Metabolomics, Metabonomics and Functional Nutrition: The Next Step in Nutritional Metabolism and Biotherapeutics

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Abstract: Metabonomics is the newest member of the convergent "-omics" family. This multi-parametric technique is used for studying metabolites in biological fluids for the purpose of metabolic profiling for biomarker and diagnostic applications, and to study the effects of environmental stimuli (diet, weight loss, exercise) and other therapeutic applications (lifestyle modification and drug effects) on the metabolome. Metabonomics has the advantage for defining biological phenotypes, but has a close relationship to investigation of the proteome, driven by advances in NMR and MS. The technique has proved highly informative in applications that include toxicology, pharmacology, and the biomedical sciences. This review elaborates on the paradigm shift that is emerging from this discovery process.

Keywords: Genomics, Functional genomics, Proteomics, Metabolic Pathways, Metabolome, Metabonomics, Metabolic Syndrome, Inflammatory Process, Cancer, Iron Metabolism, Obesity, Fat Metabolism.

HIGHLIGHTS

1. Metabolomics is a part of an integrated approach to biological sciences that is grouped with an "OMICS" family of genomics, proteomics, transcriptomics, translational medicine, and pharmacogenomics

2. The biological sciences that evolved in the 19th century was largely tied to the development of the periodic table, anatomic and morbid science, and the foundation for physiology, mainly, the circulation and respiration, and microbiology

3. This was expanded in the 20th century with the emergence of organic chemistry and physical chemistry leading to the discovery of the genetic code, metabolites, metabolic pathways, biochemistry of plant and animal metabolism, and the ontogeny of differentiated organ systems

4. The 21st century challenges our knowledge comprehension with an explosive development in the computational technology for modeling biological systems and studying the interaction between the genome, the ribosome, protein synthesis, protein folding, and metabolic regulation

INTRODUCTION

The human genome may encode over 30,000 genes, and generates more than 100,000 proteins, the function of many of either is yet unknown. Understanding the interrelationships among genes, gene products, and dietary habits is fundamental to identifying those who will benefit most from or be placed at risk by intervention strategies. Unraveling the multitude of nutrigenomic, proteomic, and metabolomic patterns that arise from the ingestion of foods or their bioactive food components is expected to provide insights into an individualized approach to diet and health. The use of new and innovative technologies, such as separation and spectrometric methods, microarrays, RNA interference, and nanotechnologies, is already providing insights into molecular targets for specific bioactive food components [1].

Nutrigenetics is directed at how the individual as expressed through single nucleotide polymorphisms, copy-number polymorphisms and epigenetic phenomena is affected by diet, as exemplified by the biochemical disorders phenylketonuria and maple syrup urine disease.

Nutrigenomics explores how diet influences gene transcription, protein expression and metabolism. A major methodological challenge to nutrigenomics is integrating genomics (gene analysis), transcriptomics (gene expression analysis), proteomics (protein expression analysis) and metabonomics (metabolite profiling) to define a "healthy" phenotype. The work of nutrigenomics might lead to defining more personalized nutrition prescriptions [2].

Heretofore, we have had little understanding of how genetic variation and epigenetic events alter nutrient requirements. However, we now have powerful methods that enable profiling almost all of the products of metabolism in a single sample of blood or urine. Relations between diet and genetically regulated

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metabolic and signaling pathways and between specific profiles and health have become important components of research that would impact clinical practice in nutrition.

Our historical base of nutrition knowledge assumes that all persons have average dietary requirements, and these studies were not planned for determining subsets of the population who differ in requirements for a nutrient. Moreover, the baseline took into account active essential metabolites involved in anabolic and catabolic functions in the catagories of carbohydrates, fats, and proteins, macronutrients, and vitamins and minerals, micronutrients, deficiencies of which may coexist and which have characteristic symptomatology in deficiency states. Large variances in responses that occur when such a population exists can result in statistical analyses that argue for a null effect. If nutrition studies could better identify responders and differentiate them from nonresponders on the basis of nutrigenomic or metabolomic profiles, the ability to detect differences between groups could be greatly increased, and the resulting dietary recommendations could be better targeted [3]. Moreover, there is a class of metabolically active chemicals derived from natural products that have no “food value”, but which has become identified with the modulation of gene-signaling pathways that regulate inflammation, repair, and growth, or have pharmacological value. Examples that interact with human body reactions include Foxglove (digitalis leaf), dicoumerol (benzopyrene derivative), vitamin K antagonists (papain, quinine, garlic, leafy vegetables (collard, kale, cabbage, brussel sprouts, brocolli)), soybean, flax seed, and snake venom toxins. In transitioning from classical epidemiology and physiology to molecular biology and genetics, nutrigenomics emerges as the field that aims to elucidate how diet can influence human health. Since bioactive food compounds can interact with genes affecting transcription factors, protein expression and metabolite production, the study of these complex interactions requires the development of advanced analytical approaches combined with computational biology. Thus, Transcriptomics, Proteomics and Metabolomics approaches when employed together to carry out these studies brings an illuminating integration of the information that they provide [4]. Metabonomics is the extension of metabolomics, the elucidation of the metabolome (the expression of the genome) to metabolic classification of individuals with the asset of quantitative, non-invasive analysis of easily accessible human body fluids such as urine, blood and saliva. This feature also relies significantly on Proteomics, with the dependence that the latter has on sample stability. Nevertheless, Proteomics provides a platform for identifying markers for targeting of interventions and their effects. Application of these emerging technologies will drive the understanding of interrelated and interacting pathways in healthy and pathological conditions, and will help to manipulate molecular 'switchboards' the effect of which is measured by disease related biomarkers. This is essential for developing preventive and therapeutic strategies for both pharmacological and nutritional interventions [5].

Of the factors affecting human health, diet and inherited genes play important roles. Food constituents, including secondary metabolites of fruits and vegetables, may interact directly with DNA *via* methylation and changes in expression profiles (mRNA, proteins) which results in metabolite content changes. Many studies have shown that food constituents may affect human health (as the examples above) and the exact knowledge of genotypes and food constituent interactions with both genes and proteins may delay or prevent the onset of diseases. Many high throughput methods have been employed to get some insight into the whole process and there are several examples of successful research in the field of genomics and transcriptomics. Studies on epigenetics and translational genome-metabolic interactions are noteworthy. Proteomics and metabolomics need to encompass large numbers of experiments and linked data. Due to the nature of the proteins, as well as due to the properties of various metabolites, experimental approaches require the use of comprehensive high throughput methods and a sufficiency of tissue or body fluid samples [6]. New experimental tools that investigate gene function at the subcellular, cellular, organ, organ system, and ecosystem level are being used. New bioinformatics tools to analyze and extract meaning from increasingly systems-based datasets are already available. An important and revolutionary aspect of “The 2010 Project” is that it implicitly endorses the allocation of resources to explore the function of genes that have no known function (recall that 99% of the genome is termed nonfunctional). This represents a significant departure from the common practice of defining and justifying a scientific goal based on the biological phenomena (the experimental basis for a clinical trial: hypothesis testing as distinct from hypothesis generation). The rationale for endorsing this radical change is that for the first time it is feasible to envision a whole-systems approach to gene and protein function. This whole-systems

approach promises to be orders of magnitude more efficient than the conventional approach [7].

PUBLIC POLICY IMPORTANCE FOR NUTRITION IN HUMAN HEALTH

The Institute of Medicine convened a workshop in 2006 to review the state of the various domains of nutritional genomics research and policy and to provide guidance for further development and translation of this knowledge into nutrition practice and policy. The report of a 2012 meeting identifies best practices to enhance development, evaluation, and translation of omics-based tests while simultaneously reinforcing steps to ensure that these tests are appropriately assessed for scientific validity before they are used to guide patient treatment in clinical trials [8]. Nutritional genomics holds the promise to revolutionize both clinical and public health nutrition practice and facilitate the establishment of (a) genome-informed nutrient and food-based dietary guidelines for disease prevention and healthful aging, (b) individualized medical nutrition therapy for disease management, and (c) better targeted public health nutrition interventions (including micronutrient fortification and supplementation) that maximize benefit and minimize adverse outcomes within genetically diverse human populations. As the field of nutritional genomics matures and bridges gaps in knowledge of nutrient-genome interactions in health and disease and demonstrates the potential benefits of customizing nutrition prescriptions, registered dietitians will be faced with the opportunity of making evidence-based dietary recommendations.

The new era of nutrition research translates empirical knowledge to evidence-based molecular science. Modern nutrition research focuses on promoting health, preventing or delaying the onset of disease, optimizing performance, and assessing risk. Personalized nutrition is a conceptual analogue to personalized medicine and means adapting food to individual needs. Nutrigenomics and nutrigenetics build the science foundation for understanding human variability in preferences, requirements, and responses to diet and may become the future tools for consumer assessment motivated by personalized nutritional counseling for health maintenance and disease prevention [9]. The primary aim of “omic” technologies is the non-targeted identification of all gene products (transcripts, proteins, and metabolites) present in a specific biological sample. By their nature, these technologies reveal unexpected properties of biological systems. A second and more challenging aspect of “omic” technologies is the refined analysis of quantitative dynamics in biological systems. Metabolomics characterizes individuals metabolic features and delivers metabolic endpoints possibly related to health or disease. Proteomics is most challenging for assessing nutritional adaptations because it delivers not only markers but also targets of intervention, such as enzymes or transporters, and it is the platform of choice for discovering bioactive food proteins and peptides [10]. Deployed in an integrated fashion, the science may elucidate biomarkers for defining an individual's susceptibility to diet in nutritional interventions and for assessing food ingredient efficacy [11].

ADVANCING POTENTIAL APPLICATIONS OF METABOLOMICS

Either individually or grouped as a profile, metabolites are detected by either nuclear magnetic resonance spectroscopy or mass spectrometry. There is potential for a multitude of uses of metabolome research, including the early detection and diagnosis of cancer and as both a predictive and pharmacodynamic marker of drug effect. However, the knowledge regarding metabolomics, its technical challenges, and clinical applications is unappreciated even though when used as a translational research tool, it can provide a link between the laboratory and clinic.

Precise numbers of human metabolites is unknown, with estimates ranging from the thousands to tens of thousands. Metabolomics encompasses several types of analyses, including (*a*) metabolic fingerprinting, which measures a subset of the whole profile with little differentiation or quantitation of metabolites; (*b*) metabolic profiling, the quantitative study of a group of metabolites, known or unknown, within or associated with a particular metabolic pathway; and (*c*) target isotope-based analysis, which focuses on a particular segment of the metabolome by analyzing only a few selected metabolites that comprise a specific biochemical pathway.

WHAT IS THE TECHNOLOGY BASE?

For metabolomics, gas and liquid chromatography coupled to mass spectrometry are well suited for coping with high sample numbers in reliable measurement times with respect to both technical accuracy and the identification and quantitation of small-molecular-weight metabolites. This potential is a prerequisite for the analysis of dynamic systems. For those who are unfamiliar with the methods of analytical measurements let us examine these methods. One can

also argue that the processing times required for routine analysis of many clinical specimens is still too slow. The principle here is a separation step that fractionates the species of analyte, whether it be a group of proteins, lipids, amino acides, or organic acids. The volatile chemical species are separated well by gas chromatography, routinely used in bacterial identification.

The species migrate at different rates through a column matrix based on their volatility. Liquid chromatography carries out a separation as the molecules differ by their adsorption to and elution from a solid matrix, which is dependent on the binding to the matrix and solubility in the solvent eluate, modified by ph, ionic concentration, and specific conditions needed for recovery. These methods were perfected after years of purification of protein and smaller molecules in laying down the foundation for biochemistry of proteins, peptides, substrates, hormones, and nucleotides. The serial extraction and identification of different species was another problem. Figure **1** identifies the methodology used.

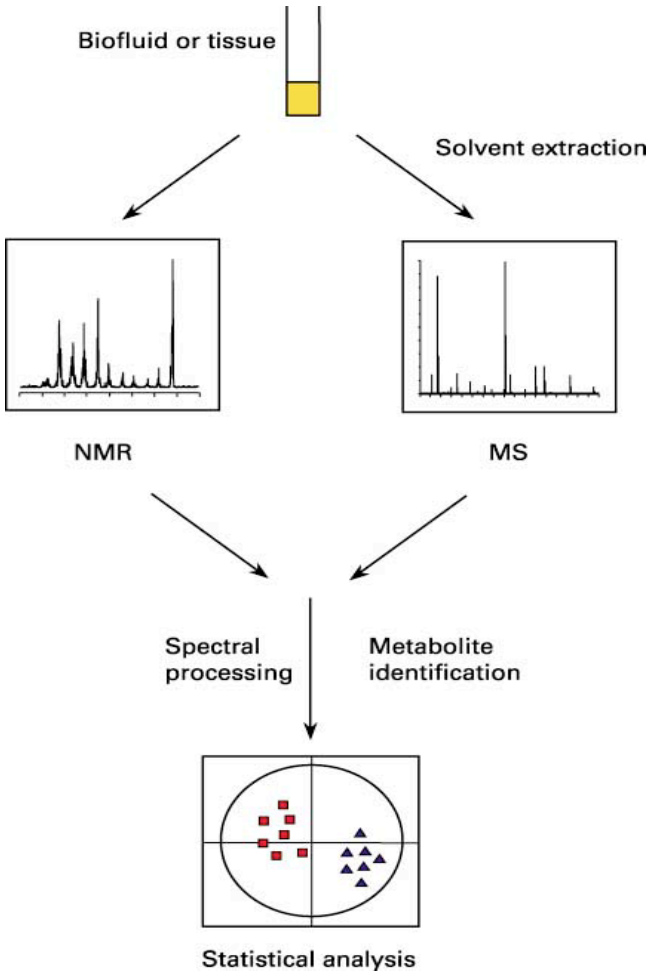

Figure 1: The methods of metabolite identification.

In carrying out the identification, there have been many types of detectors at the end of the separation. These could be photometric, fluorescent, light scattering, electromagnetic, and mass spectrometry or even magnetic resonance. The signal for inspection is a collection of wave forms that have peaks corresponding to individual substances that have characteristic migration rates, and that are quantified by their peak height. The instruments used today have computers that have libraries of reference information enabling the rapid identification of species in each specimen. But that doesn't explain the identification of a series of specimens from different sources with respect to identifying a metabolic state. The advances that have been applied to this specific problem are a result of more than 20 years of development of applied mathematics in refined regression, discriminant function, simulation, structural equations modeling, and a large amount of computational capability only available recently.

In the genetic sphere there are microarrays robotically driven and read in sequence.

In modern nutrition research, mass spectrometry has developed into a tool to assess health, sensory as well as quality and safety aspects of food. In this review, we focus on health-related benefits of food components and, accordingly, on biomarkers of exposure (bioavailability) and bioefficacy. Current nutrition research focuses on unraveling the link between dietary patterns, individual foods or food constituents and the physiological effects at cellular, tissue and whole body level after acute and chronic uptake. The bioavailability of bioactive food constituents and dose-effect correlations are key information to understand the impact of food on health outcomes. Both strongly depend on use of the analytical tools mentioned to identify and quantify minute amounts of individual compounds in highly complex matrices--food or biological fluids--and to monitor molecular changes in the biological subject in a highly specific and sensitive manner. Based on these requirements, mass spectrometry has become the analytical method of choice with broad applications throughout all areas of nutrition research [12].

Recent advances in high data-density analytical techniques offer unrivalled promise for improved medical diagnostics in the coming decade. Genomics, proteomics and metabonomics (as well as a whole slew of less well known “omics” technologies) provide a detailed descriptor of each individual. Relating the large

quantity of data on many different individuals to their current (and possibly even future) phenotype is a task not well suited to classical multivariate statistics. The datasets generated by "omics" techniques very often violate the requirements for multiple regression. However, other statistical approaches exist, already well established in areas such as medicinal chemistry and process control, but which are unfamiliar to medical diagnostics, that can overcome these problems.

One such approach, called [magavariate] analysis (MVA), has the potential to revolutionize medical diagnostics in a broad range of diseases. This refers to multivariate regression models that predict binding affinity for a combination of features of the ligand and protein. Variable subset selection usually has relied on using all available examples, a situation encountered in microarray gene expression data analysis [13]. In a clinical study the method was used to examine how depression, anxiety, catastrophizing, and disability were intercorrelated and specific patterns of the subscales of self-efficacy corresponded to specific patterns of negative factors for the outcome of disability, quality of life, and health [14]. It opens up the possibility of expert systems that can diagnose the presence of many different diseases simultaneously, and even make exacting predictions about the future diseases an individual is likely to suffer from [15].

There are yet other approaches that are being used. To be sure, a BIG DATA revolution has erupted to address complex problems. This is because business, economics, and bioinformatics encounters data sets which have choices or combinations or outcomes that are so numerous that they are impossible to process manually. A mathematical model has to be constructed that is a representation of the key characteristics and attributes of the data which deciphers the make-up of the system by breaking it down into its usable components for examination. There are a number of such modeling agents or services now available because of the pressure to innovate, not just in biomedicine. Some of these are IBM Cognos, STAR analytical services (Bedford, MA), NCSS (Kaysville, UT), Statistical Innovations, Inc. (Correlated Component Regression [COREXpress] and Latent GOLD 5.0) (Belmont, MA), Ingenuity IPA Systems (Analyze and integrate data from gene expression and SNP microarrays, proteomics, metabolomics, and genotyping experiments). A serious problem for prediction that has to be overcome is the presence of many predictors and insufficient data. It results in overfitting the data. Magidson goes through the issues of validation and cross-validation in a 2012 Statistical Innovations online course on CCR using the genetics of acute myelogenous leukemia [16]. He indicates that a portion of sample cases are not used for estimation for purposes of validation, but holding out many cases for validation is not justified when the sample size for estimation would be too small. Cross-validation (CV) is a popular alternative to validation. In CV, all cases are used for both estimation and validation. The most popular CV method is called M-fold cross-validation.

Rypka describes a method used for microbial classification [17], S-clustering, that uses the entropy principle to extracting features from endogenous data that amplify or maximize structural information to create distinctive classes. Recall that the organisms divide into subclasses, which requires metabolic differentiation in growth media. Antibiotic sensitivities and genetic analyses would be add-ons to the features used. His method owes much to the discovery of Kullback-Liebler distance or "information" [18] and Akaike [19], who established a relationship between information theory and Fisher's maximized log-likelihood function (20). Rudolph, Bernstein and Babb [21] used syndromic classification to amplify use of cardiac markers for the diagnosis of acute myocardial infarction. Another classification method used anomaly identification to interpret the hemogram [22], and it has also been applied to malnutrition screening.

REGULATION OF TRANSCRIPTION AND METABOLIC PATHWAYS

There is an abundance of current research that reveals that upregulation and downregulation of synthetic pathways as well as catabolic processes are affected by genetic, nutritional, and environmental factors amenable to experimental manipulation involving signaling proteins that interact with the genome [23]. Figure **2** illustrates the schema for metabolic regulation controlled by a network of systems unfolding with the evolving "OMICs" research. Table **1** shows a classification of the signaling pathways and their intimate association with specific cells and activities. There are really more than the three pathways that are used here.

Table **1** illustrates impressively a cross-linkage to innate immunity:

1) Detection of the foreign: pathogen-associated molecular patterns (PAMPs) signal the presence of the agent upon binding to Toll-like receptors (i.e., Lipopolysaccharides).

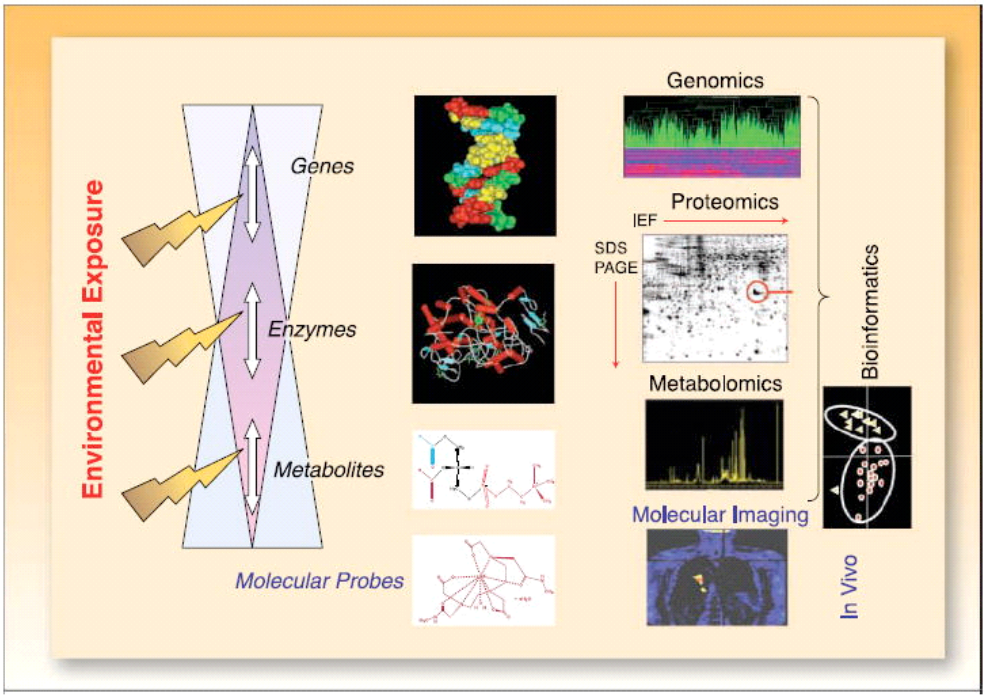

Figure 2: Dynamic Construct of the –Omics.

Table 1: Key Concepts Underly the Tie between Genome Regulation, Protein Synthesis, and the Expression of Metabolic Pathways

Receptor Type	Receptor	Cell Distribution	Cell Location
Toll-Like Receptors (TLRs)	NF-kB, IRF, MAPK	Immune Cells, DCs, NK Cells, B Cells, T Cells	Surface or Endosomes
C-Type Lectin Receptors (CLRs)	MAPK	Immune Cells, DCs, Monocytes, Macrophages, Endothelial Cells	Surface
RIG-I-Like Receptors (RLRs)	IRF, MAPK	Cells characterized mostly in Fibroblasts & DCs	Cytoplasm
NOD-Like Receptors (NLRs)	NOD1 NOD2: Inflammasomes	Innate Immune Cells: Macrophages, Neutrophils	Cytoplasm
	Caspase-1 NALPs: Ipaf, NAIP	Intestinal Epithelial Cells & others	

2) Verification of pathogenicity: damage-associated molecular patterns (DAMPs) signal cell injury using different receptors.

Toll-like receptors (TLRs), Nod-like and RLR receptors are key receptors in Table **1** that recognize structures in pathogens and have a central role in antigen-specific adaptive immunity [24-28].

Two reviews by Corella and Ordovas provide extensive examples [29, 30]. For example, individuals with a common genetic variation in the *APOA1* gene have a greater low-density lipoprotein cholesterol (LDL-C) response to dietary fat manipulation than those without the variation. In a second example, the *MTHFR* gene encodes the enzyme 5,10-methylenetetrahydrofolate reductase that activates folate, which in turn serves as a critical methyl donor as well as being essential to maintaining desirable levels of homocysteine. Individuals with the 677C>T variation in the *MTHFR* gene are at risk for impaired MTHFR enzymatic activity when folate intake is low.

"Genetic variation" (or "gene variant") is used to describe a mutation that does not have an impact on function strong enough on its own to cause disease. "Nutritional epigenomics" asks how gene expression can be altered without changing the DNA sequence, how diet (food and dietary supplements) influences this type of control of gene expression, and how (whether?) the changes are inherited the epigenetic markings are sensitive to signals from the environment, and nutrition is a powerful tool for changing an individual's

epigenetic signature the nutrition influence appears to be felt for multiple generations [23].

A new molecule, analogue of Staurosporine, selected from a family of compounds discovered by combinatorial biosynthesis of indolocarbazole biosynthesis genes, whose mechanism of action (MoA) relies on potent and selective inhibition of Ikkb kinase, a validated target of the IKK complex, and as such, a disruptor of the NF-kB signaling pathway, well known by its involvement in inflammation and cancer diseases.

The goal is to develop selective and potent inhibitors of kinases involved in signal transduction pathways related to a variety of diseases, especially those unlocking new drug targets. Chemical modifications to DNA bases [31, 32], or epigenetic marks, ensures gene expression. Two widely known chemical modifications are 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC). What they do can now be better understood with the first tool to measure, with single-base resolution, their relative amounts. Researchers have known that when cytosine bases are methylated, the gene is silenced. The precise role of 5hmC is still debated, but it's clear it has enormous biological importance, most researchers believe that 5hmC marks are an epigenetic "on" switch for genes. Others think that 5hmC is an intermediate in so-called active demethylation. cytosine bases on the newly combined DNA are rapidly de-methylated to create an embryonic stem cell.

A collaborative study performed at MIT and Stanford found that chaperonins promote the proper folding of newly translated proteins and proteins that have been stress-denatured—meaning they've lost their structure—by encapsulating them inside a protective chamber formed from two rings of molecular complexes stacked back-to-back [33]. There are two classes of chaperonins, group I found in prokaryotes; and group II found in eukaryotes. Much of the basic architecture has been evolutionarily preserved across these two classes but they do differ in how the protective chamber is opened to accept proteins and closed to fold them. Whereas group I chaperonins require a detachable ring-shaped molecular lid to open and close the chamber, group II chaperonins have a built-in lid.

In bacteria, the so-called RecA protein is responsible for conducting a search operation. In *E. coli* bacteria, a filament of RecA protein formed on DNA, searches and pairs a sequence within a second DNA molecule. To do so, individual molecules of RecA first come together to form a filamentous structure on the broken DNA. The filament's secondary DNA-binding site interacts with a single strand of the incoming double-stranded DNA with recognition occurring upon binding of both strands of the incoming DNA to each of two DNA-binding sites in the filament. Three-letter DNA "words" specify an amino acid, and a few codons tell the cell when to stop adding amino acids to a protein chain. The researchers targeted a "stop" codon, which spells TAG, delete them and then deleted the cell machinery that reads the TAG codon, thereby enabled to encode a novel amino acid. They used a linker probe containing an ester group to connect arylsulfonamide to the protein and to 7-hydroxy-4-methylcoumarin, a fluorescent dye. The 7-hydroxy-4-methylcoumarin attached to the linker's ester group stays dark when hit with ultraviolet light. The dye fluoresces when it is cleaved from the linker's ester [34].

DIET, THE INFLAMMATORY COMPONENT AND CARDIOVASCULAR DISEASE

Cardiovascular diseases are the leading cause of morbidity and mortality in Western countries. Although coronary thrombosis is the final event in acute coronary syndromes, there is increasing evidence that inflammation also plays a key role in development of atherosclerosis and its clinical manifestations, such as myocardial infarction, stroke, and peripheral vascular disease. The inflammatory component was indicated by the epidemiological studies of elevated serum levels of high sensitivity C-reactive protein [35], that eventually led to the demonstration of a benefit from reduction of CRP in individuals without characteristic lipidemia in a major clinical trial [36], and which drew a relationship between diabetes, obesity, and disordered inflammatory response in the causation of coronary artery disease, aortic valve and artery disease, carotid artery and peripheral vascular disease.

The more elaborate tools offered by metabolomics opened the door to exploring an active role played by adipose tissue that is affected by diet, race, sex, and probably age and activity. When the multifactorial is brought into play, and the effect of changes in diet and activities studied we leave the study of metabolomics and enter the world of "metabonomics". Adiponectin and adipokines arrive [37-44].

The beneficial cardiovascular health effects of diets rich in fruits and vegetables are widely accepted. The benefit is in part mediated by their flavanol content. This concept is supported by findings from small-scale

intervention studies with surrogate endpoints including endothelium-dependent vasodilation, blood pressure, platelet function, and glucose tolerance. Mechanistically, short term effects on endothelium-dependent vasodilation following the consumption of flavanol-rich foods, as well as purified flavanols, have been linked to an increased nitric oxide bioactivity, and any active mechanism for reducing oxidative stress is beneficial. The critical biological target(s) for flavanols have yet to be identified [44], but we are beginning to see over the horizon.

Anemia

Hepcidin is a key hormone governing mammalian iron homeostasis and may be directly or indirectly involved in the development of most iron deficiency/overload and inflammation-induced anemia. The anemia of chronic disease (ACD) is characterized by macrophage iron retention induced by cytokines and hepcidin regulation. Hepcidin controls cellular iron efflux on binding to the iron export protein ferroportin. While patients present with both ACD and iron deficiency anemia (ACD/IDA), the latter results from chronic blood loss. Iron retention during inflammation occurs in macrophages and the spleen, but not in the liver. In ACD, serum hepcidin concentrations are elevated, which is related to reduced duodenal and macrophage expression of ferroportin. Individuals with ACD/IDA have significantly lower hepcidin levels than ACD subjects. ACD/IDA patients, in contrast to ACD subjects, were able to absorb dietary iron from the gut and to mobilize iron from macrophages. Hepcidin elevation may affect iron transport in ACD and ACD/IDA and it is more responsive to iron demand with IDA than to inflammation. Hepcidin determination may aid in selecting appropriate therapy for these patients [45].

There is correlation between serum hepcidin, iron and inflammatory indicators associated with anemia of chronic disease (ACD), ACD, ACD concomitant iron-deficiency anemia (ACD/IDA), pure IDA and acute inflammation (AcI) patients. Hepcidin levels in anemia types were statistically different, from high to low: ACD, AcI > ACD/IDA > the control > IDA. Serum ferritin levels were significantly increased in ACD and AcI patients but were decreased significantly in ACD/IDA and IDA. Elevated serum EPO concentrations were found in ACD, ACD/IDA and IDA patients but not in AcI patients and the controls. A positive correlation exists between hepcidin and IL-6 levels only in ACD/IDA, AcI and the control groups. A positive correlation between hepcidin and ferritin was marked in the control group, while a negative correlation between hepcidin and ferritin was noted in IDA. The significant negative correlation between hepcidin expression and reticulocyte count was marked in both ACD/IDA and IDA groups. If the hepcidin role in pathogenesis of ACD, ACD/IDA and IDA, it could be a potential marker for detection and differentiation of these anemias [46].

Cancer

Because cancer cells are known to possess a highly unique metabolic phenotype, development of specific biomarkers in oncology is possible and might be used in identifying fingerprints, profiles, or signatures to detect the presence of cancer, determine prognosis, and/or assess the pharmacodynamic effects of therapy [47].

When platelets come into contact with tumor cells, they somehow activate the NF-kappa-b pathway, which is involved in regulating the immune response to infection. Both of the signals, NF-kappa-b activation and TGF-beta, are necessary for the switch to occur.

HDM2, a negative regulator of the tumor suppressor p53, is over-expressed in many cancers that retain wild-type p53. Consequently, the effectiveness of chemotherapies that induce p53 might be limited, and inhibitors of the HDM2–p53 interaction are being sought as tumor-selective drugs. A binding site within HDM2 has been dentified which can be blocked with peptides inducing p53 transcriptional activity. A recent report demonstrates the principle using drug-like small molecules that target HDM2 [48].

OBESITY, CRP, INTERLEUKINS, AND CHRONIC INFLAMMATORY DISEASE

Elevated CRP levels and clinically raised CRP levels were present in 27.6% and 6.7% of the population, respectively. Both overweight (body mass index [BMI], 25-29.9 kg/m^2) and obese (BMI, 30 kg/m^2) persons were more likely to have elevated CRP levels than their normal-weight counterparts (BMI, <25 kg/m^2). After adjusting for potential confounders, the odds ratio (OR) for elevated CRP was 2.13 for obese men and 6.21 for obese women. In addition, BMI was associated with clinically raised CRP levels in women, with an OR of 4.76 (95% CI, 3.42-6.61) for obese women. Waist-to-hip ratio was positively associated with both elevated and clinically raised CRP levels, independent of BMI. Restricting the analyses to young adults (aged 17-39 years) and excluding smokers, persons with inflammatory disease, cardiovascular

disease, or diabetes mellitus and estrogen users did not change the main findings [49].

A study of C-reactive protein and interleukin-6 with measures of obesity and of chronic infection as their putative determinants related levels of C-reactive protein and interleukin-6 to markers of the insulin resistance syndrome and of endothelial dysfunction. Levels of C-reactive protein were significantly related to those of interleukin-6 (r=0.37, P<0.0005) and tumor necrosis factor-a (r=0.46, P<0.0001), and concentrations of C-reactive protein were related to insulin resistance as calculated from the homoeostasis model and to markers of endothelial dysfunction (plasma levels of von Willebrand factor, tissue plasminogen activator, and cellular fibronectin). A mean standard deviation score of levels of acute phase markers correlated closely with a similar score of insulin resistance syndrome variables (r=0.59, P<0.00005) and the data suggested that adipose tissue is an important determinant of a low level, chronic inflammatory state as reflected by levels of interleukin-6, tumor necrosis factor-a, and C-reactive protein [50].

A number of other studies have indicated the inflammatory ties of visceral obesity to adipose tissue metabolic profiles, suggesting a role in "metabolic syndrome". There is now a concept of altered liver metabolism in "non-alcoholic" fatty liver disease (NAFLD) progressing from steatosis to steatohepatitis (NASH) [51,52].

These unifying concepts were incomprehensible 50 years ago. It was only known that insulin is anabolic and that insulin deficiency (or resistance) would have consequences in the point of entry into the citric acid cycle, which generates 16 ATPs. In *fat catabolism*, *triglyceride*s are *hydrolyzed* to break them into *fatty acid*s and *glycerol*. In the liver the *glycerol* can be converted into glucose *via* dihydroxyacetone phosphate and glyceraldehyde-3-phosphate by way of gluconeogenesis. In the case of this cycle there is a tie in with both *catabolism* and *anabolism*.

Aerobic glucose and acetate metabolism is shown in Figure **3** [53].

For bypass of the **Pyruvate Kinase reaction** of Glycolysis, cleavage of **2 ~P** bonds is required. The free energy change associated with cleavage of one ~P bond of ATP is insufficient to drive synthesis of phosphoenolpyruvate (PEP), since PEP has a higher negative ΔG of phosphate hydrolysis than ATP.

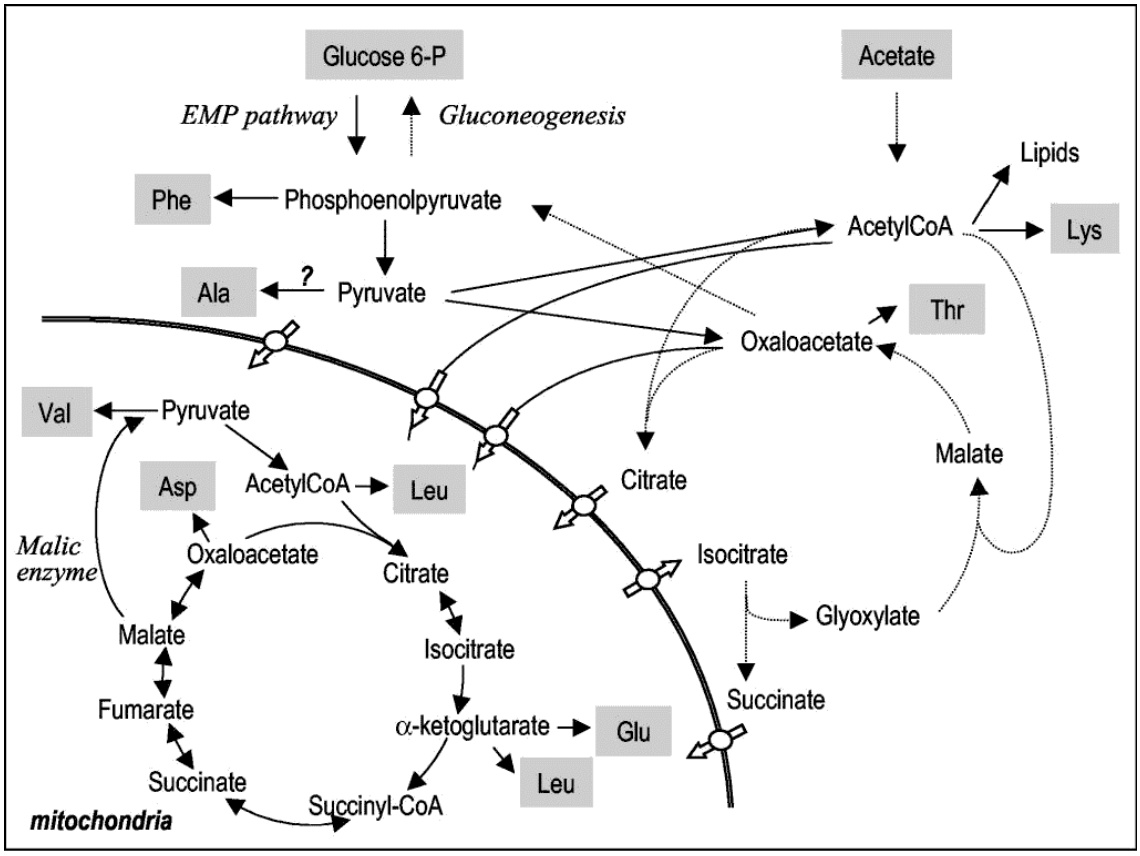

Figure 3: Aerobic glucose and acetate metabolism (from dos Santos MM, *et al*. *EUKARYOTIC CELL* 2003; 2:599–608).

The two enzymes that catalyze the reactions for bypass of the Pyruvate Kinase reaction are the following:

(a) Pyruvate Carboxylase (Gluconeogenesis) catalyzes:

pyruvate + HCO_3^- + ATP → oxaloacetate + ADP + P_i

(b) PEP Carboxykinase (Gluconeogenesis) catalyzes:

oxaloacetate + GTP → phosphoenolpyruvate + GDP + CO_2

The concept of anomalies in the pathways with respect to diabetes was sketchy then, and there was much to be filled in. This has been substantially done, and is by no means complete. However, one can see how this comes into play with diabetic ketoacidosis accompanied by gluconeogenesis and in severe injury or sepsis with peripheral proteolysis to provide gluconeogenic precursors. The reprioritization of liver synthetic processes is also brought into play with the conundrum of protein-energy malnutrition.

The picture began to be filled in with the improvements in technology that emerged at the end of the 1980s with the ability to profile tissue and body fluids by NMR and by MS. There was already a good inkling of a relationship of type 2 diabetes to major indicators of CVD [54,55]. And a long suspected relationship between obesity and type 2 diabetes was evident. But how did it tie together?

END STAGE RENAL DISEASE AND CARDIOVASCULAR RISK

Mortality is markedly elevated in patients with end-stage renal disease. The leading cause of death is cardiovascular disease. As renal function declines, the prevalence of both malnutrition and cardiovascular disease increase. Malnutrition and vascular disease correlate with the levels of markers of inflammation in patients treated with dialysis and in those not yet on dialysis. The causes of inflammation are likely to be multifactorial. CRP levels are associated with cardio-vascular risk in the general population. The changes in endothelial cell function, in plasma proteins, and in lipids in inflammation are likely to be atherogenic. That cardiovascular risk is inversely correlated with serum cholesterol in dialysis patients, suggests that hyperlipidemia plays a minor role in the incidence of cardiovascular disease.

Hypoalbuminemia, ascribed to malnutrition, has been one of the most powerful risk factors that predict all-cause and cardiovascular mortality in dialysis patients. The presence of inflammation, as evidenced by increased levels of specific cytokines (interleukin-6 and tumor necrosis factor [TNF] a) or acute-phase proteins (C-reactive protein and serum amyloid A), however, has been found to be associated with vascular disease in the general population as well as in dialysis patients. Patients have loss of muscle mass and changes in plasma composition—decreases in serum albumin, prealbumin, and transferrin levels, also associated with malnutrition. Inflammation alters lipoprotein structure and function as well as endothelial structure and function to favor atherogenesis and increases the concentration of atherogenic proteins in serum. In addition, pro-inflammatory compounds, such as advanced glycation end products, accumulate in renal failure, and defense mechanisms against oxidative injury are reduced, contributing to inflammation and to its effect on the vascular endothelium [56,57].

Endogenous copper can play an important role in postischemic reperfusion injury, a condition associated with endothelial cell activation and increased interleukin 8 (IL-8) production. Excessive endothelial IL-8 secreted during trauma, major surgery, and sepsis may contribute to the development of systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS), and multiple organ failure (MOF). No previous reports have indicated that copper has a direct role in stimulating human endothelial IL-8 secretion. Copper did not stimulate secretion of other cytokines. Cu(II) appeared to be the primary copper ion responsible for the observed increase in IL-8 because a specific high-affinity Cu(II)-binding peptide, d-Asp-d-Ala-d-Hisd-Lys (d-DAHK), completely abolished this effect in a dose-dependent manner. These results suggest that Cu(II) may induce endothelial IL-8 by a mechanism independent of known Cu(I) generation of reactive oxygen species [58].

Blood coagulation plays a key role among numerous mediating systems that are activated in inflammation. Receptors of the PAR family serve as sensors of serine proteinases of the blood clotting system in the target cells involved in inflammation. Activation of PAR_1 by thrombin and of PAR_2 by factor Xa leads to a rapid expression and exposure on the membrane of endothelial cells of both adhesive proteins that mediate an acute inflammatory reaction and of the tissue factor that initiates the blood

coagulation cascade. Other receptors that can modulate responses of the cells activated by proteinases through PAR receptors are also involved in the association of coagulation and inflammation together with the receptors of the PAR family. The presence of PAR receptors on mast cells is responsible for their reactivity to thrombin and factor Xa, essential to the inflammation and blood clotting processes [59].

The understanding of regulation of the inflammatory process in chronic inflammatory diseases is advancing. Evidence consistently indicates that T cells play a key role in initiating and perpetuating inflammation, not only *via* the production of soluble mediators but also *via* cell/cell contact interactions with a variety of cell types through membrane receptors and their ligands. Signaling through CD40 and CD40 ligand is a versatile pathway that is potently involved in all these processes. Many inflammatory genes relevant to atherosclerosis are influenced by the transcriptional regulator nuclear factor κ B (NFκB). In these events T-cells become activated by dendritic cells or inflammatory cytokines, and these T-cells activate, in turn, monocytes / macrophages, endothelial cells, smooth muscle cells and fibroblasts to produce pro-inflammatory cytokines, chemokines, the coagulation cascade *in vivo*, and finally matrix metalloproteinases, responsible for tissue destruction. Moreover, CD40 ligand at inflammatory sites stimulates fibroblasts and tissue monocyte/macrophage production of VEGF, leading to angiogenesis, which promotes and maintains the chronic inflammatory process.

NFκB plays a pivotal role in co-ordinating the expression of genes involved in the immune and inflammatory response, evoking tumor necrosis factor α (TNFα), chemokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-8, matrix metalloproteinase enzymes (MMP), and genes involved in cell survival. A complex array of mechanisms, including T cell activation, leukocyte extravasation, tissue factor expression, MMP expression and activation, as well induction of cytokines and chemokines, implicated in atherosclerosis, are regulated by NFκB.

Expression of NFκB in the atherosclerotic milieu may have a number of potentially harmful consequences. IL-1 activates NFκB upregulating expression of MMP-1, -3, and -9. Oxidized LDL increases macrophage MMP-9, associated with increased nuclear binding of NFκB and AP-1. Expression of tissue factor, initiating the coagulation cascade, is regulated by NFκB. In atherosclerotic plaque cells, tissue factor antigen and activity were inhibited following over-expression of IκBα and

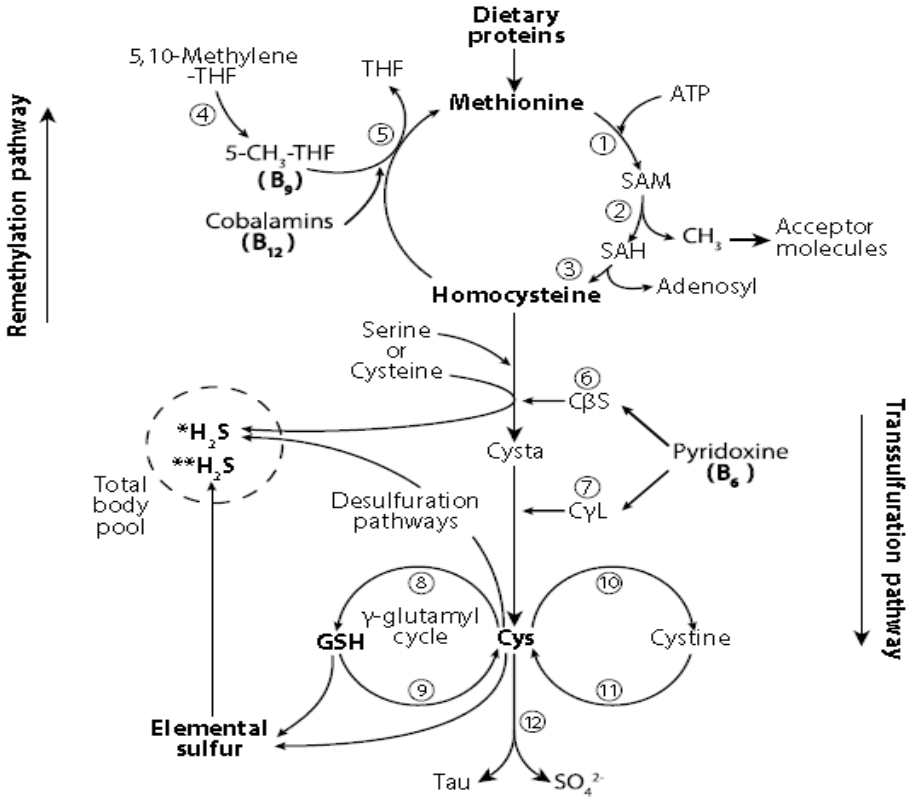

Figure 4: Diagram of Met and Hcy converting pathways [65].

dominant-negative IKK-2, but not by dominant negative IKK-1 or NIK. Tis supports the concept that activation of the "canonical" pathway upregulates pro-thrombotic mediators involved in disease. Many of the cytokines and chemokines which have been detected in human atherosclerotic plaques are also regulated by NFκB. Over-expression of IκBα inhibits release of TNFα, IL-1, IL-6, and IL-8 in macrophages stimulated with LPS and CD40 ligand (CD40L). This report describes how NFκB activation upregulates major pro-inflammatory and pro-thrombotic mediators of atherosclerosis [60-64].

I have given some detail on diabetes, obesity, malnutrition, and SIRS and commonalities of serious disease states. However, I have not mentioned the increasing visibility of methylation reactions in protein synthesis, and a relationship of total body sulfur or lack thereof to protein loss and to cardiovascular risk. This has recently been shown to be a critical finding by Ingenbleek and others [65-70]. It appears to be related to a fundamental ratio of S:N, lower in plants than in animals, and a poor intake of sulfur, or methionine deficiency has an adverse effect with on the catabolic state. This implicates the role that S has in methyl transfer and phosphorylation. Figure **4** is a picture of this unexpected finding, which could lead to homocysteinemia from methionine insufficiency.

This review is both focused and comprehensive. The details of evolving methods are avoided in order to build the argument that a very rapid expansion of discovery has been evolving depicting disease, disease mechanisms, disease associations, metabolic biomarkers, study of effects of diet and diet modification, and opportunities for targeted drug development. The extent of future success will depend on the duration and strength of the developed interventions, and possibly the avoidance of dead end interventions that are unexpectedly bypassed. I anticipate the prospects for the interplay between genomics, metabolomics, metabonomics, and personalized medicine may be realized for several of the most common conditions worldwide within a few decades [71-73].

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