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Metabolomic does not predict response to cardiac resynchronization therapy in patients with heart failure

Luigi Padeletti[a,b], Pietro A. Modesti[a], Stella Cartei[a], Luca Checchi[a], Giuseppe Ricciardi[a], Paolo Pieragnoli[a], Stefania Sacchi[a], Margherita Padeletti[c], Brunetto Alterini[a], Pietro Pantaleo[a], Xiaoyu Hu[d], Leonardo Tenori[d,e] and Claudio Luchinat[e,f]

Aims **Metabolomic, a systematic study of metabolites, may be a useful tool in understanding the pathological processes that underlie the occurrence and progression of a disease. We hypothesized that metabolomic would be helpful in assessing a specific pattern in heart failure patients, also according to the underlining causes and in defining, prior to device implantation, the responder and nonresponder patient to cardiac resynchronization therapy (CRT).**

Methods **In this prospective study, blood and urine samples were collected from 32 heart failure patients who underwent CRT. Clinical, electrocardiography and echocardiographic evaluation was performed in each patient before CRT and after 6 months of follow-up. Thirty-nine age and sex-matched healthy individuals were chosen as control group. For each sample, 1H-NMR spectra, Nuclear Overhauser Enhancement Spectroscopy, Carr-Purcell-Meiboom-Gill and diffusion edited spectra were measured.**

Results **A different metabolomic fingerprint was demonstrated in heart failure patients compared to healthy controls with high accuracy level. Metabolomics fingerprint was similar between patients with ischemic and nonischemic dilated cardiomyopathy. At 6-month follow-up, metabolomic fingerprint was different from baseline. At follow-up, heart failure patients' metabolomic fingerprint remained significantly different from that of healthy controls, and accuracy of cause discrimination remained low. Responders and nonresponders had a similar metabolic fingerprint at baseline and after 6 months of CRT.**

Conclusion **It is possible to identify a metabolomic fingerprint characterizing heart failure patients candidate to CRT, it is independent of the different causes of the disease and it is not predictive of the response to CRT.**

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Keywords: cardiac resynchronization therapy, heart failure, metabolomic

[a]Department of Cardiology, University of Florence, [b]Department of Cardiology, Cliniche Gavazzeni, Bergamo, [c]Department of Cardiology, University of Siena, [d]FiorGen Foundation, [e]Magnetic Resonance Center (CERM), University of Florence and [f]Department of Chemistry, University of Florence, Sesto Fiorentino, Firenze, Italy

Correspondence to Professor Luigi Padeletti, MD, V.le Morgagni 85, Florence, Italy
Tel: +39 0557947514; e-mail: lpadeletti@interfree.it

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Introduction

Metabolomic is the systematic study of small-molecule metabolites that are by-products of cellular metabolism. The metabolomic fingerprint, derived from specific protein enzymatic processes, directly correlates with the clinical phenotype of the diseases.[1–3] Cardiac resynchronization therapy (CRT) has shown a clear clinical benefit in heart failure patients by improving symptoms, quality of life, exercise capacity and cardiac function, significantly reducing morbidity and mortality,[4–9] but up to 40% of heart failure patients are nonresponder to CRT.[10,11] As an emerging discipline for molecular profiling, metabolomic may increase understanding of human diseases and clinical risk because changes in metabolite levels provide a real-time estimate of disease state and reflect the integrated effects of genomic, transcriptomic and proteomic variation.[3] The purpose of this prospective study was to assess if there is a specific metabolic profile in heart failure patients compared to healthy individuals; to investigate whether there is a difference in metabolic profile between patients with ischemic and nonischemic dilated cardiomyopathy; and, finally, to assess if there is a different metabolic pattern at baseline and at follow-up in responders and nonresponders to CRT.

Methods

Study population

Patients with ischemic and nonischemic dilated cardiomyopathy who underwent implantation of CRT at our institute were enrolled in the study. Dilated cardiomyopathy was diagnosed based on clinical history, echocardiographic examination, cardiac catheterization, and coronary angiography. Inclusion criteria were 6 months of optimal medical therapy, New York Heart Association

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(NYHA) II–IV functional class with left-ventricular ejection fraction (LVEF) 35% or less. Exclusion criteria were end-stage renal disease, clinical and hemodynamic instability, and tumor with a life expectancy of less than 1 year. Thirty-nine healthy blood donors were used as control. The study conforms to the principles outlined in the Declaration of Helsinki. The institutional review board approved the protocol and all patients gave written informed consent.

Study protocol

All patients underwent clinical and physical examination: NYHA functional class, ECG, echocardiogram with tissue Doppler imaging (TDI), Short Form-36 questionnaire of quality of life were assessed.[12] Peripheral blood and urine sample were collected at baseline, before pacemaker implantation, and after 6 months of CRT. Thirty-one patients had biventricular defibrillator (CRT-D device); only one patient had a biventricular pacemaker (CRT-P device). All patients were implanted through cephalic or subclavian left vein into right atrium, apex of right ventricle and into the coronary sinus to pace left ventricle lateral wall. Biventricular pacing parameters were optimized 1 week after implantation on the basis of the myocardial performance index (MPI), calculated as the sum of isovolumic contraction and relaxation times divided by ejection time. Optimum atrioventricular delay and interventricular delay were identified by the minimum MPI in each patient.[13] Echocardiographic measurements were repeated at 6-month follow-up. CRT responders at the 6-month follow-up were defined as those with a reduction of left-ventricular end-systolic volume (LVESV) more than 15%; patients were defined as nonresponders if LVESV at follow-up remained unchanged or was reduced by less than 15% compared with baseline.[13–16]

Blood and urine samples management

Venous blood samples from heart failure patients and controls were collected into plastic tubes (BD Vacutainer, Franklin Lakes, New Jersey, USA). Serum samples were then centrifuged at 4°C at 5000 r.p.m. for 15 min. Aliquots of 400 μl were finally transferred in cryovial (Bruker BioSpin, Milan, Italy), frozen and stored at −80°C until used. Freshly voided urine samples from heart failure patients were centrifuged at 4°C at 5000 r.p.m. for 15 min. Aliquots of 800 μl were transferred in cryovial, immediately frozen and stored at −80°C until used.

NMR samples preparation

Frozen serum and urine samples were thawed at room temperature and shaken. Three-hundred microliter of sodium phosphate buffer was added to equal quantity of serum sample, and 450 μl of this mixture was pipetted into a 4.25-mm NMR tube (Bruker BioSpin) for analysis. Seventy microliter of sodium phosphate buffer was added to 630 μl of urine. Samples were centrifuged at $1.4 \times 10^4 g$ for 5 min and 600 μl supernatant was transferred into 4.25-mm NMR tubes (Bruker BioSpin).

NMR analysis and spectral processing

One-dimensional 1H-NMR spectra were measured on a Bruker 600 MHz spectrometer using standardized protocols. Nuclear Overhauser Enhancement Spectroscopy (1D-NOESY)[17] spectra were acquired for serum and urine samples; Carr-Purcell-Meiboom-Gill (1D-CPMG)[18] and diffusion edited (DIFF)[19] spectra were acquired for serum samples only. 1D-CPMG spectra contain signals arising mostly from low-molecular-mass metabolites, DIFF spectra contain signals arising mostly from macromolecules, whereas 1D-NOESY spectra contain both kinds of signals. Each spectrum in the region 10.00–0.02 ppm (excluding the water region) was segmented into 416 0.02-ppm chemical shift bins (buckets) prior to any statistical analysis. Bucketing is a means to reduce the number of total variables and to compensate for small shifts in the signals. Serum spectra were not normalized,[20] whereas urine spectra were normalized to the total area intensity.

Statistical analysis

Continuous variables were expressed as mean ± SD. Continue variables were analyzed with the Student's *t*-test and one-way analysis of variance (ANOVA), when appropriate.

The statistical procedure employed for classification is the orthogonal partial least squares discriminant analysis (OPLS-DA).[21] OPLS is a newly developed variant of partial least squares (PLS) analysis. Briefly, it is a projection technique aimed at building a discrimination space (obtained by linear combinations of the original predictors) where the groups of interest are maximally divided. Predictive and uncorrelated information is kept separated in the model. After the training model is build, new samples can be predicted by projecting it in the discriminant space. This was done in Fig. 2 to assess whether follow-up samples are more similar to baseline samples or to controls. The accuracy for classification was assessed by means of a double cross-validation scheme.[22,23] The original data set was split into a training set (80%) and a test set (20%) randomly before any step of statistical analysis. The number of OPLS components (3–40 components) was chosen on the basis of a five-fold cross-validation performed on the training set only, and the best model was used to predict the samples in the test set. The whole procedure was repeated 200 times with a Monte Carlo cross-validation scheme, and the results averaged. To assess the significant differences of metabolites (i.e. NMR peaks) from different groups, a univariate Wilcoxon test was used. A total of 41 NMR peaks for serum and 29 for urine spectra were tested. A *P*-value of 0.05 or less (not corrected for multiple test) was considered statistically significant. Two kinds of comparisons

were performed: the comparison between patients and healthy controls (32 vs. 39 samples) and the comparison between baseline patients and 6-month follow-up patients (32 vs. 32 samples). Following the Cohen formulation of power calculation, and using a *t*-test as model, these numbers were sufficient, in the first case, to detect moderate effects ($d = 0.7$) at a significance level of 0.05 with a statistical power higher than 0.8. For the second analysis, because the patients were the same before and after follow-up, and so the test was paired, the statistical power was higher than 0.95, at the same significance level and assuming the same moderate effect. Alternatively, this means that we can detect even smaller effects ($d = 0.5$) still with a sufficient power (0.82).

We avoided the use of Bonferroni correction because of the rise of the risk of false-negatives; anyway metabolites still statistically significant ($P \leq 0.001$ for serum and $P \leq 0.002$ for urines) after this correction were also reported.

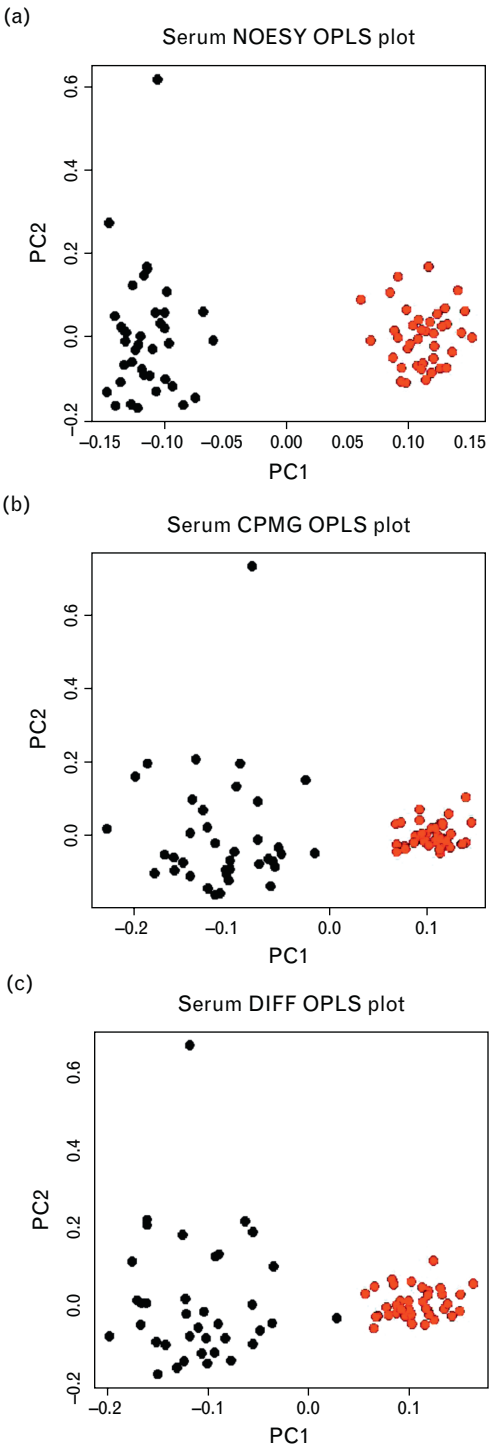
All resonances of interest were then manually checked, and signals were assigned on template one-dimensional NMR profiles by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (Version 2-0-0; Bruker BioSpin) reference database[24] and published literature when available. All calculations for metabolomic purpose analysis were made using home-made scripts written in our lab using the R language.[25]

Table 1 Demographic and clinical characteristics of the studied population values are reported as number, percentage or median ± SD

	Controls	HF patients (baseline)
Sex (W/M)	11/28	9/23
Age (years)	68.7 ± 1.9	70.7 ± 12.5
Weight (kg)	78.1 ± 11.7	78.8 ± 17.7
HF cause, ischemic (*n*)	–	12
NYHA class (II/III/IV) (*n*)	–	11/16/5
LVEF (%)	> 55	29 ± 6
QRS duration (ms)	< 120	142 ± 36
SBP (mmHg)	133 ± 16	124 ± 18
Laboratory:		
Hemoglobin (g/dl)	14.8 ± 0.9	12.6 ± 1.8
Leucocytes ($\times 10^3$/ml)	6.0 ± 1.2	7.4 ± 2.7
Cholesterol, total (mg/dl)	210 ± 29	154 ± 39
Cholesterol, LDL (mg/dl)	138 ± 19	90.7 ± 31.2
Triglycerides (mg/dl)	108 ± 40	118 ± 53
Glucose (g/dl)	0.87 ± 0.2	0.95 ± 0.18
Creatinine (mg/dl)	0.85 ± 0.17	1.14 ± 0.58
sMDRD (ml/min)	96 ± 19	74 ± 27
Medications		
ACE-I (*n*)	–	17
ARB (*n*)	–	11
β-blockers (*n*)	–	27
Aldosterone antagonists (*n*)	–	11
Diuretics (*n*)	–	28
Allopurinol (*n*)	–	5
Statins (*n*)	–	19

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; HF, heart failure; LDL, low-density lipoprotein; LVEF, left-ventricular ejection fraction; M, men; NYHA, New York Heart Association; sMDRD, simplified modification of diet in renal disease; W, women.

Fig. 1



Descriptive clustering of heart failure patients at baseline (black dots) and controls (red dots) obtained by use of OPLS method on serum NOESY (a), CPMG (b) and DIFF spectra. CPMG, Carr-Purcell-Meiboom-Gill; DIFF, diffusion edited; NOESY, Nuclear Overhauser Enhancement Spectroscopy; OPLS, orthogonal partial least squares.

Results

Study population

Between October 2010 and October 2012, 32 patients (23 men, nine women) who underwent implantation of CRT were enrolled. During follow-up, two patients died because of heart failure complications. Baseline demographic and clinical characteristics are listed in Table 1. No significant difference was found between controls and patients in terms of age and sex.

Molecular signatures at baseline

A different metabolomic fingerprint was demonstrated in serum of heart failure patients compared to healthy controls with high accuracy level (NOESY: 93.3%; CPMG: 99.1%; DIFF: 95.7%), as shown in Fig. 1. Heart failure patients were characterized by significantly lower levels of lactate, methionine and higher levels of formate, phenylalanine, glucose, serine, acetate, dimethylsulfone, hypoxanthine, creatinine + creatine, and trimethylamine-N-oxide compared to healthy controls (Table 2). No significant difference was demonstrated between ischemic and nonischemic patients before CRT. The accuracy of cause discrimination at baseline was only 63.4% for NOESY, 59.2% for CPMG, and 59.4% for DIFF serum spectra. Cause discrimination remained low when considering urine spectra (58.2% for NOESY).

Molecular signatures at 6 months follow-up

After 6 months of CRT, OPLS analysis showed that none of the patients had a metabolomic fingerprint in the area of healthy controls (Fig. 2). Likewise, the accuracy of discrimination between heart failure causes remained low both for serum (64.6% for NOESY, 61.1% for CPMG and 62.5% for DIFF) and urine spectra (65.3%for NOESY). Pair-wise multivariate statistics indicated that metabolomic fingerprint at this time could be discriminated from those at baseline with suboptimal accuracy both for serum (71.8% for NOESY, 81.4% for CPMG and 72.6% for DIFF) and urine spectra (70.1% for NOESY). Serum spectra revealed a significant increase of tyrosine, lactate, proline, alanine and lipid (–CH=CH–) (Table 3). In urine spectra, the levels of hippurate and trigonelline were reduced, whereas threonine levels were increased when compared to baseline (Table 3). Echocardiographic criteria allowed identifying 19 responder and 11 nonresponder patients. No significant difference was observed in responders' metabolomic fingerprint with respect to nonresponders at baseline (accuracy levels: 45.2% for NOESY spectra, 46.7% for CPMG spectra, 50.8% for DIFF spectra; 43.7% for urine NOESY spectra) and after 6 months follow-up (accuracy levels: 35.3% for NOESY spectra, 32.1% for CPMG spectra, 35.7% for DIFF spectra; 44.5% for urine NOESY spectra).

Discussion

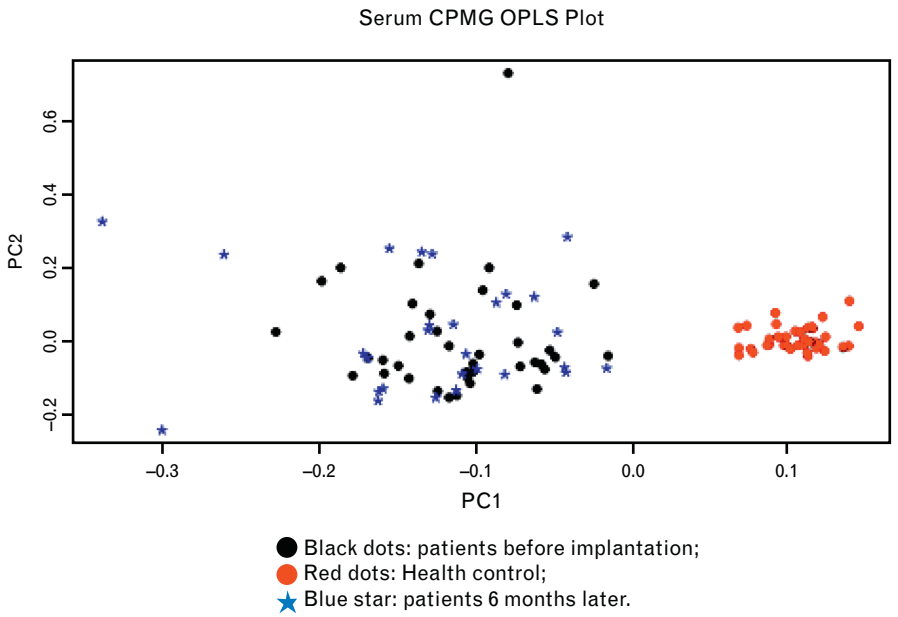
In this study, we demonstrated the existence of a specific metabolomic fingerprint that characterizes patients with dilated cardiomyopathy candidate to CRT compared with healthy individuals. Metabolomic fingerprint of heart failure patients resulted to be characterized by lower levels of lactate, methionine and by higher levels of formate, phenylalanine, glucose, serine, acetate, dimethylsulfone, hypoxantine, creatine + creatinine, and trimethylamine-N-oxide (Table 2). Other authors have shown a specific metabolomic fingerprint in heart failure patients. In the study by Lin *et al.*,[26] who used the same techniques as our study, the metabolomic fingerprint was characterized by higher levels of acetoacetate and urea and by lower levels of threonine, glycine, ethanol, histidine, alanine and tyrosine, results which were significantly different from our heart failure patient population. In the study by Dunn *et al.*,[27] who employed gas chromatography and mass spectrometry, pseudopurine and 2-oxoglutarate were identified as two good indicators of heart failure. These metabolites were different from those reported by Lin *et al.* and in our studies. These results could be due to the different characteristics of patients investigated (Lin *et al.*[26] analyzed end-stage heart failure patients just before transplantation) or to the different samples analysis technique performed (Dunn *et al.*[27] used gas chromatography). It is possible to affirm that metabolomic approach is able to identify a fingerprint characterizing heart failure patients, but a univocal and reproducible pattern is still not emerged in the literature.

Table 2 Metabolites found to be statistically different in serum of HF patients at baseline with respect to the control group[a]

	CHF		Healthy control		
Serum metabolites	Mean	95% CI	Mean	95% CI	*P*-value
Formate	10.11	3.81–15.25	8.99	4.64–19.12	0.014
Phenylalanine	156.91	97.49–239.16	132.69	112.42–164.35	0.005
Glucose	2419.9	1768.1–3634.5	2142.5	1759.1–2984.2	0.022
Lactate	1609.3	662.24–3476.95	1893.65	1055.9–2857.3	0.019
Serine	622.16	460.05–813.67	510.54	421.79–627.49	7.7e-7*
Acetate	309.69	214.89–492.73	252.76	198.82–318.37	0.02
Methionine	12.97	0.01–75.55	52.42	0.02–264.96	4.4e-6*
Dimethylsulfone	71.37	31.88–151.89	54.74	16.78–150.04	0.002
Hypoxanthine	15.91	4.33–38.08	8.58	0.02–16.51	8.4e-5*
Trimethylamine-N-oxide	83.97	5.62–288.74	31.09	2.85–66.94	2.9e-4*
Creatinine + creatine	418.88	249.98–669.61	309.77	242.26–422.88	3.37e-5*

CI, confidence interval; HF, heart failure. [a]Values are given in arbitrary units together with confidence intervals at 95%. * Still significant after Bonferroni correction ($P \leq 0.001$).

Fig. 2



Predictive analysis of serum CPMG spectra of patients after 6 months of CRT. Follow-up spectra are projected on the discriminant model built on the 39 controls' and 32 baseline patients' spectra. CPMG, Carr-Purcell-Meiboom-Gill; CRT, cardiac resynchronization therapy; OPLS, orthogonal partial least squares.

This poor reproducibility of the result impairs the validity of the method. Similar to the study by Lin *et al.*[26], we could not assess a different metabolic pattern in patients with different ischemic and nonischemic cardiomyopathy at baseline and after 6 months of CRT. This implies the existence of a common final pathway irrespective of the cause of the dilated cardiomyopathy. Therefore, metabolomic approach is not helpful in improving the diagnostic process necessary to define the cause of heart failure.

After 6 months of CRT, we observed a totally different metabolic pattern from baseline, which resulted to be further different from the fingerprint of healthy controls. In none of the patients, metabolomic fingerprint returned in the area of healthy controls at 6 months of follow up (Fig. 2). Analyzing samples obtained at this time, we identified another specific pattern, even if with a suboptimal level of accuracy (above 70%). The levels of serum metabolites – tyrosine, lactate, proline, alanine and lipid (–CH=CH–) – were significantly higher when compared to baseline (Table 3); similarly, at 6 months of follow up, results showed that urine metabolite levels of hippurate andtrigonelline were significantly lower, whereas levels of threonine were significantly higher (Table 3). These differences in metabolomic spectra could be considered as the expression of complex cellular and molecular modification induced by CRT. In fact, as Chakir and Kass[28] have observed, CRT can get the failing heart to contract more and perform more work, improving ion channel function involved with electrical repolarization, enhancing

Table 3 Metabolites found to be statistically different in serum and urine of HF patients at the 6-month follow-up with respect to baseline[a]

	CHF before CRT		CHF 6 months after CRT		
	Mean	95% CI	Mean	95% CI	*P*-value
Serum metabolites					
Tyrosine	138.72	103.63–193.02	161.57	112.46–216.55	0.002
Lactate	1640.77	662.24–3476.95	2602.43	1125.55–4871.36	0.002
Proline	531.03	268.35–797.93	603.41	374.91–980.81	0.04
Alanine	1141.17	722.22–1751.14	1336.38	848.19–1999.55	0.02
Lipid (-CH=CH-)	8554.31	4924.54–14253.7	9743.96	5575.39–21072.9	0.03
Urine metabolites					
Hippurate	0.011	0.002–0.026	0.019	0.007–0.069	0.01
Trigonelline	0.0023	0.0002–0.0044	0.0028	0.0009–0.0053	0.04
Threonine	0.0018	0.001–0.0033	0.0013	0.0008–0.0019	0.001*

CI, confidence interval; CRT, cardiac resynchronization therapy; HF, heart failure. [a]Values are given in arbitrary units together with confidence intervals at 95%. * Still significant after Bonferroni correction ($P \leq 0.002$).

sarcomere function and calcium handling, inducing the up-regulation of beta-adrenergic responses and improving mitochondrial energetic efficiency.

According to the present literature, up to 40% of patients may not experience any improvement in clinical status and/or reversal of cardiac remodeling after CRT.[29] An increase in LVEF above 7.5% during low-dose dobutamine echocardiography exhibited a sensitivity of 76% and a specificity of 86% in predicting response to CRT.[30] The burden and the transmural extension of myocardial scar measured by cardiovascular MRI have been shown to be associated with a poor response rate to CRT,[31] similar to a high scar burden quantified by single photon emission computed tomography myocardial perfusion imaging.[32] Other clinical, electromechanical and electrophysiological issues before device implantation have been identified by Mullens *et al.*[29] In the present study, we demonstrated that metabolomic is unable to predict the outcome of CRT patients: NOESY, CPMG and DIFF spectra for both serum and urine samples showed very low discrimination accuracy (from 43.7 to 50.8%) that precludes its use in common clinical practice.

Conclusion

Although it is possible to identify a metabolomic fingerprint that characterizes heart failure patients candidate to CRT, this pattern is quite different in different studies and is unaffected by the different causes of the disease. Furthermore, this approach seems unable to identify a metabolic profile predictive of a favorable response to CRT. Therefore, at the current state of the art, the use of this method is not justified in patients undergoing CRT.

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