A Novel Association Between Primary Biliary Cholangitis and Idiopathic Pulmonary Arterial Hypertension

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Abstract

Background and Hypothesis

Idiopathic Pulmonary Arterial Hypertension (IPAH) and Primary Biliary Cholangitis (PBC) are both rare diseases that are progressive and may be fatal when left untreated. Furthermore, mitochondrial dynamics are fundamental to the pathogenesis of both conditions. An association between the two in the form of an overlap syndrome has never been previously described. Despite this, observations from the Pulmonary Hypertension clinic at the University of Alberta indicate a number of patients with both conditions, raising suspicion for a possible overlap syndrome. We hypothesize that IPAH and PBC may co-exist in some patients as an overlap syndrome as opposed to simply co-occurring by chance.

Methods

The PBC patient database at the University of Alberta was reviewed, identifying patients with echocardiograms showing a right ventricular systolic pressure (RVSP) of >30mmHg. Within these cases we identified PBC patients with IPAH by identifying and subsequently excluded those with left heart disease, chronic lung disease, connective tissue disease, hyperdynamic circulation secondary to liver disease, and portal hypertension since these are all causes of secondary pulmonary hypertension. We used a control group of patients with primary sclerosing cholangitis (PSC), as this is a chronic immune mediated cholestatic liver disease, much like PBC.

Results

A total of 211 patients were analyzed, 134 with PBC and 77 with PSC. 5 patients with PBC also had concurrent IPAH (3.73%), compared to none in the PSC group (0%). Compared to the prevalence of IPAH in the general population of roughly 0.005%, the prevalence of IPAH in PBC patients was over seven hundred times higher. Of all patients with RVSP >30mmHg, 60.4% of PBC patients had portal hypertension, compared to 89% in the PSC group. Of patients without hyperdynamic circulation or heart disease, and RVSP>30mmHg, and secondary signs of PAH on echocardiogram, portopulmonary hypertension was observed at a proportion of 50% in PBC compared to 0% in PSC.

Conclusions

Given a 700 fold increased prevalence of IPAH in PBC compared to the general population, and also when compared to PSC, it is likely that PBC and IPAH can co-exist in a previously undescribed overlap syndrome. The underlying pathogenesis is unclear, but may involve mitochondrial suppression and a differential response of the immune system in the pulmonary arteries and biliary epithelial cells. Further epidemiological studies are warranted to confirm our findings of an overlap between IPAH and PBC, as well as laboratory studies to explore the underlying pathophysiology of such a condition.

Preface

This thesis is an original work by Jason An. The research project received ethics approval from the University of Alberta Research Ethics Board, project name “A Novel Association Between Primary Biliary Cholangitis and Idiopathic Pulmonary Arterial Hypertension”.

No part of this thesis has been previously published.

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Contents

List of Abbreviations

AMA – anti-mitochondrial antibodies
ATP – adenosine triphosphate
BEC – Biliary Epithelial Cell
BMPR2 - Bone morphogenetic protein receptor type II
HIF1α – hypoxia induced factor 1 alpha
HIV – human immunodeficiency virus
IPAH – idiopathic pulmonary arterial hypertension
PBC – primary biliary cholangitis
PDC – pyruvate dehydrogenase complex
PDK – pyruvate dehydrogenase kinase
PSC – Primary Sclerosing Cholangitis
RV – right ventricle
RVSP – Right ventricular systolic pressure
SIRT3 – sirtuin 3
TNF – Tumor Necrosis Factor
UCP2 – uncoupling protein 2

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INTRODUCTION

Idiopathic pulmonary arterial hypertension (IPAH) and primary biliary cholangitis (PBC) are both rare, progressive, and ultimately fatal diseases when left untreated. This study examines the possibility of a never before described association between the two entities, born out of a clinical encounter of patients presenting to the PAH clinic both with severe IPAH (as diagnosed by right heart catheterization) as well as PBC. As these two diseases are individually very rare, the question arose whether they developed in the same patients by pure coincidence, or whether they may actually coexist in a previously unrecognized overlap syndrome. This possibility was further driven by the observation that a mitochondrial enzyme, pyruvate dehydrogenase complex (PDC) plays a central role in both diseases as we will explore in detail below.

Pulmonary Arterial Hypertension

What is Pulmonary Arterial Hypertension?

Pulmonary arterial hypertension (PAH) is a condition characterized by elevated pulmonary arterial pressures, and very high morbidity and mortality. PAH is a subset of pulmonary hypertension, termed 'Group 1 Pulmonary Hypertension' where the disease predominantly arises within the pulmonary arterioles (as opposed to increases in pulmonary pressures secondary to other primary diseases like chronic lung disease, thromboembolic lung disease, valvular heart disease or left ventricular dysfunction) (Figure 1). PAH is diagnosed when specific hemodynamic parameters are met on right heart catheterization. The updated 2019 hemodynamic definition of PAH by the World Symposium on Pulmonary Hypertension Task Force (shown in Figure 1) requires a mean pulmonary artery pressure exceeding 20mmHg, pulmonary artery wedge pressures of less than 15mmHg, and pulmonary vascular resistance greater than 3 woods units (Simonneau et al., 2019). Aside from hemodynamic parameters, the individual diseases making up Group 1 Pulmonary Hypertension (i.e. PAH) also share histologic features. PAH is characterized by a vascular remodeling of the small-to-medium size pulmonary arteries, consisting of increase in the media thickness, increased muscularization of pre-capillary arterioles and plexogenic arteriopathy; which is a unique lesion consisting of an anarchous proliferation of pulmonary artery smooth muscle cells, pulmonary endothelial cells, fibroblasts as well as heavy infiltration of immune

cells (Wagenvoort, 1994). Overall, this proliferative vascular remodeling leads to intima and medial thickening, and subsequent obliteration of the pulmonary arterial lumen. Physiologically, this is reflected by increased pressures of the pulmonary arteries, which lead to increased strain and ultimately failure of the right ventricle. This right ventricular failure is the basis of both the morbidity (decreased cardiac output, dyspnea, fluid retention and exertional syncope) and mortality in this disease.

The underlying etiologies of PAH (Figure 1) are classified into heritable causes, toxin induced, associated diseases (such as HIV, portal hypertension, schistosomiasis, connective tissue diseases, congenital heart disease), pulmonary venous occlusive disease, persistent pulmonary hypertension of the newborn, and idiopathic pulmonary arterial hypertension (IPAH) which is the focus of this study (Simonneau et al., 2019). While in a small number of cases, specific mutations have been described, this is not the case for the majority of IPAH cases.

Although the exact prevalence of IPAH is remains unknown, estimates in the general population have placed it to be roughly 1/20,000, or 0.005% of the general population (Sutendra & Michelakis, 2014). The estimated prevalence of associated PAH is greater. For example, PAH has been reported to exist in 0.5% of HIV cohorts, and up to 12% in patients with systemic sclerosis (Sutendra & Michelakis, 2014).

1 PAH
- 1.1 Idiopathic PAH
- 1.2 Heritable PAH
- 1.3 Drug- and toxin-induced PAH (table 3)
- 1.4 PAH associated with:
  - 1.4.1 Connective tissue disease
  - 1.4.2 HIV infection
  - 1.4.3 Portal hypertension
  - 1.4.4 Congenital heart disease
  - 1.4.5 Schistosomiasis
- 1.5 PAH long-term responders to calcium channel blockers (table 4)
- 1.6 PAH with overt features of venous/capillaries (PVOD/PCH) involvement (table 5)
- 1.7 Persistent PH of the newborn syndrome

2 PH due to left heart disease
- 2.1 PH due to heart failure with preserved LVEF
- 2.2 PH due to heart failure with reduced LVEF
- 2.3 Valvular heart disease
- 2.4 Congenital/acquired cardiovascular conditions leading to post-capillary PH

3 PH due to lung diseases and/or hypoxia
- 3.1 Obstructive lung disease
- 3.2 Restrictive lung disease
- 3.3 Other lung disease with mixed restrictive/obstructive pattern
- 3.4 Hypoxia without lung disease
- 3.5 Developmental lung disorders

4 PH due to pulmonary artery obstructions (table 6)
- 4.1 Chronic thromboembolic PH
- 4.2 Other pulmonary artery obstructions

5 PH with unclear and/or multifactorial mechanisms (table 7)
- 5.1 Haematological disorders
- 5.2 Systemic and metabolic disorders
- 5.3 Others
- 5.4 Complex congenital heart disease

PAH: pulmonary arterial hypertension; PVOD: pulmonary veno-occlusive disease; PCH: pulmonary capillary haemangiomatosis; LVEF: left ventricular ejection fraction.

Figure 1: World Symposium on Pulmonary Hypertension Classification of Pulmonary Hypertension, adapted from (Simonneau et al., 2019).

Pathogenesis of IPAH

Understanding the pathogenesis of IPAH is crucial to exploring the possible biological mechanisms that may link it with PBC. As its name suggests, the underlying pathogenesis of IPAH remains incompletely elucidated. Although initially thought to be a vasoconstrictive disease of the pulmonary arteries, research over the past decades has shown that IPAH is a multifaceted disease with multiple pathogenic disease mechanisms. Two theories have been proposed to comprehensively describe the pathogenesis of IPAH; the Metabolic Theory, and the Inflammatory Theory.

The Metabolic Theory of IPAH suggests that a primary mitochondrial abnormality is the basis of the proliferative and anti-apoptotic environment of the remodeled PAH pulmonary arteries. This is because suppressed mitochondrial function typically results in suppressed apoptosis in a manner similar to cancer, allowing an unopposed proliferation of cells (Paulin & Michelakis, 2014). This theory may also explain why

PAH is restricted in the pulmonary arteries, sparing all systemic arteries, because significant differences between the pulmonary versus systemic arterial smooth muscle cells have been previously described. In addition, this theory explains other intriguing aspects of the disease. For example, skeletal muscle metabolic changes (i.e. insulin resistance) have been described in PAH, in the absence of obesity or diabetes. The metabolic theory suggests that a primary mitochondrial abnormality is present throughout the body but is primarily expressed in the pulmonary circulation because this circulation is much more sensitive to mitochondria-derived signaling than the systemic circulation. Finally, the metabolic theory also describes a potential predisposition of immune cells to be activated (e.g. a decrease in mitochondrial glucose oxidation is known to be an early event in T cell activation and sufficient in triggering their activation) (Gerritje J.W. van der Windt, 2012). In fact, inflammation is now known to be a very prominent feature of the disease and the Inflammatory Theory of PAH has been proposed as an alternate comprehensive theory of PAH (Rabinovitch, Guignabert, Humbert, & Nicolls, 2014). Whether PAH is better explained by a primary metabolic disturbance with secondary immune cell activation, or primary immune dysregulation with secondary metabolic changes remains unknown, but both are fundamental features of PAH.

PAH and Autoimmunity

Like many autoimmune disorders, a female preponderance exists in IPAH patients. There have been several theories put forth to explain this and most have centered around the differential role of hormones between the sexes. Interestingly however, when males are affected, they often have more severe disease. Recent studies are now revealing that the X-chromosome may play a pivotal role in the pathogenesis of IPAH, and explain the worse severity in males (Osman, & Michelakis, 2018). The X chromosome contains many critical elements of the immune system, and the second X chromosome in females is sometimes only partially inactivated. This means that some immune elements in females are expressed at double the male allelic levels resulting in a more robust immune response against foreign pathogens or self-antigens. For example, some of these elements encode factors that control T-regulatory cells which interact with endothelial cells to modulate their survival and secretion of vasodilatory substances.

Furthermore, antinuclear antibodies which are most classically known for their association with autoimmune connective tissue diseases are often also found in patients with IPAH (P. Dorfmuller, F. Perros, K. Balabanian, 2003). Certain HLA haplotypes such as HLA-DR6 and HLA-DR52, which have been linked to autoimmune diseases, are also associated with a higher risk of developing PAH in patients with

HIV (Pellicelli et al., 2001). Specifically, HLA-DR6 has been associated with myasthenia gravis (Shinomiya, Nomura, & Segawa, 2004), and HLA-DR52 to connective tissue diseases, such as systemic sclerosis (Kuwana, Kaburaki, Okano, Inoko, & Tsujill, 1993) and inflammatory myositis (Garlepp, Laing, Zilko, & Olliert, 1994). This observation may suggest a role for genetic predisposition to immune dysregulation in the pathogenesis of PAH (at least in the HIV and connective tissue disease patients).

PAH is often associated with connective tissue diseases. Preliminary prospective trials have found some benefit with intense immunosuppression (glucocorticoid and cyclophosphamide) combined with vasodilator therapy, compared to vasodilator therapy alone for patients with early PAH associated with connective tissue diseases (Saori Miyamichi-Yamamoto et al, 2011). It is important to note however, that PAH associated with scleroderma specifically, appeared less likely to respond to immunosuppression compared with non-scleroderma connective tissue diseases. This is consistent with other studies and postulated to be due to the more intense fibrosis in scleroderma (Laura C. Price, S. John Wort, Peter Dorfmüller , Alice Huertas, & Sylvia Cohen-Kaminsky, 2012). It is possible that the benefits of immunosuppression on PAH are due to treating the underlying disease as opposed to directly treating the PAH. It would be important to know therefore, whether immunosuppression works on IPAH, or PAH associated with non-inflammatory conditions. To our knowledge, there have been no published studies on using immunosuppressive therapy on human IPAH patients. Nonetheless, these studies showing response to immunosuppression in patients with PAH associated with connective tissue diseases support the idea that immune dysregulation is important in PAH pathogenesis in this population. Immune dysregulation is likely not the *sole* driver of PAH pathogenesis however given the lack of response in scleroderma associated PAH.

As discussed above, histologic sections in patients with IPAH show inflammatory cell infiltration within and around the remodeled pulmonary arteries and plexogenic lesions (Laura C. Price, S. John Wort et al., 2012). These inflammatory cells consist mainly of T-cells and macrophages, and to a lesser extent B-cells and dendritic cells. Tertiary lymphoid follicles are an organized structure of stromal cells and compartmentalized lymphocytes and are found in the end organs of many recognized autoimmune diseases (Shipman, Dasoveanu, & Lu, 2017). For example, they are found in the inflamed synovium of rheumatoid arthritis, renal sections of lupus nephritis, secretory glands in Sjogren's, thymus in myasthenia gravis, central nervous system in multiple sclerosis, and gut in inflammatory bowel disease. Their exact role in these diseases is unclear. It is known they can induce and harbor pathologic T-cells but can also

sequester aberrant lymphocytes and prevent further inflammatory cell infiltration into surrounding tissues/ organs. Hence it is interesting that tertiary lymphoid follicles have also been identified in the pulmonary arteries in IPAH and again suggest the possibility of immune dysregulation as a component in IPAH pathogenesis.

The Role of Mitochondria in PAH

Mitochondria are the remnants of aerobic bacteria that invaded eukaryotic cells over a billion years ago (Dromparis & Michelakis, 2013). They are highly sensitive detectors and coordinators of cellular energy stores, and their most widely known function is to synthesize adenosine triphosphate (ATP) for the cell. In cellular respiration, glucose is converted to pyruvate in the cytoplasm via glycolysis. The pyruvate dehydrogenase complex (PDC) enzyme within the mitochondrial matrix serves as a gatekeeper for the conversion of pyruvate into acetyl CoA. This enters the Krebs cycle, where the electron donors NADH and $FADH_2$ are generated. These compounds enter the electron transport chain where their electrons reduce oxygen to form water. Simultaneously, protons are secreted into the mitochondrial intermembrane space generating a mitochondrial membrane potential. These protons then flow down their electric gradient, which is utilized by the ATP synthetase to create ATP for cellular use (**Figure 2**). Since the mitochondrial membrane potential is created by the products of the Krebs cycle and sustained by a functioning electron transport chain, it has been used as a surrogate marker for mitochondrial function and activation status (Dromparis, Sutendra, & Michelakis, 2010).

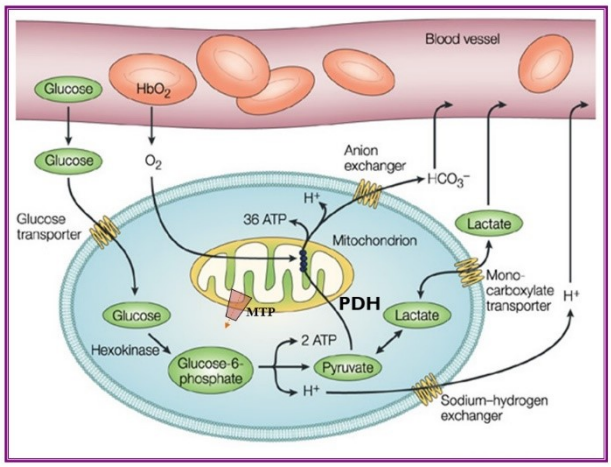


Figure 2: The mitochondria are organelles responsible for cellular respiration. Following glycolysis where glucose is converted to pyruvate, pyruvate enters the mitochondrial matrix and is converted to acetyl-CoA via pyruvate dehydrogenase. Acetyl CoA is fed through the Krebs cycle, ultimately generating adenosine triphosphate through the electron transport chain.

Although mitochondria are most classically known for their role in respiration, they serve many other functions critical to overall cellular homeostasis (Dromparis & Michelakis, 2013). They are able to sense

danger signals from invading pathogens and initiate inflammatory responses through the formation of the inflammasome or the release of mitochondrial reactive oxygen species. When conditions for cellular survival become exceedingly taxing for the overall organism, mitochondria can release factors normally sequestered in its matrix to trigger apoptosis.

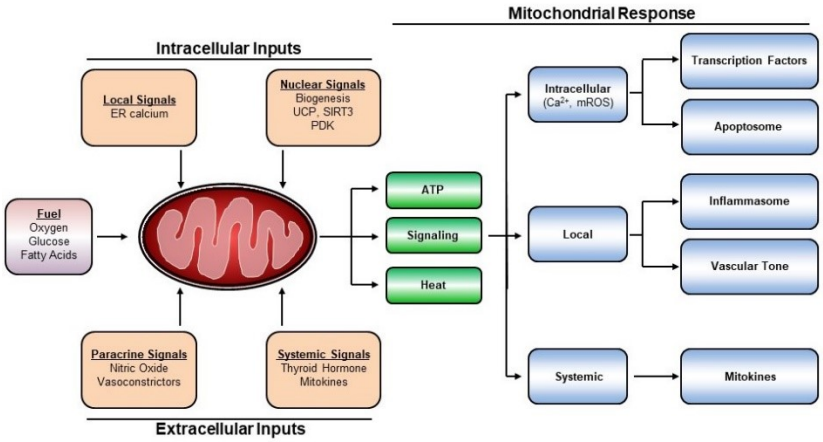


Figure 3: Mitochondria perform a vast diversity of cellular functions outside of respiration. Adapted from (Dromparis & Michelakis, 2013).

Mitochondria can react differently to the same stimuli in a tissue-specific manner. For example, mitochondria respond differently to hypoxia in the pulmonary compared to the systemic arteries (Sutendra & Michelakis, 2014). In pulmonary artery smooth muscle cells, a unique phenomenon known as Hypoxic Pulmonary Vasoconstriction allows for perfusion to be reduced in areas of the lung that are hypoxic, thereby optimizing ventilation perfusion matching. Hypoxia is sensed by components of the electron transport chain which results in reduced production of mitochondrial reactive oxygen species, closure of cell membrane redox-sensitive K+ channels and as a result, cellular depolarization. This is followed by opening of voltage-gated calcium channels leading to calcium influx into the cell and subsequent vasoconstriction.

In contrast to the pulmonary arteries, hypoxia leads to reduced cytosolic calcium and vasodilation in systemic arteries to enhance oxygenation to target tissues. The differential response of vascular smooth muscle cells to hypoxia in the pulmonary versus systemic arteries likely reflects differences in mitochondria between the lungs and other organs (Sutendra & Michelakis, 2013) (Michelakis et al., 2002). For example, inhibiting electron transport chain complexes 1 and 3 in systemic vascular mitochondria

reproduces the hypoxic response seen in the pulmonary vascular mitochondria which suggest there may be differences in expression or function of electron transport chain components between mitochondria residing in different tissues (Sutendra & Michelakis, 2013). There are a few possible explanations for why mitochondria may be different between pulmonary and systemic vasculature. Pulmonary arteries have a different embryonic origin compared to the systemic arteries. Alternatively, the pulmonary and systemic arteries diverged in their developmental trajectory in response to different redox conditions; i.e. much higher oxidative stress in the pulmonary arteries because of the much higher levels of oxygen in the lungs, compared to the lower levels of oxygen (and thus reactive oxygen species) in all the systemic arteries and organs (Michelakis et al., 2002).

This tissue-dependent mitochondrial diversity has important implications for our study because we know that mitochondria may be involved in both PBC and IPAH. As discussed later in PBC, antibodies are formed against the PDC enzyme of mitochondria. In IPAH, the PDC of mitochondria is inhibited through complex processes described in the following sections. This raises the question of why only specific organ systems are affected in IPAH and PBC; namely the pulmonary arteries and biliary ducts respectively, when mitochondria exist in all cells throughout the body? Based on the observations discussed earlier on how mitochondria behave differently in the systemic versus pulmonary vasculature, it is interesting to postulate that mitochondrial diversity may underlie the diversity in organ involvement in IPAH and PBC.

In other words, intrinsic differences between mitochondria residing in different organs may explain why some organs are affected in disease while others are spared. Conversely, conserved features between mitochondria residing in different organs may explain why separate organs may be affected in the same disease. For example, the effect of PDC inhibition in the pulmonary artery smooth muscle cells may be similar to that in the biliary epithelial cells (BECs), and yet different from that in other organs. Such a scenario of conserved mitochondrial physiology may explain how two individually rare diseases (IPAH and PBC) may cluster in the same patients. In general, mitochondrial suppression promotes cellular growth through suppression of apoptosis, as well as promoting inflammation, both of which are features of PAH and PBC. Exploring the possibility of such a theory will require an understanding of mitochondrial involvement in PAH and specifically the inhibition of PDC, which we discuss below.

Suppression of PDC in PAH

Within the past decade, mitochondria in pulmonary artery smooth muscle cells have been observed to be

suppressed in PAH, resulting in an imbalance favoring glycolysis over glucose oxidation. Mitochondrial and specifically PDC suppression can be due to several intra and extra mitochondrial factors.

Two important intra-mitochondrial factors include deficiencies in uncoupling protein 2 (UCP2) and sirtuin 3 (SIRT3) (Sutendra & Michelakis, 2014). UCP2 is a mitochondrial calcium transporter and deficiencies lead to reduced mitochondrial calcium. Since many mitochondrial enzymes including PDC are calcium dependent, UCP2 inhibition leads to suppressed PDC (Michelakis et al., 2017). UCP2 deficiency induces expression of hypoxia inducible factor 1α (HIF1α) despite normoxic conditions (Sutendra & Michelakis, 2014). HIF1α activates pyruvate dehydrogenase kinase (PDK), which phosphorylates PDC at serine 293 resulting in PDC inhibition (Michelakis et al., 2017). UCP2 knockout mice are prone to developing spontaneous pulmonary arterial hypertension.

SIRT3 is a mitochondrial deacetylase which activates a number of mitochondrial enzymes including PDC, and electron transport chain complexes (Paulin et al., 2014). Deficiencies have been associated with PAH, metabolic syndrome, as well as malignancy. As described in a 2017 study, the collective status of UCP2 and SIRT3 are important factors that influence the activity of PDC (Michelakis et al., 2017). The administration of dichloroacetate to humans with IPAH was shown to suppress PDK, disinhibit PDC, and improve hemodynamic parameters as well as functional capacity (Michelakis et al., 2017). In the proportion of patients that did not robustly respond to treatment, it was shown that the degree of response was highly dependent on the single nucleotide polymorphism status of the UCP2 and SIRT3 genes. Specifically, these single nucleotide polymorphisms were associated with reduced function of their respective proteins, which in turn impairs the function of PDC in a genetic dose-dependent manner.

Extra-mitochondrial factors that inhibit PDC include HIF1α and tyrosine kinases (both of which activate PDK) and are prominently activated under hypoxic and inflammatory conditions. Extra-mitochondrial factors may also exert their inhibition on PDC by inducing endoplasmic reticulum stress (Sutendra & Michelakis, 2014). This activates the reticulon protein Nogo which increases the separation between the endoplasmic reticulum and mitochondria. As mitochondrial function is very dependent on calcium released from the endoplasmic reticulum, increased endoplasmic reticulum to mitochondrial distance reduces the calcium available to mitochondria and thus downregulates PDC.

As the gatekeeping enzyme in glucose oxidation**,** PDC suppression diminishes glucose oxidation through

the Krebs cycle and subsequently oxidative phosphorylation that occurs in the electron transport chain (Sutendra & Michelakis, 2014). Since mitochondrial reactive oxygen species are produced in the electron transport chain complexes during oxidative phosphorylation, PDC suppression results in reduced mitochondrial reactive oxygen species. Reduced mitochondrial reactive oxygen species in turn lead to inhibition of membrane bound voltage gated K^+ channels. Since these K^+ channels regulate membrane potential as well as voltage gated calcium channels, their inhibition leads to an increase in intracellular calcium which leads to vasoconstriction as well as proliferation in the pulmonary artery smooth muscle cells (Dromparis et al., 2010).

Reduced activity of the Krebs cycle naturally leads to a reduction in the mediators produced by the Krebs cycle such as a-ketoglutarate (αKG). αKG is a co factor of prolyl hydroxylases which inhibit HIF1α (Sutendra & Michelakis, 2014). Reduction of αKG destabilizes prolyl hydroxylases and leads to increased HIF1α activity. HIF1α is a master transcription factor and enhances the downstream expression of angiogenic growth factors as well as PDK (Sutendra et al., 2013). Increased PDK in turn further inhibits PDC in a self-perpetuating positive feedback loop. Decreased mitochondrial reactive oxygen species also leads to increased intracellular calcium which upregulates nuclear factor of activated T-cells (NFAT) and directly promotes pulmonary artery smooth muscle cell proliferation in PAH (Sutendra & Michelakis, 2014).

Suppression of PDC results in glycolysis being upregulated to maintain ATP synthesis. Cytoplasmic ATP accumulates outside the mitochondrial membranes, creating an electrochemical gradient preventing intramitochondrial ATP from exiting the organelle (Paulin & Michelakis, 2014). This phenomenon prevents protons from re-entering the mitochondria from the cytosol, increasing the mitochondrial potential and closing the mitochondrial transition pore. Pro-apoptotic factors within the mitochondria are prevented from escaping; specifically, cytochrome C which activates the apoptosis-inducing caspase system in the cytoplasm (Schuler, Bossy-wetzel, Goldstein, Fitzgerald, & Green, 2000), and Apoptosis Inducing Factor which traverses to the nucleus leading to chromatin condensation, DNA fragmentation and caspase independent apoptosis (Candé et al., 2002). Collectively, the reduced egress of these mitochondrial pro-apoptotic factors leads to apoptotic resistance in pulmonary artery smooth muscle cells.

With hyperproliferation and apoptotic resistance, pulmonary artery smooth muscle cells in IPAH resemble cancer cells in phenotype, and indeed, there are now well recognized histologic and metabolic similarities between the two entities. Both display the Warburg effect, whereby glycolysis becomes the predominant

source of cellular energy in the context of suppressed oxidative phosphorylation. Cancer cells show an increased uptake of ^{18}F-labelled deoxy-glucose, a radiotracer with structural homology to glucose. Similarly, this is also observed in the pulmonary arteries of IPAH patients, indicating an increase in glucose uptake and a shift to a glycolytic predominant source of energy production (Paulin & Michelakis, 2014).

PAH Diagnosis

PAH can only be diagnosed definitively by right heart catheterization. Measurements used as diagnostic thresholds are a mean pulmonary artery pressure of ≥20mmHg, pulmonary vascular resistance of ≥3 Woods units, and a pulmonary artery occlusion pressure of ≤15mmHg (to exclude elevated left sided filling pressures as a cause of pulmonary hypertension), in a patient with clinical symptoms suggestive of PAH.

Due to the invasive nature of right heart catheterization, patients suspected of PAH often undergo initial assessment with echocardiogram. Right ventricular systolic pressure (RVSP) is used as a surrogate marker for systolic pulmonary artery pressures, and is generally correlated with systolic pulmonary artery pressures (and by extension potentially to mean pulmonary artery pressure). The RVSP is calculated by adding a measured tricuspid regurgitant jet velocity with an estimated right atrial pressure.

The 2009 Guidelines for the Diagnosis and Treatment of Pulmonary Hypertension suggest diagnostic risk stratification based on echocardiographic data (i.e. tricuspid regurgitant jet velocity, RVSP, and structural features suggestive of pulmonary hypertension) (Hoeper et al., 2009). It is important to note however that the thresholds for these echo features were somewhat arbitrary and based on only a few studies.

Furthermore, the estimation of systolic pulmonary artery pressure can be problematic as it can be confounded by a hyperdynamic circulation such as that seen in cirrhosis. Unlike true PAH, flow-induced pulmonary hypertension has a normal pulmonary vascular resistance index (Simonneau et al., 2019). Thus in these cases, a right heart catheterization is indicated to differentiate true PAH from that secondary to hyperdynamic circulation. In addition, the structural changes that are a result of raised pulmonary vascular resistance in PAH such as right ventricular hypertrophy, right atrial enlargement, and tricuspid regurgitation are absent in flow induced PAH. The advantage of echocardiogram therefore, is that it allows for the detection of these structural sequelae of primary PAH, which helps differentiate (at least late stage) PAH from elevated pulmonary artery pressures simply due to a high flow state. Moreover, the echocardiogram is useful in identifying underlying cardiac disease (e.g. valvulopathy, left ventricle

dysfunction, congenital heart disease) which would argue against PAH.

Perhaps the other major advantage of echocardiogram is the relative accessibility, as well as noninvasive nature of the test. Indeed, it is this practicality that has led to its widespread use as a surrogate marker in the diagnosis of pulmonary hypertension, such as that by Gladwin et al in their seminal paper in the New England Journal of Medicine on PAH in patients with sickle cell disease (Gladwin et al., 2004). Thus, despite its limitations, echocardiography is an important tool in the investigation of suspected PAH and is the reason for its use in our study.

PAH Therapy

Currently, the available therapies for PAH were developed with the model that vasoconstriction was the driving pathogenic mechanism. The major classes of medications include endothelin receptor antagonists, prostacyclin agonists, phosphodiesterase 5 inhibitors, and soluble guanylate cyclase inhibitors (Gurtu & Michelakis, 2015). Although their mechanisms of action vary, they all converge on vasodilating the pulmonary arteries. With recent advances in our understanding of how PAH may be better described by cell proliferation within the pulmonary arterial lumen leading to structural vascular remodeling, a completely different approach to treatment is being explored. As alluded to previously, dichloroacetate is a small molecule originally designed to treat lactic acidosis in patients with congenital mitochondrial disorders. It works by inhibiting PDK which subsequently results in disinhibition of PDC, and activation of mitochondria. Dichloroacetate has indeed been shown to reduce pulmonary vascular resistance and right ventricular hypertrophy in mouse models of IPAH, as well as more recently in human trials (Michelakis et al., 2017).

Primary Biliary Cholangitis

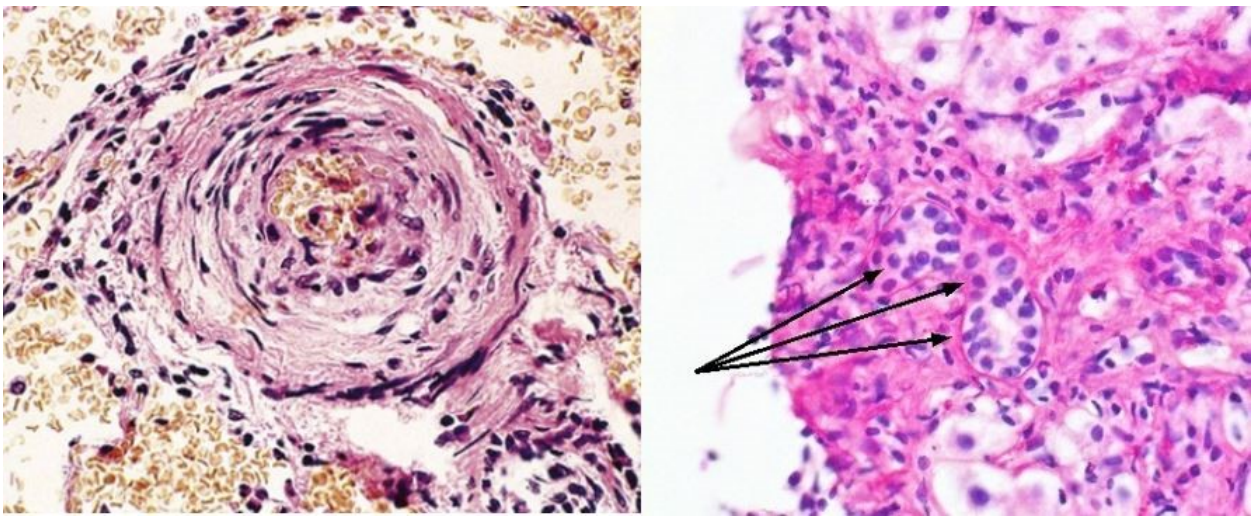
What is Primary Biliary Cholangitis?

Primary Biliary Cholangitis (PBC) is a chronic progressive cholestatic disease characterized by intrahepatic ductal obliteration and ultimate liver failure. Its prevalence is around 30-400/million (Assassi et al., 2009) (Rigamonti et al., 2011), and is often diagnosed incidentally in asymptomatic patients being worked up for other conditions (Rigamonti et al., 2011). PBC is thought to have components of autoimmunity and can co-exist with other autoimmune conditions in up to 84% of cases. Like many autoimmune diseases,

PBC has a significant preponderance towards females in a ratio of 9:1. (Marzorati, Lleo, Carbone, Eric, & Invernizzi, 2016)

PBC is histologically characterized by CD4+ and CD8+ T-cell infiltrates within the liver. In a manner intriguingly similar to the vascular cell proliferation in the pulmonary arteries in PAH, there is an increased proliferation of cells in the intrahepatic ducts in PBC (Figure 4).

Figure 4 *Left*: concentric pulmonary artery smooth muscle cell proliferation seen in pulmonary arterial hypertension. *Right*: Intraductal cell proliferation upstream of intrahepatic bile duct destruction seen in primary biliary cholangitis. Images adapted from *Robbins & Cotran Pathologic Basis of Disease*.



Pathogenesis of PBC

Although the exact etiological triggers and pathogenic process of PBC remains unknown, various possibilities can be postulated based on histologic observations in combination with our knowledge of how the immune system functions. Aside from ductal proliferation as discussed earlier, the other major characteristics include inflammation, ductopenia and necrosis, cholestasis, fibrosis and ultimately cirrhosis.

Initially, the inflammatory infiltrate include T helper and cytotoxic cells, natural killer cells which kill biliary epithelial cells (BEC) via activating CD40, Fas receptors, and Tumor Necrosis Factor (TNF) (Poupon, 2010). Infiltrated areas show hepatocyte shrinkage, edema, and apoptosis. Interface hepatitis is observed, where destruction occurs at the border between the liver parenchyma and the portal tracts or septa. Subsequently, a repair mechanism is initiated where fibroblasts among other cells are recruited to the area. The result is fibrosis which over time becomes cirrhosis.

Both ductal proliferation and ductal necrosis can be seen simultaneously, in different areas (Poupon, 2010). Both of these processes can then lead to cholestasis- in the former case, BEC proliferation obstructs the ductal lumen, while in the latter, the outflow tract for bile is lost to necrosis.

Other studies of PBC have revealed that the ductopenia is a result of cytotoxic T cells that specifically target and kill BECs (Poupon, 2010) that abnormally express a mitochondrial epitope on its cell surface (Tsuneyama, Coppel, & Gershwin, 1995). We know that B-cells are also involved, and produce antibodies directed against the E2 component of the PDC enzyme in mitochondria. Despite mitochondria being ubiquitous across all cell types, only BECs are targeted for killing.

These lines of observation raise a few interesting questions:

1) First, what triggers BECs to present a mitochondrial antigen on its cell surface?
2) What are the consequences of this?
3) Are AMA implicated in the pathogenesis or are they simply a marker of other underlying processes?
4) Why are BECs the only victims in PBC if mitochondrial elements are the supposed targets and are ubiquitous across all cell types?

A theoretical model that can provide answers to all these questions involves three major stages with positive feedback loops in between. These are:

1) The development of a mitochondrial phenotype.
2) Immune recognition and targeted killing of BECs.
3) BEC death and collective ductopenia.

Development of the Mitochondrial Phenotype

Liver sections of PBC patients have been found to exhibit a 'mitochondrial phenotype', where BEC abnormally express a mitochondrial protein on their cell surface (Tsuneyama et al., 1995). The identity of this protein has been found to bear striking similarity to the E2 component of PDC within mitochondria. Since PDC-E2 is normally hidden within cells, its aberrant appearance on the outside of cells could trigger immune recognition and destruction of the BEC that is presenting it. This raises the question of what might trigger the BEC's to present PDC-E2 on their apical surface in the first place. Here we discuss three possible mechanisms; viral infection, mistrafficking of PDC, and exposure to toxins or xenobiotics.

Intriguing studies over recent years by Mason et al., have shown evidence suggesting that PBC may be triggered by infectious agents- specifically, human beta retrovirus. The presence of this retrovirus has been identified in lymph node and liver specimens via electron microscopy, as well as specific RT-PCR studies in PBC patients (Mason, 2011). Antibody reactivity against components of human beta retrovirus has been recorded in PBC patients. A close relative to human beta retrovirus is the mouse mammary tumor virus, which can induce cholangitis in the Nodc3.c4. mouse model of PBC. In vitro experiments have shown that lymph node extracts from PBC patients can induce the mitochondrial phenotype of PBC. It is unclear how exactly this occurs, but one possibility is that exogenous viral particles incorporates or binds PDC-E2 as it inserts itself into the cell surface prior to budding (Wasilenko, Mason, & Mason, 2009). This would not be the first instance where viruses were suspected to induce translocation of intracellular antigens to the cell surface. Experiments even in 1989 showed that when epithelial cells in vitro were infected with adenovirus, the nuclear antigen 'La', was induced to translocate from its usual nuclear location to the cell membrane (Baboonian et al., 1989). The authors postulated this allowed circulating immune cells to detect the normally hidden antigen and form Anti-La antibodies which are characteristic of the autoimmune disease we know as Sjogren's Syndrome.

As our work aims to find unifying ground between PBC and PAH, it is interesting to note that the presence of viral material has also been linked to PAH. In 2003, a New England Journal of Medicine paper described an association between human herpes virus 8 (HHV-8; the etiological agent for the cutaneous growths of Kaposi's sarcoma) and primary PAH (Serls et al., 2003). The authors observed HHV-8 markers by using immunofluorescence to visualize Latency Associated Nuclear Antigen-1, as well as polymerase chain reaction to detect viral cyclin within the disorganized endothelial cell layers of pulmonary hypertension lesions. These viral markers were not detected in patients with secondary pulmonary hypertension nor in patients with HIV-1 infection (which is an important consideration as HIV is already an established as a cause of PAH). Mechanistically, Latency Associated Nuclear Antigen-1 inhibits p53 dependent apoptosis, while Viral cyclin inhibits the retinoblastoma gene, allowing for unchecked cell cycle progression (Serls et al., 2003). The logical result is constitutive proliferation of the infected cells which in this case were the endothelial cells of the pulmonary arteries. It is indeed interesting to note therefore, that there were histologic similarities between the lesions of Kaposi's sarcoma, and the plexiform structures of PAH.

Collectively, these studies of human beta retrovirus in PBC and HHV-8 in PAH raise the possibility that both disorders may also be linked by viral infection (potentially of the same or similar viruses).

The aberrant expression of PDC-E2 on the surface of BEC may also be related to intracellular mistrafficking of the protein. Experiments from the Michelakis lab have shown that contrary to longstanding belief, PDC can migrate out of the mitochondria and translocate into the nucleus where it plays a key role in cell cycle progression in mammalian cells (Sutendra et al., 2014). Inter-organelle translocation is always a complex process, especially in the case of PDC as it is a large enzyme with multiple components. Thus one could speculate that if PDC or its components are incorrectly trafficked to the nuclear membrane and end up instead near the cell membrane, they may become accessible for the immune system to detect and ultimately generate autoreactivity. The work by Sutendra et al also showed that nuclear translocation of PDC could be triggered by administration of rotenone, a known inhibitor of the electron transport chain. These findings suggest that mitochondrial suppression may be one trigger for PDC translocation and possible mistrafficking to the cell surface.

Aside from infectious agents and PDC mistrafficking, toxic exposures may also play a role in promoting the mitochondrial phenotype. PBC has been associated with a number of environmental exposures such as toxins in waste facilities, nail polish, and smoking (Shimoda, 2014). Results from a study by Tanaka et al in 2017 found that the initial trigger may be xenobiotics containing/resembling lipoic acid, or PDC-E2 that was modified with xenobiotic exposure (Tanaka et al., 2017). In this study, the authors generated monoclonal antibodies from plasmablasts (the immature form of a plasma cell) isolated from serum of PBC patients. They found monoclonal antibodies reacting exclusively to PDC-E2, as well as monoclonal antibodies with cross reactivity also to xenobiotics. Specifically, these xenobiotics were 2-octynoic acid, a substance commonly found in cosmetics and perfumes, and 6-8 bis acetylthio octanoic acid, an acetaminophen metabolite which is a modified form of lipoic acid. PDC-E2 is one of few human proteins that are naturally lipoylated, and both xenobiotic agents can alter the lipoyl domain of PDC-E2, forming a neoantigen. Genetic analysis revealed a higher frequency of somatic hypermutations in antibodies reactive only to PDC-E2 compared to antibodies cross reactive to modified PDC-E2. Reversion of the highly mutated monoclonal antibody genes to the germline sequence resulted in elimination of specific reactivity to PDC-E2 with preserved reactivity to the xenobiotics. The authors thus concluded that initial exposure to xenobiotics that mimic or modify the lipoyl domain of PDC-E2 primes naïve B cells which raise monoclonal antibodies targeting the neoantigen. Somatic hypermutation of immunoglobulin genes then allow monoclonal antibodies to expand its target to include the endogenous PDC-E2 molecule itself. Indeed, studies showing a higher affinity of AMA to xenobiotic modified PDC-E2 compared to the native PDC-E2 itself suggests that such exogenous molecules may have been the initial ‘molecular mimic’ (Zhang & Lleo, 2015).

It is unclear how exactly PDC modification by xenobiotics occurs. One possibility is that xenobiotics enter the enterohepatic circulation through the GI tract, and are taken up by the BEC. Once inside the BEC, they modify PDC-E2. Whether this occurs in the mitochondria or cytoplasm (during nucleus bound translocation) is unknown. The modified PDC-E2 may now be recognized as a foreign entity and translocated to the cell surface for presentation to immune cells. Alternatively, the xenobiotics might modify PDC-E2 only after its placement into the cell surface. In this case, some mechanism (such as PDC mistrafficking, or retroviral infection) may promote the mitochondrial phenotype. With the mitochondrial protein now on the cell surface exposed to the circulating xenobiotics, it can be modified. It turns out that the modified parts of PDC-E2 i.e. the lipoyl or lipoamide conjugates are intense immune adjuvants and may thus serve to augment the immune response to the mitochondrial phenotype (Water, Fregeau, Davis, Ansari, & Danner, 1988).

Immune Recognition and targeted death of Biliary Epithelial Cells

Due to its intracellular location, PDC-E2 is not normally visible to the circulation, and thus any unexpected exposure to the circulation may trigger recognition by immune cells. It is interesting that not only do BEC express PDC-E2 on their surface, but experiments have also shown increased expression of MHC class II molecules on the BEC surface(Pape, Hoffmann, Spengler, & Ller, 1988). This is unexpected as MHC II molecules are generally exclusive to professional antigen presenting cells, for which BEC's are not. This may further support the autoimmune nature of PBC, as non-professional antigen presenting cells have been known to aberrantly express MHC II on their surfaces in other autoimmune diseases; thyrocytes in Graves disease, epithelial cells in Sjogren's syndrome, as well as capillary endothelium and beta cells in type one diabetes (Bottazzo et al., 2010). Furthermore, MHC II expression on BEC precedes lymphocytic infiltration on histologic examinations over the course of disease, supporting the notion that MHC II on BEC surfaces promotes immune destruction of bile ducts (Pape et al., 1988).

It is unclear whether these MHC II molecules are in fact presenting PDC-E2. If so, a predictable immune response can occur whereby CD4+ T helper cells react with these BEC's, and in turn activate downstream components of the adaptive immune system such as cytotoxic T cells, natural killer cells, and B cells. Cytotoxic CD8+ T-cells will identify the antigen (PDC-E2) and the cell presenting it (BEC in this case) and destroy the BEC. As discussed earlier, this is indeed observed in the histology of PBC slides where T-cell infiltrates surround dying BEC's.

This mechanism offers an explanation for why the damage is limited to BEC in PBC, even though the mitochondrial protein (i.e. PDC) exists in all cells. The specific mitochondrial phenotype stimulates a specific response in the adaptive immune system that only destroys cells presenting this cryptic antigen.

In addition to cytotoxic T-cells, T-helper cells also activate B cells. B cells also detect the culprit antigen (in this case PDC-E2), and form anti-mitochondrial antibodies (AMA). AMA are quite specific and are present in 90-95% of cases of PBC (Norman et al., 2009). The appearance of serum AMA helps make the diagnosis, and sometimes precedes the diagnosis in some cases for up to three years (Lleo et al., 2009). One of the key questions in PBC has been whether AMA contributes to pathogenesis, or whether it is just a bystander product of the underlying disease process.

Studies have shown that AMA may trigger caspase induced apoptosis in cholangiocytes, although these experiments have been carried out in vitro (Poupon, 2010). Since mitochondrial suppression is a cornerstone of PAH pathogenesis and may be an early event leading to PDC mistrafficking as explored above, it would be important to know whether AMA has any inhibitory effects on PDC, in PBC patients. Interestingly, Van de Water et al in 1988 showed that PBC sera (containing AMA) not only recognized PDC-E2, but were able to inhibit the enzyme activity of PDC solution in vitro (Water et al., 1988). Furthermore, some studies have noted the ability of IgA antibodies (produced by plasmablasts) which are directed against PDC-E2 to be transcytosed from the basolateral to apical membranes of biliary epithelial cells (Gershwin, Ansari, Mackay, Nakanuma, & Nishio, 2000). During their intracellular transit, these IgA antibodies colocalized with PDC-E2 autoantigen in the cytoplasm (likely just following their synthesis and prior to shuttling into the mitochondria). It was unclear whether PDC and more generally mitochondrial function was affected. It is certainly plausible however, that IgA antibodies may interact with PDC-E2 and disrupt metabolic function of mitochondria specifically in biliary epithelial cells.

Despite this, other observations suggest that AMA's do not contribute to the pathogenesis of PBC. Patients who are AMA negative by high sensitivity assays experience an identical disease course to AMA positive patients from clinical, serological, and histologic perspectives (Shimoda, Miyakawa, Nakamura, & Ishibashi, 2008). Shimoda et al in 2008 were able to show that the most likely reason for the lack of AMA in some PBC patients are secondary to a defect in B-cell responsiveness as opposed to a defect in T-helper cells. This further supports the theory that the damage done to bile ducts in PBC is T-cell mediated and that AMA generation by B-cells are simply a bystander phenomenon. Studies have also been conducted where different species of mammals were immunized with purified PDC-E2. The test animals developed

AMA's as predicted, but none of them developed PBC from serological and histologic examination over an 8 month period (Krams et al., 1988).

Biliary Epithelial Cell Death and Collective Ductopenia

Another factor contributing to BEC injury may be deficient bicarbonate secretion into bile ducts (Molinaro & Marschall, 2017). In healthy individuals, cholangiocytes harbor a HCO_3^-/Cl^- anion exchanger which secretes bicarbonate into the bile ducts. In PBC patients, there are increased levels of miRNA506 in BEC which disables anion exchanger mRNA with subsequent reduced bicarbonate secretion. Toxic bile salts directly contact BEC and stimulate calcium release from the endoplasmic reticulum. This in combination with increased intracellular bicarbonate induces BEC apoptosis. Interestingly, mice lacking anion exchanger also develop histological features of PBC as well as AMA positivity. This injured 'bicarbonate umbrella' may be one factor which may explain why immunosuppression in PBC is ineffective. Once apoptosis is induced or BEC death exposes intact PDC-E2, autoimmunity is generated against PDC-E2. Even if immune cell infiltration is dampened by immunosuppression, toxic bile salts continue their onslaught on the ductal epithelium.

Once apoptosis has been induced, (regardless of whether it was through T-cells or toxic bile salts), dysregulated cell death may further stimulate immune cells to destroy BEC in a positive feedback loop. Normally when cells undergo apoptosis, its intracellular constituents undergo glutathiolation, cleavage into its sub components and packaging into apoptotic blebs before being discharged outside the cell (Shimoda, 2014). This process is critical to escape detection by the immune system, which would otherwise lead to recognition of intact intracellular particles which could trigger immune destruction of self-antigens (Lleo et al., 2009). Dysregulated apoptosis and inability to remove cellular remains has been demonstrated in autoimmune diseases such as systemic lupus. In PBC, it was shown that PDC-E2 in apoptotic blebs of BEC remained intact, while in other cell lines it was appropriately glutathiolated and cleaved (Lleo et al., 2009). In line with this theory, other studies in PBC have demonstrated overexpression of Bcl-2 (which inhibits glutathiolation) within apoptotic BECs (Odin, Huebert, Casciola-rosen, Larusso, & Rosen, 2001). Surprisingly, AMA reacted with PDC-E2 without artificial permeabilization of cell walls in apoptotic BEC (Lleo et al., 2009), supporting the idea that antibodies were able to detect and react with an antigen inside an intact apoptotic bleb. It is not clear however, whether immune cells themselves could detect intact PDC-E2 within apoptotic blebs. If so, this may perpetuate the immune recognition and targeting of PDC-E2 as well as the BEC's expressing this antigen on its surface.

Finally, genetics are likely to play a considerable role in the pathogenesis of PBC, as suggested by familial studies showing a significantly increased concordance in monozygotic twins (Zhang & Lleo, 2015). AMA positivity is much higher at 13% between first degree relatives, compared to prevalence in the general population (being less than 1%). HLA alleles have been studied extensively in many autoimmune diseases including PBC. HLA variants such as DRB1*08 have been found to confer risk, while others such as DRB1*11 and DRB1*13 were protective against developing PBC (Joshita, Umemura, Tanaka, & Ota, 2017). Epigenetic mechanisms such as DNA methylation, histone post translational modification, and like IPAH-dysregulation of X-chromosome inactivation have also been associated with PBC (Marzorati et al., 2016). It is possible that these genetic determinants may be involved in any of the above pathogenic processes in the evolution of PBC; from the development of the mitochondrial phenotype, to enhanced recognition of PDC-E2 by immune cells, to dysregulated apoptosis and immune mediated destruction of BEC.

Treatment of PBC

The mainstay of treatment for PBC is ursodeoxycholic acid, a remedy that dates back to textbooks of traditional Chinese medicine. It is currently the only medication that has been shown to alter the natural progression of disease, delaying liver transplant and reducing mortality (Andreani et al., 2008). Mechanistically, ursodeoxycholic acid is a secondary bile acid that dissolves intestinal cholesterol but has also been described to have immunomodulatory and anti-apoptotic effects on BECs through stabilization of the mitochondrial membrane (Ward et al., 2017)(Solá, Aranha, & Clifford, 2002). Interestingly, it has also been shown to decrease titres of AMA (Poupon, 2010). Unfortunately however, up to 40% of PBC patients do not respond to ursodeoxycholic acid with worsening biochemical, histologic, and clinical status ultimately requiring liver transplant (Andreani et al., 2008).

Patients who inadequately respond to ursodeoxycholic acid can be managed with a newer agent; obeticholic acid, which was first approved by the Food and Drug Administration in 2016 (Lindor, Bowlus, Boyer, Levy, & Mayo, 2019). Obeticholic acid is an agonist of the farnesoid X receptor and modulates bile acid synthesis, secretion, absorption, metabolism and transport. It has shown benefit in PBC patients in combination with ursodeoxycholic acid, or as monotherapy (Kowdley et al., 2018).

Beyond bile acid modulators, various other therapies have been trialled without reproducible success, including colchicine, methotrexate, steroids, mycophenolate mofetil, and anti-retroviral medications (Molinaro & Marschall, 2017).

It is unclear why immunosuppression is not effective in PBC. PBC is likely an entity that joins a small list of other autoimmune conditions like Sjogren's, Hashimoto's, and type one diabetes, that do not respond to immunosuppression despite their immune mediated pathogenesis.

Development of Pulmonary Hypertension in PBC

Portal hypertension seen in cirrhotic patients is a risk factor for developing pulmonary hypertension, with 2% of patients with portal hypertension having PAH (Liberal et al., 2015). Conversely, 5-10% of patients with PAH also have portal hypertension (Liberal et al., 2015). Individuals who develop PAH in the setting of portal hypertension are diagnosed with portopulmonary hypertension, an entity first described by Mantz et al in 1951. Presently, the prognosis of portopulmonary hypertension is grim without liver transplant, with a 5-year survival of under 30%. The predominant theory that remains to be proven, involves the shunting of vasoactive substances through a malfunctioning cirrhotic liver into the right heart and pulmonary vasculature where it leads to pulmonary vasoconstriction and local hypertension (Raevens et al., 2015). Metabolites that have been implicated in this process include ET-1A, thromboxane A2, IL-1, IL-6, angiotensin-1. Despite its biological plausibility, this theory is unlikely to completely explain the development of pulmonary hypertension in the setting of PBC as there are PBC patients without portal hypertension who develop pulmonary hypertension (Yoshida et al., 1994) (Shen, Zhang, & Zhang, 2009).

Patients with cirrhosis but without portal hypertension are also prone to developing elevated pulmonary artery pressures due to a hyperdynamic circulation (Liberal et al., 2015). Here, cirrhosis induced splanchnic vasodilation, volume overload and subsequent increased left ventricular filling pressures induce a mild increase in mean pulmonary artery pressure. Unlike portopulmonary hypertension or IPAH, the mildly elevated pulmonary artery pressures from a hyperdynamic circulation do not portend to worse mortality. Importantly, elevated pulmonary artery pressures due to a hyperdynamic circulation can be differentiated from portopulmonary hypertension or IPAH by measuring the pulmonary vascular resistance which is normal in hyperdynamic circulation but raised in portopulmonary hypertension and IPAH (Kuo et al., 1997).

Is there a possible association between Primary PAH and PBC?

Given the observation of mitochondrial involvement in both disorders, it is not surprising that there have been previous studies examining the co-existence of PBC and pulmonary hypertension (Shen et al., 2009) as well as a case report of PBC patients without portal hypertension developing what appears to be PAH

(Yoshida et al., 1994). However, Shen et al reported the association of pulmonary hypertension in general with PBC, and not PAH specifically, and also did not provide a potential mechanistic association between the two rare diseases.

PAH and PBC are both rare disorders with prevalence's of 15-50 per million, and 30 per million respectively (Sutendra & Michelakis, 2013) (Assassi et al., 2009). They share unusual features of mitochondrial abnormalities where PDC function of mitochondria are downregulated in IPAH; and components of PDC are aberrantly expressed on the cell surface in PBC. This study thus aims to address the provocative question of whether these two seemingly unrelated rare diseases may in fact be associated. We hope that the results obtained from our study can help generate new hypotheses for future investigations on pathogenesis and potential therapies for these serious diseases.

Our study was a retrospective review of patient records in both the PAH as well as PBC clinic databases. Cases of pulmonary hypertension were identified in PBC patients, and the potential causes of PAH were explored. Some PBC patients receive an echocardiogram at some point, particularly during the work up for transplantation. After exclusion of secondary causes, patients with PAH were identified and the prevalence of PAH within PBC patients was explored. In our study, we used a control group comprised of patients with primary sclerosing cholangitis (PSC) as a comparator to our PBC cohort.

Primary Sclerosing Cholangitis

A PSC comparator was used as this condition is also believed to be an autoimmune disease that results in cholestasis and progressive liver failure, but there is no association known between PSC and mitochondrial abnormalities. Genetic predispositions and environmental triggers are also important in PSC although the specific culprits are not proven (Konstantinos N. Lazaridis, 2016). Like PBC, it is initially asymptomatic, and suspicion is raised when alkaline phosphatase is elevated on bloodwork usually done for other purposes. PSC is then diagnosed by imaging which shows classic extrahepatic biliary structuring. Histologically, PSC is characterized by intra as well as extrahepatic biliary inflammation, fibrosis and destruction. Unlike PBC however, there is no treatment; ursodeoxycholic acid given in higher doses in PSC trials resulted in higher rates of cirrhosis and its complications, liver transplantation, and mortality. However, both PBC and PSC could potentially lead to portal hypertension and potential portopulmonary hypertension, or potential cirrhosis-driven increase in cardiac output and flow-induced pulmonary

hypertension. Our study hypothesized that there would be an association between PBC and PAH, but not PSC and PAH.

In contrast to the usual process in translational medicine whereby molecular work at the bench inspires the exploration of new clinical and therapeutic avenues, our study takes the reverse approach. Observations by the bedside lead us to explore molecular mechanisms that may be driving clinical manifestations and the association between two very rare disorders. The findings of this small proof-of-principle study provided a strong argument that these two rare diseases (PBC and PAH) were more likely to be clinically associated as opposed to co-existing by pure chance. We discuss how future molecular experiments may help decipher the mechanisms behind the development of such an overlap syndrome between PAH and PBC. Finally, we conclude by suggesting how new therapies being developed for PAH which target the mitochondria, may be a tantalizing new drug target for patients with PBC.

METHODS

We examined the University of Alberta database of PBC patients many of whom were being referred to transplant, with the aim to ultimately identify patients with PBC and coexisting PAH. Ethics approval was attained to review charts from the liver clinic (which included patients with PBC and PSC) as well as the PAH clinic at the University of Alberta. Our study was based on a retrospective review of clinical data collected at the University of Alberta Hospital between 2009 and 2018. Echocardiograms were assessed and PBC patients with RVSP of over 30 were shortlisted for further analysis. The cutoff of RVSP of >30mmHg was used based to the fact that has been used is similar well-received clinical studies, like the study providing an association between sickle cell anemia and PAH, published in the New England Journal of Medicine (Gladwin et al., 2004).

These echocardiograms were individually read by a cardiologist with expertise in pulmonary arterial hypertension to identify studies with hyperdynamic circulation due to liver disease, as well as intrinsic cardiac disease such as congestive heart failure (due to systolic or diastolic dysfunction) or valvular disease. Patients with such features on their echocardiogram were excluded.

Subsequently, discharge summaries, pulmonary function tests, and chest CT scans were reviewed to identify significant obstructive small airways disease, thromboembolic lung disease, restrictive interstitial lung disease, or significant obstructive sleep apnea. Discharge transcriptions, immunology serologies, and consult letters on the provincial electronic health record were reviewed to identify patients with connective tissue diseases. Patients with these conditions were excluded from further analysis as these are all established causes of secondary (i.e. associated) PAH. Following this, we reviewed the charts of remaining patients and identified evidence suggestive of portal hypertension. As hepatic vein pressure gradient studies are invasive and not routinely available, surrogate markers for portal hypertension were used. Such standard markers included a combination of platelets < 150 x 10^9/L, transient elastography of >20kPa, the presence of varices on gastroscopy, reversed flow on abdominal ultrasound, and splenomegaly. Portal hypertension was ruled in if there were varices, splenomegaly, or a combination of any 2 or more of the above factors if gastroscopy results were unavailable. If gastroscopy was unavailable or negative for varices and splenomegaly, then portal hypertension was ruled out by the absence of all the other factors (e.g. platelet count and transient elastography). Platelet count was considered here since PSC patients with platelets below 150k/mm^3, and PBC patients with platelets below 140k/mm^3 were associated with varices reflective of clinically significant portal hypertension (Levy et al., 2007). If available, transient elastography was also used in combination with platelet count as platelets of over 150 x 10^9/L and transient elastography of < 20kPa have been shown to be reliable (albeit not perfect) in excluding clinically significant portal hypertension in cirrhotic patients (Franchis & Vi, 2015).

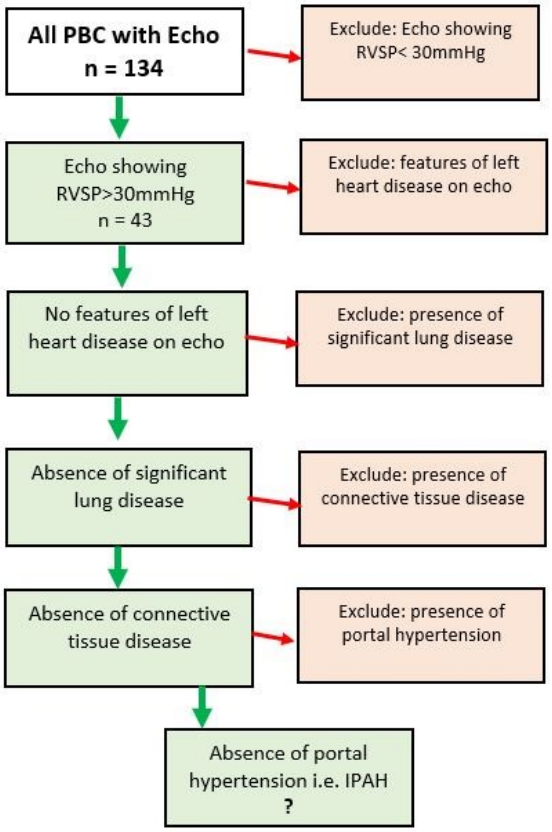


Figure 5: Flow diagram of how PBC patients with echo data in our cohort were analyzed to find the proportion of patients with concurrent IPAH.

The number and proportion of PBC patients with pulmonary arterial hypertension with and without portal hypertension were then derived as shown in Figure 5.

In the same manner as for PBC, echocardiograms of PSC patients were reviewed and those with RVSP > 30 selected. Of these patients, exclusions were made on the basis of hyperdynamic circulation secondary to cirrhosis, left heart disease, significant pulmonary disease, or connective tissue disorders. As above, we underwent an extensive chart review of the remaining patients to determine the presence of portal hypertension. The number and proportion of PSC patients with pulmonary arterial hypertension, with and without portal hypertension were then calculated to find the prevalence of PSC patients with IPAH.

In the process of building our dataset, other relevant clinical parameters were collected including liver enzymes, liver function, renal function, electrolytes, and blood counts. The Model for End stage Liver Disease – sodium (MELD-Na) scores include composite measures that assess severity of liver disease based on a patient's creatinine, INR, bilirubin, and sodium (Kim et al., 2008). These scores were calculated for subgroups of PBC and PSC patients to ensure similarity of baseline liver disease severity.
Specifically, the MELD score was calculated with the use of a standard formula, which adds multiples of the natural logarithm (ln) of the values for the INR, creatinine, and bilirubin as follows: $11.2 \times \ln(\text{INR}) + 9.57 \times \ln(\text{creatinine, in milligrams per deciliter}) + 3.78 \times \ln(\text{bilirubin, in milligrams per deciliter}) + 6.43$ (an intercept), with a lower limit of 1 for all variables and with creatinine capped at 4; creatinine was set at 4 if the patient was receiving renal dialysis.

After compiling our data, we aimed to answer three questions:

1) is PAH (in the absence of portal hypertension) more prevalent in PBC compared to the general population?
2) is PAH more prevalent in PBC compared to PSC?
3) is portopulmonary hypertension more prevalent in PBC as compared to PSC?

Although these three questions are slightly different, they ultimately converge on elucidating whether patients with PBC have a higher risk of pulmonary vasculature remodelling leading to PAH, compared to PSC patients and the healthy population.

Statistical Analysis

Comparison of proportions were performed by 2 sample Z-test for proportions. Categorical data was analyzed with Chi-squared test, and numeric data was analyzed with 2-tailed T-Test. A p-value of 0.05 was used to indicate statistical significance.

RESULTS

A total of 211 patients with echocardiographic data were included where 134 had PBC and 77 had PSC. As expected, the major difference between these two groups were sex. Otherwise, the other characteristics including the MELD-Na scores between the two groups were similar, indicating that the PSC group was an appropriate control for the PBC study group (Table 1).

	PBC	**PSC**	**P-value**
n	134	77	
Male	17	47	<0.05
Female	117	30	<0.05
Age	60±0.93	47.8±1.81	<0.05
ALT	55.6±9.31	72.3±8.27	0.23
AST	61.3±7.45	85.5±14.23	0.1
ALP	257±24.96	292.6±26.14	0.37
Bilirubin	39.6±6.30	67.8±14.87	<0.05
Albumin	35.9±0.62	34.7±0.86	0.23
INR	1.5±0.29	1.2±0.03	0.56
Hemoglobin	120±1.78	120.1±2.63	0.96
Platelets	203±9.97	178.3±11.4	0.12
Creatinine	94.4±8.7	86.4±5.3	0.52
Sodium	137.9±0.34	137.7±0.41	0.71
MELD-Na[1]	16	15	0.07

Table 1: Baseline characteristics of PBC and PSC patients with echocardiogram data. Numbers following '±' sign indicate standard error of the mean. [1] *MELD-Na*: Model for End stage Liver Disease – Sodium score.

Of 134 PBC patients with recorded echocardiograms, 43 had an RVSP>30 (Table 2). Of these 43 patients, 21 showed signs of hyperdynamic circulation, valvular disease, or left sided heart disease on echocardiogram and were excluded from further analysis. The remaining 22 lacked evidence of the above

conditions and had additional signs of PAH on echocardiogram such as RV hypertrophy/ dilation, or right atrial enlargement.

Of these 22 patients, 3 had evidence of significant pulmonary disease on chart review and were hence excluded from further analysis. Of the remaining 19 patients, 3 had connective tissue diseases (2 Sjogren's syndrome and 1 systemic sclerosis) on chart review and were hence excluded from further analysis. Of the remaining 16 patients, 11 had evidence suggesting portal hypertension for which the PAH may be attributed to. The remaining 5 patients with PBC therefore had PAH that was not associated with any secondary cause. In other words, we found 5 patients with IPAH in our PBC cohort and thus the **prevalence of IPAH in our PBC cohort was 5/134, equivalent to 3.73%** as illustrated in Figure 6.

Of the 77 PSC patients with recorded echocardiograms, 18 had RVSP>30. Unlike the PBC patients however, none had secondary features expected in true PAH such as RV hypertrophy/dilation or right atrial enlargement. In all these cases, the raised RVSP were either due to a hyperdynamic circulation, LV dysfunction (systolic or diastolic), or valvular disease. The absence of secondary features listed above, and the presence of left heart disease/hyperdynamic circulation on echocardiogram argues against a diagnosis of PAH (which by extension also excludes IPAH).

As there were no cases of IPAH in the PSC cohort, IPAH was thus more prevalent in our PBC (3.73%) compared to PSC (0%) patients. This however, did not reach statistical significance using a two sided Chi-squared test, with $p = 0.086$.

	PBC	PSC	p-value
n	43	18	
Male	7	13	<0.05
Female	36	5	<0.05
Age	64±1.67	52±4	<0.05
ALT	54±17	86±23	<0.05
AST	55±6.5	125±48	<0.05
ALP	257±37.8	328±49	0.29
Bilirubin	49±10	70±26	0.41
Albumin	35±1.1	32±1.1	0.16
INR	2.1±0.9	1.3±0.1	0.46
Hemoglobin	119±3.4	113±5.9	0.34
Platelets	142±13	117±20	0.21
Creatinine	91±7.4	88±12	0.75
Sodium	138±0.7	138±1	0.73
MELD-Na	14.6±1.26	15.5±1.34	0.38
RVSP	45.1±1.96	37.7±1.54	<0.05
Left Heart disease	18	13	n/a
Significant Lung disease[1]	11	0	n/a
Connective Tissue disease[2]	5	1	n/a
Portal Hypertension	26	16	n/a
HIV[3]	0	0	n/a
Primary PAH	5	0	n/a

Table 2: Subpopulation of PBC and PSC patients with RVSP > 30mmHg. Numbers following '±' sign indicate standard error of the mean.

[1] Includes diseases causing chronic hypoxia and thromboembolic disease.

[2] These 5 cases included Sjogren's syndrome and systemic sclerosis.

[3] HIV status was assessed and included here as HIV is an associated cause of PAH.

The prevalence of portal hypertension in PBC patients with RVSP>30 was 26/43 (60.4%). The prevalence of portal hypertension in PSC pts with RVSP>30 was 16/18 (89%) (**Table 3**).

	PBC	PSC
Patients with RVSP>30 without hyperdynamic circulation, valvular disease, or left sided heart disease on echocardiogram	22	0
Significant Lung disease	3	0
Connective Tissue disease	3	0
Portal Hypertension	11	0
HIV	0	0
IPAH	**5**	0

Table 3: Subpopulation of PBC and PSC patients with RVSP > 30mmHg ***and*** secondary signs of established PAH on echocardiogram (RV hypertrophy, RA dilation, tricuspid regurgitation). Note: in PBC, the total number of patients with RVSP>30 (without hyperdynamic circulation, valvular disease, or left sided heart disease on echocardiogram) was 22. These 22 patients were broken down into the subgroups of the rows below. There were no PSC patients that had RVSP > 30mmHg ***and*** secondary signs of PAH on echocardiogram (RV hypertrophy, RA dilation, tricuspid regurgitation) since no PSC patients had secondary signs of PAH on echocardiogram.

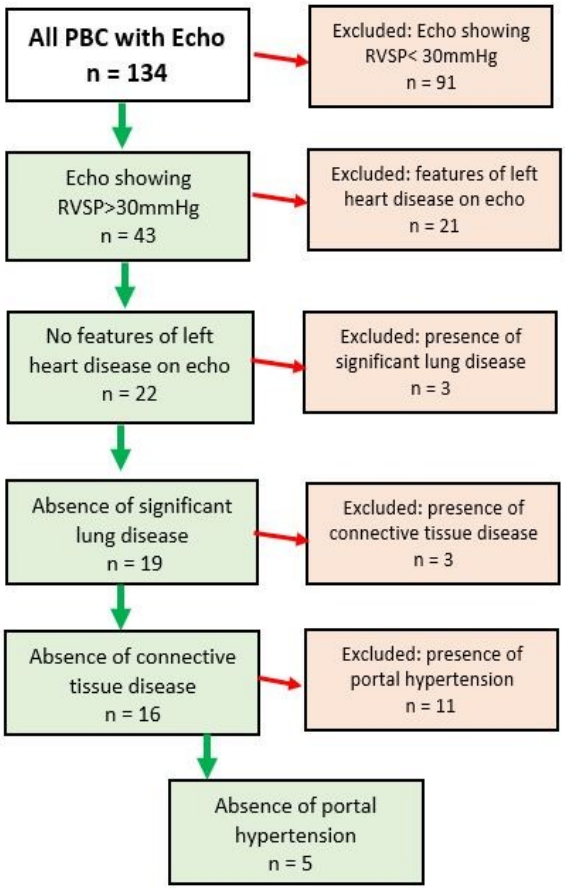


Figure 6: Of 143 PBC patients with echocardiographic data, 5 were found to have PAH without any secondary cause i.e. IPAH.

If the PAH was driven by portal hypertension (i.e. portopulmonary hypertension) in these patients, we would expect the prevalence of PAH to be greater in PSC. However, there were no cases of PAH in the PSC group despite PSC patients having more portal hypertension. In the PBC group, there were 11 patients with PAH and portal hypertension. Although it is possible that all 11 patients had portopulmonary hypertension, this would be unlikely as PAH is known to be a *rare* complication of portal hypertension with a prevalence of only 2% in some studies (Liberal et al., 2015). Furthermore, there were no cases of PAH in the PSC patients (which had higher rates of portal hypertension) which further argues against portal hypertension being a strong trigger for PAH.

A range of estimates for the incidence and prevalence of portopulmonary hypertension have been quoted depending on the study population. For example, 0.73% of cirrhotic patients had

evidence of pulmonary hypertension in a study of 17,901 autopsied patients above the age of 1 (Liberal et al., 2015). In a prospective study of 500 inpatients with portal hypertension but without known PAH, 2% were found to have PAH after right heart catheterization was performed on all 500 individuals. In contrast, portopulmonary hypertension was found in 16% of patients with refractory ascites (Liberal et al 2015).

In our study, with the population being 134 PBC patients with available echo data, PAH with portal hypertension was present in 11 patients (8.2%). This is comparable to the proportion of portopulmonary hypertension found in hospitalized cirrhotic patients, at 10% (Chen, Xing, Xu, & Yang, 2013). The prevalence of portal hypertension within PBC patients with PAH was 11/22 (50%) in our study (Table 3). HIV which in rare cases can cause PAH, was negative for the five patients with IPAH and PBC.

DISCUSSION

Our study was conceived after observing a few cases of PBC in the pulmonary hypertension clinic at the University of Alberta. We hence set forth to explore the idea of whether there could be an association between these two extremely rare diseases- IPAH and PBC, considering the potential molecular similarities between these two rare diseases.

Previous groups had indeed identified an association between pulmonary hypertension and PBC. In 2009, Shen et al examined the co-occurrence of pulmonary hypertension and PBC in a cohort of 178 PBC patients. Pulmonary hypertension was diagnosed by echocardiography, where they found 11.8% of PBC patients also had pulmonary hypertension (Shen et al., 2009). They found no differences in age, gender, and disease duration between PAH-PBC overlap patients and those with PBC alone. The incidence of interstitial lung disease and Raynaud's phenomenon were both increased in the PAH-PBC overlap group compared to PBC alone. Importantly, Shen et al did not attempt to characterize PAH by excluding patients with collagen vascular disease, portopulmonary hypertension or diseases of the left ventricle, like we did in our case.

For example, it is well known that PBC, PAH, interstitial lung disease and Raynaud's phenomenon are manifestations of connective tissue diseases such as systemic sclerosis. Shen et al did not assess or report the prevalence of systemic sclerosis within the overlap group, so it remains unclear whether parenchymal

lung disease and Raynaud's reflected possible underlying systemic sclerosis, or whether they represent a true overlap syndrome (distinct from systemic sclerosis).

In addition, portal hypertension was higher in the overlap group (66.7%) compared to PBC alone (42%) which could account for more PAH in the overlap group via the mechanism of portopulmonary hypertension. All such aforementioned causes of secondary pulmonary hypertension were not addressed and hence the prevalence of true IPAH was not clearly delineated in their population.

Our study takes the idea of a PBC-PAH overlap disorder a step further to not only identify PBC patients with pulmonary hypertension, but IPAH specifically. To address this question, we asked whether IPAH was more likely to occur in PBC compared to the general population. If IPAH and PBC were indeed associated, we asked further whether this could be attributed to cholestasis or cirrhosis in general, and thus used a control group comprised of patients with PSC which is a cholestatic disease. Finally, we compared the rates of portopulmonary hypertension between PBC and PSC.

Given that the prevalence of IPAH in our PBC cohort was 5/134 (3.73%), in contrast to the prevalence of IPAH in the general population which is roughly 0.005% (Sutendra & Michelakis, 2014), this *seven hundred fold* difference makes it extremely unlikely that this association occurred by chance. (Statistical analysis with 2-tailed z-statistic for 2 population proportions yielded $p<0.00001$). Note that this is a conservative estimate, and assumes that for the 11 PBC patients with PAH and portal hypertension, their PAH was due to their portal hypertension (i.e. portopulmonary hypertension). Since it is impossible to definitively attribute PAH to portal hypertension, it is possible that for these 11 patients, the PAH was unrelated to their portal hypertension. In such a scenario, we would have a maximum prevalence of IPAH in our PBC cohort as 16/134 (11.9%). Thus we believe that at least in our cohort, a true clinical association between IPAH and PBC exists.

Was this association of IPAH specific to PBC? Or could it occur in other causes of cirrhosis? PSC was chosen as a control group given its similarities to PBC in biochemistry, histology, and autoimmune pathogenesis. Our results showed that there was in fact no association at all between IPAH and PSC as all PSC patients with RVSP greater than 30 also had hyperdynamic circulation and/or signs of secondary PAH such as left heart disease. Thus it appears that the association between IPAH and PBC is specific, and did not occur simply due to cirrhosis, or even autoimmune cholestatic cirrhosis.

Portopulmonary hypertension is histologically identical to IPAH and belongs in the same category as IPAH in the World Health Organization classification of pulmonary hypertension. It occurs as a rare complication of cirrhosis, with a pathogenesis that remains unknown. A multicenter case control study identified certain factors associated with higher risk of portopulmonary hypertension, such as female sex and autoimmune hepatitis (Kawut et al., 2008). In contrast, hepatitis C was inversely associated with the risk of portopulmonary hypertension. Several factors logically suspected to affect risk of portopulmonary hypertension were not found to be significantly associated, including severity of liver disease by MELD score, alcohol use, race, and age. PSC was negatively associated with portopulmonary hypertension (OR 0.44) but was statistically insignificant (p=0.45). Interestingly, PBC tended to increase risk for portopulmonary hypertension with an odds ratio of 3.63 but also failed to reach statistical significance.

In our study, the prevalence of portopulmonary hypertension in PBC was 11 out of 134 patients (8.2%) compared to 0 out of 77 in the PSC control group, although this finding was statistically non significant. A logical additional question would be whether portopulmonary hypertension is more likely in PBC compared to other cirrhotic diseases. There are different prevalence's of portopulmonary hypertension reported in the literature depending on the population and diagnostic criteria. According to one study of 100 hospitalized patients with cirrhosis, the prevalence of portopulmonary hypertension was 10% (Chen et al., 2013). In this study, the majority of cirrhosis was secondary to hepatitis virus B or C, and all patients were Chinese. Given these limitations in generalizability, our prevalence for portopulmonary hypertension in PBC patients of 8.2% was similar.

It is important to note however, that the challenge with diagnosing portopulmonary hypertension is that beyond requiring both PAH and portal hypertension, it is largely one of exclusion. It is currently impossible to definitively attribute a patients' PAH to their portal hypertension as numerous studies have found no correlation between the PAH to the presence, or severity of their portal hypertension (Liberal et al., 2015). Some studies have found that the two conditions can be differentiated on right heart catheterization as IPAH exhibited significantly higher pulmonary and systemic vascular resistance, but unfortunately we do not have data from right heart catheterization in our patients (Kuo et al., 1997).

The association observed is nevertheless unexpected given the rarity of these two diseases. It raises the question of whether PBC or IPAH develop in parallel as a potential overlap syndrome potentially based on a global primary mitochondrial abnormality or driven by genetic or immunologic factors.

As with all other complex diseases, a 'multi-hit model' of genetic predisposition with an environmental trigger may be a plausible explanation. In light of recent observations that IPAH patients harboring loss-of-function single nucleotide polymorphisms in SIRT3 or UCP2 lead to mitochondrial suppression, it is possible that asymptomatic patients with these genetic alterations may carry a higher baseline risk of developing IPAH and/or PBC. In such a patient, any additional 'hit' to the mitochondria may precipitate IPAH and PBC at a lower threshold compared to the general population. For example, this individual may be exposed to an environmental trigger such as xenobiotics. Xenobiotics prime B and T cells to develop antibodies against the E2 component of PDC. BECs are selectively injured due to their intrinsically reduced glutathiolation of PDC-E2 within apoptotic blebs, allowing them to be continually recognized as foreign material by the hepatic immune surveillance system and targeted for destruction. An alternative trigger may be infection with viruses, leading to the pro-proliferative and anti-apoptotic state of pulmonary vessels or biliary epithelial cells.

Importantly, this contrasts to our PSC controls who do not have evidence of mitochondrial involvement and were found not to have an elevated risk of PAH compared to the general population beyond that due to portopulmonary hypertension.

Limitations of the Study

Only patients with echocardiograms were assessed for possible PAH which may represent a selection bias as there may have already been a suspicion of PAH in these patients. Despite this, there are of course a vast number of other reasons an echocardiogram may have been performed, such as suspicion of concurrent heart failure, or a new murmur picked up on clinical exam. This selection bias is unlikely to influence our comparisons with the PSC control group as these patients were also included only if they had echocardiograms done. Furthermore, all PBC patients would eventually receive an echocardiogram as part of their transplant workup, so it is impossible to delineate how many of these patients received imaging as routine part of transplant workup versus due to a clinical suspicion for PAH. A future study may circumvent this issue by performing echocardiograms on all PBC and PSC patients prospectively to evaluate the true prevalence of PAH in these populations. Nevertheless, one can speculate that if we had

echocardiograms in all patients with PBC, we would only detect even more PAH, not less. Certainly a 700-fold increase in the chance of observing PAH in PBC patients (compared to the normal population) is hard to dismiss as “random”.

PAH can only be diagnosed definitively by right heart catheterization. However, the invasive nature of this diagnostic procedure meant that it was not consistently available, including the 5 PBC patients with IPAH. Thus, RVSP from echocardiography was used as a surrogate marker for mean pulmonary artery pressures. Although the importance of RVSP in estimating true pulmonary arterial pressures is somewhat limited, it has been used in well cited studies for specific patient populations. For example, echocardiographic data was the sole measure in a study published in the New England Journal of Medicine which showed that PAH was strongly associated with sickle cell disease (Gladwin et al., 2004). Furthermore, the presence of additional PAH features in our PBC echocardiograms, like right ventricular hypertrophy, right atrial enlargement or tricuspid regurgitation, further increases the sensitivity and specificity of echocardiograms in the diagnosis of true PAH in our PBC patients.

The echocardiograms were interpreted and reported by cardiologists prior to the conception of this study. In addition to the blinded readings by cardiologists, equivocal echocardiograms were later confirmed by a cardiologist who was also an expert in PAH. Ultimately, echocardiogram was chosen as the mainstay of PAH diagnosis in our study due to its ability in assessing secondary causes of PAH such as left ventricular hypertrophy, diastolic dysfunction, valvular and congenital heart disease- all of which exclude IPAH.

Portal hypertension is defined as a hepatic vein portal gradient of greater than 5mmHg. Clinically significant portal hypertension is >10mmHg, where the risk of formation of varices and subsequent hemorrhage increases significantly. As the measurement of hepatic wedge pressures requires an invasive procedure, most clinicians elect to rely on indirect markers of portal hypertension. Our study utilized a variety of surrogate markers as discussed earlier, such as a combination of platelets < 150 x 10^9/L with transient elastography of >20kPa, the presence of varices on gastroscopy, reversed flow on abdominal ultrasound, and splenomegaly. Although these surrogate markers for portal hypertension are helpful, we recognize they are indirect indicators compared to the gold standard of hepatic wedge pressure.

Connective tissue diseases were excluded in our analysis as they are established secondary causes of PAH i.e. not IPAH. There has been some data suggesting that a large proportion of PBC patients have features

of Sjogren's syndrome. Depending on the study, up to 73% of PBC patients can report sicca symptoms, and Sjogren's syndrome was diagnosed in 36.2% in a study of 322 PBC patients (Wang et al., 2013). In a smaller study of 32 patients with PBC, 38% were found to have antibodies to SSB (an antibody associated with Sjogren's) (Hansen et al., 1988). In a subset of 15 PBC patients where only a third had sicca symptoms, focal lymphocyte infiltration from a lip biopsy was actually found in 14. A control sample of 20 Sjogren's patients were all positive for the same histological finding.

Conversely, a proportion of Sjogren's patients also have liver involvement suggestive of PBC. A study of 300 Sjogren's patients revealed AMA positivity in 6.6%, and histological findings of PBC in 82% of these AMA positive patients (Skopouli, Barbatist, & Moutsopoulos, 1994).

These findings raise the possibility of (at least some) of these patients having two disease labels for the same underlying process as opposed to having two distinct diseases. This is further supported by the observation that both conditions are characterized by immune mediated injury of ductal epithelial cells; the bile ducts in PBC, and lacrimal/salivary glands in Sjogren's (Wang et al., 2013). If Sjogren's and PBC truly were the same pathological process, then Sjogren's patients perhaps should have been included in the IPAH cases, bringing the total number of IPAH patients to 7 in our cohort of 134 PBC cases.

However, it still remains more likely although PBC and Sjogren's often co-exist, they are two distinct disorders nonetheless. Serologic markers such as SSB were significantly higher in those with primary Sjogren's (67%) compared to PBC (38%), ($P<0.05$) (Hansen et al., 1988). There are also lower levels of HLA B8, DR3, and DRW52 in these PBC-Sjogren's patients compared to isolated primary Sjogren's patients (Wang et al., 2013). All PBC patients also had significant IgM deposits in salivary gland specimens which was not present in Sjogren's syndrome suggesting the histologic process was not identical. Given this, we chose to take the more conservative approach and not include Sjogren's patients in our IPAH group.

In patients who have both rare disorders, it would be interesting to delineate which condition came first. Although our study had access to electronic medical records on testing dates for patient's first positive AMA serology as well as echocardiograms, it was not possible to definitively conclude a chronological relationship due to the fact that testing was done in some cases at a time that preceded the implementation of electronic medical records.

Finally, a significant drawback of the study was that conclusions on IPAH in PBC vs healthy population were not able to be extended to patients with PSC as statistical significance was not achieved. It is possible that there is not a true difference between PBC and PSC regarding rates of IPAH. Alternatively, this finding may simply reflect an inadequate sample size as there were approximately half as many patients in the PSC group as compared to PBC. With the hugely significant difference in IPAH rates between PBC patients compared with the general population and possible mechanisms of mitochondrial dysfunction that link IPAH with PBC (but not with PSC), it would be premature to rule out a truly distinct overlap disorder between IPAH and PBC that is not present with PSC.

CONCLUSIONS

We conclude that our patients with PBC had a statistically higher risk of developing pulmonary arterial hypertension and pulmonary vasculature remodelling compared to the general population, and a non statistically significant higher risk of developing IPAH compared to patients with PSC. The mechanism in which this overlap disorder may occur in an additive injury model, whereby patients with subclinically inhibited mitochondria (due to a genetic polymorphism) may be triggered by an environmental agent that converges on the mitochondrial PDC enzyme, either inhibiting or driving its ectopic expression on the plasma membrane. Due to the phenomenon of mitochondrial diversity, this mitochondrial suppression manifests in a tissue specific manner- as IPAH in the pulmonary arteries, and as PBC in the liver.

Future Directions

Our work suggests that larger sample sizes are necessary to confirm the association of IPAH with PBC as compared with other similar conditions such as PSC. Prospective multicenter studies are needed on patients with PBC ideally with right heart catheterization and invasive assessment of portal hypertension to definitively confirm such an association between PBC and IPAH on a larger scale. Such studies should also collect enough data to examine the temporal relationship between the onset of PBC and IPAH as this would provide clues to the pathogenesis of such an overlap disorder. They should also follow these patients for a sufficient period of time to see if a connective tissue disease may eventually develop. If so, this would suggest that perhaps the co-existence of PBC and IPAH were simply manifestations of an underlying connective tissue disease as opposed to a unique overlap disorder. Routinely checking IPAH patients for AMA may also yield further evidence supporting/refuting mitochondrial involvement in pathogenesis of IPAH.

Unlike the usual approach of bench to bedside, our work is based on clinical observations which can guide experiments with animal models of PDC dysregulation. Future experiments should examine whether this association is also true in appropriate animal models. For example, ongoing work at the Michelakis lab is showing that mice heterozygous for both SIRT3 and UCP2 have very severe PAH. These mice appear to be the only mouse model of PAH that also exhibits plexogenic lesions in the lungs. These mice should also be examined for the presence of AMA, early PBC histology, or combined with existing models of PBC in order to express this novel overlap PAH/PBC phenotype. Also, ongoing studies in the Mason lab show that cultured biliary epithelial cells from patients with PBC do exhibit suppression of glucose oxidation with hyperpolarized mitochondria and an upregulation of glycolysis (i.e. Warburg effect), i.e. features that are present in pulmonary artery smooth muscle cells of all known models of PAH.

If these animal models or in vitro human cell experiments indeed demonstrate mitochondrial inhibition as a central component of the pathogenesis of PBC, then compounds that activate mitochondria may become candidates for therapy in PBC. It is possible that the same therapy that has been studied in IPAH and malignancy may also be beneficial in PBC. As discussed earlier, dichloroacetate is a small molecule that inhibits PDK and thus activates PDC in mitochondria. It has been shown to downregulate HIF1α and NFAT which are key players contributing to pulmonary vascular proliferation and remodeling in IPAH (Gurtu & Michelakis, 2015). Such a compound may offer benefit to patients with both PBC and IPAH by activating PDC and restoring apoptotic homeostasis in the hyperproliferative intrahepatic biliary duct cells as well as in the pulmonary arteries.

Larger prospective studies of PBC patients are needed also to evaluate whether the single nucleotide polymorphism status of genes like SIRT3 and UCP2 could be predictive of concurrent or future development of IPAH. The implications of such data are clinically important and may affect resource allocation, as PBC patients who are at higher risk of developing severe IPAH may be less ideal candidates for liver transplant. Conversely, prophylactic therapies might be started in PBC patients with these single nucleotide polymorphisms to lower their risk of developing IPAH and ultimately improve their eligibility for life saving liver transplant.

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