

Researcher Perspective



## Stable Isotopes in Drug Development and Personalized Medicine: Biomarkers that Reveal Causal Pathway Fluxes and the Dynamics of Biochemical Networks

**Marc Hellerstein, MD, PhD**

Professor of Human Nutrition (Calloway Chair), University of California at Berkeley  
Professor of Endocrinology, Metabolism and Nutrition, University of California at San Francisco  
Co-Founder and Chief of SAB, KineMed, Inc., Emeryville, CA 94608 USA

The combination of stable isotope labeling with powerful mass spectrometric analytic techniques is providing increasingly important diagnostic tools for drug development and clinical diagnostics in the emerging era of personalized medicine.

### The Problem: High Attrition Rates in Contemporary Drug Development

Although it is widely believed that we live in a golden era of breakthroughs in new medicines, the opposite is true. Recent years have witnessed the lowest rate of new drug approvals in a generation, despite greatly increased pharmaceutical industry investment.<sup>1</sup> These disappointing facts hold true for all classes of disease, but are particularly worrisome for growing epidemics of chronic disease, such as Alzheimer's disease, diabetes, osteoarthritis and obesity-related disorders.

The problem is not a lack of molecular targets or candidate drugs. The molecular target-based approach to drug discovery, which has dominated pharmaceutical research for the past 20 years, has generated huge lists of genes, proteins and potential drug therapies.

The problem is that the attrition rate of drug leads has gotten worse, not better, with >98% of leads now failing for efficacy or safety reasons, including 90% failure rates in human trials.<sup>2,3</sup> This attrition is largely responsible for the high cost of each successful drug eventually approved.

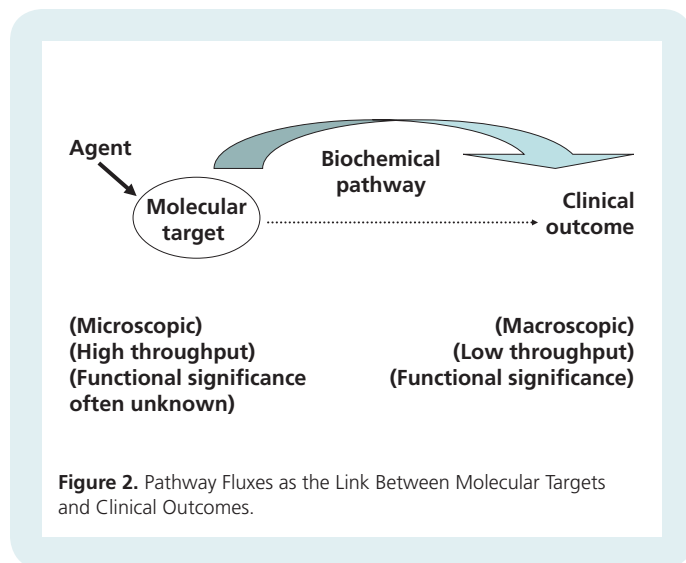
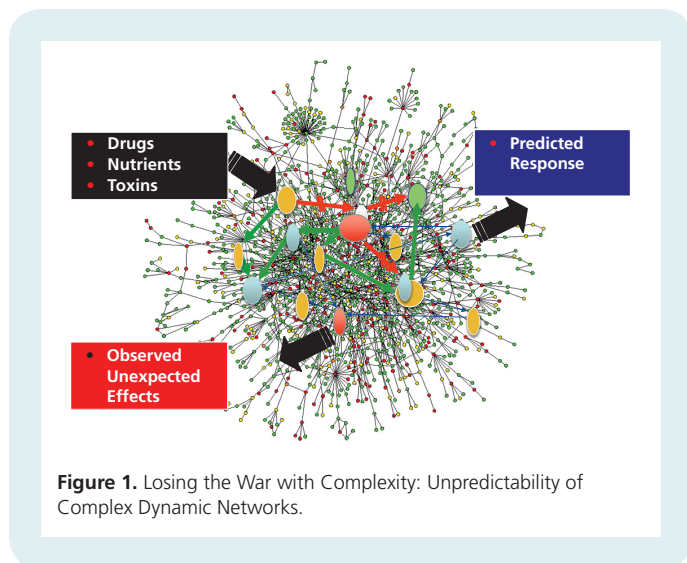
### Losing the War with Complexity

Attrition, in turn, is largely due to the unpredictability of the complex networks that comprise living systems in response to targeted interventions at specific nodes.<sup>2</sup> Unanticipated functional consequences of targeted interventions, both undesirable and beneficial, are the rule rather than the exception in such systems (Figure 1). Pathogenic heterogeneity among individuals within each disease magnifies this problem, requiring different intervention strategies for different subsets of patients. The latter issue is embodied by the notion of personalized medicine.

### The Missing Link: Metrics for Navigating through the Complex Biology of Disease

The key missing factors for navigating through the complex biology of disease are objective measures that guide drug developers toward the goals of safe and efficacious outcomes.<sup>4</sup> These metrics, called biomarkers, must be predictive of clinical outcomes and translatable from preclinical models into humans. The most reliable way to achieve these goals is to capture the underlying biologic processes driving each disease (*i.e.* the disease modifying pathways or underlying pathogenesis). Metrics of this type can serve to guide rational drug discovery and development and allow monitoring of clinical response.

Nowhere will this need for functionally informative biomarkers be greater than in the field of "personalized medicine"—the right



(continued)

patient, the right drug, at the right time, and in the right dose. Companion diagnostic tests are extremely high value examples of this trend.

### Stable Isotopes Are Essential for a New Class of Biomarkers: Tests that Predict Clinical Outcomes by Revealing Functionally Interpretable Information about Underlying Disease Processes

A new class of biomarkers is needed that are predictive of clinical outcomes.<sup>4,5</sup> The biologic pathways that underlie chronic diseases – the causal processes responsible for initiation, progression, severity and therapeutic reversal of disease – generally involve the flow of molecules through a pathway that is itself complex and influenced by numerous factors<sup>5-8</sup> (Figure 2).

Stable isotopic techniques have made all of these causal pathways measurable in higher organisms.

#### What Stable Isotopes Bring to Diagnostic Biomarkers

In the following discussion, the underlying principles and recent examples of stable isotope-based biomarkers will be briefly reviewed.

Stable isotopes allow fluxes through metabolic pathways and the dynamics of global biochemical networks to be measured, without toxicity and often non-invasively, for two reasons: first, experimental administration of stable isotopes introduces an “asymmetry” in the dimension of time (label not present, then present), which allows the timing of dynamic processes to be measured; and, second, biochemical research over the past century has established the pathways that link molecules in cells and organisms, allowing the fates of labeled substrates to be traced *in vivo*.

Importantly, stable isotopes have been used for over 70 years in humans and experimental animals and have almost no known toxicities. The FDA policy toward stable isotope-labeled products is clear and has been consistent for decades: no regulatory approval is required to administer stable isotope-labeled compounds, beyond what is needed to administer their natural abundance congeners (sterility, pyrogenicity, etc.). It should be noted that stable isotopic-mass spectrometric biomarkers are not radiographic imaging techniques, but require a sample from the body (blood, urine, CSF, tissue, saliva).

#### Two Broad Categories of Stable Isotope-Based Kinetic Biomarkers Are Available

There are two broad categories of stable isotope-based biomarkers that are most useful in drug development and diagnostics: (1) Kinetics of targeted causal pathways and, (2) Interrogation of network dynamics for unbiased discovery of kinetic signatures and unanticipated causal pathways. Both types are available and useful in drug discovery and development.<sup>5-16</sup>

**Table 1. Examples of Causal Pathways:**

#### A) Neurobiology

- Cargo transport through axons
- Amyloid beta synthesis and plaque turnover
- Neurogenesis
- Myelination/remyelination
- Neurotransmitter release and turnover
- Neuronal mitochondrial biogenesis
- Neuroinflammation, microglia activation
- Cytokine release
- Huntingtin protein turnover
- Prion turnover
- Synaptic plasticity

#### B) Obesity / T2DM

- Pancreatic beta cell proliferation and mass
- Insulin-mediated glucose uptake
- Hepatic glucose production
- Adipogenesis and TG deposition
- Adipose tissue fatty acid oxidation/brown fat transition
- Adipose tissue remodeling
- Hepatic TG synthesis and release
- Atheroma cholesterol removal and deposition
- Adipose tissue macrophage proliferation and activation
- Muscle mitochondrial beta-oxidation and biogenesis

#### C) Cancer / Neoplasia

- Tumor cell proliferation and death rate
- Angiogenesis
- Lymphangiogenesis/metastatic spread
- Tumor-specific T-cell proliferation
- DNA methylation/demethylation
- Ribonucleotide reductase activity
- Histone deacetylation
- Precancer evolution to aggressive phenotypic
- Extracellular matrix turnover

#### Kinetics of Targeted Causal Pathways as Biomarkers for Drug Discovery and Development

Some common examples of causal pathways in disease are shown (Table 1). These include: synthesis of collagen and extracellular matrix in fibrotic diseases; myelin synthesis and metabolism in multiple sclerosis; turnover of amyloid plaque and synthesis of amyloid beta 1-42 in Alzheimer's disease; synthesis of muscle myosin and biogenesis of mitochondria in sarcopenia; angiogenesis and proliferation and death of tumor cells in cancer; transport of cargo molecules through axons in neurodegenerative conditions; autophagic flux in Huntington's, Parkinson's and other diseases characterized by protein aggregates; clot formation and lysis in thromboembolic diseases; insulin-mediated glucose uptake and pancreatic beta cell proliferation in insulin-resistant states; adipose tissue lipid dynamics and remodeling in obesity; reverse cholesterol transport in atherosclerosis; activation of the complement cascade in inflammatory states; HIV replication and turnover of CD4+T-cells in AIDS; and many others.

The ability to measure the activity of any of these functionally relevant processes that are believed to play causal roles in disease is potentially transformative for drug discovery and development in these fields (e.g. Parkinson's Disease.<sup>10,11</sup>).

### Interrogation of Network Dynamics

Perhaps the most exciting advance in stable isotope biomarkers in recent years is the emergence of "Network Dynamics": unbiased interrogation of the dynamic behavior of complex biochemical networks that comprise living systems. This has been successfully applied to preclinical models and humans for the dynamics of the global proteome, or Dynamic Proteomics.<sup>12,13</sup> This provides a new type of systems biology, with great potential as an unbiased screening tool for biomarker discovery.

Dynamic Proteomics represents the most functionally interpretable of the "omics" technologies – *i.e.*, providing not just heat maps or informatics, but functionally interpretable systems biology information. The operational flow chart for measuring the dynamics of a proteome is shown (Figure 3). This approach has been applied with great success to questions such as the effects of calorie restriction of cellular proteostasis, including mitochondrial biogenesis and mitophagy; the proteome dynamic signature of poor prognosis in chronic lymphocytic leukemia tumor cells; differentiating between pancreatic islets successfully compensating for insulin resistance in obese animals vs. islets that are failing and becoming "exhausted"; the effects of exercise on muscle proteome turnover; the effects of neuro-inflammation on CSF proteome turnover; the dynamics of the high-density lipoproteins (HDL) proteome in dyslipidemic states; and other questions of interest in physiology and pathophysiology.

### 'Virtual Biopsy' Approach for Non-Invasive Biomarkers of Intracellular Pathways

Unbiased screening of proteome dynamics in a tissue can also lead to discovery of targeted protein biomarkers that are accessible to sampling in a body fluid. Called the "virtual biopsy" technique (Figure 4), this is a powerful method for measuring the rate of protein synthesis or protein breakdown in an inaccessible tissue of origin, such as skeletal muscle, heart, brain, kidney, liver, or a cancer tissue, through a measurement made from an accessible body fluid, such as blood, cerebrospinal fluid, saliva or urine. The method comprises administering a stable isotope tracer (e.g. Deuterium oxide (D, 70%) (DLM-4-70); L-Leucine (<sup>13</sup>C<sub>6</sub>, 99%) (CLM-2262); Glycine (<sup>15</sup>N, 98%) (NLM-202); Spirulina whole cells (lyophilized powder) (U-<sup>15</sup>N, 98%+) (NLM-8401)) that is metabolically incorporated into newly synthesized proteins. These proteins then escape into an accessible body fluid, from which they are isolated and analyzed for isotopic content or pattern. The measured replacement rate of the escaped protein reflects the synthesis or breakdown rate of the protein back in the tissue of origin. A "virtual biopsy" of the tissue of origin has thereby been carried out.

The "virtual biopsy" method has utility for discovering and validating biomarkers for use in drug discovery and development, for identifying disease subsets in personalized medicine and for clinical diagnosis and management of patients. This approach has been developed and applied to blood-based measurements of tissue fibrosis and skeletal muscle protein synthesis and CSF-based measurements of axonal transport of cargo<sup>10</sup> and neuro-inflammation. An example is plasma creatine kinase-MM (derived from skeletal muscle), for measuring skeletal muscle protein anabolism from a blood test. Many other applications can be envisioned.

### In Situ Kinetic Histochemistry: Combining Histopathology with Stable Isotopes and Mass Spectrometry

It is also now possible to visualize the kinetics of targeted molecules of interest spatially, within a histopathologic specimen.<sup>14</sup> Linking spatial histologic information with molecular flux rates provides a remarkable new dimension to pathologic diagnosis and monitoring of disease. This can be carried out by either laser microdissection or physical microdissection of slides (Figure 5). An example of tissue microdissection after introducing stable isotopes has been published for prostate cancer. The proliferative gradient of prostate cells, for example, has been shown to correlate closely with histologic grade in biopsy specimens from men with prostate cancer and is reflected by the proliferative rate of prostate epithelial cells isolated from seminal fluid, as a potential non-invasive biomarker.<sup>14</sup>

### Kinetic Imaging of Tissue Samples

Kinetic or metabolic flux imaging is now possible by combining stable isotope labeling with mass spectrometric imaging of tissues, through NIMS or MALDI-based spatial visualization of histologic slides. Spatially defined kinetic lipidomics in cancer models has revealed anatomic differences in tumor behavior that correlate with *in vivo* aggressiveness in mouse mammary cancer models.<sup>15</sup>

### Practical Uses of Stable Isotope-Based Biomarkers in Drug Development

There are many uses for stable isotope-based biomarkers in drug discovery and development (Table 2). These include target validation; translating preclinical results rapidly into man; "quick-kill" of agents or classes with poor activity against the targeted pathway; identifying the right subsets of patients for treatment; identifying optimal dose, regime, measurement end-points and inter-subject variability of response; medical personalization (companion diagnostics); and anticipating toxicities or avoiding toxicities through dose-adjustment. Translational markers that are predictive of disease outcomes also allow the selection of animal models that best reflect human disease, or the de-emphasis or even gradual elimination of animal models from the drug-development process.

**Table 2. Applications of Causal Pathway Metrics**

Less guessing about:

1. Picking targets
2. Choosing chemical class and best compound in class
3. Identifying the right patients (excluding nonresponders subsets at risk for toxicities)
4. Finding the best dose and regimen for clinical trials
5. Selecting intermediate end-points to measure and variability to expect in patients
6. Dosing to avoid minimize toxicities
7. Testing whether personalization can improve response
8. Deciding whether to get out early (quick kill)

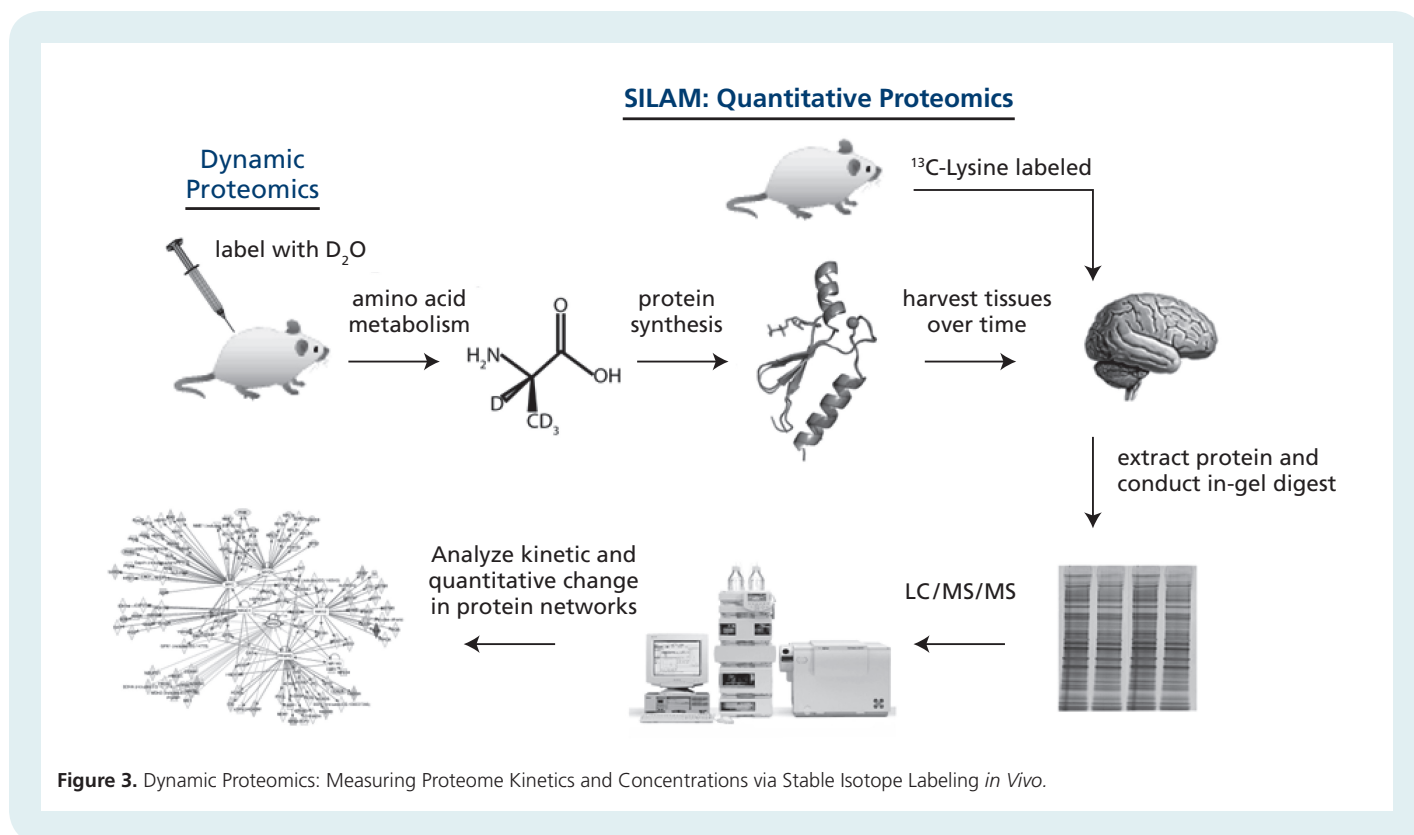
### Stable Isotope-Based Kinetic Biomarkers Have Advantages over but Are Complementary to Static Biomarkers

Traditional static biomarkers provide information about the concentration, presence or structure of molecules in a living system. In contrast, kinetic biomarkers reveal the dynamic behavior of the pathways that lead to and from these molecules. The amount of collagen in a tissue, for example, does not reveal the rate at which collagen is being synthesized (fibrogenesis) in a disease setting or after starting a therapeutic intervention. Nor does the content of mitochondrial proteins tell us the degree to which mitochondrial biogenesis or mitophagy was induced by an intervention. Similarly, the concentration of a protein in the cerebrospinal fluid does inform us the efficiency at which neurons

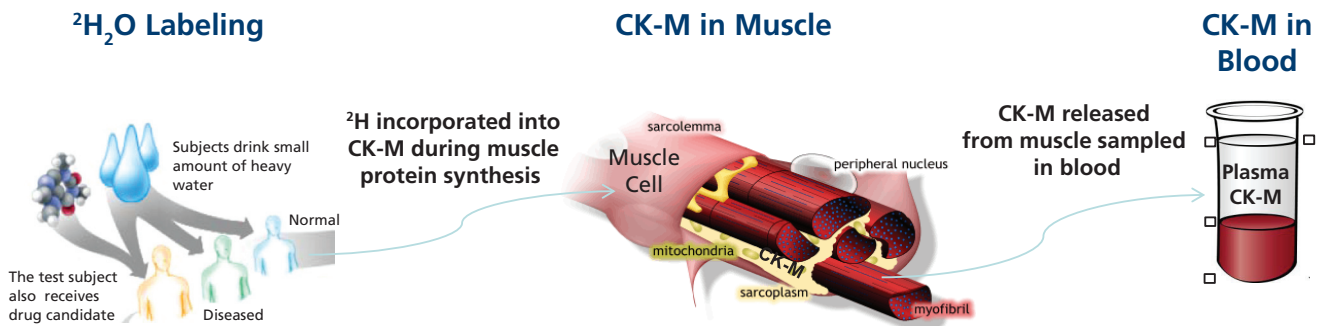
in the brain transported this molecule through axons to nerve terminals. These latter processes all involve, at their core, the flux of molecules through often complex pathways and networks.

The activity of these pathogenic processes or disease pathways are in principle the metrics most closely related to the initiation, severity, progression and therapeutic reversal of a disease. The only way to measure molecular flux rates is by the introduction of isotopic labels, as noted above. Although static parameters can provide key complementary information, such as pool size and net gain or loss of a molecular component, the functional activity of underlying pathogenic processes can only be revealed through kinetic measurements.

The same considerations apply to “Network Dynamics,” such as dynamic proteomics, when compared to static “-omics” biomarkers, but with an additional point that is worth noting. Protein synthesis and breakdown rates typically represent a pro-active decision by a cell or organism that is functionally interpretable in context of health or disease. By way of example for proteins, ubiquitin-proteasome-based removal, transcription factor-stimulated synthesis, assembly during biogenesis of an organelle, packaging and secretion in vesicles, modulation through the unfolded protein response, deposition as extracellular matrix, induction as part of a protein signaling cascade, etc. – these can all be thought about in functional terms by physiologists, toxicologists and clinicians. The same cannot always be said for



**Figure 3.** Dynamic Proteomics: Measuring Proteome Kinetics and Concentrations via Stable Isotope Labeling *in Vivo*.



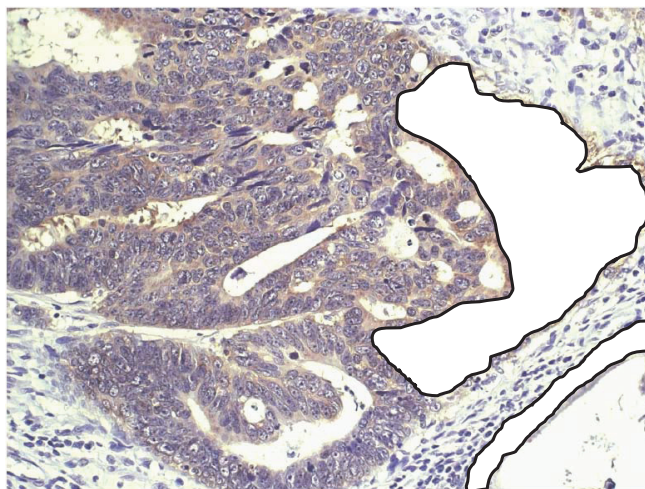
**Figure 4.** "Virtual Biopsy" Technique for Kinetic Biomarkers. Example of Skeletal Muscle Protein Synthesis from Plasma Creatine Kinase M-type (CK-M).

the simple presence or concentration of a protein. Because of this marriage between intrinsic functional significance and broad, hypotheses-free screening, dynamic proteomics is a particularly powerful technology for biomarker and target discovery.

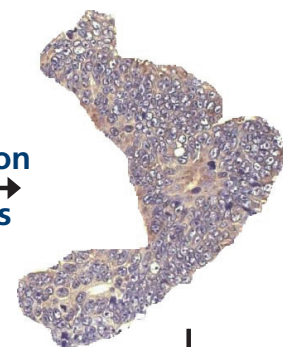
### Summary and Conclusions

In summary, the recent addition of stable isotope-based biomarkers to the diagnostic repertoire has brought a new and rapidly expanding dimension to drug development. These biomarkers provide functionally interpretable, decision-

relevant information about the underlying biology of disease, capturing the activity of causal pathways that are the driving forces underlying disease and therapy. Kinetic biomarkers thereby predict clinical response and its relation to target engagement or the effects of a clinical treatment regimen. Stable isotope-based kinetic biomarkers are particularly powerful additions in the emerging era of personalized medicine.



Micro-dissection  
of tumor cells



Into microfuge tube

Derivatize & analyze

**Figure 5.** Micro-dissection of Normal and Tumor Tissues for Mass Spectrometric Kinetic Analysis.

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