Review

Challenging homeostasis to define biomarkers for nutrition related health

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A primary goal of nutrition research is to optimize health and prevent or delay disease. Biomarkers to quantify health optimization are needed since many if not most biomarkers are developed for diseases. Quantifying "normal homeostasis" and developing validated biomarkers are formidable tasks because of the robustness of homeostasis and of inter-individual diversity. In this paper, we discuss the science, strategies, and technologies for measuring parameters that define individual health. The following concepts are central to define the physiology of the healthy individual: (i) responses to a challenge of homeostasis will be more informative than static homeostatic measures; (ii) processes involved in maintaining homeostasis usually are multi-factorial and require quantitative analyses of the many individual components involved; (iii) health includes a large variation in "normality" and the effects of nutritional interventions may remain hidden in this "diversity of robustness," if incompletely analyzed. Specifically, comprehensive multi-parameter ("omics") analysis may identify key parameters (biomarkers) and lead to a greater understanding of health supporting processes. Perturbation tests that accurately target aspects of the overarching drivers of health (metabolism, oxidation, inflammation, and psychological stress) may be instrumental in creating knowledge for maintaining health and preventing disease through nutrition.

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1 Introduction

Determining optimal dietary intakes to maintain health requires a means of assessing the physiological effects of macro- and micronutrients, toxins, and non-nutritional bioactives. Identifying biomarkers which can be used to determine health *in individuals* has proven difficult, because "health" is usually described by the absence of diagnostic parameters above a disease threshold. This definition may not be adequate for the specific purpose of optimizing health because: (i) processes involved in disease and disease progression are not necessarily the same as those involved in health optimization or disease prevention, (ii) homeostasis acts to maintain levels of many functional biomarkers within a limited range, masking early effects or predispositions under "normal" or "resting" conditions, and (iii) large inter-individual differences in "normal" values exist.

Ideally, biomarkers of health should quantify the subtle but relevant effects in the healthy status that precede the onset of disease, specifically identify predispositions, or predict our capacity to deal with environmental (including dietary) and age-related stresses. Recently, nutrigenomics and related novel analytical methods have been postulated [1, 2] to assist in the identification of new biomarker profiles or patterns among metabolites, proteins and transcripts, to generate accurate health assessment diagnostics. However, in general these approaches were applied to the resting state and the results primarily provided measures of the robustness of homeostasis rather than predictors of disease initiation or progression. In addition, the application of

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Abbreviations: HR, Heart rate; **OGTT,** oral glucose tolerance test

Table 1. Definitions in biomarker discussions

Accuracy	Closeness of agreements between the value of measured and the true concentration of the measured in that sample	[5]
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention	[30, 31]
Clinical endpoint	A disease, symptom, or sign that constitutes one of the target outcomes of the trial	[30, 31]
Diagnostic predictability	Ability of the test to predict presence or absence of disease for a given test result and is 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, the negative predictive value are the proportion of patients with negative test who do not have the disease	[5]
Homeostasis	The steady states of systems and physiologies in an organism – the constancy of the internal environment in two separate states – sleep and awake	[60, 98]
Hormesis	A dose – response relationship phenomenon characterized by low-dose stimulation and high-dose inhibition	[99]
Nutritional phenotype	Defined and integrated set of genetic, proteomic, metabolomic, functional, and behavioral factors that form the basis for assessment of nutritional status	[100]
Prognostic factor	Individuals with disease have biomarkers that are predictive over time and require evidence for validity. Comparative and equivalent to risk factor	[32]
Reliability/Repeatability	Ability to replicate tests to yield the same results under the same measurement conditions	[4, 5]
Reproducibility	The degree to which measurements under different conditions show the same results	[5]
Risk factor	Individuals without disease have biomarkers that are predictive over time and require evidence for validity. Comparative and equivalent to prognostic factor. A risk factor is a variable associated with an increased risk of a certain outcome	[31]
Sensitivity	Proportion of individuals who test positive for a given biomarker and reflects the true positive rate	[5, 6]
Specificity	Proportion of individuals without symptoms who yield negative results and reflects false-positive rate	[5, 6]
Surrogate endpoint (or outcome)	A biomarker intended to substitute for a clinical endpoint. A clinical investigator uses epidemiologic, therapeutic, pathophysiologic, or other scientific evidence to select a surrogate endpoint to predict clinical benefit, harm, or lack of benefit or harm	[31, 33]
Trueness	Closeness of agreement between the average measured value of different samples and the true concentration value	[5]

omics technologies showed that the utility and validity of some biomarkers vary with age, further emphasizing inter-individuality [3]. These two bottlenecks (robustness of homeostasis and inter-individuality) may be addressed by combining a nutrigenomics approach with homeostatic challenge tests. This concept is proposed and discussed in this paper.

2 The state of biomarkers of disease

Since the early 1990s [4], considerable effort has been made in discovering, qualifying, verifying, and validating [5] biomarkers (see Table 1) with diagnostic or prognostic utility for cancer (*e.g.*, [6]), obesity (*e.g.*, [7]), oxidative damage (*e.g.*, [8]), diabetes [9], cardiovascular diseases [10], and neurodegeneration (*e.g.*, [11, 12]). Metabolites (*e.g.*, cholesterol, calcium, homocysteine), proteins or complexes (LDL or HDL particles), or conditions (*e.g.*, blood pressure) have long been used as clinical biomarkers. Genomic-based technologies (single nucleotide polymorphisms (SNP) in candidate genes, transcriptomics) provided additional tools to develop multiple biomarkers for pathologies [13–19].

Many of these studies and initiatives developed biomarkers without assessing the effect of environmental influences or their suitability to address these, even though it is known that dietary chemicals alter the expression of genetic information (see below). This was taken up by nutrition researchers who applied these biomarkers to their research by, for example, linking nutrient intakes to genomic health [20], the nutritional modulation of gut health [21], effects of SNPs–nutrient interactions on one carbon metabolism [22] and related SNPs [23], the influence of fatty acids on SNP–phenotype associations (*e.g.*, [24]), and best practices for integrating nutrition and proteomic [25, 26], metabolomic [27], and transcriptomic [28] studies on human health.

While the development of biomarkers for personalized nutrition is complex, the apparent much more straightforward translation of new biomarkers into clinical disease assessments and personalized medicine has been slow [29]. Various disciplines have formed working groups to improve quantitative and statistical methods for verifying and validating biomarkers ([4, 6, 30–32], and http://www.c-path.org) or formed collaborative networks for improving gene–disease association studies [33], a prerequisite for a marker with a disease. Best practices and standardization of surro-

gate endpoints and development paths will improve reliability, reproducibility, trueness, and diagnostic predictability of biomarker measurements (see Table 1 and [32]).

3 Identification of biomarkers in nutrition research

The development of nutrigenomic knowledge as well as biomarker development for disease are complicated because of genetic heterogeneity, environmental diversity, and physiological complexity [34]. Developing biomarkers for health status also faces several significant obstacles. Nevertheless, it may become approachable if we take the following three aspects into consideration; overarching processes, complexity of the healthy-related biomarkers, and individuality.

3.1 Overarching processes

Balanced nutrient intakes for individual genotypes are necessary for health optimization and prevention of disease. Although many diseases are caused by the effects of unbalanced nutrition, the molecular pathways causing disease progression and final phenotypes, which are typically analyzed for biomarker development, may not necessarily be the same as the gene–nutrient interactions that maintain health. More specifically, we propose that many diseases are initiated by imbalances of major "overarching processes" where nutrition has a function in maintaining homeostasis. We define four overarching processes: (i) metabolism, (ii) oxidative stress, (iii) inflammation, and (iv) psychological stress (Fig. 1), which are closely related on cellular, organ, and organism levels.

An example is colon cancer. While it is well established that the hereditary form (~5–6% of the total cases) is caused by multi-step mutations in specific genes [35], the majority of cases are caused by complex and unbalanced gene–nutrient interactions in many different pathways. Hence, strategies to prevent familial or sporadic colon cancer are limited to general advice such as increase in intake of fiber, vegetables, fruit, and lowering intake of refined foods and meat (http://www.coloncancerfoundation.org/prevention.htm). Few studies have examined genetic susceptibility that cause differences in individual risk of developing the disease (*e.g.* interaction was seen between genotype and glycemic index for colon cancer risk [36]) or the overarching processes that may contribute to cellular and genomic stability. Host–microbe interactions and intestinal cell proliferation, both influenced by diet [37, 38], may promote a milieu that is "pro-carcinogenic." In addition, inflammatory processes such as those associated with inflammatory bowel diseases [39] may also play a role by stimulating or contributing to susceptibility or tumor progression [40]. In support of this, evidence is accumulating

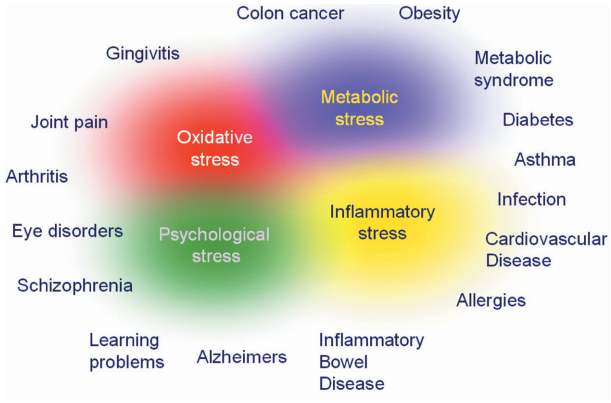

Figure 1. Overarching processes. Health is not the absence of disease but the maintenance of overarching processes controlling health status. The interaction of metabolic, oxidative, inflammatory and psychological processes determines major components of the health status. Related stress causes development of many related diseases.

that non-steroidal anti-inflammatory drugs may prevent colon cancer [41]. Obesity and its related pathologies also have a strong inflammatory component and neurodegenerative pathologies are linked through nutrition by these overarching processes (*e.g.*, [42, 43]). These processes are linked networks whose robustness and individual variability are poorly understood. Analyzing their interactions and quantifying their status in relation to nutrient intakes is a perquisite to guiding health choices in individuals.

3.2 Health-related biomarkers

Since most complex diseases are late onset, biomarkers are typically associated with surrogate endpoints which, ideally, would be equivalent to the clinical endpoint [30]. However, the same clinical endpoint can result from imbalances in different organs, pathways, and genes, which has been acknowledged as a confounder in the development of single biomarkers [6]. For example, the control of serum glucose levels depends upon secretion of insulin from the pancreas, tissue responsiveness to its presence, glucose uptake and subsequent metabolism by multiple organs and tissues [44, 45]. Patients may have imbalances in one or more of these pathways, have different surrogate endpoints, and yet still have the same clinical endpoint. Multiple biomarkers will be needed for each chronic disease [6] and perhaps stages of each disease. Subsets of the pathways and genes for each surrogate endpoint may be differentially affected by individual genetic differences, diet, and other environmental factors [34, 46], which may alter the reliability, reproducibility, trueness, and diagnostic predictability of their measurement. An example of the complexity is the demonstration that changes in gut microbiota affect plasma lipopolysaccharide levels and inflammation [47]. Alterations in microbiota in the intestine also controlled meta-

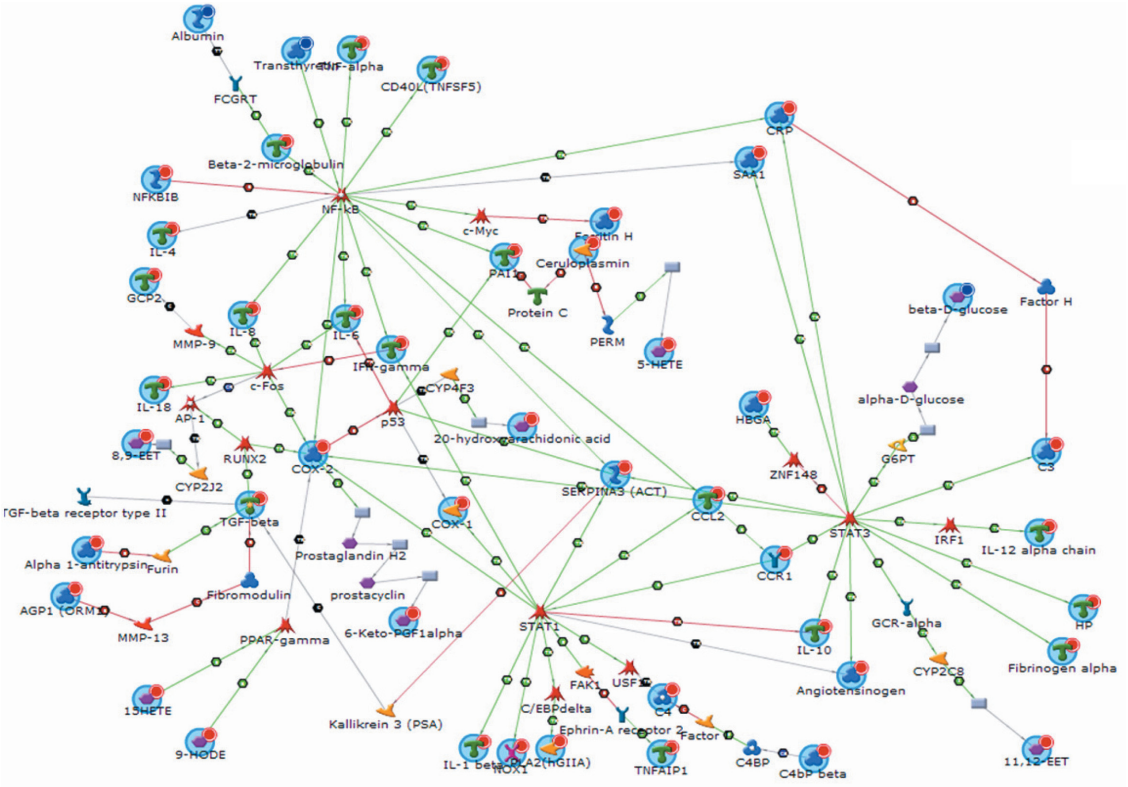

Figure 2. Complexity. Overarching processes are (by nature) complex as the human organism constantly needs to subtly fine-tune these processes in response to changing and challenging environment. This complexity can be visualized and the responses can be analyzed using pathway tools. One example is the human plasma inflammation interactome map which shows a large number of proteins and metabolites in plasma involved in inflammatory processes and their biological interaction. The figure has been generated by the Genego Metacore™ software. While the map focuses on inflammation, it includes nodes (*e.g.* p53) which are not usually associated with inflammation. This illustrates interdependency of different processes.

bolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice [47]. Endotoxemia induced by antibiotic treatment or high fat feeding altered metabolism in a manner to initiate obesity and insulin resistance [48]. While these studies focus on the microbiome, a network of pathways will be influenced as a consequence (Fig. 2 as an example). Genetic variation and the interaction of dietary factors (and other environmental factors) will influence the health of individuals differently. Health therefore is a continuous trait involving multiple organs, pathways, and genes interacting to maintain homeostasis, exposed to and reacting to a very varying environment. Physiological adaptation to support a healthy status of functional homeostatic parameters will involve adaptation of different genes, pathways and tissues and will depend on environment, age, sex, and the genetic constitution.

Inflammation is now recognized as one of the drivers for the onset of many nutrition related diseases. The ability to react appropriately to inflammatory stress thus is one of the overarching processes involved in maintaining optimal (nutrition related) health. Inflammatory status is usually quantified by markers like C-reactive protein, interleukin 1, TNF-alpha, and fibrinogen. Yet, many more plasma proteins and metabolites are altered by inflammation. As an example, the long chain omega-3 fatty acids ecosapentanoic acid (EPA) and docohexanoic acid (DHA) affect inflammation pathways in multiple organs (liver, adipose, muscle, endothelium, gut), have acute versus chronic responses, and alter concentration of lipid mediators and feedback regulators. The large number of inflammatory factors, most of them detected in plasma, with their interrelationships are shown in Fig. 2.

Health-related biomarkers have an added level of complexity, as (apart from the reasons above), most of the factors will have small deviations from "normal" values in different individuals and physiological adaptation may involve many different pathways.

Added strength must therefore be gained from the use of combinations of single biomarkers; that is, "biomarker profiles" based on additivity or multiplicity of effects. So far, these profiles have been used as statistical observations for group differences without a mechanistic or functional linkage and usually in the absence of knowledge of long-term

nutritional status. The biologically and nutritionally relevant profile or interactome biomarker will only emerge if these relationships are established (Fig. 2) through quantitative assessment of the overarching processes.

3.3 Individuality

Our health is determined by our genes and environment, and George Orwell's words "All animals are equal, but some animals are more equal than others" also holds true for genotype and phenotype. Genetic individuality is well established and a subset of the >11 million known SNPs (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD = Preview&DB = snp) contributes to the heterogeneity that is observed in the diversity of health states. Associating SNPs or haplotypes with phenotypes (disease or HDL, glucose levels, etc) or with a response to a nutrient identifies genes that may cause or contribute to the complex trait. Many of the relevant variants of these disease genes likely to be involved in the key overarching processes and constitute a part of the biomarker panels for a given phenotype. These gene variants and biomarkers validated in genetic or environmental epidemiological studies produce population attributable risk (PAR) factors which may or may not predict risk for an individual of different genetic make-up and lifestyle [49].

Apart from genetic make-up, diet contributes to inter-individual differences by altering gene – nutrient interactions directly [46] or through multiple pathways: (i) epigenetic changes caused by DNA and chromatin protein modifications produce phenotypic variation [50, 51]; (ii) Direct diet – transcriptome interactions complement the variation [52 – 54]; (iii) many other gene-environment interactions (*e.g.*, drugs, toxins, exercise) contribute to phenotypic individuality. A classical nutritional example demonstrates the result of all of the above arguments: plasma concentrations of most vitamins vary within a healthy population but also within a single person over time [55]. The resulting variation in "healthy" biomarker ranges poses problems to the classical approaches but may also provide new opportunities in analyzing subpopulations that may respond differently than other subpopulations [56 – 58].

4 Challenging homeostasis

In general, diet related diseases are caused by chronic exposure to unbalanced diets and not by acute exposures. Physiology may cope with a single high fat, high salt, and high caloric meal through various feedback mechanisms, the buffering capacity of homeostasis, and, if necessary, repair mechanisms. Adaptation to repeated consumption of such a diet-or diets with less extreme but nonetheless unbalanced composition-modulates the acute response and produces less dramatic alterations in molecular and physiological processes. Claude Bernard stated in 1866 that constancy of the internal environment is a condition for free life and the processes responsible were governed by self-organization [59]. Cannon, who summarized and further extended self-organization as the concept of homeostasis in 1929 [60] recognized that there were many homeostatic systems: glucose, lipids, calcium, and others. More recently, Young [61] proposed additional systems: organic acids involved in the Krebs cycle, purine, and pyrimidine precursors and metabolites, urea cycle metabolites, polyunsaturated fatty acids (PUFA), and hydroxyeicosatetraenoic acid (HETE) oxidation products, pro-oxidant and antioxidant capacity in relation to trace elements, and ribose and deoxyribose as cell turnover time markers. This longer list can be summarized under the processes of metabolism, oxidative stress, inflammation, and physiological stress (Fig. 1), all of which are linked to health and pathologies such as brain and immune dysfunctions, gingivitis, cardiovascular diseases, metabolic disorders, and certain (if not all) cancers. These adaptive responses attempt to keep physiology within an individual's "normal" range.

The robustness of homeostasis has led to the conclusion that homeostatic biomarkers may be of less value than the same biomarkers measured in a condition of homeostatic perturbation. While physiological adaptation will result in maintenance of functional markers within the healthy range, these markers may show a very different response in one healthy individual versus another, more susceptible, individual. The best known of a perturbation test is the oral glucose tolerance test (OGTT), which monitors the ability of the body to respond to glucose intake. [62]. The OGTT is used as one of the key surrogate endpoints for diagnosing type 2 diabetes [63 – 65] and is used to assess effects of nutrition (*e.g.* [66]). Recently, impaired glucose tolerance in midlife was shown to be a risk factor for the occurrence of Alzheimer 30 years later [67]. The usefulness of challenge test is illustrated by the Women's Health Study, where postprandial plasma triglyceride concentrations were associated with increased cardiovascular events, while fasting state fatty acids showed no correlation [68]. This was simultaneously reported in a Danish cohort [69]. While perturbation may be obtained with a metabolic challenge, such as glucose or a meal, also other stresses may be used, such as exercise [70] and other functions may be analyzed. In a recent example, a mental stress test was applied in combination with a lipid challenge to assess the effects of a 7 day high fructose diet [71].

5 Challenge tests

In principle, two types of challenges can be applied in nutrition research:

(i) A nutritional challenge, producing a "nutrient stress." Examples are short-term intakes of high fat, high carbohydrates, or high antioxidants.

(ii) A functional challenge, testing the robustness and elasticity of functions involved in maintaining relevant homeostatic functions. Examples are anti-oxidant capacity by an oxidative challenge like acetaminophen, immune function by a challenge with LPS or interleukins, endurance using a treadmill, cognitive functions by behavioral, and learning tests.

Physiology may need to be measured before, during, and after the homeostatic challenge in multiple organs or subprocesses because the responses are "whole body" or integrative responses. Exercise, for example, provides a means to assess the interaction of autonomic nervous system with the cardiovascular system by measuring:

(i) Functional (*e.g.*, aerobic) capacity–timed treadmill tests at known percentages of maximal oxygen uptake (VO_{2max}) with simultaneous measures of ethane and pentane in air exhalations

(ii) Kinetics of heart rate (HR) recovery to baseline, HR response to exercise (reserve), HR recovery after exercise, and multiple components of HR variability both at rest and with exercise [72]

(iii) Damage and repair–oxidative stress markers in urine or serum, such as increases in epinephrine and other catecholamines, O,O-dityrosine as a measure of protein oxidation, 8-hydroxy-20-deoxyguanosine (8-OHdG) for DNA, and malondialdehyde for lipids [73].

(iv) Metabolic functions measured by levels of serum creatinine, uric acid, albumin and glucose [74] or other metabolites.

(v) Inflammatory responses including C-reactive protein, serum amyloid A, interleukin-6, tumor necrosis factor-α and anti-inflammatory factors, such as adiponectin [75]. Since training reduces inflammatory markers [76], challenges of homeostasis also will require repeated sampling at rest.

6 Challenging homeostasis in a nutrigenomics context

With the capabilities systems biology, the concept of robustness of homeostasis is being revisited, both from a fundamental, intracellular perspective [77] and from an organism perspective. One example is the critical pathways for cytokine regulation in CD4+ cells that contribute critically to immune function [78, 79]. Such critical core pathways are represented by the "bow-tie" architectural motif [80]. A bow-tie representation can be visualized by the many metabolites that funnel (as if a fan structure) into activated intermediates (the knot in the tie–*e.g.* ATP, NADH and NADPH, glucose 6-phosphate, fructose 6-phosphate, phosphoenolpyruvate, and pyruvate), which are building blocks for many metabolites and macromolecules (the opposite fan structure [80]. Other intracellular processes such as the endoplasmic reticulum stress response show intricate mechanisms of homeostatic control, involving hundreds of molecular components [81]. Hence, we are beginning to understand the molecular machinery behind the physiological observations related to control of homeostasis in a systems perspective. This may allow combining "the best of two worlds": (i) perturb homeostasis in order to quantify the systems robustness and (ii) measure all relevant components that describe the system and discriminate between individuals, thus analyzing the complexity and individuality obstacles.

Nutrition studies performing this combination of nutrigenomics and challenge tests have only recently been published so far. Examples approaching the concept are available:

(i) A single breakfast showed differential PBMC transcriptomes between a high carbohydrate and high protein breakfast [52].

(ii) Fasting also induced PBMC transcriptome changes in humans [82].

(iii) Three-day high fat diet induced muscle transcriptome changes in humans which were confirmed by long term exposure in mouse experiments [83].

(iv) A one-day CLA feeding in mice resulted in 5400 transcript changes in adipose tissues in mice [84].

(v) Mice intestinal epithelial transcriptome changes were similar between a PPRA agonist and omega-3 fatty acids after acute exposure [85].

(vi) The human urinary metabolome showed strong changes after a 2 day high phytochemical diet [54].

(vii) The acute phase plasma proteome response was studied after a single LPS administration in humans [86].

(viii) LPS stimulation in mice was quantified with an accurate transcriptome time course [87].

(ix) In a recent study employing exercise, hyperinsulinemic euglycemic clamps and metabolomics analysis two plasma metabolites were found to be better markers better for insulin sensitivity than glucose. While this was true for men, another as yet unidentified compound was more predictive in women [88].

(x) A prime example of a combined nutrigenomics–challenge test in humans was a study combining the OGTT with plasma metabolomics [89]. Selected plasma metabolomics were analyzed over time following glucose administration. Four major insulin sensitive responses (proteolysis, lipolysis, ketogenesis, and glycolysis) inter-individual differences in responses, and novel metabolic responses that had not been previously reported were observed.

In most of the human examples above, inter-individual response varied, and changes between persons were larger than intra-individual response. All studies reported multiple processes to be affected by the challenge, and thus provide more information than the classical single endpoint analysis measured in homeostasis.

Most studies did not explicitly report on subgroup differences (clusters of study subjects with differential responses in one or more biological process), but the statistical methods available would allow for this type of analyses (see below).

The above studies describe a (nutri)genomics response to an acute homeostatic perturbation. Only a very few studies have analyzed the challenge response with an accurate time course, allowing area under the curve (AUC) analysis of all parameters. Examples of plasma metabolomics in this context have not yet been published. These would closely approach the classical OGTT and oral lipid tolerance test (OLTT). A few studies are in process of publication. The European Nutrigenomics Organisation (www.nugo.org) is currently performing and the U.S. FDA National Center for Toxicological Research is planning a series of nutritional challenge test studies in mice and human, with detailed time curves, to fill this gap and provide a proof of concept.

7 Solutions to improve the identification of health-related biomarkers

The complexity of gene–nutrient interactions in genetically, environmentally, and biologically heterogeneous individuals, together with our ability to maintain homeostasis, contributed to disease-centered phenotyping. This has been a beneficial approach so far, but may not be the best approach for health-oriented biomarker development. In overcoming this obstacle, three paths emerge in human nutrition research:

7.1 Quantification of the complexity

Ideally, phenotypic description includes the integration of the multi-parameter molecules (RNA, proteins, gene variants, metabolites, and genetic ancestry) analyzed by focused omics methods and physiology focused methods, including newly emerging whole body imaging techniques. Creating a functional database (bioinformatics) from data generated by the relevant analytical methods is among the first steps of this process.

7.2 Personalization

The differences among healthy phenotypes demonstrated that the current "one size fits all" approach does not necessarily lead to better health for the majority of individuals. We propose that personalization can be met from two different approaches: (i) study design in which the effects of defined and standardized nutritional challenges on health will be examined in cohorts with minimal but measured inter-individual variation, together with the acceptance, inclusion and computational assessment of the complexity mentioned above, and (ii) product development where the efficacy of (functional) food products may be established and claimed for subpopulations. Both of these approaches require replication in culturally and genetically different populations.

7.3 Challenge tests as biomarkers

Because maintenance of homeostasis has a wide spectrum of "normal values" in different individuals [90], imposing nutrient or functional challenges on different homeostatic systems will most likely provide more robust and reliable information for determining how each individual responds to nutrients (and other lifestyle choices) and therefore a means to develop paths for personalizing nutrition and medicine. The methodological foundation of these new and improved experimental strategies requires the integration of "omics" technologies with more comprehensive analyses of physiological responses in response to homeostatic challenges. These approaches and data will better define an individual's health and susceptibility to disease

Combining challenge test with nutrigenomics will allow the analysis of multiple processes and subgroup identification [49]. A series of tools are becoming available. Recently, statistical methods have been developed and applied in dealing with transcriptome data analysis exploiting these biological relationships. Gene set enrichment analysis (GSEA, [91–93] uses gene ontologies to exploit common biological features. Bayesian network analysis exploits prior (biological) knowledge in statistical analysis (*e.g.*, [94, 95]). Analogously, metabolite set enrichment analysis is also being developed which may rely on the extensive databases like the human metabolome database (www.hmdb.ca).

Correlational network analysis is another powerful tool to analyze subgroup behavior in a challenge test setting [96]. Pearson correlation matrixes between AUC of challenged parameters (*e.g.*, plasma metabolites from a metabolomics analysis) reveals subgroups of parameters, possibly related to subgroups in the study population. The use of biological network visualization as presented in Fig. 2 helps to cope in understanding the complexity. Next to commercial software, open source tools are becoming available for transcriptome and metabolome networks (www.pathvisio.org). In addition to pathway tools, statistical tools that make use of interaction between genes are being employed and seem well suited for the analysis of nutritional datasets, which are characterized by multiple small effects [97].

8 Conclusion

While conceptually simple, a coherent strategy for developing a series of challenges that test surrogate endpoints linked to health or clinical endpoints is necessary for producing meaningful surrogate endpoints. Selecting the key set of challenges that defines the most important and informative physiological responses will require additional input from physiologists, nutritionists, molecular biologists, geneticists, and clinical researchers.

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