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Molecular Risk Factors of Pulmonary Arterial Hypertension

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MOLECULAR RISK FACTORS OF PULMONARY ARTERIAL HYPERTENSION

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by

Hamza Assaggaf

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To: Dean Tomás R. Guilarte
Robert Stempel College of Public Health and Social Work

This dissertation, written by Hamza Assaggaf, entitled Molecular Risk Factors of Pulmonary Arterial Hypertension, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read the dissertation and recommend that it be approved.

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ABSTRACT OF THE DISSERTATION

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Professor Quentin Felty, Co-Major Professor

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The overall objective of the research presented in this dissertation was to investigate molecular risk factors of susceptibility to estrogenic chemicals, polychlorinated biphenyls (PCBs), hormone replacement therapy, and oral contraceptives and how that leads to the development of pulmonary arterial hypertension (PAH). Environmental and molecular risk factors for PAH are not clearly understood. This is a major hurdle for the development of new therapy against PAH as well as understanding individual susceptibility to this disease. Gender has been shown to impact the prevalence of PAH. Although controversial, estrogens have been implicated to be a risk factor for PAH. Thus, we hypothesize that women exposed to estrogenic chemicals are at increased risk of developing PAH when endocrine disrupting chemicals interact with unopposed estrogen to worsen pulmonary arterial disease. In support of this hypothesis, we have accomplished the following: Microarray data on PAH were collected and subsequent meta-analysis was conducted using genome-wide association and environment-wide association approaches on published

studies as well as GEO and NHANES data. All PCB geometric mean concentrations found higher levels in people at risk of PAH than people not at risk of PAH. The sum of non-dioxin-like PCBs and the sum of dioxin-like PCBs were significantly higher in people at risk of PAH than people not at risk of PAH. Also, different levels of LOD (including PCBs concentration >LOD, > 50^{th} percentile, 50^{th}-75^{th} percentile, and $\geq 75^{th}$ percentile) were significantly higher in people at risk of PAH than people not at risk of PAH. We reported that females used estrogen pills and oral contraceptive were associated with risk of PAH. However, females used progestin and estrogen/progestin pills were not at risk of PAH. Molecular risk factor analysis using machine learning approaches revealed that VAMP2, LAMA5, POLR2C, VEGFB, and PRKCH genes are causal genes of PAH pathogenesis. Gene ontology and pathway analysis of PAH showed that genes involved in the apoptosis pathway, p53 pathway, Ras Pathway, T-cell activation, TGF-beta pathway, VEGF pathway, and Wnt pathway appear to be significantly associated with PAH. Documenting the exposure to estrogenic chemicals among the general U.S. population, and identifying agents and molecular risk factors associated with PAH have the potential to fill research gaps and facilitate our understanding of the complex role environmental chemicals play in producing toxicity in the lungs.

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overexpressed PRKCH variable to see the effect on other variables. C) We knockdown PRKCH variable to see the effect on other variables

ABBREVIATIONS AND ACRONYMS

BMI	Body Mass Index
BML	Benign Metastasizing Leiomyoma
BMP	Bone Morphogenic Protein
BMPR2	Bone Morphogenic Protein Receptor Type II
BN	Bayesian Network
BPA	Bisphenol A
CDC	Centers for Disease Control and Prevention
CF	Cystic Fibrosis
CHD	Congenital Heart Disease
CHD	Coronary Heart Disease
CI	Confidence Intervals
COPD	Chronic Obstructive Pulmonary Disease
CTD	Comparative Toxicogenomics Database
CTD	Connective Tissue Disease
CTEPH	Chronic Thromboembolic Pulmonary Hypertension
CYP19	Cytochrome P-450 Enzyme Aromatase
DAGs	Directed Acyclic Graphs
DNA	Deoxyribonucleic Acid
E1	Estrone
E2	17β-estradiol
E3	Estriol
EC	Endothelial Cells
EDCs	Endocrine Disrupting Chemicals
ERs	Estrogen Receptors
ERα	Estrogen Receptor α

ERβ	Estrogen Receptor β
FPAH	Familial Pulmonary Arterial Hypertension
FXR	Farnesoid X Receptor
GEO	Gene Expression Omnibus
GM	Geometric Means
GO	Gene Ontology
GSE	Geometric Standard Errors
HDL	High Density Lipoproteins
HIV	Human Immunodeficiency Virus
HRT	Hormone Replacement Therapy
IP	Interstitial Pneumonia
IPAH	Idiopathic Pulmonary Arterial Hypertension
IR	Insulin Resistance
IS	Insulin Sensitive
LAM	Lymphangioleiomyomatosis
LOD	Limit of Detection
lss-PAH	Limited Systemic Sclerosis-PAH
MCT	Monocrotaline
MEC	Mobile Evaluation Clinic
mRNA	messenger RNA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAHNES	National Health and Nutrition Examination Survey
NCBI	National Center for Biotechnology Information
NCHS	National Center for Health Statistics
NIH	National Institute of Health
NSCLC	Non-Small Cell Lung Cancer
ORs	Odds Ratios

PAH	Pulmonary Arterial Hypertension
PAH	Pulmonary Hypertension
PANTHER	Protein ANalysis THrough Evolutionary Relationships, Classification System Data Base
PASMCs	Pulmonary Artery Smooth Muscle Cells
PCBs	Polychlorinated biphenyls
PCBs	Polychlorinated Biphenyls
PoPH	Portopulmonary Hypertension
REVEAL	The Registry to Evaluate Early and Long-term PAH Disease Management
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SMC	Smooth Muscle Cells
SR	Sex Ratio
Ssc-PAH	Systemic Sclerosis
U.S.	United States
VSD-PAH	Ventricular Septal Defect-PAH
VSMCs	Vascular Smooth Muscle Cells
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Exposure to toxic environmental chemicals have been known to cause many health effects over a period of time. Pulmonary arterial hypertension (PAH) is a rare, complex disease that affects the vascular system and leads to increases in pulmonary artery pressure and causes thickness of their walls. This thickness has been associated with different mechanisms including angiogenesis and vasculogenesis (Tofovic, 2010). Pathogenesis of PAH has been associated with many pathways that interact at different molecular levels (Tuder, Marecki, Richter, Fijalkowska, & Flores, 2007). Some molecular level interactions that are involved in PAH pathogenesis include: inflammation, uncontrolled vascular growth, and formation of obliterative vascular lesion (Irey & Norris, 1973). Females are associated with PAH and are reported to be at higher risk to the disease than males. This has been suggested to be a result of the higher exposure to estrogen (Austin et al., 2009). Moreover, many IPAH registries report such differences between females and males including REVEAL and French PAH registry with 4.1:1 and 1.9:1, respectively (Badesch et al., 2010; Humbert et al., 2006). Estrogen causes multiple effects in PAH-associated pathways such as alveologenesis (through estrogen α and β receptors, ERα, ERβ) (Massaro, Clerch, & Massaro, 2007; Patrone et al., 2003). Furthermore aromatase and mutation of BMPR2 are also associated with the exposure to estrogen and the formation of the plexiform lesion and cause PAH (Cakan, Aldemir, Topcuoglu, & Altuğ, 2009; Machado et al., 2009).

Vascular changes are known to be associated with the exposure to different endocrine disrupting chemicals which are available in the environment in different

products. Also, they can be found in plastic products, pesticides, and flame retardants; and different routes of exposure were reported for PCB including inhalation, ingestion, and absorption through skin (Balabanič, Rupnik, & Klemenčič, 2011; J. R. Roy, Chakraborty, & Chakraborty, 2009). Even though EDCs are presented in small quantities in the environment, there were reported to accumulate in the body and causing many health and side effects including cancer, neurological and immunological effect, and reducing fertility. High exposure in household products was suggested to cause embryonic exposure to EDCs as well as during breast feeding (Yang, Kim, Weon, & Seo, 2015). Overall, the effect of these compounds on estrogen activity were suggested through many pathways, which all lead to increased levels of circulating estrogens; such as the inhibition of sulfotransferase by PCBs leads to elevation of estradiol in circulation (Kester et al., 2000). The combined effect of EDCs and estrogen reported to have about 1000 times higher synergic effect than the individual effect (Kortenkamp & Altenburger, 1998).

Many researchers reported synergic effect either through direct or indirect effect, starting from ingestion, and exposure to EDC such as PCBs to molecular gene expression alteration. High exposure levels to PCBs cause lung toxicity and hypertension (Andersson & Brittebo, 2012). Furthermore pathways-associated with PAH such as inflammation reported modification of a number of genes due to EDC exposure. For example, PCB 153 modified AHR, IL6, and IL1B; while PCB 126 modified CXCL2, SOD2, and TNF. BPA-modified inflammation genes are PARP1, TNF, and IFNG; and Dibutyl phthalate, diethyl-hexyl phthalate modified genes are PARP1, MIF, CSF2, MMP9 and HMOX1 (D. Roy, Cai, Felty, & Narayan, 2007).

Notably, EDCs showed association with estrogen receptors on disease pathways. For example, activation of mitogen-activated protein kinase pathway resulted from high levels of exposure to BPA which activate ERs in breast cancer (Li, Zhang, & Safe, 2006). Some data suggested that obesity, which is a risk factor of PAH, is linked to EDC exposure (Grün & Blumberg, 2009). Also, adipogenesis is a known risk factor of obesity, and PCBs were reported to increase adipogenesis (Arsenescu, Arsenescu, King, Swanson, & Cassis, 2008). BPA exposure showed an association with a high risk of breast cancer, asthma, and different neurological effects (Rezg, El-Fazaa, Gharbi, & Mornagui, 2014). Phthalates were associated with an increased risk of cardiovascular disease, through alteration of PPARγ gene expression (Yang et al., 2015).

Pathways associated with PAH induce estrogen activity, and effects of exposure to EDCs have not been reviewed. However, it is suggested that reviewing common associated pathways would provide better understanding in disease pathogenesis. Different expressions of a number of genes were associated with PAH and its multiple pathways and their associated genes. TGF-β family receptors which cause BMPR2 mutation is known to be associated with FPAH (Thomson et al., 2000). Also, mutation in Bax, angiopoietin-1, and Smads are associated with a high risk of PAH (Rajkumar et al., 2010). Moreover, many pathways are associated with PAH and its pathogenesis including: MAPK signaling pathway, Apoptosis signaling pathway, VEGF signaling pathway, and Wnt signaling pathway (Sitbon & Morrell, 2012).

Common PAH associated pathways and genes were collected from literature and from GEO microarray gene expression datasets. Using this information, we will perform

Bayesian network analysis and gene ontology search to find common pathways and genes associated with PAH. This will result in a markov blanket network with a number of genes could be plausible pathway of PAH.

Using NHANES survey data will help us to better understand the association between exposure to EDC and how this exposure would affect subjects identified at risk of PAH. The National Health and Nutrition Examination Survey (NHANES) provide information about the U.S population in demographic, dietary, physical examination, laboratory, and questionnaire data (CDC Centers for Disease Control and Prevention, 2017). NHANES reports data in 2-years cycles since 1999 with an average of 5,000 participants each year. We will evaluate the effect of different PCBs including PCB 74, 99, 118, 138, 153, and 180 on population ≥ 20 years old at identified risk of PAH. We will compare different LODs to evaluate what concentrations of PCB would affect the risk of PAH. We will evaluate the effect of use of estrogen pills only, use of progestin pills only, use of estrogen/ progestin combo pills, and use of birth controls on females ≥ 20 years old at identified risk of PAH. We used REVEAL scoring methods to create new variable "risk of PAH" to include subjects at risk of PAH since it was not available in NHANES (Benza et al., 2012). We used available variables hypertension, diabetes, thyroid problems, uric acid level, and insulin status.

The main objective of this research was to use NHANES data from 1999-2004 and perform statistical analysis to evaluate the associations between EDCs concentrations, hormone replacement therapy, and oral contraceptives among subjects identified at risk of PAH. Also, we will support this finding with the plausible genes resulted from the meta-

analysis preformed using GEO datasets of microarray gene expressions of PAH. We will provide an overall evaluation of the effect of EDC on estrogen, effect of different genes and pathways on risk PAH, and how these different analyses would lead to increased identified risk of PAH.

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CHAPTER 2

LITERATURE REVIEW

GENDER, ESTROGEN, AND OBLITERATIVE LESIONS IN THE LUNG

Abstract

Gender has been shown to impact the prevalence of several lung diseases such as cancer, asthma, chronic obstructive pulmonary disease, and pulmonary arterial hypertension (PAH). Controversy over the protective effects of estrogen on the cardiopulmonary system should be of no surprise as clinical trials of hormone replacement therapy have failed to show benefits observed in experimental models. Potential confounders to explain these inconsistent estrogenic effects include the dose, cellular context, and systemic versus local tissue levels of estrogen. Idiopathic PAH is disproportionately found to be up to 4 times more common in females than in males, however, estrogen levels cannot explain why males develop PAH sooner and have poorer survival. Since the sex steroid hormone 17β-estradiol is a mitogen, obliterative processes in the lung such as cell proliferation and migration may impact the growth of pulmonary tissue or vascular cells. We have reviewed evidence for biological differences of sex specific lung obliterative lesions and highlighted cell-context specific effects of estrogen in the formation of vessel lumen-obliterating lesions. Based on this information, we provide a biological based mechanism to explain the sex difference in PAH severity as well as propose a mechanism for the formation of obliterative vascular lesions by estrogens.

CHAPTER 2

LITERATURE REVIEW

GENDER, ESTROGEN, AND OBLITERATIVE LESIONS IN THE LUNG

INTRODUCTION

Lung disease is not only responsible for more than 349,000 deaths per year in the United States, but also is a chronic condition with more than 35 million Americans living with chronic lung disease according to the American Lung Association. The increased prevalence in women of certain lung diseases such as asthma, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD) suggest that sex specific hormones have detrimental effects on the lung (Tam et al., 2011). The lung is a target tissue of estrogen. Since the lung expresses estrogen receptor (ER) subtypes, ERα and ERβ, estrogen has been implicated as a risk factor. The controversy over whether estrogen is protective or detrimental to the cardiopulmonary system should be of no surprise as clinical trials have failed to show cardiovascular benefits from hormone therapies. The Women's Health Initiative, reported that long-term use of estrogen may have increased, risk of cardiovascular disease while a significant increase of coronary heart disease was observed among men receiving estrogens in The Coronary Drug Project (JAMA, 1970, 1973; Rossouw, 2005). Since the sex hormone 17β-estradiol (E2) is a mitogen, a possible explanation may be that exposure to E2 contributed to atherosclerotic lesions, which have been proposed to occur as a result of the monoclonal expansion of a mutated vascular cell (Benditt & Benditt, 1973).

The dose of estrogens reportedly used in experimental models and clinically may offer a potential explanation for the estrogen paradox. On the one hand, estrogen at low doses acts as a pro-oxidant whereas higher doses act to suppress oxidative stress (Felty, Xiong, et al., 2005; Felty & Roy, 2005; Felty, Singh, & Roy, 2005; Konings et al., 2017; Parkash, Felty, & Roy, 2006). In order to understand the actions of estrogen in lung cells, it is important that we understand estrogen actions which we have summarized in brief. The classical paradigm of estrogen mechanism of action is through the ER which have been extensively reviewed; therefore we have limited our discussion in this area. Estrogen supports cell growth via interaction with estrogen receptors alpha and beta (ERα and ERβ) by directly binding to estrogen response elements, or through non-genomic pathways. The non-genomic action of estrogen very often includes ligand-dependent activation of GPR30 at the plasma membrane and leads to the activation of signaling pathways such as ERK/MAPK, protein kinases A and C, and calcium pathways (Marino, Galluzzo, & Ascenzi, 2006). Together these genomic and non-genomic pathways can contribute to obliterative lesions via cell proliferation. Alternatively, reactive oxygen species (ROS) generated from redox cycling of both stilbene and catechol estrogens can act as signaling messengers also that are also involved in cell growth (Felty, Xiong, et al., 2005; Liehr & Roy, 1990; D. Roy & Liehr, 1988). We have shown that physiologically achievable E2 concentrations, corresponding to the estrogenic menstrual peak, induces formation of ROS. Importantly, the ROS produced as a result of estrogen stimulation does not require estrogen receptors, as the ER-negative cell line produces a similar amount of ROS as the ER-positive cell lines (Felty, Xiong, et al., 2005). These studies suggest that estrogen induces oxidative stress, in part, by both ER-dependent and ER-independent pathways. Therefore, estrogen-

induced ROS through influencing cell signaling pathways may contribute to the growth of estrogen exposed lung cells.

Clinically, estrogen is given at a “low dose” to minimize thrombotic risk and hormone-dependent malignancies. Few in vitro and in vivo studies have studied the adverse effects of low dose estrogen exposure. For example, high concentrations of E2 (10 μM) have been shown to act as antioxidants in vitro (Behl et al., 1997), which may explain certain beneficial effects. Also, the exogenous administration of estrogen may not mimic the endogenous estrogen response because of differences in pulsatile versus continuous cell exposure. It has been argued that estrogens perhaps through antioxidant activity scavenges lipid peroxyl radicals and thus interrupting lipid peroxidation. Estrogen has been suggested to scavenge hydroxyl radicals at higher doses, and inhibit superoxide radical generation (Abplanalp et al., 2000). Estrogen can also produce its antioxidant actions through suppressing inflammatory cytokines or modulating antioxidant enzyme status. For instance, the apoptotic oxidative effects of cytokine TNF-α which include ROS generation, lipid peroxidation, antioxidant enzyme consumption, and disruption of mitochondrial membrane potential may be countered by estrogen (Mok et al., 2006). The chemical structure of estrogens contain a phenolic ring. In the presence of an oxidant-generating environment, the phenolic hydroxyl group present at the C3 position of the A ring of estrogens or catechol estrogens accept electrons, and gets oxidized by either accepting these electrons or losing a proton (Liehr & Roy, 1990; D. Roy & Liehr, 1988). This may help explain the antioxidant function of estrogens or estrogenic chemicals. In contrast to antioxidant effects, estrogens have been described to induce an inflammatory response with an increase of chemokines such as IL-8 (P. Comeglio et al., 2014). On the contrary,

androgens have been demonstrated to have potent anti-inflammatory effects, reducing secretion of cytokines and chemokines which are related to Th1 inflammatory response (Vignozzi et al., 2013). Testosterone was able to blunt the inflammatory response induced by potent pro-inflammatory stimuli such as TNFα, LPS, activated CD4 (+) lymphocytes (Vignozzi et al., 2012). Hence, the counteractive effects of these two sex steroid hormones might justify the relative increased incidence of pulmonary diseases in females as compared to males as well as help to explain the paradoxical effects of estrogens.

Besides the dose, the capability of lung tissue to biosynthesize estrogen from circulatory testosterone by the cytochrome P-450 enzyme aromatase (CYP19) raises the question of whether a local imbalance between testosterone and E2 levels influences the development of lung disease. Lastly, cell-context specific effects may also determine whether physiological or pharmacological concentrations of E2 stimulates cell proliferation, hypertrophy, or survival of obliterative vascular leisons found in severe pulmonary arterial hypertension (PAH). Understanding the biological and biochemical differences of sex specific lung diseases poses a major challenge in clinical research because of the predominant use of male cell lines and animals models. This has garnered the attention of NIH which has implemented an initiative to reduce sex bias in research (Clayton & Collins, 2014). This review will discuss the general state of knowledge of estrogens in lung disease with a focus on vessel-lumen obliterative lesions that are found in PAH. This will include a description of estrogens and xenoestrogens in lung tissue and disease, review of sex bias in obliterative lung disease, explanation for the sex differences in PAH, and a proposed mechanism for the formation of obliterative vascular lesions by estrogenic stress.

Estrogens and the lung

Three major steroidal estrogens in women: estrone (E1), estradiol (E2), and estriol (E3) are produced by the ovary from cholesterol. The steroidogenesis pathway also produces ovarian androgens, specifically testosterone and androstenedione, which are aromatized to E2 by the enzyme aromatase. The cytochrome P-450 enzymes CYP1A1 and CYP1B1 metabolize E2 into two catechol estrogens, 4-hydroxyestradiol (4-OHE2) and 2-hydroxyestraidol (2-OHE2) which are further metabolized to methoxyestrogens via catechol-O-methyltransferase (Liehr & Roy, 1990; D. Roy & Liehr, 1988). Out of the three estrogens, E2 has the highest estrogenic activity and is the most abundant in the bloodstream during reproductive years. Women experience normal fluctuations in estrogen throughout their lifetime and in their reproductive years. Premenopausal circulating E2 levels range 40-400 pg/ml with a considerable drop after menopause to approximately 10-20 pg/ml (DiMarco, 2000). During the menstrual cycle E2 increases in the follicular phase (days 0-14) in the range of 40-100 pg/ml that ends with a surge of E2 ranging from 100-400 pg/ml on day 14. Estradiol levels lower during the luteal phase 40-250 pg/ml and return to lower levels prior to menstruation. Men also produce estrogen, but at lower levels than women. The adult testis convert testosterone to E2 by aromatase in Leydig cells and germ cells (Hess, 2003). Once in the bloodstream estrogen can exist in two forms, bound or unbound to a protein carrier. Between 20-40% of circulating estradiol is bound to sex hormone-binding globulin (SHBG) which retains them in the circulation where they are considered to be inactive (Dunn, Nisula, & Rodbard, 1981). Estradiol that is unbound can diffuse directly through the cell membrane where it binds to estrogen receptors to regulate transcriptional processes. In addition, membrane-bound estrogen receptors mediate both

genomic and non-genomic effects on target cells. Sex differences in fetal lung development and maturation of adult lung tissue have been attributed to estrogen (Becklake & Kauffmann, 1999). The formation of alveoli in females depends on estrogens which modulate alveologeneiss by ERα and ERβ (Massaro, Clerch, & Massaro, 2007; Patrone et al., 2003). The production of surfactant in the fetal lung can be increased by E2 treatment (Chu & Rooney, 1985), which may contribute to more rapid lung maturation in female fetuses than in the male fetus (Torday & Nielsen, 1987). Although alveolar volume and number of alveoli per unit area do not differ between male and female, males develop larger lungs with larger conducting airways in adulthood (Martin, Castile, Fredberg, Wohl, & Mead, 1987).

Several lung diseases are more common in women than in men; and estrogen has been implicated as a risk factor. Since the most biologically active estrogen is E2; we reviewed concentrations of E2 reported in pathological conditions of the human lung. In patients with PAH, it has been recommended to avoid pregnancy. Levels of E2 tend to rise in the bloodstream up to 7,200 pg/ml during pregnancy which may exacerbate lung pathology (Carranza-Lira et al., 1998). A recent study reported a significantly higher level of circulatory E2 [42 pg/ml] and E2/testosterone ratio in men with PAH (Ventetuolo et al., 2016). Aromatase was shown to be expressed by human pulmonary arterial smooth muscle cells in both PAH patients and controls(Mair et al., 2014). Since E2/testosterone ratio has been considered to be correlated with aromatase activity (Cakan, Aldemir, Topcuoglu, & Altuğ, 2009), it is possible that the localized expression of aromatase may elevate E2 in the pulmonary artery. With regard to local E2 concentrations, lung tissue concentrations of 20 pg/g in non-small cell lung cancer (NSCLC) have been reported to be 2.2-fold higher

than those found in corresponding non-neoplastic lung tissues (Niikawa et al., 2008). E2 concentration of 79 pg/g was reported in interstitial pneumonia (IP) which was 2.8-fold higher than in normal lung (Taniuchi et al., 2014). A significant immunolocalization of aromatase in IP tissues implicate a role of local metabolism in causing local estrogen overexposure in the lung. In pre-menopausal women, the major sources of circulatory estrogens are the ovaries. However, estrogens are produced locally in various reproductive and non-reproductive tissues in both post-menopausal women and men by enzymatic conversions of serum androgens and adrenal-cortex steroids. The production of E2, the most potent estrogen, from the precursor E1 is a major conversion pathway dependent on the enzyme 17-beta-hydroxysteroid dehydrogenases (17β-HSDs) (Labrie, 2003). The enzyme CYP19A1 aromatase, mentioned previously, also catalyzes the aromatization of androstenedione to E1 and testosterone to E2. Evidence from a recent study of COPD showed that the local production of E2 in the lung had increased levels of enzymes involved in local estradiol synthesis (Konings et al., 2017). Since chronic inflammation is a major hall mark of lung diseases such as COPD and pulmonary hypertension we provide a summary of pro-inflammatory effects as it pertains to estrogen in the following section.

Pro-inflammatory effects of estrogen in the lung:

The function of estrogen in inflammation is complex because on one hand, suppression of inflammation with increased estrogen occurs in chronic inflammatory diseases while on the other hand estrogen produces pro-inflammatory effects in some chronic autoimmune diseases. Estrogen induces pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), and a number of other

inflammation associated genes, which were also associated with exposure to endocrine disrupting chemicals (EDCs) (Deodutta Roy et al., 2015). How estrogen induced inflammation may play a role in lung disease is not clear. One of the mechanisms includes inflammation-mediated oxidative stress. For example, inflammatory genes are associated among estrogen, EDCs, and several chronic diseases. Polychlorinated biphenyls (PCBs) congener 126 and congener 153 modify the following inflammation related genes—AHR, CXCL2, HMOX1, IFNG, IL6, PTGS2, SOD2, and TNF; AHR, CXCL8, HMOX1, IL1B, IL6, MMP9, NOS2, NOS3, PARP1, PTGS2, and TNF; and AHR, IFNG, IL1B, PARP1, PTGS2, and TNF, respectively. Dibutyl phthalate, diethyl-hexyl phthalate and BPA-modified inflammation genes are AHR, CXCL8, HMOX1, IL1B, IL6, MIF, MMP9, PARP1, SOD2, TFRC, and TNF; AHR, CSF2, CXCL8, IFNG, LEP, MMP9, SOD2, and TNF; and AHR, CSF2, HMOX1, IFNG, IL1B, IL6, LEP, MIF, MMP9, NOS2, NOS3, PARP1, PTGS2, SOD2, and TNF, respectively. In addition to the direct effect of estrogen on mitochondria and the redox cycling of catechol estrogen, estrogen-induced pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α can also generate reactive oxygen and nitrogen species (RO/NS) (Deodutta Roy, Cai, Felty, & Narayan, 2007). In the pathogenesis of estrogen-dependent lung diseases, the role of IL-6 and IL-1β are implicated in cell proliferation, angiogenesis and cell adhesion. The concentration of the peptide IL-1β seems to determine its stimulatory or inhibitory paracrine and/or autocrine signals that regulate the growth of estrogen dependent disease (Deodutta Roy, Sarkar, & Felty, 2006). IL-6 is an important cytokine involved in the pathogenesis of PAH. Clinical data showed an association between higher levels of IL-6 in PAH patients that also correlated with patient survival (Groth et al., 2014). Furthermore, IL-6 has been shown to impact the

development of pulmonary hypertension in COPD patients (Chaouat et al., 2009). In the transgenic mouse model, overexpression of IL-6 resulted in obliterative neointimal lesions consisting of endothelial cells (Steiner et al., 2009). It is important to note that estrogen differentially regulates IL-6 production in various cell types, however, estrogen has been shown to stimulate IL-6 production in mice and humans (Isse et al., 2010). Taken together these evidences support the pro-inflammatory contribution of estrogens to obliterative lung lesions in chronic disease.

Xenoestrogens, endocrine disruptors, and the lung:

Endogenous estrogens are known to strongly regulate angiogenesis and vascular modeling by influencing the growth of both vascular endothelial and smooth muscle cells. Exogenous estrogen exposures may also be important factors to consider in sex specific lung diseases. Pharmacological exposure to hormone replacement therapy (HRT) or oral contraceptives have been shown to exacerbate PAH (Irey & Norris, 1973; Kleiger, Boxer, Ingham, & Harrison, 1976; Morse, Horn, & Barst, 1999; Sweeney & Voelkel, 2009), LAM (Shen, Iseman, Waldron, & King, 1987; Wahedna et al., 1994), and NSCLC (Chlebowski et al., 2016). There is also a growing body of evidence in support of estrogenic endocrine disruptors including occupational exposure to chlorinated solvents in PAH (Montani et al., 2015). High levels of PCBs have been reported in human lung tissue (Rallis et al., 2014). Inhalation exposure to vapor-phase PCBs was demonstrated to be even more important than ingestion under some circumstances (Carpenter, 2015). Epidemiological studies have shown that chronic exposure to PCBs including its estrogenic congeners are associated with lung toxicity (Hansen et al., 2016) and hypertension (Kreiss et al., 1981). Prenatal

exposures to PCBs have been associated with decreased lung function in 20-year old offspring (Hansen et al., 2016). Moreover, population-based studies have provided evidence that PCBs are damaging to the vascular system (Goncharov et al., 2008; Gustavsson & Hogstedt, 1997; Sergeev & Carpenter, 2005; Tokunaga & Kataoka, 2003). In vivo animal studies have shown that PCBs produce placental vascular lesions and trophoblastic lesions (Bäcklin, Persson, Jones, & Dantzer, 1998). We have reported that physiological levels of E2 and estrogenic PCB153 [1ng/ml] at a level found in human serum [0.60 - 1.63 ng/ml] (Charlier, Albert, Zhang, Dubois, & Plomteux, 2004), altered pulmonary endothelial as well as smooth muscle cell phenotypes (Charlier et al., 2004). PCB153's effects on both endothelial cells are even more pronounced than E2 with respect to vasculosphere formation and vasculogenesis. Another endocrine disrupting chemical, 4,4′-methylenedianiline, used in the synthesis of polyurethanes has been shown to increase hyperplasia of pulmonary arteries exclusively in female rats (Carroll-Turpin et al., 2015). In vitro, human pulmonary smooth muscle cells were shown to proliferate when exposed to 4,4′-methylenedianiline and this was inhibited by treatment with the estrogen receptor antagonist ICI 182,780. Another well-known xenoestrogen, bisphenol A, has been reported to enhance development of asthma (Midoro-Horiuti, Tiwari, Watson, & Goldblum, 2010). Environmentally relevant concentrations of bisphenol A have been shown to elicit proangiogenic effects in human endothelial cells (Andersson & Brittebo, 2012). Taken together, these studies suggest that exposure to xenoestrogens and/or endocrine disruptors as a potential risk factor for obliterative lung lesions.

Sex bias in lung disease:

Asthma: Gender has been shown to play a role in the diseased lung. We will summarize sex differences in major lung diseases at times highlighting how estrogens contribute to obliterative processes in the lung such as cell proliferation and migration. Female hormones in allergic disease have been extensively studied in asthma. After puberty the prevalence of asthma is greater in girls than boys (Akinbami, Moorman, & Liu, 2011). The prevalence of asthma is greater in women than men during early to middle adulthood (Leynaert et al., 2012). Severity of asthma is also more severe in women with a higher likelihood of death compared to men (Tam et al., 2011). Modulation of lung inflammation by estrogen may partly explain this association. In asthma, inflammation enhances airway smooth muscle cell contractility, proliferation, and extracellular matrix production. Estrogens are known to modulate immune cells such as macrophages, lymphocyte, and mast cells some of which express ERs and the estrogen membrane receptor GPR30 (Bonds & Midoro-Horiuti, 2013), which may contribute to smooth muscle hyperplasia that obliterate the airway.

Chronic obstructive pulmonary disease (COPD): Chronic obstructive pulmonary disease is a progressive disease that includes emphysema and chronic bronchitis. The incidence of COPD in women has been reported to be increasing (Han et al., 2007). For example, smoking is a major risk factor for COPD, but females tend to develop COPD faster than males even though they smoke less cigarettes (Gold et al., 1996). In nonsmokers, females make up two-thirds of cases with COPD (Salvi & Barnes, 2009). Cell proliferation has been shown to contribute to the intimal thickening of pulmonary arteries in both smokers and patients with mild COPD (Harness-Brumley, Elliott, Rosenbluth, Raghavan, & Jain,

2014). The early appearance of obliterative vascular lesions in COPD suggest that the pathology is not a late complication of pulmonary hypertension. Rather the growth promoting effects of estrogen on smooth muscle cells may be involved in the early development of COPD. Besides receptor mediated pathways, oxidative stress from estrogen metabolism in the lung may contribute to the growth of these cells. Estrogens have been shown to be hydroxylated to catechol estrogens, and catechol estrogens participate as a substrate in cytochrome P450 catalyzed redox reactions (Liehr & Roy, 1990; D. Roy & Liehr, 1988). Thus, estrogen potentiation of oxidative stress may confer susceptibility of female smokers to COPD. Cystic fibrosis is a rare genetic disorder that affects both men and women; and is characterized by a buildup of mucus in the lungs. This abnormal level of mucus leads to repeated, serious lung infections that over time severely damage lungs. Women have shown a higher prevalence of severe cystic fibrosis and exacerbations coincide with estrogen peak in the menstrual cycle (Chotirmall et al., 2012; Harness-Brumley et al., 2014). Estrogen has been demonstrated to upregulate the MUC5B gene, a major mucin in the human airway (Choi et al., 2009). A potential mechanism by which estrogen may exacerbate cystic fibrosis in women may be by increasing MUC5B expression.

Lymphangioleiomyomatosis (LAM): Pulmonary lymphangioleiomyomatosis (LAM) is a progressive and eventually fatal disease that primarily affects premenopausal women and can be exacerbated by pregnancy (Henske & McCormack, 2012). Estrogen can be considered a risk factor for LAM because disease severity worsens with estrogen therapy (Yano, 2002). LAM is associated with abnormal proliferation and invasion of smooth muscle cells that destroys the lung parenchyma. Small clusters of cells characterize lung

lesions in LAM which are located along pulmonary bronchioles, blood vessels, and lymphatics. Clumps of LAM cells in lymph vessels leads to thickening of the vessel wall and obliteration of the lumen. Immunohistochemical data has also shown higher levels of estrogen synthesizing enzyme aromatase in LAM cells (Adachi et al., 2015). Lung cancer is a leading cause of cancer related deaths in women (Jemal, Siegel, Xu, & Ward, 2010). A greater female predominance of NSCLC in both smokers and nonsmokers suggests that differences in sex hormones contribute to its pathogenesis (Shim et al., 2013). A worse prognosis in women with lung cancer has been associated with the expression of aromatase (Mah et al., 2007). Hence, the pro-proliferative effects of estrogen along with its known genotoxic effects may explain the sex bias observed in both LAM and NSCLC.

Pulmonary arterial hypertension (PAH): Pulmonary arterial hypertension is clinically classified as Group 1 in the World Health Organization (WHO) system. Uncontrolled vascular cell growth has been postulated as the major mechanism involved in PAH pathogenesis (Rubin M. Tuder, Marecki, Richter, Fijalkowska, & Flores, 2007), which results in vessel obliteration. Most epidemiological studies have determined the effect of gender on prevalent PAH cases. Group 1 PAH includes idiopathic PAH, heritable PAH, drug- and toxin-induced PAH, and PAH associated with conditions such as connective tissue disease (CTD)-PAH, HIV-PAH, congenital heart disease (CHD)-PAH, and schistosomiasis. The Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL) is a database used in an ongoing observational cohort study of PAH designed to enroll prevalent and/or incident patients in the United States with Group 1 PAH. This study reported the highest female to male ratio of 4.1:1 in IPAH patients as compared to the French registry (1.9:1) and the National Institutes of Health registry (1.7:1) (Badesch

et al., 2010; Humbert et al., 2006; Rich et al., 1987). A female bias was also reported in other sub-categories of Group 1 PAH which include CHD-PAH (2.8:1), CTD-PAH (9.1:1), and Drugs/toxins-PAH (5.4:1) (Badesch et al., 2010). We have provided a descriptive table of female to male ratios reported from these PAH registries (Table 1.).

Table 1: Summary of PAH Registry Female to Male Ratios

Registry	Time	Cohort	No. Patients	Female : Male Ratio	References
REVEAL	2006-2007	Mean age 53 yr IPAH, HPAH, APAH, Drug/toxin-induced PAH	2525	4.1:1 IPAH 3.8:1 APAH 5.4:1 Drug/toxin-PAH	(Badesch et al., 2010)
French	2002-2003	Mean age 50 yr IPAH, HPAH, Drug/toxin-induced PAH	674	1.9:1	(Humbert et al., 2006)
NIH	1981-1985	Mean age 36 yr IPAH, HPAH	187	1.7:1	(Rich et al., 1987)
IPAH, idiopathic PAH; HPAH, heritable PAH; APAH, associated PAH					

Estrogen as a risk factor in PAH:

In human studies, pulmonary hypertension (Kleiger et al., 1976) and vessel lumen-obliterating lesions (Irey & Norris, 1973) have been associated with oral contraceptives. Hormone replacement therapy has also been associated with severe PAH in post-menopausal women (Taraseviciute & Voelkel, 2006). While these hormone therapies contain estrogens, the contribution of estrogen to PAH has been debated because of paradoxical gender effects observed in animal models. The chronic hypoxia-induced pulmonary hypertension model showed that male rats are more susceptible than females while estrogen treatment was shown to protect against monocrotaline (MCT)-induced

pulmonary hypertension [88,89]. In contrast, there are reports of chronic E2-induced hypoxic pulmonary hypertension in ovariectomized female rats (Artem'eva, Kovaleva, Medvedev, & Medvedeva, 2015; Kovaleva, Artem'eva, Medvedev, & Medvedeva, 2013a, 2013b). The contradictory effects of E2 in the MCT-induced model may be partly due to differences in pulsatile versus continuous E2 exposure which cannot fully recapitulate what occurs in the human body. Another factor that may complicate our understanding comes from the assumption that exogenous and endogenous E2 act similarly on the pulmonary vasculature. Recently, a study has shown that reduction of endogenous E2 by ovariectomy or aromatase inhibitor treatment, decreased vessel muscle thickening or vessel obliterative lesions (Mair et al., 2014). This study used both the hypoxic mouse and the Sugen 5416 plus chronic hypoxia (SuHx) rat model of PAH. In the SuHx model, rats are given a single injection of the VEGF receptor blocker Sugen 5416 and exposed to hypoxia for several weeks (Abe et al., 2010). The protection observed with the anastrazole treatment of the previous study was corroborated by a study with metformin treatment which reversed PAH and decreased pulmonary vascular remodeling via aromatase inhibition (Dean, Nilsen, Loughlin, Salt, & MacLean, 2016). E2 treatment was reported to improve heart function in the SuHx model (Frump et al., 2015), but its effect on the development of plexiform lesions, a hallmark of human PAH reproduced in the SuHx rat model, was not reported. Further studies on the development of obliterative intimal lesions in a chronic E2 treated SuHx model would be helpful because of the previously mentioned reports of chronic E2-induced pulmonary hypertension in ovariectomized female rats.

Other rodent models of PAH have reported a female bias toward PAH. Anorectic drugs such as dexfenfluramine (Dfen) have been shown to induce PAH in only female mice

(Dempsie et al., 2013). Treatment of rats with 4, 4'-methylenedianiline (DAPM) induced female specific smooth muscle hyperplasia of the pulmonary vessels (Carroll-Turpin et al., 2015). Genetic based mouse models have also shown sex differences in PAH susceptibility. Female mice overexpressing calcium binding protein S100A4/Mts1 (Mts1) were more susceptible to develop PAH and developed plexiform-like lesions (Dempsie et al., 2011). In mice overexpressing the serotonin transporter (SERT), only female SERT+ mice developed PAH (White et al., 2011). Since E2 treatment increased the severity of PAH in female SERT+ mice, it is plausible that estrogen is a significant risk factor for the development of PAH. Furthermore, the inhibition of obliterative vascular lesions by aromatase inhibitor anastrozole in the SuHx model supports the idea that E2 mediates its adverse effects by increasing the formation of plexiform lesions in PAH. We have provided a summary table of the discussed in vivo models that support a role of female sex and/or estrogen in PAH (Table 2).

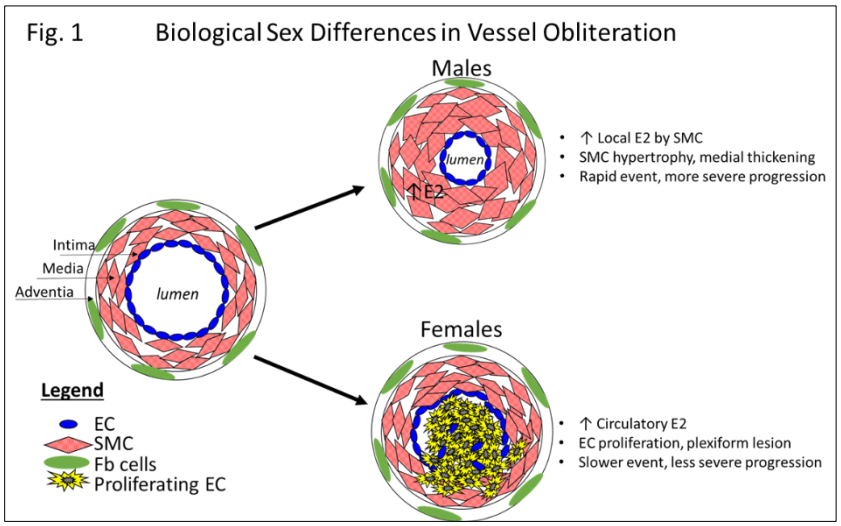
Table 2: Models of PAH that support female sex bias and/or detrimental effect of estrogen

Model	**Species**	**Findings**	**References**
Chronic Hx + E2	rat	Female develop hypoxic pulmonary hypertension; E2 detrimental	(Kovaleva et al., 2013a, 2013b)
SuHx	rat, mouse	Male and female develop PAH Aromatase inhibition protective	(Abe et al., 2010; Dean et al., 2016; Mair et al., 2014)
Dexfenfluramine	mouse	Female only develop PAH; Ovx protective	(Dempsie et al., 2013)
4,4'-methylenedianiline	rat	Female only develop PAH	(Carroll-Turpin et al., 2015)
Mts1+	mouse	PAH in female > male Ovx protective	(Dempsie et al., 2011)
SERT+	mouse	Female only develop PAH	(White et al., 2011)

		Ovx protective; E2 detrimental	
Hx, hypoxia; E2, 17β-estradiol; SuHx, Sugen 5416 plus hypoxia; Mts1+, overexpression of calcium binding protein S100A4/Mts1; SERT+, overexpression of serotonin transporter; Ovx, ovariectomized.			

Biological based mechanisms for sex differences in PAH

Circulatory levels of E2 cannot explain why males who have lower levels of E2 than females develop PAH much sooner and have poorer survival. A potential explanation may lie in different characteristics of the vascular pathology which obliterate the pulmonary artery. Blood vessels are composed of an outer layer of adventitial fibroblasts, a middle layer of smooth muscle cells (SMC), and an inner layer of endothelial cells (EC). The medial thickening of pulmonary arteries is considered the earliest pathological change in PAH (Rubin, 1997). Chronic hypoxia-induced PAH is characterized by medial thickening (Meyrick & Reid, 1980; M. Rabinovitch et al., 1986). Experimental data from rodent models attribute the thickening to pulmonary arterial SMC hypertrophy and extracellular matrix deposition in proximal pulmonary arteries (Kobs, Muvarak, Eickhoff, & Chesler, 2005; Pak, Aldashev, Welsh, & Peacock, 2007; Stenmark, Fagan, & Frid, 2006). In contrast, severe IPAH is characterized by clustered proliferation of EC that results in concentric obliteration of the lumina by vascular structures called plexiform



lesions, which consist of the monoclonal proliferation of EC and are reported in the late stages of PAH (R. M. Tuder, Groves, Badesch, & Voelkel, 1994). Three-dimensional analysis of the plexiform lesion indicated that plexiform lesion is functionally important in pathogenesis because blood flow is severely obstructed along the entire length of a vessel affected by a single plexiform lesion (Cool et al., 1999). Although both human pulmonary arterial SMCs and ECs have been shown to proliferate when exposed to E2 (Tofovic et al., 2008; White et al., 2011), a difference between these cell types from PAH patients has been shown with the expression of an estrogen synthesizing enzyme. Pulmonary arterial SMC were shown to highly express aromatase in PAH patients, but it was absent in human pulmonary arterial EC (Cakan et al., 2009). Thus, the cell-context specific difference in aromatase expression can help to explain why men have more severe PAH. Since men are ill equipped to defend against a higher body burden of E2 when compared to women, we propose that the local concentration of E2 in pulmonary arteries is higher in men with PAH. This difference in lung concentration of E2 contributes to the reported faster progression and severity of PAH in men. Although proliferative changes in pulmonary arteries play a significant role in the development of PAH, evidence from the SuHx model of PAH suggest that fibrosis is a determining factor in the poor survival rate of male patients with PAH (Rafikova et al., 2015). In this study, female rats with PAH primarily showed vasculoproliferative changes in the pulmonary artery while males showed severe fibrosis in the adventitia and media of the pulmonary artery. Severe fibrosis observed in male pulmonary arteries including myocardial fibrosis was associated with impaired heart function and lower survival rates compared to females.

Unlike SMCs exposed to the local synthesis of E2 by aromatase, the proximity of EC to the bloodstream allows these cells to be directly exposed to circulatory E2. The possibility that estrogen is involved in the growth of EC in the plexiform lesion is suggested by the increased incidence (2.8-fold) in female PAH patients of plexiform lesions compared to their male counterparts (Stacher et al., 2012). A plausible mechanism for estrogen's involvement in plexiform lesion growth comes from evidence that infantile hemangiomas, a different type of vascular lesion, are reported with increased incidence in females with elevated levels of circulating E2 (Sasaki, Pang, & Wittliff, 1984). The combination of hypoxia and estrogen has been demonstrated in vitro to synergistically enhance EC proliferation (Kleinman et al., 2007), which we postulate also contribute to the growth of plexiform lesions. Higher circulatory E2 may therefore explain the predominance of plexiform lesions in women with PAH because it acts directly on EC prolfieration. Plexiform lesions are considered to be a late pathological event compared to the much earlier pathology of pulmonary arterial SMC hypertrophy. This suggests that the plexiform lesions in women PAH patients can take more time to obstruct the pulmonary artery unlike the more rapid hypertrophy of SMCs that occurs in men, which can help to explain sex differences in disease severity. A summary scheme of the sex difference in vessel obliteration is shown in Fig. 1.

Estrogen-induced obliterative vascular lesions:

Vessel obliterating lesions have been reported in female biased lung diseases including idiopathic interstitial pneumonia (Hallowell, Reed, Fraig, Horton, & Girgis, 2012), COPD (Santos et al., 2002), and IPAH (R. M. Tuder et al., 1994). Eearly appearance

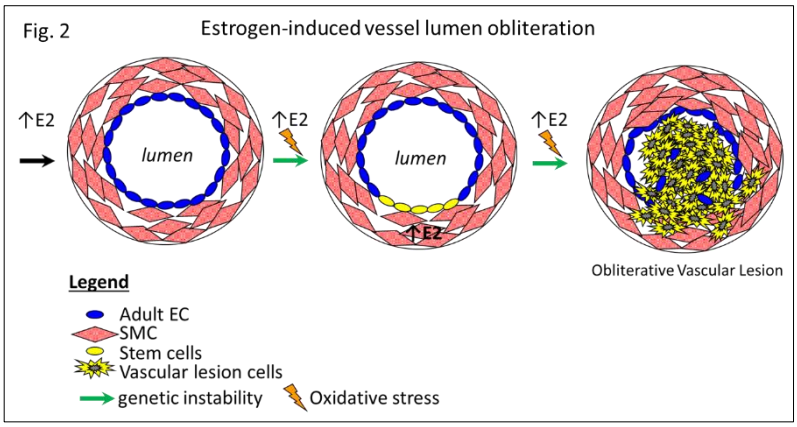
of obliterative vascular lesions observed in mild cases of COPD, mentioned previously, suggests that the growth of vascular lesions occurs much earlier than at the end stage of PAH. Uncontrolled vascular cell growth has been postulated as the major mechanism involved in PAH pathogenesis (Rubin M. Tuder et al., 2007). More specifically, the hypertrophic growth of SMC is responsible for progressive thickening of blood vessels of the lung that ends in obstruction (Marlene Rabinovitch, 2008). Proliferative endothelial lesions that result from a focal budding of EC are also reported to be an aggressive cell phenotype associated with a poor prognosis in NSCLC and severe IPAH (Rojiani & Dorovini-Zis, 1996; Tanaka et al., 2003; R. M. Tuder et al., 1994). Despite progress in understanding IPAH, current therapy (epoprostenol and derivatives, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors) has become a major clinical barrier for the treatment of patients with end-stage IPAH. Median survival for IPAH patients in the United States was reported to be only 2.8 years without treatment (D'Alonzo et al., 1991). Although these drugs allow clinical, functional, and hemodynamic improvements, the prognosis of patients remains poor because a critical aspect of end-stage IPAH is the continual growth of vascular lesion cells which eventually obliterate the lumen. Anti-proliferative agents such as tyrosine kinase inhibitors have been investigated in IPAH, however, safety concerns have restricted the clinical application of these drugs and therefore the need to identify new therapeutic targets has remained.

The molecular pathogenesis of vessel-lumen obliterating lesions in humans remains unknown. Largely the focus has been on loss-of-function mutations in the BMPR2 gene observed in approximately 80% of familial PAH and in 20% of patients with sporadic PAH (Machado et al., 2009). In addition to BMPR2, estrogen receptor signaling has been

implicated to be involved in the pathogenesis of obliterative vascular lesions. However, these studies have not been consistently focused on investigating target cells (vascular lesion "initiating" cells) that are susceptible to genetic and epigenetic instability and ultimately progress into the plexiform lesion. Investigators have conveniently used either adult EC or SMC without considering the in vivo plexiform lesion histopathology. Histopathology of both human and animal model obliterative vascular lesions suggests they are multicellular and just like solid tumors contain stem cells that may be involved in the pathogenesis of IPAH (Hanahan & Weinberg, 2011). Surprisingly, there are numerous clinical and experimental data of vessel stem cells in the blood and the lungs of various forms of PAH (Felty, Sakao, & Voelkel, 2015). Although several different cell types, including vascular SMCs, inflammatory cells, and fibroblasts are involved in the vasculoproliferative process; we recognize EC to be the initial site of injury. Previously we showed that E2 treatment leads to an increase in macrophage cell proliferation and secretion of TNF-α (Felty & Roy, 2002; D. Roy & Cai, 2002) which could contribute to vascular lesion formation via paracrine effects with other cell types in the vessel wall. Estrogen involvement in immune responses in lung diseases described previously support an inflammatory role in PAH.

Endothelial and smooth muscle cells are directly involved in the pathology of plexiform lesions. Pulmonary arterial SMC express aromatase which allows for the local production of E2 whereas human pulmonary arterial microvascular EC do not possess this enzyme (Mair et al., 2014). Higher aromatase activity in pulmonary arterial SMCs may lead to locally produced estrogen that acts in an autocrine or paracrine manner, with

possible cross talk between SMC and EC. Besides estrogen synthesis, the metabolism of E2 by another enzyme CYP1B1 may contribute to the formation of lumen obliterating vascular lesions. CYP1B1 expression is increased in pulmonary arterial SMCs from patients with IPAH (White et al., 2012). Cytochrome P450 family member CYP1B1 is a key enzyme involved in the metabolism of E2 to catechol estrogens and expressed in the lung. Oxidation of E2 produces 2 catechol estrogens that, in turn, are further oxidized to the quinones, which can react with DNA resulting in depurinating adducts that can lead to mutagenesis. Genetic instability usually associated with pathological disorders and referring to a range of genetic alterations from mutations to chromosome rearrangements may contribute to the quasi-malignant vascular lesions observed in PAH patients. In support of this concept, chromosomal abnormalities and increased DNA damage have been observed in vessel-lumen obliterating lesions from PAH patients (Federici et al., 2015) and we have shown a positive correlation of oxidative DNA damage (8-OHdG) in benign and malignant vascular tissues (Das & Felty, 2014). In vivo experimental evidence in support of genotoxic damage in PAH was shown in the SERT+ model of PAH, female SERT+ mice showed increased levels of 8-OHdG (Johansen et al., 2016). We have provided a hypothetical mechanism by which chronic estrogenic stress-induces genetic instability in stem cells that progresses to form the obliterative vascular lesion (Fig. 2).



Conclusion:

Mitogenic and genotoxic effects of estrogen may be a common pathogenic mechanism to explain the presence of obliterative lesions in lung tissue and vessels. Estrogen has been shown to promote lung disease in experimental models of PAH, lung cancer, LAM, and benign metastasizing leiomyoma (BML) (Mair et al., 2014; Márquez-Garbán, Chen, Goodglick, Fishbein, & Pietras, 2009; Shim et al., 2013; Yu, Astrinidis, Howard, & Henske, 2004). Studies have reported associations between estrogen concentrations in lung disease. Lung tissues from interstitial pneumonia are reported with 2.8-fold higher levels of E2 (Taniuchi et al., 2014), NSCLC has high intratumoral E2 concentration associated with aromatase expression (Márquez-Garbán et al., 2009); and more recently, higher concentrations of E2 have been associated with the risk of PAH in men (Ventetuolo et al., 2016). Furthermore, higher aromatase activity and circulatory E2 have been reported to increase the risk of PAH in patients with portopulmonary hypertension (Roberts et al., 2009). Based on the evidences discussed in this review, female gender bias towards obliterative lung disease may be attributed to the hormone estrogen.

Even though women have a 3-4 times higher prevalence than men of PAH, circulatory E2 levels cannot explain why men develop PAH much sooner and have poorer survival. Pulmonary arterial SMC hypertrophy that contributes to medial thickening is considered one of the earliest pathological changes observed in chronic hypoxia-induced PAH. We postulate that the severity of PAH in males is due to high local concentration of E2 produced by pulmonary arterial SMC, which leads to hypertrophy, vasoconstriction, and vessel obstruction. Since males cannot defend against a higher body burden of E2

unlike females, males succumb to a rapid and more severe progression of vascular obliteration in PAH. Females are more susceptible to develop pulmonary vascular disease characterized by obliterative hyper-proliferative vascular lesions because EC are directly exposed to circulatory E2 from the bloodstream. Higher circulatory E2 found in women can therefore explain the predominance of plexiform lesions in female PAH patients. The molecular mechanisms that underlie sex differences in vessel-lumen obliterating lesions remain largely unknown and this is a major hurdle to identifying novel sex-dependent molecular targets to treat obliterative vascular lesions. Understanding the molecular basis of this gender disparity in PAH may offer a new treatment paradigm in this devastating disease that currently has a high unmet clinical need.

Emerging evidence suggest that a local imbalance between testosterone and E2 levels influences the development of lung disease in COPD and PAH. In light of this information, we propose that novel therapies targeted against local tissue production of estrogen may be of clinical benefit and lead to novel therapeutic strategies in treating estrogen dependent lung diseases. The activation of the farnesoid X receptor (FXR) has been reported to inhibit aromatase at the level of mRNA, protein, and enzymatic activity (Catalano et al., 2010) and represents a novel therapeutic mechanism to reduce local tissue estrogen production in the lung. The potential inhibitory effect of FXR on aromatase is significant because a new class of drugs (FXR agonist, such as obeticholic acid, OCA) was recently shown to prevent monocrotaline-induced PAH (Vignozzi et al., 2017). Similar cardiopulmonary protective effects of OCA treatment have been demonstrated also in blemycin-induced pulmonary fibrosis (Paolo Comeglio et al., 2017). FXR activation by treatment with OCA was shown to protect against bleomycin-induced lung damage by

suppressing epithelial-to-mesenchymal transition (EMT), inflammation, and collagen deposition. This may be of major benefit in the treatment of PAH. Endothelial-to-mesenchymal transition (EndMT) a process similar to EMT has been implicated to contribute to obliterative vascular remodeling in idiopathic PAH (Hopper et al., 2016). Furthermore, the release of cytokines IL-1β, IL-6, TNF-alpha, and IL-10 by macrophages present in pulmonary lesions are suggested to play an important role in the pathogenesis of PAH [41]. Since FXR activation was shown to suppress EMT as well as cause a dose-dependent reduction of proinflammatory cytokines, the FXR class of drugs are highly innovative therapeutic agents for the treatment of estrogen dependent obliterative lung diseases including PAH.

Hypothesis

Estrogenic chemicals influencing ID3 target genes contribute to susceptibility of PAH

Specific Aims

Specific Aim 1: Collect retrospective environmental exposure data from NHANES database and gene expression data from the GEO database.

Specific Aim 2: Investigate the association between exposure to estrogenic chemicals and subjects at risk for PAH.

Specific Aim 3: Identify ID3 regulated molecular gene networks within PAH pathways.

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CHAPTER 3

MANUSCRIPT 1

EXPOSURE TO POLYCHLORINATED BIPHENYLS AND RISK OF PULMONARY ARTERIAL HYPERTENSION IN THE UNITED STATES POPULATION: ANALYSES OF NHANES DATA 1999-2004

ABSTRACT

Background:

Pulmonary arterial hypertension (PAH) is a disease with higher risk in women. Endocrine disrupting chemicals (EDC) are associated with increase of estrogen activity and metabolism which leads to increase the risk of PAH. Polychlorinated biphenyls (PCBs) are known to be endocrine disrupters and to alter estrogenic activity in human.

Objectives:

The objective of this study was to evaluate the association between exposure to PCBs and risk of PAH among U.S. population.

Methods:

Using Centers for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) data from 1999-2004, we selected subjects identified at risk of PAH and ≥ 20 years old with available blood samples data. We used lipid adjusted serum levels of six PCBs congeners (including PCB 74, 99, 118, 138, 153, and 180), the sum of non-dioxin-like PCBs (PCB 099 + 138 + 153+187), and the sum of dioxin-like PCBs (PCB 074 + 118) along with other variables provided from different health examinations. We included gender, age, BMI, and race and confounders in our analysis.

PCB concentration geometric means were analyzed in subjects identified ≥ 20 years old at risk of PAH and not at risk of PAH.

Results:

Different analysis showed that all PCBs congeners were significantly higher in subjects identified at risk of PAH than subjects identified not at risk of PAH. Multiple limit of detections (LODs) were used to evaluate PCBs associations with the risk of PAH. All LOD showed higher association of PCBs congeners concentrations in subjects identified at risk of PAH including (PCBs concentrations >LOD, > 50^{th} percentile, 50^{th}-75^{th} percentile, and ≥75^{th} percentile).

Conclusions:

Using NHANES data from 1999-2004, we found that exposure to PCBs increases the risk of PAH in the U.S. population ≥ 20 years old. However, epidemiological studies need to be conducted to provide better explanation of PCBs association and other diseases.

Keywords: PCBs, NHANES, pulmonary arterial hypertension, PAH

MANUSCRIPT 1

EXPOSURE TO POLYCHLORINATED BIPHENYLS AND risk of PULMONARY ARTERIAL HYPERTENSION IN THE UNITED STATES POPULATION: ANALYSES OF NHANES DATA 1999-2004

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a vascular disease that affects pulmonary arteries and causes increase in the mean arterial pressure (Rich et al., 1987). PAH has poor prognosis with duration time of 2.8 years, from the beginning of the symptoms (D'Alonzo et al., 1991). PH surveillance reported that prevalence of 5.4

/100,000 population which was reported for the year 2002 in the U.S. and the same report showed that the rate of death was decreased among men while increased among women, which reflect the higher incidence of PAH among women. Data from NCHS reported 5.3% of deaths because of cardiopulmonary diseases in 2008 (NHLBI, 2012). However, women reported higher cases than men during the same period. Also, black race reported the highest death rates comparing with other race groups with 7.3 /100,000 population. Elderly, > 85 years was the highest age group with number of death with 63.2 /100,000 population (CDC, 2005b).

Besides gender, many risk factors were reported to be associated with increased risk of PAH including: hypertension, abnormal lung function, diabetes, uric acid level, age, insulin status, obesity, thyroid problems, and female sex hormones. Insulin resistance (IR) was reported to be associated with PAH and poor survival rate in females (Zamanian et al., 2009). Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL) showed significant association between obesity (BMI > 30, P-value=0.004) and PAH comparing with normal and underweight patients (Burger et al., 2011). Zhang and colleagues showed that serum uric acid levels in PAH patients (405±130 μmol/L) were higher than control subjects (344±96 μmol/L; $P<0.05$). Reference values were 360 μmol/L and 420 μmol/L for females and males, respectively (Zhang, Ma, & Wang, 2013). Older age was reported to be a risk factor of PAH comparing with younger patients. Schachna reported that elderly > 60 years old had 2 times higher risk of PAH than patients < 60 years old (OR, 2.30; 95% CI, 1.32 to 3.99) (Schachna et al., 2003). This also supports the finding of the age specific death rate for elderly by CDC in 2005 (CDC, 2005b). Many studies showed high prevalence of thyroid

problems and risk of PAH. In 2007, Li and colleagues reported that risk of PAH was 2 times higher in people who had thyroid diseases than others (OR, 2.53; 95% confidence interval, 1.55 to 4.08; $p < 0.001$) (J. H. Li et al., 2007). Diabetes showed high prevalence in patients with PAH than control (OR, 1.53; 95% CI, 1.45 to 1.60; $p < 0.001$). Gender difference was reported in France national registry that females had higher risk of PAH than males (OR 1.9; $P < 0.035$) (Marc Humbert et al., 2006). Also, REVEAL study reported the highest female to male ratio of 4.1:1 in IPAH (Badesch et al., 2010). Moreover, oral contraceptives and hormone replacement therapies were reported to be associated with severity of PAH and formation of obliterative lesions in idiopathic-PAH (IPAH) (Irey & Norris, 1973; Taraseviciute & Voelkel, 2006).

Polychlorinated biphenyls (PCBs) are group of complex chemical contaminants in the environment that have different effects on the human body including neurodevelopmental effects (Ribas-Fito, Sala, Kogevinas, & Sunyer, 2001). They were widespread into the industries by 1920's as transformers of many products. PCBs were known to have high resistant of degradation by chemical and biological factors (Jensen, 1987). Even though they were banned during the 1970's, their exposure remained in the environment by many ways. Exposure to PCBs was reported in wildlife tissues, animal fat, and even in human serum with small amounts (Dewailly et al., 1999). PCBs are known to be endocrine disrupting chemicals (EDCs) which have different effects on health by altering cellular activity, pathways and metabolism. They can be found in plastic products, pesticides, and flame retardants (J. R. Roy et al., 2009).

Estrogen and EDCs were associated with inducing many molecular level processes such as inflammation. Estrogen reported to have direct effect on inflammatory

cytokines such as TNF-α, IL-6, and IL-1β (D. Roy et al., 2007). Various EDCs such as PCB126 congener are associated with modifications of many induced-inflammation genes including IL6, TNF, and CXCL8; while PCB 153 is associated with modifications of SOD2, AHR, and IL6. Bisphenol A is shown to be associated with inflammatory induced genes including PARP1, MMP9, HMOX1, and IL1B. Nevertheless, PCB153 was also reported to alter smooth muscle cell phenotypes and pulmonary endothelial (Charlier, Albert, Zhang, Dubois, & Plomteux, 2004).

In this study, we evaluated the association between 6 PCB congeners, the sum of dioxin-like PCBs, and the sum of non-dioxin-like PCBs in subjects at risk of PAH in the National Health and Nutrition Examination Survey (NHANES) between the years 1999-2004. We aimed to 1) describe the mean of PCB concentration in population ≥ 20 years of age who were identified at risk of PAH compared with subjects identified not at risk of PAH; and 2) use different LOD levels of PCBs' to evaluate the higher concentration of PCBs and the risk of PAH.

METHODS

Study Design

The main objective of this study was to investigate the association between exposure to EDCs such as PCB and risk of PAH and pulmonary diseases in the U.S. general population. To clarify and confirm the suggested role of EDCs, we used NHANES data from 1999-2004 provided by Centers for Disease Control and Prevention (CDC) to conduct a secondary statistical analysis. National Health and Nutrition Examination Survey (NHANES) is a program developed by National Center for Health Statistics (NCHS) to assess the nutritional and health status for children and adults in the

United States. NHANES started in the early 1960s producing different surveys for many health issues as well as many age groups. Since 1999, NHANES started producing annually continuous interviews and surveys targeting different health and nutritional measurements for evaluation purposes including demographic, dietary, health-related questions, socioeconomic, dental, medical, physiological measurements, and laboratory tests. An average of 5,000 participants enrolled every year and 15 counties across the nation are visited every year. To acquire a representative sampling of the population, many parameters needed to be taken into consideration for oversampling. Oversampling would be sub-grouped, stratified in strata's and then divided into different locations. Oversampling includes age (adolescents (12-19 years), adults (≥ 60 years)) and ethnicity/race (non-Hispanic black and Mexican Americans). After being eligible, the participants undergo series of interviews and examinations in a specially equipped mobile evaluation clinic (MEC). MEC examination consist of collections of urine and blood samples, personal interviews, and dental and physical examinations. Urine is collected from participant if ≥ 6 years, and blood samples collected from participant if ≥ 1 year. CDC institutional board approved the study protocol and all participants signed a consent before joining the NHANES. All data are strictly confident and coded in different identification factors to keep privacy. Transportation, personal care (baby/ elderly) and cash payment for participating are included (CDC, 2012).

Laboratory Methods

Exposure to environmental chemicals can be through different routes including dermal, ingestion, or inhalation. Thus, measurements of these chemicals or their

metabolites in blood and urine can reflects the amount enters the body. About 2,500 subsamples participated in each 2-years cycle for blood serum and urine samples. CDC used many procedures to measure environmental chemical exposure including isotope dilution mass spectrometry, graphite furnace atomic absorption spectrometry, or inductively coupled plasma mass spectrometry. These procedures or protocols are provided by Division of Laboratory Sciences, National Center for Environmental Health, and CDC's Environmental Health Laboratory. Serum blood samples are measured per whole weight or per gram of total lipid to reflect the amount of PCBs stored in body fat. During the MEC exam and transit location, both urine and blood serum samples are stored in 4 ◦C or froze at -20 ◦C before shipping to the Division of Laboratory Sciences, National Center for Environmental Health, CDC's Environmental Health Laboratory (CDC, 2013b).

Serum PCB

One-third sampling procedure was used to measure PCB levels in blood serum samples of participant's ≥ 12 years old in 1999-2000 and 2003-2004 survey cycles. In 2001-2002 survey cycle, NHANES used age inclusion to be ≥ 20 years old. PCBs were analytically reported on a lipid-adjusted basis (ng/g or ppb) and a whole weight (ng/g or ppb).

Questionnaire Methods

Multiple time points included collection of demographic, reproductive health, and medical health data. Intensive two weeks training program including exercises, training interview, and training on personal audio computer interview was conducted and supervised by NCHS and accompanied staff. Also, multiple quality control monitoring

procedure were conducted through full pilot test before starting questionnaire. During a household interview, people ≥ 16 years old provided demographic data. During the personal interview in Mobile Examination Center (MEC), females ≥ 12 years old provided reproductive health data. For both males and female (constriction by age and gender), medical health data was provided during the MEC interview (CDC, 2012).

Selection of Participants

Inclusion criteria used for the study included people during MEC were ≥ 20 years old who completed and provided, personal interview and urine and blood samples, respectively. A participant will be included in the study population if PCB measurements were available. In addition, people at risk of PAH were selected based on an inclusion criteria specifically for this variable. We created the Risk of PAH variable based on any risk factors that reported individually to be associated with PAH in literature. As mentioned earlier in introduction, these risk factors included hypertension, diabetes, thyroid problems, uric acid level, insulin status, and female hormone (only for female participants). Risk of PAH variable was created after sum of all risk factor scoring to be at risk of PAH if > 2 for male participants and to be 3 for female participants (CDC, 2012). Participant will be deleted from the dataset if any of these risk factors answer or level were missing.

Sample Weights and Limits of Detection

Sample weight is required when merging NHANES complex survey data to avoid bias from non-response selection. As mentioned earlier PCB measurements were collected in one third selection procedure, so weighting these environmental chemical is

also required. The weight variable was provided in each 2-year cycle from 1999-2004 "for all variables; and it was created for the environmental chemical based on NHANES analytic guidelines (CDC, 2013b). Detection limit for each of the environmental chemicals was divided into 0 if below detection limit and 1 if above detection limit for PCB. However, if PCB serum level was below the detection limit, it is required to be divided by the square root of 2. CDC improved their procedure and techniques yearly, which resulted in changing in the detection limit of the environmental chemicals (CDC, 2013). Individual PCB congener has their own LOD because they have different sample volumes. Thus, calculations for each of the environmental chemicals is required based on sample volume for each serum and urine sample to be analytically sensitive. Serum samples environmental chemical LOD was calculated per amount of lipid and urine samples environmental chemical LOD was calculated per volume of urine (CDC, 2013b).

EDC Exposure Assessment

PCB Variables.

Number of available PCB data was varied in different NHANES cycles (1999-2000, 2001-2002, and 2003-2004) and to avoid bias of low detection limit samples, we selected 6 PCB congeners which had measurements above 60% of the population samples. Based on this inclusion criteria, the selected PCB congeners to find the association with the risk of PAH were: 2,4,4',5-tetrachlorobiphenly (PCB 74), 2,2'4,4',5-pentachlorobiphenyl (PCB 99), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 2,2',3,4,4',5-hexachlorobiphenyl (PCB 138), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), and 2,2',3,4,4,5,5'-heptachlorobiphenyl (PCB 180). We also analyzed the sum of

dioxin-like PCBs (PCB 74 and PCB 118) and of non-dioxin-like PCBs (PCB 99, PCB 138, PCB 153, PCB 180). Using complex survey NHANES data for 1999-2004 merged year cycles, we analyzed the association of these PCBs and the risk of PAH in ≥ 20 years old.

Reproductive and Medical Health Variables

Data was collected from medical health questionnaire, reproductive health questionnaire, and laboratory data to analyze the association between serum PCB and the risk of PAH.

Medical Health Questionnaire.

Using medical health questionnaire, the participant > 20 years old had to provide a response to risk factors of PAH including "have you ever been told by a doctor or health professional that you have diabetes?", "Has a doctor or other health professional ever told that you had a thyroid disease?" Diabetes risk factor was scored 0 if answer was NO, and scored 1 if answer was YES. Thyroid problem risk factor was scored 0 if answer was NO, and scored 1 if answer was YES. Each of these variables was transformed into 0 if no risk of PAH and 1 if at risk of PAH.

Reproductive Health Questionnaire.

Using reproductive health questionnaire, female participants > 20 years old had to provide a response to risk factor of PAH "have you ever used female hormones such as estrogen and progesterone?" Female hormone risk factors (female participants only) was

scored 0 in the female participant answered No, and scored 1 if the female participant answered YES.

Laboratory data.

Using laboratory data measurements, uric acid level should be available for the included participant, as well as measurements of both (triglycerides and HDL-Cholesterol) to calculate insulin status (insulin sensitive or insulin resistance). Uric acid risk factor is a continuous variable, so males and females have different references. Males participants scored 0 if any had uric acid level < 419 mmol/L, and scored 1 if any male participant had uric acid level > 420 mmol/L. Females participants scored 0 if any had uric acid level < 359 mmol/L, and scored 1 if any male participant had uric acid level > 360 mmol/L. Insulin status was calculated by dividing triglycerides level by HDL-cholesterol level to be < 1.99 “insulin sensitive (IS)” or > 2.00 “insulin resistance (IR)”. Insulin status scored 0 if IS and scored 1 if IR. Risk of PAH variable was created after sum of all risk factor scoring to be at risk of PAH if > 2 for male participants and to be 3 for female participants (CDC, 2012). Participant will be deleted from the dataset if any of these risk factors answer or level were missing.

Statistical Analysis

We selected people > 20 years old and had available data for PCBs, medical health, and reproductive health to be included in the analysis to find the association with the risk of PAH. Because non-normal distribution of the PCBs measurements, they were log transformed before the analysis. Also, data was weighted using the required weight by the National Center for Health Statistics guidelines to be representative to the whole

population. We used stratum and PSA to estimate variance of the demographic data. We calculated geometric means (GM), geometric standard errors (GSE), and proportions for all PCBs and their associated with the risk of PAH. We used Student's t-test GM to compare PCBs level's mean and the association with the risk of PAH. To find the associated risk of PAH with each of the PCBs', we used logistic regression models to calculate the odds ratios (ORs) and their 95 % confidence intervals (CI).

Because of small number of participants who had risk of PAH, we used NCHS guidelines to categorize the limit of detection (LOD) into many levels to perform different analysis based on the LOD (51). These LOD including 1) < LOD vs. ≥ LOD; 2) < LOD to 50th percentile (reference) vs. ≥ 50th percentile, and 3). < LOD to 50th percentile vs. 50th percentile to 75th percentile vs. ≥ 75th percentile. Statistical analysis were performed using SPSS software (release 20) for windows and SAS software for windows (release 9.4; SAS Institute Inc. Cary, N.C.). We used 5% ($P \leq 0.05$) as significance level for all analysis.

Potential Confounding Variables.

We included many potential confounding variables as categorical variables which are gender, age, body mass index (BMI, kg/m^2), annual family income, smoking status, alcohol consumption, race, and education level. Gender were categorized into male (1) and female (2), age was categorized into three age groups including 20-59 years (1), 60-74 years (2) and ≥ 75 years (3). BMI was categorized into > 25 kg/m^2 (1), 25-30 kg/m^2 (2), and ≥ 30 kg/m^2 (3). Annual family income was categorized into 0-$24,999 (1), $25,000-$54,999 (2), $55,000-$74,999 (3), and ≥ $75,000 (4). Smoking status was categorized into smokers (1) and non-smokers (2), and alcohol consumption was

categorized into consumers (1) non-consumers (2). Race was categorized into non-Hispanic White (1) and others (2), and education level was categorized into three categories < 12^{th} grade (1), 12^{th} grade (2), and > 12^{th} grade (3). All confounding variable data included in the analysis to find the association of PCB and risk of PAH was used from the demographic file. Categorization of age and race into specific ranges was to avoid bias in the results due to low number of people at risk of PAH in younger ages and other races.

RESULTS

Descriptive Statistics.

The study population who completed medical health questionnaire, reproductive health questionnaire; and had available laboratory data to analyze the association between serum PCB and the risk of PAH were 4,495 participants ≥ 20 years old. Among the included population, 166 (3.7%) of them identified at risk to PAH and 4,329 (96.3) of them were not at risk of PAH (Table 3). Males reported 25 (0.41%) of them identified at risk to PAH, while 141 (2.96%) females were identified at risk to PAH. However, 2092 (47.78%) males and 2237 (48.86%) females were not at risk of PAH, respectively (Table 3). Race distribution reported 94 (2.72%) of people at risk of PAH to be non-Hispanic white and 69 (0.65%) were others. However, people not at risk of PAH were 2197 (69.13%) non-Hispanic white and 2132 (27.5%) were others (Table 3). Age was categorized into 3 group; where 47 (1.45%) of people at risk of PAH and 2888 (76.09%) of people not at risk of PAH were in 20-59 years old group. Most participants at risk of PAH among all age groups were in 60-74 years old group with 78 (1.28%), while same age group reported 906 (13.67%) participant not to be at risk of PAH. Elderly > 75 years

old were the smallest number among all age groups with 41 (0.63%) at risk of PAH and 535 (6.86%) not at risk of PAH (Table 3). Obese participant reported the highest number among people at risk of PAH with 83 (2.32%), followed by 54 (1.39%) and 19 (0.58%) for overweight and normal weight, respectively. However, people not at risk of PAH were 1079 (35%), 1149 (33.35%), and 970 (27.35%) for normal weight, overweight, and obese, respectively (Table 3). Annual family income reported 0-$24,999 group to represent the highest number of population in both risk of PAH 81 (1.29%) and 1692 (28.12%) not at risk of PAH. In the group of $25,000-$54,999, there were 47 (1.17%) at risk of PAH and 1242 (28.66%). However, $55,000-74,999 group reported the lowest number of people at risk of PAH and people not at risk of PAH with 10 (0.39%) and 390 (11.01%), respectively (Table 3). No significant difference in distribution of people at risk of PAH in education variable, however, people with high education level $\geq 12^{th}$ grade reported to be the highest not at risk of PAH with 1943 (53.28%) (Table 3). Most subjects at risk of PAH were not smokers with 62 (3.24%), while 29 (1.52%) were smokers. Subjects not at risk pf PAH reported 711 (46.64%) to be smokers and 899 (48.59%) were not smokers (Table 3). Alcohol users, 20 (0.77%) were not at risk of PAH, while no cases reported to be at risk of PAH. Non-alcohol users were 165 (4.41%) at risk of PAH and 3258 (94.82%) were not at risk of PAH (Table 3).

Individual PCB geometric mean (GM) for people at risk of PAH and not at risk of PAH was represented in (Table 4) with their geometric standard error (GSE). An average number of people at risk of PAH was 153 in all PCB included in the analysis which were above LOD concentration (Table 4). Generally, people at risk of PAH reported significant higher GM for all PCBs than people not at risk. Among GM of all PCB for

Table 3. Descriptive statistics for Pulmonary Arterial Hypertension (PAH) status and selected covariates among population ≥ 20 years old of age, NHANES 1999-2004.

Variable	Risk of PAH n (%)	No Risk of PAH n (%)
Total Population (n, %)	284 (5.08%)	4210 (94.92%)
Gender		
Male	187 (3.16 %)	1930 (45.02%)
Female	97 (1.92 %)	2280 (49.9 %)
Race		
Non-Hispanic White	161 (4.07%)	2133 (67.78%)
Other	123 (1.01 %)	2077 (27.14%)
Age (years)		
20-59	48 (2.38 %)	2851 (75.17%)
60-74	130 (1.79%)	854 (13.17%)
≥75	70 (0.91%)	505 (6.58%)
BMI (kg/m^2)		
Normal Weight	36 (0.71%)	1062 (34.88%)
Overweight	91 (1.99%)	1112 (32.76%)
Obese	140 (3.78%)	913 (25.89%)
Income (yearly family income)		
0-$24,999	140 (1.91%)	1633 (28.49%)
$25,000-$54,999	73 (1.53%)	1216 (28.30%)
$55,000-74,999	15 (0.53%)	385 (10.96%)
≥75,000	56 (1.11%)	976 (27.16%)
Education		
<12th Grade	112 (1.42%)	1342 (18.97%)
12th Grade	72 (1.54%)	963 (23.59%)
≥ 12th Grade	100 (2.12%)	1898 (52.34%)
Smoking		
Yes	47 (2.15%)	693 (46.02%)
No	139 (6.45%)	824 (45.38%)
Alcohol use		
Yes	0	20 (0.77%)
No	282 (6.59%)	3140 (92.63%)

Estimated percent distribution after applying NHANES sampling weights.

people at risk of PAH, GM of PCB 153 was the highest concentration with 51.28 ng/g, while PCB 99 reported the lowest GM concentration among all PCB in people at risk of

PAH with 8.79 ng/g (Table 4). As well, people not at risk of PAH reported lowest GM in PCB 99 with 6.06 ng/g and the highest GM was in PCB 153 with 33.02 ng/g (Table 4).

Table 4. Geometric Mean PCB levels (ng/g) by risk of PAH status among population ≥ 20 years of age with PCB concentration above the LOD, NHANES 1999-2004

		Geometric mean[2] (ng/g) (GSE)			
Analyte[1]	**Non-cases/ Cases**	**No Risk of PAH**	**Risk of PAH**	**No Risk of PAH[3]**	**Risk of PAH[3]**
PCB 74	2904/ 272	7.49 (0.25)	12.76 (0.70)	6.08 (0.20)	8.62 (0.72)
PCB 99	2874/ 267	6.03 (0.15)	8.43 (0.33)	5.29 (0.14)	6.35 (0.37)
PCB 118	2904/272	8.85 (0.26)	15.43 (0.84)	7.25 (0.20)	10.11 (0.85)
PCB 138	2908/ 272	22.54 (0.50)	34.35 (1.59)	19.13 (0.44)	23.56 (1.65)
PCB 153	2908/ 273	32.64 (0.73)	51.13 (2.42)	27.39 (0.63)	35.48 (2.55)
PCB 180	2905/ 273	16.79 (0.59)	33.86 (2.39)	12.75 (0.49)	19.45 (2.22)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)
[2] Geometric means calculated after applying NHANES sampling weights.
[3]Age Adjusted

We analyzed the association between our study population by different age groups and all PCBs GM > LOD. All GM PCBs, in people at risk of PAH and with no risk of PAH, were markedly increased with age with some exceptions in PCBs 99, 153, and 180 in age group 60-74 years, and PCB 180 an age group of ≥ 75 (Table 5). However, people at risk of PAH reported that PCB 153 had the highest GM while PCB 99 reported the lowest GM among all PCBs in the age group of 20-59 years old with 36.89 ng/g and 6.90 ng/g, respectively (Table 5). No significant difference was reported within GM of PCB in the study population in the other age groups 60-74 years old and ≥ 75 years old (Table 5).

We analyzed the association between age adjusted PCB concentrations in study population race groups. Using lipid adjusted PCB concentration, participants categorized as “non-Hispanic white” had higher GM concentrations of all PCBs than people categorized as “other” for not at risk of PAH population (Table 6). However, population

at risk of PAH reported that people categorized as "other" had higher concentrations of PCB 99, 138, 153, and 180, while non-Hispanic white category had higher PCB 74 and 118 (Table 6).

Table 5. Geometric Mean PCB levels (ng/g) by age group and risk of PAH status among population with PCB concentration above the LOD, NHANES 1999-2004

	Geometric mean[2] (ng/g) (GSE, n)					
	Age: 20-59 years		Age:60-74 years		Age: >75 years	
Analyte[1]	No Risk of PAH	Risk of PAH	No Risk of PAH	Risk of PAH	No Risk of PAH	Risk of PAH
PCB74	6.08 (0.20, 2031)	8.62 (0.72, 80)	15.15 (0.80, 572)	15.32 (1.19, 125)	22.65 (1.24, 301)	24.75 (2.01, 67)
PCB99	5.29 (0.13, 2011)	6.35 (0.38, 76)	9.43 (0.45, 564)	9.55 (0.78, 123)	11.68 (0.64, 299)	13.15 (1.14, 68)
PCB118	7.25 (0.20, 2035)	10.11 (0.85, 80)	17.51 (1.04, 569)	19.11 (1.93, 125)	24.71 (1.62, 300)	30.54 (2.66, 67)
PCB138	19.13 (0.44, 2033)	23.56 (1.65, 80)	40.17 (1.64, 574)	43.87 (2.42, 124)	50.31 (2.83, 301)	56.73 (3.57, 68)
PCB153	27.39 (0.63, 2032)	35.47 (2.55, 80)	60.88 (2.08, 574)	64.06 (3.49, 125)	76.17 (3.62, 302)	84.56 (5.28, 68)
PCB180	12.75 (0.49, 2036)	19.45 (2.22, 79)	47.01 (1.39, 571)	51.16 (3.22, 126)	59.19 (3.34, 298)	61.65 (5.31, 68)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)
[2] Geometric means calculated after applying NHANES sampling weights.

For all 6 PCB congeners, there were 150 participants at risk of PAH, 154 participants at risk of PAH in dioxin-like PCBs (74+ 118) (Table 7), and non-dioxin-like PCBs (99+138+153+180) (Table 8). We analyzed the Dioxin-like PCBs (74+118) serum concentration to evaluate their association with the risk of PAH in our study population (Table 7). Dioxin-like PCB concentration in serum was significantly higher in people at risk of PAH with 3.58 ng/g, than people not at risk of PAH with 2.81 ng/g (Table 7). We categorized limit of detection of dioxin –like PCBs into < LOD to 50 % and > 50%. People not at risk of PAH reported mean of dioxin-like PCB < LOD to be 2.59 ng/g, while LOD ≥ 50% with 3.53 ng/g (Table 7). However, people at risk of PAH reported mean of dioxin-like PCB < LOD to be 2.61 ng/g, while LOD ≥ 50% with 3.81 ng/g (Table 7).

Table 6. Age standardized geometric Mean PCB levels (ng/g) by race/ethnicity among population at risk of PAH ≥20 years of age with PCB concentration above the LOD, NHANES 1999-2004

	Geometric mean[2] (ng/g) (GSE, n)			
	No Risk of PAH		Risk of PAH	
Analyte[1]	Non-Hispanic White	Other	Non-Hispanic White	Other
PCB74	8.13 (0.33, 1438)	6.17 (0.17, 1466)	12.86 (0.82, 155)	12.39 (1.08, 115)
PCB99	6.11 (0.19, 1425)	5.81 (0.17, 1449)	7.97 (0.35, 152)	10.46 (1.04, 115)
PCB118	9.11 (0.30, 1438)	8.25 (0.28, 1466)	15.07 (0.95, 154)	16.86 (1.66, 118)
PCB138	23.14 (0.59, 1445)	21.14 (0.75, 1463)	32.63 (1.55, 154)	41.59 (3.56, 118)
PCB153	34.03 (0.85, 1444)	29.52 (1.03, 1464)	49.02 (2.49, 155)	59.89 (4.89, 118)
PCB180	18.87 (0.79, 1437)	12.69 (0.63, 1468)	33.45 (2.78, 154)	35.41 (3.53, 119)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)
[2] Geometric means calculated after applying NHANES sampling weights.

All covariates reported higher mean dioxin-like PCBs in population at risk of PAH than population not at risk of PAH except in race covariate, people categorized as "other" reported higher dioxin-like PCB mean to be lower in people at risk of PAH (Table 7). Overall, the highest mean of dioxin-like PCB was reported in people ≥ 75 with risk of PAH, while the lowest mean of dioxin-like PCBs was in race categorized as "others" in people at risk of PAH (Table 7).

Non-dioxin-like PCB included the sum of PCBs (99+138+153+180) was analyzed to evaluate the association with the risk of PAH by mean of concentration and 95% CI (Table 8). People at risk of PAH reported higher mean of non-dioxin-like PCBs that people not at risk of PAH with 4.87 ng/g and 4.41 ng/g, respectively (Table 8). We also evaluated the association of covariates with risk of PAH and their association with mean concentration of non-dioxin-like PCBs including gender, race, age, BMI, annual family income, education level, smoking, and alcohol use. Most covariates reported higher mean of non-dioxin-like PCBs in people at risk of PAH than people not at risk of PAH. However, concentration < LOD to 50% of non-dioxin-like PCBs was higher in people

not at risk of PAH (Table 8). Nevertheless, concentration of non-dioxin-like PCBs

Table 7. Serum Levels of dioxin-like PCB (ng/g) in the study population, ≥ 20 years of age, NHANES 1999-2004.

		Mean (ng/g)[1] (95% CI)	
Variable	**Non-cases/ Cases**	**No Risk of PAH**	**Risk of PAH**
Dioxin-like PCBs[2]	2915/ 274	2.81 (2.75-2.87)	3.36 (3.25-3.46)
Dioxin-like PCBs_50[3]			
< LOD to 50 %	362/ 22	2.59 (2.57-2.61)	2.62 (2.56-2.68)
≥ 50 %	1379/ 218	3.52 (3.49-3.56)	3.73 (3.65-3.82)
Gender			
Male	1304/ 182	2.64 (2.56-2.70)	3.16 (3.02-3.29)
Female	1611/ 92	2.96 (2.89-3.03)	3.69 (3.55-3.84)
Race			
Non-Hispanic White	1446/ 155	2.86 (2.79-2.93)	3.35 (3.23-3.48)
Other	1469/ 119	2.68 (2.62-2.75)	3.38 (3.19-3.57)
Age			
20-59	2039/ 80	2.61 (2.55-2.66)	2.95 (2.79-3.11)
60-74	574/ 126	3.49 (3.39-3.61)	3.55 (3.37-3.73)
≥75	302/ 68	3.87 (3.76-3.99)	4.01 (3.86-4.17)
BMI			
Normal Weight	985/ 33	2.78 (2.69-2.86)	3.44 (3.18-3.70)
Overweight	1031/ 85	2.79 (2.71-2.86)	3.36 (3.13-3.59)
Obese	838/ 139	2.87 (2.79-2.95)	3.29 (3.16-3.42)
Income			
0-$24,999	1094/ 136	2.84 (2.77-2.92)	3.41 (3.23-3.56)
$25,000-$54,999	838/ 69	2.77 (2.69-2.83)	3.33 (3.08-3.57)
$55,000-74,999	275/ 14	2.72 (2.59-2.84)	3.12 (2.77-3.47)
≥75,000	708/ 55	2.86 (2.78-2.93)	3.41 (3.15-3.66)
Education Level			
<12th Grade	945/ 109	2.85 (2.75-2.96)	3.49 (3.27-3.71)
12th Grade	628/ 71	2.81 (2.72-2.90)	3.13 (2.92-3.33)
≥ 12th Grade	1336/ 94	2.79 (2.72-2.86)	3.44 (3.26-3.62)
Smoking			
Yes	643/ 44	2.56 (2.49-2.63)	3.08 (2.79-3.36)
No	746/ 135	2.96 (2.86-3.07)	3.37 (3.20-3.53)
Alcohol use			
Yes	20/ 0	2.62 (2.27-2.97)	
No	2892/ 272	2.81 (2.75-2.87)	3.36 (3.25-3.46)

[1]Means calculated after applying NHANES sampling weights.
[2] Sum of Dioxin-like PCBs = (47+118); Lipid adjusted and log transformed PCBs.
[3] Serum Dioxin-like Levels: < 50th percentile vs ≥ 50th percentile

remained the same in age groups 60-74 and > 75 with 5.10 ng/g and 5.33, respectively, for both risk of PAH groups of non-dioxin-like PCBs. Overall, the highest concentration of non-dioxin-like PCBs was reported in age group > 75 years with 5.33 ng/g, while the lowest concentration was reported in people at risk of PAH at < LOD to 50% with 4.16 ng/g (Table 8).

We analyzed the LOD for all PCBs concentrations with the study population to evaluate their association with the risk of PAH with different LOD. Small number of people at risk of PAH was reported with all PCBs concentration < LOD comparing to number of people not at risk of PAH (Table 9). We reported GM with 95% CI for LOD level of each PCB and all PCB concentrations > LOD were higher in people at risk of PAH than people not at risk of PAH (Table 9). However, PCB concentration < LOD remained the same among people at risk and not at risk of PAH (Table 9).

People at risk of PAH reported PCB > LOD concentration GM at 16.94 ng/ng for PCB 74, 11.5 ng/ng for PCB99, 22.87 ng/ng for PCB118, 40.56 ng/ng for PCB138, 54.02 ng/ng for PCB153, and 42.66 ng/ng for PCB180 (Table 9). However, people not at risk of PAH reported PCB > LOD concentration GM at 11.54 ng/ng for PCB 74, 8.41 ng/ng for PCB99, 15.36 ng/ng for PCB118, 33.89 ng/ng for PCB138, 47.33 ng/ng for PCB153, and 38.31 ng/ng for PCB180 (Table 9).

We also analyzed all PCBs concentration with another division of LOD because small number of people at risk in table 1.7, and we used < LOD to 50% and > 50% percentiles to be reported in (Table 10). Also, small number of people at risk of PAH was reported for all PCB with concentration < LOD to 50 % with 4, 11, 11, 15, 22, and 21 for

PCBs 74, 99, 118, 138, 153, and 180, respectively (Table 10). All GM for different levels

Table 8. Serum Levels of non-dioxin-like PCB (ng/g) in the study population, ≥ 20 years of age, NHANES 1999-2004.

		Mean (ng/g) [1] (95% CI)	
Variable	**Non-cases/ Cases**	**No Risk of PAH**	**Risk of PAH**
Non-Dioxin-like PCBs[2]	2916/ 275	4.40 (4.36-4.45)	4.88 (4.78-4.98)
Non-Dioxin-Like-PCBs 50[3]			
< LOD to 50 %	416/ 33	4.26 (4.24-4.28)	4.29 (4.21-4.36)
≥ 50 %	1404/ 217	5.12 (5.08-5.15)	5.23 (5.16-5.29)
Gender			
Male	1304/ 183	4.41 (4.35-4.47)	4.83 (4.69-4.97)
Female	1612/ 92	4.39 (4.35-4.45)	4.96 (4.84-5.07)
Race			
Non-Hispanic White	1446/ 155	4.46 (4.40-4.51)	4.84 (4.73-4.95)
Other	1470/ 120	4.27 (4.19-4.35)	5.00 (4.85-5.16)
Age			
20-59	2040/ 80	4.21 (4.16-4.26)	4.48 (4.33-4.62)
60-74	574/ 127	5.09 (5.02-5.15)	5.14 (5.02-5.25)
≥75	302/ 68	5.31 (5.22-5.40)	5.41 (5.28-5.53)
BMI			
Normal Weight	985/ 34	4.43 (4.36-4.51)	4.83 (4.58-5.08)
Overweight	1032/ 85	4.41 (4.36-4.46)	5.06 (4.89-5.23)
Obese	838/ 139	4.34 (4.26-4.42)	4.74 (4.61-4.86)
Income			
0-$24,999	1095/ 136	4.36 (4.29-4.42)	4.94 (4.78-5.09)
$25,000-$54,999	838/ 69	4.37 (4.29-4.44)	4.86 (4.67-5.05)
$55,000-74,999	275/ 14	4.37 (4.28-4.46)	4.60 (4.27-4.94)
≥75,000	708/ 56	4.49 (4.44-4.56)	4.92 (4.76-5.09)
Education Level			
<12th Grade	945/ 109	4.49 (4.39-4.59)	5.08 (4.89-5.27)
12th Grade	629/ 71	4.38 (4.29-4.47)	4.69 (4.49-4.90)
≥ 12th Grade	1336/ 95	4.38 (4.33-4.43)	4.88 (4.71-5.04)
Smoking			
Yes	643/ 45	4.31 (4.25-4.38)	4.77 (4.50-5.04)
No	746/ 135	4.63 (4.55-4.70)	4.93 (4.78-5.09)
Alcohol use			
Yes	20/ 0	4.24 (3.83-4.64)	
No	2893/ 273	4.40 (4.36-4.45)	4.88 (4.78-4.98)

[1]Means calculated after applying NHANES sampling weights.
[2] Sum of Non-Dioxin-like PCBs = (99+138+153+180); Lipid adjusted and log transformed PCBs.
[3] Serum Dioxin-like Levels: < 50th percentile vs ≥ 50th percentile

of PCB LOD concentrations were higher in people at risk of PAH than people not at risk of PAH, except PCB118 > 50% and PCB180 (Table 10).

People at risk of PAH reported PCB > 50% concentration GM at 18.03 ng/ng for PCB 74, 12.74 ng/ng for PCB99, 25.69 ng/ng for PCB118, 47.49 ng/ng for PCB138, 64.89 ng/ng for PCB153, and 48.37 ng/ng for PCB180 (Table 10). However, people not at risk of PAH reported PCB > LOD to 50% concentration GM at 15.22 ng/ng for PCB 74, 10.58 ng/ng for PCB99, 7.22 ng/ng for PCB118, 43.87 ng/ng for PCB138, 63.86 ng/ng for PCB153, and 49.90 ng/ng for PCB180 (Table 10).

Table 9. Geometric Mean PCB levels (ng/g) by risk of PAH among population ≥20 years of age, NHANES 1999-2004

			Geometric mean[2] (ng/g) (95% CI)	
Analyte[1]	**No. Non-cases**	**No. Cases**	**No Risk of PAH**	**Risk of PAH**
PCB74				
< LOD	1039	27	3.40 (3.40-3.40)	3.40 (3.40-3.40)
≥ LOD	1865	245	11.48 (10.84-12.16)	15.91 (14.45-17.53)
PCB99				
< LOD	916	41	2.94 (2.94-2.94)	2.94 (2.94-2.94)
≥ LOD	1958	226	8.36 (8.02-8.72)	10.99 (9.96-12.14)
PCB118				
< LOD	1195	38	4.24 (4.24-4.24)	4.24 (4.24-4.24)
≥ LOD	1709	234	15.29 (14.65-15.95)	21.47 (19.13-24.11)
PCB138				
< LOD	1008	32	10.68 (10.68-10.68)	10.68 (10.68-10.68)
≥ LOD	1900	240	33.58 (32.34-34.87)	42.17 (38.56-46.13)
PCB153				
< LOD	909	21	14.00 (14.00-14.00)	14.00 (14.00-14.00)
≥ LOD	1999	252	46.79 (44.96-48.69)	58.30 (53.75-63.24)
PCB180				
< LOD	1083	25	3.86 (3.86-3.86)	3.86 (3.86-3.86)
≥ LOD	1822	248	37.90 (36.56-39.31)	45.49 (41.36-50.02)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)
[2] Geometric means calculated after applying NHANES sampling weights.

Overall, no significance difference was reported between GM PCB concentrations of people at risk of PAH and people not at risk of PAH (Table 10).

To get more accurate LOD, we performed another analysis with more divided LOD for PCB concentrations using < LOD to 50th percentile, 50th percentile to 75th percentile, and ≥ 75th percentile (Table 11). Higher number of people at risk of PAH were reported among LOD ≥ 75th percentile than < LOD to 50th percentile and 50th percentile to 75th percentile (Table 11). All LOD of PCB concentration were higher in people at risk of PAH than people not at risk of PAH, except GM of PCB 138 < LOD to 50th percentile, GM of PCB180 50th percentile to 75th percentile, and ≥ 75 % percentile (Table 11).

Table 10. Geometric Mean PCB levels (ng/g) by risk of PAH among population ≥20 years of age, NHANES 1999-2004

			Geometric mean[2] (ng/g) (95% CI)	
Analyte[1]	**No. Non-cases**	**No. Cases**	**No Risk of PAH**	**Risk of PAH**
PCB074				
< LOD to 50 %	499	22	5.83 (5.74-5.92)	6.27 (5.89-6.68)
≥ 50 %	1366	223	1517 (14.48-15.89)	17.73 (16.17-19.44)
PCB099				
< LOD to 50 %	497	26	4.64 (4.59-4.69)	4.68 (4.52-4.84)
≥ 50 %	1461	200	10.53 (10.08-11.00)	12.59 (11.56-13.71)
PCB118				
< LOD to 50 %	328	23	7.23 (7.12-7.35)	7.43 (7.10-7.78)
≥ 50 %	1381	211	19.01 (18.24-19.82)	24.21 (21.96-26.69)
PCB138				
< LOD to 50 %	502	26	18.05 (17.79-18.31)	18.89 (17.98-19.83)
≥ 50 %	1398	214	43.59 (41.98-45.27)	48.84 (44.69-53.36)
PCB153				
< LOD to 50 %	586	33	25.43 (25.02-25.84)	27.06 (25.53-29.23)
≥ 50 %	1413	219	63.33 (61.34-65.38)	69.33 (64.66-74.33)
PCB180				
< LOD to 50 %	451	30	19.99 (19.69-20.29)	20.13 (18.38-22.05)
≥ 50 %	1371	218	49.33 (47.68-51.04)	53.89 (49.86-58.23)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)
[2] Geometric means calculated after applying NHANES sampling weights.

The most significant GM was reported in PCB 118 of LOD ≥ 75 % percentile with 33.89 for people not at risk of PAH and 39.02 for people at risk of PAH (Table 11).

We calculated estimated odds ratio (OR) and 95% confidence intervals to evaluated all PCBs concentration association with risk of PAH (Table 12). Because of low number of subjects at risk of PAH, we used < LOD to 50th percentile (reference

Table 11. Geometric Mean PCB levels (ng/g) by risk of PAH among population ≥20 years of age, NHANES 1999-2004

			Geometric mean[2] (ng/g) (95% CI)	
Analyte[1]	No. Non-cases	No. Cases	No Risk of PAH	Risk of PAH
PCB074				
< LOD to 50 %	499	22	5.83 (5.74-5.92)	6.27 (5.89-6.82)
50 - 75 %	708	92	10.48 (10.27-10.69)	10.93 (10.21-11.69)
≥ 75 %	658	131	25.57 (24.65-26.53)	27.01 (24.96-29.62)
PCB099				
< LOD to 50 %	497	26	4.64 (4.59-4.68)	4.67 (4.52-5.84)
50 - 75 %	758	73	7.07 (6.95-7.19)	7.48 (7.22-7.75)
≥ 75 %	703	127	17.45 (16.57-18.37)	18.43 (16.85-20.15)
PCB118				
< LOD to 50 %	340	11	7.23 (7.11-7.35)	7.43 (7.10-7.78)
50 - 75 %	758	44	12.52 (12.26-12.79)	13.72 (12.95-14.54)
≥ 75 %	700	90	34.14 (32.62-35.73)	36.02 (32.43-39.99)
PCB138				
< LOD to 50 %	502	26	18.05 (17.79-18.31)	18.89 (17.99-19.83)
50 - 75 %	712	82	30.28 (29.75-30.82)	30.99 (29.39-32.68)
≥ 75 %	686	132	70.46 (67.69-73.34)	72.14 (66.12-78.71)
PCB153				
< LOD to 50 %	586	33	25.43 (25.02-25.84)	27.06 (25.05-29.23)
50 - 75 %	713	88	45.42 (44.55-46.30)	47.09 (45.28-48.98)
≥ 50 %	700	131	99.09 (95.46-102.86)	104.39 (96.94-112.41)
PCB180				
< LOD to 50 %	451	30	19.99 (19.69-20.29)	20.13 (18.38-22.05)
50 - 75 %	690	90	36.37 (35.58-37.19)	36.73 (34.75-38.82)
≥ 75 %	681	128	75.99 (73.65-78.40)	81.67 (77.15-86.44)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)
[2]Geometric means calculated after applying NHANES sampling weights.

group) and ≥ 50th percentile. We calculated unadjusted OR; gender adjusted OR; age,

race, and BMI adjusted OR; and age, race, BMI, and gender adjusted OR. In Unadjusted OR, all PCBs were significantly associated with identified risk of PAH in (≥ 50th percentile) when we compare it to the reference group (< LOD to 50th percentile) (Table 12). After gender adjusting, all PCB concentration were significantly associated with risk of PAH. The highest OR was found in PCB 74 with [OR of 4.00; 95% CI: 3.98-4.01] (Table 12).

We calculated estimated OR and 95% confidence intervals for PAH with PCB concentrations divided into < LOD to 50th percentile (reference group), 50th to 75th percentile, and ≥ 75th percentile (Table 13). In unadjusted calculated OR, all PCB

Table 12. Estimated ORs (95% CI) for risk of PAH by concentration of PCB levels among population ≥20 years of age, NHANES 1999-2004

Analyte[1]	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)	Adjusted OR[4] (95% CI)
PCB74						
< LOD to 50 %	502	22	1	1	1	1
≥ 50 %	1406	223	3.38 (3.37-3.40)	4.00 (3.98-4.01)	1.95 (1.94-1.96)	2.31 (2.30-2.32)
PCB99						
< LOD to 50 %	603	26	1	1	1	1
≥ 50 %	1845	200	2.32 (2.31-2.32)	2.38 (2.37-2.39)	1.44 (1.43-1.44)	1.50 (1.49-1.50)
PCB118						
< LOD to 50 %	338	23	1	1	1	1
≥ 50 %	1411	211	2.54 (2.53-2.55)	2.83 (2.82-2.84)	1.51 (1.50-1.51)	1.67 (1.66-1.68)
PCB138						
< LOD to 50 %	615	26	1	1	1	1
≥ 50 %	1837	214	2.19 (2.18-2.20)	2.22 (2.22-2.23)	1.27 (1.27-1.28)	1.28 (1.27-1.28)
PCB153						
< LOD to 50 %	738	33	1	1	1	1
≥ 50 %	1835	219	2.20 (2.19-2.21)	2.18 (2.17-2.19)	1.38 (1.380-1.389)	1.37 (1.36-1.37)
PCB180						
< LOD to 50 %	594	30	1	1	1	1
≥ 50 %	1832	218	1.98 (1.97-1.99)	1.95 (1.95-1.96)	1.37 (1.37-1.38)	1.30 (1.29-1.30)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)

[2] Adjusted for gender; Risk of PAH Cases/Non-cases: 245/1908 in 74; 226/2448 in 99; 234/1749 in 118; 240/2452 in 138; 252/2573 in 153; 248/2426 in 180

[3] Adjusted for age, race, and BMI; Risk of PAH Cases/Non-cases: 229/1846 in 74; 212/1941 in 99; 218/1693 in 118; 223/1878 in 138; 235/1980 in 153; 231/1803 in 180

[4] Adjusted for age, race, BMI, gender; Risk of PAH Cases/Non-cases: 229/1846 in 74; 212/1941 in 99; 218/1693 in 118; 223/1878 in 138; 235/1980 in 153; 231/1803 in 180

concentrations were significantly associated with identified risk of PAH. The highest OR was reported with PCB 74 with [OR of 4.32; 95% CI: (4.31-4.34)]. In gender adjusted

OR model, all PCB concentrations were higher than un adjusted model except for PCB 180 reported lower OR than in unadjusted model (Table 13). In age, race, and BMI adjusted OR; and age, race, BMI, and gender adjusted OR, all PCB concentration were associated with identified risk of PAH. However, no significant difference was reported within the two models (Table 13).

Table 13. Estimated ORs (95% CI) for risk of PAH by concentration of PCB levels among population ≥20 years of age, NHANES 1999-2004

Analyte[1]	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)	Adjusted OR[4] (95% CI)
PCB074						
< LOD to 50 %	502	22	1	1	1	1
50 - 75 %	725	92	2.70 (2.69-2.71)	3.02 (3.01-3.04)	1.94 (1.93-1.95)	2.18 (2.17-2.19)
≥ 75 %	681	131	4.32 (4.31-4.34)	5.82 (5.79-5.84)	1.98 (1.78-1.99)	2.65 (2.63-2.66)
PCB099						
< LOD to 50 %	603	26	1	1	1	1
50 - 75 %	953	73	1.77 (1.76-1.77)	1.78 (1.77-1.78)	1.38 (1.37-1.38)	1.40 (1.40-1.41)
≥ 75 %	892	127	3.00 (2.99-3.01)	3.18 (3.17-3.19)	1.52 (1.51-1.52)	1.62 (1.61-1.63)
PCB118						
< LOD to 50 %	338	23	1	1	1	1
50 - 75 %	737	76	1.81 (1.80-1.82)	1.93 (1.92-1.94)	1.34 (1.34-1.36)	1.43 (1.42-1.44)
≥ 75 %	674	135	3.55 (3.53-3.57)	4.37 (4.35-4.39)	2.55 (2.54-2.56)	2.20 (2.56-2.59)
PCB138						
< LOD to 50 %	615	26	1	1	1	1
50 - 75 %	941	82	1.82 (1.81-1.83)	1.84 (1.83-1.85)	1.21 (1.21-1.22)	1.22 (1.21-1.23)
≥ 75 %	896	132	2.67 (2.66-2.68)	2.74 (2.73-2.75)	1.37 (1.37-1.38)	1.37 (1.36-1.38)
PCB153						
< LOD to 50 %	738	33	1	1	1	1
50 - 75 %	938	88	1.98 (1.97-1.99)	1.99 (1.99-2.00)	1.42 (1.41-1.43)	1.43 (1.42-1.44)
≥ 75 %	897	131	2.46 (2.45-2.47)	2.49 (2.48-2.50)	1.30 (1.30-1.31)	1.26 (1.25-1.27)
PCB180						
< LOD to 50 %	597	30	1	1	1	1
50 - 75 %	935	90	1.75 (1.75-1.76)	1.75 (1.74-1.76)	1.31 (1.31-1.32)	1.29 (1.28-1.29)
≥ 75 %	897	128	2.32 (2.31-2.33)	2.26 (2.25-2.27)	1.51 (1.50-1.52)	1.33 (1.32-1.34)

[1]Lipid adjusted and log transformed polychlorinated biphenyls (ng/g)

[2] Adjusted for gender; Risk of PAH Cases/Non-cases: 245/1908 in 74; 226/2448 in 99; 234/1749 in 118; 240/2452 in 138; 252/2573 in 153; 248/2426 in 180

[3] Adjusted for age, race, and BMI; Risk of PAH Cases/Non-cases: 229/1846 in 74; 212/1941 in 99; 218/1693 in 118; 223/1878 in 138; 235/1980 in 153; 231/1803 in 180

[4] Adjusted for age, race, BMI, gender; Risk of PAH Cases/Non-cases: 229/1846 in 74; 212/1941 in 99; 218/1693 in 118; 223/1878 in 138; 235/1980 in 153; 231/1803 in 180

We calculated estimated OR and 95% confidence intervals for PAH and dioxin-like and non-dioxin-like PCBs < LOD to 50th percentile (reference group) and ≥ 50th percentile (Table 14). In unadjusted calculated OR, dioxin-like and non-dioxin-like PCBs were significantly associated with identified risk of PAH [OR of 2.64; 95% CI: (2.63-

2.66)] and 1.58 (1.79-1.59), respectively. In the age, race, and BMI adjusted model; and

Table 14. Estimated ORs (95% CI) for risk of PAH by concentration of PCB levels among population ≥20 years of age, NHANES 1999-2004

	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[1] (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)
Dioxin_Like_PCBs _50[5,6]						
< LOD to 50 %	370	22	1	1	1	1
≥ 50 %	1415	218	2.64 (2.63-2.66)	2.95 (2.93-2.96)	1.53 (1.52-1.54)	1.73 (1.72-1.74)
Dioxin_Like_PCBs _LOD_to75[4,7]						
< LOD to 75 %	673	139	1	1	1	1
≥ 75 %	1112	101	2.51 (2.51-2.52)	2.95 (2.95-2.97)	1.51 (1.51-1.52)	1.75 (1.75-1.76)
Non_Dioxin_Like_ PCBs_50[5,6]						
< LOD to 50 %	543	33	1	1	1	1
≥ 50 %	1841	217	1.58 (1.79-1.59)	1.59 (1.58-1.60)	1.00 (1.00-1.01)	0.99 (0.99-0.99)
Non_Dioxin_Like_ PCBs_LOD_to75[5,7]						
< LOD to 75 %	896	132	1	1	1	1
≥ 75 %	1487	118	1.51 (1.50-1.51)	1.52 (1.52-1.53)	1.00 (0.99-1.00)	0.51 (0.95-0.96)

[1] Adjusted for gender [2]
[2] Adjusted for age, race, and BMI
[3] Adjusted for age, race, BMI, gender
4 Dioxin-like PCBs: Sum of lipid adjusted and log transformed PCB Congeners (74+118)
5 Non-Dioxin-like PCBs: Sum of lipid adjusted and log transformed PCB Congeners (99+138+153+180)
6 Serum PCB levels <50th percentile vs ≥ 50th percentile.
7 Serum PCB levels <75th percentile vs ≥ 75th percentile.

age, race, BMI, and gender adjusted OR, non-dioxin-like PCBs at levels ≥ 50th percentile and levels ≥ 75th percentile were not associated with identified risk of PAH (Table 14).

DISCUSSION

The fact that EDCs can affect estrogen activity in different tissue in human body, encouraged us to evaluate PCBs and their effect on the risk of PAH. It is known that estrogen, oral contraceptive, and female bias has been suggested to be risk factors in the pathogenesis of PAH. This is the first cross-sectional study that evaluate the exposure of PCBs and their association with the risk of PAH. We represented the significant association of exposure to 6 PCB 74, 99, 118, 138, 153, and 180 congeners individually

and high risk of PAH in the U.S. population > 20 years old using NHANES data 1999-2004. Furthermore, we demonstrated the sum of dioxin-like PCBs (PCB 74 and 118), and non-dioxin-like PCBs (PCB 118, 138, 153, and 180) and their association with high risk of PAH.

Our results are supported with the known association between PAH and its risk factors which is high estrogen activity, as well the bias in female gender (Badesch et al., 2010). Females were six times significantly higher in population at risk of PAH than males. We found that PRKCH is a gene resulted from the meta-analysis of gene expression of PAH might have plausible role in pathogenesis of PAH. Also it was reported that PRKCH induce ERs transcriptional activity resulted from exposure to high levels of EDCs (Cho & Katzenellenbogen, 1993). Also, adipogenesis is known risk factor of obesity, and EDCs were reported to increase the PPARγ and promote adipogenesis (Charlier et al., 2004). Obese people were at higher risk of PAH than normal and overweight people. This result is also supported by REVEAL results that reported significant association between obese people and PAH (BMI > 30, P-value=0.004 (Burger et al., 2011). GM of all individual PCB levels was significantly increased in people at risk of PAH than people not at risk of PAH. Another NHANES study of PCBs association with hypertension reported 11 PCBs to risk factor including PCBs (PCBs 126, 74, 118, 99, 138/158, 170, and 187) (Everett, Mainous, Frithsen, Player, & Matheson, 2008). Nevertheless, PCBs reported to promote vascular endothelial cell activity and increase the risk of cardiovascular diseases (Ha, Lee, & Jacobs, 2007; Lim, Smart, Toborek, & Hennig, 2007).

GM of all PCBs concentration in people at risk of PAH was higher in different age groups than people not at risk of PAH with some exception such as GM of PCB 180 was lower in people at risk of PAH in age groups 60-74 years old and > 75 years old. Also, GM of all PCBs at people at risk of PAH was significantly higher in both races (non-Hispanic white and other) than people not at risk. However, there is no significant difference in GM of PCBs concentration within non-Hispanic white and others. Sum of Dioxin-like PCBs (74+118) and non-Dioxin-like PCBs (99+138+153+180) concentration were higher in people at risk of PAH than people not at risk of PAH. All confounders including gender, age, BMI, annual family income, educational level, smoking, and alcohol use reported higher concentration of Dioxin-like PCBs and non-Dioxin-like PCBs in people at risk of PAH than people not at risk of PAH, except race. GM of PCBs > LOD were significantly higher in people at risk of PAH than people not at risk of PAH. Also, GM of all PCBs LOD > 50%, 50-75%, and > 75% were higher in people at risk of PAH than people not at risk of PAH, except PCB 180. Risk of PAH appeared to be the highest in subjects identified at risk of PAH with the highest PCB concentrations in unadjusted models. PCB 74 reported the highest association among all PCBs in all models. However, some exception for higher concentrations of dioxin like PCBs and non-dioxin like PCBs were found no associated risk with PAH age, race, and BMI adjusted model; and age, race, BMI, and gender adjusted model.

There is limited on data of PCB and its association with PAH. However, estrogen has high affinity to the lung because of high ER receptors activity. Although, sex hormone 17β-estradiol (E2) is associated with the formation of atherosclerotic lesions and vascularization (Benditt & Benditt, 1973). As well, estrogen toxicity is significantly

associated with high levels of inflammatory cytokine, which indeed lead to PAH (D. Roy et al., 2007). High levels pf PCB were reported in the human lung (Rallis et al., 2014). Although, lung toxicity and hypertension are associated with high levels of PCBs and their estrogenic congeners (Hansen et al., 2016; Kreiss et al., 1981). Moreover, PCBs are associated with damaging of cardiovascular system (Goncharov et al., 2008; Sergeev & Carpenter, 2005). Given that pulmonary smooth muscle and endothelial cells reported human serum level of E2 was found at [0.60 - 1.63 ng/ml] of estrogenic PCB 153 [1ng/ml] (Charlier et al., 2004).

There are some limitations in our study including that we created a variable named risk of PAH because there was no PAH variable included in the NHANES survey data. This increases the risk of misclassification to people at risk and not at risk of PAH. Also, a low number of people at risk of PAH in the study population resulted in low significance comparison within different analysis. Given that limited data on PCBs exposure and their association with PAH affected our comparison and analysis of the results. However, using NHANES cross-sectional survey data including representative sample size, medical health data, reproductive health data, and laboratory data of the U.S. population provided us with strength in our study.

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CHAPTER 4

MANUSCRIPT 2

HORMONE REPLACEMENT THERAPY AND ORAL CONTRACEPTIVES AND RISK OF PULMONARY ARTERIAL HYPERTENSION IN THE UNITED STATES FEMALES: ANALYSES OF NHANES 1999-2004

ABSTRACT

Background: Females are known to be predominant of pulmonary arterial hypertension (PAH). Estrogens and oral contraceptives were reported to be associated with PAH. In this study, we will evaluate the association between hormone replacement therapy and oral contraceptives with risk of PAH.

Objectives: The objective of this study was to evaluate the association between hormone replacement therapy and oral contraceptives and risk of PAH among females ≥ 20 years old in U.S. population.

Methods: We used Centers for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) data from 1999-2004 for females ≥ 20 years old. We selected females identified at risk of PAH with available data on hormones and birth control. We used data on use of estrogen pills only, progestin pills only, estrogen/progestin pills combined, and birth control pills. We included age, race, and BMI as confounders in our analysis. We calculated odds ratios (OR) for the association between each variable and the risk of PAH.

Results: We found 97 (3.7%) females at risk of PAH and 2,280 (96.295) not at risk of PAH. Females used hormone pill with estrogen reported [OR of 1.24; 95% CI 1.23-1.25]. Females used hormone pills with progestin and estrogen/progestin pills reported no risk

of PAH with [OR of 0.57; 95% CI 0.56-0.58] and [OR of 0.59; 95%CI 0.59-0.60], respectively. However, all variables (estrogen, progestin, and estrogen/ progestin) reported significant OR with risk of PAH in elderly and obese females. Also, females used birth control pills reported significant association with risk of PAH [OR of 2.47; 95%CI 2.46-2.48], elderly [OR 5.84; 95% CI 5.81-5.88], and obese [OR 7.43; 95% CI 7.38-7.48].

Conclusions:

Using NHANES data from 1999-2004, we found that use of estrogen pills and birth controls have a significant association with risk of PAH in females ≥ 20 years old. However, progestin only pills and estrogen/progestin combo pills did not show any association with risk of PAH.

Keywords: Hormone replacement therapy, Oral Contraceptives, NHANES, pulmonary arterial hypertension, PAH

MANUSCRIPT 2

HORMONE REPLACEMENT THERAPY AND ORAL CONTRACEPTIVES AND RISK OF PULMONARY ARTERIAL HYPERTENSION IN THE UNITED STATES FEMALES: ANALYSES OF NHANES 1999-2004

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease and is frequently fatal (Yuan & Rubin, 2005). PAH is known by an increase in pulmonary arterial pressure, proliferation and remodeling of vascular cells; which leads to right heart failure (Price et

al., 2012). PAH was known to be more dominant in females with ration of 1.9:1 to 4.1:1 in various studies with no clear understanding of gender bias (Badesch et al., 2010; Rich et al., 1987). Thus, PAH patients were encouraged to avoid pregnancy. Multiple interactions are involved in the pathogenesis of PAH including environmental exposure (hypoxia and drugs), genetics, cellular interaction, and sex hormones (Pugh & Hemnes, 2010). Although it was suggested that gender bias in PAH could be explained with testosterone acts as protective factor of pulmonary vasculature, estrogens acts as destroying factor. Estrogen is known to promote growth of smooth muscle cells and vascular endothelial cells by regulating vascular remodeling and angiogenesis. As well, estrogen is known to induce inflammatory chemokines such as IL-8 (Comeglio et al., 2014). However, as we explained in our review, estrogen could have an antioxidant effect and be a protective factor for superoxide radical generation (Assaggaf & Felty, 2017). Hormonal changes usually comes with pregnancy and PAH pregnant reported high mortality rates as 50% (Weiss, Zemp, Seifert, & Hess, 1998). Usually, contraceptives are recommended for PAH females at childbearing age, but estrogen containing contraceptives are not recommended (Bonnin et al., 2005; Humbert et al., 2001). Progestin is known to prevent estrogen activity and expression. This was found in uterine cells and prevented their proliferation (Slayden, Hirst, & Brenner, 1993). Progestin was added to hormone replacement therapy (HRT) to mimic estrogen activity in breast cancer (Hirvonen, 1996). However, the mechanistic association between estrogen and progestin is not fully understood.

Animal models with induced hypoxia and monocrotaline reported that estrogen acutely increased vasorelaxation of pulmonary artery (Lahm et al., 2007). Another PH

animal model showed that females have less vascular remodeling than males (Rabinovitch, Gamble, Miettinen, & Reid, 1981). Mutation in bone morphogenetic protein receptor type 2 (*BMPR2*) gene, is known as familial PAH. This type of PAH is significantly associated with females and specifically young females (35.8 ± 15.4, $p < 0.0001$) (Eric D Austin et al., 2009). In BMPR2 mutation, females reported 2.4 higher risk than males and it was suggested to be because of estrogen metabolism (E. D. Austin et al., 2009). Many studies reported that oral contraceptives and HRT worsen PAH (Morse, Horn, & Barst, 1999; Sweeney & Voelkel, 2009). Conversely, cohort HRT study reported that patients developed PAH at low rates (Beretta et al., 2006). Thus, oral contraceptives are contradicted with PAH and extensive research is recommended. Taken together, estrogen and sex hormones are suggested to play an unknown important role with risk of PAH. Thus, we hypothesized that estrogen to be risk factor of high risk of PAH in females.

In this study, we evaluated the association between use of hormone pills with estrogen, use of hormone pills with progestin, use of hormone pills with estrogen/progestin, and use of birth control pills in females at risk of PAH in the National Health and Nutrition Examination Survey (NHANES) between the years 1999-2004. We calculated Odd Ratio (OR) of each variable to evaluate its association with risk of PAH.

METHODS

Study Design. The main objective of this study was to investigate the association between hormone replacement therapy (estrogen, progestin, estrogen/progestin) and oral contraceptives and risk for PAH and pulmonary diseases in females > 20 years old in the U.S. To clarify and confirm the suggested role of hormone replacement therapy and oral

contraceptives, we used NHANES data from 1999-2004 provided by Centers for Disease Control and Prevention (CDC) to conduct a secondary statistical analysis. National Health and Nutrition Examination Survey (NHANES) is a program was developed by National Center for Health Statistics (NCHS) to assess the nutritional and health status for children and adults in the United States. NHANES started in the early 1960s producing different surveys for many health issues as well as many age groups. Since 1999, NHANES started producing annually continuous interviews and surveys targeting different health and nutritional measurements for evaluation purposes including demographic, dietary, health-related questions, socioeconomic, dental, medical, physiological measurements, and laboratory tests. An average of 5,000 participants enrolled every year and 15 counties across the nation are visited every year. To acquire a representative sampling of the population, many parameters needed to be taken into consideration for oversampling. Oversampling would be sub-grouped, stratified in strata's and then divided into different locations. Oversampling includes age (adolescents (12-19 years), adults (≥ 60 years)) and ethnicity/ race (non-Hispanics black and Mexican Americans). After being eligible, the participants undergo series of interviews and examinations in a specially equipped mobile evaluation clinic (MEC). MEC examination consist of collections of urine and blood samples, personal interviews, and dental and physical examinations. Urine is collected from participant if ≥ 6 years, and blood samples collected from participant if ≥ 1 year. CDC institutional board approved the study protocol and all participants signed a consent before joining the NHANES. All data are strictly confident and coded in different identification factors to keep privacy.

Transportation, personal care (baby/ elderly) and cash payment for participating are included (CDC, 2012).

Questionnaire Methods. Multiple time points included collection of demographic, reproductive health, and medical health data. Intensive two weeks training program including exercises, training interview, and training on personal audio computer interview was conducted and supervised by NCHS and accompanied staff. Also, multiple quality control monitoring procedure were conducted through full pilot test before starting questionnaire. During a household interview, people ≥ 16 years old provided demographic data. During the personal interview in Mobile Examination Center (MEC), females ≥ 12 years old provided reproductive health data. For both males and female (constriction by age and gender), medical health data was provided during the MEC interview (CDC, 2012).

Selection of Participants. Inclusion criteria used for the study included females during MEC were ≥ 20 years old who completed and provided personal interview. Subjects with defined risk of PAH were selected based on an inclusion criteria. We created the Risk of PAH variable based on any risk factors that reported individually to be associated with PAH in literature. As mentioned earlier in introduction, these risk factors included hypertension, abnormal lung function, diabetes, thyroid problems, uric acid level, insulin status, and female hormone. Risk of PAH variable was created after sum of all risk factors scoring to be at risk of PAH if > 3 for female participants (CDC, 2012). Participant will be deleted from the dataset if any of these risk factors answer or level were missing.

Sample Weights. Sample weight is required when merging NHANES complex survey data to avoid bias from non-response selection. The weight variable was provided in each 2-year cycle from 1999-2004 "for all variables (CDC, 2013b).

Reproductive and Medical Health Variables. Data was collected from medical health questionnaire and reproductive health questionnaire to analyze the association between hormone replacement therapy and oral contraceptives and the risk of PAH.

Medical Health Questionnaire. Using medical health questionnaire, females > 20 years old had to provide a response to risk factors of PAH including "have you ever been told by a doctor or health professional that you have diabetes?", "Has a doctor or other health professional ever told that you had a thyroid disease?" Diabetes risk factor was scored 0 if answer was NO, and scored 1 if answer was YES. Thyroid problem risk factor was scored 0 if answer was NO, and scored 1 if answer was YES. Each of these variables was transformed into 0 if no risk of PAH and 1 if at risk of PAH.

Reproductive Health Questionnaire. Using reproductive health questionnaire, female participants > 20 years old had to provide a response to risk factor of PAH "have you ever used female hormones such as estrogen and progesterone?" Female hormone risk factor was scored 0 in the female participant answered NO, and scored 1 if the female participant answered YES.

Hormone replacement therapy and Oral Contraceptives. We used 4 questions to evaluate the association between hormone replacement therapy and oral contraceptive and PAH. For hormone therapy, we used questions "{Have you/Has SP} ever taken female hormone pills containing estrogen only?", "{Have you/Has SP} taken female hormone pills

containing progestin only?", "{Have you/Has SP} taken female hormone pills containing both estrogen and progestin?", and "Now I am going to ask you about {your/SP's} birth control history. {Have you/Has SP} ever taken birth control pills for any reason?" Use hormone pills w/estrogen only scored 1 if YES, and 2 if NO. Used hormone pills w/progestin only scored 1 if YES, and 2 if NO. Used estrogen/progestin combo pills scored 1 if YES, and 2 if NO. Ever taken birth control pills scored 1 if YES, and 2 if NO.

Laboratory data. Using laboratory data measurements of uric acid level should be available for the included participant, as well as measurements of both (triglycerides and HDL-Cholesterol) to calculate insulin status (insulin sensitive or insulin resistance). Uric acid risk factor is a continuous variable, so males and females should have different references. Thus, female participants scored 0 if any had uric acid level < 359 mmol/L, and scored 1 if any male participant had uric acid level > 360 mmol/L. Insulin status was calculated by dividing triglycerides level by HDL-cholesterol level to be < 1.99 "insulin sensitive (IS)" or > 2.00 "insulin resistance (IR)". Insulin status scored 0 if IS and scored 1 if IR. "Subjects at risk of PAH" variable was created after sum of all risk factor scoring to be "Subjects at risk of PAH" if > 3 for female participants (CDC, 2012). Participant will be deleted from the dataset if any of these risk factors answer or level were missing.

Statistical Analysis. We selected females ≥ 20 years old and had available data for hormones and reproductive health to be included in the analysis to find the association with the risk of PAH. Data was weighted using the required weight by the National Center for Health Statistics guidelines to be representative to the whole population. We used stratum and PSA to estimate variance of the demographic data. To find the associated risk of PAH

with hormone therapy and oral contraceptives, we used logistic regression models to calculate the odds ratios (ORs) and their 95 % confidence intervals (CI). Statistical analysis was performed using SPSS software (release 20) for windows and SAS software for windows (release 9.4; SAS Institute Inc. Cary, N.C.). We used 5% ($P \leq 1.05$) as significance level for all analysis.

Potential Confounding Variables. We included many potential confounding variables as categorical variables which are age, body mass index (BMI, kg/m^2), annual family income, smoking status, alcohol consumption, race, and education level. Age was categorized into three age groups including 20-59 years (1), 60-74 years (2) and ≥ 75 years (3). BMI was categorized into > 25 kg/m^2 (1), 25-30 kg/m^2 (2), and ≥ 30 kg/m^2 (3). Annual family income was categorized into 0-$24,999 (1), $25,000-$54,999 (2), $55,000-$74,999 (3), and ≥ $75,000 (4). Smoking status was categorized into smokers (1) and non-smokers (2), and alcohol consumption was categorized into consumers (1) non-consumers (2). Race was categorized into non-Hispanic White (1) and others (2), and education level was categorized into three categories < 12^{th} grade (1), 12^{th} grade (2), and > 12^{th} grade (3). All confounding variable data included in the analysis to find the association of hormones and oral contraceptives and subjects at risk of PAH was used from the demographic file. Categorization of age and race into specific ranges was to avoid bias in the results due to low number of people at risk of PAH in younger ages and other races.

RESULTS

***Descriptive Statistics*.** The study population who completed medical health questionnaire, reproductive health questionnaire; and had available laboratory data to analyze the association between hormones and oral contraceptives and subjects at risk of PAH were

2,377 participants ≥ 20 years old. Among the included population, 97 (3.7%) of them identified at risk to PAH and 2,280 (96.3) of them were not at risk of PAH (Table 15). Race distribution reported 58 (2.97%) of people at risk of PAH to be non-Hispanic white and 39 (0.73%) were others. However, people not at risk of PAH were 1129 (68.16%) non-Hispanic white and 1151 (28.13%) were others. Age was categorized into 3 group; where 26 (1.39%) of people at risk of PAH and 1550 (37.89%) of people not at risk of PAH were in 20-59 years old group. Most participants at risk of PAH among all age groups were in

Table 15. Descriptive statistics for Pulmonary Arterial Hypertension (PAH) status and selected covariates among Females ≥ 20 years old of age, NHANES 1999-2004.

Variable	Risk of PAH n (%)	No Risk of PAH n (%)
Total Population (n, %)	97 (3.70%)	2280 (96.29%)
Race		
Non-Hispanic White	58 (2.97%)	1129 (68.16%)
Other	39 (0.73 %)	1151 (28.13%)
Age (years)		
20-59	26 (1.39 %)	1550 (73.89%)
60-74	43 (1.48%)	467 (14.78%)
≥75	28 (0.82%)	263 (7.62%)
BMI (kg/m^2)		
Normal Weight	10 (0.54%)	608 (40.28%)
Overweight	29 (1.37%)	519 (26.91%)
Obese	53 (2.83%)	577 (28.07%)
Income (yearly family income)		
0-$24,999	48 (1.47%)	944 (32.78%)
$25,000-$54,999	24 (1.14%)	627 (27.98%)
$55,000-74,999	6 (0.42%)	198 (10.03%)
≥75,000	19 (0.68%)	511 (25.51%)
Education		
<12th Grade	29 (0.99%)	733 (19.62%)
12th Grade	31 (1.32%)	527 (24.37%)
≥ 12th Grade	37 (1.40%)	1018 (52.31%)
Smoking		
Yes	17 (2.16%)	316 (47.02%)
No	37 (4.02%)	372 (46.79%)
Alcohol use		
Yes	0	9 (0.65%)
No	97 (4.81%)	1744 (94.54%)

Estimated percent distribution after applying NHANES sampling weights.

60-74 years old group with 43 (1.48%), while same age group reported 467 (14.78%) participant not to be at risk of PAH. Elderly > 75 years old were 28 (0.82%) at risk of PAH and 263 (7.62%) not at risk of PAH. Obese participant reported the highest number among people at risk of PAH with 53 (2.83%), followed by 29 (1.37%) and 10 (0.54%) for overweight and normal weight, respectively. However, people not at risk of PAH were 608 (40.28%), 519 (26.91%), and 577 (28.07%) for normal weight, overweight, and obese, respectively. Annual family income reported 0-$24,999 group to represent the highest number of population in both risk of PAH 48 (1.47%) and 944 (32.78%) not at risk of PAH. In the group of $25,000-$54,999, there were 24 (1.14%) at risk of PAH and 627 (27.98%). However, $55,000-74,999 group reported the lowest number of people at risk of PAH and people not at risk of PAH with 6 (0.42%) 198 (10.03%), respectively. No significant difference in distribution of people at risk of PAH in education variable, however, people with high education level ≥ 12^{th} grade reported to be the highest not at risk of PAH with 1018 (52.31%). Most Subjects at risk of PAH were not smokers with 37 (4.02%), while 17 (2.16%) were smokers. Subjects not at risk of PAH reported 316 (47.02%) to be smokers and 372 (46.79%) were not smokers (Table 15). Alcohol users, 97 (4.81%) were not at risk of PAH, while no cases reported to be at risk of PAH. Non-alcohol users were 97 (4.81%) at risk of PAH and 1744 (94.54%) were not at risk of PAH (Table 15).

Estimated ORs and 95% confidence intervals for the risk of PAH and the use of hormone pills with estrogen are shown in (Table 16). Results are presented for five logistic regression models: unadjusted, age-adjusted; BMI-adjusted; race-adjusted; and age, BMI, and race adjusted. In unadjusted model, use of hormone pills with estrogen was associated with higher risk of PAH [OR of 1.24; 95%CI 1.23-1.25]. After adjusting age, it remained

associated with risk of PAH but with less significant [OR 1.04; 95% CI 1.03-1.05].

Table 16. Estimated ORs (95% CI) for risk of PAH by Use hormone pills with estrogen only among Females ≥20 years of age, NHANES 1999-2004

Variable	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[1] (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)	Adjusted OR[4] (95% CI)
Use hormone pills with estrogen only							
No	60	12	1	1	1	1	1
Yes	257	54	1.24 (1.23-1.25)	1.04 (1.03-1.05)	2.41 (2.41-2.43)	1.23 (1.23-1.25)	1.10 (1.09-1.11)
By Age group							
20-59			1.02 (1.01-1.03)				
60-74			2.15 (1.14-2.16)				
≥ 75			4.48 (4.45-4.51)				
By Race							
Non-Hispanic White			0.91 (0.91-0.92)				
Other			1.22 (1.21-1.23)				
By BMI							
Normal			1.28 (1.28-1.30)				
Obese			6.25 (6.21-6.29)				
Overweight			3.01 (2.99-3.04)				

1 Adjusted for age
2 Adjusted for BMI
3 Adjusted for race
4 Adjusted for age race BMI

However, when we adjusted for BMI, it was significantly associated with higher risk of PAH [OR 2.42; 95% CI 2.41-2.43]. After adjusting all variable (age, race, and BMI), use of hormone pills with estrogen was associated with higher risk of PAH [OR of 1.10; 95%CI 1.09-1.11]. We further investigated the association between use of hormone pills with estrogen and risk of PAH within each group of adjusted variables age, race, and BMI. Age reported gradual increase in OR risk of PAH with older age. Age group 20-59 years old OR was [1.02; 95% CI 1.01-1.03], 60-74 years old {OR 2.15; 95% CI 1.14-2.16], and age group > 75 reported [OR 4.48; 95% CI 4.45-4.51]. Race was not associated with risk of PAH in Non-Hispanic white [OR 0.91; 95% CI 0.91-0.92]; however, race group "other"

Table 17. Estimated ORs (95% CI) for risk of PAH by Used hormone pills with progestin only among Females ≥20 years of age, NHANES 1999-2004

Variable	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[1] (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)	Adjusted OR[4] (95% CI)
Used hormone pills with progestin only							
No	224	56	1	1	1	1	1
Yes	93	10	0.57 (0.56-0.58)	0.74 (0.74-0.75)	0.57 (0.57-0.58)	0.57 (0.56-0.57)	0.72 (0.72-0.73)
By Age group							
20-59			0.73 (0.73-0.74)				
60-74			2.04 (2.03-2.05)				
≥ 75			4.11 (4.08-4.14)				
By Race							
Non-Hispanic White			0.57 (0.56-0.57)				
Other			0.91 (0.90-0.92)				
By BMI							
Normal			0.57 (0.57-0.58)				
Obese			6.33 (6.29-6.38)				
Overweight			2.91 (2.89-2.94)				

1 Adjusted for age
2 Adjusted for BMI
3 Adjusted for race
4 Adjusted for age race BMI

was associated with high risk of PAH [OR 1.22; 95%CI 1.21-1.23]. All BMI groups were associated with risk of PAH. Normal BMI was [OR 1.28; 95% CI 1.28-1.30]; obese was [OR 6.25; 95% CI 6.21-6.29]; and overweight was [OR 3.01; 95% CI 2.99-3.04].

Estimated ORs and 95% confidence intervals for the risk of PAH and the use of hormone pills with progestin are shown in (Table 17). Results are presented for five logistic regression models: unadjusted, age-adjusted; BMI-adjusted; race-adjusted; and age, BMI, and race adjusted. All models, unadjusted and adjusted were not associated with risk of PAH. In unadjusted model, use of hormone pills with progestin was not associated with

risk of PAH [OR of 0.57; 95%CI 0.56-0.58]. After adjusting age, it remained not associated with risk of PAH [OR 0.74; 95% CI 0.74-0.75].

We further investigated the association between use of hormone pills with progestin and risk of PAH within each group of the adjusted variables age, race, and BMI. Age was associated with age groups 60-74 and > 75 years old with [OR 2.04; 95% CI 2.03-2.05] and [OR 4.11; 95% CI 4.08-4.14], respectively. Race was not associated with risk of PAH in both groups Non-Hispanic white and other wit [OR 0.57; 95% CI 0.56-0.57] and [OR 0.91; 95%CI 0.90-0.92], respectively. Subjects used hormone pills with progestin and had normal BMI were not associated with risk of PAH. However, subjects with obese and overweight BMI were associated with higher risk of PAH [OR 6.33; 95% CI 6.29-6.38] and [OR 2.92; 95% CI 2.89-2.94], respectively.

Estimated ORs and 95% confidence intervals for the risk of PAH and the use of hormone pills with estrogen/progestin are shown in (Table 18). Results are presented for five logistic regression models: unadjusted, age-adjusted; BMI-adjusted; race-adjusted; and age, BMI, and race adjusted.

All models, unadjusted and adjusted were not associated with risk of PAH. In unadjusted model, use of hormone pills with estrogen/progestin were not associated with risk of PAH [OR of 0.59; 95%CI 0.59-0.60]. After adjusting age, it remained not associated with risk of PAH [OR 0.65; 95% CI 0.65-0.66]. We further investigated the association between use of hormone pills with estrogen/progestin and risk of PAH within each group of the adjusted variables age, race, and BMI. Age was associated with age groups 60-74 and > 75 years old with [OR 2.05; 95% CI 2.04-2.06] and [OR 4.38; 95% CI 4.35-4.41], respectively. Race was not associated with risk of PAH in both groups Non-Hispanic white and other

Table 18. Estimated ORs (95% CI) for risk of PAH by Used estrogen/progestin combo pills among Females ≥ 20 years of age, NHANES 1999-2004

Variable	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[1] (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)	Adjusted OR[4] (95% CI)
Used estrogen/progestin combo pills							
No	247	56	1	1	1	1	1
Yes	70	10	0.59 (0.59-0.60)	0.65 (0.65-0.66)	0.56 (0.56-0.57)	0.60 (0.59-0.60)	0.60 (0.60-0.61)
By Age group							
20-59			0.65 (0.65-0.66)				
60-74			2.05 (2.04-2.06)				
≥ 75			4.38 (4.35-4.41)				
By Race							
Non-Hispanic White			0.90 (0.90-0.91)				
Other			0.60 (0.60-0.61)				
By BMI							
Normal			0.55 (0.55-0.56)				
Obese			6.73 (6.69-6.78)				
Overweight			3.17 (3.15-3.19)				

1 Adjusted for age
2 Adjusted for BMI
3 Adjusted for race
4 Adjusted for age race BMI

white [OR 0.90; 95% CI 0.90-0.91] and [OR 0.60; 95%CI 0.60-0.61], respectively. Subjects used of hormone pills with estrogen/ progestin and had normal BMI were not associated with risk of PAH. However, subjects with obese and overweight BMI were associated with higher risk of PAH [OR 6.73; 95% CI 6.69-6.78] and [OR 3.17; 95% CI 3.15-3.19], respectively.

Estimated ORs and 95% confidence intervals for the risk of PAH and the use of birth control pills are shown in (Table 19). Results are presented for five logistic regression models: unadjusted, age-adjusted; BMI-adjusted; race-adjusted; and age, BMI, and race adjusted. All models, unadjusted and adjusted were associated with risk of PAH. In

unadjusted model, use of birth control pills was associated with higher risk of PAH [OR of

Table 19. Estimated ORs (95% CI) for risk of PAH by Ever taken birth control pills among Females ≥ 20 years of age, NHANES 1999-2004

Variable	No. Non-cases	No. Cases	Unadjusted OR (95% CI)	Adjusted OR[1] (95% CI)	Adjusted OR[2] (95% CI)	Adjusted OR[3] (95% CI)	Adjusted OR[4] (95% CI)
Ever taken birth control pills							
No	645	60	1	1	1	1	1
Yes	974	37	2.47 (2.46-2.48)	1.24 (1.23-1.25)	2.46 (2.45-2.47)	2.56 (2.55-2.57)	1.23 (1.22-1.24)
By Age group							
20-59			1.18 (1.18-1.19)				
60-74			5.14 (5.12-5.17)				
≥ 75			5.84 (5.81-5.88)				
By Race							
Non-Hispanic White			1.86 (1.85-1.87)				
Other			2.55 (2.54-2.56)				
By BMI							
Normal			2.44 (2.43-2.45)				
Obese			7.43 (7.38-7.48)				
Overweight			3.72 (3.69-3.75)				

1 Adjusted for age
2 Adjusted for BMI
3 Adjusted for race
4 Adjusted for age race BMI

2.47; 95%CI 2.46-2.48]. After adjusting age, it remained associated with risk of PAH [OR 1.24; 95% CI 1.23-1.25]. However, when we adjusted for BMI, it was significantly associated with higher risk of PAH [OR 2.46; 95% CI 2.45-2.47]. The highest OR was reported after adjusting race [OR 2.56; 95% CI 2.55-2.57]. After adjusting all variable (age, race, and BMI), use of birth control pills was associated with higher risk of PAH [OR of 1.23; 95%CI 1.22-1.24]. We further investigated the association between use of birth control pills and risk of PAH within each group of the adjusted variables age, race, and BMI. All age groups were associated with higher risk of PAH 20-59, 60-74 and > 75 years

old with [OR 1.18; 95% CI 1.18-1.19], [OR 5.14; 95% CI 5.12-5.17], and [OR 5.84; 95% CI 5.81-5.88], respectively.

Race was associated with higher risk of PAH in both groups Non-Hispanic white and other wit [OR 1.86; 95% CI 1.86-1.87] and [OR 2.55; 95%CI 2.54-2.56], respectively. All BMI groups were associated with higher risk of PAH when use birth control pills. Subjects with normal and overweight BMI were associated with higher risk of PAH [OR 2.44; 95% CI 2.43-2.45] and [OR 3.72; 95% CI 3.69-3.75], respectively. However, obese reported the highest [OR 7.43; 95% CI 7.38-7.48].

DISCUSSION

This is the first study to date that used NHANES data to evaluate hormone and oral contraceptive in subject with risk of PAH. Our results support the known high risk of PAH with estrogen exposure. We showed estrogen to be associated with OR of 1.24 in subjects at risk of PAH. This result support the hypothesis and confirm the known risk of estrogen in PAH. In fact, many studies reported high risk of PAH with high estrogen in circulation (Roberts et al., 2009). Also, it was suggested that estrogen induce pathogenesis of PAH (E. D. Austin et al., 2009; West et al., 2008). Proliferation of pulmonary artery smooth muscle cells is known in PAH; and Estrogen metabolites 16α-hydroxyestrone showed to increase PASMC proliferation (White et al., 2012). Estrogen is associated with higher risk of thromboembolism (Humbert et al., 2001). Moreover, this association was linked with up regulation of estrogen metabolizing enzyme cytochrome P450 1B1 (CYP1B1) (White et al., 2011).

However, with BMI adjusting, the OR risk of PAH and estrogen exposure increase to 2.41, which reflect a key role of BMI in PAH. This finding support the significant

association between obesity and PAH (BMI > 30, P-value=0.004) in REVEAL (Burger et al., 2011). We found elderly to be at higher risk of PAH with OD up to 4.48 when exposed to estrogen. Schachna reported that elderly > 60 years old had 2 times higher risk of PAH than patients < 60 years old (OR, 2.30; 95% CI, 1.32 to 3.99) (Schachna et al., 2003). This also supports the finding of the age specific death rate for elderly by CDC in 2005 (CDC, 2005).

Progestin is found in birth control and it have been recommended as a safe medication to prevent pregnancy for PAH females (Santiago-Munoz, 2011; Thorne et al., 2006). Our result showed no risk of PAH in females used progestin pills only with OR of 0.75. Thorne and colleagues found no association of progestin only use with PAH and it was recommended to use for risk of venous thrombotic (Thorne et al., 2006). However, age and BMI showed contradicted high OR of up to 4.11 and 6.33, respectively, in females used progestin pills only. This might explain the contradicted effect of progestin reported to increase thromboembolism in some PAH cases (Thorne et al., 2006).

Combined estrogen/ progestin contraceptives are known to have contradicted effect on PAH (Montani et al., 2013). Our results showed OR of 0.59 for estrogen/ progestin pills use and risk of PAH. Combined therapy has been used for PAH, with some side effects of high risk of cardiovascular diseases (Lara et al., 2012). In MCT-induced PAH, combined estrogen/ progestin was reported to prevent development of PAH and right ventricular hypertrophy (Jones, Zhang, Jackson, & Tofovic, 2006). However, randomized clinical trial reported estrogen/ progestin may increase risk of coronary heart disease (CHD) in postmenopausal females (Manson et al., 2003). We reported OR of 4.38 for elderly using estrogen/ progestin pills. The same association of age was reported with estrogen/ progestin

use and risk of CHD. Also, we showed obese females using estrogen/ progestin pills to have the highest risk of PAH with OR of 6.73.

Our results showed that use of birth control increase risk of PAH in females with OR of 2.47. PAH association with oral contraceptive been reported in pre- and postmenopausal females (Kleiger, Boxer, Ingham, & Harrison, 1976). We also showed birth control female users have high risk of PAH in all age groups, race, and BMI. No reports found for race and its association with PAH. Taken together, our study is the first cross-sectional study that use NHANES data to evaluate the association between hormone therapy and oral contraceptive. Our results support the known findings that females using estrogen and oral contraceptive are at higher risk of PAH.

There are some limitations in our study including that we used scoring method for PAH risk factors such as REVEAL scoring methods (Benza et al., 2012). Because of limited data in NHANES, we used available variables hypertension, diabetes, thyroid problems, uric acid level, and insulin status. Thus, we created a variable named risk of PAH because there was not PAH variable included in the NHANES survey data. This may increase the risk of misclassification to people at risk and not at risk of PAH. However, using NHANES cross-sectional surveys including representative sample size, medical health data, and reproductive health data of the U.S. population provided us with strength in our study.

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CHAPTER 5

MANUSCRIPT 3

Meta-analysis of Pulmonary Arterial Hypertension Microarray Expression Data Reveals Significant Aberrations of Gene Networks Involved in PAH Associated Pathways

ABSTRACT

Pulmonary arterial hypertension (PAH) causes many vascular changes including smooth-muscle cell and endothelial-cell proliferation, vasoconstriction, and thrombosis. PAH reported to associated with many genes such as BMPR2 and ALK1. It is important to understand the mechanism behind the onset of PAH to find an effective treatment and early diagnosis of the disease. Many disrupted pathways represented the complexity of PAH and difference in gene expression could prove the effect of how important to understand pathways elements. Thus, we performed meta-analysis on a published gene expression microarray datasets comparing gene expression in PAH samples and control samples. Six microarray datasets were included in our meta-analysis. Bioinformatics and Bayesian network analysis statistically identifies genes that are associated with PAH from our meta-analysis results, gene ontology databases, and literature. The most significant pathways associated with PAH were Apoptosis signaling pathway, p53 pathway, Ras Pathway, T-cell activation, TGF-beta signaling pathway, VEGF signaling pathway, Wnt signaling pathway. Using BN analysis, we found VAMP2, LAMA5, POLR2C, VEGFB, and PRKCH to be a plausible pathway of PAH.

MANUSCRIPT 3

Meta-analysis of Pulmonary Arterial Hypertension Microarray Expression Data Reveals Significant Aberrations of Gene Networks Involved in PAH Associated Pathways

Introduction

Pulmonary arterial hypertension (PAH) is a disease that affects pulmonary arteries leading to increase in mean pulmonary arterial pressure. This increase in arterial pressure cause many vascular changes including smooth-muscle cell and endothelial-cell proliferation, vasoconstriction, and thrombosis (Rubin M. Tuder et al., 2007). Although, neointimal lesion formation cause intraluminal block which characterized by abnormal proliferation of smooth muscle cells (SMCs) (Marc Humbert et al., 2004). Human studies suggested that only women especially young women were more susceptible of developing PAH (Tofovic, 2010). Notably, familial PAH with mutated bone morphogenic protein receptor type 2 (BMPR2) had a higher penetration of disease in women because of the alteration in E2 metabolism (E. D. Austin et al., 2009). Recently, REVEAL reported that female-to-male ratio for IPAH is 4.1:1 (Badesch et al., 2010). In France, data were collected from 674 adults with PAH and followed up for 3 years from 2003-2006. This registry has confirmed that female are predominant in most PAH subtypes with a sex ratio (SR) female/ male of 1.9 for all PAH.

Pulmonary hypertension can be caused by different medical conditions, majority of these conditions are demonstrated histopathologically by hypertrophy of the pulmonary arterioles (Rubin M. Tuder et al., 2009). Explanation of this high susceptibility remains unclear; however sex hormones and estrogen were reported to play a role in pathogenesis

of pulmonary hypertension. Indeed, females' longer survival was referred to estrogen activity in PAH. On the other hand, mutations in Bone Morphogenic Protein (BMP) receptor type II (BMPR2) were reported in about 23% of all PAH cases (Newman, Phillips, & Loyd, 2008). Mutations in BMPR2 were associated with the increase vascular smooth muscle cells proliferation by downregulating of BMP signaling (Morrell et al., 2001).

Genomic and non-genomic pathways were reported to have an effect on estrogen receptors α and β. For example, prostacyclin release and nitric oxidelo production were reported to be higher by rapid non-genomic mechanisms, which increase endothelial nitric oxide synthase activity and endothelial nitric oxide synthase messenger RNA (mRNA) levels and (Sherman et al., 2002). Notably, HIF-1α regulate several genes that have significant role in angiogenesis, vascular reactivity and remodeling, and cell proliferation (Tofovic, 2010). The importance of HIF-1α was linked to many human and experimental models including severe PAH in humans where HIF-1α was up regulated in obliterative endothelial lesions; and high levels of HIF-1α were associated with vascular remodeling and development of PAH.

Murine models were studied by Mair and colleagues and they have reported overexpression of MTS1 and serotonin transporter gene (SERT1) in mice (Dempsie et al., 2011). Pulmonary shunting, is the less oxygen in alveoli, was reported to cause alterations of the endothelin pathway in piglets and young lambs. These alterations resulted in high expression of endothelin-1 and endothelin-A receptor; and with chronic shunting, increase endothelial nitric oxide synthesis (eNOS). Indeed, these alterations were associated with increased blood flow in pulmonary arteries (Rondelet et al., 2012). Sugen/hypoxia rat

model showed that pulmonary shunts were associated with high expression of VEGF in IPAH (Geiger et al., 2000).

Hypoxia and oxidative stress have a significant role in PAH, human and experimental. By down regulating expression of CYPIA, which control E2 metabolism, hypoxia and oxidative stress cause proliferation of pulmonary ECs and VSMCs, as well as pulmonary vascular remodeling (Kleinman et al., 2007). Hypoxia has been associated to decrease VEGF protein and gene expression, however, the down-regulation cause VEGFR blockade and endothelial cell injuries followed by plexiform lesion formation (Voelkel, Vandivier, & Tuder, 2006). Vascular endothelial growth factor (VEGF) has proliferative and high levels of VEGF and VEGF receptor-2 expressions have been reported in IPAH patients' lung samples. VEGF and VEGFR2 are expressed by ECs within the plexiform lesions in lungs from patients with PAH (Malenfant et al., 2013). However, mutations in these molecules were linked with PAH pathogenesis and lesion formation. Plexiform lesion in IPAH showed up regulation in some genes including TGF-1, HIF1a, THBS1, MMP9, and cKIT (Jonigk et al., 2011). Jonigk and colleagues found a significant up regulation mediators in plexiform lesion such as THBS1, HIF1a, Ang-1, VEGF and its receptors (VEGFR1 and VEGFR2), and Tie-2 (Jonigk et al., 2011).

Genetic mutation and risk of PAH have been reported with many genes and pathways. Transforming growth factor β (TGF-β) family receptors are the most known pathway to cause gene mutation in PAH. From the TGF-β, two genes were reported to cause PAH including BMPR2 and activin-receptor–like kinase 1 (ALK1) (Foletta et al., 2003). More than 45 mutations have been identified in familial type of PAH patients because the inheritance role of BMPR2. However, BMPR2 mutation cannot express the

disease alone. Mutation in ALK1 is rare, however it was reported to cause IPAH (Farber & Loscalzo, 2004).

Other somatic gene mutations linked to PAH development including prostacyclin, Bax, thromboxane, serotonin transporter, angiopoietin-1, plasminogen activator inhibitor, Smads, and Von Willebrand factor (Rajkumar et al., 2010). Moreover, molecular studies reported that down regulation of the voltage-gated K_ 1.5 potassium channel and up regulation of the transient receptor potential channel calcium channels were associated with PAH. These abnormal regulations cause pulmonary artery smooth muscle cells to proliferate, contract and resist to apoptosis (Bonnet et al., 2007). On the other hand, abnormal expression of NADPH oxidase 4 (NOX4) and interleukin-6, were reported to cause vasoconstriction and remodeling in PASMCs. In chronic hypoxia mice, 2.5-fold up regulation of NOX4 were reported to be associated with IPAH than controls (Mittal et al., 2007). Moreover, supporting the role of vascular remodeling, NOX2 was reported to be as 3.5-fold up regulated in human lung PAH (Rajkumar et al., 2010). Abnormal proliferation and growth of vascular smooth muscle cells in PAH were also associated with abnormal expression of PPP2CA and MYBL1 genes (Rajkumar et al., 2010). Although, Shelat and colleagues reported that overexpression of E2F1 (a transcription factor which control cell cycle) and NCOA2 were associated with PAH (Shelat et al., 2001).

Serotonin is a vasoconstrictor which stimulate smooth muscle cell hyperplasia and hypertrophy (Farber & Loscalzo, 2004). High levels of serotonin have been associated to increase the proliferation of cultured pulmonary vascular smooth-muscle cells in IPAH patients (S. Eddahibi et al., 2001). Furthermore anorexigens (appetite suppressants) such as aminorex, dexfenfluramine, and the fenfluramine and phentermine (fen/phen) have role

in plexiform lesions and serotonin pathogenesis (Wideman & Hamal, 2011). High incidence of IPAH was reported among patients who took dexfenfluramine elevate the secretion of serotonin from platelets and prevent its reuptake. Serotonin promotes cell growth and vasoconstriction; and in plexiform lesion formation, serotonin high levels affect signaling pathways downregulation of mutated BMPR2; which was observed in mice as well (X. Yang et al., 2005). Anorexigens trigger IPAH by: increasing serotonin release from cellular stores which stimulate plasma serotonin levels and serotonin receptors and trigger the formation of plexiform lesions (Saadia Eddahibi & Adnot, 2002)(Launay et al., 2002). In up to 80% of IPAH patients, serotonin role cause rapid increase in PVR that lead to vasoconstriction followed remodeling in smooth muscle cell proliferation. This acute pulmonary vasoconstriction usually mediated by the expression of receptors on vascular smooth muscle cells including 5-HT2A, 5-HT2B, and 5- HT1B/1D (Rodat-Despoix, Crevel, Marthan, Savineau, & Guibert, 2008).

It was reported that lack in expression of TGF-β receptor 1, TGF-β receptor 2, and their signaling smad(s) 1-4, including the phosphorylated smad 1/5/8 and 2 had a central role in plexiform lesions formation (Rubin M. Tuder et al., 2007). The lack of expression of these smads indicated the absence of their signaling through TGF-β or BMP as well, which allowed plexiform cells to abnormally proliferate (Richter et al., 2004). In general many signaling pathways when interrupted cause proliferation and angiogenesis, which are the leading cause of lesion formation. Many cellular and molecular mechanisms and pathways involve in the initiation and progression of IPAH. Different factors and molecules can cause plexiform lesion formation, either by increase proliferation or by decreasing apoptosis. Most of these mechanisms and pathways were applied on laboratory

experiments as animal models. Plexiform lesions have been defined as active angiogenic lesion caused by irregular or neoplastic-like endothelial proliferation and myofibroblast infiltration (Berger, Geiger, Hess, Bogers, & Mooi, 2001)(R. M. Tuder et al., 2001).

Even though TGF-β signaling molecules are not expressed in the central core of plexiform lesion, they are actively expressed outside of the plexiform lesion (Abe et al., 2010). Moreover, other pathways inducing vasoconstrictor production are over-expressed within the lesion including thromboxane, leukotrienes, and endothelin (Budhiraja, Tuder, & Hassoun, 2004); and vasodilator production by prostacyclin and nitric oxide (Berger et al., 2001).

Plexiform lesion growth was reported to be caused by mononuclear inflammatory cells surround vascular sites (P. Dorfmüller, Perros, Balabanian, & Humbert, 2003). Moreover, high levels of pro-inflammatory cytokines were reported around plexifonn lesions in PAH including macrophages and B and T lymphocytes. Inflammatory processes on pulmonary endothelial cells can cause proliferative pulmonary vasculopathy. Pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor alpha (TNF-a), as well as macrophages and lymphocytes were reported to increase aromatase activities and peripheral E2 synthesis (Tofovic, 2010). This linkage was associated with high levels of E2 as well as pulmonary vascular remodeling and progression of PAH (Peter Dorfmüller et al., 2011).

It is important to understand the mechanism behind the onset of PAH to find an effective treatment and early diagnosis of the disease. Many disrupted pathways represent the complexity of PAH and difference in gene expression could prove the effect of how important to understand pathways elements. This study aims to show what is the effect of

the difference in gene expression on PAH using microarray expression data of PAH and non-PAH from published studies. We further extend our aim to investigate what genes make this difference and what pathways are associated with the disease and these genes. Moreover, to know how this combination of significant associated genes with PAH would lead us to discover new pathway of PAH. This will be applied using BN modeling of gene networks.

Materials and Methods

Microarray dataset

National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) was searched for gene expression profiles and datasets of Pulmonary Arterial Hypertension. Synonyms combination key terms were used for this comprehensive systematic search including: Pulmonary Arterial Hypertension (PAH), Idiopathic, case control, study. No language, subject, and/or period restriction were applied. All age period were included (pediatric, children, and adults); and both gender (males and females) were included. All types of pulmonary hypertension (pulmonary arterial hypertension (PAH), congenital heart disease (CHD-PAH), connective tissue disease (CTD-PAH), chronic thromboembolic pulmonary hypertension (CTEPH), idiopathic pulmonary arterial hypertension (IPAH), systemic sclerosis (Ssc-PAH), limited systemic sclerosis-PAH (lss-PAH), portopulmonary hypertension (PoPH), and ventricular septal defect (VSD-PAH). Data extracted included study title, year of publication, disease of interest, study population, if the study included healthy controls, gender of participants (if available), mean age, age of each participant (if available). Sixteen datasets were found with different types of PAH (IPAH, Ssc-PAH, and Lss-PAH) and control subjects. The inclusion criteria

we used were: article to be published, PAH subjects, all datasets files available to download from GEO, clear information attached to submitted files, less than 50% missing gene expressions. All datasets were normalized and discretized using z-score normalization method (the value – mean / standard deviation), and (z<-1=0; -1<z<1=1; z=>1, 2), respectively.

Datasets were tested for random distribution using 10% of genes in SPSS. Because we have different biological material locations, datasets were separated into blood and tissue samples. We combined all dataset to find out how many genes are common among all six datasets. We checked that ID1-4 gene expression are available, and we calculated their fold change as well. Statistical t-test was performed to identify genes that significantly expressed in PAH blood and tissue samples from GEO datasets, and we selected the first quartile of significant p-value of 0.05. Statistical correlation analysis was performed on ID3 correlated genes in blood and tissue from GEO datasets, and we selected the first quartile of correlated genes. We matched the common genes that were significantly expressed in PAH blood and tissue samples and ID3 correlated genes in blood and tissue from GEO datasets; and target gene list were created for blood and tissue.

Gene ontology and pathway analysis

We used (PANTHER, Protein ANalysis THrough Evolutionary Relationships, Classification System DataBase) for gene ontology analysis to understand the functions of the gene list. PANTHER provides comprehensive function information about genes, and was designed to also facilitate analysis of large numbers of gene associations (http://pantherdb.org/). Statistical enrichment test for each of biological process, molecular function, protein class, cellular component, and pathway were evaluated according to the

likelihood that their numerical values were drawn randomly from the overall distribution of values. The Mann-Whitney U Test (Wilcoxon Rank-Sum Test) was used to determine the P-value (the chromatin packaging and remodeling genes have random values relative to overall list of values that were input).

Protein-protein interaction network construction

We used STRING database (http:// string-db.org), which provides functional profile of each gene, to identify the association in the gene list. Also, to demonstrate functional and canonical pathway analysis associated with the list of genes provided in network maps. STRING provided data from published identified functions and interactions.

Bayesian network analysis (Bene):

The idea behind BN is to record how many times that different values of the variable (X) occur together with different vectors (V) in the data. In our analysis, X = variable (genes, disease, age, gender,...etc.), V= sample (patient or control) (Silander & Myllymaki, 2012). BN are directed acyclic graphs (DAGs) which represent an association among different variables, and variable can have more than one state (Heckerman, 1998). The association between these variables, which represented by the best BN, resulted through Bene software are based on five logical steps which (Silander and Myllynaki) explained in their published article (Silander & Myllymaki, 2012). “These five steps are: 1) Calculate the local scores for all n2n−1 different (variable, variable set)-pairs, 2) Using the local scores, find best parents for all n2n−1 (variable, parent candidate set)-pairs, 3) Find the best sink for all 2n variable sets, 4) Using the results from Step 3, find a best ordering of the variables, and 5) Find a best network using results computed in Steps 2 and 4”. Using discretized data, Bene

first constructed a best net using BDe score (with equivalent sample size 1.0) and a best net using the BIC score (Silander & Myllymaki, 2012) and (Yoo, 2012). The aim of using BN analysis is to develop a model which examine the association of gene expression levels in PAH samples and control samples. This might determine the risk of PAH with different expression of genes.

Genie

GeNIe 2.1 is a software used to validate and manipulate BN structure resulted from Bene. It helped us to find out what genes and expression affect PAH or control samples.

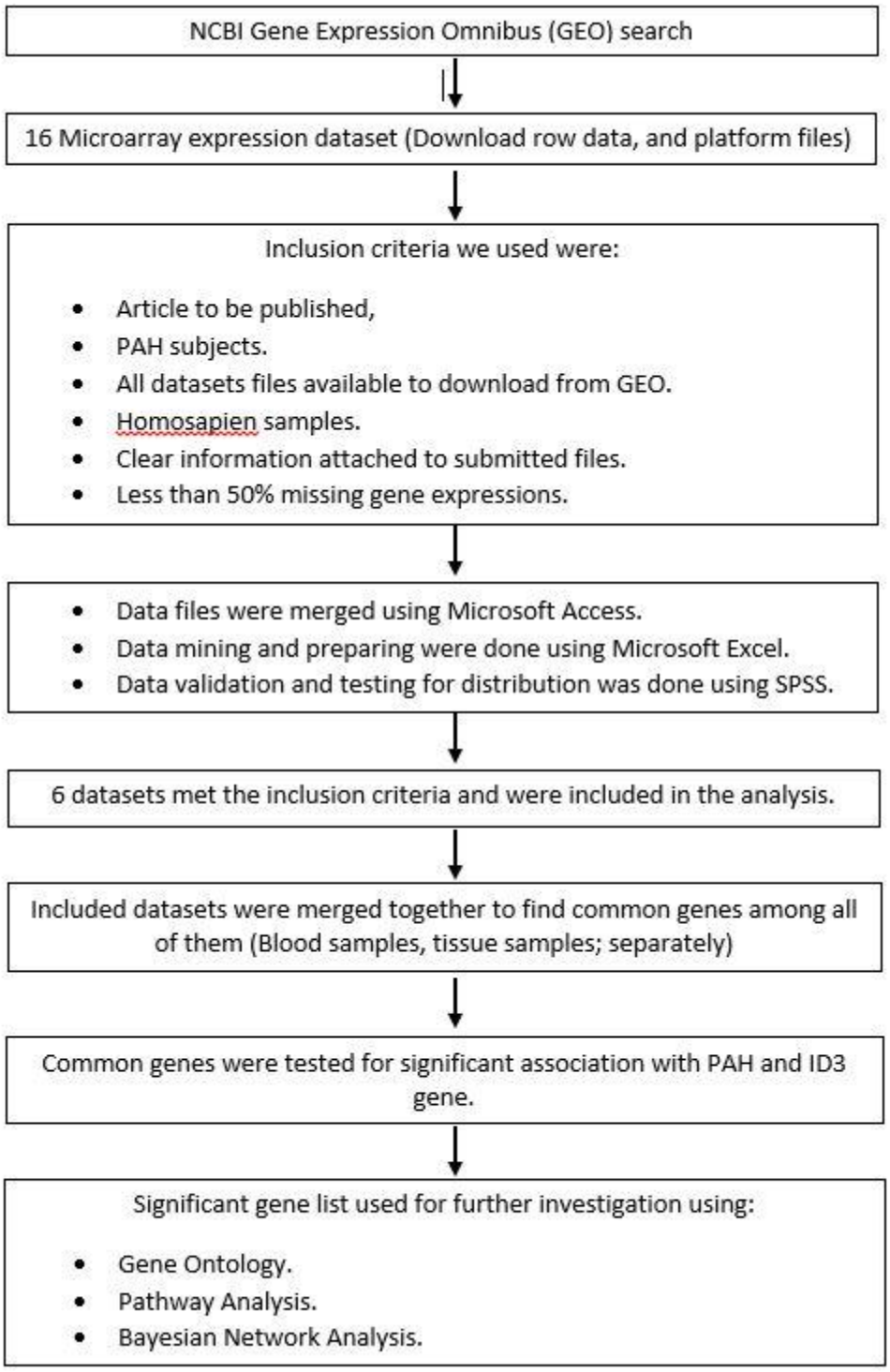

Figure 3 Flow chart summarizing the steps was carried out through this study

RESULTS

Six PAH microarray datasets met our inclusion criteria and were included in the analysis (Table 20). Samples and controls were divided into two groups based on the biological material location, blood and tissue (Table 21). Common genes were matched among six datasets and 10,716 genes found to be common in all datasets (supplement1).

Table 20. Six Pulmonary Arterial Hypertension (PAH) gene expression microarray datasets from Gene Expression Omnibus (GEO) were included. Information about publication year, Organism, subjects, gender, age, biological material, target molecule, geneChip a *and platform, country, and GEO link included.*

Dataset	Year	Organism	Subject cohort	Gender M/F	Mean age/ yrs	Biological material	Target molecule	GeneChip/ Platform	Reference	Country	GEO link
1	2010	Homo Sapiens	18 PAH, 13 normal control	7/11, 5/8 - (Ethnicity/Race: 17 White/1 Native American, 12 White/1 African American)	44, 60	LH, total RNA	mRNA	Agilen/ GPL6480	1	USA	GSE15197
2	2011		8 IPAH, 10 SSc-PAH, 9 normal control	2/6, 4/5, 4/5- Smokers/ Never smoke (4/4,3/3)	35, 52, 53	LH, total RNA		Illumina/ GPL16221	2		GSE48149
3	2008		4 PAH (BMPR2 mutation), 3 Normal Control (BMPR2 mutation)	2/2, 1/2	30, 73	Cultured lymphocytes, total RNA		Affymetrix / GPL570	10		GSE10767
4	2010		8 IPAH, 10 Normal Control	20 females	62, 51	PBMCs, total RNA		Affymetrix / GPL570	12		GSE22356
5	2014		30 IPAH, 42 SSc=PAH, 41 normal control	5/25, 9/33, 7/34 – (Ethnicity/Race: 115 Caucasian, 20 African Amirecan, 4 Asian, 1 Hispanic)	52, 60, 45	PBMCs, total RNA		Illumina/ GPL6947	13		GSE33463
6	2014		2 PAH (BMBR2 mutation), 2 Control			heart right ventricular wall, totalRNA		Affymetrix / GPL6244	15		GSE67492

Table 21. Distribution of GEO samples included in the analysis (PAH, control, and gender), based on 2 biological material location (Tissue and Blood).

	PAH	Control	Total
Tissue	38	24	62
Blood	84	54	138
Total	122	78	200
Male/ Female	29/ 82	17/ 49	46/ 131

Statistical analysis t-test of the 10,716 common genes showed that 2,598 genes in blood samples and 2,542 genes in tissue samples were significantly expressed in PAH samples (P-value < 0.05). Correlation analysis of ID3 correlated genes showed that 2,679 gene in blood samples and 2,699 genes in tissue samples, were in the first quartile of ID3 correlated genes (Supplement 2).

We matched the results of significant genes from the two statistical analysis tests, t-test and ID3 correlation. Matching results showed that 701 genes in blood and 573 genes in tissue, were common in t-test and ID3 correlation test (Table 22 and Table 23) (Figure 1 and 2). Only 28 genes were common significantly expressed among blood and tissue samples (Table 24) (Figure 3 diagram). All Common genes represented in blood samples, tissue samples, t-test significant with PAH, and ID3 correlated genes showed in (Figure 4).

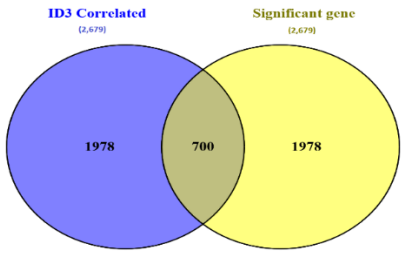

Figure 4 Venn diagram represent number of ID3 correlated genes (2,679) and number of significantly expressed genes in PAH tissue samples from GEO datasets (2,679), in Blood samples. 700 genes are common between ID3 correlated genes and significantly expressed genes in PAH tissue samples from GEO datasets.

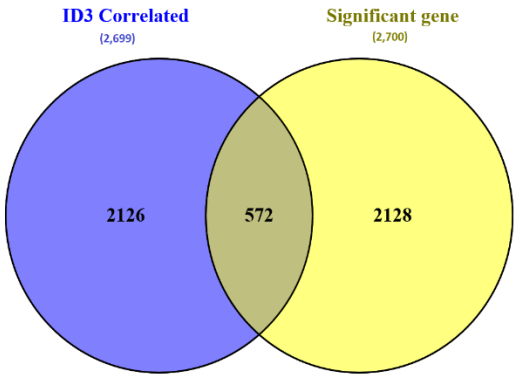

Figure 5 Venn diagram represent number of ID3 correlated genes (2,699) and number of significantly expressed genes in PAH tissue samples from GEO datasets (2,700), in Tissue samples. 572 genes are common between ID3 correlated genes and significantly expressed genes in PAH tissue samples from GEO datasets.

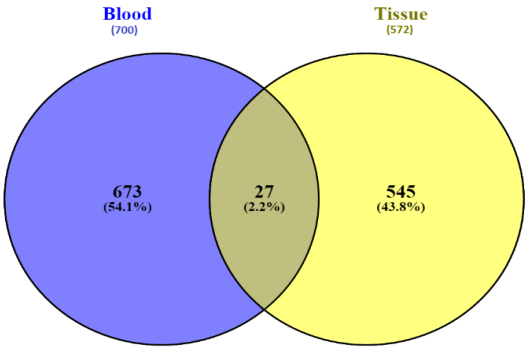

Figure 6 Venn diagram represent number genes (27) are common between ID3 correlated genes and number of significantly expressed genes in PAH from GEO datasets, blood samples (700) genes and tissue samples (572) genes.

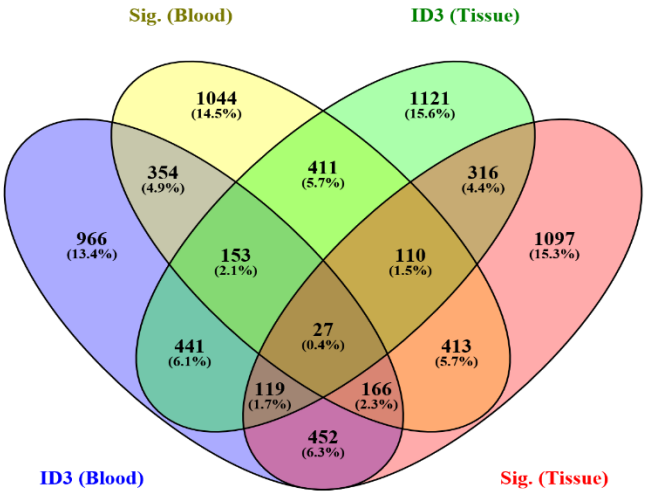

Figure 7 Venn diagram represent overall number of genes are common between different list of correlate with ID3 and number of genes are significantly expressed in PAH blood and tissue samples from GEO datasets. Yellow represent number of genes are significantly expressed in PAH blood samples from GEO datasets (2,679). Green represent number of genes are ID3 correlated in PAH tissue samples from GEO datasets (2,699). Blue represent number of genes are ID3 correlated in PAH blood samples from GEO datasets (2,679). Red represent number of genes are significantly expressed in PAH tissue samples from GEO datasets (2,700).

Table 22. Genes that are significantly expressed in PAH samples; and significantly correlated with ID3 in blood samples (701 genes) from the significant gene list provided from GEO PAH datasets

Gene Symbol	Gene Name
KIFC2	kinesin family member C2
LDHB	lactate dehydrogenase B
FHIT	fragile histidine triad gene
OXA1L	oxidase (cytochrome c) assembly 1-like
SLC2A4RG	SLC2A4 regulator
RPL19	ribosomal protein L19; ribosomal protein L19 pseudogene 12
TUBB2A	tubulin, beta 2A
H1FX	H1 histone family, member X
CLK1	CDC-like kinase 1
MYLIP	myosin regulatory light chain interacting protein
ACBD6	acyl-Coenzyme A binding domain containing 6
WDR74	WD repeat domain 74
BRPF1	bromodomain and PHD finger containing, 1
SH3YL1	SH3 domain containing, Ysc84-like 1 (S. cerevisiae)
WDR73	WD repeat domain 73
MALL	mal, T-cell differentiation protein-like
MC1R	tubulin, beta 3; melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)
RPLP0	ribosomal protein, large
CCR10	chemokine (C-C motif) receptor 10
SH3BGR	SH3 domain binding glutamic acid-rich protein
FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed
STAG3	stromal antigen 3
LSM4	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)
ZNF395	zinc finger protein 395
DHX35	DEAH (Asp-Glu-Ala-His) box polypeptide 35
RPL12	ribosomal protein L12 pseudogene 2
ADAM7	ADAM metallopeptidase domain 7
CDCA4	cell division cycle associated 4
GNG7	guanine nucleotide binding protein (G protein), gamma 7
STAG1	stromal antigen 1
KLRB1	killer cell lectin-like receptor subfamily B, member 1
CGRRF1	cell growth regulator with ring finger domain 1
LIG1	ligase I, DNA, ATP-dependent
ESPL1	extra spindle pole bodies homolog 1 (S. cerevisiae)
RAD1	RAD1 homolog (S. pombe)
UXT	ubiquitously-expressed transcript
NME3	non-metastatic cells 3, protein expressed in
MAGEE1	melanoma antigen family E, 1
CD320	CD320 molecule
MAPK6	mitogen-activated protein kinase 6
RPS17	ribosomal protein S17

RPS14	ribosomal protein S14
SURF2	surfeit 2
RPS11	ribosomal protein S11
HSPBAP1	HSPB (heat shock 27kDa) associated protein 1
CRTC2	CREB regulated transcription coactivator 2
RAD23B	RAD23 homolog B (S. cerevisiae)
WDR60	WD repeat domain 60
DUSP12	dual specificity phosphatase 12
ACP1	acid phosphatase 1, soluble
PPP1R3D	protein phosphatase 1, regulatory (inhibitor) subunit 3D
ARIH1	ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila)
IL17D	interleukin 17D
SLC29A2	solute carrier family 29 (nucleoside transporters), member 2
DUSP16	dual specificity phosphatase 16
PABPC1	poly(A) binding protein, cytoplasmic pseudogene 5; poly(A) binding protein, cytoplasmic 1
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
KIAA1683	KIAA1683
SSSCA1	Sjogren syndrome/scleroderma autoantigen 1
ZNF622	zinc finger protein 622
MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A
ZNF529	zinc finger protein 529

TCF7	transcription factor 7 (T-cell specific, HMG-box)
ZC3H15	zinc finger CCCH-type containing 15
KLF12	Kruppel-like factor 12
KLF13	Kruppel-like factor 13
NXF1	nuclear RNA export factor 1
RPS5	ribosomal protein S5
RPS7	ribosomal protein S7
C1ORF64	chromosome 1 open reading frame 64
PKP2	plakophilin 2
RGS3	regulator of G-protein signaling 3
HEBP2	heme binding protein 2
PPRC1	peroxisome proliferator-activated receptor gamma, coactivator-related 1
TGFBR3	transforming growth factor, beta receptor III
DHX40	similar to DEAH (Asp-Glu-Ala-His) box polypeptide 40; DEAH (Asp-Glu-Ala-His) box polypeptide 40
SYTL1	synaptotagmin-like 1
SGSH	N-sulfoglucosamine sulfohydrolase
ELF2	E74-like factor 2 (ets domain transcription factor)
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2
ZNF18	zinc finger protein 18
KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2
UBE2G1	ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast)
UFC1	ubiquitin-fold modifier conjugating enzyme 1

EIF2A	eukaryotic translation initiation factor 2A, 65kDa
KCNK12	potassium channel, subfamily K, member 12
LSM14A	LSM14A, SCD6 homolog A (S. cerevisiae)
HSF2	heat shock transcription factor 2
USP12	ubiquitin specific peptidase 12
USP11	ubiquitin specific peptidase 11
GKAP1	G kinase anchoring protein 1
CLDND1	claudin domain containing 1
MASTL	microtubule associated serine/threonine kinase-like
DPP9	dipeptidyl-peptidase 9
TWISTNB	TWIST neighbor
ERRFI1	ERBB receptor feedback inhibitor 1
SERTAD1	SERTA domain containing 1
FGF3	fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homolog)
SERTAD2	SERTA domain containing 2
TTC32	tetratricopeptide repeat domain 32
ZNF548	zinc finger protein 548
EXOSC9	exosome component 9
ARHGEF1	Rho guanine nucleotide exchange factor (GEF) 1
BRF1	BRF1 homolog, subunit of RNA polymerase III transcription initiation factor IIIB (S. cerevisiae)
EXOSC6	exosome component 6
BRF2	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like

GMEB2	glucocorticoid modulatory element binding protein 2
SDK2	sidekick homolog 2 (chicken)
SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
MRPL9	mitochondrial ribosomal protein L9
PAPD5	PAP associated domain containing 5
GLCCI1	glucocorticoid induced transcript 1
ZNF335	zinc finger protein 335
LRPAP1	low density lipoprotein receptor-related protein associated protein 1
CARD11	caspase recruitment domain family, member 11
PFDN1	prefoldin subunit 1
BRWD1	bromodomain and WD repeat domain containing 1
ZNF134	zinc finger protein 134
BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2
EIF4A2	similar to eukaryotic translation initiation factor 4A2; eukaryotic translation initiation factor 4A, isoform 2
IL12A	interleukin 12A
CAND2	cullin-associated and neddylation-dissociated 2 (putative)
OSBPL10	oxysterol binding protein-like 10
KCNH8	potassium voltage-gated channel, subfamily H (eag-related), member 8

ZNF551	zinc finger protein 551
ARL4C	ADP-ribosylation factor-like 4C
ZNF484	zinc finger protein 484
ASTE1	asteroid homolog 1 (Drosophila)
GRASP	GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein
KIAA1429	KIAA1429
ARL4A	ADP-ribosylation factor-like 4A
ZNF276	zinc finger protein 276
MIDN	midnolin
EXOC8	exocyst complex component 8
CREM	cAMP responsive element modulator
MKNK2	MAP kinase interacting serine/threonine kinase 2
RPL36	ribosomal protein L36
EEA1	early endosome antigen 1
CBLL1	Cas-Br-M (murine) ecotropic retroviral transforming sequence-like 1
CCL28	chemokine (C-C motif) ligand 28
ZNF653	zinc finger protein 653
ZNF34	zinc finger protein 34
ITM2A	integral membrane protein 2A
PLEKHG4	pleckstrin homology domain containing, family G (with RhoGef domain) member 4
WDR19	WD repeat domain 19
SNRK	SNF related kinase
RPL32	small nucleolar RNA, H/ACA box 7A; small nucleolar RNA, H/ACA box 7B; ribosomal protein L32

PPP3CC	protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform
ARMCX2	armadillo repeat containing, X-linked 2
TCEA2	transcription elongation factor A (SII), 2
PCSK6	proprotein convertase subtilisin/kexin type 6
GALNT11	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 11 (GalNAc-T11)
GALNT12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)
EXOC2	exocyst complex component 2
IL6	interleukin 6 (interferon, beta 2)
SPTY2D1	SPT2, Suppressor of Ty, domain containing 1 (S. cerevisiae)
ZNF160	zinc finger protein 160
EXPH5	exophilin 5
ZNF24	zinc finger protein 24
RPL24	ribosomal protein L24; ribosomal protein L24 pseudogene 6
ZNF668	zinc finger protein 668
ETF1	eukaryotic translation termination factor 1
5-Mar	membrane-associated ring finger (C3HC4) 5
PHYH	phytanoyl-CoA 2-hydroxylase
ZNF165	zinc finger protein 165
FEM1C	fem-1 homolog c (C. elegans)
DUSP5	dual specificity phosphatase 5
RLF	rearranged L-myc fusion

MEF2D	myocyte enhancer factor 2D
P2RY10	purinergic receptor P2Y, G-protein coupled, 10
RASSF5	Ras association (RalGDS/AF-6) domain family member 5
ZNF671	zinc finger protein 671
DUSP2	dual specificity phosphatase 2
TFRC	transferrin receptor (p90, CD71)
WDR26	WD repeat domain 26
DUSP1	dual specificity phosphatase 1
C21ORF2	chromosome 21 open reading frame 2
RPL22	ribosomal protein L22 pseudogene 11; ribosomal protein L22
PTP4A1	protein tyrosine phosphatase type IVA, member 1
PTP4A2	protein tyrosine phosphatase type IVA, member 2
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1
SLFN13	schlafen family member 13
ZBTB5	zinc finger and BTB domain containing 5
GAMT	guanidinoacetate N-methyltransferase
ZBTB3	zinc finger and BTB domain containing 3
ZNF256	zinc finger protein 256
WNT7A	wingless-type MMTV integration site family, member 7A
WDR20	WD repeat domain 20
SRP14	signal recognition particle 14kDa (homologous Alu RNA binding protein) pseudogene 1; signal recognition particle

	14kDa (homologous Alu RNA binding protein)
ZNF581	zinc finger protein 581
PRC1	protein regulator of cytokinesis 1
RBM3	RNA binding motif (RNP1, RRM) protein 3
PASK	PAS domain containing serine/threonine kinase
TGFB3	transforming growth factor, beta 3
LNX2	ligand of numb-protein X 2
KIAA1328	KIAA1328
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
SLC7A6	solute carrier family 7 (cationic amino acid transporter, y+ system), member 6
TCEAL3	transcription elongation factor A (SII)-like 3
IDH3G	isocitrate dehydrogenase 3 (NAD+) gamma
LRRC57	leucine rich repeat containing 57
MFHAS1	malignant fibrous histiocytoma amplified sequence 1
AAK1	AP2 associated kinase 1
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1
PDE4B	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)
DDX24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24
EIF1	similar to eukaryotic translation initiation factor 1; eukaryotic translation initiation factor 1

PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1
DDX20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20
ZNF446	zinc finger protein 446
LONRF1	LON peptidase N-terminal domain and ring finger 1
IMP4	IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)
IDUA	iduronidase, alpha-L-
NET1	neuroepithelial cell transforming 1
RPL35A	ribosomal protein L35a
SPTLC1	serine palmitoyltransferase, long chain base subunit 1
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
RELB	v-rel reticuloendotheliosis viral oncogene homolog B
ENC1	ectodermal-neural cortex (with BTB-like domain)
PDE4D	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)
YTHDF3	YTH domain family, member 3
TATDN2	TatD DNase domain containing 2
WEE1	WEE1 homolog (S. pombe)
CCDC6	coiled-coil domain containing 6
RCL1	RNA terminal phosphate cyclase-like 1
PODXL2	podocalyxin-like 2

IGBP1	chromosome 14 open reading frame 19; immunoglobulin (CD79A) binding protein 1
VSNL1	visinin-like 1
RNF138	ring finger protein 138
TESK2	testis-specific kinase 2
MFAP3	microfibrillar-associated protein 3
VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)
MFAP4	microfibrillar-associated protein 4
ZNF586	zinc finger protein 586
ARFGAP3	ADP-ribosylation factor GTPase activating protein 3
HDLBP	high density lipoprotein binding protein
EID3	EP300 interacting inhibitor of differentiation 3
ADAMTSL 2	similar to ADAMTS-like 2; ADAMTS-like 2
MAPKAPK 5	mitogen-activated protein kinase-activated protein kinase 5
SETD1A	SET domain containing 1A
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1
BEX2	brain expressed X-linked 2
LRIG2	leucine-rich repeats and immunoglobulin-like domains 2
SESN1	sestrin 1
RABGGTB	Rab geranylgeranyltransferase, beta subunit
ATF2	activating transcription factor 2
ISYNA1	inositol-3-phosphate synthase 1

TUBB	tubulin, beta; similar to tubulin, beta 5; tubulin, beta pseudogene 2; tubulin, beta pseudogene 1
ZNF324	zinc finger protein 324
ZNF326	zinc finger protein 326
POLE3	polymerase (DNA directed), epsilon 3 (p17 subunit)
ICOS	inducible T-cell co-stimulator
SYAP1	synapse associated protein 1, SAP47 homolog (Drosophila)
JUND	jun D proto-oncogene
RPL3	ribosomal protein L3; similar to 60S ribosomal protein L3 (L4)
ZNF329	zinc finger protein 329
RPL10A	ribosomal protein L10a pseudogene 6; ribosomal protein L10a; ribosomal protein L10a pseudogene 9
TRAF3	TNF receptor-associated factor 3
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1
PRAF2	PRA1 domain family, member 2
OVGP1	oviductal glycoprotein 1, 120kDa
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
TAF6	TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 80kDa
DOCK9	dedicator of cytokinesis 9
TNFRSF13C	tumor necrosis factor receptor superfamily, member 13C
NR4A1	nuclear receptor subfamily 4, group A, member 1
DNHD1	dynein heavy chain domain 1
RPL23A	ribosomal protein L23a
THUMPD2	THUMP domain containing 2
CACNA2D2	calcium channel, voltage-dependent, alpha 2/delta subunit 2
MMAA	methylmalonic aciduria (cobalamin deficiency) cblA type
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67); activating transcription factor 4C
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
PLK3	polo-like kinase 3 (Drosophila)
ZNF419	zinc finger protein 419
EAF1	ELL associated factor 1
ARMC8	armadillo repeat containing 8
CDC42SE2	CDC42 small effector 2
GTF2F1	general transcription factor IIF, polypeptide 1, 74kDa
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa
GTF2F2	general transcription factor IIF, polypeptide 2, 30kDa
HIST1H3A	histone cluster 1, H3a
TUBD1	tubulin, delta 1
FBXO30	F-box protein 30

RHBDL1	rhomboid, veinlet-like 1 (Drosophila)
HIVEP1	human immunodeficiency virus type I enhancer binding protein 1
ZNF414	zinc finger protein 414
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1
IER2	immediate early response 2
USPL1	ubiquitin specific peptidase like 1
IER5	immediate early response 5
LRRC8A	leucine rich repeat containing 8 family, member A
HP1BP3	heterochromatin protein 1, binding protein 3
DPH5	DPH5 homolog (S. cerevisiae)
WWC2	WW and C2 domain containing 2
DPH3	DPH3, KTI11 homolog (S. cerevisiae); DPH3B, KTI11 homolog B (S. cerevisiae)
ALG9	asparagine-linked glycosylation 9, alpha-1,2-mannosyltransferase homolog (S. cerevisiae)
RPS3	ribosomal protein S3 pseudogene 3; ribosomal protein S3
SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)
PAX7	paired box 7
PTDSS1	phosphatidylserine synthase 1
PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform
ANKS1A	ankyrin repeat and sterile alpha motif domain containing 1A
SIT1	signaling threshold regulating transmembrane adaptor 1

PTGER4	prostaglandin E receptor 4 (subtype EP4)
ARID5A	AT rich interactive domain 5A (MRF1-like)
CCNL1	cyclin L1
CDK9	cyclin-dependent kinase 9
MID1IP1	MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish))
RPS4X	ribosomal protein S4X pseudogene 6; ribosomal protein S4X pseudogene 13; ribosomal protein S4, X-linked
NCL	nucleolin
PMM1	phosphomannomutase 1
SIGLEC6	sialic acid binding Ig-like lectin 6
ZFAND2A	zinc finger, AN1-type domain 2A
RRAS2	related RAS viral (r-ras) oncogene homolog 2; similar to related RAS viral (r-ras) oncogene homolog 2
CA6	carbonic anhydrase VI
UBC	ubiquitin C
SLC41A1	solute carrier family 41, member 1
INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa
UBAP1	ubiquitin associated protein 1
OTUD4	OTU domain containing 4
BCLAF1	similar to Bcl-2-associated transcription factor 1 (Btf); BCL2-associated transcription factor 1
CABP1	calcium binding protein 1
NR3C2	nuclear receptor subfamily 3, group C, member 2
BTN2A2	butyrophilin, subfamily 2, member A2

PPM1B	protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform
TSPYL5	TSPY-like 5
RNF125	ring finger protein 125
RNF126	ring finger protein 126
TFAM	transcription factor A, mitochondrial
HIC2	hypermethylated in cancer 2
TSC22D2	TSC22 domain family, member 2
TSPYL2	TSPY-like 2
AP3M2	adaptor-related protein complex 3, mu 2 subunit
ECD	ecdysoneless homolog (Drosophila)
CD6	CD6 molecule
SCNN1D	sodium channel, nonvoltage-gated 1, delta
GCHFR	GTP cyclohydrolase I feedback regulator
CD7	CD7 molecule
CNNM2	cyclin M2
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
EBP	emopamil binding protein (sterol isomerase)
VHL	von Hippel-Lindau tumor suppressor
SNX25	sorting nexin 25
GDPD5	glycerophosphodiester phosphodiesterase domain containing 5
MAL	mal, T-cell differentiation protein
DRG2	developmentally regulated GTP binding protein 2
FBL	fibrillarin
PWP1	PWP1 homolog (S. cerevisiae)

ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
ZBED4	zinc finger, BED-type containing 4
SDF2L1	stromal cell-derived factor 2-like 1
BAX	BCL2-associated X protein
JAK1	Janus kinase 1
UCKL1	uridine-cytidine kinase 1-like 1
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
GGA1	golgi associated, gamma adaptin ear containing, ARF binding protein 1
SCAND1	SCAN domain containing 1
NELL2	NEL-like 2 (chicken)
SNIP1	Smad nuclear interacting protein 1
BBX	bobby sox homolog (Drosophila)
RNASEH1	ribonuclease H1
LUZP1	leucine zipper protein 1
CRY2	cryptochrome 2 (photolyase-like)
H2AFV	H2A histone family, member V
CD44	CD44 molecule (Indian blood group)
WWP2	WW domain containing E3 ubiquitin protein ligase 2
TRAK1	trafficking protein, kinesin binding 1
INSIG1	insulin induced gene 1
COL12A1	collagen, type XII, alpha 1
FAM53C	family with sequence similarity 53, member C
STAM	signal transducing adaptor molecule (SH3 domain and ITAM motif) 1
FAM103A1	family with sequence similarity 103, member A1

FAM53B	family with sequence similarity 53, member B
DDX10	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10
SUPT5H	suppressor of Ty 5 homolog (S. cerevisiae)
CRY1	cryptochrome 1 (photolyase-like)
BCL7B	B-cell CLL/lymphoma 7B
CD3G	CD3g molecule, gamma (CD3-TCR complex)
CD3E	CD3e molecule, epsilon (CD3-TCR complex)
STRN3	striatin, calmodulin binding protein 3
TBRG4	transforming growth factor beta regulator 4
KRT10	keratin 10
PIM3	pim-3 oncogene
GCC1	GRIP and coiled-coil domain containing 1
DEPDC5	DEP domain containing 5
VEGFB	vascular endothelial growth factor B
CHRAC1	chromatin accessibility complex 1
CCR7	chemokine (C-C motif) receptor 7
PRDM4	PR domain containing 4
ABT1	activator of basal transcription 1
MLLT10	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10
MNT	MAX binding protein
TGFBRAP1	transforming growth factor, beta receptor associated protein 1
RAB12	RAB12, member RAS oncogene family
BIN1	bridging integrator 1
MRPL46	mitochondrial ribosomal protein L46
CDV3	CDV3 homolog (mouse)

ELL	elongation factor RNA polymerase II
GPBP1L1	GC-rich promoter binding protein 1-like 1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
AFAP1L2	actin filament associated protein 1-like 2
ANGEL2	angel homolog 2 (Drosophila)
COIL	coilin
TIPRL	TIP41, TOR signaling pathway regulator-like (S. cerevisiae)
TJAP1	tight junction associated protein 1 (peripheral)
SLC11A2	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2
EPHB6	EPH receptor B6
STX18	syntaxin 18
NGRN	neugrin, neurite outgrowth associated
MRPL54	mitochondrial ribosomal protein L54
SUPV3L1	suppressor of var1, 3-like 1 (S. cerevisiae)
FBXO8	F-box protein 8
TSEN54	tRNA splicing endonuclease 54 homolog (S. cerevisiae)
TP53BP2	tumor protein p53 binding protein, 2
SMAD7	SMAD family member 7
CRIP2	cysteine-rich protein 2
CREBBP	CREB binding protein
ANKMY1	ankyrin repeat and MYND domain containing 1
SMYD3	SET and MYND domain containing 3
CELSR1	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)
ZBTB44	zinc finger and BTB domain containing 44

U2AF1L4	U2 small nuclear RNA auxiliary factor 1-like 4
OCIAD2	OCIA domain containing 2
DLX3	distal-less homeobox 3
EPHA4	EPH receptor A4
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
TEX13A	testis expressed 13A
LAMA5	laminin, alpha 5
DCP1A	DCP1 decapping enzyme homolog A (S. cerevisiae)
NIPSNAP1	nipsnap homolog 1 (C. elegans)
ZIK1	zinc finger protein interacting with K protein 1 homolog (mouse)
COPS2	COP9 constitutive photomorphogenic homolog subunit 2 (Arabidopsis)
HIP1R	huntingtin interacting protein 1 related
CCNT1	cyclin T1
SPINK2	serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)
UVRAG	UV radiation resistance associated gene
VPS37B	vacuolar protein sorting 37 homolog B (S. cerevisiae)
TERF2IP	telomeric repeat binding factor 2, interacting protein
SPINK7	serine peptidase inhibitor, Kazal type 7 (putative)
FLT3LG	fms-related tyrosine kinase 3 ligand
BAG4	BCL2-associated athanogene 4
C4ORF36	chromosome 4 open reading frame 36

ACTR6	ARP6 actin-related protein 6 homolog (yeast)
BAG3	BCL2-associated athanogene 3
SEMA3E	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G
COL11A2	collagen, type XI, alpha 2
NT5E	5'-nucleotidase, ecto (CD73)
TFIP11	tuftelin interacting protein 11
ZFP36	zinc finger protein 36, C3H type, homolog (mouse)
PRPF31	PRP31 pre-mRNA processing factor 31 homolog (S. cerevisiae)
ZBTB20	zinc finger and BTB domain containing 20
ZCCHC10	zinc finger, CCHC domain containing 10
POLR1E	polymerase (RNA) I polypeptide E, 53kDa
POGZ	pogo transposable element with ZNF domain
SLC25A5	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5; solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 pseudogene 8
FAM35A	family with sequence similarity 35, member A
POLR1C	polymerase (RNA) I polypeptide C, 30kDa
HERC2	hect domain and RLD 2
ARRDC2	arrestin domain containing 2
ZBTB24	zinc finger and BTB domain containing 24
ZBTB25	zinc finger and BTB domain containing 25
RHBDD2	rhomboid domain containing 2

RAB11FIP4	RAB11 family interacting protein 4 (class II)
GZMM	granzyme M (lymphocyte met-ase 1)
CTNNBIP1	catenin, beta interacting protein 1
RAB11FIP2	RAB11 family interacting protein 2 (class I)
GZMK	granzyme K (granzyme 3; tryptase II)
C6ORF106	chromosome 6 open reading frame 106
CHMP1B	chromatin modifying protein 1B
AVEN	apoptosis, caspase activation inhibitor
RBM38	RNA binding motif protein 38
SUSD3	sushi domain containing 3
GADD45B	growth arrest and DNA-damage-inducible, beta
FKBP11	FK506 binding protein 11, 19 kDa
SLC27A5	solute carrier family 27 (fatty acid transporter), member 5
NXT1	NTF2-like export factor 1
BCAT2	branched chain aminotransferase 2, mitochondrial
YPEL5	yippee-like 5 (Drosophila)
POLR2J	polymerase (RNA) II (DNA directed) polypeptide J, 13.3kDa
STARD10	StAR-related lipid transfer (START) domain containing 10
POLR2D	polymerase (RNA) II (DNA directed) polypeptide D
ZBTB17	zinc finger and BTB domain containing 17
POLR2C	polymerase (RNA) II (DNA directed) polypeptide C, 33kDa
SUMO3	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae) pseudogene; SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae); SMT3 suppressor of mif two 3 homolog 3 (S. cerevisiae)
ZFP36L2	zinc finger protein 36, C3H type-like 2
C12ORF45	chromosome 12 open reading frame 45
PLCL1	phospholipase C-like 1
ZDHHC23	zinc finger, DHHC-type containing 23
TOE1	target of EGR1, member 1 (nuclear)
NR1D2	nuclear receptor subfamily 1, group D, member 2
ANKRD39	ankyrin repeat domain 39
BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)
APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2
BCL9L	B-cell CLL/lymphoma 9-like
BCAS2	breast carcinoma amplified sequence 2
CSNK1A1	casein kinase 1, alpha 1
JARID2	jumonji, AT rich interactive domain 2
C10ORF35	chromosome 10 open reading frame 35
BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide
CD1C	CD1c molecule
BIRC6	baculoviral IAP repeat-containing 6
ILF3	interleukin enhancer binding factor 3, 90kDa
ITPR3	inositol 1,4,5-triphosphate receptor, type 3
APRT	adenine phosphoribosyltransferase
KHK	ketohexokinase (fructokinase)

MRPL22	mitochondrial ribosomal protein L22
ILF2	interleukin enhancer binding factor 2, 45kDa
HELB	helicase (DNA) B
IFT57	intraflagellar transport 57 homolog (Chlamydomonas)
RBM15	RNA binding motif protein 15
TOB1	transducer of ERBB2, 1
PBX4	pre-B-cell leukemia homeobox 4
CBX4	chromobox homolog 4 (Pc class homolog, Drosophila)
SAE1	SUMO1 activating enzyme subunit 1
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
VIPR1	vasoactive intestinal peptide receptor 1
KLHL3	kelch-like 3 (Drosophila)
MRPS30	mitochondrial ribosomal protein S30
DMPK	dystrophia myotonica-protein kinase
KLHL7	kelch-like 7 (Drosophila)
DMXL1	Dmx-like 1
GATA3	GATA binding protein 3
SRRM2	serine/arginine repetitive matrix 2; hypothetical LOC100132779
TARDBP	TAR DNA binding protein
TFB2M	transcription factor B2, mitochondrial
ROBO3	roundabout, axon guidance receptor, homolog 3 (Drosophila)
GABPB2	GA binding protein transcription factor, beta subunit 2
SATB1	SATB homeobox 1
CA11	carbonic anhydrase XI

ERP29	endoplasmic reticulum protein 29
SOCS6	suppressor of cytokine signaling 6
MFGE8	milk fat globule-EGF factor 8 protein
SLC25A36	solute carrier family 25, member 36
C8ORF58	chromosome 8 open reading frame 58
CLPP	ClpP caseinolytic peptidase, ATP-dependent, proteolytic subunit homolog (E. coli)
SNRPA	small nuclear ribonucleoprotein polypeptide A
RNF26	ring finger protein 26
SLC38A1	solute carrier family 38, member 1
AKAP8	A kinase (PRKA) anchor protein 8
EMP2	epithelial membrane protein 2
PRPS1	phosphoribosyl pyrophosphate synthetase 1; phosphoribosyl pyrophosphate synthetase 1-like 1
PACS1	phosphofurin acidic cluster sorting protein 1
DERL2	Der1-like domain family, member 2
MYL5	myosin, light chain 5, regulatory
BLM	Bloom syndrome, RecQ helicase-like
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)
COX7C	cytochrome c oxidase subunit VIIc
GIPC1	GIPC PDZ domain containing family, member 1
ZMYND8	zinc finger, MYND-type containing 8
LLGL1	lethal giant larvae homolog 1 (Drosophila)
KARS	lysyl-tRNA synthetase

FXYD7	FXYD domain containing ion transport regulator 7
RRAGC	Ras-related GTP binding C
DAZAP1	DAZ associated protein 1
PRR7	proline rich 7 (synaptic)
PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B
LAPTM5	lysosomal multispanning membrane protein 5
UBE2D2	ubiquitin-conjugating enzyme E2D 2 (UBC4/5 homolog, yeast)
BLOC1S2	biogenesis of lysosomal organelles complex-1, subunit 2
MTCH1	mitochondrial carrier homolog 1 (C. elegans)
HARS2	histidyl-tRNA synthetase 2, mitochondrial (putative); D-tyrosyl-tRNA deacylase 1 homolog (S. cerevisiae)
LMTK3	lemur tyrosine kinase 3
BTF3	basic transcription factor 3; basic transcription factor 3, like 1 pseudogene
NPM3	nucleophosmin/nucleoplasmin, 3
GLO1	glyoxalase I
RNF10	ring finger protein 10
UBAP2L	ubiquitin associated protein 2-like
STRBP	spermatid perinuclear RNA binding protein
AXIN2	axin 2
CTRL	chymotrypsin-like
PARD6A	par-6 partitioning defective 6 homolog alpha (C. elegans)
REEP6	receptor accessory protein 6

OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF
GABARAPL1	GABA(A) receptors associated protein like 3 (pseudogene); GABA(A) receptor-associated protein like 1
GNRH1	gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)
AK1	adenylate kinase 1
PTPN4	protein tyrosine phosphatase, non-receptor type 4 (megakaryocyte)
ZMYM5	zinc finger, MYM-type 5
GALT	galactose-1-phosphate uridylyltransferase
EPHX2	epoxide hydrolase 2, cytoplasmic
AK2	adenylate kinase 2
TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)
ABHD14B	abhydrolase domain containing 14B
EVL	Enah/Vasp-like
TSN	translin
FOXP4	forkhead box P4
C8ORF33	chromosome 8 open reading frame 33
ATF7IP2	activating transcription factor 7 interacting protein 2
EIF4B	similar to eukaryotic translation initiation factor 4H; eukaryotic translation initiation factor 4B
PHF3	PHD finger protein 3
TXNDC11	thioredoxin domain containing 11
PHF1	PHD finger protein 1
FCAR	Fc fragment of IgA, receptor for

EIF4H	eukaryotic translation initiation factor 4H
CYFIP2	cytoplasmic FMR1 interacting protein 2
GLMN	glomulin, FKBP associated protein
SPTBN1	spectrin, beta, non-erythrocytic 1
CIRBP	cold inducible RNA binding protein
COQ10A	coenzyme Q10 homolog A (S. cerevisiae)
KIAA0355	KIAA0355
PTPN1	protein tyrosine phosphatase, non-receptor type 1
CD79A	CD79a molecule, immunoglobulin-associated alpha
SCMH1	sex comb on midleg homolog 1 (Drosophila)
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1
HPS1	Hermansky-Pudlak syndrome 1
ECHDC2	enoyl Coenzyme A hydratase domain containing 2
HUS1B	HUS1 checkpoint homolog b (S. pombe)
CXCR3	chemokine (C-X-C motif) receptor 3
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide-like 1; ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
RSRC2	arginine/serine-rich coiled-coil 2
PDCD2	programmed cell death 2
PHC3	polyhomeotic homolog 3 (Drosophila)
CD96	CD96 molecule
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
PCGF2	polycomb group ring finger 2

PLEKHB1	pleckstrin homology domain containing, family B (evectins) member 1
KLHL26	kelch-like 26 (Drosophila)
PCBP2	poly(rC) binding protein 2
NECAP2	NECAP endocytosis associated 2
NUDT16L1	nudix (nucleoside diphosphate linked moiety X)-type motif 16-like 1
NECAP1	NECAP endocytosis associated 1
CNTNAP1	contactin associated protein 1
CDK10	cyclin-dependent kinase 10
LTB	lymphotoxin beta (TNF superfamily, member 3)
DUS1L	dihydrouridine synthase 1-like (S. cerevisiae)
PHC1	polyhomeotic homolog 1B (Drosophila); polyhomeotic homolog 1 (Drosophila)
PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8
PIGA	phosphatidylinositol glycan anchor biosynthesis, class A
PSMD9	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9
NRIP3	nuclear receptor interacting protein 3
UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)
ALDH5A1	aldehyde dehydrogenase 5 family, member A1
ZNF92	zinc finger protein 92
MPP6	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)
PRKCH	protein kinase C, eta

PIGT	phosphatidylinositol glycan anchor biosynthesis, class T
LAS1L	LAS1-like (S. cerevisiae)
ARHGAP25	Rho GTPase activating protein 25
NCR3	natural cytotoxicity triggering receptor 3
MCM6	minichromosome maintenance complex component 6
CCDC51	coiled-coil domain containing 51
IFNAR2	interferon (alpha, beta and omega) receptor 2
JOSD1	Josephin domain containing 1
CTSK	cathepsin K
BTG2	BTG family, member 2
BTG1	B-cell translocation gene 1, anti-proliferative
BTG3	BTG family, member 3
PEBP1	phosphatidylethanolamine binding protein 1
KPNA5	karyopherin alpha 5 (importin alpha 6)
PRNP	prion protein
PPP1R15B	protein phosphatase 1, regulatory (inhibitor) subunit 15B
C3ORF38	chromosome 3 open reading frame 38
MATR3	matrin 3
CTSF	cathepsin F
GLTSCR2	glioma tumor suppressor candidate region gene 2; glioma tumor suppressor candidate region gene 2 pseudogene
GLTSCR1	glioma tumor suppressor candidate region gene 1
SPOCK2	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2

TNFRSF25	tumor necrosis factor receptor superfamily, member 25
TRA2A	transformer 2 alpha homolog (Drosophila)
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase
WBSCR22	Williams Beuren syndrome chromosome region 22
DNAH1	dynein, axonemal, heavy chain 1
HECA	headcase homolog (Drosophila)
CCDC59	coiled-coil domain containing 59
SCARF2	scavenger receptor class F, member 2
MOAP1	modulator of apoptosis 1
CCDC66	coiled-coil domain containing 66
FAM107B	family with sequence similarity 107, member B
SH3GLB2	SH3-domain GRB2-like endophilin B2
SAFB	scaffold attachment factor B
MSI2	musashi homolog 2 (Drosophila)
ENO3	enolase 3 (beta, muscle)
PIK3R5	phosphoinositide-3-kinase, regulatory subunit 5
PLCD1	phospholipase C, delta 1
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
DUS3L	dihydrouridine synthase 3-like (S. cerevisiae)
DTX1	deltex homolog 1 (Drosophila)
L3MBTL3	l(3)mbt-like 3 (Drosophila)
MAP1A	microtubule-associated protein 1A
DTX3	deltex homolog 3 (Drosophila)
TUBGCP2	tubulin, gamma complex associated protein 2

CCDC69	coiled-coil domain containing 69
RALGDS	ral guanine nucleotide dissociation stimulator
GPS2	G protein pathway suppressor 2
ATXN7L2	ataxin 7-like 2
CCDC77	coiled-coil domain containing 77
TOX	thymocyte selection-associated high mobility group box
TDP1	tyrosyl-DNA phosphodiesterase 1
ANXA11	annexin A11

PSPC1	paraspeckle component 1; paraspeckle protein 1 pseudogene
SLC5A6	solute carrier family 5 (sodium-dependent vitamin transporter), member 6
DUS4L	dihydrouridine synthase 4-like (S. cerevisiae)
RNF41	ring finger protein 41
IGFBP5	insulin-like growth factor binding protein 5
NKX3-1	NK3 homeobox 1
HLA-DMA	major histocompatibility complex, class II, DM alpha

Table 23. Genes that are significantly expressed in PAH samples; and significantly correlated with ID3 in tissue samples (573 genes) from the significant gene list provided from GEO PAH datasets.

Gene Symbol	Gene Name
PDCD7	programmed cell death 7
ADM	adrenomedullin
IGFBP5	insulin-like growth factor binding protein 5
KCNK17	potassium channel, subfamily K, member 17
DCUN1D2	DCN1, defective in cullin neddylation 1, domain containing 2 (S. cerevisiae)
UBP1	upstream binding protein 1 (LBP-1a)
C19orf25	chromosome 19 open reading frame 25
ARMCX6	similar to armadillo repeat containing, X-linked 6; armadillo repeat containing, X-linked 6
NOTCH4	Notch homolog 4 (Drosophila)

ORAOV1	oral cancer overexpressed 1
ADIPOR1	adiponectin receptor 1
ZDHHC7	zinc finger, DHHC-type containing 7
ADAMTS7	similar to hCG1991431; similar to COMPase; ADAM metallopeptidase with thrombospondin type 1 motif, 7
LCE3D	late cornified envelope 3D
NES	nestin
PCDH10	protocadherin 10
CLPTM1	cleft lip and palate associated transmembrane protein 1
MCM3AP	minichromosome maintenance complex component 3 associated protein
ASB8	ankyrin repeat and SOCS box-containing 8
GIMAP6	GTPase, IMAP family member 6

DALRD3	DALR anticodon binding domain containing 3
LOXL3	lysyl oxidase-like 3
RHOT2	ras homolog gene family, member T2
SORBS2	sorbin and SH3 domain containing 2
ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10
FADS3	fatty acid desaturase 3
POLR2C	polymerase (RNA) II (DNA directed) polypeptide C, 33kDa
LRRC25	leucine rich repeat containing 25
GFM1	G elongation factor, mitochondrial 1
EXTL3	exostoses (multiple)-like 3
NSD1	nuclear receptor binding SET domain protein 1
STMN1	stathmin 1
TMEM80	transmembrane protein 80
MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)
ANK1	ankyrin 1, erythrocytic
ETFB	electron-transfer-flavoprotein, beta polypeptide
NMUR1	neuromedin U receptor 1
FOXA2	forkhead box A2
FHOD1	formin homology 2 domain containing 1
SEMA4G	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G

HIST1H1E	histone cluster 1, H1e
PAOX	polyamine oxidase (exo-N4-amino)
IDH3B	isocitrate dehydrogenase 3 (NAD+) beta
DNAL4	dynein, axonemal, light chain 4
PRRG1	proline rich Gla (G-carboxyglutamic acid) 1
RASSF3	Ras association (RalGDS/AF-6) domain family member 3
PKP4	plakophilin 4
SNX1	sorting nexin 1
AMPH	amphiphysin
USP21	ubiquitin specific peptidase 21
COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)
LUC7L	LUC7-like (S. cerevisiae)
DUT	deoxyuridine triphosphatase
CWF19L1	CWF19-like 1, cell cycle control (S. pombe)
IL2	interleukin 2
ZNF503	zinc finger protein 503
ARHGAP6	Rho GTPase activating protein 6
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
IER2	immediate early response 2
B3GALNT1	beta-1,3-N-acetylgalactosaminyltransferase 1 (globoside blood group)
MRPL45	mitochondrial ribosomal protein L45
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial

AFF2	AF4/FMR2 family, member 2
ZFP37	zinc finger protein 37 homolog (mouse)
SOX13	SRY (sex determining region Y)-box 13
RGL2	ral guanine nucleotide dissociation stimulator-like 2
GUCY1B3	guanylate cyclase 1, soluble, beta 3
CSF3R	colony stimulating factor 3 receptor (granulocyte)
SSU72	SSU72 RNA polymerase II CTD phosphatase homolog (S. cerevisiae)
MAPKAP1	mitogen-activated protein kinase associated protein 1
PARVB	parvin, beta
GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1
RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
AKR7A3	aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)
FBXO17	F-box protein 17
OTOS	otospiralin
TBX4	T-box 4
MRE11A	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
ZNF117	zinc finger protein 117
PAXIP1	PAX interacting (with transcription-activation domain) protein 1

EFNA4	ephrin-A4
FGF12	fibroblast growth factor 12
DCAKD	dephospho-CoA kinase domain containing
TRAF3IP3	TRAF3 interacting protein 3
STAT5B	signal transducer and activator of transcription 5B
HEXA	hexosaminidase A (alpha polypeptide)
FAM127B	family with sequence similarity 127, member B
REM1	RAS (RAD and GEM)-like GTP-binding 1
NKD1	naked cuticle homolog 1 (Drosophila)
NRG2	neuregulin 2
STAB1	stabilin 1
DAP	death-associated protein
ACSS2	acyl-CoA synthetase short-chain family member 2
FKBP8	FK506 binding protein 8, 38kDa
RNASE3	ribonuclease, RNase A family, 3 (eosinophil cationic protein)
RABEP1	rabaptin, RAB GTPase binding effector protein 1
CDH24	cadherin-like 24
CLK2	CDC-like kinase 2
AHNAK	AHNAK nucleoprotein
LDOC1	leucine zipper, down-regulated in cancer 1
GNL3	guanine nucleotide binding protein-like 3 (nucleolar)

ANGPT1	angiopoietin 1
EIF2B1	eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa
ZNF687	zinc finger protein 687
USP5	ubiquitin specific peptidase 5 (isopeptidase T)
GABBR1	gamma-aminobutyric acid (GABA) B receptor, 1
DLGAP3	discs, large (Drosophila) homolog-associated protein 3
CSRP1	cysteine and glycine-rich protein 1
PRKCDBP	protein kinase C, delta binding protein
EXTL2	exostoses (multiple)-like 2
RFC4	replication factor C (activator 1) 4, 37kDa
FBXW4	F-box and WD repeat domain containing 4
FOXK1	forkhead box K1
TMEM98	similar to transmembrane protein 98; transmembrane protein 98
GPRC5C	G protein-coupled receptor, family C, group 5, member C
BCL9L	B-cell CLL/lymphoma 9-like
ADCY6	adenylate cyclase 6
RERE	arginine-glutamic acid dipeptide (RE) repeats
SLC25A23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23
AGER	advanced glycosylation end product-specific receptor

CTTNBP2NL	CTTNBP2 N-terminal like
MRGPRF	MAS-related GPR, member F
AKT1	v-akt murine thymoma viral oncogene homolog 1
TSKS	testis-specific serine kinase substrate
ADCY4	adenylate cyclase 4
TTLL7	tubulin tyrosine ligase-like family, member 7
TAF1C	TATA box binding protein (TBP)-associated factor, RNA polymerase I, C, 110kDa
EHBP1	EH domain binding protein 1
CAMK1	calcium/calmodulin-dependent protein kinase I
MAN2A2	mannosidase, alpha, class 2A, member 2
PIGO	phosphatidylinositol glycan anchor biosynthesis, class O
RNF43	ring finger protein 43
TNRC6C	trinucleotide repeat containing 6C
TLCD1	TLC domain containing 1
ZCCHC9	zinc finger, CCHC domain containing 9
ATP5G3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)
TMCO3	transmembrane and coiled-coil domains 3
PTDSS1	phosphatidylserine synthase 1
XYLT2	xylosyltransferase II
AGGF1	angiogenic factor with G patch and FHA domains 1

RAVER1	ribonucleoprotein, PTB-binding 1
CDT1	chromatin licensing and DNA replication factor 1
SLC35A5	solute carrier family 35, member A5
TRIM32	tripartite motif-containing 32
EPDR1	ependymin related protein 1 (zebrafish)
RSPO2	R-spondin 2 homolog (Xenopus laevis)
PPCDC	phosphopantothenoylcysteine decarboxylase
NEK3	NIMA (never in mitosis gene a)-related kinase 3
NEDD4	neural precursor cell expressed, developmentally down-regulated 4
AGTR1	angiotensin II receptor, type 1
LDLRAP1	low density lipoprotein receptor adaptor protein 1
ZNF429	zinc finger protein 429
ZNF672	zinc finger protein 672; hypothetical LOC100130262
POLE	polymerase (DNA directed), epsilon
IMPA2	inositol(myo)-1(or 4)-monophosphatase 2
ZNF781	zinc finger protein 781
GNPDA1	glucosamine-6-phosphate deaminase 1
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
FCN3	ficolin (collagen/fibrinogen domain containing) 3 (Hakata antigen)
TFAP4	transcription factor AP-4 (activating enhancer binding protein 4)
MAGEH1	melanoma antigen family H, 1

TINAGL1	tubulointerstitial nephritis antigen-like 1
ELOVL5	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)
NFYB	nuclear transcription factor Y, beta
VPS26B	vacuolar protein sorting 26 homolog B (S. pombe)
UBE2Q1	ubiquitin-conjugating enzyme E2Q family member 1
PECR	peroxisomal trans-2-enoyl-CoA reductase
SMG6	Smg-6 homolog, nonsense mediated mRNA decay factor (C. elegans)
HIST1H4L	histone cluster 1, H4l
HIST1H4D	histone cluster 1, H4d
SCAMP2	secretory carrier membrane protein 2
STX6	syntaxin 6
LOXL4	lysyl oxidase-like 4
GNPAT	glyceronephosphate O-acyltransferase
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
PEX16	peroxisomal biogenesis factor 16
CDC25B	cell division cycle 25 homolog B (S. pombe)
GNS	glucosamine (N-acetyl)-6-sulfatase
METTL4	methyltransferase like 4
IGF2R	insulin-like growth factor 2 receptor
DCN	decorin
GANAB	glucosidase, alpha; neutral AB

AP2M1	adaptor-related protein complex 2, mu 1 subunit
CCL26	chemokine (C-C motif) ligand 26
DNAJC15	DnaJ (Hsp40) homolog, subfamily C, member 15
GPS2	G protein pathway suppressor 2
SCAMP1	secretory carrier membrane protein 1
ADRA1B	adrenergic, alpha-1B-, receptor
MMRN1	multimerin 1
PCTP	phosphatidylcholine transfer protein
OBFC1	oligonucleotide/oligosaccharide-binding fold containing 1
MRPS18A	mitochondrial ribosomal protein S18A
MRPS14	mitochondrial ribosomal protein S14
TUBAL3	tubulin, alpha-like 3
TXN2	thioredoxin 2
MUS81	MUS81 endonuclease homolog (S. cerevisiae)
STX10	syntaxin 10
SF3B1	splicing factor 3b, subunit 1, 155kDa
COG1	component of oligomeric golgi complex 1
SLC44A1	solute carrier family 44, member 1
METAP1	methionyl aminopeptidase 1
EVC	Ellis van Creveld syndrome
DOCK6	dedicator of cytokinesis 6
GPR161	G protein-coupled receptor 161
TK2	thymidine kinase 2, mitochondrial
TCEAL4	transcription elongation factor A (SII)-like 4

RAB40C	RAB40C, member RAS oncogene family
DUSP7	dual specificity phosphatase 7
SLCO4C1	solute carrier organic anion transporter family, member 4C1
NSUN5	NOL1/NOP2/Sun domain family, member 5
PARS2	prolyl-tRNA synthetase 2, mitochondrial (putative)
C6orf106	chromosome 6 open reading frame 106
TUSC2	tumor suppressor candidate 2
OPN3	opsin 3
CYP4F12	similar to cytochrome P450, family 4, subfamily F, polypeptide 12; cytochrome P450, family 4, subfamily F, polypeptide 12
TSPAN7	tetraspanin 7
ZFP2	zinc finger protein 2 homolog (mouse)
EPAS1	endothelial PAS domain protein 1
ACTN4	actinin, alpha 4
STRN4	striatin, calmodulin binding protein 4
VPS41	vacuolar protein sorting 41 homolog (S. cerevisiae)
IRF2	interferon regulatory factor 2
SCN1B	sodium channel, voltage-gated, type I, beta
SEC14L3	SEC14-like 3 (S. cerevisiae)
TPM1	tropomyosin 1 (alpha)
SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F

VEGFB	vascular endothelial growth factor B
ZMAT2	zinc finger, matrin type 2
TMEM11	transmembrane protein 11
FMO2	flavin containing monooxygenase 2 (non-functional)
VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)
SEMA6C	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6C
PTPRB	protein tyrosine phosphatase, receptor type, B
TMLHE	trimethyllysine hydroxylase, epsilon
TEX261	testis expressed 261
TRIM44	tripartite motif-containing 44
PHF2	PHD finger protein 2
GGT6	gamma-glutamyltransferase 6
ATP6V1E1	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E1
RBP1	retinol binding protein 1, cellular
ZNF311	zinc finger protein 311
CEP63	centrosomal protein 63kDa
FASTKD3	FAST kinase domains 3
IRS2	insulin receptor substrate 2
TRIB2	tribbles homolog 2 (Drosophila)
DSCR3	Down syndrome critical region gene 3
DNASE1L1	deoxyribonuclease I-like 1
RBM5	RNA binding motif protein 5
ZNF512	zinc finger protein 512

MAP3K11	mitogen-activated protein kinase kinase kinase 11
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
MORC3	MORC family CW-type zinc finger 3
VPS52	vacuolar protein sorting 52 homolog (S. cerevisiae)
TMUB2	transmembrane and ubiquitin-like domain containing 2
APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1
MMACHC	methylmalonic aciduria (cobalamin deficiency) cblC type, with homocystinuria
COPS5	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)
MED12	mediator complex subunit 12
ZFHX4	zinc finger homeobox 4
BANF1	similar to barrier-to-autointegration factor; barrier to autointegration factor 1
LZIC	leucine zipper and CTNNBIP1 domain containing
DYSF	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)
TTC13	tetratricopeptide repeat domain 13
NFKBIL1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1
ANKRD49	ankyrin repeat domain 49
CHD8	chromodomain helicase DNA binding protein 8
CSN3	casein kappa

RALA	v-ral simian leukemia viral oncogene homolog A (ras related)
SPPL2B	signal peptide peptidase-like 2B
CASKIN1	CASK interacting protein 1
RSRC1	arginine/serine-rich coiled-coil 1
GPAA1	glycosylphosphatidylinositol anchor attachment protein 1 homolog (yeast)
CAMKK2	calcium/calmodulin-dependent protein kinase kinase 2, beta
CEP68	centrosomal protein 68kDa
IL17RE	interleukin 17 receptor E
GMEB2	glucocorticoid modulatory element binding protein 2
ZFP64	zinc finger protein 64 homolog (mouse)
PLEKHA6	pleckstrin homology domain containing, family A member 6
CPT1A	carnitine palmitoyltransferase 1A (liver)
ZW10	ZW10, kinetochore associated, homolog (Drosophila)
NKTR	natural killer-tumor recognition sequence
LASP1	LIM and SH3 protein 1
GJB1	gap junction protein, beta 1, 32kDa
PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C
CXXC4	CXXC finger 4
TOP2B	topoisomerase (DNA) II beta 180kDa
RBM23	RNA binding motif protein 23
RETSAT	retinol saturase (all-trans-retinol 13,14-reductase)
SPTBN1	spectrin, beta, non-erythrocytic 1

DFFB	DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase)
DMPK	dystrophia myotonica-protein kinase
GAB3	GRB2-associated binding protein 3
TTC9C	tetratricopeptide repeat domain 9C
INPP5A	inositol polyphosphate-5-phosphatase, 40kDa
THYN1	thymocyte nuclear protein 1
RIMS3	regulating synaptic membrane exocytosis 3
PHB	prohibitin
DECR2	2,4-dienoyl CoA reductase 2, peroxisomal
CANX	calnexin
ARHGAP9	Rho GTPase activating protein 9
LRRC28	leucine rich repeat containing 28
BSDC1	BSD domain containing 1
STK38	serine/threonine kinase 38
SIRT5	sirtuin (silent mating type information regulation 2 homolog) 5 (S. cerevisiae)
PDE5A	phosphodiesterase 5A, cGMP-specific
SRP14	signal recognition particle 14kDa
SLC12A4	solute carrier family 12 (potassium/chloride transporters), member 4
LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase
EPRS	glutamyl-prolyl-tRNA synthetase
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)

DHX16	DEAH (Asp-Glu-Ala-His) box polypeptide 16
ALG6	asparagine-linked glycosylation 6, alpha-1,3-glucosyltransferase homolog (S. cerevisiae)
TMEM109	transmembrane protein 109
CENPB	centromere protein B, 80kDa
USP46	ubiquitin specific peptidase 46
NOTUM	notum pectinacetylesterase homolog (Drosophila)
TCF12	transcription factor 12
SPINT2	serine peptidase inhibitor, Kunitz type, 2
SHPRH	SNF2 histone linker PHD RING helicase
OSGEPL1	O-sialoglycoprotein endopeptidase-like 1
ATP10D	ATPase, class V, type 10D
ALDH1L2	aldehyde dehydrogenase 1 family, member L2
TTC23	tetratricopeptide repeat domain 23
KRT10	keratin 10
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
SH3KBP1	SH3-domain kinase binding protein 1
SLC26A6	solute carrier family 26, member 6
DLL4	delta-like 4 (Drosophila)
MRPL46	mitochondrial ribosomal protein L46
BFAR	bifunctional apoptosis regulator
SERINC3	serine incorporator 3
CRNKL1	crooked neck pre-mRNA splicing factor-like 1 (Drosophila)
ST5	suppression of tumorigenicity 5

LCMT2	leucine carboxyl methyltransferase 2
ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2
SCRN2	secernin 2
CA4	carbonic anhydrase IV
BPGM	2,3-bisphosphoglycerate mutase
HAAO	3-hydroxyanthranilate 3,4-dioxygenase
SLC7A9	solute carrier family 7 (cationic amino acid transporter, y+ system), member 9
TCF19	transcription factor 19
PPM1M	protein phosphatase 1M (PP2C domain containing)
WDR20	WD repeat domain 20
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2
HIC2	hypermethylated in cancer 2
PCSK7	proprotein convertase subtilisin/kexin type 7 pseudogene; proprotein convertase subtilisin/kexin type 7
RPL8	ribosomal protein L8; ribosomal protein L8 pseudogene 2
SH3BGRL	SH3 domain binding glutamic acid-rich protein like
GAPVD1	GTPase activating protein and VPS9 domains 1
SLC5A5	solute carrier family 5 (sodium iodide symporter), member 5
SLC43A1	solute carrier family 43, member 1
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2

NGRN	neugrin, neurite outgrowth associated
CHRNB2	cholinergic receptor, nicotinic, beta 2 (neuronal)
CARD10	caspase recruitment domain family, member 10
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
ZNF211	zinc finger protein 211
CTDSP2	similar to hCG2013701; CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2
SMOC1	SPARC related modular calcium binding 1
TMEM9	transmembrane protein 9
COG2	component of oligomeric golgi complex 2
PLCD3	phospholipase C, delta 3
CDYL	chromodomain protein, Y-like
ZNF630	zinc finger protein 630
STARD3NL	STARD3 N-terminal like
TFEB	transcription factor EB
ATP11C	ATPase, class VI, type 11C
BBS1	Bardet-Biedl syndrome 1
SAE1	SUMO1 activating enzyme subunit 1
ACACB	acetyl-Coenzyme A carboxylase beta
FIS1	fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)
OLFM4	olfactomedin 4
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)

ALS2CL	ALS2 C-terminal like
FGD5	FYVE, RhoGEF and PH domain containing 5
CASP2	caspase 2, apoptosis-related cysteine peptidase
CCDC8	coiled-coil domain containing 8
GATA2	GATA binding protein 2
CPNE2	copine II
PSRC1	proline/serine-rich coiled-coil 1
KCTD15	potassium channel tetramerisation domain containing 15
IL22RA1	interleukin 22 receptor, alpha 1
CARD8	caspase recruitment domain family, member 8
C11orf49	chromosome 11 open reading frame 49
PECAM1	platelet/endothelial cell adhesion molecule
MSC	musculin (activated B-cell factor-1)
CECR6	cat eye syndrome chromosome region, candidate 6
CYP4V2	cytochrome P450, family 4, subfamily V, polypeptide 2
NDRG4	NDRG family member 4
SF3B2	splicing factor 3b, subunit 2, 145kDa
RREB1	ras responsive element binding protein 1
PKN1	protein kinase N1
TTYH3	tweety homolog 3 (Drosophila)
PTPN9	protein tyrosine phosphatase, non-receptor type 9
ILVBL	ilvB (bacterial acetolactate synthase)-like

RPA2	replication protein A2, 32kDa
COPS6	COP9 constitutive photomorphogenic homolog subunit 6 (Arabidopsis)
NIF3L1	NIF3 NGG1 interacting factor 3-like 1 (S. pombe)
SYT17	synaptotagmin XVII; synaptotagmin VII
TMEM69	transmembrane protein 69
SYPL1	synaptophysin-like 1
EMP1	epithelial membrane protein 1
SAMM50	sorting and assembly machinery component 50 homolog (S. cerevisiae)
CCT7	chaperonin containing TCP1, subunit 7 (eta)
TM7SF2	transmembrane 7 superfamily member 2
GJA5	gap junction protein, alpha 5, 40kDa
CYBRD1	cytochrome b reductase 1
ARHGAP4	Rho GTPase activating protein 4
TDRD10	tudor domain containing 10
GPR107	G protein-coupled receptor 107
ASF1A	ASF1 anti-silencing function 1 homolog A (S. cerevisiae)
HARS2	histidyl-tRNA synthetase 2
VPS37D	vacuolar protein sorting 37 homolog D (S. cerevisiae)
GSTA4	glutathione S-transferase alpha 4
PHACTR2	phosphatase and actin regulator 2
TSSC1	tumor suppressing subtransferable candidate 1
DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12

NRGN	neurogranin (protein kinase C substrate, RC3)
DRD4	dopamine receptor D4
MAP3K13	mitogen-activated protein kinase kinase kinase 13
CYP2R1	cytochrome P450, family 2, subfamily R, polypeptide 1
DIXDC1	DIX domain containing 1
NOSTRIN	nitric oxide synthase trafficker
LRMP	lymphoid-restricted membrane protein
ACOX3	acyl-Coenzyme A oxidase 3, pristanoyl
TXNDC11	thioredoxin domain containing 11
ZNF333	zinc finger protein 333
ARHGAP1	Rho GTPase activating protein 1
LHFP	lipoma HMGIC fusion partner
GTF2E1	general transcription factor IIE, polypeptide 1, alpha 56kDa
RASGRP2	RAS guanyl releasing protein 2 (calcium and DAG-regulated)
LBH	limb bud and heart development homolog (mouse)
PTPN6	protein tyrosine phosphatase, non-receptor type 6
CIDEB	cell death-inducing DFFA-like effector b
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
LTK	leukocyte receptor tyrosine kinase
DNAJC4	DnaJ (Hsp40) homolog, subfamily C, member 4
UGDH	UDP-glucose dehydrogenase

RASSF1	Ras association (RalGDS/AF-6) domain family member 1
HYAL1	hyaluronoglucosaminidase 1
HES7	hairy and enhancer of split 7 (Drosophila)
LETMD1	LETM1 domain containing 1
STON2	stonin 2
SCARF1	scavenger receptor class F, member 1
MANEA	mannosidase, endo-alpha
TOB2	transducer of ERBB2, 2
C1orf116	chromosome 1 open reading frame 116
COG4	component of oligomeric golgi complex 4
PPIE	peptidylprolyl isomerase E (cyclophilin E)
RCBTB1	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1
C10orf10	chromosome 10 open reading frame 10
RHBDF1	rhomboid 5 homolog 1 (Drosophila)
CPS1	carbamoyl-phosphate synthetase 1, mitochondrial
ABLIM3	actin binding LIM protein family, member 3
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1
FADS2	fatty acid desaturase 2
EXOC4	exocyst complex component 4
SLC4A2	solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1)

TNRC6B	trinucleotide repeat containing 6B
RGL1	ral guanine nucleotide dissociation stimulator-like 1
BTG3	BTG family, member 3
PURA	purine-rich element binding protein A
2-Mar	membrane-associated ring finger (C3HC4) 2
PTGIS	prostaglandin I2 (prostacyclin) synthase
COPS7A	COP9 constitutive photomorphogenic homolog subunit 7A (Arabidopsis)
KCNJ10	potassium inwardly-rectifying channel, subfamily J, member 10
MPPE1	metallophosphoesterase 1
H1FX	H1 histone family, member X
TMCO6	transmembrane and coiled-coil domains 6
SLC16A11	solute carrier family 16, member 11 (monocarboxylic acid transporter 11)
ZNF76	zinc finger protein 76 (expressed in testis)
AVPI1	arginine vasopressin-induced 1
ZNF609	zinc finger protein 609
FIGNL1	fidgetin-like 1
TRAF7	TNF receptor-associated factor 7
DVL3	dishevelled, dsh homolog 3 (Drosophila)
MAN2C1	mannosidase, alpha, class 2C, member 1
ADAT1	adenosine deaminase, tRNA-specific 1
FOXF2	forkhead box F2
RAB26	RAB26, member RAS oncogene family
OR8B8	olfactory receptor, family 8, subfamily B, member 8

CTNS	cystinosis, nephropathic
RXRB	retinoid X receptor, beta
PEX11A	peroxisomal biogenesis factor 11 alpha
SNTA1	syntrophin, alpha 1 (dystrophin-associated protein A1, 59kDa, acidic component)
NISCH	nischarin
ATOH8	atonal homolog 8 (Drosophila)
PRMT2	protein arginine methyltransferase 2
CD1D	CD1d molecule
GRTP1	growth hormone regulated TBC protein 1
RNF146	ring finger protein 146
JAG2	jagged 2
LAMA5	laminin, alpha 5
DCTN1	dynactin 1 (p150, glued homolog, Drosophila)
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
ZSWIM1	zinc finger, SWIM-type containing 1
ZFYVE19	zinc finger, FYVE domain containing 19
PTPRM	protein tyrosine phosphatase, receptor type, M
SLC35D2	solute carrier family 35, member D2
SLCO2A1	solute carrier organic anion transporter family, member 2A1
ATG16L2	ATG16 autophagy related 16-like 2 (S. cerevisiae)
SS18L2	synovial sarcoma translocation gene on chromosome 18-like 2

ESD	esterase D/formylglutathione hydrolase
NPM2	nucleophosmin/nucleoplasmin, 2
PIGC	phosphatidylinositol glycan anchor biosynthesis, class C
ANGEL1	angel homolog 1 (Drosophila)
SFXN3	sideroflexin 3
USP19	ubiquitin specific peptidase 19
CCNB1IP1	cyclin B1 interacting protein 1
SDS	serine dehydratase
PRKCH	protein kinase C, eta
C11orf71	chromosome 11 open reading frame 71
PODXL	podocalyxin-like
ARRDC1	arrestin domain containing 1
HDAC1	histone deacetylase 1
CX3CR1	chemokine (C-X3-C motif) receptor 1
TSPAN10	tetraspanin 10
NEK8	NIMA (never in mitosis gene a)- related kinase 8
SDHB	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
RB1	retinoblastoma 1
MVD	mevalonate (diphospho) decarboxylase
ASH2L	ash2 (absent, small, or homeotic)-like (Drosophila)
CXXC1	CXXC finger 1 (PHD domain)
INPP5B	inositol polyphosphate-5-phosphatase, 75kDa
WDR45	WD repeat domain 45
MYH9	myosin, heavy chain 9, non-muscle

HPS3	Hermansky-Pudlak syndrome 3
JAM3	junctional adhesion molecule 3
PPP2R5A	protein phosphatase 2, regulatory subunit B', alpha isoform
ZFAND2B	zinc finger, AN1-type domain 2B
CHMP2A	chromatin modifying protein 2A
ETV5	ets variant 5
PDE9A	phosphodiesterase 9A
GLO1	glyoxalase I
TEX264	testis expressed 264
SYNCRIP	synaptotagmin binding, cytoplasmic RNA interacting protein
PHKB	phosphorylase kinase, beta
CYFIP1	cytoplasmic FMR1 interacting protein 1
CCS	copper chaperone for superoxide dismutase
ZBTB4	zinc finger and BTB domain containing 4
HLF	hepatic leukemia factor
DPF2	D4, zinc and double PHD fingers family 2
AES	amino-terminal enhancer of split
EIF4B	similar to eukaryotic translation initiation factor 4H; eukaryotic translation initiation factor 4B
TACC3	transforming, acidic coiled-coil containing protein 3
SDF4	stromal cell derived factor 4
ZCCHC3	zinc finger, CCHC domain containing 3
NR2F1	nuclear receptor subfamily 2, group F, member 1

RAVER2	ribonucleoprotein, PTB-binding 2
CRYZL1	crystallin, zeta (quinone reductase)-like 1
PTGDR	prostaglandin D2 receptor (DP)
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
ADARB1	adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)
PAX4	paired box 4
CDC37	cell division cycle 37 homolog (S. cerevisiae)
PUM2	pumilio homolog 2 (Drosophila)
LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)
CNNM3	cyclin M3
SPSB3	splA/ryanodine receptor domain and SOCS box containing 3
MGAT1	mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase
RACGAP1	Rac GTPase activating protein 1 pseudogene; Rac GTPase activating protein 1
GOT2	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)
PDE7A	phosphodiesterase 7A
PON3	paraoxonase 3
DNASE1L2	deoxyribonuclease I-like 2
TIMP3	TIMP metallopeptidase inhibitor 3
TMEM42	transmembrane protein 42

CACNA1E	calcium channel, voltage-dependent, R type, alpha 1E subunit
LRP6	low density lipoprotein receptor-related protein 6
FARP1	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived)
UROD	uroporphyrinogen decarboxylase

Table 24. Common genes between (significantly expressed genes in PAH and ID3 correlated, blood samples), and ((significantly expressed genes in PAH and ID3 correlated, tissue) (28 genes) from the significant gene list provided from GEO PAH datasets

Gene Symbol	Gene Name
MRPL46	mitochondrial ribosomal protein L46
HIC2	hypermethylated in cancer 2
PTDSS1	phosphatidylserine synthase 1
BCL9L	B-cell CLL/lymphoma 9-like
TXNDC11	thioredoxin domain containing 11
IER2	immediate early response 2
HARS2	histidyl-tRNA synthetase 2, mitochondrial (putative); D-tyrosyl-tRNA deacylase 1 homolog (S. cerevisiae)
SRP14	signal recognition particle 14kDa (homologous Alu RNA binding protein) pseudogene 1; signal recognition particle 14kDa (homologous Alu RNA binding protein)
BTG3	BTG family, member 3
IGFBP5	insulin-like growth factor binding protein 5
POLR2C	polymerase (RNA) II (DNA directed) polypeptide C, 33kDa
SPTBN1	spectrin, beta, non-erythrocytic 1
EIF4B	similar to eukaryotic translation initiation factor 4H; eukaryotic translation initiation factor 4B
GPS2	G protein pathway suppressor 2
H1FX	H1 histone family, member X
VEGFB	vascular endothelial growth factor B
PRKCH	protein kinase C, eta
VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)
DMPK	dystrophia myotonica-protein kinase
NGRN	neugrin, neurite outgrowth associated
C6orf106	chromosome 6 open reading frame 106
SAE1	SUMO1 activating enzyme subunit 1
GLO1	glyoxalase I
KRT10	keratin 10
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
GMEB2	glucocorticoid modulatory element binding protein 2
LAMA5	laminin, alpha 5

WDR20	WD repeat domain 20

Blood Results: (GeneOntology, pantherdb.org)

For blood sample analysis, we imported the significant gene list including 701 genes into the panther database. However, some genes had different matched IDs, and the database included both IDs. Thus, 735 gene IDs were included. To further evaluate the gene list, we performed different comprehensive functions analysis including biological process, cellular component, Molecular function, GO biological process, GO cellular component, GO molecular function, pathways, and protein class. In biological process, different functions were involved with the significant gene list provided from GEO PAH datasets including cellular process, metabolic process, localization, developmental process, cellular component organization or biogenesis, response to stimulus, biological regulation, multicellular organismal process, immune system process, reproduction, biological adhesion, locomotion, cell killing (Figure 8). Cellular process and molecular process reported the most number of genes involved with 333 and 301 genes, respectively.

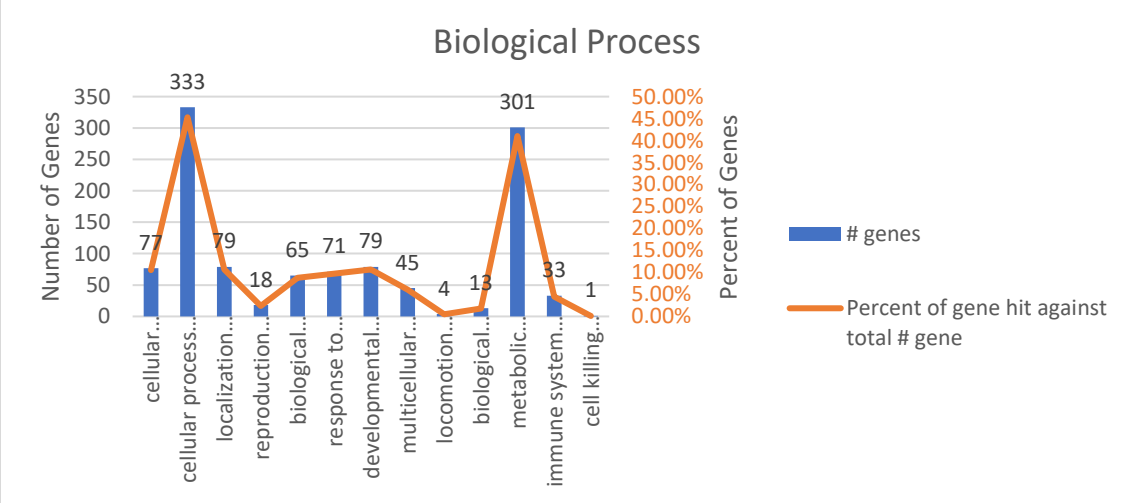

Figure 8 Biological process functional analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from panther database gene ontology analysis.

In cellular component, the functional analysis: cell junction, membrane, macromolecular complex, extracellular matrix, cell part, organelle, and extracellular region were involved with the significant gene list provided from GEO PAH datasets; however, cell part and organelle had the highest number of genes involved with 192 and 134 genes, respectively (Figure 9).

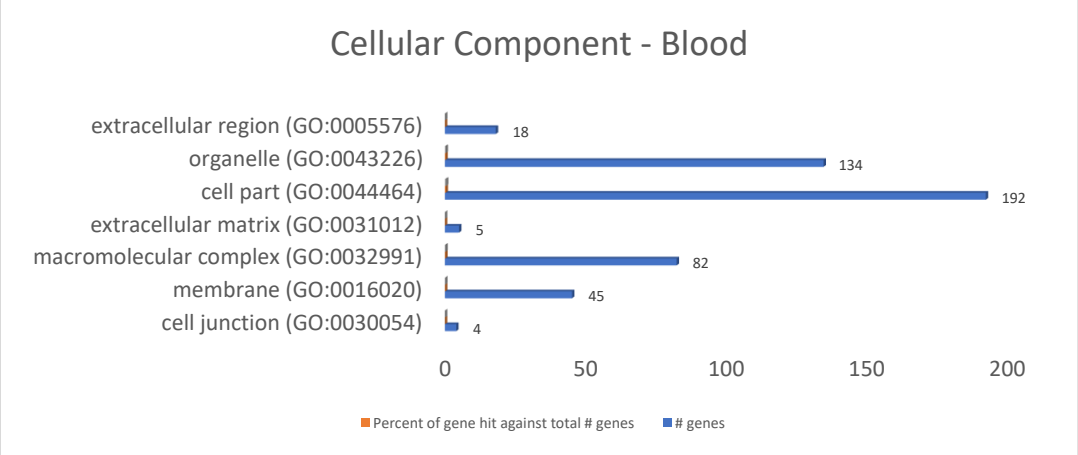

Figure 9 Cellular component functional analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from panther database gene ontology analysis.

In GO molecular function analysis, rRNA binding, structural constituent of ribosome, RNA binding, nucleic acid binding, DNA binding, enzyme binding, heterocyclic compound binding, organic cyclic compound binding, protein binding, binding, molecular function, G-protein coupled receptor activity were involved with the significant gene list provided from GEO PAH datasets (Figure 10). Moreover, molecular function, binding, and protein binding were the most functions with number of genes involved 684, 632, and 531 genes, respectively. Interestingly, molecular functions with small number of genes associated had higher fold enrichment.

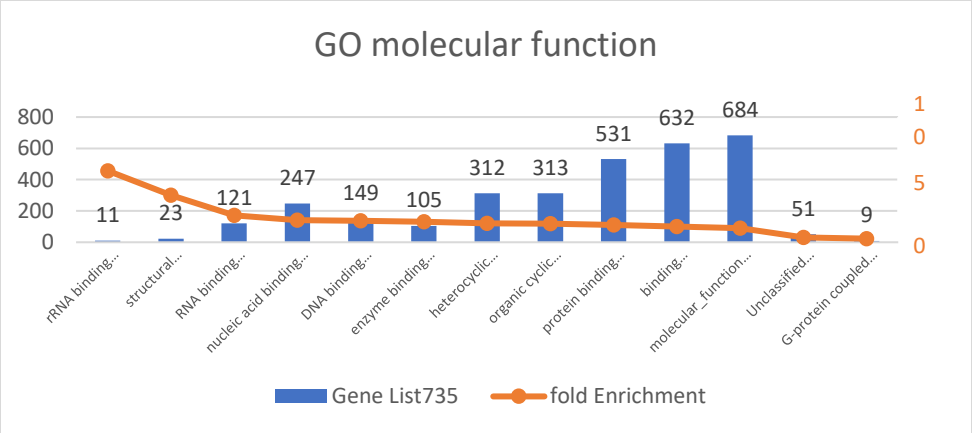

Figure 10 GO Molecular functional analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from panther database gene ontology analysis.

Pathways analysis reported 95 pathways to be involved in our significant gene list provided from GEO PAH datasets (Figure 11). Gonadotropin-releasing hormone receptor pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway, Wnt signaling pathway, Huntington disease, and CCKR signaling map represented the highest number of genes involved among all pathways with 17, 16, 12, 11, 11, and 10 genes, respectively.

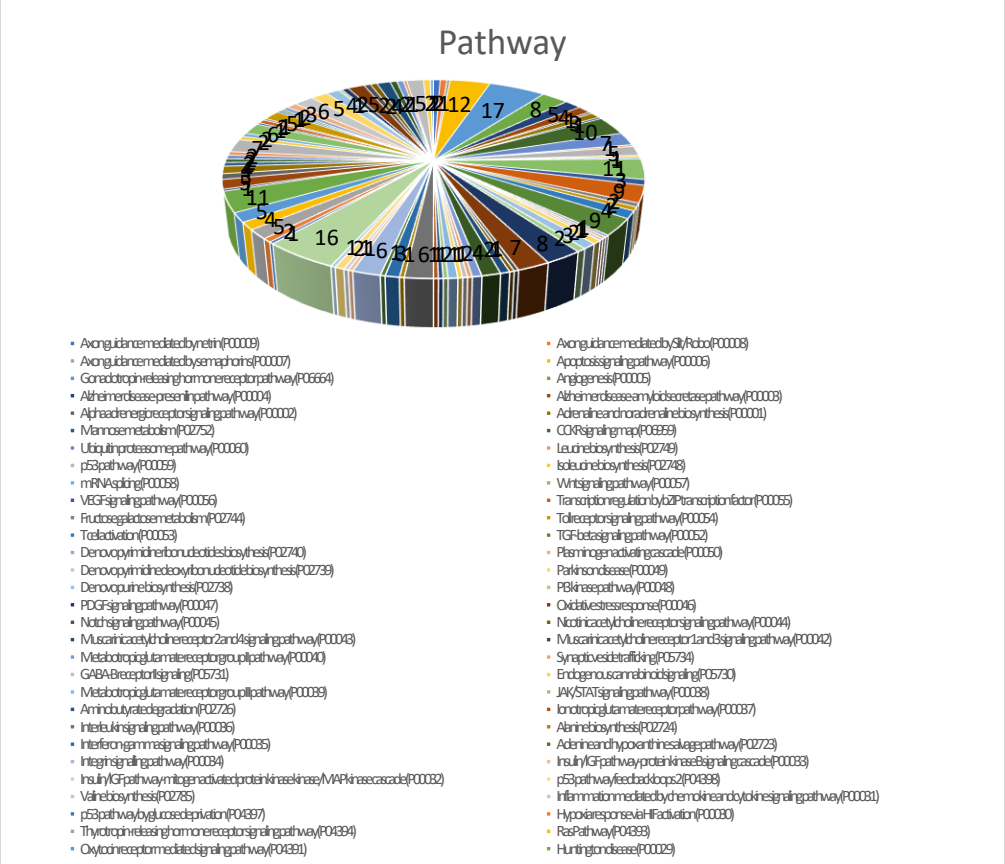

Figure 11 Pathways functional analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from panther database gene ontology analysis.

Protein class reported that ribosomal protein, RNA binding protein, transcription factor, and nucleic acid binding were involved with the significant gene list provided from GEO PAH datasets (Figure 12). Number of genes reported in each class were 25, 74, 93, and 151, respectively. Although, ribosomal protein, the class with small number of genes associated, showed the highest fold enrichment.

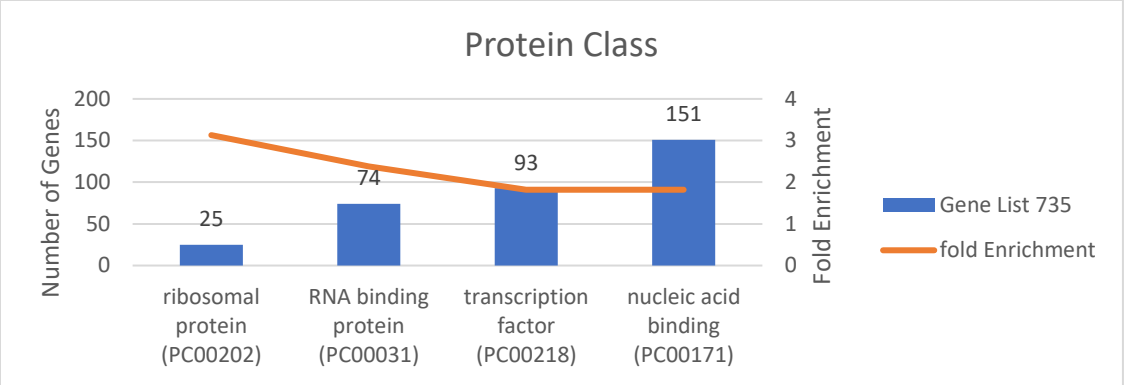

Figure 1. Protein Class functional analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from panther database gene ontology analysis.

String (Functional Enrichments)

String database predict protein-protein interactions and provide functional enrichments for biological process, molecular function, cellular component and KEGG pathways associated with the significant gene list provided from GEO PAH datasets. For biological process, 180 GO terms were observed to have set of genes involved. Cellular process, metabolic process, organic substance metabolic process, primary metabolic process, and cellular metabolic process had the highest number of genes in their pathways, with 429, 370, 366, 365, and 357 genes, respectively (supplement 3).

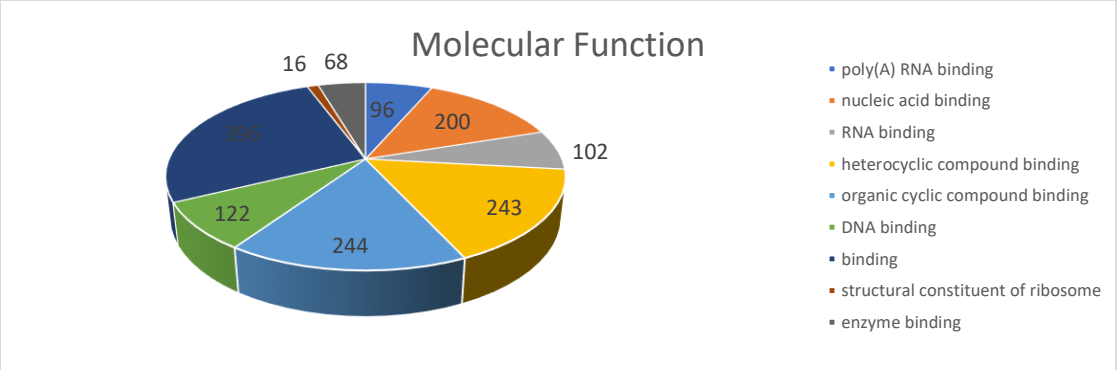

Figure 2. GO Molecular functional analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

GO of molecular function observed 9 pathways to be involved including poly(A) RNA binding, nucleic acid binding, RNA binding, heterocyclic compound binding, organic cyclic compound binding, DNA binding, binding, structural constituent of ribosome, and enzyme binding to be involved with the significant gene list provided from GEO PAH datasets (Figure 13). Organic cyclic compound binding, heterocyclic compound binding, and nucleic acid binding reported the largest number of genes involved with 244, 243, and 200 respectively (Supplement 4 for full gene list). Cellular component showed that 43 GO pathways to be involved with the significant gene list provided from GEO PAH datasets (supplement 5). KEGG pathways showed that 8 pathways to be associated with the significant gene list provided from GEO PAH datasets including Ribosome, HTLV-I infection, RNA degradation, Purine metabolism, RNA polymerase, MAPK signaling pathway, Pathways in cancer, and HIF-1 signaling pathway. Of these pathways, HTLV-I infection and Pathways in cancer reported 25 and 24 genes to be involved, respectively (Figure 14).

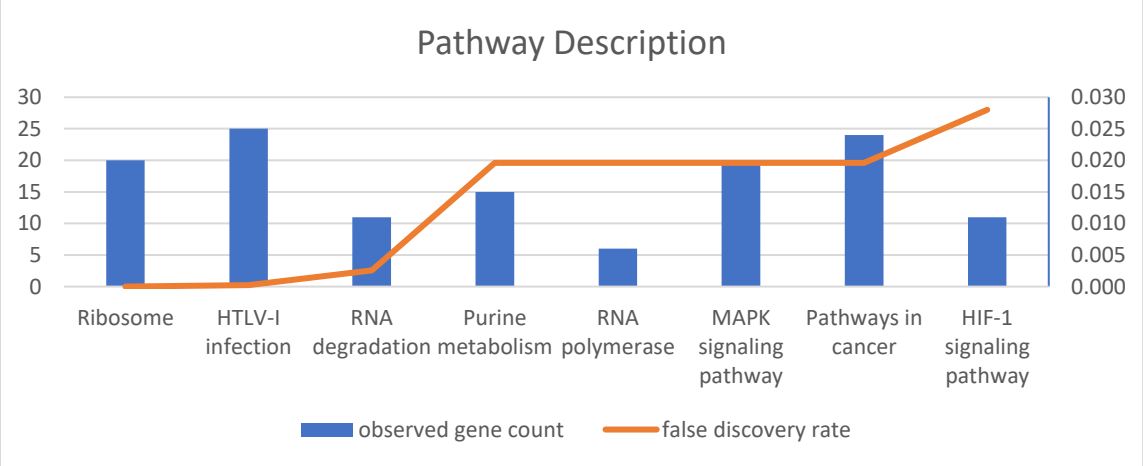

Figure 3. Pathways description analysis involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

An interaction network map was generated based on protein-protein interaction data provided by the STRING database. It concludes functional interactions between each of the 701 core genes and generated the network map which represent the interactions of these genes and their protein products (Figure 15). Another network map was generated using the significant gene list provided from GEO PAH datasets, however, it represent the highest confidence level of interaction between these genes and their protein products (Figure 16).

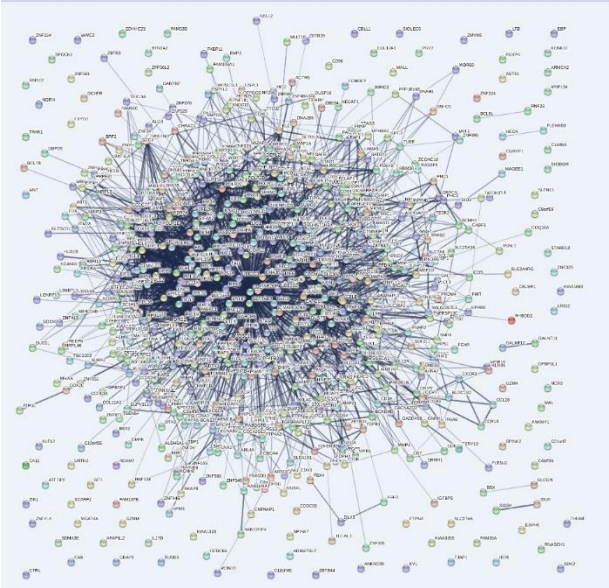

Figure 4. An interaction network map was generated based on protein-protein interaction data using the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

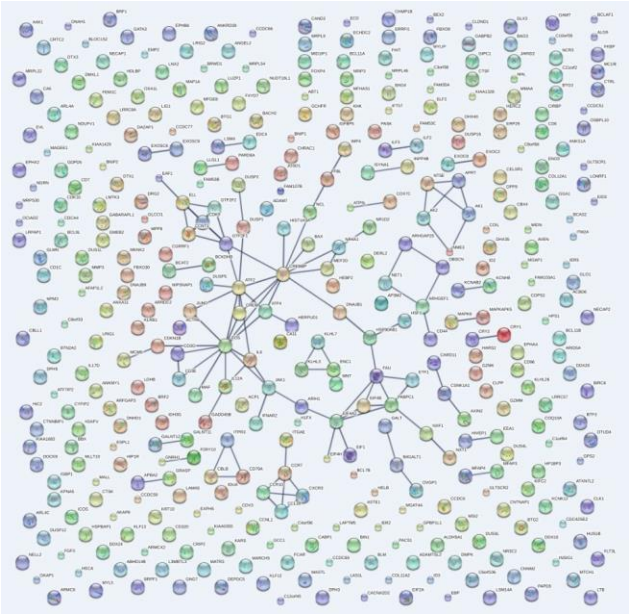

Figure 5. An interaction network map was generated based on the highest confidence level of protein-protein interaction involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from STRING functional enrichment database.

Tissue Samples Results:

For tissue sample analysis, we imported the significant gene list provided from GEO PAH datasets including 573 genes into the database. However, some genes had different matched IDs, and the database included both IDs. Thus, 602 gene IDs were included. To further evaluate the gene list, we performed different comprehensive functions analysis including biological process, cellular component, molecular function, GO biological process, GO cellular component, GO molecular function, pathways, and protein class. In biological process, different functions were involved with the significant gene list provided from GEO PAH datasets including cellular component organization or biogenesis, cellular process, localization, reproduction, biological regulation, response to stimulus, developmental process, multicellular organismal process, biological adhesion,

locomotion, metabolic process, and immune system process (Figure 17). Cellular process and metabolic process reported the most number of genes involved with 303 and 258 genes, respectively.

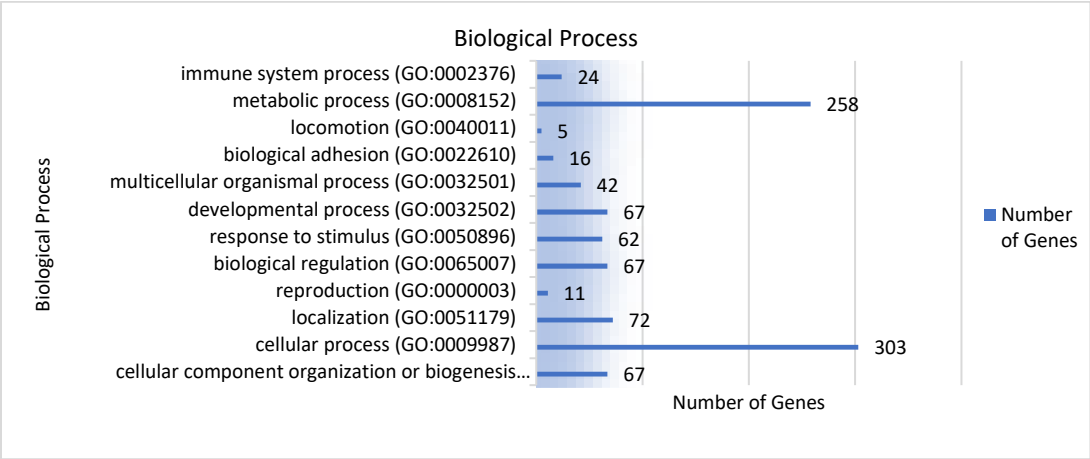

Figure 6. Biological process functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

In cellular component, synapse, cell junction, membrane, macromolecular complex, extracellular matrix, cell part, organelle, and extracellular region were involved with the significant gene list provided from GEO PAH datasets; however, cell part and organelle had the highest number of genes involved with 163 and 90 genes, respectively (Figure 18). In GO biological process, small molecule metabolic process, cellular metabolic process, single-organism cellular process, organic substance metabolic process, metabolic process, primary metabolic process, cellular process, single-organism process, biological process, detection of stimulus involved in sensory perception, and sensory perception of chemical stimulus were involved with the significant gene list provided from GEO PAH datasets. Moreover, cellular process and single-organism process were the most functions with number of genes involved 510 and 433 genes, respectively. Small molecule

metabolic process, cellular metabolic process reported the highest fold enrichment among other biological process with 1.69 and 1.28 fold enrichment, respectively (Figure 18).

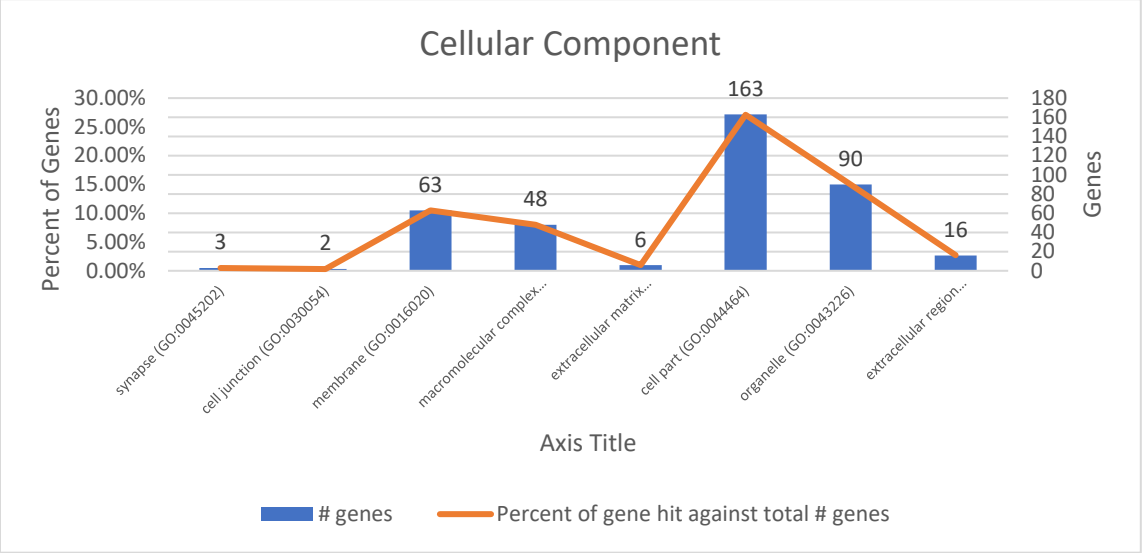

Figure 7. Cellular component functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

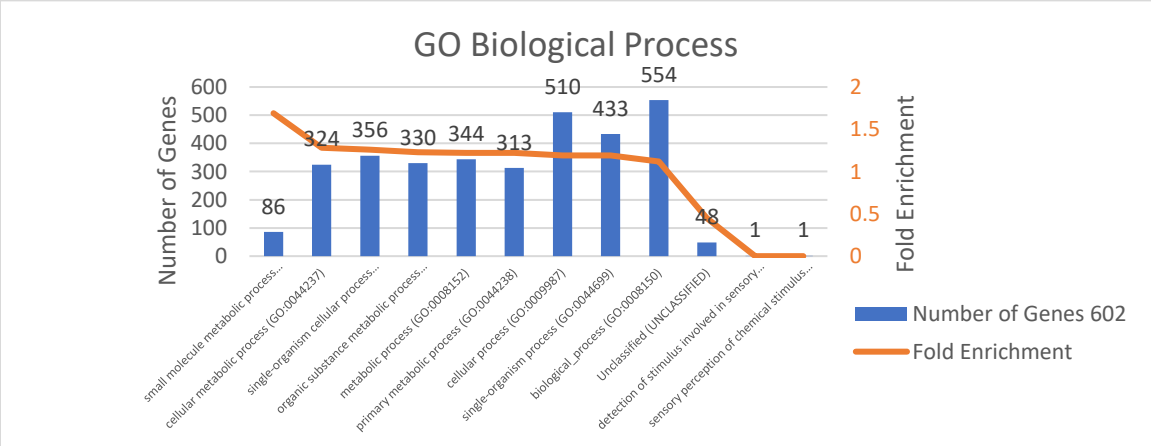

Figure 8. GO Biological Process functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

In GO of cellular component functional analysis, cytosol, intracellular organelle part, organelle part, cytoplasmic part, cytoplasm, intracellular membrane-bounded organelle, intracellular, intracellular organelle, intracellular part, membrane-bounded

organelle, organelle, cell part, cell, and cellular component were involved with the significant gene list provided from GEO PAH datasets (Figure 20). Moreover, cell and cell part, intracellular, and intracellular part represented the highest number of gene involved with 543, 500, and 486 genes, respectively. Cytosol and intracellular organelle part were the highest in fold enrichment with 1.32 and 1.3, respectively.

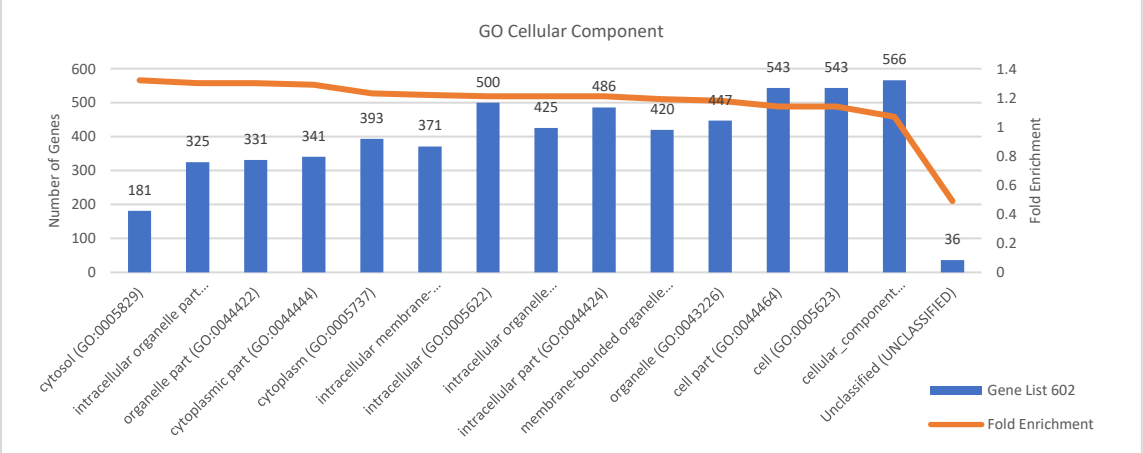

Figure 20. GO of Cellular component functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

In GO molecular functional analysis, cation binding, metal ion binding, protein binding, catalytic activity, binding, and molecular function were involved with the significant gene list provided from GEO PAH datasets (Figure 21). Moreover, protein binding and catalytic activity represented the highest number of genes involved with 417 and 220 genes, respectively. Cation binding and metal ion binding were the highest in fold enrichment with 1.37 and 1.36, respectively. In molecular function, translation regulator activity, binding, receptor activity, structural molecule activity, signal transducer activity, catalytic activity, antioxidant activity, and transporter activity were involved with the significant gene list provided from GEO PAH datasets (Figure 22). Molecular function of

binding and catalytic activity reported 194 and 190 genes to be involved, respectively. Pathway analysis reported 90 pathways to be involved in the significant gene list provided from GEO PAH datasets (Figure 23). Gonadotropin-releasing hormone receptor pathway, Angiogenesis, Wnt signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, and EGF receptor signaling pathway represented the highest number of genes involved among all pathways with 13, 11, 10, 10, and 7 genes, respectively.

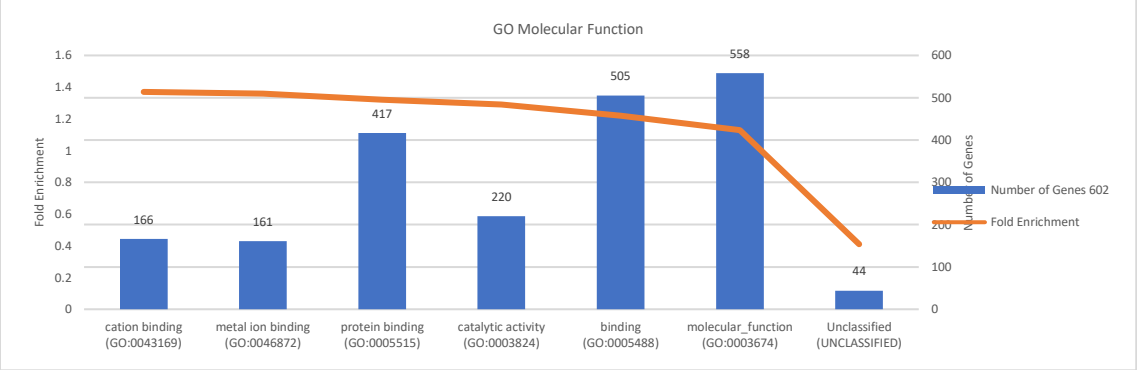

Figure 21. GO of Molecular functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

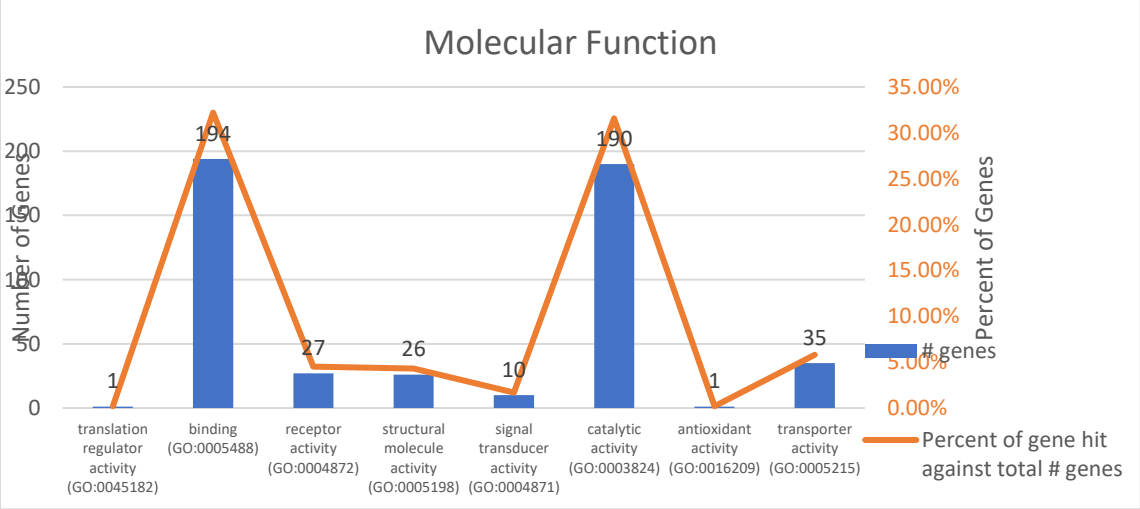

Figure 22. Molecular functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

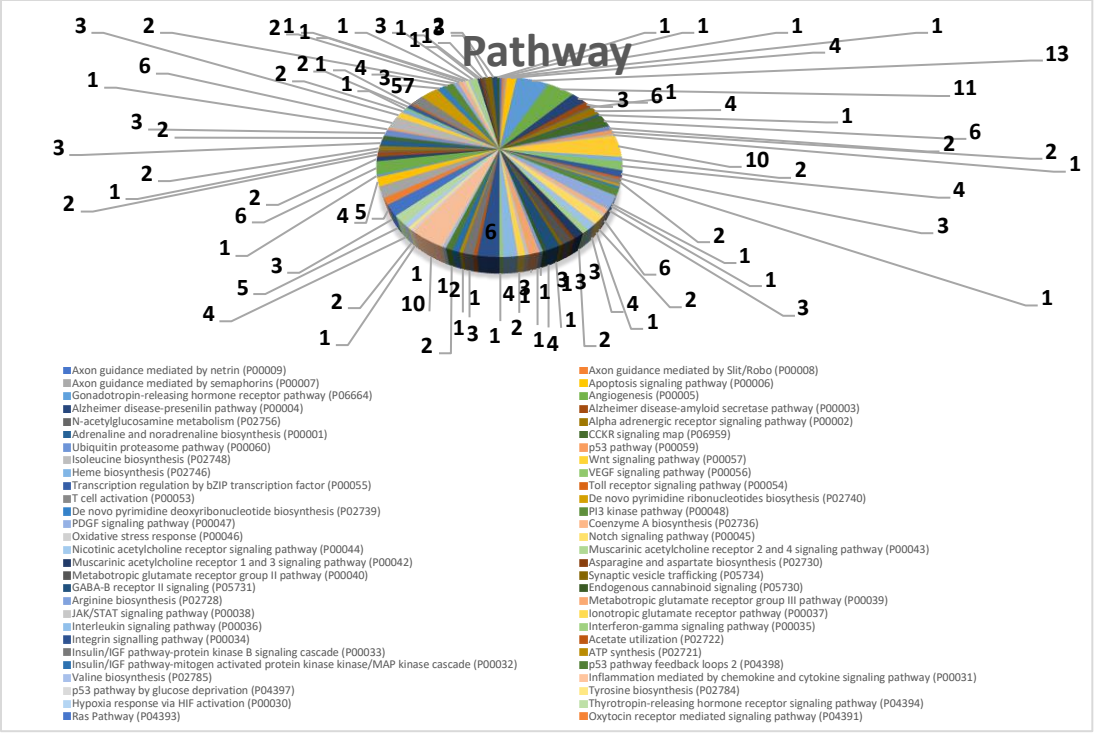

Figure 23 Pathway functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

Protein class analysis reported 20 protein class to be involved with the significant gene list provided from GEO PAH datasets (Figure 24). Nucleic acid binding, enzyme modulator, hydrolase, transferase, and transcription factor represented the highest number of genes involved with 91, 54, 51, 46, and 45 genes respectively.

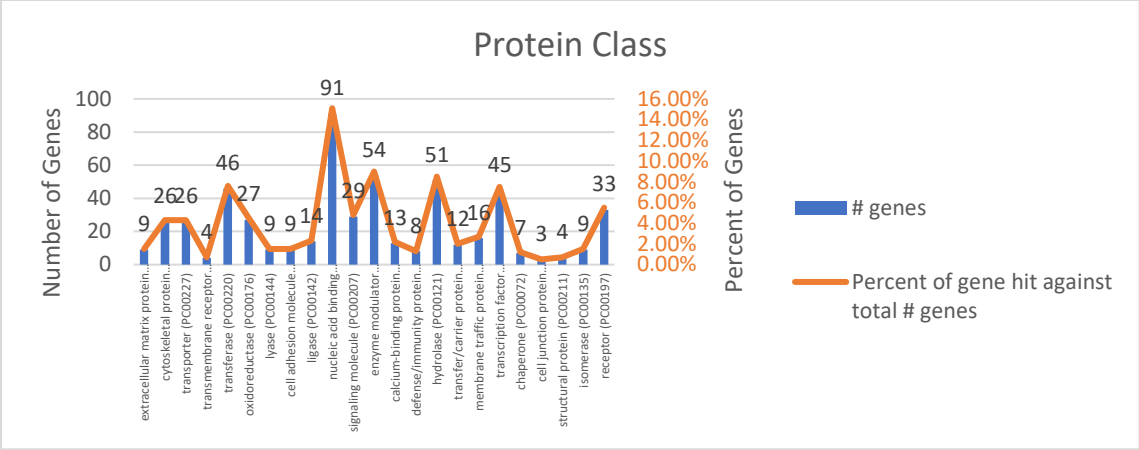

Figure 9. Protein Class functional analysis involved with the significant gene list provided from GEO PAH datasets – (Tissue samples) from panther database gene ontology analysis.

Analyzing tissue samples using STRING database for biological process reported 23 pathways to be involved in the significant gene list provided from GEO PAH datasets (Figure 25). GO Cellular process, single-organism cellular process, single-organism process, and metabolic process showed the highest number of gene with 367, 313, 310, and 295 genes, respectively. GO of Enrichment component showed 16 components to be involved with the significant gene list provided from GEO PAH datasets (Figure 26). Cell part, intracellular part, and organelle represented the highest number of genes involved with 429, 395, and 367 genes, respectively. GO of molecular function observed 8 pathways to be involved including binding, ion binding, catalytic activity, protein binding, cation binding, metal ion binding, transition metal ion binding, and phosphoric ester hydrolase activity. Binding, ion binding, and catalytic activity reported the largest number of genes involved with 320, 205, and 180, respectively (Figure 27) (Supplement attached for full gene list).

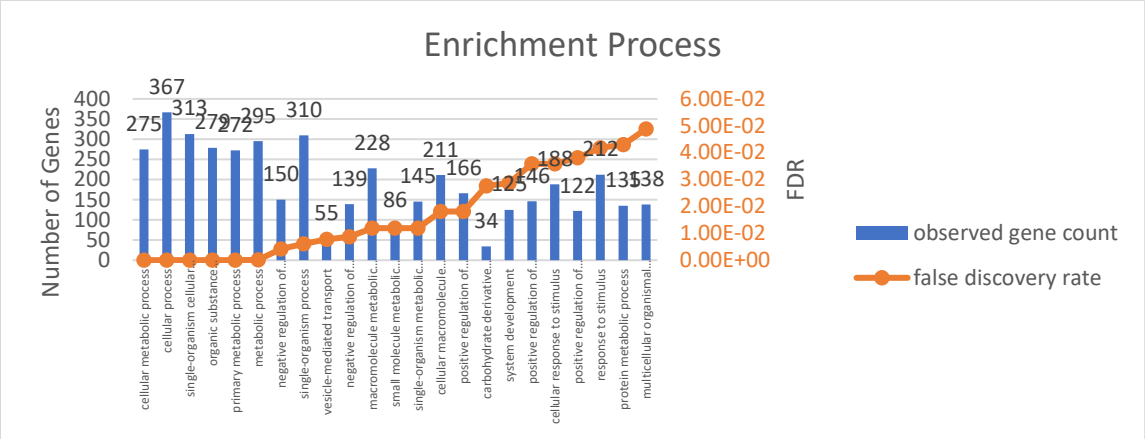

Figure 25. Biological process reported 23 pathways involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

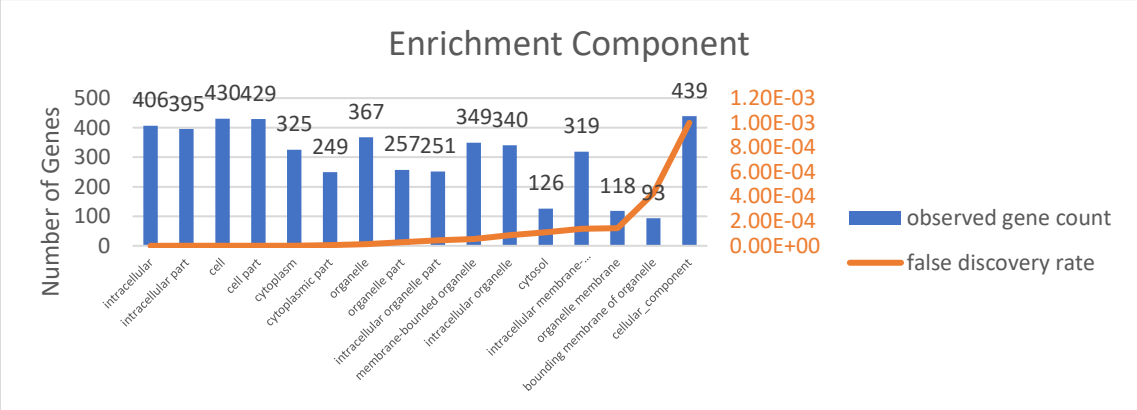

Figure 26. GO of Enrichment component reported 16 components to be involved with the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

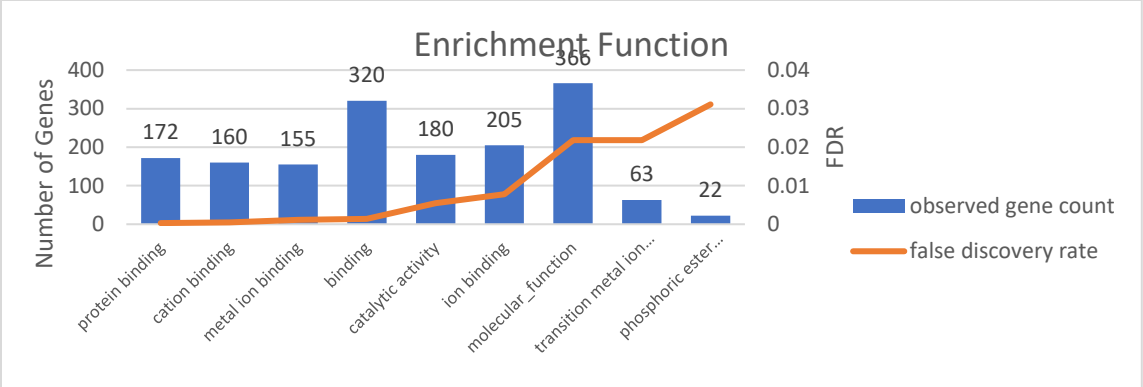

Figure 27. GO of molecular function observed 8 pathways to be with the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

An interaction network map was generated based on protein-protein interaction data provided by the STRING database. It concludes functional interactions between each of the 573 core genes and generated the network map which represent the interactions of with the significant gene list provided from GEO PAH datasets and their protein products (Figure 28). Another network map was generated; however, it represents the highest confidence level of interaction between these genes and their protein products (Figure 29).

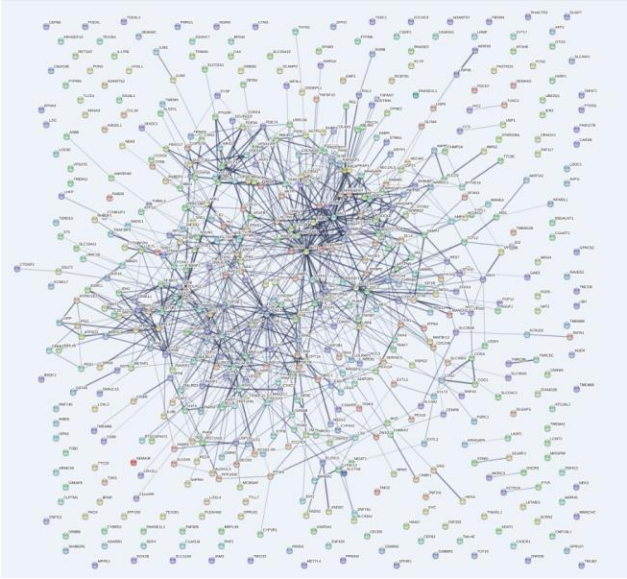

Figure 28. An interaction network map was generated based on protein-protein interaction data provided of the significant gene list provided from GEO PAH datasets – (Blood samples) from STING functional enrichment database.

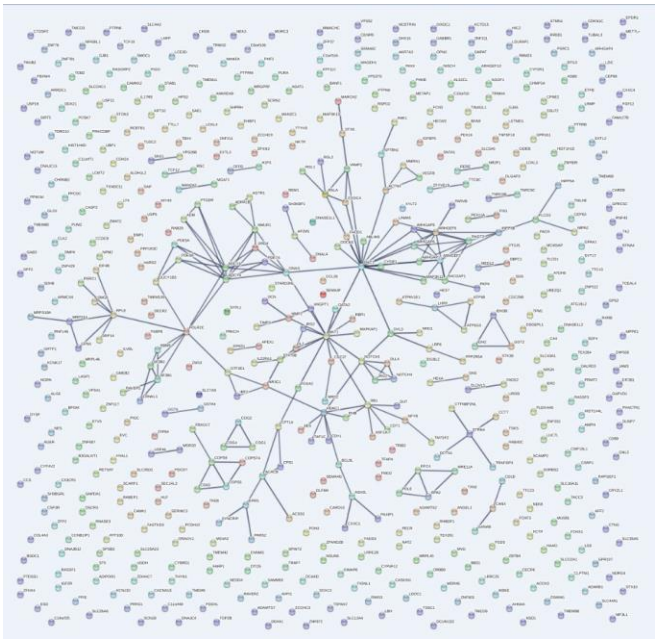

Figure 29. An interaction network map for Tissue samples was generated based on the highest confidence level of protein-protein interaction.

We searched through panther web-tool gene ontology for pathways associated with blood samples and tissue samples, and compared these pathways from STRING database. Common pathways were selected for further analysis. Also, we searched the literature for PAH associated pathways and genes associated with PAH. We searched through all pathways, and pathways with 3< genes involved were included, and common genes were included in a separate gene list.

29 genes found to be common in blood samples, and 18 genes in tissue samples (Table 28 and Table 29). This gene list was imported into Bene software to find out and validate the association between these genes with PAH in blood and tissue samples in a structure of BN which represent the best score in association between variables. The best structure of BN of tissue samples including genes associated with PAH in Figure 30.

Table 25 Genes associated with PAH; and PAH pathways from Gene Ontology, STRING, and literature (Blood Samples) (29 genes)

Gene Symbol	Gene Name
ATF4	activating transcription factor 4(ATF4)
AXIN2	axin 2(AXIN2)
BAX	BCL2 associated X, apoptosis regulator(BAX)
CD3E	CD3e molecule(CD3E)
CREM	cAMP responsive element modulator(CREM)
ATF2	activating transcription factor 2(ATF2)
CREBBP	CREB binding protein(CREBBP)
FKBP11	FK506 binding protein 11(FKBP11)
SNIP1	Smad nuclear interacting protein 1(SNIP1)
BAG3	BCL2 associated athanogene 3(BAG3)
BAG4	BCL2 associated athanogene 4(BAG4)
FOS	Fos proto-oncogene, AP-1 transcription factor subunit(FOS)
JUND	JunD proto-oncogene, AP-1 transcription factor subunit(JUND)
SMAD7	SMAD family member 7(SMAD7)
LTB	lymphotoxin beta(LTB)
EIF2A	eukaryotic translation initiation factor 2A(EIF2A)
GADD45B	growth arrest and DNA damage inducible beta(GADD45B)
MAPK6	mitogen-activated protein kinase 6(MAPK6)
PRKCH	protein kinase C eta(PRKCH)

PPP3CC	protein phosphatase 3 catalytic subunit gamma(PPP3CC)
DCP1A	decapping mRNA 1A(DCP1A)
EXOC2	exocyst complex component 2(EXOC2)
RELA	RELA proto-oncogene, NF-kB subunit(RELA)
RELB	RELB proto-oncogene, NF-kB subunit(RELB)
RALGDS	ral guanine nucleotide dissociation stimulator(RALGDS)
SUMO3	small ubiquitin-like modifier 3(SUMO3)
TGFB3	transforming growth factor beta 3(TGFB3)
RRAS2	related RAS viral (r-ras) oncogene homolog 2(RRAS2)
PIK3R5	phosphoinositide-3-kinase regulatory subunit 5(PIK3R5)

Table 26 Genes associated with PAH and PAH pathways from Gene Ontology, STRING, and literature (Tissue Samples) (18 genes)

Gene Symbol	**Gene Name**
TNFSF10	tumor necrosis factor superfamily member 10(TNFSF10)
AES	amino-terminal enhancer of split(AES)
AKT1	AKT serine/threonine kinase 1(AKT1)
ARHGAP1	Rho GTPase activating protein 1(ARHGAP1)
AP2M1	adaptor related protein complex 2 mu 1 subunit(AP2M1)
DVL3	dishevelled segment polarity protein 3(DVL3)
HDAC1	histone deacetylase 1(HDAC1)
IGF2R	insulin like growth factor 2 receptor(IGF2R)
PCDH10	protocadherin 10(PCDH10)
LRP6	LDL receptor related protein 6(LRP6)
PRKCH	protein kinase C eta(PRKCH)
PPP2R5A	protein phosphatase 2 regulatory subunit B'alpha(PPP2R5A)
CDH24	cadherin 24(CDH24)
RALA	RAS like proto-oncogene A(RALA)
RGL2	ral guanine nucleotide dissociation stimulator like 2(RGL2)

RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)(RAC1)
NKD1	naked cuticle homolog 1(NKD1)
RGL1	ral guanine nucleotide dissociation stimulator like 1(RGL1)

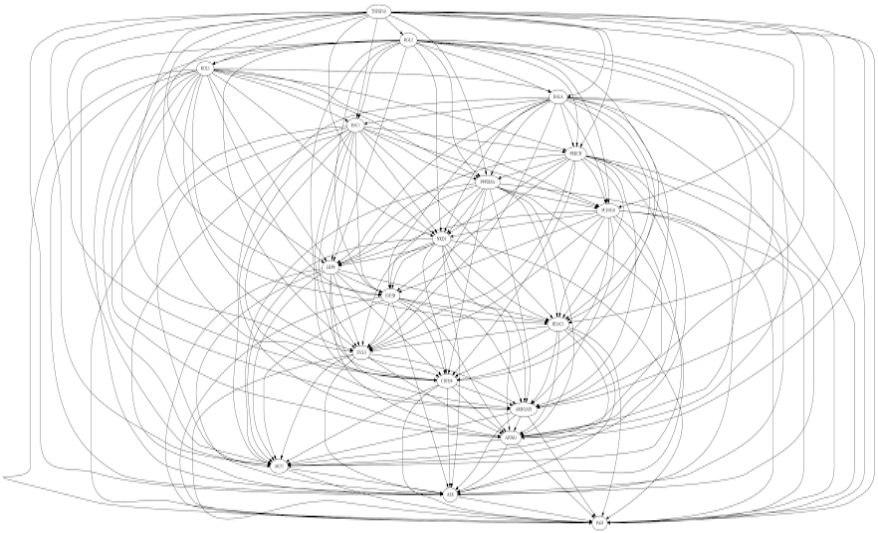

Figure 30. The best structure of BN of tissue samples including genes associated with PAH using Bene software (20 variables)

We searched the Comparative Toxicogenomics Database (http://ctdbase.org/) for association between the identified PAH and ID3 target genes and PCBs associated genes. We used the gene list of PAH and ID3 target genes (701 genes) in blood samples and PCBs associated genes curated from CTD (648). The common genes in blood sample analysis were 34 genes (Table 27). We used the gene list of PAH and ID3 target genes (573 genes) in tissue samples and PCBs associated genes curated from CTD (648). The common genes in tissue sample analysis were 19 genes (Table 27).

Table 27 Common (34) genes between PAH and ID3 target genes (701 genes) in blood samples and PCBs associated genes (648). Common (19) genes between PAH and ID3 target genes (573 genes) in tissue samples and PCBs associated genes (648) using CTD database.

Common (34) genes between PAH and ID3 target genes (701 genes) in blood samples and PCBs associated genes (648)	Common (19) genes between PAH and ID3 target genes (573 genes) in tissue samples and PCBs associated genes (648)
ALDH5A1, BTG1, CDKN1B, CIRBP, CLK1, COL12A1, CTSK, DNAJB9, DUSP1, EBP, EMP2, ENC1, FOS, HSP90AB1, ID3, IL6, ILF2, LNX2, MASTL, MCM6, MMP3, NECAP1, PEBP1, PFKFB3, PLEKHB1, PRC1, PRNP, PTGER4, RAD23B, RNASEH1, SAFB, TNFRSF25, TSEN54, YPEL5	AKT1, ARHGEF10, CASP2, CDT1, CLK2, CSF3R, HIST1H4L, ID3, IL2, MAN2C1, NR3C1, POLE, RACGAP1, NF146, SMG6, STMN1, TACC3, TIMP3, UROD

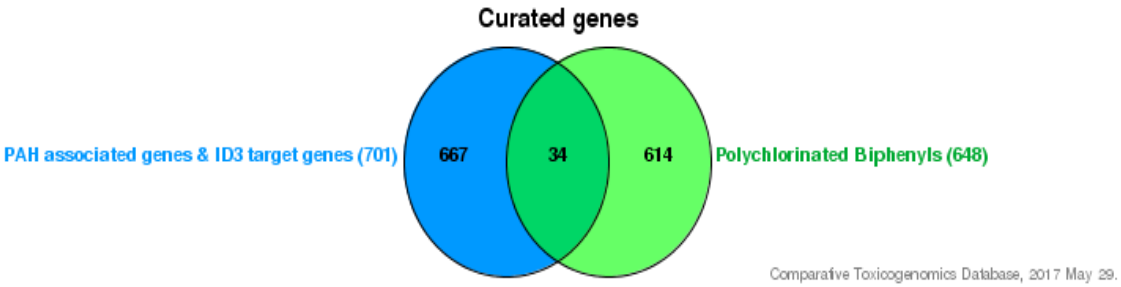

Figure 31 Common (34) genes between PAH and ID3 target genes (701 genes) in blood samples and PCBs associated genes (648).

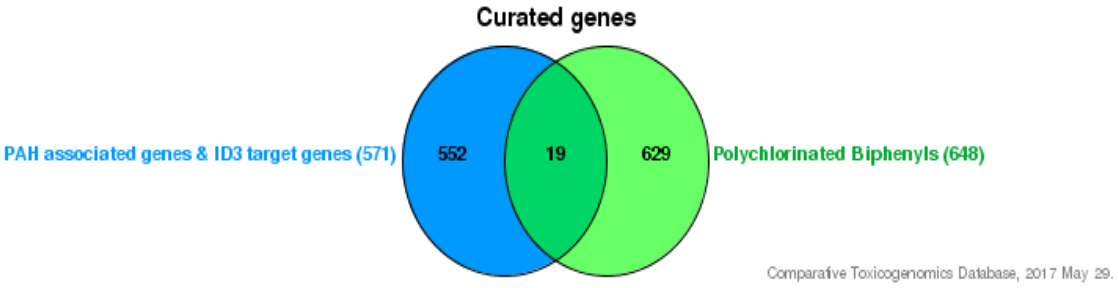

Figure 32 Common (19) genes between PAH and ID3 target genes (573 genes) in tissue samples and PCBs associated genes (648).

We used the gene list of PAH-associated genes; and genes associated with PAH pathways in literature and we found 5 genes to be common among different pathways including VEGFB, PRKCH, VAMP2, POLR2C, and LAMA5. Then, we used the expression data we have for VEGFB, PRKCH, VAMP2, POLR2C, LAMA5, location, and PAH; and we run it into Genie software to see how these genes are expressed in the data and what would be the relationship between them. We run the analysis many times and each we changed the status of one of the variables. For genes (low, normal, and high expression), location (blood and tissue), and PAH (control and Disease) (Figure 33-37).

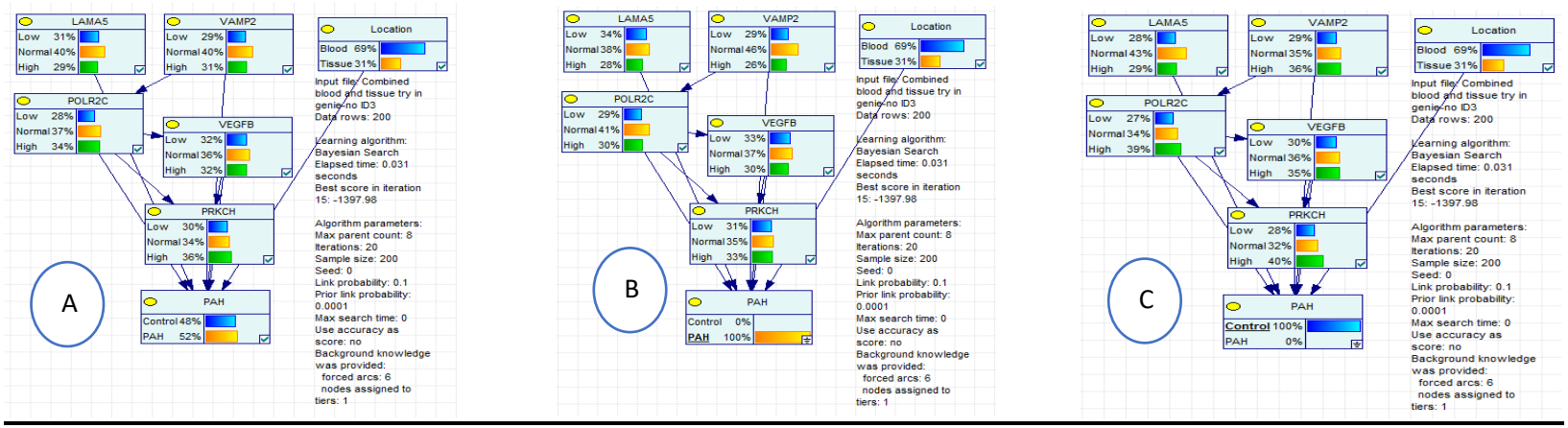

Figure 33 Genie software analysis: A) Original expression of genes, PAH, and location variables using our meta-analyzed microarray datasets. B) We overexpressed PAH variable to see the effect on its parent variable. C) We knockdown PAH variable to be all control sample expression.

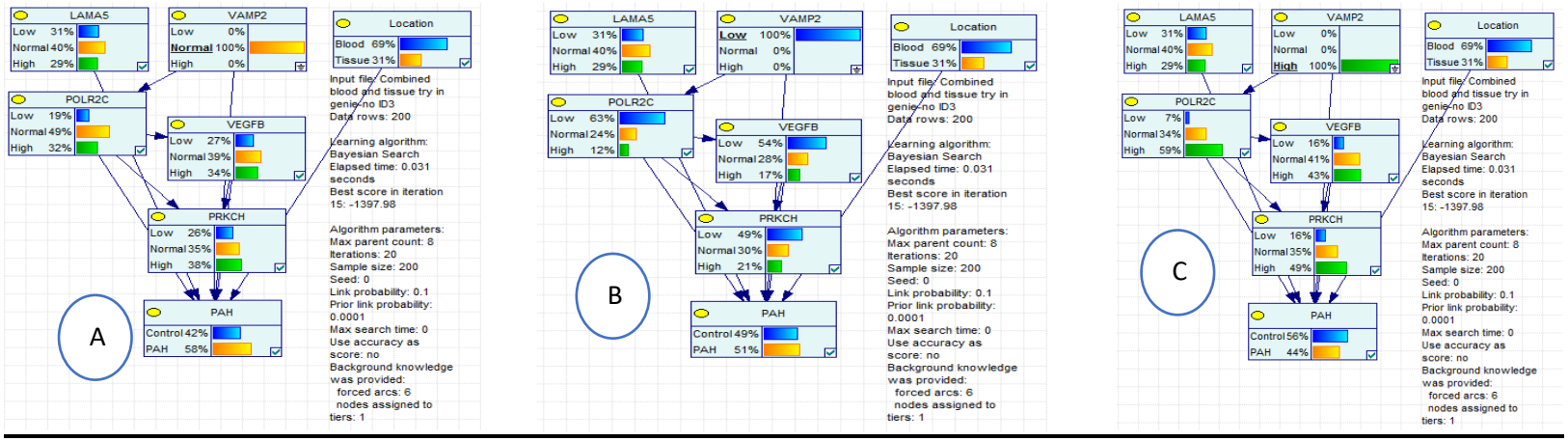

Figure 34 Genie software analysis: A) Normal expression of VAMP2 gene and its effect on other variable using our meta-analyzed microarray datasets. B) We overexpressed VAMP2 variable to see the effect on other variables. C) We knockdown VAMP2 variable to see the effect on other variables.

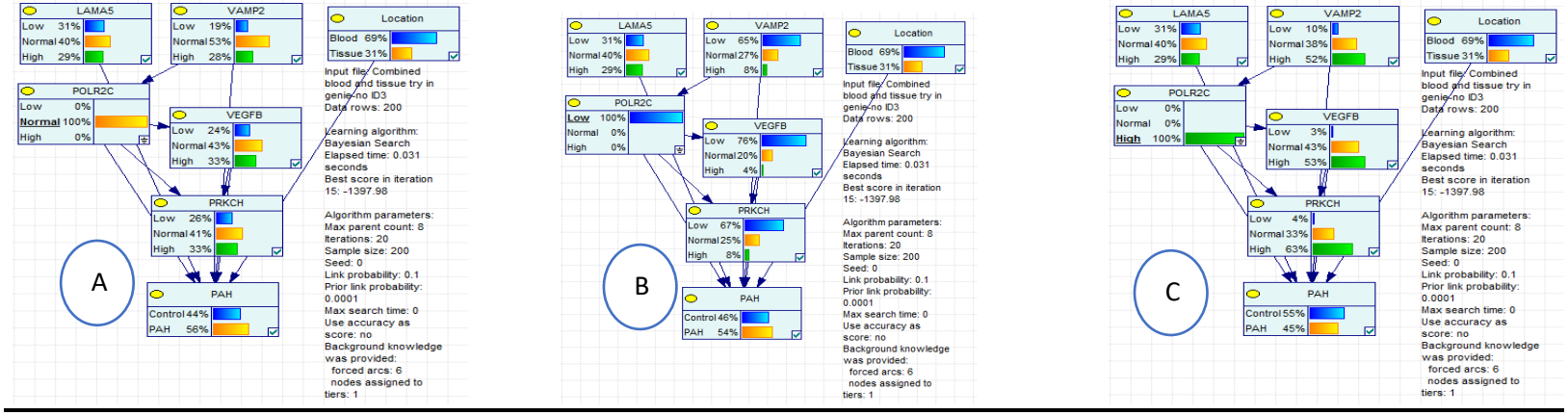

Figure 35 Genie software analysis: A) Normal expression of POLR2C gene and its effect on other variable using our meta-analyzed microarray datasets. B) We overexpressed POLR2C variable to see the effect on other variables. C) We knockdown POLR2C variable to see the effect on other variables

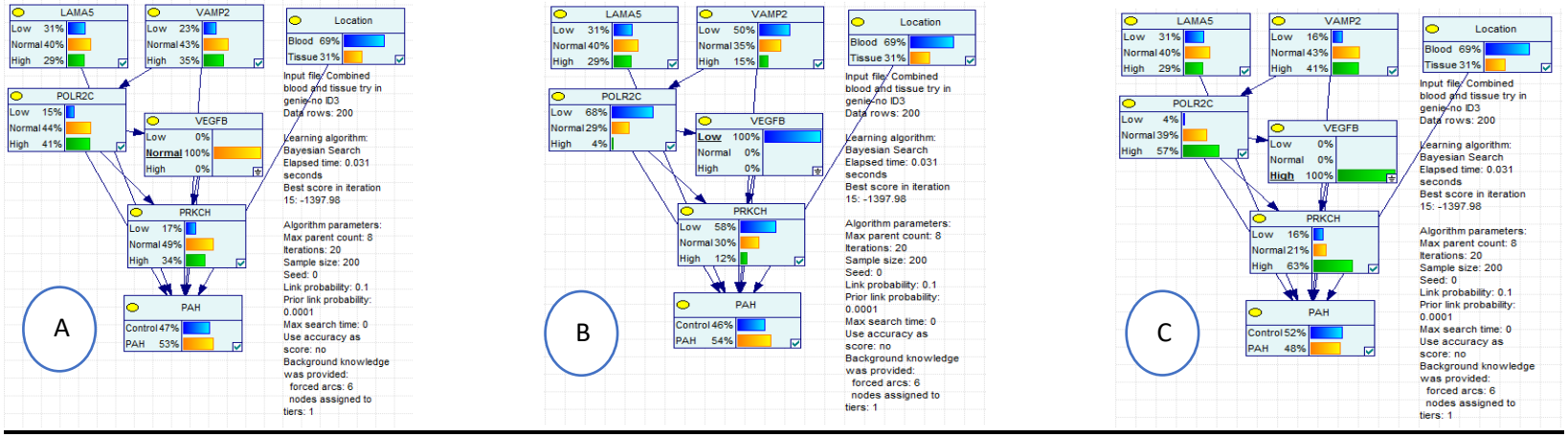

Figure 36 Genie software analysis: A) Normal expression of VEGFB gene and its effect on other variable using our meta-analyzed microarray datasets. B) We overexpressed VEGFB variable to see the effect on other variables. C) We knockdown VEGFB variable to see the effect on other variables.

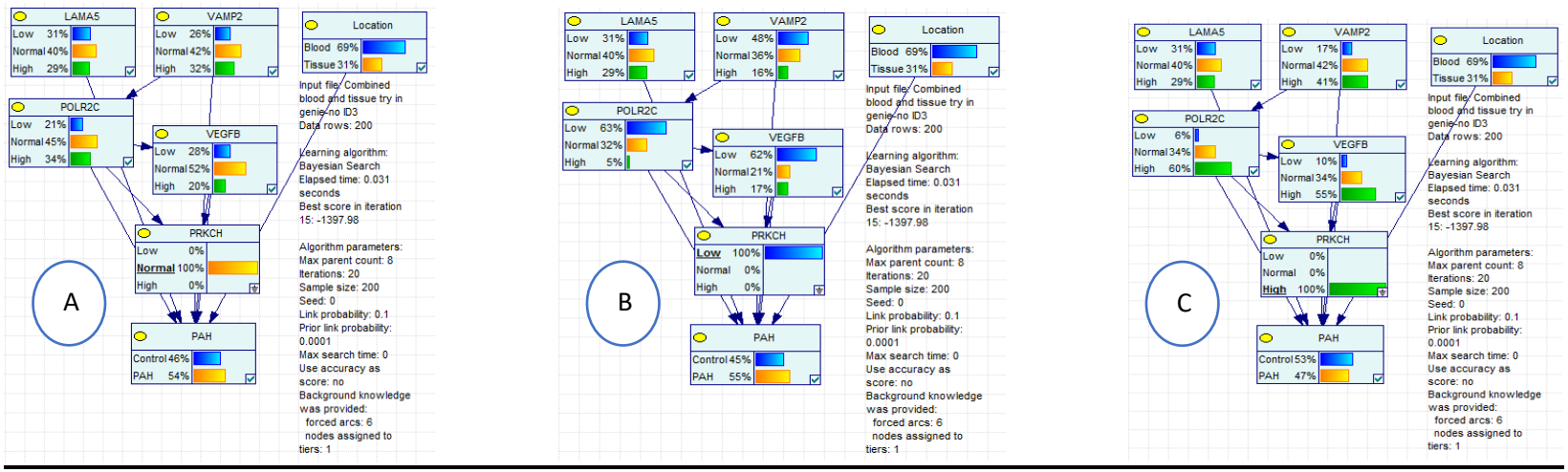

Figure 37 Genie software analysis: A) Normal expression of PRKCH gene and its effect on other variable using our meta-analyzed microarray datasets. B) We overexpressed PRKCH variable to see the effect on other variables. C) We knockdown PRKCH variable to see the effect on other variables

DISCUSSION

Our meta-analysis of PAH microarray datasets published in GEO would be the first meta-analysis on PAH microarray expression data. We identified the key gene network that significantly associated with PAH and different biological material including blood and tissues. We used those genes involved in pathways associated with PAH using different sources including literature, PANTHER gene Ontology, and STRING web tools. All pathways that had more than 3 genes involved were used, and genes were combined together.

Many pathways and genes are known to be associated with PAH. ACVRL1, ALK1, BMPR1B, BMPR2, CAV1, CBLN2, EIF2AK4, ENG, KCNA5, KCNK3, SMAD9, TBX, TBX, CYP1B1, ACE, 5-HTT, PGIS, MMP2, MMP9, Bax, TGFBR2, VIP, ET1, and angiopoietin 1. While using STRING database, many pathways showed an association with PAH through gene association, and the most significant pathways were Apoptosis signaling pathway, p53 pathway, Ras Pathway, T cell activation, TGF-beta signaling pathway, VEGF signaling pathway, Wnt signaling pathway.

In blood sample analysis using STRING database, apoptosis signaling pathway reported that ATF2, EIF2A, FOS, PRKCH, RELA, RELB, LTB, BAX, ATF4, BAG4, BAG3, and CREM to be associated with PAH. p53 pathway reported that PIK3R5, CREBBP, GADD45B, BAX, and SUMO3 to be associated with PAH. Moreover, Ras Pathway reported that ATF2, RRAS2, RALGDS, and EXOC2 to be associated with PAH. T cell activation reported that CD3, FOS, PPP3CC, and CD3E to be associated with PAH. TGF-beta signaling pathway reported ATF2, RRAS2, CREBBP, TGFB3, SMAD7, DCP1A, JUND, SNIP1, and FKBP11 to be associated with PAH. VEGF signaling pathway

reported PRKCH, MAPK6, and RRAS2 to be associated with PAH. Wnt signaling pathway reported that ATF2, RRAS2, CREBBP, TGFB3, SMAD7, DCP1A, JUND, SNIP1, FKBP11, AXIN2, and PRKCH to be associated with PAH.

In tissue sample analysis using STRING database, Wnt signaling pathway reported that AES AP2M1, CDH24, DVL3, HDAC1, LRP6, NKD1, PCDH10, PPP2R5A, and PRKCH to be associated with PAH. Ras Pathway reported that RAC1, RGL2, AKT1, RALA, and RGL1 to be associated with PAH. Apoptosis signaling pathway reported that PRKCH, IGF2R, TNFSF10, and AKT1 to be associated with PAH. VEGF signaling pathway reported that RAC1, PRKCH, AKT1, and ARHGAP1 to be associated with PAH. p53 pathway and T cell activation reported that HDAC1 and AKT1; RAC1 and AKT1 to be associated with PAH, respectively.

PRKCH is a gene that encodes the η isozyme of protein kinase C (PKCη) and it is known to play a significant role in signal transduction that control T-cell activation (Teixeira et al., 2008). PRKCH showed significant association with rheumatoid arthritis as it was expressed in lower levels than normal control. Our results showed that Low expression of PRKCH resulted in higher probability of PAH, and high expression of PRKCH decreases the probability of PAH (Figure 34 B and C).

VEGF reported to be increased in lung tissue with PAH and VEGFB is another isoform of it (Malenfant et al., 2013). VEGFB controls many non-angiogenic functions such as facilitating fatty acid transport in EC and preventing angiotension 2 (which increase cardiac diastolic dysfunction) (Voelkel & Gomez-Arroyo, 2014). Low expression of VEGFB was found in the right ventricle of SuHx rats (Al-Husseini et al., 2015). Our results showed that knock-down VEGFB is significantly associated with decrease the expression

of VAMP2, POLR2C, and PRKCH. Whereas, over expression of VEGFB, cases increase in expression of same genes. However, it causes decrease in probability of PAH status comparing with normal and low expression of VEGFB. In summary, our meta-analysis showed that different expression in VAMP2, LAMA5, POLR2C, VEGFB, and PRKCH are the most significantly associated genes with PAH and PAH regulated pathways. Although, these genes suggested to be highly influencing the pathogenesis of PAH.

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CHAPTER 6

OVERALL CONCLUSIONS

The main goal of this dissertation was to study the molecular determinant of PAH and to evaluate the exposure to EDC (PCB) and their association with the risk of PAH in a representative NHANES 1999-2004 data of the U.S population. Molecular level interactions that are associated with PAH were studied by meta-analyzing 6 microarray gene expression datasets of PAH from GEO. The resulted gene list was used to determine gene ontology and PAH-associated pathways using Panther and string database. Combined results from our meta-analysis, Panther and String databases were used to determine the plausible markov blanket genes network using Bayesian network analysis.

Meta-analysis of gene expression microarray datasets from GEO resulted in 10,716 genes to be common in all PAH studied in blood and tissue samples. ID3 correlated genes were matched with 701 and 573 genes from blood and tissue samples, respectively. GO pathway analysis showed that Gonadotropin-releasing hormone receptor pathway, inflammation mediated by chemokine and cytokine signaling pathway, and apoptosis signaling pathway had the highest number of gene involved from blood samples. However, Gonadotropin-releasing hormone receptor pathway, Angiogenesis, and Wnt signaling pathway had the highest number of genes involved from tissue samples. Common genes in pathways associated with PAH from gene ontology databases resulted in 29 genes and 18 genes common for blood and tissue samples, respectively. These genes were matched with literature PAH pathway associated genes and resulted in 5 genes to be common among different pathways including VEGFB, PRKCH, VAMP2, POLR2C, and LAMA5.

Supported with information that females have higher risk of PAH, we evaluated PCBs serum concentrations and their association with risk of PAH using NHANES 1999-2004 survey data. All 6 PCBs (74, 99, 118, 138, 153, and 180) concentration were higher in people at risk of PAH than people not at risk of PAH in the U.S. population > 20 years old. Also, higher risk of PAH was reported in the sum of dioxin-like PCBs (PCB 74 and 118), and non-dioxin-like PCBs (PCB 118, 138, 153, and 180) concentration in the U.S. population > 20 years old. All confounders (gender, age, BMI, annual family income, educational level, smoking, and alcohol use) were associated with the higher risk of PAH except older age groups. Different LOD of PCBs (> LOD, > 50%, 50-75%, and >75%) were significantly higher in people at risk of PAH except PCB 180.

Overall, we are representing genes (VEGFB, PRKCH, VAMP2, POLR2C, and LAMA5) that were associated with PAH risk factors using bioinformatics and gene-ontology. Also, we provide plausible gene network pathway that could lead to PAH. Additionally, we evaluated different PCBs serum concentrations and their association with the risk of PAH in the U.S population.

LIMITATIONS

Risk of PAH variable that was created using PAH associated risk factors remains a limitation in our study. Also, small number of people at risk of PAH in NHANES 1999-2004 data, resulted in decreasing the significance risk of PAH and exposure to PCBs results.

Future Studies

Even though our results showed association between PCBs and risk of PAH, epidemiologic studies need to be conducted to report the association between exposure to

EDCs and development of PAH. Multiple pathways associated with PAH, its related estrogen toxicity, and at the same time continuous exposure to EDCs, support the needs to conduct epidemiological studies. Lastly, RNA sequence studies need to update findings of such deep level analysis and its association of development of PAH; and further validate by what mechanism estrogen would increase risk of females.

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