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Review

Metabolic analysis as a driver for discovery, diagnosis, and therapy

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SUMMARY

Metabolic anomalies contribute to tissue dysfunction. Current metabolism research spans from organelles to populations, and new technologies can accommodate investigation across these scales. Here, we review recent advancements in metabolic analysis, including small-scale metabolomics techniques amenable to organelles and rare cell types, functional screening to explore how cells respond to metabolic stress, and imaging approaches to non-invasively assess metabolic perturbations in diseases. We discuss how metabolomics provides an informative phenotypic dimension that complements genomic analysis in Mendelian and non-Mendelian disorders. We also outline pressing challenges and how addressing them may further clarify the biochemical basis of human disease.

INTRODUCTION

Many phenotypes in humans and other organisms involve reprogramming of metabolism. Disease-associated metabolic perturbations can be fixed (e.g., defined by germline mutations) or reversible (e.g., nutritional deficiencies, transient tissue hypoxia) and may involve simple defects confined to particular cell types or complex alterations of systemic homeostasis. Most of the common causes of death in the developed world—heart disease, stroke, diabetes, cancer, and others—are characterized by metabolic changes that contribute to tissue dysfunction. The importance of metabolism in basic cellular processes and the large fraction of the genome devoted to the metabolic network explain why altered metabolism is so prominent in disease.

These connections make it appealing to identify metabolic features related to phenotypic variation. Recent advances have made it possible to characterize disease-associated metabolic alterations in detail. This has involved applying emerging technologies to metabolism research and repurposing established techniques such as stable isotope tracing to probe disease-associated metabolic perturbations *in vivo*. These efforts have produced new therapeutic targets and insights about the mechanistic basis of metabolic diseases. A case in point involves human cancers with mutations in isocitrate dehydrogenase-1 or -2 (IDH1, IDH2). Cancer-associated mutations in IDH1 and IDH2 occur in gliomas and other malignancies (Mardis et al., 2009; Parsons et al., 2008). Metabolomic profiling revealed that mutant IDH1 and IDH2 convert α-ketoglutarate to D-2-hydroxyglutartate (D-2HG), resulting in massive accumulation of this usually scarce metabolite (Dang et al., 2009). D-2HG interferes with histone and DNA demethylases that use α-ketoglutarate as a cosubstrate (Figueroa et al., 2010), and failure to activate gene expression programs required for differentiation is thought to promote malignancy in IDH-mutant cells (Rohle et al., 2013; Wang et al., 2013). It is now possible to follow D-2HG levels clinically to track disease progression (Choi et al., 2016; Intlekofer et al., 2018), and drugs that inhibit the mutant enzymes are used to treat leukemia (DiNardo et al., 2018). Therefore, discovery of a disease-associated metabolic alteration produced insights into pathophysiology and changed clinical care.

This review aims to provide biologists with a survey of recent methodological advances in metabolism, emphasizing four with particular relevance to human disease and potential to enable many new discoveries in the coming decade. We also discuss challenges that need to be addressed to fuel the next wave of breakthroughs.

Advance 1: Small-scale metabolomics to assess metabolite levels in discrete, biologically important cellular subsets

Metabolomics on bulk tissues has provided a wealth of knowledge about metabolic perturbation in disease. However, it is clear from single-cell RNA studies that an appreciation for the metabolism of individual cells within a complex microenvironment is critical to understanding pathogenesis (Xiao et al., 2019). This is important for four reasons. First, different cell types within the same tissue have specialized metabolic properties. Metabolic compartmentation between neurons and astrocytes in the brain is an example of this concept. Second, the location of cells within a microenvironment influences their metabolism. Examples include compartmentalized metabolic fluxes within the hepatic lobule, and the impact of proximity to the blood supply on cancer cell metabolism within tumors. Third, some scarce

cells, e.g., stem and progenitor cells, are thought to be metabolically distinct from the rest of the tissue, but it is nearly impossible to infer their metabolic activities from bulk analysis. Fourth, even within a single cell, metabolism is compartmentalized within organelles and other structures to achieve precise regulation. Recent advances have addressed the challenge of assessing metabolism at small scale.

Single cells and rare cell populations

Given that traditional metabolomics approaches require 10^5–10^6 cells, small-scale metabolomics has been driven by the need to characterize rare cell populations. Rare cells can be analyzed through rapid enrichment by flow cytometry at cold temperatures, sorting directly into extraction medium followed by mass spectrometry, yielding up to 160 metabolites in hematopoietic stem cells and circulating tumor cells (DeVilbiss et al., 2021). This approach revealed the surprising stability of many metabolites during the 12-min preparation and demonstrated that purine monophosphates become depleted when melanoma cells enter the circulation, possibly reflecting adaptation to stresses encountered after escape from the tumor.

Metabolomics can be coupled to sensitive metabolic flux measurements, including isotope tracing with hyperpolarized nuclear magnetic resonance (NMR). This provides insight into quantitative rates of metabolite turnover. Recent work has shown that this can provide flux measurements in as few as 9,000 cells and be applied to the study of flow-sorted cancer stem cells when coupled to a microcoil system (Jeong et al., 2017).

Beyond direct metabolite measurements, other profiling approaches can provide information about the metabolic network, sometimes at single-cell resolution. Single-cell RNA sequencing can determine the levels of metabolically relevant genes within complex tissues (Argüello et al., 2020; Artyomov and Van den Bossche, 2020). Proteomic methods such as cytometry/time of flight (TOF) mass spectrometry (CyTOF) provide a richer appreciation for metabolic heterogeneity within immune cell populations. Other work has exploited optical approaches using FRET sensors to quantify metabolites like amino acids and redox cofactors (Cameron et al., 2016; Tao et al., 2017; Wu et al., 2021). Together with live-cell imaging, this provides real-time information about how metabolic features relate to cell biology. These approaches require cellular engineering and are generally incompatible with high-throughput screening. Traditional optical imaging (e.g., two-photon microscopy) for endogenous metabolites provides a "probeless" strategy and can be used to interrogate redox cofactors such asnicotinamide adenine dinucleotide NAD(H), and flavin adenine dinucleotide (FADH[H]) (Blacker et al., 2014; Walsh et al., 2013). Although these approaches require further development, coupling them with rapidly developing spatial mass spectrometry imaging (MSI) methods could aid in the assessment of real-time flux in small-scale applications.

Spatially resolved metabolism

Isolation provides a means of enriching cells of interest but eliminates the ability to spatially map metabolites within a tissue. Multiple approaches have been pioneered in the last 10 years to address this need. Leveraging the increased sensitivity of modern mass spectrometry systems, many imaging approaches have been placed in tandem to raster scan and generate spatial metabolomic data. Matrix-assisted laser-desorption ionization (MALDI) has become the most widely utilized spatial mass spectrometry approach and is typically conducted by sectioning a sample to 5–10-μm thickness and placing the section on a moving stage where a mass spectrum is recorded in a rasterized manner to reconstruct a 3D dataset (2D spatial and 1D spectral) (Chaurand et al., 2006). The spatial resolution (50–100 μm) continues to be limiting for true single-cell analysis. Interesting methods have been developed for cells in culture with application to tissue sections that attempt to infer single-cell measurement (e.g., SpaceM; Rappez et al., 2021), and this will be a key area for further development.

Spatially resolved MSI using MALDI was instrumental in assessing novel metabolism in both the setting of normal brain function and disease. After the surprising finding that glucosamine constitutes a significant fraction of the monosaccharides composing glycogen in the mouse brain, MALDI MSI revealed that defects in glycogen breakdown resulted in co-localized glycogen accumulation and depletion of N-linked glycans (Sun et al., 2021). In genetic mouse models of squamous cell carcinoma, MALDI MSI provided evidence for a subset of CD34+ cells containing increased glutathione abundance, conferring oxidative stress resistance and tumorigenic potential *in vivo* (Choi et al., 2021). MALDI MSI has pushed into isotope tracing with recent studies annotating metabolite labeling in regions of the mouse brain (Wang et al., 2022). It is still challenging to infer true metabolic flux, given the static nature of these experiments, but a wealth of comparisons can now be made in the same region.

Metabolic flux is driven by substrate and cofactor concentrations that can vary dramatically across compartments of the cell. Although the holy grail of spatial MSI approaches would be submicron resolution to assess distinct compartments within the cell, this is not currently possible. However, intermediate approaches have been developed and proven to be informative. Building on approaches to "pull-down" cells of interest, multiple groups have taken advantage of immunopurification to enrich organelles for metabolic analysis. Mitochondrial immunopurification (MITO IP) quantifies the abundance of metabolites within the mitochondria (Chen et al., 2016). Artifacts associated with conventional, time-consuming mitochondrial enrichments using centrifugation and other techniques are minimized by the rapid (under 15 min) immunopurification approach used by MITO IP (Chen et al., 2017). Mitochondrial metabolomics has aided in the discovery of mitochondrial transporters for glutathione (Wang et al., 2021) and NAD (Kory et al., 2020). The technique has been extended to lysosomes (Abu-Remaileh et al., 2017) and peroxisomes (Ray et al., 2020). These approaches require large numbers of cells (e.g., 10^7 cells), although it is likely that this can be improved with some of the high-sensitivity mass spectrometry approaches used in rare cell applications.

Altogether, the methods discussed in this section have improved our understanding of metabolism by making it possible to explore metabolic features of discrete cellular populations and subcellular compartments. The conceptual advance emerging from these studies is that despite the undeniable utility of performing metabolic analyses on bulk tissues, some crucial aspects of metabolism are only observable through advanced

techniques that allow the investigator to focus on metabolically distinct regions within complex environments.

Advance 2: Open-ended techniques to identify new mechanisms of metabolic regulation

The reactions that comprise the metabolic network have been known for decades. However, questions persist about how context-dependent metabolic requirements are established. Screening methods have become increasingly common in metabolism research, and the open-ended nature of these approaches has produced many non-intuitive discoveries. This section discusses two screening techniques: functional genomics using libraries optimized to probe metabolism and chemical library screens tailored to identify metabolic vulnerabilities.

Functional genomics

The large number of "metabolic" genes—over 2,000 in humans (Gillespie et al., 2022)—means that a thorough analysis demands high-throughput methods. Techniques in mammalian cell functional genomics allow investigators to identify metabolic vulnerabilities in an unbiased fashion without pre-existing hypotheses about which pathways are most important. An early example used a lentiviral short-hairpin RNA library tailored to target metabolic enzymes and transporters (Possemato et al., 2011). The library was expressed in breast cancer cells; then, the cells were implanted orthotopically in mice. After tumor formation, massively parallel sequencing revealed that the gene encoding phosphoglycerate dehydrogenase (*PHGDH*) is required for tumor growth in this model. The genomic region containing *PHGDH* is amplified in over half of estrogen-receptor-negative breast cancers and some other cancers as well (Locasale et al., 2011; Possemato et al., 2011). PHGDH catalyzes a reaction in *de novo* synthesis of serine and glycine, intermediates that contribute to cancer cell growth. Many subsequent studies have explored the importance of PHGDH in cancer, including the recent finding that a paucity of serine in the brain microenvironment increases dependence on PHGDH and renders brain metastases sensitive to PHGDH inhibition (Ngo et al., 2020).

CRISPR-based screens (Figure 1) have been used to identify many metabolic liabilities, sometimes solving long-standing conundrums in cell biology. Although oxidative phosphorylation (OxPhos) by the electron transport chain (ETC) supports many metabolic functions, cells with defective OxPhos can proliferate if provided with exogenous pyruvate (King and Attardi, 1989). Why pyruvate is so important under these circumstances had been puzzling. A CRISPR screen in Jurkat cells containing guide RNAs (gRNAs) against ~3,000 metabolic genes revealed that GOT1, the cytosolic glutamate-oxaloacetate transaminase that produces aspartate from oxaloacetate, becomes essential during OxPhos inhibition (Birsoy et al., 2015). Aspartate levels decline in the absence of pyruvate, but exogenous aspartate compensates either for pyruvate depletion or GOT1 deficiency. This led to the surprising conclusion that a major function of the ETC in proliferating cells is to produce aspartate rather than energy. Pyruvate serves as an electron acceptor that ultimately enables production of oxaloacetate for transamination (Birsoy et al., 2015; Sullivan et al., 2015). Subsequent studies revealed that hypoxic regions of tumors contain insufficient levels of aspartate to support cell growth (Garcia-Bermudez et al., 2018). Similar screens have identified enzymes that produce lethal toxin-induced oxidative stress and new mechanisms of redox homeostasis (Garcia-Bermudez et al., 2019; Reczek et al., 2017). All these discoveries relied on the unbiased, global assessment of metabolic dependencies provided by functional genomics.

The tumor microenvironment (TME) influences metabolism; hence, many enzymes required for cell growth *in vivo* are dispensable in culture and vice versa (Davidson et al., 2016). Two recent studies compared liabilities from parallel screens in pancreatic ductal adenocarcinoma (PDAC) cells conducted in culture and mice (Biancur et al., 2021; Zhu et al., 2021). After introducing the library, the cells were either allowed to proliferate in culture or implanted subcutaneously to form tumors in immunocompetent mice. Surprisingly, many liabilities during *in vivo* growth were also observed in culture. However, other pathways were selectively required for growth *in vivo*. Among these, heme biosynthesis was detected by both teams, indicating that heme availability limits PDAC cell growth *in vivo* but not in culture.

Modulating metabolism with small molecules

A few hundred inhibitors against metabolic enzymes and nutrient transporters exist as tool compounds or, in some cases, drugs in the clinical arena. Chemical libraries enriched for these inhibitors have been used to identify synthetically lethal interactions, uncovering unexpected roles for metabolism in the cellular responses to blockade of other processes. For example, this chemical screening approach revealed that glutathione depletion synergizes with deubiquitinase inhibition in breast cancer, perhaps reflecting the importance of deubiquitination in mitigating the accumulation of misfolded proteins during oxidative stress (Harris et al., 2019). Inhibition of nicotinamide phosphoribosyltransferase (NAMPT), an enzyme in NAD^+ salvage, sensitized triple-negative breast cancer cells to apoptosis by B cell leukemia/lymphoma-2 homology-3 domain (BH3) mimetics (Daniels et al., 2021). The NAD^+ depletion caused by NAMPT inhibition reduces adenine levels and primes cells for apoptosis.

Metabolites bind allosteric sites on enzymes, and this is a common mechanism to regulate pathway activity. Although many such interactions are already known, an unbiased approach to search for metabolite-protein interactions could uncover novel modes of regulation. One recent approach used equilibrium dialysis and mass spectrometry to detect metabolites from a library that can bind to purified proteins (Hicks et al, 2021). Screening 33 proteins uncovered hundreds of interactions and several new regulatory sites resolved through atomic-resolution co-crystal structures containing the metabolite and protein. In one such interaction, long-chain acyl-CoAs were found to bind lactate dehydrogenase A (LDHA) and inhibit its activity at low-micromolar concentrations. The findings imply a new mode of regulation between lactate and fatty acid metabolism, exerted at the level of LDHA. Many other protein-metabolite interactions uncovered by this screening assay remain to be explored.

The primary conceptual advance of these genetic and chemical screening methods over the last decade is that they enable detailed, efficient queries of the metabolic network without a priori hypotheses about interactions and liabilities. In unbiased screens like these, the benchmark of success is that they produce

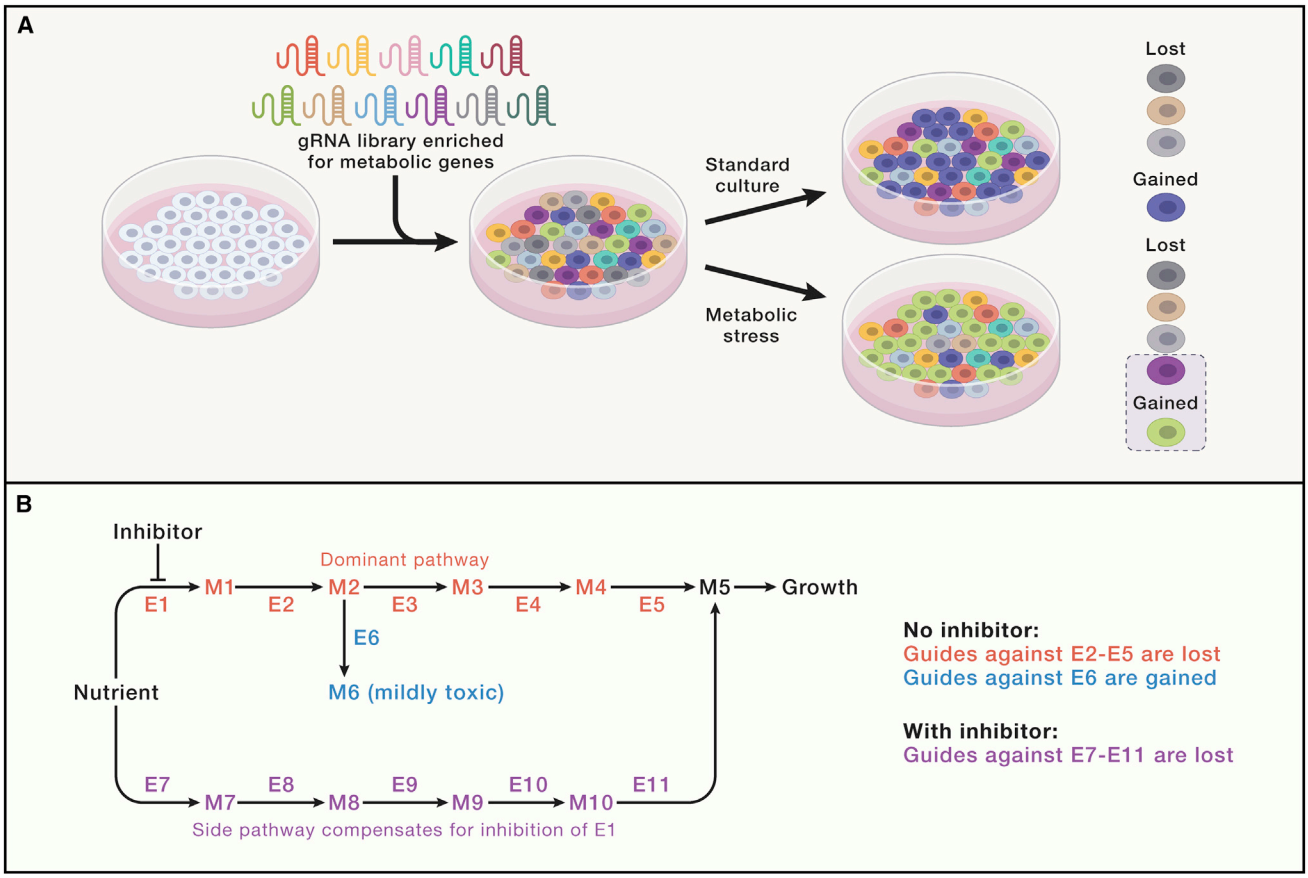

Figure 1. High-throughput screening to identify mechanisms by which cells respond to metabolic stress

(A) Here a guide RNA (gRNA) library induces loss-of-function mutations against metabolic genes. The library is introduced to cultured cells, producing a mixed population. The cells are grown in the presence or absence of a metabolic stress, and sequencing identifies gRNAs whose abundance changes selectively under stress. In this example, several gRNAs are depleted (lost) during standard culture. These gRNAs target genes required for growth under standard conditions. Blue cells increase in number (gain) because the gRNA in these cells targets a growth-suppressive gene. A different distribution occurs in cells exposed to metabolic stress, highlighted by the dashed box, with some gRNAs lost (purple) or gained (green) only under stress.

(B) Cells use the red pathway to produce a metabolite, M5, required for growth. The pathway also produces the mildly toxic M6. The purple pathway is dispensable in conventional culture but can produce M5 when levels of the nutrient increase, for example, if E1 is mutated or blocked with an inhibitor. A CRISPR screen like the one in (A) would generate the results shown on the right.

discoveries that would not have arisen purely from existing knowledge. The discoveries cited above demonstrate ways that unbiased screening has enriched our understanding of metabolism.

Advance 3: Combining genomics and metabolomics to understand the genetic basis of metabolic variability and disease

Outbred populations contain a remarkable amount of metabolic heterogeneity, much of it genetically defined and some associated with disease. It has been a long-standing challenge to identify genomic variants that contribute to metabolic heterogeneity and relate them to disease. Measuring a few key metabolites is a long-established component of the metabolic disease workup. However, more recently, analytical advances in genomics and metabolomics have made it possible to perform a holistic and integrated assessment of the genetic basis of metabolic variation. This section describes conceptual and practical advances in this area.

Genetic modifiers of metabolism in mice and humans

Inbred mice are instrumental in defining the basis of metabolic phenotypes. However, these strains are useful specifically because of the lack of genomic and metabolic heterogeneity among individuals. To capitalize on the mouse as a model system to understand genetic determinants of metabolism, eight inbred strains representing most of the genetic diversity in commonly used lab mice were intercrossed to create the diversity outbred (DO) stocks (Threadgill et al., 2011). Individual DO mice differ from each other by millions of single nucleotide polymorphisms, making it possible to use genetics to identify variants associated with metabolic features. More than 300 DO mice were phenotyped for over 3,000 plasma lipidomic features. Lipid abundance was then mapped to quantitative trait loci in the genome, de-orphaning many lipid species and identifying new aspects of regulation (Linke et al., 2020). In another study, pancreatic islets from DO mice were analyzed *ex vivo* for their ability to release insulin in response to secretagogues, revealing

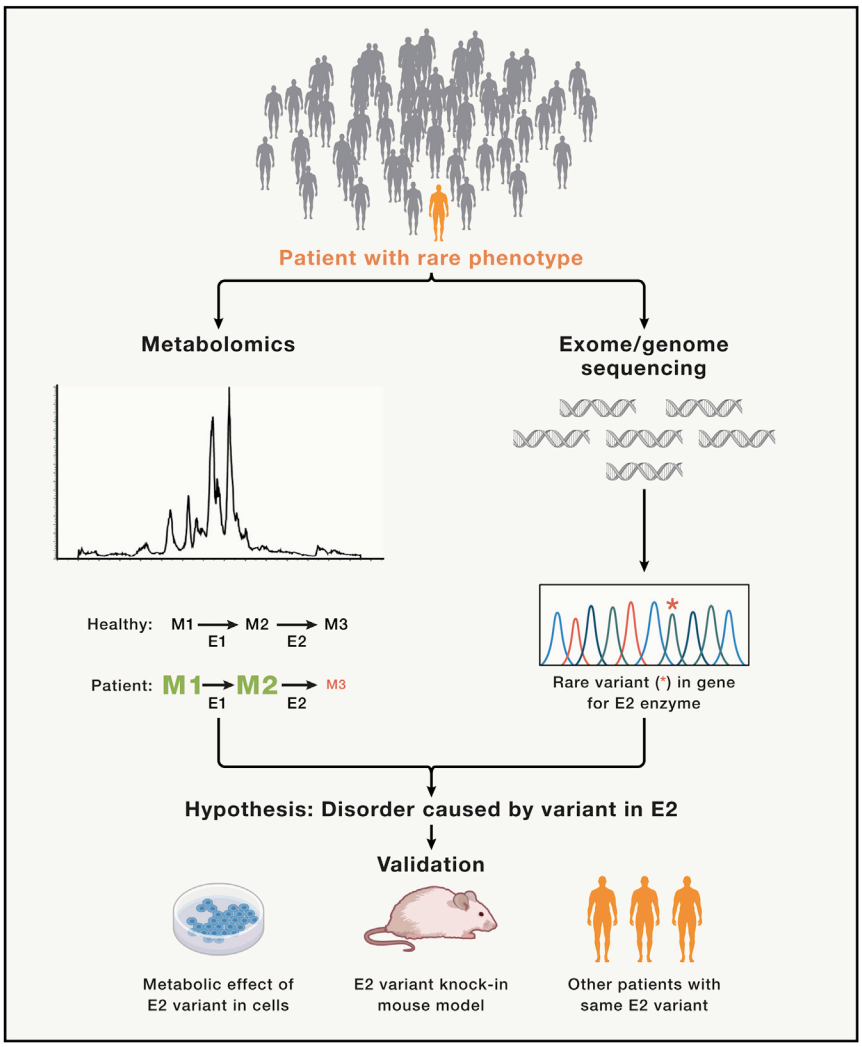

Figure 2. Integrating metabolomics and genomics to identify the molecular basis of metabolic anomalies

Broad metabolomic profiling provides an orthogonal approach to whole-exome and whole-genome sequencing in patients whose phenotypes make it difficult to pinpoint the responsible gene or pathway. This is valuable when sequence variants of uncertain significance coincide with metabolomic anomalies in a related pathway, increasing confidence that the sequence variants alter gene function. In the illustrated scenario, exome/genome sequencing reveals that the patient has a variant of uncertain significance in the gene encoding enzyme E2. In the patient's blood, accumulation of metabolites M1 and M2 upstream of E2 and depletion of metabolite M3 downstream of E2 imply that the variant impairs E2's function. This hypothesis could be validated by performing experiments to directly test the impact of the E2 variant in cells and mice and by identifying other patients with the same metabolomic-genomic findings.

several loci that contribute to variability in insulin secretion (Keller et al., 2019).

Similar approaches have been used in humans, capitalizing on natural variation across populations and combining genome-wide association studies with metabolomics. These efforts demonstrated that polymorphisms in genes encoding metabolic enzymes account for a substantial fraction of natural variation in metabolite abundance in plasma from healthy people (Gieger et al., 2008; Illig et al., 2010). Many effects are phenotypically silent beyond metabolite abundance, but some genotype-metabolite associations involve genes previously connected to common, multifactorial disorders like coronary artery disease, hypertension, and diabetes (Suhre et al., 2011).

The Dallas Heart Study (DHS), a large multiethnic population-based probability sample, has for 20 years used deep metabolic phenotyping and genome-wide association studies to detect variants accounting for disease-associated metabolic perturbations (Victor et al., 2004). This approach led to the discovery that loss-of-function variants in the gene encoding proprotein convertase subtilisin/kexin type 9 (*PCSK9*) are associated with reduced plasma low-density lipoprotein levels and protection against coronary heart disease (Cohen et al., 2006). This insight stimulated development of PCSK9 inhibitors, which are now used to treat refractory hypercholesterolemia (Sabatine et al., 2017). Another DHS advance was the association of variants in the gene encoding patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), which encodes a triacylglycerol lipase, with hepatic fat content and liver inflammation, hallmarks of nonalcoholic fatty liver disease (NAFLD) (Romeo et al., 2008). This discovery launched hundreds of studies investigating the mechanisms linking PNPLA3 to hepatic fat, and how to develop NAFLD therapies by targeting PNPLA3.

Metabolomics in rare human diseases

Metabolomics is increasingly used to characterize rare human phenotypes, particularly as whole-exome sequencing and whole-genome sequencing (WES, WGS) have become clinically available to diagnose Mendelian diseases. WES and WGS are unmatched in their ability to detect potentially pathogenic genomic variants. However, a challenge with sequence-first approaches is to interpret variants of uncertain significance—that is, sequence variants that differ from the consensus but with unknown effects on gene function. In this scenario, metabolomics provides a useful dimension of deep phenotyping. Conventional workup for inborn errors by clinical biochemical laboratories focuses on a few dozen metabolites, but modern metabolomics can report hundreds of metabolites at once. This makes it possible to observe rare patterns of metabolic anomalies in patients with nonspecific phenotypes and to relate these to rare genomic variants uncovered by WES/WGS (Figure 2).

In a recent example, we observed a pattern of plasma metabolic abnormalities that were difficult to ascribe to a defect in any single enzyme. WES revealed variants in *LIPT1*, which encodes the lipoyltransferase that adds a covalently bound cofactor to several 2-ketoacid dehydrogenases. The pattern of metabolomic abnormalities was then easily recognizable as a combined defect in lipoylation-dependent enzymes, and this was confirmed in functional assays in patient-derived cells (Ni et al., 2019). Metabolomic-assisted pathway screening (MAPS) is a clinical diagnostic test based on metabolomic profiling in plasma and envisioned as a companion to WES/WGS. In a series of 170 patients assessed by both WES and MAPS, the metabolomics data contributed to interpretation of genomic variants in over 40% of cases (Alaimo et al., 2020). Confirming the metabolic effects of sequence variants—or lack thereof—is of diagnostic value because it facilitates interpreting these variants in future patients with unexplained phenotypes.

Newborn screening identifies pre-symptomatic patients with treatable inborn errors and other diseases by testing all babies for diagnostic biomarkers (often metabolites). Because most disorders covered by these programs have a genetic basis, it is reasonable to compare DNA analysis to conventional metabolite detection in terms of diagnostic yield. A re-analysis of samples from over 1,000 babies with positive screens for inborn errors of metabolism found that biochemical analysis outperformed sequencing as a stand-alone test, both in sensitivity and in specificity (Adhikari et al., 2020). The DNA analysis' lack of sensitivity, largely caused by patients lacking variants that could be detected by WES, is problematic because the goal of newborn screening is to identify every baby eligible for treatment. Metabolomics in patients with known inborn errors could help uncover new biomarkers amenable to newborn screening. This would be useful in treatable diseases not included on newborn screens because they lack diagnostic biomarkers and in diseases that may become treatable in the future.

Overall, these examples of combining genomics with metabolic profiling have provided insights into the basis of heritable metabolic heterogeneity and in some cases led to new therapies for common metabolic diseases such as dyslipidemia. In the case of rare monogenic disorders, broad metabolomic profiling has improved the utility of clinical genomics, providing a deep phenotyping dimension that clarifies the significance of genomic variants.

Advance 4: Translational metabolic imaging

Although metabolic mechanisms can be readily teased out in experimental models, most analytical methods are destructive or require long periods of data acquisition, limiting feasibility in patients. New approaches to *in vivo* metabolic imaging have provided a means to leverage such metabolic changes to better understand human biology. These encompass approaches in nuclear medicine (herein predominantly positron emission tomography [PET]) and magnetic resonance that have the ability to resolve metabolic fluxes in humans.

Radioactive methods for metabolic imaging

Historically, PET has been the standard for probing human metabolism. From the onset of the most widely used tracer, ^{18}F-fluorodeoxyglucose (FDG), PET scans have been leveraged to infer changes in glucose metabolism (Ido et al., 1978), particularly in oncology and cardiology. Although the radioactive dose administered for a PET scan is quite low, it limits the number of PET scans that can be used in a given patient, particularly in children. Recent work has led to the development of whole body PET scanners (EXPLORER) (Badawi et al., 2019) that provide enhanced sensitivity and a 10-fold reduction in radioactive dose. The gain in signal strength can be utilized to acquire time-dependent data as opposed to the summation of accumulated ion counts in traditional PET. Moreover, with time varying signals throughout the body and improved sensitivity, one can better localize the signal using TOF PET imaging (Surti, 2015). Since localization of the radionuclide is a function of the accuracy of measuring coincident photons hitting the PET detector 180 degrees apart, TOF PET imaging leverages a highly accurate measurement of the timing of photon detection (on the order of picoseconds) to improve localization. Bringing these advances together and complementing them with new reconstruction approaches, TOF PET in the EXPLORER has the potential to fit kinetic rate models at the systems level across a wide range of tissues, promising total body measurements.

FDG PET detects radionuclide uptake and retention but is limited in its ability to resolve specific metabolic reactions, leading to ambiguity in its interpretation. FDG is "metabolically trapped" after phosphorylation by hexokinase (Gallagher et al., 1978) and does not proceed through glycolysis due to the lack of the C2 hydroxyl necessary for isomerization to fructose-6-phosphate (F6P). This results in accumulation as glucose-6-phosphate (G6P), further metabolism into the pentose phosphate pathway (PPP) or dephosphorylation and export. Recent work has focused on the development of new tracers able to probe other, potentially more specific metabolic reactions (e.g., ^{18}F-glutamine [Lieberman et al., 2011], ^{18}F-FACBC [Shoup et al., 1999], and ^{18}F-acetate [Ponde et al., 2007]). Understanding how these tracers accumulate is key for the interpretation of these images. For example, ^{18}F-glutamine is not metabolically converted to other products and informs on glutamine abundance rather than glutamine metabolism (Venneti et al., 2015). In the setting of glutaminase inhibition, ^{18}F-glutamine accumulates in response to on-target inhibition, suggesting that the pool size increases (Abu Aboud et al., 2017; Viswanath et al., 2021; Zhou et al., 2017). With the appropriate context, such readouts can be used to inform on the metabolic consequences of perturbation *in vivo* but critically require a full biochemical understanding of the probe's mechanism of action.

^{18}F analogs, although harboring fairly long lifetimes, are limited in that they do not exhibit the same metabolism as the natural substrates of metabolic reactions. For this reason, ^{13}N and ^{11}C analogs have been re-explored for use in metabolic imaging studies. For example, ^{11}C acetate has the potential not only to inform on fatty acid metabolism but also varying degrees of acetylation, now of increasing interest considering the tethering of epigenetics to metabolism (Comerford et al., 2014). Moreover, although this approach is under-utilized, radioisotope labeling at specific positions can further inform on flux selection to better interpret metabolic images. For example, incorporation of ^{11}C at the C1 position of lactate is retained in tissues with a large

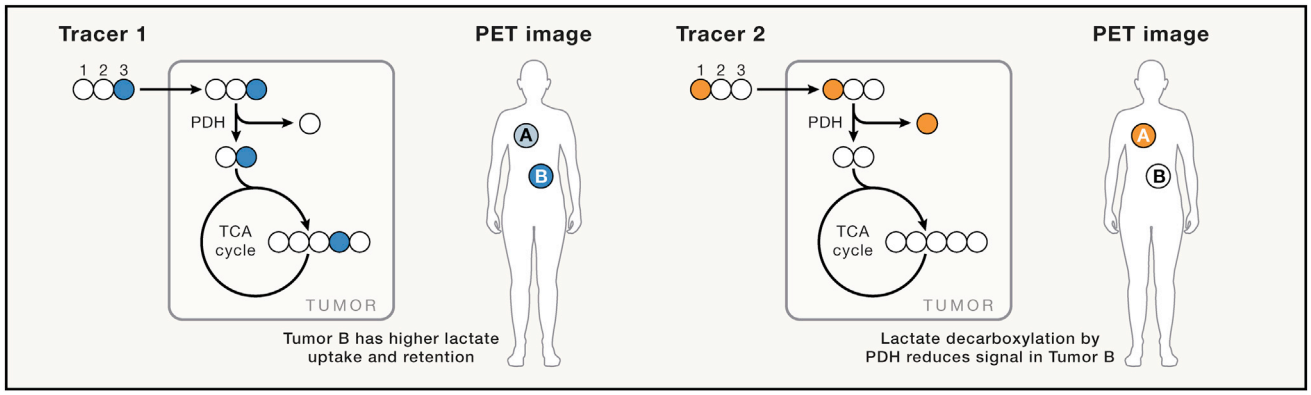

Figure 3. Isotopomer-selective *in vivo* PET imaging as a means to reveal differential metabolism

Many molecular imaging approaches are geared toward *in vivo* annotation of metabolism. Radioactive approaches, although extremely sensitive, suffer from the inability to discern specific metabolites. This can be circumvented by using isotopomers where the radionuclide is positioned at different locations within the molecule, designed to report different aspects of metabolic activity. In the example, lactate is labeled with ^{11}C in either C3 (Tracer 1, blue) or C1 (Tracer 2, orange) to illustrate the loss of $^{11}C1$ signal when PDH-mediated decarboxylation takes place, releasing $^{11}CO_2$. This would produce ^{11}C PET images of differential intensity at different locations in the same patient, e.g., in individual lesions suspected to be malignant. Blue lesions would be the highest intensity, and the relative intensity of the orange lesions would reflect the percent of the flux incorporated into the TCA cycle through PDH as opposed to total lactate oxidation.

lactate pool, but upon generation of pyruvate and subsequent metabolism through pyruvate dehydrogenase (PDH), it is lost as $^{11}CO_2$, heavily weighting the signal to lactate pool size. In contrast, incorporation of ^{11}C at the C3 position of lactate provides a means of incorporating lactate metabolism in the tricarboxylic acid (TCA) cycle as the label is retained beyond PDH (Figure 3). These approaches have the potential to provide high sensitivity for metabolic trapping *in vivo*, although their nuclear half-lives can be limiting (^{13}N $t_{1/2} \sim 10$ min and ^{11}C $t_{1/2} \sim 20.3$ min). This makes it essential that these tracers be radio-synthesized on site (Parent and McConathy, 2018). Ultimately, position matters and sophisticated chemical methods are needed to rapidly synthesize and purify such precursors.

Stable isotope methods for metabolic imaging

PET methods provide high sensitivity but relatively low specificity and spatial localization. MRI in contrast provides exquisite spatial resolution and specificity but lacks the sensitivity typically needed to quantify metabolic processes. Several MR methods pioneered in recent years have sought to address the growing need to study metabolism in humans. Stable isotope tracing is a versatile approach that provides information about metabolic activity *in vivo*. Recent efforts using stable isotope-labeled nutrients have revealed new aspects of disease-related metabolic activity, including the unexpected role of serine in hepatic lipogenesis in mice and the use of lactate as a fuel in aggressive human lung tumors (Faubert et al., 2017; Zhang et al., 2021). Conveniently, the same isotopes (^{2}H, ^{13}C, and ^{15}N) used for tracing are detectable using MRI and magnetic resonance spectroscopy (MRS). Although sensitivity is still limited for such tracing approaches, work in the setting of cancer as well as other diseases has demonstrated the ability to infuse isotopically labeled nutrients and follow their metabolic conversion non-invasively. For example, in the brain, fluxes from substrates such as ^{13}C-acetate and ^{13}C-glucose can be used to measure recycling rates of glutamine and glutamate (Boumezbeur et al., 2010; Lebon et al., 2002). This can be extended further to measuring TCA cycle flux and anapleorsis in human liver (Befroy et al., 2014). ^{2}H is transferred from ^{2}H-glucose to ^{2}H-lactate in human brain tumors, whereas ^{2}H-glx (a combination of glutamine, glutamate, and GSH) is a more prominent product in normal brain (De Feyter et al., 2018). Although these approaches provide a means of measuring a rate, like conventional MRS, they continue to suffer from both spatial resolution and time required to acquire such data due to low signal to noise ratio (SNR). Newer approaches combining stable isotope tracers with indirect ^{1}H MRS detection may be able to overcome this in small fields of view in the brain, although extending outside of the brain is extremely challenging (Rich et al., 2020). Long scan times, motion artifacts, and field inhomogeneities can severely limit SNR and make it challenging to extend these approaches to large fields of view. More work is needed to develop the MRI detection coils and pulse sequences required to overcome these limitations, although this is an emerging field of metabolic research that has promise.

By far, the most exciting advance in the area of imaging metabolic activity *in situ* with isotope tracers has been the development of hyperpolarized magnetic resonance imaging (Keshari and Wilson, 2014; Kurhanewicz et al., 2019). Hyperpolarization refers to a massive increase in the spin polarization of a target nucleus beyond its Boltzmann state for a given magnetic field. These hyperpolarized spins are detected using a conventional MRI system, enhancing the signal by thousands of fold. Although there are now a multitude of ways to generate hyperpolarized nuclei, the end goal of all such approaches has been to generate a hyperpolarized nutrient that can be infused into a living system to measure enzymatic conversion. Recent work has shown that such approaches can be leveraged to follow the metabolic conversion of many substrates including glucose (Rodrigues et al., 2014), fructose (Keshari et al., 2009), glutamine (Salamanca-Cardona et al., 2017), and most significantly pyruvate (Golman and Petersson, 2006; Nelson et al., 2013) in tumors. ^{13}C pyruvate labeled in the C1 position has proven capable of revealing fluxes

both through reduction to lactate and oxidation to CO_2 and further equilibration to bicarbonate *in vivo*, providing the potential to measure rate constants for these reactions at spatial resolutions that rival radionuclide approaches (Cunningham et al., 2016). Most notably, ^{13}C pyruvate reduction to lactate has been shown to correlate with cancer grade (Granlund et al., 2020), and oxidation to bicarbonate has provided exquisite maps of cardiac function with potential applications in heart disease. Other approaches are in the process of translation, including ^{13}C dehydroascorbate to image oxidative stress (Keshari et al., 2011) and ^{13}C fumarate to assess necrosis (Gallagher et al., 2009). The major limitation of HP MRI is the lifetime of the substrate and its products *in vivo*, currently on the order of a few minutes, thus restricting both the range of molecules that can be probed and the reaction kinetic lifetime that can be observed.

Taken together, these rapidly evolving nuclear and magnetic resonance approaches are approaching prime time. In the last 10 years, true breakthroughs in these techniques have provided new opportunities to study metabolic dimensions of disease. Stable isotope tracing using deuterium MRI in the brain has provided a means to annotate glycolytic utilization without the need for radioactivity (De Feyter et al., 2018). HP MRI has created a platform that can measure flux to a specific product *in vivo*, defining the source of the oncometabolite 2-HG (Salamanca-Cardona et al., 2017), connecting altered metabolic flux to loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in prostate cancer patients (Granlund et al., 2020), and uncovering control of LDH expression by FOXM1 in breast cancer (Ros et al., 2020).

Opportunities for future discovery

Building on a legacy of discovery that defined the human metabolic network, the technological and conceptual advances described above have given rise to new insights into the interface between metabolism and disease. These advances have occurred across scales, leveraging tools to study metabolism from the subcellular level all the way to human populations. There are many exciting opportunities for future discoveries across these scales as well, and a few are discussed below.

Localization of pathways to membrane-enclosed organelles like the mitochondria is a well-known component of metabolic regulation. However, there is increasing appreciation that some pathways are also associated into "metabolons," macromolecular complexes that are not otherwise sequestered from the rest of the cell. Organizing pathways in this manner is appealing because it implies several ways to regulate flux. The intimate co-localization of enzymes catalyzing sequential reactions could facilitate substrate channeling or increase pathway efficiency by eliminating the need for intermediates to diffuse from one enzyme to the next. Post-translational modifications could regulate protein inclusion or exclusion into these metabolons, thereby altering pathway activity. As an example, the purinosome is a biomolecular condensate of enzymes from *de novo* purine biosynthesis. It is formed through liquid-liquid phase separation in cultured cells and proposed to facilitate metabolite channeling (Pedley et al., 2022). We do not know the full set of functional mammalian metabolons, how they are regulated to match metabolic supply and demand, and which if any are relevant to disease. Research into biomolecular condensates and other mechanisms of subcellular localization contributing to metabolism may be aided by advanced cellular imaging methods to assess protein localization and macromolecular structures in live tissues, and new intracellular sensors that can visualize metabolite distribution.

Understanding how metabolism is regulated within tissues, particularly how metabolic interactions among functionally distinct cell types contribute to overall performance of the tissue, will require better resolution of metabolic and orthogonal properties *in situ*. MALDI and related MSI techniques already allow some metabolic features to be localized within tissue sections, but it is still difficult to assign features to specific cell types within complex tissues. Metabolic MSI would benefit from further method development to superimpose several spatially resolved features in the same sample, essentially multiplexing transcriptomic, proteomic, metabolomic, and possibly even isotope enrichment features with high-resolution histological data. In principle, this could provide information about metabolism, potentially at the single-cell level and assigned to individual cell types.

Finally, because identifying and quantifying metabolic mechanisms drives our understanding of disease, localizing metabolic perturbations in patients is key to our ability to leverage these findings to address disease. Rapid advances in clinical metabolic imaging have already begun, and we anticipate that new developments will allow us to illuminate pathways in an unprecedented way. Given the complementary nature of clinical imaging tools, e.g., metabolic MRI and PET, the fusion of these modalities with each other and the use of a broad range of metabolic probes could provide a holistic view of systems metabolism. Facilitating the interrogation of metabolism across scales, from organelles to patients, has the potential to transform how we approach research, diagnostics, and therapy in metabolic disease.

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AUTHOR CONTRIBUTIONS

R.J.D. and K.R.K. decided together on the topics most important to cover. They also wrote the paper and designed the figures.

DECLARATION OF INTERESTS

R.J.D. is a founder and advisor at Atavistik Bio and serves on the Scientific Advisory Boards of Agios Pharmaceuticlas, Vida Ventures, Droia Ventures, and Nirogy Therapeutics. K.R.K. is co-founder of Atish Technologies and serves on the Scientific Advisory Boards of NVision Imaging Technologies and Imaginostics. He holds patents related to imaging and leveraging cellular metabolism.

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