Metabolic signatures in nutrition and health : short-term diet response, sexual dimorphism and hormone chronobiology

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Chapter 1

General introduction and aim of the thesis

GENERAL INTRODUCTION

Personalized nutrition, which originated from gene and nutrient interaction research [1], has received much attention in health-oriented marketplaces due to increased technological capabilities to sensitively measure unique differences between individuals. Public interest in individualizing health has grown as various sectors in society offered more and improved technologies and capabilities to empower individuals to move from one-size-fits-all to the ability to quantify and control their personal lives. Consequently, new research must be designed to assess individualized health trajectories and optimize individualized diagnostic and therapeutic decision-making [2, 3]. In order to realize the power of personalized nutrition, research needs to be conducted on healthy people with highly sensitive metabolic markers capable of diagnosing the impact of environmental stressors, such as diet, and physiologic stressors, such as changing sex hormones and the menstrual cycle. Obvious phenotypic differences, such as sex and gender, should be evaluated as they represent easily targeted subtypes with unique physiologies for which personalized nutrition can be prescribed.

The power of personalized health diagnosis from which personalized nutrition therapies can be prescribed, relies not only on individual variations in genotype, but the environmental and physiologic responses manifested through transcriptomic, metabolomic and proteomic measurements, as well as personal psycho-social factors, that can be measured and modified. The aim of this thesis was to study healthy women and men and their metabolic response to nutrition and/or natural hormone dynamics, monitored by clinical and metabolomics biomarkers, as a way to better understand human metabolic health.

What is metabolic health?

Metabolism is the sum of all biochemical processes in the body mediated by cells that maintain life. It includes the transformation of foods to energy, so that cellular processes can be fueled; and waste elimination. The energy comes from the digestion and transformation of the food we eat. Optimal health is the resilient capacity to adapt when presented with physiological and environmental (social and emotional) challenges to the body's homeostatic state [4]. Thus, metabolic health can be defined as a state of resilient physical and chemical cellular physiologic functioning adequately supported by the digestion and transformation of food into energy. Health must be expansive and systemic and not fixated on singular aspects of cellular function. Naturally, by ingesting a variable multitude of nutrients and cofactors, we feed all aspects of the body's cellular

function concurrently; supporting the body's metabolic physiologic needs on a systemic level to maintain health.

An individual's metabolic health status is determined by the levels of certain biomarkers such as high cholesterol or high fasting glucose that are compared to clinically accepted "normal" value ranges. The concept of health was codified by the World Health Organization in 1948 as the complete physical, mental, and social well-being and not merely the absence of disease or infirmity [5]. Virtually no human could meet the definition of being completely healthy and objective measures of health status are lacking. Indeed, abnormal levels of disease biomarkers may not adequately define health or disease states because:

1) processes involved in disease are not the same as those involved in health optimization or disease prevention,
2) homeostasis acts to maintain levels of many conventionally accepted clinical biomarkers within a limited range, masking early predispositions and indications of disease initiation under "normal" or "resting" conditions,
3) large inter-individual differences in "normal" values exist [6, 7] and
4) disease threshold values are based on population risk factors which may not apply to the individual [2, 3, 8-11].

Unbalanced nutrition decreases metabolic flexibility and leads to disease process induction. A high fat, high calorie meal challenge has been used to demonstrate subtle improvements in vasculature, systemic stress and metabolic flexibility. The ingestion of the meal challenge temporarily disturbs the body's homeostasis and the human system's capacity to restore that homeostasis is monitored during the hours post-ingestion. The degree to which the body is able to return to homeostasis determines the degree of flexibility [12]. Using this meal challenge, metabolomics differences have been identified between glucose, lipid, amino acid, vitamin and metabolic stress markers and young lean subjects versus elderly subjects with higher adiposity; increased body fat and degree of metabolic flexibility; and healthy versus type 2 diabetic subjects [13, 14].

Can healthy diet challenges lead to knowledge for personalized nutrition and reduction of disease incidence?

Since homeostatic measurements are of limited value in defining the health state, Huber and colleagues suggested the ability to adapt to the physical, emotional, and social challenges of life may provide a more reasonable and useful definition of health. Others extended this concept to Earth's environmental health and healthy diets [4]. Physiologists and nutritionists had previously developed flexibility or adaptability concepts to define metabolic health [15, 16]. The research in this thesis uses the

definition of metabolic health as a state of resilient physiological functioning supporting digestion and transformation of food into energy and substrates for biological processes. These interlinked processes are a system of interacting pathways and networks rather than reductionistic, singular, necessary and important steps in pathways involved in cellular or organ function. These systems' processes and the energy necessary to drive metabolism are derived from the many and varied chemicals, nutrients, and cofactors used in all aspects of the body's processes.

The physiological diagnosis of adaptable metabolic health relies on the ability to examine changes in endogenous small-molecule metabolites and proteins in response to a perturbation. The simplest and most straightforward perturbation is an acute intake of nutrients whose effect can be measured by differences in levels of certain biomarkers from the homeostatic state (e.g., fasting levels) compared to the levels of those markers following a dietary challenge. The ingestion of the meal challenge temporarily disturbs the homeostasis and the capacity to restore that homeostasis is monitored during the hours post-ingestion. The degree to which the body is able to return to homeostasis determines the degree of flexibility [12].

Metabolomics analysis following a mixed-nutrient (e.g., protein, lipid, carbohydrate) meal challenge identified differences between levels of glucose, lipid, amino acid, vitamin and metabolic stress markers in young lean subjects versus elderly subjects with higher adiposity; increased body fat, and healthy versus type 2 diabetic subjects [13, 14]. These differences led to the conclusion that metabolic flexibility was decreased in individuals with diabetes and presumably other chronic conditions (IBD). Gestational diabetes (GDM) is diagnosed using the oral glucose tolerance test (OGTT) and presents an example of using acute food challenges to predict long-term health. Women who acquire GDM during pregnancy have between a 5- and 10-fold greater risk of developing T2DM within 5 years of the pregnancy depending upon ancestral background [17]. The stress of the pregnancy reveals an underlying susceptibility to metabolic imbalances that may result in disease.

Understanding the trajectory of flexibility to inflexibility for each individual may permit development of a range of nutritional solutions to abate the progress to disease. However, the efficacy of a habitual diet for an individual based on the responses to acute challenges of homeostasis has not been experimentally proven even though such a conclusion is conceptually consistent with the effect of long-term diets on health. For example, a lifestyle modification program that included a healthy, low fat diet and exercise with a 7 percent weight loss goal was shown to be more effective than the pharmaceutical, metformin, for reduction of diabetes incidence [18]. In addition,

advances in analytical technologies over the past decade have extended the quantification from scores to hundreds or thousands of metabolites and proteins in biofluids which could lead to a deeper understanding of metabolic processes.

Metabolomics and proteomics sensitively identify these additional markers of processes involved in health and disease and hold the potential to play an important role in developing personalized diagnosis, disease susceptibility assessment, health monitoring and preventive healthcare [19-21]. The correlation between novel proteomic- and metabolomics-based biomarkers to established clinical biomarkers, such as glucose and lipids, is of relevance to determine degrees of metabolic adaptation at a higher granularity.

Which healthy diet strategy is best for the individual?

However, how do we know the best diet to choose for an individual? Many different diets have been associated with health including the Mediterranean, Nordic, Okinawan, vegan, vegetarian, and DASH diets [22-27] and considered by the World Health Organization to be responsible for metabolic disease prevention and treatment [28]. These diet recommendations are based on statistical averages from population based data. Individual differences in genotypes, environments, and their interactions may affect health outcomes even among those complying with these dietary recommendations [11]. As an example, unique postprandial blood glucose responses were found between different individuals who ate identical carbohydrate based meals [29]. By extension, a Nordic diet with 50% kilocalories from carbohydrates [30] may be the right choice for one individual and the Mediterranean diet with 40% kilocalories from carbohydrates [31] may be ideal for another. With so many healthy diet options to choose from, developing a facile and rapid method to measure metabolic flexibility and create a personalized nutrition plan is needed.

Stratifying individuals based on molecular biomarker profiling is a key step toward evaluating response and non-response to diet therapies. For example, metabolic responses to short-term diet challenges [32] that are considered “healthier” (i.e., improved nutrient density or balance) can be used to identify approaches that optimize health response. Metabolomics analyses can measure responses to diet so that a nutrition therapeutic strategy can be developed consistent with acute challenge results. For example, changes in the levels of metabolites linked to specific organ functions may identify the “primary” cause of metabolic dysfunction and thus may lead to improved personalization of diets. Furthermore, insulin resistance caused by liver dysfunction may require diets for glucose and insulin control that differ in macronutrient composition from dysfunction caused by muscle processes. For example, low amounts

of carbohydrates with a low glycemic index may improve muscle metabolism while carbohydrates in high fiber foods may preferentially improve liver function without the need to reduce intake of total carbohydrates [11, 33].

Personalized medicine and nutrition therapies will ultimately require the integration of genomic data with frequent monitoring of transcriptomic, proteomic, metabolomic and clinical biomarker profiles (e.g., bloods sugar, insulin, triglycerides). These approaches are being refined through novel study methods, such as n-of-1 clinical trials that are non-population-based and designed to collect enough information on any one individual over time to draw conclusions about individual response [2, 29, 32, 34]. The most detailed n-of-1 analysis used high-throughput -omic measurement methods as well as autoantibody profiles monitored almost daily in a single individual over a 14-month period. Type II diabetes risk was identified and dynamic changes in molecular and biological pathways, such as infection and stress response, were elucidated over healthy and diseased conditions of a single individual [35]. Such exhaustive phenotypic profiling that relies on interdependency and interconnectedness of markers that are multileveled (e.g., clinical, metabolite, transcript) in nonlinear relationships will become part of the baseline reference for health monitoring, diagnosis and treatment of the individual [34].

Of note, these biological systems analyses (that is, within the body measurements) also require inclusion of improved measures of not only nutritional intakes, but also built environment, and social determinants of health [36]. Population growth and climate change will threaten the development of sustainable, healthy diets and intensify the need for faster and improved nutrition research studies and their application to society [37].

Why are sex and gender differences important?

Sex and gender based variations may alter the population averages of age of onset, symptoms and disease severity [38, 39]. Sex differences are ultimately due to differences in chromosomal content and gene expression (e.g., gene x sex interactions) between males and females [40] although differences in epigenetic (in this case DNA methylation) regulation may also contribute [41]. These structural differences cascade through gene regulatory mechanisms and hence expression of genetic information causing not only dimorphic sexual traits but also differences in many physiological processes and outcomes [40]. These include differences in gut microbe-brain axis [42], immune function [43], lipid kinetics [44], and food related neuronal responses to foods [44]. Gender is often interchangeably used with sex in basic science literature although it increasingly is reserved for behavioral, cultural, and or psychological traits that can

be expressed by either biological sex. Gender in the social sense has been linked to alterations in physiological outcomes [45]. Gender and sex in this thesis are used interchangeably based primarily on the historical use in the literature.

The genomic and genome x environment interactions that often result in differences in metabolites and proteins are dimorphic between the sexes [46]. Proton NMR analysis of the lipid region of plasma metabolites was thought to be a strong predictor of coronary artery disease subphenotypes but was not replicated due to confounders including gender, not previously contemplated [34, 47, 48]. One hundred and two of 131 metabolites including phosphatidlycholines, sphingomyelines, acylcarnitines and C6-sugars had concentration differences that differed by sex [38]. Elevated concentrations of glycine were observed in females and a single nucleotide polymorphism in the carbamoyl-phosphate synthase 1 (CPS1) locus impacted glycine concentrations in a sexually dimorphic manner, an example of gene x sex interactions. Although metabolism and outcomes result from the complexity of interactions between genetic and environmental factors, sex hormones are perhaps the central regulators of structural and metabolic dimorphisms. For example, women homozygous for the 43 base pair insertion (LL) polymorphism in the serotonin transporter-linked polymorphic region (5-HTTLPR) had responses to anti-depressant medication that were influenced by levels of their sex hormones. Non-menopausal women with the LL genotype showed significant improvement in depression scores from anti-depressive medication measured by the HAMD Hamilton Depression Rating Scale, whereas menopausal women with the same genotype showed the opposite response. An age effect was also observed in the women that was not observed in the men [49].

Drug response also differs between sexes: of the top 10 pharmaceutical drugs taken off the market due to life threatening drug reactions between 1997 and 2000, 8 were more harmful for women [50, 51]. In 2013, the FDA called for reduced doses of immediate-release zopidem products (Ambien and Edluar) due to slower metabolism of the products and increased risks to women [52]. Inclusion of females and more effective research strategies are necessary to understand dimorphic differences in response that would have increased the safety of these pharmaceuticals in advance of commercialization.

Many human clinical research studies were done either on males only, or both males and females, controlling for sex differences by averaging data as opposed to determining differences between sexes or genders. While a “convenience factor” (controlling for the menstrual cycle is challenging) for using only one sex cannot be discounted, U.S. legislation in the early 1970s to improve human research ethics guidelines codified the

concept of vulnerable populations. Women were included as vulnerable because of adverse effects of certain drugs [53] taken during pregnancy and therefore to protect against fetal injury when pregnancy status was unknown [54]. The U.S. National Institute of Health Revitalization Act was passed in 1993 required that each NIH-funded study include a representative of subpopulations (ancestral populations and women) unless their exclusion could be justified [55].

Although these policies have been in place for over 25 years, women are still under-represented in clinical studies which may be due, at least in part, to a patriarchal culture of some scientists and administrators [56]. Some progress in transitioning to more inclusive research has started. For example, Stanford University developed a Gendered Innovations program to assist scientists with practical methods for sex and gendered analysis and innovation [51]. The Food and Drug Administration (FDA) Office of Women's Health promotes research to facilitate FDA regulatory decisions related to advancing knowledge of sex and gender differences and unique health conditions to women [57]. The National Institutes of Health (NIH) has also taken a role to provide education on methods and techniques for sex and gender research at the cellular level as well as in animals and humans and offers an online course on sex and gender differences [58].

Why is a broad perspective on women's hormonal health and nutrition important?
Women's health research programs focused mainly on pregnancy, lactation and infant nutrition and dietary guidelines are published for these areas. However, research in adolescent and nonpregnant, premenopausal women has been sparse. For example, a review of PubMed literature 25 June 2018 revealed a total of 5741 published research articles on pregnancy and lactation since 1941; and a total of 583 published research articles on menstrual hormonal health and nutrition, the first of which was in 1972 [59]. Sex hormone rhythmicity and dysrhythmicity may be associated with premenstrual syndrome of various severities including abdominal bloating, menstrual cramps, mastalgia, acne, food cravings, constipation, diarrhea, or headache, among others [60]. However, these symptoms and underlying physiology occur in specific phases of the cycle. For example, premenstrual syndrome and mastalgia occur during the luteal phase, followed by menorrhagia and cramping during the menstrual phase. Dysmenorrhea [61], infertility [62, 63] and polycystic ovarian syndrome [63] are all associated with a loss of rhythmicity. The transition to menopause, a 10-year timeframe on average, is characterized by a loss of rhythmicity manifested by varying menstrual cycle lengths and reduction of sex hormone concentrations leading to hot flashes, abdominal weight gain, headaches, forgetfulness, fatigue and depression [64]. Limited dietary guidelines and accepted nutrition therapies have been published for Western

medicine and nutrition practices. A PubMed search (accessed 25 June 2018) revealed a total of 9 publications on this topic between 2002 and 2018 with an emphasis on the use of soy for symptom relief and dietary prevention of osteoporosis [59]. Early research in small pilot studies suggests vitamin B6 and magnesium supplementation decrease the symptoms of premenstrual syndrome [65, 66]. Nutrient requirements, such as lysine [67], and protein [68] may vary throughout the menstrual cycle indicating it is important to examine how supporting the nutrient x hormone connection in women could be a remedy for the uncomfortable and sometimes debilitating symptoms that coincide with natural physiological hormone changes.

The hormonal changes that occur during the normal menstrual cycle alter biomarkers of health and disease [69] demonstrating that knowledge of hormonal phase is crucial in developing diagnostics. Standards and recommended methods for determining the menstrual phase have been published [70]. For example, one study showed twice as many women had elevated cholesterol in the follicular versus luteal phases. This same study demonstrated C-reactive protein, a marker associated with inflammation and cardiovascular disease risk, to be most elevated during the menstrual phase [71].

The luteal phase of the menstrual cycle may be a normal stress during which physiological imbalances are easier to detect and these imbalances may be a predictor of long term health trajectories. For example, women with type 1 diabetes experience an increased risk of hyperglycemia during the luteal phase which is associated with decreased insulin sensitivity [72]. As noted previously, gestational diabetes is diagnosed with oral glucose tests and associated with future risk for T2D. The luteal phase also represents a time when women are more likely to overeat and crave unhealthy foods with excess fat [73] and added sugars [74, 75], increasing risk of excess body fatness or decreasing success with healthful diet modifications [76]. In fact, a weight loss program adapted to the menstrual cycle and tailored to counteract food cravings and metabolic changes has been shown to increase weight loss success [77].

Scope and outline of this thesis

Using clinical biomarkers, metabolomics, and diet interventions with intake analyses, we evaluate the metabolic impact of vegan and diet interventions in a new research study using fasting plasma samples after 48 hours and using postprandial plasma samples after meals and snacks. Sex and gender differences in response are evaluated using proteomics and pathway analyses in two larger, sex-balanced cohorts. Finally, clinical biomarker and metabolomics are assessed across the menstrual cycle phases using samples from a previously published study [69]. This fundamental information

may provide a foundation for future novel personalized nutrition strategies for women and men.

Like the OGTT and the meal challenges described, it is also possible to perform a diet challenge with the purpose of challenging metabolism with healthy foods not typically consumed. In **Chapters 2** and **3**, we describe diet intervention research in which we designed vegan and animal diets personalized to the energy needs of each individual with the same percentage of macronutrients from energy and the same food choices. Meals and snacks are provided on a short-term (3 days) basis to participants in a semi-controlled environment, compliance recorded, and nutrient composition intake calculated. Daily menus are repeated each day to strengthen the intensity of the response to those foods. The small size of our pilot study maked it possible to have a great deal of control over food intake. We provide results from standard clinical biochemistry and molecular phenotyping using liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS).

In **Chapter 2**, the concept of a healthful diet challenge is introduced in a healthy, gender-balanced population. A vegan diet regimen is evaluated for its 48-hour impact on modulating metabolic signatures. The comparison diet is an animal based diet regimen mimicking the foods typically eaten. A cross-over strategy is employed so the same individual phenotypes are exposed to both diet types.

In **Chapter 3**, the results build on **Chapter 2**, by comparing the impact of both vegan and animal meals on postprandial response. Glycemic, lipid and related metabolites demonstrate the nutritional advantages of both diet types.

In **Chapter 4**, a proteomic and network analysis strategy is used to evaluate baseline metabolic gender dimorphic differences. Aptamer-based affinity assays are used to assess the presence of low abundant serum proteins in a healthy cohort of Irish women and men. Pathway over-representation and functional pathway enrichment analyses are performed using WikiPathways, Kyoto Encyclopedia of Genes and Genomics (KEGG) and Reactome databases. The findings are then evaluated in a larger, pan-European cohort.

In **Chapter 5**, metabolomics is used to evaluate menstrual phase variations linked to hormone physiology in healthy menstruating women. A wide panel of small molecules meaurements are included, such as clinical chemistry, metabolomics, lipidomics and vitamin levels. Contrast comparisons are made across 5 menstrual phases to identify metabolic phase signatures.

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