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[1]H NMR-based metabonomic study on the metabolic changes in the plasma of patients with functional dyspepsia and the effect of acupuncture

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ABSTRACT

Functional dyspepsia (FD) is a common gastrointestinal disorder with multiple pathogenic mechanisms seen in clinical practice, and acupuncture may potentially be an alternative therapy for it. In order to investigate the biological effects of FD and the effect of acupuncture on metabolism, [1]H nuclear magnetic resonance (NMR)-based metabonomic techniques have been used to compare the plasma metabolic profiles of six female FD patients with those of six female healthy control subjects. Plasma metabolic profiles of FD patients treated by acupuncture at the Foot-Yangming Meridian were also collected and compared. Data obtained from NMR spectroscopy were subjected to principal components analysis (PCA). The results show that there are relatively higher levels of glucose, acetate, high-density lipoprotein (HDL), and phosphatidylcholine (PtdCho), and lower levels of lactate, leucine/isoleucine, N-acetyl glycoprotein (NAc), and low-density lipoprotein/very low-density lipoprotein (LDL/VLDL) in FD patients than in healthy controls. Acupuncture treatment of FD patients significantly changed the levels of leucine/isoleucine, lactate and glucose, and slightly changed lipids level towards those of the healthy controls, demonstrating its therapeutic effects on the relief of FD symptoms. Due to the limited number of subjects, the present work is just a proof-of-principle study and further researches with larger number of subjects are needed. Our work shows the potential of an NMR-based metabonomic approach in the study of biological effects of acupuncture.

1. Introduction

Functional dyspepsia (FD) is a common functional gastrointestinal disorder in clinical gastroenterology with multiple pathogenic mechanisms. Using Rome diagnostic criteria, it is estimated that the prevalence of this disorder is as high as 70% in the general population [1]. FD is characterized by epigastric pain, early satiety, nausea, vomiting, abdominal distension, and bloating and/or anorexia in the absence of organic diseases [2]. Many possible factors including abnormal neurohormonal function, autonomic dysfunction, visceral hypersensitivity or mechanical distention, *Helicobacter pylori* infection, acute gastrointestinal infection, and psychosocial comorbidity and stress [3–5] are to be considered in the pathogenesis of FD. The real causes are still unclear and the outcome of treatment is controversial [2–6]. Although the symptom-based diagnostic criteria for functional gastrointestinal disorders have been suggested [7,8], drug treatment of FD is often unsatisfactory [9], and alternative therapies are attractive to both patients and practitioners [10].

Acupuncture, an important therapeutic method based on meridian theory in traditional Chinese medicine (TCM), has been shown to be effective for the treatment of various functional diseases and for the manipulation of abnormal physiological conditions in the human body [10,11]. According to the TCM theory, in the human body, there are network systems called meridians, the paths through which the body energy flows, and many points (acupoints) on each meridian which can reflect the status of related part of the body or internal organs and also can be stimulated to regulate the status. The acupoints located on the Stomach Meridian of Foot-Yangming (ST) is the most commonly used meridian in the treatment of stomach and intestinal disorder [12–14]. The main roles of acupuncture in treating FD include reducing dyspeptic symptoms, gastric pain, improving gastric dysrhythm and accelerating solid gastric empting [15–17]. In a clinical trial on 103 cases, acupuncture was reported effective in reducing dyspeptic symptoms, mainly epigastric pain, in 95% of the patients [18]. Recent researches have shown that acupuncture can improve

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the impaired gastric motility, delayed gastric emptying through stimulating vagal efferent activity [19–21]. Although a lot of information and experience have been accumulated in clinical practice, acupuncture has been considered to be an empirical, holistic, comprehensive, dynamic, and personalized method of treatment. The lack of fundamental research on acupuncture has restricted its further development and acceptance internationally. New techniques and methods in the biomedical field should be applied to acupuncture to uncover its underlying mechanisms.

Recent developments in metabonomics have shown its great potential in high-throughput profiling of metabolites in biofluids and tissues [22,23]. As a combination of high-throughput analytical techniques such as NMR and gas chromatography/mass spectrometry (GC/MS), and multivariate statistical analysis, metabonomic approaches provide a global overview of the integrated response of an organism to a stimulus [24,25]. This feature is consistent with the principle of TCM, which is a kind of integrative medicine in which live organisms are seen as a whole. In this study, we used ^{1}H NMR-based metabonomics to characterize the changes in the metabolic profiles in the plasma of patients with FD before and after treatment with acupuncture.

The objectives of the present study were (i) to partially characterize the alteration of metabolites in patients with FD using an NMR-based metabonomic approach and (ii) to evaluate the role of metabonomics tools and multivariate statistical analysis in acupuncture research.

2. Materials and methods

2.1. Study design and sample collection

Six female patients with mean age 23.5 ± 1.8 (range 21–26) and body mass index 18–24 were diagnosed with FD on the basis of Rome III criteria [26] and were assigned to the acupuncture treatment group. Six healthy females matched for age and body mass index were recruited as a control group. Both patients and healthy subjects had been free of acute illness, and did not smoke or drink alcohol, coffee, or tea for 2 weeks before the study [27,28]. In addition, patients and controls were required to abstain from eating cheese and hot food, and performing heavy exercise from 2 weeks before the study through the end of research, as there is evidence that these factors can interfere with the metabolism of the human body [29–32]. All dietary intake and exercise were recorded.

Six acupoints, all belonging to the Stomach Meridian of Foot-Yangming, were selected for the treatment. Patients received acupuncture treatment for 6 days. Fasting venous blood samples was collected at 8:00 am on the day before acupuncture treatment began and on the second day after the final treatment.

The study was approved by the institutional ethics committee and each patient understood her involvement in a study protocol and signed a written informed consent agreement to use resulting information for medical publications. No patient refused the 6 days of acupuncture treatments.

2.2. NMR experiments

Venous blood (3 ml) was collected into a heparin sodium tube and the plasma was collected by centrifugation at 1000 × *g* at 4 °C for 10 min. An aliquot of 300 μl of plasma was mixed with 250 μl D_2O and 50 μl 3-trimethylsilyl-$^{2}H_4$-propionic acid sodium salt (TSP) in D_2O (1 mg/ml) in a 5 mm NMR tube. The D_2O provided a field-frequency lock solvent for the NMR spectrometer and the TSP served as an internal reference of chemical shift.

^{1}H NMR spectra of the plasma samples were acquired on a Varian INOVA 600 MHz NMR spectrometer at 27 °C by using Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence with a total spin–spin relaxation delay ($2n\tau$) of 320 ms. The free induction decays (FIDs) were collected into 32K data points with a spectral width of 8000 Hz and 64 scans. The FIDs were zero-filled to double size and multiplied by an exponential line-broadening factor of 0.5 Hz prior to Fourier transformation (FT). In addition, diffusion-edited experiments were also carried out with bipolar pulse pair-longitudinal eddy current delay (BPP-LED) pulse sequence [27,28]. The gradient amplitude was set at 35.0 G/cm, with a diffusion delay of 100 ms. A total of 128 transients and 16K data points were collected with a spectral width of 8000 Hz. A line-broadening factor of 1 Hz was applied to FIDs before Fourier transformation.

All plasma ^{1}H NMR spectra were manually phased and baseline-corrected using VNMR 6.1C software (Varian, Inc.). For CPMG spectra, each spectrum over the range of δ 0.4–4.4 was data-reduced into integrated regions of equal width (0.01 ppm). For BPP-LED data, each spectrum over the range of δ 0.1–6.0 was segmented into regions of equal width (0.01 ppm). The regions containing the resonance from residual water (δ 4.6–5.1) were excluded. The integral values of each spectrum were normalized to a constant sum of all integrals in a spectrum in order to reduce any significant concentration differences between samples [29,30]. Identification of metabolites in spectra was accomplished based on information in the literature and the Chenomx NMR Suite 5.0 (Chenomx, Calgary, Canada).

2.3. Multivariate analysis of NMR data

The resulting integral data were imported into SIMCA-P (version 10.04; Umetrics, Umeå, Sweden) for multivariate analysis. The CPMG data were mean-centered and Pareto-scaled while the BPP-LED data were only mean-centered prior to analysis. Partial least square discriminant analysis (PLS-DA) was used to find differential metabolites between groups. The results were visualized by two-dimensional scores plots representing the distribution of samples and the corresponding loadings plots providing information on the contribution of each variable to the pattern in the scores plots. When group separation was not satisfied based on PLS-DA, the data were further preprocessed using orthogonal signal correction (OSC) to remove the variations not correlated to the group membership [31,32], followed by PLS analysis, as indicated in the text and figure legends.

2.4. Statistical analysis

The normalized integrals of selected regions representing specific metabolites were subjected to two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. ^{1}H NMR spectra of plasma

Plasma comprises both low molecular weight metabolites and high molecular weight protein and lipoproteins. Therefore, standard 1D ^{1}H NMR spectrum of plasma is dominated by the broad resonance peaks from these high molecular weight components. In order to emphasize the small metabolites in plasma by attenuating the resonances from macromolecules, a CPMG pulse sequence was used to acquire the spectra. Fig. 1 shows low-frequency regions of typical CPMG spectra of plasma samples from patient and control groups. Most parts of the signals from macromolecules were eliminated, leaving only some residual signals from the methyl and methylene groups from lipids of lipoproteins. Based on literature reports [33,34] and by using Chenomx NMR Suite software,

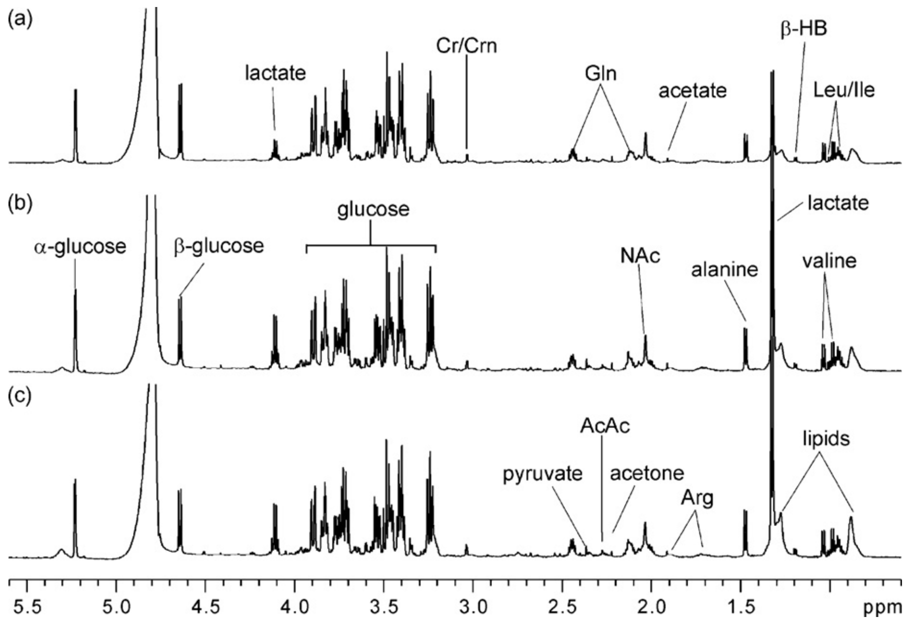

Fig. 1. Aliphatic regions of typical ^{1}H NMR CPMG spectra of plasma samples from FD patients (a) before and (b) after acupuncture treatment and from (c) controls. NAc, N-acetyl methyl groups of glycoproteins; AcAc, acetoacetate; Gln, glutamine; Cr/Crn, creatine/creatinine; β-HB, β-hydroxybutyrate; Leu/Ile, leucine/isoleucine.

major metabolites in plasma were identified, including amino acids (leucine/isoleucine, valine, alanine, arginine, and glutamine), organic acids (β-hydroxybutyrate, lactate, acetate, acetoacetate, citrate, pyruvate, creatine, and creatinine), and glucose (Fig. 1). The diffusion-edited NMR spectra of plasma samples, presenting only broad peaks from the lipids of lipoproteins and N-acetyl (NAc) groups of glycoproteins, are shown in Fig. 2. Visual inspection of these spectra shows subtle differences between groups. Further analysis should be carried out using multivariate statistical analysis to reveal the metabolic changes between FD patients and healthy controls and the changes caused by acupuncture treatments.

3.2. Metabolic profiles of plasma samples from FD patients and the effects of acupuncture

The results of pair-wise PLS-DA of the ^{1}H CPMG NMR data of plasma samples from all groups are shown in Fig. 3. The FD patients

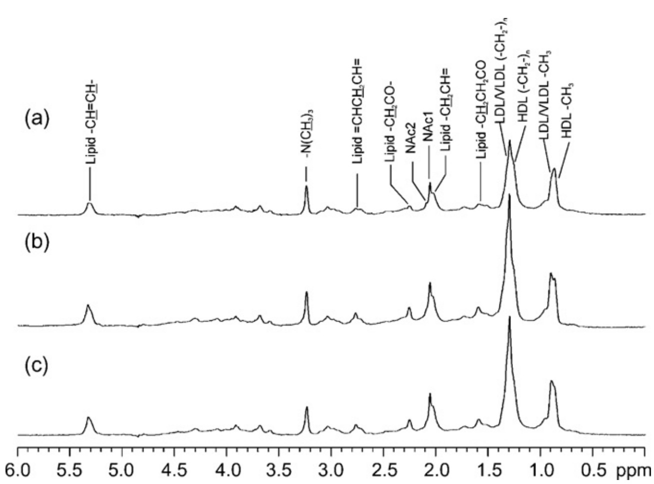

Fig. 2. Typical ^{1}H NMR BPP-LED spectra of plasma samples from FD patients (a) before and (b) after acupuncture treatment and from (c) controls. NAc, N-acetyl groups of glycoproteins; $-N(CH_3)_3$, N-methyl groups of phosphotidylcholine; HDL, high-density lipoprotein; LDL/VLDL, low-density lipoprotein/very low-density lipoprotein.

were clearly separated from controls along PC1 with $R^2X = 61\%$, $R^2Y = 78.7\%$ and $Q^2 = 72.1\%$ (Fig. 3A), and the corresponding loadings plot (Fig. 3B) showed an apparent higher level of glucose and lower level of lipids and lactate in the plasma samples of FD patients than in controls. Moreover, slightly higher levels of acetate (δ 1.91), and slightly lower levels of valine and leucine/isoleucine were observed in FD patients than in controls.

The effects of acupuncture on FD patients are shown in Fig. 3C ($R^2X = 55.2\%$, $R^2Y = 82\%$ and $Q^2 = 42.5\%$). After 6-day acupuncture treatments, all patients exhibited different plasma metabolic profiles. The loadings plot (Fig. 3D) was very similar to that between FD patients and controls except for the different changes in alanine, arginine and lipids, suggesting that acupuncture reversed the changes in plasma metabolites caused by the disease. However, further comparison between the treated and the control groups revealed that there were still some differences in metabolic profiles between them (Fig. 3E, $R^2X = 58.3\%$, $R^2Y = 92.92\%$ and $Q^2 = 43.2\%$), as demonstrated by the higher levels of glucose, glutamate/glutamine, acetate, arginine and alanine, and lower levels of lipids and N-acetyl glycoproteins in the plasma of acupuncture-treated FD patients than in those of the control group (Fig. 3F). The levels of lactate, valine and leucine/isoleucine were almost the same in these two groups.

Simple PCA or PLS-DA of diffusion-edited NMR spectra of plasma samples resulted in marginal separation of FD or the treated group from controls, but could not result in useful model for FD vs the treated groups (negative Q^2 value for the first PC). Therefore, OSC-pretreatment was performed on all LED data to maximize the possible differences between groups. The results (Fig. 4A) present clear differences between the lipid profiles of plasma from FD patients and controls along PC1 ($R^2X = 68\%$, $R^2Y = 98.4\%$, $Q^2 = 97.9\%$). Changes in lipid composition could be roughly revealed in the loadings plot (Fig. 4B). Lipoproteins can be classified according to their particle sizes into HDL, LDL and VLDL. In spite of the severe overlap between signals from these lipoproteins, a slight difference in chemical shifts of the methyl and methylene groups between HDL, LDL and VLDL could be observed, with size dependently increased chemical shifts [35,36]. This difference in chemical shift was caused by the magnetic susceptibility anisotropy of the surface shell of the lipoprotein particles [33,36]. Splittings in the loadings plot at the

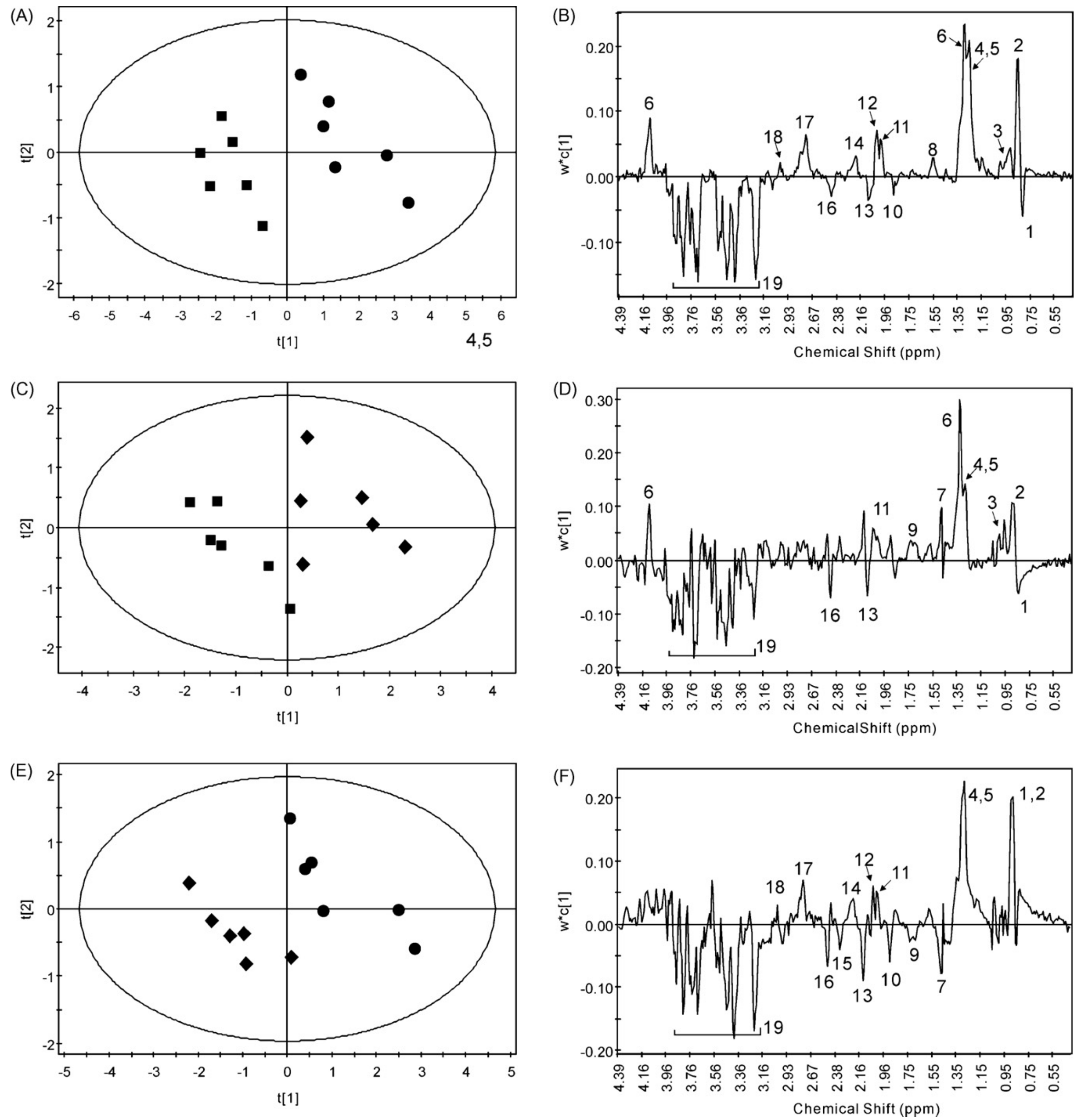

Fig. 3. Pair-wise PLS-DA of ^{1}H CPMG NMR data of plasma samples of (A and B) FD patients and controls, (C and D) FD patients before and after acupuncture treatment and (E and F) FD patients after acupuncture treatment and controls. 1, methyl protons of HDL; 2, methyl protons of LDL/VLDL; 3, valine and leucine/isoleucine; 4, methylene protons of HDL; 5, methylene protons of LDL/VLDL; 6, lactate; 7, alanine; 8, lipid $-CH_2CH_2CO$; 9, arginine; 10, acetate; 11, lipid $-CH_2CH{=}CH-$; 12, N-acetyl glycoproteins; 13, glutamine/glutamate; 14, lipid $-CH_2CO$; 15, glutamate; 16, glutamine; 17, lipid$=CH-CH_2-CH=$; 18, creatine; 19, glucose.

positions of methyl (∼δ 0.85) and methylene (∼δ 1.30) therefore indicate the different compositions of lipoproteins in the plasma samples of FD patients and controls. The plasma of FD patients has higher levels of HDL and phospholipids, and lower levels of VLDL/LDL (Fig. 4B). The total lipid concentration in the plasma of FD patients, however, is lower than that in the plasma of controls, as indicated by the positive loadings of resonances at δ 2.26 and δ 1.60, contributed from the C2 and C3 protons of fatty acids. In addition, negative loadings at δ 3.22 suggest a higher level of phosphatidylcholine (PtdCho) in FD patients than in controls. This observation is consistent with the higher level of HDL in FD patients since PtdCho is the most predominant lipid in the HDL fraction [37–39]. Furthermore, the signal at δ 2.05 arising from the N-acetyl methyl groups of glycoproteins shows a slightly negative loading, indicating a higher level of glycoproteins in the plasma of FD patients.

Acupuncture treatment caused small changes in the plasma lipid profile of FD patients, as demonstrated by the separation between them along PC2 (Fig. 4C). The loadings plot (Fig. 4D) showed increased level of total lipid content and decreased level of phospholipid in FD patients after acupuncture treatment. Fig. 4E and F demonstrates the differences existing between the treated group and the control group. After treatment, the FD patients still

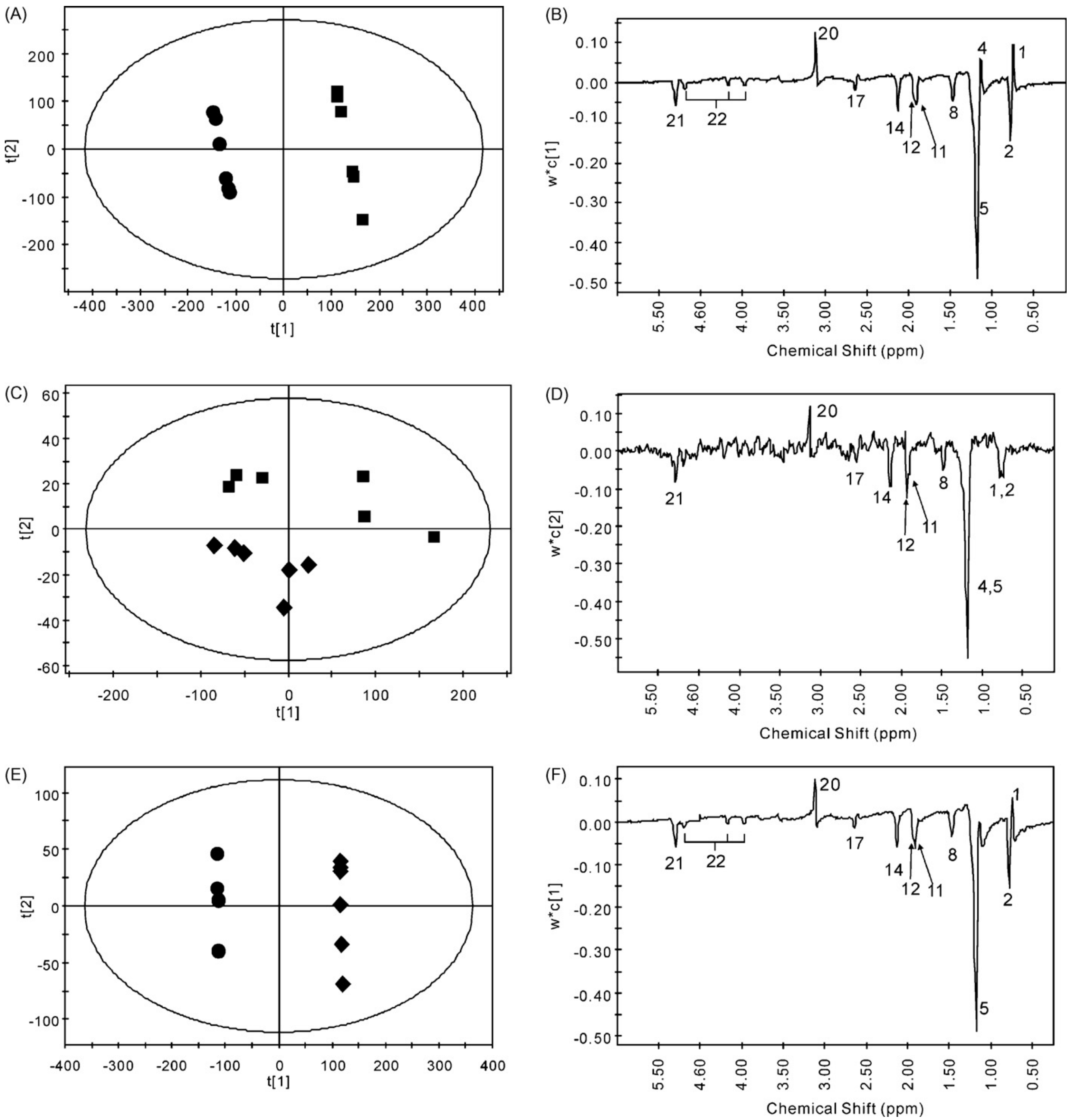

Fig. 4. Pair-wise OSC-PLS analysis of ^{1}H BPP-LED NMR data of plasma samples of (A and B) FD patients and controls, (C and D) FD patients before and after acupuncture treatment and (E and F) FD patients after acupuncture treatment and controls. The identifications of peaks 1–19 are the same as labeled in Fig. 3; 20, phosphatidylcholine; 21, lipid –CH=CH–; 22, glycerol of triglyceride.

had higher levels of HDL and phospholipids, and lower levels of LDL/VLDL, total lipids and N-acetyl glycoproteins than the control group (Fig. 4F).

The changes revealed by PLS-DA and OSC-PLS in plasma metabolites of FD patients before and after acupuncture treatment were summarized in Table 1. A two-tailed *t*-test was conducted on the normalized integral data of selected regions representing these metabolites to detect the significance of these changes. The results of *t*-test showed that the *P* values for some cases are very large, which seemed to be inconsistent with the results of multivariate analysis. These discrepancies were probably caused by the inaccurate peak integrals due to signal overlap and the small changes in chemical shifts of peaks from spectrum to spectrum. Careful inspections, however, on the loadings plot indicated that some large *P* values do correspond to very small loading values. For example, there was almost no loading peak for alanine in Fig. 3B, which was consistent with a large *P* value of 0.79. The integral data of BPP-LED spectra representing the $-(CH_2)_n-$ and $-CH_3$ regions of HDL and LDL/VLDL were not subjected to *t*-test due to the severe overlap of these signals. The results showed that the concentrations of leucine/isoleucine, lactate, acetate, N-acetyl glycoproteins and glucose were significantly different between FD patients and

Table 1
Changes of metabolites in FD patients relative to healthy controls.

Metabolites	Peak regions (δ)	FD vs control		Treated vs FD		Treated vs control	
		Changes	*P*	Changes	*P*	Changes	*P*
Valine	0.98–0.99, 1.03–1.04	↓	0.26	–	0.86	↓	0.37
Leucine/isoleucine	0.92–0.96, 1.00–1.01	↓	0.043	↑	0.043	–	0.69
Lactate	1.32, 1.33	↓	4.6E−4	↑	0.011	↓	0.47
Alanine	1.47–1.48	–	0.79	↑	0.13	↑	0.16
Acetate	1.91	↑	0.040	↑	0.26	↑	0.10
NAc	2.03–2.04	↓	0.08	↑	0.16	↓	0.57
Glutamine	2.09–2.14, 2.42–2.46	↑	0.14	↑	0.81	↑	0.17
Glucose	3.2–3.9	↑	7.9E−05	↓	0.0063	↑	0.0072
PtdCho	3.18–3.24	↑	0.0037	↓	0.29	↑	0.037
HDL	0.81–0.86, 1.20–1.26	↑		↓		↑	
LDL/VLDL	0.87–0.91, 1.27–1.36	↓		↑		↓	

Note: FD and treated represent groups of FD patients before and after acupuncture treatment, respectively. NAc, N-acetyl glycoproteins; PtdCho, phosphatidylcholine.

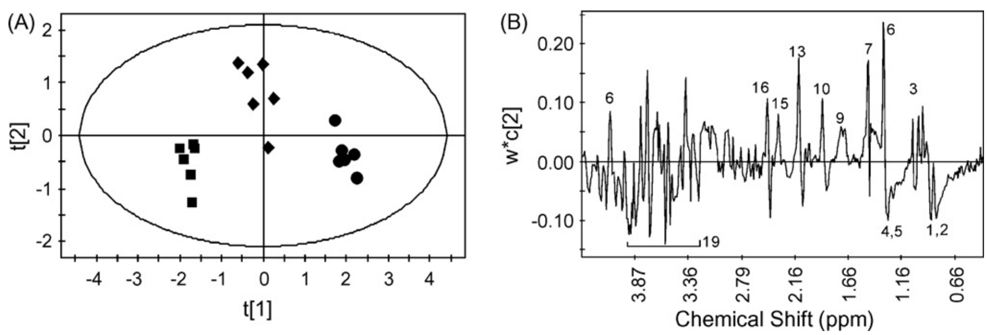

Fig. 5. OSC-PLS results of [1]H NMR CPMG spectra of plasma from FD patients before (■) and after (♦) acupuncture treatments and healthy controls (●). (A) Scores plots; (B) loadings plots corresponding to the second component. The identifications of peaks are the same as labeled in Fig. 3.

the controls. Acupuncture treatment caused significant changes in reverse direction in these metabolites except acetate, but the glucose level was still significantly different from those in the control group. The lipid contents were also very different between FD patients and the controls. Acupuncture treatment slightly increased the total lipid level in the plasma of FD patients.

Higher glucose and lower lactate and total lipid levels in FD patients suggest that the rate of glycolysis is reduced in patients and the energy consumption is switched to lipid oxidation. Acupuncture treatment can reduce these changes to some extent.

In order to further compare the effects of acupuncture, the plasma samples of three groups were also compared together. In the scores plots of OSC-PLS models (Fig. 5) for CPMG data ($R^2X = 59.4\%$, $R^2Y = 81.6\%$, $Q^2 = 64.9\%$), the acupuncture-treated group is located between the control and the untreated FD groups along the PC1 axis, a direct evidence reflecting the effect of acupuncture on FD patients to relieve the metabolic changes.

On the other hand, there were differences between the acupuncture-treated group and both untreated FD patients and controls along PC2 in scores plots (Fig. 5A), which can be temporarily attributed to the needling effects of acupuncture treatment on the physiological condition of all patients. The acupuncture-treated group had higher plasma levels of lactate, alanine, acetate, arginine, glutamine and glucose (Fig. 5B), but all these differences are not statistically significant by *t*-test. This kind of effects was not observed in BPP-LED data (data not shown).

4. Conclusion

In this study, [1]H NMR has been used to examine the metabolic profiles of plasma from FD patients before and after treatment by acupuncture. Despite the small data set, this study does detect profound changes between FD patients and healthy subjects in both small metabolites and lipoproteins. The results show that there are relatively higher levels of glucose, acetate, HDL and PtdCho, and lower levels of lactate, leucine/isoleucine, N-acetyl glycoproteins and LDL/VLDL in FD patients than in healthy controls. Acupuncture treatment appears to reverse the levels of most metabolites affected in FD patients, with significant changes in the levels of leucine/isoleucine, lactate and glucose towards those of the healthy controls. Despite of the limited number of subjects, the observed changes in FD patients after acupuncture treatment are more likely to be due to the therapeutic effect of acupuncture than its generic needling effect. This generic effect was temporarily assigned to the changes of some metabolites, though without statistical significance. Since the number of subjects in this study is very limited (6 patients and 6 controls), it is difficult to draw conclusions about what metabolites (or biomarkers) are closely related to FD. A trial with more subjects and more detailed investigation is being planned. These metabolic changes provide new clues in understanding the metabolic effects of acupuncture on diseases, and may provide a powerful tool for future research in this field.

Acknowledgments

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