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Age and Sex Effects on Plasma Metabolite Association Networks in Healthy Subjects

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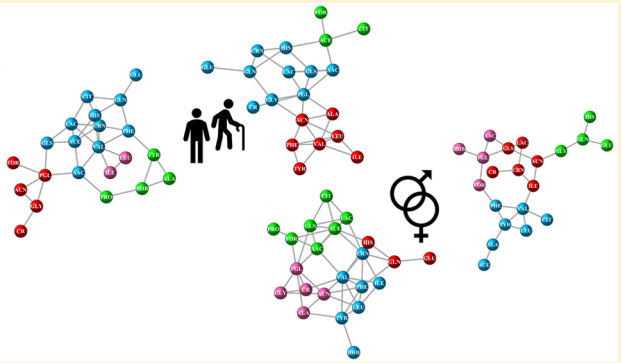
Supporting Information

ABSTRACT: In the era of precision medicine, the analysis of simple information like sex and age can increase the potential to better diagnose and treat conditions that occur more frequently in one of the two sexes, present sex-specific symptoms and outcomes, or are characteristic of a specific age group. We present here a study of the association networks constructed from an array of 22 plasma metabolites measured on a cohort of 844 healthy blood donors. Through differential network analysis we show that specific association networks can be associated with sex and age: Different connectivity patterns were observed, suggesting sex-related variability in several metabolic pathways (branched-chain amino acids, ketone bodies, and propanoate metabolism). Reduction in metabolite hub connectivity was also found to be associated with age in both sex groups. Network analysis was complemented with standard univariate and multivariate statistical analysis that revealed age- and sex-specific metabolic signatures. Our results demonstrate that the characterization of metabolite−metabolite association networks is a promising and powerful tool to investigate the human phenotype at a molecular level.



KEYWORDS: *NMR, metabolomics, differential network analysis, metabolism, network inference*

INTRODUCTION

Simple types of information like sex and age can prove valuable information within a precision medicine approach to investigate and to define disease risk and susceptibility in the human population. Although the molecular mechanisms are yet to be fully understood, it has long been known that differences between the two sexes affect manifestation, epidemiology, and pathophysiology of many widespread diseases and therefore, require different approaches to health care:[1] For instance, drug efficacy and toxicity profiles are affected by sexual dimorphisms[2] as well as the levels and associations of insulin, cholesterol, very low density lipoprotein (VLDL), and certain triacylglycerols.[3] Consistently, the human metabolome is also affected by endogenous factors such as sex and age, whose effects have been investigated through metabolomics-based studies.

Differences in the concentration levels of several urine and blood metabolites (creatinine, citrate, glycine, and hippuric acid) or class of metabolites (breached-chain amino acids and lipids) have been found to be different between men and women.[4−7]

Age-related considerations are increasingly being taken into account in medical therapy, such as in the case of cancer treatment;[8] but whereas sexual dimorphisms is a dichotomic variable providing a clear discrimination of the groups of interest, age and aging are elusive concepts, and new insights into systemic metabolic patterns associated with this variable are needed. Indeed, not only does the definition of young and old and the discriminant among them depend on socio-economic and geographical considerations but also the metabolomics study of aging requires large-scale longitudinal studies with replication due to the high variability observed among subjects in terms of metabolic phenotypes.[8−13] Metabolites such as creatinine, creatine, aromatic amino acids, glycine, and glutamate have been found to be associated with age-related differences, and the correlation between metabolome and metabolic modification with blood leukocyte telomere length (LTL) and epigenetic modifications have been also investigated, identifying several classes of metabolites (glycerophosphoethanolamines, glycerophosphocholines, glyc-

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erolipids, bile acids, isoprenoids, fatty amides, and L-carnitine esters) significantly associated with LTL.[14−16] Attempts have also been made to characterize the real "metabolic" age via metabolomic approaches.[7,17,1−6] These findings provide ample evidence that the human metabolome is highly influenced by this factor whose effects sum with those induced by environment, stress conditions, and other exogenous factors, contributing to the shaping of the human metabolic phenotype.[9−11,18]

However, despite the large efforts spent in characterizing age and sex-related characteristics of (part of) the human metabolome, a clear mechanistic interpretation and explanation of these findings is still missing. This reflects, in part, our limited understanding and knowledge of secondary metabolism and, in part, the fact that most metabolomics studies are exploratory in nature. In the attempt to move one step further toward mechanistic understanding, in this study we take a systems biology approach to investigate sex- and age-specific differences in the plasma metabolite profiles of healthy subjects by considering a group of young and middle-aged subjects of both sexes; participants were from a larger cohort of healthy blood donor volunteers who were analyzed for their plasma metabolite concentration profiles using nuclear magnetic resonance (NMR) spectroscopy.[19]

In contrast with other age-related metabolomics studies, our data analysis strategy is not limited to standard univariate and multivariate analysis[20] to explore the patterns of variation of metabolites among the two age classes but deploys metabolite−metabolite association network inference and analysis.

Networks and network analysis are fundamental tools in systems biology and are being exploited more and more often to analyze, understand, and interpret the complex patterns observed in metabolomics data.[21,22,19,13] The rationale underlying the use of network representation and analysis is that metabolite concentrations change in an orchestrated fashion in such a way that the association among metabolite inferred from measured concentrations in biofluids can be considered, to some extent, related to the underlying structure of the biological networks. Indeed, different metabolite correlation patterns have been found to be associated with sexual dimorphism[23] or to change upon treatment or dietary intervention.[24,25]

The utility of network-based approaches to analyze metabolomics data relies on these two key concepts; in a metabolomics context networks are best exploited when compared across different conditions in a so-called differential-network analysis approach: different network characteristics and different patterns of association between metabolites can highlight possibly affected molecular mechanisms. When investigating age- and sex-related effects, we expect these endogenous factors to influence not only the metabolite levels but also the patterns of metabolite associations whose changes can be more significant than those of levels alone, as previously shown in phenotyping studies.[23,25] Through network analysis in a metabolomics context, it was possible, for instance, to identify possible mechanisms of action underlying latent cardiovascular risk status in healthy subjects[19] or model the variability observed in the human urinary metabolic phenotype.[13]

MATERIALS AND METHODS

Data Description

The study consists of 844 adult healthy volunteers (661 males, 183 females, median age 41 ± 12 years; see Table 1 for an overview) recruited in collaboration with the Tuscany section of the Italian Association of Blood Donors (AVIS) in the Transfusion Service of the Pistoia Hospital (Ospedale del Ceppo, AUSL 3 - Pistoia, Italy). According to AVIS rules for blood donations, volunteers had to not take (or not have recently taken) drugs for a variable period of time according to the active substance, the pharmacokinetics of the prescribed drug, and the disease being treated.

Table 1. Demographic Characteristics of the Healthy Blood Donor Cohort Examined in This Study

	subjects	median age (years)
female (total)	183	43
young female (years <37)	63	28
old female (years >48)	60	55
male (total)	661	40
young male (years <35)	234	30
old male (years >45)	213	52

Plasma samples were obtained after overnight fasting, and ethylenediaminetetraacetic acid (EDTA) was used as anticoagulant, but its presence does not significantly affect the quality of the samples for the NMR analysis.[26] Plasma samples were collected and immediately stored at −80 °C according to the Standard Operating Procedures (SOPs) described in Bernini et al.[27] NMR spectra and associated clinical data were retrieved from the Open-Access Database Repository MetaboLights[28] with the accession number MTBLS147 (http://www.ebi.ac.uk/metabolights). Twenty samples from the original data set were excluded from this analysis because sex or age information were missing. Age distributions for both sexes are reported as histograms in Figure 1.

In the original paper,[26] multivariate and univariate analyses were applied to infer a pattern of metabolic alterations that correlates with cardiovascular risk. The same data set was then reanalyzed in a following paper[19] to define metabolite probabilistic networks specific for low and high latent cardiovascular risk using a new approach based on systems biology and metabolite−metabolite correlation networks. Starting from the data collected in the former and using the computational methodology developed in the latter, the present paper shifts the focus from cardiovascular risk to age and sex characterization. Consistent with previous observations of sex-specific differences at the metabolic level,[22] we performed the analysis separately for males and females to minimize confounding effects.

NMR Sample Preparation

Frozen plasma samples were thawed at room temperature and shaken before use.[27] A total of 300 μL of a sodium phosphate buffer (10.05 g $Na_2HPO_4{\cdot}7H_2O$; 0.2 g NaN_3; 0.4 g sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TMSP) in 500 mL of H_2O with 20% (v/v) ^2H_2O; pH 7.4) was added to 300 μL of each plasma sample, and the mixture was homogenized by vortexing for 30 s. A total of 450 μL of this mixture was transferred to a 4.25 mm NMR tube (Bruker BioSpin srl) for the analysis.

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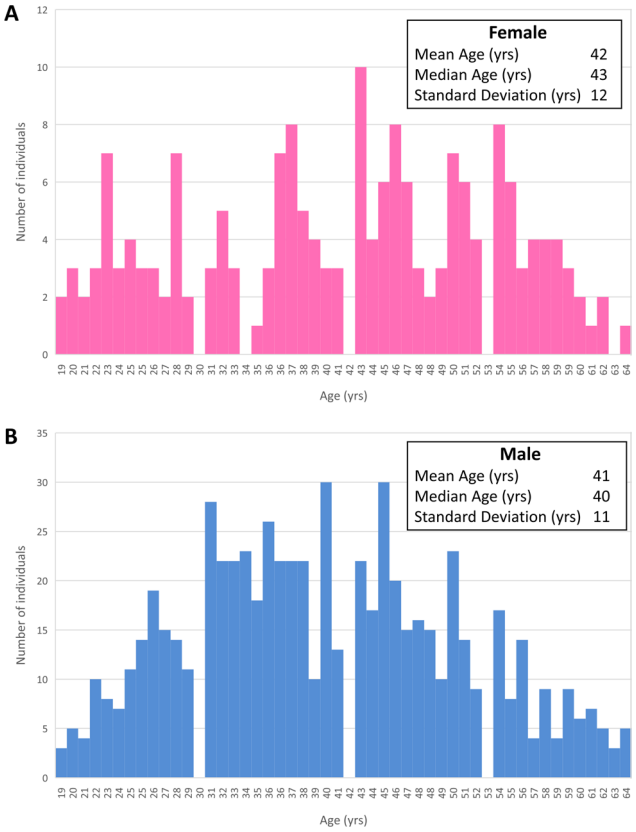

Figure 1. Age distribution for (A) female and (B) male. Median age, mean age, and standard deviation are also reported.

NMR Analysis and Processing

Monodimensional ^{1}H NMR spectra for all plasma samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm CPTCI ^{1}H-^{13}C-^{31}P and ^{2}H-decoupling cryoprobe including a *z*-axis gradient coil, an automatic tuning-matching (ATM), and an automatic sample changer. A BTO 2000 thermocouple served for temperature stabilization within an uncertainty of ~0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probehead for temperature equilibration at 310 K.

Water-suppressed Carr−Purcell−Meiboom−Gill (CPMG)[29] spin echo pulse sequence (RD-90°-(τ-180°-τ)n-acq) with a total spin echo (2nτ) of 300 ms was used to obtain monodimensional ^{1}H NMR spectra in which broad signals from high-molecular-weight metabolites (i.e., proteins, lipids, and lipoproteins) are attenuated. 64 FIDs were collected into 73 728 data points over a spectral width of 12 019 Hz, with a relaxation delay (RD) of 4 s and acquisition time of 3.1 s. Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transformation. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (anomeric glucose doublet at 5.24 ppm) using TopSpin 3.2 (Bruker Biospin srl).

For the multivariate statistical analysis, each 1D spectrum in the range 0.2−10.00 ppm was segmented into 0.02 ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX 3.8.4 software (Bruker BioSpin srl). The region between 4.2 and 6.0 ppm containing the residual water signal was removed, and thus, the dimension of the system was reduced to 451 bins. The total spectral area was calculated on the remaining bins, and the total area normalization was carried out on the data prior to pattern recognition.

Metabolite Identification and Quantification. The spectral regions related to the metabolites were assigned in the CPMG spectra retrieved from Metabolights repository by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (Bruker BioSpin), freely available data set, that is, Human Metabolome DataBase (HMDB),[30,31] and published literature when available. The metabolite quantifications were performed using an in-house developed software in MATLAB programming suite (Mathworks, MATLAB version R2014b). The algorithm is based on the unconstrained nonlinear minimization (fitting) of the metabolites NMR signals, employing a combination of Lorentzian−Gaussian functions. With this approach, each NMR region of interest is decomposed and deconvoluted into its component parts and then integrated to obtain the metabolite concentrations in arbitrary units. A list of the quantified metabolites together with the corresponding spectral region of interest and signal multiplicity is given in Supplementary Table S1.

Statistical Analysis

Univariate Analysis. Wilcoxon rank-sum test[32] was used to assess statistically the existence of differences in metabolite concentrations between the groups. Bonferroni correction[33] was applied to reduce the risk of false positive due to multiple testing. An adjusted *P*-value <0.05 was deemed significant. Cliff's *delta* (Δ)[34] was calculated using the R package "effsize" (https://cran.r-project.org/web/packages/effsize/index.html) to obtain a nonparametric estimation of the effect size, used to complement the *P* values obtained from Wilcoxon rank-sum test. Univariate analysis was applied exclusively on the quantified metabolite concentrations.

Multivariate Analysis. Standard partial least-squares discriminant analysis[35] (PLS-DA) was applied to discriminate the groups of interest: PLS models were built to discriminate between males and females, between young males and old males, and between young females and old females. Both the full NMR CPMG spectra and the set of 22 quantified metabolites were used to build a total of six discriminant PLS models.

PLS-DA is a PLS regression where the continuous response Y vectos is substituted with a vector containing dummy variables (−1, 1) indicating group belonging of the samples. PLS regression is a generalization of multiple regression where a set of new variables (component) is defined that explains as much as possible of the covariance between the predictor (X) and the response Y. The optimal number of PLS components is optimized using a double cross-validation (2CV) strategy as detailed in ref 36. In brief, the 2CV scheme consists of two nested loops CV1 and CV2: The CV1 is used to optimize the number of components to be used in the PLS-DA model while the CV2 is to assess final model performance. In the outer loop (CV2) the complete data set is split into a test set and a rest set: The test set is set aside, and the rest set is used in the loop CV1. Within CV1 the rest set is split into a validation set and a training set. The training set is used to develop a series of PLS-DA models with different number of latent variables from which the samples in the validation set are predicted: The optimal number of components is chosen that maximizes the

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AUROC (i.e., maximize the prediction power of the model). AUROC is the Area Under the Receiver Operating Curve and summarizes both the sensitivity and specificity of the discrimination model and ranges between 0 and 1, indicating perfect discrimination. The AUROC has been found to be the most appropriate measure to optimize PLS-DA model.[36]

Finally, a PLS-DA model is built using the optimal number of components on the data in the test set to assess the quality of the PLS-DA model. Using a 2CV scheme, calibration and quality assessment of the model are kept separated: In this way the samples used to assess the performance of the model are not used to calibrate the model itself, and this guarantees unbiased model calibration and assessment. For an overview of the 2CV calibration procedure, see figure 1 in ref 36. We used a 4:3 data splitting, meaning that the full data was split in four parts: One part was retained as test set (CV2), and the remaining three (the rest set) were split into three parts in the CV1 where a 3:2 split was used for model calibration. The overall 2CV procedure was repeated 100 times to account for possible differences due to the random data splitting. AUROC and accuracy for a given discrimination model are given as average values over the 100 repetitions. Significance of the classification results was assessed by means of a permutation test using 10^3 permutations.[36]

In the case of the comparison of male versus females, the group size is different. To compensate for this, 150 male samples and 150 female samples are randomly chosen from the full data set and subjected to PLS-DA modeling, as described above. The resampling procedure is then performed 100 times to account for variability in the sampling procedure. The AUROC values reported are average over the 100 resamplings.

Network Analysis

Network Reconstruction. Three different algorithms were used to infer metabolite−metabolite association networks together with the standard correlation approach. A brief description of the methods is presented here: We refer to the original publications for more details.[19,37,38] The following synthetic description is based, with minimal adaptation, on the one provided in ref 38. Networks were built using only the quantified metabolites.

Method Based on Correlations (CORR). The association between any pair of metabolites is measured through the absolute value of Pearson's correlation.

CLR Algorithm. The CLR (Context Likelihood of Relatedness) algorithm[39] uses mutual information as a measure of the similarity between the profiles of any two metabolites in the data that is then compared against the local context for each possible interaction: In this way possible spurious (indirect) associations are removed. This results in a weighted adjacency matrix that can be transformed into a binary adjacency matrix imposing a threshold of 0 (default value) on its entries.

ARACNE Algorithm. As CLR, ARACNE (Algorithm for the Reconstruction of Accurate Cellular Networks)[40] uses mutual information (MI) as a measure of the similarity between the profiles of any two metabolites. The properties of MI are used to prune the network of spurious interactions. Specifically, edges for which mutual independence cannot be ruled out at a given level are removed from the network. The default 0 threshold has been used.

PCLRC Algorithm. The PCLRC[19] (Probabilistic Context Likelihood of Relatedness) algorithm is a modification of the CLR algorithm (using correlation instead of MI to measure similarity between metabolite profiles), and on iterative sampling the data set results in a weighted adjacency matrix containing an estimate of the association likeliness between any two metabolites. The default values of 25−75% data split and 90% confidence level have been used. An R implementation of this algorithm is available at semantics.systemsbiology.nl.

Construction of Plasma Metabolite Networks. The plasma metabolite−metabolite association network takes a wisdom of crowd approach as detailed in ref 13. For each set of samples (males, females, old M, young M, old F, young F), adjacency matrices $(a_{ij})_m$ (with m = 1, 2, ..., 4) were obtained using the above-described methods. The entries of such matrices matrix are real numbers in the range $[-1, 1]$ for correlation matrices, in the $[0, +\infty]$ range for mutual information matrices, or $[0, 1]$ for probabilistic networks, indicating the strength or the likelihood of the metabolite−metabolite associations. These matrices are binarized to 0 and 1, imposing a threshold on the values[9−11,13]

$$\{a_{ij}\} \rightarrow \begin{cases} 1 & \text{if } a_{ij} > \tau_m \\ 0 & \text{otherwise} \end{cases} \quad (1)$$

The values of τ_m depend on the method considered: 0 for ARACNE and CLR, 0.95 for PCLRC, and 0.6 for the correlation map, as previously detailed.[37] The four networks were then superimposed

$$\{a_{ij}\} \rightarrow \sum_{m=1}^{4} \{a_{ij}\}_m \quad (2)$$

The final adjacency matrix, representing the metabolite network, was defined by retaining only those links inferred by three or more methods, as suggested in ref 37

$$\{a_{ij}\} \rightarrow \begin{cases} 1 & \text{if } a_{ij} \geq 3 \\ 0 & \text{otherwise} \end{cases} \quad (3)$$

In total, six networks were defined: males, females, old M, young M, Old F, and Young F.

Network vertexes are colored according to their modularity calculated using the R package "igraph".[41] The modularity of each graph with respect to vertex type measures how separated the different vertex types are from each other. It is defined as

$$Q = \frac{1}{2m} \sum_{i,j}^{m} \left(A_{ij} - \frac{k_i \cdot k_j}{2m} \right) \cdot \delta(c_i c_j) \quad (4)$$

where m is the number of edges, A_{ij} is the element of the adjacency matrix $\mathbf{A}$ in row i and column j, k_i is the degree of i, k_j is the degree of j, c_i is the type (or component) of i, c_j is that of j, the sum goes over all i and j pairs of vertices, and $\delta(x, y)$ is 1 if $x = y$ and 0 otherwise.

Ethical Issues

The original data were collected in accordance with the 1964 Helsinki declaration and its later amendments.

RESULTS AND DISCUSSION

Sex-Related Effects on Blood Metabolite Profiles

Discrimination (PLS-DA) analysis among the NMR profiles of males and females was performed using PLS-DA (Figure 2A). The data set was unbalanced, with far more males (n = 661) than females (n = 163), reflecting the sex bias observed among

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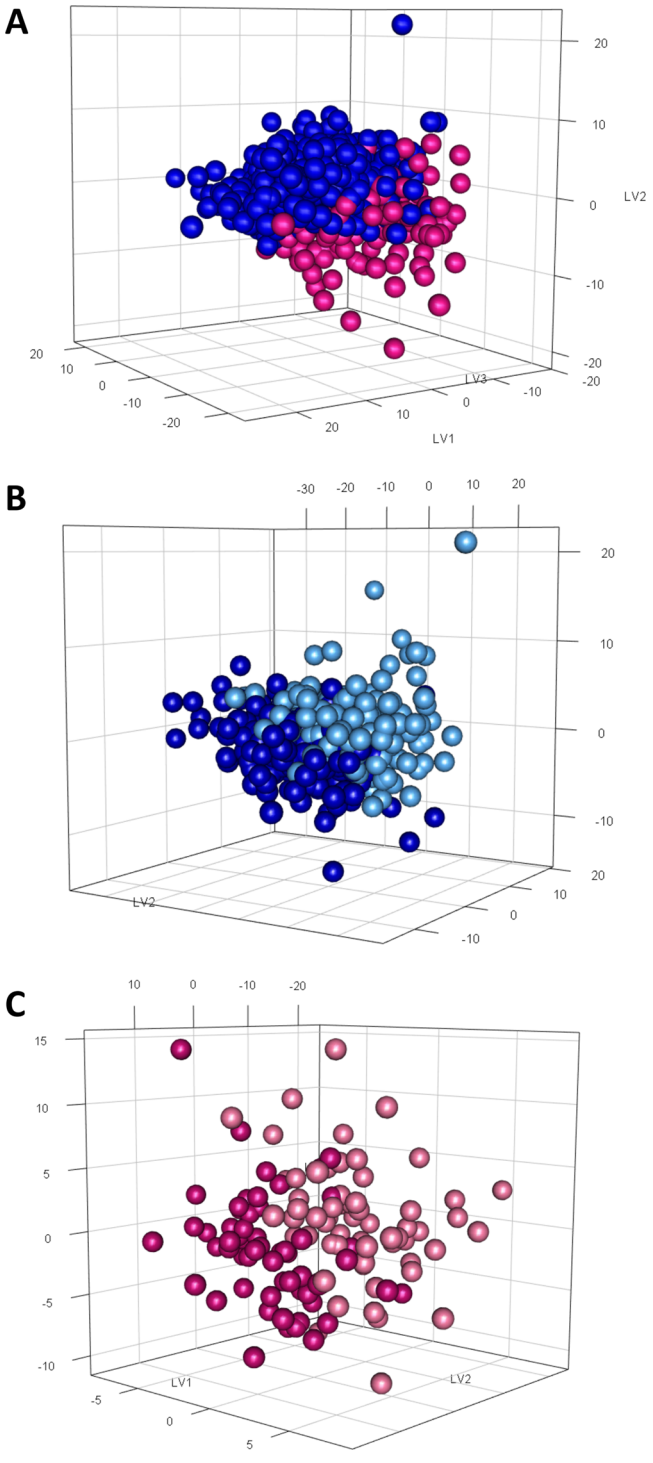

Figure 2. Scatter plot of the first two latent variables (LVs) of the PLS-DA models on NMR plasma metabolite concentration profiles of: (A) male (blue dots, n = 661) and female (pink dots, n = 183); (B) young (light blue, n = 234) and old (dark blue, n = 213) male subjects; and (C) young (light pink, n = 63) and old (dark pink, n = 60) female subjects. The corresponding discrimination accuracy obtained using kNN for each different model is given in Table 2.

blood donor volunteers in Italy.[42] To avoid possible bias resulting from different sample size of the two groups, the analysis was performed by resampling n = 150 subjects from both the male and female groups and taking the average over 10^2 repetitions.

We obtained excellent discrimination between the plasma profiles (binned NMR spectra) of males (n = 150) and females (n = 150) (see Table 2), a result in line with previous observations.[22] Discrimination accuracy was also high when only a reduced set of 22 quantified metabolites was used, indicating that sex-specific biological information is thoroughly represented by a limited number of metabolites, consistent with what is observed in the case of metabolite and metabolic profiling in urines.[13]

Table 2. Average AUROC (Area Under the Receiver Operative Curve) and Accuracy for the PLS-DA Model[a]

PLS-DA	AUROC	accuracy	P-value
Full NMR CPMG Spectra			
males vs females	0.97 ± 0.01	0.95 ± 0.02	0.001
young vs old males	0.97 ± 0.01	0.92 ± 0.02	0.001
young vs old females	0.98 ± 0.02	0.93 ± 0.04	0.001
Quantified Metabolites			
males vs females	0.95 ± 0.01	0.85 ± 0.03	0.001
young vs old males	0.78 ± 0.02	0.71 ± 0.02	0.001
young vs old females	0.85 ± 0.05	0.76 ± 0.05	0.001

[a]Values are given together with the standard error calculated over 10^2 repetitions of the double cross-validation procedure (2CV); in the case of the PLS-DA model of males vs females a 100× resampling procedure has been utilized to compensate for group unbalancedness (see Material and Methods for more details). The significance (P-value) of the discriminant model has been assessed by mean of permutation test (= 10^3 permutations).

Univariate analysis was applied only on the quantified metabolites, and several were found to be statistically different between males (using the full cohort n = 661) and female (n = 163) (P-value <0.05 after Bonferroni correction): Creatine showed higher levels in females; instead, phenylalanine, glutamine, proline, histidine, glutamate, tyrosine, valine, propylene glycol, leucine, isoleucine, creatinine, and acetone were higher in males (see Supplementary Table S2 for a summary). Higher levels of creatinine in males have been reported for a long time and have been found to relate to muscle mass. Overall we observed systematic higher plasma concentrations of amino acids, especially BCAA, in males, which may be linked to differences in muscle mass metabolism,[43] to the larger muscle mass of men,[44] or to the higher protein intake of men with respect to women.[45]

Our results are in line with those of Krumsiek and coworkers,[22] who addressed sex-specific differences in the metabolism of healthy subjects, although two different analytical approaches were applied (mass spectrometry versus NMR spectroscopy) and the study size was different. Interestingly, we found creatinine and tyrosine trends to be consistent with their findings but in contrast with what was reported by Dunn et al. in their U.K. population study,[46] where lower levels of creatinine and tyrosine in males were observed. Moreover, we did not observe sex-related differences in glucose and lactate plasma levels.

Sex-Related Effects on Plasma Metabolite Association Networks

Plasma metabolite−metabolite association networks were estimated taking a so-called "wisdom of crowd" approach, that is, combing the results of four different methods to avoid bias induced by the choice of a particular method, following an established practice.[13,37,47] Sex-specific networks are shown in

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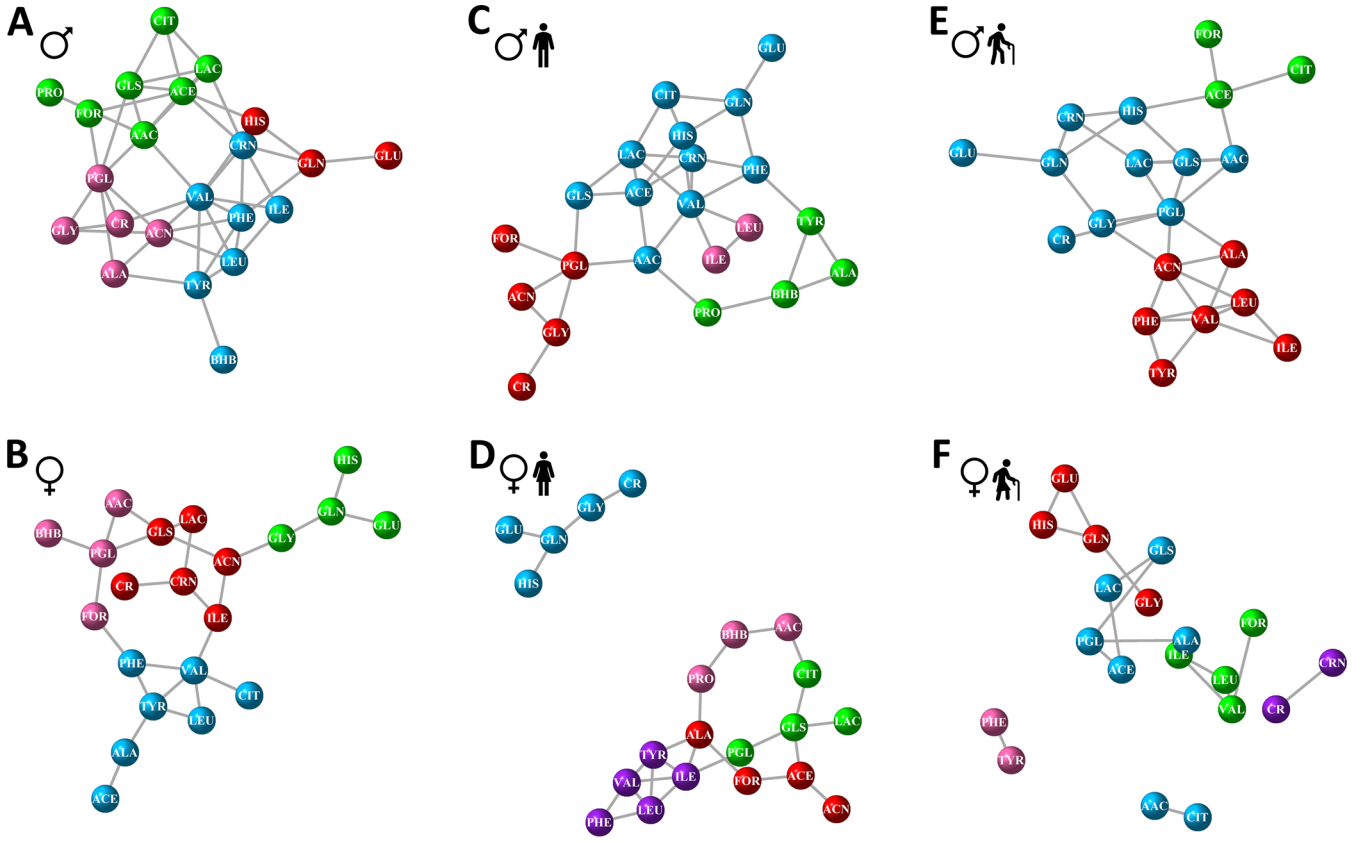

Figure 3. Metabolite−metabolite association networks reconstructed from the plasma metabolite concentration profiles of: (A) males, (B) females, (C), young males, (D) young females, (E) old males, and (F) old females. Vertexes are colored according to metabolite modularity (see eq 4). Abbreviations: AAC (acetoacetate), ACE (acetate), ACN (acetone), ALA (alanine), BHB (3-hydroxybutyrate), CIT (citrate), CR (creatine), CRN (creatinine), FOR (formate), GLN (glutamine), GLS (glucose), GLU (glutamate), GLY (glycine), HIS (histidine), ILE (isoleucine), LAC (lactate), LEU (leucine), PGL (propylene glycol), PHE (phenylalanine), PRO (proline), TYR (tyrosine), and VAL (valine).

Figure 3A (males, constructed using n = 661 samples) and Figure 3B (females, n = 163 s). Networks were arranged and colored according to metabolite modularity (see eq 4). In both sex-specific networks two amino acidic clusters are visible; moreover, in males also metabolites related to glucose/energetic metabolism formed a cluster. This evidence is in line with that observed by Krumsiek and coworkers.[22]

We observed that, in general, the female-specific network is less densely connected than the male-specific network (see Figure 3 for network representation and Figure 4 for an overview of metabolite connectivity), and this different topology is likely to reflect underlying metabolic differences, but it could also be affected by the differences in the sample size. Highly connected metabolites, the so-called "hubs", play a special role in biological network and network analysis because in many cases, for example, in gene coexpression and regulatory networks,[48,49] metabolic networks, protein−protein interaction networks,[50−52] and cell−cell interaction networks,[53] there is evidence of few highly connected nodes that are considered to play crucial biological roles; for instance, it has been shown that, in yeast, proteins that are highly connected are essential for survival.[48,50]

Consistent with previous approaches,[54] in the first stage, we considered as hubs those metabolites with a degree >5 and clustering coefficient <0.03. In male-specific networks only leucine, glucose, lactate, valine, acetoacetate, and creatinine satisfied these selection criteria, whereas in the female-specific network only valine was found to be a hub according to this classification. Interestingly, valine is a hub metabolite in both networks, yet it was more connected in males than in females (nine vs five connections); four connections are in common (isoleucine, leucine, phenylalanine, and tyrosine) while citrate is a connection found only in females, and acetoacetate, creatinine, creatine, acetone, and histidine are connected with valine only in males. It is worth noting that citrate excretion levels differ significantly in males and females, and, in particular, the sex differences have been found to increase with age.[55] We used high connectivity and low clustering coefficient to exclude metabolites that could be highly interconnected because they participate in the same molecular machine, such as amino acids deriving from protein metabolism and catabolism, while focusing on metabolites that could be pivotal in discriminating between males and females network topology. Indeed, according to this criterion, isoleucine and leucine, whose levels are also dependent on protein intake, are excluded.

The hub metabolites in the male-specific network can be related to the propionate metabolism and the valine, leucine, and isoleucine degradation. Branched amino acids cannot be synthesized de novo, so their homeostasis is maintained by degradation and dietary intake only,[56] which we speculate may be differentially regulated in males and females given the different connectivity observed in the sex-specific networks. Whether protein metabolism and catabolism are different in males and females is an open question, and there are conflicting

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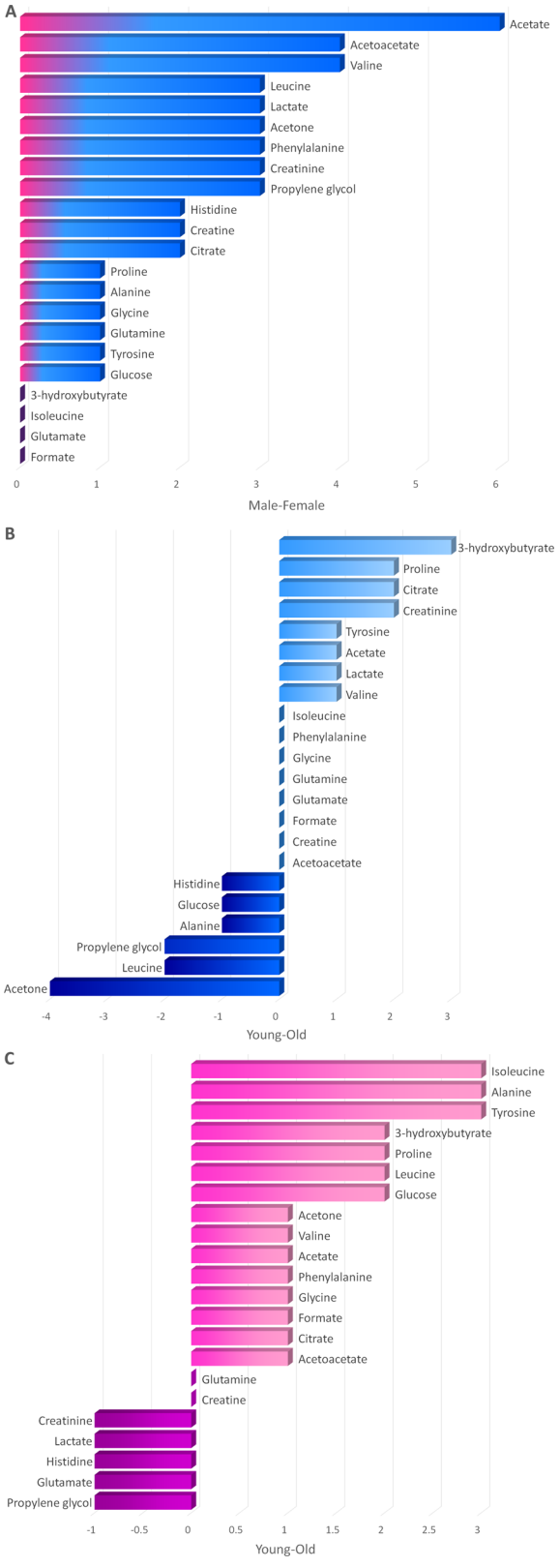

Figure 4. Differences in metabolite connectivity (node degree) between (A) males and females, (B) young and old males, and (C) young and old females.

results, also given the different methodologies used in such studies. However, the present results seem to suggest the existence of sex-specific differences in protein metabolism: Whether these differences are due to different body mass composition or different protein intake or are attributable to sex hormones, as suggested,[57,58] has to be ascertained.

Propionate and BCAA metabolism overlap at the gene level while propanoate is involved in BCAA metabolism but is also involved in the short-chain fatty acid metabolism: this result can be reconciled with the hypothesis of sexual dimorphism in human lipid metabolism,[59] for which evidence has also been provided in metabolomics studies.[23,60]

Similar to previous studies,[54] we also analyzed the networks using a hub definition of connectivity greater than 5 without considering the clustering coefficient, and we focused solely on those metabolites for which the difference Δ between a metabolite connectivity in the male and female specific network was >2. In addition to the valine, leucine, and isoleucine catabolism and propanoate metabolism, we found two other pathways possibly associated with sex-specific metabolite connectivity differences, namely, synthesis and degradation of ketone bodies and pyruvate metabolism. The fact that ketone bodies catabolism emerges as a discriminant between males and females is interesting; blood samples have been collected after overnight fasting, and it is known that during short-term fasting the decrease in plasma glucose and the increase in ketone body levels are greater in females than in males,[61−65] and evidence has been given for the existence of sexual dimorphism for what concerns lipid metabolism in response to short-term fasting.[61] However, as for the case of protein metabolism, the causes are not known, and a role of sexual hormones has been proposed.[61] Pyruvate is produced during glycolysis, and it has been suggested that females have a significantly lower overall capacity for aerobic oxidation and for anaerobic glycolysis than males.[66,67]

Age Effects on Blood Metabolite Networks

Whereas sexual dimorphism provides a clear discriminant for a comparative analysis, dividing the population under investigation in age groups is less straightforward. According to the World Health Organization,[68] the chronological age of 65 years is used for the definition of "elderly" or older person. However, in this study, the cohort is made of blood donor volunteers, and in Italy the maximum age for blood donation is set to 65. Here the average age is 41 ± 11 years for males and 42 ± 12 years for females; we are thus in the presence of a relatively young, healthy, and homogeneous population. To set boundaries for discriminant analysis, we take a pragmatic approach, by taking the lower (L) and upper (U) tertiles of the age distribution and labeling as "old" those individual with age > U and as "young" those with age < L. For males we had L = 35 and U = 45 years and for females L = 37 and U = 48 years (see Table 1 for the size of the resulting subgroups).

Discriminant analysis was performed using PLS-DA on these age-defined groups (Figure 2B,C: young males (n = 234) vs old males (n = 213) and young females (n = 63) vs old females (n = 60)). As shown in Table 2 we found good discrimination accuracy between the two age classes, as previously reported,[7,69,70] with discrimination higher in males than in females when analyzing both binned spectra and the array of 22 metabolites. This can be due to the smaller sample size in the case of females (447 males vs 123 females), which can affect the discrimination power of the statistical model, or reflect the fact that males age faster than females,[71] resulting in larger age-related differences in males in respect with females. Furthermore, in women, oral contraceptive use and menopausal state have been found to significantly alter the metabolome,

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proving an additional source of variability and possible confounding factors.[72,73] However, given the relatively young age of the study volunteers, the hypothesis of menopausal status can be probably ruled out.

Univariate analysis of the metabolite concentrations was also performed for age comparisons in both sexes: acetate and histidine exhibited statistically higher concentrations in young males; conversely, alanine and creatine were elevated in old males (Supplementary Table S3). For the female cohort, glucose, glutamine, glycine, tyrosine, and creatine presented statistically higher concentrations in old females (Supplementary Table S4). Interestingly, only in women we can observe an increasing of glucose with the age; this evidence could imply an impairment of the glucose metabolism and therefore a latent risk of developing Type II diabetes.

The analysis of the metabolite connectivity (Δ degree) was also performed for the comparison of young males (234 subjects) versus old males (213 subjects) and of young females (63 subjects) versus old females (60 subjects). For males, 3-hydroxybutyrate, proline, citrate, and creatinine showed increased connectivity in the young cohort ($\Delta \geq 2$); instead, acetone, leucine, and propylene glycol decreased their connectivity with age (Figure 4B). Isoleucine, phenylalanine, glycine, glutamine, glutamate, formate, creatine, and acetoacetate did not display any connectivity variations in the male cohort ($\Delta = 0$).

For females, isoleucine, alanine, tyrosine, 3-hydroxybutyrate, proline, leucine, and glucose were found to be more connected in the young cohort ($\Delta \geq 2$); instead, glutamine and creatine did not exhibit any changes in connectivity (Figure 4C).

As a general remark, we can observe a shrinkage of the number of connections in the older individuals, for both males (Figure 3C,E) and females (Figure 3D,F). Older males showed two hubs (valine and acetone) that are not present in younger ones; conversely, no hubs were found in females independent of age. Interestingly, Soltow et al.[74] in their study on metabolism and aging in common marmosets (*Callithrix jacchus*, a premiere primate model for studies of aging) reported that metabolite connectivity decreases with age, and these results are in line with what we are reporting in this paper. This evidence could indicate that further efforts in this direction and using these types of analyses (global metabolic profiling combined with network analysis) in well-designed models may reveal biomarkers associated with age-related phenotype and disease.

Valine was found to be a hub in older males but not in younger ones; although valine is not a hub in the networks of both young and old women, a decrease in connectivity is also present (see Figures 3 and 4). Studies in yeast[75] have revealed that the addition of serine, threonine, and valine in growth media promotes cellular sensitization and aging by activating different interconnected pathways; furthermore, the restriction of the intake of proteins or certain amino acids, including valine, has been associated with extended longevity and reduced incidence or progression of multiple age-related diseases.[76] Consistently, our data could imply a pivotal role of valine in the molecular mechanism of aging, with an increase in its "metabolic importance" with the age.

CONCLUSIONS

We have presented network reconstruction and analysis of experimentally identified relationships between metabolites and applied a differential network approach to analyze sex and age differences in a cohort of healthy subjects. Our results show significant differences between males and females for what concerns both metabolite concentrations and connections implying variations in the regulation of metabolic activities involving branches amino acids, lipids, and ketone bodies. As in the case of previous studies,[6,7,14−17] providing a rationale for the explanation of the functional role of the metabolites for which concentrations change due to age or sexual dimorphism is complicated, but we can hypothesize that hormonal and body composition differences between men and women are the underlying causes for many of our observations, as already observed for the proteome.[3]

The network approach seems to provide more insights than the standard approach, showing a decrease in the connectivity with age in both sex groups, demonstrating that this evidence is peculiar of aging even if the biological meaning of our result needs to be further investigated.

Although the age range is limited and sex is unequally biased toward males, this study provides important information on how common variables influence expression of the metabolic phenotype. Both age and sex are recognized confounders, and understanding these differences will be a critical component for the development of metabolomics-based systems biology as a population screening and precision medicine platform.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00404.

Supplementary Table S1. List of metabolites identified on 1D ^{1}H NMR spectra of AVIS healthy blood donors. Supplementary Table S2. Pairwise comparison of plasma metabolite concentration levels in males and females. Supplementary Table S3. Pairwise comparison of plasma metabolite concentration in young and old male subjects. Supplementary Table S4. Pairwise comparison of plasma metabolite concentration in young and old female subjects. (PDF)

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Author Contributions

C.L., E.S., L.T., and A.V. designed the study. E.S. and A.V. performed statistical data analyses. C.L., E.S., L.T., and A.V. interpreted the data and results, prepared the manuscript, and were responsible for its final content. All authors read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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