Global metabolomics and lipidomics approaches to probe virus-host interactions
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Chapter 1

General introduction and scope

Introduction

Viral infections and infectious diseases

The HIV and COVID-19 pandemics have drawn attention to the ongoing threat posed by viral infections [1,2]. Infection by a virus can result in a range of symptoms, from mild to severe. An infection starts when the virus enters host cells and fuses with them, passing through the host cell membrane. The virus then settles near the nucleus, where it generates new membrane-like structures called "replication organelles" and synthesizes viral replication components, including RNA and proteins. These components are then transported to the ER-Golgi intermediate compartment (ERGIC), where they are assembled into new viruses before being released into the extracellular space to infect new cells [3].

Viruses have evolved to manipulate host cell metabolism to support their replication and survival (shown in **Figure 1**). The entry points of the viruses to the cell are micro domains on the cell membrane called lipid rafts, consisting of cholesterol, sphingolipids, and specific proteins [4–7]. A virus could exploit these lipid rafts by interacting with receptors and co-receptors located within these specialized membrane domains to facilitate viral entry into the host cell [4–7]. The viral infection interacts with lipid raft entry gateways and the cell membrane, leading to dysregulation of lipid metabolism and synthesis of host cells for viral propagation [8]. By inducing alterations in metabolic pathways, viruses can increase the availability of nucleotides and amino acids, essential building blocks for viral genome replication and virion assembly, respectively. Enveloped viruses may also require increased lipid material for the envelopment of viral particles. Moreover, viruses can induce specific substrate requirements, such as increased glycoproteins for viral envelopes, by altering cellular metabolism. Additionally, metabolic changes may be necessary to provide the high energy required for genome replication and packaging. Alteration of host cell metabolism may also benefit the infected cells during viral infection. Extensive efforts have been made to identify effective antiviral drugs to treat viral infections and their associated complications. Understanding the interactions between the virus and host cells during the infection process and the biochemical pathways involved is a crucial step in the development of more effective therapies for viral infections.

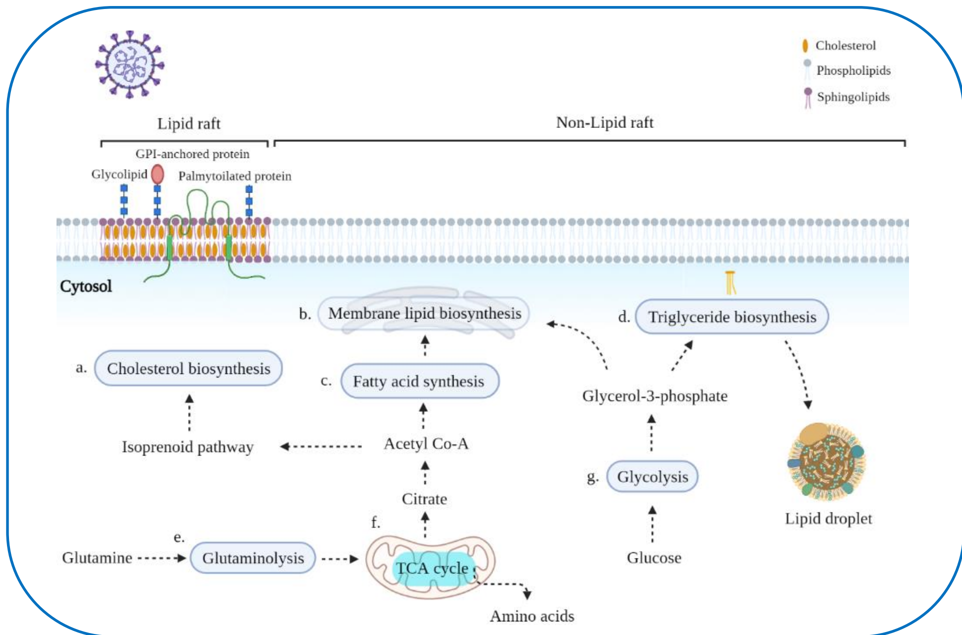


Figure 1. Metabolism and viral needs. A simplified overview of interlinks between metabolic pathways for viral cycle. (Figure designed with material from BioRender.com)

Host metabolism and therapeutical targets

Host metabolism plays a critical role in viral life and replication, making it a potential target for antiviral drug development [9–13]. The host metabolism can be targeted for therapeutic intervention in viral infections. Some viruses, such as human cytomegalovirus (HCMV), rely on host glucose metabolism for their replication, and inhibiting glucose metabolism with drugs like 2-DG or metformin can reduce HCMV replication [14]. Amino acid metabolism is also important for viral replication, and inhibiting enzymes involved in amino acid metabolism, such as glutamine synthetase, can reduce HSV replication [15]. Lipid metabolism is another host process that can be targeted. Targeting host lipid metabolism and host membrane trafficking offers greater flexibility in response to viral resistance compared to targeting viral mutations directly [16,17]. For instance, drugs such as statins [4], phytosterols, chlorpromazine, chloroquine, and Arbidol can inhibit viral infectivity by altering the membrane microdomain formation and composition [18–20]. Orlistat, a fatty acid synthase inhibitor, is a promising drug candidate for the prevention and treatment of

COVID-19 [21]. Glycerophospholipids have been suggested as a simple biochemical parameter to evaluate disease severity and could be potential therapeutic targets for COVID-19 [22]. LJ001 is a broad-spectrum antiviral drug that selectively alters the viral membrane properties of a wide range of enveloped viruses including HIV, influenza, and Ebola, thus offering protection against phospholipid hydroperoxides in host cellular membranes [23]. In light of the potential targets for antiviral drug development mentioned above, it is crucial to consider the role of metabolomics and lipidomics in identifying compounds that can inhibit viral entry, membrane fusion, or endocytosis, as well as fatty acid and cholesterol synthesis [24].

Metabolomics and lipidomics

Metabolomics is an emerging field that focuses on analyzing and quantifying all the metabolites in an organism to understand their biological functions [25]. It has been widely used in health and disease investigations to identify biomarkers for diagnosis, predict disease severity, elucidate pathogenic metabolism, and test therapeutic interventions. Metabolome studies provide a comprehensive understanding of interacting biological pathways, and many studies have been reported on viral infections using metabolomics to gain a better understanding of the virus-host interaction at the metabolic level [26,27]. Integrative systems biology approaches are also useful in understanding the interlinkages of host metabolism and their immunological responses. However, metabolomics analysis can be challenging due to the diverse compositional structures, physicochemical properties, and wide dynamic range of metabolite concentrations in biological systems. Thus, tailor-made metabolomics approaches are developed for certain metabolite classes to achieve accurate metabolic readouts.

Lipidomics is a subfield of metabolomics that focuses specifically on studying the lipidome, or the complete set of lipids in a biological system. Recent advances in mass spectrometry methodologies have led to the identification of hundreds or even thousands of different lipid species in biological samples, making lipidomics an increasingly important tool for studying the role of lipids in biological processes [28]. Lipids have been shown to play crucial roles in virus-host interactions, as evidenced by the significantly altered lipidome observed in Ebola-infected patients [29] and the correlation between altered lipid

metabolism and disease severity in COVID-19 patients [30]. However, understanding the complex pathways of lipid metabolism and the function of lipids in biological systems requires a deep understanding of how lipids are generated and interact with other molecules.

Lipid classes and complexity

Lipid classes has been classified into eight categories according to their hydrophobic or amphipathic chemical physical properties, including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids [31]. The four main categories of fatty acid-derived lipid families are glycerolipids, glycerophospholipids, sphingolipids, and sterol lipids. Typically, these lipid families have long fatty acyl chains and backbones [32]. Glycerolipids and glycerophospholipids both contain a glycerol backbone and side chains. Monoglycerides, diglycerides, and triglycerides are the primary types of glycerolipids, depending on the number of fatty acid chains on the hydroxyl positions of the backbone. Glycerophospholipids contain a phosphate group in the sn-3 position, with one or two fatty acids in the rest of the two hydroxyl positions. Sphingolipids are composed of sphingosine or sphinganine backbones and one fatty acid tail that is esterified with an amine group. Cholesterol esters are the esters of a series of free fatty acids with the hydroxyl group of cholesterol. Human plasma samples typically contain fatty acid tails with 12-26 carbons and 0-6 double bonds. The different combinations of fatty acid tails and head groups result in more than 18,000 lipid species.

Diverse lipid classes in biological systems are interconnected through three main metabolic pathways: the glycerolphospholipid metabolic pathway, sphingolipid pathway, and phospholipid pathway [33]. Wenk emphasizes the importance of lipid metabolism in maintaining cellular homeostasis and regulating biological processes [34]. To fully characterizing the lipidome, it is essential to have advanced analytical methods and tools to study lipid metabolism in depth, and the potential for lipidomics to uncover new insights into the complex interplay between lipids, proteins, and other biomolecules in biological systems. To meet these challenges, lipidomics methods must have the ability to 1) thoroughly elucidate the diverse compositional structures of lipids, and 2) accommodate the wide dynamic range of biological concentrations of lipids [35]. Thus, establishing a high-coverage lipidomics method with proper quantitative and exhaustive analysis is crucial.

Analytical challenges

Lipids are a distinct class of metabolites that present enormous challenges for identification and quantification in pre-analytics and advanced multi-omics analyses, as shown in **Figure 2**. There are two primary challenges in lipidomics analysis: identification and quantification [28,36–40].

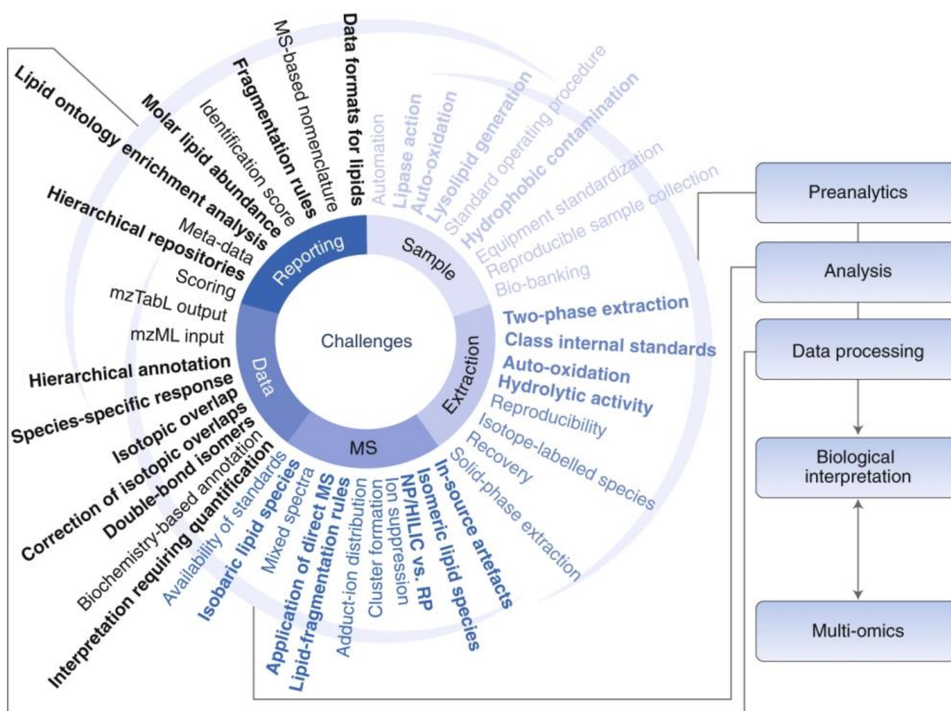
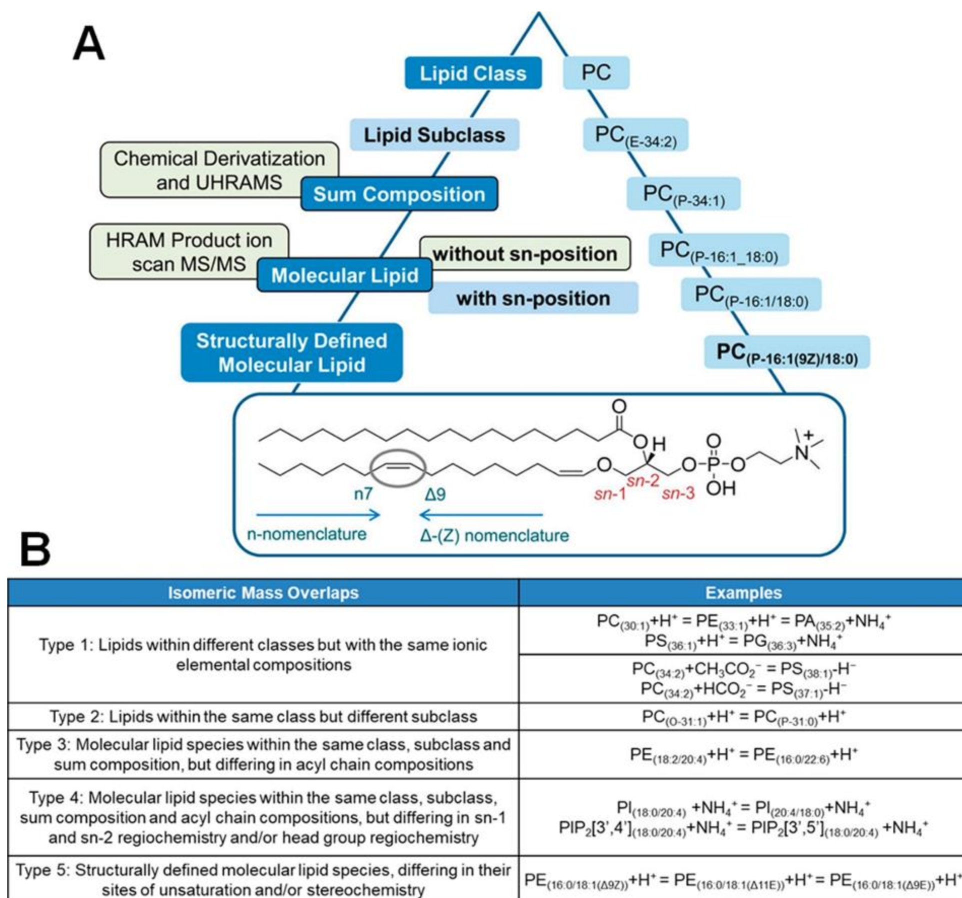


Figure 2. Analytical challenges in lipidomics workflows. Terms in bold are particularly important in lipidomics workflows. MS, mass spectrometry; NP/HILIC, normal phase/hydrophilic-interaction liquid chromatography; RP, reversed-phase. *Reproduced with permission from Liebisch G. et al. Nat Metab 2019; 1:745–747* [40].

The first challenge is due to the extreme structural complexity of lipids, including isobars, isomers, multiple adducts, and artifacts generated from in-source fragmentation [41]. **Figure 3A** illustrates how lipids can vary due to differences in fatty acyl chain position and double bond position, resulting in the same mass, while **Figure 3B** shows how isomeric mass lipid overlaps can hinder structural characterization at various annotation levels.



Isomeric Mass Overlaps	Examples
Type 1: Lipids within different classes but with the same ionic elemental compositions	$PC_{(30:1)}+H^+ = PE_{(33:1)}+H^+ = PA_{(35:2)}+NH_4^+$ $PS_{(36:1)}+H^+ = PG_{(36:3)}+NH_4^+$
	$PC_{(34:2)}+CH_3CO_2^- = PS_{(38:1)}-H^-$ $PC_{(34:2)}+HCO_2^- = PS_{(37:1)}-H^-$
Type 2: Lipids within the same class but different subclass	$PC_{(O\text{-}31:1)}+H^+ = PC_{(P\text{-}31:0)}+H^+$
Type 3: Molecular lipid species within the same class, subclass and sum composition, but differing in acyl chain compositions	$PE_{(18:2/20:4)}+H^+ = PE_{(16:0/22:6)}+H^+$
Type 4: Molecular lipid species within the same class, subclass, sum composition and acyl chain compositions, but differing in sn-1 and sn-2 regiochemistry and/or head group regiochemistry	$PI_{(18:0/20:4)}+NH_4^+ = PI_{(20:4/18:0)}+NH_4^+$ $PIP_2[3',4']_{(18:0/20:4)}+NH_4^+ = PIP_2[3',5']_{(18:0/20:4)}+NH_4^+$
Type 5: Structurally defined molecular lipid species, differing in their sites of unsaturation and/or stereochemistry	$PE_{(16:0/18:1(\Delta 9Z))}+H^+ = PE_{(16:0/18:1(\Delta 11E))}+H^+ = PE_{(16:0/18:1(\Delta 9E))}+H^+$

Figure 3. (A) Annotation hierarchy for lipid identification and characterization based on the structural information derived from the analytical method, and (B) types of isomeric mass lipid overlaps that can hinder structural characterization at various of these annotation levels. *Reprinted with permission from Rustam YH, Reid GE. Analytical Challenges and Recent Advances in Mass Spectrometry Based Lipidomics. Anal Chem 2018; 90:374–397. Copyright 2018 American Chemical Society* [28].

The second challenge is achieving absolute quantification of lipids in a biological context. This can be challenging due to the dynamic range of concentration, which can span six to eight orders of magnitude. In addition, experimental factors such as extraction efficiencies from sample preparation and differential ionization efficiencies and/or ion suppression effects can influence the measurement of actual abundances. It is not possible to have corresponding deuterated internal standards for each endogenous lipid species to correct this error [42]. Therefore, "accurate" quantitation is a more appropriate term than absolute

quantitation to describe attempts to quantify lipid molecular species at the concentration level, giving insights to biological changes [42–44].

Analytical methods

Over the past three decades, the field of lipidomics has rapidly expanded due to advances in LC (with/without)-mass spectrometry technologies, enabling the comprehensive study of the lipidome regarding lipid coverage and structure resolution. To address the aforementioned challenges, a myriad of analytical methods were developed.

There are several methods that have been used to improve the lipid coverage using liquid chromatography in lipidomics research. Reverse phase chromatography (RPLC) separates lipid species according to their fatty acid chain lengths and cannot adequately resolve inter-class isobaric interference [45,46]. Normal phase chromatography is an alternative chromatography technique employed for the class separation of lipids which uses non-polar solvents for the elution [47]. The major drawback associated with normal phase chromatography is the low conductivity and high resistance of non-polar solvents, hampering ionization in electrospray ionization (ESI) [48]. There are many LC-MS methods which either focus on neutral lipids or on glycerophospholipid species [49,50]. In all the previously mentioned methods, measuring the non-polar to polar lipid classes in a single analytical run is very challenging due to the significant differences in their physical and chemical polarities. Hydrophilic Interaction Liquid Chromatography (HILIC) is a promising technique that addresses these challenges by using a polar stationary phase in combination with mass spec compatible mobile phases [54,55]. This technique offers superior quantitative performance compared to RPLC, as it allows for close elution of internal standards and corresponding lipid molecular species, thus reducing differential matrix effects [53]. HILIC has been shown to be effective in lipidomics studies [54–57], although it is limited in coverage of a broad range of lipid classes and species.

Mass Spectrometry analysis provides information on structural resolution. The intact molecule can be identified using MS with high mass accuracy. High mass accuracy can be achieved with either triple-Tof or orbitrap, with high mass accuracy exceeding 30,000 and 80,000 respectively. Other approaches, such as data-dependent acquisition (DDA) and data-

independent acquisition (DIA) strategies, using collision-induced dissociation (CID) or higher energy collision-induced dissociation (HCD) MS/MS, can be used for fatty acyl chain information [58–60]. Newly developed techniques, such as Electron-Induced Dissociation (EID) [60,61], Electron Impact Excitation of Ions from Organics (EIEIO) [61], Ozone-Induced Dissociation (OzID) [62,63], the釘aternò–Büchi reaction [64], and Ultra-Violet Photo-Dissociation (UVPD) [65,66], have also been used for in-depth lipid structural characterization, e.g.double bond position and backbone type. In addition, orthogonal techniques, such as ion mobility and its variants, can be used to resolve isomeric lipid species and for accurate quantification [67–71]. Different combinations of chromatography (with/without) and mass spectrometry can be employed for different purposes, such as resolving lipid structure or achieving absolute quantification.

Scope and outline of the thesis

The outbreaks of AIDS and COVID-19 showed clearly how infectious viruses can influence people's lives. Investigating the changes in the host metabolism may provide a paradigm shift to consider immune-metabolic interactions as therapeutic targets. The aim of this thesis is to examine the interplay between the immune system and metabolism during viral infections, such as HIV and coronavirus. These investigations will utilize metabolomic and lipidomic mass spectrometry techniques to gain a comprehensive understanding of the metabolic changes that occur during viral infections. To enhance the coverage of the lipidome, a new method will be developed.

In **Chapter 2** we focus on a vulnerable population, HIV-exposed uninfected (HEU) infants, who face additional risks due to compromised immune systems. Small for gestational age (SGA) is exacerbated side effect of HEU infants born in HIV infected women receiving combination antiretroviral therapy (cART). This chapter focuses on using advanced lipidomics methods to investigate the immunometabolic changes in SGA infants compared to those born non-SGA infants. Comprehensive Luminex and metabolomics methods are used to analyze the plasma samples of HIV-suppressed pregnant women during the early 3rd trimester and the cord blood of their paired cART-HEU infants at birth. By characterizing these samples using a differential ion mobility based lipidomics method, this

research provides valuable insights into the role of lipid metabolism in SGA and HIV-related pregnancy complications.

In **Chapter 3**, we present a follow-up study to **Chapter 2**, where we aim to provide new insights into the immune-metabolic dysregulation over time in children born to HIV-infected women. To achieve this, we conducted longitudinal immune-metabolomic analyses of plasma samples from 32 pregnant women living with HIV (WLHIV) and 12 uninfected women and their children up to 1.5 years of age. We categorized cART exposure as cART initiation preconception (long), cART initiation post-conception up to 4 weeks before birth (medium), and cART initiation within 3 weeks of birth (short). The analyses of plasma were performed using liquid chromatography-mass spectrometry platforms that include amino acids, positive lipids, Signaling lipids and a multiplex bead assay that includes immune mediators (e.g., cytokines).

In **Chapter 4**, we extended our lipidomics capabilities by developing a hydrophilic interaction liquid chromatography-tandem MS (HILIC-MS/MS)-based method. This new method allowed us to expand the target list with additional lipid classes and improve the separation and quantitation of lipids compared to RPLC-HRMS and differential ion mobility (DMS)-based shotgun lipidomics methods. We developed models of chromatographic retention behavior that considered lipid class, chain length, and degree of unsaturation, and applied them along with in-source fragmentation for unambiguous identification. The targeted lipidomics method was validated with satisfactory analytical characteristics in terms of linearity, precision, reproducibility, and recovery for lipidomics profiling. We were able to achieve accurate quantitation of the SRM 1950 NIST plasma using a multi-internal standards per class approach and post hoc correction. By resolving lipid concentrations at the fatty acyl level, our method enabled us to report precise concentrations for phospholipids and glycerolipids in the NIST plasma sample, thus extending the current databases.

In **Chapter 5**, the developed method was applied to identify metabolite candidates for disease severity prediction in the context of the COVID-19 pandemic. The study hypothesized that disrupted lipid levels, potentially resulting from metabolic comorbidities, could be associated with severe COVID-19. The research focused on characterizing the

dysregulation of PUFA-containing lipid mediator precursors in patients with severe COVID-19 and identifying potential targets for modulating the associated aberrant immune response and increased morbidity.

The thesis concludes in **Chapter 6** with general conclusions of our work and future perspectives of improvements for optimization and application of the global lipidomics-based workflows in clinical studies. Overall, the goal of this thesis is to use cutting-edge metabolic and lipidomic techniques to uncover new insights into the immune-metabolic interaction during viral infections. By doing so, we hope to identify new targets for therapeutic intervention and improve our understanding of the underlying mechanisms driving viral pathogenesis.

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