Aging and the Plasma Metabolome: Relation to Physiological Function

by

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ABSTRACT

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Aging and the Plasma Metabolome: Relation to Physiological Function

Thesis directed by College Professor of Distinction Douglas R. Seals

Advancing age is associated with declines across numerous physiological systems, leading to an increased risk of chronic disease and disability. Whereas aging itself is inevitable, the rate at which physiological impairments occur is highly variable among individuals. Differences in the trajectory of physiological decline are due to complex biological processes; however, the molecular mechanisms underlying physiological impairments are not well understood. Metabolomics provides the unique opportunity to investigate systemic molecular changes *in vivo* by capturing perturbations in metabolic signaling linked to changes in physiological function. Therefore, the purpose of this dissertation was to determine the relation between metabolomic signatures and age-associated physiological declines in humans.

Global metabolomics profiling of plasma from young and older adults demonstrated changes in metabolomic signatures with advancing age. Pathway analysis of altered metabolites identified amino acid and lipid metabolism as primary pathways modified with age, and targeted metabolomic approaches confirmed these differences. Furthermore, changes in small molecule profiles were related to numerous clinically relevant indicators of human healthspan.

Additionally, in a systems biology approach to further investigate the molecular underpinnings of age-associated physiological declines, metabolomics analyses were applied to a model of biological aging. In a longitudinal study of older adults, biological age was quantified by integrating numerous clinical and physiological measures of human health. Importantly, biological age was significantly related to plasma metabolomic profiles, and these small molecule signatures were also associated with, and predictive of, rates of biological aging.

Collectively, these studies indicate that changes to the plasma metabolome with advancing age are related to physiological dysfunction in older adults. Moreover, these findings not only present valuable insight into pathways that may modulate healthy aging, our results also provide evidence for easily accessible blood-based markers of age-associated physiological declines in humans.

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Chapter II

Introduction

Age is the primary risk factor for the development of physiological dysfunction (Richardson *et al.* 2015), which can lead to clinical disorders and chronic disease (Franceschi & Campisi 2014; Kennedy *et al.* 2014). Given the dramatic increase in the number of middle-aged and older adults expected in the coming decades (Statisitics 2008), identifying strategies to prevent, delay, lessen and/or treat age-associated physiological dysfunction and reduce disease risk is one of the most important biomedical research goals (Fontana *et al.* 2014; Kennedy *et al.* 2014). Such efforts would, in turn, extend healthspan, the period of life associated with independence, productivity, and well-being, while simultaneously compressing dysfunction and disability into late life (Fries 1980; Kirkland & Peterson 2009). As such, understanding underlying pathways that are associated with, and even predictive of, physiological declines could benefit millions of adults at risk for age-associated chronic disease by identifying individuals who are most likely to progress to clinical conditions.

Previous research suggests that aging is tightly associated with the deterioration of metabolic processes in numerous organs and physiological systems (Ma *et al.* 2015). Assessing global metabolic function provides a unique opportunity to gain a systems biology understanding of molecular phenotypes. The metabolome, defined as all small molecules characterizing a biological system (Boccard *et al.* 2010; Barderas *et al.* 2011), is the downstream result of genomic and proteomic activity, and therefore provides important insight into multiple levels of physiological regulation (Soltow *et al.* 2010; Barallobre-Barreiro *et al.* 2013). Therefore, metabolomics is a promising tool for identifying biomarkers and elucidating mechanisms in diverse physiological and pathophysiological states by allowing for the exploration and integration of multiple pathways and networks (Soltow *et al.* 2010; Yu *et al.* 2012). Until recently, technological limitations have constrained the number of small molecules measured in any one analysis. With the advancement of novel methods, over 50,000 unique small molecules have

been detected and logged into current databases (Wishart *et al.* 2013). Complemented by the advancement of powerful computing platforms and generation of innovative statistical approaches (Xia & Wishart 2016), metabolomics analyses have the capacity to comprehensively measure countless biochemical reactions, and better characterize physiological systems (Mishur & Rea 2012).

Preclinical and clinical studies have demonstrated that the metabolome is modified with advancing age (Lawton *et al.* 2008; Houtkooper *et al.* 2011; Yu *et al.* 2012; Calvani *et al.* 2014; Ishikawa *et al.* 2014; Montoliu *et al.* 2014; Cheng *et al.* 2015), providing novel insight into the numerous physiological processes altered by aging. In an effort to focus on pathways central to aging, metabolomics analyses have been applied to populations of long-lived individuals who typically show higher levels of function into later years of life. Analyses of "longevity" cohorts (Cheng *et al.* 2015) and centenarians (Montoliu *et al.* 2014) have identified small molecule patterns that differentiate long lived populations from normal aged groups, and provide insight into metabolomic changes associated with healthy aging.

In addition to understanding metabolic pathways modified with advancing age, association of these metabolic perturbations to functional declines presents a unique opportunity to gain insight into the molecular mechanisms of age-associated physiological declines. Previous research has demonstrated that metabolomic profiles are associated with functional outcomes, and that alterations to the metabolome with intervention are related to changes in function. In preclinical models, caloric restriction and high aerobic capacity prevent age-associated changes in the serum and plasma metabolome (De Guzman *et al.* 2013; Falegan *et al.* 2016), whereas the administration of exogenous compounds reverse metabolic perturbations (Yan *et al.* 2009; Chin *et al.* 2014). Importantly, these findings translate to humans in which metabolomic modifications with intervention are related to improvements in numerous functional domains (Lustgarten *et al.* 2014; DeVan *et al.* 2015; Jablonski *et al.* 2015; Justice *et al.* 2015), sometimes independent of changes in clinical blood markers. Extending beyond

subclinical dysfunction, molecular profiles are associated with, and predictive of, chronic diseases such as cardiovascular disease and insulin resistance (Newgard 2012; Shah *et al.* 2012), and specific metabolite species are predictive of future cognitive decline and pheno-conversion from healthy/mild cognitive impairment to Alzheimer's Disease up to 5 years in advance of clinical diagnoses in elderly patients (Mapstone *et al.* 2014). Taken together, these findings suggest that the plasma metabolome can provide novel insight into the molecular events that underlie functional changes with aging and predict future dysfunction. As such, Chapter III of this dissertation focuses on the metabolomic signatures associated with advancing age and their relation to adverse changes to healthspan indicators in healthy older adults.

An effective approach to reduce disease burden is to delay the age of onset of physiological decline and related clinical conditions (Burch *et al.* 2014). Although advancing age is associated with a progressive reduction in physiological functions (Kennedy *et al.* 2014), the rate at which these declines occur is highly variable (Brooks-Wilson 2013). This discrepancy occurs as the result of numerous interactions among complex biological mechanisms that underlie physiological declines, including genetic, lifestyle, and environmental factors (Wahl *et al.* 2012). Although chronological age is one of the most important risk factors when predicting adverse clinical outcomes, its lack of plasticity limits its predictive capability. Alternatively, biological age is highly malleable and therefore a superior metric by which to assess an individual's chronic disease risk (Levine 2013). Whereas previous research has focused on generating models of biological aging based upon the association of molecular markers with chronological age (Hannum *et al.* 2013; Horvath 2013), these analyses are not necessarily associated with physiological status. Therefore, we developed a method to quantify biological age that is reliant on clinical and physiological measures. To identify pathways associated with biological aging and provide an avenue for clinical application, in Chapter IV we examine small molecules associated with, and predictive of, biological aging in humans.

CHAPTER III

Changes in plasma metabolomic patterns and signatures related to amino acid and lipid metabolism with aging are related to indicators of healthspan in humans

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ABSTRACT

Advancing age is associated with impairments in numerous physiological systems, leading to an increased risk of chronic disease and disability, and reduced healthspan (the period of high functioning, healthy life). However, the molecular mechanisms underlying physiological declines in the absence of disease are poorly understood. The plasma metabolome is thought to reflect changes in the activity of physiological systems that influence healthspan. Accordingly, we utilized an untargeted metabolomics analysis of plasma collected from healthy young and older individuals to characterize global changes in small molecule abundances with age. Using a weighted gene correlation network analysis, similarly expressed metabolites were grouped into modules that were related to indicators of healthspan. Investigation of metabolic classes represented within each module revealed amino acid and lipid metabolism as significantly associated with age and healthspan indicators. A separate targeted metabolomic analysis was used to confirm these initial findings. Overall, these results demonstrate that plasma metabolomics profiles in general, and amino acid and lipid metabolism in particular, are associated with clinical and physiological indicators of healthy aging.

Keywords: Aging, metabolomics, weighted gene correlation network analysis, healthspan

INTRODUCTION

Aging is associated with declines in multiple physiological systems, resulting in increased risk for the development of chronic disease and disability, and consequent reduction in healthspan, i.e., the period of life associated with good function and health (Franceschi & Campisi 2014; Kennedy *et al.* 2014; Seals *et al.* 2016). Given the dramatic increase in the number of middle-aged and older adults in the coming decades (Statisitics 2008), identifying and understanding the underlying mechanisms that determine healthspan is an important priority in biomedical research (Fontana *et al.* 2014). While aging itself is inevitable, the trajectory of physiological decline with advancing age is highly variable among individuals and is likely due, in part, to interactions among complex biological systems (Brooks-Wilson 2013). The metabolome, defined as all small molecules characterizing a biological system (Boccard *et al.* 2010; Barderas *et al.* 2011), is the downstream result of genomic and proteomic activity and is therefore representative of overall physiological status (Soltow *et al.* 2010; Barallobre-Barreiro *et al.* 2013). Because the plasma metabolome is reflective of global biological processes, it may provide unique insight into pathways that contribute to healthy aging.

The plasma metabolome is altered with advancing age (Lawton *et al.* 2008; Yu *et al.* 2012; De Guzman *et al.* 2013), and is predictive of disease in older, at risk populations (Shah *et al.* 2012; Mapstone *et al.* 2014). However, the relation between age-associated changes in the plasma metabolome and physiological and clinical indicators of healthspan is largely unknown. In the present study we identified unique plasma metabolomic patterns of aging in adults without disease that are associated with clinical and physiological indicators of healthspan. Using a combination of untargeted and targeted analyses we identified modules of related metabolites, as well as specific signatures of amino acid and lipid metabolism, that were associated with multiple determinants of healthspan in humans. Healthspan indicators included physiological assessments of maximal oxygen consumption ($\dot{V}O_2$max; aerobic exercise capacity), brachial artery flow-mediated dilation (vascular endothelial function), estimated glomerular filtration rate

(kidney function), body composition (fat and lean body mass), and key risk factors for chronic disease and disability including circulating glucose and insulin, plasma lipids, body mass index, arterial blood pressure, and circulating markers of inflammation.

RESULTS

Subject Characteristics and Indicators of Healthspan

To determine the effects of aging on the plasma metabolome, we performed an untargeted metabolomics analysis on groups of healthy young (Young, 18-30 years, n=14) and older (Older, 45-74 years, n=29) adults who were free of clinical disease or disability. All subjects underwent a medical history and physical examination, and were phenotyped for various indicators of healthspan (Table 1). All clinical and physiological measures were within normal ranges, with some expected age-related differences observed between the Young and Older groups. Differences observed included age-related increases in percent body fat, systolic blood pressure, body mass index, low density lipoprotein (LDL)- and total cholesterol, and reductions in $\dot{V}O_2$max and vascular endothelial function in the Older group compared with Young.

Construction of Biologically Relevant Metabolomic Modules

To determine patterns of change within the plasma metabolome associated with age, untargeted metabolomics analysis was performed using plasma collected from subjects in the Young and Older groups. Of the 4,681 small molecule features identified, 2,957 were determined to be present in at least 50% of Young or Older groups, and used to perform a weighted gene correlation network analysis (WGCNA) (Langfelder & Horvath 2008). The WGCNA method utilizes an unsupervised network–based approach to group metabolites into “modules” based on similarities in their abundances, without taking age or other phenotypic factors into consideration. This sophisticated data reduction step allows for meaningful

information to be extracted from cross-sectional studies because it drastically reduces the number of comparisons made, absolves issues of co-linearity, and provides a platform for a more practical statistical approach aimed at understanding and interpreting systems biology data. The WGCNA analysis identified 20 unique modules of co-expressed metabolites. Per WGCNA convention, each module was assigned an arbitrary color for naming purposes, and a module "score" (module eigengene) was calculated for each individual based on the first principal component score of all metabolites within a module, allowing for comparison of module expression between groups and for relating modules of metabolites to indicators of human healthspan.

Selection of Age-Associated Metabolomic Patterns and their Relation to Indicators of Healthspan

To determine if any of these modules were associated with aging, mean module scores were compared between Young and Older groups using unpaired t-tests. To understand the potential physiological and clinical relevance of metabolite modules that demonstrate age-associated differences, module scores were then related to indicators of healthspan. Because this was an exploratory analysis intended to identify potential molecular patterns associated with aging, and given the robust data reduction step employed using WGCNA, an unadjusted p-value of <0.05 was used to identify modules significantly different with age.

Of the 20 distinct modules generated using WGCNA, the Magenta (97 metabolites), Purple (87 metabolites), Blue (349 metabolites), and Green (155 metabolites) modules were found to be both different between Young and Older individuals (Figure 1) and associated with multiple indicators of healthspan (Table 2). Regarding the associations between modules and individual healthspan indicators, it is important to note for purposes of interpretation that some modules (Magenta, Blue, and Green) were related to indicators of healthspan that displayed no age-associated changes, including lean mass, glucose, insulin, interleukin-6, and HDL-

cholesterol. This observation suggests that these modules are independently related to the healthspan markers in question rather than via secondary associations with age. Based on the outcome of this untargeted analysis, the Magenta, Purple, Blue and Green modules were prioritized for further analysis, as described next.

Detected Pathways and their Association with Healthspan Indicators

In an effort to isolate the metabolic pathways associated with aging, a pathway analysis was performed on metabolites within each of the four modules of interest. Because we performed an untargeted metabolomics analysis, many of the metabolites within these four modules remain to be characterized. Therefore, unidentified metabolites were eliminated and only known metabolites from each module were included in the pathway analysis using Human Metabolome Database (HMDB) (Wishart *et al.* 2013) identifiers and KEGG curated pathways (Kanehisa *et al.* 2017) through Metaboanalyst software (Xia & Wishart 2016).

Thirty-four (34) unique metabolic sub-pathways were identified among the four modules of interest (Supplemental Table 1). To gain insight into high-level changes in metabolic regulation that may occur with age, sub-pathways were organized by their higher order "class", of which eight were identified using KEGG databases (Figure 2). Of the pathway classes identified, amino acid and lipid metabolism were strongly represented, accounting for 66% of the total class distribution among the four modules.

Targeted Metabolomics Confirms Changes in Amino Acid and Lipid Metabolism with Age

To confirm the results of our untargeted global analysis of the plasma metabolome, a targeted analysis was used to identify metabolites and pathways associated with amino acid and lipid metabolism in Young and Older individuals (Table 3). Of 43 amino acids and amino acid derivatives measured, 13 demonstrated age-related changes when compared using unpaired t-tests, seven of which are associated with protein biosynthesis (Asp, Met, Ala, Pro,

Phe, Trp, cystine). Of the proteogenic amino acids and their derivatives, all demonstrated lower concentrations with aging, except cystine, which was higher with age (Supplemental Figure 1). Alternatively, six non-proteinogenic amino acids and derivatives (3-methylhistidine, citrulline, ornithine, β-aminoisobutyric-acid, α-amino-N-butyric-acid, and cystathionine) were found to increase with age (Supplemental Figure 1). For lipid metabolism, we measured 12 fatty acids, 11 acylcarnitines, and 12 ceramides. Age-related increases were observed in six fatty acids, six acylcarnitines, and six medium- and long-chain ceramides (Supplemental Figures 2 and 3).

Age-Associated Changes in Amino Acid and Lipid Metabolism-Linked Metabolites are Related to Indicators of Healthspan

After confirming age-associated differences in numerous signatures of amino acid and lipid metabolism, we next investigated the potential relations between these metabolites and indicators of human healthspan. Metabolites most different with age ($P<0.01$ in Older vs Young) were related to clinical and physiological outcomes using correlation analysis and are presented in a heatmap matrix (Figure 3). For clarity, visualization of the relations between individual metabolites and indicators of healthspan, the clinical and physiological markers were categorized as “adverse” if higher values are associated with higher risk of age-related dysfunction/disease or “protective” if higher values are associated with lower risk.

All metabolites demonstrated significant relations to multiple indicators of healthspan. Of note, metabolites positively related to adverse indicators of healthspan were negatively associated with protective indicators, and vice versa. Certain classes of metabolites (i.e. amino acids, fatty acids, ceramides, and acylcarnitines) typically clustered their associations around certain healthspan indicators. For example, amino acids showed associations with systolic blood pressure, total cholesterol, estimated glomerular filtration rate, and $\dot{V}O_2$max. Fatty acids, in general, were related to measures of body composition and $\dot{V}O_2$max, whereas acylcarnitines primarily demonstrated associations with systolic blood pressure and $\dot{V}O_2$max. The group of

ceramide metabolites, known for their role in inflammatory and apoptotic pathways (Verheij *et al.* 1996; Nixon 2009), were particularly strongly correlated to $\dot{V}O_2$max, in addition to clinical blood markers such as triglycerides, cholesterols, and C-reactive protein.

DISCUSSION

We employed a novel systems biology approach to elucidate the relations between age-associated patterns of metabolite expression, individual metabolites, and meaningful clinical and physiological determinants of human healthspan. Our results demonstrate specific patterns of change in the plasma metabolome with age, and that these changes may be associated with multiple indicators of healthspan in adults free of disease or disability. This study also is the first to our knowledge to utilize both untargeted and targeted metabolomics approaches to explore biologically significant changes in small molecule signatures, and relate them to a diverse set of healthspan markers. By using pathway analysis and complementary targeted approaches, we were able to show that changes in specific metabolic pathways, primarily those related to amino acid and lipid metabolisms, are most consistently related to healthspan markers. These healthspan markers, in turn, are well-established risk factors for impaired physiological function linked to future disability (e.g., mobility limitations), as well as chronic cardio-metabolic diseases with advancing age.

As this analysis was exploratory in nature, the reported changes in metabolites of lipid and amino acid metabolism, in particular, and their associations with markers of healthy aging, are meant to guide future hypothesis generation. In this context, one clinically important future direction would be examining the relations between changes in these metabolic networks and $\dot{V}O_2$max. $\dot{V}O_2$max declines with age and is among the most important indicators of future risk of morbidity and mortality in older adults (Fitzgerald *et al.* 1997; Sui *et al.* 2007; Betik & Hepple 2008). A recent scientific statement by the American Heart Association emphasizes the need for increased testing of cardiorespiratory fitness as a critical determinant of human health and

disease risk (Ross *et al.* 2016). Therefore, identifying the molecular processes that influence $\dot{V}O_2$max is a high biomedical priority.

In the present study, we found that age-associated differences in metabolites involved in fatty acid and acylcarnitine metabolism, which are involved in beta-oxidation and mitochondrial function (Koves *et al.* 2008), were related to $\dot{V}O_2$max. In addition to the negative association between $\dot{V}O_2$max and increased fatty acid metabolites, several proteinogenic amino acids demonstrated positive associations with $\dot{V}O_2$max, whereas 3-methylhistidine, previously identified as a marker of muscle wasting (Bilmazes *et al.* 1978; Sheffield-Moore *et al.* 2014), was negatively associated with this important measure of integrative physiological function with aging. Similarly, changes in several metabolites of these pathways with aging were significantly related to vascular (flow-mediated dilation) and renal (estimated glomerular filtration rate) function, and numerous clinical risk factors for cardio-metabolic disease and/or disability, including body fatness, lean body mass, blood pressure, glucose-insulin function, circulating inflammatory proteins, and plasma lipids (Figure 3). Overall, these observations provide insight into some of the molecular events related to age-associated declines in physiological function and increases in risk of clinical disorders.

Our findings demonstrate that small molecule profiles from plasma can provide unique insight into the connections between metabolic processes, physiological function and risk factors for disease with aging. As such, this approach could be used in future investigations to identify other candidate pathways that influence human healthspan.

Given the limitations of this initial, hypothesis-generating set of analyses, we cannot determine whether the relations observed in metabolomics signatures, pathways and patterns reported here reflect causal influences on associated healthspan indicators or simply represent metabolic markers secondary to current physiological and clinical status. We were, however, able to identify metabolomic patterns that were related to indicators of healthspan that were not altered with aging, suggesting that the associations were not simply driven by the effects of aging.

Although more work is necessary to confirm and extend these observations, our integrated untargeted and targeted analyses provide an approach for the use of systems biology data to uncover the molecular underpinnings of healthy aging in humans.

METHODS

Study Design and Subjects

All experimental procedures were reviewed and approved by the University of Colorado Boulder Institutional Review Board. Clinical and physiological measurements were performed at the University of Colorado Boulder Clinical Translational Research Center (CTRC). Written informed consent was obtained from all study participants after the nature, benefits and risks of the study were explained. Subjects were screened for smoking status and determined free of clinical disease as assessed by medical history, physical examination, blood chemistries, and resting and exercise ECG. Older women were postmenopausal for at least one year, while premenopausal women were studied during the early follicular phase of their menstrual cycle to control for the effects of hormonal variation on function. All subject testing followed a 12-hour fast and 24-hour abstention from alcohol, exercise, and prescription medication.

Indicators of human healthspan

Body mass index was measured using anthropometry (Lohman 1988). Body composition measures were measured using dual-energy X-ray absorptiometry (DEXA-GE, Lunar Prodigy Advance; software version 5.6.003). Arterial systolic and diastolic blood pressures were measured in triplicate over the brachial artery at rest using a semi-automated device (Dinamap XL, Johnson & Johnson). Fasting glucose was measured in plasma (Ortho Clinical Diagnostics) and fasting plasma insulin was measured by radioimmunoassay (Millipore), and HOMA-IR was calculated (fasting plasma glucose (mg/dL) x fasting plasma insulin (μU/mL)/405)(Matthews *et al.* 1985). Circulating IL-6 and TNF-α were measured in serum by ELISA (R&D Systems), and

serum high-sensitivity C-reactive protein was measured by immunoturbidimetry (Beckman Coulter). Fasting serum lipids were measured by Boulder Community Hospital Clinical Laboratory with standard assays, as previously described (DeVan *et al.* 2015). Estimated glomerular filtration rate (eGFR) was calculated using the MDRD 4 equation in the R statistical platform ('nephro' package, Version 1.1) (Virga *et al.* 2007). Maximal oxygen consumption ($\dot{V}O_2max$) was measured using incremental treadmill exercise testing to exhaustion using open circuit spirometry (Balke protocol), as described previously (Evans *et al.* 1995). Endothelial function was assessed using brachial artery flow-mediated dilation in response to reactive hyperemia after a 5-minute occlusion of blood flow, as previously described (DeSouza *et al.* 2000; Pierce *et al.* 2009).

Metabolomics Analysis

Untargeted Metabolomics Analysis:

Fasting EDTA-treated plasma was collected, frozen, and stored at -80 degrees Celsius until analysis. Untargeted metabolomic profiling of plasma was performed using an Ultra High Performance Liquid Chromatograph (Infinity 1290 UHPLC, Agilent Inc. USA) coupled to 6550 Quadruple Time-of-Flight mass spectrometer (Q-ToF MS, Agilent Inc. USA). Data was acquired both in positive and negative electrospray ionization (ESI) modes in the mass range of m/z 100-1600 at a resolution of 10,000. Chromatographic separation was achieved using hydrophilic interaction liquid chromatography (HILIC) and reverse phase (C18) liquid chromatography separately. The metabolite extraction and instrument settings were performed as described previously (Dutta *et al.* 2012; Dutta *et al.* 2016) with minor modifications. Plasma (50 µl) samples was deproteinated with 80% cold acetonitrile: methanol (1:1), followed by centrifugation at 18000xg for 30 minutes at 4^0C. U-$13C_6$-phenylalanine (3 µl at 250ng/µl) was added as internal standard to each sample and QCs. The supernatants was divided into 4 aliquots and dried down using a stream of nitrogen gas for analysis on a Quadruple Time-of-

Flight Mass Spectrometer (Agilent Technologies 6550 Q-TOF) coupled with an Ultra High Pressure Liquid Chromatograph (1290 Infinity UHPLC Agilent Technologies). Profiling data was acquired in scan mode under both positive and negative electrospray ionization (ESI) conditions over a mass range m/z of 100 - 1700 at a resolution of 10,000. Metabolite separation was achieved using two columns of differing polarity, a hydrophilic interaction column (HILIC, ethylene-bridged hybrid 2.1 x 150 mm, 1.7 mm; Waters) and a reversed-phase C18 column (high-strength silica 2.1 x 150 mm, 1.8 mm; Waters) with gradient described previously using a flow rate of 400µl/min (Dutta *et al.* 2012; Trushina *et al.* 2013; Dutta *et al.* 2016). Samples were randomized, and a total of four runs per sample were performed to give maximum coverage of metabolites. Samples were injected in duplicate, and a pooled quality control (pooled QC) sample, made up of all of the samples from each study was injected ~10 times during a run. A separate plasma quality control (QC) sample was analyzed with pooled QC to account for analytical and instrumental variability. Samples were reconstituted in running buffer and analyzed within 48 hours of reconstitution.

Untargeted Metabolomics Data and Pathway Analysis:

All raw data files were converted to compound exchange file (CEF) format using MassHunter Profinder (version B08.00) software (Agilent). Mass Profiler Professional (Agilent Inc, USA) was used to convert metabolite features from each data file (m/z x intensity x time) into a matrix of detected peaks for compound annotations and statistical analysis (Dutta *et al.* 2012; Dutta *et al.* 2016). In total, 4,681 features were detected (aqueous positive: 1,244 features; aqueous negative: 1,559 features; lipid positive: 1,072 features; lipid negative: 806 features). To reduce the chance of false discovery, only metabolites present in at least 50% of Young or Older groups were included in the analysis, as described previously (DeVan *et al.* 2015; Justice *et al.* 2015). Metabolites were annotated as previously described (DeVan *et al.* 2015; Justice *et al.* 2015). Allocation of known metabolites to pathways was performed using HMDB identifiers in

MetaboAnalyst, and assigned to KEGG curated pathways (Wishart *et al.* 2013; Xia & Wishart 2016; Kanehisa *et al.* 2017).

Targeted Metabolomics Analysis:

Quantitative measurements of free fatty-acids (FFA), ceramides, acyl carnitines (C0-C18:1) and 45 amino acid metabolites in plasma were performed by tandem mass spectometry (MS/MS) (full list and %CV values, Table S2). Briefly, plasma samples were spiked with internal standards then deproteinized with cold methanol followed by centrifugation at 10,000 g for 5 minutes. The supernatant was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate according to Waters' MassTrak kit. Both derivatized standards and samples were analyzed on a triple quadrupole mass spectrometer (TSQ Quantum Ultra, ThermoFisher, USA) coupled with UPLC using selected ion monitor (SRM). Concentrations of 43 analytes of each unknown were calculated against each respective calibration curve (Lanza *et al.* 2010). Plasma ceramides (C16:0, C18:0, C20:0, C22:0, C24:1, C24:0) sphinganine, sphingosine, sphingosine-1-phosphate (S1P), were measured as previously described (Blachnio-Zabielska *et al.* 2012). Acyl carnitines (C0-C18:1) were measured by LCMS/MS using selective ion monitoring (SRM) on Thermo TSQ Quantiva mass spectrometer (West Palm Beach, FL) coupled with a Waters Acquity UPLC system (Milford, MA). Briefly, 25uL of plasma was spiked with a purchased internal standard consisting of isotopically labeled acyl carnitines (DL-Carnitine Hydrochloride, Fisher Scientific, Hampton, NH; DL-Lauroylcarnitine, Myristoyl-DL-carnitine chloride, Palmitoyl-DL-Carnitine, Palmitoyl-L-carnitine Chloride, Palmitoyl-L-carnitine HCL, Sigma-Aldrich, St. Louis, MO; Heptadecanoyl-L-carnitine in HCL, Elaidoyl L-carnitine-HCL, Oleoyl -$^{13}C_{18}$-L-carnitine HCL 5.7%, Oleoyl-L-^{13}C-carnitine HCL, Isotec, Miamisburg, OH; Stearoly-L-carnitine chloride, Linoleoyl L-carnitine chloride, Chem-Impex, Wood Dale, IL; Oleoly L-carnitine, Avanti Polar Lipids, Alabaster, AL; Arachidoyl-DL-carnitine chloride, Santa Cruz, Dallas, TX). The samples were then extracted with cold MeOH:DCM (1:1) followed by centrifugation at 12,000 g for 10

minutes. The supernatant was dried down and reconstituted in running buffer. Concentrations of each analyte were calculated against their respective standard curves (Chace *et al.* 2001). All quantitative measurements using GC-MS and LC-MS/MS were done against 12-point calibration curves that underwent the same derivatization with internal standard (Figure S4).

Statistics

Weighted Gene Correlation Network Analysis: Log transformed metabolite abundances from the untargeted metabolomics analysis were evaluated with a weighted gene correlation network analysis using the WGCNA package in R (Langfelder & Horvath 2008). This analysis generates a correlation matrix between all metabolites across all samples to identify groups of metabolites that are highly related. Under default conditions, *goodSamplesGenes* excluded 434 metabolites from network calculations. Correlation matrix soft-thresholding power was raised to $\beta = 7$, emphasizing stronger correlations (Langfelder & Horvath 2008). Key settings include: *softThresholdPower = 7, minModuleSize = 30*, and *mergeCutHeight = 0.25*. Metabolites were grouped into 20 modules containing highly correlated metabolites, and each individual subject was assigned a module eigengene (ME) "score", determined as the first principal component of the module, which represents the metabolomic pattern within each module for that subject. ME's can then be used for further statistical analyses. To determine differences with age, MEs were compared between Young and Older groups using independent t-tests. Additionally, unpaired t-tests were used to assess differences in targeted metabolite abundances between young and older groups. Metabolite expression (both modules and independent metabolites from the targeted analysis) was related to indicators of healthspan using independent linear regression.

Acknowledgements

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Author Contributions

L.C.J., C.R.M., and D.R.S. conceived and developed the overall study design. J.S.P., C.J.B., and T.R.S. collected data used for indicators of healthspan. L.C.J. performed metabolomics statistical analysis and interpretation. L.C.J., C.R.M., M.B.M. conceptualized the analytical approach and contributed technical support. L.C.J. and D.R.S. drafted the manuscript with assistance from C.R.M. All authors edited and approved the final version.

Table 1: Subject Characteristics

Subject Characteristics	Young	Older
Sex (M/F)	7/7	13/16
Age (years)	23 ± 1	61 ± 1*
Body Mass (kg)	65 ± 3	70 ± 2
Body Fat (%)	22 ± 3	28 ± 2*
Lean Mass (%)	73 ± 3	69 ± 2
Systolic Blood Pressure (mmHg)	104 ± 3	119 ± 2*
Diastolic Blood Pressure (mmHg)	68 ± 2	72 ± 1
Body Mass Index (kg/m^2)	21 ± 1	24 ± 1*
Fasting Blood Glucose (mg/dL)	84 ± 2	90 ± 2
Fasting Insulin (mIU/L)	7.3 ± 1	7.8 ± 1
HOMA-IR	1.6 ± 0.2	1.8 ± 0.2
Triglycerides (mg/dL)	78 ± 9	98 ± 12
Total Cholesterol (mg/dL)	157 ± 12	196 ± 7*
LDL-C (mg/dL)	88 ± 11	113 ± 7*
VLDL-C (mg/dL)	18 ± 3	16 ± 2
HDL-C (mg/dL)	54 ± 2	64 ± 4
TNFα (pg/mL)	1.3 ± 0.4	1 ± 0.1
C-Reactive Protein (mg/L)	0.8 ± 0.2	0.8 ± 0.1
IL-6 (pg/mL)	0.8 ± 0.1	0.9 ± 0.1
eGFR ($mL/min/1.73m^2$)	93 ± 4	76 ± 3*
VO_2max (ml/kg/min)	44.4 ± 2.3	32.7 ± 1.3*
Endothelial Function (%Δ FMD)	7.4 ± 0.6	4.3 ± 0.3*

Data are mean ± SEM; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; VO_2max, maximal oxygen consumption. * $P<0.05$ vs. Young.

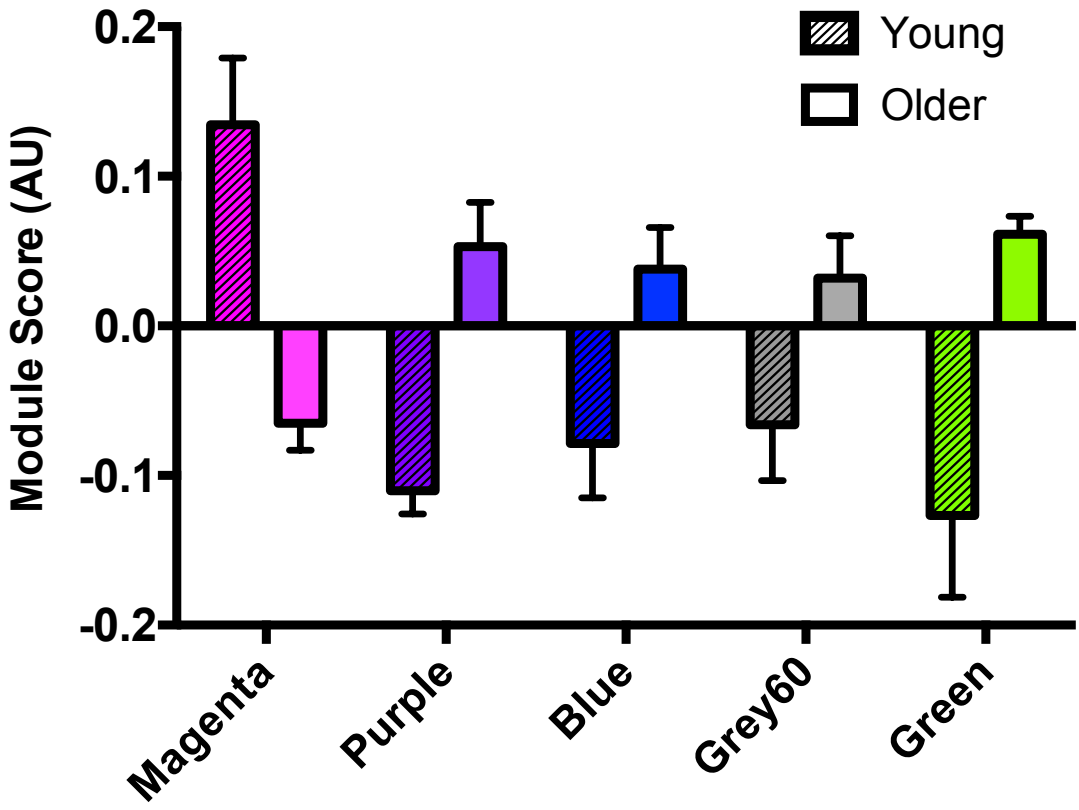


Figure 1: To detect differences in metabolomic expression patterns with age, module scores were compared between Young and Older groups, with five modules (Magenta, Purple, Blue, Grey60, and Green) demonstrated significant differences. Figure includes mean module score ± SEM for Young and Older groups. *P < 0.05 vs. Young.

Table 2: To determine if modules demonstrating differences in metabolite expression patterns with age are associated with physiological measures, modules significantly different between Young and Older groups were assessed for their relation to 19 markers of healthspan. Only significant correlations are shown (Pearson Coefficient, P<0.05).

Module	Healthspan Marker	Pearson Coefficient	P-Value
Magenta	Percent Body Fat	-0.52	<0.001
	Total Cholesterol	-0.39	0.01
	Lean Mass	0.39	0.01
	VO_2max	0.64	<0.001
	Endothelial Function	0.40	0.009
Purple	Systolic Blood Pressure	0.35	0.02
	eGFR	-0.33	0.03
	VO_2max	-0.37	0.02
Blue	Glucose	0.37	0.02
	Interleukin-6	-0.38	0.01
	HDL-Cholesterol	0.31	0.04
	eGFR	-0.50	<0.001
Green	Systolic Blood Pressure	0.43	0.004
	Insulin	0.32	0.04
	Total Cholesterol	0.32	0.04
	eGFR	-0.39	0.009
	VO_2max	-0.36	0.02

eGFR, estimated glomerular filtration rate; VO_2max, maximal oxygen consumption.

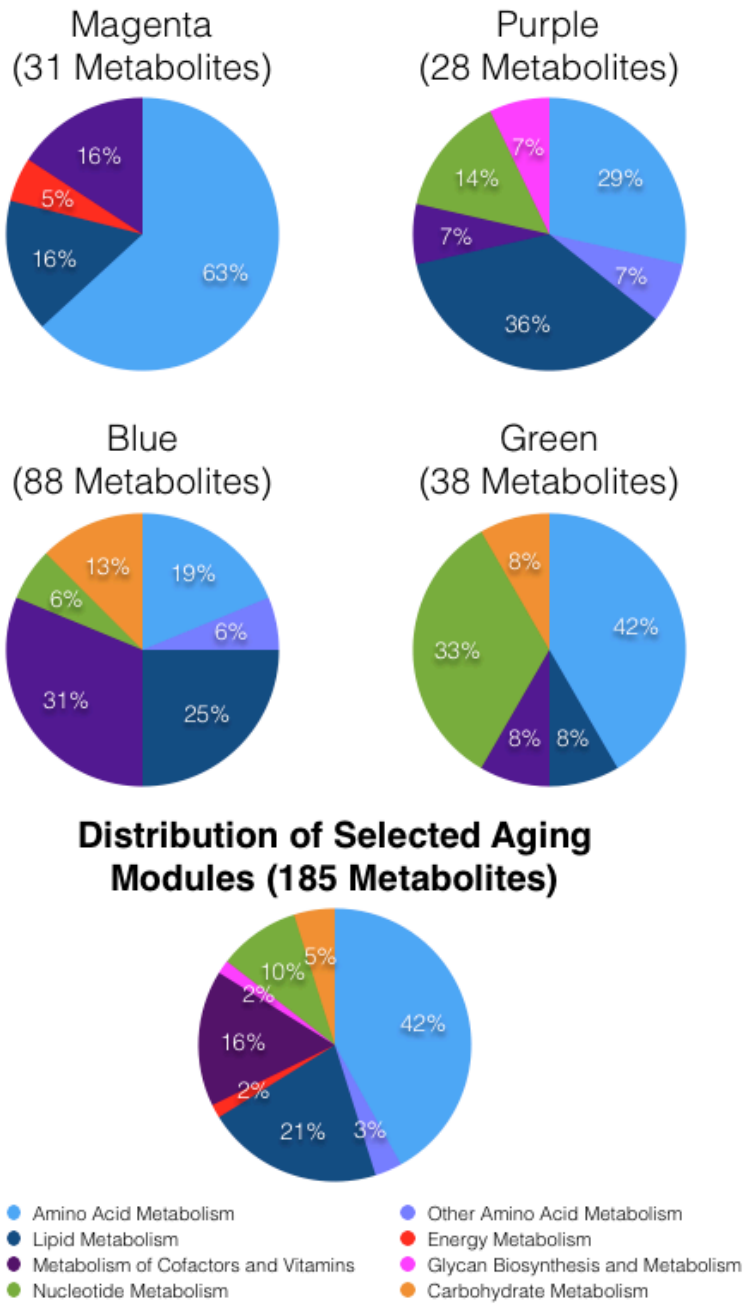


Figure 2: Distribution of identified metabolites, isolated from modules significantly different with age and related to multiple markers of healthspan, within specific classes of metabolic pathways from KEGG databases. To understand the key pathways most associated with aging and function, the two pathways most highly represented in this analysis (amino acid and lipid metabolism) were selected for targeted analyses.

Table 3: Concentrations of metabolites significantly different with age from targeted analyses of amino acid and lipid metabolism.

	Metabolite	Young	Older
Amino Acids	Aspartic Acid (uM)	4.6 ± 1.10	1.90 ± 0.35**
	Methionine (uM)	23.92 ± 1.12	20.59 ± 3.82**
	Alanine (uM)	372.71 ± 21.54	288.12 ± 53.50***
	Proline (uM)	185.98 ± 16.61	135.78 ± 25.21***
	Phenylalanine (uM)	57.27 ± 2.91	51.33 ± 9.53*
	Tryptophan (uM)	55.14 ± 2.43	44.13 ± 8.19***
	Citrulline (uM)	30.69 ± 1.72	37.22 ± 6.91**
	Ornithine (uM)	39.64 ± 2.11	46.32 ± 8.60*
	Cystine (uM)	61.92 ± 10.88	91.42 ± 16.98*
	3-Methylhistidine (uM)	1.48 ± 0.17	2.66 ± 0.49***
	β-Aminoisobutyric-acid (uM)	1.06 ± 0.16	1.92 ± 0.36**
	α-Amino-N-butyric-acid (uM)	16.38 ± 1.34	21.25 ± 3.95**
	Cystathionine (uM)	0.04 ± 0.02	0.25 ± 0.05*
Fatty Acids	Linolenic Acid (uM)	3.68 ± 0.34	5.47 ± 1.02*
	Myristic Acid (uM)	2.96 ± 0.37	4.85 ± 0.90**
	Palmitoleic Acid (uM)	4.39 ± 0.75	8.39 ± 1.56**
	Linoleic Acid (uM)	30.94 ± 3.58	43.42 ± 8.06**
	Palmitic Acid (uM)	42.86 ± 5.68	66.65 ± 12.38**
	Oleic Acid (uM)	66.95 ± 9.06	118.86 ± 22.07***
Ceramides	C16-Ceramide (uM)	0.21 ± 0.02	0.28 ± 0.05**
	C18-Ceramide (uM)	0.06 ± 0.01	0.10 ± 0.02*
	C20-Ceramide (uM)	0.07 ± 0.01	0.09± 0.02**
	C22-Ceramide (uM)	0.24 ± 0.02	0.27 ± 0.01*
	C24:1-Ceramide (uM)	0.52 ± 0.04	0.64 ± 0.12*
	C24-Ceramide (uM)	1.38 ± 0.11	1.73 ± 0.32***
Acylcarnitines	Acetylcarnitine (uM)	8.74 ± 0.68	13.59 ± 2.52***
	Octanoylcarnitine (uM)	0.24 ± 0.02	0.31 ± 0.06*
	Myristoylcarnitine (uM)	0.11 ± 0.004	0.12 ± 0.002**
	Palmitoylcarnitine (uM)	0.24 ± 0.01	0.29 ± 0.05**
	Oleoylcarnitine (uM)	0.30 ± 0.02	0.43 ± 0.08***
	Stearoylcarnitine (uM)	0.15 ± 0.01	0.18 ± 0.03*

All concentrations are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Young

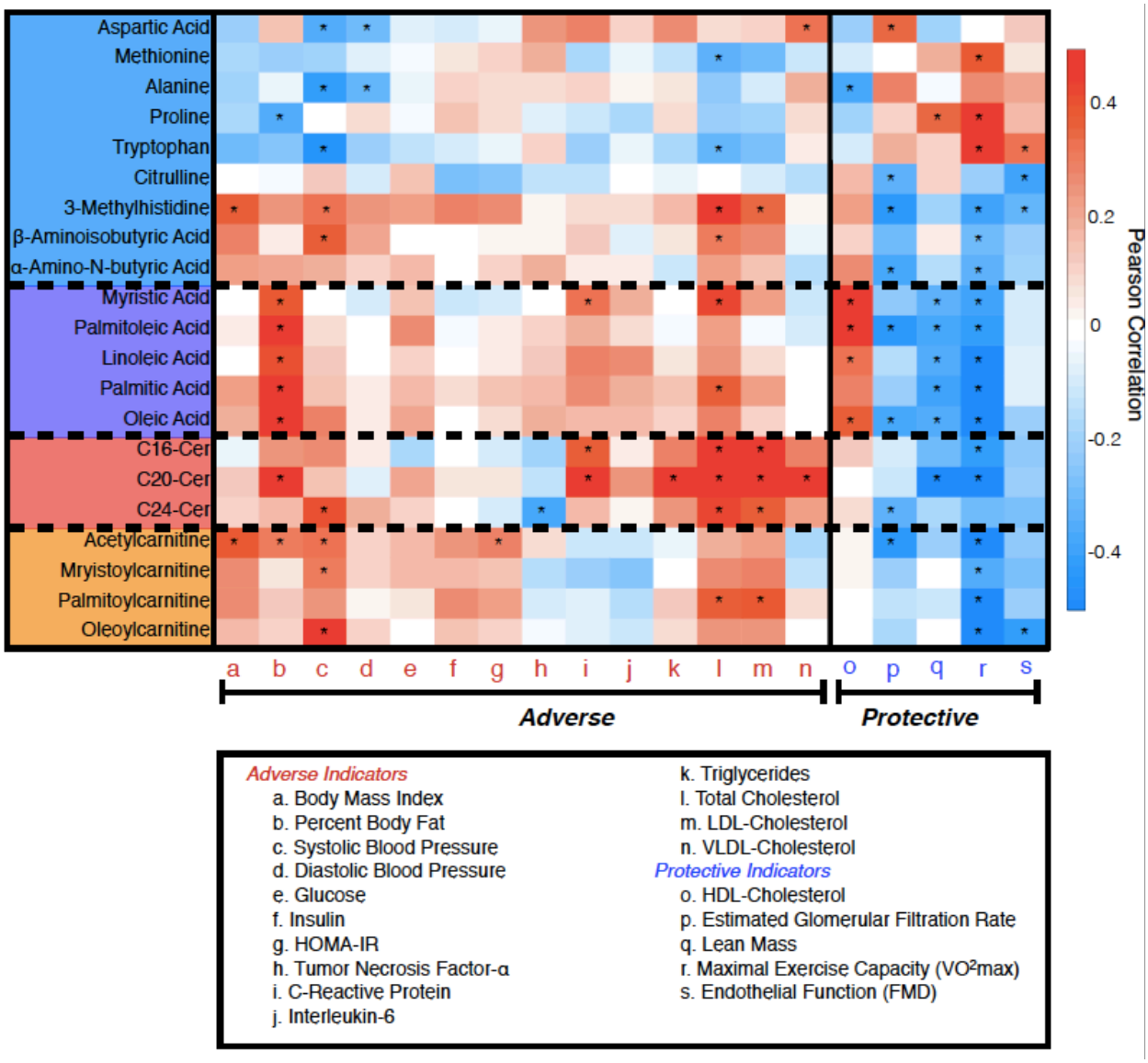


Figure 3: Correlation heatmap assessing the relation between specific metabolites found to be strongly associated with age (P<0.01 in Older vs. Young) from a targeted metabolomics analysis and indicators of healthspan. Color is indicative of positive or negative correlation, while color intensity demonstrates significance of correlation (Pearson Coefficient, *P < 0.05).

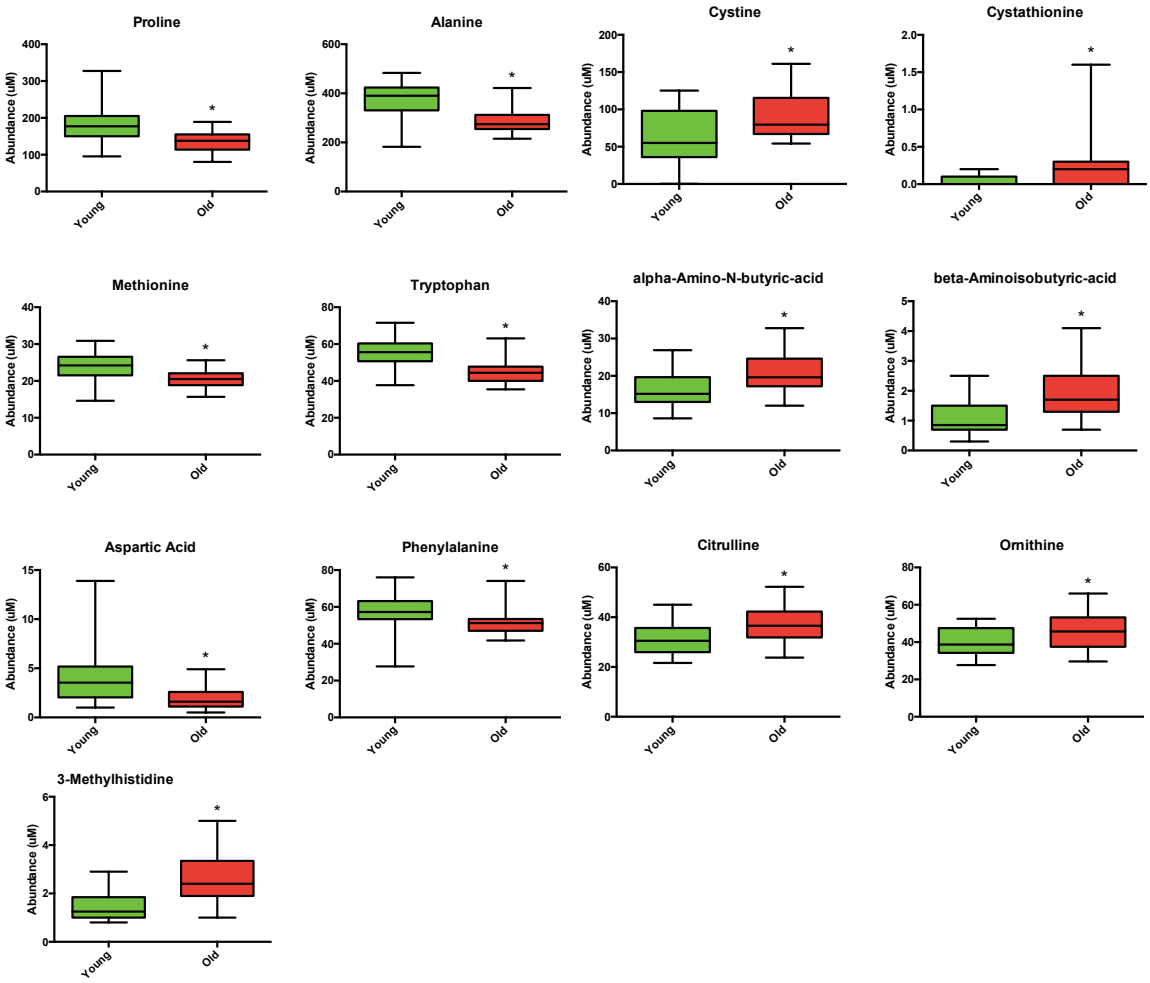
Supplemental Table 1: Metabolic sub-pathways identified within four modules of metabolites that were different with age and associated with multiple indicators of human health span. Pathway analysis was accomplished by mapping metabolites from an untargeted analysis using Metaboanalyst 3.0 and KEGG curated pathways.

Amino Acid Metabolism	Arginine and proline metabolism
	Tryptophan metabolism
	Valine, leucine and isoleucine degradation
	Lysine degradation
	Glycine, serine and threonine metabolism
	Phenylalanine, tyrosine and tryptophan biosynthesis
	Valine, leucine and isoleucine biosynthesis
	Tyrosine metabolism
	Histidine metabolism
	Phenylalanine metabolism
	Cysteine and methionine metabolism
Other Amino Acid Metabolism	Taurine and hypotaurine metabolism
	D-Arginine and D-ornithine metabolism
Lipid Metabolism	Glycerophospholipid metabolism
	Steroid hormone biosynthesis
	Linoleic acid metabolism
	alpha-Linolenic acid metabolism
	Fatty acid metabolism
	Arachidonic acid metabolism
Glycan Biosynthesis and Metabolism	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis
Energy Metabolism	Nitrogen metabolism
Metabolism of Cofactors and Vitamins	Retinol metabolism
	Lipoic acid metabolism
	One carbon pool by folate
	Nicotinate and nicotinamide metabolism
	Riboflavin metabolism
	Pantothenate and CoA biosynthesis
	Vitamin B6 metabolism
	Porphyrin and chlorophyll metabolism
Nucleotide Metabolism	Pyrimidine metabolism
	Purine metabolism
Carbohydrate Metabolism	Citrate cycle (TCA cycle)
	Starch and sucrose metabolism
	Glyoxylate and dicarboxylate metabolism

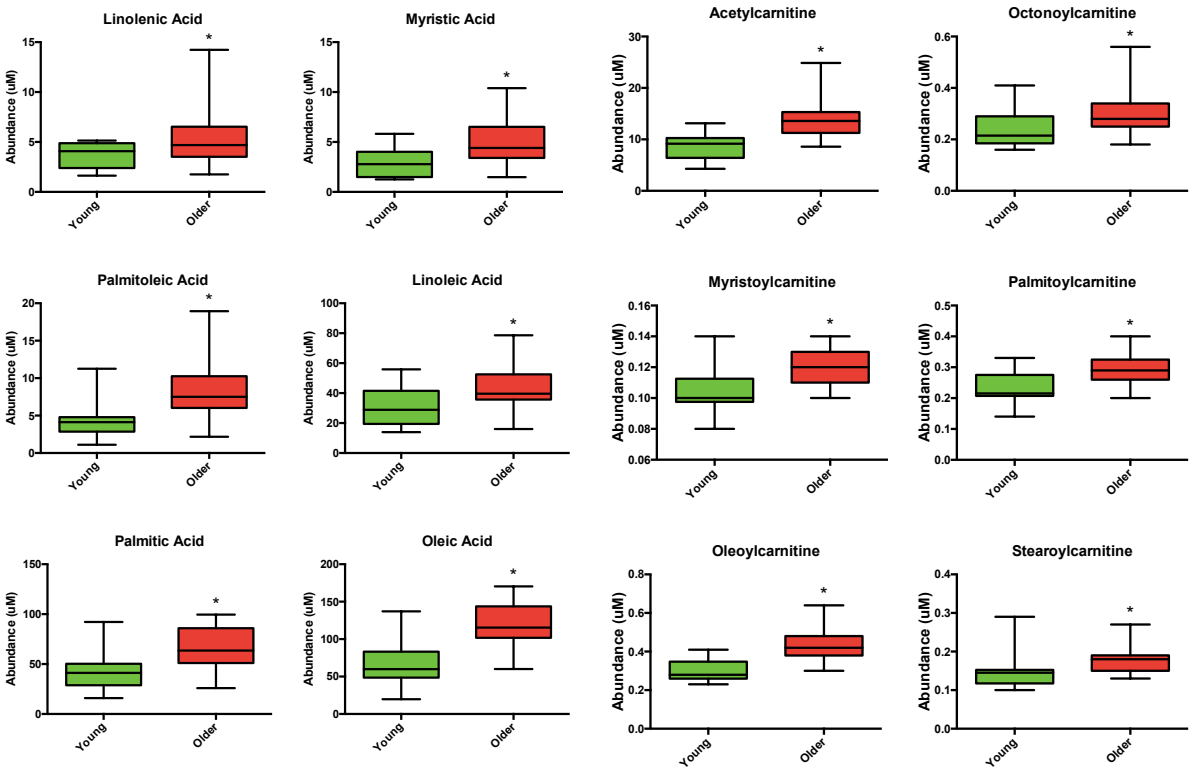
Supplemental Table 2: List of all targeted metabolites measured in 4 metabolite classes related to amino acid and lipid metabolism and critical values assessed for quality control. Concentration values are average ± SEM.

Metabolite	Young (uM)	Older (uM)	%CV (area)
Carnitine	42.62±4.39	44.78±1.98	0.04
Acetylcarnitine	8.75±0.68	13.59±0.63	0.01
Propionylcarnitine	0.57±0.06	0.58±0.03	0.03
Butyrylcarnitine	0.17±0.03	0.16±0.01	0.05
Isovalerylcarnitine	0.17±0.01	0.17±0.01	0.01
Octanoylcarnitine	0.23±0.02	0.31±0.02	0.05
Lauroylcarnitine	0.06±0.01	0.08±0.00	0.02
Myristoylcarnitine	0.11±0.00	0.12±0.00	0.02
Palmitoylcarnitine	0.23±0.01	0.29±0.01	0.01
Oleoylcarnitine	0.30±0.02	0.43±0.02	0.02
Stearoylcarnitine	0.15±0.01	0.18±0.01	0.11
EPA	1.43±0.68	2.15±0.28	0.02
linolenic	3.68±0.34	5.47±0.51	0.01
DHA	1.84±0.72	2.13±0.27	0.00
myristic	2.96±0.37	4.85±0.41	0.01
palmitoleic	4.39±0.75	8.39±0.82	0.04
arachidonic	1.54±0.12	1.77±0.10	0.00
palmitelaidic	0.00±0.00	0.00±0.00	0.00
linoleic	30.94±3.58	43.42±2.59	0.00
palmitic	42.86±5.68	66.65±3.85	0.04
oleic	66.95±9.06	118.86±5.07	0.02
elaidic	3.25±0.36	3.31±0.23	0.01
stearic	45.25±6.86	50.98±2.57	0.01
Sphingosine-1-Phosphate	0.65±0.05	0.65±0.04	0.07
Sphinganine	0.01±0.00	0.01±0.00	0.18
Sphingomyelin	0.02±0.00	0.01±0.00	0.05
C8-Ceramide	0.00±0.00	0.00±0.00	0.00
C14- Ceramide	0.01±0.00	0.01±0.00	0.06
C16- Ceramide	0.21±0.02	0.28±0.01	0.05
C18:1- Ceramide	0.01±0.00	0.01±0.00	0.40
C18- Ceramide	0.06±0.01	0.10±0.01	0.04
C20- Ceramide	0.06±0.01	0.09±0.00	0.01
C22- Ceramide	0.24±0.02	0.27±0.01	0.03
C24:1- Ceramide	0.51±0.04	0.64±0.03	0.10
C24- Ceramide	1.38±0.11	1.73±0.04	0.03
Histidine	33.26±3.58	29.93±2.57	0.04
Hydroxyproline	10.26±0.62	8.67±0.93	0.02
1-Methylhistidine	9.54±3.93	20.95±3.86	0.04

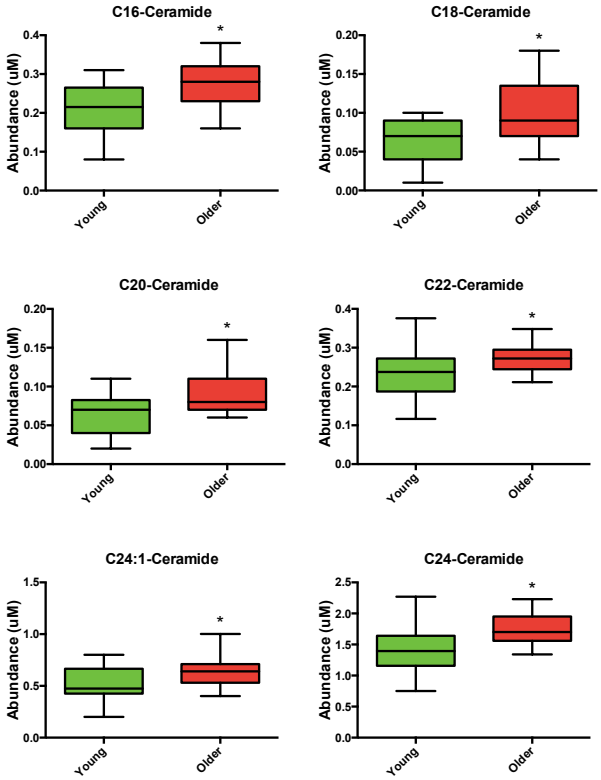
3-Methylhistidine	1.48±0.17	2.65±0.19	0.13
Phosphoethanolamine	2.51±0.54	2.23±0.37	0.10
Carnosine	0.00±0.00	0.00±0.00	0.00
Anserine	0.00±0.00	0.00±0.00	0.00
Ethanolamine	5.25±0.37	5.55±0.15	0.02
Aspartic Acid	4.69±1.10	1.88±0.20	0.01
Sarcosine	9.57±1.16	9.00±0.21	0.00
beta-Alanine	11.69±1.49	10.83±0.32	0.06
gamma-Amino-N-butyric-acid	0.03±0.01	0.03±0.00	0.00
alpha-Aminoadipic-acid	0.50±0.08	0.62±0.05	0.10
beta-Aminoisobutyric-acid	1.06±0.16	1.92±0.15	0.01
Hydroxylysine 1	0.00±0.00	0.00±0.00	0.00
Hydroxylysine 2	0.07±0.02	0.05±0.01	0.18
alpha-Amino-N-butyric-acid	16.38±1.34	21.25±1.03	0.06
Cystathionine 1	0.05±0.02	0.24±0.06	0.00
Cystathionine 2	0.00±0.00	0.00±0.00	0.00
Methionine	23.92±1.11	20.59±0.47	0.01
allo-Isoleucine	1.16±0.21	1.52±0.16	0.10
Homocystine	0.00±0.00	0.00±0.00	0.00
Asparagine	50.35±2.56	47.10±1.14	0.00
Arginine	73.72±6.03	68.19±2.98	0.02
Taurine	87.67±10.33	104.24±11.08	0.06
Serine	93.93±6.68	96.06±2.48	0.01
Glutamine	507.79±28.87	527.89±13.17	0.06
Glycine	240.71±14.93	266.05±15.36	0.02
Citrulline	30.70±1.72	37.22±1.39	0.02
Glutamic Acid	49.96±9.68	41.92±4.72	0.03
Threonine	122.64±7.28	108.76±3.43	0.01
Alanine	372.70±21.54	288.12±8.49	0.04
Proline	185.99±16.61	135.77±5.33	0.02
Ornithine	39.64±2.11	46.33±1.89	0.01
Lysine	144.24±8.89	162.22±4.54	0.02
Cystine	61.91±10.89	91.41±5.81	0.11
Tyrosine	58.55±3.37	55.41±1.51	0.02
Valine	204.07±12.30	211.76±7.09	0.01
Isoleucine	60.70±3.38	53.48±2.17	0.00
Leucine	109.53±5.46	109.14±3.53	0.00
Phenylalanine	57.28±2.91	51.33±1.16	0.02
Tryptophan	55.14±2.44	44.13±1.04	0.01



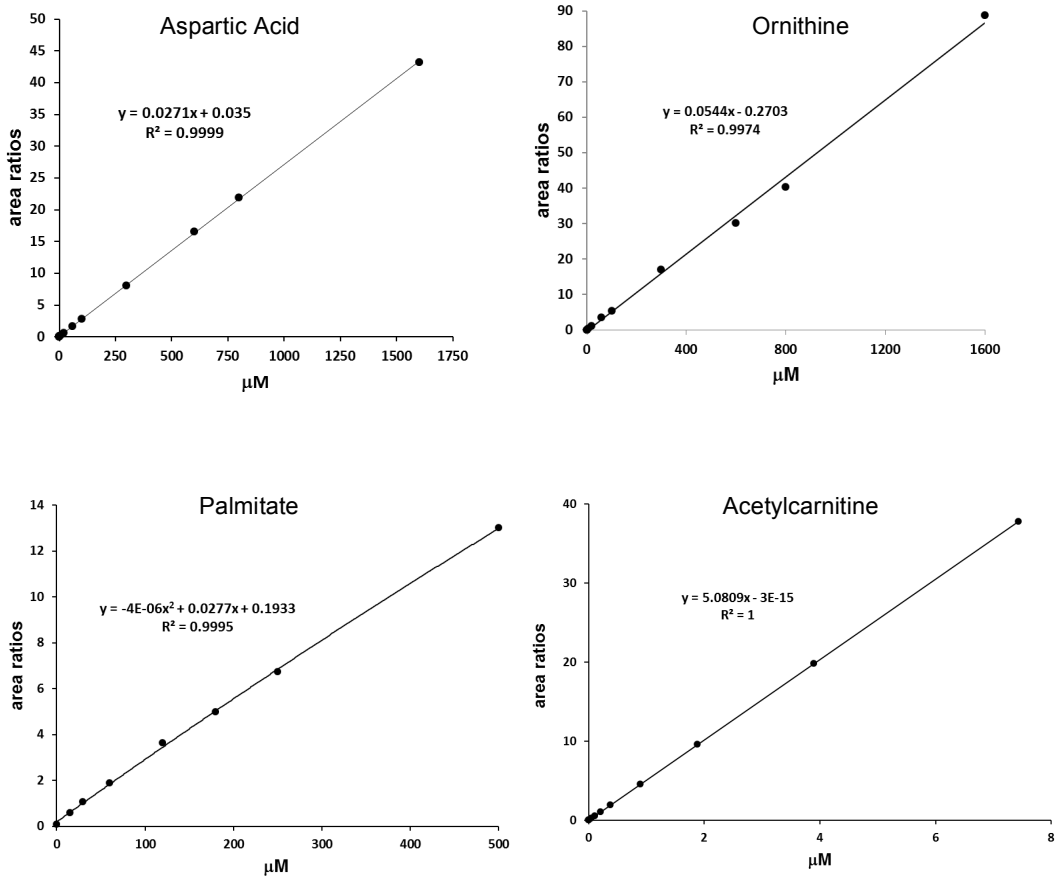
Supplemental Figure 1: Box and whiskers plots (avg. ± max/min) of amino acid concentrations in Young vs Older groups. * indicates P<0.05.



Supplemental Figure 2: Box and whiskers plots (avg. ± max/min) of fatty acid and acylcarnitine concentrations in Young vs Older groups. * indicates P<0.05.



Supplemental Figure 3: Box and whiskers plots (avg. ± max/min) of ceramide concentrations in Young vs Older groups. * indicates P<0.05.



Supplemental Figure 4: Representative standard curves for targeted metabolomics analysis.

Chapter IV

The plasma metabolome as a predictor of biological aging in humans

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ABSTRACT

Chronological age is an important predictor of morbidity and mortality, however it is unable to account for heterogeneity in the decline of physiological function and health with advancing age. Several attempts have been made to instead define a “biological age” using multiple physiological parameters in order to account for variation in the trajectory of human aging; however, these methods require extensive measurement time and technical expertise and are therefore unlikely to be implemented into clinical practice. Identifying circulating biomarkers that are associated with biological age would circumvent the need for costly physiological measurements allowing biological age to be assessed in clinical settings. In the present study, we generated a model of biological age using clinical and physiological measures obtained from a large group of healthy adults across a wide age-range, and applied it to a longitudinal cohort of healthy middle-aged and older adults. To identify minimally invasive molecular biomarkers of biological aging, we identified unique plasma metabolomic signatures that are associated with the rate of biological aging in our longitudinal cohort. These results not only have clinical implications by employing a simple blood-based assay to predict individuals at risk for faster aging, but also provide insight into the molecular mechanisms of human aging.

INTRODUCTION

Chronological age is one of the most important risk factors for many of the chronic diseases and disabilities affecting contemporary societies (Lunenfeld & Stratton 2013). The link between advancing age and increased chronic disease risk is predominately mediated by the progressive decline of multiple physiological systems (Franceschi & Campisi 2014; Kennedy *et al.* 2014). While aging itself is inescapable, the rate at which physiological functions decline with advancing age is highly variable among individuals and is the combined result of genetic and non-genetic factors including lifestyle behaviors (e.g., diet and physical activity) and other environmental or occupational exposures (e.g., sun damage or proximity to volatile chemicals) (Brooks-Wilson 2013; Jiang *et al.* 2013). As such, individuals of the same age may differ considerably with respect to their physiological function and overall health status, thus limiting the predictive capacity of chronological age in determining disease risk.

In order to address this issue, recent attempts have been made to define a "biological age" that is more reflective of the inherent heterogeneity of human aging than chronological age (Nakamura 1991; Cho *et al.* 2010; Levine 2013; Mitnitski *et al.* 2013; Belsky *et al.* 2015; Sebastiani *et al.* 2017). Central to this approach is the integration of multiple age-related "biomarkers" that are modifiable by lifestyle behaviors and other environmental exposures, and therefore more reflective of physiological status. Biological age, determined using clinical and physiological parameters, predicts morbidity and mortality better than chronological age alone (Levine 2013); however, the clinical utility of this approach is limited by the substantial cost, time, specialized equipment and training required to measure each biomarker. As such, the development of a surrogate blood-based measure of biological age would eliminate the burden of making multiple clinical and physiological assessments and more rapidly identify individuals at risk for faster aging. Moreover, such biomarkers may offer insight into the underlying mechanisms of faster aging and provide new targets for therapies aimed at improving human healthspan.

The metabolome, defined as all small molecules characterizing a biological system, is altered with age and reflective of age-related changes in physiological function; thus providing a unique window into systemic molecular changes (Lawton *et al.* 2008; Houtkooper *et al.* 2011; Mapstone *et al.* 2014). In the present study, we utilized the plasma metabolome to identify the molecular events associated with biological aging in healthy adults. After generating a model of biological age from clinical and physiological measures in a large cohort of healthy adults, we applied our algorithm to a longitudinal cohort representative of healthy aging. By analyzing metabolites within plasma samples collected at baseline and follow-up, we identified small molecule signatures that are associated with biological aging in middle-aged and older adults, including metabolites that are predictive of faster vs. slower aging.

RESULTS

Selection of Biomarkers and Calculation of Biological Age

Thirteen (13) clinical and physiological indicators of human healthspan (Table 1) were used to generate a model of biological age using a similar multi-biomarker approach as previously described (Klemera & Doubal 2006; Belsky *et al.* 2015). Briefly, each clinical and physiological measure was evaluated for its relation to chronological age using linear regression and each slope, intercept, and standard deviation of the residuals for each regression was incorporated into an equation that predicted biological age (see “Methods”). The selection of biomarkers was based on their association with chronological age in an independent training cohort (Figure S1) and/or their relevance to age-related disease risk (Calle *et al.* 1999). The training cohort consisted of 604 healthy adults (aged 18-80 years), who had previously undergone testing in our laboratory between 2003 and 2017. Importantly, all subjects were rigorously screened to be free of clinical disease and disability at the time of testing as confirmed by a medical history and physical examination and all biomarkers were within normal healthy ranges, providing a unique opportunity to study the effects of primary aging (Table 1).

Due to inherent sex-related differences of several biomarkers included in our model such as body composition and maximal aerobic capacity (Fleg *et al.* 2005; Wells 2007), separate models of biological age were created from our training cohort allowing us to compare men and women on the same scale for all downstream analyses. Biological age was significantly correlated with chronological age (Figure 1A, $R^2 = 0.68$, $P < 0.0001$; both sexes combined); however, the variability among subjects of similar age, as represented by the average deviation from chronological age, reflects the expected heterogeneity of biological aging (Figure 1B) and provides substrate for additional analyses aimed at understanding the aging process.

Rate of Biological Aging in an Independent Longitudinal Cohort

Using the sex-specific regression coefficients derived from our training models, we calculated biological age in a separate cohort of 31 healthy participants. These individuals had previously been screened for disease status and undergone testing in our laboratory between 5-10 years ago, remained mostly healthy over time to follow-up, and agreed to return to the laboratory for follow-up assessments, thus allowing us to assess how biological age changes as a result of primary aging. Only late middle-aged and older adults (aged 57-82 years) were included in this longitudinal cohort to maximize our ability to detect changes in biological age within a relatively short follow-up period, which averaged 8.6 years. Overall, we observed an average increase in biological age in our longitudinal cohort from baseline to follow-up of approximately six years, with considerable variability among individuals (Figure 2A). To better assess this variability, we also calculated each individual's deviation in age (defined as biological age - chronological age) to determine if they were younger or older than their chronological age. To understand if individuals who were biologically younger/older than their chronological age at baseline were likely to remain relatively younger/older than their chronological age at follow-up, we investigated the relation between deviations in biological age from chronological age at the two time points. Interestingly, a significant relation between

deviation in biological age from chronological age at baseline and follow-up was detected, suggesting that trajectories of biological aging are consistent within individuals (Figure 2B). Collectively, increases in biological age from baseline to follow-up were driven by expected age-related changes in the individual biomarkers (LDL- and total cholesterol decreased due largely to initiation of cholesterol-lowering medications) (Table 2).

To account for differences in follow-up time among individuals, we normalized changes in biological age to changes in chronological age. This resulted in a ratio in which a value above or below one (1) is indicative of faster vs. slower biological aging, respectively. We observed a continuous distribution in the rate of biological aging across our longitudinal cohort, with approximately half of all subjects exhibiting either faster or slower biological aging (Figure 3A).

The Plasma Metabolome as a Predictor of Biological Aging

To identify potential circulating metabolites that may serve as novel biomarkers of biological aging, we measured the abundance of 360 individual metabolites in the plasma of our longitudinal subjects at both time points. Eighty-one (81) metabolites were significantly altered from baseline to follow-up, confirming that age-related changes are detectable in the plasma metabolome over a relatively short period of time (Figure S2). To determine if these changes were also indicative of biological aging, we related the calculated rate of biological aging to changes in 28 metabolites (normalized to follow-up time) (Figure 3B). These changes included alterations in amino acid, fatty acid, acylcarnitine, sphingolipid, and nucleotide metabolites.

Although determining an individual's actual rate of biological aging holds potential clinical value (e.g., precision medicine), it is time consuming, technically challenging, and requires repeat assessments of multiple clinical and physiological parameters making it difficult to implement into clinical practice. Therefore, our ultimate goal was to identify baseline metabolomic signatures indicative of A) one's deviation in biological age from their chronological

age at baseline (i.e., if someone is biologically "younger" or "older" than their actual age) and B) one's future rate of biological aging (i.e., if someone is at risk for faster vs. slower aging).

Predicting Deviation in Biological Age from Chronological Age

To determine if plasma metabolite concentrations at baseline predict biological age in our model, we explored whether baseline metabolite concentrations are predictive of an individual's deviation in biological age from chronological age at baseline. In total, 17 metabolites were related to deviation from chronological age, 11 of which were endogenous or secondary metabolites of microbe metabolism (Table 3). Greater abundances of metabolites associated with amino acid (L-aspartate, L-phenylalanine), ethanolamine (2-acyl-sn-glycero-3-phosphoethanolamine, N-tetradecanoyl-ethanolamine), nucleotide (ADP, nicotinamide, 5-hydroxyisourate), and gamma-glutamyl (gamma-L-Glutamyl-L-cysteine) metabolism were observed in individuals who were biologically older than their chronological age. Alternatively, metabolites from pantothenate (pantetheine), fatty acid (10-hydroxydecanoic acid), and galactose (phenylgalactoside) pathways were associated with a lower biological age compared to chronological age.

Predicting Faster vs. Slower Biological Aging

Finally, to identify individuals at risk for faster biological aging we sought to determine if the concentrations of specific metabolites among individuals at baseline could predict if an individual will undergo faster or slower biological aging. Fourteen (14) metabolites measured at baseline predicted the rate of biological aging in our longitudinal cohort (Table 4). Two metabolites associated with glycolysis/TCA cycle metabolism, oxaloacetate and oxalosuccinate, were positively associated with faster aging, whereas metabolites related to glycolysis (2-phospho-D-glycerate), nucleotide (3',5'-Cyclic IMP, nicotinamide, phosphate), steroid biosynthesis (geranyl diphosphate), bile acids (glycochenodeoxycholate), caffeine metabolism

(5-acetylamino-6-formylamino-3-methyluracil), amino acid metabolism (selenohomocystine, α-D-gulcosamine 1-phosphate) and one exogenous metabolite (4-Hydroxy-2',3,5,5'-tetrachlorobiphenyl) were indicative of slower aging. Interestingly, other than nicotinamide, none of the metabolites predictive of faster vs. slower aging were the same as those associated with one's deviation from chronological age at a single time-point.

DISCUSSION

Despite a growing body of literature, there is little consensus regarding the most appropriate method for determining biological age. One approach has been to predict chronological age using circulating molecular biomarkers, such as DNA methylation levels (Hannum *et al.* 2013; Horvath 2013); however, these models are less indicative of true physiological changes that impact human healthspan. An alternative approach has been to define biological age using clinical and physiological parameters that are closely associated with age-related disease risk. Such models predict mortality better than those based only on age-related molecular markers (Levine 2013; Belsky *et al.* 2015; Kim *et al.* 2017), but are more costly and require specialized training and equipment to measure. In the present study, we developed a hybrid approach in which we identified novel sets of circulating metabolites that are associated with, and predictive of, biological aging derived from clinical and physiological parameters.

Because biological age is most valuable when compared with chronological age, (i.e., to determine if an individual is "younger" or "older" than their age would suggest), we identified metabolites associated with the *deviation* from chonological age, allowing us to derive meaningful information about one's biological aging process from a single blood sample. Several amino acids were associated with the deviation from chronological age, including metabolites involved in protein homeostasis and/or anabolic and catabolic signaling (Timmerman & Volpi 2008). Systemic and cellular energy status is tightly regulated, and its

dysregulation has been implicated as a central mechanism of age-associated physiological declines. Our observation that metabolites involved in energy homeostasis (e.g., ADP and nicotinamide) are related to deviation from chronological age aligns with previous observations that energy homeostasis may be central to aging processes (Finkel 2015; López-Otín *et al.* 2016).

A distinctive aspect of the present study is our determination of biological age from a large cross-sectional training cohort and subsequent follow-up with a longitudinal cohort of healthy (disease free) middle-aged and older adults, which allowed us to study "primary" biological aging. Furthermore, our unique longitudinal design enabled us to identify metabolites associated with the rate of biological aging, including some that were predictive of future faster or slower aging based on a single baseline blood sample. One such pathway was amino acid metabolism, in which circulating levels of L-methionine were positively associated with an increased rate of biological aging. This observation supports previous findings that L-methionine restriction improves physiological function in preclinical models (Sun *et al.* 2009; Hasek *et al.* 2010). Also associated with rate of biological aging were metabolites involved in energy homeostasis, such as the nicotinamide adenine dinucleotide (NAD+) intermediate, nicotinamide. Declines in NAD+ bioavailability have been implicated as a central mechanism in the development of age-related physiological dysfunction (Kujoth *et al.* 2005; Houtkooper *et al.* 2010; Hardie *et al.* 2012; Lopez-Otin *et al.* 2013; Imai & Guarente 2014; Verdin 2015; Wang & Hekimi 2015; de Picciotto *et al.* 2016; Wiley *et al.* 2016). The relation between lipid metabolism, including beta-oxidation intermediates, glycolysis, and TCA cycle pathways and an individual's rate of biological aging further supports the association between biological aging and mitochondrial dysfunction (Koves *et al.* 2008). Paired with the relation of energy status and sensing metabolites to deviation in biological age, these findings converge upon basic energy sensing mechanisms conserved across numerous organisms.

The use of blood-based markers of biological aging has both biomedical and clinical applications, and this analysis demonstrates that circulating plasma metabolomic profiles are associated with the rate at which changes in biological age occur, which is important in determining the molecular underpinnings of aging. For research settings, we have developed blood based markers of biological age that could allow investigators to assess biological age in large population studies, where the assessment of physiological function is not feasible. In clinical populations, our molecular markers may also be useful to efficiently determine individuals at risk of faster aging. Future analyses are needed to validate our findings in diverse patient populations; however, our model of biological aging and unique metabolomic analyses have established a novel approach to investigate the molecular foundation of biological aging in humans.

METHODS

Study Design and Subjects

All study procedures were reviewed and approved by the University of Colorado Boulder Institutional Review Board. Clinical and physiological measurements were performed at the University of Colorado Boulder Clinical Translational Research Center (CTRC). All study participants provided written informed consent after the nature, benefits and risks of the study were explained. Subjects from the longitudinal cohort were re-contacted after at least five years and provided an option to re-enroll. All subjects were non-smokers, determined to be free of clinical disease as assessed by medical history, physical examination, blood chemistries, and resting and exercise ECG. To control for their menstrual cycle, premenopausal women were studied during the early follicular phase, while older women were postmenopausal for at least one year. All subjects followed a 12-hour fast and 24 hour abstention from alcohol, exercise, and prescription medication prior to testing.

Model of Blological Age

Biological age was calculated using the basic Klemera-Doubal equation without chronological age as a marker (Klemera & Doubal 2006). Parameters for the 13 clinical and physiological measures used in the model were estimated from cross-sectional data in 355 men and 249 women (Table 1), generating separate equations for biological age in males and females due to basic differences in physiology. Specifically, a linear relationship with chronological age was estimated in men and women for each measure by individual linear regressions. Biological age (BA_E) for an individual with measurements (x_j) was calculated by the Klemera-Doubal equation with $m = 13$:

$$BA_E = \frac{\sum_{j=1}^{m}(x_j - q_j)\frac{k_j}{{s_j}^2}}{\sum_{j=1}^{m}\left(\frac{k_j}{s_j}\right)^2},$$

where k_j is the slope, q_j the intercept, and s_j the standard deviation of residuals for the corresponding regression. Once the model was trained on our initial cohort of 604 individuals, the equation was applied to a longitudinal cohort of 31 individuals to determine rates of aging, calculated as the ratio of (Δ biological age:Δ chronological age). Deviation in biological age from chronological age was calculated by assessing the difference in calculated biological age from chronological age at baseline.

Metabolomics Analysis

Sample Preparation

Plasma was isolated from subjects and stored at -80°C until analysis. Prior to LC-MS analysis, samples were diluted 1:10 (v/v) with methanol:acetonitrile:water (5:3:2, v:v). Suspensions were vortexed continuously for 30 min at 4°C. Insoluble material was removed by centrifugation at 10,000 g for 10 min at 4°C and supernatants were isolated for metabolomics analysis by UHPLC-MS.

UHPLC-MS analysis

Analyses were performed as previously published (Nemkov *et al.* 2017). Briefly, the analytical platform employs a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Plasma extracts (10 µl) were resolved over a Kinetex C18 column, 2.1 x 150 mm, 1.7 µm particle size (Phenomenex, Torrance, CA, USA) equipped with a guard column (SecurityGuard™ Ultracartridge – UHPLC C18 for 2.1 mm ID Columns – AJO-8782 – Phenomenex, Torrance, CA, USA) using an aqueous phase (A) of water and 0.1% formic acid and a mobile phase (B) of acetonitrile and 0.1% formic acid. Samples were eluted from the

column using either an isocratic elution of 5% B flowed at 250 µl/min and 25ºC or a gradient from 5% to 95% B over 1 minute, followed by an isocratic hold at 95% B for 2 minutes, flowed at 400 µl/min and 30ºC. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated independently in positive or negative ion mode, scanning in Full MS mode (2 µscans) from 60 to 900 m/z at 70,000 resolution, with 4 kV spray voltage, 15 shealth gas, 5 auxiliary gas. Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data was then converted from .raw to .mzXML file format using Mass Matrix (Cleveland, OH, USA). Samples were analyzed in randomized order with a technical mixture injected after every 15 samples to qualify instrument performance. Metabolite assignments, isotopologue distributions, and correction for expected natural abundances of deuterium, ^{13}C, and ^{15}N isotopes were performed using MAVEN (Princeton, NJ, USA) (Clasquin *et al.* 2012). Metabolic pathway analysis was performed using the MetaboAnalyst 3.0 package (www.metaboanalyst.com) (Xia & Wishart 2016).

Statistics

The relation between chronological age and biological age in our initial training cohort was established using Pearson Correlation analysis. Additional comparisons between measures made at baseline and follow-up in the longitudinal cohort were performed using paired t-tests and deemed significant at $P<0.05$. For metabolomics analysis, features between the two time-points in the longitudinal cohort with p-value < 0.05 resulting from a two-tailed t-test and a false-discovery rate (FDR) < 0.1 were classified as significant. To identify metabolites related with rate of aging, differences in metabolite concentrations were calculated and associated with rate of biological aging. Subsequent Independent linear regressions were employed to understand the relation between metabolite abundances and future rate of aging and deviation of biological age from chronological age.

Acknowledgements

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Author Contributions

L.C.J. and C.R.M. conceived and developed the overall study design and collected data used in the biological aging model. K.P. established the mathematical models used to quantify biological age. K.P. and B.F.A. developed and packaged R scripts to execute the biological age algorithm. T.G.N. and A.D. conducted metabolomics analysis and contributed technical support. L.C.J. performed statistical analysis. L.C.J. and C.R.M. D.R.S. contributed critital input towards study design and manuscript development. All authors edited and approved the final version.

Table 1: Training cohort subject characteristics.

Subject Characteristics	Male	Female
Subjects (n)	355	249
Avg. Age (range)	52 (18-79)	55 (18-80)
Body Mass Index (kg/m^2)	26 ± 0.21	24 ± 0.25
Waist-to-Hip Ratio	0.89 ± 0.003	0.76 ± 0.004
Body Fat (%)	24 ± 1	34 ± 1
Bone Mineral Density (g/cm^2)	1.27 ± 0.01	1.14 ± 0.01
Systolic Blood Pressure (mmHg)	122 ± 1	116 ± 1
Diastolic Blood Pressure (mmHg)	74 ± 1	69 ± 1
Glucose (mg/dL)	90 ± 1	87 ± 1
Total Cholesterol (mg/dL)	189 ± 2	200 ± 2
LDL-C (mg/dL)	116 ± 2	116 ± 2
HDL-C (mg/dL)	50 ± 1	66 ± 1
eGFR ($mL/min/1.73m^2$)	78 ± 1	75 ± 1
Max Heart Rate (bpm)	173 ± 1	168 ± 1
VO_2max (mL/kg/min)	37.9 ± 0.5	30.7 ± 0.5

Data are mean ± SEM; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; VO_2max, maximal oxygen consumption.

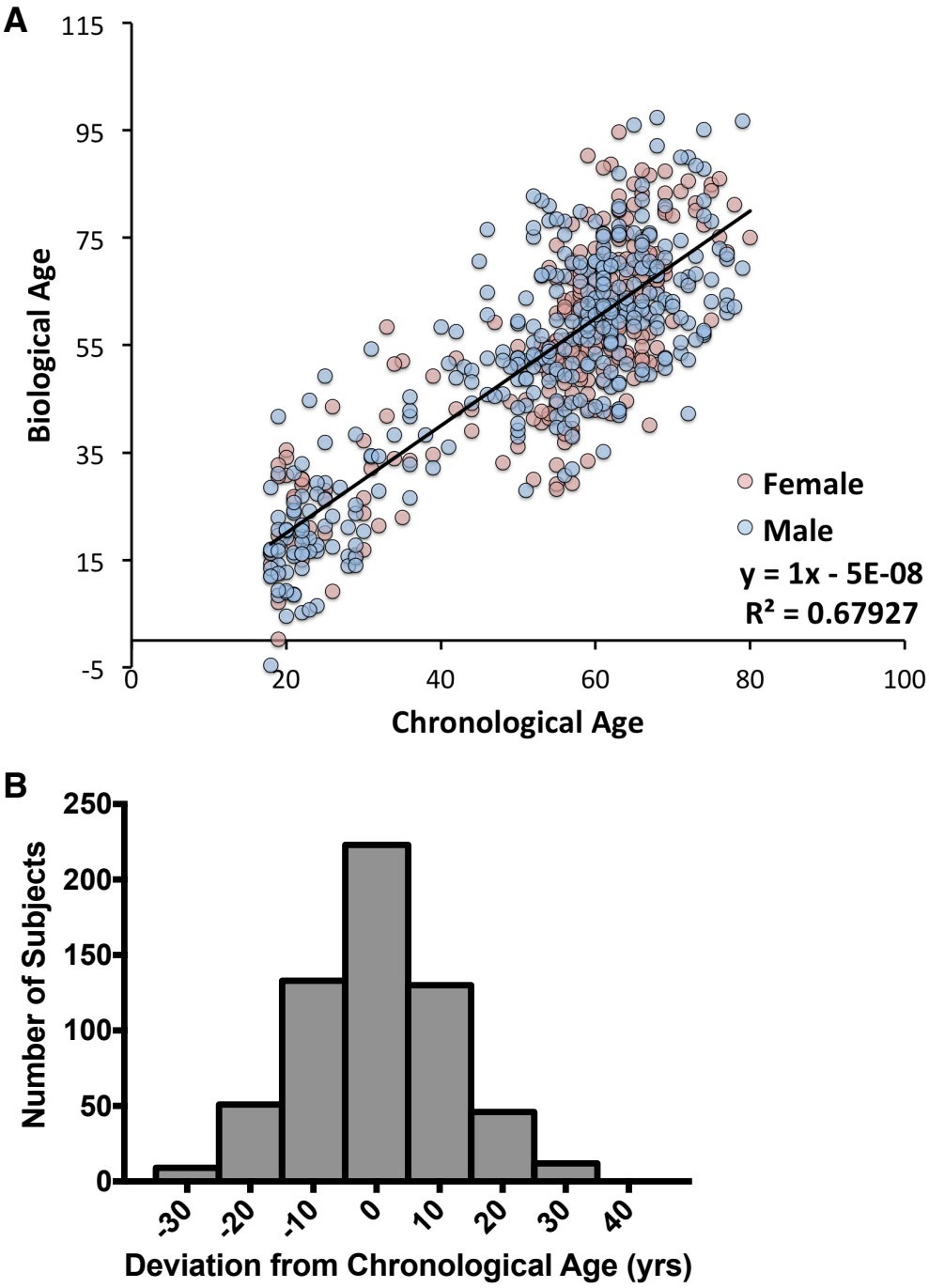

Figure 1: (A) Biological age and chronological age are significantly correlated in our training cohort of 604 healthy adults (R^2 = 0.68, P-value < 0.0001). (B) Deviation of biological age from chronological age was calculated, and although most individual's biological age is within 5 years of their chronological age, many demonstrate greater differences between biological age and chronological age.

Table 2: Subject Characteristics of our longitudinal cohort.

Subject Characteristics	Baseline	Follow-Up
Sex (M/F)	20/11	
Age (yrs)	59 ± 1	68 ± 1*
Body Mass Index (kg/m^2)	25 ± 1	25 ± 1
Waist-to-Hip Ratio	0.86 ± 0.02	0.86 ± 0.02
Body Fat (%)	26 ± 2	27 ± 2
Bone Mineral Density (g/cm^2)	1.23 ± 0.02	1.21 ± 0.02*
Systolic Blood Pressure (mmHg)	119 ± 2	123 ± 2
Diastolic Blood Pressure (mmHg)	73 ± 2	73 ± 1
Glucose (mg/dL)	89 ± 2	85 ± 1*
Total Cholesterol (mg/dL)	204 ± 5	172 ± 5*
LDL-C (mg/dL)	124 ± 4	100 ± 4*
HDL-C (mg/dL)	57 ± 3	54 ± 3*
eGFR (mL/min/1.73m^2)	71 ± 2	78 ± 3
Max Heart Rate (bpm)	168 ± 2	156 ± 3*
VO_2max (mL/kg/min)	36.1 ± 1.7	31.5 ± 1.5*

Data are mean ± SEM; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; VO_2max, maximal oxygen consumption. *P<0.05 vs baseline.

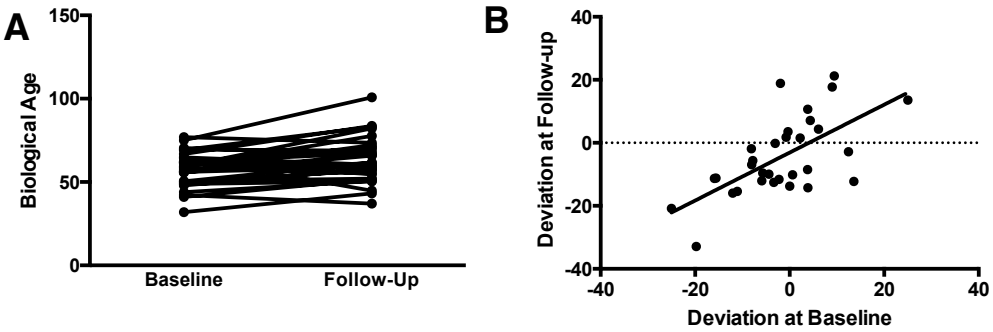

Figure 2: (A) Change in biological age, calculated from clinical and physiological measures, in our longitudinal cohort. Although the change in biological age is significant (P<0.01), the trajectories of aging are highly variable. (B) Significant relation between deviation in biological age from chronological age at baseline and follow-up (P<0.001).

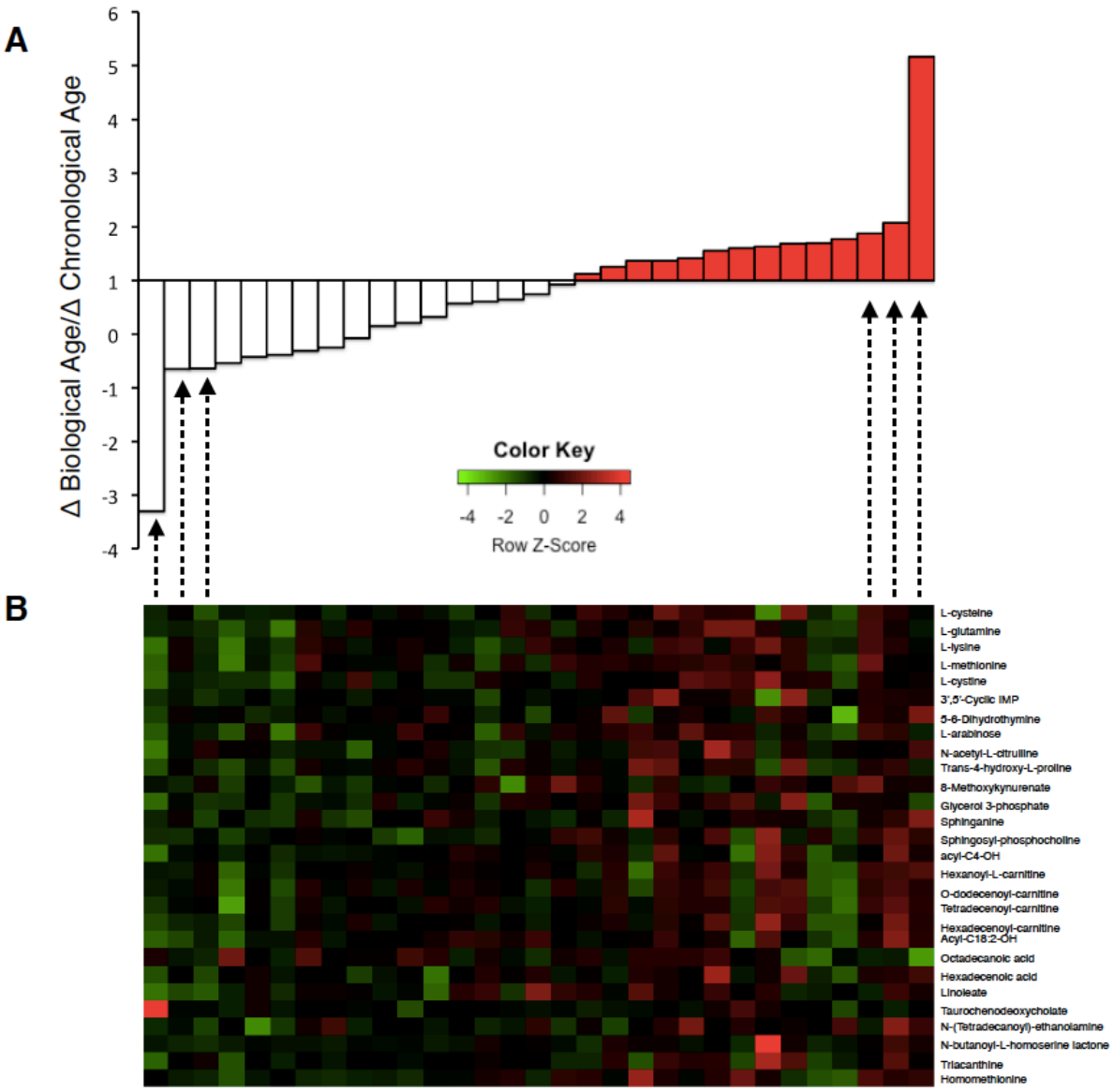

Figure 3: (A) Rate of bological aging for each individual in the longitudinal cohort. A ratio smaller than one indicates slower aging, while a ratio greater than one indicates faster aging. (B) Heatmap of the change in abundance of 28 metabolites significantly associated with rate of biological aging. Individuals are aligned in columns, metabolites in rows.

Table 3: Association of baseline metabolite concentrations with deviation (in years) of bological age from chronological age. Non-endogenous metabolites notated in italic text.

Metabolite	Estimate	Error	P-Value
Greater Values Indicative of Increased Biological Age vs Chronological Age			
N-(Tetradecanoyl)-ethanolamine	3.17E-04	1.15E-04	0.01
gamma-L-Glutamyl-L-cysteine	8.78E-04	3.30E-04	0.01
L-phenylalanine	4.13E-07	1.68E-07	0.02
L-aspartate	9.59E-06	3.99E-06	0.02
5-Hydroxyisourate	1.01E-05	4.37E-06	0.03
2-acyl-sn-glycero-3-phosphoethanolamine	3.19E-05	1.38E-05	0.03
ADP	3.57E-04	1.56E-04	0.03
Nicotinamide	3.62E-05	1.60E-05	0.03
4-Nitroaniline	*2.98E-06*	*7.30E-07*	*0.0003*
Salicylate	*3.87E-04*	*1.65E-04*	*0.03*
Felbamate	*4.03E-06*	*1.32E-06*	*0.005*
Greater Values Indicative of Reduced Biological Age vs Chronological Age			
Pantetheine	-5.27E-06	2.17E-06	0.02
10-Hydroxydecanoic acid	-1.02E-04	4.39E-05	0.03
Phenylgalactoside	-1.67E-06	7.94E-07	0.04
4-Hydroxybenzoate	*-1.22E-07*	*5.75E-08*	*0.04*
Theogallin	*-4.83E-06*	*1.45E-06*	*0.002*
6-Thioxanthine 5'-monophosphate	*-5.08E-05*	*1.80E-05*	*0.009*

Table 4: Metabolite concentrations at baseline are significantly associated with future faster or slower rate of aging. Non-endogenous metabolites notated in italic text.

Metabolite	Estimate	Error	P-Value
Greater Values Indicative of Future Faster Aging			
Oxalosuccinate	2.19E-06	8.84E-07	0.02
Oxaloacetate	6.63E-06	2.93E-06	0.03
Greater Values Indicative of Future Slower Aging			
3',5'-Cyclic IMP	-4.51E-07	1.60E-07	0.009
Phosphate	-9.82E-08	3.82E-08	0.02
2-Phospho-D-glycerate	-1.56E-05	6.08E-06	0.02
Selenohomocystine	-8.58E-07	3.56E-07	0.02
4-Hydroxy-2',3,5,5'—tetrachlorobiphenyl	-4.66E-07	1.95E-07	0.02
Geranyl diphosphate	-7.13E-09	3.02E-09	0.03
5-Acetylamino-6-formylamino-3-methyluracil	-4.20E-07	1.81E-07	0.03
Nicotinamide	-1.76E-06	7.79E-07	0.03
α-D-Glucosamine 1 phosphate	-1.30E-05	5.86E-06	0.03
Glycochenodeoxycholate	-4.18E-07	2.00E-07	0.04
Ethylenethiourea	*-7.38E-07*	*2.39E-07*	*0.004*
Octylamine	*-3.29E-07*	*1.15E-07*	*0.008*

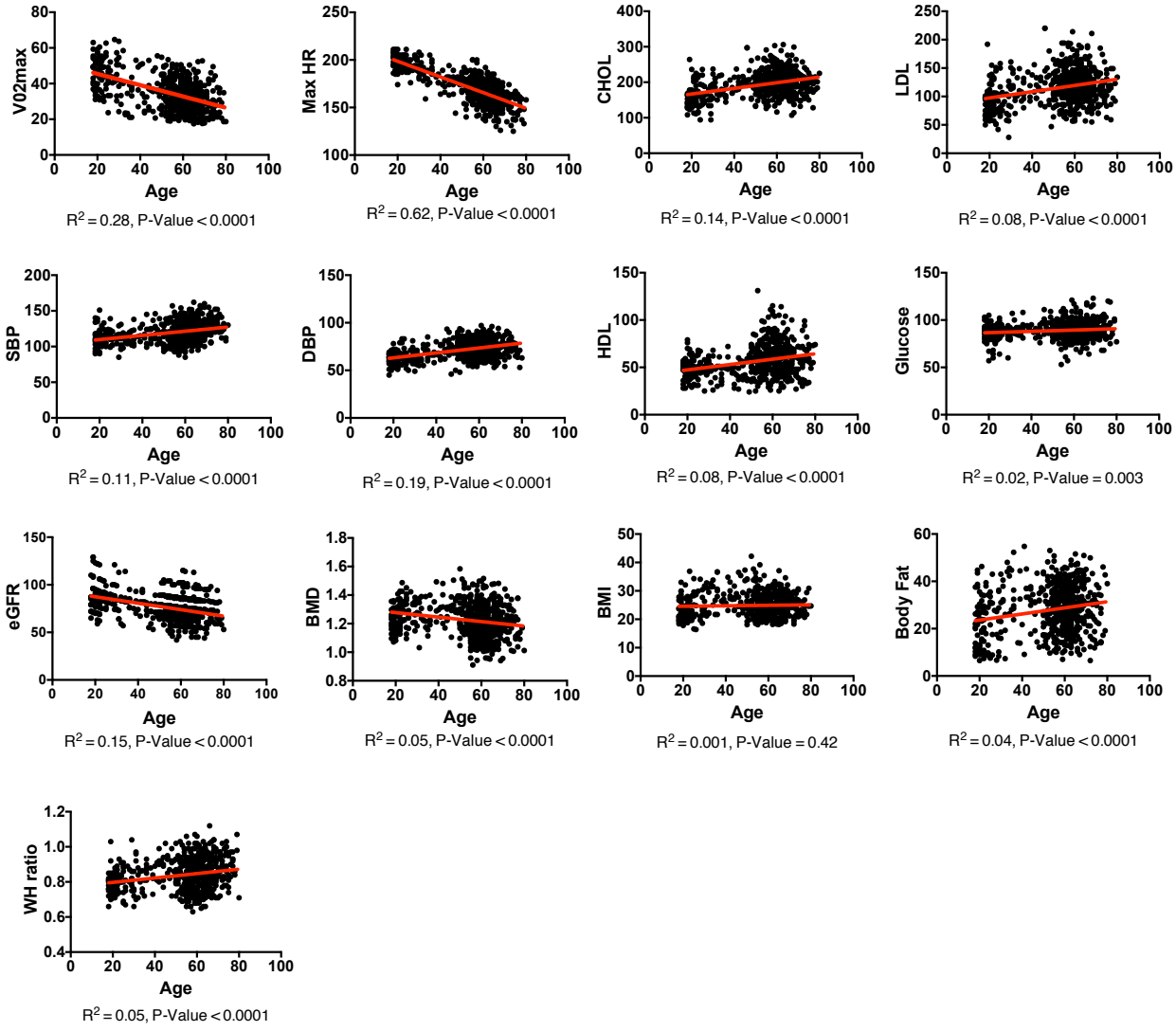

Figure S1: Regressions demonstrating the relation of clinical and physiological markers with age.

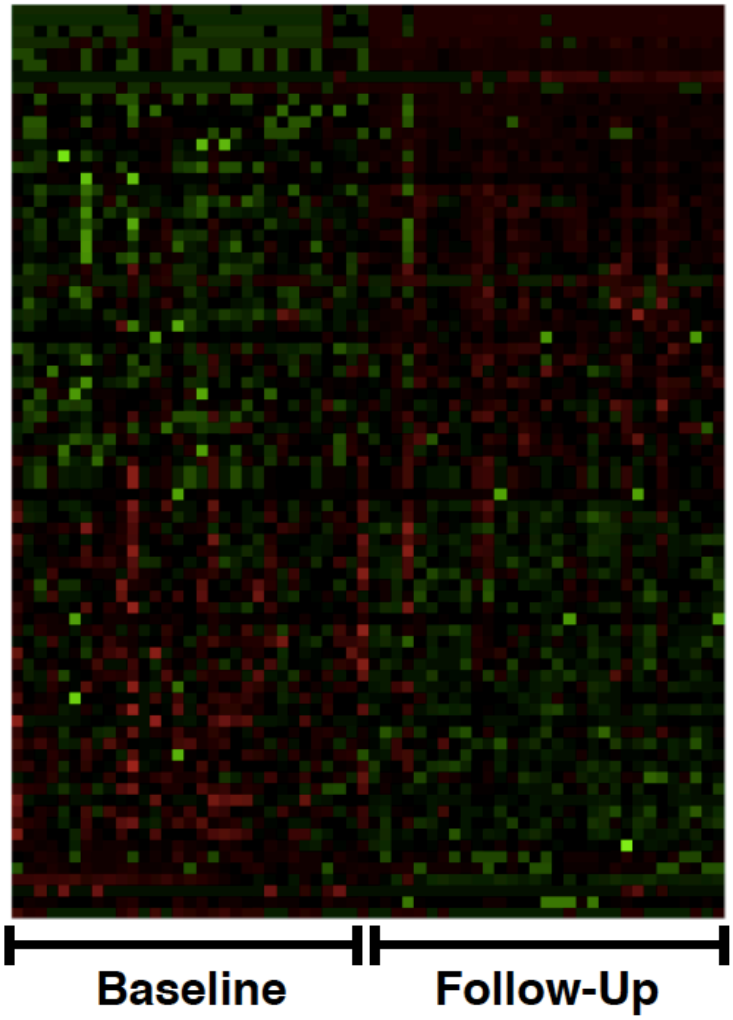

Figure S2: 81 metabolites (of 360 measured) in plasma of individuals from the longitudinal cohort were significantly different between baseline and follow-up at $P < 0.05$, $FDR < 0.1$.

Chapter V

Conclusion

The purpose of this dissertation was to gain novel insight into the molecular mechanisms of age-associated physiological declines by understanding key metabolic pathways related to physiological dysfunction in healthy older adults.

Global, exploratory metabolomics analysis of human plasma from healthy young and older adults demonstrated numerous shifts in small molecule abundances with primary aging. We discovered physiologically relevant clusters of similarly expressed small molecules that were significantly altered with advancing age and, as a group, were related to clinical and physiological outcomes. Pathway analysis determined that these metabolites were primarily associated with amino acid and lipid metabolism. Additional targeted analyses confirmed changes in amino acid and lipid metabolism with age, and the relation of individual metabolites from these pathways to healthspan indicators.

Metabolomics was also used to characterize the molecular signatures associated with biological aging. Clinical and physiological measures were combined to collectively produce a model of biological aging, which was applied to a longitudinal cohort of older adults. Our analysis determined that metabolomic profiles were associated with biological aging. Specifically, small molecule abundances were associated with current and future rates of biological aging and an individual's difference in biological age from their chronological age in older populations.

The results of this dissertation indicate that age-associated changes to the plasma metabolome are associated with adverse changes to healthspan indicators with advancing age, and that metabolomic profiles are associated with, and predictive of, rates of biological aging in healthy populations. Collectively, these findings provide novel insight into the molecular mechanisms underlying age-associated physiological declines.

Future Directions

In the present studies, we identified small molecule patterns and specific metabolic pathways associated with physiological declines. However, despite our best efforts to positively identify each molecular feature measured by our metabolomics platforms, numerous small molecules have yet to be characterized and therefore remain unknown. The continuous measurement and classification of novel metabolites is paramount to successfully exploring the biochemical reactions within biological organisms.

In addition, this work was exploratory in nature and leveraged associative models to implicate molecular pathways associated with age-related physiological dysfunction. Future studies should be performed to confirm and expand on these observations, followed with mechanistic investigations to establish cause-and-effect relations between changes in metabolic pathways and age associated physiological declines. These results would offer insight into ho changes to the plasma metabolome with age are causal to declines in physiological processes or simply a reflection of current dysfunction within various systems.

Finally, physiological declines occur in systems beyond those that were incorporated into our analyses. Therefore, further investigations into the relation between metabolomic patterns and declines in other functional domains, such as cognitive function, should be considered. Additionally, to better understand the relation of these molecular signatures to morbidity and mortality risk, robust longitudinal studies should be performed to assess transitions between healthy aging and disease phenotypes.

Chapter VI

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