

**EVALUATING ROLES OF MIRNAS IN CARDIAC FIBROSIS: A
META-ANALYSIS**

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META-ANALYSIS**

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When you have exhausted all possibilities, remember this- you haven't.

Thomas Edison

Dedicated to my parents, Vineeta and Pankaj, my brother, Sanchit, my aunt and uncle,
Sunita and Sharad, and the rest of my family and friends.

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LIST OF ACRONYMS

COL1A1	Collagen type I alpha 1 chain
COL1A2	Collagen type I alpha 2 chain
COL3A1	Collagen type III alpha 1 chain
CPC	Cardiac Progenitor Cell
CTGF	Connective Tissue Growth Factor
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ESC	Embryonic Stem Cell
HSC	Hematopoietic Stem Cell
IL-4	Interleukin-4
IPMCs	intracellular plasma membrane-connected compartments
miRISC	miRNA induced silencing complex
miRNA	microRNA
mRNA	messenger RNA
MSC	Mesenchymal Stem Cell
MVB	multivesicular bodies
PLSR	Partial least squares regression
RNA	ribonucleic acid
RNase	Ribonuclease
TGF-β	Transforming growth factor beta
VIM	Vimentin
VIP	Variable importance in projection

SUMMARY

Cardiovascular diseases are the leading cause of mortality globally. Cardiac fibrosis is an essential component of changes that occur in heart's size, shape, and composition, in response to cardiac disease or cardiac damage. Exosomes are extracellular vesicles that aid cell-cell communication and carry proteins, metabolites, nucleic acids, etc. miRNAs are small non-coding ribonucleic acid (RNA) molecules that can be transported by exosomes and are uniquely capable of facilitating long-term repair by altering the targeted cells' transcriptome. Prior studies have demonstrated relationships between exosomal miRNA content and fibrosis in the heart. In this research, self-built scoring models and PLSR modeling were used to find miRNAs that can downregulate cardiac fibrosis. miR-21, miR-33, miR-125b, miR-155-5p, miR-34a were identified as profibrotic miRNAs and miR-29b, miR-29a, miR-26a, miR-30c, miR-29c were identified as antifibrotic miRNAs. Selected under-studied miRNAs were also identified that might be important regulators of cardiac fibrosis. Computational models were built to predict the extent of cardiac fibrosis with miRNAs' fold-changes as inputs. A computational workflow was developed to predict the extent of cardiac fibrosis when exosomes with custom-designed packages of miRNA content will be injected into animal models. This analysis consolidates relationships between selected miRNAs and cardiac fibrosis and can be used to inform experimental studies of cardiac remodeling.

CHAPTER 1

BACKGROUND

1.1 Cardiac fibrosis

Cardiac fibrosis is characterized by an imbalance in extracellular matrix (ECM) production and degradation, resulting in accumulation of proteins in cardiac interstitium, causing cardiac dysfunction in a wide array of cardiac diseases. Injury to heart can activate fibrotic responses, distorting heart's shape and compromising physiological cardiac functions. Cardiac fibrosis stiffens the heart which causes problems with cardiac cycle by affecting the contraction and relaxation of the heart during systole and diastole, respectively [1]. In response to different types of injuries and stimuli, there are two types of cardiac fibrosis [2]:

1. Replacement/ reparative fibrosis: Ischemia, inflammation, toxic insult, etc. can cause cardiomyocyte necrosis, inducing replacement of cardiac muscles with fibrous tissue.
2. Reactive fibrosis: Pressure, volume overload, aging, cardiomyopathies, etc. can cause excessive deposition of ECM in expanded interstitial and perivascular spaces without significant loss of cardiomyocytes.

Cardiac fibroblasts are involved in the formation of ECM. In response to cardiac injury, fibroblasts differentiate into myofibroblasts to generate an inflammatory response and produce high levels of ECM proteins. Collagen is the main structural protein found in the ECM inside the body and helps in maintaining tissue integrity [3]. Collagen has been shown to be upregulated in pathological conditions like myocardial infarction, aging and diabetes [4, 5, 6]. Connective Tissue Growth Factor (CTGF) is a cysteine rich protein which is highly expressed in fetal myocardium. In adult heart, it is only expressed in atria and large blood

vessels, and its increased expression has been associated with cardiac pathologies [7, 8, 9]. Vimentin protein has been identified as a marker of cardiac fibroblasts, can label them with high sensitivity and check purity of their isolation. Heart's fibrotic response to a stimulus can be divided into three stages [10, 11]:

1. Initiating stage: Stimulus triggers a fibrotic response by increasing profibrotic growth factors and cytokines inside the myocardium and in circulation.
2. Effective stage: Signaling pathways (like Wnt signaling) and transcriptional factors (like Smad) are activated when pro-fibrotic growth factors and cytokines bind to their receptors, ultimately transforming cardiac fibroblasts into myofibroblasts (Figure 1.1).
3. Amplificative stage: Pro-fibrotic growth factors and cytokines form a positive feedback loop, resulting in the amplification of fibrosis.

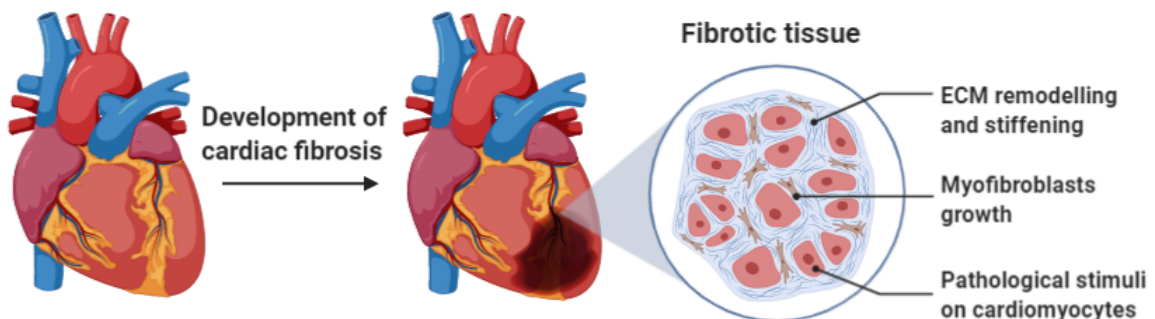


Figure 1.1: Process of cardiac fibrosis development

During the development of cardiac fibrosis, pathological stimuli cause cardiomyocytes necrosis and myofibroblasts growth. This leads to ECM remodeling and stiffening of the heart as shown with the example of fibrotic tissue in the diagram. Image created using BioRender.com.

Cardiac fibrosis can be measured using non-invasive magnetic resonance techniques and staining of tissue biopsies. T1 mapping, a cardiac magnetic resonance-based method has been recently developed for quantifying fibrosis in heart [12]. For histological assessment of fibrosis, extracted tissue can be stained with Masson Trichrome or Picrosirius red

stain which contain dyes that bind to collagen to give blue and red color, respectively [13, 14]. Other techniques for quantifying cardiac fibrosis include using CNA35, Hydroxyproline assay and Sircol assay [15].

Cell death during myocardial infarction initiates phases of reparative fibrosis in which fibroblasts and myofibroblasts generate a fibrotic scar to replace the damaged tissue [16]. This reparative process is beneficial to prevent the rupturing of ventricular wall, however, it is followed by reactive fibrosis in uninjured myocardium which causes stiffness in the heart, hindering normal cardiac functions [17]. Cardiac (atrial) fibrosis causes heterogeneity in conduction of impulses, contributing to the development of atrial fibrillation [18].

Age related accumulation of ECM in elderly patients causes cardiac dysfunction [5]. Unlike conventional fibrosis, aging associated cardiac fibrosis is caused due to degenerative changes like apoptotic cell death, loss of cardiomyocyte, thereby promoting collagen deposition [19]. Aging derived continued stress response causes low grade systemic inflammation, resulting in increase of certain cytokines and proteins which have been linked to fibrosis [20, 21]. Research shows that there is a close relationship between cardiac fibrosis and hypertension, a common disease among elderly patients [22].

Independent of atherosclerosis or hypertension, diabetic patients have been diagnosed with perivascular and interstitial fibrosis [23]. Upregulated levels of collagen type III with increased interstitial myocardial fibrosis has been found in hearts of diabetic patients in comparison to non-diabetic patients [24]. Without any history of myocardial infarction, replacement fibrosis has been observed in diabetic patients [25]. Diabetes has been related to increased fibrotic responses when coupled with other cardiac conditions like aortic stenosis [26].

There is ongoing research for better understanding of the mechanisms of cardiac fibrosis and for developing its treatment. Histone deacetylases inhibitors have been shown to inhibit inflammatory cytokines and impede fibroblast signaling during cardiac stresses like hypertension and myocardial infarction, thus alleviating cardiac fibrosis [27]. Renin-

angiotensin-aldosterone-system, Transforming growth factor beta (TGF- β) signaling pathway, injectable biomaterials, Cardiac Progenitor Cell (CPC) transplantation therapy, reprogramming of fibroblasts, non-coding RNAs like miRNAs, epigenetic modifiers, etc. are being explored as potential therapeutic options for cardiac fibrosis [28].

1.2 Stem cells, exosomes, and microRNAs

1.2.1 Stem cells

Stem cells are special cells that have the ability to continuously divide and differentiate into various cell types. They are capable of proliferating and self-renewing themselves for long periods of time and have the potential to give rise to specialized cells like cardiomyocytes, neurons, hepatocytes, etc. As shown in Figure 1.2, based on their potency, stem cells have been majorly divided into three types:

1. Totipotent stem cells: They can differentiate into all cell types and are found in an embryo as well as in all extra-embryonic cells (like placenta). For example, cells from 1-3 days old embryo [29].
2. Pluripotent stem cells: They can differentiate into all cell types in the body. For example, cells of 5-14 days old blastocyst [30].
3. Multipotent stem cells: They can differentiate into a limited number of cell types in a particular lineage. For example, adult stem cells and cells found in fetal tissue and cord blood [31].

Progenitor cells are partially specialized cells that can give rise to particular cell types and unlike stem cells, can divide only a limited number of times. For example, cardiac progenitor cells and endothelial progenitor cells.

Stem cells are being increasingly researched to understand disease pathways, generate healthy cells to replace diseased ones and for testing of new drugs. Stem/progenitor cell-

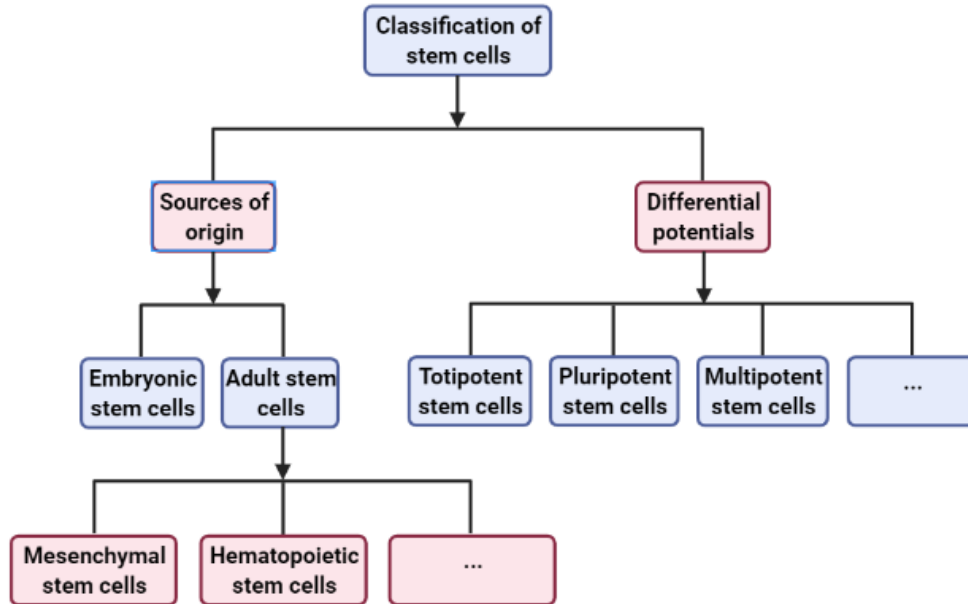


Figure 1.2: **Classification of stem cells based on sources of origin and differential potentials**

Stem cells have been classified based on their source of origin into embryonic stem cells and adult stem cells, and based on their differential potentials into totipotent stem cells, pluripotent stem cells, multipotent stem cells, etc. Adult stem cell can be further classified into mesenchymal stem cell, hematopoietic stem cells, etc.

based therapies can greatly impact the field of regenerative medicine and have attracted researchers' attention due to their efficacy in treating diseases in preclinical transplantation, tissue injury models and human clinical trials [32, 33, 34, 35]. Stem cells have been classified based on their source and function in Figure 1.2. Many stem/progenitor cells that can be used to repair organs and improve organ functions are described below:

- **CPC:** These cells can be isolated from juvenile heart and have the ability to differentiate into cells of three cardiac lineages: cardiomyocytes, smooth muscle cells and endothelial cells. CPCs have been shown to repair heart and improve its function post cardiac injury [36, 37].
- **Embryonic Stem Cell (ESC):** They are derived from the inner cell mass of blastocyst and can renew indefinitely. They can give rise to any of the three embryonic germ layers- endoderm, mesoderm and ectoderm, and can further differentiate into

cardiomyocytes, neural tissue, adipocytes, etc. ESCs have been used to improve locomotion post spinal cord injury, in developing cure for Parkinson's disease and in attempts of lung regeneration [38, 39, 40, 41, 42].

- **Mesenchymal Stem Cell (MSC):** These cells can be isolated from many different sources like bone marrow, muscle, umbilical cord, adipose tissue, etc. and can differentiate into lineages like osteocytes, chondrocytes, monocytes, adipocytes and neurons. Their roles have been investigated in Alzheimer's disease, multiple sclerosis and retinal degenerative disease [43, 44, 45, 46].
- **Hematopoietic Stem Cell (HSC):** These cells can be found inside the bone marrow and have the potential to regenerate the whole hematopoietic system. There are many intermediate progenitor cells between HSCs and mature blood cells. Blood disorders such as leukemia, myeloma and lymphoma require HSC transplantation therapy [47, 48, 49].

Cardiac fibrosis and stem cells

CardioChimeras, synthesized by the fusion of CPCs and MSCs, have been shown to alleviate fibrosis when injected in the border zone region of an acutely injured mouse heart [50]. Cellular transplantation of bone marrow derived MSCs have been shown to reduce fibrosis in porcine myocardial infarction model [51]. Previous research has demonstrated that MSCs are able to modulate the antifibrotic activity of cardiac fibroblasts, inhibit myocardial fibrosis in rat model of dilated cardiomyopathy, prevent cardiac fibrosis in isoproterenol induced global heart failure rat model and reduce myocardial fibrosis in canine chronic ischemia model [52, 53, 54, 55]. Transplantation of biodegradable and nanofibrous patches seeded with MSCs into epicardium of infarcted regions attenuated cardiac fibrosis in a rat model [56].

1.2.2 Exosomes

Exosomes are 30 to 200 nm diameter long, single-membrane, lipid bilayer extracellular vesicles. They have roles in protein quality control, driving anterior–posterior cell polarity, ECM modulation, cell-cell signaling and molecular transfer [57]. As shown in Figure 1.3, exosome biogenesis takes place through the following three modes:

1. Vesicles bud into discrete endosomes that mature into multivesicular bodies (MVB), and release exosomes into extracellular space upon plasma membrane fusion [58].
2. Exosomes directly bud from plasma membrane which result in their immediate release outside the donor cell [59].
3. Vesicles bud into intracellular plasma membrane–connected compartments (IPMCs) and are then be released by de-constriction of IPMCs’ necks. This mode of biogenesis usually results in delayed release of exosomes due to lengths of IPMCs’ necks [60].

During cell-cell signaling, exosomes transport proteins, lipids, nucleic acids, etc. from donor cells to recipient cells as shown in Figure 1.3 [61]. Exosomes are involved in inter-cellular transport and play significant roles in multiple facets of human health and disease, including development, tissue homeostasis, neurodegenerative diseases and viral invasion [57].

Exosomes facilitate communication between immune cells and mediate immune suppressive and immune-activating modulation [62]. In brain, exosomes aid cell-cell communication between neurons and other cell types that ultimately supports axon integrity, microglia and myelination [63]. Changes in exosome number and content has been seen in cardiovascular diseases where certain proteins and deoxyribonucleic acid (DNA)/RNA are transferred only under stress conditions like hypoxia [64, 65, 66].

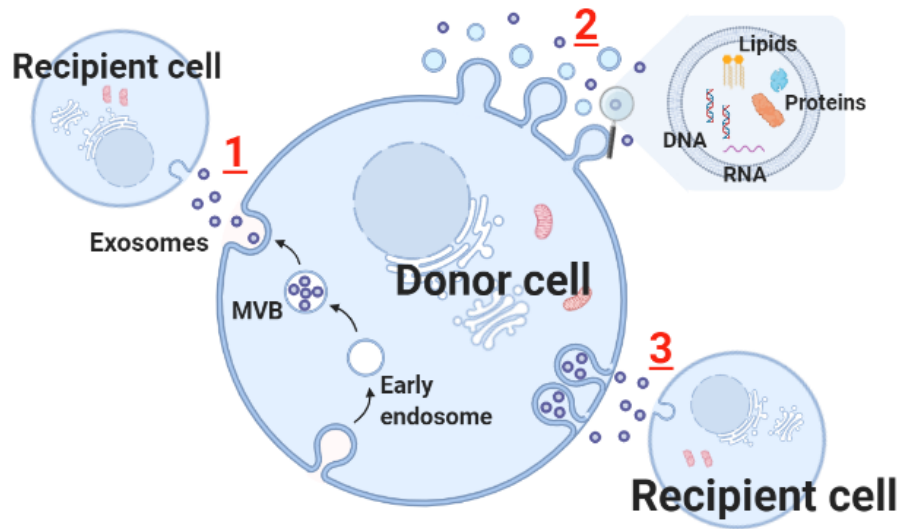


Figure 1.3: **Exosome biogenesis and exosome mediated intercellular transport**

Three modes of exosome biogenesis are shown with numbers 1,2, and 3, in correspondence with the number of modes mentioned in the text above. Exosomes are transporting proteins, lipids, nucleic acids, etc. from donor cell to recipient cells, aiding in cell-cell signaling. Image created using BioRender.com.

Along with being responsible for normal physiology, exosomes are also involved in many pathological conditions. Neurodegenerative diseases like Alzheimer’s disease and Parkinson’s disease are caused by amyloid plaque formation and increase in aggregation of α -synuclein, respectively. In Alzheimer’s disease, exosomes spread amyloid- β to other neuronal cells to form amyloid plaque within the patient’s brain [67]. Parkinson’s disease progression has been linked to neuroblastoma exosomes which reduce the catalysis of α -synuclein, leading to an increase in its aggregated levels [68].

In cardiovascular diseases, exosomes are being researched for working as diagnostic biomarkers and therapeutic agents. Exosome mediated cell-cell communication has been shown to play a significant role in each phase of atherosclerosis [69, 70]. Prior research has reported that myocardium can be protected from ischemia/reperfusion injury by using exosomes from human and rat plasma. [71] Inter-cellular signaling using exosomes between cardiac fibroblasts and cardiomyocytes plays an important role in cardiac hypertrophy and heart failure [72].

Previous studies have identified roles of exosomes in progression of cancer, tumor, as

well as investigated their curative functions in these diseases [73, 74, 75]. Exosomes have been shown to convert benign epithelial cells into malignant cancer cells [73]. Exosome mediated endocrine and paracrine signaling has been associated with cancer metastasis-ECM remodeling, invadopodia formation and Epithelial-to-mesenchymal transition (EMT) [76]. Exosomes may be used as cancer biomarkers in liquid biopsies to aid in the diagnosis of many cancers, including pancreas, ovarian, prostate and melanoma [77, 78, 79, 80].

Cardiac fibrosis and exosomes

The role of exosomes in mediating cardiac fibrosis is being increasingly explored by researchers. ESC derived exosomes have been shown to reduce fibrosis when injected in a myocardial infarction mouse model, improving cardiac function [81]. Exosome mediated paracrine regulation was reported to be the basis of regulation of fibrosis by induced pluripotent stem cell derived cardiomyocytes [82]. Injection of cardiac stem cell derived exosomes significantly suppressed cardiac fibrosis in a dilated cardiomyopathy model [83]. Reduction of cardiac fibrosis was observed when human umbilical cord MSC derived exosomes were injected into a myocardial infarction rat model [84]. CPC derived exosomes have shown improve cardiac function and alleviate fibrosis in a rat model of ischemia-reperfusion [85]. Exosomes from adipose derived stem cells decreased cardiac fibrosis in a myocardial infarction rodent model [86].

1.2.3 microRNAs

miRNAs are small non-coding RNA molecules that are 19-24 nucleotides in length and are capable of post-transcriptional gene silencing, thereby regulating gene expression levels. One miRNA can influence the expression of hundreds of genes, therefore miRNAs collectively regulate more than half of the protein-coding genes in humans and are involved in many diseases pathways [87, 88]. They have roles in cell proliferation, differentiation, death, etc. and their aberrant expression can result in diseased states like cancer, cardiovascular diseases and neurodevelopmental disorders [89].

Genes coding for miRNAs are present in the DNA within the nucleus of cells. These genes are transcribed by RNA polymerase II to make messenger RNA (mRNA), which are called primary miRNA in this case and have a hairpin loop structure [90]. Ribonuclease (RNase) III endonuclease Drosha as well as its cofactor, DGCR8, attach to the primary miRNA and cleave ends of the stem of hairpin loop to make precursor miRNA. A transporter molecule Exportin-5 carries the precursor miRNA into the cytoplasm where it is recognized by a RNase protein called DICER [91]. DICER cleaves the stem loop and forms the double-stranded miRNA intermediate [92]. Then AGO2 binds to the miRNA, unwinds it and one strand is selected [93]. Some other proteins combined with this miRNA and AGO2 form the miRNA induced silencing complex (miRISC) complex. The miRISC complex is guided by the single-stranded mature miRNA to its target sites for inactivating genes [94].

The mRNA of the target gene is complementary to the nucleotide sequence of miRNA so they can bind together. As shown in Figure 1.4, there are two ways in which miRISC can inactivate the mRNA [95]:

1. In case of partially complementary mRNA, miRISC complex attaches to the target mRNA which prevents ribosome from binding, inhibiting the translation of mRNA.
2. For perfectly complementary mRNA targets, miRISC complex cleaves the mRNA and the cleaved mRNA is further degraded by the cell.

As far as nomenclature is concerned, miRNAs are named sequentially. For example, if a novel miR-231 is discovered, the next novel miRNA discovered will be called miR-232. The first three letters in the name of miRNA indicates the organism from which the miRNA has been extracted, for example, hsa-miR-231 is a human miRNA and mmu-miR-231 is a mouse miRNA. miR-231 with the capital 'R' refers to the mature form of the miRNA whereas mir-231 with small 'r' indicates the miRNA gene and the miRNA primary transcript's stem loop. If different precursor sequences mature into same miRNA, they

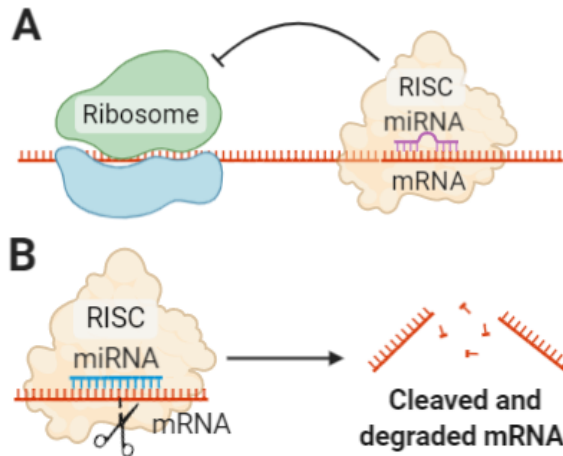


Figure 1.4: **miRNA gene silencing mechanism**

A) Translational repression: miRISC complex binds to partially complementary target site on mRNA and blocks mRNA's translation. B) mRNA cleavage: miRISC complex binds to perfectly complementary target site on mRNA and cleaves mRNA which is further degraded by the cell. Image created using BioRender.com.

are named as miR-231-1 and miR-231-2. Closely related mature sequences are designated by lettered suffixes like miR-231a and miR-231b. When the mature sequence of one out of the two miRNAs originating from the same precursor miRNA has significantly higher abundance, it will be called miR-231, whereas the mature miRNA from the opposite arm will be called miR-231*. If there is no predominant mature miRNA, they are named like miR-231-5p (from the 5' arm) and miR-231-3p (from the 3' arm). These conventions are followed in most cases but there are a few exceptions like nomenclature of plant and viral miRNAs [96].

To overexpress or underexpress a miRNA, agomirs or antagomirs can be injected. Agomirs are double-stranded nucleotide sequences that mimic endogenous miRNAs and regulate the biological function of the target gene. On the other hand, antagomirs are single-stranded and designed in a way that they can bind to the mature miRNA, inhibiting the expression of the endogenous miRNA.

Physiological quantities of miRNA are essential for the normal functioning of the body. miRNAs have been identified as potential biomarkers of aging, cellular senescence, and Alzheimer's disease and can regulate diverse pathways of cellular differentiation, growth,

and apoptosis [97, 98]. Important roles of miRNAs have been identified in the cardiovascular system and provided new insights on various cardiac pathologies like hypertrophy, heart failure, myocardial infarction and atherosclerosis [99]. They also have important functions in nervous system and its development, and their roles as mediators of plasticity are being explored [100].

Aberrant expression of miRNA can result in pathological conditions. Neurodegenerative diseases like Alzheimer's disease is identified by memory loss, cognitive impairments, and is caused by phosphorylated tau formation, synaptic loss, and amyloid plaque formation [97]. Dysfunction of miRNAs has been shown to cause hyper phosphorylation of tau and formation of toxic tau, and overexpression of certain miRNAs has resulted in improved synaptic transmission [101, 102, 103]. Several miRNAs have been associated with increased production of amyloid- β [104, 105].

Research has confirmed that a variety of cancers are caused by aberrant expression of miRNAs, with miRNAs functioning as both oncomiRs and tumor suppressors [106]. Dysregulation of miRNA biogenesis protein DICER, Drosha and AGO2 have been linked to the prognosis of cancer patients [107, 108, 109]. Many types of cancers cause downregulation of tumor suppressive miRNA families like miR-34, let-7, miR-520, etc. and owing to multiple consolidated evidences on the same, some of this research has also progressed to clinical trials [110, 111, 112, 113]. miRNAs with oncogenic functions like miR-21, miR-155, miR-210, etc. are also upregulated in cancerous tissue in comparison to healthy tissue [114, 115, 116].

In heart, miRNAs are dysregulated in response to stress conditions like hypoxia and restoration of miRNAs to the normal levels post injury have resulted in cardiac healing [117, 118]. Some miRNAs like miR-765, miR-25, miR-24 are responsible for contractile regulation, which is a hallmark of heart failure [119, 120, 121]. Age related dysregulation of miRNAs (miR-1 and miR-133) has also shown to play important roles in problems related to rhythm of the heartbeat like arrhythmias [122].

Cardiac fibrosis and microRNAs

Research in the field of miRNA is still in its nascent stages and their relation to cardiac fibrosis is being increasingly explored. miR-30 and miR-133 have been shown to target CTGF and collagen expression is influenced by miR-29 activity [123, 124]. Adenoviral based transport of miR-1 in myocardium in a rat model of pressure overload-induced hypertrophy reduced cardiac fibrosis, thereby improving cardiac function [125]. MSCs transfected with miR-133a decreased cardiac fibrosis in a rat myocardial infarction model more than MSCs without miR-133a transfection [126]. miRNA families can also regulate cardiac fibrosis like miR-15 family inhibits TGF- β pathway, affecting cardiac fibrosis [127, 128]. A major challenge with using miRNAs for treating cardiac fibrosis is their multiplicity of gene targets and their synergistic effect makes it difficult to target specific genes [129].

1.2.4 Interplay between stem cells, exosomes, and microRNAs

It has been reported that exosomes secreted from stem cells have a significant role in the paracrine signaling of transplanted stem cells [130]. Injection of exosomes derived from CPCs into insulted mouse heart have shown regenerative effects similar to CPC transplantation [131]. MSC exosomes have shown protective effects in mouse kidney injury model by inhibiting apoptosis and increasing cell proliferation [132]. A variety of tissue regeneration effects of stem cell derived exosomes have been explored, such as, wound healing, [133], skeletal muscle regeneration, [134], limb ischemia repair [135], etc. MSC derived exosomes have been shown to alleviate liver fibrosis, increase hepatic regeneration in drug-induced liver injury models and suppress the growth of hepatocellular carcinoma in a rat model [136, 137, 138].

miRNA are essential for stem cell renewal, maintenance, function and are important regulators of post-transcriptional gene expression [139]. miRNAs have been shown to regulate senescence of neural stem cells, MSCs, and HSC, by targeting genes responsible for their epigenetic changes, metabolism and DNA damage [140, 141, 142]. Certain families

of miRNAs play major roles in ESC cycle regulation by influencing the transition from G1 to S phase [143]. miRNAs have been shown to regulate self-renewal and proliferation of gastric cancer stem cells as well [144]. miR-205 is highly expressed in skin stem cells and has an important role in their neonatal expansion. Highly compromised epidermal and hair follicle growth accompanied by neonatal lethality is caused by genetic deletion of miR-205 [145].

Exosomes are used for communication by many cell types in the body. Certain miRNAs are secreted by Interleukin-4 (IL-4)-activated macrophages to promote the invading potential of breast cancer cells [146]. A miRNA composition analysis of serum exosomes from prostate cancer patients and healthy individuals revealed differential expression of 12 miRNAs in prostate cancer [147]. Exosome mediated transport of miR-375 from pancreas has been shown to regulate insulin secretion [148]. Exosomes secreted by vascular endothelial cells contain miR-214 which is capable of resisting vascular aging and promoting angiopoiesis [149]. Exosomes secreted by cardiomyocytes contains miRNAs which can reduce myocardial fibrosis and increase myocardial regeneration [150]. A few miRNAs in exosomes released by endothelial cells play significant roles in angiogenesis and anti-atherosclerosis [151, 149].

As shown in Figure 1.5, stem cells use exosomes for communicating with other cells, and miRNAs are transported inside these exosomes along with other proteins, nucleic acids, lipids, etc. In exosomes secreted from MSCs, more than 800 unique gene products and 151 distinct miRNAs have been found [152, 153]. Exosomes isolated from CPCs contain several cardio-protective and pro-angiogenic miRNAs [154]. MSC derived exosomes containing miR-21 have been shown to significantly improve cardiac function after acute myocardial infarction by reducing apoptosis, increasing neovascularization and inducing expression of vascular endothelial growth factor in heart [155]. CPC derived exosomes isolated from neonates enhanced angiogenesis, decreased fibrosis, improved hypertrophy, thereby ameliorating cardiac function in rats after ischemia– reperfusion injury. A compu-

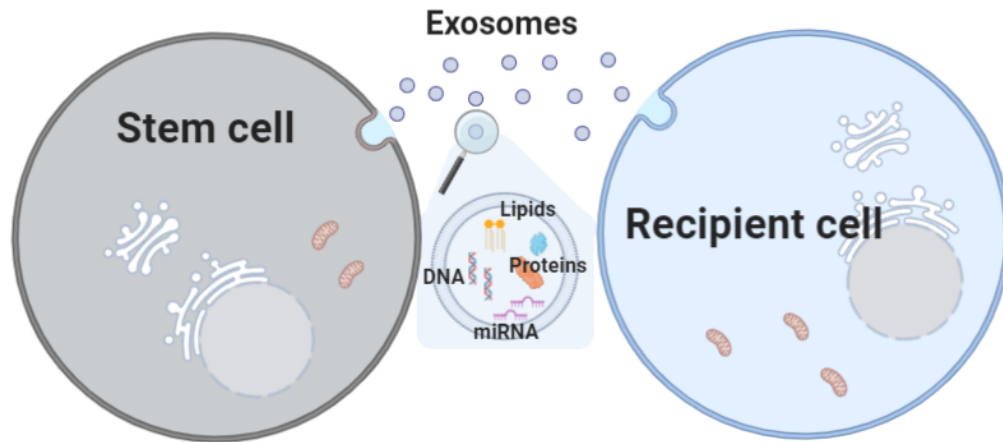


Figure 1.5: Schematic of exosomes aiding cell-cell communication between stem cell and recipient cell

Along with lipids, proteins, and nucleic acids, miRNAs are packaged inside exosomes and transported from stem cell to recipient cell for gene expression regulation in the recipient cell. Image created using BioRender.com.

tational model was built to relate miRNA levels in exosomes with cardiac outcomes like angiogenesis, fibrosis, ejection fraction [156].

1.3 Exosome therapy

Exosomes have drawn attention as valuable biomarkers in many pathophysiological states, as indicators of disease progression and provide disease stage-specific information [157, 158, 159, 160]. Differences in composition of isolated exosomes and their size reflects the type and state of the source cell. They can be secreted naturally, under cellular stress or by activation signals and have the potential to modulate processes in which they are involved [161]. Since these exosomes aid in cell-cell communication between distant or local cells by transferring metabolites, nucleic acids, and lipids, they also possess the potential to impact treatment of diseases [162, 163]. Exosomes can be personalized targeted drug delivery vehicles of the future [164].

The double layered membrane and nano size of exosomes protect exosome cargoes from damage or fast clearance by the complement fixation or macrophages, prolonging

their half-life in circulation and enhancing their biological activity. Being natural carriers with unique structural, compositional and morphological characteristics and fascinating biochemical and physicochemical properties, exosomes have many other beneficial features for therapeutic delivery like low immunogenicity, biocompatibility, biological barrier permeability and low toxicity [165]. Exosomes released directly by budding from plasma membrane contain surface-bound proteins of the cell from which they originated. So exosomes from tumor cells, antigen presenting cells, and dendritic cells are a promising source for vaccine development. The innate therapeutic capabilities of exosomes can be further enhanced by chemical or biological modification of their lipid bilayers, i.e., exosome engineering. Hence, treatment of diseases can be facilitated by exosome mediated drug transfer [166].

The ability to produce custom designed exosomes and modify their migratory itinerary is incredibly important for the development of exosome therapy [167]. Parent cells can be engineered to secrete modified exosomes by using viral and non-viral methods or contents of secreted exosomes can be directly manipulated. Taking advantage of exosomes' porous structure, electroporation can be used to modify secreted exosomes, however, this method has poor efficiency in DNA transfer [168, 169]. Basic incubation can also be used to modify exosomes parental cells or secreted exosomes can be incubated with a drug [168]. Other techniques include lipofection, sonication, genetic engineering using viral vectors and direct transfection [170, 167].

Currently, exosomes are being researched for aiding in drug delivery, nucleic acid delivery, protein delivery and immunotherapy. Scientists have engineered exosomes with required proteins and metabolites for drug delivery in cancer therapy [171]. For treatment of Parkinson's disease, biopharmaceutical-encoding mRNAs were inserted inside exosomes which attenuated neuroinflammation of patients when delivered to recipient cells [172]. A genetically engineered exosome based nanosystem with immunostimulatory DNA and endogenous tumor antigens has been shown to enhance cancer immunotherapy [173]. Hu-

man umbilical vein endothelial cell derived exosomes, when treated with chemotherapeutic agents, induced antitumor effects during breast cancer chemotherapy [174]. miR-314 was highly downregulated in breast cancer tissues in comparison to healthy tissue. Exosomes loaded miR-134 were able to reduce migration and invasion of breast cancer cells and increased their sensitivity to cancer drugs [175].

Although exosome-based therapies are showing promising therapeutic effects, they are a recent emergence and many detailed and thorough investigations are warranted to move this therapy to clinic. For example, more research is required to find the best combinations of exosome producing cells and target cells. A defined and uniform methodology for exosome isolation and characterization is required for validating numerous findings reported on therapeutic effects of exosomes [176]. Another challenge is loading exosomes efficiently, but without changing the structure and content of their lipid bilayer membrane [177]. On the bright side, exosome therapy has the potential to offer unparalleled efficiency in treatment of life-threatening diseases like cancer, cardiovascular diseases, including others with lacking effective pharmacotherapy.

1.4 Partial Least Squares Regression

Partial Least Squares Regression (PLSR) is a multivariate regression method that is used for correlating the information in one data matrix (X) to the information in another data matrix (Y). PLSR can be used to predict or explain the behavior of hard-to-measure response variables (Y) using controllable or easy-to-measure variables (X) [178]. With too many independent variables (X), a multiple regression model would over-fit the sample data, but will not be able to predict new data. In such scenarios, although there are many independent variables (X), only a few latent variables are able to explain the most variation in response variables (Y). Basically, PLSR extracts these latent variables and explains/predicts the responses without over-fitting. PLSR finds the latent variables that explain the most variation in independent variables (X), the latent variables that explain the most vari-

ation in responses (Y), and the fundamental relations between independent variables (X) and responses (Y) [179].

Principal components, like latent variables mentioned before, are constructed as a combination of initial variables that capture the most important information in the data. Loadings vector is a row vector that contains the combination of initial variables that define the principal component. Scores vector is a column vector that contains the projection of observations in the original dataset, on the principal component. The following equations show the general model of PLSR:

$$X = TP^T + E$$

$$Y = UR^T + F$$

where X is a $n \times m$ matrix of independent variables, Y is a $n \times p$ matrix of responses, l being the number of principal components, T and U are $n \times l$ scores matrices of X and Y, respectively, P and R are $m \times l$ and $p \times l$ loadings matrices of X and Y, respectively, and E and F are error terms [178]. PLSR decomposes the X and Y matrices into its scores and loading vectors such that the scores of X and Y have the maximum covariance. This way, the scores of Y can be predicted using the scores of X, and subsequently Y values can be predicted [180]. This process has been schematically explained in Figure 1.6.

There are several advantages of using PLSR. It is easy to fit PLSR models and determine their statistical properties as they are fairly simple models. PLSR generates visual results that makes it easy to interpret the results. Loadings plot shows the influence of variables on principal components and also relationships between the variables. Scores plot visualizes the observations with respect to principal components, thereby helping in identifying clusters or outliers in observations. PLSR is especially useful when the number of observations is lesser than the number of variables (Linear regression fails in such cases) [181].

PLSR modeling was used to explore relations between air pollutant levels, cytokines, immune cell types, and blood pressure in adolescents and found that markers of acute in-

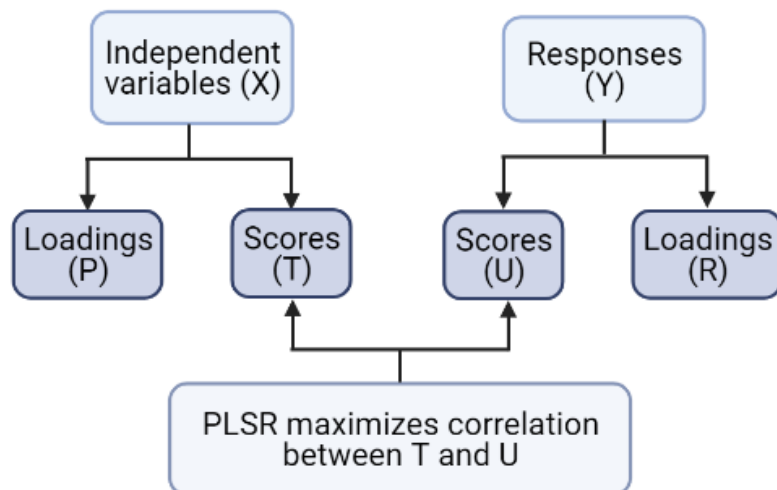


Figure 1.6: **PLSR strategy**

Matrices of independent variables and responses are decomposed into scores and loadings vectors in a way that the scores of X and Y have the maximum covariance.

inflammation, oxidative stress, and monocytes, explained the most variation in air pollution [182]. CPC RNA sequencing results analyzed with PLSR were used to make a predictive model for cellular behaviors like migration and proliferation [183]. PLSR recognized novel mRNA targets of miRNAs when analyzing miRNA and mRNA microarray expression profiles from human colon tumor tissues and normal tissues. [184] miRNA array analysis of CPCs exosomes using PLSR revealed their miRNA-mediated reparative potential in a rat model of ischemia–reperfusion injury [156].

CHAPTER 2

IDENTIFICATION OF MICRORNAS FOR DOWNREGULATING CARDIAC FIBROSIS USING COMPUTATIONAL MODELING: A META-ANALYSIS

2.1 Introduction

Cardiovascular diseases are the leading cause of mortality in the US and more than 655,000 Americans die from cardiac diseases each year, that is, one in every four deaths [185]. Cardiac remodeling changes shape, size, and composition of the heart in response to cardiac damage or disease. Myocardial fibrosis is an important component of cardiac remodeling and refers to the excess deposition of extracellular matrix in the cardiac muscle. Cardiac fibrosis can lead to a stiffer myocardium, erratic heartbeats, and eventually lead to heart failure or death [186].

Stem cells/progenitor cells use exosomes for communicating with other cells, and miRNAs are transported inside them. As explained in the previous chapter, exosomes are extracellular vesicles that aid cell-cell communication and carry proteins, metabolites, nucleic acids, etc. miRNAs are small non-coding RNA molecules that can be transported by exosomes and are uniquely capable of facilitating long-term repair by altering the targeted cells' transcriptome. Previous research has demonstrated relationships between exosomal miRNA content and fibrosis in the heart [156].

Prior research by our group (in collaboration with the Davis lab at Emory) showed that neonate derived CPC treatment significantly reduced fibrosis compared to saline treatment in a rat model of juvenile heart failure [37]. Masson's trichrome staining in hearts of rats with myocardial infarction showed lesser fibrosis when injected with CPCs compared to cardiosphere-derived cells and controls [187]. Exosomes isolated from hypoxia treated CPCs alleviated cardiac fibrosis when injected in a rat myocardial infarction model [85].

miRNA content of CPC derived exosomes was related to its antifibrotic response *in vitro* and was used to predict fibrotic responses to exosome treatment *in vivo* [188]. miRNA content of CPC derived exosomes varied with donor age, and thereby showed different degrees of antifibrotic effects when injected into athymic rats [156]. Together, these studies suggest that miRNAs play a role in determining the antifibrotic effect of exosomes, and exosomes are instrumental to the therapeutic effect of CPCs.

As next steps, our group would like to design exosomes with custom miRNA content that can downregulate cardiac fibrosis. To achieve this goal, we need to select miRNAs that can downregulate fibrosis and predict fibrosis responses with the miRNA signals. To choose miRNAs and specifically to perform predictive modeling, we would need large amount of data relating miRNA levels with cardiac fibrosis markers. So we decided to benefit from the existing research in this field and aggregate data from the large amount of published literature.

There are over 900 publications that studied the relationships between miRNAs and fibrosis markers in the heart. In this meta-analysis, expression levels of miRNAs and quantities of fibrosis markers were extracted from these papers and analyzed. PLSR models were built to form a consensus on relationships between miRNAs and cardiac fibrosis, identify miRNAs that can downregulate cardiac fibrosis, and to predict cardiac fibrosis with miRNAs' fold-changes as inputs. In future studies, the data extracted in this meta-analysis can be used to make PLSR models to analyze relations of a set of chosen miRNAs with cardiac fibrosis and predict the extent of cardiac fibrosis when exosomes with custom-designed packages of miRNAs are injected into animal models. This research has the potential to inform experimental studies of cardiac remodeling.

2.2 Methodology

Literature search and categorization of publications

The following PubMed search term was used to find publications relating expression

levels of miRNAs with cardiac fibrosis- '(miRNA or microRNA) and fibrosis and (heart or cardiac)'. The process of screening and characterizing search results is explained below and demonstrated in Figure 2.1.

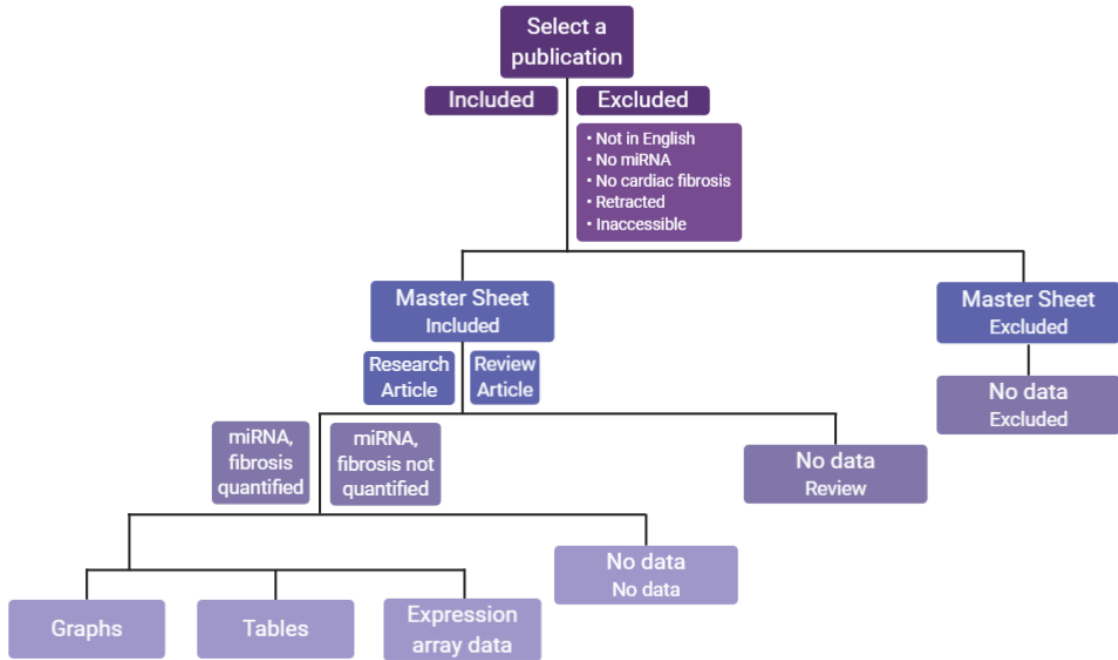


Figure 2.1: Flowchart for the process of characterizing PubMed results

This flowchart describes the process of characterizing publications into six types- Graphs, Tables, Expression array data, No data, Review and Excluded. Master sheet has two tabs- included and excluded, and No data sheet has three tabs- Excluded, Review and No data. Graphs, Tables, and Expression array data are three separate sheets.

If a publication was not written in English, did not discuss miRNAs, did not investigate cardiac fibrosis, was retracted or inaccessible, it was directly excluded from this meta-analysis. The excluded publications were listed in the excluded tab of Master sheet and in the excluded tab of No data sheet with their reason of exclusion. If a publication was included, the following details were documented in the included tab of the Master sheet- publication title, summary of the paper, miRNA name, correlation with cardiac fibrosis, measure of fibrosis and data availability. If the included publication was a review article, it was listed in the review tab of the No data sheet. On the other hand, if it was a research article, it was further evaluated for inclusion in the meta-analysis. If miRNA and cardiac

fibrosis were not quantified for same experimental conditions, this paper was listed in the No data tab of the No data sheet with a reason for the same. If miRNA and cardiac fibrosis were quantified for same experimental conditions, depending on the available data type, publications were put into these three categories- Graphs, Tables, Expression array data. If multiple types of data is available in the paper, it was put into a category using the following priority order: Expression array data > Tables > Graphs. This priority order was used as maximum data should be extracted from publications for the meta-analysis. The process of categorizing publications into these sheets has been schematically explained in Figure 2.1. Reasons of allotting publications into specific categories have also been summarized in Table 2.1.

Table 2.1: Reasons for categorizing PubMed search results into specific categories

Reasons for categorizing PubMed search results into specific sheets and tabs are summarized.

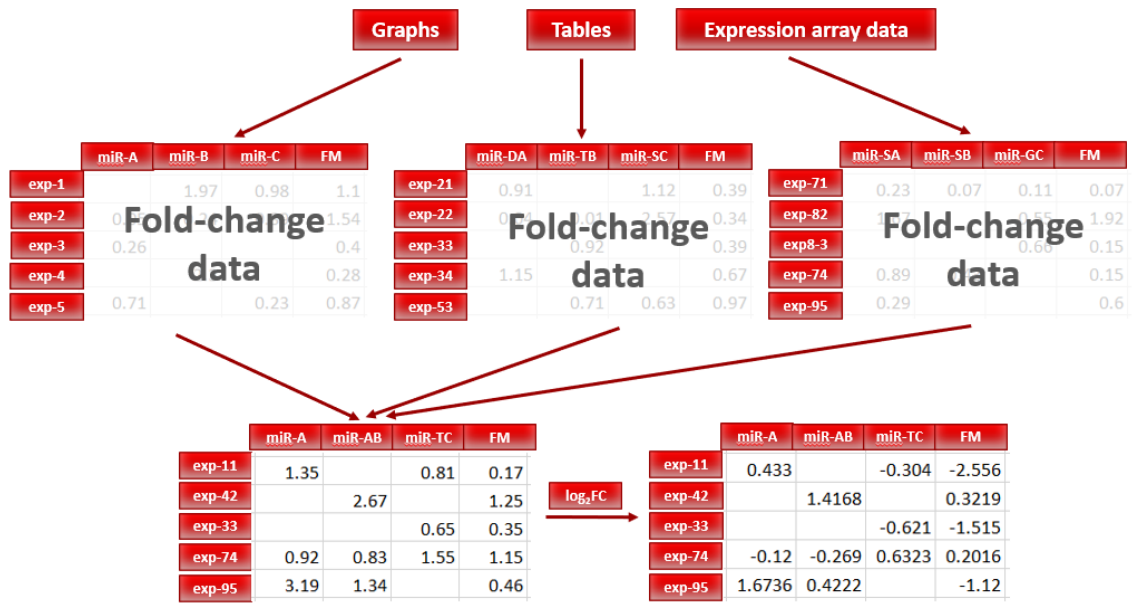
Sheet name	Tab name	Reason
Master sheet	Included	The publication is written in English, discusses miRNAs and cardiac fibrosis, is not retracted and is accessible.
Master sheet	Excluded	The publication is not written in English, does not discuss miRNAs or cardiac fibrosis, is retracted or inaccessible.
No data	No data	The publication is included but has not quantified miRNA, cardiac fibrosis, or both of them have not been quantified for same experimental conditions.
No data	Review	The publication is included but it is a review article
No data	Excluded	Same as in the excluded tab of Master sheet
Graphs		The publication is included, is a research article and has quantified both miRNA and fibrosis for same experimental conditions in a graphical format.
Tables		The publication is included, is a research article and has quantified both miRNA and fibrosis for same experimental conditions. At least one of them has been presented in a tabular format.
Expression array data		The publication is included, is a research article and has quantified both miRNA and fibrosis for same experimental conditions. Entire expression array data is available at least for miRNAs.

Data Extraction and consolidation- Fold change and p-value

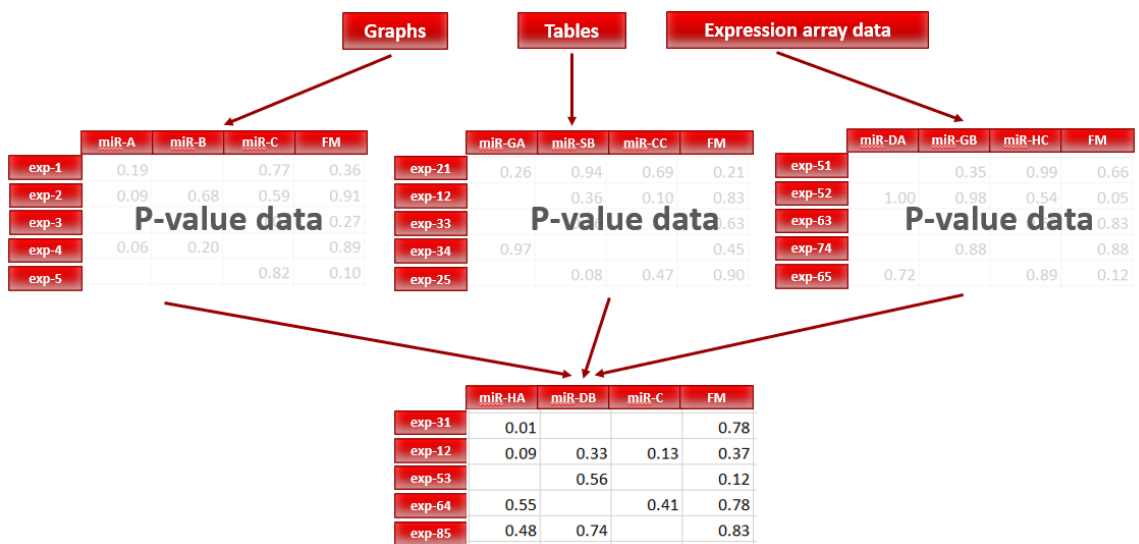
For extracting data from graphs, snippets of graphs were uploaded into graphreader.com, axis were adjusted and labeled, and values were noted by hovering the cursor over the graph. Data from graphs was extracted into excel sheets with rows being the variables (miRNAs, mRNAs, proteins, etc.) and columns being the experiments (observations). Then fold changes were calculated for the experiments that had significantly different (p -value <0.05) values for both miRNAs and fibrosis markers. P-values were also noted parallelly with fold changes. Fold changes and p-values were readily available for data in tabular format. Therefore, when extracting data from tables, for each experiment, fold changes and p-values of variables were directly copied and pasted into excel sheets for both miRNAs and fibrosis markers. When the entire expression array data was available for an experiment, it was first copied into an excel sheet. Fold changes and p-values were calculated in excel for both miRNAs and fibrosis markers. Fold change was calculated by taking ratio of mean expression values for any two conditions. P-values for the same conditions were calculated using two-tailed Student's t-test where expression values of the two conditions were input as array one and array two, respectively.

Fold changes from all the graphs were compiled into one sheet with rows being the experiments from publications (observations) and columns being miRNAs and fibrosis markers (variables). The same process was followed for data in tables, and in expression array format as shown in Figure 2.2a. These three sheets were then consolidated into one single sheet and converted to log-base 2 scale. Similarly, p-values from excel sheets of graphs, tables, and expression array were compiled into one sheet as shown in Figure 2.2b. Further analysis for establishing relationships between miRNAs and fibrosis markers used the two files with consolidated fold changes and p-values data from all selected publications.

All miRNAs found were included in the analysis but only the following fibrosis markers were considered: Masson trichrome staining, Picrosirius red staining, Collagen type I alpha 1 chain (COL1A1) mRNA, Collagen type I alpha 2 chain (COL1A2) mRNA, Collagen type III alpha 1 chain (COL3A1) mRNA, CTGF mRNA, Vimentin (VIM) mRNA, Collagen 1



(a) Extraction and consolidation process of fold change data



(b) Extraction and consolidation process of p-value data

Figure 2.2: Extraction and consolidation process of fold change and p-value data from publications selected for meta-analysis

Extraction and consolidation process of fold change and p-value data of miRNAs and fibrosis markers- starting from data in form of graphs, tables, expression array data in publications selected for meta-analysis, to one final sheet for fold change and p-value data, respectively.

protein, Collagen 3 protein, CTGF protein and Vimentin protein. The fibrosis markers were selected based on prior research and were also in sync with the previous experiments conducted as a part of the collaboration (with Davis lab at Emory) this project is a part of.

A list of miRNAs with their number of experiments was made using the consolidated fold change file. Similarly, a list was compiled for fibrosis markers with their number of experiments. To find the number of publications that studied each miRNA, the following data was documented and grouped together- miRNA name, correlation with cardiac fibrosis and source publication title. miRNAs with same precursor miRNA were placed with each other, for example, miR-29, miR-29a-3p, and miR-29b were grouped together.

Scoring formulae weighting for fold change, p-value and miRNAs’ number of experiments were developed to select highly correlated miRNAs with cardiac fibrosis

Three scoring models were used to rank ~5000 miRNAs based on the magnitude and consistency of their correlation with cardiac fibrosis- each having different weights for fold-change, p-value and miRNAs’ number of experiments. Model 1 scoring formulae were unbiased towards miRNAs’ number of experiments, model 2 scoring formulae were biased towards well-studied miRNAs and model 3 scoring formulae were biased towards under-studied miRNAs. The formulae for the three scoring models are shown below:

Model 1:

$$\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) + Pvalue(y)} \right)$$

$$\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) \times Pvalue(y)} \right)$$

Model 2:

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) + Pvalue(y)} \right) \right] \times \left(\frac{numExpts}{maxNumExpts} \right)$$

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) + Pvalue(y)} \right) \right] \times \sqrt{\frac{numExpts}{maxNumExpts}}$$

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) \times Pvalue(y)} \right) \right] \times \left(\frac{numExpts}{maxNumExpts} \right)$$

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) \times Pvalue(y)} \right) \right] \times \sqrt{\frac{numExpts}{maxNumExpts}}$$

Model 3:

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) + Pvalue(y)} \right) \right] \times \left(1 - \frac{numExpts}{maxNumExpts} \right)$$

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) + Pvalue(y)} \right) \right] \times \sqrt{1 - \frac{numExpts}{maxNumExpts}}$$

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) \times Pvalue(y)} \right) \right] \times \left(1 - \frac{numExpts}{maxNumExpts} \right)$$

$$\left[\sum_{Expts} \left(\frac{\log_2 FC(x) \times \log_2 FC(y)}{Pvalue(x) \times Pvalue(y)} \right) \right] \times \sqrt{1 - \frac{numExpts}{maxNumExpts}}$$

where $x \rightarrow$ miRNA,

$y \rightarrow$ fibrosis marker,

FC \rightarrow fold-change,

Pvalue \rightarrow p-value,

numExpts \rightarrow number of experiments the miRNA was analysed in and

maxNumExpts \rightarrow maximum number of experiments any miRNA was analysed in.

Each scoring formula calculates one number for each miRNA, based on miRNA's fold

changes and p-values, fold changes and p-values of corresponding fibrosis markers, and the number of experiments that miRNA has been studied in. This correlation number is then used to rank the miRNAs for that formula.

miRNAs having positive correlation with cardiac fibrosis had a positive score and miRNAs having negative correlation with cardiac fibrosis had a negative score. For both positively and negatively correlated miRNAs, top 200 miRNAs having the highest absolute value of correlation were noted for each scoring formula. For each scoring model, two Venn diagrams were made to show the number of miRNAs which were common among the top 200 of each formula, separately for positively and negatively correlated miRNAs. The miRNAs that were common among top 200 miRNAs of all scores, for either positive or negative correlation, were noted separately. Fold change data for these miRNAs was extracted from the previously consolidated fold change data sheet and was used for further analysis.

Partial Least Squared Regression (PLSR) modeling and optimization based on model fitting and prediction accuracy values

PLSR modeling was used to quantify the relationships between fold changes of miRNAs and fibrosis markers in the heart. In PLSR models, miRNAs were as x-variables, fibrosis markers were y-variables, and experiments were observations. SIMCA-P software (Umetrics, Sartorius Stedim Biotech, Ume^oa, Sweden) was used to build PLSR models for a variety of conditions and to calculate their model fitting and prediction accuracy values.

For each scoring model, a PLSR was built from the miRNAs selected by that model and fibrosis markers. To increase the model fitting and prediction accuracy of this PLSR model, a couple of selection criterion were applied on miRNAs and fibrosis markers. Fibrosis markers that had the maximum absolute value of PLSR coefficient with miRNAs greater than 0.1 were selected. For each chosen fibrosis marker, miRNAs were ranked based on their absolute value of PLSR coefficient. miRNAs belonging to the intersection of top 10% miRNAs for each selected fibrosis marker were selected. A PLSR model was trained on

these selected miRNAs and fibrosis markers.

Variable importance in projection (VIP) score is a measure of a variable's importance in a PLSR model and demonstrates its contribution to the model. To further optimize the PLSR model, miRNAs having a VIP score greater than 1 and lower limit of VIP standard error above 0 were selected. These miRNAs were used to calculate model fitting and prediction accuracy value for each fibrosis marker. The model fitting and prediction accuracy values for each fibrosis marker was documented in a table. Fibrosis markers with the best model fitting and prediction accuracy values were selected. The PLSR model made with the VIP miRNAs and selected fibrosis markers had the maximum model fitting and prediction accuracy values. A loadings plot was made for this PLSR model to visualize the relationships between the selected miRNAs and fibrosis markers.

Literature validation of meta-analysis results using publications selected for qualitative analysis and miRTarBase.

For the miRNAs that were identified to downregulate cardiac fibrosis, following values were calculated based on the data aggregated from literature for qualitative analysis- number of research and review articles that reported a relationship between the miRNA and cardiac fibrosis, number of publications in which the miRNA caused or inhibited cardiac fibrosis, number of experiments in which the expression of miRNA varied by more than two folds in cardiac fibrosis versus control condition, and number of papers which were published from the collaborative project with Davis lab and identified the miRNA to be correlated with cardiac fibrosis. To go in further detail on how the miRNA caused/inhibited cardiac fibrosis, the number of publications in which the miRNA was forcefully overexpression/ underexpressed were documented. For the same miRNAs, scoring models that identified that they could downregulate cardiac fibrosis and their effect on cardiac fibrosis as concluded from the meta-analysis were also noted. This entire aggregated data was condensed into a form of a table.

While making the optimized PLSR for miRNAs from scoring model 3 (biased to-

wards under-studied miRNAs), many miRNAs that were in first set of chosen miRNAs (selected using miRNA-fibrosis marker PLSR coefficients) were not present in the optimized model. To explore if these miRNAs had cardiac fibrosis related target genes, the miRNAs were queried in miRTarBase [189] for *homo sapiens*, *mus musculus*, and *rattus norvegicus* species. For each miRNA, the following cardiac fibrosis markers were searched in its target genes: COL1A1 mRNA, COL1A2 mRNA, COL3A1 mRNA, CTGF mRNA, and VIM mRNA. A table was made with these miRNAs and their cardiac fibrosis related target genes.

PLSR models were used to predict fibrosis markers in *in silico* experiments with downregulated profibrotic miRNAs and upregulated antifibrotic miRNAs

The optimized PLSRs from each scoring model were used to predict the levels of the following fibrosis markers: Masson trichome stained area, COL1A1 mRNA, and Collagen 1 protein. Profibrotic miRNAs were downregulated and antifibrotic miRNAs were upregulated for 30 *in silico* experiments. miRNA data was randomly generated using RAND() function in excel for three models. For upregulation, $\log_2(\text{fold-change})$ value was between 0 and 5 and for downregulation, $\log_2(\text{fold-change})$ value was between -5 and 0. Fibrosis markers were predicted for the three PLSR models and the results were compared using two-tailed Student's t-test.

2.3 Result

Searched PubMed, screened search results, and selected 248 articles for meta-analysis

PubMed was searched for publications relating expression levels of miRNAs with cardiac fibrosis and 911 search results were obtained. These publications were screened using multiple criteria as shown in Figure 2.3. 189 articles were excluded in the first step due to one of the following reasons- not written in English, did not discuss miRNAs, did not investigate cardiac fibrosis, was retracted or inaccessible. Remaining 511 research articles and 211 review articles were included in the qualitative study. For the meta-analysis, data

was extracted from 248 research articles that quantified miRNA and cardiac fibrosis for same experimental conditions.

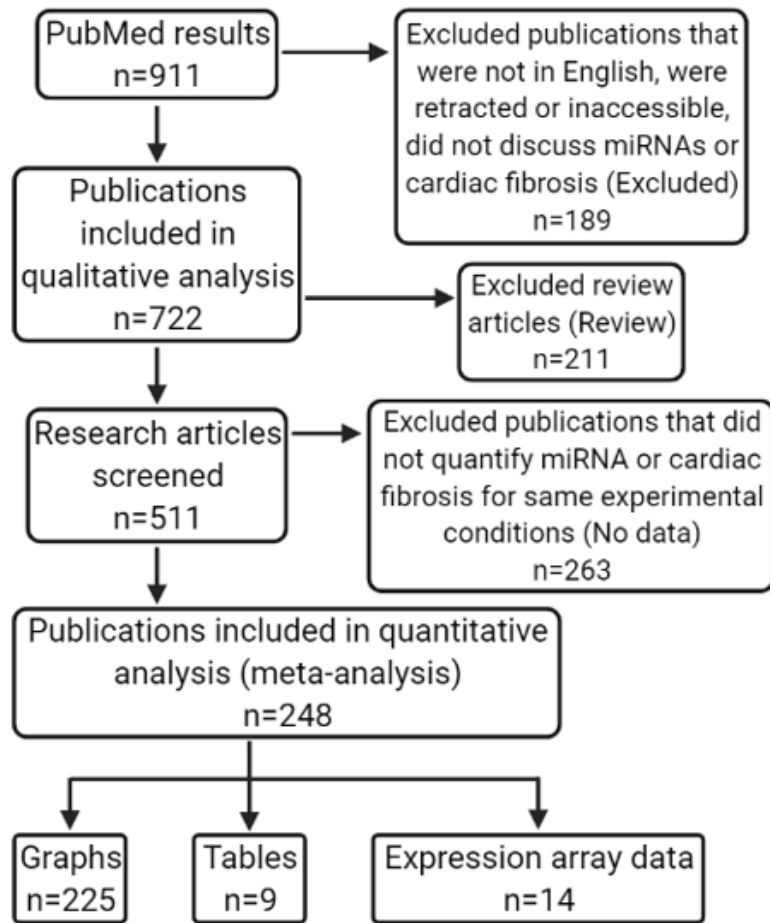


Figure 2.3: Flow diagram describes the process of screening PubMed search results and selecting 248 articles for meta-analysis

911 PubMed results were obtained using the search term- “(miRNA or microRNA) and (heart or cardiac) and fibrosis”. These publications were screened, and finally 248 papers were selected for computational modeling. The flow diagram shows 911 initial PubMed results, 722 publications included for qualitative analysis, 511 research articles and 248 publications selected for meta-analysis analysis- with data in graphical, tabular and expression array data format.

List of the most frequently analyzed miRNAs with their number of experiments in publications selected for meta-analysis is shown in Table 2.2. The relationships of miR-21, miR-29 family, miR-133a with cardiac fibrosis have been analyzed in the maximum number of experiments. A list of the most frequently analyzed fibrosis markers with their num-

ber of experiments in publications selected for meta-analysis is shown in Table 2.3. Collagen mRNAs, proteins and Masson Trichrome staining have been mostly used to quantify cardiac fibrosis in experiments.

Table 2.2: List of the most frequently analyzed miRNAs with their number of experiments in publications selected for meta-analysis

miRNA name	Number of experiments	miRNA name	Number of experiments	miRNA name	Number of experiments
miR-21	102	miR-378	28	miR-29b-3p	22
miR-29b	85	miR-15b	27	miR-423-3p	22
miR-29a	67	miR-142-3p	26	miR-27a	22
miR-29c	62	miR-10a	26	miR-331-3p	21
miR-133a	55	miR-25	26	miR-328	21
miR-132	39	miR-30a	26	miR-455	21
miR-1	36	miR-125b	26	miR-199a-3p	20
miR-26b	36	miR-145	26	miR-29c-3p	20
miR-26a	33	miR-214	26	miR-339-5p	20
miR-30c	32	miR-19b	25	miR-345-5p	20
miR-34a	30	miR-199a-5p	24	let-7c	20
miR-133b	29	miR-221	24	miR-22	20
miR-155	29	miR-143-3p	23	miR-27b	20
miR-223	29	miR-130a	23	miR-140-3p	19
miR-155-5p	28	miR-125a-5p	22	miR-151-5p	19

Table 2.3: List of the most frequently analyzed fibrosis markers with their number of experiments in publications selected for meta-analysis

Fibrosis marker name	Number of experiments
COL1A1 mRNA	363
Collagen 1 protein	246
COL3A1 mRNA	242
Masson Trichrome	229
Collagen 3 protein	143
CTGF mRNA	115
CTGF protein	78
Picrosirius red	66
COL1A2 mRNA	31
VIM mRNA	22
Vimentin protein	7

Scoring models selected miRNAs that were highly correlated with cardiac fibrosis

Data for over 5000 miRNAs was extracted from the publications selected for meta-analysis. Model 1 had two scoring formulae and was unbiased towards miRNAs' number of experiments. Models 2 and 3 had 4 scoring formulae each and were biased towards well-studied and under-studied miRNAs, respectively. Every scoring model was used to rank ~5000 miRNAs based on the magnitude and consistency of their correlation with cardiac fibrosis- each formula having different weights for fold-change, p-value and miRNAs' number of experiments.

Venn diagrams in Figure 2.4a, Figure 2.4b show that 184 highly positively correlated miRNAs were selected, and 123 highly negatively correlated miRNAs were selected for PLSR modeling with scoring model that was unbiased towards miRNAs' number of experiments. 170 (positive) and 121 (negative) miRNAs were selected using the scoring model biased towards well-studied miRNAs (Figure 2.5a, Figure 2.5b). Similarly, 186 highly positively correlated and 120 highly negatively correlated miRNAs were selected with the scoring model that was biased towards under-studied miRNAs (Figure 2.6a, Figure 2.6b). Fold change data of these selected miRNAs was extracted from previously consolidated fold change data sheet and was used for PLSR analysis.

PLSR modeling identified miRNAs that can downregulate cardiac fibrosis

Relationships between expression levels of miRNAs and quantities of fibrosis markers in the heart were quantified with PLSR modeling for the three scoring models. Model fitting and prediction accuracy values were used to measure the optimization of the model.

For scoring model 1, PLSR model made with 307 miRNAs selected using scoring formulae and 11 fibrosis markers had model fitting value of 0.44 and prediction accuracy value of 0.30. To improve the model fitting and prediction accuracy of this model, 8 fibrosis markers having the maximum absolute value of PLSR coefficient with miRNAs greater than 0.1 in this PLSR model were selected. Subsequently, miRNAs were ranked based

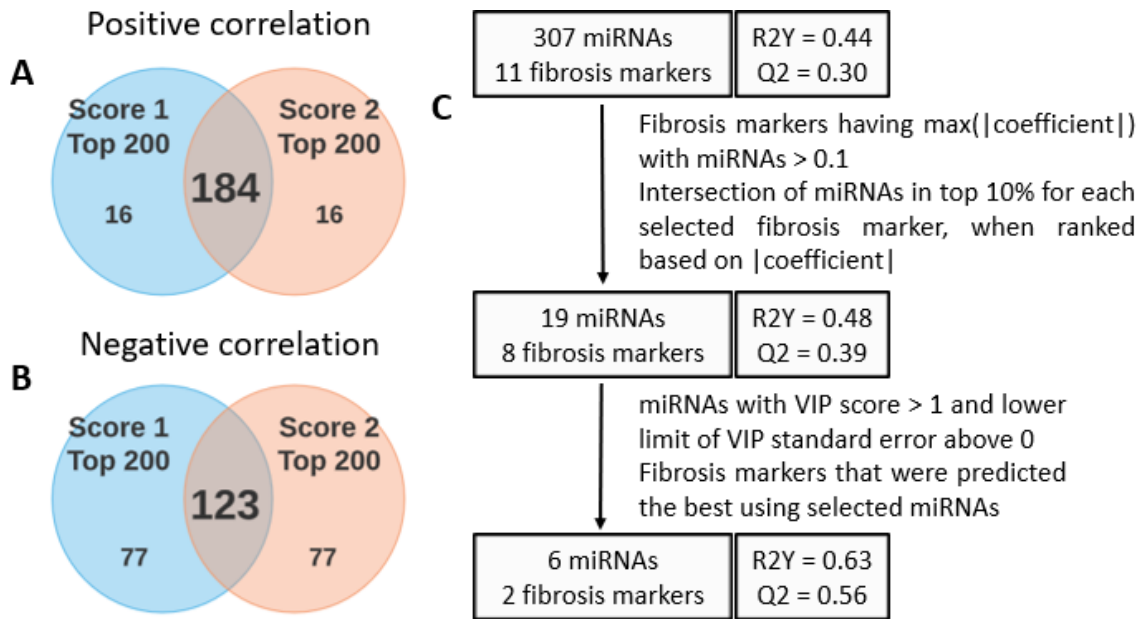


Figure 2.4: **PLSR modeling identified six miRNAs that can downregulate cardiac fibrosis using scoring formulae unbiased towards miRNAs' number of experiments**

Model 1 had two scoring formulae that were used to rank ~5000 miRNAs based on the magnitude and consistency of their correlation with cardiac fibrosis- each having different weights for fold-change, p-value and were unbiased towards miRNAs' number of experiments. A) 200 miRNAs with the highest positive correlation were selected from each scoring formula, and the overlap in the Venn Diagram shows 184 common miRNAs that were used in further analysis. B) Similarly, 123 miRNAs were selected as highly negatively correlated miRNAs. C) Together for 307 miRNAs and fibrosis markers, PLSR models were trained using experimental data extracted from publications selected for meta-analysis. Based on miRNA-fibrosis marker PLSR coefficients and VIP scores, PLSR modeling found 6 miRNAs (miR-33, miR-26a, miR-30c, miR-125b, miR-155-5p, miR-34a) that can explain and predict cardiac fibrosis (COL1A1 mRNA, Collagen 1 protein). R2Y → model fitting value and Q2 → prediction accuracy value.

on their absolute value of PLSR coefficient for each chosen fibrosis marker. 19 miRNAs in the intersection of top 10% miRNAs for each selected fibrosis marker were selected. The PLSR model trained on these selected miRNAs and fibrosis markers had improved model fitting (0.48) and prediction accuracy (0.39) values. To optimize this model further, 6 miRNAs with VIP score greater than 1 and lower limit of VIP standard error above 0 were selected from this model. Then, a PLSR model was made for each fibrosis marker from the selected miRNAs. PLSR model fitting and prediction accuracy values for each fibrosis marker have been shown in Table 2.4. COL1A1 mRNA and Collagen 1 protein were

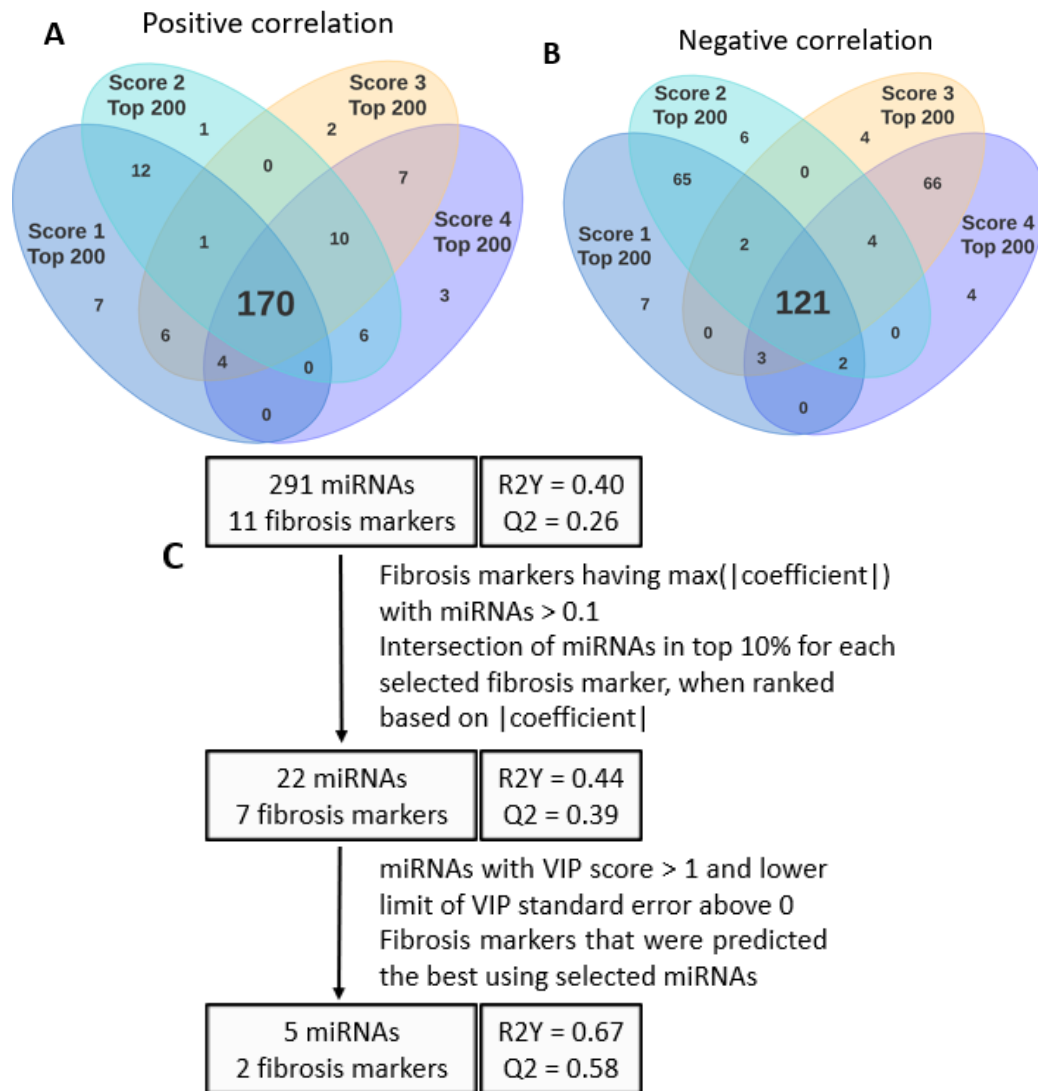


Figure 2.5: PLSR modeling identified five miRNAs that can downregulate cardiac fibrosis using scoring formulae biased towards well-studied miRNAs

Model 2 had four scoring formulae that were used to rank ~5000 miRNAs based on the magnitude and consistency of their correlation with cardiac fibrosis- each having different weights for fold-change, p-value and were biased towards miRNAs' number of experiments. A) 200 miRNAs with the highest positive correlation were selected from each scoring formula, and the overlap in the Venn Diagram shows 170 common miRNAs that were used in further analysis. B) Similarly, 121 miRNAs were selected as highly negatively correlated miRNAs. C) Together for 291 miRNAs and fibrosis markers, PLSR models were trained using experimental data extracted from publications selected for meta-analysis. Based on miRNA-fibrosis marker PLSR coefficients and VIP scores, PLSR modeling found 5 miRNAs (miR-21, miR-29b, miR-29a, miR-26a, miR-33) that can explain and predict cardiac fibrosis (CTGF mRNA, Collagen 1 protein). R2Y → model fitting value and Q2 → prediction accuracy value.

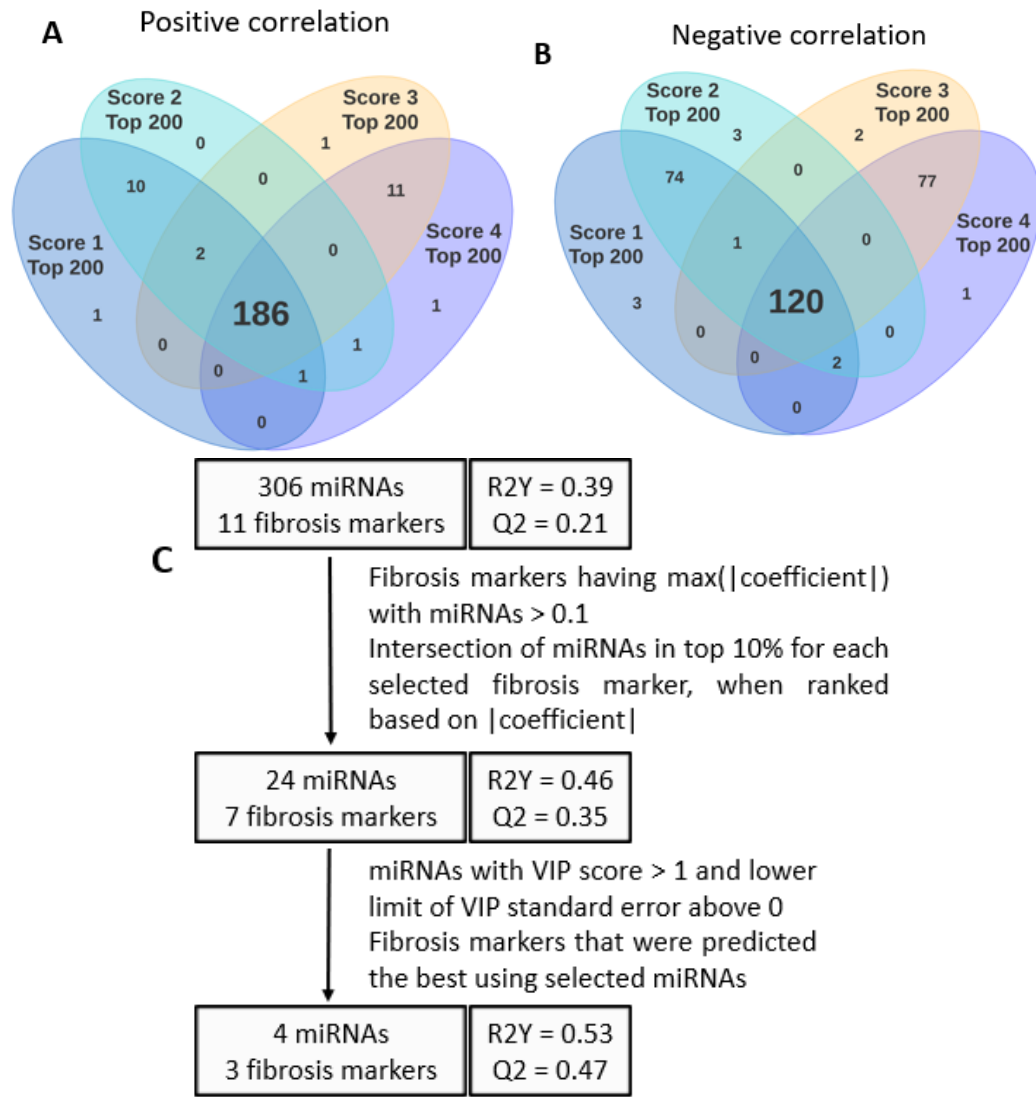


Figure 2.6: PLSR modeling identified four miRNAs that can downregulate cardiac fibrosis using scoring formulae biased towards under-studied miRNAs

Model 3 had four scoring formulae that were used to rank ~5000 miRNAs based on the magnitude and consistency of their correlation with cardiac fibrosis- each having different weights for fold-change, p-value and were biased against miRNAs' number of experiments. A) 200 miRNAs with the highest positive correlation were selected from each scoring formula, and the overlap in the Venn Diagram shows 186 common miRNAs that were used in further analysis. B) Similarly, 120 miRNAs were selected as highly negatively correlated miRNAs. C) Together for 306 miRNAs and fibrosis markers, PLSR models were trained using experimental data extracted from publications selected for meta-analysis. Based on miRNA-fibrosis marker PLSR coefficients and VIP scores, PLSR modeling found 4 miRNAs (miR-33, miR-155-5p, miR-29a, miR-29c) that can explain and predict cardiac fibrosis (CTGF mRNA, Collagen 1 protein, Collagen 3 protein). R2Y → model fitting value and Q2 → prediction accuracy value.

predicted significantly better than other cardiac fibrosis markers and were used in the final model. The optimized PLSR model made with the 6 VIP miRNAs and 2 selected fibrosis markers had the best model fitting (0.63) and prediction accuracy (0.56) values. The flow and results of this optimization has been demonstrated in Figure 2.4c. Similarly, the PLSR optimization results for scoring models 2 and 3 are shown in Figure 2.5c and Figure 2.6c.

Table 2.4: Model fitting and prediction accuracy values for fibrosis markers from PLSRs made using miRNAs that can downregulate cardiac fibrosis

Model 1 scoring formulae were unbiased towards miRNAs' number of experiments, model 2 scoring formulae were biased towards well-studied miRNAs and model 3 scoring formulae were biased towards under-studied miRNAs. After the first round of selection of miRNAs and fibrosis markers for each scoring model, miRNAs with VIP score > 1 and lower limit of VIP standard error above 0 were chosen. These miRNAs were used to make a PLSR model for each fibrosis marker with the experimental data extracted from publications selected for meta-analysis. Fibrosis markers with the highest PLSR model fitting and prediction accuracy values were used in the optimized PLSR model as markers of cardiac fibrosis and their values been marked in bold. R2Y \rightarrow model fitting and Q2 \rightarrow prediction accuracy

Fibrosis marker name	Model 1		Model 2		Model 3	
	R2Y	Q2Y	R2Y	Q2Y	R2Y	Q2Y
COL1A1 mRNA	0.57	0.53	0.43	0.38	0.43	0.37
Collagen 1 protein	0.73	0.67	0.66	0.57	0.47	0.46
COL3A1 mRNA	0.51	0.43	0.44	0.35	0.49	0.45
Masson Trichrome	0.43	0.37	0.49	0.47	0.36	0.23
Collagen 3 protein	0.59	0.41	0.45	0.18	0.66	0.68
CTGF mRNA	0.51	0.49	0.66	0.64	0.54	0.41
Picrosirius red	0.43	0.13	0.28	0.15	0.08	0.21
CTGF protein	0.53	0.49	NA	NA	NA	NA

Loadings plot from the final PLSRs, corresponding to the three scoring models, have been shown in the Figure 2.7. They visualize relationships between the miRNAs and fibrosis markers. For the loadings plot from scoring model 1, the principal component 1 explained 71% of the variability, much greater than that explained by the second principal component (18%). Hence, it is evident that miRNAs on the right side of y axis are negatively correlated with cardiac fibrosis whereas miRNAs on the left side of y axis are positively correlated with cardiac fibrosis. Similarly, profibrotic and antifibrotic miRNAs

can be seen in loadings plots from scoring models 2 and 3.

In summary, the three scoring models and PLSR modeling identified miR-21, miR-33, miR-125b, miR-155-5p, miR-34a as profibrotic miRNAs and miR-29b, miR-29a, miR-26a, miR-30c, miR-29c as antifibrotic miRNAs.

Literature validation of meta-analysis results and miRNA analysis on miRTarBase revealed under-studied miRNAs that can downregulate cardiac fibrosis

Meta-analysis miRNA findings were validated from literature. Publications that were initially selected for qualitative analysis were used to find information on the miRNAs that have been identified to downregulate cardiac fibrosis. Many values related to miRNAs' relationship with cardiac fibrosis were documented as described in the Methods section and are shown in Table 2.5. Based on the aggregated data, it can be observed that miRNAs from scoring model 2 have been studied more in the literature as scoring model 2 was biased towards well-studied miRNAs. However, there was not much difference in the number of publications that studied miRNAs corresponding to scoring models 1 and 3. miR-29 and miR-34a have been shown to inhibit cardiac fibrosis by both overexpression and underexpression in different conditions. Most of these identified miRNAs have been observed to affect cardiac fibrosis in the experiments performed as a part of the collaborative project with the Davis lab.

After the first round of selection of miRNAs and fibrosis markers in scoring model 3, 24 miRNAs were selected. Out of them, only 4 miRNAs were present in the optimized PLSR model. Table 2.6 shows the fibrosis related target genes of the remaining 20 miRNAs. miR-29b-3p and miR-29c-3p target collagen mRNAs, 8 miRNAs target CTGF mRNA, and 6 miRNAs target VIM mRNA.

PLSR models predicted downregulation of cardiac fibrosis when profibrotic miRNAs were downregulated and antifibrotic miRNAs were upregulated

The level of fibrosis markers was predicted using the optimized PLSR models from the

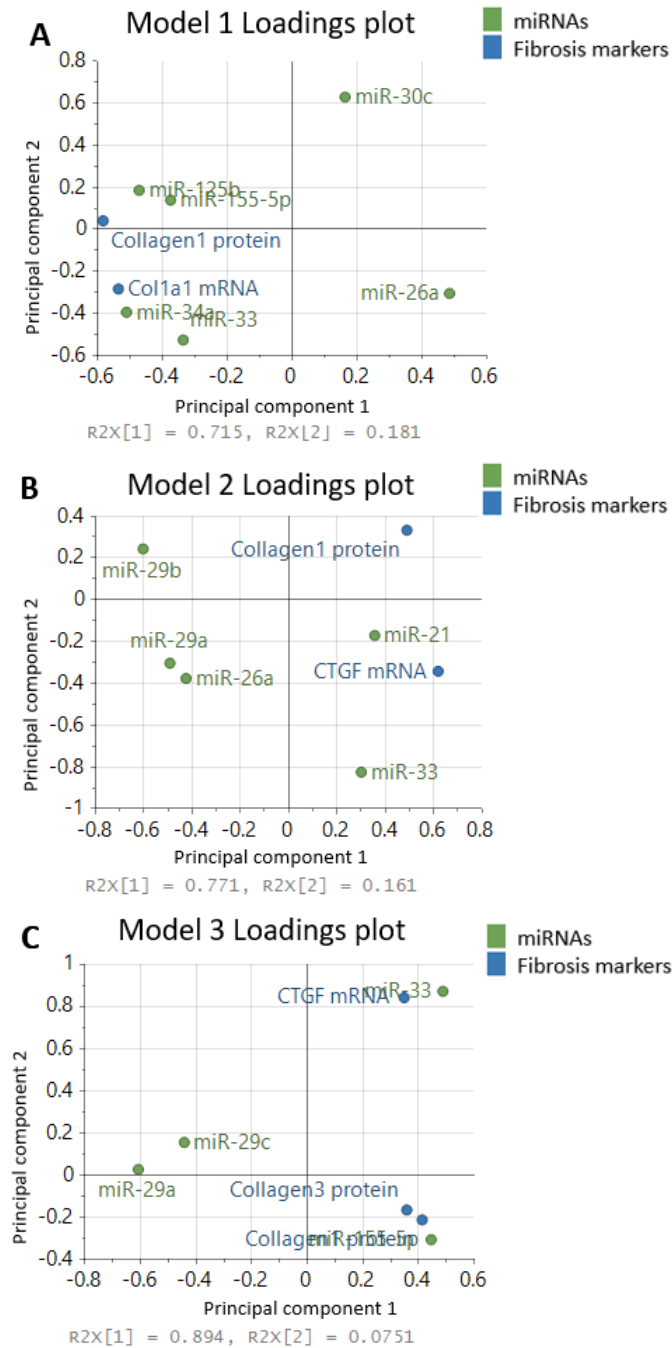


Figure 2.7: PLSR loadings plots shows profibrotic and antifibrotic miRNAs from three scoring models differently biased toward miRNAs' number of experiments

After the first round of selection of miRNAs and fibrosis markers for each scoring model, miRNAs with VIP score > 1 and lower limit of VIP standard error above 0 were chosen. Subsequently, the fibrosis markers that were best predicted by these miRNAs were selected. An optimized PLSR was made for each scoring model using the selected miRNAs and fibrosis marker. In the three PLSR models, principal component 1 explains the most variability. Hence, loadings plots from these PLSR models shows that miRNAs on one side of y axis (with fibrosis markers) are positively correlated with cardiac fibrosis whereas miRNAs on the other side of y axis are negatively correlated with cardiac fibrosis.

Table 2.5: Literature validation of meta-analysis results revealed under-studied miRNAs that can downregulate cardiac fibrosis

Meta-analysis miRNA findings were validated from publications selected for qualitative analysis. Following was documented for the miRNAs that were identified to downregulate cardiac fibrosis- scoring models that identified that the miRNA can downregulate cardiac fibrosis, effect on cardiac fibrosis as concluded from the meta-analysis, number of publications they were studied in, number of experiments where they caused or inhibited cardiac fibrosis, number of experiments in which the expression of miRNA varied by more than two folds in cardiac fibrosis versus control condition, and number of papers which were published from the collaborative project with Davis lab and identified the miRNA to be correlated with cardiac fibrosis. Literature analysis shown in the table gives a list of under-studied miRNAs that might be important regulators of cardiac fibrosis and should be investigated further to consolidate their roles in cardiac fibrosis. Number of research and review articles are separated by a ‘+’ sign. KO: Knockout, expts: experiments, FC: fold-change, N.: Number of, M1: Scoring model 1, M2: Scoring model 2, M3: Scoring model 3, Pro: Profibrotic, Anti: Antifibrotic, Ago: Agomir, Antago: Antagomir

miRNA name	Scoring models	Effect on cardiac fibrosis	N. papers	N. Cardiac fibrosis causation		N. Cardiac fibrosis inhibition		N. expts with $ \log_2 FC > 2$	N. Davis Platt papers
				Ago	Antago / KO	Ago	Antago / KO		
miR-21	M2	Pro	75+111	10			21	29	0
miR-29b	M2	Anti	45+101		9	9	1	11	2
miR-29a	M2, M3	Anti	37+101		5	6	2	5	1
miR-26a	M1, M2	Anti	8+15		2	4		4	1
miR-33	M1, M2, M3	Pro	2+1				2	4	2
miR-30c	M1	Anti	11+43		3	2		6	1
miR-125b	M1	Pro	6+14	2			3	8	3
miR-155-5p	M1, M3	Pro	17+6	4			9	1	0
miR-34a	M1	Pro	11+22	2		1	6	5	1
miR-29c	M3	Anti	28+93		2	3	1	12	1

three scoring models. 30 *in silico* experiments were conducted to downregulate cardiac fibrosis, as described in the Methods section, and the results were compared using two-tailed Student’s t test as shown in Figure 2.8. Masson Trichrome stained area, COL1A1 mRNA expression level and Collagen 1 protein level were predicted to be downregulated in all models. $\log_2(\text{fold-change})$ values of fibrosis markers, as predicted by the three PLSR models, were significantly different from each other.

Table 2.6: Selected under-studied miRNAs have cardiac fibrosis-related target genes

After the first round of selection of miRNAs and fibrosis markers in scoring model 3, 24 miRNAs were selected. Out of them, only 4 miRNAs were present in the optimized PLSR model. This table shows the fibrosis related target genes of the remaining 20 miRNAs. These miRNAs were queried in miRTarBase [189] for *homo sapiens*, *mus musculus*, and *rattus norvegicus* species. For each miRNA, the following cardiac fibrosis markers were searched in its target genes : COL1A1 mRNA, COL1A2 mRNA, COL3A1 mRNA, CTGF mRNA, and VIM mRNA. The miRNAs in this table with listed target genes might have important roles in cardiac fibrosis. However, they were not selected as regulators of cardiac fibrosis in this meta-analysis, mostly because they did not have enough data in selected publications. Hence, these miRNAs should be investigated further to explore their roles in cardiac fibrosis.

miRNA name	Target genes	miRNA name	Target genes
miR-26b	CTGF, COL1A2, VIM	miR-122	CTGF
miR-143-3p	COL1A1, COL3A1, CTGF	miR-214-3p	CTGF
miR-125b	None	miR-133	CTGF, COL1A1
miR-145	CTGF	miR-223	VIM
miR-34a	None	miR-30c-5p	CTGF, VIM
miR-29b-3p	COL1A1, COL1A2, COL3A1	miR-208b-3p	None
miR-30a	VIM	miR-30c-1-3p	None
miR-30c	CTGF, VIM	miR-181d	None
miR-455	COL3A1	miR-29c-3p	COL1A1, COL1A2, COL3A1
miR-378	VIM	miR-433	None

Build a PLSR model to analyze relationships between a set of chosen miRNAs and cardiac fibrosis using meta-analysis data

Following steps should be followed to build a customized PLSR model:

1. Choose a set of miRNAs
2. From the $\log_2(\text{fold-change})$ datasheet, select columns of chosen miRNAs and all fibrosis markers
3. Remove the remaining columns of miRNAs and the experiments that do not contain any data of chosen miRNAs
4. The PLSR model will be built on this tailored dataset to find relationships between the chosen set of miRNAs and cardiac fibrosis- assign miRNAs as x variables, fibrosis markers as y-variables, and experiments as observations

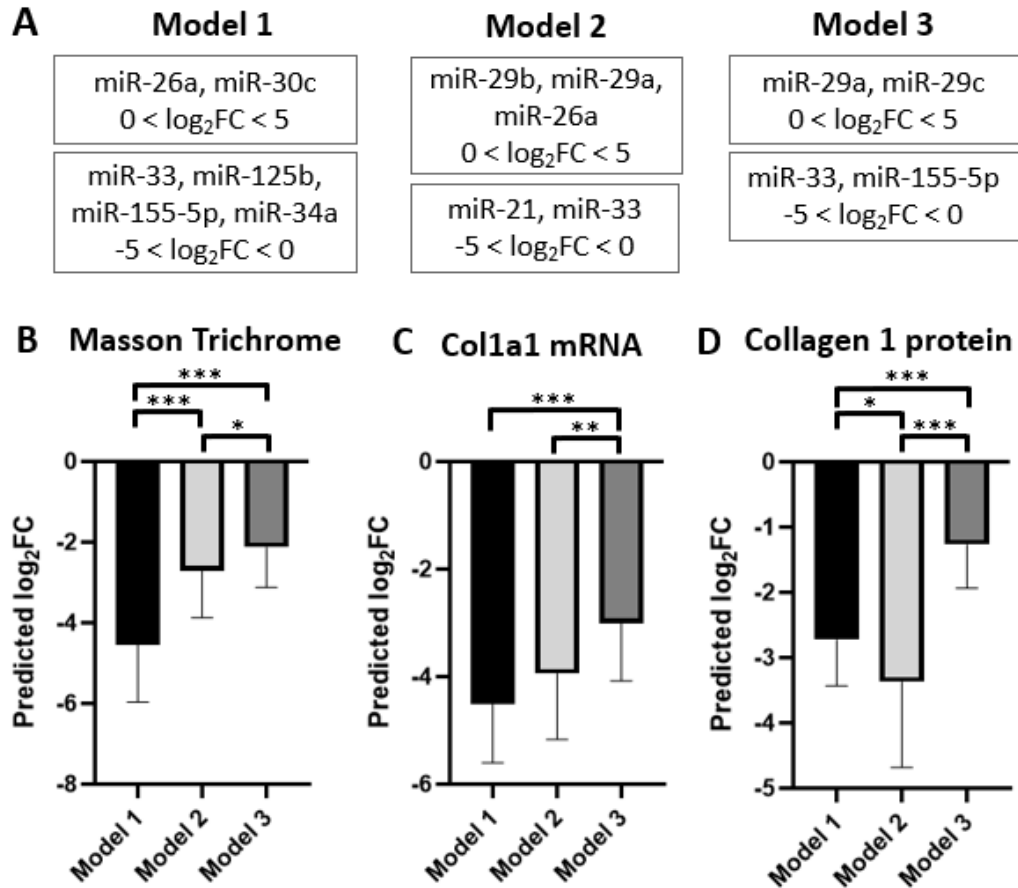


Figure 2.8: PLSR models predicted downregulation of cardiac fibrosis when profibrotic miRNAs were downregulated and antifibrotic miRNAs were upregulated

A) Data was randomly generated for miRNAs that were identified to downregulate cardiac fibrosis in the three scoring models. Downregulated profibrotic and upregulated antifibrotic miRNAs' inputs, constrained within the \log_2FC limits mentioned in the figure (number of experiments = 30), were used to predict the following three fibrosis markers: B) Masson Trichrome stained area, C) COL1A1 mRNA expression level and D) Collagen 1 protein level were predicted to be downregulated by all models. \log_2FC values of fibrosis markers, as predicted by the three PLSR models, were significantly different from each other. All data are represented as mean \pm SD. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Data were analyzed using two-tailed Student's t-Test. FC: fold-change.

This process has been demonstrated in Figure 2.9. This workflow can be used to analyze relationships between a set of chosen miRNAs and cardiac fibrosis, and eventually to predict cardiac fibrosis using the trained PLSR model, given the input fold-change data for the set of chosen miRNAs.

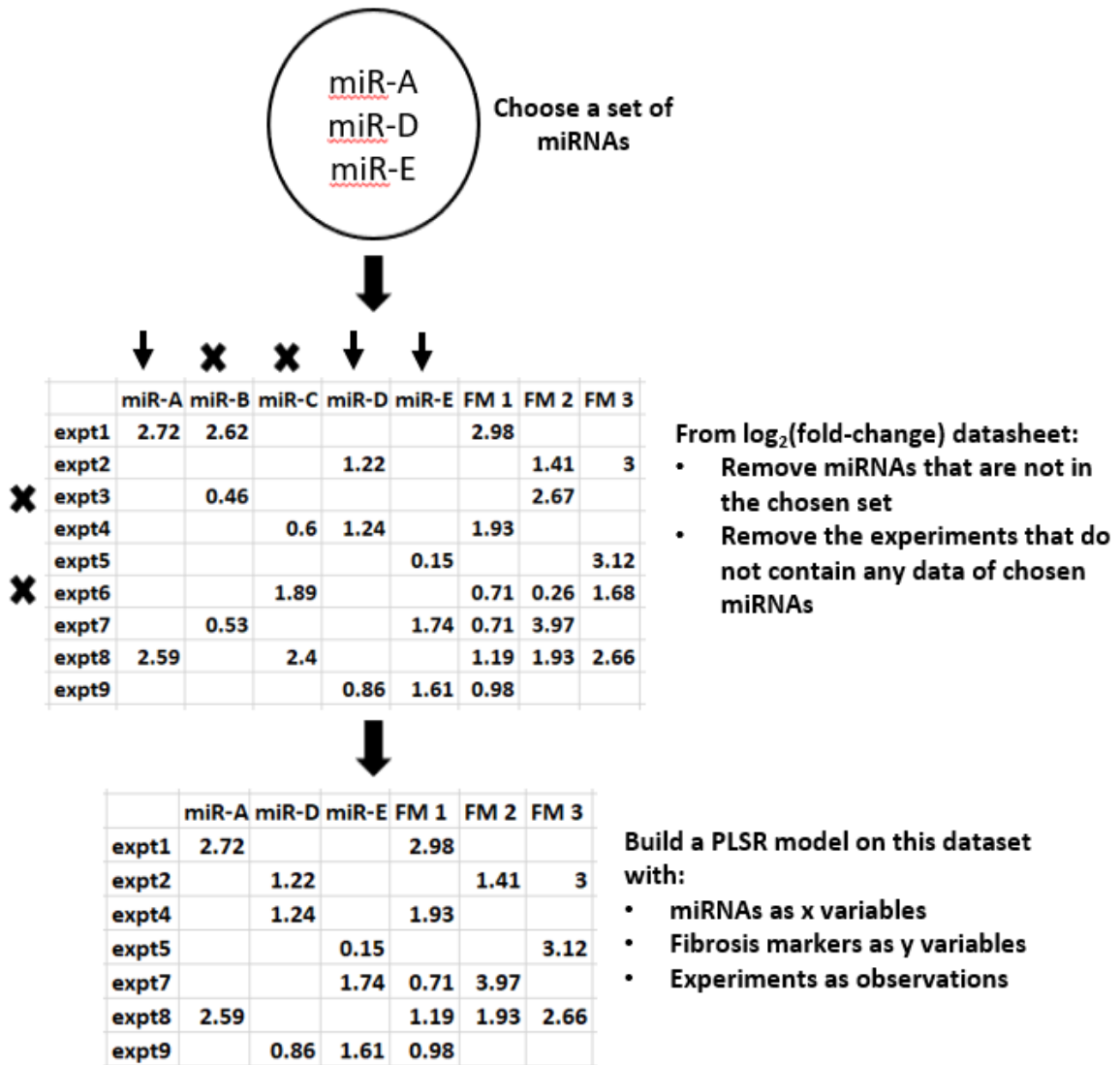


Figure 2.9: **Build a PLSR model to analyze relationships between a set of chosen miRNAs and cardiac fibrosis using meta-analysis data**

Choose a set of miRNAs. From the $\log_2(\text{fold-change})$ datasheet, select columns of chosen miRNAs and all fibrosis markers. Remove the remaining columns of miRNAs and the experiments that do not contain any data of chosen miRNAs. The PLSR model will be built on this tailored dataset to find relationships between the chosen set of miRNAs and cardiac fibrosis. For PLSR modeling, assign miRNAs as x variables, fibrosis markers as y-variables, and experiments as observations. Expt: Experiment, FM: fibrosis marker.

2.4 Discussion

This chapter demonstrated the methods and results of a meta-analysis relating expression levels of miRNAs with quantities of fibrosis markers in the heart using data extracted from

published research articles. PubMed generated 911 search results when searched for publications relating expression levels of miRNAs with cardiac fibrosis and approximately 80% of these results were included in the qualitative analysis. Only 27% of the search results were used in the meta-analysis because many research papers did not quantify miRNA and fibrosis markers for the same experimental conditions. Most of the publications selected for meta-analysis had data in graphical format. Very few papers published their expression array data.

Three scoring models were made to find miRNAs that were highly correlated with cardiac fibrosis- model 1 was unbiased towards miRNAs' number of experiments, model 2 was biased towards well-studied miRNAs and model 3 was biased towards under-studied miRNAs. In models, each scoring formula weighted fold change, p-value and miRNAs' number of experiments differently. For example, in model 2, formulae 2 and 4 has square root of numExpts, hence decreasing its importance in determining the correlation value. In model 3, formulae 3 and 4 multiples p-values in the denominator, thereby giving more weight to p-values in comparison to formulae 1 and 2. So a combination of these scoring formulae covers multiple aspects of analyzing the consistency and magnitude of correlation between miRNAs and cardiac fibrosis for each scoring model. Correlation scores were calculated for ~5000 miRNAs and only top 200 positively and negatively correlated miRNAs were selected from each formula. For the three scoring models, positively correlated miRNAs had a greater overlap among the top 200 miRNAs from constituent formulae, in comparison to negatively correlated miRNAs. The formulae that have a lesser overlap of top 200 negatively correlated miRNAs have different weights of p-values, as evident from the Venn diagrams of scoring models. Hence, we can infer that p-values had greater influence in determining the correlation coefficient for negatively correlated miRNAs than positively correlated miRNAs with cardiac fibrosis.

PLSR modeling was used to quantify the relationships between miRNAs and fibrosis markers in the heart. For each scoring model, miRNAs and fibrosis markers were se-

lected using multiple criteria to make the most optimized PLSR model with the maximum model fitting and prediction accuracy value. The optimized PLSR model obtained from the miRNAs from scoring model 2 had the best model fitting and prediction accuracy value, followed by model 1 and model 3. This is because model 2 was biased towards well-studied miRNAs that had more experimental data to train the PLSR model than the under-studied miRNAs of model 3 as shown in Table 2.2. It is also possible that the miRNAs that have been studied more with respect to cardiac fibrosis, actually target cardiac fibrosis related genes well. The fibrosis markers selected in the optimized PLSR models (based on selection criteria) for the three scores were the frequently analyzed fibrosis markers in experiments from publications selected for meta-analysis as shown in Table 2.3.

Publications that were initially selected for qualitative analysis were used to validate meta-analysis findings. Publications validated the meta-analysis results: overexpression of profibrotic miRNAs from the meta-analysis (like miR-21) have been shown to increase cardiac fibrosis [190] and their underexpression have been shown to inhibit cardiac fibrosis [191]. Similar observations were made for antifibrotic miRNAs like miR-26a [192, 193]. Literature analysis revealed that some miRNAs, for example miR-33, that might be important regulators of cardiac fibrosis and should be investigated further to consolidate their roles in cardiac fibrosis. The analysis of under-studied miRNAs using miRTarBase [189] indicated that the miRNAs with cardiac fibrosis related target genes might have important roles in cardiac fibrosis like miR-122. However, they were not selected as regulators of cardiac fibrosis in this meta-analysis, mostly because they did not have enough data in selected publications. Hence, these miRNAs should be investigated further to explore their roles in cardiac fibrosis.

For each scoring model, its optimized PLSR was used to predict the $\log_2(\text{fold-change})$ of Masson Trichrome stained area, COL1A1 mRNA and Collagen 1 protein when profibrotic miRNAs were downregulated and antifibrotic miRNAs were upregulated. Model 3 that was biased towards the under-studied miRNAs showed the least downregulation of

cardiac fibrosis markers. Lack of experimental data for the under-studied miRNAs might be the reason behind this prediction. It is also possible that under-studied miRNAs do not target cardiac fibrosis that well.

Five profibrotic and five antifibrotic have been reported in this meta-analysis. All antifibrotic miRNAs target collagen genes and profibrotic miR-34a, miR-155-5p, miR-125b target genes of ECM-degrading MMP enzymes [194]. miR-21 has shown to cause fibrosis after myocardial infarction via TGF- β /Smad7 signaling pathway [195]. miR-29b decreased myocardial fibrosis in a mouse model of hypertension by targeting TGF- β /Smad7 pathway [196]. It has been reported that miR-33 promotes cardiac fibrosis by stimulating p38 MAPK signaling and inhibiting MMP16 [197]. Antifibrotic miR-26a targets collagen 1 and CTGF, and inhibits inflammatory cytokine producing NF- κ B [198]. miR-125b has been shown to cause fibrosis by inhibiting p53 to induce fibroblast proliferation, and causes fibroblast-to-myofibroblast transition [199]. miR-155-5p has been shown to promote cardiac fibrosis by targeting TGF- β -inhibiting c-Ski [200]. miR-34a has been reported to cause cardiac fibrosis post myocardial infarction by targeting Smad4, involved in TGF- β signaling [201].

Tissue remodeling and fibrotic scarring can occur in other organs like lung, kidney and liver post injury. Along with cardiac fibrosis, upregulated miR-21 has been reported to be involved in pulmonary fibrosis, renal fibrosis and kidney fibrosis [202, 203, 204]. On the other hand, downregulated miR-29 family has been shown to cause renal fibrosis, pulmonary fibrosis, and hepatic fibrosis [205, 206, 207]. miR-26a has been shown to decrease pulmonary fibrosis and lens fibrosis [208, 209]. Inhibition of miR-33 downregulates renal fibrosis [210]. miR-30c is involved in decreasing hepatic and renal fibrosis [211, 212]. miR-125 promotes hepatic fibrosis [213]. miR-155-5p is involved in renal and pulmonary fibrosis. Upregulation of miR-155-5p inhibits pulmonary fibrosis but causes renal fibrosis [214, 215] miR-34a promotes pulmonary, hepatic and renal fibrosis [216, 217, 218]. It can be observed that mostly the relations between miRNA and cardiac fibrosis concluded from

this meta-analysis are true for fibrosis in other organs as well. However, miRNAs need to be carefully chosen for decreasing cardiac fibrosis as sometimes they can increase fibrosis in other organs. This indicates that after computational selection of miRNAs, another level of literature verification is needed to ensure that changing expression levels of a miRNA for decreasing cardiac fibrosis does not lead to another disease.

To the best of our knowledge, this meta-analysis is the first of its kind. No meta-analysis has been performed to consolidate relationships between miRNAs and cardiac fibrosis. However, some meta-analyses have established relationships between miRNAs and other diseases. Diagnostic performance of circulating miR-122 was evaluated for hepatocellular carcinoma using 13 studies with data for over 900 patients and 1200 controls [219]. 37 studies of miRNA expression profiles and 6 studies of diagnostic accuracy were used to identify miR-34a, miR-122 and miR-192, as potential diagnostic markers of non-alcoholic fatty liver and non-alcoholic steatohepatitis diseases [220]. 7 highly significant and consistently dysregulated miRNAs were identified in 20 studies of renal fibrosis [221]. 6 out of these 7 miRNAs have been analysed in over 20 experiments of cardiac fibrosis (Table 2.2). 5 of these miRNAs are consistently correlated with cardiac fibrosis according to scoring models but only 2 miRNAs are present in the list of our 10 miRNAs selected for downregulating cardiac fibrosis (Table 2.5). Other miRNAs were excluded based either on their inconsistent correlation with cardiac fibrosis or during computational screening for best correlated miRNAs with cardiac fibrosis. For future studies, a combination of computational analysis and biological reasoning for miRNA selection might yield more comprehensive results. Anyhow, the presence of overlapping miRNAs indicates that a few common miRNAs might be responsible for causing renal and cardiac fibrosis.

This meta-analysis has some limitations. Although PubMed is huge database with quality control and is maintained by National Institutes of Health (NIH), other databases like Embase and Web of Science can be explored for additional articles. Publications in other languages can be translated. miRNAs have synergistic effect, that is, one miRNA targets

multiple genes and affects many biological pathways [222]. miRNAs that downregulate cardiac fibrosis might disturb the synergistic network and cause another disease. Hence, a broader analysis, considering a wide array of diseases is required. For future studies, machine learning methods like Random Forest can be tested for miRNA selection. Random forests can be used for classification and regression analysis and trains a combination of decision trees, and then mode or mean of decision tree results can be used for prediction. PLSR and Random Forest have been used together in publications to get better insights into variables' importance in the model. In comparison to linear models, Random Forest models have sometimes shown better predictability [223, 224, 225]. More scoring models can be explored, for example, for under-studied miRNAs, the penalty on number of experiments can be increased by squaring the multiplication factor. Other combinations of mathematical manipulations with biological reasons can be used to conduct this meta-analysis from another perspective. To account for the synergistic effect of miRNAs, a tool can be developed that uses the data aggregated in this meta-analysis and existing miRNA-mRNA interaction data to provide a detailed report on miRNAs' roles in biological pathways, with a focus on cardiac fibrosis. Fibrosis in several organs like lung, liver and kidney can be investigated.

This meta-analysis concluded that downregulation of miR-21, miR-33, miR-125b, miR-155-5p, miR-34a and upregulation of miR-29b, miR-29a, miR-26a, miR-30c, miR-29c can downregulate cardiac fibrosis. Literature analysis validated the meta-analysis results and it is proposed that under-studied miRNAs, like miR-33 and miR-122, should be analyzed more for their roles in cardiac fibrosis. Computational models were made to predict cardiac fibrosis using miRNA fold-changes as inputs. The fold-change dataset from this meta-analysis can be used to establish relationships between any set of miRNAs and cardiac fibrosis, and to predict the extent of cardiac fibrosis when exosomes with custom-designed packages of miRNAs will be injected into animal models.

CHAPTER 3

CONCLUSIONS AND FUTURE WORK

Stem/progenitor cell-based therapies can greatly impact the field of regenerative medicine and have attracted researchers' attention due to their efficacy in treating diseases in preclinical transplantation, tissue injury models and human clinical trials. Experimental studies have shown that exosomes secreted from stem cells have a significant role in the paracrine signaling of transplanted stem cells. Along with lipids, proteins, and nucleic acids, miRNAs are packaged inside exosomes and transported from stem cell to recipient cell for gene expression regulation in the recipient cell. Prior research suggests that miRNAs play a role in determining the reparative effect of exosomes, and exosomes are instrumental to the therapeutic effect of stem cells.

Cardiac fibrosis is characterized by an imbalance in ECM production and degradation, resulting in accumulation of proteins in cardiac interstitium, causing cardiac dysfunction in a wide array of cardiac diseases. Injury to heart can activate fibrotic responses, distorting heart's shape and compromising physiological cardiac functions. The reparative effects of cardiac progenitor cells-derived exosomal miRNAs are being explored on cardiac fibrosis. As a part of the Platt-Davis lab collaboration, our groups have been exploring methods for treating myocardium post injury- decreasing myocardial fibrosis, increasing angiogenesis in the damaged area, increasing proliferation of cardiac progenitor cells, etc. miRNAs and exosomes are studied for identifying therapeutic RNA clusters in human cardiac progenitor cells, engineering exosome like vesicles and evaluating effects of exosome delivery on heart function and regeneration. With the knowledge of miRNAs that can downregulate cardiac fibrosis, exosome therapy can be explored as a potential treatment- candidate miRNAs can be packaged inside exosomes and injected for alleviating cardiac fibrosis.

There are over 900 publications that have studied the relationships between miRNAs

and fibrosis markers in the heart. Using these publications and computational modeling, this research was aimed at finding miRNAs that can downregulate cardiac fibrosis, identifying under-studied miRNAs that might be important regulators of cardiac fibrosis and predicting cardiac fibrosis using miRNA inputs. Another goal was to develop a computational workflow that will allow us to analyze relationships between a set of chosen miRNAs and cardiac fibrosis, and eventually to predict cardiac fibrosis given the input data for the set of chosen miRNAs.

In summary, this meta-analysis identified 10 miRNAs that can downregulate cardiac fibrosis. It was proposed that under-studied miRNAs, like miR-33 and miR-122, should be analyzed more for their roles in cardiac fibrosis. Computational models were made to predict cardiac fibrosis using miRNA fold-changes as inputs. Using the fold-change dataset from this meta-analysis, a computational workflow has been developed to establish relationships between any set of miRNAs and cardiac fibrosis, and to predict cardiac fibrosis given the input data for the set of chosen miRNAs. This analysis consolidates relationships between selected miRNAs and cardiac fibrosis and can be used to inform experimental studies of cardiac remodeling.

Cardiac fibrosis can be caused due to a variety of reasons like myocardial infarction, diabetes, atherosclerosis, to name a few. The articles included in this meta-analysis covered many causes of cardiac fibrosis and therefore, the conclusions of this meta-analysis are applicable to cardiac fibrosis in general. To the best of my knowledge, no one has published such a comprehensive analysis of the relationships between miRNAs and cardiac fibrosis. The miRNAs that have been identified to downregulate cardiac fibrosis in this meta-analysis can be used to downregulate cardiac fibrosis, independent of its cause. For experiments targeted at finding more miRNAs that can be used for decreasing cardiac fibrosis, the list of under-studied miRNAs with cardiac-fibrosis related gene targets can be useful. The extent of decrease in cardiac fibrosis can be predicted using the developed computational workflow, if miRNA fold change data is available. If some correlation can be made between

the amount of miRNA mimic or inhibitor that is supplied within the injected exosome and their expression in target tissues, the prediction workflow developed in this research can be used to fine tune the miRNA concentration needed to achieve the desired cardiac fibrosis outcome. This would save time and resources of the experimentalists. This kind of generalised prediction of cardiac fibrosis markers is possible only because in this meta-analysis huge amounts of data was taken from approximately 250 publications studying cardiac fibrosis.

Synergistic effect of miRNAs has not been considered in this analysis and might be a challenge while conducting experiments. One miRNA has multiple mRNA targets, and thereby one miRNA can be involved in many biological processes. miR-34a was identified as profibrotic miRNA in this study. It has been reported that miR-34a decreases angiogenesis and causes cellular senescence in endothelial progenitor cells [226]. So, downregulation of miR-34a has several simultaneous benefits: decrease cardiac fibrosis, increase angiogenesis, and inhibit cellular senescence. The profibrotic miRNA, miR-21, has been shown to prevent cardiomyocytes apoptosis under oxidative stress [227]. In a case of cardiac injury, upregulation of miR-21 to decrease cardiomyocyte apoptosis can in-turn increase cardiac fibrosis, leading to impaired cardiac functions. Similarly, miR-29 family has been identified as antifibrotic in this analysis, but upregulation of miR-29 family has been shown to cause apoptosis in hearts post ischemia/reperfusion injury [228]. mRNA targets of the selected miRNAs should be studied and pathway analysis should be performed to have a comprehensive outlook of the advantageous and disadvantageous effects of altering the expression levels of miRNAs to decrease cardiac fibrosis. Further analysis should be performed to study the synergistic effect of cardiac fibrosis-related miRNAs on other biological pathways. Therefore, there is a need to develop a platform that can inform researchers about the potential side-effects of using a miRNA to regulate a particular biological process.

An all-encompassing tool can be developed to evaluate the roles of cardiac fibrosis-related miRNAs in other biological pathways. The aggregated data from this analysis,

existing information on miRNA-mRNA interactions and their roles in biological pathways can be taken from online databases to get comprehensive insights into other biological outcomes like angiogenesis, migration, proliferation, along with cardiac fibrosis. When changing miRNA levels for decreasing cardiac fibrosis, such an analysis would help in accessing the effect of modified miRNA level on other biological pathways as well. The computational workflow for analyzing relationships between chosen miRNA expression levels and cardiac fibrosis, explained in the previous section can be automated. Query pipeline can be developed to find mRNA targets and target pathways from online resources for the chosen miRNAs. This tool can be manually updated by adding the extracted data from recently published articles to the uploaded meta-analysis data. This tool would be beneficial in understanding the impact of changing miRNA levels on cardiac fibrosis, their effect on other pathways, and ultimately help researchers in making informed decisions while designing experiments for improving heart functions post injury.

The results of this research can be used to inform experimental studies of cardiac remodeling. Exosomes can be engineered and injected to decrease cardiac fibrosis- agomirs of antifibrotic miRNAs and antagomirs of profibrotic miRNAs identified in this meta-analysis, can be transfected into exosomes. The computational workflow developed in this thesis can be used to predict the extent of cardiac fibrosis when engineered exosomes with custom-designed packages of miRNA content will be injected into animal models. Such experiments can be explored to develop potential therapeutic options for cardiac fibrosis. If this meta-analysis is extended for other organs, and some miRNAs are identified that can downregulate fibrosis independent of the target organ, antifibrotic synthetic exosomes can be developed and explored as potential therapeutic options for downregulating fibrosis in the body.

Appendices

APPENDIX A

ONE

Python scripts written for data extraction and score calculation are shown below.

'miRColumns' should be replaced by the number of miRNAs in the aggregated data.

1. Concatenate fold-change and p-value data extracted from graphs, tables, and expression array, into one data sheet

```
# import packages
import pandas as pd

### concatenate fold-change data
# load fold-change data from graphs, tables, and expression
array data sheet
Study1 = pd.read_excel(ExpressionArraySheet_FC, sheet_name='Sheet1')
Study2 = pd.read_excel(TablesSheet_FC, sheet_name='Sheet1')
Study3 = pd.read_excel(GraphsSheet_FC, sheet_name='Sheet1')
# concatenate the fold-change data and save
df_FC = pd.concat([Study1, Study2, Study3], axis=0, join='outer',
ignore_index=True, sort=False)
df_FC.to_excel('ConcatenatedSheet_FC.xlsx')

### concatenate p-value data
# load p-value data from graphs, tables, and expression array
data sheet
Study4 = pd.read_excel(ExpressionArraySheet_Pval, sheet_name='Sheet1')
```

```

Study5 = pd.read_excel(TablesSheet_Pval, sheet_name='Sheet1')
Study6 = pd.read_excel(GraphsSheet_Pval, sheet_name='Sheet1')
# concatenate the p-value data and save
df_Pval = pd.concat([Study4, Study5, Study6], axis=0, join='outer',
ignore_index=True, sort=False)
df_Pval.to_excel('ConcatenatedSheet_Pval.xlsx')

```

2. Calculate scores from fold-change and p-value data for scoring model 1

2.1 Score Formula 1

```

# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:, 1:miRColumns]
FC_fibrosis = FC.iloc[:, miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:, 1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)

```

```

Pval_fibrosis = Pval.iloc[:,miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

    if len(df.index)>1:

```

```

        z = np.sum([df.iloc[:,2],df.iloc[:,3]], axis = 0)
        mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)]
        ,axis=0)
        corr = np.sum(mult)
elif len(df.index) == 1:
        corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2]+
        df.iloc[:,3],-1.)
        corr = float(corr)

if len(df.index)>=1:
        if corr>=0:
                correlation_pos[miR_name] = corr
                points_pos[miR_name] = po
        else:
                correlation_neg[miR_name] = corr
                points_neg[miR_name] = po

# save miRNAs with positive and negative correlation values with
fibrosis markers separately
writer = pd.ExcelWriter('SM1_Score1.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

2.2 Score Formula 2

```
# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:, 1:miRColumns]
FC_fibrosis = FC.iloc[:, miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:, 1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:, miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
```

```

cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

    if len(df.index)>1:
        z = np.prod([df.iloc[:,2],df.iloc[:,3]], axis = 0)
        mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)]
            ,axis=0)
        corr = np.sum(mult)
    elif len(df.index) == 1:
        corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2],-1.)
            *np.power(df.iloc[:,3],-1.)
        corr = float(corr)

```

```

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

# save miRNAs with positive and negative correlation values with
# fibrosis markers separately
writer = pd.ExcelWriter('SM1_Score2.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

2.3 Find common miRNAs between scoring formulae

```

# import packages
import numpy as np
import pandas as pd

# Function to find common elements in arrays
def commonElements(arr):

```

```

    result = set(arr[0])
    for currSet in arr[1:]:
        result.intersection_update(currSet)
    return list(result)

# In score excel sheets, sort miRNAs in decreasing order of their
absolute values

# read the excel sheets
pos_1 = pd.read_excel("SM1_Score1.xlsx", sheet_name='positive')
pos_2 = pd.read_excel("SM1_Score2.xlsx", sheet_name='positive')
neg_1 = pd.read_excel("SM1_Score1.xlsx", sheet_name='negative')
neg_2 = pd.read_excel("SM1_Score2.xlsx", sheet_name='negative')

# select the top 200 miRNAs from each sheet
n_pos_1 = pos_1.head(200).iloc[:,0]
n_pos_2 = pos_2.head(200).iloc[:,0]
n_neg_1 = neg_1.head(200).iloc[:,0]
n_neg_2 = neg_2.head(200).iloc[:,0]
n_pos_1 = n_pos_1.to_numpy()
n_pos_2 = n_pos_2.to_numpy()
n_neg_1 = n_neg_1.to_numpy()
n_neg_2 = n_neg_2.to_numpy()

# finding overlapping positive miRNAs and extracting their
fold-change data
ABCD = [n_pos_1, n_pos_2]
res_all = commonElements(ABCD)

```

```

FC = pd.read_excel(Fold-change sheet, sheet_name='Log base 2')
data_pos = FC[FC.columns.intersection(res_all)]

# finding overlapping negative miRNAs and extracting their
fold-change data
ABCD = [n_neg_1, n_neg_2]
nres_all = commonElements(ABCD)
FC = pd.read_excel(Fold-change sheet, sheet_name='Log base 2')
data_neg = FC[FC.columns.intersection(nres_all)]

# save data of overlapping miRNAs
writer = pd.ExcelWriter('SM1_DataTop200.xlsx', engine='xlsxwriter')
data_pos.to_excel(writer, sheet_name = 'positive')
data_neg.to_excel(writer, sheet_name = 'negative')
writer.save()

```

3. Calculate scores from fold-change and p-value data for scoring model 2

3.1 Score Formula 1

```

# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')

```

```

Pval = pd.read_excel(P-value file,sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:,1:miRColumns]
FC_fibrosis = FC.iloc[:,miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:,1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:,miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

```

```

fib = FC_fibrosis.iloc[:,0]
fib_pval = Pval_fibrosis.iloc[:,0]
df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
df.replace("", nan_value, inplace=True)
df.dropna(inplace=True)
po = len(df.index)

if len(df.index)>1:
    z = np.sum([df.iloc[:,2],df.iloc[:,3]], axis = 0)
    mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)],
axis=0)
    corr = np.sum(mult)
elif len(df.index) == 1:
    corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2]+
df.iloc[:,3],-1.)
    corr = float(corr)

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0

```

```

for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():
    num = points_pos[key]
    Int = num/max_num
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]
    Int = num/max_num
    corr = float(correlation_neg[key])*float(Int)
    correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
# fibrosis markers separately
writer = pd.ExcelWriter('SM2_Score1.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])

```

```
df.to_excel(writer, sheet_name = 'negative')
writer.save()
```

3.2 Score Formula 2

```
# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:, 1:miRColumns]
FC_fibrosis = FC.iloc[:, miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:, 1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:, miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
```

```

correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

    if len(df.index)>1:
        z = np.sum([df.iloc[:,2],df.iloc[:,3]], axis = 0)
        mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)],
            axis=0)
        corr = np.sum(mult)
    elif len(df.index) == 1:

```

```

corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2]+
df.iloc[:,3],-1.)
corr = float(corr)

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():
    num = points_pos[key]
    Int = np.sqrt(num/max_num)
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():

```

```

num = points_neg[key]
Int = np.sqrt(num/max_num)
corr = float(correlation_neg[key])*float(Int)
correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
# fibrosis markers separately
writer = pd.ExcelWriter('SM2_Score2.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

3.3 Score Formula 3

```

# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
# in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

```

```

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:,1:miRColumns]
FC_fibrosis = FC.iloc[:,miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:,1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:,miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

```

```

fib = FC_fibrosis.iloc[:,0]
fib_pval = Pval_fibrosis.iloc[:,0]
df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
df.replace("", nan_value, inplace=True)
df.dropna(inplace=True)
po = len(df.index)

if len(df.index)>1:
    z = np.prod([df.iloc[:,2],df.iloc[:,3]], axis = 0)
    mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)],
axis=0)
    corr = np.sum(mult)
elif len(df.index) == 1:
    corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2],-1.)
    *np.power(df.iloc[:,3],-1.)
    corr = float(corr)

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():

```

```

    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():
    num = points_pos[key]
    Int = num/max_num
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]
    Int = num/max_num
    corr = float(correlation_neg[key])*float(Int)
    correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
# fibrosis markers separately
writer = pd.ExcelWriter('SM2_Score3.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')

```

```
writer.save()
```

3.4 Score Formula 4

```
# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:, 1:miRColumns]
FC_fibrosis = FC.iloc[:, miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:, 1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:, miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
```

```

points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

    if len(df.index)>1:
        z = np.prod([df.iloc[:,2],df.iloc[:,3]], axis = 0)
        mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)]
            ,axis=0)
        corr = np.sum(mult)
    elif len(df.index) == 1:
        corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2],-1.)

```

```

    *np.power(df.iloc[:,3],-1.)
    corr = float(corr)

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():
    num = points_pos[key]
    Int = np.sqrt(num/max_num)
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]

```

```

Int = np.sqrt(num/max_num)
corr = float(correlation_neg[key])*float(Int)
correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
# fibrosis markers separately
writer = pd.ExcelWriter('SM2_Score4.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

3.5 Find common miRNAs between scoring formulae

```

# import packages
import numpy as np
import pandas as pd

# Function to find common elements in arrays
def commonElements(arr):
    result = set(arr[0])
    for currSet in arr[1:]:
        result.intersection_update(currSet)
    return list(result)

```

```

# In score excel sheets, sort miRNAs in decreasing order of their
absolute values

# read the excel sheets
pos_1 = pd.read_excel("SM2_Score1.xlsx", sheet_name='positive')
pos_2 = pd.read_excel("SM2_Score2.xlsx", sheet_name='positive')
pos_3 = pd.read_excel("SM2_Score3.xlsx", sheet_name='positive')
pos_4 = pd.read_excel("SM2_Score4.xlsx", sheet_name='positive')
neg_1 = pd.read_excel("SM2_Score1.xlsx", sheet_name='negative')
neg_2 = pd.read_excel("SM2_Score2.xlsx", sheet_name='negative')
neg_3 = pd.read_excel("SM2_Score3.xlsx", sheet_name='negative')
neg_4 = pd.read_excel("SM2_Score4.xlsx", sheet_name='negative')

# select the top 200 miRNAs from each sheet
n_pos_1 = pos_1.head(200).iloc[:,0]
n_pos_2 = pos_2.head(200).iloc[:,0]
n_pos_3 = pos_3.head(200).iloc[:,0]
n_pos_4 = pos_4.head(200).iloc[:,0]
n_neg_1 = neg_1.head(200).iloc[:,0]
n_neg_2 = neg_2.head(200).iloc[:,0]
n_neg_3 = neg_3.head(200).iloc[:,0]
n_neg_4 = neg_4.head(200).iloc[:,0]

n_pos_1 = n_pos_1.to_numpy()
n_pos_2 = n_pos_2.to_numpy()
n_pos_3 = n_pos_3.to_numpy()
n_pos_4 = n_pos_4.to_numpy()
n_neg_1 = n_neg_1.to_numpy()
n_neg_2 = n_neg_2.to_numpy()

```

```

n_neg_3 = n_neg_3.to_numpy()
n_neg_4 = n_neg_4.to_numpy()

# finding overlapping positive miRNAs and extracting their
fold-change data
ABCD = [n_pos_1,n_pos_2,n_pos_3,n_pos_4]
res_all = commonElements(ABCD)
ABC = [n_pos_1,n_pos_2,n_pos_3]
res_ABC = commonElements(ABC)
ABD = [n_pos_1,n_pos_2,n_pos_4]
res_ABD = commonElements(ABD)
ACD = [n_pos_1,n_pos_3,n_pos_4]
res_ACD = commonElements(ACD)
BCD = [n_pos_2,n_pos_3,n_pos_4]
res_BCD = commonElements(BCD)
AB = [n_pos_1,n_pos_2]
res_AB = commonElements(AB)
AC = [n_pos_1,n_pos_3]
res_AC = commonElements(AC)
AD = [n_pos_1,n_pos_4]
res_AD = commonElements(AD)
BC = [n_pos_2,n_pos_3]
res_BC = commonElements(BC)
BD = [n_pos_2,n_pos_4]
res_BD = commonElements(BD)
CD = [n_pos_3,n_pos_4]
res_CD = commonElements(CD)

```

```

FC = pd.read_excel(Fold-change sheet,sheet_name='Log base 2')
data_pos = FC[FC.columns.intersection(res_all)]

# finding overlapping negative miRNAs and extracting their
fold-change data
ABCD = [n_neg_1,n_neg_2,n_neg_3,n_neg_4]
nres_all = commonElements(ABCD)
ABC = [n_neg_1,n_neg_2,n_neg_3]
nres_ABC = commonElements(ABC)
ABD = [n_neg_1,n_neg_2,n_neg_4]
nres_ABD = commonElements(ABD)
ACD = [n_neg_1,n_neg_3,n_neg_4]
nres_ACD = commonElements(ACD)
BCD = [n_neg_2,n_neg_3,n_neg_4]
nres_BCD = commonElements(BCD)
AB = [n_neg_1,n_neg_2]
nres_AB = commonElements(AB)
AC = [n_neg_1,n_neg_3]
nres_AC = commonElements(AC)
AD = [n_neg_1,n_neg_4]
nres_AD = commonElements(AD)
BC = [n_neg_2,n_neg_3]
nres_BC = commonElements(BC)
BD = [n_neg_2,n_neg_4]
nres_BD = commonElements(BD)
CD = [n_neg_3,n_neg_4]
nres_CD = commonElements(CD)

```

```

FC = pd.read_excel(Fold-change sheet, sheet_name='Log base 2')
data_neg = FC[FC.columns.intersection(nres_all)]

# save data of overlapping miRNAs
writer = pd.ExcelWriter('SM2_DataTop200.xlsx', engine='xlsxwriter')
data_pos.to_excel(writer, sheet_name = 'positive')
data_neg.to_excel(writer, sheet_name = 'negative')
writer.save()

```

4. Calculate scores from fold-change and p-value data for scoring model 3

4.1 Score Formula 1

```

# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:, 1:miRColumns]
FC_fibrosis = FC.iloc[:, miRColumns:miRColumns+1]

```

```

Pval_miR = Pval.iloc[:,1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:,miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

```

```

if len(df.index)>1:
    z = np.sum([df.iloc[:,2],df.iloc[:,3]], axis = 0)
    mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)],
axis=0)
    corr = np.sum(mult)
elif len(df.index) == 1:
    corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2]+
df.iloc[:,3],-1.)
    corr = float(corr)

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}

```

```

for key in correlation_pos.keys():
    num = points_pos[key]
    Int = 1-num/max_num
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]
    Int = 1-num/max_num
    corr = float(correlation_neg[key])*float(Int)
    correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
# fibrosis markers separately
writer = pd.ExcelWriter('SM3_Score1.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
    columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
    columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

4.2 Score Formula 2

```

# import packages
from scipy import stats
import numpy as np

```

```

import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:, 1:miRColumns]
FC_fibrosis = FC.iloc[:, miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:, 1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:, miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

```

```

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

    if len(df.index)>1:
        z = np.sum([df.iloc[:,2],df.iloc[:,3]], axis = 0)
        mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)],
            axis=0)
        corr = np.sum(mult)
    elif len(df.index) == 1:
        corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2]+
            df.iloc[:,3],-1.)
        corr = float(corr)

    if len(df.index)>=1:
        if corr>=0:
            correlation_pos[miR_name] = corr

```

```

        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():
    num = points_pos[key]
    Int = np.sqrt(1-num/max_num)
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]
    Int = np.sqrt(1-num/max_num)
    corr = float(correlation_neg[key])*float(Int)
    correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
fibrosis markers separately

```

```

writer = pd.ExcelWriter('SM3_Score2.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

4.3 Score Formula 3

```

# import packages
from scipy import stats
import numpy as np
import pandas as pd

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet
# load fold-change and p-value data
FC = pd.read_excel(Fold-change file, sheet_name='Log base 2')
Pval = pd.read_excel(P-value file, sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:,1:miRColumns]
FC_fibrosis = FC.iloc[:,miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:,1:miRColumns]

```

```

Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:,miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

```

```

if len(df.index)>1:
    z = np.prod([df.iloc[:,2],df.iloc[:,3]], axis = 0)
    mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)],
axis=0)
    corr = np.sum(mult)
elif len(df.index) == 1:
    corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2],-1.)
    *np.power(df.iloc[:,3],-1.)
    corr = float(corr)

if len(df.index)>=1:
    if corr>=0:
        correlation_pos[miR_name] = corr
        points_pos[miR_name] = po
    else:
        correlation_neg[miR_name] = corr
        points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():

```

```

    num = points_pos[key]
    Int = 1-num/max_num
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]
    Int = 1-num/max_num
    corr = float(correlation_neg[key])*float(Int)
    correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
fibrosis markers separately
writer = pd.ExcelWriter('SM3_Score3.xlsx', engine='xlsxwriter')
df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

4.4 Score Formula 4

```

# import packages
from scipy import stats
import numpy as np
import pandas as pd

```

```

# convert fibrosis data into one column using vertical concatenation
in both fold-change and p-value data sheet

# load fold-change and p-value data
FC = pd.read_excel(Fold-change file,sheet_name='Log base 2')
Pval = pd.read_excel(P-value file,sheet_name='Sheet1')

# select miRNA and fibrosis columns
# exclude data points where p-value=0
nan_value = float("NaN")
FC_miR = FC.iloc[:,1:miRColumns]
FC_fibrosis = FC.iloc[:,miRColumns:miRColumns+1]
Pval_miR = Pval.iloc[:,1:miRColumns]
Pval_miR.replace(0, nan_value, inplace=True)
Pval_fibrosis = Pval.iloc[:,miRColumns:miRColumns+1]
Pval_fibrosis.replace(0, nan_value, inplace=True)

# initialise
correlation_pos = {}
correlation_neg = {}
points_pos = {}
points_neg = {}
cols_miR = len(FC_miR.columns)
cols_fibrosis = len(FC_fibrosis.columns)

# calculating scoring values

```

```

for i in range(cols_miR):

    miR = FC_miR.iloc[:,i]
    miR_pval = Pval_miR.iloc[:,i]
    miR_name = list(FC_miR.columns.values)[i]

    fib = FC_fibrosis.iloc[:,0]
    fib_pval = Pval_fibrosis.iloc[:,0]
    df = pd.concat([miR, fib, miR_pval, fib_pval], axis=1)
    df.replace("", nan_value, inplace=True)
    df.dropna(inplace=True)
    po = len(df.index)

    if len(df.index)>1:
        z = np.prod([df.iloc[:,2],df.iloc[:,3]], axis = 0)
        mult = np.prod([df.iloc[:,0],df.iloc[:,1],np.power(z,-1.)]
            ,axis=0)
        corr = np.sum(mult)
    elif len(df.index) == 1:
        corr = df.iloc[:,0]*df.iloc[:,1]*np.power(df.iloc[:,2],-1.)
            *np.power(df.iloc[:,3],-1.)
        corr = float(corr)

    if len(df.index)>=1:
        if corr>=0:
            correlation_pos[miR_name] = corr
            points_pos[miR_name] = po

```

```

        else:
            correlation_neg[miR_name] = corr
            points_neg[miR_name] = po

max_num = 0
for key in points_pos.keys():
    if max_num < points_pos[key]:
        max_num = points_pos[key]
for key in points_neg.keys():
    if max_num < points_neg[key]:
        max_num = points_neg[key]
correlation_norm_pos = {}
for key in correlation_pos.keys():
    num = points_pos[key]
    Int = np.sqrt(1-num/max_num)
    corr = float(correlation_pos[key])*float(Int)
    correlation_norm_pos[key] = corr
correlation_norm_neg = {}
for key in correlation_neg.keys():
    num = points_neg[key]
    Int = np.sqrt(1-num/max_num)
    corr = float(correlation_neg[key])*float(Int)
    correlation_norm_neg[key] = corr

# save miRNAs with positive and negative correlation values with
fibrosis markers separately
writer = pd.ExcelWriter('SM3_Score4.xlsx', engine='xlsxwriter')

```

```

df = pd.DataFrame.from_dict(correlation_pos, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'positive')
df = pd.DataFrame.from_dict(correlation_neg, orient='index',
columns=['Fibrosis'])
df.to_excel(writer, sheet_name = 'negative')
writer.save()

```

4.5 Find common miRNAs between scoring formulae

```

# import packages
import numpy as np
import pandas as pd

# Function to find common elements in arrays
def commonElements(arr):
    result = set(arr[0])
    for currSet in arr[1:]:
        result.intersection_update(currSet)
    return list(result)

# In score excel sheets, sort miRNAs in decreasing order of their
absolute values
# read the excel sheets
pos_1 = pd.read_excel("SM3_Score1.xlsx", sheet_name='positive')
pos_2 = pd.read_excel("SM3_Score2.xlsx", sheet_name='positive')
pos_3 = pd.read_excel("SM3_Score3.xlsx", sheet_name='positive')
pos_4 = pd.read_excel("SM3_Score4.xlsx", sheet_name='positive')

```

```

neg_1 = pd.read_excel("SM3_Score1.xlsx", sheet_name='negative')
neg_2 = pd.read_excel("SM3_Score2.xlsx", sheet_name='negative')
neg_3 = pd.read_excel("SM3_Score3.xlsx", sheet_name='negative')
neg_4 = pd.read_excel("SM3_Score4.xlsx", sheet_name='negative')

# select the top 200 miRNAs from each sheet
n_pos_1 = pos_1.head(200).iloc[:,0]
n_pos_2 = pos_2.head(200).iloc[:,0]
n_pos_3 = pos_3.head(200).iloc[:,0]
n_pos_4 = pos_4.head(200).iloc[:,0]
n_neg_1 = neg_1.head(200).iloc[:,0]
n_neg_2 = neg_2.head(200).iloc[:,0]
n_neg_3 = neg_3.head(200).iloc[:,0]
n_neg_4 = neg_4.head(200).iloc[:,0]
n_pos_1 = n_pos_1.to_numpy()
n_pos_2 = n_pos_2.to_numpy()
n_pos_3 = n_pos_3.to_numpy()
n_pos_4 = n_pos_4.to_numpy()
n_neg_1 = n_neg_1.to_numpy()
n_neg_2 = n_neg_2.to_numpy()
n_neg_3 = n_neg_3.to_numpy()
n_neg_4 = n_neg_4.to_numpy()

# finding overlapping positive miRNAs and extracting their
fold-change data
ABCD = [n_pos_1, n_pos_2, n_pos_3, n_pos_4]
res_all = commonElements(ABCD)

```

```

ABC = [n_pos_1,n_pos_2,n_pos_3]
res_ABC = commonElements(ABC)
ABD = [n_pos_1,n_pos_2,n_pos_4]
res_ABD = commonElements(ABD)
ACD = [n_pos_1,n_pos_3,n_pos_4]
res_ACD = commonElements(ACD)
BCD = [n_pos_2,n_pos_3,n_pos_4]
res_BCD = commonElements(BCD)
AB = [n_pos_1,n_pos_2]
res_AB = commonElements(AB)
AC = [n_pos_1,n_pos_3]
res_AC = commonElements(AC)
AD = [n_pos_1,n_pos_4]
res_AD = commonElements(AD)
BC = [n_pos_2,n_pos_3]
res_BC = commonElements(BC)
BD = [n_pos_2,n_pos_4]
res_BD = commonElements(BD)
CD = [n_pos_3,n_pos_4]
res_CD = commonElements(CD)

FC = pd.read_excel(Fold-change sheet,sheet_name='Log base 2')
data_pos = FC[FC.columns.intersection(res_all)]

# finding overlapping negative miRNAs and extracting their
fold-change data
ABCD = [n_neg_1,n_neg_2,n_neg_3,n_neg_4]
nres_all = commonElements(ABCD)

```

```

ABC = [n_neg_1,n_neg_2,n_neg_3]
nres_ABC = commonElements(ABC)
ABD = [n_neg_1,n_neg_2,n_neg_4]
nres_ABD = commonElements(ABD)
ACD = [n_neg_1,n_neg_3,n_neg_4]
nres_ACD = commonElements(ACD)
BCD = [n_neg_2,n_neg_3,n_neg_4]
nres_BCD = commonElements(BCD)
AB = [n_neg_1,n_neg_2]
nres_AB = commonElements(AB)
AC = [n_neg_1,n_neg_3]
nres_AC = commonElements(AC)
AD = [n_neg_1,n_neg_4]
nres_AD = commonElements(AD)
BC = [n_neg_2,n_neg_3]
nres_BC = commonElements(BC)
BD = [n_neg_2,n_neg_4]
nres_BD = commonElements(BD)
CD = [n_neg_3,n_neg_4]
nres_CD = commonElements(CD)

FC = pd.read_excel(Fold-change sheet,sheet_name='Log base 2')
data_neg = FC[FC.columns.intersection(nres_all)]

# save data of overlapping miRNAs
writer = pd.ExcelWriter('SM3_DataTop200.xlsx', engine='xlsxwriter')
data_pos.to_excel(writer, sheet_name = 'positive')
data_neg.to_excel(writer, sheet_name = 'negative')

```

```
writer.save()
```

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