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METABOLOMIC PROFILE OF MINI BRONCHOALVEOLAR LAVAGE FLUID FROM CHRONIC OBSTRUCTIVE PULMONARY DISEASE PATIENTS TO INVESTIGATE THE EFFECTS OF ENDOTRACHEAL INTUBATION AND MECHANICAL VENTILATION USING NMR SPECTROSCOPY

Anesthesiology

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ABSTRACT

Introduction: COPD patients are exposed to endotracheal intubation and mechanical ventilation during general anaesthesia for various surgical interventions. In order to delineate the pathophysiology at molecular level, mini bronchoalveolar lavage (BAL) fluid was collected from these patients during mechanical ventilation and metabolomics profile of this was analyzed using nuclear magnetic resonance spectroscopy.

Methods: 20 adult COPD patients (GOLD stage II and III) were enrolled in this prospective observational pilot study, which is the first study in which mini BAL fluid was utilized for assessing metabolomics of COPD patients undergoing mechanical ventilation during general anaesthesia for various surgical procedures. During NMR spectroscopy, several 1D and 2D spectra such as 1H, COSY and HSQC were carried out to identify various metabolites.

Results: NMR spectra between 2 groups of samples (after intubation and before extubation) showed a slight difference on visual inspection in H1 D spectral analysis. There was slight increase in concentration of some metabolites (betaine, serine, aspartate, lysine/arginine and glucose) in "before extubation" samples. But 2D assignment techniques demonstrated no significant difference between 2 groups of samples.

Conclusion: No change in lung metabolomic profile of COPD patients could be detected after endotracheal intubation and mechanical ventilation during surgery of 3 to 5 hours duration. We can also infer that optimized COPD grade II and grade III patients do not undergo metabolomic change in lungs during 3 to 5 hours of mechanical ventilation.

KEYWORDS

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the most common chronic adult respiratory diseases around the world. A significant number of these patients are exposed to the risk of anaesthesia and surgery during various elective and emergency situations, where they are exposed to various drugs and invasive procedures which can have variable effects at molecular level on the airways of these patients who already have chronic inflamed lungs. To quantify and to delineate the pathophysiology at molecular level in these patients we need to apply upcoming and new techniques.

Importance of bio fluids[1,2,3.] in exploring disease pathophysiology has been well established. Composite bio fluids analysis can give information regarding onset of disease, drug effects, disease course and outcome, which comprise an intricate network of endogenous metabolites with polar and soluble components[1,2].

Metabolites hold importance in the arena of clinical applications as an indicator of the physiological state and possible index to the biological pathway involved in various disease processes.[3,4,5]

The analytical platform of metabolomic has been employed to monitor and ascertain disease susceptibility correlated with the altered and aberrant metabolite concentration[6]. Such metabolic studies have been carried out extensively in bio fluids to decipher the information on the lung microenvironment in the state of disease and debilitation.[7,8]

Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as a powerful analytical platform with an edge over other tools by being non-destructive, nonselective, reproducible, and conducive for timely analysis and requiring minimal sample preparation[9].The metabolites and their composition and interaction for putative biomarkers can be ascertained from a single biological sample[10].

Intubation and mechanical ventilation is an invasive procedure which has the potential to induce local as well as systemic metabolomic changes and these changes can be more pronounced in patients with chronic obstructive pulmonary disease.

COPD patients have hyper reactive and inflamed airways; mechanical ventilation during general anaesthesia can alter the pathophysiology of the primary lung disease. There is a need to identify the metabolomic changes associated with intubation and ventilation in COPD patients which can guide in anaesthetic management of the patient and may prevent morbidity associated with endotracheal intubation and ventilation.

Therefore, we planned this study to apply NMR spectroscopy based metabolomic on mini BAL (Broncho Alveolar Lavage) fluid collected from patients of COPD and to demonstrate the airway metabolomic changes during general anaesthesia after endotracheal intubation and mechanical ventilation. Primary objective of this study was to compare the metabolomic analysis of mini BAL fluids just after intubation and immediately before extubation in COPD patients who were mechanically ventilated during general anaesthesia while undergoing elective surgical procedures.

Our secondary objective was to apply mini BAL metabolomic analysis technique to detect any metabolomic markers which may detect inflammation in airways and lungs of these patients.

MATERIALS AND METHODS:

It was a prospective observational pilot study which was conducted in a tertiary care medical institute in collaboration with Centre of Biomedical Research. Approval from institutes ethical committee (IEC code No.2015-77-MD-85) and informed patient consent was taken before sample collection. Since it was a pilot study, we enrolled 20 adult COPD patients, aged more than 18 years who were planned to

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undergo elective surgery under general anaesthesia with end otracheal intubation. We decided to enrol those patients in whom expected duration of surgery was more than 3 hours but less than 5 hours.

The diagnosis of COPD was based on clinical and spirometry criteria (post-bronchodilator ratio of FEV1/FVC<0.7). The degree of airflow obstruction was graded according to GOLD guidelines as mild, moderate, severe, or very severe if FEV1 was 80, 50–80,30–49 %, or <30 % of the predicted value, respectively (Table No. 1).

Table 1: GOLD grading for severity of COPD

Stage I	Mild COPD	FEV1/FVC<0.70	FEV1≥ 80% of normal
Stage II	Moderate COPD	FEV1/FVC<0.70	FEV1 50-79% of normal
Stage III	Severe COPD	FEV1/FVC<0.70	FEV1 30-49% of normal
Stage IV	Very severe COPD	FEV1/FVC<0.70	FEV1<30% of normal or <50% of normal with presence of chronic respiratory failure

Data collection included demographic profile, clinical characteristics and illness severity scores like GOLD grading of COPD. Patients in whom it was not advisable to disconnect ventilator due to risk of hypoxemia, those with duration of surgery less than 3 hours & more than 5 hours, and those with active respiratory infections were excluded.

Patients were enrolled for study at the time of pre-anaesthetic examination. They were advised to continue their bronchodilator therapy till the time of surgery and resume in post-operative period. Age, gender of patient, duration of COPD, pulmonary function test values, medical therapy received, co-morbidities, and duration of surgery (time period between skin incision and wound closure) was recorded.

Standard anaesthetic management of the patients was done which included premedication with oral alprazolam 0.5 mg and ranitidine 150 mg. Anesthesia was induced with injection fentanyl 2-3 mcg/kg and injection propofol 1.5 – 2.0 mg/kg. Injection vecuronium bromide 0.12 mg/kg was administered to facilitate endotracheal intubation and surgical muscle relaxation. Mechanical ventilation of lungs was continued with a mixture of 50% air in oxygen and sevoflurane. Peak airway pressures were measured during the entire surgery. Tidal volume and respiratory rate were adjusted to maintain normocarbia (end tidal CO_2 35-40 mm Hg). During the maintenance of anaesthesia, intermittent doses of fentanyl and vecuronium bromide were administered. Monitoring included ECG, pulse oximetry (Spo2), non-invasive blood pressure, airway pressure, temperature and urine output.

Sample collection: Bronchoalveolar lavage sample was collected using mini bronchoalveolar lavage technique using "catheter in catheter" technique[11], as shown in figure 1, five minutes after intubation and approximately 15 minutes prior to estimated extubation. This "catheter in catheter" method has been validated in previous study[11] as a method of mini BAL, which is less invasive, economical and readily available. Earlier studies have shown that mini BAL and bronchoscopic alveolar fluids collections yield similar diagnostic results[12].

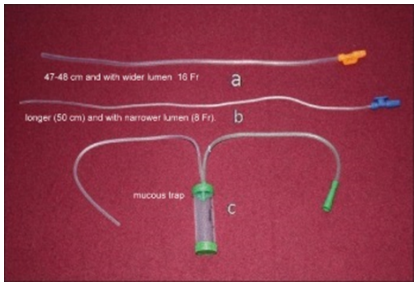


Figure1. The apparatus used for non-bronchoscopic mini BAL. It comprises two suction catheters (a, b) and one mucus trap ©. The suction catheters have different lengths and luminal diameters. The outer suction catheter (a) is shorter in length (about 47-48 cm) with a wider lumen (16 Fr). The inner catheter (b) is longer (50 cm) with narrower lumen (8 Fr).

Mini BAL fluid 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 degree C to remove cellular debris and bacteria and then supernatant stored at -80 degree C till NMR experiments were performed.

At the end of surgery, anaesthesia was terminated and residual effect of muscle relaxant was reversed with a mixture of Neostigmine 0.05 mg/kg and Glycopyrrolate 0.01 mg/Kg, intravenously. On reversal of residual effect of muscle relaxant and satisfactory respiratory parameters endotracheal extubation was performed. Patients were followed up in the post-operative units for any complications and discharged from PACU as per the institutional protocol.

Processing of Samples:

NMR spectroscopy: To minimize the variation in pH 200 µL of a buffer solution (0.1 M Na_2HPO_4/NaH_2PO_4, pH 7.0) and 350 µL of mini BAL Fluid 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, 13C, 15N, and 2H lock) cryogenic probe.

All 1D ^{1}H NMR (noesypr1Din Bruker library) spectra with water suppression were recorded with 128 scans, 64 K data points, spectral width of 20 ppm, relaxation delay 5 seconds. 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.

Statistical analysis:

Demographic data was analyzed using SPSS version 22. A total of 40 spectra corresponding to 20 COPD patients who underwent elective surgery under general anaesthesia underwent multivariate analysis. Principal component analysis (PCA) was carried out by 'The Unscrambler X' software package (Version 10.0.1, Camo ASA, Norway). PCA was performed to transform a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called principal components. Binning of NMR data was performed using AMIX software (version3.7.10, 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 has been excluded from the study to avoid variability from water suppression. A total 750 continuous integral segments of equal width of 0.01 ppm were collected for the further analysis. The data obtained was mean centred scaled to total intensity.

Observations and Results:

20 patients were enrolled in this observational pilot study. Their demographic and clinical characteristics are shown in table 2 and 3 respectively.

Table 2: Demographic characteristics of patients

	Mean ± SD	Range
Age (years)	54.35±12.33	39 – 81
Gender (percentage)	Male: 15 (75%), Female: 5(25%)	
Weight (Kg)	62.1± 16.87	40-90
Body Mass Index (Kg/M2)	25.06±5.68	17.3-36.7
Associated co-morbidities (number of patients)	Hypertension: 7 (35%) Diabetes mellitus: 5 (25%) None: 8 (40%)	

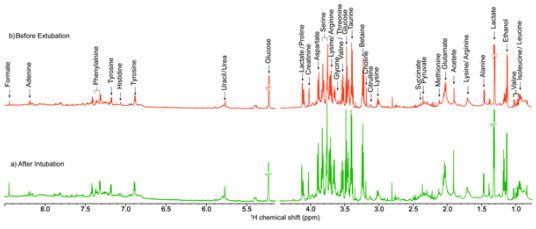
Table 3: Clinical characteristics of patients

	Mean ± SD	Range
Duration of COPD (years)	7.90 ± 5.06	1-20
Post-bronchodilatation FEV1	0.59 ± 0.10	0.44-0.75
FEV1/FVC	0.57 ± 0.07	0.45-0.68
Duration of surgery (Hours)	3.89 ± 0.80	3-5
Severity of COPD (number of patients, as per GOLD protocol)	COPD Grade II: 14 (70%) COPD Grade III: 6 (30%)	
Distribution of bronchodilators (Number of patients)	Salmeterol: 9 (45%) Formoterol: 6 (30%) Ipratropium bromide:5 (25%)	
Distribution of inhaled steroids (Number of patients)	Budesonide: 10 (50%) Fluticasone: 9 (45%) None: 1 (5%)	

The 1H NMR spectra of mini BAL fluid from COPD patients are shown in the Figure 12. Spectra shown in green and red correspond to mini BAL fluid obtained just after intubation and just before extubation respectively.

These spectra were calculated from the mean of different spectra in each group. The acquired spectra were phased and baseline-corrected. The distinctive chemical shifts in the frequency domain originating from sets of spectral measurements were assigned for respective metabolites. Low molecular- weight metabolite resonances present in 1D spectrum (Figure 2) were identified using biological magnetic resonance data bank (BMRB) database, human metabolome database (HMDB), MetaboMiner, established literature values, and reference spectra from standard compounds.

Figure 2. The 1H NMR spectra of COPD mini BAL Fluid samples are shown in this diagram. Spectra shown in green and red correspond to mini BAL fluid just after intubation and before extubation respectively. Numbers of signals indicate how many "different kinds" of protons are present. Position of signals indicates about chemical shift and magnetic (electronic) environment of protons .Splitting of signals indicates the number of nearby nuclei (spin spin coupling) usually protons.



Further confirmation of metabolites was achieved using 2D spectra including Correlation Spectroscopy (COSY) (Figure 3) and Heteronuclear Single Quantum Correlation (HSQC) (Figure 4).

Figure 3. COSY plot of mini BAL fluid .This 2D correlation spectral analysis is applied for assignment of molecules on the basis of 1H -1H coupling.

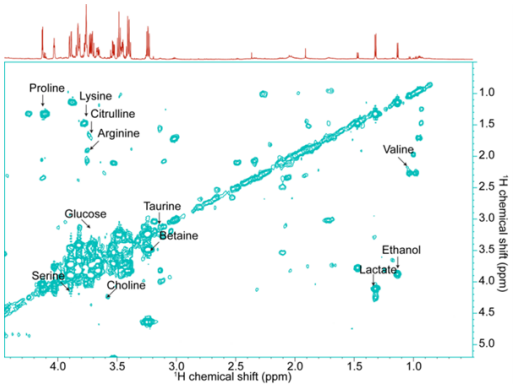
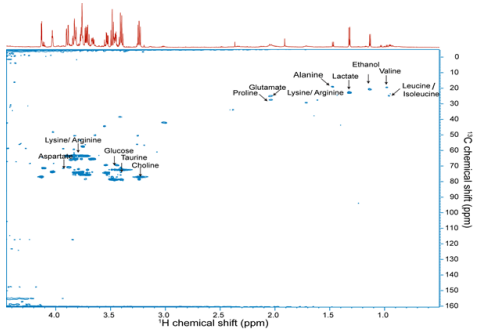


Figure 4. HSQC plot of mini BAL fluid samples. HSQC is a 2D technique which is used for better assignment of the molecules and it uses the interaction between 13C and 1H nuclei by spin-spin coupling mechanism.



Several metabolites, with high and low level concentration were observed in the spectra. Many metabolites signals were visible and large numbers of overlapped metabolites resonances were also found. Several 1D and 2D spectra such as 1H, COSY and HSQC were carried out to identify those molecules. 2D spectra were recorded to separate the overlapping chemical shift and to find the connectivity of different spin systems. Both homonuclear (COSY) and heteronuclear (HSQC) were recorded to find the connectivity between proton and carbon.

Various metabolites such as amino acids, glucose, and small molecules are identified in mini BAL Fluid spectrum representing metabolomic information about lungs of COPD patients. The 1D 1H NMR spectroscopy (Figure 2) of human BAL Fluid is mostly dominated by low-molecular-weight metabolites like short chain fatty acids, branched chain amino acids, monosaccharides, aliphatic and aromatic amino acids, tricarboxylic acid cycle intermediates, purine and pyrimidine metabolites, and organic acids. The aromatic portion constituting 6−9 ppm is evident from formate, histidine, phenylalanine, tyrosine, urea, and uracil, which make up the nucleic acid breakdown products. The majority of the crowded and overlapped regions were observed in the 3 to 4 ppm range with predominant forms of energy derivatives like glucose, creatine, lactate and amino acids, and water-soluble nutrients, which were resolved and assigned by 2D approach. 2D techniques have been employed to remove strong coupling artefacts that result in additional peaks and to overcome the ambiguities in the complex overlapped regions of aliphatic methyl groups along with the cluster of multiplets in the mid-frequency range of 3 to 4.5 ppm.

NMR spectra between 2 groups of samples (after intubation and before extubation) showed a slight difference on visual inspection in H^1 D spectral analysis. There was slight apparent increase in concentration of some metabolites (betaine, serine, aspartate, lysine/arginine and glucose) in “before extubation” samples as compared to “after intubation” samples. (Figure 2). This slight rise in the above-mentioned metabolites in 1D spectra can be due to artefacts and overlapping and thus further 2D assignment techniques was used.

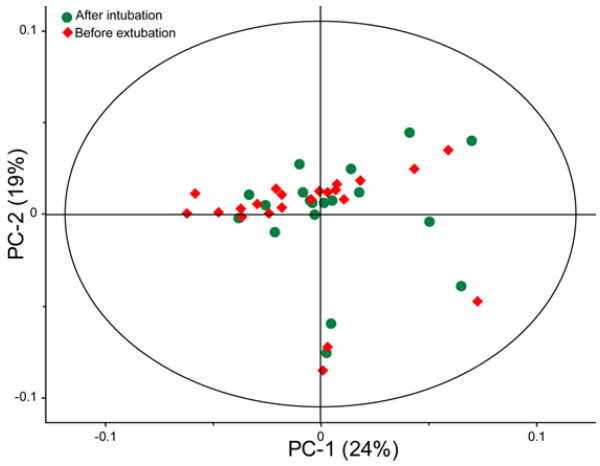
Unambiguous assignment, which is severely limited by the spectral overlap of 1D NMR is overcome by 2D NMR methods which addresses the high chemical shift degeneracy and incomplete information on reference spectra from databases encountered in 1D NMR methods. The combined approach of higher field and more dispersive 2D spectroscopy enabled us to recognise the individual metabolites even within complex and heterogeneous mini BAL Fluid samples of COPD patients. Precise assignment techniques specific and peculiar to sample dynamics like heterogeneity, viscosity, motional artefacts, different nuclear spin relativities, diffusion, and compartmentation were taken into consideration before optimization and identification. Substantial assignment of mini BAL Fluid using multidimensional 2D approaches measured and assessed the candidate biomarkers, which validates the metabolites difference, if any between the two sample groups.

By employing HSQC, metabolite assignments are made more feasible due to the 1H−13C chemical shift correlation, distinct chemical shift assignments and larger 13C chemical shift evolution. The loading plot indicates how variables were associated with principal components. Principal Component Analysis (PCA) was first performed based on the normalized NMR spectral data obtained from two groups of samples. We performed PCA because the numbers of variables were much higher than the number of samples. The first and second principal components (PC1 and PC2) were calculated for the models of comparing two groups of “after intubation” and “before extubation” mini BAL fluid samples.

By plotting the PC-1 versus PC-2 we got the separation in “after intubation” (green circles) and “before extubation” (red diamonds) as shown in the Figure 5. The first principal component (PC1) separated the sample and accounted for the 24 % of the variance within the data. The second principal component accounted for 19% of variance. The corresponding loading plot shows the variation in the spectral region among the sample. The PCA loading plot did not show dynamic change of metabolites of human lungs of COPD patients undergoing surgery under general anaesthesia for duration of 3 to 5 hours.

Figure 5. Principal Component Analysis . The first principal component (PC1) separated the sample and accounted for the 24 % of the variance within the data. The second principal component

accounted for 19% of variance.



There was significant superimposition of the two groups of samples, and no separation between these two groups was found in the PC1 vs. PC2 scores scatter plots.

DISCUSSION:

Any invasive intervention like endotracheal intubation has a likelihood of predisposition to enhancement of the inflammation of airways in COPD patients. Moreover, surgery itself along with various intravenous and inhaled anaesthetics used can have various effects on the respiratory system of these patients. Patient characteristics, type and duration of surgery can also influence the inflammation of the airways in these patients. Patient factors like duration, severity and grade of disease as well as treatment profile can influence the degree of airway inflammation. External factors like anaesthetic drugs, type and duration of surgery may also be important in the process of airway changes.

Surgical factors which could alter the inflammatory status of airways are duration and type of surgeries. We included patients of gastrointestinal and Urological surgeries which will have minimal impact on airways. Head and neck surgery were not included to avoid any exaggeration in airway manipulation and possible inflammation. Average duration of these surgery were 3.89 hours and too short procedures were not included to allow sufficient time for inflammation, if any, by the endotracheal tube in the airway.

The patient factors which could affect the airway inflammation are duration and grade of disease, bronchodilator therapy, anti-inflammatory therapy and associated comorbidities. The average duration of COPD was 7.90 ± 5.06 years. All patients were either of COPD grade II (70 %) or grade III (30%) in our study. All patients were on long acting bronchodilators and 95 % were on inhalational steroid therapy. Steroids can suppress airway inflammation but no patient required steroids intraoperatively. Systemic inflammation, free radical injury and hyperglycaemia can alter the natural history of COPD[13]. Five patients were diabetic which were optimized for glycaemic control before surgery. Premedication, intraoperative anaesthetic drug usages and extubation protocols were kept similar in order to negate the differences of drugs on the airway inflammation. No bronchodilators therapy was needed in any patient intraoperatively. Mechanical ventilation was performed with 7-9 ml/kg of tidal volume adjusted to achieve end tidal C02 of 35-45mm Hg. Average peak airway pressure was 24.40 ± 5.33 cm of H_2O.High airway pressures were avoided to prevent barotrauma and any further injuries to lungs.
Mini bronchoalveolar fluid has been proven to be an excellent biological fluid for studying lung injuries in previous studies as it is extracted from the vicinity of lungs[14,15] . This is the first study of mini BAL in COPD patients. NMR spectroscopy has also emerged as a new technology and already been validated in various studies in literature as a tool to study metabolomic of diseased lungs. The same principle has also been applied in exploring the metabolomic profile in urine[16] and exhaled breath condensate[17] of COPD patients but never in bronchoalveolar fluid.

The purpose of the study was to search for metabolomic profile changes in airways of COPD patients when they are exposed to invasive procedure like endotracheal intubation and mechanical ventilation during surgery. We analysed 40 mini BAL fluid samples. Serial and stepwise processing of the samples by NMR did not reveal any significant change in the metabolomic profile in between post intubation and pre extubation group of samples. Initially, 1D spectrum was done and multiple chemical groups were identified after assignment. This included branches chain amino acids, monosaccharides, TCA (tricyclic acid) cycle intermediates product and various other metabolic products. On initial analysis of 1D spectra the metabolites assignment and distribution seemed similar to normal lungs. But close observation revealed there were some changes in metabolites in two groups of mini BAL samples. After assignment in 2D spectra like COSY and HSQC no changes in spectra of two groups of samples was found.

Metabolomic studies have been done previously in obstructive lung patients using various specimens like serum[18], urine[16] and exhaled breath condensate[17]. In a study of metabolomic applied to exhaled breath condensate in childhood asthma[19] peaks in 3.2 to 3.4 ppm range of NMR spectra was observed representing oxidative compounds. Our study also showed peak in the range of 3 to 4 ppm in 1 D spectral analysis which suggests the oxidative stress in COPD patients. In an another study by de laurentis[20] ,pyruvate was found to have increased intensity in exhaled breath condensate in COPD patients but succinate, choline and glutamine were absent. Our study showed the presence of pyruvate, succinate and choline in mini BAL of COPD patients but glutamine was absent. These differences may be because of the diversity of metabolomic profile of different specimen of COPD patients. Intensities of branched chain amino acids like leucine and isoleucine can be indicator of increased muscle breakdown which is consistent with previous studies[14]. Betaine and choline intensities were also found in the 1D spectra, conversion of choline to betaine can be due to action of bacterial enzymes which is a feature of airways of COPD[21].Taurine intensities signify role of smooth muscle relaxation in COPD and airway neutrophilia and oxidative stress[21]. Above mentioned metabolomic profile of COPD patients in our study is comparable to previous studies but we did not observe any changes between metabolomics of post intubation and pre extubation samples.
In a previous study by Singh et al[18] a number of metabolites were found to be increased in cases of ALI/ARDS in intensive care units. The predominant metabolites which showed elevated concentrations were lactate, branched chain amino acids, threonine and nucleotide degradation products. Increases in these metabolites were explained by the acute nature of acute lung injury and severe oxidative stress. But our patients had chronically diseased lungs and were not in any acute stress. It can be deduced from this study that the severity of inflammation caused by endotracheal intubation and ventilation in a chronically obstructed inflamed airway, is not exaggerated during surgery. But further studies are needed to explore new metabolomic in other varying COPD characteristics and external factors.

There were some limitations in our study. All our patients were in COPD GOLD grade II and grade III category. These were well optimized before elective surgery and were not in acute exacerbations at the time of sample collection. Well optimized patients before surgery may have a lesser degree of airway inflammation compared to an un-optimised or patient with acute exacerbation of COPD. No changes in metabolomic during intubation and ventilation of these patients can be due to control of airway inflammation and decrease in reactiveness due to long term therapy. Patients who are in acute exacerbation can be expected to have different metabolomic profile and further studies are needed to explore the metabolomic profile of such patients by the same techniques. COPD grade I and grade IV patients were not present in the group. A different metabolic profile of patients can also be expected in COPD grade IV patients, which can be explored in future studies.

We conclude that no change in lung metabolomic profile of COPD patients occur due to endotracheal intubation and mechanical ventilation during surgery of 3 to 5 hours duration. We can also infer that optimized COPD grade II and grade III patients do not undergo metabolomic change in lungs during 3 to 5 hours of mechanical ventilation and thus, general anaesthesia with endotracheal intubation and mechanical ventilation for duration up to 5 hours may not increase perioperative risk of respiratory complications.

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