Lipidomics study in liver metabolic diseases

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Chapter 1

General introduction and scope

Introduction

Liver metabolic diseases encompass a group of conditions arising from a multitude of factors including environmental, lifestyle choices, viral infections, or genetic mutations resulting in enzyme deficiencies, affecting the normal metabolic processes associated with the liver. Lipidomics, a branch of metabolomics, plays a pivotal role in advancing our understanding of liver metabolic diseases by identifying, quantifying and characterizing lipids in the liver. By examining the diverse lipid species present in the liver, elucidating their structural variations, and deciphering their functional implications, lipidomics provides valuable insights into the underlying mechanisms of these diseases. This approach not only aids in the identification of potential biomarkers but also provides a deeper comprehension of the complex interplay between lipids and metabolic dysregulation in the liver, paving the way for more targeted and effective therapeutic interventions.

Metabolomics and Lipidomics

Systems biology is a multidisciplinary approach that aims to understand the interactions in biological systems by interlinking biochemical networks from the molecular level to the entire organism level. The development of various omics technologies such as genomics, proteomics, transcriptomics and metabolomics advanced our understanding of the biological system. **Figure 1** shows the hierarchy of systems biology. Metabolomics serves as a reporter for downstream processes in systems biology and involves the measurements of small molecules (metabolites) which are end products of biological processes. Understanding changes in these metabolites can assist in unraveling the complex interactions between genes, transcriptional activators, proteins and enzymes, to gain insights into the overall functioning of biological systems [1,2]. Liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been the most commonly used method for metabolomics studies as this technique provides high sensitivity, selectivity and broad coverage of metabolites such as amino acids, organic acids, amines, acylcarnitines, flavonoids, etc.

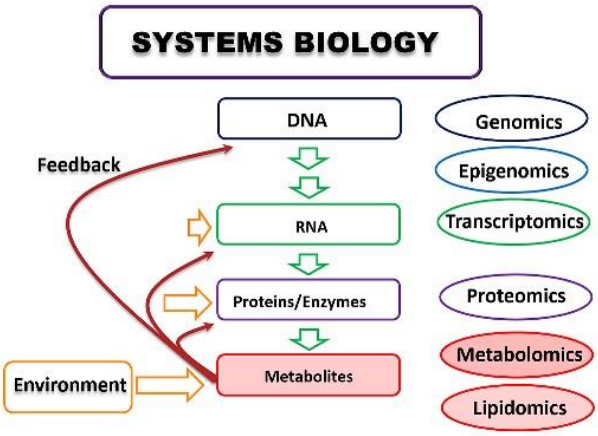

Figure 1. Hierarchy of systems biology showing the connection between different omics approaches [1]. Figure was reprinted under the Creative Commons Attribution License.

Lipidomics is a branch of metabolomics that focuses on the comprehensive analysis of lipids present in biological systems. A typical metabolomics/lipidomics workflow is depicted in **Figure 2**. The ultimate aim of lipidomics analysis is to identify and quantify the lipid biomarkers that can differentiate between healthy subjects and patients. These biomarkers can also contribute to understanding the biochemical and pathophysiological mechanisms of disease pathogenesis. Understanding deviations in the lipid profile due to a disease state is not only beneficial for diagnosis but also essential for monitoring the disease and evaluating a treatment's outcome [3,4].

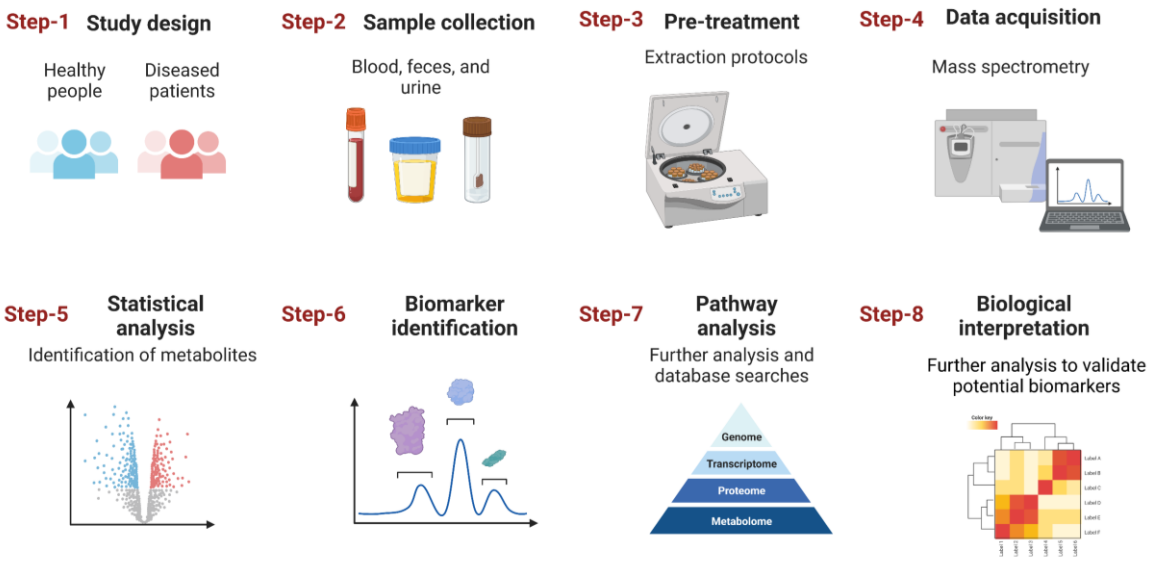

Figure 2. Metabolomics/lipidomics workflow for biomarker identification. (Figure designed with material from BioRender.com).

Advances in liquid chromatography coupled to mass spectrometry (LC-MS) techniques have facilitated lipidomics profiling in complex biological matrices, thus helping in the discovery of novel diagnostic biomarkers.

Lipids classification and biosynthetic pathway

Approximately 70% of entries in the Human Metabolome Database (HMDB) are classified as lipids [5]. The lipidome consists of tens to hundreds of thousands of entities that arise due to variations in the head groups, fatty acid chains, type of linkage, unsaturation, isomerism, etc. [6]. There are more than 44,800 lipid features present in the LIPID MAPS database [3]. The International Lipid Classification and Nomenclature Committee (ILCNC) under the LIPID MAPS consortium developed the ‘‘Comprehensive Classification System for Lipids’’ [7,8]. Based on the biosynthesis and structure of lipids, lipids are divided into eight categories including glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, polyketides and fatty acyls. Saccharolipids and polyketides are found only in plants and bacteria while the other six classes are found in all organisms. This thesis will focus on five categories, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and fatty acyls. Each category is further divided into several classes and/or subclasses and typically consists of a backbone and fatty acid chain [6]. Glycerophospholipids consist of a polar head group, glycerol backbone and one or two fatty acid chains [8]. This category includes classes such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, etc. Glycerolipids consist of a glycerol backbone with one, two or three fatty acid chains esterified at the hydroxyl group of glycerol. Examples of this category include monoglycerides, diglycerides and triglycerides [8]. Sphingolipids are another category of complex lipids that contain a sphingosine backbone N-acylated with fatty acid chain and include classes such as ceramides and sphingomyelin [9]. Cholesteryl esters are members of sterol lipids and are formed by esterification of the hydroxyl group of cholesterol with the free fatty acids [10]. Acyl-coenzyme A (acyl-CoA) belongs to the group of thioester compounds and comes under the category of fatty acyl lipids [11]. Acyl-CoA molecules consist of a fatty acid chain linked to the coenzyme A moiety through a thioester bond. These molecules play a crucial role in fatty acid metabolism.

Lipids are involved in numerous physiological functions including important constituents for various tissues and organs, contributing to cell membrane structure, energy metabolism, signaling, cell-cell interactions, etc. [5,12]. The role of lipids has been established in various

diseases such as cancer, Alzheimer's, atherosclerosis, diabetes, obesity and also in the recent COVID-19 pandemic [13–17].

Lipid biosynthetic and catabolic pathways are highly influenced by dietary processes. In high glucose conditions, glycolysis in the cytosol converts glucose to pyruvate, which is then transformed into acetyl-CoA through oxidative decarboxylation [18]. Acetyl-CoA enters the Krebs cycle for ATP production. Citrate derived from the Krebs cycle returns to the cytosol where it regenerates acetyl-CoA via ATP citrate lyase. This acetyl-CoA serves as a precursor for fatty acid and lipid biosynthesis. In low glucose (starvation) conditions, lipids break down into free fatty acids which enter mitochondrial fatty acid beta-oxidation (FAO) to generate acetyl-CoA. **Figure 3** depicts the metabolic pathway of lipids and provides a clear depiction of the interconnected nature of lipid biosynthesis. The modification in the profile of one lipid class will significantly influence the profile of other classes. Therefore, comprehensive analysis of the majority of classes involved in the lipid metabolism pathway is crucial to gain a deeper understanding of the pathophysiology underlying behind various disorders.

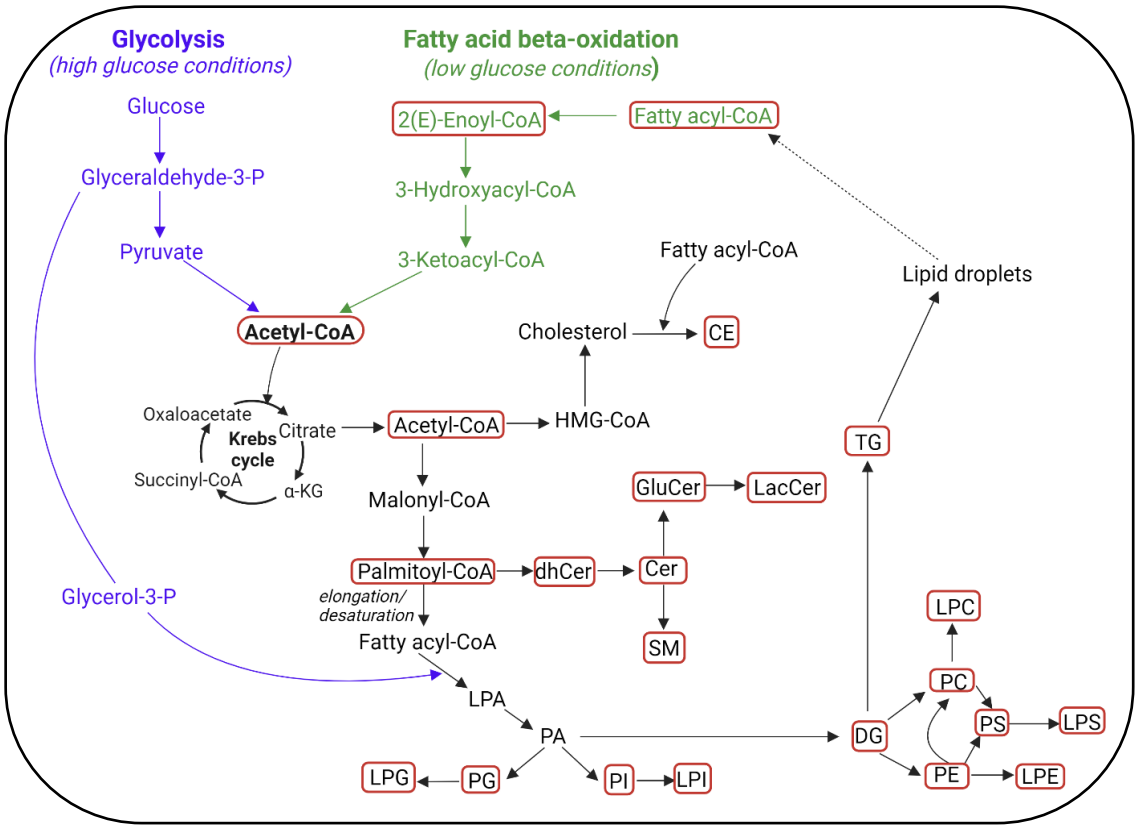

Figure 3. Lipid biosynthetic pathway. (Figure designed with material from BioRender.com).

In the cytosol, acetyl-CoA and malonyl-CoA combine to form longer fatty acyl-CoA through acetyl-CoA carboxylase. Glycerophospholipids are created by attaching fatty acyl groups to glycerol-3-phosphate, leading to LPA and PA in the endoplasmic reticulum. PA splits into pathways, one producing PI and PG, and the other generating DG, PC and PE via the Kennedy pathway. PG is formed in mitochondria, while the rest are synthesized

in the endoplasmic reticulum [19]. PC and PE can form PS through a base-exchange reaction with serine. Lysophospholipids are created through phospholipase-mediated hydrolysis [20]. TG is produced in the endoplasmic reticulum from DG through diacylglycerol transferase. Cholesterol synthesis starts with cytosolic acetyl-CoA, leading to cholesterol formation in the endoplasmic reticulum. CE results from cholesterol esterification with long-chain fatty acyl-CoA. Sphingolipid synthesis begins in the endoplasmic reticulum with serine and palmitoyl-CoA, progressing to ceramides [21], which then transform into SM, GluCer and LacCer in the Golgi apparatus [22–24].

Glyceraldehyde-3-P, Glyceraldehyde-3-Phosphate; α-KG, alpha-Ketogluatrate; HMG-CoA, 3-hydroxy-3-methyl glutaryl CoA; CE, Cholesteryl esters; Glycerol-3-P, Glycerol-3-Phosphate; LPA, Lysophosphatidic acids; PA, Phosphatidic acids; PG, Phosphatidylglycerol; LPG, Lysophosphatidylglycerol; PI, Phosphatidylinositol; LPI, Lysophosphatidylinositol; DG, Diglycerides; TG, Triglycerides; PC, Phosphatidylcholine; LPC, Lysophosphatidylcholine; PE, Phosphatidylethanolamine; LPE, Lysophosphatidylethanolamine; PS, Phosphatidylserine; LPS, Lysophosphatidylserine; dhCer, Dihydroceramides; Cer, Ceramides; SM, Sphingomyelin; GluCer, Glucosylceramides; LacCer, Lactosylceramides. The classes/species encircled in the red box are covered in this thesis.

Challenges in lipidomics analysis

Complex lipids

Complex lipids such as glycerophospholipids, glycerolipids and sphingolipids, differ significantly in their polarities. For instance, glycerolipids like TG require extremely non-polar conditions for extraction and analysis, while glycerophospholipids such as LPS require more polar conditions for the same processes. As a result, diverse analytical strategies are frequently utilized to effectively study and characterize different lipid classes. There are several types of LC-MS techniques that have been reported for the analysis of lipids. Reversed-phase chromatography (RPLC) [25], normal phase chromatography (NPLC) [26] and hydrophilic interaction liquid chromatography (HILIC) [27] have been used for the chromatographic separation of lipids but the existing methods have limitations in covering a broad range of lipid classes in one method. Mass spectrometry detection methods include both targeted and untargeted approaches. Triple quadrupole (QqQ) and quadrupole ion trap (QTRAP) instruments are typically employed for targeted analysis where multiple reaction monitoring (MRM) transitions are predetermined. Commonly used instruments for untargeted analysis include quadrupole time of flight (QTOF) and orbitrap, which acquire data across the entire mass range. The complexity in the structure of lipids is a major complication in lipidomics analysis which can lead to inaccurate identification and quantitation of lipid species. Multiple lipid species may share identical molecular masses, resulting from factors like isomers, isobars, adducts, in-source fragmentation, and variations in the position of fatty acyl chains and double bonds. In the absence of complete chromatographic resolution, this similarity in molecular mass can potentially lead to the misidentification of lipid species [28–30]. Ion-mobility mass

spectrometry, electron-activated dissociation (EAD) techniques, Ultra-Violet Photo-Dissociation (UVPD) are some of the techniques that have been introduced for characterization of isomeric lipid species and identification of double bonds positions in fatty acyl chains.

Absolute quantitation of metabolites is essential for accurately determining their concentrations in biological samples. However, in lipidomics, this can be challenging due to the vast number of existing endogenous lipids, and the limited availability of commercial standards. Experimental factors like extraction efficiency, ionization efficiency and matrix effects can influence the calculated concentrations of endogenous species. These factors are typically corrected using corresponding deuterated (internal) standards, however, the unavailability of these standards can lead to inaccurate correction, thus affecting the accuracy of the measured concentrations. As a result, achieving absolute quantitation for every lipid species is currently not feasible. The use of multiple standards enables the pairing of each endogenous species with the internal standards that closely match their fatty acyl chain composition and level of unsaturation. Although this approach does not provide absolute quantitation for each lipid species, it provides a practical and effective means of achieving accurate quantitation in lipidomics research [31–33]. Harmonization is another significant challenge in lipidomics studies as diverse extraction methods and analytical workflows across different laboratories can lead to variation in results making it difficult to compare data between research groups and hindering the reproducibility of findings.

Acyl-CoAs

Acyl-CoA species have distinct structures and properties as compared to complex lipids and are highly polar in nature. These compounds are not routinely used as diagnostic biomarkers for metabolic disorders due to several problems associated with their analysis such as instability and low endogenous level. The extreme variation in the physicochemical properties of acyl-CoA species often required multiple chromatographic methods [34,35], or other strategies such as derivatization [36] or ion pairing [37] to cover full range of species within this class. Moreover, measuring acyl-CoAs in readily available matrices such as plasma and urine is challenging due to their intracellular location. Consequently, tissue or biopsy samples are required for their analysis.

Lipidomics in liver metabolic diseases

The liver plays a central role in lipid metabolism and transport, making the lipidome responsive to genetic and environmental influences. Consequently, disorders related to the liver will

primarily affect the lipid metabolism pathway and as a result, will influence lipid levels in the body. There are numerous studies conducted to understand the role of lipids in various liver metabolic diseases. Metabolic dysfunction-associated steatotic liver disease (MASLD), previously known as non-alcoholic fatty liver disease (NAFLD) is one of the most common liver metabolic diseases with a global prevalence of 32% [38,39]. Although MASLD is mostly related to dietary factors, there are studies that have reported its genetic connection as well [40,41]. Fat accumulation in the liver is one of the important indications associated with MASLD. This condition arises due to several factors, including increased *de novo* lipogenesis, enhanced uptake of fatty acids and elevated *de novo* fatty acid synthesis in the liver due to insulin resistance [42,43]. The accumulated free fatty acids can either undergo fatty acid beta-oxidation to produce ATP or be esterified to form triglycerides (TG). These TGs are subsequently transported as very low-density lipoprotein (VLDL) to other tissues in the body [43]. The accumulation of triglycerides (TG), diglycerides (DG), cholesteryl esters (CE), ceramides (Cer) and decrease in phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) are common observations associated with development and progression of MASLD [44–48]. Liver biopsy is the gold standard for assessing the severity of MASLD. Yet, the prevalence of MASLD in large populations highlights the need for reliable non-invasive biomarkers to identify high-risk patients. Lipidomics holds promise in identifying these biomarkers, enhancing early detection and monitoring the progression of MASLD.

Inborn errors of metabolism (IEMs) are another major cause of liver metabolic diseases. The concept of IEMs was first introduced in 1908 by Sir Archibald Garrod [49]. IEMs are rare inherited genetic disorders occurring due to defects in the enzymes or transporters in the biological pathways and are most commonly inherited as autosomal recessive disorders [50,51]. These defects cause disruptions in metabolic pathways leading to the accumulation of toxic substances or deficiencies in essential metabolites. IEMs can vary depending on the affected enzymes, organelles and accumulated metabolites [50]. Based on pathophysiology, they are classified into three groups [52,53]:

1. IEMs leading to toxicity because of the accumulation of toxic substances resulting from defects in the biochemical metabolic pathway, eg., maple syrup urine disease;
2. IEMs causing deficiency in energy, eg., mitochondrial disorders;

3. IEMs involving the synthesis or catabolism of complex molecules in the cellular organelles, eg., lysosomal storage disorders.

Glycogen storage diseases (GSD) and fatty acid oxidation disorders (FAOD) are two of the most widely recognized metabolic disruptions associated with liver. GSD is a rare condition characterized by the impaired functioning of the enzyme responsible for glycogen metabolism, resulting in the accumulation of glycogen in the liver. There are 14 known types of GSD, with the most prevalent being Type I or von Gierke disease, Type III or Cori disease (or Forbes disease), and Type IV or Anderson disease. Complications associated with GSD include hypoglycemia pancreatitis and hepatic adenomas [54,55]. In GSD I, hyperlipidemia is a significant indication characterized by elevated levels of both cholesterol (hypercholesterolemia) and triglycerides (hypertriglyceridemia) [56]. Sidorina et al. have reported abnormal phospholipid metabolism in GSD II [57]. GSD III has been associated with hepatomegaly and hyperlipidemia as well [58]. Furthermore, a separate study has reported an increase in short-chain acylcarnitines in GSD I [59]. These findings shed light on the lipid metabolism disturbances in various types of GSDs and their potential implications in disease pathophysiology.

Mitochondrial fatty acid oxidation (FAO) is a crucial pathway that serves as an energy source during starvation or strenuous exercise. FAO generates acetyl-CoA, which plays a crucial role in ketogenesis, a vital energy source for the brain [60,61]. FAOD are inherited metabolic diseases resulting from deficiencies in certain enzyme activities and transporter proteins essential for mitochondrial fatty acid metabolism [62–64]. Consequently, there is an accumulation of acyl-CoAs and their carnitine and glycine conjugates. The accumulation of these compounds can result in lipid peroxidation, hepatic steatosis and impaired energy production. FAOD can also disrupt various metabolic pathways such as glucose metabolism, lipid synthesis and detoxification, thus compromising liver functions. The severity of symptoms associated with FAOD varies from mild hypoglycemia to sudden and unexpected death. Currently, the diagnosis for FAOD relies on analyzing the plasma acylcarnitines using tandem mass spectrometry alongwith genetic and enzyme analysis [65]. Acyl-CoA compounds are directly involved in the FAO pathway and are primary biomarkers associated with FAOD. However, there are several technical challenges associated with the analysis of acyl-CoA, which has led to the use of acylcarnitines as alternative biomarkers for FAOD. Different FAODs are characterized by distinct profiles of acylcarnitine species, allowing for differentiation and precise diagnosis of the specific disorder [66]. In cases of medium-chain acyl-CoA

dehydrogenase deficiency (MCADD), there have been reports of the accumulation of medium-chain acylcarnitines, including C6, C8, C10 and C10:1 [67,68]. On the other hand, very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is associated with the accumulation of long-chain acylcarnitines such as C12, C12:1, C14:1, C14:2, C16 and C16:1 [64,69]. In addition to acylcarnitines, other complex lipids have been reported to be altered in FAOD. For instance, in MCADD, the levels of oxidized phospholipids were found to be increased [70], whereas in VLCADD, phospholipids such as SM, PC, LPE and LPC were found to be elevated [71]. There have been limited reports regarding the analysis of complex lipids in FAOD. Most of the studies have used metabolomics approaches while lipidomics approaches were limited to specific lipid classes [72]. This shows further research is needed for comprehensive lipid analysis to unveil the lipid alterations in advancing and managing FAOD.

Diagnosis of liver metabolic diseases

Diagnosing liver metabolic diseases typically involves a combination of blood tests for liver function, liver enzymes and the lipid profile such as cholesterol and triglyceride levels, as well as imaging tests to assess structural liver changes. In some cases, a liver biopsy may be required for liver histology or the identification of accumulating metabolites. For inherited diseases such as FAOD, newborn screening (NBS) is recommended shortly after birth to screen for IEMs. The introduction of tandem mass spectrometry (MS/MS) has significantly improved the speed and accuracy of IEMs detection. Metabolite measurement using mass spectrometry remains so far the preferred method for newborn screening compared to genome sequencing due to several advantages. The metabolite measurements enable quick clinical decisions as after a metabolic disorder is detected, healthcare providers can immediately start treatment, dietary interventions, and additionally, these measurements can be used to monitor interventions. Whole-genome population screening has been reported to detect variants of uncertain clinical significance in the majority of people screened [73]. Currently, targeted next-generation sequencing is the first diagnostic tool to detect disorders for which a metabolite is not measurable [74]. The combination of genome sequencing with metabolite measurements has the potential of improving the diagnostic rate of IEMs.

Currently, the NBS program collects a small amount of blood samples from the baby's heel, followed by the detection of specific metabolites or the ratios between two metabolites. These metabolites or ratios serve as specific biomarkers for detecting and identifying the type of IEMs. The disorders targeted in the screening procedure vary across different regions. A single

MS/MS injection can diagnose approximately 45 different disorders [75]. The diagnosis of liver metabolic diseases can be further improved by discovering and analyzing a broader range of biomarkers that demonstrate changes in their concentration levels during the diseased condition, indicating the presence of the disease. Identifying a wide range of biomarkers can better capture disruptions in biochemical pathways and the interplay among multiple pathways. These biomarkers can guide the selection of appropriate treatments and monitor therapy responses. The biomarker profile should also help to understand how genetic variations affect disease progression and susceptibility, facilitating personalized diagnosis based on individual characteristics. Furthermore, these biomarkers should provide insights into complex disorders, adapt to emerging health conditions, and offer comprehensive metabolic profiles for early detection of issues, thus aiding in the prediction of future disease risks.

Scope and outline of the thesis

Liver metabolic diseases, influenced by genetic and environmental factors have a substantial impact on the lipid profile. Considering this, lipidomics can play a critical role in facilitating the diagnosis and monitoring of these diseases by identifying and quantifying disease-specific lipid biomarkers. The correlation of these biomarkers with metabolic pathways has the potential to offer valuable insights into the underlying disease mechanisms. Therefore, lipidomics can contribute to personalized diagnosis based on individual patient profiles, thereby assisting in treatment strategies through a 'systems medicine' approach.

The ideas underlying this thesis are that lipidomics may improve the diagnosis of liver metabolic diseases, and can provide further insights into the underlying pathophysiology of these diseases. However to achieve this, the measurement of lipids should be further improved by enabling comprehensive coverage, accurate identification and quantitation. Therefore, in this thesis, new analytical methodologies using LC-MS for the accurate detection and quantitation of lipids are developed. These methods are then applied to MCADD samples to identify lipid biomarkers, which are further correlated with gene expression analysis to understand the underlying biochemical pathways.

Firstly, we have developed an LC-MS/MS-based lipidomics method for comprehensive coverage of complex lipid classes in **Chapter 2**. The aim of this chapter was to develop a hydrophilic interaction liquid chromatography-tandem MS (HILIC-MS/MS)-based lipidomics method covering 1200 lipid features across 19-(sub) classes in human plasma, including both non-polar and polar lipid classes. Several cross-class and within-class interferences were

evaluated to avoid over-reporting and confirm lipid features. A strategy was developed to assign scores showing confidence in the identification of species. To optimize and validate accurate quantitation, SRM 1950 NIST plasma samples were analyzed using multi-internal standards per class and post hoc correction approach. This targeted lipidomics method was validated and applied to COVID-19 plasma samples for biomarker discovery. To evaluate whether this method is efficient in providing a comprehensive coverage of the lipidome, the method was applied in subsequent chapters.

The investigation of liver metabolic diseases requires a biological model that can mimic hepatocyte-like liver functions, helps to understand the pathophysiology associated with liver metabolic diseases and subsequently can assist in the development of novel therapies. In **Chapter 3,** we compared the metabolic capabilities of different *in vitro* models of human hepatocytes. Primary human hepatocytes (PHH) are the gold standard for conducting metabolic liver disease research. However, they have several limitations such as rapid de-differentiation, limited availability and donor-to-donor variation. These limitations show the need of hepatocyte-like cell lines that can replicate defects and dysfunctions associated with the diseases. In this chapter, we compared stem cell-derived hepatocytes (iPSC-Hep), HepG2 cells, upcyte-hepatocytes (Upcyte-Hep) and adult donor-derived liver organoids with PHH in terms of energy metabolism. The goal was to evaluate the production of secreted glucose and induction of gluconeogenesis-related genes in challenged (starvation) conditions. To assess the lipid profile of these matrices and the alterations in intracellular lipid composition in challenged conditions, the HILIC-MS/MS-based lipidomics method, developed in **Chapter 2,** was used.

In **Chapter 4**, the importance of acyl-CoAs in diagnosing FAOD was emphasized and this chapter delved into the evolution of analytical techniques for the identification and quantitation of these compounds. Acyl-CoAs serve as primary biomarkers for FAOD, however, there are several analytical challenges associated with their analysis which restrict their use as the diagnostic biomarker. This chapter highlights these technical difficulties, such as low endogenous levels, diverse physicochemical properties and instability. Further, the chapter describes how LC-MS techniques are advantageous in terms of sensitivity and selectivity for acyl-CoA measurements. This chapter also suggests measures that clinics and hospitals can adopt to overcome the challenges associated with the quantitation of acyl-CoAs.

As **Chapter 4** highlights the importance of acyl-CoAs as diagnostic biomarkers for FAOD, in **Chapter 5**, the development of an analytical method utilizing HILIC-MS/MS for acyl-CoA

analysis was described. The analytical method development of acyl-CoAs is challenging due to significant variations in their polarity leading to the need of multiple chromatographic methods for the coverage of their entire species, which consequently increases complexity and analysis time. To overcome this issue, we have developed a method to cover free CoA and short- to long-chain acyl-CoA species in a single analytical run with a zwitterionic HILIC column. Firstly, a high-resolution instrument (QTOF) was employed for species identification and targeting retention time. After initial optimization, a targeted HILIC-MS/MS method was created using a QTRAP instrument in scheduled multiple reaction monitoring (MRM) mode. This targeted method showed satisfactory performance for various validation parameters such as linearity, precision, recovery and matrix effect. This method was evaluated for the study of acyl-CoA profile in wildtype HepG2 cells cultured in supplemented and starved state. As the method demonstrated significant changes in the acyl-CoA profile in the starved state, the method was used in **Chapter 6** for the study of MCADD.

Further, in **Chapter 6** we applied the HILIC-MS/MS method (developed in **Chapter 5**) for studying CoA metabolism in medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most prevalent form of fatty acid oxidation disorder (FAOD). Within this chapter, we utilized *in silico*, *in vitro* and *in vivo* models to explore the systemic changes triggered by MCADD and the potential compensatory mechanisms involved. Our observations unveiled an accumulation of medium-chain acyl-CoA and acylcarnitine metabolites in MCADD models, along with a reduction in free CoA levels. These findings were further correlated with gene expression analysis followed by an *in silico* demonstration of the involvement of multiple compensatory mechanisms to mitigate the disorder.

The thesis concludes in **Chapter 7** with a summary of the work reported in this thesis, discussion of the results and includes future perspectives and potential avenues for further investigating the role of lipids in samples related to liver metabolic diseases.

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