Metabolomics meets lipidomics: Assessing the small molecule component of metabolism

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Abstract
Metabolomics, including lipidomics, is emerging as a quantitative biology approach for the assessment of energy flow through metabolism and information flow through metabolic signaling; thus, providing novel insights into metabolism and its regulation, in health, healthy ageing and disease. In this forward-looking review we provide an overview on the origins of metabolomics, on its role in this postgenomic era of biochemistry and its application to investigate metabolite role and (bio)activity, from model systems to human population studies. We present the challenges inherent to this analytical science, and approaches and modes of analysis that are used to resolve, characterize and measure the infinite chemical diversity contained in the metabolome (including lipidome) of complex biological matrices. In the current outbreak of metabolic diseases such as cardiometabolic disorders, cancer and neurodegenerative diseases, metabolomics appears to be ideally situated for the investigation of disease pathophysiology from a metabolite perspective.

KEYWORDS
energy metabolism, lipidomics, metabolic profiling, metabolic signaling, metabolite activity, metabolomics, quantitative biology approach

INTRODUCTION: RE-EXPLORING THE METABOLISM WITH METABOLOMICS

Metabolomics, along with lipidomics, has evolved as an analytical science with a holistic approach to allow for ’omic scale metabolite analysis and an integrated view on the metabolism as a whole.[1,2] The root of terms metabolomics, metabolites and metabolism was derived from the Greek “metabolê” meaning “change” and refers to the definition of metabolism as a complete set of biochemical “conversions” that are involved in maintenance and reproduction of life. The concept of metabolism was first described by the Arabian physician Ibn al-Nafis in 13th century who stated that our body is in a continuous state of “dissolution and nourishment” or undergoing a permanent change. The biochemical understanding of metabolism has started in the beginning of the 19th century with a discovery of first small molecules (i.e., citric acid and lactic acid) and has been evolving rapidly ever since the first enzymes were discovered in the beginning of the 20th century. The understanding of biochemical pathways that comprise human metabolism is considered as one of the major achievements in the past century of research in life sciences. It went from the discovery of first cycles (i.e., glycolysis, urea cycle and Krebs cycle in 1930s) to the first hand drawn metabolic chart, containing around 20 pathways, and the definition of information flow in the central dogma of molecular biology.[3] These discoveries in biochemistry and molecular biology were essentially driven by the advancements in different analytical technologies. The introduction of highly sensitive electrospray ionization mass spectrometry (ESI-MS, Figure 1A) technology in late 1980s finally paved the way for the ’omic scale metabolite analyses and metabolomics as a new analytical approach. The fundamental potential of metabolomics, as “the last piece of the ’omics puzzle” lies in
FIGURE 1  (A) Resolving and characterizing the chemical composition of complex biological samples using hyphenated MS techniques. Complex biological matrices, such as different biofluids, cell and tissue lysates, contain a multitude of chemically highly diverse polar and lipid metabolites. Three common separation techniques, liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE), are used to resolve these complex extracts prior to molecular mass measurement using a mass spectrometer. Following the metabolite separation, the molecular mass of each metabolite is measured by the mass spectrometer, whose main constituents comprise the ion source, mass analyzer and detector. In order to measure its mass, a molecule needs to be charged—gas-phase ions need to be generated in the ion source. High resolution mass spectrometers, with the time-of-flight (TOF) or an Orbitrap analyzer, allow for accurate mass measurement (down to 1 ppm error). The data recorded are represented by a chromatogram (or electropherogram) and include the metabolite retention and/or migration time (depending on separation technique used), accurate mass to charge ratio (m/z up to four decimals) and the abundance of its signal (or peak). The additional information on metabolite fragmentation pattern, acquired by collision-induced dissociation (in mass analyzer), is often necessary to validate metabolite identity. (B) Chemical composition of human plasma. Relative distribution of polar and lipid metabolites in human plasma is based on number of species reported in Human Metabolome Database. Out of 114,105 metabolites detected in human blood plasma (including the metabolites of exogenous microbial, plant and food origin) more than 70% are lipids and lipid-like metabolites. As shown, the human plasma metabolome is composed of numerous chemically diverse classes of lipids and polar metabolites, which biological roles range from fuels in cellular energetics and building blocks of macromolecules and cellular organelles to signaling, hormone-like mediators of cellular processes.
diseases (i.e., cancer, obesity, diabetes, cardiovascular disease and neurodegenerative diseases) from a metabolite perspective, in addition to gene perspective, is providing the necessary information on the metabolic activity that has taken place and thus the phenotype at the molecular level.

This information stored in metabolic signatures or metabolic profiles provides the additional insights into the functional status of a biological system and contributes to understanding of the pathophysiological mechanisms for more efficient diagnosis, treatment and ultimately the disease prevention.

Following significant advancements in technology, computing power and bioinformatic solutions, we can today measure not only the metabolites implicated in energy production and storage (i.e., highly abundant nutrients, energy currency metabolites, metabolic by-products, structural lipids and lipid reserves) but also the low abundant metabolites, present in trace amounts, that are responsible for the information flow through chemical signaling. These signaling molecules provide us the additional insights into the metabolic signaling and the regulation of essential biological processes, from cell growth, differentiation and activation to cell proliferation and apoptosis.

Several recent metabolomics data-driven studies clearly demonstrate that “the metabolites are perhaps the body’s most important signaling molecules” ([David Wishart]. Following the paramount evidence on how metabolites act as signaling molecules and modulate protein activities, RNA metabolism and gene expression, and most importantly the disease phenotype, the metabolite role and activity presents a gold-mine yet to be explored.

In this review, we are providing an overview on MS-based technologies, as the most versatile systems to resolve and analyze the chemically highly diverse metabolome and lipidome. We discuss the advantages of different methodological approaches and provide a critical opinion on the future developments and requirements. Finally, we highlight the importance of metabolomics application to reveal metabolite role and activity and provide us new means for the modulation of metabolic processes and health outcomes.

**“ONE SIZE DOESN’T FIT ALL”—RESOLVING METABOLITE CHEMICAL DIVERSITY**

The metabolome represents a small molecule complement (<1500 Da) of a cell, tissue or a biofluid. Its main constituents comprise polar metabolites (e.g., carbohydrates or sugars, amino acids and their derivatives, short peptides, other carboxylic acids, purines and pyrimidines and their nucleosides and nucleotides) and lipids and lipid-like metabolites, from free fatty acids, acylcarnitines, bile acids and steroids, to more complex glycerolipids, glycerophospholipids, sterol lipids, sphingolipids, oxylipins, etc. (see Figure 1B). These “primary” metabolites are highly conserved across different phyla and species and play essential role for organismal survival as fuels for cellular energetics, building blocks of structural components of cells and (bio)active and signaling molecules. More diverse and specific, exogenous metabolome encompasses the xenobiotics coming from diet or the environment, such as drugs, food additives, pollutants, toxicants, and natural products. The latter ones, also called “secondary” metabolites, are products of specialized metabolism in plants, fungi, microorganisms and animals (e.g., sessile marine invertebrates, insects), serving mainly as chemical defenses with the ecological role in improving the organismal fitness.

The tremendous chemical diversity and wide concentration ranges (spanning at least 11 orders of magnitude) contained in complex biological matrices represent the aim and the challenge of omic scale metabolite analysis. There are no limits on how metabolites can be assembled from the structural point of view and there is no universal technique or even combination of techniques that can be used to assess the entire metabolome, along with the lipidome, present in biofluids, cell and tissue lysates, etc. To resolve the chemical diversity, multiple metabolite extraction protocols, measurement technologies, approaches and modes of analysis are combined to make use of their complementarity and thus expand the coverage of polar metabolome and complex lipidome.

The metabolite extraction protocol, depending on the affinity of organic solvent and the reproducibility of the protocol itself, will determine the data quality with respect to the scope of extraction and introduced analytical bias (i.e., due to sample handling, spontaneous oxidation of certain metabolites, etc.). The adequate protocol should efficiently quench the metabolism (to arrest the residual enzymatic activity), extract the broadest range of metabolites (of interest) and remove proteins. The metabolite extraction is sample type and analyte dependent although generic methods are used in untargeted assays, not to bias for or against specific classes of analytes.

The sample preparation also depends on the measurement technology used a posteriori. While derivatization is usually necessary to prepare the samples for GC-MS analysis (see Table 1), it is only rarely used prior to LC-MS analysis, mainly for the measurement of low abundant and poorly ionizable metabolites, such as oxysterols and phosphoinositides for example.

Among the technological platforms used to resolve chemical diversity, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) constitute two main, widely applied technologies for broad-range metabolite and lipid analysis. While NMR has the advantage of being quantitative, non-destructive and highly reproducible—mainly due to the lack of direct interaction between the sample and the instrument, its important drawbacks are the lack of sensitivity and spectral resolution or overlapping signals making unambiguous metabolite identification and quantification difficult when analyzing complex biological matrices. NMR is widely used for the analysis of urinary metabolites, present in high concentrations, in the context of large-scale population studies, in view of its high robustness over time. Although less standardized, it can also be efficiently used for the quantitative analysis of highly abundant cellular metabolites in cell lysates and their supernatants. Despite of robustness and quantitative capacity of NMR, its application remains limited when compared to MS-based technologies that made the most significant imprint in metabolomics and lipidomics, mainly due to the most comprehensive breath of coverage, low instrumentation footprint as well as their cost-effectiveness. Major improvements in
**TABLE 1** Technological platforms used to resolve polar and lipid metabolite diversity. The most common and successful applications are described.

| Technological platform | Advantage | Disadvantage | Applications |
|------------------------|-----------|--------------|--------------|
| Nuclear magnetic resonance (NMR) | Non-destructive technique, Minimal sample preparation, Well suited to ‘dirty’ samples (i.e., urine, plasma), Highly reproducible over time—good for large-scale population studies, Quantitative approach, Comprehensive database for metabolite identification | Low sensitivity, Lack of spectral resolution (i.e., overlapping signal—ambiguous identification), Expensive instrumentation, High footprint | Polar metabolites: Amino acids and derivatives, organic acids and derivatives, phosphorylated metabolites, sugars (31–36) |
| Flow-injection analysis (FIA), Shotgun lipidomics (SL), Multidimensional shotgun lipidomics (MDMS-SL) | High-throughput, Well suited for in real-time analysis (i.e., cell cycle), Shotgun lipidomics (SL)—suited for rapid analysis of highly abundant lipids, Quantitative approach | HRMS necessary, Strong matrix effects and ion suppression, Complex spectra, Lack of sensitivity and selectivity, Identification issues | Polar metabolites: Amino acids and derivatives, organic acids and derivatives (37) |
| Surface ionization techniques, MALDI-MS, DESI-MS, SIMS, REIMS, NIMS | High-throughput, Imaging—added lateral resolution about the local metabolite distribution within the tissue, Ex vivo/in vivo tissue analysis, Rapid multivariate pattern recognition analysis | Lack of sensitivity and specificity, Identification issues, Suitable agent is required to assist the ionization – matrix-related peaks for MALDI, Lack of quantitative capacity | Polar metabolites: Amino acids and derivatives (41–43) |
| Gas chromatography mass spectrometry (GC-MS including GC-CI MS and GC×GC MS) | High chromatographic resolution, High sensitivity, High reproducibility, Wide dynamic range, Quantitative approach, Comprehensive database for metabolite identification, Relatively cheap | Limited to volatile and temperature stable molecules – sample preparation requires derivatization and is time-consuming, Potential metabolite transformations, Low-throughput—relatively long analysis time | Polar metabolites: Amino acids and derivatives, organic acids and derivatives, phosphorylated metabolites, sugars, short chain fatty acids, ketone bodies, nucleosides, nucleotides (49–51) |
| Liquid chromatography mass spectrometry (LC-MS including LC-ESI MS, LC-APCI MS and LC-IMS) | Simple sample preparation (usually no derivatization needed), Short analysis time—relatively high-throughput, Breadth of coverage, Soft ionization (ESI), less fragmentation, High sensitivity, Wide dynamic range, High reproducibility, Quantitative approach, Good chromatographic resolution | Lack of comprehensive and publically available (i.e., downloadable) spectral libraries for metabolite identification, Buffers needed to improve chromatographic resolution—source of ion suppression | Polar metabolites: Amino acids and derivatives, organic acids and derivatives, phosphorylated metabolites, sugars (57–61) |
| Capillary electrophoresis mass spectrometry (CE–MS) | High separation efficiency based on electrophoretic mobility, Predictable selectivity, Small sample size (1 μL), High-throughput, High sensitivity, Quantitative approach, Low solvent consumption | Low reproducibility, Carryover effect, Issues with low abundant and insoluble compounds, Only suitable for charged molecules | Polar metabolites: Amino acids and derivatives, organic acids and derivatives, phosphorylated metabolites, sugars (67–70) |
FIGURE 2  The scope of untargeted and targeted assays. The breadth of coverage of untargeted versus targeted assays is illustrated by the “top of the iceberg” illusion. Although the untargeted assays are unbiased in their approach to detect as many metabolites as possible, in a generic way, without an a priori hypothesis (sample preparation and analysis are performed in a way that should not favor any specific group of metabolites), their coverage is often limited to the most abundant metabolites present in biological samples in mM and μM concentrations (such as building blocks of cell membranes and “fuel” metabolites involved in energy production and storage); thus, constituting the “top of the iceberg.” Low abundant, signaling metabolites (present in fM to nM concentrations) often need to be measured using targeted assays to significantly increase the measurement sensitivity and specificity. These assays need to be tailored specifically for metabolites belonging to one class or one pathway of interest.

Mass spectrometry (MS)-based technologies, approaches, and applications

How do we resolve chemical diversity and identify metabolites using mass spectrometry?

Mass spectrometer measures the mass-to-charge ratio (m/z) of a molecular ion. The metabolites must be ionized (i.e., charged) for their mass to be recorded and thus the ions need to be generated in the ion source of the mass spectrometer prior to the mass measurement by the mass analyzer (Figure 1A). Several different types of mass analyzers can be used for metabolite analysis, depending on their resolution, accuracy, scan speed and dynamic range. Most commonly used analyzers comprise low-resolution triple quadrupoles (QqQ) and high-resolution hybrid quadrupoles time-of-flight (Q-TOF) and quadrupole Orbitraps (Figure 1A).[71,72] Mass spectrometer with high resolving power (Q-TOF and Q-Orbitrap) will allow for accurate m/z measurement, with errors <1 ppm. Although higher MS resolving power facilitates the resolution of chemical diversity (e.g., to distinguish between isobars—or metabolites of similar molecular mass), many small molecule-metabolites have the same elemental composition and thus, no matter how high the MS resolution is (e.g., Orbitrap Fusion Lumos Tribrid MS reaches ultra-high resolution of 1,000,000 FWHM [FWHM – Full Width at Half Maximum – measure of mass resolving power on an extracted ion chromatogram (EIC) at m/z 200]), it is not sufficient to assign the unique metabolite identity.[73] Yet, the majority of metabolites have the highly specific fragmentation patterns that can be generated by collision-induced dissociation (CID). They represent the metabolite MS/MS signatures composed of product ions, which constitute the essential information to validate metabolite identity with high level of confidence.[74,75] The high-resolution instruments (HRMS) with high scanning speeds (from 20 Hz Orbitrap to 100 Hz Q-TOF) allow for subsequent collection of high amount and quality of MS (precursor ion) and MS/MS (product ion) spectra, thereby facilitating peak definition and integration for metabolite quantification.
as well as metabolite identification using MS/MS data. However, the current scanning speeds of HRMS instruments are still insufficient for a good definition of MS/MS peaks and the quantification at the MS/MS level. The low-resolution tandem mass spectrometers (i.e., QqQ) remain the standard for this highly selective quantification at the MS/MS level with significantly lower signal-to-noise ratio and enhanced sensitivity.

The experimentally acquired MS/MS data, on pure standards or by data-dependent (DDA) or independent assays (DIA) on the sample extracts, are recorded and stored in a multitude of spectral databases and libraries and used for MS/MS matching. The content of spectral libraries maintained by the community is publicly available and downloadable (e.g., MassBank) while the most comprehensive and well-curated libraries remain proprietary and searchable only online (e.g., METLIN, mzCloud). It is worth emphasizing that the fragmentation patterns of polar metabolites are difficult to predict due to extremely diverse and species-specific physicochemical properties, whereas lipids have consistent and class-specific predictive fragmentation patterns. Beyond mass, the additional chemical information can be acquired using hyphenated techniques, such as retention time (RT) and ion mobility (expressed as cross collisional section or CCS value) to facilitate the metabolite identification (see section below).

Direct injection-based mass spectrometry strategies

Most of the MS-based technologies are endowed with high-throughput capacity, thus facilitating the analysis of large batches of samples, up to thousands in human population studies. Direct injection strategies, such as flow injection analysis (FIA) are the most high-throughput, and of particular interest for in real time metabolite profiling; however, they suffer from matrix effects and thus the poor reproducibility, as well as lack of specificity for metabolite identification. Contrary to its limited application for polar metabolite analysis, the direct infusion-based shotgun lipidomics (SL) is widely used in lipidomics community and has recently evolved towards multidimensional mass spectrometry—shotgun lipidomics (MDMS-SL), applying multiple acquisitions in full and MS/MS scan modes, for more robust lipid quantification and identification. As such it has become relatively low-throughput and still remains subject to ion suppression, thus limiting the analysis to highly abundant lipid species.

Surface ionization techniques: mass spectrometry imaging

Mass spectrometry imaging applies direct surface ionization to gain insights into spatial distribution of endogenous lipids, polar metabolites, drugs and their metabolites, across profiled tissue sections. Commonly used techniques for in situ tissue imaging include matrix-assisted laser desorption ionization or MALDI–MS, desorption electrospray ionization or DESI–MS, matrix-free nanostructure initiated MS or NIMS and secondary ion – MS or SIMS. While relying on different ionization principles, all these techniques, through ion detection as a function of position, yield spatially resolved chemical maps of tissue sections. The generated maps have been particularly useful for the metabolic characterization of heterogeneous tissues, such as tumor tissue.

MALDI is the most widely used tissue imaging technique with broad coverage of polar and lipid metabolites; however, the presence of organic matrices can interfere with the small molecule detection. DESI is an ambient ionization technique that allows for the direct analysis of unaltered tissue sections with the improved signal in low mass range (<500 m/z) and has an important application in spatial mapping of tumor metabolism. Also in the context of tumor tissue analysis, but with the foremost aim to facilitate intraoperative tumor tissue identification, as an alternative to frozen section histology, rapid evaporative ionization MS or REIMS has been recently developed for in vivo tissue analysis. It was straight off applied in clinical practice during surgical interventions (i.e., iKnife) for supervised cancerous tissue removal. Although MS imaging offers this lateral resolution on local distribution of metabolites within tissue (vs. the analysis of a homogenized tissue extracts as a whole) and its spatial resolution was improved remarkably to allow for imaging at single-cell and even subcellular level, several challenges, mainly regarding the low ionization yields of still broad-range of metabolites, identification and quantification issues remain to be addressed prior to its wider application in biomedical and clinical research.

Hyphenated MS techniques: why separation matters

Despite the resolving power of mass spectrometry, and notably high-resolution mass analyzers (HRMS) to distinguish between metabolites based on m/z ratio, the structural diversity and dynamic concentration range of metabolites present in complex biological samples cannot be resolved uniquely by mass spectrometry. Therefore, the separation techniques such as gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) are commonly coupled to mass spectrometry to improve the sensitivity, selectivity and dynamic range of metabolite measurement (Figure 1A, Table 1). In addition, a supercritical fluid chromatography (SFC) has recently received a lot of attention for lipid analysis, thanks to the development of robust commercial solutions and high separation efficiency and short analysis time compared to commonly used LC-MS. In general, the specified separation techniques are powerful in reducing the matrix effects to minimize the ion-suppression and therefore maximize the MS signal intensity. They will also maximize the measurement specificity by providing the complementary data, such as retention time, for metabolite identification.

Gas Chromatography coupled to mass spectrometry (GC-MS)

Gas chromatography uses a gaseous phase to transport volatile metabolites that are separated along temperature gradient in very long columns (up to 60 m) depending on strength of chemical interaction with column's stationary phase. GC-MS is well-suited for the analysis...
of polar metabolites (e.g., sugars, amino acids and other organic acids, short and long chain fatty acids, cholesterol and its derivatives) that are made volatile by chemical derivatization.

In addition to improving the compound volatility, the derivatization will also reduce their polarity and increase their thermal stability, to facilitate the GC-MS analysis. Gas chromatography provides high separation capacity (peak width < 5s) and is therefore fairly efficient for the highly sensitive and selective analysis of complex mixtures. The major weaknesses of GC-MS are its limited analytical coverage (volatile molecules, thermally stable molecules and molecules that can be derivatized) and fastidious sample preparation resulting in higher measurement variability. Sample preparation constitutes a critical step, and extraction protocol and derivatization are mainly applied depending on the targeted functional groups in different classes of compounds. Among different derivatization protocols the two steps, methoximation followed by a silylation (using Methyl(trimethylsilyl)trifluoroacetamide – MSTFA or Bis(trimethylsilyl)trifluoroacetamide – BSTFA) are the most commonly applied due to their efficiency to induce chemical alterations in a wide range of metabolites presenting functional chemical groups such as hydroxyls, ketones, carboxylic acids, thiols and amines. Oximation improves the detection of ketones by their conversion to oximes, which are then rapidly derivatized with trimethylsilyl (TMS) reagents. The requirement of organic solvents in derivatization protocols increases the complexity of sample preparation, and the reproducibility of derivatization is crucial to assure the precision and accuracy of the analytical measurement.

Most widely used GC-MS platforms operate with electronic impact (EI) ionization source (at 70 eV) owing to the high analysis robustness across different instruments, allowing for metabolite identification (i.e., structural elucidation) by matching against common mass spectral libraries (e.g., FiehnLib, NIST). The transposability of spectral libraries facilitates metabolite identification and constitutes one of the greatest advantages of GC-MS based metabolomics.

**Liquid Chromatography coupled to mass spectrometry (LC-MS)**

Liquid chromatography coupled to mass spectrometry is the most versatile and commonly used separation technique in MS based metabolomics and lipidomics, for both targeted and untargeted assays. It uses liquid phase to carry analytes for the separation along solvent gradient in columns (generally from 5 to 15 cm long) as a function of chemical interaction between the column’s stationary phase and the mobile phase or solvent. The introduction of ultra-high-performance liquid chromatography (UHPLC) in the last decade, using sub-2 μm particle size columns and porousil particles, has improved the resolution and sensitivity of LC-MS analysis and significantly increased the number of metabolites detected in complex biological matrices. In addition, the UHPLC methods allow for the operation at high flow rates (>400 μL min⁻¹) with highly reproducible retention times hence increasing the throughput (i.e., number of samples analyzed per batch). In general, UHPLC represents a good compromise with high (although still limiting) mass spectral acquisition (scanning) rates. It significantly maximizes the measurement specificity by separation of isobars and isomers, and by providing the retention time identifiers. Most commonly used stationary phases can be classified in the following three groups, depending on their chemistry and separation mechanisms: (i) reversed phase (RP), (ii) ion (cation or anion) exchange and (iii) hydrophilic interaction liquid chromatography (HILIC). Metabolite interaction with these different stationary phases and their retention is based on their hydrophobicity, positive and negative charges and hydrophilicity. While RPLC has the broadest applicability due to its robustness, HILIC separation mode is becoming increasingly popular as complementary method to RPLC for the analyses of polar and highly hydrophilic ionic compounds. HILIC stationary phases are classified based on the functional groups (e.g., diol, amide, amino) present on their surface and their charge. They can be divided in unbounded and bonded phases, and the latter ones are classified as neutral and charged or zwitterionic phases.

The LC system is generally coupled to the MS system using electrospray ionization source (ESI) operating in both positive and negative ionization mode. In most of the labs worldwide UHPLC-MS analytical methodologies, requiring minimal (and rapid) sample preparation, are commonly used to reduce the analysis time and measure the greatest number of metabolites in a single run. However, these methodologies also face the issue of ion suppression, affecting analysis reproducibility and accuracy. This phenomenon occurs due to the presence of buffer additives, sample matrix components and still insufficient chromatographic separation. Although ion suppression is inherent to LC-MS technique, different strategies, including the optimization of sample preparation protocols, gain in resolving power by using small particle size columns and elution gradient optimization, can be employed to minimize this issue.

**Ion mobility coupled to mass spectrometry (IMS)**

Even though the described separation techniques (i.e., LC and GC), maximize the sensitivity and specificity of MS analysis, they still cannot resolve the high chemical diversity of structural isomers (e.g., sugars, complex lipids) present in complex biological matrices. The ion mobility spectrometry (IMS) has recently emerged as an additional dimension of separation offering an orthogonal resolving power to the existing analytical setup that further facilitates the metabolite identification and expands the metabolite coverage.

The technique is based on the separation of ions according to their shape, conformation and size, allowing for the measurement of the collisional cross section (CCS) values for each compound, depending on the IMS design (e.g., drift tube ion mobility MS or DTIMS and travelling wave ion mobility MS or TWIMS). The CCS values can be obtained with high reproducibility, independently of analytical conditions used, thus providing a valuable additional chemical information to improve the annotation and identification of polar metabolites, lipids, glycans and proteins. Besides, the separation of ions according to their size and conformation prior to MS/MS data acquisition, will also improve the quality of MS/MS data with respect to spectral clarity and fragmentation specificity. In the past few years the IMS instrumentation, using different operation modes, has been introduced to the market, and the acquired CCS values are recorded in databases which growth will undoubtedly contribute to resolving the bottleneck of metabolite identification.
**Capillary electrophoresis coupled to mass spectrometry (CE-MS)**

Capillary electrophoresis coupled to mass spectrometry (CE-MS) is a powerful technique with high resolving power still underused in the field of metabolomics. Several advantages, including high separation efficiency based on electrophoretic mobility, relatively short analysis time, low sample and solvent consumption, and simple instrumentation, make this technique particularly compelling for the application in metabolomics. In addition, with its ability of analysis in aqueous media, the CE represents an alternative to LC and GC for the analysis of polar, highly polar and ionic metabolites. However, as every analytical technique, CE has some drawbacks with the critical one being its robustness that still prevents its larger scale applicability. Regardless, CE-MS performance is being constantly improved by recent technological advancements making this technique suitable for polar metabolome profiling, especially when only very low sample amounts are available.[104–106]

**Targeted or untargeted, that is the question**

To cope with metabolite chemical diversity and wide concentration range in which metabolites are present in complex biological matrices, two main approaches are used for metabolomic and lipidomic assays, the targeted approach that typically focuses on one pathway or class of metabolites of interest, and untargeted approach that tends to detect as many metabolites as possible without an a priori hypothesis (Figure 2). Targeted approach has been employed in clinical chemistry since its beginnings in 1950s, dominated by GC-MS methodology, while the untargeted approach has evolved relatively recently (first untargeted experiment was performed in 1970s by Pauling and Robinson[107,108]) following the advancement in MS technologies (i.e., enhancement in sensitivity with the introduction of ESI) and the systems biology concepts to complement the data acquired by other ‘omic technologies. Both of these approaches, targeted and untargeted, have high-throughput phenotyping capacity and can be applied from model systems to human population studies.

**Untargeted profiling without an a priori hypothesis**

The concept of global untargeted analysis has evolved with the aim to assess the levels of the broadest range possible of polar metabolites and complex lipids to draw as complete picture possible of the metabolism as a whole. Metabolic diseases, such as type 2 diabetes and cancer are great examples why it is important to have an integrated look at the metabolism as a whole, including multiple pathways, rather than focusing on specific pathway out of its metabolic network context. For both of these disease states it is well-known that the origin of disease is not directly associated with abnormal glucose metabolism, which should be regarded as a consequence of deregulated lipid metabolism.[109,110]

The untargeted profiling is a powerful discovery approach that may highlight yet unknown changes in (un)known metabolic pathways, associated with the investigated phenotype due to disease, drug treatment or environmental factors. It is considered “unbiased” because without an a priori hypothesis although the metabolite and/or lipid coverage will be biased by the applied extraction protocol and the analytical conditions of data acquisition. This bias is often reflected in the detectable set of metabolites—representing only the “top of the metabolome iceberg” (Figure 2), due to the ion-suppression related issues and the reduced sensitivity in a full scan acquisition mode (compared to targeted acquisition modes). To minimize the bias introduced through sample preparation, the protocols usually require minimal sample handling and rely only on the addition of organic solvents for protein precipitation. To maximize the metabolome coverage, data are usually acquired in several chromatographic and ionization modes, ideally HILIC for polar metabolites, and RPLC for complex lipids (using isopropanol for elution). Data acquisition is performed on high-resolution mass spectrometers (Q-TOF and Q-Orbitrap) in a full scan mode, where the instrument is constantly scanning the entire mass range, from 50 to 1500 Da. To alleviate the fastidious metabolite identification, the MS/MS data—of high quality and volume, are acquired (simultaneously to MS data) in DDA and/or DIA mode, on pooled quality control (QC) samples—representative of the entire sample set (see section below on “relative quantification”). Untargeted data processing and analysis workflow, including the open-access tools and databases have been extensively reviewed elsewhere.[23,111]

The untargeted experiments are semi-quantitative, based on group comparison to evaluate the relative abundance (i.e., increase or decrease) of each metabolite (feature) to the average abundance measured in the control group. The untargeted experiment can also be coupled with the targeted quantification of specific class of relatively abundant metabolites, whose quantification can be performed in a full scan mode.[60] In this way the quantitative data can be generated while benefiting from the retrospective exploration of full scan data on other detected metabolites. While untargeted approach can reveal the unanticipated metabolic alterations, the obtained results should be considered as hypothetical and must be validated by targeted quantification that will allow for the normalization of the inherent analytical variability (related to matrix effects).

**From single pathway to broad-coverage multiple pathway targeted assays**

The relative lack of sensitivity of full scan instruments compared to tandem mass spectrometry (MS/MS), together with the bottleneck of (true) metabolite identification that it is vital for data interpretation have motivated the development of broad-coverage targeted assays to screen for small molecule intermediates in multiple pathways, including those present at low concentration levels in biological samples[112,113] (see Figure 2). These multiple pathway targeted assays with a comprehensive coverage of several hundred metabolites have therefore emerged as a surrogate to untargeted assays, taking the advantage of high sensitivity of triple quadrupole instruments operating in multiple reaction monitoring (MRM) or scheduled/dynamic MRM...
Relative or absolute quantification?

Relative quantification of detected metabolites is based on group comparison or evaluation of relative metabolite abundance to control group using the peak areas or ion abundances expressed in arbitrary units (A.U.). In these semi-quantitative assays without an internal reference (due to difficulty to cover with internal standards all chemically diverse metabolites that behave differently in the same analytical conditions) the use of pooled samples as quality controls (QC) is essential to monitor and correct for the analytical variability during the sample analysis. Pooled QC samples are used for system conditioning and to correct for within- and between-batch variation of signal (i.e., signal intensity drift). Guidelines and considerations applied for quality control and batch correction in semi-quantitative assays have been covered in many more details in a review by Ivanisevic and Want[123] and Broadhurst et al.[124] The results of these untargeted semi-quantitative assays lack the quantitative accuracy (i.e., the peak area in not proportional to metabolite concentration) and thus cannot be compared across different studies, laboratories, etc. Hence, we strongly recommend that the data generated by relative quantification approaches (untargeted and multiple pathway targeted profiling in the absence of internal standards) must be validated by an absolute targeted quantification approach as detailed below.

In quantitative mass spectrometry or absolute quantification approaches the calibration curves and stable isotope-labeled standards (deuterated, $^{13}$C or $^{15}$N) are used for quantification purpose, to translate peak areas to metabolite concentrations. This is necessary because the MS response (i.e., peak area) depends on compound structure and ionization efficiency, in addition to being affected by the matrix effect produced by all the other components in the complex biological mixture. The internal standard spike will correct for this matrix effect and the bias introduced by sample preparation and yield of extraction (Figure 3). To determine the absolute concentration of a specific metabolite in a biological sample the internal standard is added at defined known concentration to all the samples and a standard mixture dilution series (i.e., calibration curve), thus allowing for the correction of matrix effects and ionization efficiency. The accuracy, precision, repeatability and limits of detection of methods used for absolute quantification of metabolites of interest need to be validated prior to their application. The translation of peak areas to concentrations and normalization for inherent analytical variability facilitates the cross-laboratory and cross-study comparisons and the establishment of reference (or “normal”) concentration ranges, essential for clinical research and practice.

Isotopic profiling or stable isotope-assisted (UN)targeted metabolite analysis

Untargeted and targeted metabolomic assays allow for the relative and absolute measurement of intracellular and extracellular metabolite levels. However, these measurements do not provide the information on relative pathway activities depending on investigated conditions. The increase in level of specific metabolite can be due to the enhanced activity of metabolite producing enzymes or the decreased activity of metabolite consuming enzymes. The inability of standard untargeted and targeted assays to provide this information on pathway utilization in different conditions has led the development of stable
Targeted quantification assay. Internal standard spike or the addition of stable isotope-labeled standard (IS) mixture to the sample is necessary to translate the signal abundance (i.e., peak area) to metabolite concentration. Signal abundance is not an equivalent (or proportional) to metabolite concentration because it depends on the ionization efficiency of each metabolite. Metabolites producing higher signal intensities ionize well but do not necessarily represent the most abundant metabolites. The metabolite concentration will be calculated based on the calibration curve by reporting the area of detected endogenous metabolite peak to the area of its internal standard (IS) spiked at known concentration. This IS spike allows for the correction of analytical variability during the sample preparation (i.e., variation due to metabolism quenching and potential metabolite transformations) and analysis (i.e., due to matrix effects), and is mandatory for an accurate and precise quantification of metabolite concentrations and cross-laboratory comparisons.

Isotopic profiling or stable isotope-assisted analysis. Isotopic profiling is applied to track the fate of labelled nutrient and understand the changes in its utilization in different conditions (e.g., WT vs. KO, CTRL vs. treated)—to identify the pathways that are actively used for labelled nutrient metabolism. The experiment can be performed in vitro (by the addition of labelled metabolite to cell culture media) or in vivo (by bolus injection or drinking water to mice) and is based on group comparison. Data can be acquired in several different analytical settings, in untargeted and in targeted fashion. The identification of 13C or 15N enriched metabolites (mainly based on carbon or nitrogen transfer) is based on the distribution calculation of isotopologue abundances (i.e., one, two, three, four, etc. carbon-labeled isotopologues), corrected for natural isotope abundances. In the example showed on the figure, the 13C-labeled glucose was metabolized via pyruvate and TCA cycle and the succinate enriched in 13C label was detected. Bar plot was reproduced from data generated for Kanellopoulos et al. 2020 (https://doi.org/10.1016/j.cell.2020.02.044) as an example of differential pathway utilization in different conditions (mutant/treatment). Values shown are mean plus or minus SEM, ***p<0.001, Sidak’s multiple comparisons test.

Isotope-resolved metabolomic approaches or isotopic tracer analysis (Figure 4). Tracing metabolite fate can be done by isotopic profiling in steady state experiments to determine qualitative changes in pathway contributions to labeled nutrient utilization, or by quantitative, flux analysis to determine metabolite production and consumption rates. The latter requires computational data modeling based on known stoichiometry of the metabolic network. The review by Buescher et al. describes the difference between qualitative 13C tracer analysis and quantitative flux analysis with respect to experimental design and applications. Both types of these stable isotope-assisted analysis are essential to understand the mechanisms of metabolic regulations, like for example in reprogrammed cancer metabolism. In these experiments a biological system is fed with one or more metabolic substrates or nutrients labeled with stable heavy isotopes.
From model systems to human population studies. Due to its high-throughput and phenotyping capacity, different metabolomic and lipidomic approaches, from untargeted screening to targeted quantification and isotopic profiling, can be applied to different model systems, demanding lower number of independent biological replicates, to clinical research studies, demanding high number of participants due to high human inter-individual variability and multiple confounding factors. Estimated number of biological replicates necessary for the appropriate experimental design—to yield relevant conclusions with sufficient statistical power—is indicated below each studied system (with the assumption of same genotype in model organisms). It is mandatory to validate the results of untargeted profiling by targeted quantification, particularly in human population studies, also to allow for the cross-study comparability.

(\textsuperscript{13}C, \textsuperscript{15}N, \textsuperscript{2}H, \textsuperscript{18}O). These labeled nutrients can be metabolized by the studied system through different metabolic pathways (depending on different conditions of growth, genotypes, etc.) and the isotope labels spread in a time, reaction rate and pathway dependent manner will generate characteristic labelling patterns that can be measured and identified using MS or NMR techniques. In MS-based isotopic profiling the propagation of a label from the tracer to a given metabolite or label (e.g., \textsuperscript{13}C) enrichment is quantified as the fractional abundance of isotopologues (relative to the total metabolite abundance). Isotopologues are molecules that have the same molecular formula and structure but differ in their isotopic composition through the substitution of one or more atoms with different isotopes (e.g., H\textsubscript{2}O/D\textsubscript{2}O, Glucose/\textsuperscript{13}C\textsubscript{6}-Glucose, Glutamine/\textsuperscript{13}C\textsubscript{5}-Glutamine). The measured isotopologue distribution needs to be corrected for the abundances of naturally occurring isotopes (that depend on the molecular composition).

The appropriate time-series experimental design is crucial to obtain solid conclusions from an isotopic tracer experiment. The isotopic profiling applications, tracers to use, metabolite readouts and biological data interpretation are extensively covered in two reviews, by Jang et al. on isotope tracing and metabolomics, and by Belcells et al. on metabolic flux analysis.

A very important aspect in isotopic tracer experiments is the choice of a tracer or labeled substrate. Generally, \textsuperscript{13}C-Glucose tracer constitutes the best compromise for determining fluxes in upper metabolism (glycolysis and pentose phosphate pathway), while \textsuperscript{13}C-Glutamine tracers are typically used to resolve metabolite passage through the TCA cycle and reductive carboxylation. The most powerful approach is to perform labeling experiments with different tracers in parallel and then integrate the data into a single flux study. In general, completely (100\%) and uniformly \textsuperscript{13}C-labeled substrates (\textsuperscript{13}C\textsubscript{6}-Glucose, \textsuperscript{13}C\textsubscript{5}-Glutamine) are preferred for identifying (i.e., qualitatively) their fate in a cellular system. The use of \textsuperscript{13}C-labeled substrates in combination with unlabeled ones is widely used to assess multiple fluxes or contribution from multiple sources (or pathways) in central carbon metabolism with a single experiment. The combination of labeled and unlabeled carbon backbones will lead to partly labeled metabolite forms, and allow for the differentiation of pathways used for their production. Although the interpretation of labeling data can be fairly fastidious, and the cost of isotopic tracer experiments high, they complement the standard measurements of metabolite levels with the clues on the origins of metabolic alterations.

CONCLUSIONS AND PERSPECTIVES

Metabolomics, including lipidomics, is focusing the metabolism research back to metabolites, in addition to genes and their function that were in the spotlight during the past era of biochemical genetics.
Metabolic profile constitutes a dynamic and sensitive measure of phenotype at the molecular level, integrating the gene-defined metabolic capacity and the response to environmental exposures. The sensitivity, specificity, dynamic range and reproducibility of MS-based techniques are evolving at a constant pace; thus, expanding the polar and lipid metabolome coverage and offering the accuracy and precision for its quantification. With these continuous technological improvements, the metabolite analysis has gone beyond the measurement of highly abundant metabolites involved in energy production and storage, and towards the measurement of low abundant signaling metabolites implicated in the metabolic signaling and regulation (neurotransmitters, steroids, lipid mediators such as eicosanoids, oxysterols, phosphoinositides, etc.). These high-throughput quantitative methodologies, in combination with experimental biological approaches, allow us to revisit the metabolism, from model systems to human population studies (Figure 5), and gain new insights into the far-reaching metabolite role and (bio)activity, instead of reducing metabolites to biomarkers. The generation of quantitative data on metabolite concentrations across clinically well characterized and genotyped human population(s) will also advance the hypothesis on molecular mechanisms behind complex metabolic traits and data interpretation in the physiologically relevant context of body cycles, and energy homeostasis regulation. The use of this information to understand the health outcome and pathophysiological processes will ultimately help us to modulate the metabolic disease phenotype. While the untargeted approach is a valuable discovery approach, the isotopic profiling and toward the measurement of low abundant signaling metabolites implicated in the metabolic signaling and regulation, and towards the interpretation in a biologically relevant context.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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