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

Draper, C.F.

Metabolic signatures in nutrition and health:

Short-term diet response, sexual dimorphism and hormone chronobiology

Cover design: Magali Eggli, Michel Combes
Thesis design technology: Michel Combes
Thesis lay-out: Colleen Fogarty Draper
Printing: Proefschriftenprinten.nl

ISBN: ISBN/EAN:978-94-92679-63-5

Metabolic signatures in nutrition and health:

Short-term diet response, sexual dimorphism and hormone chronobiology

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op donderdag, 20 December 2018

klokke 12:30 uur

door

Colleen Fogarty Draper

geboren te West Islip, N.Y., U.S.A. in 1969

Promotor: Prof. dr. Thomas Hankemeier

Prof. dr. Jan van der Greef

Co-promotor: Dr. Sofia Moço

Promotiecommissie:

Prof. dr. Hubertus Irth substitute of the Dean: Scientific Director (or subsitute) (Chair)

Prof. dr. Joke Bouwstra affiliated with Faculty of Science, UL (Secretary)

Prof. dr. Hannelore Daniel affiliated with TUM/Germany

Prof. dr. Lorraine Brennan affiliated with UCD Dublin/Ireland

Prof. dr. Hanno Pijl affiliated with LUMC/Netherlands

Prof. dr. Ron de Kloet affiliated with LUMC/Netherlands

The research described in this thesis was performed at the division of Systems Biomedicine and Pharmacology of the Leiden Academic Centre for Drug Research (LACDR), Leiden University (Leiden, The Netherlands), the Nestle Institute of Health Sciences, Nestle Research Center and University College Dublin. The research was financially supported by Nestle Institute of Health Sciences and Nestle Research.

Perseverance, persistence, creativity and flexibility in spite of all obstacles, discouragements and impossibilities: It is this in all things that distinguishes the strong and resilient soul from the weak…and creates a life of limitless possibilities.

Modified extensively from Thomas Carlyle

Dedicated to Madeleine, Corinne and Sophia

Contents

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.

REFERENCES

[1] DeBusk, R. M., Fogarty, C. P., Ordovas, J. M., Kornman, K. S., Nutritional genomics in practice: where do we begin? *Journal of the American Dietetic Association* 2005, *105*, 589-598.
[2] Schork, N. J., Goetz, L. H., Single-Subject Studies in Translational Nutrition Research. *Annual review of nutrition* 2017, *37*, 395-422.
[3] Kaput, J., Morine, M., Discovery-based nutritional systems biology: developing N-of-1 nutrigenomic research. *Int J Vitam Nutr Res* 2012, *82*, 333-341.
[4] Tilman, D., Clark, M., Global diets link environmental sustainability and human health. *Nature* 2014, *515*, 518-522.
[5] Huber, M., Knottnerus, J. A., Green, L., van der Horst, H., Jadad, A. R., Kromhout, D., Leonard, B., Lorig, K., Loureiro, M. I., van der Meer, J. W., Schnabel, P., Smith, R., van Weel, C., Smid, H., How should we define health? *BMJ* 2011, *343*, d4163.
[6] Williams, R. P., *Biochemical Individuality: The Basis for the Genetrophic Concept* Keats Publishing, New Canaan, CT 1956.
[7] Williams, R. J., Biochemical approach to individuality. *Science* 1948, *107*, 459.
[8] Bachmann, O. P., Dahl, D. B., Brechtel, K., Machann, J., Haap, M., Maier, T., Loviscach, M., Stumvoll, M., Claussen, C. D., Schick, F., Haring, H. U., Jacob, S., Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. *Diabetes* 2001, *50*, 2579-2584.
[9] Baek, S., Moon, H., Ahn, H., Kodell, R. L., Lin, C. J., Chen, J. J., Identifying high-dimensional biomarkers for personalized medicine via variable importance ranking. *Journal of biopharmaceutical statistics* 2008, *18*, 853-868.
[10] Benjamini, Y., Discovering the false discovery rate. *Journal of the Royal Statistical Society. Series B (Statistical Methdology)* 2010, *72*, 405-416.
[11] van Ommen, B., van den Broek, T., de Hoogh, I., van Erk, M., van Someren, E., Rouhani-Rankouhi, T., Anthony, J. C., Hogenelst, K., Pasman, W., Boorsma, A., Wopereis, S., Systems biology of personalized nutrition. *Nutr Rev* 2017, *75*, 579-599.
[12] Stroeve, J. H. M., van Wietmarschen, H., Kremer, B. H. A., van Ommen, B., Wopereis, S., Phenotypic flexibility as a measure of health: the optimal nutritional stress response test. *Genes Nutr* 2015, *10*, 13.
[13] Wopereis, S., Stroeve, J. H. M., Stafleu, A., Bakker, G. C. M., Burggraaf, J., van Erk, M. J., Pellis, L., Boessen, R., Kardinaal, A. A. F., van Ommen, B., Multi-parameter comparison of a standardized mixed meal tolerance test in healthy and type 2 diabetic subjects: the PhenFlex challenge. *Genes Nutr* 2017, *12*, 21.
[14] van den Broek, T. J., Bakker, G. C. M., Rubingh, C. M., Bijlsma, S., Stroeve, J. H. M., van Ommen, B., van Erk, M. J., Wopereis, S., Ranges of phenotypic flexibility in healthy subjects. *Genes Nutr* 2017, *12*, 32.
[15] Storlien, L., Oakes, N. D., Kelley, D. E., Metabolic flexibility. *Proc Nutr Soc* 2004, *63*, 363-368.
[16] van Ommen, B., Keijer, J., Heil, S. G., Kaput, J., Challenging homeostasis to define biomarkers for nutrition related health. *Molecular nutrition & food research* 2009, *53*, 795-804.

[17] Xiang, A. H., Li, B. H., Black, M. H., Sacks, D. A., Buchanan, T. A., Jacobsen, S. J., Lawrence, J. M., Racial and ethnic disparities in diabetes risk after gestational diabetes mellitus. *Diabetologia* 2011, *54*, 3016-3021.
[18] Knowler, W. C., Barrett-Connor, E., Fowler, S. E., Hamman, R. F., Lachin, J. M., Walker, E. A., Nathan, D. M., Diabetes Prevention Program Research, G., Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *The New England journal of medicine* 2002, *346*, 393-403.
[19] Chen, R., Snyder, M., Promise of personalized omics to precision medicine. *Wiley Interdiscip Rev Syst Biol Med* 2013, *5*, 73-82.
[20] Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., Haqq, A. M., Shah, S. H., Arlotto, M., Slentz, C. A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B. R., Yancy, W. S., Jr., Eisenson, H., Musante, G., Surwit, R. S., Millington, D. S., Butler, M. D., Svetkey, L. P., A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009, *9*, 311-326.
[21] Pastor, M. D., Nogal, A., Molina-Pinelo, S., Melendez, R., Salinas, A., Gonzalez De la Pena, M., Martin-Juan, J., Corral, J., Garcia-Carbonero, R., Carnero, A., Paz-Ares, L., Identification of proteomic signatures associated with lung cancer and COPD. *J Proteomics* 2013, *89*, 227-237.
[22] Darwiche, G., Hoglund, P., Roth, B., Larsson, E., Sjoberg, T., Wohlfart, B., Steen, S., Ohlsson, B., An Okinawan-based Nordic diet improves anthropometry, metabolic control, and health-related quality of life in Scandinavian patients with type 2 diabetes: a pilot trial. *Food Nutr Res* 2016, *60*, 32594.
[23] Ohlsson, B., Darwiche, G., Roth, B., Hoglund, P., Alignments of endocrine, anthropometric, and metabolic parameters in type 2 diabetes after intervention with an Okinawa-based Nordic diet. *Food Nutr Res* 2018, *62*.
[24] Ruiz-Canela, M., Guasch-Ferre, M., Toledo, E., Clish, C. B., Razquin, C., Liang, L., Wang, D. D., Corella, D., Estruch, R., Hernaez, A., Yu, E., Gomez-Gracia, E., Zheng, Y., Aros, F., Romaguera, D., Dennis, C., Ros, E., Lapetra, J., Serra-Majem, L., Papandreou, C., Portoles, O., Fito, M., Salas-Salvado, J., Hu, F. B., Martinez-Gonzalez, M. A., Plasma branched chain/aromatic amino acids, enriched Mediterranean diet and risk of type 2 diabetes: case-cohort study within the PREDIMED Trial. *Diabetologia* 2018.
[25] Pawlak, R., Vegetarian Diets in the Prevention and Management of Diabetes and Its Complications. *Diabetes Spectr* 2017, *30*, 82-88.
[26] Trepanowski, J. F., Varady, K. A., Veganism Is a Viable Alternative to Conventional Diet Therapy for Improving Blood Lipids and Glycemic Control. *Critical reviews in food science and nutrition* 2015, *55*, 2004-2013.
[27] Razavi Zade, M., Telkabadi, M. H., Bahmani, F., Salehi, B., Farshbaf, S., Asemi, Z., The effects of DASH diet on weight loss and metabolic status in adults with non-alcoholic fatty liver disease: a randomized clinical trial. *Liver Int* 2016, *36*, 563-571.
[28] Organization, W. H., Fostering healthier and more sustainable diets - learning from the Mediterranean and NEw Nordic experience. 2018.
[29] Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T., Lotan-Pompan, M., Suez, J., Mahdi, J. A., Matot, E., Malka, G., Kosower, N., Rein, M., Zilberman-Schapira, G., Dohnalova, L., Pevsner-Fischer, M., Bikovsky, R., Halpern, Z., Elinav, E., Segal, E., Personalized Nutrition by Prediction of Glycemic Responses. *Cell* 2015, *163*, 1079-1094.

[30] Mithril, C., Dragsted, L. O., Meyer, C., Tetens, I., Biltoft-Jensen, A., Astrup, A., Dietary composition and nutrient content of the New Nordic Diet. *Public Health Nutr* 2013, *16*, 777-785.
[31] Thanopoulou, A., Karamanos, B., Angelico, F., Assaad-Khalil, S., Barbato, A., Del Ben, M., Djordjevic, P., Dimitrijevic-Sreckovic, V., Gallotti, C., Katsilambros, N., Migdalis, I., Mrabet, M., Petkova, M., Roussi, D., Tenconi, M. T., Nutritional habits of subjects with Type 2 diabetes mellitus in the Mediterranean Basin: comparison with the non-diabetic population and the dietary recommendations. Multi-Centre Study of the Mediterranean Group for the Study of Diabetes (MGSD). *Diabetologia* 2004, *47*, 367-376.
[32] Mathias, M. G., Coelho-Landell, C. A., Scott-Boyer, M. P., Lacroix, S., Morine, M. J., Salomao, R. G., Toffano, R. B. D., Almada, M., Camarneiro, J. M., Hillesheim, E., de Barros, T. T., Camelo-Junior, J. S., Campos Gimenez, E., Redeuil, K., Goyon, A., Bertschy, E., Leveques, A., Oberson, J. M., Gimenez, C., Carayol, J., Kussmann, M., Descombes, P., Metairon, S., Draper, C. F., Conus, N., Mottaz, S. C., Corsini, G. Z., Myoshi, S. K. B., Muniz, M. M., Hernandes, L. C., Venancio, V. P., Antunes, L. M. G., da Silva, R. Q., Laurito, T. F., Rossi, I. R., Ricci, R., Jorge, J. R., Faga, M. L., Quinhoneiro, D. C. G., Reche, M. C., Silva, P. V. S., Falquetti, L. L., da Cunha, T. H. A., Deminice, T. M. M., Tambellini, T. H., de Souza, G. C. A., de Oliveira, M. M., Nogueira-Pileggi, V., Matsumoto, M. T., Priami, C., Kaput, J., Monteiro, J. P., Clinical and Vitamin Response to a Short-Term Multi-Micronutrient Intervention in Brazilian Children and Teens: From Population Data to Interindividual Responses. *Molecular nutrition & food research* 2018, *62*, e1700613.
[33] Blanco-Rojo, R., Alcala-Diaz, J. F., Wopereis, S., Perez-Martinez, P., Quintana-Navarro, G. M., Marin, C., Ordovas, J. M., van Ommen, B., Perez-Jimenez, F., Delgado-Lista, J., Lopez-Miranda, J., The insulin resistance phenotype (muscle or liver) interacts with the type of diet to determine changes in disposition index after 2 years of intervention: the CORDIOPREV-DIAB randomised clinical trial. *Diabetologia* 2015.
[34] van der Greef, J., Hankemeier, T., McBurney, R. N., Metabolomics-based systems biology and personalized medicine: moving towards n = 1 clinical trials? *Pharmacogenomics* 2006, *7*, 1087-1094.
[35] Chen, R., Mias, G. I., Li-Pook-Than, J., Jiang, L., Lam, H. Y., Chen, R., Miriami, E., Karczewski, K. J., Hariharan, M., Dewey, F. E., Cheng, Y., Clark, M. J., Im, H., Habegger, L., Balasubramanian, S., O'Huallachain, M., Dudley, J. T., Hillenmeyer, S., Haraksingh, R., Sharon, D., Euskirchen, G., Lacroute, P., Bettinger, K., Boyle, A. P., Kasowski, M., Grubert, F., Seki, S., Garcia, M., Whirl-Carrillo, M., Gallardo, M., Blasco, M. A., Greenberg, P. L., Snyder, P., Klein, T. E., Altman, R. B., Butte, A. J., Ashley, E. A., Gerstein, M., Nadeau, K. C., Tang, H., Snyder, M., Personal omics profiling reveals dynamic molecular and medical phenotypes. *Cell* 2012, *148*, 1293-1307.
[36] *Accounting for Social Risk Factors in Medicare Payment: Identifying Social Risk Factors*, Washington (DC) 2016.
[37] Barrett, B., Charles, J. W., Temte, J. L., Climate change, human health, and epidemiological transition. *Prev Med* 2015, *70*, 69-75.
[38] Mittelstrass, K., Ried, J. S., Yu, Z., Krumsiek, J., Gieger, C., Prehn, C., Roemisch-Margl, W., Polonikov, A., Peters, A., Theis, F. J., Meitinger, T., Kronenberg, F., Weidinger, S., Wichmann, H. E., Suhre, K., Wang-Sattler, R., Adamski, J., Illig, T.,

Discovery of sexual dimorphisms in metabolic and genetic biomarkers. *PLoS Genet* 2011, *7*, e1002215.
[39] Kautzky-Willer, A., Harreiter, J., Pacini, G., Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus. *Endocr Rev* 2016, *37*, 278-316.
[40] Rawlik, K., Canela-Xandri, O., Tenesa, A., Evidence for sex-specific genetic architectures across a spectrum of human complex traits. *Genome Biol* 2016, *17*, 166.
[41] Hartman, R. J. G., Huisman, S. E., den Ruijter, H. M., Sex differences in cardiovascular epigenetics-a systematic review. *Biol Sex Differ* 2018, *9*, 19.
[42] Jasarevic, E., Morrison, K. E., Bale, T. L., Sex differences in the gut microbiome-brain axis across the lifespan. *Philos Trans R Soc Lond B Biol Sci* 2016, *371*, 20150122.
[43] Klein, S. L., Flanagan, K. L., Sex differences in immune responses. *Nat Rev Immunol* 2016, *16*, 626-638.
[44] Santosa, S., Jensen, M. D., The Sexual Dimorphism of Lipid Kinetics in Humans. *Front Endocrinol (Lausanne)* 2015, *6*, 103.
[45] Sciences, N. A. o., *Accounting for Social Risk Factors in Medicare Payment: Identifying Social Risk Factors*, Washington (DC) 2016.
[46] Al-Daghri, N. M., Al-Attas, O. S., Johnston, H. E., Singhania, A., Alokail, M. S., Alkharfy, K. M., Abd-Alrahman, S. H., Sabico, S. L., Roumeliotis, T. I., Manousopoulou-Garbis, A., Townsend, P. A., Woelk, C. H., Chrousos, G. P., Garbis, S. D., Whole serum 3D LC-nESI-FTMS quantitative proteomics reveals sexual dimorphism in the milieu interieur of overweight and obese adults. *J Proteome Res* 2014, *13*, 5094-5105.
[47] Kirschenlohr, H. L., Griffin, J. L., Clarke, S. C., Rhydwen, R., Grace, A. A., Schofield, P. M., Brindle, K. M., Metcalfe, J. C., Proton NMR analysis of plasma is a weak predictor of coronary artery disease. *Nat Med* 2006, *12*, 705-710.
[48] Brindle, J. T., Antti, H., Holmes, E., Tranter, G., Nicholson, J. K., Bethell, H. W., Clarke, S., Schofield, P. M., McKilligin, E., Mosedale, D. E., Grainger, D. J., Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. *Nat Med* 2002, *8*, 1439-1444.
[49] Gressier, F., Verstuyft, C., Hardy, P., Becquemont, L., Corruble, E., Menopausal status could modulate the association between 5-HTTLPR and antidepressant efficacy in depressed women: a pilot study. *Arch Womens Ment Health* 2014, *17*, 569-573.
[50] United States General Accounting Office, 2001. GAO-01-286R Drugs Withdrawn From Market. https://www.gao.gov/assets/100/90642.pdf. 13 April 2018.
[51] Stanford University, 2011. Gendered Innovations: in Science, health and MEdicine, Engineering, and Environment. http://genderedinnovations.stanford.edu/what-is-gendered-innovations.html. 13 April 2018.
[52] United States Food and Drug Administration, 2013. FDA Drug Safety Communications: FDA approves new label chagnes and dosing for zolpidem products and a recommendation to avoid driving the day after using Ambien CR. https://www.fda.gov/drugs/drugsafety/ucm352085.htm. 13 April 2018.
[53] Collins, T. F., History and evolution of reproductive and developmental toxicology guidelines. *Curr Pharm Des* 2006, *12*, 1449-1465.
[54] Institute of Medicine, 1999. Women and Health Research: Ethical and Legal Issues of Including Women in Clinical Studies: Volume 1. https://www.ncbi.nlm.nih.gov/pubmed/25144106. 25 June 2018.

[55] National Institues of Health, 1993. S.1- National Institutes of Health Revitalization Act of 1993. Subtitle B--Clinical Research Equity Regarding Women and Minorities. https://orwh.od.nih.gov/sites/orwh/files/docs/NIH-Revitalization-Act-1993.pdf. 25 June 2018.
[56] Saini, A., *Inferior: how science got women wrong and the new research that's rewriting history*, Beacon Press, Boston 2017.
[57] United States Food and Drug Administration, 2018. Women's Health Research. https://www.fda.gov/ScienceResearch/SpecialTopics/WomensHealthResearch/default.htm. 18 May 2018.
[58] National Institutes of Health, 2018. NIH Office of Research on Women's Health (ORWH). https://orwh.od.nih.gov/sex-gender. 25 June 2018.
[59] National Institutes of Health, 2018. PubMed. https://www.ncbi.nlm.nih.gov/pubmed. 25 June 2018.
[60] Yonkers, K. A., Simoni, M. K., Premenstrual disorders. *Am J Obstet Gynecol* 2018, *218*, 68-74.
[61] Osayande, A. S., Mehulic, S., Diagnosis and initial management of dysmenorrhea. *Am Fam Physician* 2014, *89*, 341-346.
[62] Koroma, L., Stewart, L., Infertility: evaluation and initial management. *J Midwifery Womens Health* 2012, *57*, 614-621.
[63] Spritzer, P. M., Polycystic ovary syndrome: reviewing diagnosis and management of metabolic disturbances. *Arq Bras Endocrinol Metabol* 2014, *58*, 182-187.
[64] Santoro, N., Epperson, C. N., Mathews, S. B., Menopausal Symptoms and Their Management. *Endocrinol Metab Clin North Am* 2015, *44*, 497-515.
[65] Ebrahimi, E., Khayati Motlagh, S., Nemati, S., Tavakoli, Z., Effects of magnesium and vitamin b6 on the severity of premenstrual syndrome symptoms. *J Caring Sci* 2012, *1*, 183-189.
[66] Fathizadeh, N., Ebrahimi, E., Valiani, M., Tavakoli, N., Yar, M. H., Evaluating the effect of magnesium and magnesium plus vitamin B6 supplement on the severity of premenstrual syndrome. *Iran J Nurs Midwifery Res* 2010, *15*, 401-405.
[67] Kriengsinyos, W., Wykes, L. J., Goonewardene, L. A., Ball, R. O., Pencharz, P. B., Phase of menstrual cycle affects lysine requirement in healthy women. *American journal of physiology. Endocrinology and metabolism* 2004, *287*, E489-496.
[68] Calloway, D. H., Kurzer, M. S., Menstrual cycle and protein requirements of women. *The Journal of nutrition* 1982, *112*, 356-366.
[69] Wallace, M., Hashim, Y. Z., Wingfield, M., Culliton, M., McAuliffe, F., Gibney, M. J., Brennan, L., Effects of menstrual cycle phase on metabolomic profiles in premenopausal women. *Hum Reprod* 2010, *25*, 949-956.
[70] Allen, A. M., McRae-Clark, A. L., Carlson, S., Saladin, M. E., Gray, K. M., Wetherington, C. L., McKee, S. A., Allen, S. S., Determining menstrual phase in human biobehavioral research: A review with recommendations. *Exp Clin Psychopharmacol* 2016, *24*, 1-11.
[71] Schisterman, E. F., Mumford, S. L., Sjaarda, L. A., Failure to consider the menstrual cycle phase may cause misinterpretation of clinical and research findings of cardiometabolic biomarkers in premenopausal women. *Epidemiol Rev* 2014, *36*, 71-82.
[72] Brown, S. A., Jiang, B., McElwee-Malloy, M., Wakeman, C., Breton, M. D., Fluctuations of Hyperglycemia and Insulin Sensitivity Are Linked to Menstrual Cycle Phases in Women With T1D. *J Diabetes Sci Technol* 2015, *9*, 1192-1199.

[73] McNeil, J., Cameron, J. D., Finlayson, G., Blundell, J. E., Doucet, E., Greater overall olfactory performance, explicit wanting for high fat foods and lipid intake during the mid-luteal phase of the menstrual cycle. *Physiol Behav* 2013, *112-113*, 84-89.
[74] Gorczyca, A. M., Sjaarda, L. A., Mitchell, E. M., Perkins, N. J., Schliep, K. C., Wactawski-Wende, J., Mumford, S. L., Changes in macronutrient, micronutrient, and food group intakes throughout the menstrual cycle in healthy, premenopausal women. *Eur J Nutr* 2016, *55*, 1181-1188.
[75] Yen, J. Y., Chang, S. J., Ko, C. H., Yen, C. F., Chen, C. S., Yeh, Y. C., Chen, C. C., The high-sweet-fat food craving among women with premenstrual dysphoric disorder: emotional response, implicit attitude and rewards sensitivity. *Psychoneuroendocrinology* 2010, *35*, 1203-1212.
[76] Buffenstein, R., Poppitt, S. D., McDevitt, R. M., Prentice, A. M., Food intake and the menstrual cycle: a retrospective analysis, with implications for appetite research. *Physiol Behav* 1995, *58*, 1067-1077.
[77] Geiker, N. R., Ritz, C., Pedersen, S. D., Larsen, T. M., Hill, J. O., Astrup, A., A weight-loss program adapted to the menstrual cycle increases weight loss in healthy, overweight, premenopausal women: a 6-mo randomized controlled trial. *Am J Clin Nutr* 2016, *104*, 15-20.

Chapter 2

A 48-hour vegan diet challenge in healthy women and men induces a branch-chain amino acid related, health associated, metabolic signature

Based on

Colleen Fogarty Draper[1] Irene Vassallo, Alessandro Di Cara, Cristiana Milone, Ornella Comminetti, Irina Monnard, Jean-Philippe Godin, Max Scherer, MingMing Su, Wei Jia, Seu-Ping Guiraud, Fabienne Praplan, Laurence Guignard, Corinne Ammon Zufferey, Maya Shevlyakova, Nashmil Emami, Sofia Moco, Maurice Beaumont, Jim Kaput, Francois-Pierre Martin

A 48-hour vegan diet challenge in healthy women and men induces a branch-chain amino acid related, health associated, metabolic signature

Reproduced with full permission, License 4456371118756o, from John Wiley and Sons from *Molecular nutrition & food research* 2018;62(3)

doi: 10.1002/mnfr.201700703

ABSTRACT

Research is limited on diet challenges to improve health. A short-term, vegan protein diet regimen nutritionally balanced in macronutrient composition compared to an omnivorous diet was hypothesized to improve metabolic measurements of blood sugar regulation, blood lipids, and amino acid metabolism. This randomized, cross-over, controlled vegan versus animal diet challenge was conducted on 21 (11 female,10 male) healthy participants. Fasting plasma was measured during a 3 day diet intervention for clinical biochemistry and metabonomics. Intervention diet plans met individual caloric needs. Meals were provided and supervised. Diet compliance was monitored. The vegan diet lowered triglycerides, insulin and homeostatic model assessment of insulin resistance (HOMA-IR), bile acids, elevated magnesium levels, and changed branched-chain amino acids (BCAAs) metabolism ($p < 0.05$), potentiating insulin and blood sugar control after 48 h. Cholesterol control improved significantly in the vegan versus omnivorous diets. Plasma amino acid and magnesium concentrations positively correlated with dietary concentrations. Polyunsaturated fatty acids and dietary fiber inversely correlated with insulin, HOMA-IR, and triglycerides. Nutritional biochemistries, BCAAs, insulin, and HOMA-IR were impacted by sexual dimorphism. A health-promoting, BCAA-associated metabolic signature was produced from a short-term, healthy, controlled, vegan diet challenge when compared with a healthy, controlled, omnivorous diet.

INTRODUCTION

Vegan diets are plant-based regimens that exclude meat, eggs, dairy products, and any other animal-derived foods and ingredients. In contrast, a vegetarian diet emphasizes plant-based foods but can also include dairy, eggs, honey, and fish. Populations who lack access to animal protein or cultures with historical or religious traditions have a higher percentage of vegetarians: about 35% of the Indian population eats strictly plant-based diets. Approximately 10% of all vegetarians are vegan but an increasing number of people are adopting a non-animal product diet [1].

While epidemiologic evidence published in the 1980s and 1990s supports the benefits of vegetarian diets, skepticism remains largely because of concerns about specific nutrient deficiencies of plant-based foods. Both vegan and vegetarian diets can be healthful for all life stages with appropriate selection of plant-based foods that adequately meet requirements for protein, iron, n-3 fatty acids, iodine, zinc, calcium, and vitamin B12 [2]. An intermittent vegan diet regimen that is alternated within a habitual, balanced omnivorous diet can also meet these nutritional requirements.

The growing demand for unsustainable animal-based products by an expanding and wealthier global population is negatively effecting the planet [3]. Plant-based food production requires less energy and has less of an impact on non-renewable environmental resources. Policies that promote adoption of plant-based diets may help protect the planet while improving the health of individuals [4].

The health benefits of nutrients or foods are typically analyzed after weeks or months of consuming experimental diets. Epidemiological evidence suggests habitual intake of plant-based diets (vegan and vegetarian) reduces risk of diabetes, lipid disorders, and metabolic syndrome [5]. For example, the prevalence of type 2 diabetes mellitus (T2DM) in Seventh-Day Adventists who respect a strict vegan diet is 45% of the incidence in the general population [6]. Meta-analysis of vegan diet studies show improved glycemic control compared to American Diabetes Association (ADA)'s dietary recommendations in T2DM individuals [7]. A dietary portfolio (a vegan regimen with specific amounts of plant sterols, viscous fibers, soy protein, and nuts) reduced blood lipids more than the National Cholesterol Education Program (NCEP) in individuals with hypercholesterolemia [7] using plant-based interventions that ranged from 2 to 104 weeks. Consistency in nutrient content and participant compliance are difficult to maintain in long-term studies, which may be confounded by unmeasured environmental variables that differ between participants.

Our study measured metabolic changes associated with consumption of vegan- compared to animal-based diets in the short time period of 48 hours. Diets were rigorously planned, intake compliance monitored, and clinical parameters as well as a diverse panel of plasma metabolites analyzed. The results indicated significant health-promoting benefits of a short-term, healthy vegan diet exposure.

MATERIALS AND METHODS

Study population and ethical approval

This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki, approved by the Ethical Committee of Lausanne University School of Medicine (CER-VD, ref no. 222/14), and registered on ClinicalTrials.gov with the identifier NCT02223585. All participants provided written informed consent for study participation and were offered financial compensation agreed by the ethical committee (3,200 CHF) for time spent and schedule inconveniences.

A total of 56 healthy male and female volunteers were pre-screened at information sessions held at the Metabolic Unit, Nestlé Research Center (Lausanne, Switzerland). Out of the 32 participants who signed informed consent, 26 were enrolled in the study (6 screening failures), 5 dropped out and 21 healthy participants (10 men, 11 women) completed this pilot study. Two participants dropped out because of non-serious adverse events, and another 3 decided not to proceed with the study. All participants habitually ate a heterogeneous diet including animal and vegan proteins before entrance into the study.

Study inclusion criteria were age (from 18 to 55 years), regular bowel movement (at least once every 1-2 days), body mass index (BMI, from 18.5 to 27 kg.m-2). Health status was assessed by a physician during a screening visit as a standard medical visit with blood chemistry analysis. Exclusion criteria included special diets (vegetarian, high protein, and low cholesterol or weight loss program), pregnancy, food allergy, smoking, high alcohol consumption (more than 2 drinks per day), and excessive physical exercise (more than 5 moderate physical exercises per week).

Animal and vegan dietary interventions

The energy provided by vegan and animal meals was personalized for each participant according to their calculated resting energy requirements and level of physical activity. Energy requirements were calculated as a function of height, weight, age, and activity level, based on the Harris Benedict Equation [8]. Eighteen (9 animal and 9 vegan) meal

plans were designed with different caloric values, ranging from 1600 to 3000 calories (**Supplementary Figure 1**). Macronutrient composition was matched between animal and vegan-based meals, and was calculated based on 20% protein, 50% carbohydrate and 30% fat of total calories within ±5% of calculated needs of each participant.

Clinical trial design

The clinical trial was a randomized, open label, cross-over, controlled study. Study participants were randomly assigned to the animal and plant protein challenges using Medidata Balance with dynamic allocation [9].The study lasted five weeks (**Figure 1**) following a one week run-in phase (Week 1 = W1) that defines baseline of the participant's normal diet and lifestyle. Participants were then randomly assigned to either animal or vegan meals for 3 consecutive days (Tuesday, Wednesday and Thursday). Fasting blood samples were obtained after an overnight fast on each of the 3 days (days 0,1,2) of the intervention diets as indicated in **Figure 1**. The third week (W3) was a washout period during which participants resumed their usual diets. The cross-over intervention occurred during the 4th week and lasted three consecutive days also during the middle of the week. Participants were monitored during week 5 (W5) to determine if they returned to their usual health and dietary status. During each 3-day intervention, participants ate the same meals on each day, including breakfast, morning snack, lunch, afternoon snack, and dinner. All meals and snacks were prepared and provided to study participants under supervision by the Metabolic Unit staff with the exception of the dinner meal, which was packaged for home consumption. During the study period, the participants were told to avoid consumption of alcoholic beverages and limit caffeinated and sugary beverages (no more than 2 cups of coffee, black or green tea per day). Fasting blood samples were obtained after an overnight fast as indicated in **Figure 1**.

Descriptions of the materials and methods used for the diet diaries, compliance questionnaires and diet intake analysis; anthropometric, clinical data, and blood sample collection; amino acid analysis, bile acid analysis, and metabonomics analysis are found in **Chapter 2**.

Statistical analysis

Statistical significance of observed differences across groups was calculated using a Wilcoxon Rank-Sum test. A Wilcoxon Signed rank test was used for paired comparison. To determine if dietary intake was correlated with plasma metabolite levels we calculated the Spearman correlation coefficients and Spearman's rho statistic to test the significance of the association. All p-values were corrected for multiple testing using false discovery rate, and values < 0.10 are reported.

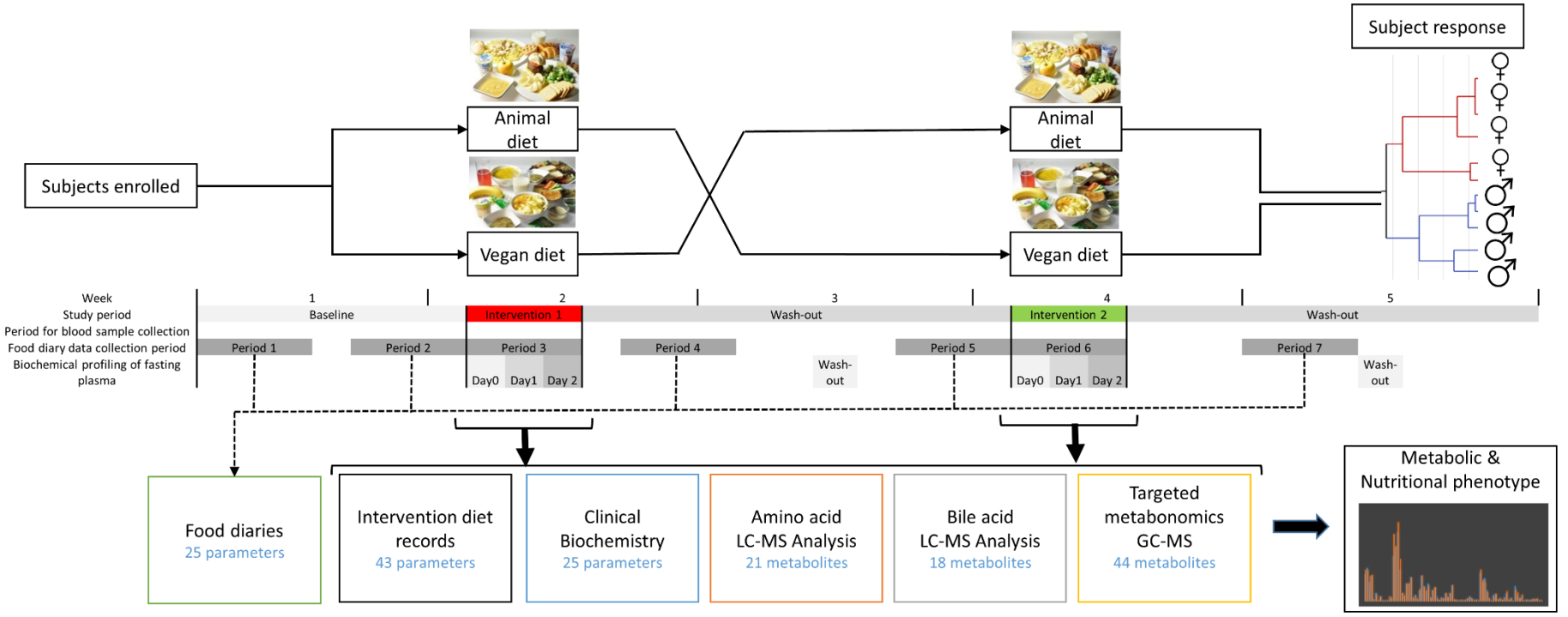


Figure 1. Overview of the experimental design and the analytical strategy. **A)** The clinical trial was a randomized, open label, cross-over, controlled study that lasted five weeks. Study participants were randomly assigned to the animal and plant protein diets to which they were challenged for three consecutive days. **B)** Dietary intake was assessed using three day food diaries each week during run-in periods, before, and after dietary interventions. During intervention periods, accurate dietary intake and metabolic status were monitored. Extensive metabolic phenotyping covered standard biochemical and nutritional measures, as well as amino acid, bile acid and targeted metabolic profiling. **C** Impact of vegan diets on individual metabolic and nutritional status was investigated in comparison to the animal dietary intervention as well as to subjects' free living dietary habits.

However, due to the small sample size, and exploratory nature of the study, the non-corrected statistical significance was used for interpretation of results

The crossover nature of this study required assessing potential carryover effects of each diet. A model was fit to assess the interaction between each metabolite and the diet sequence. A p-value below 0.05 was interpreted as a significant carry-over. To test the association between the type of diet and the changes in metabolite levels a linear mixed effect models (Model 1) was fit for each metabolite separately.
Further description of statistical methods can be found in **Chapter 2**.

Table 1. Baseline anthropometric and clinical parameters by gender

Clinical marker	Females (n=11)		Males (n=10)		p-Value
	Mean	SD	Mean	SD	
Age (years)	34.0	9.1	35.0	9.9	6.72E-01
Height (cm)	162.3	4.9	179.6	5.6	1.56E-04
Weight (kg)	58.9	8.3	75.7	10.9	3.08E-03
BMI (kg/m^2)	22.4	3.0	23.4	2.82	4.60E-01
HDL (mmol/L)	1.6	0.3	1.5	0.2	8.05E-01
Albumin (Kg/m^3)	39.7	3.8	43.7	2.9	**1.51E-02**
Alanine aminotranferase (U/L)	20.5	4.5	31.3	9.0	**1.36E-03**
Aspartate aminotransferase (U/L)	18.7	2.7	24.5	8.7	**2.19E-02**
Total cholesterol (mmol/L)	4.5	0.7	4.3	0.9	4.60E-01
Chol/HDL	2.8	0.5	3.0	1.0	6.99E-01
Creatine kinase inhibitor (mmol/L)	71.9	20.9	223.4	186.4	**6.37E-04**
Chloride (mmol/L)	105.0	1.3	104.5	2.0	7.20E-01
Creatinine (µmol/L)	67.0	11.4	93.4	10.9	**3.26E-04**
Ferritin (µg/L)	66.6	49.4	178.3	75.3	**1.06E-03**
Glucagon (pM/L)	6.2	3.1	9.3	5.4	2.18E-01
Glucose (mmol/L)	5.2	0.5	5.5	0.4	9.80E-02
Insulin (µU/mL)	17.5	6.6	13.5	5.5	2.31E-01
Iron (µmol/L)	13.8	4.2	16.9	4.3	2.18E-01
Potassium (mmol/L)	4.1	0.2	4.2	0.3	5.02E-01
LDL (mmol/L)	2.6	0.6	2.8	0.9	4.38E-01
Magnesium (mmol/L)	0.8	0.1	0.8	0.0	**2.20E-02**
Sodium (mmol/L)	141.4	1.1	142.6	1.2	**4.67E-02**
Pre-albumin (g/m^3)	288.0	39.9	369.1	69.4	**6.71E-03**
Phosphate (mmol/L)	1.1	0.2	1.2	0.2	7.51E-01
c-reactive protein (g/m^3)	1.6	1.6	1.9	3.4	2.18E-01
Total bilirubin (µmol/L)	7.2	3.2	17.0	7.0	**2.76E-03**
Triglycerides (mmol/L)	0.8	0.2	0.9	0.6	3.07E-01
Total protein (Kg/m^3)	72.7	4.9	75.2	3.7	2.91E-01
Tranferrin (Kg/m^3)	2.5	0.5	2.2	0.2	1.05E-01
Non-esterified fatty acids (µmol/L)	385.1	191.1	390.5	194.1	9.16E-01

The p-values are calculated by performing a Wilcoxon rank sum test. SD = standard deviation.

RESULTS

Population characteristics

A total of 21 (11 females, 10 males) healthy participants completed the clinical trial (**Table 1**). Individuals were similar in age and BMI . Several clinical parameters showed strong

gender specificities, including plasma clinical biochemistries (albumin, pre-albumin, ferritin, Mg, Na), hepatic functions (ALAT, ASAT, total bilirubin), and others (creatinine, creatine kinase inhibitor), as reported in **Table 1**.

Diet intervention

Forty-three nutrients were analyzed from the diet records from each 2-day intervention for the vegan and animal intervention diets (**Table 2**). Statistical analysis was performed using the average nutrient intakes from all participants over 2 days using Wilcoxon signed rank test. The 2 controlled-intervention diets were matched for macronutrient (carbohydrate, protein and fat) intakes within a pre-determined 5% variation since different food sources have unique nutrient compositions (**Supplementary Figure 1**). Menus were not matched for micronutrient intakes. Since diet intake may be altered by individual preferences, appetite, and satiety, diet intake compliance was monitored to quantify actual intake differences across the 2 intervention diets. Total carbohydrate and fat density (percentage of total calories) intakes were not significantly different between diets. Protein intake in the animal diet was 3% higher compared to vegan diet for females, and 1.3% higher for males. Intake of total calories, saturated fat, B2, B6, sodium, phosphorus, selenium, and potassium was higher on the animal diet. Intake of polyunsaturated fat, fiber, magnesium, iron, copper, vitamin A, vitamin C, vitamin B1 and folate was higher on the vegan diet. Non-heme iron, which is less easily absorbed than the heme-iron found in red meat, was also higher on the vegan diet. Although vitamin B12 was not consumed in the vegan diet, its absence is unlikely to alter the results since the half-life of this vitamin is 6 days in plasma [10]. Overall, nutrient intakes were higher in males compared to females (**Table 2**).

Diet intervention and habitual diet intake

Three-day diet diaries were used to assess habitual diet intake for comparison with the 2 controlled diet interventions (**Supplementary Table 1**). Habitual diet intakes were significantly higher in vitamin C, fat, % calories from fat, and sodium compared to mean intakes in both the animal and vegan diets. Participants demonstrated full compliance with intake of vegan and animal meals and snacks, although there were some intake differences when satiety was reached before meal completion. Participants on the vegan diet increased their intake of vitamin A, vitamin E, iron, folate, magnesium, relative calorie intake from protein, polyunsaturated fats, and total dietary fiber; and decreased their intake of vitamin B12, carbohydrates, calories, and monounsaturated and saturated fats compared to their habitual diets. Participants on the animal diet also significantly increased intakes of vitamins B2 and B6 and decreased intakes of vitamin E, potassium, protein, relative calorie intake from protein, polyunsaturated fatty acids, and total dietary fiber compared to their habitual diets. The direction of these differences was

consistent across genders. No significant differences in intake were noted between the vegan/animal or animal/vegan washout periods (**Supplementary Table 2**).

Table 2. Significant diet intake differences between vegan and animal intervention diets

	Animal diet means		Vegan diet means			Gender differences**	
Diet Variable	**F**	**M**	**F**	**M**	**P-value***	**Vegan P-**	**Animal P-value**
Kilocalories (Kcal)	1758.00	2619.75	1508.91	2255.47	**1.32E-05**	**2.86E-04**	**2.18E-04**
Alanine (mg)	4168.60	6214.95	2585.01	4098.12	**6.40E-05**	**2.86E-04**	**2.86E-04**
Cysteine (mg)	1339.82	1935.35	706.64	1194.12	**6.40E-05**	**2.17E-04**	**2.86E-04**
Glycine (mg)	3827.37	5628.07	2324.53	3706.53	**6.40E-05**	**2.86E-04**	**2.86E-04**
Histidine (mg)	2476.30	3661.50	1493.30	2368.32	**6.40E-05**	**2.86E-04**	**2.86E-04**
Isoleucine (mg)	4278.14	6398.27	2382.80	3873.30	**6.40E-05**	**2.86E-04**	**2.17E-04**
Leucine (mg)	6880.82	10366.10	3468.68	6072.80	**6.40E-05**	**1.64E-04**	**2.17E-04**
Lysine (mg)	5618.20	8634.38	3060.98	4908.35	**6.40E-05**	**2.86E-04**	**2.86E-04**
Methionine (mg)	1984.23	3029.90	793.07	1298.65	**6.40E-05**	**2.17E-04**	**2.86E-04**
Phenylalanine (mg)	3820.75	5816.07	2625.02	4257.70	**6.40E-05**	**2.17E-04**	**2.17E-04**
Proline (mg)	17998.58	24831.60	2116.40	3689.31	**6.40E-05**	**1.64E-04**	**2.86E-04**
Threonine (mg)	3418.18	5190.48	2184.59	3549.60	**6.40E-05**	**2.17E-04**	**2.86E-04**
Tryptophan (mg)	1157.75	1689.03	570.14	873.45	**6.40E-05**	**3.75E-04**	**2.86E-04**
Tyrosine (mg)	2870.95	4404.55	1773.75	2872.97	**6.40E-05**	**2.86E-04**	**2.17E-04**
Valine (mg)	4444.27	6769.45	2750.23	4454.65	**6.40E-05**	**2.17E-04**	**2.17E-04**
Polyunsaturated fat (g)	13.29	17.52	22.42	36.71	**6.41E-05**	**1.64E-04**	5.40E-03
Protein (g)	85.60	128.27	62.54	102.91	**6.41E-05**	**2.17E-04**	**2.87E-04**
Saturated fat (g)	21.89	37.44	9.48	13.92	**6.41E-05**	**5.55E-04**	**1.65E-04**
Total fiber (g)	14.67	20.32	30.52	49.02	**6.41E-05**	**2.14E-04**	**3.76E-04**
Iron (mg)	7.39	10.45	12.71	19.00	**7.41E-05**	**9.23E-04**	**2.86E-04**
Protein %	19.50	19.58	16.47	18.26	**7.42E-05**	**4.89E-04**	9.16E-01
Vitamin B12 (ug)	3.48	5.38	0.00	0.00	**1.41E-04**	NA	1.58E-03
Selenium (ug)	46.33	57.75	13.14	11.78	**1.43E-04**	4.79E-01	2.14E-03
Copper (ug)	927.11	1015.05	1872.39	2528.43	**1.43E-04**	3.43E-03	5.48E-02
Potassium (mg)	4.42	6.49	1.80	2.29	**1.43E-04**	1.65E-02	**2.76E-04**
Vitamin E (mg)	6.39	8.33	13.38	17.48	**1.43E-04**	3.75E-02	3.73E-02
Magnesium (mg)	202.25	290.25	424.75	589.12	**1.43E-04**	**1.06E-03**	**5.20E-04**
Phosporus (mg)	1331.64	2054.53	801.05	1121.75	**1.43E-04**	2.18E-03	**2.80E-04**
Vitamin B2 (mg)	1.06	1.70	0.60	0.81	**1.43E-04**	3.43E-03	**9.39E-04**
Vitamin A (mg)	0.17	0.30	4.62	3.51	**1.65E-04**	5.24E-01	**2.01E-04**
Vitamin C (mg)	40.92	49.58	61.23	80.80	**1.68E-04**	1.11E-02	1.63E-03
Vitamin B1 (mg)	0.71	1.05	0.94	1.31	**1.68E-04**	4.30E-03	**2.76E-04**
Vitamin B6 (mg)	1.60	2.27	1.20	1.42	**1.68E-04**	5.35E-03	**7.03E-04**
Folate (ug)	197.83	258.32	281.98	351.43	**1.97E-04**	2.64E-02	**3.80E-04**
Sodium (mg)	1899.36	2725.60	1423.84	2011.90	**2.31E-04**	2.18E-03	**9.44E-04**
Carbohydrate (g)	208.48	312.80	191.23	267.18	**1.23E-03**	**3.75E-04**	**2.87E-04**
FAT (g)	59.93	88.08	52.73	79.28	1.56E-03	**6.32E-04**	**1.65E-04**
FAT %	30.71	30.32	31.31	31.51	1.01E-02	9.72E-01	1.00E+00
Monounsaturated fats (g)	21.43	28.67	17.18	27.96	1.12E-02	**2.82E-04**	**2.15E-04**
Serine (mg)	3236.43	4909.48	2870.00	4586.06	1.65E-02	**2.86E-04**	**2.17E-04**
Vitamin D (ug)	1.30	1.67	1.15	0.86	3.11E-02	2.60E-01	**5.30E-04**
CHO %	47.38	47.70	50.95	47.50	7.63E-02	**1.35E-03**	1.00E+00
Arginine (mg)	4612.57	6672.82	4315.64	7102.65	8.08E-01	**2.17E-04**	**2.17E-04**

*Conventional P-values for animal vs. vegan diets calculated using Wilcoxon signed-rank test. F, female; M, male. Significant p values for gender are in bold. A Wilcoxon rank sum test was used to calculate gender differences.

Clinical response and nutritional status

Subjects on the vegan and animal dietary interventions showed small but significant changes in several plasma biochemistry parameters related to glucose and lipid

metabolisms within 48 hours (**Table 3, Figure 2**). The most significant metabolic effect was a decrease in triglycerides and cholesterol/HDL ratio in participants consuming the vegan diet with no gender interaction. However, participants consuming the vegan diet had a significant decrease in insulin and HOMA-IR [11] that differed by gender. A marked increase in plasma magnesium, slight increase in sodium, but decreased phosphate occurred in individuals consuming the vegan diet (**Table 3**).

Effect of the dietary intervention on plasma amino acid metabolic status

Compared to the animal diet, the vegan diet intervention was associated with a decrease within 48 hours of 10 plasma amino acid levels, including total BCAAs (driven by leucine and valine), total essential amino acids (EAAs) (driven by leucine, valine, threonine, tryptophan, methionine, and lysine), and an increase in arginine and glycine (Table 3, Figure 2). The BCAA and EAA effects differed by gender with the males having significantly higher plasma levels of valine and leucine. The alpha-keto acid analogue of isoleucine (a BCAA), 3-methyl 2 oxovaleric acid, was found to be significantly increased during the vegan diet with no gender specific effects. Lysine, tryptophan, arginine, and methionine levels showed gender differences at baseline that did not carry over to the intervention response (See **Supplementary Table 3**).

Effect of the dietary intervention on plasma fatty acid profiles

Significant increases in the concentration of 3 saturated and 2 monounsaturated fatty acids, specifically dodecanoic acid (C12:0), myristic acid (C14:0), capric acid (C10:0), 5-dodecenoic acid (C12:1) and myristoleic acid (C14:1) occurred in participants consuming the vegan diets. None of these effects were gender dependent (**Table 3**).

Effect of the dietary intervention on plasma bile acids

Participants consuming the vegan diet had significantly decreased bile acids at 24hrs (GUDCA, and GCA) and at 48 hours (GUDCA, DCA, and HDCA) (**Table 3**). A trend in decreased plasma concentrations of GUDCA occurred in males consuming the vegan diet relative to the animal diet at 24 and 48 hours.

Relationships between dietary intake and plasma metabolites

Spearman's rank correlation analysis was performed to analyze changes in diet intake against blood biochemical and metabolite concentrations (**Table 4**). Strong positive correlations were identified between intakes of 11 dietary amino acids and their amino acid concentrations in the plasma. Positive correlations were also found between intakes of vitamins B2, B6, B12, and B1 and total plasma amino acids and between dietary and plasma magnesium. In addition, dietary polyunsaturated fats and total dietary fiber were negatively correlated with plasma insulin, HOMA-IR, and triglycerides.

Table 3: Vegan verses animal diet intervention response, clinical and metabonomic biomarkers

Panel	MARKER	Diet Day	Animal F* Mean	Animal M* Mean	Vegan F Mean	Vegan M Mean	Estimate DIET	P-value DIET**	P-value GENDER***
Amino Acid									
	Proline (nmol/ml)	Day 3	133.69	157.05	87.65	102.67	-5.30E-01	**1.87E-11**	1.43E-01
	Valine (nmol/ml)	Day 3	189.44	250.42	157.50	218.91	-3.21E+01	**2.20E-08**	**2.64E-05**
	EAAs (nmol/ml)	Day 3	829.65	963.39	734.15	867.82	-9.52E+01	**1.63E-06**	**8.26E-04**
	BCAAs (nmol/ml)	Day 3	332.68	454.43	294.94	413.18	-5.58E+00	**1.74E-06**	**5.80E-05**
	Lysine (nmol/ml)	Day 3	154.14	175.17	136.12	150.24	-2.18E+01	**2.38E-05**	7.14E-01
	Citrulline (nmol/ml)	Day 3	23.62	29.47	20.35	26.42	-2.66E+00	**3.82E-05**	7.90E-02
	Threonine (nmol/ml)	Day 3	140.30	120.94	112.54	103.85	-1.14E-01	**5.92E-04**	6.48E-01
	Tryptophan (nmol/ml)	Day 3	52.29	56.01	46.69	48.78	-5.44E+00	**7.91E-04**	8.90E-01
	Alanine (nmol/ml)	Day 3	278.92	301.04	242.58	262.26	-3.31E+01	2.62E-03	5.15E-01
	Arginine (nmol/ml)	Day 3	68.76	75.00	66.50	87.34	9.38E+00	2.88E-03	5.99E-01
	Methionine (nmol/ml)	Day 3	23.18	25.29	19.93	22.96	-2.44E+00	3.57E-03	2.30E-01
	3 methyl 2 oxovaleric acid (nmol/ml)	Day 3	168.88	99.96	219.16	209.77	1.45E+02	7.71E-03	4.00E-01
	Leucine (nmol/ml)	Day 3	228.54	311.67	220.17	163.29	-1.63E+00	1.03E-02	**4.49E-03**
	Tyrosine (nmol/ml)	Day 3	47.42	55.06	40.62	53.44	-5.21E+00	1.80E-02	1.51E-01
	Glycine (nmol/ml)	Day 3	167.30	172.06	162.56	182.50	2.60E+01	3.86E-02	9.18E-01
Clinical									
	Magnesium ($mM.L^{-1}$)	Day 3	0.79	0.85	0.87	0.89	6.40E-02	**5.19E-05**	3.40E-01
	Triglycerides ($mM.L^{-1}$)	Day 3	0.83	1.22	0.72	0.58	-4.01E-01	**1.61E-04**	7.75E-01
	CHOL/HDL	Day 3	2.79	3.08	2.67	2.75	-2.98E-02	**3.42E-04**	7.06E-01
	Sodium ($mM.L^{-1}$)	Day 3	140.48	141.32	140.84	142.88	1.01E+00	4.76E-03	3.79E-01
	Phosphate ($mM.L^{-1}$)	Day 3	1.11	1.17	1.02	1.05	-8.01E-02	8.56E-03	2.36E-01
	Insulin ($\mu U.mL^{-1}$)	Day 3	18.56	12.48	15.40	9.18	-2.98E-01	3.04E-02	**2.97E-03**
	HOMA-IR	Day 3	4.24	3.04	3.49	2.16	-2.42E-01	3.95E-02	**1.07E-02**
Fatty Acids									
	Dodecanoic acid (C12:0) (ng/ml)	Day 3	721.40	801.41	1338.69	1564.94	1.26E+02	**4.08E-05**	2.55E-01
	5-Dodecanoic acid (C12:1) (ng/ml)	Day 3	989.79	1255.54	1806.04	2234.53	8.79E+02	9.77E-04	1.79E-01
	Myristic acid (C14:0) (ng/ml)	Day 3	3067.98	3056.96	4357.64	4233.59	1.18E+03	2.27E-03	8.65E-01
	Capric acid (C10:0) (ng/ml)	Day 3	686.56	603.76	1285.24	1408.98	6.94E+02	3.09E-03	9.94E-01
	Myristoleic acid (C14:1) (ng/ml)	Day 3	3067.98	3056.96	4357.64	4233.59	2.14E+03	1.92E-02	2.43E-01
Bile Acids									
	Glycoursodeoxycholic acid (GUDCA) (nmol/L)	Day 2	0.08	0.08	0.08	0.04	-0.4904	**6.42E-04**	3.50E-01
	Glycocholate (GCA) (nmol/L)	Day 2	0.21	0.16	0.07	0.08	-0.778	**9.58E-04**	8.77E-02
	Glycoursodeoxycholic acid (GUDCA) (nmol/L)	Day 3	0.09	0.12	0.09	0.06	-6.75E-01	1.46E-02	5.01E-02
	Deoxycholic acid (DCA) (nmol/L)	Day 3	0.26	0.38	0.31	0.21	-3.35E-01	3.63E-02	5.82E-01
	Hyodeoxycholic acid (HDCA) (nmol/L)	Day 3	0.05	0.06	0.09	0.10	1.57E-01	4.66E-02	4.87E-01

*F=female; M=male. The model is a mixed effect model for each day and marker separately. A random effect per subject was used. A t test (null hypothesis: coeff = 0) was used. **The p-values correspond to the coefficient of interest in the regression model. ***P-values correspond to gender main term effect. P-values bolded if False Discovery Rate <0.10.

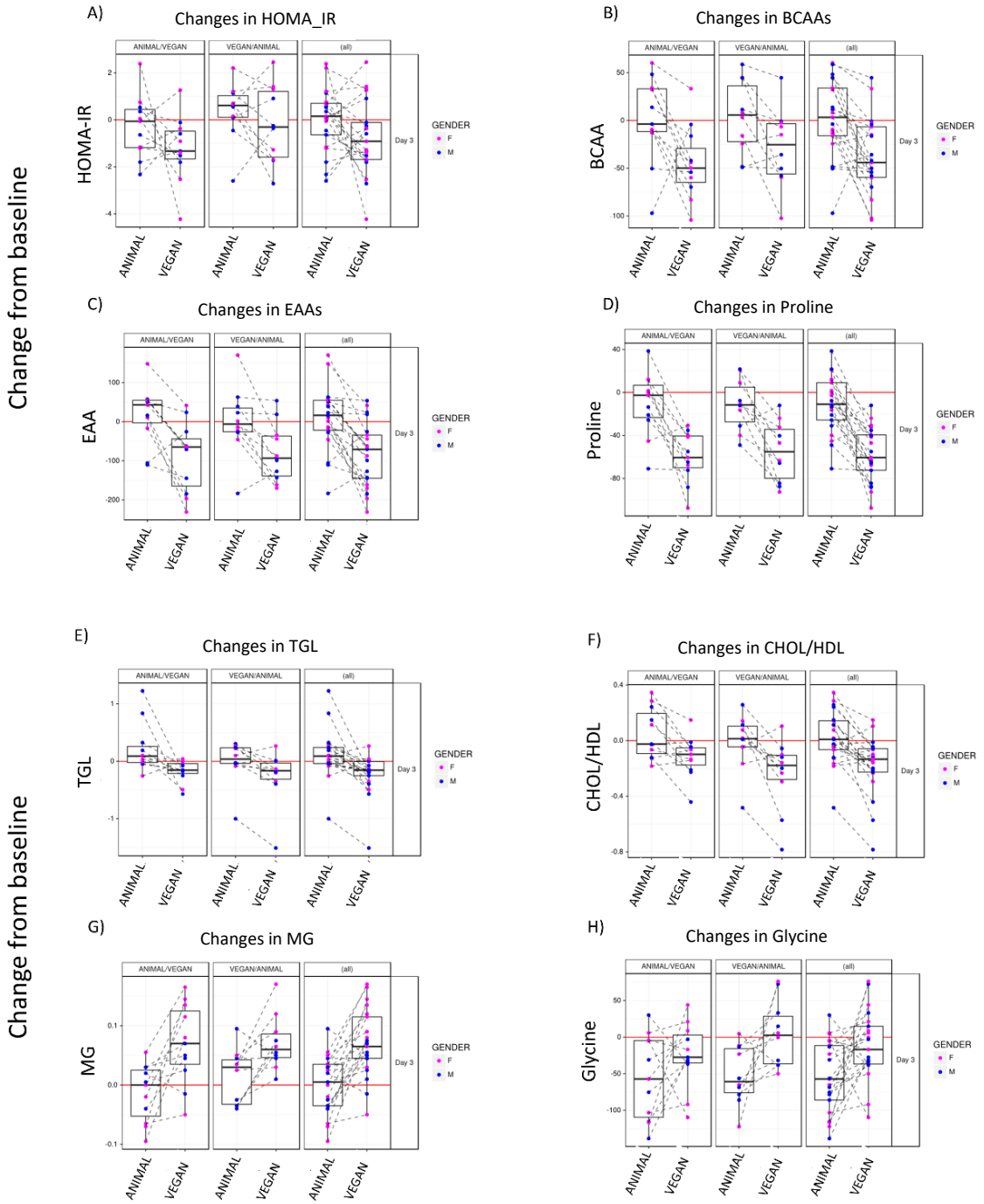


Figure 2A-H. Vegan vs. animal diet metabolic response signature by diet day and diet sequence. Key metabolite differences are graphically depicted showing day-3 changes from baseline for both diets, both directions (animal, washout, then vegan and vice versa). Gender differences shown with pink (female) and blue (male) dots. A mixed linear model was used to evaluate significant differences. A) HOMA-IR p=3.95E-02 (diet) p=1.07E-02 gender; B) BCAAs p (diet)=1.74E-06 p (gender)=5.80E-05; C) EAAs p(diet)=1.63E-06 p(gender)=8.26E-04; D) Proline p(diet)=1.87E-11; E) TGL p(diet)=1.61E-04; Chol/HDL p(diet)=3.42E-04; F) Mg p(diet)=5.19E-05; G) Glycine p(diet)=3.86E-02. BCAAs=branched chain amino acids; EAA=essential amino acids; TGL=triglycerides; Chol/HDL=cholesterol to high density lipoprotein ratio; MG=magnesium; HOMA-IR is calculated by glucose*insulin/22.5.

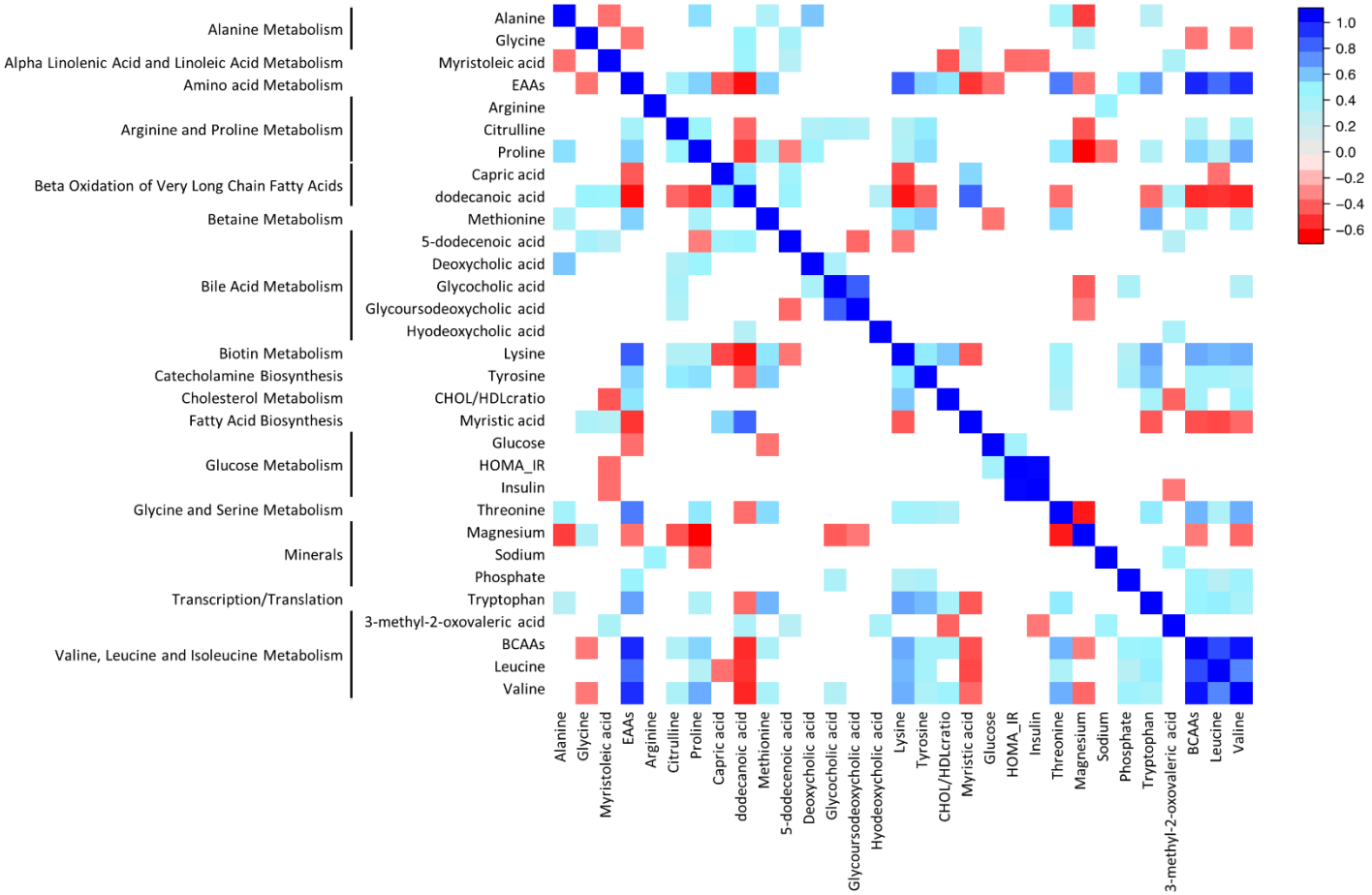


Figure 3. Heatmap with color gradients related to the similarity between blood biochemical variables based on spearman correlation coefficients, using a statistical significant treshold at 95% confidence interval. A blue cell represents a positive correlation between the corresponding biochemical species with a value ranging from 0.3 to 1; red represents an anti-correlation between the variables, with a value ranging from -0.3 to 0.60; a white cell indicates no correlation. Variables were ordered with biochemical pathways or classes.

Correlation analyses between blood plasma biochemical species

In addition to the correlations identified between biochemical metabolite classes (e.g., correlations of an amino acid with another amino acid, or of one bile acid with another), strong correlations between metabolites of different molecular classes were found: individual bile acids, fatty acids, and lipids correlated with individual amino acids (Figure 3). The branched-chain amino acids were negatively correlated with myristic acid and magnesium but positively correlated with the Chol/HDL ratio and phosphorus. The essential amino acids were negatively correlated with dodecanoic acid, myristic acid, capric acid, HDL, glucose, and glycine and positively correlated with Chol/HDL ratio, phosphorus, and triglycerides. The metabolite 3-methyl-2-oxovaleric acid had an inverse correlation with insulin, Chol/HDL ratio, and TGs and a positive correlation with the myristoleic and dodecaonic acids and hyodeoxycholic acid (HDCA).

DISCUSSION

Vegan diet patterns have been historically associated with diabetes prevention, promotion of blood sugar control, improved insulin sensitivity, decreased total and LDL cholesterol, and higher levels of HDL [5, 12], To our knowledge, this is the first short-term, plant-based (vegan) diet study focused on metabolic health that identified improvement in clinical and metabolic parameters associated with insulin resistance in healthy subjects.

A well-balanced vegan diet improves lipid and insulin metabolic status

Participants enrolled in this study were not vegans and habitually consumed animal protein-based diets typical of the Swiss culture. The animal- and vegan-protein foods provided in this intervention were intentionally designed to be more nutritious than typical diets and were balanced in macronutrient content. The foods were consumed in a semi-controlled, supervised environment and intake measured to reduce uncertainty in nutrient consumption. Participants in our study showed statistically significant improvements in insulin and HOMA-IR (33% reduction vegan vs. animal), TGs, and the Chol/HDL ratio after only 48 hours of consuming the vegan diet. Elevated serum lipids, including total CHOL and TGs and a high cholesterol/HDL ratio have been associated with insulin resistance [13-15]. Improvements in these metabolic syndrome-associated risk factors were likely related to higher fiber content (39.8 g and 17.5 g from the vegan and animal based protein diets consecutively), micronutrient density, and non-oxidized polyunsaturated fats (PUFAs), and lower glycemic index food choices found in a healthful vegan diet pattern. Inverse correlations were also found between dietary PUFA intake and total dietary fiber with insulin, HOMA-IR, and TGs, consistent with higher PUFA and fiber intakes (**Table 2, Table 4**). Major contributors of PUFAs from the vegan

diet include cashew butter, rapeseed oil, sunflower oil, hummus, hemp protein powder, and soy yoghurt (**Supplementary Figure 1**).

A significant increase in plasma magnesium from the vegan diet was found and a highly significant positive correlation between dietary magnesium intake and plasma magnesium, insulin, and HOMA-IR was detected (**Tables 3 and 4**), cashew butter, hummus, lentils, and kidney beans eaten as part of the vegan diet regimen in this study are rich sources of magnesium. Increased intake of magnesium has been shown to reduce the risk of impaired glucose tolerance and insulin metabolism [16, 17] consistent with the improved insulin control and decreased triglyceride levels observed in this study (**Table 2, Figure 4**).

In addition to the changes in blood lipid profile, the saturated fatty acids dodecanoic, capric, and myristic acids were significantly elevated after the vegan diet. These saturated fatty acids (SFAs) likely reflect the fatty acid composition of the coconut milk provided at dinner each evening over the 2 days. The monounsaturated species of these fatty acids, 5-dodecanoic and myristoleic acids, were also significantly elevated after the vegan diet and reflect the digestion and metabolism of their dietary precursors. Intake of dodecanoic acid may decrease the ratio of total to HDL cholesterol ratio by raising HDL [18], [19]. Although only a trend between dodecanoic acid HDL was seen in the present study, a positive correlation between capric acid and HDL and an inverse correlation between Chol/HDL ratio and myristoleic acid was found, suggesting that these fatty acids may improve the lipoprotein profile (**Figure 3**).

In relation to observed lipid changes, some bile acid species were higher after consuming the animal protein relative to the vegan diets, consistent with their role in the digestion and absorption of dietary fat and cholesterol; and their synthesis in the liver from cholesterol. This effect may be explained by the higher saturated fat and cholesterol content in the animal diet (red meat) and the higher fiber in the vegan diet may have resulted in a relative decrease in bile acid absorption. Furthermore, bile acids are also essential metabolic integrators and signalling factors, far beyond their role as lipid solubilizers and simple regulators of bile-acid homeostasis[20]. In particular, it is now well established how glucose and insulin can enlarge bile acid pool size and their blood circulating levels, by modulating Cholesterol 7 alpha-hydroxylase, a rate limiting enzyme in bile acid synthesis[21]. Blood bile acid concentrations have been associated with fasting and postprandial insulin and glucose, with further implication in diabetes and obesity research [22, 23].The higher circulating levels of certain bile acids (and secondary bile acid DCA) and insulin observed in individuals on the animal diet compared to the vegan diet may therefore be directly related. It may be envisioned that

a vegan diet strategy may offer protective benefits against insulin resistance, as noted here with improved HOMA-IR, and lowered circulating insulin and specific bile acid species [24]. Future evaluation of the alternating plant – animal protein diet in insulin resistant individuals is warranted to determine if this strategy is beneficial for improving metabolic control.

Table 4: Spearman's rank correlations between plasma concentrations and dietary nutrient intakes

Diet	Plasma	r	P-value*
Amino acids (AAs)			
PRO	PRO	0.74	**2.42E-08**
VAL	VAL	0.73	**5.35E-08**
LYS	LYS	0.62	**1.05E-05**
LEU	LEU	0.59	**3.94E-05**
TYR	TYR	0.52	**4.04E-04**
MET	MET	0.52	**4.82E-04**
PHE	PHE	0.47	**1.74E-03**
ARG	ARG	0.46	**2.27E-03**
ILE	ILE	0.42	**5.61E-03**
TRP	TRP	0.42	**6.01E-03**
ALA	ALA	0.36	**1.77E-02**
B2	Total AAs	0.71	**3.28E-07**
B6	Total AAs	0.71	**3.54E-07**
B12	Total AAs	0.60	**4.22E-05**
B1	Total AAs	0.34	3.23E-02
Mg	Mg	0.54	**3.48E-04**
PUFA	Insulin	-0.55	**1.56E-04**
PUFA	HOMA-IR	-0.54	**2.07E-04**
PUFA	TG	-0.49	**8.76E-04**
Saturated Fat	TG	0.30	**4.80E-02**
TDF	Insulin	-0.51	**5.55E-04**
TDF	HOMA-IR	-0.50	**7.38E-04**
TDF	TG	-0.49	**1.10E-03**

*Conventional p-values are shown and those marked in bold were significant after False Discovery

A vegan diet may improve insulin sensitivity by modulating AA bioavailability

Elevated fasting BCAA and aromatic amino acids have been associated with higher metabolic risk of insulin resistance and obesity (21-29). The vegan diet induced a strong modulatory effect within 48 hours on the circulating levels of EAAs and BCAAs (Figure 2). In addition, blood concentrations of EAAs were directly correlated with intake of these amino acids suggesting this improved metabolic health status was associated with dietary protein (**Table 4, Figure 4**). Several studies captured specific AA signatures related to HOMA-IR conditions in lean and obese subjects [25], Chinese and Asian-Indian males [26], and in a weight loss cohort study of 500 men and women [27]. These reports showed a similar elevated AA signature in high versus low HOMA-IR status

including BCAAs (valine, leucine, isoleucine), aromatic (phenylalanine, tyrosine), alanine, glutamine, asparagine, and arginine which were positively associated with insulin resistance. The same AA differences from the animal vs vegan diets were found in this study with the exception of phenylalanine and asparagine. In a 6-month weight loss intervention study of 500 men and women, change in HOMA-IR was not strongly associated with amount of weight lost but rather with an AAs signature that included alanine, proline, BCAAs (valine leucine/isoleucine), methionine, aromatic amino acids (ArAAs) (phenylalanine, tyrosine), glutamine and ornithine [27]. The association of this AAs profile and HOMA-IR in different populations is consistent with the associations found in our participants consuming the vegan diet. Increased concentrations of the circulating essential BCAAs resulted from diet intake and protein catabolism. However, BCAA may increase insulin resistance when consumed with high fat diets, at least in rats [25]. The animal protein diet in the study reported here was slightly higher in overall fat content and significantly higher in saturated fat and BCAAs, which may potentiate an increase in HOMA-IR (**Table 3**). The vegan diet produced reductions in, but not strong statistical correlations between HOMA-IR, BCAAs, and ArAAs, which may be related to the short duration of our study (**Table 3, Figure 3**). Longer term (1 and 6 week) vegetarian plus fish diets significantly reduced and showed strong correlations between HOMA-IR, BCAAs, and ArAAs [28]. Plasma concentrations of 3-methyl-2-oxovaleric acid, a keto-acid product of isoleucine metabolism, decreased on the animal protein diet which may be due to increased activity of branched-chain keto acid dehydrogenase (BCKDH) [29].

Gender dimorphism may influence the impact of dietary modulation of glucose and insulin metabolism

Twelve amino acids, including the BCAAs, showed significant gender differences at baseline (**Supplementary Table 3**). The BCAAs, insulin and HOMA-IR showed gender differences post intervention (**Table 3**). Recent studies found that males had significantly higher BCAAs, phenylalanine, tyrosine, alanine, proline, methionine, glutamine and ornithine [30]. In contrast, insulin and HOMA-IR showed gender differences in intervention response with plasma concentrations slightly higher in females in the study presented here (**Table 3**). These results were inconsistent with published literature on correlations between gender dimorphism and insulin resistance [31, 32]. However, gender differences in insulin concentrations and calculated HOMA-IR may be augmented by consuming defined short-term or long-term diets or by differences in estrogen levels between studies based on age or unknown menstrual cycle status, or any other difference in study conditions. Males were also found to have slightly higher magnesium levels (**Table 3**), consistent with published literature [33].

Flexitarian dieting for metabolic health improvement?

Intermittent or periodic fasting has been proposed as a novel approach to weight management and modulation of markers of metabolic syndrome. A similar flexitarian approach of alternating between animal and vegan protein choices requires only periodic alteration of an individual's dietary habits. Reducing the amount of habitual change on a frequent basis may increase adherence to dietary lifestyle change which has been shown to be the most important factor for diet success [34]. Additionally, a flexitarian diet optimizes nutrient intake from all protein sources and prevents potential micronutrient deficiencies from an exclusively vegan diet. Based on the results shown here, an intermittent vegan diet with healthful macronutrient and micronutrient balance may beneficially modulate blood insulin, lipids, and amino acids (**Figure 4A**).

Study limitations & opportunities

This was a small, pilot study of 21 participants with concomitant limitations of sample size and risk of false positives. Even though vegan meals appeared to have a higher volume of food, caloric intake was less on this diet compared to animal protein diet due to the satiating effect of the high fiber containing plant foods and despite efforts to match calories and macronutrient contents (**Table 2**). We did not distinguish between the different types of fiber (soluble, insoluble, inulin) and phytosterols such as plant stanols and sterols that are known to have an effect on lipids/cholesterol, bile acids, and glucose. However, the complexity of metabolite-metabolite and varying genotype-metabolite interactions challenge simplistic single metabolite interpretations of the phenotypic response to the diet interventions. Even though many nutrient parameters were recorded, this study relied on food diaries with manual database entry for analysis which highlights the lack of availability of high quality, reliable nutrition data capture and measurement technologies. This was a study of non-obese healthy participants without insulin resistance. However, the results presented here suggest that this diet strategy be tested in a non-healthy population to examine its beneficial effects on insulin and AA metabolism.

The strength of the study was the development and testing of a specific diet strategy that more closely resembled habitual animal protein-based diets compared to a vegan diet comprised of easily accessible food choices in a controlled, cross-over design. These results can be translated more easily to a diet strategy to promote health improvement compared to single food ingredient interventions. Moreover, the small size of the study permitted a focused, quality controlled measurement and analysis of dietary intake.An intermittent, high protein vegan diet may not only impact weight control but may be more environmentally sustainable than current animal-based protein diets.

A

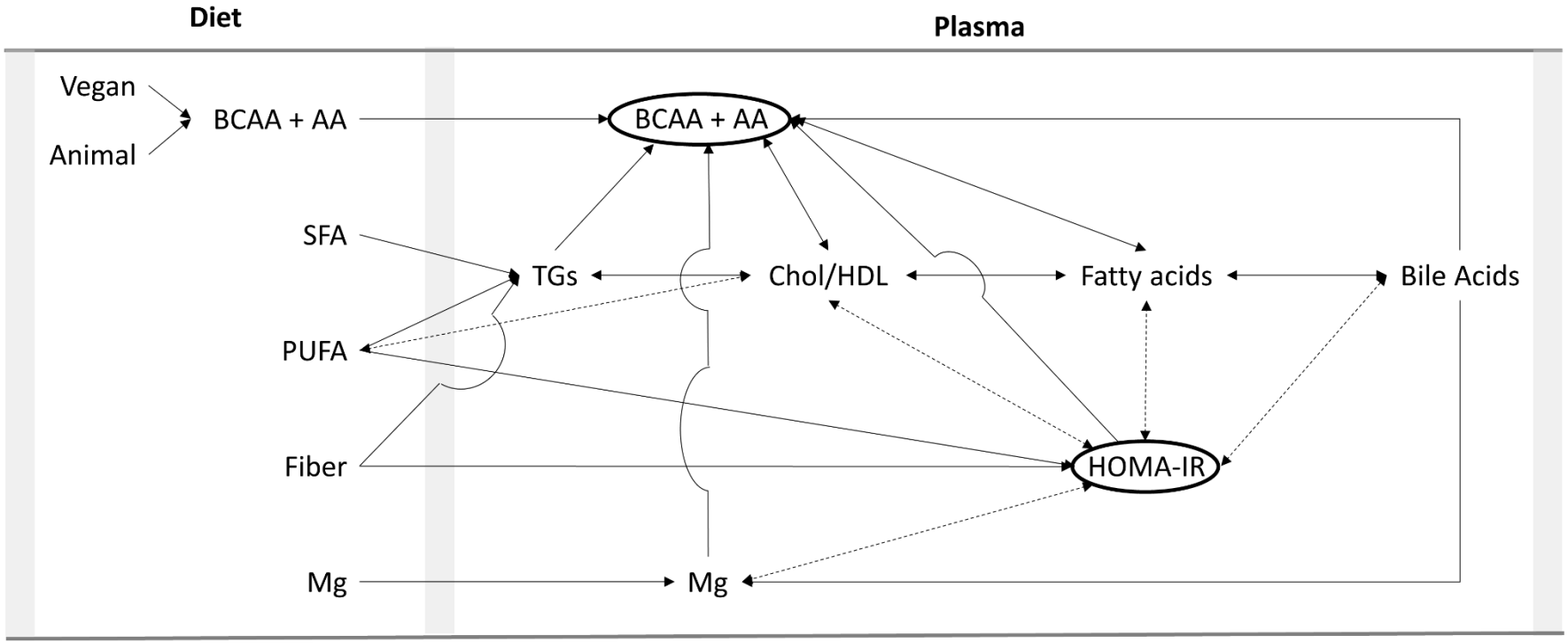


Figure 4A - The vegan diet decreased plasma AA ,HOMA-IR, Chol/HDL, and bile acids while increasing Mg; with the animal diet having opposing effects. Dietary SFAs increased TGs in the animal diet; high dietary PUFA and fiber intakes from the vegan diet reduced HOMA-IR and TGs. Dietary AA increased 11 plasma AA. Solid lines and arrows depict significant Spearman's correlations between diet and plasma variables and between plasma metabolites; dotted lines depict known associations. Biomarkers that showed significant gender dimorphic responses to the diet interventions are circled. AA=amino acid; BCAA=branched chain amino acids; TGs=triglycerides; Chol/HDL=cholesterol to high density lipoprotein ratio; Mg=magnesium; HOMA-IR is calculated by glucose*insulin/22.

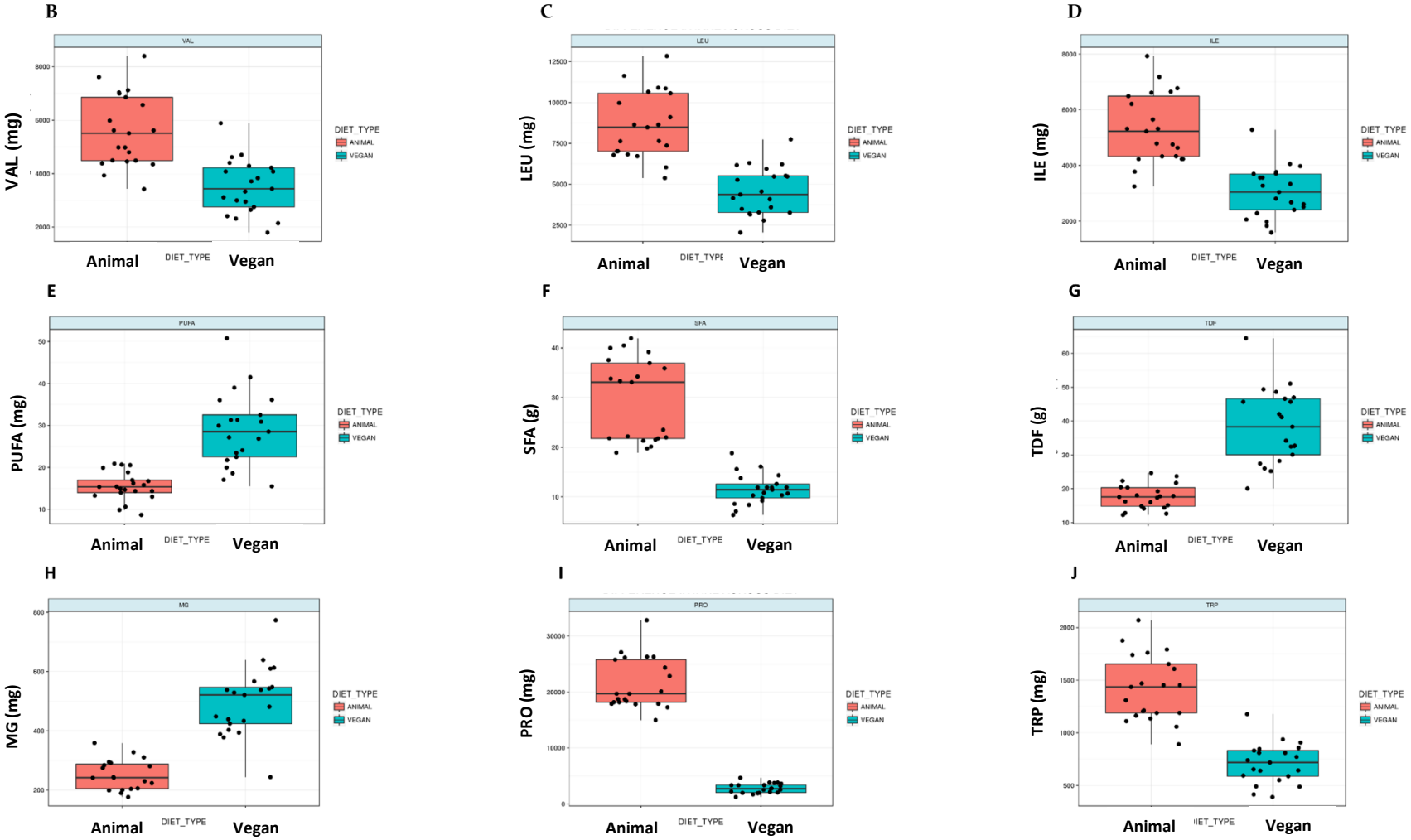


Figure 4B-J - Dietary intake differences across diet interventions for correlated nutrients. Nutrient intake differences in animal vs. vegan diets using a Wilcoxon signed-rank test. A subset of nutrients also found to be correlated with significant metabolites is shown here. B) VAL (valine) p=6.40E-05; C) LEU (leucine) p=6.40E-05; D) ILE (Isoleucine) p=6.40E-05; E) PUFA (polyunsaturated fatty acids) p=6.41E-05; F) SFA (saturated fatty acids) p=6.41E-05; G) TDF (total dietary fiber) p=6.41E-05; H) MG (magnesium) p=1.43E-04; I) PRO (protein) p=6.41E-05; J) TRP (tryptophan) p=6.40E-05. All nutrient intakes were significantly higher in males vs. females (p<0.05).

CONCLUSION

We analyzed the effects of vegan- versus animal protein-based diets on metabolic health parameters. A slightly higher than average protein intake was chosen for both diets to optimize metabolic health impact. We produced a branched-chain amino acid-associated metabolic signature from a short term, healthy, vegan diet challenge (Figure 4). These results suggest an improvement in the ability to adapt to changes in intake of different nutrients or levels of nutrients while maintaining a healthy metabolism [35]. Intermittently substituting vegan meals in otherwise animal-based diets may decrease the unsustainable environmental impact of animal-based diets. Future research should be conducted to evaluate the benefits of this diet strategy in an obese, insulin-resistant population.

ACKNOWLEDGEMENTS

We would like to thank the metabolic team at the clinical development unit for their role in running the study at the metabolic unit (MU) (Corine Nielsen Moennoz, Frederik Delodder, Sylviane Oguey-Araymon), at Nestle Research Center (NRC); Richard Cote from Nestlé institute of Health Sciences SA (NIHS) for support in nutritional data integration; Frederic Ronga from NIHS for his assistance on the diet intervention analysis. Jorg Hager, Martin Kussmann, Denis Barron, Ivan Montoliu and Peter Sperisen at NIHS, and Leonidas Karagounis, Christophe Schmitt, Christian Darimont at NRC for scientific discussions, Stephanie Blum-Sperisen, Emil Chuang and Eric Rolland for their advice on study design. Special thanks to Kei Sakamoto for reviewing and providing feedback on this manuscript.

REFERENCES

[1] Leitzmann, C., Vegetarian nutrition: past, present, future. Am J Clin Nutr 2014, 100 Suppl 1, 496S-502S.
[2] Craig, W. J., Mangels, A. R., American Dietetic, A., Position of the American Dietetic Association: vegetarian diets. Journal of the American Dietetic Association 2009, 109, 1266-1282.
[3] Tilman, D., Clark, M., Global diets link environmental sustainability and human health. Nature 2014, 515, 518-522.
[4] Sabate, J., Soret, S., Sustainability of plant-based diets: back to the future. Am J Clin Nutr 2014, 100 Suppl 1, 476S-482S.
[5] Chiu, Y. F., Hsu, C. C., Chiu, T. H., Lee, C. Y., Liu, T. T., Tsao, C. K., Chuang, S. C., Hsiung, C. A., Cross-sectional and longitudinal comparisons of metabolic profiles between vegetarian and non-vegetarian subjects: a matched cohort study. Br J Nutr 2015, 114, 1313-1320.
[6] Snowdon, D. A., Phillips, R. L., Does a vegetarian diet reduce the occurrence of diabetes? American journal of public health 1985, 75, 507-512.
[7] Trepanowski, J. F., Varady, K. A., Veganism Is a Viable Alternative to Conventional Diet Therapy for Improving Blood Lipids and Glycemic Control. Critical reviews in food science and nutrition 2015, 55, 2004-2013.
[8] Harris, J. A., Benedict, F. G., A Biometric Study of Human Basal Metabolism. Proc Natl Acad Sci U S A 1918, 4, 370-373.
[9] Lebowitsch, J., Ge, Y., Young, B., Hu, F., Generalized multidimensional dynamic allocation method. Statistics in medicine 2012, 31, 3537-3544.
[10] Adams, J. F., Biological half-life of vitamin B12 in plasma. Nature 1963, 198, 200.
[11] Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., Turner, R. C., Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985, 28, 412-419.
[12] Robberecht, H., De Bruyne, T., Hermans, N., Effect of various diets on biomarkers of the metabolic syndrome. Int J Food Sci Nutr 2017, 68, 627-641.
[13] Bulum, T., Duvnjak, L., Insulin resistance in patients with type 1 diabetes: relationship with metabolic and inflammatory parameters. Acta Clin Croat 2013, 52, 43-51.
[14] Ren, X., Chen, Z. A., Zheng, S., Han, T., Li, Y., Liu, W., Hu, Y., Association between Triglyceride to HDL-C Ratio (TG/HDL-C) and Insulin Resistance in Chinese Patients with Newly Diagnosed Type 2 Diabetes Mellitus. PLoS One 2016, 11, e0154345.
[15] Li, N., Fu, J., Koonen, D. P., Kuivenhoven, J. A., Snieder, H., Hofker, M. H., Are hypertriglyceridemia and low HDL causal factors in the development of insulin resistance? Atherosclerosis 2014, 233, 130-138.
[16] Grober, U., Schmidt, J., Kisters, K., Magnesium in Prevention and Therapy. Nutrients 2015, 7, 8199-8226.
[17] Hruby, A., Meigs, J. B., O'Donnell, C. J., Jacques, P. F., McKeown, N. M., Higher magnesium intake reduces risk of impaired glucose and insulin metabolism and progression from prediabetes to diabetes in middle-aged americans. Diabetes Care 2014, 37, 419-427.

[18] Mensink, R. P., Zock, P. L., Kester, A. D., Katan, M. B., Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am J Clin Nutr 2003, 77, 1146-1155.
[19] Lagrost, L., Mensink, R. P., Guyard-Dangremont, V., Temme, E. H., Desrumaux, C., Athias, A., Hornstra, G., Gambert, P., Variations in serum cholesteryl ester transfer and phospholipid transfer activities in healthy women and men consuming diets enriched in lauric, palmitic or oleic acids. Atherosclerosis 1999, 142, 395-402.
[20] Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., Schoonjans, K., Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov 2008, 7, 678-693.
[21] Chiang, J. Y., Bile acids: regulation of synthesis. J Lipid Res 2009, 50, 1955-1966.
[22] Cariou, B., Chetiveaux, M., Zair, Y., Pouteau, E., Disse, E., Guyomarc'h-Delasalle, B., Laville, M., Krempf, M., Fasting plasma chenodeoxycholic acid and cholic acid concentrations are inversely correlated with insulin sensitivity in adults. Nutr Metab (Lond) 2011, 8, 48.
[23] Andersen, B. L., Tarpley, H. T., Regen, D. M., Characterization of beta-hydroxybutyrate transport in rat erythrocytes and thymocytes. Biochim Biophys Acta 1978, 508, 525-538.
[24] Prawitt, J., Caron, S., Staels, B., Bile acid metabolism and the pathogenesis of type 2 diabetes. Curr Diab Rep 2011, 11, 160-166.
[25] Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., Haqq, A. M., Shah, S. H., Arlotto, M., Slentz, C. A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B. R., Yancy, W. S., Jr., Eisenson, H., Musante, G., Surwit, R. S., Millington, D. S., Butler, M. D., Svetkey, L. P., A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009, 9, 311-326.
[26] Tai, E. S., Tan, M. L., Stevens, R. D., Low, Y. L., Muehlbauer, M. J., Goh, D. L., Ilkayeva, O. R., Wenner, B. R., Bain, J. R., Lee, J. J., Lim, S. C., Khoo, C. M., Shah, S. H., Newgard, C. B., Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. Diabetologia 2010, 53, 757-767.
[27] Shah, S. H., Crosslin, D. R., Haynes, C. S., Nelson, S., Turer, C. B., Stevens, R. D., Muehlbauer, M. J., Wenner, B. R., Bain, J. R., Laferrere, B., Gorroochurn, P., Teixeira, J., Brantley, P. J., Stevens, V. J., Hollis, J. F., Appel, L. J., Lien, L. F., Batch, B., Newgard, C. B., Svetkey, L. P., Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. Diabetologia 2012, 55, 321-330.
[28] Elshorbagy, A., Jerneren, F., Basta, M., Basta, C., Turner, C., Khaled, M., Refsum, H., Amino acid changes during transition to a vegan diet supplemented with fish in healthy humans. Eur J Nutr 2016.
[29] Soumeh, E. A., Hedemann, M. S., Poulsen, H. D., Corrent, E., van Milgen, J., Norgaard, J. V., Nontargeted LC-MS Metabolomics Approach for Metabolic Profiling of Plasma and Urine from Pigs Fed Branched Chain Amino Acids for Maximum Growth Performance. J Proteome Res 2016, 15, 4195-4207.
[30] Patel, M. J., Batch, B. C., Svetkey, L. P., Bain, J. R., Turer, C. B., Haynes, C., Muehlbauer, M. J., Stevens, R. D., Newgard, C. B., Shah, S. H., Race and sex differences in small-molecule metabolites and metabolic hormones in overweight and obese adults. OMICS 2013, 17, 627-635.

[31] Geer, E. B., Shen, W., Gender differences in insulin resistance, body composition, and energy balance. Gend Med 2009, 6 Suppl 1, 60-75.
[32] Escalante Pulido, J. M., Alpizar Salazar, M., Changes in insulin sensitivity, secretion and glucose effectiveness during menstrual cycle. Arch Med Res 1999, 30, 19-22.
[33] De Leeuw, I., Vansant, G., Van Gaal, L., Magnesium and obesity: influence of gender, glucose tolerance, and body fat distribution on circulating magnesium concentrations. Magnes Res 1992, 5, 183-187.
[34] Thom, G., Lean, M., Is There an Optimal Diet for Weight Management and Metabolic Health? Gastroenterology 2017, 152, 1739-1751.
[35] Stroeve, J. H., van Wietmarschen, H., Kremer, B. H., van Ommen, B., Wopereis, S., Phenotypic flexibility as a measure of health: the optimal nutritional stress response test. Genes Nutr 2015, 10, 459.

SUPPLEMENTARY INFORMATION

ANIMAL DIET Menu

Breakfast
Croissant
Strawberry jam
Coffee with milk and sugar

Snack
Yogurt Nature
Fresh apple

Lunch
Beef Hamburger with mustard and fresh tomato slices
Chips
Cucumber salad
Applesauce

Snack
Crackers

Dinner
Dijon chicken with pasta
Mixed lettuce with balsamic vinaigrette
Apricot tart

Drinks
Water

"Emergency Snack"
Yogurt, skimmed with fruit
10 Raw plain almonds

Kcals
1600
1800
2000
2200
2400
2600
2800
3000

VEGAN DIET Menu

Kcals
1600
1800
2000
2200
2400
2600
2800
3000

Breakfast
Healthy quinoa rice milk müesli with cashew butter
Hemp protein powder
Black coffee

Snack
Homemade hummus + Hemp protein powder
Carrots, cucumber
Gluten-free bread, Kombucha drink

Lunch
Brown rice and red beans salad with baby spinach, white balsamic vinaigrette
Gluten Free Bread
Green Tea

Snack
Fresh banana with soy yogurt

Dinner
Asian lentil coconut curry +Hemp protein powder
Gluten-free bread
Ginseng and green tea

Drinks
Water

"Emergency Snack"
Soy Yogurt Nature (Coop Naturaplan)

Supplementary Figure S1. The animal and vegan diet menu plans were the same each day for 2 days. Food portions were provided based on 8 personalized calorie plans in accordance with the caloric needs of the individual participants. Hemp protein powder was used for the vegan diet to boost total protein.

Supplementary Table S1. Mean diet intake differences between baseline and intervention diets

Diet Variable	Baseline vs. vegan P-value*	Baseline vs. animal P-value*
Vitamin B12 (ug)	**6.41E-05**	NS
Sodium (mg)	**6.41E-05**	**1.68E-04**
Polyunsaturated fat (g)	**6.41E-05**	**2.29E-04**
Saturated fat (g)	**6.41E-05**	NS
Magnesium (mg)	**7.42E-05**	NS
Fat (g)	**8.56E-05**	**3.01E-04**
Folate (ug)	**8.57E-05**	NS
Kilocalories (Kcal)	**8.58E-05**	NS
Protein (%)	**1.14E-04**	**6.41E-05**
Vitamin C (mg)	**2.29E-04**	**1.68E-04**
Vitamin A (mg)	**3.01E-04**	NS
Carbohydrate (g)	**4.77E-04**	NS
Vitamin B2 (mg)	**6.58E-04**	**1.38E-03**
Fat%	**1.56E-03**	**6.59E-04**
Vitamin E (mg)	**1.76E-03**	**6.73E-04**
Monounsaturated fats (g)	**3.92E-03**	NS
Total fiber (g)	**3.92E-03**	**8.48E-04**
Iron (mg)	**4.87E-03**	NS
Protein (%)	NS	NS
Phosporus (mg)	NS	**1.43E-04**
Protein (g)	NS	**1.52E-04**
Vitamin C (mg)	NS	NS
Sodium (mg)	NS	NS
Polyunsaturated fat (g)	NS	NS
Fat (g)	NS	NS
Vitamin B6 (mg)	NS	**5.00E-04**
Vitamin E (mg)	NS	NS
Fat%	NS	NS
Total fiber (g)	NS	NS
Vitamin B2 (mg)	NS	NS

*Wilcoxon signed-rank test comparing paired average animal and vegan diet intakes with corresponding baseline and all comparisons are bolded as they also met a false discovery rate of P<0.10.

Supplementary Table S2. Comparison of baseline and washout diet intakes

Diet Variable	P-value*
Carbohydrate	**6.36E-03**
Total fiber	**1.23E-02**
Magnesium	1.65E-02
Kilocalories	1.99E-02
Sodium	2.85E-02

*Wilcoxon signed-rank test comparing paired average washout food diaries with corresponding baseline average and those marked in bold were after False Discovery Rate (P<0.10).

Supplementary Table S3. Baseline plasma amino acid status by gender

Amino Acid (nmol/ml)	Female	SD	Male	SD	P-value
BCAAs	322.879	43.869	457.996	42.248	**1.65E-04**
Leucine	94.528	12.853	141.503	11.265	**1.65E-04**
Valine	174.545	25.471	244.347	30.226	**2.18E-04**
Isoleucine*	53.806	9.205	72.146	7.75	**4.91E-04**
EAAs	786.518	80.857	962.85	88.423	**1.36E-03**
Ornithine*	32.559	10.591	45.087	6.989	**4.35E-03**
Phenylalanine*	45.7	6.158	55.726	5.765	**4.35E-03**
Methionine	22.111	3.387	26.377	4.035	**5.41E-03**
Lysine	137.13	30.114	174.895	29.075	**1.24E-02**
Glutamic acid*	16.452	8.07	33.702	19.751	**1.83E-02**
Glutamine*	471.3	59.72	562.596	81.189	**1.83E-02**
Tyrosine	47.073	11.555	59.167	8.034	**2.65E-02**
Arginine	67.603	19.984	82.202	14.837	**4.48E-02**
Tryptophan	52.314	7.933	59.029	7.642	**4.48E-02**
Alanine	302.367	37.867	337.712	65.431	5.28E-02
Proline	137.206	28.536	168.398	42.841	8.45E-02
Citrulline	23.213	5.957	29.073	6.732	9.80E-02
Glycine	187.707	100.015	213.71	68.119	1.30E-01
Asparagine	41.154	6.475	46.753	10.575	1.93E-01
Threonine	128.34	41.449	110.838	23.28	5.50E-01
Serine	91.633	15.776	95.653	19.314	7.51E-01
Histidine	78.044	8.474	77.989	7.283	9.16E-01
Cystine	49.772	21.201	53.412	11.122	1.00E+00

Conventional P-values calculated using Wilcoxon signed-rank test.
*Amino acids with gender difference at baseline but no significant post-intervention
BCAAs=branched chain amino acids, EAAs=essential amino acids
Significant P-values <0.05 in bold

Chapter 3

Vegan and animal meal composition and timing influence glucose and lipid related postprandial metabolic profiles

Based on

Colleen Fogarty Draper, Giulia Tini, Irene Vassallo, Jean Philippe Godin, MingMing Su, Wei Jia, Maurice Beaumont, Sofia Moco, Francois-Pierre Martin

Vegan and animal meal composition and timing influence glucose and lipid related postprandial metabolic profiles

Submitted for Publication 2018

ABSTRACT

Flexitarian dieting is increasingly associated with health benefits. The study of postprandial metabolic response to vegan and animal diets is essential to decipher how specific diet components may mediate metabolic changes. Therefore, a randomized, cross-over, controlled vegan versus animal diet challenge was conducted on 21 healthy participants. Postprandial metabolic measurements were conducted at six timepoints during breakfast and lunch. Area under the curve analysis of the vegan diet response demonstrated higher glucose (EE 0.35), insulin (EE 0.38), triglycerides (EE 0.72) and 9 amino acids at breakfast (EE 4.72 to 209.32); and 6 lower health-promoting fatty acids at lunch (EE -0.1035 to -0.13) ($p < 0.05$). Glycemic and lipid parameters varied irrespective of diet type, demonstrating vegan and animal meals contained both health promoting and suboptimal nutrient combinations. The vegan breakfast produced the same elevated branched chain amino acid-associated metabolic signature as the animal diet from our fasting results, reflecting the low protein load in the animal and the higher branched chain amino acid load of the vegan breakfasts. Liberalization of the vegan menu to vegetarian and the animal menu to a Nordic-based diet could result in optimal metabolic signatures for both flexitarian diet strategies in future research.

INTRODUCTION

Vegan, vegetarian and animal-based omnivorous diets are naturally health promoting if they are well-balanced with sufficient macro and micronutrients to meet dietary requirements [1-6]. Mediterranean, Nordic and flexitarian (semi-vegetarian) diets are omnivorous diets with an emphasis on plant-based foods and have been shown to correlate with reduced risk of diabetes and cardiovascular disease onset and promote glucose and lipid control [7-13]. Healthful and macronutrient balanced vegan diets are naturally high in dietary soluble fibers, well- known for a multitude of health promoting benefits, such as glucose and lipid control [14-16]. Similarly, animal-based diets that include a high intake of soluble fiber containing fruits and vegetables, while minimizing red meat intake can also be health promoting. We previously studied the metabolic non-equivalence between balanced vegan and animal diets in healthy subjects that mediated an insulin, lipid and amino acid signature at fasting known to be associated with diabetes risk after 48 hours [17].

Postprandial studies permit the comprehensive examination of complex interactions between the food matrices, components and human metabolism after meal ingestion to shed light on metabolic response and adaptation [18]. Such research is fundamental to substantiate personalized nutritional approaches. For example, postprandial lipaemia (hypertriglyceridemia) from a high fat meal, known to worsen in individuals with type 2 diabetes, is associated with cardiovascular disease risk and acute cardiovascular events [19-21]. Postprandial lipaemia can be lowered with higher intake of dietary fiber, polyphenols, medium chain fatty acids and long-chain n-3 polyunsaturated fatty acids [22-25]. Postprandial dysmetabolism is distinguished by elevated glucose and lipids and associated with the onset of cardiovascular events [26]. In fact, postprandial dysmetabolic responses are useful to demonstrate individual resilience to high fat and high glucose challenges, known as phenotypic flexibility [27-29] .

Comparison of postprandial responses to vegan and animal meals holds the potential to provide a deeper understanding of the cumulative impact of meals, snacks and timing on fasting results. For the present analysis, we investigated the quantitative and correlative impact of meal nutrient composition in vegan and animal diets on postprandial metabolic response as a follow-on analysis from the same study represented in our first publication. This was achieved by evaluating clinical variables, such as insulin, glucose, triglycerides, amino acid, fatty acid and bile acid responses to meals and snacks from each diet type. This investigation was conducted to describe which meal compositions contributed optimal clinical and metabolomic biomarker

results in order to make recommendations for nutrient composition improvement using either vegan or animal based diets.

EXPERIMENTAL SECTION

This study is a follow on analysis of postprandial results from a study previously published which evaluated the fasting results from the vegan and animal diet interventions on day 3 [17]. Further description of the methods can be found in **Chapter 2**.

Study Population and Ethical Approval

This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki, approved by the Ethical Committee of Lausanne University School of Medicine, Switzerland (CER-VD, ref no. 222/14), and registered on ClinicalTrials.gov with the identifier NCT02223585. All participants provided written informed consent for study participation and were offered financial compensation agreed by the ethical committee for time spent and schedule inconveniences.

A total of 56 healthy male and female volunteers were pre-screened at information sessions held at the Metabolic Unit, Nestlé Research Center (Lausanne, Switzerland). Out of the 32 participants who signed informed consent, 26 were enrolled in the study (6 screening failures), 5 dropped out and 21 healthy participants (10 men, 11 women) completed this pilot study (**Figure S1**). Two participants dropped out because of non-serious adverse events, and another three decided not to proceed with the study. All participants habitually ate a heterogeneous diet including animal and vegan proteins before entrance into the study.

Study inclusion criteria were age (from 18 to 55 years), regular bowel movement (at least once every 1-2 days), and body mass index (BMI, from 18.5 to 27 kg.m-2). Health status was assessed by a physician during a screening visit as a standard medical visit with blood chemistry analysis. Exclusion criteria included special diets (vegetarian, high protein, and low cholesterol or weight loss program), pregnancy, food allergy, smoking, high alcohol consumption (more than 2 drinks per day), and excessive physical exercise (more than 5 moderate physical exercises per week).

Diet Interventions

All meals and snacks were provided by the Metabolic Unit (MU). Participant compliance with breakfast, lunch, morning and afternoon snacks were supervised by

the MU. The energy provided by vegan and animal meals was personalized for each participant according to their calculated resting energy requirements from anthropometrics, gender and level of physical activity [30]. Macronutrient composition was matched for the day between animal and vegan-based menus, and was calculated based on 20% protein, 50% carbohydrate and 30% fat of total calories within ±5% of calculated needs of each participant. However, the macronutrient composition for the individual meals and snacks differed within and across diets even though total compositions were matched (**Table S1**).

Clinical Trial Design

The clinical trial was a randomized, open label, cross-over, controlled study. Study participants were randomly assigned to the animal and plant protein challenges using Medidata Balance with dynamic allocation [31].The study lasted five weeks following a one week run-in phase (Week 1 = W1) that defines baseline of the participant's normal diet and lifestyle. Participants were randomly assigned to either animal or vegan meals for three consecutive days (Tuesday, Wednesday and Thursday). During each 3-day intervention, participants ate the same meals on each day, including breakfast, morning snack, lunch, afternoon snack, and dinner (**Figure S2**). All meals and snacks were prepared and provided on site (MU) to study participants under supervision by the MU staff with the exception of the dinner meal, which was packaged for home consumption. Fasting analysis results of the 3 day interventions have been previously published [17]. This study analyzes the results from plasma drawn at 7 time-points on day 3 after breakfast and lunch, to evaluate post-prandial response of each diet type. The timeframes between meals and snacks were approximately 2 ½ hours between breakfast and the morning snack, 2 hours between the morning snack and lunch, and 4 hours between lunch and the afternoon snack. As indicated in **Figure 1,** the considered timepoints are: breakfast minus 15 minutes [T0], breakfast plus 1 hour [T1], breakfast plus 2 hours [T2], lunch minus 15 minutes [T3], lunch plus 1 hour [T4], lunch plus 2 hours [T5], lunch plus 4 hours [T6], and lunch plus 6 hours [T7].

Amino acids, bile acids, clinical biomarkers and metabonomics analyses

Small molecule analysis (amino acids, clinical biomarkers, and metabonomics) of plasma samples was conducted using the same methodologies previously reported [29] (see Methods Supplement). For bile acids analysis, a method providing a broader coverage of targeted biochemical species was employed, differing from the quantification of 18 major bile acids used for the fasting plasma sample analyses. Plasma samples were extracted and prepared according to previously published methods [32, 33]. Briefly, all standards were obtained from Steraloids Inc. (Newport, RI, USA) and TRC Chemicals (Toronto, ON, Canada), and 9 stable isotope-labeled bile acid standards

were used as internal standards. An ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA) was used to quantitate bile acids in the human plasma Data acquisition was performed using MassLynx version 4.1 and quantification was performed using TargetLynx version 4.1 (Waters, Milford, MA, USA).

STATISTICAL ANALYSIS

Study of differences in diet intake at breakfast, lunch and dinner
Statistical significance of observed nutrient composition differences across meals and snacks was calculated using a Wilcoxon signed rank-test using the p value <0.05 as an initial threshold of significance. False Discovery Rate (FDR) <0.10 was used to further assess the statistical significance of the results.

Study of post-breakfast and post-lunch time diet effects in plasma using the AUC model
A linear mixed effect model was used to assess the association between genders, diet type and diet sequence with the total changes in metabolites before and after lunch. The model was fit separately for each metabolite and for each time-period. The total metabolite changes between post-breakfast (breakfast, morning snack) and post-lunch (lunch, afternoon snack) periods were assessed with Area Under the Curve (AUC). AUCs were computed using the trapezoidal rule and their normality was assessed with the Shapiro-Wilk test: those showing a Shapiro-Wilk p-value ≤ 0.05 were transformed using the Box-Cox method, prior to fitting the model. The carry-over effect was assessed, as done previously [29].

Study of time specific diet effects post-breakfast, post-lunch and fasting day 3 post-dinner day 2 in plasma using ANOVA
To compare the metabolic effects of the diet type at specific timepoints, the interaction between time and diet was fit for each metabolite with a linear mixed effect model. With an ANOVA test, gender, diet and diet sequence affected metabolic measurements at fasting. Thus, the mixed model was applied to concentration values previously corrected for the baseline (fasting visit at day 3 of diet). For metabolites showing a significant time/diet interaction term (p-value≤0.05), a post-hoc analysis was performed. Least squares means were used to determine the difference between the vegan and the animal (baseline) diet at each time point. False Discovery Rate (FDR) <0.10 was used to assess the statistical significance of the results.

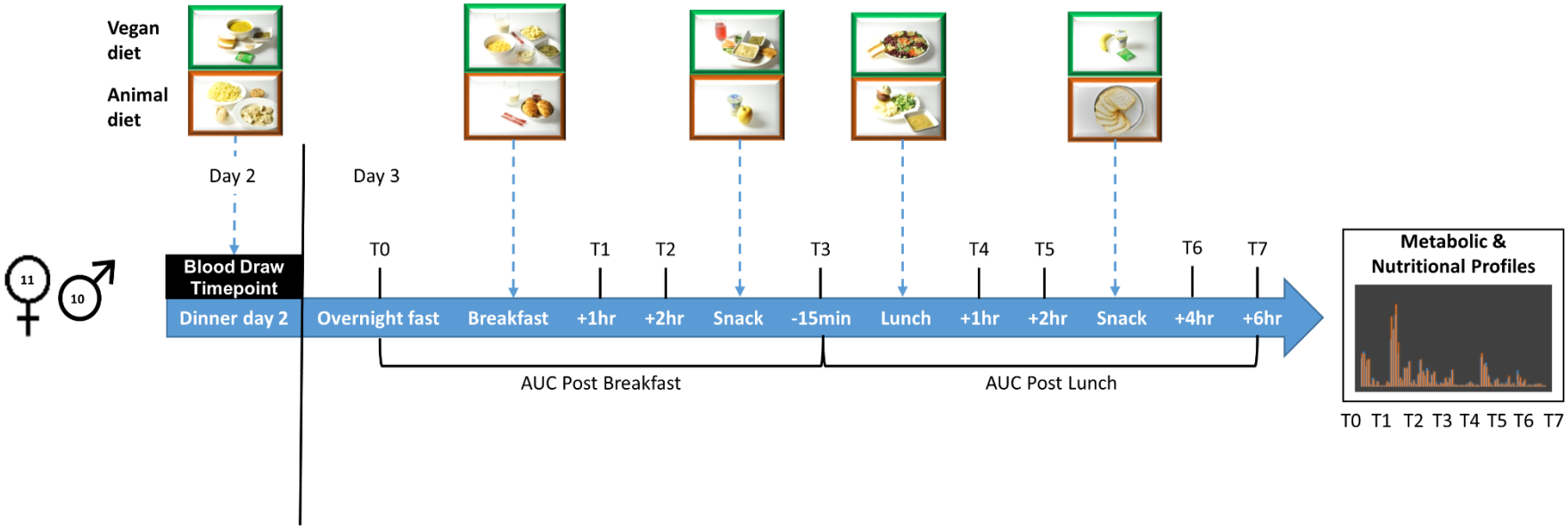


Figure 1. Schema. This randomized, controlled, cross-over pilot study compared 21 (10 men, 11 women) participants. Vegan and animal diets were tailored to individual kilocalorie needs and matched over the day for macronutrient composition but not between meals and snacks. Each diet type was fed for 3 days with 7 postprandial measurements on day 3 during breakfast and lunch. Clinical, amino acid, fatty acid and bile acid metabolic biomarkers were measured and compared at each timepoint and correlated with dietary intake. To Fasting = breakfast minus 15 min? T1 Breakfast +1 hour,T2 Breakfast +2 hours, T3 Lunch minus 15 minutes ,T4 Lunch + 1 hour, T5 Lunch + 2hours, T6 Lunch + 4 hours, T7 Lunch + 6 hours Note: post breakfast and post lunch snacks outside of 2 hour sampling timepoints. Post lunch snack generally 3-4 hours after lunch.

Correlation between dietary intake and metabolite levels

A paired Wilcoxon signed-rank test was used to investigate the relationships between the nutrient intakes on the concentration of plasma metabolites. For each meal, intakes were compared across diets, with results providing FDR q-value <0.10 considered significant. To better explore the results obtained with the AUC analysis, also the intakes of "combined meals" (breakfast+morning snack, lunch+afternoon snack, dinner+emergency snack) were compared across diets. The correlation between dietary intake and plasma metabolite levels was calculated with the Spearman correlation. Spearman's rho statistic was used to test the significance of the association. P-values were corrected for multiple testing using FDR <0.10.

RESULTS

Population Characteristics

A total of 21 (11 females, 10 males) healthy participants completed the clinical trial. Individuals were similar in age and BMI. Baseline characteristics have been described previously [29].

Meal and Snack Compositions

Supervised meal and snack nutrient compositions were analyzed from participant compliance records to quantify actual intake differences due to individual preferences, appetite and satiety. Of the 43 nutrients analyzed from the diet intervention intakes during dinner day 2, and breakfast and lunch day 3; we have highlighted 24 nutrient compositions for each meal and snack due to their significant influence on the postprandial metabolite response (**Table S1**). Statistical analysis was completed using mean nutrient intakes for: i) breakfast and morning snacks from day 3; ii) lunch and afternoon snacks from day 3; and, ii) dinner and emergency snacks from day 2 (**Table 1**).

All mean dinner and evening snack intakes from day 2 were statistically significantly greater from the animal diet with the exception of percent kilocalories (kcal) from protein (PROT %), total dietary fiber (TDF), percent kilocalories from carbohydrate (CHO %), percent kilocalories from fat (Fat %) and polyunsaturated fatty acids (PUFA). Particularly notable was the large difference in kcal (915.24 vs. 400.10 kcals in animal vs. vegan diets) and protein (PROT) (63.18 vs. 21.74 g in the animal vs. vegan diets). The vegan diet maintained statistically significantly higher TDF and PUFA contents (**Table 1, Table S1**).

Breakfast and morning snack intakes from day 3 (e.g. Kcals, CHO, PROT, AA, fat) were statistically significantly greater for the vegan diet with the exception of saturated fatty acids (SFA) which were statistically significantly greater in the animal diet (FDR<0.10). Lysine (LYS), methionine (MET), proline (PRO) and CHO % differences did not reach statistical significance (**Table 1, Table S1**).

Lunch and afternoon snack intakes were statistically significantly higher from the animal diet for Kcal, fat, fat %, MUFA, PUFA, PROT, PROT % and 9 amino acids (AA). Total dietary fiber and methionine contents were higher from the vegan diet and statistically significant (**Table 1, Table S1**).

Clinical Response to Meal and Snack Compositions

Out of the 24 metabolic parameters analyzed, statistically significant differences were observed in insulin, triglycerides and glucose AUCs between diets. Insulin, triglycerides and glucose AUCs were significantly elevated from the vegan diet during the post-breakfast timeperiod. Insulin decreased on the vegan diet and became elevated from the animal diet during the lunch period. Glucose remained significantly elevated from the vegan diet during the post-lunch timeperiod (**Figure 2, Table S2**). Temporal analysis using ANOVA (timepoints To-T7) revealed statistically significant differences at T1, T2 and T7 for triglycerides (TGs) with the elevated TGs from the vegan diet being most notable at T1 and T2. A part from T1, all the timepoints were statistically significant for insulin, with T4 most notable for an elevated peak from the animal diet. Timepoint T3 was statistically significant for glucose and the elevation between T3 and T4 is most notable (**Table S3**). Insulin and TG responses showed gender dimorphism (see **gender dimorphism supplement, Table S4**).

The elevated TG and insulin from the vegan breakfast demonstrated a strong statistically significant correlation between the TG and insulin AUCs, and CHO intake, elevated from the vegan diet (**Table 2, Figure S4**).

The prolonged elevated glucose from the vegan diet at lunch correlated significantly with TDF, suggesting a correlation with slowed absorption of glucose over time. Fasting plasma day 3 insulin was significantly inversely correlated with TDF intake from dinner day 2. In this case, insulin had been elevated from the animal diet and fiber intake was higher from the vegan diet [29] (**Table 2, Figure S5**).

Elevated triglycerides and insulin from the vegan breakfast demonstrated a significant correlation with their respective AUCs for total fat intake, consistent with the higher fat intake from the vegan breakfast (**Table 1, Table 2, Figure S4**).

Table 1. Significant diet intake differences between AUC timeperiods.

		Means						Significance					
Nutrient		**PB**	**PB Vegan**	**PL Animal**	**PL**	**PD**	**PD**	**P value**	**FDR PB**	**P value**	**FDR PL**	**P value**	**FDR PD**
Kilocalories	**Kcal**	598.35	895.51	674.21	556.26	915.24	400.10	**1.91E-06**	**5.15E-05**	**1.22E-03**	**3.30E-02**	**6.40E-05**	**1.73E-03**
Protein (g)	**PROT**	15.89	36.80	27.92	22.23	63.18	21.74	**6.40E-05**	**1.73E-03**	**3.42E-04**	**9.23E-03**	**6.40E-05**	**1.73E-03**
Percent protein	**PROT %**	23.07	33.32	28.60	31.10	26.51	21.25	**1.91E-06**	**5.15E-05**	**1.37E-03**	**3.70E-02**	**1.81E-02**	4.89E-01
Alanine (mg)	**ALA**	665.18	1389.77	1230.18	1004.30	3294.94	870.64	**6.40E-05**	**1.73E-03**	**7.44E-04**	**2.01E-02**	**6.40E-05**	**1.73E-03**
Arginine (mg)	**ARG**	689.86	2392.93	1271.14	1501.08	3674.33	1681.07	**6.40E-05**	**1.73E-03**	**7.81E-03**	2.11E-01	**6.40E-05**	**1.73E-03**
Cysteine (mg)	**CYS**	206.14	472.50	437.60	213.29	992.49	240.14	**6.40E-05**	**1.73E-03**	**6.37E-05**	**1.72E-03**	**6.40E-05**	**1.73E-03**
Glycine (mg)	**GLY**	442.16	1185.10	1250.76	949.95	3026.74	807.36	**6.40E-05**	**1.73E-03**	**1.51E-04**	**4.07E-03**	**6.40E-05**	**1.73E-03**
Histidine (mg)	**HIS**	403.89	754.92	718.19	635.98	1945.85	497.95	**6.40E-05**	**1.73E-03**	**2.61E-02**	7.04E-01	**6.40E-05**	**1.73E-03**
Isoleucine (mg)	**ILE**	862.68	1259.65	1110.30	932.10	3372.88	854.80	**3.43E-04**	**9.27E-03**	**3.12E-03**	**8.43E-02**	**6.40E-05**	**1.73E-03**
Leucine (mg)	**LEU**	1582.60	2221.90	1921.19	1173.86	5139.06	1220.38	**2.62E-04**	**7.09E-03**	**6.37E-05**	**1.72E-03**	**6.40E-05**	**1.73E-03**
Lysine (mg)	**LYS**	1164.13	1372.62	1359.37	1334.99	4604.08	1169.70	**2.39E-02**	6.44E-01	6.76E-01	1.00E+00	**6.40E-05**	**1.73E-03**
Methionine (mg)	**MET**	422.46	475.89	491.77	299.85	1593.38	244.21	**4.38E-02**	1.00E+00	**6.37E-05**	**1.72E-03**	**6.39E-05**	**1.72E-03**
Phenylalanine (mg)	**PHE**	844.86	1337.51	1049.30	1056.30	2932.96	957.98	**9.90E-05**	**2.67E-03**	8.62E-01	1.00E+00	**6.40E-05**	**1.73E-03**
Proline (mg)	**PRO**	1747.60	1454.31	1540.73	626.24	4356.06	734.66	**4.37E-03**	1.18E-01	**6.37E-05**	**1.72E-03**	**6.40E-05**	**1.73E-03**
Serine (mg)	**SER**	966.74	1435.11	1180.25	1175.40	1948.72	1027.00	**1.74E-04**	**4.70E-03**	8.35E-01	1.00E+00	**6.40E-05**	**1.73E-03**
Threonine (mg)	**THR**	685.98	1124.94	907.19	866.57	2713.07	803.98	**8.57E-05**	**2.31E-03**	3.66E-01	1.00E+00	**6.40E-05**	**1.73E-03**
Tryptophan (mg)	**TRP**	193.17	267.54	335.40	255.04	894.69	184.07	**3.92E-04**	**1.06E-02**	**1.51E-04**	**4.07E-03**	**6.40E-05**	**1.73E-03**
Tyrosine (mg)	**TYR**	746.24	940.77	822.38	650.39	2081.37	677.18	**3.50E-03**	**9.46E-02**	**3.41E-04**	**9.22E-03**	**6.40E-05**	**1.73E-03**
Valine (mg)	**VAL**	1065.40	1436.82	1221.48	1056.06	3336.85	1022.83	**5.09E-04**	**1.37E-02**	**6.69E-03**	1.81E-01	**6.40E-05**	**1.73E-03**
Carbohydrate (g)	**CHO**	68.57	97.64	73.31	81.83	119.14	47.10	**6.40E-05**	**1.73E-03**	7.62E-02	1.00E+00	**6.40E-05**	**1.73E-03**
Percent carbohydrate	**CHO %**	92.40	92.45	115.98	120.46	50.80	47.70	6.09E-01	1.00E+00	3.44E-01	1.00E+00	3.66E-01	1.00E+00
Total fiber (g)	**TDF**	4.65	15.14	6.80	14.72	6.03	8.80	**6.37E-05**	**1.72E-03**	**6.37E-05**	**1.72E-03**	**1.38E-03**	**3.74E-02**
Fat (g)	**Fat**	27.31	36.90	29.15	14.63	17.10	13.30	**1.99E-04**	**5.38E-03**	**6.36E-05**	**1.72E-03**	**2.79E-03**	**7.54E-02**
Percent fat	**Fat %**	79.45	66.83	54.41	45.44	19.10	30.13	**6.68E-06**	**1.80E-04**	**1.81E-03**	**4.88E-02**	**2.18E-02**	5.88E-01
Polyunsaturated	**PUFA**	1.02	13.20	9.16	8.12	5.03	7.45	**6.40E-05**	**1.73E-03**	1.80E-01	1.00E+00	**1.08E-03**	**2.93E-02**
Monounsaturated	**MUFA**	5.99	15.98	11.83	4.35	6.94	1.98	**7.37E-05**	**1.99E-03**	**6.35E-05**	**1.72E-03**	**6.35E-05**	**1.71E-03**
Saturated fatty acids	**SFA**	18.32	6.81	7.84	2.14	3.56	2.59	**6.40E-05**	**1.73E-03**	**6.37E-05**	**1.72E-03**	**1.72E-02**	4.65E-01

The p values were calculated by performing a Wilcoxon signed rank-test. PB, post-breakfast; PL, post-lunch; PD, post-dinner. Significant p values in bold ($p<0.05$). Significant values in bold that meet false discovery rate FDR<0.1

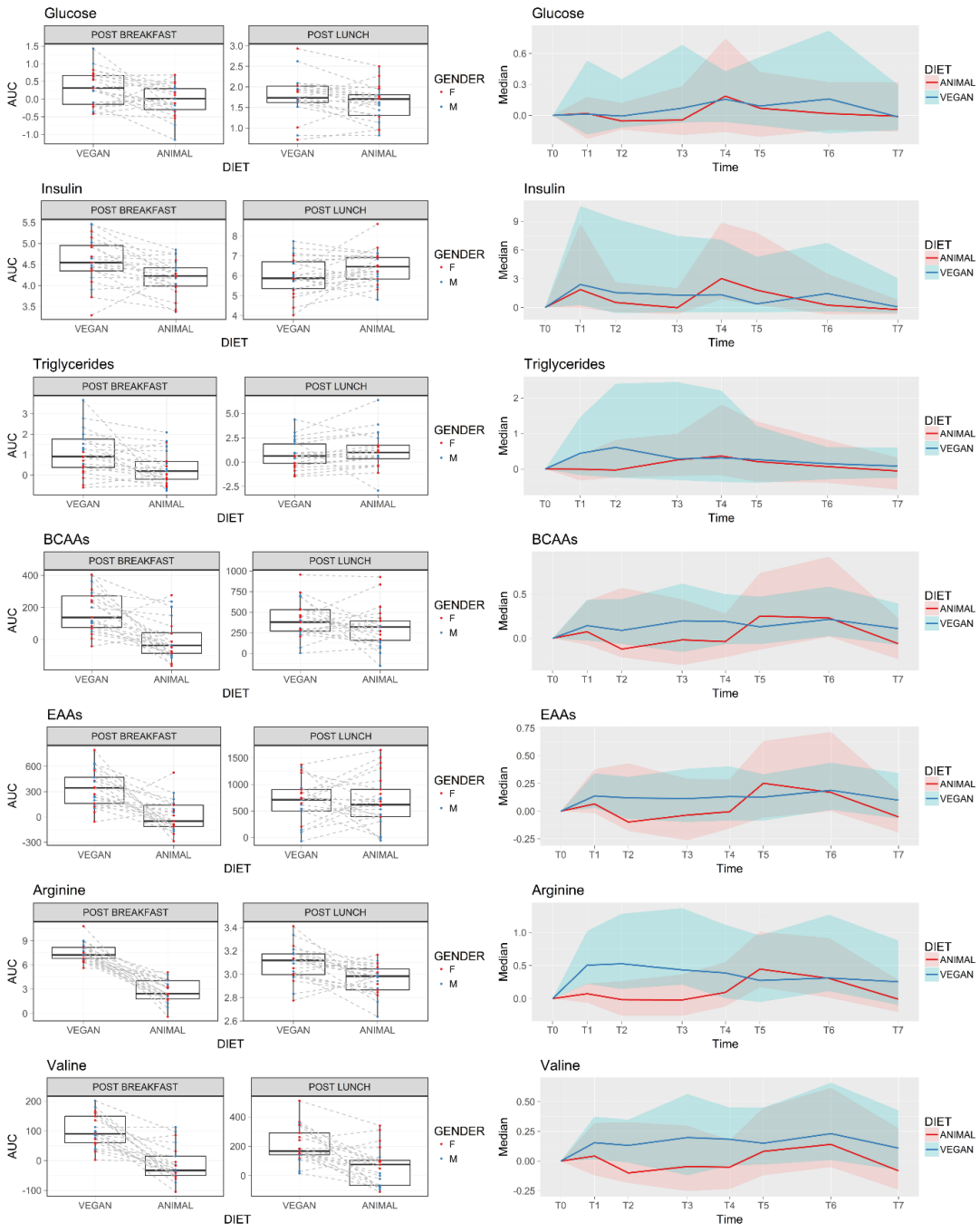


Figure 2. Clinical biomarkers TG, insulin, glucose changes according to meal composition timing. Amino acids changes according to meal composition timing. Figures on the left represent AUC changes across diets and meal period. Gender differences are shown with red (female) and blue (male) dots. Figures on the right display timepoint changes, with blue and red lines represent respectively the vegan and the animal diet. To Fasting = breakfast minus 15 min? T1 Breakfast +1 hour T2 Breakfast +2 hours T3 Lunch minus 15 minutes T4 Lunch + 1 hour T5 Lunch + 2hours T6 Lunch + 4 hours T7 Lunch + 6 hours Note: post breakfast and post lunch snacks outside of 2 hour sampling timepoints. Post lunch snack generally 3-4 hours after lunch. PB, post breakfast; PL, post lunch. A) glucose PB FDR=1.29E-02, PL FDR=5.60E-02; B) glucose T3 FDR<0.10; C) insulin PB FDR=6.54E-03, PL FDR<=5.60E-02; D) insulin T2-T7 FDR<0.10; E) triglycerides PB FDR=8.70E-03, PL FDR=3.75E-01; F) triglycerides T1-T2,T7 FDR<0.10; G) BCAAs (branch chain amino acids) PB FDR=2.09E-03, PL FDR=3.46E-01; H) branch chain amino acids T2-T3,T7; I) EAAs (essential amino acids) PB FDR=1.69E-04, PL FDR=9.48E-01; J) essential amino acids T2-T5,T7; K) arginine PB FDR=2.98E-09, PL FDR=1.57E-02; L) arginine T1-T5,T7; M) valine PB FDR=1.65E-04, PL FDR<=1.38E-03; N) valine T1-T5,T7.

Elevated glucose from the vegan lunch demonstrated a significant inverse correlation with the lunch AUC for total fat intake, consistent with the significantly lower fat intake from the vegan lunch (**Table 1, Figure S4**). Elevated triglycerides, insulin and glucose from the vegan breakfast demonstrated significant positive correlations with the breakfast AUC for total PUFA intake, found to be significantly elevated in the vegan breakfast (**Table 2, Figure S4**). Insulin and TG, elevated in plasma fasting day 3 from the animal dinner day 2 [29], inversely correlated with PUFA intake, consistent with the higher PUFA intake from the vegan dinner (**Table 1, Table 2**).

Plasma Amino Acid Response to differences in sources and Timing of Dietary Protein
From a targeted quantification of 21 amino acids (AA), a total of 14 individual amino acids (arginine, ornithine, phenylalanine, asparagine, valine, tryptophan methionine, proline, citrulline, lysine, leucine, isoleucine, threonine, histidine), including the branched chain amino acids (BCAAs) and essential amino acids (EAA), demonstrated statistically significant higher AUCs from the vegan diet during the breakfast timeperiod. This result reflects the higher intake of BCAAs from the vegan protein hemp supplement and the extremely low intake of foods rich in protein on the animal diet. Proline demonstrated significantly higher AUCs at breakfast and lunch from the naturally high proline animal diet. Valine) continued with a statistically significantly higher AUC from the vegan breakfast to lunch timeperiods despite the high protein animal lunch. Three AAs (lysine, methionine and proline) demonstrated significantly higher AUCs from the animal lunch timeperiod reflecting the differences in AA protein compositions between the two diet types (**Figure 2, Figure S3, Table S2**). All 21 amino acids demonstrated statistically significant timepoint interactions. Significant peaks can be easily visualized in **Figure 2**, especially between T4 and T5 for the animal diet. These same types of peaks were not visualized in the vegan diet in which the AAs, particularly the EAAs, appear more stable (**Figure 2, Table S3, Figure S3**). Amino acid responses showed gender dimorphism (See **gender dimorphism supplement**, **Table S4**).

A total of 11 plasma AA (arginine, proline, alanine, phenylalanine, valine, leucine, tryptophan, isoleucine, lysine, threonine, methionine) demonstrated statistically significant correlations with breakfast and lunch amino acid intakes. Protein intake was strongly and statistically significantly correlated with elevated total plasma BCAA and EAA AUCs from the high protein, vegan breakfast (FDR<0.10) (**Table 2, Figure S4**) Of the plasma AAs found to be statistically significant in a previous publication [29], EAAs and BCAAs showed a strong, statistically significant correlation with the high protein intake from the animal dinner on day 2 and seven individual plasma AAs significantly correlated with their respective AA intakes, from the animal diet dinner (**Table 2, Table 1**).

Table 2. Spearman's rank correlations (r) between plasma concentrations and dietary nutrient intakes for breakfast

Plasma	Diet	r PB	*P* value	r PL	*P* value	r FD3	*P* value
Amino acids							
ARG	ARG	0.75	**8.46E-09**	0.42	**5.94E-03**	0.09	5.75E-01
BCAAs	PROT %	0.70	**2.94E-07**	0.67	**2.72E-06**	0.28	6.93E-02
EAAs	PROT %	0.66	**1.81E-06**	0.69	**7.33E-07**	0.32	4.04E-02
EAAs	PROT	0.68	**9.04E-07**	-0.31	4.47E-02	0.72	**8.61E-08**
BCAAs	PROT	0.65	**3.34E-06**	-0.30	4.99E-02	0.62	**1.05E-05**
PRO	PRO	0.67	**1.51E-06**	0.11	4.75E-01	0.74	**2.60E-08**
ALA	ALA	0.54	**2.52E-04**	0.19	2.20E-01	0.38	1.22E-02
PHE	PHE	0.50	**8.60E-04**	-0.01	9.28E-01	0.33	3.48E-02
VAL	VAL	0.49	**9.31E-04**	-0.27	8.93E-02	0.67	**1.06E-06**
LEU	LEU	0.41	**6.29E-03**	-0.54	**2.51E-04**	0.54	**2.34E-04**
TRP	TRP	0.42	**6.13E-03**	-0.18	2.41E-01	0.44	**3.28E-03**
ILE	ILE	0.41	**7.32E-03**	-0.18	2.43E-01	0.36	1.85E-02
LYS	LYS	0.35	**2.33E-02**	0.05	7.76E-01	0.58	**6.51E-05**
THR	THR	0.33	**3.55E-02**	-0.09	5.50E-01	0.22	1.65E-01
MET	MET	0.33	**3.40E-02**	0.09	5.74E-01	0.46	**2.33E-03**
CYS	CYS	0.26	1.00E-01	0.43	**5.02E-03**	0.06	7.17E-01
GLY	GLY	-0.14	3.84E-01	-0.38	**1.44E-02**	0.06	7.07E-01
Fatty acids							
Capric acid (C10:0)	PUFA	-0.77	**2.26E-09**	0.16	3.09E-01	0.27	8.44E-02
Dodecanoic acid (C12:0)	PUFA	-0.74	**2.81E-08**	0.11	4.78E-01	0.50	**7.37E-04**
Myristic acid (C14:0)	PUFA	-0.67	**1.55E-06**	-0.01	9.68E-01	0.22	1.59E-01
Caprylic acid (C8:0)	PUFA	-0.52	**4.05E-04**	0.28	6.79E-02	0.10	5.41E-01
Pentadecanoic acid (C15:0)	PUFA	-0.59	**3.78E-05**	-0.17	2.85E-01	0.15	3.52E-01
5-dodecanoic acid (C12:1)	PUFA	-0.47	**1.48E-03**	0.06	7.23E-01	0.45	**2.59E-03**
Myristoleic acid (C14:1)	PUFA	-0.38	**1.20E-02**	-0.02	8.89E-01	0.29	5.79E-02
Heptadecanoic acid (C17:1)	PUFA	-0.32	**4.20E-02**	-0.20	2.13E-01	0.20	1.99E-01
Capric acid (C10:0)	SFA	0.73	**3.47E-08**	0.62	**1.10E-05**	-0.20	1.99E-01
Dodecanoic acid (C12:0)	SFA	0.61	**1.50E-05**	0.63	**9.28E-06**	-0.17	2.85E-01
Myristic acid (C14:0)	SFA	0.55	**1.78E-04**	0.56	**1.20E-04**	-0.16	3.07E-01
Caprylic acid (C8:0)	SFA	0.60	**2.94E-05**	0.48	**1.38E-03**	-0.08	6.25E-01
Pentadecanoic acid (C15:0)	SFA	0.36	**1.87E-02**	0.37	**1.47E-02**	-0.06	6.94E-01
5-dodecanoic acid (C12:1)	SFA	0.32	**4.16E-02**	0.51	**4.84E-04**	-0.01	9.38E-01
Clinical biomarkers							
Insulin	CHO	0.45	**2.60E-03**	0.23	1.35E-01	0.11	4.82E-01
TG	CHO	0.74	**1.84E-08**	0.42	**5.26E-03**	0.34	2.83E-02
TG	CHO %	-0.32	**3.81E-02**	0.28	7.87E-02	0.24	1.31E-01
TG	FAT	0.72	**7.12E-08**	-0.01	9.33E-01	0.04	7.81E-01
Glucose	FAT	0.16	3.13E-01	-0.46	**2.13E-03**	0.16	3.00E-01
Insulin	FAT	0.41	**7.31E-03**	-0.13	4.06E-01	-0.21	1.78E-01
Insulin	FAT %	-0.32	**3.92E-02**	-0.21	2.02E-01	-0.18	2.62E-01
TG	PUFA	0.62	**1.42E-05**	0.13	3.98E-01	-0.33	3.33E-02
Insulin	PUFA	0.44	**3.16E-03**	0.06	7.09E-01	-0.50	**7.10E-04**
Glucose	PUFA	0.32	**3.89E-02**	-0.06	7.13E-01	0.03	8.42E-01
Glucose	SFA	-0.26	1.02E-01	-0.51	**5.65E-04**	0.18	2.60E-01

Table 2. Spearman's rho statistic was used to test the significance of the association PB, post-breakfast; PL, post-lunch; FD3, fasting day 3; r, correlation coefficient. Conventional p values are shown and significant false discovery rates in bold (FDR<0.10). Kcal, kilocalories; PROT, protein, PROT%, percent kilocalories from protein; ARG, arginine; BCAAs, branched chain amino acids; EAAs, essential amino acids; PRO, proline; ALA, alanine; PHE, phenylalanine; VAL, valine; LEU, leucine; TRP, tryptophan; ILE, isoleucine; LYS, lysine; THR, threonine; MET, methionine; CYS, cysteine; GLY, glycine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglycerides; CHO, carbohydrates; CHO%, percent kilocalories from carbohydrates; TDF, total dietary fiber.

Plasma Bile Acid Response to Composition Differences and Timing of Meals and Snacks

We analyzed 38 plasma bile acids for a comprehensive view of the postprandial phase response [29]. From this analysis, three primary bile acids demonstrated statistically significant AUC changes during the breakfast and lunch periods. Cholic acid (CA) demonstrated statistically significantly higher AUCs and □-Chenodeoxycholic acid (bCDCA) demonstrated statistically significantly lower AUCs from the vegan diet during breakfast and lunch. The chenodeoxycholic acid (CDCA) AUC was statistically significantly higher from the vegan diet during lunch only. Ten secondary bile acids showed statistically significant AUC changes. Four of these (12-Ketolithocholic acid [12-ketoLCA], 7-Ketolithocholic acid [7-ketoLCA], Glycolithocholic acid-3-sulfate [GLCA-3S] and, Lithocholic acid-3-sulfate [LCA-3S]) significantly increased from the vegan diet after breakfast and lunch. Four tertiary bile acids demonstrated statistically significant AUC changes from lunch only (**Figure 3, Table S2**). Analyses of timepoint results (ANOVA, To-T7) revealed 5 primary, 14 secondary, and 8 tertiary bile acids with complex temporal profiles (**Table S3, Figure S6**).

Of the bile acids with statistically significant AUCs, TDF was positively correlated with GLCA-3S at breakfast and lunch, but not dinner; The KCAL, CHO, TDF, PROT, 14 AAs, fat, PUFA and MUFA with positive correlations with bile acids (GLCA-3S and LCA-3S) at breakfast, were reversed at lunch, reflecting the change in relative concentrations of the vegan diet (**Figure 4, Table 1**).

Plasma Fatty Acid Response to Sources and Timing of Dietary Fat Intake
Out of 31 fatty acids analyzed, a total of 7 (5-dodecanoic, capric, caprylic, dodecanoic, 1,2-methylpentanoic, myristic, and pentadecanoic acids), showed elevated AUC from the animal diet during the breakfast period. Of those, AUC of capric, dodecanoic, myristic and pentadecanoic acids remained elevated from the animal diet during the lunch period.

AUC of eicosenoic and myristoleic acids were statistically significantly elevated from the animal diet during the lunch period only. Temporal analysis using ANOVA further revealed 8 fatty acids with statistically significant timepoint differences across the two diets (vegan vs. animal). Of note, caprylic acid was elevated at T2 (breakfast period) and myristoleic acid was higher at T5. (**Figure 5, Table S3**).

Dietary intake of saturated fatty acids (SFA), elevated on the animal diet, demonstrated statistically significant correlations with 6 plasma fatty acids (capric, dodecanoic, myristic, caprylic, pentadecanoic, and 5-dodecenoic acids) during the breakfast and lunch timeperiods (**Table 2, Table 1, Figure S4, Figure S5, Table 1**).

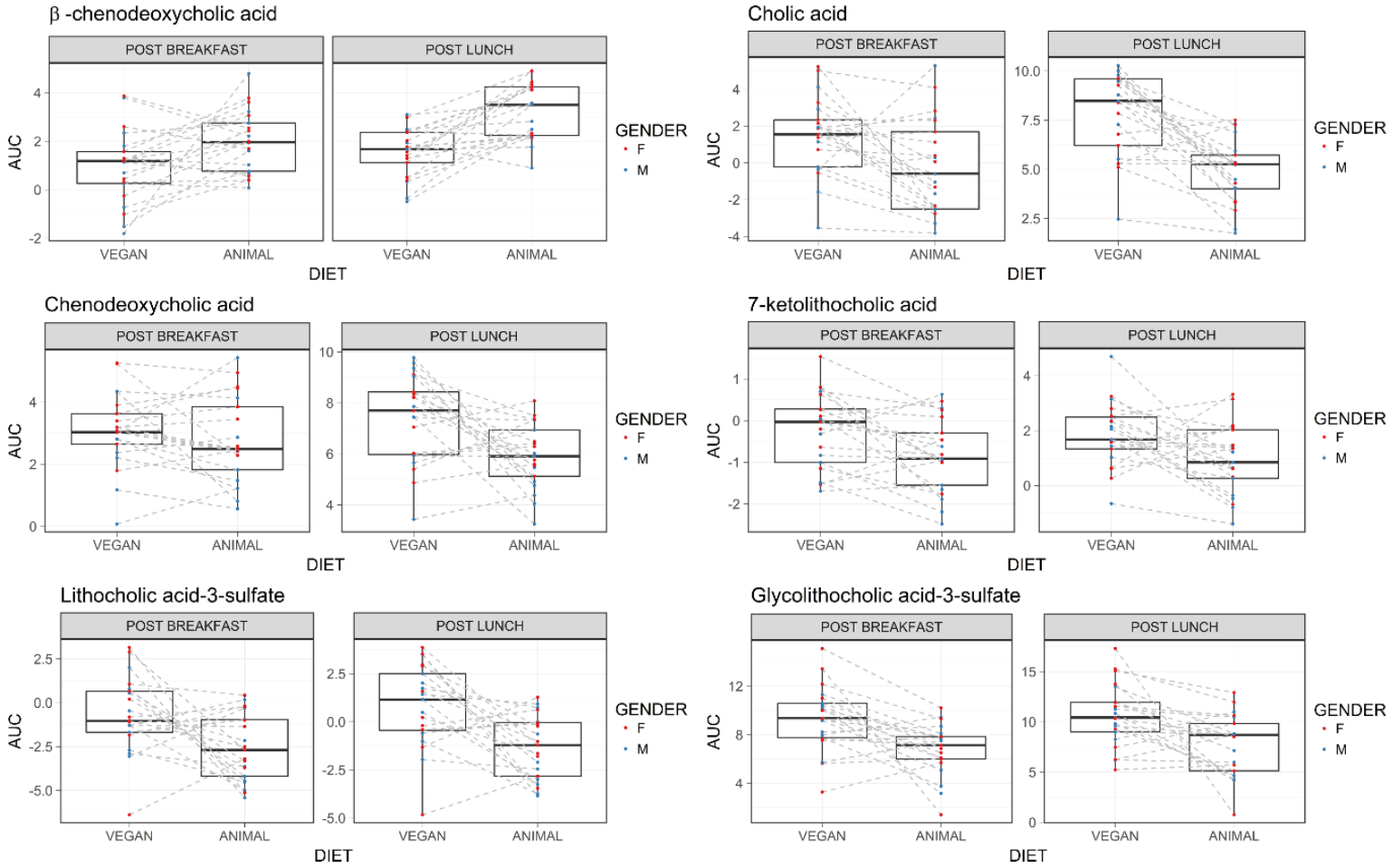


Figure 3. Representative bile acids that showed statistically significant different AUC responses post-breakfast and post-lunch to the vegan vs. animal diets are graphically depicted. Gender differences are shown with red (female) and blue (male) dots. β-chenodeoxycholic acid post-breakfast FDR=8.74E-02, post-lunch FDR=5.53E-04; Cholic acid post-breakfast FDR=8.68E-02, post-lunch FDR=5.52E-04; Chenodeoxycholic acid post-breakfast FDR=7.4E-01, post-lunch FDR=2.85E-02; 7-ketolithocholic acid post-breakfast FDR=8.68E-02, post-lunch FDR=6.12E-02; Lithocholic acid 3 sulfate post-breakfast FDR=8.44E-02, post-lunch FDR=1.42E-02; Gglycholithocholic acid post-breakfast FDR=8.68E-02, post-lunch FDR=8.88E-01.

DISCUSSION

The metabolic impact of vegan and animal meals and snacks was compared in a cross-over study design. Daily intakes were matched for energy densities of CHO, PROT and fat (50%, 20%, and 30%). The vegan diet contained approximately twice the amount of TDF than the animal diet (39 and 18 grams respectively). The macronutrient and TDF compositions of the vegan diet were similar to the Nordic diet (51% CHO,17% PROT, 32% fat, 41g TDF) [34]. Meals and snacks were not matched for macronutrient composition. This allowed us to observe the limitations, benefits and opportunities to improve metabolic signatures with both diet types. Comparison of postprandial signature responses to fasting signatures previously published on this cohort provided a deeper understanding of the cumulative impact of meals, snacks and timing on fasting results [29].

Lipid and glycemic responses are elevated from both animal and vegan meal combinations
Postprandial TG and insulin are known to be higher in individuals with coronary artery disease and may play a role in the development of atherosclerosis, a risk factor for cardiovascular disease [35, 36]. Hypertriglyceridemia is also a common abnormality observed in obesity, metabolic syndrome and diabetes [37]. In a previous paper on this same cohort, fasting results after 48 hours of following the supervised diets demonstrated lower TG and insulin plasma levels from the vegan diet. In the present study, the higher kcals, CHO and fat from the vegan breakfast produced elevated plasma TG, insulin and glucose AUCs (**Figure 2, Table S2**). The peak in TG from the vegan diet two hours after breakfast (T2) reflected the high CHO content of the muesli and high fat content of the cashew butter (**Table S1**).

Glucose remained mildly elevated with a peak in glucose and insulin 4 hours post-lunch (T6), reflecting the trend toward higher CHO and TDF intakes from the vegan beans, rice and banana snack (**Figure 2, Figure S2**).

The continual rise in glucose from 2 hours after breakfast (T2) until 1-hour post-lunch (T4), may have been a remnant from the sugar contained in the drink taken during the morning snack on the vegan diet mixed with the slowed digestion and short chain fatty acid production from the soluble dietary fiber (**Figure 2**). This would have sustained glucose levels and prevented the large plasma glucose peak often seen after fast acting sugar intake [38-40]. Vegan diet soluble fiber sources included hemp protein powder, hummus, red beans, banana and lentils (**Figure S2**). The glucose and insulin peaks visualized from the animal diet at T4 (1 hour post-lunch) reflected the higher saturated fat intake from the hamburger known for its association with inflammation and insulin resistance (**Figure 2, Table S3**) [41].

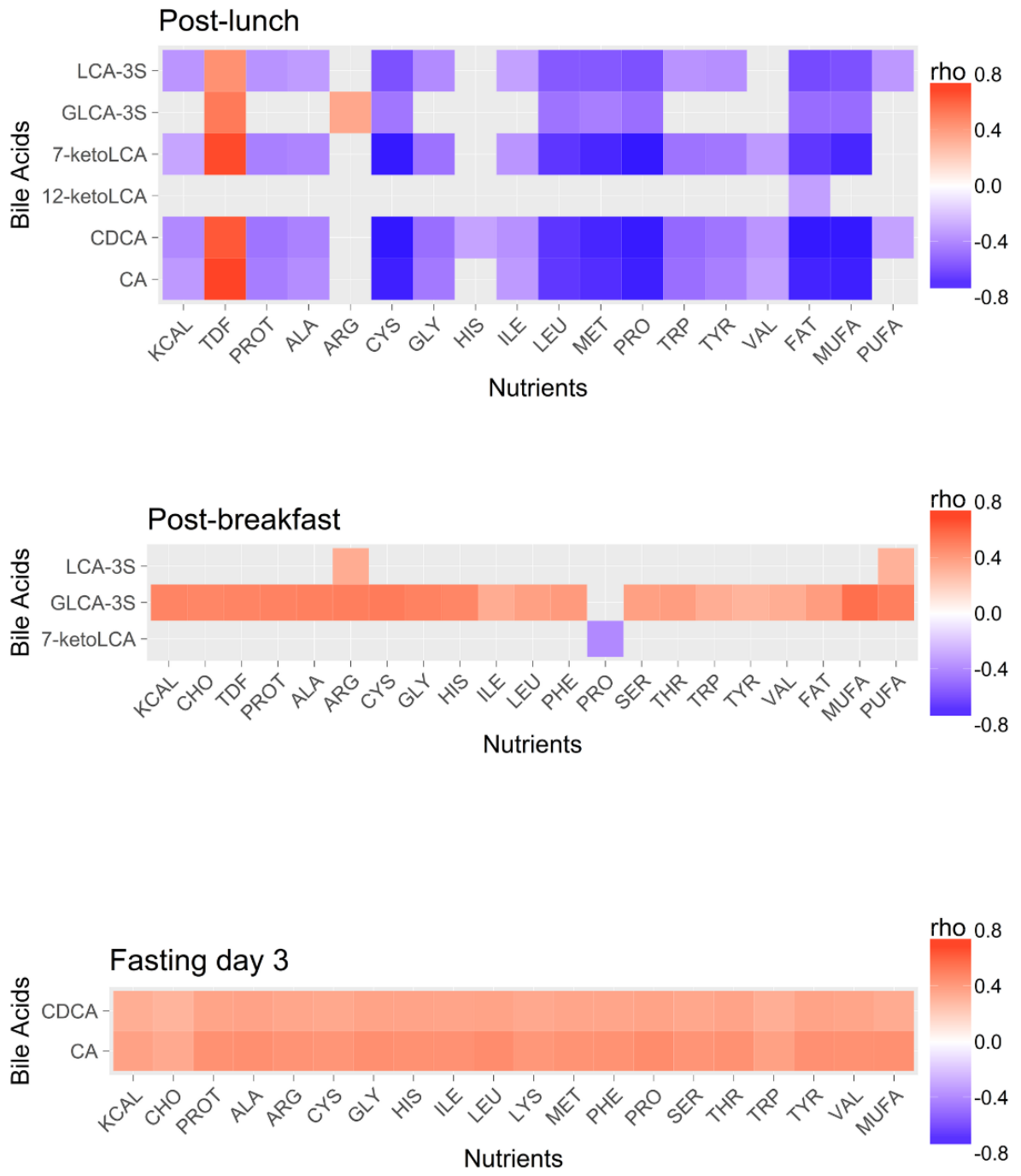


Figure 4. (A-C) Spearman correlations between significant plasma bile acids and diet intake are graphically represented with a heatmap for the post-breakfast, post-lunch and dinner day 2 AUC responses. KCAL, kilocalories; TDF, total dietary fiber; PROT, protein,; ALA, alanine; ARG, arginine; CYS, cysteine; GLY, glycine; HIS, histidine; ILE, isoleucine; LEU, leucine; MET, methionine; PRO, proline; TRP, tryptophan; TYR, tyrosine; VAL, valine; FAT, total fat; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CHO, carbohydrate; PHE, phenylalanine; SER, serine; THR, threonine.

Together, these results demonstrated the immediate metabolic influence of alterations in nutrient combinations of both diet types.

Postprandial amino acid plasma response varies with dietary AA composition irrespective of diet type

Elevated AA concentrations are associated with higher risk of insulin resistance and obesity [29, 42]. In a previous publication, we demonstrated the association of a suboptimal BCAA associated metabolic signature at fasting after 2 days following the supervised animal based protein diet [29]. Additionally, postprandial BCAAs were previously associated with fasting glucose and insulin concentrations [43]. In the present study, significantly elevated plasma AAs from the vegan diet breakfast resulted from the hemp protein supplement and produced a similar AA signature to the 2 day animal diet response suggesting a large difference in protein intake from either animal or vegan food sources could produce the same, suboptimal metabolic signature (**Figure 2, Figure S3, Table S2**).

Strong correlations in the data between plasma AA concentrations and dietary AA intake substantiate the rapid and direct impact of dietary intake on metabolism (**Table 2, Figure S4, Figure S5**). Plasma lysine, methionine and proline were elevated after the animal diet lunch due to significant concentrations in the hamburger; and may have accumulated from the morning yogurt animal diet snack. Notably, the BCAAs were not elevated, despite their large quantity in the animal diet foods, due to the addition of BCAAs in the form of Hemp protein powder to the vegan diet (**Figure S5, Table S2, Figure S2**). Dietary methionine may worsen insulin sensitivity while lysine attenuates glucose response in the absence of any effect on insulin [44, 45]. Thus, the insulin peak 1 hour after the animal lunch in the absence of a significant glucose peak may have been influenced by the lysine and methionine content of the hamburger lunch (**Figure 2, Figure S2).**

Bile acids are elevated and absorption is prolonged from the TDF content of the vegan meals

Bile acids facilitate postprandial lipid digestion, transport and metabolism. As nutrient signaling hormones, they interact with insulin to regulate nutrient metabolism in the liver. Elevated bile acid concentrations are associated with improved glucose homeostasis and lipid profiles [46-48].

The majority of the bile acids were elevated after the vegan breakfast (secondary bile acids) and lunch timeperiods (primary and secondary bile acids) (**Figure 3, Figure S6, Table S2**). Dietary fiber intake slows CHO, AA and lipid absorption reducing the risk of

hyperlipidemia, hypercholesterolemia and hyperglycemia [51]. It also shifts gut microbial populations by facilitating bacterial fermentation [7]. Significant bile acid correlations were observed with TDF across these two timeperiods suggesting TDF may have had the biggest impact on the bile acid concentrations, particularly with GLCA-3S, a conjugated secondary bile acid from bacterial colonic activity (**Figure 4**). It is possible that the additional presence of the TDF, prominent in the vegan diet, facilitated GLCA-3S colonic conjugation causing its significant elevation from the vegan diet. The elevated cholic acid observed at both mealtimes, with a significant peak 4 hours post-lunch (T6), may be associated with increased energy expenditure and could be related to the decreased body mass index seen in vegetarians (**Figure 3, Table S2, Table S3**). Further research is needed to explore this potential effect in a postprandial state [52, 53].

Elevated plasma fatty acid concentrations correlate with saturated fatty acid intake from the animal breakfast and lunch timeperiods

Saturated fats are known to be hypercholesterolemic and insulin resistance promoting relative to their less saturated counterparts [41, 54]. However, certain saturated fats have health benefits. Capric and caprylic acid are both saturated fats and medium chain fatty acids that may reduce plasma cholesterol through its excretion and are inversely correlated with pancreatic cancer and ischemic heart disease [55-57]. Saturated fatty acid dietary intake was significantly greater for the animal diet and positively correlated with elevated plasma fatty acid concentrations for both breakfast and lunch timeperiods (**Table 1, Table 2, Table S2**).

The large capric acid plasma concentration seen in participants on the animal diet is consistent with the high capric acid intake from animal fats, such as the butter in the croissant, yogurt and hamburger eaten during the breakfast and lunch periods on the animal diet (**Figure 5, Figure S2, Table S3**). Coconut milk fed to the study participants on the vegan diet at dinner also produced elevated fasting plasma capric acid the morning after, further substantiating the rapid sensitivity of plasma fatty acid response to a well-controlled diet [29].

Caprylic acid is found in animal fats in smaller quantities than capric acid as reflected in the time-point fatty acid variation (**Figure 5**) where we can visualize a 2 hour post-breakfast (T2) peak that reflects the butter content of the croissant and milk from the animal diet breakfast (**Figure 5**). The 5-dodecenoic acid (monounsaturated form of dodecanoic acid) peak reflects butter and milk in the animal diet breakfast as well (Figur**e 5, Figure S2**). Coconut is also a good source as reflected in the post dinner fasting results previously reported [29]. Myristoleic acid, the monounsaturated form of

myristic acid, showed strong peaks reflecting the fat content of the animal diet at the breakfast and lunch meals and seemed to fall quickly in between meals. It appears the lunch-time hamburger meat had the largest impact on the myristoleic acid (**Figure 5, Table S3, Figure S2**).

Here we highlight the significantly increased fatty acids from the animal diet and potential health benefits. However, in our previous publication on fasting results from this study, we observed elevated fatty acids from the coconut milk used in the dinner meal on the vegan diet [29]. The results in this paper, when combined with the former, suggest that optimal healthy fatty intake can be achieved from either a carefully planned vegan or animal diet. A combination of the two options in a flexitarian approach may be optimal for health maintenance to prevent the accumulation of longer chain SFAs that present more health challenges [41, 54].

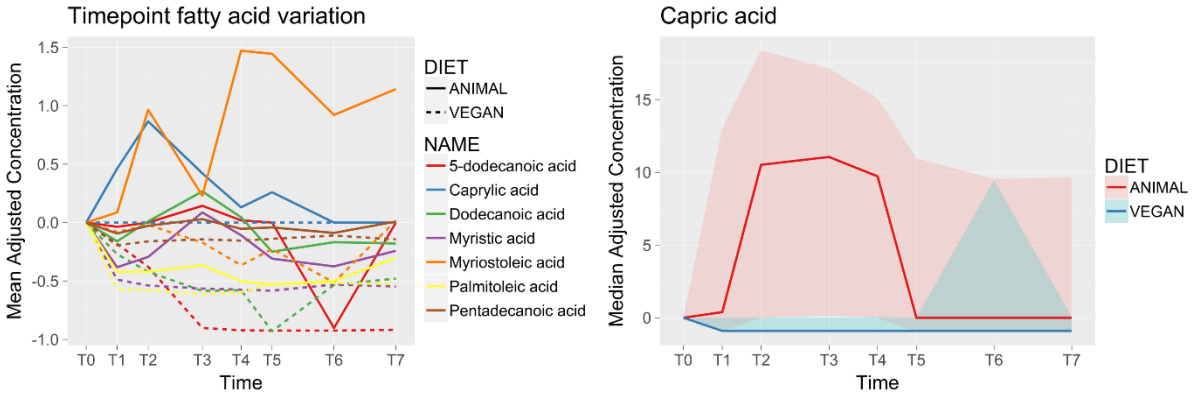


Figure 5. Fatty acid timepoint variation. All fatty acids with significant timepoint variations are graphically depicted. To compare the metabolic effects of the diet type at specific time-points, the interaction between time and diet was fit for each metabolite with a linear mixed effect model. 5-dodecanoic acid FDR<0.10 for T2-T7; caprylic acid FDR<0.10 for T1-T5; dodecanoic acid FDR<0.10 for T2-T7; myristic acid FDR<0.10 T1-T7; myristoleic acid FDR<0.10 for T2-T7; pentadecanoic acid FDR<0.10 for T1-T5,T7; palmitoleic acid FDR<0.10 for T1-T4,T7; capric acid FDR<0.10 for T1-T7.

Study limitations and Opportunities

Carbohydrate, fat and protein macronutrient compositions were matched across diet types for daily intake but not between individual meals and snacks. If macronutrient compositions were matched at meal times, investigation of micronutrient impact on metabolic health signatures of different diet types may have been feasible. Thus, this descriptive study is mainly focused on the impact of different meal compositions that naturally occur from habitual intake of the different diet types. In order to match protein composition across diets, hemp protein was added to the vegan regimen. While a strict vegetarian diet has been shown to improve risk factors associated with metabolic diseases; it is unknown whether a high calorie high protein vegan dinner would have produced a similar fasting metabolic signature as the animal diet previously published [9, 29]. Additional research is needed to understand if a 2-day meal plan with a high

protein and BCAA vegan dinner, matched to the animal dinner composition, would produce the same fasting metabolic signature or if a lower protein animal dinner would produce a more optimal metabolic signature.

Liberalization of the animal and vegan meal plans used in our research could improve the metabolic impact of the meals and snacks. The Nordic diet is characterized by a high content of fruits and vegetables, plants from the countryside, whole grains, nuts, seafood, free-range livestock and game [34].The macronutrient composition of the animal and vegan diets and TDF content of the vegan diet match the Nordic diet, which could represent the liberalization of our animal menu plan and which has been shown to reduce diabetes risk [13]. The flexitarian or semi-vegetarian diet represents an alternative approach to the liberalization of the animal diet that also emphasizes plant foods with only periodic animal protein intake [16]. See Diet Personalization Supplement.

CONCLUSIONS

We expected to observe a sub-optimal metabolic postprandial profile from the animal meals that would reflect our previous fasting findings in this same cohort. However, the high BCAA protein supplemented vegan breakfast produced the same elevated BCAA-associated metabolic signature as the animal diet produced from our fasting results, reflecting the lower BCAA protein load in the animal breakfast. Our postprandial analysis demonstrated glucose, insulin, AA, TG, bile acid and fatty acid plasma biomarkers varied with diet nutrient composition irrespective of diet type; and that fasting analysis alone is not sufficient to diagnose diet impact. Additionally, our results suggest BCAA content, irrespective of diet type and protein source, may have a negative impact on metabolic health. Liberalization of both diet strategies to optimize metabolic signatures should be tested in future research.

ACKNOWLEDGEMENTS

We would like to thank Cristiana Milone for her work generating the menus and diet analyses; the Metabolic team at the Clinical Development Unit for their role in running the study at the metabolic unit (MU) and Irina Monnard at NRC; Richard Cote (NIHS) for support in nutritional data integration and Frederic Ronga (NIHS) for his assistance on the diet intervention analysis. Jorg Hager and Jim Kaput (NIHS) for scientific discussions. Jan van der Greef for his scientific review and discussions; Eugenia Migliavacca for her statistical insights.

REFERENCES

[1] Pawlak, R., Vegetarian Diets in the Prevention and Management of Diabetes and Its Complications. *Diabetes Spectr* 2017, *30*, 82-88.
[2] Trepanowski, J. F., Varady, K. A., Veganism Is a Viable Alternative to Conventional Diet Therapy for Improving Blood Lipids and Glycemic Control. *Critical reviews in food science and nutrition* 2015, *55*, 2004-2013.
[3] Najjar, R. S., Moore, C. E., Montgomery, B. D., A defined, plant-based diet utilized in an outpatient cardiovascular clinic effectively treats hypercholesterolemia and hypertension and reduces medications. *Clin Cardiol* 2018, *41*, 307-313.
[4] Satija, A., Hu, F. B., Plant-based diets and cardiovascular health. *Trends Cardiovasc Med* 2018.
[5] Estruch, R., Martinez-Gonzalez, M. A., Corella, D., Basora-Gallisa, J., Ruiz-Gutierrez, V., Covas, M. I., Fiol, M., Gomez-Gracia, E., Lopez-Sabater, M. C., Escoda, R., Pena, M. A., Diez-Espino, J., Lahoz, C., Lapetra, J., Saez, G., Ros, E., Investigators, P. S., Effects of dietary fibre intake on risk factors for cardiovascular disease in subjects at high risk. *J Epidemiol Community Health* 2009, *63*, 582-588.
[6] Weickert, M. O., Pfeiffer, A. F. H., Impact of Dietary Fiber Consumption on Insulin Resistance and the Prevention of Type 2 Diabetes. *The Journal of nutrition* 2018, *148*, 7-12.
[7] Satija, A., Bhupathiraju, S. N., Spiegelman, D., Chiuve, S. E., Manson, J. E., Willett, W., Rexrode, K. M., Rimm, E. B., Hu, F. B., Healthful and Unhealthful Plant-Based Diets and the Risk of Coronary Heart Disease in U.S. Adults. *J Am Coll Cardiol* 2017, *70*, 411-422.
[8] Ruiz-Canela, M., Guasch-Ferre, M., Toledo, E., Clish, C. B., Razquin, C., Liang, L., Wang, D. D., Corella, D., Estruch, R., Hernaez, A., Yu, E., Gomez-Gracia, E., Zheng, Y., Aros, F., Romaguera, D., Dennis, C., Ros, E., Lapetra, J., Serra-Majem, L., Papandreou, C., Portoles, O., Fito, M., Salas-Salvado, J., Hu, F. B., Martinez-Gonzalez, M. A., Plasma branched chain/aromatic amino acids, enriched Mediterranean diet and risk of type 2 diabetes: case-cohort study within the PREDIMED Trial. *Diabetologia* 2018.
[9] Torres-Pena, J. D., Garcia-Rios, A., Delgado-Casado, N., Gomez-Luna, P., Alcala-Diaz, J. F., Yubero-Serrano, E. M., Gomez-Delgado, F., Leon-Acuna, A., Lopez-Moreno, J., Camargo, A., Tinahones, F. J., Delgado-Lista, J., Ordovas, J. M., Perez-Martinez, P., Lopez-Miranda, J., Mediterranean diet improves endothelial function in patients with diabetes and prediabetes: A report from the CORDIOPREV study. *Atherosclerosis* 2018, *269*, 50-56.
[10] Ohlsson, B., Darwiche, G., Roth, B., Hoglund, P., Alignments of endocrine, anthropometric, and metabolic parameters in type 2 diabetes after intervention with an Okinawa-based Nordic diet. *Food Nutr Res* 2018, *62*.
[11] Darwiche, G., Hoglund, P., Roth, B., Larsson, E., Sjoberg, T., Wohlfart, B., Steen, S., Ohlsson, B., An Okinawan-based Nordic diet improves anthropometry, metabolic control, and health-related quality of life in Scandinavian patients with type 2 diabetes: a pilot trial. *Food Nutr Res* 2016, *60*, 32594.
[12] Lacoppidan, S. A., Kyro, C., Loft, S., Helnaes, A., Christensen, J., Hansen, C. P., Dahm, C. C., Overvad, K., Tjonneland, A., Olsen, A., Adherence to a Healthy Nordic Food Index Is Associated with a Lower Risk of Type-2 Diabetes--The Danish Diet, Cancer and Health Cohort Study. *Nutrients* 2015, *7*, 8633-8644.

[13] Derbyshire, E. J., Flexitarian Diets and Health: A Review of the Evidence-Based Literature. *Front Nutr* 2016, *3*, 55.
[14] Simpson, H. L., Campbell, B. J., Review article: dietary fibre-microbiota interactions. *Aliment Pharmacol Ther* 2015, *42*, 158-179.
[15] Adam, C. L., Thomson, L. M., Williams, P. A., Ross, A. W., Soluble Fermentable Dietary Fibre (Pectin) Decreases Caloric Intake, Adiposity and Lipidaemia in High-Fat Diet-Induced Obese Rats. *PLoS One* 2015, *10*, e0140392.
[16] Kim, M. S., Hwang, S. S., Park, E. J., Bae, J. W., Strict vegetarian diet improves the risk factors associated with metabolic diseases by modulating gut microbiota and reducing intestinal inflammation. *Environ Microbiol Rep* 2013, *5*, 765-775.
[17] Draper, C. F., Vassallo, I., Di Cara, A., Milone, C., Comminetti, O., Monnard, I., Godin, J. P., Scherer, M., Su, M., Jia, W., Guiraud, S. P., Praplan, F., Guignard, L., Ammon Zufferey, C., Shevlyakova, M., Emami, N., Moco, S., Beaumont, M., Kaput, J., Martin, F. P., A 48-Hour Vegan Diet Challenge in Healthy Women and Men Induces a BRANCH-Chain Amino Acid Related, Health Associated, Metabolic Signature. *Molecular nutrition & food research* 2018, *62*.
[18] Moazzami, A. A., Shrestha, A., Morrison, D. A., Poutanen, K., Mykkanen, H., Metabolomics reveals differences in postprandial responses to breads and fasting metabolic characteristics associated with postprandial insulin demand in postmenopausal women. *The Journal of nutrition* 2014, *144*, 807-814.
[19] Sondergaard, E., Johansen, R. F., Jensen, M. D., Nielsen, S., Postprandial VLDL-TG metabolism in type 2 diabetes. *Metabolism: clinical and experimental* 2017, *75*, 25-35.
[20] Iso, H., Naito, Y., Sato, S., Kitamura, A., Okamura, T., Sankai, T., Shimamoto, T., Iida, M., Komachi, Y., Serum triglycerides and risk of coronary heart disease among Japanese men and women. *Am J Epidemiol* 2001, *153*, 490-499.
[21] Nordestgaard, B. G., Benn, M., Schnohr, P., Tybjaerg-Hansen, A., Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *JAMA* 2007, *298*, 299-308.
[22] Cara, L., Dubois, C., Borel, P., Armand, M., Senft, M., Portugal, H., Pauli, A. M., Bernard, P. M., Lairon, D., Effects of oat bran, rice bran, wheat fiber, and wheat germ on postprandial lipemia in healthy adults. *Am J Clin Nutr* 1992, *55*, 81-88.
[23] Annuzzi, G., Bozzetto, L., Costabile, G., Giacco, R., Mangione, A., Anniballi, G., Vitale, M., Vetrani, C., Cipriano, P., Della Corte, G., Pasanisi, F., Riccardi, G., Rivellese, A. A., Diets naturally rich in polyphenols improve fasting and postprandial dyslipidemia and reduce oxidative stress: a randomized controlled trial. *Am J Clin Nutr* 2014, *99*, 463-471.
[24] Aoyama, T., Nosaka, N., Kasai, M., Research on the nutritional characteristics of medium-chain fatty acids. *J Med Invest* 2007, *54*, 385-388.
[25] Miyoshi, T., Noda, Y., Ohno, Y., Sugiyama, H., Oe, H., Nakamura, K., Kohno, K., Ito, H., Omega-3 fatty acids improve postprandial lipemia and associated endothelial dysfunction in healthy individuals - a randomized cross-over trial. *Biomed Pharmacother* 2014, *68*, 1071-1077.
[26] Pappas, C., Kandaraki, E. A., Tsirona, S., Kountouras, D., Kassi, G., Diamanti-Kandarakis, E., Postprandial dysmetabolism: Too early or too late? *Hormones (Athens)* 2016, *15*, 321-344.
[27] Kardinaal, A. F., van Erk, M. J., Dutman, A. E., Stroeve, J. H., van de Steeg, E., Bijlsma, S., Kooistra, T., van Ommen, B., Wopereis, S., Quantifying phenotypic

flexibility as the response to a high-fat challenge test in different states of metabolic health. *FASEB J* 2015, *29*, 4600-4613.
[28] van Ommen, B., van der Greef, J., Ordovas, J. M., Daniel, H., Phenotypic flexibility as key factor in the human nutrition and health relationship. *Genes Nutr* 2014, *9*, 423.
[29] Wopereis, S., Stroeve, J. H. M., Stafleu, A., Bakker, G. C. M., Burggraaf, J., van Erk, M. J., Pellis, L., Boessen, R., Kardinaal, A. A. F., van Ommen, B., Multi-parameter comparison of a standardized mixed meal tolerance test in healthy and type 2 diabetic subjects: the PhenFlex challenge. *Genes Nutr* 2017, *12*, 21.
[30] Harris, J. A., Benedict, F. G., A Biometric Study of Human Basal Metabolism. *Proc Natl Acad Sci U S A* 1918, *4*, 370-373.
[31] Lebowitsch, J., Ge, Y., Young, B., Hu, F., Generalized multidimensional dynamic allocation method. *Statistics in medicine* 2012, *31*, 3537-3544.
[32] Xie, G., Zhong, W., Li, H., Li, Q., Qiu, Y., Zheng, X., Chen, H., Zhao, X., Zhang, S., Zhou, Z., Zeisel, S. H., Jia, W., Alteration of bile acid metabolism in the rat induced by chronic ethanol consumption. *Faseb J* 2013, *27*, 3583-3593.
[33] Zhao, L., Ni, Y., Su, M., Li, H., Dong, F., Chen, W., Wei, R., Zhang, L., Guiraud, S. P., Martin, F. P., Rajani, C., Xie, G., Jia, W., High Throughput and Quantitative Measurement of Microbial Metabolome by Gas Chromatography/Mass Spectrometry Using Automated Alkyl Chloroformate Derivatization. *Anal Chem* 2017, *89*, 5565-5577.
[34] Mithril, C., Dragsted, L. O., Meyer, C., Tetens, I., Biltoft-Jensen, A., Astrup, A., Dietary composition and nutrient content of the New Nordic Diet. *Public Health Nutr* 2013, *16*, 777-785.
[35] Nakamura, A., Monma, Y., Kajitani, S., Kozu, K., Ikeda, S., Noda, K., Nakajima, S., Endo, H., Takahashi, T., Nozaki, E., Different postprandial lipid metabolism and insulin resistance between non-diabetic patients with and without coronary artery disease. *J Cardiol* 2015, *66*, 435-444.
[36] Boren, J., Matikainen, N., Adiels, M., Taskinen, M. R., Postprandial hypertriglyceridemia as a coronary risk factor. *Clin Chim Acta* 2014, *431*, 131-142.
[37] Subramanian, S., Chait, A., Hypertriglyceridemia secondary to obesity and diabetes. *Biochim Biophys Acta* 2012, *1821*, 819-825.
[38] Ulmius, M., Johansson, A., Onning, G., The influence of dietary fibre source and gender on the postprandial glucose and lipid response in healthy subjects. *Eur J Nutr* 2009, *48*, 395-402.
[39] Hu, G. X., Chen, G. R., Xu, H., Ge, R. S., Lin, J., Activation of the AMP activated protein kinase by short-chain fatty acids is the main mechanism underlying the beneficial effect of a high fiber diet on the metabolic syndrome. *Med Hypotheses* 2010, *74*, 123-126.
[40] Stanhope, K. L., Griffen, S. C., Bremer, A. A., Vink, R. G., Schaefer, E. J., Nakajima, K., Schwarz, J. M., Beysen, C., Berglund, L., Keim, N. L., Havel, P. J., Metabolic responses to prolonged consumption of glucose- and fructose-sweetened beverages are not associated with postprandial or 24-h glucose and insulin excursions. *Am J Clin Nutr* 2011, *94*, 112-119.
[41] Sears, B., Perry, M., The role of fatty acids in insulin resistance. *Lipids Health Dis* 2015, *14*, 121.
[42] Newgard, C. B., An, J., Bain, J. R., Muehlbauer, M. J., Stevens, R. D., Lien, L. F., Haqq, A. M., Shah, S. H., Arlotto, M., Slentz, C. A., Rochon, J., Gallup, D., Ilkayeva, O., Wenner, B. R., Yancy, W. S., Jr., Eisenson, H., Musante, G., Surwit, R. S., Millington, D.

S., Butler, M. D., Svetkey, L. P., A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009, *9*, 311-326.
[43] Ottosson, F., Ericson, U., Almgren, P., Nilsson, J., Magnusson, M., Fernandez, C., Melander, O., Postprandial Levels of Branch Chained and Aromatic Amino Acids Associate with Fasting Glycaemia. *J Amino Acids* 2016, *2016*, 8576730.
[44] Yu, D., Yang, S. E., Miller, B. R., Wisinski, J. A., Sherman, D. S., Brinkman, J. A., Tomasiewicz, J. L., Cummings, N. E., Kimple, M. E., Cryns, V. L., Lamming, D. W., Short-term methionine deprivation improves metabolic health via sexually dimorphic, mTORC1-independent mechanisms. *FASEB J* 2018, fj201701211R.
[45] Kalogeropoulou, D., LaFave, L., Schweim, K., Gannon, M. C., Nuttall, F. Q., Lysine ingestion markedly attenuates the glucose response to ingested glucose without a change in insulin response. *Am J Clin Nutr* 2009, *90*, 314-320.
[46] Zhou, H., Hylemon, P. B., Bile acids are nutrient signaling hormones. *Steroids* 2014, *86*, 62-68.
[47] Li, T., Francl, J. M., Boehme, S., Ochoa, A., Zhang, Y., Klaassen, C. D., Erickson, S. K., Chiang, J. Y., Glucose and insulin induction of bile acid synthesis: mechanisms and implication in diabetes and obesity. *The Journal of biological chemistry* 2012, *287*, 1861-1873.
[48] Fouladi, F., Mitchell, J. E., Wonderlich, J. A., Steffen, K. J., The Contributing Role of Bile Acids to Metabolic Improvements After Obesity and Metabolic Surgery. *Obes Surg* 2016, *26*, 2492-2502.
[49] Grundy, M. M., Edwards, C. H., Mackie, A. R., Gidley, M. J., Butterworth, P. J., Ellis, P. R., Re-evaluation of the mechanisms of dietary fibre and implications for macronutrient bioaccessibility, digestion and postprandial metabolism. *Br J Nutr* 2016, *116*, 816-833.
[50] Reddy, B. S., Diet and excretion of bile acids. *Cancer Res* 1981, *41*, 3766-3768.
[51] Kaczmarczyk, M. M., Miller, M. J., Freund, G. G., The health benefits of dietary fiber: beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and colon cancer. *Metabolism: clinical and experimental* 2012, *61*, 1058-1066.
[52] Watanabe, M., Houten, S. M., Mataki, C., Christoffolete, M. A., Kim, B. W., Sato, H., Messaddeq, N., Harney, J. W., Ezaki, O., Kodama, T., Schoonjans, K., Bianco, A. C., Auwerx, J., Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* 2006, *439*, 484-489.
[53] Zietak, M., Kozak, L. P., Bile acids induce uncoupling protein 1-dependent thermogenesis and stimulate energy expenditure at thermoneutrality in mice. *American journal of physiology. Endocrinology and metabolism* 2016, *310*, E346-354.
[54] Nicolosi, R. J., Dietary fat saturation effects on low-density-lipoprotein concentrations and metabolism in various animal models. *Am J Clin Nutr* 1997, *65*, 1617S-1627S.
[55] Jansen, R. J., Robinson, D. P., Frank, R. D., Anderson, K. E., Bamlet, W. R., Oberg, A. L., Rabe, K. G., Olson, J. E., Sinha, R., Petersen, G. M., Stolzenberg-Solomon, R. Z., Fatty acids found in dairy, protein and unsaturated fatty acids are associated with risk of pancreatic cancer in a case-control study. *International journal of cancer* 2014, *134*, 1935-1946.
[56] Praagman, J., Beulens, J. W., Alssema, M., Zock, P. L., Wanders, A. J., Sluijs, I., van der Schouw, Y. T., The association between dietary saturated fatty acids and ischemic

heart disease depends on the type and source of fatty acid in the European Prospective Investigation into Cancer and Nutrition-Netherlands cohort. *Am J Clin Nutr* 2016, *103*, 356-365.
[57] Xu, Q., Xue, C., Zhang, Y., Liu, Y., Wang, J., Yu, X., Zhang, X., Zhang, R., Yang, X., Guo, C., Medium-chain fatty acids enhanced the excretion of fecal cholesterol and cholic acid in C57BL/6J mice fed a cholesterol-rich diet. *Biosci Biotechnol Biochem* 2013, *77*, 1390-1396.

GENDER DIMORPHISM SUPPLEMENT

Most cellular and human clinical research has been performed either on males only or both males and females, controlling for sex and gender differences to homogenize the data as opposed to differentiating between sex or gender specific responses. We feel it is important to acknowledge the gender and sex differences observed in our research in order to expose potential hypotheses for future testing, the results of which can be used to strengthen clinical practice. This is necessary to improve the healthcare of both genders.

Gender dimorphic postprandial response
Breakfast AUC results for plasma Insulin, TGs, PRO, and ALA were significantly lower for women whose lower calorie and nutrient needs necessitated lower intakes from CHO, fat and PROT. Lunch AUC results for plasma TGs were, also, significantly lower for women. Essential amino acids, BCAAs and 8 AAs were significantly higher for women at lunch which was not consistent with differences in intake (FDR<0.10). Five bile acids were elevated in women after breakfast and ten bile acids were higher for women after lunch ($p < 0.05$, FDR>0.10) (**Figure 2, Figure 4**,**Table S4, Table 1**) .

Gender dimorphic postprandial response leads to more insight on personalization
Triglyceride and insulin responses in women at breakfast and lunch were lower, likely reflecting lower nutrient intakes; however, differences in physiology and metabolism may have played a role. For example, a lower TG response may also relate to known increased postprandial skeletal muscle clearance of TG in women, which leads to lower plasma concentrations (**Figure 2,Table S4**) [1]. Estrogen regulates insulin sensitivity in females and may have further augmented the lower plasma insulin response to the diet interventions seen in the premenopausal women in our study (**Table S2**) [2, 3]. Additionally, previously published research suggests females may have been more responsive to the triglyceride and insulin lowering effects of higher soluble fiber in the vegan diet (**Figure S6**) [4].

Analysis of gender differences in dietary intake during the breakfast, lunch and dinner timeperiods revealed men had significantly higher nutrient intakes, as expected. This is due to higher calorie and nutrient needs of men as the composition of the meals provided were matched to age, height, weight and sex (**Table S4**). This was reflected in significantly greater plasma AUC AA concentrations in men at breakfast, however, women had higher plasma AUC AA at lunch (**Table S2**). Elevated plasma AA in women despite lower protein intake may reflect an accumulation of plasma AA over time due

to slower female mechanisms that regulate AA utilization and protein metabolism and a gender specific response to the prolonged nutrient digestion effects of TDF [4, 5].
The trend of elevated bile acids in women after breakfast and lunch despite lower dietary fat intake, may be linked to the known effect of sex hormones and increased expression of Cyp7a1, as well as a known differential bile acid response of females to high TDF intake (**Figure 4, Table S2, Table S3**) [4, 6-8]. Thus, consideration of differences in gender response is an important diagnostic component of personalized diet assessment and therapy.

CONCLUSION

Gender dimorphism in metabolic signatures was observed. Lower nutrient intakes in women led to lower TG and insulin responses. Lower insulin and elevated bile acid responses may have been further augmented by estrogen. Prolonged plasma amino acid elevation in women may have been influenced by a gender specific response to soluble TDF and gender specific differences in metabolic rate. Thus, consideration of differences in gender response is an important diagnostic component of personalized diet assessment and therapy.

DIET PRESCRIPTION FOR PERSONALIZATION

Measurement of postprandial response creates opportunities for diet personalization. The results from our study suggest liberalization of the animal and vegan diet plans used in our research could improve the metabolic impact of the meals and snacks. The following are examples of recommended modifications to both strategies to personalize the diet prescription based on diet type and postprandial response.

Modification of the vegan diet to vegetarian
A reduction in the muesli, cashew and hemp protein portions would reduce calories and nutrients at breakfast to moderate TG, insulin, glucose and AA response. Inclusion of cheese at lunch would increase plasma fatty acid concentrations. A decrease in the portion of rice and beans at lunch and removal of the Kombucha drink would reduce CHO and blood glucose.

Modification of the animal diet to include vegetarian components
An increase in TDF at breakfast with the addition of fruit, such as berries, may increase bile acid concentrations. The addition of TDF at lunch obtained by replacing chips with cooked vegetables, apple sauce with a fresh apple, as well as the hamburger roll with a whole wheat variety, could help prolong the absorption of amino acids from the

hamburger meal, reducing the amino acid spike observed after lunchtime. A reduction in the portion of chicken at dinner, replacement of standard pasta with a whole wheat variety and replacement of the apricot tart with fresh fruit and nuts would decrease the high amino acid and simple sugar intakes. Indeed, those may have contributed to the elevated AA, insulin and TG observed from the day 3 fasting results [9]. The addition of cottage to cheese to the afternoon snack would replace the protein intake removed from dinner.

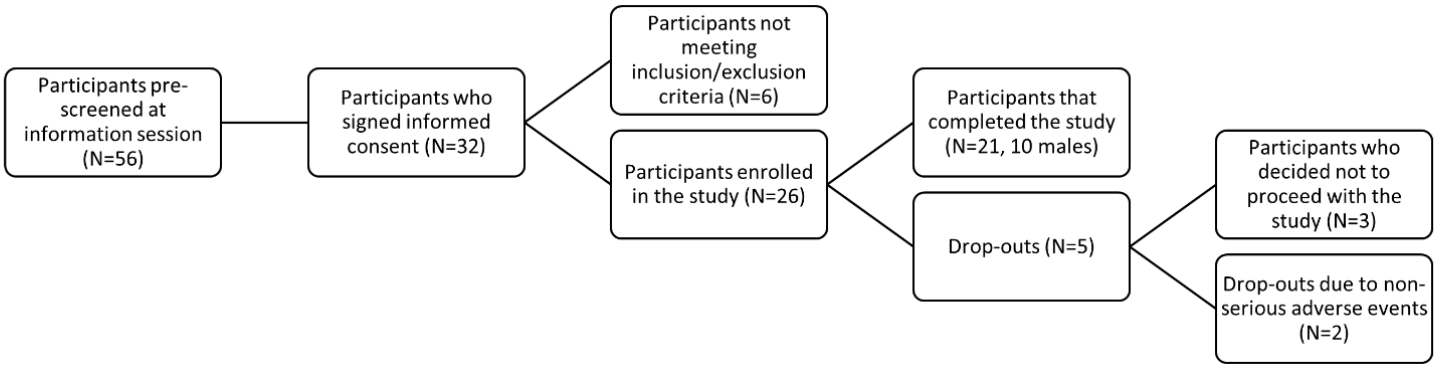
CONCLUSION

The Nordic diet is characterized by a high content of fruits and vegetables, plants from the countryside, whole grains, nuts, seafood, free-range livestock and game [10]. The macronutrient composition of the animal and vegan diets and TDF content of the vegan diet match the Nordic diet, which could represent the liberalization of our animal menu plan and which has been shown to reduce diabetes risk [11]. The flexitarian or semi-vegetarian diet represents an alternative approach to the liberalization of the animal diet that also emphasizes plant foods with only periodic animal protein intake [12].

SUPPLEMENTARY REFERENCES

[1] Horton, T. J., Commerford, S. R., Pagliassotti, M. J., Bessesen, D. H., Postprandial leg uptake of triglyceride is greater in women than in men. *American journal of physiology. Endocrinology and metabolism* 2002, *283*, E1192-1202.
[2] Shen, M., Kumar, S. P., Shi, H., Estradiol regulates insulin signaling and inflammation in adipose tissue. *Horm Mol Biol Clin Investig* 2014, *17*, 99-107.
[3] Rettberg, J. R., Yao, J., Brinton, R. D., Estrogen: a master regulator of bioenergetic systems in the brain and body. *Front Neuroendocrinol* 2014, *35*, 8-30.
[4] Ulmius, M., Johansson, A., Onning, G., The influence of dietary fibre source and gender on the postprandial glucose and lipid response in healthy subjects. *Eur J Nutr* 2009, *48*, 395-402.
[5] Comitato, R., Saba, A., Turrini, A., Arganini, C., Virgili, F., Sex hormones and macronutrient metabolism. *Critical reviews in food science and nutrition* 2015, *55*, 227-241.
[6] Kern, F., Jr., Everson, G. T., DeMark, B., McKinley, C., Showalter, R., Erfling, W., Braverman, D. Z., Szczepanik-van Leeuwen, P., Klein, P. D., Biliary lipids, bile acids, and gallbladder function in the human female. Effects of pregnancy and the ovulatory cycle. *The Journal of clinical investigation* 1981, *68*, 1229-1242.
[7] Turley, S. D., Schwarz, M., Spady, D. K., Dietschy, J. M., Gender-related differences in bile acid and sterol metabolism in outbred CD-1 mice fed low- and high-cholesterol diets. *Hepatology* 1998, *28*, 1088-1094.
[8] Fu, Z. D., Csanaky, I. L., Klaassen, C. D., Gender-divergent profile of bile acid homeostasis during aging of mice. *PLoS One* 2012, *7*, e32551.
[9] Draper, C. F., Vassallo, I., Di Cara, A., Milone, C., Comminetti, O., Monnard, I., Godin, J. P., Scherer, M., Su, M., Jia, W., Guiraud, S. P., Praplan, F., Guignard, L., Ammon Zufferey, C., Shevlyakova, M., Emami, N., Moco, S., Beaumont, M., Kaput, J., Martin, F. P., A 48-Hour Vegan Diet Challenge in Healthy Women and Men Induces a BRANCH-Chain Amino Acid Related, Health Associated, Metabolic Signature. *Molecular nutrition & food research* 2018, *62*.
[10] Mithril, C., Dragsted, L. O., Meyer, C., Tetens, I., Biltoft-Jensen, A., Astrup, A., Dietary composition and nutrient content of the New Nordic Diet. *Public Health Nutr* 2013, *16*, 777-785.
[11] Ohlsson, B., Darwiche, G., Roth, B., Hoglund, P., Alignments of endocrine, anthropometric, and metabolic parameters in type 2 diabetes after intervention with an Okinawa-based Nordic diet. *Food Nutr Res* 2018, *62*.
[12] Derbyshire, E. J., Flexitarian Diets and Health: A Review of the Evidence-Based Literature. *Front Nutr* 2016, *3*, 55.

SUPPLEMENTARY FIGURES



Supplementary Figure S1. Study flow chart.

ANIMAL DIET Menu

Breakfast
Croissant
Strawberry jam
Coffee with milk and sugar

Snack
Yogurt Nature
Fresh apple

Lunch
Beef Hamburger with mustard and fresh tomato slices
Chips
Cucumber salad
Applesauce

Snack
Crackers

Dinner
Dijon chicken with pasta
Mixed lettuce with balsamic vinaigrette
Apricot tart

Drinks
Water

"Emergency Snack"
Yogurt, skimmed with fruit
10 Raw plain almonds

Kcals
1600
1800
2000
2200
2400
2600
2800
3000

VEGAN DIET Menu

Kcals
1600
1800
2000
2200
2400
2600
2800
3000

Breakfast
Healthy quinoa rice milk müesli with cashew butter
Hemp protein powder
Black coffee

Snack
Homemade hummus + Hemp protein powder
Carrots, cucumber
Gluten-free bread, Kombucha drink

Lunch
Brown rice and red beans salad with baby spinach, white balsamic vinaigrette
Gluten Free Bread
Green Tea

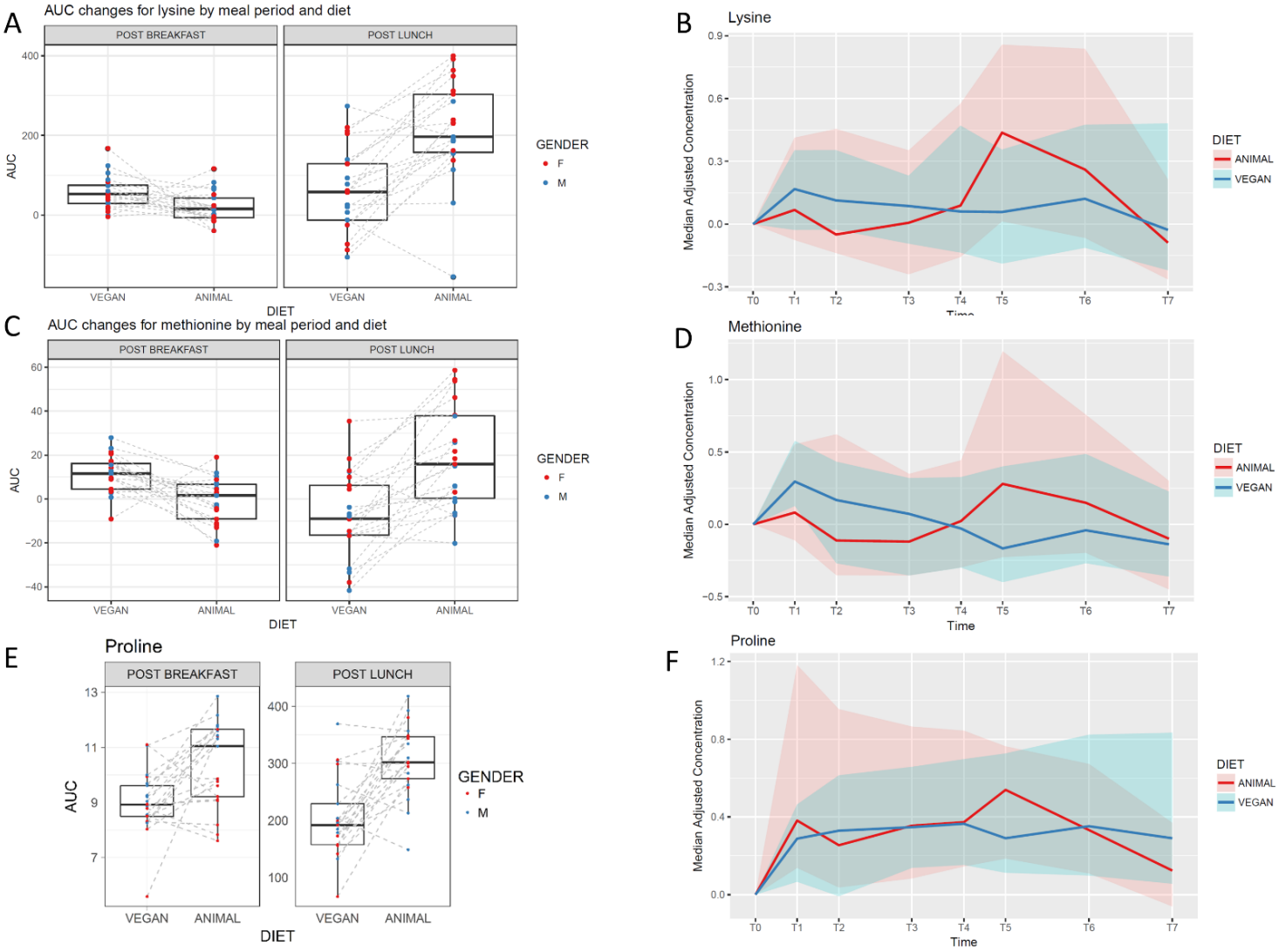
Snack
Fresh banana with soy yogurt

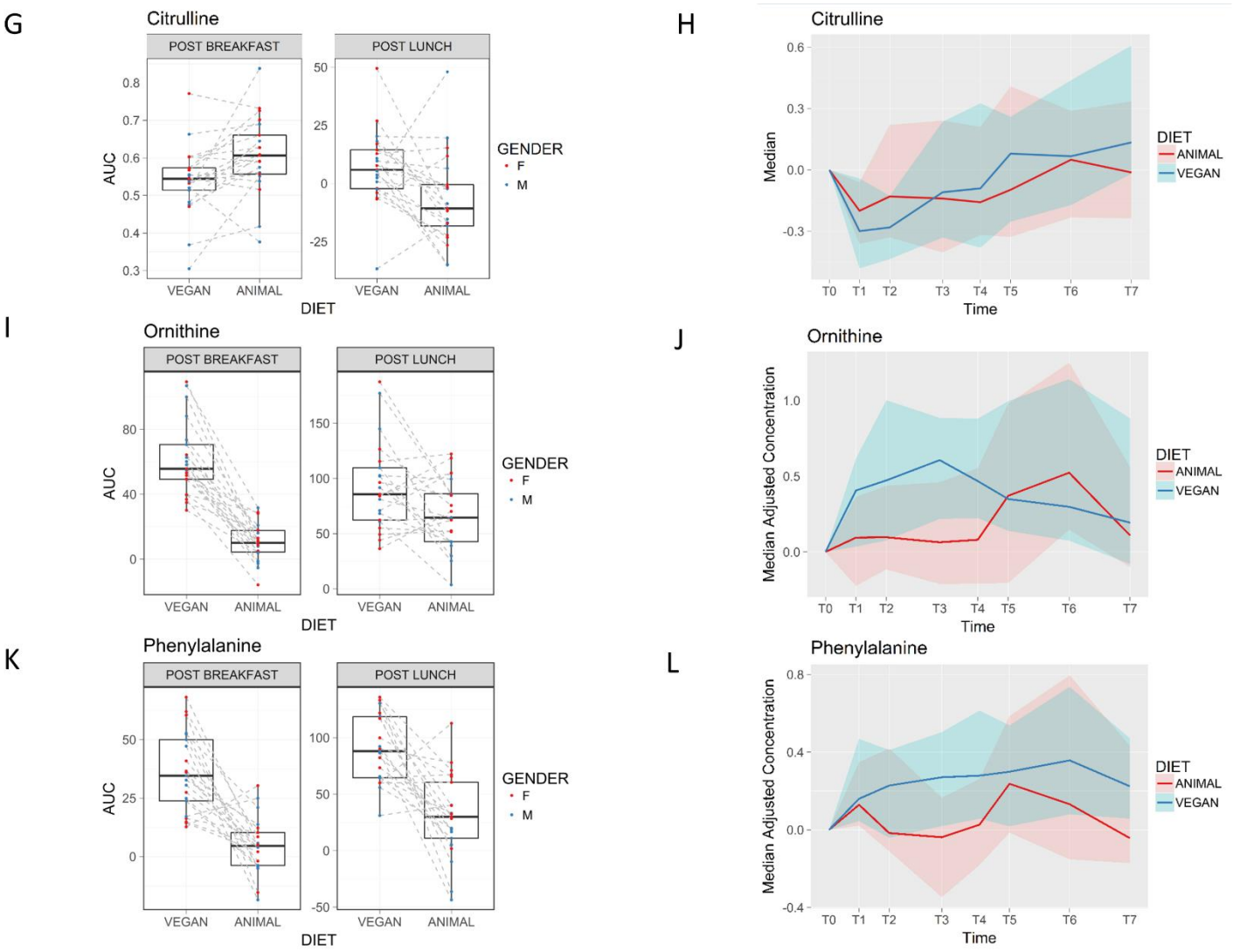
Dinner
Asian lentil coconut curry +Hemp protein powder
Gluten-free bread
Ginseng and green tea

Drinks
Water

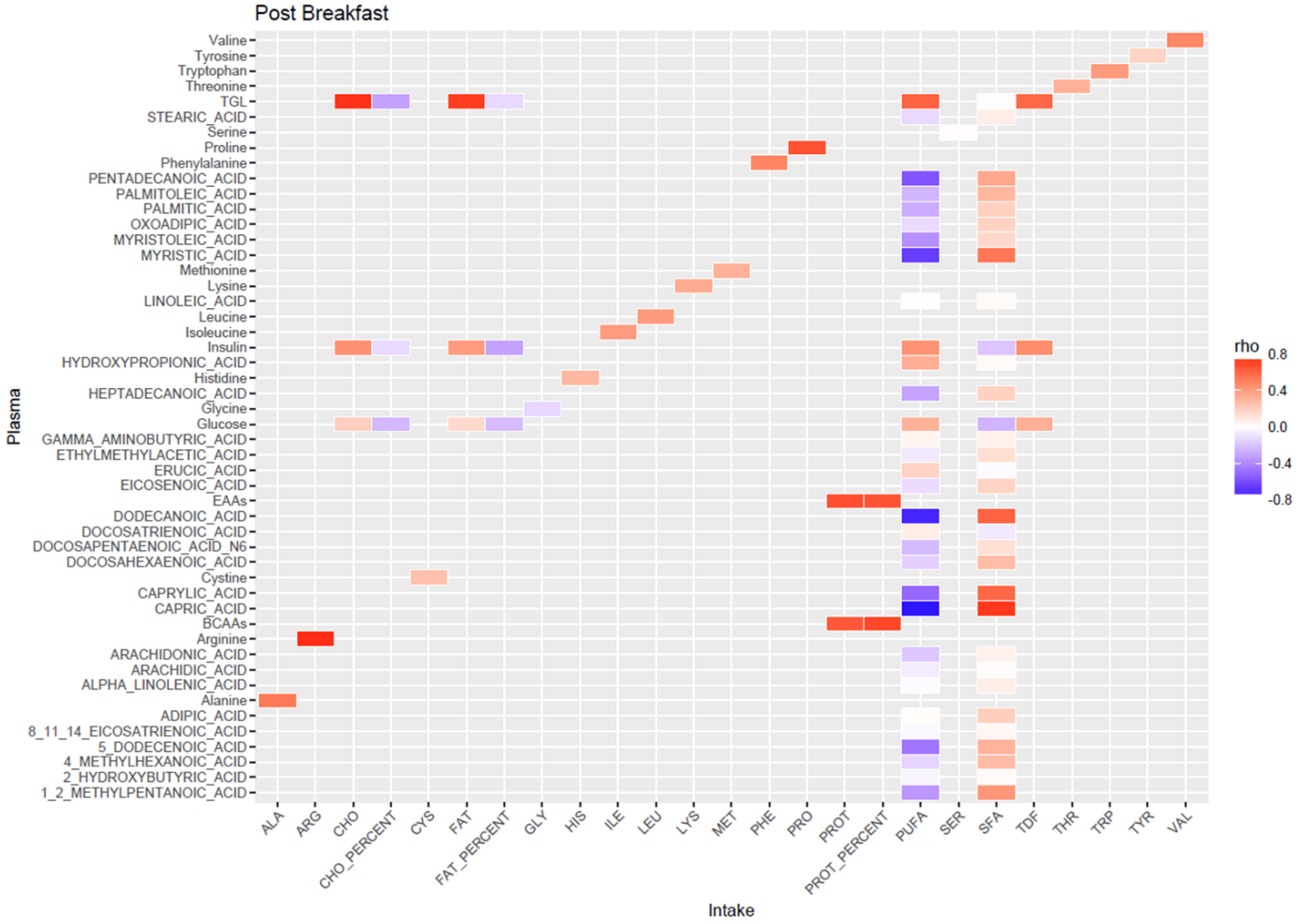
"Emergency Snack"
Soy Yogurt Nature (Coop Naturaplan)

Supplementary Figure S2. The animal and vegan diet menu plans were the same each day for 2 days. Food portions were provided based on 8 personalized calorie plans in accordance with the caloric needs of the individual participants. Hemp protein powder was used for the vegan diet to boost total protein.

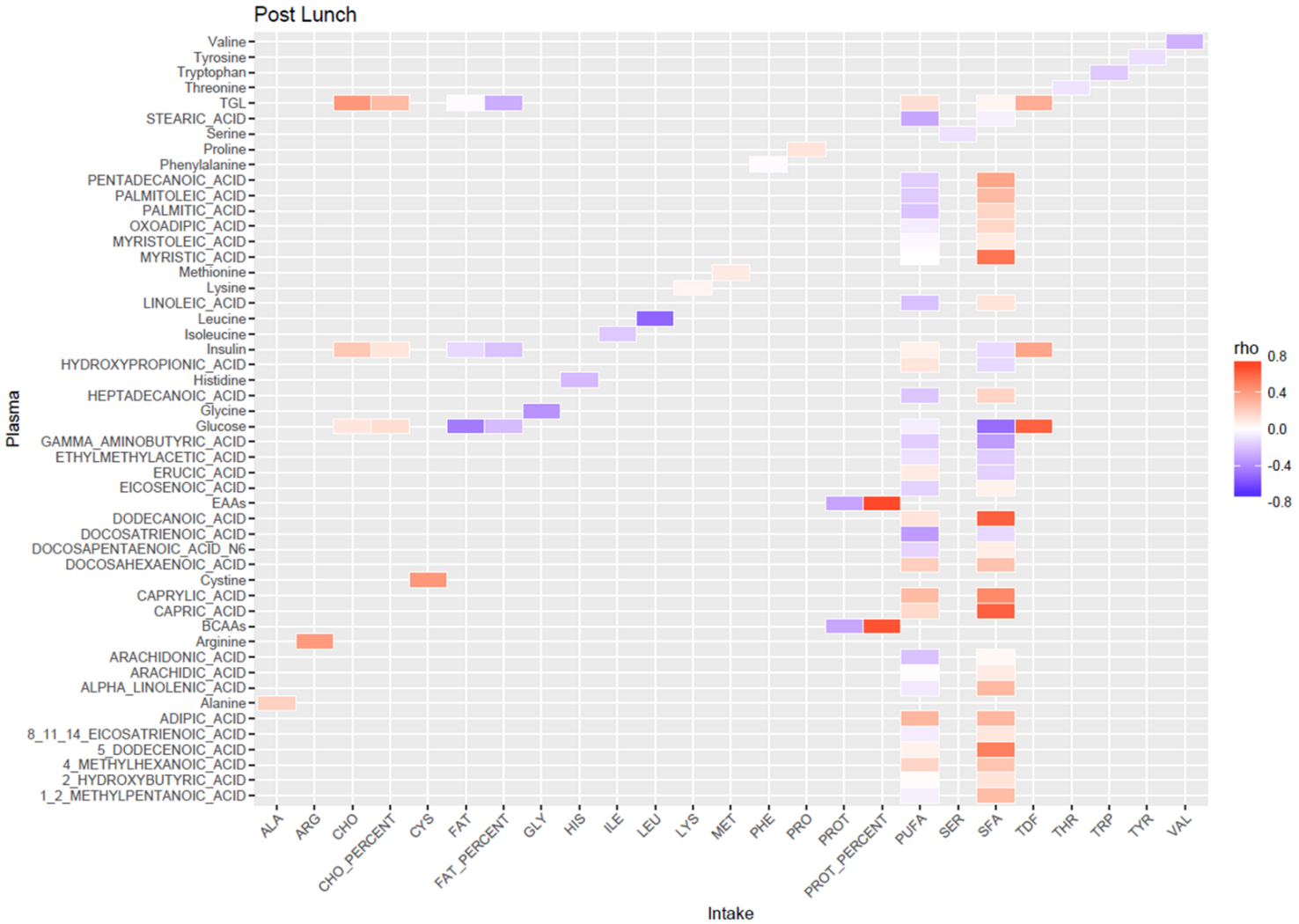

A
AUC changes for lysine by meal period and diet
POST BREAKFAST
POST LUNCH
AUC
DIET
VEGAN
ANIMAL
GENDER
F
M
B
Lysine
Median Adjusted Concentration
DIET
ANIMAL
VEGAN
T0
T1
T2
T3
T4
T5
T6
T7
C
AUC changes for methionine by meal period and diet
D
Methionine
Time
E
Proline
F
Proline



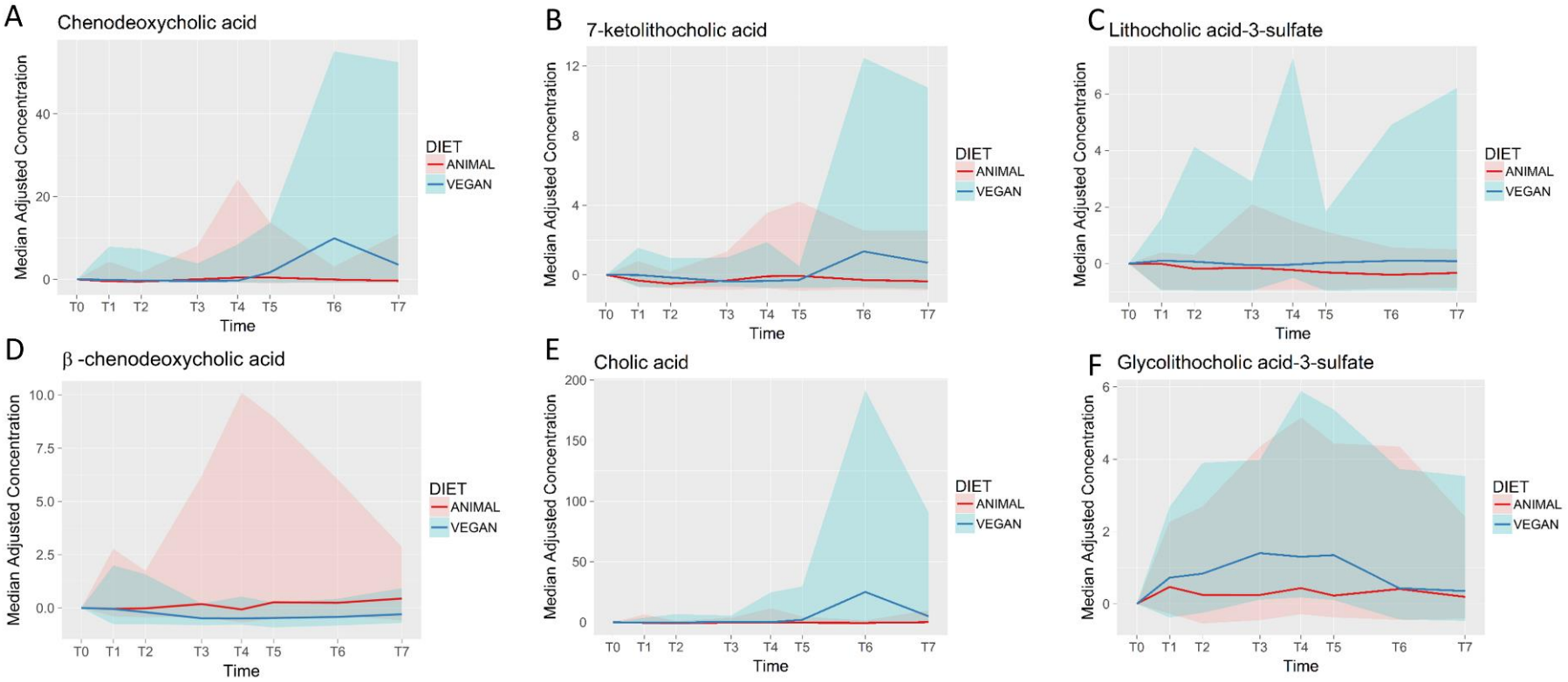
Supplementary Figure S3. (A-L) Amino acids with significant differences observed in AUC timeframes comparing vegan and animal breakfast, vegan and animal lunch, vegan vs animal changes from breakfast to lunch and from ANOVA timepoint analyses. Statistics can be found in Table S2. Metabolic signatures differ according to timing (AUC comparisons); and Table S3. Metabolic signatures differ according to timing (Timepoint comparisons).



Supplementary Figure S4. Spearman correlations between significant plasma metabolites and diet intake are graphically represented with a heat map for the post-breakfast AUC response. Statistics can be found in Table 2. Plasma biomarker x diet correlations.



Supplementary Figure S5. Spearman correlations between significant plasma metabolites and diet intake are graphically represented with a heat map for the post-lunch AUC response. Statistics can be found in Table 2. Plasma biomarker x diet correlations.



Supplementary Figure S6. (A-F) Line graphs of the medians of representative bile and fatty acids demonstrating statistically significant changes from the vegan versus animal diets throughout the postprandial timeperiods. Statistical results can be found in Table S3. Metabolic signatures differ according to timing (Timepoint comparisons).

SUPPLEMENTARY TABLES

Supplementary Table 1. Meal and snack comparisons

	Breakfast			Morning snack			Lunch			Afternoon snack			Dinner		
Nutrient	**Animal diet means**	**Vegan diet means**	***p*-value***	**Animal diet means**	**Vegan diet means**	***p*-value***	**Animal diet means**	**Vegan diet means**	***p*-value***	**Animal diet means**	**Vegan diet means**	***p*-value***	**Animal diet means**	**Vegan diet means**	***p*-value***
Kilocalories (Kcal)	405.81	596.37	**1.49E-04**	192.54	299.14	5.72E-03	569.50	344.60	**6.37E-05**	104.71	211.67	**4.04E-05**	851.65	351.56	**6.38E-05**
Protein (g)	8.90	22.40	**6.16E-05**	6.99	14.40	2.22E-03	24.98	14.72	**6.37E-05**	2.94	7.52	**4.73E-05**	59.62	18.58	**6.37E-05**
% Kcals from protein	8.41	15.14	**6.04E-05**	14.66	18.18	4.37E-03	17.68	17.51	6.51E-01	11.30	13.59	1.25E-03	28.14	20.20	**7.38E-05**
Alanine (mg)	350.38	834.63	**7.14E-05**	314.80	555.14	5.42E-03	1133.65	670.47	**6.36E-05**	96.54	333.83	**4.04E-05**	3142.47	735.26	**6.35E-05**
Arginine (mg)	436.33	1320.26	**6.24E-05**	253.52	1072.67	**1.31E-04**	1165.76	931.25	1.08E-03	105.38	569.83	**4.04E-05**	3470.18	1430.94	**6.35E-05**
Cysteine (mg)	152.33	285.62	**7.23E-05**	53.81	186.88	**1.50E-04**	374.88	152.95	**6.35E-05**	62.71	60.33	1.42E-01	957.74	210.80	**6.35E-05**
Glycine (mg)	267.16	679.39	**6.16E-05**	175.00	505.71	**1.51E-04**	1131.40	641.36	**6.36E-05**	119.36	308.58	**4.73E-05**	2904.32	680.99	**6.35E-05**
Histidine (mg)	222.43	418.63	**7.14E-05**	181.46	336.29	2.21E-03	661.62	386.48	**6.37E-05**	56.57	249.50	**2.95E-05**	1854.68	416.13	**6.35E-05**
Isoleucine (mg)	432.95	632.39	**2.22E-04**	429.73	627.26	4.75E-02	997.77	612.43	**6.37E-05**	112.52	319.67	**4.73E-05**	3163.58	720.35	**6.35E-05**
Leucine (mg)	849.90	1325.48	**1.46E-04**	732.69	896.43	1.86E-01	1702.81	1075.52	**6.37E-05**	218.38	98.33	**6.44E-05**	4837.73	1181.73	**6.35E-05**
Lysine (mg)	605.90	643.88	6.26E-01	558.23	728.74	1.54E-01	1301.32	894.65	**6.36E-05**	58.05	440.33	**3.45E-05**	4373.74	983.80	**6.35E-05**
Methionine (mg)	244.52	298.68	2.59E-02	177.94	177.21	9.72E-01	446.77	213.35	**6.35E-05**	45.00	86.50	**6.44E-05**	1519.57	208.36	**6.35E-05**
Phenylalanine (mg)	468.10	742.58	**1.46E-04**	376.76	594.93	2.38E-02	907.87	732.30	2.21E-03	141.43	324.00	**5.52E-05**	2762.67	818.12	**6.35E-05**
Proline (mg)	936.02	915.70	9.72E-01	811.58	538.61	2.49E-03	15042.56	577.58	**6.36E-05**	364.77	48.67	**4.73E-05**	4060.44	710.22	**6.35E-05**
Serine (mg)	534.38	796.54	**1.46E-04**	432.36	638.58	4.75E-02	1029.08	769.57	**3.41E-04**	151.17	405.83	**4.73E-05**	1785.19	858.44	**6.30E-05**
Threonine (mg)	380.38	612.80	**1.46E-04**	305.60	512.14	1.23E-02	822.52	573.57	**9.85E-05**	84.67	293.00	**4.04E-05**	2584.56	680.11	**6.35E-05**
Tryptophan (mg)	112.43	156.23	7.26E-04	80.74	111.31	7.62E-02	301.45	159.54	**1.50E-04**	33.95	95.50	**5.52E-05**	863.38	149.21	**6.35E-05**
Tyrosine (mg)	392.10	492.65	2.82E-02	354.14	448.12	1.64E-01	735.05	399.39	**6.35E-05**	87.33	251.00	**4.73E-05**	1942.92	570.93	**6.35E-05**
Valine (mg)	528.90	834.54	**1.46E-04**	536.50	602.29	5.31E-01	1094.67	714.06	**6.35E-05**	126.81	342.00	**4.04E-05**	3115.46	883.45	**6.35E-05**
Carbohydrate (g)	45.96	62.93	**2.52E-04**	22.61	34.71	2.19E-03	52.69	46.83	**6.36E-05**	20.62	35.00	**3.45E-05**	111.36	41.98	**6.37E-05**
% Kcals from carbohydrate	45.83	42.40	**1.43E-04**	46.57	50.06	5.78E-01	37.08	52.82	1.14E-01	79.18	67.65	1.45E-03	51.99	48.98	7.62E-02
Total fiber (g)	2.01	7.41	**6.14E-05**	2.63	7.73	**1.72E-04**	5.86	10.47	**6.36E-05**	0.94	4.25	**2.95E-05**	5.37	7.60	2.21E-03
Total fat (g)	19.67	27.52	**1.92E-04**	7.64	9.38	2.81E-01	27.96	10.46	**6.35E-05**	1.19	4.17	1.03E-03	15.38	11.51	**2.23E-04**
% Kcal from fat	43.45	41.30	**1.90E-04**	35.99	25.53	**9.90E-05**	44.02	28.89	**6.36E-05**	9.73	16.55	1.68E-03	16.31	29.48	**6.38E-05**
Polyunsaturated fat (g)	0.83	7.21	**6.16E-05**	0.19	6.00	**7.34E-05**	8.73	6.02	3.90E-03	0.43	2.10	8.31E-04	4.69	6.53	3.90E-03
Monounsaturated fats (g)	3.67	14.77	**6.16E-05**	2.31	1.21	**5.63E-04**	11.58	3.43	**6.35E-05**	0.26	0.92	**5.62E-05**	5.86	1.55	**6.13E-05**
Saturated fat (g)	14.37	5.27	**6.16E-05**	3.95	1.54	**8.47E-05**	7.44	1.37	**6.37E-05**	0.40	0.77	8.31E-04	3.38	2.39	1.11E-02

***p values bolded for q<0.10**

Supplementary Table S2. Metabolic signatures differ according to AUC comparisons

	AUC Post-Breakfast			AUC Post-Lunch		
Marker	**Gender *P* value***	**Diet effect***	**Diet *P* value***	**Gender *P* value***	**Diet effect***	**Diet *P* value***
Amino acids (nmol/ml)						
Phenylalanine	8.99E-01	31.32	**1.43E-06**	**1.16E-02**	60.06	**4.81E-06**
Lysine	2.52E-01	36.78	**9.06E-03**	**4.02E-02**	-145.93	**2.24E-05**
Methionine	1.75E-01	12.40	**4.56E-04**	**9.01E-03**	-26.35	**3.96E-05**
Proline	**7.85E-04**	-1.44	**6.55E-04**	7.28E-01	-102.95	**5.04E-05**
Valine	6.32E-01	120.44	**3.59E-05**	**2.69E-03**	147.27	**2.99E-04**
Arginine	1.77E-01	4.76	**1.29E-10**	9.87E-01	0.14	**4.10E-03**
Citrulline	8.27E-02	-0.07	**7.56E-03**	7.23E-01	13.94	**1.62E-02**
Ornithine	1.52E-01	50.89	**2.58E-09**	6.70E-01	24.92	**2.27E-02**
Threonine	9.11E-01	31.84	**1.99E-02**	**1.66E-02**	33.94	1.80E-01
BCAAs	5.30E-01	209.32	**9.08E-04**	**3.88E-03**	91.70	1.81E-01
Leucine	3.78E-01	38.96	**1.24E-02**	**6.21E-03**	-27.68	2.21E-01
Acetyltryptophan				2.88E-02	-0.42	2.52E-01
Tyrosine				**1.62E-03**	11.23	3.22E-01
Glycine				**3.40E-02**	27.82	6.84E-01
EAAs	4.06E-01	326.33	**5.14E-05**	**7.10E-04**	7.78	9.48E-01
Asparagine	9.92E-01	0.10	**7.01E-06**			
Tryptophan	9.28E-01	24.70	**4.64E-05**			
Isoleucine	4.18E-01	26.77	**1.34E-02**			
Histidine	9.93E-01	13.17	**2.80E-02**			
Alanine	**6.57E-03**	47.09	1.96E-01			
Fatty acids (ng/ml)						
Dodecenoic acid (C12:1)	1.34E-01	-2489.25	**2.94E-06**	1.11E-01	-0.23	**1.38E-06**
Capric acid (C10:0)	7.95E-01	-0.39	**1.27E-09**	9.00E-01	-0.35	**1.05E-05**
Myristic acid (C14:0)	2.06E-01	-5854.76	**2.22E-03**	3.09E-01	-0.13	**5.14E-05**
5-Dodecenoic acid (C12:1)	2.57E-01	-2626.74	**3.36E-03**	3.22E-01	0.00	**2.97E-04**
Myristoleic acid (C14:1)				1.03E-01	-881.28	**1.86E-03**
Pentadecanoic acid (C15:0)	2.28E-01	-0.10	**3.86E-05**	2.90E-02	-0.14	**6.96E-03**
Eicosenoic acid (C20:1)				3.04E-02	-1035.60	3.69E-01
1,2-Methylpentanoic acid	8.01E-01	-0.45	**6.77E-03**			
Caprylic acid (C8:0)	9.22E-01	-1872.33	**2.94E-04**			
Heptadecanoic acid (C17:1)	9.33E-01	-0.87	2.84E-02			
Docosahexaenoic acid (C22:6)	4.36E-01	-3886.31	4.15E-02			
Clinical variables						
Insulin (µU/mL)	**4.62E-02**	0.38	**3.27E-03**	3.98E-01	-0.45	**5.11E-02**
Glucose (mM/L)	8.75E-01	0.35	**1.29E-02**	7.15E-01	0.16	**5.60E-02**
TGL (mM/L)	**3.96E-04**	0.72	**8.70E-03**	**6.90E-03**	-0.31	3.75E-01
Metabolites						
Norleucine				2.65E-01	-10.55	**5.45E-03**
3-methyl-2-oxovaleric acid	3.87E-01	-0.20	**7.73E-04**	3.67E-01	-0.03	2.87E-02
Pimelic acid				1.56E-01	-1.96	3.62E-02
Citric acid	8.62E-01	-7.47	2.96E-02	5.01E-01	-1.13	4.15E-02
Oxoadipic acid				1.88E-01	-0.31	2.03E-02
Bile acids (nmol/L)						
b-Chenodeoxycholic acid (bCDCA)	4.82E-01	-1.04	**1.84E-02**	3.64E-02	-1.66	**2.82E-05**
Cholic acid (CA)	1.09E-01	1.64	**1.60E-02**	7.31E-01	2.90	**2.91E-05**
Hyocholic acid (HCA)	5.66E-02	1.88	3.01E-02	5.52E-02	3.08	**7.62E-05**
b-dehydrochenodeoxycholic acid (bDHCDCA)				2.79E-02	-1.13	**1.41E-03**
Lithocholic acid-3-sulfate (LCA-3S)	2.40E-01	1.85	**8.88E-03**	6.05E-01	2.14	**1.87E-03**
7-Dihydrocholestanoic acid (7-DHCA)	3.73E-02	0.36	7.62E-02	4.18E-03	1.08	**2.42E-03**
Glycolithocholic acid-3-sulfate (GLCA-3S)	7.94E-01	2.57	**1.98E-03**	4.39E-01	2.99	**5.59E-03**
Chenodeoxycholic acid (CDCA)				3.68E-01	1.45	**6.01E-03**
Ursodeoxycholic acid (bUDCA)				1.52E-01	-0.99	**7.53E-03**
Nor deoxycholic acid (NorDCA)				9.09E-02	0.65	**1.78E-02**
Deoxycholic acid (DCA)	2.52E-02	0.35	3.14E-01	1.24E-01	410.43	**1.96E-02**
7-Ketolithocholic acid (7-ketoLCA)	6.37E-02	0.57	**1.22E-02**	1.26E-01	0.91	**1.97E-02**
b-Deoxycholic acid (bDCA)				8.47E-03	-0.27	**2.09E-02**
Ursodeoxycholic acid (UDCA)	3.93E-02	0.31	2.97E-01	4.41E-02	0.78	7.42E-02
Taurohyocholic acid (THCA)				4.99E-02	0.15	6.19E-01
Hyodeoxycholic acid (HDCA)				7.68E-03	0.21	8.17E-01
Taurodeoxycholic acid (TDCA)	4.75E-02	0.45	4.36E-01	3.32E-02	-0.02	9.34E-01
Tauorlithocholic acid (TLCA)	7.65E-01	1.02	**6.39E-04**			
Isoithocholic acid (IsoLCA)	4.63E-02	-1.01	**7.13E-03**			
7-Ketolithocholic acid (7-ketoLCA)	6.37E-02	0.57	**1.22E-02**			
Gycolithocholic acid (GLCA)	5.01E-02	1.35	**1.46E-02**			

AUC, Area Under the Curve. *Bolded for q<0.10. **Vegan vs. animal. Blank cells signify no significant gender or diet differences.

Supplementary Table S3. Metabolic signatures differ according to timing (Timepoint comparisons)

Marker	T0	T1	T2	T3	T4	T5	T6	T7
Amino acids (nmol/ml)								
Alanine	**1.00E+00**	9.80E-01	**1.53E-04**	3.60E-01	**1.17E-03**	**1.72E-03**	1.54E-01	**8.74E-09**
Arginine	**1.00E+00**	**9.33E-15**	**0.00E+00**	**0.00E+00**	**4.07E-07**	**3.40E-02**	5.32E-01	**2.08E-08**
Asparagine	**1.00E+00**	**6.90E-03**	**5.48E-10**	**7.11E-08**	1.24E-01	4.26E-01	**5.56E-03**	**2.69E-08**
BCAAs	**1.00E+00**	1.79E-01	**3.15E-07**	**5.10E-07**	**4.60E-07**	3.96E-01	7.96E-01	**4.81E-04**
Citrulline	**1.00E+00**	**6.90E-03**	**8.36E-06**	3.89E-01	3.13E-01	**2.06E-04**	3.83E-01	**2.29E-06**
Cystine	**1.00E+00**	2.06E-01	5.37E-02	2.99E-01	5.20E-01	**2.08E-03**	1.31E-01	1.02E-01
EAAs	**1.00E+00**	1.70E-01	**7.40E-08**	**1.49E-06**	**5.27E-04**	**5.50E-03**	5.83E-01	**4.44E-06**
Glutamine	**1.00E+00**	6.97E-01	5.37E-02	1.27E-01	**3.76E-02**	5.17E-01	1.35E-01	**1.41E-04**
Histidine	**1.00E+00**	9.22E-01	**6.14E-04**	**4.91E-02**	3.09E-01	**4.01E-07**	6.14E-01	**2.08E-08**
Isoleucine	**1.00E+00**	9.43E-01	**2.96E-04**	**4.06E-04**	**3.44E-03**	**3.90E-03**	7.99E-02	2.61E-01
Leucine	**1.00E+00**	9.80E-01	**8.71E-05**	**7.39E-04**	**2.90E-03**	**4.67E-03**	2.72E-01	**9.00E-02**
Lysine	**1.00E+00**	5.23E-02	**8.71E-05**	3.09E-01	7.18E-01	**1.09E-08**	**1.45E-03**	**2.59E-02**
Methionine	**1.00E+00**	**1.49E-02**	**1.18E-05**	**2.41E-03**	6.49E-01	**1.43E-12**	**2.30E-05**	7.36E-01
Ornithine	**1.00E+00**	**2.41E-10**	**9.56E-14**	**0.00E+00**	**0.00E+00**	5.17E-01	**1.45E-03**	**6.77E-03**
Phenylalanine	**1.00E+00**	1.70E-01	**7.40E-08**	**0.00E+00**	**7.26E-11**	1.10E-01	**7.05E-05**	**4.65E-10**
Proline	**1.00E+00**	**1.49E-02**	1.02E-01	9.67E-01	3.13E-01	**1.54E-03**	7.30E-01	**1.21E-08**
Serine	**1.00E+00**	4.28E-01	1.35E-01	2.24E-01	7.73E-01	**3.40E-02**	7.99E-02	**2.29E-06**
Threonine	**1.00E+00**	9.22E-01	**1.15E-04**	**2.32E-02**	2.16E-01	1.27E-01	3.27E-01	**2.47E-06**
Tryptophan	**1.00E+00**	**9.24E-03**	**1.77E-10**	**5.75E-08**	**5.27E-04**	**5.50E-03**	1.39E-01	**6.88E-07**
Tyrosine	**1.00E+00**	9.92E-01	**3.48E-03**	**2.31E-03**	**2.08E-02**	4.71E-01	8.18E-01	**1.54E-05**
Valine	**1.00E+00**	**5.54E-03**	**1.77E-10**	**2.66E-11**	**8.25E-13**	**3.43E-02**	1.54E-01	**2.52E-08**
Fatty acids (ng/ml)								
5-Dodecanoic acid (C12:1)	**1.00E+00**	6.31E-01	**1.68E-03**	**2.04E-08**	**1.03E-04**	**1.69E-02**	**3.05E-02**	**1.16E-02**
Capric acid (C10:0)	**1.00E+00**	**0.00E+00**	**0.00E+00**	**0.00E+00**	**0.00E+00**	**6.84E-14**	**6.47E-03**	**5.38E-06**
Caprylic acid (C8:0)	**1.00E+00**	**4.32E-05**	**1.50E-04**	**4.40E-06**	**1.03E-04**	**3.32E-02**	5.29E-01	4.52E-01
Dodecanoic acid (C12:0)	**1.00E+00**	4.50E-01	**4.75E-07**	**0.00E+00**	**6.89E-10**	**1.60E-02**	**2.09E-02**	**3.32E-03**
Myristic acid (C14:0)	**1.00E+00**	**1.19E-02**	**5.05E-08**	**0.00E+00**	**6.89E-10**	**9.42E-05**	**1.79E-02**	**2.64E-06**
Myristoleic acid (C14:1)	**1.00E+00**	6.79E-01	**4.39E-03**	**1.02E-03**	**2.33E-05**	**3.10E-04**	**6.47E-03**	**1.91E-02**
Palmitoleic acid (C16:1)	**1.00E+00**	**1.29E-02**	**1.68E-03**	**1.41E-04**	**4.31E-02**	6.59E-01	7.34E-01	**1.16E-02**
Pentadecanoic acid (C15:0)	**1.00E+00**	**5.72E-05**	**1.11E-03**	**1.03E-08**	**1.44E-03**	**3.35E-02**	2.25E-01	**1.98E-04**
Clinical variables								
Insulin (µU/mL)	**1.00E+00**	7.18E-01	**5.06E-02**	**3.52E-05**	3.82E-01	3.52E-01	8.20E-02	4.92E-01
Glucose (mM/L)	**1.00E+00**	1.78E-01	**1.83E-03**	**1.51E-07**	**4.39E-04**	**4.44E-04**	**2.73E-03**	**3.43E-02**
TGL (mM/L)	**1.00E+00**	**5.92E-07**	**1.01E-07**	4.91E-01	2.91E-01	9.57E-01	7.15E-01	**1.31E-02**
Metabolites								
Citric acid	**1.00E+00**	**4.77E-02**	1.40E-01	**7.46E-03**	**9.88E-05**	**2.91E-04**	9.94E-01	**1.16E-02**
Methylsuccinic acid	**1.00E+00**	6.31E-01	6.68E-01	**7.46E-03**	**5.02E-04**	1.63E-01	**3.05E-02**	9.01E-01
Succinic acid	**1.00E+00**	6.18E-01	9.91E-01	**9.71E-02**	**5.15E-05**	**3.35E-02**	**4.67E-02**	8.84E-01
Bile acids (nmol/L)								
12-ketoLCA_hawaii	**1.00E+00**	2.28E-01	4.01E-01	6.84E-01	**8.15E-03**	**4.70E-02**	**7.83E-06**	2.14E-01
3-Dihydrocholestanoic acid (3-DHCA)	**1.00E+00**	**1.30E-02**	**8.79E-02**	6.84E-01	7.85E-01	8.58E-01	**1.57E-04**	**1.98E-02**
7-Dihydrocholestanoic acid (7_DHCA)	**1.00E+00**	2.88E-01	3.91E-01	7.20E-01	7.85E-01	2.18E-01	**5.54E-06**	**2.98E-03**
7-Ketolithocholic acid (7-ketoLCA)	**1.00E+00**	**4.91E-02**	**2.71E-02**	9.11E-01	1.75E-01	2.46E-01	**6.06E-06**	**9.00E-09**
b-Chenodeoxycholic acid (bCDCA)	**1.00E+00**	7.16E-01	**9.99E-02**	**6.01E-07**	**1.93E-05**	**4.47E-10**	**1.13E-09**	**1.30E-05**
b-Deoxycholic acid (bDCA)	**1.00E+00**	9.02E-01	1.03E-01	**3.71E-04**	**7.60E-03**	**2.50E-05**	**6.02E-02**	4.56E-01
b-Dehydrochenodeoxycholic acid (bDHCDCA)	**1.00E+00**	**2.98E-02**	6.28E-01	**1.57E-02**	**1.93E-05**	**3.67E-09**	4.75E-01	**9.79E-02**
b-Ursodeoxycholic acid (bUDCA)	**1.00E+00**	8.13E-01	5.73E-01	1.31E-01	1.31E-01	**3.85E-03**	**2.55E-04**	**3.53E-05**
Cholic acid (CA)	**1.00E+00**	5.72E-02	**6.77E-02**	**7.22E-02**	4.41E-01	**2.50E-05**	**0.00E+00**	**1.46E-07**
Chenodeoxycholic acid (CDCA)	**1.00E+00**	**4.91E-02**	1.33E-01	1.18E-01	**4.07E-02**	4.11E-01	**2.94E-06**	**2.14E-06**
Chenodeoxycholic acid-3-glycine (CDCA-3G)	**1.00E+00**	8.63E-01	5.38E-01	1.38E-01	**2.33E-02**	**6.50E-03**	6.41E-01	2.20E-01
Deoxycholic acid (DCA)	**1.00E+00**	**4.91E-02**	3.81E-01	1.31E-01	6.34E-02	9.23E-01	**2.98E-06**	**2.93E-06**
Dehydrolithocholic acid (dehydroLCA)	**1.00E+00**	**4.23E-05**	**3.95E-02**	7.35E-01	8.38E-01	9.23E-01	9.59E-01	8.40E-01
GCA_hawaii	**1.00E+00**	**5.95E-09**	**2.61E-02**	2.88E-01	5.06E-01	4.24E-01	**2.25E-02**	1.13E-01
GHCA_hawaii	**1.00E+00**	**8.42E-05**	1.33E-01	1.31E-01	2.79E-01	**6.47E-03**	9.29E-01	7.85E-01
Glycolithocholic acid-3-sulfate (GLCA-3S)	**1.00E+00**	1.74E-01	**8.34E-04**	**8.71E-04**	**1.93E-05**	**1.56E-06**	6.64E-01	2.38E-01
Hyocholic acid (HCA)	**1.00E+00**	4.01E-01	1.02E-01	3.67E-01	8.38E-01	1.45E-01	**6.93E-11**	**3.48E-09**
Hyodeoxycholic acid (HDCA)	**1.00E+00**	1.63E-01	5.73E-01	3.85E-01	1.31E-01	3.16E-01	**6.22E-02**	**5.45E-02**
Isoithocholic acid (IsoLCA)	**1.00E+00**	6.66E-01	**2.61E-02**	**5.42E-05**	**1.22E-02**	1.04E-01	4.37E-01	9.34E-01
Lithocholic acid (LCA)	**1.00E+00**	**4.91E-02**	3.94E-01	7.35E-01	**3.36E-02**	**9.44E-02**	9.59E-01	8.71E-01
Lithocholic acid-3-sulfate (LCA-3S)	**1.00E+00**	**4.91E-02**	**8.97E-03**	2.76E-01	**2.33E-02**	**3.02E-02**	**6.73E-06**	**4.06E-06**
Taurocholic acid (TCA)	**1.00E+00**	**3.20E-09**	**2.61E-02**	6.46E-01	8.27E-01	9.23E-01	**1.24E-02**	1.42E-01
Taurochenodeoxycholic acid (TCDCA)	**1.00E+00**	**4.37E-07**	**2.61E-02**	3.79E-01	8.38E-01	8.58E-01	**2.25E-02**	1.91E-01
Taurodeoxycholic acid (TDCA)	**1.00E+00**	**1.48E-08**	**8.97E-03**	1.25E-01	1.59E-01	9.23E-01	**7.87E-03**	**5.61E-02**
Taurohyocholic acid (THCA)	**1.00E+00**	**3.81E-04**	**8.79E-02**	5.29E-01	9.78E-01	1.45E-01	2.57E-01	7.66E-01
Tauorlithocholic acid (TLCA)	**1.00E+00**	**1.98E-04**	**1.02E-02**	3.67E-01	8.38E-01	9.23E-01	3.14E-01	8.99E-01
Ursodeoxycholic acid (UDCA)	**1.00E+00**	**5.17E-02**	1.68E-01	3.67E-01	1.31E-01	6.73E-01	**6.19E-05**	**1.54E-05**

Comparison is vegan verses animal. Anova analysis. False discovery rate adjusted values shown. Bolded for q<0.10.

Supplementary Table S4. Diet intake analysis by gender for breakfast, lunch and dinner timeperiods

	Mean Intake						Adjusted *p* value Female vs. Male*		
	Breakfast		Lunch		Dinner		Breakfast	Lunch	Dinner
Diet variable	Male	Female	Male	Female	Male	Female			
Alanine (mg)	2582.71	1575.18	2580.63	1919.80	5151.85	3268.98	**4.43E-03**	**4.35E-03**	**3.32E-03**
Arginine (mg)	3857.60	2378.41	3191.13	2391.41	6727.73	4107.84	**1.32E-02**	**2.18E-02**	**3.32E-03**
Total carbohydrate(g)	203.68	132.16	182.53	130.25	200.15	135.41	**1.07E-03**	**7.58E-03**	**4.43E-03**
Cysteine (mg)	846.90	525.68	736.75	572.82	1542.50	950.93	**5.86E-03**	**3.27E-03**	**3.32E-03**
Total fat (g)	82.00	48.05	48.88	39.16	36.88	24.50	**3.32E-03**	5.90E-01	**2.78E-02**
Glycine (mg)	2076.56	1218.81	2525.81	1905.16	4732.78	3017.12	**5.86E-03**	**4.35E-03**	**3.32E-03**
Histidine (mg)	1482.20	864.82	1526.78	1197.25	3028.40	1912.34	**4.41E-03**	**1.30E-02**	**3.32E-03**
Isoleucine (mg)	2722.85	1576.41	2308.23	1800.73	5234.50	3312.39	**4.43E-03**	**2.18E-02**	**3.32E-03**
Kilocalories (Kcal)	1874.80	1147.55	1421.20	1057.09	1608.60	1048.73	**1.53E-04**	**2.18E-02**	**4.43E-03**
Leucine (mg)	4834.95	2867.73	3561.60	2670.91	7986.05	4880.70	**3.34E-03**	**7.58E-03**	**3.32E-03**
Lysine (mg)	3314.45	1829.75	3102.70	2323.14	7112.85	4556.45	**3.34E-03**	**7.58E-03**	**3.32E-03**
Methionine (mg)	1141.75	677.09	911.45	682.68	2274.93	1440.02	**3.32E-03**	**4.35E-03**	**3.32E-03**
Monounsaturated fat (g)	27.04	17.35	19.17	13.47	10.44	7.53	**7.55E-03**	**3.23E-03**	1.79E-01
Phenylalanine (mg)	2787.85	1631.93	2437.10	1804.23	4839.40	3028.70	**3.34E-03**	**9.94E-03**	**3.32E-03**
Proline (mg)	4028.80	2450.20	18090.64	14163.50	6371.41	3926.45	**3.34E-03**	**7.58E-03**	**3.32E-03**
Protein (mg)	66.76	39.92	57.37	43.60	106.97	64.88	**4.43E-03**	**1.30E-02**	**3.34E-03**
Polyunsaturated fat (g)	18.97	9.91	19.17	15.57	16.16	9.14	**4.41E-03**	1.00E+00	**1.71E-02**
Serine (mg)	3071.08	1793.46	2703.41	2039.51	3718.66	2300.33	**3.34E-03**	**1.30E-02**	**3.32E-03**
Saturated fat (g)	32.89	18.07	11.23	8.85	7.40	5.02	**3.32E-03**	**1.30E-02**	**3.61E-02**
Total fiber (g)	24.95	15.10	25.11	18.26	19.42	10.68	**7.58E-03**	**3.59E-02**	**5.86E-03**
Threonine (mg)	2320.15	1347.98	2038.25	1533.32	4377.88	2734.48	**4.43E-03**	**1.69E-02**	**3.32E-03**
Tryptophan (mg)	590.75	342.48	664.35	523.25	1311.38	867.30	**3.32E-03**	**2.18E-02**	**3.32E-03**
Tyrosine (mg)	2164.45	1252.98	1687.68	1277.41	3428.85	2149.18	**3.34E-03**	**7.58E-03**	**3.32E-03**
Valine (mg)	3169.80	1895.34	2624.40	1962.20	5428.55	3387.98	**3.34E-03**	**4.35E-03**	**3.32E-03**

Mean diet intakes analyzed by gender for breakfast, lunch and dinner. *Adjusted p values bolded for q<0.10.

Chapter 4

Sexual dimorphism, age and fat mass are key phenotypic drivers of proteomic signatures

Based on

Colleen Fogarty Draper, Aoife M Curran, Marie-Pier Scott-Boyer, Armand Valsesia, Helen M Roche, Miriam F Ryan, Michael J Gibney, Martina Kutmon, Chris T Evelo, Susan L Coort, Arne Astrup, Wim H Saris, Lorraine Brennan, Jim Kaput

Sexual dimorphism, age and fat mass are key phenotypic drivers of proteomic signatures

doi: 10.1021/acs.jproteome.7b00501

ABSTRACT

Validated protein biomarkers are needed for assessing health trajectories, predicting and sub-classifying disease, and optimizing diagnostic and therapeutic clinical decision making. The sensitivity, specificity, accuracy, and precision of single or combinations of protein biomarkers may be altered by differences in physiological states limiting the ability to translate research results to clinically useful diagnostic tests. Aptamer based affinity assays were used to test whether low abundant serum proteins differed based on age, sex and fat mass in a healthy population of 94 males and 102 females from the MECHE cohort. The findings were replicated in 217 healthy male and 377 healthy female participants in the DiOGenes consortium. Of the 1129 proteins in the panel, 141, 51 and 112 proteins (adjusted $p<0.1$) were identified in the MECHE cohort and significantly replicated in DiOGenes for sexual dimorphism, age, and fat mass, respectively. Pathway analysis classified a subset of proteins from the 3 phenotypes to the complement and coagulation cascades pathways and to immune and coagulation processes. These results demonstrated that specific proteins were statistically associated with dichotomous (male v female) and continuous phenotypes (age, fat mass) which may influence the identification and use of biomarkers of clinical utility for health diagnosis and therapeutic strategies.

INTRODUCTION

The concentrations of proteins in the blood vary dynamically in the healthy state but may also change during the trajectory toward the onset of disease. Robust technologies that accurately measure protein levels have an increasingly important role in investigating and advancing health research and clinical practice [1, 2]. The full promise of protein diagnostics has yet to be realized in the clinical setting. The majority of protein diagnostic tests are based on single proteins for acute conditions (e.g., myocardial infarction) or cancers. Protein signature tests consisting of multiple markers may be needed to achieve an optimal level of sensitivity and specificity for assessing complex health and disease processes [3].

Variations in phenotype during aging, physiology (e.g., obesity or other physical conditions), or by sex dimorphism may independently affect protein levels making it difficult to optimize the utility of clinical diagnostics, especially in genetically and culturally diverse individuals. Many of the well-accepted risk factors for cardio-metabolic disease risk have defined phenotypic cut-offs. For example, HDL cholesterol levels less than 40 mg/dL (1.0 mmol/L) are used to assess increased risk of heart disease for men but that cut-off is 50 mg/dL (1.3 mmol/L) for women. Sex differences and other risk factors such as LDL cholesterol levels may independently increase risk of heart disease or other chronic medical conditions [4]. Previous work in the field of proteomics has identified 40 low-abundant proteins which differed in serum between 12 males and 12 females [5] and more than 60 plasma proteins differed by over 2 standard deviations in 29 and 30 overweight and obese women and men, respectively [6]. Age, body mass index (BMI), body fat mass, and other physiological parameters may also influence the serum proteome and therefore utility and veracity of diagnostic markers. Serum proteomic and metabolomic approaches were combined to identify circulating proteins and metabolites that differed between 5 healthy lean and 5 healthy obese men [7]. That study, albeit small, established a link between the complement system and obesity and both novel and previously reported markers of alterations in body fat mass were identified. Considering age, physiological (such as, body fat mass), dietary, and other environmental variations, additional research into sex dimorphic plasma and serum protein modulations will be needed before sex specific medical and nutritional recommendations are implemented.

One of the main challenges for analyzing the blood proteome is the large dynamic range in protein concentrations [8]. New technologies have been developed and successfully implemented to overcome this challenge [9]. Chemically modified single-stranded DNA aptamers (SOMAmers) have high specificity as affinity capture reagents for use with

undiluted and diluted plasma and serum samples to quantify low and high abundant proteins. SOMAmers are used in multiplex assays similar to DNA microarrays allowing for the simultaneous analysis of over 1000 proteins in small amounts (~65 ul) of serum. Improvements in mass spectroscopy pipelines and analysis[10] are also being made in blood proteomic analysis although these approaches require expensive equipment and expertise in the technologies.

The primary aim of the research reported here was to identify the impact of sex, age and body fat mass on the proteomic signature and replicate the findings in an independent cohort. Furthermore, the identified proteins were mapped using pathway analysis methods to provide context and a greater understanding of the biological processes that differ by phenotype. The results of this study provide a foundational understanding of the effect of these 3 phenotypic variables on protein markers.

METHODS

Study population

The research described here extended the Metabolic Challenge (MECHE) study which is part of a national research program by the Joint Irish Nutrigenomics Organisation[11]. Briefly, the MECHE study enrolled 214 participants aged 18-60 y who underwent an oral glucose tolerance test (OGTT) and/or oral lipid tolerance test (OLTT). Clinical measures, body composition, and dietary habits were assessed in the fasted state (baseline) and at multiple time points following each challenge[11]. Demographic parameters obtained at baseline were used for analysis. Height was obtained using a wall mounted Harpenden stadiometer (Holtain Limited, UK) and weight was measured using a calibrated beam balance platform scale (SECA 888, Germany). Total fat mass was determined using DXA scanning (Lunar iDXA, GE Healthcare, UK). Individuals were informed about the purpose of the study and the experimental procedures, prior to giving written consent. Good health was defined as the absence of any known chronic or infectious disease and this was verified by a number of fasting blood tests. Individuals with a BMI below 18.5 kg/m2, a low blood haemoglobin concentration (<12 g/dL), an elevated fasting plasma glucose (≥7 mM), an elevated cholesterol concentration (>7.5 mM), an elevated triglyceride concentration (>3.8 mM) and elevated enzyme indicators of liver or kidney function, any of which warranted pharmaceutical intervention, were excluded. Details of the study have been published elsewhere[11-13]. The study was registered at clinicaltrials.gov under NCT01172951. Ethical approval was obtained from the Research Ethics Committee at University College Dublin (LS-08-43-Gibney-Ryan) and the study was performed according to the Declaration of Helsinki. For the present

study, participants from the MECHE study who had proteomic data were included (n=200) (**Table 1**).

Table 1. Clinical characteristics of study participants

	MECHE (n=196)	DiOGenes (n=594)
Sex (m/f)	94/102	217/377
Age(y)	31 ± 10*	42 ± 6 **
Weight (kg)	74.42 ± 15.99	99.87 ± 17.8
BMI (kg/m^2)	24.7 ± 4.8	34.2 ± 4.8
WHR	0.84 ± 0.1	-
BP SYS (mm/Hg)	122.93 ± 12.37	-
BP DIA (mm/Hg)	73.69 ± 10.52	-
Glucose (mmol/L)	5.21 ± 0.58	-
HDL-c (mmol/L)	1.35 ± 0.35	1.2 ± 0.33
TAG (mmol/L)	1.04 ± 0.62	1.37 ± 0.65
Insulin (µIU/mL)	8.71 ± 6.93	-
HOMA-IR	1.87 ± 1.51	3.16 ± 2.56
Body fat %	25.76 ± 10.92	-
DEXA fat mass (kg)	28.28 ± 9.72	39.72 ± 11.1

Data are presented as means ± standard deviation (SD); BMI: body mass index; WHR: waist to hip ratio; HDL-c: high-density lipoprotein cholesterol; TAG: triacylglycerol; HOMA: homeostasis model assessment.*18-60 y **23-58 y.

Proteomics analysis

1,129 proteins were quantified in fasting (at least 12 hours) serum samples of 200 MECHE participants using the proteomic platform SOMAmer™ (Slow Off-rate Modified Aptamer) as previously described [9]. Dataset is available upon request. This technology has a dynamic range of more than 8 logs, allowing quantification of both low and high abundant proteins which might otherwise be missed. Pre-processing of the proteomic data included log transformation of the abundance of each protein. Principal component analysis (PCA) did not reveal any significant batch effect across the proteins analyzed. PCA identified four individuals as outliers whose data were removed. Therefore, the final proteomic dataset included 196 individuals and 1,129 proteins. Proteins measured by SOMAmers are found in the blood as secreted (431), external membrane origin (275), and intracellular proteins (423). Proteins are often shed from membranes by proteolytical cleavage and intracellular proteins may be released from cells as a part of normal or abnormal physiological cell turnover.

Replication cohort

Participants were recruited from 8 cities in 8 European counties that were healthy, overweight/obese with a BMI between 27 and 45 kg/m2 and aged <65 y. Informed consent was obtained from all participants and the study was approved by the local

Medical Ethical Committees in the respective research centers, in accordance with the Helsinki Declaration [14]. The DiOGenes intervention study was registered on Clingov (NCT00390637). The DiOGenes cohort were selected as a suitable replication cohort for this analysis due to the availability of SOMAlogic data and a large sample size. SOMAlogic proteomic data were analyzed in serum of 594 participants: 377 females and 217 males of the DiOGenes consortium, age 16-63 y, mean BMI 34 ± 4.8 kg/m2 (0 individuals with BMI<25, 122 individuals with BMI 25-30, and 472 individuals with BMI>30 [15].

Statistical methodology

Analysis for study population characteristics was carried out using IBM SPSS Statistics 20. Data are expressed as means ± standard deviation. Multivariate statistical analysis was performed using Simca-P+ software (version 14.0; Umetrics, Umea, Sweden). Prior to data analyses the MECHE dataset was scaled using UV scaling. PCA and PLS-DA was carried out to explore differences in protein levels between males and females. Orthogonal partial last-squares discriminant analysis (OPLS-DA) was performed which improves interpretation and separation between classes on a scores plot by filtering unwanted variation. A S-plot was generated from which potential proteins of interest were extracted. A value for p (corr) > 0.15 was used to select proteins that differed significantly between males and females to enhance identification of pathways.

Robust regression (R package limma) [16] was used to identify proteins that were significantly associated with either age or total body fat measured by DEXA (fat mass in kg). Robust regression was chosen over linear regression since it is less sensitive to outliers. Proteins levels were first transformed to the residuals from a linear regression on sex to correct for this covariate. The threshold of significance of Benjamini Hochberg (BH) adjusted p-values was 0.1. Baseline serum samples from DiOGenes were analyzed to test replication of the effect of sex, age and fat mass on protein levels. Robust regressions corrected for the collection center and sex, when analyzing age and fat mass.

Pathway annotation

Pathway over-representation analysis was performed with the human pathway collection from WikiPathways (curated collection with 276 pathways downloaded on 26 January 2016) using PathVisio (version 3.2.4) [17]. Permuted p-value is calculated by performing a permutation test. The data is permuted 1000 times and a rank is calculated of the actual Z score compared to the permuted Z Scores. The Z scores are calculated based on the changed proteins in a pathway out of the total proteins in the pathway that have been measured in the uploaded dataset. Pathways with a Z-score of >1.96 and a permuted p-value < 0.05 are considered important. Functional pathway enrichment

analyses were also performed with KEGG and Reactome databases with the R packages HTSAnalyzer [18] and ReactomePA, [19] respectively. The analyses were conducted with all ENTREZ proteins/genes as background and with an adjusted p-value threshold of 0.05. Pathway analyses were conducted only with proteins found to vary significantly in the same direction in both cohorts.

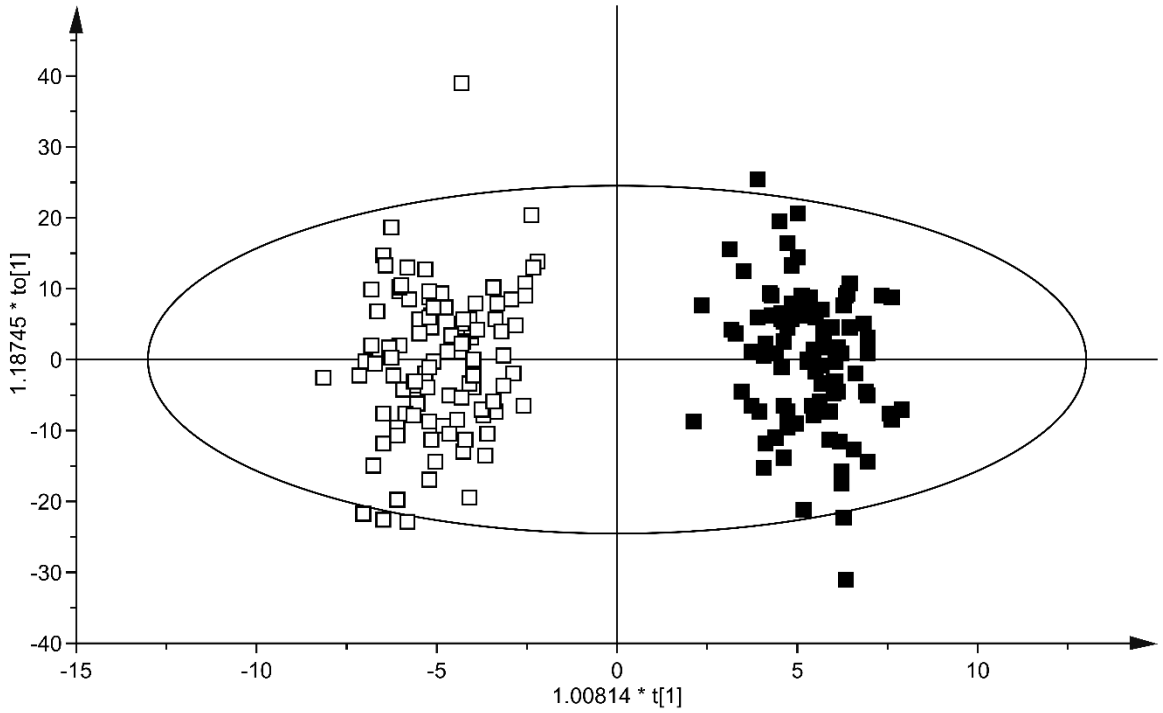


Figure 1. OPLS-DA of males vs females derived from proteomic data of MECHE participants (n = 196). (■) Males, (□) Females, R2Y = 0.945; Q2 =0.765.

RESULTS/DISCUSSION

Sexual Dimorphism

The 317 differentially expressed proteins between male and female in the MECHE cohort account for 28% of the total proteins analyzed (**Table 2** and **3, Figure 1**). From there 141 proteins were replicated in the DiOGenes cohort (**Table S5a and S5b**). The top 10 most statistically significant over-expressed proteins for each sex were compared for known physiological functions and associations with sex hormones, metabolic disease, diet, and previously characterized sex dimorphism (**Table S1** and **S2**).

The 8 most significant secreted proteins elevated in females have known associations with sex hormone metabolism (SHBG, leptin, thyroxin-binding globulin, adiponectin, angiotensinogen, fetuin B, immunoglobulin M, trefoil factor 3) (**Table 3**) [20-25]. Each of these proteins is involved in at least one diet related metabolic disease (except

immunoglobulin M) (**Table S1**). These proteins were affected by or affect glucose and insulin metabolism (SHBG, leptin, adiponectin, fetuin B, trefoil factor 3), metabolic rate (thyroxin binding globulin), and dietary carbohydrate intake (SHBG, leptin, adiponectin), and salt sensitive hypertension (angiotensinogen) [21, 22, 26-30]. Increased immunoglobulin M expression has been associated with gluten and dietary protein intakes [31, 32]. Recent studies suggest SHBG, adiponectin, angiotensinogen, and fetuin B may be involved in diabetes development [28, 33-35]. The cell-membrane located immune proteins, LAMP, and collectin placenta 1 were upregulated in females. These proteins stimulate neural growth and provide host defense with no previously described difference by sex or metabolic disease associations [36, 37]. Of note, collectin placenta 1 is the only protein within this group that was not replicated in the DiOGenes data.

Nine of the top 10 most significant proteins more abundant in males are secreted (exception is myoglobin located in the exosome - **Table 2**). Of these 10, several are associated with sex hormone metabolism (myoglobin, matrix metalloproteinase 3, serum amyloid P, tissue factor pathway inhibitor, protein S, interleukin 1 receptor like 1, LEAP) [38-46]. These top 10 proteins function in connective tissue development and growth, amyloid deposit development, blood coagulation, inflammation modulation, anti-microbial immunity and iron metabolism, as well as, immune cell migration and adhesiveness. These proteins are involved in processes contributing to metabolic diseases, specifically myocardial infarction (myoglobin), cardiovascular disease (matrix metalloproteinase), atherosclerosis (serum amyloid P, tissue factor pathway inhibitor, protein S), diabetes (ficolin-3), and iron overload (LEAP-1) [47-52]. Certain nutrients alter the abundances of some of these proteins: iron (myoglobin, LEAP), lipids (myoglobin), monounsaturated fatty acids, and n-3 PUFA (tissue factor pathway inhibitor), vitamin K (protein S), and vitamin A (LEAP) although the effect of diet was not analyzed in the MECHE cohort. The liver expressed chemokine (HCC-4) may be induced by total fat and calorie intake [53-60]. All 10 significant proteins found in MECHE were replicated in the same direction with robust regression in the DiOGenes data (**Table S5a**).

Interpreting the role of a protein by its activity, association to a disease process, or association to an individual provides information on physiological states. However, our results suggest a more holistic difference between males and females since the coagulation pathway cross-talks with and cross-regulates the immune system to maintain homeostasis [61]. Serpin Family D Member 1, a1-antitrypsin and plasminogen mapped to the complement and coagulation cascades pathway (**Figure 3**) in females.

Table 2: Proteomics results depict sexual dimorphism – proteins higher in males

Full Protein Name	UniProt ID	Protein	OPLS-DA	p (corr)	Cellular
Myoglobin	P02144	MB	0.118	0.613	Exosome
Matrix metalloproteinase 3	P08254	MMP3	0.110	0.574	Secreted
Bone morphogenetic protein-1	P13497	BMP-1	0.105	0.549	Secreted
Serum amyloid P	P02743	SAP	0.100	0.522	Secreted
Tissue factor pathway inhibitor	P10646	TFPI	0.099	0.516	Secreted
Protein S	P07225	Protein S	0.096	0.501	Secreted
Ficolin-3	O75636	Ficolin-3	0.094	0.491	Secreted
Interleukin-1 receptor-like 1	Q01638	IL-1 R4	0.093	0.483	Secreted
LEAP-1 (Hepcidin)	P81172	LEAP-1	0.090	0.470	Secreted
Liver-expressed chemokine HCC-4	O15467	HCC-4	0.088	0.459	Secreted

[a] Data presented as first 10 significant proteins out of 173 total in males using OPLS-DA with a 0.15 cut-off. [c] describes the direction of the difference in males vs. females.

Table 3: Proteomics results depict sexual dimorphism – proteins higher in females

Full Name	Uniprot ID	Protein	OPLS-DA	p (corr)	Cellular
Sex hormone-binding	P04278	SHBG	-0.102	-0.531	Secreted
Leptin (OB)	P41159	Leptin	-0.098	-0.513	Secreted
Thyroxine-binding	P05543	Thyroxine Binding	-0.097	-0.507	Secreted
Adiponectin	Q15848	Adiponectin	-0.089	-0.466	Secreted
Angiotensinogen	P01019	Angiotensinogen	-0.079	-0.414	Secreted
Fetuin B	Q9UGM5	FETUB	-0.073	-0.381	Secreted
Immunoglobulin M	P01871	IgM	-0.072	-0.378	Secreted
Trefoil factor 3 - Intestinal trefoil	Q07654	TFF3	-0.071	-0.369	Secreted
Limbic system-associated	Q13449	LSAMP	-0.069	-0.362	Membrane
Collectin Placenta 1[b]	Q5KU26	COLEC12	-0.067	-0.351	Membrane

[a] Data presented as first 10 significant proteins out of 144 total in females using OPLS-DA with a 0.15 cut-off. [b] Only protein not replicated in DiOGenes data set. [c] Describes the direction of the difference in males vs. females.

The same pathway was identified in males but through different proteins (tissue factor pathway inhibitor, thrombin activatable fibrinolysis inhibitor, plasminogen activator, serpin family A member 5, serpin family C member 1 and Protein S). Tissue factor pathway inhibitor is present in free and lipoprotein-associated forms [62] while protein S is more frequently (60% of total) bound to C4B which abolishes its anticoagulant properties [63]. Bound and free Protein S were more abundant in males compared to females [64]. This protein also is involved in phagocytosis of apoptotic cells [65]. Others studies identified Serpin Family D Member 1, Serpin Family C Member 1, Serpin Family A Member 1 and protein S among 27 significant proteins that differed in the complement and coagulation cascades between males and females [6]. Toll- like receptors, immune- and adipo-cytokine proteins were more abundant in females.

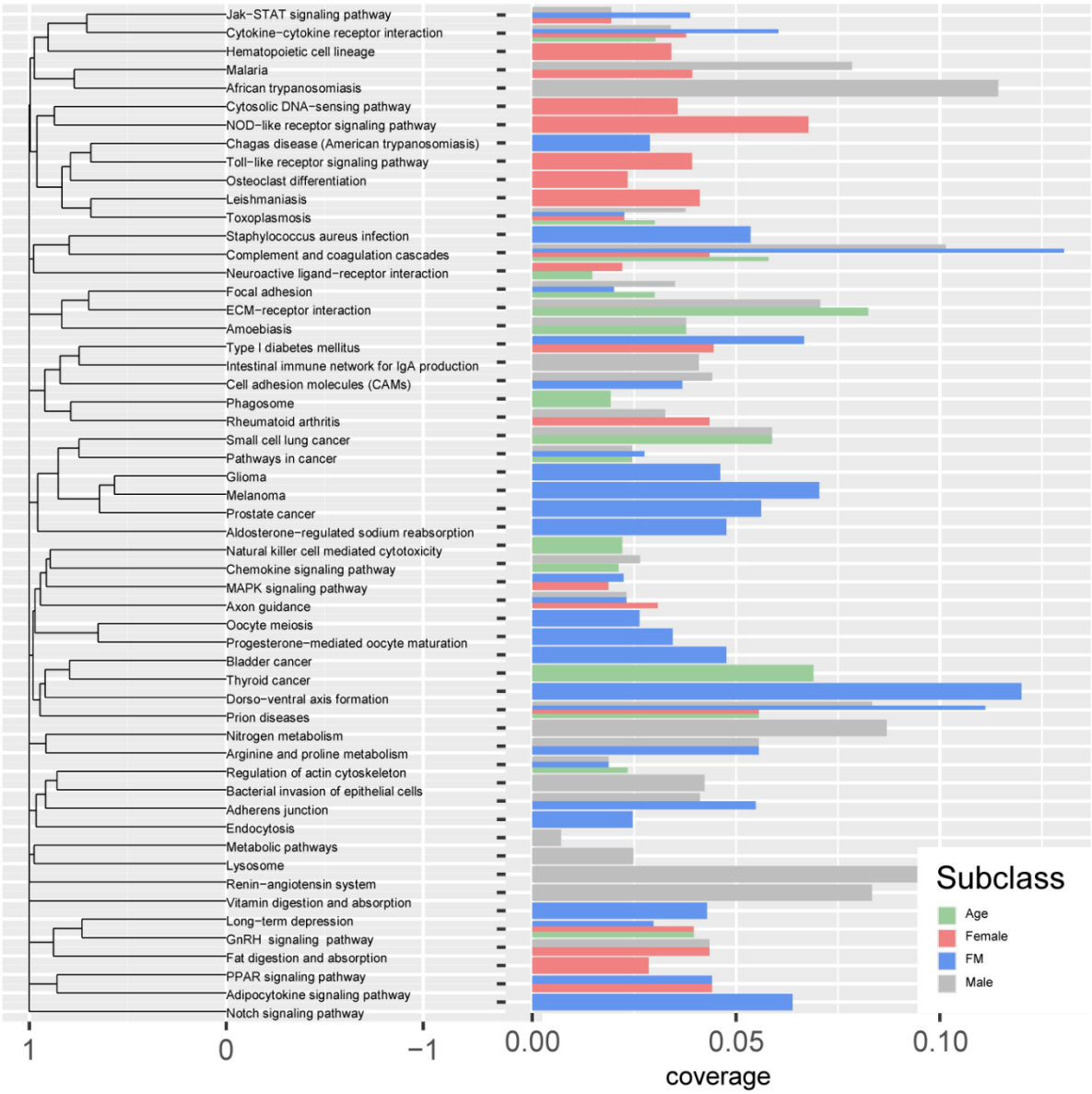


Figure 2. Overview of KEGG pathway enrichment. Bar graph displaying KEGG pathway enrichments by classes (proteins significant for male,female, age and fat mass). The size of the bar graph represent the coverage of the pathway (number significant proteins in the pathway/number of total proteins in the pathway). The dendrogram groups similar pathways (pathways that include similar genes).

These proteins mapped to pathways (**Figures 2, Figure S1, Table 6, Tables S6-S8**) previously identified as belonging to the inflammasome [1], a system of interacting networks regulating acute and chronic inflammatory conditions.

The individual proteins mapped to these pathways (e.g., members of the interleukin family) have been linked with diseases associated with chronic inflammation in (for example) obesity and T2DM [2], the pathogenesis for which differs between men and women [3, 4]. This connected complement-immune system may result from and

contribute to metabolic differences of sex dimorphism. How these related systems are regulated will require more comprehensive analysis of components of these pathways in future studies.

Age

Regression analysis revealed 167 proteins (15% of 1129 proteins) significantly associated (adjusted p-value <0.1) with age (range: 20-60 y). Fifty-one of these protein - age associations were replicated in the DiOGenes data (**Table S5c**). Coefficients of the regression for age can also be found in **Table S5c**. The top 10 proteins associated with age were IL1RL2, FSHB, ADMTS5, CHIT1 (all positively correlated with age) and AGRP, OMD, RET, CDON, IGFBP3 and IGFBP5 (negatively correlated with age) (see **Table 4**), however OMD was not significantly associated in DiOGenes. The levels of the majority of these proteins, with the exception of IL1RL2 and CDON, were previously associated with age [5-11]. The identified proteins are involved in diseases and sub-optimal states of health in relation to aging including (i) inflammation (IL1RL2, CHIT1) [10-12], (ii) arthritis (ADAMTS5, IGFBP3) [13, 14], (iii) vertebral fractures (IGFBP3) [15] (iv) bone metabolism (IGFBP5,AGRP,OMD) [16-18], (v) weight homeostasis (AGRP,CDON) [19, 20], (vi) lean body mass (IGFBP3) [21], (vii) cancer development (RET,CDON) [22, 23] and prevention (IGFBP3, IGFBP5) [24, 25], and (viii) muscle metabolism (IGFBP3, IGFBP5, CDON) [26, 27]. Levels of FSHB and AGRP were positively influenced by caloric restriction and a high-fat diet, while IGFBP3 was impacted by supplementing the diet with n-3 PUFA [28-30].

Table 4: Proteomics results significantly associated with age[a]

Full Protein Name	UniProt ID	Protein	P.Value	adj.P.Val	Cellular
Interleukin 1 receptor like 2	Q9HB29	IL1RL2	1.85E-13	2.09E-10	Membrane
alpha polypeptide - follicle	P01215	CGA	1.12E-11	6.34E-09	Secreted
Metallopeptidase with	Q9UNA0	ADAMTS5	1.41E-10	5.29E-08	Secreted
Chitinase 1	Q13231	CHIT1	2.08E-10	5.88E-08	Secreted
Agouti related neuropeptide	O00253	AGRP	4.51E-10	1.02E-07	Secreted
Osteomodulin[b]	Q99983	OMD	6.89E-10	1.30E-07	Secreted
Ret proto-oncogene 2	P07949	RET	2.85E-09	4.60E-07	Membrane
Cell adhesion associated,	Q4KMG0	CDON	1.32E-07	1.86E-05	Membrane
Insulin like growth factor	P17936	IGFBP3	1.83E-07	2.29E-05	Secreted
Insulin like growth factor	P24593	IGFBP5	2.04E-07	2.30E-05	Secreted

[a] Data presented as top 10 significant proteins out of 166 total using robust regression with correlations calculated using residuals following correction for sex. [b] Only protein not replicated in DiOGenes data set.

Annotation of the MECHE proteins statistically significant in the DiOGenes cohort identified a number of associated pathways (**Figure 2, Figure S1, Table 6, Tables S6-**

S8). Four proteins associated with aging emerged in the complement and coagulation cascade pathway, of which SERPING1 (c1 esterase inhibitor) was specific to aging. Increases in C1 esterase inhibitor are observed during inflammation [31]. All of these proteins with the exception of CCL21 were positively associated with increased age. The chemokine pattern found in this study (CCL21 lower and other CCL's higher) was consistent with other studies showing increased expression of CCL27 in senescent cells [32], levels of CCL11 and CCL7 in aged animals or humans [33], and decreased levels of CCL21 [34]. Further pathways of interest were identified through KEGG and Reactome (**Tables S6-7**). Collectively, these proteins and the pathways in which they act are processes consistent with inflammation, the interconnected processes that result from lifelong insults to the immune system resulting in chronic low-grade inflammation and immunosenescence [35].

Fat Mass

Regression analysis identified 21% of SOMAscan proteins significantly associated (adjusted p-value <0.1) with body fat mass (range: 8-58.2kg). Of these 232 proteins, 112 were replicated at an adjusted p-value <0.1 in the DiOGenes cohort, with coefficients of regression displayed in **Table S5d**. The top 10 proteins associated with body fat mass are LEP, PLAT and C1S (all positively correlated with fat mass) and IGFBP1, TFF3, SHBG, MMP2, WFIKKN2, HFE2 and TF (negatively correlated with fat mass) (**Table 5**). All 10 proteins were replicated in the DiOGenes cohort. The physiologic functions of these top proteins include inflammation, glucose metabolism, defense response, blood coagulation, regulation of cell growth, along with angiogenesis and iron homeostasis (**Table S4**). Leptin was strongly associated with fat mass [36, 37] and elevated in females, consistent with its known role in regulation of body weight and energy balance [38, 39].

Table 5: Proteomics results significantly associated with fat mass[a]

Full Protein Name	UniProt	Protein	P.Value	adj.P.Val	Cellular
Leptin	P41159	Leptin	1.57E-18	1.78E-15	Secreted
Tissue-type plasminogen	P00750	PLAT	2.92E-13	1.32E-10	Secreted
Insulin-like growth factor-binding	P08833	IGFBP1	3.51E-13	1.32E-10	Secreted
Trefoil factor 3	Q07654	TFF3	2.09E-12	5.90E-10	Secreted
Sex hormone-binding globulin	P04278	SHBG	7.32E-11	1.65E-8	Secreted
Matrix metalloproteinase 2	P08253	MMP2	3.56E-10	6.70E-8	Secreted
WAP, kazal, immunoglobulin,	Q8TEU8	WFIKKN2	4.80E-10	7.74E-8	Secreted
Complement C1s	P09871	C1S	9.65E-9	1.36E-6	Exosome
Hemojuvelin	Q6ZVN8	HFE2	2.16E-8	2.71E-6	Membrane
Transferrin	P02787	TF	2.44E-8	2.75E-6	Secreted

[a] Data presented as top 10 significant proteins out of 233 total using robust regression with correlations calculated using residuals following correction for sex. All proteins replicated with DiOGenes data set.

Increased BMI and fat mass are known risk factors for diseases such as metabolic syndrome and cardiovascular disease (CVD). Three proteins associated with fat mass in the MECHE/DiOGenes cohorts (tPA, IGFBP-1, and TFF3) have been associated with metabolic conditions. High levels of tPA antigen independently predicted cardiovascular events both in a healthy population and in individuals with prevalent coronary disease [40]. Elevated plasma tPA antigens were associated with insulin resistance, T2D, and obesity. Decreased abundance of plasma tPA (approximately 29%) was observed following a 12 week energy restricted diet in overweight women with metabolic syndrome [41]. Insulin like growth factor binding protein 1 (IGFBP-1) is negatively associated with fat mass in the MECHE/DiOGenes cohorts. Lower levels of IGFBP-1 at baseline was associated with the combination of increased percentage body fat and plasma insulin levels [42]. Trefoil factor 3 (TFF3) was negatively associated with fat mass but positively associated with female sex. Increased levels of TFF3 were observed to improve glucose tolerance in a diet-induced obesity mouse model, which supports previous reports that TFF3 plays a role in energy metabolism [43].

The 112 differentially abundant proteins associated with fat mass were mapped to KEGG, WikiPathways and Reactome pathways (**Figure 2, Figure S1, Table 6, Tables S6-S8**). Seven proteins significantly associated with fat mass mapped to the complement and coagulation cascade pathway (**Figure 3**). The complement and coagulation cascade pathway is associated with chronic disease risk [44]. In this pathway, abundances of TFPI, coagulation factor IX, tPA, Factor H and C1s were higher while anti-thrombin III and C7 were less abundant as fat mass increased. Although not directly tested in this study, enzymatic activity of thrombin would be maintained in conditions of decreased levels of anti-thrombin III with the result that coagulation would be increased. The association between increased coagulation factor IX, which is also inhibited by anti-thrombin III, and increased fat mass found in the MECHE/DiOGenes cohorts is consistent with more active coagulation processes. Evidence from cell culture demonstrated that a subset of proteins expressed in the complement pathway were altered in adipose cells from insulin resistant humans and in animal models of obesity [45]. These proteins associated with fat mass and the pathways to which they belong suggest a link between insulin resistance, T2D, and coagulation processes.

Fifteen proteins, including adiponectin, insulin, and leptin mapped to the Reactome development biology pathway (**Figure S1, Table S7**) and to KEGG cytokine - cytokine receptor interactions (**Figure 2, Table S6**).

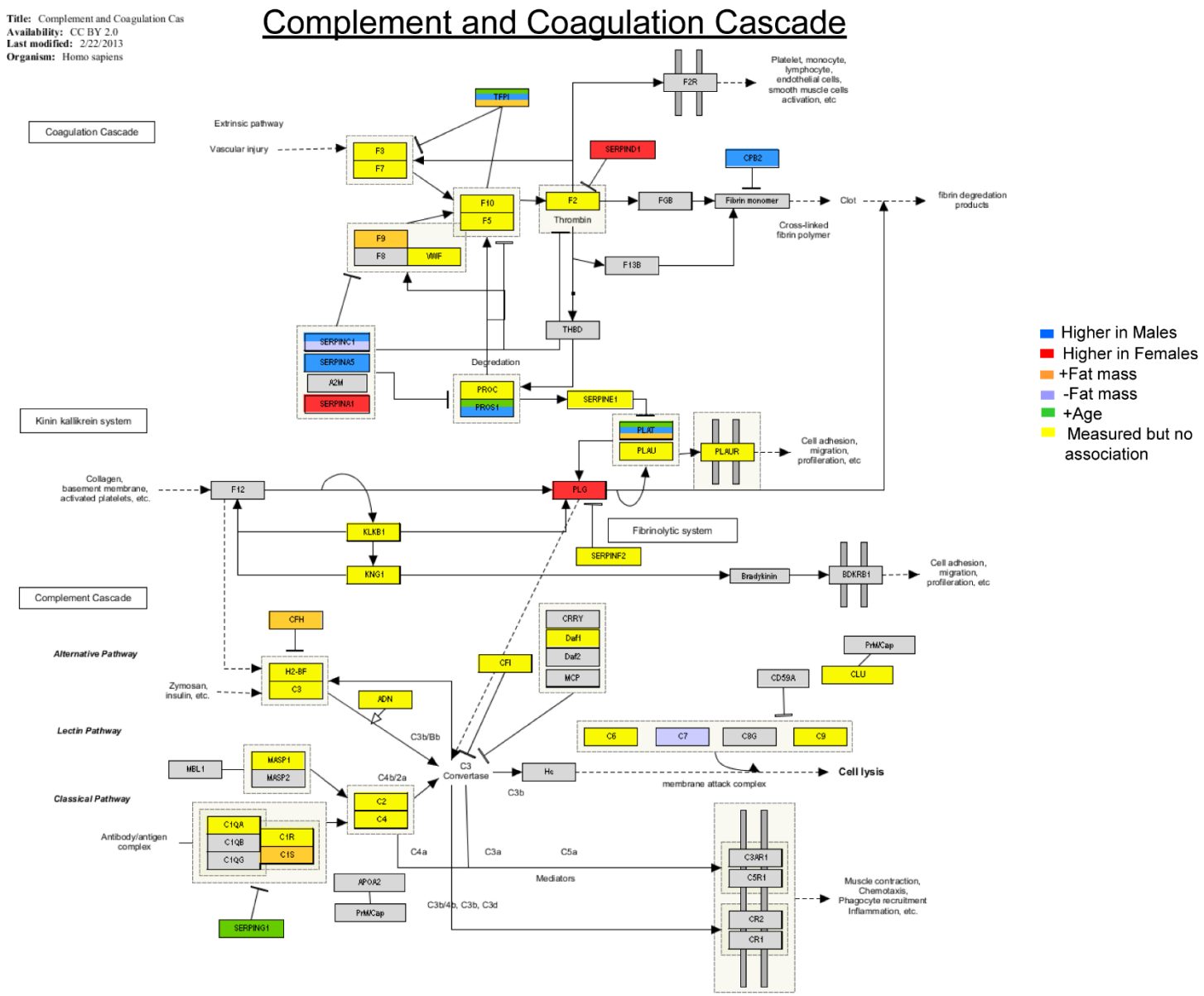


Figure 3. Complement and coagulation cascades pathway obtained from WikiPathways displaying proteins differentially expressed across sex, age, and fat mass phenotypes.

Eleven other proteins, which included ECM proteins (e.g., NCAM1, MMP2, CHL1 negative associations with FM) and growth factors (EGFR, FGFR1, negative associations with FM) were also assigned to the axon guidance pathway, a participant in Reactome's developmental biology pathway. The axon guidance pathway was identified in a transcriptomic analysis of fatty hearts in miniature pigs fed a high energy diet [1] suggesting that dysregulation of these genes may not be specific to neuronal tissues. Decreased levels of axon guidance proteins (e.g., UNC5D, RGMB CHL1) may alter neuro-adipose junctions involved in leptin regulation [2]. The mapping of individual proteins to multiple pathways also identified potential processes associated with increased fat mass. For example, NOTCH1 was found in 11 of the 33 significantly enriched Reactome pathways. NOTCH1 was inversely associated with fat mass in the present study. Decreases in levels of endothelial NOTCH1 may be a risk factor for vascular inflammation and promotion of diet-induced atherosclerosis [3]. Therefore, examination of pathways related to fat mass provides a platform for further investigation of associated biological processes.

Phenotypic variables have an impact on protein levels

Examining the impact of phenotypic variables revealed the importance of considering sex, age and fat mass in proteomics studies. Aptamer based binding assays were used to quantify low abundant serum proteins at baseline in healthy participants of the MECHE (11) and DiOGenes (15) cohorts based on sex, body fat, and age. Forty four percent of sex proteins (51% male, 35% female), 31% age proteins and 49% fat mass proteins identified as significant in the MECHE cohort were replicated in the DiOGenes cohort. The differences in replicated proteins for each phenotype group likely reflects known differences between the two cohorts. DiOGenes participants were older in age (41.6 ± 6.1 vs. 31 ± 10 y in MECHE) with a higher BMI (34.2 ± 4.8 vs. 24.7 ± 4.8 kg/m2 in MECHE) and more body fat (39.7 ± 11.1 vs. 25.76 ± 10.9 kg in MECHE) (**Table 1**). The difference in these parameters is considered a strength since the same proteins were significant in a slightly older and more obese cohort, which extends the use of these proteins in studies of individuals with wider age and BMI ranges. Histogram plots of age and sex for both cohorts are in **Figures S2a-f**. Two proteins overlapped all 3 phenotype groups, 6 proteins between age and sex, 30 proteins between sex and fat mass and 4 proteins between age and fat mass (**Figure S3**). Scatterplots of proteins related to age and fat mass respectively can be found in **Figure S6** and **Figure S7**. The present results highlight the need for including phenotypic parameters in proteomics studies and make a case for the development of phenotypic specific cut offs. Differences in sex, age and fat mass may independently induce quantitative changes in the proteins thought to be specific for a biological process or disease phenotype. That is, underlying differences in phenotype (sex, age, fat mass) may confound the identification of disease-specific

markers. The successful identification of proteins related to gender, age and fat mass in this study will allow for promising biomarkers to be tested for potential confounding factors prior to progression into a clinical setting. In addition, findings from this analysis will contribute to improved statistical modelling by including the identified proteins as confounding factors in future biomarker discovery studies.

The strengths of this study include testing whether proteins identified in the MECHE cohort were replicated in the larger DiOGenes cohort. Pathway analysis was also performed using several different pathway analysis software platforms and provided further insights into the functions of the proteins. While the present study represents an important advancement for proteomics there are a number of limitations worth noting. The proteins were identified using SOMAlogic assays which are a subset of the total protein pool. Subsequent versions of SOMAscan or mass spectroscopic methods may identify additional proteins and pathways for each of the phenotypes studied here. In addition, mapping proteins to KEGG, Reactome, and WikiPathways to create meaningful interpretation of the proteomics data is constrained by the depth and publication biases of pathway databases.

CONCLUSIONS

Phenotypic characteristics such as sex, age and body fat mass have independent associations with the levels of certain serum proteins. Mapping these proteins to pathways identified biological processes differing across phenotypic measures. Importantly, the findings were replicated in an independent cohort. Gender and sex specific health care is emerging as differences in trajectories towards disease and therapeutic responses between males and females are identified. Many of the most significant proteins identified in this study had known relationships with sex hormone metabolism indicating sex hormones play key roles in influencing metabolic health. Additionally, age and fat mass are well-established risk factors for disease. These results are relevant to the development of diagnostic and prognostic markers of health and disease trajectories. The present results will be an important consideration in the development of protein signatures for use in the clinical setting.

Table 6: Overview of pathways related to sex, age and fat mass using WikiPathways

	Pathway	Positive	Measured	Z Score	*P*	ID
Males	Complement and Coagulation Cascades	7	40	2.27	0.039	WP558
	Selenium Micronutrient Network	7	27	3.49	0.003	WP15
	Vitamin B12 Metabolism	7	24	3.88	0.002	WP1533
	Folate Metabolism	6	26	2.88	0.011	WP176
	Statin Pathway	3	4	4.95	0.001	WP430
	Urea cycle and metabolism of amino groups	3	4	4.95	0.001	WP497
Females	Adipogenesis	5	23	4.05	<0.01	WP236
	Aryl Hydrocarbon Receptor	3	19	2.41	0.032	WP2586
	FAS pathway and Stress induction of HSP regulation	3	15	2.93	0.021	WP314
	Myometrial Relaxation and Contraction Pathways	3	22	2.1	0.031	WP289
Aging	Complement and Coagulation Cascades	4	40	2.01	0.044	WP558
	Senescence and Autophagy in Cancer	4	39	2.07	0.047	WP615
Fat mass	Spinal cord injury	8	47	1.78	0.052	WP2431
	Complement and coagulation Cascades	7	40	1.74	0.053	WP558
	Adipogenesis	6	23	2.72	0.006	WP236
	Cardiac Progenitor Differentiation	5	15	3.15	0.008	WP2406
	Neural Crest Differentiation	5	18	2.65	0.017	WP2064
	Complement Activation	4	17	1.97	0.038	WP545
	AMPK signaling	3	12	1.82	0.049	WP1403
	Differentiation of white and brown adipocyte	3	3	5.33	<0.01	WP2895
	Notch Signaling Pathway	3	7	3	0.013	WP268
	NOTCH1 regulation of human endothelial cell calcification	3	10	2.2	0.039	WP3413

Table 6. Pathways obtained from pathway statistics using PathVisio software, using the curated WikiPathways directory. Pathways with a Z-Score of >1.96, a p-value of <0.05 and who have 3 or more proteins differentially expressed are considered important. P-value is permuted. Sorted by number of differentially expressed proteins in pathway.

FUNDING

This work was conducted as part of a clinical trial (NCT01172951), funded under the Food for Health Research Initiative (NDP 2007–2013; 07FHRIUCD1) by the Department of Agriculture, Fisheries and Food (http://www.agriculture.gov.ie/). This research was funded in part by Food for Health Ireland, through Enterprise Ireland Grant No. TC20130001. The DiOGenes project was supported by a contract (FP6-2005- 513946) from the European Commission Food Quality and Safety Priority of the Sixth Framework Program.

ACKNOWLEDGEMENTS

Authors would like to acknowledge participants of both the MECHE and DiOGenes studies. Special thanks to Kei Sakamoto, Sofia Moço, and Loic Dayon from Nestle Institute of Health Sciences and Jan van der Greef from Leiden University for their review of the manuscript.

REFERENCES

[1] Hanash, S., Disease proteomics. *Nature* 2003, *422*, 226-232.
[2] Anderson, N. L., Anderson, N. G., The human plasma proteome: history, character, and diagnostic prospects. *Molecular & cellular proteomics : MCP* 2002, *1*, 845-867.
[3] Zethelius, B., Berglund, L., Sundstrom, J., Ingelsson, E., Basu, S., Larsson, A., Venge, P., Arnlov, J., Use of multiple biomarkers to improve the prediction of death from cardiovascular causes. *The New England journal of medicine* 2008, *358*, 2107-2116.
[4] American Diabetes Association, Cardiovascular disease and risk management. *Diabetes Care* 2015, *38* S49-57.
[5] Miike, K., Aoki, M., Yamashita, R., Takegawa, Y., Saya, H., Miike, T., Yamamura, K., Proteome profiling reveals gender differences in the composition of human serum. *Proteomics* 2010, *10*, 2678-2691.
[6] Al-Daghri, N. M., Al-Attas, O. S., Johnston, H. E., Singhania, A., Alokail, M. S., Alkharfy, K. M., Abd-Alrahman, S. H., Sabico, S. L., Roumeliotis, T. I., Manousopoulou-Garbis, A., Townsend, P. A., Woelk, C. H., Chrousos, G. P., Garbis, S. D., Whole serum 3D LC-nESI-FTMS quantitative proteomics reveals sexual dimorphism in the milieu interieur of overweight and obese adults. *J Proteome Res* 2014, *13*, 5094-5105.
[7] Oberbach, A., Bluher, M., Wirth, H., Till, H., Kovacs, P., Kullnick, Y., Schlichting, N., Tomm, J. M., Rolle-Kampczyk, U., Murugaiyan, J., Binder, H., Dietrich, A., von Bergen, M., Combined proteomic and metabolomic profiling of serum reveals association of the complement system with obesity and identifies novel markers of body fat mass changes. *J Proteome Res* 2011, *10*, 4769-4788.
[8] Chandramouli, K., Qian, P. Y., Proteomics: challenges, techniques and possibilities to overcome biological sample complexity. *Hum Genomics Proteomics* 2009, *2009*.
[9] Gold, L., Ayers, D., Bertino, J., Bock, C., Bock, A., Brody, E. N., Carter, J., Dalby, A. B., Eaton, B. E., Fitzwater, T., Flather, D., Forbes, A., Foreman, T., Fowler, C., Gawande, B., Goss, M., Gunn, M., Gupta, S., Halladay, D., Heil, J., Heilig, J., Hicke, B., Husar, G., Janjic, N., Jarvis, T., Jennings, S., Katilius, E., Keeney, T. R., Kim, N., Koch, T. H., Kraemer, S., Kroiss, L., Le, N., Levine, D., Lindsey, W., Lollo, B., Mayfield, W., Mehan, M., Mehler, R., Nelson, S. K., Nelson, M., Nieuwlandt, D., Nikrad, M., Ochsner, U., Ostroff, R. M., Otis, M., Parker, T., Pietrasiewicz, S., Resnicow, D. I., Rohloff, J., Sanders, G., Sattin, S., Schneider, D., Singer, B., Stanton, M., Sterkel, A., Stewart, A., Stratford, S., Vaught, J. D., Vrkljan, M., Walker, J. J., Watrobka, M., Waugh, S., Weiss, A., Wilcox, S. K., Wolfson, A., Wolk, S. K., Zhang, C., Zichi, D., Aptamer-based multiplexed proteomic technology for biomarker discovery. *PLoS One* 2010, *5*, e15004.
[10] Cominetti, O., Nunez Galindo, A., Corthesy, J., Oller Moreno, S., Irincheeva, I., Valsesia, A., Astrup, A., Saris, W. H., Hager, J., Kussmann, M., Dayon, L., Proteomic Biomarker Discovery in 1000 Human Plasma Samples with Mass Spectrometry. *J Proteome Res* 2016, *15*, 389-399.
[11] Morris, C., O'Grada, C., Ryan, M., Roche, H. M., Gibney, M. J., Gibney, E. R., Brennan, L., Identification of differential responses to an oral glucose tolerance test in healthy adults. *PLoS One* 2013, *8*, e72890.
[12] Wallace, M., Morris, C., O'Grada, C. M., Ryan, M., Dillon, E. T., Coleman, E., Gibney, E. R., Gibney, M. J., Roche, H. M., Brennan, L., Relationship between the

lipidome, inflammatory markers and insulin resistance. *Molecular bioSystems* 2014, *10*, 1586-1595.
[13] Ryan, M. F., O'Grada, C. M., Morris, C., Segurado, R., Walsh, M. C., Gibney, E. R., Brennan, L., Roche, H. M., Gibney, M. J., Within-person variation in the postprandial lipemic response of healthy adults. *Am J Clin Nutr* 2013, *97*, 261-267.
[14] Larsen, T. M., Dalskov, S., van Baak, M., Jebb, S., Kafatos, A., Pfeiffer, A., Martinez, J. A., Handjieva-Darlenska, T., Kunesova, M., Holst, C., Saris, W. H., Astrup, A., The Diet, Obesity and Genes (Diogenes) Dietary Study in eight European countries - a comprehensive design for long-term intervention. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 2010, *11*, 76-91.
[15] Papadaki, A., Linardakis, M., Plada, M., Larsen, T. M., van Baak, M. A., Lindroos, A. K., Pfeiffer, A. F., Martinez, J. A., Handjieva-Darlenska, T., Kunesova, M., Holst, C., Saris, W. H., Astrup, A., Kafatos, A., Diet, O., Genes, P., A multicentre weight loss study using a low-calorie diet over 8 weeks: regional differences in efficacy across eight European cities. *Swiss medical weekly* 2013, *143*, w13721.
[16] Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., Smyth, G. K., limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research* 2015, *43*, e47.
[17] Kutmon, M., van Iersel, M. P., Bohler, A., Kelder, T., Nunes, N., Pico, A. R., Evelo, C. T., PathVisio 3: an extendable pathway analysis toolbox. *PLoS computational biology* 2015, *11*, e1004085.
[18] Wang, X., Terfve, C., Rose, J. C., Markowetz, F., HTSanalyzeR: an R/Bioconductor package for integrated network analysis of high-throughput screens. *Bioinformatics* 2011, *27*, 879-880.
[19] Yu, G., He, Q. Y., ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. *Molecular bioSystems* 2016, *12*, 477-479.
[20] Liu, S., Sun, Q., Sex Differences, Endogenous Sex-hormone Hormones, Sex-hormone Binding Globulin (SHBG), and Exogenous Disruptors in Diabetes and Related Metabolic Outcomes. *Journal of diabetes* 2016.
[21] Geber, S., Brandao, A. H., Sampaio, M., Effects of estradiol and FSH on leptin levels in women with suppressed pituitary. *Reproductive biology and endocrinology : RB&E* 2012, *10*, 45.
[22] Tahboub, R., Arafah, B. M., Sex steroids and the thyroid. *Best practice & research. Clinical endocrinology & metabolism* 2009, *23*, 769-780.
[23] Komukai, K., Mochizuki, S., Yoshimura, M., Gender and the renin-angiotensin-aldosterone system. *Fundamental & clinical pharmacology* 2010, *24*, 687-698.
[24] Floehr, J., Dietzel, E., Neulen, J., Rosing, B., Weissenborn, U., Jahnen-Dechent, W., Association of high fetuin-B concentrations in serum with fertilization rate in IVF: a cross-sectional pilot study. *Hum Reprod* 2016, *31*, 630-637.
[25] Strauss, J. F. B., Robert L., *Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management, Seventh Edition*, Saunders, Philadelphia, Pennsylvania 2014.
[26] Friedman, J., The long road to leptin. *The Journal of clinical investigation* 2016, *126*, 4727-4734.
[27] Volp, A. P., Barbosa, K. B., Bressan, J., Nutrients can modulate the adiponectin concentrations in apparently healthy young adult. *Nutricion hospitalaria* 2016, *33*, 264.

[28] Meex, R. C., Hoy, A. J., Morris, A., Brown, R. D., Lo, J. C., Burke, M., Goode, R. J., Kingwell, B. A., Kraakman, M. J., Febbraio, M. A., Greve, J. W., Rensen, S. S., Molloy, M. P., Lancaster, G. I., Bruce, C. R., Watt, M. J., Fetuin B Is a Secreted Hepatocyte Factor Linking Steatosis to Impaired Glucose Metabolism. *Cell Metab* 2015, *22*, 1078-1089.
[29] Jiang, H., Przybyszewski, J., Mitra, D., Becker, C., Brehm-Stecher, B., Tentinger, A., MacDonald, R. S., Soy protein diet, but not Lactobacillus rhamnosus GG, decreases mucin-1, trefoil factor-3, and tumor necrosis factor-alpha in colon of dextran sodium sulfate-treated C57BL/6 mice. *The Journal of nutrition* 2011, *141*, 1239-1246.
[30] Burnier, M., Urinary angiotensinogen and salt sensitivity of blood pressure: the challenge of finding biomarkers of salt-sensitivity. *Journal of hypertension* 2015, *33*, 1368-1370.
[31] Montenegro, L., Piscitelli, D., Giorgio, F., Covelli, C., Fiore, M. G., Losurdo, G., Iannone, A., Ierardi, E., Di Leo, A., Principi, M., Reversal of IgM deficiency following a gluten-free diet in seronegative celiac disease. *World journal of gastroenterology* 2014, *20*, 17686-17689.
[32] Kim, K. O., Park, H., Kim, H. S., Effects of High-Protein Diet and/or Resveratrol Supplementation on the Immune Response of Irradiated Rats. *Preventive nutrition and food science* 2014, *19*, 156-163.
[33] Kische, H., Gross, S., Wallaschofski, H., Volzke, H., Dorr, M., Nauck, M., Haring, R., Clinical correlates of sex hormones in women: The study of health in Pomerania. *Metabolism: clinical and experimental* 2016, *65*, 1286-1296.
[34] Hara, K., Yamauchi, T., Kadowaki, T., Adiponectin: an adipokine linking adipocytes and type 2 diabetes in humans. *Curr Diab Rep* 2005, *5*, 136-140.
[35] Joyce-Tan, S. M., Zain, S. M., Abdul Sattar, M. Z., Abdullah, N. A., Renin-Angiotensin System Gene Variants and Type 2 Diabetes Mellitus: Influence of Angiotensinogen. *Journal of diabetes research* 2016, *2016*, 2161376.
[36] Pimenta, A. F., Zhukareva, V., Barbe, M. F., Reinoso, B. S., Grimley, C., Henzel, W., Fischer, I., Levitt, P., The limbic system-associated membrane protein is an Ig superfamily member that mediates selective neuronal growth and axon targeting. *Neuron* 1995, *15*, 287-297.
[37] Hansen, S. W., Ohtani, K., Roy, N., Wakamiya, N., The collectins CL-L1, CL-K1 and CL-P1, and their roles in complement and innate immunity. *Immunobiology* 2016, *221*, 1058-1067.
[38] Manttari, S., Anttila, K., Jarvilehto, M., Testosterone stimulates myoglobin expression in different muscles of the mouse. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology* 2008, *178*, 899-907.
[39] Grandas, O. H., Mountain, D. J., Kirkpatrick, S. S., Rudrapatna, V. S., Cassada, D. C., Stevens, S. L., Freeman, M. B., Goldman, M. H., Effect of hormones on matrix metalloproteinases gene regulation in human aortic smooth muscle cells. *The Journal of surgical research* 2008, *148*, 94-99.
[40] Katou, M., [Change of serum amyloid P component concentrations in women]. *Nihon Sanka Fujinka Gakkai zasshi* 1996, *48*, 481-487.
[41] Ali, H. O., Stavik, B., Dorum, E., Iversen, N., Sandset, P. M., Skretting, G., Oestrogen induced downregulation of TFPI expression is mediated by ERalpha. *Thrombosis research* 2014, *134*, 138-143.

[42] Jin, H., Lin, J., Fu, L., Mei, Y. F., Peng, G., Tan, X., Wang, D. M., Wang, W., Li, Y. G., Physiological testosterone stimulates tissue plasminogen activator and tissue factor pathway inhibitor and inhibits plasminogen activator inhibitor type 1 release in endothelial cells. *Biochemistry and cell biology = Biochimie et biologie cellulaire* 2007, *85*, 246-251.
[43] Glueck, C. J., Friedman, J., Hafeez, A., Hassan, A., Wang, P., Testosterone therapy, thrombophilia, and hospitalization for deep venous thrombosis-pulmonary embolus, an exploratory, hypothesis-generating study. *Med Hypotheses* 2015, *84*, 341-343.
[44] Dieplinger, B., Egger, M., Poelz, W., Gabriel, C., Haltmayer, M., Mueller, T., Soluble ST2 is not independently associated with androgen and estrogen status in healthy males and females. *Clinical chemistry and laboratory medicine* 2011, *49*, 1515-1518.
[45] Guo, W., Bachman, E., Li, M., Roy, C. N., Blusztajn, J., Wong, S., Chan, S. Y., Serra, C., Jasuja, R., Travison, T. G., Muckenthaler, M. U., Nemeth, E., Bhasin, S., Testosterone administration inhibits hepcidin transcription and is associated with increased iron incorporation into red blood cells. *Aging cell* 2013, *12*, 280-291.
[46] Yang, Q., Jian, J., Katz, S., Abramson, S. B., Huang, X., 17beta-Estradiol inhibits iron hormone hepcidin through an estrogen responsive element half-site. *Endocrinology* 2012, *153*, 3170-3178.
[47] Yang, X. S., Huang, D. X., Li, Z. J., The value of radioimmunoassay of myoglobin in the diagnosis of acute myocardial infarction. *Acta cardiologica* 1982, *37*, 441-449.
[48] Yamada, Y., Izawa, H., Ichihara, S., Takatsu, F., Ishihara, H., Hirayama, H., Sone, T., Tanaka, M., Yokota, M., Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. *The New England journal of medicine* 2002, *347*, 1916-1923.
[49] Song, Z., Cai, L., Guo, L., Tsukamoto, Y., Yutani, C., Li, X. A., Accumulation and expression of serum amyloid P component in human atherosclerotic lesions. *Atherosclerosis* 2010, *211*, 90-95.
[50] Chen, D., Xia, M., Hayford, C., Tham el, L., Semik, V., Hurst, S., Chen, Y., Tam, H. H., Pan, J., Wang, Y., Tan, X., Lan, H. Y., Shen, H., Kakkar, V. V., Xu, Q., McVey, J. H., Dorling, A., Expression of human tissue factor pathway inhibitor on vascular smooth muscle cells inhibits secretion of macrophage migration inhibitory factor and attenuates atherosclerosis in ApoE-/- mice. *Circulation* 2015, *131*, 1350-1360.
[51] Shang, Q., Feng, L., Yu, W., Xu, J., Liu, X., Wang, J., [Proteomics study on ficolin 3 in the human plasma of type 2 diabetics]. *Wei sheng yan jiu = Journal of hygiene research* 2016, *45*, 8-13.
[52] Powell, L. W., Seckington, R. C., Deugnier, Y., Haemochromatosis. *Lancet* 2016, *388*, 706-716.
[53] Hagler, L., Askew, E. W., Neville, J. R., Mellick, P. W., Coppes, R. I., Jr., Lowder, J. F., Jr., Influence of dietary iron deficiency on hemoglobin, myoglobin, their respective reductases, and skeletal muscle mitochondrial respiration. *Am J Clin Nutr* 1981, *34*, 2169-2177.
[54] Moreno-Navarrete, J. M., Moreno, M., Puig, J., Blasco, G., Ortega, F., Xifra, G., Ricart, W., Fernandez-Real, J. M., Hepatic iron content is independently associated with serum hepcidin levels in subjects with obesity. *Clinical nutrition* 2016.

[55] Schlater, A. E., De Miranda, M. A., Jr., Frye, M. A., Trumble, S. J., Kanatous, S. B., Changing the paradigm for myoglobin: a novel link between lipids and myoglobin. *Journal of applied physiology* 2014, *117*, 307-315.
[56] Perez-Jimenez, F., Castro, P., Lopez-Miranda, J., Paz-Rojas, E., Blanco, A., Lopez-Segura, F., Velasco, F., Marin, C., Fuentes, F., Ordovas, J. M., Circulating levels of endothelial function are modulated by dietary monounsaturated fat. *Atherosclerosis* 1999, *145*, 351-358.
[57] Berrettini, M., Parise, P., Ricotta, S., Iorio, A., Peirone, C., Nenci, G. G., Increased plasma levels of tissue factor pathway inhibitor (TFPI) after n-3 polyunsaturated fatty acids supplementation in patients with chronic atherosclerotic disease. *Thrombosis and haemostasis* 1996, *75*, 395-400.
[58] Grober, U., Reichrath, J., Holick, M. F., Kisters, K., Vitamin K: an old vitamin in a new perspective. *Dermato-endocrinology* 2014, *6*, e968490.
[59] Citelli, M., Bittencourt, L. L., da Silva, S. V., Pierucci, A. P., Pedrosa, C., Vitamin A modulates the expression of genes involved in iron bioavailability. *Biological trace element research* 2012, *149*, 64-70.
[60] Moro, T., Nakao, S., Sumiyoshi, H., Ishii, T., Miyazawa, M., Ishii, N., Sato, T., Iida, Y., Okada, Y., Tanaka, M., Hayashi, H., Ueha, S., Matsushima, K., Inagaki, Y., A Combination of Mitochondrial Oxidative Stress and Excess Fat/Calorie Intake Accelerates Steatohepatitis by Enhancing Hepatic CC Chemokine Production in Mice. *PLoS One* 2016, *11*, e0146592.
[61] Ricklin, D., Lambris, J. D., Complement in immune and inflammatory disorders: pathophysiological mechanisms. *Journal of immunology* 2013, *190*, 3831-3838.
[62] Kokawa, T., Abumiya, T., Kimura, T., Harada-Shiba, M., Koh, H., Tsushima, M., Yamamoto, A., Kato, H., Tissue factor pathway inhibitor activity in human plasma. Measurement of lipoprotein-associated and free forms in hyperlipidemia. *Arteriosclerosis, thrombosis, and vascular biology* 1995, *15*, 504-510.
[63] Griffin, J. H., Gruber, A., Fernandez, J. A., Reevaluation of total, free, and bound protein S and C4b-binding protein levels in plasma anticoagulated with citrate or hirudin. *Blood* 1992, *79*, 3203-3211.
[64] Liberti, G., Bertina, R. M., Rosendaal, F. R., Hormonal state rather than age influences cut-off values of protein S: reevaluation of the thrombotic risk associated with protein S deficiency. *Thrombosis and haemostasis* 1999, *82*, 1093-1096.
[65] Anderson, H. A., Maylock, C. A., Williams, J. A., Paweletz, C. P., Shu, H., Shacter, E., Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. *Nature immunology* 2003, *4*, 87-91.
[66] Triantafilou, M., Hughes, T. R., Morgan, B. P., Triantafilou, K., Complementing the inflammasome. *Immunology* 2016, *147*, 152-164.
[67] Logue, J., Walker, J. J., Colhoun, H. M., Leese, G. P., Lindsay, R. S., McKnight, J. A., Morris, A. D., Pearson, D. W., Petrie, J. R., Philip, S., Wild, S. H., Sattar, N., Scottish Diabetes Research Network Epidemiology, G., Do men develop type 2 diabetes at lower body mass indices than women? *Diabetologia* 2011, *54*, 3003-3006.
[68] Geer, E. B., Shen, W., Gender differences in insulin resistance, body composition, and energy balance. *Gend Med* 2009, *6 Suppl 1*, 60-75.
[69] Feldman, H. A., Longcope, C., Derby, C. A., Johannes, C. B., Araujo, A. B., Coviello, A. D., Bremner, W. J., McKinlay, J. B., Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the

Massachusetts male aging study. *The Journal of clinical endocrinology and metabolism* 2002, *87*, 589-598.
[70] Menni, C., Kiddle, S. J., Mangino, M., Vinuela, A., Psatha, M., Steves, C., Sattlecker, M., Buil, A., Newhouse, S., Nelson, S., Williams, S., Voyle, N., Soininen, H., Kloszewska, I., Mecocci, P., Tsolaki, M., Vellas, B., Lovestone, S., Spector, T. D., Dobson, R., Valdes, A. M., Circulating Proteomic Signatures of Chronological Age. *The journals of gerontology. Series A, Biological sciences and medical sciences* 2015, *70*, 809-816.
[71] Kurt, I., Abasli, D., Cihan, M., Serdar, M. A., Olgun, A., Saruhan, E., Erbil, M. K., Chitotriosidase levels in healthy elderly subjects. *Annals of the New York Academy of Sciences* 2007, *1100*, 185-188.
[72] Verma, P., Dalal, K., ADAMTS-4 and ADAMTS-5: key enzymes in osteoarthritis. *Journal of cellular biochemistry* 2011, *112*, 3507-3514.
[73] Zhao, C. Q., Zhang, Y. H., Jiang, S. D., Li, H., Jiang, L. S., Dai, L. Y., ADAMTS-5 and intervertebral disc degeneration: the results of tissue immunohistochemistry and in vitro cell culture. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2011, *29*, 718-725.
[74] Malaguarnera, L., Ohazuruike, L. N., Tsianaka, C., Antic, T., Di Rosa, M., Malaguarnera, M., Human chitotriosidase polymorphism is associated with human longevity in Mediterranean nonagenarians and centenarians. *Journal of human genetics* 2010, *55*, 8-12.
[75] Teumer, A., Qi, Q., Nethander, M., Aschard, H., Bandinelli, S., Beekman, M., Berndt, S. I., Bidlingmaier, M., Broer, L., Group, C. L. W., Cappola, A., Ceda, G. P., Chanock, S., Chen, M. H., Chen, T. C., Chen, Y. D., Chung, J., Del Greco Miglianico, F., Eriksson, J., Ferrucci, L., Friedrich, N., Gnewuch, C., Goodarzi, M. O., Grarup, N., Guo, T., Hammer, E., Hayes, R. B., Hicks, A. A., Hofman, A., Houwing-Duistermaat, J. J., Hu, F., Hunter, D. J., Husemoen, L. L., Isaacs, A., Jacobs, K. B., Janssen, J. A., Jansson, J. O., Jehmlich, N., Johnson, S., Juul, A., Karlsson, M., Kilpelainen, T. O., Kovacs, P., Kraft, P., Li, C., Linneberg, A., Liu, Y., Loos, R. J., Body Composition Genetics, C., Lorentzon, M., Lu, Y., Maggio, M., Magi, R., Meigs, J., Mellstrom, D., Nauck, M., Newman, A. B., Pollak, M. N., Pramstaller, P. P., Prokopenko, I., Psaty, B. M., Reincke, M., Rimm, E. B., Rotter, J. I., Saint Pierre, A., Schurmann, C., Seshadri, S., Sjogren, K., Slagboom, P. E., Strickler, H. D., Stumvoll, M., Suh, Y., Sun, Q., Zhang, C., Svensson, J., Tanaka, T., Tare, A., Tonjes, A., Uh, H. W., van Duijn, C. M., van Heemst, D., Vandenput, L., Vasan, R. S., Volker, U., Willems, S. M., Ohlsson, C., Wallaschofski, H., Kaplan, R. C., Genomewide meta-analysis identifies loci associated with IGF-I and IGFBP-3 levels with impact on age-related traits. *Aging cell* 2016, *15*, 811-824.
[76] Johnston, A., Xing, X., Guzman, A. M., Riblett, M., Loyd, C. M., Ward, N. L., Wohn, C., Prens, E. P., Wang, F., Maier, L. E., Kang, S., Voorhees, J. J., Elder, J. T., Gudjonsson, J. E., IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *Journal of immunology* 2011, *186*, 2613-2622.
[77] Fosang, A. J., Little, C. B., Drug insight: aggrecanases as therapeutic targets for osteoarthritis. *Nature clinical practice. Rheumatology* 2008, *4*, 420-427.
[78] Evans, D. S., Cailotto, F., Parimi, N., Valdes, A. M., Castano-Betancourt, M. C., Liu, Y., Kaplan, R. C., Bidlingmaier, M., Vasan, R. S., Teumer, A., Tranah, G. J., Nevitt, M. C., Cummings, S. R., Orwoll, E. S., Barrett-Connor, E., Renner, J. B., Jordan, J. M.,

Doherty, M., Doherty, S. A., Uitterlinden, A. G., van Meurs, J. B., Spector, T. D., Lories, R. J., Lane, N. E., Genome-wide association and functional studies identify a role for IGFBP3 in hip osteoarthritis. *Annals of the rheumatic diseases* 2015, *74*, 1861-1867.
[79] Yamaguchi, T., Kanatani, M., Yamauchi, M., Kaji, H., Sugishita, T., Baylink, D. J., Mohan, S., Chihara, K., Sugimoto, T., Serum levels of insulin-like growth factor (IGF); IGF-binding proteins-3, -4, and -5; and their relationships to bone mineral density and the risk of vertebral fractures in postmenopausal women. *Calcified tissue international* 2006, *78*, 18-24.
[80] Rutter, M. M., Markoff, E., Clayton, L., Akeno, N., Zhao, G., Clemens, T. L., Chernausek, S. D., Osteoblast-specific expression of insulin-like growth factor-1 in bone of transgenic mice induces insulin-like growth factor binding protein-5. *Bone* 2005, *36*, 224-231.
[81] Kim, J. G., Sun, B. H., Dietrich, M. O., Koch, M., Yao, G. Q., Diano, S., Insogna, K., Horvath, T. L., AgRP Neurons Regulate Bone Mass. *Cell reports* 2015, *13*, 8-14.
[82] Wendel, M., Sommarin, Y., Heinegard, D., Bone matrix proteins: isolation and characterization of a novel cell-binding keratan sulfate proteoglycan (osteoadherin) from bovine bone. *The Journal of cell biology* 1998, *141*, 839-847.
[83] Argyropoulos, G., Rankinen, T., Neufeld, D. R., Rice, T., Province, M. A., Leon, A. S., Skinner, J. S., Wilmore, J. H., Rao, D. C., Bouchard, C., A polymorphism in the human agouti-related protein is associated with late-onset obesity. *The Journal of clinical endocrinology and metabolism* 2002, *87*, 4198-4202.
[84] Corfitsen, H. T., Drago, A., Insight gained from genome-wide interaction and enrichment analysis on weight gain during citalopram treatment. *Neuroscience letters* 2017, *637*, 38-43.
[85] Waters, D. L., Yau, C. L., Montoya, G. D., Baumgartner, R. N., Serum Sex Hormones, IGF-1, and IGFBP3 Exert a Sexually Dimorphic Effect on Lean Body Mass in Aging. *The journals of gerontology. Series A, Biological sciences and medical sciences* 2003, *58*, 648-652.
[86] Kheiroddin, P., Rasihashemi, S. Z., Estiar, M. A., Mahmudian, B., Halimi, M., Mousavi, F., Nemati, M., Sakhinia, E., RET Gene Analysis in Patients with Medullary Thyroid Carcinoma. *Clinical laboratory* 2016, *62*, 871-876.
[87] Huang, S. P., Levesque, E., Guillemette, C., Yu, C. C., Huang, C. Y., Lin, V. C., Chung, I. C., Chen, L. C., Laverdiere, I., Lacombe, L., Fradet, Y., Chang, T. Y., Lee, H. Z., Juang, S. H., Bao, B. Y., Genetic variants in microRNAs and microRNA target sites predict biochemical recurrence after radical prostatectomy in localized prostate cancer. *International journal of cancer* 2014, *135*, 2661-2667.
[88] Naspi, A., Zingariello, M., Sancillo, L., Panasiti, V., Polinari, D., Martella, M., Rosa Alba, R., Londei, P., IGFBP-3 inhibits Wnt signaling in metastatic melanoma cells. *Molecular carcinogenesis* 2017, *56*, 681-693.
[89] Butt, A. J., Dickson, K. A., McDougall, F., Baxter, R. C., Insulin-like growth factor-binding protein-5 inhibits the growth of human breast cancer cells in vitro and in vivo. *The Journal of biological chemistry* 2003, *278*, 29676-29685.
[90] Spangenburg, E. E., Abraha, T., Childs, T. E., Pattison, J. S., Booth, F. W., Skeletal muscle IGF-binding protein-3 and -5 expressions are age, muscle, and load dependent. *American journal of physiology. Endocrinology and metabolism* 2003, *284*, E340-350.

[91] Kang, J. S., Mulieri, P. J., Hu, Y., Taliana, L., Krauss, R. S., BOC, an Ig superfamily member, associates with CDO to positively regulate myogenic differentiation. *The EMBO journal* 2002, *21*, 114-124.
[92] Lokiec, K., Blonska, A., Walecka-Kapica, E., Stec-Michalska, K., [Effect of treatment with diet on reducing levels of sex hormones in perimenopausal women with overweight and obesity]. *Polski merkuriusz lekarski : organ Polskiego Towarzystwa Lekarskiego* 2016, *40*, 362-368.
[93] Briggs, D. I., Lockie, S. H., Wu, Q., Lemus, M. B., Stark, R., Andrews, Z. B., Calorie-restricted weight loss reverses high-fat diet-induced ghrelin resistance, which contributes to rebound weight gain in a ghrelin-dependent manner. *Endocrinology* 2013, *154*, 709-717.
[94] Gholamhosseini, S., Nematipour, E., Djazayery, A., Javanbakht, M. H., Koohdani, F., Zareei, M., Djalali, M., omega-3 fatty acid differentially modulated serum levels of IGF1 and IGFBP3 in men with CVD: a randomized, double-blind placebo-controlled study. *Nutrition* 2015, *31*, 480-484.
[95] Caliezi, C., Wuillemin, W. A., Zeerleder, S., Redondo, M., Eisele, B., Hack, C. E., C1-Esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol Rev* 2000, *52*, 91-112.
[96] Andriani, G. A., Almeida, V. P., Faggioli, F., Mauro, M., Tsai, W. L., Santambrogio, L., Maslov, A., Gadina, M., Campisi, J., Vijg, J., Montagna, C., Whole Chromosome Instability induces senescence and promotes SASP. *Sci Rep* 2016, *6*, 35218.
[97] Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., Stan, T. M., Fainberg, N., Ding, Z., Eggel, A., Lucin, K. M., Czirr, E., Park, J. S., Couillard-Despres, S., Aigner, L., Li, G., Peskind, E. R., Kaye, J. A., Quinn, J. F., Galasko, D. R., Xie, X. S., Rando, T. A., Wyss-Coray, T., The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* 2011, *477*, 90-94.
[98] Lefebvre, J. S., Maue, A. C., Eaton, S. M., Lanthier, P. A., Tighe, M., Haynes, L., The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. *Aging cell* 2012, *11*, 732-740.
[99] Monti, D., Ostan, R., Borelli, V., Castellani, G., Franceschi, C., Inflammaging and human longevity in the omics era. *Mechanisms of ageing and development* 2016.
[100] Friedman, J. M., Halaas, J. L., Leptin and the regulation of body weight in mammals. *Nature* 1998, *395*, 763-770.
[101] Baile, C. A., Della-Fera, M. A., Martin, R. J., Regulation of metabolism and body fat mass by leptin. *Annual review of nutrition* 2000, *20*, 105-127.
[102] Spiegelman, B. M., Flier, J. S., Obesity and the regulation of energy balance. *Cell* 2001, *104*, 531-543.
[103] Jequier, E., Leptin signaling, adiposity, and energy balance. *Annals of the New York Academy of Sciences* 2002, *967*, 379-388.
[104] Al-Hamodi, Z., Ismail, I. S., Saif-Ali, R., Ahmed, K. A., Muniandy, S., Association of plasminogen activator inhibitor-1 and tissue plasminogen activator with type 2 diabetes and metabolic syndrome in Malaysian subjects. *Cardiovascular diabetology* 2011, *10*, 23.
[105] Svendsen, O. L., Hassager, C., Christiansen, C., Nielsen, J. D., Winther, K., Plasminogen activator inhibitor-1, tissue-type plasminogen activator, and fibrinogen:

Effect of dieting with or without exercise in overweight postmenopausal women. *Arteriosclerosis, thrombosis, and vascular biology* 1996, *16*, 381-385.
[106] Ahmed, R. L., Thomas, W., Schmitz, K. H., Interactions between insulin, body fat, and insulin-like growth factor axis proteins. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2007, *16*, 593-597.
[107] Ge, H., Gardner, J., Wu, X., Rulifson, I., Wang, J., Xiong, Y., Ye, J., Belouski, E., Cao, P., Tang, J., Lee, K. J., Coberly, S., Gupte, J., Miao, L., Yang, L., Nguyen, N., Shan, B., Yeh, W. C., Veniant, M. M., Li, Y., Baribault, H., Trefoil Factor 3 (TFF3) Is Regulated by Food Intake, Improves Glucose Tolerance and Induces Mucinous Metaplasia. *PLoS One* 2015, *10*, e0126924.
[108] Hertle, E., Stehouwer, C. D., van Greevenbroek, M. M., The complement system in human cardiometabolic disease. *Molecular immunology* 2014, *61*, 135-148.
[109] Zhang, J., Wright, W., Bernlohr, D. A., Cushman, S. W., Chen, X., Alterations of the Classic Pathway of Complement in Adipose Tissue of Obesity and Insulin Resistance. *The FASEB Journal* 2007, *21*, A294-A294.
[110] Xia, J., Zhang, Y., Xin, L., Kong, S., Chen, Y., Yang, S., Li, K., Global Transcriptomic Profiling of Cardiac Hypertrophy and Fatty Heart Induced by Long-Term High-Energy Diet in Bama Miniature Pigs. *PLoS One* 2015, *10*, e0132420.
[111] Zeng, W., Pirzgalska, R. M., Pereira, M. M., Kubasova, N., Barateiro, A., Seixas, E., Lu, Y. H., Kozlova, A., Voss, H., Martins, G. G., Friedman, J. M., Domingos, A. I., Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell* 2015, *163*, 84-94.
[112] Briot, A., Civelek, M., Seki, A., Hoi, K., Mack, J. J., Lee, S. D., Kim, J., Hong, C., Yu, J., Fishbein, G. A., Vakili, L., Fogelman, A. M., Fishbein, M. C., Lusis, A. J., Tontonoz, P., Navab, M., Berliner, J. A., Iruela-Arispe, M. L., Endothelial NOTCH1 is suppressed by circulating lipids and antagonizes inflammation during atherosclerosis. *The Journal of experimental medicine* 2015, *212*, 2147-2163.

SUPPLEMENTARY INFORMATION

The Supporting Information for **Chapter 4** is available free of charge on the ACS Publications website (https://pubs.acs.org/) at DOI: 10.1021/acs.jproteome.7b00501 (https://pubs.acs.org/doi/abs/10.1021/acs.jproteome.7b00501).

Tables S1–S8
(https://pubs.acs.org/doi/suppl/10.1021/acs.jproteome.7b00501/suppl_file/pr7b00501_si_001.pdf)

Figures S1–S7
(https://pubs.acs.org/doi/suppl/10.1021/acs.jproteome.7b00501/suppl_file/pr7b00501_si_001.pdf)

Supporting Information available
Table S1. Summary of proteins that are associated with females
Table S2. Summary of proteins that are associated with males
Table S3. Summary of proteins that are associated with age
Table S4. Summary of proteins that are associated with body fat mass
Table S5a. Proteins associated with males in MECHE and replicated in DiOGenes
Table S5b. Proteins associated with females in MECHE and replicated in DiOGenes
Table S5c. Proteins associated with age in MECHE and replicated in DiOGenes
Table S5d. Proteins associated with fat mass in MECHE and replicated in DiOGenes
Table S6. Summary of KEGG pathway analysis
Table S7. Summary of Reactome pathway analysis
Table S8. Summary of WikiPathway analysis
Figure S1. Overview of Reactome Pathway Enrichment
Figure S2.a-f. Histogram plots of age and sex per cohort
Figure S3. Venn diagram displaying number of proteins which overlap across phenotypes
Figure S4. OPLS-DA for age of <34yrs vs >35yrs derived from proteomic data of MECHE participants (n=196).
Figure S5. OPLS-DA for fat mass of <25.4kg vs >25.5kg derived from proteomic data of MECHE participants (n=196).
Figure S6. Scatterplot of proteins related to age obtained from regression analysis.
Figure S7. Scatterplot of proteins related to fat mass obtained from regression analysis.

Chapter 5

Menstrual cycle rhythmicity: metabolic patterns in healthy women

Based on

Draper CF, Duisters K, Weger B, Chakrabarti A, Harms AC, Brennan L, Hankemeier T, Goulet L, Konz T, Martin FP, Moco S, van der Greef J

Menstrual cycle rhythmicity: metabolic patterns in healthy women

doi: 10.1038/s41598-018-32647-0

ABSTRACT

The menstrual cycle is an essential life rhythm governed by interacting levels of progesterone, estradiol, follicular stimulating, and luteinizing hormones. To study metabolic changes, biofluids were collected at four timepoints in the menstrual cycle from 34 healthy, premenopausal women. Serum hormones, urinary luteinizing hormone and self-reported menstrual cycle timing were used for a 5-phase cycle classification. Plasma and urine were analyzed using LC-MS and GC-MS for metabolomics and lipidomics; serum for clinical chemistries; and plasma for B vitamins using HPLC-FLD. Of 397 metabolites and micronutrients tested, 208 were significantly ($p<0.05$) changed and 71 reached the FDR 0.20 threshold showing rhythmicity in neurotransmitter precursors, glutathione metabolism, the urea cycle, 4-pyridoxic acid, and 25-OH vitamin D. In total, 39 amino acids and derivatives and 18 lipid species decreased (FDR<0.20) in the luteal phase, possibly indicative of an anabolic state during the progesterone peak and recovery during menstruation and the follicular phase. The reduced metabolite levels observed may represent a time of vulnerability to hormone related health issues such as PMS and PMDD, in the setting of a healthy, rhythmic state. These results provide a foundation for further research on cyclic differences in nutrient-related metabolites and may form the basis of novel nutrition strategies for women.

INTRODUCTION

The monthly menstrual cycle represents one of many physiological rhythms essential for life. The heartbeat and daily sleep-wake cycle represent obvious rhythms. Less obvious are the physiological processes inside the body such as the rhythmicity of the sex hormones that drive the menstrual cycle and others that regulate growth and metabolism [1]. These rhythms also interact with each other through synchronization of cellular activities with the external environment through feedback mechanisms that promote dynamic stability, such as the interaction between circadian rhythms, sleep and the menstrual cycle [2, 3]. Perturbations of the body's rhythmic processes are associated with disorders[4] such as disturbed circadian rhythmicity with premenstrual dysphoric disorder (PMDD)[5] or abnormal expression of the circadian clock gene and spontaneous abortion[6].

The first half of the menstrual cycle is comprised by the menstrual and follicular phases during which time estrogen levels are low (menstrual phase) and rise (follicular phase) and ends with the periovulatory phase in which follicular stimulating hormone (FSH) and luteinizing hormones (LH) peak. The second half of the cycle is comprised by the luteal (during which time estrogen level rises with a progesterone peak) and the pre-menstrual phases during which time estrogen and progesterone levels fall (**Fig. 1, Supplementary Fig. 1**) [7-9]). However, it is during this time that women experience worsening of chronic diseases such as diabetes and inflammatory bowel disease, bloating, poor sleep quality, and premenstrual syndrome (PMS) or PMDD [10-16]. Moreover, the luteal phase is also accompanied by decreasing amino acid levels and elevated nitrogen utilization [17, 18]. Women with PMS and PMDD have an increased appetite, food cravings and excess calorie intake which are associated with cyclical changes in serotonin during this period [12, 19-21]. These biochemical changes suggest nutrient utilization is affected by changing sex hormones between phases. The luteal phase of the menstrual cycle may be considered a normally stressed physiology which amplifies differential responses between individuals to environmental stressors such as diet intake. These differential responses might predict future chronic health issues.

In order to characterize baseline metabolic rhythmicity in the menstrual cycle, advanced metabolomic profiling, clinical and nutrient biochemistries were analyzed for rhythmic variations throughout a healthy menstrual cycle in 34 healthy women. Samples were analysed from five different phases of the menstrual cycle obtained from a previously published stud y[17]. The results are interpreted in light of metabolic differences that may represent vulnerability to sex hormone related disorders and

nutritional needs, as well as diagnostic and therapeutic approaches that vary across the menstrual cycle (**Fig. 2**).

METHODS

Study design

All methods were performed in accordance with the relevant guidelines and regulations. Ethical approval was received from both the Research Ethics Committee, University College Dublin (UCD) and the Commission Cantonale D'Ethique de la Recherche sur L'Etre Humain (CER-VD) in Switzerland. All participants provided written informed consent before study participation. Participants visited the clinic at 4 different timepoints for blood and urine collection at different menstrual cycle phases (**Fig. 2**). Participants were instructed to keep a menstrual calendar for 1 month prior to sample collection in order to estimate the length of their cycle. Using a guide for menstrual phase length [17], urine and blood samples were collected from each woman at 4 different time points in one menstrual cycle; estimated to represent 4 stages of the menstrual cycle: menstrual, follicular, luteal and premenstrual (**Fig. 1**). Participants were instructed to use a luteinizing hormone (LH) urine dip strip test kit (Medimpex Ltd Inc) at home in order to determine the date of ovulation.

Following serum hormonal analysis, classification of the phases was refined to define 5 stages of the menstrual cycle (menstrual, follicular, periovulatory, luteal and premenstrual) [17].

Study participants

Thirty four healthy, premenopausal women at UCD, Dublin with a mean age of 26.6 years, standard deviation (SD) of +/- 5.9; and a mean body mass index (BMI) (Kg/m^3) of 22.9 +/- 3.5 volunteered to participate in the study. Participants were excluded with a BMI <18 or >30 Kg/m^3, iron deficiency anemia (hemoglobin < 11.5 g/dl), diagnosis with a medical condition and use of prescribed medication or hormonal contraceptives (**Fig. 2**).

Blood and urine collection

Prior to blood and urine collection, participants were instructed to fast for 12 h, limit tea and coffee consumption and abstain from fish, alcohol and vigorous exercise for 24h. On the morning of collection, volunteers collected their first void urine at home in a chilled graduated container and then immediately delivered it to the laboratory on ice. Sample processing and serum hormone analyses were measured at the Biochemistry Department, National Maternity Hospital, Dublin, as previously described[17].

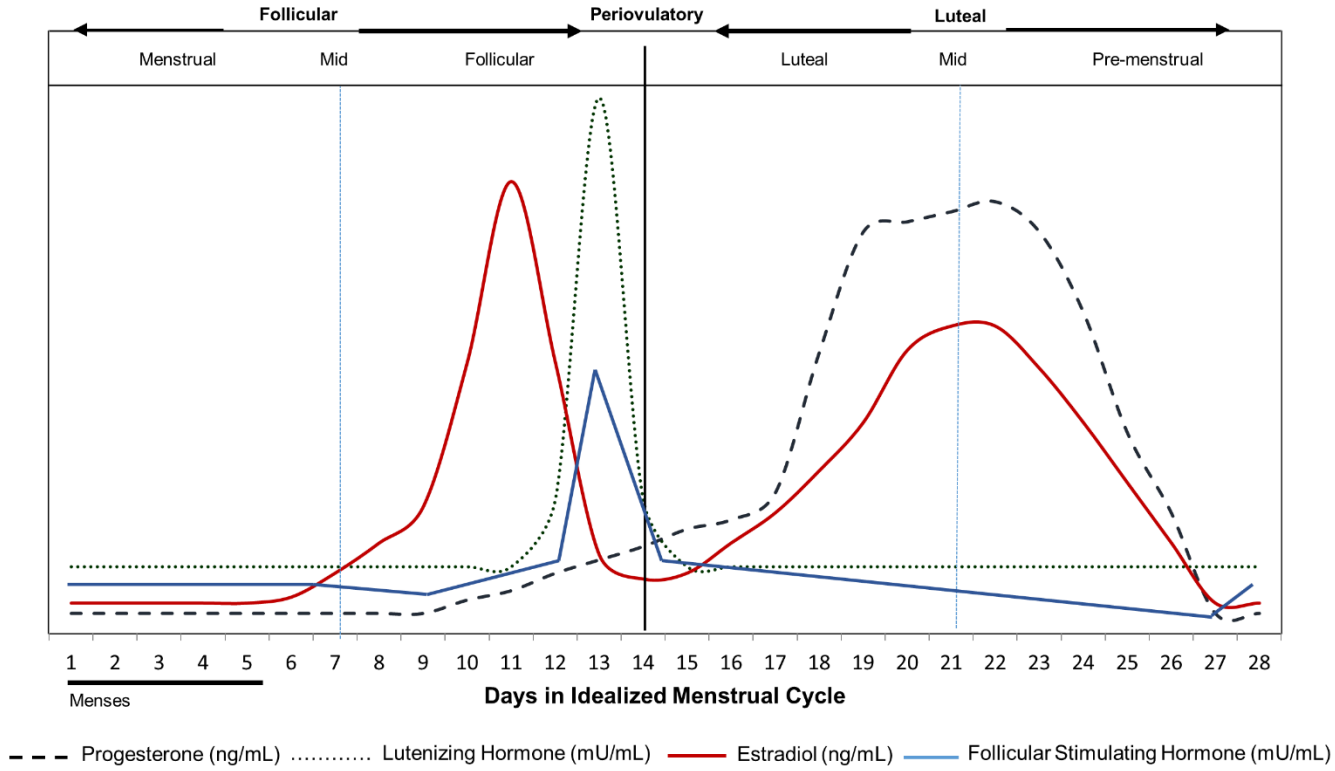


Fig. 1. Hormone levels according to menstrual cycle phase. Changing concentrations of female sex hormones (progesterone, luteinizing hormone, follicular stimulating hormone, estradiol) that characterize the 5 phases (menstrual, follicular, periovulatory, luteal and pre-menstrual) of the menstrual cycle (adapted with permission 7). Follicular stimulating hormone concentration changes overlayed. Adapted with permission from Allen, A. M. *et al.* Determining menstrual phase in human biobehavioral research: A review with recommendations. *Exp Clin Psychopharmacol* **24**, 1-11, doi:10.1037/pha0000057 (2016).

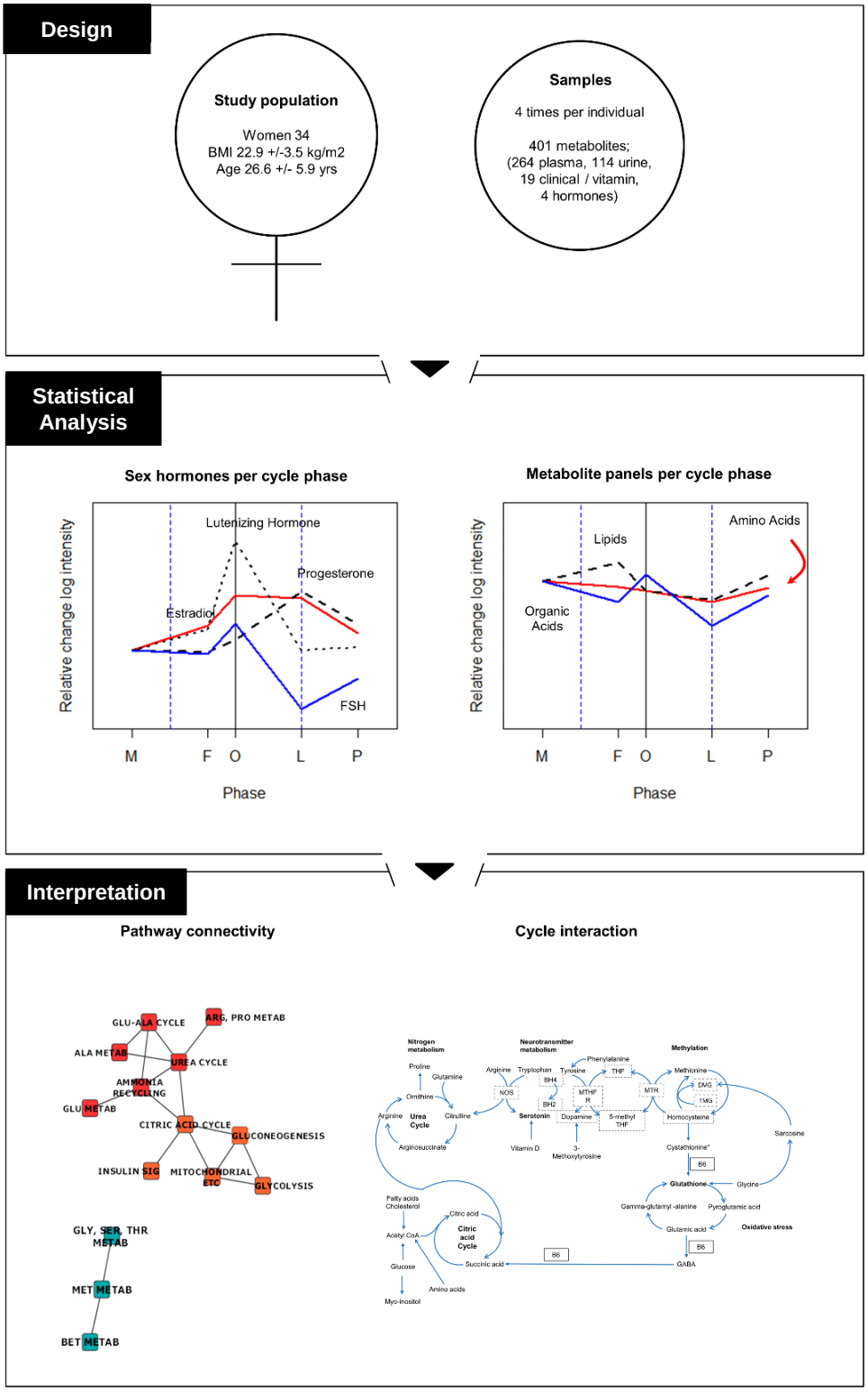


Fig. 2. Study schema. Thirty-four women (BMI 22.9 +/-3.5 kg/m², age 26.6+/-5.9 yrs) provided 4 blood and urine samples that each uniquely fit into 1 of 5 phase timepoints based on 4 sex hormone measurements (LH, FS, estradiol, and progesterone). A total of 401 metabolites were measured including 263 plasma, 114 urine, and 19 clinical and vitamin analyses. Metabolite profiling was conducted and rhythmicity is depicted for the amino acid, lipid and organic acid panels. Biochemical pathway interconnectivity was identified between the urea cycle, 1 carbon metabolism, glutathione metabolism and the citric acid cycle. M-menstrual, F-follicular, O-Periovulatory, L-luteal, P-premenstrual phases.

Metabolite profiling analysis

Metabolite profiling was done by the Biomedical Metabolomics Facility, Leiden University, Leiden, The Netherlands. All samples (plasma and urine) were randomized and analyzed in batches, which included calibration lines, quality control (QC) samples and blanks. QC samples were prepared from pooled plasma and urine available in the laboratory and were analyzed every 10 samples for data quality and instrument response correction. Blank samples were used to correct for background signal and in-house developed algorithms were applied using the pooled QC samples to compensate for time-dependent drifts of the sensitivity of the mass spectrometer. Data was reported as ratio of analyte signal to internal standard.

The amine platform analyzed 74 amino acids and biogenic amines in plasma and urine using liquid chromatography coupled to a mass spectrometer (LC-MS) employing an AccQ-Tag derivatization strategy adapted from the protocol supplied by Waters (Etten-Leur, The Netherlands) [1]. One µL of the reaction mixture was injected into the ACQUITY UPLC System (Waters, Etten-Leur, The Netherlands) on an AccQ-Tag Ultra column (Waters) for chromatographic separation coupled to a triple quadrupole mass spectrometer (AB SCIEX Qtrap 6500, Framingham, MA USA). Acquired data were evaluated using MultiQuant Software for Quantitative Analysis (AB SCIEX, Version 3.0.2). After quality control correction [2], 54 plasma amines and 60 urine amines complied with the acceptance criteria of RSDqc <15%.

The lipid platform analyzed 185 compounds in 9 lipid classes in plasma using LC-MS as described[3]. Chromatographic separation was achieved on a ACQUITY UPLC™ (Waters, Etten-Leur, The Netherlands) with a HSS T3 column which was coupled to a ESI-Q-TOF (Agilent 6530, Jose, CA, USA) using reference mass correction[3]. The raw data were pre-processed using Agilent MassHunter Quantitative Analysis software (Agilent, Version B.04.00). After QC and blank correction, 139 compounds comply with the acceptance criteria RSDQC <30% and blank effect <40 %. Twenty six organic acids were analyzed in urine by gas chromatography coupled to mass spectrometry (GC-MS). After QC correction and considering blank effects, 23 urinary and 16 plasma organic acids compounds complied with the acceptance criteria RSDQC <30% and blank effect <20 %. The plasma and urine metabolites were measured by gas chromatography on an Agilent Technologies 7890A equipped with an Agilent Technologies mass selective detector (MSD 5975C) and MultiPurpose Sampler (MPS, MXY016-02A, Gerstel, Germany). Chromatographic separations were performed on a HP-5MS and detected using a quadrupole mass spectrometer. The raw data were pre-processed using Agilent MassHunter Quantitative Analysis software (Agilent, Version B.05.01).

The endocannabinoid profiling platform analyzed 24 compounds in plasma, as previously described [4]. Chromatographic separation was achieved by an ACQUITY UPLC System (Waters, Etten-Leur, The Netherlands) on an ACQUITY UPLC HSS T3 Column. The UPLC was coupled to electrospray ionization on a triple quadrupole mass spectrometer (AB SCIEX Qtrap 6500, Framingham, MA USA). Acquired data were evaluated using MultiQuant Software for Quantitative Analysis (AB SCIEX, Version 3.0.2). After quality control correction 19 endocannabinoids complied with the acceptance criteria of RSDqc <15%.

The acylcarnitine platform analyzed 45 acylcarnitines as well as trimethylamine-N-oxide, choline, betaine, deoxycarnitine and carnitine in plasma and urine. Chromatographic separation was achieved by UPLC (Agilent 1290, San Jose, CA, USA) on an AccQ-Tag Ultra column (Waters, Etten-Leur, The Netherlands) coupled to electrospray ionization on a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA). Acquired data were evaluated using Agilent MassHunter Quantitative Analysis software (Agilent, Version B.05.01). After QC correction, 25 urinary and 27 plasma acylcarnitines complied with the acceptance criteria of RSDqc <15%.

The method validation used for the metabolite profiling fits with the acceptance criteria for precision (15% RSD, 20% RSD near LLOQ and accuracy (bias within +/- 15% of the accepted reference value, within 20% near LLOQ) specified by the Conference Reports have been widely accepted in bio-analysis [5]. Recommended guidelines were followed for most metabolites, however, some were accepted which were close to the LOQ.

Clinical and vitamin biochemistry

Standard clinical routine analysis of human serum samples was performed by the Molecular Nutrition laboratory at NIHS. The applied Architect plus ci4100 platform from Abbott Laboratories (Lake Bluff, IL, USA), consists of a chemistry and an immunoassay module [6]. Glucose, insulin, vitamin B12, holotranscobalamin, folate, 25-hydroxy vitamin D and cortisol were determined by chemiluminescent microparticle immunoassays (CMIA) while cholesterol, HDL, triglycerides, high sensitivity C-reactive protein (hsCRP), ceruloplasmin, copper and magnesium were analyzed using the ARCHITECT cSystems assays developed by Abbott Laboratories (Wiesbaden, Germany) [6].

Plasma analysis was completed by Vitas Analytical Services, Oslo, Norway for B1 (thiamin and thiamin mondphosphate), B2 (FAD, FMN), and B6 (4-pyridoxic acid, pyridoxal 5 phosphate). The analysis was performed with an an Agilent LC-FLD 1200 system using a fluorescence detector (FLD). Separation of the analytes was achieved by

a Phenomenex Kinetex® column (2.6µm C18 100 Å 100 x 4.6 mm). Unknowns were calibrated against known standards from Sigma-Aldrich, and reported as nmol/l.

Data analysis

Univariate data analysis was used to evaluate changes in metabolite concentrations, clinical, and vitamin data (biochemical species) over five menstrual cycle phases. A mixed model approach was used in which all data was natural log transformed to meet normality assumptions.

In the analysisY_{ijt} is the log transformed level of participant i in (1,..,34) for biochemical species j in (1,..,397) at menstrual phase t in (1,...,5). Data were analyzed using the following Linear Mixed Model[7].

$$Y_{ijt} = b_{ij} + \mu_{jt} + \varepsilon_{ijt} \quad (1)$$

The random intercept, $b_{ij} \sim N(0, \sigma_j^b)$, accounts for the dependence of observations from the same participant, μ_{jt} is the (mean biochemical species j) fixed effect in phase t and $\epsilon_{ijt} \sim N(0, \sigma_j^{\varepsilon})$ is an independent, normally distributed error term.
Of interest are pairwise (phase-phase) differences, which we call "contrasts", defined as $(\mu_{jt} - \mu_{jt'})$ where t and t' represent two different phases, e.g. luteal and menstrual. The five phases yield ten unique pairwise differences per biochemical species j, delivering a total of 3970 (two-sided Student's t-test) p-values, denoted p_j^c for contrast c in (1,...,10) in biochemical species j.

Multiple testing adjustments were made using Bonferroni *within* biochemical species by transforming p_j^c to $\hat{p}_j^c = \min(10 \times p_j^c, 1)$, and *across* biochemical species by controlling the false discovery rate contrast-wise using Benjamini-Hochberg (BH) [8]. For each contrast c we provide the BH procedure with the $\hat{p}_j^c$ values of all biochemical species from the same panel (amino acids, lipids, et cetera) and sample (plasma, urine, et cetera) resulting in a corresponding set of FDR controlled values q_j^c. A pairwise difference c for metabolite j is considered significantly different from zero if $q_j^c < 0.20$. All biochemical species with at least one significant contrast are labeled as "entities" and studied further. This threshold is sufficiently liberal to ensure the consecutive pathway analysis is conducted on a rich set of exploratory biochemical species. All statistical analyses were conducted in the R programming language (version R 3.4.0 for Windows) using packages lme4 (1.1-13), lmtest (0.9-35) and multcomp (1.4-6) [9-11].

Metabolite reactions and subsystems analyses

The Human Genome Scale Metabolic Model or RECON 2.2.[12-14] was used for overlaying the metabolite concentration data to map changes in metabolism at different phases to a global/systems scale. Metabolites were input into the RECON model using

corresponding KEGG identities[15]. Reactions in the RECON model were identified and filtered for those with meaningful metabolic impact. Impacted metabolic subsystems were then identified based on participating reactions. Interconnectivities and differences between menstrual cycle phases were visualized using custom MATLAB (Mathwork Inc.) scripts and edited using yEd (yWorks GmbH). (**Supplementary Fig. 2a,b, and 3**).

RESULTS

Analysis of the plasma levels of estradiol, progesterone, luteal hormone and follicular stimulating hormone followed the expected temporal concentration profiles of cycle phases, however, the estradiol concentration in the follicular phase was lower than the luteal phase peak (**Supplementary Fig. 1 vs. Fig. 1**). This variation may represent the small number of women in a narrow age range in this study.

The 4 sampling windows of the original study were timed to capture the menstrual (M), follicular (F), periovular (O), luteal (L) and pre-menstrual (P) phases of the cycle. All participants were observed during one monthly cycle. A total of 33, 31, 15, 27, and 11 samples were available for the M,F,O,L,P phases respectively; and 117 samples were available for analysis of all metabolites (**Supplementary Table 1**).

Since the overall research goal was to investigate menstrual cycle metabolic rhythmicity, it is natural to assess phase dynamics of individual biochemical species. To do so, calculated phase means of each biochemical species were compared, while taking the participant-specific nature of the data into account. Each phase-phase difference, or contrast, is tested for statistical significance before ($p<0.05$) and after multiple testing control of the false discovery rate ($q<0.20$; details in the Methods section). Logarithmically transformed statistically significant metabolite patterns, per cycle phase, meeting q value threshold $q<0.20$ can be visualized in **Fig. 3**. Mean log intensity and individual variability of metabolites with 2 or more statistically significant contrast comparisons meeting $q<0.20$ are depicted in **Fig. 4**.

Plasma amino acids and biogenic amines were significantly lower in the luteal phase.
Out of the 54 amino acids and derivatives and biogenic amines detected, 48 reached statistical significance (p-value<0.05) in the 5 phase contrast comparisons: luteal-follicular (L-F), luteal-menstrual (L-M), luteal-periovulatory (L-O), premenstrual-luteal (P-L) and peri-ovular-menstrual (O-M) (**Table 1** and **Supplementary Table 1**.)

Ornithine, arginine, alanine, glycine, methionine, and proline were statistically significant in all 5 phase contrast comparisons, with the luteal phase showing a statistically significant reduction in amines relative to the other phases. After correction for multiple testing and using a q value threshold < 0.20, 37 amines reached statistical significance in the L-M contrast. Nineteen of these same amines met the q value threshold <0.20 for the L-F, 4 for L-O and 9 for P-L (**Table 2** and **Fig. 4**). Total glutathione was statistically significant only for L-F. Threonine, ornithine, and serine showed significance across the 4 phase contrast comparisons; L-M, L-F, L-O, and P-L (q<0.20) (**Table 1**).

Analysis of the same amino acids and biogenic amines in urine yielded data for 60 compounds. Twenty amino acids were statistically significant (p<0.05, **Table 1**) between phases for at least 1 phase contrast (L-F, L-M, L-O, O-M) with L-O and O-M having the highest number of statistically significant differences (**Table 1** and **Supplementary Table 1**).Threonine differed in 3 comparisons and was the only amino acid that reached the FDR threshold in urine (q<0.20 for L-O).

Plasma phospholipids were significantly reduced in the luteal phase.
Of the 139 lipid species with detectable plasma levels, 57 reached statistical significance, (p-value <0.05), for 1 to 5 phase contrast comparisons: L-F, L-M, L-O, P-L and O-M (specific p-values in **Table 2**). Thirty eight percent of the lipid species tested (53/139) consistently showed a statistically significant decrease in the luteal phase relative to the follicular and in some cases, relative to the menstrual phase (16/139) with 7 compounds showing a decrease in comparison to the premenstrual phase and 2 in comparison to the periovulatory phase. One compound, LPE 22:6, showed a statistically significant difference in 4 out of the 5 phase contrasts: L-F, L-M, L-O and P-L. After multiple testing, at q<0.20, 17 lipid species met this threshold for L-F including 6 LPCs, 10 PCs and 1 LPE. One other LPC met this threshold for O-M (**Table 2**).

Vitamin D and pyridoxic acid increased in the menstrual phase.
Nineteen clinical parameters were tested including eight B vitamins, cofactors and metabolites. C reactive protein (CRP) was statistically significant in L-F (p<0.05), while high density lipoprotein (HDL), triglycerides and cholesterol were statistically significant in L-F (p<0.05). Glucose showed rhythmicity with a statistically significant decrease in the luteal phase in comparison to menstrual, pre-menstrual and periovulatory phases (p<0.05). Magnesium showed a statistically significant decrease in L-M and O-M and riboflavin showed a statistically significant decrease in luteal vs. pre-menstrual phases. However, these results were not significant when corrected for multiple testing (q<0.20).

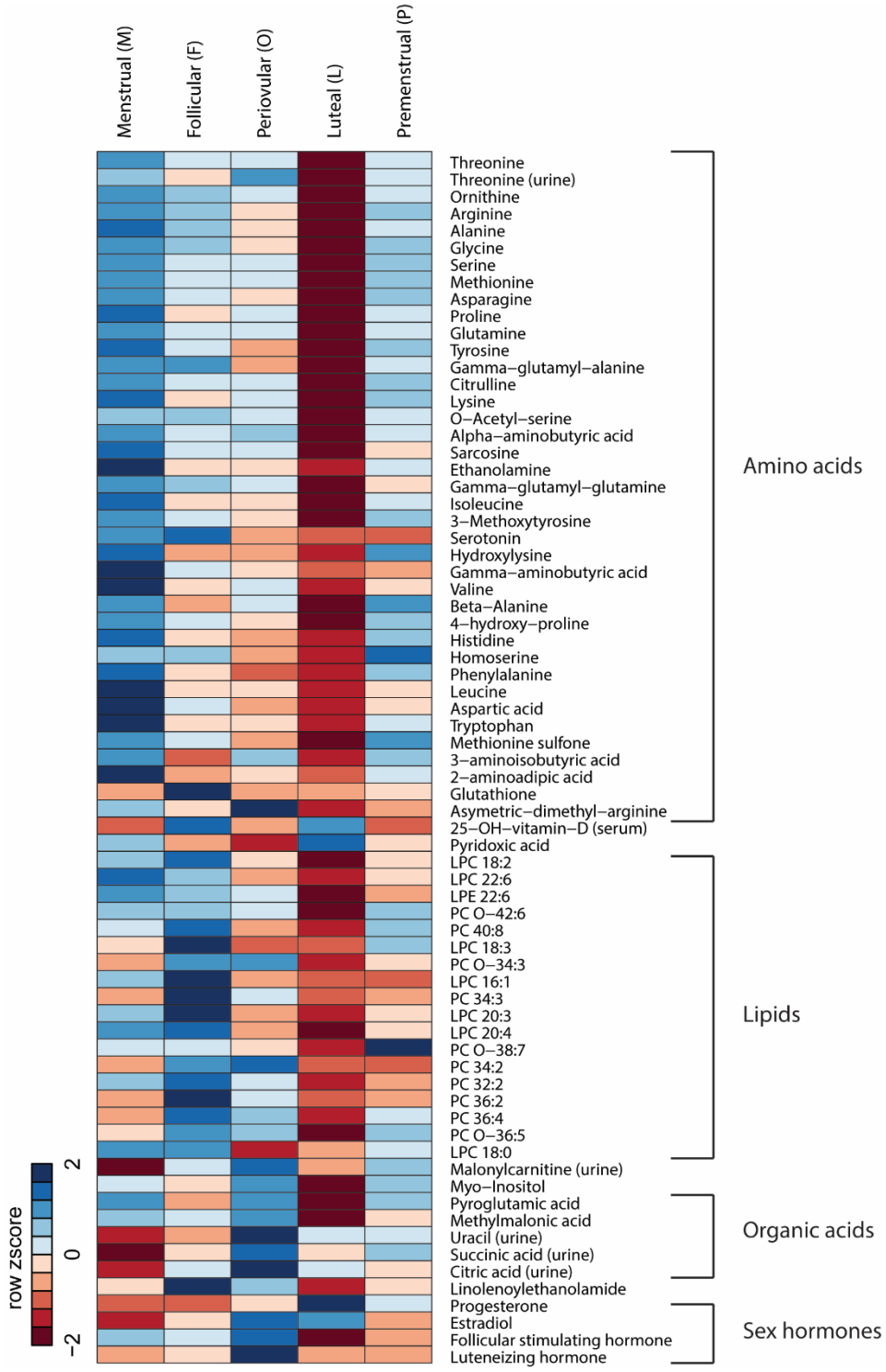


Fig. 3. Metabolites vary across menstrual cycle phase. This heatmap with color gradients indicates rhythmicity across the menstrual cycle. Lower amino acid and lipid metabolite concentrations are visualized in the luteal phase. Phase means of logarithmically transformed metabolite data are row standardized in the heatmap to obtain Z scores. Two cells that are close in color represent similar Z scores, ranging from blue (Z equals minus 2) to red (Z equals plus 2). Amino acid, lipid, organic acid and sex hormone variables are ordered according to main biochemical pathways or classes and depicted at q <0.20 after contrast analyses. Menstrual (M), Follicular (F), Periovulatory (O), Luteal (L), Premenstrual (P) phases are depicted. LPC- Lysophosphatidylcholine, LPE- Lysophosphatidylethanolamine, PC- phosphatidylcholine.

Vitamin D (25-OH vitamin D) showed significant decreases in L-F, L-M and O-M with L-M and O-M and met the multiple testing threshold q<0.20). Moreover, the menstrual phase consistently showed higher levels of vitamin D. Pyridoxic acid also showed an elevation in the menstrual phase compared to the periovulatory (q<0.20, **Table 2**)

Plasma and urine acylcarnitines showed an increase in the periovulatory phase.
Of the 50 compounds tested in the acylcarnitine panel, 19 were statistically significant in the plasma and 16 statistically significant in urine (p<0.05). The majority of plasma and urine metabolites were altered in O-M with an increase in the periovulatory phase. However, only urinary malonylcarnitine reached the q<0.20 threshold for multiple testing (**Table 2 and Supplementary Table 2**).

Organic acids and endocannobinoids showed different patterns between phases.
Sixteen organic acid metabolites had concentration levels above the respective limit of quantification in plasma out of which 10 reached statistical significance, p<0.05. Inositol, pyroglutamic acid and methylmalonic acid reached the multiple testing threshold (q<0.20) for L-M for all 3 metabolites and L-O for inositol and pyroglutamic acid **(Table 2)**.

Twenty-three organic acid metabolites were analyzed in urine, of which 14 were statistically significant, p<0.05, in various contrasts. Uracil, succinic acid and citric acid reached the multiple testing threshold q<0.20 for O-M (**Table 2**).

In plasma, 19 endocannabinoids had detectable levels, of which 5 demonstrated statistical significance p<0.05 across L-F, L-M, L-O, and O-M; and LEA reached statistical significance for L-F,L-M and L-O and met the multiple testing threshold (q<0.20) for L-F (**Table 2**).

Metabolite reactions and subsystems analyses demonstrated interconnectivity and differentiation between menstrual cycle phases
The KEGG database and Human RECON model were used to enrich functional understanding of the 71 compounds that met multiple testing q-value (<0.20). KEGG currently maps 18,111 metabolites to 519 pathways and RECON 2.2 uses 5324 metabolites and 7785 primarlly intracelluar reactions. Sixty-two of the 71 compounds in this study were identified in the KEGG pathways and 41 of these 62 entitites could be mapped in RECON 2. These 41 metabolites participate in 1213 reactions (out of the 7440 reactions tabulated). We do not consider the 710 reactions for extracellular transport and the 84 reactions for exchange/demand. Thus, 419 reactions remained for further analysis and

interpretation. Metabolite subsystem analyses of the 41 metabolic entities in the 419 reactions identified showed reduced glutathione, succinate, L-histidine and glycine followed by L-lysine, L-alanine, L-arginine and L-serine impact or are impacted by the most number of metabolic reactions (**Supplementary Fig. 2A**).

The 34 impacted sub-systems in the metabolic landscape include amino acid metabolism/synthesis; such as glutathione metabolism and the urea cycle; eicosanoid metabolism; citric acid cycle and bile acid synthesis. (**Supplementary Fig. 2b**) show the global, significant impact of changes in menstrual phase on metabolism.

A deeper pathway analysis in which interconnected, amino acid phase contrasts were compared, revealed minor differences between the 2 significant phase contrast categories. The luteal menstrual contrast did not show a significant difference in glutathione levels like the luteal follicular contrast (**Supplementary Fig. 3**).

DISCUSSION

The present study demonstrates the rhythmic synchronicity of the menstrual cycle with healthy metabolism. Using deep molecular phenotyping of 5 menstrual cycle phases, paired with sex hormone rhythmicity, 67 biochemical species of amino acid, lipid, carbohydrate, energy and vitamin metabolism significantly changed between phases; particularly, with a decrease in the luteal relative to menstrual and follicular phases (**Fig. 3**). Much fewer changes were observed in the urine in comparison to the plasma. These biochemical species comprise major biochemical pathways which impact physiological functioning and may increase vulnerability to sex-hormone related disorders, such as PMS, PMDD, and polycystic ovarian syndrome (PCOS).

Luteal phase protein, lipid, steroid, endometrial biosynthesis and increased energy utilization may lead to reduced biomarkers relative to menstrual and follicular phases. The decrease in amino acid plasma levels observed in the luteal phase, particularly in comparison to the menstrual phase, may be associated with progesterone's upregulation of cell cycle progression and growth and the associated protein biosynthesis required for endometrial thickening to prepare the uterus for pregnancy [16] (**Table 1** and **Fig. 3** and **Fig. 4**). The decreased amino acid concentrations that participate in the urea cycle (arginine, ornithine and citrulline) suggest reduced ammonia waste in the luteal phase, which supports progesterone's anabolic amino acid use (**Fig. 5**). Sex hormone regulation of nitrogen utilization through nitrogen excretion fluctuation [17, 18] and reduced concentrations of amino acids in the luteal phase suggest the intake of a higher protein load might, in certain instances, be advantageous to support additional nitrogen needs.

Table 1. Sex hormones and amino acids

	Effect Size**					FDR q values****				
Biomarkers	**L-F***	**L-M**	**L-O**	**P-L**	**O-M**	**q L-F**	**q L-M**	**q L-O**	**q P-L**	**q O-M**
Sex Hormones, serum										
Progesterone (ng/L)	2.88	2.80	2.30	-1.51	0.50	0	0	0	**2.00E-11**	**7.87E-02**
Estradiol (pmol/L)	0.94	1.79	-0.10	-1.19	1.89	**1.32E-09**	0		**2.88E-08**	0
Follicular stimulating hormone (FSH) (IU/L)	-0.75	-0.80	-1.16	0.42	0.36	0	0	0	**1.37E-03**	**3.52E-03**
Luteneizing hormone (LH) (IU/L)	-0.27	0.00	-1.40	0.04	1.39	2.72E-01		0		0
Amines, plasma										
L-Threonine	-0.45	-0.59	-0.46	0.43	-0.13	**6.73E-09**	**0**	**2.24E-05**	**1.67E-03**	
Ornithine	-0.35	-0.47	-0.31	0.31	-0.16	**2.12E-05**	**2.10E-11**	**4.67E-02**	**9.32E-02**	
L-Arginine	-0.34	-0.47	-0.26	0.28	-0.21	**5.51E-04**	**1.87E-08**	3.47E-01	3.62E-01	
L-Alanine	-0.35	-0.45	-0.24	0.35	-0.21	**3.93E-04**	**3.29E-08**	4.59E-01	**9.32E-02**	
Glycine	-0.31	-0.40	-0.23	0.34	-0.17	**4.36E-04**	**6.16E-08**	3.71E-01	**9.32E-02**	
L-Serine	-0.26	-0.37	-0.25	0.28	-0.11	**2.50E-03**	**9.20E-08**	**1.93E-01**	**1.01E-01**	
L-Methionine	-0.25	-0.37	-0.22	0.28	-0.15	**4.95E-03**	**9.20E-08**	3.47E-01	**1.01E-01**	
L-Asparagine	-0.27	-0.37	-0.22	0.30	-0.15	**2.89E-03**	**5.24E-07**	4.52E-01	**9.32E-02**	
L-Proline	-0.21	-0.38	-0.18	0.29	-0.20	**1.30E-01**	**1.57E-06**		2.05E-01	
L-Glutamine	-0.24	-0.32	-0.21	0.24	-0.11	**4.21E-03**	**2.58E-06**	3.47E-01	**1.94E-01**	
L-Tyrosine	-0.18	-0.32	-0.12	0.25	-0.20	**1.74E-01**	**1.32E-05**		3.04E-01	
Gamma-L-glutamyL-L-alanine	-0.49	-0.51	-0.23	0.33	-0.28	**3.93E-04**	**1.34E-05**		7.02E-01	
Citrulline	-0.22	-0.31	-0.23	0.26	-0.09	**2.11E-02**	**1.34E-05**	3.47E-01	**1.87E-01**	
L-Lysine	-0.18	-0.32	-0.21	0.23	-0.11	2.26E-01	**1.34E-05**	4.59E-01	4.63E-01	
O-AcetyL-L-serine	-0.31	-0.33	-0.25	0.24	-0.07	**5.51E-04**	**2.12E-05**	2.90E-01	3.82E-01	
L-Alpha-aminobutyric acid	-0.25	-0.37	-0.32	0.23	-0.05	**4.81E-02**	**2.33E-05**	**1.74E-01**	9.54E-01	
Sarcosine	-0.17	-0.32	-0.09	0.17	-0.23	3.69E-01	**5.34E-05**			
Ethanolamine	-0.11	-0.23	-0.07	0.11	-0.16		**4.98E-04**			
Gamma-glutamylglutamine	-0.34	-0.41	-0.28	0.22	-0.13	**3.51E-02**	**7.35E-04**			
L-Isoleucine	-0.14	-0.24	-0.08	0.16	-0.16	5.87E-01	**1.09E-03**			
3-Methoxytyrosine	-0.20	-0.25	-0.16	0.21	-0.09	**7.40E-02**	**1.17E-03**		6.48E-01	
Serotonin	-0.71	-0.57	-0.12	0.06	-0.46	**1.17E-03**	**6.95E-03**			
Hydroxylysine	-0.08	-0.22	-0.05	0.17	-0.17		**1.53E-02**			
Gamma-aminobutyric acid	-0.10	-0.22	-0.05	0.02	-0.17		**1.58E-02**			
L-Valine	-0.11	-0.21	-0.08	0.11	-0.13		**1.66E-02**			
Beta-Alanine	-0.05	-0.11	-0.06	0.09	-0.05		**1.72E-02**			
L-4-hydroxy-proline	-0.26	-0.32	-0.19	0.35	-0.13	2.42E-01	**1.78E-02**		5.12E-01	
L-Histidine	-0.09	-0.19	-0.06	0.14	-0.13		**3.89E-02**			
L-Homoserine	-0.23	-0.24	-0.09	0.34	-0.15	**1.61E-01**	**4.08E-02**		**1.49E-01**	
L-Phenylalanine	-0.08	-0.19	-0.01	0.14	-0.17		**4.79E-02**			
L-Leucine	-0.07	-0.18	-0.05	0.09	-0.13		**4.79E-02**			
L-Aspartic acid	-0.15	-0.28	-0.07	0.10	-0.21		**1.00E-01**			
L-Tryptophan	-0.08	-0.18	-0.05	0.10	-0.13		**1.06E-01**			
Methionine sulfone	-0.14	-0.17	-0.09	0.16	-0.08	7.41E-01	**1.12E-01**			
DL-3-aminoisobutyric acid	-0.07	-0.19	-0.21	0.11	0.02		**1.74E-01**			
L-2-aminoadipic acid	-0.06	-0.18	-0.05	0.10	-0.14		**1.91E-01**			
Glutathione	-0.33	-0.01	0.00	0.01	-0.02	**1.61E-01**				
Assymetric dimethyl arginine (ADMA)	-0.08	-0.14	-0.22	0.03	0.08		**1.96E-01**	2.36E-01		
Amines, urine										
L-Threonine	-0.29	-0.40	-0.54	0.19	0.14	1	3.54E-01	**1.79E-01**		

Measured sex hormone (progesterone, estradiol, follicular stimulating hormone, lutenizing hormone) and 39 amino acid effect sizes and q values are listed for all significant phase comparison categories. *Luteal/follicular (L-F), luteal-menstrual (L-M), luteal-periovulatory (L-O), premenstrual-luteal (P-L) and peri-ovular-menstrual (O-M); ** All results are based on natural log transformed data. Effect sizes are estimated phase-phase differences ("contrasts"). *** Student's t-test p-values of the contrasts are controlled for multiple testing within metabolites by Bonferroni and across metabolites using Benjamini-Hochberg FDR, resulting in q values as listed. Significant q values are in bold. Values above 0.50 are intentionally left blank.

Table 2. Vitamins, lipids, acylcarnitines, organic acids, endocannabinoids

	Effect Size					FDR q values				
Biomarkers	**L-F**	**L-M**	**L-O**	**P-L**	**O-M**	**q L-F**	**q L-M**	**q L-O**	**q P-L**	**q O-M**
Vitamins										
25-OH-vitamin-D - serum (nmol/L)	-0.06	-0.09	0.01	0.00	-0.10	4.82E-01	**1.21E-02**			**3.85E-02**
Pyridoxic acid - plasma (nmol/L)	0.05	-0.16	0.29	0.18	-0.45					**1.18E-01**
Lipids, plasma										
LPC 18.2	-0.26	-0.17	-0.08	0.22	-0.09	**4.78E-04**	2.89E-01			
LPC 22.6	-0.20	-0.18	-0.09	0.17	-0.09	**1.76E-02**	2.89E-01			
LPE 22.6	-0.34	-0.27	-0.21	0.27	-0.06	**1.45E-02**	2.89E-01			
PC O.42.6	-0.13	-0.09	-0.06	0.10	-0.03	**1.11E-02**	9.34E-01			
PC 40.8	-0.15	-0.07	-0.01	0.09	-0.06	**2.41E-03**				
LPC 18.3	-0.30	-0.09	0.02	0.26	-0.11	**1.76E-02**				
PC O.34.3	-0.14	-0.02	-0.09	0.09	0.08	**2.64E-02**				
LPC 16.1	-0.20	-0.11	-0.02	0.05	-0.08	**1.76E-02**				
PC 34.3	-0.19	-0.02	-0.04	0.10	0.02	**3.30E-02**				
LPC 20.3	-0.16	-0.07	0.02	0.07	-0.10	**3.83E-02**				
LPC 20.4	-0.17	-0.12	-0.06	0.12	-0.06	**1.76E-02**				
PC O.38.7	-0.09	-0.02	-0.04	0.11	0.02	**7.74E-02**				
PC 34.2	-0.08	-0.02	-0.06	0.02	0.03	**3.30E-02**				
PC 32.2	-0.26	-0.19	-0.05	0.17	-0.14	**3.30E-02**				
PC 36.2	-0.09	-0.02	-0.02	0.03	0.00	**9.41E-02**				
PC 36.4	-0.08	-0.01	-0.04	0.03	0.02	**1.26E-01**				
PC O.36.5	-0.13	-0.03	-0.07	0.10	0.04	**1.26E-01**				
LPC 18.0	-0.11	-0.11	0.08	0.03	-0.19	4.78E-01				**1.60E-01**
Acylcarnitines, urine										
Malonylcarnitine	-0.18	0.18	-0.43	0.19	0.61					**1.96E-01**
Organic Acids, plasma										
Inositol	-0.20	-0.30	-0.40	0.30	0.10		**9.53E-02**	**9.62E-02**		
Pyroglutamic acid	-0.14	-0.39	-0.46	0.23	0.07		**2.11E-02**	**9.62E-02**		
Methylmalonic acid	-0.12	-0.18	-0.13	0.06	-0.04		**9.53E-02**			
Organic Acids, urine										
Uracil	0.27	0.39	-0.23	-0.28	0.61		4.11E-01			**3.74E-02**
Succinic acid	-0.03	0.26	-0.30	0.07	0.57					**9.67E-02**
Citric acid	0.00	0.17	-0.16	-0.03	0.33					**1.80E-01**
Endocannabinoids, plasma										
Linolenoylethanolamide (LEA)	-0.15	-0.08	-0.11	0.02	0.03	**1.92E-02**				

Measured vitamins (25-OH-vitamin D, pyridoxic acid) ,lipids (7 LPC, 1 LPE, 10 PC), malonylcarnitine, plasma organic acids (inositol, pyroglutamic acid, methylmalonic acid), urine organic acids (uracil, succinic acid, citric acid) and linolenylethanolamide are listed for all significant phase comparison categories. *Luteal/follicular (L-F), luteal-menstrual (L-M), luteal-periovulatory (L-O), premenstrual-luteal (P-L) and peri-ovular-menstrual (O-M); ** All results are based on natural log transformed data. Effect sizes are estimated phase-phase differences ("contrasts"). *** Student's t-test p-values of the contrasts are controlled for multiple testing within metabolites by Bonferroni and across metabolites using Benjamini-Hochberg FDR, resulting in q values as listed. Significant q values are in bold.Values above 0.50 are intentionally left blank. LPC, Lysophosphatidylcholine, LPE-Lysophosphatidylethanolamine, PC-phosphatidylcholine.

Women have higher energy expenditure and compensate by eating more in the luteal phase, particularly protein; suggesting the anabolism in this phase could be greater than the degree of difference we observe using the tested metabolomics technologies [19, 20].

The anabolic effect of the luteal phase does not appear to be limited to amino acids, as certain lipids decreased in the luteal relative to follicular phases suggesting a higher utilization of fat for lipid or steroid synthesis, and/or an increase in fat absorption with less need for anabolism in the follicular phase (**Table 2** and **Fig. 3**). Previous research demonstrates total phospholipid content of the endometrium is increased in the luteal phase by 26% relative to the periovulatory time-period[21]. A 10-fold increase of phospholipase A_2 in endometrial tissue has previously been identified in the luteal phase[22]. Partial hydrolysis of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) by phospholipase A_2 produces lysophosphatidlycholines (LPC) and lysophosphatidlyethanolamines (LPE).

PE and LPC are minor phospholipids found in cell membranes such as myelin sheaths and erythrocytes; playing roles in cell signaling and enzyme activation. Our findings of decreased PCs, LPCs, PEs and LPEs are consistent with their anabolic use for endometrial tissue thickening for pregnancy preparation during the luteal phase. Previous research identified PCs, LPCs and LPEs are further reduced in the luteal phase of PCOS patients versus healthy controls [23, 24]. Thus, lower phospholipids in the luteal phase could be physiologically normal. However, certain diseases may perturb this state further, suggesting augmentation of physiologic vulnerability and highlighting the importance of studying hormonal rhythmicity in health and disease.

Endocannabinoids are known to interact with sex hormones and cytokines to regulate fertility [25]. Conversely, changes in levels of sex hormones are known to alter endocannabinoid signaling [26]. The central nervous system is a rich source of endocannabinoids, highly sensitive to inflammation and this interaction is implicated in PMDD [27]. Additionally, endocannabinoids are generated from membrane phospholipids [25, 28]. In the present study, one endocannabinoid, linolenoyl ethanolamide (LEA), demonstrated luteal phase rhythmicity, which has not been demonstrated previously (**Table 2**). The low concentration of LEA observed may be a result of phosphotidylethanolamine use for endometrium development. This results in less LEA available for endocannabinoid generation.

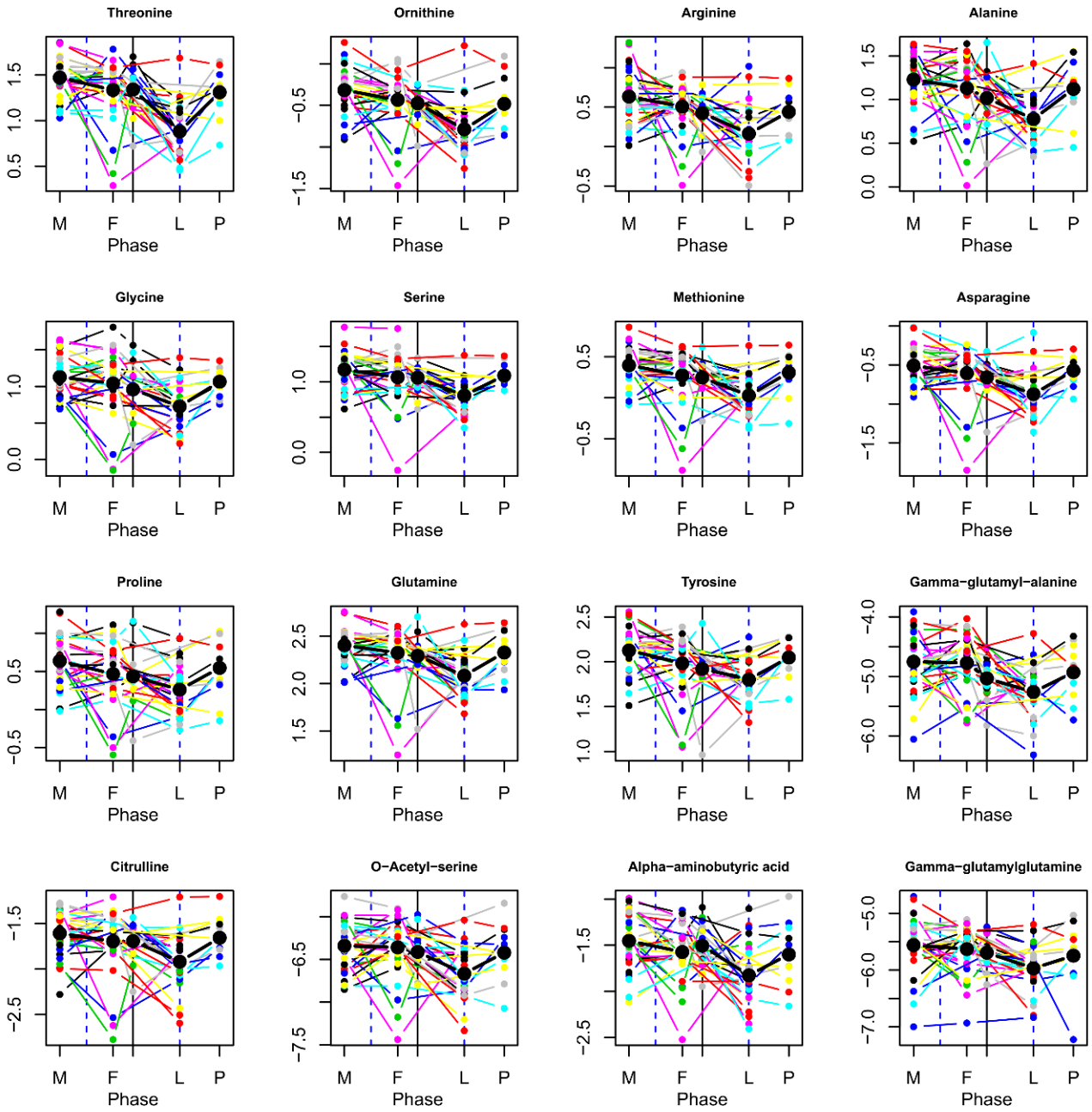


Fig. 4. Amino acid variability by cycle phase. Mean log intensity is depicted along with individual variability for threonine, ornithine, arginine, alanine, glycine, serine, methionine, asparagine, proline, glutamine, tyrosine, gamma-glutamyl-alanine, citrulline, o-acetyl-serine, alpha-aminobutyric acid, and gamma-glutamylglutamine at one time point for each of the 5 menstrual phases (M=menstrual, F=follicular, O=periovular, L=luteal, p=premenstrual). Each colored line represents an individual. Amino acids are depicted which have 2 or more contrast comparisons meeting the multiple testing threshold of q<0.20. Statistically significant luteal phase reductions can be observed.

Further research is needed to identify if this can increase vulnerability to a sub-optimal stress response in individuals susceptible to PMS or PMDD, particularly when combined with low concentrations of amino acid precursors of neurotransmitter metabolism.

Medium and longchain acylcarnitines are formed from fatty acid oxidation, and elevated in inflammation, menopause and lower in PCOS [1, 2]. In our healthy, premenopausal population, a trend of upregulated acylcarnitines was observed in the periovular phase from urine and plasma which may reflect a higher state of inflammation and demand for beta oxidation and energy utilization [3] (**Supplementary Table 2**).

Clinical laboratory diagnostics used in practice can vary with the menstrual cycle due to increased anabolic demands in the luteal phase, for example, and should be interpreted with caution. Cholesterol is a key constituent of sex hormones and is utilized during the luteal phase for progesterone and estrogen synthesis. In the present study, cholesterol and HDL showed significant trends with reductions in the luteal relative to the follicular phases and, consistent with prior literature [4, 5]. Triglyceride concentrations are known to be reduced in the luteal phase, as was observed in our study; and, more specifically, are reduced 30% from estradiol treatment (but not progesterone) due to accelerated VLDL-TG plasma clearance [6, 7]. Thus, caution is warranted when interpreting cholesterol and triglyceride laboratory results (**Table 2**).

While not yet accepted as a biomarker for clinical practice, inositol is produced by the human body from glucose and may be in high demand to meet the anabolic requirement of luteal phase pregnancy preparation. It plays a key role in insulin signal transduction, lipid transport and catabolism, oocyte maturation, embryonic development and cytoskeleton assembly which influence the steroidogenesis process [8-10]. We observed a significant reduction in luteal phase myo-inositol (**Table 2**). The trend observed of reduced glucose concentration in the luteal phase may have led to the reduction in inositol production (**Supplementary Table 2**).

Significant neurotransmitter, amino acid and B vitamin precursor rhythmicity may influence susceptibility to the cyclical stress, anxiety and depression implicated in PMS and PMDD. Women are more affected than men by depressive disorders during the time between menarche and menopause suggesting this increase in depression risk is sex hormone related [11-13]. For example, γ-amino-butyric-acid (GABA) inhibition has been implicated in depression pathophysiology, differs between men and women, and can be modulated by progesterone and estrogen [14, 15]. We have observed a significant reduction of the neurotransmitter serotonin and several neurotransmitter metabolic

precursors (tyrosine, tryptophan, 3-methoxytyrosine, GABA, L-phenylalanine) which is consistent with previous research that demonstrates reduced mood enhancing neurotransmitter metabolite levels in the luteal phase, such as, 5-hydroxyindoleacetic acid (5-HIAA), the serotonin metabolite [16] (**Table 1**, and **Fig. 5**).

Four-pyridoxic acid is one of two major vitamin B-6 compounds present in plasma. It is a cofactor in sex hormone gene expression and neurotransmitter metabolism through the conversion of tryptophan to serotonin [17]. It is also a cofactor for GABA synthesis (**Fig. 5**). Supplementation with B6 has been shown to improve the psychiatric symptoms of PMS [18, 19]. In our study, 4-pyridoxic acid was significantly lower in the periovulatory phase, which may increase susceptibility to premenstrual syndrome in vulnerable individuals should there be insufficient B6 for tryptophan to serotonin conversion in the brain (Table 2). The periovulatory reduction in 4-pyridoxic acid may lead to the cystathionine depletion observed in the luteal phase as B6 is a cofactor for the cystathione-β–synthase conversion to cystationine (**Table 2** and **Fig. 5**).

Rhythmicity in glutathione and associated metabolites may lead to oxidative stress and impaired liver detoxification and may be associated with sex hormone influences in oxidative stress and drug metabolism [20-22]. Sex hormones have been shown to be correlated with redox balance during the menstrual cycle in the endometrium through modulation of glutathione metabolism [23]. In the present study, plasma total glutathione and its' precursors; glycine, γ-glutamyl-alanine, and pyroglutamic acid showed significant differences across the menstrual cycle with the precursors following the same pattern of lowest concentrations in the luteal phase (**Table 1** and **Fig. 5**). In the present study, the reduced glutathione precursors in the luteal phase significantly increased in the menstrual phase, which may be necessary to precede glutathione's successful follicular phase regeneration (**Supplementary Fig. 3**). Previous findings correlate elevated glutathione and glutathione peroxidase activity with the estrogen peak in the follicular phase [24-26]; and may be one mechanism through which estrogens attenuate oxidative stress [27, 28]. Individuals with PMS have been shown to have an imbalance in oxidant/antioxidant status and may be more susceptible in a state of low glutathione metabolic activity in the luteal phase [29].

Vitamin D supplementation, when combined with calcium, has been used to improve weight loss, menstrual regularity, hyperandrogenism and, possibly, fertility; in women with PCOS [30]. High dietary intake of vitamin D may reduce risk of PMS which may be related to its capacity to activate serotonin synthesis [31, 32]. Vitamin D regulates calcium and bone health, sex steroidogenesis, and interacts with progesterone to regulate the immune system through T cell induction of the vitamin D receptor [33].

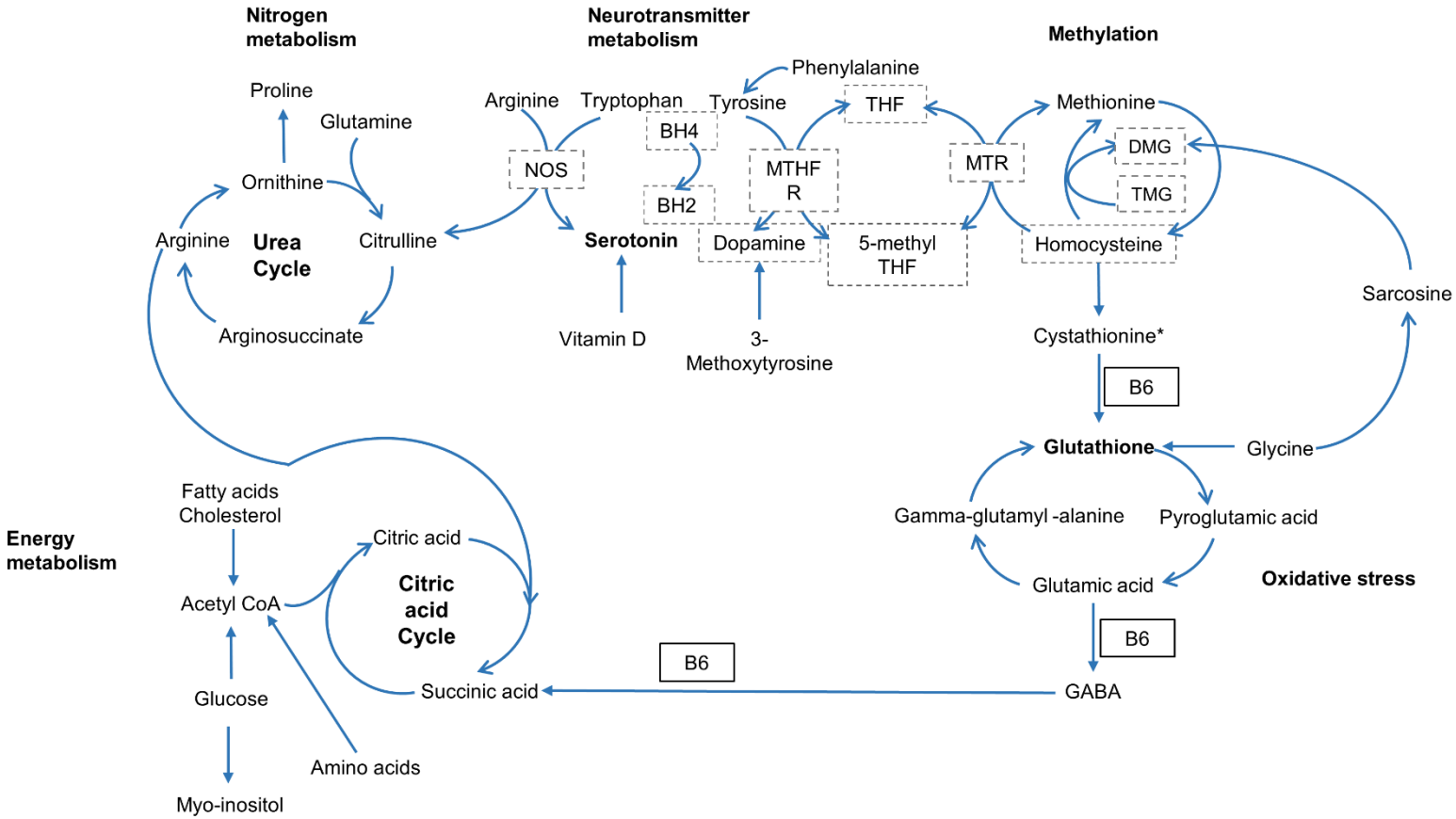


Fig. 5. Rhythmic metabolites in the urea cycle, neurotransmitter metabolism connect with 1 carbon, glutathione metabolism and the citric acid cycle. The metabolites with FDR controlled rhythmicity participate in inter-related, biochemical pathways including nitrogen metabolism (the urea cycle), neurotransmitter metabolism, methylation (1 carbon metabolism), oxidative stress (glutathione metabolism) and energy metabolism (citric acid cycle). NOS = Nitric oxide synthase; BH_4 = Tetrahydrobiopterin; BH_2 = Bihydrobiopterin; MTHFR = Methylenetetrahydrofolate reductase; THF = Tetrahydrofolate; MTR = Methionine synthase; DMG = Dimethylglycine; TMG = Trimethylglycine; B6 = Vitamin B6. Compounds boxed with dotted lines (NOS, BH_4, BH_2, MTHFR, THF, MTR, 5-methyl THF, DMG, TMG, homocysteine) were not evaluated or not significant (dopamine). All metabolites without dotted lines met the multiple testing threshold q<0.20. *Cystathionine was statistically significant with p value <0.05, but did not meet the multiple testing threshold.

A reduction of 25-hydroxyvitamin D (25OH-vitamin D) associated with a decrease in estradiol is seen in post-menopause and likely related to the sensitive interdependency between changes in estrogen levels and vitamin D binding protein[1]. In human follicular cells, it alters FSH sensitivity and participates in folliculogenesis and progesterone production, indicating a possible role in follicular development and luteinization[2]. In our study, the significant reduction of vitamin D in the luteal and periovulatory phases may reflect a higher utilization for folliculogenesis in the periovulatory phase and progesterone synthesis during the luteal phase, which has been suggested in prior research [3] (**Table 2**).

Fatty acids contained in the phospholipids found reduced in the luteal phase of the present study participate in inflammation modulation through eicosanoid signaling, including linoleic (18:2,ω6), docosahexaenoic (22:6,ω3), stearic (18:0ω6), linolenic (18:3,ω6), arachidonic (20:4,ω6), acids (**Table 2**). It has been suggested conversion of linoleic to α-linolenic acid is reduced in PMS [4]. Thus, a state of low linolenic acid could be further augmented in the luteal phase in individuals susceptible to PMS leading to inflammation [5]. PMS symptoms related to inflammation include mood, abdominal cramps, back pain, breast tenderness, appetite cravings, weight gain and bloating [6]. Symptoms of PMS are associated with elevated CRP, which was also observed in the luteal phase [6]; and the elevated luteal phase acylcarnitines may also potentiate a hyperinflammatory state [7].

Health can be defined as the ability of a living being to adapt and to self manage [8]. The healthy, physiologic state of rhythmicity must be defined to understand the perturbations that need adaptation and management. Identification of biochemical variations in a healthy menstrual cycle can provide a foundation of comparison for future deep phenotyping, such as phenotypic challenges of adaptation [9, 10], for sex hormone related disorders, such as PMS, and PMDD.

This data obtained from healthy women highlights the importance of deeper research on metabolism and sex hormone rhythmicity to understand how therapeutic strategies could be developed to treat challenging medical conditions such as PMS and PMDD. Perturbations in rhythmicity caused by diet, stress and environmental toxins may impact sex hormone related health challenges and result in a loss of rhythmicity and, thus, therapeutic strategies, such as dietary change, may be optimal for restoration. The dietary implications of this study's findings deserve further testing in a population vulnerable to insufficient diet intake to sustain healthy rhythmicity such as the large population of women with symptoms of PMS and PMDD. Higher protein load, phosphatidylcholine, omega 3 [11] and omega 6 [5] fatty acid intakes may be implicated

in the luteal phase along with assurance of sufficient vitamin D intake [12]/sun exposure, B6 [13] sulfur containing vegetables to promote glutathione metabolism [14], and antioxidant food sources [15] intake throughout the cycle.

The following summarizes the key limitations in our study. Dietary intake has a significant impact on metabolomics results [16]. While participants limited tea, coffee, fish, alcohol and vigorous exercise 24h prior to sampling[17]; dietary data were not collected throughout the cycle to assess the associations between differences in food intake and biochemical changes in the blood, plasma and urine. This study was limited in its capacity to detect significant vitamin differences due to missing values from limited sample volumes. In order to develop effective, sensitive diagnostics using metabolomics technologies, more time point measurements could enhance the granularity of conclusions about biochemical changes. Participants were excluded if they were diagnosed with a health condition, however, women who may have had recurrent PMS symptoms in the setting of a healthy menstrual cycle, insufficient to necessitate a medical diagnosis, may not have been excluded.

Our study is, to the best of our knowledge, the first of its kind to conduct a deep phenotyping of the metabolomic, lipidomic, and nutrient biomarker differences across menstrual cycle phases in healthy women. Significant changes in levels of several amino acid and lipid metabolites were identified in addition to those characterized previously [18]. Amino acid and lipid metabolites were reduced in the luteal phase, suggesting differences in anabolic requirements related to changing hormone levels. Rhythmic differences in neurotransmitter related amino acid precursors, vitamin cofactors and stress related metabolites may influence predisposition for anxiety and depression related PMS or PMDD. Glutathione and associated amino acid precursors show rhythmic differences suggesting a greater propensity for oxidative stress throughout the menstrual cycle. The reduction of amino acid levels in the luteal phase combined with prior research on cyclical nitrogen fluctuation, increased energy metabolism and food intake may suggest the intake of a higher protein load as a portion of the increased calorie intake would be advantageous. A menu plan that optimizes protein intake, B6, omega 3 and omega 6 fatty acid and glutathione metabolism deserves further testing in a population at risk of PMS or PMDD. The information generated from this study provides the foundation for research on differences in menstrual cycle, sex hormone related metabolism and clinical biomarker interpretations. Furthermore, it forms the basis to test novel nutrition strategies for women with an emphasis on health issues impacted by rhythmic variability.

ACKNOWLEDGEMENTS

We are grateful to University College Dublin for providing plasma, serum and urine samples for this study; to Leiden University for their support of metabolomics and statistical analyses; and to Nestle Institute of Health Sciences for funding support. The authors acknowledge the scientific support of M. Wallace, J. Kaput, T. Kloots, B. Gonzalez, M.M. Nieman, M. Martinez-Zapata, H.L. Elfrink, K. Sakamoto, J. Hager, T. Gunderson, M. Kussmann, M., C. Gobet, F. Gachon, A. Valsesia and Juliana Haber leading to the completion of this study. S.M. is a participant of the EU-funded COST Action FA1403 POSITIVe (interindividual variation in response to consumption of plant food bioactives and determinants involved).

REFERENCES

[1] Quabbe, H. J., Chronobiology of growth hormone secretion. *Chronobiologia* 1977, *4*, 217-246.
[2] Baker, F. C., Driver, H. S., Circadian rhythms, sleep, and the menstrual cycle. *Sleep Med* 2007, *8*, 613-622.
[3] Van Reen, E., Kiesner, J., Individual differences in self-reported difficulty sleeping across the menstrual cycle. *Arch Womens Ment Health* 2016, *19*, 599-608.
[4] Glass, L., Synchronization and rhythmic processes in physiology. *Nature* 2001, *410*, 277-284.
[5] Shechter, A., Lesperance, P., Ng Ying Kin, N. M., Boivin, D. B., Pilot investigation of the circadian plasma melatonin rhythm across the menstrual cycle in a small group of women with premenstrual dysphoric disorder. *PLoS One* 2012, *7*, e51929.
[6] Li, R., Cheng, S., Wang, Z., Circadian clock gene plays a key role on ovarian cycle and spontaneous abortion. *Cell Physiol Biochem* 2015, *37*, 911-920.
[7] Allen, A. M., McRae-Clark, A. L., Carlson, S., Saladin, M. E., Gray, K. M., Wetherington, C. L., McKee, S. A., Allen, S. S., Determining menstrual phase in human biobehavioral research: A review with recommendations. *Exp Clin Psychopharmacol* 2016, *24*, 1-11.
[8] Strauss, J. F. B., Robert L., *Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management, Seventh Edition*, Saunders, Philadelphia, Pennsylvania 2014.
[9] Voet, D. e. a., *Biochemistry*, John Wiley & Sons, U.S.A., & Canada 1995.
[10] Gugapriya, T. S., Karthick, S., Nagarjuna, B., A Prospective Study of Variability in Glycemic Control during Different Phases of the Menstrual Cycle in Type 2 Diabetic Women Using High Sensitivity C - Reactive Protein. *Journal of clinical and diagnostic research : JCDR* 2014, *8*, CC01-04.
[11] Varlamov, O., Bethea, C. L., Roberts, C. T., Jr., Sex-specific differences in lipid and glucose metabolism. *Frontiers in endocrinology* 2014, *5*, 241.
[12] Yen, J. Y., Chang, S. J., Ko, C. H., Yen, C. F., Chen, C. S., Yeh, Y. C., Chen, C. C., The high-sweet-fat food craving among women with premenstrual dysphoric disorder: emotional response, implicit attitude and rewards sensitivity. *Psychoneuroendocrinology* 2010, *35*, 1203-1212.
[13] McNeil, J., Cameron, J. D., Finlayson, G., Blundell, J. E., Doucet, E., Greater overall olfactory performance, explicit wanting for high fat foods and lipid intake during the mid-luteal phase of the menstrual cycle. *Physiol Behav* 2013, *112-113*, 84-89.
[14] Shechter, A., Varin, F., Boivin, D. B., Circadian variation of sleep during the follicular and luteal phases of the menstrual cycle. *Sleep* 2010, *33*, 647-656.
[15] Shechter, A., Boivin, D. B., Sleep, Hormones, and Circadian Rhythms throughout the Menstrual Cycle in Healthy Women and Women with Premenstrual Dysphoric Disorder. *International journal of endocrinology* 2010, *2010*, 259345.
[16] Kiesner, J., One woman's low is another woman's high: Paradoxical effects of the menstrual cycle. *Psychoneuroendocrinology* 2011, *36*, 68-76.
[17] Wallace, M., Hashim, Y. Z., Wingfield, M., Culliton, M., McAuliffe, F., Gibney, M. J., Brennan, L., Effects of menstrual cycle phase on metabolomic profiles in premenopausal women. *Hum Reprod* 2010, *25*, 949-956.

[18] Fong, A. K., Kretsch, M. J., Changes in dietary intake, urinary nitrogen, and urinary volume across the menstrual cycle. *Am J Clin Nutr* 1993, *57*, 43-46.
[19] Reed, S. C., Levin, F. R., Evans, S. M., Changes in mood, cognitive performance and appetite in the late luteal and follicular phases of the menstrual cycle in women with and without PMDD (premenstrual dysphoric disorder). *Horm Behav* 2008, *54*, 185-193.
[20] Frackiewicz, E. J., Shiovitz, T. M., Evaluation and management of premenstrual syndrome and premenstrual dysphoric disorder. *J Am Pharm Assoc (Wash)* 2001, *41*, 437-447.
[21] Dye, L., Blundell, J. E., Menstrual cycle and appetite control: implications for weight regulation. *Hum Reprod* 1997, *12*, 1142-1151.
[22] Noga, M. J., Dane, A., Shi, S., Attali, A., van Aken, H., Suidgeest, E., Tuinstra, T., Muilwijk, B., Coulier, L., Luider, T., Reijmers, T. H., Vreeken, R. J., Hankemeier, T., Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis. *Metabolomics* 2012, *8*, 253-263.
[23] van der Kloet, F. M., Bobeldijk, I., Verheij, E. R., Jellema, R. H., Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. *J Proteome Res* 2009, *8*, 5132-5141.
[24] Hu, C., van Dommelen, J., van der Heijden, R., Spijksma, G., Reijmers, T. H., Wang, M., Slee, E., Lu, X., Xu, G., van der Greef, J., Hankemeier, T., RPLC-ion-trap-FTMS method for lipid profiling of plasma: method validation and application to p53 mutant mouse model. *J Proteome Res* 2008, *7*, 4982-4991.
[25] Kantae, V., Nahon, K. J., Straat, M. E., Bakker, L. E. H., Harms, A. C., van der Stelt, M., Hankemeier, T., Jazet, I. M., Boon, M. R., Rensen, P. C. N., Endocannabinoid tone is higher in healthy lean South Asian than white Caucasian men. *Sci Rep* 2017, *7*, 7558.
[26] Peters, F. T., Drummer, O. H., Musshoff, F., Validation of new methods. *Forensic Sci Int* 2007, *165*, 216-224.
[27] Abbott Laboratories, D. D., Abbott Park, IL 60064 USA
2016.
[28] McCullogh, C. E., Searle, S.R., & Neuhause, J.M, *Generalized linear and mixed models, 2nd Edition*, John Wiley & Sons, Hoboken, NJ 2008.
[29] Benjamini, Y. H., Y. , Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Metholodological)* 1995, *57*, 289-300.
[30] Hothorn, T., Bretz, F., Westfall, P., Simultaneous inference in general parametric models. *Biom J* 2008, *50*, 346-363.
[31] Bates, D. M. M. B. B. S. S., Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 2015, *67*, 1-48.
[32] T., Z. A. H., Diagnostic checking in regression relationships. *R News* 2002, *2*, 7-10.
[33] Thiele, I., Swainston, N., Fleming, R. M., Hoppe, A., Sahoo, S., Aurich, M. K., Haraldsdottir, H., Mo, M. L., Rolfsson, O., Stobbe, M. D., Thorleifsson, S. G., Agren, R., Bolling, C., Bordel, S., Chavali, A. K., Dobson, P., Dunn, W. B., Endler, L., Hala, D., Hucka, M., Hull, D., Jameson, D., Jamshidi, N., Jonsson, J. J., Juty, N., Keating, S., Nookaew, I., Le Novere, N., Malys, N., Mazein, A., Papin, J. A., Price, N. D., Selkov, E., Sr., Sigurdsson, M. I., Simeonidis, E., Sonnenschein, N., Smallbone, K., Sorokin, A., van Beek, J. H., Weichart, D., Goryanin, I., Nielsen, J., Westerhoff, H. V., Kell, D. B.,

Mendes, P., Palsson, B. O., A community-driven global reconstruction of human metabolism. *Nat Biotechnol* 2013, *31*, 419-425.
[34] Swainston, N., Mendes, P., Kell, D. B., An analysis of a 'community-driven' reconstruction of the human metabolic network. *Metabolomics* 2013, *9*, 757-764.
[35] Swainston, N., Smallbone, K., Hefzi, H., Dobson, P. D., Brewer, J., Hanscho, M., Zielinski, D. C., Ang, K. S., Gardiner, N. J., Gutierrez, J. M., Kyriakopoulos, S., Lakshmanan, M., Li, S., Liu, J. K., Martinez, V. S., Orellana, C. A., Quek, L. E., Thomas, A., Zanghellini, J., Borth, N., Lee, D. Y., Nielsen, L. K., Kell, D. B., Lewis, N. E., Mendes, P., Recon 2.2: from reconstruction to model of human metabolism. *Metabolomics* 2016, *12*, 109.
[36] Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., Morishima, K., KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic acids research* 2017, *45*, D353-D361.
[37] Calloway, D. H., Kurzer, M. S., Menstrual cycle and protein requirements of women. *The Journal of nutrition* 1982, *112*, 356-366.
[38] Gorczyca, A. M., Sjaarda, L. A., Mitchell, E. M., Perkins, N. J., Schliep, K. C., Wactawski-Wende, J., Mumford, S. L., Changes in macronutrient, micronutrient, and food group intakes throughout the menstrual cycle in healthy, premenopausal women. *Eur J Nutr* 2016, *55*, 1181-1188.
[39] Webb, P., 24-hour energy expenditure and the menstrual cycle. *Am J Clin Nutr* 1986, *44*, 614-619.
[40] Montane, J. L., Perez-Ballester, B., Cyclic changes in phospholipid content and composition in human endometrium during the menstrual cycle. *J Reprod Fertil* 1985, *73*, 317-321.
[41] Bonney, R. C., Measurement of phospholipase A2 activity in human endometrium during the menstrual cycle. *J Endocrinol* 1985, *107*, 183-189.
[42] Haoula, Z., Ravipati, S., Stekel, D. J., Ortori, C. A., Hodgman, C., Daykin, C., Raine-Fenning, N., Barrett, D. A., Atiomo, W., Lipidomic analysis of plasma samples from women with polycystic ovary syndrome. *Metabolomics* 2015, *11*, 657-666.
[43] Zhao, Y., Fu, L., Li, R., Wang, L. N., Yang, Y., Liu, N. N., Zhang, C. M., Wang, Y., Liu, P., Tu, B. B., Zhang, X., Qiao, J., Metabolic profiles characterizing different phenotypes of polycystic ovary syndrome: plasma metabolomics analysis. *BMC Med* 2012, *10*, 153.
[44] Battista, N., Meccariello, R., Cobellis, G., Fasano, S., Di Tommaso, M., Pirazzi, V., Konje, J. C., Pierantoni, R., Maccarrone, M., The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. *Mol Cell Endocrinol* 2012, *355*, 1-14.
[45] Gorzalka, B. B., Dang, S. S., Minireview: Endocannabinoids and gonadal hormones: bidirectional interactions in physiology and behavior. *Endocrinology* 2012, *153*, 1016-1024.
[46] Carrier, E. J., Patel, S., Hillard, C. J., Endocannabinoids in neuroimmunology and stress. *Curr Drug Targets CNS Neurol Disord* 2005, *4*, 657-665.
[47] Ahn, K., McKinney, M. K., Cravatt, B. F., Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chem Rev* 2008, *108*, 1687-1707.
[48] Mai, M., Tonjes, A., Kovacs, P., Stumvoll, M., Fiedler, G. M., Leichtle, A. B., Serum levels of acylcarnitines are altered in prediabetic conditions. *PLoS One* 2013, *8*, e82459.
[49] Rutkowsky, J. M., Knotts, T. A., Ono-Moore, K. D., McCoin, C. S., Huang, S., Schneider, D., Singh, S., Adams, S. H., Hwang, D. H., Acylcarnitines activate

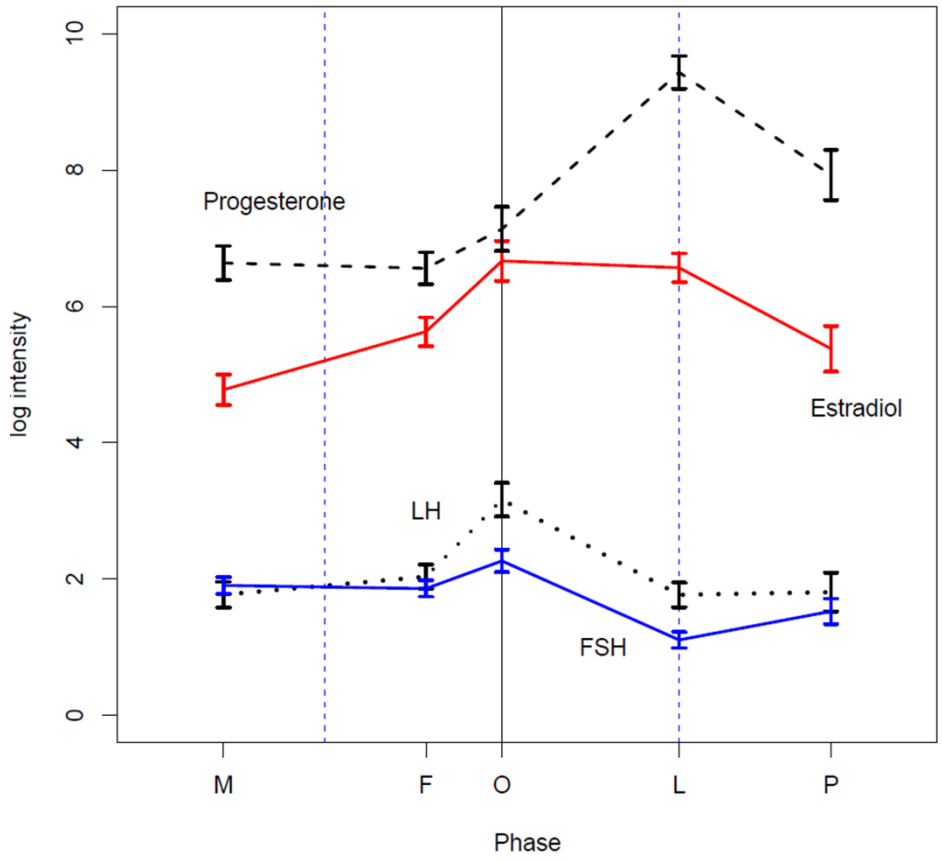
proinflammatory signaling pathways. *American journal of physiology. Endocrinology and metabolism* 2014, *306*, E1378-1387.
[50] Ke, C., Hou, Y., Zhang, H., Yang, K., Wang, J., Guo, B., Zhang, F., Li, H., Zhou, X., Li, Y., Li, K., Plasma Metabolic Profiles in Women are Menopause Dependent. *PLoS One* 2015, *10*, e0141743.
[51] Barnett, J. B., Woods, M. N., Lamon-Fava, S., Schaefer, E. J., McNamara, J. R., Spiegelman, D., Hertzmark, E., Goldin, B., Longcope, C., Gorbach, S. L., Plasma lipid and lipoprotein levels during the follicular and luteal phases of the menstrual cycle. *The Journal of clinical endocrinology and metabolism* 2004, *89*, 776-782.
[52] Schisterman, E. F., Mumford, S. L., Sjaarda, L. A., Failure to consider the menstrual cycle phase may cause misinterpretation of clinical and research findings of cardiometabolic biomarkers in premenopausal women. *Epidemiol Rev* 2014, *36*, 71-82.
[53] Ahumada Hemer, H., Valles de Bourges, V., Juarez Ayala, J., Brito, G., Diaz-Sanchez, V., Garza-Flores, J., Variations in serum lipids and lipoproteins throughout the menstrual cycle. *Fertil Steril* 1985, *44*, 80-84.
[54] Smith, G. I., Reeds, D. N., Okunade, A. L., Patterson, B. W., Mittendorfer, B., Systemic delivery of estradiol, but not testosterone or progesterone, alters very low density lipoprotein-triglyceride kinetics in postmenopausal women. *The Journal of clinical endocrinology and metabolism* 2014, *99*, E1306-1310.
[55] Dinicola, S., Chiu, T. T., Unfer, V., Carlomagno, G., Bizzarri, M., The rationale of the myo-inositol and D-chiro-inositol combined treatment for polycystic ovary syndrome. *J Clin Pharmacol* 2014, *54*, 1079-1092.
[56] Rago, R., Marcucci, I., Leto, G., Caponecchia, L., Salacone, P., Bonanni, P., Fiori, C., Sorrenti, G., Sebastianelli, A., Effect of myo-inositol and alpha-lipoic acid on oocyte quality in polycystic ovary syndrome non-obese women undergoing in vitro fertilization: a pilot study. *J Biol Regul Homeost Agents* 2015, *29*, 913-923.
[57] Carlomagno, G., Nordio, M., Chiu, T. T., Unfer, V., Contribution of myo-inositol and melatonin to human reproduction. *Eur J Obstet Gynecol Reprod Biol* 2011, *159*, 267-272.
[58] Payne, J. L., Palmer, J. T., Joffe, H., A reproductive subtype of depression: conceptualizing models and moving toward etiology. *Harv Rev Psychiatry* 2009, *17*, 72-86.
[59] Payne, J. L., Roy, P. S., Murphy-Eberenz, K., Weismann, M. M., Swartz, K. L., McInnis, M. G., Nwulia, E., Mondimore, F. M., MacKinnon, D. F., Miller, E. B., Nurnberger, J. I., Levinson, D. F., DePaulo, J. R., Jr., Potash, J. B., Reproductive cycle-associated mood symptoms in women with major depression and bipolar disorder. *J Affect Disord* 2007, *99*, 221-229.
[60] Perich, T. A., Roberts, G., Frankland, A., Sinbandhit, C., Meade, T., Austin, M. P., Mitchell, P. B., Clinical characteristics of women with reproductive cycle-associated bipolar disorder symptoms. *Aust N Z J Psychiatry* 2017, *51*, 161-167.
[61] Vigod, S. N., Strasburg, K., Daskalakis, Z. J., Blumberger, D. M., Systematic review of gamma-aminobutyric-acid inhibitory deficits across the reproductive life cycle. *Arch Womens Ment Health* 2014, *17*, 87-95.
[62] Amin, Z., Mason, G. F., Cavus, I., Krystal, J. H., Rothman, D. L., Epperson, C. N., The interaction of neuroactive steroids and GABA in the development of neuropsychiatric disorders in women. *Pharmacol Biochem Behav* 2006, *84*, 635-643.

[63] Clayton, A. H., Keller, A. E., Leslie, C., Evans, W., Exploratory study of premenstrual symptoms and serotonin variability. *Arch Womens Ment Health* 2006, *9*, 51-57.
[64] Tully, D. B., Allgood, V. E., Cidlowski, J. A., Modulation of steroid receptor-mediated gene expression by vitamin B6. *FASEB J* 1994, *8*, 343-349.
[65] Kashanian, M., Mazinani, R., Jalalmanesh, S., Pyridoxine (vitamin B6) therapy for premenstrual syndrome. *Int J Gynaecol Obstet* 2007, *96*, 43-44.
[66] Doll, H., Brown, S., Thurston, A., Vessey, M., Pyridoxine (vitamin B6) and the premenstrual syndrome: a randomized crossover trial. *J R Coll Gen Pract* 1989, *39*, 364-368.
[67] Damoiseaux, V. A., Proost, J. H., Jiawan, V. C., Melgert, B. N., Sex differences in the pharmacokinetics of antidepressants: influence of female sex hormones and oral contraceptives. *Clin Pharmacokinet* 2014, *53*, 509-519.
[68] Beierle, I., Meibohm, B., Derendorf, H., Gender differences in pharmacokinetics and pharmacodynamics. *Int J Clin Pharmacol Ther* 1999, *37*, 529-547.
[69] Prediger, M. E., Gamaro, G. D., Crema, L. M., Fontella, F. U., Dalmaz, C., Estradiol protects against oxidative stress induced by chronic variate stress. *Neurochem Res* 2004, *29*, 1923-1930.
[70] Serviddio, G., Loverro, G., Vicino, M., Prigigallo, F., Grattagliano, I., Altomare, E., Vendemiale, G., Modulation of endometrial redox balance during the menstrual cycle: relation with sex hormones. *The Journal of clinical endocrinology and metabolism* 2002, *87*, 2843-2848.
[71] Massafra, C., Gioia, D., De Felice, C., Picciolini, E., De Leo, V., Bonifazi, M., Bernabei, A., Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle. *J Endocrinol* 2000, *167*, 447-452.
[72] Ha, E. J., Smith, A. M., Plasma selenium and plasma and erythrocyte glutathione peroxidase activity increase with estrogen during the menstrual cycle. *J Am Coll Nutr* 2003, *22*, 43-51.
[73] Sheng-Huang, C., Chieh-Hsin, C., Mu-Chun, Y., Wen-Tung, H., Chia-Ying, H., Ya-Ting, H., Wan-Ling, S. U., Jiuan-Jen, S., Chih-Yang, H., Jer-Yuh, L., Effects of estrogen on glutathione and catalase levels in human erythrocyte during menstrual cycle. *Biomed Rep* 2015, *3*, 266-268.
[74] Almeida, M., Martin-Millan, M., Ambrogini, E., Bradsher, R., 3rd, Han, L., Chen, X. D., Roberson, P. K., Weinstein, R. S., O'Brien, C. A., Jilka, R. L., Manolagas, S. C., Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERalpha. *J Bone Miner Res* 2010, *25*, 769-781.
[75] Abbas, A. M., Elsamanoudy, A. Z., Effects of 17beta-estradiol and antioxidant administration on oxidative stress and insulin resistance in ovariectomized rats. *Can J Physiol Pharmacol* 2011, *89*, 497-504.
[76] Duvan, C. I., Cumaoglu, A., Turhan, N. O., Karasu, C., Kafali, H., Oxidant/antioxidant status in premenstrual syndrome. *Arch Gynecol Obstet* 2011, *283*, 299-304.
[77] Pal, L., Berry, A., Coraluzzi, L., Kustan, E., Danton, C., Shaw, J., Taylor, H., Therapeutic implications of vitamin D and calcium in overweight women with polycystic ovary syndrome. *Gynecol Endocrinol* 2012, *28*, 965-968.

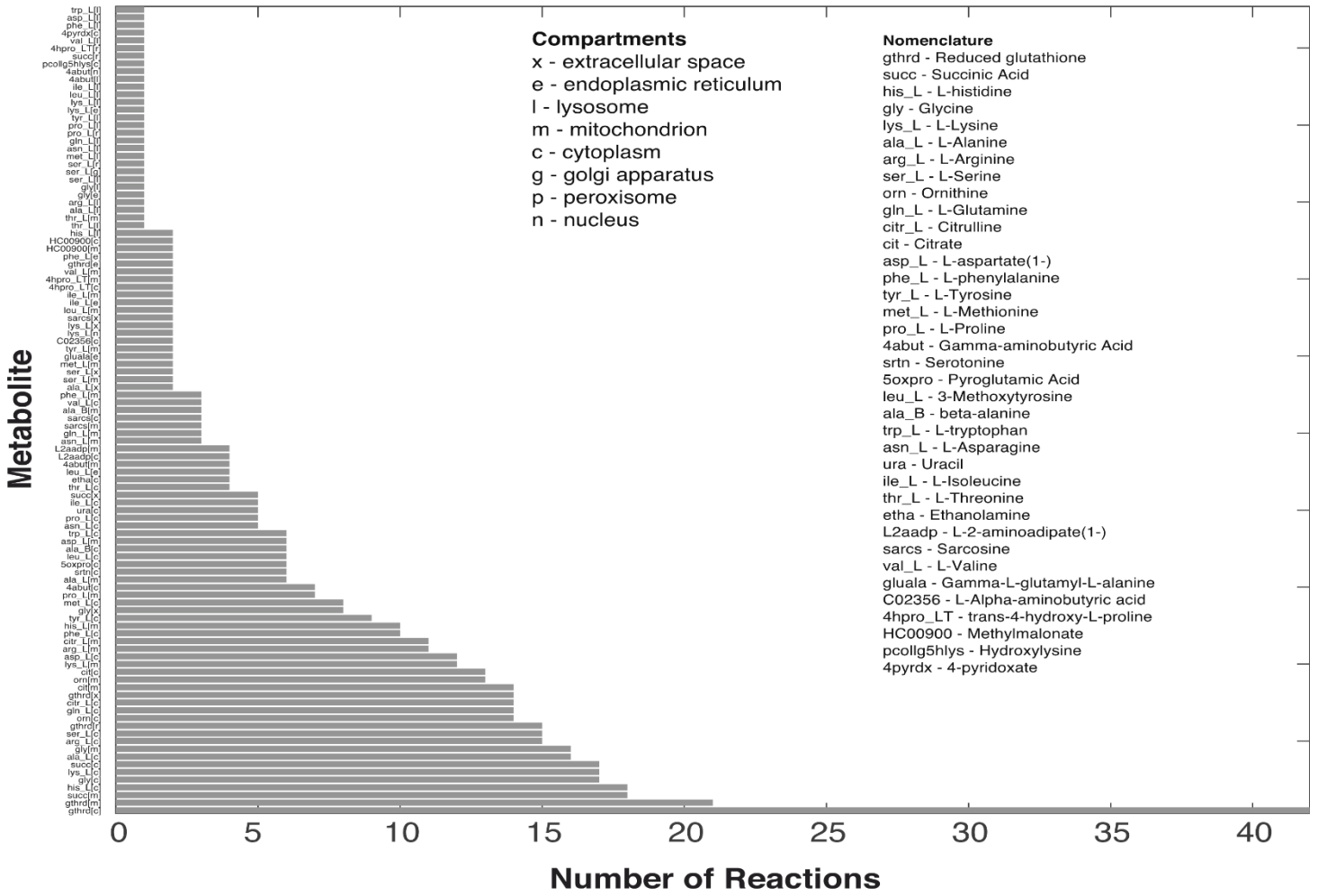
[78] Bertone-Johnson, E. R., Chocano-Bedoya, P. O., Zagarins, S. E., Micka, A. E., Ronnenberg, A. G., Dietary vitamin D intake, 25-hydroxyvitamin D3 levels and premenstrual syndrome in a college-aged population. *J Steroid Biochem Mol Biol* 2010, *121*, 434-437.
[79] Patrick, R. P., Ames, B. N., Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism. *FASEB J* 2014, *28*, 2398-2413.
[80] Kim, C. H., A functional relay from progesterone to vitamin D in the immune system. *DNA Cell Biol* 2015, *34*, 379-382.
[81] Mendoza, C., Ortega, E., Ruiz, E., Carreras, A., Osorio, C., Calcium metabolism in post-menopausal women. *Rev Esp Fisiol* 1985, *41*, 447-450.
[82] Irani, M., Merhi, Z., Role of vitamin D in ovarian physiology and its implication in reproduction: a systematic review. *Fertil Steril* 2014, *102*, 460-468 e463.
[83] Knight, J. A., Wong, J., Blackmore, K. M., Raboud, J. M., Vieth, R., Vitamin D association with estradiol and progesterone in young women. *Cancer Causes Control* 2010, *21*, 479-483.
[84] Brush, M. G., Watson, S. J., Horrobin, D. F., Manku, M. S., Abnormal essential fatty acid levels in plasma of women with premenstrual syndrome. *Am J Obstet Gynecol* 1984, *150*, 363-366.
[85] Montserrat-de la Paz, S., Garcia-Gimenez, M. D., Angel-Martin, M., Perez-Camino, M. C., Fernandez Arche, A., Long-chain fatty alcohols from evening primrose oil inhibit the inflammatory response in murine peritoneal macrophages. *J Ethnopharmacol* 2014, *151*, 131-136.
[86] Gold, E. B., Wells, C., Rasor, M. O., The Association of Inflammation with Premenstrual Symptoms. *J Womens Health (Larchmt)* 2016, *25*, 865-874.
[87] Huber, M., Knottnerus, J. A., Green, L., van der Horst, H., Jadad, A. R., Kromhout, D., Leonard, B., Lorig, K., Loureiro, M. I., van der Meer, J. W., Schnabel, P., Smith, R., van Weel, C., Smid, H., How should we define health? *BMJ* 2011, *343*, d4163.
[88] Stroeve, J. H. M., van Wietmarschen, H., Kremer, B. H. A., van Ommen, B., Wopereis, S., Phenotypic flexibility as a measure of health: the optimal nutritional stress response test. *Genes Nutr* 2015, *10*, 13.
[89] van Ommen, B., van der Greef, J., Ordovas, J. M., Daniel, H., Phenotypic flexibility as key factor in the human nutrition and health relationship. *Genes Nutr* 2014, *9*, 423.
[90] Sohrabi, N., Kashanian, M., Ghafoori, S. S., Malakouti, S. K., Evaluation of the effect of omega-3 fatty acids in the treatment of premenstrual syndrome: "a pilot trial". *Complement Ther Med* 2013, *21*, 141-146.
[91] Wyatt, K. M., Dimmock, P. W., Jones, P. W., Shaughn O'Brien, P. M., Efficacy of vitamin B-6 in the treatment of premenstrual syndrome: systematic review. *BMJ* 1999, *318*, 1375-1381.
[92] Bianchini, F., Vainio, H., Isothiocyanates in cancer prevention. *Drug Metab Rev* 2004, *36*, 655-667.
[93] Lenz, E. M., Bright, J., Wilson, I. D., Hughes, A., Morrisson, J., Lindberg, H., Lockton, A., Metabonomics, dietary influences and cultural differences: a 1H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J Pharm Biomed Anal* 2004, *36*, 841-849.
[94] Wallace, M., Morris, C., O'Grada, C. M., Ryan, M., Dillon, E. T., Coleman, E., Gibney, E. R., Gibney, M. J., Roche, H. M., Brennan, L., Relationship between the

lipidome, inflammatory markers and insulin resistance. *Molecular bioSystems* 2014, *10*, 1586-1595.

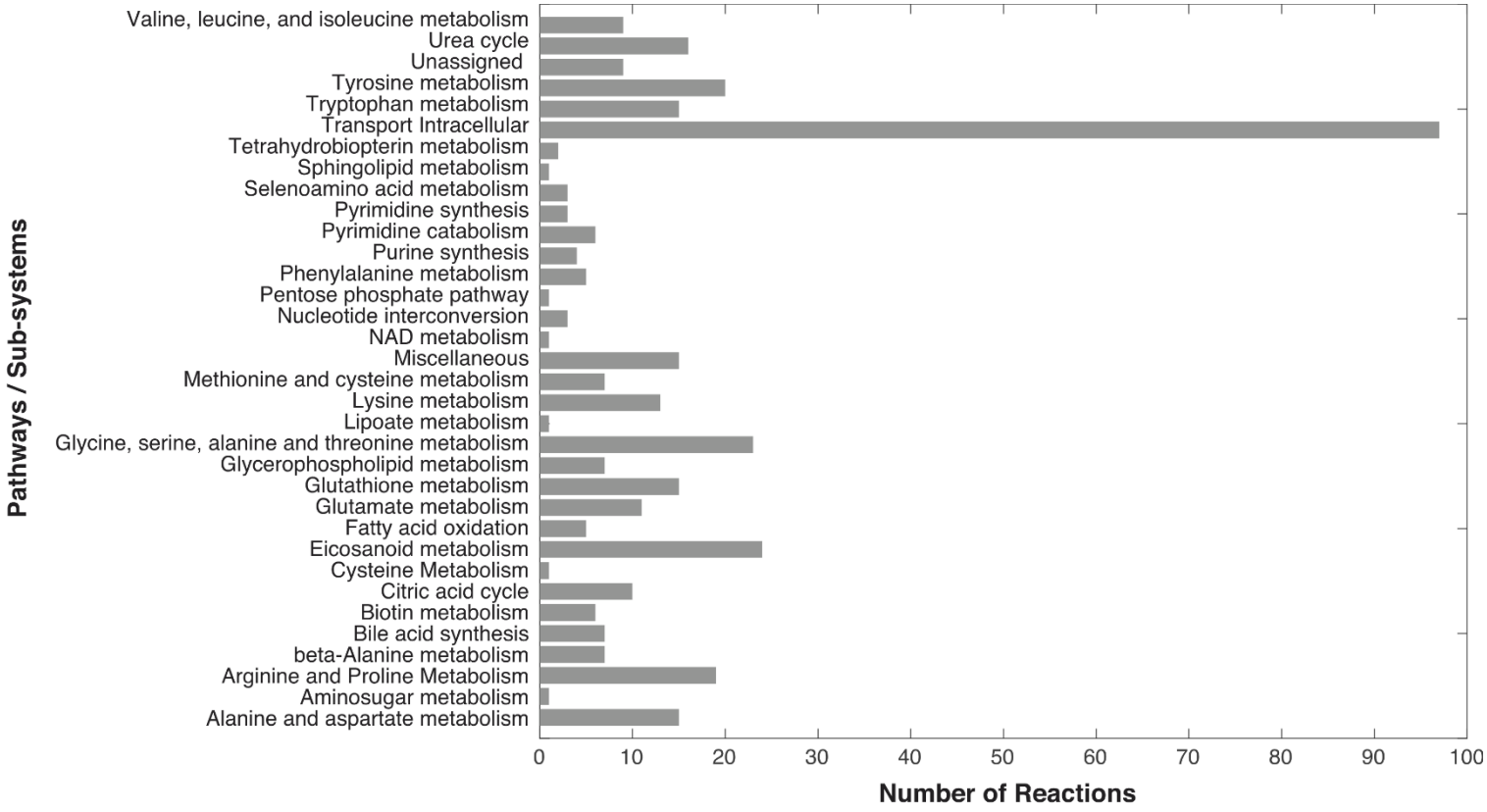
SUPPLEMENTARY INFORMATION



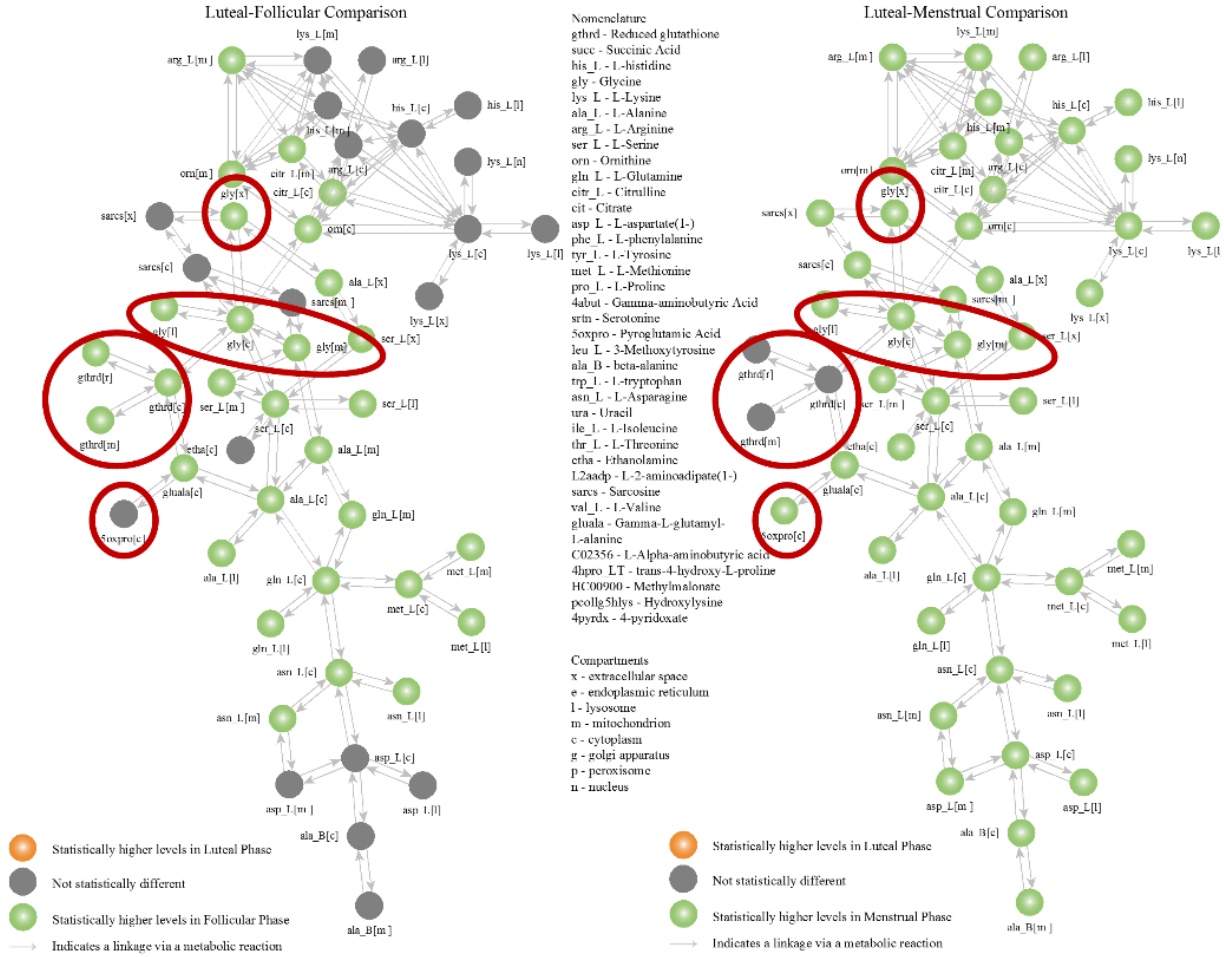
Supplementary Fig. 1. Sex hormones per cycle phase. Illustrates the concentration of 4 sex hormone levels across phases in the current study. Serum hormone levels, urinary luteninizing hormone (LH) for date of ovulation and self-reported menstrual cycle length were used to determine menstrual cycle phase. FSH = follicular stimulating hormone; M = menstrual; F = follicular; O = peri-ovulatory; L = luteal; P = premenstrual phases.



Supplementary Fig. 2a. Reactions per metabolite and impacted sub-systems. The connectivity of the 41 metabolic entities within the 419 reactions was analyzed using the Human Genome Scale Metabolic Model or RECON 2.2. Reduced glutathione, succinate, L-histidine and glycine followed by L-lysine, L-alanine, L-arginine and L-serine are impacting/impacted by the most number of reactions.



Supplementary Fig. 2b. Thirty four subsystems which comprise the 419 reactions were identified within the metabolic landscape using the Human Genome Scale Metabolic Model or RECON 2.2. The spread across different pathways is shown. Affected sub-systems include amino acid metabolism/synthesis, the urea cycle, eicosanoid metabolism, citric acid cycle and bile acid synthesis.



Supplementary Fig. 3. Interconnected metabolites (L-F, L-M). A metabolite connectivity network is illustrated to assess how the differentially observed metabolites are dependent on each other and accordingly express an interdependent change. Any metabolite is connected to another metabolite, if they are participating in a reaction, i.e. they are either products/reactants and vice versa. Interconnectivities and differences between menstrual cycle phases were visualized using custom MATLAB (Mathwork Inc.) scripts and edited using yEd (yWorks GmbH). To visualize and analyze the interconnections within the reactome, any line with an arrow denotes a direct relationship between the two reactions. The maximum number of differences are observed between the menstrual and the luteal phase, with the key set of metabolites methionine, asparagine, □-alanine, glutamine, alanine, serine, glycine, ornithine, arginine and lysine forming the core backbone of interconnected metabolites impacted by Luteal-Follicular and Luteal-Menstrual phase differences. Red circles are used to denote glutathione and its' related precursors.

Supplementary Table 1. Number of observations per cycle phase

Sample collection	M	F	O	L	P	Total
A	33	0	0	0	0	33
B	0	31	2	0	0	33
C	0	0	13	16	1	30
D	0	0	0	11	10	21
Total	33	31	15	27	11	**117**

Number of participants observed for each metabolite per sample and per phase, including the sample to phase classification. Blood and urine collection timepoints are denoted as A,B,C,D. Each of the 4 sample timepoints were assigned to 1 of 5 cycle phases. Number of observations are denoted based on cycle phase and collection timepoint. M=menstrual, F=follicular, O=periovular, L=luteal, P=premenstrual. Refer to Wallace, et.al. for details[17].

Supplementary Table 2. Significant clinical and acylcarnitine metabolites

	Effect Size					P values				
Biomarkers	**L-F**	**L-M**	**L-O**	**P-L**	**O-M**	**raw p L-F**	**raw p L-M**	**raw p L-O**	**raw p P-L**	**raw p O-M**
Clinical, serum										
Glucose	-0.02	-0.03	0.00	0.04	-0.03	1.37E-01	**5.21E-03**	9.79E-01	**1.01E-02**	**2.35E-02**
CRP	0.65	0.18	0.56	-0.36	-0.38	**5.80E-03**	4.68E-01	8.15E-02	2.70E-01	2.08E-01
HDL	-0.04	-0.02	-0.02	-0.02	0.00	**2.43E-02**	2.09E-01	3.34E-01	4.71E-01	9.97E-01
Magnesium	-0.01	-0.03	0.00	0.00	-0.03	6.10E-01	**3.92E-02**	7.90E-01	9.82E-01	**4.82E-02**
Triglycerides	-0.11	-0.05	-0.03	-0.03	-0.02	**3.32E-02**	3.69E-01	7.06E-01	7.14E-01	7.34E-01
Riboflavin - plasma	-0.10	-0.05	-0.02	0.22	-0.03	1.36E-01	4.61E-01	7.97E-01	**3.78E-02**	7.54E-01
Cholesterol	-0.05	-0.04	-0.04	0.01	0.00	**3.86E-02**	1.18E-01	2.51E-01	7.88E-01	9.45E-01
Acylcarnitines, plasma										
Linoleylcarnitine	-0.11	-0.01	-0.17	0.01	0.16	**7.86E-03**	7.86E-01	**1.32E-03**	8.23E-01	**1.52E-03**
Acetylcarnitine	-0.14	0.00	-0.23	0.05	0.22	**2.44E-02**	9.58E-01	**2.94E-03**	5.86E-01	**1.98E-03**
Carnitine	-0.11	-0.04	-0.14	0.08	0.10	**2.13E-02**	3.23E-01	**1.43E-02**	2.37E-01	7.59E-02
Palmitoylcarnitine	0.00	0.02	-0.09	-0.08	0.11	9.80E-01	5.70E-01	6.48E-02	1.55E-01	**1.62E-02**
Oleylcarnitine	-0.09	0.00	-0.18	-0.02	0.18	6.45E-02	9.55E-01	**4.90E-03**	8.18E-01	**2.58E-03**
Myristoilcarnitine	-0.07	0.02	-0.19	-0.10	0.21	2.69E-01	7.28E-01	**1.30E-02**	2.45E-01	**3.71E-03**
Choline	-0.05	-0.18	0.04	-0.10	-0.22	4.88E-01	**1.41E-02**	7.05E-01	3.21E-01	**1.65E-02**
Hexanoylcarnitine	-0.20	-0.05	-0.34	0.01	0.28	**3.27E-02**	5.53E-01	**4.45E-03**	9.58E-01	**1.17E-02**
Butyrylcarnitine	-0.16	-0.11	-0.20	0.13	0.09	**4.39E-03**	**4.34E-02**	**5.75E-03**	1.18E-01	1.97E-01
Hexadecenoylcarntine	-0.09	-0.01	-0.24	-0.03	0.22	2.03E-01	8.50E-01	**8.80E-03**	7.44E-01	**9.04E-03**
Isovalerylcarnitine	-0.05	-0.17	0.00	0.04	-0.17	4.98E-01	**6.80E-03**	9.96E-01	6.59E-01	**2.93E-02**
Tetradecadienylcarntine	-0.22	-0.01	-0.28	-0.02	0.27	5.62E-02	9.08E-01	**4.59E-02**	9.05E-01	**4.44E-02**
Tetradecenoylcarnitine	-0.14	0.02	-0.30	-0.07	0.32	2.08E-01	8.38E-01	**3.30E-02**	6.57E-01	**1.58E-02**
Tiglylcarnitine	-0.05	-0.13	0.01	0.02	-0.14	3.23E-01	**1.54E-02**	8.35E-01	8.26E-01	**2.97E-02**
Decenoylcarnitine	-0.20	-0.05	-0.25	0.01	0.19	**4.16E-02**	5.51E-01	**3.91E-02**	9.45E-01	9.02E-02
Octenoylcarnitine	-0.11	0.03	-0.17	0.09	0.20	1.78E-01	7.04E-01	9.38E-02	4.45E-01	**3.81E-02**
Isobutyrylcarnitine	-0.02	-0.03	0.17	0.01	-0.20	8.07E-01	6.71E-01	5.21E-02	9.46E-01	**1.68E-02**
Propionylcarnitine	-0.12	-0.03	-0.05	0.01	0.01	**3.37E-02**	5.53E-01	5.22E-01	8.80E-01	8.46E-01
2 methylbutyroylcarnitine	-0.05	-0.08	-0.04	0.03	-0.04	1.69E-01	**2.48E-02**	4.28E-01	6.18E-01	3.30E-01
Acylcarnitines, urine										
Dodecenoylcarnitine	-0.17	0.06	-0.36	0.15	0.42	2.53E-01	6.59E-01	5.34E-02	4.78E-01	**1.76E-02**
Tetradecadienylcarntine	-0.18	0.04	-0.43	0.16	0.48	2.27E-01	7.66E-01	**1.92E-02**	4.41E-01	**7.28E-03**
Octanoylcarnitine	-0.32	0.01	-0.39	0.15	0.40	**2.77E-02**	9.59E-01	**3.45E-02**	4.85E-01	**2.45E-02**
Pivaloylcarnitine	-0.16	0.10	-0.43	-0.17	0.53	2.86E-01	5.11E-01	**2.69E-02**	4.45E-01	**4.45E-03**
Carnitine	-0.35	0.15	-0.55	0.42	0.71	1.24E-01	4.93E-01	5.47E-02	1.98E-01	**1.04E-02**
Butenylcarnitine	-0.15	0.15	-0.28	0.17	0.44	2.71E-01	2.50E-01	1.04E-01	3.86E-01	**8.49E-03**
Hexanoylcarnitine	-0.24	0.03	-0.39	0.10	0.42	8.02E-02	8.10E-01	**2.53E-02**	5.93E-01	**1.14E-02**
Glutarylcarnitine	-0.18	0.10	-0.44	0.27	0.55	2.94E-01	5.35E-01	**3.99E-02**	2.68E-01	**8.08E-03**
Octenoylcarnitine	-0.09	0.32	-0.27	0.19	0.58	6.64E-01	1.12E-01	2.99E-01	5.05E-01	**1.75E-02**
Acetylcarnitine	-0.31	0.33	-0.43	0.48	0.77	3.03E-01	2.66E-01	2.55E-01	2.63E-01	**3.60E-02**
Tetradecenoylcarnitine	-0.21	0.02	-0.47	0.16	0.50	1.82E-01	8.91E-01	**1.92E-02**	4.95E-01	**1.06E-02**
Hexenoylcarnitine	-0.14	0.27	-0.27	0.24	0.53	4.92E-01	1.68E-01	2.88E-01	4.02E-01	**2.57E-02**
Decanoylcarnitine	-0.19	0.02	-0.34	0.03	0.36	1.41E-01	8.62E-01	**3.85E-02**	8.60E-01	**2.13E-02**
CholinE caution	0.23	0.13	0.19	-0.42	-0.05	9.16E-02	3.25E-01	2.82E-01	**3.46E-02**	7.45E-01
Deoxycarnitine	-0.03	0.02	-0.30	0.09	0.32	8.13E-01	8.54E-01	6.86E-02	6.14E-01	**4.08E-02**

Clinical and acycarnitine metabolites q>0.10.

Chapter 6

Conclusions and Perspectives

CONCLUSIONS

The clinical nutrition practitioner, such as a registered dietitian, seeks to optimize nutritional therapies for the individual. Such experts rely on clinical practice guidelines translated from scientific publications as realistic recommendations for nutritional diagnosis and therapeutic recommendations. For example, the Academy of Nutrition and Dietetics in the U.S. has over 100 guidelines for common conditions (e.g., nursing) to specific diseases such as emergency medicine, allergy and immunology, obstetrics and gynecology, podiatry, or urology [1]. However, much of the scientific literature in the area of nutrition, and indeed, biomedical research, relies on statistical averages of selected groups (e.g., cases versus controls) which by definition cannot represent larger, more varied populations or an individual. These averages are used to supplement disease diagnostic practices; and translating this corpus of literature results to personalized nutrition recommendations evidence is currently not possible.

Two broad strategies are needed to overcome the challenges of improving population as well as personal health. The first tactic is to develop n-of-1 research strategies that are capable not only of deeply analyzing individuals [2-4] but also aggregating data to expand recommendations beyond the studied population [4]. The second approach expands the reductionism currently used to analyze data from drug or nutrition studies to a more holistic analysis and interpretation of multiple biological and environmental scales (e.g., data from within the body plus the environment of individuals [5]. Systems biology [6, 7] approaches that produce data from the microscales within the body, such as metabolomic, proteomic, and network pathway analyses, hold the potential to assist the clinical nutritionist in developing a more personalized approach. However, the application of systems biology to nutrition in practice is still in its infancy. To date, these approaches rely on aggregating individual reductionistic analysis since the systems view of the whole metabolite-profile has not yet been elucidated. The integration of a systems view of nutrition with a systems view of metabolomics, and a systems view of proteomics, genomics, clinical, and laboratory metabolomics data sets has not yet been attained. Interpreting these systems integrations in the individual's environmental, social, and psychological contexts is at the fore-front of healthcare but has not yet been initiated in academic, government, and all but a few isolated examples.

The challenge remains…how do we integrate the various –omics technologies that are increasingly available from academic and industry research with nutrient measurements and nutrition interventions to generate the statistical evidence

necessary to help people become healthier and prevent disease? This thesis has touched on just a few elements of this challenge, using metabolomics and proteomics to characterize subgroups and diet response.

In **Chapter 2,** we demonstrated it is possible to use a short-term healthy vegan diet to challenge metabolism and produce a metabolic signature conducive to optimal blood sugar, insulin and lipid control after only 48 hours. Our tightly controlled diet intervention resulted in strong correlations between dietary nutrients and plasma metabolites, supporting the notion food intake was closely linked to metabolites measured. Finally, we observed that nutritional biochemistries and the metabolite results, insulin and branched chain amino acids, were impacted by gender dimorphism.

In **Chapter 3,** we continued to build the story begun with **Chapter 2**. We evaluated postprandial responses with glycemic, lipid and related metabolites on day three of the vegan and animal diets and demonstrated both diet types can have health advantages with flexitarian modifications. The vegan diet breakfast resulted in a less optimal metabolic signature despite apparently healthful food choices. However, the fiber content of the vegan diet may have reduced metabolite peaks and promoted bile acid concentrations that have positive health implications. The animal diet produced undesirable insulin and glucose peaks after lunch but a more favorable fatty acid profile from both mealtimes. We concluded that liberalization of the vegan meal plan to vegetarian; and the animal meal plan to a Nordic-based diet with increased focus on vegetable-based foods could result in improved metabolic signatures for both diet strategies. Insulin, triglyceride, amino acid and bile acid results showed gender dimorphic responses in these analyses.

In **Chapter 4**, we further explored the influence of sexual dimorphism plasma profiles using aptamer-based proteomics combined with network analysis in a healthy cohort of women and men. Twenty eight percent of the total proteins analyzed were differentially expressed in a sexually dimorphic manner. These results were then successfully replicated in a larger cohort. The top eight most significant proteins elevated in females had known associations with sex hormone metabolism and each protein was involved in at least one diet- related metabolic disease. These proteins were all involved in glucose and insulin metabolism, metabolic rate, carbohydrate intake and salt sensitive hypertension. Of the top proteins more highly expressed in males, some were also involved in sex hormone metabolism with a focus on such areas as blood coagulation, inflammation and iron metabolism and overload and cardiovascular disease risk. Iron, total lipids, monounsaturated fatty acids, omega 3

polyunsaturated fatty acids, and vitamins K and A are known to play key roles in the proteins found to be highly expressed in males.

In **Chapter 5**, we analyzed vitamins, metabolomics and clinical chemistries during the five menstrual cycle phases in women to uncover the sex hormone related metabolic differences, which influence the sexual dimorphism seen in **Chapter 4**. Fifty percent of the metabolites tested showed significant differences in rhythmicity across cycle phases and were enriched in neurotransmitter, glutathione (oxidative stress), urea cycle (nitrogen), vitamin B6 and vitamin D metabolism. Thus, we demonstrated the importance of accounting for menstrual cycle phase and sex hormone concentration differences in performing routine health diagnosis. Additionally, the luteal phase demonstrated the most significant decreases in amino acids and lipids, which may be caused by the anabolic effect of the progesterone peak. The changes in level of these metabolites could be linked to current biochemical and physiologic knowledge on biomarker changes in menstrual conditions such as premenstrual syndrome (PMS) and premenstrual dysphoric disorder (PMDD). Since individuals differ genetically, socially, and in environmental exposures, a larger population sample might identify those susceptible to PMDD and PMS and allow for strategic dietary interventions to alleviate symptoms.

FUTURE PERSPECTIVES

Innovative research designs, such as n-of-1 research [8] approaches that capture individual variability, need to be combined with controlled diet interventions to effectively translate –omics results to diet prescriptions. Challenge studies can be conducted alongside diet interventions to examine short-term changes in phenotypic flexibility.

As a part of this thesis project, **Figure 1** depicts a conceptual experimental framework that was developed to analyze utilization of a combinatorial glucose and fructose (e.g., disaccharides in sugar) tolerance challenge test during different phases of the menstrual cycle. Specifically, this model experiment may determine how a sugar bolus (often consumed in excess in the luteal phase) alters carbohydrate, lipid, and protein metabolic effects, over a 10-hour time period in pre-diabetic and diabetic women. Introducing a protein shake, supplemented with essential fatty acids, may alter metabolism during the luteal phase to compensate for the increased protein and lipid requirements that may be relevant to the cell regeneration and recycling described in **Chapter 5**.

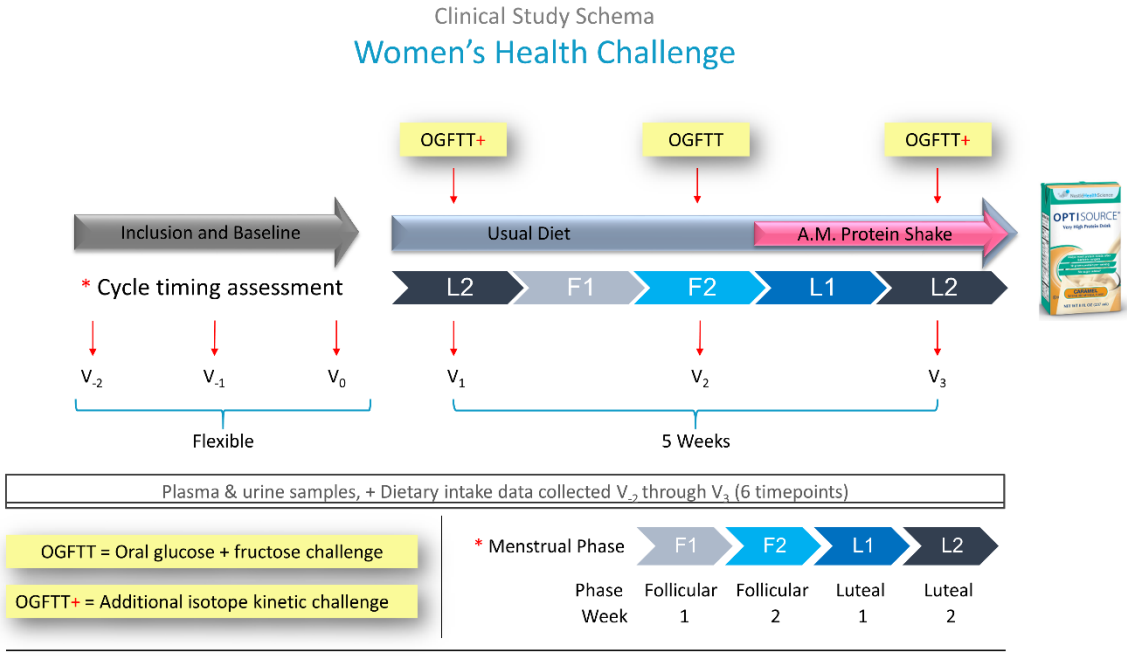


Figure 1: Hypothetical diet challenge study to evaluate the metabolic impact of an increase in protein intake during the luteal phase of the menstrual cycle.

The challenge test would be done during the follicular and luteal phases when the participants are ingesting a usual diet; and then repeated after the daily ingestion of a high protein shake during the luteal phase. The results of the tolerance test would be compared between the follicular phase, which is the more stable phase for blood sugar control, and the luteal phase to examine the differences in the individual's blood sugar control during this more vulnerable timeperiod. Finally, the luteal phase response of the individual to the luteal phase protein shake would be compared to examine the utility of this type of periodic intervention.

Multiple plasma samples would be collected for clinical biomarker and metabolomics assessments. Creatinine clearance from 24-hour urine may be used to evaluate differences in protein utilization during these 3 phase specific timepoints. Dietary intake data would also be collected and evaluated for differences in habitual intake across individuals that are known to influence blood sugar control (**Figure 1**). This type of research strategy may create opportunities to use sex hormone subtyping for diet design that is translatable to practice and deserves further exploration.

Hence, menstrual phase subgroups and sexually dimorphic data, may launch personalized nutrition into future healthcare practice. Metabotypes, which are identified through cluster analysis of individuals based on similar metabolic phenotypes that can be identified using clinical and metabolomic markers [9], provide the conceptual basis to differentiate dietary intervention response.

Metabotype response then translates to targeted diet prescriptions. This type of analysis on the data generated in **Chapters 1** and **2** in response to the vegan and animal test diets would have been appropriate. However, a sample size of 21 was too small to locate significant metabotype clusters.

As one example of how metabotyping works, in a study of 1500 individuals, 3 metabotypes were identified: 1) high HDL (high density lipoprotein) cholesterol, low glucose, low triglycerides; 2) low cholesterol; 3) high trigyclerides, high total cholesterol, low HDL and high insulin resistance. Dietary advice was targeted to metabotypes 1 and 3 to lower the characteristic biomarkers. This simplified targeted approach showed strong agreement with the individualized dietary advice provided by the dietitian (2).

Another study used metabotyping to further analyze metabolic syndrome marker reduction with Vitamin D supplementation. That study analysis demonstrated an increase in Vitamin D concentration but no significant metabolic syndrome marker changes in the responder group.

However, the use of a metabotype clustering approach identified a sub-group of vitamin D responsive patients that demonstrated significant decreases in fasting insulin, homeostatic model assessment score, and C-reactive protein (3). LDL level, fasting glucose, and cholesterol subgroups were also identified based on response to a longer term (6 week) micronutrient intervention in teens and adolescents [4] further buttressing the use of challenges tests to identify metabotypes. This type of approach could be used to analyze response to semi-vegetarian (flexitarian) diets, a concept that emerged from the vegan diet studied in this thesis.

Study approaches need to join the rapid pace of technology development in order to ensure new technologies can help more people sooner. For example, the research results represented in **Chapters 1** and **2** were generated from five years of work; including study design, recruitment, laboratory measurements, data analysis, manuscript generation and publication. The cost was close to 5 million francs. Research takes an average of 17 years to translate evidence into practice [10, 11]. This is too long given the rapid pace of climate change, population growth, food systems changes, and healthcare crises faced by modern society [12].

Self-quantification emerged following the development of smart phones and apps which allow for self-monitoring, data collection, and analysis to track activities, nutrient intakes, and health status. Self-quantifiers reflect on their personal health

data to gain a better understanding of their body, health behavior and interaction with their environment [13]. A worldwide community exists to leverage homemade tools and experiences [14]. For example, smartphone applications, such as Clue [15] are widely used by women to easily track related symptoms and phases of their menstrual cycle, fertility and perimenopausal transition. These types of easy to use applications provide the inputs for individuals to track the results of their own self-experimentation, such as the impact of changing diet strategies on menstrual cycle symptoms. The individual is empowered by the opportunity to test the effectiveness for themselves of an alternate diet strategy, such as the luteal phase protein shake suggested in the Women's Health Challenge future study design (**Figure 1**). This type of self-centered research can be conducted more efficiently than currently accepted standard approaches to human clinical research.

The challenge lies in aggregating this kind of data from a variety of tools collected by individuals in a consistent way so that humanity can benefit. Health information sharing websites, such as PatientsLikeMe [16] empower individuals to share their health conditions, data and unique experiences and connect with other patients like them. Participants have the opportunity to find new ideas and solutions to their unique health challenges that may not be known from published scientific research. Their data is then aggregated and sold to companies that wish to produce new products and services in diagnostics and therapeutics. Other companies, such as Arivale [17], Human Longevity, Inc. [18], and Molecularyou [19] target big data diagnostics analyzing blood, saliva and urine for genomics, metabolomics, microbiome analysis, proteomics, and lifestyle information. Advances in machine learning and artificial intelligence computing are likely to uncover new therapeutic solutions. Health action plans and personalized coaching are provided. Participants pay to provide their samples and receive their analyses. Data are aggregated and research discoveries made on a rolling basis. An example of using public domain data is analysis of 9896 users who self-recorded 587,187 food diary pages of the MyFitnessPal app with machine learning algorithms to discover under and over-reporting of food intake goals [20]. Thus, big data and self-quantification could potentiate translation of valuable nutrition research findings to advance healthcare more quickly by overcoming some of the timeline and cost hurdles associated with the more traditional approaches to clinical trials research.

Challenges with integrating –omics technology and nutrition remain. This thesis has taken some steps in this direction by evaluating healthy women and men to obtain metabolomics and proteomics signatures. Personalized nutrition subtypes were examined using the menstrual cycle phases and gender differences. Short-term diet

challenges were evaluated using metabolomics technology and demonstrated short-term health improvement. Harnessing the power of nutrition by integrating it with new scientific and information technologies will launch us into a new era of preventive healthcare in which we can more effectively use metabolism and nutrition to diagnose and optimize health.

REFERENCES

[1] American Academy of Nutrition and Dietetics, Copyright 2017 American Medical Association. Guideline Summaries. https://www.guidelinecentral.com/summaries/organizations/academy-of-nutrition-and-dietetics/?start=80. 21 June 2018.
[2] Stanberry, L., Mias, G. I., Haynes, W., Higdon, R., Snyder, M., Kolker, E., Integrative analysis of longitudinal metabolomics data from a personal multi-omics profile. *Metabolites* 2013, *3*, 741-760.
[3] Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T., Lotan-Pompan, M., Suez, J., Mahdi, J. A., Matot, E., Malka, G., Kosower, N., Rein, M., Zilberman-Schapira, G., Dohnalova, L., Pevsner-Fischer, M., Bikovsky, R., Halpern, Z., Elinav, E., Segal, E., Personalized Nutrition by Prediction of Glycemic Responses. *Cell* 2015, *163*, 1079-1094.
[4] Mathias, M. G., Coelho-Landell, C. A., Scott-Boyer, M. P., Lacroix, S., Morine, M. J., Salomao, R. G., Toffano, R. B. D., Almada, M., Camarneiro, J. M., Hillesheim, E., de Barros, T. T., Camelo-Junior, J. S., Campos Gimenez, E., Redeuil, K., Goyon, A., Bertschy, E., Leveques, A., Oberson, J. M., Gimenez, C., Carayol, J., Kussmann, M., Descombes, P., Metairon, S., Draper, C. F., Conus, N., Mottaz, S. C., Corsini, G. Z., Myoshi, S. K. B., Muniz, M. M., Hernandes, L. C., Venancio, V. P., Antunes, L. M. G., da Silva, R. Q., Laurito, T. F., Rossi, I. R., Ricci, R., Jorge, J. R., Faga, M. L., Quinhoneiro, D. C. G., Reche, M. C., Silva, P. V. S., Falquetti, L. L., da Cunha, T. H. A., Deminice, T. M. M., Tambellini, T. H., de Souza, G. C. A., de Oliveira, M. M., Nogueira-Pileggi, V., Matsumoto, M. T., Priami, C., Kaput, J., Monteiro, J. P., Clinical and Vitamin Response to a Short-Term Multi-Micronutrient Intervention in Brazilian Children and Teens: From Population Data to Interindividual Responses. *Molecular nutrition & food research* 2018, *62*, e1700613.
[5] Kaput, J., van Ommen, B., Kremer, B., Priami, C., Monteiro, J. P., Morine, M., Pepping, F., Diaz, Z., Fenech, M., He, Y., Albers, R., Drevon, C. A., Evelo, C. T., Hancock, R. E., Ijsselmuiden, C., Lumey, L. H., Minihane, A. M., Muller, M., Murgia, C., Radonjic, M., Sobral, B., West, K. P., Jr., Consensus statement understanding health and malnutrition through a systems approach: the ENOUGH program for early life. *Genes Nutr* 2014, *9*, 378.
[6] van Ommen, B., Nutrigenomics: exploiting systems biology in the nutrition and health arenas. *Nutrition* 2004, *20*, 4-8.
[7] Saqi, M., Pellet, J., Roznovat, I., Mazein, A., Ballereau, S., De Meulder, B., Auffray, C., Systems Medicine: The Future of Medical Genomics, Healthcare, and Wellness. *Methods Mol Biol* 2016, *1386*, 43-60.
[8] Kaput, J., Morine, M., Discovery-based nutritional systems biology: developing N-of-1 nutrigenomic research. *Int J Vitam Nutr Res* 2012, *82*, 333-341.
[9] Fiamoncini, J., Rundle, M., Gibbons, H., Thomas, E. L., Geillinger-Kastle, K., Bunzel, D., Trezzi, J. P., Kiselova-Kaneva, Y., Wopereis, S., Wahrheit, J., Kulling, S. E., Hiller, K., Sonntag, D., Ivanova, D., van Ommen, B., Frost, G., Brennan, L., Bell, J., Daniel, H., Plasma metabolome analysis identifies distinct human metabotypes in the postprandial state with different susceptibility to weight loss-mediated metabolic improvements. *FASEB J* 2018, fj201800330R.

[10] Green, L. W., Ottoson, J. M., Garcia, C., Hiatt, R. A., Diffusion theory and knowledge dissemination, utilization, and integration in public health. *Annu Rev Public Health* 2009, *30*, 151-174.
[11] Jonathan Grant, L. G., Barbara Mason, Basic research and health: a reassessment of the scientific basis for the support of biomedical science. *research evaluation* 2003, *12*, 217-224.
[12] Kaput, J., Kussmann, M., Mendoza, Y., Le Coutre, R., Cooper, K., Roulin, A., Enabling nutrient security and sustainability through systems research. *Genes Nutr* 2015, *10*, 462.
[13] Almalki, M., Gray, K., Martin-Sanchez, F., Activity Theory as a Theoretical Framework for Health Self-Quantification: A Systematic Review of Empirical Studies. *J Med Internet Res* 2016, *18*, e131.
[14] Quantified Self Labs, Quantified Self (QS): self knowledge through numbers. http://quantifiedself.com/about/. 4 June 2018.
[15] Clue, 2018. Clue. https://helloclue.com/. 4 June 2018.
[16] Patientslikeme, patientslikeme. https://www.patientslikeme.com/. 4 June 2018.
[17] Arivale, 2018. Arivale. www.arivale.com. 4 June 2018.
[18] Human Longevity, Inc., 2018. www.humanlongevity.com. 4 June 2018.
[19] Molecularyou, 2018. www.molecularyou.com. 4 June 2018.
[20] Weber, I., Achananuparp, P., Insights from Machine-Learned Diet Success Prediction. *Pac Symp Biocomput* 2016, *21*, 540-551.

Appendix

SAMENVATTING

Metabolische gezondheid kan worden gedefinieerd als een toestand van veerkrachtig fysiek en chemisch cellulair fysiologisch functioneren, adequaat ondersteund door de spijsvertering en transformatie van voedsel in energie. Gezondheid moet breed en systematisch worden bezien, en niet gefixeerd zijn op enkelvoudige aspecten van cellulaire functie. Van nature voeden we alle aspecten van de cellulaire functie van ons lichaam gelijktijdig door het innemen van een variabele veelvoud aan voedingsstoffen en cofactoren. Zodoende worden de metabole fysiologische behoeften van het lichaam op een systematisch niveau ondersteund om de gezondheid te behouden. Omgekeerd vermindert onevenwichtige voeding de metabolische flexibiliteit en leidt deze tot het ontstaan van ziekteprocessen.

Geslachts- en genderverschillen evenals een verandering in dynamiek van het vrouwelijke geslachtshormoon beïnvloeden gezondheidstrajecten, genexpressie en voedingsbehoeften. Er is onderzoek gedaan naar gezondheid van vrouwen, met name gericht op zwangerschap, borstvoeding en kindervoeding, waaruit voedingsrichtlijnen zijn gepubliceerd. Echter, onderzoek onder adolescente, niet-zwangere vrouwen is over het algemeen schaars. De luteale fase van de menstruatiecyclus is mogelijkerwijs een normale stressor, waarbij fysiologische onevenwichtigheden gemakkelijker te detecteren zijn, en deze onevenwichtigheden zouden een voorspeller van gezondheidstrajecten op lange termijn kunnen zijn. Dynamiek in geslachtshormonen, of juist een gebrek aan dynamiek, staan mogelijk in verband met diverse vormen van premenstrueel syndroom (PMS), die zich voordoen in verschillende fasen van de cyclus, maar voornamelijk in de luteale fase. Voedingsbehoeften kunnen variëren gedurende de menstruatiecyclus, maar er zijn slechts beperkte voedingsrichtlijnen en geaccepteerde voedingstherapieën gepubliceerd voor Westerse geneeskunde en diëtiek in de praktijk. Het gebruik van geslacht of gender en vrouwelijke hormonale cycli identificeert subgroepen voor wie gepersonaliseerde voeding kan worden ontwikkeld en ingezet.

De kracht van gezondheidsdiagnose op maat waaruit gepersonaliseerde voedingstherapieën kunnen worden voorgeschreven, is afhankelijk van zowel individuele variaties in genotype als milieu- en fysiologische reacties die tot uiting komen in transcriptomics, metabolomics en proteomics-metingen. Tevens kunnen persoonlijke psychosociale factoren worden gemeten en erbij betrokken worden. Het doel van dit proefschrift was om gezonde vrouwen en mannen en hun metabole respons op voeding en/of natuurlijke hormonale dynamiek te bestuderen, gemonitord met

klinische en metabole biomarkers, als een manier om menselijke metabolische gezondheid beter te begrijpen.

In Hoofdstuk 2 werd het concept van een voedingsrijke dieetuitdaging geïntroduceerd in een gezonde, gender-gebalanceerde populatie. Een veganistisch dieet werd geëvalueerd op basis van een 48-uurs impact op een modulerende metabole signatuur. Het vergelijkingsdieet was een dierlijk dieet gebaseerd op alledaagse voeding. We hebben aangetoond dat het mogelijk is om een kortdurend gezond veganistisch dieet te gebruiken om het metabolisme te prikkelen, zodat al na 48 uur een metabolische signatuur ontstaat die bevorderlijk is voor een optimale bloedsuikerspiegel, insuline en lipidencontrole. Onze streng gecontroleerde dieetinterventie resulteerde in sterke correlaties tussen voedingsnutriënten en plasmametabolieten, hetgeen het idee ondersteunde dat voedselinname nauw verbonden was met de metabolieten onder studie. Ten slotte hebben we vastgesteld dat de voedingsbiochemie en de metabolietresultaten, insuline- en vertakte keten aminozuren, beïnvloed werden door geslachtsdimorfisme, wat suggereert dat dieetstrategieën genderspecifiek zijn.

Hoofdstuk 3 bouwt voort op de resultaten van Hoofdstuk 2, door de impact te vergelijken van zowel veganistische als dierlijke maaltijden op de postprandiale respons. Postprandiale respons met glycemische, lipide en gerelateerde metabolieten werden op dag 3 van de veganistische- en dierlijke diëten geëvalueerd. Beide soorten voeding met flexitarische aanpassingen kunnen gezondheidsvoordelen hebben. Het veganistische dieetontbijt resulteerde ondanks een ogenschijnlijk gezonde voedingskeuze in een minder optimale metabole signatuur. Het vezelgehalte van het veganistische dieet kan mogelijk metaboliet concentraties verlagen en galzuurconcentraties verhogen, die positieve gezondheidsimplicaties hebben. Het dierlijk dieet produceerde na de lunch ongewenste insuline en glucose pieken, maar resulteerde in een gunstiger vetzuurprofiel. Liberalisering van het veganistische maaltijdplan naar vegetarisch en van het dierlijke plan naar een Scandinavisch dieet met meer aandacht voor plantaardig voedsel, zou kunnen resulteren in verbeterde metabole signaturen voor beide voedingsstrategieën. Resultaten van insuline, triglyceriden, aminozuren en galzuren vertoonden gender-dimorfe respons in deze analyses, hetgeen wederom suggereert dat voedingsstrategieën genderspecifiek zijn.

In Hoofdstuk 4 hebben we de invloed van seksueel dimorfisme op plasmaprofielen verder onderzocht met behulp van aptameer-gebaseerde proteomics in combinatie met netwerkanalyse in een gezond cohort van vrouwen en mannen. Pathway over-representation en functionele pathway-enrichment analyse werden uitgevoerd met behulp van WikiPathways, Kyoto Encyclopedia of Genes and Genomics (KEGG) en

Reactome-databases. Achtentwintig procent van de totale geanalyseerde eiwitten werden differentieel uitgedrukt op een seksueel dimorfe manier. Deze resultaten werden vervolgens met succes gerepliceerd in een groter cohort. De top acht van de meest significante eiwitten die zijn verhoogd bij vrouwen, hadden associaties met het geslachtshormoonmetabolisme en elk eiwit was betrokken bij ten minste één dieet-gerelateerde metabole ziekte. Deze eiwitten waren betrokken bij glucose- en insulinemetabolisme, metabole snelheid, inname van koolhydraten en zoutgevoelige hypertensie. Van de meest significante eiwitten, die hoger tot expressie waren bij mannen, waren sommigen ook betrokken bij het metabolisme van geslachtshormonen met een focus op gebieden als bloedcoagulatie, ontsteking en ijzermetabolisme, overbelasting en cardiovasculaire ziekterisico's. Deze resultaten suggereren dat seksueel dimorfisme van eiwit-expressie op een geslachts-specifieke manier kan worden beïnvloed door verschillende dieetcomponenten.

In Hoofdstuk 5 werd metabolomics gebruikt om variaties in de menstruatie gerelateerd aan hormoonfysiologie te evalueren bij gezonde menstruerende vrouwen. Vitaminen, metabolieten en klinische chemie werden tijdens de vijf fasen van de menstruatiecyclus bij vrouwen gemeten om de metabolische verschillen in geslachtshormonen bloot te leggen, die het seksuele dimorfisme zoals bestudeerd in Hoofdstuk 4 beïnvloeden. Vijftig procent van de geteste metabolieten vertoonden significante verschillen in dynamiek gedurende cyclusfasen met name in neurotransmitter, glutathione (oxidatieve stress), ureumcyclus (stikstof), vitamine B6 en vitamine D-metabolisme. Zodoende is het belang aangetoond om rekening te houden met de menstruatiecyclus-fase en geslachtshormoonconcentratie bij het uitvoeren van routinematige gezondheidsdiagnoses. Bovendien vertoonde de luteale fase de meeste significante verlagingen van aminozuren en lipiden, die veroorzaakt zouden kunnen worden door het anabole effect van de progesteron stijging. Omdat individuen genetisch verschillend zijn en bloot staan aan verschillende sociale en andere omgevingsfactoren, kunnen in een grotere steekproef met personen die vatbaar zijn voor premenstruele dysfore stoornis (PMDD) en PMS identificeren, mogelijk strategische dieetinterventies ontdekt worden om de symptomen te verlichten.

Onderzoeksbenaderingen moeten aansluiten bij het snelle tempo van technologische ontwikkeling om ervoor te zorgen dat nieuwe technologieën meer mensen eerder kunnen helpen. Zo werden de onderzoeksresultaten die zijn weergegeven in Hoofdstukken 1 en 2 bijvoorbeeld gegenereerd op basis van vijf jaar werk en een budget van 5 miljoen Zwitserse frank. Het is mogelijk dat de hoge kosten en lange tijdlijnen die nodig zijn om klinisch onderzoek uit te voeren op mensen verminderd kunnen worden door zelfmeting. Zelfmeting is ontstaan na de ontwikkeling van smartphones en apps,

die monitoring, gegevensverzameling en analyse van activiteiten, voedingsinname en gezondheidsstatus mogelijk maken. Dit soort eenvoudig te gebruiken applicaties geven individuen de gelegenheid om de resultaten van hun eigen experimenten bij te houden. Deze zelf-geïnitieerde onderzoeksaanpak kan efficiënter worden uitgevoerd dan de huidige standaardbenaderingen voor klinisch onderzoek bij mensen. Uitdagingen rond de integratie van –omics technologie in voedingsonderzoek blijven echter bestaan.

Dit proefschrift heeft enkele stappen in een verbetering hiervan in gang gezet door gezonde vrouwen en mannen te evalueren om zo inzicht te krijgen in metabolomics en proteomics-signaturen. Voedingssubtypes werden onderzocht op basis van de fasen van de menstruatiecyclus en genderverschillen. Kortdurende dieetuitdagingen werden geëvalueerd met behulp van metabolomics-technologie en toonden gezondheidsverbetering op korte termijn aan. Door gebruik te maken van de kracht van voeding en deze kennis te integreren met nieuwe wetenschappelijke- en informatietechnologieën, zullen we een nieuw tijdperk van preventieve gezondheidszorg ingaan waarin we metabolisme en voeding effectiever kunnen gebruiken om gezondheid te diagnosticeren, optimaliseren en personaliseren.

ACKNOWLEDGEMENTS

My profound thanks to Dr. Ir. Ben van Ommen for his enthusiastic support and mentorship of my interests in improving clinical nutrition practice with -omics technologies during which time he connected me with Prof. Dr. Jan van der Greef.

I am profoundly grateful for working with and receiving creative inspiration and support from Prof. Dr. Jan van der Greef and Prof. Dr. Thomas Hankemeier to execute on my PhD while managing my work at Nestle Institute of Health Sciences.

Special thanks to Dr. Edward Baetge, Eric Rolland and Nestle Institute of Health Sciences for believing in me and agreeing to financially support my PhD work. I am extremely thankful fto Dr. Jim Kaput, Dr. Sofia Moco, Dr. Francois-Pierre Martin and Dr. Kei Sakamoto for their extensive effort supporting me throughout the execution of my PhD over the last 4 ½ years. In particular, Kei was a political magician and able to remove the many blocks we faced for execution. I am grateful to Sofia, in particular, who was a profound source of daily inspiration and strength. Special thanks to Francois-Pierre and Jim who invested a lot of time helping me evolve my concepts and stories for each paper. Thanks to the early direction and inspiration from Dr. Martin Kussmann to initiate my writing efforts and Dr. Jorg Hager for helping me focus my work.

I am extremely grateful for the opportunity to work with Prof. Dr. Lorraine Brennan for the open generosity with which she executed on our collaboration, providing free access to the womens' health and JINGO samples for our research and her influence and mentorship with me on writing the papers.

I would also like to profoundly thank PhD student Kevin Duisters for his patience, persistence, and commitment to quality during our work on the menstrual cycle data analyses and paper writing.

Thanks to Dr. Bernhard Sonderegger and Dr. Eugenia Migliavacca for their tireless advice on everything from statistical approaches to software use and data management.

Thanks to Prof. Dr. Marie-Pier Scott-Boyer, Dr. Giulia Tini and COSBI for their teachings on statistical concepts and network analysis approaches.

Special thanks to Dr. Amy Harms and her scientific team for their hard work conducting metabolomics analysis of the womens menstrual cycle study samples.

Thanks to NuGO and Wageningen for their graduate student coursework.

Thanks to Maarten Warndorff for his dedicated contract negotiation support. Thanks to Loes Beijersbergen-Addink for her wonderful assistance with the execution of Leiden's requirements for my PhD.

A deep and special thanks to Dr. Paula Nenn, for her continued encouragement of me to pursue a PhD that started 8 years ago. Profound thanks to Michel Combes for his tireless encouragement, kindness, and graphics design and creative support.
Thanks to Magali Eggli for her generous design work of the thesis graphics cover.

Special thanks to Tanya, Vera, Lynne and Julianne for helping me stay harmonious.

Finally, I express my deepest and most profound gratitude to my Family - Dan, Madeleine, Corinne, Sophia; my parents (Bette and John Fogarty); and my brother (John) for their tenacious support, patience, encouragement, and unconditional love.

CURRICULUM VITAE

Colleen Fogarty Draper was born on May 6, 1969 in West Islip, New York, U.S.A., and raised in Merritt Island and Coral Springs, Florida, U.S.A. Colleen received her Bachelor of Science (BS) degree in Food and Nutrition at Florida State University in 1991 and went on to Tufts University in Boston, Massachusetts to receive her Masters of Science (MS) degree in 1993. She completed her dietetic internship with the Frances Stern Nutrition Center and New England Medical Center Hospitals in conjunction with her graduate work in 1993. Colleen successfully passed her exams to become a Registered Dietitian by the Commission on Dietetic Registration, U.S.A. during this same year.

Colleen has 27 years of experience in the field of nutrition of which she has spent 16 years in personalized nutrition and genetic technology translation, 21 years of in clinical trials research, and 11 years in traditional clinical dietetics and private functional medicine practice. Over the last 13 years, she has been an invited lecturer in personalized nutrition, womens' health and the integration of dietetics with new technology and functional medicine. Colleen has developed clinical practice and scientific expertise in a vast array of health areas including metabolic health, women's health, gastrointestinal health and brain health; as well as food allergies and sensitivities, pediatrics, personalized nutrition, metabolomics, genetics, diet intake and analysis technology development and oncology. She has worked in the academic hospital setting, as well was the food and pharmaceutical industries.

Colleen worked closely with Dr. Sofia Moco, Dr. Francois-Pierre Martin and Dr. Jim Kaput at the Nestle Institute of Health Sciences as a scientific researcher; while pursuing her PhD in life sciences with Prof. Dr. Jan van der Greef and Prof. Dr. Thomas Hankemeier in the research group Analytical BioSciences and Metabolomics at Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.

LIST OF PUBLICATIONS

Draper CF, Tini G, Vassallo I, Godin JP, Su M, Jia W, Beaumont M, Moco S, Martin FP. Vegan and animal meal composition and timing influence glucose and lipid related postprandial metabolic profiles, *in review*.

Draper CF, Duisters K, Weger B, Chakrabarti A, Harms AC, Brennan L, Hankemeier T, Goulet L, Konz T, Martin FP, Moco S, van der Greef J. Menstrual cycle rhythmicity: metabolic patterns in healthy women, Sci Rep 2018;8(1).doi:10.1038/s41598-018-32647-0.

Wei R, Ross AB, Su M, Jingye Wang, Guiraud SP, **Draper CF**, Beaumont M, Jia, W, Martin FP. Metabotypes related to meat and vegetable intake reflect microbial, lipid and amino acid metabolism in healthy people. Molecular nutrition and food research 2018; e1800583. doi: 10.1002/mnfr.201800583.

Draper CF, Vassallo I, Di Cara A, Milone C, Comminetti O, Monnard I, Godin JP, Scherer M, Su M, Jia W, et al. A 48-Hour Vegan Diet Challenge in Healthy Women and Men Induces a BRANCH-Chain Amino Acid Related, Health Associated, Metabolic Signature. Molecular nutrition & food research 2018;62(3). doi: 10.1002/mnfr.201700703.

Mathias MG, Coelho-Landell CA, Scott-Boyer MP, Lacroix S, Morine MJ, Salomao RG, Toffano RBD,Almada M, Camarneiro JM, Hillesheim E, , de Barros TT, Camelo-Junior J1, Giménez EC, Meisser K, Goyon A, Bertschy E, Lévêques 4, Oberson JM, Giménez C, Carayol J, Kussmann M, Descombes P, Métairon 5, **Draper CF**, et al. Clinical and Vitamin Response to a Short-Term Multi-Micronutrient Intervention in Brazilian Children and Teens: From Population Data to Interindividual Responses. Molecular nutrition & food research 2018;62(6):e1700613. doi: 10.1002/mnfr.201700613.

Draper CF, Curran AM, Scott-Boyer MP, Valsesia A, Roche HM, Ryan MF, Gibney MJ, Kutmon M, Evelo CT, Coort SL, et al. Sexual Dimorphism, Age, and Fat Mass Are Key Phenotypic Drivers of Proteomic Signatures. J Proteome Res 2017;16(11):4122-33. doi: 10.1021/acs.jproteome.7b00501.

Kaput, J, **Draper, CF,** Descombes, P, Rezzi, S, Kussmann M. Targeted (Personalized) Nutrition. Regulatory Focus 2016; 1–17./www.raps.org.

Moco S, Candela M, Chuang E, **Draper CF**, Cominetti O, Montoliu I, Barron D, Kussmann M, Brigidi P, Gionchetti P, et al. Systems biology approaches for inflammatory bowel disease: emphasis on gut microbial metabolism. Inflammatory Bowel Diseases 2014;20(11):2104-14. doi: 10.1097/MIB.0000000000000116.

DeBusk RM, **Fogarty CP**, Ordovas JM, Kornman KS. Nutritional genomics in practice: where do we begin? Journal of the American Dietetic Association 2005;105(4):589-98. doi: 10.1016/j.jada.2005.01.002.

LIST OF BOOK CHAPTERS

Mullin, GE. **Draper, CF**, Munsell MA. Inflammatory Bowel Disease. Advancing Medicine with Food and Nutrients. (Editor Kohlstadt, Ingrid) Chapter 15. 279-304. 2013.

Draper, CF. Nutritional Genomics in Research and Practice. AAPI's Nutrition Guide to Optimal Health: Using Principles of Functional Medicine and Nutritional Genomics. American Association of Physicians of Indian Origin. Chapter 2. 13-24. 2012.

Kornman K., **Fogarty C**. Nutrigenomics: Opportunities and Challenges. Chapter 1. Phytonutrient Conference; CRC Press: 2006.