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Challenges in Translating Clinical Metabolomics Data Sets from the Bench to the Bedside

Candice Z. Ulmer[a,*], **Anthony Maus**[b], **Jolaine Hines**[c], **Ravinder Singh**[b,c]

[a]Clinical Chemistry Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

[b]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

[c]Immunochemical Core Laboratory, Mayo Clinic, Rochester, MN, USA

Metabolomics encompasses the comprehensive study of metabolism as it pertains to a biological system in response to a stimulus or pathophysiological condition. Lipidomics, a subset discipline of metabolomics, encompasses the study of lipid pathways, networks, functions, and interactions. While the term “metabolome” was coined in 1998 and the metabolomics field was considered emerging until recently, metabolic profiles have been studied in biological fluids for centuries. Metabolomics methodologies are currently divided into 2 main categories: targeted and untargeted studies. Targeted metabolomics studies apply single to multianalyte quantitative approaches to analyze a predefined selection (1 to 100s) of compounds originating from a specific metabolic pathway. Untargeted metabolomics studies apply chemometric approaches to focus on the comprehensive or global analysis of all detectable metabolites in a biological sample in a hypothesis-generating manner. The goal of targeted and untargeted metabolomics is to use the metabolic profile to infer biological functions within a system and better understand biochemical responses. In addition, the workflows for both approaches include bioanalysis (e.g., sample collection, sample preparation, and data acquisition), data processing, compound identification, and biological interpretation.

Since their formal introduction into the scientific community, targeted metabolomics workflows have carved out an essential role in clinical laboratory testing. As a result, the clinical applications of these targeted metabolomic analyses are innumerable and include diagnosing and monitoring diseases, detecting metabolites of therapeutic drugs and drugs of abuse, identifying inborn errors of metabolism, and quantitating various clinical biomarkers. While targeted assays are routine and well-integrated in the clinical space, untargeted

[*]Address correspondence to this author at: 4770 Buford Hwy NE, MS S110-2, Atlanta, GA 30331, USA. Fax 770-488-0333; culmer@cdc.gov.

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metabolomics assays are considered to still be in infancy stages. However, the adoption of untargeted metabolomics assays in clinical laboratories should not be overlooked. These assays allow for new opportunities in precision medicine with an array of metabolites being detected in a single test. In addition, these assays allow for the discovery of novel biomarkers for various elusive diseases. However, untargeted metabolomics assays have unique challenges in designing the study of interest, locating appropriate internal standards, analyzing and interpreting complicated data sets, and ensuring precise results. To maximize the potential of this powerful technique in routine clinical labs and allow for the successful translation of untargeted metabolomics studies from the bench to the bedside, these challenges must be addressed.

As indicated in numerous reviews, the execution of a successful metabolomics study requires careful consideration of the study design (e.g., sample size, grouping, and collection), sample type (e.g., serum/plasma, urine, tissue, saliva, exhaled breath, etc.), optimal analytical technique(s), and data processing workflow (1). These considerations are important for targeted and untargeted studies. However, targeted metabolomics studies can follow consensus-based standards and guidelines, which are not available in the untargeted metabolomics space. As a result, the decisions made to address the previously mentioned considerations are left to the discretion of the investigator(s). These considerations are further exacerbated by the demands placed on clinical researchers to identify novel biomarkers for elusive diseases that are not as well-defined. Nevertheless, failure to appropriately address each step of the biomarker discovery workflow can result in an inaccurate biological interpretation, disease misdiagnosis, or wrong treatment profile for the patient. Therefore, it is important in metabolomics workflows to apply appropriate sample sizes, ensure sample integrity, minimize biases, and report accurate data interpretations as clinical researchers translate a feature (i.e., m/z and retention time) into a putative biomarker with associated biological function(s) in an effort to better understand disease etiology.

Analytical advancements in the field of nuclear magnetic resonance and mass spectrometry have allowed for the analysis of complex matrices and metabolomes through extended dynamic ranges, higher selectivity, and greater sensitivity. The coupling of these techniques with various separation methodologies (e.g., liquid chromatography, gas chromatography, capillary electrophoresis, and the rapidly developing variations of ion mobility) has also contributed to the growth of the field of metabolomics. With this increasing interest in the field, sample preparation techniques, post-acquisition data processing approaches, metabolite annotation software, and data interpretation/visualization tools have been similarly expanding in capabilities and usage. While this is quite promising, the progress in the field of metabolomics should be appropriately coupled with the rise in harmonized workflows. The lack of harmonized workflows in the metabolomics field has resulted in an undesirable increased risk of incorrect metabolite annotations and biological interpretations. In a recent article by Ghosson et al. (2), the authors highlight risks associated with biomarker determination in metabolomics. The authors describe the role of ion suppression on the inaccurate classification of potential biomarkers. Ion suppression or enhancement, often introduced by matrix components in the sample (e.g., proteins and phospholipids present in blood, serum, and/or plasma), affects the sensitivity, precision, and specificity of LC-MS/MS measurements. Ion suppression most often occurs due to the presence of a

coeluting species that alters droplet formation and the ultimate formation of gaseous ions. Ion suppression can be quite prevalent in mass spectrometry platforms that incorporate electrospray ionization as it is a soft, competitive ionization technique capable of ionization and desolvation of a plethora of thermally labile biomolecules, including polar and semipolar metabolites and lipids. While Ghosson et al. reported that numerous biomarkers were identified by their original methodology, manual crosschecking revealed an abundance of false positives caused by ion suppression from coeluting molecules, the implications of which were only apparent when samples were diluted. This is a timely example of one of the many challenges facing the field of metabolomics, which is exacerbated by the lack of reference materials and defined parameters to assess data quality.

Ion suppression and enhancement are widely known limitations of electrospray ionization–tandem mass spectrometry, which are typically quantified and corrected for when performing targeted metabolomics studies. Quantitative tests generally utilize multiple levels of correction to minimize the impact of suppression/enhancement through the use of coeluting isotopically labeled internal standards and calibration curves prepared in an analyte-depleted sample matrix. In addition, studies that assess the prevalence and impact of ion suppression using post-column infusion or post-extraction spiking experiments are an essential part of the validation of targeted LC-MS/MS tests (3, 4). In a relevant example published by Ismaiel et al., 100% ion enhancement for diphenhydramine and 70% suppression for diphenhydramine were observed by post-column infusion (5). Methods developed in the Protein Biomarker and Clinical Reference Laboratories at the CDC focus on reducing ion suppression to ensure consistency of measurements, especially at the lower limit of quantitation. Mayo Clinic also performs post-extraction spiking experiments when validating new tests. More specifically, Mayo Clinic observed a 20% mean suppression for challenging metabolites such as vitamin B6, 1,25 hydroxyvitamin D, and leukotriene E4. However, the suppression tests indicated that the isotopically labeled internal standards experienced similar suppression, so the impact on patient results was negligible. This assumption is always corroborated with precision and accuracy data.

The establishment of a practical and harmonized approach for identifying ion suppression or enhancement when performing untargeted metabolomics experiments will improve data quality and provide further impetus for the translation of a detected biomarker from a research study into routine clinical laboratory testing. It should be noted that the ramifications of ion suppression or enhancement can be easily overlooked when performing untargeted metabolic analyses. Matrix components that cause ion suppression or enhancement are often not easily detected because the interfering compounds' *m/z* falls outside the experimental scan range. In addition, commonly used data analysis techniques and software packages are not well-suited for the assessment of ion suppression/enhancement. Fundamental principles from the previously outlined evaluation and mitigation techniques, which are cornerstones of targeted testing, should be heavily scrutinized for potential adoption in the validation of untargeted metabolomics workflows. For example, dilution or linearity experiments similar to those described by Ghosson et al. (2), wherein samples are serially diluted and quantitative results are compared to expected results based on the analyte concentration in the neat (undiluted) sample, can be implemented in untargeted metabolomics validation studies. Not only does this experiment

allow for characterization of ion suppression/enhancement, dilution/linearity tests aid in evaluating the specificity and accuracy of a measurement, making it a foundational component of test development. In cases where ion suppression adversely affects results, the situation may be addressed by enhancing sample purification, altering chromatographic conditions, and/or manipulating the mass spectrometer source conditions.

Organizations such as the Metabolomics Quality Assurance and Quality Control Consortium, the International Lipidomics Society, and the International Metabolomics Society are eagerly creating interlaboratory studies, ring trials, best practice guidelines, suitable reference materials, and data standards to ensure data quality in metabolomics data sets and push toward the harmonization of metabolomics workflows. However, until these guidelines are fully vetted and external quality assurance programs for untargeted clinical metabolomics applications are established, researchers should be mindful that the translation of a feature into a clinically relevant biomarker is influenced by the entire metabolomics workflow. This will require open and early communication between clinical chemists and researchers, alignment of goals, and a collective understanding of the challenges facing the field. Careful consideration of these challenges will not only maximize the efficiency of translation, but will also benefit researchers, clinical chemists, and, most important, patients.

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