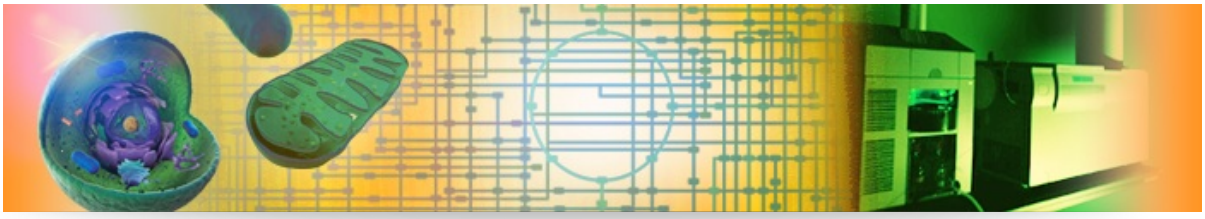

HMT Newsletter

Friends and Colleagues,

We are very happy to present a special report to you this month from our General Manager and Vice President for Research Planning, Douglas Osei-Hyiaman, MD, PhD. Dr. Osei-Hyiaman joined HMT last year to help develop our Biomarker Pipeline and Strategy. With the success HMT is having with the development of Phosphoethanolamine (PEA) for Major Depressive Disorder, Douglas will share with you his vision for metabolomics in Biomarker Development and the critical steps for success.

Sincerely,

Alexander Buko, PhD
Vice President
Human Metabolome Technologies America

Special Report

HMT Strategy for Biomarker Development

Douglas Osei-Hyiaman, MD, PhD

General Manager, Vice President for Research Planning
Human Metabolome Technologies Inc.

I. Patient Stratification

Virtually, every stage of a patient's care can be revealed by a biomarker, thereby providing the clinician with the right information to evaluate disease likelihood, diagnosis of the disease, determine the severity and progression of the disease, and drive decision on optimal therapeutic approach, as well as monitoring of therapeutic response.

Based on the circumstances of their applications, biomarkers can be classified into four main classes;

1. predisposition biomarkers

2. diagnostic biomarkers
3. prognostic biomarkers
4. predictive biomarkers

The different classes of biomarkers, all serve different purposes throughout the clinical stages to;
1. determine disease risk
2. diagnose early, a disorder / disease / condition
3. predict how a disease/disorder may develop or progress
4. stratify patients that are more likely to better respond to a specific therapeutic

Metabolomics biomarkers are therefore becoming increasingly important to;
1. provide opportunities to diagnose based on metabolites that are closely linked to phenotype
2. make a diagnosis that was previously difficult or impossible

HMT biomarker strategic approach covers the four main classes of biomarkers. Through in-house research and collaborative initiatives, HMT applies the unique technology of metabolomics to identify biomarkers, and assesses its passage and transition from biomarker candidate to a diagnostic tool. Upon thorough validation of the biomarker, it can be applied in disease diagnosis or in tailor-made therapeutics in personalized medicine for individual patients.

II. Omics Approach

Across biology and medicine, metabolomics has rekindled interest in metabolism, especially in the area of functional genomics and biomarker discovery; with significant potential advantages over classical diagnostic approaches and conventional clinical biomarkers, in terms of sensitivity and specificity. Unlike the classical monovariate approach, where single biomolecules are explored one at a time as biomarkers in a trial and error, time and labor intensive fashion, the -omics tools of genomics, transcriptomics, and proteomics utilizes the multivariate approach where multiple biomolecules are simultaneously identified and explored in relation to the disease phenotype in question.

The problems that arise when biomarkers are discovered with these three tools are two-fold: First, it is difficult to identify reliable markers of the phenotype, and to avoid false positives as a result of chance correlations; Secondly, identifying all candidate markers that fully characterize the effect or phenotype is time and labor intensive. Moreover, while DNA, RNA, or protein derived biomarkers may be good indicators of future risk of disease, they are not amenable to early diagnosis of an impending disposition to a disease phenotype.

The -omics tool of metabolomics at HMT, however, employs the multivariate approach to screen biological samples with an added advantage to identify near real time metabolites that can be further evaluated, and developed as biomarkers that better reflects the disease phenotype.

III. Biomarker Qualifications

For a biomarker to transition to a diagnostic tool, collection of the sample material must be less invasive, with little to no discomfort to the patient. Sample materials may be blood, urine, saliva, sputum, synovial fluid, ear wax, cerebrospinal fluid, tears, or nasal fluid etc. In order for the biomarker to effectively support the rapid initiation of therapy, it is crucial that the speed with which the biomarker test results are obtained is as fast as possible. An optimal rapid test results within a few minutes, hours, and days is essential: Depending on the disorder or disease, the turnaround time of the biomarker test may be different.

Generally, for readily lethal diseases/conditions, a quick biomarker test turnaround is key. However, for chronic diseases with prolong onset, a biomarker test turnaround time of days and weeks could be acceptable. Most importantly however, the detection method for the biomarker must be as accurate and as easy to carry out as possible; and inter-laboratory results must not be

significantly different from each other. Above all, the biomarker must have been independently well validated with proven effectiveness for diagnosis of the disease in question. Using our specific tools of metabolomics, **HMT is well positioned in the discovery and development of biomarkers for effective applications**.

IV. Metabolomic Strategies

Metabolomics studies can be sorted into two general categories:

1. Studies that seek to understand and **clarify biological mechanisms**
2. Studies aimed at the **development of biomarkers**.

The primary focus of mechanism based metabolomics studies is to gain an enhanced knowledge of biological processes through metabolic profile analysis, that is supported by a combination of statistical and machine learning tools, where interesting metabolites are derived post hoc. On the other hand, in metabolomics biomarker studies metabolites selections are determined prior to the derivation of a definitive multivariate predictive model. Furthermore, while a long metabolite list, or a multivariate model generating hundreds of molecular features may be useful for understanding the pathways and mechanisms of a biological process, they are however, not useful for the cost-effective development of a biomarker test. Instead, from the metabolite pool, a final list of about 1 to 10 biomarker candidates, is considered mathematically realistic and for a practical clinical testing purpose.

Potential biomarker researchers and developers need to understand this purpose difference before initiating their studies. **HMT has the platform and the expertise to support either need**. Obviously, it is also possible that after a biomarker has been selected a clear understanding of the mechanism by which the biomarker plays a role in the phenotype is necessary, then metabolomics based mechanisms studies could follow. This article provides a brief overview of HMT's strategy for biomarker screening and development; from metabolomics to the development of target assay, sample size for feasibility and target validation studies; and to finally highlight some current **HMT biomarker pipeline** from in-house as well as collaborative research outputs, including an update on HMT's diagnostic biomarker for major depressive disorder (MDD).

V. The 3 Keys to the HMT Strategy

Metabolomics to development of target assay: The HMT strategy for metabolomics biomarker discovery, from screening to development follows three key essential steps that ensure marker accuracy: The general study design principle includes additional intercalated stringencies at various vantage points in the process to maintain quality, consistency, and overall end result integrity for an unbiased decision on the marker. Key features of the strategy consists of,

1. **Maintenance of sample source adequacy and integrity**: After defining the intended use of the biomarker, cases and controls are prospectively enrolled, and sample collection and outcomes determined for clinically relevant populations: From this cohort, retrospective random selection of cases and controls are made. Throughout the discovery process, specimen handling and assays are carefully blinded; and finally, the resulting metabolomics data is evaluated with relevant commercial, as well as, proprietary statistical methods and presentation support tools to make it easier for the client to use the wealth of information.

2. **Establishment of biomarker performance measures**: First, a quantitative index of biomarker effectiveness (M) is established within the intended clinical application; a value for a useful biomarker (M1), and a value for a useless biomarker (M0).

3. **Robust Group size calculation**: How would the biomarker effectiveness be estimated from the study data? What criteria based on the biomarker effectiveness and sampling variability would the biomarker be judged and considered potentially promising? Determine the proportion of useful markers expected to pass filter criteria (**discovery power**), along with proportion of

useless markers that will pass the filter criteria (**False Lead Expected percent**), and finally, use variable group sizes and acceptable criterion to derive acceptable values for *Discovery power and FLE%.*

VI. Group Size is critical for success

Group size for feasibility and validation studies: In order to evaluate the accuracy of a diagnostic biomarker, the group size plays a critically important role in estimating or testing accuracy. Using a small group size will yield an imprecise estimation of accuracy resulting in a wide confidence interval, which for clinical decision making, is non-informative. On the other hand, overly large group size is a waste of resources especially when the cost of the diagnostic test from the biomarker is going to be expensive. Unfortunately, very few clinical investigators of diagnostic biomarker studies report group size calculations, and most clinicians are quite oblivious about this lack of essential information for end users. Oftentimes researchers arbitrary decide their group size based on convenience, or on previously published article. It is critical to consider the prevalence of the phenotype (disorder, disease, or condition) in the formula for group size calculation to estimate sensitivity and specificity of the potential biomarker. Group size without knowledge of the prevalence of the phenotype may be adequate for estimating specificity or sensitivity, but would be inadequate for both, because of the lack of actual knowledge of true phenotype/disease status during sampling from the target population.

Several factors affect group size for diagnostic biomarker accuracy: Based on a general statistical rule of thumb for group size calculation, because both sensitivity and specificity are proportions, they are used to guide the four essential elements of group size calculation: First, since the standard error of sensitivity or specificity depends on its value, it is important to pre-determine the value of sensitivity (or specificity) based on existing information such as data from the published literature, or based on the clinical judgement; Second, the confidence level (1-α) of statistical judgement where " α " is the probability of type 1 error; Third, the precision estimates of sensitivity (or specificity), which is the maximum difference between the estimated sensitivity (or specificity) and the true value. Above all, the investigators must be aware of the disease/phenotype prevalence in the population, and this value should be considered in the group size determination. Essentially, the goal is to estimate the number of subjects affected as well as the unaffected separately so as to yield a total number of subjects that is large enough for each group. In actual practice however, it is the sensitivity as opposed the specificity that determines the total number of subjects for the study. When the true condition or disease status is known prior to inclusion of subjects for the new diagnostic biomarker, the prevalence of the phenotype is no longer considered in the group size calculation for sensitivity/specificity.

Moreover, for testing purposes, sensitivity difference under the null and alternate hypothesis is required (probability of type II error). Finally, one must be aware that generally, with higher precision in accuracy estimation, and a detection of a small difference of effect in testing of accuracy with higher power, a larger group size is required.

VII. Our HMT Biomarker Pipeline

Currently the HMT biomarker pipeline targets **major depressive disorder, colorectal cancer, non-alcoholic steatohepatitis (NASH), diabetic nephropathy and infection-associated encephalopathy**. While most are still under development; HMT, in collaboration with clinicians and academic researchers, identified a metabolite Phosphoethanolamine (PEA) in the plasma of patients with MDD.

Ethanolamine phosphate level was found to be consistently low in patients with clinically diagnosed MDD. When tested alone, PEA has over 90% sensitivity and specificity, yet in conjunction with a clinical score for depression, PEA achieves about 100% sensitivity and specificity for MDD. Low plasma level of PEA distinguishes MDD from other forms of mental disorders including schizophrenia, bipolar disorder, and other forms of anxiety disorders. An international multi-center clinical studies in the US, Japan, Europe, and China is underway to

validate the diagnostic utility of PEA in MDD patients. HMT has recently released a beta version of its PEA clinical assay kit for large scale clinical studies. Major depressive disorder is a serious global condition with unmet medical needs. It is hoped that this type of diagnostic biomarker could play a role in early detection of patients at risk for depression in the primary care setting and referred to mental health specialists for professional support before full-blown depression complications ensue.

VIII. In Conclusion

In this era of increasing constraints on resources, HMT recognizes the need for reliable partnerships for biomarker research and development. In the Biopharmaceutical and biotechnology fields metabolite biomarkers of target biology and therapeutic efficacy are driving the drug development process. Furthermore, in toxicology, metabolomics application is the most closely linked to the classical knowledge of dysregulated biochemical processes. Based on in depth understanding and experience with metabolomics service, and research on clinically relevant metabolite biomarkers for diagnostics, therapeutic response, and drug discovery; **HMT metabolomics technology and expertise can contribute significantly to your biomarker discovery** from target identification, validation, and development of target assay throughout the clinical development phases moving forward. As metabolomics advances, small molecule metabolites will play an essential role in biological systems, and represents attractive candidates for understanding disease phenotypes.

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HMT Updates

Press release

HMT and Toyobo have established production of Ethanolaminephosphate phospho-lyase for clinical assay relating Major Depression Disorder (MDD)

On October 19, 2017, HMT and Toyobo Co., Ltd. have announced that they have

collaboratively established mass production technology for the enzyme Ethanolamine-phosphate phospho-lyase used for measurement of a depression-related biomarker.

More details

Featured articles

Plasma Nervonic Acid Is a Potential Biomarker for Major Depressive Disorder: A Pilot Study

Kageyama Y., *et al., Int. J. Neuropsychopharmacol., in press*.

For the screening of diagnostic biomarkers of major depressive disorder (MDD), bipolar disorder (BD), and schizophrenia (SZ), a comprehensive metabolome analysis was performed for plasma from drug-free patients with MDD, BD, SZ, and matched healthy controls. A significant effect of diagnosis was found for nervonic acid and cortisone, but nervonic acid showed the most significantly alteration. The reproducibility and effects of psychotropic medication on nervonic acid were verified in an independent sample set of medicated patients and controls (n = 90), and confirmed that plasma nervonic acid levels were increased in the depressive state in patients with MDD compared with the levels in the remission state in patients with MDD and the depressive state in patients with BD.

Distinct transcriptional and metabolic profiles associated with empathy in Buddhist priests: a pilot study

Ohnishi J., *et al., Hum. Genomics.,* 11: *21*.

Spiritual/religious involvement may have beneficial effects on both psychological and physical functions, and accompany wide range of biological markers, including transcripts and metabolites. The blood from ten professional Buddhist priests and 10 matched non-priest controls were analyzed by gene expression and metabolic profiles. The metabolomics analysis revealed some metabolites were elevated in the Buddhist priests whereas there was no significant difference of healthy lifestyle behaviors and daily nutrient intakes between the priests and the controls in this study. Spearman's rank correlation analysis showed that empathy aspects in the priests were significantly correlated with the certain transcripts and metabolites.

Identification of Characteristic Components and Foodstuffs in Healthy Japanese Diet and the Health Effects of a Diet with Increased use Frequency of these Foodstuffs

Iwagaki Y., *et al., Mol. Nutr. Food Res., in press*.

Recent study showed that the 1975 Japanese diet exhibited strong health benefits. To develop a diet with even higher health benefits, mass spectrometry for analysis of Japanese diets was performed to determine the characteristic components in the 1975 diet. PCA proposed14 characteristic components found in fish, fruits, vegetables, seaweed, soybean foods, soup stock "dashi", and fermented seasoning. Based on the list, the modified diet was prepared

and fed to mice. It induced decrease in liver total cholesterol and serum LDL cholesterol compared with feeding original 1975 diet. In addition, the modification also resulted in the increase of serum adiponectin and decrease of serum TBARS and IL-6. The result proposes potential approach to improve health benefits of food.

Edited by Takushi Oga, PhD

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