Serum metabolomics reveals many novel metabolic markers of heart failure, including pseudouridine and 2-oxoglutarate

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There is intense interest in the identification of novel biomarkers which improve the diagnosis of heart failure. Serum samples from 52 patients with systolic heart failure (EF $< 40\%$ plus signs and symptoms of failure) and 57 controls were analyzed by gas chromatography – time of flight – mass spectrometry and the raw data reduced to 272 statistically robust metabolite peaks. 38 peaks showed a significant difference between case and control ($p < 5\times10^{-5}$). Two such metabolites were pseudouridine, a modified nucleotide present in t- and rRNA and a marker of cell turnover, as well as the tricarboxylic acid cycle intermediate 2-oxoglutarate. Furthermore, 3 further new compounds were also excellent discriminators between patients and controls: 2-hydroxy, 2-methylpropanoic acid, erythritol and 2,4,6-trihydroxypyrimidine. Although renal disease may be associated with heart failure, and metabolites associated with renal disease and other markers were also elevated (e.g. urea, creatinine and uric acid), there was no correlation within the patient group between these metabolites and our heart failure biomarkers, indicating that these were indeed biomarkers of heart failure and not renal disease *per se*. These findings demonstrate the power of data-driven metabolomics approaches to identify such markers of disease.

KEY WORDS: heart failure; metabolomics; biomarkers; pseudouridine; 2-oxoglutarate.

1. Introduction

Biomarkers are essential tools in diagnosing disease and monitoring progression as well as response to therapy. In heart failure, BNP has proved its usefulness as a diagnostic marker and there are some limited data on monitoring response to therapy (Troughton *et al.*, 2000; Zaphiriou *et al.*, 2005). Other markers such as TNF alpha, IL-1beta and IL-6 have also been shown to be elevated and are likely to reflect peripheral processes distinct from cardiac overload and/or hypertrophy which is predicated by BNP secretion (Pan *et al.*, 2004).

There is intense interest in the identification of further biomarkers in the syndrome of heart failure. Distinct patterns of the ensemble of several such markers may eventually help in identifying specific classes of the syndrome with improved predictive power in terms of diagnosis, prognosis and treatment options.

In a first step towards this goal, we here present a new approach to identifying biomarkers by focusing on metabolic changes during heart failure. Metabolomics is the data-driven study of the different patterns of metabolites within living organisms, in which we seek measurements that are as comprehensive as possible. Conceptually, metabolomics lends itself ideally to studying heart failure and other diseases. In contrast say to malignancy, where tissue is readily available for transcriptomic and proteomic analysis, routine laboratory tests for heart failure are limited to serum samples. Furthermore, metabolic alterations have been well documented in the heart (Neubauer *et al.*, 1997; Finck and Kelly, 2006) and peripheral tissues (Drexler *et al.*, 1992; De Sousa *et al.*, 2000) in heart failure and other diseases (Orešič *et al.*, 2006; van der Greef *et al.*, 2006). Metabolomics is also expected on theoretical grounds to be more discriminatory than proteomics (Oliver *et al.*, 1998; Raamsdonk *et al.*, 2001; Kell, 2004; Kell, 2006a, b).

The present metabolomic study of serum from patients with heart failure and appropriate controls detected 272 candidate metabolite peaks, of which 38 showed highly significant differences between cases and controls. At least two of these metabolites, pseudouridine and 2-oxoglutarate, have the potential, alone or together, to improve on or add to BNP in terms of their sensitivity and specificity as biomarkers.

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2. Methods

2.1. Participants

The study was approved by the local ethics committee for clinical studies. Patients attending the heart failure clinic at the South Manchester University Hospital Regional Cardiothoracic Centre and Heart Transplant Unit were invited to participate in the study by providing samples of venous blood. 52 patients were recruited with an established diagnosis of left ventricular dysfunction (LVD, EF < 40%) and heart failure. Ejection fraction was determined echocardiographically. All patients belonged to NYHA (New York Heart Association) classes II-IV. The age-matched control group were mainly taken from an ENT clinic, though some were from an ophthalmology clinic and others were the healthy partners of the patients. They comprised subjects with no history of cardiac disease, and were examined clinically to this end at recruitment. Subjects with acute or chronic inflammatory conditions, malignancies and significant respiratory pathology were excluded. See Table 1 for the patient characteristics.

2.2. Sample collection

Venous blood was collected from the antecubital veins by venepuncture, left to coagulate for 10 min, followed by centrifugation (2200 g, 15 min). The supernatant serum was then aliquoted into 2.5 mL cryotubes and stored at − 80°C until required.

Table 1
Summary of patient characteristics. IQR = inter-quartile range

	Cases HF (n = 52)	Controls (n = 57)
Age, ± 1SD, range	68, 10, 46–86	67, 9, 44–87
Sex M:F	43:9	22:35
Median NYHA Class (IQR)	2 (0)	0
Median Ejection Fraction (IQR)	25(5)	
Mean Ejection Fraction (IQR)	27(6)	
Etiology ischemic:non-ischemic	33:19	
Bean BMI ± 1SD	27(5)	26(5)
Hypertensive: normotensive	8:44	15:42
DM:nonDM	9:43	4:53
Smoker:non-smoker	5:47	8:49
Na +, mean, ± 1SD, Range	141, 3, 133–145	140, 3, 132–146
K +, mean, ± 1SD, range	4.2, 0.7, 3.3–5.2	4.2, 0.3, 3.7–5.2
Urea, mean, ± 1SD, range	12, 8, 3 – 56	6, 2, 3–12
Creatinine, mean, ± 1SD, range	139, 57, 70–352	81, 18, 51–142
Hemoglobin, mean, ± 1SD, range	142, 13, 110–182	144, 14, 111–152
Beta-blockers Y:N	44:4	7:52
ACE inhibitors Y:N	43:8	6:50
Diuretics Y:N	44:7	7:49

DM = Diabetes mellitus. The *p*-value for the difference in creatinine between cases and controls was 2.5×10^{-12}. Spearman's rank correlation coefficient between creatinine and BNP was 0.66 with a significance *p*-value of 8×10^{-13}

2.3. Sample preparation and GC-TOF-MS analysis

Serum samples were prepared for Gas Chromatography-Time of Flight-Mass Spectrometry (GC-tof-MS) analysis as described (Kenny *et al.*, 2005; O'Hagan *et al.*, 2005; Underwood *et al.*, 2006). 200 μL serum was spiked with 100 μL internal standard solution (0.18 mg.mL^{-1} succinic d$_4$ acid; Sigma-Aldrich, Gillingham, UK) and vortex mixed for 15 s. Protein precipitation was performed by addition of 600 μL acetonitrile and vortex mixing for 15 s followed by centrifugation (13,385 g, 15 min). The supernatant was transferred to an Eppendorf tube and lyophilised (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences, Basingstoke, UK). To increase metabolite volatility and thermal stability a two-stage chemical derivatisation procedure was performed. 50 μL 20 mg.mL^{-1} O-methylhydroxylamine solution was added and heated at 40°C for 90 min followed by addition of 50 μL MSTFA (*N*-acetyl-*N*-(trimethylsilyl)-trifluoroacetamide) and heating at 40°C for 90 min. 20 μL of a retention index solution (3 mg.mL^{-1} n-decane, n-dodecane, n-pentadecane, n-nonadecane, n-docosane dissolved in hexane) was added and the samples were analysed using an Agilent 6890 N gas chromatograph and 7683 autosampler (Agilent Technologies, Stockport, UK) coupled to a LECO Pegasus III electron impact time-of-flight mass spectrometer (LECO Corporation, St Joseph, USA) employing previously described optimised instrumental conditions for serum (O'Hagan *et al.*, 2005). Initial data processing of raw data was undertaken using LECO ChromaTof v2.25 software to construct a data matrix (metabolite peak vs. sample no.) including response ratios (peak area metabolite/peak area succinic-d$_4$ internal standard) for each metabolite peak in each sample. The number of peaks in this matrix is 1467, based on a series of studies of serum samples additional to but including those in the present sample set (and note that some metabolites can produce more than one derivative). This matrix was then further reduced by removing any peak which had more than 60% missing values. In this way if any class was not being consistently matched with a given peak, that peak was considered not robust enough for further statistical analysis.

2.4. Pro-BNP

N-terminal pro-BNP was measured on the Roche E170 immunoassay analyser using the manufacturer's chemiluminescence kit according to their instructions.

2.5. Urea and creatinine

Urea and creatinine were measured enzymatically in serum using a Roche analyser according to the manufacturer's instructions.

2.6. Statistical analyses

Univariate statistical analysis was performed in order to assess the characteristics of each independent metabolite peak generated using the above protocol. As the experimental design employed in this study was that of a matched case–control study (Rothman and Greenland, 1998), single-factor analysis of class distribution was performed for each metabolite peak. Before choosing which significance test to apply, each metabolite peak was checked for within-class kurtosis, and for within-class goodness of fit to a normal distribution using the Lilliefors test. For a given metabolite peak, if either control or case samples had kurtosis > 3, or failed the Lilliefors test (where the null hypothesis, that the sample set has a normal distribution, is rejected if the *p*-value < 0.05) then the non parametric rank-based Wilcoxon rank sum (Mann–Whitney) test was used (Hollander and Wolfe, 1973); otherwise the classical one-way ANOVA test was used. For each metabolite, the null hypothesis (that the sample metabolite concentrations from both classes came from populations with the same mean) was tested. Generally if a *p*-value of less than 0.01 is calculated then the two sample populations for that metabolite are deemed to be significantly different; however, when many parallel tests are performed care has to be taken regarding Type I errors (i.e. falsely rejecting the null hypothesis). To help reduce the possibility of Type I errors the critical *p*-value was modified using Bonferroni correction. Bonferroni correction is considered to be overzealous in its reduction of Type I errors (Bland and Altman, 1995; Perneger, 1998; Broadhurst and Kell, 2006), leading to the conclusion that any peak found to have a *p*-value below this level is clearly significant. Nonetheless, Type I errors are an ever-present danger in this type of study (Wacholder *et al.*, 2004). Thus, with 272 statistically usable peaks detected (note that some metabolites have more than one peak, though not all metabolites are identified), the modified critical (*p*-) value was set conservatively to 0.01/200, or, 5×10^{-5}.

All statistical analyses were carried out using the Matlab® scripting language (http://www.mathworks.com/). All algorithms used are implemented such that any missing values are ignored. Scripts are available upon request.

3. Results

Of the 272 metabolite peaks produced by the pre-processing protocol, 38 showed a significant difference between case and control (*p*-value < 5.10^{-5}) (figure 1, Table 2), indicating changes in several metabolic pathways. We note that there was some gender bias between the cases and controls (Table 1), but that this is not responsible for the discriminating biomarkers observed (Table 2) (cf. (Kirschenlohr *et al.*, 2006) and see also

(Ransohoff, 2005; Broadhurst and Kell, 2006) for reviews). There was also inevitable bias in the use of pharmaceutical drugs between cases and controls (Table 1) but this did not affect the distribution of the biomarkers detected (see later, and all relevant data and metadata will be made available electronically as supplementary information). Analysis of these peaks indicated that the metabolites that they represent came from many different parts of metabolism including energy metabolism and nitrogen metabolism. While it is known that metabolites such as urea, uric acid and creatinine are often raised in heart failure, reflecting impaired renal excretion (Smith *et al.*, 2006), a number of 'novel' metabolites which have not previously been implicated in heart failure were also observed to be to be present at considerably different levels in cases and controls. Five such metabolites were identified chemically and considered to be of particular biological interest: pseudouridine, 2-oxoglutaric acid, 2-hydroxy, 2-methylpropanoic acid, erythritol, and 2,4,6-trihydroxypyrimidine. Figure 1a demonstrates the ROC curves for these metabolites, as well as for BNP which is presently considered a 'gold standard' heart failure biomarker (Zaphiriou *et al.*, 2005). It is evident that pseudouridine and 2-oxoglutaric acid are at least as good indicators of heart failure as is BNP, while 2-hydroxy,2-methylpropanoic acid, erythritol and 2,4,6-trihydroxypyrimidine are almost as good. The area under the ROC curve and the statistical *p*-value are not entirely unrelated, but they do nevertheless measure different properties; ROC curves are more sensitive to the *actual* class distributions across the total range, rather than being encoded as an estimate of this distribution as calculated by variance-based significance tests. The relationship between these two values for a series of metabolites is illustrated in figure 2b. Figure 1 highlights the ability of the technologies employed to identify metabolites from the mass spectra produced, in this example, that of pseudouridine. There are also two 'unknowns' (peaks with mass spectra very different from any of those in currently available mass spectral libraries) which have areas under the ROC curve greater than 0.85 (one being the third highest), which may be taken to indicate that metabolomics is still a youthful science (Harrigan and Goodacre, 2003; van der Greef *et al.*, 2004).

Figure 3a demonstrates the corresponding 'box and whisker' plots for the top 5 novel metabolites plus BNP. A model combining pseudouridine and 2-oxoglutaric acid discriminates visually between case and control with only 3 false negatives (figure 3b). Furthermore, the patients misclassified by this model differ from those misclassified using BNP.

Bias or the influence of uncontrolled variables can present a major headache in many omics studies, including those seeking biomarkers (Ransohoff, 2005; Broadhurst and Kell, 2006; Kirschenlohr *et al.*, 2006;). In a case–control study such as the present one, it occurs
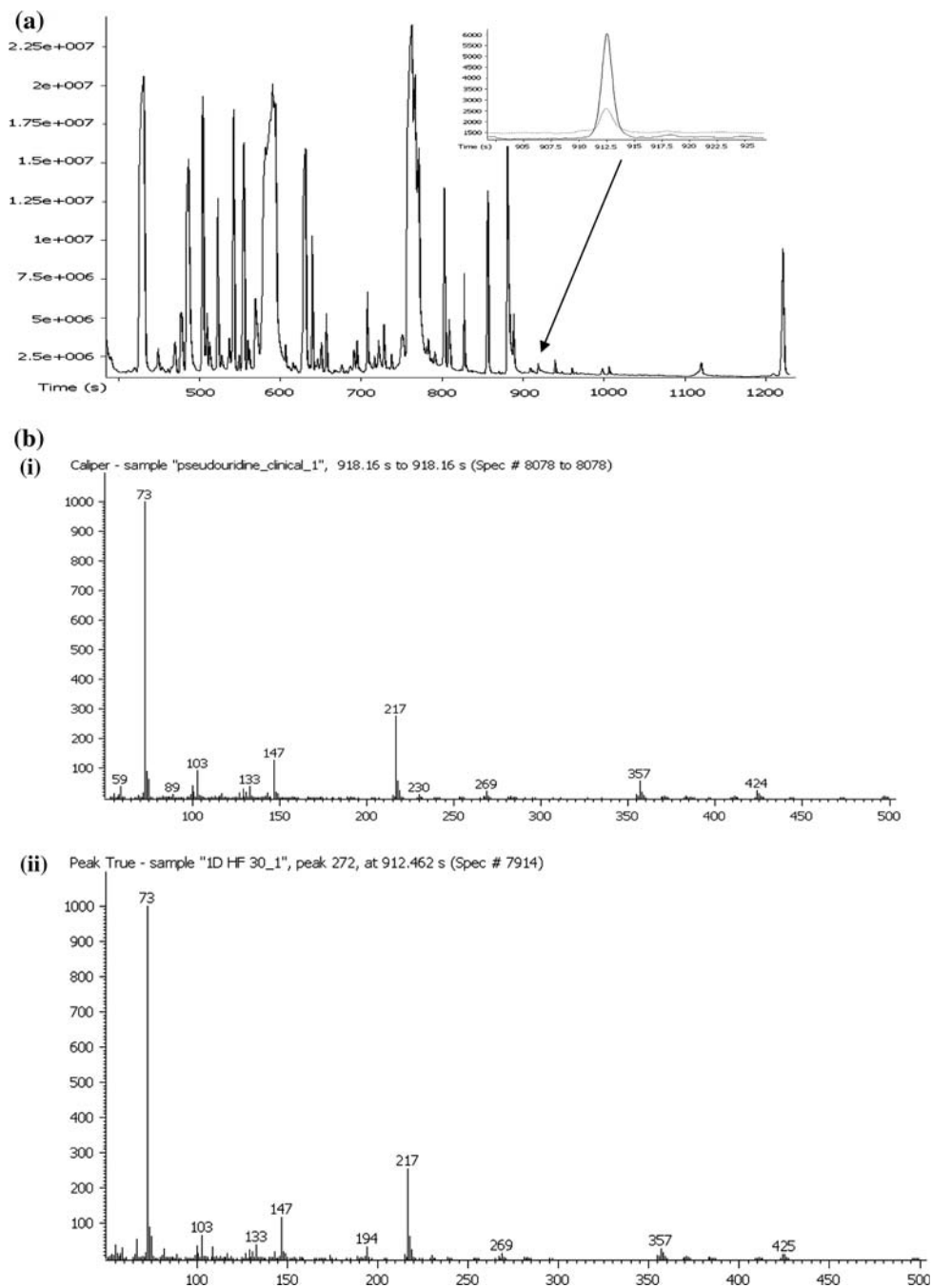
Figure 1. GC-tof-MS analysis of pseudouridine. (a) Total Ion Chromatogram as a function of time obtained by GC-tof-MS for a diseased subject. The inset shows an enlarged single ion chromatogram for pseudouridine (m/z 357) from a subject with heart failure (full line) and a control subject (dotted line) showing the difference in peak area and therefore concentration between the two classes. (b) Mass spectra of a peak identified as pseudouridine obtained from (i) clinical serum samples and (ii) an authentic standard. Note that several metabolites appear more than once because one metabolite can form multiple derivatives during sample preparation. For (b) the ordinate is in count normalised to the highest peak while the abscissa is the mass-to-charge ratio.

in particular when variables other than those of primary interest are unevenly distributed between the cases and the controls. It is then possible, and certainly possible to argue, that the changed distribution of any proposed biomarkers is not due to the presence of the disease but to the differential distribution of these 'confounding' variables. One immediate potential source of bias arises from the straightforward fact that 'cases' are (presumably considerably) more likely to be taking pharmaceutical medication than are controls. In the present work, however, it transpired that while the distributions of those taking particular classes of medication between cases and controls is not at all even, simple inspection of the data shows that quite a considerable fraction of the

Table 2
Discriminatory metabolite peaks

Metabolite	Median value C (Female, Male)	Median abs. dev. C (Female, Male)	Median value HF (Female, Male)	median abs. dev. HF (Female, Male)	p-value M–W test	ROC Convex hull area
Pseudouridine *	(0.048, 0.048)	(0.0077, 0.0047)	(0.094, 0.11)	(0.015, 0.037)	1.78E-15	0.96
N-BNP	(8.5, 7.5)	(2.5,4.5)	(291, 305)	(125,241)	9.99E-16	0.93
2-Oxoglutaric Acid *	(0.028, 0.03)	(0.0034, 0.0044)	(0.045, 0.054)	(0.0065, 0.0091)	1.61E-13	0.93
Unknown	(0.058, 0.059)	(0.02, 0.016)	(0.16, 0.12)	(0.11, 0.08)	4.59E-05	0.89
2-hydroxy,2-methylpropanoic acid *	(0.039, 0.036)	(0.0063,0.0059)	(0.19, 0.11)	(0.13, 0.042)	7.09E-10	0.88
Erythritol *	(0.0099, 0.011)	(0.0017, 0.0027)	(0.015, 0.022)	(0.0018, 0.0071)	1.37E-10	0.88
Uric acid*	(0.015, 0.014)	(0.0039, 0.0039)	(0.031, 0.026)	(0.0079, 0.01)	9.06E-11	0.87
Unknown	(0.016, 0.016)	(0.0047, 0.0034)	(0.036, 0.034)	(0.016, 0.013)	7.75E-10	0.87
Erythritol *	(0.074, 0.076)	(0.014, 0.018)	(0.11, 0.16)	(0.017, 0.048)	4.37E-10	0.87
Unknown	(0.0039, 0.0055)	(0.0014, 0.0017)	(0.0054, 0.0096)	(0.0023, 0.004)	1.16E-08	0.87
2,4,6-Trihydroxypyrimidine	(0.013, 0.015)	(0.0018, 0.0028)	(0.023, 0.026)	(0.0037, 0.0064)	2.13E-10	0.87
Threonic acid	(0.034, 0.033)	(0.0058, 0.0067)	(0.056, 0.092)	(0.0049, 0.031)	5.68E-10	0.85
Uric acid *	(0.41, 0.56)	(0.11, 0.15)	(0.83, 0.87)	(0.089, 0.17)	8.83E-10	0.85
Myo-inositol *	(0.55, 0.57)	(0.062, 0.097)	(0.7, 0.86)	(0.14, 0.25)	1.42E-08	0.84
Para-hydroxyphenylacetic acid	(0.0028, 0.0033)	(0.00079, 0.0011)	(0.0064, 0.0056)	(0.0022, 0.0023)	5.07E-08	0.83
Unknown	(0.0029, 0.0027)	(0.00072, 0.0012)	(0.0047, 0.0055)	(0.0015, 0.0023)	4.92E-07	0.83
Xylitol or ribitol	(0.024, 0.022)	(0.0054, 0.0074)	(0.038, 0.046)	(0.018, 0.016)	1.39E-08	0.83
Unknown	(0.021, 0.015)	(0.0065, 0.004)	(0.012, 0.009)	(0.0067, 0.0042)	2.41E-05	0.82
Uric acid*	(1.5,2)	(0.56, 0.66)	(3.2,3.4)	(0.43, 0.6)	1.82E-07	0.82
Xylose or isomer	(0.024, 0.03)	(0.0054, 0.011)	(0.038, 0.048)	(0.018, 0.017)	6.87E-08	0.81
Disaccharide	(0.0056, 0.0082)	(0.0021, 0.0025)	(0.0086, 0.013)	(0.0022, 0.005)	3.68E-09	0.80
Para-cresol	(0.088, 0.13)	(0.04, 0.041)	(0.15, 0.26)	(0.026, 0.12)	1.09E-06	0.80
Disaccharide	(0.0025, 0.0027)	(0.00041, 0.00029)	(0.0032, 0.0051)	(0.00065, 0.0015)	5.09E-06	0.80
Unknown	(0.0061, 0.0055)	(0.0013, 0.0024)	(0.012, 0.0092)	(0.0034, 0.0028)	6.69E-06	0.79
Unknown	(0.067, 0.072)	(0.017, 0.02)	(0.08, 0.13)	(0.022, 0.044)	1.60E-05	0.79
Indole-3-acetic acid *	(0.043, 0.054)	(0.0098, 0.022)	(0.08, 0.091)	(0.033, 0.026)	5.25E-06	0.79
Glyceric acid	(0.091, 0.094)	(0.012, 0.014)	(0.12, 0.14)	(0.02, 0.043)	1.81E-05	0.78
2,4-Dihydroxybutanoic Acid	(0.014, 0.018)	(0.003, 0.0031)	(0.029, 0.034)	(0.0035, 0.013)	3.00E-09	0.78
Unknown	(0.038, 0.064)	(0.011, 0.03)	(0.099, 0.1)	(0.022, 0.03)	2.37E-05	0.77
Sucrose *	(0.019, 0.019)	(0.011, 0.011)	(0.035, 0.062)	(0.012, 0.043)	1.78E-05	0.77
Unknown	(0.025, 0.029)	(0.004, 0.0037)	(0.041, 0.037)	(0.0037, 0.0047)	2.26E-06	0.77
Tetronic acid	(0.035, 0.027)	(0.0095, 0.0068)	(0.043, 0.048)	(0.011, 0.018)	4.59E-05	0.76
Dehydroascorbic acid	(0.22, 0.21)	(0.08, 0.07)	(0.15, 0.1)	(0.078, 0.061)	3.59E-05	0.76

Peaks are listed when there is significant discrimination between case (HF) and control, i.e. the area under the ROC (receiver operator characteristic) curve ≥ 0.75 and the Wilcoxon rank sum (Mann–Whitney = MW) test p-values $< 5\times10^{-5}$. Median absolute deviation (abs.dev.) is used in place of the usual standard deviation as it is less sensitive to outliers and non-normal distributions. Identification of the metabolites was performed initially by mass spectral library searches employing NIST/EPA/NIH (02) and an author's (WBD) prepared mass spectral libraries (requiring a similarity and reverse match score >700 for a peak to be identified). Further definitive identification (indicated by *) was performed by analysis of authentic standards with identical analytical conditions and identification was confirmed if the retention index ($\pm$ 10) and mass spectra (similarity and reverse matches > 750) of metabolite peak in sample and standard were equivalent (as illustrated in figure 1 for pseudouridine). C = Control; HF = Heart failure
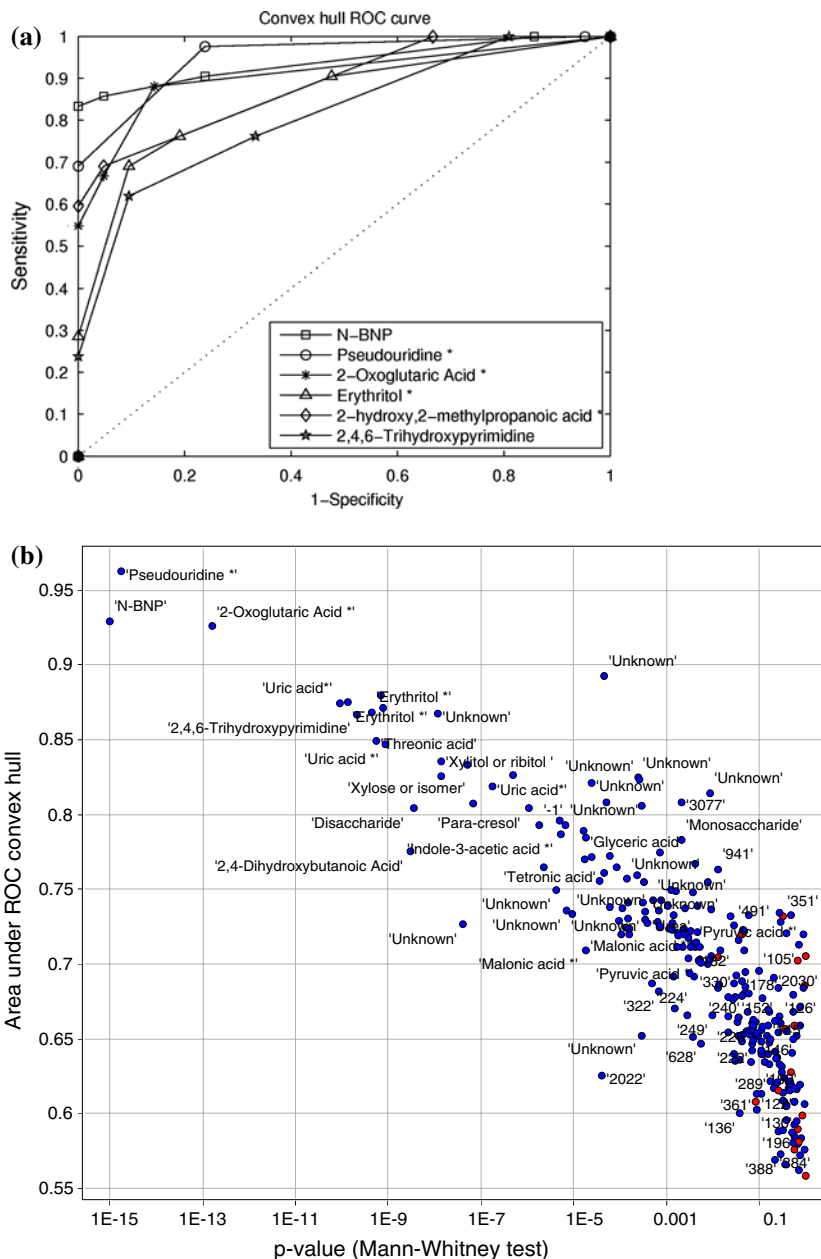
Figure 2. Receiver-operator characteristic (ROC) values for various metabolite peaks (a) ROC curves for BNP and five of the metabolites determined in the present study, (b) relationship between area under ROC curve and p-values for the various metabolites. * indicates that the metabolite has been chemically identified by analysing authentic chemical standards and determining that retention indices (± 10) and mass spectra (match > 750) of metabolites and authentic standards are similar. In (b), the colour of the symbol indicates whether the metabolite levels are greater (blue) or smaller (red) in the disease than the control.

controls were in fact also receiving the medications that one might expect in heart failure patients (Table 1), and we give the data for beta-blockers, ACE inhibitors, diuretics and statins in figure 4 (and for these and other metadata in tabulated form in the Supplementary information). It is equally clear from the data shown in figure 4 that the two main markers are still much larger in the disease than in the control patients, although for instance some of the patients taking ACE inhibitors do have values of Psi lower than those who are not (figure 4c), possibly providing evidence of the efficacy of

these drugs. By contrast, diuretics have no (or rather even a negative) effect in terms of lowering Psi (figure 4e) or 2OG (figure 4f) in patients, either indicating that excretion has less of a controlling influence on the steady-state values of these metabolites or (perhaps less plausibly) that the drugs happen to have been given only to patients with lower values of these metabolites in the first place.

Another kind of potential for bias or misinterpretation can come where A causes B and A causes C and it might then be interpreted that C causes B as they
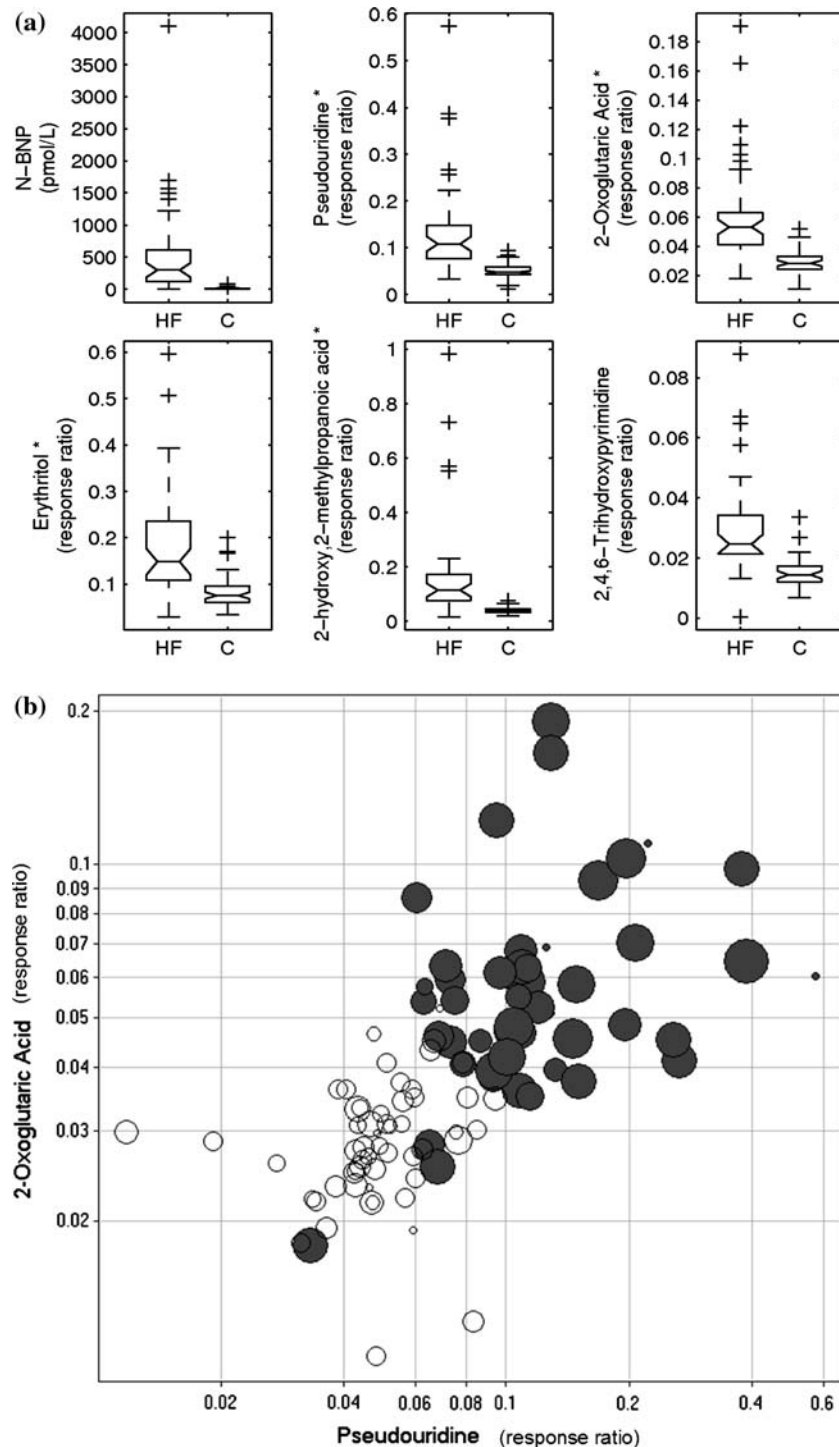
Figure 3. Metabolomics biomarkers for heart failure. (a) Box-whisker plots of the distribution of the most discriminating chemically identified markers in heart failure described in this study. HF: Heart failure; C: Controls. In the ordinates, N-BNP is in units of pmol/L while the other metabolites are unitless and described as a response ratio (peak area-metabolite/peak area-internal standard). The lower and upper lines of the "box" are the 25th and 75th percentiles of the sample. The distance between the top and bottom of the box is the interquartile range. The line in the middle of the box is the sample median. If the median is not centred in the box, that is an indication of skewness. The "whiskers" are lines extending above and below the box. They show the extent of the rest of the sample (unless there are outliers). Assuming no outliers, the maximum of the sample is the top of the upper whisker. The minimum of the sample is the bottom of the lower whisker. By default, an outlier is a value that is more than 1.5 times the interquartile range away from the top or bottom of the box. The notches in the box are a graphic confidence interval about the median of a sample. A side-by-side comparison of two notched box plots provides a graphical way to determine which groups have significantly different medians. (b) Relationship between 2-oxoglutarate and pseudouridine in controls (open circles) and heart failure patients (closed circles). The BNP concentration from the same individuals is encoded via the size of the symbols (small = lower, large = higher), and serves to illustrate that the three markers visually misclassify different samples and thus give different information.
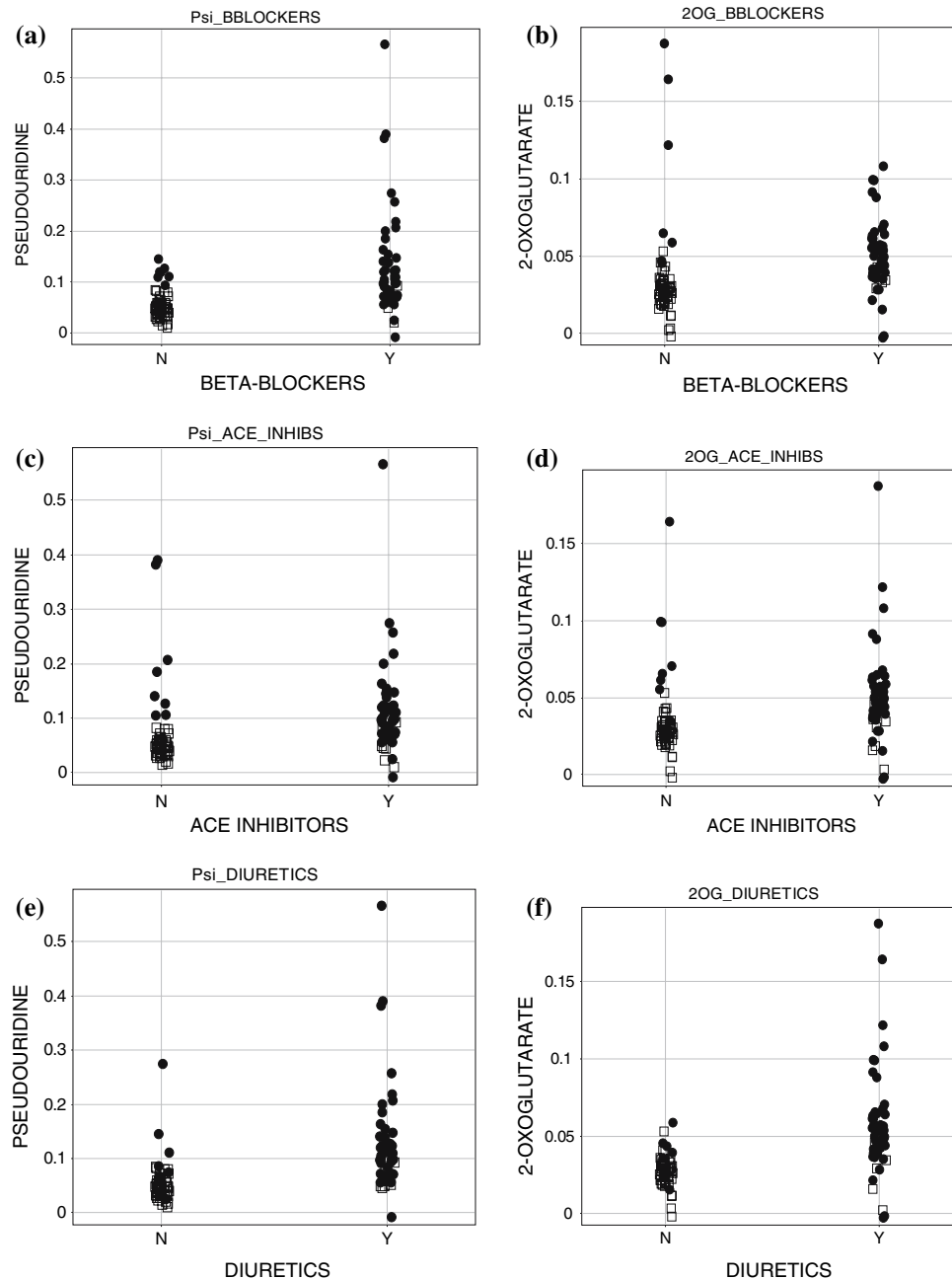
Figure 4. Lack of effect of pharmaceutical medications in increasing the values of pseudouridine (a, c, e, g) or 2-oxoglutarate (b, d, f, h) in patients relative to controls. The medications involved were beta-blockers (a, b), ACE inhibitors (c, d), Diuretics (e, f) and statins (g, h), although because of the numbers of samples involved these were not stratified further into individual drugs. Closed circles patients, open squares controls. Drug status indicated by N/Y. Data points have been 'jittered'.

will tend to be correlated (Pearl, 2000; Mackay, 2003). As mentioned above, it is, for instance, known that patients with heart failure may tend to have problems with renal function (Tsutamoto *et al.*, 2006), and that the impaired renal function might then be 'responsible' for an inference that biomarker X is a biomarker for heart disease when it is 'really' a biomarker for impaired renal function (and known serum biomarkers for renal dysfunction include creatinine and urea). The concentration of any metabolite is clearly a function of its synthesis, degradation, sequestering and excretion, such that changes in its excretion might automatically be expected to raise its concentration. Of course almost any small molecule might then be raised due to renal dysfunction, but the specificity that we see for our biomarkers relative to other molecules makes this kind of explanation *a priori* unlikely. There are several other reasons why we do not consider that our new heart failure biomarkers such as Psi and 2OG fall into this category of renal dysfunction co-variates. First, these
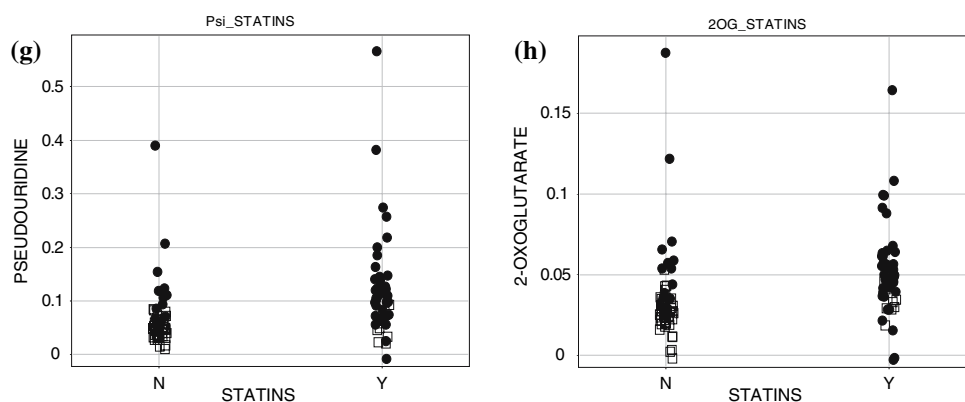
Figure 4. continued

patients do primarily have, and indeed presented with, heart failure. A combined aetiology that goes renal failure causes our biomarkers causes heart failure is not realistic. Secondly, as before (Kenny *et al.*, 2005), to distinguish this type of phenomenon it is appropriate to look within as well as between classes. Thus although they are reasonably well correlated (see also (Niwa *et al.*, 1998)), the relationship between Psi and urea and between Psi and creatinine (both measured enzymatically in the same serum samples) is little different between cases and controls (figure 5a, b), and it is not suggested that the controls are suffering from renal failure. Similarly, within a class, there is no relationship at all between 2OG and these same renal function markers (figure 5c, d). It is then even harder to argue somehow that *both* Psi and 2OG should therefore be renal dysfunction markers. This contrasts markedly with the close relationship between the two known renal dysfunction biomarkers urea and creatinine both within and between classes (figure 5e). Finally, we mention again the relative lack of impact of diuretics (figure 4e, f) on our biomarkers relative to their apparent effect on creatinine (figure 5f).

Uric acid has been suggested as a marker of cardiovascular mortality and its level is known to increase in patients with heart failure (Leyva *et al.*, 1998a, b; Anker *et al.*, 2003 Sakai *et al.*, 2006), albeit not, it is claimed, when adjusted for diuretic use (Culleton *et al.*, 1999b). It is probably a measure of impaired oxidative capacity (Leyva *et al.*, 1997, b) and it is of interest that it too was observed as being among the most significant biomarkers in our metabolomics data (figure 2, Table 2). In this case, the GC method showed three separate derivatives to be discriminatory (figure 2) and the data for the two more significant ones are given in figure 5g. Again, there is little relationship between uric acid and creatinine within a class (i.e. controls or cases, figure 5h) (and the uric acid levels in this case seem little influenced by diuretic usage; data not shown). The same is true for BNP against creatinine and urea (figure 5i, j).

4. Discussion

The principal findings of this study are represented by the identification of a fingerprint of serum metabolites that characterize heart failure, and stress the potential of pseudouridine and 2-oxoglutaric acid (α-ketoglutarate in the traditional nomenclature) as novel diagnostic markers of heart failure. Furthermore, this study highlights the potential of metabolomic analysis and, more generally, of the systems biology approach (Kell, 2004, 2006a), in the evaluation of a disease condition.

An advantage of the metabolomic approach in the identification of novel biomarkers is that it is not limited by our current knowledge, and instead seeks to measure the maximum number of metabolites in a given sample; the total number in the native human metabolic network is unknown (although leaving aside combinatorial lipids a number around 3000 is a reasonable starting estimate (Kell, 2006b; Duarte *et al.*, 2007)) and a more restricted number, a 'metabolic profile', is usually obtained. The most common analytical technologies employed in metabolic profiling applications (Dunn and Ellis, 2005) involve chromatographic separation (gas chromatography, liquid chromatography and capillary electrophoresis) followed by sensitive mass spectrometric detection (so-called hyphenated techniques). Gas chromatography – time of flight-mass spectrometry (GC-tof-MS), which was used in this study, probably provides the highest one-dimensional chromatographic resolution, with detection from nM to mM concentrations, and with metabolite identification being provided via electron-impact ionisation mass spectrometry. Two-dimensional GCxGC can reveal yet more metabolites (O'Hagan *et al.*, 2007).

Pseudouridine is a modified nucleoside that is found in ribosomal and transfer RNA and is produced post-transcriptionally (Charette and Gray, 2000; Ofengand, 2002) such that it is then 'fixed' inside these macromolecules. It is not further metabolised other than by RNA hydrolysis, and its appearance in blood and urine is therefore considered to be an excellent measure for
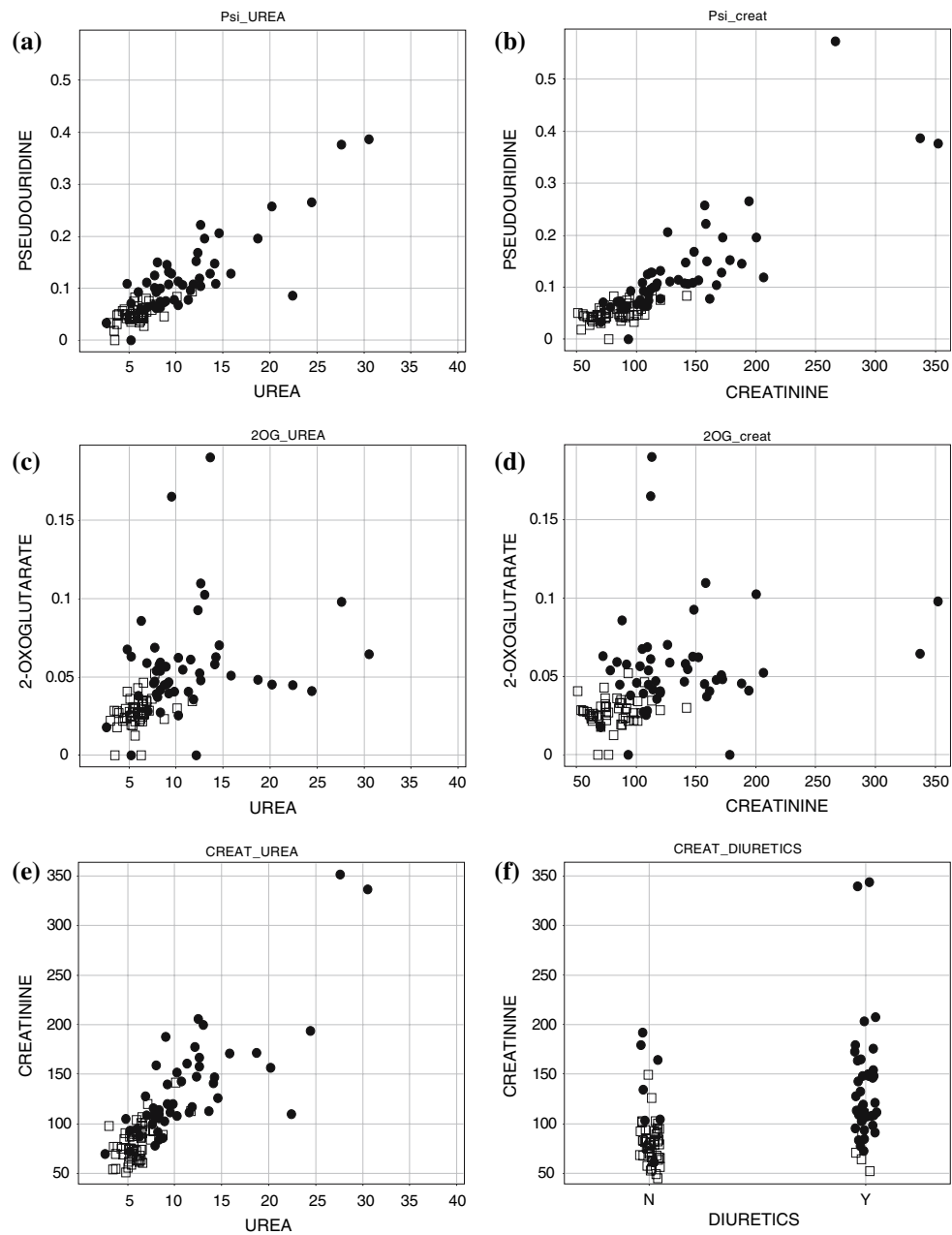
Figure 5. Imperfect relationships between novel and existing heart failure biomarkers and known renal dysfunction biomarkers. Pseudouridine against (a) urea and (b) creatinine. 2-oxoglutarate against (c) urea and (d) creatinine. (e) urea vs creatinine. (f) creatinine as a function of diuretic usage. (g) the top two peaks from uric acid are plotted against each other, also showing that occasionally individual peaks can 'disappear'. (h) uric acid vs creatinine. BNP against (i) creatinine and (j) urea. Note that when urea is shown the scale has been increased for visual clarity, leading to the disappearance of 3 samples (2 case, 1 control) whose urea levels were greater than 60. All data are in the supplementary information. Cases closed circles, controls open squares, as in Figure 4(f) data are jittered for clarity.

RNA degradation and thus of cell turnover. Tumour cells exhibit an unusually high turnover, and consequently it has also been proposed as a tumour marker (e.g. (Higley *et al.*, 1982; Salvatore *et al.*, 1983; Russo *et al.*, 1984; Tamura *et al.*, 1987; Amuro *et al.*, 1988; Xu *et al.*, 2000; Zheng *et al.*, 2005)) where it can have significant prognostic value (Pane *et al.*, 1993). In heart failure its raised level may in part reflect the remodelling process in the heart itself (Morgan *et al.*, 1987), the exact dynamics of which are unknown, but a substan-

tially raised turnover of peripheral cells including skeletal muscle seems a more likely explanation. Remodelling processes in peripheral muscle are well described in heart failure (Drexler *et al.*, 1992; De Sousa *et al.*, 2000) and are thought to be in part responsible for symptoms such as fatigue and shortness of breath. Conceptually, a biomarker for peripheral cell turnover would therefore be a valuable addition to a matrix of markers classifying subgroups of patients. It is of interest that the differences in pseudouridine between
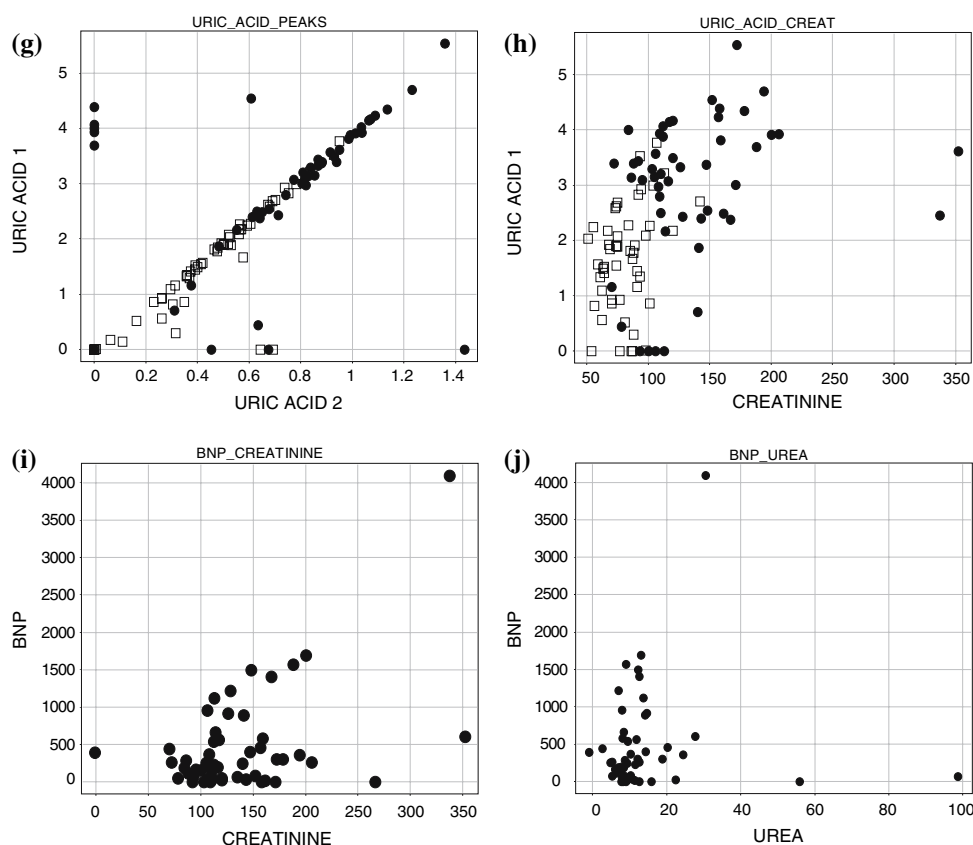
Figure 5. continued

cases and controls also increased somewhat with age, although the levels in the controls were essentially unchanged with age, as was also true for creatinine (data not shown, see also (Culleton *et al.*, 1999a)).

We note that 2,4,6-trihydroxypyrimidine is often displayed and described as its tautomer malonylurea or barbituric acid. Its origin in mammals has not been fully elucidated (http://www.genome.ad.jp/dbget-bin/ show_pathway?map00240 ± C02067), but the fact that we identify it as a marker in an important syndrome such as heart failure points to the value of further research into this issue.

We note, too, that it is also possible that pseudouridine and other metabolites whose concentrations are

Table 3
Bootstrap-calculated Pearson's correlation coefficients (*r*)

		2-Oxoglutaric Acid *	N-BNP	Uric acid*	Urea	Creatinine *
	Pseudouridine*	0.219 ± 0.110	0.561 ± 0.177	0.299 ± 0.139	-0.086 ± 0.218	0.489 ± 0.188
2-Oxoglutaric Acid *	0.005–0.409	2-Oxoglutaric Acid*	0.268 ± 0.109	0.247 ± 0.108	0.103 ± 0.104	0.041 ± 0.118
N-BNP	0.297–0.926	0.018–0.444	N-BNP	-0.122 ± 0.119	0.164 ± 0.263	0.207 ± 0.116
Uric acid*	−0.070–0.505	0.029–0.448	−0.344–0.125	Uric acid*	0.138 ± 0.159	0.291 ± 0.112
Urea	−0.454–0.399	−0.108–0.304	−0.245–0.674	−0.186–0.431	Urea	-0.103 ± 0.173
Creatinine *	0.007–0.751	−0.168–0.296	0.017–0.494	0.068–0.497	−0.430–0.248	Creatinine *

If *r* = 0 this indicates that there is no correlation; r = 1 indicates that the two compared measurements are perfectly positively correlated; r = −1 indicates perfect negative correlation. The bootstrap procedure (see (Efron and Gong, 1983; Efron and Tibshirani, 1993)) involved repeatedly (p times) choosing n random samples with replacement from the disease only data set (where n = the total number of available disease samples; thus a particular data point from this original data set could appear multiple times in a given bootstrap sample set) and analyzing each sample the same way – in this case using Pearson's correlation coefficient equation. For this table p was set to 5,000. The upper right quadrant of the table shows the mean of the 5,000 r values calculated for each pair-wise metabolite correlations, together with +/− the bootstrap standard error. The lower left quadrant describes the same correlation data but in terms the 95% confidence interval for r for each pair-wise metabolite correlation (calculated using the bias corrected and accelerated percentile method; see (DiCiccio and Efron, 1996)). The correlation data indicates that all the metabolites are positively correlated with each other (i.e. *r* > 0 for all comparisons). However, there are no strong correlations (*r* > 0.7). However, even the relatively high correlation between Pseudouridine and N-BNP (rest = 0.561 +/− 0.177) is questionable upon inspection of such a small data set

raised in heart failure are not merely innocent marker metabolites but participate in the pathophysiology of the syndrome, e.g. by inducing cellular alterations in the heart or in peripheral tissues. Such may be the case for uric acid (Sakai et al., 2006).

Pseudouridine is also related to creatinine in our overall dataset. This might be expected, given that a reduction in renal function is part of the pathophysiology of heart failure and indeed, reduced renal function has been considered to contribute to the increase in BNP in heart failure (Tsutamoto et al., 2006).

Conversely, reduced renal function contributes to the progression of heart failure. The mechanisms of the intimate link between renal and cardiac function, sometimes referred to as the 'cardiorenal syndrome' (Bongartz et al., 2005), are unclear, but our data raise the interesting hypothesis that pseudouridine or the other metabolites identified by us contribute to the deterioration in renal function in heart failure. More research is needed, but our present data clearly provide novel and testable hypotheses to address these issues.

Overall, however, when we look at the heart failure class alone, the correlation between BNP and creatinine was weak (Table 3), as it was between any proposed heart failure marker and any renal marker, and it appears unlikely that a decrease in renal function is the dominant explanation for the increase in pseudouridine, 2OG and the other heart markers.

As with other marker studies, it cannot be completely excluded a priori that drug therapy influenced the level of metabolites. Our analysis makes this highly unlikely, but the ultimate proof of measuring the markers in patients left untreated for several weeks is clearly not ethical and this question may therefore be addressed further by a combination of animal experiments and indirect clinical investigations in future, e.g. by measuring these parameters in patients with similar medication but other diseases, such as hypertension. By definition, however, it will be impossible to identify a non-heart failure group with identical combination of all drugs in a large patient cohort and without the use of inferential methods some questions may remain unresolved in the human situation, in close analogy to BNP in human heart failure.

2-oxoglutarate is a major intermediate of the tricarboxylic acid cycle (also known as citric acid or Krebs cycle) that occupies a central place in energy metabolism and is one of the 12 major precursors for the synthesis of most biochemical substances (Csete and Doyle, 2004)). It was also substantially raised in the heart failure patients. In recent years, it has become increasingly clear that heart failure is characterized by alterations in energy metabolism. In humans, the reduction of the flux rate from creatine phosphate to ATP is characterized best as it is amenable to in vivo NMR measurements (Neubauer et al., 1997). The most straightforward hypothesis therefore is that the raised levels of 2-oxo-glutarate reflect a decreased flux through the Krebs cycle and overflow of some metabolites into the circulation. (In bacteria, 2-oxoglutarate is a classical product of 'overflow metabolism' in microbes (Neijssel and Tempest, 1976) where as a partial oxidation product of carbohydrate metabolism it signifies an insufficient oxidative capacity.) Recent findings using metabolomics to investigate alterations during cardiac ischemia showed decreases in several constituents of the Krebs cycle (though 2-oxoglutarate was not specifically named) (Sabatine et al., 2005). This suggests that the metabolic state of the heart and/or peripheral tissues is at least in part reflected in serum metabolites, which can be harnessed as markers of disease. Further mechanistic studies regarding this issue are warranted.

The origin of the sugar alcohol erythritol in serum is unclear. Although there are a few reports of its measurement in serum and urine (e.g. (Bultitude and Newham, 1975; Pitkänen, 1972; Schoots et al., 1979; Roboz et al.,1984, 1990;Verhoeven et al., 2001; Wamelink et al., 2005)) it is not entirely certain whether its origin is exogenous/dietary (as well as being a component of various plant and animal tissues it is a permitted food additive and pharmaceutical congener), by biosynthesis within human tissues (it does not yet appear in the human metabolic network reconstruction (Duarte et al., 2007)), or from gut microflora (a significant contributor to the metabolome (Nicholson et al., 2005)). However, as a reducing compound (Jauniaux et al., 2005) it shares with 2-oxoglutarate the potential implication of an impaired oxidative capacity. Consistent with this, two other sugar alcohols (xylitol, myo-inositol) also appear in the list of most discriminatory metabolites (Table 2). The biological significance of 2-hydroxy, 2-methyl propanoic acid is unknown, although it too may be of microbial origin (see (Hierro et al., 2005) for some closely related metabolites).

In conclusion, an unbiased, hypothesis-generating (Kell and Oliver, 2004) metabolomics strategy has uncovered numerous novel candidate markers for heart failure. Targeted analyses of these (using a method with greater analytical precision) may be expected to prove even more discriminatory, and a mechanistic biochemical and longitudinal analysis of their role as prognostic indicators and in the aetiology of heart failure is clearly warranted.

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