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Clearing the Logjam for Biomarkers with Global Metabolomics

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Abstract

The search for biomarkers is a hotly studied and active pursuit in a variety of industries. In fact, the future of personalized health interventions rests squarely on the ability to discover and validate new biomarkers. For example, PLX4032, a drug that has been in the clinic for less than three years, successfully completed a 675 patient Phase 3 clinical trial, with a reported response rate for metastatic melanoma of over 80%. The reason for this remarkably rapid drug development story is twofold: The drug is 10,000-fold more selective to the mutant V600 BRAF protein that is present in the majority of metastatic tumors, and a companion diagnostic genetic test was used to select only those individuals with the V600 BRAF mutation for inclusion in the trials. Remarkable stories of this type are driving increasing interest in new biomarkers and our ability to tailor medical interventions to the individual based on his or her genetics, lifestyle or other individualized information.

The biomarkers themselves can be nearly anything that distinguishes one individual from another. They can be based on a diagnostic test (e.g., glucose or cholesterol measurements), physical characteristic (e.g., BMI), genetics (e.g., SNPs) or any other distinguishing characteristic (e.g., age, diet). Unfortunately, general screening methods for the discovery of new biomarkers have been very challenging with few success stories. The challenges are mathematical, technological and/or limited availability of proper sample types and number. One recent promising technology with potential to overcome a number of these issues is metabolomics.

Glossary of Abbreviations
α-HB: Alpha-Hydroxy Butyrate
α-KB: Apha-Ketobutyrate
BMI: Body Mass Index
CVD: Cardiovascular Disease
FFM: Fat-Free Body Mass
HI Clamp: Hyperinsulinemic Euglycemic Clamp
IFG: Impaired Fasting Glucose
IGT: Imparied Glucose Tolerance
IR: Insulin Resistance
IS: Insulin Sensitivity
LDH: Lactose Dehydrogenase
T2D: Type 2 Diabetes

Introduction

Biomarker discovery and validation is a relatively new and evolving concept that typically involves, as a first step, the deployment of a discovery technology. Whether it's a genomics, proteomics, metabolomics or some other discovery technology, the process starts with an idea of the type of biomarkers that are needed. For example, insulin resistance (IR) is a well-studied condition that can lead to a number of increased health risks, including diabetes and cardiovascular and metabolic diseases. However, simple fasting blood biomarkers or tests don't currently exist for IR or the assessment of the degree of IR. New biomarkers are needed to better manage this growing concern in humans and companion animals. If insulin resistance is the clinical test idea, then the next step is the biomarker discovery and feasibility study to understand whether it is possible to identify new biomarkers (Figure 1). Clinical biomarker discovery studies usually

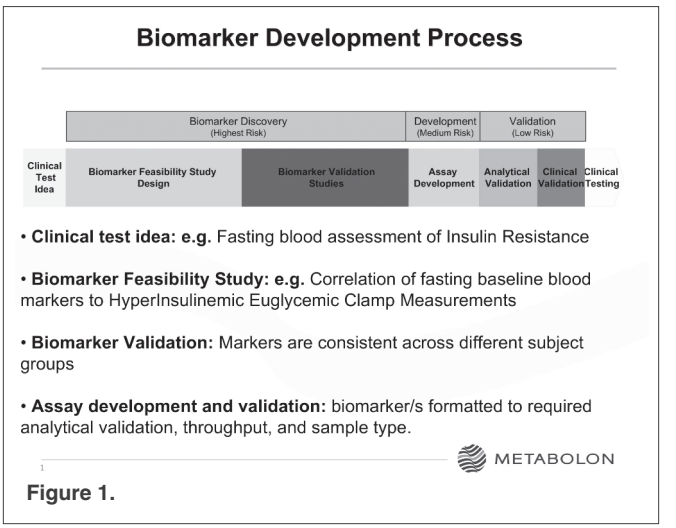

Figure 1.

involve the identification of the appropriate study populations that distinguish individuals with the disease from the nondisease individuals, identify whether appropriate sample types in sufficient numbers with the necessary meta-data can be obtained, and in many cases, involve the development of clinical research agreements with academic and/or health-care organizations.

As with many initial research studies, the outcome of the biomarker feasibility study can be mixed, despite being well-designed and sufficiently powered. Unfortunately, these types of studies can be complicated by the unforeseen impacts of age, gender or other differences (e.g., diet, medications, lifestyle, etc.) of the groups confounding the analysis. In addition, when a very large number of measurements (e.g., 1 million SNPs) are made on only a small number of subjects per group (e.g., 50 individuals per group), the probability of separating the groups by chance alone is enormous and often leads to "false" discovery. As a result, more than one feasibility study may be required to become more confident in the data and sufficiently comfortable to make the investment in the resources required for biomarker validation.

Once it seems reasonable that the sample type for the biomarker is suitable (blood, urine, etc), then larger well-powered clinical studies are warranted. In most cases, these studies will contain sufficient numbers of subjects to not only assess the reproducibility of the biomarkers but to do so on an independent test set of subjects, that is, subjects who haven't been used to discover the biomarkers. Another important consideration in biomarker discovery and validation is how demographically suitable the subjects in the discovery population are relative to those for whom the test will be applied. For instance, using only European populations for the discovery and development of the biomarkers may be a concern if the test will also be employed in Japan.

Analytical assay development and validation aspects of biomarker discovery and validation have significantly less risk associated with developing the biomarker but can be equally time-consuming. Sufficient numbers of samples from the test population are still necessary to analytically validate the assays. In addition, the assay throughput requirements or the assay setting (e.g., hospital, point-of-care, central laboratory) often require specific formats for the biomarker measurement and may differ from the discovery assays. As an example, gene chips are often used for the discovery analysis of genetic biomarkers, but to satisfy the throughput, cost and quantitation requirements, other methods, such as PCR-based assays, have been developed.

Advantages of Metabolomics for Biomarker Discovery

The word "metabolomics" (or "metabonomics") first appeared in journal articles in 2000. Only a few metabolomic scientific papers were published that year, but by 2009, that number rose to over 1,300 published scientific papers reporting metabolomics results. In fact, metabolomics publications are one of the fast-growing areas of scientific publications in the past few years. Although analytical chemists and biochemists have been identifying small molecules in biological samples since long before 2000, as a robust, nontargeted discovery tool, the technology is new and rapidly evolving. In addition to the increasing number of publications, several significant biomarker reports using metabolomics that include supporting validation data for these discoveries have been published.[1-3]

The major challenge for metabolomics has been to develop a technology that can extract, identify and quantitate the entire spectrum of the small molecules (MW<1500Da) in any biological sample. While this is clearly a significant goal, the exact number of small molecules in biological samples is a hotly debated subject. Some databases of metabolites have as many as 6,000 metabolites listed as comprising the human metabolome. However, a deeper evaluation of these metabolites reveals that several thousand molecules can be simply grouped as different combinations of complex lipids or small peptides.[4,5] From a primary metabolism standpoint, and disregarding the combinations of complex lipids or peptides and xenobiotic metabolites from drugs, diet and the like, there are very likely fewer than 3,000 human metabolites of significance for understanding metabolism and metabolic effects. Importantly, in any one sample matrix (i.e., blood, urine, tissue, etc.), there always will be dramatically fewer metabolites than the total number synthesized in the entire organism.

Most importantly, this number (less than 3,000) is much smaller than the other "omics" technologies, such as genomics or proteomics, and may represent a significant advantage for metabolomics in biomarker discovery. A smaller number of total observed measurements for any individual allows the application of more robust statistical testing methodologies and results in fewer false discoveries; two factors that have plagued other biomarker discovery technologies. This simple mathematical effect, called false discovery, is often overlooked, but it represents a severe limitation for generally profiling genes and proteins.

The underlying math is actually quite simple to explain. As the total number of observations per individual subject increases, the likelihood of separating groups of individ-

uals purely by chance goes up significantly. For instance, in a study of two groups of 100 subjects, the likelihood of separating these groups by random measurements is significantly higher if measuring 100,000 variables per individual compared to measuring 1,000 variables per individual. The false discovery problem is much less significant in metabolomics than any other "omic" technology simply due to the fact that the total number of unique biochemicals is so much smaller than the 35,000 or so genes or >100,000 different proteins.[6]

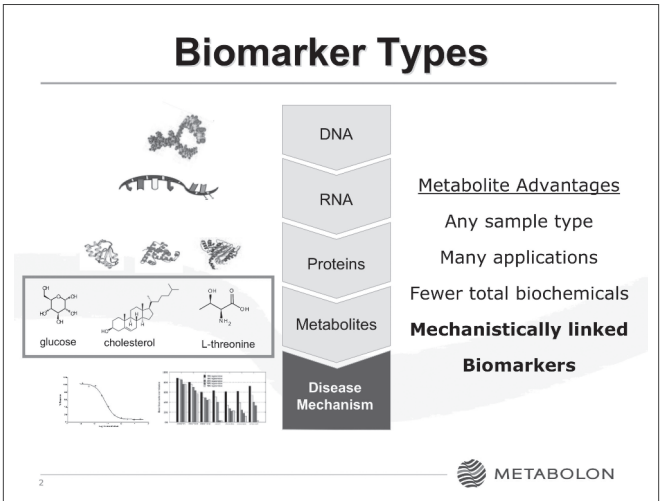

Figure 2.

Based on the analysis of small molecules, metabolomics analysis and interpretation are grounded in biochemistry. This provides a significant advantage to the interpretation of metabolomics experiments. Biochemistry is a mature, more highly developed field of science compared to molecular biology and proteomics. While the newer genomic/proteomic technologies have received the vast majority of attention in recent years, it is noteworthy that many Nobel Prizes in medicine before the 1960s were awarded in biochemistry.

Today, we routinely depend upon metabolite-based disease diagnosis. For instance, high glucose in urine was one of the earliest tests for diabetes, and cholesterol is used to measure the risk of heart disease. Metabolic panels of fatty acids, bile acids, sugars, creatine, creatinine, urea, etc. are routinely used clinically to assess organ function or risk for many diseases as well as for disease diagnosis. Clearly, the field of metabolomics is positioned to take advantage of this repository of biochemical pathway knowledge.

Another important advantage of metabolomics, especially for developing tests that rely on more noninvasive types of samples, is that essentially any type of sample can be analyzed. Metabolites can be routinely measured in urine, feces, sweat, saliva, blood, tissue, etc., most of which can often be challenging sources for obtaining genetic or protein information. A number of papers have been recently published describing the use of metabolomics assessment of more creative sample types for disease.[7-9]

The development of metabolomics as a tool to leverage this body of knowledge has not been straightforward. Most laboratories have focused on targeted metabolomic analysis, specializing in the measurement of 20 to 100 different metabolites, which, most often, are within a common class of compounds. For example, a number of companies and academic labs have developed methods for detecting lipid compounds. Although lipids represent a smaller set of the total biologically relevant metabolites, this data has proved useful for biomarker discovery efforts.[10]

Other groups have focused on methods to truly investigate all the small molecules in samples. "Global" or "unbiased" metabolomics has been plagued by difficulties stemming from the diverse physical properties of small molecules. These properties can vary greatly, with significant differences in solubilities and molecular weights ranging from 20 to >1000 Da.

It is therefore difficult to develop a single chromatography method to separate all the compounds and even more difficult to analyze individual compounds without chromatographic separation. Further complications arise if studies are expected to be completed with a reasonable turn-around time. These issues currently are being addressed through advanced multi-system approaches, where the best separation and detection instrument technologies are being developed to run in tandem. This approach allows for a comprehensive solution achieved by combining principles offered by various best-in-breed technologies. As this new technology develops and its use in biomarker detection studies increases, it is rapidly becoming clear that metabolomics will likely represent a high-impact technology in various health care related fields, such as the diagnosis of disease, identification of drug targets, evaluation of the effects of drugs, and selection of patients most likely to respond to drug therapy (i.e., personalized medicine).

Many biological effects of drugs and disease result from the overall health of an individual, as well as his or her environment, lifestyle and diet. While genetics can play an important part in predisposing an individual to drug side effects or disease, the biochemistry of an individual is likely a more informative measurement of an individual's current state and condition. Combining genetic predisposition with environmental and health status measurements that can be achieved with metabo-

lomics will likely be a very important biomarker discovery method of the future.

Global Metabolomics

Metabolon Inc. launched its commercial fee-for-service platform in 2004, following years of pioneering work in software development and mass spectrometry engineering.[11-13] To date, Metabolon has completed over 700 studies and analyzed over 70,000 study samples. The number of completed metabolomic studies has more than doubled every year since 2004. In addition, Metabolon owns an extensive portfolio of patents covering its analytical platform, technology and biomarker discovery using global metabolomics.[14-17]

Metabolon's process operates in essentially four steps, as shown in Figure 3. Step one is extraction of the small molecules from the biological sample. Step two is the chromatography coupled with mass spectrometry and data collection. Step three is the automated and manual QC analysis of the data using Metabolon's proprietary software. Step four, the final step, is the statistical and biological interpretation of the data itself. Metabolon has published methods of extracting a wide range of very polar to nonpolar compounds from as little as 50ul of blood plasma.[13] Extracted samples are split into four aliquots for different chromatography and mass spectrometry platforms, two UHPLC methods and one GC method, with one aliquot held in reserve. These three chromatography and MS systems complement each other in the range of biochemicals measured and provide an enhanced biochemical coverage of each sample. Approximately 70% to 80% of the biochemicals are measured on more than one platform, with 30% to 40% measured on all three platforms. For compounds observed on multiple platforms, the platform with the best analytical characteristics (e.g., fewest interfering peaks or highest signal to noise) is generally used for the analysis of that compound. In general, the GC method provides better separation of molecules that tend to be more difficult to separate using a typical reverse phase LC method (e.g., carbohydrates).

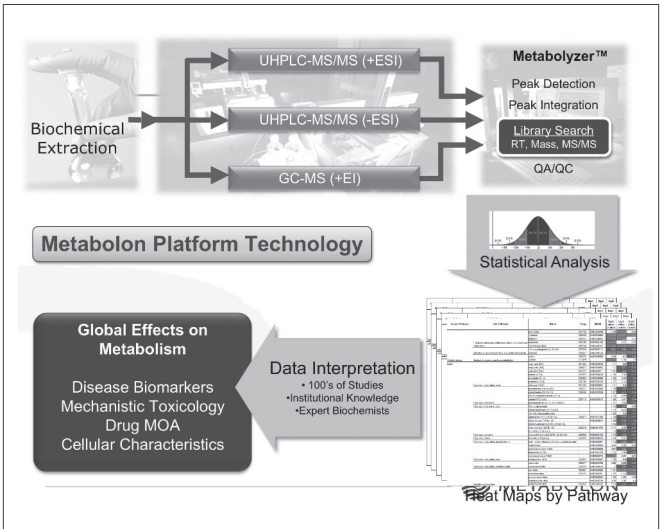

Figure 3.

After the raw data has been acquired from the instruments, Metabolon has developed a suite of software packages that automatically integrates each ion across retention time and then uses that ionic information, which may include additional MS/MS fragmentation information and retention time, to identify the compound. After a compound is identified in a sample, one of the characteristic and stronger ions is used to determine a relative concentration of that compound in each sample. This approach assures that the compound will be represented only once in the subsequent statistical analysis. When the software has finished analyzing the samples, all the data is loaded into a visual user interface that allows Metabolon scientists to curate the data for QC purposes, visually inspect how well each compound was identified, and verify only those compounds with the highest degree of confidence for inclusion in the final data set.[12]

A variety of statistical approaches can be applied to the final data set at that point, including ANOVA, t-tests, random forest, PCA, etc. The goal of these types of statistical treatments is to identify the biochemicals that best represent the most significant changes in concentration among the groups in the study. One advantage of biochemistry is that multiple compounds in a particular biochemical pathway may often be significantly altered, giving an even higher degree of confidence to the importance of that biochemical change. In this respect, it is important to point out that most statistical treatments assume independent variables when, in fact, we know that certain biochemicals are related to the same or similar pathways. Metabolon is developing a large database of these types of biochemical changes as well as those that result from toxicity, drug mechanism, disease, etc. This knowledge enhances Metabolon's ability to provide a biological interpretation for each study it performs.

Recently, Metabolon has started to build research and commercialization groups to develop and market diagnostic products in the health sciences that were discovered using its proprietary technology. While the technology can be applied for many types and stages of diagnostic development, Metabolon has primarily focused its diagnostic development in the area of patient management in cancer and diabetes. The principal goals for any of these diagnostic development projects are unmet medical need, commercial opportunity and applicability of the technology. As a result, Metabolon's metabolomic

platform represents a significant opportunity to bring a novel and highly tested technology to discover and develop new diagnostic assays in order to better aid patients in their medical care.

The Need for Simple Insulin Resistance (IR) Biomarkers

IR is a well-established risk factor for type 2 diabetes (T2D) and cardiovascular disease (CVD) progression.[18-24] IR and compensatory hyperinsulinemia are often associated with obesity. When coupled with ß-cell dysfunction, IR is a major pathophysiological determinant of dysglycemia (impaired fasting glycemia [IFG]), impaired glucose tolerance (IGT) and T2D.[25,26] Conditions of high CVD risk, such as hypertension, dyslipidemia and atherosclerosis, also have been associated with IR.[25-28]

Unfortunately, traditional clinical fasting plasma measurements do not directly assess IR. The gold standard for assessing insulin resistance is the hyperinsulinemic euglycemic clamp (HI clamp), which involves glucose and insulin infusions and requires insertion of two catheters into the patient and the patient to remain immobilized for up to six hours. As a result, the procedure is typically performed in a research setting and its clinical utility is limited due to cost and time constraints. Fasting insulin and derived indices (HOMA, QUICKI) have been widely used[29] to assess IR, but difficulties with insulin standardization and accuracy have prevented adoption in routine clinical practice. The identification of novel IR markers for detection of subjects at risk of IR and to stratify the risk of progression to T2D and/or CVD in subjects with IR in order to implement effective strategies for prevention, as well as for monitoring treatment response, remains an unmet need.

To discover new IR biomarkers, we obtained fasting blood-plasma samples from 399 HI-clamped subjects, all of whom were nondiabetic and clinically healthy. Roughly half the subjects were male and half were female; all were matched to age and their body mass index (BMI). Each clamped subject had his or her insulin sensitivity assessed resulting in a measured insulin-mediated glucose disposal rate, umol x min-1 x kg FFM-1. Subjects with less than 45 umol x min-1 kg FFM-1 were defined as insulin-resistant and belonged to the bottom tertile of the cohort analyzed (Figure 4).

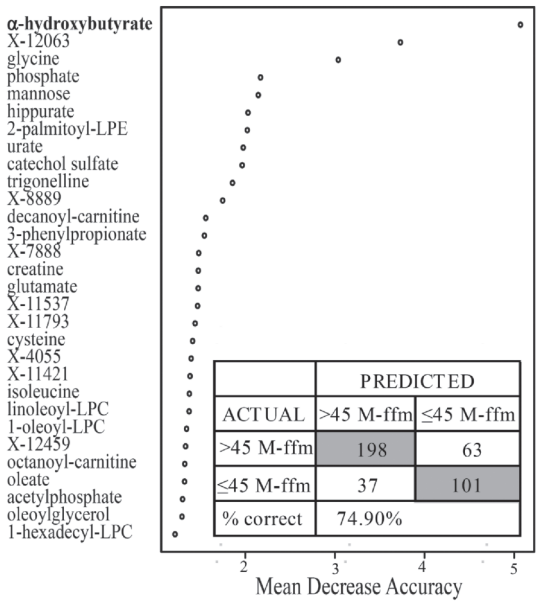

Figure 4.

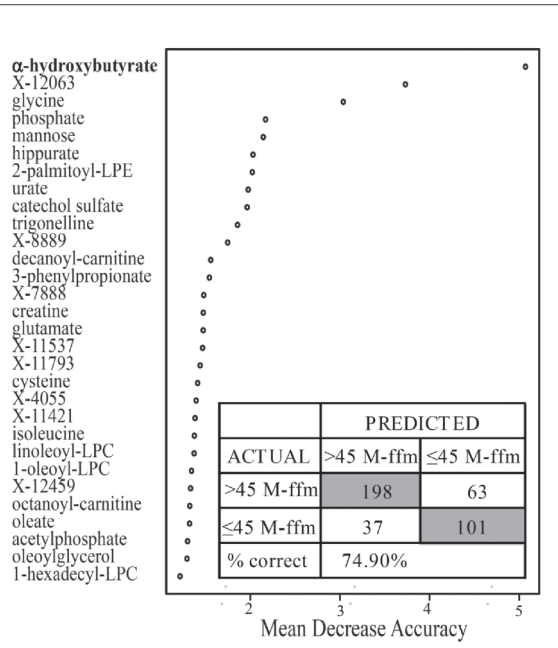

Figure 5.

Biomarker Discovery for IR Using a Global Metabolomics Analysis

A total of 471 metabolites were measured in the blood plasma samples collected from the 399 HI clamp subjects and analyzed for IR biomarkers as previously described.[30] The median relative standard deviation (MRSD), a quality assurance metric of quantification and a measure of instrument variability, was determined to be 8% for a panel of 30 internal standards. Overall process variability (i.e., extraction, recovery, resuspension and instrument performance) for endogenous biochemicals within technical replicate plasma samples was calculated to be 15% MRSD. These values reflect normal levels of variability for overall process and instrumentation of the screening platform.

To assess the ability to classify subjects as IS or IR, random forest analysis was performed using the entirety of the screening data (i.e., 471 compounds). As shown in Figure 5, α-hydroxybutyrate (α-HB) was the

top metabolite in the resulting importance plot, which ranks the classifiers based upon the contribution of each to the separation of the subjects into groups. In this analysis, the subjects were classified as either insulin sensitive (IS) or IR. IR was defined as less than 45 umol x min-1 x kg FFM-1, and IS was greater than 45 umol x min-1 x kg FFM-1. This result did not change when normalizing the M value for kg of body weight rather than kg of fat-free mass (FFM). A separate univariate correlation analysis of the data from the biochemical profiling screen also ranked α-HB as the metabolite with the highest correlation to the glucose disposal rate. The results are summarized in Tables 1 and 2. From a biomarker success criteria, it was very important that two completely independent statistical treatments, random forest and correlation analysis, resulted in the same top metabolite and further confirmed the likely importance of α-HB as a biomarker for IR.

Table 1. Summary of Top-Ranking Insulin Sensitivity Biomarkers from Global Biochemical Screen

Biochemical	Correlation Coefficient (r) M_{FFM}	*p*-Value
α-hydroxybutyrate (α-HB)	-0.45	1.40E-21
X-12063	-0.36	7.92E-14
glycine	0.33	2.79E-11
urate	-0.31	3.90E-10
X-12816	0.3	1.24E-09
α-ketobutyrate (α-KB)	-0.28	1.54E-08
catechol-sulfate	0.27	6.16E-08
trigonelline (N-methylnicotinate)	0.26	7.86E-08
phosphate	0.24	8.18E-07
decanoylcarnitine	0.24	8.89E-07

Table 2. Biochemicals that Separate NGT-IS from NGT-IR and IGT

Biochemical	NGT-IS vs. NGT-IR Rank	NGT-IS vs. IGT Rank
2-Hydroxybutyrate	1	1
creatine	2	2
1-Linoleoylglycerophosphocholine	3	25

Since the initial analyses were based upon data obtained from the nontargeted biochemical profiling technology, a targeted assay was developed to provide absolute quantitative results. As shown in Figure 6, whether measured by the screening platform or the targeted isotopic dilution assay, α-HB was consistently higher in IR than IS subjects ($p<0.0001$, for both the screening and targeted data).

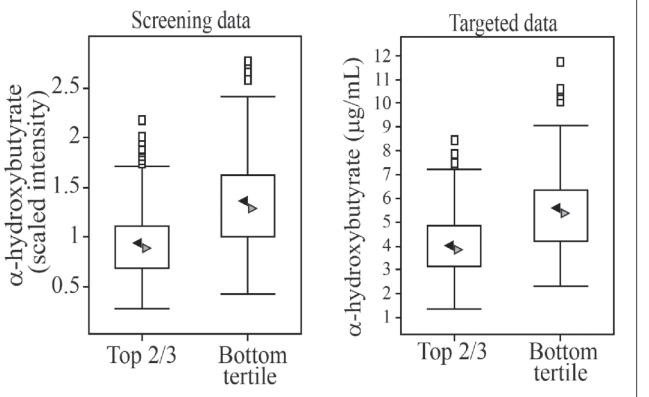

Figure 6.

A completely independent validation of α-HB was investigated by a random forest analysis of the oral glucose tolerance test that was performed on each individual in addition to the HI clamp. OGTT is a completely independent assessment of glucose metabolism (dysglycemia) that also is associated with IR and, although not as accurate as the HI Clamp measurement, is actually used more often clinically. In the OGTT analysis, individuals were classified as having normal or impared glucose tolerance, which is another indicator of insulin resistance. In this completely independent assessment of IR, α-HB was again consistently the highest ranking metabolite/biomarker in the importance plot from a random forest analysis of the two groups.

Next Steps for Developing an IR Biomarker

α-HB is an organic acid derivative of α-ketobutyrate (α-KB). Both the catabolism of threonine and methionine and the anabolism of cysteine lead to α-KB formation.[31] α-KB is subsequently metabolized to propionyl-CoA and carbon dioxide.[31] Lactate dehydrogenase (LDH) or α-hydroxybutyrate dehydrogenase (α-HBDH), an LDH isoform present in the heart,[32] catalyze the formation of α-KB, with α-HB being produced as a byproduct (Figure 5). It has been proposed that *in vivo* the LDH-catalyzed conversion of α-KB into α-HB occurs when either (1) the rate of α-KB formation exceeds the rate of its catabolism, which leads to substrate accumulation, or (2) there is product inhibition of the dehydrogenase that catalyzes the conversion of α-KB to propionyl-CoA.[31,33]

At least two mechanisms may lead to elevated levels of α-HB. In the first, increased lipid oxidation leads to an elevated NADH/NAD+ ratio, and in the second, there is an increased demand for glutathione due to elevation of hepatic glutathione stress. Increased lipid oxidation is a metabolic feature of IR and is indexed by the insulin-inhibited FFA concentration.[27,34] Our finding of a positive

association between clamp FFA and plasma α-HB concentrations in the whole cohort supports the possibility that an increased NADH/NAD+ ratio favors reduction of α-KB to α-HB. The second mechanism likely contributes to increased α-HB formation by supplying more α-KB substrate from increased cysteine anabolism (Figure 5).

Whatever the mechanism of increased α-HB, further work to validate the marker and demonstrate its utility for detecting IR and for monitoring intervention is needed. Most recently, the entire cohort of over 1,300 subjects who were clamped in the EGIR study, was assessed, and again, α-HB was the most significant biomarker correlated to glucose utilization. α-HB is unlikely to be as difficult to measure from fasting plasma samples, and its variation may be lower than other markers such as insulin. As shown in this study, a specific assay has now been developed and other biomarkers may enhance the assessment of IR from fasting blood samples. Given the unmet need for a practical clinical test that accurately measures IR in individuals, identification of α-HB may be an important new analyte for such a diagnostic test. In addition, a general metabolomics analysis has shown utility in the discovery of this new biomarker and further supports this new technology for biomarker discovery.

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