Meta-analysis and estimation of gene expression to establish the role of *SFTPD* in COPD

Project work submitted to the Central University of Punjab

For the award of

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In

Life Sciences with Specialization in Human Genetics

BY

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DECLARATION

I declare that all the changes suggested by the external examiner in the dissertation/thesis entitled "**Meta-analysis and estimation of gene expression to establish the role of *SFTPD* in COPD** " submitted by me for the award of degree of **Master of Life Sciences with Specialization in Human Genetics** in the **Department for Human Genetics and Molecular Medicine** has been incorporated in the dissertation/thesis.

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CERTIFICATE

I declare that the project work entitled "**Meta-analysis and estimation of gene expression to establish the role of *SFTPD* in COPD** " has been prepared by me under the guidance of Dr. Sabyasachi Senapati, Assistant Professor, **Department for Human Genetics and Molecular Medicine**, School of Life Sciences, Central University of Punjab. No part of this project work has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Miss. Debparna Nandy has prepared her project work entitled "**Meta-analysis and estimation of gene expression to establish the role of *SFTPD* in COPD** ", for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the **Department of Human Genetics and Molecular Medicine,** School of Life Sciences, Central University of Punjab.

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ABSTRACT

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COPD is considered to be third leading cause of death by 2020. So far, there is no proper biomarker available for the diagnosis of all the different sub-phenotypes. Multiple GWAS studies have reported the role of *SFTPD* (both genetic and serum protein) as a diagnosis biomarker. This current study aims to systemic – review and meta-analyse the association of anthropometric parameter (Smoking status, gender), Protein biomarker (serum SFTPD) and genetic biomarker (SFTPD rs721917) with COPD and its other phenotypes. To support the secondary findings, this study also aims to analyse the expression of SFTPD gene among COPD cases and healthy control from North Indian population. So far bronchodilators therapy are available for providing temporary relief to the patients. But bronchodilators are found to be associated with multiple number of side-effects. Indian Traditional medicine options like Ayurveda and yoga has lot to offer in this case. Many local Indian herbs are found to be significantly effective against COPD condition. Most importantly this herbs don't have any side effects. *Pranayama* and *Dhyana* have also been found to be improving Lung functions in multiple studies.

Debparna Nandy **Dr. Sabyasachi Senapati**

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Sr. No.	Full form	Abbreviation
1.	Chronic Obstructive Pulmonary Disease	COPD
2.	Tuberculosis	TB
3.	Genome Wide Association Study	GWAS
4.	Surfactant Protein D	SFTPD
5.	Acute Exacerbation COPD	AECOPD
6.	Matrix Metalloproteinase	MMP
7.	Extracellular matrix	ECM
8.	Chronic Bronchitis	CB
9.	Enzyme linked Immunosorbent Assay	ELISA
10.	Alpha-antitrypsin	AAT
11.	Receptor for advanced Glycation End Product	RAGE
12.	Intracellular Adhesion molecule	ICAM
14.	Congenital diaphragmatic hernia	CDH
16.	C-reactive protein	CRP
17.	Clara cell	CC-16
18.	Interlukin 6	IL-6
19.	Interlukin 8	IL-8
20.	Tumour Necrosis factor	TNF-α
21.	Matrix Metalloprotease	MMP-9
22.	Medical Subject Headings	MeSH
23.	Hardy Weinberg Equillibrium	HWE
24.	Review Manager	REVMAN
25.	Odds ratio	OR
26.	Standard Deviation	SD
27.	Mean deviation	MD
28.	Global Initiative for Chronic Obstructive Lung Disease	GOLD
29.	World Health Organisation	WHO
30.	Traditional Chinese Medicine	TCM
31.	Long Term Oxygen Treatment	LTOT
32.	Long Acting β_2 Agonists	LABA
33.	Short Acting β_2 Agonists	SABA
34.	Short Acting Muscarinic Antagonists	SAMA
35.	Short Acting Anti-Cholinergics	SACS
36.	Ultra- Long Acting β_2 Agonists	Ultra-LABA
37.	Forced Vital Capacity	FVC
38.	Forced Expiratory Flow	FEF
39.	Peak expiratory flow rate	PEFR
40.	Cyclic adenosine monophosphate	CAMP
41	Disodium cromoglycates	DSCG
42.	Mucin 5AC	MUC5AC
43.	cAMP response element-binding protein	CREB
44.	Highly sensitive quantification of CRP	hsCRP
45.	Respiratory muscle strength	RMS

46.	ST. George Respiratory Questionnaire	SGRQ
47.	Licorice flavonoid	LF
48.	Licorice flavonoid oil	LFO
49.	Forced Expiratory volume in 1sec	FEV1
50.	Maximal inspiratory pressure	PIMax
51.	Maximal expiratory pressure	PEMax

Chapter 1
Introduction and Review of literature

Chapter 2
Systemic Review and Meta-analysis

Chapter 3
Gene expression analysis

Chapter 4
COPD: Alternative medicines

1.1 Introduction

COPD (Chronic Obstructive Pulmonary Disease) is a progressive disorder which is characterized by multiple phenotypes that ultimately leads to respiratory problem within the diseased persons (Dickens *et al*, 2011, Søndergaard and Halling, 2014). Major COPD sub-phenotypes include chronic bronchitis, induced sputum, emphysema, chronic cough, mucus hypersecretion, muscle wasting, depression, cardiac diseases airway obstruction, acute- exacerbation and dyspnea (Dickens *et al*, 2011, Søndergaard and Halling, 2014, Carolan *et al*, 2014, Kohli *et al*, 2015). Different phenotype has different pathological induction, which further complicates the diagnosis criteria, thereby making COPD, one of the leading global diseases responsible for large scale mortality and morbidity (Liu *et al*, 2014). In fact, it is projected to be the 3rd leading cause of death by 2020 (Dickens *et al*, 2011).

India, one of the major developing countries is also not safe from the grasp of such deadly disease. According to Bhome, 2012 , India is also not very far from its western and Asian compatriots in terms of COPD mortality number. Death tolls are found to be rising every day and COPD has now very clearly left behind other diseases like TB and malaria (Bhome, 2012). According to the , Indian Study on Epidemiology of Asthma, Respiratory Symptoms and Chronic Bronchitis in Adults (INSEARCH), overall prevalence of COPD is found to be 3.49% among total population of 1,69,575 people (Vijayan *et al*, 2013; Jindal *et al*., 2012).

COPD is known for its progressive nature, where the onset of the disease takes place 45 years onwards (Carolan *et al*, 2014). Although, nowadays younger people are also experiencing COPD like symptoms but most cases are found among the aged people experiencing extreme exacerbation (Lock-Johansson *et al*., 2014). Many GWAS studies has been done for the identification of the responsible genetic loci but proper disease pathology has so far not clearly been outlined. Genetic locus mainly related to pulmonary pneumotic protein is found to be altered. CC16 and sftpd loci are amongst the most affected regions found to be associated with the disease from the GWAS study (Kim *et al*, 2012).

Various biomarker study has also been done in order to identify the disease pathology of COPD. Most of this studies has assessed the biomarker level by using ELISA techniques. Biomarkers like fibrinogen, RAGE, CC16, SFTPD are

also found to be significantly correlated with the disease (Carolan *et al*, 2014). *SFTPD* also known as Surfactant protein D is a pneumoprotein, that mainly acts as surfactant (Sims *et al*, 2008). In numerous study, SFTPD is been found to be correlated with COPD overall as well as with individual phenotypes (Shakoori *et al*, 2009; Shakoori *et al*, 2012, Ishi *et al*, 2012). So far different types of results are been obtained, which are clearly indicative of the heterogeneity of the sampling been involved. It could be speculated from these studies that, single protein can interact with different inducers (for different phenotypes) to give different outcome in a single disorder. For example, emphysematous model of mice is found to be sftpd negative, while in case of induced sputum and acute exacerbation, sftpd level is found to be elevated among the diseased persons (Sims *et al*, 2008;Shakoori *et al*, 2012) . Both emphysema, induced sputum and acute exacerbation are phenotypes of COPD but the level of sftpd is found to be altered positively in one case and negatively in others (Shakoori *et al*, 2012; Ishii *et al*, 2012).

In a case – control study among 81 participants, stable COPD patient, AECOPD and control subjects were assessed for their level of sftpd in their serum. The level of serum sftpd (ng/ml) among stable COPD patient, AECOPD and control subjects are found to be 127 ± 65, 151 ± 83 and 227 ± 120. From this result, it is very much evident that AECOPD patient has significantly ($p = 0.023$) higher sftpd protein present in their serum (Shakoori *et al*, 2012). Ju *et al*, 2012 also confirmed similar results among 171 Chinese participants divided into three groups : control (n=60), stable COPD (n= 71) and acute exacerbation COPD (n=40). Here also sftpd level is found to be significantly elevated in acute exacerbation group (Ju *et al*, 2012).

Apart from assessing the level of sftpd in disease phenotype, the serum and bronchoalveolar lavage fluid (BALF) sftpd level association has also been examined among smokers and non- smokers of different ages. In a cross – sectional study (n=60), BALF level is found in the order young smoker < elderly smoker < non-smoker, while opposite results are obtained from serum sftpd level (young smoker > elderly smoker > non-smoker) (Winkler *et al*.,2011). Ozyurek *et al*., 2013 has also came across similar study results, where serum sftpd level were found to be most among GOLD stage III and IV patient and least among contols,

while sputum sftpd level is found to be most in the control group (Ozyurek *et al*, 2013).

1.2 Pathology

a.) Inflammatory cells

Higher number of inflammatory cells are observed amongst COPD patient. For e.g. Neutrophil, macrophages, interlukin as well as other immune system cell come into play by mediating their action through secretory cytokines which further aids in the detoriation of pulmonary conditions and different aspects of airway exchange within lung (Kim *et al*., 2012).

b.) Anomaly in Protease/Antiprotease secretion

Protease are enzymes which degrades proteins while antiprotease functions to antagonize the action of protease. Both Protease and Anti-protease play a major role in maintaining proper cellular integrity thereby leading to proper homeostatic condition of the organs. Protease for e.g: MMP (Matrix metalloprotease) is known to cause degradation of ECM (extra-cellular matrix), which is known to be as cementing material for the cells. Therefore over secretion of such proteases is often found to be associated with COPD like conditions. COPD association with antiprotease has also been established. Antiprotease inhibits protease function thereby promoting enhanced cellular integrity condition. But due to its lower secretion amongst COPD patients, protease action upon lung alveolus did not get checked, as a result degeneration of tissue starts taking place (MacNee, 2016).

c.) Oxidative imbalance

Balance between oxidants and anti-oxidants play an important role in maintaining proper physiological activity. Abrupt rise in the amount of cellular oxidants is a sign of diseased condition (MacNee, 2016). COPD conditions are often experienced by increased oxidative stress. These increased oxidant level is mainly caused due to smoking and irregular diet habits. High oxidant level not only deregulates cellular function but also causes progressive aging. It is also one of the reason that COPD is mainly observed among patient with age > 45 years. Not only oxidative balance but pathological condition are also very much prominent at a greater age.

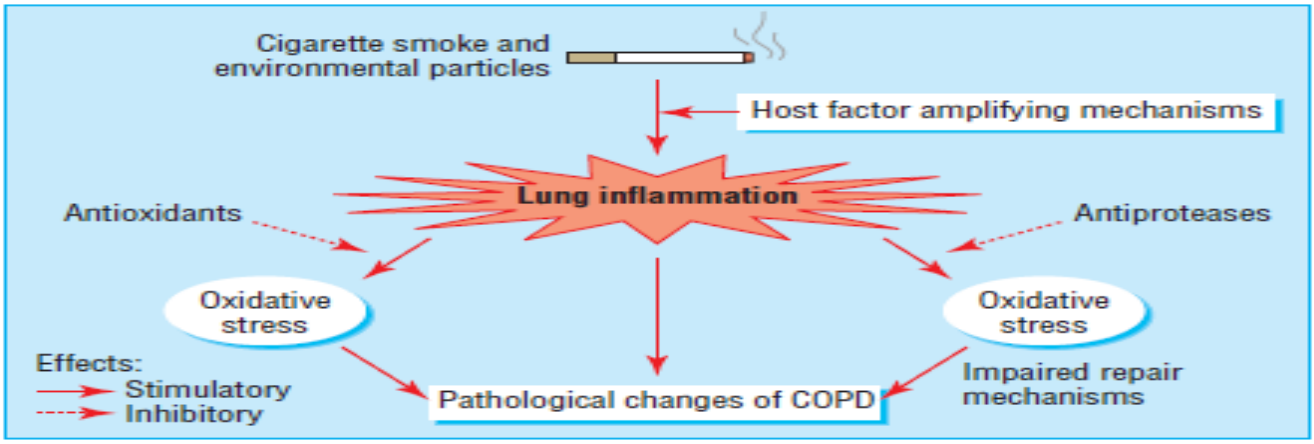

Fig 1a:- Different factor responsible for the disease condition (MacNee, 2016).

1.3 Types and sub-types

COPD can be divided into various sub-types, where each is been characterized by different as well as overlapping phenotypes. Each of these phenotypes again forms a sub – group within themselves, such that there is marker association and variation found from one type to another and hence it is expected to get treated better within sub-group (Lahousse *et al*, 2017).

- **Emphysema** – Emphysema is a COPD sub-type which is characterized by change in alveolar morphology beyond terminal bronchiole of the lungs leading to its collapse which ultimately creates hindrance in the process of respiration (Carolan *et al*, 2014). Emphysematous condition has also been found to get transformed into lung cancer cases thereby ultimately leading to increased mortality rate (Ishii *et al*, 2012). Although α-antitrypsin has been associated with Emphysematous condition but it is only responsible for 1% of the cases, therefore indicating that other numerous unknown existing factors are responsible for the pathogenesis and disease progression (Carolan *et al*, 2014).

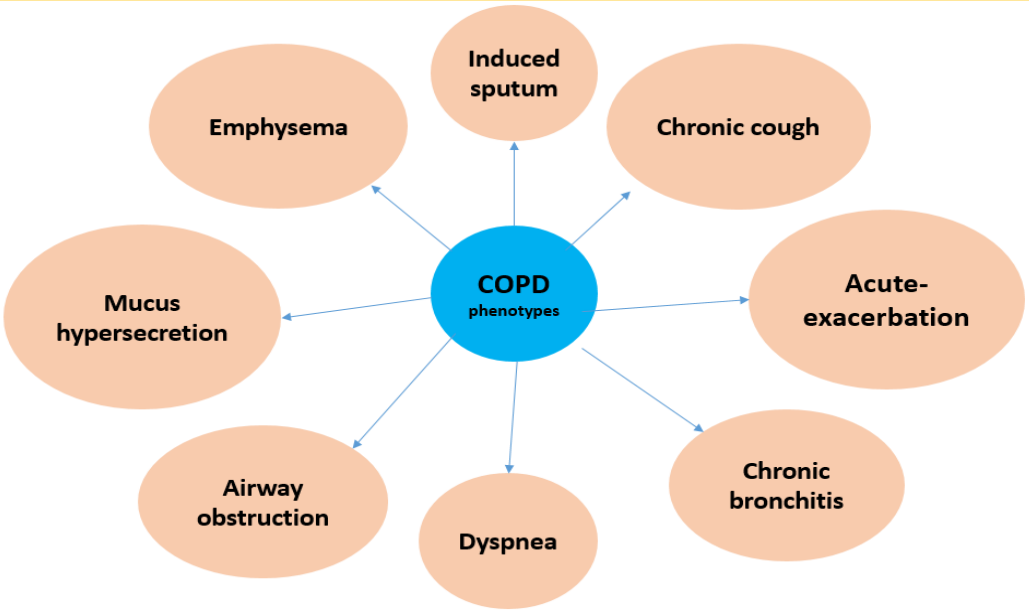

Fig 1b:- Different sub-phenotypes of COPD.

- **Chronic Bronchitis** – Chronic Bronchitis also known as CB accounts for another major proportion amongst COPD patients. This condition is mainly characterized by airway obstruction caused by mucus hyper-section by Goblet cells (Kim and Criner, 2013). So far, the CB has been correlated with decline in Lung – function as well as with acute exacerbation and mortality (Lahousse *et al*, 2017). According to a cohort study, done amongst 972 participants (CB^+ = 172, CB^-=752), CB^+ patients are found to have more severe pulmonary status and are more prone to acute – exacerbation (OR 4.0, 95% CI 2.7–5.9; p<0.001) (Lahousse *et al*, 2017).
- **Acute exacerbation** – Also known by the name of AECOPD, acute exacerbation is found to be rising widely now a days. This phenotype is characterized by sudden detoriation in the respiratory function of an individual with increased vulnerability starting from airway obstruction, frequent coughing to changed phlem colour (Cai *et al*, 2014). It has been estimated that nearly every COPD patients has an exacerbation phase from 0.5 to 3.5 times (Cai *et al*, 2014).
- **Chronic cough** – Chronic cough is something which can be seen widely for numerous disorders. COPD patients frequently suffers from this condition (Smith and Woodcock, 2006). So far cigarette smoking has been mainly associated with cough like conditions (Smith and Woodcock, 2006). Cough along with phlegm together is found to be associated with mortality rate (HR 1.27, 95% CI 1.02–1.59) (Putcha *et al*., 2014).

- **Induced sputum** – Different sputum level is found to be associated with different types of COPD phenotypes. Numerous studies has been done correlating this phenotype with major COPD condition and therefore can be easily considered as an important biomarker. The association of Surfactant protein D with sputum level is always considered as an important marker for the disease diagnosis where high sputum level is found in exacerbation condition and lower sputum level is associated with emphysema like condition (Ozyurek *et al*, 2013; Winkler *et al*., 2011).
- **Mucus hypersecretion** – Mucus is normal part of our immune system that helps in the elimination of pathogen but when its production recedes in a greater way than its production, it starts attracting infectious material thus leading to COPD like condition (Allinson *et al*, 2015).
- **Airway obstruction** – Blockage in the respiratory pathway is often experienced by COPD patients. Although this phenotype is overlapping with the symptoms for other respiratory disorder but within COPD itself it is a huge domain and stands solely as a subtype.
- **Dyspnea**- Dyspnea is the term given to acute phases of breathlessness. COPD patients often experiences period of dyspnea. Such condition mainly arise due to “respiratory hunger” in the lungs which causes an urge for breathing more air, ie, it’s a neurophysiological. So far numerous breathing exercises are been considered as therapy for dyspnea.

1.4 Biomarkers

Biomarkers can be defined as a biomolecule which can differ in their expression among cases and controls, such that the marker can be associated with the disease pattern. Proper biomarker expression demarcation is widely important as they play an important role in the diagnosis procedure. Proper diagnosis is an important part of disease management. As far as COPD is concerned, various studies has reported the association of various biomarkers but due to heterogeneity of the disease, their associations are found to be contrasting for different sub-phenotypes (Sims *et al*, 2008; Zemans *et al*., 2017).

Biomarkers can be differentiated into three types (Agusti *et al*., 2016):-

1. **Cellular biomarkers-** Various blood cells are so far used as a part of cellular biomarkers. Blood cells like neutrophils and eosinophils are found to be widely associated with COPD conditions (Agusti *et al*., 2016). Earlier studies has shown that such cellular acquaintances are quite common for other inflammatory disorder thus rejecting the chances of neutrophils and eosinophils as an effective biomarker for COPD phenotypes.
2. **Protein biomarkers –** So far protein biomarkers are one of the best method for disease diagnosis purpose. Diagnosis can be done widely from serum or plasma samples by means of procedure like ELISA. Serum protein level plays an important role when its value is altered in diseased condition. So far, GWAS study done among COPD cohorts have found various serum protein biomarkers association with the disease. A study done by Carolan et. al, 2014, reports the use of 114 plasma biomarkers for emphysema sub-phenotype of COPD. Most widely used protein biomarkers for COPD are SFTPD, CRP, fibrinogen, IL-6 and CCL18.
3. **Genetic biomarkers –** Genetic biomarkers are stretches of genomic regions which varies (SNPs, deletion, insertion) between control and cases. Numerous Genome Wide SNPs are been found to be associated with the disease (Fakih *et al*, 2017, Shakoori *et al*, 2012, Ishii *et al* , 2012). Ishii *et al*., 2012 reported the association of SNP rs721917 along with COPD sub-phenotype emphysema. This SNP is also reported by other studies where its correlation has been found with overall COPD phenotype (Shakoori *et al*, 2012). Apart from this various other SNPs are also found and reported in multiple studies.

1.5 SFTPD

SFTPD also known as Surfactant Protein D belongs to hydrophilic Surfactant protein family in lung (Moreno et al., 2014). It is a mixture of lipo-protein that weighs around 28-35 KDa. It is produced by alveolar type II cells. It forms a monolayer around cell to provide innate immunity by not allowing pathogens to enter the respiratory system while its secondary role is to prevent lung alveoli from collapsing (Moreno et al., 2014). So far many studies has indicated the

role of surfactant protein D in COPD. Altered amount of SFTPD has been seldom observed and reported by multiple studies (Zemans *et al*., 2017, Carolan *et al*.,2014). But the amount of SFTPD variation has produced contrasting result in contrasting phenotypes as well as in different populations (Shakoori *et al*., 2009, Ishii *et al*., 2012). Like in emphysematous condition sftpd concentration is found to be significantly lower than normal participants. But in case of acute exacerbation or induced sputum phenotype sftpd concentration is found to be significantly more than normal healthy controls (Shakoori *et al*, 2012). Thus these variations in sftpd concentration must be studied properly in order to identify the proper disease induction mechanism in different phenotypes.

2.1 Introduction

COPD or Chronic Obstructive Pulmonary Disorder is a disorder which exhibits irreversible airflow condition that leads to improper respiratory functioning (Carolan *et al*, 2014). It is a global phenomenon which accounts for 3 million deaths annually (Zemans *et al*, 2017). It is responsible for the increase in worldwide mortality and morbidity (Dickens *et al*, 2011). COPD is projected to be the third leading cause of death by 2020 (Dickens *et al*, 2011). It has multiple sub-phenotypic conditions like emphysema, lean body mass, mucus hypersecretion and acute exacerbation (Carolan *et al*, 2014; Dickens *et al*, 2011; Shakoori *et al*, 2012). Each sub- phenotype is considered to be the outcome of different pathways (Ishii *et al*, 2012). Smoking ceasation and Oxygen therapy are the mainly used strategies undertaken for disease management although there are fewer drugs that currently available as a therapeutic option (Zemans *et al*, 2017). The main problem of this disease lie in its heterogeneity of different sub-types and their mode of disease progression (Zemans *et al*, 2017).

As different sub-phenotypes are coming into picture, focus is now moving towards the identification of biomarkers which can ease the disease management by means of proper diagnosis. So far the most important biomarker found to be associated with Emphysema is α- antitrypsin (AAT) (Stoller *et al*, 2012). But still it accounts for only 1% of the COPD cases (Stoller *et al*, 2012). Therefore it sheds light about the current scenario of absence of any particular biomarker for COPD diagnosis.

So far multiple Genome Wide Association Studies were done for the identification probable biomarker (both serum level and genetic) (Kim *et al*., 2012; Zemans *et al*, 2017). With numerous biomarker coming into picture, it might be cumbersome to understand which one is effective in which cases. As discussed before, COPD is a highly heterogenic disorder, therefore it become essential to have a clear idea regarding disease pathology along with the disease progression. So, far the serum protein biomarkers found to be associated with COPD and its various sub-phenotypes are RAGE, ICAM, CCM20, CDH1, CDH 13, SERPINA7, Fibrinogen, CRP, SFTPD, CC16, IL-6, IL-8, TNF-α, MMP-9 (Carolan *et al*, 2014, Zemans *et al*, 2017; Kim *et al*., 2012; Dickens *et al*, 2011).

2.2 Surfactant Protein D (SFTPD) as an inflammatory biomarker

Surfactant protein D is a glycoprotein secreted by Type II alveolar cell and non-ciliated Clara cell (Shakoori *et al*, 2012; AKIKI *et al*, 2016). It is a multimeric protein that takes part in immune regulation and lung maintainance (Ju *et al*, 2012). This glycoprotein belongs to Lectin super family (Moreno *et al*, 2014). SFTPD protein is found to have three domains namely: Collagen like domain, neck domain and carbohydrate domain (Moreno *et al*, 2014). The Carbohydrate binding domain is responsible for the maintenance of innate immune function within the Lung. It's a Calcium dependent protein, which upon binding calcium can able to cross talk with defensin and other immunoregulatory molecule (Moreno *et al*, 2014, Jäkel *et al*, 2013; Crouch and Wright 2001). SFTPD can be easily isolated from serum and endovascular cells. Main advantage of SFTPD over other marker is its stability. It stable over six months (Holmskov *et al*, 2013; Hoegh *et al*., 2010).

Serum SFTPD is often found to have correlation with different Lung Function parameters (Liu *et al*, 2014). Besides COPD, SFTPD is found to be associated with multiple pulmonary and other multifactorial diseases comprising Lung Cancer, Interstitial pneumonia, asthma, viral infection and other acute respiratory syndrome (Ishi *et al*., 2012, Carolan *et al*., 2014; Zemans *et al* 2017). Changes in the level of serum protein during disease condition is an indicator for the built up of disease condition. Hence serum SFTPD level can be useful in the diagnosis of various pulmonary conditions.

Emphysema – a COPD sub-phenotype is often characterized by the destruction of alveolar wall in the distal end of the terminal bronchiole (Carolan *et al*., 2014). SFTPD negative mice were found to develop emphysema (Wert *et al*., 2000; Ishii *et al*., 2012). In SFTPD negative mice, MMP (matrix-metaloproteases) are found to have wider impact in the destruction of the surfactant protein (Wert *et al*., 2000). In human, emphysema patients are also found to have lower level of serum SFTPD. Ishii *et al*, conducted a case-control study amongst Japanese emphysema (n=160), COPD (n=188) and control subjects (n=1053) (Ishii *et al*., 2012). Serum SFTPD were found to be lowest amongst the cases (74.6±58.0 ng/ml) (Ishii *et al*., 2012).

Most of the COPD people belongs to SCOPD category (Stable COPD) followed by AECOPD (Acute exacerbation). Acute exacerbation is a COPD condition that is experienced by sudden worsening of respiratory condition including secretion of greenish phlegm (Shakoori *et al*, 2009). It has been reported that the serum level SFTPD is found to be more elevated amongst AECOPD (227 ± 120ng/ml) patient followed by SCOPD (151 ± 83ng/ml) (Shakoori *et al*, 2009). Control group (127 ± 65ng/ml) in this case is found to have least amount serum SFTPD (Shakoori *et al*, 2009). In another case-control study, done among Chinese SCOPD (n=71), AECOPD (n=40) and control (n=60) subjects also found to have similar data (ju *et al*., 2012). The serum SFTPD level is found to be significantly ($p<0.001$) highest among AECOPD (235.22 ± 48.27 ng/ml)> SCOPD (153.54 ± 45.21ng/ml)> Control subjects (103.05 ± 24.97ng/ml).

SFTPD has well also established itself as a good genetic biomarker amongst different population (Shakoori *et al*., 2012; Fakih *et al*., 2017). SNP variants in rs721917 is found to be responsible for the production of different level of SFTPD among different condition (Foreman *et al*., 2011). Upon genotyping it was confirmed that, that a C>T change at 11^{th} location of the SFTPD is responsible for differential serum SFTPD output. C allele at 11^{th} position codes for methionine while T allele at the same position codes for threonine (Shakoori *et al*., 2012). C/C genotype is found to be associated with least amount of serum SFTPD while T/T genotype is found to be associated with highest amount of serum SFTPD (Fakih *et al*., 2017). Heterozygous C/T genotype while found to be associated with medium level of serum SFTPD. In another study done by Shakoori *et al*, similar results are obtained. Genotype T/T (175.95 ± 86.00 ng/ml) is found to have significantly ($p<0.001$) highest amount of serum SFTPD followed by genotype C/T (127.24± 81.31 ng/ml) and C/C (112.12 ± 43.23 ng/ml).

2.3 Objective

The main aim of these chapter is to review systematically all the literatures related to association of serum SFTPD with COPD as well as its sub-phenotypes and to meta – analyse the various anthropometric variables (male, female, current and ex-smoker), serum SFTPD biomarker level and genetic biomarker (rs721917) from SFTPD gene in order to increase the study power and to evaluate the significance of SFTPD as diagnostic biomarker.

2.4 Materials and Method

i. Identification and eligibility of relevant studies

Search for eligible papers were made till 12th Feb, 2018. Databases used for the retrieval of eligible papers are PubMed (along with MESH database) and Google Scholar. Searches were made using the keywords “COPD” AND “Surfactant protein D” OR “Emphysema” AND “Surfactant protein D” OR “Acute exacerbation” AND “Surfactant protein D”. Apart from these entire phrases like “COPD association with surfactant protein D”, “Emphysema association with surfactant protein D” and “Acute exacerbation association with surfactant protein D”, “SFTPD polymorphisms in COPD”, “SFTPD polymorphisms in Emphysema” and “SFTPD polymorphisms in Acute Exacerbation” were also used for searching. Papers with the desirable keywords were selected. Further paper were added from the cross-references of retrieved articles.

ii. Study inclusion/exclusion criteria

Study design for inclusion/exclusion criteria were made in multiple stages. In primary stage, scanning of articles with relevant titles and abstracts, only case-control, population based studies are included encompassing various COPD sub-phenotypes. In the next stage for detailed evaluation, only studies encompassing the criterias like age group>40 years and smokers control are included. For, Serum level protein biomarker, studies were selected comprising both male and female as well as only male participants. While for genetic polymorphisms, studies having information about rs721917 (Met11Thr; C<T) are selected. For genetic polymorphisms, study on emphysema patients are excluded. Since among emphysema patients, C allele at 11th codon is found to be risk allele while in other

COPD sub-phenotypes, T allele is found to be associated (Ishii *et al*., 2012; Shakoori *et al*., 2009). As the number of papers are more from overall COPD and acute exacerbation cases (risk allele is T at codon 11), so they are selected for meta analysis. Final analysis includes the rejection of papers obtained due to duplication, review, methodological analysis and other respiratory diseases with overlapping symptoms.

iii. Data extraction

Following data were extracted from the finally selected papers on the basis of three parameters:-

a.) Anthropometric parameters

It includes study number, author name, participants number, sex (M/F) and smoking status (ex/current smoker).

b.) Protein biomarker

It includes study number, author name, participants number, SFTPD serum/plasma level mean value (cases and control).

c.) Genetic biomarker (rs721917)

It includes study number, author name, participants number, genotypic frequency, allelic frequency.

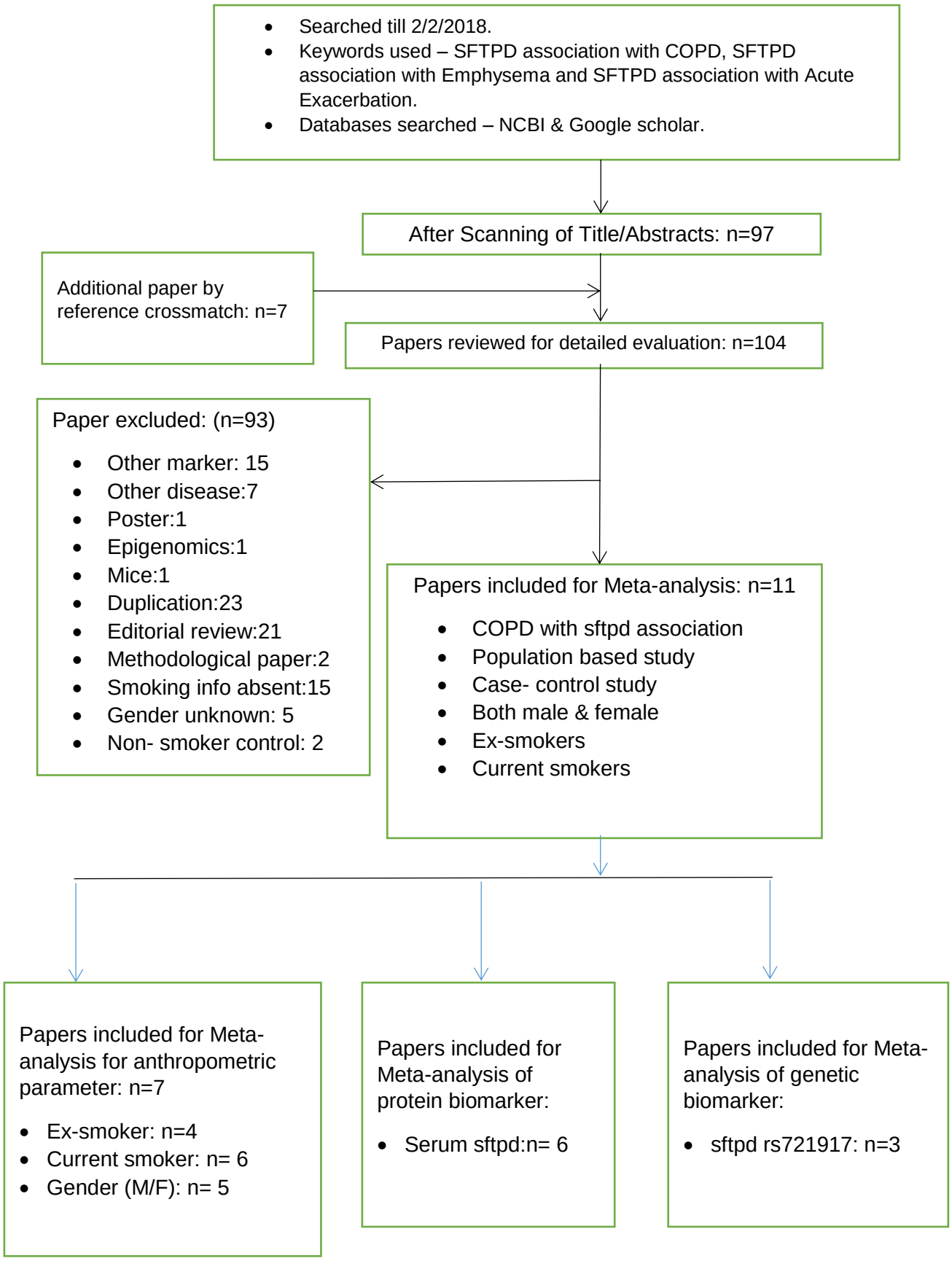

Fig 2a: Flow- diagram for the article procedure chosen for systemic review. (Adapted from the PRISMA flow diagram for meta-analysis)

2.5 Statistical Analysis

For statistical analysis, Revman (version 5.3.0, The Cochrane Collaboration) software is used. For anthropometric data different types of statistical tests are done on the basis of whether the data variables are dichotomous or continuous. Data variables for the parameters like Smokers (current/ ex-smokers) and sex (Male/Female) are dichotomous in nature, therefore, odds ratio were calculated for the assessment of result. As serum biomarker level and genetic biomarker are continuous and discrete variables respectively, therefore mean difference and odds ratio for pooled data are calculated respectively.

For Genetic biomarker study, first Hardy-Weinberg Equilibrium (HWE) was calculated for the control groups of all the studies for comparing observed and expected Genotypic Frequency. Evaluation of the individual models were first done in random model, where upon obtaining significant result analysis were done in fixed model. Genetic models used for analysis are Allelic model, Dominant model, Recessive model and Additive model. Finally Chi^2 test for heterogeneity and i^2 test were used for the analysis of pooled data. While, Z-test are done for the assessment of overall effect.

2.6. Result

Study characteristics are present in the table 2a, 2b, 2c and 2d for individual parameters. For the association of gender parameter along with COPD patients, five studies met the inclusion criteria. These studies indicated significant association of the disease with male sub-group ($p<0.0001$ & $i^2 = 0\%$), where the $OR_{pooled} = 1.61$ (in the confidence interval of 95%) indicated the pre-disposition of the disease among males, while no significant association were observed among females.

On the basis of smoking status, four and six studies respectively were selected for ex-smoker and current - smoker association with COPD. No significant association were found among current smoker. Ex- smokers were found to be significantly associated COPD condition ($p<0.00001$, $i^2=18\%$, $OR_{pooled} = 7.87$).

Serum level SFTPD value were found to be significantly ($p<0.00001$; $i^2= 11\%$; Mean Difference=39.26) associated with COPD condition. Six studies m*et al*l the inclusion criteria.

For the association of SFTPD polymorphisms (rs721917; Met11Thr; C<T) with overall COPD as well as Acute exacerbation sub-phenotype, three and two studies were included respectively. All of these three studies are done in Asian population (Pakistan, Lebanon and China). Cases in Chinese and Lebanese population were diagnosed both according to American Thoracic Society and GOLD Criteria, while the Pakistani population were diagnosed solely on the basis of GOLD criteria.

The association of COPD in various genetic model is given in table 2g. Only those model with significant p-value (<0.05) and heterogeneity (<25%) are being selected. In overall COPD, significant association were obtained in allelic model (T vs C) of sftpd with COPD (O.R=1.34, 95% CI= 1.07-1.67). Statistically similar results were obtained for recessive model, CC vs CT/TT (OR = 1.88; 95% CI= 1.01 - 2.23) and in homozygote comparison (TT vs CC) under Additive model (OR=1.88; 95% CI= 1.15-3.09). No such significant association of sftpd polymorphisms with overall COPD is found in Dominant model TT vs CT/CC (OR=1.41; 95% CI= 1.00 - 1.99) & in heterozygote comparison under additive model, TT vs CT (OR=1.30; 95% CI= 0.9-1.86) and CT Vs CC (OR= 1.51; 95% CI =0.96 – 2.38).

Significant associations were also observed when stratification for Acute-exacerbation were done. SFTPD polymorphisms were found to be significantly associated with Acute exacerbation phenotype in Allelic model, T vs C (OR= 1.44; 95% CI=1.09 – 1.83), Dominant model TT vs CT/CC (OR=1.50; 95% CI= 1.01 – 2.23) and in homozygote comparison under Additive model TT vs CC (OR=2.10; 95% CI= 1.18-3.75). No significant association for sub- phenotype Acute-exacerbation with SFTPD polymorphisms were observed in Recessive Model, CC vs CT/TT (OR= 0.55; 95% CI= 0.90 – 2.06) & CT vs CC (OR=1.63, 95% CI= 0.95-2.78).

Sr no.	Author name	Subjects (M/F)	Characteristics of COPD patients
1.	Lomas *et al*., 2009	2385 (1383/1002)	Age (40-75 yrs), Smokers (>10 pack years, GOLD (stage II, III, IV)
2.	Ishii *et al*., 2012	270 (243/27)	Age (>60 yrs), Smokers, coughing, expectoration, and/or dyspnea
3.	Ozyurek *et al*., 2013	60 (49/11)	Age (>40 yrs), Smokers (>10 pack years), GOLD (stage I, II, III, IV)
4.	Wei Liu *et al*., 2014	91(83/8)	Age (>60 yrs), Smokers
5.	Alabdeen *et al*., 2016	84 (68/16)	Age (40-75 yrs), Smokers (ex/current/non), GOLD criteria, Stable and Acute exacerbation

Table 2a:- Study characteristics for the anthropometric parameter (Gender)

Sr no.	Author name	Subjects (Ex-smoker)	Characteristics of COPD patients
1.	Shakoori *et al*., 2009	81(15)	Stable and Acute exacerbation,
2.	Ozyurek *et al*., 2013	60 (32)	Age (>40 yrs), Smokers (>10 pack years), GOLD (stage I, II, III, IV)
3.	Alabdeen *et al*., 2016	84 (20)	Age (40-75 yrs), Smokers (ex/current/non), GOLD criteria, Stable and Acute exacerbation
4.	Fakih *et al*., 2017	313 (34)	Smokers (Non/current/ex), GOLD criteria

Table 2b:- Study characteristics for the anthropometric parameter (Ex-smoker)

Sr no.	Author name	Subjects	Characteristics of COPD patients
1.	Shakoori *et al*., 2009	81	Stable and Acute exacerbation
2.	Lomas *et al*., 2009	2385	Age (40-75 yrs), Smokers (>10 pack years, GOLD (stage II, III, IV)
3.	Ju *et al*., 2012	171	Stable and Acute exacerbation, GOLD criteria
4.	El Deek *et al*., 2013	90	GOLD criteria, Smoker (non/current)
5.	Ozyurek *et al*., 2013	60	Age (>40 yrs), Smokers (>10 pack years), GOLD (stage I, II, III, IV)
6.	Shakoori *et al*., 2012	221	Lung function test by GOLD criteria, smokers (non/current/ex)

Table 2c:- Study characteristics for the protein biomarker (Serum sftpd level)

Sr no.	Author	Country	Ethnicity	Subjects	COPD Characteristics	HWE
1.	Shakoori *et al*., 2012	Pakistan	Asian	165	AECOPD, SCOPD patients diagnosed according to GOLD criteria	1.271
2.	C-Y-ou *et al*., 2015	China	Asian	320	AECOPD, SCOPD patients diagnosed according to GOLD criteria and American Thoracic Society.	0.031
3.	Fakih *et al*., 2017	Lebanon	Asian	177	Patients diagnosed according to GOLD criteria and American Thoracic Society	1.459

Table 2d:- Study characteristics for the Genetic biomarker (rs721917; Met11Thr).

Sr no.	Anthropometric parameter	Study no.	Cases/ Control	OR (95% CI)	P	I^2	$P_{heterogeneity}$	Effect model
1.	Gender (male)	5	2189/452	1.61 [1.29, 2.02]	<0.0001	0	0.44	Fixed
2.	Gender (female)	5	2189/452	0.62 [0.49, 0.78]	<0.0001	0	0.44	Fixed
3.	Smoking status (Ex-smoker)	4	151/325	7.87 [4.17, 14.83]	<0.00001	18	0.30	Fixed
4.	Smoking status (Current-smoker)	6	526/787	1.86 [0.97, 1.74]	0.08	12	0.34	Fixed

Table 2e:Result for anthropometric parameter

Sr.no.	**Srudy no.**	**Stratification**	**Cases/Control**	**MD (95% CI)**	**P**	**I^2**	**$P_{heterogeneity}$**	**Effect model**
1a.	6	Overall COPD	2109/464	39.26 [36.97, 41.54]	<0.0001	11	0.34	Fixed
1b.	2	Acute exacerbation	53/114	130.41[114.62, 46.20]	<0.00001	0	0.36	Fixed

Sr. no.	Genetic model	Stratification	Study numbers	Cases/Control	OR (95 % CI)	P	I^2 (%)	$P_{heterogeneity}$	Effect model
1.	Allelic model (T vs C)	Total	3	668/656	1.34 [1.07,1.67]	0.01	0	0.73	Fixed
		AE	2	544/426	1.41 [1.09, 1.83]	0.009	0	0.98	Fixed
2.	Dominant model (TT vs CT/CC)	Total	3	334/328	1.41 [1.00, 1.99]	0.05	0	0.72	Fixed
		AE	2	272/213	1.50 [1.01, 2.23]	0.04	0	0.58	Fixed
3.	Recessive model (CC vs CT/TT)	Total	3	334/328	0.60 [0.39, 0.94]	0.02	22	0.28	Fixed
		AE	2	272/213	0.55 [0.33, 0.92]	0.02	54	0.14	Fixed
4a.	Additive model Homozygote comparison (TT vs CC)	Total	3	162/150	1.88 [1.15, 3.09]	0.01	0	0.49	Fixed
		AE	2	135/99	2.10 [1.18, 3.75]	0.01	0	0.34	Fixed
4b.	Additive model Heterozygote comparison (TT vs CT)	Total	3	293/264	1.30 [0.90, 1.86]	0.16	0	0.62	Fixed
		AE	2	241/172	1.36 [0.90, 2.06]	0.14	0	0.14	Fixed
4c.	Additive model Heterozygote comparison (CT vs CC)	Total	3	213/140	1.51 [0.96, 2.38]	0.08	37	0.20	Fixed
		AE	2	168/153	1.63 [0.95, 2.78]	0.08	66	0.08	Fixed

Table 2g.) Result for the genetic model (SFTPD gene) association with COPD

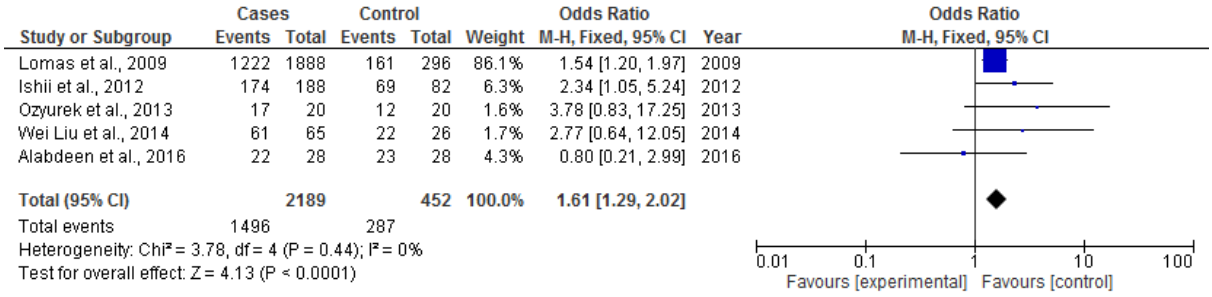

Fig 2b:- Meta- analysis of Anthropometric parameter (Male) with COPD.

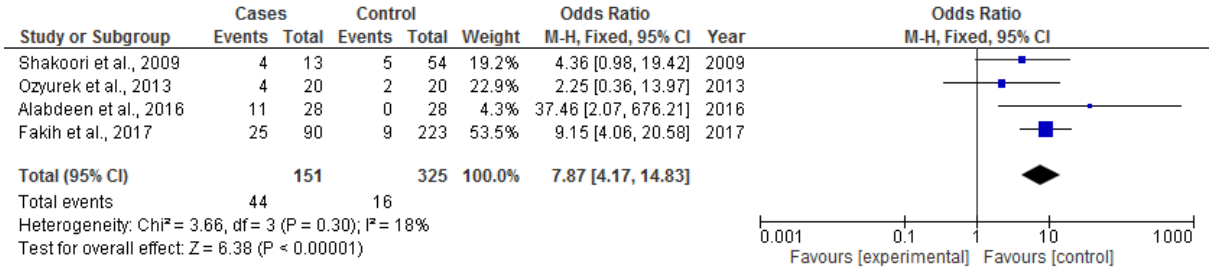

Fig 2c:- Meta- analysis of Anthropometric parameter (Ex-smoker) with COPD

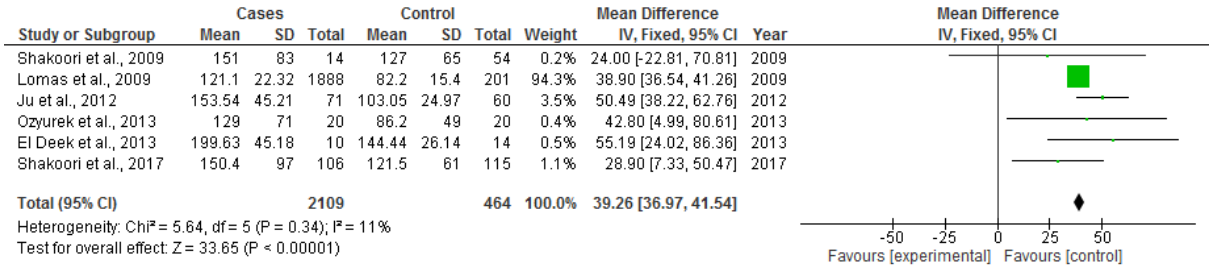

Fig 2d:- Meta- analysis of serum protein biomarker (SFTPD level) with COPD

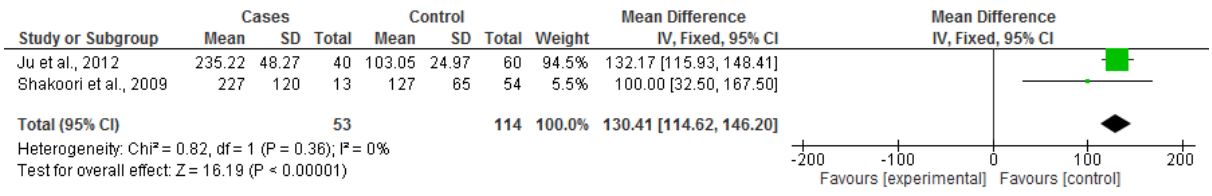

Fig 2e:- Meta- analysis of serum protein biomarker (SFTPD level) with COPD (sub- phenotype Acute exacerbation)

Genetic biomarker (Overall COPD)

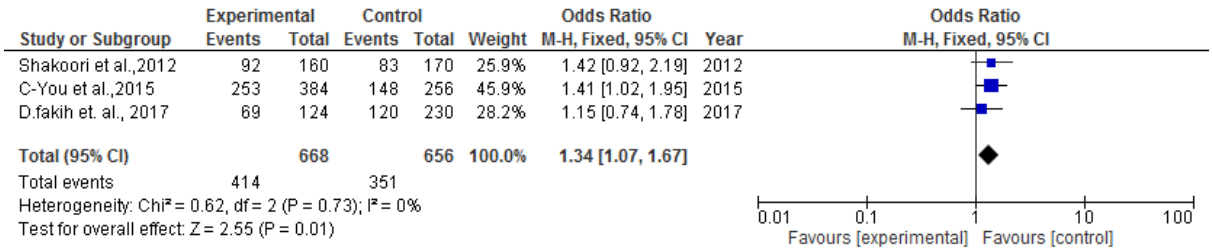

Fig 2f:- Meta-analysis with a fixed-effect model (Allelic model) for the ORs of COPD risk associated with SFTPD polymorphism rs721917 (T vs C).

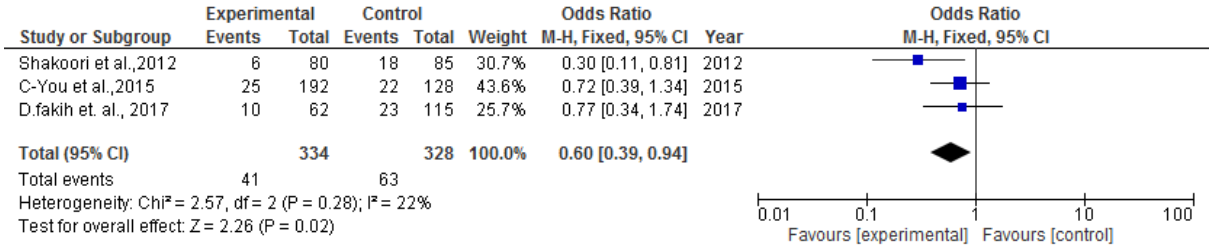

Fig 2g:- Meta-analysis with a fixed-effect model (Recessive model) for the ORs of COPD risk associated with SFTPD polymorphism rs721917 (CC vs CT/TT).

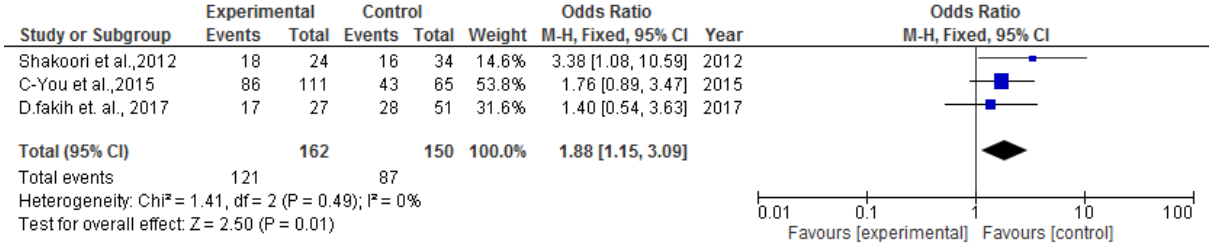

Fig 2h:- Meta-analysis with a fixed-effect model (Additive model; homozygote comparision) for the ORs of COPD risk associated with SFTPD polymorphism rs721917 (TT vs CC).

Genetic biomarker (sub- phenotype Acute- Exacerbation)

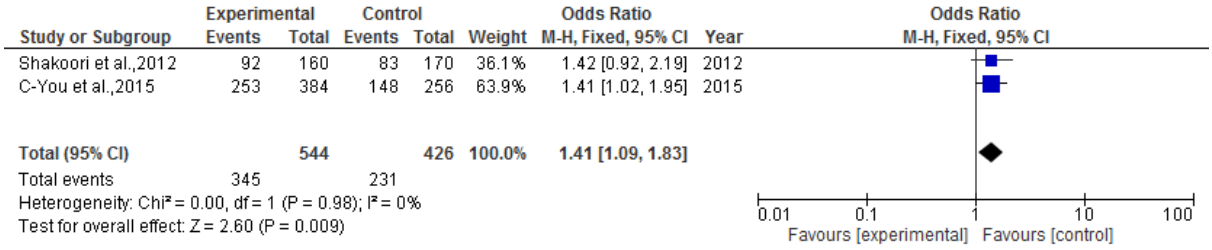

Fig 2i:- Meta-analysis with a fixed-effect model (Allelic model) for the ORs of sub phenotype Acute-exacerbation associated with SFTPD polymorphism rs721917 (T vs C).

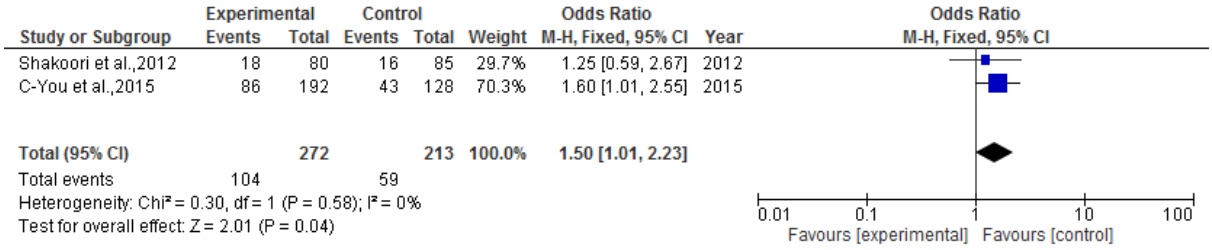

Fig 2j:- Meta-analysis with a fixed-effect model (Dominant model) for the ORs of sub phenotype Acute-exacerbation associated with SFTPD polymorphism rs721917 (TT vs CT/CC).

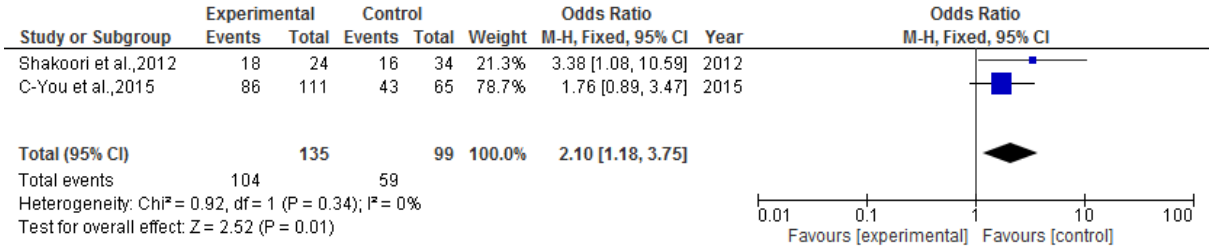

Fig 2k:- Meta-analysis with a fixed-effect model (Additive model: Homozygote comparision) for the ORs of sub phenotype Acute- exacerbation associated with SFTPD polymorphism rs721917 (TT vs CC).

2.7. Publication bias

For checking publication bias, Funnel plot tests were done for individual parameters. No biasness were observed for any of the anthropometric parameters male and ex-smoker. While asymmetric funnel plot were obtained for serum biomarker (SFTPD) in COPD as well as sub – phenotype Acute exacerbation, thus depicting presence of biasness.

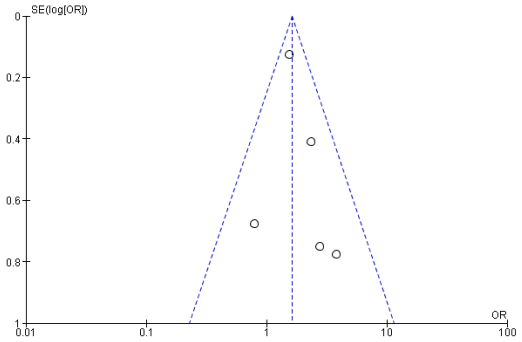

Fig:- 2l

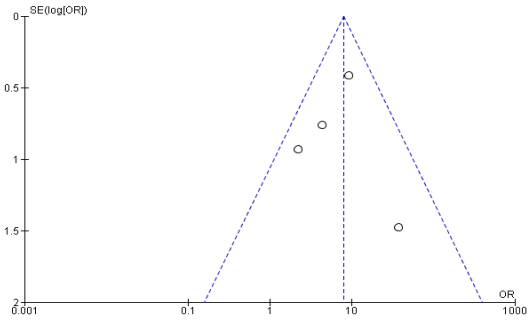

Fig:- 2m

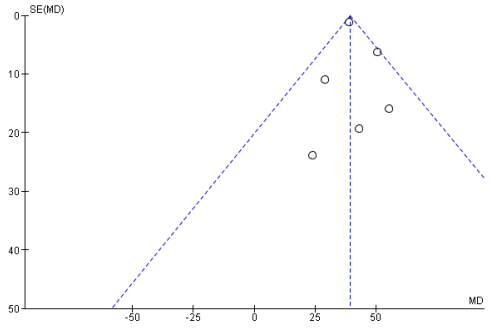

Fig:- 2n

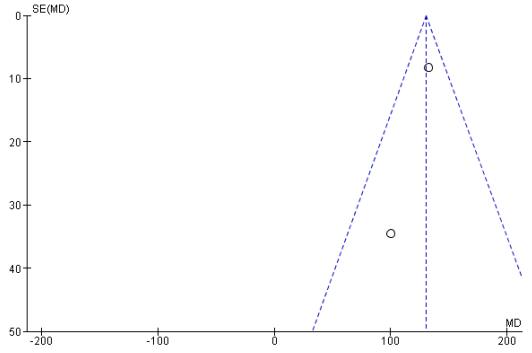

Fig:- 2o

Fig 2: Funnel plot (Publication bias test) for l.) Anthropometric parameter (Male) association with COPD m.) Anthropometric parameter (Ex-smoker) association with COPD n.) Serum SFTPD association with COPD and o.) Serum SFTPD association with Acute exacerbation

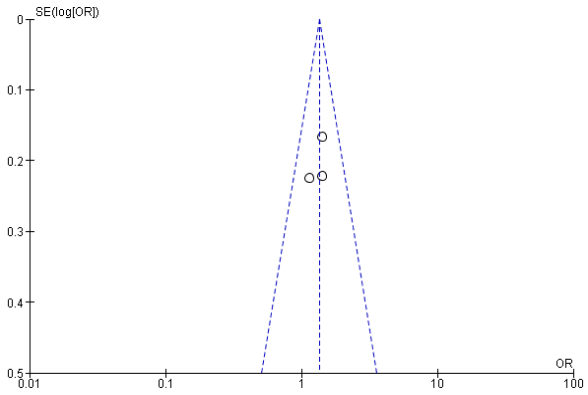

Fig:- 2p

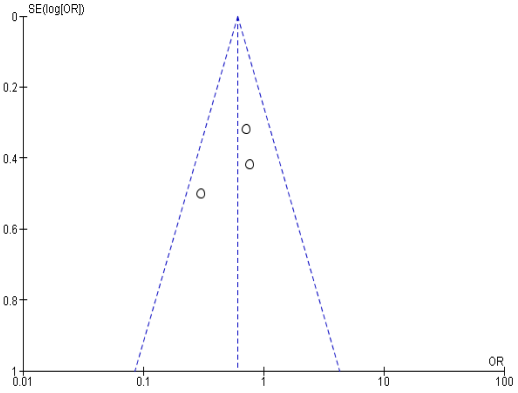

Fig:- 2q

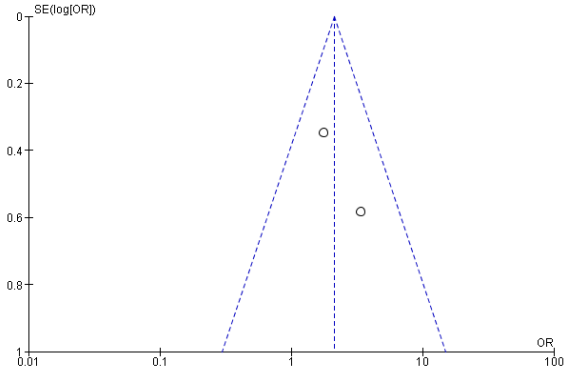

Fig:- 2r

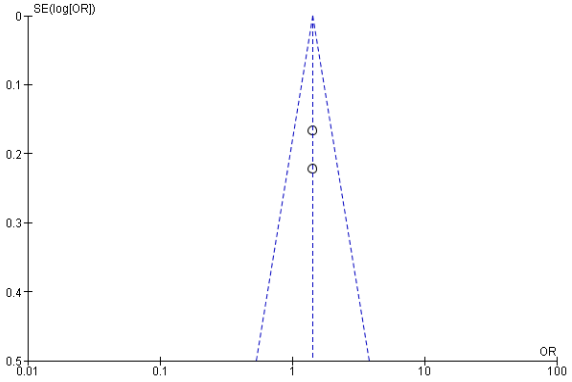

Fig:- 2s

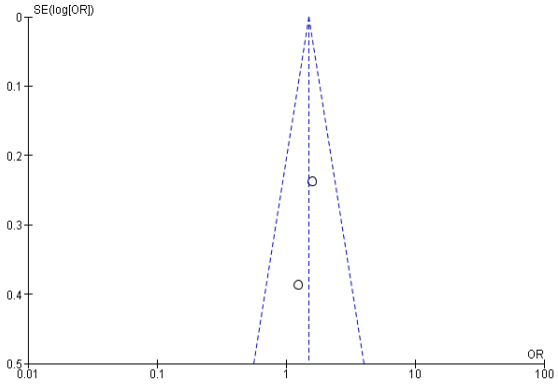

Fig:- 2t

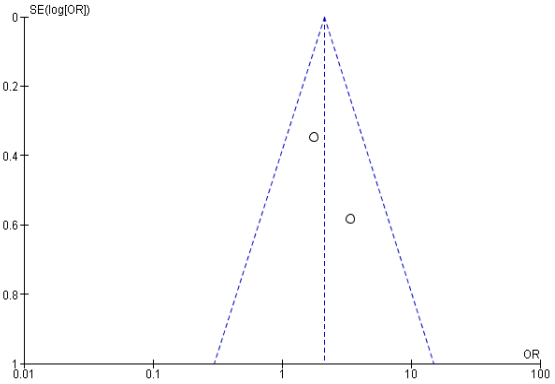

Fig:- 2u

Fig 2: Funnel plot (Publication bias test) for p.) SFTPD Allelic model (T vs C) association with overall COPD q.) SFTPD Recessive model (CC vs CT/TT) association with overall COPD r.) SFTPD Additive model, homozygote comparison (TT vs CC) association with overall COPD and s.) SFTPD Allelic model (T vs C) association with Acute exacerbation t.) SFTPD Dominant model (TT vs CT/CC) association with Acute exacerbation u.) SFTPD Additive model, homozygote comparison (TT vs CC) association with Acute exacerbation

2.7. Discussion

As mentioned in the beginning, SFTPD gene is associated with COPD as well as its sub-phenotype by numerous GWAS study. But so far meta- analysis study indicating the relation of allelic component with the disease severity has not been documented. However this study, tries to fill this gap. Role of anthropometric parameters like gender and smoking status were already been documented by multiple studies. Our findings are also consistent with the datas for anthropometric parameter. Males and ex-smokers are found to be more prone to COPD condition than females and current smokers. It might be due to the fact that, males are more exposed to smokes and other environmental hazards. Smoking is already found to be a potent cause of diseases like COPD and Lung carcinoma. Thus, it could be easily concluded from the result that smoking condition leads to predisposition of COPD. As far the current smoker is related, although they are currently not suffering from COPD but they tend to acquire it with progressing time and age.

Serum protein SFTPD is already found to be an important biomarker for COPD conditions. The significant difference in SFTPD concentration among the cases and control can be used as criteria for disease diagnosis. So far, except for emphysema no biomarkers are available in the market for the diagnosis of COPD. By means of serum SFTPD biomarker one can easily assess the severity of the disease or any particular phenotype like emphysema. Therefore, the increasing order for serum SFTPD concentration is Emphysema< Healthy individual< Stable COPD< Acute- Exacerbation COPD.

SFTPD is also an important genetic biomarker. So far multiple studies has documented the role of rs721917 (Met11Thr; C927) in COPD conditions. Allele C is found to be associated with low serum SFTPD and is mainly found among the emphysematous people. While T allele is found to be associated with more serum SFTPD. So it is found to be associated with Stable COPD and most importantly with Acute- Exacerbation COPD. Although for genetic biomarker we have concluded this result, but verification is also required as only 3 studies with lesser participant number is included.

3.1 Objective

The objective of this chapter is to analyse differential SFTPD gene expression level among cases and control.

3.2 Materials and Method

Study Population

A total of 13 participants are recruited for the Case-Control analysis of SFTPD gene expression (Cases=3; Control=10). All the participants are above 40years of age, includes both male and female, non-smoker, non-farmer, North Indian population. Cases are diagnosed according to GOLD criteria while control samples are recruited from healthy donor.

RNA Isolation

RNA isolation was done by Trizol method. Peripheral venous blood (2ml) were calculated from the participants and the samples are processed instantly or within an hour, for maintaining the integrity of the samples. Samples were washed with RBC lysis buffer followed by PBS in order to extract leucocytes. Further leucocytes are treated with Tri-reagent (Invitrogen) according to the RNA isolation protocol is mentioned in Appendix i.

Quantitative Analysis of RNA

Concentration and purity of RNA were checked by using Nanodrop machine.

RNA Working Sample

For downstream processing of RNA templates, its very important to maintain different stock and working solution. Thus, for the preparation RNA working solution, main stock is diluted to 200ng per 15 μl.

cDNA synthesis

For the preparation of cDNA from RNA working, i-transcript kit is used. Detail about the procedure of cDNA synthesis is given in Appendix ii.

Real-Time PCR

For gene-expression analysis, Real time PCR is used. SYBr Green fluorescent probes were used for the detection of amplification. Primer sequence (SFTPD)

used for the reaction is mentioned below. Detail about the Real-time protocol is mentioned in the Appendix iii.

Gene primer	**Accession no.**	**Primer sequence 5'- 3'**	**Amplicon base pairs**
SFTPD forward	NM_003019	TGCTTTCCTGAGCATGACTG	164
SFTPD backward		AAGCCCTGTCATTCCACTTG	

Table 3a: Detail about the real time primer sequence used for gene expression analysis (Wojcik *et al*, 2016).

3.3 Calculation

ΔC_t value is calculated for each individual samples, where $\Delta C_t = C_t^{SFTPD} - C_t^{\beta\text{-Actin}}$. Then $\Delta C_t^{Cases\_Av}$ and $\Delta C_t^{Control\_Av}$ were calculated by taking the mean of ΔC_t samples for respective groups (Cases and Control) and $\Delta\Delta C_t$ were calculated by taking difference between them ($\Delta\Delta C_t = \Delta C_t^{Cases\_Av} - \Delta C_t^{Control\_Av}$). In order to calculate fold change, $2^{\wedge(-\Delta\Delta Ct)}$ formula is used. Relative mRNA expression for cases and control are analysed by plotting 2 log $\Delta C_t^{Cases\_Av}$ and 2 log $\Delta C_t^{Control\_Av}$ (Livak and Schmittgen, 2001). Association study for both the groups were done by unpaired student's t-test with 2-tailed P-value in online GraphPad software.

3.4 Result

Details about the ΔC_t, $\Delta\Delta$ Ct and $2^{\wedge(-\Delta\Delta Ct)}$ were given in table 3b. Real time PCR indicated 2.18 fold upregulation of sftpd gene among cases than control. Relative mRNA expression were given in the figure- 3a. Although significant association ($p>0.05$) of sftpd among cases were not observed by unpaired student's 't' – test ($t=0.823$, $df=11$). Insignificant association might be due to the fact, that samples size is less.

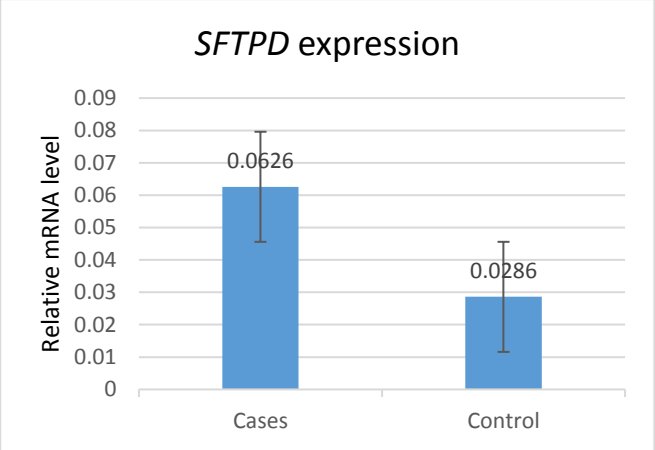

Fig 3a:- Relative mRNA expression of SFTPD gene among cases and control

3.5 Discussion

As it has been already established by multiple GWAS study that, SFTPD is an important biomarker for COPD diagnosis. This overall study aims to associate SFTPD along with COPD and its various sub-phenotype patients. In previous chapter we have seen, how meta-analysis result is significantly validating the role of SFTPD among COPD patients. We also tried to assess the role of SFTPD among north Indian COPD patients with respect to control of similar ethnicity background.

In current study, we found positive association between sftpd mRNA level and COPD patients. There was 2.18 fold increased level of SFTPD mRNA among the COPD patients. This data is consistent with the multiple studies which also showed the similar trends of SFTPD mRNA level among COPD patients.

Gene expression analysis indicated upregulation of SFTPD gene among cases with respect to control. Although significantly higher level of SFTPD expression from lecocytes cells, were not observed by unpaired student's 't' – test ($p=0.425$). Such results may appear owing to the small sample size where deviation from the original result is highly possible. Small sample size often leads to inconclusive data due to low Power of the study.

Serial no.	Sample type	CT-Mean Endogeneous	CT-Mean SFTPD	Delta CT	Delta CT average	Delta Delta CT	old Change
1.	Control-1	24.96205521	30.56804276	5.605987549			
2.	Control-2	22.93728638	26.63724518	3.699958801			
3.	Control-3	23.13980103	28.11284637	4.973045349			
4.	Control-4	22.26511383	26.25141907	3.986305237			
5.	Control-5	23.25256348	27.71042252	4.457859039			
6.	Control-6	20.75678062	25.58328247	4.826501846		-1.128968684	2.187023441
7.	Control-7	16.96703911	28.37766457	11.41062546			
8.	Control-8	30.3429184	34.55121231	4.208293915			
9.	Control-9	27.20201683	31.22269058	4.020673752			
10.	Control-10	29.85000992	33.9076767	4.057666779	5.124691772		
11.	Cases-1	22.29334641	26.01131821	3.717971802			
12.	Cases-2	23.52915955	27.32784271	3.798683167			
13.	Cases-3	18.43478203	22.90529633	4.470514297	3.995723089		

Table 3b:- Calculation of Real time data obtained from 13 samples.

4.1 Introduction:

Chronic Obstructive Pulmonary Disease (COPD) is a progressive malfunctioning of the bronchial airways and the lung walls, leading to the manifestation of different disease phenotypes such as chronic cough with/without mucus secretion, panting condition during physical exercises and higher rate of respiratory tract infection (Kim *et al*., 2013). COPD broadly includes two disease conditions, namely emphysema and chronic bronchitis. Emphysema is a COPD sub-phenotype and characterized by the destruction of gentle alveolar wall (Kim *et al*., 2013). In case of chronic bronchitis inflammation occurs in the bronchial tubes, which results in more mucus production by goblet cells (Kim *et al*., 2013).

According to World Health Organisation (WHO), 65 million people are under the burden of this disease. Nearly 3 million deaths have been reported due to COPD in 2015 and by 2030 it is projected to be the third leading cause of death (WHO repository). Middle income country such as India, COPD has already emerged as second most frequent cause of death by any non-infectious diseases (Bhome, 2012). COPD is an adult onset disease with age of onset being 45- 65 years (Kim *et al*., 2013).COPD was earlier found to be associated mainly with males due to the frequent use of tobacco but recent reports suggested rapid increase of COPD prevalence among female due to increase in tobacco consumption and exposure to smoke and fume (Christine *et al*, 2017). Majority (~90%) of the COPD cases are found in middle to low income countries indicating their poor health status and increased level of air pollution due to rapid industrialisation (Christine *et al*, 2017). Beside, environmental conditions, predisposition to familial α-antitrypsin deficiency and emphysema have been reported as risk factor for COPD (Hemminki *et al*, 2005).

Conventional treatment of COPD includes use of bronchodilators and in severe cases steroid drugs (Montuschi, 2006). However, variable efficiency and efficacy, adverse side effects of these drugs and very wide range of responsiveness among patients have widened the scope for identifying safer alternative treatment regimen (Montuschi, 2006). Alternative or complementary treatment could be defined as an independent approach for the treatment of disease in comparison to regular

medications. Traditional medicine formulation such as *Ayurveda* (Indian), Traditional Chinese medicine (Chinese), *Unani* (Middle-East, India, South Asia) and other disease management therapeutic approaches such as *yoga*, physiotherapy are being used to cure different pulmonary malfunctions (Sharma *et al*., 2010, Feng *et al* 2016, Gupta *et al*., 2014). These clinical studies have highlighted better efficacy and specificity of these medications and therapies. Furthermore, very limited side-effects of such treatment approach could make them first choice of treatment over conventional medicine in COPD, where long term use of bronchodilators or immune-suppressants are shown to have negative effect on the health of patients (Gupta *et al*., 2008).

In this review article we will briefly discuss conventional COPD treatments and their shortcoming and traditional Indian treatment regimen that could be useful to treat COPD and pulmonary rehabilitation.

4.2 Conventional COPD treatment:

COPD is a chronic inflammatory and non-curable disease. Conventional drug treatments aid in lowering the disease severity. Treatment is often done to manage the disease progression, ease symptoms and prevent complications to avoid advance stage fatality and improve the quality of life (chee *et al*., 2011). Primary medication supplemented with oxygen therapy (recommended in a patient with chronic hypoxemia), surgery and meditation are commonly recommended treatment for COPD (Stoller *et al*., 2010; Geffen *et al*., 2017, Brill *et al*., 2014). Oxygen treatment is given when the patients are in the severe stage of the COPD and with moderate hypoxemia. Long Term Oxygen Treatment (LTOT) increases cardiac output, decreases hyperinflation, which helps in improved systemic oxygen delivery and enhances respiratory muscle function of COPD patients (Brill *et al*., 2014).

Management and treatment of COPD are based on the (GOLD) classification of severity of the disease. The primary treatment for COPD includes bronchodilators, such as theophylline and albuterol (Hanania s*et al*., 2011). Bronchodilator therapy is the mainstay of COPD treatment that helps in relaxing the action of airway muscles and aids in easier breathing. However, nearly 20-30% COPD patients do not respond to bronchodilators (Hanania s*et al*., 2011; Bokov *et al*., 2017).

Bronchodilator treatment involves both substantial cost and chance of adverse drug reactions (Montuschi, 2006). We have briefly discussed commonly used types of bronchodilators.

β_2 agonists – COPD patients are commonly given both Short Acting β_2 Agonists (SABA) and/or Long Acting β_2 Agonists (LABA)(Cazzola *et al*.,2012; Maesen *et al*., 1995) SABA (such as salbutamol, terbutaline, etc.) starts to work within 5 minutes of inhalation while it remains functionally active upto 4 hours while LABA (such as salmeterol, formoterol, etc.) remains functional till 12 hours after inhalation (Maesen *et al*., 1995). LABA are useful to relief short term symptoms of COPD via relaxing the bronchial smooth muscle to stimulate β_2 receptor (Cazzola *et al*.,2012). Activation of β_2 adrenergic leads to increased intracellular cAMP concentration which results bronchodilation. SABAs such as albuterol or Short Acting Muscarinic Antagonists (SAMAs) such as ipratropium are used to treat acute symptoms of COPD. Similarly, LABAs such as salmeterol, formoterol or Long-Acting Muscarinic Antagonists (LAMAs) such as aclidinium, glycopyrronium are given to moderate or more severe COPD patient (Montuschi, 2006). Use of LABAs and Short Acting Anti-Cholinergics (SAACs) reduce symptoms and improve exercise capacity and thus produce a significant increase in forced expiratory volume at first second (FEV_1) (Montuschi, 2006). Apart from this there is another class of long acting β_2 agonists, Ultra-LABA (e.g:- indacaterol) which remains functional for a day (Cazzola *et al*.,2012).

Anti-cholinergic anti-muscarinic agents – Anti-cholinergic anti-muscarinic drugs are mainly quaternary ammonium compounds (Montuschi, 2006). Anticholinergic bronchodilators include the short-acting ipratropium bromide, which has a half-life of fewer than 6 hours (Cazzola *et al*.,2012). Whereas long-acting anti-cholinergic agent, such as tiotropium is more potent than ipratropium bromide. This is because tiotropium dissociates very slowly from lung muscarinic receptors and in airway smooth muscles (Cazzola *et al*.,2012). They work by inhibiting broncho-constriction as well as mucus secretion and thus subsides COPD symptoms. Tiotropium has also been shown to significantly enhance lung function by maintaining FEV_1, forced vital capacity (FVC), forced expiratory flow (FEF) and peak expiratory flow rate (PEFR) (Maesen *et al*., 1995). There are also certain novel long acting anticholinergic agent used for the treatment of COPD, such as

glycopyronnium bromide, which is found to have faster action and slower release of the drug (Montuschi, 2006).

Xanthines –Xanthine compound such as Theophylline used as inhaled bronchodilators prescribed to those patients who are suffering from extremely severe COPD conditions (Barnes, 2010). It also relaxes the smooth muscle of airway by an increase in cyclic AMP through inhibition of phosphodiesterase, which results in reduced hyperinflation and dyspnea (Barnes, 2010). Theophylline treatment reduced the level of IL-6, IL-8, neutrophils and TNF-α in sputum of COPD patients. IL-6 and IL-8 release by lung fibroblast which involves the abnormal inflammation in COPD patient (Zhang *et al*., 2012). Low-dose theophylline (400mg/day) alleviates the efficiency of inhaled corticosteroid in a patient with COPD and decreases the occurrence of exacerbations (Zhang *et al*., 2012).

Apart from commonly used bronchodilators, anti-inflammatory drugs are also used to quicker management of severe disease symptoms, which includes:

Glucocorticoid – Glucocorticoid such as beclomethasone dipropionate, budesonide etc. are used as compounds of inhaler for the better management of COPD. Glucocorticoid is found to act indirectly with proteins such as Heat shock protein, thereby leading to the formation of complex, which can further aid in the transcription regulation by acting as an transcription inhibitor (Montuschi, 2006).

Roflumilast –Roflumilast is an approved inhibitory drug for the enzyme Phosphodiesterase-4 which helps in bronchodilation by increasing the level of intracellular cAMP in smooth muscle and inflammatory cells (Gauvreau *et al*., 2012).

4.3 Side effects of commonly used COPD drugs:

As a chronic disease, COPD needs continuous treatment and close monitoring of vital parameters to avoid emergency and sudden collapse of the patients. Major hurdle in treatment management is unavoidable side-effects and in some cases adverse responses. Gupta and Mahony, 2008 has reported about the various adverse effects that results due to bronchodilators treatment (Gupta and Mahony, 2008). Cardiomyopathy and arythemias are often observed due to the effect of β_2

agonists which have ionotropic compounds. β_2 agonists are also found to be associated with osteoporosis (Gupta and Mahony, 2008). Beside these, reports suggested incidence of xerostomia and cognitive imbalance among COPD patients during treatment (Gupta and Mahony, 2008). Long term use of β_2 agonists is found to be associated with tachycardia, decrease in partial pressure of arterial oxygen (PO_2), vasodilation, increased respiratory mortality, hospitalization, hypokalemia and academia (Montuschi, 2006, Frei *et al*., 2014). β_2 agonists are also found to be involved with glycogenolysis and diabetes (Philipson *et al*., 2003). Major dropout of β_2 agonist treatment is that it leads to drug tolerance and poor disease management in long run (Suissa *et al*., 2003). Treatment with long-acting β2-adrenoreceptor agonists or tiotropium are believed to increase risk of acute myocardial ischemia or cardiomyopathy, electrolyte disturbances, therefore, contribute to arrhythmias or heart failure, stroke, osteoporosis and pneumonia (Suissa *et al*., 2003). Muscarinic acetylcholine receptor is found to be associated with blurred vision and pupillary dilation (Montuschi, 2006). Glucocorticoid is also found to be associated with high morbidity besides its low responsive rate. Short or long term use of corticosteroids has been found to have several side effects such as pneumonia, hyperglycemia, glaucoma, skinbruising, reduction in bone mineral density, osteoporosis, osteonecrosis, neuropathy, cardiovascular effects, risk of cataract, panniculitis, pharyngitis (Weinstein, 2012). Xanthines are also found to be associated with arrhythmias and artrial fibrillation due to depolarization and electrolyte depletion (Huerta *et al*., 2005).

Wide array of non-specific action and several adverse side effects of conventional COPD drugs potentially imposed huge health burden which frequently leads to poor treatment outcome mainly among elderly patients. Therefore, to reduce non-specific drug reactions and implement efficient and safer therapy, there is a need to formulate alternative treatment strategies. Further we have discussed and highlighted specific uses of traditional Indian therapies, namely, *Ayurveda* and *Yoga* which can be used for efficient treatment intervention for COPD.

4.4 Alternative treatment strategies: Indian traditional therapies

Large number of countries possesses wealth of traditional medicine knowledge which has shown promise not only to improve the treatment efficacy but also to

reduce the long term side-effects of regular drugs. Such therapies had been practised for several centuries but restricted within a small fraction of population with tropical countries (Sharma *et al*., 2010, Feng *et al* 2016, Gupta *et al*., 2014). While describing origin and development of "*Ayurveda"* (an ancient Indian medicine system), Dr. V Narayanaswami said *"All primitive societies have had a collection of remedies for common illness, evolved through trial and error methods, accident or by inspiration. But such remedies were purely empirical and not based on any logical understandings of illness or of drugs"*. This pointed out the evidence based implementation of ancient medicine, however also warranted for extensive research and clinical trials to re-establish its efficacy and understand its pharmacology (Narayanaswamy, 1981). Traditional medicines and other therapeutics from different countries have come up with their own strove of knowledge which could be of immensely significant for improving COPD treatment regimen.

A. Ayurveda based treatment:

'*Ayurveda'* is considered as one of the oldest ancient medicine and treatment system conceptualized and implemented sometime during 6th century BC in India. *Ayush* (life) and *Veda* (knowledge) are the two fundamental components of *Ayurveda*, which means "knowledge of life" (Mishra *et al*., 2004). It emerged from *Vedic* treatment methodology originally written by *Charaka* (written in *Charaka Samhita*) and *Sushruta* (in *Sushruta Samhita*). *Ayurveda* is probably the only therapeutic strategy where personalized medicine had been practiced since its foundation. Stratification of individuals into "*Prakriti*" constitution based on their three "*Doshas*" such as, *Vata*, *Pitta* and *Kapha* is basic criteria for ayurvedic treatment. These stratification is done based on one's body's natural constitution (physical, physiological and psychological) (Jayasundar, 2010; Valiathan 2003; Valiathan 2009). The '*tridosha*' play key roles in cellular metabolism and function, while *Vata*, *Pitta* and *Kapha* govern pranic life energy, digestion-nutrition and cellular health respectively. Different course of treatment for same disease in different prakriti is practised to improve efficacy (Patwardhan, 2008). People classified under *Vata* stratification is considered to have functional property equivalent to wind and space. It considers people with thinner body frame, quick mind, creativity, forgetfulness and restlessness (Purandare, 2013). *Pitta* (signifies

fire and water) includes people with medium body frame, better appetite, digestion, nutrition, intelligence (Purandare, 2013). The *Kaphas* (large body frame) are considered to be equivalent to soil and water. It considers people with strength, endurance, obese, calm, tolerant and lethargic people (Purandare, 2013). Frequently ayurvedic medicines are formulated on the basis of these prakritis. Ayurveda believes that different *prakritis* functions differentially, so the treatment should balance their individual functionality (Jaiswal *et al*., 2017).

In *Ayurveda* '*Shwasa Roga*' has been described as disease of '*Pranavaha srotas*' (Tracheo-bronchial tree) and fundamentally same as COPD. In *Charaka Samhita*, *Shwasa Roga was* explained as the abnormal, rapid, difficulty in breathing and when *Prana Vayu* (inhaled air) get obstructed by *Kapha* due to their impurity and moves towards the opposite (upward) direction, thus hinder normal breathing function (Brahmananda, 2002). Several natural extracts (as oil or soluble powder) and fume inhalation treatments have been reported to be effective for treating COPD. In this review we will be discussing seven major ayurvedic formulation/medicines which have been studied in recent time and tried on human in interventional study.

a. ***Vasadi kwatha* (*Vasadi syrup*):** This is generally taken as syrup as mentioned in *Yogaratnakara* (Laxmipati,, 1993). This herbal mixture contains natural extracts from medicinal plants as *kwatha dravya* (kwatha ingredients) and mixed with *Maricha churna* (Black pepper) as *prakshepa dravya* (chemical conjugate) in equal proportion. *Kwatha dravya* contains extracts from medicinal plants such as *Vasa*, *Haridra*, *Dhanyaka*, *Bharangi*, *Guduchi*, *Shunthi*, *Kantakari* and *Pippali*. A Vasadi syrup formulation from Ayush Herbs Pharmaceutical, Kangra, Himachal Pradesh, India was used for a clinical trial at Rajiv Gandhi Government Post-Graduate Ayurvedic College & Hospital, Paprola, Himachal Pradesh, India. In that study the role and efficacy of this formulation was evaluated . In this study a cohort of 24 COPD patients was evaluated, who were given 15 ml of this syrup thrice daily for 30 days. Significant improvement was observed for four COPD sub-phenotypes such as breathlessness, cough, expectoration and heaviness in chest ($p<0.001$); cyanosis ($p<0.01$) and edema ($p<0.05$). Statistical significance was also observed for improvement of $FEV_{1(L)}$

($p<0.05$) and FEV_1/FVC% ($p<0.01$). Overall improvement was also observed for breath holding time and power of exertion ($p<0.001$). This trial identified vasadi kwatha as effective formulation for curing COPD symptoms with no symptomatically adverse effects on patients (Sharma *et al*., 2010).

***b. Shwasaghna dhuma*:** It is a herbal dry powder and given to patients to inhale as mentioned in '*Anubhutayoga prakaran*' (Laxmipati, 1993). It is an equiproportional mixture of grinded seeds of *Kantakari*, dry leaves of *Dhatura*, *Ajowan*, *Khurasani ajowan*, *Kalmishora*, *Haridra* and *Bhanga*. *Shwasaghna dhuma* is known for its anti-inflammatory, mucolytic and expectorant property (Sharma *et al*., 2010). This has shown to be effective in significantly improving breathlessness, heaviness in chest, breath holding time and power of exertion ($p<0.01$); cough and expectoration ($p<0.001$). However, no significant improvement was observed for FVC_L, $FVC_{1(L)}$ and FEV_1/FVC% when in a cohort of 10 COPD patients dose of (2 puffs/1g twice daily) *shwasaghna dhuma* was given for 30 days (Sharma *et al*., 2010).

c. Adhatoda Vasica (Vasa or Adulsa): *Adhatoda Vasica* (Vasa or Adulsa) commonly known as Malabar Nut, is an evergreen indigenous plant of India and has been extensively used in Ayurvedic medicine to treat respiratory disorders. Essential oils and quinazoline alkaloids vasicine, vasicinone, deoxyvasicine and maiontone are the key components present in the plant leaves and have medicinal values (Claeson *et al*., 2010; chemixil, 1992). These compounds and their oxidised forms promote bronchodilation and respiratory stimulation (Cambridge *et al*, 1962; Gupta *et al*, 1977). Bromhexine (Bisolvon) and ambroxol are two semisynthetic derivatives of visicine and were found to have broncho-mucolytic, anti-inflammatory and antiodidant property and have been used to treat chronic pulmonary disease (Gupta *et al*, 1977, Grange *et al*., 1996; Štětinová *et al*., 2004). In a randomized placebo controlled study on chronic obstructive lung disease (COLD, also known as COPD) bromhexin treatment (30 mg bid for 14 days) found to improve sputum volume ($p<0.01$) and quality ($p<0.01$), facility of expectoration ($p<0.01$), cough ($p<0.01$), auscultatory thoracic symptoms ($p<0.01$), FEV ($p<0.01$) (Valenti *et al*., 1959). *In vitro* experimentation with

ambroxol along with N-acetyl cysteine (NAC) has been found to regulate expression of IL-12 and IL-10 secreted from alveolar macrophages (primary culture of A-MFs). Both of these ILs are immunomodulatory in function and regulate lung function through cell mediated immunity (Aihara *et al*., 2000). Ambroxol was also found to inhibit histamine secretion from mast cells derived from various tissues that help in down regulation of allergic reactions (Gibbs *et al*., 1999)

d. ***Katuki (Picrorhiza kurroa):*** This is a perennial herb mostly found in the Himalaya Mountains. Roots and rhizomes of this plant is used in *Ayurveda* to treat various illness including respiratory disorders. Recent studies have identified presence of anti-oxidant and anti-inflammatory compounds (picrorhiza acid, picrorhizoside-A/B/C and triterpenoid compounds) even in their seeds (Kutkin, 2001; Zhang, 2004). While administered with DSCG disodium cromoglycate) extracts from *Katuki* has been found to significantly inhibit allergic histamine secretion from pulmonary must cells (Dorsch *et al*., 1991). A commercial formulation known as AKL1 (AKL International, Ltd, Guernsey, UK) prepared from extracts of Katuki, *Gingo biloba* and *Zingiberofficinale* (ginger) has been used for a recent trial, where it was found not to have any significant effects on lung function or treat COPD symptoms (Brockwell *et al*., 2014).

e. ***Rhodiola spp.:*** *Rhodiola crenulata* and *Rhodiola rosea* are both known as *rasayana* herbs in *Ayurveda* and well known in ancient Chinese medicine that grows in high altitude and cold environment (Chan *et al*., 2012; Rege *et al*., 1999; Gupta *et al*., 2007). Plant rhizome contains several medicinal compounds such as rhodioloside (salidroside), glycosidic compound and a class of rosavins such as, rosavin, rosarin and rosin. Mucus overproduction is a major clinical hallmark of acute exacerbation of COPD/COLD. Excess production of cold-induced mucin (MUC) through over expression of MUC5AC (Mucin 5AC), TRPM8 (transient receptor potential melastatin 8) and CREB (cAMP response element-binding protein) has been shown to be associated with COPD/COLD. A recent *in vitro* study on airway epithelial cell line (HEB16) shown that salidroside treatment (50 µM and 100 µM) repressed the expression of MUC5AC, TRPM8 and CREB and thus could be used as intervention to treat COPD (Li *et al*., 2013). Another recent

study identified treatment supplementation with *R. crenulata* significantly ($p<0.05$) decreases INF-γ and $CD8^+$ T cell count in blood among COPD patients in comparison to healthy individuals. Both INF-γ and $CD8^+$ T induce inflammation and involve in COPD pathogenesis. Intervention with *R. crenulata*was also shown to be associated ($p<0.05$) with lower CAT score (COPD activity score), decreased titre of high-sensitivity C-reactive protein (hs-CRP) which are prognostic markers for COPD (Chen *et al*., 2015).

f. Mulethi (Glycyrrhiza uralensis or licorice): Also known as "*Yashtimadhu*" in Sanskrit, is an ayurvedic medicine and also used extensively in traditional Chinese medicine (Aoki *et al*., 2007; Fiore *et al*., 2005; Xie *et al*., 2009). Root of the plant has several medically useful licorice flavonoid (LF) such as liquiritin, liquiritinapioside, liquiritigenin and isoflavan compound glabridin (Xie *et al*., 2009). *In vitro* study confirmed anti-inflammatory role (through suppression of neutrophil, macrophage and lymphocyte accumulation) of LF when treated to LPS induced pulmonary inflamed in mice. Administration of LF (30mg/kg body weight in mice) also reported to reduce expression of TNFα and IL-1β which otherwise induce lung inflammation associated with COPD. Its effect on human tissues has not been studied *in vitro*, however a recent study has shown that licorice favonoid oil (LFO: Kaneka Glavonoid Rice Oil) is safe when administered once daily up to 1.2gm (Fiore *et al*., 2005).

***g. Nigella sativa*:** Known by the name of Magical Herb and Black seed, both *N. sativa* seed and its oil is found to have high significance in the field of *Ayurveda*, *Unani* (traditional Greco-Arabian system of medicine) and *Tibb-e-Nabwi* (Prophetic Medicine) (Al-Bukhari, 1976). Besides India, many other South-Asian and Middle-Eastern countries have also explored the medicinal benefit of this thymoquinone containing herb (Al-Bukhari, 1976). Thymoquinone, cuminaldehyde, γ-terpinene, β-pipene, *p*-mentha-1,3-diene-7-al, *p*-mentha-1,4-diene-7-al are the main photochemicals which have medicinal values (Kunnumakkara *et al*., 2018). This high thymoquinone containing herb is known widely for its anti- inflammatory, mucolytic and bronchodilation activity and is mainly used for respiratory clearance, which falls within the broad symptoms of COPD (Ahmad *et al*., 2013). However, till

date there is no evidence available on its specific role in COPD treatment or management.

B. Yoga based therapy and rehabilitation:

Yoga practice and theory was first described in '*Yoga Sutras*' by Patanjali (Desikachar *et al*, 2005). The word '*yoga'* originated from Sanskrit work '*Yuj'* which means 'to join' or 'to unite'. Yoga has been described as a fundamental process of balancing and harmonizing the body, mind and emotions and thus has been used as effective treatment or rehabilitation when there is imbalance between these components (in any disease condition). Yoga is found to rejuvenate health condition by means of various sorts of precise exercises such as '*Asana'* (relaxation exercise), '*Pranayama'* (breathing exercise), '*Dhyana'* (meditation/concentration), 'Mudra' (gesture), 'Bandha' (body lock) and 'Shatkarma' (cleansing practice) (**See table. 1**) (Salmon *et al*., 2009; Saraswati *et al*., 1996). Specifically *pranayama*, which is a type of breathing exercise which regulate alternative increase and decrease in lung volume, thereby increasing elasticity of the pulmonary muscles besides increasing the oxygen input into the body, blood supply into different organ at increased rate, finally maintaining healthy body condition by achieving homeostasis. *Pranayama* is also found to reduce dead spaces within the lung alveoli, where air normally don't reaches during normal breathing (Saraswati *et al*., 1996).

Yoga	Description
Asana	It is a steady and comfortable posture for longer period of time, which is necessary to perform meditation. This body position believes to open the energy channels to psychic centres, which improves awareness and control on self body.
Pranayama	*Pranayama* ('*prana'* is vital energy + '*yama'* control) is generally defined as breathing exercise which is stated as to utilizes breathing to influence the flow of vital energy in the energy channels of the body.
Dhyana	Usually translated as meditation indicating a state of long lasting calm without conscious efforts. *Dhyana* can only be

	experienced once body stays upright keeping spinal cord straight for some time.
Mudra:	A Sanskrit word meaning 'gesture' or 'attitude'. Gesture or attitude could be psychic, emotional, devotional and aesthetic. In practical it is meant to strengthened awareness and concentration through combination of controlled physical movements.
Bandha	This is a Sanskrit word that means 'tighten' or 'lock', fundamentally a traditional practice of *mudra* as well as *pranayama* when taught by *gurus*. This a physical action by which vital energy can be locked in a particular section of one's spiritual life.
Shatkarma	*Shatkarma*, a Sanskrit word (s*hat* = six + *karma* = action) that aims to purify physical and mental balance. It consists of six group of purification practice. It also used to balance three *doshas* in the body: *vata* (wind); *pitta* (bile) and *kapha* (mucus). This is generally practiced before specific *yoga* to purify body from toxins and ensure safe and satisfactory practice.

Table 4a: Different classes of yoga.

Several recent studies have already established that yoga therapy can be used therapeutically to improve symptoms of several neurological malfunctions, stress, post-traumatic stress disorder and promote well-being, happiness and self satisfaction (Li *et al*., 2012; Woodyard, 2011). Different forms of *yogas* are mentioned in *vedas* for the treatment of different disorders (Ross and Thomas, 2010). Several recent studies have shown effectiveness of yoga therapy in treatment and management of several chronic as well as acute diseases, such as cardiovascular disease, type-2 diabetes, neurological disorders (Bijlani *et al*., 2005; Gordon *et al*., 2012; Oken *et al*., 2014). It is worth mentioning that physical training (PT) has been found to be effective in improving lung function and over all wellbeing among COPD patients, however physiotherapy breathing exercises have failed in this regard but have been observed as key method for COPD rehabilitation (Alpert *et al*., 1974, Chester *et al*., 1977, Guazzi *et al*., 2004, Emirgil

et al.,1974, Holland *et al*., 2010, Valenza *et al*., 2014, Cross *et al*., 2010). In this review article a brief description of different forms of yoga will be given those have been found significant in treating/managing COPD or related lung function/health disorder.

An initial study on COPD patients from Austrailia had reported specific yogic practice was effective in management of COPD symptoms, which is significantly more effective than physiotherapy based breathing exercise (Tandon *et al*., 1978). Statistically significant improvements were observed for improved exertion tolerance ($p<0.003$), quicker recovery after exertion ($p<0.026$), Easier control of severe dyspnoea ($p<0.006$), overall improved chest condition ($p<0.005$) (Tandon *et al*., 1978.

Practice of *asanas* and *visama vritti pranayama* (timed breathing) for twice weekly for 12 weeks have been found to be safe for chronically ill older (age 69.9±9.5 yrs) COPD patients (n=29). Lung function measured by 6-minute walk test (6MWT) and self-reported functional performance had increased significantly ($p<0.05$) after the yoga therapy (. Donesky-Cuenco *et al*., 2009).

A preliminary cross sectional study conducted by Fulambarker *et al*., 2016, in the United States have showed significant improvement of quality of life (QOL) (<0.0001), pulmonary function (FVC_L=0.02) and respiratory muscle strength (RMS) (PIMax = 0.001; PEMax = 0.02) in a cohort of 33 COPD patients (as per GOLD criteria) (Fulambarker *et al*., 2012). Lung function was evaluated by FVC, FEV_1, maximal inspiratory pressure (PIMax) and maximal expiratory pressure (PEMax) and QOL was evaluated using St George respiratory questionnaire (SGRQ) (Jones, 2001). Asanas, Dhyana, Kapalbharti and Pranayama were the different classes of yoga techniques which were given to the patients to practice for 1 hr daily for thrice a week for 6 weeks.

A cross-sectional COPD cohort (n=50) study on north Indian population confirmed that pranayama is effective to treat COPD symptoms and could be used for therapeutic intervention for long term benefit. In that study, 25 patients each were chosen for pranayama intervention and routine COPD drug treatment respectively for three months. Statistically significant improvements were observed for COPD assessment score (CAT) ($p<0.001$) and 6-min walk test (6MWT) ($p<0.05$).

Notably, pranayama intervention could not improve FEV_1 significantly (Gupta *et al*., 2014). Effectiveness of *pranayama* was evaluated in another similar study on north Indian COPD cohort (n=48). This study reported three months pranayama intervention significantly improved peak expiratory flow (PEF%) ($p<0.05$), 6MWT ($p<0.05$), SGRQ impact scores (COPD symptoms [$p<0.03$], activity [$p<0.005$], impact [$p<0.008$] and total score [$p<0.02$]). Significant improvement was not seen among the COPD patient group who were given regular COPD treatment during this study period (Katiyar and Bihari, 2006).

Case-control based yoga intervention study on 60 mild-to-moderate COPD patients (age 30-60 yrs) showed that pranayama and various yoga posture for 45 min/day for 45 days could be a very effective therapeutic intervention and rehabilitation strategy. Statistically significant improvement was observed for the transfer factor of the lung for carbon monoxide (TLCO) ($p<0.001$) (Soni *et al*., 2012).

Kulpati and Kamath (1982) performed a hospital based cohort study (follow-up 12 weeks) where they segregate COPD patients into three groups (drug-only vs drug + breathing exercise vs drug + yogasana). Patients undergoing additional yogasana intervention showed marked improvement over drug-only group. Significant improvements were observed for respiratory rate ($p<0.001$) and heart rate (0.01) (Kulpati and Kamat, 1982).

Another COPD cohort study (n=11) established that yoga lesson is well tolerated among patients with severe COPD and helps in modifying breathing pattern and oxygen saturation among patients. Significant ($p<0.05$) improvements were observed for oxygen saturation (SaO2%), Tidal volume (V_T), rapid shallow breathing index (f/V_T), total breath time (T_t), inspiratory time (T_i). This signified yoga modified the breathing pattern towards deeper and slower pattern which helps in rehabilitation (Pomidori *et al*., 2009).

4.5 Conclusion

The number of COPD patient is rising at an alarming rate and it has been considered as third leading cause of death by 2030 (WHO repository) Although drugs like β_2– agonists, glucocorticoids are currently administered to the patients suffering from COPD, but they have also got side effects like arrythemias, optical problems, seizurres etc (Gauvreau *et al*., 2011). Traditional medicinal like Ayurveda has vast strove of knowledge which is practised among very small group of people. Due to lack of traditional knowledge among urban dwellers, most of the COPD patients depends on chemically formulated drug instead of local floras. Thus, awareness regarding traditional knowledge must be implemented so that people might come to know about these effective and economic sources of medicine. In addition to that, there is very low expertise and data available in these fields. More number of clinical trials with effective population number is required to prove the significance of such medicine in treating COPD. Even future indication of “Probiotic” treatment for COPD progression should also be considered. If upon administration of healthy microbiome enhances breathing phenomenon among COPD subjects than studies involving such parameters have to be done amongst large number of participants, in order to prove its significance

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Student Approval Form

Name of the author	Debparna Nandy
Centre	Human Genetics and Molecular Medicine
Degree	M.Sc. Life Sciences with specialization in Human Genetics
University	Central University Of Punjab
Guide	Dr. Sabyasachi Senapati
Thesis title	**Meta-analysis and estimation of gene expression to establish the role of *SFTPD* in COPD**
Year of award	2018

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Appendix – i

RNA Isolation Protocol

Pre- requisite for RNA isolation

1. Switch on the ice machine (bucket full of ice needed).
2. Cleaning of the working area properly with 70% alcohol (multiple no. of times).
3. Wipe of the pipettes with 70% alcohol.
4. Preparation of 1x solutions from 10x.
5. Autoclaved water.
6. Autoclaved tips.
7. Autoclaved centrifuge tubes (1.5 & 2 ml).
8. 15 ml falcons.
9. Stands

Main Protocol

1. Transfer contents of the tube into 15ml polypropylene tube.
2. Add RBC Lysis Buffer (3 times of initial blood volume; for 2ml blood, 6ml RBC lysis buffer, total = 8ml). Let stand at ice for 20 min.
3. Shake vigorously.
4. Centrifuge at 1500rpm/ 10min/ RT.
5. Remove supernatant.
6. Gently re-suspend the pellet in 6 ml of RBC Lysis Buffer. Let stand for 5 minutes. Repeat the step until RBC's layer not gone.
7. Centrifuge at 3000rpm/ 5min/ RT
8. Remove supernatant
9. Re-suspend the pellet in 1.5 ml of sterile DPBS in 1.5 ml centrifuge tube.
10. Centrifuge at 3000rpm/ 5min/ RT
11. Remove supernatant.

12. Add 300 µl of TRIzol solution for 2ml blood starting volume. Pipette it up and down and leave it at room temperature for 5 min. then spin @ 12,000 rpm/10 min/4°C.
13. Add 150 µl of Chloroform ($CHCl_3$) and vortex each tube for 15 sec, ONE AT A TIME or invert up and down. Incubate at RT for 5 min.
14. Centrifuge the samples at 10,000 rpm for 5 minutes at 4°C.
15. Remove the upper phase and transfer to a clean micro-centrifuge tube (1.5 ml). Be careful not to remove any of the white interface while collecting the upper phase of the extraction.
16. To this upper phase, add an equal volume/ 75 µl of chilled isopropanol and invert to mix.
17. The samples can be placed in a -20°C freezer to precipitate.
18. Samples are centrifuged at 12,000 rpm for 10 minutes at 4°C. **Note:** you may be able to see a small white pellet of RNA at the bottom of the tube after this step.
19. Carefully decant the supernatant, and rinse the pellet with 0.5 ml of ice-cold 70% ethanol. The 70% EtOH should be prepared RNase-free and stored at -20 C.
20. Centrifuge the samples at 2,000 rpm for 5 min at 2-8°C (2 times).
21. Decant the supernatant.
22. Using a pipettor, carefully remove all of the remaining liquid in the bottom of the tube without disturbing RNA pallet.
23. Allow the pellet to air- dry (Laminar air-flow) for 5 to 10 minutes to remove any remaining ethanol.
24. Dissolve the RNA pellet by adding 20 µl of RNAse-free H_2O to each sample.
25. RNA should be quantitated within 2 hours of elution. It can be held temporarily at -20 until permanent storage at -80. Repeated freeze-thaws are to be avoided, so RNA should be aliquoted for transfer as soon as possible after quantitation.

Appendix- ii

cDNA Synthesis

For reaction mix, we need ,

5x Reaction Mix- 4µl

Reverse Transcriptase- 1 µl

Nuclease free water- 12 µl

RNA -3 µl

Total -20 µl

Incubate the entire reaction mix, according to following temperatures

Priming – 5min @25°

Reverse Transcription- 20min@40°

RT inactivation- 1min @95°

Optional step- Hold @ 4°

Store the cDNA in -20 freezer for future use.

Appendix iii

Real Time PCR protocol

For Negative controls

Beta-actin forward primer /SFTPD forward primer = 0.25 µl

Beta- actin reverse primer/ SFTPD reverse primer= 0.25 µl

SYBRMix= 5 µl

Water= 4.5 µl

Total= 10 µl

For other samples,

Beta-actin forward primer /SFTPD forward primer = 0.25 µl

Beta- actin reverse primer/ SFTPD reverse primer= 0.25 µl

SYBRMix= 5 µl

cDNA = 2 µl

Water= 2.5 µl

Total= 10 µl

Appendix iV

List of equipment used

- Centrifuge
- Vortex
- Thermal cycler
- Real time PCR

List of reagents used

1x RBC Lysis Buffer

1. Simply dilute the 10x stock solution 1:10 with ddH_2O. Stable for 1 week at room temperature.

10x RBC Lysis Buffer

89.9 g NH_4Cl

10.0 g $KHCO_3$

2.0 ml 0.5 M EDTA

Dissolve the above in approximately 800 ml ddH_2O and adjust pH to 7.3. This solution is stable for 6 months at 2 – 8° C in a tightly closed bottle.

Human papillomavirus infection induced cervical cancer: Indian scenario

Journal:	*Journal of Infection Prevention*
Manuscript ID	JIP-RV-18-0051
Manuscript Type:	Reviews
Keyword:	human papillomavirus, cervical cancer, serotype, Treatment, Prevention
Abstract:	Human papillomavirus (HPV), the major causative agent for cervical cancer is found to be playing havoc all over the world. One of the world's largest mortality burden is due to cervical cancer which tolls nearly 274,000 deaths annually. Concurrent association has been reported between HPV infection and various cancers such as head, neck, oropharynx and anogenital region besides the most common cervical cancer. The most prevalent HPV serotypes are HPV16 followed by HPV18. Several epidemiological studies highlighted the association of socio-economic condition, sexual behaviour, opportunistic infections and other life style linked factors with high-risk HPV infection induced cervical cancer. Vast majority of HPV infection induced cervical cancer patients resides in India and significant proportion of women are at risk. Although there are no available curative treatments but preventive measures such as regular screening of risk population group at early age, vaccination, recommended hygiene maintenance can make the condition much better.

Manuscript Details

Manuscript number	JAIM_2018_335
Title	Alternative treatments for chronic obstructive pulmonary disease (COPD): Lesson from Indian traditional therapies
Short title	Indian traditional therapies for COPD
Article type	Review Article

Abstract

COPD is common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles and gases (as per GOLD guideline 2018). Various molecular events associated with onset of COPD include hyper-production of mucus from goblet cells, alveolar wall destruction, alteration in surfactant protein secretion from alveolar type II cells etc. Currently there is no cure for COPD, however three main groups of drugs are available for management of stable COPD, namely inhaled beta2-agonists, anticholinergics and inhaled corticosteroids (ICS). These therapies suffer from frequent non-responsiveness and may impose adverse side effects. Several traditional medicines on the other hand are found to be affective besides having very limited or no side-effects. These alternative treatment options are non-invasive, low-cost and already available which can be implemented to improve overall COPD disease burden. Indian traditional ayurvedic medicines like vasadi kwatha, shwasaghuna dhuma, adhatoda vasica eta are long part of traditional knowledge which showed effective results in several recent interventional studies when administered orally to COPD patients. Beside ayurveda, specific yoga therapies are often advised as a part of treatment. Different types of yoga-asana and pranayama have been established to be effective in controlling COPD progression and effective rehabilitation for any age group. However, large scale, multi-centric cohort study is warranted to re-confirm these findings to establish these as reliable alternative treatment for COPD.

Keywords	COPD; alternative treatment; ayurveda; yoga; pranayama.
Manuscript category	Reviews
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Systematic review and meta-analysis to establish the association of common genetic variations in vitamin D binding protein with chronic obstructive pulmonary disease

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Urkund Analysis Result

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