MTECH BIOINFO THESSIS

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ABSTRACT

Gallstone disease (cholelithiasis) is one of the most common hepatobiliary metabolic disorders, which are characterized by the presence of solid deposits within the biliary tract or gallbladder, caused by a disruption in the cholesterol metabolism, bile acid homeostasis, gallbladder motility and inflammatory regulation. The disease is closely linked with obesity, dyslipidemia and insulin resistance, metabolic syndrome and disturbed gut microbiota, all of which result in cholesterol supersaturation and lithogenic bile formation. While treatment with ursodeoxycholic acid and cholecystectomy can offer symptomatic relief, the success of these treatments is short-term and/or can be complicated by surgery or the potential for recurrence of symptoms.

The major objective in the current study was to discover and prioritize the possible candidates for repurposing for gallstone treatment through an integrated metabolomics guided computational process. To close the gap in the current therapeutic prioritization scenario, the research adopted a metabolomics, pathway enrichment, network pharmacology, molecular docking, molecular dynamics simulation, and pharmacokinetic validation strategy was used. The data sets of serum metabolomics for both patients with gallstones and healthy volunteers were downloaded from the Metabolomics Workbench database (ST004186), followed by pre-processing, normalization, differential metabolite analysis and pathway enrichment analysis. Metabolites that were found to be significant were linked to major genes in their related pathways, including those involved in bile acid metabolism, cholesterol transport, fatty acid metabolism, and inflammatory signaling.

A network-based gene-drug interaction analysis was then conducted to select some compounds that may be therapeutically relevant for targeting some key gallstone-associated genes, such as NR1H4 (FXR), ABCB11, CYP7A1, PPARA, PPARG, GPBAR1 and SLC10A2. Drugs that were selected for further investigation were given a highest priority when they were both biologically relevant, pathway associated, network topologically related and had thermodynamic potential. The molecular docking and molecular dynamics simulation were performed for further validation of the protein–ligand binding affinity, stability and dynamic behavior of selected drug–target complexes. Drug-likeness and the therapeutic feasibility were also evaluated by ADME and pharmacokinetic analyses.

Bile acid (BA) biosynthesis, lipid metabolism, fatty acid degradation, inflammatory signaling and hepatobiliary transport pathways were found to be significantly dysregulated. Favorable interaction profile between a number of candidate compounds with the main therapeutic targets related to gallstone disease was observed. Pathway oriented metabolic modulators and bile acid regulatory drugs showed strong binding affinity, stable molecular interactions, and promising pharmacokinetic properties and could potentially have relevance for the management of gallstones. Further, the interconnected systems-level analysis

presented the strong point of interacting metabolic and inflammatory pathways, as opposed to targeting a single molecular target.

The results of this work show the value of metabolomics, systems biology, and computational drug repurposing as tools to discover new therapeutic targets in complex metabolic diseases. The results can help guide precision medicine approaches to gallstone disease and inform the treatment strategies for biologically responsive therapies. The computational predictions obtained in the present study however, should be further tested in vitro, in vivo and in the clinic. Future studies could involve combining multi-omics datasets, machine learning-based therapeutic prediction models, personalized metabolic profiling, and comprehensive molecular simulations for heightened therapeutic precision and clinical relevance in hepatobiliary disorders.

LIST OF PUBLICATION

IEEE Global Symposium on Emerging and Communication Technologies (GSEACT 2026)

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To: Yasha Hasija <yashahasija@dtu.ac.in>

Dear Yasha Hasija,

Delhi Technological University
Paper ID: 1486
Paper Title: Integrating Metabolomics and Network Biology to Prioritize Repurposable Drug for Gallstone Disease
Conference Record Number: 68539

We are delighted to inform you that your paper has been accepted for presentation at the 1st International Conference on Global Symposium on Emerging and Communication Technologies (GSEACT 2026).

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TABLE OF CONTENTS

TITLE i

ACKNOWLEDGEMENT ii

CANDIDATE'S DECLARATION iii

CERTIFICATE iv

ABSTRACT v

LIST OF PUBLICATIONS vi

TABLE OF CONTENTS vii

LIST OF FIGURES viii

LIST OF TABLES ix

LIST OF ABBREVIATIONS x

CHAPTER 1 – INTRODUCTION 1

1.1 GALLSTONE DISEASE OVERVIEW 1

1.2 BIOLOGICAL BASIS OF GALLSTONE FORMATION 3

1.2.1 CHOLESTEROL AND BILE ACID DYSREGULATION 3

1.2.2 GALLBLADDER DYSFUNCTION AND METABOLIC FACTORS 4

1.2.3 GUT MICROBIOTA AND ENTEROHEPATIC INTERACTION 5

1.3 CURRENT DIAGNOSTIC AND THERAPEUTIC APPROACHES 7

1.3.1 DIAGNOSTIC APPROACHES 7

1.3.2 THERAPEUTIC STRATEGIES 8

1.4 COMPUTATIONAL DRUG REPURPOSING 10

1.5 RESEARCH GAP AND OBJECTIVES 11

1.5.1 RESEARCH GAP 11

1.5.2 AIM AND OBJECTIVES 12
CHAPTER 2 – LITERATURE REVIEW 13
2.1 MOLECULAR MECHANISMS AND THERAPEUTIC TARGETS 13
2.1.1 CHOLESTEROL METABOLISM 13
2.1.2 BILE ACID SIGNALING AND FXR/TGR5 14
2.1.3 GENETIC AND METABOLIC REGULATION 15
2.2 MULTI-OMICS INSIGHTS IN GALLSTONE DISEASE 17
2.2.1 GENOMICS AND TRANSCRIPTOMICS 17
2.2.2 METABOLOMICS AND PROTEOMICS 18
2.2.3 GUT MICROBIOTA AND METABOLIC INTERACTION 19
2.3 CURRENT AND EMERGING THERAPEUTIC STRATEGIES 20
2.3.1 URSODEOXYCHOLIC ACID (UDCA) THERAPY 20
2.3.2 STATINS AND LIPID-LOWERING STRATEGIES 21
2.3.3 FXR AGONISTS AND BILE ACID MODULATORS 22
2.3.4 SURGICAL AND MINIMALLY INVASIVE APPROACHES 23
2.3.5 LIMITATIONS OF CURRENT THERAPIES 24
2.4 COMPUTATIONAL THERAPEUTIC PRIORITIZATION APPROACHES 26
2.4.1 NETWORK PHARMACOLOGY 26
2.4.2 MOLECULAR DOCKING AND VIRTUAL SCREENING 27
2.4.3 MOLECULAR DYNAMICS AND ADME PREDICTION 28
2.4.4 AI BASED DRUG REPURPOSING – EMERGING TECHNOLOGY 29
2.5 RESEARCH GAPS IN EXISTING COMPUTATIONAL STUDIES 30
CHAPTER 3 – METHODOLOGY 31
3.1 STUDY DESIGN OVERVIEW 31

3.2 DATASET ACQUISITION 32
3.3 DATA PREPROCESSING 32
3.4 DIFFERENTIAL METABOLITE ANALYSIS 33
3.5 PATHWAY ENRICHMENT ANALYSIS 33
3.6 METABOLITE–GENE MAPPING 34
3.7 GENE–DRUG INTERACTION MAPPING 34
3.8 INTEGRATED NETWORK CONSTRUCTION 35
3.9 NETWORK TOPOLOGY ANALYSIS AND DRUG PRIORITIZATION 35
CHAPTER 4 – RESULTS 37
4.1 METABOLOMICS PREPROCESSING 37
4.2 DIFFERENTIAL METABOLITE ANALYSIS 37
4.3 PATHWAY ENRICHMENT ANALYSIS 39
4.4 METABOLITE–GENE MAPPING RESULTS 41
4.5 GENE–DRUG MAPPING RESULTS 42
4.6 INTEGRATED NETWORK ANALYSIS 43
4.6.1 SUBNETWORK ANALYSIS OF ATORVASTATIN CALCIUM TRIHYDRATE 44
4.6.2 SUBNETWORK ANALYSIS OF RIFAMPIN 45
4.6.3 SUBNETWORK ANALYSIS OF ROSUVASTATIN 46
4.6.4 SUBNETWORK ANALYSIS OF FENOFIBRATE MICRONIZED 47
4.6.5 SUBNETWORK ANALYSIS OF ASPIRIN 48
4.6.6 SUBNETWORK ANALYSIS OF CURCUMIN 50
4.6.7 SUBNETWORK ANALYSIS OF SIMVASTATIN 51
4.6.8 SUBNETWORK ANALYSIS OF QUERCETIN 52

4.6.9 SUBNETWORK ANALYSIS OF INDOMETHACIN 53
4.6.10 SUBNETWORK ANALYSIS OF CELECOXIB 54
4.6.11 SUBNETWORK ANALYSIS OF COLCHICINE 55
4.6.12 SUBNETWORK ANALYSIS OF PIOGLITAZONE HYDROCHLORIDE 56
4.6.13 SUBNETWORK ANALYSIS OF ODEVIXIBAT 57
4.6.14 SUBNETWORK ANALYSIS OF HESPERETIN 58
4.6.15 SUBNETWORK ANALYSIS OF APIGENIN 59
4.7 NETWORK TOPOLOGY ANALYSIS AND DRUG PRIORITIZATION 60
CHAPTER 5 – CONCLUSION AND FUTURE SCOPE 65
5.1 CONCLUSION 65
5.2 LIMITATIONS 67
5.3 FUTURE SCOPE 68
REFERENCES 70
APPENDICES 79
PLAGIARISM REPORT 87

LIST OF TABLES

Table No.	Title
Table 1.1	Classification Of Gallstones
Table 1.2	Comparison Of Diagnostic Modalities in Gallstone Disease
Table 1.3	Comparison Of Pharmacological and Surgical Strategies in Gallstone Disease
Table 2.1	Key Molecular Therapeutic Targets in Gallstone Disease
Table 3.1	Dataset Characteristics
Table 4.1	Dataset Preprocessing Summary
Table 4.2	Significant Differential Metabolites List
Table 4.3	Metabolite Gene Mapping
Table 4.4	Gene Drug Mapping
Table 4.5	Prioritized Drug Based on Network Centrality

LIST OF FIGURES

Figure No.	Title
Fig 1.1	Major Types of Gallstones
Fig 1.2	Integrated Mechanism of Cholesterol Metabolism, Dysregulation, And Gallstone Formation
Fig 1.3	Major Metabolic Factors Contributing to Gallstone Formation
Fig 1.4	Gut Microbiota-Bile Acid Axis in Gallstone Disease
Fig 1.5	Current Therapeutic Strategies for Gallstone Disease
Fig 2.1	FXR And TGR5 Signaling Pathways in Bile Acid and Metabolic Regulation
Fig 2.2	Emerging Therapeutic and Computational Strategies in Gallstone Disease
Fig 3.1	Integrated Computational Workflow
Fig 4.1	Volcano Plot of Differential Metabolite Analysis Between Gallstone Disease and Healthy Controls
Fig 4.2	Metabolites Set Enrichment Analysis of Differential Metabolites
Fig 4.3	Subnetwork Analysis of Atorvastatin Calcium Trihydrate
Fig 4.4	Subnetwork Analysis of Rifampin
Fig 4.5	Subnetwork Analysis of Rosuvastatin
Fig 4.6	Subnetwork Analysis of Fenofibrate Micronized
Fig 4.7	Subnetwork Analysis of Aspirin
Fig 4.8	Subnetwork Analysis of Curcumin
Fig 4.9	Subnetwork Analysis of Simvastatin
Fig 4.10	Subnetwork Analysis of Quercetin
Fig 4.11	Subnetwork Analysis of Indomethacin
Fig 4.12	Subnetwork Analysis of Celecoxib
Fig 4.13	Subnetwork Analysis of Colchicine
Fig 4.14	Subnetwork Analysis of Pioglitazone Hydrochloride

Fig 4.15	Subnetwork Analysis of Odevixibat
Fig 4.16	Subnetwork Analysis of Hesperetin
Fig 4.17	Subnetwork Analysis of Apigenin

LIST OF ABBREVIATIONS

CYP7A1 – Cholesterol 7α-Hydroxylase

FXR – Farnesoid X Receptor

TGR5 – Takeda G-Protein Coupled Receptor 5

RCT – Reverse Cholesterol Transport

MRCP – Magnetic Resonance Cholangiopancreatography

CT – Computed Topography

USG - Ultrasonography

UDCA – Ursodeoxycholic Acid

PUFA – Polyunsaturated Fatty Acids

HMGCR - 3-Hydroxy-3-Methylglutaryl-Coa Reductase

NPC1L1 – Niemann-Pick C1-Like 1

HDL – High Density Lipoprotein LDL – Low Density Lipoprotein CA – Cholic Acid

CDCA – Chenodeoxycholic Acid

GWAS – Genome Wide Association Study

TUDCA – Tauroursodeocycholic Acid

LDL-C – Low Density Lipoprotein Cholesterol

OCA – Obeticholic Acid

LCBDE – Laparoscopic Common Bile Duct Exploration

ERCP – Endoscopic Retrograde Cholangiopancreatography

PTCS – Percutaneous Transhepatic Choledochoscopic Lithotripsy PPI – Protein-Protein Interaction

IPA – Integrated Pathway Analysis

CAR – Constitutive Androstane Receptor

MD – Molecular Dynamics

RMSD – Root Mean Square Deviation

RMSF – Root Mean Square Fluctuation

AI – Artificial Intelligence

HMDB – Human Metabolome Database

NVT – Number, Volume, Temperature

NPT – Number, Pressure, Temperature

PME – Particle Mesh Ewald

Rg – Radius of Gyration

SASA – Solvent Accessible Surface Area IBAT/ASBT – Ileal Bile Acid Transporter GLA – Gut-Liver-Axis

CHAPTER 1

INTRODUCTION

1.1 GALLSTONE DISEASE OVERVIEW

Gallstone disease (also called cholelithiasis) is a very common hepatobiliary disorder that results from an imbalance in the composition of bile and leads to the formation of solid deposits inside the gallbladder or biliary tract. Gallstones can consist of cholesterol, bilirubin, calcium salts or a combination of these and range in size from microscopic crystals to large calculi that can obstruct the bile duct and lead to serious complications [1], [2].

Gallstones can be broadly categorized into cholesterol stones, pigment stones and mixed stones. Cholesterol stones are the most common and are closely linked with metabolic changes and changes in the composition of bile. The formation of pigment stones is associated with bilirubin metabolism, haemolytic disorders and biliary infection, liver disease [1], [3]. Major types of gallstones are shown in Fig 1.1 and detailed classification and clinical characteristics are summarized in Table 1.1.

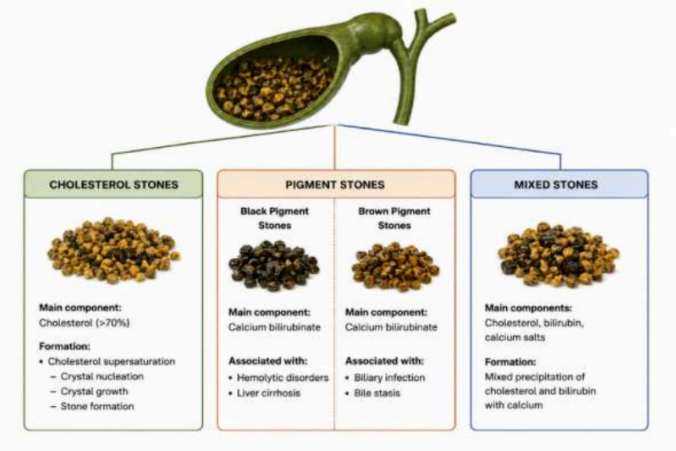


Fig 1.1 Major Types of Gallstones

Table 1.1 Classification of Gallstones

Gallstone Type	Composition	Clinical Features
Cholesterol Stones	Mainly composed of cholesterol crystals in the form of monohydrate crystals with small amounts of calcium salts, bile pigments, mucin and proteins	Most common kind of gallstones; usually yellow to yellow-green, associated with cholesterol supersaturation, obesity, insulin resistance, metabolic syndrome and impaired motility of the gallbladder
Black Pigment Stones	Mainly composed of calcium bilirubinate polymers, mostly with calcium phosphate and carbonate salts	Hard, brittle stones that are dark in colour, and are usually associated with chronic haemolytic disorders, liver cirrhosis and raised levels of unconjugated bilirubin
Brown Pigment Stones	Contain calcium bilirubinate, fatty acid soaps, cholesterol and bacterial components	Softer and brown in colour, usually associated with biliary infection, bile stasis and bacterial β-glucuronidase activity, and are more frequently seen in cases of biliary tract infection
Mixed Stones	Combination of cholesterol, calcium salts, bile pigments, phospholipids and mucin components	Show the features of both cholesterol and pigment stones associated with multifactorial metabolic and biliary abnormalities

Gallstone disease is now well known to be a metabolic disorder that is closely linked to obesity, dyslipidemia, insulin resistance, type 2 diabetes mellitus and metabolic syndrome. These conditions affect the composition of bile and motility of the gallbladder and thus increase the risk of cholesterol supersaturation and lithogenesis [2], [4].

Gallstone disease has a geographical variation and its prevalence rises with age. Women are more likely to be affected than men and there has been a gradual rise in its incidence with the general growth of obesity and metabolic disease around the world [1], [5]. Gallstone disease also places a significant burden on healthcare resources as it continues to be a common cause of gastrointestinal hospitalisation and surgery.

Many gallstones are asymptomatic, but if they are symptomatic, they can cause the following symptoms: biliary colic, acute cholecystitis, choledocholithiasis, cholangitis,

pancreatitis, and biliary obstruction [6], [7]. Several systemic conditions have also recently been linked to gallstone disease, such as cardiovascular disease and non-alcoholic fatty liver disease, which further supports the association of gallstone disease with metabolic dysfunction [8].

Gallstones are a complex, metabolically active, hepatobiliary disorder with many interactions between cholesterol metabolism, bile acid regulation, gallbladder dysfunction and systemic metabolic disturbances occurring overall.

1.2 BIOLOGICAL BASIS OF GALLSTONE FORMATION

1.2.1 CHOLESTEROL AND BILE ACID DYSREGULATION

A disturbance in the regulation of cholesterol and bile acid metabolism is the main cause of the formation of cholesterol gallstones. In normal physiological conditions the liver balances the production of cholesterol, the absorption by the small intestine, the secretion into the bile, the formation of bile acids from cholesterol and the elimination of cholesterol. This imbalance can trigger the production of lithogenic bile with high levels of cholesterol in relation to bile acids and phospholipids [9], [10].

Overproduction of hepatic cholesterol can occur as a result of excessive de novo synthesis, excess intestinal absorption or failure to eliminate cholesterol from the liver. Higher cholesterol level in the liver leads to excessive bile cholesterol production and cholesterol supersaturation in bile, which can cause bile to precipitate [11], [12]. If the concentration of cholesterol surpasses the ability of bile acids and phospholipids to solubilize it, cholesterol monohydrate crystals begin to nucleate and aggregate, which will lead to the formation of gallstones [13].

The major pathway of cholesterol catabolism and elimination is bile acid synthesis. The rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1), regulates conversion of cholesterol to primary bile acids and regulates the size of the bile acid pool [14], [15]. This leads to less bile acid synthesis and less solubilisation of cholesterol in the bile, which may help to cause lithogenic bile.

Other bile acid signaling pathways, such as farnesoid X receptor (FXR) and Takeda G-protein coupled receptor 5 (TGR5), also play roles in bile acid homeostasis, cholesterol metabolism and enterohepatic circulation. Dysregulated FXR and TGR5 signaling is likely to disrupt the cholesterol secretion/bile acid synthesis balance, making gallstones more likely to form [14], [16].

In addition, there are important roles played by cholesterol transport mechanisms in the pathogenesis of gallstones. An increase in the cholesterol absorption and biliary cholesterol secretion drives cholesterol accumulation and lithogenicity to the liver [11]. Reverse cholesterol transport (RCT) also has an effect on hepatic clearance of cholesterol and biliary cholesterol balance.

The integrated mechanism between cholesterol synthesis, bile acid imbalance and lithogenic bile formation is presented in Fig 1.2. In summary, the development of cholesterol gallstones is due to a combination of factors that include increased cholesterol supply, decreased bile acid production, disturbances in cholesterol transport and bile acid signaling. All of these abnormalities contribute to the supersaturation of cholesterol, crystal nucleation and lithogenic bile.

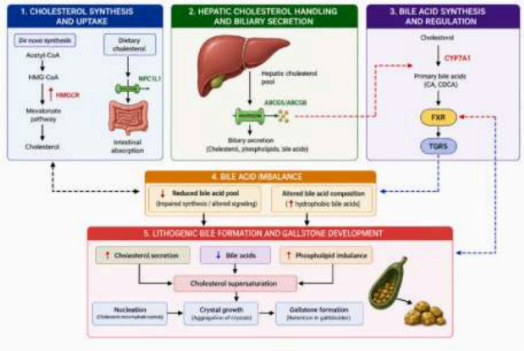


Fig 1.2. Integrated Mechanism of Cholesterol Metabolism, Bile Acid Dysregulation, and Gallstone Formation

1.2.2 GALLBLADDER DYSFUNCTION AND METABOLIC FACTORS

Once a person's cholesterol is supersaturated and bile acids are out of balance, gallbladder dysfunction is a key factor in gallstone progression. A healthy gall bladder contracts normally, which helps store bile and causes it to empty from time to time to avoid the build-up of bile, and help remove cholesterol crystals. The poor function of the gallbladder leads to bile stasis, retention of bile crystals and gallstones [17], [18].

Gallbladder hypomotility increases the time that cholesterol rich bile resides in the gallbladder lumen, which increases the concentration of cholesterol, thus favouring the nucleation of cholesterol crystals. These cholesterol microcrystals are easily retained and this is helpful to the aggregation of the crystals and the formation of larger stones. This is further facilitated by an overproduction of mucin, which creates a matrix of mucin that binds cholesterol crystals and increases crystal stability [19], [20].

Metabolic disorders play a significant role in the dysfunction of the gallbladder and in medium and high lithogenicity. Metabolic syndrome is associated with increased hepatic

cholesterol secretion, and reduced gallbladder contractility [21], [22]. These metabolic changes help to cause the bile to become stagnant and increase the supersaturation of cholesterol.

Obesity and metabolic syndrome are also linked to chronic low-grade inflammation, which further damages the function of the gall bladder, causing it to become more sluggish and leading to excessive mucin secretion and increased bile retention and crystal formation [21]. Other factors, such as hormones and genetics, can also lead to a change in gallbladder movement, and predispose people to developing gallstones [18], [23].

The important metabolic factors which predispose to gallstone formation are summarized in Fig 1.3. In general, gallbladder hypomotility can be considered a key mechanistic link between metabolic dysfunction and the progression of gallstones. The combined effects of bile stasis, mucin accumulation and crystal retention lead to the conversion of cholesterol supersaturation to mature cholesterol stones.

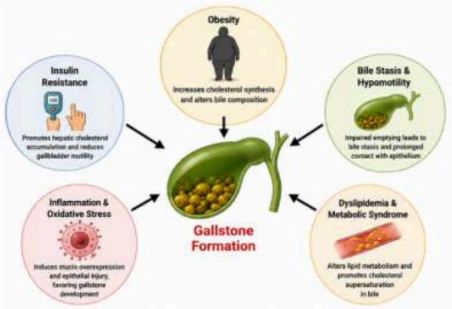


Fig 1.3. Major Metabolic Factors Contributing to Gallstone Formation

1.2.3 GUT MICROBIOTA AND ENTEROHEPATIC INTERACTION

The composition of gut microbiota is proven to be significant in regulating the metabolism of bile acids, cholesterol homeostasis and enterohepatic circulation. Imbalance of the gut microbial composition can cause abnormal recycling of bile acids and facilitate the development of metabolic disorder that leads to gallbladder stones [17], [24].

The enterohepatic circulation involves the bi-directional recirculation between the liver, the intestine and the biliary system. Gut microflora alters primary bile acids through pathway including deconjuation, dehydroxylation and other enzyme-catalyses, leading

to the generation of secondary bile acids, which differ from primary bile acids in metabolic characteristics [15].

Microbial transformation of bile acids is a big part in determining the composition of bile acids and affecting the solubilisation and lithogenicity of cholesterol. Disruption in the formation of secondary bile acids might mean an alteration in the composition of bile acids, phospholipids and cholesterol, which can favour precipitation and formation of crystals.

Various researches have demonstrated gut microbiota dysbiosis in the gallstone disease, with decreasing beneficial bacteria and rising pathogenic microbes which participated in disturbed metabolism of bile acids [17], [24]. Dysbiosis led to changed composition of bile acids in the portal circulation and make a contribution to digestive bile acids imbalance and cholesterol supersaturation. Fig 1.4 shows gut microbiota dysbiosis, microbial bile acid transformation, altered bile acid pool, impaired FXR/TGR5 signaling, and their contribution to gallstone formation.

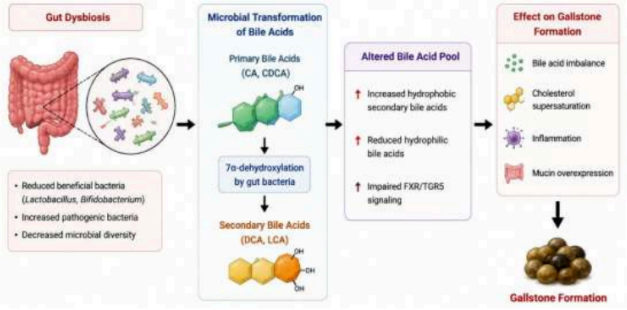


Fig 1.4. Gut Microbiota–Bile Acid Axis in Gallstone Disease

Gut microbiota also affects cholesterol absorption, lipids metabolism, signals of cell inflammation, and overall metabolic homeostasis. As showed in experimental studies, the shifts of gut microbiota composition were tied to the rise of cholesterol accumulation and more gallstones while bile acids approaches might fresh the balance of microbes and diminish lithogenicity [25], [26].

In short, gut microbiota dysbiosis mediates the development of cholelithiasis via its impact on conversion of bile acids, enterohepatic circulation, cholesterol homeostasis, and metabolic regulation. The combined occurrences lead to the formation of lithogenic bile and the nucleation of cholesterol crystals.

1.3 CURRENT DIAGNOSTIC AND THERAPEUTIC APPROACHES

1.3.1 DIAGNOSTIC APPROACHES

Timely diagnosis of gallstone disease is vital to detect biliary abnormalities and intervenes to prevent acute complications such as acute cholecystitis, cholangitis, pancreatitis and biliary obstruction [27].

The first-line imaging modality continues to be ultrasonography, given its high sensitivity combined with ease of use, low cost, and no ionizing radiation. It enables for fast preliminary imaging of gallbladder thickening, and sludge/gallstones [28]. However, very small numbers of stones and complications caused by imaging in obesity may reduce the accuracy of diagnosis.

Magnetic resonance cholangiopancreatography (MRCP) is a non-invasive and high-resolution imaging of the biliary tree that has been shown to accurately identify choledocholithiasis, as well as its utility in cases of complicated gallstone disease [27]. Computed tomography (CT) imaging is largely reserved for complicated cases of inflammation, perforation, pancreatitis or malignancy suspicion, and cholesterol stones may be not well visualized on CT.

Evaluation of biliary obstruction and inflammatory complication is supported by biochemical investigations including liver function tests, bilirubin, alkaline phosphatase, transaminases and inflammatory markers [29], [30]. Table 1.2 summarizes a comparison of the primary diagnostic modalities, including their benefits and drawbacks. In conclusion, correlation of imaging findings with biochemical markers augments the accuracy of diagnosis and adds to clinical management in gall stone disease.

Table 1.2 Comparison of Diagnostic Modalities in Gallstone Disease

Diagnostic Method	Clinical Application	Advantages	Limitations
Ultrasonography (USG)	Initial evaluation of possible gallstones or gall bladder inflammation	Non-invasive, inexpensive, quick, very sensitive to detect gall bladder stones and sludge; helpful to assess gall bladder wall thickening and biliary dilation	Less sensitivity for small common bile duct stones and in obese patients
Magnetic Resonance Cholangiopancreatography (MRCP)	Assessment of biliary obstruction and choledocholithiasis	No use of X-rays; high diagnostic accuracy for stones in the bile duct	Costly, less easily available and longer examination time

Computes Tomography (CT)	Evaluation of complications such as pancreatitis, perforation, and severe inflammation	Helpful in the assessment of abdominal complications and adjacent tissues	Reduced sensitivity for cholesterol stones; radiation exposure
Biochemical Markers	Evaluation of hepatobiliary function, cholestasis and inflammation	Liver enzymes and inflammatory markers help to evaluate the disease as well as to assess complications	Does not provide enough information or is not specific enough to be used to make a diagnosis without imaging

1.3.2 THERAPEUTIC STRATEGIES

Current therapeutic strategies for gallstone disease mainly aim at lowering cholesterol supersaturation, getting the bile acid balance more aligned, easing biliary obstruction, and then preventing annoying complications in the long run [3]. The therapeutic options available for the management of gallstone disease are shown in Fig 1.5.

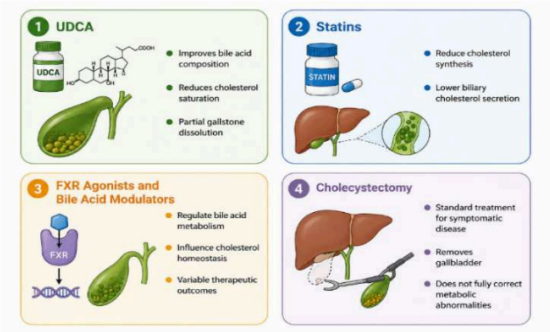


Fig 1.5. Current Therapeutic Strategies for Gallstone Disease

Ursodeoxycholic acid (UDCA) is still considered the principal pharmacological option for cholesterol gallstones. UDCA reduces the saturation of cholesterol in bile, tweaks the

bile acid composition, and supports cholesterol solubilization [31]. Still, the dissolution process is often slow, and it can be incomplete or partial, then after discontinuation recurrence shows up quite frequently, like it never really went away.

Other cholesterol-lowering approaches, including statins, have been examined as well, because they can reduce hepatic cholesterol synthesis and may lower biliary cholesterol secretion [32]. In parallel, bile acid–oriented treatments that involve FXR agonists can modulate bile acid synthesis and cholesterol handling, even though certain agents might end up creating lithogenic conditions, unintentionally [33].

Even with these medications, outcomes are not always consistent, since gallstone disease is not just one pathway. It involves multiple, connected metabolic and inflammatory routes that keep feeding each other. So, in the end, current pharmacological therapies tend to offer only limited long-term control of the condition.

However, it remains the gold standard for symptomatic gallstone disease, especially for laparoscopic cholecystectomy, as it eliminates the gallbladder and in practice, decreased recurrent biliary issues [3], [34]. However, surgery cannot completely repair the underlying metabolic imbalance associated with gallstones. The disruption of the normal mechanism of cholesterol metabolism and bile acid regulation can also occur after cholecystectomy despite the removal of the gallbladder's stone [35]. Table 1.3 summarizes comparison of pharmacological and surgical strategies including their mechanism of action, advantages and limitations for the gallstone disease. In summary, current therapeutic approaches provide symptomatic treatment and disease control in the short-term but are not enough for long-term metabolic control and prevention of recurrence of the disease.

Table 1.3. Comparison of Pharmacological and Surgical Strategies in Gallstone Disease

Therapy / Strategy	**Mechanism of Action**	**Advantages**	**Limitations**
Cholecystectomy	Gallbladder removal (cholecystectomy) to treat gallstones that are causing symptoms and to prevent the stones from blocking the bile ducts again	Standard therapeutic approach to gallstone disease when symptomatic; very effective for acute and recurrent complications	Invasive procedure; cannot completely correct underlying metabolic dysfunction; digestive problems may occur after the procedure, and metabolic abnormalities may return.
Ursodeoxycholic Acid (UDCA)	Increases the hydrophilicity of bile acids, decreases the cholesterol saturation in bile and facilitates the progressive	Non-invasive pharmacological treatment; useful in patient with good gallbladder function if the stone is small	Slow response to treatment, treatment needs to be continued for a long time, less effective in large, calcified or

	dissolution of cholesterol gallstones	and radiolucent with cholesterol; enhances the composition of bile	pigment stones, reoccur frequently after treatment stops
Statins	Inhibit HMG-CoA reductase (HMGCR) and decrease liver (hepatic) cholesterol production and removal from the liver (biliary) secretion	Reduce the level of cholesterol in the blood; may lower the risk of gallstones forming and supersaturation of cholesterol during long term therapy	Mostly preventative rather than remedial, may not be effective in established gallstones, may respond differently depending on the metabolic status of the patient
FXR Agonists / Bile Acid Modulators	Modulate bile acid metabolism, cholesterol metabolism and hepatobiliary metabolic signalling via FXR-dependent pathways	Restore the balance of bile acid and metabolic regulation; therapeutic significance in hepatobiliary metabolic diseases	Little long-term clinical data; receptor selectivity and adverse effects are still major issues
Combination Metabolic Therapy (e.g., UDCA + PUFA)	Stabilizes bile acids, along with lipid metabolic modulation to aid in improving cholesterol dissolution and bile composition	Better gallstone dissolution and metabolic control than with monotherapy in experimental and clinical trials	Lacks of standardization in clinical practice; need additional validation in larger patient population

1.4 COMPUTATIONAL DRUG REPURPOSING

Due to the shortcomings of existing pharmacological and surgical treatments, there has been a growing interest in computational approaches for drug repurposing for gallstone disease. Drug repurposing involves the discovery of novel indications for established drugs that have defined pharmacokinetic and safety profiles, and as a result have shorter timescales, lower costs and greater clinical probability of success in the development process [36], [37].

Gallstone disease is characterized by a complex interaction of dysregulation of cholesterol homeostasis, bile acid metabolism and inflammation and the hepatobiliary signaling. Systems biology approaches have gained significant attention in gallstone disease [38].

Metabolomics-facilitated prioritization helps to recognize co-variant metabolic pathways that are associated with gallstone disease like bile acids metabolism, lipid metabolism and inflammation reflexed signaling [39]. Combining metabolomics data with pathway analysis and network bioinformatics enhances biological understanding of metabolomics data for pathway-based therapy prioritization.

Network pharmacology allows to test the interactions between genes proteins pathways and drugs within a network of interconnected systems. It aids in the discovery of drugs which can be used back into clinical practice to impact more than one of the pathways involved in disease [38].

Molecular docking, molecular dynamics simulation and ADME prediction are also part of computational pipelines to evaluate the drug-target interaction, stability of interaction as well as the pharmacokinetic acceptability of the identified compounds [40], [41].

In general, computational repurposing integrated strategies offer a biologically driven approach to identifying potential therapeutic candidates in gallstone disease and help guide the development of pathway-oriented precision therapeutics.

1.5 RESEARCH GAP AND OBJECTIVES

1.5.1 RESEARCH GAP

Despite the advances in the knowledge of gallstone disease, some important areas have yet to be answered. The studies that have been conducted have suggested changes in cholesterol metabolism, bile acid regulation, inflammation, intestinal microbiota and metabolic pathways linked to gallstone development. While there are numerous omics studies, most often they focus on a single biomarker or metabolite and do not provide sufficient systems-level interpretation [24], [29].

Likewise, there is weak integration of metabolomics, pathway analysis, network biology and therapeutic prioritization in current computational methods. However, because gallstone formation is a complex disease that is linked to multiple metabolic and hepatobiliary processes, single computational analyses may not fully account for disease progression or biologically relevant therapeutic candidates.

Networks-based repurposing strategy has shown that it has therapeutic relevance in hepatobiliary disease [38], but integrated metabolomics repurposing strategies have not been studied in gallstone disease.

In this context, metabolomics, pathway enrichment, network pharmacology, and therapeutic prioritization are biologically integrated computational approaches that can be used to find potential repurposable drug candidates in the context of gallstone disease.

1.5.2 AIM AND OBJECTIVES

AIM

To discover and suggest potential drug candidate that may be repurposed to treat gallstones using an integrated metabolomics-guided pathway analysis, network biology.

OBJECTIVES

1. Data preprocessing and analysis for identification of significant differential metabolites related to gallstone disease.

2. To carry out pathway enrichment and metabolite–gene mapping to identify pathways and molecular targets of interest from a biological perspective.

3. To build networks of gene–drug and gene–metabolite interactions for systems-level therapeutic analysis and prioritization.

4. To identify therapeutically relevant, gallstone disease pathway associated drug candidates for therapeutic use using network metrics.

CHAPTER 2

LITERATURE REVIEW

2.1 MOLECULAR MECHANISMS AND THERAPEUTIC TARGETS

2.1.1 CHOLESTEROL METABOLISM

The role of cholesterol metabolism in the pathogenesis of gallstones and hepatobiliary lipid homeostasis is central. The liver controls cholesterol balance by coordinated processes of hepatic cholesterol synthesis, intestinal cholesterol absorption, lipoprotein transport, biliary secretion and cholesterol elimination. Disturbances of these mechanisms leads to hepatic cholesterol accumulation, biliary cholesterol supersaturation, and cholesterol gallstone formation [9], [11].

Hepatic cholesterol synthesis takes place via the mevalonate pathway, with the key rate-limiting enzyme of cholesterol biosynthesis [11] being HMG-CoA reductase (HMGCR). Higher HMGCR activity boosts cholesterol availability in the liver, and helps to cause overproduction of cholesterol in the bile. The intestinal cholesterol absorption also plays a role in hepatic cholesterol load by Niemann–Pick C1-like 1 (NPC1L1), a protein that regulates absorption of cholesterol from the intestinal lumen into enterocytes. High levels of cholesterol absorption in the intestine leads to a rise in cholesterol transport to the liver and to the formation of lithogenic bile [4].

Lipoprotein-mediated transport also plays a role in the maintenance of cholesterol homeostasis. The high-density lipoprotein (HDL) also plays a role in reverse cholesterol transport, bringing cholesterol back to the liver for elimination, while the low-density lipoprotein (LDL) transports cholesterol to peripheral tissues. Dyslipidemia changes the flow of cholesterol in the liver and leads to the development of gallstones [11]. ABCG5 and ABCG8 are ATP-binding cassette transporters that pump cholesterol out into bile, and are the main regulators of hepatobiliary cholesterol secretion. The upregulation of these transporters leads to increased cholesterol saturation in the bile and cholesterol crystal nucleation [9], [10].

The process of cholesterol secretion from the bile has to balance the secretion of bile acids and phospholipids to maintain the solubility of the cholesterol. When the production of cholesterol is excessive and bile acid is not available, it causes cholesterol to precipitate and forms lithogenic bile [13]. These disturbances are further exacerbated by metabolic abnormalities, such as obesity, insulin resistance and dyslipidemia, which leads to an increase in hepatic cholesterol synthesis and the impaired elimination pathways [4]. In summary, coordinated disruption of cholesterol synthesis, transport and biliary secretion plays a direct role in the cholesterol supersaturation and gallstone formation.

2.1.2 BILE ACID SIGNALING AND FXR/TGR5

In addition to lipid digestion, bile acids control hepatobiliary metabolism via the receptor-mediated signaling pathway. The bile acid receptors most important for bile acid homeostasis, enterohepatic regulation, glucose metabolism, lipid metabolism, and inflammatory signaling are farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) [14].

The main pathway of catabolism of cholesterol is bile acid synthesis. Classical bile acid synthesis pathway begins with cholesterol 7α-hydroxylase (CYP7A1), which is the key rate-limiting enzyme that converts cholesterol to primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) [14], [15]. Under metabolic and hepatic stress conditions, an alternate pathway through the actions of CYP27A1 and CYP7B1 also plays a role in the regulation of the bile acid pool. The proper control of these pathways is crucial to keep the composition of bile acids and the solubility of cholesterol of bile within normal limits.

FXR is the major bile acid synthesis and enterohepatic feedback regulator. FXR activation, either by the SHP-dependent signaling pathway or the intestinal FGF15/19 signaling pathway, decreases bile acid biosynthesis by downregulating the expression of the rate-limiting enzyme in bile acid biosynthesis, CYP7A1 [42], [43]. FXR also regulates the transport proteins which play a role in bile acid secretion and phospholipid transport, thereby maintaining hepatobiliary lipid balance and preventing hepatobiliary lipid toxicity. Dysfunctions of FXR signaling contribute to impaired bile acid homeostasis, reduced bile acid cholesterol solubilisation and increased bile acid supersaturation and gallstone formation [43].

The membrane bound bile acid receptor is TGR5, which is expressed in intestinal epithelial cells, macrophages, gall bladder epithelial cells and adipose tissue. Glucose metabolism, mitochondrial energy expenditure and inflammatory responses are regulated by cyclic AMP dependent signaling pathways through activation of TGR5 [44]. TGR5 signaling also suppresses the production of inflammatory cytokines and promotes metabolic homeostasis of hepatobiliary tissues. FXR and TGR5 are linked in a single signaling pathway, which regulates bile acid metabolism, lipid homeostasis and inflammatory balance shown in Fig 2.1.

Owing to their central metabolic roles, FXR and TGR5 have become interesting therapeutic targets in hepatobiliary diseases. While FXR agonists are capable of disrupting the bile acid homeostasis and prohibiting lithogenic bile formation, experimental TGR5 agonists have anti-inflammatory and metabolic regulatory effects [45], [46]. In therapeutic development however, receptor selectivity and long-term safety is a major drawback. In general, bile acid defects and defects in bile acid receptor-mediated signaling are important factors in the progression of gallstones and metabolic targets in therapy.

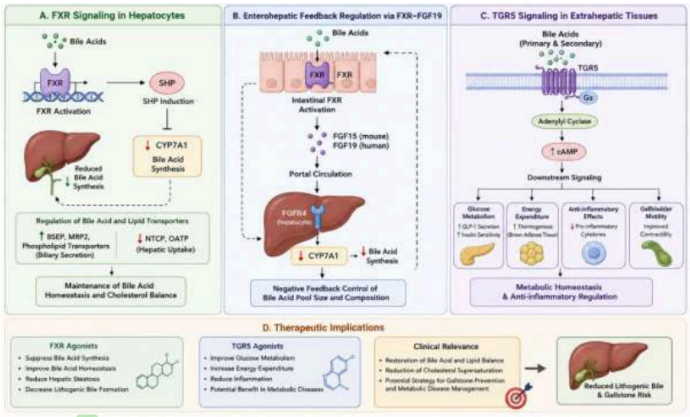


Fig 2.1. FXR and TGR5 Signaling Pathways in Bile Acid and Metabolic Regulation

2.1.3 GENETIC AND METABOLIC REGULATION

The formation of gallstones is a process involving interactions between genetic susceptibility and metabolic dysfunction. Multiple lithogenic genes related to cholesterol transport, phospholipid secretion, bile acid transport and hepatobiliary lipid regulation were identified by the genome-wide association study (GWAS) [47]. These genetic changes affect the composition of the bile and make it more prone to becoming saturated with cholesterol and forming gallstones.

The ABC transporters are important regulators of hepatobiliary cholesterol transport. ABCG5 and ABCG8 regulate cholesterol efflux into bile and gain-of-function variants lead to increased biliary cholesterol secretion and gallstone risk [19]. ABCB4 also converts phosphatidylcholine into bile and thus stabilizes the micelles containing cholesterol, thus ensuring the solubility of the cholesterol. Mutations in ABCB4 decrease the concentration of phospholipids in bile and increase its lithogenicity [47]. ABCB11 also plays a role in bile acid excretion and bile acid-dependent solubilisation of cholesterol.

However, NPC1L1 also plays a major role in the pathogenesis of gallstones by controlling the absorption of cholesterol in the intestine. Changes to NPC1L1-mediated cholesterol uptake affect the availability of cholesterol in the liver and consequently affect biliary cholesterol saturation [12]. The other susceptibility genes discovered through GWAS are related to pathways of lipoprotein metabolism, inflammatory response, glucuronidation, and hepatic metabolic signaling [18], [48]. These results

validate the polygenicity and metabolically integrated of gallstone disease. Table 2.1 summarizes the major molecular therapeutic targets in gallstones.

Table 2.1. Key Molecular Therapeutic Targets in Gallstone Disease

Gene/Regulator	Biological Function	Relevance with Gallstones	Therapeutic Relevance
HMGCR	Rate-limiting enzyme in the mevalonate pathway in hepatic cholesterol biosynthesis	Increased cholesterol production leads to a supersaturation of cholesterol in the bile and formation of lithogenic bile	Statins targets to reduce cholesterol synthesis in the liver and to decrease the lithogenic risk
CYP7A1	Catalyzes conversion of cholesterol into primary bile acids in the bile acid synthesis	Disruption of Bile Acid Synthesis Affects Cholesterol Elimination and Causes Bile Acid Imbalance	Modulation of bile acid synthesis, cholesterol homeostasis and lithogenicity
ABCG5/ABCG8	ATP-binding cassette transporters that facilitate biliary cholesterol secretion	Increased secretion of biliary cholesterol promotes cholesterol crystal nucleation and gallstone formation	Regulating targets for cholesterol transport and lithogenic bile formation
NPC1L1	Intestinal cholesterol transporter active in dietary cholesterol absorption	High cholesterol absorption results in greater cholesterol available to the liver and secretion into the bile	Inhibition blocks the absorption of cholesterol and could lower the risk of gallstones
FXR (Farnesoid X Receptor)	Nuclear receptor regulating bile acid synthesis, transport and enterohepatic circulation	FXR signaling is dysregulated and this disrupts bile acid homeostasis, which results in lithogenic bile formation.	FXR activation enhances the bile acid homeostasis and prevents the formation of lithogenic bile
TGR5 (GPBAR1)	G-protein-coupled bile acid receptor involved in metabolic and inflammatory regulation	Regulates bile acid signaling, glucose metabolism, gallbladder motility and inflammatory responses in gallstones disease	Could be used as a therapeutic target for metabolic modulation and anti-inflammatory drugs
ABCB4	Phosphatidylcholine transporter involved in biliary phospholipid secretion	Preserves the solubility of cholesterol and the stability of the biliary micelle	Dysfunction leads to lithogenic bile and hepatobiliary injury

ABCB11 (BSEP)	The bile salt export pump is the key component to the secretion of bile acids into the bile canaliculi	Preserves bile acid-dependent solubilization of cholesterol and bile acid homeostasis	Therapeutic modulation could favour hepatobiliary lipid balance and bile acid transport.
LDL Receptor / Lipoprotein Regulation	Regulates the uptake of LDL cholesterol in the liver	Affects systemic cholesterol transport and hepatic cholesterol accumulation	Lipid-lowering treatments restore cholesterol homeostasis and decrease lithogenic circumstances.
FGF15/19 – FGFR4 Axis	Signaling pathway that controls bile acid biosynthesis, in the intestine	Inhibits bile acid production via FXR signalling in the intestine	Important regulatory pathway for bile acid targeted therapeutic intervention

Other factors, such as metabolic abnormalities like obesity, insulin resistance, dyslipidemia, and hepatic steatosis, further interact with genetic susceptibility to accelerate gallstone formation. These conditions stimulate hepatic cholesterol synthesis, stimulate biliary cholesterol secretion and impair hepatobiliary transport regulation. The Mendelian randomization analyses also showed that pathways regulating cholesterol metabolism have differential impacts on the susceptibility to gallstones, based on their effects on handling cholesterol in the bile [12]. In summary, a combination of dysregulation of cholesterol transport genes and metabolic regulatory pathways is responsible for hepatobiliary lipid imbalance and gallstone development.

2.2 MULTI-OMICS INSIGHTS IN GALLSTONE DISEASE

2.2.1 GENOMICS AND TRANSCRIPTOMICS

Increased knowledge about gallstone disease has emerged from genome-wide and transcriptome-wide analysis which has led to the discovery of susceptibility genes, regulators and molecular signatures of hepatobiliary metabolic dysfunction. These studies highlight that gallstone disease is a polygenetic disorder characterized by coordinated disruptions in cholesterol transport, bile acid regulation, inflammatory signaling and hepatobiliary homeostasis [18], [48].

Several susceptibility loci for regulation of cholesterol metabolism and biliary transport have been identified by genome-wide association studies. Of these, ABCG5 and ABCG8 are important susceptibility genes for gallstone formation as they control cholesterol efflux and biliary cholesterol secretion. The polymorphisms in these transporters elevate cholesterol supersaturation and the formation of lithogenic bile [18]. Similarly, NPC1L1-associated pathways modulate cholesterol absorption in the intestine and cholesterol availability in the liver, which are important to the development of gallstones [49].

A transcriptomic analysis also showed major changes in the expression of hepatobiliary genes and miRNA regulation. These genes and dysregulated miRNAs involved in bile secretion, ABC transporter regulation, immune signaling, and lipid metabolism were identified by Yang et al. (2015)[49]. MiR-210, miR-200c, miR-194 and miR-192 were found to be differentially expressed and associated with dysregulated hepatobiliary transport and cholesterol metabolism. Peripheral transcriptomic profiling studies also found oxidative stress, DNA repair, mucin associated and transporter related dysregulation in gallstone disease [50].

Together, genomics and transcriptomics analyses suggest that the pathogenesis of gallstone disease is characterized by coordinated abnormalities of cholesterol transport, inflammatory signaling, and hepatobiliary metabolic regulation. Such molecular signatures may serve as a valuable basis for multi-omics integration and pathway-driven biological interpretation in the study of gallstone disease.

2.2.2 METABOLOMICS AND PROTEOMICS

Metabolomics and proteomics are now becoming important systems biology tools to understand biochemical and metabolic changes related to gallstone formation. In contrast to genetic observations, metabolomics and proteomics provide a complete picture of bile acid metabolism, cholesterol levels homeostasis, inflammatory responses, and hepatobiliary transport dysfunction that accompany the disease [29], [51].

The most consistent metabolomic finding in gallstone disease is changes in bile acid metabolism. Han et al. (2024) showed that several metabolites of bile acids were significantly dysregulated, including taurocholic acid, glycocholic acid, glycochodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid and deoxycholic acid[51]. In gallstone patients, serum total bile acids were elevated while bile total bile acids were decreased, suggesting a dis-regulation of enterohepatic bile acid circulation and the impaired bile acid homeostasis. Other bile acid metabolites were additionally suggested as possible markers for early diagnosis of gallstones as they were related to metabolic changes in the formation of cholesterol crystals and instability in the bile.

In addition to bile acid metabolism, metabolomic analysis was applied to a range of metabolic pathways associated with lipids, indicating disturbances in these processes as well. According to Uche-Anya et al. (2024) triacylglycerols and diacylglycerols were positively correlated with the risk of gallstone disease, whereas plasmalogens and cholesterol esters were inversely correlated with the risk of gallstone disease[52]. The close relationship between systemic lipid dysregulation and gallstone pathogenesis was further highlighted by pathway enrichment analysis, which revealed a set of changes in pathways of de novo lipid biosynthesis and cholesterol metabolic regulation.

Furthermore, coordinated dysregulation of inflammatory, oxidative stress associated, and energy metabolism proteins in gallstone disease were also revealed in integrated metabolomic and proteomic profiling studies [29]. A number of changes in arachidonic acid metabolism, lipid associated metabolites, inflammatory proteins and hepatobiliary

metabolites were noted to be significant. The integrated network analysis also identified relevant connections between metabolic modules and clinical biochemical parameters, indicating a strong interaction between metabolic dysfunction and systemic liver function.

A proteomic analysis of bile and gall bladder tissue also produced significant protein dysregulation, including inflammation, oxidative stress, mitochondrial activity, transport regulation, and cellular metabolism. In cholesterol and pigment gallstone patients, Jing et al. (2024) have shown enrichment of pathways involved in amino acid metabolism, vitamin B6 metabolism, porphyrin metabolism, and riboflavin metabolism[53]. Likewise, previous work showed proteins associated with oxidative stress and cytoskeletal injuries, which are related to gallstone formation, are involved [54].

More recent bile proteomic studies also showed that bile expression of catabolic metabolism-related proteins was suppressed while expression of inflammatory signaling proteins was increased, along with impaired transport-related proteins in gallstone disease [55]. There were more severe metabolic disturbances in the samples of acute cholelithiasis than in the chronic disease samples, which suggests that the disease advanced, causing more metabolic dysfunction.

In summary, metabolomics and proteomics research shows that gallstone disease is a system-level metabolic disease characterised by coordinated abnormalities in bile acid metabolism, cholesterol transport, lipid biosynthesis, oxidative stress regulation, inflammatory signalling and hepatobiliary transport activity. The confluence of these molecular signatures offers significant insights into the pathogenesis of gallstones and serve as a useful tool for prioritizing target pathways for diagnosis and therapy in hepatobiliary metabolic disorders.

2.2.3 GUT MICROBIOTA AND METABOLIC INTERACTION

Recent systems biology studies suggest gut microbiota plays a major role in the pathogenesis of gallstone disease via its effects on bile acid metabolism, cholesterol homeostasis and regulation of host metabolism. This dysbiosis affects the bile acid composition and the metabolism of lipids, inflammatory balance and hepatobiliary metabolic activity, which favour the supersaturation of cholesterol and the formation of gallstones [24], [25].

In patients with cholesterol gallstone disease, several studies indicated that there is significant gut microbiota dysbiosis. Wang et al. (2025) found a decrease in microbial diversity along with changes in the amino acid metabolism, lipid metabolism, carbohydrate metabolism and bile acid-related metabolic pathways associated with bacteria[24]. Metabolomic analysis also showed that the bile acid profile was changed and amino acid metabolism was disrupted, which suggests that the disruption of microbiota associated metabolic balance is not restricted to bile metabolism, but it also impacts on the regulation of amino acid metabolism in a systemic manner.

The primary role of gut microbiota in the pathogenesis of gallstones is through bile acid transformation and secondary bile acid production. Primary bile acids are modified by intestinal microbial enzymes, which perform deconjugation and dehydroxylation reactions to produce secondary bile acids, such as deoxycholic acid, lithocholic acid, ursodeoxycholic acid, and hyodeoxycholic acid [15]. These changes modify the hydrophobicity, enterohepatic circulation, cholesterol solubility and metabolic signaling ability of the bile acids.

Experimental studies also showed that microbiota-associated metabolic regulation regulates hepatic and intestinal cholesterol metabolism. Lu et al. (2021) reported that feeding of tauroursodeoxycholic acid (TUDCA) changed the gut microbial structure, lowered the level of hepatic cholesterol accumulation, and prevented the formation of gallstones in a high-fat diet induced mouse model[26]. The research also revealed that there was abnormal expression of genes that play a role in cholesterol transport and in the production of bile acids (bile salts), such as ABCG5, ABCG8, ACAT2, CYP27 and ABCB11.

Likewise, Shen et al. (2023) showed that co-treatment with hyodeoxycholic acid reduced the risk of cholesterol gallstones by simultaneously regulating the composition of gut microbiota and bile acid metabolism[25]. Overall, these results are a further indication that gut microbiota modulate hepatobiliary metabolism through the dynamic microbiota–bile acid interaction that can amplify cholesterol imbalance and lithogenic conditions.

In summary, microbiota induced metabolic dysregulation plays a significant role in the progression of gallstone disease and its effects include changes in bile acid transformation, the production of secondary bile acids, cholesterol metabolism and systemic metabolic signaling. Thus, microbiome and metabolomic studies provide important data on the role of gut microbiota as a key metabolic regulator in hepatobiliary disease.

2.3 CURRENT AND EMERGING THERAPEUTIC STRATEGIES

2.3.1 URSODEOXYCHOLIC ACID (UDCA) THERAPY

The most common bile acid–based drugs used for the treatment of cholesterol gallstone disease are bile acid–based drugs. Bile acid–based drugs are the most commonly used drugs for the treatment of cholesterol gallstone disease. UDCA is a hydrophilic bile acid which inhibit bile supersaturation with cholesterol and induces progressive dissolution of cholesterol gallstones [31]. Oral litholysis by bile acids is the main non-surgical treatment for selected individuals with radiolucent cholesterol stones and intact gallbladder function.

The therapeutic action of UDCA is principally due to its action on bile composition and its cholesterol solubility. Raising the hydrophilicity of the bile decreases the tendency of cholesterol crystals to nucleate and aggregate, which helps to prevent formation of

gallstones and promotes dissolution of stones [31]. Also, UDCA enhances flow of bile and regulates bile acid homeostasis to decrease lithogenicity.

Experimental research also indicated that bile acid-based drugs affect cholesterol transport and bile secretion pathways [26]. These results suggest that the UDCA-induced bile acid modulation is not only needed to dissolve cholesterol, but also it may play a role in other metabolic regulation aspects related to gallstone disease.

From a clinical point of view, the main indications for UDCA are patients with small, non-calcified cholesterol stones and a functional gallbladder. The response to treatment will vary with stone size, composition, gallbladder motility and treatment duration. Cholesterol dissolution, however, is a slow process and is often administered for long periods of time over a number of months [31].

Studies in recent years have examined combination therapies to increase the effectiveness of therapy. UDCA in combination with n-3 polyunsaturated fatty acids (PUFA) resulted in improved gallstone dissolution and radiological response rates than UDCA alone [56]. Experimental results indicated that PUFA supplementation could decrease the cholesterol saturation and improve the composition of phospholipids in bile, which would increase the solution of cholesterol.

Although clinically useful, there are limitations to the use of UDCA therapy. Treatment is effective only in patients with large or calcified stones, or pigment stones, which are rare. Furthermore, recurrence following the end of treatment is frequent, since UDCA does not completely reverse the metabolic disturbances that cause gallstone formation [31]. Therapeutic failure in long-term use is thus still hindered by persistent obesity, dyslipidemia, insulin resistance and the changed composition of bile acids.

Overall, UDCA has been the most well-established medical drug therapy for cholesterol gallstone disease, since it is capable of altering the composition of cholesterol in the gallstones. There are however still limitations in the long-term effectiveness of this treatment such as slow therapeutic response, incomplete dissolution and recurrence.

2.3.2 STATINS AND LIPID-LOWERING STRATEGIES

Statins are a significant therapeutic approach for lowering blood lipids that are also known to prevent gallstones and regulate blood metabolism. These drugs are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme of hepatic cholesterol biosynthesis [11]. Decreased hepatic cholesterol synthesis results in decreased level of low-density lipoprotein cholesterol (LDL-C) in the blood stream and less cholesterol available for biliary secretion.

This mechanism may be clinically significant as it can directly contribute to cholesterol supersaturation and lithogenic bile formation. Multiple research studies showed that statins could decrease biliary cholesterol level and thereby decrease the risk of cholesterol gallstone development by regulating hepatobiliary cholesterol metabolism [32].

A large meta-analysis of 590,086 patients found that long-term statins were associated with reduced gallstone disease risk but short-term statins had limited benefit [32]. The results indicate that long-term cholesterol lowering might gradually lead to resolution of the long-term lithogenic environment and to improvement in the biliary cholesterol homeostasis, over time.

Evidence has also emerged in recent years that statins have wider metabolic effects than simply on cholesterol. Statins produce anti-inflammatory and metabolic regulatory effects which can indirectly impact gallstone pathogenesis [17]. Long term statin therapy was associated with decreased prevalence of gallstones and gut microbiota dysbiosis in dyslipidemic patients indicating that lipid lowering drugs may be able to modulate both cholesterol metabolism and the microenvironment of the gut associated with gallstone formation.

These studies further demonstrated the importance of cholesterol-lowering via HMGCR in the prevention of gallstones in genetic and pathway-based studies. Using Mendelian randomization, the authors found that genetic variants linked to statin-mediated HMGCR inhibition were linked to reduced risk of gallstone disease [12]. The observations highlight the significance of pathway-specific metabolic control for prevention of gallstones.

Even so, there are some drawbacks when using statins in the treatment of gallstones. The overwhelming evidence is that it is a preventative, not a curative, agent and statins have only modest success at dissolving existing gallstones. The clinical response may also differ depending on obesity, insulin resistance, duration of treatment, and hepatobiliary dysfunction [32].

In general, statins are metabolic regulatory agents which decrease the availability of cholesterol and have an inhibitory effect on lithogenic risk. But the failure of lipid-lowering monotherapy to offer a satisfactory reduction in cholesterol-lipid-induced gallstones remains a challenge because of the persistence of metabolic dysfunction and the multifactorial pathogenesis of gallstones.

2.3.3 FXR AGONISTS AND BILE ACID MODULATORS

Therapies for hepatobiliary diseases have now gone from symptom management to receptor-mediated metabolic regulation, with recent advances in hepatobiliary pharmacology. Of these, FXR agonists and bile acid modulators have proven to be promising therapeutic targets to address metabolic abnormalities that drive gallstones.

FXR agonists modulate the synthesis of bile acids, the metabolism of cholesterol, lipid homeostasis and enterohepatic bile acid circulation. Cholestyramine and bile acid chelators activate FXR to inhibit overproduction of bile acids and achieve metabolic homeostasis in the gut–liver axis. Also, FXR signaling decreases hepatic cholesterol accumulation, clears triglycerides and increases insulin sensitivity. These effects are especially important as cholesterol supersaturation and metabolic dysfunction are direct contributors to the development of gallstones.

The semi-synthetic bile acid derivative Obeticholic acid (OCA) is one of the most widely studied FXR agonists. OCA has been shown to be therapeutic in cholestatic and metabolic liver diseases, validated FXR as clinically relevant metabolic target. Clinical studies demonstrated that OCA treatment stimulates fibroblast growth factor 19 (FGF19) pathway and inhibits endogenous bile acid synthesis.

Receptor-mediated modulation, however, has significant therapeutic drawbacks. It was clinically shown that treatment with OCA can lead to an increased risk of gallstones in persons who are prone to them. FXR activation by OCA decreased the levels of bile acids, increased the cholesterol saturation index, and increased the hydrophobicity of bile acids, which had the effect of reducing the solubility of cholesterol in bile [33]. Increased biliary FGF19 level might also contribute to gallbladder relaxation and bile stasis, which leads to an increased lithogenic potential.

Due to these restrictions, research efforts have been directed towards the development of more selective FXR modulators with better safety characteristics. The use of conventional bile acid derivatives may lead to unwanted side effects due to activation of both FXR and membrane bile acid receptor, TGR5. Dual FXR/TGR5 activation may be beneficial to insulin sensitivity and lipid metabolism, but over-activation could disrupt biliary physiology.

TGR5 is also an emerging receptor of bile acid which plays a significant role in regulation of glucose, lipids, mitochondrial energy expenditure and inflammatory signaling. Experimental TGR5 agonists were shown to have potential in controlling obesity, insulin resistance and inflammatory response linked to hepatobiliary dysfunction.

FXR agonists and BAs represent an important shift towards metabolic receptor therapy as a whole. These drugs have a mechanistically directed strategy for metabolic correction of gallstone disease, as they target pathways in bile acid homeostasis, lipid metabolism and inflammatory control. Yet, their use in the clinic is still constrained by issues of long-term safety, lithogenic risk and adverse effects on pathways.

2.3.4 SURGICAL AND MINIMALLY INVASIVE APPROACHES

The treatment for gallstone disease has gradually been shifted from open surgery to less-invasive and endoscopy surgical procedures. While medicines can help clear up the symptoms or change the make-up of the bile, surgery is the best way to treat gallstones that cause symptoms and gallstone problems. Despite the success of other therapies in preventing recurrent biliary events and the beneficial long-term effects, cholecystectomy, especially laparoscopic cholecystectomy, remains the accepted treatment [3].

Laparoscopic cholecystectomy has largely replaced open surgery techniques due to decreased surgical trauma, decreased postoperative pain, shorter hospital stays and decreased recovery time. At present, laparoscopic cholecystectomy is the preferred treatment for acute cholecystitis and symptomatic gallstones [34]. The early cholecystectomy also showed better results than the delayed or conservative approach,

with reduced recurrence of the biliary pain, cholangitis and overall biliary complications [57].

Although laparoscopic cholecystectomy is effective, it does have complications. Clogged bile ducts, infection, bleeding, leakage of bile and postoperative symptoms of the abdomen continue to be significant issues. Recurrent biliary symptoms occur after surgery in some patients, and complex anatomy of the bile duct and inflammation can make surgery more difficult and sometimes necessitate conversion to open surgery.

Endoscopic procedures and combined laparoscopic-endoscopic procedures are now routinely being used in the minimally invasive management of cholecystectomy. Common strategies for common bile duct stones include laparoscopic common bile duct exploration (LCBDE) and endoscopic retrograde cholangiopancreatography (ERCP) along with laparoscopic cholecystectomy. Comparative studies showed comparable results in terms of success rates, morbidity and residual stone rate between the two methods [58].

The use of ERCP-based interventions was especially significant because they enabled the removal of stones without significant surgery. Advancements in endoscopic visualization and instrumentation has improved the accuracy of the procedure and reduced operative trauma [35]. Percutaneous transhepatic choledochoscopic lithotripsy (PTCS) was also found to be an alternative option for stone extraction from the bile duct in the case of difficult or refractory stones, which can be directly visualized during the stone extraction and may result in lower rates of residuals stones [35].

Percutaneous cholecystostomy can be used to temporarily relieve gallbladder inflammation and stabilize for high-risk surgical patients. Systematic reviews showed however that generally, laparoscopic cholecystectomy reduces the mortality rates and readmission rates compared to percutaneous drainage alone [59].

In general, surgery and minimally invasive treatments continue to play a key role in the treatment of gallstones. Laparoscopic cholecystectomy is a proven long-term treatment for symptom control, and endoscopic and percutaneous techniques have increased the treatment options for complex and high-risk patients. However, therapeutic results remain suboptimal due to postoperative complications, problems related to recurrence and patient-specific factors.

2.3.5 LIMITATIONS OF CURRENT THERAPIES

The current treatment options for gallstone disease resulted in better symptom control and decreased acute complications, but several limitations remain to limit long-term management of the disease. Current treatment strategies are mainly aimed at the removal of stones or at relieving the symptoms of gallstones, and do not address the underlying metabolic abnormalities which cause the stones to form in the first place.

Medication is not widely applicable and is not effective in many patients for extended periods of time. Ursodeoxycholic acid (UDCA) therapy is primarily reserved for patients with small stones, cholesterol stones and intact gallbladder function [31]. Furthermore,

prolonged administration of therapy is often required and incomplete dissolution can occur. Another problem of the same nature is that recurrence after therapy is also frequent, due to the fact that the post-therapy environment is still full of factors that can create lithogenic conditions, namely persistent obesity, insulin resistance, dyslipidemia and altered bile composition.

Pharmacological interventions are also limited by therapeutic side effects. Previously, bile acid treatments were associated with hepatotoxicity, diarrhea and an unfavorable lipid profile; more recent receptor-targeted treatments are not associated with these effects, but may actually contribute to an increased lithogenic risk. Metabolic interventions that induce activation of FXR increased the biliary cholesterol saturation and bile acid hydrophobicity, indicating that metabolic regulation could be simultaneously improved and susceptibility to gallstone formation increased by activating FXR [33].

Surgical management – especially laparoscopic cholecystectomy – is the first choice in the treatment of symptomatic gallstones as it is an effective way to prevent recurrent biliary events. But the systemic metabolic abnormalities that occur from gallstone disease cannot be reversed completely with surgery. Obesity, dyslipidaemia, insulin resistance and disturbed cholesterol metabolism can persist after gallbladder removal.

Postoperative morbidity is also clinically relevant. Bile leakage, bile duct injury, retained stones and the presence of ongoing abdominal symptoms remain a problem with a certain percentage of patients [60]. Likewise, the use of minimally invasive techniques like ERCP and PTCS demands high technical skills and can cause significant complications such as pancreatitis, biliary reflux, bleeding, and the presence of residual stones [35].

The response to the therapy is also dependent on the disease activity, metabolic profile, anatomical features, age and co-morbid conditions. Percutaneous biliary drainage and other non-surgical methods are useful in providing temporary stabilization in high-risk patients, but may be associated with increased mortality and readmission rates compared to definitive surgical management [59].

Another clear shortcoming is the absence of successful disease prevention. Most existing strategies are reactive and are only started when symptomatic disease occurs. Conservative management can be considered to avoid surgery but carries a risk of recurrence of the biliary complication and common bile duct stone [60]. This means that current treatments only help to a certain extent to tackle the many factors involved in cholesterol metabolism, bile composition, inflammation and genetic susceptibility. Fig 2.2 represents the emerging therapeutic approaches and computational strategies for gallstone disease management.

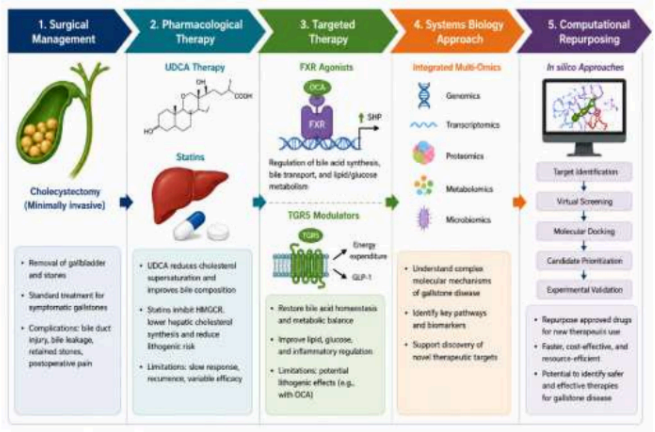


Fig 2.2 Emerging Therapeutic and Computational Strategies in Gallstone Disease

Overall, there remains a lack of long-term treatment success in gallstone disease, with recurrence, incomplete metabolic correction, treatment-related complications, and patient response variability contributing to this. The limitations emphasize the importance of biological targeting therapeutic strategies that will be able to target the molecular and metabolic events involved in gallstone pathogenesis.

2.4 COMPUTATIONAL THERAPEUTIC PRIORITIZATION APPROACHES

2.4.1 NETWORK PHARMACOLOGY

It is known that network pharmacology is one of the more important systems-level approaches to understanding complex diseases and finding therapeutic strategies by using biological network analysis. Network pharmacology is different from traditional pharmacology, which primarily targets a single target, and can study the interaction between genes, proteins, signaling pathways, metabolites, and therapeutic drugs in the networks of complex living systems. This framework is especially important in multifactorial metabolic disorders where disease progression often entails a coordinated dysfunction in a number of pathways or processes rather than a dysfunction in a single target.

Complex hepatobiliary diseases are related to lipid metabolism, inflammatory signaling, pathways leading to fibrosis, bile acid homeostasis, and immune regulation. To tackle this complexity, network pharmacology has emerged as a new concept that involves constructing biological interaction networks for systems-level interpretation of disease mechanisms and therapeutic opportunities [38].

The protein–protein interaction (PPI) network is a significant part of network pharmacology. These networks reveal functional relationships between proteins associated with disease progression and uncover nodes with high connectivity, clusters of molecules, and pathways that are associated with disease, which are useful for therapeutic prioritization [38]. The integrated pathway analysis (IPA) carried out with the help of various resources like KEGG and Reactome further supports the identification of biologically relevant therapeutic pathways and multi-target therapeutic interpretation.

Machine learning based systems also improve the therapeutic prediction by allowing the integration of the biological, genomic, chemical and clinical data. Kamya et al. (2024) described platforms that integrate with AI and databases from the omics, pathway analysis, and biomedical text-mining for therapeutic target discovery and biomarker prioritization[39]. Likewise, Lu et al. (2024) and Pugliese et al. (2025) emphasized the increasing importance of AI in predictive modeling and therapeutic development in metabolic and liver-related diseases[61], [62].

In summary, network pharmacology offers a biological holistic perspective for the comprehension of complex metabolic diseases and target identification at a system level by pathway analysis.

2.4.2 MOLECULAR DOCKING AND VIRTUAL SCREENING

Molecular docking and virtual screening are important components of structure-based drug discovery and computational therapeutic prioritization. At the structural level, these approaches assess small molecule-target protein interactions and aid in the identification of biologically relevant therapeutic candidates.

Structure Based Drug Discovery is based on the three-dimensional structure of protein to understand ligand binding and molecular interactions. Molecular docking is used to predict the preferred orientation of a ligand in the protein binding site and also gives an estimation of the stability of the interactions between the ligand and the protein through the calculation of binding affinity. According to Jose et al. (2023), docking is a computational approach that can efficiently use a protein–ligand interaction analysis and virtual screening in order to find therapeutic candidates[40].

The strength of the interaction and receptor modulation is supported by protein–ligand interactions, including hydrogen bonding, hydrophobic interactions, van der Waals forces, and electrostatic contacts. Jose et al. (2023) showed that the binding of the ligands was stable in the active site of Farnesoid X Receptor (FXR) and the hydrogen bonds and hydrophobic interactions were related to the favourable protein–ligand stability[40].

Virtual screening is a docking-based method that can be expanded to systematically screen multiple compounds for a particular disease-associated target. Recently, Zhou et al. (2015) showed that pharmacophore-based virtual screening could be used to discover new agonist molecules against the Takeda G-protein receptor 5 (TGR5), which is linked to the regulation of bile acid and metabolism[46].

The docking approaches also facilitate therapeutic repurposing strategies. In this regard, docking-based virtual screening was used by Jose et al. (2023) to select potential FXR agonists among FDA-approved compounds and by Sharma et al. (2024) to show that scoparone is a good ligand for interaction with the Constitutive Androstene Receptor (CAR) that is important for the activation of the receptor, which is linked to cholesterol metabolism[40], [41].

Despite all these benefits, the prediction of docking is still a calculation approximation due to the dynamic nature of proteins. Thus, interactions with the docking domain need to be validated in biological and experimental studies [41]. However, Molecular docking and Virtual screening are still useful tools for prioritization of therapeutic targets based on protein structure analysis and interaction profiling.

2.4.3 MOLECULAR DYNAMICS AND ADME PREDICTION

Molecular dynamics (MD) simulation and ADME prediction are important steps in computational therapeutic validation. Molecular docking gives the static picture of the interactions between protein and ligand, whereas MD simulation gives the prediction of the behavior and stability of the molecular complexes in dynamic physiological environment.

MD simulation examines the movements and flexibility of molecules over time. MD simulation is considered an important method for stabilizing docked complexes and analyzing the interaction between the receptor and the ligand under dynamic conditions, as described by Sharma et al. (2024)[41]. These analyses provide increased confidence in the interactions determined by docking by assessing the structural stability of the interactions over the course of the simulation.

The measure of structural stability during simulation is usually root mean square deviation (RMSD) and that of flexibility of the residue level and local structural fluctuations is usually root mean square fluctuation (RMSF). Generally, the lower the RMSD or RMSF values are, the more conformationally stable the molecule is [41]. Hydrogen bond stability also contributes to the interpretation of the persistence of molecular recognition and interaction during simulation studies.

Dynamic stability analysis is thus a more physiologically plausible description of molecular properties and can complement computational therapeutic prioritization.

In addition to structural validation, ADME prediction analyses if the candidate molecules have suitable pharmacokinetic properties needed for therapeutic use. Absorption, toxicity, metabolic instability and bioavailability are continuing challenges for drug development.

The drug-likeness of the drug is further supported by pharmacokinetic filtering, which involves determining molecular descriptors like lipophilicity, molecular weight, polarity, hydrogen bonds and solubility. These computational filtering methods do help to remove compounds that are likely to have poor therapeutic properties. Furthermore, Sardar et al. (2024) showed that molecular fingerprints and structural characteristics can have an effect on the permeability and solubility properties of membranes, which can affect drug delivery behaviour[63].

The overall trend of MD simulation and ADME prediction contributes to the computational therapeutic validation by combining the stability analysis of the structure with the pharmacokinetic assessment prior to experimental and clinical testing.

2.4.4 AI BASED DRUG REPURPOSING – EMERGING TECHNOLOGY

The use of artificial intelligence (AI) is becoming a more significant part of computational drug discovery and therapeutic repurposing. AI tools can sift through vast amounts of biological data, uncover unseen connections in molecules and aid in the prediction of new drug candidates. AI and machine learning approaches are gaining significance in the field of drug repurposing, as they help speed up the process of target identification and drug–target interactions prediction, according to Anokian et al. (2024)[64].

Repurposing of drugs is an alternative approach to traditional drug discovery, which involves new therapeutic uses of compounds already used for other purposes that have proven to be safe in the past. Farghali et al. (2021) highlighted that using AI-assisted methods can shorten and cut the cost of therapeutic development, by predicting and prioritizing therapeutics[65].

Therapeutic prediction is also enhanced using machine learning-based systems, which enable the integration of biological, genomic, chemical and clinical data. Kamya et al. (2024) outlined platforms that harness AI and incorporate omics data, pathway analysis, and biomedical text mining for therapeutic target discovery and biomarker prioritization[39]. Likewise, Lu et al. (2024) and Pugliese et al. (2025) emphasized the increasing importance of AI in predictive modeling and therapeutic development in metabolic and liver-related diseases[61], [62].

Despite these improvements, AI systems still rely on the quality of the datasets, biological interpretation, and the transparency of computation. As omics complexity grows, data curation and data interpretation based on evidence is critical, as noted by Anokian et al. (2024), and algorithmic bias and model interpretability is also a concern, per Chustecki (2024)[64], [66].

2.5 RESEARCH GAPS IN EXISTING COMPUTATIONAL STUDIES

Despite the advancement in understanding gallstone disease using computational biology and omics, there are several major drawbacks in the existing studies. Existing studies tend to examine individual biological layers (e.g. metabolomics, proteomics, microbiome analysis or machine learning based prediction) separately rather than in integrated systems-level frameworks. This disjointed strategy narrows the understanding of the multifaceted molecular interactions that underlie the development of gallstones.

A metabolomic and proteomic analysis, for example, has revealed changes in lipid metabolism, inflammatory pathways and potential biomarkers for cholelithiasis [29]. Likewise, microbiome-based research has shown that dysbiosis of intestinal flora, disbalance of bile acids, and metabolic disorders play a crucial role in the development of cholesterol gallstones [24]. Such results, however, are frequently presented in isolation, and there has been little incorporation of results from these different disease networks into any integrated model of the disease network that can explain biologic interactions in a more complete manner.

The other key missing link is the absence of system pharmacology investigation specifically for gallstones. Existing machine learning approaches mainly focus on disease prediction and biomarker identification using clinical or metabolomic datasets [67]. Such models have better classification performance, but often lack of mechanistic interpretation and pathway-oriented therapeutic analysis. Network-based drug repurposing approaches have been shown to be useful in related hepatobiliary disease, and have integrated disease-associated genes, protein interaction networks, and pathway enrichment strategies [38]. Similar biologically integrated models are not widely used in the research of gallstone disease.

The use of computational repurposing methods is also inadequate in terms of biological filtering and validation. Numerous AI/machine learning models produce massive lists of potential targets or drug associations, but fail to account for disease-specific metabolic perturbations, pathway, or systems-level validation. Reviews on AI-driven drug repurposing also highlight issues with data quality, interpretability, reproducibility, and poor biological integration [64], [68].

Integrated computational frameworks that integrate metabolomics, systems biology, network pharmacology, and therapeutic prioritization in a biologically meaningful context, therefore, still have an unmet need. These strategies could also help better identify mechanistically relevant therapeutic targets and enhance translational interpretation in the research on gallstone disease.

CHAPTER 3

METHODOLOGY

3.1 STUDY DESIGN OVERVIEW

In this study, an integrated metabolomics-driven network pharmacology and computational drug repurposing approach was adopted to screen candidate drugs for gallstone disease shown in Fig 3.1.

The untargeted serum metabolomics data were collected and pre-processed for the detection of metabolites which were significantly changed. Pathway enrichment and metabolite–gene mapping analyses were then conducted to investigate disease-associated molecular mechanisms. Then, gene–drug interaction analysis and integrated network construction was performed to prioritize candidate drugs by the network topology parameters.

The best compounds were secondly confirmed by molecular docking and molecular dynamics simulation to investigate the stability of protein–ligand binding and interaction behaviour.

This systems-level workflow facilitated the therapeutic prioritization by incorporating metabolomics, pathway biology and network pharmacology approaches, analogous to computational drug repurposing studies that have been previously reported [36], [38], [69].

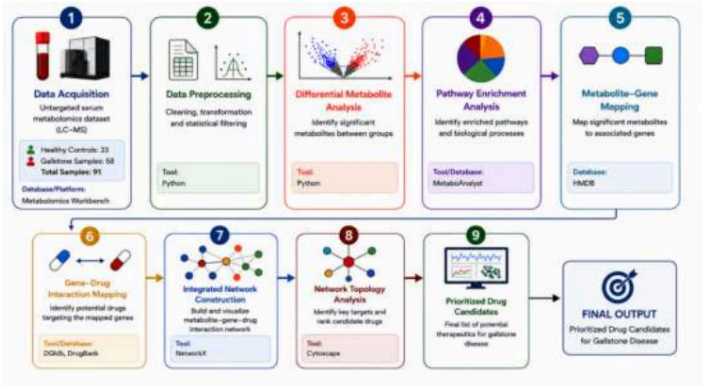


Fig 3.1. Integrated Computational Workflow

3.2 DATASET ACQUISITION

The metabolomics data set, used in this study, was acquired from the Metabolomics Workbench database (ST004186)[70]. The data were untargeted serum metabolomics profiles obtained from the serum of patients with gallstone disease and healthy controls through LC–MS metabolomics. The details and characteristics of the dataset has been given in the Table 3.1.

To ensure metabolite coverage and reliability of analyses, both positive ion mode and negative ion mode datasets were included and analysed separately during the pre-processing step. In the present analysis, the samples that were designated as LQS and LGDH were combined into a single cohort of gallstone disease to make disease-based comparisons.

Table 3.1 Dataset Characteristics

Parameter	**Description**
Database Source	Metabolomics Workbench
Dataset Accession ID	ST004186
Study Type	Untargeted serum metabolomics
Disease	Gallstone disease (Cholelithiasis)
Organism	*Homo sapiens*
Sample Type	Blood serum
Analytical Platform	LC – MS
Ion Modes	Positive and Negative ion mode
Study Group	Healthy Controls (HC), Liver Qi Stagnation (LQS), Liver-Gallbladder Damp-Heat (LQDH)
Total Samples	91 samples
Healthy Controls	33 samples
LQS Samples	30 samples
LGDH Samples	28 samples

3.3 DATA PREPROCESSING

Downstream analysis was done on the pre-processed positive and negative ion mode datasets separately. There were some duplicate entries and unknown metabolites that were removed to enhance the quality of the dataset and non-numeric features were simply discarded. Low quality features were eliminated before statistical analysis to make the data reliable. Features with more than 20% missing data were removed due to the fact that features with too much missing data cannot be reliably quantified and cause bias in downstream testing, which is a commonly used threshold applied to metabolomics literature.

In addition, a QC based relative standard deviation (RSD) filter was performed, keeping only features that had an RSD value of less than 30% in the QC samples, which would exclude features where the signal in QC samples was not reproducible enough between technical replicates and would be more likely to be instrument noise rather than true biological variation.

After filtering, any remaining missing values were replaced with half of the minimum observed intensity as is standard in metabolomics when a signal is sub-detected it is considered low abundance rather than a true absence. Samples were then normalized using Probabilistic Quotient Normalization (PQN) which corrects for between-sample differences in total metabolite concentration (including dilution effects) by scaling each sample relative to a reference profile obtained as the median of all the samples, which is more robust than simple sum normalization.

Lastly, the wide dynamic range of LC-MS intensities (from ~1,000 to more than 53 billion in the raw data) was log2 transformed to compress the range, to stabilize variance across the intensity spectrum and to make the data approximately normally distributed, which is a necessary assumption for valid parametric statistical testing of the data. Many preprocessing pipelines have been applied to metabolomics research on gallstone disease and metabolic disorders, such as [29], [67].

3.4 DIFFERENTIAL METABOLITE ANALYSIS

To find the metabolites that were significantly different between the gallstone disease samples and the healthy controls, a differential metabolite analysis was conducted. Student's t-test or Mann–Whitney U test was used to perform statistical analysis according to the characteristics of data distribution.

Statistically significant metabolites were found using fold change analysis and Benjamini–Hochberg false discovery rate correction. The metabolites with adjusted p-value < 0.05 and |log2 fold change| < 0.5 were considered as significant ones.

The expression patterns of metabolites and differential metabolite distribution between groups were visualized with volcano plots. Previous metabolomics studies of gallstone disease have reported similar statistical metabolomics workflows [52], [53].

3.5 PATHWAY ENRICHMENT ANALYSIS

The metabolites that were significantly changed in the differential analysis were analysed with MetaboAnalyst for pathway enrichment analysis[71].

Pathway impact analysis and over-representation analysis were conducted to determine and select metabolic pathways that are relevant to gallstone disease in biological context. The pathways studied were amino acid metabolism, arachidonic acid metabolism, glutathione metabolism, fatty acid metabolism and bile acid metabolism.

Pathway-level analysis was used to gain systems-level understanding of disease associated metabolic dysregulation and to facilitate metabolite to gene mapping analysis downstream. Enriching pathways has been applied in previous metabolomics and bile acid metabolism studies [14], [15], [25].

3.6 METABOLITE–GENE MAPPING

Differential metabolite analysis yielded significant metabolites, which were mapped to biologically associated genes to elucidate the molecular mechanisms impacting on gallstone disease through metabolites. The Human Metabolome Database (HMDB) database was used to find metabolite–gene associations[72].

The databases were employed to identify genes associated with altered metabolites that have been shown by experimentation to be enzymes, transporters or metabolic regulators or to play a role in signalling. Such metabolite interaction mapping methods have been used in the study of gallstones and metabolic diseases [24], [55].

After filtering, only those interactions that are biologically relevant and supported by the databases such as GWAS Catalog, Open Targets and literature were retained[73], [74]. Genes involved in bile acid transport, lipid metabolism, fatty acid oxidation and inflammatory signalling were prioritized.

Key genes associated with gallstones were included in the final curated gene list, including ABCB11, SLC10A2, NR1H4, CYP7A1, PPARA, PPARG, ABCC2, GPBAR1, CPT2 and FABP1. Analogous identification strategies based on metabolites have been described in other molecular investigations of gallstones [19], [47]. The analysis created metabolite–gene interaction tables, which were then used to perform the downstream therapeutic interaction mapping.

3.7 GENE–DRUG INTERACTION MAPPING

The genes were mapped and gene-drug interaction analysis was carried out to find compounds that would be therapeutically relevant for targeting genes associated with the formation of gallstones. The databases used to retrieve drug–gene interactions were DGIdb[75].

Only drugs approved by FDA and/or investigated by them with direct target interactions were kept for downstream analysis. New network-based therapeutic studies, such as computational drug repurposing and systems pharmacology, have been recently employed [38], [69].

Other biological filtering favoured drugs that are related to bile acid regulation, cholesterol metabolism, fatty acid metabolism, and hepatobiliary signalling. The genes targeted for higher priority were those known to interact with candidate drugs that have

been shown to be relevant to the mechanism of gallstone disease, including ABCB11, NR1H4, SLC10A2, PPARA, and PPARG.

3.8 INTEGRATED NETWORK CONSTRUCTION

An integrated metabolite–gene–drug interaction network was built to facilitate systems-level analysis of biological and therapeutic interactions of gallstone disease. The network was constructed by incorporating metabolite–gene and gene–drug interaction data in Python and NetworkX. Tripartite network pharmacology methods are also reported in systems biology studies and computational repurposing [36], [38].

The network comprised three types of nodes: metabolites, genes and drugs. Metabolite–gene and gene–drug associations are shown by interaction edges. Low connectivity nodes were removed to enhance network quality and interpretability. Pathway-focused subnetworks were generated and networks were visualized using the Cytoscape software. This produced integrated interaction networks, sub-networks and interaction topology maps which were used for downstream topology analysis.

3.9 NETWORK TOPOLOGY ANALYSIS AND DRUG PRIORITIZATION

Network topology analysis was used to identify biologically important drug candidates with high connectivity between each other within the integrated interaction network. Using the NetworkX library, the centrality analysis was performed and four major parameters of topology were calculated: degree centrality, betweenness centrality, closeness centrality, and eigenvector centrality. Topology-based prioritization methods have been quite popular in network pharmacology and computational drug repurposing research [38], [39].

Network centrality measures were normalized using the min-max normalization technique. To prioritize the candidate drugs, weighted composited score was calculated using below given formula based on their topological importance within the metabolite-gene-drug interaction network

Normalized centrality values score:

Final score = 0.3 * (Degree) + 0.2 * (Betweenness) + 0.2 * (Closeness) + 0.3 * (eigenvector)

Network topology alone is not enough to choose the final candidate for drug development as, for example, a highly connected hub could not be correlated to biliary or hepatic biology or already be clinically used for gallstone disease. To solve this, a multi-criteria scoring approach was used that combines five complementary criteria: quantitative network final score based on centrality analysis, relevance of the drug's mechanism of action to pathways that are enriched in the dataset, literature evidence of involvement in

gallstone/biliary disease from experimental or epidemiological sources, confirmed regulatory approval status and novelty relative to existing gallstone therapeutics. This combination of statistical support through network data and biological plausibility, clinical feasibility, and novelty in the field of gallstone disease management will ensure that the final prioritized candidate is both statistically and biologically supported.

CHAPTER 4

RESULTS

4.1 METABOLOMICS PREPROCESSING

To ensure high-quality data and minimize technical variation, as well as to stabilize metabolite intensity distributions, all metabolomics data were pre-processed for statistical analysis downstream. The preprocessing workflow was effective in minimizing technical variability, making samples more comparable, and stabilizing the metabolomics data for subsequent differential metabolite and pathway enrichment analyses.

The data was also stable as shown in the Table 4.1. The number of metabolite features were kept for 182 in the positive ionization mode after preprocessing, while in the negative ionization mode minimal preprocessing had an effect on the number of features (from 363 to 362). After combining the data, 544 metabolite features were obtained, showing that the preprocessing procedure resulted in a successful preservation of most metabolite information, whilst excluding signals that were less reliable.

Table 4.1 Dataset Preprocessing Summary

Step	**Feature remaining**
Raw positive metabolites	182
Raw negative metabolites	363
After preprocessing positive metabolites	182
After preprocessing negative metabolites	362
Final positive metabolites	182
Final negative metabolites	362
Combined dataset	544

4.2 DIFFERENTIAL METABOLITE ANALYSIS

Differential metabolite analysis was conducted to discover metabolites that were significantly abundant in samples of gallstone disease compared to healthy controls. The volcano plot showed good separation between significant and non-significant metabolites, suggesting that gallstone disease is related to significant metabolic dysregulation as shown in Fig 4.1. In fact, there were significant metabolites both up and

down-regulated, indicating a general metabolic imbalance, more than just biochemical changes.

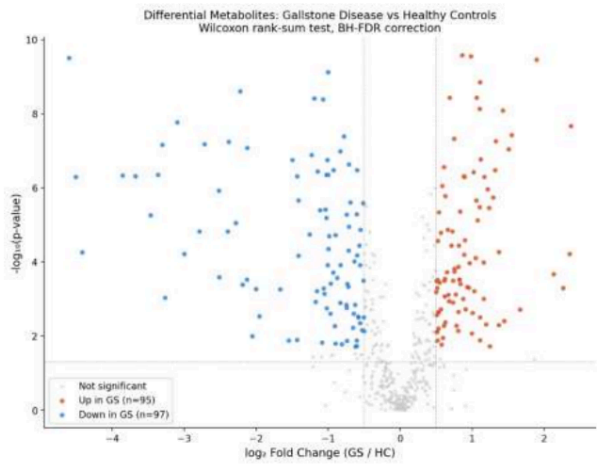


Fig 4.1 Volcano Plot of Differential Metabolite Analysis Between Gallstone Disease and Healthy Controls

The significant metabolite table showed that there were several metabolites that changed that were involved in fatty acid metabolism, amino acid metabolism, bile acid metabolism, oxidative stress, and lipid transport pathways as shown in Table 4.2 and detailed significant differential metabolites list is provided in the Appendix-I. A series of fatty acid related metabolites were found to have different abundance patterns such as palmitoleic acid, oleic acid, tetradecenoic acid and eicosadienoic acid derivatives. These metabolites are highly related to lipid utilization and cholesterol homeostasis. Cholesterol supersaturation and abnormal lipid metabolism play important roles in the development of gallstones, and the fatty acid dysregulation observed in this study is consistent with an altered hepatic and biliary lipid metabolism in gallstone disease.

Table 4.2 Significant Differential Metabolites List

Metabolite	**log2FC**	**pvalue**	**adjusted p-value**
Ursodeoxycholic acid (UDCA)	-2.191	0.0004	0.0015
Isoursodeoxycholic acid	-2.396	0.000014	0.0001
Hyodeoxycholic acid	-2.129	0.0002	0.0012

Deoxycholic acid glycine conjugate	-1.707	0.006	0.016
Glycohenodeoxycholate-3-sulfate	2.265	0.0005	0.0018
Glycoursodeoxycholate-3-sulfate	2.132	0.0002	0.0009
3β-Hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate	2.369	0.00000002	0.0000007
LysoPE(18:1/0:0)	-1.193	0.000000003	0.0000001
LysoPE(0:0/20:4)	-1.0023	0.0000000007	0.00000008
Oleic acid (FFA 18:1n9)	1.327	0.00000005	0.000001

A number of bile acid-related metabolites, such as glycoursodeoxycholic acid, hyodeoxycholic acid, isoursodeoxycholic acid, and deoxycholic acid glycine conjugates, were also significantly dysregulated. The solubility of cholesterol, composition of bile, and enterohepatic circulation are controlled by bile acids. The disturbed bile acid profiles thus indicate disturbances in biliary metabolism which can directly play a role in gallstone formation. Additionally, metabolites that indicated oxidative stress and altered amino acid metabolism were found, namely methionine sulfone and gamma-glutamyl derivatives. Large amounts of acylcarnitines also were found, demonstrating a disturbance of fatty acid oxidation and mitochondrial metabolic activity.

Microbial-host associated metabolites were also identified from the metabolite analysis, such as hippuric acid, phenol sulfate, pyrocatechol sulfate and p-cresol glucuronide. These metabolites are associated with gut microbial metabolism and may suggest that metabolism between the gut and liver is different in gallstone disease. Also, significant metabolites identified include spermidine, which indicates changes in the regulation of cell stress and inflammatory pathways.

In conclusion, the differential metabolite profile showed that there are interrelated metabolic changes linked to gallstone disease that are related to fatty acid metabolism, bile acid regulation, amino acid turnover, oxidative stress and mitochondrial metabolism. The metabolites identified demonstrated good statistical separation between disease and control groups and were used to inform downstream pathway enrichment analysis.

4.3 PATHWAY ENRICHMENT ANALYSIS

The significant metabolites identified by the differential metabolite analysis were analyzed using pathway enrichment analysis with MetaboAnalyst to identify biological pathways associated with the metabolites. The top 25 enriched pathways are plotted in a bubble plot shown in Fig 4.2 with the x-axis showing statistical significance as (−log10(p-value)), the bubble size showing the enrichment ratio, and colour ranging from yellow (least statistically significant) to deep red (most statistically significant) according to the magnitude of the p-value.

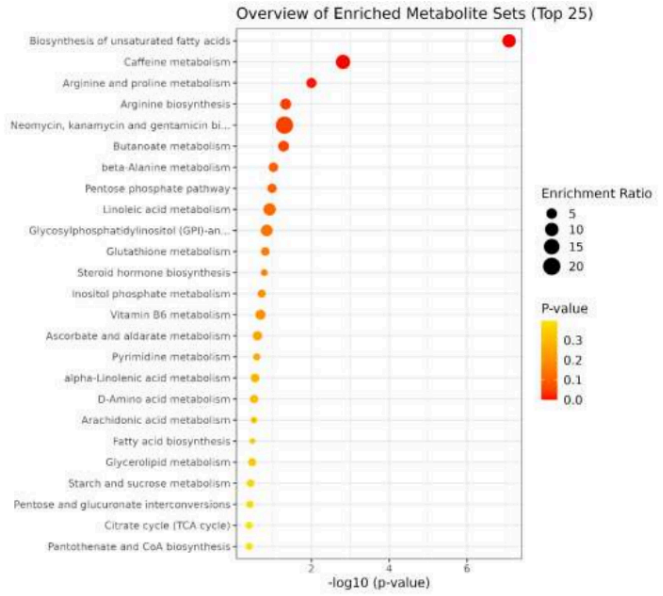


Fig 4.2. Metabolite Set Enrichment Analysis of Differential Metabolites

Biosynthesis of unsaturated fatty acids was the most statistically significant pathway, being the largest and darkest bubble at the right-hand side of the plot, with both a high enrichment ratio and a close to null p-value. Enrichment of the pathways involving amino acid metabolism (such as arginine metabolism, proline metabolism, and beta-alanine metabolism) grouped in the moderate to high significance range, reflecting their known association with metabolic regulation in the cell, response to stress, and regulation of inflammation.

These enrichments were corroborated by a differential metabolite analysis of individual metabolites, which led to the identification of metabolites that differed in abundance between control and the treated group and were associated with amino acids. In addition, glutathione metabolism was identified among enriched pathways, indicating increased oxidative stress and redox regulation in gallstone disease, which was also evidenced by the enrichment of oxidative metabolites like methionine sulfone and gamma-glutamyl-associated metabolites.

A number of further lipid metabolism pathways were enriched with lesser, still significant, effects (in the yellow to orange range): these pathways include linoleic acid metabolism, fatty acid biosynthesis, glycerolipid metabolism, and alpha-linolenic acid metabolism, all contributing to cholesterol handling dysfunction, bile composition

imbalance, and inflammatory cascades that are central to gallstone pathogenesis. The combination of the enrichment landscape shown in the bubble plot reveals a highly interconnected network of dysregulation in amino acid metabolism, the glutathione-mediated antioxidant system, and fatty acid metabolism in gallstone disease.

4.4 METABOLITE → GENE MAPPING RESULTS

Metabolite–gene mapping analysis revealed biologically relevant relationships between relevant metabolites and disease-associated genes related to bile acid transport, lipid metabolism and metabolic signalling pathways. The altered metabolites were highly associated with genes involved in bile secretion, cholesterol homeostasis, inflammatory signalling and fatty acid metabolism, as evidenced by the mapping results as shown in Table 4.3 and detailed metabolite-gene mapping table is provided in Appendix-II. These results established a systems-level connection between metabolite alterations and molecular processes related to gallstone disease.

Table 4.3 Metabolite Gene Mapping

Metabolite	**Genes**
Deoxycholic acid glycine conjugate	GLYAT, GLYATL1, GLYATL2, GLYATL3
Glycoursodeoxycholate-3-sulfate	GLYAT, GLYATL1, GLYATL2, GLYATL3
Glycochenodeoxycholate-3-sulfate	SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLCO1A2, SLCO4A1, FABP6
Isoursodeoxycholic acid	SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLCO1A2, SLCO4A1, FABP6
3beta-Hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate	SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLCO1A2, SLCO4A1, FABP6
Ursodeoxycholic acid	AKR1C1, AKR1C2, SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLC10A1A2, SLCO4A1, FABP6
Oleic acid (FFA 18:1n9)	FASN, BAAT, ACOT7, ACOT2, ACOT2, ACOT8, FAAH, MOGAT2, FAAH2, ACOT1, OLAH

Several metabolites of biological relevance were identified, which interact with genes. Deoxycholic acid was associated with ABCB11, an important bile salt export transporter for the export of bile acid into the bile. ABCB11 may affect the transport of bile acids and cholesterol balance in the bile and thus play a role in gallstone formation. Likewise,

Isoursodeoxycholic acid showed association with NR1H4, the Farnesoid X Receptor (FXR), an important regulator of bile acid synthesis and enterohepatic circulation. FXR signaling plays an important role in maintaining hepatic cholesterol and bile acid homeostasis.

Some more mapped genes were related to fatty acid metabolism and PPAR signalling pathways, which confirmed the enrichment results seen earlier. A number of metabolites also engaged genes related to oxidative stress control and inflammatory signalling pathways, indicating a link between metabolic and inflammatory dysregulation of gallstone disease.

The overall metabolite–gene mapping results showed that the genes involved in lipid metabolism, bile acid transport and inflammatory signalling pathways are closely associated with altered metabolites. These interactions improved the understanding of gallstone disease progression through a biological point of view and laid a solid groundwork for the downstream gene–drug interaction analysis and therapeutic prioritization.

4.5 GENE → DRUG MAPPING RESULTS

Within the genes that were mapped to metabolites, gene–drug interactions were analyzed to find drugs which may be of therapeutic relevance. The interaction table revealed several biologically relevant associations between disease associated genes and drugs targeting inflammatory signalling pathways, lipid metabolism and bile acid regulation as shown in Table 4.4 and detailed gene-drug mapping table is provided in Appendix-III. The results identified the potential drug repurposing opportunities for gallstone disease in terms of the systems level therapeutics targeting.

Table 4.4 Gene Drug Mapping

Gene	Drug
ABCB11	ODEVIXIBAT, ROSUVASTATIN
NR1H4	APIGENIN
SLCO1B1	ATORVASTATIN CALCIUM TRIHYDRATE, EZETIMIBE, FENOFIBRATE MICRONIZED, RIFAMPIN, ROSUVASTATIN
CYP2C9	ASPIRIN, CELECOXIB, COLCHICINE, FENOFIBRATE MICRONIZED, HESPERETIN, INDOMETHACIN, PIOGLITAZONE HYDROCHLORIDE, QUERCETIN, RIFAMPIN, SIMVASTATIN
ABCB1	ATORVASTATIN CALCIUM TRIHYDRATE, COLCHICINE,

	CURCUMIN, FENOFIBRATE MICRONIZED, RIFAMPIN, SIMVASTATIN
SLC10A2	MARALIXIBAT, MARALIXIBAT CHLORIDE, ODEVIXIBAT, ODEVIXIBAT SESQUIHYDRATE

As shown in Table 4.4, the gene–drug mapping results indicated biologically relevant associations among important disease-related genes and candidate therapeutic drugs. Bile salt export pump (ABCB11) was mapped with Rosuvastatin, and with Odevixibat, with which it has an interaction mechanism of particular interest because it is an IBAT inhibitor that targets bile acid transport. Interestingly, SLC10A2, which encodes the apical sodium-dependent bile acid transporter involved in the reabsorption of bile acids from the intestine, was only mapped to Odevixibat and its formulations, further confirming the specificity of Odevixibat's interaction with the enterohepatic circulation axis.

The gene NR1H4, which encodes the transcriptional regulator of bile acid synthesis, the Farnesoid X receptor (FXR), was mapped to Apigenin. SLCO1B1 related to Atorvastatin Calcium Trihydrate, Ezetimibe, Fenofibrate Micronized, Rifampin and Rosuvastatin was the most clustered of the drug interactions and is known as a common liver uptake transporter for several different classes of drugs.

Cytochrome P450-mediated hepatic oxidative metabolism appeared to be the predominant pathway over engagement of the disease pathway as the two most promiscuous gene nodes in the mapping were CYP2C9 and ABCB1, which showed interactions with the highest number of drugs, such as Aspirin, Celecoxib, Colchicine, Fenofibrate Micronized, Hesperetin, Indomethacin, Pioglitazone Hydrochloride, Quercetin, Rifampin, and Simvastatin.

Similarly, ABCB1 mapped to several drugs, such as Atorvastatin, Colchicine, Curcumin, Fenofibrate Micronized, Rifampin and Simvastatin, indicating P-glycoprotein-mediated efflux interactions. These broadly promiscuous metabolic gene interactions are further evidence of the mechanistic specificity of the network positioning of Odevixibat and highlight its potential to serve as the most metabolism-dysregulating candidate directly related to bile acid dysregulation pathways identified in this study.

4.6 INTEGRATED NETWORK ANALYSIS

A metabolite-gene-drug interaction network was built in Cytoscape to investigate the systems-level biological connections between gallstones and metabolites, genes and drugs, and to connect the differentially expressed metabolites with their gene targets and potential drug targets. The resulting network was highly interconnected across all three node types, highlighting the complex multi-layered structure of metabolic dysregulation in pathogenesis of gallstones.

Of particular interest in this regard were nodes with significantly higher connectivity, suggesting the existence of interaction hubs of biological relevance. The genes associated with these high-degree nodes were largely related to the biosynthesis of bile acids, lipid metabolism, fatty acid degradation and PPAR-mediated inflammatory signalling, all of which were highly enriched in the preceding metabolite set enrichment analysis (MSEA) analysis of the metabolomics data, and consistent with the interpretation of the systems-level data.

Multiple genes were identified in several drug nodes that co-targeted multiple pathway genes, indicating pathway-level therapeutic integration and no single-target engagement. The integrated network thus constructed therefore served as a computational framework to prioritize drug–target relationships relevant to biology for downstream analysis.

The integrated network was then further divided into 15 pathway-specific subnetworks each focused on a candidate drug and its direct interactors in the form of metabolites and genes, for further fine-grained analysis of individual drug–target interactions. Each of these subnetworks is described separately and the relevant biological relationships and therapeutic significance associated with each drug highlighted in its context with gallstone disease.

4.6.1 SUBNETWORK ANALYSIS OF ATORVASTATIN CALCIUM TRIHYDRATE

The Atorvastatin Calcium Trihydrate subnetwork presented a wide and complex network structure with six gene targets interacting with different sets of metabolites, all of which are associated with gallstone pathogenesis shown in Fig 4.3. The most notable interaction point was with lipid metabolites, such as arachidonic acid, palmitic acid, and linoleic acid, and metabolites of the xanthine pathway, such as caffeine, theophylline, and paraxanthine, with CYP3A4.

A prominent association of hepatic uptake transporter, SLCO1B1, with a plethora of bile acid metabolites, such as chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, and glycochenodeoxycholate-3-sulfate, highlighted its critical role in the handling of bile acids in the liver. The UGT family genes (UGT1A1, UGT1A4, UGT1A6) were correlated with metabolites involved in glucuronidation, including glucuronic acid, androsterone O-glucuronide and p-cresol glucuronide, which are associated with detoxification and conjugation activity. The activity of atorvastatin on lipid metabolism, bile acid transport and conjugation pathways are in accordance with the pleiotropic effects of atorvastatin on the liver and its ability to modulate the composition of bile and inhibit cholesterol saturation in bile [17], [32].

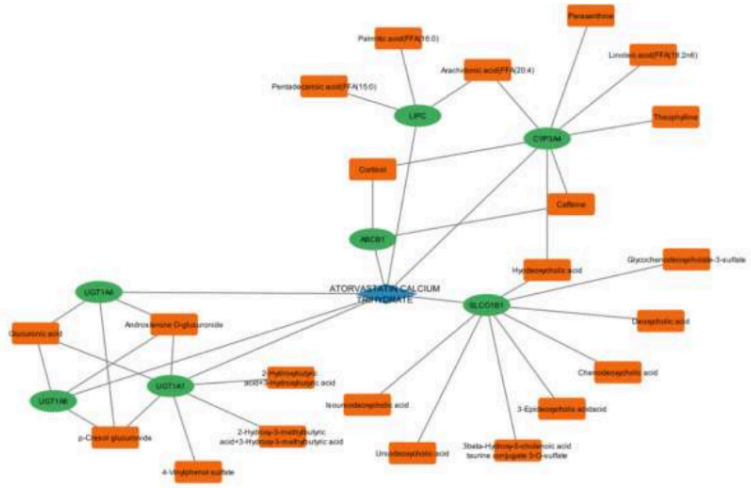


Fig 4.3 Subnetwork Analysis of Atorvastatin Calcium Trihydrate

4.6.2 SUBNETWORK ANALYSIS OF RIFAMPIN

The Rifampin subnetwork was one of the best represented of all 15 subnetworks, with five gene hubs (SLCO1B1, SLCO1B3, CYP3A4, CYP2C9, and ABCB1) that participated in a significant number of interactions with a large metabolite set of bile acid metabolism and hepatic drug processing shown in Fig 4.4. The cluster of bile acid transporters SLCO1B1 and SLCO1B3 was very densely connected and interacted with chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, isoursodeoxycholic acid, 3-epideoxycholic acid, glycochenodeoxycholate-3-sulfate, and 3beta-hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate, indicating that they were involved in the uptake of bile acids in the liver.

The CYP3A4 was found to interact with xanthine metabolites such as caffeine, theophylline and paraxanthine, as well as arachidonic acid, linoleic acid, cortisol, and hyodeoxycholic acid; the CYP2C9 and ABCB1 provided additional metabolite links. The extensive network interactions are consistent with a known mechanism of action as an agonist of the pregnane X receptor (PXR), which transcriptionally regulates the expression of the related proteins such as CYP3A4, drug transporters, and bile acid conjugating enzymes, and are in line with its known clinical efficacy in cholestatic liver disease, driven by improved hepatic clearance of bile acids [76], [77].

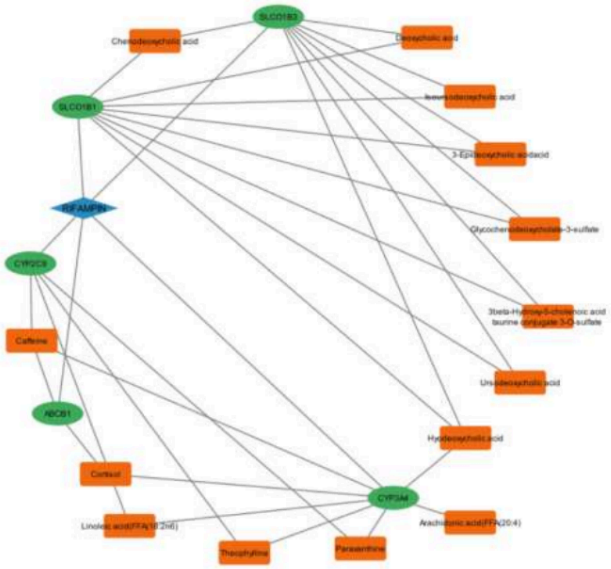


Fig 4.4 Subnetwork Analysis of Rifampin

4.6.3 SUBNETWORK ANALYSIS OF ROSUVASTATIN

The Rosuvastatin subnetwork had one of the largest connectivity profiles of any of the 15 subnetworks, with four major gene hubs (SLCO1B1, SLC10A1, ABCB11, CYP3A4) interacting with a large number of metabolites, representing bile acid metabolism and hepatic lipid processing shown in Fig 4.5. Both SLC1B1 and SLC10A1 were associated with a cluster of bile acid metabolites that share common proteins, such as deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, isoursodeoxycholic acid, 3-epideoxycholic acid, glycochenodeoxycholate-3-sulfate, and 3beta-hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate, indicating their overlapping roles as hepatic organic anion transporters that facilitate sinusoidal bile acid uptake.

ABCB11 was involved in a shared interaction with these transporters, confirming the dimension of canalicular bile acid secretion of the subnetwork, while CYP3A4 had extended interactions with xanthine metabolites and polyunsaturated fatty acids including arachidonic acid, linoleic acid, caffeine, theophylline and cortisol. This multi-transporter interaction is in line with the known pharmacokinetics of rosuvastatin as a

SLCO1B1 substrate, and with previous observations of effects on hepatic bile acid transport that might help prevent the development of gallstones [17], [32].

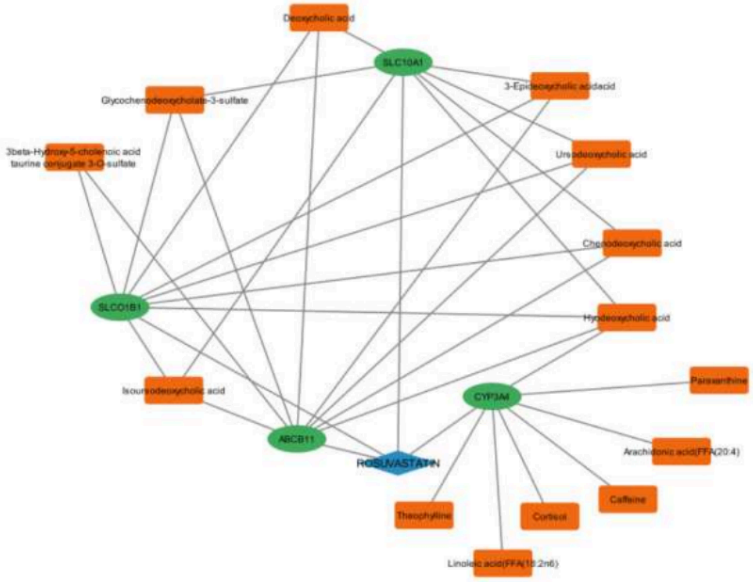


Fig 4.5 Subnetwork Analysis of Rosuvastatin

4.6.4 SUBNETWORK ANALYSIS OF FENOFIBRATE MICRONIZED

The Fenofibrate Micronized subnetwork showed a well-distributed interaction topology among four gene hubs (SLCO1B1, CYP3A4, CYP2C9 and ABCB1), which interact with the compound and its metabolites related to bile acid transport and hepatic lipid metabolism shown in Fig 4.6. SLCO1B1 was the most central metabolite hub with the highest number of metabolite connections, interacting with a broad range of bile acid metabolites such as chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, isoursodeoxycholic acid, 3-epideoxycholic acid, glycochenodeoxycholate-3-sulfate, and 3beta-hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate, indicative of its important role in hepatic bile acid uptake.

CYP3A4 and CYP2C9 interacted with the polyunsaturated fatty acids such as arachidonic acid, linoleic acid, theophylline, paraxanthine and caffeine, and ABCB1 with cortisol. Mechanistically, this network architecture fits the PPARα agonistic properties of fenofibrate, which inhibit hepatic triglyceride synthesis, stimulate fatty acid oxidation and modulate bile acid composition — although, importantly, fenofibrate has been shown to stimulate biliary cholesterol secretion in some circumstances, which should be taken into account when it comes to its potential therapeutic use in gallstones [78].

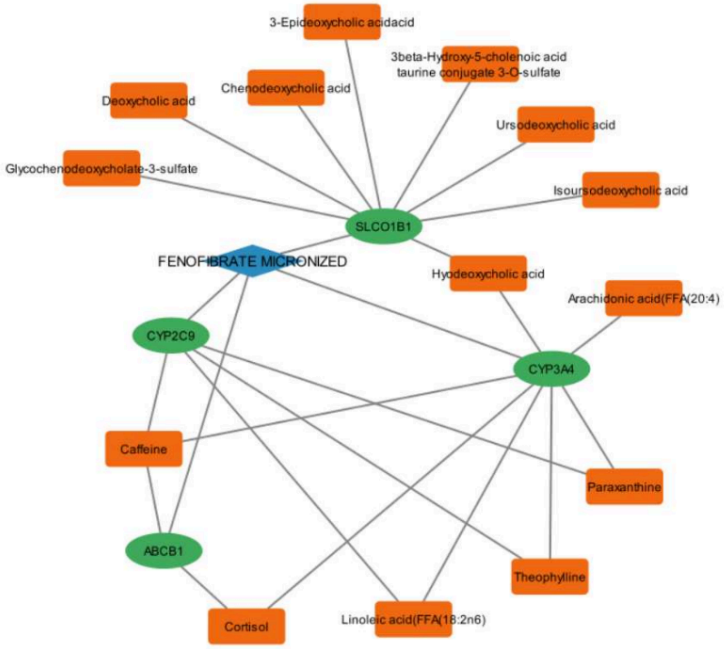


Fig 4.6 Subnetwork Analysis of Fenofibrate Micronized

4.6.5 SUBNETWORK ANALYSIS OF ASPIRIN

The Aspirin subnetwork was organized around four gene hubs — CYP3A4, CYP2C9, PTGS2 and UGT1A6 — all related to metabolic pathways that involve aspirin and inflammation, as well as detoxification pathways that are known to be involved in gallstone disease shown in Fig 4.7. Both CYP3A4 and CYP2C9 bound to a set of xanthine metabolites and lipid-derived fatty acids, such as arachidonic acid and linoleic

acid, in accordance with their common functions in the metabolism of drugs and lipids in the liver. PTGS2, which encodes cyclooxygenase-2, was directly linked to the levels of arachidonic acid and eicosapentaenoic acid, major intermediates in the prostaglandin biosynthesis pathway, thus illustrating the above-mentioned mechanism of irreversible inhibition of PTGS2 by Aspirin and the subsequent inhibition of pro-inflammatory eicosanoids.

The metabolites of glucuronidation, such as glucuronic acid, androsterone O-glucuronide and p-cresol glucuronide, were associated with UGT1A6, demonstrating hepatic phase II conjugation activity. This multi pathway interaction is in keeping with epidemiological and experimental evidence of protective effects of regular aspirin on the risk of gallstones; these are mediated by the ability to inhibit bile prostaglandin synthesis and modulate bile cholesterol crystallization [79].

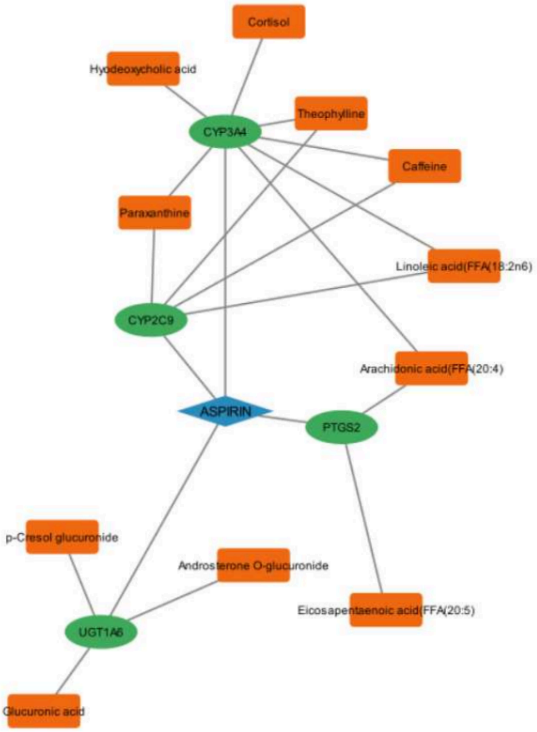


Fig 4.7 Subnetwork Analysis of Aspirin

4.6.6 SUBNETWORK ANALYSIS OF CURCUMIN

The Curcumin subnetwork had a multi-hub architecture with four gene nodes (CYP3A4, XDH, ABCB1, and PTGS2) that linked the drug to a large network of metabolites involved in lipid metabolism, xanthine catabolism, and inflammatory pathways shown in Fig 4.8. In the case of CYP3A4 interactions were observed with fatty acid and xanthine related metabolites such as arachidonic acid, linoleic acid, caffeine, theophylline, paraxanthine whereas in XDH, purine catabolism metabolites like inosine, spermine, theobromine and paraxanthine were seen which are indicative of oxidative metabolism by xanthine oxidase.

ABCB1 bound Curcumin and related to the effects on cortisol, indicating modulation of drug efflux and steroid transport, whereas PTGS2 bound arachidonic acid and eicosapentaenoic acid, indicating anti-inflammatory activity by inhibiting COX-2. The wide network interactions of Curcumin are consistent with its already well-documented pleiotropic bioactivities, including anti-inflammatory, antioxidant, choleretic properties, which have been shown in previous studies to have a direct impact on gallstone prevention by reducing the concentration of cholesterol in the bile, increasing bile flow, and decreasing gallbladder inflammation [80], [81], [82].

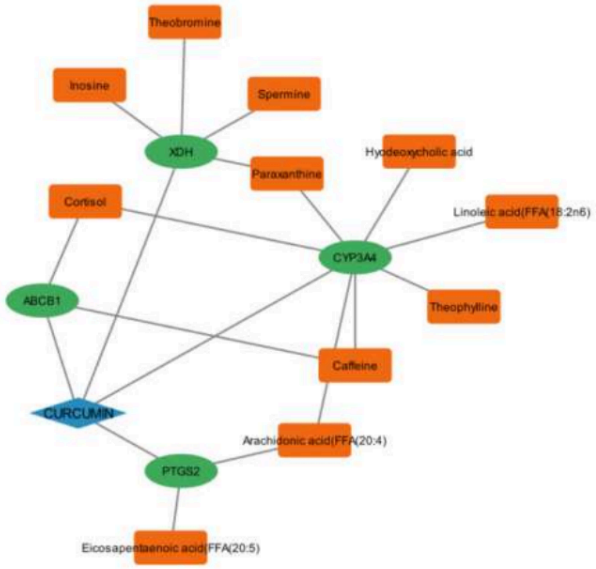


Fig 4.8 Subnetwork Analysis of Curcumin

4.6.7 SUBNETWORK ANALYSIS OF SIMVASTATIN

The Simvastatin subnetwork was found to have a compact but well-connected topology with four gene nodes (CYP3A4, CYP2C9, LIPC, ABCB1) that were interconnected and also linked together in the dense cluster of metabolite interactions shown in Fig 4.9. Given the diversity of lipids and metabolites involved in the metabolism of this drug, as well as xanthine-related metabolites like caffeine, theophylline, and paraxanthine, CYP3A4 and CYP2C9 had multiple metabolites in common.

Both LIPC and ABCB1 interacted with fatty acid metabolites, indicating potential roles in transporting and regulating fatty acid efflux. The subnetwork topology is consistent with the known effects of Simvastatin on hepatic lipid metabolism and bile acid composition, which are known to be beneficial for decreasing the risk of cholesterol gallstone formation [17], [32].

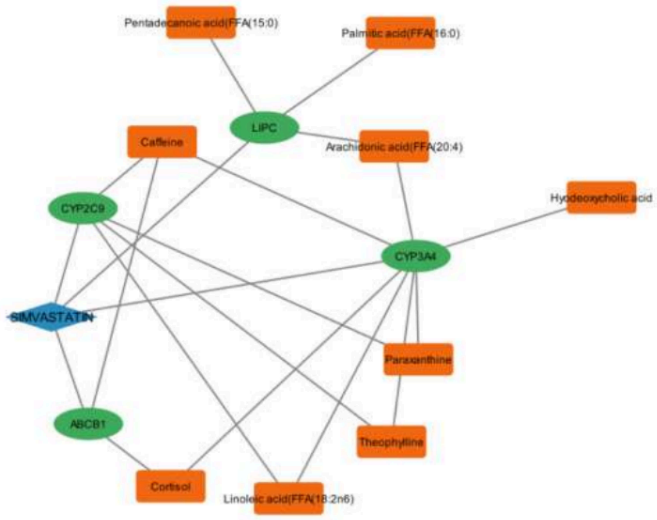


Fig 4.9 Subnetwork Analysis of Simvastatin

4.6.8 SUBNETWORK ANALYSIS OF QUERCETIN

The Quercetin subnetwork showed a multipoint hub architecture involving three gene nodes (CYP3A4, CYP2C9, XDH) that linked the drug to metabolites involved in hepatic lipid metabolism, xanthine catabolism and polyamine pathways shown in Fig 4.10. The most highly connected hub was CYP3A4, which interacted with arachidonic acid, hyodeoxycholic acid, cortisol, caffeine, theophylline, paraxanthine, and linoleic acid, and CYP2C9 was also highly connected, interacting with caffeine, theophylline, paraxanthine and linoleic acid, indicating the extensive metabolism of this abundant dietary flavonoid through cytochrome P450.

XDH showed a strong binding with paraxanthine, spermine, theobromine and inosine, which corresponds to the known inhibitory effect of Quercetin on the activity of xanthine oxidase and its antioxidant properties, which are achieved by the ability to inhibit the generation of reactive oxygen species. The ability of Quercetin to interact with both cytochrome P450 enzymes and XDH puts it in the crosshairs of hepatic lipid metabolism and oxidative stress that are both linked to the pathogenesis of gallstones, and evidence from experimental models of cholesterol gallstone formation has shown that Quercetin lowers biliary cholesterol saturation, decreases hepatic oxidative damage, and alters bile acid composition [83], [84].

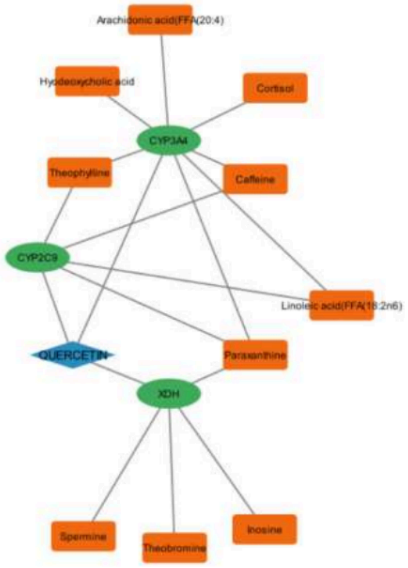


Fig 4.10 Subnetwork Analysis of Quercetin

4.6.9 SUBNETWORK ANALYSIS OF INDOMETHACIN

Indomethacin subnetworks demonstrated significant structural similarity to Aspirin subnetworks, each of which focused on three gene nodes: CYP3A4, CYP2C9 and PTGS2, which were involved in common sets of metabolites that are involved in hepatic lipid metabolism and inflammatory signalling shown in Fig 4.11. The cytochrome P450-mediated metabolic processing of xanthine derivatives (caffeine, theophylline, paraxanthine) and of polyunsaturated fatty acids such as arachidonic acid and linoleic acid was the shared cytochrome P450-mediated metabolic processing.

PTGS2 was the predominant pharmacological target node that was linked to arachidonic acid and eicosapentaenoic acid, reflecting the effect of Indomethacin, which has a strong inhibitory effect against cyclooxygenase enzymes, and hence prostaglandin biosynthesis. Aspirin lacks glucuronidation associated gene nodes as compared to the other NSAIDs, which may be due to variations in metabolic fate and conjugation pathways of these two drugs. The network topology is consistent with the biology behind the mechanism of COX inhibition as relevant to gallstone pathogenesis, including the actions of prostaglandin to promote gallbladder smooth muscle relaxation and secretion of mucin, both of which are factors favouring gallstone nucleation [79].

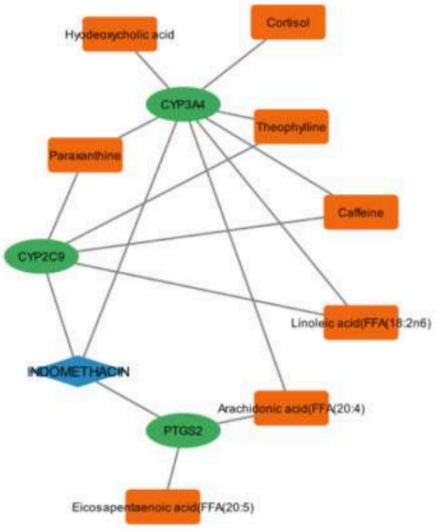


Fig 4.11 Subnetwork Analysis of Indomethacin

4.6.10 SUBNETWORK ANALYSIS OF CELECOXIB

The Celecoxib subnetwork was structurally similar to the Indomethacin network, and the three main gene hubs (CYP3A4, CYP2C9 and PTGS2) connecting the drug to the relevant metabolite groups were the same shown in Fig 4.12. The metabolism of this selective COX-2 inhibitor is characteristic of liver, where CYP3A4 and CYP2C9 form a shared metabolite pool for the metabolism of caffeine, theophylline, paraxanthine, cortisol, arachidonic acid, linoleic acid and hyodeoxycholic acid. Celecoxib's selective cyclooxygenase-2 inhibitory mechanism and its downstream attenuation of pro-inflammatory prostaglandin synthesis, led to the engagement of arachidonic acid and eicosapentaenoic acid in the course of the investigation of PTGS2.

In contrast to non-selective NSAIDs, Celecoxib is uniquely connected to the network because of its preferential binding to PTGS2, while at the same time binding to other cytochrome P450 enzymes involved in the network. This is supported by the fact that selective COX-2 inhibition could be beneficial in decreasing biliary inflammation and mucin hypersecretion that are thought to play a role in gallstone nucleation without gastrointestinal side effects seen with the non-selective NSAIDs [79].

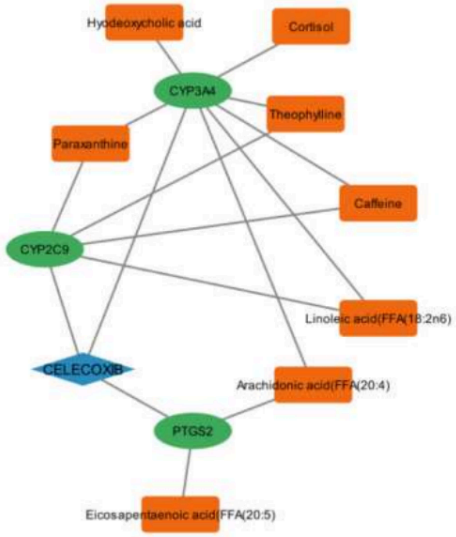


Fig 4.12 Subnetwork Analysis of Celecoxib

4.6.11 SUBNETWORK ANALYSIS OF COLCHICINE

The Colchicine subnetwork was structured around three gene hubs: CYP3A4, CYP2C9 and ABCB1, which were tightly interconnected and shared a common set of metabolites, all associated with hepatic drug metabolism and inflammation shown in Fig 4.13. CYP3A4 has the greatest number of interactions with other genes, including with hyodeoxycholic acid, arachidonic acid, paraxanthine, caffeine, theophylline, linoleic acid and cortisol, consistent with CYP3A4's role as the major enzyme in the oxidative metabolism of Colchicine in the liver. CYP2C9 had multiple interactions with metabolites such as caffeine, paraxanthine, linoleic acid, and cortisol, while ABCB1 (P-glycoprotein) interacted with cortisol, suggesting the established association of P-glycoprotein with Colchicine efflux transport.

The nodes that do not occur in the subnetwork of anti-inflammatory drugs, including the bile acid transporter nodes, are absent in the Colchicine subnetwork, suggesting that it acts mechanistically in a different way from the anti-inflammatory NSAIDs and bile acid targeting drugs; instead, the topology of the nodes is more consistent with its tubulin-polymerization inhibitory mechanism and with its established anti-inflammatory properties, attributed to suppression of NLRP3 inflammasome activation, which has been proposed as a relevant pathway in the process of biliary inflammation and cholesterol crystallization in gallstone disease [85].

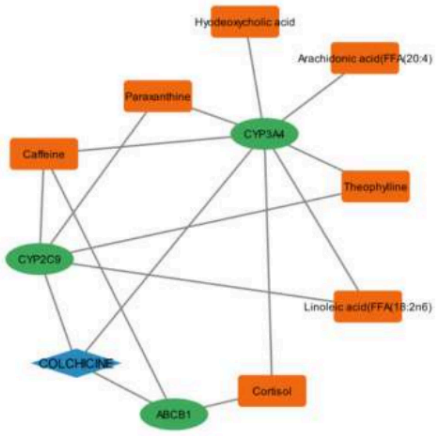


Fig 4.13 Subnetwork Analysis of Colchicine

4.6.12 SUBNETWORK ANALYSIS OF PIOGLITAZONE HYDROCHLORIDE

The Pioglitazone Hydrochloride subnetwork was organized around three gene hubs — CYP3A4, CYP2C9 and LPL — that link the drug to metabolites that are products of hepatic cytochrome P450-mediated metabolism and lipid hydrolysis shown in Fig 4.14. These enzymes CYP3A4 and CYP2C9 interacted with the xanthine-pathway metabolites, such as caffeine, theophylline, and paraxanthine, as well as arachidonic acid and linoleic acid, cortisol, hyodeoxycholic acid, all of which are known to undergo extensive hepatic CYP-mediated oxidative metabolism, consistent with the pattern of hepatic CYP-mediated oxidative metabolism typical of thiazolidinediones.

LPL, involved in the coding of lipoprotein lipase, had a peripheral, but biologically important, interaction with palmitic acid, which is known to be PPARγ-mediated upregulation of LPL activity and as such, its effects on triglyceride hydrolysis and free fatty acid redistribution. This multi-enzyme network architecture is in keeping with the known action of Pioglitazone as a PPARγ agonist, a molecule which acts on lipid metabolism in the liver and peripheral tissues; evidence suggests that activation of PPARγ may be of relevance to gallstone pathogenesis due to its role in modulating bile acid synthesis and cholesterol homeostasis [86].

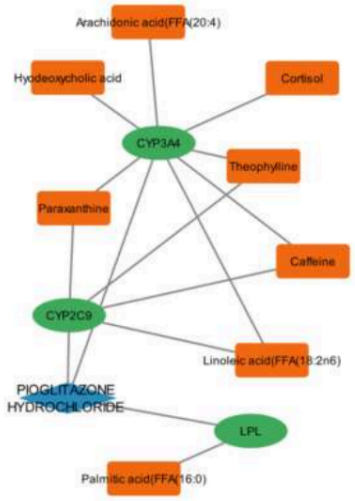


Fig 4.14 Subnetwork Analysis of Pioglitazone Hydrochloride

4.6.13 SUBNETWORK ANALYSIS OF ODEVIXIBAT

The Odevixibat subnetwork was organized into a gene network of three gene nodes (ABCB11, SLC10A2, ATP8B1) that were clustered in a focused subnetwork of metabolites, most of which were bile acids shown in Fig 4.15. ABCB11 (encoding bile salt export pump (BSEP)) and SLC10A2 (encoding the apical sodium-dependent bile acid transporter (ASBT)) were both highly connected to other bile acid metabolites, such as but not limited to ursodeoxycholic acid, chenodeoxycholic acid, deoxycholic acid, isoursodeoxycholic acid, 3-epideoxycholic acid, glycochenodeoxycholate-3-sulfate, and 3beta-hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate, which is consistent with their known involvement in canalicular bile acid secretion and intestinal bile acid reabsorption, respectively.

ATP8B1 made one peripheral interaction with LysoPE(16:0p/0:0) indicating its potential role in membrane phospholipid asymmetry and hepatocyte membrane integrity in the presence of bile acid. The subnetwork topology supports Odevixibat's proposed mechanism as an ASBT inhibitor, which disrupts enterohepatic bile acid recycling, lowers pool recirculation of bile acids, and thus may be able to restore the dysregulated bile acid homeostasis at the core of gallstone pathogenesis [87].

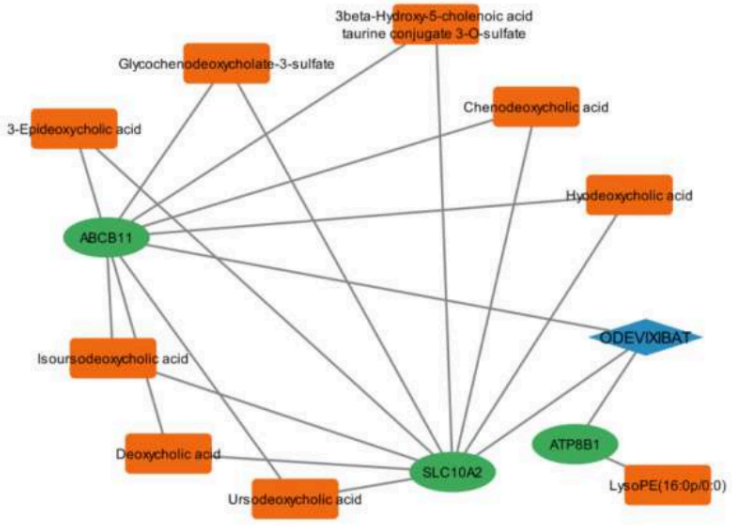


Fig 4.15 Subnetwork Analysis of Odevixibat

4.6.14 SUBNETWORK ANALYSIS OF HESPERETIN

The Hesperetin subnetwork was one of the smaller networks of the 15 that clustered around two gene hubs, CYP3A4 and CYP2C9, and had a tightly knit group of xanthine and lipid-derived metabolites shown in Fig 4.16. Hepatic metabolism of this flavonoid compound CYP3A4 emerged as the main interaction node, which was connected to hyodeoxycholic acid, arachidonic acid, cortisol, theophylline, caffeine, paraxanthine, and linoleic acid.

There were a number of metabolite relationships shared between CYP2C9 an87d CYP3A4, such as, for caffeine, the presence of paraxanthine and theophylline and for linoleic acid. Hesperetin's subnetwork differs from the bile acid transporter and COX pathway gene nodes and is related to the metabolites of bile acids and lipids, which is in line with reports that dietary flavonoids modulate hepatic lipid metabolism, decrease oxidative stress, and have choleretic activity and may be protective against the formation of cholesterol gallstones [83], [84].

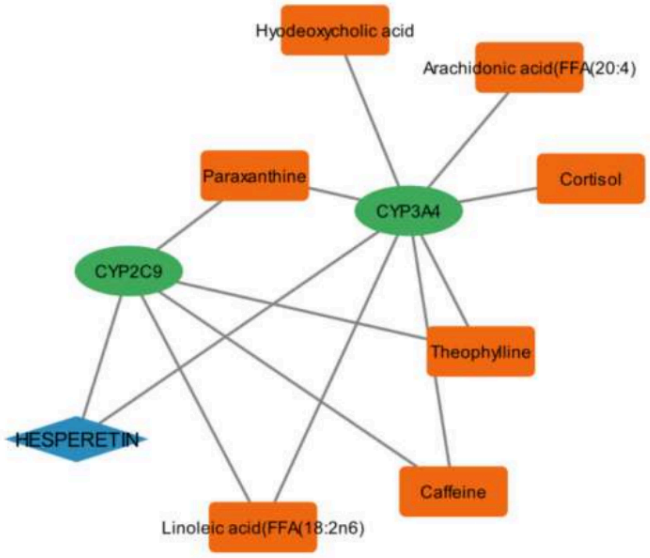


Fig 4.16 Subnetwork Analysis of Hesperetin

4.6.15 SUBNETWORK ANALYSIS OF APIGENIN

The Apigenin subnetwork was composed into two gene nodes: one for hepatobiliary regulation (CYP3A4) and one for metabolic regulation (NR1H4) Fig 4.17. CYP3A4 was the major hub, where it interacted with a large number of metabolites such as arachidonic acid, hyodeoxycholic acid, linoleic acid, caffeine, theophylline, paraxanthine, cortisol and theophylline, which were typical of hepatic oxidative metabolism mediated by CYP3A4. The farnesoid X receptor (FXR) encoded by NR1H4 was specifically associated with deoxycholic acid, and provides a biologically relevant interaction with the major nuclear receptor involved in bile acid synthesis and enterohepatic circulation.

The activation of FXR has been shown to inhibit the synthesis of bile acid in the liver, to increase the expression of canalicular bile acid transporters and to decrease secretion of biliary cholesterol, all of which are direct mechanisms relevant to cholesterol gallstone prevention. The ability of Apigenin to simultaneously target CYP3A4 and FXR is in line with the growing evidence of the hepatoprotective and choleretic effects of Apigenin via FXR-mediated transcriptional regulation of bile acid [83], [84].

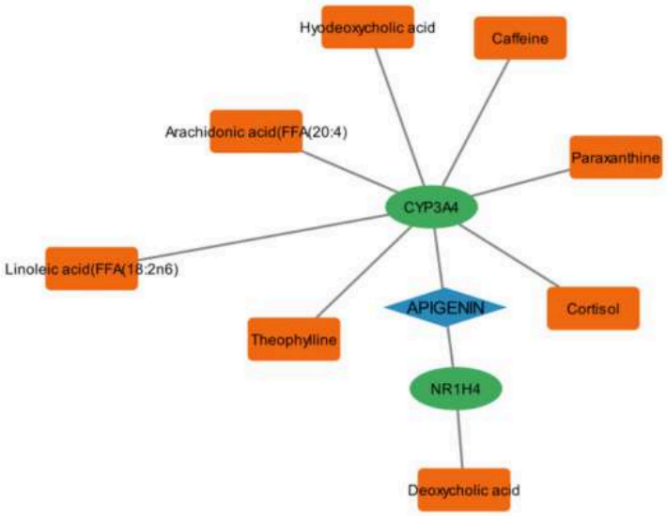


Fig 4.17 Subnetwork Analysis of Apigenin

4.7 NETWORK TOPOLOGY ANALYSIS AND DRUG PRIORITIZATION

Interaction connectivity and centrality were used for network topology analysis to select therapeutically relevant candidate drugs for gallstone disease, based on the integrated metabolite-gene-drug network. The built network revealed several drugs to be well-positioned in terms of connectivity within the interaction network, which suggested their potential involvement in important metabolic and signalling pathways involved in the pathogenesis of gallstones. The top ranked compounds were linked to bile acid transport, lipid metabolism, fatty acid degradation and PPAR-mediated signalling pathways which were biologically relevant pathways that are associated with the formation and progression of gallstones.

The prioritized drugs were shown in Table 4.5. A final score was created by normalizing the four metrics of network which are degree, betweenness, closeness and eigenvector centrality to rank the drugs. More the degree centrality means several interactions with disease-associated genes, while more betweenness centrality means important pathway bridging role in the network. Likewise, closeness and eigenvector centrality reveal higher global connectivity and overall influence of the integrated biological system.

Table 4.5 Prioritized Drug Based on Network Centrality

Drug	**Degree**	**Betweenness**	**Closeness**	**Eigenvector**	**Final score**
ATORVASTATIN CALCIUM TRIHYDRATE	1	1	1	0.675	0.9025
RIFAMPIN	0.666667	0.197866	0.760176	0.966652	0.681604
ROSUVASTATIN	0.5	0.217778	0.800308	1	0.653617
FENOFIBRATE MICRONIZED	0.5	0.095673	0.74063	0.708811	0.529904
ASPIRIN	0.5	0.183173	0.70253	0.375337	0.439742
CURCUMIN	0.5	0.186001	0.683961	0.335151	0.424538
SIMVASTATIN	0.5	0.04981	0.612702	0.429049	0.411217
QUERCETIN	0.333333	0.094635	0.612702	0.336321	0.342364
INDOMETHACIN	0.333333	0.045139	0.612702	0.350445	0.336702
CELECOXIB	0.333333	0.045139	0.612702	0.350445	0.336702

COLCHICINE	0.333333	0.007211	0.562227	0.397907	0.33326
PIOGLITAZONE HYDROCHLORIDE	0.333333	0.088916	0.578783	0.320269	0.329621
ODEVIXIBAT	0.333333	0.215745	0.063559	0.505074	0.307383
HESPERETIN	0.166667	0.001796	0.545932	0.312245	0.253219
APIGENIN	0.166667	0.070145	0.562227	0.222525	0.243232

All 15 candidate drugs exhibited network interactions with biologically relevant groups and metabolite–gene–drug network centrality scores were not enough for the selection of further drugs to be studied. Four other criteria were used to assess each drug on a case-by-case basis, including: Mechanism-specific relevance to gallstone pathogenesis; Clinical safety profile; Therapeutic novelty in relation to gallstone disease; Availability of supportive literature evidence. The remaining section outlines the reasoning behind the exclusions of each of the drugs, and the justification for Odevixibat as the last selected drug.

The highest composite score (0.903) was obtained by atorvastatin calcium trihydrate, which obtained perfect degree centrality, betweenness centrality and closeness centrality. However, it has an inconsistent body of evidence for gallstones. A recent meta-analysis of 590,086 patients revealed that statins actually had a very weak association with decreasing the incidence of gallstones, with patients using statins for short and medium terms of use having a paradoxically increased risk of gallstones. Moreover, Atorvastatin's mechanism of action is mainly HMG-CoA reductase inhibition and there is not any novelty and therapeutic specificity in its use for dyslipidaemia and gallstone disease.

Rifampin was ranked 2nd (0.682) and had high eigenvector centrality (0.967) indicating wide hepatic transporter interactions. Not used for the treatment of biliary disease, however, Rifampin is an antibiotic mainly used in the treatment of tuberculosis. It has been reported to cause hepatotoxicity upon chronic consumption via endoplasmic reticulum stress mediated mechanisms, and is known to cause the suppression of bile salt export pump (BSEP/ABCB11), counterintuitively increasing bile acid levels in the liver. It not only has an antimicrobial indication, but a well-documented hepatotoxic potential, making it unsuitable as a possible gallstone therapeutic candidate.

Rosuvastatin had the highest eigenvector centrality (1.000) and high closeness centrality (0.800) and was ranked third in terms of their scores (0.654), indicating a strong connection to bile acid transporter genes such as SLCO1B1, SLC10A1, and ABCB11. Unlike Atorvastatin, Rosuvastatin is a member of the statin class and its mechanism of action is to inhibit cholesterol synthesis in the liver; this act directly on an enterohepatic circulation that was identified as a main driver of the dysregulated pathway by the metabolomics data from this study. There is the same degree of inconsistency with it found in all statins and it has no new mechanism of action beyond other statins that have already been ruled out in the context of gallstone prevention.

The fenofibrate micronized was ranked fourth (score: 0.530), and is known to be a PPARα agonist with known actions on bile acid glucuronidation and lipid metabolism. The treatment of fibates, however, has been known to cause compositional changes in the biliary lipids and bile acid, and fibates, such as fenofibrate, are known to have an effect on stimulating the secretion of cholesterol into the bile — an effect that is detrimental in the case of cholesterol gallstone disease. It is used mainly to treat dyslipidaemia and cholestasis of the liver (like primary biliary cholangitis) with a different mechanism of action to the bile acid transport impaired by the development of gallstones.

Aspirin was the fifth (score: 0.440) and it is the most important target of aspirin in terms of irreversible cyclooxygenase inhibition, COX-2 (PTGS2). There is some epidemiological evidence that aspirin can decrease the risk of gall stones by decreasing the production of PGs in the bile, but the supporting evidence is only limitedly observational and inconclusive, and the known gastrointestinal toxicity profile of aspirin—most prominently, ulceration and bleeding— poses a significant risk in patients with hepatobiliary disease. Does not directly target the bile acid transport axis that was the focus of this study.

Curcumin was found to be multi-hub network engaging as a top-six member of the network (score: 0.425) and was found to engage PTGS2, CYP3A4, XDH, and ABCB1. However, Curcumin suffers from very low oral bioavailability, due to its very low aqueous solubility, fast metabolism and high first-pass elimination. These limitations of pharmacokinetics have always limited its clinical translation and till now there is no validated pharmaceutical formulation of curcumin for therapeutic use in a biliary disease.

Simvastatin was the seventh ranked drug (0.411), and shares the same mechanistic and evidential limitations as Atorvastatin and Rosuvastatin, which are all HMG-CoA reductase inhibitors (statins). Other than the ambiguous gallstone data observed in statins, Simvastatin has a narrower bile acid transporter interaction profile than Rosuvastatin, and offers no further mechanistic rationale. It is excluded straight from the statin class exclusions mentioned earlier.

Quercetin scored at the 8th place (0.342), binding to CYP3A4, CYP2C9 and XDH and its xanthine and lipid metabolites. Quercetin is a dietary flavonoid that has been shown to exhibit hepatoprotective and antioxidant effects in animal studies; however, it does not have a Drug License in any country or region. However, due to its bioavailability after oral administration (which is not constant and is poor in humans) with no clinical trial data in the field of gallstone disease at this time it is not suitable as a compound to be therapeutically used at this point of development.

Indomethacin was the ninth (score: 0.337) with non-selective COX inhibition through CYP3A4, CYP2C9 and PTGS2. Indomethacin inhibits both COX-1 and COX-2, and is a non-selective NSAID, the most potent gastrointestinal toxin in its class. It has been reported to be associated with important gastrointestinal ulcer, renal failure and hepatotoxicity, which makes it unsuitable as a candidate for a chronic biliary disease condition, like gallstone disease. It interacts with many of the same targets as Aspirin and Celecoxib, and does not provide much specificity on its mechanism.

Celecoxib was the 10th compound out of 5025 compounds screened as a selective COX-2 inhibitor that engaged PTGS2 and CYP3A4 and CYP2C9 (score: 0.337). While

Celecoxib is not associated with gastrointestinal effects, the drug has proven cardiovascular side effects with the use of long-term therapy, especially in patients with a high metabolic risk in whom the drug is being used. The mechanism of action is not directly involved in the bile acid transport and/or the cholesterol maladjustment in bile central to the pathogenesis of gallstones in this study like Aspirin and Indomethacin.

Colchicine was the eleventh (0.333) drug that was found to interact with the enzymes CYP3A4, CYP2C9 and ABCB1. Colchicine is an anti-inflammatory drug mostly used to treat gout and pericarditis, which works by inhibiting the polymerization of tubulin and the suppression of the NLRP3 inflammasome. Inflammatory pathways are involved in gallbladder disease, but the mechanism of action of Colchicine is not related to the bile acid synthesis, transport or cholesterol secretion pathways directly involved in gallstone disease. Its network connectivity was mostly due to metabolic interactions with CYP, rather than due to specific pathways, providing a weak biological basis for further exploration in this context.

Pioglitazone Hydrochloride was twelfth (0.330) and is a PPARγ agonist that acts on CYP3A4, CYP2C9 and LPL. Thus, although the activation of PPARγ can influence hepatic lipid metabolism and some benefits in patients with NAFLD have been demonstrated, its well-documented side effects of weight gain, fluid retention and increased bladder cancer risk with chronic administration are serious concerns for safety. In addition, it has only been effective in the treatment of Type 2 Diabetes Mellitus (T2DM) and relevance to the particular bile acid transport defect found in gallstone disease is likely to be indirect, if present at all.

Hesperetin was ranked fourteenth (0.253), it had only limited metabolism pathways and metabolized by CYP3A4 and CYP2C9. Hesperetin is a dietary flavanone that does not have a drug approval and has limited and variable in vivo bioavailability. The subnetwork was one of the smallest networks of the fifteen networks studied with no interaction with any disease-specific gene nodes (bile acid transporters or FXR-related regulators). Due to its small network engagement and the lack of clinical evidence in the case of gallstone disease, further prioritization is not warranted.

Apigenin was the last (15th) (score: 0.243), and only interacted with two proteins, namely CYP3A4 and NR1H4 (FXR). The interaction of FXR is biologically significant as FXR controls bile acid synthesis and is a legitimate target for pharmacological manipulation, however the dietary flavone Apigenin has not been regulated, has low bioavailability and there is no clinical evidence for it in gallstone disease. It has the lowest score in the multi-criteria framework, as well as regulatory and pharmacokinetic restrictions, resulting in it being the lowest in the prioritizing hierarchy.

For these reasons, Odevixibat was chosen as the final candidate, as it was uniquely targeted to the correct mechanism, had direct targets on the most disease-relevant gene nodes in the network (ABCB11 and SLC10A2), had a growing clinical evidence base, and met all five multi-criteria selection parameters including its therapeutic novelty in the treatment of gallstone disease. Odevixibat is a selective IBAT inhibitor, thus, directly inhibiting enterohepatic bile acid recirculation at the intestinal level, decreasing bile acid reabsorption and terminating the biliary cholesterol supersaturation that is the underlying cause of gallstone nucleation. New data has been published which has demonstrated the effectiveness of IBAT inhibition in lowering systemic bile acid and its safety in

cholestatic conditions. Critically, Odevixibat has not been investigated or applied in cholesterol gallstone disease, meeting novelty requirement and resulting in a most rational choice for further computational analysis in this study, a drug that is mechanistically precise, clinically approved, and has a directly relevant biological rationale.

CHAPTER 5

CONCLUSION AND FUTURE SCOPE

5.1 CONCLUSION

In this work, metabolomics guided computational framework for drug repurposing was used to search for potential therapeutic drugs for gallstone disease. Gallstone disease is a complex hepatobiliary disorder which is closely connected with cholesterol metabolism disturbances, bile acid homeostasis, lipid metabolism, inflammation and metabolic imbalance. Having limited effective pharmacological therapies for disease prevention and disease progression, the study was directed towards elucidating disease-associated molecular mechanisms and prioritizing potential disease targets for therapeutic selection by systems-level computational approaches.

The study started with pre-processing and differential metabolite analysis of serum metabolomics data (untargeted) from gallstone disease patients and healthy controls. The metabolites related to fatty acid metabolism, bile acid metabolism, amino acid metabolism, oxidative stress and gut microbial metabolism were found to have significant metabolic changes. These results showed that cholesterol accumulation is not the only part of gallstone disease and there is extensive metabolic dysregulation. The metabolisms associated to bile acids, lipid metabolism and inflammatory metabolites have also been reported previously in studies of gallstones metabolomics [29], [51], [67].

Bile acid metabolism pathways, lipid metabolism pathways, arachidonic acid metabolic pathways and fatty acid metabolic pathways were also significantly dysregulated in gallstone disease as revealed by metabolite pathway enrichment analysis. These included bile acid and lipid metabolism pathways as the key pathways associated with diseases. The disruption of pathways involved in bile acid metabolism—such as cholesterol solubility, enterohepatic circulation and hepatobiliary metabolic balance—may directly play a role in cholesterol supersaturation and gallstone formation. The results thus shed light on the central role played by bile acid and cholesterol dysregulation in the pathogenesis of gallstone disease [11], [14], [15].

Through metabolite–gene interaction mapping and gene enrichment analysis, several genes associated with disease were identified that were linked to bile secretion, cholesterol metabolism, inflammatory signalling, regulation of oxidative stress, and lipid homeostasis. The integrated metabolite–gene network showed that the disease of gallstones is not a result of a single molecular defect, but rather controlled by a complex network of metabolites and signalling pathways. Furthermore, the gut–liver metabolic axis was suggested to play a role in the progression of gallstone disease by the enrichment of pathways associated with gut microbes and liver. The effect of bile acid dysregulations and altered microbial metabolism in the gut has also been shown in previous studies to play a crucial role in cholesterol gallstone formation [24].

Therapeutic candidate prioritization was then performed via gene–drug interaction analysis and integrated network pharmacology based on pathways and genes related to the disease. Analysis of pathway-level biological interactions and drug-target relationships led to an improvement in therapeutic prioritization through network-based analysis. The systems level analysis allowed for identification of compounds that have therapeutic potential on the basis of bile acid regulatory mechanisms and hepatobiliary metabolic pathways that were previously reported in studies of network pharmacology in hepatobiliary diseases [38].

The metabolite-gene-drug integrated network constructed in Cytoscape highlighted the systems-level architecture of this dysregulation and identified highly connected hub nodes of genes related to bile acid biosynthesis, lipid metabolism, fatty acid degradation and PPAR-mediated signalling. By dissecting the integrated network into fifteen DRM specific subnetworks, individual drug–target–metabolite interactions could be examined to gain granular insights into disease-relevant biology that each of the candidate drugs acted on through unique, but overlapping, mechanistic axes. Multiple subnetworks contained recurring hubs such as CYP3A4, SLCO1B1, ABCB11, SLC10A2, PTGS2, and NR1H4, further highlighting the hepatically mediated drug metabolism, bile acid transport, eicosanoid signalling in inflammation and farnesoid X receptor-mediated transcriptional regulation as primary pharmacological interaction domains of gallstone disease.

Multi-criteria network centrality scoring (combining the four centrality measures: degree, betweenness, closeness, eigenvector) placed Atorvastatin Calcium Trihydrate at the top of the list, followed by Rifampin, Rosuvastatin and Fenofibrate Micronized. But network topology was not enough to make it to the final selection of candidates. Systematic evaluation of all these drugs based on the literature identified significant drawbacks: As strong network players statins exhibited inconsistent and paradoxical associations in large meta-analyses, with short-term users being at higher and not lower risk of developing gallstones; their HMG-CoA reductase inhibitory mechanism does not directly target the bile acid transport dysfunction at the core of the gallstone pathogenesis.

The highest eigenvector centrality score is awarded to rifampin, but it has documented hepatotoxic liability via ER stress-induced BSEP suppression, making it unhelpful for gallstone-directed therapy. Mechanistically, the PPARα agonism of fenofibrate is relevant to lipid metabolism, but is linked to increased biliary cholesterol lithogenicity (unwanted effect) in gallstone disease and its main use is in dyslipidaemia and cholestasis unrelated to gallstones. Anti-inflammatory drugs such as Aspirin, Indomethacin and Celecoxib were not used in the exclusion process because they have gastrointestinal toxicity, cardiovascular risk and a lack of direct connection with the bile acid transport axis. The broad spectrum of cytochrome P450 inhibition caused significant issues with its well-known oral bioavailability problems and lack of pharmaceutical product approval.

The dietary flavonoids Quercetin, Hesperetin and Apigenin, although showing interesting interactions with both FXR and xanthine oxidase, have not been approved for clinical use, have very inconsistent bioavailability and have not yet been clinically evaluated in gallstone disease. The PPARγ-mediated mechanism of action of pioglitazone would indirectly affect lipid metabolism, and the potential for

development of bladder cancer and heart failure render it ineligible. The anti-inflammatory mechanism of colchicine, by inhibiting tubulin and suppressing NLRP3 inflammasome, is not directly involved in the bile acid synthesis and transport pathways that were revealed in this study as the major pathways that are dysregulated in biliary inflammation.

After this multi-layered evaluation, odevixibat was identified as the last prioritized candidate with the unique combination of mechanistic specificity, disease pathway relevance, network-level support, and therapeutic novelty. Both ABCB11 (the bile salt export pump) and SLC10A2 (the apical sodium-dependent bile acid transporter) were direct mediators of the enterohepatic bile acid recirculation pathway, which was shown to be disrupted in the metabolomics data of this study and were uniquely represented in its subnetwork.

Odevixibat is a selective IBAT inhibitor that directly blocks the reabsorption of bile acids in the intestine, lowering bile acid pool recirculation and directly targeting the specific pathophysiological mechanism driving the supersaturation of bile acids and gallstone nucleation, which no other candidate in the network could do with the same precision. It has a validated safety and pharmacological basis, because of its approved clinical use in cholestatic liver disorders, and is a genuinely new therapeutic opportunity in gallstone disease that has not been explored in any previous clinical therapy.

To summarize, the metabolomics guided systems pharmacology framework used in the current study was able to successfully combine altered metabolite signatures, enriched biological pathways and integrated drug-gene-metabolite network topology, thus identifying Odevixibat as the most biologically plausible and computationally supported repurposable candidate to treat gallstone disease. The results highlight bile acid transport and enterohepatic circulation as the two most therapeutically targetable pathways in the pathogenesis of gallstones and offer an extensive computational basis for future experimental validation of Odevixibat in this pathobiological context. Overall, these findings show the potential of integrated systems pharmacology as a rational drug repurposing hypothesis generating approach to target and repurpose existing drugs in complex metabolic diseases.

5.2 LIMITATIONS

While implementing the integrated metabolomics-guided computational framework for therapeutic prioritisation in the context of gallstone disease, the present study still faces a few limitations while interpreting the results. Restrictions are prevalent in computational systems pharmacology research and emphasize the need for additional biological validation.

The only drawback of the study is that it is a computation only study. In silico analysis, such as metabolomics analysis, pathway analysis, network pharmacology was used to generate all the findings. Although these methods are useful for the discovery of disease-associated pathways, and the prioritization of therapeutic candidates, they cannot fully

capture the complexity of physiological systems. Hence, therapeutic candidates identified need to be viewed as hypothesis generating and should need experimental validation by in vitro, in vivo and clinical studies before therapeutic applicability can be confirmed [38].

An additional constraint is that reliance on publicly available metabolomics datasets is needed. Metabolite profiles and pathway interpretation may be affected by variability associated with sampling, patient samples or variables in analytical platforms and sample pre-processing. Furthermore, due to the limitations of the instrument's sensitivity and metabolite annotation of untargeted metabolomics analyses, do not cover all biologically relevant metabolites. Thus, some metabolic pathways associated with the diseases may not have been completely captured by the current analysis [29].

The pathway enrichment and metabolite–gene interaction analyses are also reliant on databases of biological networks (e.g., HMDB, DrugBank), which can include incomplete or generalized information. Therefore, the molecular networks built might not constitute the full complexity of the disease pathways in gallstones.

The inherent limitation of network pharmacology and therapeutic prioritization approaches is that network connectivity and pathway proximities do not necessarily represent biological activities under the real physiological environment. The completeness and accuracy of the prioritization relies on the coverage and the currency of the curated databases employed to construct the network, which might not contain all the disease relevant interactions or new characterised molecular interactions. Although the multi-criteria scoring system includes four different centrality measures, it is still a quantitative approximation of biological relevance and not a direct measure of therapeutic efficacy. Moreover, pathway enrichment analysis depends on the completeness of metabolite annotation and statistical testing and feature filtering thresholds, which bring analytical assumptions that can affect the biological interpretation of the results. These limitations that are intrinsic to computational therapeutic studies are all factors that contribute to the boundaries of how the findings should be interpreted as potential hypothesis-generating evidence, which should be further tested and validated in experiments and translation research.

5.3 FUTURE SCOPE

The current study provides a computational basis for the study of translational gallstone disease and several important directions arise from the results. First, experimental validation of Odevixibat as the prioritized candidate, in vitro and in vivo studies to investigate the role of Odevixibat on bile acid transport, enterohepatic circulation, biliary cholesterol saturation and hepatobiliary signalling pathways (biological axes identified in metabolomics analysis in this study as being dysregulated).

The integration of multi-omics analysis is also a future direction of research, which could be achieved by integrating the metabolomics data presented here with the metabolomics, transcriptomics, and proteomics data available from patient groups with gallstones. This integration could offer a more complete systems-level characterization of the molecular

mechanisms of gallstone pathogenesis, aid in the better delineation of disease associated biomarkers, and help to enrich the therapeutic target landscape beyond that provided by metabolomics.

The integrated network expanded in this study with more and larger patient-derived datasets would further enhance the biological representativeness of the drug–gene–metabolite interaction architecture. Sex-specific, age-stratified and comorbidity-adjusted data sets may reveal interactions in the network that are context-dependent and may have implications for therapeutic prioritization in patient subpopulations.

Another focus for the future is the gut–liver axis as gut microbiota plays a key role in bile acid biotransformation and cholesterol regulation. The microbiome profiling coupled with metabolomics and network pharmacology framework established in this study might help to explain the mechanism of intestinal bile acid transporter inhibition by Odevixibat and how the metabolized bile acid profile is associated with the conjugated and sulphated bile acid dysregulation found in the study, and suggest that microbiome-targeted interventions could be coupled with Odevixibats mechanism of action.

Machine learning and artificial intelligence techniques introduced to the integrated data set produced in this work may help to better rank therapeutic candidates, refine the prediction of drug–target interaction scores, as well as uncover disease-specific molecular signatures that current network centrality measures could miss. Metabolic and molecular profiling of patients at the level of the individual could be used to further drive personalized therapeutic strategies based on the specific bile acid and lipid dysregulation profile of an individual gallstone patient.

Finally, the automated computational workflow developed in this study, from metabolomics preprocessing, through pathway enrichment, network construction, subnetwork analysis, to multi-criteria drug prioritization, is readily applicable to other hepatobiliary and metabolic diseases involving bile acid dysregulations, lipid metabolic dysfunction and inflammatory signalling disruption such as primary biliary cholangitis, non-alcoholic fatty liver disease and cholestatic liver diseases. Adaptation of this pipeline to other contexts of related disease could facilitate computational drug repurposing for the entire hepatobiliary field.

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APPENDICES

Appendix-I

Differential Metabolite Analysis Results

Metabolite_name	pvalue	log2FC	FDR
4-Methylcatechol 1-sulfate	3.08E-10	-4.596606265	4.64E-08
Docosadienoate(FFA(22:2)	2.63E-10	0.858447965	4.64E-08
Hexanoylglutamine	3.43E-10	1.896154485	4.64E-08
Hexadecenoylcarnitine(AcCa(16:1)	2.77E-10	0.981005085	4.64E-08
LysoPE(0:0/20:4)	7.54E-10	-1.002375321	8.16E-08
Eicosadienoic acid(FFA(20:2n6)	1.40E-09	1.106150847	1.26E-07
Salicyluric acid	2.46E-09	-2.224309403	1.90E-07
Docosapentaenoic acid(FFA(22:5n6)	3.67E-09	1.062554657	2.00E-07
LysoPE(0:0/20:3)	4.06E-09	-1.07016441	2.00E-07
LysoPE(18:1/0:0)	3.86E-09	-1.193500443	2.00E-07
Oleoylcarnitine(AcCa(18:1)	3.67E-09	0.688450072	2.00E-07
Eicosenoic acid(FFA(20:1)	7.35E-09	1.10448257	3.31E-07
2-Hydroxybutyric acid+3-Hydroxybutyric acid	8.11E-09	1.427841896	3.37E-07
Pyrocatechol sulfate	1.68E-08	-3.096912308	6.48E-07
3beta-Hydroxy-5-cholenoic acid taurine conjugate 3-O-sulfate	2.13E-08	2.369597195	7.68E-07
Spermidine	3.75E-08	1.548009653	1.27E-06
LysoPI(18:1)	4.12E-08	-0.780169721	1.31E-06
Arachidonic acid(FFA(20:4)	4.74E-08	0.746249508	1.42E-06
4-Vinylphenol sulfate	5.71E-08	-2.380765492	1.54E-06
Oleic acid(FFA(18:1n9)	5.45E-08	1.327352292	1.54E-06
Thymol Sulfate	6.55E-08	-2.71624445	1.69E-06
Caffeine	6.86E-08	-3.301338172	1.69E-06
2_6-Dihydroxybenzoic acid	8.24E-08	-2.125651453	1.94E-06
Palmitoleic acid(FFA(16:1)	9.03E-08	1.503788547	2.04E-06
LysoPA(0:0/18:2)	1.04E-07	-0.832913705	2.24E-06
2-Hydroxycapric acid(FFA(10:0-OH)	1.30E-07	-1.232111406	2.70E-06
N1-Acetylspermidine+N8-Acetylspermidine	1.70E-07	1.11590355	3.31E-06
15_16-DiHODE	1.78E-07	-1.49783859	3.31E-06
Phe-Trp	1.78E-07	-1.009397955	3.31E-06
LysoPA(18:2/0:0)	2.32E-07	-0.71107073	4.18E-06
2-Methylguanosine	2.76E-07	0.609818337	4.82E-06
gamma-Glu-His	3.29E-07	1.318757774	5.24E-06

LysoPC(17:0/0:0)	3.29E-07	-0.925348562	5.24E-06
5-Hydroxyhexadecanoic acid	3.29E-07	-0.598045811	5.24E-06
12_13-DHOME	3.59E-07	-1.144781319	5.55E-06
N_N_N-Trimethyl-alanyl-proline betaine	3.75E-07	1.018909644	5.64E-06
LysoPE(18:0p/0:0)	4.46E-07	-1.02412349	5.97E-06
Ethyl beta-D-glucopyranoside	4.46E-07	-3.363670203	5.97E-06
Theophylline	5.08E-07	-4.507059529	5.97E-06
Biliverdin isomer3	5.08E-07	0.894513464	5.97E-06
Heptadecenoic acid(FFA(17:1)	5.08E-07	1.170210215	5.97E-06
Paraxanthine	4.66E-07	-3.854426383	5.97E-06
2-Hydroxyoctanoic acid(FFA(8:0-OH)	4.46E-07	-1.000665642	5.97E-06
Nonadecenoic acid(FFA(19:1)	4.87E-07	0.882341751	5.97E-06
9_10-DHOME	4.87E-07	-1.429729783	5.97E-06
4-Acetylphenol sulfate	4.87E-07	-3.678458453	5.97E-06
Glu-Trp	8.84E-07	0.582737667	1.02E-05
Human Fibrinopeptide B residual [1-9_ XGVNDNEEG]	1.09E-06	1.21452666	1.20E-05
O-Methoxycatechol-O-sulphate	1.18E-06	-2.519007608	1.28E-05
1-Methylinosine	1.65E-06	0.626126847	1.72E-05
Pregnanediol disulfate	1.79E-06	1.289201945	1.83E-05
Linoleic acid(FFA(18:2n6)	2.20E-06	1.054985381	2.16E-05
Tyr-Glu	2.20E-06	-1.411240557	2.16E-05
Caprylic acid(FFA(8:0)	2.48E-06	-0.690660625	2.40E-05
Behenic acid(FFA(22:0)	2.58E-06	-0.51261139	2.45E-05
3beta-Hydroxy-5-cholenoic acid glycine conjugate 3-O-sulfate	3.29E-06	1.104391383	2.97E-05
Tetradecenoic acid(FFA(14:1n9)	3.43E-06	1.236799777	3.04E-05
Arachidylcarnitine(AcCa(20:0)	3.86E-06	-1.037750952	3.37E-05
Glycerol 3-phosphate	4.02E-06	-1.1150657	3.45E-05
Docosahexaenoic acid(FFA(22:6)	4.35E-06	0.831191128	3.68E-05
Nervonic acid(FFA(24:1)	4.52E-06	0.539731013	3.77E-05
LysoPE(18:0/0:0)	5.09E-06	-0.605658844	4.17E-05
LysoPC(20:3/0:0)	5.30E-06	-0.741977098	4.28E-05
Theobromine	5.51E-06	-3.468720191	4.38E-05
Nonanoylcarnitine(AcCa(9:0)	6.44E-06	-1.022626524	4.98E-05
Biliverdin isomer2	7.52E-06	1.076840512	5.73E-05
Trigonelline	8.77E-06	-2.283278578	6.59E-05
LysoPE(16:0p/0:0)	1.10E-05	-0.709386093	8.07E-05
LysoPE(0:0/18:0)	1.33E-05	-0.551013806	9.49E-05
Palmitic acid(FFA(16:0)	1.33E-05	0.660941089	9.49E-05
Isoursodeoxycholic acid	1.49E-05	-2.396832161	0.000100911
Methylpyrogallol sulfate	1.49E-05	-2.786650787	0.000100911

Appendix-II

Metabolite-Gene Mapping Table

Metabolite Name	Associated Genes
Hexadecenoylcarnitine(AcCa(16:1)	CPT1B, CPT1A, CPT2, CPT1C, ACSL1
LysoPE(0:0/20:4)	ENPP2
Salicyluric acid	GLYAT, GLYATL1, GLYATL2, GLYATL3
LysoPE(0:0/20:3)	ENPP2
LysoPE(18:1/0:0)	ENPP2
Spermidine	SAT1, ACAA2, PAOX, TXNRD1, CHOH, DMGDH, SMS, SRM, DHPS, PCCA, ALDH9A1, BBOX1, NTAP, AMD1, GATM, BHMT, SARDH, HDC, OPC1, SLC22A4, SLC22A1, SMOX, AGMAT, TMLHE, BHMT2, PDPR, OAZ1, AILE3, PMF1
4-Vinylphenol sulfate	UGT1A1, SULT1A3
Caprylic acid(FFA(8:0)	CROT, CPT2, SLC22A11, SLC25A20, SLC22A6
Caffeine	PDE4B, CYP3A4, CYP2C9, CYP2C19, CYP2E1, CYP3A43, CYP1B1, CYP2D6, CYP2C18, CYP2F1, CYP4X1, CYP2B6, CYP3A5, CYP1A1, CYP2A13, CYP3A7, CYP4B1, CYP4Z1, CYP1A2, CYP19A1, CYP2C8, CYP2S1, CYP2J2, CYP2A7, CYP2A6, ADORA1, ADORA2A, ABCB1, CYP1A1, RYR1, CYP2D6
LysoPA(0:0/18:2)	PLD2, ENPP2, DGKQ, PPAP2, DGKA, DGKD, PPAP2A, DGKB, DGKI, PPAP2B, DGKZ, CDS2, CDS1, LPGAT1, PTK2, PTK2B, AGPAT3, AGPAT2, AGPAT1, GPLD1, LPAR2, LPAR1, AGPAT5, AGPAT6, DGKH, AGPAT4, LPCAT1, LPCAT2, LCLAT1, LPAR3, LPAR4, LPAR5, LPCAT4, AGPAT9, ACPB, LPAR6, LIPH, LIP1, LPPR4, AGK, AGPAT6, DGKE, LPAR1
N1-Acetylspermidine+N8-Acetylspermidine	SAT2, SAT1, PAOX
2-Hydroxycapric acid(FFA(10:0-OH)	OLAH
LysoPA(18:2/0:0)	PLD2, ENPP2, DGKQ, PPAP2, DGKA, DGKD, PPAP2A, DGKB, DGKI, PPAP2B, DGKZ, CDS2, CDS1, LPGAT1, PTK2, PTK2B, AGPAT3, AGPAT2, AGPAT1, GPLD1, LPAR2, LPAR1, AGPAT5, AGPAT6, DGKH, AGPAT4, LPCAT1, LPCAT2, LCLAT1, LPAR3, LPAR4, LPAR5, LPCAT4, AGPAT9, ACPB, LPAR6, LIPH, LIP1, LPPR4, AGK, AGPAT6, DGKE, LPAR1
LysoPC(17:0/0:0)	PLA2G4A, CLC, PLA2G7, PAFAH1B3, PAFAH1B2, PAFAH2, FFAR1, LPCAT3, LPCAT1, LPCAT2, ASPG, PLB1, GPR119, GPR55, GPR4
Theophylline	PDE3A, PDE4B, PDE4A, PDE5A, ADA, CYP3A4, CYP2C9, CYP2E1, CYP1B1, CYP2D6, CYP1A1, CYP1A2, CYP2C8, ADORA1, ADORA2, ADORA2A, ADORA2B, SLC22A7

Biliverdin isomer3	HMOX2, HMOX1, BLVRA, BLVRB, AHR
Paraxanthine	NAT1, NAT2, XDH, CYP3A4, CYP2C9, CYP2C19, CYP2E1, CYP3A43, CYP1B1, CYP2C18, CYP2F1, CYP4X1, CYP2B6, CYP3A5, CYP2A13, CYP3A7, CYP4B1, CYP4Z1, CYP1A2, CYP19A1, CYP2C8, CYP2S1, CYP2J2, CYP2A7, CYP2A6, ANAT2, CYP1A1, CYP2D6
Eicosatrienoic acid(FFA(20:3n6)	BAAT, ACOT7, ACOT2, ACOT4, ACOT8, ACOT1
Glycerol 3-phosphate	GIPD2, GK2, GK, GIPD1, GPAM, GK3B, AGPAT6, PGS1, GIPDL, AGPAT9, DKFZP4B1B1115, GIDE1, GPAT2, GK5, ESCO1, ADPRM, GIPCPD1
LysoPE(18:0/0:0)	ENPP2
LysoPC(20:3/0:0)	LYPLA1, PLA2G15, PLA2G5, PLA2G2F, PLA2G4A, PLA2G4B, PLA2G12B, PLA2G10, PLA2G2E, LYPLA2, PLA2G6, LCAT, CLC, PLA2G2D, PLA2G7, PAFAH1B3, PAFAH1B2, PLA2G3, PAFAH2, FFAR1, LPCAT3, LPCAT1, LPCAT2, ASPG, PLB1, PLA2G4B, GPR119, GPR55, GPR4
Theobromine	XDH, PDE4B, CYP2E1, CYP1A2, ADORA1, ADORA2A
Docosahexaenoic acid(FFA(22:6)	ACOT7, ACOT2, ACOT4, CYP2U1, ACOT1
LysoPE(16:0p/0:0)	PLD2, PLD1, ATP11C, ATP11A, ATP10A, ATP8B1, ATP9A, ATP10D, ATP8A2, ATP8A1, ATP8B4, ATP11B, ATP8B3, PLSCR1, PISD, PLTP, CEPT1, PLD3, PLD4, PLSCR2, PLSCR3, PLSCR4, PLSCR5, ATP10B, ATP8B2, ABHD4, NAPEPLD, PEBP1, PEBP4, EPT1
LysoPE(0:0/18:0)	ENPP2
Isoursodeoxycholic acid	SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLCO1A2, SLCO4A1, FABP6
5_6-Dihydrouridine	DUS2L, DUS1L, DUS3L, DUS4L, PP35
Porphobilinogen	HMBS, ALAD
Cortisol	CYP11B1, AKR1D1, HSD11B2, HSD11B1, CYP3A4, CYP3A5, CYP3A7, CYP2C8, ABCB1, ABCG2, NR3C2, SLCO1A2, HSD3B1, HSD3B2, CYP11B2, P450-CYP21B, ANXA1, CYP21A2, CYP21A2
LysoPC(20:2/0:0)	LYPLA1, PLA2G15, PLA2G5, PLA2G2F, PLA2G4A, PLA2G1B, PLA2G12B, PLA2G10, PLA2G2E, LYPLA2, PLA2G6, LCAT, CLC, PLA2G2D, PLA2G7, PAFAH1B3, PLA2G3, PAFAH2, FFAR1, LPCAT3, LPCAT1, LPCAT2, ASPG, PLB1, PLA2G4B, GPR119, GPR55, GPR4
Corticosterone	CYP11B1, AKR1D1, HSD11B2, HSD11B1, NR3C2, CYP11B2, P450-CYP21B, CYP21A2, DEFA4, CYP21A2
Spermine	SAT2, SAT1, XDH, CHOH, DMGDH, SMS, SRM, ALDH9A1, BBOX1, ABP1, MTAP, AMD1, GATM, BHMT, SARDH, HDC, OPC1, SLC22A4, SLC22A1, SMOX, AGMAT, TMLHE, BHMT2, PDPR, OAZ1, PMF1
3-Hydroxyhippuric acid	GLYAT, GLYATL1, GLYATL2, GLYATL3
Hyodeoxycholic acid	SLCO1B3, SLCO1B1, CYP3A4, ABCC3, ABCB11, SLC10A2, SLC10A1, SLC10A1A2, SLCO4A1, FABP6
Oxoglutaric acid	DLD, ABAT, PHYH, TAT, GOT1, GOT2, ALDH2, ALDH3A2, ALDH1B1, BCAT1, PYHA2, PYHA1, AADAT, BBOX1,

	AGXT2, OAT, OGDH, PLOD1, PLOD2, PLOD3, CCBL1, BCAT2, GLUD2, GLUD1, AGXT, GPT, PSAT1, AASS, IDH2, IDH3B, IDH1, IDH3A, IDH3G, PHGDH, ASPH, HIF1AN, SLC25A11, D2HGDH, L2HGDH, AADAT, TMLHE, GPT2, ADHFE1, AASS, ALKBH2, LEPRE1, LEPREL1, LEPREL2, GOT1L1, CCBL2, DHTKD1, OGDHL, PYHA3, KDM2A, KDM2B, EGLN1, EGLN2, EGLN3, PYHTM, ALKBH1, KDM8, MINA, NIT2, NO66, PHF8, TET1, TET2, TET3
3-Oxotetradecanoic acid	FASN, OXSM
LysoPC(22:4/0:0)	LYPLA1, PLA2G15, PLA2G5, PLA2G2F, PLA2G4A, PLA2G1B, PLA2G12B, PLA2G10, PLA2G2E, LYPLA2, PLA2G6, LCAT, CLC, PLA2G2D, PLA2G7, PAFAH1B2, PLA2G3, PAFAH2, FFAR1, LPCAT3, LPCAT1, LPCAT2, ASPG, PLB1, PLA2G4B, GPR119, GPR55, GPR4
N-Acetylornithine	ACY1
Androsterone sulfate (1)	SULT2B1, STS
Ursodeoxycholic acid	AKR1C1, AKR1C2, SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLC10A1A2, SLCO4A1, FABP6
Glycochenodeoxycholate-3-sulfate	SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLCO1A2, SLCO4A1, FABP6
Hippuric acid	GLYAT, GLYATL1, GLYATL2, GLYATL3
Glucuronic acid	UGT2B28, UGT2B4, UGT1A4, UGT2B10, UGT2B7, UGT2B15, UGT2A1, UGT1A1, UGT1A9, UGT1A3, UGT1A10, UGT2B17, UGT1A6, UGT1A5, UGT2B11, UGT1A7, GUSB, MIOX, UGT2A3, KL, DSE, ABHD10
Ribose 5-phosphate	PRPS1L1, NUDT5, PRPS1, PRPS2, NUDT9, TKTL1, TKT, RBKS, PGM1, PGM3, RPIA, TRPM2, PGM2, TKTL2, PRPS2, ADPRM
Inosine	NT5E, NT5C1B, NT5C1A, NT5C, NT5M, XDH, ADA, SLC29A4, NT5C3, NT5C2, PNP, ADAT1, CECR1
2-Oxoarginine	ABP1
Oleic acid(FFA(18:1n9)	FASN, BAAT, ACOT7, ACOT2, ACOT2, ACOT8, FAAH, MOGAT2, FAAH2, ACOT1, OLAH
2-Hydroxybutyric acid+3-Hydroxybutyric acid	DLD, SULT2B1, UGT1A1, LDHAL6A, LDHB, LDHC, LDA, LDHAL6B, BDH1, BDH2, DKFZP434G1411
Linoleic acid(FFA(18:2n6)	PLA2G5, PLA2G2F, PLA2G4A, PLA2G1B, PLA2G12B, PLA2G10, PLA2G2E, PLA2G12A, PLA2G6, PLA2G2A, PLA2G2D, PLA2G4C, PLA2G3, BAAT, ALOX5, ALOX15B, ALOX15, CYP3A4, CYP2C9, CYP2C19, ACOT7, ACOT2, ACOT4, ACOT8, CYP2E1, CYP3A43, CYP1B1, CYP2C18, CYP2F1, CYP4X1, CYP2B6, CYP3A5, CYP2A13, CYP3A7, CYP4B1, CYP4Z1, CYP1A2, CYP19A1, CYP2C8, CYP2S1, CYP2J2, CYP2A7, CYP2A6, PLB1, CYP1A1, PLA2G4D, PLA2G4E, PLA2G4F, PLA2G4B, CYP2D6, ACOT1, PLA2G16
Eicosapentaenoic acid(FFA(20:5)	BAAT, ACSL4, ACSL3, ACOT7, ACOT2, ACOT4, ACOT8, FFAR1, PPARG, TRPV1, PPARD, FADS1, SLC8A1, ACOT1, PTGS2, PTGS1
Arachidonic acid(FFA(20:4)	FASN, PLA2G5, PLA2G2F, PLA2G4A, PLA2G1B, CYP3A4, PLA2G12B, PLA2G10, ACOT7, ACOT2, LGALS3, PLA2G2E, CYP4F3, ACOT4, ACOT8, PLA2G12A, PLA2G6, CLC,

	ASAH1, HIPK1, LIPC, PNLIPRP1, PNPLA3, LIPE, CYP2E1, CYP4F11, LIPG, CYP4A11, CES1, CES2, CYP3A43, CYP1B1, BCHE, PLA2G4C, LIPE, PLA2G3, CYP2C18, CYP4B1, BAAT, ACYP2, ACYP1, ACY1, CYP2F1, CYP4Z1, ASPA, ACY3, ALOX5, ALOX15B, CYP4X1, ALOX12, PTGS2, PTGS1, ALOX15, CYP2B6, CYP2S1, CYP4F2, CYP3A5, CYP2A13, CYP3A7, CYP4B1, CYP19A1, CYP2C8, CYP4F8, CYP2J2, CYP2A7, CYP2A6, PPARG, SLC27A6, FAAH, ALOX12B, SLC27A3, PNPLA4, SLC27A1, SLC02A1, SLC27A4, CYP4A22, ASPG, PLB1, PNPLA2, CYP1A1, PLA2G4D, PLA2G4E, PLA2G4F, PNPLA8, CYP4F22, PLA2G4B, ASAH2C, ACER2, ASAH2, FAAH2, CYP2D6, CES5A, ACER1, PNPLA6, PNLIPRP3, ACOT1, CES3, ADFP, CPT1B, PPARG, CPT1B, PLA2G16
Glycoursodeoxycholate-3-sulfate	GLYAT, GLYATL1, GLYATL2, GLYATL3
Palmitic acid(FFA(16:0)	FASN, LYPLA1, PLA2G3, PLA2G2F, PLA2G4A, PLA2G1B, PLA2G10, LGALS3, PLA2G2E, LYPLA2, PLA2G12A, PLA2G6, CLC, PLA2G2A, PLA2G2D, PNLIP, LIPC, PNLIPRP1, PNPLA3, LIPE, LIPG, CEL, PNLIPRP2, LPL, CES1, CES2, BCHE, PLA2G4C, LIPE, PLA2G3, BAAT, ACYP2, ACYP1, ACY1, ASPA, ACY3, ACSL4, ACSL1, ACSL6, ACSL3, ACOT7, ACOT2, ACOT4, ACOT8, PPT1, PPT2, ASAH1, HIPK1, PPARG, SLC27A6, SLC27A3, PNPLA4, SLC27A1, SLC27A4, ASPG, PLB1, PNPLA2, PLA2G4D, PLA2G4E, PLA2G4F, PNPLA8, PLA2G4B, ASAH2C, ACER2, ASAH2, CES5A, ACER1, PNPLA6, PNLIPRP3, CES3, ADFP, CPT1B, PPARG, CPT1B, OLAH, THEM5, THEM4
Hexose phosphate(Glucose 6-phosphate etc)	GCK, HK2, HK1, HK3, G6PD, H6PD, ADPGK, PYGB, PYGL, PYGM, G6PC, GPI, PGM1, SLC37A4, GLCKR, PGM2, G6PC3, G6PC2, ISYNA1, HKDC1
Alpha-Linolenic acid(FFA(18:3n3)	PLA2G5, PLA2G2F, PLA2G4A, PLA2G1B, PLA2G12B, PLA2G10, PLA2G2E, PLA2G12A, BAAT, PLA2G6, PLA2G2A, PLA2G2D, PLA2G4C, PLA2G3, ACOT7, ACOT2, ACOT4, ACOT8, TRPV1, FADS1, FADS2, ELOVL4, PLB1, PLA2G4D, PLA2G4E, PLA2G4F, PLA2G4B, ACOT1, PLA2G16
Gamma-Linolenic acid(FFA(18:3n6)	BAAT, ACOT7, ACOT2, ACOT4, ACOT8, ACOT1
Pentadecanoic acid(FFA(15:0)	FASN, PLA2G5, LIPC, PLA2G2F, PLA2G4A, PLA2G1B, PLA2G10, LGALS3, PLA2G2E, PLA2G12A, PLA2G6, CLC, PLA2G2A, PLA2G2D, PNLIP, PNLIPRP1, PNPLA3, LIPE, CES1, CES2, BCHE, PLA2G4C, LIPE, PLA2G3, ACYP2, ACYP1, ACY1, ASPA, ACY3, ASAH1, HIPK1, PPARG, SLC27A6, SLC27A3, PNPLA4, SLC27A1, SLC27A4, ASPG, PLB1, PNPLA2, PLA2G4D, PLA2G4E, PLA2G4F, PNPLA8, PLA2G4B, ASAH2C, ACER2, ASAH2, CES5A, ACER1, PNPLA6, PNLIPRP3, CES3, ADFP, CPT1B, PPARG, CPT1B
Tetradecenoic acid(FFA(14:1n12)	FASN
3-Epideoxycholic acidacid	SLCO1B3, SLCO1B1, ABCC3, ABCB11, FABP6, SLC10A2, SLC10A1, SLC10A1A2, SLCO4A1

4-Hydroxyproline	DAO, PYHA2, PYCR1, PYHA3, PYCR2, DPPY4, PRODH, PYCRL, SLC1A4, TRPM1, PYHA3, EGLN1, EGLN2, EGLN3, PYHTM
1-Methylhistidine	CNDP1, PRMT3, METTL9
Pantothenic acid	PANK4, PANK2, PANK1, PANK3, VNN1, VNN2, VNN3
4-Guanidinobutanoic acid	GATM
Serotonin	AANAT, MAOB, MAOA, DDC, AKR1C3, INMT, IDO1, PRMT3, SLC6A4, HTR4, HTR7, SP1, HTR2B, HTR1B, HTR1F, HTR1A, HTR2A, HTR3A, HTR1D, IDO2, HTR3B, HTR1E, HTR5A, HEMK1, METTL2B, METTL6, WBSCR22
4-Pyridoxic acid	AOX1
Taurolithocholic acid 3-sulfate	SULT2A1
15-HETE	GPX7, GPX5, GPX6, GPX1, GPX4, GPX3, GPX2
Deoxycholic acid	SLCO1B3, SLCO1B1, ABCC3, ABCB11, SLC10A2, SLC10A1, SLCO1A2, NR1H4, SLCO4A1, FABP6
Dehydroepiandrosterone sulfate	SULT2B1, STS
Cytidine	NT5E, NT5C1B, NT5C1A, NT5C, NT5M, UCK1, GINE, UCK2, AID, NT5C3, NT5C2, CDA, UCKL1, APOBEC3H, ZNF638, CELF2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, AICDA
Androsterone O-glucuronide	UGT2B28, UGT2B4, UGT1A4, UGT2B10, UGT2B7, UGT2B15, UGT2A1, UGT1A1, UGT1A9, UGT1A3, UGT1A10, UGT2B17, UGT1A6, UGT1A5, UGT2B11, UGT1A7, GUSB, UGT2A3, UGT1A10, UGT1A8, UGT1A7
2-Hydroxy-3-methylbutyric acid+3-Hydroxy-3-methylbutyric acid	SULT2B1, UGT1A1
Glycoursodeoxycholic acid	GLYAT, GLYATL1, GLYATL2, GLYATL3

Appendix-III

Gene-Drug Mapping Table

Gene	**Associated_Drugs**
ABCB1	ATORVASTATIN CALCIUM TRIHYDRATE, COLCHICINE, CURCUMIN, FENOFIBRATE MICRONIZED, RIFAMPIN, SIMVASTATIN
ABCB11	ODEVIXIBAT, ROSUVASTATIN
ATP8B1	ODEVIXIBAT
CYP2C9	ASPIRIN, CELECOXIB, COLCHICINE, FENOFIBRATE MICRONIZED, HESPERETIN, INDOMETHACIN, PIOGLITAZONE HYDROCHLORIDE, QUERCETIN, RIFAMPIN, SIMVASTATIN

CYP3A4	APIGENIN, ASPIRIN, ATORVASTATIN CALCIUM TRIHYDRATE, CELECOXIB, COLCHICINE, CURCUMIN, FENOFIBRATE MICRONIZED, HESPERETIN, INDOMETHACIN, MELATONIN, PIOGLITAZONE HYDROCHLORIDE, QUERCETIN, RESVERATROL, RIFAMPIN, ROSUVASTATIN, SIMVASTATIN
LIPC	ATORVASTATIN CALCIUM TRIHYDRATE, SIMVASTATIN
LPL	PIOGLITAZONE HYDROCHLORIDE
NR1H4	APIGENIN
PTGS2	ASPIRIN, CELECOXIB, CURCUMIN, INDOMETHACIN, INDOMETHACIN SODIUM
SLC10A1	ROSUVASTATIN
SLC10A2	MARALIXIBAT, MARALIXIBAT CHLORIDE, ODEVIXIBAT, ODEVIXIBAT SESQUIHYDRATE
SLCO1B1	ATORVASTATIN CALCIUM TRIHYDRATE, EZETIMIBE, FENOFIBRATE MICRONIZED, RIFAMPIN, ROSUVASTATIN
SLCO1B3	RIFAMPIN
UGT1A1	ATORVASTATIN CALCIUM TRIHYDRATE
UGT1A4	ATORVASTATIN CALCIUM TRIHYDRATE
UGT1A6	ASPIRIN, ATORVASTATIN CALCIUM TRIHYDRATE
XDH	CURCUMIN, QUERCETIN
ABCB1	ATORVASTATIN CALCIUM TRIHYDRATE, COLCHICINE, CURCUMIN, FENOFIBRATE MICRONIZED, RIFAMPIN, SIMVASTATIN

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PAGE 4

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PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13

PAGE 14

PAGE 15

PAGE 16

PAGE 17

PAGE 18

PAGE 19

PAGE 20

PAGE 21

PAGE 22

PAGE 23

PAGE 24

PAGE 25

PAGE 26

PAGE 27

PAGE 28

PAGE 29

PAGE 30

PAGE 31

PAGE 32

PAGE 33

PAGE 34

PAGE 35

PAGE 36

PAGE 37

PAGE 38

PAGE 39

PAGE 40

PAGE 41

PAGE 42

PAGE 43

PAGE 44

PAGE 45

PAGE 46

PAGE 47

PAGE 48

PAGE 49

PAGE 50

PAGE 51

PAGE 52

PAGE 53

PAGE 54

PAGE 55

PAGE 56

PAGE 57

PAGE 58

PAGE 59

PAGE 60

PAGE 61

PAGE 62

PAGE 63

PAGE 64

PAGE 65

PAGE 66

PAGE 67

PAGE 68

PAGE 69

PAGE 70

PAGE 71

PAGE 72

PAGE 73

PAGE 74

PAGE 75

PAGE 76

PAGE 77

PAGE 78

PAGE 79

PAGE 80

PAGE 81

PAGE 82

PAGE 83

PAGE 84

PAGE 85

PAGE 86

PAGE 87

PAGE 88

PAGE 89

PAGE 90

PAGE 91

PAGE 92

PAGE 93

PAGE 94

PAGE 95

PAGE 96