



Harnessing Metabolomics to Advance Epilepsy Research

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Abstract

Metabolomics is the laboratory analysis and scientific study of the metabolome—that is, the entire collection of small molecule chemicals in an organism. The metabolome represents the functional state of an organism and provides a multifaceted readout of the aggregate activity of endogenous (cellular) and exogenous (environmental) processes. In this review, we discuss how the integrative and dynamic properties of the metabolome create unique opportunities to study complex pathologies that evolve and oscillate over time, like epilepsy. We explain how the scientific progress and clinical applications of metabolomics remain hampered by biological and technical challenges, and we propose best practices to overcome these challenges so that metabolomics can be used in a rigorous and effective manner to further epilepsy research.

Keywords

biomarkers, chromatography, epileptogenesis, mass spectrometry, metabolomics, seizure forecasting

Introduction

The metabolome comprises the entire collection of small molecule chemicals in an organism or biological sample and includes various compounds like amino acids, organic acids, sugars, steroids, nucleotides, and lipids (Figure 1). The latter category reflects a subset of the metabolome known as the lipidome. Metabolomics or chemical profiling entails the laboratory analysis and scientific study of the metabolome.

The terms metabolome¹ and metabolomics² were not widely used until about 20 years ago, even though chemical profiling of biological samples by paper chromatography was underway since the late 1940s.^{3,4} Several technical innovations in automated analyzers, chromatography, and mass spectrometry in the 1970s dramatically expanded the analytical capacity of earlier methods, allowing for the rapid detection of numerous different chemicals in a biological sample.⁵ These innovations increased the efficiency of clinical laboratories, expanded newborn

screening programs for genetic disorders, and greatly facilitated detection of toxins and drugs in clinical and forensic settings. Motivated by the emerging fields of genomics and proteomics, scientists recognized the potential of these innovations and began promoting metabolomics as a “new” and powerful discipline in the “omics” field. The idea was that metabolomics can elucidate complex biological mechanisms, identify biomarkers of disease, and monitor therapeutic efficacy, in a more effective, high-throughput manner than previously possible.⁶⁻⁸

While metabolomics has been useful for a variety of clinical, forensic, and research scenarios, the scientific progress and clinical applications remain hampered by challenges related to the instability of the metabolome,^{9,10} the laboratory techniques,¹¹ and the analysis and interpretation of the data.¹²⁻¹⁴ This brief review will discuss these challenges and propose best practices to incorporate metabolomics in epilepsy research. The article will also highlight possible applications and new developments in the field.



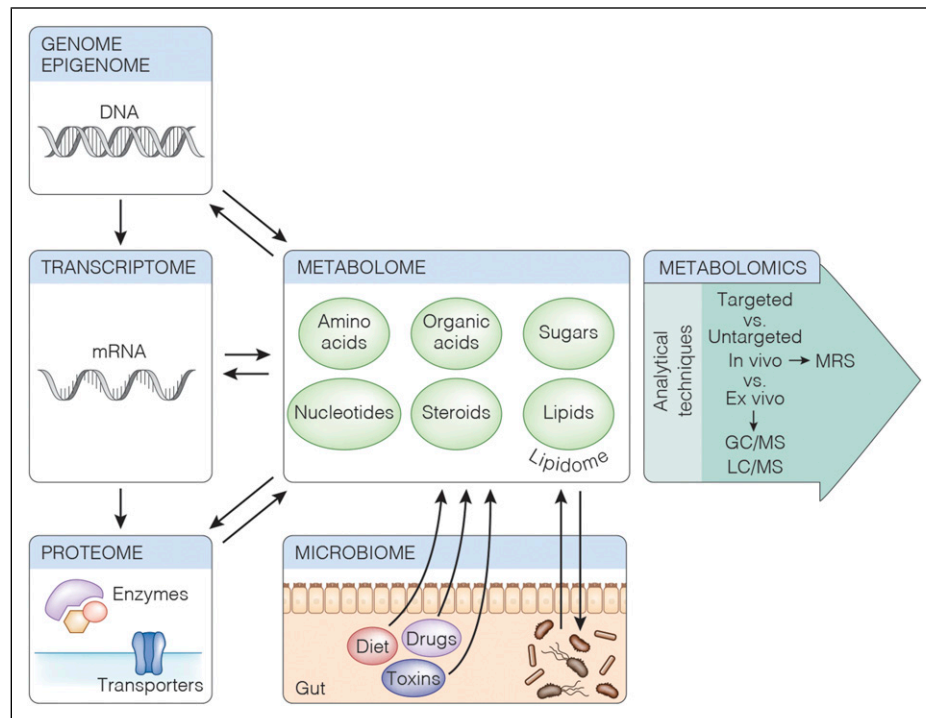


Figure 1. The metabolome integrates endogenous and exogenous events. The metabolome is the entire collection of small molecule chemicals in an organism, and the drawing illustrates how the metabolome (center) influences and is influenced by the genome and epigenome, the transcriptome, the proteome, the microbiome, and external factors such as the diet, drugs, and toxins. Metabolomics (right arrow) is the scientific study and laboratory analysis of the metabolome. Metabolomics can be done in a targeted or untargeted fashion, in living organs *in vivo* (using MRS), or in a biological sample *ex vivo* (using MRS, GC/MS, or LC/MS). See text for details. Abbreviations: GC/MS, gas chromatography coupled with mass spectrometry; LC/MS, liquid chromatography coupled with mass spectrometry; MRS, magnetic resonance spectroscopy. Art credit: Katie Vicari.

Metabolomics Is Well-Suited for Epilepsy Studies

The metabolome represents the aggregate chemical output from multiple internal and external factors affecting the body (e.g., genes, proteins, diet, microbiota, drugs, and toxins; Figure 1). The individual variability in these factors reflects the metabolic phenotype for each person, and fluctuates in response to physiological or pathological events, such as nutrition,¹⁵ biological rhythms,¹⁶ gut microbial changes,¹⁷ or infection and inflammation.¹⁸ Below, we discuss how the integrative and dynamic properties of the metabolome create a unique approach to study complex pathologies that evolve and oscillate over time, like epilepsy.

Certain brain insults, such as prolonged seizures or head trauma, can lead to epilepsy in some, but not all people.¹⁹ The disparate outcomes suggest that epileptogenesis is a multifactorial process that requires a “perfect storm” of genetic, physiological, and environmental events. A biomarker that can be used clinically to diagnose and track the epileptogenic process will significantly advance patient care, research, and novel therapeutic strategies.²⁰ While no such biomarker is available for human use, we posit that repetitive, prospective measurements of the metabolome after an epileptogenic insult could lead to the discovery of relevant markers. Since effective interventions are limited, in-depth knowledge about metabolomic changes during epileptogenesis could provide new insights into the pathophysiological mechanisms and seed the development of effective antiepileptogenic strategies.

Thus, far, only a small number of studies have used metabolomics to investigate epileptogenesis.^{21–24} Heischmann et al²² explored the hippocampal and plasma metabolome in the rat kainic acid model of mesial temporal lobe epilepsy (MTLE) at different time points during epileptogenesis. The authors found that disease development was associated with perturbations in vitamin D3 metabolism, which is important for mitochondrial function, red-ox processes, and immune modulation. They also reported changes in lipids, adenosine, citrulline, bile acids, and steroids. A recent study by Alqurashi et al used the mouse kainic acid and pilocarpine models of MTLE combined with analysis of mRNA, proteins, and metabolites in the hippocampus. They found enhanced aerobic glycolysis (the Warburg phenomenon) in the epilepsy hippocampus along with increased signaling through the Wnt, the 5' adenosine monophosphate-activated protein kinase (AMPK), and the mammalian target of rapamycin (mTOR) pathways.²⁵ The above publications support the notions from other studies that perturbations involving energy metabolism,²⁶ red-ox homeostasis,²⁷ and signaling molecules (e.g., adenosine)²⁸ may play important roles in epileptogenesis.

Patients with drug resistant epilepsy live in constant fear of when their next seizure will occur,²⁹ and the field requires more effective approaches that can forecast and prevent spontaneous seizures.³⁰ Although our mechanistic insight into spontaneous seizure triggers remains limited, studies suggest that chemical changes likely contribute. Using continuous brain chemistry

sampling from humans with focal epilepsies and animal models, extracellular concentrations of several endogenous compounds, such as glutamate,³¹ isoleucine,³² γ -aminobutyric acid (GABA),³³ lactate,³⁴ and adenosine,³⁵ were found to change in relation to a seizure. Moreover, rapid fluctuations in serum chemicals like ammonia³⁶ and sodium³⁷ can trigger seizures. We propose that frequent monitoring of the metabolome at the seizure focus *in vivo* or in readily accessible biological samples collected during the pre-seizure period will result in additional biomarkers. These markers may be used to forecast seizures prior to onset and permit “on demand” preventative therapeutic strategies involving antiseizure medications or brain stimulation approaches.

There are many other possible applications of metabolomics in epilepsy. As a precision medicine approach, a patient’s metabolome tracked over time could reveal a personalized, predictive biomarker of therapy efficiency.³⁸ Somewhat related to this application, Olson et al³⁹ showed that the gut microbiota drove responses to a ketogenic diet and led to changes in the brain metabolome (e.g., glutamate and GABA) in a mouse model of epilepsy. Other studies have used metabolomics to explore the roles of the ketogenic diet⁴⁰ and inflammation⁴¹ in the pathogenesis of epilepsy.

Pros and Cons in Metabolomics Research

The dominant methods for metabolomic research are liquid or gas chromatography combined with mass spectrometry (LC/MS or GC/MS)⁴² and magnetic resonance spectroscopy (MRS, Figure 1).⁴³ *Ex vivo* analysis of biological samples heavily relies on LC/MS and GC/MS, given their high sensitivity and specificity, high throughput, low cost per reported metabolite, and widespread availability. While MRS has significant drawbacks (low sensitivity, low throughput, cost-prohibitive, and limited availability), it can provide spatial mapping of the metabolome in live organs *in vivo*. This is beneficial for regions that cannot be readily sampled for *ex vivo* measurements, such as the brain. The method has been used to map brain chemistry changes in patients with different types of epilepsy, mostly emphasizing high-abundance chemicals such as glutamate, glutamine, N-acetyl aspartate (NAA), phosphocreatine, and myo-inositol. Many of these studies find evidence of impaired energy metabolism in the seizure onset zone of the brain.²⁶

Additionally, MRS has advantages for metabolic flux analysis. Petroff et al infused ¹³C glucose to patients who were treated with hippocampal resection for refractory MTLE and measured the incorporation of ¹³C in different brain metabolites using *ex vivo* MRS. The authors found that sclerotic hippocampi were characterized by reduced glutamate-glutamine cycling, decreased glutamine content, and a relative increase in glutamate levels.⁴⁴ Later studies have suggested that a loss of astroglial glutamine synthetase explains the reduced glutamate-glutamine cycling in MTLE and that the enzyme deficiency is implicated in the pathogenesis of MTLE.⁴⁴⁻⁴⁷

While mass spectrometry- and MRS-based methods are well-suited to study the metabolome, caution is warranted to ensure accuracy, reproducibility, and scientific validity of the results, as discussed below.

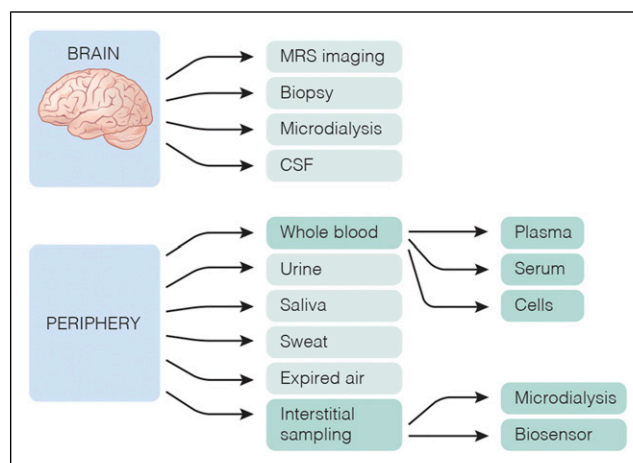


Figure 2. Sampling of the metabolome. The brain metabolome can be interrogated by *in vivo* MRS imaging, brain biopsy, brain microdialysis, and CSF collection. Many other sample types can be used, including blood, urine, saliva, sweat, expired air, and interstitial (subcutaneous) fluid. Each sample type has distinct strengths and limitations and requires an informed choice for the best experimental design. Sample collection and processing are critical steps that require careful planning and execution to avoid erroneous results. Art credit: Katie Vicari.

Study Design and Preanalytical Issues

Careful research design is critical for metabolomics studies. Several components of the metabolome fluctuate rapidly and markedly in response to biological and environmental factors,^{9,10} and studies of these fluctuations may be a critical part of the experiment. However, some of these fluctuations may be unwanted and result in experimental noise. Factors known to influence the metabolome include age,⁴⁸ sex,⁴⁹ genotype,² medications,⁵⁰ diet,¹⁵ time of day,¹⁶ estrous cycle,⁴⁹ and stress⁵¹ (Figure 1). The effects can be substantial and sometimes greatly exceed the variation caused by the analytical method. Controlling these factors requires great care and involves matching of study subjects and standardization of sample collection. Statistical corrections of confounders may also occur during data analysis; however, this approach may require a larger sample size.

The site, type, and collection time of the biological sample are another important consideration (Figure 2). Ideal samples for epilepsy studies may derive from a tissue biopsy at the site of seizure onset and a sample from the same brain region of a “perfectly matched” healthy control. Unless laboratory animals are used, such samples are difficult to obtain, and even then, the specimen only reflects a “snapshot” of the metabolome at the time of biopsy. Longitudinal sampling can utilize brain microdialysis, cerebrospinal fluid, serum/plasma, urine, saliva, expired air, or sweat (Figure 2). Each sample type has distinct strengths and limitations and requires an informed choice for the best experimental design.

It is often assumed that analysis of peripheral samples has limited utility for brain disorders; however, many chemicals effectively cross the blood brain barrier (BBB) via various transporter systems for polar metabolites and simple diffusion for lipid soluble compounds. Concentration changes in many peripheral metabolites are therefore likely to be followed by

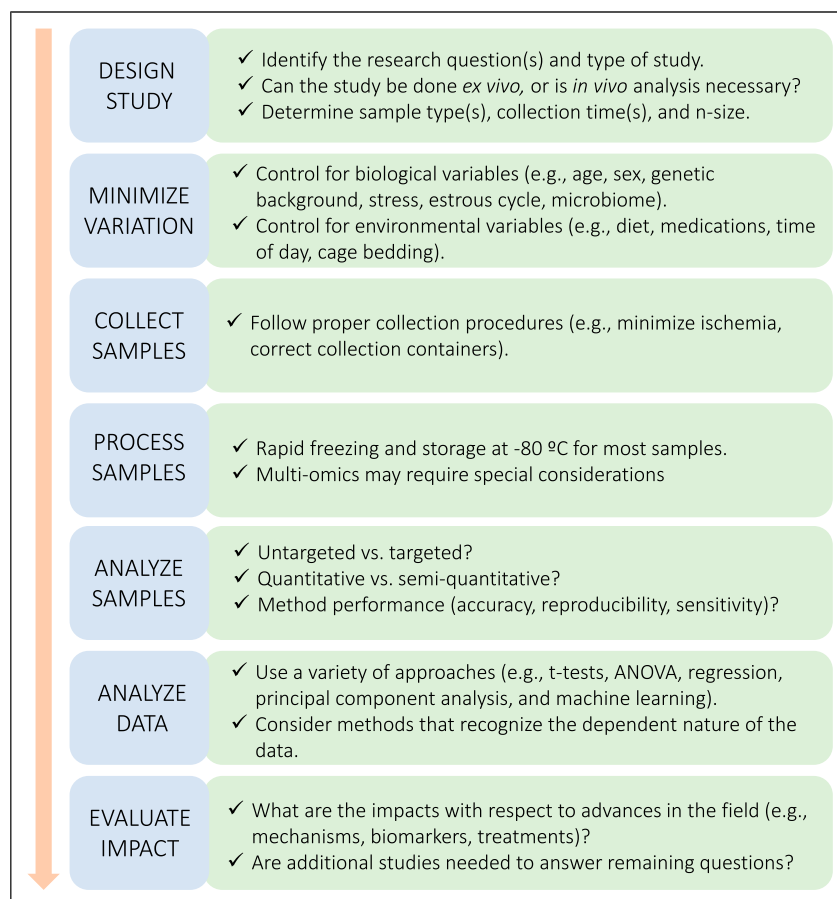


Figure 3. Planning and execution of metabolomics studies. Successful studies of the metabolome require careful planning and consideration of issues related to unwanted preanalytical variables, the performance of the laboratory methods, and the analysis and interpretation of the data. The diagram is meant to help investigators in this process by providing a series of considerations and best practices for such studies.

corresponding changes in the brain—and vice versa. Studies in patients with Alzheimer’s disease and relevant animal models have identified numerous metabolomic alterations in both the blood and brain,⁵² and peripheral samples, such as blood,⁵³ sweat,⁵⁴ and urine,⁵⁵ are increasingly used in metabolomic studies of epilepsy.


Sample collection and processing remain tantamount.⁵⁶ Brief periods of ischemia during tissue biopsy or blood collection can change the concentration of many metabolites.⁵⁷ Approaches such as focal brain microwave irradiation (in laboratory animals),⁵⁸ and minimal use of tourniquet during phlebotomy, can minimize the ischemic effects. Additionally, chemical reactions may continue to occur after sample collection.⁵⁶ Thus, all collected samples, except for whole blood, generally require rapid freezing and storage at -80°C . Whole blood should remain at room temperature after collection and centrifuged as soon as possible, followed by immediate transfer of serum/plasma to -80°C .⁵⁶ Other factors that are known to artifactually change the metabolome include the choice of anticoagulant in blood collection tubes and *ex vivo* hemolysis due to improper collection and specimen handling techniques.⁵⁶

Laboratory Analysis

While the genome can be mapped by one analytical technique, no single approach can fully chart the metabolome. The metabolome is distributed in a heterogenous fashion throughout the body and is composed of several thousand chemicals with varying physio-chemical properties and concentrations, ranging from less than 0.000,000,001 mmol/L to over 1 mmol/L. Several different methods are therefore required to comprehensively analyze the metabolome.

Metabolomic approaches fall into two categories: untargeted and targeted, each with their own pros and cons.⁴² Untargeted approaches usually detect several hundred chemicals in a single “run,” which can best serve exploratory studies. However, the chemical identity is sometimes unknown and requires considerable time and effort to be resolved. It may also be difficult to provide accurate quantitative data. Targeted approaches can detect fewer metabolites; however, the chemical identities are known, and accurate quantitation is feasible.

Another issue is the performance of the analytical method, that is, its accuracy, reproducibility, and sensitivity.⁵⁹ Accuracy refers to the closeness of a measured value to a standard or



known value. Reproducibility is the capability of producing the same results when the same sample is tested on different times or by different laboratories. Sensitivity refers to the lowest concentration the method can reliably detect a given chemical. While these parameters are critical in clinical and diagnostic settings and regulated through the Clinical Laboratory Improvement Amendments Act (CLIA88) in the US, we recommend that these parameters are considered for research. The National Institutes of Health have several initiatives to enhance rigor and reproducibility in scientific research, underscoring the need for accurate and reproducible laboratory methods.

Data Analysis

Metabolomics is a “big-data” discipline that poses unique challenges for data processing and interpretation. Various analytical approaches can be employed, such as principal component analysis that reduces the multiple dimensions of the data for analysis and visualization; t-tests, analysis of variance (ANOVA), and regression analysis, all of which investigate the association between metabolites and variables of interest; and different machine learning algorithms for clustering and classification.^{60,61} Many statistical tests assume that each variable is independent. However, this assumption does not universally apply to the metabolome, which is highly interconnected through enzymatic reactions and transport mechanisms. The significance criteria for many statistical tests may therefore be too conservative for metabolomic analysis, and alternative methods that recognize the dependent nature of the data should also be considered.⁶²

Another critical step is to assess the biological and translational relevance of the results. Many metabolomics studies yield large amounts of “statistically significant” data; however, some of these findings may not be biologically or translationally important. For example, a statistically significant change in the concentration of a metabolite may not be large enough to significantly contribute to a disease process because of effective compensatory (homeostatic) mechanisms, and a potential biomarker of epileptogenesis or spontaneous seizures may not be useful in a clinical setting due to poor sensitivity/specificity, or long result turnaround time, respectively. A great deal of intellectual effort may be required to establish the biological and translational relevance of the results, and supplementary studies are often necessary. Animal experiments using targeted approaches (e.g., knockouts or overexpression of specific enzymes, transporters, or receptors) may be done to establish the relevance of specific metabolites in disease mechanisms, and clinical trials are usually necessary to determine the benefits of novel biomarkers for patient care.

Future Directions and Conclusion

While MRS and MS-based methods remain the mainstay of *in vivo* and *ex vivo* metabolomics, several alternative approaches exist, each with their unique set of impactful applications.

- *Multi-omics studies.* By careful planning and stringent sample processing procedures, it is possible to perform

several different omics experiments on the same sample. A protocol for lipidomic and transcriptomic analysis of brain punches from mice with kainic acid induced seizures was published by Lerner et al.⁶³

- *Imaging mass spectrometry.* Using various MS-based methods, multi-omics studies, including metabolomics, can be carried out on tissue sections at micrometer resolution.⁶⁴ Ajith et al⁶⁵ used desorption electrospray ionization MS imaging on 15 μm thick, frozen brain sections from patients with different types of focal epilepsies and found changes in phosphatidylcholine and phosphatidylethanolamine levels among different patient categories.
- *Implantable or external biosensors.* Continual advances in miniaturized chemical sensing technologies are expected to expand the *in vivo* monitoring capabilities for several metabolites present in interstitial fluids, saliva, sweat, and expired air, allowing for continuous or intermittent tracking of biomarkers over time.⁶⁶ Such sensors are already approved for glucose monitoring in patients with diabetes,⁶⁷ and handheld breathalyzer devices for acetone detection may be used to monitor people on a ketogenic diet.⁶⁸

In conclusion, we propose that metabolomics is well-suited to conduct studies of epilepsy. However, successful studies require careful planning and consideration of issues related to unwanted preanalytical variables, the performance of the laboratory methods, and properly incorporating “big data” statistical approaches. To help investigators in this process and to advance the field, we provide a checklist of considerations and best practices for metabolomics of biological samples from humans and laboratory animals (Figure 3).

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