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Original Article

Age- and Sex-Dependent Changes of Free Circulating Blood Metabolite and Lipid Abundances, Correlations, and Ratios

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Abstract

In this study, we investigated how the concentrations, pairwise correlations and ratios of 202 free circulating blood metabolites and lipids vary with age in a panel of n = 1 882 participants with an age range from 48 to 94 years. We report a statistically significant sex-dependent association with age of a panel of metabolites and lipids involving, in women, linoleic acid, α-linoleic acid, and carnitine, and, in men, monoacylglycerols and lysophosphatidylcholines. Evaluating the association of correlations among metabolites and/or lipids with age, we found that phosphatidylcholines correlations tend to have a positive trend associated with age in women, and monoacylglycerols and lysophosphatidylcholines correlations tend to have a negative trend associated with age in men. The association of ratio between molecular features with age reveals that decanoyl-L-carnitine/lysophosphatidylcholine ratio in women "decrease" with age, while L-carnitine/phosphatidylcholine and L-acetylcarnitine/phosphatidylcholine ratios in men "increase" with age. These results suggest an age-dependent remodeling of lipid metabolism that induces changes in cell membrane bilayer composition and cell cycle mechanisms. Furthermore, we conclude that lipidome is directly involved in this age-dependent differentiation. Our results demonstrate that, using a comprehensive approach focused on the changes of concentrations and relationships of blood metabolites and lipids, as expressed by their correlations and ratios, it is possible to obtain relevant information about metabolic dynamics associated with age.

Keywords: Correlation analysis, Gender differences, Human aging, Lipids, Metabolomics

Aging is a very complex process, influenced by genetic, environmental, and lifestyle factors (1,2), and involves progressive systemic dysregulation, affecting all levels of an organism, from molecules to organs (3,4). Metabolomics, that is the comprehensive analysis of small molecule profiles measured in a biological sample like blood or urine (5,6), is an excellent approach to obtain a global representation of the metabolic status of an organism with respect to a healthy status or a particular pathophysiological condition (7–9). The analysis of metabolomic profiles obtained from participants of different ages, performed using an integrative systems biology approach (10), allows the comprehensive description of the metabolic dynamics and can help to quantify and decipher the relationships between molecular features and aging process (4,11). Studies have been conducted in humans, highlighting how the metabolome is sex and age-dependent, indicating sex-specific association of certain genetic loci with several metabolites and lipid species: the levels of many metabolites (among them fatty acids, including 10 long-chain fatty acids, polyunsaturated fatty acids, glutamine, tyrosine, and histidine) and variation thereof are highly dependent on sex and age, and that sex differentially influences the levels and variation over time of many metabolites (12,13).

Correlations and ratios among molecules, and not only their levels, bear relevant biological information: Because molecules behave in an orchestrated way through metabolic pathways, changes in their association patterns, as represented by correlations and ratios (14,15), can provide information on the remodulation of

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biochemical reaction networks and metabolic pathways associated with age or sexual dimorphism, thus suggesting mechanisms through which molecules may modify cell membranes and affect hormonal activities, mitochondrial metabolism, and cell responses to oxidative stress (11,16).

In this study, making use of publicly available data, we took a comprehensive system biology approach, focusing on the association of the blood circulating unconjugated metabolites and lipids with age and sex in a large population cohort with an age range between 48 and 98 years (17). We investigated how metabolite and lipid abundances correlate with age groups, but also how the correlation and the ratios between metabolites and lipids change in groups of participants of different (increasing) ages.

Material and Methods

Experimental Data

We used data from the TwinGene project (17) that includes a longitudinal cohort from the Swedish Twin Register and a matched subcontrol cohort stratified on age and sex. The cohort was selected by Ganna et al. (17). This data set is a valid representation of a population consisting of not related participants and with a wide age range. It contains 202 quantified blood metabolites and lipids measured on n = 2 139 participants (n_W = 921 women [43%] and n_M = 1 218 men [57%]) with an overall age range of 47.6–93.9 years (women age range = 48.4–93.9 years and men age range = 47.6–93.3 years) and with an overall average age of 68.8 (women average age = 68.8 years and men average age = 68.7 years). This data set was used to identify potential molecular features and metabolic pathways associated with the sex-related aging process. Data were downloaded from the MetaboLights database (https://www.ebi.ac.uk/metabolights/) with accession number MTBLS93. Briefly, metabolomic profiling was performed on ultra-performance liquid chromatography to quadrupole time-of-flight mass spectrometry with an atmospheric electrospray interface operating in positive ion mode. The first step was the detection, alignment, grouping, and assignment of metabolites, performed by Ganna et al. (17), using the XCSM software. For the metabolic annotation, 4 approaches were performed by the authors: (a) based on matching accurate mass, fragmentation pattern, and retention time with their in-house spectral library of authentic standards collected; (b) based on spectrum and/or *m/z* similarities, but not retention time, and the annotation relies on the information of public databases; (c) based on the combination of spectral data, accurate mass, and retention time to assign the metabolite to a specific chemical class; (d) the other approaches failed in the annotation of the metabolite and the metabolite was annotated as "unknown." Combining these approaches, m = 202 molecular features, divided into m_1 = 36 metabolites and m_2 = 166 lipids and lipid precursors, were assigned in the original publication (Supplementary Table 1).

For further details, we refer the reader to the original publication (17).

Data Preprocessing

Removal of outliers

To obtain a uniform study population, we removed those participants showing outlying blood metabolites and lipid profiles under the assumption of the presence of possibly undiagnosed pathophysiological conditions. Outliers were removed using a Principal Components Analysis (PCA) based approach. Hotelling's T^2 values were calculated from PCA scores; samples whose T^2 values exceeded the 95% confidence ellipsis were considered outliers and were removed from subsequent analysis. The optimal number of significant principal components to be retained (at the $\alpha = 0.05$ level) was determined using a statistical test based on the Tracy–Widom distribution (18). A total of 117 women (18%) and 140 men (11%) were removed from the analysis. This left n = 1 882 (n_W = 804 women, 43%, n_M = 1 078 men, 57%) samples/participants available for further analysis.

Subject stratification

The n_W = 804 women and the n_M = 1 078 men were separately stratified by age in 20 groups, W_t (for women) and M_t (for men) with t = 1,2, …, 20 of size w_i and m_i by taking the 20 quantiles QT_1, QT_2, …, QT_{20} of the women and men age distributions, reflecting the 5th, 10th, …, 95th, and 100th percentiles of the sex-specific age distribution. Consequently, each W_t group and M_t group had approximately 5% of the sex-specific sample (≅40 for women and ≅54 for men). The age characteristics for each women and men group are given in Supplementary Table 2. A graphical illustration is shown in Figure 1. For each W_t and M_t group, we defined the corresponding data matrices $\mathbf{W}_t$ and $\mathbf{M}_t$ of size $w_i \times p$ and $m_i \times p$ containing the concentrations of the p = 202 metabolites and lipids measured on the w_i and m_i participants in the corresponding group. Each set of data matrices is associated with a 1 × 20 vector $\mathbf{t}_M$ (respectively $\mathbf{t}_F$) containing the average age of the $M_1, M_2, \ldots, M_{20}$ group (respectively $W_1, W_2, \ldots, W_{20}$).

Statistical Analysis

Estimation of the average concentration of molecular features specific to age groups

For each data set $\mathbf{W}_t$ and $\mathbf{M}_t$ we calculated the mean abundance m_i between each molecular feature x_i. As for the correlation case, we obtained thus 20 values for each metabolite–lipid, representing the changes of the average abundance of molecular feature x_i associated with the age groups (a graphical representation is shown in Figure 2A):

We considered the standard mean estimation:

$$a_i = \frac{1}{n}\sum_{k=1}^{n} x_i \quad (1)$$

For each feature, we thus obtained 20 mean values:

$$\mathbf{A}(x_i) = \{a_{i\ (t=1)},\ a_{i\ (t=2)},\ \ldots,\ a_{i\ (t=20)}\} \quad (2)$$

Estimation of correlations between molecular features specific to age groups

For each data set $\mathbf{W}_t$ and $\mathbf{M}_t$ of size $w_i \times p$ and $m_i \times p$, we calculated the correlation r_{ij} between each pair of molecular features x_i, x_j. For each pair, we obtained thus 20 correlation values, representing the evolution of the strength of the relationship between molecular features x_i, x_j associated with different age groups (Figure 2A):

$$\mathbf{C}(x_i,\ x_j) = \{r_{ij\ (t=1)},\ r_{ij\ (t=2)},\ \ldots,\ r_{ij\ (t=20)}\} \quad (3)$$

We used Winsorized correlation coefficients that are robust toward the shape, sample size, and outliers in the metabolite concentration distribution (19) to estimate the correlation r_{ij} within molecular features pairs. The Winsorized correlation coefficient is obtained by replacing the k smallest observations with the $(k + 1)$st smallest

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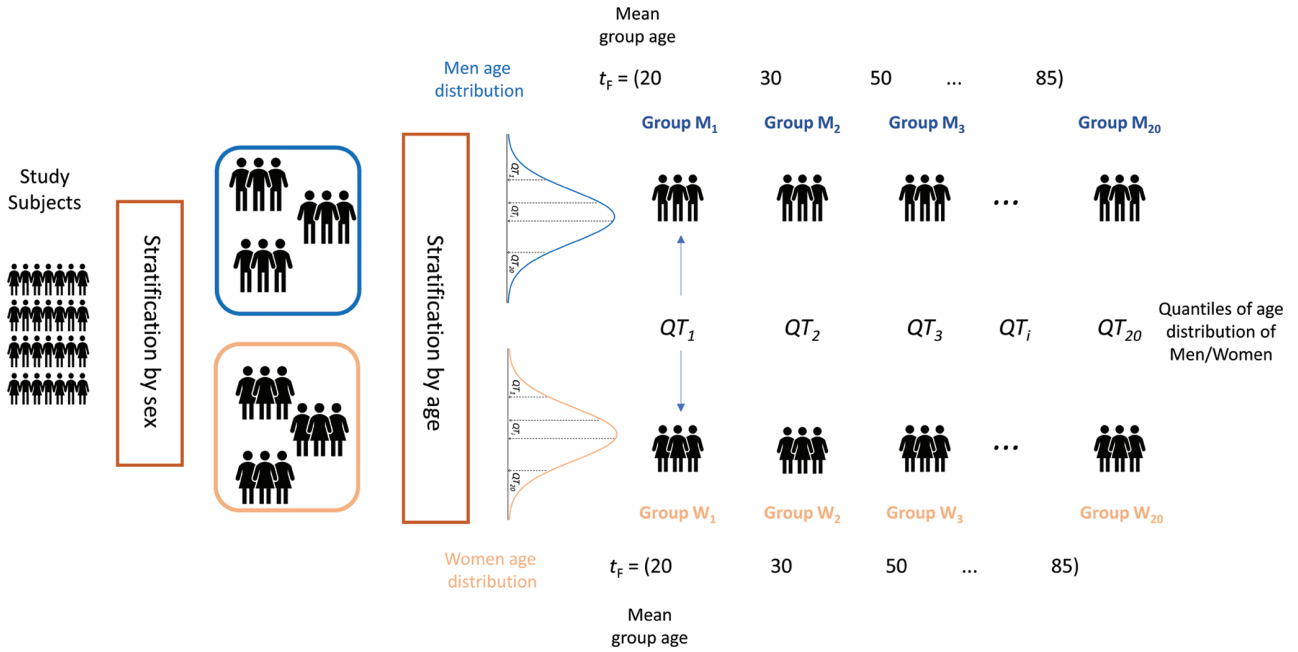


Figure 1. Overview of stratification of the study participants. Participants are first stratified by sex and then by age. Women and men are divided into 20 groups according to the 20 quantiles obtained from the age distribution of the 2 sex-specific groups.

observation, and the k largest observations with the $(k + 1)$st largest observation. In this way, the observations are Winsorized at each end of both x_i, and x_j. The Pearson's correlation coefficient is then calculated on the Winsorized variables (20). A 10% Winsorization was used. Among the $\frac{1}{2}p(p-1)$ possible correlations we retained for further analysis only those pairs of molecular features for which the correlation r_{ij} *was* found to be significant at the $\alpha = 0.01$ level in at least 10 of the 20 data sets $\mathbf{W}_t$ and $\mathbf{M}_t$.

Estimation of ratios between molecular features specific to age groups

For each data set $\mathbf{W}_t$ and $\mathbf{M}_t$, we calculated the ratio q_{ij} between each pair of molecular features x_i, x_j. As for the correlation case, we thus obtained 20 values for each pair, representing the evolution of the ratio magnitude of molecular features x_i and x_j (Figure 2A). We considered the unbiased ratio estimator proposed by van Kempen and van Vliet (21) which is defined as:

$$q_{ij} = \frac{\overline{x_i}}{\overline{x_j}} - \frac{1}{n}\left(\frac{\overline{x_i}}{\overline{x_j}^3}\mathrm{var}(x_i) - \frac{\mathrm{cov}(x_i,x_j)}{\overline{x_j}^2}\right) \quad (4)$$

where $\overline{x_i}$ is the mean of x_i, $\overline{x_j}$ is the mean of x_j, $\mathrm{var}(x_i)$ is the variance of x_i, $\mathrm{cov}(x_i,x_j)$ is the covariance between x_i and x_j, and n is the sample size. For each ratio, we thus obtained 20 ratio values:

$$Q(x_i, \quad x_j) = \{q_{ij \quad (t=1)}, \quad q_{ij \quad (t=2)}, \quad \ldots, \quad q_{ij \quad (t=20)}\} \quad (5)$$

Because we were looking for ratio values varying over the 20 age groups, we retained for further analysis only those ratios q_{ij} for which the relative variation between $q_{ij}(t = 1)$ and $q_{ij}(t = 20)$ was larger than 10%.

Estimation of the association with average group age of the correlation and ratios among molecular features

The association $rc_A(x_i, x_j)$ of the correlation of each pair of molecular features x_i, x_j with the average group age t_M was estimated by taking the Winsorized correlation between the vectors of correlations $\mathbf{C}(x_i, x_j)$ defined in eqn (3) and the average group age vector t_M (respectively, t_F):

$$rc_A(x_i, \quad x_j) = \mathrm{corr}(C(x_i, \quad x_j), \ t_M) \quad (6)$$

The association $rq_A(x_i, x_j)$ of the ratio of each pair of molecular features x_i, x_j with the average group age t_M was estimated in a similar fashion:

$$rq_A(x_i, \quad x_j) = \mathrm{corr}(Q(x_i, \quad x_j), \ t_M) \quad (7)$$

The association $ra_A(x_i, x_j)$ of the mean abundance of each molecular features x_i with the average group age t_M was estimated as:

$$ra_A(x_i) = \mathrm{corr}(A(x_i), \ t_M) \quad (8)$$

We considered to be associated with age only the correlations, ratios, or mean abundances of those molecular features for which $|rc_A(x_i, x_j)| \geq 0.65$, $|rq_A(x_i, x_j)| \geq 0.65$ and $|ra_A(x_{ij})| \geq 0.65$ and $p < .01$ after correction (*fdr*) for multiple testing with the Benjamini–Hochberg method. Correction for multiple testing (Benjamini–Hochberg) was applied at all analysis stages. This choice is based on both statistical and biological considerations. There are 20 age groups, which means that the sample size available to estimate the correlation between metabolite concentrations and associations (correlations and ratios) is 20: With 20 observations, it is possible to assess significance at $\alpha = 0.01$ with 80% power only of correlations $|r| \geq 0.65$. In addition, there are ~20 participants per age group, thus metabolite–metabolite correlation $|r| \geq 0.65$ can be estimated. Biologically the 0.65 threshold is justified by considering that the majority of correlations observed in metabolomics studies are below 0.6 (22,23): Setting a higher threshold allows to focus on correlations that really stand out of the background correlation.

Validation of the results

To validate the results of the analysis described in the previous sections, that is, the existence of an association between average

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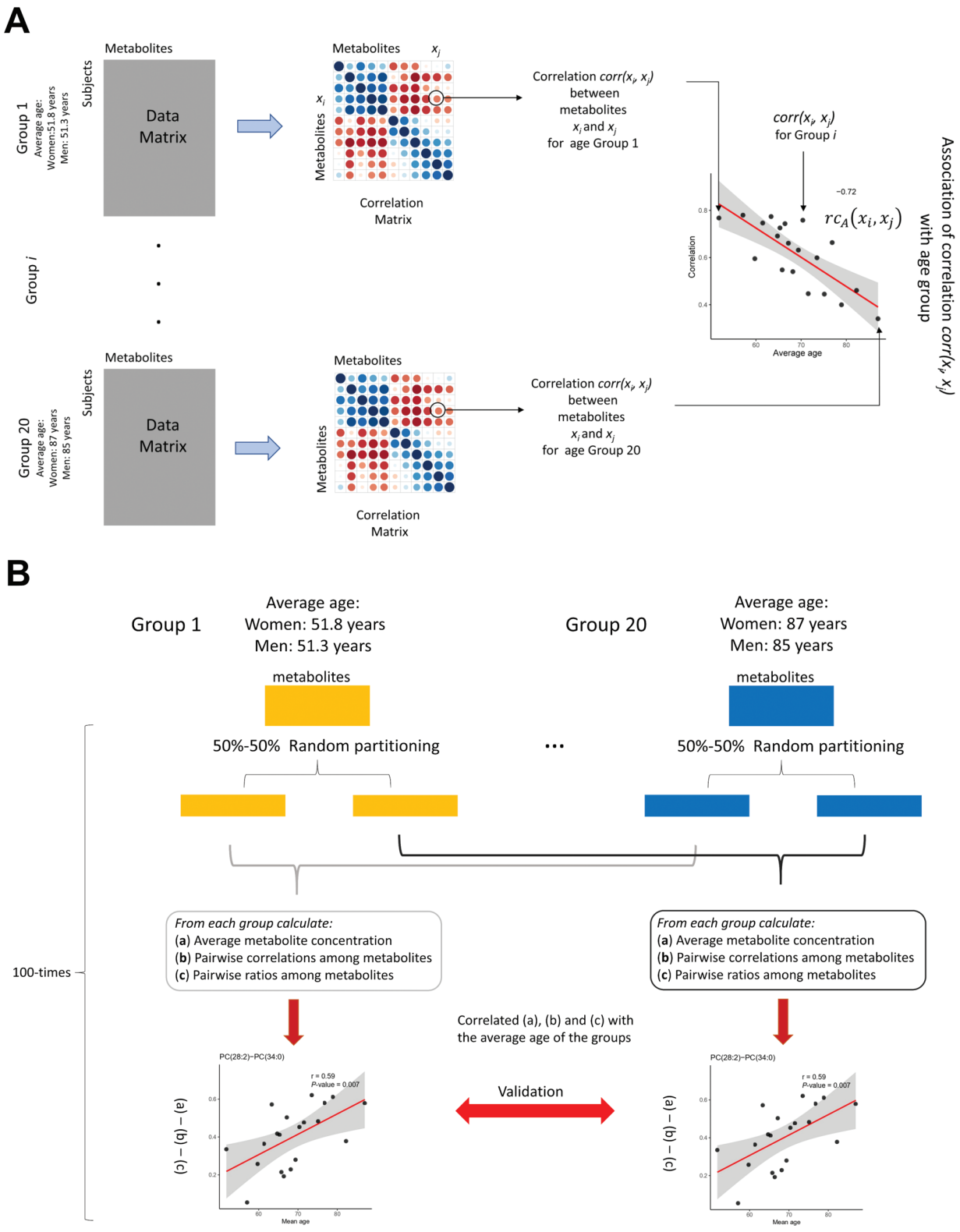


Figure 2. (**A**) Overview of the statistical procedure used to establish the association $rc_A(x_i, x_j)$ (eqn (6)) between the metabolite and lipids pairwise correlations $C(x_i, x_j)$ (eqn (3)), with the group age (Figure 1). In the case of ratios (eqn (7)), the correlation matrix is replaced with the matrix of pairwise ratios, for average abundances is replaced by the vectors of means (eqn (8)). (**B**). Overview of the data-splitting procedure used to validate the results. Each subject group is randomly split into 2 halves, obtaining 2 sets of 20 groups. The analysis is performed on the first set, while the second set is used for validation. The procedure is repeated 100 times: Only results validated more than 50% of the times are considered significant.

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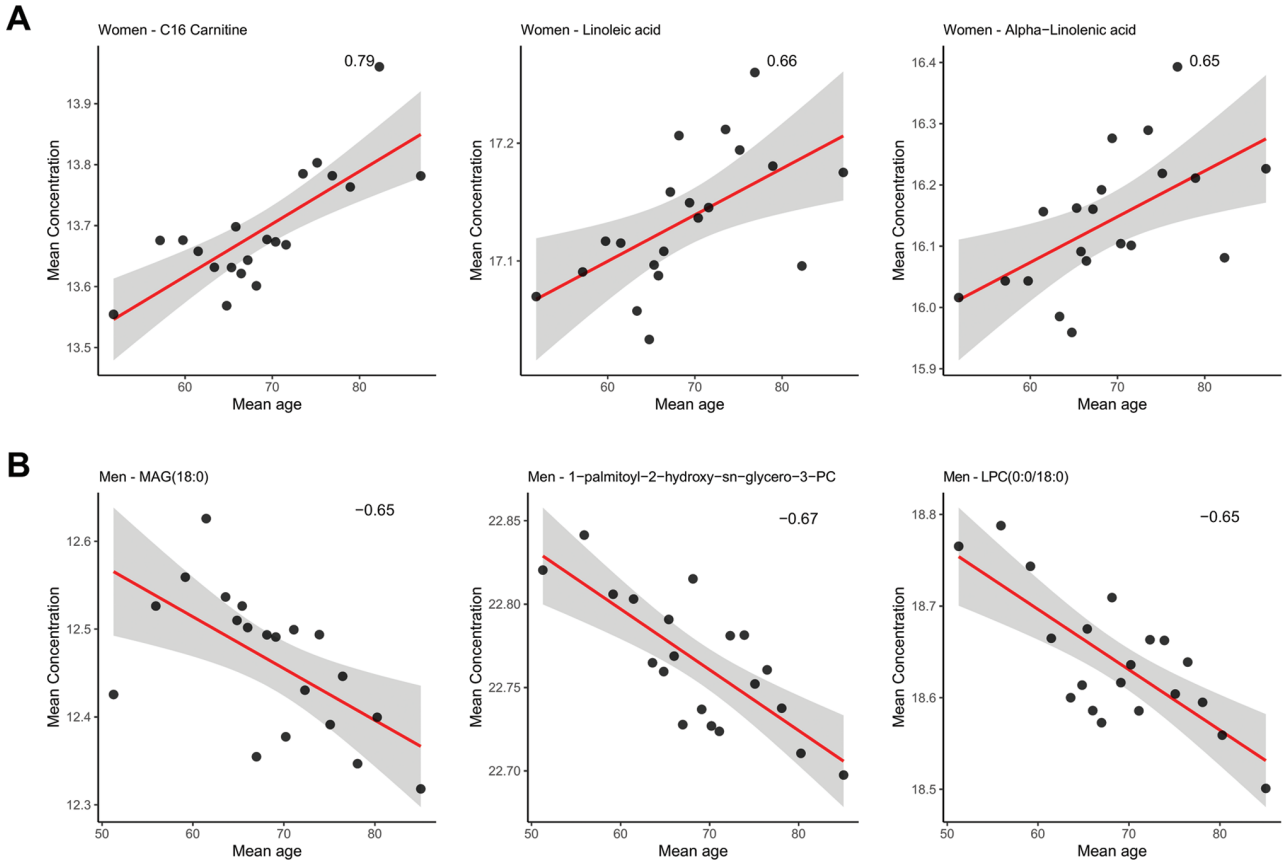


Figure 3. Correlations between average metabolites and lipids concentrations and the average age of the 20 subject groups: women (**A**) and men (**B**). LPC = lysophosphatidylcholine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; MAG = monoacylglycerol. See Figure 2 for an overview of the statistical procedure.

age group and metabolite and lipid concentration (eqn (8)), correlations (eqn (6)), and ratios (eqn (7)), we implemented a data splitting approach (24,25). Basically, we randomly split each of the 20 age groups into 2 halves and performed the analysis independently on the 2 data splits to ascertain if the results could be reproduced. To consider the variability due to the random splitting, the overall procedure was repeated generating $k = 100$ different pairs of data splits: Analysis was repeated on the 100 pairs of data. We considered to be valid only those results that were confirmed in at least 50% of the splits (Figure 2B). In this way, we could obtain an estimation of the reproducibility and robustness of the results by mimicking validation in an external cohort: A portion of the data is used to suggest a hypothesis, and a second, independent portion is used to test it. Note that this approach can be rephrased in an inferential setting and implies that Type I error (ie, the risk of false positives) is controlled (conservatively) at the 0.01 level (26) after correction for multiple testing. The downside of such an approach is a potential loss of power, due to the reduction of the sample size used to estimate correlation. However, this approach is effective in giving valid inference after the selection of a hypothesis, estimating nuisance parameters, and avoiding overfitting (26).

Software

All calculations and plots were performed in R (version 3.3.2). The function "win.cor," implemented in WRS2 package, was used to calculate the Winsorized correlations.

Results

Association of Metabolite and Lipid Abundances With Age

Starting from a total of $n = 202$ metabolites and lipids, a total of $p_W = 3$ (women) and $p_M = 3$ (men) compounds were found statistically significant (adjusted $p \leq .01$ and absolute value of $ra_A \geq 0.65$, see eqn (8)) in more than 50% of splits obtained performing the validation method.

In particular, in the women cohort, we observed a positive correlation of the concentrations of carnitine with $ra_A = 0.79$ and an adjusted $p = .0009$ in the 79% of validation splits, linoleic acid with $ra_A = 0.66$ and an adjusted $p = .001$ in the 59% of validation splits, and α-linoleic acid with $ra_A = 0.65$ and an adjusted $p = .01$ in the 66% of validation splits with the average age of women group (Figure 3A).

The age groups that we used here are data-driven and are not physiologically informed. In particular, the first group of women (W1) corresponds to a 6-year age bin that likely represents perimenopausal women, given that the average age of menopause in women in the Western world is 51 years (27). Although this does not affect statistical analysis, we shall consider that menopausal transition aligns with age.

In the men cohort, we observed negative correlation with age of monoacylglycerol (MAG), especially MAG (18:0) with $ra_A = -0.65$ and an adjusted $p = .005$ in the 53% of validation splits, and lysophosphatidylcholines (LPCs), especially

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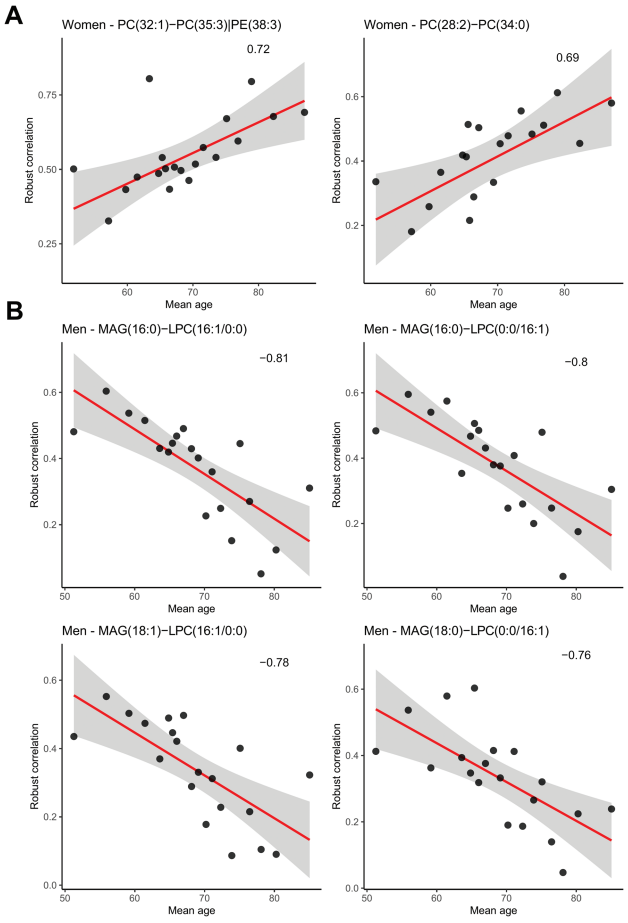


Figure 4. Correlations between metabolites and lipids correlations and the average age of the 20 subject groups: women (**A**) and men (**B**). LPC = lysophosphatidylcholine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; MAG = monoacylglycerol. See Figure 2 for an overview of the statistical procedure.

1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC [16:0]) with $ra_A = -0.67$ and an adjusted $p = .005$ in the 58% of validation splits, and LPC (0:0/18:0) with $ra_A = -0.65$ and an adjusted $p = .008$ in the 62% of validation splits (Figure 3B).

For a complete overview of the results for all metabolites see Supplementary Table 3.

Association of the Correlation Among Molecular Features With Age

Starting from a total of $n = 20\,301$ metabolites and lipids correlations, a total of $c_W = 2$ (women) and $c_M = 4$ (men) correlations among molecules result to be statistically significant (adjusted $p \leq .01$ and absolute value of $rc_A \geq 0.65$, see eqn (6)) in more than 50% of splits after the validation method.

In the women cohort, the correlations between phosphocholines (PCs), especially between (a) PC (28:2)–PC (32:1) with $rc_A = 0.69$ and an adjusted $p = .008$ in the 57% of validation splits and (b) PC (32:1)–PC (35:3) | PE (38:3) with $rc_A = 0.72$ and an adjusted $p = .008$ in the 54% of validation splits, tend to increase with age (Figure 4A).

In Figure 4B, correlations between MAG and LPC, especially (a) MAG (16:0)–LPC (16:1/0:0) with $rc_A = -0.81$ and an adjusted $p = .002$ in the 53% of validation splits, (b) MAG (16:0)–LPC (0:0/16:1) with $rc_A = -0.80$ and an adjusted $p = .002$ in the 59% of validation splits, (c) MAG (18:1)–LPC (16:1/0:0) with $rc_A = -0.78$ and an adjusted $p = .003$ in the 60% of validation splits, and (d) MAG (18:0)–LPC (0:0/16:1) with $rc_A = -0.76$ and an adjusted $p = .004$ in the 53% of validation splits, decrease with the average age of men-specific groups. The levels of these lipids vary in a similar fashion, decreasing with the age.

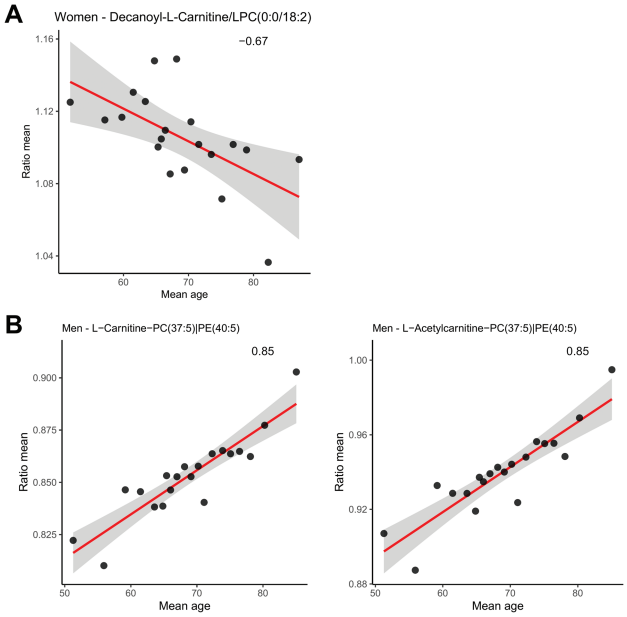


Figure 5. Correlations between average metabolites and lipids ratios and the average age of the 20 subject groups: women (**A**) and men (**B**). GCA = glycocholic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; MAG = monoacylglycerol. See Figure 2 for an overview of the statistical procedure.

For a complete overview of the results for all metabolites see Supplementary Table 3.

Association of the Ratios Among Molecular Features With Age

Alterations in the ratios between 2 single lipids and/or metabolites may point at perturbations in pathways relevant for a certain specific phenotype and they could influence the physiological course of aging. In this light, pairwise ratios may serve as potential biomarkers of the aging process (28,29).

Starting from a total of $n = 20\,301$ metabolites and lipids ratios, after the validation method, we found only $q_W = 1$ (women) and $q_M = 2$ (men) ratios between molecules whose variation is significantly associated with the average age (adjusted $p \leq 0.01$ and absolute value of $rq_A \geq 0.65$, see eqn (7)). In particular, the ratio between decanoyl-L-carnitine/LPC (0:0/18:2) with $rq_A = -0.67$ and an adjusted $p = .002$ in the 56% of validation splits shows a negative association with the average age of the women cohort (Figure 5A).

In Figure 5B, the ratios between L-carnitine/PC (37:5) with $rq_A = 0.85$ and an adjusted $p = 1 \times 10^{-4}$ in the 55% of validation splits and L-acetylcarnitine/PC (37:5) with $rq_A = 0.85$ and an adjusted $p = 2 \times 10^{-4}$ in the 51% of validation splits tend to be positively correlated with the average age of men-specific groups.

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For a complete overview of the results for all metabolites see Supplementary Table 3.

Discussion

To shed light on the molecular mechanisms possibly associated with age, we studied how the concentration, correlations, and ratios of and among circulating blood metabolites and lipids vary with subject age groups, considering men and women separately to highlight possible dependencies on sex. Using different approaches, in the original paper, Ganna et al. (17) demonstrated that LPC (18:1) and LPC (18:2) are not directly associated with coronary heart disease (CHD), but they found an age-dependent negative trend of these 2 lipids in association with CHD risk. Moreover, MAG (18:2) and sphingomyelin (28:1) have a positive correlation with the CHD risk. Our results support the usefulness of the metabolomic analysis conjugated with a system biology approach for the identification of age-related metabolites and their association patterns, providing additional information compared to what is already known from the literature.

In women, the levels of carnitine, linoleic acid, and α-linoleic acid show a positive correlation with (group) age. These significant correlations are of particular interest because previous studies showed that the age-dependent carnitine serum levels increase more with age in adult women than men (30,31), and the endogenous biosynthesis of carnitine depends on the production, by lysosomal protein degradation, of trimethyl-lysine (32) whose homeostasis is regulated by dietary intake, intestinal absorption, and renal reabsorption. Carnitine also plays an important role in carnitine-shuttle biochemical reactions and in the energy pool metabolism, inducing an expression of intramitochondrial alterations (30,33), fundamental in linoleic acid metabolism. Previous studies report that the reduction of estrogens activity and the increase of testosterone levels induce modification of the rate of conversion of linoleic acid and α-linolenic acid into $n - 3$ long-chain polyunsaturated fatty acids, inducing changes in cell membrane composition and in cell cycle mechanisms (34,35). Endogenous biosynthesis of carnitine depends on the production, by lysosomal protein degradation, of trimethyl-lysine (32). The homeostasis of this molecule is regulated by dietary intake, intestinal absorption, and renal reabsorption. Carnitine also plays an important role in carnitine-shuttle biochemical reactions and in the energy pool metabolism, inducing an expression of intramitochondrial alterations (30,33), fundamental in linoleic acid metabolisms, whose activity shows age-dependent dysregulation (36). In addition to the role of polyunsaturated fatty acids as energy sources, they have several functions, as cellular signaling pathways (37) and as structural components of cell membranes (38), inducing age-dependent changes (39).

The negative correlation of LPCs concentrations with age, molecularly associated with the reduction of MAGs levels by the MAG lipase enzyme activity (36,40), induces a skeletal muscle mitochondrial dysfunction (41); the decreasing of LPCs is, generally, also associated with the increase of body mass index (BMI) but, in an older population, this effect is associated, firstly, with the increasing of age-dependent inflammation, depending on an overall remodulation of cell membrane and mitochondrial dysfunction.

Because the pairwise correlations among molecules can be used as a proxy to describe the underlying metabolic network (10), here we consider the correlations observed as the result of the combination of all reactions and regulatory processes occurring in the metabolic network (18,42) at a given age.

In women, the correlations between PC (28:2)–PC (32:1) and PC (32:1)–PC(35:3) | PE(38:3) tend to increase with age. During the menopause period, a global dysregulation on liver enzymes is induced, causing the synthesis of PCs from choline (43,44). The interactions of PCs are associated with the remodulation of membranes integrity, promoting their conservation and directly affecting the membrane permeability, increasing the fluidity of the bilayer and protecting it from peroxidative damage (38,45), a frequent phenomenon in advanced age (46). Correlations between MAG (16:0)–LPC (16:1/0:0), MAG (16:0)–LPC (0:0/16:1), MAG (18:1)–LPC (16:1/0:0), and MAG (18:0)–LPC (0:0/16:1) decrease with age in men, and this has been related to the increase of the MAG lipase enzyme activity that determines the hydrolysis of MAG into glycerol and fatty acid alkyl ester (36,40) and to the impaired mitochondrial oxidative capacity associated with low levels of LPCs in advanced age (36,41).

The alterations in the ratios between 2 single lipids and/or metabolites may point at perturbations in pathways relevant for a certain specific phenotype. We considered the pairwise ratios as potential biomarkers (28,29) of the aging process. We found that only the ratio between decanoyl-L-carnitine/LPC(0:0/18:2) shows a negative association with the average age in women, and, at best of our knowledge, this association has never been reported. We can speculate that decreasing levels of LPCs and the increasing levels of decanoyl-L-carnitine induce, synergistically, a mitochondrial dysfunction (36,41), contributing to age-dependent metabolic changes and being an indirect result of aging (47). In contrast, the ratios between L-carnitine/PC(37:5) and L-acetylcarnitine/PC(37:5) tend to be positively correlated with the average age of men-specific groups.

Little is known about these molecular ratios. As said before, carnitine plays a role in carnitine-shuttle biochemical reactions: carnitine palmitoyltransferase 1 enzyme is involved in the reversible acylation of L-carnitine, producing L-acetylcarnitine, and this event is fundamental in fatty acid beta-oxidation, maintenance of acyl-coenzyme A pools, and energy metabolism (30). The carnitine-shuttle activity could generate a specific remodeling of mitochondrial fatty acids oxidation, promoting a modification in the mitochondrial membrane lipidome (48), increasing PCs fraction (49,50). Although, actually, the overall aging molecular mechanisms are unclear, our results show that lipids (ie, LPC, MAG, PC, PE, linoleic acid) and carnitine are fundamental in the age-related metabolic pathways.

Strengths and Limitations

One of the strengths of this study is a large number of patients with a very wide age range (47.6–93.9 years) whose metabolome was analyzed. We implemented a stringent validation of the results using a repeated data resampling to account for varaibility and to obtain robust estimate of metabolite concentrations, correlations, and ratios calculated at the age group level to eliminate subject-to-subject variability.

One limitation of this study is the lack of availability of the clinical data (ie, BMI, waist circumference, systolic and diastolic blood pressures) associated with the participants' metabolite data, publicly available on the MetaboLights public database, resulting in an incomplete representation of the pathophysiological conditions of the cohort, indicating that we could not correct at the individual level for such factors in the analysis.

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Conclusions

In this study, we presented a comprehensive biology approach to highlight potential molecular features concentrations, associations, and ratios directly associated with the increase of the age of a sex- and age-matched population. We showed that linoleic acid, α-linoleic acid, and carnitine have, in the women cohort, a positive correlation trend with age, while MAGs and LPCs have, in the men cohort, a negative correlation trend with age. These results highlight, in women, the effect of the reduction of estrogens activity and the increase of testosterone levels on the linoleic acid metabolism and on the energy pool metabolism that induces the overall changes in cell membrane composition and cell cycle mechanisms. In men, low levels of LPCs concentrations are directly connected with the reduction of MAGs levels by the MAG lipase enzymatic activity that induces mitochondrial dysfunction.

Analyzing the pairwise correlations among molecules, we observed that PCs/PCs correlations tend to have a positive trend associated with the average ages of women, while MAGs/LPCs correlations tend to have a negative trend associated with men average ages. These results, in both cases, suggest an age-dependent remodeling of fatty acid metabolism that induces, overall, remodeling of cell and mitochondrial membranes and modification in terms of fluidity of membranes bilayers.

We studied the pairwise ratios as potential biomarkers of aging. In women, the decanoyl-L-carnitine/LPC ratio has a negative association with the increasing of the average ages, while in men the ratios between L-carnitine/PC and L-acetylcarnitine/PC have a positive association with the increase of age, suggesting, in both cases, a radical remodeling of the dynamic membrane fluidity and carnitine-shuttle activity.

This study brings forward the concept that correlation and ratios among molecular features, and not only abundances along, could be used to investigate the dynamic of molecular mechanisms and their association with age.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

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References

1. Karasik D, Demissie S, Cupples LA, Kiel DP. Disentangling the genetic determinants of human aging: biological age as an alternative to the use of survival measures. *J Gerontol A Biol Sci Med Sci*. 2005;60(5):574–587. doi:10.1093/gerona/60.5.574
2. Kerber RA, O'Brien E, Cawthon RM. Gene expression profiles associated with aging and mortality in humans. *Aging Cell*. 2009;8(3):239–250. doi:10.1111/j.1474-9726.2009.00467.x
3. Hoffman JM, Lyu Y, Pletcher SD, Promislow DEL. Proteomics and metabolomics in ageing research: from biomarkers to systems biology. *Essays Biochem*. 2017;61(3):379–388. doi:10.1042/EBC20160083
4. Jové M, Maté I, Naudí A, et al. Human aging is a metabolome-related matter of gender. *J Gerontol A Biol Sci Med Sci*. 2016;71(5):578–585. doi:10.1093/gerona/glv074
5. Vignoli A, Ghini V, Meoni G, et al. High-throughput metabolomics by 1D NMR. *Angew Chem Int Ed Engl*. 2019;58(4):968–994. doi:10.1002/anie.201804736
6. Eckhart AD, Beebe K, Milburn M. Metabolomics as a key integrator for "omic" advancement of personalized medicine and future therapies. *Clin Transl Sci*. 2012;5(3):285–288. doi:10.1111/j.1752-8062.2011.00388.x
7. Vignoli A, Tenori L, Luchinat C, Saccenti E. Age and sex effects on plasma metabolite association networks in healthy subjects. *J Proteome Res*. 2018;17(1):97–107. doi:10.1021/acs.jproteome.7b00404
8. Vignoli A, Tenori L, Giusti B, et al. NMR-based metabolomics identifies patients at high risk of death within two years after acute myocardial infarction in the AMI-Florence II cohort. *BMC Med*. 2019;17(1):3. doi:10.1186/s12916-018-1240-2
9. Vignoli A, Paciotti S, Tenori L, et al. Fingerprinting Alzheimer's disease by 1H nuclear magnetic resonance spectroscopy of cerebrospinal fluid. *J Proteome Res*. 2020;19(4):1696–1705. doi:10.1021/acs.jproteome.9b00850
10. Rosato A, Tenori L, Cascante M, De Atauri Carulla PR, Martins Dos Santos VAP, Saccenti E. From correlation to causation: analysis of metabolomics data using systems biology approaches. *Metabolomics*. 2018;14(4):37. doi:10.1007/s11306-018-1335-y
11. Yu Z, Zhai G, Singmann P, et al. Human serum metabolic profiles are age dependent. *Aging Cell*. 2012;11(6):960–967. doi:10.1111/j.1474-9726.2012.00865.x
12. Darst BF, Koscik RL, Hogan KJ, Johnson SC, Engelman CD. Longitudinal plasma metabolomics of aging and sex. *Aging (Albany NY)*. 2019;11(4):1262–1282. doi:10.18632/aging.101837
13. Mittelstrass K, Ried JS, Yu Z, et al. Discovery of sexual dimorphisms in metabolic and genetic biomarkers. *PLoS Genet*. 2011;7(8):e1002215. doi:10.1371/journal.pgen.1002215
14. Petersen AK, Krumsiek J, Wägele B, et al. On the hypothesis-free testing of metabolite ratios in genome-wide and metabolome-wide association studies. *BMC Bioinformatics*. 2012;13(1):120. doi:10.1186/1471-2105-13-120
15. Altmaier E, Ramsay SL, Graber A, Mewes HW, Weinberger KM, Suhre K. Bioinformatics analysis of targeted metabolomics—uncovering old and new tales of diabetic mice under medication. *Endocrinology*. 2008;149(7):3478–3489. doi:10.1210/en.2007-1747
16. Barbieri M, Boccardi V, Papa M, Paolisso G. Metabolic journey to healthy longevity. *Horm Res*. 2009;71(suppl 1):24–27. doi:10.1159/000178032
17. Ganna A, Salihovic S, Sundström J, et al. Large-scale metabolomic profiling identifies novel biomarkers for incident coronary heart disease. *PLoS Genet*. 2014;10(12):e1004801. doi:10.1371/journal.pgen.1004801
18. Saccenti E. Correlation patterns in experimental data are affected by normalization procedures: consequences for data analysis and network inference. *J Proteome Res*. 2017;16(2):619–634. doi:10.1021/acs.jproteome.6b00704
19. Tuğran E, Kocak M, Mirtagioğlu H, Yiğit S, Mendes M. A simulation based comparison of correlation coefficients with regard to type I error rate and power. *J Data Anal Inf Process*. 2015;3(3):87–101. doi:10.4236/jdaip.2015.33010
20. Wilcox RR. Some results on a Winsorized correlation coefficient. *Br J Math Stat Psychol*. 1993;46(2):339–349. doi:10.1111/j.2044-8317.1993.tb01020.x
21. van Kempen GM, van Vliet LJ. Mean and variance of ratio estimators used in fluorescence ratio imaging. *Cytometry*. 2000;39(4):300–305. doi:10.1002/(sici)1097-0320(20000401)39:4<300::aid-cyto8>3.0.co;2-o

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22. Camacho D, Fuente ADL, Mendes P. The origins of correlations in metabolomics data. *Metabolomics*. 2005;1(1):53–63. doi:10.1007/s11306-005-1107-3
23. Jahagirdar S, Saccenti E. On the use of correlation and MI as a measure of metabolite–metabolite association for network differential connectivity analysis. *Metabolites*. 2020;10(4):171. doi:10.3390/metabo10040171
24. Cox DR. A note on data-splitting for the evaluation of significance levels. *Biometrika*. 1975;62(2):441–444. doi:10.1093/biomet/62.2.441
25. Rubin D, Dudoit S, van der Laan M. A method to increase the power of multiple testing procedures through sample splitting. *Stat Appl Genet Mol Biol*. 2006;5:Article19. doi:10.2202/1544-6115.1148
26. DiCiccio CJ, DiCiccio TJ, Romano JP. Exact tests via multiple data splitting. *Stat Probab Lett*. 2020;166:108865. doi:10.1016/j.spl.2020.108865
27. Lindh-Åstrand L, Hoffmann M, Järvstråt L, Fredriksson M, Hammar M, Spetz Holm AC. Hormone therapy might be underutilized in women with early menopause. *Hum Reprod*. 2015;30(4):848–852. doi:10.1093/humrep/dev017
28. Zelezniak A, Sheridan S, Patil KR. Contribution of network connectivity in determining the relationship between gene expression and metabolite concentration changes. *PLoS Comput Biol*. 2014;10(4):e1003572. doi:10.1371/journal.pcbi.1003572
29. Krumsiek J, Stückler F, Suhre K, et al. Network-based metabolite ratios for an improved functional characterization of genome-wide association study results. *bioRxiv*, doi:10.1101/048512, 13 April 2016, preprint: not peer reviewed.
30. Mitchell SL, Uppal K, Williamson SM, et al. The carnitine shuttle pathway is altered in patients with neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2018;59(12):4978–4985. doi:10.1167/iovs.18-25137
31. Malaguarnera G, Catania VE, Bonfiglio C, Bertino G, Vicari E, Malaguarnera M. Carnitine serum levels in frail older subjects. *Nutrients*. 2020;12(12):3887. doi:10.3390/nu12123887
32. Koves TR, Ussher JR, Noland RC, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab*. 2008;7(1):45–56. doi:10.1016/j.cmet.2007.10.013
33. Judit B, Andras S, Katalin K, Bela M. Mass spectrometric analysis of L-carnitine and its esters: potential biomarkers of disturbances in carnitine homeostasis. *Curr Mol Med*. 2020;20(5):336–354. doi:10.2174/1566524019666191113120828
34. Janssen CI, Kiliaan AJ. Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: the influence of LCPUFA on neural development, aging, and neurodegeneration. *Prog Lipid Res*. 2014;53:1–17. doi:10.1016/j.plipres.2013.10.002
35. Cybulska AM, Skonieczna-Żydecka K, Drozd A, et al. Fatty acid profile of postmenopausal women receiving, and not receiving, hormone replacement therapy. *Int J Environ Res Public Health*. 2019;16(21):4273. doi:10.3390/ijerph16214273
36. Johnson AA, Stolzing A. The role of lipid metabolism in aging, lifespan regulation, and age-related disease. *Aging Cell*. 2019;18(6):e13048. doi:10.1111/acel.13048
37. Sokoła-Wysoczańska E, Wysoczański T, Wagner J, et al. Polyunsaturated fatty acids and their potential therapeutic role in cardiovascular system disorders—a review. *Nutrients*. 2018;10(10):1561. doi:10.3390/nu10101561
38. Li Z, Agellon LB, Allen TM, et al. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab*. 2006;3(5):321–331. doi:10.1016/j.cmet.2006.03.007
39. Chung KW. Advances in understanding of the role of lipid metabolism in aging. *Cells*. 2021;10(4):880. doi:10.3390/cells10040880
40. Grabner GF, Zimmermann R, Schicho R, Taschler U. Monoglyceride lipase as a drug target: at the crossroads of arachidonic acid metabolism and endocannabinoid signaling. *Pharmacol Ther*. 2017;175:35–46. doi:10.1016/j.pharmthera.2017.02.033
41. Semba RD, Zhang P, Adelnia F, et al. Low plasma lysophosphatidylcholines are associated with impaired mitochondrial oxidative capacity in adults in the Baltimore Longitudinal Study of Aging. *Aging Cell*. 2019;18(2):e12915. doi:10.1111/acel.12915
42. Steuer R, Kurths J, Fiehn O, Weckwerth W. Observing and interpreting correlations in metabolomic networks. *Bioinformatics*. 2003;19(8):1019–1026. doi:10.1093/bioinformatics/btg120
43. Auro K, Joensuu A, Fischer K, et al. A metabolic view on menopause and ageing. *Nat Commun*. 2014;5:4708. doi:10.1038/ncomms5708
44. Cui X, Yu X, Sun G, et al. Differential metabolomics networks analysis of menopausal status. *PLoS One*. 2019;14(9):e0222353. doi:10.1371/journal.pone.0222353
45. Rabini RA, Moretti N, Staffolani R, et al. Reduced susceptibility to peroxidation of erythrocyte plasma membranes from centenarians. *Exp Gerontol*. 2002;37(5):657–663. doi:10.1016/s0531-5565(02)00006-2
46. Akila VP, Harishchandra H, D'souza V, D'souza B. Age related changes in lipid peroxidation and antioxidants in elderly people. *Indian J Clin Biochem*. 2007;22(1):131–134. doi:10.1007/BF02912896
47. Haas RH. Mitochondrial dysfunction in aging and diseases of aging. *Biology*. 2019;8(2):48. doi:10.3390/biology8020048
48. Lum H, Sloane R, Huffman KM, et al. Plasma acylcarnitines are associated with physical performance in elderly men. *J Gerontol A Biol Sci Med Sci*. 2011;66(5):548–553. doi:10.1093/gerona/glr006
49. Burstein MT, Titorenko VI. A mitochondrially targeted compound delays aging in yeast through a mechanism linking mitochondrial membrane lipid metabolism to mitochondrial redox biology. *Redox Biol*. 2014;2:305–307. doi:10.1016/j.redox.2014.01.011
50. Janikiewicz J, Szymański J, Malinska D, et al. Mitochondria-associated membranes in aging and senescence: structure, function, and dynamics. *Cell Death Dis*. 2018;9(3):332. doi:10.1038/s41419-017-0105-5

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