Ritika MSc Thesis

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Abstract

Non-alcoholic fatty liver disease, Type 2 diabetes mellitus and dyslipidemia share common pathological mechanisms including chronic inflammation, insulin resistance, extracellular matrix remodeling, and endothelial dysfunction. However, the integrated molecular signatures that link these conditions remain incompletely defined. An integrated systems and computational biology method to identify common molecular markers among different metabolic diseases. To identify shared hub genes, liver and pancreatic gene data were subjected to tissue-specific gene coexpression assessment, followed by construction of gene-miRNA and transcription factor regulatory networks using NetworkAnalyst. ICAM1 and COL3A1 were identified as key hub genes commonly dysregulated in both liver and pancreatic tissues, implicating their respective roles in inflammatory and fibrotic processes. Gene-miRNA network analysis identified miR-335-5p and the miR-26b-5p as central regulators based on node degree filtering. Across NAFLD, T2DM, and dyslipidemia, a reciprocal dysregulation pattern was observed wherein hsa-miR-335-5p was consistently upregulated while hsa-miR-26b-5p was consistently downregulated. Transcription factor-gene interaction analysis further identified RELA, NFKB1, and SP1 as key transcriptional regulators, with NFKB1 and RELA influencing ICAM1 expression and SP1 regulating COL3A1 expression. The successful clinical translation of RNA interference therapeutics including inclisiran, patisiran, and givosiran has established the feasibility of oligonucleotide-based strategies for metabolic diseases. Based on the network analyses conducted, a combination therapy comprising antagomiR-335-5p and miR-26b-5p mimic is proposed as a rational treatment strategy, given the dual mechanism of action involving inhibition of pathogenic miR-335-5p alongside restoration of physiological miR-26b-5p levels to indirectly normalize RELA/NFκB, SP1, ICAM1, and COL3A1 function. These results indicate that ICAM1, COL3A1, and their associated miRNAs may serve as promising novel biomarkers and therapeutic targets for the development of integrated therapies for metabolic diseases.

Keywords: Non-alcoholic fatty liver disease, Type 2 diabetes mellitus, Dyslipidemia, microRNA, ICAM1, COL3A1, NF-κB, SP1, RNA interference therapeutics, antagomiR, Integrative network biology.

Chapter1

Introduction

Metabolic disorders encompass a broad spectrum of conditions arising from impaired biochemical pathways involved in nutrient utilization and energy regulation, ultimately disturbing glucose, lipid, and protein balance within the body. Diseases such as type 2 diabetes mellitus (T2DM), metabolic dysfunction-associated steatotic liver disease (MASLD; previously referred to as non-alcoholic fatty liver disease, NAFLD), obesity, dyslipidemia, and gout have become major global health concerns due to their rapidly increasing prevalence and long-term complications. T2DM, which constitutes more than 90% of diabetes cases worldwide [1], is primarily associated with insulin resistance in hepatic and skeletal muscle tissues along with progressive pancreatic β-cell dysfunction. Epidemiological evidence indicates that diabetes currently affects over 10% of the global population [2], with projections suggesting a substantial increase of nearly 25% by 2030 and more than 50% by 2045 [3].MASLD is among the most prevalent chronic liver disorders and is characterized by excessive lipid accumulation in more than 5% of hepatocytes. Persistent hepatic fat deposition contributes to lipotoxic stress and may progress to non-alcoholic steatohepatitis (NASH), an inflammatory form of liver injury. If left untreated, the disease can further advance to fibrosis, cirrhosis, and hepatocellular carcinoma. Current reports estimate the worldwide prevalence of MASLD to range between 22.10% and 28.65% [4].Dyslipidemia represents an abnormal lipid profile in circulation and is typically characterized by elevated levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TGs), accompanied by reduced concentrations of high-density lipoprotein cholesterol (HDL-C). The prevalence of dyslipidemia varies widely across populations, with global estimates ranging from 20% to 80% [5].MicroRNAs (miRNAs) are evolutionarily conserved, single-stranded non-coding RNAs approximately 20–24 nucleotides in length that exhibit tissue-specific expression patterns. These regulatory molecules modulate gene expression at the post-transcriptional level through binding to complementary sequences located predominantly within the 3′-untranslated regions (3′-UTRs) of target messenger RNAs (mRNAs), thereby influencing diverse physiological and pathological processes [6,7]. The transcription of ICAM1 mRNA is triggered by the recruitment of the NF-κB p65/RelA to the enhancer and promoter regions of the ICAM1 gene and experiments have shown that SP1 inhibition efficiently leads to the suppression of COL3A1 transcription, validating SP1 as a key factor in activating COL3A1 transcription. miRNAs that inhibit p65 or SP1, like miR-26b that inhibits RELA or miR-335-5p that inhibits SP1, can serve as a treatment approach to inhibit ICAM1 or COL3A1 expression.

A diet rich in calories, sedentary behavior, inherited genes, and epigenetic changes can all contribute to metabolic diseases. Additionally, psychological factors such as depression, anxiety, chronic stress and behavioural factors like disruption in sleep cycle, sedentary lifestyle, smoking leads to development of these diseases. Insulin resistance, oxidative stress, chronic inflammation, mitochondrial dysfunction, and adipokine imbalance are among the molecular pathways that overlap across type 2 diabetes, nonalcoholic fatty liver disease, and dyslipidemia. Insulin resistance causes hyperglycemia and aberrant lipid metabolism,

whereas oxidative stress and inflammatory cytokines cause hepatocellular damage and disrupted glucose homeostasis. These interrelated pathways form a vicious metabolic cycle that promotes disease development while increasing cardiovascular and metabolic implications. One such miRNA that has been shown to be pathogenic and consistently elevated in all three metabolic diseases is miR-335-5p. In pancreatic β-cells, miR-335-5p negatively affects the process of glucose-stimulated insulin release via its inhibition of proteins critical for exocytosis, such as SNAP25, STXBP1, and SYT11. In the liver, miR-335-5p promotes adipocyte differentiation and hepatic steatosis by modulating the expression of the genes that produce fatty acids. Finally, in dyslipidemia, miR-335-5p affects the cholesterol metabolism by inhibiting HMGCR, CYP7A1, and PCSK9, which plays important role in cholesterol biosynthesis, degradation, and elimination.

On contrary, miR-26b-5p is considered a protective miRNA because it is consistently reduced in all three metabolic diseases under study. One such beneficial function of miR-26b is its role in improving insulin sensitivity by targeting the tumor suppressor PTEN protein involved in PI3K/AKT signaling pathways, which results in increased GLUT4 translocation. Also, due to its antifibrotic effects, miR-26b targets TGFβ1 and SMAD2, thus preventing Current treatment strategies for NAFLD, T2DM, and dyslipidemia mostly concentrate on metabolic anomalies unique to the diseases rather than the common regulatory mechanisms of inflammation and fibrosis that contribute to the development of these conditions. Moreover, limited research has been done on the therapeutic potential of miRNA-mediated regulation of inflammatory and fibrotic pathways across metabolically associated organs. The current approaches employed in treating NAFLD, T2DM, and dyslipidemia focus primarily on managing the metabolic disturbances peculiar to each of the diseases while ignoring the common regulatory pathways of inflammation and fibrosis that play a role in their pathogenesis. Besides, there is less literature that investigates the possibility of targeting these pathways by regulating miRNA activity in metabolically-related organs.

This thesis explores current understanding of the molecular mechanisms linking insulin resistance to T2DM, NAFLD, and dyslipidemia, emphasizing the integrated pathophysiology that has profound implications for diagnosis, prevention, and treatment. The findings presented herein provide a mechanistic framework for understanding metabolic diseases as a network disorder and offer prioritized therapeutic targets for future experimental validation and clinical translation.

CHAPTER2

LITERATURE REVIEW

2.1 Insulin Resistance: The Metabolic Nexus Linking T2DM, NAFLD, and Dyslipidemia

A condition of reduced responsiveness in insulin-targeting tissues to elevated levels of insulin, is termed as insulin resistance [8].
Insulin resistance represents a central pathological mechanism underlying several chronic metabolic disorders, including obesity, type 2 diabetes mellitus (T2DM), polycystic ovarian syndrome (PCOS), metabolic dysfunction-associated steatotic liver disease (MASLD), and cardiovascular diseases. Under physiological conditions, insulin secreted by pancreatic β-cells plays a critical role in maintaining glucose equilibrium by suppressing hepatic gluconeogenesis, promoting glucose uptake in skeletal muscle and adipose tissues, and inhibiting lipolysis within adipocytes.

In insulin-resistant states, the responsiveness of peripheral tissues such as the liver, skeletal muscle, and adipose tissue to insulin becomes markedly impaired, resulting in reduced glucose utilization despite elevated insulin levels. Concurrently, hepatic glucose production remains abnormally elevated, contributing to persistent hyperglycemia. Initially, pancreatic β-cells compensate for this metabolic imbalance through increased insulin secretion, leading to compensatory hyperinsulinemia. However, progressive β-cell dysfunction gradually diminishes this adaptive response, ultimately aggravating glucose intolerance and chronic hyperglycemia[9]. These signaling pathways are hindered in insulin resistance by inflammatory cytokines, oxidative stress, excess free fatty acids (FFAs), and lipid intermediates such ceramides and diacylglycerol (DAG). This disturbance contributes to metabolic dysfunction by lowering glucose absorption and promoting hepatic glucose synthesis. Insulin resistance results in increase in inflammatory signalling, promotes lipid accumulation, activates NF-κB and worsens fibrosis

2.1.1. Molecular mechainsm of Insulin Signaling Pathways in Metabolic Tissues

Insulin exerts its metabolic functions through a tightly regulated intracellular signaling network initiated by the interaction of insulin with the insulin receptor (INSR). Binding of insulin promotes autophosphorylation of tyrosine residues on the receptor, leading to the recruitment and phosphorylation of insulin receptor substrate (IRS) proteins. Activated IRS subsequently stimulates phosphatidylinositol 3-kinase (PI3K), resulting in the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). This lipid second messenger facilitates the activation of phosphoinositide-dependent kinase-1 (PDK1) and Akt, also known as protein kinase B. Activated Akt regulates several downstream targets responsible for insulin-mediated metabolic responses. These include translocation of GLUT4 transporters to the plasma membrane in skeletal muscle and adipose tissue to enhance glucose uptake, inhibition of glycogen synthase kinase-3 (GSK-3) to promote glycogen synthesis, and phosphorylation-mediated nuclear exclusion of FOXO1 in hepatocytes, thereby suppressing hepatic gluconeogenesis.

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2.1.2 Insulin resistance in T2DM

In type 2 diabetes mellitus (T2DM), insulin resistance (IR) refers to the impaired responsiveness of skeletal muscle, adipose tissue, and hepatic cells to physiological concentrations of insulin. This dysfunction is driven by multiple factors, including genetic susceptibility and ectopic lipid deposition–induced lipotoxicity, which promote the activation of stress-related serine kinases such as protein kinase C (PKC) and c-Jun N-terminal kinase (JNK). These kinases disrupt insulin signaling by inducing serine phosphorylation of insulin receptor substrate-1 (IRS-1) instead of the normal tyrosine phosphorylation, thereby inhibiting subsequent PI3K/Akt pathway activation.

Consequently, GLUT4 translocation fails (reducing glucose uptake by ~40%), glycogen synthase remains inactive, and FoxO1 is unrestrained—driving hepatic gluconeogenesis. Elevated levels of pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and resistin, together with reduced adiponectin expression, contribute significantly to metabolic dysfunction. These molecular alterations impair insulin signaling cascades and promote a state of chronic low-grade inflammation. In addition, mitochondrial abnormalities and oxidative stress intensify insulin resistance by disrupting cellular bioenergetics and enhancing the generation of reactive oxygen species (ROS). Sustained hyperglycemia further induces glucotoxic effects, leading to progressive pancreatic β-cell impairment and depletion of insulin secretory capacity.

2.1.3 Insulin Resistance in Non-Alcoholic Fatty Liver Disease (NAFLD)

The role of the resistance to insulin in pathogenesis of the NAFLD is dual. First, resistance of insulin in adipose tissue promotes lipolysis and increases circulating FFAs, which are then shuttled to liver. Second, paradoxically, hepatic insulin resistance does not lead to a defect in lipogenesis, leading to increased triglyceride synthesis and storage while hyperinsulinemia compensatory to

the resistance directly activates SREBP-1c to stimulate a DNL. The result is excessive triglyceride accumulation within hepatocytes .Excess lipid accumulation in hepatocytes leads to lipotoxicity, oxidative stress, mitochondrial dysfunction and inflammatory reactions that may have a role in the progression of simple steatosis into cirrhosis, fibrosis, and non-alcoholic steatohepatitis (NASH)[10]. A major molecular mechanism linking NAFLD to insulin resistance involves intracellular accumulation of diacylglycerol (DAG), which activates (PKCε). PKCε interferes with insulin receptor signaling and impairs hepatic insulin sensitivity. Ceramides and inflammatory mediators also contribute to defective insulin signaling pathways. Furthermore, endoplasmic reticulum stress and altered gut microbiota have been implicated in NAFLD progression and worsening insulin resistance.

2.1.4 Insulin resistance in dyslipidemia

In insulin resistant adipose tissue, increased lipolysis results in an excessive release of FFAs into the circulation. The liver utilizes these FFAs to synthesize triglycerides and overproduce VLDL particles. Concurrently, insulin resistance inhibits lipoprotein lipase activity, reducing the clearance of triglycerides from plasma. Increased secretion of VLDL by the liver also favors formation of the LDL particles , which are more susceptible to oxidation and vascular injury.

In addition, insulin resistance lowers HDL-C levels by promoting the exchange of triglycerides between lipoproteins via cholesteryl ester transfer protein (CETP)-mediated pathways. These changes together lead to endothelial dysfunction, atherosclerosis, and increased cardiovascular morbidity.

NAFLD further aggravates dyslipidemia through impaired hepatic lipid handling and increased de novo lipogenesis. Hence, insulin resistance establishes a metabolic network between glucose intolerance, hepatic steatosis and lipid abnormalities[10].

2.1 Endothelial Inflammation and ICAM1 in Metabolic Disease

Intercellular adhesion molecule-1 (ICAM-1) has emerged as a critical mediator of leukocyte recruitment and vascular inflammation.

2.1.1 Diabetes Mellitus : ICAM-1 Upregulation

The molecular pathway from hyperglycaemia to ICAM-1 expression is a cascade of intracellular events converging to activate the NF-κB. Chronic hyperglycemia promotes excessive generation of reactive oxygen species (ROS) through multiple mechanisms, including glucose autoxidation, advanced glycation end product (AGE) formation, and impairment of the mitochondrial electron transport chain. Increased ROS production functions as an intracellular signaling trigger that activates the IκB kinase (IKK) complex. Activated IKK phosphorylates the inhibitory protein IκBα, leading to its degradation and subsequent release of the NF-κB complex from cytoplasmic sequestration. Following IκBα degradation, the NF-κB p50/RELA (p65) heterodimer translocates into the nucleus, where it binds to κB regulatory elements located within the ICAM-1 promoter region. Two functional NF-κB binding motifs positioned between −225 to −215 and −95 to −85 upstream of the transcription initiation site are essential for optimal cytokine- and glucose-mediated ICAM-1 transcriptional activation. Consequently, NF-κB signaling represents a major molecular mechanism responsible for hyperglycemia-induced upregulation of ICAM-1 expression in diabetic endothelial cells.In parallel, oxidative stress associated with hyperglycemic conditions also activates additional transcriptional regulators, including activator protein-1 (AP-1) and specificity protein-1 (SP-1), which interact with adjacent response elements within the ICAM-1 promoter and further enhance transcriptional activity. Within the diabetic pancreas, elevated ICAM-1 expression facilitates recruitment and infiltration of inflammatory immune cells into the islets of Langerhans, thereby promoting insulitis, β-cell injury, and progressive impairment of insulin secretion[11]. Numerous clinical trials have found an increase in the levels of soluble ICAM-1 (sICAM-1) in diabetic patients as compared with the control group. Soluble ICAM-1 is released from membrane ICAM-1 through proteolytic cleavage and can be detected in blood plasma and used as a proxy of endothelial activation. Notably, levels of sICAM-1 are associated with haemoglobin A1c (HbA1c), a measure reflecting average glucose levels in the past 2-3 months, indicating a positive correlation between the degree of activation of the endothelium and the magnitude of hyperglycemia. Furthermore, high levels of sICAM-1 can act as predictors of diabetic complications. In prospective cohort studies, patients diagnosed with T2DM with high sICAM-1 levels, Compared to other patients, those in the highest quartile were more likely to experience proliferative retinopathy, diabetic nephropathy, and cardiovascular issues. Importantly, the association held true even after adjusting for conventional cardiovascular risk factors, implying that soluble ICAM-1 can provide unique insight into vascular health[12].

2.2.2.ICAM-1 in NAFLD

The deposition of lipids in hepatocytes (steatosis) causes cellular stress that leads to production of DAMPs and pro-inflammatory cytokines, thus activating LSECs, which

transform from a dormant, non-adhesive to a pro-inflammatory. High expression ICAM-1, VCAM-1, and E-selectin indicates an active state.

Activated liver sinusoidal endothelial cells (LSECs) exhibiting elevated surface expression of ICAM-1 promote monocyte adhesion through interactions between ICAM-1 and leukocyte integrins, particularly lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1).The subsequent migration of the monocytes results in the transendothelial migration and differentiation of the monocytes into macrophages. These recruited monocyte-derived macrophages together with the resident Kupffer cells get activated and secrete pro-inflammatory cytokines, thereby augmenting the inflammatory process and contributing to hepatocellular damage. Recruitment of inflammatory monocytes is especially important for progressing from steatosis to the steatohepatitis (NASH), the inflammatory and fibrotic subtype of NAFLD[13]. Soluble ICAM-1 (sICAM-1), Clinical investigations have demonstrated that this potential non-invasive biomarker is significantly elevated in patients with NAFLD and exhibits a positive association with disease progression and severity.

2.2.3 ICAM1 in Dyslipidemia

Dyslipidemia acts as a direct promoter of inflammation in the vasculature through the oxidation and accumulation of lipoproteins in the vessel wall. Endothelium serves as the first point of contact of lipoprotein accumulation since it forms the innermost layer of blood vessels. In cases of dyslipidemia, LDL accumulates within the subendothelial space and gets oxidized, thus forming oxLDL. Instead of simply being deposited as lipid, oxLDL is an active agent in causing inflammation as well. It activates the endothelium, up-regulates cell adhesion molecules like ICAM-1, and leads the way for the development of atherosclerosis through interaction with LOX-1 and This process initiates multiple intracellular signaling cascades that culminate in NF-κB activation. Reactive oxygen species generated through LOX-1 signaling, together with NIK-dependent stimulation of the IKK complex, promote phosphorylation and degradation of IκBα. The resulting release of NF-κB enables its translocation into the nucleus, where it induces transcriptional upregulation of ICAM-1.

Experimental studies further demonstrate that inhibition of inflammatory signaling pathways can reduce ICAM1 expression and monocyte-endothelial interactions. This observation supports the concept that ICAM1 is functionally involved in vascular inflammation rather than serving solely as a passive biomarker. Dyslipidemia-associated endothelial activation therefore represents a major mechanistic link between metabolic disturbances and cardiovascular disease[14].

2.3 Extracellular Matrix Remodeling and COL3A1 in Fibrosis

2.3.1.COL3A1 in Pancreatic Islet Fibrosis: A Hidden Contributor to Beta-Cell Failure

There is an interplay between the formation of collagen and the occurrence of beta cell dysfunction. Whereas the presence of fibrosis reduces insulin secretion by the beta cells, the absence of insulin production itself could enhance fibrotic activity. Studies indicate that insulin possesses antifibrotic actions, and the reduction in the amount of insulin due to dysfunctional beta cells would limit such actions, which would then stimulate collagen

formation. The recognition of islet fibrosis as a key contributor to beta-cell failure has important therapeutic implications. Interventions that prevent or reverse COL3A1 deposition within the islet could potentially preserve beta-cell mass and function and results in slow progression of diabetes.

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In patients with type 2 diabetes mellitus, pancreatic β-cells experience endoplasmic reticulum stress as a consequence of sustained demand for elevated insulin secretion. Under these stress conditions, islet amyloid polypeptide (IAPP) undergoes a conformational transition from its native random-coil structure to an antiparallel cross β-sheet configuration. This structural alteration represents an early event in the amyloidogenic process, promoting aggregation of IAPP molecules and subsequent formation of amyloid fibrils[15]. The amyloid deposits trigger an inflammatory and fibrotic response resulting in progressive loss of islet architecture. A capsule that surrounds the amyloid deposits consists mainly of types I and III collagen and serves to isolate the surviving beta cells from their blood supply and prevent the interactions needed for insulin secretion.

2.3.2. Collagen III in Liver Fibrosis

Collagen type III, also referred to as collagen type III alpha 1 chain (COL3A1), is a homotrimeric protein forming thin reticular fibers. Normally, this type of fiber is responsible for providing a flexible matrix to accommodate the metabolic activity of the liver. After liver injury from various factors, such as metabolic insults like in NAFLD, viral hepatitis, or toxins, there comes a time when the regenerative capacity of the liver gets surpassed, resulting in scar formation in place of functioning parenchymal tissue. Hepatic stellate cells reside within the space of Disse and primarily serve as the principal storage site for vitamin A in the liver.Following the activation by different stimulants, such as reactive oxygen species, inflammatory cytokines, and adipocytokines like leptin and angiotensin II, these cells dramatically change their phenotype. The cells lose their vitamin A stores, gain a myofibroblast-like morphology characterized by alpha-smooth muscle actin expression, and become the main producers of collagen in damaged liver tissue. COL3A1 gene expression is regulated mainly at the transcriptional level.

Bataller & Brenner explain that activated HSCs exhibit higher stability of collagen mRNA, thus enabling continuous synthesis of collagen despite the lack of ongoing stimulation of its transcription. The process is regulated by specific sequences in collagen mRNA's 3'UTR and may be considered as a potential pharmacologic target.The transformation of simple steatosis into NASH in NAFLD patients reflects a change from a purely metabolic to an inflammatory and fibrogenic disease[16].

2.3.2.COL3A1 in dyslipidemia

Type III collagen (COL3A1) has been identified as one of the significant components in the pathogenesis of vascular dysfunction, adipose tissue dysfunction, and progression of atherosclerotic cardiovascular disease and dyslipidemia. Dyslipidemia is linked to upregulation of COL3A1 due to its association with various factors that contribute to its increased expression. These factors include inflammation, oxidative stress, and extracellular matrix remodeling. LDL and oxidized LDL lead to endothelial dysfunction and trigger inflammation signaling pathways, which include the TGFB-1 pathway. This Signaling pathway leads to activation of fibroblasts and vascular smooth muscles. This further leads to

enhanced production of the extracellular matrix protein type III collagen coded for by COL3A1. In essence, the increased expression of COL3A1 signifies vascular fibrosis and ECM remodeling during the pathogenesis of atherosclerosis and metabolic conditions characterized by dyslipidemia. Another way in which COL3A1 upregulation occurs involves phenotypic transformation of vascular smooth muscles into a synthetic phenotype, leading to excessive deposition of collagen in the atherosclerotic lesions, thereby increasing arterial stiffness. Consequently, it should be noted that COL3A1 acts as a biomarker in dyslipidemia.

2.4. Dysregulated expression of miRNA

2.4.1. miR-335-5p – The Metabolic Disruptor

miR-335-5p as one of the pathophysiological factors which remain upregulated in three metabolic diseases: T2DM, NAFLD, and dyslipidemia. The pathological effect extends to impairment in insulin-secreting β cells, hepatic lipogenesis, and cholesterol regulation in the body.It involves in the disruption of both pancreatic insulin secretion and peripheral insulin sensitivity[17].

Insulin resistance induction: Besides β-cell dysfunction, miR-335-5p induces insulin resistance as well. In the case of mice that had gestational diabetes mellitus, miR-335-5p overexpression induced insulin resistance by decreasing VASH1 (Vasohibin-1) expression, leading to activation of TGF-β signaling pathway, it thus caused the blood's fasting glucose levels to rise, high HOMA-IR, and decreased glucose infusion rate in hyperglycemic clamp studies. Furthermore, additional mechanisms indicate that miR-335-5p contributes to the progression of T2DM by repressing the gene expression of SLC2A4 (GLUT4) to reduce glucose uptake[18]. In the diabetic prone New Zealand Obese (NZO) mice, the adipose tissue evaluated several weeks prior to the development of T2DM exhibits a substantial decrease in miR-335-5p levels compared to diabetic resistant mice; both clinical and experimental data indicate that the initial repression of this miRNA could be considered a protective response[19]. Furthermore, a 2024 study confirmed that miR-335-5p expression, along with BMI, triglycerides, fasting blood glucose, and HOMA-IR, represents an independent risk factor for metabolic syndrome occurrence in obese populations[20] .

In NAFLD, miR-335-5p is an important regulator of adipogenic and hepatic steatosis development. Experimental studies conducted on mice with obesity phenotypes shows that miR-335 expression is significantly higher in the liver and white fat tissue of these models, and there is a direct association between miR-335 overexpression, an increase in the weight of liver and adipose tissue, and an increase in hepatic triglycerides [21].

Regulation of adipocyte differentiation and lipids accumulation: In experimental studies carried out with the use of 3T3-L1 cells, it is shown that miR-335-5p is a key factor of adipogenic switch. At day 3 of differentiation, miR-335 overexpression initiates the process of early triacylglycerol accumulation, marked with the induction of fatty acids synthesis genes (SCD2, ENPP2) and repression of lipid metabolism related factors (FABP5, PNPLA2, FASN). Later, at day 6 of differentiation, miR-335 overexpression leads to activation of the expression of SCD1, SCD2, GPD1, FABP4, and glycolysis related genes (ALDOA, PGK1), with the notable induction of GLUT4 (SLC2A4) Notably, treatment with miR-335-5p mimics causes an

increase in triglyceride deposition as early as day 3 in 3T3-L1 cells, establishing its involvement in the promotion of fat deposition.

In dyslipidemia, miR-335-5p plays role in the regulating cholesterol levels in body through its modulation of multiple genes involved in the synthesis and clearance of cholesterol.

Cholesterol metabolism gene targets: Through high-throughput RNA-seq analysis in a model of atherosclerosis using $ApoE^{-/-}$ mice, Studies have demonstrated a significant upregulation of miR-335-5p expression in hepatic tissue under high-fat diet conditions. Notably, both miR-335-5p and its complementary strand, miR-335-3p, have been implicated in the regulation of multiple genes associated with cholesterol metabolism, including at least three key metabolic regulators: HMGCR is a enzyme for cholesterol biosynthesis,CYP7A1 enzyme for cholesterol catabolism to bile acid, PCSK9 (a protein promoting LDLR degradation)

LDL clearance is regulated by modulating PCSK9 expression, miR-335-5p can influence LDL receptor-mediated cholesterol uptake and processing. Normal conditions see LDLR clearing LDL from the blood. However, binding of PCSK9 to LDL receptors leads to their lysosomal degradation and hence impairs LDL clearance. Hence, the increased expression of miR-335-5p in cases of dyslipidemia may lead to hypercholesterolemia through the regulation of the PCSK9-LDLR axis [22].

2.4.2. miR-26b-5p- The Metabolic Protector

miR-26b-5p has been consistently recognized as a protective molecule in the metabolic organs. The described data allow classifying miR-26b-5p as an all-round metabolic guardian working via multiple integrated pathways: (1) improved insulin sensitivity through PTEN inhibition and PI3K/AKT signaling; (2) restricted progression of hepatic fibrosis owing to TGFβ1/SMAD2 inhibition; (3) lowered plasma concentration of LDL-cholesterol as well as decreased synthesis of fatty acids because of PCSK9, FASN, and SREBP1c targeting; and (4) protection of β-cells. The decrease in expression of miR-26b in obesity, T2DM, NAFLD, and dyslipidemia indicates a general feature of metabolic diseases, and its up-regulation can provide a therapy for such diseases[23].

Anti-Fibrotic Action

The miR-26b is an effective anti-fibrotic molecule due to the downregulation of several profibrotic factors. Through targeting the TGFβ1 and SMAD2 genes, which act as downstream targets of TGF-β signaling, miR-26b suppresses the activation of hepatic stellate cells as well as the deposition of ECM. The anti-fibrotic effect of the miR-26b molecules is especially important in the case of NAFLD since progressive hepatic fibrosis is one of the crucial factors defining the prognosis of the disease. The miR-26b-TGFβ/SMAD pathway is one of the most common in fibrosis studies; however, the hepatic aspects of the pathway were investigated for the close relative – miR-26a.

Lipid Metabolism Regulator: PCSK9, FASN, and SREBP1c Silencing

Cholesterol level regulation in LDL by miR-26b is a direct regulator of PCSK9. The silencing of PCSK9 by the miR-26b leads to increased stabilization of LDL receptors (LDLRs) at the cell membrane. In normal circumstances, PCSK9 interacts with LDLR and promotes its proteolysis within the lysosome. However, inhibition of PCSK9 by miR-26b promotes

upregulation of the LDLR and thereby lowers circulating LDL-cholesterol concentration.

Hepatic fat metabolism regulation by miR-26b regulates fat metabolism through suppressing de novo lipogenesis by directly silencing the master regulators of fat metabolism such as FASN (fatty acid synthase) and SREBP1c (sterol regulatory element-binding protein 1c). While FASN is the enzyme responsible for the formation of fatty acids, SREBP1c acts as a transcription factor responsible for lipid biosynthetic processes.

2.5. Transcriptional Regulation of ICAM1 and COL3A1 as a Node for miRNA-Mediated Targeting

Transcriptional regulation plays a critical role in controlling intercellular adhesion molecule-1 (ICAM1) expression. Activation of ICAM1 transcription is mediated through the binding of the NF-κB subunit p65 (RelA) to enhancer and promoter elements within the ICAM1 gene, thereby promoting mRNA synthesis [24]. Consequently, any factor that reduces the availability or activity of p65/RelA would be predicted to suppress ICAM1 expression, even without directly binding to the ICAM1 3′ untranslated region (UTR).

A similar regulatory logic governs collagen type III alpha 1 chain (COL3A1) expression.The transcription factor Specificity Protein 1 (SP1) binding motifs are found in the COL3A1 promoter and transcriptional activation of COL3A1 is dependent on SP1 occupancy at these sites [25]. Functional studies have further demonstrated that targeting SP1 effectively blocks COL3A1 expression [26].

Based on these transcriptional dependencies, we hypothesized that microRNAs (miRNAs) could be harnessed to indirectly downregulate ICAM1 and COL3A1 by directly targeting their requisite transcription factors. This strategy bypasses the need for direct miRNA–mRNA interactions with the ICAM1 or COL3A1 transcripts. Two miRNAs are particularly well-suited for this purpose:

1. **miR-26b** has been reported to directly target the 3′UTR of RELA (encoding p65), leading to reduced p65 protein levels. By lowering p65 abundance, miR-26b diminishes the availability of this transcription factor for binding to the ICAM1 enhancer and promoter, thereby suppressing ICAM1 transcription.

2. **miR-335** targets the SP1 transcript, reducing SP1 protein expression. Given the dependence of COL3A1 transcription on promoter-bound SP1, miR-335-mediated knockdown of SP1 results in reduced COL3A1 mRNA expression.

Thus, these miRNAs provide an indirect yet potent mechanism to modulate the expression of ICAM1 and COL3A1 by acting at the level of transcription factor regulation, rather than through direct post-transcriptional silencing of the target genes themselves.

2.6. Therapeutic strategies

The conventional pharmacological therapy which works by targeting individual targets has proven to be ineffective in treating diseases because of the interrelatedness of these pathways. The introduction of RNA interference therapy provides a whole new approach to addressing such pathways using networks. This literature review presents evidence on a new form of

RNAi treatment involving hub genes ICAM1 and COL3A1, deregulated miRNAs miR-335-5p (upregulation) and miR-26b-5p (downregulation), as well as transcription factors NFκB, RELA, and SP1 in metabolic diseases. RNA interference (RNAi) has been identified by small non-coding RNAs as a critical regulatory mechanism for post-transcriptional gene silencing, which has important therapeutic implications. It disrupts gene expression in a number of ways, such as via recruiting de-adenylation/de-capping enzymes or by endonucleolytically cleaving target mRNA.The mediators of RNA interference (RNAi), which may potentially silence any gene (disease-associated) in a sequence-specific way, are non-coding RNAs such as miRNA, circRNA, lncRNA, siRNA. This makes them a prospective therapeutic approach. The RISC (RNA induced silencing complex), which binds to complementary sequences in target mRNAs, causes mRNA instability, degradation, and ultimately translational suppression. Despite their modest distinctions, they play diverse roles in pharmacological practice.

Some of the potential advantages RNAi therapeutics have over traditional small molecule drugs include: High level of sequence specificity for modulating specific targets, Ability to modulate "undruggable" protein and noncoding RNA targets, Quick development cycles once target sequences are identified, Longevity of effect (weeks/months after single administration),Combinatorial oligonucleotide delivery.

Our bioinformatic and experimental data identified a core regulatory network involving **ICAM1** and **COL3A1** as hub genes, dysregulated **miR-335-5p (up)** and **miR-26b-5p (down)**, and key transcription factors **NFKB**, **RELA**, and **SP1**. These elements form a positive feedback loop amplifying metabolic inflammation and fibrosis. Therefore, a rational RNA interference (RNAi) strategy should aim to (1) silence pathogenic hub genes, (2) restore protective miRNA levels, and (3) indirectly modulate transcription factor activity using **antagomirs** and **miRNA mimics**.

AntagomiR-335-5p shows Sequence-specific binding and sequestration of mature miR-335-5p, preventing its interaction with target mRNAs (e.g., IRS1, SIRT1). Mir-26 mimics restores miR-26b-5p levels, enhancing translation of its metabolic targets (e.g., PTEN, GPAM), improving insulin sensitivity and reducing lipogenesis.

NFKB/RELA are central inflammatory drivers. Both miR-335-5p upregulation and ICAM1 overexpression are NFKB-dependent. AntagomiR-335-5p breaks this feedback loop, reducing p65 nuclear translocation and subsequent pro-inflammatory cytokine transcription.

SP1 drives COL3A1 expression. Our strategy uses indirect modulation by reducing inflammation (via antagomiR-335-5p + miR-26b-5p), SP1 acetylation and transcriptional activity decrease. If needed, a direct siRNA against *SP1* can be added, but this increases off-target risk.

Strong preclinical evidence supports ICAM-1 as a viable therapeutic target for hepatic inflammation—a key driver of T2DM, NAFLD, and dyslipidemia. In a comprehensive study using multiple murine models of acute hepatitis (LPS/D-galactosamine-induced, carbon tetrachloride-induced, dimethylnitrosamine-induced, and ischemia-reperfusion injury), researchers delivered ICAM-1 siRNA using mannose-modified bubble liposomes combined with ultrasound exposure[27]. The key findings were:

Table 2.6.2.key results obtained after delivering ICAM-1 siRNA using mannose-modified

bubble liposomes

ICAM-1 mRNA/protein	Significantly suppressed in hepatic endothelial cells
Serum ALT/AST	Markedly reduced across all models
Neutrophil infiltration	Substantially decreased (asessed by naphthol AS-D chloroacetate staining)

Clinical correlate: **Alicaforsen** [28]-It is a first-generation antisense oligonucleotide investigated as a therapeutic agent for inflammatory bowel disorders, including ulcerative colitis, Crohn's disease and chronic refractory pouchitis. Its mechanism of action involves targeted suppression of intercellular adhesion molecule-1 (ICAM-1) expression, a key mediator of leukocyte recruitment and intestinal inflammatory responses. By hybridizing with ICAM-1 mRNA, alicaforsen inhibits its translation, thereby limiting inflammatory cell infiltration and attenuating tissue inflammation.

By binding to ICAM-1 messenger RNA, alicaforsen reduces inflammatory cell recruitment and suppresses the inflammatory response in intestinal tissues. Clinical studies have shown that topical formulations, particularly rectal enemas, may provide therapeutic benefits in patients with distal ulcerative colitis and pouchitis, although results in Crohn's disease have been inconsistent. Due to its targeted mechanism and relatively favorable safety profile, alicaforsen has attracted interest as a potential biological therapy for inflammatory bowel diseases.

Mithramycin A (SP1 inhibitor)-shows 65% reduction in COL3A1 expression

Curcumin (indirect SP1 inhibitor)- shows 50% reduction in expression of COL3A1 in high-fat diet fed mice.

2.7. Approved siRNA Therapeutics: Clinical Validation of RNAi in Human Disease

Approved small interfering RNA (siRNA) therapeutics represent a major advancement in RNA-based medicine by enabling selective gene silencing through RNA interference (RNAi). These therapies function by binding to complementary mRNA molecules, leading to their degradation and preventing the production of disease-causing proteins. The first approved siRNA drug, Patisiran. Since then, several additional siRNA therapeutics have been approved, including Givosiran, Lumasiran, Inclisiran, and Vutrisiran. Most approved siRNA drugs utilize lipid nanoparticles or N-acetylgalactosamine (GalNAc) conjugates to achieve targeted delivery to hepatocytes in the liver. These therapeutics have demonstrated high specificity, prolonged duration of action, and the potential for infrequent dosing, making them valuable for treating genetic, metabolic, and cardiovascular diseases. Their success has established RNA interference as an important platform for precision medicine and has

accelerated the development of next-generation nucleic acid therapeutics.

Patisiran represents the first clinically approved small interfering RNA (siRNA)-based therapeutic and is indicated for the management of hereditary transthyretin amyloidosis associated with polyneuropathy. The drug utilizes lipid nanoparticle-mediated delivery to selectively transport siRNA into hepatocytes, where it silences transthyretin (TTR) gene expression, thereby decreasing TTR protein synthesis and limiting amyloid accumulation. Clinical studies demonstrated significant improvement in neurological impairment and quality of life in affected patients.

Givosiran is an siRNA-based therapy approved for acute hepatic porphyria, a metabolic disorder characterized by toxic accumulation of heme synthesis intermediates. The drug targets ALAS1 messenger RNA in the liver, thereby decreasing the production of neurotoxic metabolites responsible for acute attacks. Its use has been associated with a marked reduction in the frequency of porphyria episodes and improved disease management.

Lumasiran is an RNA interference–based therapeutic developed for the management of primary hyperoxaluria type 1, a rare inherited metabolic disorder characterized by excessive oxalate accumulation and progressive renal impairment. The drug functions through targeted silencing of the hydroxyacid oxidase 1 (HAO1) gene, which encodes the hepatic enzyme glycolate oxidase, thereby reducing endogenous oxalate production in the liver. Clinical investigations have demonstrated marked decreases in urinary oxalate excretion along with improvements in kidney-related clinical parameters.

Inclisiran is a small interfering RNA (siRNA) therapy designed to lower circulating low-density lipoprotein cholesterol (LDL-C) in individuals with hypercholesterolemia and elevated cardiovascular risk. Its mechanism involves selective inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) synthesis within hepatocytes, leading to enhanced hepatic clearance of LDL cholesterol from circulation. Owing to its prolonged pharmacological activity, inclisiran requires maintenance dosing only twice annually following the initial treatment regimen.

Vutrisiran is a next-generation siRNA therapeutic approved for hereditary transthyretin-mediated amyloidosis with polyneuropathy. Similar to patisiran, it suppresses hepatic production of transthyretin protein; however, it utilizes GalNAc conjugate technology for targeted liver delivery and subcutaneous administration. The therapy demonstrated sustained TTR reduction, improved neurological function, and enhanced patient convenience due to less frequent dosing.

CHAPTER 3
MATERIALS AND METHODOLOGY

3.1 Software and Tools Used:

Comparative Toxicogenomics Database (CTD) (https://ctdbase.org/):- It is a powerful, freely accessible scientific database for manually establishing connections among environmental agents, genes, and diseases in order to elucidate molecular pathways for the impact of environmental factors on health and disease.

Network analyst(https://www.networkanalyst.ca/) :-NetworkAnalyst is a user-friendly, online analytical tool that focuses on gene expression and proteomic profiling. This system creates interaction and regulatory networks and coexpression networks using proteins without necessitating robust computation on your computer.

Google colab (https://colab.research.google.com/)**: -** used to find out common genes among the metabolic diseases.

Microsoft excel : - This was used to sort and filter significant dysregulated miRNAs according to their degree values.

3.2 Data Acquistion

In order to build a strong foundation that would be used later for further multi-omics studies, gene sets associated with the disease of interest were gathered from the CTD, which provides manually curated interactions between chemicals and human genes/diseases [29]. The first goal was to find reliable data about three diseases that had an important biochemical connection.

Since CTD can provide many results that may not be experimentally confirmed and include various types of associations, it was necessary to filter these data in order to keep only genes for which biological relevance had been confirmed via direct or indirect experiments. Thus, all genes were selected that either participated in known signaling pathways, interacted with other proteins or had phenotype-modulating effects. On average, 500 genes were kept for each disease to optimize computations and avoid redundant data collection. Such a filtering procedure aimed at achieving two goals: (i) avoiding redundancy resulting from paralogues, and (ii) keeping only genes that have been confirmed to play an important role in the

pathology of the disease.

3.3Identification of Common Genes Across Three Metabolic Diseases

Once the data had been collected, the next step involved performing an intersection analysis on the three disease-associated gene lists (T2DM, NAFLD, and dyslipidemia). The process was completed using custom Python code written by the author, which was run using the "Google Colaboratory platform (https://colab.research.google.com/)". It is worth noting that the Google Colab platform is a cloud-based Jupyter notebook that allows users to write reproducible code without necessarily needing access to high-performance local computing systems.

As such, finding a common gene list between the three diseases was motivated by the molecular overlap between the conditions. For instance, T2DM, NAFLD, and dyslipidemia often coexist within patients as part of metabolic syndrome, implying the presence of common molecular pathways such as insulin resistance, chronic inflammation. Therefore, the aim of finding common genes was to identify a core transcriptional network behind their pathogenesis. A total of 151 common genes were found from the process, and this gene list was taken to be the starting point for the network analyses.

3.4 Construction of Tissue-Specific Co-Expression Networks

In order to establish the context-specificity of 151 metabolic genes, the analysis of their tissue-specific gene co-expression network was conducted using NetworkAnalyst web server (https://www.networkanalyst.ca/). NetworkAnalyst is a web-based gene/protein interaction network visualization and analysis software with an integrated InetModels gene/protein network resource[30]. This computational framework allows studying interactions between genes/proteins within specific tissues, based on expression profiles from the large-scale compendium of public transcriptional datasets.

Two tissues were chosen for the study: the liver and the pancreas. The rationale behind this choice lies in their crucial role and complementarity in the regulation of systemic metabolism. Specifically, the liver is the main organ of de novo lipogenesis, gluconeogenesis, and lipoprotein metabolism, and is thus associated with the pathogenesis of NAFLD and dyslipidemia. Meanwhile, the pancreas participates in the development of T2DM via its endocrine function (the production of insulin and glucagon). Thus, comparing the co-expression network of metabolic genes in two tissues enabled separating the commonly expressed metabolic genes from those with tissue-specific coexpression.

In case of each tissue, the list of 151 commonly shared genes was mapped on pre-existing tissue specific expression data in NetworkAnalyst tool. Co-expression network analysis was performed individually for the liver and pancreas tissues using Pearson's correlation approach. In these networks, genes acted as nodes and co-expressions among them were represented as edges. This process helped identify the presence of tissue-specific gene modules and interaction hubs.

3.5 Identification of Significant Hub Genes

For each tissue-specific co-expression network, hub genes were identified as the network nodes with high degree connectivity – such genes being the ones that are connected to a disproportionately larger number of genes in the network compared to others. The hub genes

are of significance from a biological perspective as they usually code for proteins that act as signaling bottlenecks, transcriptional regulators, or scaffolds whose alteration could lead to extensive downstream effects on cellular function.

The degree centrality measure for all genes in the networks of both liver and pancreas was computed using in-built network analysis methods. Initially, all genes found within the top percentile in terms of degree distribution were designated as potential hub genes. Nevertheless, to increase functional significance in our research, we adopted the strategy of considering for analysis only the genes that met the criteria for being a hub gene in both networks of liver and pancreas.

3.6 Construction of Gene-miRNA Interaction Network

The above-listed 151 commonly shared genes were analyzed using the miRTarBase database (version 9.0), which is incorporated in NetworkAnalyst. miRTarBase is an experimental interaction database containing miRNA-target gene associations documented in the published literature and experimentally verified using various techniques such as reporter gene assay, western blotting, and CLIP-seq (crosslinking, followed by immunoprecipitation, and sequencing). Only those associations that were experimentally supported, such as luciferase-based reporter gene assays or immunoblotting, were considered to reduce the number of false positives typically seen in algorithmic predictions. The generated network was a bipartite graph consisting of two classes of nodes: the commonly listed genes and miRNAs.

3.7 Identification of Key Regulatory miRNAs

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Based on the generated gene-miRNA network, a ranking based on the degree measure was carried out to select miRNAs having the most target genes connected to them. In the current situation, the degree means the number of unique common genes regulated by a particular miRNA experimentally. The miRNAs with degrees within the top 10% of all miRNA degrees in the network were classified as hub miRNAs. miRNAs with such a degree measure may be regarded as having high regulatory significance, because they have the capacity to regulate several genes at once from the same disease gene set.

3.8 Transcription Factor (TF)-Gene Network

Along with the miRNA regulatory network, we created a TF-gene network to illustrate the transcriptional regulatory mechanism. As before, we used NetworkAnalyst to generate the network; however, this time, we downloaded data from the TRRUST database. TRRUST is a manually curated database that describes transcriptional regulatory relationships between transcription factors and their target genes in terms of activation and inhibition in human and mouse species.

This was done with the same set of 151 overlapping genes in mind. In particular, for every gene, the TRRUST database was searched to find all transcription factors that had been either experimentally validated or manually curated as regulators of that gene. In this way, we have created an oriented TF-target gene network that illustrates a hierarchical structure of transcriptional regulation. Contrary to co-expression networks, TF-gene networks in TRRUST were generated using causal evidence (e.g., ChIP, EMSA, reporter gene assays).

3.9 **Identification of Key Regulatory Transcription Factors**

In order to determine which TFs would have the most influence on the disease gene set, the degree centrality of the TFs within the network was also determined. For this analysis, the out-degree of each TF (i.e., the number of unique targets from the gene set it regulates) was used as a key criterion within the directed network. Hub transcription factors were considered as those whose out-degree was found to lie within the top 10% of the distribution. It is proposed that hub transcription factors play the role of master regulators, regulating several genes involved.

CHAPTER 4

RESULTS

4.1 . Tissue-Specific Gene Co-Expression Network And Identification Of Hub Genes

Tissue-Specific Gene Co-Expression Network were constructed for all of the three diseases under consideration using iNET model as shown in fig 1and 2 (Nodes represents genes, and edges represents significant co-expression of the genes (specific to liver). Node size/color represents degree or connectivity weight), and hub genes were identified for each.

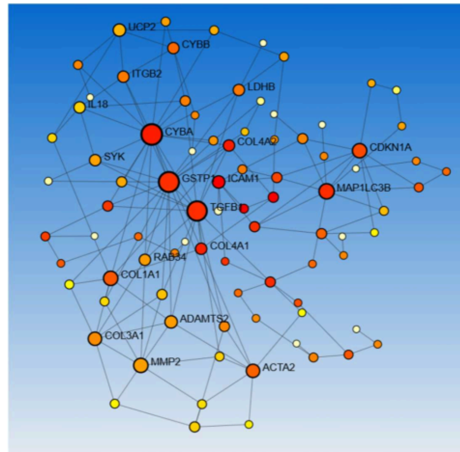

Figure 4.1.1: Tissue Specific Co-Expression Network For Liver Constructed Using Network Analyst iNET Model

Upon analysing the co-expression network of genes in the liver, we found that there were certain hubs which were unique to the liver. The major hubs of interest **CYBA, GSTP1, TGFB1, ICAM1, MAP1LC3B, CDKN1A, COL1A1, COL4A1, COL3A1 .** These genes

have been extensively studied and their known involvement in metabolic diseases is stated below

Key hub genes (strongest hubs)

The **CYBA** protein was identified as one of the most significant hub genes as a result of being located centrally in the network with the largest node size. The prominent topology implies the involvement of CYBA in various biological pathways related to immunity, oxidative stress, and extracellular matrix regulation.

GSTP1 is another protein with many connections that is also located near the center of the network. The GSTP1 hub involves detoxification and oxidation resistance pathways along with structural and inflammatory modules which is consistent with the known functions of the protein involved in protecting cells from glutathione.

TGFB1 has a big size in terms of node and serves as a connection between signaling components and components of extracellular matrix such as collagen. The hub topology of TGFB1 indicates the function of this protein as the master of inflammation and fibrosis in the liver.

Secondary hubs

ICAM1 shows multiple connections with other network nodes in all types of modules including structural and immune pathways.

MAP1LC3B serves as a central node in the autophagy/cell stress subnetwork. This node mediates cell responses related to mechanisms of cell survival and autophagy flux, thus playing an important role in integrating stress responses in hepatocytes.

CDKN1A acts as a hub in the cell cycle and stress response subnetwork. The hub shows relatively less interconnectedness with respect to other hubs such as CYBA and TGFB1 but still demonstrates high interconnectivity within that specific network module.

Structural/ECM-related hubs

COL4A1 has many interconnections between ECM structure genes and signaling hubs. Thus, COL4A1 functions as a structural hub, possibly mediating matrix-cell contacts in liver pathology through maintaining basement membranes.

COL1A1 acts as a hub within collagen/ECM subnetwork. Here, COL1A1 connects collagen matrix with the regulatory genes, such as TGFB1, which are important for matrix production in fibrotic pathways.

COL3A1 encodes type III collagen, a major extracellular matrix component expressed in the liver, adipose tissue, vascular wall, skeletal muscle. COL3A1-driven extracellular matrix remodeling contributes to tissue fibrosis, which exacerbates insulin resistance and impairs pancreatic β-cell function.

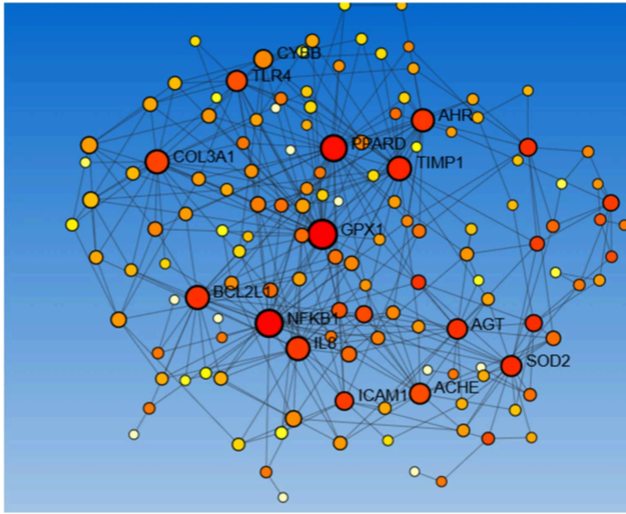

Figure 4.1.2: Tissue Specific Co-Expression Network For Pancreas Constructed Using Network Analyst iNET Model

Upon analysing the co-expression network of genes in the pancreas , we found that there were certain hubs which were unique to the pancreas compared to those identified in the liver due to their pathophysiological significance. The major hubs of interest are **NFKB1, GPX1, ICAM1, COL3A1, AGT, SOD2, TIMP1, IL6, and AHR.** These genes have been extensively studied and their known involvement in metabolic diseases is stated below.

NFKB1 – A master regulator transcription factor, which regulates gene expression for inflammatory mediators. The activation of NFKB1 in pancreatic tissue is critical in mediating inflammation, insulin resistance and development of T2DM. Prolonged NF-κB 22ignalling causes beta-cell dysfunction and apoptosis.

GPX1 (Glutathione Peroxidase 1) – An enzyme that catalyzes reactions of hydrogen peroxide and lipid hydroperoxides involved in detoxification processes in beta-cells. Decreased levels of GPX1 can cause impaired insulin production and an increased risk of diabetes while dysregulation may indicate metabolic stress.

ICAM1 (Intercellular Adhesion Molecule 1) – This molecule is responsible for leukocytes' ability to attach to other cells. Increased expression of ICAM1 is associated with insulinitis and is involved in beta-cell damage by immune cells.

COL3A1 (collagen type III alpha 1 chain) – A constituent of the extracellular matrix. In metabolic diseases, the accumulation of COL3A1 in the process of developing pancreatic fibrosis is caused by prolonged periods of hyperglycemia and hyperlipidemia, which lead to the development of exocrine and endocrine dysfunction. COL3A1 is a sign of activated pancreatic stellate cells.

AGT (angiotensinogen) – The precursor of angiotensin II, the link between the renin-angiotensin system (RAS) and metabolism. RAS activation leads to impaired insulin secretion, decreased pancreatic blood flow, and fibrosis development. Excessive expression of AGT is associated with obesity-induced pancreatic dysfunction and T2DM.

SOD2 (superoxide dismutase 2, mitochondrial) – An antioxidant enzyme in mitochondria. The enzyme prevents oxidative stress in pancreatic beta-cells as a consequence of hyperglycemia and excess of fatty acids. Dysfunction of SOD2 is associated with mitochondrial impairment and decreased insulin release.

TIMP1 (tissue inhibitor of metalloproteinases 1) – An agent responsible for regulating the degradation of extracellular matrix. High expression of TIMP1 during metabolic diseases stimulates pancreatic fibrosis through inhibition of the matrix degradation process. In addition, it possesses anti-apoptotic activity towards pancreatic stellate cells. Their levels in the blood serum is associated with the severity of metabolic syndrome.

IL6 (interleukin 6) – A multi-functional pro-inflammatory cytokine. Persistent elevation of IL6 in pancreatic tissues is characteristic of low-grade inflammation caused by obesity. IL6 disrupts insulin 23ignalling in beta-cells, causes infiltration of immune cells, and triggers beta-cell apoptosis in patients with T2DM.

There are two Hub genes which are common in both the liver and pancreatic tissue are **ICAM1** and **COL3A1**.

4.2 Identification Of Dysregulated miRNAs as Potential Shared Biomarkers in NAFLD, T2DM, and Dyslipidemia

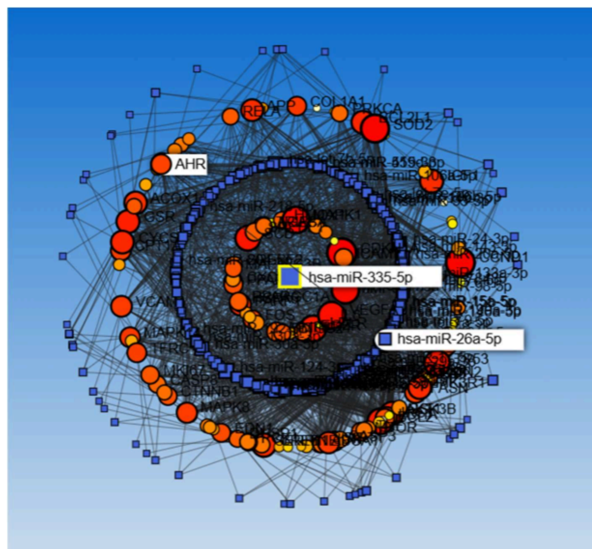

Figure 4.2.1 Integrated gene–miRNA regulatory interaction network

Table 4.2.1 . Node table showing 2 primary miRNAs dysregulated in the diseases with highest degree

ID	LABEL	DEGREE	BETWEENESS
MIMAT0000765	hsa-miR-335-5p	41	5739.4
MIMAT0000083	hsa-miR-26b-5p	34	3867.74
MIMAT0000646	hsa-miR-155-5p	34	3728.67
MIMAT0000070	hsa-miR-17-5p	29	2636.96
MIMAT0000069	hsa-miR-16-5p	28	2728.08
MIMAT0000255	hsa-miR-34a-5p	26	2032.59
MIMAT0000422	hsa-miR-124-3p	25	1902.3
MIMAT0000680	hsa-miR-106b-5p	22	1789.76
MIMAT0000080	hsa-miR-24-3p	22	1638.13

MIMAT0000093	hsa-miR-93-5p	22	1442.1
MIMAT0000416	hsa-miR-1-3p	21	1719.99
MIMAT0000103	hsa-miR-106a-5p	21	1472.45
MIMAT0000092	hsa-miR-92a-3p	21	1448.03

Network analysis identified miR-335-5p, miR-26b-5p as key regulatory microRNAs associated with NAFLD, T2DM, and dyslipidemia, indicating their potential utility as cross-disease biomarkers due to consistent and detectable dysregulation.

Analysis Reveals two shared microRNAs miR-335-5p and miR-26b-5p as Biomarkers for NAFLD, T2DM and Dyslipidemia

Based on the current analysis of networks, two microRNAs (miR-335-5p , miR-26b-5p) were identified as novel and potential common biomarkers for NAFLD, T2DM, and dyslipidemia due to the consistent detection of their dysregulation in these three metabolic diseases.

The dysregulation of hsa-miR-335-5p in the form of its upregulation has been associated with NAFLD [19]. With regard to T2DM, overexpression of this microRNA has been demonstrated to negatively impact insulin secretion [20,21]. In addition, increased expression levels of this microRNA have also been noted in patients suffering from dyslipidemia [22]. Regarding the mechanisms of action, the upregulated microRNA has been shown to cause harm via direct regulation of important hub genes such as intercellular adhesion molecule 1 (ICAM1) [23].

On the contrary, hsa-miR-26b-5p was found to be consistently downregulated under all three conditions, namely, NAFLD [25], T2DM [26], and dyslipidemia [27]. Importantly, The anti-inflammatory impact of hsa-miR-26b-5p is attributed to its involvement in the control of the NF-κB signaling pathway [28]. Furthermore, because miR-26b may directly target COL3A1, it has anti-fibrotic characteristics [29]. Consequently, this microRNA's downregulation may result in the absence of defense mechanisms against the emergence of metabolic diseases. In summary, the network analysis findings show that hsa-miR-335-5p and hsa-miR-26b-5p are common biomarkers for T2DM, dyslipidemia, and NAFLD.

4.3 Identification Of Transcription factors Regulating The Expression Of Hub Genes

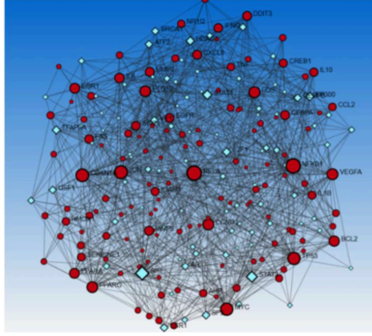

Figure 4.3.1.Transcription factor–gene regulatory interaction network.

Table4.3.1. Node table showing top hub transcription factors based on highest degree.

ID	LABEL	DEGREE	BETWEENESS
5970	RELA	64	2500.47
4790	NFKB1	63	2330.41
6667	SP1	55	3178.26
3725	JUN	48	2258.06
1026	CDKN1A	45	2112.12
4609	MYC	42	1633.63
7157	TP53	41	1368.46
7422	VEGFA	31	1035.08
2353	FOS	31	100.72
6774	STAT3	31	771.17
595	CCDN1	31	761.45

The transcription factor (TF)-gene interaction network analysis identitifies RELA, NFKB1, and (SP1) function as master transcriptional regulators of the hub genes ICAM1 and COL3A1.

The NF-κB transcription factor family comprises five structurally related proteins, including RelA (p65) and NF-κB1 (p105/p50). Mechanistically, it has been shown that NF-κB

p65/RelA recruitment to the ICAM1 gene's enhancer and promoter regions directly increases ICAM1 mRNA expression. [30].

Regarding COL3A1 regulation, the promoter region of the COL3A1 gene contains specific binding sequences for the transcription factor SP1 [31]. Functional studies have demonstrated that targeting SP1 effectively blocks COL3A1 expression, further confirming its regulatory role [32].

Collectively, these findings establish RELA, NFKB1, and SP1 as key upstream regulators controlling the expression of the hub genes ICAM1 and COL3A1 within the proposed regulatory network.

4.4 Discussion

The major finding of the network analysis is that ICAM1 and COL3A1 have been identified to be hub genes in both liver and pancreatic co-expression networks. These findings are very pathophysiologically important. The identification of ICAM1 as a common hub between the two types of tissue signifies the fact that vascular dysfunction can be seen as one common feature between different types of metabolic disease in organs. On the same lines, COL3A1 can be termed as a common feature of fibrosis that takes place in the two organs.

Based on the transcription factor-gene regulatory network, it was determined that the transcription factors that were found to be key regulators occupying the highest regulatory hierarchy levels included RELA, NFKB1, and SP1. ICAM1 was taken into consideration. RELA and NFKB1, key constituents of the NF-κB signaling cascade, are major regulators of inflammatory gene transcription. Previous studies have demonstrated that the p65 (RELA) subunit of NF-κB interacts directly with enhancer and promoter regions of the ICAM1 gene, thereby contributing to transcriptional activation and regulation of ICAM1 expression.

The gene-microRNA network, which was constructed exclusively by means of experimentally verified microRNA-target interactions recorded in miRTarBase, showed two microRNAs with maximum degree centrality: miR-335-5p (up-regulation) and the miR-26b-5p (down-regulation). The inverse relationship between gain of one type of microRNA and reduction of another one is a classic feature.

Upregulation of miR-335-5p is in line with the well-characterized functions of this miRNA in blocking insulin release through SNAP25, STXBP1, SYT11, induction of liver lipogenesis, and disruption of cholesterol synthesis using HMGCR, CYP7A1, and PCSK9. In contrast, downregulation of miR-26b-5p strips away all of the inhibition by the miRNA, including PI3K/AKT signaling by inhibiting PTEN (decreasing insulin sensitivity), fibrosis through inhibiting TGFβ1/SMAD2, and dyslipidemia via PCSK9, FASN, and SREBP1c. Notably, the literature analysis confirmed that miR-26b inhibits RELA (p65) via targeting the 3'UTR sequence of the transcription factor,

This research makes a significant contribution to network medicine, an emerging discipline, by showing that even complicated metabolic diseases affecting multiple organs can be simplified to core regulatory pathways. Identification of common hubs among different diseases and tissues indicates that one and the same therapy is able to improve the inflammatory and fibrotic pathology of both the liver and pancreas. Overall, the application of a network correction approach, involving treatment with miRNA mimics and antagomiRs to achieve homeostasis in regulatory networks, presents a novel paradigm in comparison with targeted drug development. Together, patisiran, givosiran, lumasiran, and vutrisiran are examples showing that chemically altered siRNAs are capable of producing prolonged silencing effect with satisfactory safety outcomes in different disease indications.

In case of antagomiRs, miravirsen (an antagomiR-122 against HCV) and MRG-201 (a miR-29 mimic targeting fibrosis) have shown proof of concept regarding safety issues for both types of therapeutic approaches for miRNAs. Neither drug has received approval for the treatment of metabolic diseases but they show that the technological advancement of miRNA technology is sufficient and the safety issues have been successfully managed.

The combination therapy of an antagomiR-335-5p and miR-26b-5p is a network-based, not a single-target, therapy since both miRNAs should be simultaneously inhibited and upregulated in order to restore the proper function and activity level of the network. This strategy corresponds to modern concepts of system pharmacology when the effectiveness of drug action depends on changes within the network dynamics rather than individual molecules.

In addition to therapeutic potentials, the results hold promise for biomarker discovery. Elevated serum concentration of soluble ICAM1 indicates endothelial cell activation and is indicative of diabetic comorbidity as well as NAFLD severity based on several observational cohort analyses. Likewise, the N-terminal propeptide of type III collagen, generated through COL3A1 processing, has been found as an indicator of active fibrosis. This current study implies that measurement of both markers simultaneously could complement one another in evaluating different aspects of inflammatory and fibrotic diseases.

miR-335-5p and miR-26b-5p can be used as circulating biomarkers themselves since both are measurable in plasma and exosomes, along with other bodily fluids. The inverse relationship between their expression can be useful in identifying patients with metabolic syndrome from others. Yet, the issue of the tissue origin of circulating miRNAs needs to be clarified in further studies.

Whereas other works have examined individual disease states or particular biochemical pathways, in the present study there is explicit mention of overlapping regulatory points in the three metabolism-related diseases studied. Whereas one could expect, for example, miR-335-5p to be associated with NAFLD and miR-26b-5p to have been implicated in insulin resistance, it is shown here that miRNAs act in concert in a common transcriptional network, namely RELA/NFKB1 involved in regulation of ICAM1 and SP1 involved in regulation of COL3A1. In addition, the co-expression analysis at the tissue level allows the distinction between liver- and pancreas-specific hubs.

CHAPTER 5
CONCLUSION

This comprehensive research extensively explored the intricate gene-miRNA regulatory processes underlying metabolic diseases driven by insulin resistance. The investigation systematically uncovered a novel regulatory network between ICAM1, COL3A1, miR-335-5p, miR-26b-5p, RELA/NFKB1, and SP1 connecting the liver and pancreas in metabolic diseases has been uncovered using an integrative network-based approach, thus laying down the biological foundation for therapeutic interventions in the future.

From the results obtained through this research, there are a number of directions that need to be addressed in the future. Experimental validation of the results is required through qPCR, Western Blotting, and ChIP-qPCR to demonstrate the expression of ICAM1 and COL3A1 and their transcription factors. In vitro studies will include transfection of HepG2 and EndoC-βH1 cells with antagomiR-335-5p and miR-26b-5p mimics to assess expression changes in ICAM1 and COL3A1, NF-κB activity, and collagen deposition. In vivo studies will entail feeding mice with high-fat diets and use of db/db and ApoE^{-}/$^{-}$ mice models and GalNAc conjugated miRNAs therapeutics to observe metabolic parameters, histological observations, and insulin resistance. Studies of biomarkers should involve determination of serum levels of sICAM1, PRO-C3, and miR-335-5p/miR-26b-5p in patients' cohorts. Optimization of therapies should include studies into different modifications of the miRNAs, delivery systems, administration, and safety profiles. Finally, further studies into the network will entail using additional tissues like fat and muscle and multi-omics including metabolomics and proteomics together with single-cell approaches.

Ritika MSc Thesis

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