. Williams, P. Saxena, S. Coffey and P.P. Jones, '"Impaired relaxation despite upregulated calcium-handling protein atrial myocardium from type 2 diabetic patients with preserved ejection fraction," *Cardiovasc.Diabetol.*, vol. 13, April 05, pp. 72-72.

[178] T.A. Wynn, '"Cellular and molecular mechanisms of fibrosis," *J.Pathol.*, vol. 214, no. 2, January 01, pp. 199-210.

[179] A. Schmitt-Graff, A. Desmouliere and G. Gabbiani, '"Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity," *Virchows Arch.*, vol. 425, no. 1, pp. 3-24.

[180] A. Hanna and N.G. Frangogiannis, '"The Role of the TGF-beta Superfamily in Myocardial Infarction," *Front.Cardiovasc.Med.*, vol. 6, September 18, pp. 140.

[181] C.J. Roberts, T.M. Birkenmeier, J.J. McQuillan, S.K. Akiyama, S.S. Yamada, W.T. Chen, K.M. Yamada and J.A. McDonald, '"Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts," *J.Biol.Chem.*, vol. 263, no. 10, April 05, pp. 4586-4592.

[182] S. Rosenkranz, M. Flesch, K. Amann, C. Haeuseler, H. Kilter, U. Seeland, K.D. Schluter and M. Bohm, '"Alterations of beta-adrenergic signaling and cardiac hypertrophy in transgenic mice overexpressing TGF-beta(1)," *Am.J.Physiol.Heart Circ.Physiol.*, vol. 283, no. 3, September 01, pp. 1253.

[183] N.G. Frangogiannis, '"Cardiac fibrosis," *Cardiovasc.Res.*, vol. 117, no. 6, May 25, pp. 1450-1488.

[184] R. Li and N.G. Frangogiannis, '"Chemokines in cardiac fibrosis," *Curr.Opin.Physiol.*, vol. 19, February 01, pp. 80-91.

[185] N. Venteclef, V. Guglielmi, E. Balse, B. Gaborit, A. Cotillard, F. Atassi, J. Amour, P. Leprince, A. Dutour, K. Clement and S.N. Hatem, '"Human epicardial adipose tissue induces fibrosis of the atrial myocardium through the secretion of adipo-fibrokines," *Eur.Heart J.*, vol. 36, no. 13, April 01, pp. 795-805a.

[186] P. Haemers, H. Hamdi, K. Guedj, N. Suffee, P. Farahmand, N. Popovic, P. Claus, P. LePrince, A. Nicoletti, J. Jalife, C. Wolke, U. Lendeckel, P. Jais, R. Willems and S.N. Hatem, '"Atrial fibrillation is associated with the fibrotic remodelling of adipose tissue in the subepicardium of human and sheep atria," *Eur.Heart J.*, vol. 38, no. 1, January 01, pp. 53-61.

[187] S. Greulich, B. Maxhera, G. Vandenplas, D.H. de Wiza, K. Smiris, H. Mueller, J. Heinrichs, M. Blumensatt, C. Cuvelier, P. Akhyari, J.B. Ruige, D.M. Ouwens and J. Eckel, '"Secretory products from epicardial adipose tissue of patients with type 2 diabetes mellitus induce cardiomyocyte dysfunction," *Circulation*, vol. 126, no. 19, November 06, pp. 2324-2334.

[188] H. Lin, E. Lee, K. Hestir, C. Leo, M. Huang, E. Bosch, R. Halenbeck, G. Wu, A. Zhou, D. Behrens, D. Hollenbaugh, T. Linnemann, M. Qin, J. Wong, K. Chu, S.K. Doberstein and L.T. Williams, "'Discovery of a cytokine and its receptor by functional screening of the extracellular proteome," *Science*, vol. 320, no. 5877, May 09, pp. 807-811.

[189] H. Shoji, S. Yoshio, Y. Mano, E. Kumagai, M. Sugiyama, M. Korenaga, T. Arai, N. Itokawa, M. Atsukawa, H. Aikata, H. Hyogo, K. Chayama, T. Ohashi, K. Ito, M. Yoneda, Y. Nozaki, T. Kawaguchi, T. Torimura, M. Abe, Y. Hiasa, M. Fukai, T. Kamiyama, A. Taketomi, M. Mizokami and T. Kanto, "'Interleukin-34 as a fibroblast-derived marker of liver fibrosis in patients with non-alcoholic fatty liver disease," *Sci.Rep.*, vol. 6, July 01, pp. 28814.

[190] A. Kuzumi, A. Yoshizaki, S. Toyama, T. Fukasawa, S. Ebata, K. Nakamura, T. Yamashita, R. Saigusa, S. Miura, M. Hirabayashi, A. Yoshizaki, Y. Asano and S. Sato, "'Serum interleukin-34 levels in patients with systemic sclerosis: Clinical association with interstitial lung disease," *J.Dermatol.*, vol. 45, no. 10, October 01, pp. 1216-1220.

[191] R. Tao, Q. Fan, H. Zhang, H. Xie, L. Lu, G. Gu, F. Wang, R. Xi, J. Hu, Q. Chen, W. Niu, W. Shen, R. Zhang and X. Yan, "'Prognostic Significance of Interleukin-34 (IL-34) in Patients With Chronic Heart Failure With or Without Renal Insufficiency," *J.Am.Heart Assoc.*, vol. 6, no. 4, April 01, pp. 10.1161/JAHA.116.004911.

[192] G. Muller-Newen, C. Kohne, R. Keul, U. Hemmann, W. Muller-Esterl, J. Wijdenes, J.P. Brakenhoff, M.H. Hart and P.C. Heinrich, "'Purification and characterization of the soluble interleukin-6 receptor from human plasma and identification of an isoform generated through alternative splicing," *Eur.J.Biochem.*, vol. 236, no. 3, March 15, pp. 837-842.

[193] C. Ancey, P. Corbi, J. Froger, A. Delwail, J. Wijdenes, H. Gascan, D. Potreau and J.C. Lecron, "'Secretion of IL-6, IL-11 and LIF by human cardiomyocytes in primary culture," *Cytokine*, vol. 18, no. 4, May 21, pp. 199-205.

[194] Z. Dong, X. Zhao, W. Tai, W. Lei, Y. Wang, Z. Li and T. Zhang, "'IL-27 attenuates the TGF-beta1-induced proliferation, differentiation and collagen synthesis in lung fibroblasts," *Life Sci.*, vol. 146, February 01, pp. 24-33.

[195] G.M. Coppock, L.R. Aronson, J. Park, C. Qiu, J. Park, J.H. DeLong, E. Radaelli, K. Susztak and C.A. Hunter, "'Loss of IL-27Ralpha Results in Enhanced Tubulointerstitial Fibrosis Associated with Elevated Th17 Responses," *J.Immunol.*, vol. 205, no. 2, July 15, pp. 377-386.

[196] S. Xu, J. Zhang, J. Liu, J. Ye, Y. Xu, Z. Wang, J. Yu, D. Ye, M. Zhao, Y. Feng, W. Pan, M. Wang and J. Wan, "'The role of interleukin-10 family members in cardiovascular diseases," *Int.Immunopharmacol.*, vol. 94, May 01, pp. 107475.

[197] K.L. Tsai, W.C. Chou, H.C. Cheng, Y.T. Huang, M.S. Chang and S.H. Chan, "'Anti-IL-20 Antibody Protects against Ischemia/Reperfusion-Impaired Myocardial Function through

Modulation of Oxidative Injuries, Inflammation and Cardiac Remodeling," *Antioxidants (Basel)*, vol. 10, no. 2, February 10, pp. 10.3390/antiox10020275.

[198] Y. Wu, L. Tan, L. Shi, Z. Yang, Y. Xue, T. Zeng, Y. Shi, Y. Lin and L. Liu, "'Interleukin-22 is elevated in the atrium and plasma of patients with atrial fibrillation and increases collagen synthesis in transforming growth factor-beta1-treated cardiac fibroblasts via the JNK pathway," *Exp.Ther.Med.*, vol. 20, no. 2, August 01, pp. 1012-1020.

[199] Y.L. Han, Y.L. Li, L.X. Jia, J.Z. Cheng, Y.F. Qi, H.J. Zhang and J. Du, "'Reciprocal interaction between macrophages and T cells stimulates IFN-gamma and MCP-1 production in Ang II-induced cardiac inflammation and fibrosis," *PLoS One*, vol. 7, no. 5, pp. e35506.

[200] M.F. Desentis-Desentis, J.D. Rivas-Carrillo and S. Sanchez-Enriquez, "'Protective role of osteocalcin in diabetes pathogenesis," *J.Bone Miner.Metab.*, vol. 38, no. 6, November 01, pp. 765-771.

[201] S.L. Rowland, K.F. Leahy, R. Halverson, R.M. Torres and R. Pelanda, "'BAFF receptor signaling aids the differentiation of immature B cells into transitional B cells following tonic BCR signaling," *J.Immunol.*, vol. 185, no. 8, October 15, pp. 4570-4581.

[202] M. Yarchoan, W.J. Ho, A. Mohan, Y. Shah, T. Vithayathil, J. Leatherman, L. Dennison, N. Zaidi, S. Ganguly, S. Woolman, K. Cruz, T.D. Armstrong and E.M. Jaffee, "'Effects of B cell-activating factor on tumor immunity," *JCI Insight*, vol. 5, no. 10, May 21, pp. 10.1172/jci.insight.136417.

[203] Y. Nakamichi, N. Udagawa and N. Takahashi, "'IL-34 and CSF-1: similarities and differences," *J.Bone Miner.Metab.*, vol. 31, no. 5, September 01, pp. 486-495.

[204] K. Zorena, O. Jachimowicz-Duda and P. Waz, "'The cut-off value for interleukin 34 as an additional potential inflammatory biomarker for the prediction of the risk of diabetic complications," *Biomarkers*, vol. 21, no. 3, pp. 276-282.

[205] E.J. Chang, S.K. Lee, Y.S. Song, Y.J. Jang, H.S. Park, J.P. Hong, A.R. Ko, D.Y. Kim, J.H. Kim, Y.J. Lee and Y.S. Heo, "'IL-34 is associated with obesity, chronic inflammation, and insulin resistance," *J.Clin.Endocrinol.Metab.*, vol. 99, no. 7, July 01, pp. 1263.

[206] S.J. Hwang, B. Choi, S.S. Kang, J.H. Chang, Y.G. Kim, Y.H. Chung, D.H. Sohn, M.W. So, C.K. Lee, W.H. Robinson and E.J. Chang, "'Interleukin-34 produced by human fibroblast-like synovial cells in rheumatoid arthritis supports osteoclastogenesis," *Arthritis Res.Ther.*, vol. 14, no. 1, January 20, pp. R14.

[207] E. Franze, I. Monteleone, M.L. Cupi, P. Mancia, F. Caprioli, I. Marafini, A. Colantoni, A. Ortenzi, F. Laudisi, G. Sica, P. Sileri, F. Pallone and G. Monteleone, "'Interleukin-34 sustains inflammatory pathways in the gut," *Clin.Sci.(Lond)*, vol. 129, no. 3, August 01, pp. 271-280.

[208] S. Zwicker, G.L. Martinez, M. Bosma, M. Gerling, R. Clark, M. Majster, J. Soderman, S. Almer and E.A. Bostrom, "'Interleukin 34: a new modulator of human and experimental inflammatory bowel disease," *Clin.Sci.(Lond)*, vol. 129, no. 3, August 01, pp. 281-290.

[209] X. Unamuno, J. Gomez-Ambrosi, A. Rodriguez, S. Becerril, G. Fruhbeck and V. Catalan, "'Adipokine dysregulation and adipose tissue inflammation in human obesity," *Eur.J.Clin.Invest.*, vol. 48, no. 9, September 01, pp. e12997.

[210] T. Kawai, M.V. Autieri and R. Scalia, "'Adipose tissue inflammation and metabolic dysfunction in obesity," *Am.J.Physiol.Cell.Physiol.*, vol. 320, no. 3, March 01, pp. C375-C391.

[211] S. Torres, E. Fabersani, A. Marquez and P. Gauffin-Cano, "'Adipose tissue inflammation and metabolic syndrome. The proactive role of probiotics," *Eur.J.Nutr.*, vol. 58, no. 1, February 01, pp. 27-43.

[212] S.R. Anthony, A.R. Guarnieri, A. Gozdiff, R.N. Helsley, A. Phillip Owens and M. Tranter, "'Mechanisms linking adipose tissue inflammation to cardiac hypertrophy and fibrosis," *Clin.Sci.(Lond)*, vol. 133, no. 22, November 29, pp. 2329-2344.

[213] I. Abe, Y. Teshima, H. Kondo, H. Kaku, S. Kira, Y. Ikebe, S. Saito, A. Fukui, T. Shinohara, K. Yufu, M. Nakagawa, N. Hijiya, M. Moriyama, T. Shimada, S. Miyamoto and N. Takahashi, "'Association of fibrotic remodeling and cytokines/chemokines content in epicardial adipose tissue with atrial myocardial fibrosis in patients with atrial fibrillation," *Heart Rhythm*, June 13.

[214] A.C.T. Ng, M. Strudwick, van der Geest, R J, A.C.C. Ng, L. Gillinder, S.Y. Goo, G. Cowin, V. Delgado, W.Y.S. Wang and J.J. Bax, "'Impact of Epicardial Adipose Tissue, Left Ventricular Myocardial Fat Content, and Interstitial Fibrosis on Myocardial Contractile Function," *Circ.Cardiovasc.Imaging*, vol. 11, no. 8, August 01, pp. e007372.

[215] H.H. Hu, D.Q. Chen, Y.N. Wang, Y.L. Feng, G. Cao, N.D. Vaziri and Y.Y. Zhao, "'New insights into TGF-beta/Smad signaling in tissue fibrosis," *Chem.Biol.Interact.*, vol. 292, August 25, pp. 76-83.

[216] H. Khalil, O. Kanisicak, V. Prasad, R.N. Correll, X. Fu, T. Schips, R.J. Vagnozzi, R. Liu, T. Huynh, S.J. Lee, J. Karch and J.D. Molkentin, "'Fibroblast-specific TGF-beta-Smad2/3 signaling underlies cardiac fibrosis," *J.Clin.Invest.*, vol. 127, no. 10, October 02, pp. 3770-3783.

[217] M. Dobaczewski, W. Chen and N.G. Frangogiannis, "'Transforming growth factor (TGF)-beta signaling in cardiac remodeling," *J.Mol.Cell.Cardiol.*, vol. 51, no. 4, October 01, pp. 600-606.

[218] X. Cui, L. Chang, Y. Li, Q. Lv, F. Wang, Y. Lin, W. Li, J.D. Meade, J.C. Walden and P. Liang, "'Trivalent soluble TNF Receptor, a potent TNF-alpha antagonist for the treatment collagen-induced arthritis," *Sci.Rep.*, vol. 8, no. 1, May 09, pp. 7327-w.

[219] J. Palomo, D. Dietrich, P. Martin, G. Palmer and C. Gabay, "'The interleukin (IL)-1 cytokine family--Balance between agonists and antagonists in inflammatory diseases," *Cytokine*, vol. 76, no. 1, November 01, pp. 25-37.

[220] M. Mihara, M. Hashizume, H. Yoshida, M. Suzuki and M. Shiina, "'IL-6/IL-6 receptor system and its role in physiological and pathological conditions," *Clin.Sci.(Lond)*, vol. 122, no. 4, February 01, pp. 143-159.

[221] S. Rose-John, "'The Soluble Interleukin 6 Receptor: Advanced Therapeutic Options in Inflammation," *Clin.Pharmacol.Ther.*, vol. 102, no. 4, October 01, pp. 591-598.

[222] S. Rose-John, "'IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6," *Int.J.Biol.Sci.*, vol. 8, no. 9, pp. 1237-1247.

[223] D.A. Siwik, D.L. Chang and W.S. Colucci, "'Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro," *Circ.Res.*, vol. 86, no. 12, June 23, pp. 1259-1265.

[224] S.H. Ki, O. Park, M. Zheng, O. Morales-Ibanez, J.K. Kolls, R. Bataller and B. Gao, "'Interleukin-22 treatment ameliorates alcoholic liver injury in a murine model of chronic-binge ethanol feeding: role of signal transducer and activator of transcription 3," *Hepatology*, vol. 52, no. 4, October 01, pp. 1291-1300.

[225] R. Mo, R. Lai, J. Lu, Y. Zhuang, T. Zhou, S. Jiang, P. Ren, Z. Li, Z. Cao, Y. Liu, L. Chen, L. Xiong, P. Wang, H. Wang, W. Cai, X. Xiang, S. Bao and Q. Xie, "'Enhanced autophagy contributes to protective effects of IL-22 against acetaminophen-induced liver injury," *Theranostics*, vol. 8, no. 15, July 30, pp. 4170-4180.

[226] G. Perriard, A. Mathias, L. Enz, M. Canales, M. Schluep, M. Gentner, N. Schaeren-Wiemers and R.A. Du Pasquier, "'Interleukin-22 is increased in multiple sclerosis patients and targets astrocytes," *J.Neuroinflammation*, vol. 12, June 16, pp. 119-3.

[227] J.A. Dudakov, A.M. Hanash and van den Brink, M R, "'Interleukin-22: immunobiology and pathology," *Annu.Rev.Immunol.*, vol. 33, pp. 747-785.

[228] J. Ye, Q. Ji, J. Liu, L. Liu, Y. Huang, Y. Shi, L. Shi, M. Wang, M. Liu, Y. Feng, H. Jiang, Y. Xu, Z. Wang, J. Song, Y. Lin and J. Wan, "'Interleukin 22 Promotes Blood Pressure Elevation and Endothelial Dysfunction in Angiotensin II-Treated Mice," *J.Am.Heart Assoc.*, vol. 6, no. 10, October 03, pp. 10.1161/JAHA.117.005875.

[229] J. Ye, Q. Ji, J. Liu, L. Liu, Y. Huang, Y. Shi, L. Shi, M. Wang, M. Liu, Y. Feng, H. Jiang, Y. Xu, Z. Wang, J. Song, Y. Lin and J. Wan, "'Interleukin 22 Promotes Blood Pressure Elevation and Endothelial Dysfunction in Angiotensin II-Treated Mice," *J.Am.Heart Assoc.*, vol. 6, no. 10, October 03, pp. 10.1161/JAHA.117.005875.

[230] Q. Kong, Y. Xue, W. Wu, F. Yang, Y. Liu, M. Gao, W. Lai and X. Pan, "'IL-22 exacerbates the severity of CVB3-induced acute viral myocarditis in IL-17A-deficient mice," *Mol.Med.Rep.*, vol. 7, no. 4, April 01, pp. 1329-1335.

[231] H. Yoshida, M. Nakaya and Y. Miyazaki, "'Interleukin 27: a double-edged sword for offense and defense," *J.Leukoc.Biol.*, vol. 86, no. 6, December 01, pp. 1295-1303.

[232] W. Jin, Y. Zhao, W. Yan, L. Cao, W. Zhang, M. Wang, T. Zhang, Q. Fu and Z. Li, "'Elevated circulating interleukin-27 in patients with coronary artery disease is associated with dendritic cells, oxidized low-density lipoprotein, and severity of coronary artery stenosis," *Mediators Inflamm.*, vol. 2012, pp. 506283.

[233] K. Tang, C. Zhang, Y. Zhang, Y. Zhang, H. Du, B. Jin and Y. Ma, "'Elevated plasma interleukin 34 levels correlate with disease severity-reflecting parameters of patients with haemorrhagic fever with renal syndrome," *Infect.Dis.(Lond)*, vol. 51, no. 11-12, December 01, pp. 847-853.

[234] R. Wang, G. Han, J. Wang, G. Chen, R. Xu, L. Wang, X. Li, B. Shen and Y. Li, "'The pathogenic role of interleukin-27 in autoimmune diabetes," *Cell Mol.Life Sci.*, vol. 65, no. 23, November 01, pp. 3851-3860.

[235] H.J. Chen, S.W. Tas and de Winther, M P J, "'Type-I interferons in atherosclerosis," *J.Exp.Med.*, vol. 217, no. 1, January 06, pp. 10.1084/jem.20190459.

[236] K. Chen, J. Liu and X. Cao, "'Regulation of type I interferon signaling in immunity and inflammation: A comprehensive review," *J.Autoimmun.*, vol. 83, September 01, pp. 1-11.

[237] V. Deretic and B. Levine, "'Autophagy balances inflammation in innate immunity," *Autophagy*, vol. 14, no. 2, pp. 243-251.

[238] L.B. Ivashkiv and L.T. Donlin, "'Regulation of type I interferon responses," *Nat.Rev.Immunol.*, vol. 14, no. 1, January 01, pp. 36-49.

[239] S.J. Noppert, K.A. Fitzgerald and P.J. Hertzog, "'The role of type I interferons in TLR responses," *Immunol.Cell Biol.*, vol. 85, no. 6, September 01, pp. 446-457.

[240] S.P. Levick and P.H. Goldspink, "'Could interferon-gamma be a therapeutic target for treating heart failure?" *Heart Fail.Rev.*, vol. 19, no. 2, March 01, pp. 227-236.

[241] L. Marko, H. Kvakan, J.K. Park, F. Qadri, B. Spallek, K.J. Binger, E.P. Bowman, M. Kleinewietfeld, V. Fokuhl, R. Dechend and D.N. Muller, "'Interferon-gamma signaling inhibition ameliorates angiotensin II-induced cardiac damage," *Hypertension*, vol. 60, no. 6, December 01, pp. 1430-1436.

[242] K. Gee, C. Guzzo, N.F. Che Mat, W. Ma and A. Kumar, "'The IL-12 family of cytokines in infection, inflammation and autoimmune disorders," *Inflamm.Allergy Drug Targets*, vol. 8, no. 1, March 01, pp. 40-52.

[243] E.D. Tait Wojno, C.A. Hunter and J.S. Stumhofer, "'The Immunobiology of the Interleukin-12 Family: Room for Discovery," *Immunity*, vol. 50, no. 4, April 16, pp. 851-870.

[244] C.S. Hsieh, S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra and K.M. Murphy, "'Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages," *Science*, vol. 260, no. 5107, April 23, pp. 547-549.

[245] S.E. Macatonia, N.A. Hosken, M. Litton, P. Vieira, C.S. Hsieh, J.A. Culpepper, M. Wysocka, G. Trinchieri, K.M. Murphy and A. O'Garra, "'Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells," *J.Immunol.*, vol. 154, no. 10, May 15, pp. 5071-5079.

[246] K.R. Bastos, J.M. Alvarez, C.R. Marinho, L.V. Rizzo and M.R. Lima, "'Macrophages from IL-12p40-deficient mice have a bias toward the M2 activation profile," *J.Leukoc.Biol.*, vol. 71, no. 2, February 01, pp. 271-278.

[247] K.R. Bastos, de Deus Vieira de Moraes, L, C.A. Zago, C.R. Marinho, M. Russo, J.M. Alvarez and M.R. D'Imperio Lima, "'Analysis of the activation profile of dendritic cells derived from the bone marrow of interleukin-12/interleukin-23-deficient mice," *Immunology*, vol. 114, no. 4, April 01, pp. 499-506.

[248] K.R. Bastos, R. Barboza, L. Sardinha, M. Russo, J.M. Alvarez and M.R. Lima, "'Role of endogenous IFN-gamma in macrophage programming induced by IL-12 and IL-18," *J.Interferon Cytokine Res.*, vol. 27, no. 5, May 01, pp. 399-410.

[249] D. Fairweather, S. Frisancho-Kiss, S.A. Yusung, M.A. Barrett, S.E. Davis, R.A. Steele, S.J. Gatewood and N.R. Rose, "'IL-12 protects against coxsackievirus B3-induced myocarditis by increasing IFN-gamma and macrophage and neutrophil populations in the heart," *J.Immunol.*, vol. 174, no. 1, January 01, pp. 261-269.

[250] H. Blumberg, D. Conklin, W.F. Xu, A. Grossmann, T. Brender, S. Carollo, M. Eagan, D. Foster, B.A. Haldeman, A. Hammond, H. Haugen, L. Jelinek, J.D. Kelly, K. Madden, M.F. Maurer, J. Parrish-Novak, D. Prunkard, S. Sexson, C. Sprecher, K. Waggie, J. West, T.E. Whitmore, L. Yao, M.K. Kuechle, B.A. Dale and Y.A. Chandrasekher, "'Interleukin 20: discovery, receptor identification, and role in epidermal function," *Cell*, vol. 104, no. 1, January 12, pp. 9-19.

[251] B.E. Rich, "'IL-20: a new target for the treatment of inflammatory skin disease," *Expert Opin.Ther.Targets*, vol. 7, no. 2, April 01, pp. 165-174.

[252] H. Cucak, L. Hoj Thomsen and A. Rosendahl, "'IL-20 contributes to low grade inflammation and weight gain in the Psammomys obesus," *Int.Immunopharmacol.*, vol. 45, April 01, pp. 53-67.

[253] N.P. Kumar, V.V. Banurekha, D. Nair, P. Kumaran, C.K. Dolla and S. Babu, "'Type 2 diabetes - Tuberculosis co-morbidity is associated with diminished circulating levels of IL-20 subfamily of cytokines," *Tuberculosis (Edinb)*, vol. 95, no. 6, December 01, pp. 707-712.

[254] T.W. Kragstrup, T. Andersen, L.D. Heftdal, M. Hvid, J. Gerwien, P. Sivakumar, P.C. Taylor, L. Senolt and B. Deleuran, "'The IL-20 Cytokine Family in Rheumatoid Arthritis and Spondyloarthritis," *Front.Immunol.*, vol. 9, September 25, pp. 2226.

[255] H.H. Wang, Y.H. Hsu and M.S. Chang, "'IL-20 bone diseases involvement and therapeutic target potential," *J.Biomed.Sci.*, vol. 25, no. 1, April 24, pp. 38-z.

[256] T.Y. Lin, C.J. Chiu, C.H. Kuan, F.H. Chen, Y.C. Shen, C.H. Wu and Y.H. Hsu, "'IL-29 promoted obesity-induced inflammation and insulin resistance," *Cell.Mol.Immunol.*, vol. 17, no. 4, April 01, pp. 369-379.

[257] J.H. Zhou, Y.N. Wang, Q.Y. Chang, P. Ma, Y. Hu and X. Cao, "'Type III Interferons in Viral Infection and Antiviral Immunity," *Cell.Physiol.Biochem.*, vol. 51, no. 1, pp. 173-185.

[258] C.H. Hung, C.H. Huang, L. Wang, C.C. Huang, M.C. Wu, Y.Y. Chin, C.Y. Lin, K. Chang, D.C. Wu and Y.H. Chen, "'IL-28 and IL-29 as protective markers in subject with dengue fever," *Med.Microbiol.Immunol.*, vol. 206, no. 3, June 01, pp. 217-223.

[259] C.H. Hung, C.H. Huang, L. Wang, C.C. Huang, M.C. Wu, Y.Y. Chin, C.Y. Lin, K. Chang, D.C. Wu and Y.H. Chen, "'IL-28 and IL-29 as protective markers in subject with dengue fever," *Med.Microbiol.Immunol.*, vol. 206, no. 3, June 01, pp. 217-223.

[260] T.Y. Lin, C.J. Chiu, C.H. Kuan, F.H. Chen, Y.C. Shen, C.H. Wu and Y.H. Hsu, "'IL-29 promoted obesity-induced inflammation and insulin resistance," *Cell.Mol.Immunol.*, vol. 17, no. 4, April 01, pp. 369-379.

[261] A. Mizokami, T. Kawakubo-Yasukochi and M. Hirata, "'Osteocalcin and its endocrine functions," *Biochem.Pharmacol.*, vol. 132, May 15, pp. 1-8.

[262] G. Karsenty, "'Update on the Biology of Osteocalcin," *Endocr.Pract.*, vol. 23, no. 10, October 01, pp. 1270-1274.

[263] J. Li, H. Zhang, C. Yang, Y. Li and Z. Dai, "'An overview of osteocalcin progress," *J.Bone Miner.Metab.*, vol. 34, no. 4, July 01, pp. 367-379.

[264] T. Komori, "'Functions of Osteocalcin in Bone, Pancreas, Testis, and Muscle," *Int.J.Mol.Sci.*, vol. 21, no. 20, October 12, pp. 10.3390/ijms21207513.

[265] F.L. Bilotta, B. Arcidiacono, S. Messineo, M. Greco, E. Chiefari, D. Britti, T. Nakanishi, D.P. Foti and A. Brunetti, "'Insulin and osteocalcin: further evidence for a mutual cross-talk," *Endocrine*, vol. 59, no. 3, March 01, pp. 622-632

APPENDIX

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
4N-64 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** · **www.ecu.edu/ORIC/irb**

Notification of Amendment Approval

From: Biomedical IRB
To: Jacques Robidoux
CC:

Date: 9/14/2017
Re: Ame8 UMCIRB 09-0669
UMCIRB 09-0669
MYOCARDIAL REDOX STATUS, CATECHOLAMINE METABOLISM AND POST-OPERATIVE ARRHYTHMIA

Your Amendment has been reviewed and approved using expedited review for the period of 9/13/2017 to 8/22/2018. It was the determination of the UMCIRB Chairperson (or designee) that this revision does not impact the overall risk/benefit ratio of the study and is appropriate for the population and procedures proposed.

Please note that any further changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. A continuing or final review must be submitted to the UMCIRB prior to the date of study expiration.The investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document	Description
Informed consent POAF JR 9-05-2017(0.01)	Consent Forms

PI changed: E. Anderson to J. Robidoux; E. Anderson added as subinvestigator
Study team member added: D. Madipally

The Chairperson (or designee) does not have a potential for conflict of interest on this study.