ORIGINAL RESEARCH

Diastolic Left Ventricular Function in Relation to Circulating Metabolic Biomarkers in a General Population

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***Background*—**The metabolic signature associated with subclinical diastolic left ventricular (LV) dysfunction in the population remains ill defined.

***Methods and Results*—**In 711 randomly recruited Flemish (50.8% women; mean age, 50.8 years), we assessed echocardiographic Doppler indexes of diastolic LV function in relation to 44 circulating metabolites determined by nuclear magnetic resonance spectroscopy. In multivariable-adjusted regression analysis with Bonferroni correction of significance levels applied, peak a' decreased ($P \leq 0.048$) and e'/a' increased ($P \leq 0.044$) with circulating tyrosine, high-density lipoprotein apolipoproteins, glucose+glutamine, and an unidentified molecule. Effect sizes expressed per 1-SD increment in the metabolite ranged from −0.277 to −0.203 cm/s for peak a' and from +0.047 to +0.054 for e'/a'. In addition, peak a' decreased ($P \leq 0.031$) with glucose+2-aminobutyrate (−0.261 cm/s) and glucose+2-phosphoglycerate (−0.209 cm/s). In partial least square discriminant analysis (PLS-DA), metabolites associated with normal diastolic LV function (n=538) included glucose+glutamine, glucose+2-aminobutyrate, and glucose+2-phosphoglycerate, whereas those siding with abnormal function encompassed 4-aminobutyrate, 4-hydroxybutyrate, creatinine, and phosphocholine. In receiver operating characteristics plots, adding 3 latent factors identified by PLS-DA to prohormone brain natriuretic peptide increased ($P<0.0001$) the area under the curve from 0.64 (95% CI, 0.58–0.68) to 0.73 (0.68–0.78).

***Conclusions*—**In a general population, circulating metabolites indicative of energy substrate utilization and protection against oxidative stress differentiated normal from abnormal diastolic LV function. These findings improve our understanding of the pathophysiology underlying deterioration of diastolic LV function and potentially point to new targets for prevention and treatment of this condition. **(*J Am Heart Assoc*. 2016;5:e002681 doi: 10.1161/JAHA.115.002681)**

Key Words: biomarker • diastolic left ventricular function • metabolomics • nuclear magnetic resonance spectroscopy • population science

Prevalence of heart failure (HF) among adults living in Europe amounts to 15 million[1] and 5 million in the United States,[2] of whom approximately half have diastolic HF with a 5-year mortality rate in excess of 50%.[1,3] In population studies,[4–6] frequency of asymptomatic diastolic left ventricular (LV) dysfunction, as diagnosed by echocardiography, is as high as 27%. Availability of biomarkers helping to understand the underlying pathophysiology and identify the large pool of individuals with subclinical diastolic LV dysfunction at high risk of HF is key to prevention and timely treatment.[1]

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Metabolomics is the systematic study of small-molecule metabolite profiles in biological fluids.[7,8] Studies of HF patients matched with healthy controls showed a different metabolic signature in serum[9] and urine.[9,10] Recently, Cheng et al. demonstrated that over and beyond classical risk factors and B-type natriuretic peptide (BNP), circulating plasma metabolites had diagnostic value in assessing HF-related metabolic disturbances and predicting hospitalizations and mortality.[8] However, the utility of metabolite profiling in HF remains uncertain.[11] Findings in symptomatic patients cannot be extrapolated to subclinical disease, in which biomarkers would have the greatest diagnostic or prognostic meaning.[12] In keeping with these considerations,[11,12] we examined the metabolic signature of diastolic LV dysfunction in a random population sample enrolled in the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO).[13]

Methods

Study Participants

The Ethics Committee of the University of Leuven approved FLEMENGHO protocol.[13] Recruitment started in 1985 and continued until 2004. The initial participation rate was 78.0%.

Table 1. Characteristics of 711 Participants by Diastolic LV Function Class

Characteristic	Normal	Abnormal	*P* Value
No. in class	538	173	
No. of subjects (%)			
Women	262 (48.7)	99 (57.2)	0.051
Smokers	115 (21.4)	29 (16.8)	0.19
Drinking alcohol	391 (72.7)	96 (55.5)	<0.0001
Hypertension	158 (29.4)	129 (74.6)	<0.0001
Antihypertensive treatment	88 (16.4)	89 (51.5)	<0.0001
Diabetes mellitus	5 (1.0)	4 (2.3)	0.16
Mean (SD) of characteristic			
Age, y	46.7±13.8	63.4±13.2	<0.0001
Body mass index, kg/m^2	25.8±4.0	28.6±4.5	<0.0001
Waist-to-hip ratio	0.86±0.08	0.90±0.08	<0.0001
Office blood pressure, mm Hg			
Systolic pressure	125.3±14.9	140.5±17.8	<0.0001
Diastolic pressure	79.1±9.0	81.6±10.0	0.002
Mean arterial pressure	94.5±9.9	101.2±10.2	<0.0001
Heart rate, beats per minute	60.5±9.0	63.0±10.9	0.005
Biochemical data			
Serum creatinine, μmol/L	82.2±13.3	87.5±21.2	0.002
eGFR, mL/min per 1.73 m^2	83.4±16.2	71.8±14.6	<0.0001
Total cholesterol, mmol/L	5.16±0.92	5.48±0.94	0.0001
HDL cholesterol, mmol/L	1.44±0.36	1.38±0.35	0.054
Total-to-HDL cholesterol ratio	3.77±1.02	4.18±1.06	<0.0001
Plasma glucose, mmol/L	4.86±0.63	5.20±1.05	<0.0001
γ-Glutamyltransferase, units/L	22 (12–48)	26 (13–53)	0.005
Insulin, pmol/L	33.0 (14.4–71.8)	43.1 (21.5–93.3)	<0.0001
NT-proBNP, pmol/L	192 (91–395)	242 (117–493)	<0.0001

Diastolic dysfunction includes an abnormally low age-specific transmitral E/A ratio without increased LV filling pressures (E/e' ≤8.5) or an elevated LV filling pressure (E/e' >8.5) with normal or low age-specific E/A (see references 4 and 5). Office blood pressure was the average of 5 consecutive readings. Hypertension was an office blood pressure of ≥140 mm Hg systolic, or ≥90 mm Hg diastolic, or use of antihypertensive drugs. For γ-glutamyltransferase, insulin and NT-proBNP values are geometric mean (interquartile range). NT-proBNP was measured in 488 and 161 participants in the normal and abnormal LV function group, respectively. Diabetes mellitus was a self-reported diagnosis, a fasting glucose level of at least 7 mmol/L, or use of antidiabetic agents. eGFR indicates estimated glomerular filtration rate derived by the Chronic Kidney Disease Epidemiology Collaboration equation formula; HDL, high-density lipoprotein; LV, left ventricular; NT-proBNP, N-terminal of the prohormone brain natriuretic peptide;

Table 2. Echocardiographic Measurements by Diastolic LV Function Class

Characteristic	Normal	Abnormal
Number in class	538	173
Conventional echocardiography		
Left atrial volume, mL	41.2±12.4	48.1±15.5
Left atrial volume index, mL/m^2	21.9±5.40	26.0±7.54
Left ventricular mass, g	166.8±44.9	194.6±56.8
Left ventricular mass index, g/m^2	88.8±19.1	104.5±25.5
Doppler data		
Deceleration time, ms	160.2±31.0	190.2±44.0
Isovolumetric relaxation time, ms	94.4±14.0	107.2±18.6
E peak, cm/s	77.3±14.9	68.2±17.2
A peak, cm/s	59.1±13.9	82.3±15.7
E/A ratio	1.39±0.46	0.85±0.24
e' peak, cm/s	12.6±3.26	7.80±1.95
a' peak, cm/s	9.78±2.11	11.1±1.92
e'/a' ratio	1.42±0.66	0.73±0.26
E/e' ratio	6.38±1.35	9.13±2.74

Diastolic dysfunction includes an abnormally low age-specific transmitral E/A ratio without increased LV filling pressures (E/e' ≤8.5) or an elevated LV filling pressure (E/e' >8.5) with normal or low age-specific E/A (see references 4 and 5). By definition, all *P* values for the between-group differences were significant. LV indicates left ventricular.

Participants were repeatedly followed up.[13] From May 2005 until May 2010, we mailed an invitation letter to 1208 former participants for a follow-up examination. However, 153 were unavailable because they had died (n=26), had been institutionalized or were too ill (n=27), or had moved out of the area (n=100). Of the remaining 1055 former participants, 828 renewed informed consent. At the stage of re-examination, the participation rate was therefore 78.5%. We excluded 117 participants from analysis because serum samples were unavailable (n=96), metabolites had extreme values dissociated from the distribution (n=3), atrial fibrillation or paced heart rhythm made assessment of diastolic LV function impossible (n=7), or poor echocardiographic image quality (n=11). Thus, the number of participants statistically analyzed totaled 711.

Echocardiography

Data acquisition

One observer (T.K.) did the ultrasound examination,[4] using a Vivid7 Pro (GE Vingmed, Horten, Norway) device interfaced with a 2.5- to 3.5-MHz phased-array probe. For offline analysis, she recorded at least 5 heart cycles according to the recommendations of the American Society of Echocardiography.[14] M-mode echocardiograms of the left ventricle were recorded from the parasternal long-axis view under control of the 2-dimensional image. The ultrasound beam was positioned just below the mitral valve at the level of the posterior tendinous chords. To record mitral and pulmonary vein (PV) flow velocities from the apical window, the observer positioned the Doppler sample volume at the mitral valve tips, in the right superior PV, and between the LV outflow and mitral inflow, respectively. From the apical window, the observer positioned a 5-mm Doppler sample at the septal, lateral, inferior, and posterior sites of the mitral annulus to record low-velocity, high-intensity myocardial signals at a high frame rate (>190 frames per second), while ensuring parallel alignment of the ultrasound beam with the myocardial segment of interest.

Off-line analysis

One reader (T.K.) analyzed the digitally stored images, averaging 3 heart cycles, using a workstation running EchoPac software (version 4.0.4; GE Vingmed, Horten, Norway). LV internal diameter and interventricular septal and posterior wall thickness were measured at end-diastole from the 2-dimensionally guided M-mode tracing. When optimal orientation of M-mode ultrasound beam could not be obtained, the reader performed linear measurements on correctly oriented 2-dimensional (2D) images. End-diastolic LV dimensions were used to calculate LV mass by an anatomically validated formula.[14] Left atrial (LA) volume was calculated using the prolate-elipsoid method from the LA dimensions in 3 orthogonal planes and indexed to body surface area.[14] From the transmitral flow signal, the reader determined peak early diastolic velocity (E), peak late diastolic velocity (A), E/A ratio, and transmitral A flow duration. From the PV flow signal, she measured the duration of PV reversal flow during atrial systole (AR). From the tissue Doppler imaging recordings, the reader measured peak early (e') and peak late (a') diastolic mitral annular velocities, and the e'/a' ratio at the 4 acquisition sites (septal, lateral, inferior, and posterior).

Intraobserver reproducibility was the 2-SD interval about the mean of the relative differences across pair-wise readings. Intraobserver reproducibility for the tissue Doppler peak velocities across the 4 sampling sites ranged from 4.5% to 5.3% for e' and from 4.0% to 4.5% for a'.[4] Reproducibility was 2.2% for internal end-diastolic LV diameter, 4.6% for LV wall thickness, and 4.3% for LV mass.[15]

We dichotomized diastolic LV function into normal and abnormal as described in previous publications.[4,5] Diastolic dysfunction included: (1) patients with an abnormally low age-specific transmitral E/A ratio indicative of impaired relaxation, but without evidence of increased LV filling pressures (E/e' ≤8.5); (2) patients with mildly to moderately elevated LV filling pressure (E/e' >8.5) and an E/A ratio within the normal age-specific range; and (3) and patients with an elevated E/e'

Table 3. List of Plasma Metabolic Biomarkers

Amino Acids	1H Shift
2-Aminobutyrate (C4H9NO2)	1.86
4-Aminobutyrate (C4H9NO2)	3.00
Alanine (C3H7NO2)	1.46
Aspartate (C4H7NO4)	2.81
Glutamate (C5H9NO4)	2.36
Glycine (C2H5NO2)	3.55
Glutamine (C5H10N2O3)	2.42
Isoleucine (C6H13NO2)	1.00
Leucine (C6H13NO2)	1.71
Phenylalanine (C9H11NO2)	3.26
Threonine (C4H9NO3)	3.58
Tyrosine (C9H11NO3)	6.88
Valine (C5H11NO2)	0.97
Lipids	**1H Shift**
Fatty acids with α-CH2	2.23
Fatty acids with (-CH2-)n	1.25
Fatty acids with =CH-CH2-CH2= +citrate	2.69
Fatty acids with –CH3	0.84
Fatty acids with =CH-CH2-CH2-	2.00
Fatty acids with –CH=CH	5.31
HDL apolipoproteins	6.50
Cholesterol (C27H46O)	0.66
Valerate (C5H10O2)	1.28
Fatty acids with β-CH2	1.57
3-Hydroxybutyrate (C4H8O3)	1.20
Carbohydrates	**1H Shift**
α-Glucose (C6H12O6)	5.23
Glucose+taurine (C6H12O6+C2H7NO3S)	3.24
Glucose (C6H12O6)	3.47
Glycoprotein	2.04
Organic Acids	**1H Shift**
Acetate (C2H4O2)	1.91
4-Hydroxybutyrate (C4H8O3)	1.78
Lactate (C3H6O3)	1.33
2-Oxobutyrate (C4H6O3)	2.75
Other Metabolites	**1H Shift**
Creatinine (C4H7N3O)	4.05
Choline (C5H14NO)	3.21
Ethanolamine (C2H7NO)	3.41
Trimethylamine (C3H9N)	3.37
Glycerol (C3H8O3)	3.61
Ethanol (C2H6O)	3.64
Creatine+creatine-phosphate (C4H9N3O2+C4H10N3O5P)	3.92
Phosphocholine (C5H15NO4P)	3.22
Glucose+2-aminobutyrate (C6H12O6+C4H9NO2)	3.72
Glucose+glutamine (C6H12O6+C5H10N2O3)	3.78
Glucose+2-phosphoglycerate (C6H12O6+C3H7O7P)	3.80
Unknown molecule	3.87

HDL indicates high-density lipoprotein.

ratio and an abnormally low age-specific E/A ratio (combined dysfunction). To confirm elevation of LV filling pressure, the reader of the offline echocardiographic images checked the differences in the duration between the transmitral A flow (Ad) and the reverse flow in the pulmonary veins (ARd) during atrial systole (Ad<ARd+10) and the left atrial volume index (≥28 mL/m^2).

Nuclear Magnetic Resonance Spectroscopy

For nuclear magnetic resonance (NMR) spectroscopy,[7,16,17] 500 μL of plasma were mixed with 50 μL of D_2O (as a field lock). A total of 500 μL of the mixture of each sample was then individually transferred into a 5-mm high-resolution NMR tube. All proton NMR (^{1}H NMR) spectra were acquired using a standard 1-dimensional pulse sequence with water suppression (Bruker Avance 600 spectrometer operating at 600.13 MHz with a 5-mm 1H/13C/15N TXI probe). A total of 256 FIDs (free induction decay) were collected into 64 k data points with a spectral width of 14 ppm and a recycle delay (RD) of 1 second. The water signal was saturated with weak irradiation during the RD. Before Fourier transformation, the FID was multiplied by a 0.3-Hz exponential line broadening.

Spectral chemical shift referencing on the alanine CH3 doublet signal at 1.475 ppm was performed in all spectra. Resonances in these spectral regions were assigned using the literature[18] and selected 2D NMR (especially for long chain metabolites) spectra. We normalized all spectra to total spectral area. Thus, individual peak intensities were normalized to total metabolite content for a better comparison between samples. Spectral regions belonging to EDTA resonances (2.52–2.57 and 3.06–3.17 ppm) were removed from spectra for subsequent analysis. We used available spectral databases and 2D NMR experiments to aid structural identification of relevant metabolites. All spectra were processed using Topspin 1.3 (Bruker Biospin GmbH, Rheinstetten, Germany) and transferred to MATLAB (2006; The MathWorks, Inc., Natick,

Table 4. Levels of Metabolic Biomarkers by Diastolic LV Function Class

Metabolic Biomarker	Normal (n=538)	Abnormal (n=173)	*P* Value
Amino acids			
2-Aminobutyrate	17.7±1.09	17.4±0.82	0.0003
4-Aminobutyrate	6.97±0.50	6.80±0.50	0.0001
Alanine	12.3±0.63	12.2±0.51	0.0058
Aspartate	5.46±0.73	5.46±0.58	0.95
Glutamate	9.26±1.12	9.06±0.77	0.0088
Glycine	1.28±0.16	1.29±0.16	0.62
Glutamine	10.4±1.48	10.2±1.18	0.018
Isoleucine	12.6±0.59	12.5±0.57	0.075
Leucine	33.4±2.51	32.7±1.86	0.0002
Phenylalanine	2.92±0.62	2.91±0.58	0.83
Threonine	2.31±0.27	2.35±0.24	0.098
Tyrosine	2.36±0.35	2.29±0.25	0.0045
Valine	9.65±0.46	9.51±0.46	0.0009
Lipids			
Fatty acids with α-CH2	10.6±0.89	10.8±0.78	0.0057
Fatty acids with (-CH2-)n	51.2±6.59	51.3±5.51	0.81
Fatty acids with =CH-CH2-CH2= +citrate	16.7±1.89	17.0±1.34	0.019
Fatty acids with –CH3	64.8±6.83	64.2±4.52	0.20
Fatty acids with =CH-CH2-CH2-	36.1±1.23	36.0±1.09	0.52
Fatty acids with –CH=CH	11.1±2.62	11.8±2.42	0.0016
HDL apolipoproteins	46.0±6.96	44.6±5.65	0.0099
Cholesterol	22.6±4.88	21.9±2.73	0.012
Valerate	35.7±7.91	38.5±8.03	<0.0001
Fatty acids with β-CH2	39.7±2.00	39.7±1.22	0.75
3-Hydroxybutyrate	18.4±2.30	18.5±3.59	0.82
Carbohydrates			
α-Glucose	2.27±0.42	2.39±0.56	0.010
Glucose+taurine	9.31±1.21	9.26±1.19	0.61
Glucose	20.2±2.18	20.6±2.67	0.048
Glycoprotein	15.5±0.79	15.9±1.09	0.0003
Organic acids			
Acetate	5.36±0.31	5.29±0.42	0.037
4-Hydroxybutyrate	16.0±1.29	15.6±0.91	<0.0001
Lactate	13.7±2.49	14.7±2.47	<0.0001
2-Oxobutyrate	4.67±0.62	4.83±0.49	0.0006
Other metabolites			
Creatinine	2.84±0.21	2.83±0.16	0.26
Choline	3.31±1.09	3.21±0.38	0.055
Ethanolamine	0.81±0.15	0.83±0.13	0.89
Trimethylamine	0.55±0.13	0.54±0.12	0.45
Glycerol	1.91±0.38	1.91±0.16	0.72
Ethanol	4.55±0.52	4.52±0.38	0.50
Creatine+creatine-phosphate	1.49±0.14	1.47±0.12	0.027
Phosphocholine	6.50±1.09	6.17±1.05	0.0006
Glucose+2-aminobutyrate	6.16±0.57	6.29±0.73	0.044
Glucose+glutamine	6.08±0.56	6.16±0.53	0.11
Glucose+2-phosphoglycerate	1.42±0.13	1.43±0.13	0.31
Unknown molecule	3.56±0.31	3.55±0.29	0.55

Diastolic dysfunction includes an abnormally low age-specific transmitral E/A ratio without increased LV filling pressures (E/e' ≤8.5) or an elevated LV filling pressure (E/e' >8.5) with normal or low age-specific E/A (see references 4 and 5). *P* values denote the significance of the differences between groups. LV indicates left ventricular.

MA) using in-house scripts for data analysis. Signals belonging to selected metabolites were integrated and quantified, using semiautomated in-house MATLAB peak-fitting routines. These fitting routines were based on Levenburg-Marquard optimization procedures. The target function for the optimization included experimental spectra measured for standard solutions of selected metabolites with complex multiplet patterns and theoretically generated Lorentzian-shape signals for those metabolites with simpler spectral patterns.

Other Measurements

Blood pressure was the average of 5 consecutive auscultatory readings obtained according to European guidelines with a standard mercury sphygmomanometer with the participant resting in the seated position for at least 5 minutes.[19] Hypertension was a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or use of antihypertensive drugs. Body mass index (BMI) was weight in kilograms divided by height in meters squared. Venous blood samples were drawn after at least 8 hours of fasting for measurement of plasma glucose and serum total and high-density lipoprotein (HDL) cholesterol, creatinine, γ-glutamyltransferase (biomarker of alcohol intake), and insulin. The homeostatic model assessment (HOMA) index was computed from glucose and insulin.[20] Glomerular filtration rate (GRF) was derived by the Chronic Kidney Disease Epidemiology Collaboration equation.[21] Diabetes mellitus was a self-reported diagnosis, a fasting glucose level of at least 7 mmol/L, or use of antidiabetic agents.[22] In 649 participants, N-terminal of the prohormone BNP (NT-proBNP) was measured in plasma by a

competitive enzyme immunoassay (EIA) for research use (Biomedica Gruppe, Vienna, Austria).

Statistical Analysis

For database management and statistical analysis, we used the SAS system (version 9.3; SAS Institute Inc., Cary, NC). Means were compared using the large-sample *z*-test or ANOVA and proportions by Fisher's exact test. We normalized the distributions of γ-glutamyltransferase, insulin, and NT-proBNP by a logarithmic transformation. Our statistical methods also included multivariable linear regression with, as dependent variables, the echocardiographic indexes of diastolic LV function. We identified variables to be retained in the analyses by a step-wise regression procedure with *P* value for variables to enter and stay in the models set at 0.15. Statistical significance was a 2-sided *P* level of 0.05. Where appropriate, we applied Bonferroni correction for multiple testing by multiplying *P* values by 44 (the number of metabolites tested). The annotation *P* and P_B refer to significance levels unadjusted and adjusted for multiple testing.

We constructed a −log10 probability plot for the multivariable-adjusted associations of the diastolic LV function indexes with the metabolites. In the next step of our analyses, we dichotomized the study population in 538 participants with normal LV function and in 173 with subclinical diastolic LV dysfunction[4,5] and used a binary dummy variable indicating membership to these 2 groups as a dependent variable in a partial least squares discriminant analysis (PLS-DA) to identify a set of independent latent factors (LFs) that were linear combinations of the metabolites and that maximized the covariance between the metabolites and LV diastolic dysfunction. We retained the smallest number of latent factors for which the predicted residual sums of squares (PRESS; calculated using leave-one-out cross-validation) did not differ significantly ($P>0.10$) from the model with the minimum PRESS value as assessed by the van der Voet T^2 statistic. The importance of each metabolite in the construction of the PLS factors was assessed from the Variable Importance in Projection (VIP) scores of Wold.

Finally, we evaluated the capability of the LFs to discriminate between participants with and without diastolic LV dysfunction by constructing the receiver operating characteristic (ROC) curve and by calculating the area under the ROC curve (AUC). The 95% CI of the AUC was calculated by the DeLong method.[23] The discriminative capability of the latent factors on top of NT-proBNP was assessed by comparing the AUCs from a basic model including NT-proBNP and an extended model including NT-proBNP plus the LFs. We tested the statistical significance of the change in AUC attributable to adding the LFs to NT-proBNP by the Delong test for paired ROC curves.[23]

Results

Characteristics of Participants

Of 711 participants, 361 (50.8%) were women. All subjects were white Europeans. Mean values (SD) in all participants

Table 5. Covariates Selected by Step-wise Regression

Variables	E Peak	A Peak	E/A	e'	a'	e'/a'	E/e'
R^2	0.27	0.68	0.66	0.75	0.50	0.72	0.46
Female sex (0, 1)	9.284§	5.944§	—	−0.354†	−0.950§	0.078‡	1.109§
Age (+15.4 y)	−6.478§	9.972§	−0.316§	−2.380§	1.248§	−0.453§	0.811§
Body mass index (+4.3 kg/m^2)	—	3.188§	−0.077§	−0.630§	0.385§	−0.138§	0.291§
Mean arterial pressure (+10.4 mm Hg)	—	1.943§	−0.024†	−0.308§	—	−0.035†	0.320§
Heart rate (+9.6 bpm)	−3.085§	3.554§	−0.131§	−0.252§	0.689§	−0.117§	−0.122*
Total cholesterol (+0.95 mmol/L)	−1.127†	—	−0.029‡	−0.200‡	0.173‡	−0.063§	—
Log γ-glutamyltransferase (×2)	—	−5.843*	—	−2.571§	0.938*	−0.290†	—
Fasting plasma glucose (+0.77 mmol/L)	0.835*	—	—	0.115*	—	—	0.109*
LVMI (+21.9 g/m^2)	—	—	—	−0.332§	−0.237‡	—	0.360§
On treatment with diuretics (0, 1)	—	—	—	−0.452*	−0.420*	—	0.932§
On treatment with β-blockers (0, 1)	—	2.302*	−0.068†	−0.457†	−0.698§	—	—
On treatment with RAAS inhibitors (0, 1)	−4.858†	—	—	—	—	—	−0.495†

Variables considered for entry into the models included sex, age, body mass index, mean arterial pressure, heart rate, total cholesterol, γ-glutamyltransferase (as index of alcohol intake), fasting plasma glucose, LVMI, and treatment with diuretics (thiazides, loop diuretics and aldosterone antagonists), β-blockers, inhibitors of the renin-angiotensin system (angiotensin-converting enzyme inhibitors or angiotensin type-1 receptor blockers), and vasodilators (calcium-channel blockers and α-blockers). *P* values for variables to enter and stay in the regression models were set at 0.15. LVMI left ventricular mass index; RAAS, renin-angiotensin-aldosterone system.
Significance of the association: *$0.05<P\leq0.15$; †$P\leq0.05$; ‡$P\leq0.01$; §$P\leq0.001$.

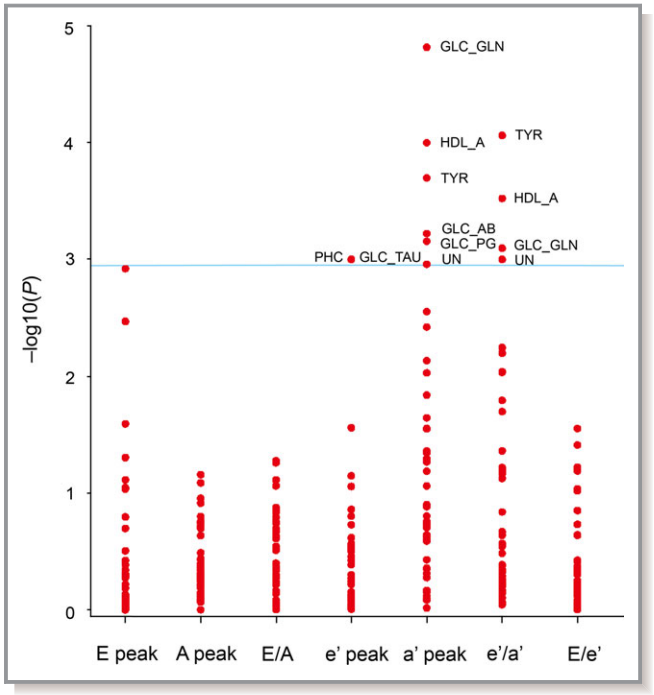

Figure 1. −Log10(*P*) probability plot of the multivariable-adjusted associations of various indexes of diastolic left ventricular dysfunction (see Table 6) with the metabolic biomarkers. TYR, HDL_A, GLC_TAU, PHC, GLC_AB, GLC_GLN, GLC_PG, and UN, respectively, indicate tyrosine, high-density lipoprotein apolipoproteins, glucose+taurine, phosphocholine, glucose+2-aminobutyrate, glucose+glutamine, glucose+2-phosphoglycerate, and unknown molecule. The adjustment accounted for sex, age, body mass index, mean arterial pressure, heart rate, total cholesterol, γ-glutamyltransferase, fasting plasma glucose, treatment with diuretics, β-blockers, and inhibitors of the renin-angiotensin system. The horizontal line denotes the significance level with Bonferroni correction applied.

combined were 50.8 (15.4) years for age, 129.0 (17.0) and 79.7 (9.3) mm Hg for systolic and diastolic blood pressure, 26.5 (4.3) kg/m^2 for BMI, and 5.24 (0.95) mmol/L for total cholesterol. Of all participants, 287 (40.4%) had hypertension, of whom 177 (61.7%) were on antihypertensive drug treatment, and 9 (1.3%) had diabetes. The prevalence of diastolic LV dysfunction amounted to 173 (24.3%), because of impaired relaxation in 70 (40.5%) or an elevated filling pressure in the presence of a normal (79 [45.7%]) or low (24 [13.9%]) age-specific E/A ratio.

Associations of Diastolic LV Function With Metabolites

Unadjusted analyses

Comparing with participants with normal function (Table 1), age, BMI, central obesity, blood pressure, heart rate, serum creatinine, γ-glutamyltransferase and insulin, plasma glucose, total-to-HDL cholesterol ratio, and NT-proBNP all increased ($P \leq 0.005$), whereas estimated GFR (eGFR) decreased

Table 6. Multivariable-Adjusted Associations of Tissue Doppler indexes With Metabolites

Metabolic Markers (SD)	Estimate (95% CI)	P_B Value
Tyrosine (0.327)		
a' peak	−0.225 (−0.419, −0.030)	0.009
e'/a'	0.054 (0.010, 0.099)	0.004
HDL apolipoproteins (6.689)		
a' peak	−0.229 (−0.425, −0.034)	0.004
e'/a'	0.050 (0.005, 0.095)	0.013
Glucose+taurine (1.209)		
e' peak	0.250 (0.005, 0.496)	0.044
Phosphocholine (1.091)		
e' peak	0.255 (0.003, 0.507)	0.044
Glucose+2-aminobutyrate (0.616)		
a' peak	−0.261 (−0.509, −0.014)	0.026
Glucose+glutamine (0.552)		
a' peak	−0.277 (−0.483, −0.070)	0.0007
e'/a'	0.049 (0.001, 0.097)	0.035
Glucose+2-phosphoglycerate (0.131)		
a' peak	−0.209 (−0.407, −0.010)	0.031
Unknown molecule (0.307)		
a' peak	−0.203 (−0.404, −0.002)	0.048
e'/a'	0.047 (0.001, 0.093)	0.044

All estimates were adjusted for sex, age, body mass index, mean arterial pressure, heart rate, total cholesterol, γ-glutamyltransferase, plasma glucose, LV mass index, and treatment with diuretics, β-blockers, and inhibitors of the renin-angiotensin system. Estimates express the change in the dependent variable for a 1-SD increase in circulating metabolites. *P* values and 95% CIs account for testing 44 metabolites according to the Bonferroni approach. HDL indicates high-density lipoprotein; LV, left ventricular.

($P<0.0001$), in participants with diastolic LV dysfunction. Table 2 shows that LA volume, LA volume index, LV mass, LV mass index (LVMI), deceleration time, isovolumetric relaxation time, A and a' peak velocities, and E/e' ratio increased ($P<0.0001$) in participants with diastolic LV dysfunction, whereas the opposite was the case ($P<0.0001$) for E and e' peak velocities and the E/A and e'/a' ratios. Table 3 lists the circulating metabolites subdivided into amino acids, lipids, carbohydrates, organic acids, and other molecules. Table 4 shows that of 44 non-overlapping metabolites, 24 showed significant differences between 2 groups. Generally, amino acids were lower in diastolic LV dysfunction group (8 of 8 significant associations), whereas fatty acids were higher (4 of 6 significant associations).

Multivariable-adjusted regression

Variables considered for entry and retained for multivariable adjustment of the indexes of diastolic LV function are listed in

Table 7. Factor Loadings of the Metabolic Biomarkers in Analyses Contrasting Normal With Diastolic LV Dysfunction

Metabolic Biomarker	LF1	LF2	LF3
Amino acids			
2-Aminobutyrate	−0.25	0.13	−0.10
4-Aminobutyrate	−0.23	0.16	−0.14
Alanine	−0.26	0.04	−0.03
Aspartate	−0.02	0.15	−0.27
Glutamate	−0.13	0.15	−0.26
Glycine	−0.07	0.23	−0.01
Glutamine	−0.13	0.16	−0.27
Isoleucine	−0.11	−0.11	0.12
Leucine	−0.24	0.13	−0.12
Phenylalanine	−0.01	0.16	−0.26
Threonine	−0.08	0.26	0.02
Tyrosine	−0.15	0.06	−0.05
Valine	−0.18	−0.13	0.13
Lipids			
Fatty acid with α-CH2	0.21	0.01	−0.21
Fatty acid with (-CH2-)n	0.12	−0.21	0.14
Fatty acid with =CH-CH2-CH2= +citrate	0.01	0.14	−0.23
Fatty acid with –CH3	−0.03	−0.19	0.24
Fatty acid with =CH-CH2-CH2-	0.05	−0.18	0.10
Fatty acid with –CH=CH	0.24	−0.07	−0.07
HDL apolipoproteins	−0.15	0.08	−0.08
Cholesterol	−0.08	−0.12	0.27
Valerate	0.31	−0.10	−0.05
Fatty acid with β-CH2	0.05	−0.02	−0.22
3-Hydroxybutyrate	−0.01	−0.13	0.25
Carbohydrates			
α-glucose	0.11	0.07	0.05
Glucose+taurine	−0.06	0.05	−0.07
Glucose	−0.04	0.26	−0.02
Glycoprotein	0.19	0.09	−0.04
Organic acids			
Acetate	−0.25	0.08	0.06
4-Hydroxybutyrate	−0.25	0.10	−0.13
Lactate	0.28	−0.03	−0.12
2-Oxobutyrate	0.13	0.08	−0.23
Other metabolites			
Creatinine	0.01	0.15	−0.24
Choline	−0.06	0.03	0.04
Ethanolamine	−0.10	0.16	−0.08
Trimethylamine	−0.01	0.14	−0.18
Glycerol	−0.09	0.16	−0.04
Ethanol	−0.14	0.14	0.12
Creatine+creatine-phosphate	−0.15	0.21	−0.14
Phosphocholine	−0.14	−0.08	−0.01
Glucose+2-aminobutyrate	−0.03	0.23	0.12
Glucose+glutamine	−0.10	0.28	0.02
Glucose+2-phosphoglycerate	−0.13	0.24	0.09
Unknown molecule	−0.14	0.22	0.06

The study population was dichotomized in 538 participants with normal LV function and 173 with subclinical diastolic LV dysfunction.[4,5] LF1, LF2, and LF3 refer to the first, second, and third latent factors derived by partial least square discriminant analysis of 44 metabolites. LV indicates left ventricular.

Table 5. We adjusted the associations of the indexes of diastolic LV function for sex, age, BMI, mean arterial pressure, heart rate, total cholesterol, γ-glutamyltransferase, plasma glucose, LVMI and treatment with diuretics, β-blockers, and inhibitors of the renin-angiotensin system and applied Bonferroni correction of the significance levels. Under these conditions, the transmitral blood flow Doppler indexes were not correlated with any circulating metabolite. There was a positive correlation of e' peak velocity with glucose+taurine and phosphocholine (Figure 1; P_B=0.044 for both). Significance was also preserved (Figure 1) for the negative correlations of a' peak velocity with tyrosine, HDL apolipoproteins, glucose+2-aminobutyrate, glucose+glutamine, glucose+2-phosphoglycerate, and an unknown molecule ($P_B \leq 0.048$) and the positive correlations of e'/a' with tyrosine, HDL apolipoproteins, glucose+glutamine, and an unknown molecule ($P_B \leq 0.044$). Table 6 lists quantitative estimates for the association sizes expressing the differences in the diastolic LV function indexes per 1-SD increment in the circulating metabolites with 95% CIs rescaled to the Bonferroni significance level. The results reported in Figure 1 and Table 6 remained consistent, if models were additionally adjusted for insulin or if glucose was replaced by the HOMA index.[20] However, additional adjustment for HDL cholesterol or replacing total cholesterol by the total-to-HDL cholesterol ratio removed the significance of the associations of e' peak velocity with glucose+taurine and phosphocholine.

Partial Least Square Discriminant Analysis

We dichotomized the study population in 538 participants with normal LV function and in 173 with subclinical diastolic LV dysfunction.[4,5] The PLS-DA procedure identified 3 latent factors accounting for 19.5%, 21.3%, and 13.6% of the

Table 8. VIP Scores in Analyses Contrasting Normal With Diastolic LV Dysfunction

Metabolomic Biomarker	VIP Score
Lactate	1.433
4-Aminobutyrate	1.411
Glycoprotein	1.403
Valerate	1.398
Creatinine	1.271
4-Hydroxybutyrate	1.269
Alanine	1.267
Valine	1.247
Glucose+glutamine	1.201
Glucose	1.187
Glucose+2-aminobutyrate	1.185
Glucose+2-phosphoglycerate	1.172
Acetate	1.143
Phosphocholine	1.142
Threonine	1.126
2-Aminobutyrate	1.112
Leucine	1.112
Fatty acid with –CH=CH	1.106
2-Oxobutyrate	1.027
Fatty acid with α–CH2	1.024
Creatine+creatine-phosphate	1.024
α-Glucose	1.007

Variable Importance in Projection (VIP) scores estimate the importance of each variable in the projection used in a partial least square (PLS) model and is often used for variable selection. A variable with a VIP score greater than 1 can be considered important in a given model. LV indicates left ventricular.

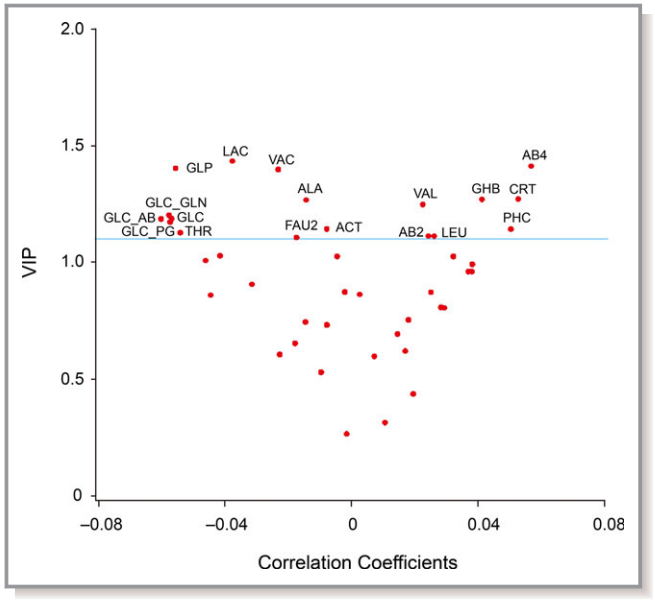

Figure 2. After dichotomizing the study population in 538 participants with normal LV function and 173 with subclinical diastolic LV dysfunction, V-plots were generated for the PLS-DA-derived VIP scores versus the centered and rescaled correlation coefficients. Spots indicating metabolites with a VIP score higher than 1.1 were labeled. Spots associated with normal diastolic LV function (left), ordered by descending VIP score include lactate (LAC), glycoprotein (GLP), valerate (VAC), alanine (ALA), glucose+glutamine (GLC-GLN), glucose (GLC), glucose+2-aminobutyrate (GLC-AB), glucose+2-phosphoglycerate (GLC-PG), acetate (ACT), threonine (THR), and fatty acid with –CH=CH (FAU2). Spots associated with diastolic LV dysfunction (right), ordered by ascending VIP score, include leucine (LEU), 2-aminobutyrate (AB2), phosphocholine (PHC), valine (VAL), 4-hydroxybutyrate (GHB), creatinine (CRT), and 4-aminobutyrate (AB4). LV indicates left ventricular; PLS-DA, partial least square discriminant analysis; VIP, Variable Importance in Projection.

variance in the metabolites and 54.4% in total. Table 7 presents the factor loadings of the circulating metabolites on LF1, LF2, and LF3. As listed in Table 8, metabolites with a VIP score greater than 1 included phosphocholine (1.142), glucose+2-aminobutyrate (1.185), glucose+glutamine (1.201), and glucose+2-phosphoglycerate (1.172), but not tyrosine (0.808), HDL apolipoproteins (0.806), glucose+taurine (0.265), and unknown molecule (0.733). Figure 2 shows the V-plot for the PLS-DA-derived VIP scores versus the centered and rescaled correlation coefficients. The dependent variable in this analysis was diastolic LV dysfunction. The metabolites associated with normal diastolic LV function (left side of the V-plot in Figure 2) included, among others, glucose+glutamine, glucose+2-aminobutyrate, and glucose+2-phosphoglycerate. Metabolites siding with abnormal diastolic LV function (right side of the V-plot in Figure 2) encompassed 4-aminobutyrate, 4-hydroxybutyrate, creatinine, and phosphocholine.

Receiver Operating Curves

Measurement of NT-proBNP in plasma is currently the standard in diagnosing LV dysfunction and monitoring its treatment. Figure 3 shows that combining the 3 latent factors identified by PLS-DA to NT-proBNP increased ($P<0.0001$) the AUC from 0.64 (95% CI, 0.58–0.68) to 0.73 (0.68–0.78).

Discussion

In the current study, we applied 2 different methods to investigate association of diastolic LV function with circulating metabolites. In multivariable-adjusted regression models with Bonferroni correction of significance levels applied, a' was inversely and e'/a' was positively correlated with circulating tyrosine, HDL apolipoproteins, glucose+glutamine, and an unknown molecule, whereas a' was also inversely associated

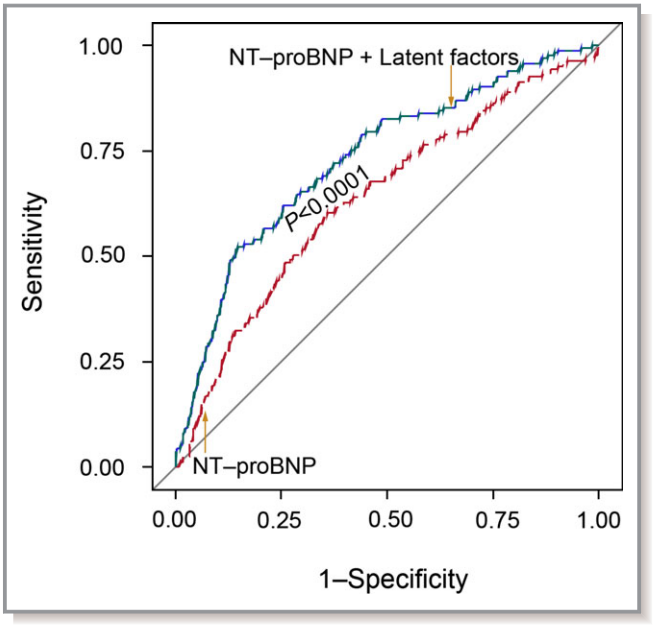

Figure 3. Receiver operating characteristic (ROC) curves for discriminating between normal and abnormal diastolic LV function using NT-proBNP (red line) or NT-proBNP plus 3 latent factors identified by PLS-DA (green line). Combining the 3 latent factors with NT-proBNP increased (P<0.0001) the AUC from 0.64 to 0.73. AUC indicates area under the curve; LV, left ventricular; NT-proBNP, N-terminal of the prohormone brain natriuretic peptide; PLS-DA, partial least square discriminant analysis.

with glucose+2-aminobutyrate and glucose+2-phosphoglycerate (Table 6). In PLS-DA, metabolites associated with normal diastolic LV function included glucose+glutamine, glucose+2-aminobutyrate, and glucose+2-phosphoglycerate, whereas those siding with abnormal function encompassed 4-aminobutyrate, 4-hydroxybutyrate, creatinine, and phosphocholine (Figure 2).

In multivariable-adjusted regression analyses (Table 6), the early diastolic e' peak was positively associated with glucose+taurine and with phosphocholine. The late diastolic a' peak velocity, either as single measurement or as denominator in the e'/a' ratio, was the other trait associated with the circulating metabolites. Impaired relaxation, as reflected by the early diastolic e' peak velocity, is usually the first step in the progression toward diastolic LV dysfunction, but it is also part of the normal aging process.[4,5] Stiffening of the LV occurs later and requires a greater contribution of the atrial contraction to late diastolic LV filling. This might explain why, in multiple linear regression, fewer metabolites were associated with e' than a'. This interpretation is also in keeping with the observation that in our study population, in contrast to hospitalized patients with end-stage HF, only 11 participants, all with high LV filling pressure, had an e'/a' ratio higher than unity.

Extruding calcium from cardiomyocytes during early diastole to facilitate cardiac relaxation and diastolic filling requires high amounts of energy.[24] Higher availability of energy substrate might improve LV relaxation and reduce the atrial contribution to LV filling. Glucose+2-phosphoglycerate and glucose+glutamine, 2 metabolic markers involved in the generation of energy, were associated with lower a' peak velocity (Figure 1 and Table 6) and better diastolic LV function (Figure 2). As shown in Figure 4, 2-phosphoglycerate is an intermediate metabolite in the cytosolic anaerobic glycolysis pathway, which converts 1 glucose molecule into 2 pyruvate moieties, with a net production of ATP.[25] This pathway also generates reduced NADH. Transferred to mitochondria, NADH links the citric acid cycle to oxidative phosphorylation, the major source of ATP.[25] Oxygen being available, pyruvate produced by glycolysis is converted to acetyl-CoA (coenzyme A) and runs through the citric acid cycle, in which acetyl-CoA is completely oxidized generating ATP through oxidative phosphorylation. Glutamine is an alternative substrate (Figure 4) that, in the presence of glucose, can enter the citric acid cycle and generates energy by aerobic oxidation.[26]

The antioxidant, glutathione (GSH), is the main hepatic protection system against systemic oxidative stress.[27] When hepatic availability of cysteine is limited and GSH is depleted, 2-aminobutyrate enters an alternative pathway driven by the same enzymes leading to the synthesis of ophthalmate.[27,28] In our current study, a performant diastolic function, as exemplified by lower a' peak in the multivariable-adjusted regression analysis (Table 6) or a VIP-to-correlation-coefficient spot associated with normal diastolic LV function (left side of the V-plot in Figure 2) was associated with a higher glucose+2-aminobutyrate signal. In multivariable-adjusted regression analyses (Table 6), the early diastolic e' peak correlated positively with glucose+taurine and the e'/a' ratio with HDL apolipoproteins, whereas the late diastolic a' peak was inversely related to tyrosine and HDL apolipoproteins. Protection against oxidative stress might underlie better diastolic LV function associated with these 2 markers reflecting amino acid (tyrosine) and lipid (HDL apolipoproteins) metabolism (Figure 4). Major sources of the amino acid, taurine, are hepatic biosynthesis from cysteine and dietary intake.[29] Although the underlying mechanisms require further elucidation, several studies suggest that taurine inhibits the generation of ROS.[29] Japanese studies,[30] confirmed by other investigators,[31,32] demonstrated that daily taurine administration to HF patients improved end-diastolic LV volume,[31] key symptoms,[30] and exercise capacity.[32] Tyrosine, a product of phenylalanine degradation, is a precursor of dopamine and melanin.[33] At the start of the pathway, phenylalanine upregulates expression and activity of guanosine-5′-triphosphate cyclohydrolase I, which is the first and rate-controlling enzyme for synthesis of tetrahydrobiopterin, an essential cofactor for nitric oxide (NO) synthase and NO synthesis.[34] Near the end of the tyrosine chain, dopamine and melanin reduce the

synthesis of proinflammatory cytokines, including tumor necrosis factor alpha and interleukins 1b, 6, and 10, and induce production of anti-inflammatory mediators by leucocytes.[34] Apolipoprotein (Apo) A1 and apoA2 are the major protein component of HDL lipoproteins and account for over two thirds of the protein content of HDL. Both HDL lipoproteins and HDL cholesterol possess anti-inflammatory and -adhesive properties.[35] Adjustment for HDL cholesterol or the total-to-HDL cholesterol ratio in the multivariable-adjusted regression analysis did not remove the associations of a' and e'/a' with HDL apolipoproteins.

Phospholipid bilayers, in which phosphatidylcholine is the main constituent, maintain the structure and functionality of cellular membranes. Phosphocholine is an intermediate metabolite in the cytidine diphosphocholine pathway that synthesizes phosphatidylcholine from choline (Figure 4), an essential nutrient primarily provided by the diet. Carnitine is a chemical analog of choline and mediates the transport of long-chain fatty acids into the mitochondrial matrix for beta-oxidation and has proven antioxidant and -inflammatory activity. In contrast, choline deficiency is associated with increased oxidative stress.[36] Compared to controls, patients with diastolic HF have lower serum concentrations of phosphatidylcholines.[37] L-carnitine is a nutritional supplement approved by regulatory agencies for use in cardiovascular and cardiac disease, including HF.[38] In multiple linear regression, e' increased with higher circulating phosphocholine, but in the PLS-DA, that relied on various Doppler velocities other than e', the VIP-to-correlation-coefficient spot (right side of the V-plot in Figure 2) was associated with diastolic LV dysfunction. Adjustment for HDL-cholesterol or the total-to-HDL cholesterol ratio weakened the positive association of e' with phosphocholine in the multivariable-adjusted regression analysis, perhaps because HDL-lipoproteins are acceptors in the efflux of phospholipids and free cholesterol from peripheral cells.[35]

Strong points of our study are the availability of Doppler indexes of early subclinical diastolic LV dysfunction, application of 2 different approaches in the statistical analysis, and the population-based character of our research. The epidemiological angle enhances the relevance of our findings over and beyond that of case-control studies involving selected HF patients who represent the end stage of a long pathogenetic process confounded by multiple comorbidities and polymedication.[11] However, our present study must also be interpreted within the context of its limitations. First, our findings originate from a cross-sectional analysis and therefore reflect a snapshot of a long-lasting process in each individual participant. Moreover, the pathogenetic drivers leading to

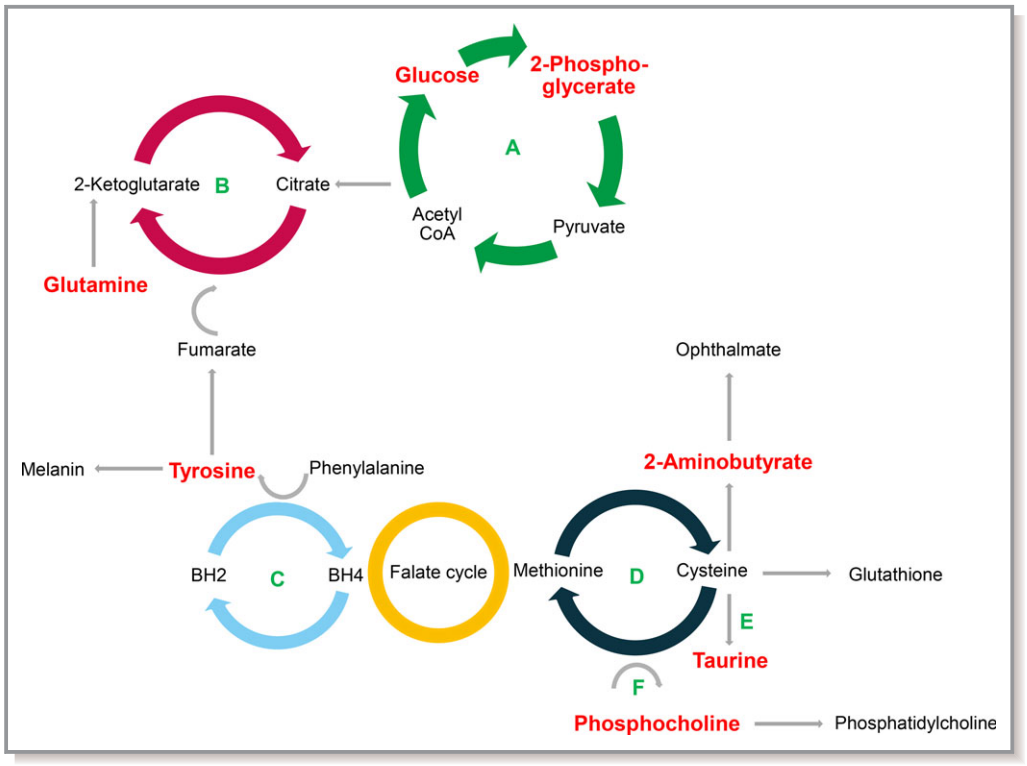

Figure 4. Simplified representation of metabolic pathways potentially involved in diastolic LV dysfunction. Metabolites significantly associated with diastolic LV function are in red color. Depicted cycles are (A) glycolysis; (B) citric acid (Krebs) cycle; (C) biopterin cycle; (D) 5-methylthioadenosine/methionine cycle; (E) transsulfuration (taurine); and (F) methylation (phosphatdylcholine). BH2 and BH4 indicate dihydrobiopterin and tetrahydrobiopterin, respectively; CoA, coenzyme A; LV, left ventricular.

diastolic LV dysfunction are multifaceted, each with different contributions among people at risk. Whether or not the metabolic markers can predict the course, over time, of diastolic LV dysfunction and associated cardiovascular complications remains to be proven in longitudinal studies. Second, keeping in mind the redundancy and bidirectionality in many metabolic pathways,[25] studies like ours cannot determine whether levels of circulating metabolites vary because of changes in production or degradation or release into or clearance from the circulation, or because of a combination of these mechanisms. Third, epidemiological studies only demonstrate association. Our proposal of molecular pathways (Figure 4) linking diastolic LV dysfunction to the metabolic markers rests on our interpretation of the available literature. Fourth, our findings need replication in other cohorts and validation in molecular studies. Finally, NMR spectroscopy is fast and keeps samples separated from the instrument, but produces crowded spectra that cannot always be reliably deconvoluted to single metabolites.[16]

Conclusions

In this first population study of its kind, we identified a profile of circulating metabolites, indicative of energy substrate utilization and protection against oxidative stress, associated with diastolic LV function. In ROC curve analyses, adding the LFs to NT-proBNP increased the AUC. Given the current state of this newly emerging field of research, we believe that, in the near future, the principal application of metabolic markers might lie in the characterization of biochemical pathways leading to diastolic LV dysfunction rather than in diagnosing this condition. One line of research that we are currently pursuing in this respect is linking the metabolic profiles to markers of mitochondrial function.[39] Deeper insights in the pathogenetic mechanisms causing diastolic HF will potentially identify new targets for prevention and treatment at a subclinical and still reversible stage.

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Disclosures

None.

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