Lipidomics study in liver metabolic diseases
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Chapter 7

Conclusion and perspectives

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The liver, one of the body's largest organs, plays a pivotal role in all metabolic processes. It governs energy metabolism during both fed and fasted states, regulating essential functions such as glycolysis, lipogenesis, glycogenolysis, gluconeogenesis, fatty acid oxidation and ketogenesis [1]. These processes play key roles in maintaining lipid homeostasis through various cellular, biochemical and signaling pathways [2]. Disruptions in the normal metabolic processes can result from factors such as genetics and environmental influences, leading to liver metabolic diseases. Lipids have diverse roles in various essential cellular functions that are closely interconnected within chemical and genetic networks. As liver is the central organ for lipid metabolism, any deviation in the normal liver metabolic process will directly impact the lipid concentration levels. Hence, lipids are important biomarkers for elucidating the underlying pathophysiological mechanism associated with liver metabolic diseases, as previously discussed in chapter 1. The goal of this thesis is to develop technologies facilitating the measurement of different lipid classes and apply these technologies in biological models of liver metabolic diseases, to enhance our understanding of lipid biomarkers and associated biochemical pathways. The first part of thesis (**Chapter 2 and 3**) was focused on the development of methods for analyzing complex lipid classes and their application in cell models. The aim was to overcome shortcomings of current lipidomics methods and to improve coverage and quantitation. The developed method employed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) and was subsequently applied to different *in vitro* liver matrices to compare their metabolic capabilities. The second part of thesis (**Chapter 4, 5 and 6**) centered on acyl-coenzyme A (acyl-CoA), belonging to the category of fatty acyl lipids. In this part, the emphasis was to study the significance of acyl-CoAs as diagnostic biomarkers for fatty acid oxidation disorders (FAOD) and the challenges associated with the measurement of these compounds. Further, we developed a targeted method for acyl-CoA analysis using LC-MS/MS to improve coverage for acyl-CoA profiling and applied this method to evaluate CoA metabolism in medium-chain acyl-CoA dehydrogenase deficiency (MCADD, a type of FAOD) and to explore its effect on relevant metabolic networks. The upcoming sections will provide a summary, discussion and future perspectives of this thesis.

Chapter 2 aimed at the development and validation of a hydrophilic interaction liquid chromatography-tandem MS (HILIC-MS/MS)-based lipidomics method, covering both non-polar and polar lipid classes and allows for the quantitation of lipid species at fatty acyl chain level. This method provided a comprehensive coverage of 1200 lipid transitions across 19-

(sub)classes belonging to categories glycerolipids, glycerophospholipids, sphingolipids and sterol lipids. The lipid species within these categories were evaluated on various cross-class (such as isobars, isomers and in-source fragments) and within-class interferences (including isotopes, different ion types and chromatographic separation) for unambiguous identification and to prevent over-reporting. The scores were assigned to indicate confidence in lipid identification. This HILIC-MS/MS method was validated on various parameters; the accurate quantitation strategy was evaluated by quantifying 608 lipid species with a high confidence score (score 4) in NIST SRM 1950 plasma samples using multiple internal standards per class followed by post-hoc correction. We demonstrated that our quantitation results shows good correlation (R^2 ranging from 0.64-0.84) with other reported studies [3–5]. The developed method was applied to coronavirus (COVID-19) patient samples to monitor the lipidome changes for disease severity assessment.

Future improvements in our HILIC method could include the ability to determine the positional isomers (sn-1 and sn-2 positions of fatty acyl chain), identifying double bonds locations in fatty acyl chains, and addressing cis-trans isomerization, as distinct isomers have different functions and properties with diverse biological implications [6]. Ion-mobility-mass spectrometry techniques are recognized for their ability to differentiate between isomeric or isobaric lipid species [7]. Recently, electron-activated dissociation (EAD) techniques, such as Electron-Induced Dissociation (EID) and Electron-Impact Excitation of Ions from Organics (EIEIO), have been introduced for the purpose of identifying the positions of double bonds within fatty acyl chains [8]. Ultraviolet Photodissociation (UVPD) activation mode has also been employed to identify sn-positional isomers and the positions of double bonds [9]. Calculating the variation in response factors based on differences in head group and fatty acyl chain compositions among various isomers is a crucial aspect for achieving absolute quantitation, especially due to the limited availability of commercial (internal)standards. Furthermore, there is a critical need to enhance high-throughput capabilities, particularly in the context of analyzing over 1000 lipid species and dealing with a large number of clinical samples. The traditional methods of manual sample preparation and peak integration are not only time-consuming but are also labor-intensive. These processes can cause significant delays in overall data analysis. Therefore, implementing automated tools for sample preparation and peak integration has the potential to streamline the sample analysis and preprocessing, and reduce the time required for analysis.

Research in liver metabolic diseases needs *in vitro* models that can effectively replicate the complex functions associated with liver. These models should be physiologically relevant and

accurate in mimicking metabolic processes and functions of liver. **Chapter 3** aimed at the characterization and evaluation of alternative *in vitro* models for energy metabolism studies in comparison to primary human hepatocytes (PHH) by assessing their metabolic capabilities. PHH are regarded as the gold standard liver-based *in vitro* models as they can replicate the metabolic processes and functionality of the human liver. However, there are several limitations associated with their culture such as loss of liver-specific functions, weak proliferation ability and rapid de-differentitation *in vitro* [10]. Therefore, there is a need for alternative sources that can perform functions at a similar level to PHH. We compared the metabolic capabilities of PHH with stem cell-derived hepatocytes (iPSC-Hep), human hepatocellular carcinoma cells (HepG2), immortalized upcyte-hepatocytes (Upcyte-Hep) and adult donor-derived liver organoids. These cells were cultured under fed conditions (supplemented with glucose) and glucose production (GP) challenge conditions (culturing them without glucose). These cell models were assessed on the basis of production of secreted glucose and induction of gluconeogenesis-related genes in GP-challenged conditions. The HILIC-MS/MS-based lipidomics method (developed in **Chapter 2**) was applied to analyze the lipid profile of these cell models and to observe changes in intracellular lipid composition in GP-challenged conditions. Among the cell models investigated, it was observed that when exposed to GP-challenging conditions, PHH exhibited the highest levels of glucose production and secretion, both intracellularly and extracellularly. Organoids and iPSC-Hep followed PHH in terms of intracellular glucose production, while Upcyte-Hep and HepG2 showed glucose production below the detection limit. Extracellularly, the ranking from highest to lowest glucose production was PHH, followed by organoids, iPSC-Hep, Upcyte-Hep and HepG2. Gluconeogenesis is the process of glucose production from non-carbohydrate sources after depletion of glycogen stores. Glucose-6-phosphatase (*G6PC*), phopshoenolpyruvate carboxykinase 1 (*PCK1*) and fructose 1,6-biphosphatase (*FBP1*) are the three key enzymes involved in the gluconeogenesis process. We observed that both G6PC and PCK1 were upregulated in all cell models when cultured in a GP medium compared to a fed medium. However, the regulation of FBP1 differed among the cell types. In PHH, iPSC-Hep and Upcyte-Hep, FBP1 expression showed minimal changes, while in organoids and HepG2 cells, it was downregulated. The lipid profiles of PHH closely resemble to those of organoids. Under GP-challenged conditions, both PHH and organoids exhibited decreased triglyceride (TG) levels, while minimal changes were observed in iPSC-Hep and HepG2 cells. Conversely, Upcyte-Hep cells showed a significant increase in several TG species under the same conditions. These findings suggested a correlation with glucose production and fatty acid oxidation rates. The reduction in TG levels implies the

activation of the fatty acid beta-oxidation pathway, potentially explaining the higher net glucose production in PHH and organoids. In contrast, the TG profiles in iPSC-Hep, HepG2, and Upcyte-Hep indicated a decrease in fatty acid beta-oxidation, reflecting reduced glucose production rates compared to PHH and organoids. Furthermore, there was significant alteration in the level of phospholipids, specifically phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in GP conditions. PHH exhibited a decrease in PE, organoids demonstrated a reduction in both PC and PE, while an increase was observed in iPSC-Hep and Upcyte-Hep. This suggests an interconnection with metabolic processes aimed at maintaining energy homeostasis. The results presented in this chapter indicate that organoids may serve as a potential substitute for PHH. They have demonstrated the second highest net glucose production after PHH and exhibit a lipid profile similar to PHH. However, it is essential to conduct additional research to validate whether organoid models accurately represent fully mature, differentiated and metabolically proficient hepatocytes, and are suited to study liver metabolic diseases.

Future research in this direction should concentrate on exploring various experimental conditions and measuring metabolites involved in central carbon metabolism to deepen the understanding of energy metabolism in these hepatocyte models. One aspect would be to determine the ideal experimental parameters for these liver cell models, enabling the activation of both fatty acid oxidation and glucose production, and interpret the correlation between them. This may need the introduction of an initial fasting period to deplete the glycogen reserves or the application of fatty acid treatments to increase the cellular triglyceride levels before putting the matrices under glucose production challenge. Furthermore, there is need of additional studies that can enhance our understanding behind the complex process of lipid metabolism and the regulation of energy balance including the aspect of hormonal regulation. Different cell models require specific culture media tailored to their needs, which subsequently regulate the cell growth and proliferation. Examination of the effect of the medium on the profile of fatty acid chains in lipids, alterations in these chains at different stages of cell proliferation and maturation over various days and the correlation with the enzymes responsible for elongating and desaturating fatty acyl chains of lipid molecules can be another aspect of future research. The study of these three-dimensional (3D)-cell culture models have several challenges that should be carefully considered. Organoids are cultivated within Matrigel domes, requiring the extra washing steps prior to analysis due to the potential presence of residual glucose even after repeated washes. This residual glucose can compromise the accuracy of glucose production

assays. This chapter also highlighted the concerns about glucose and Matrigel traces during the incubation period of organoids, and further investigation needs to be conducted to establish robust glucose production assays for these three-dimensional (3D)-cell models. These hepatocyte models can be further investigated to study the disruption in energy metabolism and subsequently affected metabolic networks in liver metabolic diseases and can also be used for the assessment of treatment effectiveness, ultimately aiding in the development of novel therapies for these diseases.

Fatty acid oxidation disorders (FAOD) are a group of liver metabolic diseases that result from a deficiency in the activity of enzymes or transporter proteins involved in the fatty acid oxidation (FAO) pathway. The FAO pathway is crucial for human survival as it serves as an energy source in conditions of low glucose availability. Acyl-CoA molecules are central to this pathway and in FAOD, these molecules accumulate depending on the type of disorder. Acyl-CoAs, despite being the primary biomarkers, are not commonly used for diagnostic purposes. Instead, hospitals and clinics rely on secondary biomarkers such as acylcarnitines. The main reason for this preference is the significant technical challenges associated with the analysis of acyl-CoAs. **Chapter 4** provides a comprehensive overview on the technical challenges, recent analytical advancements for the measurement of these compounds, and potential measures that could be taken for improving the analysis. A major limitation in the analysis of acyl-CoAs is their intracellular location, making them inaccessible for measurement in readily available matrices like plasma and urine. Other obstacles include low endogenous level, instability and extreme variation in the physicochemical properties often leads to the need of using multiple chromatographic separation methods. To address these challenges, numerous efforts had been undertaken to improve the extraction of acyl-CoA from biological samples and perform their analysis. Protein precipitation, liquid-liquid extraction, solid-phase extraction or the combination of these techniques had been used for the extraction of acyl-CoA from biological matrices. Further, various methods had been explored for the separation and detection of these compounds including enzymatic assays, liquid chromatography with ultraviolet detection (LC-UV), gas chromatography coupled with mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Among all these approaches, LC-MS is the most sensitive method, offering high resolution and comprehensive coverage, and is currently the most commonly employed technique. In recent years, several attempts such as ion-pairing [11], derivatization [12] and chemical ligation [13] have been made to improve the separation, peak shape and detection of these compounds. This chapter also emphasizes on the use of correct

internal standards for accurate quantitation of acyl-CoAs. Stable isotope labeling by essential nutrients in cell culture (SILEC) had been developed for the generation of labeled isotopic standards for acyl-CoA. These isotopic standards can be used as internal standards for the quantitation of corresponding endogenous acyl-CoA species. The generation of labeled internal standards is crucial for quantitation of unstable compounds like acyl-CoAs as both internal standards and endogenous species will undergo through the same process of degradation. In conclusion, this chapter outlines recent advancements in analytical techniques for acyl-CoA measurements and the need for further studies focusing on ways to increase the stability and simplifying the analytical strategies of these compounds.

Chapter 5 in this thesis addressed one of the technical challenges associated with the analysis of acyl-CoAs. As stated in **Chapter 4**, acyl-CoAs differ considerably in their physiochemical properties, often requiring multiple chromatographic methods or alternative strategies such as use of ion-pairing reagents or derivatization. However, these techniques also have disadvantages, ion-pairing are known to cause contamination in the mass spectrometers, while derivatization increases the complexity and analysis time, thus affecting the desired high-throughput of analysis. In **Chapter 5**, we developed an analytical method that can cover the entire range of acyl-CoA species in one analytical run. We employed a HILIC-MS/MS approach with the use of zwitterionic ZIC-cHILIC column. The initial method development was performed on a QTOF instrument to identify acyl-CoA species and determine their retention times. Various factors such as buffer concentration, effect of cell matrix and injection solvents were considered to optimize the chromatography. The ZIC-cHILIC column is composed of both negative and positive charged moieties, resulting in weak electrostatic interaction between the analyte and stationary phase, hence a lower concentration of buffer is needed for elution. After optimization of various parameters, 5 mM ammonium acetate was chosen for both organic and aqueous phase with a flow rate of 0.25 mL/min. The reconstitution solvent consisted of methanol:water:isopropanol (1:1:1, v/v/v). Further, a targeted method was created on a QTRAP instrument in scheduled multiple reaction monitoring mode. This method was successful in covering free CoA and short- to long-chain acyl-CoAs in one run. The performance of the method was evaluated on parameters such as linearity, precision, recovery and matrix effect for acyl-CoA quantitation in HepG2 cells. The method was further applied in wildtype (WT) HepG2 cells cultured in supplemented (high in carbon sources) and starved state (fewer carbon sources) to evaluate acyl-CoA profile. We observed an increase in the

concentration of short-, medium- and long-chain acyl-CoAs while decrease in the level of free CoA in starved state, thus indicating an activation in FAO process.

Our HILIC-MS/MS method for acyl-CoA analysis can be further explored for increasing the species coverage and separation of isomers. The current method was targeted on coverage of short- to long-chain acyl-CoAs, as our primary focus was on MCADD. However, the coverage of this method can further be extended to measure very long-chain acyl-CoA species (>20 carbon atoms) and can be applied to assess the acyl-CoA profile in very long-chain acyl-CoA dehydrogenase deficiency (VLCADD). Furthermore, ion-mobility mass spectrometry, EAD and UVPD techniques could be employed to distinguish between isomeric species, for instance, distinguishing between succinyl-CoA and methylmalonyl-CoA, and to determine the position of double bonds in species like C16:1 and C18:1. The endogenous concentrations of acyl-CoAs are very low which necessitates the use of highly sensitive techniques. In the future, there is potential for the utilization and further exploration of micro-LC-MS/MS and nano-LC-MS/MS techniques to achieve even greater sensitivity [14,15]. Additionally, it has been reported that acyl-CoAs exhibit suboptimal recovery rates when analyzed in tissue samples, attributed to their tendency to form anhydrides and S-acyl glutathione [13]. Consequently, it is essential to initiate efforts aimed at delving deeper into the recovery issue and gaining a comprehensive understanding of the factors contributing to the low recovery and degradation of acyl-CoAs. To address potential issues related to degradation, SILEC can be a valuable tool for achieving absolute quantitation of acyl-CoAs. This approach can help compensate for degradation problems and enhance the accuracy of the analysis, thus providing a reliable diagnostic approach. Presently, this method has been applied for the analysis of acyl-CoA species in HepG2 cells. In the future, its applicability can be extended to human samples, particularly tissue samples to have clinically relevant insight into liver metabolic diseases. Given that acyl-CoAs are primarily intracellular, obtaining tissue samples can be challenging, especially in the case of newborns. Therefore, alternative matrices like human fibroblasts, whole blood and platelets should be considered as a more accessible approach for biomarker analysis.

In **Chapter 6**, we extensively explored *in silico*, *in vitro* and *in vivo* models to gain insights into the CoA metabolism and systemic alterations caused by MCADD, and also to identify potential compensatory mechanisms. An *in silico* model of the human liver was developed to analyze changes in saturated and even-chain acyl-CoAs between the MCAD-knockout (KO) and WT conditions. The MCAD-KO model shows a marked increase in C6-CoA and C8-CoA levels, while all other species, including free CoA, decrease significantly. *In silico* simulations were

corroborated with experimental data, by culturing HepG2 MCAD-KO and WT cells in *high-fat low-glucose medium* (containing glucose, pyruvate, glutamine, palmitate and L-carnitine) and *high-fat no-glucose medium* (contains only palmitate and L-carnitine). We determined the intracellular levels of free CoA and acyl-CoA in HepG2 MCAD-KO and WT models using the HILIC-MS/MS method (developed in **Chapter 5**). Additionally, acylcarnitines were quantified using a RPLC (reversed-phase liquid chromatography)-MS/MS method. In both media formulations, MCAD-KO clones exhibited significant increases in medium-chain (C6-C10) acyl-CoA levels, particularly in the case of C8-CoA. In contrast, other saturated even-chain species and free CoA levels were reduced in all MCAD-KO compared to WT cells. The acylcarnitines exhibited a similar trend as acyl-CoA, with an increase in species containing (C6-10) chains and a decrease in other acylcarnitine esters and free carnitine in MCAD-KO samples. Further, we investigated the effect of MCAD-KO on CoA biosynthesis. We treated cells in a *high-fat low-glucose medium* with labeled pantothenate for 24 h. We observed an increase in label incorporation in newly synthesized CoA after 24 h indicating an increase in CoA biosynthesis, which were similar for both WT and MCAD-KO cells, suggesting an active CoA biosynthesis pathway in MCAD-KO conditions. In the same experiment, MCAD-KO cells exhibited lower unlabeled free CoA compared to WT, while total CoA remained unchanged. We conducted similar *in vivo* experiments under physiologically relevant conditions, involving MCAD-KO and WT mice in three states: fed, 14 h overnight fasted, and 14 h overnight fasted with additional 4 h of cold exposure. Total CoA levels and CoA biosynthesis intermediates were increased in both WT and MCAD-KO mice during fasting, with no significant difference between the two groups, indicating a comparable CoA biosynthesis rate. However, MCAD-KO mice exhibited a significant rise in total CoA levels when subjected to both fasting and cold conditions. These findings imply that higher metabolic stress tends to lead to an increase in total CoA levels.

We examined gene expression levels in both HepG2 cells and mouse samples across all study conditions. The upregulation of CoA biosynthesis enzymes and carnitine acyltransferases aligns with our findings from *in silico*, *in vitro* and *in vivo* experiments, suggesting that the accumulation of medium-chain acyl-CoAs could deplete the free CoA pool. This depletion might stimulate the production of more free CoA, however this increased production of free CoA, in turn, leads to further accumulations of acyl-CoAs. Another key finding was the upregulation of acyl-CoA thioesterases (ACOTs) enzymes in MCAD-KO conditions, suggesting an attempt to replenish the free CoA pool and mitigate the excessive buildup of acyl-

CoA esters. We used *in silico* experiments to show this compensatory mechanisms. Our computational model demonstrated that the simultaneous increase in total CoA and upregulating ACOTs in the MCAD-KO model effectively restored free CoA levels while reducing toxic C8-CoA, highlighting the concurrent activation of multiple compensatory mechanisms. These findings confirm the validity of MCADD as <u>Co</u>A sequestration, <u>to</u>xicity, and <u>re</u>distribution (CASTOR) disease.

The future directions of this study involve exploring various models for metabolic network analysis for MCADD. While HepG2 cells are readily accessible and convenient for use, they are not a perfect representation of human liver cells. In **Chapter 3**, we had already demonstrated their distinct response to PHH in terms of energy metabolism and response to challenged conditions. As discussed in **Chapter 3**, organoids and iPSC-Hep cells closely resemble to PHH. Therefore, it is recommended that future research places a priority on using these models for studying MCADD, as they offer a more accurate representation of the human hepatocyte system and allow for direct involvement of MCADD patient cells. Another limitation in the current study is the lack of broader physiological context, MCAD deficiency affects the entire body, which could influence the way the condition manifests. Patient studies such as "Fasting Tolerance in MCADD-infants (FiTtINg MCADD)" hold promise in providing human data collected under controlled conditions, which may offer deeper insights [16]. It is possible that various MCAD-KO cell lines, animals, and different MCADD patients may employ distinct compensatory mechanisms, offering valuable insights into inter-patient heterogeneity. Unraveling this heterogeneity and examining its precise effects on acyl-CoA and free CoA levels is another potential future direction for this study.

Final Perspectives

Understanding the lipid complexity and unveiling their involvement in metabolic disorders needs substantial amounts of research to fully explore the potential of lipidomics in deciphering the dynamics of lipid metabolism for early diagnosis of diseases, monitoring disease progression and efficiency of an intervention. To delve deeper into this field, it is important to engage in interdisciplinary research that incorporates other omics studies. The integration of multi-omics approaches will contribute significantly in gaining a systematic understanding of the pathological aspects related to liver metabolic diseases. Next-generation sequencing (NGS) has made it easier to identify disease-associated mutations, yet the diagnosis is only possible in (30-40)% cases with presumed hereditary abnormality. Many cases result in variants of unknown significance (VUS) without confirmed disease associations. The integration of NGS

with lipidomics can offer a promising approach to establish the pathogenicity of VUS and improve diagnostic accuracy. This combined approach has the potential to increase the diagnostic yield of NGS to (50-90)% [17].

Modern technologies for comprehensive coverage, better resolution, automation and quantitation of lipids are continuously being developed by researchers. These new technologies (including high-throughput technologies) support lipidomics research and broaden the scope of lipidomics from identification of disease-specific biomarkers to also include the monitoring of drug efficacy in the treatment of disorders, potentially leading to novel therapeutic approaches such as understanding the difference between responder and non-responders to interventions. An emerging field, tracer-based lipidomics, holds the potential for flux analysis, enabling a deeper understanding of reaction rates in lipid metabolism at molecular levels and aiding the reconstruction of metabolic pathways. These approaches allow us to link alterations in a patient's lipid profile with their specific gene variants, thereby revealing the connections between various metabolic networks. This knowledge can support in the development of personalized medicine, providing treatments based on individual needs and ensuring precise and effective healthcare.

This thesis primarily focuses on *in vitro* samples, but the next phase involves incorporating the technologies developed in **Chapters 2** and **5** into analysis of clinical samples. We have recently initiated the analysis of plasma and liver samples from patients at various stages of metabolic (dysfunction)-associated steatotic liver disease (MASLD) using the HILIC-MS/MS method described in **Chapter 2**. Patients have been categorized based on their SAF (steatosis, activity, and fibrosis) score, characterizing the disease severity [18]. Preliminary findings indicate promising lipid accumulation patterns linked to disease severity. The comprehensive dataset and associated biomarkers will be of great interest once the analysis is completed. Furthermore, correlating this data with a planned transcriptomics study in the future will enhance our understanding of the underlying pathophysiological mechanisms of MASLD. These analytical methods can also be extended to other liver metabolic diseases, including glycogen storage diseases (GSD) and various other types of fatty acid oxidation disorders.

In the present scenario, plasma is the most accessible and commonly utilized matrix in clinical studies. However, **Chapter 4** has highlighted the significance of acyl-CoA in cellular metabolic health. The challenge arises from the intracellular location of these compounds, making their analysis in clinical samples quite challenging. Therefore, there is urgent requirement for

investigation of alternative matrices for these metabolites. Harmonization in lipid quantitation is another crucial aspect. Lipid concentrations reported (even for the same sample) frequently differ between laboratories, posing an obstacle in establishing reference ranges. The standardized, or at least harmonized, workflows are necessary for data reproducibility and inter-laboratory comparability.

Overall, the research described in this thesis contributes to our understanding of how lipidomics can enhance our knowledge of liver metabolic diseases. We have developed analytical techniques to comprehensively study lipid molecules and to link them with biochemical pathways. These techniques have been applied to various *in vitro* models to gain insights into liver metabolism and to quantify alterations in lipid species in liver metabolic disease samples. In the future, this research shows potential for integrating lipidomics with multi-omics data, paving the way to a systems biology approach that could extend the possibilities of personalized medicine.

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