Metabolomics assisted with stable-isotope labeling: exploring neuronal metabolism related to Parkinson's disease

Huang, L.

Chapter 1:

General introduction and scope

Introduction

Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder of the central nervous system that mainly affects the motor system, finally leading to symptoms such as bradykinesia, rigidity, resting tremor, and postural instability. As the disease worsens, patients can further develop cognitive and behavioral problems such as depression, anxiety, apathy, and dementia [1]. It is generally regarded as a disease of old age and affects roughly 1% of the population over the age of 60 and up to 5% of the population over the age of 85 [2]. However, 4% of patients present early-onset PD symptoms before the age of 50 [3]. The majority of these early-onset cases are linked to various forms of genetic mutations, such as dominantly inherited mutations including SNCA, LRRK2, recessively inherited mutations including Parkin, PINK1, DJ-1 and GBA [4,5]. In addition, epidemiological research indicates environmental factors associated with an increased risk of PD, such as herbicides and pesticides (e.g., paraquat, rotenone, and maneb), metals (e.g., manganese and lead), head trauma, and well water [6]. The etiological discoveries have prompted subsequent research questions about how these risk factors contribute to the loss of dopaminergic neurons in the mid-brain, notably targeting the substantia nigra (SN). Two major hypotheses have been proposed regarding the pathogenesis of the disease. One hypothesis claims that neuron demise can be triggered by protein misfolding and aggregation, whereas the other hypothesis proposes this process is provoked by mitochondrial dysfunction and the consequent oxidative stress [7].

To address the underlying pathogenic mechanism, associated PD studies have been carried out using patient biofluids, postmortem tissue, in vitro cell models and animal models. In vitro patient-derived neuronal models using human induced pluripotent stem cells (iPSC) technology are still young, yet they offer unique advantages for studying specific neuronal subtypes represented by human genetics [8]. Disease modeling in either genomic or epigenetic base helps to reveal unique or common routes leading to the consequences of clinical PD symptoms. The convergence and interactions of genetic predispositions, advancing age, and environmental factors on the impairment of metabolism network can play a crucial role in progressive neurodegeneration. I believe investigating the affected metabolism network caused by various individual risk factors and their interactions can provide new insights into disease cause and hint at potential treatment strategies or possible early-intervention therapies (**Figure 1**).

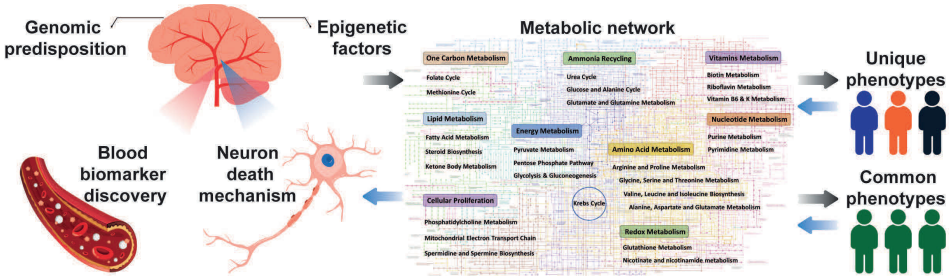

Figure 1. A graphic overview of PD risk factors associated with genomic predispositions and epigenetic factors (due to e.g. environmental stress and aging) that cause unique and common clinical PD symptoms through various metabolism dysregulations. Studies on patient blood or patient-derived neuron analysis can be performed with the goal of identifying disease biomarkers or understanding neuron death mechanisms. Metabolic network adapted from Cao et al [9].

System biology

System biology integrates experimental and computational approaches to perform a comprehensive and systematic analysis and evaluation of complex biological systems [10,11]. Constructing a hierarchical molecular network topology is a fundamental key to understanding cellular function in disease-specific or cell-specific conditions at the molecular level, which also requires knowledge of diverse biological components and sufficient collection of biological data [12,13]. Advanced developments in quantitative measurement technology covering genomics, transcriptomics, proteomics, and metabolomics in a high-throughput manner highly improve the quality and efficiency of metabolic model construction [14,15]. Recon3D is the latest updated and expanded human metabolic network reconstruction, accounting for 3,288 open reading frames, 13,543 metabolic reactions involving 4,140 unique metabolites, and 12,890 protein structures [16]. With multi-layers of biological data integration into a generic human metabolic model, context-specific models at the genome scale can be generated and characterized for a certain cell or tissue type. Applications have been shown for hepatocytes [17], liver cancer stem cells [18], fibroblasts [19], and peripheral blood mononuclear cells [20,21]. This comprehensive modeling approach can also be utilized to help in PD research, thereby, our group generated a comprehensive, high-quality, thermodynamically constrained model named iDopaNeuro, representing the normal metabolism in iPSC-derived human dopaminergic neurons [22]. The iDopaNeuro model can simulate changes in metabolic phenotypes brought on by any neurotoxin or drug intervention, providing directions for new biochemical experiments and insights into a systematic understanding of PD pathogenesis.

The metabolic process involves thousands of metabolites that are exchanged or transformed through biochemical reactions in a metabolic network. It can be greatly influenced by genetic or environmental factors and reflects the global outcomes of gene expression, protein kinetics, and regulations in a biological system [23]. Tracking the changes in the dynamic metabolism pattern is currently of great interest [10]. Metabolomics has emerged as a powerful tool to target the changes in metabolism at its sources, intermediates, and products. Furthermore, it can offer metabolite-relevant data as constraints used for genome-scale modeling analysis; Alternatively, it can also be utilized as a validation dataset to assist biological interpretation and refine the model's predictive fidelity.

Metabolomic analysis

Metabolomics is focused on the systematic analysis of small biochemical molecules in biological organisms [24]. These small molecules, also referred to as metabolites, are characterized by a molecular weight below 1500 Da, diverse physicochemical properties and a broad concentration range from millimolar to picomolar [25,26]. Based on the compound partition coefficient, represented by LogP, metabolites can be categorized into three groups: polar ($LogP < 0$); medium-polar ($0 < LogP < 5$); non-polar ($LogP > 5$) [27]. Polar metabolites generally include amino acids, nucleotides, carbohydrates, and carnitines. Medium-polar metabolites are represented by classes of fatty acids, steroids, benzenes, prenol lipids, ketones, some of the amino acids, and glycerophospholipids. Non-polar metabolites generally include glycerolipids, sphingolipids, steroids, some of the prenol lipids, and glycerophospholipids [27].

Metabolomics measurement is generally achieved using two main analytical techniques: mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. MS is more widely used than NMR due to its superior detection sensitivity, wide dynamic range, and capacity for accurate metabolite identification [28]. Recent advancements of mass spectrometry technology in ionization versatility, detector sensitivity and resolution intimately promote high-throughput metabolome analysis [29,30]. MS can be coupled with different chromatography separations. According to the physico-chemical properties of metabolite targets, suitable chromatography among gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) can be selected for sufficient metabolite separations. GC is suitable for volatile and thermally stable metabolites (eventually after derivatization), and CE is robust for polar and charged metabolite analysis [31]. Compared to them, LC has the widest metabolite coverage, with a combined utilization of hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC). On top of these, a recent innovative technique of coupling ion

mobility spectrometry to mass spectrometry allows supplementary chromatographic separation on the basis of compound size, shape, charge, and mass [32].

A query performed across various human metabolome databases reported that around 3278 metabolites have been detected via LC-MS analysis and collected with experimental spectra. The most frequent biological sample types used in LC-MS-based metabolomics analysis are blood (plasma or serum), tissue, cells, urine, and feces [27]. Blood carries diverse metabolites through vessels and maintains a homeostatic correspondence with tissues and cells in the body [33]. Typically, receiving nutrients or hormones from the blood, tissues and cells release metabolic waste products, organic waste, or send hormone signal regulation to neighboring tissues. This also describes the role of blood as a crucial hub for metabolite exchange throughout the body [34]. The study of paired arterial and venous plasma profiling shows additional benefits for revealing subtle changes related to tissue-specific metabolism [33]. Unsurprisingly, the metabolome composition of plasma and cells was found with considerable overlap, except for the lipids, which are found in higher enrichment in cells due to the need for composing membrane structure [27]. From an analytical perspective, sample preparation and the LC-MS method, with minor tweaks, can be shared between these two sample types. From a biological perspective, global metabolic profiling for plasma and cell samples can offer a data-driven research approach to disease biomarker discovery. While targeted analysis of metabolites belonging to specific compound classes, metabolic pathways, or modules, especially for tissue and cell samples, can provide a hypothesis-driven research approach in disease mechanism study [35]. Selecting the appropriate approach and sample type depends on the biological questions that need to be answered in any metabolomics study.

Use of stable isotopes in metabolite quantification and identification

Stable-isotopes have an unreplaceable role in targeted metabolite quantification. Instrumental variations and complex matrix effects are inevitable issues and easily lead to signal suppression during LC-MS analysis. To circumvent this, stable-isotope labeled metabolites possessing with the same retention and ionization behavior can be used as internal standards (**Figure 2.a**). This method is known as the stable-isotope dilution and has already become the gold standard for accurate quantification. However, due to the high cost of stable-isotope labeled standard, it is not practical to get an internal standard for each individual metabolite in targeted metabolomics analysis. Many alternative strategies have been implemented, for instance, 1) select a single or small number of internal standards per metabolite class [36,37] (**Figure 2.b**). 2) generate labeled intracellular metabolome as internal standards reference by culturing with labeled substrates, applicable for

metabolomics study not only in cell level, but also in plant, animal and human level [38–41] (**Figure 2.b**). 3) prepare internal standards by derivatizing standards with isotope labeling reagents, only applicable for the derivatization strategy [42,43] (**Figure 2.c**).

One major challenge that hinders biological interpretation based on global metabolic profiling is metabolite identification. For untargeted MS-based metabolomics analysis, detected features generally consist of biological signals, contaminants, non-metabolite-related noise, and background signals [44,45]. Unique biological features were reported to account for approximately 10% of the signals in the electrospray ionization mode [46,47] This adds difficulties to the reliable feature extraction and annotation of putative metabolites of biological origin. To tackle this problem, stable-isotope labeling becomes a novel approach for feature identification. The basic principle is a cultivation of an organism group with highly isotopic labeling and a group with isotopic natural abundance. The labeled samples are extracted and mixed into a whole labeled metabolome pool, later divided into aliquots, and added to a non-labeled metabolome extract [48]. The analysis of mixtures of native and labeled metabolome leads to labeling-specific isotopic distributions of both the non-labeled and labeled metabolite ions in the mass spectra, which helps to filter biologically derived metabolites [49–51] (**Figure 2.d**). Studies have reported a comprehensive identification of true metabolite-related features in microorganisms [49,52–54], plants [49–51] with the help of feeding fully ^{13}C labelled nutrients. However, the same approach is not often utilized in mammalian organisms since mammalian cells require a complex mixture of nutrients and rarely reach full ^{13}C labeling. A proof-of-principle study on human cancer cells managed to realize "deep labeling" using a custom growth medium where glucose and all amino acids were fully ^{13}C labeled, while vitamins and serum components were ^{12}C. Due to the fact that the isotopic distributions of metabolites can be in a non-fully carbon-labeled state, there is difficulty in determining the carbon numbers for unknown metabolic features. But more importantly, endogenous metabolic features from de novo synthesis can be fully identified via ^{13}C incorporation [55].

Either through improving metabolite quantification or metabolite identification towards full metabolome annotation in a given cell or tissue type, it can be highly beneficial in gaining a broader and deeper picture of human metabolism. Additionally, it will also help refine a context-specific genome-scale model to achieve more accurate metabolic prediction and comprehension of mechanisms. More effort has to be made to reach this ultimate goal since the analytical challenges still remain. In this thesis, we aim to make efforts focusing on the fundamental evaluation of a robust HILIC-MS method for polar metabolome analysis with high feature coverage and excellent separation that is applicable for global metabolite

profiling and transferable to metabolite quantification, as demonstrated in **Chapter 2** and **Chapter 3**.

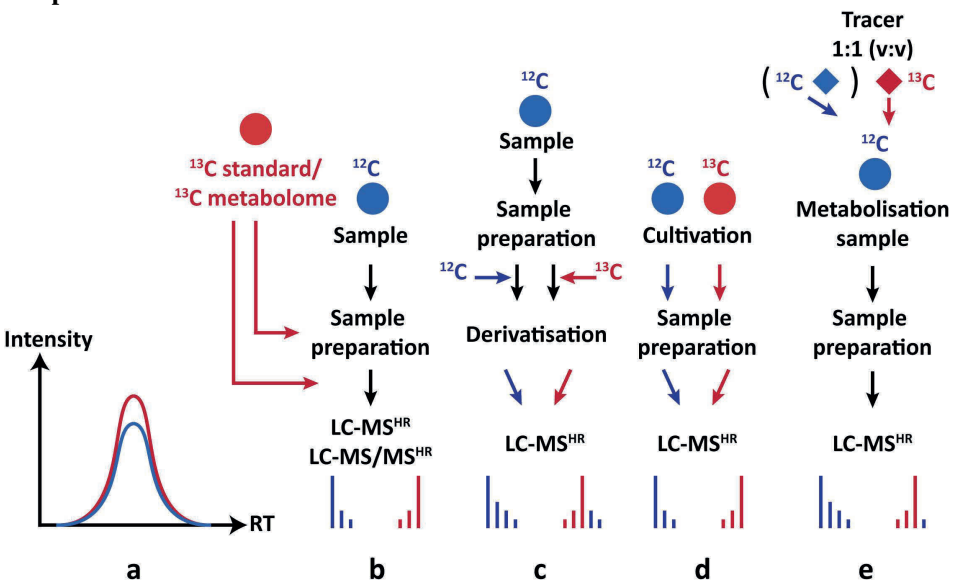

Figure 2. Different strategies for using stable isotopes to assist metabolomics studies. **a.** ^{13}C, ^{15}N, and ^{34}S-enriched substances are not chromatographically separated from the corresponding natural isotopologues, thus the non-labeled (blue) and the labeled isotopologues (red) elute at the same retention time with identical peak profiles. **b.** Absolute compound quantification using an authentic, labeled standard or relative quantification using a stock of globally labeled sample extract of the same organism for inter-experiment comparison. The extracts are subsequently mixed and measured with high resolution LC–MS (LC-MS^{HR}). **c.** Derivatization using non-labeled and labeled derivatization agents enables rapid recovery of many metabolites belonging to the same chemical groups (e.g. alcohols, acids …). **d.** For non-targeted annotation of an organism's metabolome, the organism can be cultivated in parallel using differently isotopologue-enriched nutrition sources (e.g., ^{12}C and ^{13}C glucose as sole carbon source). The resulting data pattern helps in the extraction of true biological signals. **e.** Metabolism experiment using natural and fully labeled tracer substances enables metabolism studies and greatly helps to separate products of metabolism from other biological signals. In contrast with metabolism studies, fluxomics (tracer-based metabolomics) experiments only spike with the labeled tracer. Referred to Bueschl et al [56].

Stable-isotope labeling in tracing cellular metabolism activity (Tracer-based metabolomics)

Cellular metabolism is a self-maintenance and regulation process that provides energy, generates building blocks, and tunes signaling pathways for cell survival and growth. Metabolism also supports cell-to-cell communication, helping to maintain an active microenvironment and contributing to the whole organism's survival. Due to the intrinsic network feature of metabolic pathways complexity, redundancy and reaction reversibility, metabolite concentrations generally exhibit strong robustness to any genetic or enzymatic changes [57]. Many uncertainties remain for the interpretation of cell metabolism based on the static metabolic phenotype data. Metabolic flux, also known as metabolite turnover over time or metabolic reaction rate, starts to gain more attention because of its ability in representing functional pathway activities. Stable or radioactive isotope labeling shows a unique advantage in studying metabolic fluxes and elucidating the structure of metabolic pathways and networks. A given isotopic tracer fed to living cells can be metabolized via enzymatic reactions. In a reaction process, a number of molecular bonds broken and reformed, the isotopic atoms are rearranged and incorporated into downstream metabolites within the metabolic network [58]. The specific labeling pattern of intermediate metabolites derived from stable isotopic tracer can be measured by mass spectrometry (**Figure 2.e**). Mass isotopologues refers to molecules that differ only by the number of isotopic substitutions [59]. Mass isotopologue distribution (MID) records the relative abundance for all mass isotopologue peaks. Here, we will introduce qualitative and quantitative applications involving metabolite isotopologue data interpretation and modeling for exploring cellular metabolism, as well as the remaining challenges for tracer-based metabolomics.

Probing pathway activity

With stable isotopic labeling, we can quantify the utilization of certain carbon or nitrogen sources in the targeted downstream products. ^{13}C-glucose, ^{13}C-glutamine and $^{15}N_2$-glutamine have been frequently used to monitor the nutrient dependence of tumor tissue. Through infusing $^{13}C_6$-glucose into human lung cancer patients, higher ^{13}C-enrichment in lactate, alanine, succinate, glutamate, aspartate, and citrate was observed in the tumors compared to non-cancerous tissues, suggesting more active glycolysis and the tricarboxylic acid cycle relying on glucose in the tumor tissues [60]. Glutamine was reported to be an important nutrient for most cancer cells in culture [61]. However, an in-vivo lung tumor study in mice showed low ^{13}C-glutamine utilization by both tumors and normal tissue. Genetic deletion and pharmacological inhibition of glutaminase showed no influence on the tumor growth [62]. ^{13}C labeled substrates can also be used to determine their contribution

to lipogenic acetyl-CoA production by measuring the isotopic enrichment in fatty acids (palmitate, stearate, oleate, etc.) [63].

Specific enzyme activation over certain pathways can be characterized through labeled enrichment analysis of intermediate metabolites or quantification of particular isotopologue. Pyruvate carboxylase (PC) catalyzes an important anaplerotic reaction that creates oxaloacetate from pyruvate. The resulting presence of $^{13}C_3$-citrate, $^{13}C_5$-citrate, $^{13}C_3$-malate, and $^{13}C_3$-aspartate from $^{13}C_6$-glucose indicates high PC activity in human lung tumors [60,64]. The last step of glycolysis, converting phosphoenolpyruvate to pyruvate, is catalyzed by the M2 isoform of pyruvate kinase (PKM2). By quantifying the labeled abundance of $^{13}C_6$-glucose-derived metabolite isotopologues ($^{13}C_3$-phosphoenolpyruvate, $^{13}C_3$-pyruvate, $^{13}C_2$-citrate, $^{13}C_3$-serine, $^{13}C_2$-glycine, $^{13}C_3$-lactate), human colon carcinoma HCT116 cells demonstrated PKM2 silencing in response to serine deprivation, shown with more pyruvate diverted into mitochondria and shifting more carbon flux into serine biosynthesis [65]. Similar approaches were employed for reporting a highly activated phosphoglycerate dehydrogenase in some cancer cells, which largely diverts glycolytic flux carbon into serine and glycine metabolism [66].

Many enzymatic reactions are bidirectional, reaction reversibility adds more flexibility to metabolic network regulation. By feeding cells with a designed tracer, a reversible reaction direction can be identified when the expected labeling pattern is observed in certain reaction products [67]. In addition, stable-isotope tracing shows its advantages in studying compartment-specific pathways. By tracing the isotope labeled hydrogen (^2H) in compartmentalized reactions that use NADPH as a cofactor and produce ^2H-labeled 2-hydroxyglutarate by mutant isocitrate dehydrogenase enzymes, Lewis et al. successfully differentiated pathway-specific NADPH production in the cytosol and mitochondria [68].

Delineating the metabolite labeling pattern associated with two relevant pathways can help determine their relative pathway flux activity. One classical application is determining the relative flux through glycolytic versus pentose phosphate pathway (PPP) catabolism using 1,2-$^{13}C_2$-glucose [69]. The ratio of lactate with M+1 labeling and M+2 labeling implies the ratio of PPP overflow to glycolysis. A relative contribution of oxidative PPP to non-oxidative PPP to ribose-5-phosphate and thus nucleotide synthesis can be quantified by a relative comparison of M+1 and M+2 labeling [70]. A similar approach was employed for reporting the flux contribution of glutamine to palmitate synthesis derived from two distinct pathways, the glutaminolysis and reductive carboxylation pathways, in brown adipocyte cells [71].

Discovering novel pathways

Isotope tracing through known pathways or metabolome-wide analysis may facilitate the discovery of novel metabolic flux routes [57,72]. Tracing the carbon conversion of $^{13}C_6$-glutamine in the tricarboxylic acid (TCA) cycle resulted in the identification of a novel pathway for tumorigenesis, which is the reductive carboxylation of transforming α-ketoglutarate into citrate, followed by a conversion into acetyl-CoA for lipid synthesis [73,74]. Another breakthrough discovery found in lung and pancreatic cancer studies showed ^{13}C-lactate extensively labels TCA cycle intermediates, indicating a lactate oxidation flux into the TCA cycle [75,76]. However, tracing targeted pathways using specifically designed tracers is generally slow and random in discovering new pathways that have important physiological relevance. Very few studies have performed a metabolome-wide analysis with stable isotope labeling due to the difficulties in complex data analysis and interpretation. Puchalska et al. combined stable isotope tracing with untargeted metabolomics and identified a set of mitochondrial and cytoplasmic metabolic pathways related to the utilization of ketone bodies, acetoacetate in macrophages. They revealed an acetoacetate shuttle that connects the metabolism of hepatocytes to neighboring macrophages and protects the liver from high-fat diet-induced fibrosis [77]. To fully exploit their ability to discover new pathways based on broad-scope metabolomes, ongoing efforts should be made in the areas of unknown feature identification and dedicated data analysis pipelines for labeled data processing [78].

Quantitative metabolic flux analysis

For a metabolic network, metabolite concentrations and metabolic fluxes are regarded as the integrated functional response to the intertwined regulations at the genetic, protein modification, allosteric, and kinetic levels [79]. Quantifying network-flux distributions gives a complementary characterization of metabolic phenotypes in cells under particular conditions. The intracellular reaction rates are not measurable directly but can be inferred computationally using stable-isotopically labeled isotopologue distribution data. ^{13}C-based tracers are most commonly used for experimental flux quantification, known as ^{13}C metabolic flux analysis. The network model scope is basically determined based on specific research hypotheses. Most studies so far have focused on the central carbon metabolism and the related amino acid and fatty acid metabolism. A curated network model including metabolic reactions of interest and the respective carbon atom transitions is needed to be built as a prerequisite. Following an isotope labeling experiment, the isotope labeling distribution of intermediate metabolites, and external rates of substrate uptake and product secretion are measured and used as model constraint inputs. The model flux simulation generally starts with a set of free fluxes with random initial values. The simulated labeling

distribution is then compared with the experimental labeling distribution. This computational fitting step restarts continuously until it reaches the minimized differences between simulated and experimental measurements, thereby, the final simulated flux distribution is approaching the in vivo fluxes [80].

Simulation of the ^{13}C metabolic flux is typically based on several assumptions [81]. For example, cultured cells are maintained under a metabolic steady state with constant metabolic fluxes and metabolite pools during the labeling experiment. It is also assumed that enzyme activity displays no kinetic differences between natural substrate and isotopically labeled substrate. When isotopic steady state is reached, the labeling distribution data can be used for stationary metabolic flux analysis. While in many cases, it takes rather long time to reach constant labeling distribution for some metabolites. Isotopically non-stationary metabolic flux analysis can be performed with additional inputs of intracellular metabolite pool size together with dynamic labeling distribution at multiple sampling time points [82,83]. The precision and accuracy of metabolic flux estimation are statistically evaluated by verifying the goodness of fit and determining confidence intervals for the fluxes, also called sensitivity analysis.

Quantitative metabolic flux analysis has helped in characterizing metabolic rewiring and understanding disease phenotypes [84,85]. During the process of detachment from monolayer culture and growth as anchorage-independent tumor spheroids, Jiang et al quantified the reduction in glycolysis, pyruvate dehydrogenase flux, and glucose/glutamine oxidation but enhancement in reductive isocitrate dehydrogenase flux in spheroids [85]. Metabolic flux quantitative analysis also helped guide cell bioengineering to produce valuable products from renewable resources [79]. For instance, ^{13}C flux analysis was performed in Chinese hamster ovary cells to evaluate the effectiveness of a newly designed medium variant in reducing ammonia production. The metabolic effect showed an effective reduction of toxic product (ammonia) production and no significant alteration in the bioenergetic fluxes [86].

Challenges in tracer-based metabolomics

A well-established metabolic pathway for a particular cell type is crucial for compelling interpretation of either metabolite concentrations or MID data toward pathway activity. For a well-studied metabolic pathway, for instance, metabolite connection and transformation in the classical central carbon metabolism are usually taken for granted. New reactions or unknown reaction reversibility can be confirmed using a designed tracer. In other words, this also means the labeling information can help support and validate the metabolic pathway reconstruction. Technically, time-of-flight (TOF) or Orbitrap mass spectrometry

analysis at high mass resolution could obtain the labeling information, including the labeled atom number and total isotopic enrichment, based on the intact structure level. However, the positional labeling information embedded in the metabolite substructure or moiety can be missed. Although a tandem quadrupole-based mass spectrometry analysis using multiple reaction monitoring (MRM) can offer partial substructure information, it has the significant drawback of losing detection sensitivity with increased metabolite targets and paired ion transitions [87–90]. Tandem MS-based approaches via parallel reaction monitoring (PRM/MRM^{HR}) [91], SWATH [92], and all-ion fragmentation techniques (MS^{All}) [93] in high resolution have shown their advantages in terms of recording the entire fragmentation spectrum and increasing sensitivity by reducing the detection cycle time. Application based on ultrahigh-resolution MS^{All} on an Orbitrap Fusion Tribrid MS has been shown to confirm the reconstruction of purine and pyrimidine metabolism using not only the MID of an intact metabolite but also the MID of its moiety [93]. Changes in intact and moiety MIDs could also be collectively used to infer specific enzyme activity [94]. A labeled metabolite containing the same number of isotopes but with different labeling positions can indicate distinct enzyme regulations. This method, however, lost the specific link between fragments and their given precursor isotopologue, making it impossible to distinguish different labeling positions for the same precursor isotopologue. Thereby, further method development is required to improve the part of LC-MS measurement (step 1 in **Figure 3**) in order to fully capture labeled metabolite information at the intact molecule and moiety level with good sensitivity in a single analytical run.

Quantitative metabolic flux analysis into understanding human cellular metabolism still has relatively few applications. One bottleneck can be the tedious work related to tracer-based metabolomics data processing (step 2 in **Figure 3**). The other bottleneck is that the model scope is often limited to the central carbon metabolism with established methods for ^{13}C metabolic flux analysis. It is faced with highly challenging computation in order to integrate tracer-based MID data into a larger network model and further to a genome-scale level (step 3 in **Figure 3**). A recently developed mathematical and computational method, named moiety fluxomics, has shown its ability to infer metabolic reaction flux at genome scale, given mass isotopologue distribution data [95]. This remains to be tested in the iDopaNeuro model for human dopaminergic neurons. Flux outputs have to be viewed with skepticism until the model has passed many rounds of flux estimates, flux accuracy assessment, and experimental study validation. This can be a lengthy cycle before finally reaching a compelling biological conclusion.

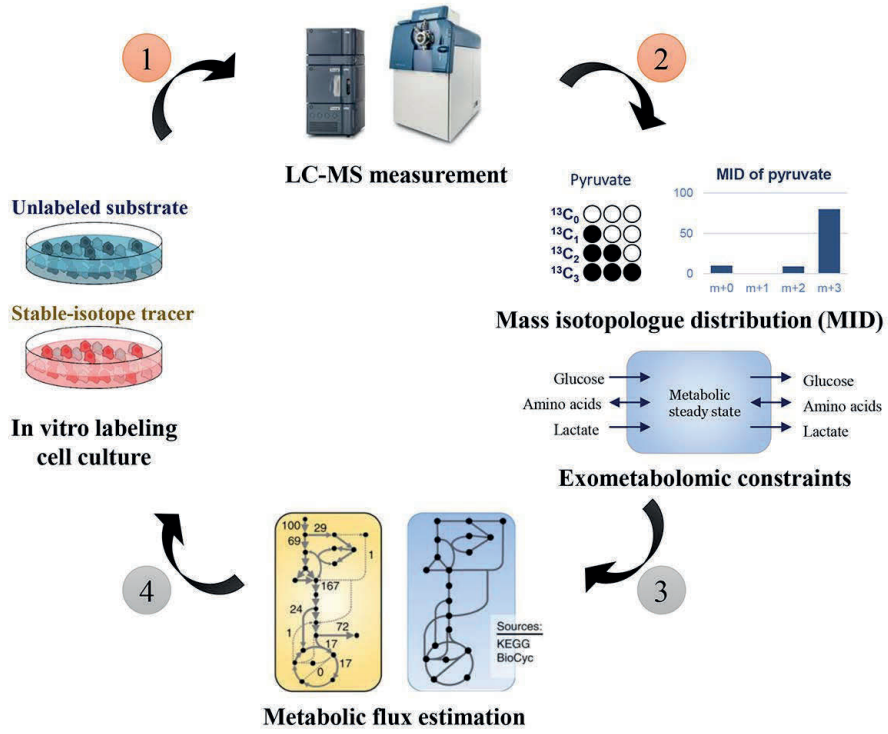

Figure 3. In a typical ^{13}C metabolic flux analysis workflow, in-vitro labeled samples can be measured using LC-MS techniques (step 1), MS raw data needs to be processed metabolite by metabolite (step 2), and the calculated MID data, together with exometabolomic constraints, are next fed into a constructed metabolic model for estimating the optimal flux distribution (step 3). The obtained flux results can guide the next tracer experiment design (step 4). Metabolic network model adapted from Long et al [81].

Scope of this thesis

Based on the hypothesis that mitochondrial dysfunction and the resulting oxidative stress is one of the drivers of Parkinson's disease (PD), the basic goal of our research is to study the role of mitochondrial dysfunction in the onset of Parkinson's disease through monitoring the underlying molecular events. Both variations in metabolite pool size and metabolite transformation or transport (turnover) rate should be taken into account for a comprehensive characterization of metabolic regulation over biochemical pathways. However, as was discussed in the introductory section, there is currently still a lack of suitable analytical measurement, computational processing, and modeling techniques. The study of mitochondrial dysfunction requires (i) the robust measurement of polar metabolites, (ii) the

study of metabolic fluxes at a larger scale. For the latter, sensitive MS/MS analysis coupled with high-resolution MS analysis and a proper computational workflow for tracer-based mass spectrometry data processing and quantitative flux analysis in an atomically resolved genome-scale model are needed.

Therefore, the aim of this thesis was to develop a robust LC-MS method to analyze a wide range of polar metabolites, a tracer-based metabolomics analytical method, and a computational workflow for metabolic flux analysis within a human-specific genome-scale metabolic model. The other aim of this thesis was to apply these methods to investigate metabolic dysregulation of dopaminergic neurons due to genetic and environmental factors.

The aim in **Chapter 2** was to systematically evaluate polar stationary phases for global polar metabolome analysis, moreover, to offer valuable guidance on determining an optimal chromatography column for various biological matrices. We compared the neutral phase of the Waters BEH-amide column with the zwitterionic phase of the Merck ZIC-cHILIC column for 9 classes of polar compounds using 54 authentic standards at three pH conditions. The ZIC-cHILIC column outperformed BEH-amide in terms of chromatographic peak performance and selectivity of critical isomers. Investigation into the retention mechanism demonstrated mixed mode interactions in neutral and zwitterionic phases, specifically with a strong electrostatic interaction present in ZIC-c at neutral pH condition. A matrix-related assessment covering matrix effect, salt effect, intra- and inter-batch repeatability was carried out using human plasma, which was followed by a practical metabolomics study using plasma samples with diverse phenotypes. ZIC-c enhanced plasma feature coverage and improved their retention distribution, which is highly advantageous for global profiling of plasma samples and assisting new biomarker discovery. In the following chapters, we validated, adapted, and further applied the established ZIC-c HILIC-MS method to cellular metabolomics analysis.

To achieve a comprehensive picture of metabolic dysregulation related to individual and combined effects of genetic and environmental factors (PINK1 mutation and rotenone) for PD and facilitate the pathogenesis mechanism understanding, **Chapter 3** employed multiple targeted metabolomics platforms covering polar and non-polar metabolomes covering central carbon metabolism, acylcarnitine and polyunsaturated fatty acid metabolism. The patient-specific and isogenic human induced pluripotent stem cell (iPSC)-derived mid-brain neurons with and without PINK mutation were utilized as the in vitro experimental model, which received additional treatments with rotenone exposure or NAD+ supplementation. The study revealed overlapping and compensating metabolome disturbances induced by individual factors and their contributions to a broad metabolic dysregulation indicative of neurodegeneration. The supplementation of NAD+ to the dual factor-influenced neurons

was evaluated with limited improvement in neuronal energy production relying on the enhanced branched chain amino acid metabolism.

In **Chapter 4**, a new LC-MS method is proposed for stable-isotope labeled mass isotopologue analysis at both MS^1 and MS^2 level. Given the fact that the detection duty cycles for the orthogonal injection TOF-MS are typically less than 30%, this unavoidable cause of sensitivity loss always exists for current high-resolution tandem TOF-MS method. A recent revolutionary technique, Zeno-pulsing, has been reported to increase the duty cycle up to 100%, thus achieving significant detection improvement [96]. The combination of HILIC separation coupled to high-resolution MRM detection with Zeno trap pulsing allowed for wide coverage of polar metabolome analysis and excellent sensitivity at the MS^2 level. In comparison with the conventional SWATH and MRM^{HR} methods, the HILIC-Zeno MRM^{HR} method achieved a higher sensitivity gain. Meanwhile, it maintained isotope fidelity for precursor and fragment isotopologue distribution as well as specificity linking a given precursor isotopologue to its generated fragments. Tracing labeled atoms at the moiety level clearly illuminates the reaction connections through metabolite transformation. The method was applied to a human-derived mid-brain neuronal model and revealed a new elucidation of glutathione metabolism regulation in response to rotenone stress via interpreting labeling pattern changes from both intact metabolites and moieties. Aside from capturing the static metabolite level or concentration, dynamic pathway activity provides a complementary perspective for a more complete understanding of metabolic phenotyping.

Quantitative flux inference from metabolite labeling patterns remains a big challenge, especially at a genome-scale. Besides, there is still a lack of an automated processing pipeline to make this procedure more efficient and turn it into a standardized workflow. **Chapter 5** aims to construct an automated data processing pipeline for quantitative flux analysis in a genome-scale model, termed *fluxTram*. The pipeline is composed of two essential modules: the processing of tracer-based mass spectrometry data into standardized mass isotopologue distribution and the generation of metabolite structure and reaction databases over a genome-scale model. As a demonstration of the pipeline, *fluxTram* processed ^{13}C-labeled metabolomics data collected from an *in vitro* iPSC-derived mid-brain neuron model, which assisted a conventional ^{13}C metabolic flux analysis within a central carbon (core) metabolism model. In parallel, *fluxTram* resolved the atom mappings of a genome-scale, dopaminergic neuronal metabolic model (iDopaNeuroC). The combination of the *fluxTram* outputs allowed us to conduct a moiety fluxomics analysis in the iDopaNeuroC model. An integrative metabolic flux analysis involving core model flux solution, moiety flux solution, and results from two other in silico genome-scale flux analysis methods: entropy flux solution and flux balance analysis became possible, enabling

a comparison of neuronal metabolic function inference and prediction using competing methods. Furthermore, atom mapping assisted in determining the labeling configuration of a tracer used in a subsequent tracer experiment.

Finally, in **Chapter 6** a general conclusion of the studies described in this thesis is provided. Perspectives and recommendations on future improvements and applications of the proposed LC-MS methods and data processing pipelines are also discussed.

References

1. Kouli A, Torsney KM, Kuan W-L. Parkinson's Disease: Etiology, Neuropathology, and Pathogenesis. Exon Publications. 2018;3–26.
2. Fahn S. Description of Parkinson's disease as a clinical syndrome. Ann N Y Acad Sci. 2003;991:1–14.
3. Van Den Eeden SK, Tanner CM, Bernstein AL, Fross RD, Leimpeter A, Bloch DA, et al. Incidence of Parkinson's Disease: Variation by Age, Gender, and Race/Ethnicity. American Journal of Epidemiology. 2003;157:1015–22.
4. Reeve A, Simcox E, Turnbull D. Ageing and Parkinson's disease: Why is advancing age the biggest risk factor? Ageing Research Reviews. 2014;14:19–30.
5. Farrer MJ. Genetics of Parkinson disease: paradigm shifts and future prospects. Nature Reviews Genetics. 2006;7:306–18.
6. Tsuboi Y. Environmental-Genetic Interactions in the Pathogenesis of Parkinson's Disease. Exp Neurobiol. 2012;21:123–8.
7. Dauer W, Przedborski S. Parkinson's Disease: Mechanisms and Models. Neuron. 2003;39:889–909.
8. Tran J, Anastacio H, Bardy C. Genetic predispositions of Parkinson's disease revealed in patient-derived brain cells. npj Parkinsons Dis. Nature Publishing Group; 2020;6:1–18.
9. Cao G, Song Z, Hong Y, Yang Z, Song Y, Chen Z, et al. Large-scale targeted metabolomics method for metabolite profiling of human samples. Analytica Chimica Acta. 2020;1125:144–51.
10. Kitano H. Systems Biology: A Brief Overview. Science. American Association for the Advancement of Science; 2002;295:1662–4.
11. Kitano H. Computational systems biology. 2002;420:21.
12. Ideker T. Systems biology 101–what you need to know. Nature Biotechnology. 2004;22:473–5.
13. Aderem A. Systems Biology: Its Practice and Challenges. Cell. Elsevier; 2005;121:511–3.
14. Fischer HP. Towards quantitative biology: Integration of biological information to elucidate disease pathways and to guide drug discovery. Biotechnology Annual Review [Internet]. Elsevier; 2005 [cited 2022 Nov 1]. p. 1–68.
15. Pinu FR, Beale DJ, Paten AM, Kouremenos K, Swarup S, Schirra HJ, et al. Systems Biology and Multi-Omics Integration: Viewpoints from the Metabolomics Research Community. Metabolites. Multidisciplinary Digital Publishing Institute; 2019;9:76.
16. Brunk E, Sahoo S, Zielinski DC, Altunkaya A, Dräger A, Mih N, et al. Recon3D enables a three-dimensional view of gene variation in human metabolism. Nat Biotechnol. Nature Publishing Group; 2018;36:272–81.
17. Mardinoglu A, Agren R, Kampf C, Asplund A, Uhlen M, Nielsen J. Genome-scale metabolic modelling of hepatocytes reveals serine deficiency in patients with non-alcoholic fatty liver disease. Nat Commun. Nature Publishing Group; 2014;5:3083.
18. Hur W, Ryu JY, Kim HU, Hong SW, Lee EB, Lee SY, et al. Systems approach to characterize the metabolism of liver cancer stem cells expressing CD133. Sci Rep. Nature Publishing Group; 2017;7:45557.
19. Wegrzyn AB, Herzog K, Gerding A, Kwiatkowski M, Wolters JC, Dolga AM, et al. Fibroblast-specific genome-scale modelling predicts an imbalance in amino acid metabolism in Refsum disease. The FEBS Journal. 2020;287:5096–113.
20. Zhang X, Mardinoglu A, Joosten LAB, Kuivenhoven JA, Li Y, Netea MG, et al. Identification of Discriminating Metabolic Pathways and Metabolites in Human PBMCs Stimulated by Various Pathogenic Agents. Frontiers in Physiology [Internet]. 2018 [cited 2022 Nov 2];9.
21. Cho JS, Gu C, Han TH, Ryu JY, Lee SY. Reconstruction of context-specific genome-scale metabolic models using multiomics data to study metabolic rewiring. Current Opinion in Systems Biology. 2019;15:1–11.
22. Preciat G, Moreno EL, Wegrzyn AB, Willacey CCW, Modamio J, Monteiro FL, et al. Mechanistic model-driven exometabolomic characterisation of human dopaminergic neuronal metabolism [Internet]. bioRxiv; 2021 [cited 2022 Oct 15]. p. 2021.06.30.450562.
23. Nicholson JK, Lindon JC. Systems biology: Metabonomics. Nature. 2008;455:1054–6.
24. Beger RD, Dunn W, Schmidt MA, Gross SS, Kirwan JA, Cascante M, et al. Metabolomics enables precision medicine: "A White Paper, Community Perspective." Metabolomics. 2016;12:149.
25. Fiehn O. Metabolomics – the link between genotypes and phenotypes. Plant Mol Biol. 2002;48:155–71.

26. Weckwerth W. Metabolomics: an integral technique in systems biology. Bioanalysis. 2010;2:829–36.
27. Roca M, Alcoriza MI, Garcia-Cañaveras JC, Lahoz A. Reviewing the metabolome coverage provided by LC-MS: Focus on sample preparation and chromatography-A tutorial. Analytica Chimica Acta. 2021;1147:38–55.
28. Pan Z, Raftery D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem. 2007;387:525–7.
29. Wang S, Blair IA, Mesaros C. Analytical Methods for Mass Spectrometry-Based Metabolomics Studies. In: Woods AG, Darie CC, editors. Advancements of Mass Spectrometry in Biomedical Research [Internet]. Cham: Springer International Publishing; 2019 [cited 2021 Aug 24]. p. 635–47.
30. Alves S, Paris A, Rathahao-Paris E. Chapter Four - Mass spectrometry-based metabolomics for an in-depth questioning of human health. In: Makowski GS, editor. Advances in Clinical Chemistry [Internet]. Elsevier; 2020 [cited 2021 Aug 24]. p. 147–91.
31. Ren J-L, Zhang A-H, Kong L, Wang X-J. Advances in mass spectrometry-based metabolomics for investigation of metabolites. RSC Advances. Royal Society of Chemistry; 2018;8:22335–50.
32. Kanu AB, Dwivedi P, Tam M, Matz L, Hill Jr. HH. Ion mobility–mass spectrometry. Journal of Mass Spectrometry. 2008;43:1–22.
33. Ivanisevic J, Elias D, Deguchi H, Averell PM, Kurczy M, Johnson CH, et al. Arteriovenous Blood Metabolomics: A Readout of Intra-Tissue Metabostasis. Sci Rep. Nature Publishing Group; 2015;5:12757.
34. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, et al. The Human Serum Metabolome. PLoS One. 2011;6:e16957.
35. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nature Reviews Molecular Cell Biology. 2012;13:263–9.
36. Cífková E, Holčapek M, Lísa M, Ovčačíková M, Lyčka A, Lynen F, et al. Nontargeted Quantitation of Lipid Classes Using Hydrophilic Interaction Liquid Chromatography–Electrospray Ionization Mass Spectrometry with Single Internal Standard and Response Factor Approach. Anal Chem. American Chemical Society; 2012;84:10064–70.
37. Hines KM, Herron J, Xu L. Assessment of altered lipid homeostasis by HILIC-ion mobility-mass spectrometry-based lipidomics. J Lipid Res. 2017;58:809–19.
38. Srivastava A, Kowalski G, Callahan D, Meikle P, Creek D. Strategies for Extending Metabolomics Studies with Stable Isotope Labelling and Fluxomics. Metabolites. 2016;6:32.
39. You L, Zhang B, Tang YJ. Application of Stable Isotope-Assisted Metabolomics for Cell Metabolism Studies. Metabolites. 2014;4:142–65.
40. Bennett BD, Yuan J, Kimball EH, Rabinowitz JD. Absolute quantitation of intracellular metabolite concentrations by an isotope ratio-based approach. Nature Protocols. 2008;3:1299–311.
41. Evers B, Gerding A, Boer T, Heiner-Fokkema MR, Jalving M, Wahl SA, et al. Simultaneous Quantification of the Concentration and Carbon Isotopologue Distribution of Polar Metabolites in a Single Analysis by Gas Chromatography and Mass Spectrometry. Anal Chem. 2021;93:8248–56.
42. Willacey CCW, Naaktgeboren M, Lucumi Moreno E, Wegrzyn AB, van der Es D, Karu N, et al. LC–MS/MS analysis of the central energy and carbon metabolites in biological samples following derivatization by dimethylaminophenacyl bromide. Journal of Chromatography A. 2019;1608:460413.
43. Guo K, Li L. High-Performance Isotope Labeling for Profiling Carboxylic Acid-Containing Metabolites in Biofluids by Mass Spectrometry. Anal Chem. American Chemical Society; 2010;82:8789–93.
44. Covey TR, Thomson BA, Schneider BB. Atmospheric pressure ion sources. Mass Spectrom Rev. 2009;28:870–97.
45. Trötzmüller M, Guo X, Fauland A, Köfeler H, Lankmayr E. Characteristics and origins of common chemical noise ions in negative ESI LC-MS. J Mass Spectrom. 2011;46:553–60.
46. Keller BO, Sui J, Young AB, Whittal RM. Interferences and contaminants encountered in modern mass spectrometry. Anal Chim Acta. 2008;627:71–81.
47. Sindelar M, Patti GJ. Chemical Discovery in the Era of Metabolomics. J Am Chem Soc. American Chemical Society; 2020;142:9097–105.
48. Clendinen CS, Stupp GS, Ajredini R, Lee-McMullen B, Beecher C, Edison AS. An overview of methods using 13C for improved compound identification in metabolomics and natural products. Frontiers in Plant Science. 2015;6:611.
49. Bueschl C, Kluger B, Lemmens M, Adam G, Wiesenberger G, Maschietto V, et al. A novel stable isotope labelling assisted workflow for improved untargeted LC–HRMS based metabolomics research. Metabolomics. 2014;10:754–69.
50. Kluger B, Bueschl C, Neumann N, Stückler R, Doppler M, Chassy AW, et al. Untargeted Profiling of Tracer-Derived Metabolites Using Stable Isotopic Labeling and Fast Polarity-Switching LC–ESI-HRMS. Analytical Chemistry. 2014;86:11533–7.
51. Hegeman AD, Schulte CF, Cui Q, Lewis IA, Huttlin EL, Eghbalnia H, et al. Stable Isotope Assisted Assignment of Elemental Compositions for Metabolomics. Anal Chem. 2007;79:6912–21.
52. Stupp GS, Clendinen CS, Ajredini R, Szewc MA, Garrett T, Menger RF, et al. Isotopic Ratio Outlier Analysis Global Metabolomics of Caenorhabditis elegans. Anal Chem. American Chemical Society; 2013;85:11858–65.
53. Qiu Y, Moir R, Willis I, Beecher C, Tsai Y-H, Garrett TJ, et al. Isotopic Ratio Outlier Analysis of the S. cerevisiae Metabolome Using Accurate Mass Gas Chromatography/Time-of-Flight Mass Spectrometry: A New Method for Discovery. Anal Chem. American Chemical Society; 2016;88:2747–54.

54. Mahieu NG, Huang X, Chen Y-J, Patti GJ. Credentialing Features: A Platform to Benchmark and Optimize Untargeted Metabolomic Methods. Anal Chem. American Chemical Society; 2014;86:9583–9.
55. Grankvist N, Watrous JD, Lagerborg KA, Lyutvinskiy Y, Jain M, Nilsson R. Profiling the Metabolism of Human Cells by Deep 13C Labeling. Cell Chemical Biology [Internet]. 2018 [cited 2018 Oct 2];
56. Bueschl C, Krska R, Kluger B, Schuhmacher R. Isotopic labeling-assisted metabolomics using LC–MS. Analytical and Bioanalytical Chemistry. 2013;405:27–33.
57. Jang C, Chen L, Rabinowitz JD. Metabolomics and Isotope Tracing. Cell. 2018;173:822–37.
58. Hiller K, Metallo C, Stephanopoulos G. Elucidation of cellular metabolism via metabolomics and stable-isotope assisted metabolomics. Curr Pharm Biotechnol. 2011;12:1075–86.
59. Trefely S, Ashwell P, Snyder NW. FluxFix: automatic isotopologue normalization for metabolic tracer analysis. BMC Bioinformatics. 2016;17:485.
60. Fan TW, Lane AN, Higashi RM, Farag MA, Gao H, Bousamra M, et al. Altered regulation of metabolic pathways in human lung cancer discerned by 13C stable isotope-resolved metabolomics (SIRM). Molecular Cancer. 2009;8:41.
61. Mayers JR, Vander Heiden MG. Famine versus feast: understanding the metabolism of tumors in vivo. Trends in Biochemical Sciences. 2015;40:130–40.
62. Davidson SM, Papagiannakopoulos T, Olenchock BA, Heyman JE, Keibler MA, Luengo A, et al. Environment Impacts the Metabolic Dependencies of Ras-Driven Non-Small Cell Lung Cancer. Cell Metabolism. 2016;23:517–28.
63. Tumanov S, Bulusu V, Kamphorst JJ. Analysis of Fatty Acid Metabolism Using Stable Isotope Tracers and Mass Spectrometry. Methods Enzymol. 2015;561:197–217.
64. Sellers K, Fox MP, Bousamra M, Slone SP, Higashi RM, Miller DM, et al. Pyruvate carboxylase is critical for non–small-cell lung cancer proliferation. J Clin Invest. American Society for Clinical Investigation; 2015;125:687–98.
65. Chaneton B, Hillmann P, Zheng L, Martin ACL, Maddocks ODK, Chokkathukalam A, et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature. 2012;491:458–62.
66. Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, et al. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. Nat Genet. 2011;43:869–74.
67. Park JO, Rubin SA, Xu Y-F, Amador-Noguez D, Fan J, Shlomi T, et al. Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. Nat Chem Biol. 2016;12:482–9.
68. Lewis CA, Parker SJ, Fiske BP, McCloskey D, Gui DY, Green CR, et al. Tracing Compartmentalized NADPH Metabolism in the Cytosol and Mitochondria of Mammalian Cells. Molecular Cell. 2014;55:253–63.
69. Lee WN, Boros LG, Puigjaner J, Bassilian S, Lim S, Cascante M. Mass isotopomer study of the nonoxidative pathways of the pentose cycle with [1,2-13C2]glucose. Am J Physiol. 1998;274:E843-851.
70. Ghergurovich JM, Lang JD, Levin MK, Briones N, Facista SJ, Mueller C, et al. Local production of lactate, ribose phosphate, and amino acids within human triple-negative breast cancer. Med (N Y). 2021;2:736–54.
71. Yoo H, Antoniewicz MR, Stephanopoulos G, Kelleher JK. Quantifying Reductive Carboxylation Flux of Glutamine to Lipid in a Brown Adipocyte Cell Line. J Biol Chem. 2008;283:20621–7.
72. Chokkathukalam A, Kim D-H, Barrett MP, Breitling R, Creek DJ. Stable isotope-labeling studies in metabolomics: new insights into structure and dynamics of metabolic networks. Bioanalysis. 2014;6:511–24.
73. Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature. 2012;481:380–4.
74. Mullen AR, Wheaton WW, Jin ES, Chen P-H, Sullivan LB, Cheng T, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. Nature. 2012;481:385–8.
75. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, et al. Lactate Metabolism in Human Lung Tumors. Cell. 2017;171:358-371.e9.
76. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. 2017;551:115–8.
77. Puchalska P, Martin SE, Huang X, Lengfeld JE, Daniel B, Graham MJ, et al. Hepatocyte-Macrophage Acetoacetate Shuttle Protects against Tissue Fibrosis. Cell Metab. 2019;29:383-398.e7.
78. Weindl D, Wegner A, Hiller K. Metabolome-Wide Analysis of Stable Isotope Labeling—Is It Worth the Effort? Front Physiol [Internet]. 2015 [cited 2019 Feb 24];6.
79. Sauer U. Metabolic networks in motion: 13C-based flux analysis. Molecular Systems Biology [Internet]. 2006 [cited 2018 Sep 28];2.
80. Wiechert W, Niedenführ S, Nöh K. A Primer to 13 C Metabolic Flux Analysis. In: Villadsen J, editor. Fundamental Bioengineering [Internet]. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2015 [cited 2019 Nov 27]. p. 97–142.
81. Long CP, Antoniewicz MR. High-resolution 13C metabolic flux analysis. Nat Protoc. 2019;14:2856–77.
82. Wiechert W, Nöh K. Isotopically non-stationary metabolic flux analysis: complex yet highly informative. Current Opinion in Biotechnology. 2013;24:979–86.
83. Jazmin LJ, Young JD. Isotopically Nonstationary 13 C Metabolic Flux Analysis. 2013.
84. Jiang L, Boufersaoui A, Yang C, Ko B, Rakheja D, Guevara G, et al. Quantitative metabolic flux analysis reveals an unconventional pathway of fatty acid synthesis in cancer cells deficient for the mitochondrial citrate transport protein. Metab Eng. 2017;43:198–207.

85. Jiang L, Shestov AA, Swain P, Yang C, Parker SJ, Wang QA, et al. Reductive carboxylation supports redox homeostasis during anchorage-independent growth. Nature. 2016;532:255–8.

86. Pereira AGM, Walther JL, Hollenbach M, Young JD. 13C Flux Analysis Reveals that Rebalancing Medium Amino Acid Composition can Reduce Ammonia Production while Preserving Central Carbon Metabolism of CHO Cell Cultures. Biotechnology Journal. 2018;13:1700518.

87. Antoniewicz MR. Tandem mass spectrometry for measuring stable-isotope labeling. Current Opinion in Biotechnology. 2013;24:48–53.

88. Choi J, Antoniewicz MR. Tandem mass spectrometry: A novel approach for metabolic flux analysis. Metabolic Engineering. 2011;13:225–33.

89. Yuan J, Bennett BD, Rabinowitz JD. Kinetic flux profiling for quantitation of cellular metabolic fluxes. Nature Protocols. 2008;3:1328–40.

90. Rühl M, Rupp B, Nöh K, Wiechert W, Sauer U, Zamboni N. Collisional fragmentation of central carbon metabolites in LC-MS/MS increases precision of 13C metabolic flux analysis. Biotechnol Bioeng. 2012;109:763–71.

91. Mairinger T, Hann S. Implementation of data-dependent isotopologue fragmentation in 13C-based metabolic flux analysis. Anal Bioanal Chem. 2017;409:3713–8.

92. Jaiswal D, Prasannan CB, Hendry JI, Wangikar PP. SWATH Tandem Mass Spectrometry Workflow for Quantification of Mass Isotopologue Distribution of Intracellular Metabolites and Fragments Labeled with Isotopic 13 C Carbon. Analytical Chemistry. 2018;90:6486–93.

93. Sun Q, Fan TW-M, Lane AN, Higashi RM. An Ion Chromatography–Ultrahigh-Resolution-MS 1 /Data-Independent High-Resolution MS 2 Method for Stable Isotope-Resolved Metabolomics Reconstruction of Central Metabolic Networks. Anal Chem. 2021;93:2749–57.

94. Fan TW-M, Sun Q, Higashi RM. Ultrahigh resolution MS1/MS2-based reconstruction of metabolic networks in mammalian cells reveals changes for selenite and arsenite action. Journal of Biological Chemistry. 2022;298:102586.

95. Fleming RMT, Haraldsdottir HS, Preciat G, Huang L, Thiele I, Harms A, et al. Conserved moiety fluxomics.

96. Chernushevich IV, Merenbloom SI, Liu S, Bloomfield N. A W-Geometry Ortho-TOF MS with High Resolution and Up to 100% Duty Cycle for MS/MS. J Am Soc Mass Spectrom. 2017;28:2143–50.