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Untargeted Metabolomics for Inborn Errors of Metabolism: Development and Evaluation of a Sustainable Reference Material for Correcting Inter-Batch Variability

Article:
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Guest: Dr. Roy Peake is the Director of the Biochemical Genetics Laboratory in the Department of Laboratory Medicine at Boston Children's Hospital, and an Assistant Professor of Pathology at Harvard Medical School.

Bob Barrett: This is a podcast from *Clinical Chemistry*, a production of the Association for Diagnostics & Laboratory Medicine. I'm Bob Barrett. Most test methods used in the clinical laboratory are designed to accurately measure a specific analyte or group of analyte. For instance, glucose assays determine the glucose concentration but provide no information about other substances present in the patient's sample. This works well in most cases, but can be considered a major limitation when screening for inborn errors of metabolism because targeted metabolic testing looking for the presence of specific compounds can detect only a fraction of all known metabolic conditions. On the other hand, untargeted metabolomics can identify many more compounds, allowing the diagnosis of conditions that otherwise would have been missed by targeted metabolic testing. While this carries obvious appeal, untargeted metabolomics is hindered by inter-batch variability, making it difficult to compare samples tested in different analytical runs and even interpret individual patient results.

A new research article appearing in the December 2024 issue of *Clinical Chemistry* introduces a new way to prepare reference material for untargeted metabolomics and demonstrates its impact on routine screening for inborn errors of metabolism. Will this solution unleash the potential of untargeted metabolomics and allow for more widespread implementation? In this podcast, we are joined by the article's senior author. Dr. Roy Peake is the Director of the Biochemical Genetics Laboratory in the Department of Laboratory Medicine at Boston Children's Hospital, and an Assistant Professor of Pathology at Harvard Medical School. His current research focus involves the application of high-resolution mass spectrometry-based metabolomics to the study of inborn errors of metabolism and rare disease.

So, Dr. Peake, let's start with the basics. First, what is metabolomics?

Roy Peake: Well, the term metabolomics was first coined actually in the late 1990s. It's in many ways, the new kid on the block, the newest member of the omics groups. And by definition, it

essentially represents the total global comprehensive analysis of small molecular weight compounds in a single specimen. These small molecular weight compounds are typically less than a thousand Daltons. These could be substrates or intermediates or a product of cellular metabolism, and they could be anything from amino acids, organic acids, sugars, lipids, and so on. So, in essence, metabolomics provides a snapshot of the metabolic flux within an organism and this is done through the global assessment of huge numbers of compounds for disparate physical chemical properties.

Bob Barrett: So how can metabolomics be used to investigate inborn errors of metabolism?

Roy Peake: Well, we tend to think about inborn errors of metabolism, or IEMs for short, as enzyme deficiencies causing blocks in metabolic pathways, and this leads to severe disease, which is true to some extent, but that's only part of the story. We now know there's well over a thousand recognized inborn errors of metabolism of extremely diverse clinical phenotypes, and likely there's probably many more that have to be discovered. So having more comprehensive metabolite coverage and pathway analysis through more global metabolomics profiling would undoubtedly improve the discovery rate of IEMs.

And also from a diagnostic perspective, only a fraction of the total number of IEMs are screened for at birth through population newborn screening programs so the rest have to be detected by clinical evaluation, together with laboratory testing, whether that be through traditional biochemical testing or molecular diagnostics. So, if we consider traditional biochemical testing for a moment, these are tests like amino acids, organic acid profiles. So, in truth, we've been performing metabolomic profiling and biochemical genetics labs for decades using these tests, but it's now known as targeted metabolomics, this is what we call these tests nowadays. Because we have a predetermined hypothesis about the condition, the testing itself is targeted to a particular metabolite class.

So, firstly, if we consider these traditional biochemical screening assays for a moment, the problem with this approach is that the diagnostic yield is very low, we know this. And you know, this may be due to a large number of specialist biochemical tests that are available, and so selecting the right one in every patient is challenging, even for metabolic specialists, and this is in part due to the vast phenotypic spectrum of inborn errors metabolism. So therefore, having a more global and more comprehensive metabolite screen through metabolomics available up front, would not only take some of the pressure of providers ordering these tests, but would also help us detect even more

of these disorders in the pre-symptomatic phase and enable earlier initiation of treatment.

And the hope is that this will be achieved through what we call untargeted metabolomics, where there is no hypothesis about the testing before the testing is conducted, and everything from the analyte capture right through the analytics and data processing is essentially untargeted. And secondly, molecular diagnostics we're talking here in the primarily about whole genome, whole exome sequencing is becoming a major route of discovery of IEMs. However, what we're seeing in the biochemical laboratory is a greater need for functional characterization of molecular variants of unknown clinical significance, or VUS, and we know that information flows from genes to transcripts to proteins to metabolites. And out of all of these, metabolites are arguably the closest to the clinical phenotype because, unlike genes or proteins, metabolites are not subject to modification. So, I think this is an area where global comprehensive and, dare I say, untargeted metabolomics could help fill this gap and provide a means of functional assessment of equivocal genomics data.

Bob Barrett: Doctor, what are the major barriers to implementing untargeted metabolomics into the clinical laboratory?

Roy Peake: Well, first, it should be noted that untargeted metabolomics is already established in a small number of clinical laboratories to investigate IEMs, and these pioneering individuals deserve a lot of credit for taking the idea forward. But that said, there remains much skepticism amongst the clinical chemistry fraternity that untargeted metabolomics is still not suitable for routine diagnostics for a number of reasons.

Firstly, there's the issue of measuring hundreds of compounds of vastly differing physical chemical properties and that comes with its own challenges. Untargeted metabolomics requires high-resolution mass spectrometry, which is a form of mass spec that is not really mainstream in clinical labs. And there's also concerns over the vast quantities of data that are generated and how to condense this into meaningful, reportable data sets that can be used for clinical decision making. And there's also a perception that this is a research tool and is only useful for large numbers of test samples and matched controls, and therefore is not amenable to routine analysis where we analyze samples in singleton. But perhaps one of the most challenging aspects is variability in high-resolution mass spec-based metabolomics.

Bob Barrett: Well, you mentioned specifically variability there, why is this such a problem and what can be done to fix it?

Roy Peake: Absolutely, this is mostly related to inter batch variability associated with untargeted experiments using high resolution mass spec. Now, this unwanted variability arises from various sources, for instance, from extraction procedures, instrument variation, mostly related to analyte response variability, which may be due to differences in mobile phase composition. For example, column degradation, detector response, changes in consumables, of course, analyst performance. And this variability really adversely impacts the ability to directly compare batch data from separate analytical runs.

So, from a routine clinical workflow perspective, this makes the analysis of random specimens very, very difficult indeed. And the way we generally fix this variability is through the use of normalization against a biological matrix matched reference material. Now, there's a couple of accepted ways to do this. Firstly, you can use commercial reference materials, such as the SRM 1950 from NIST. However, this is difficult because of the high cost involved and the limited availability, and so they're not really a viable and sustainable option for a routine clinical lab workflow. Another way involves the use of a pool of specimens but there's questions over the stability of specimen pools, and there's also the finite nature of the specimen pool, which may present a traceability conundrum, in that future formulations may not be comparable to previous versions. And this requires bridging runs using both the new and previous materials. So there's clearly a need to have a readily available, stable, and sustainable biological reference material for use in routine clinical laboratory workflows.

Bob Barrett: Could you briefly describe this new approach that you and your colleagues have developed?

Roy Peake: Sure. Well, the approach we took was based on the iterative batch averaging method, or IBAT. And this approach was originally described by Gouveia and researchers who initially developed the IBAT concept to create an *E. coli* reference material that was robust to changes over time. And we thought that this would be perfect for generating biological reference materials.

And so, we developed this concept with the goal of creating a sustainable plasma-based reference material for use in clinical diagnostics. Now the process is fairly simple and essentially involves the use of specimen remnants left over from routine testing, and we pool these individual specimens to create a batch. Then individual batches are combined to create a reference material. And as the reference material reserves deplete, successive iterations create new reference material. So, in this way, the reference material is

continuously expanded by combining previously frozen batches of the current reference material with a batch of new specimens.

So, ultimately, by incorporating these little changes over time, a large homogeneous pool is created while exhibiting minimal variance between iterations. It is sort of a rolling calibrator approach, and the real appeal about this IBAT reference material is that it's potentially infinite and therefore sustainable. So, it's really perfect for routine testing purposes.

Bob Barrett: That sounds wonderful. Does it work?

Roy Peake: Yes, it certainly does. We demonstrated that inter-batch variability observed with the IBAT reference material was significantly less than with individual specimens used for its creation. We also demonstrated the superiority of the IBAT reference material compared with the traditional specimen pool. When we used plasma amino acid analysis as a surrogate metabolite, the IBAT demonstrated significantly less variability over a 12-week period for all amino acids tested compared with the specimen pool, and this was an important step for us. And finally, and possibly most importantly, we assess the ability of the IBAT reference material in a metabolomic screening workflow used to identify patients with an inborn error of metabolism.

For this part of the study, we studied how the reference material was able to correct inter-batch variability in a cohort of phenylketonuria patients, or PKU patients. We chose PKU patients because it's the most common inborn error of metabolism and will be a good model to study. So, we analyze specimens from 30 PKU subjects with 146 non-PKU controls in one batch using high-resolution mass spec, and again, one month later. And *z* scores for both the PKU and control groups were calculated and compared. And when the reference material was used for inter-batch normalization, all of the PKU specimens were correctly classified according to their appropriate category based on their plasma phenylalanine concentrations and their current treatment regimens. And interestingly, when the reference material was not used for normalization, over half of PKU patients would have been misclassified. So clearly the IBAT-based reference material is effective in both reducing unwanted variability, and most importantly, in its use in a routine clinical workflow for the identification of an inborn error of metabolism.

Bob Barrett: Well, finally, Dr. Peake, what's the potential impact of this work in clinical diagnostics?

Roy Peake: Well, it's my hope that in a few years from now, we will be using metabolomics to identify many more patients with rare disease, and for many of these patients, end their diagnostic odyssey. Our primary goal with this work, really, what we set out to achieve was to provide a blueprint for clinical labs doing biochemical testing to develop their own reference materials. And hopefully, this would bring comprehensive metabolite screening using high-resolution mass spec closer to the mainstream routine testing environment. Due to economic and practical limitations, it is simply no longer possible for biochemical labs to offer a full menu of targeted biochemical assays. However, I do think it would be possible to use robust high-resolution mass spec-based metabolomic platform to screen for IEMs, together with genomics data, which I predict will markedly increase the diagnostic rate for rare disorders. And particularly when you consider that genomics for newborn screening is already here, I think this is the future for biochemical laboratories.

Bob Barrett: That was Dr. Roy Peake from Boston Children's Hospital in Boston, Massachusetts. He wrote a new research article on improving untargeted metabolomics for inborn errors of metabolism in the December 2024 issue of *Clinical Chemistry*, and he has been our guest in this podcast on that topic. I'm Bob Barrett. Thanks for listening.