

# Shreya MSc Thesis

*by Shreya Msc*

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# IDENTIFICATION OF SIGNATURE MOLECULAR BIOMARKERS FOR THERAPEUTIC TARGETING FOR CARDIOVASCULAR DISEASES BY NETWORK-BASED ANALYSIS

## ABSTRACT

The Cardiovascular diseases are the age-related diseases and their genesis is not only affiliated with the accumulation of lipids in the cardiac vessels but more to the inflammatory process. In this study, while focusing on the therapeutic significance, a systems and computational biology strategy was utilized to examine biomarkers central to myocardial inflammation and analyze various regulatory networks monitoring the process. In tissue-specific gene co-expression assessment, CCL2, IL6 and IL8 were revealed as the central hub genes in atrial appendage and CCL2 was shown to be the major hub gene in left ventricle. Consequently, CCL2 was the master gene since it was expressed by both of the cardiac tissues. Moreover, in gene-miRNA interaction analysis, the post-transcriptional regulator hsa-miR-155-5p was identified as the primary regulator, furthermore, in gene-transcription factor (TF) interaction examination, NFkB1 was found to be the key regulator. The expression of CCL2, IL6 and IL8 (all cytokines) creates an intensified inflammatory cycle. The hub post-transcriptional regulator hsa-miR-155-5p and transcription factor NFkB1 are observed to be associated with CCL2 and IL6 so, can be considered as crucial regulatory points and hub biomarkers which can be targeted for therapeutics as all are playing major role in inflammatory signalling pathways. The CCL2-CCR2 and IL6-IL6R signalling axes can be obstructed by designing CCL2 and IL6 specific antibodies which can neutralize them or designing CCL2R and IL6R antagonists, and hsa-miR-155-5p and NFkB1 can also be targeted by designing their inhibitor molecules. Moreover, the drugs- Tocilizumab, Canakinumab, Adalimumab, Methotrexate, Colchicine and Rosuvastatin addresses these biological markers which are associated with inflammation. Therefore, these can be the novel and potential biomarkers which can be targeted for achieving broad-spectrum therapeutics and by targeting these signature biomarkers, we can intervene the inflammatory signalling pathways at multiple levels thus, reducing the risk of pathogenesis of major fatal CVDs.

## CHAPTER 1

### INTRODUCTION

#### 1.1 Ageing and Inflammation

The global procedure of ageing commenced around three billion years back in time, when the concept of life first emerged. The build-up of various detrimental alterations in cells and tissues due to biological flaws, environmental factors and illness processes that raise the likelihood of illness and death with age is known as ageing. It is associated with multifactorial processes such as- genetic abnormalities and steady deterioration of biological processes taking place at the molecular, cellular and tissue levels. The likelihood of mortality for a certain age is the indicator of the typical number of ageing modifications acquired among people of that age group, also known as physiological or biological age. Improving overall circumstances or quality of life reduces the risk of fatality. It is also defined by the surge of senescent or aged cells within the human physique. ROS, AMPK, SIRT6, mTOR, IGF-1, and p53 are important regulators of metabolic control that link ageing to the pathways that lead to diseases which include oxidative stress, inflammation, mitochondrial dysfunction, deregulated autophagy and cellular replication brought on by telomere shortening might also cause senescence.

The global number of people across the threshold across the age of 70 is expected to increase progressively during the subsequent period of time escalating from 11% in 2010 to 21% in 2040, as reported by the figures from the United Nations (UN). Person's standard of living doesn't necessarily become better as their longevity expands. Age – Related Diseases (ARDs) are the diseases which occur in the human as the body gets older and cardiovascular diseases (CVDs), Alzheimer's disease, type 2 diabetes, osteoporosis, arthritis and cataract are some common examples of these ARDs. These ailments were responsible for approximately 70% of fatalities, with cardiac diseases accounting for 40% out of the whole, reported in 2010.

The free radical concept of ageing proposes that reactive oxygen species (ROS) promotes oxidative impairment in macromolecules within the cells which is responsible for the reduction in bodily activity with age. In the mammals, ROS is produced by a variety of mechanisms such as- respiration in the mitochondria, NADPH oxidase, NO synthase, peroxidase and cytochrome p450s. Numerous ROS forms comprises of single or unpaired electrons, referred to as free radicals. This category encompasses species such as-  $\text{H}_2\text{O}_2$  (hydrogen peroxide),  $\text{O}_2^{\cdot-}$  (superoxide),  $\text{HO}^{\cdot}$  (hydroxyl),  $\text{NO}^{\cdot}$  (nitric oxide),  $\text{ONOO}^-$  (peroxynitrite),  $\text{HOCl}$  (hypochlorous acid) which boost oxidative deterioration.

The mitochondria generate the majority of harmful radicals throughout the senescence. Mitochondria convert  $\text{O}_2$  into  $\text{H}_2\text{O}$ , a reaction that when insufficient culminates in the excessive generation of superoxide radicals, eventually results in the elevated levels of mitochondrial reactive oxygen species (mtROS), ultimately contributing to the build-up of mtDNA alterations, which pushes organelle malfunction triggering senescence.

Excess synthesis of ROS is a prerequisite for AP-1 and NF- $\kappa$ B activation via kinase stress, including ERKs, JNKs, p38 MAPK, PKC, PI3K, Akt, and Src family kinases. TNF- $\alpha$ , IL-6, and IL-1 $\beta$  are the primary proinflammatory substances and are predominantly mediated by transcriptional regulators such as AP-1 and NF- $\kappa$ B.

## 1.2 Cardiovascular Diseases

CVDs are the issues which occurs inside the myocardium and vessels of the heart including Atherosclerosis, Coronary Artery Disease (CAD), Heart Failure, Hypertension, and other heart diseases.

Nowadays, combined with the modern-day consuming habits and technologically-oriented lifestyle, the shift to commercialization and urbanization is seeking more sedentary occupations than the physical ones, which is associated with increased office hours and decreased relaxation time for leisure pursuits. Lack of exercise, a diet containing excessive calories, saturated and trans fatty acids, carbohydrates are linked to the progression of atherosclerotic plaques. This could clarify the considerable and consistent surge in CVD incidences during the past years. Non-changeable characteristics such as- genetic background, sex, age factor are recognized as distinct risk elements.

In United States of America, CVDs continue to be the most prevalent causes of mortality accounting for more than 6 lakh fatalities since 1975. In 2014, heart illness was the primary reason of morbidity second only to cancer-associated deaths. In 2016, as stated by WHO (World Health Organization), CVD is the most widespread contributor of mortalities across the globe, accounting for approximately 18 million fatalities.

Atherosclerosis is the pathological phenomenon within the arterial system which may induce damage as the outcome of diminished or zero vascular circulation resultant due to the narrowing of blood vessels. It consists of several variables, including dyslipidaemia, immunologic abnormalities, inflammatory response along with endothelial cell damage. Such factors are thought to cause the creation of fatty patches which are the characteristics of the progression of atherosclerotic lesion. The mechanism begins with the layering and swelling in the inner cellular layer, then the implantation of foam cells i.e., the lipid filled macrophages as well as the aggregate formation and the development of smooth muscles cells which create the plaque. As the plaques grow, programmed cell death of the underlying tissue could take place, contributing in the greater foam cell influx which might get crystallized and proceed to the formation of atherosclerotic lesions. Further processes such as- vascular remodelling and haemorrhage in the internal plaque, serves a crucial function in the prolonged and exacerbated course of atherosclerosis.

The most dreaded consequence of heart diseases is terminal illness and it continues to be the most prominent causes of fatalities worldwide due to its worrisome predominance among individuals. Further issues, including since the requirement for prolonged hospital stay, medical incapacity, and elevated service expenses, are vital and are currently being addressed by medical service officials, given that they are projected to escalate further in subsequent generations.

### 1.3 Atherosclerosis

The form with the greatest prevalence in cardiovascular disorder is atherosclerosis, which is characterised by cholesterol deposition as well as dysfunction of the main vascular artery systems, which is capable of causing medical disorders such as cardiac infarction and haemorrhage. Atherosclerosis is defined as the continual deposition of substances such as fats, pro-inflammatory cells and cellular debris in the inner region of the wall of vessels of the heart forming plaques or lesions which results in reduction of flow of blood and can induce angina attacks, especially during physical activity or stressful situations. Plaques, especially those with lipid and proinflammatory constitutions, are susceptible to becoming eruptive and may burst. When this takes place within the arteries in the heart, it might generate a site-specific coagulation clot that entirely hinders blood circulation, resulting in a cardiac infarction. In addition, if the blood clot exits the cardiac chamber and migrates to the brain, can also lead to stroke.

The excessive buildup of cholesterol, fibrous substances, and hardening in major blood vessels defines the condition known as atherosclerosis. The entire procedure begins with endothelial stimulation, and is accompanied with a series of phenomena that includes shrinkage of blood vessels and the stimulation of signalling pathways involving inflammation. Overall, the above procedures yield heart-related issues, which continue to be the most widespread reason of fatalities globally.

Atherosclerosis begins with dysfunction of the endothelial cells, followed by low-density lipoprotein (LDL) persistence. Altered low-density lipoprotein molecules, along with extra atherogenic stimuli, activate endothelial cells (ECs), resulting in influx of monocytes into the inner layers, which readily collect the LDL molecules, facilitating the production of foam cells. Furthermore, various proinflammatory pathways are engaged, triggering the production of fatty streaks, which are considered as the first manifestations of atherosclerotic plaques. LDLs concentrate within the endothelial zone where they undergo modifications in the beginning stages of the development of atherosclerotic lesions. Vascular smooth muscle cells (VSMCs) treated with transformed LDLs produce chemical attractant molecules such as CCL2 and CCL5, those increase monocyte infiltration. Furthermore, ox-LDL can cause a stimulating inflammatory reflex in endothelial cells and macrophages, exacerbate endothelial destruction, and infiltrate cells called leukocytes. Moreover, the modified LDLs may attach onto the TLR2 receptors and Class 4 of PRRs and trigger the release of cytokines that promote inflammation which are IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in a NF-kB-mediated manner. oxLDL absorption directed by CD36 stimulates the inflammasome known as the NLRP3, leading to the increased production of IL-1 $\beta$ 36. Additionally, oxLDL may associate to specific cellular antibodies, producing immunological complexes which activate the phagocytes called as macrophages and dendritic cells. During the initial phases of atherosclerosis, the expulsion of CCL2 and T-cell attracts lymphocytes as well as monocytes towards the interior artery lining, wherein monocytes develop into the foam cells derived from macrophages. When a plaque of lipid-streak forms, TNF- $\beta$  and IFN- $\gamma$  are produced by T-lymphocytes which promote VSMC recruitment and growth. Metalloproteinase (MMP) synthesis via macrophages within the plaque is also promoted by the activated T-lymphocytes. Crystallization of the lipid crystals trigger the inflammasome NLRP3 by generating lysosomal disruption. The activated endothelial cells enhance the expression of cellular attachment proteins on their surfaces, including VCAM-1, ICAM-1, E-selectin, and P-selectin which serve as the primary means of attracting leukocytes towards the plaques associated with atherosclerosis.

## 1.4 <sup>8</sup> Coronary Artery Disease

Coronary Artery Disease (CAD), also known as Ischemic Heart Disease (IHD) is a frequently occurring cardiovascular disorder caused by the accumulation of lesion caused by atherosclerosis residing in the vascular cavity and is distinguished by an inappropriate influx of blood along with oxygen reaching the cardiac chamber. This medical disorder is caused by blockage in coronary artery, which creates a type of unequal distribution in oxygen demand and supply. It is primarily characterised by the growth of deposits within the passageway of arteries in the heart, which obstruct the circulation of blood. Atherosclerosis-associated lesion development serves as the defining feature of CAD pathology, which is the accumulation of lipids which clogs the artery opening and obstructs circulating blood. The development of a fatty-streak through sub-endothelial sedimentation of lipid-packed foam cells is the initial phase in this mechanism. Whenever a cardiovascular injury is experienced, the inner membrane zone ruptures, allowing monocytes to propagate toward the sub-endothelial area and differentiate into macrophages, which ingest oxidised particulates of LDL, resulting in the production of foam cells. To assist the procedure of inflammation, the stimulated T-lymphocytes synthesise molecules called cytokines. Growth factors trigger smooth muscle fibres, that absorb oxidised LDL fragments alongside collagen molecules, implant them beside stimulated macrophages, enhancing the density of foam cells, eventually establishing the plaque. When not any subsequent injury is observed, the calcification may develop or stabilise, which leads to the development of a fibrous cap. As time progresses, the plaque's haemodynamic significance may increase. Whenever the vascular aperture is clogged not less than 75%, coronary circulation could turn inadequate, thereby causing symptoms of chest pain especially in the instances of high activity such as- physical activity and workouts.

An investigation revealed that CAD accounted for approximately 3% of the worldwide burden of disease and about 33% of heart illnesses.

It is a complex disease which depends on multiple variables which are categorised as - Changeable and Non-Changeable. Changeable characteristics encompasses- elevated blood pressure, cigarette smoking, being overweight, cholesterol concentrations. Non-changeable characteristics are such as- sexual orientation, physical age, ancestry, and biological traits. A more rapid-paced living in this developed world has contributed to a greater consumption of fried and fatty food items, thereby establishing the road for a surge in the overall incidence of the disease and cigarette continues to be the most prevalent causes of vascular illness. Men are more susceptible towards this disease than women. Having high cholesterol continues to be a major hazard underlying it. Elevated low-density lipoproteins (LDL) levels enhance CAD susceptibility, while higher high-density lipoproteins (HDL) reduce CAD development. Inflammatory determinants also represent important warning indicators of it.

## 1.5 Heart Failure

Heart failure impacts approximately 3% of the people globally. It is a persistent condition having a yearly rate of death of approximately 12% in which the most prevalent causes are abrupt cardiac demise and organ failure owing to inadequate blood supply.

It is a type of medical condition defined by usual manifestations such as- difficulty in breathing, swelling in limbs and tiredness alongside symptoms like- hypertension, bronchial cracks, or peripheral pulmonary swelling attributed to cardiovascular anomaly, resulting in a decreased heart rate or increased cardiovascular pulses. Accelerated oxygen-mediated damage

has been linked to heart failure, and reduced scavenger function in deteriorating heart muscle enhances oxidative strain.

Diabetes, a familial background, overweight, long-term lung conditions, inflammatory conditions, persistent infections, metabolic ailments, cardiovascular-toxic medication and drinking alcohol are the most important risk variables.

It has also been reported that redox-regulated signalling mechanisms such as- MAPK as well as transcriptional regulators such as- NF $\kappa$ B are also stimulated. ROS can trigger various variables that regulate contractions, including substances that promote inflammation, ischaemia, and catecholamine auto-oxidative reactions. Free radicals caused by oxygen may possess an immediate detrimental influence on cardiac physiology and working.

## **1.6 Hypertension**

Hypertension is described as an elevation in the pressure of blood towards vessel walls. More than 80% of people in developed nations are at danger of developing this disorder i.e., blood pressure above 120/80 mmHg over the course of their lives.

It is often associated with additional risk variables involving getting older, being overweight, having diabetes, and high cholesterol levels. Mild organ impairment, including left ventricular expansion, a condition called microalbuminuria, as well as intellectual impairment, occurs at the beginning of the progression of hypertension, whereas serious incidents including as cardiac arrest, stroke, kidney dysfunction typically occur after chronic episodes of untreated hypertensive condition.

## CHAPTER 2

### LITERATURE REVIEW

#### **2.1 Analysis of Drugs for Targeting Potential Inflammatory Biomarkers and their Mechanisms of Action**

##### **2.1.1 Tocilizumab**

Tocilizumab (TCZ) is an antagonist of IL-6 Receptor (IL-6R) which suppresses the concentrations of CRP via inhibiting IL-6 activation. Significant amounts of CRP and molecules that cause inflammation like- IL-6 and TNF- $\alpha$ , may contribute to the prevalence of atherosclerosis.

Since inflammatory reactions leads to the advancement of vascular disease, this drug's therapy could potentially safeguard people with heart-related complications as it suppresses inflammatory processes and slows the onset of plaque formation in the vessels.

It inhibits the LDL catabolism amplification as well as diminish the synthesis of LDL receptor through a PCSK9-dependent route. It further enhances endothelial activity, lowers the inflammation caused by ROS, regulates lipids, inhibits leukocyte blood clotting and inflamed manifestations, and causes shortened NETosis.

##### **2.1.2 Canakinumab**

Canakinumab is an anti-inflammatory drug that addresses the IL-1 $\beta$  intrinsic immune system axis and dramatically reduces the frequency of subsequent cardiac maladies.

NLRP3 stimulates IL-1 $\beta$ , which is triggered through lipoprotein clumps, leukocyte extrinsic clumps, low cell oxygenation, as well as vascular circulation dynamics. Eventually, these factors contribute to the progression of vascular diseases. There have been studies which linked the induction of IL-1 $\beta$  with the descending IL-6R communication channel, suggesting its contribution to cardiac thrombosis. It has various functions in facilitating the formation atherosclerotic lesions, among which are stimulating coagulation signalling, promoting macrophage and lymphocyte attachment onto endothelial cells in the bloodstream, and stimulating the proliferation of VSMCs.

### 2.1.3 Adalimumab

Adalimumab is a monoclonal antibody drug that binds to the cytokine TNF- $\alpha$ , thereby hindering its attachment to its respective receptor, and eliminates each of the free and membrane-bound protein.

It inhibits TNF- $\alpha$ 's proinflammatory impacts which causes stimulation of endothelial cells, attachment of macrophage as well as permeability, thus broadening the possibilities of treatment to alleviate arterial inflammatory responses.

This TNF- $\alpha$  antagonist substantially inhibits the influence of TNF- $\alpha$  generated by activated oxLDL and monocytes which includes enhanced vascular attachment protein synthesis, macrophage aggregation, along with endothelium spillage and such mechanisms can be defined as preliminary occurrences in the progression of cardiovascular lesions. Therefore, it effectively inhibits the secretion of TNF- $\alpha$  out of the monocytes within the atherosclerotic region in the face of oxLDL, consequently reducing its subsequent anti-atherosclerosis implications.

The medication has been linked to strengthen the activity of endothelium tissue, reduction of stiffening of inner layer membrane, and reduced concentrations of CRP.

### 2.1.4 Methotrexate

Methotrexate can reduce inflammation-related as well as atherogenesis-related mediators like TNF- $\alpha$ , IL-1, and IL-6 while increasing anti-atherosclerosis molecules like IL-10, although it directly targets the enzyme- dihydrofolate reductase (DHFR). This could provide a competitive benefit over existing medications which solely address a single specific inflammation-causing mediator.

A number of processes are being proposed to rationalise the potential vasculoprotective properties of the drug for instance, interleukin alterations, adenine buildup, stimulation of the enzyme AMPK (5'-Adenosine Monophosphate-Activated Protein Kinase), along with the regulation of equilibrium redox.

### 2.1.5 Colchicine

Colchicine is believed to interfere with cytoskeleton integrity and targets  $\beta$ -tubulin, which affects various internal activities like as cell division, cell engulfment, as well as transportation, regulate of stimulation of NLRP3 axis which influences the synthesis of inflammation-associated mediators. The aforementioned actions have immediate influence on the inflammation mechanisms of the vascular cells resulting in a variety of circulatory advantages. The cumulative impression upon lesion status and development influences the physical symptoms of cardiovascular disease, lowering the likelihood of substantial detrimental cardiac complications, implying that adding the drug for treatment atherosclerosis may be advantageous.

### **2.1.6 Rosuvastatin**

Although the drug Rosuvastatin directly addresses the enzyme- HMG-CoA-Reductase, the receivers of this drug experienced substantial drops in the concentrations of CRP, fibrinogen, IL-6, TNF- $\alpha$ , INF- $\gamma$ . It has also been shown to have anti-oxidative abilities and to improve endothelial health along with thrombocytes.

Its administration drastically decreased the proportion of macrophages clinging onto the epidermal layer indicating that lower levels of vascular cell stimulation is associated with the prevention of atherosclerosis activity. It significantly diminished the size of atherosclerotic patches that contained macrophages.

## CHAPTER 3

### MATERIALS AND METHODOLOGY

#### 3.1 Data Source and Gene Retrieval

- **Tool Used: Comparative Toxicogenomics Database (CTD)**
- It is a freely accessible, human-curated library that brings together data from scholarly literature and offers excellent-quality associations pertinent to toxicogenomics research and pathological biology. It gathers data on gene-disease, gene-chemical and disease-chemical interactions.
- By exploring for the phrases associated with the cardiovascular diseases, corresponding genes became apparent and top 100 genes with proven relationships and involvement in the pathogenesis in each of the 4 diseases – Atherosclerosis, CAD, Heart Failure and Hypertension were included in the selected dataset. The retrieved gene list was considered as the primary feed for subsequent computational and network evaluation.

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Comparative Toxicogenomics Database

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1. What human diseases are associated with a [gene/protein](#)? [\(Example\)](#)
2. What human diseases are associated with a [chemical](#)? [\(Example\)](#)
3. What genes/proteins interact with a [chemical](#)? [\(Example\)](#)
4. What chemicals interact with a [gene/protein](#)? [\(Example\)](#)
5. What references report a [chemical-gene/protein interaction](#)? [\(Example\)](#)
6. What cellular functions (GO terms) are affected by a [chemical](#)? [\(Example\)](#)

**109,241,671 TOXICOGENOMIC RELATIONSHIPS!**

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**Updated Chemicals**

1-aminomethylphosphonic acid Alkatriin B1 aristolochic acid 1 benzo(a)anthracene  
**Benzo(a)pyrene** benzo(b)fluoranthene bisphenol A Cadmium Cadmium Chloride chrysene deoxyvalenol Dexamethasone Dextran Sulfate Dietary Fats dodecylmethylamine oxide gamma-sitosterol Isopropenol Lipopolysaccharides Lithocholic Acid Loperamide Palmitic Acid Particulate Matter Potassium Dichromate Swainsonine **Tobacco Smoke Pollution**

**Updated Diseases**

Airway Obstruction Birth Weight Cardiomegaly Cardiotoxicity  
**Chemical and Drug Induced Liver Injury** Colitis, Ulcerative Dermatitis, Atopic Diabetes, Gestational Dysbiosis Emphysema Fatty Liver Hepatomegaly Kidney Diseases Liver Diseases, Alcoholic Lung Injury Myocardial Infarction Nociceptive Pain overweight Pheochromocytoma Pericardial Effusion Premature Birth Prenatal Exposure Delayed Effects Pulmonary Disease, Chronic Obstructive Pulmonary Fibrosis Splenomegaly Teratozoospermia Testicular Diseases ventricular dysfunction, left **Weight Loss**

**News**

- 1 April 2026
- New data available**
- Latest Features
  - [New Chemical Vocabulary Report Cross-Reference Field!](#)
- [New Reports!](#)
  - [Curated Chemical–Disease Associations!](#)
  - [Curated Gene–Disease Associations!](#)
- [New Chord Diagram Tool for CTD Tetramers!](#)

Figure 3.1-Interface of Comparative Toxicogenomics Database (CTD)

### 3.2 Identification of Common Genes by Computational Analysis

- Tool Used: Google Colab and a Python Code
- Employing the programming language Python, a computational processing methodology was implemented in order to narrow down the set of genes responsible for cardiovascular illness and determine the most significant and potential outcomes along with Google Colab, a cloud-based system designed to provide a collaborative environment for executing python coding scripts, was utilised for the purpose of investigation.
- The dataset of genes was uploaded in the python framework, put together into hierarchical and set-driven operations were executed to identify typical commonly occurring genes in the collected data. This approach permitted a successful and accurate determination of the genes which existed throughout the dataset.
- The retrieved collection of the typical commonly occurring genes was further utilised for network-driven evaluation which includes tissue-specific gene co-expression, gene-miRNA, gene-TF interaction analysis research.

### 3.3 Network Construction and Analysis

- Tool Used: Network Analyst
- It is a web-based bioinformatics tool that aggregates numerous excellent archives and facilitate tools for sophisticated and complete network modelling and assessment. With the aim to generate distinct interaction network models, the filtered collection of genes was uploaded onto it.

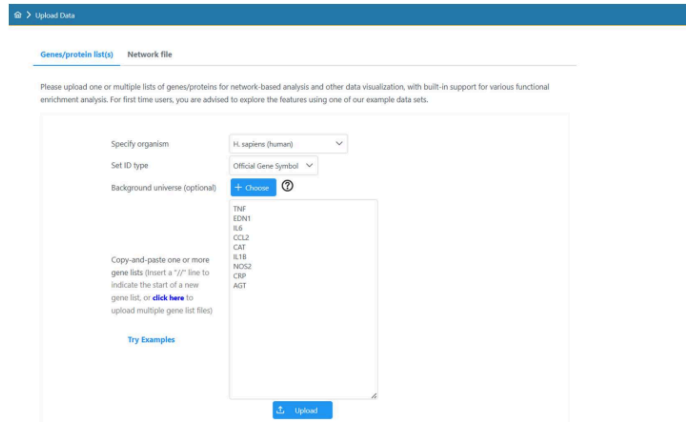


Figure 3.2-Interface of Network Analyst Database

### 3.3.1 Tissue-Specific Gene Co-Expression Network

- Tool Used: iNetModels Database
- This database is a thorough foundation for building standardised genetic interaction and communication networks build around rigorously validated datasets which are plugged into the platform. In order to achieve high level of accuracy and biological value, it mainly relies on data of protein-protein interactions (PPIs) from carefully selected libraries. By projecting user-supplied gene sets against pre-existing networks relationship models, it makes it easier to identify hub genes. This tool eliminates erroneous correlations and delivers a robust framework for conducting systemic-scale molecular webs exploration by emphasising on connections that have been verified by experiments.
- To examine functional links among genes according to their expression trends in specific myocardial tissues, gene co-expression analysis was conducted. The networks were built for 2 cardiac tissues- Atrial Appendage and Left Ventricle, in order that the localised gene associations could potentially be assessed.
- Co-expression analysis was performed utilising freely accessible expression databases merged with the software. Correlation-dependent relationships among patterns of genetic expressions were utilised to establish networks. In the networks, genes were represented by nodes and co-expression interactions were represented by edges.
- To figure strongly connected nodes (hub genes) that might be essential to biological processes, topological metrics were assessed. The generated networks shed light on the functional grouping of genes implicated in cardiovascular illness as well as tissue-specific regulation mechanisms.

### 3.3.2 Gene-miRNA Network

- Tool Used: miRTarBase Database
- This database is a well-known repository providing scientifically verified interactions that occur between miRNAs and their and their desired genes. Using high-throughput sequencing approaches for instance, qPCR, CLIP-seq, western blotting and luciferase reporter assays, it compiles interaction information substantiated by solid evidence from experiments. It enables researchers to categorise highly reliable regulatory links by classifying associations with respect to the degree of experiment-based confirmation. In network-mediated investigations, it constitutes as a vital strategy for uncovering miRNAs that govern a variety of target genes exposing post-transcriptional gene regulatory cascades. The primary goal upon documented linkages optimises the biological accuracy and precision underlying gene-miRNA relationships.
- Leveraging experimentally verified associations incorporated into the database, gene-miRNA target interaction webs were created to examine post-transcriptional regulators.
- To recognise regulatory miRNAs which target various genes, the inputted gene dataset was compared to libraries of miRNA interconnections. In the derived network structure, genes were represented by circular nodes, miRNAs by blue-square nodes while regulatory interactions were represented by edges.
- To figure strongly connected miRNAs (hub miRNAs) which could behave as crucial controllers modulating numerous desired genes being targeted

concurrently, topological network modelling was implemented. The generated networks provide insights regarding the way in which these post transcriptional-regulators modulate expression of genes in cardiovascular conditions.

### 3.3.3 Gene-Transcription Factor Network

- Tool Used: TRRUST Database
- It is a comprehensive tool spanning gene-TF associations in human beings and mice. It incorporates data acquired from scientific publications via text extraction algorithms alongside human curation to assure maximum range and correctness. It supplies precise data regarding regulation-associated connections especially, mode of regulation such as- activation and suppression. The data contained herein is notably important for searching for transcriptional regulators that alter gene manifestation. For the purpose of network assessment, it permits the establishment of gene-TF relationship structures, yielding details about regulatory domains and determining chief regulators engaged in pathological mechanisms.
- To determine transcriptional regulatory components influencing activity of genes, gene-TF target interaction webs were constructed.
- A regulatory network structure from a combination of transcriptional regulatory elements and their intended genes was constructed by mapping the genes to previously identified gene-TF relationships. In the derived network structure, genes were represented by red nodes, TFs by blue nodes while regulatory interactions were represented by edges.
- To figure strongly connected TFs (hub TFs) with significant connection implying their potential involvement as master regulatory agents, network-based method was applied. Understanding the transcriptional regulatory mechanisms driving proinflammatory signalling in cardiac disorders was rendered by this approach.

## CHAPTER 4

### RESULTS

#### 4.1 Gene Retrieval and Identification of Common Genes

- The initial batch of genes comprised of the most prominent 100 genes linked with proven relationships and involvement in the pathogenesis in each of the 4 diseases – Atherosclerosis, CAD, Heart Failure and Hypertension obtained from CTD.
- To fine-tune this collection of data Python-powered examination was executed via Google Colab aiming to find common genes shared across all the diseases.
- This approach of filtering condensed the dataset to a segment of '9' commonly occurring genes that reflected the most frequently-related genes spanning the entire collection of data and were employed as the input data for subsequent evaluation.

| Common_Genes |
|--------------|
| TNF          |
| EDN1         |
| IL6          |
| CCL2         |
| CAT          |
| IL1B         |
| NOS2         |
| CRP          |
| AGT          |

Figure 4.1-The '9' Common Genes Involved in the Pathogenesis of – Atherosclerosis, CAD, Heart Failure and Hypertension

#### 4.2 Tissue Specific Gene Co-Expression Network Analysis

- These 9 reported genes were subsequently being investigated in different cardiac tissues to determine their co-expression patterns. In order to analyse spatial variations in the trends of interaction of genes tissue-specific gene co-expression network webs were designed for two heart tissues- atrial appendage and left ventricle.

#### 4.2.1 Gene Co-Expression in Heart Atrial Appendage and Determination of Hub Genes

- The tissue-specific gene co-expression network was created by utilizing iNetModels Database for the tissue – Atrial Appendage and “3” central hub genes were identified demonstrated as red nodes:-
  - ✓ CCL2
  - ✓ IL6
  - ✓ IL8

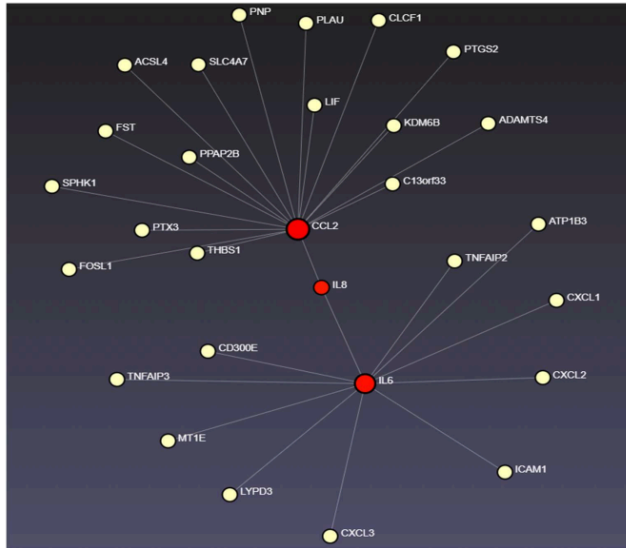


Figure 4.2-Tissue-Specific Gene Co-Expression Network in the Tissue - Atrial Appendage Showing Central Hub Genes CCL2, IL6 and IL8

#### 4.2.2 Gene Co-Expression in Heart Left Ventricle and Determination of Hub Genes

- The tissue-specific gene co-expression network was created by utilizing iNetModels Database for the tissue – Left Ventricle and “1” central hub gene was identified demonstrated as red node:-
  - ✓ CCL2

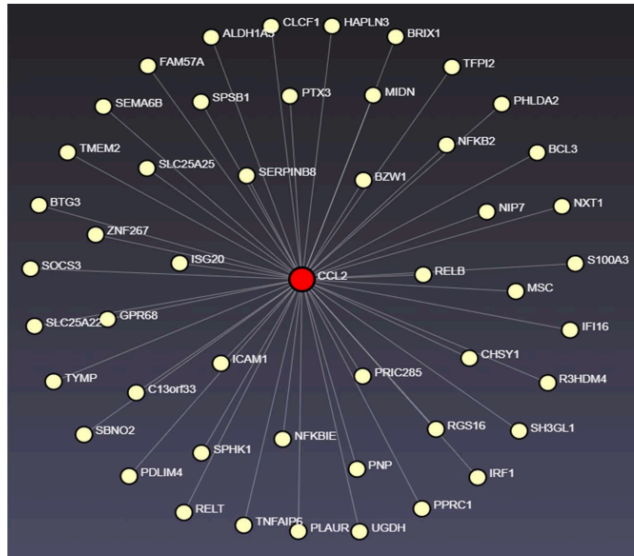


Figure 4.3-Tissue-Specific Gene Co-Expression Network in the Tissue – Left Ventricle Showing Central Hub Gene CCL2

#### 4.2.3 Identification of Master Gene

- The gene “CCL2” was revealed as the typical shared master gene involved across all the diseases studied, expressed in both the tissues.

#### 4.3 Gene-miRNA Interaction Network Analysis and Determination of Hub miRNAs

- The gene-miRNA interaction network was built by utilizing miRTarBase Database in which “9” primary miRNAs (blue nodes) were found to be dysregulated, with hsa-miR-155-5p having the highest degree ‘6’ showing its maximum contribution in regulating the networks involved in the disease.

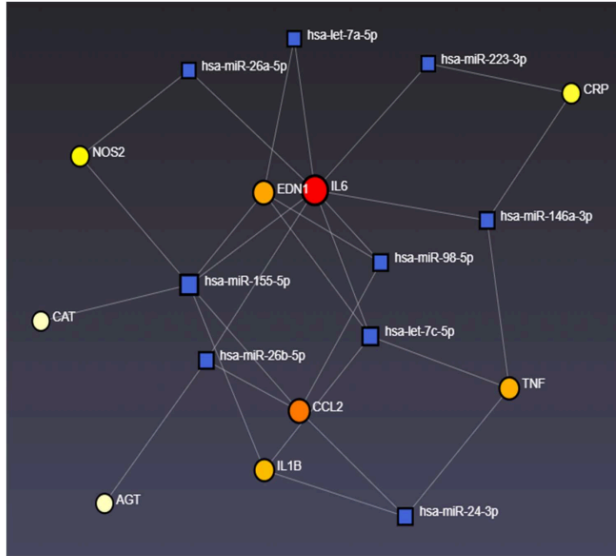


Figure 4.4-Gene-miRNA Interaction Network Analysis Showing hsa-miR-155-5p as the Key Hub miRNA Regulating Central Hub Genes IL6 and CCL2

Table 4.1-The '9' Primary miRNAs Dysregulated in the Diseases

| miRNA           | Degree |
|-----------------|--------|
| hsa-miR-155-5p  | 6      |
| hsa-let-7c-5p   | 4      |
| hsa-miR-26b-5p  | 3      |
| hsa-miR-146a-3p | 3      |
| hsa-miR-24-3p   | 3      |
| hsa-miR-98-5p   | 3      |
| hsa-miR-223-3p  | 2      |
| hsa-miR-26a-5p  | 2      |
| hsa-let-7a-5p   | 2      |

#### 11 4.4 Gene-TF Interaction Network Analysis and Determination of Hub TFs

- 11 The gene-TF interaction network was constructed by utilizing TRRUST Database in which “8” primary TFs (blue nodes) were found to be affected, with NFKB1 having the highest degree ‘8’ showing its maximum regulatory effects on the genes contributing to the disease.

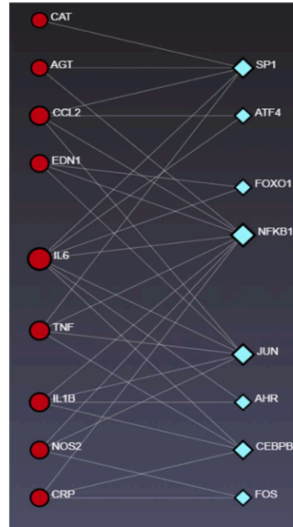


Figure 4.5-Gene-TF Interaction Network Analysis Showing NFKB1 as the Key Hub TF Regulating Central Hub Genes IL6 and CCL2

Table 4.2-The ‘8’ Primary TFs Affected in the Diseases

| Transcription Factor | Degree |
|----------------------|--------|
| NFKB1                | 8      |
| JUN                  | 6      |
| SP1                  | 5      |
| CEBPB                | 4      |
| FOXO1                | 2      |
| AHR                  | 2      |
| FOS                  | 2      |
| ATF4                 | 2      |

## 4.5 Analysis of Most Potential Identified Biomarkers

### 4.5.1 CCL2

- Another name for CCL2 is Monocyte Chemoattractant Protein-1 (MCP-1), a polypeptide which consists of 76 amino acids and includes 2 adjoining cysteine residues at amino (-NH<sub>2</sub>) terminal. It falls under CC-motif chemokine family and has a molecular weight of 13 kDa.
- It is typically produced by immunological cells; however, Smooth Muscle Cells (SMCs), Endothelial Cells (ECs), thylakoid cells, and fibroblasts can also produce CCL2. Its synthesis can be induced by several cytokines, including IL-1, IL-4, IL-6, TNF- $\alpha$ , TGF- $\beta$ , and IFN- $\gamma$ . It controls the influx of several immune cells such as monocytes, macrophages, memory-T cells and natural killer cells. It also regulates the movement of monocytes from the area of bone marrow to the site of atherosclerotic lesion via CCL2-CCR2 interaction. On coupling of CCL2-CCR2, the production of Monocyte Chemotactic Protein-1 Inducible Protein-1 (MCP-1), which then initiates the production of the genes IL-1, CCL2, TNF and activation of wide range of signalling pathways such as PI3K/MAPKs, JAK/STAT and PI3K/Akt/ERK/NFKB, which further stimulates the expression of various cytokines, proliferation and development of cells, cell maintenance, transit and death, vascular development, and inflammatory responses. The recruitment of monocytes triggered by CCL2 arterial surface is a crucial factor underlying the progression of vascular atherosclerosis. The activity of CCL2 is regulated by NFKB. The CCL2-CCR2 circuit recruits monocytes that are travelling in the blood stream to lesions produced by atherosclerosis, where they develop into macrophages, grow and multiply, develop into foam cells, and drive the process of inflammation. Each of the above findings point to a strong link between CCL2-CCR2 axis and atherosclerosis.

### 4.5.2 IL6

- IL-6 controls immunological and inflammatory responses. It is synthesised via a variety of cell kinds in reaction to illnesses and injury to tissue, and it also boosts the synthesis of proteins for instance, C-reactive protein (CRP), that is widely used as a medical diagnostic of inflammatory conditions. Excessive levels of IL-6 in patients suffering from CVD have been correlated with a greater likelihood of heart attack, cardiac failure, and total morbidity and also aids towards the advancement of atherosclerotic disease by stimulating cell damage in endothelial cells and lesion formation.
- The coupling of IL6-IL6R is capable of stimulating various subsequent signalling channels, which include the JAK/STAT axis, which results in the activation of genes that contribute to inflammatory processes and proliferation of cells. Frequent signalling of IL-6 leads to the preservation of a favourable inflammatory milieu, worsening dysfunction in the endothelial system and promoting the formation of fibrous lesions causing atherosclerosis.

#### 4.5.3 IL8

- IL-8, a versatile cytokine coming from the C-X-C type of cytokine supergene group, has been implicated in the emergence of a number of medical conditions. It chemotactically activates T-cells and neutrophils. It can boost attachment of monocytes to endothelial cells as well. It is chemotactic agent for smooth muscle cells in vascular tissues thus might serve an active part in the recruitment of smooth muscle cells across the medium towards the inner membrane of vascular walls.
- A defining characteristic of an atherosclerotic plaque is the presence of foam cells produced from macrophages. It has also been proven that oxidised low-density lipoprotein (oxLDL) activate macrophages to secrete IL-8 while also triggering the expression of CCL2.
- Macrophage is a predominant cell that secretes IL-8. Macrophages' inflammatory cytokine synthesis in plaques caused by cardiovascular disease could play a role in the emergence and escalation of inflammatory events in vascular disease called atherosclerosis.
- In humans, foam cells isolated from atherosclerotic plaques exhibit greater amounts of IL-8 than monocytes circulating in the blood and macrophages derived from monocytes and the aforementioned cell types are likely still competent to be responsive to stimuli that cause inflammation. The lipid oxidised compounds called as oxysterols, can modulate the synthesis of IL-8, which potentially help promote the establishment of vascular lesions.

#### 4.5.4 TNF

- TNF is primarily synthesised on the cytoplasmic membrane of the T-cells, monocytes, macrophages as a 26kDa inactivated molecule known as pro-TNF, which is then processed by the enzyme called as matrix metalloproteinases, ultimately resulting in a dissolved derivative of 17 kDa activated protein.
- Endothelial cells foster inflammatory events by the stimulation of TNF via exhibiting a variety of binding proteins for white blood cells, such as E-selectin, ICAM-1, and VCAM-1. Such reactions, in conjunction with the production of IL-8, CCL2 and IP-10, results in the influx of aggregates of white blood cells that are not antigen-specific.
- Reactions of TNF- $\alpha$  are triggered through interactions of ligands via 2 uniquely constructed receptors, namely TNF-RI or p55 while TNF-RII or p75, that can be observed upon cellular membranes of every kind of cell excluding red blood cells. Both of these sites varied greatly in terms of interacting properties and inner cell signalling mechanisms. The inner cellular region of TNF-RI attaches with the TNF Receptor-Associated Death Domain (TRADD) protein, thereby triggering one of the death signalling pathways through "Fas-Associated Death Domain" (FADD) or the inflammatory signalling pathway through "TNF Receptor-Associated Factor 2" (TRAF2) and receptor-binding proteins, eventually triggering NF $\kappa$ B stimulation.
- TNF- $\alpha$ , along with numerous other molecules that promote inflammation, contributes to the progression of vascular atherosclerotic plaques by encouraging adhesion proteins in endothelial tissue, recruiting as well as activating cells

associated with inflammation, and launching the cycle of inflammation residing within the vascular system.

#### 4.5.5 IL1 $\beta$

- IL-1 $\beta$  serves as an inflammatory mediator which is currently being investigated as a potential medical candidate in the management of atherosclerosis. It is now extensively examined for inducing a state of inflammation in endothelial tissues and stimulating the buildup as well as infiltration of immunological cells, particularly macrophages, towards the inner layer lining vessels, contributing to an elevated mediator of inflammation.
- The latest study has revealed that IL-1 $\beta$  serves a crucial part in the development of atherosclerotic disease via stimulating the production of lipid cells, that infiltrate the wall of an arterial cavity subsequently regulating the lesion formation.
- Investigations have shown a spike in the expression of IL-1 $\beta$  in macrophages subjected to oxLDL. Cholesterol, chylomicrons, and triglycerides are the examples of fats that might trigger IL-1 $\beta$  production. Elevated cholesterol levels stimulate macrophages to produce more IL-1 $\beta$ , which contributes to the progression of the atherosclerosis and caspase-1 promotes to the atherosclerosis through NLRP3 inflammasome assembly.

#### 4.5.6 CRP

- CRP, for short, C-Reactive Protein is a protein with 5 homologous monomers organised into circular pentameric- structure.
- Its concentrations rise in heart disorders with even mild inflammatory responses, including vascular atherosclerosis. Investigations have demonstrated that mediators such as IL-1 $\beta$  and IL-6 stimulate the expression of CRP in atherosclerotic plaque cells, monocytes, endothelial cells, and smooth muscle cells.
- The breakthrough of CRP's fat-flocking activity provided initial confirmation regarding a potential link to atherosclerosis. CRP's ability to coagulate fats is caused by its association with cholesterol, moreover, clustered derivatives of the original CRP structure were found to adhere to LDL molecules.
- CRP is being discovered to be accumulated within atherosclerosis -related plaques, in which it colocalizes alongside cholesterol and monocytes. The occurrence of CRP near the areas of plaque were not surprising given the established relationship that exists between LDL and CRP. Despite localised CRP production by artery cells as well as monocytes was previously demonstrated, it was found that CRP expressed within the plaques was transferred through the bloodstream.

#### 14 4.5.7 hsa-miR-155-5p

- hsa-miR-155-5p derives primarily from the B-cell Integration Cluster (BIC) locus found on the 21st chromosome.
- The transcription of hsa-miR-155-5p is promoted by NF- $\kappa$ B in the presence of pro-inflammatory stimuli.
- It is a key miRNA regulating immunological as well as inflammatory reactions. It is regulated via an array of transcriptional regulatory variables and shows considerable elevation after the stimulation of T cells, that is required during the division and development of lymphocytes.
- It serves a significant function in controlling macrophage inflammatory mechanisms and constitutes a component of a complicated pathological cascade encompassing several variables, as well as being implicated in the progression of atherosclerotic disease through modifying distinct communication channels. In the past decades, investigation has emphasised the importance of hsa-miR-155-5p in the Renin-Angiotensin-Aldosterone System (RAAS) which stimulates the assembly of inflammation-related cells around atherosclerotic spots. More precisely, the Angiotensin II stimulation occurring in this framework triggers the expulsion of factors associated with inflammation as well as the accumulation of Reactive Oxygen Species (ROS), whereas simultaneously minimising the synthesis of Nitric Oxide (NO), a combination of which facilitate the emergence of lesions associated with atherosclerosis.

#### 4.5.8 NFKB1

- NF- $\kappa$ B represents a dimeric-type transcriptional component which attaches with the  $\kappa$ B domain located within the promoter portion found in the light molecule chain of the antibody. It was identified as an activated B lymphocyte-associated component. It modulates immunological and inflammation-associated processes.
- It has a key role in the development of atherosclerosis. Its stimulation boosts the synthesis of cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and CCL2, resulting in induction of atherosclerotic lesions as it is found in the nucleus of macrophage cells of atherosclerotic plaques, revealing its induction potentially contribute to the process of atherogenesis.
- The stimulation of NF $\kappa$ B triggers transcriptional components which induce inflammatory responses, including leukocyte attachment molecules, cellular cytokines, and chemokines, which operate via both classical and non- classical routes.
- It is among the several physiological oxidative mechanisms that contribute to prolonged inflammation-associated atherosclerosis. It stimulates the enzyme inhibitor of kappa-B ( $\kappa$ B) kinase in the face of triggers associated with inflammation. Either of the signalling routes- classical or non- classical, cause NF $\kappa$ B to express the inflammatory proteins in the cells such as- macrophages, monocytes, and T/B lymphocytes, macrophages, monocytes, etc.
- The classical channel initiates in response to events, thereby boosting cytokines that induce inflammation for example IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , which ultimately causes cellular death.

Table 4.3-The Most Potential Drugs and Their Targets

| Potential Drug | Target (Mode of Action)   |
|----------------|---|
| Tocilizumab    | IL-6R antagonist  |
| Canakinumab    | Inhibits IL-1 $\beta$   |
| Adalimumab     | Inhibits TNF- $\alpha$  |
| Methotrexate   | Inhibits various inflammatory molecules such as- IL-6 and TNF- $\alpha$ |
| Colchicine     | Inhibits the stimulation of NLRP3 inflammasome                          |
| Rosuvastatin   | Inhibits IL-6, TNF- $\alpha$ , INF- $\gamma$ and CRP                    |

## CHAPTER 5

### DISCUSSION

Over many years, the cardiovascular disorders such as- **atherosclerosis, coronary artery disease, heart failure**/ ischemic heart disease, **hypertension**, etc. are been investigated and been proven to be at the top of the list among other chronic and age-related diseases for the most deleterious fatalities on the globe.

Even after significant and top-notch technological breakthroughs in the field of diagnostics, therapies and medications, these heart illnesses are truly complicated as they propagate via various mechanisms, channels and molecules underlying them because of which the molecular biomarkers such as- genes, miRNAs, transcription factors and other proteins are need to be recognised which will further assist the researchers and scientists developing the advancements for early diagnosis of the diseases as well as designing targeted treatments for instance, developing personalised medications for different individuals.

In this investigation, we attempted to recognise pathogenic biomarkers underlying the cardiovascular disorders using a systems biology and bioinformatics approach, which recognised the 9 common genes among the all aforementioned illnesses which were- TNF, EDN1, IL-6, CCL2, CAT, IL1- $\beta$ , NOS2, CRP and AGT, out of which the primary molecular biomarkers were found to be- CCL2 (a chemoattractant molecule), IL-6 (an inflammatory cytokine), hsa-miR-155-5p (an overexpressed post-transcriptional regulator) and NFKB1 (an overexpressed transcriptional regulator) which were playing a crucial function in the genesis and modulation of the diseases.

The expression of CCL2, IL6 and IL8 (all cytokines) creates an intensified inflammatory cycle. In the **gene-miRNA and gene-TF** web networks, the hub **post-transcriptional** regulator hsa-miR-155-5p and transcription factor NFKB1 are observed to be associated with CCL2 and IL6 so, can be considered as crucial regulatory points and hub biomarkers which can be targeted for therapeutics as all are playing major role in inflammatory signalling pathways.

CCL2-CCR2 and IL6-IL6R signalling axes can be obstructed by designing CCL2 and IL6 specific antibodies which can neutralize them or CCL2R and IL6R antagonists, thereby preventing the activation of inflammatory pathways such as- **JAK/STAT and PI3K/Akt/ERK/NFKB**. hsa-miR-155-5p and NFKB1 can also be targeted by designing their inhibitor molecules as they are regulating hub and other various inflammatory genes. This can serve to reduce the production of ROS and activation of pro-inflammatory cells and genes such as- CCL2, IL6, **TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1**.

Moreover, an analysis was performed by reviewing the literature to identify the drugs for targeting the most potential identified biomarkers which were- Tocilizumab (IL-6R antagonist), Canakinumab (inhibits IL-1 $\beta$ ), Adalimumab (inhibits TNF- $\alpha$ ), Methotrexate (inhibits various inflammatory molecules such as- IL-6 and TNF- $\alpha$ ), Colchicine (inhibits the

stimulation of NLRP3 inflammasome) and Rosuvastatin (inhibits IL-6, TNF- $\alpha$ , INF- $\gamma$  and CRP).

Therefore, by targeting these signature biomarkers, we can intervene the inflammatory signalling pathways at multiple levels thus, reducing the risk of pathogenesis of major fatal CVDs.

## CHAPTER 6

### CONCLUSION

In this investigation, we attempted to recognise pathogenic biomarkers underlying the cardiovascular disorders using a systems biology and bioinformatics approach by analysing tissue-specific gene co-expression network, gene-miRNA interaction network and gene-transcription factor interaction network. CCL2 and IL6 emerged as the central hub genes with hsa-miR-155-5p as the key miRNA and NFKB1 as the key transcriptional factor involved in the pathogenesis of cardiovascular disorders. After performing a literature review, they were found to be responsible primarily for exaggerating the inflammation-associated mechanisms.

Moreover, the drugs for targeting the aforementioned biomarkers were also recognised, which were- Tocilizumab, Canakinumab, Adalimumab, Methotrexate, Colchicine and Rosuvastatin. Although, these drugs have not been approved for the treatment of cardiovascular diseases but these can be developed as a medication of the heart-associated disorders as they target the biological markers which are associated with inflammation, which is the major underlying cause of CVDs.

In conclusion, this study unveiled that these can be the novel and potential biomarkers which can be targeted for achieving broad-spectrum therapeutics.

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