Introduction

The prevalence of heart failure (HF) is increasing despite major therapeutic advances, and HF is now considered as one of the major public health burdens both in developed and developing countries [1]. It has been well known that HF is, in part, the result of the progression of chronic hypertension as well as acute and chronic cardiac injury including myocardial infarction [2]. Considering the aging of populations and the pandemic of cardiovascular disease (CVD) all over the world, the importance of its prevention is magnified [3]. As such, the prevention of HF is an urgent public health need with national and global implications that deserves high priority.

The etiology of HF is very complex and correspondingly numerous studies have aimed to define the underlying pathophysiology of HF using recent advances in the systems biology approach, which enables the diagnosis of disease state [4]. Metabolomic analysis is a comprehensive tool for simultaneously measuring the amounts of metabolites of multiple metabolic pathways. Global metabolite profiles of complex samples are used to identify potential biomarkers, which can provide new and unexpected insights into biological processes [5]. Indeed, emerging data have been reported by studies of metabolomics to define perturbations in metabolic pathways and networks in human disease including diabetes [6], cancer [7], and neurodegenerative disorders [8,9]. In the application of metabolomics to cardiovascular pathway discoveries, there have been several attempts to identify metabolic phenotypes differentiating the metabolic pathways of myocardial ischemia [10], planned myocardial infarction [11], and a reperfusion model [12]. However, metabolic investigations into urinary metabolites in human heart failure have not been performed.

In the present study, we attempted to determine the differences in urinary metabolites of HF patients versus healthy controls.

Objective: To identify metabolic pathways characterizing human heart failure (HF) using 1H nuclear magnetic resonance (NMR) based urinary metabolomic analysis in conjunction with multivariate statistics.

Design and methods: Patients with systolic HF of ischemic origin (n = 15) and healthy controls (n = 20) participated in this study. Patients with type 2 diabetes mellitus were excluded.

Results: The results showed that the urine of the HF patients had higher levels of metabolites for acetate (p < 0.05) and acetone (p < 0.01) compared to the healthy controls. In addition, there was a perturbation in methylmalonate metabolism as shown by increased urinary levels of methylmalonic acid (p < 0.001) in the HF patients. HF patients also had increased urinary levels of cytosine (p < 0.01) and phenylacetylglycine (p < 0.01) and decreased 1-methyl nicotinamide (p < 0.05) compared to healthy controls.

Conclusions: TCA cycle metabolites and fatty acid metabolism were modified in the HF patients, indicating altered energy metabolism. Moreover, perturbations of metabolism in nucleotide and methylmalonate were observed.
Materials and methods

Subjects

For this study, 15 chronic stable HF patients from Yonsei Cardiovascular Hospital and 20 age- and gender-matched healthy controls participated in the study. The inclusion criteria for HF were: (1) a diagnosis of systolic HF of ischemic origin and a left ventricular ejection fraction (LVEF) ≤ 50%, (2) ≤ 80 years of age, and (3) stable HF (New York Heart Association Classes I–II) with medication for at least 1 month prior to inclusion. The exclusion criteria were: (1) a diagnosis of HF with a preserved ejection fraction (>50%) or non-ischemic origin, (2) diagnosed type 2 diabetes mellitus and acute myocardial infarction 3 months prior to inclusion, and (3) severe cognitive impairment. At the time of initial enrollment, HF patients underwent a complete physical examination including blood pressure and heart rate and laboratory assessment. Body mass index (BMI) was calculated as weight in kg divided by height in meters squared. Medical history, including diagnosis, underlying disease, etiology of HF, and drug use were obtained. All patients were given written informed consent, and the Institutional Review Board at Yonsei University Medical Center approved the study protocol. Venous bloods were collected for further analysis after a fasting period. Urine was collected in polyethylene tubes in the morning after 12 h of fasting. The tubes were immediately stored at −70 °C until analysis.

Biochemical measurements

Biochemical measurements including total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, free fatty acid, creatinine, uric acid and glucose were analyzed by enzymatic methods with commercially available kits with automatic analyzer (Advia 1650, Siemens, Germany). Plasma B-type natriuretic peptide (BNP) concentrations were measured with a Triage BNP test kit (Biosite Diagnostics, San Diego, CA, USA).

NMR spectroscopic measurement of urine

The urine samples were thawed, vortexed, and allowed to stand for 10 min prior to mixing aliquots (540 μL) with an internal standard solution [60 μL, consisting of 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), 0.2% sodium azide in 99% deuterium water (D2O)], and then centrifuged at 12,000 rpm for 10 min. The supernatant from each urine sample was adjusted to pH 7.0 and an aliquot of the prepared urine sample (600 μL) was transferred into a 5-mm NMR tube (Wilmad, Buena, NJ). D2O and DSS provided a field frequency lock and chemical shift reference (1H, δ 0.00), respectively. The 1H NMR spectra were acquired on a Varian VNMRS-600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) operating at a 599.84 MHz 1H frequency and a temperature of 298 K, using a triple resonance 5-mm HCN salt tolerant cold probe. A noesypresat pulse sequence was applied to suppress the residual water signal. For each sample, FIDs were collected with 64 transients into 32 K data points, using a spectral width of 9615.4 Hz with a relaxation delay of 2 s, an acquisition time of 4 s, and a mixing time of 400 ms. All spectra were multiplied by an exponential weighting function corresponding to a line-broadening of 0.5 Hz.

NMR data preprocessing and multivariate statistical analysis

All NMR spectra were phased and baseline corrected using Chenomx NMR suite 4.6 software, professional edition (Chenomx Inc.). The NMR spectral data were reduced into 0.005 ppm spectral buckets and the regions corresponding to water/HOD and urea (4.32–6.32) were removed from all the spectra. The spectral data were then normalized to the total spectral area and converted to the ASCII format. The ASCII format files were imported into MATLAB (R2006a, Mathworks, Inc., Natick, MA), and all spectra were aligned using the Correlation Optimized Warping (COW) method [13]. The resulting data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) and Pareto scaled for multivariate statistical analysis. Principal components analysis (PCA), an unsupervised pattern method, was performed to examine the intrinsic variation in the dataset. To maximize the separation between samples, the orthogonal projections to latent structures method or an orthogonal partial least-squares discriminant analysis (OPLS-DA) model was applied to maximize the covariance between the measured data (X variable, peak intensities in the NMR spectra) and the response variable (Y variable, predictive classifications), and simultaneously to remove non-correlated variation in X variables to Y variables or variability in X that is orthogonal to Y [14]. Hotelling’s T2 region [15], shown as an ellipse in the scores plot, defines the 95% confidence interval of the modeled variation. The quality of the models was described by R2 and Q2 values. R2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q2 is defined as the proportion of variance in the data predictable by the model, and indicates predictability.

Quantification of urinary metabolites

Quantification of the metabolite concentrations was achieved using Chenomx NMR Suite 5.1 (Chenomx Inc. Edmonton, Canada) [15]. A reference compound (DSS) was used as an internal standard for the chemical shifts (set to 0 ppm) as well as a reference signal for quantification. Quantification was done by comparing the integral of a known reference signal (DSS-d6) with the signals derived from a library of compounds containing chemical shifts and peak multiplicities. Identifiable metabolites were chosen for quantification by matching to the Chenomx 600 MHz Library. Each urinary metabolite concentration was normalized to creatinine (μM/mM creatinine) in each urine samples. The metabolite concentration data sets were then imported into Spotfire Decision site 9.2 software (Tibco software, CA, USA). For pattern detection, heat-map analysis was performed to characterize and to verify the metabolite concentrations of the urinary metabolites. Differences in metabolite concentration and blood parameters were evaluated using Student’s t-test with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). A two-tailed value of P<0.05 was considered statistically significant.

Results

This study consisted of 15 patients with HF of ischemic origin (12 NYHA I and 3 NYHA II) and 20 age- and gender-matched healthy controls. The mean ejection fraction, systolic blood pressure, diastolic blood pressure and heart rate were 37.1 ± 8.7%, 121.7 ± 15.9 mmHg, 76.0 ± 8.9 mmHg and 74.8 ± 9.1 beats/min in the HF patients, respectively. Medical treatment for the HF patients consisted of diuretics (53.3%), digitalis (0%), β-blocker (53.3%), angiotensin converting enzyme inhibitors and/or angiotensin II receptor blocker (69.2%), anti-platelet agents (100.0%), and hypolipidemic agents (80.0%). The proportions of HF patients with hypertension, hyperlipidemia, and chronic renal failure were 40.0%, 33.3% and 13.3%, respectively. Table 1 presents baseline characteristics and biochemical parameters of the subjects. Age, gender distributions and BMI were not statistically different between the two groups. Serum levels of total cholesterol and LDL-cholesterol were lower in HF patients than in healthy controls, whereas creatinine, uric acid and BNP levels were higher in HF patients than in healthy controls.
NMR spectroscopy findings in controls and HF patients

The 600 MHz $^1$H NMR spectra of representative urine samples obtained from the healthy controls and patients with HF are shown in Fig. 1. The spectral resonances of the metabolites were assigned according to the literatures [15] and the 600 MHz library of the Chenomx NMR suite 5.1 (Chenomx Inc., Edmonton, Canada). The ambiguous peaks due to overlap or slight shifts were confirmed by spiking samples with the respective standard compounds. As noted in Fig. 1, the dominant metabolites identified in the majority of urine samples included adenine, creatinine, hippurate, taurine, betain, trimethylamine N-oxide (TMAO), dimethylamine, lactate, 3-hydroxybutyrate, phenylacetylglycine (PAG), glycine, citrate, succinate, alanine, and 2-hydroxyisobutyrate.

Metabonomic analysis of human urine in controls and HF patients

Fig. 2 presents the results of O-PLS-DA of the NMR spectra of urine samples in the healthy control and HF patient. Fig. 2A is a score plot on the first two LVs (Latent Viables) of the $^1$H NMR spectra of the control and HF patient groups. Fig. 2A shows the significant metabolic differences of the control and HF patient groups ($R^2 = 0.78; Q^2 = 0.39$). The unsupervised PR method, PCA, was initially applied to the urinary NMR spectra obtained from the control and HF patient groups to observe any inherent separation between the groups (data not shown). However, no significant separation was found between them. O-PLS-DA was therefore used for the classification of the NMR data of the urine samples. OPLS-DA comparisons between spectra obtained from the control and HF patient groups were carried out with a pareto scaling strategy. The O-PLS-DA models comparing the spectral data of the groups were built using the NMR data as the X-matrix, and class information (i.e., control or HF patient groups) as the Y matrix. A model was constructed in which one PLS component and one orthogonal component were calculated, using the spectral data scaled to pareto (Fig. 2B). A clear indication of some degree of separation between the control and HF patient groups (Fig. 2A) was observed. The O-PLS-DA loading plot (Fig. 2B) indicates that levels of hippurate, PAG, betain, TMAO, and dimethylamine were increased in the HF patients. In contrast, levels of 2-hydroxyisobutyrate, citrate, creatinine, valine, and lactate were decreased in the HF patients.

Fig. 3 shows a heat-map for the metabolite concentrations, which were quantified using targeted profiling of the urinary samples from the two groups. The metabolite concentrations were determined using the 600-MHz library of Chenomx NMR Suite 5.1, which compares the integral of a known reference signal (DSS-d6) with signals derived from a library of compounds. The changes in metabolite levels were represented by heat-map analysis using Spotfire software to investigate the overall metabolic patterns between the control and HF patients group. The color of each cell in the heat-map represents the level of metabolic concentration.

Table 1

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Healthy control (n = 20)</th>
<th>Heart failure (n = 15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>67.3 ± 11.5</td>
<td>71.2 ± 6.1</td>
<td>0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>14:6</td>
<td>12:3</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.4 ± 2.4</td>
<td>21.6 ± 2.9</td>
<td>0.15</td>
</tr>
<tr>
<td>Waist circumferences (cm)</td>
<td>78.9 ± 7.3</td>
<td>81.3 ± 8.9</td>
<td>0.15</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>126.6 ± 47.9</td>
<td>106.6 ± 31.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>186.1 ± 34.2</td>
<td>139.2 ± 30.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>50.1 ± 10.2</td>
<td>45.7 ± 11.2</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>110.7 ± 26.4</td>
<td>72.2 ± 22.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Free fatty acid (mg/dL)</td>
<td>403.6 ± 229.6</td>
<td>455.4 ± 237.1</td>
<td>NS</td>
</tr>
<tr>
<td>creatinine (mg/dL)</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>4.5 ± 0.9</td>
<td>5.7 ± 1.8</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>90.7 ± 9.6</td>
<td>94.1 ± 13.5</td>
<td>NS</td>
</tr>
<tr>
<td>BNP (pg/mL)</td>
<td>16.0 (8.8–22.4)</td>
<td>41.5 (16.9–160.6)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Mean ± SD. BNP: B-type natriuretic peptide, Results of plasma BNP levels are expressed as the median value (25th to 75th percentile range). $^1$χ$^2$ test.
Fig 2. OPLS-DA score plot (A) and loadings plot (B) of urinary NMR spectra obtained from healthy controls and patients with HF. The loading plot reveals metabolites responsible for differentiating the control group and HF group. The upper section of the loadings plot represents metabolites increased in the control group, whereas the lower section represents metabolites increased in the HF group. The goodness of fit and predictability for the models was evaluated by values of $R^2$ of 0.78 and $Q^2$ of 0.39.

Fig 3. Heat-map of 24 urinary metabolites showing metabolic difference between HF patient and control groups. Metabolite concentrations were measured by targeted profiling through the use of Chenomx NMR Suite 5.1 (Chenomx Inc. Edmonton, Canada) and concentrations were determined using the 600-MHz library from Chenomx NMR Suite, which compares the integral of a known reference signal (DSS-d6) with signals derived from a library of compounds containing chemical shifts and peak multiplicities.
indicating different metabolic patterns between the control and HF patients group. The metabolite concentrations of 1-methyl nicotinamide (MNM amide), 2-hydroxyisobutyrate, 2-oxoglutarate, adenine, cis-acconitate, citrate, glutamine, histidine, phenylalanine, and pyruvate were relatively lower in the HF patients group compared to the control group, whereas concentrations of acetate, acetoacetate, acetyl, betain, cytokine, dimethylamine, glutarate, hippurate, methylmalonate, and PAG were relatively higher in the HF patients group. The metabolites responsible for the observed differences between the two groups are summarized in Table 2, along with the relative concentrations of metabolites in the groups.

Quantification of metabolites in control and HF patients

To further analyze the metabolic changes, we performed the comparison of the urinary metabolites normalized by creatinine using Student’s t-test between the control and HF patient groups. The results showed that urinary metabolite levels of acetate (p < 0.05), acetone (p < 0.01), cytosine (p < 0.01), methylmalonate (p < 0.001), and PAG (p < 0.01) were significantly higher in the HF patients compared to the control group, whereas levels of 1-MNM amide (p < 0.01) were significantly lower in the HF patients compared to the healthy controls, which could possibly lead to reduced levels of urinary succinate as shown in this study. It is possible that the subjects of this study had potentially depleting stores required for homocysteine metabolism. As cellular cobalamin deficiency or by cataplerosis in normal cardiomyocytes [19], the TCA cycle intermediates may support the previous notion of ischemia-related TCA cycle impairment and reflect the relative maintenance of the myocardial TCA cycle. Although an alteration in myocardial substrate metabolism in the failing heart has been suggested, the molecular mechanisms and consequences of metabolic alterations in myocardial substrate utilization are poorly understood and remain to be elucidated.

Energy metabolism in HF

The metabolite profiles of the HF patients were characterized by increased urinary excretion of acetone, acetocacete, and acetate, and decreased urinary excretion of TCA cycle intermediates such as citrate, succinate, 2-oxo-glutarate, and cis-acconitate. It has been recognized that the failing heart is characterized by alterations in myocardial energy metabolism, including mitochondrial dysfunction and a reduction in the fatty acid (FA) oxidation rate, which is partially compensated by an increase in glucose utilization [16]. The rate of metabolism along these different pathways is determined by various enzymes and substrate-product relationships including pyruvate dehydrogenase (PDH) and palmitoyltransferase-1 (CPT-1) [16,17]. Alternatively, it has been postulated that a reduction in the expression of genes involved in fatty acid metabolism may be associated with cardiac hypertrophy and heart failure [16]. Indeed, several mitochondrial β-oxidative enzymes have reduced activity [18] in heart failure, and are partly responsible for the reduction in fatty acid metabolism. It may be that increases in urinary ketone bodies shown in the present study could possibly be due to alterations in myocardial utilization.

Cardiomyocyte levels of TCA cycle intermediates are regulated to provide adequate throughput of substrates which were derived from glycolysis and β-oxidation of fatty acids [10]. While there is a constant rate of mitochondrial efflux of TCA cycle intermediates as evidenced by cataplerosis in normal cardiomyocytes [19], the TCA cycle intermediates in the setting of acute ischemia are preserved to defend ATP production [10]. In the present study, the patients were all diagnosed with ischemia-origin HF; thus, the depressed pattern of urinary TCA cycle intermediates may support the previous notion of ischemia-related TCA cycle impairment and reflect the relative maintenance of the myocardial TCA cycle. Although an alteration in myocardial substrate metabolism in the failing heart has been suggested, the molecular mechanisms and consequences of metabolic alterations in myocardial substrate utilization are poorly understood and remain to be elucidated.

Methylmalonate metabolism in HF

Methylmalonate (MMA) is the metabolite that is converted to the TCA cycle intermediate succinate with the help of the cofactor vitamin B12. It was previously demonstrated that plasma and urinary concentrations of MMA and homocysteine will rise in cases of intra-cellular cobalamin deficiency [20], and MMA is now considered a biomarker for vitamin B12 deficiency. In the present study, significantly higher levels of urinary MMA were observed in the HF patients compared to the healthy controls, which could possibly lead to reduced levels of urinary succinate as shown in this study. It is possible that the subjects of this study had potentially deficient vitamin B12 status from either nutritional B vitamin deficiency or by the depletion of stores required for homocysteine metabolism. As a result, hyperhomocysteinemia can occur in HF patients, which was not experimentally examined in the present study. Given that hyperhomocysteinemia is a predictive risk marker of HF [21] as well as associated with the severity of HF [22], adequate intake of B
vitamins needs to be placed as a priority for preventive measure in HF and the pathogenic role of vitamin B12 deficiency in HF needs to be further determined. Moreover, MMA was reported to inhibit succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake [23], therefore it can be speculated that the accumulation of MMA would be associated with impaired TCA cycle flux.

With regard to the adequacy of the subjects' vitamin B12 intakes in this study, the results showed that about 38% of the total subjects had inferior intakes according to the dietary reference intakes (DRIs); however, their actual daily intakes met the DRIs for Koreans (data not shown). This indicates that the proportion of low intakes was substantial, which could lead to potential nutritional complications in these HF patients.

**Nucleotide metabolism in HF**

In the present study, urinary levels of 1-methylnicotinamide were significantly lower and urinary levels of cytosine were significantly higher in the HF patients compared with healthy controls. N-methylnicotinamide (NMN amide) is involved in the tryptophan-NAD+ pathway, which supplies pyridine nucleotides to the liver [24], and NMN amide has been suggested as a urinary and plasma biomarker of peroxisome proliferation in rats [25]. The observed pattern for reduced NMN amide may simply reflect niacin deficiency in the HF patients. Alternatively, it is also possible that the tryptophan-NAD+ pathway was modified in the HF patients, resulting from the alteration of gene transcription of encoding key enzymes in the tryptophan-NAD+ pathway with relation to PPARs [25,26].

On the other hand, the urinary excretion of modified nucleosides including cytidine, pseudouridine, and methylguanosine is considered to be indicators of RNA degradation and cell turnover [27]. It has been reported that elevated urinary levels of modified nucleosides were found in patients with leukemia and lymphoma [28], and several types of cancer [29–31]. In the present study, urinary levels of cytosine, a metabolite of cytidine, were significantly higher in the HF patients than in the healthy controls. This is in line with a recent report demonstrating that serum levels of pseudouridine were raised in documented HF patients, suggesting that it reflects the

![Fig 4. Quantification of identified metabolites in healthy controls (green) and HF (red) patients. Data are given as means ± SD. Urinary metabolite concentrations were normalized to creatinine (μM/mM creatinine). *P < 0.05; **P < 0.01; ***P < 0.001.](image-url)
remodeling process in the heart itself or increased catabolic activity in the peripheral tissues [32]. Along with the perturbations in nucleotide metabolism, increases in PAG were also observed in the HF patients compared to the healthy controls. In addition, levels of methylamines such as dimethylamine, TMAO, and tyramine tended to be increased in the HF patients, possibly indicating infection, disturbances in gut microflora, and drug-induced phospholipids in these patients [33].

This study has the limitation that it is difficult to draw conclusions about dynamic processes including flux rates and influences of drugs. Moreover, it should be acknowledged that urinary metabolite profiling may not account the changes in myocardial metabolites for all. Furthermore, given our relatively small sample size, our observation still remains to be verified in larger studies. Further study is needed to characterize the differential metabolite profiling according to the grade of HF with large cohort enough to distinguish metabolite pattern. Finally, subjects confined only to ischemic HF may limit the observed results to generalize in HF patients with whole spectrum of etiologies. However, global measurement of metabolite changes using simple urine specimen seems to be a useful tool to detect metabolic perturbations, in addition, the multivariate statistical approach considers all of the metabolic changes simultaneously.

To conclude, we identified metabolites by comparing stable HF patients and healthy controls using NMR spectroscopy. The metabolite profiles of the HF patients exhibited increased urinary levels of ketone bodies accompanied by reduced levels of TCA cycle intermediates, suggesting altered energy metabolism including changes in the TCA cycle and fatty acid metabolism. Furthermore, we observed perturbations in metabolic pathways involved in methylmalonate and nucleotide metabolism. Further studies are needed to investigate whether or not these metabolic changes are associated with the potential pathogenesis of HF.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.clinbiochem.2010.11.010.

References