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Metabolomics, metabolic flux analysis and cancer pharmacology

Lingfan Liang [a], Fei Sun [a], Hongbo Wang [b,*], Zeping Hu [a,**]

[a] School of Pharmaceutical Sciences; Tsinghua-Peking Joint Center for Life Sciences; Beijing Frontier Research Center for Biological Structure, Tsinghua University, Beijing 100084, China
[b] Key Laboratory of Molecular Pharmacology and Drug Evaluation (Yantai University), Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Yantai University, Yantai 264005, China

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ABSTRACT

Metabolic reprogramming is a hallmark of cancer and increasing evidence suggests that reprogrammed cell metabolism supports tumor initiation, progression, metastasis and drug resistance. Understanding metabolic dysregulation may provide therapeutic targets and facilitate drug research and development for cancer therapy. Metabolomics enables the high-throughput characterization of a large scale of small molecule metabolites in cells, tissues and biofluids, while metabolic flux analysis (MFA) tracks dynamic metabolic activities using stable isotope tracer methods. Recent advances in metabolomics and MFA technologies make them powerful tools for metabolic profiling and characterizing metabolic activities in health and disease, especially in cancer research. In this review, we introduce recent advances in metabolomics and MFA analytical technologies, and provide the first comprehensive summary of the most commonly used isotope tracing methods. In addition, we highlight how metabolomics and MFA are applied in cancer pharmacology studies particularly for discovering targetable metabolic vulnerabilities, understanding the mechanisms of drug action and drug resistance, exploring potential strategies with dietary intervention, identifying cancer biomarkers, as well as enabling precision treatment with pharmacometabolomics.

Contents

1. Introduction

Proliferating cancer cells usually present considerable metabolic alterations that utilize more nutrients to build new biomass and produce bioenergy compared to non-cancerous cells. Huge successes in biochemistry research in the 20th century offered researchers abundant information about metabolic enzymes and pathways as well as the regulation of metabolism. The Warburg effect, also known as aerobic

* Corresponding author.
** Correspondence to: Z. Hu, School of Pharmaceutical Sciences, Tsinghua University, Beijing 100084, China.
E-mail addresses: hongbowang@ytu.edu.cn (H. Wang), zeping_hu@tsinghua.edu.cn (Z. Hu).

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Abbreviations

AML	acute myeloid leukemia
BCAA	branched chain amino acid
CML	chronic myeloid leukemia
EGFR	epidermal growth factor receptor
GC	gas chromatography
IDH	isocitrate dehydrogenase
LC	liquid chromatography
MFA	metabolic flux analysis
MS	mass spectrometry
NMR	nuclear magnetic resonance
NSCLC	non-small cell lung cancer
OxPPP	oxidative pentose phosphate pathway
PC	pyruvate carboxylase
PDAC	pancreatic ductal adenocarcinoma
PDH	pyruvate dehydrogenase
PHGDH	phosphoglycerate dehydrogenase
PKM2	pyruvate kinase isozyme type M2
PSA	prostate specific antigen
SCC	squamous cell carcinoma
SHMT	serine hydroxymethyltransferase
THF	tetrahydrofolate
TOF	time of flight
2HG	R(−)-2-hydroxyglutarate

glycolysis, is characterized by increased glycolysis and lactate production even with the presence of oxygen (Warburg, 1925; Warburg, 1956). This discovery eventually led to development of the clinical diagnostic technology flurodeoxyglucose positron emission tomography (FDG-PET), which enables live tumor imaging in cancer patients (DeBerardinis & Chandel, 2020; Hsu & Sabatini, 2008; Vander Heiden, Cantley, & Thompson, 2009). Beyond aerobic glycolysis, glutamine metabolism was also widely studied, with its importance suggested by the cell's capability of utilizing glutamine to produce TCA cycle intermediates via both oxidative and reductive carboxylation (DeBerardinis et al., 2007; Le et al., 2012; Metallo et al., 2011; Mullen et al., 2011; Mullen et al., 2014). In addition, studies on tumor metabolism in the past two decades provide us essential insights into how the global network of metabolic pathways is regulated to meet the demands of excessive energy and biosynthetic precursors in cancer. A well-known example is the discovery of mutated isocitrate dehydrogenase (IDH), which generates 2-hydroxyglutarate (2-HG), an oncometabolite contributing to the development of acute myeloid leukemia (AML) and gliomas (Dang et al., 2009). These findings led to the development of small molecule inhibitors targeting the mutated IDH (DiNardo et al., 2018; Rohle et al., 2013; Yen et al., 2017). To date, targeting cancer metabolism has become increasingly appealing for new drug discovery and treatment optimization.

Owing to the rapid development of analytical technologies, metabolomics has become a powerful tool for global profiling of metabolites in biological systems, providing researchers with static data at a certain time point. On the other hand, metabolic flux analysis (MFA) by isotope tracing methods explores the metabolic activities of turnover in a dynamic manner (Jang, Chen, & Rabinowitz, 2018). Metabolomics and MFA, when used in combination, enable a more comprehensive and complementary understanding of changes and interplay in the regulation of metabolic networks.

In the past decade, emerging evidence of the benefits of applying metabolomics in cancer pharmacology have been observed, from identifying novel therapeutic targets to uncovering mechanisms underlying drug resistance, as well as precision medicine enabled by pharmacometabolomics. In this review, we first briefly introduce the recent advances in metabolomics and isotope-tracing based MFA, and then emphasize their applications in cancer drug discovery and pharmacology.

2. Metabolomics and metabolic flux analysis technologies

2.1. Mass spectrometry-based metabolomics

Metabolomics reveal metabolites at a large scale. While metabolomics can be categorized into targeted and untargeted approaches (Pang, Jia, & Hu, 2019), three steps are typically present in the experimental procedures: sample preparation, data acquisition, and data processing and analysis (Fig. 1).

Sample preparation requires quickly quenching enzymes and metabolic activities to obtain an accurate and unbiased metabolite profile. Cold temperature is usually applied to quickly freeze cultured cells or tissue samples and then to extract metabolites using various organic solvents. Potential metabolite degradation or interconversion requires special attention. Drying should be used with caution regarding redox-active species (Lu et al., 2017).

The realm of metabolite measurement has been propelled by the advances of various analytical methodologies, mainly including mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Fan et al., 2012). MS first ionizes and then detects the mass/charge (m/z) ratio of metabolites or fragments. Due to its high sensitivity, high throughput and capability of coupling with chromatography, for example liquid chromatography (LC) or gas chromatography (GC), to enhance the separation and quantification of metabolites, MS is now more widely used (Emwas, 2015). Tandem GC or LC with MS are referred to as GC-MS or LC-MS. In LC methods, chromatographic approaches are chosen according to the polarity of metabolites, where reversed-phase chromatography uses a C18 column to separate nonpolar metabolites while hydrophilic interaction chromatography (HILIC) is better suited to analysis of water-soluble metabolites. For those metabolites that are not retained well on LC, GC-MS coupled with pre-column chemical derivatization for increasing the stability and volatility is usually used.

In terms of MS, high resolution mass analyzers such as orbitrap and time of flight (TOF) are usually preferred for accurate mass detection. Quadrupoles with relatively low-resolution are often coupled in a hybrid TOF (Q-TOF) or in a triple quadruple way (Dieterle et al., 2011). The rapid advancements in MS instruments, ionization techniques and data analysis software enormously enlarge the scope of metabolites detected in both targeted and untargeted experiments. By utilizing a MS-based large-scale targeted metabolomics platform with high sensitivity, we were able to explore metabolism within the context of tumor progression, such as the targetable liabilities in KRAS/LKB1 mutant lung cancer cells (Kim et al., 2017), the distant metastasis of melanoma cells (Piskounova et al., 2015) as well as subtype-selective metabolic vulnerabilities in small cell lung cancer (Huang et al., 2018). While typical metabolomics methods greatly facilitate understanding of cancer metabolism (Mullen et al., 2014), particularly when combined with quantitative metabolic flux analysis (Jiang et al., 2017), improvements and optimizations in sensitivity have led to some exciting discoveries. For example, to address the long-standing difficulty in comparing metabolite levels within rare cell populations in tissues, we have developed an ultra-sensitive LC-MS-based targeted method coupled with flow cytometry. With this method, we were able to detect around 60 metabolites from 10,000 hematopoietic stem cells (HSCs) and further revealed the unexpected role of ascorbate in regulating HSC (Agathocleous et al., 2017).

Another advancement in metabolomics enables the profiling of metabolic dynamics in mitochondria, which is achieved by rapidly and specifically isolating intact mitochondria in tandem with a database of predicted mitochondrial metabolites ("MITObolome") (Chen et al., 2016). The development of subcellular metabolomics technologies will greatly facilitate the characterizations of metabolism in different organelles, providing information not reflected in whole-cell analysis.

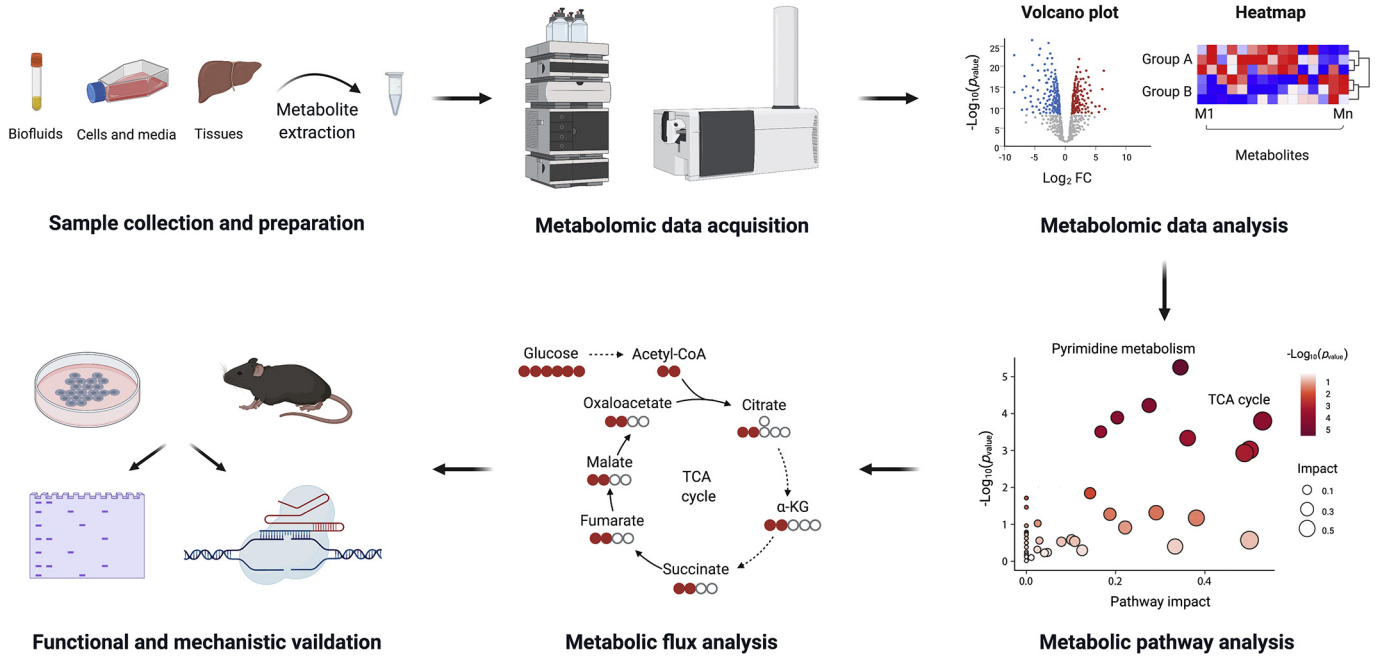


Fig. 1. Workflow of LC-MS based metabolomics. Samples (biofluids, cells and media or tissues) from diverse sources need to be collected and stored appropriately. After sample preparation, metabolites can be extracted and then separated by chromatography. Separated metabolites can be detected by mass spectrometry to acquire m/z ratio, MS^2 spectrum and retention time data. The processed data can be analyzed and presented as a heat map and volcano plot. Then metabolic pathway analysis can be used to reveal significantly changed pathways. Isotope tracing based metabolic flux analysis (optional) is usually used to probe dynamic fluxes of these pathways of interest. Moreover, functional and mechanistic validation can be performed using *in vitro* and *in vivo* models.

Given the cell heterogeneity in many contexts, single cell analysis including metabolomics are necessary and essential emerging technologies for understanding biology at the single cell scale (Rubakhin, Lanni, & Sweedler, 2013). However, due to the limited sample volume and low metabolite abundances in single cells, more sophisticated sampling and higher sensitivity of the detection technologies are needed. To address these issues, novel single cell sampling and ionization methods have been developed to make metabolomics analysis feasible at the single cell level, thus promoting further insights into biological research (Zhu et al., 2018).

Despite great advances, MS is still facing great challenges such as annotation of unknown metabolites and large-scale absolute quantitation for metabolites. Most metabolites are measured in relative abundances in metabolomics studies, which cannot meet the requirement of many clinical applications. For MS analysis, due to many confounding factors (e.g., ionization efficiency, ion suppression, and matrix effects) (Lu et al., 2017), ion signal intensity cannot reflect the absolute concentration. To address this problem, isotopic internal standards (ISs) with known concentrations can be added into samples during extraction. The absolute quantity of the analytes can be deduced by their peak area ratios against calibration curves (Bennett, Yuan, Kimball, & Rabinowitz, 2008). However, many isotopic ISs are not commercially available for many metabolites and are prohibitively expensive. Combining NMR and MS with chemical derivatization has emerged as a new strategy, which significantly reduces matrix effects in MS and improves quantitative accuracy (Fei et al., 2019).

2.2. NMR-based metabolomics

Although NMR suffers from lower sensitivity when compared with MS, it still has irreplaceable advantages, including high reproducibility and quantitative capability, particularly its superiority in identifying chemicals with identical masses and providing structural information (Markley et al., 2017). Considerable efforts have been made to improve the sensitivity of NMR, specifically by using ultra-high-field magnets and hyperpolarization (Markley et al., 2017). Furthermore, approaches exploiting the combination of MS and NMR were developed to achieve better separation and accurate mass measurement (Marshall et al., 2015). The use of complementary methods offers us more comprehensive metabolic information, thus better explaining the biological functions and mechanisms (Nagana Gowda and Raftery, 2017). Given that NMR can determine the labeled atom positions, it has also been used for the analysis of isotopomer distribution in stable isotope resolved metabolomics (SIRM) studies (Lin, Lane, & Fan, 2019).

2.3. Isotope tracing and MFA

While metabolomics generates valuable information about metabolite abundances, it fails to reveal the dynamic activities in metabolic pathways, which is critical for understanding metabolism as either faster production or slower consumption can lead to the accumulation of certain metabolites (Jang et al., 2018). Stable isotope tracing-based MFA can determine the flux rates by measuring downstream metabolites derived from the labeled tracer at multiple time points. The integrals of peak area indicating the abundance of metabolites of interest can be used to plot the pattern of labeling, which is informative for tracing flux. Many tracers have been used to measure specific metabolic activities. For instance, [1-^{2}H] and [3-^{2}H]-glucose can both be used to quantitatively measure synthesis of NADPH from oxidative production pathways such as oxidative pentose phosphate pathway (OxPPP) (Chen et al., 2019; Fan et al., 2014) and NAD synthesis-breakdown fluxes (Liu et al., 2018). Recently, a comprehensive isotopic targeted MS (CIT-MS) method was reported for reliable MFA analysis with broad coverage of metabolic pathways (Shi et al., 2020). Here we provide a comprehensive summary and graphic interpretation of the commonly used isotopic tracers in probing pathway activities in Table 1 and Fig. 2.

In MFA, there are two common experimental designs: steady-state and pseudo steady-state MFA (Zamboni et al., 2015). The advantage of steady-state MFA is in collecting labeling patterns independent of

Table 1
Isotopic tracers in metabolic flux analysis

Tracer	Probe	Metabolite readout	Graphic interpretation
Glucose tracer			
[U-^{13}C]-glucose	Glycolysis activity	Glycolytic intermediates	
	Serine biosynthesis	[U-^{13}C]serine	Fig. 2A
	Pentose phosphate pathway	M+5 ribose, nucleotides	
[U-^{13}C]-glucose	PC activity	M+3 malate, oxaloacetate	Fig. 2B
	PDH activity	M+2 citrate	
[1-^{14}C]-glucose	TCA cycle	$^{14}CO_2$ (radioactive)	Fig. 2C
[6-^{14}C]-glucose	OxPPP and TCA cycle		
[1-^{2}H]-glucose	OxPPP	NADP^{2}H	Fig. 2D
[3-^{2}H]-glucose			
[4-^{2}H]-glucose	ME1 activity	NADP^{2}H	Fig. 2E
Fructose tracer			
[U-^{13}C]-fructose	Fructose metabolism	Glycolytic&TCA intermediates, *de novo* lipogenesis in liver	Fig. 2F
Amino acid tracer			
[U-^{13}C]-glutamine	Glutamine metabolism	TCA intermediates	
	Forward TCA cycle	M+4 succinate, fumarate, aspartate	Fig. 2G
	Reverse TCA cycle	M+5 citrate, M+3 malate	
[U-^{13}C]-serine	THF pools	M+1 5,10-methenyl THF	
	Serine metabolism	M+2 glycine	
	Pyrimidine synthesis	M+1 TTP	Fig. 2H
	Purine synthesis	M+1, M+2, M+3, M+4 IMP, AMP, GMP	
[3-^{13}C]-serine	Pyrimidine synthesis	M+1 dTTP	Fig. 2I
[2,3,3-^{2}H]-serine	Location of serine catabolism to make cytosolic 1C units	M+1, M+2 dTTP	Fig. 2J
[U-^{13}C]-glycine	Purine synthesis	M+1, M+2, M+3, M+4 AMP, GMP	Fig. 2K
[U-^{13}C]-essential amino acids	Protein synthesis	[^{13}C]-protein	Fig. 2L
Fatty acid tracer			
[U-^{13}C]-fatty acids	Fatty acid catabolism	TCA intermediates	Fig. 2M
[U-^{13}C]-acetate	Acetate metabolism; ACSS1/2 activity	TCA intermediates; lipids	Fig. 2N
Organic acid tracer			
[U-^{13}C]-formate	Purine synthesis	M+1, M+2 AMP, GMP	Fig. 2O
[1-^{13}C]-pyruvate	PC flux	M+1 malate, oxaloacetate	Fig. 2P
[3-^{13}C]-pyruvate	PC+PDH flux	M+1 malate, oxaloacetate	
Ketone tracer			
[1,2-^{13}C]-β-hydroxylbutyrate	Interconversion rate; Cell redox state; ketogenesis	[AcAc]/[BHB] ratio; [^{13}C]Acetyl-CoA	Fig. 2Q
[3,4-^{13}C]- acetoacetate	Interconversion rate; Cell redox state; ketogenesis	[AcAc]/[BHB] ratio; [^{13}C]Acetyl-CoA	
H_2O tracer			
[^{2}H]-H_2O	Fatty acid synthesis	[^{2}H]-fatty acids	Fig. 2R
[^{2}H]-H_2O	Protein synthesis	[^{2}H]-nonessential amino acids	Fig. 2S

metabolite levels; however, it usually takes a long time, sometimes up to hours, to achieve the steady state. Pseudo steady-state MFA overcomes this limitation with rapid labeling that enables researchers to calculate fluxes within linear pathways, but requires substantially more time points and heavily depends on intracellular metabolite levels, which thus requires quantitative analysis (Zamboni et al., 2015). Pseudo steady-state MFA also established the concept of isotopically non-stationary MFA (INST-MFA), which assumes metabolic steady state, not isotopic steady state (Dai & Locasale, 2017). Kinetic flux profiling (KFP), a special case in INST-MFA, is carefully described in concept (Yuan, Bennett, & Rabinowitz, 2008) and has been applied to measure OxPPP flux (Fan et al., 2014).

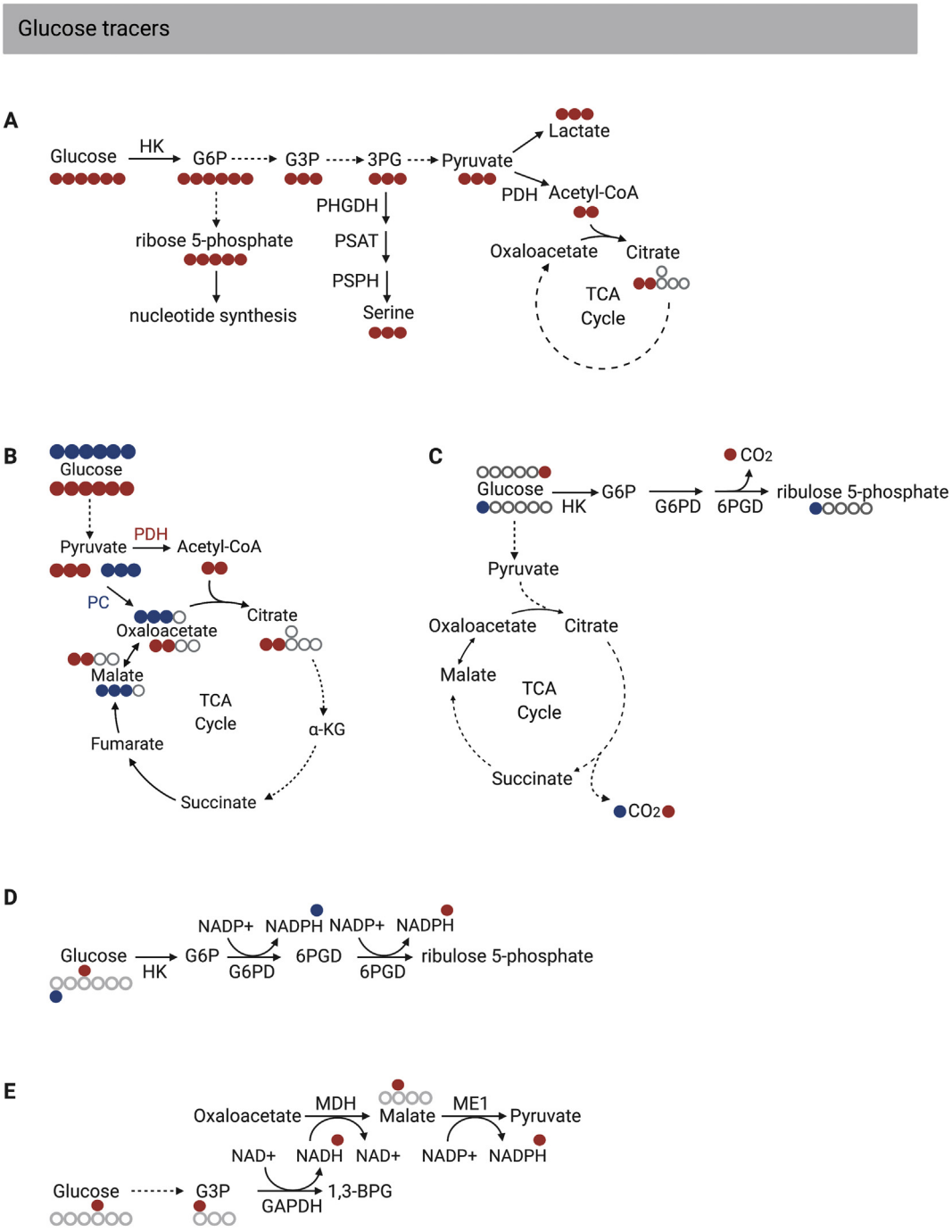
The labeling patterns of metabolites and extracellular or inter-organ metabolite exchange can then be fitted into a mathematic model for metabolic flux measurement (Fernández-García, Altea-Manzano, Pranzini, & Fendt, 2020), which is now widely used to interpret mammalian cell metabolism within both *in vitro* and *in vivo* contexts. In addition to profiling cancer metabolism in cultured cells (Mullen et al., 2011) and spheroids (Jiang et al., 2016), quantitative analysis of metabolites exchange is also conducted at a whole-body level to study dietary impacts in pigs (Jang et al., 2019) and in laboratory mice (Hui et al., 2020). Furthermore, MFA was also successfully used in non-small cell lung cancer (NSCLC) patients to track metabolic changes in tumors (Faubert et al., 2017; Hensley et al., 2016), exhibiting great potential for exploring tumor metabolism in human. The rapid advancements in analytical technologies and increasing implementation of isotope tracers have greatly accelerated our understanding of cancer metabolism.

3. Identification of targetable metabolic vulnerabilities in cancer

The characteristic metabolic needs of cancer cells make them vulnerable to strategies that restrain their metabolic dependencies and lead to the discovery of specific enzyme targets. The identification of oncometabolite 2-hydroxyglutarate (2HG) generated by cytosolic isocitrate dehydrogenase (IDH1) marks the significance of exploiting metabolic vulnerabilities to selectively target cancer (Dang et al., 2009). So far, metabolomics and MFA have achieved great success in probing these metabolic vulnerabilities, for which glucose, glutamine, serine and lipid metabolism are highlighted.

3.1. Glycolysis and the TCA cycle

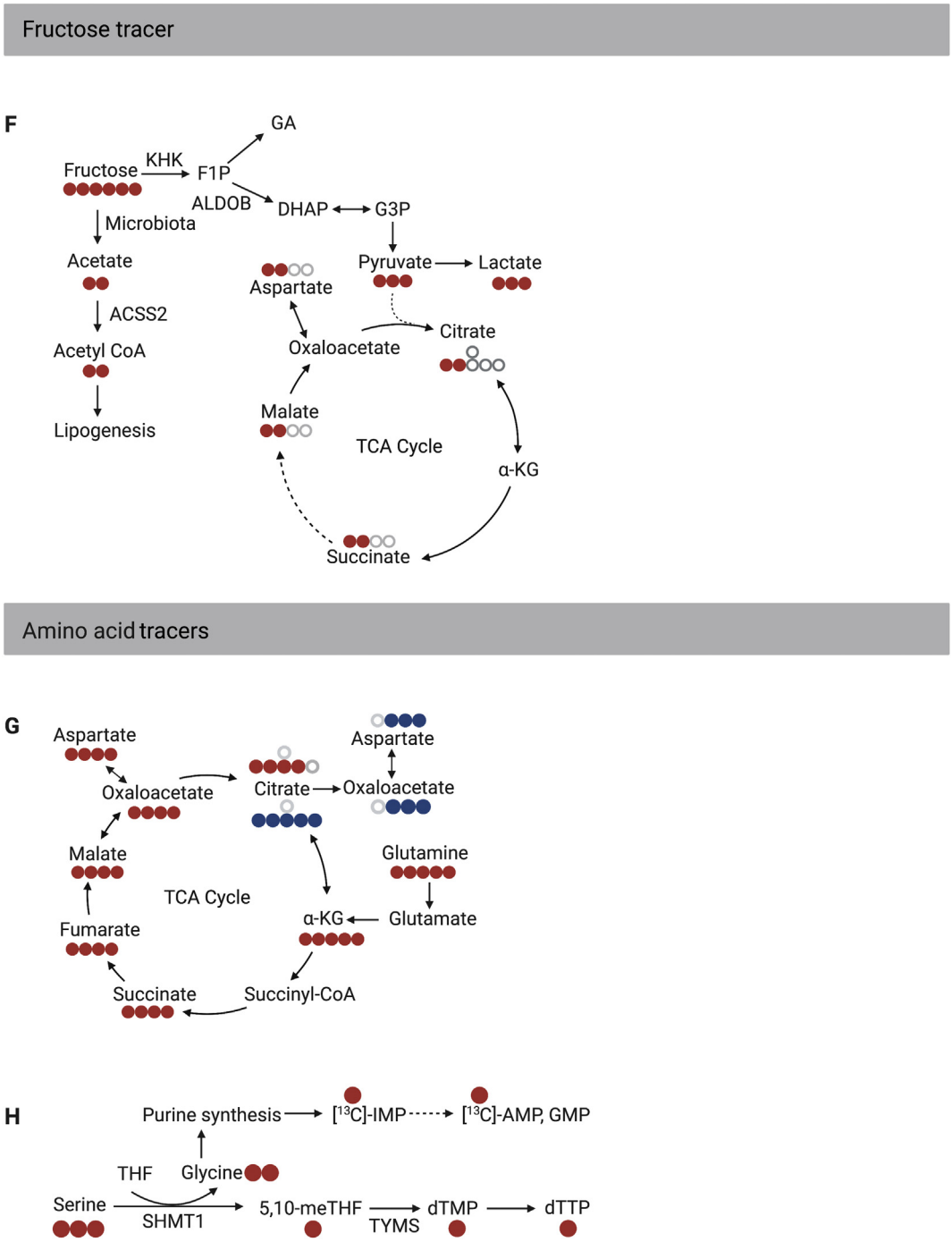
Some tumors may exhibit preferences for certain nutrients to fuel biosynthetic pathways. Glucose and lactate, the two most abundant circulating carbon sources (Rabinowitz & Enerbäck, 2020), have been extensively studied.



The M2 isoform of pyruvate kinase (PKM2), a key enzyme in glucose metabolism, has received extensive attention in cancer research. It was first reported as essential for shifting to aerobic glycolysis and contributes to tumorigenesis *in vitro* by supporting anabolic metabolism (Christofk et al., 2008; Vander Heiden et al., 2010). LC-MS-based metabolomics analysis revealed that succinylaminoimidazolecarboxamide ribose-5′-phosphate (SAICAR) is the regulator of PKM2, which interacts with the latter and promotes cell survival under glucose depletion (Keller, Tan, & Lee, 2012). Still, its role in tumorigenesis is controversial: PKM2 is reported to lack protein kinase activity (Hosios, Fiske, Gui, & Vander Heiden, 2015) and to be dispensable for PDAC development (Hillis et al., 2018). An isotope tracing study with [U-^{13}C]-glucose and [U-^{13}C]-glutamine indicated that PKM1 activates central glucose metabolism while PKM2 metabolizes glutamine more robustly (Morita et al., 2018).

Glycolysis reprogramming is identified in cancer driven by genetic mutations. Metabolomics analysis of the oncogenic Kras^{G12D} mutation driven pancreatic ductal adenocarcinoma (PDAC) mouse model showed that such mutation regulated tumor metabolism by diverting glycolytic intermediates into nonoxidative pentose phosphate pathways and thereby promoting ribose biogenesis (Ying et al., 2012). Besides genetic mutations, epigenetic modifiers can also promote tumorigenesis and induce metabolic reprogramming in lung cancer. For instance, the lung specific loss of histone methyltransferase KMT2D induced aberrant metabolic reprogramming, mainly upregulating glycolysis (Alam et al., 2020).

Lung tumors show an increased TCA flux from glucose relative to adjacent normal tissue, in both mouse models (Davidson et al., 2016) and human tissues (Hensley et al., 2016). Pyruvate dehydrogenase (PDH), the essential enzyme that converts pyruvate to acetyl-CoA and commits

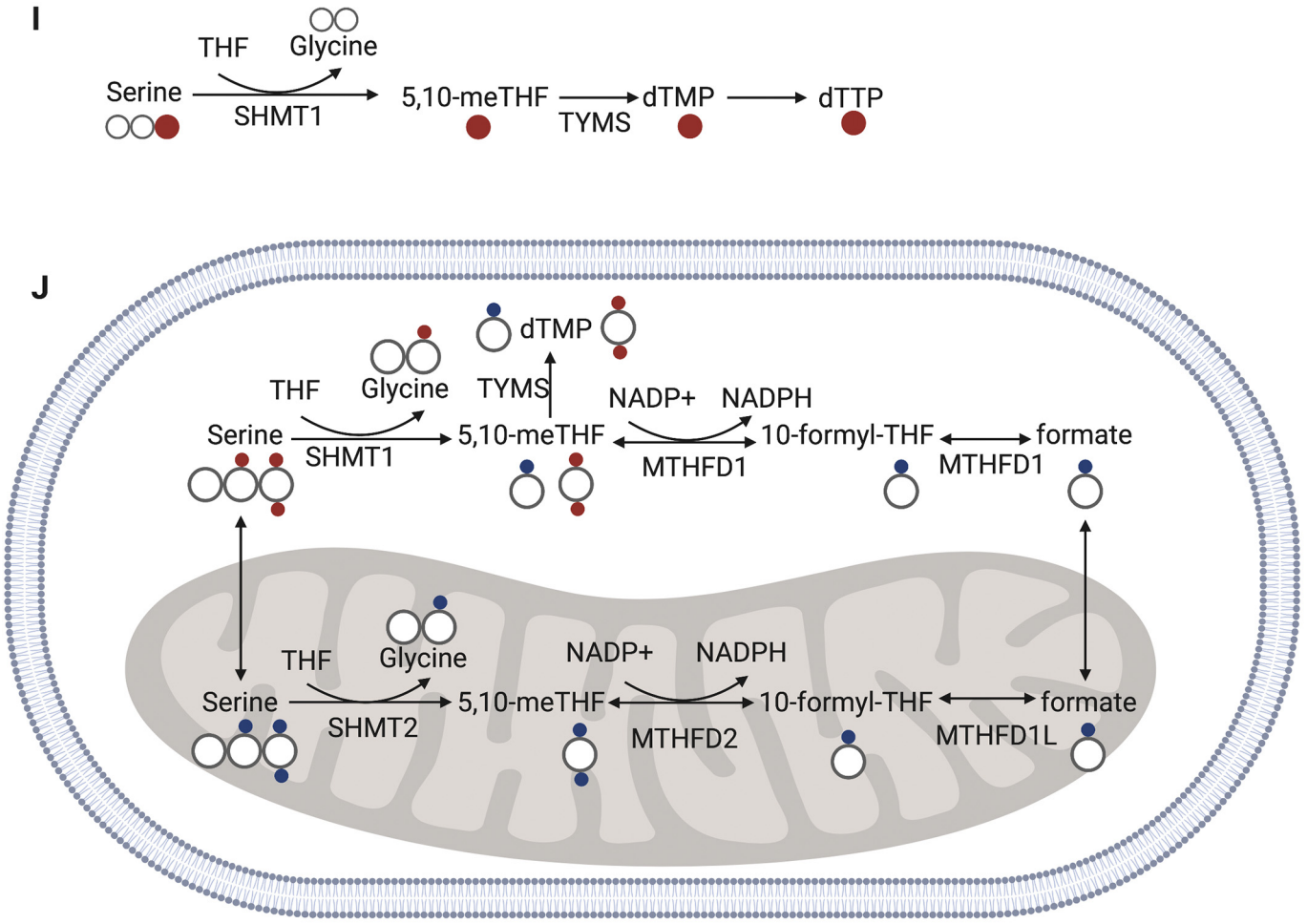


glycolytic intermediates to the TCA cycle, is required for this increased TCA flux. Deletion of the E1α subunit of PDH (*Pdha1*) disrupts the initiation and proliferation of tumors *in vivo* and suggests metabolic dependency on this pathway in lung tumors. The TCA cycle also depends on the activity of pyruvate carboxylase (PC), the enzyme that coverts pyruvate to oxaloacetate, since knockdown of PC perturbed the flux of glucose and glutamine through the TCA cycle in NSCLC cells (Sellers et al., 2015). Pyruvate is also reported to support acetate production, which is further coupled to mitochondrial function (Liu, Cooper, et al., 2018).

Lactate serves as another carbon source to fuel the TCA cycle in NSCLC (Faubert et al., 2017) and pancreatic cancer (Hui et al., 2017) while under physiological conditions, glucose is reported as the major nutrient feeding the TCA cycle (Liu, Dai, Cooper, Kirsch, & Locasale, 2020). The *in vivo* findings challenge the long-standing view of cancer switching from oxidative glucose metabolism to aerobic glycolysis. Besides its metabolic roles, lactate is reported to be involved in lactylation of histone lysine residues as an epigenetic modification (Zhang et al., 2019).

3.2. Glutamine metabolism

Glutamine is another essential nutrient for tumor growth, which can feed the TCA cycle independent of glucose. A [U-^{13}C,^{15}N]-glutamine labeling study showed that MYC-inducible human Burkitt lymphoma displays persistent glutamine metabolism and contribution of carbons to the TCA cycle under hypoxia, whereas glucose entry into the TCA cycle is suppressed (Le et al., 2012). In Ras and Akt oncogene activated cancer cells, glutamine mainly fuels the oxidative phosphorylation and accounts for the majority of ATP production and oxygen consumption (Fan et al., 2013). Glutamine also contributes greatly to protein

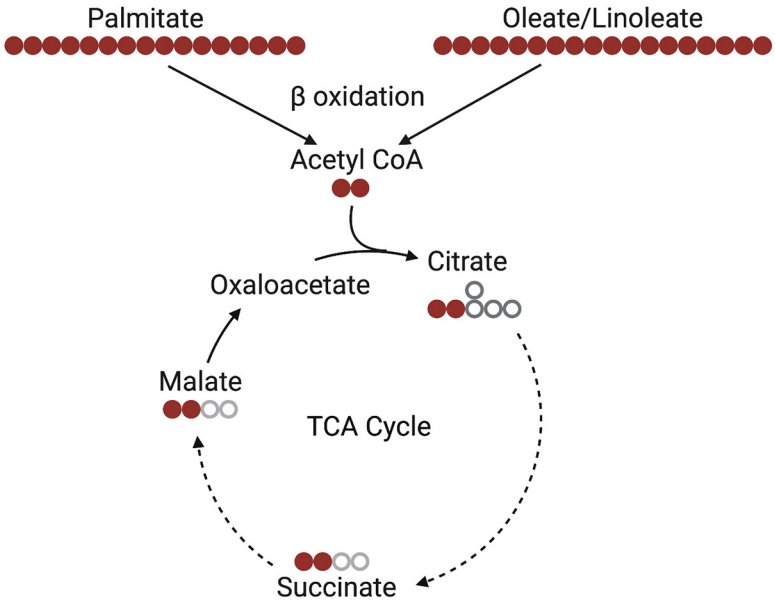


synthesis in rapidly proliferating mammalian cells (Hosios et al., 2016) and supports the glutathione pathway in high grade renal cell carcinoma (Wettersten et al., 2015) and NADPH production in proliferating glioblastoma cells (DeBerardinis et al., 2007). When glutamine is limited, cytosolic aspartate availability is required to sustain cellular survival and cellular redox homeostasis (Alkan et al., 2018).

Despite the fact that *in vitro* data supports the metabolic dependency on glutamine, *in vivo* studies show controversial findings. Isotope

Fatty acid tracers

M



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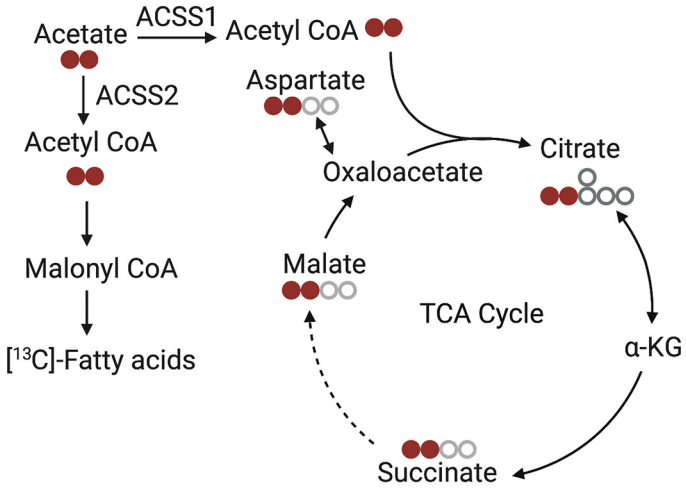
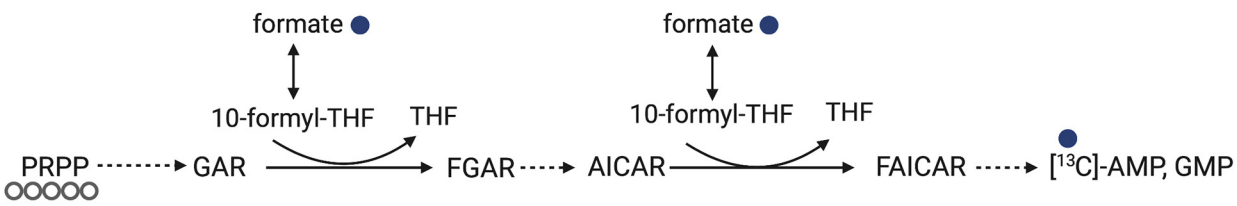
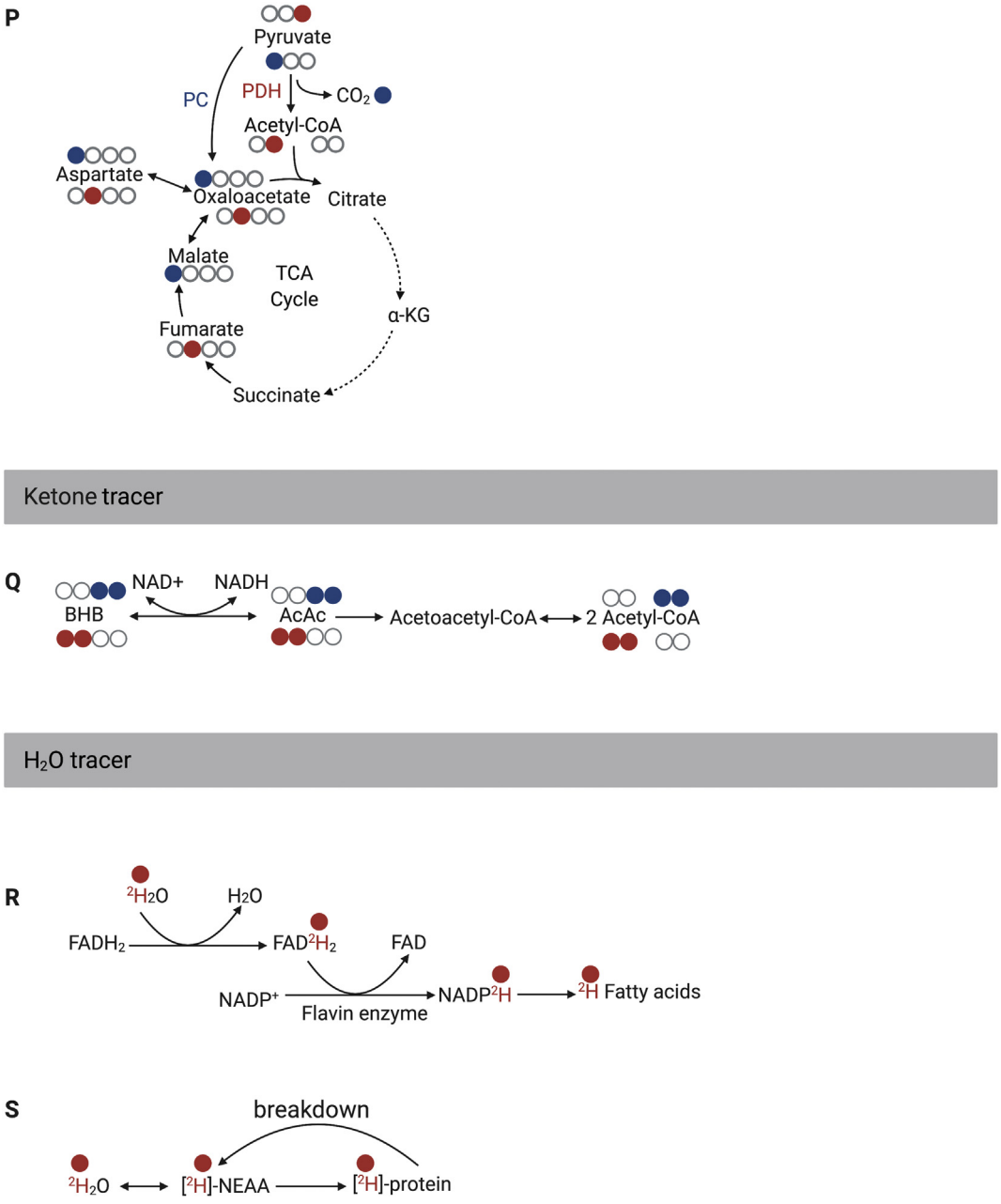


Organic acid tracers

O





tracing reveals no significant difference of glutamine carbon contribution to the TCA cycle between Kras-driven lung tumors and adjacent lung in mouse models, and Kras-driven lung tumors are less dependent on glutaminase than cultured cells (Davidson et al., 2016). Different preferences of nutrients between cancer cells in culture and tumors, especially regarding glutamine metabolism, highlight the importance of studying cancer metabolism under a specific physiologic context. *In vivo* metabolic tracing of [U-^{13}C]-glucose and [U-^{13}C]-glutamine in lung squamous cell carcinoma (SCC) showed that treatment of catalytic mTOR kinase inhibitor MLN128 lowers the contribution of glucose to the TCA cycle while enhancing the glutamine-driven TCA cycle (Momcilovic et al., 2018). This metabolic reliance of lung SCC on both glucose and glutamine *in vivo* demonstrated the delicacy of nutrient utilization in tumors. Therapeutically, glutamine blockade is exploited together with tumor immunotherapy, which modulates the metabolism of cancer cells and immune cells differentially based on the metabolic plasticity of each cell type (Leone et al., 2019). This also highlights the importance of the combination of targeted therapies on both immune and metabolic checkpoints.

3.3. One carbon metabolism

Recently, extensive studies have suggested that one carbon metabolism is essential in tumor growth. Using a combination of MS and NMR with stable isotope labeling, researchers observed the diversion of glycolytic flux into serine and glycine biosynthesis in H1299 cells, along with phosphoglycerate dehydrogenase (PHGDH) amplification (Locasale et al., 2011). NRF2 was reported to regulate serine biosynthesis, which supports glutathione and nucleotide production in NSCLC (DeNicola et al., 2015). Notably, activation of the serine biosynthesis pathway promotes tumorigenesis in NSCLC. An *in vivo* study indicated that physiologic serine levels are limiting for tumor growth and PHGDH dependent serine biosynthesis promotes growth of human breast cancer xenografts (Sullivan et al., 2019). Given the critical role

of serine in tumor progression, dietary serine restriction can potently slow tumor growth by inducing the accumulation of deoxysphingolipids. The inhibition of serine palmitoyltransferase (SPT), an enzyme that catalyzes the *de novo* biosynthesis of sphingolipids, was reported to rescue xenograft growth in mice fed with restricted serine and glycine (Muthusamy et al., 2020). Glycine metabolism, a key part involved in one carbon metabolic network, was shown to correlate with rapid cancer cell proliferation and to support *de novo* purine synthesis (Jain et al., 2012). So far, a large body of evidence has suggested one carbon metabolism as an essential network with great potential to target cancer cells, for which the enzyme serine hydroxymethyltransferase (SHMT) serves as a promising drug target (Ducker et al., 2017; Lee et al., 2021).

3.4. Lipid metabolism

The recognition of metabolic reprogramming in lipids as a novel hallmark of malignancy has brought much attention to the regulation of lipid metabolism, in which sterol regulatory element-binding proteins (SREBPs) have an essential role (Cheng, Geng, Cheng, & Guo, 2018; Cheng, Li, & Guo, 2018). As the master regulator of lipid synthesis and uptake, SREBPs are activated in glioblastomas and promote tumor survival (Guo et al., 2009), which further leads to the identification of a targetable pathway (Guo et al., 2011). An LC-MS/MS-based lipidomic analysis showed the positive correlation between *de novo* lipogenesis and the aggressiveness and metastatic potential of human prostate cancer (CaP) as well as the distinctive profile of lipid species in *Pten* and *Pml* double-null CaP, which appears to be part of an aberrant SREBP-dependent lipogenic program (Chen et al., 2018). mTORC2, a key metabolic regulator in tumors, was revealed by lipidomic and metabolomic analyses to promote tumor development by increasing *de novo* fatty acid and lipid synthesis, and therefore inhibiting mTORC2 is a potential strategy for treating hepatocellular carcinoma (Guri et al., 2017). LC–MS lipidomics analysis also identified accumulation of arachidonic acid and subsequent synthesis of PGE_2 in polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), a class of pathologically activated neutrophils in cancer, whose immunosuppressive function is mediated by fatty acid transport protein 2 (FATP2) (Veglia et al., 2019). A recent lipidomics-based study showed that synthesis of polyunsaturated ether phospholipids (PUFA-ePLs) induced ferroptosis by acting as substrates for lipid peroxidation, whose downregulation could be exploited by cancer cells to evade ferroptosis (Zou et al., 2020).

Overall, the global profiling of lipid metabolism has led to identification of multiple regulatory pathways, which provide comprehensive insights into cancer biology and offer great therapeutic potentials.

3.5. Other pathways

Metabolomic studies have also revealed many other metabolic vulnerabilities, such as glutathione metabolism (Ogiwara et al., 2019), branched chain amino acid (BCAA) metabolism (Gu et al., 2019), nonessential amino acid dependency (LeBoeuf et al., 2020) and fructose utilization (Chen et al., 2016). In mutant KRAS plus LKB1 loss (KL) lung cancer cells, our metabolomics study revealed that altered urea metabolism and loss of the urea cycle enzyme carbamoyl phosphate synthetase-1 (CPS1) led to pyrimidine depletion and resultant cell death (Kim et al., 2017), which identified CPS1 as a potential metabolic vulnerability for cancer treatment. In another study, by using metabolomics we revealed distinctive metabolomic subsets in small cell lung cancers (SCLCs), which correlated with the expression levels of the Achaete-scute homolog-1 (ASCL1) transcription factor. Further functional study showed that inhibition of inosine monophosphate dehydrogenase (IMPDH) impaired the growth of SCLC with low expression of ASCL1, suggesting IMPDH as a potential therapeutic target (Huang et al., 2018). All these studies provide a large number of vulnerabilities in metabolic pathways that could be exploited to treat tumors (Fig. 3), showing the power of metabolomics in identification of therapeutic targets.

4. Understanding the mechanisms of drug action

Metabolomics and MFA have become increasingly essential tools for understanding the modes of drug action. A well-known example is the study of imatinib (Gleevec, STI571), a competitive inhibitor of the BCR-Abl kinase. Imatinib prevents the catalytic kinase activity of BCR-Abl and leads to the inhibition of tyrosine phosphorylation of downstream proteins. Using the [1,2-$^{13}C_2$]-glucose tracer in cultured K562 chronic myeloid leukemia (CML) cells treated with increasing doses of imatinib, researchers found that decreased glucose uptake and oxidation resulted in restricted glucose carbon incorporation into *de novo* nucleic acid and fatty acid synthesis, which revealed how imatinib inhibits leukemia cell glucose utilization and mediates its anti-proliferative effect (Boren et al., 2001). A later study, focusing on different doses of imatinib-mediated changes in glucose metabolism,

Fig. 2. Graphic interpretation of isotopic tracers in probing metabolic pathway activities. The tracer is metabolized into downstream metabolites, which are consequently labeled in certain positions. By analyzing the amounts of labeled metabolites in a certain period of time, the metabolic activity of a pathway of interest can be calculated. The isotope labeled atom is demonstrated in color. The one-step reaction is represented by a solid line while the multi-step reactions are in dotted lines. The enzymes catalyzing the reactions are shown by arrows. [U-^{13}C]-glucose (here U indicates uniformly labelled) can be metabolized to downstream glycolytic intermediates and TCA cycle intermediates. Glucose also serves as a substrate for serine biosynthesis and the pentose phosphate pathway, which further contributes to nucleotide synthesis. [U-^{13}C]-glucose metabolized by PDH results in M+2 TCA intermediates, including M+2 malate, citrate and oxaloacetate while the same tracer metabolized by PC results in M+3 TCA intermediates. The combined use of positionally labeled glucose tracers reveals OxPPP activity. The [1-^{14}C]-glucose is metabolized fully to CO_2 via the TCA cycle and the [6-^{14}C]-glucose is metabolized fully to CO2 via both OxPPP and the TCA cycle. The deduction reflects OxPPP activity. [1-^{2}H]-glucose and [3-^{2}H]-glucose measures the OxPPP activity by labeling the NADPH generated. [4-^{2}H]-glucose measures the ME1 activity through a series of enzymatic reactions and labels the NADPH generated by ME1. [U-^{13}C]-fructose is metabolized to downstream two-carbon metabolites, for example M+2 acetyl-CoA, and participates in the TCA cycle via the entrance of M+3 pyruvate. [U-^{13}C]-glutamine can enter the TCA cycle. In the forward TCA cycle, it is metabolized into the generation of M+4 succinate; in the reverse TCA cycle, it is converted to M+5 citrate. [U-^{13}C]-serine can label dTTP synthesis via one carbon metabolism. [3-^{13}C]-serine can generate M+1 dTTP. [2,3,3-^{2}H]-serine can distinguish the cellular compartments 1C units come from. When [2,3,3-^{2}H]-serine is metabolized in mitochondria, resultant M+1 formate contributes to cytosolic M+1 dTMP synthesis. When [2,3,3-^{2}H]-serine is metabolized in cytosol, M+2 dTMP is synthesized. [U-^{13}C]-glycine labels AMP and GMP synthesis. [U-^{13}C]-labeled essential amino acids label protein turnover, including synthesis and breakdown. A [U-^{13}C]-labeled fatty acid tracer, for example palmitate, oleate or linoleate, goes through β oxidation and generates M+2 acetyl-CoA, which later enters the TCA cycle. [U-^{13}C]-acetate tracer is metabolized to M+2 acetyl-CoA, which enters the TCA cycle or lipid biosynthesis. [U-^{13}C]-formate labels AMP and GMP synthesis. Positionally labeled pyruvate tracers measure the PC and PDH activity. [1-^{13}C]-pyruvate loses its labeled carbon atom as CO_2 via PDH and contributes to M+1 TCA intermediates only via the PC pathway. [3-^{13}C]-pyruvate enters the TCA cycle via both PC and PDH. [1,2-^{13}C]-BHB (in red) and [3,4-^{13}C]-AcAc (in blue) tracers can be metabolized to downstream M+2 acetyl-CoA. [^{2}H]-H_2O tracer labels the fatty acid synthesis via the H-D exchange catalyzed by flavin enzymes. [^{2}H]-H_2O tracer labels the non-essential amino acid synthesis and thus protein synthesis. G6P, glucose-6-phosphate; G3P, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; α-KG, α-ketoglutarate; 1,3-BPG, 1,3-bisphosphoglycerate; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; R5P, ribose-5-phosphate; F1P, fructose-1-phosphate; THF, tetrahydrofolate; 5,10-methylene-THF, 5,10-methylene-tetrahydrofolate; 10-formyl-THF, 10-formyl-tetrahydrofolate; dTMP, thymidine 5'-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, N-formylaminoimidazole-4-carboxamide ribonucleotide; BHB, β-hydroxybutyrate; AcAc, acetoacetate; NEAA, nonessential amino acids; HK, hexokinase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; PHGDH, phosphoglycerate dehydrogenase; PKM2, pyruvate kinase M2; KHK, ketohexokinase; ACSS2, acetyl-CoA synthetase 2; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; IDH, isocitrate dehydrogenase; ME, malic enzyme; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; TYMS, thymidylate synthase.

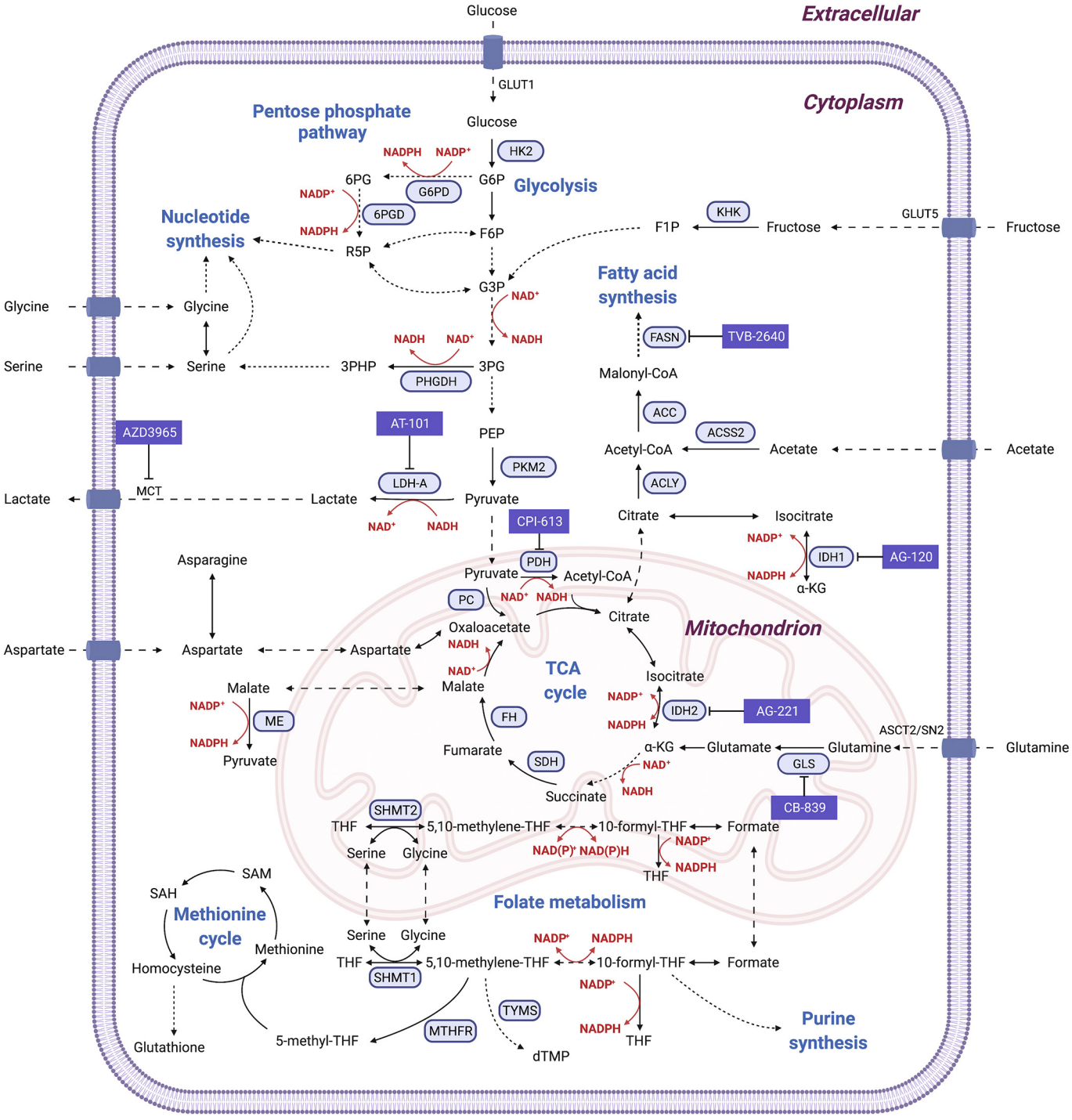


Fig. 3. Metabolic pathways that promote cell growth. Cells generate biomolecules by related catabolic and anabolic pathways. Extracellular glucose is taken up through the GLUT1 transporter and enters glycolysis. Glycolysis intermediates such as G6P and 3PG can be converted to R5P and serine for the pentose phosphate pathway and nucleotide synthesis. Through anaerobic glycolysis, glucose can be metabolized to pyruvate and then to lactate by LDH-A. Aerobic metabolism of glucose takes place in the mitochondria by the TCA cycle. Glutamine enters the cell through the ASCT2/SN2 transporter and can be converted to glutamate and then to α-KG, which undergoes reductive metabolism to form citrate, contributing to the synthesis of fatty acids. Acetate can enter the cell and be converted to Acetyl-CoA by ACSS2. In addition, serine and glycine are incorporated into folate metabolism in both mitochondria and cytoplasm. Folate metabolism is coupled to the methionine cycle. The conversions of NAD+ and NADH as well as NADP+ and NADPH are required to maintain the redox balance. Important metabolic enzymes that catalyze the reactions are marked for reference, and are the promising metabolic targets for cancer therapy. The targeted inhibitors that have been in prospective clinical studies are shown. The enzymes are shown in ovals and the inhibitors of enzymes are in rectangles. The transporters on the cell membrane are cylindrical. The one-step reaction is represented by a solid line while the multi-step reactions are in dotted lines. Transportation between cell membrane and mitochondrial membrane are in longer dotted lines and transporters on the mitochondrial membrane are omitted. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; 6PG, 6-phosphogluconate; 3PG, 3-phosphoglycerate; R5P, ribose-5-phosphate; 3PHP, 3-phosphohydroxypyruvate; PEP, phosphoenolpyruvate; F1P, fructose-1-phosphate; α-KG, α-ketoglutarate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate; 5,10-methylene-THF, 5,10-methylene-tetrahydrofolate; 10-formyl-THF, 10-formyl-tetrahydrofolate;5-methyl-THF, 5-methyl-tetrahydrofolate; dTMP, thymidine 5'-phosphate; GLUT, glucose transporter; HK2, hexokinase 2; ASCT2/SN2, glutamine transporter, G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; PHGDH, phosphoglycerate dehydrogenase; PKM2, pyruvate kinase M2; KHK, ketohexokinase; ACSS2, acetyl-CoA synthetase 2; LDH-A, lactate dehydrogenase A; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; ACC, acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; MCT, monocarboxylate transporter; FASN, fatty acid synthase; IDH, isocitrate dehydrogenase; ME, malic enzyme; SDH, succinate dehydrogenase; FH, fumarate hydratase; GLS, glutaminase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; TYMS, thymidylate synthase.

systemically demonstrated that glucose metabolism was changed from anaerobic glycolysis (lactate production) to the mitochondrial TCA cycle after 96 hours of imatinib treatment in BCR-Abl–positive cells (Gottschalk, Anderson, Hainz, Eckhardt, & Serkova, 2004). These data offer us valuable insights into the underlying mechanisms of this small-molecule kinase inhibitor.

Proliferating cells require not only ATP but also macromolecules such as nucleotides, which results in heavy exploitation of the folate biosynthesis pathway in chemotherapy. The chemotherapeutic drug methotrexate acts by inhibiting the enzyme dihydrofolate reductase (Farber & Diamond, 1948) and the resultant generation of tetrahydrofolate (THF). Since THF is essential for nucleotide synthesis, depletion of the cellular THF pool leads to cell death. In addition, methotrexate was also reported to induce an increase in the AMP/ATP ratio, activate AMP kinase (AMPK), and inhibit protein and glutathione synthesis (Tedeschi et al., 2015). However, the clinical use of methotrexate is limited by its efficacy and key determinants on its sensitivity remained unclear. By utilizing metabolomics and MFA, researchers examined the THF pools and monitored the nucleotide synthesis from [U-^{13}C]-serine, thereby elucidating that depletion of the enzymes formimidoyltransferase cyclodeaminase (FTCD) and histidine ammonia lyase (HAL), which are involved in histidine degradation pathway, is able to maintain THF pools and nucleotide synthesis even when cancer cells were treated with methotrexate (Kanarek et al., 2018). These findings shed light on the role of the histidine degradation pathway in cellular sensitivity to methotrexate. A LC-MS/MS based targeted metabolomics study revealed the mechanism of action of irinotecan in 13 patients with solid tumors (Bao, Wu, Kim, LoRusso, & Li, 2019). Irinotecan is a prodrug of 7-ethyl-10-hydroxycamptothecin (SN-38), a potent inhibitor of DNA topoisomerase I. Following intravenous infusion of irinotecan in cancer patients, elevations in plasma levels of metabolites such as amino acids, acylcarnitine derivatives and pyrimidine/purine nucleobases were detected. The time-dependent metabolic signature was well replicated in cell culture experiments, which together shed light on the global metabolic change induced by irinotecan.

Rewired cancer metabolism opens up the possibility for antidiabetic drugs to be repurposed for treating cancer. The biguanide metformin is widely-used for the treatment of type 2 diabetes. Although its primary use is to decrease blood glucose level, further studies indicate that metformin inhibits mitochondria respiratory chain complex I (El-Mir et al., 2000) and requires LKB-1 dependent AMPK activation for its hepatic glucose-lowering action (Shaw et al., 2005). Interestingly, LKB-1 also acts as a tumor suppressor, which links the physiologic network of general metabolism and cancer biology. However, LKB-1 expression in tumors is not essential for the antineoplastic activity of metformin *in vivo* (Algire et al., 2011). Multiple studies provided evidence of metformin's anti-tumor activity *in vitro* and *in vivo*. Metformin is reported to activate fatty acid β-oxidation and stimulate glycolysis in a p53-dependent manner in HCT116 cells and greatly inhibits growth of HCT116 p53$^{-/-}$ xenograft (Buzzai et al., 2007). Metabolic profiling of obese, nondiabetic endometrial cancer patients demonstrated that metformin has differential effects in the endometrial tumors of responders versus nonresponders, where in responders, the lipid metabolism was more pronouncedly altered, with elevated 3-hydroxybutyrate and palmitoleate levels (Schuler et al., 2015). In human ovarian tumors from patients taking metformin, lower levels of TCA intermediates and short chain acyl carnitines indicated the altered mitochondria metabolism caused by metformin (Liu, Romero, Litchfield, Lengyel, & Locasale, 2016). Metformin is also exploited in combination therapies. Combinatorial therapy of metformin and doxorubicin, a chemotherapeutic agent, killed both cancer stem cells and non-stem cancer cells in culture, reduced tumor mass and prevented relapse much more effectively than either drug alone in a xenograft mouse model (Hirsch, Iliopoulos, Tsichlis, & Struhl, 2009). Similarly, metformin suppressed growth of xenograft triple-negative breast cancer (TNBC) tumors with BACH1-depletion (a haem-binding transcription factor) whereas neither BACH1-depletion nor metformin alone altered tumor size (Lee et al., 2019). These combinatorial therapeutic strategies demonstrate the potential of targeting central metabolism and exploiting anti-diabetic agents in breast cancer treatment.

5. Elucidation of metabolic mechanisms underlying drug resistance

Drug resistance is a major cause of failed cancer treatment. Despite great efforts over the past decades, our understanding of the underlying mechanisms of drug resistance is still elusive. Recently, metabolomics has offered novel insights into mechanisms of drug resistance in subjects ranging from antibiotic-resistant bacteria (Peng et al., 2015) to cancer patients (Griffin & Shockcor, 2004; Kaushik & DeBerardinis, 2018). The metabolic alterations and mechanisms associated with drug resistance have drawn increasing research attention.

5.1. Resistance to cytotoxic chemotherapy

Cytotoxic chemotherapeutic agents remain a traditional and principal component of the treatment of cancer. After years of development, chemotherapeutic agents can be divided into various categories, including antimetabolites, platinum compounds, topoisomerase I and II inhibitors and microtubule inhibitors (Holohan, Van Schaeybroeck, Longley, & Johnston, 2013). However, effectiveness of these chemotherapies has been constrained by drug resistance. Given that cancer metabolism has intrinsic plasticity, the changes in certain metabolic processes, which can be revealed by metabolomic profiling, may be exploited by the cancer cells to overcome the drug treatment-induced stress.

A typical class of chemotherapeutic agents, for example platinum, can directly intercalate DNA and induce DNA damage, and are thus widely used in the treatment of ovarian cancer. However, resistance to platinum hinders successful treatment. Various aspects of platinum resistance mechanisms have been examined, including increased DNA repair (Masuda et al., 1988), secondary BCAR1 mutations (Swisher et al., 2008), and increased expression of Annexin A3 (Yan et al., 2010). Metabolomics revealed an altered methionine degradation pathway as a distinct metabolic feature between platinum sensitive A2780 and resistant C200 ovarian cancer cell lines (Poisson et al., 2015). Another widely-used DNA intercalating agent is doxorubicin (also referred as adriamycin), which is an inhibitor on topoisomerase II. In TNBC cells, *in vitro* and *in vivo* metabolite profiling revealed increased activity of the *de novo* pyrimidine synthesis pathway and resultant elevation of pyrimidine nucleotides following doxorubicin treatment (Brown, Spinelli, Asara, & Toker, 2017), which suggests that the combination of targeting pyrimidine synthesis and chemotherapy is a promising strategy to improve clinical responses. Similarly, daunorubicin-resistant leukemia cells displayed increased glucose dependence, reduced glutamine dependence, and altered fatty acid metabolism (Staubert et al., 2015), which represents rewired central metabolism accompanied with drug resistance status.

Antimetabolites, such as cytarabine and gemcitabine that target thymidylate synthase or DNA synthesis, have a wide range of implications in cancer treatment. Either response or resistance to these antimetabolites is closely associated with cellular metabolic energy status. For example, *in vivo* chemoresistant leukemic cells (RLCs) in AML displayed elevated levels of ROS and a high oxidative phosphorylation state in mitochondria after cytarabine treatment (Farge et al., 2017). Metabolite profiling of the gemcitabine-resistant pancreatic cells showed increased glucose metabolism, increased glucose contribution to the non-oxidative pentose phosphate pathway and increased *de novo* pyrimidine synthesis, leading to increased levels of deoxycytidine triphosphate (dCTP) that affect gemcitabine efficacy (Shukla et al., 2017). These studies may offer a deeper understanding of how these agents rewire cancer metabolism to acquire resistance, which may help to improve the responses in patients.

5.2. Resistance to targeted therapy

The development of targeted therapies in treatment of leukemia is a milestone in anticancer drug discovery. However, poor clinical responses associated with these therapies remain a great challenge due to acquired drug resistance. Imatinib resistance in CML was associated with HIF-1α induction of increased glucose flux through the non-oxidative arm of the pentose phosphate pathway for ribose synthesis (Zhao et al., 2010). The combination of imatinib and oxythiamine — the latter is an analog of thiamine that inhibits the pyruvate dehydrogenase complex and transketolase — restores imatinib sensitivity *in vitro* and *in vivo*.

AML is a hematopoietic neoplasm characterized by the accumulation of aberrant hematopoietic stem cells that fail to differentiate and proliferation of myeloid blasts in the bone marrow and peripheral blood (Bonnet & Dick, 1997). BCL-2 inhibitor venetoclax is an approved treatment for AML, which releases proapoptotic proteins to induce apoptosis upon binding to BCL2. Multiple studies demonstrated that resistance to venetoclax is associated with over expression of MCL-1, one of the BCL2 family members (Guieze et al., 2019; Konopleva et al., 2016). Other drivers of resistance included increased oxidative phosphorylation (Guieze et al., 2019) and induced expression of mitochondrial chaperonin *CLPB* (Chen et al., 2019). Multiple studies have demonstrated that mitochondrial metabolism and oxidative phosphorylation are partial drivers of drug resistance in various cancers, including breast cancer (Lee et al., 2017) and leukemia (Kuntz et al., 2017; Skrtic et al., 2011). These metabolic reprogramming and cellular metabolism associated changes revealed by metabolomics provide us potential targets to overcome resistance.

Despite the enlarging scope of oncogenic drivers identified and clinically effective treatments developed, the therapeutic resistance associated with NSCLC severely hinders the progress towards overall cure and survival (Rotow & Bivona, 2017). The oncogenic alterations in epidermal growth factor receptor (EGFR) identified in NSCLC led to the development of tyrosine kinase inhibitors (TKIs) (Herbst, Morgensztern, & Boshoff, 2018). Regarding resistance mechanisms of TKIs, metabolic reprogramming can be an important player in addition to second-site mutations. For example, a study used an UHPLC-qTOF-MS based analysis to reveal the higher level of GSH in sublethal TKI adapted cells (STACs) compared to parental cells while knockdown of branched-chain amino acid aminotransferase 1 (BCAT1) decreased GSH levels. Together, these results lend support to the notion that BCAT1 confers resistance to EGFR TKIs through attenuating ROS accumulation via GSH synthesis (Wang et al., 2019). The EGFR/PI3K/AKT axis was also reported to promote glioblastoma growth (Guo et al., 2011), and the heterogeneity of EGFR expression compromises the effectiveness of EGFR targeted therapies (Furnari, Cloughesy, Cavenee, & Mischel, 2015). The overexpressed wild-type IDH1 in glioblastoma modulated lipid biosynthesis and redox balance and promoted tumor growth, which represents an opportunity to use IDH1 inhibitors in combination with targeted therapies (Calvert et al., 2017). Taken together, interventions in metabolic reprogramming can improve the clinical applications of EGFR targeted therapies.

6. Exploring the potential strategies of dietary intervention

6.1. Dietary supplementation and restriction

The tempting concept of supplementing certain nutrients in the diet as a part of anticancer therapy has recently received greater attention. In the 1970s, supplemental ascorbate (vitamin C) was reported to function in prolonging survival times in terminal human cancer (Cameron & Pauling, 1976). Strikingly, high-dose vitamin C can impair tumor growth in *Apc/Kras*^{G12D} mutant mouse intestinal cancers in *in vivo* studies (Yun et al., 2015). Human colorectal cancer cells harboring *KRAS* or *BRAF* mutations were selectively killed when exposed to high levels of vitamin C due to increased uptake of an oxidized form of vitamin C, dehydroascorbate (DHA) via GLUT1, which causes cellular oxidative stress and depletes glutathione (Yun et al., 2015). Promising as it seems, a controversy of this high-dose vitamin C therapy revolves around the administration route to reach a plasma concentration that is cytotoxic to cancer cells (Chen et al., 2005; Levine et al., 1996). Nevertheless, considering its low toxicity and relatively low financial cost, vitamin C is still worth testing in further trials (Ngo, Van Riper, Cantley, & Yun, 2019).

Dietary control of amino acids can substantially improve the efficacy of traditional anticancer therapy. The histidine degradation pathway functions in determining cellular sensitivity to methotrexate (Kanarek et al., 2018). Asparagine bioavailability can mediate progression in breast cancer (Knott et al., 2018). Serine starvation can cause significant change to central metabolism (Maddocks et al., 2013) and enhance the anti-tumor activity of phenformin (Gravel et al., 2014). Dietary removal of the nonessential amino acids serine and glycine reduces tumor growth in xenograft and allograft models (Maddocks et al., 2017). Methionine restriction displayed therapeutic responses in patient-derived xenograft models of chemotherapy-resistant RAS-driven colorectal cancer. Metabolomics revealed that the therapeutic effects were due to the disruption of flux through one-carbon metabolism and vulnerabilities involving redox and nucleotide metabolism, which thus interact with the antimetabolite treatment or radiation intervention (Gao et al., 2019). Methionine restriction *in vivo* alters the methionine cycle and histone methylation status (Mentch et al., 2015). Another study regarding methionine restriction focused on the interaction between environmental factors and genomic alterations. Depletion of methylthioadenosine phosphorylase (MTAP), an enzyme in the methionine salvage pathway, in various cultured cancer cell lines is not predictive of cellular responsiveness to availability of methionine and other nutrient components involved in one-carbon metabolism (Sanderson, Mikhael, Ramesh, Dai, & Locasale, 2019).

Excessive fructose consumption is closely linked with the incidence of obesity and metabolic syndrome (Dhingra et al., 2007; Johnson et al., 2007). Recently, emerging evidence suggests that cancer cells may confer the metabolic advantage of utilizing fructose for their malignant growth. Within the APC mutant colorectal cancer, fructose was converted to fructose-1-phosphate, leading to accelerated glycolysis and increased synthesis of fatty acids, which lends support to the notion that high-fructose corn syrup can enhance tumorigenesis in mice (Goncalves et al., 2019). This evidence of fructose's contribution to intestinal tumor growth in laboratory mice suggests that it can be extrapolated to use in humans. Inhibitors of fructose transporter or fructose metabolic enzymes, or dietary fructose control serve as therapeutic strategies for cancer. A recent [2-^{13}C] fructose tracing study revealed unexpected metabolism of fructose specifically in AML, in which fructose fueled the *de novo* serine synthesis pathway (Jeong et al., 2021). Dietary intervention disrupts the essential metabolic backbone of restricted nutrients and therefore vulnerabilities are created and can be further exploited to target critical metabolic needs in cancer cells. Determining how diet intervention improves cancer outcomes is a quickly-expanding territory, where further detailed investigations will be highly appreciated (Kanarek, Petrova, & Sabatini, 2020).

6.2. Calorie restriction

An even more aggressive approach, dietary restriction, has displayed its utility in delaying the incidence and decreasing the growth of various tumors. First described in the beginning of the 20th century (Rous, 1914), dietary restriction has shown substantial and reproducible anti-tumor effects in laboratory mice, which are usually achieved by around 10 to 50% decrease in calorie intake (Tannenbaum & Silverstone, 1949, 1953; Weindruch & Walford, 1982). However, human tumor xenografts exhibit differential sensitivities to dietary restriction. Interestingly, the differential activation status of PI3K served

as an indicator in xenograft models of their differential sensitivities to dietary restriction (Kalaany & Sabatini, 2009), which suggests that activating mutations in the PI3K pathway may influence the response of cancers to dietary restriction.

6.3. Ketogenic diet

A ketogenic diet was also reported to be effective against various cancers, including advanced endometrial adenocarcinoma, bladder cancer, breast cancer and AML, when supplemented with PI3K inhibitors, by reducing blood insulin (Hopkins et al., 2018). A comprehensive panel of 15 isotope tracers used in various mouse models revealed persistent circulatory carbohydrate fluxes and pyruvate cycling across tissues under a ketogenic diet (Hui et al., 2020). Similar with dietary restriction, a ketogenic diet may exhibit variable effects in different tumor mouse models, which should be closely examined in a case-by-case manner.

6.4. Fasting

Fasting asks for no or a minimal amount of food and caloric beverages for defined periods and is thus different from caloric restriction, given that the latter still maintains meal frequency (Longo & Mattson, 2014). There are different forms of fasting, including intermittent fasting (IF), such as fasting for 2 days a week, and periodic fasting (PF) lasting three days or longer every 2 or more weeks (Longo & Mattson, 2014). The mechanisms and applications of fasting have been discussed under physiological and pathological settings (Longo & Mattson, 2014) and particularly in cancer (Nencioni, Caffa, Cortellino, & Longo, 2018). IF prevented carcinogenesis in p53-deficient mice, which are naturally prone to sarcoma and lymphoma due to the loss of one allele of the suppressor gene (Berrigan, Perkins, Haines, & Hursting, 2002). Fasting was also shown to augment the efficacy of certain chemotherapy strategies in breast cancer and neuroblastoma (Lee et al., 2012). How fasting improves the response to chemotherapy was related to the level of insulin-like growth factor-1 (IGF1). Specifically, the reduced level of systemic IGF1 protected normal cells but not cancer cells, which explains the differential sensitivity to chemotherapies (Lee et al., 2010). Despite these results, fasting is a harsh approach that lacks feasibility for wide application in patients (Kanarek et al., 2020).

7. Identifications of biomarkers

Like other omics technologies, metabolomics has been extensively used in biomarker discovery for the diagnosis and prognosis of diseases.

7.1. Diagnostic biomarkers

Mutations in certain genes that encode metabolic enzymes can significantly affect enzyme activity and disrupt their physiologic roles. In this way, a mutated enzyme can either lose its function or instead generate specific downstream metabolites that do not appear in the physiologic condition. These metabolites can thus be referred to as onco-metabolites, of which 2-HG was one of the first identified in the formation and progression of brain tumors (Dang et al., 2009). The single amino acid R132H mutation of the cytosolic isocitrate dehydrogenase (IDH1) is recognized as an early event in brain tumors (Watanabe, Nobusawa, Kleihues, & Ohgaki, 2009) and leads to the disruption of its catalytic ability (Zhao et al., 2009). The mutated IDH1 lacks the ability to convert isocitrate to α-ketoglutarate (αKG), but gains the ability to catalyze αKG to 2HG, in a NADPH dependent manner. A LC-MS-based metabolomics study revealed elevated 2HG levels in IDH1 mutant expressing cells (Dang et al., 2009). Excess accumulation of 2HG was also found in human brain tumor samples harboring IDH1 mutation and is associated with the malignant progression of cancer. Another study used proton magnetic resonance spectroscopy and six metabolites (tetraethyl amines, phosphocreatine and creatine, N-acetyl groups, alanine, lactate and lipids) were identified to be characteristic and able to discriminate normal brains from brain tumors (Preul et al., 1996). This *in vivo* analysis of brain tumor allows for accurate biochemical identification of tissues as well as proper characterization of pathological states.

Metabolomics is essential in identifying biomarkers for the early detection of prostate cancer. The detection of human prostate cancer has been challenging. Despite the fact that the prostate specific antigen (PSA) blood test is helpful for detecting human prostate cancer at early and asymptomatic stages (Andriole et al., 2009; Schröder et al., 2009), PSA values are not cancer specific and even benign prostate conditions may elevate PSA readings. Definitive diagnosis of prostate cancer relies on biopsies, which still carry high false-negative rates due to the heterogeneity of cancer in the prostate (Hansel et al., 2007). Metabolomic imaging, however, has great potential to identify the presence of prostate cancer and indicate tumor pathological states. Metabolomic profiling features higher sensitivity and specific correlations with tumor histology (Wu et al., 2010).

Similarly, metabolic profiling uncovers metabolic signatures for early detection of breast cancer. A GC-MS based serum metabolic profile of pre-operative breast cancer patients and healthy controls revealed seven metabolites (tetradecane, alpha-D-glucopyranoside, methyl stearate, dodecane, 1-4-benzene, D-galactose and octadecanoic acid) for breast cancer diagnosis (Hadi et al., 2017). Metabolomics is also used to identify biomarkers for other types of cancer, including the elevation of circulating BCAAs as an early event in PDAC (Mayers et al., 2014), diacetylspermine as a novel pre-diagnostic serum biomarker in NSCLC (Wikoff et al., 2015), and diagnostic metabolites (lysophosphatidylethanolamine, lysophosphatidylinositol etc.) in early-stage ovarian cancer (Gaul et al., 2015).

7.2. Prognostic biomarkers

The metabolic characteristics of cancer cells can serve as a pretreatment prediction of the tumor inherent response to chemotherapy. For instance, the combinational use of ^{1}H-NMR spectroscopy and neural networks revealed that metabolite spectra could be predictive of cellular responses to a chemotherapeutic drug (El-Deredy et al., 1997). Accumulation of polyunsaturated fatty acids was detected in rat glioma receiving a robust ganciclovir-thymidine kinase gene therapy induced programmed cell death (Griffin et al., 2003). Based on metabolic profile and metabolite markers, a predictive model was established with better sensitivity and specificity and revealed the differential metabolic signature in BRCA1-like and non BRCA1-like breast cancer profiles, for example N6-methyladenosine and 1-methylguanine, which demonstrated the heterogeneity of breast cancer (Roig et al., 2017).

Metabolomics has a high capacity for probing cancer progression. LC-MS based metabolomics profiling identified sarcosine, an N-methyl derivative of the amino acid glycine, as a key metabolite that was highly increased in prostate cancer progression and metastasis and non-invasively detectable in urine (Sreekumar et al., 2009). Another metabolomics-based profile, combined with NMR and two-dimensional gas chromatography-mass spectrometry (GCxGC-MS), identified eleven metabolite markers (formate, histidine, proline, choline, tyrosine, 3-hydroxybutyrate, lactate, glutamic acid, N-acetyl-glycine, 3-hydroxy-2-methyl-butanoic acid and nonanedioic acid) as the contributing factors for breast cancer recurrence (Asiago et al., 2010). The highlighted papers from section 2 to 7 are summarized in Table 2.

8. Enabling precision treatment with pharmacometabolomics

The concept of precision medicine has been thriving in the healthcare field in the past decade. Precision medicine emphasizes tailoring treatment strategies to each individual in an attempt to maximize therapeutic effects and minimize toxicities (Hastings, O'Donnell, Fey, &

Table 2
Summary of major findings in highlighted papers

	Method/Mechanism	Cancer type	Reference
Method advancements			
High sensitivity	An ultra-sensitive LC-MS based method coupled with flow cytometry	HSC	Agathocleous et al., 2017
Subcellular metabolomics	Isolation of intact mitochondria in tandem with LC-MS based metabolomics	HeLa	Chen et al., 2016
Metabolic vulnerabilities			
Glycolysis and the TCA cycle	$Kras^{G12D}$ mutation diverts glycolytic intermediates into nonoxidative PPP and promotes ribose biogenesis	PDAC	Ying et al., 2012
	Lung tumors show increased TCA flux from glucose relative to adjacent normal tissue	Lung tumors	Davidson et al., 2016; Hensley et al., 2016
	Lactate fuels the TCA cycle	NSCLC	Faubert et al., 2017
		Pancreatic cancer	Hui et al., 2017
Glutamine metabolism	Glutamine supports TCA cycle	Glioblastoma	DeBerardinis et al., 2007
	Glutamine supports TCA cycle under hypoxia	Burkitt lymphoma	Le et al., 2012
One carbon metabolism	Diversion of glycolytic flux into serine and glycine biosynthesis	Lung cancer	Locasale et al., 2011
	Serine biosynthesis promotes tumor growth	Breast cancer	Sullivan et al., 2019
	Glycine metabolism correlates with cancer cell proliferation	Various cancers	Jain et al., 2012
Lipid metabolism	mTORC2 promotes lipid synthesis and tumorigenesis	Hepatocellular carcinoma	Guri et al., 2017
	Cancer cells downregulate the synthesis of polyunsaturated ether phospholipids to evade ferroptosis	Various cancers	Zou et al., 2020
Nucleotide metabolism	A subtype of SCLC depends on IMPDH for guanosine nucleotides synthesis and tumor growth	SCLC	Huang et al., 2018
Drug mode of action			
Methotrexate	Histidine catabolism determines methotrexate sensitivity	Erythroleukemia	Kanarek et al., 2018
Imatinib	Imatinib inhibits leukemia cell glucose utilization	CML	(Boren et al., 2001)
	Glucose metabolism switches from aerobic oxidation to TCA cycle	CML	Gottschalk et al., 2004
Metformin	Metformin alters mitochondria metabolism	Ovarian tumors	Liu et al., 2016
	Targeting mitochondrial metabolism and BACH1 sensitizes breast cancer to mitochondria inhibitors	Breast cancer	Lee et al., 2019
Drug resistance			
Doxorubicin	Doxorubicin treatment increases *de novo* pyrimidine synthesis	Breast cancer	Brown et al., 2017
Cytarabine	Cytarabine treatment elevates levels of ROS and oxidative phosphorylation state.	AML	Farge et al., 2017
Gemcitabine	Increased levels of dCTP affects gemcitabine efficacy	Pancreatic cancer	Shukla et al., 2017
Imatinib	Imatinib resistance is related to HIF-1α induced metabolic reprogramming	CML	Zhao et al., 2010
Venetoclax	Cancer cells escape BCL-2 inhibition via mitochondria reprogramming	CML	Guieze et al., 2019
EGFR TKIs	BCAT1 mediated metabolic reprogramming contributes to resistance to EGFR TKIs	Lung cancer	Wang et al., 2019
Dietary intervention			
Dietary restriction & supplementation	Vitamin C in high dose kills KRAS and BRAF mutant colorectal cancer cells	Colorectal cancer	Yun et al., 2015
	Methionine restriction influences chemotherapy-resistant RAS-driven colorectal cancer	Colorectal cancer	(Gao et al., 2019)
	High-fructose corn syrup can enhance tumorigenesis	Colorectal cancer	Goncalves et al., 2019
Calorie restriction	PI3K status predicts the sensitivity to calorie restriction	Various cancers	Kalaany & Sabatini, 2009
Ketogenic diet	Ketogenic diet improves responses to PI3K inhibitors	Various cancers	Hopkins et al., 2018
Biomarkers identification			
2HG	2-hydroxyglutarate generated by mutant IDH can be noninvasively detected	Gliomas	Choi et al., 2012

Croucher, 2020). The advent of high throughput technologies has revolutionized our access to collectible, large volumes of data and opened a way to understand the dynamics of biological systems under health and disease. The generation of large-scale omics data has made collecting patient-specific information easier and more realizable, and personal omics profiling is expected to bring forward the development of precision treatment.

The fast-growing omics techniques, when applied in the pharmacology field, have generated pharmacomics, which greatly expands our knowledge in characterizing and evaluating disease state and drug responses (Milward, Daneshi, & Johnstone, 2012). Metabolomics is increasingly being used in precision medicine (Agren et al., 2014), together with other omics approaches (Chen et al., 2012). In particular, pharmacometabolomics, the application of metabolomics in pharmacology, tackles how metabolic states are affected when exposed to drug treatment. It can enable precision medicine by contributing to identifying biomarkers, predicting drug response, toxicities and pharmacokinetic profile of drugs (James, 2013; Kaddurah-Daouk et al., 2014; Kaddurah-Daouk et al., 2015).

Pharmacometabolomics has shown its importance in studying drugs such as statins (Krauss, Zhu, & Kaddurah-Daouk, 2013), selective serotonin (5-HT) reuptake inhibitors (SSRIs) (Kaddurah-Daouk et al., 2013), and antiplatelet therapy (Lewis et al., 2013). Recently, it is also increasingly applied in the studies of anti-cancer drugs. Circulating spermidine and tryptophan are metabolite biomarkers that can differentiate good and poor responders to trastuzumab-paclitaxel neoadjuvant therapy in HER-2 positive breast cancer patients (Miolo et al., 2016). Patients with higher levels of spermidine and lower serum levels of tryptophan had a better response to the therapy, which implies that an individual patient's metabolism may guide the selection of patients that are more responsive. Tissue metabotypes of 25 PDAC patients who underwent gemcitabine-based adjuvant therapy identified 19 marker metabolites potentially associated with chemoresistance, including lactic acid, proline and pyroglutamate (Phua et al., 2018). Using NMR-based metabolomics, researchers identified serum levels of formate and acetate as potential markers that can predict the response to gemcitabine-carboplatin chemotherapy in 29 metastatic breast cancer (MBC) patients (Jiang, Lee, & Ng, 2018). In addition, pharmacometabolomics can also be used to predict the gastrointestinal toxicity induced by irinotecan in rats (Gao, Li, et al., 2019). All the above findings indicate that pharmacometabolomics will open up broad prospects for precision medicine.

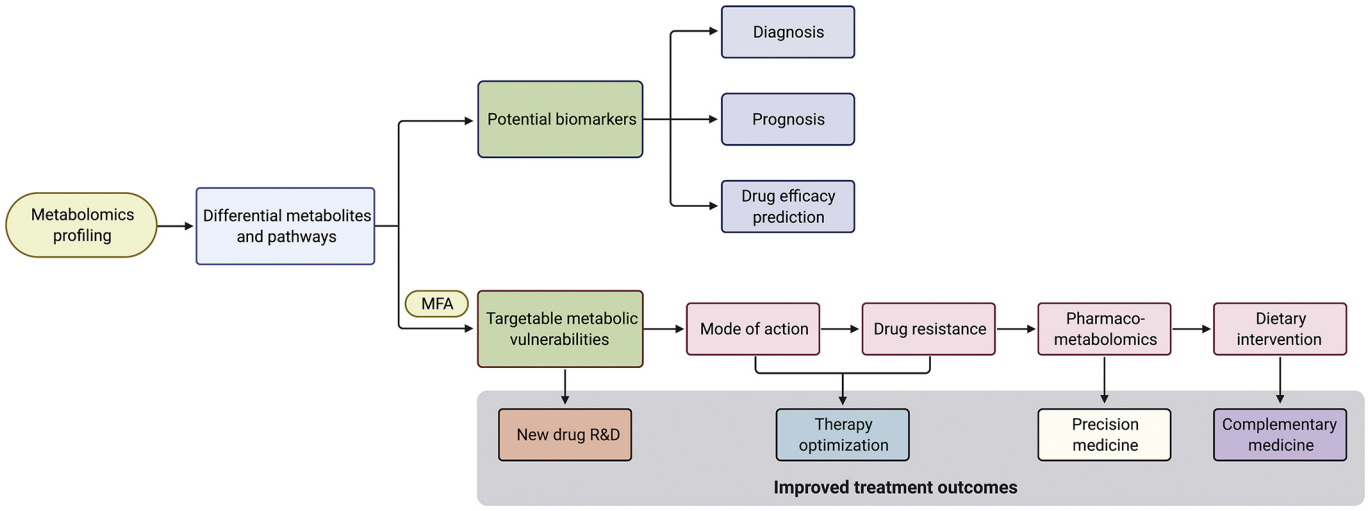


Fig. 4. The applications of metabolomics and MFA in cancer drug discovery and pharmacology. After metabolomics profiling, differential metabolites and pathways can be identified, which may lead to the identification of potential biomarkers and targetable metabolic vulnerabilities. Biomarkers can facilitate cancer diagnosis and prognosis as well as prediction of anti-cancer drug efficacy. Metabolic vulnerabilities can serve as targets for new drug research and development (R&D) and facilitate other cancer pharmacology studies for therapy optimization, including understanding drug modes of action and circumventing drug resistance. Pharmacometabolomics on the basis of predictive biomarkers can enable precision medicine while dietary intervention can work as complementary medicine. All these studies may eventually contribute to improved outcomes including maximized efficacies and minimized toxicities.

Interestingly, pharmacometabolomics has also shown exciting potential in predicting the treatment outcomes in patients receiving immunotherapy. Metabolomics analysis of gut microbiota in 11 NSCLC patients receiving nivolumab, an antibody against programmed cell death protein 1 (PD-1), suggested that gut microbiota associated metabolites can be indicators of response duration (Botticelli et al., 2020). Kynurenine was significantly up-regulated in serum metabolite profiling of advanced melanoma and renal cell carcinoma patients treated with nivolumab and the increase in the ratio of serum kynurenine/tryptophan was identified as an adaptive resistance mechanism related to worse overall survival (Li, Bullock, Gurjao, Braun, & Giannakis, 2019). A recent study used metabolomics to classify TNBC patients into three heterogenous categories based on their distinctive metabolic features. While these metabolic-pathway-based subtypes respond to metabolic inhibitors differently, one of the subtypes, MPS2, which is defined as the glycolytic subtype with upregulated carbohydrate and nucleotide metabolism, is prone to anti-PD-1 immunotherapy when lactate dehydrogenase is inhibited (Gong et al., 2021). Together, these studies suggest the possibility of predicting the effects of immunotherapy by pharmacometabolomics.

As discussed above, metabolomics is increasingly applied in almost every stage of cancer drug discovery and development as well as pharmacological research (Fig. 4).

9. Future directions

Here, we discuss how metabolomics and MFA help to shed light on cancer biology and provide us with new insights into the frontiers of cancer pharmacology. While significant improvements have been achieved in this area, there are still several challenges to overcome. Regarding the analytical technologies, it remains critical to improve the accuracy and precision of metabolite detection and to achieve a better understanding of the entire metabolome. Advancements in the standardization procedures spanning sample collection to data analysis are required to enable more reproducible and reliable metabolic analyses (Salek et al., 2015). In addition, cell heterogeneity and subcellular compartmentalization call for detection technologies with sufficient sensitivity that could enable reliable and extensive coverage of metabolites at single cell or subcellular organelle levels. Even though many studies have been reported on single cell metabolomics, most of them are focused on single cell isolation and sample transfer to an ion source (Zhang et al., 2018; Zhu et al., 2017). It is still difficult to separate the subcellular compartments while maintaining their metabolic state. Substantial efforts are still needed to break these technical bottlenecks particularly to achieve ultra-high sensitivity of detection for broader coverage of the metabolome. This is even more important for MFA analysis of a limited number of biological samples even in single cells, as isotope enrichment analysis requires even more challenging sensitivity of the detection approaches. Another important direction is to understand the spatial organization and regulation of metabolism. Developments of novel imaging MS techniques can reveal new information and possibly broaden the field (Bodzon-Kulakowska & Suder, 2016). Mass spectrometry imaging (MSI) approaches using matrix-assisted laser desorption ionization (MALDI) or desorption electrospray ionization (DESI) can identify spatial distribution of metabolites across tissues and reveal the metabolic crosstalk in the specific tumor microenvironment, offering researchers abundant information of metabolic phenotypes and regulation *in situ*.

The next stage of cancer metabolism research will need to address increasingly important questions about how identified metabolic phenotypes can be integrated, exploited and translated into better cancer therapies. The metabolic interplay among various cell types including tumor, stromal and immune cells in the complex tumor microenvironment has drawn increasing attention (Vander Heiden & DeBerardinis, 2017). Dissecting their potential metabolic competitions and dependencies may greatly advance our understanding of the mechanisms underlying metabolic regulation, facilitate development of new therapies and improve efficacies of currently available medications particularly immunotherapies. Yet there still remain great challenges to isolate different types of cells from digested tumor tissues without introducing additional metabolic disturbances. *In vitro* 3-D culture systems that could recapitulate biological events in the complex *in vivo* context while minimizing artificial effects are also needed. Moreover, the advances in multi-omics integrative analyses of cancer may help to extend and deepen our understanding of cancer biology and exploit altered metabolism, thereby to accelerate and improve drug discovery and development as well as to enable better anti-cancer therapy.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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