EVALUATION OF ASSOCIATION OF FOLIC ACID METABOLISM IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Research Project submitted to the Central University of Punjab

For the award of

Master of Science

In

Life Sciences with Specialization in Human Genetics

By

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May, 2018

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Declaration

I declare that all the changes suggested by the external examiner in the dissertation/thesis entitled "**Evaluation of association of folic acid metabolism in Chronic Obstructive Pulmonary Disease** " 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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ABSTRACT

EVALUATION OF ASSOCIATION OF FOLIC ACID METABOLISM IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Key words: COPD, Occupational hazard, Micronutrient, Folic acid, Homocysteine

COPD is characterized by increasing breathlessness. It is the fourth cause of death in the world and it is currently presenting a major global public health challenge, causing premature death from pathophysiological complications. It continues to be an important cause of morbidity, mortality, and health-care costs worldwide. It is a global health issue, with cigarette smoking being an important risk factor universally with several other factors, such as exposure to indoor and outdoor air pollution, occupational hazards, and infections. As the global population ages, the burden of COPD will increase in years to come. Folic acid or vitamin B9 is a water soluble vitamin plays a major role in metabolism. Since, COPD is a disease characterized by complex nutritional abnormalities and lower level of vitamin B9 and B12. Homocysteine or total homocysteine is a sulfur containing amino acid, acts as an intermediate in the metabolism of methionine in the folic acid or folate pathway. As observed among general population hyperhomocysteinaemia (mild to moderate), is mainly associated with vitamin B deficiencies, which are considered to be essential cofactors for the level and catabolism of hcy. Assessment of level of micronutrients is useful in establishing effects in COPD.

Deepti Chaudhary Dr. Sabysachi Senapati

ACKNOWLEDGEMENTS

I express my intense sense of gratitude to my supervisor **Dr. Sabyasachi Senapati,** Assistant Professor, Department for Human Genetics and Molecular Medicine, Central University of Punjab, Bathinda whose guidance was always available to me. Whenever I ran into a trouble spot or had a question about my research or writing, he steered me in the right direction. I am grateful to him for his resourceful guidance, constant encouragement, imperative suggestions and support.

I am extremely thankful to **Dr. Anjana Munshi,** Professor and HOD, Department for Human Genetics and Molecular Medicine, Central University of Punjab, Bathinda for her constant encouragement. I am also grateful honorable Vice Chancellor, **Prof. R.K. Kohli**.

I would also like to thank **Dr. Vitull Gupta** (Kishori Ram Hospital), for providing us the samples. I am also thankful to him for sharing his knowledge about his area of expertise. I would also like to give heartfelt gratitude to the participants of the study.

I extend my thanks to all the faculty members of Department for Human Genetics and Molecular Medicine namely **Dr. Preeti Khetarpal, Dr. Sandeep Singh, Dr. Harish Chander,** for their kind cooperation and support whenever required. I am tremendously grateful to our laboratory assistant **Mr. Roshan Kumar,** whose help is unfailingly available to me at any time. I am very much thankful to my friends Kamlesh, Debparna, Swati, Manas, Harkanwaljit, Debasis and all my classmates along with I am very much thankful to my seniors Balraj Gill, Satej Kaul, Sishir Upadhayay, Vicky Sibbal, Anil Jaglan, Abhilash Ludhiadch, Navgeet Gill, Richa Mehra, Shruti Choudhary, Ramanpreet kaur Gill, Nidhi Sharma, Kanika Vasudeva who supported me during my work and making it beautiful a journey.

Finally, I express my very profound thankfulness to the almighty**,** and my father, mother ,my sister Sakshi Chaudhary and entire family, for providing me with unfailing support and continuous encouragement throughout my two years of study and through the process of research and writing this Project Work. This accomplishment would not have been possible without them. Thank you.

Deepti Chaudhary

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LIST OF ABBREVIATIONS

S. NO.	Full Form	Abbreviation
1.	Chronic Obstructive Pulmonary Disease	COPD
2.	World Health Organization	WHO
3.	Environmental Tobacco Smoke	ETS
4.	De-oxy ribonucleic acid	DNA
5.	Forced expiratory Volume	FEV
6.	Forced Vital Capacity	FVC
7.	Global initiative for Chronic Obstructive Pulmonary Disease	GOLD
8.	Alpha-1 Antitrypsin	AATD
9.	Natural Killer Cells	NK cells
10.	C- reactive Protein	CRP
11.	Tumor Necrosis factor α	TNF α
12.	Inter leukin 6	IL6
13.	Inter leukin 1-β	IL 1-β
14.	Chemokine CXC motif 8 / Inter leukin 8	CXCL8/ IL 8
15.	25-hydro-vitamin D	25-OHD
16.	Inflammatory Bowel Disease	IBD

17.	Rheumatoid Arthritis	RA
18.	Body Mass Index	BMI
19.	Tetra hydro-folic acid	THF
20.	Di-hydro Folate	DHF
21.	Methylene tetra hydrofolate	MTHF
22.	Methionine synthase reductase	MSR
23.	Di-hydroxy Folate Reductase	DHFR
24.	Methyl-tetra-hydro-folate Reductase	MTHFR
25.	Homocysteine	Hcy
26.	Total Homocysteine	tHcy
27.	Hyper-Homocysteinaemia	hHcy

CHAPTER I

INTRODUCTION

1.1 INTRODUCTION

Predicted by 2020, COPD will the third most pandemic disease, across the globe (Seemungal *et al.*, 2007). The term chronic obstructive pulmonary disease (COPD) is used to describe progressive lung diseases involving emphysema, refractory asthma, chronic bronchitis, and some forms of bronchiectasis. Being the fourth cause of death in the world, it is currently presenting a major global public health challenge, causing premature death from pathophysiological complications and increased breathlessness (Naushad *et al.,* 2016). The development of this disease occurs through a combination of factors including exposure to pollutants and cigarette smoke, which presents a combination of both emphysema and chronic obstructive bronchitis, which causes lung airflow limitations that are not fully reversible by bronchodilators (Barnes, 2009). In COPD, oxidative stress plays an important and key role in the maintenance and amplification of inflammation in tissue injury, and also induces DNA damages. When the DNA molecule is damaged, the enzymatic mechanism acts in order to repair the DNA molecule. There are specific mechanisms to repair oxidative damages (nitrogen base modifications, or larger DNA damages, such as double-strand breaks). Additionally, there is an enzymatic mechanism for controlling the length of telomere. All these mechanisms contribute to cell viability and homeostasis (Nunomiya *et al.*, 2012). The modulation based therapies of genomic stability and DNA repair could be effective in the improvement of recovery and repair of lung tissue in COPD patients. It continues to be an important cause of morbidity, mortality, and health-care costs worldwide. Universally Cigarette smoking is considered as an important risk factor for influencing COPD along with several other factors including exposure to indoor and outdoor air pollution, occupational hazards, and infections, makes it a global health issue (Hirayama *et al.*, 2010). With the increase in the global population, the burden of COPD will increase in the upcoming years. The estimated prevalence of this disorder has shown a considerable variability across populations, suggesting that risk factors can affect populations differently. With time there are advances in our understanding of COPD such as increased recognition of the importance of comorbid disease, identification of different COPD phenotypes, and understanding how factors other than lung function can affect outcome in our

patients. The challenge we will all face in the next few years will be implementation of cost-effective prevention and management strategies to stem the tide of this disease and its cost (Baliastas *et al.*, 2017; Halbert *et al*, 2006).

As studied in the literature, earlier many definitions of COPD have stressed on the terms "emphysema" and "chronic bronchitis" which are no longer included in the definition of COPD. Emphysema is defined as deteoriation of the alveolar surface, which is one of the well-known structural abnormalities present in COPD patients. On other hand, in chronic bronchitis there is production of cough and sputum for at least 3 months in each of two consecutive years, and it remains epidemiologically and clinically a useful term (Boschetto *et al.*, 2006). This disorder does not have any clinical sub-category it is clearly identified as occupational, because the condition develops slowly and it is given that the airflow limitation turns chronic, and there are no chances of the reversal even if the exposure is discontinued. Based on the Epidemiological studies, the identification of occupational COPD is based on observing the excess occurrence among exposed workers. Occupational COPD can be described using certain examples, such as work-related variable airflow limitation may occur with occupational exposure to organic dusts like jute, cotton, sisal, etc. Such organic dust induces the development of the airway diseases and is sometimes classified as asthma like disorder. However, both chronic bronchitis accompanied with poorly reversible airflow limitation can develop with the chronic exposure. Two other conditions, namely bronchiolitis obliterans and irritant-induced asthma often overlap clinically with work-related COPD (Boschetto *et al.,* 2006).

1.2 STAGES OF COPD

According to WHO (World Health Orgnaization), COPD is categorized in four stages, abbreviated as GOLD (Global Initiative for Chronic Obstructive Lung Disease) stages of COPD.

GOLD is a criterion to differentiate the stages of COPD and aids in reducing mortality and morbidity of the disease. GOLD aims for the improvement in the prevention and

management of COPD through worldwide efforts of the people involved in all facets of health care and health care policies (WHO COPD, 2007). Table 1.1 briefly explains the stages of COPD according to GOLD.

TABLE 1.1: STAGES OF COPD

S.NO	STAGES	AIRFLOW LIMITATION	SYMPTOMS
1.	Mild (Stage 1)	FEV 1/ FVC < 0.70, FEV 1≥ 80%	Chronic cough & sputum (not always), patient usually unaware of lung function abnormalities.
2.	Moderate(Stage 2)	FEV 1/ FVC < 0.70, 50%≤ FEV 1 <80%	Shortness of breath developed on exertion & cough, sputum production. Patient seeks medical attention.
3.	Severe (Stage 3)	FEV 1/ FVC < 0.70, 30% ≤ FEV1 < 50%	Fatigue & repeated exacerbations with greater shortness of breath, with impact on quality of life.
4.	Very severe (stage 4)	FEV 1/ FVC < 0.70, FEV <30% or FEV≤ 80%	Chronic failure, quality of life appreciably impaired & exacerbation, may be life threatning.

CHAPTER II

REVIEW OF LITERATURE

2.1 BACKGROUND AND RISK OF COPD

The risk of developing COPD is related to the many factors, some are discussed below:

Smoking is considered to be the cause of COPD among smokers, the tobacco smoke (including cigarette, cigar and other types of tobacco, as well as environmental tobacco smoke (ETS)) is popular in many countries, and is threat in COPD(Kai *et al.*, 2006). Indoor air pollution from biomass fuel which is used for cooking and heating in poorly vented dwellings, is turning as a risk factor that particularly affects women in developing countries. Occupational exposures (organic and inorganic dusts, chemical agents and fumes), are under appreciated risk factors for COPD. According to studies, outdoor air pollution also contributes to the lungs total burden of inhaled particles, although it appears to have a relatively small effect in causing COPD, but is contributing a lot in developed countries (Boschetto *et al.*, 2006). Genetic factors also play an important role in COPD such as severe hereditary deficiency of alpha-1 antitrypsin (AATD), leads to severity of the lung function.

Age and gender causing aging and increases risk of COPD among olds and in females there is notable increase COPD risk. Factors which affect the lung growth and lung function during gestation and childhood such as low birth weight, respiratory infections, etc., are considered of having the potential to increase an individual's risk of developing COPD (Cavaille *et al*, 2013).

For Socioeconomic status, there are strong evidences from the studies, that the risk of development of COPD is inversely related to socioeconomic status, though it is not clear, however, there this pattern that reflects exposures to outdoor and indoor air pollutants, infections, poor nutrition, infections, crowding or other factors, are considered to be related to low socioeconomic status. Asthma and airway hyper-reactivity may be a risk factor for the development of airflow limitation and COPD. To check the association of the exposure with the reduced lung function and increased respiratory symptoms in adulthood, child hood infection can be considered as a source for the determination of the same (Halbert *et al.*, 2006; Duijts *et al.,*2014).

2.2 COMORBIDITIES

Cardiovascular conditions, hypertension and depression have a major contribution to the severity of the aforementioned disease. Moreover, coexisting symptoms or infections such as sleep problems and respiratory infections are highly prevalent among COPD patients. Evidences suggest that comorbid disorders and coexisting symptoms are key determinants of prolonged hospitalization, unfavorable prognosis, functional impairment, and increased healthcare costs in patients with COPD (Vestbo *et al.,* 2012). Studies have reported significant association of endotoxins and fine airborne particles (PM10) with the respiratory diseases. Livestock farms are known sources of the above mentioned components. Rapid expansion of these sources has escalated adverse health effects especially among people living in their vicinity (Vestbo *et al.,* 2012). Harting *et al.* (2010) reported that the whole blood cells of COPD patients release markers of systemic inflammation when stimulated with swine dust extract in comparison to the controls. Borlée *et al.* (2015) showed a significant association between exposures to livestock farms and respiratory symptoms among COPD patients. It was recently shown that areas with high livestock density could be a risk factor for the respiratory complications among COPD patients (Dijk *et al.,* 2016). However, fewer studies have been made on respiratory susceptible groups of people who are exposed to livestock.

At molecular level, there are evidences of systematic inflammation in COPD patients, measured either as increased circulating cytokines such as IL6, TNF-α, IL1-β, chemokines, and acute phase proteins such as CRP, fibrinogen, serum amyloid A, surfactant protein D, or as abnormalities in circulating cells such as monocyte, neutrophils, lymphocyte and NK cells (Agusti *et al.*, 2003). Cytokines such as IL6, is increased in systemic circulation of COPD patients during exacerbation and it may help in increase of acute phase protein such as CRP, inducing release of acute phase protein from liver, the effect of IL6 is not certain but according to evidences, it may be associated with weakness of skeletal muscle. TNF α is increased in COPD patients, is a soluble receptor. It is also released from circulating cells in COPD patients with

cachexia. The increased systemic TNFα has been implicated as a mechanism of cachexia, skeletal muscle, atropy and weakness in COPD. As studied at molecular level, the chemokine such as CXCL8 (IL8), including other chemokine's play important role in the recruitment of neutrophil, and monocyte but the circulating chemokine CXCL8 concentration is found to be increased in COPD patients and is related to the weakness of muscles (Barnes, 2009). Figure 2.1 describes the systematic effects and comorbidities of COPD.

2.3 MICRONUTIRNTS IN COPD

2.3.1 Vitamin D and calcium

Vitamin D, a macronutrient, has an important anti-inflammatory prospect in different respiratory diseases with COPD. Most population-based studies, which explored cross-sectional associations between serum 25-hydroxyvitamin D levels (25-OHD) and pulmonary function, found strong relationships with FEV_1, FVC and Peak Expiratory Flow rate. Conflicting evidence has been reported on relationship between 25-OHD and FEV_1/FVC ratio, lung function decline as well as with respiratory tract infections. In COPD cohorts, 25-OHD levels are correlating with disease severity and deficiency 25-OHD <20ng/ml may occur in approximately 75% of the more severe stages, GOLD III and IV (Hughes *et al.,* 2009).In addition, different meta-analyses have shown a significant effect of vitamin D supplementation of fall reduction in elderly people. Whether this is directly related to an increase in muscle strength or rather to neuromuscular control is still not clear. Similarly, it remains to be shown whether these benefits of vitamin D and calcium supplementation are also true for COPD patients (Janssens *et al.*, 2009).

2.3.2 Micro-nutritional deficiencies

A key role in the pathogenesis of COPD is played by the oxidative stresses. Population based studies have been found associated with low dietary intake of anti-oxidant vitamins (vitamin C, E, A, carotene) or higher intake of the oxidative

nitrogen's. There are observed impaired pulmonary function, increased risk of COPD and increased lung function decline. Similar effects have been attributed to wine and resveratrol intake. Two recent studies, also reported that relationship with lung function are not only determined by food intake but are directly correlated with anti-oxidant levels in the serum, thereby highlighting its potential mechanism (Laudisio *et al.*, 2016). Together, most epidemiological evidence strongly suggest that a prudent dietary pattern (rich in fresh vegetables, fruit, oily fish, wine and cereals, the so called 'Mediterranean diet') may protect against COPD especially in smokers (Laudisio *et al.,* 2016).

2.4 FOLIC ACID OR FOLATE

Other names of folic acid: 5'-methyltetrahydrofolate, 5'-MTHF, Acide Folique, Acide Ptéroylglutamique, Acide Ptéroylmonoglutamique, Acido Folico, B Complex Vitamin, Complexe de Vitamine's B, Complex'e Vitaminique B, Dihydrofolate, Folacin, Folacine, Folate, Folinic Acid, L-methylfolate, Methylfolate, Méthylfolate, Pteroylglutamic Acid, Pteroylmonoglutamic Acid, Pteroylpolyglutamate, Tetrahydrofolate, Tétrahydrofolate, Vitamin B9, Vitamine B9 (NCBI).

Vitamin B9, also called folate or folic acid, is one of 8 B vitamins. All the B vitamins are water-soluble, meaning the body does not store them. All B vitamins help the body convert food (carbohydrates) into fuel (glucose), which is used to produce energy. These B vitamins, often referred to as B complex vitamins, also help the body use fats and protein (Naushad *et al.,* 2016). B complex vitamins are needed for a healthy liver, and healthy skin, hair, and eyes. They also help the nervous system function properly. Synthetic form of vitamin B9 that is folic acid is found in supplements and fortified foods, while folate occurs naturally in food. Folic acid is crucial for the proper functioning of brain and plays an important role in mental and emotional health. It aids in the production of the genetic material such as DNA and RNA, and is especially important when cells and tissues are growing rapidly, such as in infancy, adolescence, and pregnancy. Folic acid also helps in making red blood

cells and helps iron to work properly in the body as it works closely with vitamin B12. Vitamin B9 works with vitamins B6 and B12 and other nutrients to control blood levels of the amino acid homocysteine. High levels of homocysteine are associated with heart disease, however there are certain evidences found which marks the role of folic acid in the chronic obstructive pulmonary disease. It is fairly common to have low levels of folic acid. Alcoholism, inflammatory bowel disease (IBD), and celiac disease can cause folic acid deficiency.

2.4.1 Available Forms

Vitamin B9 is found in multi-vitamins, including chewable, liquid drops, and B complex vitamins for children's, and is also sold separately. It is a good idea to take folic acid along with, a multi-vitamin because other B vitamins are needed for it to work. It is available in a variety of forms (including tablets, soft gels, and lozenges).

2.5 FOLIC ACID IN COPD

As it is already discussed that the deficiency and excess supplementation both can lead to several diseases but folic acid also plays a significant role in causing COPD.
The two gene present (DHFR and MTHFR) in the regulation of folic acid plays a key role.

2.5.1 DHFR

Dihydrofolate reductase is a protein coding gene often called as: DYR; DHFRP1 required for the conversion of dihydrofolate (DHF) into tetrahydrofolate (THF), which is a methyl group shuttle required for the de novo synthesis of purines, thymidylic acid, and certain amino acids. The functional dihydrofolate reductase gene (DHFR) has been mapped on chromosome 5q14.1, with multiple intron less processed pseudo genes or dihydrofolate reductase like genes which have been identified on separate chromosomes. The deficiency of Dihydrofolate reductase has been linked to megaloblastic anemia. DHFR is the Key enzyme in folate (folic acid) metabolism, which contributes to the de-novo mitochondrial thymidylate biosynthesis pathway and catalyzes an essential reaction for de-novo glycine and purine synthesis, and even

DNA precursor synthesis. It binds its own mRNA and to that of DHFR (PubMed; NCBI).

2.5.2 MTHFR

The Methylenetetrahydrofolate (MTHF) gene is mapped on 1p36.22, which is the short (p) arm of chromosome 1 at position 36.22. This gene provides instructions for making an enzyme called methylenetetrahydrofolate reductase (MTHFR). This enzyme plays an important role in processing of amino acids, and in the building blocks of proteins. MTHFR is important for a chemical reaction which involves forms of the vitamin folate (also called as vitamin B9). Specifically, this enzyme converts a molecule 5,10-methylenetetrahydrofolate(5,10-MTHF) to a molecule called 5-methyltetrahydrofolate (5-MTHF). This reaction is required for the conversion of the amino acid homocysteine to another amino acid, methionine. The body uses methionine to make proteins and other important compounds.

Other names of MTHFR gene: 5, 10-methylenetetrahydrofolate reductase, 5,10-methylenetetrahydrofolate reductase (NADPH), methylenetetrahydrofolate reductase (NADPH)(PubMed; NCBI)

2.5.3 FOLATE PATHWAY

MTHFR is short form for Methylenetetrahydrofolate Reductase, which is a very important enzyme in the body. This enzyme is necessarily required for Methylation to occur which is a metabolic process that switches on and off of genes, and repairs DNA and many other important things. Methylation in folic acid pathway is essential process to convert both forms of vitamin B9 i.e, folate and folic acid into its active and usable form called 5-MTHF or 5-Methyltetrahydrofolate. Figure 2.2 represents the pathway of folic acid. (Seemungal *et al.*, 2009).

Homocysteine is a sulfur containing compound, requires healthy levels of folate, vitamin B12 and vitamin B6 to be metabolized properly. Recent data however shows

that supplementation with these vitamins to lower homocysteine levels does not produce any benefit regarding cardiovascular risk reduction (Kai *et al.*, 2006; Seemungal *et al.*, 2009).

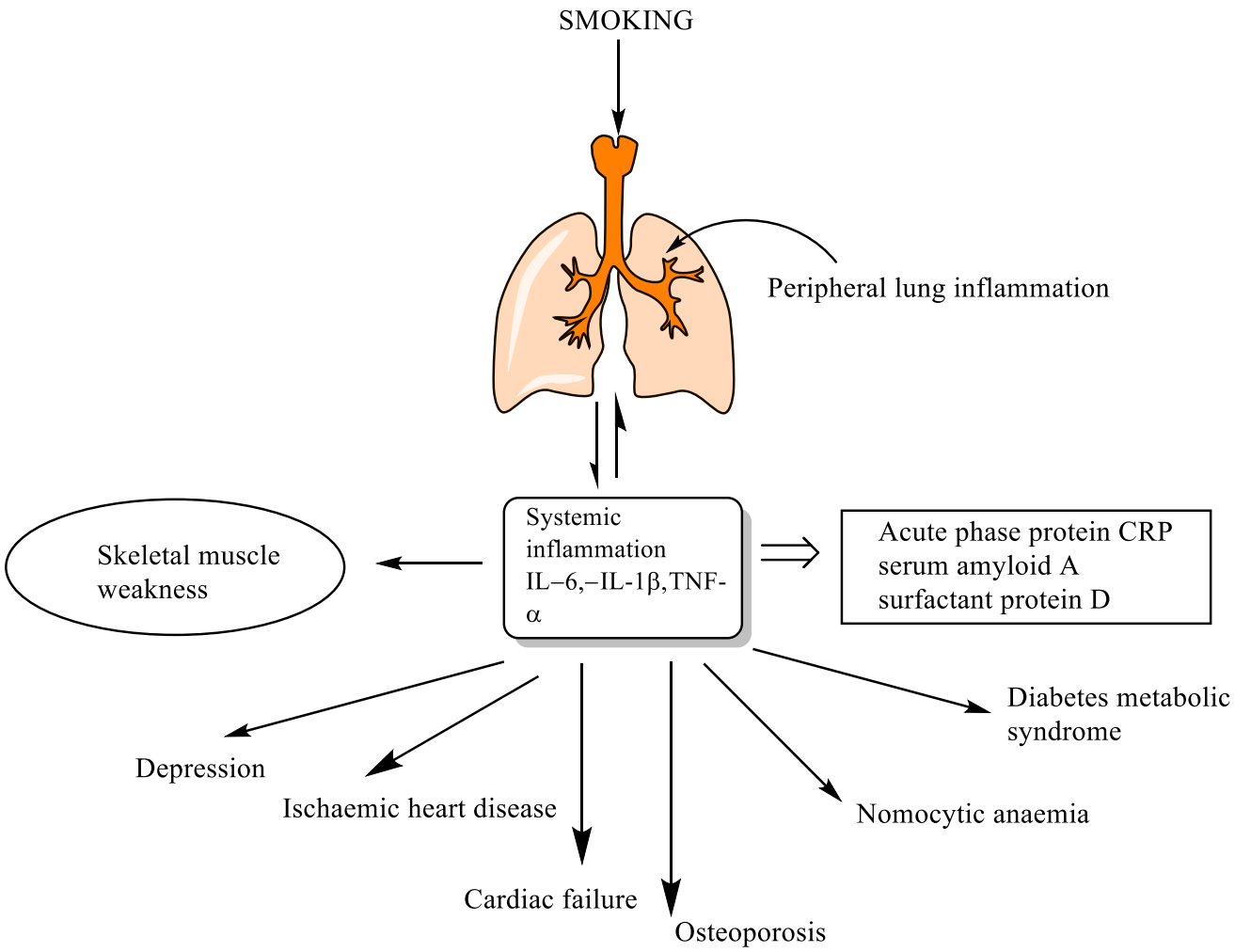


FIGURE 2.1 Systemic effects and comorbidities of COPD. Peripheral lung inflammation may cause a ‘‘spill-over’’ of cytokines, such as interlukin (IL-6, IL-1β) and tumour necrosis factor (TNF-α) into the systemic circulation, which may increase acute-phase proteins such as C-reactive protein (CRP). (Adapted, Barnes, 2009).

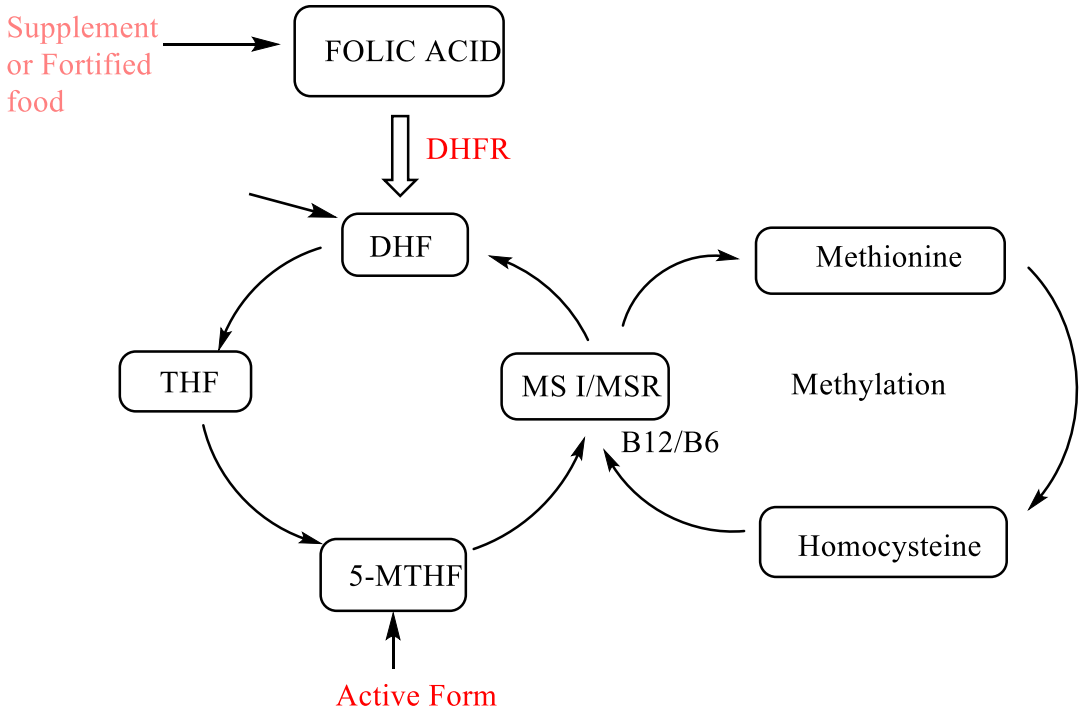


FIGURE 2.2 Folic acid pathway

CHAPTER III

SYSTEMATIC REVIEW AND META-ANALYSIS

OBJECTIVE

This study is aimed to "**evaluate the association of folic acid metabolism in COPD**".

3.1 METHODOLOGY

We intended to find the role of folic acid in COPD (Chronic Obstructive Pulmonary disease), in the patients and healthy population in association with the gene DHFR in influencing it. The overall flow of the work is described in the figure.

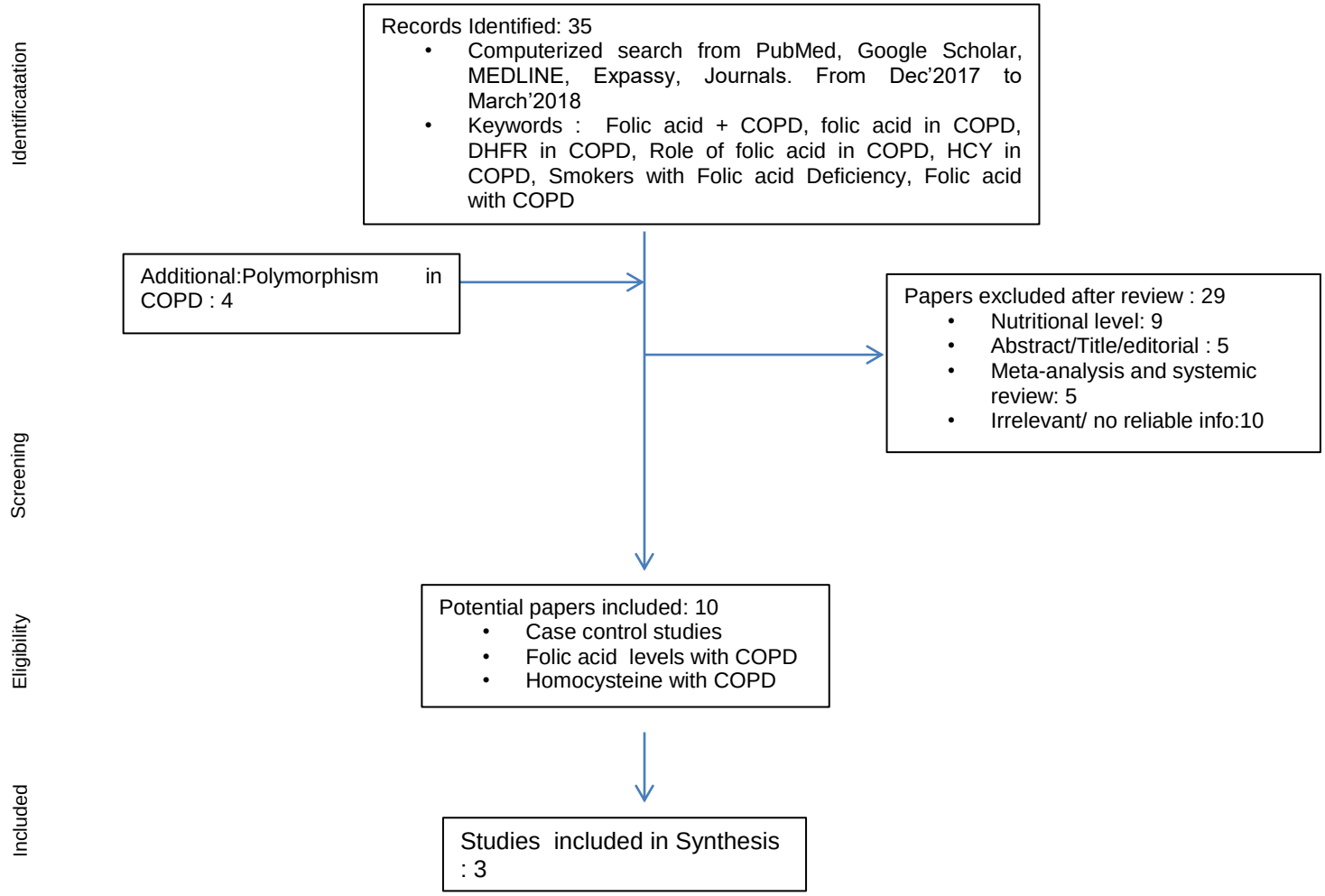


FIGURE 3.1: Overall flow chart of search and selection process

3.2 LITERATURE RETRIVAL

Literature was searched using google scholar, NCBI, PubMed, Embase taking keywords such as: folic acid, Folic acid+ COPD, B9 in COPD, smoking COPD, Folate, FA, Polymorphism in COPD, Variants in COPD, for retrieving the related literature.

The aim of the study was to find the role/function/effect of folic acid (vitamin B9) in COPD with patients having smoking and non-smoking status, as reported in the papers.

The study, further narrowed down to 3 papers as only these paper contain the related information which signifies the relation of folic acid in COPD with homocysteine (thcy) as a diagnosed exposure.

3.3 PARAMETERS

Going through the literature it was observed that the demographic data varies largely so to narrow down the heterogeneity and biasness we decided some basic parameters for both the cases and controls, taking references from the above searched literature. The inclusion and exclusion criterion remained same for both the group. Table 3.1 contains the inclusion and exclusion criterion of the smokers and non-smokers.

3.4 BACKGROUND

During the literature search it was observed that the patients having COPD may have increased risk and exposure to the level of plasma homocysteine (thcy) which can be taken as a reliable marker for in predicting folic acid or folate deficiency in patients having COPD(Fimognari *et.al*,2009). Further it was seen that plasma homocysteine is significantly elevated in COPD patient, (Seemungal *et.al*, 2007). the role of homocysteine in the form of hyper-homocystenemia was also examined in the patients having COPD and a relationship was established that the patients having COPD have poor folic acid status as a consequence they have high homocysteine concentration, though there was significant decrease in the level of thcy after folic

acid supplementation(Naushad *et.al,* 2016). These studies targeted the level of folic acid taking homocysteine as a marker in both the cases and control as there were no direct evidences of this relation. Taking references from the above studied literature we picked up the demographic data and set variables for both the cases and control, and excluded the non-reliable portions for further study. These studies included recent smokers with age group of 50-70 years with no gender biasness.

3.5 RESULTS

The abnormalities were defined with the inclusion and exclusion of parameters to maintain homogeneity of the study. We collected the pre-published data from the above literature to meet the homogeneity /heterogeneity in the results. The obtained data was separated taking the status of smoking into consideration for both the cases and control. The parameter includes details such as: age, BMI, smoking status and importantly the level of homocysteine, sex ratio was also maintained for both the cases and control to avoid the biasness in the results.

3.6 STATISTICAL ANALYSIS

Statistical analysis was performed, taking data from the pre published papers. Homocysteine was taken as a marker for the estimation of the relation with COPD. For the analysis, patients were classified as either smokers or non-smokers. All the continuous data were presented as mean ± SD. For anthropometric data diffe different types of statistical tests are done on the basis of whether the data variables are dichotomous or continuous.

The continuous data obtained from the papers was in median and range form, to pool the result of sample mean and standard deviation (SD) from the above trials, formula from the reported study was used(*Xiang Wan et al.*,2014) which used median, minimum and maximum values of range to find standard deviation.

$$S.D = \frac{b-a}{4},$$

Often called as range rule of thumb, where, b= upper range, a=lower range, and 4 is considered for the 100% accuracy. To estimate mean from median values from trials (Hozo *et al*., 2005) it was reported, using simulation, median can be used to estimate mean when sample size is larger or equal to 25.

$$\overline{X} = \frac{a+2m+b}{4},$$

Where, $\bar{X}$= mean, m = median, a= lower range, b= upper range. It was discussed that for the moderately sized samples (15< n≤ 70), formula, $\frac{range}{4}$, is the best estimator for the standard deviation (S.D).

3.7 META-ANALYSIS

The above obtained data, for both smokers and non-smokers in cases and control was then analyzed using software Revman (Review Manager 5.3), which is used to support preparing and maintaining systematic review. The forest plot was constructed to check the existence of the biasness in the publications. Further the relation of homocysteine with COPD was also seen by developing forest and funnel plot considering both the case and control. Evaluation of the individual models were first done in random model, where upon obtaining significant result analysis were done in fixed model.

In figure 3.2 and 3.3 it was observed that, for the patients and control having smoking status the heterogeneity (chi2) was 0%, 95% CI =8.11,OR=3.76 and P value= 0.0007 and was favoring controls.

The patients and control having non-smoking status, in figure 3.4 and 3.5 it was observed that the heterogeneity (chi2) was 1%, 95% CI= 0.65, OR=0.27 and the P value=0.003, the study favours the experimental as seen in forest plot.

Combined forest plot for smokers and non-smokers was constructed, figure 3.6 and 3.7, for the analysis of the heterogeneity between the two groups. The heterogeneity (chi2) for both the groups was 79%, 95% CI=1.93, OR=1.16, and the P value=0.58, which is non-significant.

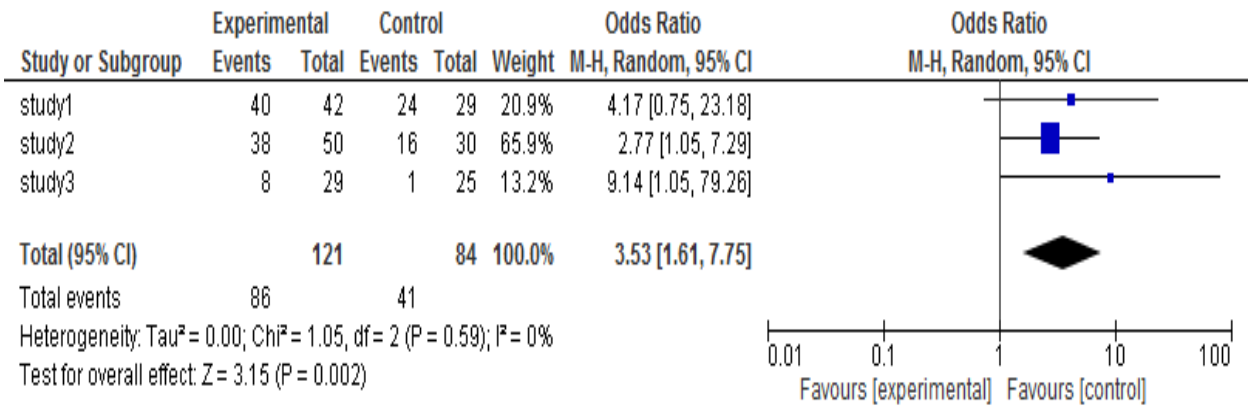
For the estimation of role of folic acid in chronic obstructive pulmonary disease (COPD), homocysteine was taken as a marker for the relation. The individual values for the level of homocysteine in the case-control was provided in the research paper, taking the total number of events, forest plot and funnel plot figure 3.8 and 3.9, was constructed and was concluded that all the studies favor control and the heterogeneity among them 0%, with 95% CI=12.10, OR=8.44 and p value to be significant i.e, $P<0.00001$.

3.8 DISCUSSION

The elevated concentration of homocystiene and the decreased concentration of folic acid have influence on COPD. The data obtained strongly recommend that hyper-homocysteinaemia may result in poor folic acid status in COPD patients, which is recognized cofactor in thcy degradation (Naushad *et al.*, 2016). Studies reported, that patients having COPD have reduced level of B vitamins, this abnormality is considered as a main factor responsible the COPD related hyper-homocysteinaemia. Studies also investigated influence on plasma hcy concentration of some important clinical factors such as B vitamins, renal function, airflow obstruction, CRP, smoking status, and comorbid diseases in COPD (Fimognari *et al.*, 2008). Seemungal *et al.* reported that thcy was not related to dietary vitamin intake (folic acid and vitamin B12, B6). The median thcy observed in COPD patients was found higher when compared with controls, and with smokers and non-smokers. It was concluded that smoking may be independent factor for the rise of thcy in COPD. In multivariate analysis, it was noted that several enzymes are directly or indirectly involved in the homocysteine metabolism in many forms such as MTHFR (methylnetetrahydrofolate reductase), MSR (methionine synthase reductase), cystathionine beta-synthase, contributing to hyperhomocysteinaemia (Seemungal *et al.*, 2007). High level of thcy in COPD when compared to healthy controls may be a marker for indicating unhealthy lifestyle. The levels of hcy were more elevated in COPD patients with GOLD stages 3 and 4 as compared to patients with stage 1 and 2, suggesting the role of pathogenesis in COPD (Naushad et al, 2016; Seemungal et al, 2007). Folic acid is considered to be the most effective vitamins among vitamin B class, which is responsible for the reduction of the elevated concentration of homocysteine, approximate reduction of 25%. Almost all studies have found that low folic acid is related to hyperhomocysteinaemia (Naushad *et al.*, 2016). The reasons for the explaining poor vitamin B status in COPD is unclear, but the depletion in vitamin B 12 adds to deficiency of folic acid. The low level of folic acid is an independent predictor of the HHcy in COPD (Fimognari *et al.,* 2009). Hence, it is concluded from above studies that the vitamin depletion may reflect or results in micro nutritional deficiencies that contributes to the increased level of homocysteine, causing hyperhomocysteinaemia

in majority of the COPD patients, as observed (Fimognari *et al.*, 2009; Naushad *et al.*, 2016).

The analysis was conducted to evaluate the association of folic acid in COPD with gene DHFR, taking Hcy as a marker. The pooled results from the above 3 studies demonstrated significant association in Hcy level between the cases and control, homogeneity and no biasness in the publication were identified. Significant difference was seen in the level of folic acid via homocysteine in both cases and control based on the pooled data from the case control studies. The rate of deficiency of homocysteine (hcy) was also compared between the pooled groups with odds ratio in case-control studies. No publication biasness was found in the analysis. Results suggested that the low level of homocysteine is not associated with COPD susceptibility, however, higher amount of homocysteine was found associated with the severity of this disease. The evidences from the demographic data from studies showed that folic acid is a risk factor for the infection. It has been hypothesized that the changes in the nutritional values and excess of supplementation was the reason for the manifestation. Our meta-analysis has several limitations. First, the studies exploring the relationship between folic acid and COPD were lacking in direct relation as we have to pick homocysteine as reliable marker for the expression of the severity. Second, some meta-analysis results were based on median and range, we have to convert them into mean± S.D, to get the forest plot to get strong results and to avoid publication biasness. While systematic analysis of the curative effects of vitamin B9 supplement was analyzed on the case-control group. Thus, more studies should be performed for better understanding of the role of folic acid in COPD patients.



. **FIGURE 3.2**: forest plot for smokers having COPD, (case-control)

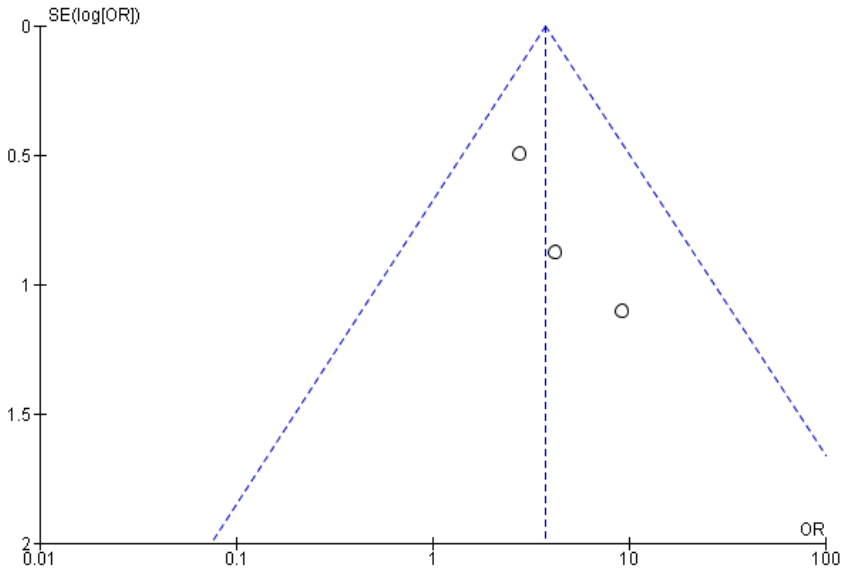


FIGURE 3.3: Funnel plot for Smokers having COPD , (case-control)

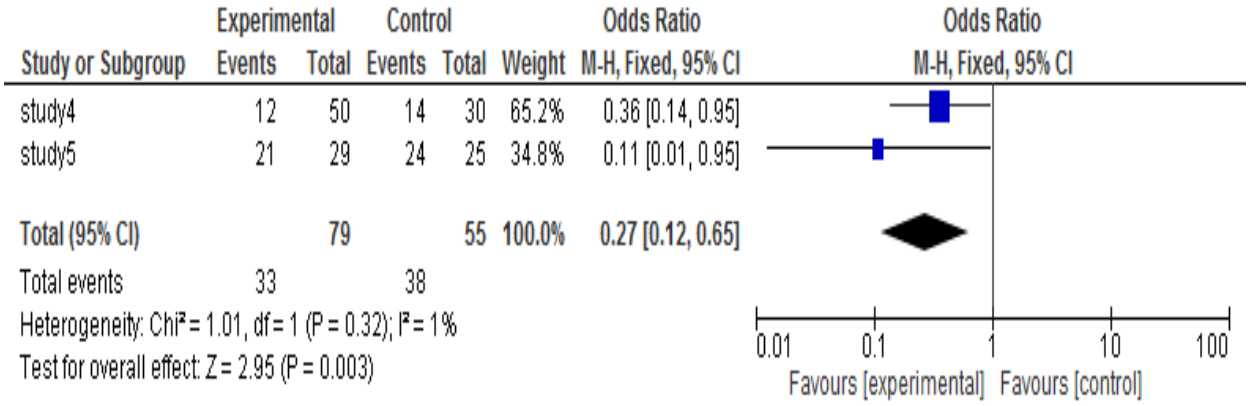


FIGURE 3.4: forest plot for non-smokers having COPD, (case-control)

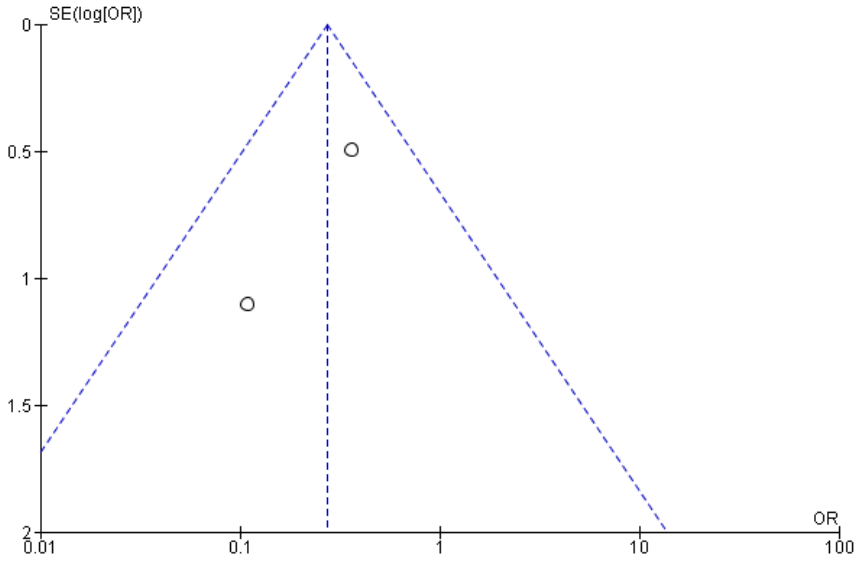


FIGURE 3.5: Funnel plot for Non-smokers having COPD,(case-control)

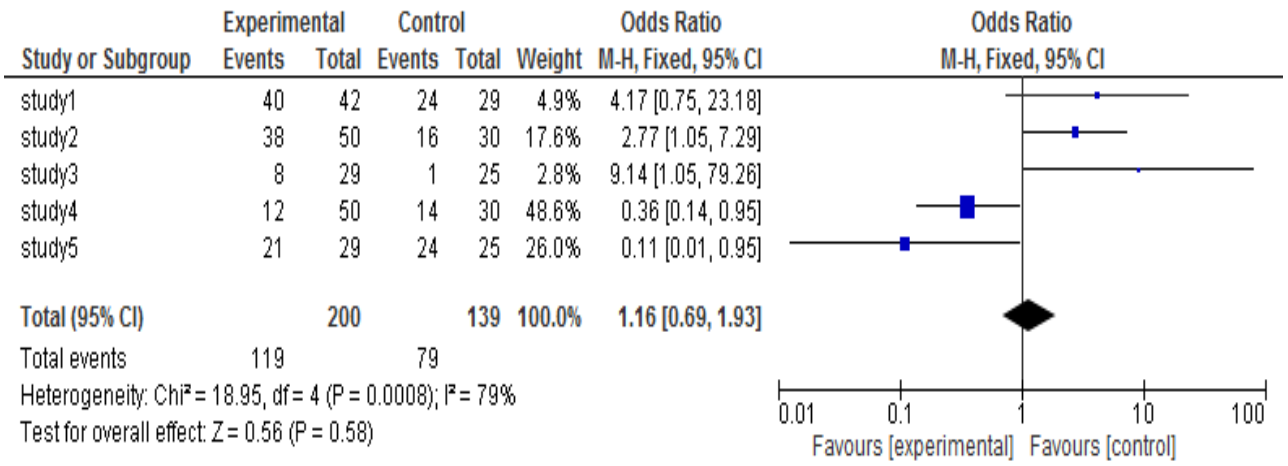


FIGURE 3.6: Forest plot for both smokers and non-smokers having COPD, (cases-controls)

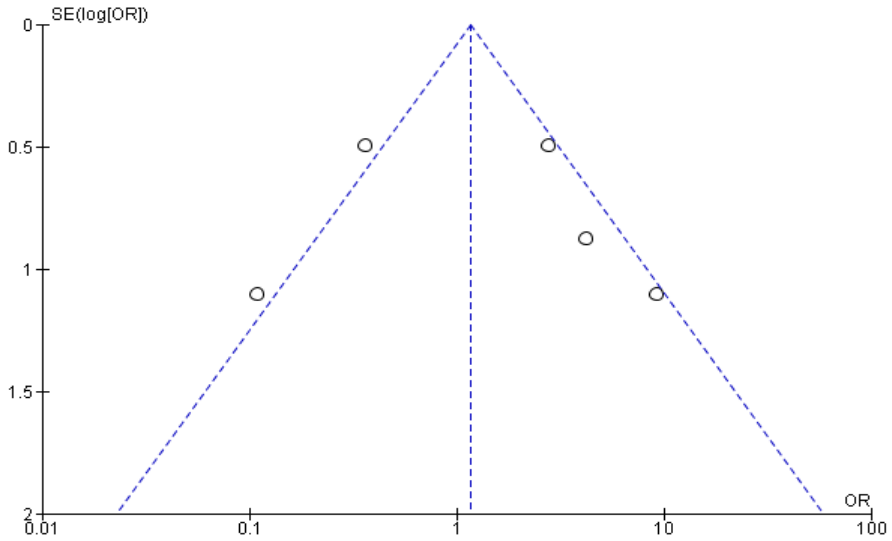


FIGURE 3.7: Funnel plot showing heterogeneity for both groups, (smokers and smokers COPD)

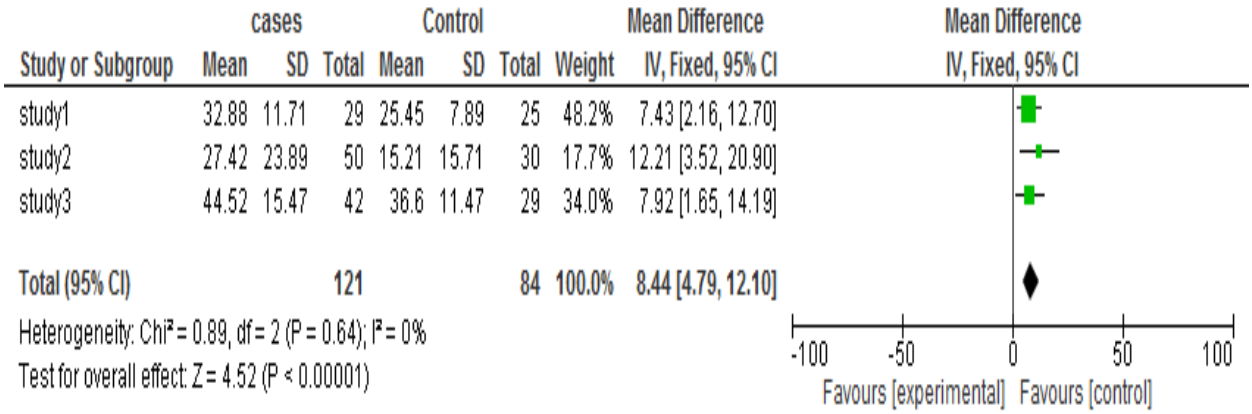


FIGURE 3.8: Forest plot for the relation taking homocysteine as a marker of folic acid with COPD

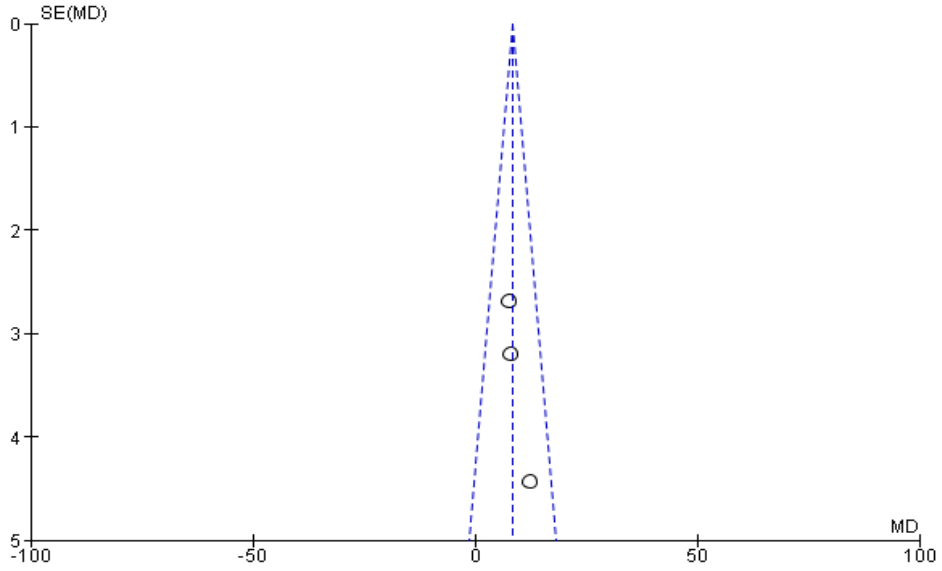


FIGURE 3.9: Funnel plot for the relation taking homocysteine as a marker of folic acid with COPD

TABLE: 3.1 Inclusion Exclusion criterion

INCLUSION	EXCLUSION
Age: 35-70 years	**Age**: > 70 years
Gender: Both male & female were considered to avoid biasness	
Smokers and Non-Smokers were included	Population exposed to occupational hazard
No autoimmune disease/supplementation	Person with autoimmune disorder

TABLE 3.2: Demographic data for both smokers and non-smokers COPD

PARAMETERS	STUDY 1		STUDY 2		STUDY 3	
PUBLICATION& YEAR	Seemungal et.al. (2007)		Fimognari *et.al*. (2008)		Naushad *et.al*. (2016)	
	CASES	CONTROL	CASES	CONTROL	CASES	CONTROL
No. of individuals	29	25	42	29	50	30
Age	69.1	64.8	71.3±88	70.6±5.8	58.3±9.2	51.5±7.6
Smokers	8	1	40	24	38	16
Non smokers	21	24	-	-	12	14
BMI	24.0	27.0	26.5±4.4	28.1±4.1	21.3±64.2	22.8±4.6
Thcy	10.96	8.22	13.9	11.5	27.42±23.89	15.21±15.71
Calculated mean (Hcy)	25.45±7.89	32.88±11.71	44.52±15.47	36.6±11.47	27.42+_23.89	15.21+_15.71

CHAPTER IV

EXPRESSION ANALYSIS OF DHFR GENE

4.1 MATERIAL AND METHODS:

4.1 SUBJECTS

Three individuals with COPD were identified by the clinician, Kishori Ram Hospital, and Adesh Hospital, Bathinda, were recruited for the study. Ethical clearance was taken for the study, with the help of consent form. The same procedure was carried out for the controls; the controls for the study were recruited from the Central University of Punjab, Bathinda, with no biasness in gender and environmental conditions to maintain homogeneity. Blood samples were collected from all the three cases (with COPD) and ten controls (without COPD)

The overall methodology of the present study has been given in Figure 4.1

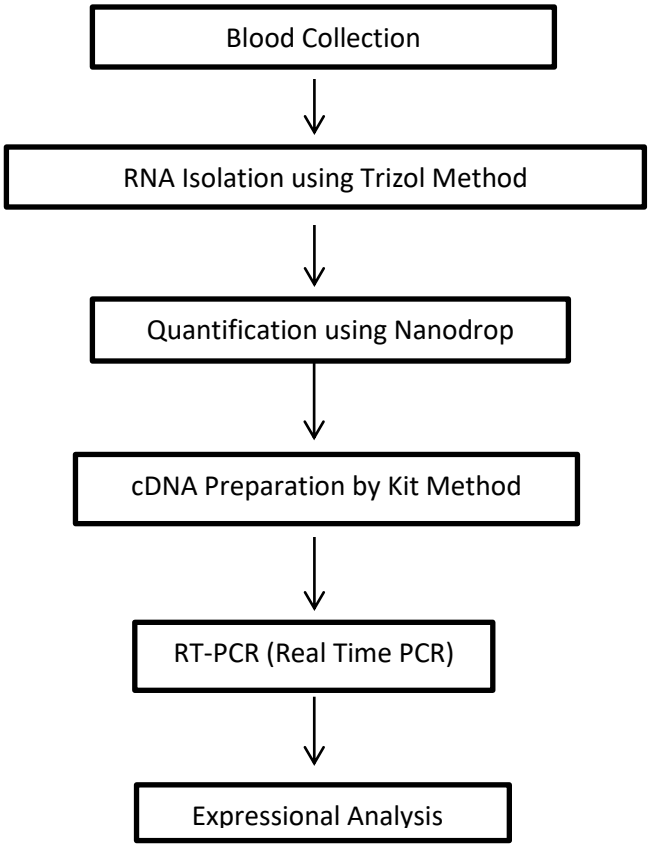


Fig.4.1: Overall work-flow of the study

4.2 Blood Sample collection

Around 2 ml peripheral blood sample was collected from each of the subject and control after venipuncture by a technician, under supervision of Dr. Vitull Gupta, Kishori Ram HospitaL and Adesh Hospital, Bathinda. The samples were collected in vacutainers with sodium EDTA as anticoagulant.

4.3 RNA Isolation

Isolation of RNA is very sensitive and can be easily degraded, by always present RNAses. Therefore it is mandatory to use RNAse free tubes and solutions. One must not ignore the need for a clean work environment when working with RNA. Autoclave all the tubes and solutions before use, and use RNAse free distilled water to avoid contamination.

4.3.1 STEPS FOR RNA ISOLATION

1. Transfer 2 ml peripheral blood cell sample from vacutainers into 15ml polypropylene tube.
2. **RBC Lysis Buffer**
   Add, 3 times of initial blood volume for 2 ml of blood, total amount 8 ml. let it stand at ice for 20 minutes.
3. Shake vigorously
4. **Centrifuge**
   Centrifuge at 1500 rpm for 10 minutes at room temperature
5. Remove the supernatant
6. Gently, re-suspend the pellet in 6 ml of RBC lysis Buffer and let it stand for 5 minutes.
7. Repeat the above step until RBC’s layer disappears.
8. **Centrifuge**
   Centrifuge at 3000rpm for 5min at room
9. Remove supernatant
10. Re-suspend the pellet in 1.5 ml of sterile DBPS in 1.5 ml centrifuge tube

11. **Centrifuge**

    Centrifuge at 3000rpm for 5min at room

12. Remove supernatant
13. Add 300 µl of TRIzol solution taking the initial amount of blood i.e, 2 ml, mix well and leave it at room temperature for 5 minutes.
14. **Spin:** Spin at 12,000 rpm for 10 minutes at 4°C.
15. Add 150 µl of chloroform and vortex it for 15 second.
16. Incubate at room temperature for 5 minutes.
17. **Centrifuge**

    Centrifuge the samples at 10,000 rpm for 5 minutes at 4°C.

18. Remove the upper phase and transfer to a clean micro-centrifuge tube (1.5 ml) cautiously, do not to remove any of the white interface while collecting the upper phase of the extraction.
19. To this upper phase add equal volume of 75 µl, chilled isopropanol and invert to mix.
20. Freeze sample at -20°C to precipitate.
21. **Centrifuge**

    Centrifuge sample at 12,000 rpm for 10 minutes at 4°C.

    (Small white pellet of RNA can be seen at the bottom of the tube)

22. Carefully decant the supernatant, rinse the pellet with 0.5 ml of ice cold 70% ethanol. The EtOH should be prepared RNAse free, store at -20°C.
23. **Centrifuge**

    Centrifuge sample at 2,000 rpm for 5 minutes at 2-8°C, repeat twice.

24. Decant the supernatant.
25. Carefully, remove all the remaining liquid without disturbing the pellet containing RNA.
26. Allow the pellet to air dry for 5-10 minute in laminar air flow, remove remaining ethanol.
27. Dissolve RNA pellet by adding 20µl RNAse free water

28. RNA should be quantitated within 2 hour of elution. Store at -80°C. Avoid repeated freeze-thaw.

4.3.2 Quantification

Quantification of RNA was done by following manufacturer's instructions using Nanodrop 200. It is used for the quantification of DNA, RNA, and Proteins.

1. The nanodrop was calibrated by double distilled autoclaved water by standard 0.1-0.5 µl. Blank was set before quantification.
2. 2 µl of isolated RNA sample was taken, and was quantified.

4.3.3 cDNA preparation

cDNA was prepared after RNA isolation, by kit method.

1. Total of 20 µl solution was prepared, using
    a) 5 × Reaction mixture = 4 µl
    b) Reverse transcriptase = 1 µl
    c) RNA sample(isolated) = approx. 3 µl

        (we can take 100fg -1 µg of total RNA, we made 200ng)

    d) Nuclease free water = 12 µl(for rest amount water is added)
2. Incubate complete reaction mixture in PCR, by applying 4 step reaction:
    a) Priming at 25°C for 5 minutes.
    b) Reverse transcription at 46°C for 20 minutes.
    c) RT inactivation at 95°C for 1 minute.
    d) Hold at 4°C (optional step)

4.3.4 RT-PCR (Real-Time PCR)

Summary of primers used, PCR reaction mixture and conditions are given in Table 4.1,
4.2 respectively. The software setup design wizard was used for RT-PCR.

PCR reaction mixture was set up to analyse the total 13 samples, which includes 3 subjects and 10 controls. Table 4.3

4.4 RESULTS

The present study consisted of 3 Indian subjects with COPD phenotype, aged 40-60years. Diagnosis of COPD was fulfilled by the clinician, with key features.

4.4.1 Demographics

A total of 3 COPD patients and 10 controls were enrolled for the study. All demographic data and baseline characteristics of the study were according to the inclusion criterion given in table 3.1. There was no difference in the environmental conditions between the COPD patients. The controls were enrolled with no difference in environmental conditions.

4.4.2 Findings among Cohort

Analysis was carried out with peripheral blood cells, RNA isolation of cases and controls. Real time PCR was used for the further analysis of the results. In our study, the mean CT value of the gene of interest and internal control were noted separately for both cases and controls. ΔCT mean was obtained by adopting formula ΔCT= (CT gene of interest - CT internal control) (Livak *et al.,* 2008).

ΔCT average for cases=3.04572 and controls=2.9774, with Δ ΔCT value=0.06827, for cohort was obtained. The Fold change = $2^{-\Delta\Delta CT}$ was also extracted by applying formula $2^{-\Delta\Delta CT}$ =[(CT gene of interest - CT internal control) sample A- (CT gene of

interest - CT internal control) sample B)] (Livak *et al.,* 2008). The details of the analysis are given in table 4.3. A histogram was also constructed, to analyze the expression of DHFR gene in cases and control, with relative mRNA level on the y-axis. Figure 4.2

4.4.3 Statistical Analysis

The obtained data was further analyzed by applying unpaired t-test; data was presented as mean ± standard deviation (SD), for cases 3.045333 ± 0.729023 and for controls 2.977013 ± 0.351090, with mean = 0.06833. The 95% CI of difference ranges from 0.64011 to 0.7767, the standard error of difference= 0.313. Although significant association ($p>0.05$) of DHFR among cases were not observed by unpaired student 't' – test ($t=0.218$, $df=9$). By conventional criterion, the level of significance was set at $p= 0.8322$, and is considered to be non-significant. Insignificant association might be due to the fact, that samples size is less.

4.4.4 Discussion

As it has been already established by multiple studies that, homocysteine is an important biomarker for COPD diagnosis. This overall study aims to associate DHFR 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 DHFR taking homocysteine as a biomarker among COPD patients. We also tried to assess the role of DHFR among north Indian COPD patients with respect to control of similar ethnicity background. In current study, we found positive association between DHFR mRNA level and COPD patients. There was 1.04 fold increased level of DHFR mRNA among the COPD patients. This data is consistent with the multiple studies which also showed the similar trends of DHFR mRNA level among COPD patients.

Gene expression analysis indicated down regulation of DHFR gene among cases with respect to control. Although significantly higher level of DHFR expression from leucocytes cells, were not observed by unpaired student't' test, $p=0.83$. 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.

Apart from these, data varies from population to population, due to different environmental condition. Hidden confounding factors are mostly present, that can act as modifier for the disease condition. COPD being a polygenic disorder has multiple modes of disease induction. So, it is very important to understand the background of disease etiology for particular populations. Since, sometimes, specific trends of disease induction were observed among particular populations for complex disorders.

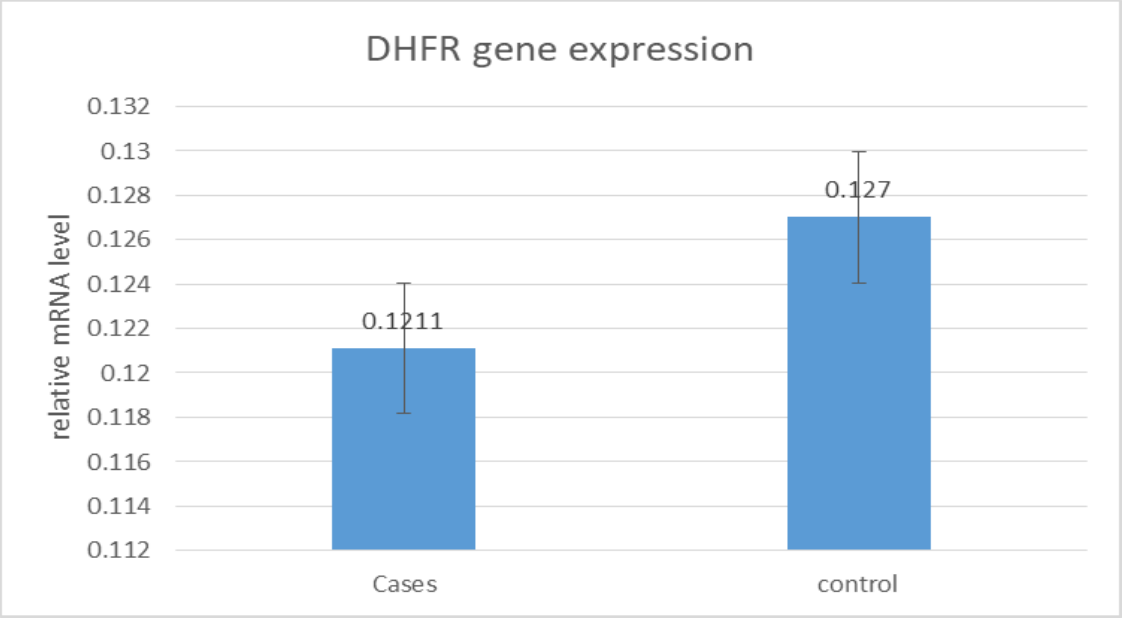


FIGURE 4.2 Relative mRNA expression of DHFR gene among cases and control

Table.4.1: Sequence of primers used

Primer	Sequence	Annealing Temperature (a°C)	Product Size	Reference
DHFR Forward Reverse	 GAATCACCCAGGCCATCTTA GCCTTTCTCCTCCTGGACAT	60°C	20bp	RTPrimer DB ID 1852 Hwang *et al.*,2002

Table.4.2: Polymerase chain reaction conditions

S. No	PCR Condition	Time	Temperature
1.	Initial Denaturation	10 min	94°C
2.	Denaturation	15 sec	95°C
3.	Annealing	1 min	60°C
4.	Extension	30 sec	72°C
5.	Part of melting curve	15 sec	60°C
6.	Part of melting curve	15 sec	95°C

Table 4.3 PCR Results

S.NO	SAMPLE	CT MEAN		ΔCT MEAN	ΔCT AVERAGE		ΔΔCT VALUE	2^ (ΔΔCT)
	CASES	β-actin	DHFR		CASES	CONTROL		
1.	Case 1	19.72791	22.78040	3.05249			0.06827	
2.	Case 2	21.06287	24.83395	3.21434	3.04572			1.04846
3.	Case 3	17.50218	19.81579	2.31360				
	CONTROL							
1.	Control 1	22.46844	25.39640	2.92795				
2.	Control 2	21.04601	24.26036	3.21434				
3.	Control 3	21.19794	23.60916	2.41121				
4.	Control 4	19.52844	22.25954	2.73111		2.9774		
5.	Control 5	20.79246	23.43308	2.64061				
6.	Control 6	19.61375	22.91415	3.30040				
7.	Control 7	20.53538	23.80665	3.27127				
8.	Control 8	27.13588	30.45854	3.32266				
9.	Control 9	Undetermined						
10.	Control 10	Undetermined						

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APPENDICES

Appendix A

Consent Form

I have been explained the possible risks and benefits and have understood the purpose for which blood sample from me/my children is being sought by the centre for _____ Central University of Punjab, Bathinda.

I am free from any pressure whatsoever and herby give my own consent/consent of my children (who are under 18 years of age today) to: (i) withdrawal of sample of about .....ml blood by veni-puncture; and (ii) to all types of analysis of my blood for non-profit research purposes for acquisition of knowledge for the benefit of mankind by Centre for _____ or their collaborators.

I will have the right to know the analyzed results for my sample (samples) and I am not giving my consent for disclosure of any personal information either direct or derived from the analysis of my sample (samples) to any one without my further consent.

I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Government agencies, and ethics committee. I understand that they may inspect my original records.

I am aware of the fact that I can opt out of the study at any time without having to give any reason and this will not affect my future treatment in the hospital. I am also aware that the investigators may terminate my participation in the study at any time, for any reason, without my consent.

I have been informed that my consent will be sought prior to any for-profit (including filing of patents) that may be taken by the Centre for _____ or their collaborators on the basis of my blood sample.

Date:

Name: Sex: Age (Yrs):

Address:

Investigator Certificate

I certify that all the elements including the nature, purpose and possible risks of the above study as described in this consent document have been fully explained to the subject. In my judgment, the participant possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate,

Signature & Name of the Investigator: _____ Dated: _____

IMMUNOGENETICS LABORATORIES

DEPARTMENT OF HUMAN GENETICS AND MOLECULAR MEDICINE

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

(Healthy control sample form)

SUBJECT DETAILS

1. Name: _____
2. (Contact: _____)
3. Age : _____ Year: _____ Month : _____
4. D.O.B : _____
5. Gender: _____
6. Height : _____(in cms)
7. Weight : _____(in kg)
8. Occupation : _____
9. Ethnicity by birth : _____
10. Smoking : Yes / No
11. If 8 yes then pack / consumption per day : _____
12. Drinking : Yes / No
13. Common disease : T2D / Hypertension / Asthma / Allergy / Arthritis (rheumatoid/osteoporosis) (specify: _____)
14. Chronic infection disease : TB / Hepatitis / UTI (specify: _____)

15. Any drug taken in last 6 months: (specify) _____

16. Any operation/ pregnancy: (specify) _____

Appendix B

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.

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.

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

List of reagents used

1x RBC Lysis Buffer

Simply dilute the 10x stock solution 1:10 with double distilled H_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

Appendix C

S.No.	Chemical Name
1.	RBC lysis Buffer
2.	TRIzol
3.	Chloroform
4.	Isopropanol
5.	EtOH (Ethanol)
6.	RNAse free water
7.	cDNA preparation Kit
8.	SYBER green

Appendix D

S.No.	Instruments Used
1.	Pipettes
2.	Centrifuge Machine
3.	Spinwin
4.	Vortex shaker
5.	Qubit
6.	Nanodrop 200
7.	Thermal cycler
8.	Ice flanking machine
9.	Real Time PCR (q-PCR)

Student Approval Form

Name of the author	Deepti Chaudhary
Department	Human genetics and Molecular Medicine
Degree	M.Sc.
University	Central university of Punjab
Guide	Dr. Sabyasachi Senapati
Thesis title	Evaluation of association of folic acid metabolism in Chronic Obstructive Pulmonary Disease
Year of award	2018

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4. View only (No downloads) (world-wide)	Not applicable

Signature of the candidate Signature and seal of the supervisor

Place

Date