Received: 4 May 2021 | Revised: 21 June 2021 | Accepted: 9 July 2021

DOI: 10.1002/nbm.4594

SPECIAL ISSUE REVIEW ARTICLE

NMR IN BIOMEDICINE WILEY

Closing the gap between in vivo and in vitro omics: using QA/QC to strengthen ex vivo NMR metabolomics

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Funding information
Nebraska Center for Integrated Biomolecular Communication through NIGMS, National Institutes of Health, Grant/Award Number: P20 GM113126; National Science Foundation, Grant/Award Number: 1660921

Metabolomics aims to achieve a global quantitation of the pool of metabolites within a biological system. Importantly, metabolite concentrations serve as a sensitive marker of both genomic and phenotypic changes in response to both internal and external stimuli. NMR spectroscopy greatly aids in the understanding of both in vitro and in vivo physiological systems and in the identification of diagnostic and therapeutic biomarkers. Accordingly, NMR is widely utilized in metabolomics and fluxomics studies due to its limited requirements for sample preparation and chromatography, its non-destructive and quantitative nature, its utility in the structural elucidation of unknown compounds, and, importantly, its versatility in the analysis of in vitro, in vivo, and ex vivo samples. This review provides an overview of the strengths and limitations of in vitro and in vivo experiments for translational research and discusses how ex vivo studies may overcome these weaknesses to facilitate the extrapolation of in vitro insights to an in vivo system. The application of NMR-based metabolomics to ex vivo samples, tissues, and biofluids can provide essential information that is close to a living system (in vivo) with sensitivity and resolution comparable to those of in vitro studies. The success of this extrapolation process is critically dependent on high-quality and reproducible data. Thus, the incorporation of robust quality assurance and quality control checks into the experimental design and execution of NMR-based metabolomics experiments will ensure the successful extrapolation of ex vivo studies to benefit translational medicine.

KEYWORDS
biomarkers, ex vivo, HR-MAS, metabolomics, NMR, quality assurance, quality control, translational medicine

1 | INTRODUCTION

Metabolite profiling (metabolomics) is a powerful tool that can aid in our understanding of human diseases and may help in identifying potential diagnostic and therapeutic biomarkers to improve disease diagnosis and prognosis.[1–4] All living organisms are constantly influenced by their local environment and health status, and will readily adjust to any disease state, nutrient limitation or environmental stressor through a rapid metabolic response. Thus, we may be able to better understand the biochemical or cellular processes being impacted by a disease state or environmental perturbation by tracing these metabolite changes.[5,6]

Abbreviations: 1D, one dimensional; 2D, two dimensional; EBM, evidence-based medicine; GC-MS, gas chromatography-mass spectrometry; HR-MAS, high-resolution magic-angle spinning; LC-MS, liquid chromatography-mass spectrometry; MIME, minimum information about metabolomics experiment; MS, mass spectrometry; NAD^+, nicotinamide adenine dinucleotide; PCA, principal component analysis; PLS-DA, projections to latent structures-discriminant analysis; PQC, pooled quality control; QA, quality assurance; QC, quality control; RCC, renal cell carcinoma; TG, triglyceride; TOCSY, total correlation spectroscopy.

Biological samples can be handled and processed in fundamentally distinct manners that will inevitability impact the outcome of any study and, consequently, determine which metabolites are still present and detectable. A metabolomics investigation may employ any combination of in vitro, in vivo, or ex vivo samples. For in vitro samples, the metabolites are typically solvent extracted from lysed cell lines, but the analysis of metabolites from whole live cells, often referred to as cellular metabolomics, is also routinely accomplished. Conversely, an in vivo analysis requires detection of metabolites from an intact, living organism. Ex vivo samples lie in between in vitro and in vivo samples and solve many of the problems of both approaches. In ex vivo studies, metabolites are detected from tissues, organs or biofluids (eg blood, urine, etc) that are removed from a living organism and can be analyzed "as is" with minimal treatment, or the metabolites can be solvent extracted from homogenized tissues or organs. Not surprisingly, each of these sample processing methods has inherent strengths and weaknesses, and can be conducted either independently or in combination to take advantage of complementary information. Notably, in vitro and ex vivo samples can be characterized using either NMR or mass spectrometry (MS). However, in vivo samples can only be analyzed using NMR. Despite the valuable information that can be obtained from metabolomics experiments, appropriate quality control (QC) and quality assurance (QA) checks are also needed to validate the biological results. In this review, the importance of QA/QC checks and validation tools to metabolomics will be discussed along with a brief comparison of metabolomics approaches, with a particular focus on the emerging field of ex vivo NMR metabolomics. The conversion of metabolic insights from bench-side observations to bedside treatments is currently confounded by the limitations of in vitro samples and the challenges of in vivo experiments. In this manner, ex vivo NMR may bridge the gap between in vitro and in vivo studies while also facilitating translational medicine.

2 | METABOLOMICS—A BRIEF PRIMER

Metabolites are low-molecular-weight organic and inorganic biochemicals that serve as reactants, intermediates or end-products of biochemical reactions. Metabolites are the basic building blocks of all biochemical and cellular components (Figure 1). Moreover, metabolites are routinely involved in signaling pathways and are used to regulate numerous cellular processes. Metabolites are a chemically diverse set of compounds due to their various and unique physicochemical properties, which include differing structural scaffolds and functional groups, and a wide range of molecular weights, hydrophobicity, hydrophilicity, acidity, basicity, stability, and volatility, among other characteristics.[7] The entire collection of metabolites from a defined biological system is referred to as the metabolome. Metabolomics is the area of science that studies the metabolome.

The metabolome can be specific to a cell line, tissue, or organ (e.g., HeLa cells, plasma or gut metabolome, etc) or it can refer to an entire biological system or organism (eg *Staphylococcus aureus*, yeast or human metabolome, etc).[8,9] In general, the size and complexity of the metabolome is dependent on the biological source. For example, *Saccharomyces cerevisiae* is estimated to contain only 1100 metabolites.[10] In contrast, the human metabolome is predicted to contain upwards of 150 000 metabolites,[8,11] not including xenobiotics (e.g., drugs, pharmaceutical medications, etc.) or persistent organic pollutants (eg poly/perfluoroalkyl substances, pesticides, etc). Consequently, the large diversity in both the

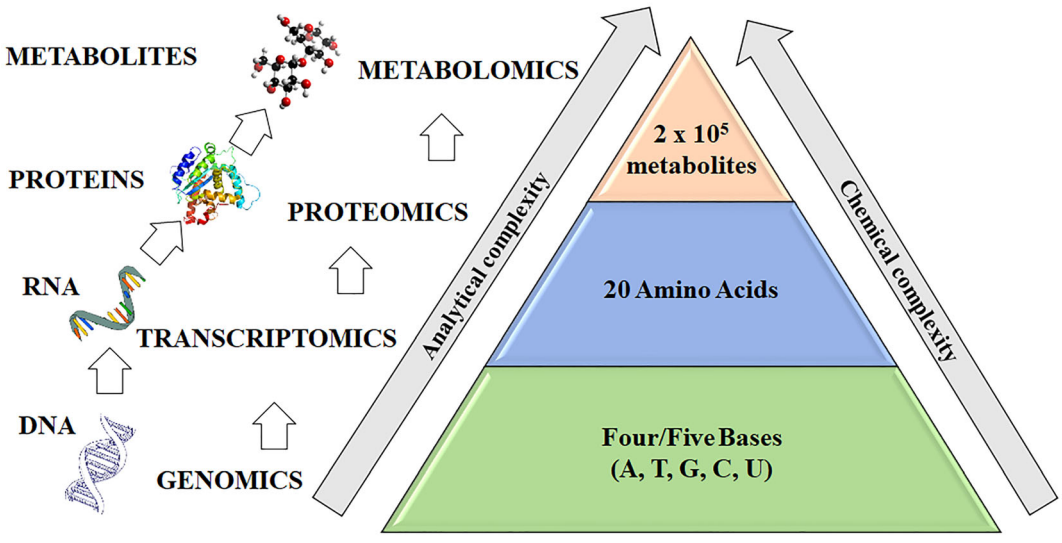

FIGURE 1 Schematic diagram of the central dogma of molecular biology. The transfer of information from DNA to the synthesis of metabolites leads to an overall rise in the complexity of the biological molecules involved in the process. Images sourced under Fair Use policy of pngtree

number and chemical characteristics of a metabolome makes metabolomics technically challenging. Metabolomics aspires to investigate the physicochemical, dynamics, functions, and interactions of *all* the metabolites in an entire biological system.[12,13] This ambitious goal is seldom achieved. Instead, metabolomics typically identifies and/or quantifies only a subset of the metabolome, which comprises the metabolites detectable by a particular analytical source.[14–16]

Metabolomics is conceptually downstream of the other traditional omics techniques (ie genomics, transcriptomics, and proteomics). Simplistically, traditional omics studies may be viewed as monitoring how changes in only four or five nucleotide bases or 20 amino acids impact a tissue, cell, or subcellular compartments. In contrast, metabolomics aims to achieve a precise picture of the entire cellular system by examining changes in over 2×10^5 metabolites (conservative estimate) (Figure 1). In this regard, perturbations in the genome, transcriptome, or proteome represent possible changes to cellular processes, but alterations in the metabolome identify changes in metabolic or signaling pathways that have occurred. Moreover, the metabolome is able to rapidly respond to and adapt to changing environmental conditions. Thus, the metabolome is more prone to be impacted or swayed by external factors (Figure 2).

While traditional omics analyses can be reliably accomplished with a single analytical platform, metabolomics commonly requires multiple analytical methods because of the physicochemical diversity of the metabolites.[14–16] As a result, a single analytical technique cannot capture the entirety of the metabolites present in the biological sample.[17–20] Currently, MS coupled with chromatography or NMR spectroscopy are the preferred analytical methods for metabolomics experiments.[21–26] MS is currently the preferred choice for conducting metabolomics studies due to its superior sensitivity, dynamic range, and resolution. Nevertheless, NMR has several unparalleled advantages. NMR is a non-destructive technique that requires little to no sample processing prior to data collection. Moreover, NMR provides a quick and robust mechanism for the accurate and reliable identification of known metabolites and methods for determining the structures of unknown compounds. Also, NMR spectral data are highly reproducible across different instruments, laboratories, users, or times. NMR has a significantly higher throughput, where one-dimensional (1D) ^{1}H NMR spectra are routinely collected in seconds or a few minutes; whereas a liquid chromatography (LC)-MS experiment may require upwards of 30 min to acquire a single spectrum. Of course, the experimental time is doubled if mass spectra are collected in both the positive and negative mode. This time sink may be resource or financially prohibitive for datasets containing thousands of samples. Recent technical advancements in material chemistry, magnet research, and probe design have ushered in ultra-high-field magnets (1.2 GHz), and cryogenically cooled small-volume microprobes (30 μL). These achievements have pushed the sensitivity and limits of detection obtainable by NMR closer to

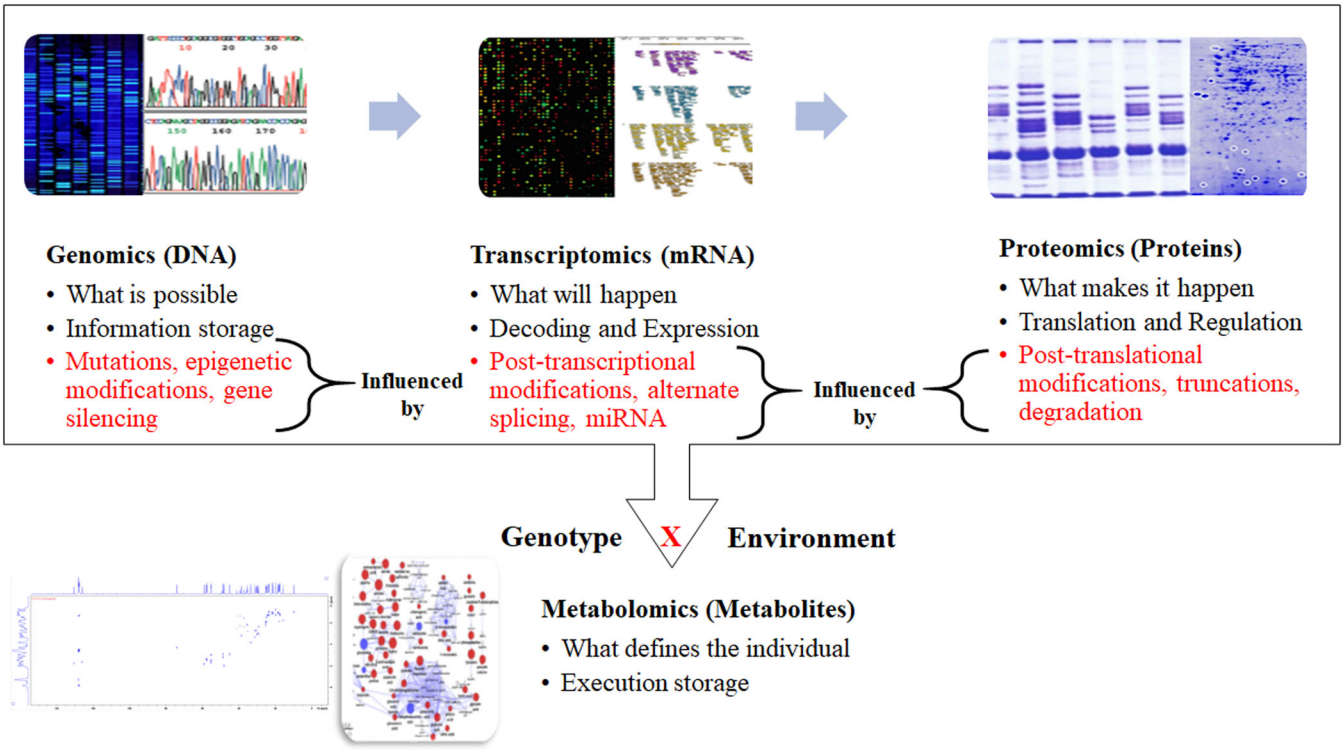

FIGURE 2 Schematic diagram of the flow of information and the role and complexity of data obtainable from the various "omics" techniques. The traditional omics techniques of genomics, transcriptomics, and proteomics provide information about the processes that are programmed to happen while metabolomics provides a definite and real-time representation of the output of these genetic and proteomic activities. The metabolome represents the response to a variety of internal (ie genetic mutations) and external (ie nutrient limitation or drug treatment) stimuli

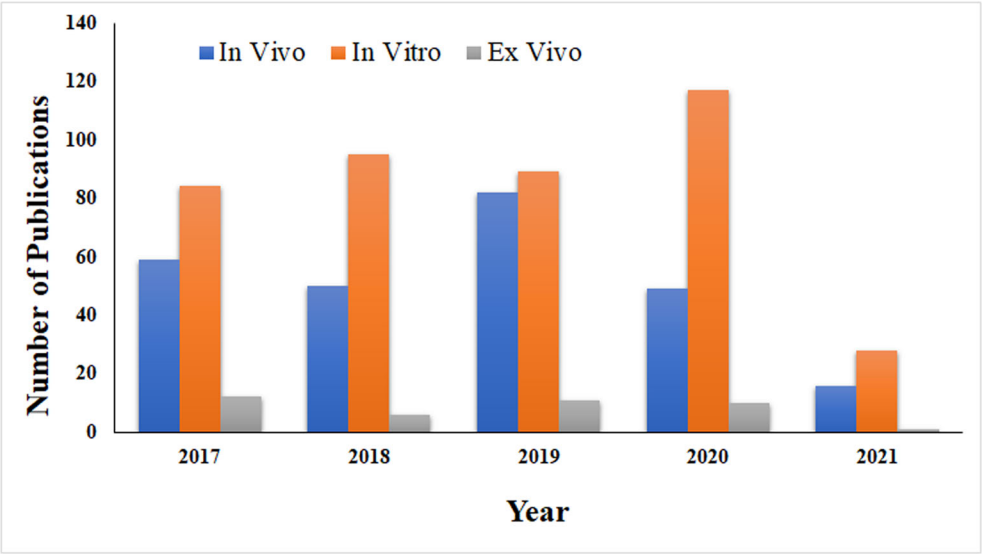

FIGURE 3 Number of manuscripts published between 2017 and 2021 that covered the topics of in vivo, in vitro, or ex vivo NMR metabolomics. The list of publications was curated from the Web of Knowledge (Clarivate Analytics; http://apps.webofknowledge.com) with the following keyword searches: "In vivo" and "NMR" and "Metabolomics", "In vitro" and "NMR" and "Metabolomics", and "Ex vivo" and "NMR" and "Metabolomics

those of MS. Taken together, NMR has been an important metabolomics platform for in vivo, in vitro, and ex vivo analysis, which can be reproducibly translated from bench-side to bedside applications (Figure 3).

3 | EX VIVO METABOLOMICS: BRINGING SCIENCE FROM BENCH-SIDE (IN VITRO) TO BEDSIDE (IN VIVO)

In vivo NMR is a powerful technique that allows for the study of naturally abundant small molecules and metabolites present in an intact living organism. In this regard, in vivo NMR enables the real-time monitoring of these metabolites while requiring minimal sample preparation, which allows for an uninterrupted data collection and the capturing of metabolic fluxes. Solution-state in vivo NMR-based metabolomics is a relatively new approach in understanding metabolic flux and serves as a promising complementary tool to the established in vitro approaches. The earliest studies on intact living organisms relied on high-resolution magic-angle spinning (HR-MAS) NMR spectroscopy,[27–29] where the organisms were spun at ~2.5 kHz at the magic angle.[30,31] However, this high-speed spinning exerts additional stress on the organisms, which may alter the metabolome and introduce stress metabolites that may confound the results. Moreover, HR-MAS techniques are not amenable to coupling with a flow system and will restrict the availability of sufficient oxygen, food, and water to the organism. Solution-state in vivo NMR-based metabolomics overcomes these limitations and may be optimized to accommodate essential living conditions.[32] However, in vivo NMR is plagued by limited resolution and relatively low sensitivity due to dipolar anisotropy, chemical shift anisotropy, and magnetic susceptibility anisotropy. In vivo metabolomics samples of intact living organisms adversely affect the homogeneity of the magnetic field. Simply, variable magnetic susceptibility across each organism and across the entire sample (ie solvent and solvent-organism interfaces) distorts the local magnetic field, resulting in broad NMR resonances. A living organism is biologically compartmentalized (eg brain, lungs, heart, etc), with each having different magnetic susceptibilities.[33] Therefore, the same metabolite but in a different organ compartment would interact with a slightly different local magnetic field, leading to variable chemical shifts and linewidths and resulting in an overall broadening of the NMR spectrum.[34] The loss in field homogeneity due to the inherent heterogeneity of an NMR tube containing a living organism will also contribute to poor shimming and further peak broadening. Similarly, the lack of random isotropic tumbling due to the constrained nature of fitting an organism in an NMR tube will lead to dipolar and chemical shift anisotropy and further spectral broadening.

Since almost all living organisms are made up of more than 50% water, the need to suppress the abundant water signal in a 1D ^{1}H NMR spectrum presents a further challenge to in vivo NMR. Hence, most in vivo NMR metabolomics studies have been restricted to heteronuclear NMR experiments (eg ^{31}P NMR).[35–37]

Another limitation with in vivo metabolomics is the size of the organism that can be studied in an NMR magnet with a standard 54 mm bore size. Small organisms, such as *Daphnia magna*, with diameters less than 10 mm can be accommodated inside standard NMR tubes using a larger-

diameter (ie 10 mm) cryoprobe.[38,39] Again, the low spectral resolution due to line broadening makes assigning individual metabolites particularly challenging, leading to limited metabolite identification. For instance, NMR resonances from individual metabolites, such as amino acids and carbohydrates, are mostly obscured by peak overlap with highly abundant lipids. This limitation may be partly overcome by employing additional two-dimensional (2D) NMR experiments, such as a two-dimensional heteronuclear single quantum correlation (2D ^{1}H-^{13}C HSQC).[32,38–40] Alternatively, Anaraki et al[33] suggested that sample homogeneity may be increased by crushing the organisms after the in vivo data have been collected. In this manner, in vivo and ex vivo NMR metabolomics data can be combined to greatly improve metabolite assignments.[33]

Conversely, in vitro NMR provides a simpler alternative to characterizing the metabolome of an intact organism. Instead of the challenges of maintaining a living organism within an NMR tube and the corresponding negative impact on the quality of the NMR spectra by the presence of these organisms, in vitro NMR eliminates the organism entirely and focuses on the extracted metabolome from lysed cells or detecting metabolites from whole live cells. Simple homogeneous solutions generated from in vitro samples (ie solvent extracted lysed cells) produce uniform and narrow NMR line-shapes. In this manner, in vitro NMR allows for a cleaner view of the metabolome obtained from bacterial cell lines, primary cell cultures, or immortalized cell lines, among others. The removal of interfering biomolecules (eg proteins, DNA, RNA), cell debris, and the overall cellular environment by means of sample processing leads to a higher sensitivity and resolution in the 1D ^{1}H NMR spectrum. The presence of these confounding elements in the NMR tube is the primary reason an in vivo NMR spectrum is lower in overall quality. In this regard, in vitro NMR may provide a better and more complete insight into the metabolic changes that might be missed by in vivo NMR.

In vitro NMR is simply easier to execute and to perform under controlled experimental settings.[41–43] For example, the extraction solvent (water or chloroform) will dictate the type of metabolite (lipids or aqueous metabolites) observed in the NMR spectrum. Similarly, temporal metabolite changes are equally easy to define in an in vivo experiment based on the chosen time(s) of cell harvesting. It is easy enough to define how long a cell line is treated with a drug or environmental stressor prior to extracting the metabolome, or what growth phase the cells have entered before the cells are collected. For in vivo NMR, all metabolic processes are on-going as long as the organisms are viable in the NMR tube, nutrients and oxygen are available, and spectral data are being collected. In vitro metabolomics has the unique advantage that it can use either live cells or extracted cellular metabolomes.[44] This flexibility expands the applicability of in vitro NMR metabolomics. However, in vitro NMR metabolomics has serious challenges regarding translating the experimental metabolomics data to real-world scenarios. Sample preparation, processing, and handling can induce biologically irrelevant alterations in metabolic profiles. Batch variability due to differences in growth medium formulation, presence of different additives, variable rates of cell proliferation, different passage numbers, or age of the cell line may all contribute to erroneous changes in the metabolome.[44,45] Thus, it is critical to validate and correlate the metabolomics data from an in vitro system to the data obtained from a native environment or an in vivo system.[46–48]

Ex vivo NMR may provide a means to bridge the gap between in vivo and in vitro NMR metabolomics while simultaneously alleviating many of the challenges associated with these two approaches. NMR can be used to obtain metabolic profiles from various sources including cell extracts, live cells, or whole organisms. In the case of ex vivo NMR, the metabolomics study commonly uses biofluids or tumor biopsies. As for cell extracts, the metabolome may be solvent extracted from the homogenized tissue to obtain a solution-state NMR spectrum. Alternatively, the intact solid tissue can be analyzed directly by HR-MAS. 1D ^{1}H NMR is the preferred NMR experiment for an ex vivo metabolomics.[49–52] Importantly, an ex vivo sample (eg biofluids) yields relatively high-quality NMR spectra and the corresponding ability to quantify metabolites with concentrations as low as 1 μM.[13] In fact, a 1D ^{1}H HR-MAS spectrum of an ex vivo sample is likely to identify many more metabolites than a similar spectrum of an in vivo sample due to the higher-quality spectrum.[53] It is possible to observe upwards of 50 to 200 different metabolites from an in vitro NMR spectrum.[24] Conversely, due to issue of severe peak overlap in in vivo NMR metabolomics, despite having close to ∼3000 peak capacity (spectral features), very few metabolites (0 to 15) can be reliably assigned in conventional in vivo ^{1}H NMR studies.[33,54,55] However, it is reported that by employing multi-dimensional NMR techniques one can significantly overcome in vivo line broadening and reduce spectral overlaps.[33] For example, Anaraki et al reported close to 30 metabolites identified in the *D. magna* metabolome using a ^{1}H-^{1}H in phase intermolecular single quantum coherence NMR experiment.[33]

An additional advantage of ex vivo NMR is the ability to analyze small pieces of intact tissue (eg plant tissues, organs from insects and small animals, etc) with minimal sample preparation or sample destruction. These tissues can still be recovered for additional studies using other analytical techniques or methods (eg biochemical analyses, proteomics, transcriptomics, etc). Overall, ex vivo NMR can be reliably used for monitoring the metabolome of a tumor or for identifying metabolic perturbations induced by genetic (eg oncogenes or tumor suppressor genes) and/or therapeutic factors (eg anti-cancer drug treatments).[56] In this manner, ex vivo NMR has been used to detect and quantify various biomolecules (eg metabolites, enzymes, macromolecules, etc), which were difficult to study by other methods.

A study by Nagana Gowda et al[57] utilized ex vivo NMR to reliably measure seven major coenzymes from complex biological mixtures obtained from a cardiac-specific mouse knockout of the mitochondrial complex I *Ndufs4* gene (cKO) and transgenic mice with cardiac-specific overexpression of the nicotinamide phosphoribosyltransferase gene (*cNAMPT*) (Figure 4). 1D ^{1}H NMR was used to quantify adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, nicotinamide adenine dinucleotide (NAD^{+}), nicotinamide adenine dinucleotide phosphate, NADH, and NADPH from heart, kidney, brain, liver, and skeletal muscle tissues harvested from the knockout and transgenic mouse models. These major coenzymes of cellular energy and redox reactions were measured using small tissue quantities (5 to 80 mg) on a Bruker Avance III 800 MHz spectrometer equipped with a cryoprobe. The metabolome was extracted from homogenized tissue samples using a mixture

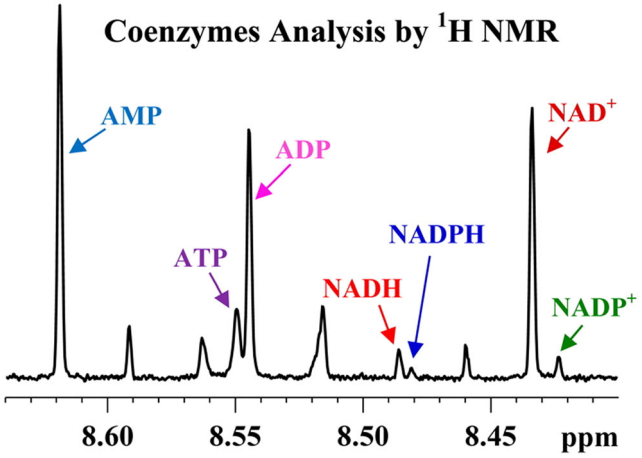

FIGURE 4 Expanded spectral region from an 800 MHz ^{1}H NMR spectrum of a mouse liver tissue extract obtained using a 5 mm sample tube showing the characteristic fingerprint of the redox and energy coenzymes. Figure reprinted with permission from Nagana Gowda GA, Abell L, Lee CF, Tian R, Raftery D. Simultaneous analysis of major coenzymes of cellular redox reactions and energy using ex vivo ^{1}H NMR spectroscopy. *Analytical Chemistry* 2016;88(9):4817-4824. Copyright 2016 American Chemical Society

of methanol and chloroform. Metabolite assignments were confirmed using two-dimensional double quantum filter-homonuclear correlation spectroscopy (2D ^{1}H-^{1}H DQF-COSY) and two-dimensional total correlation spectroscopy (2D ^{1}H-^{1}H TOCSY) NMR spectra, database searches and in-house reference spectra, and spiking of samples with authentic compounds. As expected, different NAD^+/NADH ratios were observed across the different tissues and between the knockout and transgenic mouse models. Overall, a reliable ex vivo NMR method was demonstrated for the simultaneous quantification of ubiquitous coenzymes that improved upon traditional enzymatic assays. Specifically, ex vivo NMR avoided the need for a separate sample preparation protocol to individually measure each coenzyme. The approach offered a new avenue to study the mechanistic details of cellular function in health and disease.

Ex vivo NMR has also been widely used to study the metabolome of various cancer types including gliomas and breast, esophageal, and lung cancers.[53,58–60] Gao et al applied ex vivo ^{1}H NMR metabolomics to characterize renal cell carcinoma (RCC) metastases.[61] Biopsied tumors along with adjacent tissue samples (150 mg) were collected from patients diagnosed with both metastatic and non-metastatic RCC. The tissue samples were homogenized, and the metabolome was then extracted with a mixture of methanol, chloroform, and water. 1D ^{1}H NMR combined with multivariate statistical models, principal component analysis (PCA), and projections to latent structures-discriminant analysis (PLS-DA) were used to identify metabolic differences between the various tissue samples. Two-dimensional correlation spectroscopy (2D ^{1}H-^{1}H COSY) and TOCSY spectra and literature chemical shift values were used to verify metabolite assignments. Overall, the metabolomic profiles from the PCA and PLS-DA score plots differed markedly between metastatic and non-metastatic RCC. Furthermore, the RCC metabolome, whether metastatic or not, was also distinctly different from the paired adjacent tissue samples. Specifically, the PLS-DA loadings and variable importance plot scores were used to identify 23 metabolites (15 decreased and 8 increased in RCC) that differentiated RCC from adjacent tissues. The absolute quantification of metabolite changes identified 18 metabolites with statistically significant ($p < 0.05$) concentration differences between RCC tumors and adjacent tissues. Similarly, 13 metabolites were shown to differentiate between metastatic and non-metastatic RCC. The discovery of potential metabolite biomarkers by ex vivo NMR is significant in aiding the detection, diagnosis, treatment and prognosis of RCC. Ex vivo metabolomics has been similarly employed for metabolite biomarker discovery for rare cancers, such as bladder cancer (BCa), prostate cancer, and infiltrating gliomas, in which methods for early diagnosis and/or prognosis are scarce.[62–65] For instance, ex vivo NMR was utilized by Tripathi et al to examine the metabolic signature of bladder cancer in order to distinguish it from a benign disease.[66] Frozen bladder tissues (15 to 25 mg) were surgically collected from volunteer patients and then classified according to the presence or absence of BCa and subsequently graded according to the disease stage. 1D ^{1}H HR-MAS spectra were collected on the intact tissue. PCA and PLS-DA models were then generated from the NMR spectral dataset. The resulting score plots showed a clear separation between BCa and benign tissue samples (Figure 5). 2D ^{1}H-^{1}H TOCSY spectra, loadings from the PLS-DA model, and targeted metabolite quantifications using gas chromatography (GC)-MS were then used to identify statistically significant ($p < 0.001$) metabolites that varied with BCa stage and were different compared with benign tissues. The BCa tissues, irrespective of stage, had increased levels of aqueous metabolites, such as intermediates of the glycolysis and tricarboxylic acid cycle, amino acids, taurine, glutathione, and choline-containing metabolites, and a decrease in triglyceride (TG) levels. The ex vivo NMR-based metabolic profiling showed improved performance in distinguishing BCa tissues from benign tissues, but was not able to distinguish between the various BCa disease stages.

Ex vivo NMR metabolomics may also provide a hypothesis-free approach for establishing gene ontology in model and non-model organisms.[67] Feussner and Feussner illustrated the application of ex vivo metabolomics to functionally annotate enzymes of unknown function.[67] A

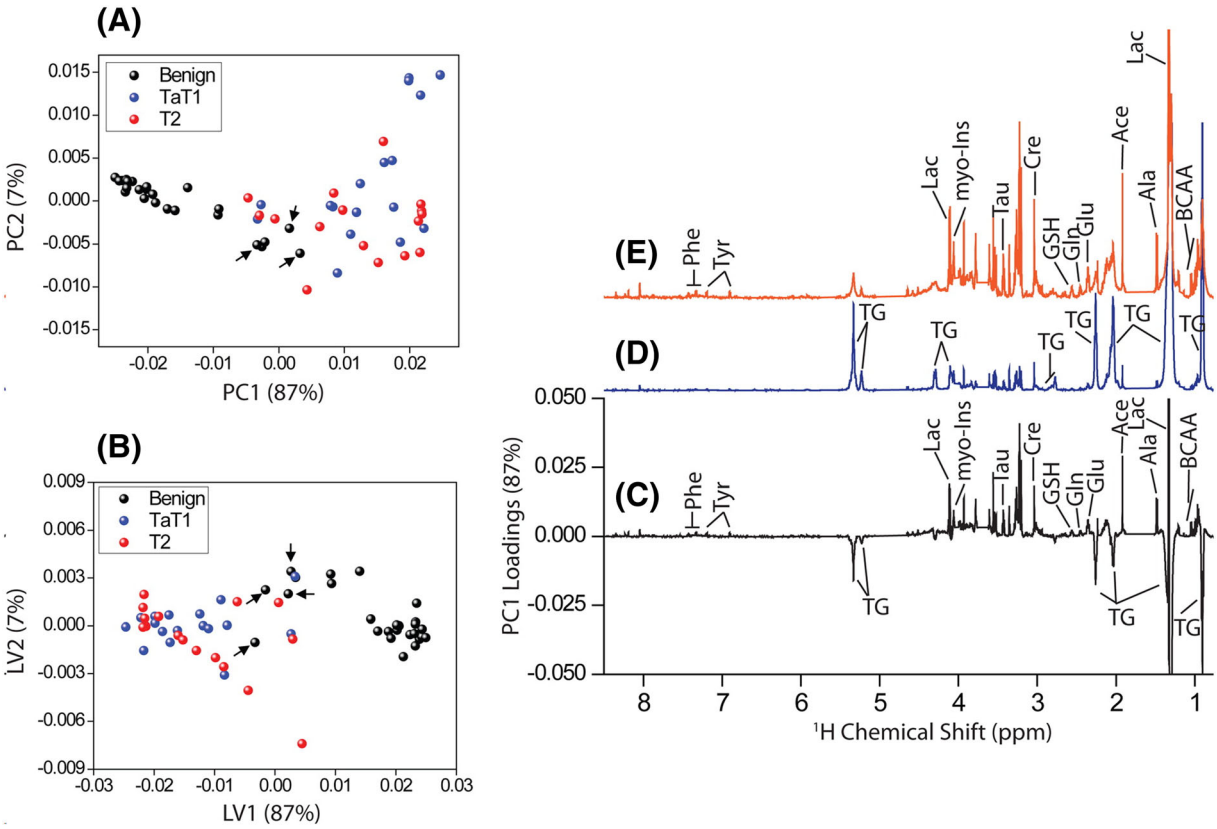

FIGURE 5 A-C, Unsupervised 2D PCA score plot (A), supervised 2D PLS-DA score plot (B), and PC1 loadings (C) of a total of 59 tissues generated from ^{1}H CPMG spectra of 26 benign, 17 bladder cancer Ta-T1, and 16 bladder cancer T2 stage or higher. Both PCA and PLS-DA score plots showed clear segregation of benign and BCa groups, mainly due to PC1 and LV1 loadings respectively. From PC1 loadings, TG levels were clearly decreased (negative loadings) while all other aqueous metabolites were elevated (positive loadings) in BCa compared with benign tissues. A few benign samples (indicated by black arrows) categorized under BCa samples were predominantly benign urothelium partially contaminated with urothelial carcinoma. D, E, The average ^{1}H CPMG spectra of all 26 benign spectra (D) and 33 bladder cancer spectra (E). The spectral difference between the two average spectra is consistent with PC1 loadings. Figure reprinted with permission from Tripathi P, Somashekar BS, Ponnusamy M, Gursky A, Dailey S, Kunju P, Lee CT, Chinnaiyan AM, Rajendiran TM, Ramamoorthy A. HR-MAS NMR tissue metabolomic signatures cross-validated by mass spectrometry distinguish bladder cancer from benign disease. *Journal of Proteome Research* 2013;12 (7):3519-3528. Copyright 2013 American Chemical Society

multi-step procedure was developed that involved the extraction of metabolites from tissues rich in the enzyme of interest. The metabolite extract was then incubated with either the active or inactivated enzyme. A comparative analysis of the metabolite mixtures by either LC-MS or NMR would reveal the potential substrate-product pair for the enzyme based on a relative change in metabolite concentrations compared with the negative control. In this manner, the substrate-product pair could be leveraged to infer a potential enzymatic function(s) for the unannotated gene.

Overall, ex vivo NMR may provide a more in-depth analysis of the metabolome because of the limited sample preparation and higher spectral quality that can be achieved relative to in vivo NMR.[34] Nevertheless, there are limitations to ex vivo NMR-based metabolomics such as the inability to measure a real-time response of an organism to a treatment. Importantly, ex vivo NMR does provide metabolomics results that are highly complementary to both in vitro and in vivo studies (Figure 6).

4 | INCREASING SCIENTIFIC RIGOR AND CONFIDENCE: ROLE OF QA/QC IN NMR METABOLOMICS

A metabolomics investigation will typically follow a standard multi-step workflow, which starts at the experimental design step and concludes at data analysis (Figure 7). The overall integrity of the study's outcome relies heavily on the quality, consistency, execution, and reliability of each procedure conducted during the entire metabolomics processes.[68] Thus, several procedural check points and controls are required to ensure that the data obtained are reproducible, precise, and accurate. QA and QC processes are often overlooked when conducting a metabolomics

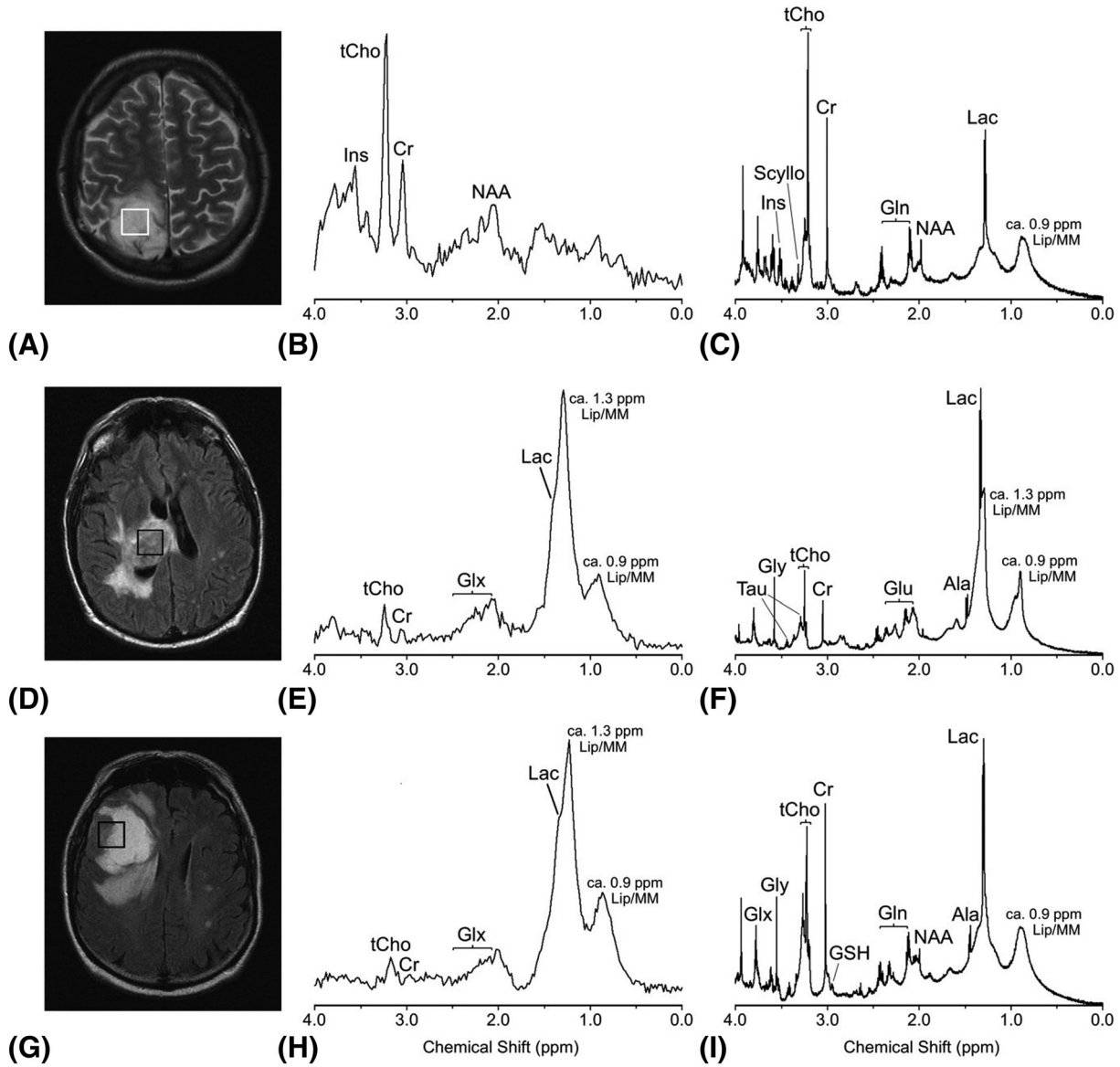

FIGURE 6 In vivo PRESS T_E 30 ms spectra (B, E, H) and their equivalent ex vivo HRMAS presaturation spectra (C, F, I) from a histopathologically verified astrocytoma grade II (A-C) and glioblastoma (D-F) showing unimodal variation of the grayscale pixel values in the voxel placement areas (A, D), and a histologically verified glioblastoma with multimodal variation of the grayscale pixel values (G-I). Major peaks of the metabolites discussed have been labeled, but for clarity not all metabolites are labeled in each spectrum (Ala, alanine; Cr, creatine; Gln, glutamine; Glu, glutamate; Glx, [Gln + Glu]; Gly, glycine; Ins, myo-inositol; Lac, lactate; Lip/MM, lipids/macromolecules; Scyllo, scyllo-inositol; Tau, taurine; tCho, total cholines). Figure reprinted with permission from Opstad KS, Wright AJ, Bell BA, Griffiths JR, Howe FA. Correlations between in vivo ^{1}H MRS and ex vivo ^{1}H HRMAS metabolite measurements in adult human gliomas. *Journal of Magnetic Resonance Imaging* 2010;31:289-297. Copyright 2010 John Wiley and Sons

investigation. Nevertheless, there is a growing awareness of the necessity of QA/QC practices within the metabolomics community. Several taskforces have been established to promote and address key issues in the use of QA and QC by metabolomics investigators. Notable efforts include the Metabolomics Quality Assurance and Quality Control Consortium (mQACC),[69] which was established in 2018, and the Metabolomics Society Data Quality Task Group (DQTG),[70,71] which was established in 2014. According to these taskforces and other stakeholders, QA has been broadly defined as the set of procedures performed prior to sample analysis that are used to improve data quality. These procedures include personnel training on safety, sample handling and processing, instrument calibration and daily system suitability tests, establishing written standard operating procedures (SOPs) for specific sample types that include consumable sources (ie vendor, part number, batch/lot number, etc), and performing regular audits. With respect to NMR-based metabolomics, the SOPs would include protocols for the proper storage and transport of biological samples, the solvent extraction of the metabolomes from different cell types, homogenized tissues, and other biological samples, or the

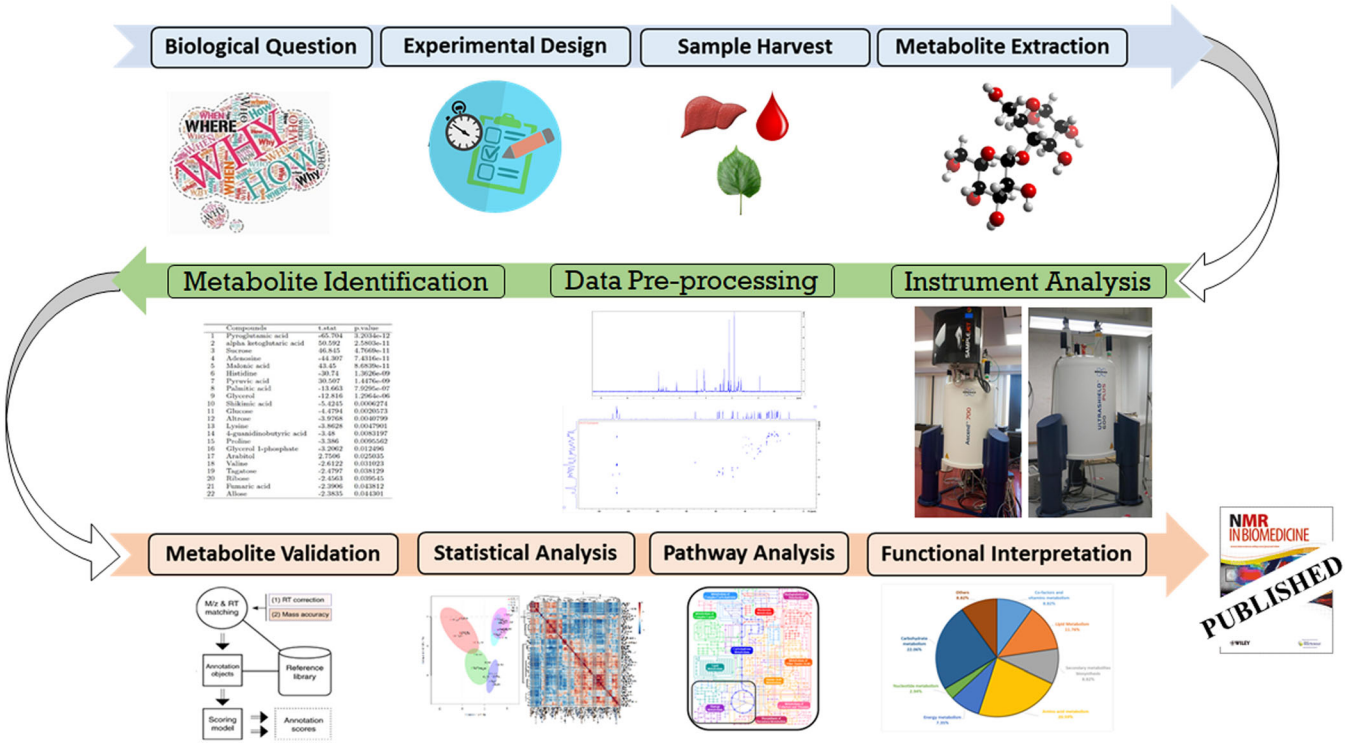

FIGURE 7 Designing a metabolomics study. The general metabolomics workflow involves formulating a biological question, setting up an experimental design to test the hypothesis, sample treatment and harvest, metabolite extraction, clean-up, sample analysis, metabolite identification, statistical validation, and functional interpretation. Adapted with permission from Maroli AS, Gaines TA, Foley ME, Duke SO, Doğramacı M, Anderson JV, Horvath DP, Chao WS, Tharayil N. Omics in weed science: a perspective from genomics, transcriptomics, and metabolomics approaches. *Weed Science* 2018;66(6):681-695. Copyright 2020 Cambridge University Press

handling and preparation of various biofluids, and the preparation of NMR samples (ie choice of pH, buffer, internal reference, NMR tube diameter, etc) from the metabolomics samples.

Likewise, QC has been defined as the set of activities that a laboratory needs to perform during or immediately after a metabolomics data acquisition and analysis to ensure the quality of the experimental data. Some of the common QC processes include the use of one or more internal standards spiked into each sample, analysis of pooled QC samples (internal or external), procedural and instrument blanks, and reporting of study-specific quality metrics. In regards to NMR-based metabolomics, some QC processes that are routinely followed by the community include the addition of chemical shift standards (eg sodium trimethylsilylpropanesulfonate, trimethylsilylpropanoic acid) to each NMR sample, monitoring laboratory temperature and humidity, incorporating gradient shimming, sample tuning and matching into automation protocols, routine calibration of experimental parameters (ie temperature, line-widths, 90-degree pulses, signal-to-noise, etc) with standard reference samples, and randomizing sample batch order.

QA/QC has been a "hot topic" in the MS metabolomics community given the well known variability and reproducibility issues with both MS instrumentation and chromatography platforms.[69,72,73] Nevertheless, the same rigorous QA/QC procedures are equally relevant and important to NMR-based metabolomics. In fact, a number of research publications have highlighted the need for including routine QA/QC checks in NMR metabolomics.[74–77] Since the intra-laboratory and inter-laboratory variations between NMR metabolomics datasets are smaller than for MS, QC may be limited to using pooled biological samples (pooled quality control; PQC) by combining together a small aliquot from each replicate in the dataset. PQC can be used to shim and tune the NMR, and can also be repeatedly analyzed at regular intervals throughout the NMR data collection to ensure system stability.

While inherently valuable to all NMR data collection, QA/QC checks may be particularly important to ensuring the confidence of ex vivo NMR data and to avoid false positive and false negative results. Simply, ex vivo NMR-based metabolomics is playing an expanding role in translational medicine. It is bridging the gap between in vitro and in vivo studies and aims to bring "bench-side" scientific insights and discoveries closer to the reality of a "bedside" break-through. In this regard, poor quality data, unreliable outcomes, and erroneous conclusions may have severe and detrimental consequences to an individual's health and well-being. Thus, there is an urgent need to establish a standardized analytical workflow for NMR-based metabolomics that can deliver comparable, reliable, high-quality, and precise

quantitative data.[78] There should be widely accepted SOPs for handling and processing each type of tissue or biofluid to ensure reproducibility across laboratories and between individual operators. These SOPs should include validated workflows that include all steps of the procedure from the pre-analytical stage (QC markers, stability markers, database selection, etc) to the post-analytical stage (identify deviations in pre-analytical QA/QC checks, data normalization, batch correction, data cleaning/filtering, confounder adjustment, outlier detection, etc).

The choice of instrument and experimental parameters may also serve as a QA check when conducting an NMR metabolomics study. Thus, establishing a set of standardized operational parameters could certainly benefit the overall robustness and uniform quality of the NMR spectral data. For example, and among other issues, an effective water suppression method is a key parameter that can have a pronounced impact on the overall quality and accuracy of the NMR spectrum. Accordingly, defining a standardized water suppression scheme with an established protocol for implementing the NMR pulse sequence would lead to a high level of consistency and reliability across multiple datasets and between different investigators. In this manner, Giraudeau et al provided a valuable tutorial with a point-by-point explanation on how to choose and optimize the right water suppression parameters for an NMR metabolomics experiment.[79] Establishing a collection of similar standard protocols and tutorials would clearly contribute to developing QA/QC checks for the NMR metabolomics community.

To ensure inter-compatibility and enable external data auditing, the acquired and processed data need to be saved and archived in standardized data formats[80] and submitted to data depositories, such as the Metabolomics Workbench (https://www.metabolomicsworkbench.org/).[81] Publicly available metabolomics datasets should include sufficient metadata (minimum information about metabolomics experiment, MIME) to allow an investigator to readily understand the biological significance and technical origins of the data in enough detail to be able to reproduce the same results.[82] The metadata may include other relevant information to satisfy QA/QC requirements and increase general confidence in the data. A concise and informative MIME would assist in extrapolating reliable data obtained from in vitro experiments to an in vivo application. The need for QA and QC in ex vivo and in vivo metabolomics approaches has risen several-fold since ex vivo metabolomics was being used to represent in vivo conditions.[83,84] Simply, leveraging a correlation between two or more distinct datasets necessitates a common framework or point of reference to enable a valid comparison. A common set of QA/QC checks provides this important framework.

Clinical practices have started to accept evidence-based medicine (EBM), and the confidence in EBM has grown with the incorporation and reporting of QA/QC in NMR ex vivo studies.[85–87] Notably, the extrapolation of in vitro and/or ex vivo data to an in vivo system has been mostly carried out in the field of toxicometabolomics. Despite these advances, LC- or GC-MS is still the tool of choice for a majority of in vitro to in vivo metabolomics extrapolation studies.[88]

5 | WHAT LIES AHEAD FOR EX VIVO NMR-BASED METABOLOMICS?

Metabolomics is widely employed by the scientific community to aid in our understanding of a variety of complex biochemical processes. As with any scientific endeavor, metabolomics aims to obtain high quality, relevant, and reproducible information about the biological system under investigation. Several key elements are needed to achieve this laudable goal: (a) asking the right biological questions; (b) designing the appropriate experiments and, most importantly, (c) ensuring effective QA/QC checks are used. QA/QC checks are necessary to assure that all measurements are reliable and that they hold up to validation assessments and scrutiny. An important step of the QA/QC process is the submission of metabolomics data to open-source databases or repositories. This will support community-wide efforts to standardize experimental protocols and MIME, while ensuring the public dissemination of information. In this manner, metabolomics datasets are available for further validation and analysis while also providing a valuable resource for the development of new methodologies and software. Ideally, the routine deposition of metabolomics datasets to public repositories would only increase the preponderance of high-quality data and reliable biological insights being reported in the scientific literature. Thus, while the deposition of metabolomics datasets to repositories, such as Metabolomics Workbench, is growing voluntarily, this process needs to become mandatory and should be enforced by granting agencies and publishers to make the investigators compliant.

QA/QC is particularly critical to untargeted metabolomics since it is typically a hypothesis-generating endeavor reliant on the screening of complex, heterogeneous mixtures comprised of both known and unknown compounds with diverse physicochemical properties. From this complexity, untargeted metabolomics strives to uncover anything from the mechanism of action of a novel drug to the identification of potential diagnostic biomarkers for human disease. However, it is also susceptible to erroneous results and misleading discoveries. To mitigate potentially negative outcomes, the analytical methods need to be robustly and routinely validated, and the experimental protocols need to be properly evaluated and standardized. While efforts are on-going, there is still a lack of well defined validation criteria and standardized operating protocols, especially for ex vivo NMR metabolomics. The variety of tissue types investigated, the complexity of the tissue microarchitecture, the variable distribution of metabolites throughout the tissue, and the diversity of the physiochemical properties of compounds that comprise the metabolome, among other challenges, makes it difficult to define validation parameters and establish uniform methodologies. Nevertheless, the metabolomics

community needs to continue its efforts in developing standardized QA/QC protocols and to promote the wide acceptance of these standards by all investigators.

The translation of scientific insights from the laboratory to clinical applications is a fundamental goal of metabolomics. As discussed throughout this review, ex vivo NMR holds the promise of bridging the gap between in vivo and in vitro studies. Simply, in vivo NMR provides a direct observation of an intact living organism, but the data quality and information content are low. Conversely, in vitro NMR provides high-quality and information-rich data, but it is distal from the natural environment of a living organism. Instead, ex vivo NMR provides high-quality and informative data on a tissue or biofluid directly removed from an intact living organism. In this regard, in vitro/ex vivo results can be extrapolated to the in vivo system. However, the success of this extrapolation approach is highly dependent on a uniform and reliable data acquisition and analysis framework. To ensure the quality of the data, the sample needs to be handled, processed, and analyzed with pre-defined SOPs. Unfortunately, divergent QA and QC practices for in vivo and ex vivo NMR have confounded this extrapolation process. Instead, a consensus is needed to identify a standardized QA/QC process for both in vivo and ex vivo NMR to strengthen the utility of ex vivo metabolomics for translational medicine.

ACKNOWLEDGEMENTS

This work was supported in part by funding from the National Science Foundation under Grant Number 1660921 and the Nebraska Center for Integrated Biomolecular Communication (P20 GM113126, NIH, NIGMS).

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Maroli AS, Powers R. Closing the gap between in vivo and in vitro omics: using QA/QC to strengthen ex vivo NMR metabolomics. *NMR in Biomedicine*. 2021;e4594. https://doi.org/10.1002/nbm.4594