UNIVERSIDADE D
COIMBRA

Francisco Ribeiro Coelho de Melo Costa

miRNAs IN PERICARDIAL FLUID AND THEIR ROLE IN CARDIAC FIBROSIS

CONVERSATIONS INSIDE US AND HOW THEY CHANGE OUR HEARTS

Dissertação no âmbito do Mestrado de Biologia Celular e Molecular orientada pela Professora Diana Esperança dos Santos Nascimento e co-orientada pela Professora Ana Luísa Carvalho e apresentada ao Departamento de Ciências da Vida

Outubro de 2021

UNIVERSIDADE D
COIMBRA

Francisco Ribeiro Coelho de Melo Costa

MIRNAS IN PERICARDIAL FLUID AND THEIR ROLE IN CARDIAC FIBROSIS

CONVERSATIONS INSIDE US AND HOW THEY CHANGE OUR HEARTS

Dissertação no âmbito do Mestrado de Biologia Celular e Molecular orientada pela Professora Diana Esperança dos Santos Nascimento e co-orientada pela Professora Ana Luísa Carvalho e apresentada ao Departamento de Ciências da Vida

Outubro de 2021

Resumo

O enfarte do miocárdio (MI), a morte celular resultante de isquemia numa porção do miocárdio, é a maior causa de mortalidade no mundo. Uma caraterística do MI é o desenvolvimento de fibrose cardíaca, uma acumulação anormal de matriz extracelular (ECM). Esta é importante para dar suporte à parede isquémica MI, mas exacerbada deposição de ECM acaba por contribuir para a disfunção cardíaca. Uma das necessidades clínicas mais emergentes é o desenvolvimento de métodos de diagnósticos precoce e terapias para controlar a formação de fibrose cardíaca. Os microRNAs (miRNAs) são considerados poderosos agentes teranósticos, tendo sido associados à regulação da fibrose cardíaca. O líquido pericárdico (PF) é composto por diferentes biomoléculas secretadas pelo coração nomeadamente miRNAs.

O objectivo deste projecto é validar o PF como uma fonte robusta de miRNAs com papel relevante na fibrose cardíaca após MI. Usando RNA-sequencing, demonstramos que o PF concentra miRNAs relacionados com remodelação de ECM e fibrose. Adicionalmente, descobrimos um conjunto de miRNAs desregulados no PF de pacientes como MI com elevação do segmento-ST (STEMI), comparado com doentes com doença coronária estável (CTRL). Fibroblastos cardíacos humanos (hCFs) demonstram níveis de expressão de genes fibróticos (ACTA2) superiores aos controlos, quando expostos a PF STEMI. Inconsistentemente, o miR-22-3p, um miRNA previamente descrito como inibidor de fibrose, está mais concentrado no PF de STEMI e diminuído na área isquémica num modelo de MI de ratinho. A sobreexpressão de miR-22-3p atenua a activação de hCFs por TGF-β, enquanto a inibição não produziu qualquer efeito.

Estes resultados indicam que o miR-22-3p é libertado após MI, concentrado no PF, e poderá afetar o coração, especificamente CFs, como parte de um mecanismo de controlo de fibrose. Este trabalho constitui assim uma prova de conceito da relevância de PF como uma fonte de potenciais biomarcadores e alvos terapêuticos no combate às doenças cardiovasculares.

Palavras-chave: Enfarte do Miocárdio; Fibrose Cardíaca, microRNA, Fluido Pericárdico, TGF-β1

Abstract

Myocardial infarction (MI), the tissue damage resulting from decreased or complete blood flow interruption to a myocardial region, is the leading cause of death among ischemic heart disease patients. A pathologic hallmark of MI is the development cardiac fibrosis, an aberrant accumulation of extracellular matrix (ECM), which is fundamental during initial cardiac remodeling, but becomes deleterious and contributes to tissue dysfunction. Thus, early and reliable diagnosis and therapies to control/restrain cardiac fibrosis is a main unmet clinical need. microRNAs (miRNAs) have been advanced as potent theranostic molecules, also in the context of myocardial fibrosis. Actually, heart-derived molecules, including niRNAs, are concentrated in pericardial fluid (PF), thus, this biofluid has recently been suggested as a potential source of relevant targets for therapy and biomarker discovery.

Our aim is to validate PF as a reliable miRNAs source with a relevant role in cardiac fibrosis after MI. Using RNA-sequencing, we showed that PF concentrates miRNAs related with ECM remodeling and fibrosis. A subset of miRNA was found to be dysregulated in the PF of ST-elevation MI (STEMI) patients when compared with coronary stable patients (CTRL). Additionally, human cardiac fibroblasts (hCFs) exposed to PF of STEMI patients have increased levels of fibrosis related genes (*ACTA2*), compared to controls. Inconsistently, miR-22-3p, a miRNA previously described to inhibit fibrosis, was found amongst the miRNAs concentrated in STEMI patients and was downregulated in the infarcted area of a MI murine model. miR-22-3p overexpression hampered *in vitro* TGF-β-mediated cardiac fibroblast activation, whilst miR-22-3p downregulation produced no effect.

These findings indicate that miR-22-3p is released after MI, concentrates in PF and may affect the heart, specifically cardiac fibroblasts, as part of a fibrosis-control-mechanism. This work constitutes a proof-of-principle study, demonstrating the relevance of PF as a source of potential biomarkers and therapeutic targets for the management of cardiovascular diseases.

Key words: Myocardial Infarction; Cardiac Fibrosis; microRNA; Pericardial Fluid; TGF-β1

Acknowledgement

First and foremost I would like to thank my supervisor Diana Nascimento, for accepting me as a student and for the unparalleled support throughout my course in the Stem Cells in Reparative Biology and Repair group. Your brilliancy as an investigator goes without mention, Diana, and as such I will take this opportunity to appreciate your remarkable humanitarian side and the conviction, patience and dedication that you showed, not only in my work, but in everything you do. As someone very dear to the both of us once said, it takes someone very down-to-earth to help people who spend their life appreciating the snowflakes that fall from the skies, and making sense out of it. You were the best person I could have asked help from in this journey, Diana, and I will always hold a special place in my heart for you. Thank you for this snowman.
I would also like to thank Professor Perpétua Pinto-do-Ó for accepting me into the group, and most of all for the understanding of my mental deambulations and idiosyncracies, sometimes without the need for words. I thank you for all the apples and the support that came in every one of them, my fellow snowflake appreciator.
Additionally, I must thank my co-supervisor Professor Ana Luísa Carvalho, for all her support during my academical journey, both during my Bachelor, as I entered my Masters, and everything henceforth. You are a standard for academic excellence, and I consider myself very lucky for having had the chance of being helped and teached by you.
Elsa, you broke me down several times, and sometimes it was not easy. But I always remember that you can't construct something new and better without removing what was there before. I might not become what you intended, but know that today I am a better version of myself, more capable of facing what lies ahead. That's the part of you that rubbed off on me. Thank you for being my supervisor and for helping me grow, most of the time at the expense of your patience.
I can't refrain myself from thanking the members of my group. I must first thank Luísa. I know I have you as a life friend, and want you to have written proof of how important your company and friendship was in these challenging months of my life, and how our conversations were an anchor to my stability. I must thank Márcia, in whom I always found support, a well-said word or advice, but most of all a reliable friend. To Rita, for all the well-spent laughs, be it to lighten the mood as a result of a joke or as a way of relieving the pressure, and for always being available to help me. To Daniel, whose work I continued, and with whose help I knew I could count on, no matter the

geographical distance. To Rita Santos and Cassilda Pereira, who never let me struggle with my academical and personal choices alone, and whose help I knew I could count on. To Diogo, with whom I shared this road, and who I know that, if need be, will find the persistence to build a new road. I can't forget the support of the scientific platforms of i3S, with special affection for the people at CCGEN. Paula, Tânia and Diana, thank you for making every qPCR plate a moment for a healthy pause and laughter. You kept my head in its right place. Thank you all!

I must also thank FCTUC for this five-year-long trip, with all its bumps and curves, the first major stepping stone in my way to become a scientist, and i3S for becoming my home and the nursery for this dissertation.

Dedicatória

A quem me acompanhou neste percurso e ajudou a construir quem sou. Aos meus amigos. Sandro, que sempre me dizes o quanto te ajudei, deixo aqui por escrito que muitas vezes me salvaste também. Tenho orgulho em chamar-te o meu melhor amigo. Alex, meu protetor, meu sábio do acaso. De ti sei que não me livro, e não podia estar mais satisfeito com isso. Ricardo e Luís, os meus pais da Academia, que tantas vezes foram mais meus irmãos e a quem devo mais do que eles sabem. Iaro e Ivan, foi um prazer dividir estes anos convosco, e os próximos que virão. Henrique, que continues a ser uma inspiração e um desafio a superar na minha vida. Anastasiya, de quem posso estar 10 anos longe, e vou sentir que nenhum tempo passou. Paula, meu texugo desatinado, que eu nunca deixe de te dar na cabeça quando me irritas, e tu nunca deixes de me irritar. Ana Rita, a quem devo a partilha de uma ansiedade que não parece ter limite, e por sempre me compreenderes. Maria João e Guilherme, que nunca deixaram, nem deixarão, de dar a mão, mesmo quando tenho as mãos cheias. Teresa, que não posso contar o número de vezes que me salvou.

A ti, Isabella, meu pequeno demónio. Por manteres vivas as duas moléculas de serotonina que ainda hoje me sustêm. Por partilhares contigo o melhor da tua vida. Por deixares que eu partilhe contigo o melhor da minha. Por seres um abraço com quem posso sempre contar.

A quem me inspirou. A ti, Diana, já acima referida, que me inspiras a vir a ser um melhor (e mais organizado) investigador. À professora Celeste, que foi um marco na minha decisão (acertada) de escolher a Biologia para a vida. Ao professor João Ramalho-Santos, cuja confiança depositada em mim sempre foi uma fonte de força. Espero vir a tornar-me no investigador que sabia que posso. À professora Ana Luísa, tamnbém referida, que nunca duvidou da minha capacidade e esteve sempre um passo à frente, tanto na sua investigação como para me ajudar tanto a mim como outro aluno que precisasse. Muito especialmente a ti, Bibiana. És a minha inspiração e o meu standard para um investigador modelo, e como se não bastasse és uma pessoa excepcional.

Ao meu pai, que, não compreendendo o meu trabalho, nunca deixou de me apoiar mesmo nas minhas alturas mais insuportáveis, seja deste trabalho, seja da vida, e ser o melhor amigo que eu podia pedir. A ti, Pedro, pai for afinidade, que sempre me trataste como um filho a sério, e foste fulcral para a criação do Homem que sou hoje. Obrigado.

Acima de tudo, a ti. À pessoa mais importante da minha vida. A ti, que me deste o Ser, e desde então não descansaste na tarefa de me dar o Tornar. Que me deste duas mãos para trabalhar, e o teu ombro para quando elas estão demasiado cansadas. Que me deste tudo. A ti, a mulher nº1 da minha vida. A ti, mãe.

Este trabalho também é vosso. Portanto, se tiverem queixas, deviam primeiro questionar o vosso papel nisto.

Espero que gostem.

"The difference between screwing around and science is writing it down"

Adam Savage

Funding

This work was funded by European Structural and Investment Fund (ESIF), under Lisbon Portugal Regional Operational Programme and National Funds through Fundação para a Ciência e Tecnologia (FCT) [POCI-01-0145-FEDER-030985] and [POCI-01-0145-FEDER-016385].

Index

Table index

Figure index

Abbreviations

ACTA2 - α-smooth muscle actin (gene)
ANG-II – Angiotensin II
Asc-2P – L-ascorbic acid 2-phosphate
BNP – B-type Natriuretic Peptide
BSA – Bovine Serum Albumine
Cav3 – Caveolin 3
CCN2 – Connective Tissue Growth Factor
cDNA – complementary DNA
CICP – C-Propeptide of type-I Procollagen
CMR – Cardiac Magnetic Resonance
COL1A1 – Collagen Type I α 1 chain
CTRL – Stable Angina
CVD –Cardiovascular Disease
DMEM – Dulbecco's Modified Eagle Medium
Erk1/2 – Extracellular signal-regulated Kinase 1/2
ET-1 – Endothelin 1
EV – Extracellular Vesicle
FBS – Fetal Bovine Serum
FGF2 – Fibroblast Growth Factor 2
FGM – Fibroblast Growth Medium
hCF – human cardiac fibroblast
HDAC4 – Histone Deacetylase 4
HMGB1 – High mobility groupbox 1
IHD – Ischemic Heart Disease
IL – Interleukin
IP – Intraperitoneal Injection
LAD – Left Anterior Descending
lncRNA – Long non-coding RNA
MAPK – Mitogen Activated Protein Kinase
MCP-1 – Monocyte Chemoattractant Protein
MI – Myocardial Infarction
MIP (1α, 1β, 2) – Macrophage Inflammatory Protein
miRNA – MicroRNA
MMP-1 – Matrix Metalloproteinase 1
mRNA – Messenger RNA
ncRNA – non-coding RNA
NF-kB – Nuclear Factor kappaB
NSTEMI – Non-ST-elevation Myocardial Infarction
OGN – Osteoglycin
P/S – Penicillin/Streptomycin
PBMC – Peripheral Blood Mononuclear Cell
PBS – Phosphate Buffer Saline
PCA – Principal Components Analysis
PCI – Percutaneous Coronary Intervention
PDGF – Platelet-derived Growth Factor
PF – Pericardial Fluid
PGC1- α - Peroxisome proliferator-activated receptor gamma coactivator 1-α
PICP – Procollagen type-I C-terminal propeptide
PIIINP – Procollagen type-III N-terminal propeptide

PPARα - Peroxisome proliferator-activated receptor α
PTFAR – Platelet-activating Factor Receptor
PURB – Purine-rich Element-Binding Protein
qRT-PCR - Quantitative Real Time Polymerase Chain Reaction
RNA-seq – RNA-Sequencing
ROS – Reactive Oxygen Species
RT – Room temperature
Sirt1 – Sirtuin-1(gene)
SMAD – Small Mothers Against Decapentaplegic
ST-2 – Soluble Interleukine 2
STEMI – ST-elevation Myocardial Infarction
TGF-β – Transforming Growth Factor β
TLR – Toll-like Receptor
TNF-α – Tumour Necrosis Factor α
TRAIL – TNF-related Apoptosis-Inducing Ligand
VEGF – Vascular Endothelial Growth Factor
α-SMA – α-smooth muscle actin (protein)

1. Introduction

1.1. An overview of the heart

The heart is a complex organ comprised of several different cell types that indulge in different purposes. The healthy adult heart is constituted by vascular and lymphatic endothelial cells, which constitute the main cell population in the heart (~60%), cardiomyocytes (~25 -35%), fibroblasts (>20%) and immune cells (~3%) (Gersch et al. 2002; Pinto et al. 2016)). However, the prevalence of each cell type may be altered with aging, health status, and between individuals (Pinto et al. 2016; Talman and Ruskoaho 2016). While the primary contractile function of the heart is supported by cardiomyocytes, the endothelial tissue regulates blood pressure, homeostasis, and provides high energy-demanding cardiomyocytes with nutrients and oxygen, along with a plethora of other processes (Brutsaert 2003).

Much of the structural resilience of the heart is supported by extracellular matrix (ECM) proteins, namely fibrillar collagens (Berk, Fujiwara, and Lehoux 2007). Type I and type III collagen accounts for approximately 85% and 10% of ECM constitution, respectively (Pinto et al. 2016). The remainder 5% of the ECM is composed of molecules such as glycosaminoglycans, proteoglycans and glycoproteins, along with stored growth factors and proteases (Frangogiannis 2019). This intricate matrix is deeply related to the fibroblast population, as fibroblasts are involved both in the synthesis and degradation of collagen, therefore regulating its turnover in the cardiac interstitium (Brown et al. 2005; Sperr et al. 1994).

1.2. Ischemic heart disease: epidemiology, current treatment and pathophysiology

Cardiovascular diseases (CVD),which are defined as a set of diseases affecting the heart and blood vessels , remain the primary cause of death in developed countries (Virani et al. 2020). In 2017, an annual estimate of 17,8 million deaths worldwide were attributed to CVD. In Europe, CVD are responsible for 45% of mortality (3,9 million deaths per year). Alone, ischemic heart disease (IHD) is the leading cause of mortality under 75 and 65 years, both in men and also in women (Wilkins et al. 2017). According to statistics from the Global Burden of Disease of 2020 (Khan et al. 2020; Virani et al. 2020), approximately 1,72% of the world population (126 million individuals) is affected

by IHD, and annually approximately 9 million deaths worldwide are attributed to IHD, with eastern Europe countries displaying the highest incidence (Wilkins et al. 2017).

IHD, also known as coronary heart disease, is characterized by deficient blood supply to an area of the heart muscle. The insufficient blood flow is usually associated with the accumulation of atherosclerotic plaques in the coronary blood vessels that can lead to total or partial blockage of the vessel or, result in thrombus formation and subsequent downstream blood vessel occlusion. The concomitant depletion of oxygen and nutrients to the muscle for >20-40 minutes (Skyschally, Schulz, and Heusch 2008) results in necrotic cell death, and subsequently an episode of myocardial infarction (MI) that if untreated has devastating functional consequences (Frangogiannis 2012).

MI is usually diagnosed with the joint resources of clinical criteria, electrocardiography, imaging studies and cardiomyocyte-associated biomarker detection, namely Troponin T and I, as well as creatinine-kinase/MB-creatine kinase (Frangogiannis 2012; Gabriel-Costa 2018; Rischpler 2016). Reperfusion of the blood vessels through percutaneous coronary intervention (PCI) with balloon, stent or alternate approved device, is the gold standard method to minimize irreversible myocardial damage (Skyschally et al. 2008). Although expeditious PCI following MI diagnosis is determinant in reducing mortality, re-infarction, and stroke rates (Wilkins et al. 2017), the procedure can induce some myocardial damage and microvascular impairment (Skyschally et al. 2008)(Frangogiannis 2012). Pharmacological measures contribute to improve prognosis and reduce complication such as the administration of β-blockers (Braunwald et al. 1983), angiotensin converting enzyme inhibitors and angiotensin receptor blockers (IJsselmuiden and Faden 1992), lipid-lowering therapy using statins (Auer, Weber, and Eber 2004) and the use of mineralocorticoid/aldosterone receptor antagonists (Cavalera, Wang, and Frangogiannis 2014; Gabriel-Costa 2018).

1.3. The repair response after myocardial infarction

At the tissue level MI initiates a reparative response resulting in the formation of a non-contractile collagenous scar to replace the lost muscle (Frangogiannis 2015; Hortells, Johansen, and Yutzey 2019). The latter is divided in three partially overlapping phases: inflammatory, proliferative and maturation phase (Figure 1).

The inflammatory phase

After an initial ischemic episode, necrotic cells initiate an inflammatory signaling cascade. The release of several intracellular biomolecules, such as high mobility

groupbox 1 (HMGB1), RNAs and nucleotides, heat-shock proteins , members of the S100 family, IL-1α , toll-like receptors (TLRs) (Frangogiannis 2012), reperfusion-derived ROS and the complement cascade (Hensley et al. 2000; Yasojima et al. 1998), promote the activation of a wide array of inflammatory responses. TLR activation leads to the activation of the transcription factor nuclear factor-kappaB (NF-kB) which induced the transcription of pro-inflammatory mediators (IL-1, IL-6, cardiotrophin-1, and oncostatin M) (Frangogiannis 2012). In parallel, IL-1 stimulates the production of several chemokines (MIP-1α, MIP-1β, MIP2 and MCP-1) (Bujak et al. 2008; Saxena et al. 2013) that recruit immune cells to the myocardium, and activate the expression of several adhesion molecules in endothelial cells (Frangogiannis 2015; Saxena et al. 2013) and consequently facilitate the infiltration of immune cells in the myocardial tissue (Frangogiannis 2015). This leukocyte recruitment to the infarcted area promotes extracellular matrix (ECM) remodeling and clearance of necrotic cells (Frangogiannis 2015). In this phase, the ECM is partially degraded, mainly due to the activation of latent collagenases, such as matrix metalloproteinases (MMPs) (Dobaczewski, Gonzalez-Quesada, and Frangogiannis 2010; Kong, Christia, and Frangogiannis 2014). Of note, the catabolic products of matrix degradation further promote the inflammatory response (Gaggar et al. 2008; Robbins and Swirski 2010) by activating monocytes and neutrophils (Frangogiannis 2015; Kania, Blyszczuk, and Eriksson 2009). These changes affect the structure of the cardiac interstitium, which becomes more permeable to cell migration and signal transduction for modulation of gene expression in different cell types. The passive clearance of apoptotic bodies and necrotic cells by macrophages leads to the production of anti-inflammatory mediators such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β) (Frangogiannis 2012).

The proliferative phase

The inflammatory phase of post-myocardial infarction leads to the second phase: the proliferative phase. In fact, several molecules produced during the late inflammatory phase such as TGF-β (Frangogiannis 2015; Kong et al. 2014), Endothelin-1 (Kong et al. 2014; Wang et al. 2015), PDGF (Zymek et al. 2006) and the activation of the renin-angiotensin-aldosterone system (Bader 2002; Blaufarb 1996), impact resident cardiac fibroblasts, leading to their activation and recruitment to the ischemic region. Fibroblasts become highly proliferative and differentiate into myofibroblasts acquiring a polygonal morphology, increased production of contractile proteins, such as α-smooth muscle actin (α-SMA), and of smooth muscle-like stress fibers (Frangogiannis 2012;

Shinde and Frangogiannis 2014). This activation shifts the activity of fibroblasts into a population capable of producing higher amounts of structural proteins like collagen, as well as protease inhibitors, such as tissue inhibitor of metalloproteinases-1 and plasminogen activator inhibitor-1 (Biernacka, Dobaczewski, and Frangogiannis 2011; Bujak et al. 2007), which promotes preservation of the newly formed ECM (Frangogiannis 2015).

The maturation phase

The formation of the collagenous ECM marks the transition from the proliferative to the maturation phase. The latter is recognized by crosslinking of structural proteins (collagen crosslinking) deposited in the infarct zone concomitantly with a downregulation of all the previous proliferation-related processes. In fact, the active myofibroblast population enters a quiescent state with less contractile fibers and matrix protein production (Ren et al. 2002), halting the growth of the collagenous scar. This is accompanied by a regression of uncoated vascular microvessels and repression of angiogenesis (Dobaczewski et al. 2004). Parallel to this, the existing extracellular matrix is subjected to the effect of secreted enzymes, such as lysolipids and collagen crosslinking occurs (Xie et al. 2012). Overall, this abnormal expansion of cardiac interstitium as a result of excessive accumulation of extracellular matrix (ECM) proteins is commonly referred as cardiac fibrosis (Frangogiannis 2015; Kong et al. 2014).

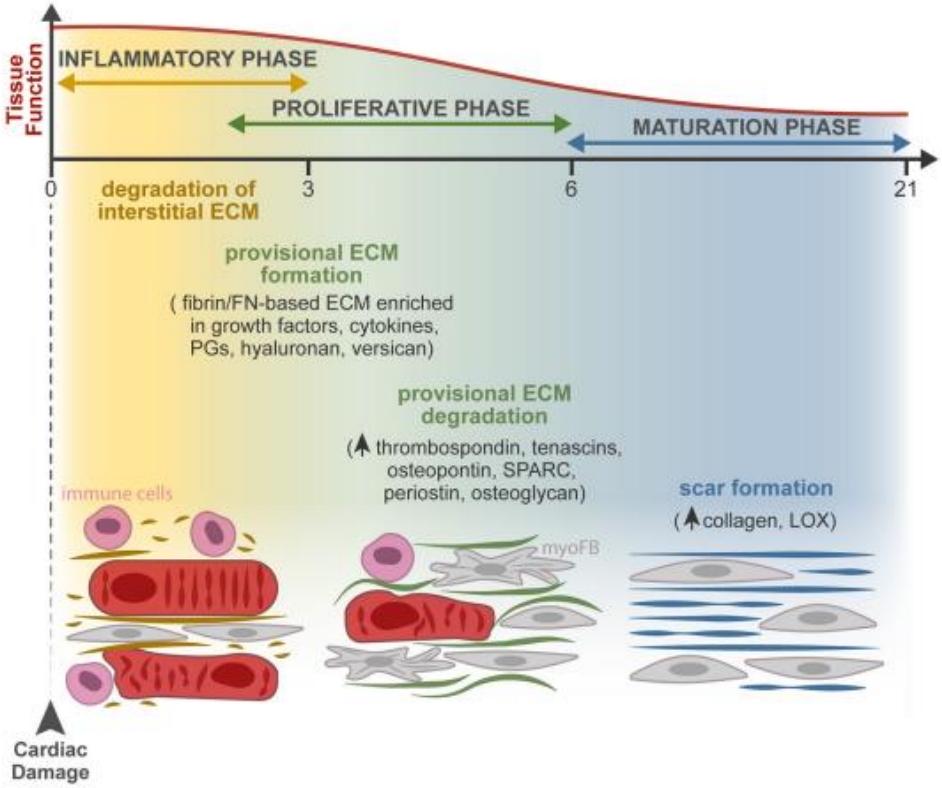

Figure 1 - Schematic representation of the reparative response to MI, highlighting the severe remodeling of the ECM (Source:(Silva et al. 2021))

1.4. Mechanisms regulating cardiac fibrosis

Cardiac fibrosis is not only formed after MI as described above, but is rather a hallmark of several other CVD or injury, such as hypertensive heart disease (Berk et al. 2007) and inflammatory heart disease (Kania et al. 2009), amongst others (Piccoli, Bär, and Thum 2016). Indeed, three distinct types of cardiac fibrosis have been described using histopathological criteria: reactive interstitial fibrosis, infiltrative interstitial fibrosis and replacement fibrosis. Reactive and infiltrative interstitial fibrosis are characterized by expansion of endomysium and perimysium, respectively (Frangogiannis 2019). Reactive fibrosis occurs without significant loss of cardiomyocytes and is associated with pressure overload and a series of cardiomyopathies (Disertori, Masè, and Ravelli 2017; Graham-Brown et al. 2017), while infiltrative fibrosis is associated with glycolipid accumulation in various cell types (Disertori et al. 2017; Graham-Brown et al. 2017). Replacement fibrosis is the end result of cardiac injury, such as ischemic injury, and is defined by the deposition of a collagen scar to compensate loss of cardiomyocytes (Frangogiannis 2019). Replacement fibrosis is essential to ensure structural integrity of the heart during remodeling prior to injury, ensuring a scaffold for structural maintenance and avoiding myocardial dysfunction or rupture (Hinderer and Schenke-Layland 2019). However, formation of myocardial fibrosis also has deleterious consequences to the heart because the inherent ECM crosslinking results in a less compliant matrix, leading to ventricular stiffness, contractile dysfunction and impaired mechano-electric coupling of cardiomyocytes (Spach and Boineau 1997; Travers et al. 2017). Not surprisingly, cardiac fibrosis is a relevant contributor to the development and progression of heart failure (Heymans et al. n.d.; Hinderer and Schenke-Layland 2019). Hence, prevention and reversion of cardiac fibrosis are key targets to advance CVD management. Apart from pharmacological control, early diagnosis of cardiac fibrosis is crucial to predict the disease outcome but current diagnostic entails evaluation of endomyocardial biopsies or expensive imaging resources as cardiac magnetic resonance (CMR) (Heymans et al. n.d.; Talman and Ruskoaho 2016) Circulating collagen biomarkers are an alternative strategy to phenotype cardiac fibrosis. Using these markers, one can discriminate collagen synthesis (procollagen type III N-terminal propeptide (PIIINP) (Klappacher et al. n.d.)and procollagen type I C-terminal propeptide (PICP) (Sa and Larman 2004)) from degradation (C-terminal telopeptide of collagen type I, CICP) and even cross-linking (matrix metalloproteinase-

1,MMP-1 (Ravassa et al. n.d.). However, their direct correlation to the degree of cardiac fibrosis is sometimes lacking or inconclusive and their tissue and organ origin are uncertain (for examples,. due to concomitant noncardiac disorders interfering with collagen metabolism) (Talman and Ruskoaho 2016). Hence, investigation of other biomarkers to target myocardial fibrosis is a main priority in the upcoming years.

Role of TGF-β in the activation of fibroblast-to-myofibroblast transition

From a pathophysiological standpoint, cardiac fibrosis has been extensively described (Figure 2). One of the major steps in fibroblast-to-myofibroblast differentiation is the activation of the TGF-β signalling cascade in fibroblasts. (Dobaczewski et al. 2010; Rosenkranz 2004). TGF-β is consistently present in both animal models of cardiac fibrosis and in human patients and is today the most well characterized growth factor involved in fibrogenesis (Biernacka et al. 2011; Kong et al. 2014). Our knowledge about its role in cardiac fibroblasts is limited to the isoform TGF-β1, the most ubiquitously expressed in the cardiovascular system, while the remaining isoforms (TGF-β2 and 3) present no reported role in the adult heart (Kong et al. 2014; Talman and Ruskoaho 2016). TGF-β1 exists in the cardiac interstitium in an inactive state and is activated following a cardiac insult (Frangogiannis 2012; Kong et al. 2014; Talman and Ruskoaho 2016). Upon activation, TGF-β1 binds to a tetrameric receptor complex usually constituted by two TβR1/ALK5 (type I) and two TβR2 (type II) receptors (Massagué 2012), which promotes the activation of Smads. Smad2 and 3 are phosphorylated by the TβR1/ALK5, whereas Smad1, Smad5 and Smad8 are activated by ALK1, a similar receptor present in endothelial cells. After phosphorylation, Smad 2 and 3 form a complex with Smad4 and are translocated into the nucleus, where they promote expression or inhibition of several genes in conjunction with a series of transcription factors and recruited coativators/corepressors. (Feng and Derynck 2005). Additionally, TβR2 can phosphorylate a series of other proteins, activating Smad-independent signalling by activating p38 MAPK and JNK pathways (by phosphorylating TAK1/Mekk1) or the Erk pathway (by phosphorylating Ras)(Derynck and Zhang 2003). Moreover, the activation of these pathways can promote both expression and inhibition of TGF-β, thus creating either a positive or negative feedback, as reviewed elsewhere (Biernacka et al. 2011; Derynck and Zhang 2003; Rosenkranz 2004).The activation of the TGF- β pathway leads, thus, to a cascade of events that culminate with *de novo* ECM synthesis by fibroblasts and myofibroblasts. In fact, Smad3-null mice display less remodelling and improved diastolic function in MI models (Bujak et al. 2007). These benefits were associated with lower functionality of migrating fibroblasts that were

accompanied with impaired activation into myofibroblasts and decreased ECM synthesis. TGF-β also regulates a Smad-independent signalling cascade, which comprises other cascades such as mitogen-activated protein kinase (MAPK) and Rho signalling (Kong et al. 2014; Park et al. 2019), and induces CCN2 expression, which has been correlated to prolonged fibrogenesis (Kong et al. 2014). Overall, TGF-β1 appears to activate α-smooth muscle actin (α-SMA) synthesis, leading to contractile bundle development and focal adhesion emergence in fibroblasts, inducing differentiation into myofibroblasts (Kong et al. 2014; Park et al. 2019). Although TGF-β1 is the most well characterized mediator of the fibrogenic process, there are other molecules playing vital roles on cardiac fibroblast activation, as is the case of angiotensin II (ANG-II). These molecules are out of the scope of the present MSc dissertation, and their roles are described elsewhere (reviewed by(Kong et al. 2014; Talman and Ruskoaho 2016; Travers et al. 2017)).

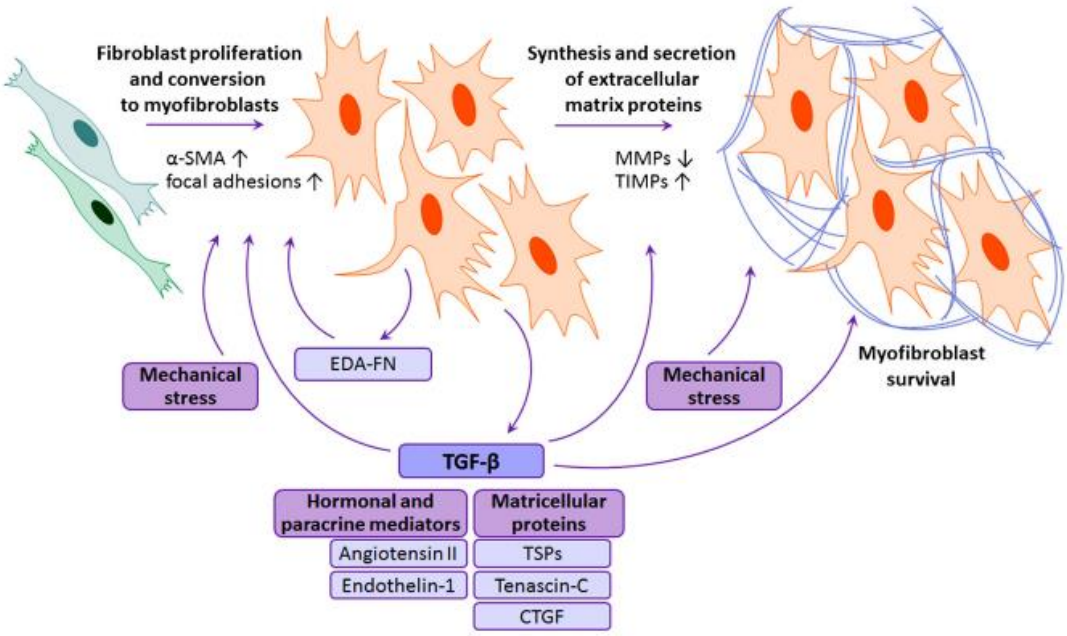

Figure 2 - Fibroblast-to-myofibroblast transition and the main extracellular stimuli involved in this process during MI. Source: (Talman and Ruskoaho 2016).

Regulation of cardiac fibrosis by non-coding RNAs

Non-coding RNAs (ncRNAs) regulate key cardiovascular cellular functions, displaying great value as therapeutic targets and, owing to their circulatory nature, also as biomarkers. Amongst ncRNAs, microRNAs (miRNAs) are the most studied in what concerns regulation of fibroblast activity as well as development and diagnosis of myocardial fibrosis. Recently, several studies revealed also the involvement of long

ncRNAs (lncRNAs) in cardiac fibrosis, an area that is only starting to emerge (Kreutzer, Fiedler, and Thum 2020).

miRNAS are sequences of approximately 21 nucleotides with a function of inhibiting protein expression. Normally, these sequences inhibit protein synthesis through base-pairing with 3' UTR of specific mRNAs (Xie et al. 2005), marking them for degradation or repressing their binding to the ribosomes through a cap-dependent mechanism (Dalmay 2013), primarily by the association with proteins such as Argonaute (Hutvagner and Simard 2008) and Dicer (Dalmay 2013; Ha and Kim 2014). An interesting relation between long non-coding RNAs (lncRNA) (>200-nucleotides sequences with no protein-coding capacity) and miRNAs has also been reported, wherein lncRNA can act as a miRNA sponge, restraining its expression by direct binding (Huang 2018; Zhou et al. 2017). Conversely, miRNA has been found to negatively modulate the expression of lncRNA (Zhang et al. 2013). Relevantly, this axis of interaction was reported to affect several CVD (Huang 2018).

A myriad of pathways related to the onset and progression of cardiac fibrosis are regulated by miRNA. miR-21 is the most well studied profibrotic miRNA. This miRNA targets sprout homologue 1 (Spry1) leading to indirect activation of p38 MAPK and subsequent upregulation of fibroblast migration, proliferation and differentiation (Brønnum et al. 2013; Thum et al. 2008). Moreover, inhibition of this miR promoted upregulation of Smad7 and, therefore, inhibiting activation of fibroblasts in an MI murine model (Yuan et al. 2017). Additionally, inhibition of a miR-15 family member promoted p38 MAPK and Smad7 upregulation in a study in COS7 cells (Tijsen et al. 2014). Particularly, the miR-15b was found to have anti fibrotic potential, inhibiting the TGF-β signaling pathway in human cardiac fibroblasts by targeting p38 MAPK, SMAD2, and SMAD3. Additionally, some miRNAs, such as the miR-29 family have dubious effects on cardiac fibrosis: whilst initially identified as cardioprotective miRNAs (Creemers and Van Rooij 2016), further studies showed that miR29a, miR29b1/2 and miR29c induce pro-fibrotic responses through activation of the Wnt signaling pathway (Sassi et al. 2017). Mcmullen and Bernardo suggested that this partial deletion of miR-29 in a murine model improved cardiac function following transverse aortic constriction and diminished cardiac fibrosis (McMullen and Bernardo 2018).

Overall, miRNAs have clearly shown their involvement in the modulation of cardiac disease and fibrosis, supporting their theranostic potential.

miR-22

Because of its relevance to the present MSc dissertation, the role of miRNA-22 (miR-22) in the heart and, particularly in the regulation of myocardial fibrosis, will be further reviewed. miR-22 is an evolutionally well conserved microRNA, whose seed sequence is unaltered from fly to human genome, and whose expression is enriched in skeletal and cardiac muscle in mammals (Huang and Wang 2014). Deep sequencing analysis identified miR-22 as the most abundant miRNA in the heart, particularly in cardiomyocytes (Hu et al. 2012; Huang et al. 2013), where it was found to act as a promoter of cardiomyocyte hypertrophy (Huang et al. 2013). Interestingly, the expression of miR-22 was found to be several times higher in adult murine cardiomyocytes when compared to neonatal counterparts (Huang et al. 2013).

Several studies point to an upregulation of miR-22 expression in the heart following various forms of cardiac stress (Huang et al. 2013; Small and Olson 2011; Tu et al. 2014), supporting that this mRNA may have a relevant role in cardiac remodeling during adverse events. Deletion of miR-22 from cardiomyocytes abrogates cellular hypertrophy in response to isoproterenol. These animals develop adverse cardiac remodeling and dilation, concomitant with cardiomyocyte death and fibrotic deposition (Huang et al. 2013). A different study further demonstrated that miR-22-null mice develop dilated cardiomyopathy in response to pressure overload (Gurha et al. 2012).

miR-22 has been both computational and experimentally validated as a repressor of a variety of genes. Notoriously, it has been found, in murine heart, to inhibit Sirt1 (Huang et al. 2013; Wen et al. 2021), HDAC4 (Huang et al. 2013), PPARα (Gupta et al. 2016; Zhang et al. 2020), PGC1-α (Wen et al. 2021), and PurB (Gurha et al. 2012). This highlights the importance of miR-22 in modulating the expression of hypertrophic genes, as well as genes related to the synthesis of contractile stress fibers and the beta-oxidation of fatty acids.

Furthermore, several other studies have delved into the role of miR-22 in cardiac fibroblast activity. In 2020, Zhao *et al* showed that miR-22-3p overexpression diminishes fibrinogenesis in Angiotensin-II-treated cardiac fibroblasts (Zhao et al. 2020) and abrogates cell proliferation and collagen deposition by targeting platelet activating factor receptor (PTAFR) (Zhao et al. 2020). Consistently, miR-22 inhibition in cultured murine cardiac fibroblasts led to increased expression of fibrillar collagens and fibrogenesis, whilst overexpression abrogated the pro-fibrotic effects of Angiotensin-II (Hong et al. 2016). In the same study, miR-22-3p was found to be dynamically downregulated in murine cardiac tissue following permanent ligation of the left anterior descending coronary artery (Hong et al. 2016). Additionally, Dong *et al* reported that

downregulation of miR-22 expression enhanced the expression of proinflammatory cytokines in a murine model of ischemic stroke (Dong, Cui, and Hao 2019), suggesting that miR-22 may play a role during the inflammatory phase of MI.

Studies in other tissues have produced similar conclusions. In a model of bleomycin-induced pulmonary fibrosis, miR-22 was increased 2-fold in circulating extracellular vesicles (Kuse et al. 2020). In the same study, overexpression of miR-22 abrogated the TGFβ1-induced activation of lung fibroblasts by inhibition of the ERK1/2 pathway (Kuse et al. 2020). Similarly, in rheumatoid arthritis miR-22 reduced fibroblast proliferation and production of pro-inflammatory cytokines by inhibiting Sirt1 (Zhang et al. 2020).

Although these reports advocate an anti-fibrotic of miR-22, other studies reported otherwise. For example, miR-22 was shown to have a pro-fibrotic effect after MI by promoting cardiac fibroblast activation, leading to increased collagen deposition, by targeting Caveolin-3 (Zhang et al. 2018). Additionally, miR-22 is upregulated in aging murine hearts, positively correlating with fibroblast senescence, activation, and migration (Jazbutyte et al. 2013). As such, our understanding of the role of miR-22 is limited and based on conflicting reports. Of note, no study has yet addressed the impact of miR-22 in the human heart.

1.5. Pericardial fluid – a new source of bioactive molecules and biomarkers

The heart is protected from shock and outer friction, as well as possible infection, by a fibrous membrane known as the pericardial sac, the pericardium (Vogiatzidis et al. 2015). This flask-shaped container involves the full surface of the heart and is conventionally divided into two distinct membranes, the parietal pericardium and visceral pericardium (Figure 3). The parietal pericardium establishes contact with the diaphragm, the pulmonary mediastinal pleura, the sternum, and the vertebral column, and is comprised of two layers: a fibrous outer layer, constituted by dense collagen bundles and elastic fibers, and a thinner, serosal layer constituted of mesothelial cells. The visceral pericardium, or epicardium, establishes contact with myocardium, and is constituted by a serosal layer similar to that of its counterpart (Vogiatzidis et al. 2015) .

The two pericardial layers surround the pericardial cavity that is filled with pericardial fluid (PF) (~15-35 mL in humans) a product of plasma ultrafiltration thought to be drained by the lymphatic capillary bed.

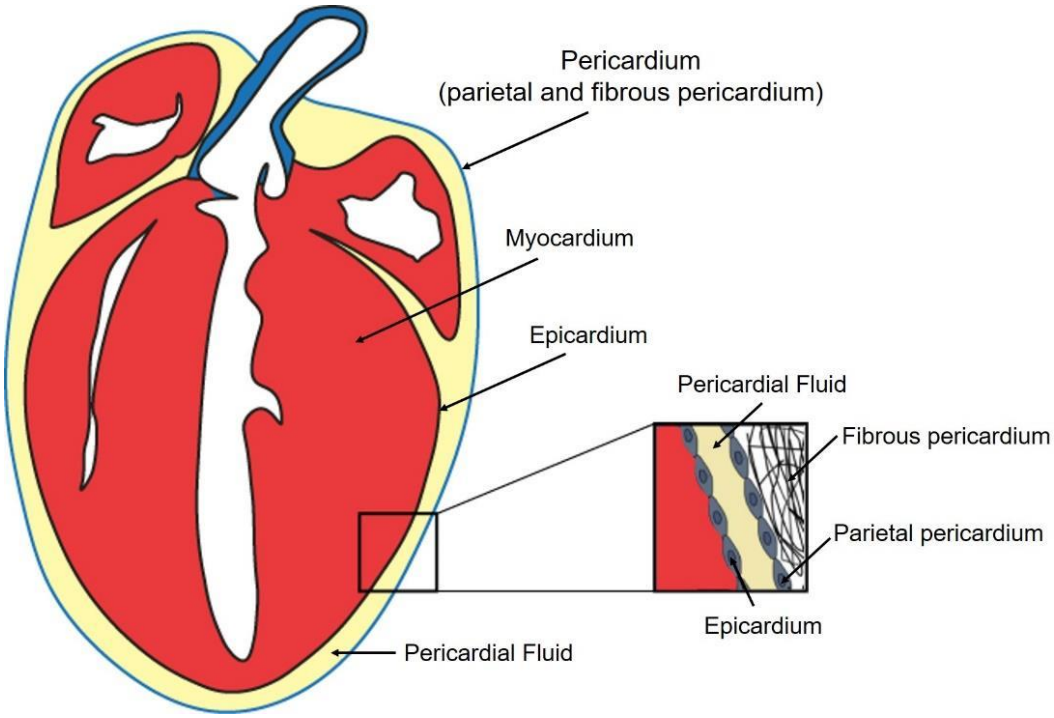

Figure 3 - Schematic representation of the pericardial layers and layers of the heart wall. Source: (Sousa 2018).

PF is responsible for stabilizing the heart by correcting its anatomical position and isolating the heart from the surrounding anatomical structures, by lubricating its moving surfaces. Furthermore, PF is also crucial in several essential processes for heart homeostasis. During diastole, it restricts excessive heart dilation, reducing endomyocardial tension. Additionally, it both contributes to the hydrostatic maintenance of end diastolic pressure at all hydrostatic levels, and prevents regression of ventriculo-atrial blood during high-end diastolic ventricular pressures. Similarly, it aids in maintaining negative endothoracic pressure and subsequent atria blood filling. Finally, it aids nervous stimulation of the heart (Vogiatzidis et al. 2015). Additionally, the biochemical components of PF modulate autonomic cardiac reflexes, myocardial contractile function, and epicardial coronary tone, namely through the action of secreted prostaglandins (Vogiatzidis et al. 2015).

Other than these cardioprotective functions, PF has been recently advanced as a regulator in cardiac function (Beltrami et al. 2017; Nemeth 2015; Yoneda et al. 2000) in normal conditions as well as in pathological scenarios (Dusting and Nolan 1981; Knabb et al. 1982; Nemeth 2015). The initial definition of PF as a plasma ultrafiltrate with mostly structural and protective functions resulted in the dismissal of its clinical relevance as both a means of diagnosis and therapy for heart disease (Gibson and Segal 1978a). However, recent studies shed light on the origin of PF as a product of

both filtration of plasma from epicardial capillaries and containing secreted products from pericardial mesothelium and myocardial interstitium (Beltrami et al. 2017; Hoit 2017; Stewart et al. 1997). These secreted products comprise a vast array of biomolecules that further impact on cardiac cells such as growth factors (Fujita et al. 1996; Liou et al. 2006; Santiago et al. 2014), cytokines (Ege et al. 2003; Ristić et al. 2013), hormones (Kuh et al. 1990; Nishikimi et al. 2004; Tanaka et al. 1998) and prostaglandins (Miyazaki, Pride, and Zipes 1990), as well as non-coding RNAs (Beltrami et al. 2017; Kuosmanen et al. 2015), (Trindade et al. 2019; Vogiatzidis et al. 2015). Likewise, PF contributes to other circulating fluids, namely blood and lymph (Boulanger et al. 1999; Gibson and Segal 1978b). In recent experiments, the lymphatic system has proven to be the major contributor in protein clearance of the PF (Boulanger et al. 1999; Gibson and Segal 1978b). Of interest, PF turnover rate is at a significantly slower rate than blood, which supports the hypothesis that PF can not only act as a reservoir of secreted biomolecules, but also as a vessel for therapy with higher retention times.

PF has previously been set aside as a diagnostic tool, due to the invasiveness of the harvest process and/or limited access during surgical procedure. Retroactively, the physiology/pathophysiology of the heart is reflected in the composition of pericardial fluid, as has been verified in several diseases, such as tuberculosis, congestive heart disease, and, importantly, ischemic heart disease (Fujita et al. 1996; Trindade et al. 2019). Indeed, the new paradigm defining PF as a net result of molecules secreted by the epicardium and myocardial insterstitium is drawing interest as a source of therapeutic targets and biomarkers for a myriad of CVDs. During an ischemic episode, the composition of pericardial fluid has been shown to shift regarding concentrations of several biomolecules (Fatehi Hassanabad et al. 2021). FGF2 concentration in PF has been found to be positively correlated to ischemic heart disease (Unstable and Stable Angina) and significantly upregulated when compared to non-ischemic patients (Fujita et al. 1996, 1998; Ikemoto et al. 1999; Kubota et al. 2004; Santiago et al. 2014; Yoneda et al. 2000). Similarly, VEGF levels have been shown to be increased in the pericardial fluid of unstable angina patients when compared to its non-ischemic counterparts (Oyama et al. 2001). In acute myocardial infarction patients, PF levels of cytokines, such as tumor necrosis factor α (TNF-α), and interleukins 1β, 6 and 8 (IL-1β, IL-6 and Il-8) and TNF-related apoptosis-inducing ligand (TRAIL) have been found to be more concentrated when compared to serum of the same patients (Oyama et al. 2001; Vogiatzidis et al. 2015). As a matter of fact, except for BNP, most biomolecules associated with cardiac pathology show stronger correlations on all parameters in

pericardial fluid analysis when compared to plasma or serum analysis (Trindade et al. 2019).

With the rising interest on the theranostic potential of miRNAS, the first studies on PF miRNA profiling are now emerging. Miyamoto *et al* evaluated the expression profiles of miRNAs miR-423-5p, miR-133a, miR-126 and miR-92a in the pericardial fluid of patients with stable or unstable angina, as well as with aortic stenosis; however, no evident differences were revealed between groups (Miyamoto et al. 2015; Trindade et al. 2019). Likewise, another study by Beltrami *et al* on a murine model of unilateral limb ischemia, validated that PF-derived, EV-contained human miRNA let-7b-5p is a key positive regulator of angiogenesis in endothelial cells (ECs). The paper assessed that 1) PF contains EVs carrying functional let-7b-5p; 2) these EVs can transfer functional let-7b-5p into ECs; 3) transferred let-7b-5p diminishes the expression of TGFβR in ECs, promoting angiogenesis (Beltrami et al. 2017). Of note, although a series of cardiac fibroblast-derived miRNAs have been reported in the PF (Bang et al. 2014; Beltrami et al. 2017), no study has yet address the role of PF in the development of myocardial fibrosis.

These findings give support that PF reflects significant changes in cardiac physiology both during and post-pathogenesis of several cardiac diseases (Trindade et al. 2019) and, importantly, works as a reservoir of bioactive molecules capable of affecting the heart as part of a positive feedback loop.

1.6. Conclusion

CVD are the world leading cause of death and a major societal health challenge. Most forms of CVDs are associated with myocardial fibrosis, characterized by exacerbated production of extracellular matrix in the heart. In the case of myocardial infarction (MI), initial fibrosis is crucial to replace the tissue defect but excessive fibrosis contributes to progressive functional decline. Hence, early detection, prevention and reversion of cardiac fibrosis are key targets to advance MI management. However, reliable diagnostic biomarkers for early and non-invasive fibrosis detection and therapies able to modify scar properties for patient benefit are two unmet clinical needs.

miRNAS are emerging as promising theranostic candidates to target myocardial fibrosis. Still, due to limited availability of human myocardial biopsies, most studies either search ncRNAs in human blood, lacking correlation with the heart, or using animal models not always reflecting the human pathology.

PF is emerging as an alternative source of both relevant disease biomarkers of disease and putative targets for therapy, namely miRNAs. Additionally, several components of PF altered with the type of CVD (Trindade et al. 2019), of which some have showed therapeutic properties (Beltrami et al. 2017). Although, the view of the PF as a new bioactive fluid is emerging, high-throughput studies on well-characterized patient cohort are yet missing and are urgently required.

2. Aims

2.1. Preliminary data supporting the present dissertation

One of the main challenges in attaining an in-depth characterization of the cardiac microenvironment during MI is the invasiveness and scarcity of myocardial biopsies. Even today, most of the knowledge regarding the pathophysiology of MI and the underlying mechanisms relies on the use of animal models (Frangogiannis 2015; Medzikovic, Aryan, and Eghbali 2019) which, albeit useful in the preliminary stages of uncovering the mechanistic behind the pathology, seldom represents the human counterpart in all faithfulness. One attractive source of biomolecules secreted by cardiac cells is the pericardial fluid (PF), which surrounds the entirety of the heart and whose composition has been found to reflect a variety of cardiovascular diseases (Fatehi Hassanabad et al. 2021; Trindade et al. 2019). With the aim of understanding the role of this biological fluid in the pathological response to MI, and in particularly in the regulation of cardiac fibrosis, the host lab has collected the PF and plasma from coronary patients undergoing coronary artery bypass grafting (Table 1). Three groups of patients were included in the study: 1- CTRL- patients with stable angina and no previous history of acute coronary syndrome; 2- NSTEMI- patients with a recent (<3 months) and first non-ST segment elevation myocardial infarction; 3- STEMI- patients with a recent (<3 months) and first ST segment elevation myocardial infarction. The groups were similar in terms of age, sex, cardiovascular risk factors (Table 1) and medication (not shown) at the time of surgery. Left ventricle ejection fraction (LVEF) was decreased in the MI groups, owning to tissue damage triggered by the blockage of coronary arteries, which contrasts with the CTRL group composed of stable coronary patients. Levels of miR-21, miR-29a and miR-29c (Figure 4A) were higher in the PF when compared to the plasma of the same patients. Likewise, levels of ST-2 (a biomarker for cardiac stress secreted during adverse cardiac remodeling, and that is associated with tissue fibrosis (Gawor et al. 2018) and procollagen type I carboxyl-terminal propeptide (PICP), which is secreted by cardiac fibroblasts as a precursor of fibrillar collagen (Jong, Veen, and Bakker 2011) were significantly higher in PF when comparing to plasma, in all cohorts (Figure 4B). Of note, PF from STEMI patients showed higher levels of ST-2 and a tendency for accumulation of PICP, when compared to PF of CTRL patients. Together, these results indicate that heart-derived biomolecules are concentrated in the PF during pathological episodes.

Table 1: Patient demographic data and cardiovascular risk factors.
Patients' cohorts are divided into 3 groups: the Control group (CTRL), comprised the patients with stable coronary disease and no record of previous acute coronary syndrome, and the two myocardial infarction (MI) cohorts, the NSTEMI cohort (comprised of patients with a recent (<3 months) and first non-ST segment elevation myocardial infarction) and the STEMI cohort (comprised of patients with a recent (<3 months) and first ST segment elevation myocardial infarction).

	CTRL (n=9)	NSTEMI (n=25)	STEMI (n=8)	ANOVA p Value	Dunn's multiple comparisons Test p Value		
					CTRL vs NSTEMI	CTRL vs STEMI	NSTEMI vs STEMI
Age (years)	65,6 ±8,8	68,8± 7,1	62,2 ±9,2	ns	ns	ns	ns
Female (%)	1(11,11)	1 (4,00)	1 (12,50)	ns	ns	ns	ns
LV EF (%)	55,75 ±5,97	45,04 ±13,07	44,75 ±10,74	0,005	0,0643	0,1405	ns
IHD Risk Factors							
BMI (Kg.m-2)	28,23 ±2,56	27,50 ±4,10	26,89 ±4,00	ns	ns	ns	ns
Diabetes melitus (%)	4 (44,44)	13 (52,00)	5 (62,50)	ns	ns	ns	ns
Hypertension (%)	5 (55,56)	21 (84,00)	6 (75,00)	ns	ns	ns	ns
Dyslipedemia (%)	8 (88,88)	18 (72,00)	7 (87,50)	ns	ns	ns	ns
Smokers (%)	1 (11,11)	4 (16,00)	2 (25,00)	ns	ns	ns	ns
Ex-smokers (%)	5 (55,56)	10 (40,00)	4 (50,00)	ns	ns	ns	ns
Previous IHD(%)	0 (0,00)	4 (16,00)	1 (12,50)	ns	ns	ns	ns

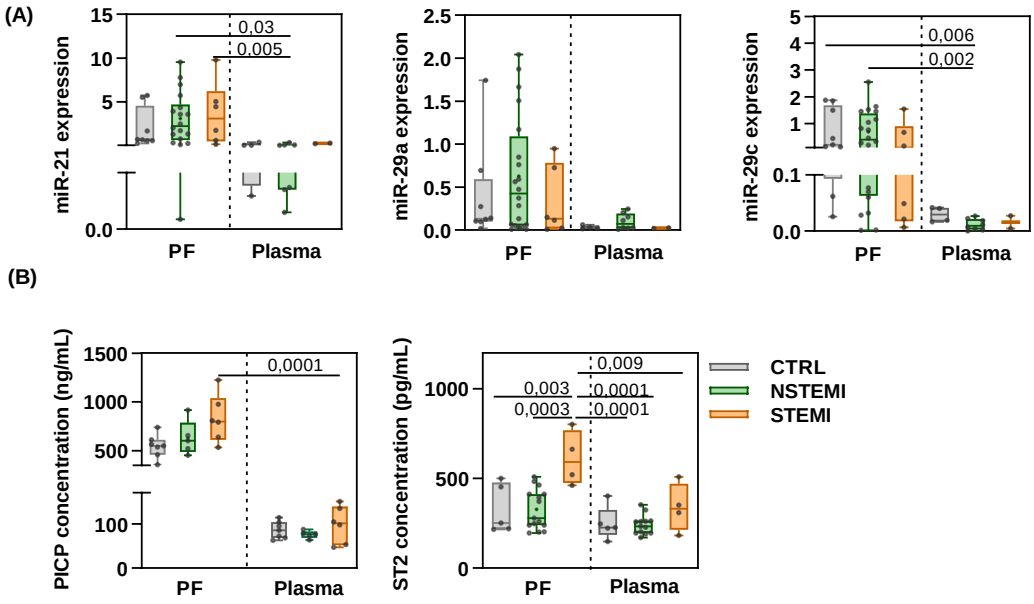

Figure 4 - **Pericardial fluid (PF) is a reservoir of fibrosis- and inflammatory-related molecules compared to plasma.**
(A) Expression of miR-21, miR29a and miR-29c in PF and plasma from patient cohorts. Expression is presented through the 2-ΔCt method, normalized to the C. elegans spike-in cel-miR-39 (PF CTRL n=8; PF NSTEMI n=18; PF STEMI n=6; Plasma CTRL n=4; Plasma NSTEMI n=7; Plasma STEMI n=2); **(B)** Enzyme-linked immunosorbent assay (ELISA) quantification of procollagen I c-terminal propeptide (PICP) and serum stimulation-2 (ST-2) in pericardial fluid and plasma from patient cohort (PF CTRL n=7; PF NSTEMI n=5; PF STEMI n=6; Plasma CTRL n=7; Plasma NSTEMI n=6; Plasma STEMI n=5).

2.2. Aims and objectives

Taking into consideration the previous findings we hypothesized that the PF may constitute a valid candidate as a screening agent for cardiac fibrosis diagnostic/prognostic biomarkers or surrogate targets of therapy. Hence, the broad aim of this project is to validate PF as a reliable source of miRNAs with a relevant role in cardiac fibrosis after MI, and to obtain further insight into how PF-contained miRNA regulates the fibrogenic process and how this can be valuable from a clinical standpoint.

To attain this main goal, we will:

1. Establish a protocol for the assessment of activation of hCFs *in vitro*, as to obtain a feasible model of cardiac fibrosis to extrapolate conclusions;
2. Assess the effect that PF from different patient cohort with varying degrees of ischemic damage exerts on hCFs *in vitro;*

3. Analyze and characterize miRNome of PF of the different patient cohorts as to assess for significant differences and establish candidates for further research. Validate those candidates by qRT-PCR;
4. Evaluate the physiological effects of inhibition and overexpression of our validated miRNA candidates through precursor- and inhibitor-transfection on hCFs, and how this correlates with the response verified in PF-treated hCFs.

3. Materials and Methods

3.1. Sample collection and processing

Peripheral blood and pericardial fluid (PF) were collected from patient cohorts undergoing coronary artery bypass grafting: i) a control, stable coronary artery patients (stable angina, without previous MI or acute coronary syndrome) and ii) a MI group, composed of patients with a recent first MI episode (maximum 3 months before surgery). The groups were characterized in terms of age, sex, cardiovascular risk factors and medication at the time of surgery (Table 1). Within two hours after blood (using EDTA as anti-coagulant) and PF collection, samples were processed as follows. Plasma and PF were separated from the buffy coat through a centrifugation at 12000 x g for 30 minutes (min) at room temperature (RT), without brake and acceleration. Then, PF and plasma were centrifuged twice at 2500 x g for 15 min at RT and the supernatants were stored at -80 ºC in 1 mL aliquots. To isolate peripheral blood mononuclear cells (PBMCs) from buffy coat, PBS was added in equal volume to buffy coat. After, the diluted buffy coat was transferred to the top of 4 mL of LymphoprepTM (STEMCELL Technologies™) and centrifuged at 800 x g for 30 minutes without brake and acceleration at RT. Then, PF cells and PBMCs were incubated with 5 mL red blood cell lysis buffer (10 mM Tris-base, 150 mM NH_4Cl) for 8 min at 37 ºC. The tube was filled with PBS and centrifuged at 300 x g at 10 min. The cell pellets obtained were resuspended in 500 µL of TRIzol (ThermoFisher Scientific) and stored at -80 ºC.

3.2. Animal Model

To perform myocardial infarction (MI), adult C57BL/6 mice with age ranging from 8 to 12 weeks were used. MI was induced by left anterior descending coronary artery ligation (LAD) coronary artery ligation, as described in (Nascimento et al. 2014). Briefly, animals were anesthetized with medetomidine ((1 mg/kg, Sededorm; ProdivetZN, Lisboa, Portugal) and ketamine (75 mg/kg, Clorketam; Vétoquinol, Lure, France) by intraperitoneal injection (IP). Then, mice were orally intubated and mechanically ventilated using small-animal respirator (Minivent 845; Harvard Apparatus, Holliston, MA, USA). During the entire procedure, and until fully recovery, animals were maintained on warming pads. Under a stereomicroscope (Leica EZ4; Leica Microsystems, Wetzlar, Germany), the heart was exposed via left thoracotomy on the

third intercostal space, and the pericardial sac was gently disrupted. The first portion of the LAD coronary artery was visualized. A nonabsorbable 7.0 suture (Silkam; B. Braun, Melsungen, Germany) was passed under the artery, and the ligation was performed. The intercostal incision was closed by an absorbable 6.0 suture (Safil; B. Braun), and surgical staples were used for skin closure. Anesthesia was reversed by 5 mg/kg ip atipamezole (Revertor; Virbac, Carros, France). Analgesia and fluid therapy were performed by IP delivery of butorphanol (1 mg/kg; Butador; Richter Pharma AG, Wels, Austria) and subcutaneous injection of 5% glucose physiologic saline (B. Braun), respectively. This procedure was repeated every 12 hours up to 72 hours after surgery or until full recovery.

3.3. Cell culture

Primary adult human cardiac fibroblasts (hCF; Cell Applications, Inc.) were cultured (passage 2 to 7) in fibroblast growth medium (FGM; Cell Applications, Inc.) and incubated in a HERAcell® 150 CO2 incubator (Heraeus®) at 37 ºC in a humidified atmosphere containing 5% CO2. Cells were routinely observed for assessing confluence and passaged when reaching approximately 80%. For that, cells were detached with 0,25% trypsin/EDTA (Sigma Aldrich, Co.), centrifuged at 300 x g for 5 min and seeded with a cell density of 10000 cells/cm^2 in FGM.

3.3.1. Human cardiac fibroblast activation assay

HCF were seeded at a density of 36500 cells/cm^2 and incubated in seeding medium (Dulbecco's Modified Eagle's Medium high glucose (DMEM; Thermofisher) supplemented with 10% fetal bovine serum (FBS; Lonza), 1% Penicillin-Streptomycin (P/S; Biowest) and 0.2 mM 2- phospho-L-ascorbic acid (Asc-2P; Sigma Aldrich, Co.)) for 24h. Then, hCF media was changed to working medium (WM) (DMEM supplemented with 0,1% FBS, 1% P/S and 0.2 mM 2Asc-2P. After 48h, cells were incubated in WM either with or without TGFβ at 10 ng/mL (PreproTech). Medium was changed every 48h.

3.3.2. Human cardiac fibroblasts culture with PF from patients

The cells were seeded and cultured as mentioned in section 2.3.1. After 48h in WM, cells media was replaced for WM containing 2% PF. Cells cultured in WM were used as negative control. Medium was changed every 48h.

3.3.3. Human cardiac fibroblasts in hypoxic conditions

HCF were seeded at a density of 36500 cells/cm^2 and incubated in seeding medium for 24h. Then, the media was changed to WM and the cells were incubated for 48h a HERAcell® 150 CO2 incubator (Heraeus®) at 37 ºC in a humidified atmosphere containing 5% CO2 and 1% O_2.

3.3.4. Transfection of human cardiac fibroblasts

HCFs were seeded at density of 15000 cells/cm^2 in FGM during 48h. Then, hCF were incubated during 6 hours with a mix of OptiMEM containing lipofectamine (RNAiMax, Thermofisher™) and the respective micro-RNA of interest that was either precursor miRNA hsa-miR-22-3p (leading strand only) or inhibitor miRNA hsa-miR-22-3p (leading strand only) (Life Technologies™) (Table 2) accordingly with manufacturer's instructions. It was used as control the respective anti miR™ miRNA inhibitor negative Control and pre miR™ miRNA precursor negative Control (Thermofisher™)). After incubation, cells were left overnight in FGM. Subsequently, media was replaced by WM either with or without TGFβ at 10 ng/mL. Medium was changed every 48h.

Table 2 – Mix components according to the transfection protocol

Reaction 1 (µl)	
Lipofectamine RNAiMax	0.3
Opti-MEM	5
Reaction 2 (µl)	
10 µM miRNA (uL)	0.1
Opti-MEM (uL)	5

3.4. RNA extraction and reverse transcription

3.4.1. miRNA extraction from PF and reverse transcription

RNA extraction from PF samples (200 µL) was performed using the miRNeasy® Serum/Plasma Kit (QIAGEN) following manufacturer instructions. During extraction, 3,5 µL of spike-in (cel-miR-39) at 1,6 x 10^8 copies/µL was added to the mixture. RNA was stored at -80 ºC. RNA quantity and quality were assessed using a NanoDropTM 1000

Spectrophotometer (ThermoFisher Scientific). The absorbance at 260 nm was used to calculate RNA concentration and ratios 260/280 and 260/230 nm measured contamination with proteins and phenols, respectively. Reverse transcription was performed to synthetize complementary DNA using miScript® II RT Kit (QIAGEN) or miRCURY® RT Kit (QIAGEN) according to manufacturer instructions as shown in Tables 3 and 4. The thermocycling protocol was run in a T100™ Thermal Cycler (BioRad) according to Table 5. Resulting cDNA was stored at -20ºC.

Tables 3 and 4 – Reverse transcription master mix components

Components	Volume
5x miScript HiSpec Buffer	4 µL
miScript Reverse Transcriptase Mix	2 µL
10x miScript Nucleics Mix	2 µL
Template TRNA	12 µL
Total Volume	20 µL

Components	Volume
5x miRCURY SYBR® Green RT Reaction Buffer	5 µL
10x miRCURY RT Enzyme Mix	1 µL
RNase-free water	1 µL
Template RNA (5 ng/µl)	2 µL
Total Volume	10 µL

Table 5– Thermocycling protocol for the synthesis of miRNA cDNA

Step	Time	Temperature (ºC)
Reverse Transcription	60 min	42
RT Inactivation	5 min	95
Storage	---	4

3.4.2. RNA extraction from cultured hCFs

3.4.2.1. mRNA extraction and reverse transcription

mRNA extraction from cultured hCFs was performed using the QIAzol Reagent (Qiagen) following manufacturer instructions. Reverse transcription was performed to synthetize complementary DNA using PrimeScript RT reagent Kit (Takara) according to manufacturer instructions as shown in Table 6. The thermocycling protocol was run in a T100™ Thermal Cycler (BioRad) according to Table 7. Resulting cDNA was diluted 1:5 in nuclease-free water and stored at -20ºC.

Table 6 – Reverse transcription master mix components.

Components	Volume
5X PrimeScript Buffer (for Real Time)	2 µL
PrimeScript RT Enzyme Mix I 1	0.5 µL
Oligo dT Primer (50 µM)	0.5 µL
Random 6 mers (100 µM)	0.5 µL
Template RNA	6.5 µL
Total Volume	10 µL

Table 7– Thermocycling protocol for the synthesis of mRNA cDNA

Step	Time	Temperature (ºC)
Reverse Transcription	15 min	38
RT Inactivation	5 sec	85
Storage	---	4

3.4.2.2. miRNA extraction and reverse transcription

miRNA extraction from cultured hCFs was performed using the miRNeasy® Mini Kit (QIAGEN) following manufacturer instructions. RNA quantity and quality were assessed using a NanoDropTM 1000 Spectrophotometer (ThermoFisher Scientific) and stored at -80 ºC. The absorbance at 260 nm was used to calculate RNA concentration and ratios 260/280 and 260/230 nm measured contamination with proteins and phenols, respectively. Reverse transcription was performed to synthetize complementary DNA using miScript® II RT Kit (QIAGEN) or miRCURY® RT Kit

(QIAGEN) according to manufacturer instructions as shown in Table 8 and 9. The thermocycling protocol was run in a T100™ Thermal Cycler (BioRad) according to Table 10. Resulting cDNA was stored at -20ºC.

Table 8 and 9 – Reverse transcription master mix components

Components	Volume
5x miScript HiSpec Buffer	4 µL
miScript Reverse Transcriptase Mix	2 µL
10x miScript Nucleics Mix	2 µL
Template TRNA	12 µL
Total Volume	20 µL

Components	Volume
5x miRCURY SYBR® Green RT Reaction Buffer	5 µL
10x miRCURY RT Enzyme Mix	1 µL
RNase-free water	1 µL
Template RNA (5 ng/µl)	2 µL
Total Volume	10 µL

Table 10– Thermocycling protocol for the synthesis of mRNA cDNA

Step	Time	Temperature (ºC)
Reverse Transcription	60 min	42
RT Inactivation	5 min	95
Storage	---	4

3.4.3. Real-time PCR (RT-PCR)

To detect miRNA, qRT-PCR was performed using the miScript® SYBR Green PCR Kit (Qiagen) and the miRCURY® SYBR Green PCR Kit (Qiagen). A master mix was prepared without cDNA as shown in Tables 11 and 12. The cDNA was then added to the wells, and the qPCR low plate was covered by a sealing tape to prevent evaporation of the reaction mix, centrifuged at 1000g for 1 min and placed in a CFX96 TouchTM Real-Time PCR Detection System (Biorad). The programmed PCR cycling was run according to manufacturer instructions and is detailed in Table 13.

Tables 11, 12 – qRT-PCR master mix components.

Components	Volume
2x QuantiTect SYBR Green PCR Master Mix	5 µL
10x miScript Universal Prime	1 µL
10x miScript Primer Assay	1 µL
RNase-free water cDNA	2 µL
cDNA	1 µL
Total Volume	10 µL

Components	Volume
2x miRCURY SYBR® Green Master Mix	5 µL
Resuspended PCR primer mix	1 µL
RNase-free water	1 µL
cDNA (1:30)	3 µL
Total Volume	10 µL

Table 13– Thermocycling protocol for qRT-PCR run

Step		Time	Temperature (ºC)
PCR initial heat activation		120 sec	95
2-step cycling Nb of cycles: 40	Denaturation	10 sec	95
	Annealing	60 sec	56
Melting Curve		---	60-95

To quantify mRNA expression, qRT-PCR was performed using iTaqTM Universal SYBR Green Supermix (Bio-Rad) accordingly with manufacturer's instructions. A master mix was prepared without cDNA as shown in Table 14. The cDNA was then added to the wells, and the qPCR low plate was covered by a sealing tape to prevent evaporation of the reaction mix, centrifuged at 1000g for 1 min and placed in a CFX96 TouchTM Real-Time PCR Detection System (Biorad). The programmed PCR cycling was run according to manufacturer instructions as detailed in Table 15. Primer sequences are available in Table 16.

Table 14– qRT-PCR master mix components

Components	Volume
iTaq Universal SYBR Green Supermix	5µL
Ressuspended Primer Mix (10mM)	0.8µL
cDNA	1 µL
RNase-free water	3.2 µL

Table 15- Thermocycling protocol for the qRT-PCR run

Step		Time	Temperature (ºC)
PCR initial heat activation		3:30 min	95
2-step cycling Nb of cycles: 40	Denaturation	20 sec	95
	Annealing	30 sec	60
Melting Curve		---	55-95

Table 16- Primer sequences for reverse and forward primers of each cDNA

Gene	Primer Sequence (5' to 3')
ACTA2	RV: GTGGTTTCATGGATGCCAGC FW: GGCAAGTGATCACCATCGGA
COL1α1	RV: CAGATCACGTCATCGCACAAC FW: GAGGGCCAAAGACGAAGACATC
CCN2	RV: CCGTCGGTACATACTCCACAGA FW: CTTGCGAAGCTGACCTGCAAGA
FAP	RV: CAGTGTGAGTGCTCTCATTGTA FW: GCTACGATGGAGGCGCTAAT
GAPDH	RV: GGACTCCCCAGCAGTG FW: CCTCCACCTTTGACGCT

3.5. RNA Sequencing (RNA-Seq) analysis

miRNA was extracted from PF described in section 2.4.2.1. Quality of RNA was assessed using a 2100 BioAnalyzer (Agilent). Samples were then sequenced using a HiSeq 2000 (Illumina) sequencer.RNA sequencing was performed at the GeneCore Scientific Platform of EMBL, Germany. Reads were then aligned to mature miRs using the bowtie2 alignment software. Unmapped reads are further aligned to the whole genome to rescue miRbase unaligned reads and count other RNA molecules.

Differential expression analysis was performed between patient cohorts. To be considered differently expressed, the normalized counts had to have a fold change (FC) of 1,5 between sample groups and the false discovery rate (FDR) adjusted p-value had to be lower than 0,05. Finally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that might be altered by the differently expressed miRs were identified. To verify the latter, the database DIANA Tarbase v8was used. Heatmaps were generated using the Heatmapper web server.

3.6. Immunofluorescence assay

hCFs were fixed with 4% paraformaldehyde PBS pH=7,4 for 12 min, and rinsed twice with PBS pH=7,4 (3min, RT). hCFs were further permeabilized with 0,1% Triton X-100 (Sigma Aldrich, Co.) PBS for 5 min, rinsed twice with PBS pH=7,4 (3min, RT), and blocked with 4% FBS/1% BSA PBS blocking solution for 1 hour at room temperature. The hCFs were then incubated with an anti-human α-SMA primary antibody (A5228, Sigma Aldrich, Co.) diluted 1:400 in blocking solution overnight at 4º C. hCFs were then rinsed twice with PBS pH=7,4 (3min, RT), incubated with Alexa Fluor® 594 donkey anti-mouse (A21203, Invitrogen) 1:1000 in blocking solution for 1 h and rinsed again as mentioned before. Following this incubation, cells were rinsed twice (3min, RT) with 0,1%Tween PBS (PBST) and incubated with DAPI 1:1000 for nuclei staining (5min, RT). After the last two washes with PBS (3min, RT), the cells were kept in PBS pH=7,4 protected from light until image acquisition using an IN Cell Analyzer 2000 (GE Healthcare Life Sciences, USA) with Nikon 20X/0.45 NA Plan Fluor objective and the 4,6-diamidino-2-phenylindole (DAPI), Texas Red and FITC filters. The number of cells per field of view was counted using Fiji software (ImageJ version 1.51n, NIH, USA). The quantification of collagen area was performed using Cell Profiler (Broad Institute, USA).

3.7. Statistical Analysis Data

Statistical analysis was done using GraphPad® Prism 8 software. Outliers were excluded by ROUT analysis, and normality of distribution was evaluated by D'Agostino-Pearson and Shapiro-Wilk test. Parametric (ANOVA for normal distribution sets of samples) or non-parametric tests (Kruskal-Wallis test for non-normal distribution sets of samples) were used to test all the obtained data. The statistical significance level chosen for all statistical tests was $p<0,05$.

4. Results

4.1. Establishment of an in vitro model to access human cardiac fibroblast activation

Owing to our preliminary data (Section 2) showing that after MI PF concentrates fibrosis-associated molecules, one can hypothesize that PF may impact the heart, as part of a feedback loop, by regulating cardiac fibroblast activation. To assess that, we developed an assay for measuring the activation of human cardiac fibroblasts (hCFs) *in vitro* in which TGF-β1 (from this point onwards referred as TGF-β) was used as a positive control. hCFs were cultured in the presence or absence of TGF-β, as detailed in Figure 5(A), and gene expression was monitored by qRT-PCR at different time points. The expression of *ACTA2*, *COL1A1*, *CCN2* and *FAP* was upregulated in hCFs cultured in response to TGF-β at all days, with α-SMA reaching significant differences at day 3 and 5 when compared to untreated cells (Figure 5(B)). To further confirm hCF activation at the culture endpoint (day7), the protein levels of α-SMA and α-1 type I collagen (α1Col I) were assessed by immunofluorescence. As expected, both activation markers were increased following TGF-β treatment (Figure 5(C) and 5(D)). In addition, TGF-β-treated hCFs were morphologically distinct from cells cultured in the absence of this growth factor, displaying well-pronounced and prominent actin stress fibers, and a larger, polygonal shape akin to the described shape of myofibroblasts (Frangogiannis 2015).

4.2. Pericardial fluid from STEMI patients triggers human cardiac fibroblast activation

To further evaluate whether PF could impact on hCF activation, the latter were challenged with PF (2% v/v) collected from our patient cohort for 72 hours (Table 1 and Figure 6(A)). Similarly, to what was observed in TGF-β-treated cells, a fraction of fibroblasts cultured in the presence of PF from the STEMI group up-regulated the expression of α-SMA and became large and polygonal, when compared to the CTRL and NSTEMI groups and cells cultured in basal media which were smaller and elongated (Figure 6(B)). Although the expression of *COL1A1* was not affected, the expression of *ACTA2* and *CCN2* showed a tendency to be upregulated, reaching statistical significance between the STEMI cohort and the two other patient groups in the case of *ACTA2* (Figure 6(C)). These findings support that PF collected from MI

patients may stimulate the differentiation of fibroblasts into myofibroblasts, contributing to the profibrotic environment created after MI.

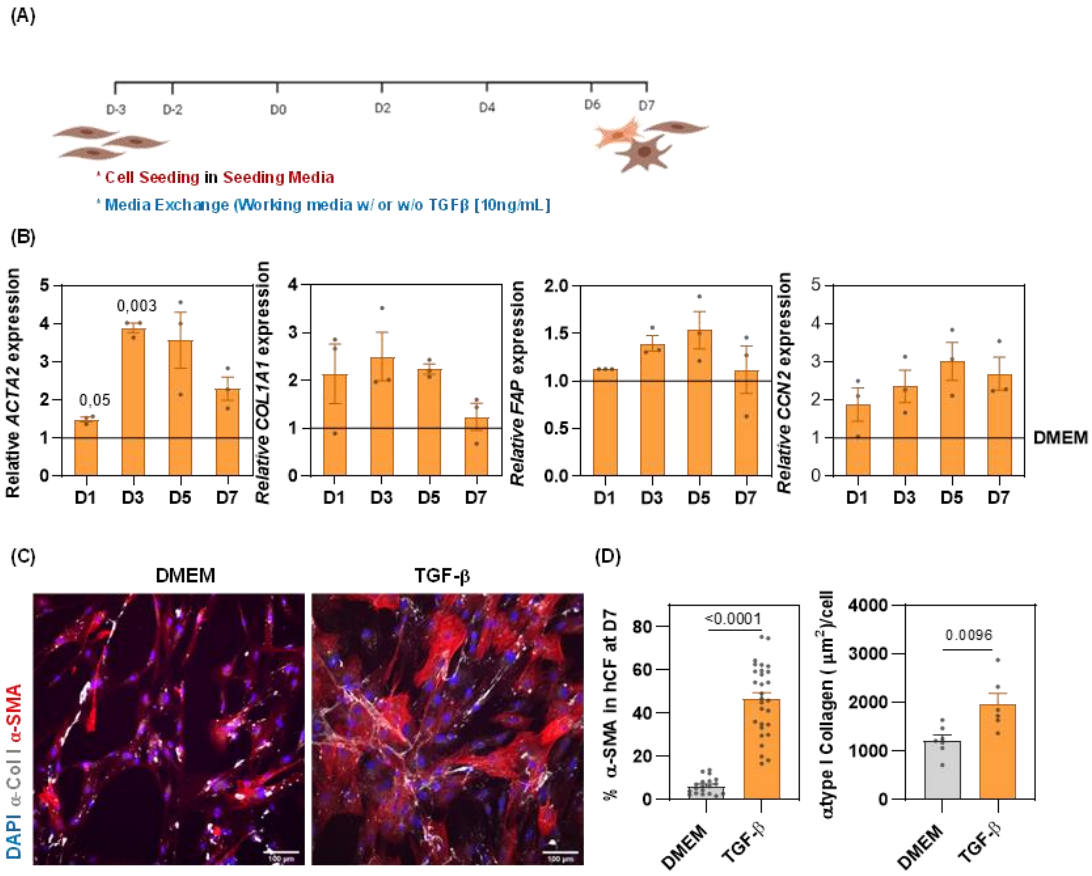

Figure 5 - Establishment of an in vitro assay to assess human cardiac fibroblast (hCF) activation.
(A) Schematic representation of the experimental design. **(B)** Expression of ACTA2, COL1A1, CCN2 and FAP at different time points. Data presented as fold-change relative to the negative control (DMEM) (Mean±SD) of three independent experiences (n=3); normalizing gene: GAPDH. **(C)** Representative images (scale: 100 µM) and subsequent quantification. **(D)** of α-SMA and α-1 type I collagen (α-Col I) immunostaining of hCFs following at 7 days of an activation assay (representative experiment of 2 and 1 independent experiments for α-SMA and α-Col I, respectively.

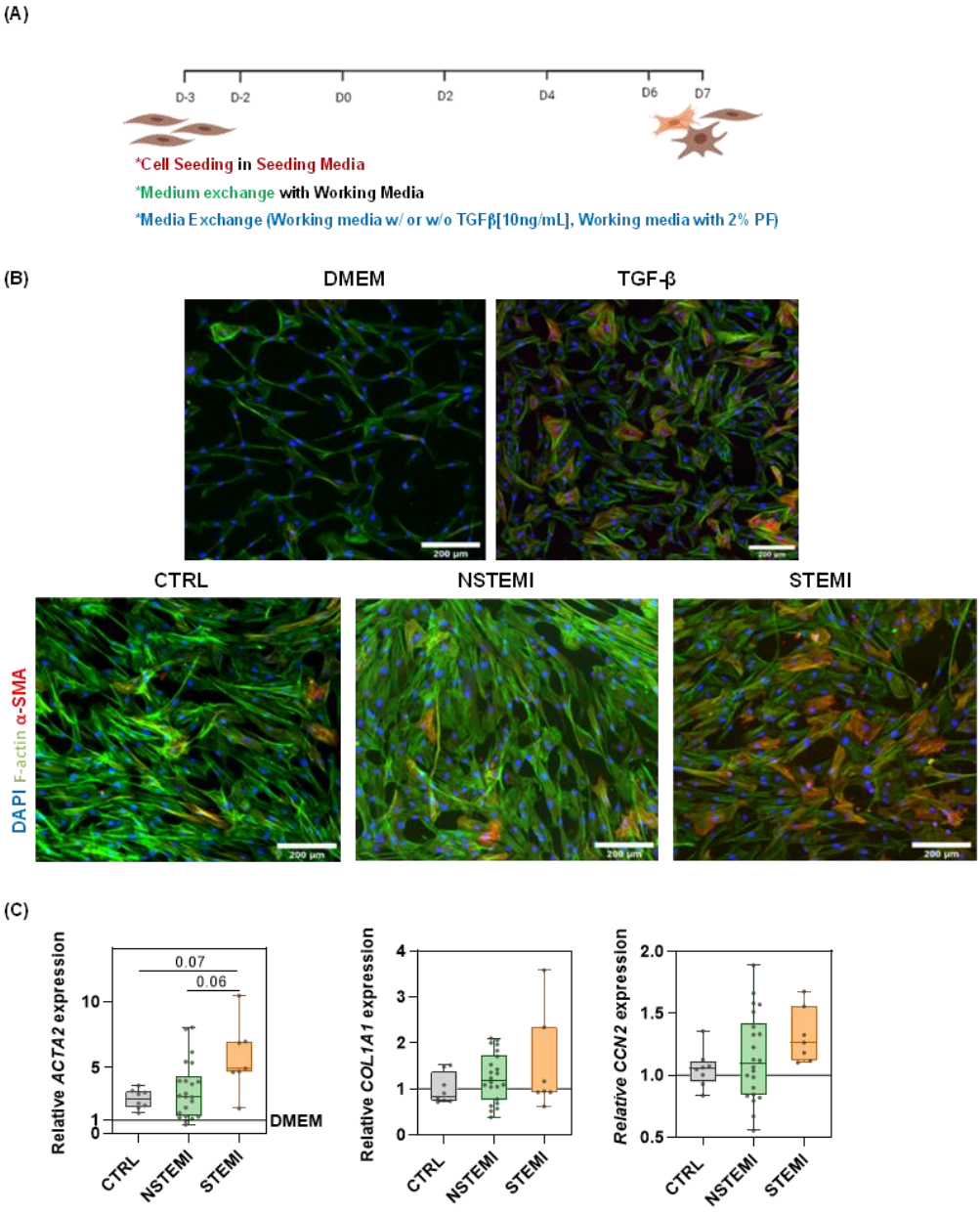

Figure 6 - Pericardial fluid from STEMI patients induces higher activation of hCF. **(A**) Schematic representation of the experimental design. **(B)** Representative images of α-SMA (red) and F-actin (green) immunostaining coupled with DAPI of human cardiac fibroblasts cultured with 2% PF from patients during 3 days (scale bar= 200 µm; CTRL and MI n=3). **(C)** Expression of ACTA2, COL1A1 and CCN2 in hCFs cultured for 72 hours in 2% pericardial fluid from each patient cohort. Expression is presented through the 2-ΔCt method, normalized to the housekeeping gene GAPDH (CTRL n=8; NSTEMI n=22; STEMI n=7).

4.3. RNA-sequencing reveals that fibrosis-related miRNAs are more abundant in the pericardial fluid of STEMI patients

The constitution of PF has been previously pointed out as a source of biomolecules of interest in a myriad of cardiovascular diseases (Trindade et al. 2019). Herein, by small RNA sequencing, we characterized the miRNome of PF collected from our patient cohorts (Table 1) to identify relevant candidates in the pathophysiology of MI. From the array of identified miRNAs, the 50 most abundant in all groups were selected and Kegg pathways prediction analysis identified extracellular matrix (ECM) receptor interactions, supporting the hypothesis that PF-contained miRNAs are possibly involved in the regulation of ECM synthesis (Figures 7(A) and 7(B)). Eleven miRNAs were differentially regulated in STEMI when compared to CTRL patients, of which 9 were upregulated and 2 were less abundant (Figure 7(C)). Kegg pathways analysis on this assortment of targets showed TGF-β signaling as one of the altered pathways (Figure 7(D)). Of note, four of the identified miRNAs have had previously established ties with cardiac fibroblast activation (miR-21-5p (Zhou et al. 2018), miR-30e-5p (Chen, Yin, and Jiang 2021), miR-203-3p (He et al. 2017), miR-22-3p (Zhao et al. 2020)). Overall, these findings demonstrate that PF works as a reservoir of miRNAs known to regulate cardiac fibrosis and whose abundance is altered upon acute ischemic events, such as MI.

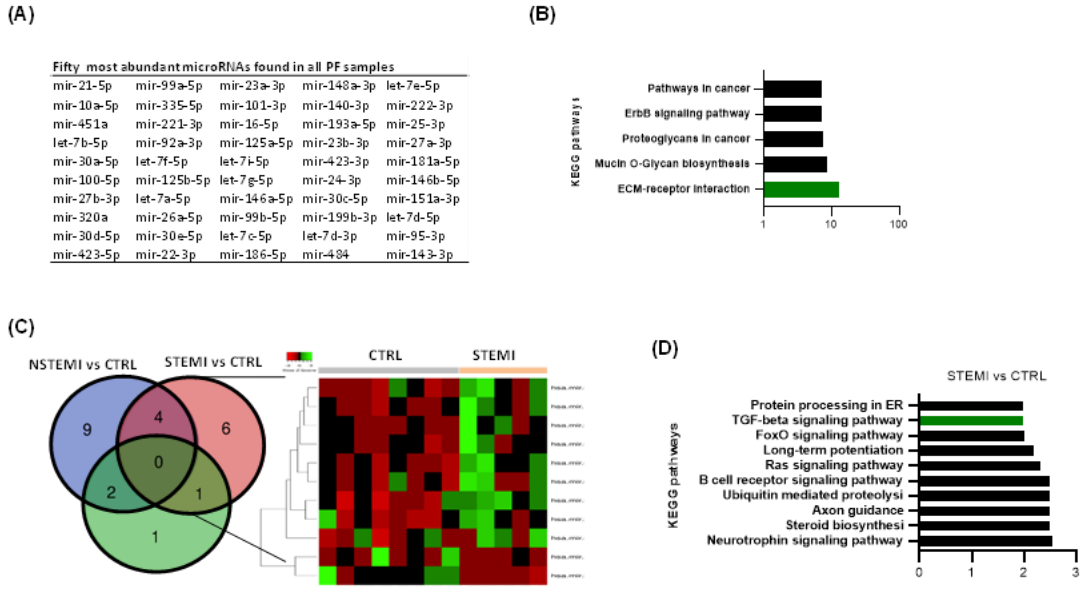

Figure 7 – Small RNA sequencing unveils that the miRNome of pericardial fluid is altered after MI.
(A) Fifty most abundant miRNAs identified in all PF samples by small RNA-sequencing (n=8, CTRL; n= 9 NSTEMI; n=5 STEMI). **(B)** KEGG pathways of altered miRNAs in STEMI patients when compared to CTRL. In green, the KEGG pathways relevant for

cardiac fibrosis and the respective miRs involved are highlighted (right). **(C)** Venn diagram with the number and intersection of differentially expressed genes in NSTEMI vs CTRL, STEMI vs CTRL and STEMI vs NSTEMI. Heat-map with the relevant altered miRNAs regarding the comparison between CTRL vs STEMI. **(D)** KEGG pathway predictions for increased the 11 miRNAs identified in STEMI group.

MiR-203a-3p and miR-22-3p were selected for validation by qPCR due to the scarcity of previous work describing their role in the context of MI-induced cardiac fibrosis. For that, and because there is limited information regarding the best miRNA for normalization in the PF, we resort to RNA-sequencing and bibliography and selected miR-99a-5p (MaClellan et al. 2014) as a normalizer miRNA. qRT-PCR demonstrated that miR-203-3p was present in the PF in lower amounts when compared to miR-22-3p (Figure 8(A)). Of note, only a tendency was observed for higher miR-203-3p levels in both MI groups, when compared to controls. Differently, miR-22-3p was significantly upregulated in the STEMI group and thus we decided to further investigate the source and role of this miRNA in the context of cardiac fibrosis.

We started by analysis the expression of miR-22-3p on hCF in vitro either in response to TGF-β stimulation as well as in response to hypoxia, two conditions that resident myocardial fibroblast are subjected to upon acute ischemic injury. The expression of miR-22-3p showed a tendency for upregulation, in a time-dependent manner, in TGF-β conditioned cells (Figure 8(B)). A similar upregulation is verified upon hypoxia treatment (Figure 8(C)), supporting that hCFs are contributing to the production miR-22-3p upon a cardiac stress stimuli. However, previous literature describes miR-22 as majorly present in skeletal and cardiac muscle, and reports expression variation during various cardiac stress situations (Huang and Wang 2014). To further investigate the expression of miR-22-3p in the myocardium after MI, we used murine hearts collected 96 hours following left anterior descending coronary artery ligation. The levels of expression were downregulated in the remote and infarcted region after MI, when compared to the respective region of sham-operated animals (Figure 8(D)). This contrasts starkly with the previously evidenced upregulation of miR-22-3p in hCFs and PF of STEMI patients.

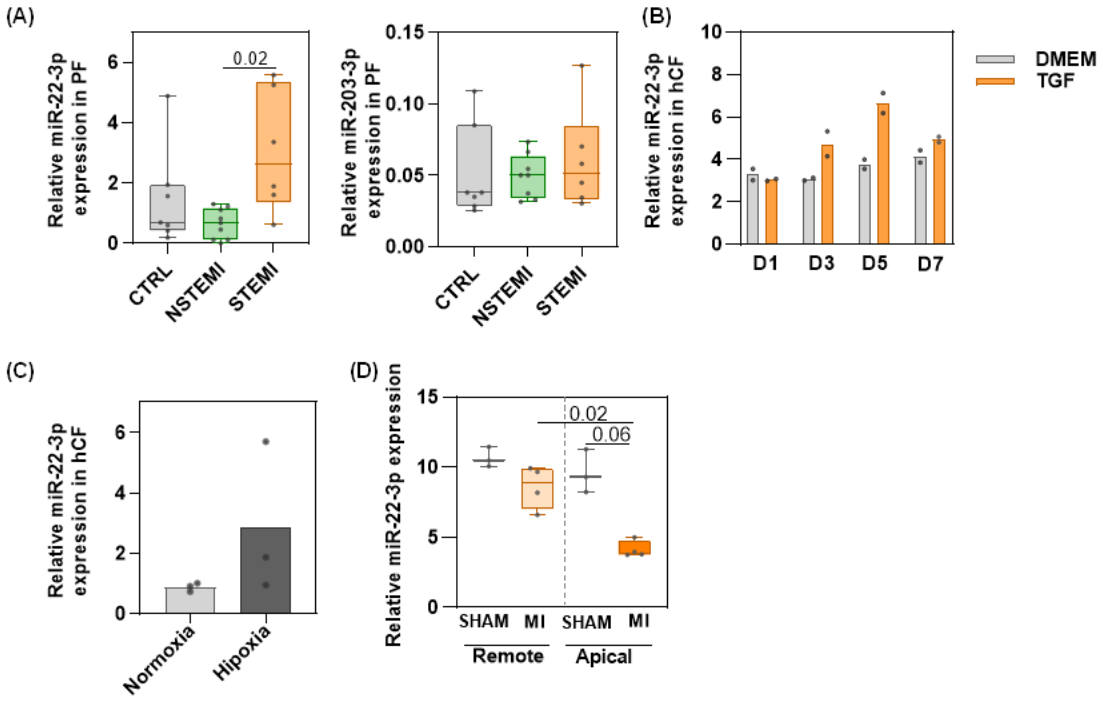

Figure 8 - miR-22-3p is upregulated in pericardial fluid of STEMI patients and downregulated in infarcted murine hearts.
(A) qRT-PCR validation of RNAseq results for mir-22-3p and miR-203-3p. Expression is presented through the $2^{-\Delta Ct}$ method, with normalization to the expression of miR-99a-5p (CTRL n=7; NSTEMI n=9; STEMI n=6) **(B)** miR-22-3p expression at different timepoints during an activation assay (n= 2 independent experiments) **(C)** miR-22-3p expression in hCFs after hypoxia treatment during 48 hours (n=3). **(D)** miR-22-3p expression in basal and apical zones of infarcted murine heart at 4 days post-surgery, normalized to the expression of miR-103a-3p (SHAM n=3; MI n=4).

4.4. miR-22-3p inhibition had no impact on hCFs activation

To assess the impact of miR-22-3p on hCF activation we performed inhibition in the presence and absence of TGF-β (Figure 9(A)). Transfection was optimized by comparing the efficiency of 100 nM and 200 nM of mir-22-3p or control (scrambled) inhibitors (Figure 9(A) and 9(B)). A 3,5-fold (100 nM) and 2-fold (200nM) reduction of miR-22 expression was obtained and, the 100 nM was selected for the following experiments. Of note, in subsequent experiments the expression of miRNA-22 was only slightly reduced 48h after transfection whereas at day 6 the expression was mostly restored (Figure 9(C)), demonstrating that the transfection protocol requires further optimization. Still, we proceeded to evaluate the effect of miR-22-3p inhibition on the expression of activation-associated genes in the presence of absence of TGF-β. No differences were observed in the expression of *ACTA2* and *COL1A1* following miR-22 inhibition (Figure 9(D)).

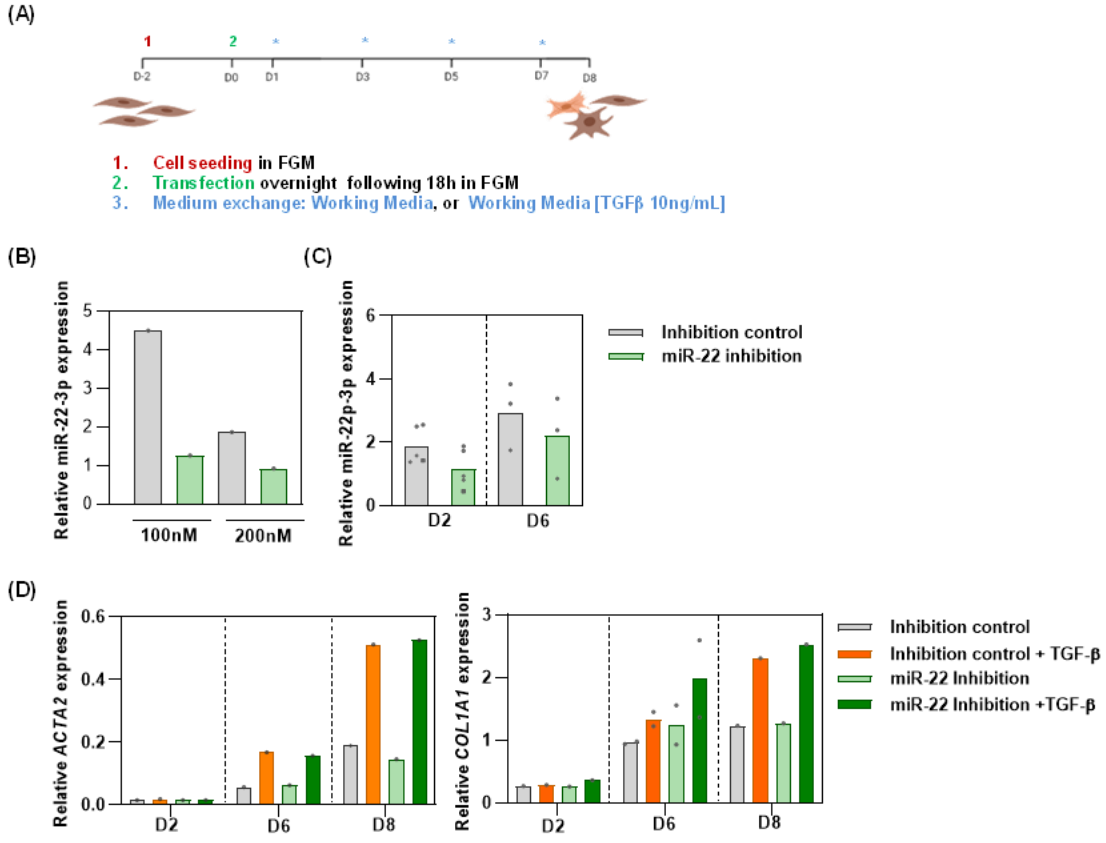

Figure 9 - miR22-3p inhibition is partial and does not affect TGF-β-mediated hCF activation.
(A) Schematic representation of the experimental design. **(B)** Optimization of the transfection protocol for miR-22-3p inhibition (n=1). Expression, calculated by the $2^{-\Delta Ct}$ method, was normalized to the expression of miR-103a-3.p **(C)** Expression of miR-22-3p in transfected hCFs 2 and 6 days post-transfection (n≥3). Expression, calculated by the $2^{-\Delta Ct}$ method, was normalized to the expression of miR-103a-3p. **(D)** Expression of *ACTA2* and *COL1A1* in hCFs during a 7-day activation assay (n≥1). Expression, calculated by the $2^{-\Delta Ct}$ method, was normalized to GAPDH.

4.5. miR-22-3p overexpression abrogates hcFb activation by TGFβ

The upregulation of miR-22-3p in the PF of STEMI patients suggests a functional role during MI. To assess if the accumulation of miR-22-3p around the heart exerts an effect on the cardiac tissue, specifically on the fibroblasts that are at the basis of fibrotic tissue formation, we analyzed the effect of miR-22-3p overexpression on hCFs in the presence and absence of TGF-β (Figure 10(A)). Transfection was optimized by comparing the efficiency of 100 nM and 200 nM of mir-22-3p or control (scrambled) precursors for the leading strand (Figure 10(B)). Overexpression was evidently attained with both conditions and therefore the lowest concentration was selected for the

following experiments. Efficiency of transfection was evaluated at different time points further demonstrating consistent overexpression of miR-32-3p of 8,3-fold and 18-fold at day 2 and 6, respectively (Figure 10(C)). To understand the role of miR-22-3p on hCFs activation, cells were stimulated with TGF-β following induction of miR-22-3p overexpression. Upon TGF-β treatment, miR-22-3p-overexpressing hCFs displayed downregulation of *ACTA2* and *COL1A1* when compared to the precursor control (Figure 10(D)). No evident effect was observed in the expression of *CCN2* (not shown). These results indicate that miR-22 may be protecting hCF from TGF-β-mediated activation. To further confirm this, an immunofluorescence assay for α-SMA microfilaments and α-1 type I collagen was performed on mir-22 precursor-transfected hCFs upon stimulation with TGF-β for 7 days. Both markers were significantly downregulated upon overexpression of miR-22-3p (Figure 10(E) and 10(F)). Of importance, cells displayed a more fusiform elongated shape, retaining a non-activated fibroblast morphology upon miR-22-3p overexpression.

Overall, these results advocate a protective role of miR-22-3p in the TGF-β-mediated hCF activation, advancing it as a possible therapeutic candidate to control cardiac fibrosis in the context of MI.

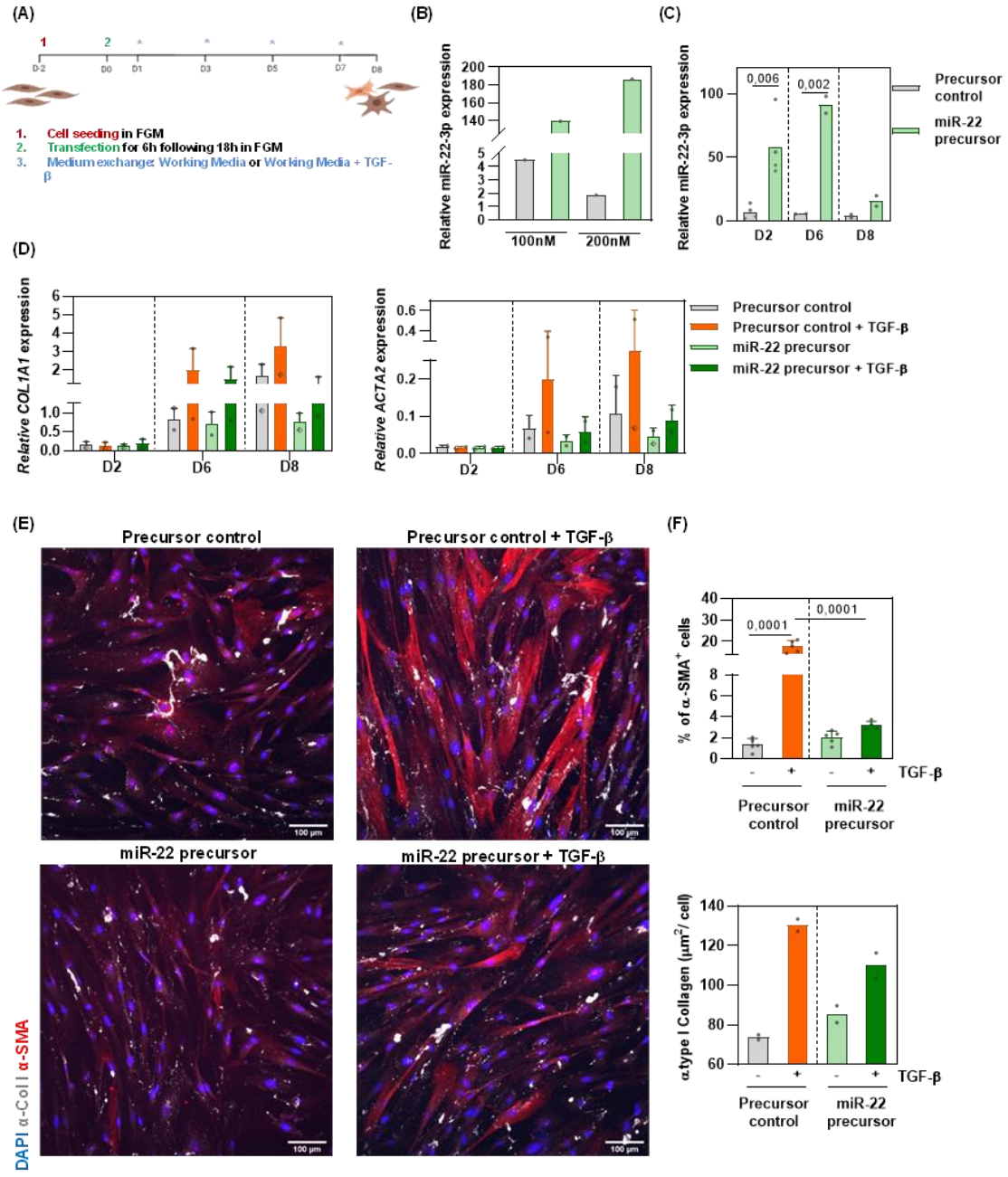

Figure 10 - miR22-3p overexpression inhibits TGF-β-mediated cardiac fibroblast activation.

(A) Schematic representation of the experimental design. **(B)** Expression of miR-22-3p in hCFs at 2, 6 and 8 days post-transfection. Expression is presented through the $2^{-\Delta Ct}$ method, with normalization to the expression of the housekeeping miR-103-3p **(C)** Expression of *ACTA2*, *COL1A1* and *CCN2* in hCFs during a post-transfection 7-day activation assay. Expression, calculated through the $2^{-\Delta Ct}$ method, was normalized to GAPDH (n=2) **(D)** Representative figures of α-smooth muscle actin (α-SMA) and αtype I Collagen (α-Col I) in overexpression-transfected hCFs following a 7-day activation assay. (E) Percentage of α-SMA^{+} cells (n=4) and area of COL1α1 per cell (n=2).

5. Discussion

Most forms of CVD are associated with myocardial fibrosis, characterized by exacerbated production of ECM in the heart. In the case of MI, initial fibrosis is crucial to replace the tissue defect, but excessive fibrosis contributes to progressive functional decline (Hinderer and Schenke-Layland 2019). Hence, early detection, prevention and reversion of cardiac fibrosis are key targets to advance MI management. However, reliable diagnostic biomarkers for early and non-invasive fibrosis detection and therapies able to modify scar properties for patient benefit are two unmet clinical needs.

This work is based on preliminary data showing that PF concentrates miRNAs and secreted molecules associated with ECM remodeling and TGF-β1 signaling. In fact, PF from STEMI patients, when compared to stable coronary artery patients, displayed higher concentration of PICP and ST-2.

Herein, we further show that PF from STEMI patients shows a different content in ECM-associated miRNA and stimulates α-SMA expression in human cardiac fibroblasts, further supporting that this fluid is signaling back to the heart by regulating key pathological mechanisms such as the development of fibrosis. Paradoxically, miR-22-3p levels, a miRNA previously demonstrated to inhibit mouse cardiac fibroblast activation (Hong et al. 2016; Zhao et al. 2020), were upregulated in the PF of STEMI patients. Of interest, this miRNA is downregulated in the infarcted zone of a mouse MI model, whilst its overexpression inhibits TFG-β-mediated human fibroblast activation. Although further investigation is required, these findings advocate that miR-22-3p is released by the heart after MI, accumulates in the PF, further affecting the heart as part of a fibrosis control mechanism.

In the scope of this project, we postulated that PF concentrates fibrosis-associated signals released by the myocardium after MI. In fact, PF has recently emerged as a bioactive fluid whose content, previously been shown to vary with cardiac diseases (Fatehi Hassanabad et al. 2021; Trindade et al. 2019), constituting a valuable source of disease biomarkers and/or therapeutic targets. We found that PF concentrates miRNAs (miR-21, miR-29a and miR-29c) and proteins (PICP and ST-2), compared to plasma of the same patients, indicating that the PF content seems to reflect the cardiac environment with a degree of precision that is unparalleled by plasma. Given the previously established correlation between the aforementioned miRNAs and IHD and cardiac fibrosis, it is reasonable to assume that their higher concentration in the PF

occurs as a result of the preceding myocardial damage (in the case of STEMI and NSTEMI patients) or corresponding affliction (in the case of patients suffering from stable coronary heart disease) and that measurable variations of these biomarkers are more likely to be detected in PF than plasma.

Considering that the PF is a product of plasma ultrafiltration and secretions from heart (either from pericardial mesothelium and myocardium interstitium), along with the reported abundancy of biomarkers of fibrosis in this biofluid, one can hypothesize that PF may be a source of prognostic and diagnostic biomolecules that reflect pathological events as is the case of cardiac fibrosis.

PF from STEMI patients stimulates the expression of activation-related genes *ACTA2* and *CCN2*, when compared to the CTRL and NSTEMI cohorts (Figure 6). This observation supports the argument that PF may contribute to the activation of fibroblasts and subsequently cardiac fibrosis and remodeling upon a transmural ischemic episode. Other works also supported that PF contains bioactive molecules that impact on cardiac cells. For example, PF from ischemia reperfusion human patients modulated the isometric tone of isolated rat carotid arteries (Nemeth 2015). This effect was mitigated, in part, by treatment with an ET-1 antagonist, implying that PF concentration of ET-1 may explain the vasoconstrictive response. This finding is of special value also in the context of fibrosis because ET-1 is an important mediator of fibroblast activation, acting downstream of ANG-II and TGF-β1 in the fibrogenic process (Kong et al. 2014; Wang et al. 2015), and is known to be secreted by failing human hearts (Tsutamoto, Wada, and Maeda 2000). Similarly, exosomes isolated from PF of aortic stenosis patients were shown to promote therapeutic angiogenesis in a mouse model of ischemia, as well as to improve survival, networking and proliferation of cultured endothelial cells (Beltrami et al. 2017). PF also exhibits modulation of cardiac function outside of pathological scenarios, as shown by Miyazaki *et al*, who assessed in 1990 that prostaglandin concentration in PF inhibits efferent sympathetic stimulation by antagonizing ANG-II at presynaptic sites (Miyazaki et al. 1990), reinforcing our assessment of PF as an active modulator of cardiac activity rather than a passive agent.

Of note, PF from NSTEMI patients was unable to stimulate activation at levels above the CTRL. This observation is somehow surprising, as one would expect that NSTEMI cohort PF would present a slightly higher pro-fibrotic potential than the non-infarcted CTRL. However, this could be attributed to unrecognized MI. Indeed, a study on patients with suspected IHD found that the preponderance of undiagnosed MI was 14,8% when assessed by cardiac magnetic resonance (CMR). These MI were not

diagnosed when considering only the clinical history and electrocardiography (Page 2020; Wagner et al. 2003; Wu et al. 2008). As such, the presence of undiagnosed MI can increase the standard deviation of the CTRL and mask results. To address this, we would increase the number of patients in the study and/or include a cohort of patients without previous cardiac afflictions undergoing open-heart surgery, which would be a very rare event.

Analysis of the PF miRNome by RNA-sequencing further corroborated that PF may regulate the extracellular environment of the heart because, when considering the 50 most expressed miRNAs in the PF, pathways pertaining to ECM receptor interactions were predicted to be targeted. While running a cohort comparison, PCA was unable to discriminate groups. In a similar study, Kuosmanen SM, et al. profiled miRNAs from patients with several heart diseases, such as MI, mitral valve insufficiency, aortic stenosis, aortic valve insufficiency and other CVDs and was unable to detect differences in the miRNA content amongst different cardiovascular diseases (Kuosmanen et al. 2015). Yet, this study presented an extended list of miRNAs abundant in the PF, from which the most abundant miRs were miR-21-5p, miR-451a, miR-125b-5p, let-7b-5p and miR-16-5p. Each of these miRNAs was also amongst the most concentrated miRNAs in our analysis.

Herein, although PCA was not able to segregate groups, a small subset of miRNAs differentially regulated amongst the different cohorts was found. Focusing our attention on the differences between STEMI and CTRL patients, we identified 11 differentially regulated miRNAs associated with the regulation of TGFβ pathways. Four of these selected miRNAs have previous experimental data correlating them to cardiac fibroblast activation regulation: miR-21-5p, miR-30e-5p, miR-203-3p and miR-22-3p (Chen et al. 2021; He et al. 2017; Yuan et al. 2017; Zhao et al. 2020; Zhou et al. 2018)).

The increase in the relative amount of miR-22-3p in the PF from STEMI patients was further confirmed by qPCR and this mRNA was selected for further investigation as its biological role in the human heart was yet to be further defined. It is mainly expressed in cardiomyocytes and plays an important role in the regulation of hypertrophic response, sarcomere organization and metabolic shift during cardiac remodeling (Huang and Wang 2014). Regarding murine cardiac fibroblasts, miR-22-3p was validated as a direct inhibitor of osteoglycin (OGN) (Gurha et al. 2012; Jazbutyte et al. 2013), of PTFAR (Zhao et al. 2020), TGFβR1 (Hong et al. 2016) and Cav3 (Zhang et al. 2018). Hence, its overall role in cardiac fibroblasts *in vivo* is still a matter of discussion. In addition, miRNA-22 had previously been shown to be increased in the

plasma of acute MI patients (Maciejak et al. 2016; Wang, Wang, and Li 2019). Likewise, miR-22 has shown to be increased in serum of heart failure patients in two separate studies (Boven et al. 2017; Goren et al. 2012). Of special relevance, we should mention that while we identified miR-22-3p in PF of ischemic patients, the aforementioned studies report an increase in miR-22-5p in plasma. These two miRNAS originate from the same hairpin structure (Maciejak et al. 2016), advocating that the corresponding miR-22-3p could present a similar expression.

miR-22-3p expression in two different pro-fibrotic environments (human cardiac fibroblasts subjected to *in vitro* activation assays and a MI-heart cell extract from murine hearts subjected to LAD coronary artery ligation) produced interesting, yet conflicting results. hCFs show upregulation of miR-22-3p expression during TGF-β-mediated activation whereas miR-22-3p is clearly downregulated in the infarcted myocardium of murine hearts.

The elevated expression of miR-22-3p in PF of the STEMI patients, whose ischemic damage is considerably superior to CTRL and NSTEMI cohorts, can therefore be justified in multiple ways, which require further insight.

One possible explanation for the apparent contradictory results on miRNA accumulation after MI could be related to passive release of this miRNA upon ischemia-induced cardiomyocyte death. In fact, cardiomyocytes have been shown to be the main source of miR-22 in the heart (Huang and Wang 2014) and to increase miR-22 expression when subjected to hypoxia/reoxygenation damage. In this scenario, this miRNA would accumulate in the PF, whereas the expression in the infarcted myocardium would be reduced as a result of cardiomyocyte death/clearance.

miRNA-22 had previously been appointed as having a role in cardiac remodeling in mice. Resorting to overexpression, our data demonstrates miR-22-3p protects hCFs from TGF-β-mediated activation. These results are in line with previously described research, where overexpression of miR-22-3p decreases cell migration and collagen deposition, while inhibiting proliferation of mouse cardiac fibroblasts upon Angiotensin-II treatment (Zhao et al. 2020). In the same study, Zhao *et al* speculated that this effect was attributed to direct inhibition of PTFAR. Inhibition of miR-22 produced no effect on hCF activation, even in the presence of TGF-β. This dissonance between overexpression and inhibition of miRNA-22 can be attributed to different factors. For example, the efficiency of mir-22 downregulation was low (on average of 0.58-fold compared to the negative control) which may not be enough to obtain a functional effect. Alternatively, this lack of response on the part of hCFs could hint at a redundant mechanism that prevents exacerbated activation in fibroblasts. However, AMO-22-

mediated inhibition of miR-22 was previously found to increase fibrillar collagen synthesis significantly in murine cardiac fibroblasts (Hong et al. 2016). To probe into this hypothesis, further research is required.

Inconsistently, overexpression of miR-22-3p in hCFs was reported to target osteoglycin synthesis *in vitro*, thus promoting senescence and fibroblast activation, and was demonstrated to promote cardiac fibrosis in ageing hearts (Jazbutyte et al. 2013). miR-22 upregulation during cardiac ageing in mice and its apparently contradictory role in hCF activation is a possible hint at the relevance of timing regarding the effect of this miRNA, and how its interaction with the surrounding environment and stimuli might alter its role. A different study also reported that *in vitro* miR-22-3p overexpression promotes murine cardiac fibroblast activation through direct inhibition of caveolin-3 (Zhang et al. 2018). Interestingly, in this latter study, cardiac-fibroblast specific expression of miR-22-3p was upregulated during ANG-II treatment, akin to our observations in hCFs. Also worthy of note, while our results and those of Hong and colleagues (Hong et al. 2016) report a clear downregulation of miR-22-3p on infarct zone upon ischemic injury, Zhang *et al* reports an upregulation. This could be justified by the timing of data collection, as both our data and that of Hong pertains to 3 and 7 days post-ligation, respectively, whereas the data from Zhang was obtained at 4 weeks post-ligation.

Herein, by combining RNA-sequencing with robust *in vitro* models we were able to demonstrate that the PF of STEMI patients is fibrogenic and concentrates a series of miRNA involved in the regulation of cardiac fibrosis. Paradoxically, miRNA-22-3p was more abundant in the PF of STEMI patients whilst seeming to protect hCF from TGF-β-mediated activation. Hence, several questions still remain to be answered: which cells are contributing to the observed accumulation of miR-22 in the PF after MI? What other biological components are present in the PF that may be responsible for its pro-fibrogenic effect? Still, this work constitutes a proof-of-principal study demonstrating that PF is a bioactive fluid that in disease circumstances accumulates biological components that impact back in heart cells, regulating important pathological mechanisms such as cardiac fibrosis.

6. Concluding Remarks and Future Directions

Our results support that miR-22-3p exerts an anti-fibrotic effect on hCFs, preventing their activation and subsequent formation of α-smooth muscle actin microfilaments and exacerbated production of fibrillar collagens. Furthermore, miRNA-22-3p upregulation in the PF of of STEMI patients comes as valuable data, allowing us to pose relevant questions regarding whether results of passive diffusion to this fluid (for example resulting from shared content from dead cardiomyocytes), or as a programmed mechanism of active secretion. Alongside, our data advances PF as a potential source of fibrosis-associated biomarkers.

Nonetheless, our research is far from over. While *in vitro* models are undoubtedly useful in understanding interactions at cellular and molecular level, their validity in representing a biological system is far from perfect. The used *in vitro* 2-D cell culture does not include interactions between different populations nor with the extracellular environment. Actually, the use of a matrix for cell adhesion *in vitro* has been shown to modulate mechano-transduction signaling, such as the PI3K/AKT pathway (Wang et al. 2013). Advances in this area have steadily been made, with the creation of polyethilene glycol-based hydrogels, which emulate the elasticity of the myocardium (Wang et al. 2013). On the other end, animal models can only partially eliminate these hurdles to our research, since the pathophysiology varies from species to species, and seldom does it reflect the human pathology faithfully.

In this project, we aimed to use PF to narrow our prospective list of theranostic targets to molecules in close relation with the cardiac environment. While this constitute the main advantage of using this biofluid when compared to the use of serum/plasma, the availability and variability of PF harvest is limited because harvesting requires open-heart surgery. To implement routine collection of PF as a means of constructing a systematic biobank with associated clinical data would be a tremendous step towards the identification possible theranostic targets for CVD management.

7. Bibliography

Auer, Johann, Thomas Weber, and Bernd Eber. 2004. “Intensive versus Moderate Lipid Lowering with Statins after Acute Coronary Syndromes.” *The New England Journal of Medicine* 351(7):714—7; author reply 714—7.

Bader, Michael. 2002. “Role of the Local Renin ± Angiotensin System in Cardiac Damage : A Minireview Focussing on Transgenic Animal Models.” 1462.

Bang, Batkai S., Dangwal S., Gupta S.K., Fischer Foinquinos A., Holzmann A., Just A., Remke J., Zimmer K., Zeug A., Ponimaskin E., Schmiedl Schober A., Yin X., Mayr M., Halder R., Fischer Foinquinos A., Engelhardt S., Wei Y., Schmiedl Schober A., Fiedler J., Thum T., Claudia Bang, Sandor Batkai, Seema Dangwal, Shashi Kumar Gupta, Ariana Foinquinos, Angelika Holzmann, Annette Just, Janet Remke, Karina Zimmer, Andre Zeug, Evgeni Ponimaskin, Andreas Schmiedl, Xiaoke Yin, Manuel Mayr, Rashi Halder, Andre Fischer, Stefan Engelhardt, Yuanyuan Wei, Andreas Schober, Jan Fiedler, and Thomas Thum. 2014. “Cardiac Fibroblast-Derived MicroRNA Passenger Strand-Enriched Exosomes Mediate Cardiomyocyte Hypertrophy.” *Journal of Clinical Investigation* 124(5):2136–46.

Beltrami, Cristina, Marie Besnier, Saran Shantikumar, Andrew I. U. Shearn, Cha Rajakaruna, Abas Laftah, Fausto Sessa, Gaia Spinetti, Enrico Petretto, Gianni D. Angelini, and Costanza Emanueli. 2017. “Human Pericardial Fluid Contains Exosomes Enriched with Cardiovascular-Expressed MicroRNAs and Promotes Therapeutic Angiogenesis.” *Molecular Therapy* 25(3):679–93.

Berk, Bradford C., Keigi Fujiwara, and Stephanie Lehoux. 2007. “ECM Remodeling in Hypertensive Heart Disease Bradford C. Berk.” *J Clin Invest* 117(3):568.

Biernacka, Anna, Marcin Dobaczewski, and Nikolaos G. Frangogiannis. 2011. “TGF-β Signaling in Fibrosis.” *Growth Factors* 29(5):196–202.

Blaufarb, Ira S. 1996. “The Renin-Angiotensin S Stem in Left Ventricular Remo Ar Cling.” 9149(96).

Boulanger, B., Z. Yuan, M. Flessner, J. Hay, and M. Johnston. 1999. “Pericardial Fluid Absorption into Lymphatic Vessels in Sheep.” *Microvascular Research* 57(2):174–86.

Boven, Nick Van, K. Martijn Akkerhuis, Sharda S. Anroedh, Dimitris Rizopoulos, Yigal Pinto, Linda C. Battes, Hans L. Hillege, Kadir C. Caliskan, Tjeerd Germans, Olivier C. Manintveld, Jan-hein Cornel, Alina A. Constantinescu, Eric Boersma, Victor A. Umans, and Isabella Kardys. 2017. “Serially Measured Circulating MiR-22-3p Is a Biomarker for Adverse Clinical Outcome in Patients with Chronic Heart Failure : The Bio-SHiFT Study.” *International Journal of Cardiology* 235:124–32.

Braunwald, Eugene, James E. Muller, Robert A. Kloner, and Peter R. Maroko. 1983. “Role of Beta-Adrenergic Blockade in the Therapy of Patients with Myocardial Infarction.” *The American Journal of Medicine* 74(1):113–23.

Brønnum, Hasse, Ditte C. Andersen, Mikael Schneider, Maria B. Sandberg, and Tilde Eskildsen. 2013. “MiR-21 Promotes Fibrogenic Epithelial-to-Mesenchymal Transition of Epicardial Mesothelial Cells Involving Programmed Cell Death 4 and Sprouty-1.” 8(2):1–13.

Brown, R. Dale, S. Kelly Ambler, M. Darren Mitchell, and Carlin S. Long. 2005. “The Cardiac Fibroblast: Therapeutic Target in Myocardial Remodeling and Failure.” *Annual Review of Pharmacology and Toxicology* 45(1):657–87.

Brutsaert, Dirk L. 2003. “Cardiac Endothelial-Myocardial Signaling: Its Role in Cardiac Growth, Contractile Performance, and Rhythmicity.” *Physiological Reviews* 83(1):59–115.

Bujak, Marcin, Marcin Dobaczewski, Khaled Chatila, Leonardo H. Mendoza, Na Li, Anilkumar Reddy, and Nikolaos G. Frangogiannis. 2008. “Interleukin-1 Receptor Type I Signaling Critically Regulates Infarct Healing and Cardiac Remodeling.” 173(1):57–67.

Bujak, Marcin, Guofeng Ren, Hyuk Jung Kweon, Marcin Dobaczewski, Anilkumar Reddy, George Taffet, Xiao Fan Wang, and Nikolaos G. Frangogiannis. 2007. “Essential Role of Smad3 in Infarct Healing and in the Pathogenesis of Cardiac Remodeling.” *Circulation* 116(19):2127–38.

Cavalera, Michele, Junhong Wang, and Nikolaos G. Frangogiannis. 2014. “Obesity, Metabolic Dysfunction, and Cardiac Fibrosis: Pathophysiological Pathways, Molecular Mechanisms, and Therapeutic Opportunities.” *Translational Research* 164(4):323–35.

Chen, Yongli, Yan Yin, and Hua Jiang. 2021. “MiR-30e-5p Alleviates Inflammation and Cardiac Dysfunction After Myocardial Infarction Through Targeting PTEN.” 44(2).
Creemers, Esther E., and Eva Van Rooij. 2016. “Function and Therapeutic Potential of Noncoding RNAs in Cardiac Fibrosis.” *Circulation Research* 118(1):108–18.
Dalmay, Tamas. 2013. “Mechanism of MiRNA-Mediated Repression of MRNA Translation” edited by M. A. Lindsay and S. Griffiths-Jones. *Essays in Biochemistry* 54:29–38.
Derynck, Rik, and Ying E. Zhang. 2003. “Smad-Dependent and Smad-Independent Pathways in TGF-.” 4.
Disertori, Marcello, Michela Masè, and Flavia Ravelli. 2017. “Myocardial Fibrosis Predicts Ventricular Tachyarrhythmias.” *Trends in Cardiovascular Medicine* 27(5):363–72.
Dobaczewski, Marcin, Spyridon Akrivakis, Kamal Nasser, Lloyd H. Michael, Mark L. Entman, and Nikolaos G. Frangogiannis. 2004. “Vascular Mural Cells in Healing Canine Myocardial Infarcts The Journal of Histochemistry & Cytochemistry The Journal of Histochemistry & Cytochemistry.” 52(8):1019–29.
Dobaczewski, Marcin, Carlos Gonzalez-Quesada, and Nikolaos G. Frangogiannis. 2010. “The Extracellular Matrix as a Modulator of the Inflammatory and Reparative Response Following Myocardial Infarction.” *Journal of Molecular and Cellular Cardiology* 48(3):504–11.
Dong, Huixiao, Benliang Cui, and Xiuzhen Hao. 2019. “MicroRNA-22 Alleviates Inflammation in Ischemic Stroke via P38 MAPK Pathways.” *Molecular Medicine Reports* 20(1):735–44.
Dusting, G. J., and R. D. Nolan. 1981. “STIMULATION OF PROSTACYCLIN RELEASE FROM THE EPICARDIUM OF ANAESTHETIZED DOGS.” 553–62.
Ege, Turan, Suat Canbaz, Volkan Yuksel, and Enver Duran. 2003. “Effect of Pericardial Fluid Pro-Inflammatory Cytokines on Hemodynamic Parameters.” *Cytokine* 23(1–2):47–51.
Fatehi Hassanabad, Ali, Anna Zarzycki, Justin F. Deniset, and Paul WM Fedak. 2021. “An Overview of Human Pericardial Space and Pericardial Fluid.” *Cardiovascular Pathology* 53.
Feng, Xin-hua, and Rik Derynck. 2005. “Specificity and Versatility in TGF- β Signaling Through Smads.”
Frangogiannis, Nikolaos G. 2012. “Regulation of the Inflammatory Response in Cardiac Repair.” *Circulation Research* 110(1):159–73.
Frangogiannis, Nikolaos G. 2015. “Pathophysiology of Myocardial Infarction.” *Comprehensive Physiology* 5(4):1841–75.
Frangogiannis, Nikolaos G. 2019. “Cardiac Fibrosis: Cell Biological Mechanisms, Molecular Pathways and Therapeutic Opportunities.” *Molecular Aspects of Medicine* 65(July):70–99.
Fujita, M., M. Ikemoto, M. Kishishita, H. Otani, R. Nohara, T. Tanaka, S. Tamaki, A. Yamazato, and S. Sasayama. 1996. “Elevated Basic Fibroblast Growth Factor in Pericardial Fluid of Patients with Unstable Angina.” *Circulation* 94(4):610—613.
Fujita, Masatoshi, Masaki Ikemoto, Terumitsu Tanaka, Shun Ichi Tamaki, Ario Yamazato, Tatsuya Sawamura, Koji Hasegawa, Yasuki Kihara, Ryuji Nohara, and Shigetake Sasayama. 1998. “Marked Elevation of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Pericardial Fluid of Patients with Angina Pectoris.” *Angiogenesis* 2(1):105–8.
Gabriel-Costa, Daniele. 2018. “The Pathophysiology of Myocardial Infarction-Induced Heart Failure.” *Pathophysiology* 25(4):277–84.
Gaggar, Amit, Patricia L. Jackson, Brett D. Noerager, Philip J. O’Reilly, D. Brent McQuaid, Steven M. Rowe, J. P. Clancy, and J. Edwin Blalock. 2008. “A Novel Proteolytic Cascade Generates an Extracellular Matrix-Derived Chemoattractant in Chronic Neutrophilic Inflammation.” *The Journal of Immunology* 180(8):5662–69.
Gawor, Monika, Mateusz Śpiewak, Agata Kubik, Aleksandra Wróbel, Magdalena Marczak, and Jacek Grzybowski. 2018. “Assessed by Cardiac Magnetic Resonance Ac Ce Pt Ed Us T.” *Biomarkers* 0(0):000.
Gersch, Christine, Oliver Dewald, Martin Zoerlein, Lloyd H. Michael, Mark L. Entman, and Nikolaos G. Frangogiannis. 2002. “Mast Cells and Macrophages in Normal C57/BL/6 Mice.” *Histochemistry and Cell Biology* 118(1):41–49.
Gibson, B. Y. A. T., and M. B. Segal. 1978a. “Of Physiology,.” 367–77.
Gibson, B. Y. A. T., and M. B. Segal. 1978b. “Pericardial Cavity To the Blood in Rabbits.” *Blood* 423–33.
Goren, Yaron, Michal Kushnir, Barak Zafrir, Sarit Tabak, Basil S. Lewis, and Offer Amir. 2012.

"Serum Levels of MicroRNAs in Patients with Heart Failure." 147–54.
Graham-Brown, M. P. M., A. S. Patel, D. J. Stensel, D. S. March, A. M. Marsh, J. McAdam, G. P. McCann, and J. O. Burton. 2017. "Imaging of Myocardial Fibrosis in Patients with End-Stage Renal Disease: Current Limitations and Future Possibilities." *BioMed Research International* 2017.
Gupta, Shashi Kumar, Ariana Foinquinos, Sabrina Thum, Janet Remke, Karina Zimmer, Christophe Bauters, Pascal de Groote, Reinier A. Boon, Leon J. de Windt, Sebastian Preissl, Lutz Hein, Sandor Batkai, Florence Pinet, and Thomas Thum. 2016. "Preclinical Development of a MicroRNA-Based Therapy for Elderly Patients With Myocardial Infarction." *Journal of the American College of Cardiology* 68(14):1557–71.
Gurha, Priyatansh, Cei Abreu-Goodger, Tiannan Wang, Maricela O. Ramirez, Ana L. Drumond, Stijn Van Dongen, Yuqing Chen, Nenad Bartonicek, Anton J. Enright, Brendan Lee, Robert J. Kelm, Anilkumar K. Reddy, George E. Taffet, Allan Bradley, Xander H. Wehrens, Mark L. Entman, and Antony Rodriguez. 2012. "Targeted Deletion of MicroRNA-22 Promotes Stress-Induced Cardiac Dilation and Contractile Dysfunction." *Circulation* 125(22):2751–61.
Ha, Minju, and V. Narry Kim. 2014. "Regulation of MicroRNA Biogenesis." *Nature Publishing Group* 15(8):509–24.
He, Q., C. Wang, J. Qin, Y. Zhang, D. Xia, and X. Chen. 2017. "Effect of MiR-203 Expression on Myocardial Fibrosis." 837–42.
Hensley, Kenneth, Kent A. Robinson, S. Prasad Gabbita, Scott Salsman, and Robert A. Floyd. 2000. "Reactive Oxygen Species, Cell Signaling, and Cell Injury." *Free Radical Biology and Medicine* 28(10):1456–62.
Heymans, Stephane, Arantxa González, Anne Pizard, Anna P. Papageorgiou, Natalia López-andrés, Frédéric Jaisser, Thomas Thum, Faiez Zannad, and Javier Díez. n.d. "Searching for New Mechanisms of Myocardial Fibrosis with Diagnostic and / or Therapeutic Potential." 6:764–71.
Hinderer, Svenja, and Katja Schenke-Layland. 2019. "Cardiac Fibrosis – A Short Review of Causes and Therapeutic Strategies." *Advanced Drug Delivery Reviews* 146:77–82.
Hoit, Brian D. 2017. "Pathophysiology of the Pericardium." *Progress in Cardiovascular Diseases* 59(4):341–48.
Hong, Yuan, Huaming Cao, Qiang Wang, Jianlin Ye, Lijun Sui, Jinhua Feng, Xiaojun Cai, Huizhu Song, Xiuhong Zhang, and Xichuang Chen. 2016. "MiR-22 May Suppress Fibrogenesis by Targeting TGFβR i in Cardiac Fibroblasts." *Cellular Physiology and Biochemistry* 40(6):1345–53.
Hortells, Luis, Anne Katrine Z. Johansen, and Katherine E. Yutzey. 2019. "Cardiac Fibroblasts and the Extracellular Matrix in Regenerative and Nonregenerative Hearts." *Journal of Cardiovascular Development and Disease* 6(3):1–17.
Hu, Yuanxin, Scot J. Matkovich, Peter A. Hecker, Yan Zhang, John R. Edwards, and Gerald W. Dorn. 2012. "Epitranscriptional Orchestration of Genetic Reprogramming Is an Emergent Property of Stress-Regulated Cardiac MicroRNAs." *Proceedings of the National Academy of Sciences of the United States of America* 109(48):19864–69.
Huang, Ying. 2018. "The Novel Regulatory Role of LncRNA-MiRNA-MRNA Axis in Cardiovascular Diseases." *Journal of Cellular and Molecular Medicine* 22(12):5768–75.
Huang, Zhan Peng, Jinghai Chen, Hee Young Seok, Zheng Zhang, Masaharu Kataoka, Xiaoyun Hu, and Da Zhi Wang. 2013. "MicroRNA-22 Regulates Cardiac Hypertrophy and Remodeling in Response to Stress." *Circulation Research* 112(9):1234–43.
Huang, Zhan Peng, and Da Zhi Wang. 2014. "MiR-22 in Cardiac Remodeling and Disease." *Trends in Cardiovascular Medicine* 24(7):267–72.
Hutvagner, Gyorgy, and Martin J. Simard. 2008. "Argonaute Proteins : Key Players in RNA Silencing." 9(january):22–33.
IJsselmuiden, Carel B., and Ruth R. Faden. 1992. "The New England Journal of Medicine Downloaded from Nejm.Org on January 31, 2011. For Personal Use Only. No Other Uses without Permission. Copyright © 1992 Massachusetts Medical Society. All Rights Reserved." 326.
Ikemoto, Masaki, Koji Hasegawa, Yasuki Kihara, Atsushi Iwakura, Masashi Komeda, Ario Yamazato, and Masatoshi Fujita. 1999. "Development of Enzyme-Linked Immunosorbent Assay for Acidic Fibroblast Growth Factor and Its Clinical Application." *Clinica Chimica Acta* 283(1–2):171–82.

Jazbutyte, Virginija, Jan Fiedler, Susanne Kneitz, Paolo Galuppo, Annette Just, Angelika Holzmann, Johann Bauersachs, and Thomas Thum. 2013. “MicroRNA-22 Increases Senescence and Activates Cardiac Fibroblasts in the Aging Heart.” *Age* 35(3):747–62.
Jong, Sanne De, Toon A. B. Van Veen, and Jacques M. T. De Bakker. 2011. “Biomarkers of Myocardial Fibrosis Biomarkers of Myocardial Fibrosis.” (March).
Kania, Gabriela, Przemyslaw Blyszczuk, and Urs Eriksson. 2009. “Mechanisms of Cardiac Fibrosis in Inflammatory Heart Disease.” *Trends in Cardiovascular Medicine* 19(8):247–52.
Khan, Moien AB, Muhammad Jawad Hashim, Halla Mustafa, May Yousif Baniyas, Shaikha Khalid Buti Mohamad Al Suwaidi, Rana AlKatheeri, Fatmah Mohamed Khalfan Alblooshi, Meera Eisa Ali Hassan Almatrooshi, Mariam Eisa Hazeem Alzaabi, Reem Saif Al Darmaki, and Shamsa Nasser Ali Hussain Lootah. 2020. “Global Epidemiology of Ischemic Heart Disease: Results from the Global Burden of Disease Study.” *Cureus* 12(7).
Klappacher, Gijnter, Patrick Franzen, Doris Haab, Mohammad Mehrabi, Michael Binder, Karina Plesch, Richard Pacher, Michael Grimm, Hans-georg Eichler, and H. Dietmar Glogar. n.d. “Measuring Extracellular Matrix Turnover in the Serum of Patients With Idiopathic or Lschemic Dilated Cardiomyopathy and Impact on Diagnosis and Prognosis.”
Knabb, Robert M., Stephen W. Ely, Alban N. Bacchus, Rafael Rubio, and Robert M. Berne. 1982. “Consistent Parallel Relationships among Myocardial Oxygen Consumption , Coronary Blood Flow , and Pericardial Infusate Adenosine Concentration with Various Interventions and ^ -Blockade in the Dog.”
Kong, Ping, Panagiota Christia, and Nikolaos G. Frangogiannis. 2014. “The Pathogenesis of Cardiac Fibrosis.” 549–74.
Kreutzer, Fabian Philipp, Jan Fiedler, and Thomas Thum. 2020. “Non-Coding RNAs: Key Players in Cardiac Disease.” *Journal of Physiology* 598(14):2995–3003.
Kubota, Tetsuya, Atsushi Namiki, Masayuki Fukazawa, Michiro Ishikawa, Masao Moroi, Kunio Ebine, and Tetsu Yamaguchi. 2004. “Concentrations of Hepatocyte Growth Factor, Basic Fibroblast Growth Factor, and Vascular Endothelial Growth Factor in Pericardial Fluid and Plasma.” *Japanese Heart Journal* 45(6):989–98.
Kuh, J. H., K. S. Kim, S. H. Kim, K. W. Cho, K. H. Seul, and G. Y. Koh. 1990. “Presence of Immunoreactive Atrial Natriuretic Peptides in Pericardial Fluid of Human Subjects with Congenital Heart Diseases.” *Life Sciences* 46(26):1977—1983.
Kuosmanen, Suvi M., Juha Hartikainen, Mikko Hippeläinen, Hannu Kokki, Anna Liisa Levonen, and Pasi Tavi. 2015. “MicroRNA Profiling of Pericardial Fluid Samples from Patients with Heart Failure.” *PLoS ONE* 10(3):1–23.
Kuse, Naoyuki, Koichiro Kamio, Arata Azuma, Kuniko Matsuda, Minoru Inomata, Jiro Usuki, Akemi Morinaga, Toru Tanaka, Takeru Kashiwada, Kenichiro Atsumi, Hiroki Hayashi, Yoshinobu Saito, Masahiro Seike, and Akihiko Gemma. 2020. “Exosome-Derived MicroRNA-22 Ameliorates Pulmonary Fibrosis by Regulating Fibroblast-to-Myofibroblast Differentiation in Vitro and in Vivo.” *Journal of Nippon Medical School* 87(3):118–28.
Liou, Jer Young, Kou Gi Shyu, Ming Jen Lu, Hung Hsin Chao, Bao Wei Wang, and Pei Liang Kuan. 2006. “Pericardial Fluid and Serum Levels of Vascular Endothelial Growth Factor and Endostatin in Patients with or without Coronary Artery Disease.” *Journal of the Formosan Medical Association* 105(5):377–83.
Maciejak, Agata, Marek Kiliszek, Grzegorz Opolski, Agnieszka Segiet, Krzysztof Matlak, and Slawomir Dobrzycki. 2016. “MiR-22-5p Revealed as a Potential Biomarker Involved in the Acute Phase of Myocardial Infarction via Profiling of Circulating MicroRNAs.” 2867–75.
MaClellan, Sara A., Calum Macaulay, Stephen Lam, and Cathie Garnis. 2014. “Pre-Profiling Factors Influencing Serum MicroRNA Levels.” *BMC Clinical Pathology* 14(1):1–11.
Massagué, Joan. 2012. “TGFβ Signalling in Context.” *Nature Reviews Molecular Cell Biology* 13(10):616–30.
McMullen, Julie R., and Bianca C. Bernardo. 2018. “Inhibition of MiR-29 Protects against Cardiac Hypertrophy and Fibrosis: New Insight for the Role of MiR-29 in the Heart.” *Non-Coding RNA Investigation* 2(March):14–14.
Medzikovic, Lejla, Laila Aryan, and Mansoureh Eghbali. 2019. “Connecting Sex Differences, Estrogen Signaling, and MicroRNAs in Cardiac Fibrosis.” *Journal of Molecular Medicine* 97(10):1385–98.
Miyamoto, Shoichi, Shunsuke Usami, Yasuhide Kuwabara, Takahiro Horie, Osamu Baba, Daihiko Hakuno, Yasuhiro Nakashima, Masataka Nishiga, Masayasu Izuhara, Tetsushi Nakao, Tomohiro Nishino, Yuya Ide, Fumiko Nakazeki, Jun Wang, Koji Ueyama, Takeshi

Kimura, and Koh Ono. 2015. “Expression Patterns of MiRNA-423-5p in the Serum and Pericardial Fluid in Patients Undergoing Cardiac Surgery.” *PLoS ONE* 10(11):1–12.
Miyazaki, T., H. P. Pride, and D. P. Zipes. 1990. “Prostaglandins in the Pericardial Fluid Modulate Neural Regulation of Cardiac Electrophysiological Properties.” *Circulation Research* 66(1):163–75.
Nascimento, Diana Santos, Diogo Mosqueira, Luís Moura Sousa, Mariana Teixeira, Mariana Filipe, Tatiana Pinho Resende, Ana Francisca Araújo, Mariana Valente, Joana Almeida, José Paulo Martins, Jorge Miguel Santos, Rita Nogueira Bárcia, Pedro Cruz, Helder Cruz, and Perpétua Pinto-do-ó. 2014. “Human Umbilical Cord Tissue-Derived Mesenchymal Stromal Cells Attenuate Remodeling after Myocardial Infarction by Proangiogenic , Antiapoptotic , and Endogenous Cell-Activation Mechanisms.” 1–14.
Nemeth, Zoltan. 2015. “Pericardial Fluid of Cardiac Patients Elicits Arterial Constriction :” (April).
Nishikimi, Toshio, Hiroshi Asakawa, Hiroshi Iida, Yasushi Matsushita, Ikuko Shibasaki, Kazuyoshi Tadokoro, Yosuke Mori, Hideaki Mori, Yoshihiko Mochizuki, Yoshitaka Okamura, Shinichiro Miyoshi, Kenji Kangawa, and Hiroaki Matsuoka. 2004. “Different Secretion Patterns of Two Molecular Forms of Cardiac Adrenomedullin in Pressure- and Volume-Overloaded Human Heart Failure.” *Journal of Cardiac Failure* 10(4):321–27.
Oyama, J., H. Shimokawa, S. Morita, H. Yasui, and A. Takeshita. 2001. “Elevated Interleukin-1beta in Pericardial Fluid of Patients with Ischemic Heart Disease.” *Coronary Artery Disease* 12(7):567—571.
Page, S. E. E. 2020. “Imaging of Clinically Unrecognized Myocardial Fibrosis in Patients With Suspected Coronary Artery Disease.” 76(8).
Park, Shuin, Ngoc B. Nguyen, Arash Pezhouman, and Reza Ardehali. 2019. “Cardiac Fibrosis: Potential Therapeutic Targets.” *Translational Research* 209:121–37.
Piccoli, Maria Teresa, Christian Bär, and Thomas Thum. 2016. “Non-Coding RNAs as Modulators of the Cardiac Fibroblast Phenotype.” *Journal of Molecular and Cellular Cardiology* 92:75–81.
Pinto, Alexander R., Alexei Ilinykh, Malina J. Ivey, Jill T. Kuwabara, Michelle L. D’antoni, Ryan Debuque, Anjana Chandran, Lina Wang, Komal Arora, Nadia A. Rosenthal, and Michelle D. Tallquist. 2016. “Revisiting Cardiac Cellular Composition.” *Circulation Research* 118(3):400–409.
Ravassa, Susana, Tobias Trippel, Doris Bach, Diana Bachran, Arantxa González, Begoña López, Rolf Wachter, Gerd Hasenfuss, Christian Delles, Anna F. Dominiczak, Burkert Pieske, Javier Díez, and Frank Edelmann. n.d. “Biomarker-Based Phenotyping of Myocardial Fibrosis Identifies Patients with Heart Failure with Preserved Ejection Fraction Resistant to the Beneficial Effects of Spironolactone : Results from the Aldo-DHF Trial.” 290–99.
Ren, Guofeng, Lloyd H. Michael, Mark L. Entman, and Nikolaos G. Frangogiannis. 2002. “Morphological Characteristics of the Microvasculature in Healing Myocardial Infarcts.” *Journal of Histochemistry and Cytochemistry* 50(1):71–79.
Rischpler, Christoph. 2016. “A Cute Myocardial Infarction.” *Quarterly Journal of Nuclear Medicine and Molecular Imaging* 60(3):236–51.
Ristić, Arsen D., Sabine Pankuweit, Ružica Maksimović, Rainer Moosdorf, and Bernhard Maisch. 2013. “Pericardial Cytokines in Neoplastic, Autoreactive, and Viral Pericarditis.” *Heart Failure Reviews* 18(3):345–53.
Robbins, Clinton S., and Filip K. Swirski. 2010. “The Multiple Roles of Monocyte Subsets in Steady State and Inflammation.” *Cellular and Molecular Life Sciences* 67(16):2685–93.
Rosenkranz, Stephan. 2004. “TGF-B1 and Angiotensin Networking in Cardiac Remodeling.” *Cardiovascular Research* 63(3):423–32.
Sa, Eloy, and Mariano Larman. 2004. “Effects of Loop Diuretics on Myocardial Fibrosis and Collagen Type I Turnover in Chronic Heart Failure.” 43(11).
Santiago, Jon Jon, Leslie J. McNaughton, Navid Koleini, Xin Ma, Brian Bestvater, Barbara E. Nickel, Robert R. Fandrich, Jeffrey T. Wigle, Darren H. Freed, Rakesh C. Arora, and Elissavet Kardami. 2014. “High Molecular Weight Fibroblast Growth Factor-2 in the Human Heart Is a Potential Target for Prevention of Cardiac Remodeling.” *PLoS ONE* 9(5).
Sassi, Yassine, Petros Avramopoulos, Deepak Ramanujam, Laurenz Grüter, Stanislas Werfel, Simon Giosele, Andreas David Brunner, Dena Esfandyari, Aikaterini S. Papadopoulou, Bart De Strooper, Norbert Hübner, Regalla Kumarswamy, Thomas Thum, Xiaoke Yin,

Manuel Mayr, Bernhard Laggerbauer, and Stefan Engelhardt. 2017. “Cardiac Myocyte MiR-29 Promotes Pathological Remodeling of the Heart by Activating Wnt Signaling.” *Nature Communications* 8(1):1–11.
Saxena, Amit, Wei Chen, Ya Su, Vikrant Rai, Olisambu U. Uche, Na Li, and Nikolaos G. Frangogiannis. 2013. “IL-1 Induces Proinflammatory Leukocyte Infiltration and Regulates Fibroblast Phenotype in the Infarcted Myocardium.” *The Journal of Immunology* 191(9):4838–48.
Shinde, Arti V., and Nikolaos G. Frangogiannis. 2014. “Fibroblasts in Myocardial Infarction: A Role in Inflammation and Repair.” *Journal of Molecular and Cellular Cardiology* 70:74–82.
Silva, Ana Catarina, Cassilda Pereira, Ana Catarina R. G. Fonseca, Perpétua Pinto-do-ó, and Diana S. Nascimento. 2021. “Bearing My Heart : The Role of Extracellular Matrix on Cardiac Development , Homeostasis , and Injury Response.” 8(January):1–18.
Skyschally, Andreas, Rainer Schulz, and Gerd Heusch. 2008. “Pathophysiology of Myocardial Infarction. Protection by Ischemic Pre- and Postconditioning.” *Herz* 33(2):88–100.
Small, Eric M., and Eric N. Olson. 2011. “Pervasive Roles of MicroRNAs in Cardiovascular Biology.” *Nature* 469(7330):336–42.
Sousa, Daniel Pereira De. 2018. “Integrated Master in Bioengineering Pericardial Fluid : From Biochemical Screening to Therapeutic Potential in Ischemic Heart Disease Daniel Pereira de Sousa.”
Spach, Madison S., and John P. Boineau. 1997. “Microfibrosis Produces Electrical Load Variations Due to Loss of Side-to-Side Cell Connections ; A Major Mechanism of Structural Heart Disease Arrhythmias.” 20(February):397–414.
Sperr, W. R., H. C. Bankl, G. Mundigler, G. Klappacher, K. Großschmidt, H. Agis, P. Simon, P. Laufer, M. Lmhof, Th Radaszkiewicz, D. Glogar, K. Lechner, and P. Valent. 1994. “The Human Cardiac Mast Cell: Localization, Isolation, Phenotype, and Functional Characterization.” *Blood* 84(11):3876–84.
Stewart, R. H., D. A. Rohn, S. J. Allen, and G. A. Laine. 1997. “Basic Determinants of Epicardial Transudation.” *The American Journal of Physiology* 273(3 Pt 2):H1408—14.
Talman, Virpi, and Heikki Ruskoaho. 2016. “Cardiac Fibrosis in Myocardial Infarction—from Repair and Remodeling to Regeneration.” *Cell and Tissue Research* 365(3):563–81.
Tanaka, Terumitsu, Koji Hasegawa, Masatoshi Fujita, Shun Ichi Tamaki, Ario Yamazato, Yasuki Kihara, Ryuji Nohara, and Shigetake Sasayama. 1998. “Marked Elevation of Brain Natriuretic Peptide Levels in Pericardial Fluid Is Closely Associated with Left Ventricular Dysfunction.” *Journal of the American College of Cardiology* 31(2):399–403.
Thum, Thomas, Carina Gross, Jan Fiedler, Thomas Fischer, Stephan Kissler, Markus Bussen, Victor Koteliansky, Andreas Rosenwald, M. Albert Basson, Jonathan D. Licht, John T. R. Pena, Sara H. Rouhanifard, Martina U. Muckenthaler, Thomas Tuschl, Gail R. Martin, and Johann Bauersachs. 2008. “MicroRNA-21 Contributes to Myocardial Disease by Stimulating MAP Kinase Signalling in Fibroblasts.” 456(December).
Tijsen, Anke J., Ingeborg Van Der Made, Maarten M. Van Den Hoogenhof, Wino J. Wijnen, Elza D. Van Deel, Nina E. De Groot, Sergey Alekseev, Kees Fluiter, Blanche Schroen, Marie Jose Goumans, Jolanda Van Der Velden, Dirk J. Duncker, Yigal M. Pinto, and Esther E. Creemers. 2014. “The MicroRNA-15 Family Inhibits the TGFβ-Pathway in the Heart.” *Cardiovascular Research* 104(1):61–71.
Travers, Joshua G., Fadia A. Kamal, Jeffrey Robbins, Katherine E. Yutzey, and C. Burns. 2017. “Cardiac Fibrosis : The Fibroblast Awakens.” 118(6):1021–40.
Trindade, Fábio, Rui Vitorino, Adelino Leite-Moreira, and Inês Falcão-Pires. 2019. *Pericardial Fluid: An Underrated Molecular Library of Heart Conditions and a Potential Vehicle for Cardiac Therapy*. Vol. 114. Springer Berlin Heidelberg.
Tsutamoto, Takayoshi, Atsuyuki Wada, and Keiko Maeda. 2000. “Transcardiac Extraction of Circulating Endothelin-1 Across the Failing Heart.” 86(mean 59).
Tu, Yingfeng, Lin Wan, Dongliang Zhao, Lihong Bu, Dandan Dong, Zheyu Yin, Zhen Cheng, and Baozhong Shen. 2014. “In Vitro and in Vivo Direct Monitoring of MiRNA-22 Expression in Isoproterenol-Induced Cardiac Hypertrophy by Bioluminescence Imaging.” *European Journal of Nuclear Medicine and Molecular Imaging* 41(5):972–84.
Virani, Salim S., Alvaro Alonso, Emelia J. Benjamin, Marcio S. Bittencourt, Clifton W. Callaway, April P. Carson, Alanna M. Chamberlain, Alexander R. Chang, Susan Cheng, Francesca N. Delling, Luc Djousse, Mitchell S. V. Elkind, Jane F. Ferguson, Myriam Fornage, Sadiya S. Khan, Brett M. Kissela, Kristen L. Knutson, Tak W. Kwan, Daniel T. Lackland, Tené T.

Lewis, Judith H. Lichtman, Chris T. Longenecker, Matthew Shane Loop, Pamela L. Lutsey, Seth S. Martin, Kunihiro Matsushita, Andrew E. Moran, Michael E. Mussolino, Amanda Marma Perak, Wayne D. Rosamond, Gregory A. Roth, Uchechukwu K. A. Sampson, Gary M. Satou, Emily B. Schroeder, Svati H. Shah, Christina M. Shay, Nicole L. Spartano, Andrew Stokes, David L. Tirschwell, Lisa B. VanWagner, Connie W. Tsao, Sally S. Wong, and Debra G. Heard. 2020. *Heart Disease and Stroke Statistics—2020 Update: A Report from the American Heart Association*.

Vogiatzidis, Konstantinos, Sotirios G. Zarogiannis, Isaac Aidonidis, Evgeniy I. Solenov, Paschalis Adam Molyvdas, Konstantinos I. Gourgoulianis, and Chrissi Hatzoglou. 2015. "Physiology of Pericardial Fluid Production and Drainage." *Frontiers in Physiology* 6(MAR):1–6.

Wagner, Anja, Heiko Mahrholdt, Thomas A. Holly, Michael D. Elliott, Matthias Regenfus, Michele Parker, Francis J. Klocke, Robert O. Bonow, Raymond J. Kim, and Robert M. Judd. 2003. "Contrast-Enhanced MRI and Routine Single Photon Emission Computed Tomography (SPECT) Perfusion Imaging for Detection of Subendocardial Myocardial Infarcts : An Imaging Study." 361:374–79.

Wang, Huan, Mark W. Tibbitt, Stephen J. Langer, Leslie A. Leinwand, and Kristi S. Anseth. 2013. "Hydrogels Preserve Native Phenotypes of Valvular Fi Broblasts through an Elasticity-Regulated PI3K / AKT Pathway." 110(48).

Wang, Xianwei, Zhikun Guo, Zufeng Ding, Magomed Khaidakov, Juntang Lin, Zhenping Xu, Shree G. Sharma, Shahanawaz Jiwani, and Jawahar L. Mehta. 2015. "Endothelin-1 Upregulation Mediates Aging-Related Cardiac Fibrosis." *Journal of Molecular and Cellular Cardiology* 80:101–9.

Wang, Yu, Yin Wang, and Peifeng Li. 2019. "Circulating MiR - 22 - 5p and MiR - 122 - 5p Are Promising Novel Biomarkers for Diagnosis of Acute Myocardial Infarction." (August 2018):4778–86.

Wen, Wanxue, Xiaoling Chen, Zhiqing Huang, Daiwen Chen, Ping Zheng, Jun He, Hong Chen, Jie Yu, Yuheng Luo, and Bing Yu. 2021. "MiR-22-3p Regulates Muscle Fiber-Type Conversion through Inhibiting AMPK/SIRT1/PGC-1α Pathway." *Animal Biotechnology* 32(2):254–61.

Wilkins, E., L. Wilson, K. Wickramasinghe, and P. Bhatnagar. 2017. "European Cardiovascular Disease Statistics 2017." *European Heart Network* 94–100.

Wu, E., J. T. Ortiz, P. Tejedor, D. C. Lee, C. Bucciarelli-Ducci, P. Kansal, J. C. Carr, T. A. Holly, D. Lloyd-Jones, F. J. Klocke, and R. O. Bonow. 2008. "Infarct Size by Contrast Enhanced Cardiac Magnetic Resonance Is a Stronger Predictor of Outcomes than Left Ventricular Ejection Fraction or End-Systolic Volume Index: Prospective Cohort Study." *Heart* 94(6):730 LP – 736.

Xie, Yuping, Jianmin Chen, Pengfei Han, Pingliang Yang, Jianglong Hou, and Y. James Kang. 2012. "Immunohistochemical Detection of Differentially Localized Up-Regulation of Lysyl Oxidase and down-Regulation of Matrix Metalloproteinase-1 in Rhesus Monkey Model of Chronic Myocardial Infarction." *Experimental Biology and Medicine* 237(7):853–59.

Xie, Zhixin, Edwards Allen, Noah Fahlgren, Adam Calamar, Scott A. Givan, and James C. Carrington. 2005. "Expression of Arabidopsis MIRNA Genes." *Plant Physiology* 138(4):2145–54.

Yasojima, Koji, Claudia Schwab, Edith G. McGeer, and Patrick L. McGeer. 1998. "Human Heart Generates Complement Proteins That Are Upregulated and Activated after Myocardial Infarction." *Circulation Research* 83(8):860–69.

Yoneda, T., M. Fujita, Y. Kihara, K. Hasegawa, T. Sawamura, T. Tanaka, M. Inanami, R. Nohara, and S. Sasayama. 2000. "Pericardial Fluid from Patients with Ischemic Heart Disease Accelerates the Growth of Human Vascular Smooth Muscle Cells." *Japanese Circulation Journal* 64(7):495—498.

Yuan, Jinxia, Hongtao Chen, Dawei Ge, Yu Xu, Haihua Xu, Yang Yang, Ming Gu, Yuhe Zhou, Jingdong Zhu, Ting Ge, Qun Chen, Yue Gao, Yanqing Wang, Xiaowei Li, and Yanfang Zhao. 2017. "Mir-21 Promotes Cardiac Fibrosis after Myocardial Infarction Via Targeting Smad7." *Cellular Physiology and Biochemistry* 42(6):2207–19.

Zhang, Chengqiang, Lihua Fang, Xiaoping Liu, Tingting Nie, Rui Li, Luping Cui, Jie Wang, and Yanhong Ji. 2020. "MiR-22 Inhibits Synovial Fibroblasts Proliferation and Proinflammatory Cytokine Production in RASF via Targeting SIRT1." *Gene* 724(76).

Zhang, Lu, Hongli Yin, Lei Jiao, Tianyi Liu, Yuqiu Gao, Yingchun Shao, Yuanyuan Zhang,

Hongli Shan, Ying Zhang, and Baofeng Yang. 2018. “Abnormal Downregulation of Caveolin-3 Mediates the Pro-Fibrotic Action of MicroRNA-22 in a Model of Myocardial Infarction.” *Cellular Physiology and Biochemistry* 45(4):1641–53.

Zhang, Z., Z. Zhu, K. Watabe, X. Zhang, C. Bai, M. Xu, F. Wu, and Y-y Mo. 2013. “Negative Regulation of LncRNA GAS5 by MiR-21.” 2200:1558–68.

Zhao, X. S., Y. Ren, Y. Wu, H. K. Ren, and H. Chen. 2020. “MiR-30b-5p and MiR-22-3p Restrain the Fibrogenesis of Post-Myocardial Infarction in Mice via Targeting PTAFR.” *European Review for Medical and Pharmacological Sciences* 24(7):3993–4004.

Zhou, Xiang, Wei Zhang, Mengchao Jin, Jianchang Chen, Weiting Xu, and Xiangqing Kong. 2017. “LncRNA MIAT Functions as a Competing Endogenous RNA to Upregulate DAPK2 by Sponging MiR-22-3p in Diabetic Cardiomyopathy.” *Cell Death & Disease* 8(7):e2929.

Zhou, Xue liang, Hua Xu, Zhi bo Liu, Qi cai Wu, Rong rong Zhu, and Ji chun Liu. 2018. “MiR-21 Promotes Cardiac Fibroblast-to-Myofibroblast Transformation and Myocardial Fibrosis by Targeting Jagged1.” *Journal of Cellular and Molecular Medicine* 22(8):3816–24.

Zymek, Pawel, Marcin Bujak, Khaled Chatila, and Anna Cieslak. 2006. “The Role of Platelet-Derived Growth Factor Signaling in Healing Myocardial Infarcts.” 48(11).

Auer, Johann, Thomas Weber, and Bernd Eber. 2004. “Intensive versus Moderate Lipid Lowering with Statins after Acute Coronary Syndromes.” *The New England Journal of Medicine* 351(7):714—7; author reply 714—7.

Bader, Michael. 2002. “Role of the Local Renin ± Angiotensin System in Cardiac Damage : A Minireview Focussing on Transgenic Animal Models.” 1462.

Bang, Batkai S., Dangwal S., Gupta S.K., Fischer Foinquinos A., Holzmann A., Just A., Remke J., Zimmer K., Zeug A., Ponimaskin E., Schmiedl Schober A., Yin X., Mayr M., Halder R., Fischer Foinquinos A., Engelhardt S., Wei Y., Schmiedl Schober A., Fiedler J., Thum T., Claudia Bang, Sandor Batkai, Seema Dangwal, Shashi Kumar Gupta, Ariana Foinquinos, Angelika Holzmann, Annette Just, Janet Remke, Karina Zimmer, Andre Zeug, Evgeni Ponimaskin, Andreas Schmiedl, Xiaoke Yin, Manuel Mayr, Rashi Halder, Andre Fischer, Stefan Engelhardt, Yuanyuan Wei, Andreas Schober, Jan Fiedler, and Thomas Thum. 2014. “Cardiac Fibroblast-Derived MicroRNA Passenger Strand-Enriched Exosomes Mediate Cardiomyocyte Hypertrophy.” *Journal of Clinical Investigation* 124(5):2136–46.

Beltrami, Cristina, Marie Besnier, Saran Shantikumar, Andrew I. U. Shearn, Cha Rajakaruna, Abas Laftah, Fausto Sessa, Gaia Spinetti, Enrico Petretto, Gianni D. Angelini, and Costanza Emanueli. 2017. “Human Pericardial Fluid Contains Exosomes Enriched with Cardiovascular-Expressed MicroRNAs and Promotes Therapeutic Angiogenesis.” *Molecular Therapy* 25(3):679–93.

Berk, Bradford C., Keigi Fujiwara, and Stephanie Lehoux. 2007. “ECM Remodeling in Hypertensive Heart Disease Bradford C. Berk.” *J Clin Invest* 117(3):568.

Biernacka, Anna, Marcin Dobaczewski, and Nikolaos G. Frangogiannis. 2011. “TGF-β Signaling in Fibrosis.” *Growth Factors* 29(5):196–202.

Blaufarb, Ira S. 1996. “The Renin-Angiotensin S Stem in Left Ventricular Remo Ar Cling.” 9149(96).

Boulanger, B., Z. Yuan, M. Flessner, J. Hay, and M. Johnston. 1999. “Pericardial Fluid Absorption into Lymphatic Vessels in Sheep.” *Microvascular Research* 57(2):174–86.

Boven, Nick Van, K. Martijn Akkerhuis, Sharda S. Anroedh, Dimitris Rizopoulos, Yigal Pinto, Linda C. Battes, Hans L. Hillege, Kadir C. Caliskan, Tjeerd Germans, Olivier C. Manintveld, Jan-hein Cornel, Alina A. Constantinescu, Eric Boersma, Victor A. Umans, and Isabella Kardys. 2017. “Serially Measured Circulating MiR-22-3p Is a Biomarker for Adverse Clinical Outcome in Patients with Chronic Heart Failure : The Bio-SHiFT Study.” *International Journal of Cardiology* 235:124–32.

Braunwald, Eugene, James E. Muller, Robert A. Kloner, and Peter R. Maroko. 1983. “Role of Beta-Adrenergic Blockade in the Therapy of Patients with Myocardial Infarction.” *The American Journal of Medicine* 74(1):113–23.

Brønnum, Hasse, Ditte C. Andersen, Mikael Schneider, Maria B. Sandberg, and Tilde Eskildsen. 2013. “MiR-21 Promotes Fibrogenic Epithelial-to-Mesenchymal Transition of Epicardial Mesothelial Cells Involving Programmed Cell Death 4 and Sprouty-1.” 8(2):1–13.

Brown, R. Dale, S. Kelly Ambler, M. Darren Mitchell, and Carlin S. Long. 2005. “The Cardiac Fibroblast: Therapeutic Target in Myocardial Remodeling and Failure.” *Annual Review of Pharmacology and Toxicology* 45(1):657–87.

Brutsaert, Dirk L. 2003. “Cardiac Endothelial-Myocardial Signaling: Its Role in Cardiac Growth, Contractile Performance, and Rhythmicity.” *Physiological Reviews* 83(1):59–115.
Bujak, Marcin, Marcin Dobaczewski, Khaled Chatila, Leonardo H. Mendoza, Na Li, Anilkumar Reddy, and Nikolaos G. Frangogiannis. 2008. “Interleukin-1 Receptor Type I Signaling Critically Regulates Infarct Healing and Cardiac Remodeling.” 173(1):57–67.
Bujak, Marcin, Guofeng Ren, Hyuk Jung Kweon, Marcin Dobaczewski, Anilkumar Reddy, George Taffet, Xiao Fan Wang, and Nikolaos G. Frangogiannis. 2007. “Essential Role of Smad3 in Infarct Healing and in the Pathogenesis of Cardiac Remodeling.” *Circulation* 116(19):2127–38.
Cavalera, Michele, Junhong Wang, and Nikolaos G. Frangogiannis. 2014. “Obesity, Metabolic Dysfunction, and Cardiac Fibrosis: Pathophysiological Pathways, Molecular Mechanisms, and Therapeutic Opportunities.” *Translational Research* 164(4):323–35.
Chen, Yongli, Yan Yin, and Hua Jiang. 2021. “MiR-30e-5p Alleviates Inflammation and Cardiac Dysfunction After Myocardial Infarction Through Targeting PTEN.” 44(2).
Creemers, Esther E., and Eva Van Rooij. 2016. “Function and Therapeutic Potential of Noncoding RNAs in Cardiac Fibrosis.” *Circulation Research* 118(1):108–18.
Dalmay, Tamas. 2013. “Mechanism of MiRNA-Mediated Repression of MRNA Translation” edited by M. A. Lindsay and S. Griffiths-Jones. *Essays in Biochemistry* 54:29–38.
Derynck, Rik, and Ying E. Zhang. 2003. “Smad-Dependent and Smad-Independent Pathways in TGF-.” 4.
Disertori, Marcello, Michela Masè, and Flavia Ravelli. 2017. “Myocardial Fibrosis Predicts Ventricular Tachyarrhythmias.” *Trends in Cardiovascular Medicine* 27(5):363–72.
Dobaczewski, Marcin, Spyridon Akrivakis, Kamal Nasser, Lloyd H. Michael, Mark L. Entman, and Nikolaos G. Frangogiannis. 2004. “Vascular Mural Cells in Healing Canine Myocardial Infarcts The Journal of Histochemistry & Cytochemistry The Journal of Histochemistry & Cytochemistry.” 52(8):1019–29.
Dobaczewski, Marcin, Carlos Gonzalez-Quesada, and Nikolaos G. Frangogiannis. 2010. “The Extracellular Matrix as a Modulator of the Inflammatory and Reparative Response Following Myocardial Infarction.” *Journal of Molecular and Cellular Cardiology* 48(3):504–11.
Dong, Huixiao, Benliang Cui, and Xiuzhen Hao. 2019. “MicroRNA-22 Alleviates Inflammation in Ischemic Stroke via P38 MAPK Pathways.” *Molecular Medicine Reports* 20(1):735–44.
Dusting, G. J., and R. D. Nolan. 1981. “STIMULATION OF PROSTACYCLIN RELEASE FROM THE EPICARDIUM OF ANAESTHETIZED DOGS.” 553–62.
Ege, Turan, Suat Canbaz, Volkan Yuksel, and Enver Duran. 2003. “Effect of Pericardial Fluid Pro-Inflammatory Cytokines on Hemodynamic Parameters.” *Cytokine* 23(1–2):47–51.
Fatehi Hassanabad, Ali, Anna Zarzycki, Justin F. Deniset, and Paul WM Fedak. 2021. “An Overview of Human Pericardial Space and Pericardial Fluid.” *Cardiovascular Pathology* 53.
Feng, Xin-hua, and Rik Derynck. 2005. “Specificity and Versatility in TGF- β Signaling Through Smads.”
Frangogiannis, Nikolaos G. 2012. “Regulation of the Inflammatory Response in Cardiac Repair.” *Circulation Research* 110(1):159–73.
Frangogiannis, Nikolaos G. 2015. “Pathophysiology of Myocardial Infarction.” *Comprehensive Physiology* 5(4):1841–75.
Frangogiannis, Nikolaos G. 2019. “Cardiac Fibrosis: Cell Biological Mechanisms, Molecular Pathways and Therapeutic Opportunities.” *Molecular Aspects of Medicine* 65(July):70–99.
Fujita, M., M. Ikemoto, M. Kishishita, H. Otani, R. Nohara, T. Tanaka, S. Tamaki, A. Yamazato, and S. Sasayama. 1996. “Elevated Basic Fibroblast Growth Factor in Pericardial Fluid of Patients with Unstable Angina.” *Circulation* 94(4):610—613.
Fujita, Masatoshi, Masaki Ikemoto, Terumitsu Tanaka, Shun Ichi Tamaki, Ario Yamazato, Tatsuya Sawamura, Koji Hasegawa, Yasuki Kihara, Ryuji Nohara, and Shigetake Sasayama. 1998. “Marked Elevation of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Pericardial Fluid of Patients with Angina Pectoris.” *Angiogenesis* 2(1):105–8.
Gabriel-Costa, Daniele. 2018. “The Pathophysiology of Myocardial Infarction-Induced Heart Failure.” *Pathophysiology* 25(4):277–84.
Gaggar, Amit, Patricia L. Jackson, Brett D. Noerager, Philip J. O’Reilly, D. Brent McQuaid, Steven M. Rowe, J. P. Clancy, and J. Edwin Blalock. 2008. “A Novel Proteolytic Cascade

Generates an Extracellular Matrix-Derived Chemoattractant in Chronic Neutrophilic Inflammation." *The Journal of Immunology* 180(8):5662–69.
Gawor, Monika, Mateusz Śpiewak, Agata Kubik, Aleksandra Wróbel, Magdalena Marczak, and Jacek Grzybowski. 2018. "Assessed by Cardiac Magnetic Resonance Ac Ce Pt Ed Us T." *Biomarkers* 0(0):000.
Gersch, Christine, Oliver Dewald, Martin Zoerlein, Lloyd H. Michael, Mark L. Entman, and Nikolaos G. Frangogiannis. 2002. "Mast Cells and Macrophages in Normal C57/BL/6 Mice." *Histochemistry and Cell Biology* 118(1):41–49.
Gibson, B. Y. A. T., and M. B. Segal. 1978a. "Of Physiology,." 367–77.
Gibson, B. Y. A. T., and M. B. Segal. 1978b. "Pericardial Cavity To the Blood in Rabbits." *Blood* 423–33.
Goren, Yaron, Michal Kushnir, Barak Zafrir, Sarit Tabak, Basil S. Lewis, and Offer Amir. 2012. "Serum Levels of MicroRNAs in Patients with Heart Failure." 147–54.
Graham-Brown, M. P. M., A. S. Patel, D. J. Stensel, D. S. March, A. M. Marsh, J. McAdam, G. P. McCann, and J. O. Burton. 2017. "Imaging of Myocardial Fibrosis in Patients with End-Stage Renal Disease: Current Limitations and Future Possibilities." *BioMed Research International* 2017.
Gupta, Shashi Kumar, Ariana Foinquinos, Sabrina Thum, Janet Remke, Karina Zimmer, Christophe Bauters, Pascal de Groote, Reinier A. Boon, Leon J. de Windt, Sebastian Preissl, Lutz Hein, Sandor Batkai, Florence Pinet, and Thomas Thum. 2016. "Preclinical Development of a MicroRNA-Based Therapy for Elderly Patients With Myocardial Infarction." *Journal of the American College of Cardiology* 68(14):1557–71.
Gurha, Priyatansh, Cei Abreu-Goodger, Tiannan Wang, Maricela O. Ramirez, Ana L. Drumond, Stijn Van Dongen, Yuqing Chen, Nenad Bartonicek, Anton J. Enright, Brendan Lee, Robert J. Kelm, Anilkumar K. Reddy, George E. Taffet, Allan Bradley, Xander H. Wehrens, Mark L. Entman, and Antony Rodriguez. 2012. "Targeted Deletion of MicroRNA-22 Promotes Stress-Induced Cardiac Dilation and Contractile Dysfunction." *Circulation* 125(22):2751–61.
Ha, Minju, and V. Narry Kim. 2014. "Regulation of MicroRNA Biogenesis." *Nature Publishing Group* 15(8):509–24.
He, Q., C. Wang, J. Qin, Y. Zhang, D. Xia, and X. Chen. 2017. "Effect of MiR-203 Expression on Myocardial Fibrosis." 837–42.
Hensley, Kenneth, Kent A. Robinson, S. Prasad Gabbita, Scott Salsman, and Robert A. Floyd. 2000. "Reactive Oxygen Species, Cell Signaling, and Cell Injury." *Free Radical Biology and Medicine* 28(10):1456–62.
Heymans, Stephane, Arantxa González, Anne Pizard, Anna P. Papageorgiou, Natalia López-andrés, Frédéric Jaisser, Thomas Thum, Faiez Zannad, and Javier Díez. n.d. "Searching for New Mechanisms of Myocardial Fibrosis with Diagnostic and / or Therapeutic Potential." 6:764–71.
Hinderer, Svenja, and Katja Schenke-Layland. 2019. "Cardiac Fibrosis – A Short Review of Causes and Therapeutic Strategies." *Advanced Drug Delivery Reviews* 146:77–82.
Hoit, Brian D. 2017. "Pathophysiology of the Pericardium." *Progress in Cardiovascular Diseases* 59(4):341–48.
Hong, Yuan, Huaming Cao, Qiang Wang, Jianlin Ye, Lijun Sui, Jinhua Feng, Xiaojun Cai, Huizhu Song, Xiuhong Zhang, and Xichuang Chen. 2016. "MiR-22 May Suppress Fibrogenesis by Targeting TGFβR i in Cardiac Fibroblasts." *Cellular Physiology and Biochemistry* 40(6):1345–53.
Hortells, Luis, Anne Katrine Z. Johansen, and Katherine E. Yutzey. 2019. "Cardiac Fibroblasts and the Extracellular Matrix in Regenerative and Nonregenerative Hearts." *Journal of Cardiovascular Development and Disease* 6(3):1–17.
Hu, Yuanxin, Scot J. Matkovich, Peter A. Hecker, Yan Zhang, John R. Edwards, and Gerald W. Dorn. 2012. "Epitranscriptional Orchestration of Genetic Reprogramming Is an Emergent Property of Stress-Regulated Cardiac MicroRNAs." *Proceedings of the National Academy of Sciences of the United States of America* 109(48):19864–69.
Huang, Ying. 2018. "The Novel Regulatory Role of LncRNA-MiRNA-MRNA Axis in Cardiovascular Diseases." *Journal of Cellular and Molecular Medicine* 22(12):5768–75.
Huang, Zhan Peng, Jinghai Chen, Hee Young Seok, Zheng Zhang, Masaharu Kataoka, Xiaoyun Hu, and Da Zhi Wang. 2013. "MicroRNA-22 Regulates Cardiac Hypertrophy and Remodeling in Response to Stress." *Circulation Research* 112(9):1234–43.

Huang, Zhan Peng, and Da Zhi Wang. 2014. “MiR-22 in Cardiac Remodeling and Disease.” *Trends in Cardiovascular Medicine* 24(7):267–72.
Hutvagner, Gyorgy, and Martin J. Simard. 2008. “Argonaute Proteins : Key Players in RNA Silencing.” 9(january):22–33.
IJsselmuiden, Carel B., and Ruth R. Faden. 1992. “The New England Journal of Medicine Downloaded from Nejm.Org on January 31, 2011. For Personal Use Only. No Other Uses without Permission. Copyright © 1992 Massachusetts Medical Society. All Rights Reserved.” 326.
Ikemoto, Masaki, Koji Hasegawa, Yasuki Kihara, Atsushi Iwakura, Masashi Komeda, Ario Yamazato, and Masatoshi Fujita. 1999. “Development of Enzyme-Linked Immunosorbent Assay for Acidic Fibroblast Growth Factor and Its Clinical Application.” *Clinica Chimica Acta* 283(1–2):171–82.
Jazbutyte, Virginija, Jan Fiedler, Susanne Kneitz, Paolo Galuppo, Annette Just, Angelika Holzmann, Johann Bauersachs, and Thomas Thum. 2013. “MicroRNA-22 Increases Senescence and Activates Cardiac Fibroblasts in the Aging Heart.” *Age* 35(3):747–62.
Jong, Sanne De, Toon A. B. Van Veen, and Jacques M. T. De Bakker. 2011. “Biomarkers of Myocardial Fibrosis Biomarkers of Myocardial Fibrosis.” (March).
Kania, Gabriela, Przemyslaw Blyszczuk, and Urs Eriksson. 2009. “Mechanisms of Cardiac Fibrosis in Inflammatory Heart Disease.” *Trends in Cardiovascular Medicine* 19(8):247–52.
Khan, Moien AB, Muhammad Jawad Hashim, Halla Mustafa, May Yousif Baniyas, Shaikha Khalid Buti Mohamad Al Suwaidi, Rana AlKatheeri, Fatmah Mohamed Khalfan Alblooshi, Meera Eisa Ali Hassan Almatrooshi, Mariam Eisa Hazeem Alzaabi, Reem Saif Al Darmaki, and Shamsa Nasser Ali Hussain Lootah. 2020. “Global Epidemiology of Ischemic Heart Disease: Results from the Global Burden of Disease Study.” *Cureus* 12(7).
Klappacher, Gijnter, Patrick Franzen, Doris Haab, Mohammad Mehrabi, Michael Binder, Karina Plesch, Richard Pacher, Michael Grimm, Hans-georg Eichler, and H. Dietmar Glogar. n.d. “Measuring Extracellular Matrix Turnover in the Serum of Patients With Idiopathic or Lschemic Dilated Cardiomyopathy and Impact on Diagnosis and Prognosis.”
Knabb, Robert M., Stephen W. Ely, Alban N. Bacchus, Rafael Rubio, and Robert M. Berne. 1982. “Consistent Parallel Relationships among Myocardial Oxygen Consumption , Coronary Blood Flow , and Pericardial Infusate Adenosine Concentration with Various Interventions and ^ -Blockade in the Dog.”
Kong, Ping, Panagiota Christia, and Nikolaos G. Frangogiannis. 2014. “The Pathogenesis of Cardiac Fibrosis.” 549–74.
Kreutzer, Fabian Philipp, Jan Fiedler, and Thomas Thum. 2020. “Non-Coding RNAs: Key Players in Cardiac Disease.” *Journal of Physiology* 598(14):2995–3003.
Kubota, Tetsuya, Atsushi Namiki, Masayuki Fukazawa, Michiro Ishikawa, Masao Moroi, Kunio Ebine, and Tetsu Yamaguchi. 2004. “Concentrations of Hepatocyte Growth Factor, Basic Fibroblast Growth Factor, and Vascular Endothelial Growth Factor in Pericardial Fluid and Plasma.” *Japanese Heart Journal* 45(6):989–98.
Kuh, J. H., K. S. Kim, S. H. Kim, K. W. Cho, K. H. Seul, and G. Y. Koh. 1990. “Presence of Immunoreactive Atrial Natriuretic Peptides in Pericardial Fluid of Human Subjects with Congenital Heart Diseases.” *Life Sciences* 46(26):1977—1983.
Kuosmanen, Suvi M., Juha Hartikainen, Mikko Hippeläinen, Hannu Kokki, Anna Liisa Levonen, and Pasi Tavi. 2015. “MicroRNA Profiling of Pericardial Fluid Samples from Patients with Heart Failure.” *PLoS ONE* 10(3):1–23.
Kuse, Naoyuki, Koichiro Kamio, Arata Azuma, Kuniko Matsuda, Minoru Inomata, Jiro Usuki, Akemi Morinaga, Toru Tanaka, Takeru Kashiwada, Kenichiro Atsumi, Hiroki Hayashi, Yoshinobu Saito, Masahiro Seike, and Akihiko Gemma. 2020. “Exosome-Derived MicroRNA-22 Ameliorates Pulmonary Fibrosis by Regulating Fibroblast-to-Myofibroblast Differentiation in Vitro and in Vivo.” *Journal of Nippon Medical School* 87(3):118–28.
Liou, Jer Young, Kou Gi Shyu, Ming Jen Lu, Hung Hsin Chao, Bao Wei Wang, and Pei Liang Kuan. 2006. “Pericardial Fluid and Serum Levels of Vascular Endothelial Growth Factor and Endostatin in Patients with or without Coronary Artery Disease.” *Journal of the Formosan Medical Association* 105(5):377–83.
Maciejak, Agata, Marek Kiliszek, Grzegorz Opolski, Agnieszka Segiet, Krzysztof Matlak, and Slawomir Dobrzycki. 2016. “MiR-22-5p Revealed as a Potential Biomarker Involved in the Acute Phase of Myocardial Infarction via Profiling of Circulating MicroRNAs.” 2867–75.
MaClellan, Sara A., Calum Macaulay, Stephen Lam, and Cathie Garnis. 2014. “Pre-Profiling

Factors Influencing Serum MicroRNA Levels." *BMC Clinical Pathology* 14(1):1–11.
Massagué, Joan. 2012. "TGFβ Signalling in Context." *Nature Reviews Molecular Cell Biology* 13(10):616–30.
McMullen, Julie R., and Bianca C. Bernardo. 2018. "Inhibition of MiR-29 Protects against Cardiac Hypertrophy and Fibrosis: New Insight for the Role of MiR-29 in the Heart." *Non-Coding RNA Investigation* 2(March):14–14.
Medzikovic, Lejla, Laila Aryan, and Mansoureh Eghbali. 2019. "Connecting Sex Differences, Estrogen Signaling, and MicroRNAs in Cardiac Fibrosis." *Journal of Molecular Medicine* 97(10):1385–98.
Miyamoto, Shoichi, Shunsuke Usami, Yasuhide Kuwabara, Takahiro Horie, Osamu Baba, Daihiko Hakuno, Yasuhiro Nakashima, Masataka Nishiga, Masayasu Izuhara, Tetsushi Nakao, Tomohiro Nishino, Yuya Ide, Fumiko Nakazeki, Jun Wang, Koji Ueyama, Takeshi Kimura, and Koh Ono. 2015. "Expression Patterns of MiRNA-423-5p in the Serum and Pericardial Fluid in Patients Undergoing Cardiac Surgery." *PLoS ONE* 10(11):1–12.
Miyazaki, T., H. P. Pride, and D. P. Zipes. 1990. "Prostaglandins in the Pericardial Fluid Modulate Neural Regulation of Cardiac Electrophysiological Properties." *Circulation Research* 66(1):163–75.
Nascimento, Diana Santos, Diogo Mosqueira, Luís Moura Sousa, Mariana Teixeira, Mariana Filipe, Tatiana Pinho Resende, Ana Francisca Araújo, Mariana Valente, Joana Almeida, José Paulo Martins, Jorge Miguel Santos, Rita Nogueira Bárcia, Pedro Cruz, Helder Cruz, and Perpétua Pinto-do-ó. 2014. "Human Umbilical Cord Tissue-Derived Mesenchymal Stromal Cells Attenuate Remodeling after Myocardial Infarction by Proangiogenic , Antiapoptotic , and Endogenous Cell-Activation Mechanisms." 1–14.
Nemeth, Zoltan. 2015. "Pericardial Fluid of Cardiac Patients Elicits Arterial Constriction :" (April).
Nishikimi, Toshio, Hiroshi Asakawa, Hiroshi Iida, Yasushi Matsushita, Ikuko Shibasaki, Kazuyoshi Tadokoro, Yosuke Mori, Hideaki Mori, Yoshihiko Mochizuki, Yoshitaka Okamura, Shinichiro Miyoshi, Kenji Kangawa, and Hiroaki Matsuoka. 2004. "Different Secretion Patterns of Two Molecular Forms of Cardiac Adrenomedullin in Pressure- and Volume-Overloaded Human Heart Failure." *Journal of Cardiac Failure* 10(4):321–27.
Oyama, J., H. Shimokawa, S. Morita, H. Yasui, and A. Takeshita. 2001. "Elevated Interleukin-1beta in Pericardial Fluid of Patients with Ischemic Heart Disease." *Coronary Artery Disease* 12(7):567—571.
Page, S. E. E. 2020. "Imaging of Clinically Unrecognized Myocardial Fibrosis in Patients With Suspected Coronary Artery Disease." 76(8).
Park, Shuin, Ngoc B. Nguyen, Arash Pezhouman, and Reza Ardehali. 2019. "Cardiac Fibrosis: Potential Therapeutic Targets." *Translational Research* 209:121–37.
Piccoli, Maria Teresa, Christian Bär, and Thomas Thum. 2016. "Non-Coding RNAs as Modulators of the Cardiac Fibroblast Phenotype." *Journal of Molecular and Cellular Cardiology* 92:75–81.
Pinto, Alexander R., Alexei Ilinykh, Malina J. Ivey, Jill T. Kuwabara, Michelle L. D'antoni, Ryan Debuque, Anjana Chandran, Lina Wang, Komal Arora, Nadia A. Rosenthal, and Michelle D. Tallquist. 2016. "Revisiting Cardiac Cellular Composition." *Circulation Research* 118(3):400–409.
Ravassa, Susana, Tobias Trippel, Doris Bach, Diana Bachran, Arantxa González, Begoña López, Rolf Wachter, Gerd Hasenfuss, Christian Delles, Anna F. Dominiczak, Burkert Pieske, Javier Díez, and Frank Edelmann. n.d. "Biomarker-Based Phenotyping of Myocardial Fibrosis Identifies Patients with Heart Failure with Preserved Ejection Fraction Resistant to the Beneficial Effects of Spironolactone : Results from the Aldo-DHF Trial." 290–99.
Ren, Guofeng, Lloyd H. Michael, Mark L. Entman, and Nikolaos G. Frangogiannis. 2002. "Morphological Characteristics of the Microvasculature in Healing Myocardial Infarcts." *Journal of Histochemistry and Cytochemistry* 50(1):71–79.
Rischpler, Christoph. 2016. "A Cute Myocardial Infarction." *Quarterly Journal of Nuclear Medicine and Molecular Imaging* 60(3):236–51.
Ristić, Arsen D., Sabine Pankuweit, Ružica Maksimović, Rainer Moosdorf, and Bernhard Maisch. 2013. "Pericardial Cytokines in Neoplastic, Autoreactive, and Viral Pericarditis." *Heart Failure Reviews* 18(3):345–53.
Robbins, Clinton S., and Filip K. Swirski. 2010. "The Multiple Roles of Monocyte Subsets in Steady State and Inflammation." *Cellular and Molecular Life Sciences* 67(16):2685–93.

Rosenkranz, Stephan. 2004. “TGF-B1 and Angiotensin Networking in Cardiac Remodeling.” *Cardiovascular Research* 63(3):423–32.
Sa, Eloy, and Mariano Larman. 2004. “Effects of Loop Diuretics on Myocardial Fibrosis and Collagen Type I Turnover in Chronic Heart Failure.” 43(11).
Santiago, Jon Jon, Leslie J. McNaughton, Navid Koleini, Xin Ma, Brian Bestvater, Barbara E. Nickel, Robert R. Fandrich, Jeffrey T. Wigle, Darren H. Freed, Rakesh C. Arora, and Elissavet Kardami. 2014. “High Molecular Weight Fibroblast Growth Factor-2 in the Human Heart Is a Potential Target for Prevention of Cardiac Remodeling.” *PLoS ONE* 9(5).
Sassi, Yassine, Petros Avramopoulos, Deepak Ramanujam, Laurenz Grüter, Stanislas Werfel, Simon Giosele, Andreas David Brunner, Dena Esfandyari, Aikaterini S. Papadopoulou, Bart De Strooper, Norbert Hübner, Regalla Kumarswamy, Thomas Thum, Xiaoke Yin, Manuel Mayr, Bernhard Laggerbauer, and Stefan Engelhardt. 2017. “Cardiac Myocyte MiR-29 Promotes Pathological Remodeling of the Heart by Activating Wnt Signaling.” *Nature Communications* 8(1):1–11.
Saxena, Amit, Wei Chen, Ya Su, Vikrant Rai, Olisambu U. Uche, Na Li, and Nikolaos G. Frangogiannis. 2013. “IL-1 Induces Proinflammatory Leukocyte Infiltration and Regulates Fibroblast Phenotype in the Infarcted Myocardium.” *The Journal of Immunology* 191(9):4838–48.
Shinde, Arti V., and Nikolaos G. Frangogiannis. 2014. “Fibroblasts in Myocardial Infarction: A Role in Inflammation and Repair.” *Journal of Molecular and Cellular Cardiology* 70:74–82.
Silva, Ana Catarina, Cassilda Pereira, Ana Catarina R. G. Fonseca, Perpétua Pinto-do-ó, and Diana S. Nascimento. 2021. “Bearing My Heart : The Role of Extracellular Matrix on Cardiac Development , Homeostasis , and Injury Response.” 8(January):1–18.
Skyschally, Andreas, Rainer Schulz, and Gerd Heusch. 2008. “Pathophysiology of Myocardial Infarction. Protection by Ischemic Pre- and Postconditioning.” *Herz* 33(2):88–100.
Small, Eric M., and Eric N. Olson. 2011. “Pervasive Roles of MicroRNAs in Cardiovascular Biology.” *Nature* 469(7330):336–42.
Sousa, Daniel Pereira De. 2018. “Integrated Master in Bioengineering Pericardial Fluid : From Biochemical Screening to Therapeutic Potential in Ischemic Heart Disease Daniel Pereira de Sousa.”
Spach, Madison S., and John P. Boineau. 1997. “Microfibrosis Produces Electrical Load Variations Due to Loss of Side-to-Side Cell Connections ; A Major Mechanism of Structural Heart Disease Arrhythmias.” 20(February):397–414.
Sperr, W. R., H. C. Bankl, G. Mundigler, G. Klappacher, K. Großschmidt, H. Agis, P. Simon, P. Laufer, M. Lmhof, Th Radaszkiewicz, D. Glogar, K. Lechner, and P. Valent. 1994. “The Human Cardiac Mast Cell: Localization, Isolation, Phenotype, and Functional Characterization.” *Blood* 84(11):3876–84.
Stewart, R. H., D. A. Rohn, S. J. Allen, and G. A. Laine. 1997. “Basic Determinants of Epicardial Transudation.” *The American Journal of Physiology* 273(3 Pt 2):H1408—14.
Talman, Virpi, and Heikki Ruskoaho. 2016. “Cardiac Fibrosis in Myocardial Infarction—from Repair and Remodeling to Regeneration.” *Cell and Tissue Research* 365(3):563–81.
Tanaka, Terumitsu, Koji Hasegawa, Masatoshi Fujita, Shun Ichi Tamaki, Ario Yamazato, Yasuki Kihara, Ryuji Nohara, and Shigetake Sasayama. 1998. “Marked Elevation of Brain Natriuretic Peptide Levels in Pericardial Fluid Is Closely Associated with Left Ventricular Dysfunction.” *Journal of the American College of Cardiology* 31(2):399–403.
Thum, Thomas, Carina Gross, Jan Fiedler, Thomas Fischer, Stephan Kissler, Markus Bussen, Victor Koteliansky, Andreas Rosenwald, M. Albert Basson, Jonathan D. Licht, John T. R. Pena, Sara H. Rouhanifard, Martina U. Muckenthaler, Thomas Tuschl, Gail R. Martin, and Johann Bauersachs. 2008. “MicroRNA-21 Contributes to Myocardial Disease by Stimulating MAP Kinase Signalling in Fibroblasts.” 456(December).
Tijsen, Anke J., Ingeborg Van Der Made, Maarten M. Van Den Hoogenhof, Wino J. Wijnen, Elza D. Van Deel, Nina E. De Groot, Sergey Alekseev, Kees Fluiter, Blanche Schroen, Marie Jose Goumans, Jolanda Van Der Velden, Dirk J. Duncker, Yigal M. Pinto, and Esther E. Creemers. 2014. “The MicroRNA-15 Family Inhibits the TGFβ-Pathway in the Heart.” *Cardiovascular Research* 104(1):61–71.
Travers, Joshua G., Fadia A. Kamal, Jeffrey Robbins, Katherine E. Yutzey, and C. Burns. 2017. “Cardiac Fibrosis : The Fibroblast Awakens.” 118(6):1021–40.
Trindade, Fábio, Rui Vitorino, Adelino Leite-Moreira, and Inês Falcão-Pires. 2019. *Pericardial*

Fluid: An Underrated Molecular Library of Heart Conditions and a Potential Vehicle for Cardiac Therapy. Vol. 114. Springer Berlin Heidelberg.
Tsutamoto, Takayoshi, Atsuyuki Wada, and Keiko Maeda. 2000. “Transcardiac Extraction of Circulating Endothelin-1 Across the Failing Heart.” 86(mean 59).
Tu, Yingfeng, Lin Wan, Dongliang Zhao, Lihong Bu, Dandan Dong, Zheyu Yin, Zhen Cheng, and Baozhong Shen. 2014. “In Vitro and in Vivo Direct Monitoring of MiRNA-22 Expression in Isoproterenol-Induced Cardiac Hypertrophy by Bioluminescence Imaging.” *European Journal of Nuclear Medicine and Molecular Imaging* 41(5):972–84.
Virani, Salim S., Alvaro Alonso, Emelia J. Benjamin, Marcio S. Bittencourt, Clifton W. Callaway, April P. Carson, Alanna M. Chamberlain, Alexander R. Chang, Susan Cheng, Francesca N. Delling, Luc Djousse, Mitchell S. V. Elkind, Jane F. Ferguson, Myriam Fornage, Sadiya S. Khan, Brett M. Kissela, Kristen L. Knutson, Tak W. Kwan, Daniel T. Lackland, Tené T. Lewis, Judith H. Lichtman, Chris T. Longenecker, Matthew Shane Loop, Pamela L. Lutsey, Seth S. Martin, Kunihiro Matsushita, Andrew E. Moran, Michael E. Mussolino, Amanda Marma Perak, Wayne D. Rosamond, Gregory A. Roth, Uchechukwu K. A. Sampson, Gary M. Satou, Emily B. Schroeder, Svati H. Shah, Christina M. Shay, Nicole L. Spartano, Andrew Stokes, David L. Tirschwell, Lisa B. VanWagner, Connie W. Tsao, Sally S. Wong, and Debra G. Heard. 2020. *Heart Disease and Stroke Statistics—2020 Update: A Report from the American Heart Association*.
Vogiatzidis, Konstantinos, Sotirios G. Zarogiannis, Isaac Aidonidis, Evgeniy I. Solenov, Paschalis Adam Molyvdas, Konstantinos I. Gourgoulianis, and Chrissi Hatzoglou. 2015. “Physiology of Pericardial Fluid Production and Drainage.” *Frontiers in Physiology* 6(MAR):1–6.
Wagner, Anja, Heiko Mahrholdt, Thomas A. Holly, Michael D. Elliott, Matthias Regenfus, Michele Parker, Francis J. Klocke, Robert O. Bonow, Raymond J. Kim, and Robert M. Judd. 2003. “Contrast-Enhanced MRI and Routine Single Photon Emission Computed Tomography (SPECT) Perfusion Imaging for Detection of Subendocardial Myocardial Infarcts : An Imaging Study.” 361:374–79.
Wang, Huan, Mark W. Tibbitt, Stephen J. Langer, Leslie A. Leinwand, and Kristi S. Anseth. 2013. “Hydrogels Preserve Native Phenotypes of Valvular Fi Broblasts through an Elasticity-Regulated PI3K / AKT Pathway.” 110(48).
Wang, Xianwei, Zhikun Guo, Zufeng Ding, Magomed Khaidakov, Juntang Lin, Zhenping Xu, Shree G. Sharma, Shahanawaz Jiwani, and Jawahar L. Mehta. 2015. “Endothelin-1 Upregulation Mediates Aging-Related Cardiac Fibrosis.” *Journal of Molecular and Cellular Cardiology* 80:101–9.
Wang, Yu, Yin Wang, and Peifeng Li. 2019. “Circulating MiR - 22 - 5p and MiR - 122 - 5p Are Promising Novel Biomarkers for Diagnosis of Acute Myocardial Infarction.” (August 2018):4778–86.
Wen, Wanxue, Xiaoling Chen, Zhiqing Huang, Daiwen Chen, Ping Zheng, Jun He, Hong Chen, Jie Yu, Yuheng Luo, and Bing Yu. 2021. “MiR-22-3p Regulates Muscle Fiber-Type Conversion through Inhibiting AMPK/SIRT1/PGC-1α Pathway.” *Animal Biotechnology* 32(2):254–61.
Wilkins, E., L. Wilson, K. Wickramasinghe, and P. Bhatnagar. 2017. “European Cardiovascular Disease Statistics 2017.” *European Heart Network* 94–100.
Wu, E., J. T. Ortiz, P. Tejedor, D. C. Lee, C. Bucciarelli-Ducci, P. Kansal, J. C. Carr, T. A. Holly, D. Lloyd-Jones, F. J. Klocke, and R. O. Bonow. 2008. “Infarct Size by Contrast Enhanced Cardiac Magnetic Resonance Is a Stronger Predictor of Outcomes than Left Ventricular Ejection Fraction or End-Systolic Volume Index: Prospective Cohort Study.” *Heart* 94(6):730 LP – 736.
Xie, Yuping, Jianmin Chen, Pengfei Han, Pingliang Yang, Jianglong Hou, and Y. James Kang. 2012. “Immunohistochemical Detection of Differentially Localized Up-Regulation of Lysyl Oxidase and down-Regulation of Matrix Metalloproteinase-1 in Rhesus Monkey Model of Chronic Myocardial Infarction.” *Experimental Biology and Medicine* 237(7):853–59.
Xie, Zhixin, Edwards Allen, Noah Fahlgren, Adam Calamar, Scott A. Givan, and James C. Carrington. 2005. “Expression of Arabidopsis MIRNA Genes.” *Plant Physiology* 138(4):2145–54.
Yasojima, Koji, Claudia Schwab, Edith G. McGeer, and Patrick L. McGeer. 1998. “Human Heart Generates Complement Proteins That Are Upregulated and Activated after Myocardial Infarction.” *Circulation Research* 83(8):860–69.

Yoneda, T., M. Fujita, Y. Kihara, K. Hasegawa, T. Sawamura, T. Tanaka, M. Inanami, R. Nohara, and S. Sasayama. 2000. “Pericardial Fluid from Patients with Ischemic Heart Disease Accelerates the Growth of Human Vascular Smooth Muscle Cells.” *Japanese Circulation Journal* 64(7):495—498.

Yuan, Jinxia, Hongtao Chen, Dawei Ge, Yu Xu, Haihua Xu, Yang Yang, Ming Gu, Yuhe Zhou, Jingdong Zhu, Ting Ge, Qun Chen, Yue Gao, Yanqing Wang, Xiaowei Li, and Yanfang Zhao. 2017. “Mir-21 Promotes Cardiac Fibrosis after Myocardial Infarction Via Targeting Smad7.” *Cellular Physiology and Biochemistry* 42(6):2207–19.

Zhang, Chengqiang, Lihua Fang, Xiaoping Liu, Tingting Nie, Rui Li, Luping Cui, Jie Wang, and Yanhong Ji. 2020. “MiR-22 Inhibits Synovial Fibroblasts Proliferation and Proinflammatory Cytokine Production in RASF via Targeting SIRT1.” *Gene* 724(76).

Zhang, Lu, Hongli Yin, Lei Jiao, Tianyi Liu, Yuqiu Gao, Yingchun Shao, Yuanyuan Zhang, Hongli Shan, Ying Zhang, and Baofeng Yang. 2018. “Abnormal Downregulation of Caveolin-3 Mediates the Pro-Fibrotic Action of MicroRNA-22 in a Model of Myocardial Infarction.” *Cellular Physiology and Biochemistry* 45(4):1641–53.

Zhang, Z., Z. Zhu, K. Watabe, X. Zhang, C. Bai, M. Xu, F. Wu, and Y-y Mo. 2013. “Negative Regulation of LncRNA GAS5 by MiR-21.” 2200:1558–68.

Zhao, X. S., Y. Ren, Y. Wu, H. K. Ren, and H. Chen. 2020. “MiR-30b-5p and MiR-22-3p Restrain the Fibrogenesis of Post-Myocardial Infarction in Mice via Targeting PTAFR.” *European Review for Medical and Pharmacological Sciences* 24(7):3993–4004.

Zhou, Xiang, Wei Zhang, Mengchao Jin, Jianchang Chen, Weiting Xu, and Xiangqing Kong. 2017. “LncRNA MIAT Functions as a Competing Endogenous RNA to Upregulate DAPK2 by Sponging MiR-22-3p in Diabetic Cardiomyopathy.” *Cell Death & Disease* 8(7):e2929.

Zhou, Xue liang, Hua Xu, Zhi bo Liu, Qi cai Wu, Rong rong Zhu, and Ji chun Liu. 2018. “MiR-21 Promotes Cardiac Fibroblast-to-Myofibroblast Transformation and Myocardial Fibrosis by Targeting Jagged1.” *Journal of Cellular and Molecular Medicine* 22(8):3816–24.

Zymek, Pawel, Marcin Bujak, Khaled Chatila, and Anna Cieslak. 2006. “The Role of Platelet-Derived Growth Factor Signaling in Healing Myocardial Infarcts.” 48(11).