ORIGINAL ARTICLE

Metabolic profiling in human lung injuries by high-resolution nuclear magnetic resonance spectroscopy of bronchoalveolar lavage fluid (BALF)

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Abstract We present a method for identifying biomarkers in human lung injury. The method is based on high-resolution nuclear magnetic resonance (NMR) spectroscopy applied to bronchoalveolar lavage fluid (BALF) collected from lungs of critically ill patients. This biological fluid can be obtained by bronchoscopic and non-bronchoscopic methods. The type of lung injury in acute respiratory failure presenting as acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), continues to challenge critical care physicians. We characterize different metabolites in BAL fluid by non-bronchoscopic method (mBALF) for better diagnosis and understanding of ALI/ARDS by NMR spectroscopy. NMR spectra of mBALF collected from 30 patients (9 controls, 10 ARDS and 11 ALI) were analyzed for the identification of biomarkers. Statistical methods such as principal components analysis and partial least square discriminant analysis were carried out on ^{1}H NMR spectrum of mBALF to identify biomarker responsible for separation among different lung injuries classes (ALI and ARDS) and normal lungs. The corresponding correlation of biomarkers with metabolic cycle has given insight into metabolism of lung injuries in critically ill patients. Our study shows statistically significant differentiation of various metabolites concentration in mBALF collected from lungs of ALI, ARDS and healthy control patients, making NMR spectroscopy as a possible new method of characterizing human lung injury.

Keywords Metabonomics · PCA · PLS-DA · Bronchoalveolar lavage fluid (BALF) · NMR spectroscopy · Metabolic profiling

Abbreviations
BALF   Bronchoalveolar lavage fluid
mBALF   Mini bronchoalveolar lavage fluid
PCA   Principal component analysis
PLS-DA Partial least square discriminant analysis

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1 Introduction

Acute respiratory failure (ARF) leading to intensive care unit (ICU) admission can develop in patients due to many clinical disorders. These disorders may be pulmonary and extra pulmonary in origin (Ashbaugh et al. 1967; Cribbs and Martin 2008; Kollef and Schuster 1995; Murray et al. 1988). According to National Heart and Lung Institute (NHLI), approximately 150,000 people are diagnosed with acute respiratory syndrome every year in U.S.A. The survival rate of these patients can be low as 34 %. A simple way of classifying severity of lung injury in these patients is based on P/F ratio (P—partial pressure of oxygen/F—fraction of inspired oxygen). Patients with P/F ratio

between 200 and 300 are classified as acute lung injuries (ALI) patients and patients with P/F ratio less than 200 are classified as acute respiratory distress syndrome (ARDS) patients. ARDS is more severe form of lung injury and carries a high ICU mortality and long term morbidity amongst survivors (Bernard et al. 1994b). More than 60 different causes of ALI/ARDS were reported in literature, and research is still continuing to find more causes of ALI/ARDS (Petty 1982). The most common causes of ALI/ARDS are severe traumatic injury (especially multiple fractures), severe head injury, and injury to the chest pneumonia, sepsis (presence of various pathogenic micro-organisms, or their toxins, in the blood or tissues), aspiration of fumes, food or stomach contents into the lung, and trauma. These conditions cause human body to produce substances that may cause inflammation in lungs. Once lungs get inflamed, the alveoli of lungs cannot perform normal oxygenation of the blood. Different diagnostic methods of lung injuries in common practice are arterial blood gas analysis, bronchoscopy, complete blood count (CBC, number of white blood cells are increased in sepsis.), chest X-ray, sputum cultures and analysis. These diagnostic methods do not suffice in prognostication, progression of disease and management of ALI/ARDS patients. For past few decades, research for the identification of biomarkers for early diagnosis, management and prognostication in patients with ALI/ARDS (Cribbs and Martin 2008; Donnelly et al. 1996; Geiser et al. 2001; Matthay and Zimmerman 2005; Matthay et al. 2003) has continued without much success. In spite of best efforts there are no specific biomarkers present to classify ALI/ARDS unless it gets in severe form. Therefore, there is a need for complementary method that may enable earlier detection of ALI/ARDS, in order to improve patient treatment and prognosis. Since lung injury results in the altered metabolism of lungs, biological fluid collected closer to the lung will be ideal candidate for the identification of biomarker. In clinical practice, broncholoalvolar lavage fluid (BALF) is collected for monitoring lung related injuries. There are two different methods for collecting BALF. One of the techniques is optical guided bronchoscopy technique in which small amount of saline water is inserted in human lung and extracted later. This technique is considered as gold standard. The other alternative technique known as non-bronchoscopic based method and fluid collected is known as mini BALF (mBALF). The method of collecting mBALF is less intensive. For the diagnosis of ventilator-associated pneumonia, it has been shown in many earlier studies that mBALF can be as effective compared to BALF (Heyland et al. 2006; Ruiz et al. 2000; Sanchez-Nieto et al. 1998; Kapil et al. 2011; Fartoukh et al. 2003; Papazian et al. 1995; Fagon et al. 2000; Kollef et al. 1995; Perkins et al.

2005). For the diagnosis of lung inflammation, mBALF does not show clear separation between ALI and ARDS if the criterion of inflammation is based on different cell counts such as bronchial epithelial cell (BEC), neutrophils, macrophages and BEC to macrophage ratio(Perkins et al. 2006). However, lung inflammation is known to produce small molecular weight metabolites. These metabolites can get dissolved in BALF and mBALF, which can be detected by spectroscopic techniques. We present here the identification of different lung metabolites in mBALF by nuclear magnetic resonance spectroscopy.

Recent developments in spectroscopic techniques have made possible for researchers to study human diseases at an unprecedented scale. 'Metabolomics' is one such recently developed field of study of different metabolites in a given biological fluid (Nicholson et al. 1999) and its correlation to various disease conditions (Kaddurah-Daouk and Krishnan 2009; Kaddurah-Daouk et al. 2008; Nicholson and Wilson 2003; Beckonert et al. 2007; Gamache et al. 2004; Lindon et al. 2004; Nicholson et al. 1999; Gehlenborg et al. 2010). Metabolomics studies typically employ either mass spectrometry or nuclear magnetic resonance (NMR) to analyze metabolic components of a tissue or biological fluids (Bollard et al. 2005; Engan et al. 1990; Gupta et al. 2009; McClay et al. 2010; Nicholson et al. 1999; Rocha et al. 2010). Now high-resolution ^{1}H NMR based metabolomics has been established as useful tool to study biological fluid. High-resolution NMR spectroscopy appears to be appropriate tool for investigating abnormal body fluid compositions too, as wide range of metabolites can be detected simultaneously with no sample preparation and many cellular biochemical processes can be probed. NMR spectroscopy of human urine (Gartland et al. 1990), serum (Tzouvelekis et al. 2005a, b; Beckonert et al. 2007), plasma (Engan et al. 1990; Stringer et al. 2011), tissue (Jayalakshmi et al. 2011; Rocha et al. 2010), follicular fluid (Pinero-Sagredo et al. 2010), and bile (Ijare et al. 2005a, b) offers a rapid and unambiguous analysis of lipids, amino acids, bile acids, phospholipids and cholesterol under normal and various pathological conditions. Here we present application of high-resolution NMR spectroscopy to achieve metabolic insight into various lung injuries in human. The application is demonstrated by NMR spectroscopy of minibronchoalveolar lavage fluid (mBALF), which is taken after saline or distilled water wash of the airways (broncho) and air sacs (alveolar) for recovery of inflammatory cells in human lungs. Till now, to the best of our knowledge, literature suggests that NMR based metabolomics on BALF has been applied in a rat model for 1-nitroonapthaline toxicity (Azmi et al. 2005) and another study on cystic fibrosis patients in which inflammatory changes have been reported (Wolak et al. 2009). In this article, we present application of high-resolution NMR metabolic profiling of

human mBALF with different lung injuries. In the present study, for the first time we are reporting the high resolution NMR metabolic profiling of mBALF to identify and characterize ALI/ARDS related metabolic changes that has provided valuable insight about biochemical and physiological condition of *human lungs*. More specifically, the aim of this study is to investigate the potential of NMR based metabolic profiling of mBALF to characterize different lung injury (ALI and ARDS). This study can be of further use for diagnosis as well as in providing novel insights about ALI/ARDS metabolism.

2 Materials and methods

2.1 Subjects

This study was conducted in ICU of a tertiary care medical centre in northern India Sanjay Gandhi Post Graduate Institute of Medical Science (SGPGIMS), Lucknow, INDIA and Centre of Biomedical Magnetic Resonance, Lucknow (CBMR), INDIA. Approval from institutes ethical committee and informed patient consent were taken before sample collection. All subjects diagnosed with ALI/ARDS at ICU admission of SGPGIMS were enrolled in the study group. Data collection includes demographic profile, clinical characteristics and illness severity scores like APACHE II and SOFA score at admission (SI Table 1).

ALI/ARDS diagnostic criteria was based on American–European Consensus conference definition published in year 1994 (Bernard et al. 1994a). The diagnostic criteria for ARDS proposed by this committee were Pao2/Fio2 $\leq$200, bilateral infiltrates on chest radiograph that need not be diffuse, and pulmonary artery occlusion pressure $\leq$18 mmHg or no clinical evidence of left arterial hypertension when a pulmonary artery catheter was not used. For the ALI, diagnostic criteria is similar expect Pao2/Fio2 $\leq$300. The control group in the study comprises of patients who underwent mechanical lung ventilation for routine elective surgeries. Exclusion criteria in present study include patients with age less than 18 years, pregnancy, chronic obstructive pulmonary diseases (COPD) (McClay et al. 2010) patients, bronchial asthma, interstitial lung disease, and other chronic respiratory ailments.

3 Experimental section

3.1 Sample collection

The technique used for sample collection is one of the non-bronchoscopic standardized techniques in ICU to collect mBALF in mechanically ventilated patients. Patient's

mBALF was collected using non-bronchoscopic technique "catheter in catheter" technique (Kapil et al. 2011) within 48 h of diagnosis. In the present study 10 ml sterile distilled water was used for sample collection for all patients. mBALF sample was transferred from mucus trap to a collection vial and immediately placed in liquid nitrogen for storage. The samples were preserved after centrifuging at 16,000 rpm for 10 min at 4 °C to remove cellular debris and bacteria and then supernatant stored at −80 °C till NMR experiments were performed. For NMR spectroscopy 350 μL sample was taken for each experiment. Thirty samples were included in the present study. Nine samples belonged to non-ALI/ARDS and served as control group for the study. Eleven samples from ALI and ten samples from the ARDS were included in the study.

3.2 NMR spectroscopy

To minimize the variation in pH and to provide a field-frequency lock, 200 μL of a buffer solution (1 M Na_2HPO_4/NaH_2PO_4, pH 7.0) and 350 μL of BALF sample were mixed for NMR experiments. Trimethylsilylproionate (TSP) (6.53 mM solution) was included in buffer for the internal chemical shift reference. All spectra were collected on a Bruker 800-MHz NMR spectrometer equipped with a triple-resonance TCI (^1H, ^{13}C, ^{15}N, and ^2H lock) cryogenic probe.

All 1D ^1H NMR (noesypr1d in Bruker library) spectra with water suppression were recorded with 64 scans, 64 K data points, spectral width of 20 ppm, relaxation delay 5 s. All 1D spectra were processed with line broadening of 0.3 Hz, manually phase and baseline corrected. The chemical shifts were internally calibrated to the TSP peak at 0.0 ppm.

For the NMR peak assignment purpose, two-dimensional 2D homonuclear and heteronuclear spectra were recorded. Details of NMR parameters are given in the supporting information.

3.3 Statistical analysis

A total of 30 spectra corresponding to 21 lung injury patients (ALI = 11, ARDS = 10) and nine controls have been considered for multivariate analysis. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) analysis were carried out by 'The Unscrambler X' software package (Version 10.0.1, Camo ASA, Norway). PCA was performed to transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called principal components and PLS-DA is performed in order to sharpen the separation between groups of observations. Binning of NMR data was performed using AMIX software (version

3.7.10, Bruker BioSpin, Switzerland) for the chemical shift regions of 0.5–4.5 and 5.1–9 ppm. The region between 4.5 and 5.1 have been excluded from the study to avoid variability from water suppression. A total 851 continuous integral segments of equal width of 0.01 ppm were collected for the further analysis. The data obtained was mean centered, scaled to total intensity. These buckets were integrated and normalized by adjusting the total area to unity to minimize the effect of variable sample dilution. To find significant variation (p value) of different metabolites among groups we have applied one-way analysis of variance (ANOVA) with post hoc multiple comparisons by Bonferroni correction. For the ANOVA analysis we have used NMR peak area scaled with respect to TSP resonance. ANOVA analysis was performed by software SPSS (Version 11.2, IBM USA).

4 Results and discussions

The ^{1}H NMR spectra of human mBALF samples are shown in the Fig. 1. Spectra shown in green, red and black correspond to ARDS, ALI and control groups respectively. These spectra were calculated from the mean of different spectra in each group. Several metabolites with high and low level concentration were observed in the spectra. Many metabolites signal were visible and large numbers of overlapped metabolites resonances were also found. Several 1D and 2D spectra such as ^{1}H, J-resolved, COSY, TOSCY and HSQC (SI Fig. 3) were carried out to identify those molecules (spectra are given in supporting information). 2D spectra were recorded to separate the overlapping chemical shift and to find the connectivity of different spin systems. Both homonuclear (COSY, TOSCY) and hetronuclear (HSQC) were recorded to find the connectivity between proton and carbon (supporting Fig. 1, 2). Most of signals were assigned either by standard solution of metabolites or BMRB NMR database. SI Table 2 shows the list of identified compounds in mBALF and their ^{1}H and ^{13}C chemical shift measured by 1D and various 2D spectra. All assignments were confirmed using BMRB NMR data base (http://www.bmrb.wisc.edu/).

The differences in NMR spectra between healthy subjects and ALI/ARDS group were apparent from the visual inspection (Fig. 1). By visual inspection it can be easily identified that concentration of various metabolites in ALI/ARDS groups were more than control group. The presence of high concentration of various metabolites in mBALF of ALI/ARDS patients represent increased metabolic functioning in human lungs, which is directly correlated with corresponding injury condition. This increase metabolic functioning is due to microbial growth, own metabolism and breakdown products due to tissue injury.

This is reflected in increase in concentration of small molecular weight metabolites. Various metabolites such as amino acids, glucose, and small molecules are identified in mBALF spectrum representing metabolic information about lungs of ALI/ARDS patients. Presence of these metabolites in altered concentration reflects severity of lung injury. Low concentration of these metabolites in NMR spectra of mBALF of control group (non ALI/ARDS) represents absence of corresponding metabolic cycles in healthy lungs (Fig. 2). The NMR spectra also show significant differences in number of metabolites in both classes. All 21 patients in present study were diagnosed as having ALI or ARDS based on several clinical parameters. But during this study we took only (P/F) ratio as single parameter variable as the diagnostic marker. To confirm the separation between these two groups multivariate statistical analysis were performed. This has enabled us to identify the metabolites, which are responsible for the maximum separation between these groups. We performed the analysis in following two parts; (a) To find difference among control, ALI and ARDS, and (b) difference between ALI and ARDS groups. In order to find the significance (p value) between metabolites among different groups, we have applied one-way analysis of variance with post hoc multiple comparisons by Bonferroni correction. List of significant metabolite with p value were given in Table 1. A p value <0.05 was considered significant for the ANOVA on the metabolites among control, ALI, and ARDS.

First we performed PCA because the numbers of variables were much higher than the number of samples. By plotting the PC-1 versus PC-2 we got the separation in control (blue square) and ARDS (green open circle) as shown in the Fig. 2a. The first principal component (PC1) separated the sample and accounted for the 76 % of the variance within the data. The second principal component accounted for 10 % of variance. The corresponding loading plot shows the variation in the spectral region among the sample (Fig. 2b). The PCA loading plot shows dynamic change of metabolome of human lungs associated with ALI/ARDS. Various metabolites were identified responsible for separation among control, ALI and ARDS. All identified metabolites were labeled in corresponding loading plot. The control group was well-separated from ARDS group. Three samples from ALI group overlapped with control group. These three members have P/F ratio closer to the control group (P/F = 300, 300, 260). The less severe group is at the top of the quadrant and more severe is at the bottom. The metabolites responsible for the separation between these groups is shown in Fig. 2b. To identify the main metabolites discriminating among control, ALI and ARDS PLS-DA analysis were also carried out. PLS-DA along with loading plot is shown in SI Fig. 4a and b respectively. The PLS-DA model explains 76 % variation
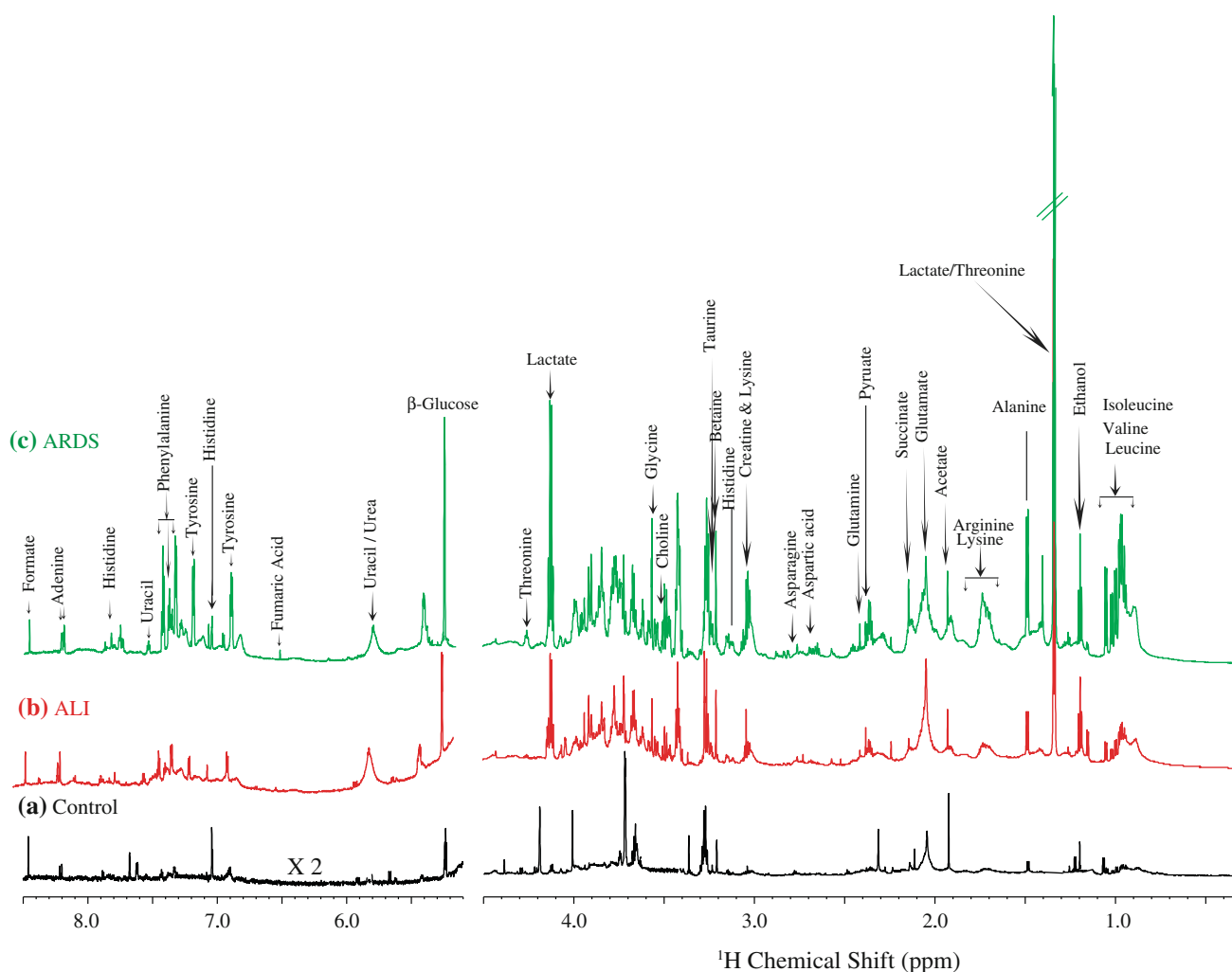
Fig. 1 Mean plot of ^{1}H NMR spectra of mBALF from different groups in the present study. The groups are **a** control, **b** ALI, and **c** ARDS patients. The assignments of various resonances are shown in the *plot*

with $R^2Y = 89$, $Q^2 = 84$. The positive intensity metabolites responsible for the separation among these three groups are isoleucine, valine, lysine, leucine, lactate, threonine, alanine, betaine, arginine and choline. On the other hand, the metabolite resonances with negative intensity responsible for separation are ethanol and proline. The loading plot indicates how variables were associated with principal components. PLS-DA analysis shows similar results as PCA showing healthy group is well separated from the ALI/ARDS, although there is slight overlap between ALI and ARDS group. PCA and PLS-DA analysis were based on latent vectors and can be similar. In PCA and PLS-DA analysis, the intensity of lactate/threonine/ethenol resonances is very high. We further performed PCA and PLS-DA analysis after removing resonances of lactate/threonine/ethanol (1.1–1.36 and 4.10–4.15 ppm). The result of PCA and PLS-DA analysis is shown in Fig. 2c, d and SI Fig. 4(c, d). After removing contributions

of lactate resonance, we find results being similar in nature and shows contributions of few metabolites, which have very less concentration. This was not possible to observe in earlier analysis. It can be easily seen from ANOVA Table 1 and supporting information Fig. 5 ethanol, betaine, proline, choline, threonine, and lactate are significant metabolites whose concentration is increasing or decreasing in separation between control and ALI. Other metabolites, which are significantly responsible for the separation between control, ALI and ARDS are given in Table 1.

Inflammatory changes in metabolic patterns between control and ALI and ARDS are expected and it gets reflected in terms of NMR peak intensity. The important aspect of present study is to find the metabolites separating classes between ALI and ARDS groups, thus finding out if NMR spectroscopy of mBALF can be used for diagnosis of inflammatory changes in lung injury. Again PCA and PLS-DA were performed between ALI and ARDS groups. The
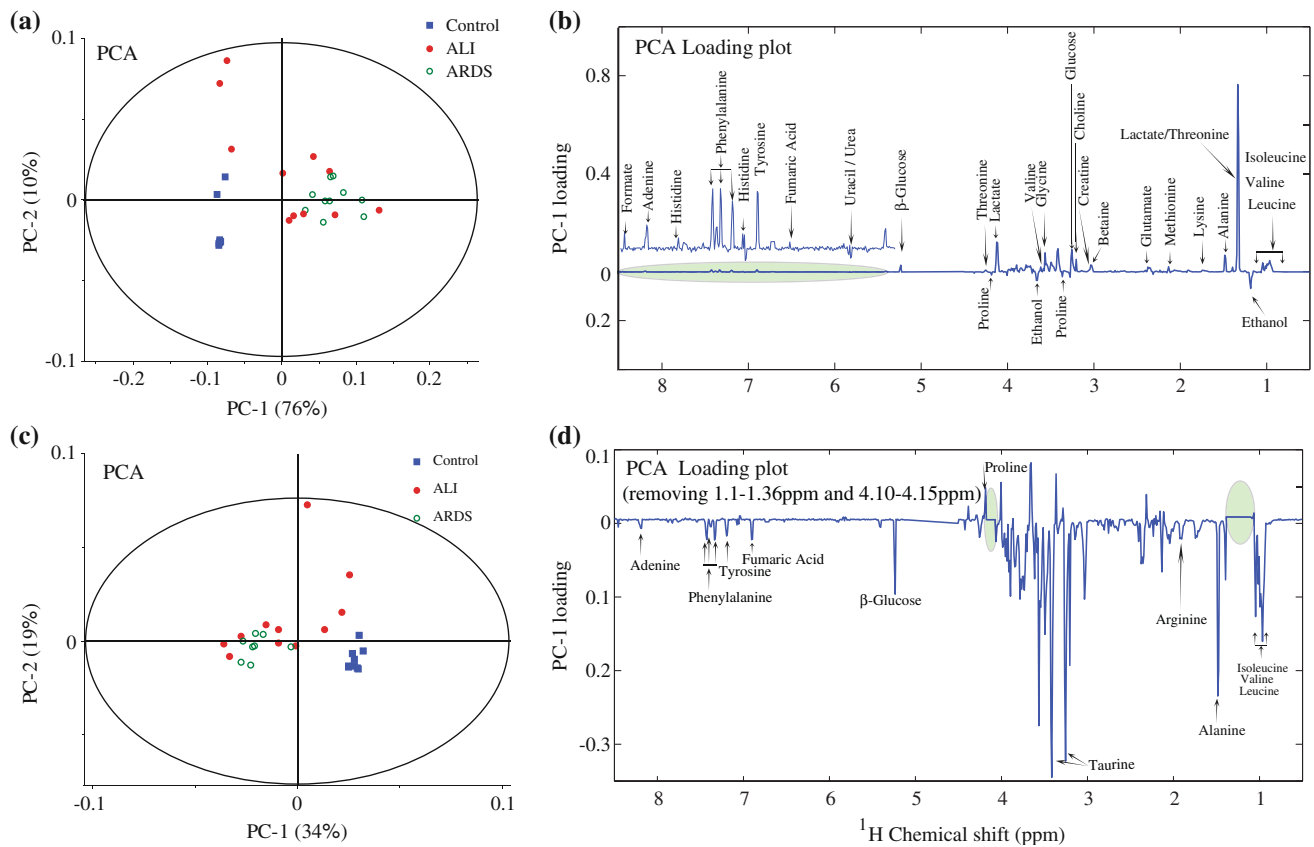
Fig. 2 a PCA scores plot (PC-1 vs PC-2) of the mBALF NMR spectra from the control group and ALI/ARDS groups along with their **b** PCA loading plots. **c, d** corresponds to similar PCA analysis after removing contributions from resonance of lactate/threonine at 1.1–1.36 and 4.10–4.15 ppm

results are shown in Fig. 3a, b. PCA plot PC-1 with 73 % variance and PC-2 with 8 % variance shows a clear difference between ALI and ARDS (Fig. 3a). ALI and ARDS are separated between two groups with minor overlap and this is expected since ARDS differs from ALI in term of severity of inflation in lungs. PLS-DA ($R^2Y = 0.50$, $Q^2 = 0.48$) (SI Fig. 5a, 5b) analysis were performed to identify metabolites whose concentration changes among groups ALI and ARDS; the results are shown in SI Fig. 5b. Metabolites with positive value of loading score are isoleucine, valine, lysine, leucine, lactate, threonine, alanine and glutamate. Metabolites with negative values of loading score are ethanol, acetate and proline. The corresponding change in concentration of various metabolites is represented in Table 1 and SI Fig. 7. The PCA and PLS-DA analysis was performed after removing resonance from 1.1 to 1.36 ppm and 4.10 to 4.15 ppm. The results are shown in Fig. 3c, d and SI Fig. 5c, d. The results are similar in nature along with contributions from low intensity metabolites. Lactate concentration is increasing in the ALI/ARDS group ($p = 0.001$) as reported in the earlier studies. This observation is consistent with earlier studies showing

lactate production in human lungs (O'Neil and Tierney 1974; Effros et al. 2002; De Backer et al. 1997). Increase in lactate concentration in lung injury cases was also reported in earlier study (De Backer et al. 1997). Lung inflammation triggers an increase in lactate production (Wolak et al. 2009). It also reflects the low oxygen environment in lungs, which triggers anaerobic metabolism and could result in increased level of lactate production. High concentration of taurine ($p = 0.032$) is observed in ARDS patients as compared to ALI group (Fig. 4h and SI Fig. 5). The increase in taurine concentration is correlated with severity of lung inflammation (Witko-Sarsat et al. 1995). It is known that taurine accumulates in epithelial cells where its function is to regulate the osmotic balance (Musante et al. 1999) and to protect the lung epithelial cells against myeloperoxidase-derived oxidants (Cantin 1994). Threonine ($p = 0.026$) is another molecule whose concentration increases considerably in ARDS group. It has been reported earlier that threonine level always increases in case of trauma or stress. Increases in threonine concentration enhance the immune system by aiding in the production of antibodies. Adenine and uracil concentration increases in

Table 1 Significance level (*p* value by Bonferroni correction after one way ANOVA)

Metabolites	Significance level (*p* value by Bonferroni correction after one way ANOVA)		
	Control versus ALI	Control versus ARDS	ALI versus ARDS
BCA	NS	0.001	0.001
Ethanol	0.001	0.001	NS
Alanine	0.001	0.001	0.001
Lysine/arginine	NS	0.001	0.001
Acetate	NS	0.001	0.001
Glutamate	NS	0.001	0.035
Aspartate	NS	0.001	0.016
Creatine	NS	0.001	0.001
Betaine	0.001	0.001	0.001
Proline	0.013	0.002	NS
Taurine	NS	0.001	0.032
Choline	0.001	0.01	NS
Threonine	0.001	0.001	0.026
Lactate	0.048	0.001	0.001
Phenylalanine	NS	0.016	NS
Glycine	NS	0.038	NS
Succinate	NS	0.001	0.001

p values were determined from one way ANOVA, only *p* < 0.05 are displayed

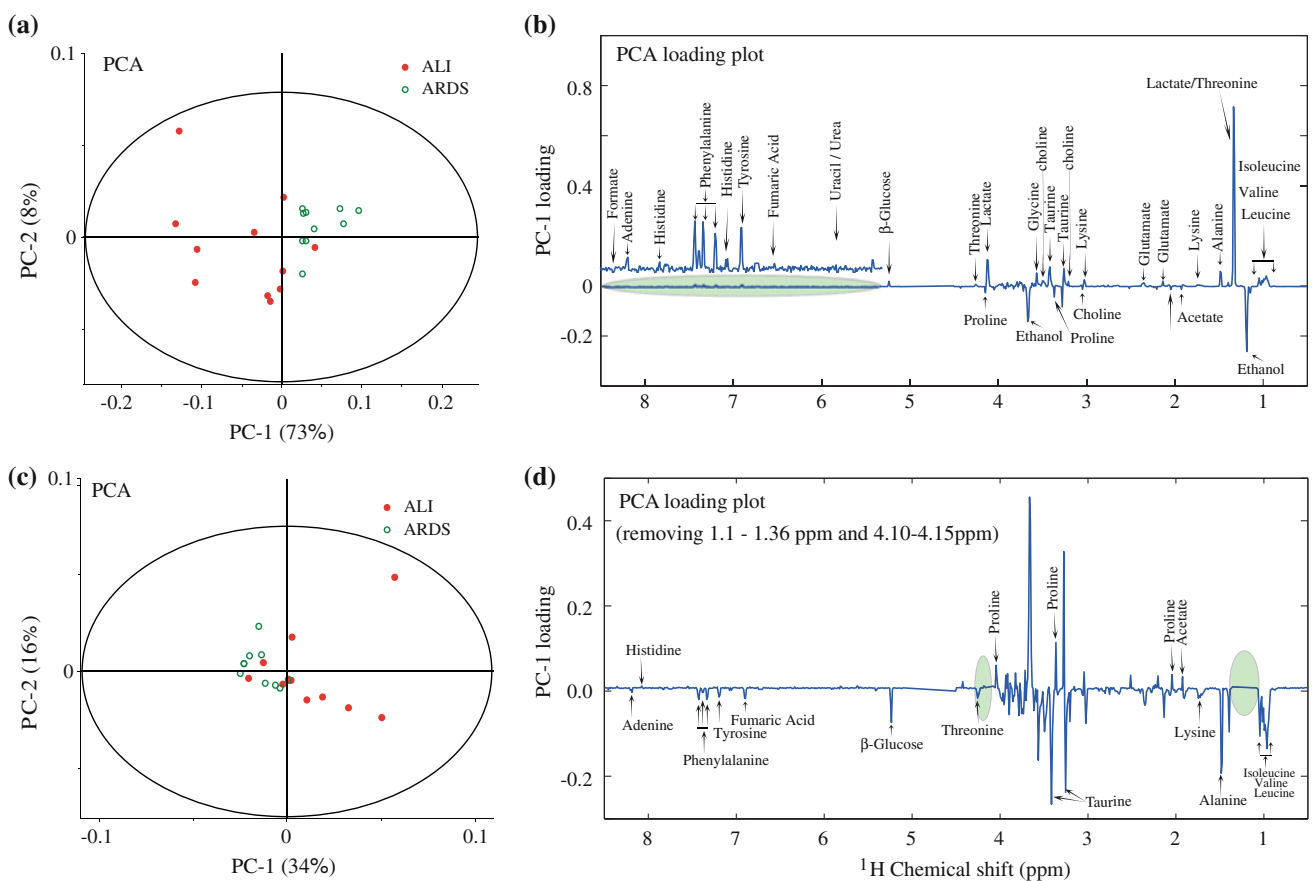*BCA* branch chain amino acids, (Leucine, Isoleucine and Valine), *NS* not significant

Fig. 3 a PCA scores plot (PC-1 vs PC-2) of the mBALF NMR spectrum from ALI and ARDS groups. The corresponding loading plot is shown in (**b**). **c** PCA score plot and **d** PCA loading plot correspond to similar PCA analysis after removing contributions from resonance of lactate/threonine at 1.1–1.36 and 4.10–4.15 ppm

ARDS group, which are end products of nucleic acid degradation. The degradation of these nucleotides by the nucleotidases may play a role in the immune response (Carson et al. 1979) or it may be used as the rapid energy source (Matsumoto et al. 1979).

We also find that, metabolites such as branch chain amino acids (leucine, valine and isoleucine) play major role in ALI and ARDS classification (Table 1). Increase in branch chain amino acids is an indicator of muscle breakdown (Garlick and Grant 1988). This is expected in case of lung injury. These results are consistent with previous study (Freund et al. 1978). These kind of metabolic changes are known to be correlated with the progression of sepsis (Siegel 1983). Other molecules such as arginine, glycine, aspartic acid, succinate, acetate, and glutamate concentration get elevated and proline decreased. Acetate is one molecule that is not reported earlier in sepsis or ALI/ARDS and our study shows increase level of acetate in case of lung injury. This molecule could be the result of oxidative decarboxylation of pyruvic acid (Kuo et al. 1989). The possible schematic representation of biochemical pathway during the lung injury is represented in SI Fig. 6. Various metabolites observed in mBALF spectra of lung injuries are correlated with the metabolic pathways.

The above analysis presents the ^{1}H NMR spectroscopy based metabolomics as an appropriate tool for the identification of potential novel biomarkers in case of human lung injuries. In summary, here we present the first metabolic profile of lung injury in mBALF of human and try to correlate clinical variables with metabolic alteration of different metabolites. Still more work is needed to establish NMR as possible tool for diagnostic as well as prognostic. We believe that NMR based metabolic profiling could potentially be used as the general method for screening severity of the lung diseases and diseases progression (Nin et al. 2011).

5 Conclusion

The present study has shown that ^{1}H NMR spectroscopy combined with statistical analysis is able to detect consistent metabolic alterations in the mBALF profile of ALI and ARDS subjects, enabling their discrimination from healthy control subjects. We identified various metabolites in mBALF of human with ALI/ARDS along with their statistical significant variations. Metabolites such as lactate, taurine, threonine, arginine, aspartic acid, glutamate, and acetate are identified as possible biomarkers for discrimination between ALI and ARDS groups. With similar studies on larger group, these metabolites can prove to be useful biomarker for early diagnosis of ARDS. These studies are currently under progress in our laboratory.

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