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Analysis of the adult human plasma metabolome

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Objective: It is well established that disease states are associated with biochemical changes (e.g., diabetes/glucose, cardiovascular disease/cholesterol), as are responses to chemical agents (e.g., medications, toxins, xenobiotics). Recently, nontargeted methods have been used to identify the small molecules (metabolites) in a biological sample to uncover many of the biochemical changes associated with a disease state or chemical response. Given that these experimental results may be influenced by the composition of the cohort, in the present study we assessed the effects of age, sex and race on the relative concentrations of small molecules (metabolites) in the blood of healthy adults.

Methods: Using gas- and liquid-chromatography in combination with mass spectrometry, a nontargeted metabolomic analysis was performed on plasma collected from an age- and sex-balanced cohort of 269 individuals. **Results:** Of the more than 300 unique compounds that were detected, significant changes in the relative concentration of more than 100 metabolites were associated with age. Many fewer differences were associated with sex and fewer still with race. Changes in protein, energy and lipid metabolism, as well as oxidative stress, were observed with increasing age. Tricarboxylic acid intermediates, creatine, essential and nonessential amino acids, urea, ornithine, polyamines and oxidative stress markers (e.g., oxoproline, hippurate) increased with age. Compounds related to lipid metabolism, including fatty acids, carnitine, β -hydroxybutyrate and cholesterol, were lower in the blood of younger individuals. By contrast, relative concentrations of dehydroepiandrosterone-sulfate (a proposed antiaging androgen) were lowest in the oldest age group. Certain xenobiotics (e.g., caffeine) were higher in older subjects, possibly reflecting decreases in hepatic cytochrome P450 activity. **Conclusions:** Our nontargeted analytical approach detected a large number of metabolites, including those that were found to be statistically altered with age, sex or race. Age-associated changes were more pronounced than those related to differences in sex or race in the population group we studied. Age, sex and race can be confounding factors when comparing different groups in clinical studies. Future studies to determine the influence of diet, lifestyle and medication are also warranted.

With the recent advances in chromatographic separation and detection technologies (e.g., mass spectrometry), coupled with statistical analysis, advanced mathematical modeling and high order computation capability, it is possible to use a nontargeted analytical approach to identify and measure the relative concentration of a large number of small molecules in a single biological sample (metabolomics). These small molecules include endogenous metabolites, peptides, xenobiotics, dietary constituents and agents of environmental exposure. The metabolomics approach has been applied to problems in disparate areas, such as pharmaceutical discovery and development, natural products research, and disease diagnosis [1–9,101,102]. Thus, metabolomics is an important emerging approach for biological research, including marker discovery [10]. In the

optimal case, the populations to be compared are balanced for age, sex and race, and diet and lifestyle factors are controlled. However, this may not always be possible. For instance, the available cohort may be small (e.g., amyotrophic lateral sclerosis [ALS] and other rare diseases), or not easily amenable to dietary and lifestyle controls (e.g., Alzheimer's disease, schizophrenia and other mental disorders). To uncover potentially confounding influences on interpretation of metabolomic results, it is important to determine the effects of age, sex and race on human plasma metabolites (i.e., plasma metabolome) in a generally healthy population that has not been subjected to lifestyle and dietary controls.

The goal of any metabolomic analysis is to extract, identify and quantify all of the metabolites in a biological sample. Thus, an unbiased

Keywords: age, biochemical, biomarker, metabolite, metabolomic, metabonomic, race, sex

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metabolomics analysis is useful to monitor in a nontargeted manner (i.e., globally) changes in metabolic profiles related to age and/or sex, and can be extended to detect dietary metabolites and xenobiotics that are present in the blood. In addition, this approach provides insight into the influence of age and sex on the relative concentrations of observed biochemicals. Besides facilitating our understanding of how these factors affect the small-molecule complement of human plasma and/or other biological samples (e.g., tissue, saliva, urine), such knowledge permits an efficient, metabolomics-based measurement process to be compared with more traditional clinical measurement determinations.

Here we report the use of a chemocentric and nontargeted metabolomics analysis using data collected in combination with liquid chromatography (LC)/mass spectrometry (MS) and gas chromatography (GC)/MS instrumentation to determine the number, identity and relative concentrations of small molecules that are present in plasma of a representative, healthy cohort consisting of 269 subjects; Caucasian, Hispanic and African-American males and females, aged 20–65 years. The implications of our findings on age-, sex- and race-associated changes in metabolism are discussed.

Materials & methods

Cohort

Metabolomic analysis was performed on 269 healthy subjects, 131 males and 138 females, ranging in age between 20 and 65 years. The subjects were divided into three age groups: 20–35 years (24.5 ± 3.3 , mean \pm standard deviation [SD]); 36–50 years (40.8 ± 5.6); 51–65 years (55.6 ± 3.7). The detailed demographic description of the cohort is displayed in Tables 1 & 2. Ethylenediaminetetraacetic acid (EDTA) plasma samples were collected by Bioreclamation, Inc. (NY, USA) after informed

consent was obtained from the participants. All Bioreclamation, Inc. human material is collected from healthy donors who have been stringently screened at US FDA licensed donor centers. All subjects underwent a complete physical exam that included hematocrit, total protein, alanine aminotransferase (ALT) levels and urinalysis. Exclusion criteria included abnormal results in any of those tests. Subjects with a positive result for HIV1, HIV2, hepatitis B surface antigen, hepatitis C virus (HCV) or HIV-1 Ag were also excluded. Qualified donors were selected after receiving a complete physical exam and passing all required viral marker tests by an FDA-approved methodology. Following collection, all plasma samples were tested again and found negative for hepatitis B surface antigen, HIV-1 and -2, HCV, and HIV-1Ag by approved FDA methods. Diet, medication and lifestyle (exercise, tobacco and alcohol intake) were not assessed in this analysis.

Sample preparation

Plasma samples (100 μ l) were extracted using an automated MicroLab STAR[®] system (Hamilton Company, UT, USA). The samples were extracted using a series of four solvent extraction steps: 400 μ l tridecanoic acid (2.5 mg/ml) in ethyl acetate:ethyl alcohol (1:1); 200 μ l methanol; 200 μ l methanol:H₂O (3:1); and 200 μ l dichloromethane:methanol (1:1). Each solvent extraction step was performed by shaking for 2 min in the presence of glass beads using a Geno/Grinder 2000 (Glen Mills, Inc. NJ, USA). After each extraction the sample was centrifuged and the supernatant removed using the MicroLab STAR[®] robotics system, followed by re-extraction of the pellet. The multiple extract supernatants were pooled and then split into two equal aliquots, one for LC/MS and one for GC/MS. Aliquots were placed on a TurboVap[®] (Zymark, Runcorn, UK) to remove the

Table 1. Cohort description.

Age (years)	20–35			36–50			51–65			All		
	M	F	Total	M	F	Total	M	F	Total	M	F	Total
Race												
Caucasian	10	10	20	4	16	20	6	15	21	20	41	61
African-American	29	24	53	19	27	46	21	15	36	69	66	135
Hispanic	9	8	17	13	12	25	20	11	31	42	31	73
All races	48	42	90	36	55	91	47	41	88	131	138	269

F: Female; M: Male.

Table 2. Cohort body mass index.

BMI	Underweight	Normal weight	Overweight	Obese
Summary by age (years)				
20–35	3 (3)	29 (32)	27(30)	31 (34.4)
36–50	0	22 (24)	38 (42)	31 (34)
51–65	0	13 (15)	48 (55)	27 (20)
Summary by sex				
Male	2 (1.5)	44 (36)	53 (40)	32 (34)
Female	1 (0.7)	20 (14.5)	60 (44)	57 (43)
Summary by race				
African–American	1	38 (28)	55 (41)	41 (31)
Caucasian	1	11 (18)	24 (39)	25 (41)
Hispanic	1	15 (21)	34 (47)	23 (30)

BMI: Underweight ≤ 18.5 ; Normal weight = 19–25; Overweight = 25–29.9; Obese ≥ 30 ; (%).

BMI: Body mass index = weight (kg)/Height (m^2).

solvent, frozen and dried under vacuum overnight. Samples were maintained at 4°C throughout the extraction process. For LC/MS analysis extract aliquots were reconstituted in 10% methanol and 0.1% formic acid. For GC/MS analysis, aliquots were derivatized using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for 1 h.

LC/MS & GC/MS analysis

LC/MS was carried out using a Surveyor High performance liquid chromatography (HPLC) (ThermoElectron Corporation, CA, USA) with an electrospray ionization [11] source coupled to an LTQ MS (ThermoElectron Corporation). The mobile phase consisted of 0.1% formic acid in H₂O (solvent A) and 0.1% formic acid in methanol (solvent B). The extract was loaded onto a 100 × 2.1 mm, 3 μm particle, Aquasil column (ThermoElectron Corporation) via an CTC autosampler (LeapTechnologies, NC, USA) and gradient eluted (0% B, 4 min; 0–50% B, 2 min; 50–80% B, 5 min; 80–100% B, 1 min; maintain 100% B, 2 min) directly into the mass spectrometer at a flow rate of 200 μl/min. The LTQ took full scan mass spectra (99–1500 m/z) while alternating polarity to monitor both negative and positive ions.

The derivatized samples for GC/MS were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS (ThermoElectron Corporation) operated at unit mass resolving power. The GC column was 20 m × 0.18 mm, initial oven temperature was 60° C ramped to 340°C, and helium was the carrier gas. GC/MS

was operated using electron impact ionization with a 50–750 atomic mass unit (amu) scan range and was tuned and calibrated daily for mass resolution and mass accuracy.

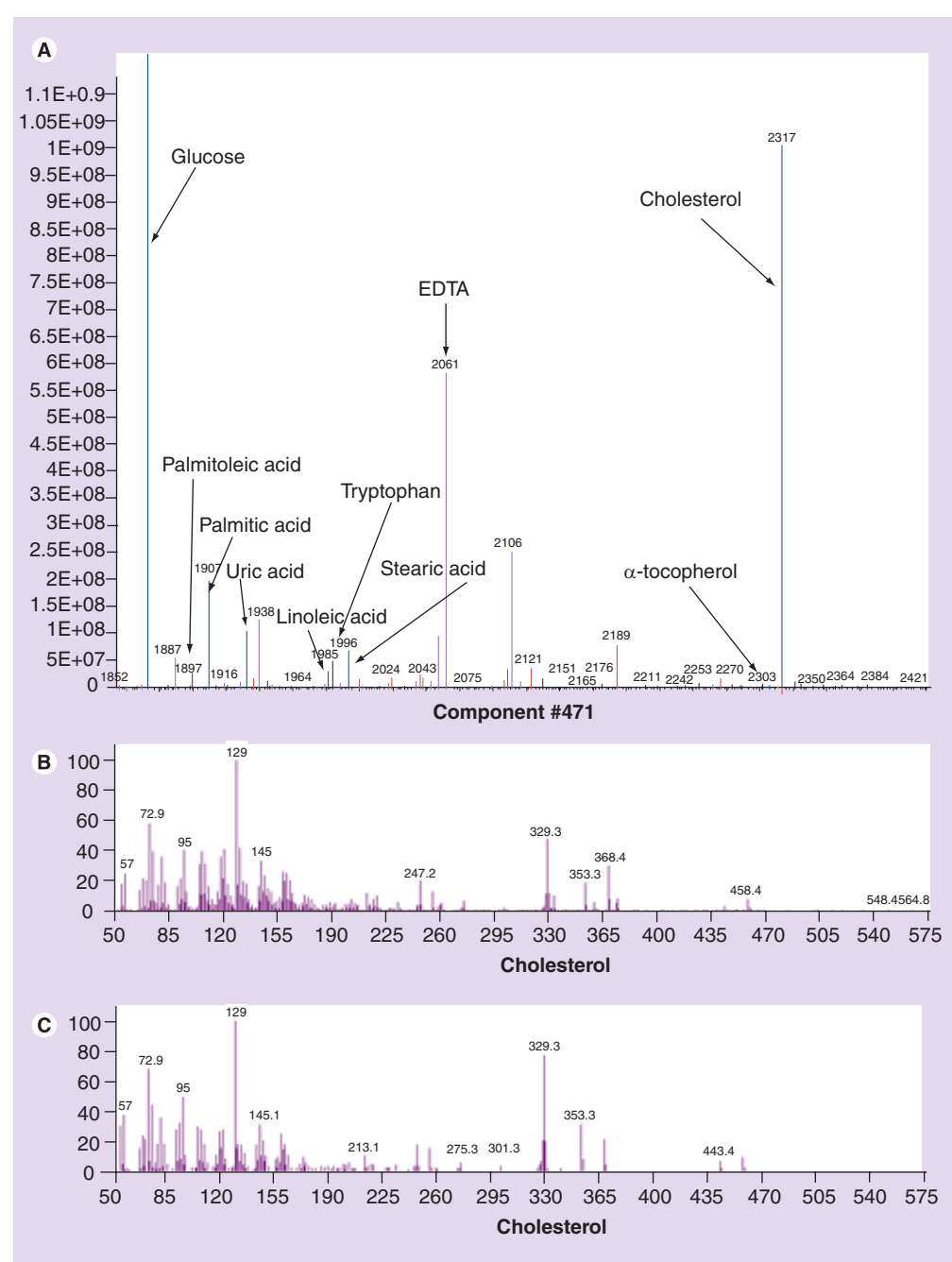
Compound identification

Compounds were identified by automated comparison to Metabolon's reference library entries using Metabolon's proprietary software developed for creating library entries from known chemical entities and then automatically fitting those spectra to experimentally derived spectra (see Figure 1). Peaks that elute from either the LC or GC method are compared with the library at a particular retention time and its associated spectra for that compound. Internal standards are primarily used in both the GC and LC methods to calibrate retention times of compounds across all of the samples in the study and for quality control of each instrument run. Identification of known chemical entities was based on comparison with Metabolon's library entries of purified external standards. Each entry that is automatically identified by the software is visually inspected with VPhil™ [103] to confirm the acceptance of that compound in the study. Peptides were identified using standard tandem mass spectrometry sequencing technique [12].

Data normalization

Raw area counts for each compound in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences. Raw area counts for a compound were divided by the median value, setting the medians equal for each day's run. Missing values were assumed to result from areas being below the

Figure 1. Compound identification.



The Metabolon library matching software compares the retention time and mass spectral characteristics of components detected in test samples with the reference library entries. **(A)** Total ion chromatogram from a representative plasma sample. Retention time is plotted on the x-axis and signal intensity is plotted on the y-axis. **(B)** Mass spectrum of component 471 from **(A)**. **(C)** Mass spectrum of cholesterol from a reference standard. For **(B)** and **(C)** the mass divided by the number of elementary charges (m/z) is plotted on the x-axis and the normalized signal is plotted on the y-axis. Signals were normalized to 100% relative to the base peak.

limits of detection. Missing values for a given compound were imputed with the observed minimum after the normalization step. Quantitative values were derived from integrated raw

detector counts of the mass spectrometers. Importantly, while peak area comparisons between samples represent relative amounts of each ion detected, different compounds and ions

have different ionization potentials. To preserve all of the variation, yet allow compounds of widely different raw peak areas to be compared directly on a similar graphical scale, the normalized intensities were scaled by their median values for each compound.

Statistical analyses

Metabolomic analysis was conducted on six populations representing three age groups and both sexes. To achieve less than a 10% Type II error with a Type I error of 0.05 (90% power), the sample size for each population was estimated to be 45 individuals, based on a power curve constructed using the total noise in the analytical systems at the time the work was initiated. In advance of analyzing the results the subjects were divided into three age groups (20–35, 36–50 and 51–65 years) with approximately equal numbers of subjects in each of the three categories.

Statistical analysis of the data was performed using JMP (SAS, [201]), a commercial software package, and 'R' [202], which is a freely available open-source, software package. For each compound an ANOVA was performed on the full factorial design with sex, race and age as factors. A log transform was applied to the observed relative concentrations for each compound because, in general, the variance increased as a function of a compound's average response. Two analyses were performed (sex and race as factors): one with age as a categorical variable (with the three groups as described earlier), and one analysis with age as a continuous variable and no interaction terms (analysis of covariance [ANCOVA]).

Results

The various metabolites examined for comparative analysis in the cohort were selected by matching their analytical signatures against that of Metabolon's in-house database of thousands of compounds. Our library matching software compares the retention time and mass spectral characteristics of components detected in test samples with the reference library entries (Figure 1). Over 300 compounds were detected in the plasma samples and visually confirmed with our software curation program VPhil™ [103]. Approximately 60% of the compounds were identified using LC/MS and 40% by GC/MS. For compounds that were detected by both platforms (approximately 10–15%), the data from the platform giving the better spectral match to authentic compound standards were selected for analysis.

We were able to identify endogenous compounds, for example amino acids, carbohydrates, fatty acids, dietary compounds and xenobiotics, vitamins and a variety of nutritional and other supplements. We focused our analysis on those uniquely identified compounds that match our library of authentic chemical standards; compounds detected but without an associated chemical standard were not further analyzed. Of these standard compounds over 100 were significantly altered with age, sex or race.

In order to be included in the statistical analysis, a compound must have a response for at least two thirds of the samples overall. In cases where a response was not detected, it was assumed that the value was missing because the compound was below the limit of detection. In those cases with a missing value, a value was imputed with the minimum response for that compound. False discovery rates (FDRs) were computed to adjust for multiple comparisons [13]. The FDRs were computed using the q-value method [14]. Compounds with $p < 0.05$, $q < 0.15$ were deemed significant.

Metabolites where the levels showed statistically significant differences according to age are presented in Table 3. To identify age-specific biochemical changes, the influences of sex and race were also evaluated. The number of age-associated metabolite changes were the most significant, and some of these age changes were affected by sex but none by race. In addition, increases and decreases in metabolites that were associated specifically with sex or race but not with age were observed and are described below.

Changes associated with age

To assess changes in metabolite levels as a function of age, the subjects were divided into three age groups: ages 20–35, 36–50 and 51–65 years. Metabolites that show statistically significant increases or decreases, ($p < 0.05$, $q < 0.15$) both in a significance test (p-value) and in a measure of false discovery rate (q-value), in relative levels according to either the model using age as a categorical variable or the model using age as a continuous variable are presented in Table 3. Based on their biochemical relevance, eight compounds (denoted by #) were included that met a more relaxed criteria of statistical significance that allowed slightly higher p-values but met the q-value criterion in at least one of the statistical models. The plasma levels of most of the compounds were higher in the oldest age group compared with the youngest.

Table 3. Age-associated changes in the plasma metabolome.

Metabolic pathway	Age group			Categorical model				Linear model			
	Mean 20–35	Mean 36–50	Mean 51–65	p-value	q-value	Fold-change 51–65/20–35	p-value	q-value	Correlation	Slope	
	Amino acids metabolism										
Leucine	0.71	1.07	1.40	<0.0001	<0.0001	1.99*	<0.0001	<0.0001	0.40	0.0224	
Isoleucine	0.72	1.03	1.35	<0.0001	<0.0001	1.87*	<0.0001	<0.0001	0.37	0.0209	
Valine [†]	0.70	1.01	1.33	<0.0001	<0.0001	1.89*	<0.0001	<0.0001	0.44	0.0208	
Histidine	0.77	1.09	1.18	<0.0001	<0.0001	1.53*	<0.0001	<0.0001	0.28	0.0125	
Lysine	0.64	1.07	1.53	<0.0001	<0.0001	2.41 [§]	<0.0001	<0.0001	0.38	0.0271	
Phenylalanine	0.96	1.02	1.05	0.0323	0.0218	1.09	0.0105	0.0096	0.21	0.003	
Tryptophan [¶]	1.00	0.95	1.03	0.0506	0.0272	1.02	0.4101	0.1613	0.06	-	
Threonine	0.82	1.04	1.28	<0.0001	<0.0001	1.56*	<0.0001	<0.0001	0.32	0.014	
Serine	0.75	1.04	1.30	<0.0001	<0.0001	1.73*	<0.0001	<0.0001	0.37	0.0164	
Alanine	0.72	1.03	1.42	<0.0001	<0.0001	1.97*	<0.0001	<0.0001	0.43	0.0194	
Glutamine [¶]	0.75	0.92	1.49	<0.0001	<0.0001	1.99*	<0.0001	<0.0001	0.42	0.0206	
Glycine	0.67	1.02	1.47	<0.0001	<0.0001	2.18 [§]	<0.0001	<0.0001	0.38	0.0228	
Glutamate	0.53	1.08	1.47	<0.0001	<0.0001	2.75 [§]	<0.0001	<0.0001	0.39	0.0315	
Tyrosine	0.97	1.00	1.06	0.1122	0.0499	1.09	0.0195	0.0115	0.15	0.0029	
Oxoprolone	0.87	1.05	1.13	<0.0001	<0.0001	1.30	0.0001	0.0001	0.22	0.0065	
Ornithine	0.56	1.10	1.73	<0.0001	<0.0001	3.07 [§]	<0.0001	<0.0001	0.45	0.0339	
Urea ^{††}	0.88	1.04	1.19	0.0004	0.0004	1.35	<0.0001	<0.0001	0.27	0.0114	
Hippurate	0.81	1.14	1.23	0.0022	0.0019	1.52*	0.0001	0.0001	0.24	0.0145	
p-cresolsulfate	0.58	0.81	1.05	0.0043	0.0035	1.79*	0.0016	0.0013	0.28	0.0164	
Creatine [†]	0.87	1.02	1.11	0.0178	0.0116	1.27	0.0014	0.0012	0.18	0.0082	
Thyroxine [¶]	1.07	0.88	0.96	0.0022	0.0019	0.90	0.0956	0.0473	-0.10	-0.0028	
p-hydroxyphenyllactate	0.77	0.93	0.92	0.0327	0.0191	1.19	0.0008	0.0007	0.20	0.0078	

*Increased 50–99%.

†Indicates compounds in which a higher BMI increased the parameter.

§Increased at least 100%.

¶Indicates quadratic coefficient, $p < 0.05$, $q < 0.1$.

#Indicates statistical trend, $q < 0.15$.

††Indicates significant age * sex interaction.

‡Decreased at least 50%.

Table 3. Age-associated changes in the plasma metabolome (cont.).

Metabolic pathway	Name	Age group			Categorical model			Linear model			
		Mean	Mean	Mean	p-value	q-value	Fold-Change	p-value	q-value	Correlation	Slope
		20–35	36–50	51–65			51–65/20–35				
Amino acids metabolism (cont.)											
	Phenyllactate	0.87	1.05	1.11	0.0331	0.0192	1.27	0.0035	0.0026	0.19	0.0084
	Indolelactate [#]	0.95	0.91	1.04	0.2003	0.0807	1.09	0.1494	0.0689	0.11	0.0031
	L-Kynurenine ^{†,†,†,†,†} **	0.98	0.95	1.16	<0.0001	<0.0001	1.19	<0.0001	0.0001	0.26	0.0056
	Histamine	0.95	0.99	1.14	0.0006	0.0006	1.21	0.0058	0.0041	0.18	0.0042
	3-methylhistidine	0.53	0.72	0.82	0.0457	0.0250	1.53*	0.0052	0.0037	0.07	0.0144
	2-hydroxybutyrate	0.90	1.07	1.08	0.0752	0.0371	1.19	0.0011	0.0009	0.18	0.0085
	Hydroxypruvic acid	1.08	0.99	0.91	<0.0001	<0.0001	0.84	<0.0001	0.0002	-0.24	-0.005
	N-acetylvaline [#]	0.83	0.87	0.92	0.1459	0.0633	1.11	0.0984	0.0485	0.08	0.0027
Energy											
	cis-Aconitate	0.99	1.06	1.24	0.0281	0.0170	1.25	0.0031	0.0024	-0.07	0.0075
	Isocitrate	0.76	1.02	1.26	<0.0001	<0.0001	1.65*	<0.0001	<0.0001	0.31	0.0165
	α-ketoglutarate ^{†,†,†} **	0.64	1.07	1.00	0.0007	0.0007	1.55*	0.0041	0.0030	0.19	0.0120
	Succinate [†]	0.76	1.08	1.09	<0.0001	<0.0001	1.43	<0.0001	<0.0001	0.25	0.0114
	Malate	0.79	1.11	1.31	<0.0001	<0.0001	1.66*	<0.0001	<0.0001	0.28	0.0174
	Orthophosphate (P _i)	0.90	0.97	1.06	<0.0001	<0.0001	1.18	<0.0001	<0.0001	0.29	0.0052
Carbohydrate metabolism											
	Glucose	0.96	0.82	0.86	0.1234	0.0621	0.90	0.1618	0.0949	-0.13	-0.0046
	Lactate	0.73	1.05	1.23	<0.0001	<0.0001	1.68*	<0.0001	<0.0001	0.38	0.0163
	Myo-inositol ^{†,†,†} **	0.83	1.09	1.18	<0.0001	<0.0001	1.42	<0.0001	<0.0001	0.44	0.0115
	Mannose ^{†,†}	1.02	1.06	0.91	0.104	0.0535	0.89	0.1087	0.0684	-0.03	-

[†]Increased 50–99%.

^{††}Indicates compounds in which a higher BMI increased the parameter.

[‡]Increased at least 100%.

^{†††}Indicates quadratic coefficient, $p < 0.05$, $q < 0.1$.

^{††††}Indicates statistical trend, $q < 0.15$.

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Metabolic pathway	Age group			Categorical model				Linear model		
	Mean 20-35	Mean 36-50	Mean 51-65	p-value	q-value	Fold-Change 51-65/20-35	p-value	q-value	Correlation	Slope
Lipid metabolism										
Glycerol-3-phosphate ^{†, **}	0.96	1.12	0.93	0.0664	0.0339	0.97	0.4171	0.1634	-0.05	-
Stearate	0.93	1.03	1.08	0.0004	0.0004	1.16	<0.0001	0.0001	0.22	0.0046
Palmitoleate [#]	0.88	1.01	0.95	0.6301	0.2054	1.08	0.1038	0.0507	0.10	0.0064
Oleate	0.89	1.05	1.03	0.1517	0.0649	1.16	0.0126	0.0080	0.13	0.0067
Nonanedioate [#]	0.97	1.04	1.01	0.4242	0.1499	1.03	0.0900	0.0447	0.09	0.0023
Arachidonic acid	0.92	0.97	1.02	0.1900	0.0774	1.11	0.0437	0.0235	0.13	0.0035
Linoleic acid	0.93	1.06	1.07	0.0415	0.0262	1.15	0.0222	0.0177	0.13	0.004
Choline ^{**}	0.99	1.02	1.11	0.0381	0.0215	1.12	0.0013	0.0011	0.09	0.0043
L-α-glycerophosphoryl choline	0.70	0.93	0.97	0.0191	0.014	1.39	0.0087	0.0082	0.11	0.010
β-hydroxybutyrate	1.02	1.38	1.29	0.1793	0.0739	1.27	0.0096	0.0062	0.02	0.0130
Cholesterol	0.92	0.95	1.12	0.0690	0.0347	1.22	0.0191	0.0113	0.13	0.0064
Dehydroepiandrosterone -sulfate	1.38	0.97	0.59	<0.0001	<0.0001	0.42 ^{††}	<0.0001	<0.0001	-0.39	-0.0235
Carnitine	0.92	0.96	1.03	0.0620	0.0321	1.12	0.0280	0.0157	0.17	0.0032
Nucleotide metabolism										
Urate [#]	0.97	1.00	1.02	0.1062	0.0478	1.04	0.0153	0.0094	0.14	0.0014
Allantoin	0.64	0.93	1.15	<0.0001	<0.0001	1.80 [*]	<0.0001	<0.0001	0.31	0.0175
Xanthine [†]	0.58	0.76	1.27	<0.0001	<0.0001	2.18 [§]	<0.0001	<0.0001	0.31	0.0202
Hypoxanthine	0.41	0.84	1.36	<0.0001	<0.0001	3.31 [§]	<0.0001	<0.0001	0.05	0.0349
Inosine	0.62	0.92	1.05	0.0047	0.0044	1.69 [*]	0.0016	0.0019	0.01	0.017
Uridine	0.89	0.98	1.18	<0.0001	<0.0001	1.33	<0.0001	<0.0001	0.29	0.0082

[†]Increased 50-99%.

^{††}Indicates compounds in which a higher BMI increased the parameter.

[§]Increased at least 100%.

^{*}Indicates quadratic coefficient, $p < 0.05$, $q < 0.1$.

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		Mean	Mean	Mean	p-value	q-value	Fold-Change	p-value	q-value	Correlation	Slope
		20–35	36–50	51–65			51–65/20–35				
Peptide	γ-glutamyltyrosine	0.90	0.83	0.77	0.2086	0.0827	0.86	0.0180	0.0108	-0.14	-0.0063
	γ-glutamylleucine	1.02	0.96	0.85	0.0055	0.0042	0.83	0.0016	0.0013	-0.23	-0.0055
	SHAXQXNNR	0.48	0.80	0.59	0.0310	0.0184	1.24	0.0368	0.0200	0.03	0.0117
	HXGXA	0.30	0.75	0.64	0.0089	0.0064	2.17 [§]	0.0036	0.0027	0.01	0.0278
	HWESASXXR**	0.38	0.91	0.83	0.0013	0.0012	2.18 [§]	0.0006	0.0005	0.18	0.0266
Cofactors and vitamins	α-tocopherol**	0.91	0.95	1.11	0.0289	0.0174	1.21	0.0119	0.0077	0.21	0.0057
	Pantothenate	0.84	1.05	1.25	<0.0001	<0.0001	1.49	<0.0001	<0.0001	0.24	0.0123
	Nicotinamide	0.52	0.81	1.05	<0.0001	<0.0001	2.03 [§]	<0.0001	<0.0001	0.29	0.0194
Xenobiotics	Theobromine	0.69	0.73	1.00	0.0665	0.0339	1.46	0.0545	0.0286	0.07	0.0098
	Paraxanthine†	0.32	0.69	0.81	<0.0001	0.0001	2.54 [§]	<0.0001	<0.0001	0.12	0.0282
	Caffeine	0.28	0.70	1.16	<0.0001	<0.0001	4.17 [§]	<0.0001	<0.0001	0.26	0.0405
	Cotinine [¶]	0.34	0.58	0.33	0.0236	0.0145	0.96	0.7729	0.2590	0.08	-
	Erythritol [¶]	0.93	0.98	1.24	<0.0001	<0.0001	1.33	<0.0001	<0.0001	0.37	0.0083
	Iminodiacetate	0.22	0.64	4.39	<0.0001	<0.0001	20.31 [§]	<0.0001	<0.0001	0.33	0.0940

^{*}Increased 50–99%.

[†]Indicates compounds in which a higher BMI increased the parameter.

[‡]Increased at least 100%.

[¶]Indicates quadratic coefficient, $p < 0.05$, $q < 0.1$.

[#]Indicates statistical trend, $q < 0.15$.

^{**}Indicates significant age*sex interaction.

^{‡‡}Decreased at least 50%.

The plasma levels of both essential and non-essential amino acids as well as several polyamines were higher in the 51–65 age group (Table 3). The change in the levels of serine, alanine, glutamine, glycine, glutamate, histidine, isoleucine, valine, leucine and lysine was most pronounced in the oldest age group. In addition, the relative concentrations of hippurate, p-cresolsulfate, 3-methylhistine and ornithine were also higher in the 51–65 years age group.

Significant changes in carbohydrate, lipid and energy metabolism were observed with increasing age (Table 3). Elevated levels of myo-inositol and lactate and lower levels of mannose were measured in the oldest age group. The relative concentrations of a number of fatty acids, β -hydroxybutyrate, and cholesterol, showed significant increases with age. As shown previously [15], dehydroepiandrosterone-sulfate (DHEA-S), was dramatically lower (>50% decrease) in older individuals (Table 3, Figure 2). Citric acid cycle intermediates also increased in older age groups with the largest increases observed in the levels of isocitrate, α -ketoglutarate, and malate. Smaller, but significant, increases in *cis*-aconitate and succinate were also observed.

Concentrations of several compounds associated with nucleotide metabolism increased as individuals aged (Table 3). High levels of allantoin, xanthine and hypoxanthine were measured in older relative to younger individuals. Inosine, urate and uridine were also higher in older subjects.

Significant differences in several cofactors and vitamins were determined (Table 3). α -tocopherol, pantothenate and nicotinamide were higher in older subjects. The amount of nicotinamide measured in the oldest age group was twice that of the youngest age group.

Several peptides (γ -glutamyltyrosine, γ -Glutamylleucine, SHAXQXNNR, HXGXA, HWESASXXR; X being either isoleucine or leucine, which have the same mass and cannot be distinguished with the present method), also increased with age (Table 3). The most pronounced increases were observed with HXGXA and HWESASXXR, which were over twofold higher in the oldest as compared with the youngest age group.

Interestingly, the levels of a number of xenobiotics varied significantly according to age. Cotinine, an indicator of exposure to cigarette smoke, was highest in the 36–50 years age group. A large increase in iminodiacetate, a xenobiotic intermediate common to both aerobic and anaerobic metabolism of nitrilotriacetate (NTA), was measured in the oldest group. Caffeine and

paraxanthine, the primary metabolite of caffeine, were highest in the oldest age group compared with the younger age groups. However, the ratio of paraxanthine to caffeine (P/C) was lower in the oldest age group (0.69 vs 1.14 in youngest group).

Influence of sex & race on age-associated changes in metabolites

The data was further analyzed to examine the influence of sex and race on age-associated changes in small molecules. A significant interaction ($p < 0.05$, $q < 0.15$) was observed between age and sex for several named compounds (Table 3). Urea was the most significant compound ($p = 0.0001$, $q = 0.0177$); the increases in the level of urea with age were more pronounced in females than in males. The other age-associated compounds where significant sex interactions were observed include: α -tocopherol, L-kynurenine, and glycerol-3-phosphate. The increase in α -tocopherol was more pronounced in females while the changes in L-kynurenine (increase with increased age) and glycerol-3-phosphate (decrease with increased age) were more pronounced in males. There were no significant interactions for named compounds between age and race, and there were no significant three-way interactions observed for age, race and sex.

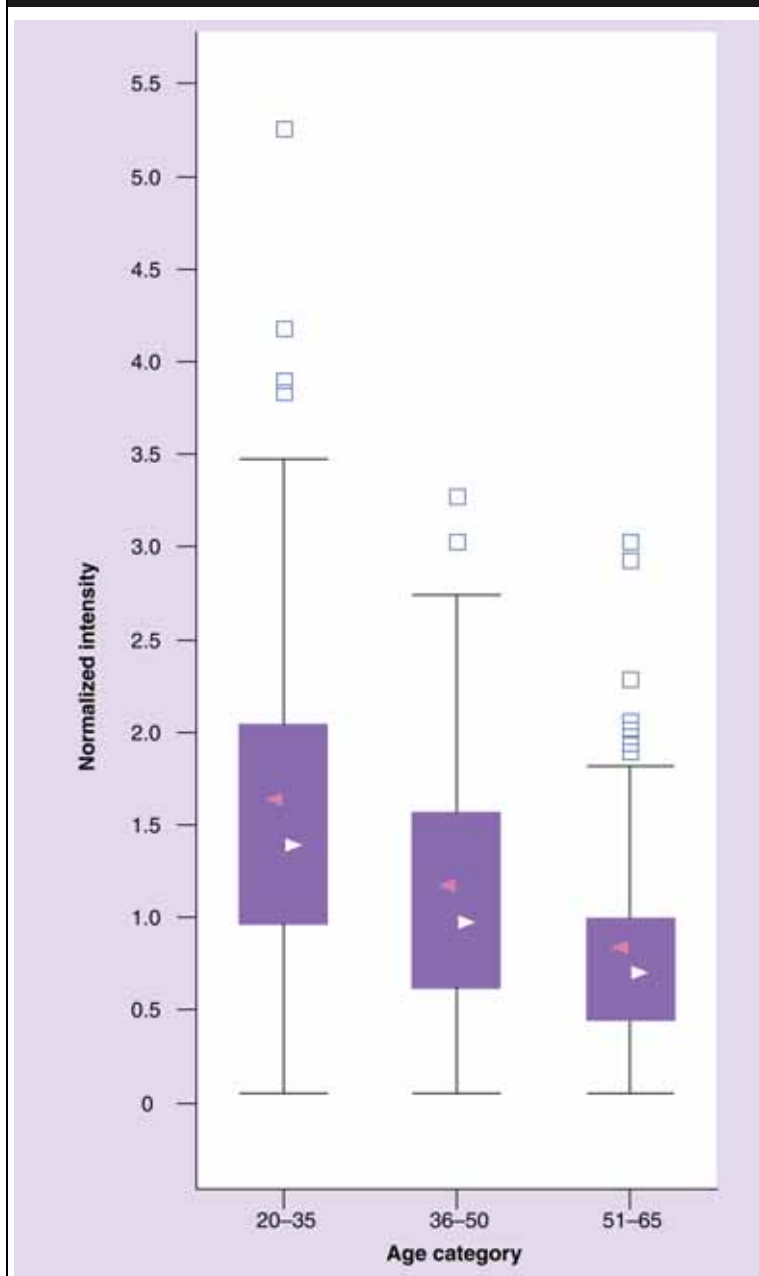
Effect of sex

Fewer changes specifically associated with sex were observed in plasma levels of metabolites. These changes were not influenced by age or race. A total of 27 metabolites were higher in males than in females, while eight were elevated in females compared with males (Table 4). Orthophosphate, α -tocopherol, creatine and biochemicals associated with lipid metabolism (nonanedioate, myristate, palmitoleate, glycerol, β -hydroxybutyrate) were higher in females. Consistent with previous findings creatinine was higher in males than in females [16], as was DHEA-S [15]. Generally, amino acid metabolism, energy metabolism and nucleotide metabolism were elevated in males relative to females. No significant differences in the levels of xenobiotics were observed.

Effect of race

Few statistically significant increases or decreases in metabolite levels were observed among African-Americans, Hispanics and Caucasians. A total of six compounds had differences of greater than 40% in relative concentration

Figure 2. Dehydroepiandrosterone-sulfate levels decrease with increasing age.



Age-associated changes in DHEA-S levels. Whisker plot showing relationship of DHEA-S levels to age. The top and bottom of the dark box represent the 75th and 25th percentile; 50% of the data points lie within the boxed region. The bars ('whiskers') represent the entire spread of the data points for each compound and group, excluding outliers, which are indicated with white squares. The mean value is represented by the gray arrow and the median value is represented by the white arrow. Intensity represents the normalized peak area from LC/MS analysis. As shown DHEA-S levels decline with age. DHEA-S: Dehydroepiandrosterone-sulfate.

between African-Americans and Caucasians. Three of these significant differences are dietary metabolites (caffeine and its metabolites,

paraxanthine and theobromine), which were much lower in African-Americans. The other three compounds (hydroxyproline, glycochenodeoxycholate/glycodeoxycholate and glycolate) are endogenously synthesized and were much higher in African-Americans. No statistically significant differences in the relative concentrations of identified metabolites were observed between Hispanics and Caucasians or between Hispanics and African-Americans, although differences were detected in observed compounds that did not have an associated chemical standard.

Influence of body mass index on metabolite levels

When BMI was treated as a continuous variable, there was a significant association between the relative concentrations of a number of metabolites and an increase in BMI (Tables 3 & 4). In some cases, an increase in BMI had a significant affect on metabolites across two or more of the main categories of age, sex and race. Higher BMI was associated with increases in creatine, L-kynurenine and urea in all three categories. Higher amounts of α -ketoglutarate, succinate and xanthine were measured with higher BMI in the age and sex categories only. The xenobiotic paraxanthine increased in age and race with increasing BMI. Other effects of BMI on metabolites were observed in only one category. In the oldest group, a higher BMI was associated with higher relative concentrations of valine, mannose and choline.

Discussion

By using sophisticated analytical methodology and data curation techniques, we have identified significant increases or decreases in a number of metabolites in human plasma that occur with age, sex or race. The most striking of these differences were associated with age. Although these changes are of considerable interest, and, in many cases, are consistent with theories of aging and published literature (e.g., oxidative stress markers increased with age, consistent with the free-radical theory of aging), the biological significance of several of these observations is difficult to discern and remains speculative.

It should be underscored that changes in plasma levels of any metabolite reflect alterations in both anabolic and catabolic processes in the individual. For example, an increase in the concentration of essential amino acids and

Table 4. Sex-associated changes in plasma metabolites.

Pathway	Name	Mean female	Mean male	p-value	q-value
Amino acid metabolism	Methionine	0.94	1.07	0.0001	0.0010
	Tryptophan	0.94	1.05	<0.0001	0.0005
	Phenylalanine*	0.97	1.04	0.0128	0.0390
	Glutamate	0.85	1.06	0.0212	0.0553
	Oxoproline	0.96	1.06	0.0271	0.0677
	Creatinine*	0.86	1.10	<0.0001	<0.0001
	Creatine	1.10	0.90	0.0047	0.0180
	p-Hydroxyphenyllactate	0.80	0.94	0.0175	0.0484
	4-Methyl-2-oxopentanoate	0.87	1.15	<0.0001	<0.0001
	Indolelactate	0.90	1.04	0.0111	0.0366
	Indoxylsulfate [‡]	0.90	1.06	0.0717	0.1253
	Phenyllactate [‡]	0.94	1.07	0.0908	0.1485
	Hippurate [‡]	0.95	1.14	0.0734	0.1270
	Kynurenine*. [§]	0.99	1.06	0.0641	0.1153
Energy metabolism	Citrate	0.98	1.18	0.0106	0.0360
	<i>cis</i> -Aconitate	0.98	1.22	0.0029	0.0137
	Isocitrate	0.90	1.10	0.0137	0.0400
	α -ketoglutarate*	0.76	1.02	0.0113	0.0366
	Malate	0.95	1.16	0.0020	0.0108
	Orthophosphate	1.02	0.93	0.0007	0.0057
Lipid metabolism	Nonanedioate	1.07	0.95	0.0026	0.0130
	Myristate	1.10	0.99	0.0537	0.1084
	Palmitoleate	1.13	0.79	0.0015	0.0102
	Glycerol*	1.13	0.96	0.0016	0.0102
	Glycocholate	0.71	0.98	0.0304	0.0749
	Dehydroepiandrosterone-sulfate	0.73	1.17	<0.0001	0.0002
	Carnitine	0.93	1.01	0.0340	0.0774
	β -hydroxybutyrate [‡]	1.40	1.06	0.0542	0.1084
Nucleotide metabolism	Hypoxanthine	0.62	0.97	0.0134	0.0400
	Xanthine*	0.71	0.95	0.0035	0.0154
	Uridine	0.94	1.08	0.0044	0.0172
	Urate*	0.95	1.04	<0.0001	<0.0001
Cofactors & vitamins	Biliverdin*. [§]	0.91	1.21	0.0005	0.0043
	α -Tocopherol [‡] . [§]	1.04	0.94	0.0930	0.1494

*Affected by Body mass index.

[‡]Indicates statistical trend, $q < 0.15$.[§]Indicates significant age*sex interaction.

3-methylhistidine may occur in response to a high protein diet or may be owing to an acute increase in protein breakdown. By contrast, the steady state levels of these metabolites (i.e., sustained change in concentration) may be related to adaptive responses in a number of other functions, such as a change in expression and function of a specific transport protein or a

decrease in the oxidative capacity of catabolic reactions, such as the citric acid cycle. Therefore, the data obtained in steady state conditions should be interpreted with some caution, since they represent the integrated response of a number of complex functions. In addition, while all subjects met healthy inclusion criterion for the study, diet and lifestyle factors (e.g., tobacco

and alcohol consumption, exercise) were not controlled in this study. Taking this into account, we discuss our observations in a conservative manner and with the intention to provide interpretation of the statistically significant biochemical alterations.

There was a significant, age-associated increase in both essential and nonessential amino acid concentration in the plasma. Further analyses show a small, but significant, effect of sex on the levels of some amino acids. Previous data from humans have shown both a decrease [17–19], and an increase [20] in the levels of amino acids in the plasma of the elderly, as compared with younger age population. The observed differences in amino acid levels among the various studies could be the consequence of the study design, the size and composition of the study cohort (e.g., age range, ethnicity, lifestyle factors, BMI) or confounding variables, such as the presence of various disorders associated with aging, such as dementia or arthritis, or owing to diversity in medication and nutrient intake.

An increased concentration of essential amino acids in the plasma could result from an increase in the rate of whole-body protein breakdown. However, this is considered unlikely since the data from several studies show that the rate of protein breakdown in the skeletal muscle is either unchanged or slightly decreased in the elderly [21].

Another possible contributor to higher plasma amino acids could be resistance to insulin action in the older age groups. Insulin resistance, related to a decreased amino acid transport across the cell membrane, could contribute to the observed increase in the concentration of amino acids in the plasma. This suggestion is supported by the increased prevalence of overweight and obese individuals in the older age groups and by the observed association between BMI and amino acids in the present study. However, glucose levels were unchanged across the age groups in the present cohort.

It is also possible that the elevation of amino acids in the plasma with increased age is associated with a decreased rate of transamination and subsequent oxidation of their carbon skeletons in the citric acid cycle. In this regard, we noted an especially prominent increase in the levels of aconitate, isocitrate and malate in the blood during aging. These compounds are intermediates of reactions of the citric acid cycle. Since the carbon skeletons of amino acids generally end up in the citric acid cycle, there could be a metabolic link between the levels of these intermediates

and the levels of amino acids observed in this study. This idea is further supported by the observed increases in alanine, serine, glutamate and glutamine and the increased level of urea in the plasma in older subjects. The catabolism of these amino acids would be expected to increase the levels of citric acid cycle anions, which, if not oxidized, could result in the accumulation of these intermediates in the blood.

The rise in urea and the urea cycle intermediate ornithine (3.1-fold) in the blood could reflect either an increase in hepatic ureagenesis or a decrease in the rate of clearance of the urea by the kidney [22]. The source of the citric acid cycle intermediates is not indicated by our measurements but we assume that it reflects the breakdown of mitochondria in the aging muscle and liver, resulting in the release of citric acid cycle anions. These observations are consistent with the reported changes in mitochondrial function with aging [23,24]. An analysis of gene expression profiles in the skeletal muscle of 30-month-old mice, as compared with 5-month-old animals, indicates that genes that control mitochondrial biogenesis or that are involved in energy generating processes such as glycolysis, the citric acid cycle, cholesterol synthesis and glycogen metabolism are markedly downregulated in older mice [24].

With age there was also a significant increase in the concentration of degradation products of purine metabolism. These include allantoin (1.8-fold), xanthine (2.2-fold), hypoxanthine (3.3-fold) and inosine (1.3-fold); the pyrimidine, uridine was also increased (1.3-fold). This may reflect an increased cellular nucleic acid turnover in older humans owing to inflammation caused by diseases of aging, such as arthritis, or by cellular necrosis. Surprisingly, the older population did not demonstrate an elevation in the levels of uric acid in the blood but did show increases in hypoxanthine and xanthine, the immediate precursors of uric acid, and allantoin, the immediate breakdown product of urate.

The change in plasma concentrations of peptides could be the result of subclinical inflammation in the older individuals. The identified peptides, HWESASXXR and SHAXQXNNR, have identity (9/9) to fragments of complement C₃ and C₄, respectively. Complement C₃ and C₄, the most and second most abundant complement proteins in human serum, are an affectation of both the innate and the acquired immune systems [25]. Increased levels of both C₃ and C₄ are linked to acute inflammatory disease and tissue inflammation [25].

We did not observe many significant differences in metabolite levels related to race. One reason for this may be because information on race was self-volunteered and did not account for family history. The results should be viewed as a preliminary metabolomic comparison of race-related differences.

Future perspective

The current study underscores the power of metabolomics to present a broad picture of the biochemical profile of a population and changes in that profile with age, sex and race. The major strength of the approach used here is the large number of metabolites that can be identified and quantified in a single plasma sample. This permits the identification of specific interactions of metabolic pathways that occur with age in males and females or as the result of other biological perturbations being investigated. As the global

metabolomic techniques are refined to identify more compounds shown to change with age, sex or race but are hitherto unidentified, the predictive power of this technology will greatly increase.

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Executive summary

- A nontargeted metabolomic analysis of a healthy cohort consisting of 269 subjects; Caucasian, Hispanic and African-American males and females, aged 20–65 years was performed.
- All subjects met an inclusion criteria for 'healthy'.
- Over 300 compounds were detected in 100 µl of human plasma for each subject.
- Nearly 100 compounds were found to be statistically altered with age and we attempted to interpret many of these biochemical alterations.
- Age-related changes were more pronounced than sex or race in this nontargeted metabolomic analysis.
- The results contained in this study suggest that a metabolomic age index could be developed through the quantitation of a biochemical subset.
- Further studies are needed to better address the 'biochemical profile' of an adult population taking into account additional information, such as lifestyle, diet and medication.

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