Journal of proteome research

Article

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Microbial−Mammalian Cometabolites Dominate the Age-associated Urinary Metabolic Phenotype in Taiwanese and American Populations

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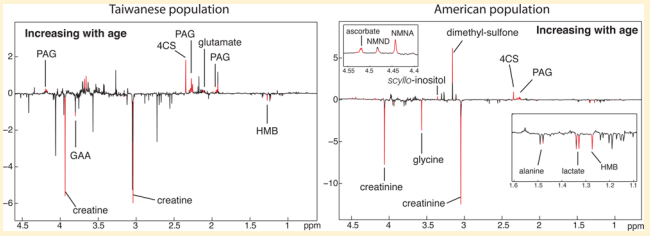
Supporting Information

ABSTRACT: Understanding the metabolic processes associated with aging is key to developing effective management and treatment strategies for age-related diseases. We investigated the metabolic profiles associated with age in a Taiwanese and an American population. ^{1}H NMR spectral profiles were generated for urine specimens collected from the Taiwanese Social Environment and Biomarkers of Aging Study (SEBAS; n = 857; age 54−91 years) and the Mid-Life in the USA study (MIDUS II; n = 1148; age 35−86 years). Multivariate and univariate linear projection methods revealed some common age-related characteristics in urinary metabolite profiles in the American and Taiwanese populations, as well as some distinctive features. In both cases, two metabolites—4-cresyl sulfate (4CS) and phenylacetylglutamine (PAG)—were positively associated with age. In addition, creatine and β-hydroxy-β-methylbutyrate (HMB) were negatively correlated with age in both populations ($p < 4 \times 10^{-6}$). These age-associated gradients in creatine and HMB reflect decreasing muscle mass with age. The systematic increase in PAG and 4CS was confirmed using ultraperformance liquid chromatography−mass spectrometry (UPLC−MS). Both are products of concerted microbial−mammalian host cometabolism and indicate an age-related association with the balance of host−microbiome metabolism.



KEYWORDS: *age, sex, metabolic profiling, NMR spectroscopy, 4-cresyl sulfate, phenylacetylglutamine*

INTRODUCTION

The chronic nature of most diseases associated with aging, coupled with the increased probability of elderly individuals presenting with multiple pathologies requiring complex therapeutic management strategies, makes analysis of age-related conditions challenging. Aging is associated with a general decline in physiological function, particularly in the intestine, where a decrease in intestinal motility, a reduction in the capacity of the immune system and changes in the beneficial and hostile gut microbiota contribute to the general decline in health. Many elegant studies in short-lived model organisms such as the nematode worm *Caenorhabditis elegans* and the mouse have contributed to our current understanding of the aging process.[1,2] However, the true complexity of aging in human populations cannot be fully characterized in these animal models, given the diverse exposure of humans to a myriad of physical, environmental and social stressors.[3,4] Thus, in parallel to exploring experimental models of aging, there is a need for research into the mechanisms and consequences of aging in human populations. Epidemiological studies investigating population differences in the prevalence of diseases across countries[5−7] and between men and women[8] offer a particularly useful resource for studying aging.

Received: January 5, 2013
Published: May 23, 2013

Metabolic phenotyping and metabolome-wide association studies (MWAS) offer a powerful new means for discovering molecular biomarkers and metabolic pathways that underlie disease risk.[9,10] This approach uses high-resolution spectroscopic techniques and mathematical modeling to generate a molecular fingerprint of a biological specimen[11] and can provide a novel framework for identifying appropriate therapeutic intervention strategies at the individual and population level. A particular strength of metabolic phenotyping lies in its ability to reveal a representative overview of host, extra-genomic and environmental contributions to metabolism.

Metabolic profiling approaches have been applied to studies on age-associated diseases in both nonhuman[2,12] and human populations, with a focus on identifying age-related changes in the biochemical composition of serum or plasma. Several groups have reported decreased serum carnitines, acylcarnitines and amino acids with age and increased free fatty acid levels in aging rodents.[13,14] In contrast, other studies have found an increase in free serum carnitine with age in humans.[15] While plasma provides a useful system-level readout of the physiological status of an organism at a given point in time, urine provides time-averaged information on the metabolic events that have occurred throughout the whole animal. The metabolic signature of urine is influenced by the host's genome and physiology but also provides a window on extrinsic input from dietary factors and the gut microbiome.

Here we apply a spectroscopic profiling approach to define the metabolic signature of aging in two distinct human populations—the Taiwanese Social Environment and Biomarkers of Aging Study (SEBAS)[16] and the Mid-Life in the USA (MIDUS II)[17] cohorts—using ^{1}H nuclear magnetic resonance (NMR) spectroscopy and ultraperformance liquid chromatography–mass spectrometry (UPLC–MS) of urine specimens. Through this approach we identify the global sources of metabolic variation and sex-specific elements within the metabolic signatures of these geographically and culturally distinct populations. In addition, we identify clear metabolic correlates of biological aging in relation to declining muscle metabolism and also age-related variation in the functionality of several pathways involved in gut microbial–host metabolic regulation.

METHODS AND MATERIALS

Description of Populations and Specimen Collections

SEBAS Study. A total of 857 urine specimens from the 2000 SEBAS study (age range 54–91; mean 68 years) were shipped from the Lombardi Comprehensive Cancer Center, Georgetown University to Imperial College London. This specimen set comprised urine from 368 females and 489 males. Specimens were stored at Imperial College at −80 °C prior to analysis.

MIDUS Study. A total of 1148 urine specimens from the MIDUS II study (age range 35–86; mean 57 years) were shipped from the Harlow laboratory, University of Wisconsin and stored at −80 °C at Imperial College prior to analysis. Participants included 651 females and 497 males. Both sample sets were 12-h overnight urine collections.

The demographic characteristics of the SEBAS and MIDUS participants are summarized in Table 1.

^{1}H NMR Spectroscopic Analysis

Quality control (QC) aliquots for NMR analysis were prepared by combining aliquots of urine from randomly selected subgroups of individuals. For each cohort, SEBAS and MIDUS, specimens were randomized and interspersed with QC aliquots (using a total of 129 QC aliquots) in order to assess data quality and variation over the analytical measurement period. Specimens were prepared and spectra acquired using in-house protocols[18] adopting a standard one-dimensional pulse sequence with suppression of the water resonance. Briefly, urine specimens were prepared by the addition of phosphate buffer made up in deuterium oxide containing 1 mM 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-propionic acid sodium salt (TSP) as an external reference and 2 mM sodium azide as a bacteriocide. For each specimen, a standard one-dimensional NMR spectrum was acquired with water peak suppression using a standard pulse sequence (recycle delay (RD)-90°-t_1-90°-t_m-90°-acquire free induction decay (FID)). A mixing time (t_m) of 100 ms was used and the RD was set at 2 s. The 90° pulse length was approximately 12 μs and t_1 was set to 3 μs. An acquisition time per scan was 2.73 s and, for each specimen, 8 dummy scans were followed by 128 scans. The spectra were collected into 64K data points using a spectral width of 20 ppm.

Table 1. Study Participant Information for SEBAS and MIDUS

	SEBAS	MIDUS
Total specimens NMR[a]	857	1148
Total specimens MS	725	1196
Age range	54–91	35–86
Sex (female/male)	368/489	651/497

[a]The number of urine specimens for NMR and MS differ due to the number of specimens excluded based on the differing analytical constraints of the two techniques. For NMR analysis, specimens were excluded if the glucose levels or ethanol concentrations were too high, which caused bias in the models. For MS specimens were excluded where there was insufficient specimen volume or where specimens contained a polyethylene glycol contaminant, possibly leached from the storage vials. Outliers in the PCA scores plots of the NMR data were evaluated using the Hotellings T ellipse and discarded where appropriate in order to remove undue influence of artifacts on the models.

Preprocessing and Modeling of the NMR Spectral Data

Spectra were phased, corrected for baseline distortions and referenced to the TSP signal at δ 0.00. The region between δ 4.70 and 6.20 containing the residual water resonance and the urea peak was removed for all spectra. For the MIDUS spectral data, the region containing the methyl resonance of acetate (δ 1.92) was removed owing to pretreatment of these aliquots with acetate. The remaining spectral variables between δ 0.70–4.70 and δ 6.20–10.00 were normalized to the sum of the spectral integral prior to analysis using principal components analysis (PCA). Data were analyzed with and without peak alignment using the algorithm defined by Veselkov et al.[19] The main sources of variation in the data were identified and further explored. Partial least-squares discriminant analysis (PLS-DA) was applied to the data with and without the application of an orthogonal filter to remove extraneous variation and to establish metabolic patterns relating to a variety of participant variables including age and sex. The predictive performance of the models was assessed using a 7-fold cross-validation approach and the Q^2Y (goodness of prediction) values are provided. Permutation testing (1000 permutations) has been performed to ensure the validity of the PLS models. Linear regression was used to measure the statistical significance of the metabolic variations. A cutoff of $p < 4 \times 10^{-6}$ was used based

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166–3180

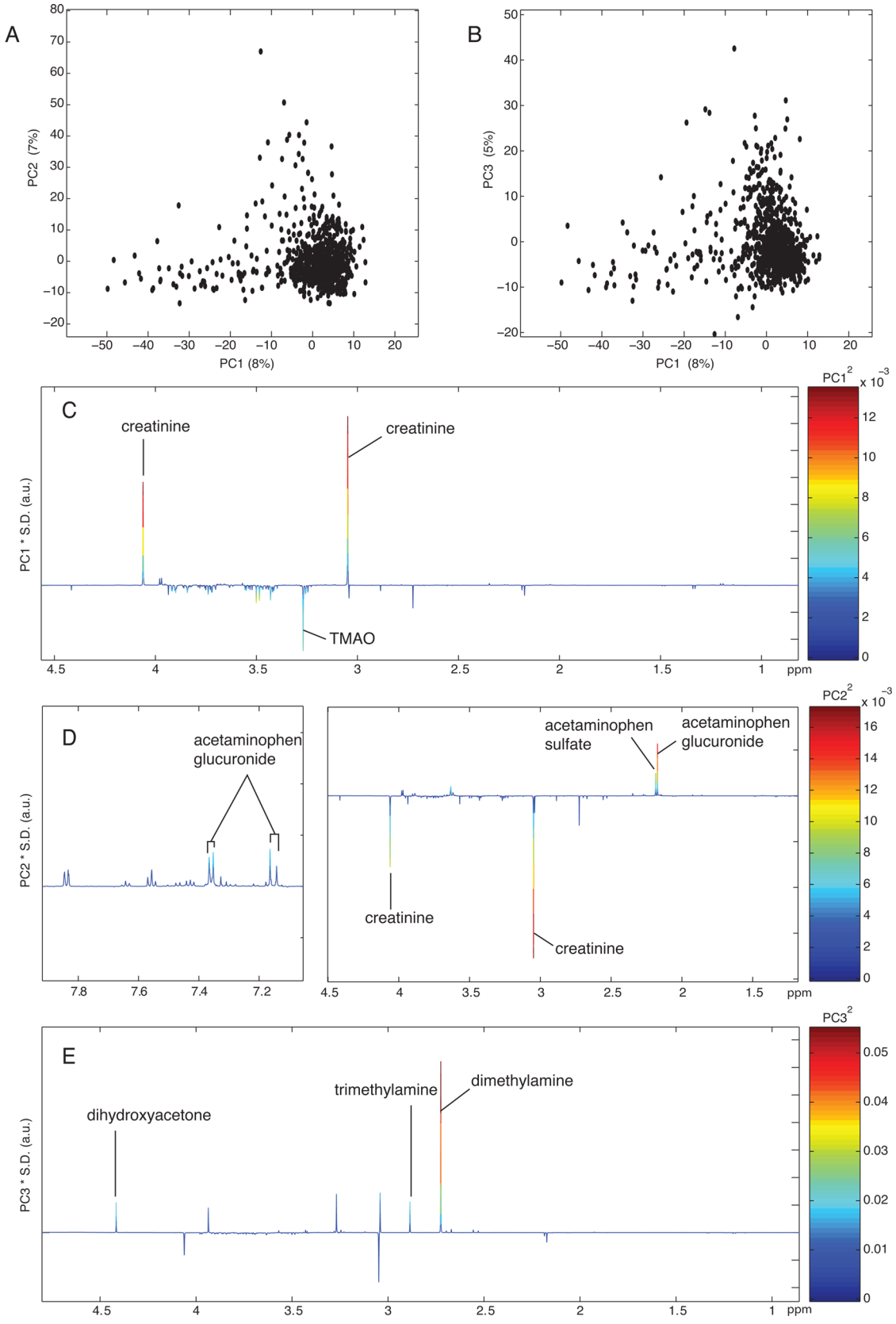


Figure 1. PCA model of the urinary profiles of all SEBAS participants. Scores plots for (A) PC1 vs PC2 and (B) PC1 vs PC3 (% variance explained in parentheses). Product of PC loadings with standard deviation of the entire data set, colored by the square of the PC shown for (C) PC1, (D) PC2 and (E) PC3.

on the method described by Chadeau-Hyam et al.[20] for selecting a suitable level of significance in metabolome wide association studies (MWAS) with an expected family wise error rate of 5% for 13 000 variables.

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166−3180

UPLC–MS Spectral Analysis

UPLC–MS analysis was performed to validate the NMR-detected correlation of PAG and 4-cresyl sulfate with age and to explore other possible age related variation in the urinary metabolome using optimized protocols for urine metabolite profiling.[21] Briefly, urine specimens were prepared by dilution (1:1) with water (Sigma, LC–MS grade), vortexed for ten seconds, and centrifuged at 16 000× *g* for 10 min. Two hundred microliters were aliquoted into 96-well 350 μL plates (Waters Corporation, Milford, MA) with cap mats (VWR, U.K.). A composite quality control (QC) aliquot was prepared by combining 50 μL from 775 randomly selected SEBAS and MIDUS specimens. The QC aliquot was subaliquoted to minimize freeze–thaw cycle effects and stored frozen until required for the analysis. Ten analyses of the QC aliquot were performed at the beginning of the analytical run for system conditioning. A single QC aliquot injection was performed at 10-aliquot intervals throughout the subsequent data acquisition to provide data for the assessment of analytical reproducibility including peak retention times and detector response. Additionally, five blanks were injected prior to the injection of QC-conditioning aliquots in order to ensure that there was no contamination from the UPLC system, and again at the end of the experiment to ensure that specimen carryover was not observed.

Metabolic profiling was performed on an Acquity UPLC system (Waters Corp., Milford, MA) coupled to an LCT Premier time-of-flight mass spectrometer (Waters Corp., Manchester, U.K.). UPLC–MS conditions were optimized in terms of peak shape, reproducibility and retention times of analytes. Chromatography was performed using an Acquity HSS T3 column, 2.1 × 100 mm column (Waters Corp., Milford, MA) held at 40 °C. Separation was performed using gradient elution with 0.1% (v/v) formic acid in H_2O (A) and 0.1% (v/v) formic acid in ACN (B) at a flow rate of 0.5 mL/min. Starting conditions were 99.9% A and 0.1% B for 1.0 min, changing linearly to 15% B over the next 2 min, and then to 50% B over the next 3 min, and finally to 95% B in the next 3 min and kept for 1 min. Afterward the solvent composition returned to starting conditions over 0.1 min, followed by re-equilibration for 2 min prior to the next injection.

Mass spectrometry was performed using electrospray in both positive and negative ionization modes (ESI+ and ESI–). The capillary voltage was 3.2 kV (ESI+) and 2.4 kV (ESI–), cone voltage was 35 V, desolvation temperature was 350 °C, and source temperature was 120 °C. The cone gas flow rate was 25 L/h, and desolvation gas flow rate was 900 L/h. The LCT Premier was operated in V optics mode with a scan time of 0.2 s and interscan delay of 0.01 s. For mass accuracy, a LockSpray interface was used with a 20 μg/L leucine enkephalin (555.2645 amu) solution (50/50 ACN/H_2O with 0.1% v/v formic acid) at 70 μL/min as the lock mass. Data were collected in centroid mode with a scan range of 50–1000 *m/z*, with lockmass scans collected every 15 s and averaged over 3 scans to perform mass correction.

Preprocessing and Modeling of the UPLC–MS Data

Since the system is not generally stable during the first injections, the first 10 QC samples were used to ensure that stability had been attained, after which the QC-conditioning aliquots were excluded from further data processing. The rest of the raw data (i.e., the target specimens plus the remaining QC aliquots) within the run were converted to netCDF format using the DataBridge tool implemented in MassLynx software (Waters Corporation, Milford, MA).

The data were preprocessed using the freely available XCMS software. The Centwave algorithm was used for peak picking with a peak width window of 3–15 s, the *m/z* width for the grouping was changed to 0.1 Da, the bandwidth parameter was kept to default (30 s) for the first grouping and was subsequently determined from the time deviation profile plot after retention time correction. An output table was obtained at the end comprising *m/z*, RT and intensity values of the detected metabolite features in each specimen.

The data were then normalized in R with an in-house script.[22] The coefficient of variation (CV = standard deviation/mean) values were calculated for all the intensities of metabolite features (mz_Rt) in the QC samples analyzed within the run (see Supporting Information for details). In the generated data sets features with a CV higher than 30% in replicated injections of the QC aliquots interspersed within the run were removed. The output table was exported into SIMCA-P+ 12.0.1 software (Umetrics, Umeå, Sweden) for multivariate analysis. Principal component analysis (PCA), partial least-squares-discriminant analysis (PLS-DA) and orthogonal projection on latent structures-discriminant analysis (OPLS-DA) were performed on all data.

Adjustment of Data Sets for Differential Age Ranges between the SEBAS and MIDUS Studies

Owing to different age ranges between the two study populations (SEBAS 54–91 years, mean 68 years; MIDUS 35–86 years, mean 57 years), auxiliary models were constructed using a restricted age range that comprised the overlap between the two studies (ages 54–86 years); the results are reported in Supporting Information (Figures S3–S5).

RESULTS

The analytical platforms and methods were robust and reliable, as indicated by the coefficients of variation for the quality control specimens. Moreover, the analytical quality of the data was good across both the NMR spectroscopy and the UPLC–MS data, obtained for both the SEBAS and the MIDUS data sets, with the one exception of ESI negative mode data for the MIDUS cohort. No adjustment of the MS data for run order was necessary. For the UPLC–MS in ESI+ ion mode, the coefficients of variation for the QC samples were 25.2 ± 19.1 and 23 ± 17.7 for SEBAS and MIDUS, respectively. ESI– ion mode gave similar results with CV values 31.8 ± 19.3 for the SEBAS study. For the MIDUS study, the CV ESI– ion values were high (50 ± 53.3); therefore, we refrained from further analysis of the negative ionization mode data set.

Global Analysis of the ^{1}H NMR Urine Data

The scores and loadings plots from the global PCA model for the SEBAS data set (Figure 1) show that the first component was dominated by creatinine and trimethylamine-*N*-oxide (TMAO), which represented the greatest sources of variation across the specimen set. Creatinine is a crude indicator of muscle mass and can vary with sex and age. TMAO is associated with consumption of certain fish and shellfish, where it functions as an antifreeze agent and an osmolyte and has been shown to be elevated in urine after consumption of diets rich in phytoestrogens, for example, soy or miso. The variance in the second component was dominated by metabolites related to acetaminophen, namely acetaminophen glucuronide and acetaminophen sulfate. Methylamines and a singlet (δ 4.41)

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166–3180

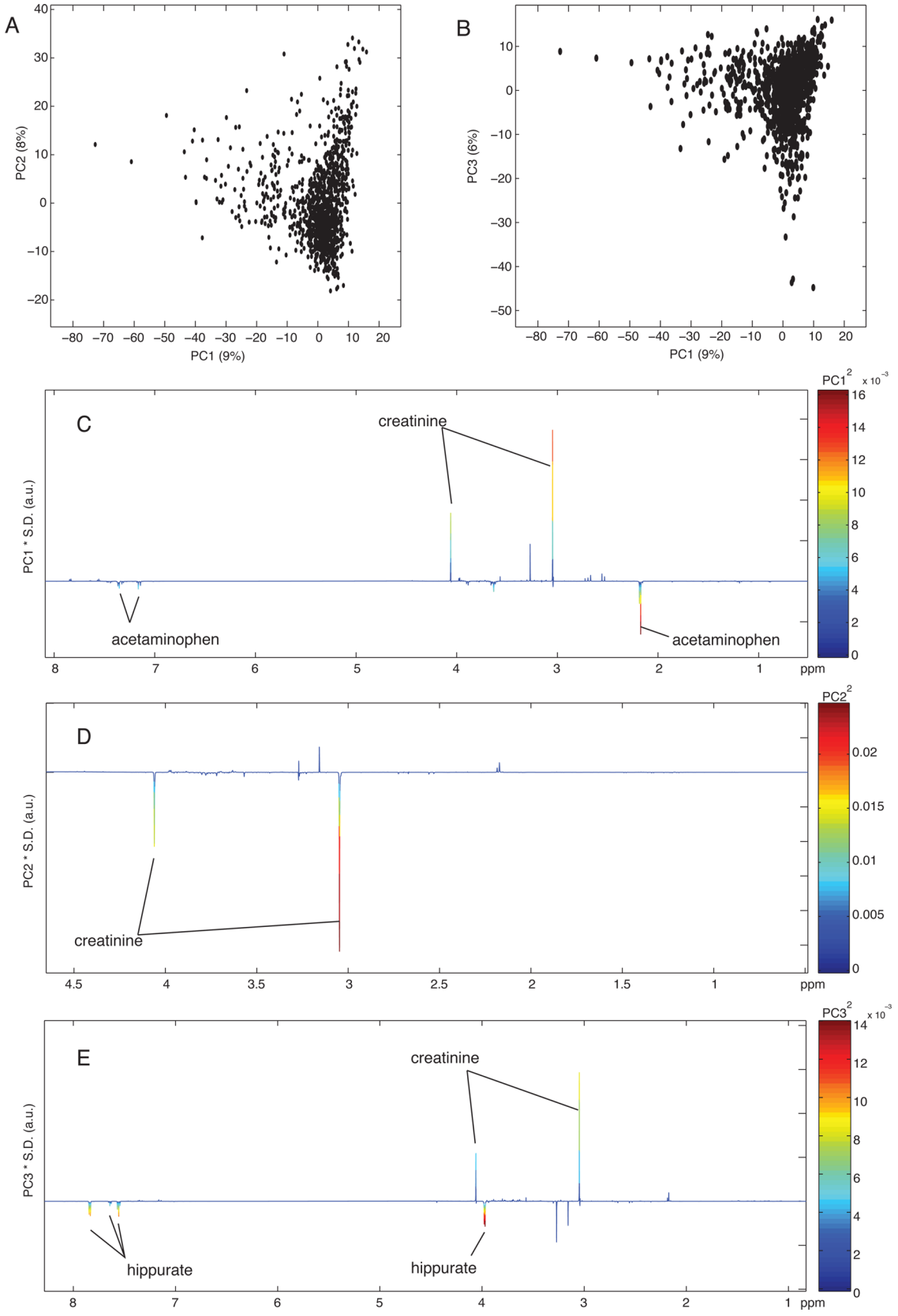


Figure 2. PCA model of the urinary profiles of all MIDUS participants. Scores plots for (A) PC1 vs PC2 and (B) PC1 vs PC3 (% variance explained in parentheses). Product of PC loadings with standard deviation of the entire data set, colored by the square of the PC shown for (C) PC1, (D) PC2 and (E) PC3.

tentatively assigned as dihydroxyacetone exerted the greatest influence on the third principal component.

Similarly to the SEBAS data set, the first component of the PCA model calculated for the MIDUS data set was strongly

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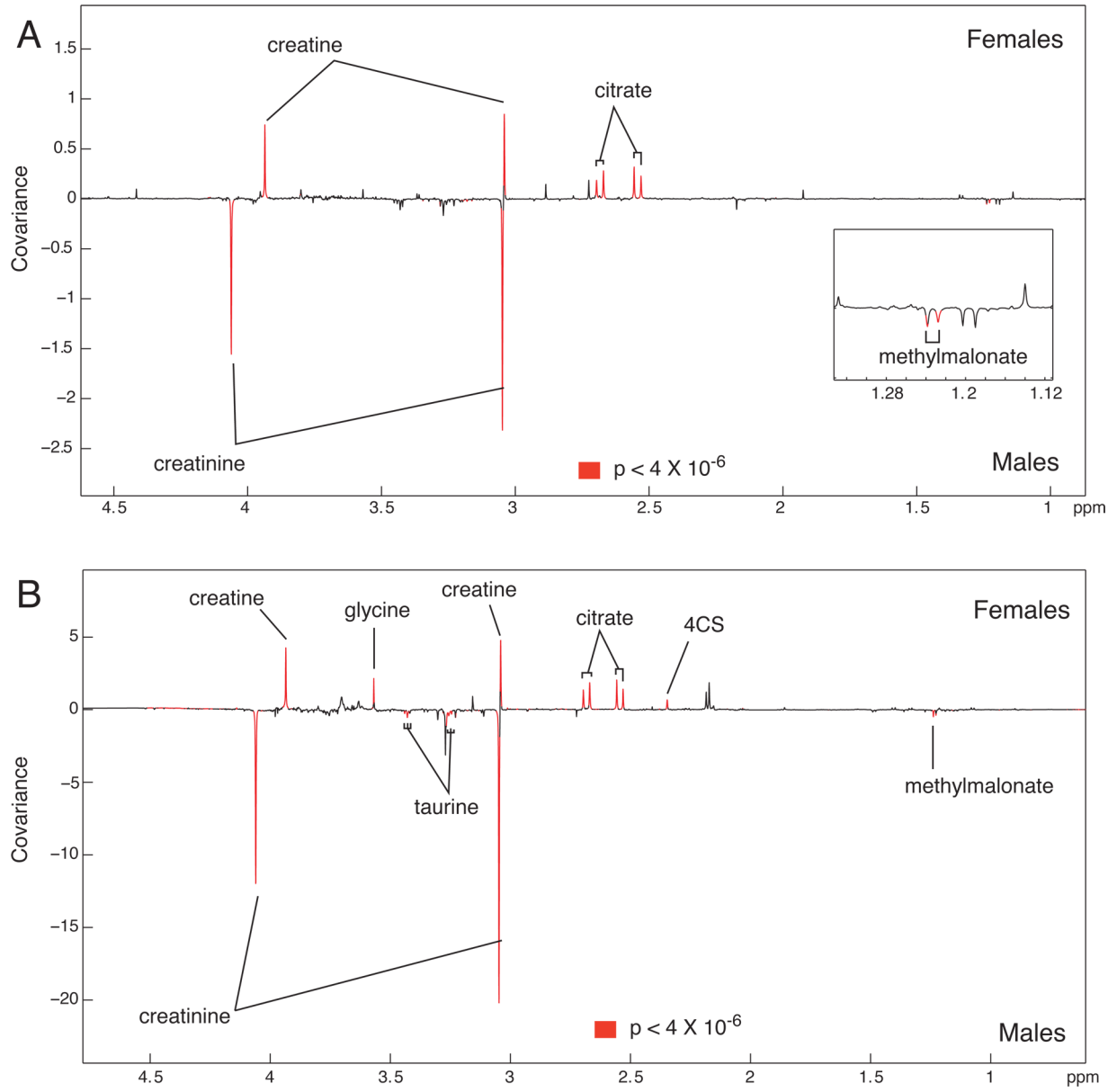

Figure 3. Linear regression analysis correlating ^{1}H NMR spectral profiles of urine with sex. Covariance plots derived from linear regression analysis for (A) SEBAS and (B) MIDUS, color-coded by significance. Significance determined by $p < 4 \times 10^{-6}$, the metabolome-wide significance level (MWSL).

influenced by creatinine (Figure 2). In addition, acetaminophen metabolites also made a substantial contribution to the first component. Although the principal components are linear and orthogonal, creatinine also dominated the second component. When a metabolite is influential in the loadings explaining more than one component, it is generally because the variance of that metabolite is determined by more than one major source of variation in the data set. The mammalian−microbial cometabolite hippurate accounted for the majority of the variance in the third component of the MIDUS II model.

Since methylamines contributed strongly to the variation in the SEBAS but not the MIDUS II data set, the urinary concentrations of trimethylamine (TMA) and dimethylamine (DMA) were calculated from the integrals at δ 2.88 and δ 2.72 respectively and found to be significantly different for the Taiwanese (mean concentration TMA = 0.11 ± 0.11 mM and DMA = 0.44 ± 0.46 mM) and American populations (mean concentration TMA = 0.02 ± 0.01 mM and DMA = 0.15 ± 0.1 mM). Because of overlap with taurine and other metabolites, the integral values for the TMAO signal were not calculated but visual inspection of the data suggested that TMAO was found in higher concentrations in the urine of Taiwanese participants.

Sex-related Differences in Urinary Metabolic Phenotypes

Because creatinine was one of the major sources of variation found in both the SEBAS and MIDUS cohorts, and is known to differ with both age and sex, the influence of sex on the NMR derived metabolic profiles was characterized prior to focusing on age-related metabolic differences. Using an unsupervised PCA approach, no clear discrimination of specimens according to sex could be seen for either the SEBAS or the MIDUS cohorts (Supporting Information Figure S1) indicating that the major sources of variation in urine composition across the populations were not sex-related.

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166−3180

Table 2. Age-related Variation in SEBAS and MIDUS Urinary Metabolic Profiles using Linear Regression[a]

	SEBAS						MIDUS					
	all		females		males		all		females		males	
metabolite	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value	*R*	*P*-value
4CS	+0.32	1.53×10^{-21}	+0.34	2.66×10^{-11}	+0.30	1.12×10^{-11}	+0.23	9.83×10^{-16}	+0.20	3.21×10^{-7}	+0.20	3.21×10^{-7}
PAG	+0.32	1.20×10^{-21}	+0.34	1.53×10^{-11}	+0.31	4.08×10^{-12}	+0.29	6.55×10^{-23}	+0.29	4.57×10^{-14}	+0.29	4.57×10^{-14}
glutamate	+0.23	1.32×10^{-11}	–	–	–	–	–	–	–	–	–	–
creatine	−0.23	3.67×10^{-12}	−0.28	1.4×10^{-6}	–	–	−0.20	2.77×10^{-11}	−0.20	2.21×10^{-7}	–	–
GAA	−0.16	3.79×10^{-6}	–	–	–	–	–	–	–	–	–	–
HMB	−0.18	2.14×10^{-6}	–	–	−0.23	1.63×10^{-7}	−0.26	1.31×10^{-19}	−0.28	5.19×10^{-13}	−0.28	5.19×10^{-13}
NMNA	–	–	–	–	–	–	+0.19	1.40×10^{-10}	+0.26	8.92×10^{-12}	–	–
NMND	–	–	–	–	–	–	+0.15	4.4×10^{-7}	+0.21	9.73×10^{-8}	–	–
4PY	–	–	–	–	–	–	+0.15	6.68×10^{-7}	–	–	–	–
scyllo-inositol	–	–	–	–	–	–	+0.21	1.29×10^{-12}	+0.28	3.12×10^{-13}	–	–
dimethyl sulfone	–	–	–	–	–	–	+0.14	1.17×10^{-6}	–	–	–	–
ascorbate	–	–	–	–	–	–	+0.18	4.47×10^{-10}	+0.25	1.42×10^{-10}	–	–
creatinine	–	–	–	–	–	–	−0.26	2.09×10^{-19}	−0.31	1.90×10^{-15}	−0.30	1.90×10^{-15}
glycine	–	–	–	–	–	–	−0.29	2.03×10^{-23}	−0.34	1.24×10^{-18}	−0.34	1.24×10^{-18}
alanine	–	–	–	–	–	–	−0.15	2.84×10^{-7}	–	–	–	–
lactate	–	–	–	–	–	–	−0.15	2.28×10^{-7}	−0.23	6.79×10^{-9}	–	–

[a]Correlation coefficients (R) and corresponding P-values are given for each metabolite significantly associated with age. Age-related variation is provided for all SEBAS and MIDUS participants and stratified by sex. 4CS, 4-cresyl-sulfate; 4PY, *N*-methyl-4-pyridone-3-carboxamide; GAA, guanidinoacetic acid; HMB, *β*-hydroxy-*β*-methylbutyrate; NMNA, *N*-methyl nicotinic acid; NMND, *N*-methyl nicotinamide; PAG, phenylacetylglutamine.

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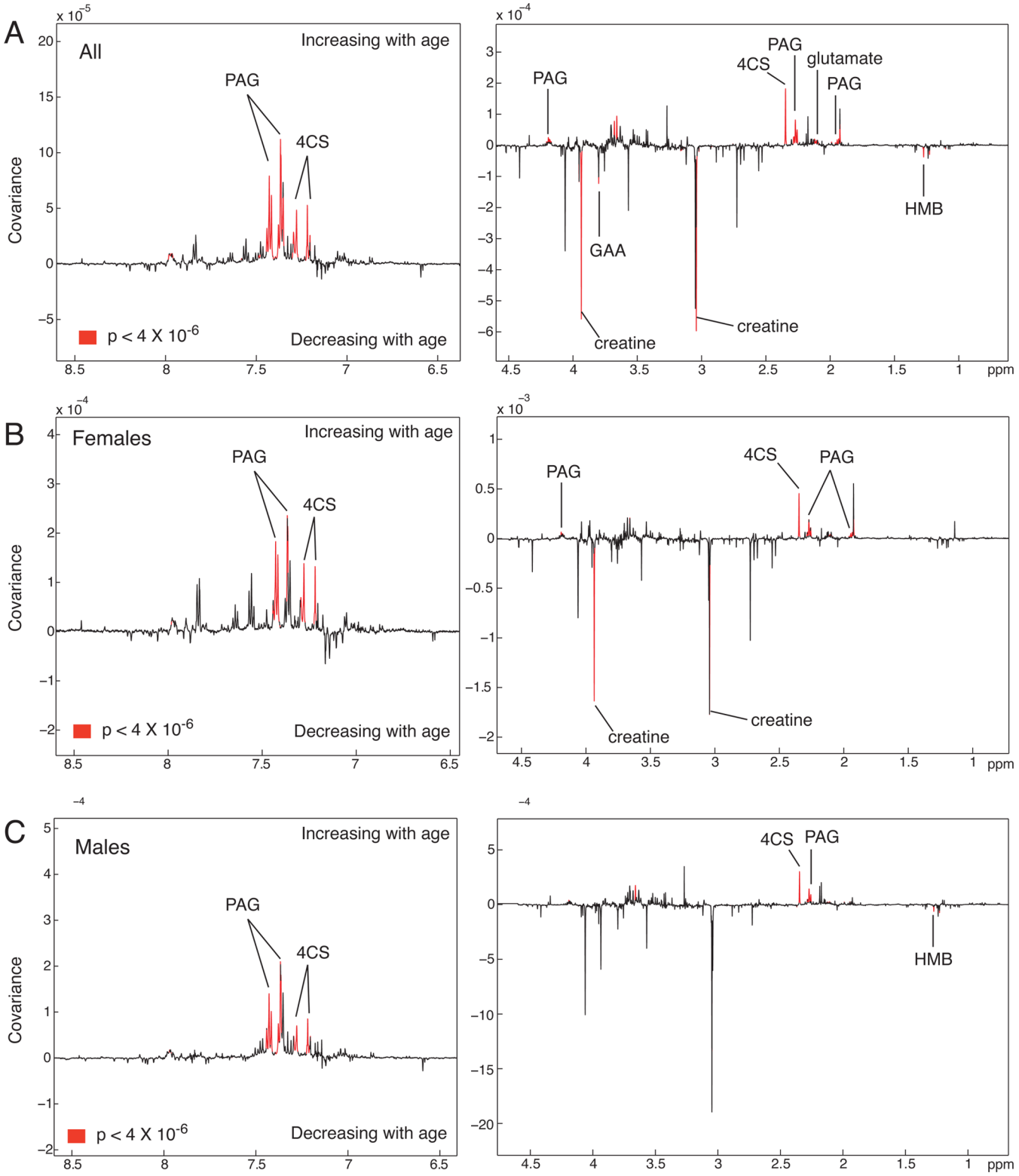

Figure 4. Age-related variation in SEBAS urinary metabolic profiles using linear regression. Covariance plots derived from linear regression analysis for (A) all SEBAS participants and stratified by sex ((B) females and (C) males). Covariance plots are colored by significance ($p < 4 \times 10^{-6}$). HMB, β-hydroxy-β-methylbutyrate; PAG, phenylacetylglutamine; 4CS, 4-cresyl-sulfate.

OPLS-DA and linear regression analysis were used to establish that systematic differences in the metabolic phenotypes of men and women existed and to extract the sex-dependent metabolic characteristics. For the SEBAS specimen set (Supporting Information Figure S2A) a model with a predictive value (Q^2Y) of 0.236 for a 1 orthogonal, 1 aligned component model was obtained. As expected, the major discriminating metabolite between men and women was creatinine, which was found to be at systematically higher concentrations in male urine. Conversely, females excreted

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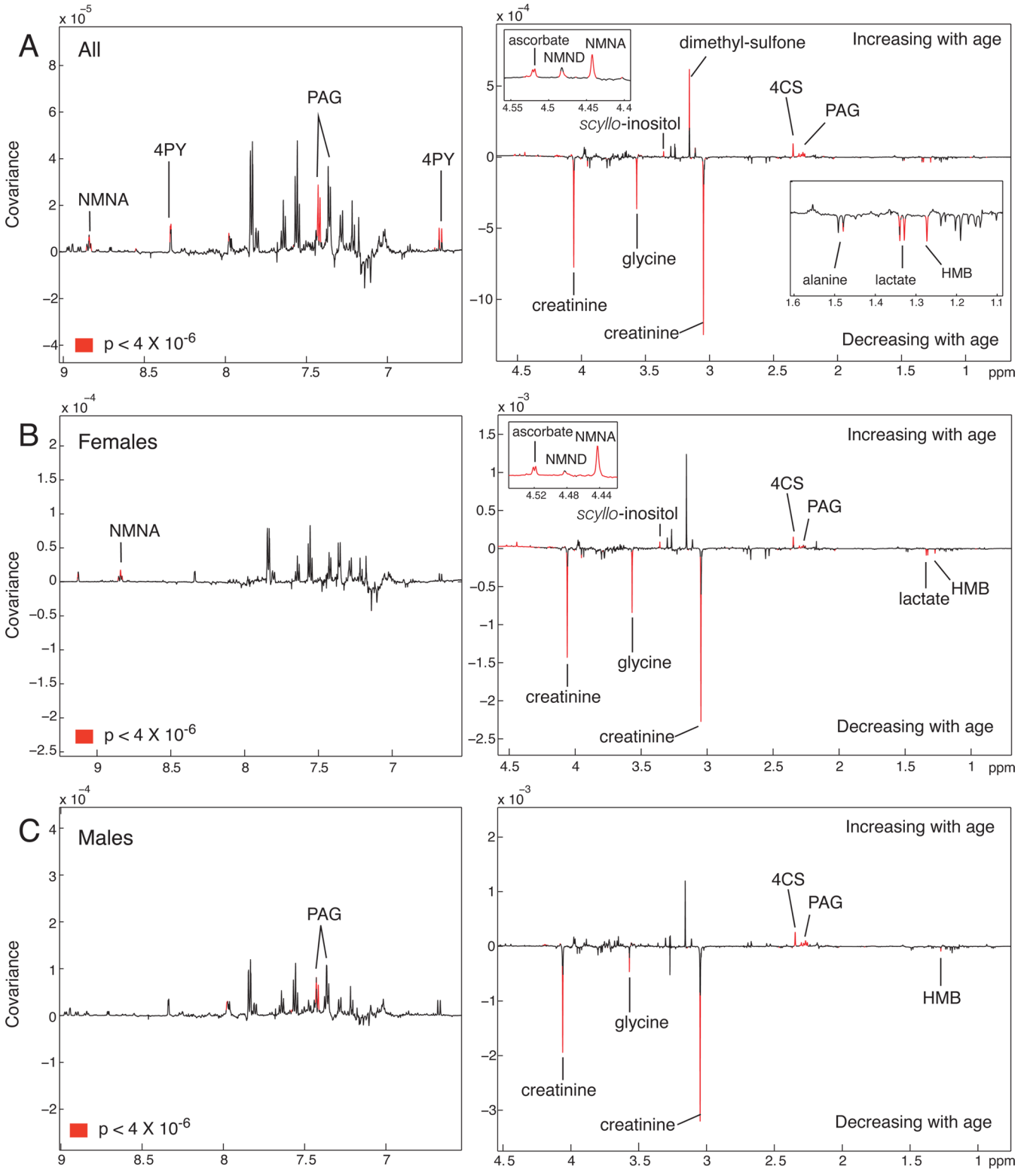

Figure 5. Age-related variation in MIDUS urinary metabolic profiles using linear regression. Covariance plots derived from linear regression analysis for (A) all MIDUS participants and stratified by sex ((B) females and (C) males). Covariance plots are colored by significance ($p < 4 \times 10^{-6}$). 4PY, *N*-methyl-4-pyridone-3-carboxamide; NMNA, *N*-methyl nicotinic acid; NMND, *N*-methyl nicotinamide; HMB, *β*-hydroxy-*β*-methylbutyrate; PAG, phenylacetylglutamine; 4CS, 4-cresyl-sulfate.

greater amounts of creatine and citrate than males. This difference is illustrated in the linear regression plot (Figure 3A). Men were also found to excrete greater amounts of a methylmalonate. Similar findings were noted in the OPLS-DA analysis between sexes in the MIDUS II specimen set (Supporting Information Figure S2B) with a $Q^2Y = 0.207$ for a 1 aligned and 1 orthogonal component model. As with the SEBAS cohort, men had higher urinary excretion of creatinine and methylmalonate and lower citrate and creatine than women. Additional sex-related differences in the US specimen

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166−3180

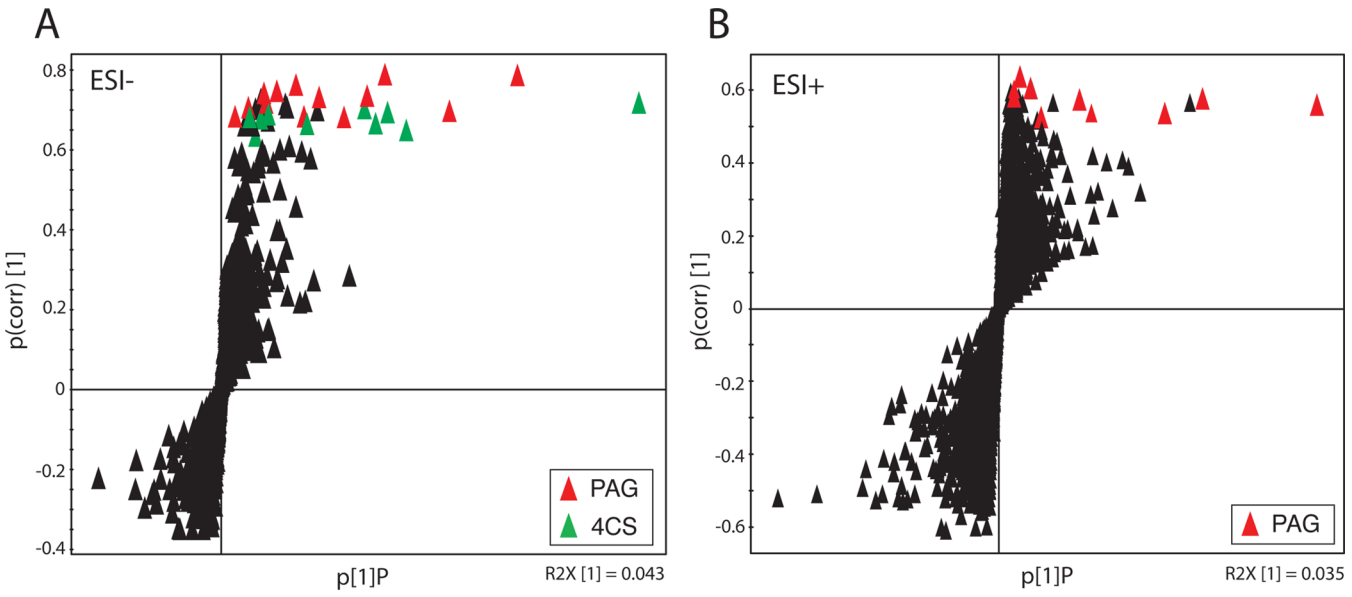

Figure 6. S-plots of the OPLS models identifying UPLC−MS derived-metabolic features associated with aging for (A) SEBAS and (B) MIDUS cohorts.

set included higher taurine in male urine and higher glycine and 4-cresyl sulfate concentrations in female urine (Figure 3B). The urinary concentration of creatinine was calculated from the CH_2 signal of creatinine at δ 4.06. The mean creatinine concentrations for men and women in the SEBAS population were 10.25 ± 5.83 mM and 7.26 ± 4.72 mM respectively and the values for the MIDUS participants were 11.07 ± 6.68 mM (men) and 10.55 ± 6.55 mM (women).

When the data sets were adjusted to align the age range for the SEBAS and MIDUS studies, some of the metabolites identified as being significantly different between men and women in the MIDUS II cohort were not sustained and the urinary metabolites differentiating between men and women were more similar for the two populations (Supporting Information Figure S3). Higher urinary concentrations of citrate and creatine were present in female urine from both SEBAS and MIDUS participants, whereas males excreted higher creatinine and methylmalonate. Additionally, for the MIDUS study, taurine was present in higher concentration in urine specimens collected from men, even after adjustment for age range.

Age-related Differences in Urinary Metabolic Phenotypes

PLS models were calculated for the SEBAS and MIDUS specimen sets independently for both the complete data sets and the age-restricted data sets as summarized in Supporting Information Table S1. Both the univariate linear regression and the OPLS regression models indicated that there was significant variation in the NMR metabolite profiles with age (summarized in Table 2). Mean signal intensities for each metabolite significantly associated with age have been calculated for youngest and oldest participants (n = 100) in the SEBAS and MIDUS studies and are provided in Supporting Information Table S2. Overall, for the SEBAS study, age was directly correlated with excretion of phenylacetylglutamine (PAG), 4-cresyl sulfate (4CS) and glutamate and was inversely correlated with excretion of creatine, β-hydroxy-β-methylbutyrate (HMB) and guanidinoacetate (GAA) (Figure 4). Further models were calculated for this data set after stratification by sex. For both sexes, the gut-microbially derived metabolites, PAG and 4CS, were directly correlated with age. There were also a few differences between the sex-specific models: HMB was inversely correlated with age for males, whereas females showed a similar trend in HMB with age but the age-related variation in urinary concentration was not significant. Women excreted lower amounts of creatine with age.

Similar patterns were observed in the MIDUS study, with PAG and 4CS excretion increasing and creatine, creatinine and HMB excretion inversely correlated with age (Figure 5A). In addition, *scyllo*-inositol, dimethyl-sulfone, *N*-methylnicotinamide (NMDA), *N*-methylnicotinic acid (NMNA), *N*-methyl-4-pyridone-3-carboxamide (4PY) and ascorbate excretion were also directly associated with age. Lower amounts of several amino acids (alanine, glycine and lactate) were excreted with increasing age. When stratified by sex, the females excreted higher PAG, 4CS, *scyllo*-inositol, NMNA, NMND and ascorbate as they aged and lower levels of HMB, creatine, creatinine, lactate and glycine (Figure 5B). Fewer metabolites were correlated with age in the male participants (Figure 5C), with PAG and 4CS positively correlated with age while HMB, creatinine and glycine were negatively correlated with age.

When the data sets were restricted to the same age range in both the MIDUS and SEBAS populations (Supporting Information Figures S4 and S5), the metabolites related to age in the complete data set persisted for SEBAS. For the MIDUS participants, the narrower age range reduced the sample size (females n = 365; males n = 297) and thus the predictive strength of the models. When male and female participants were considered together, PAG and 4CS were positively correlated with aging. In males, the higher concentration of urinary PAG was the metabolic feature most strongly associated with age. The analyses of urine from only MIDUS females yielded a model with poor predictive strength (Q^2Y = 0.008); the results from this linear regression are not shown in Supporting Information Figure S5.

UPLC−MS data indicated that the most discriminatory metabolite for both populations was PAG (Figure 6), followed by 4CS in the SEBAS population, confirming the results generated via NMR. These UPLC−MS metabolite findings

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166−3180

were identified by comparison with authentic standards. For SEBAS, PAG was discriminatory in both the negative (p(corr) range 0.68−0.79) and positive (p(corr) range 0.72−0.82) ESI mode profiles with a mean coefficient of variation of 13 ± 2.8% and 15.5 ± 4.9%, respectively. For MIDUS, the CV values of PAG were similar (16.1 ± 6.3%) in ESI+, but as noted earlier, the ESI− data were of insufficient quality. 4CS was a discriminatory metabolite in urine samples of the SEBAS population analyzed in ESI− with a mean coefficient of variation of 19.1 ± 7.0%. The S-plots for the OPLS models constructed from the SEBAS (ESI−) and MIDUS (ESI+) UPLC−MS data are provided in Figure 6.

DISCUSSION

Human metabolism is influenced by a wide variety of genetic and environmental factors, giving rise to extensive variation in the composition of biological tissues and fluids. Understanding the nature of this variation both between individuals and across populations is critical to attributing systematic changes in metabolism to physiological processes or disease and remains a challenging aspect of biomarker research. In this study, we characterized metabolic signatures associated with sex and age in representative national populations from Taiwan (SEBAS) and the USA (MIDUS). A combination of NMR spectroscopy and UPLC−MS analysis was used to probe similarities and differences in urine specimens obtained from a large number of middle-aged and older participants. The most notable source of variation associated with age in both populations was attributed to metabolites derived from gut microbial transformation of aromatic amino acids, specifically PAG and 4CS.

Global Sources of Metabolic Variation

Major sources of variation within each data set were found to be similar and comprised a mixture of endogenous, dietary, gut-microbial and xenobiotic signatures from human metabolite profiles. The general overview of the metabolic profiles provided by principal components analysis identified metabolites of dietary origin contributing to variation in the metabolic profiles and differing across the two samples. In SEBAS, the excretion of methylamines was a strong source of variation while hippurate concentrations were highly variable in the MIDUS II data set. Urinary dimethylamine (DMA) and trimethylamine (TMA) are predominantly gut microbial products of dietary choline metabolism.[23] The high concentration of TMA in fish is responsible for the characteristic odor. The significant findings in the Taiwanese data may be indicative of greater variation in fish/choline consumption across this cohort, although TMAO is also known to be a component of foods that are high in phytoestrogens such as soy and miso. This interpretation is reasonable given that no dietary restriction was required prior to specimen collection and that fish, seafood and soy are major components of the Taiwanese diet. Alternatively, choline biotransformation capacity encoded in the microbiome may vary widely in this sample. TMAO is a hepatic oxidation product of dietary amines, specifically TMA, and was noted to vary across SEBAS participants in a similar manner to its metabolic precursor. Recent work has demonstrated an association between gut microbial-produced TMA and TMAO and cardiovascular disease risk in humans,[24] where TMAO was demonstrated to be pro-atherogenic.

A further indication that gut microbial capacity may differ between the American and Taiwanese populations is the difference in the urinary variation and concentration of hippurate, a gut microbial−mammalian cometabolite, which is formed from glycine conjugation of dietary or microbially produced benzoic acid in the liver mitochondria. Hippurate was found in higher concentrations in the MIDUS cohort than the SEBAS cohort (SEBAS mean hippurate 1.4 ± 1.51 mM; MIDUS 2.15 ± 1.71 mM) and was also responsible for a large part of the variation in the PCA scores plot in the MIDUS but not the SEBAS data set (Figures 1, 2). Typical urinary concentrations of hippurate in a predominantly Caucasian population have been reported as 1.83 ± 1.24 mM.[25] Differences in the excretion pattern of hippurate and methylamines may simply reflect dietary variation—for example in the consumption of fish, coffee and other sources of benzoic acid (a precursor of hippurate)—or may partially relate to population differences in the gut microbiota and/or their activities. It has been shown that gut microbial transformations can be influenced or entrained by diet. For example, certain porphyranases from marine Bacteroidetes have been acquired by the gut microbiota of Japanese populations where sushi is a stable part of the diet but are absent from the metagenome of Americans.[26]

From the principal components analysis, creatinine was identified as the metabolite with the greatest variation across both the Taiwanese and US samples. Creatinine is known to differ between sexes, with age, with meat consumption, and to be proportional to muscle mass. It is expected, therefore, that creatinine might vary widely across these two large-scale sets of specimens. Urinary creatinine was also strongly influenced by sex, with higher concentrations found in men, in keeping with the known influence of muscle mass.

Other metabolites that exhibited a high degree of variation across the two data sets included xenobiotics such as acetaminophen metabolites, namely acetaminophen-glucuronide and acetaminophen-sulfate, an interesting reflection of prevailing medical practice and medication use across two nations. Acetaminophen metabolites (predominantly glucuronide and sulfate) emerged as strong contributors to the coefficients of the first principal component of the MIDUS PCA model and the second principal component of the SEBAS model.

Sex-dependent Metabolites in the SEBAS and MIDUS Samples

Variation attributable to sex was a major component of both the SEBAS and the MIDUS data sets. On the whole the sex-dependent urinary signature was similar for both data sets. As expected, differences in urinary creatinine proved to be the strongest discriminator with higher levels of urinary creatinine excretion in men, reflecting their greater muscle mass. Creatinine has also been shown to be directly correlated with body weight.[27] Metabolic profiling studies in Swiss (n = 84 women and 66 men),[28] American (n = 30 women and 30 men)[29] and Greek (n = 61 women and 61 men)[30] populations using ^{1}H NMR spectroscopy and multivariate statistics have also reported that creatinine dominates the models. Metabolic profiling studies in rats and mice have also reported higher urinary creatinine concentrations in male animals.[31]

Urinary citrate levels were higher in women than men, in both the SEBAS and MIDUS samples, a finding also reported in prior studies of Swiss, American and Greek populations.[28−30] Higher urinary citrate levels in females have also been found in animal studies, and it is known that urinary citrate excretion increases during pregnancy along with 2-oxoglutarate and

lactate.[32] Urinary citrate excretion in women rises during ovulation and following the administration of estrogens.[33] A comparison of the age-restricted samples suggested that the citrate variation between men and women was stronger in SEBAS ($r = 0.24$; $p = 1.21 \times 10^{-12}$) than in MIDUS ($r = 0.19$; $p = 5.99 \times 10^{-7}$). The higher levels of urinary citrate in women is thought to account for their lower risk of kidney stone formation due to citrate's inhibitory influence on calcium salt crystallization. Conversely, hypocitraturia is an important risk factor for kidney stone formation.[34]

Amino acid excretion was found to differ between sexes in the MIDUS sample only. Greater taurine excretion was observed in male participants while higher glycine excretion was noted in females. Taurine is an amino acid associated with meat intake and could thus reflect dietary preferences for meat consumption,[35] but increased excretion is also a consequence of increased tissue catabolism and protein turnover, which is known to be higher in men. Glycine is required for the biosynthesis of creatine, which was also observed to be greater in females than males. The higher excretion of glycine may therefore reflect a greater requirement for creatine synthesis in these females.

Methylmalonate (MMA) was present in greater amounts in male than in female urine. This sex effect was consistent across both the Taiwanese and US samples. This malonic acid derivative is a precursor for succinyl-CoA and its synthesis requires the cofactor, cobalamin (vitamin B_{12}). Hence, urinary MMA is known to be elevated in cobalamin-deficient individuals. Cobalamin deficiency is most common in elderly white males[36] and has been associated with cognitive impairment, anemia and peripheral neuropathy.[37]

Characterization of Age-associated Metabolites in the SEBAS and MIDUS Samples

Age-related variation was apparent in both data sets. Two notable metabolites—phenylacetylglutamine (PAG) and 4-cresyl sulfate (4CS)—were positively correlated with age, even when the samples were stratified by sex. Another variation that was consistent across both samples was lower excretion of β-hydroxy-β-methylbutyrate (HMB) and creatine in older participants.

Associations with age that were unique to the SEBAS population included a positive relationship between urinary glutamate and age and an inverse relationship with guanidinoacetic acid (GAA). For MIDUS participants, ascorbate, *N*-methylnicotinamide (NMND), *N*-methylnicotinic acid (NMNA), *N*-methyl-4-pyridone-3-carboxamide (4PY), dimethyl-sulfone and *scyllo*-inositol were directly associated with age, while creatinine, lactate, alanine and glycine were inversely correlated with age.

Through this molecular epidemiology approach we have identified potential metabolic windows into multiple age-related processes and diseases. These have great potential for understanding the biochemical basis of disease processes, early diagnostics and health implications of such diseases. Specifically, the results are relevant to the biochemical events associated with sarcopenia, neurological dysfunction and the susceptibility to gastrointestinal infection.

Creatinine, creatine and HMB are likely to be associated with muscle turnover, which declines with age. As discussed with respect to sex differences in creatinine excretion, creatinine is an index of muscle mass[27] and aging is associated with progressive loss of muscle performance and lean mass.[38] In a metabolic profiling study of aging in Labrador retriever dogs, the level of urinary creatinine rose during development through young adulthood, reached a maximum at 5−9 years old and then declined in later life.[39] Differences in creatinine concentration with age can also arise from the age-dependent decrease in renal plasma flow and glomerular filtration rate.[40] However, since the proximal tubules are responsible for the excretion of 10% of creatinine then although reduced glomerular filtration rate may contribute to the association between age and declining creatinine, it is unlikely to be the main factor influencing this event. Muscle holds a vital role in whole-body protein metabolism serving as a repository for protein and amino acids and maintaining systemic protein synthesis. Reasons for the decline in muscle mass with age include reduced exercise, poor nutrition and loss of muscle integrity. However, a definitive mechanism for muscle loss with age has not yet been established. Maintenance of muscle mass can protect against various pathologies and diseases. Age-related muscle mass atrophy (sarcopenia) can have adverse effects on protein metabolism, immune function, organ function and wound healing.[41] Proposed reasons for sarcopenia stem from a host of intrinsic and extrinsic factors including decreased hormonal activity.[42] The inverse association between HMB and age is also consistent with the progressive loss of muscle mass with age and has previously been reported as characteristic of differences between young (19−40 years) and old (41−69) in a metabolic profiling study in a small cohort of Americans.[29] HMB is a metabolite of the amino acid leucine and has a protective effect on muscle loss. It can serve as a precursor for cholesterol synthesis in muscle tissue, which can then have an important role in strengthening the cellular membrane of muscle cells. Furthermore, HMB can attenuate protein degradation and up-regulate protein synthesis in muscle tissue. Research has shown that supplementing the elderly with HMB can decrease muscle damage and increase lean body mass.[43]

Elevations in the excretion of several metabolites in the nicotinic acid pathway—*N*-methylnicotinic acid (trigonelline or NMNA), *N*-methylnicotinamide (NMND) and *N*-methyl-4-pyridone-3-carboxamide (4PY)—were positively associated with age in the American cohort. This type of metabolic dysregulation may be associated with age-related neurodegenerative conditions and cognitive dysfunction associated with aging e.g. Parkinson's and Alzheimer's disease.[6] Lower urinary 4-PY concentrations have been found in stressed rats compared with controls, and those exhibiting fatigue have perturbed nicotinate and nicotinamide metabolism.[44] Increased NMND excretion has also been observed in individuals with Parkinson's disease[45,46] and has been implicated as a mechanism mediating the death of dopamine-generating cells.[47] Similarly, brain concentrations of inositol metabolites have been linked to neurodegenerative diseases, specifically Alzheimer's dementia, and are present in greater amounts in elderly than in young individuals,[48] suggesting that the regulatory integrity for maintaining intracellular inositol concentrations may weaken with age.

Indices of Age-associated Variation in the Gut Microbiome

Mammals are now considered to be "superorganisms" or "metaorganisms" whose processes represent the sum of both genomic and microbiomic contributions. It is reasonable, therefore, to consider how aging affects the symbiotic relationship between the host and resident microbiota. Such age-associated changes are likely to be reciprocal in nature with

dx.doi.org/10.1021/pr4000152 | J. Proteome Res. 2013, 12, 3166−3180

microbial modulations being both a cause and consequence of structural and biochemical changes in the gastrointestinal tract, immunosenescence and alterations in food consumption caused by changes in appetite, taste and digestion. In addition, host factors, including reduced physical activity, oropharangeal dysphagia and changes in gut motility and immune competence in the elderly can all impact on health and the microbiota.[49] Conditions such as constipation and slow gut transit times are also more prevalent in the elderly and may lead to increased usage of various medications for chronic symptoms.[50] Elderly people are more likely than younger people to be the recipients of drug therapy of many classes, including ones that affect the gut microbiome (e.g., elderly, defined as >65 years, comprise approximately 13% of the U.S.A. population, but are the recipients of >40% of all prescription drugs[51]). Laxatives, antibiotics, and calcium channel blockers commonly lead to side-effects such as diarrhea, malabsorption and constipation.[52]

PAG and 4CS showed the strongest association with age for both populations with a correlation coefficient (r) of 0.32 ($p = 1.2 \times 10^{-21}$) and 0.32 ($p = 1.53 \times 10^{-21}$), respectively, for SEBAS and 0.29 ($p = 6.55 \times 10^{-23}$) and 0.23 ($p = 9.83 \times 10^{-16}$) for MIDUS (Figures 4 and 5). PAG and 4CS are formed from protein putrefaction of phenylalanine and tyrosine by the gut microbiota. Phenylalanine is converted to phenylacetate in the colon and subsequently conjugated with glutamine in the liver and the gut mucosa,[53] whereas 4CS is a product of microbial tyrosine breakdown *via* hydroxyphenylacetate to *4-cresyl*, followed by conjugation with sulfate.[54] Age-related variations were also observed in the bacterial fermentation product, lactic acid, being negatively associated with aging in the American sample.

The marked age-associated alteration of PAG and 4CS concentrations are consistent with known shifts in the composition of the microbiome, including increased representation from enterobacteria and decreasing proportions of anaerobes and Bifidobacteria.[55] The ratio of Firmicutes to Bacteroidetes has also been found to be lower in the elderly.[56] Decreases in anaerobes and *Bifidobacterium* spp. and increases in enterobacteria may increase susceptibility to gastrointestinal infections, and changes in the composition of gut microbiota have been implicated in many diseases such as Irritable Bowel Syndrome (IBS), Ulcerative Colitis (UC) and Crohn's disease (CD).[57] Moreover nosocomial infections such as *Clostridium difficile* are known to have greater morbidity in the elderly. The diversity of species comprising the dominant fecal microbiota increase with aging.[58] In addition to the composition changes, the interaction between the microbiota and intestinal functions likely shift with age. He et al. demonstrated that certain Bifidobacterium strains isolated from healthy adults aged 30–40 were able to bind better to the intestinal mucus than were the same bacterial strains isolated from healthy seniors (>70 years of age).[59] However, not all researchers have consistently found these age-related differences. Other studies have shown that there is a tendency for stability in the gut microbiome throughout adulthood,[60] and several studies suggest that age-related alterations in microbial composition may be dependent upon the population and geographic location.[61] Aging has been associated with an increase in enterobacteria and Clostridia in particular, while health-promoting bacteria such as the Bifidobacteria have been reported to decline in abundance and diversity of species with age.[58] Several bacteria can synthesize 4CS such as members of the Clostridia including *Clostridium difficile*.[62]

Other studies have reported associations between age and mammalian-microbial urinary cometabolites. One ^{1}H NMR-based profiling study investigating lifelong changes in the urinary metabolome of dogs under caloric restricted and nonrestricted conditions found that hippurate and 3-HPPA concentrations increased with age.[39] Urinary levels of amines, resulting from degradation of dietary choline by gut microbiota, also changed with age. This increase in gut microbial metabolites was enhanced by dietary restriction. Similar results have been shown in a study in which rats fed with chow diets were compared with rats fed with casein-rich diets.[63] Moreover, in both humans and nonhumans, clear differences in microbially derived metabolites have been shown in the urinary, fecal and plasma profiles from obese individuals with metabolites such as hippurate and PAG being associated with leaner phenotypes. Thus, it is possible that variation in the excretion of 4CS and PAG seen with age in both the SEBAS and MIDUS surveys reflect a general reduction in caloric intake by the older participants.

■ CONCLUSIONS

In summary, this work reinforces the great potential of applying metabolome-wide association studies to large-scale epidemiology studies. Through this application we have identified potential metabolic windows into later life diseases. These windows point to an underpinning dysregulation of the microbiota that may relate to increased susceptibility to GI infection in the elderly. Additionally some of the changes are suggestive of a decline in muscle mass. Specifically, we have shown significant age-related differences in the urinary metabolite profiles of Taiwanese and American populations, with the strongest effects being attributed to 4-cresyl sulfate and phenylacetylglutamine. These metabolite differences were significant in both males and females and revealed a marked shift in the functionality of the gut microbiome with age. In addition, the bacterial fermentation product, lactic acid, was negatively correlated with age in Americans. The age-related variation in these gut microbial metabolites may reflect increasing enterobacterial numbers and warrants further investigation to directly link metabolic profiles to fecal microbial composition. The appearance of functional aging observed in the microbiome was consistent across both national populations in spite of some cultural features.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Institute on Aging (grant numbers R01AG16790, R01AG16661, P01-AG020166); and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant number

R24HD047879). SEBAS was funded by the Demography and Epidemiology Unit of the Behavioral and Social Research Program of the National Institute on Aging [grant numbers R01 AG16790, R01 AG16661]. The Bureau of Health Promotion (Department of Health, Taiwan) provided additional financial support for SEBAS 2000. We acknowledge the hard work and dedication of the staff at the Center for Population and Health Survey Research (BHP), who were instrumental in the design and implementation of the SEBAS and supervised all aspects of the fieldwork and data processing. The MIDUS longitudinal follow-up was supported by the National Institute on Aging [grant number P01-AG020166]. The specimen collection was also facilitated by the General Clinical Research Centers Program [grant numbers M01-RR023942 to Georgetown University; M01-RR00865 to UCLA] and by the Clinical and Translational Science Award program of the National Center for Research Resources, National Institutes of Health [grant number 1UL1RR025011 to University of Wisconsin-Madison].

REFERENCES

(1) Piper, M. D.; Selman, C.; McElwee, J. J.; Partridge, L. Separating cause from effect: how does insulin/IGF signalling control lifespan in worms, flies and mice? *J. Intern. Med.* **2008**, *263* (2), 179−91.

(2) Wijeyesekera, A.; Selman, C.; Barton, R. H.; Holmes, E.; Nicholson, J. K.; Withers, D. J. Metabotyping of long-lived mice using (1)H NMR spectroscopy. *J. Proteome Res.* **2012**, *11* (4), 2224−35.

(3) Robert, L.; Labat-Robert, J.; Robert, A. M. Genetic, epigenetic and posttranslational mechanisms of aging. *Biogerontology* **2010**, *11* (4), 387−99.

(4) Walston, J.; Hadley, E. C.; Ferrucci, L.; Guralnik, J. M.; Newman, A. B.; Studenski, S. A.; Ershler, W. B.; Harris, T.; Fried, L. P. Research agenda for frailty in older adults: toward a better understanding of physiology and etiology: summary from the American Geriatrics Society/National Institute on Aging Research Conference on Frailty in Older Adults. *J. Am. Geriatr. Soc.* **2006**, *54* (6), 991−1001.

(5) Coe, C. L.; Love, G. D.; Karasawa, M.; Kawakami, N.; Kitayama, S.; Markus, H. R.; Tracy, R. P.; Ryff, C. D. Population differences in proinflammatory biology: Japanese have healthier profiles than Americans. *Brain Behav. Immun.* **2011**, *25* (3), 494−502.

(6) Muangpaisan, W.; Mathews, A.; Hori, H.; Seidel, D. A systematic review of the worldwide prevalence and incidence of Parkinson's disease. *J. Med. Assoc. Thai.* **2011**, *94* (6), 749−55.

(7) van den Bussche, H.; Koller, D.; Kolonko, T.; Hansen, H.; Wegscheider, K.; Glaeske, G.; von Leitner, E. C.; Schafer, I.; Schon, G. Which chronic diseases and disease combinations are specific to multimorbidity in the elderly? Results of a claims data based cross-sectional study in Germany. *BMC Public Health* **2011**, *11*, 101.

(8) Regitz-Zagrosek, V.; Lehmkuhl, E.; Weickert, M. O. Gender differences in the metabolic syndrome and their role for cardiovascular disease. *Clin. Res. Cardiol.* **2006**, *95* (3), 136−47.

(9) Holmes, E.; Loo, R. L.; Stamler, J.; Bictash, M.; Yap, I. K.; Chan, Q.; Ebbels, T.; De Iorio, M.; Brown, I. J.; Veselkov, K. A.; Daviglus, M. L.; Kesteloot, H.; Ueshima, H.; Zhao, L.; Nicholson, J. K.; Elliott, P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature* **2008**, *453* (7193), 396−400.

(10) Yap, I. K.; Brown, I. J.; Chan, Q.; Wijeyesekera, A.; Garcia-Perez, I.; Bictash, M.; Loo, R. L.; Chadeau-Hyam, M.; Ebbels, T.; De Iorio, M.; Maibaum, E.; Zhao, L.; Kesteloot, H.; Daviglus, M. L.; Stamler, J.; Nicholson, J. K.; Elliott, P.; Holmes, E. Metabolome-wide association study identifies multiple biomarkers that discriminate north and south Chinese populations at differing risks of cardiovascular disease: INTERMAP study. *J. Proteome Res.* **2010**, *9* (12), 6647−54.

(11) Nicholson, J. K.; Lindon, J. C.; Holmes, E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29* (11), 1181−9.

(12) Mishur, R. J.; Rea, S. L. Applications of mass spectrometry to metabolomics and metabonomics: detection of biomarkers of aging and of age-related diseases. *Mass Spectrom. Rev.* **2012**, *31* (1), 70−95.

(13) Houtkooper, R. H.; Argmann, C.; Houten, S. M.; Canto, C.; Jeninga, E. H.; Andreux, P. A.; Thomas, C.; Doenlen, R.; Schoonjans, K.; Auwerx, J. The metabolic footprint of aging in mice. *Sci. Rep.* **2011**, *1*, 134.

(14) Okuda, Y.; Kawai, K.; Yamashita, K. Age-related change in ketone body metabolism: diminished glucagon effect on ketogenesis in adult rats. *Endocrinology* **1987**, *120* (5), 2152−7.

(15) Takiyama, N.; Matsumoto, K. Age-and sex-related differences of serum carnitine in a Japanese population. *J. Am. Coll. Nutr.* **1998**, *17* (1), 71−4.

(16) Goldman, N.; Lin, I. F.; Weinstein, M.; Lin, Y. H. Evaluating the quality of self-reports of hypertension and diabetes. *J. Clin. Epidemiol.* **2003**, *56* (2), 148−54.

(17) Marmot, M. G.; Fuhrer, R.; Ettner, S. L.; Marks, N. F.; Bumpass, L. L.; Ryff, C. D. Contribution of psychosocial factors to socio-economic differences in health. *Milbank Q.* **1998**, *76* (3), 403−48.

(18) Beckonert, O.; Keun, H. C.; Ebbels, T. M.; Bundy, J.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat. Protoc.* **2007**, *2* (11), 2692−703.

(19) Veselkov, K. A.; Lindon, J. C.; Ebbels, T. M.; Crockford, D.; Volynkin, V. V.; Holmes, E.; Davies, D. B.; Nicholson, J. K. Recursive segment-wise peak alignment of biological (1)h NMR spectra for improved metabolic biomarker recovery. *Anal. Chem.* **2009**, *81* (1), 56−66.

(20) Chadeau-Hyam, M.; Ebbels, T. M.; Brown, I. J.; Chan, Q.; Stamler, J.; Huang, C. C.; Daviglus, M. L.; Ueshima, H.; Zhao, L.; Holmes, E.; Nicholson, J. K.; Elliott, P.; De Iorio, M. Metabolic profiling and the metabolome-wide association study: significance level for biomarker identification. *J. Proteome Res.* **2010**, *9* (9), 4620−7.

(21) Want, E. J.; Wilson, I. D.; Gika, H.; Theodoridis, G.; Plumb, R. S.; Shockcor, J.; Holmes, E.; Nicholson, J. K. Global metabolic profiling procedures for urine using UPLC-MS. *Nat. Protoc.* **2010**, *5* (6), 1005−18.

(22) Veselkov, K. A.; Vingara, L. K.; Masson, P.; Robinette, S. L.; Want, E.; Li, J. V.; Barton, R. H.; Boursier-Neyret, C.; Walther, B.; Ebbels, T. M.; Pelczer, I.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. Optimized preprocessing of ultra-performance liquid chromatography/mass spectrometry urinary metabolic profiles for improved information recovery. *Anal. Chem.* **2011**, *83* (15), 5864−72.

(23) Zhang, A. Q.; Mitchell, S. C.; Smith, R. L. Dimethylamine in human urine. *Clin. Chim. Acta* **1995**, *233* (1−2), 81−8.

(24) Wang, Z.; Klipfell, E.; Bennett, B. J.; Koeth, R.; Levison, B. S.; Dugar, B.; Feldstein, A. E.; Britt, E. B.; Fu, X.; Chung, Y. M.; Wu, Y.; Schauer, P.; Smith, J. D.; Allayee, H.; Tang, W. H.; DiDonato, J. A.; Lusis, A. J.; Hazen, S. L. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* **2011**, *472* (7341), 57−63.

(25) Saude, E. J.; Adamko, D.; Rowe, B. H.; Marrie, T.; Sykes, B. D. Variation of metabolites in normal human urine. *Metabolomics* **2007**, *3*, 439−51.

(26) Hehemann, J. H.; Correc, G.; Barbeyron, T.; Helbert, W.; Czjzek, M.; Michel, G. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. *Nature* **2010**, *464* (7290), 908−12.

(27) Davies, K. M.; Heaney, R. P.; Rafferty, K. Decline in muscle mass with age in women: a longitudinal study using an indirect measure. *Metabolism* **2002**, *51* (7), 935−9.

(28) Kochhar, S.; Jacobs, D. M.; Ramadan, Z.; Berruex, F.; Fuerholz, A.; Fay, L. B. Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics. *Anal. Biochem.* **2006**, *352* (2), 274−81.

(29) Slupsky, C. M.; Rankin, K. N.; Wagner, J.; Fu, H.; Chang, D.; Weljie, A. M.; Saude, E. J.; Lix, B.; Adamko, D. J.; Shah, S.; Greiner, R.; Sykes, B. D.; Marrie, T. J. Investigations of the effects of gender,

diurnal variation, and age in human urinary metabolomic profiles. *Anal. Chem.* **2007**, *79* (18), 6995−7004.
(30) Psihogios, N. G.; Gazi, I. F.; Elisaf, M. S.; Seferiadis, K. I.; Bairaktari, E. T. Gender-related and age-related urinalysis of healthy subjects by NMR-based metabonomics. *NMR Biomed* **2008**, *21* (3), 195−207.
(31) Stanley, E. G.; Bailey, N. J.; Bollard, M. E.; Haselden, J. N.; Waterfield, C. J.; Holmes, E.; Nicholson, J. K. Sexual dimorphism in urinary metabolite profiles of Han Wistar rats revealed by nuclear-magnetic-resonance-based metabonomics. *Anal. Biochem.* **2005**, *343* (2), 195−202.
(32) Yasuda, M.; Tsunoda, S.; Nagasawa, H. Comparison of urinary component levels in 4 strains of mice with different physiological characteristics. *In Vivo* **1997**, *11* (2), 109−13.
(33) Hammar, M. L.; Berg, G. E.; Larsson, L.; Tiselius, H. G.; Varenhorst, E. Endocrine changes and urinary citrate excretion. *Scand J Urol Nephrol* **1987**, *21* (1), 51−3.
(34) Welshman, S. G.; McGeown, M. G. Urinary citrate excretion in stone-formers and normal controls. *Br J Urol* **1976**, *48* (1), 7−11.
(35) Laidlaw, S. A.; Shultz, T. D.; Cecchino, J. T.; Kopple, J. D. Plasma and urine taurine levels in vegans. *Am. J. Clin. Nutr.* **1988**, *47* (4), 660−3.
(36) Carmel, R.; Green, R.; Jacobsen, D. W.; Rasmussen, K.; Florea, M.; Azen, C. Serum cobalamin, homocysteine, and methylmalonic acid concentrations in a multiethnic elderly population: ethnic and sex differences in cobalamin and metabolite abnormalities. *Am. J. Clin. Nutr.* **1999**, *70* (5), 904−10.
(37) Clarke, R.; Refsum, H.; Birks, J.; Evans, J. G.; Johnston, C.; Sherliker, P.; Ueland, P. M.; Schneede, J.; McPartlin, J.; Nexo, E.; Scott, J. M. Screening for vitamin B-12 and folate deficiency in older persons. *Am. J. Clin. Nutr.* **2003**, *77* (5), 1241−7.
(38) Hurley, B. F. Age, gender, and muscular strength. *J. Gerontol. A: Biol. Sci. Med. Sci.* **1995**, *50*, 41−4.
(39) Wang, Y.; Lawler, D.; Larson, B.; Ramadan, Z.; Kochhar, S.; Holmes, E.; Nicholson, J. K. Metabonomic investigations of aging and caloric restriction in a life-long dog study. *J. Proteome Res.* **2007**, *6* (5), 1846−54.
(40) Perrone, R. D.; Madias, N. E.; Levey, A. S. Serum creatinine as an index of renal function: new insights into old concepts. *Clin. Chem.* **1992**, *38* (10), 1933−53.
(41) Wolfe, R. R. The underappreciated role of muscle in health and disease. *Am. J. Clin. Nutr.* **2006**, *84* (3), 475−82.
(42) Cannon, J. G. Intrinsic and extrinsic factors in muscle aging. *Ann. N.Y. Acad. Sci.* **1998**, *854*, 72−7.
(43) Vukovich, M. D.; Stubbs, N. B.; Bohlken, R. M. Body composition in 70-year-old adults responds to dietary beta-hydroxy-beta-methylbutyrate similarly to that of young adults. *J. Nutr.* **2001**, *131* (7), 2049−52.
(44) Zhang, F.; Jia, Z.; Gao, P.; Kong, H.; Li, X.; Lu, X.; Wu, Y.; Xu, G. Metabonomics study of urine and plasma in depression and excess fatigue rats by ultra fast liquid chromatography coupled with ion trap-time of flight mass spectrometry. *Mol. Biosyst.* **2010**, *6* (5), 852−61.
(45) Willets, J. M.; Lunec, J.; Williams, A. C.; Griffiths, H. R. Neurotoxicity of nicotinamide derivatives: their role in the aetiology of Parkinson's disease. *Biochem. Soc. Trans.* **1993**, *21* (3), 299S.
(46) Williams, A.; Sturman, S.; Steventon, G.; Waring, R. Metabolic biomarkers of Parkinson's disease. *Acta Neurol. Scand. Suppl.* **1991**, *136*, 19−23.
(47) Fukushima, T.; Kaetsu, A.; Lim, H.; Moriyama, M. Possible role of 1-methylnicotinamide in the pathogenesis of Parkinson's disease. *Exp. Toxicol. Pathol.* **2002**, *53* (6), 469−73.
(48) Kaiser, L. G.; Schuff, N.; Cashdollar, N.; Weiner, M. W. Scyllo-inositol in normal aging human brain: 1H magnetic resonance spectroscopy study at 4 T. *NMR Biomed.* **2005**, *18* (1), 51−5.
(49) Dean, M.; Raats, M. M.; Grunert, K. G.; Lumbers, M. Factors influencing eating a varied diet in old age. *Public Health Nutr.* **2009**, *12* (12), 2421−7.
(50) Wald, A. Constipation in elderly patients. Pathogenesis and management. *Drugs Aging* **1993**, *3* (3), 220−31.
(51) Cho, S.; Lau, S. W.; Tandon, V.; Kumi, K.; Pfuma, E.; Abernethy, D. R. Geriatric drug evaluation: where are we now and where should we be in the future? *Arch. Intern. Med.* **2011**, *171* (10), 937−40.
(52) Triantafyllou, K.; Vlachogiannakos, J.; Ladas, S. D. Gastrointestinal and liver side effects of drugs in elderly patients. *Best Pract. Res. Clin. Gastroenterol.* **2010**, *24* (2), 203−15.
(53) Ramakrishna, B. S.; Gee, D.; Weiss, A.; Pannall, P.; Roberts-Thomson, I. C.; Roediger, W. E. Estimation of phenolic conjugation by colonic mucosa. *J. Clin. Pathol.* **1989**, *42* (6), 620−3.
(54) Ramakrishna, B. S.; Roberts-Thomson, I. C.; Pannall, P. R.; Roediger, W. E. Impaired sulphation of phenol by the colonic mucosa in quiescent and active ulcerative colitis. *Gut* **1991**, *32* (1), 46−9.
(55) Hebuterne, X. Gut changes attributed to ageing: effects on intestinal microflora. *Curr. Opin. Clin. Nutr. Metab. Care* **2003**, *6* (1), 49−54.
(56) Mariat, D.; Firmesse, O.; Levenez, F.; Guimaraes, V.; Sokol, H.; Dore, J.; Corthier, G.; Furet, J. P. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* **2009**, *9*, 123.
(57) Partridge, L.; Thornton, J.; Bates, G. The new science of ageing. *Philos. Trans. R. Soc. Lond., B: Biol. Sci.* **2011**, *366* (1561), 6−8.
(58) Hopkins, M. J.; Sharp, R.; Macfarlane, G. T. Variation in human intestinal microbiota with age. *Dig. Liver Dis.* **2002**, *34* (Suppl 2), S12−8.
(59) He, F.; Ouwehand, A. C.; Isolauri, E.; Hosoda, M.; Benno, Y.; Salminen, S. Differences in composition and mucosal adhesion of bifidobacteria isolated from healthy adults and healthy seniors. *Curr. Microbiol.* **2001**, *43* (5), 351−4.
(60) Tiihonen, K.; Ouwehand, A. C.; Rautonen, N. Human intestinal microbiota and healthy ageing. *Ageing Res. Rev.* **2010**, *9* (2), 107−16.
(61) O'Sullivan, O.; Coakley, M.; Lakshminarayanan, B.; Claesson, M. J.; Stanton, C.; O'Toole, P. W.; Ross, R. P. Correlation of rRNA gene amplicon pyrosequencing and bacterial culture for microbial compositional analysis of faecal samples from elderly Irish subjects. *J. Appl. Microbiol.* **2011**, *111* (2), 467−73.
(62) Elsden, S. R.; Hilton, M. G.; Waller, J. M. The end products of the metabolism of aromatic amino acids by Clostridia. *Arch. Microbiol.* **1976**, *107* (3), 283−8.
(63) Bell, J. D.; Sadler, P. J.; Morris, V. C.; Levander, O. A. Effect of aging and diet on proton NMR spectra of rat urine. *Magn. Reson. Med.* **1991**, *17* (2), 414−22.