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Article

Differential Network Analysis Reveals Molecular Determinants Associated with Blood Pressure and Heart Rate in Healthy Subjects

Alessia Vignoli, Leonardo Tenori, Claudio Luchinat,* and Edoardo Saccenti*

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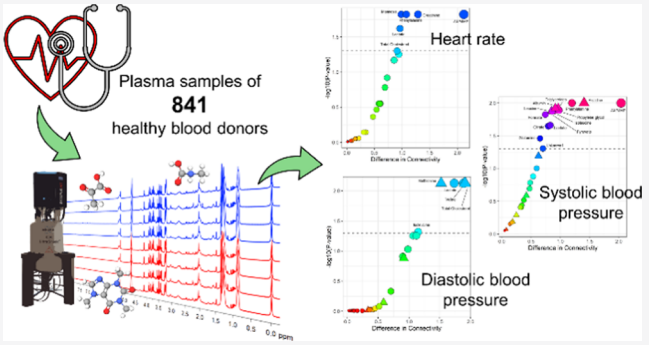
ABSTRACT: There is mounting evidence that subclinical non-pathological high blood pressure and heart rate during youth and adulthood steadily increase the risk of developing a cardiovascular disease at a later stage. For this reason, it is important to understand the mechanisms underlying the subclinical elevation of blood pressure and heart rate in healthy, relatively young individuals. In the present study, we present a network-based metabolomic study of blood plasma metabolites and lipids measured using nuclear magnetic resonance spectroscopy on 841 adult healthy blood donor volunteers, which were stratified for subclinical low and high blood pressure (systolic and diastolic) and heart rate. Our results indicate a rewiring of metabolic pathways active in high and low groups, indicating that the subjects with subclinical high blood pressure and heart rate could present latent cardiometabolic dysregulations.



KEYWORDS: *cardiovascular disease, cardiovascular risk, metabolomics, nuclear magnetic resonance*

INTRODUCTION

Elevated blood pressure represents one of the most important risk factors for cardiovascular diseases, often preceding heart failure, albeit the underlying mechanisms are still far from being clarified.[1] Increased cardiovascular risk associated with high blood pressure is not just limited to patients with overt hypertension but also affects healthy individuals with normal pressure in the higher range:[2] as a matter of fact, in a meta-analysis involving about 1 million adults (40−89 years old), the statistical evidence of an association between blood pressure and cardiovascular risk vanishes only below the threshold 115/75 mmHg.

Based on this evidence, the 2017 joint guidelines of the American Heart Association and American College of Cardiology[3] reclassified hypertension as systolic blood pressure higher than 130 mmHg and diastolic pressure ≥80 mmHg. As a consequence, hypertension prevalence increased in the general population, and it has been estimated that the number of patients suffering from hypertension is approaching the number of normotensive persons.[4] Furthermore, the 2017 guidelines also introduced the intermediate category of elevated blood pressure, defined as systolic pressure between 120 and 129 mmHg with diastolic pressure ≤80 mmHg.

The definition of this new intermediate category acknowledges what is emerging from the medical literature: exposure to mild, nonpathological, blood pressure elevation during youth and adulthood steadily increases the risk of developing a cardiovascular disease at a later stage.[5] Although many studies have analyzed the effects and consequences of high levels of blood pressure in aged individuals, it is now believed that the seeds of future cardiovascular risk are planted in early years[1] because it seems that there is cumulative damage due to elevated blood pressure over time that treatments at a later age are unable to repair or only able to repair partially. For this reason, it is important to understand the mechanisms underlying blood pressure elevation in healthy, relatively young individuals.

Similarly, heart rate is an established prognostic factor for cardiovascular, cerebrovascular, and all-cause mortality in both the general population and in patients with cerebrovascular and cardiovascular diseases.[6] Many studies discuss the relationships between high heart rate and morbidity and mortality, and the results are concordant regarding the existence of this association. Interestingly, this connection is usually less evident in women than in men. The increase in heart rate is commonly concurrent with the increase in blood

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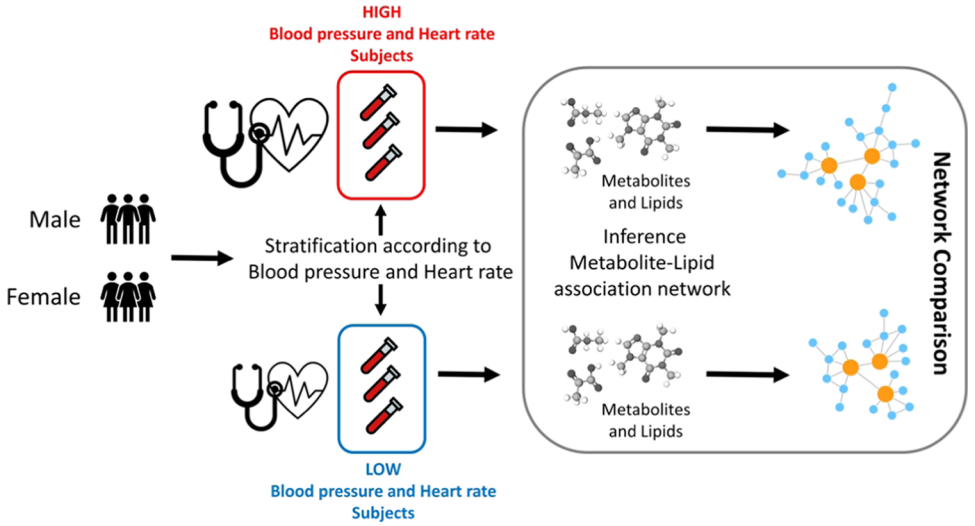

Figure 1. Overview of the study design to investigate differences between metabolite and lipid association networks of healthy subjects with high and low blood pressure (systolic and diastolic) and heart rate. Metabolite−metabolite association networks were inferred from the two groups using the PCLRC algorithm and compared to detect metabolites with differential connectivity with respect to physiological conditions (high/low pressure or heart rate).

pressure; however, these two prognostic factors have been found to be independent.[6]

In contrast with blood pressure, there is no official threshold for heart rate associated with increased cardiovascular risk and currently, a heart rate cutoff >100 beats/min is used for the diagnosis of tachycardia;[7] however, this limit was set arbitrarily when heart rate was not yet regarded as a risk factor for cardiovascular disease and was defined only with the diagnostic purpose of characterizing an overt disease state from a normal condition.[8] A medical literature survey indicates heart rate normality between 60 and 80 beats/min, with a value of around 64 beats/min the lower limit from which the cardiovascular risk starts to increase.[9]

Metabolomics has already proved to be an excellent instrument for biomedical research covering broad application areas:[10] disease diagnosis[11,12] and prognosis,[13−15] monitoring personal response to drug administrations[16,17] and lifestyle interventions,[18−20] and studying of the biochemical mechanisms underlying different pathological conditions.[21−28] Biological networks and network analysis of metabolites represent a further step in the comprehension of biological systems, since not only the singular components are considered but also their interconnections and their function as a whole.[29] Metabolite association patterns can change with the onset of pathophysiological conditions, and networks can be compared across conditions under the assumption that differences and commonalities in the biological processes are reflected in the characteristics of the reconstructed networks.[30,31]

The aim of this study was to analyze the metabolic profiles obtained by nuclear magnetic resonance (NMR) spectroscopy of blood plasma samples[32] of a large cohort of healthy adults, with limited evidence of cardiovascular risk factors. We used a metabolite−metabolite association network approach to investigate and explore the existence of possible molecular mechanisms underlying the different clinical profiles represented by high/low values of systolic and diastolic blood pressure and heart rate. We built a metabolite and lipid association network for each one of the three abovementioned clinical parameters. Data for males and females were analyzed separately, to take sex-related differences into account, and corrected for confounding factors;[33] we implemented a recently proposed statistical approach that extracts specific parts of the metabolite concentrations that are related specifically with blood pressure and heart rate, thus removing the effect of other clinical confounding factors. For each sex-specific group, we obtained six metabolite and lipid association networks corresponding to high (elevated) (>120 mmHg) and low systolic blood pressure, high (elevated) (>80 mmHg) and low diastolic blood pressure, and high (>70 bpm) and low heart rate. An overview of the study design is given in Figure 1.

Our results indicate that subclinical manifestations of high blood pressure and heart rate in healthy subjects is reflected in subtle metabolic changes that do not result in obvious blood metabolite and lipid concentrations but result in alteration rewiring of the metabolic connectivity of circulating blood metabolites pointing to cardiac energy metabolism and that such alterations are different for men and women.

MATERIALS AND METHODS

Study Population and Sample Collection

The study population comprises 841 adult healthy blood donor volunteers (659 males, 182 females) recruited in 2009 by 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). Blood donors had to adhere to the Italian regulation and guidelines for blood donation, which restricts donors of age 18−60 years, body weight >50 kg, systolic blood pressure 110−148 mmHg, diastolic blood pressure 60−100 mmHg, absence of (manifested) infectious diseases, absence of chronic diseases, no current menstruation, no consumption of medicines within 1 week before donation (bd), no common diseases (such as flu, cold, bronchitis) within 2 weeks bd, no surgery within 3 months bd, no endoscopic exams within 4 months bd, no pregnancy within 12 months bd, no abortion within 4 months bd, no travel to tropical countries within 6 months bd, and, in particular, no sport activity within 24 h bd. All samples were collected under a fasting condition. Ethylenediaminetetraacetic acid (EDTA) plasma samples were collected and handled as

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previously described[33−35] and stored at −80 °C pending nuclear magnetic resonance (NMR) analysis.

Study subjects were retrospectively divided into six groups: high (elevated) and low systolic blood pressure (setting a discriminant threshold at >120 mmHg for elevated pressure), high (elevated) and low diastolic blood pressure (discriminant threshold at >80 mmHg for elevated pressure), and high and low heart rate (discriminant threshold at >70 bpm for heart rate).

Baseline characteristics of the full cohort are given in Table 1. Characteristics of the six patient groups previously defined are summarized in Table 2.

Table 1. Demographic and Clinical Characteristics of the Study Cohort

	females (182)	males (659)	*P*-value
demographic and clinical characteristics	mean (SD)	mean (SD)	
age (years)	42 (12.0)	41 (10.7)	1.87×10^{-01}
heart rate (bpm)	72 (5.3)	70 (6.0)	1.31×10^{-03}
diastolic blood pressure (mmHg)	78 (6.9)	81 (6.9)	6.01×10^{-06}
systolic blood pressure (mmHg)	119 (10.3)	124 (10.5)	5.31×10^{-08}
albumin (g/L)	59.3 (3.1)	61.5 (12.0)	2.11×10^{-05}
glycemia (mg/dL)	87.9 (14.0)	90.0 (12.6)	9.09×10^{-02}
total protidemy (g/dL)	7.8 (0.4)	7.8 (0.4)	4.92×10^{-01}
total cholesterol (mg/dL)	212.7 (35.6)	201.7 (35.0)	6.37×10^{-03}
triglycerides (mg/dL)	89.7 (51.9)	105.4 (57.8)	6.53×10^{-05}
distributions of groups of interest	*n* (%)	*n* (%)	
heart rate (bpm) >70	136 (74.7%)	401 (60.8%)	
diastolic blood pressure (mmHg) >80	33 (18.1%)	224 (40.0%)	
systolic blood pressure (mmHg) >120	40 (22.0%)	287 (43.6%)	

NMR Experiments

One-dimensional ^{1}H NMR spectra were acquired on a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz and equipped with a 5 mm cryoprobe, an automatic tuning-matching (ATM), and an automatic sample changer. A water-suppressed Carr−Purcell−Meiboom−Gill[36] (CPMG) spin−echo pulse sequence was used to obtain spectra in which broad signals of lipids and proteins were attenuated. An extended description of instrument configuration and setting of the NMR parameters can be found in previous publications.[33−35]

NMR spectra and associated clinical data were retrieved from the MetaboLights[37] database (http://www.ebi.ac.uk/metabolights) with accession number MTBLS147; 23 samples from the original data set were excluded from this analysis because demographic or clinical information relevant to this study was missing.

Quantification of Metabolites

In all NMR spectra, 23 metabolites were unambiguously assigned using matching routines of AssureNMR (Bruker BioSpin) and the Human Metabolome Database.[38] The relative quantification of these metabolites (concentrations in arbitrary units) was performed with an in-house-developed algorithm based on standard line-shape analysis methods. No data normalization was applied. Total cholesterol and triglycerides were measured using direct enzymatic assays.[39,40] However, in our previous article,[34] we already demonstrated that there is a good correlation between lipoproteins measured via NMR and via biochemical assays. For more detailed information, we refer the reader to previous publications.[33−35]

Statistical Methods

Handling of Missing Data. Missing data were present in some of the clinical parameters and covariates: total cholesterol 17.1% missing data, triglycerides 17.9%, systolic blood pressure 3.4%, diastolic blood pressure 3.9%, heart rate 4.3%, albumin 20.5%, and total protidemy 20.6%. Missing data were imputed using the random forest approach implemented in R package "missForest".[41] Default parameters were used. No missing data were present among metabolites because only metabolites detectable in all NMR plasma spectra were quantified.

Data Preprocessing. All of the subsequent analyses were performed using log-transformed metabolite and lipid concentrations. Data were adjusted for "Age" before univariate analysis and random forest modeling.

Univariate Metabolite Analysis. To infer differences between the metabolite levels in the comparison between the groups of interest, a Wilcoxon rank-sum test was used.[42] *P*-values were adjusted (FDR) for multiple testing using the Benjamini−Hochberg correction.[43]

Random Forest Modeling. The random forest algorithm was employed for sample classification to discriminate between different high/low groups in males and females, and three classification models were built to discriminate between high and low systolic and diastolic pressure and high and low heart rate. For all calculations, the R package "Random Forest"[44] was used to grow a forest of 1000 trees and the option "strata" was used to take into account the unbalanced number of subjects in each group to be compared. For each comparison, the procedure was repeated 100 times to take into account the variability due to the resampling step used by the RF algorithm to randomly select the same number of subjects from each group and so to build the model on balanced data. The size of the resampled groups was set to 90% of the smallest group. All results are given as the mean over the 100 iterations. The resampling was nested within the cross-validation step used to assess the quality of the prediction models in an unbiased way.

The cross-validated model quality statistics (accuracy, sensitivity, and specificity and the area under the ROC, AUROC) were calculated according to standard definitions.

The statistical significance of the results was assessed by means of a permutation test. Basically, the full analysis was repeated after class labels were randomly permuted to destroy the relationship between predictors and response. Repeating this K times, a null distribution D^{perm} of model quality measures is created from which the *P*-value for each measure can be calculated by comparing the value m_0 obtained from the original, nonpermuted data with the values m_1, m_2, ..., m_K obtained from the permuted data. For instance, the *P*-value for the AUROC is calculated as

$$P - \text{value}|_{\text{AUC}} = \frac{1 + \#(D^{\text{perm}}_{\text{AUC}} \geq \text{AUC}_0)}{K} \quad (1)$$

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Table 2. Description of Cohort Characteristics Based on the Three Outcomes of Interest[a]

		female			male		
		mean LG	mean HG	*P*-value	mean LG	mean HG	*P*-value
systolic blood pressure	age (years)	40.1	48.1	0.04	38.6	43.2	1.35×10^{-7}
	glycemia (mg/dL)	87.2	90.1	1.00	88.8	91.5	0.33
	total cholesterol (mg/dL)	206.6	227.1	0.47	197.0	207.1	9.58×10^{-4}
	triglycerides (mg/dL)	82.9	93.6	0.08	98.4	116.3	5.33×10^{-4}
	total protidemy (g/dL)	7.8	7.8	1.00	7.8	7.8	0.46
	albumin (g/L)	58.8	58.9	1.00	62.2	60.7	0.14
diastolic blood pressure	age (years)	40.2	49.2	0.02	38.8	44.0	6.57×10^{-7}
	glycemia (mg/dL)	87.3	90.3	0.19	89.1	91.9	0.50
	total cholesterol (mg/dL)	206.3	231.7	0.11	198.0	208.0	0.04
	triglycerides (mg/dL)	81.8	100.4	0.02	101.2	116.0	0.01
	total protidemy (g/dL)	7.8	7.7	0.27	7.9	7.8	0.05
	albumin (g/L)	58.7	59.2	0.71	61.9	60.9	0.50
heart rate	age (years)	42.0	41.8	0.83	40.0	40.9	0.45
	glycemia (mg/dL)	87.7	87.9	0.83	89.9	90.1	0.14
	total cholesterol (mg/dL)	214.6	210.0	0.86	199.0	203.1	0.05
	triglycerides (mg/dL)	83.7	85.9	0.83	101.6	109.4	0.45
	total protidemy (g/dL)	7.8	7.8	0.83	7.8	7.9	0.14
	albumin (g/L)	59.0	58.8	0.83	61.1	61.8	0.96

[a]Study subjects were retrospectively divided into six groups: high (HG) and low (LG) systolic blood pressure (setting a discriminant threshold at >120 mmHg for elevated pressure), high (HG) and low (LG) diastolic blood pressure (setting a discriminant threshold at >80 mmHg for elevated pressure), and high (HG) and low (LG) heart rate (setting a discriminant threshold at >70 bpm for heart rate).

where #(*) indicates the number of the elements of D^{perm} satisfying the inequality. Similar formulas are used to calculate the *P*-values associated with the other measures.

Identification of Metabolic Information Related to Clinical Variables

For each blood metabolite and lipid fraction, we modeled the variation of metabolite concentrations attributable to the clinical covariates, (i.e., systolic blood pressure, diastolic blood pressure, and heart rate) using the method proposed by Bartzis et al.[45] The rationale underlying this approach is that metabolites/lipids with similar relationships with a given covariable tend to be close to each other in the network, thus giving a better representation of the underlying biological phenomena.

Briefly, let $Y^{(p)}$ be the $(n \times 1)$ vector of the concentrations of the pth metabolite or lipid component (with $p = 1, 2, ..., P$) measured on $n = 659$ male and $n = 182$ female subjects and $\mathbf{X}$ be the $(n \times M)$ matrix containing $M = 3$ covariates recorded on n subjects. Let X_m be the $n \times 1$ vector containing the values for mth clinical variables and $X^{(-m)} = \{X_1, X_2, ..., X_{(m-1)}\}$ be the remaining $m - 1$ clinical variables. The information $Y^{(p)}$ of a metabolite or lipid component p associated with a specific clinical variable, m, was estimated by regressing $Y^{(p)}$ on $\mathbf{X}$ and retaining only the main effects and interactions with covariate X_m

$$\hat{Y}^{(p)} = \hat{\beta}^{(p)} X_m + \sum_{\delta \in \Delta} \hat{\eta}_{\delta}^{(p)} X_m \circ \prod_{m-1}^{j=1} X_j^{\delta_j} \tag{2}$$

where the term $\sum_{\delta \in \Delta} \hat{\eta}_{\delta}^{(p)} X_m \circ \prod_{j=1}^{m-1} X_j^{\delta_j}$ models all main effects and second- and higher-order interactions in terms of clinical variables.

For each clinical parameter m, the procedure is repeated for all P metabolites and lipid components to obtain $M = 3n \times P$ data sets, $Y_m = \{Y_{(1)}, Y_{(2)}, ..., Y_{(p)}\}$ containing a *part of the measured* metabolite and lipid concentrations associated with each one of the three clinical parameters. The reader is referred to the original study[45] for more details on the methodology and its implementation.

Network Analysis

Reconstruction of Metabolite and Lipid Association Networks. The Probabilistic Context Likelihood of Relatedness on Correlation (PCLRC) algorithm[35] was used to build metabolite and lipid association networks. The algorithm allows for the robust estimation of correlation employing a resampling strategy in combination with a modified version of the Context Likelihood of Relatedness (CLR)[46] to remove nonsignificant background correlations. The algorithm returns a probability matrix $\mathbf{P}$ with values between 0 and 1 that was used to filter significant correlation r_{ij} between pairs of metabolites/lipids. In particular

$$r_{ij} = \begin{cases} r_{ij} & \text{if} p_{ij} \geq 0.90 \\ 0 & \text{if} p_{ij} < 0.90 \end{cases} \tag{3}$$

We built a metabolite and lipid association network for each of the $3 \times P$ (for women) and $3 \times P$ (for men) data sets $Y_m = \{Y_{(1)}, Y_{(2)}, ..., Y_{(p)}\}$ containing the part of the measured metabolite and lipid concentrations associated with each of the three clinical parameters. We analyzed data for males and females separately, obtaining six metabolite and lipid association networks for both men and women. The six networks correspond to high (>120 mmHg) and low systolic blood pressure, high (>80 mmHg) and low systolic blood pressure, and high (>70 bpm) and low heart rate (Figure 1).

Network Differential Connectivity Analysis. Given a network a belonging to category S, the connectivity $\chi_i^{a \in S}$ for metabolite/lipid i is defined as

$$\chi_i^{a \in S} = \left(\sum_{j=1}^{J} |r_{ij}| \right) - 1 \tag{4}$$

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Table 3. Univariate Metabolite Analysis (Adjusted for Age)[a]

	heart rate		systolic blood pressure		diastolic blood pressure	
	males	females	males	females	males	females
leucine	0.7668	0.9441	0.0244 (↓)	0.9202	0.0317 (↓)	0.9971
isoleucine	0.7255	0.9441	0.9995	0.9659	0.8186	0.9074
valine	0.7668	0.6682	0.0443 (↓)	0.7910	0.236	0.9074
unknown1	0.6960	0.9923	0.2683	0.9202	0.3891	0.9789
propylene glycol	0.8564	0.9571	0.4475	0.9202	0.145	0.9789
3-hydroxybutyrate	0.8564	0.9571	0.2683	0.9079	0.4479	0.9074
alanine	0.9095	0.9441	0.9995	0.9479	0.1749	0.9074
acetate	0.6960	0.9441	0.0028 (↓)*	0.8952	0.2256	0.9074
glutamate	0.6960	0.9441	0.2883	0.8646	0.2535	0.9074
pyruvate	0.6960	0.9571	0.9023	0.8952	0.7982	0.9074
glutamine	0.7255	0.9571	0.8948	0.7969	0.9673	0.9074
methionine	0.7668	0.9441	0.251	0.9079	0.0139 (↓)	0.9919
citrate	0.7255	0.9571	0.316	0.8646	0.6769	0.9074
unknown 2	0.8564	0.9923	0.1123	0.8952	0.2258	0.9971
glycine	0.9539	0.9571	0.0001 (↓)*	0.9079	0.0006 (↓)*	0.9789
creatine	0.8564	0.9571	0.9023	0.3573	0.5004	0.9074
creatinine	0.7668	0.9571	0.0081 (↓)	0.9079	0.2697	0.9074
lactate	0.7255	0.9571	0.4164	0.9079	0.4028	0.9074
mannose	0.6960	0.9571	0.6823	0.9079	0.236	0.9074
glucose	0.7668	0.9923	0.0565	0.7969	0.3971	0.9074
fumarate	0.7091	0.9441	0.2683	0.1240	0.1386	0.4897
tyrosine	0.7255	0.6682	0.1429	0.3573	0.1258	0.9074
histidine	0.8564	0.9441	0.0081 (↓)	0.9659	0.1749	0.9074
phenylalanine	0.8564	0.9441	0.157	0.9715	0.1749	0.9074
formate	0.8564	0.9441	0.1261	0.9202	0.1749	0.9074
AXP/IMP	0.7668	0.9571	0.4164	0.5898	0.3891	0.9074
total cholesterol	0.6960	0.9571	0.0073 (↑)	0.2672	0.0317 (↑)	0.4943
triglycerides	0.6960	0.9571	0.0001 (↑)*	0.2512	0.0139 (↑)	0.4897
total protidemy	0.6960	0.9571	0.316 (↑)*	0.8952	0.1258	0.9074
albumin	0.7255	0.9923	0.0427 (↓)	0.9202	0.3891	0.9074

[a]*P*-values reported are adjusted with Benjamini−Hochberg correction (FDR); for significant ones, the trend is also reported: ↑/↓ implies higher/lower levels in the high group. "*" refers to significant metabolites after adjustment for age.

Adopting a similar definition in the case of a network b, the differential connectivity ($\Delta_i^{a\in S,b\in S}$) of a metabolite/lipid i between two networks a and b belonging to the same category S is defined as

$$\Delta_i^{a\in S,b\in S} = \chi_i^{a\in S} - \chi_i^{b\in S} \quad (5)$$

Analyzing differential connectivity is interesting to understand the origin of the metabolite−metabolite connectivity. We distinguish two main cases of interest:

(1) Conserved differential connectivity: when the majority of edges is conserved between different conditions but with different weights, that is, edges that are present in both networks but with reduced or increased weight (i.e., correlation, in absolute value).
(2) Differentially conserved connectivity: when the majority of edges is not conserved between different conditions. In this case, the differential connectivity is due to different edges, which are not present in both networks.

Estimation of the Differential Network Connectivity. The statistical significance of the differential connectivity ($\Delta_{ik}^{a\in S,b\in S}$) was assessed by means of a permutation test. Briefly, the columns of every Y_m matrix were independently permuted to obtain a permutated matrix $\mathbf{X}_{(k)}$ whose column mean and variance were unchanged, but the association between the elements of different columns was destroyed.

For each metabolite/lipid, the differential connectivity was calculated for networks a and b built from the permuted data $\mathbf{X}_{(k)}$

$$\Delta_{i,k}^{a\in S,b\in S} = \chi_{i,k}^{a\in S} - \chi_{i,k}^{b\in S} \quad (6)$$

and the overall procedure was repeated $k = 100$ times to create a null distribution D_i of permutated differential connectivity values. The significance of a given differential connectivity value $\Delta_i^{a\in S,b\in S}$ (calculated on the original data) was calculated as a *P*-value using the formula[47]

$$P - \text{value} = \frac{1 + (|D_i| > |\Delta_i^{a\in S,b\in S}|)}{k} \quad (7)$$

RESULTS AND DISCUSSION

Metabolite Univariate Analysis Comparing High and Low Groups For Systolic, Diastolic Blood Pressure, and Heart Rate

The presence of sex-specific differences in human metabolism is well known and NMR metabolomics is sensitive to these differences.[33,48] For this reason, to obtain results unbiased by sex, the following analyses were performed separately for males and females.

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Table 4. Sex-Specific Random Forest Models (Adjusted for Age) for the Discrimination of High and Low Groups for Systolic Blood Pressure, Diastolic Blood Pressure, and Heart Rate

		accuracy, % (*P*-value)	sensitivity, % (*P*-value)	specificity, % (*P*-value)	AUROC (*P*-value)
males	systolic blood pressure	55.8 (0.01)	57.6 (0.01)	53.5 (0.07)	0.58 (0.02)
	diastolic blood pressure	56.2 (0.01)	55.1 (0.02)	56.2 (0.01)	0.59 (0.01)
	heart rate	49.8 (0.52)	50.4 (0.49)	49.4 (0.54)	0.51 (0.93)
females	systolic blood pressure	53.3 (0.30)	52.5 (0.34)	56.5 (0.12)	0.54 (0.62)
	diastolic blood pressure	46.5 (0.85)	46.4 (0.84)	46.7 (0.72)	0.55 (0.52)
	heart rate	48.1 (0.72)	44.8 (0.87)	49.2 (0.60)	0.55 (0.35)

Univariate analysis was performed on the 30 quantified metabolites to compare high and low groups for systolic and diastolic blood pressure and heart rate (Table 3).

No statistically significant differences in metabolite and lipid concentrations, both for males and females, were found in the comparison between high (bpm > 70) and low heart rate groups of subjects. Male subjects with systolic blood pressure higher than 120 mmHg showed significantly higher levels of total cholesterol and triglycerides and lower levels of leucine, valine, acetate, glycine, creatinine, histidine, and albumin. Of note, only acetate, glycine, triglycerides, and total protidemy were statistically significant after adjustment for age. Moreover, males with high diastolic blood pressure (P mmHg >80) presented higher concentrations of total cholesterol and triglycerides and lower concentrations of leucine, methionine, and glycine (only glycine remains significant after correction for age). Conversely, no significant difference emerged in the female group. These results can be attributed to the limited numerosity of the female group or to the fact that in women, oral contraceptive use and menopausal state could alter significantly the metabolome, providing an additional source of variability.[49,50] However, given the relatively young age of the blood donors, we speculated menopause could affect only a limited number of subjects.[33]

Although the study population can be regarded as healthy, otherwise the subjects would have not been admitted to blood donation given the strict regulations applied,[33] the glycine reduction in the high blood pressure group could be thought of as a prodromal sign of hypertension development. Glycine is involved in multiple metabolic pathways: it contributes to the reduction of oxidative stress, it promotes the availability of nitric oxide, and it plays a pivotal role in structural protein synthesis, such as collagen and elastin, the alterations of which have been associated to impaired elastic properties of vessels, a key aspect in hypertension pathogenesis.[51,52] Furthermore, circulating levels of glycine have been associated with the incidence of coronary heart diseases, especially in patients with high levels of lipoproteins.[53]

The role of lipoprotein metabolism has already been discussed:[54] we observed higher levels of cholesterol and triglycerides in the high systolic and diastolic blood pressure groups. We observed an association of total cholesterol with age (see Table 3), as previously reported.[55] The correlation between blood pressure and lipoproteins in our data set not only confirms the former hypothesis but also implicitly explains why treating both hypertension and dyslipidemia led to a stronger reduction of risk of ischemic heart disease with respect to the treatment of hypertension solely.[56,57]

Acetate, as well as other short-chain fatty acids (SCFAs) (propionate and butyrate), is produced by the gut microbiota mainly in the colon, especially after consumption of a diet rich in fibers. In our data set, acetate shows to be in lower concentrations in the high blood pressure groups (both systolic and diastolic), and this is in line with several pieces of evidence that point to a link between SCFAs and lower blood pressure levels in experimental models of hypertension, suggesting that a diet enriched in fibers and acetate could reduce blood pressure and thus could represent an effective counter move against hypertension.[58,59]

Branched-chain amino acids (BCAAs) leucine, valine, and albumin present reduced levels in the high systolic blood pressure group. This data is in contrast with the literature currently available.[60−62] However, the significance is lost after correction for age. Moreover, we did not take into account dietary habits and physical activity, both of which can influence the concentrations of leucine, valine, and albumin,[19,63] since we do not have information about these two confounding factors. However, we can exclude subjects who were engaged in physical activities 24 h before sample collection as per guidelines on blood donation (see the Materials and Methods section).

Comparison of High and Low Groups for Systolic and Diastolic Blood Pressure and Heart Rate Using Random Forest Modeling. Random forest classification was used to discriminate high and low groups for systolic and diastolic blood pressure and heart rate. Classification results are shown in Table 4. It was not possible to build classification models able to discriminate between high and low groups starting from metabolite and lipid concentrations specific to each group. This result indicates that metabolic differences underlying these subclinical phenotypes in healthy subjects are weakly reflected in metabolite/lipid concentrations, as also indicated by the results of the univariate analysis. However, as shown in the Differential Network Analysis of Metabolites Association Network Related to Blood Pressure and Heart Rate section, differential network analysis is able to discriminate between the two groups, indicating that these subclinical traits are reflected by changes in the relationships among molecular features like lipids and metabolites.

Differential Network Analysis of Metabolite Association Network Related to Blood Pressure and Heart Rate. We compared the metabolite and lipid association networks across different subject groups, i.e., high and low blood pressure (systolic and diastolic) and heart rate to explore the magnitude of metabolite/lipid connections and their variability. The rationale of this approach is that metabolites and lipids behave in an orchestrated manner and perturbations of the systems, such as those associated or induced by high/low pressure and heart rate, induce modifications in the relationships among metabolites, which is reflected in their connectivity patterns. Differential connectivity plots both for males and females are shown in Figure 2.

We observed differential connectivity of total cholesterol, lactate, mannose, phenylalanine, and AXP/IMP when compar-

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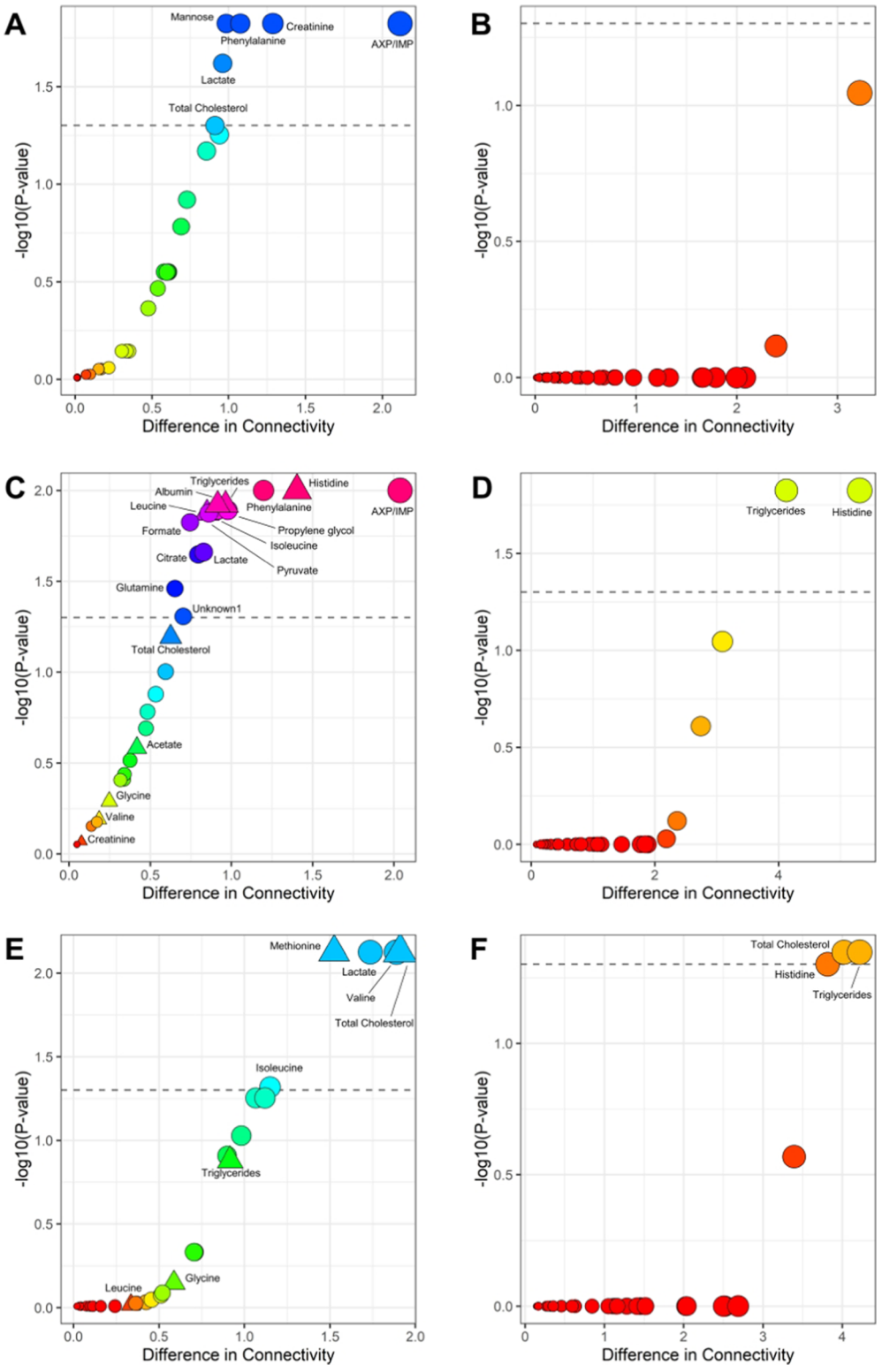

Figure 2. Differential network analysis. (A) Differentially connected metabolites between the networks specific for high and low heart rate specific for male subjects. (B) Differentially connected metabolites between the networks specific for high and low heart rate specific for female subjects. (C) Differentially connected metabolites for high and low systolic blood pressure for male subjects. (D) Differentially connected metabolites for high and low systolic blood pressure for female subjects. (E) Differentially connected metabolites for high and low diastolic blood pressure for male subjects. (F) Differentially connected metabolites for high and low diastolic blood pressure for female subjects. Only the names of differentially connected metabolites are shown. The difference in metabolite connectivity (see eq 5) is given against the corresponding *P*-value. The threshold for significance at 0.05 after Bonferroni correction is given by the horizontal line. Red to blue colors encode for the increasing difference. Triangles (▲) indicate metabolites whose concentration is different between high and low groups (see Table 3) and circles (●) indicate nondifferentially abundant metabolites.

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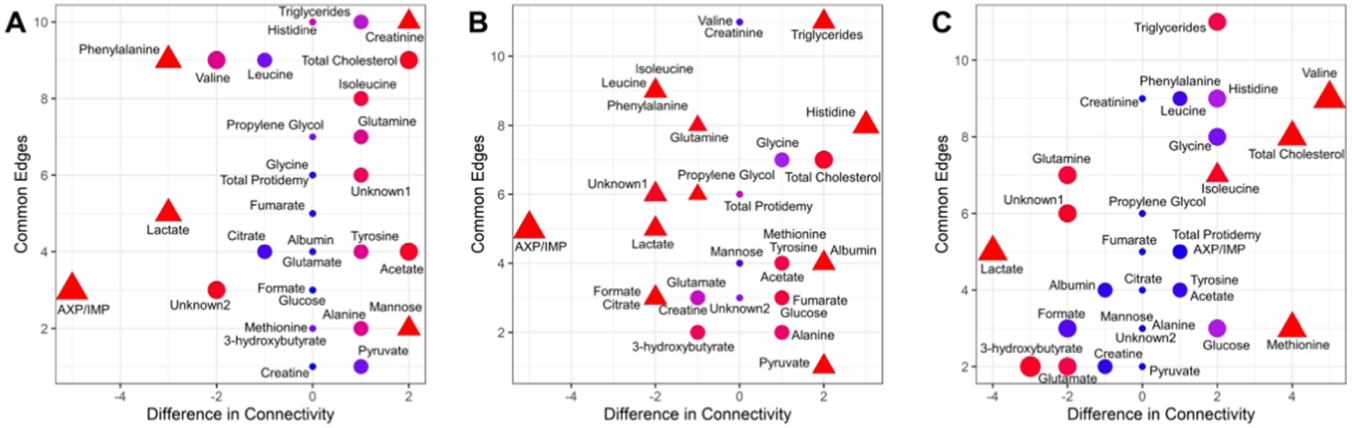

Figure 3. Conservation of metabolite/lipid connectivity across different subject groups (high/low systolic and diastolic blood pressure and heart rate). (A) Comparison between high/low systolic blood pressure groups. (B) Comparison between high/low diastolic blood pressure groups. (C) Comparison between high/low heart rate groups. Common edges indicate the number of links that are conserved between two conditions (high/low), although the edge weights (absolute value of the correlation between two metabolites/lipids) can be different. Conservation of the majority of edges between different conditions but with different weights indicates conserved differential connectivity; when the majority of edges is not conserved between different conditions and the differential connectivity is due to different edges which are not present in both networks, this refers to differentially conserved connectivity.

ing male subjects with high−low heart rates (Figure 2A), while no differences are observed for females (Figure 2B).

Differences in albumine, AXP/IMP, BCAA (valine, leucine, and isoleucine), citrate, formate, glutamine, histidine, lactate, phenylalanine, propylene glycol, pyruvate, and triglycerides were also observed in the case of high and low systolic pressure in males (Figure 2C), while for females only differences in the connectivity of histidine and triglycerides were observed (Figure 2D). For diastolic pressure, changes in the connectivity of isoleucine, lactate, methionine, total cholesterol, and valine can be observed for males (Figure 2E); for females, we observed changes in histidine, cholesterol, and triglycerides (Figure 2F).

Overall, we observed a stronger relationship between circulation metabolites and lipid fractions and blood pressure; heart rate seems to be only marginally related to overall metabolism, since both metabolite levels and metabolite connectivity present a small variation in the groups of interest.

In normal conditions, the energy requirement of the heart is elevated, and maintaining the efficiency of cardiac energy metabolism is pivotal for its biology and physiology. Furthermore, the dynamicity of its metabolism allows the heart to quickly alter its activity to maintain cardiac contraction in response to stressful stimulations, thus ensuring cardiomyocytes' survival.[64] However, when an initial adaptation in an energetically unfavorable state, in particular, related to glucose and fatty acid metabolism, turns into a prolonged metabolic shift, the maladaptation leads to progression to pathological conditions.[65−68]

Amino acid metabolism mostly occurs in the liver, but several amino acids, including BCAA and histidine, are catabolized in nonhepatic tissues, mostly neuron, kidney, and cardiac muscle.[69] BCAA, histidine, and cholesterol may be linked in a superpathway linking BCAA catabolism and glutamine metabolism, a set of pathways that are receiving renewed attention in the context of cardiovascular disease and health.

It has been shown that in normal hearts, branched amino acids inhibit the activity of pyruvate dehydrogenase complex (PDH); this results in decreased glucose oxidation and promotes fatty acid oxidation.[70]

The hexosamine pathway is one of the proposed metabolic mechanisms through which glutamine and glutamate may exert their effects on the heart. Glutamine is the co-substrate, together with fructose-6-phosphate, for glutamine fructose-6-phosphate aminotransferase (GFAT), which is the first and rate-limiting enzyme of the hexosamine pathway, and therefore is essential for its activity. It has been shown that glutamine significantly enhances the contribution of exogenous long-chain fatty acids (LCFAs) to β-oxidation and triglyceride (TG) formation and that the predominant metabolic effect of glutamine in the normoxic heart is to increase exogenous LCFA oxidation and storage.[71]

Another possible way of utilization of glutamine is through the so-called anaplerotic pathway (anaplerosis) through which glutamine is converted to glutamate, which is further converted to α-ketoglutarate in the citric acid cycle (CAC). Through this mechanism, the CAC intermediate pool can be partially replenished, when and if partially depleted in response to stress or acute increases in energy demand and thereby ensuring optimal CAC flux.[72] Although there is ample evidence of the activity of this pathway in proliferating cells, intestine, and kidney, there is no clear and supporting evidence that the heart is able to use glutamine as an anaplerotic substrate.[71,73,74]

It should be noted that our study population, being composed of blood donor volunteers, is highly homogenous for what concerns demographical and biochemical characteristics (see the Material and Methods section). The remodulation of the correlation patterns of BCAA, histidine, glutamine, and triglycerides observed when comparing subjects with high and low heart rate and blood pressure should be interpreted as a subclinical manifestation of latent cardiovascular risk.

Figure 3 shows the number of common edges, i.e., indicates the number of links that are conserved between two conditions (high/low). If between two conditions there are differences in conservation in metabolite connectivity, i.e., changes in correlation magnitude which sum up to the connectivity (see eq 2), then this can be seen as a proxy for metabolism rewiring

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or disruption. Metabolite−metabolite correlations arise from the combination of metabolic and regulatory reactions[75] and information on the underlying metabolic activity, which is encoded in both the magnitude and the sign. For instance, a rapid equilibrium condition or enzyme dominance would result in a strong positive correlation, while moiety conservation would result in a strong negative correlation.[76]

From the conservation/differential connectivity plot shown in Figure 3 (since only males showed statistically significant results, only results for male are shown), it can be seen that differential connectivity of metabolites involved in the hexosamine pathway originates from changes in the magnitude of the correlation, as indicated by a large number of common edges preserved; we thus see the presence of conserved differential connectivity, which indicates a remodulation of the hexosamine pathway rather than its disruption, which is consistent with the observed subclinical manifestations.

It has been observed that increased glucose metabolism via the hexosamine biosynthesis pathway and an associated increase in *O*-linked-*β*-*N*-acetylglucosamine (*O*-GlcNAc) levels in proteins contribute to the adverse effects on the heart at the level of the cardiomyocyte, which could contribute to contractile dysfunction and an increased risk for heart failure.[77,78]

We observed that most differentially connected metabolites are sex-specific and pertain to the male population investigated in this study (Figure 2). In general, we observed fewer differences in females than in males. Although this can be the effect of the different sizes of the two cohorts considered, and due to the inherent different power of the analysis, these results can be easily reconciled with the observation that nearly all aspects of metabolism, including energy balance as well as glucose and lipid metabolism, are regulated in a sexually dimorphic manner.[48,79]

We found that BCAA are differentially connected only in males, and indeed sex-related differences in regulation of branched-chain amino acid catabolism have been observed,[80,81] while differential connectivity of triglycerides is mostly associated with females, an observation supported by the fact that there are sex-related differences in the substrate used for (prolonged) physical activity: since males rely mostly on carbohydrate and amino acids, while females predominantly use fat. In addition, the glutamate metabolic pathway has been found to be different in men and women.[82]

Apart from the possibility for cardiac energy metabolism to be differentially regulated in males and females, the weaker results pointing to the remodulation of the activity of hexosamine in females could also be considered as evidence of the well-known fact that premenopausal females (given the relatively low age, mean age of 42 years, the females in our cohort can be considered premenopausal) have reduced incidence of cardiovascular disease when compared to age-matched males and thus remodulation effects may go underdetected in our analysis. This lower incidence (which increases after menopause) has been attributed to the protective effect of sex hormone levels, at least in part to estrogens, which exert their protective effect through many different mechanisms like reduced fibrosis, stimulation of angiogenesis, vasodilation, improved mitochondrial function, and reduced oxidative stress.

Lactate shows an increase of the differential connectivity in the male groups of high systolic and diastolic blood pressure and heart rate. Moreover, in subjects with high systolic blood pressure, we also observed an increase in pyruvate connections with respect to subjects with low pressure. Conversely, no significant changes in terms of lactate and pyruvate concentrations are detected in females. Both these pieces of evidence could indicate a variation in the activation of the metabolic pathways linked to the energetic metabolism, since lactate is mainly produced to sustain the energy needs during anaerobic conditions, which, however, do not alter the quantitative production of lactate and pyruvate. We can hypothesize that the different connectivity arrangement could be an early sign of a future cardiometabolic dysregulation that probably disrupts this metabolism and thus also changes metabolite concentrations.

CONCLUSIONS

In this paper, we have presented a differential network approach to experimentally identified metabolites to analyze their associations with blood pressure and heart rate in a population of healthy subjects, and we showed that subclinical manifestations like high/low blood pressure and heart rate in healthy subjects are better captured by analyzing changes in the correlation patterns among metabolites and lipids rather than concentrations alone.

Our results indicate that subjects with high and low blood pressure present different levels of several metabolic features and that there are even more marked differences in the pattern of connections between the different metabolites. Thus, the network approach seems to provide more insights than the standard approach. If we hypothesize that connectivity differences could represent substantial changes in the architecture of the metabolic pathways active in the two groups, we can conclude that the connectivity changes in the high-risk groups could embody an early sign of cardiometabolic dysregulations.

Although this study provides important information on the relationship between blood pressure and heart rate and circulating blood metabolites, some limitations should also be mentioned. First, our analyses do not consider diet habits and physical activities. Second, information regarding follow-up of the blood donors enrolled are missing; thus, although our results point to some early sign of cardiovascular disease or metabolic syndrome in the metabolomic profile and/or network architecture, any definitive conclusion is prevented. Third, it would be interesting to evaluate also a possible association with metabolic network architecture and prediabetes signs, but the available data did not allow us to proceed in this direction. For all of these reasons, in the future, further efforts to replicate these analyses in other study cohorts with available follow-up data are guaranteed.

AUTHOR INFORMATION

Corresponding Authors

Claudio Luchinat − *Consorzio Interuniversitario Risonanze Magnetiche di Metallo Proteine (CIRMMP), 50019 Sesto Fiorentino, Italy; Magnetic Resonance Center (CERM) and Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy;* orcid.org/0000-0003-2271-8921; Phone: +39 055 457 4296; Email: luchinat@cerm.unifi.it

Edoardo Saccenti − *Laboratory of Systems and Synthetic Biology, Wageningen University & Research, 6708 WE Wageningen, The Netherlands;* orcid.org/0000-0001-

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8284-4829; Phone: +31 (0)317 482018;
Email: edoardo.saccenti@wur.nl

Authors

Alessia Vignoli − *Consorzio Interuniversitario Risonanze Magnetiche di Metallo Proteine (CIRMMP), 50019 Sesto Fiorentino, Italy; Magnetic Resonance Center (CERM), University of Florence, 50019 Sesto Fiorentino, Italy;* orcid.org/0000-0003-4038-6596

Leonardo Tenori − *Magnetic Resonance Center (CERM) and Department of Chemistry "Ugo Schiff", University of Florence, 50019 Sesto Fiorentino, Italy;* orcid.org/0000-0001-6438-059X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jproteome.0c00882

Author Contributions

C.L., E.S., and L.T. designed the study. E.S. and A.V. performed statistical data analyses. C.L., E.S., L.T., and A.V. interpreted 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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