Systems-Level Nutrition Approaches to Define Phenotypes Resulting from Complex Gene-Environment Interactions

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Abstract
High-throughput metabolomic, proteomic, and genomic technologies have delivered 21st-century data showing that humans cannot be randomized into groups: individuals are genetically and biochemically distinct. Gene-environment interactions caused by unique dietary and lifestyle factors contribute to the heterogeneity in physiologies observed in human studies. The risk factors determined for populations (i.e. the population-attributable risk) cannot be applied to the individual. Developing individual risk/benefit factors in light of the genetic diversity of human populations, the complexity of foods, culture and lifestyle, and the variety in metabolic processes that lead to health or disease are significant challenges for personalizing dietary advice for healthy or diseased individuals.

Introduction
Two of the great advances in biomedical research over the past 100 years were the standardization of experimental designs, specifically the case-control design [1], and the one gene-one polypeptide concept that emanated from the groundbreaking work of Beadle and Tatum [2]. These contributions, in addition to a few other seminal discoveries (e.g. elucidation of the DNA structure), laid the conceptual framework for 20th-century biological research. Much of the research of the last 80 years is what Kuhn [3] called normal science. This type of research activity does not produce new concepts, but rather finds facts to match
and better explain the existing framework. Normal science standardizes experimental designs that ultimately are considered mandatory for interpreting data and publishing results. Scientific dogma holds, for example, that the only trustworthy results of human studies are from prospective randomized controlled trials (RCTs) [4, 5]. Similarly, many studies in humans or laboratory animals are reductionistic in analyzing how, for example, a single gene or a single nutrient correlates with some physiological effect [2].

Revolutions can occur when normal science produces experimental data that cannot be explained by existing paradigms [3]. The outcomes of this paradigm shift have profound implications for biomarker identification and development that are necessary to assess nutrition and lifestyle choices for maintaining or improving personal and public health.

**Analyzed Heterogeneity Supplants Randomization**

In addition to the elaborations of human experimental designs, legal developments occurred over a long period that began with the passage of the US Food, Drug, and Cosmetic Act in 1938. The Cosmetic Act required that new drugs undergo premarket safety evaluation, although the legal mandate to prove efficacy was not enacted until 1962. The Drug Amendment Act specifically required adequate and well-controlled clinical investigations with positive findings from at least two clinical studies [6]. The scientific precedents for these legal actions were built on the success of analyzing the efficacy of antibiotics [6]. Infectious bacteria (e.g. *Vibrio cholera*) are extrinsic agents that affect virtually all humans. Hence, the average response between the treated group and the control group in RCTs can provide proof of the efficacy of the treatment. In contrast, drugs, nutrition, and lifestyle choices become intrinsic factors in that they interact with and affect internal physiological functions.

Unlike extrinsic agents, intrinsic factors may be metabolized by or interact with physiological systems. Humans (and all species) not only show biochemical individuality [7] at homeostasis, but may also respond differently to drug or food chemicals [8–10]. Differential responses occur because each individual is unique [11] and genetic variations may be differently expressed in response to nutrients and other factors. Heterogeneity within a species is, of course, the fundamental basis by which natural selection acts to produce evolutionary changes. The statistical result of RCTs is the population-attributable risk (PAR), defined as the number (or proportion) of cases that would not occur *in a population* if the factor were eliminated [12] – they are not individual risk factors [13]. While PARs are applicable for large effect sizes and extrinsic agents, the utility of PARs is diminished
by the heterogeneity of many individual genetic makeups added to the calculations, the latent effects of many interacting environmental factors (dietary chemicals and activity levels), and the resulting individuality of metabolic responses [14–16]. The human system, which is comprised of a person’s genetic makeup, microbiome, diet, lifestyle, and resulting physiology, acts on (metabolizes) treatments, and the treatments differentially alter the physiology depending upon the individual. The result of this heterogeneity can be (and often is) that the distribution of metabolic measures (or responses) in the case group can overlap with the distribution of metabolic measures (or responses) in the control group [17].

Since the pregenomic era had limited methodological tools to analyze human variation at the genetic or physiological level, randomization was essential for 20th-century biomedical research. However, genome sequencing has not only demonstrated genetic individuality [reviewed in ref. 11, 18, 19] but can now be used to completely characterize genetic makeups of study participants. Recent data [20, 21] suggest that each person differs from others and the reference genomes by about 3.5 million single nucleotide polymorphisms (SNPs), almost 1,000 large copy number variants (CNVs), and large numbers of insertions and deletions. Different levels of DNA methylation and therefore epigenetic regulation have also been demonstrated [22–24]. Variation in the (epi)genetic makeup may express itself in variation in RNA abundance [25, 26] and therefore levels of proteins and enzymes. To add to this complexity, physiological variability is influenced by the human microbiome [27, 28], the combination of all microorganisms that reside on the skin, in saliva, in the oral mucosa, in the conjunctiva, in the gastrointestinal tract, and in the vagina. Each of these factors alone or in combination could alter the level of a biomarker.

Nutrition and lifestyle not only alter the expression of information encoded in the genome [29], they can also modify the epigenome [30] and the microbial composition [31]. No experiments are needed to demonstrate that individuals have heterogeneous dietary intakes and physical activity levels. The result of interactions among (epi)genetic, microbiome, nutritional, and environmental factors is that humans have heterogeneous metabolomic profiles [32–34] in health and disease states. Physiological variability has been recognized for centuries and summarized in the modern pregenomic era by Williams [7] in 1956 in a book entitled *Biochemical Individuality*, and by nutritionists of the 1960s and 1970s [35, 36]. Many (but not all) of the previously unmeasurable, molecular characteristics of individuals can now be analyzed.

Medical practitioners and health professionals treat individuals and not population groups [35]. Individual risk factors are unknown, even though the concepts of personal determinants of health were taught by Hippocrates and Galen centuries ago. In addition to Williams’ [7] treatise in 1956, others also expressed
the need and approach to analyze individuals rather than groups [35–40]. More recently, we [41–44] and others [45–47] have been promoting or using n-of-1 aggregation and analysis methods [45, 48]. The concept of n-of-1 studies is that each person serves as his or her own control. Physiological assessments are usually done before and after a treatment [39] or intervention. The results of each trial (that is, from one individual) can then be aggregated for statistical analysis [45]. For example, we aggregated results from data obtained at homeostasis to analyze group average differences between males and females [48], but also found that each individual varies in micronutrient levels. This variation could then be used to associate patterns of plasma protein levels and variations in the genetic makeup [41].

Most -omes will remain incompletely characterized because they are so complex in composition and because they vary in time and space. Nevertheless, the incomplete molecular data sets can be analyzed to generate a model that predicts new markers to test in remaining samples of the experiment, or as new markers in the follow-on experiment in an iterative process of refining the model. Regardless of the new methodological and analytical approaches, the quantitative postgenomic data demonstrating human metabolomic and physiological heterogeneity should have profound impacts on the design of human research studies and, specifically, the validity of RCTs for determining optimal nutrition or drug treatments. Hence, even though not all physiological variables can be analyzed, randomization becomes unnecessary since 21st-century technologies produce enough data to more completely characterize each individual.

**Systems Thinking Supplants Reductionism**

Biochemical research over the past 100 years identified pathway maps consisting of individual components and sequential enzymatic reactions [2] for metabolizing carbohydrates, lipids, and amino acids, for synthesizing and degrading nucleic acids, for producing energy, for transporting metabolites, proteins, and nucleic acids, and for regulating hormone status. Studies of intracellular signaling in cellular growth and death pathways extended metabolic maps to signal transduction and gene-regulatory functions. However, even these regulatory networks were reduced to 2D maps of interconnecting components. Many of these pathway maps were derived from studies of disease rather than health processes, which is not reflected in those representations. Nevertheless, the beauty and simplicity of these maps leads to the illusion of explanatory depth [49] – a concept that elegant visual figures can appear as reality rather than that they represent simple one-to-one relationships that are frozen in time and space.
Pathway maps were and continue to be highly influential in guiding the design of experiments to elucidate the mechanisms of physiological disorders such as cancer, diabetes, obesity, cardiovascular disease, and other chronic diseases. However, relying on pathway maps to design experiments explicitly reduces the complexity of a phenotype to single (or a few) reactions in a pathway regardless of the experimental model (i.e. transgenic animals, cell model systems, or humans). For example, high fasting glucose levels, a hallmark of type-2 diabetes, may be caused by overproduction of glucose through gluconeogenesis in the liver, increased absorption of dietary carbohydrates in the intestine, decreased production of insulin from the pancreas, or insulin resistance [44, 50, 51]. Understanding one of these pathways does not provide comprehensive understanding of type-2 diabetes, which results from a complex set of interactions between multiple genes and multiple environmental factors.

Many researchers in the nutrition community also adopted reductionistic experimental designs by analyzing the effects of a single nutrient on complex phenotypes including health and disease states. Since most nutrients are ingested in small amounts, exposure to safe and small increases in the test nutrient over baseline intake requires months for a physiological or anthropometric effect large enough to be measured. A nutrient, of course, cannot be given alone but must be consumed in the background of a normal diet, which is not only complex, but can vary between individuals. Nota bene – individuals deficient in a single nutrient can be successfully treated by a reductionistic approach: worldwide incidence rates of rickets (vitamin D), beriberi (thiamine), scurvy (vitamin C), pellagra (niacin), and night blindness (vitamin A) have all been reduced by single-nutrient interventions in malnourished individuals [52–55]. However, the impact of subclinical undernourishment and the needs of populations in different environments and with diverse cultural, genetic, and agricultural histories are unknown.

The focus on individual genetic variants or the identification of independent environmental factors [55–57] needs to evolve toward a more comprehensive analysis of nutrient intakes, environmental and lifestyle factors, genetic make-ups, and physiology [58]. High-throughput omic technologies have generated paradigm-shifting data that challenge the conceptual basis not only of RCTs but also experimental reductionism.

Systems-level designs and analyses of high-dimensional data have been reported for studies of cardiovascular disease, obesity, diabetes, nutrition, drug intake, toxicology, immunology, gut microbiota, medicine, health care, and health disparities [59–67]. These systems-level reports analyzed the patterns within one or at most two data type(s), i.e. the interaction network of metabolites or between metabolites and proteins. In most of these publications, the system was closed since variables such as diet intake, lifestyle, or other environmental
variables were not measured or included in the analysis. Excluding external factors that influence internal biological processes generates at best an incomplete system, and likely an inaccurate understanding of the interactions between environment and genetic makeup. From a practical standpoint, such a design misses an opportunity to identify modifiable factors that influence health. Biological processes occur in open systems [68], and ex vivo factors including nutrients and other naturally occurring chemicals in food can alter biochemical processes and signaling networks occurring within the organism [29]. Several recent reports included dietary intake variables as a part of omic-based systems [69–71] or genomic analysis [41, 48, 72]. These studies are consistent with a paradigm shift from a focus on individual genetic variants or the identification of single independent environmental factors [56–58] toward a more comprehensive analysis of nutrient intakes, environmental and lifestyle factors, genetic makeups, and physiology [58].

**Systems, Heterogeneity, and Biomarkers**

The reality of human heterogeneity and the view of physiology as a system are not abstractions, but have consequences for the design of experiments [42] and interpretation of data [41, 48], including the discovery, verification, and validation of biomarkers (table 1). Metabolites (e.g. cholesterol, calcium, and homocysteine), proteins (e.g. insulin), supramolecular complexes (LDL or HDL particles), or conditions (e.g. blood pressure) have long been used as clinical biomarkers. The omics revolution accelerated biomarker research of disease [73–78] and health [79]. Genomic technologies based on SNPs in candidate genes [80], CNVs [81], transcriptomics [71, 82, 83], and microRNAs [84, 85] provide a new means to explore multiple biomarkers for pathologies. However, the majority of biomarker discovery studies were conducted using RCTs and hence result in PAR rather than individual risk. Most individual biomarkers and additive combinations of SNPs identified by genome-wide association studies explain between 1% (obesity) and 20% (ankylosing spondylitis) of the phenotype of interest [86]. Geneticists refer to the remaining variability as missing heritability [87, 88], apparently ignoring that many phenotypes are the result of gene-gene (epistatic [89]), epigenetic (e.g. DNA methylation [90]), and gene-environment interactions [91].

Systems thinking is starting to influence nutritional [41, 48, 69–72, 91, 92] and immunological [66] research, although significant challenges remain in adapting and applying these advances to human studies [93]. More specifically, metabolic processes underlying health, disease initiation, disease progression, and disease
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>Closeness of agreements between the value of the measured and the true concentration in that sample</td>
<td>98</td>
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<tr>
<td>Biomarker</td>
<td>A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenetic processes, or pharmacologic responses to a therapeutic intervention</td>
<td>96, 100, 101</td>
</tr>
<tr>
<td>Clinical end point</td>
<td>A characteristic or variable that reflects how a patient feels or functions, or how long a patient survives</td>
<td>96, 100, 101</td>
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<tr>
<td>Diagnostic predictability</td>
<td>Ability of the test to predict the presence or absence of a disease for a given test result and determined by calculating the positive and negative predictive values Positive predictive values are the proportion of patients with positive test results who have the disease, while negative predictive values are the proportion of patients with negative test results who do not have the disease</td>
<td>98</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Differentiation of efficacy or safety of a drug within the same class</td>
<td>77</td>
</tr>
<tr>
<td>Homeostasis</td>
<td>The steady states of systems and physiologies in an organism – the constancy of the internal environment in two separate states: sleep and awake</td>
<td>102, 103</td>
</tr>
<tr>
<td>Hormesis</td>
<td>A dose-response relationship phenomenon characterized by low-dose stimulation and high-dose inhibition</td>
<td>104</td>
</tr>
<tr>
<td>Nutritional phenotype</td>
<td>Defined and integrated set of genetic, proteomic, metabolomic, functional, and behavioral factors that form the basis for the assessment of nutritional status</td>
<td>105</td>
</tr>
<tr>
<td>Prognostic factor</td>
<td>Individuals with disease have biomarkers that are predictive over time and require evidence for validity Comparative and equivalent to risk factors</td>
<td>106</td>
</tr>
<tr>
<td>Reliability/repeatability</td>
<td>Ability to replicate tests to yield the same results under the same measurement conditions</td>
<td>98</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>Describes measurements performed under different conditions</td>
<td>98</td>
</tr>
<tr>
<td>Risk factor</td>
<td>Individuals without disease have biomarkers that are predictive over time and require evidence for validity Comparative and equivalent to prognostic factors</td>
<td>106</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Proportion of individuals who test positive for a given biomarker: reflects the true positive rate</td>
<td>98, 99</td>
</tr>
<tr>
<td>Specificity</td>
<td>Proportion of individuals without symptoms who yield negative results: reflects false-positive rate</td>
<td>98, 99</td>
</tr>
<tr>
<td>Stratification</td>
<td>Select patients to increase likelihood of therapeutic success</td>
<td>77</td>
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<tr>
<td>Surrogate end point (or outcome)</td>
<td>A biomarker intended to substitute for a clinical end point A clinical investigator uses epidemiologic, therapeutic, pathophysiologic, or other scientific evidence to select a surrogate end point to predict clinical benefit, harm, or lack of benefit or harm</td>
<td>96, 101, 106</td>
</tr>
<tr>
<td>Trueness</td>
<td>Closeness of agreement between the average value of different samples and the true concentration value</td>
<td>98</td>
</tr>
</tbody>
</table>

See also [http://www.genomicglossaries.com/content/Biomarkers.asp](http://www.genomicglossaries.com/content/Biomarkers.asp).
outcome are linked networks whose robustness and individual variability are poorly understood. A likely reason is that biomarkers are discovered and analyzed individually. For example, a genome-wide association study statistically corrects p values of each SNP individually for multiple comparisons. A systems view of interacting processes is that patterns of markers will better indicate the state of the system than single markers. For most complex phenotypes, a biomarker will have a different effect size in each individual depending on gene-gene, epigenetic, and gene-environment interactions (fig. 1). This concept was explained for genetic contributions to complex disease phenotypes by Peltonen and McKusick [94].

Fig. 1. Monogenic versus complex disorders. Monogenic diseases are caused by (in this case, an autosomal dominant) mutations in a single gene and hence the genetic risk is the same for each family or individual who inherits it. Pedigrees can be used to trace the presence of the mutation. Affected individuals are shown with a black symbol with white X (mutation) in the pedigree. Complex disorders (or phenotypes) are caused by genes (e.g. A, B, C, and D) having one or more variations (SNPs, CNVs, insertions, and deletions) and epigenetic factors interacting with other genes (epistasis) and with environmental factors. Pedigrees reveal no Mendelian inheritance pattern due to the effect size of each variant in the particular genetic background of the individual (represented by the size of the letters in the pedigree). PAR factors (figure labeled population) for each variant in each gene (A, B, C, and D, with the pattern of the box matched to the risk factor in the graphs) are the average of each variation in the tested population. The phenotype in different families or individuals may be impacted differently by each of these variants (family 1, 2, and 3 in the graphs). Some genes that predispose individuals to disease might have minor or no effect in some families/individuals (gene D, family 2). This figure was adapted from Peltonen and McKusik [94].
An added complexity for biomarker discovery is the dynamic nature of health and disease processes. Since most of these diseases have a late onset, biomarkers are typically associated with surrogate end points which, ideally, would be equivalent to the clinical end point [95, 96]. However, the same clinical end point can result from imbalances in different organs, pathways, and genes, which has been acknowledged as a confounder in the development of single biomarkers [97, 98]. A case in point is type-2 diabetes mellitus, as explained earlier. Multiple biomarkers will be needed for each chronic disease [98] and likely for stages of each disease. Subsets of the pathways and genes for each surrogate end point may be differentially affected by long-term exposure to diet and other environmental factors [44, 51], which may alter the reliability, reproducibility, trueness, and diagnostic predictability of their measurement.

Health-related biomarkers have an added level of complexity since many of the parameters will have small deviations from normal values in different individuals, and added strength must be gained from the use of combinations of single biomarkers (biomarker profiles based on additivity or multiplicity of effects). So far, these profiles have been (mis)used as statistical observation for group differences without a mechanistic or functional linkage, and usually in the absence of knowledge about long-term nutritional status. The biologically (and nutritionally) relevant profile (or interactome-representing biomarker) will only emerge if these relationships are established through quantitative assessment of the overarching processes [79]. Based on these concepts, each disease or health biomarker in a panel will have a probability of diagnostic predictability and, more importantly, the panel itself will have a probability associated with its predictive efficacy. Developing these biomarker panels will require novel experimental designs for discovery [42], verification, and validation. The era of personalized health care and nutrition will not emerge until genetic heterogeneity, environmental complexity, and physiological variability are taken into account when conducting human biomedical research. However, the result of including nutrition in research will result in evidence-based knowledge that can be applied to improving human health through nutrition and lifestyle choices.

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