Omics approach towards understanding the pathophysiology of Primary sclerosing cholangitis

by

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic progressive cholestatic liver disease with unknown etiology that is characterized by inflammation and fibrosis of the intrahepatic and extrahepatic biliary tracts, leading to diffuse multifocal stricture formation. The pathogenesis of PSC is unknown and there is no effective treatment. Previously, we found evidence of serological reactivity to retroviral proteins, reverse transcriptase activity and upregulation of the viral restriction factor family of APOBEC3 deaminases in PSC patients. To investigate the hypothesis that PSC may be linked with viral infection, we searched for altered expression of genes and associated pathways involved in host defense against viruses. Peripheral blood samples from 23 PSC patients and 26 healthy controls were obtained and genes differentially expressed were identified by RNA-sequencing analysis. Proteomic studies were conducted using liquid chromatography tandem mass spectrometry on biliary epithelial cells (BEC) obtained from PSC patients and were compared with patients with non-cholestatic liver disease. We found total of 1542 overexpressed and 1074 under-expressed transcripts among PSC patients compared to healthy controls (padj<0.05). In the pathway analyses, "Viral process" was among the top overrepresented pathway (padj=4.48E-32). Several of the identified genes overlapped with known infections including HIV (padj=2.37E-14) and Influenza (padj=7.97E-12). Among the top overlapped pathways with HIV were "transport" and "intracellular-transport of virus" (24.9% and 22.49%, respectively). Among the top overlapped pathways with Influenza, we found "nuclear transcribed mRNA catabolic process, nonsense mediated decay" (50%) and "7-methylguanosine mRNA capping" (33%). String analysis of proteome of cholangiocytes showed negative regulation of "mRNA splicing via spliceosome and SRSF1, RNA recognition motif 1"- as well as decreased proteasomal degradation, confirming transcriptomics findings. We postulated that the activation

of nonsense mediated decay might be due to increased formation of premature stop codons following the activity of APOBEC3s. In addition, considering enrichment of 7-methylguanosine capping –that is found to be utilized by several viruses in order to remain undetectable in cytoplasm using a host posttranslational modification and to stabilize the viral mRNA to have a successful translation– and mRNA splicing through SRSF1–which is a potent inhibitor of HIV-1 that its deletion results in double-stranded DNA breaks and genomic instability– and upregulation of host defense components against viruses such as APOBEC3 deaminases that found among PSC patients may suggest a viral pathogenesis. Further studies are warranted to better identify the presence of virus infection in PSC.

Preface

This thesis is an original work by Kiandokht Bashiri. The research project, of which this study is a part, has the ethics approval from the HERB Biomedical, Project Name "Viral Discovery in Healthy Individuals and Individuals with Idiopathic Disorders", No "PRO00005105".

Dr. Bishoi Aziz, Dr. Bavan Sangarasivam, Hussain Syed, Bruna Dutra, Dr. Hiatem Abofayed and Dr. Kerolous Messeha aided in storage and processing of blood samples collected from clinic.

The experiments referred to in chapter 2 were designed by me with the assistance of Prof. Andrew Mason and Dr. Juan Joval. I performed RNA extraction and globin depletion for all the samples and library preparation for part of the samples with assistance of Jessica Hamilton and Sudip Subedi in The Applied Genomic Centre at the University of Alberta. Library preparation for another part of the samples and sequencing were performed by Novagen, Inc. Mapping and quantification were done by Dr. Juan Jovel. I performed annotations as well as differential expression and functional analyses and designed the graphs. Proteomic data are provided by Wei Wei Wang and Bo Meng. The functional analysis of proteomic is performed by me. The literature review is my original work. Chapters 1 and 4 of the present thesis are partly adopted from our chapter submission for publication in Translational Autoimmunity, Elsevier.

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Abbreviations

AIDS: Acquired immunodeficiency syndrome
AIH: Autoimmune hepatitis
APC: Antigen presenting cell
APOBEC3, A3: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
BCR: B-cell receptor
BEC: Biliary epithelial cell
BP: Biological pathway
CMV: Cytomegalovirus
CVID: Combined variable immunodeficiency
EBV: Epstein Barr virus
ER: Endoplasmic reticulum
ERAD: Endoplasmic reticulum-associated protein degradation
GFAP: Glial fibrillary acidic protein
GO: Gene ontology
GWAS: Genome wide association study
HBV: Hepatitis B virus
HCV: Hepatitis C virus
HEV: Hepatitis E virus
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
HPV: Human papilloma virus
HRT: Hormone replacement therapy
HTLV-1: Human T-cell leukemia virus
IBD: Inflammatory bowel disease
ICAM-1: Intercellular Adhesion Molecule 1
IFN: Interferon
IHL: Intrahepatic Lymphocyte
IL1: Interleukin 1
IL2: Interleukin 2
IL2RA: Interleukin 2 receptor
IRF3: Interferon regulatory factor 3
IS: Immune synapse
KIR: Killer cell immunoglobulin-like receptor
LFA-1: Lymphocyte function-associated antigen 1
MDR: Multidrug resistant

MF: Molecular function
MHC: Major histocompatibility complex
MMTV: Mouse mammary tumor retrovirus
MST-1: Mammalian sterile 20-like kinase 1
NASH: Non-alcoholic fatty liver disease
NEMO: nuclear factor-κB essential modulator
NF-κB: Nuclear factor κB
NGS: Next generation sequencing
NK cell: Natural killer cell
NMD: Nonsense-mediated decay
PBC: Primary biliary cholangitis
PBMC: Peripheral blood mononuclear cell
PERK: Protein kinase RNA activated like ER kinase
PSC: Primary sclerosing cholangitis
RA: Rheumatoid arthritis
RIN: RNA integrity number
ROS: Reactive oxygen species
rPSC: Recurrent PSC
RT: Reverse transcriptase
SLE: Systemic lupus erythematous
SOCS1: Suppressor of signaling 1
SRSF1: Serine and Arginine Rich Splicing Factor 1
TCR: T-cell receptor
TIRAP: Toll/IL-1 receptor adapter protein
TLR: Toll-like receptor
UC: Ulcerative colitis
UPR: Unfolded protein response
UPS: Ubiquitin-Proteasome System
VST: Variance-stabilizing transformation
WAS: Wiscott-Aldrich syndrome
XLA: X-linked agammaglobulinemia

1. Introduction

1.1. Definition

PSC is a chronic progressive cholestatic liver disease with unknown etiology that is characterized by inflammation and fibrosis of the intrahepatic and extrahepatic biliary tracts, leading to diffuse multifocal stricture formation [1, 2].

1.2. Clinical presentation

Manifestation of PSC at the time of diagnosis ranges from asymptomatic disease to cholestasis related symptoms (e.g., fatigue, pruritis, excoriation), cholangitis (jaundice, fevers, rigors, and right upper quadrant pain), and the signs associated with advanced liver disease (e.g., jaundice and hepatosplenomegaly). The diagnosis is made by visualization of multifocal strictures and dilations in bile ducts using imaging techniques in the absence of secondary causes such as infectious or ischemic cholangiopathy, surgical trauma and choledocholithiasis [3, 4].

1.3. Epidemiology

Estimated prevalence of PSC is 6 cases per 100,000, mostly affecting men in their 4th decade of life [3, 5, 6]. Based on the epidemiologic studies, coffee consumption, smoking and hormone replacement therapy (HRT) appear to be protective against PSC incidence [7-9]. In addition, dietary habits were observed to be linked with risk of PSC [9]. However, not much is known about the mechanistic correlation of these environmental factors with disease pathogenesis.

Approximately, 80% of PSC cases are associated with Inflammatory bowel disease (IBD), especially UC, while only 5% of IBD patients have concurrent PSC, representing the importance of gut-liver axis in the pathogenesis of PSC [6, 10]. PSC patients bear a significant risk of colorectal cancer and cholangiocarcinoma, and extrahepatic autoimmune diseases such as

autoimmune thyroid disease [11, 12]. Affected males have poorer survival than females and are more likely to undergo liver transplant [13]. Likewise, PSC-associated IBD is more common in men [12]. North America and Northern Europe seem to have more prevalence of PSC compared to Asia [14]. African Americans develop a more severe phenotype of PSC compared to whites and Hispanics [4, 15].

Disease recurrence after transplant occurs in ~20% of PSC cases (rPSC) and is less likely to occur in patients with colectomy before transplant, or post-transplant use of cyclosporine A [16, 17]. Interestingly, corticosteroids do not decrease the rate of rPSC and the use mycophenolate mofetil is correlated with higher incidence of rPSC [16-18].

1.4. Pathophysiology of PSC

Association of several human leukocyte antigens (HLAs) with PSC suggests an underlying immunologically mediated process [19]. Furthermore, genome wide association studies (GWAS) have shown common susceptibility loci among PSC and primary immunodeficiency diseases [20]. For instance, CD28, IL2 and IL2RA that have been found as risk loci for PSC are all parts of costimulatory signaling pathway for T cell activation [21]. Binding of CD28 on T-cell to B7 on the surface of antigen presenting cell (APC) transmits a costimulatory signal in the activated T-cell via upregulating IL-2 production [22]. Similarly, liver tissues of PSC patients have shown a lower frequency of CD28 expressing CD4+ and CD8+ T cells [23]. Impaired or absent CD28 co-stimulation leads to altered immune response against certain viral infections, such as influenza A by promoting apoptosis of virus specific effector T-cells and murine herpetic keratitis through delayed CD8 T cell response in trigeminal ganglia [24, 25]. Lack of CD28 co-stimulation also contributes to exacerbation of Theiler's murine encephalomyelitis virus-induced autoimmune demyelinating disease through spreading of cross-reactive epitopes [26]. Another example of PSC

risk loci is Mammalian sterile 20-like kinase 1 (MST1) [27]. MST1 mutation is associated with an autosomal recessive primary immunodeficiency phenotype, characterized by shortened naive T cell survival and impaired leukocyte adhesion and chemotaxis [28, 29], as well as increased susceptibility to certain human papillomavirus infections [30]. In addition, MST1 is shown to halt cellular antiviral defense via IRF3 phosphorylation followed by attenuating IRF3-mediated transcriptional responses and its deletion is linked to exaggerated antiviral response [31]. Other than the genetic risk loci, physical components of immune homeostasis are also associated with PSC progression. Reduced risk of rPSC in patients with colectomy prior to liver transplant highlights the importance of microbiome in disease progression [32, 33]. Mucosal microbiome study has revealed dominance of Gamma proteobacteria Shigella in microbial signature of patients who have not developed rPSC [34]. It has been suggested that the presence of Shigella might improve colonic mucosa through releasing mucolytic molecules and subsequent stimulation of mucin expression in both colon and theoretically cholangiocytes that creates a layer of protection against other microorganisms or insults [34-38].

Liver transplant can be effective for patients with genetic disorders affecting liver such as biliary atresia, Wilson's disease, alpha-1 antitrypsin deficiency, protein C and S deficiency and congenital metabolic disorders. However, liver transplant is not a cure for all patients with PSC. Following the advancements in HCV management, PSC is the leading cause of recurring disease after liver transplant [39]. High rate and early occurring of posttransplant recurrence in PSC is similar to the pattern of viral hepatic diseases such as HBV and HCV infections [40]. Altogether, the overlap between PSC risk loci, IBD and immunodeficiency syndromes, along post-transplant disease recurrence in a significant proportion of PSC patients, and a lack of response to immunosuppressants, suggests a hypothesis that an underlying immunodeficiency causes an

inadequate immune response to an unknown infectious agent that leads to biliary stricture formation. Interestingly, cyclosporin A, formerly known as an immunosuppressant, that in a few studies is correlated to less rPSC rate, has been found to have immunomodulatory and antiviral effects in a dose dependent manner [16, 17, 41]; for example, cyclosporine can disturb HCV binding and replication in vitro, although it has not shown to be beneficial in prevention of posttransplant recurrence of HCV [42].

1.5. Differential diagnosis

Here we review some of the leading secondary causes of sclerosing cholangitis that must be ruled out before diagnosing PSC.

1.5.1 Infections associated with sclerosing cholangitis

A wide variety of infections (mostly viral) have been linked with the phenotype of sclerosing cholangitis (Table 1). For example, during the pathogenesis of HIV-1-related cholangiopathy, other than the opportunistic infections, HIV itself has potential of inducing UPR, autophagy and apoptosis [43, 44]. For instance, HIV-Tat mediates HIV-associated neurocognitive disorders through upregulating glial fibrillary acidic protein (GFAP) expression in astrocytes that initiates UPR [45]. HIV has also capability of inducing eIF2α and BiP (a molecular chaperone located in the ER) [46] leading to UPR activation. Interaction between GP120 (an HIV envelope glycoprotein) and CD4 mediates mitochondrial membrane permeabilization and cytochrome c release through Bax-dependent apoptotic mitochondrial pathway [47]. However, the specific role of ER and mitochondrial stress in AIDS related cholangiopathy has not been studied yet.

1.5.2 Ischemic cholangiopathy

Ischemic cholangiopathy occurs as a result of impaired blood flow (e.g., in the state of hypercoagulability, iatrogenic vascular injury, use of chemotherapy agent floxuridine, etc.) in the peribiliary plexus [48-51]. Clinical manifestations and imaging features are closely resembled with PSC and a positive history suggestive of impaired blood flow helps in diagnosis (e.g. recent liver transplantation) [52]. ER stress has been shown to play an important role in cellular stress in response to ischemia and reperfusion injury [53, 54].

1.5.3 Cholestasis associated with parenteral nutrition

Parenteral nutrition associated liver disease (PNALD) as a well-known complication of prolonged parenteral nutrition is presented by cholestatic features and steatosis followed by fibrosis and cirrhosis [55, 56]. Mechanism of PNALD is yet to be known. However, catheter related infections and changes in gut microbiota have been proposed to be involved [56]. Interestingly, rats undergone total parenteral nutrition have shown higher levels of the biochemical markers linked to the ER stress and proapoptotic molecules [57]. In addition, proteome of liver tissue from patients with PNALD has revealed mitochondrial dysfunction and oxidative stress [58].

1.5.4 Chemotherapy-induced sclerosing cholangitis

Intrahepatic arterial infusion of fluoropyrimidines, and in rare cases systemic chemotherapy seem to induce direct toxicity and ischemic changes in biliary epithelium. Although the radiologic feature may resemble PSC, involvement of hepatic porta is more common than PSC (which is mainly intrahepatic) [59, 60].

1.6. The interplay between immunodeficiency, infection and PSC

Phenotype of sclerosing cholangitis has been reported in a wide variety of infections (mostly linked with viruses (Table 1) and immunodeficiency syndromes (Table 2)

The association of immunodeficiency with sclerosing cholangitis was first described by Record et.al in 1973 following the detection of sclerosing cholangitis in family members with primary immunoglobulin-deficiency [61]; Hyper IgM syndrome, combined immunodeficiency, MHC class-II deficiency and acquired immunodeficiency syndrome were reported afterwards [62-66]. Subsequently, variety of other congenital immunodeficiencies have been related to sclerosing cholangitis including natural killer cell deficiency, CD40 ligand deficiency, Wiscott-Aldrich syndrome, agammaglobulinemia, combined variable immunodeficiency and AIDS associated cholangiopathy [67]. Herein, we review the mechanism of biliary epithelial cell destruction following the immunodeficiencies that has been correlated with sclerosing cholangitis.

Table 1. Infections reported in patients with the phenotype of sclerosing cholangitis. There are several reports of HIV and cryptococcus infections that have the same manifestations with PSC. We hypothesized that PSC is caused by an unknown infection.

Viruses	Bacteria	Fungi	Parasites
HIV [68-71]	Streptococci [72]	Cryptococcus [73-76]	Strongyloides stercoralis [77]
HTLV-1[78]	Helicobacter [79, 80]	Candida [81, 82]	
CMV [83, 84]	Chlamydia [85]	Trichosporon [86, 87]	
HCV [88-92]	Bacillus cereus [93]	Isospora belli [94]	
HBV[95, 96]	Shewanella [97]		
HEV[98]	MDR Salmonella spp[99]		
Reovirus [100, 101]			

Table 2. Immunodeficiency syndromes associated with phenotype of sclerosing cholangitis support the hypothesis of an underlying immunodeficiency or inadequate immune response against an infectious agent.

Hyper IgM syndrome	Wiscott-Aldrich syndrome
Agammaglobulinemia	Natural killer cell deficiency
Good syndrome	Gain of function mutation of PIK3CD
MHC class-II deficiency	Combined variable immunodeficiency
CD40 ligand deficiency	Acquired immunodeficiency syndrome

1.6.1 AIDS-related cholangiopathy

One of the well-known infections/immunodeficiency syndromes associated with sclerosing cholangitis is human immunodeficiency virus 1 (HIV-1) infection [102]. Prior to introduction of antiretroviral therapy, cholangiopathy was a complication of AIDS; HIV-1 mediated immunodeficiency (AIDS) provides a susceptibility to opportunistic infections such as Cytomegalovirus (CMV) and Cryptosporidium parvum (C. parvum) [68-70, 103]. C. parvum is armed by a complex virulence mechanisms to activate different signaling cascades including NF-κB pathway in the host cell, which leads to apoptotic resistance in the infected cells and parasite survival [104]. Moreover, C. parvum has the capacity of upregulating FasL expression in the infected cells and initiating apoptotic cascades in bystander noninfected cells [105]. It has been suggested that HIV and C. parvum have a synergistic destructive effect on biliary system through the stimulatory effect of HIV trans-activator of transcription (Tat) on induction of Fas/FasL by C. Parvum [103, 106].

1.6.2 Natural killer cell deficiency

NK cells play a pivotal role in the innate immune system through cytolytic activity, while normally restrained by cell surface inhibitory receptors present on autologous cells [107]. Thus, any

abnormalities in NK cell maturation and function may lead to the development of autoimmune diseases. For instance, increased population of NK cells has been demonstrated in PSC compared to other liver diseases [108, 109]. Furthermore, a study by Karlsen et. al, showed reduced HLA-Bw4 and -C2 (ligands of inhibitory killer cell immunoglobulin-like receptors (KIRs)), among PSC patients [110]. Also, there is evidence suggesting NK cells involve in autoimmunity through their capacity of cytokine release [111]. Factors that are determinant of protective vs. pathogenic role of NK cells are: first, the equilibrium between the engagement of inhibitory and stimulatory receptors [112]; second, NK cell localization [113]. In a study by Langeneckert et. al, CCR7+ and CXCR3+ NK cells isolated from PSC patients demonstrated a higher functional capacity in peripheral blood rather than liver tissues; chronic activation and exhaustion by the inflamed liver might be the cause of dampened functional capacity of NK cells in the liver [114]. Besides that, There is a report of a patient with NK cell deficiency with concurrent Trichosporon infection who has presented with manifestations of sclerosing cholangitis [115], suggesting an underlying immunodeficiency that has predisposed patient to a certain infection, followed by biliary damage.

1.6.3 Hyper IgM syndrome

Another example of immunodeficiency syndrome with presentation of sclerosing cholangitis is hyper IgM syndrome, which is a form of primary immunodeficiency characterized by a defective immunoglobulin class switch recombination [116]. There are variety of molecular defects that lead to the phenotype of hyper IgM syndrome, including mutations in nuclear factor-κB essential modulator (NEMO), CD40 or CD40 ligand, and activation-induced cytidine deaminase (which is a member of APOBEC family) [117-121]. Mutations in zinc finger domain of NEMO halt CD40-CD40L mediated NF-κB inhibitor activity [120]. These mutations limit the ability of immunoglobulin class switch recombination in B cells and abolish the expression of NF-κB–

induced cytokines including IL-12 or tumor TNF-α in response to CD40L engagement. Defective CD40 signaling in hyper-IgM syndrome leads to inefficient interaction between B-cells and T-cells that affects humoral immune response and raises the host's vulnerability to encapsulated organisms such as cryptosporidium [122, 123]. In addition, inhibiting canonical NF-κB pathway in $NEMO^{LPC-KO}$ mice and the bile duct-induced cholestatic mice has led to increased apoptosis and expression of inflammatory in hepatocytes resulting in chronic liver disease [124, 125]. Noteworthy, sclerosing cholangitis has been known as a complication of 6-20% of cases with X-linked hyper IgM syndrome and some are not related to cryptococcal infection [62, 64]. In a study by Hartman et.al, recurrent sinopulmonary infections was reported in 2 family members who had gain of function mutation in p110δ catalytic subunit of the phosphatidylinositol-3-OH (PIK3CD) [126]. PIK3CD is known to predispose a defect in class switch recombination/hyper IgM syndrome [127-130].

1.6.4 X-linked agammaglobulinemia (XLA)

XLA is caused by arrest in B-cell differentiation due to a mutation in BTK gene [131]. Btk protein is a component of the B-cell receptor (BCR) and Toll-like receptors (TLR) pathway [132]. Btk interacts with TIR domains od TLR 4, 6, 8 and 9, as well as key components of TLR4 signal transduction including MyD88, MyD88 adapter-like protein or TIRAP (Toll/IL-1 receptor adapter protein), and interleukin-1 receptor-associated kinase-1 [133]. Phosphorylation of TIRAP by Btk in response to TLR4 engagement leads to its interaction with suppressor of signaling 1 (SOCS1) protein (SOCS1 is a candidate risk gene for PSC based on genome wide association studies [134]). This interaction is followed by polyubiquitination of the TIRAP and its degradation by proteasome [135-137]. Any disturbance in this cascade may lead to TIRAP-dependent NF-κB activation and prolonged proinflammatory response [138]. Missense mutation in BTK leads to deficient

expression of BTK in most cases of XLA [139, 140], and these patients demonstrate a substantial inflammatory response through TLR signaling pathway [141]. Furthermore, transgenic mice that overexpress BTK also develop increased cytokine production, antinuclear antibodies (ANA) formation and systemic autoimmune disease [142]. There are a few reports of sclerosing cholangitis in patients with XLA and patients with Good syndrome (hypogammaglobulinemia associated with thymoma) [143-146].

1.6.5 Common variable immunodeficiency

Common variable immunodeficiency (CVID) is one of the most common symptomatic primary immunodeficiencies characterized by hypogammaglobinemia, susceptibility to recurrent bacterial infections and poor vaccine response [147]. CVID is an example of how immunodeficiency and autoimmunity become intertwined as it is associated with SLE, IBD, rheumatoid arthritis (RA), hemolytic anemia, immune thrombocytopenic purpura, as well as autoimmune liver diseases [148-151]. The underlying mechanism of increased autoimmunity in CVID has not been elucidated. Multiple mutations have been associated with the pathogenesis of CVID, but not found in all cases [152]. Recent studies have focused on the role of NF-κB pathway alterations in CVID [153, 154]. Loss of function of NFKB1 variants has been introduced as the most prevalent monogenic etiology of CVID [155]. Noteworthy, NFKB1 has a regulatory function in NK cell maturation and cytotoxic activity [156]. Although NFKB1 gene has been introduced as a risk gene for PSC [134] and NFKB1 mRNA is shown to be differentially expressed in these patients [157], this finding is not disease-specific and might be found in a wide variety of inflammatory processes; more study is needed to find potential malfunctions in the protein or its localization in PSC.

1.6.6 Wiscott Aldrich syndrome

Wiscott Aldrich syndrome (WAS) also named as eczema-thrombocytopenia-immunodeficiency syndrome, is caused by a mutation in WAS gene [158, 159]. WAS protein (WASP) activates actin polymerization and roles in formation of immunological synapses (IS) [160]. Several mechanisms have been proposed for autoimmunity occurred in WAS including the incomplete pathogen clearance due to the cytoskeleton defect and defective interactions between immune cells (e.g., inability of WASP-deficient T-reg cells in suppression of T and B cells) [161]. There are a few reports of coincidence of sclerosing cholangitis and WAS [162-164]. Nevertheless, to date, other than transcriptomic data of PSC patients that has found differentially expressed WAS among PSC patients [157], there is no study focusing on the functional role of WAS protein in the pathogenesis of sclerosing cholangitis. However, huge effort has been made to correlate the expression of leukocyte adhesion molecules to PSC [165-168].

Establishment of a highly organized IS is a key step in T-cell activation and antigen presentation [169], and depends on the formation of supramolecular activation complex (SMACs) [170]. Central and peripheral regions of the SMAC are enriched in T-cell receptors and adhesion molecules (e.g. ICAM-1, LFA-1), respectively [171]. A polymorphism of ICAM-1 has been correlated with susceptibility to PSC [167]. Noteworthy, it has been observed that the expression of adhesion molecules increases in response to inflammatory cytokines [172, 173] and overexpression of adhesion molecules might be a late finding secondary to prior events [168].

1.7. Modelling of PSC as an infectious disease

All the above processes exemplify our proposed model for PSC that suggests PSC patients have underlying immunodeficiency trait caused by either genes or an unknown infectious agent that

predisposes biliary tracts to damages by other pathogens (such as gut microbiome) or environmental factors (Figure 1).

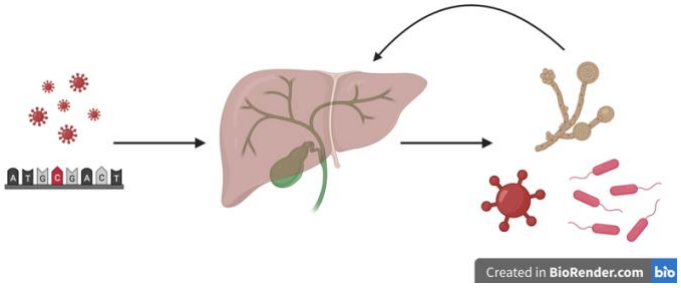


Figure 1. An underlying genetic predisposition may provide susceptibility to an infectious disease process that can damage the biliary epithelium in immune suppressed individuals.

Previously, our research group found serum reactivity to HIV-1 p24 gag among 1/3 of PSC patients in the absence of HIV infection [174] and reverse transcriptase activity in the serum of patients with PSC [175].

1.8. APOBEC3 proteins in PSC; an antiretroviral host defense mediator

In addition to the findings above, a test PCR by our group showed upregulation in IFN-γ induced "Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3" (APOBEC3, A3) proteins (Figure 2) [176]. Note that the result of this PCR is incomplete due to the lack of reference gene (the run is normalized based on starting RNA material only). APOBEC3 family of proteins consists of a group of Zn^{2+}-dependent cytidine deaminases including A3A, A3B, A3C, A3D, A3F, A3G and A3H that deaminate cytosine to uracil in the single stranded DNA or RNA, which leads to hypermutation and interfering with reverse transcription and integration of exogenous retroviruses such as lentiviruses (eg, HIV) and deltaviruses (eg, HTLV), as well as endogenous retroviruses (eg, HERV) and retrotransposons (eg, L1). A3s also plays a role in restriction of several DNA viruses including HVB, HPV, canine parvovirus, EBV and HHV-1. Noteworthy, antiviral effect

of A3s is not limited to their deaminase activity and they have shown antiviral properties in the absence of deamination as well [177-188]. Properties of different A3 subsets are summarized in Table 3.

Intriguingly, several viruses have developed mechanisms to evade the A3s. For instance, Vif as an accessory protein of lentiviruses has shown to disrupt A3 functions [189]. It has been demonstrated that in the T cells expressing A3D, A3G, A3F and A3H, each A3 is capable of mutating viral genome and restricting viral replication upon reverse transcriptase-mediated cDNA synthesis in Vif-deficient HIV-1 [190]. The uracilated cDNA will be reflected as a G to A hypermutation in the viral genome or will be destabilized and degraded. However, in the presence of Vif, an E3 ubiquitin ligase complex formed by Vif binds to the A3 directly and marks the deaminase for degradation through ubiquitin proteasome system [189, 191-193].

Table 3. Properties of APOBEC3 proteins. First column shows the class of A3, second column shows the tissues that the class of A3 is mainly expressed. Third column shows the tissues and the cells that express the class of A3 differentially based on "ProteomicsDB". Fourth column shows the number of active domains in each A3 that have deamination activity. The fifth column shows the experimentally tested interactions between A3s and HIV Vif & Gag proteins. The last column shows the viruses that are restricted by A3s based on the published studies.

Class	**Main mRNA expressing tissues [194]**	**Protein differential expression in normal tissues [195, 196]**	**Active deaminase domains**	**HIV Vif & Gag binding [197]**	**Associated viruses [198-200]**
A3A	Whole blood, spleen, salivary gland	Cervix, heart, monocytes, and spleen	1	?	HIV, HTLV-1, HBV, HCV, HPV, HHV
A3B	Whole blood	B lymphocytes, heart, PBMCs	1	?	HIV, HTLV-1, HBV
A3C	Spleen, testes, PBMCs, heart, thymus, prostate, ovary	?	1	Yes	HIV, HBV, HPV, HHV

A3D	Blood and spleen	PBMCs, heart, B lymphocytes	2	Yes	HIV
A3F	Ovary	NK cells, testis, ovary, platelets	1	Yes	HIV, HBV
A3G	Spleen, testes, ovary, PBMCs, non-permissive PBMCs,	Kidney, skin, lymph nodes, blood, spleen	1	Yes	HIV, HTLV-1, HBV, HCV, HHV
A3H	Lymphoid organs, lung, testis, ovary, fetal liver and skin	Nasopharynx, Colon, Bronchus, Connective tissue	1	Yes	HIV, HBV, HPV

Increased A3s in PSC compared to healthy controls led us to look at the evidence for the presence of a virus in disease pathogenesis from the window of cellular antiviral defense.

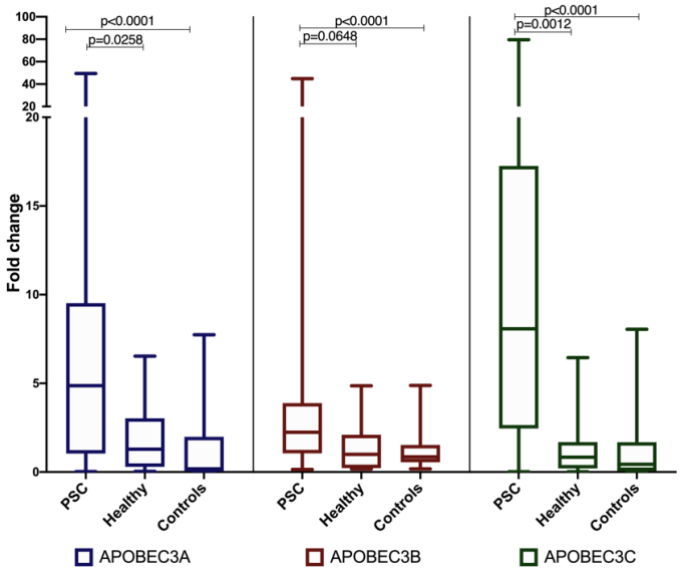


Figure 2. Increased expression of APOBEC3 deaminases using RT_PCR on peripheral blood samples obtained from PSC patients compared to healthy subjects (labeled as healthy) and patients with other liver diseases labeled as "Controls". Note that this is a result of a test PCR, and the fold changes are calculated based on delta-CT and not delta-delta CT, i.e., the run is normalized based on the starting RNA and not the reference gene.

1.9. Hypothesis

The pathogenesis of PSC is poorly understood. Limited or no response to immunosuppressive therapy, significant rate of posttransplant disease recurrence, association with immunodeficiency syndromes and resemblance of pathology to infection associated cholangiopathies raise the possibility of involvement of an infectious process.

Our lab has spent a great deal of effort on finding a possible viral etiology for autoimmune liver diseases. To date, we have found seroreactivity to retroviral antibodies, increased antiretroviral defense molecules (e.g., APOBEC3 deaminases) and reverse transcriptase activity in the serum of patients with PSC.

Taking all these into consideration, in the present study, we hypothesized that PSC is caused by a retroviral infection; therefore, we aim to test whether virus related pathways are activated in PSC patients using next generation sequencing technology.

1.10. Importance and implications

Unknown etiology of PSC has led to dearth of an effective therapy and majority of patients undergo liver transplant or death. We believe our findings will pave the road of identifying an etiology and a treatment for PSC.

2. Materials and methods

2.1. Study population and design

Peripheral blood samples were collected in PAXgene® Blood RNA Tubes, from 23 PSC patients with the exclusion criteria of having concurrent known viral hepatitis and 26 healthy controls in the Zeidler Ledcor Gastrointestinal Clinic at the University of Alberta.

2.2. RNA extraction

Whole blood RNA was extracted using E.Z.N.A.® PX Blood RNA Kit according to the manufacturer's protocol. Briefly, the cells were pelleted using centrifugation, washed and lysed by a Proteinase K containing buffer. After removing debris using a homogenizer mini column, the samples were transferred to the HiBind® RNA Mini Column. Following a brief centrifugation, genomic RNA was removed and exposed to DNaseI. After three washes, RNA was eluted in RNase-free water and was quantified by NanoDrop spectrophotometry.

2.3. Globin depletion

Since around 70% of whole blood RNA consists of globin transcripts, in order to increase the sensitivity of sequencing to the less abundant transcripts, globin transcripts were removed using GLOBINclear™ Kit, human, Invitrogen™ according to the company's instruction. In short, for each sample, 10 µg of whole blood RNA with a minimum concentration of 70 ng/µL and the RNA integrity number (RIN) above 8 (Agilent Technologies 2100 Bioanalyzer, Figure 3) was combined with 1µL of Capture Oligo Mix followed by hybridization of globin transcripts with biotinylated oligonucleotides by adding 15 µL of prewarmed 50ºC Hybridization Buffer and 15 min incubation.

Prewarmed 50ºC Streptavidin magnetic beads were then mixed with biotinylated oligonucleotides that were bound to globin mRNA and incubated for 30 min. Using a magnetic stand, the Streptavidin magnetic beads were pulled to the side of the tube and the globin-depleted mRNA, in the supernatant, was transferred to a new tube.

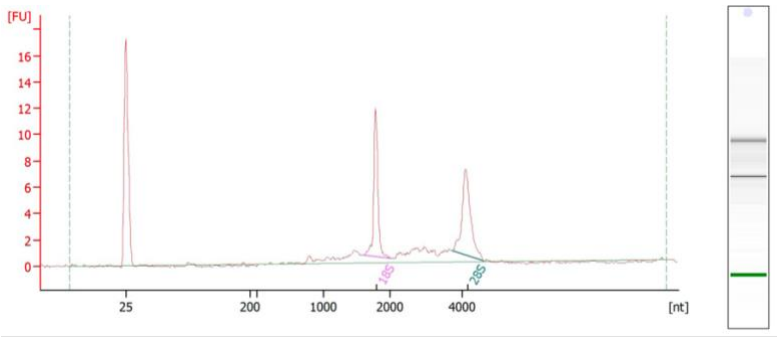


Figure 3. Starting RNA Bioanalyzer Trace. High-quality RNA shows a 28S rRNA band at 4.5 kb at twice the intensity of the 18S rRNA band at 1.9 kb. Both kb determinations are relative to an RNA 6000 ladder. The mRNA appears as a smear from 0.5–12 kb.

2.4. Library preparation

Purified globin-depleted mRNA samples with a minimum amount of 0.12 µg and RIN ≥ 7 were used for library preparation using Illumina® TruSeq® RNA Sample Preparation Kit v2 based on manufacturer's guide. All the samples passed the quantitative and qualitative criteria of library preparation. Briefly, oligo-dT attached magnetic beads were applied in order to purify poly-A containing transcripts. In the next step, transcripts were fragmented using divalent cation, primed with random hexamers and reverse transcribed into first strand cDNA by reverse transcriptase and random primers. Then, RNA templates were replaced by DNA to generate double-stranded (ds) cDNA. Afterwards, ds cDNA was separated from the second strand reaction mix by Ampure XP beads. The stretches of unpaired nucleotides in 3' end of the DNA (overhangs) were blunted using an End Repair Mix with 3' to 5' exonuclease activity. To prevent template concatenation during

the adapter ligation and to provide a primer binding site for indexing PCR, 3' end adenylation of the blunt fragments was performed. Indexing adapters were ligated to the end of ds cDNA. Adapter-ligated DNA fragments were enriched using 15 cycles of PCR followed by an Ampure bead cleanup. For each sample, to assess the library concentration, Qubit 2.0 Fluorometer was used. To ensure quality of the libraries, the bands were visualized by loading 1µL of the 1:50 dilution of product on High Sensitivity DNA chip using Agilent Technologies 2100 Bioanalyzer, Figure 4). For cluster generation, indexed DNA libraries were normalized to 10 pM and pooled in equal volumes.

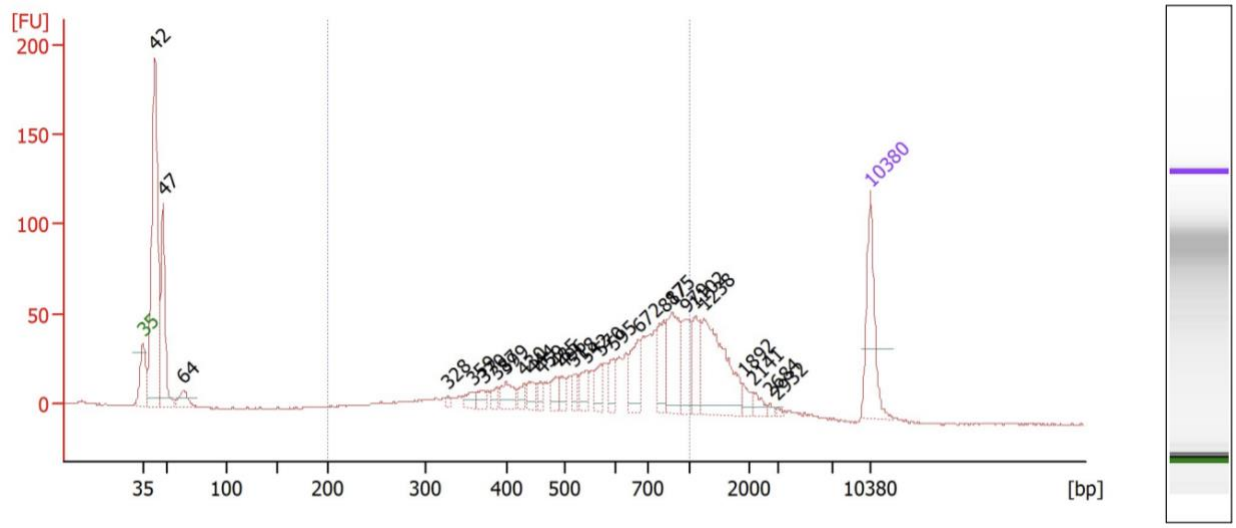


Figure 4. Gel-free library of one the samples on high sensitivity DNA chip. Height of the sample peak was in the standard range (based on the upper ladders). Libraries sized between 300-2k bp.

2.5. Sequencing

Sequencing was performed using PE 150 bp, with on average 20M reads per sample by Illumina Sequencer HiSeq 2500.

2.6. Data quality control

Inspection of sequencing quality was conducted with FastQC, and trimming of bases was done with the software fastq-mcf considering a threshold Phred score > 30 (i.e., base call accuracy:

99.9%). Distribution of sequencing error rate and distribution of A/T/G/C base were visualized (Figure 5). All the low-quality reads, the reads containing adapters and the reads with > 10% undetermined bases (if there was any) were removed (Figure 6).

2.7. Mapping and read quantification

Transcript-level abundances were estimated following pseudoalignment using Kallisto (1), with bias correction and 100 bootstraps. As reference database, Homo_sapiens.GRCh38.cdna.all.fa from Ensembl was used.

2.8. Gene expression analysis and annotation

Differential expression analysis of the counts across genes was performed using DESeq2 package (available at http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) with FDR < 0.05. DE transcripts/genes were annotated with BioMart. The script used for DESeq2 is attached in supplementary file 1. To correct the batch effect due to the variation of runs across samples, we used batch modelling in the design of DESeq2. To remove the batch effect from the individual count data we used "removeBatchEffect" in limma.

2.9. Functional analysis

For functional pathway enrichment analysis, Enrichr (2), gProfiler (3), DAVID (4) and ClueGO [201] were utilized.

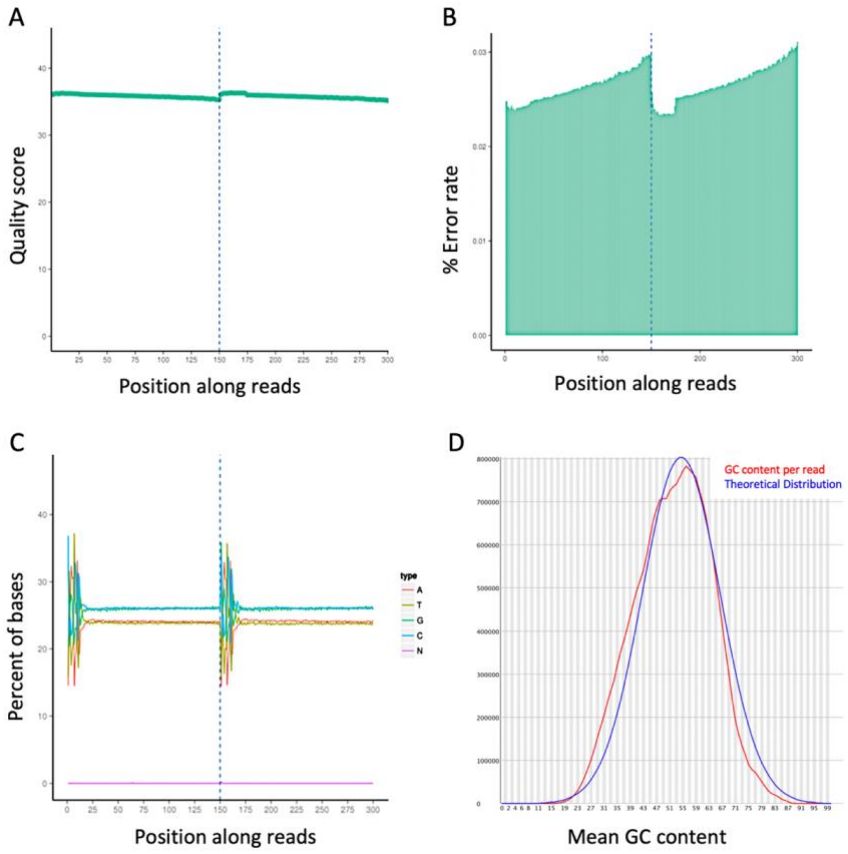


Figure 5. ***A.*** *Quality score distribution: the green line represents the mean quality, which is an indicator of base call accuracy (accuracy > 99.9% or Phred score>30),* ***B.*** *Error rate distribution or total number of incorrectly sequenced bases as a percentage of total number of total sequenced bases.* ***C.*** *Percent of bases along reads of one of the samples, According to FastQC instruction, the non-uniform distribution of bases for the first 10-15 nucleotides is considered to be normal and expected depending on the type of library kit used. "RNA-Seq data showing non-uniform base composition will always be classified as Failed by FastQC for this module even though the sequence is perfectly good".* ***D.*** *GC distribution over all sequences of one of the samples: reads form a normal distribution with a peak at the mean GC content of Homosapien (~50%).*

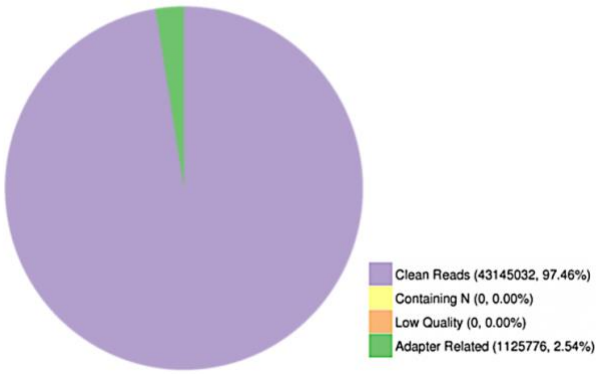


Figure 6. Classification of raw reads in one of the samples. Downstream analyses are based on clean reads. The filtering process was as follows. (1) Discard reads related to adaptor contamination. (2) Discard reads with uncertain nucleotides constitute more than 10% (there was not any in our samples). (3) Discard reads when low-quality nucleotides (Phred<20) more than 50% of the read (there was not any in our samples).

2.10. Calculation of clinical prognosis

We assigned a clinical prognostic score to patients with PSC by Mayo Risk Score (R) (5) calculated as follows: (0.0295 * (age in years)) + (0.5373 * LN(total bilirubin in mg/dL)) - (0.8389 * (serum albumin in g/dL)) + (0.5380 * LN(AST in IU/L) + (1.2426 * (points for variceal bleeding)), where $R \leq 0$ has low risk, $0 < R < 2$ has intermediate risk and $R > 2$ has high risk for having a transplant free survival. The correlation between clinical prognosis and the genes of interest were assessed using simple linear regression.

2.11. Proteomics

BEC from 3 PBC and 7 control samples (3 PSC, 2 cryptogenic cirrhosis and one each with autoimmune hepatitis and alcohol related cirrhosis) were processed for shotgun-proteomic analyses by liquid chromatography tandem mass spectrometry (LC-MS/MS). Lysates were derived from each of the BEC samples and duplicates of 25 µg protein per sample was resolved on a polyacrylamide gel and analyzed using LC-MS/MS (University of Alberta Institute of Biomolecular Design). For primary data analysis, peptide to protein matches and peptide spectral

match (PSM) values were assembled using ThermoFisher Proteome Discover v.1.4 following previously established methods. Spectral counting on all detected proteins was performed using the online QSpec Spectral Counter(http://www.nesvilab.org/qspec.php/) which calculates natural log fold changes and false discovery rate (FDR) values. For statistical analysis, samples were separated into the three groups of PBC, PSC and non-cholestatic liver disease controls. Proteins were considered to be significantly different with FDR: 0.05. Differentially expressed proteins were then separated for pathway analysis and functional enrichment analysis using the STRING database.

3. Results

Demographic data of the two groups of study are shown in Table 4. Lab and demographic data as well as Mayo score were available for 21 patients detailed in Table 5.

Table 4. Demographic data of study subjects. Patients and controls were not age and sex matched. Mean age of the controls was about 5y younger than the disease group.

Study group	PSC	Healthy control
Total number	23	26
Female	10	20
Male	13	6
Mean age (range)	39.87 (18-58)	34.789(18-66)

We found total of 2616 differentially expressed genes with 1074 down and 1542 upregulated genes (Figure 7) (Supplementary File 2).

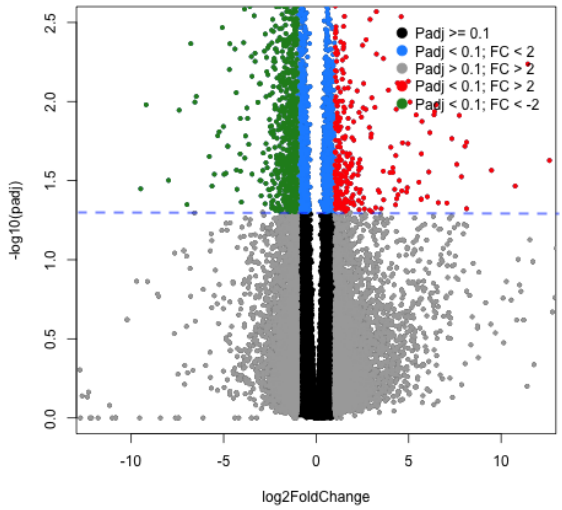


Figure 7. Volcano plot showing differential expression between PSC and healthy controls. The dashed line demonstrates where padj=0.05 and points above the line have padj<0.05. The graph is plotted using ggplot.

Pathway enrichment analysis of over and underrepresented genes is shown in (Figure 8). Top altered molecular functions (GO:MF) and biological pathways (GO:BP) of overrepresented and underrepresented genes are shown in Figure 9 (A and B, respectively). Components of ubiquitin-proteasome system that are differentially expressed in PSC patients compared to healthy controls are shown in Figure 10.

Table 5. Characteristics of PSC patients

Patient	Gender	Total bilirubin (mg/dL)	Alb	Variceal Bleeding	Age	AST	ALT	ALP	Platelet	Neutrophil	Lymphocyte	Mayo Score	Drugs
P1	m	0.1695	4.5	0	18	30	11	90	251	3.1	1.4	-2.3	Azathioprine 200 mg daily, Mesalamine 1.6 g b.i.d, vitamin D 1000 IU daily, and Ursodiol 500 mg b.i.d.
P2	m	0.113	4.5	0	30	68	73	157	297			-1.9	
P3	f	0.113	4.7	0	37	64	128	430	269			-1.8	
P4	m	0.1356	4.2	0	26	69	112	582	264	1.7	1.9	-1.7	Humira, Imuran, Ursodiol
P5	f	0.0791	4.5	0	47	39	60	190	189			-1.7	Ursodiol 750 mg daily, vitamin D, calcium, and multivitamins.
P6	m	0.1582	4.4	0	34	27	26	105	288	1.9	1.2	-1.7	40 mg weekly Humira and Imuran 200 mg and Salofalk 2 g b.i.d.
P7	f	0.1356	3.7	0	33	26	39	229	359	1.7	1.1	-1.6	
P8	f	0.113	4	0	51	18	17		133			-1.5	
P9	f	0.2034	4.6	0	28	95	46	80	75	2	2	-1.4	
P10	m	0.2034	4.6	0	43	44	80	204	171			-1.4	Vit D, calcium
P11	f	0.0565	4.5	0	58	35	41	131	271	3.9	2.3	-1.4	
P12	m	0.113	4.5	0	59	62	58	152	306	3.8	1.7	-1.1	Ursodiol 1,250 mg daily, Losec 20 mg daily, Colchicine 1.2 mg daily, Imuran 75 mg daily, calcium 300 mg b.i.d, Actone1 35 mg weekly, Lipitor 40 mg daily, Aitace 20 mg daily, Atenolol 100 mg daily, Norvasc 10 mg daily, ASA 81 mg daily, hydrochlorothiazide 25 mg daily
P13	m	0.58	45	0	36	70	149	216	286	3.3	1.8	-0.7	UDCA
P14	f	0.53	38	0	36	29	29	137	143	2.6	0.8	-0.6	Sirolimus
P15	m	0.2599	4.3	0	48	91	116	259	198			-0.4	
P16	f	0.82	40	0	28	85	77	702	193	2	1.3	-0.2	Tacrolimus
P17	m	0.1921	3.6	0	57	49	25	137	57	2.8	0.6	-0.1	Septra/Cipro on alternate weeks, Ursodiol 500 mg bid, four ADEK tablets daily, calcium supplements, Pantaloc 40 mg daily
P18	m	0.99	40	0	31	117	224	434	309	3.6	2.5	0.11	Prednisone Asacol
P19	m	0.3616	2.9	0	50	69	45	361	108	3.4	0.9	0.8	Ursodiol, Calcium, Vit D
P20	m	1.23	37	0	46	114	135	181	429	5.4	2.6	0.91	Clindamycin, Fenofibrate, Ursodiol, Loperamide
P21	m	2.2261	3.5	1	47	199	239	399	98	4.8	0.3	1.7	

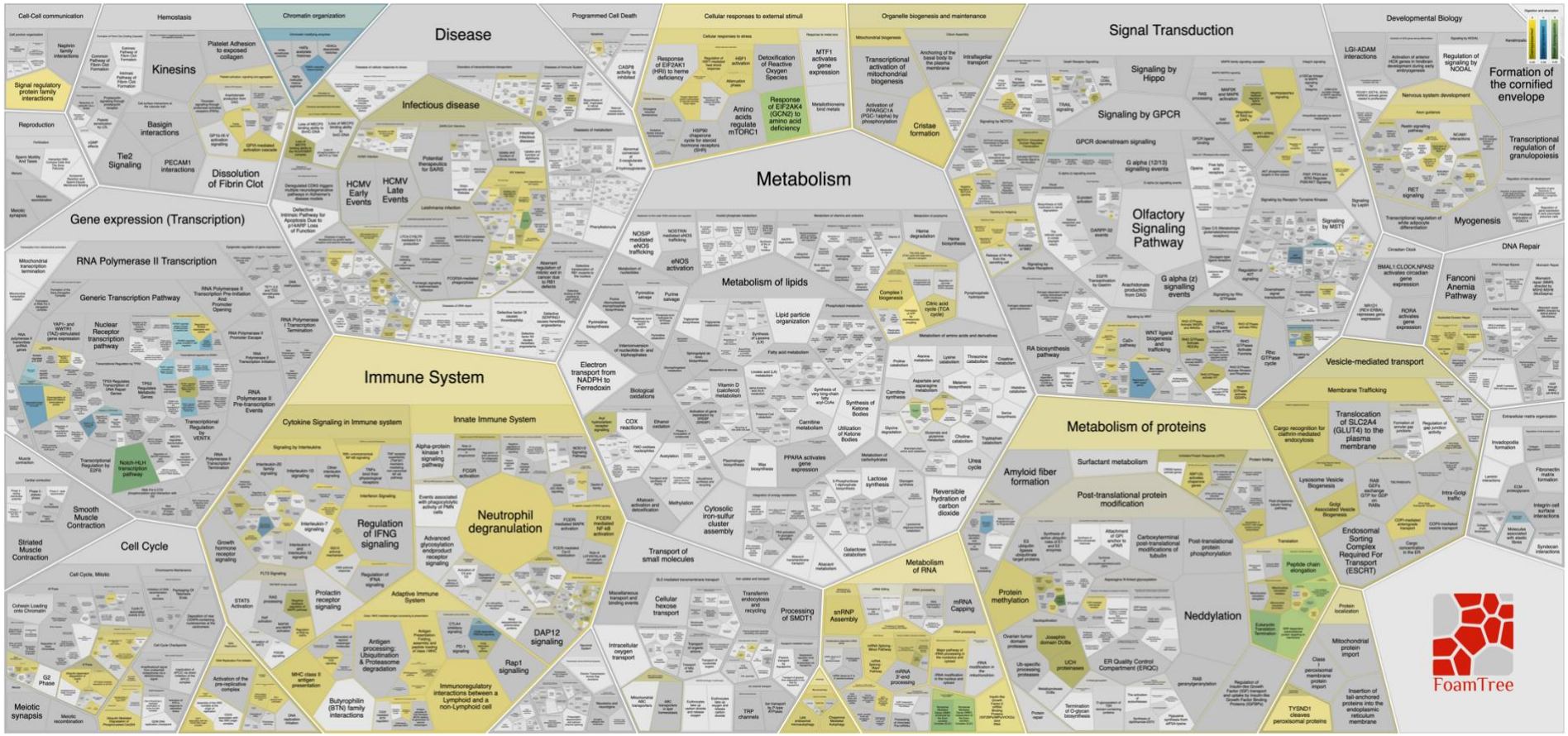
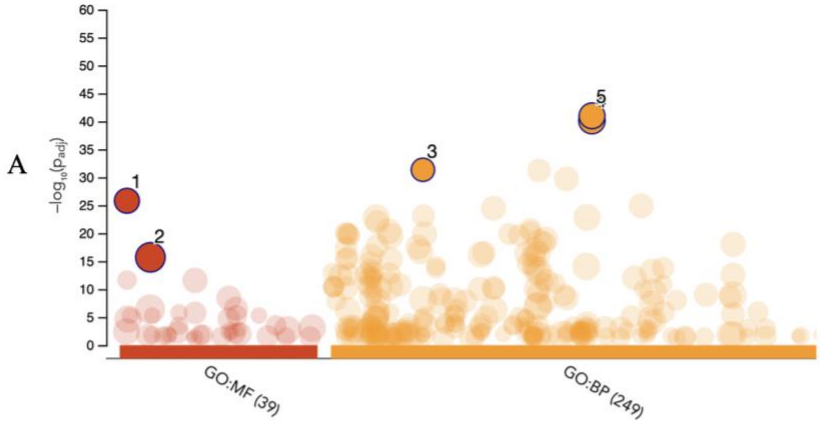
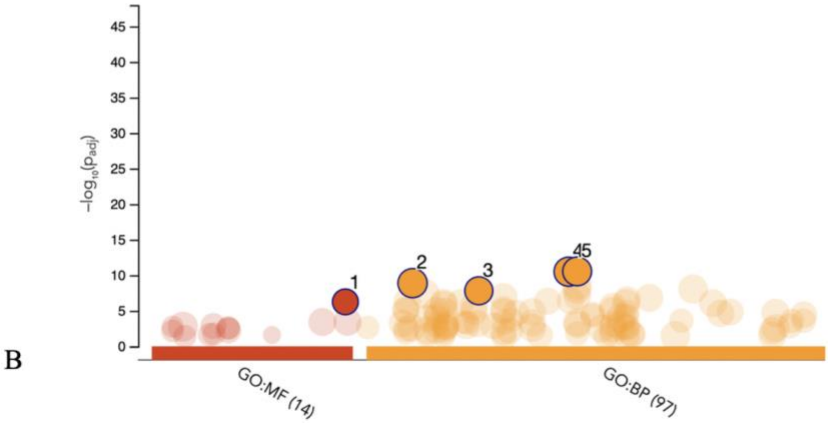


Figure 8. Pathway enrichment analysis of over (yellow) and under (blue) represented networks implicates immune system-related pathways such as neutrophil degranulation and NFKB signaling, metabolism of proteins such as translation initiation and termination, and metabolism of RNA such as mRNA splicing and nonsense mediated decay in the pathophysiology of PSC. The graph is generated by Reactom.



ID	Source	Term ID	Term Name	Padj
1	GO:MF	GO:0003723	RNA binding	1.550×10^{-26}
2	GO:MF	GO:0005515	protein binding	2.030×10^{-16}
3	GO:BP	GO:0016032	viral process	4.489×10^{-32}
4	GO:BP	GO:0051641	cellular localization	7.545×10^{-41}
5	GO:BP	GO:0051649	establishment of localization in cell	1.011×10^{-41}



ID	Source	Term ID	Term Name	Padj
1	GO:MF	GO:0140096	catalytic activity, acting on a protein	5.543×10^{-7}
2	GO:BP	GO:0006807	nitrogen compound metabolic process	1.288×10^{-9}
3	GO:BP	GO:0019538	protein metabolic process	1.528×10^{-8}
4	GO:BP	GO:0043170	macromolecule metabolic process	3.167×10^{-11}
5	GO:BP	GO:0044260	cellular macromolecule metabolic process	2.865×10^{-11}

Figure 9. Top up (A) and down (B) regulated gene ontology molecular functions (red) and biological pathways (orange). The graphs show the relative difference of padj for the selected pathways (labeled with numbers) and functions compared to the rest of enriched functions and pathways. The graphs are generated by gProfiler.

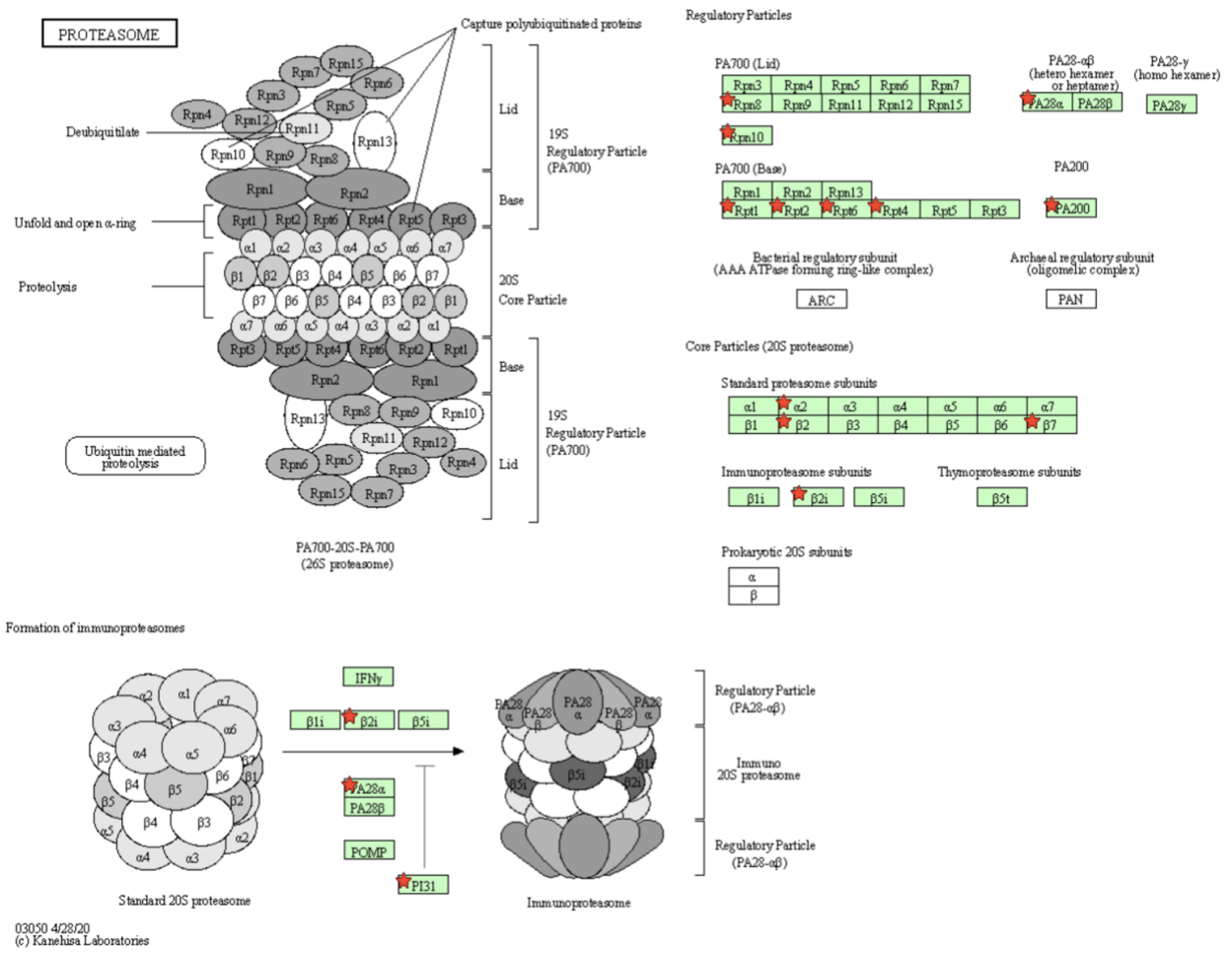


Figure 10. Components of ubiquitin-proteasome system are differentially expressed in PSC patients compared to healthy controls (marked with stars). Several components of regulatory particles such as Rpts with role in capturing polyubiquitinated proteins are dysregulated. The graph is generated by DAVID.

APOBEC3C (ENST00000361441) was significantly increased in RNA-seq with log2FC = 0.33, pvalue = 0.001, padj = 0.04 among PSC patients compared to healthy controls. Using simple linear regression, there was no significant correlation between APOBEC3C expression and Mayo score. Increase in APOBEC3A (ENST00000249116) among PSC patients compared to healthy controls was not significant, however, it was correlated with Mayo score (pvalue = 0.01, $R^2 = 0.26$) (Figure 11). Since APOBEC3 molecules are a part of interferon gamma induced immune components, we tested for correlation of IFN gamma as well as its receptors with disease progression (Figure 12).

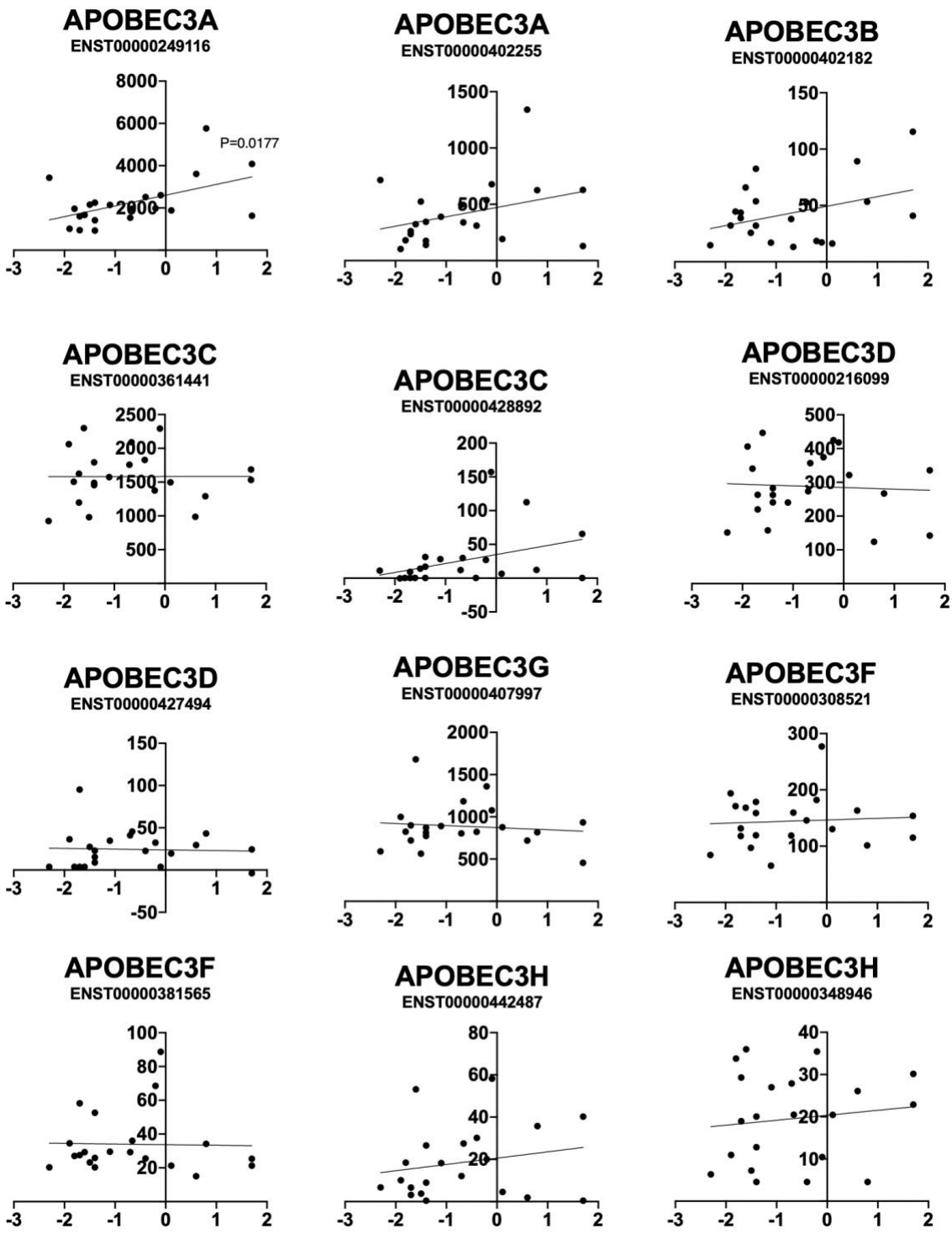


Figure 11. Correlation of APOBEC3 expression with Mayo score. X axis shows Mayo score, and Y axis shows the expression values. Each sample is shown as a point. Expression level of APOBEC3A (A) was correlated with Mayo score in PSC patients. A3B, A3C and A3H were also increased with disease progression, although the correlation was not significant. This implies that patients with poor prognosis express higher levels of different APOBEC3 subsets. The graph is generated by Prism and the details of analysis can be found in supplementary file 3.

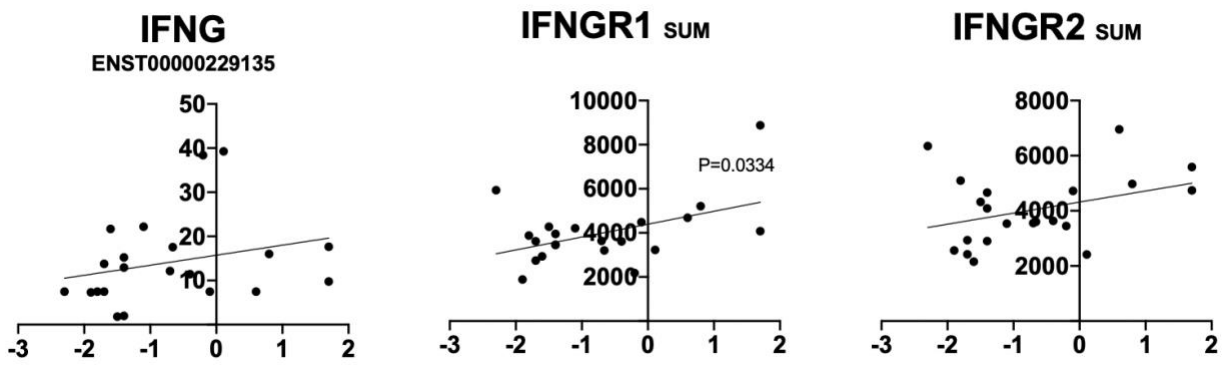


Figure 12. Correlations between expression of IFN gamma and its receptor components with Mayo score. X axis shows Mayo score, and Y axis shows the expression values. Each sample is shown as a point. The graphs are generated using simple linear regression in GraphPad prism. Complete equations and R squares for the tested correlations can be found in supplementary file 3. As shown, sum of the IFNGR1 transcripts was positively correlated with Mayo score and IFNG and IFNGR2 tend to increase with disease progression that is in line with the finding of increased APOBEC3 expression in patients with poor prognosis.

We also evaluated the correlation of Mayo score with CD4, CD8 and CD4/CD8 ratio to test potential biases of T-cells toward cytotoxic or helper immunophenotype (Figure 13). There is a trend for downregulation of CD3E (general T-cell marker) and CD4 with disease progression, although none were significant after correction by neutrophil marker to resolve the effect of hypersplenism on T-cell counts. Detailed data, equations and R squares can be found in supplementary file 3.

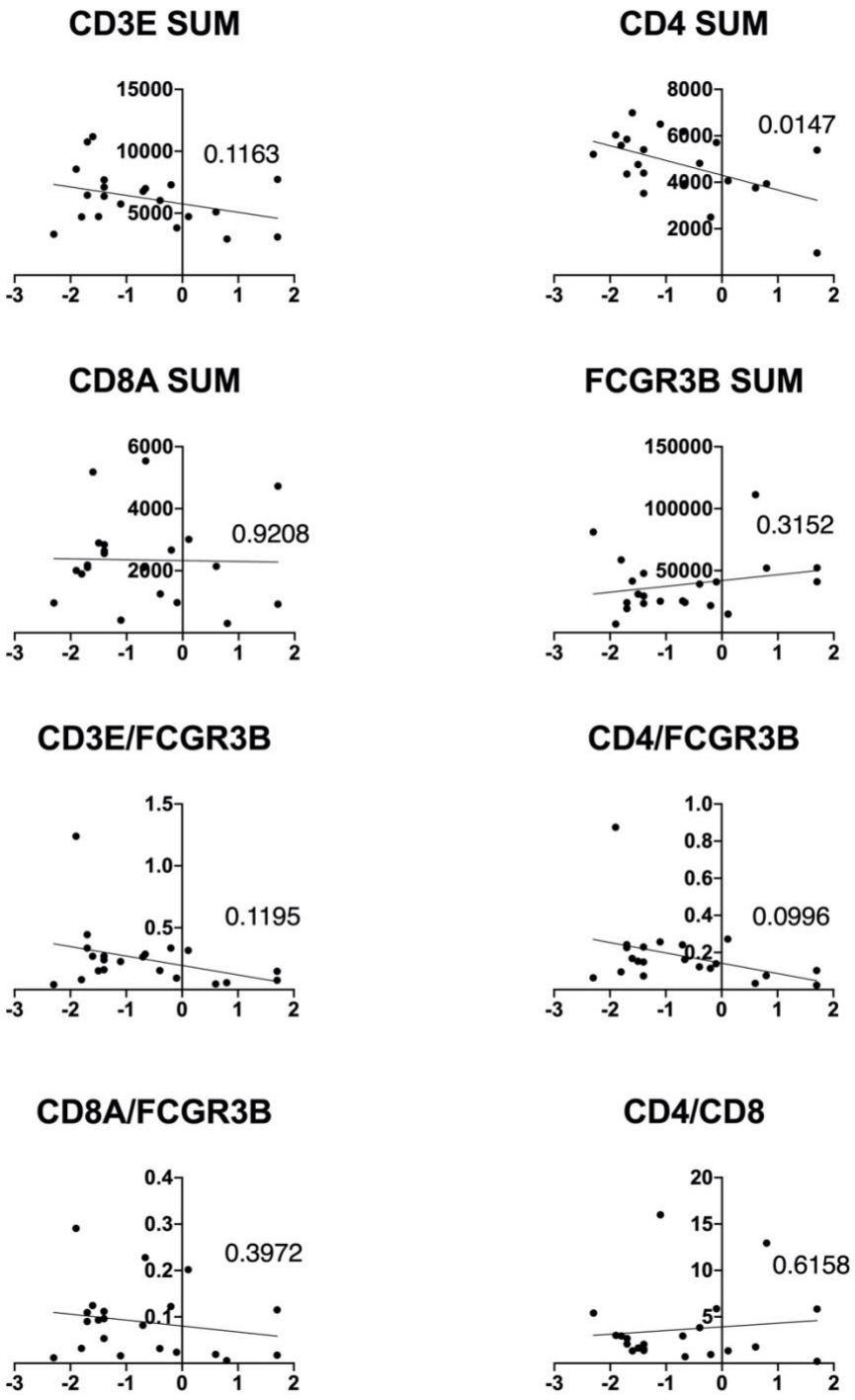


Figure 13. Simple linear regression with Mayo score using sum of protein coding transcripts of CD4, CD8, CD4/CD8 ratio and CD3E, and their corrected expression based on the expression of neutrophil marker (FCGR3B) to resolve the effect of hypersplenism on T-cell count in late-stage liver disease. After correction, although there was no significant correlation with Mayo score, there was a trend for CD3E and CD4 loss that suggest dysregulated adaptive immune response. In previous studies, CD4 T cells have shown reduced

apoptosis in PSC patients [202]. Increase in CD4/CD8 ratio highlights the importance of MHCII mediated immune response in advanced disease.

Differentially expressed genes among PSC patients compared to healthy controls, were aligned with disease related terms in Bioplanet database using Enrichr. Top 10 enriched terms are shown in Table 6.

Table 6. Overlapping genes with known disease related pathways using Bioplanet. There is a significant overlap between the differentially expressed genes in PSC compared to healthy controls with the genes that their dysregulation is involved in pathogenesis of HIV and influenza infections. Majority of these overlapping genes were the same as the overlapping genes in Cap-dependent translation initiation which can be hijacked by several viruses in order to force the host to translate viral mRNAs.

Enriched Terms	Overlap	P-value	Adjusted P-value
Disease	155/674	3.17E-22	4.78E-19
Gene expression	188/968	4.65E-18	3.51E-15
HIV infection	64/200	4.72E-17	2.37E-14
Translation	54/151	5.71E-17	2.15E-14
Influenza viral RNA transcription and replication	45/125	1.68E-14	5.09E-12
Cap-dependent translation initiation	33/73	2.56E-14	6.44E-12
Influenza infection	48/142	3.70E-14	7.97E-12
Cytoplasmic ribosomal proteins	40/108	1.62E-13	3.06E-11
HIV factor interactions with host	44/128	2.17E-13	3.64E-11
Messenger RNA processing	56/203	4.24E-12	6.40E-10

A significant overlap between differentially expressed genes in PSC patients and HIV infection (padj = 2.37E-14) and influenza infection (padj = 7.97E-12) were found.

Using the overlapping differentially expressed genes with HIV infection, most overlapping pathways were related to IL-1 mediated signalling (34.14%), transport of virus (24.9%) and intracellular transport of virus (22.49%) (Figure14).

The overlapping differentially expressed genes with influenza infection were mostly involved in nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (50%), 7-methylguanosine mRNA capping (33.33%) and ribosome assembly (12.96%) (Figure 15). Both

functional analyses (Figure14 and Figure 15) are created by ClueGo, showing the pathways with p < 0.05 and medium network specificity.

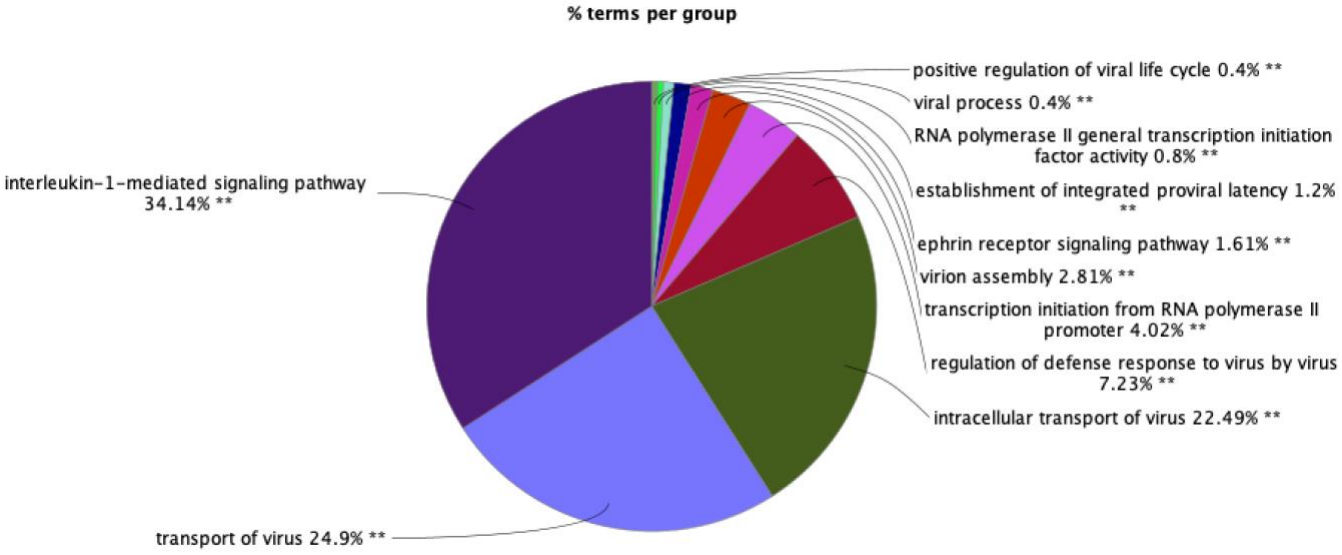


Figure 14. Enriched pathways using overlapping differentially expressed genes in PSC with HIV infection. IL-1 mediated signaling pathways and the pathways related to transport of virus are among top enriched pathways. The graph is generated by ClueGo.

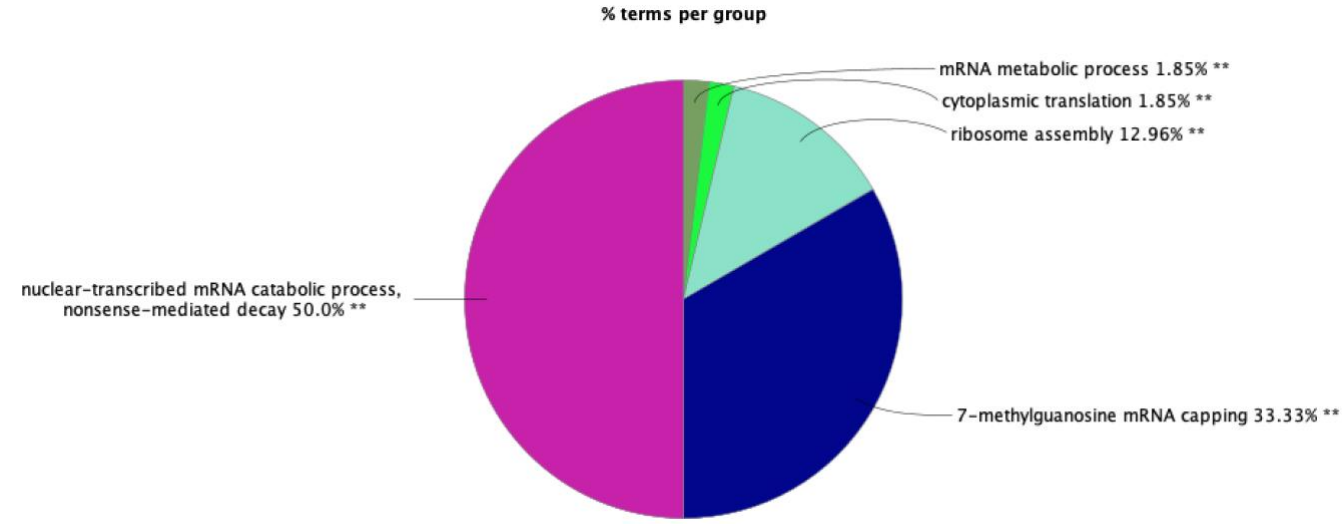


Figure 15. Enriched pathways using overlapping differentially expressed genes in PSC with influenza infection. Nuclear transcribed mRNA catabolic process, nonsense-mediated decay (NMD) and 7-mathaguanosine mRNA capping are among top enriched pathways is a potent inhibitor of retroviruses and positive-strand RNA viruses [203]. Several viruses such as Murine leukemia virus use 7-methylguanosine capping to stabilize their mRNA and evade cellular defense mechanisms with the cover of a host post-transcriptional modification [204]. The graph is generated by ClueGo.

Proteomics of biliary epithelial cells showed a significant dysregulation in proteasomal ubiquitination system among downregulated proteins (Figure 16). Among the enriched upregulated proteins in biliary epithelial cells, there was a significant overlap with the proteins affected by human papilloma virus, H5N1 Avian Influenza virus and Porcine reproductive and respiratory syndrome virus (Figure 17).

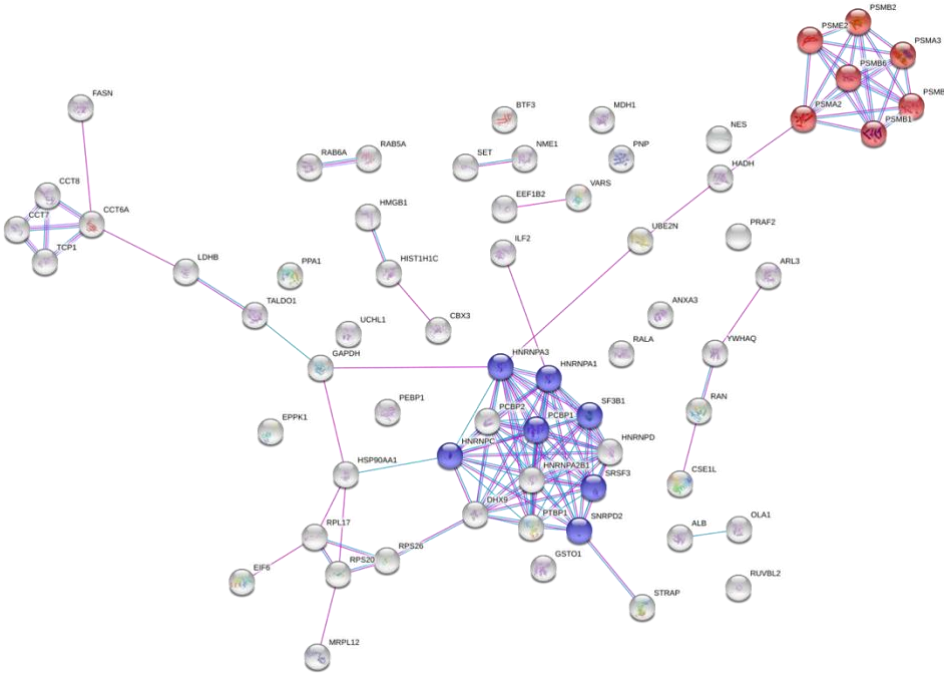


Figure 16. Functional enrichment analysis of downregulated proteins in biliary epithelial cells of PSC vs control. The red nodes show different proteasomal subunits such as PSMAs and the blue nodes show spliceosomal subunits such as HNRNPs. The graph is generated by STRING. Functional analysis showed negative regulation of "mRNA splicing via spliceosome and SRSF1, RNA recognition motif 1 as well as decreased proteasomal degradation". SRSF1 is a potent inhibitor of HIV-1 and its deletion results in inability to restrict viral replication, double-stranded DNA breaks and genomic instability [205, 206].

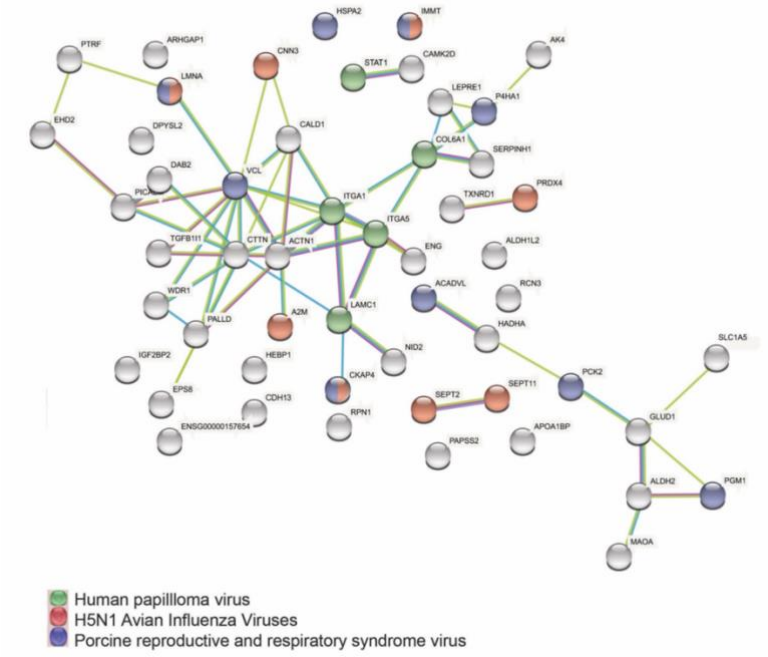


Figure 17. Functional enrichment analysis of upregulated proteins in biliary epithelial cells of PSC vs control. There are overlapping parts of the network with HPV, H5N1 Influenza virus and RSV infections. The graph is generated by STRING.

4. Discussion

In the present study, we investigated the whole genome transcriptomics of patients with PSC and compared their transcriptome signature with healthy controls. We found a marked increase in virally induced pathways that were comparable to HIV and influenza infection owing to an overlap between the differentially expressed genes with the genes involved in virion transport and assembly, halting host transcription and ubiquitin proteasome system (UPS). Expression levels of antiretroviral APOBEC3 deaminases were correlated with disease severity. Given the fact that none of these findings are solely specific to retroviral infections or capable of proving the hypothesis of virus involvement in the pathogenesis of PSC, we tried to put all the pieces together to probe a possible viral etiology for PSC. Herein, we classify dysregulated pathways in PSC compared to healthy controls into different cellular stress responses and the way they can be affected by a virus.

4.1. Endoplasmic reticulum stress response

Endoplasmic reticulum (ER) is responsible for the initial eukaryotic biosynthesis of secretory, luminal, and transmembrane proteins. Transportation of the synthetized protein from ER is conditional to proper folding and posttranscriptional modification. This process is taking place by interactions between the newly synthetized proteins and chaperons, as well as modifications such as glycosylation, and disulfide bond formation that help the protein to attain a 3D conformation [207-209]. Accumulation of misfolded or unfolded proteins inside the lumen of ER triggers a highly conserved adaptive system called unfolded protein response (UPR) [210]. Protein kinase RNA activated (PKR)-like ER kinase (PERK) is one the major UPR signaling pathways to perceive endoplasmic reticulum stress (ERS). PERK kinase mediates phosphorylation of the regulatory α subunit of eukaryotic translation initiation factor 2 (eIF2α) that leads to repression of translation and protein synthesis load [211].

In case of ER failure to restore normal folding process, the protein is translocated to the cytosol through the endoplasmic reticulum associated degradation (ERAD) pathway. In cytosol, the misfolded/unfolded protein is labeled by ubiquitin molecules and is degraded by UPS [212].

ER stress and subsequent UPR can be initiated by impaired folding process, or brisk overexpression of proteins (e.g., mutations and viral infections). In our study, there is evidence of ramping down in protein degradation and UPR. There is consistent evidence for the role of ER stress, UPR and immunoproteasome assembly either as early events or the consequences in the pathogenesis of sclerosing cholangitis and IBD [213-215]. In IBD, genetic and environmental factors (e.g., bacterial metabolites) have been speculated to be responsible for UPR activation [216-218]. In this regard, deletion of the genes involved in UPR (e.g. Xbp1 and Agr2) leads to a phenotype closely resemble to IBD in mice [216]. Whereas there is scarce data on the mechanistic explanation of ER stress in PSC. One of the proposed mechanisms for ER stress in cholestatic liver

disease is toxic injury by bile acids. This idea stems from a study in which feeding mice by bile acid led to hepatic expression of the UPR components [219, 220]. Moreover, in another study, bile duct ligation was found as a trigger of ER stress responses [221]. However, lowering bile acid concentration by ursodeoxycholic acid (UDCA) is not shown to be beneficial in treatment of PSC patients suggesting the presence of a factor more than bile-induced physical injury]222[.

There is growing evidence for a relationship between viral infection and UPR. While host utilizes the UPR to restrict the infection at a cost of blocking cellular mRNA translation, viruses attempt to manipulate the host UPR in favor of its own replication [223, 224]. Both phenomena lead to a pathogenic dysregulation in the host UPR. For instance, cells expressing phosphorylation-insensitive eIF2α have shown an increased viral protein synthesis in vesicular stomatitis virus (VSV) infection [225]; another example is the protein encoded by herpes simplex virus type I (HSV-1) that is homologous to the cellular dephosphorylation mediator of eIF2 [226]. Correspondingly, eIF2 has been shown to be downregulated in high risk compared to low risk PSC patients [213] that may suggest and inadequate cellular constraining ability against a cause of ER stress.

In addition, overrepresentation of virus-related pathways in transcriptomics of patients with PSC supports the hypothesis of virally triggered ER stress in PSC patients [214].

4.2. Interferon type I signaling pathways

UPR has the ability of direct stimulation of inflammatory signal transduction pathways and activation of major inflammatory transcription factors [227]. For example, hepatotropic viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) trigger ER stress response that leads to an upregulation in NF-κB and IFN gene regulatory elements, as well as increase in reactive oxygen species (ROS) [228-230]. IFN-γ response related pathways are overrepresented in

transcriptomics of PSC patients [214] and upregulated APOBEC3 deaminases is an example of this overrepresentation [176]. In line with our previous finding of increased APOBEC3C deaminase using RT-PCR, APOBEC3C was significantly increased in RNA-seq. Increase in APOBEC3A among PSC patients compared to healthy controls (that was found upregulated in RT-PCR), was not significant, considering different study subjects enrolled in two experiments that might be in different stages of disease. Noteworthy, persistently stressed ER releases calcium that initiates cytochrome c release from the mitochondrial matrix into the cytosol through opening of the permeability transition pores. While cytochrome c maintains a positive feedback loop on calcium release from ER, it also inhibits complex III of the electron transport chain leading to ROS formation. Hence, ER stress and mitochondria form a vicious circle and aggravate their dysfunction that eventually triggers apoptotic cascades. Other than the role of ER stress on mitochondria, several viruses have been shown to promote mitochondrial dysfunction, directly. For instance, HCV core protein, HBV X protein, Rabies virus phosphoprotein, HIV-1 viral protein R (Vpr) and Dengue virus can promote ROS-mediated mitochondrial permeability transition followed by induction of apoptosis [231-235].

Intriguingly, a substantial mitochondrial dysfunction has been shown a in both PSC and IBD [214, 236-238]. In experimental models of cholestatic diseases, impaired fatty acid oxidation and elevated acylcarnitine and ketone bodies detected in both serum and bile metabolome in PSC are proposed to be a consequence of mitochondrial dysfunction [238-242].

Besides, oxidative stress has been detected in a wide range of inflammatory diseases such as Crohn disease, ulcerative colitis (UC) and rheumatoid arthritis (RA) [243-245]. Likewise, patients with concurrent PSC and IBD have shown a significant dysregulation in antioxidant response and mitochondrial redox signaling [246]. In the same way, serum metabolome of patients with PSC

has revealed elevated biochemical markers of oxidative stress (e.g. free radical scavenger biliverdin) [239]. Moreover, in a study by Denk et.al, serum markers of apoptosis were increased among PSC patients and correlated with disease severity [247]. Nonetheless, other studies failed to show the apoptosis as a leading phenomenon in PSC [248-250].

Overall, it is not known whether the mitochondrial stress response and apoptosis are cause or consequence of inflammatory diseases, but they might occur as a result of prolonged ER stress response or direct interaction of a viral product with mitochondria. Strikingly, APOBEC3 deaminases that were upregulated with disease progression in our PSC patients, have been linked to mitochondrial stress response in different studies. First, they were found to be involved in catabolism of mitochondrial DNA that is leaked from damaged cells in chronic immune activation through generating U rich DNA that is unable to reanneal well that leads to subsequent attenuation of cytoplasmic danger signaling [251-253]. Second, it has been shown that not only interferon, but also hypoxic stress can induce APOBEC3-mediated editing events [254]. In this regard, a proposed effect of APOBEC3 during a mitochondrial stress response is to induce Warburg effect by dampening mitochondrial respiration in support of adaptation to hypoxic stress [255]. However, more studies are needed to investigate this effect experimentally. Hence, APOBEC3s should not be considered solely as antiviral components and their upregulation in PSC patients with disease progression may be a sign of cellular stress in response to wide variety of triggers not limited to viruses. Regardless of the trigger for the increase in APOBEC3 with disease progression, enhanced deamination of C can lead to formation of premature stop codons in mRNAs. Interestingly, the major enriched pathway using the overlapping differentially expressed genes in our study with influenza infection was nonsense mediated decay (NMD). NMD is a quality control system in which transcripts that harbor a premature stop codon are eliminated to prevent subsequent

formation of a nonfunctional or truncated protein [256]. In addition, viral RNAs with their multiple stop/termination codons due to the presence of multiple open reading frames are favorite substrates for NMD; several viruses especially positive-sense viruses such as alphaviruses and HCV, as well as retroviruses have been found to be restricted by NMD [203].

In short, our proposed model is the presence of a trigger such as viral infection, ischemia, mutation, or environmental factor in a genetically susceptible person that initiates ER stress and UPR with subsequent mitochondrial dysfunction that leads to apoptosis of biliary epithelial cells. Increase in APOBEC3 enzymes with disease progression may be a consequence of mitochondrial stress response to aid in catabolism of leaked mitochondrial DNA. The increase in APOBEC3 activity may lead to formation of premature stop codons and activation of nonsense mediated decay that was found in the pathway enrichment analysis.

4.3. Future direction and conclusion

Differences in immune system were identified in PSC vs controls. Aspects of this response have similarities to host-immune response to virus. However, further work is needed to show specificity to PSC and role of specific antigenic stimulation. A major limitation of this study is the difference between samples included in transcriptomics and proteomics. To substantiate the robustness of the findings, more studies with larger sample sizes with inclusion of groups with different liver diseases are warranted. Our findings may help in developing an animal model by using a virus specific to biliary epithelial cells and assessing the inflammatory responses and disease phenotype in the model in sake of replicating PSC. Advancement in deep sequencing approaches might be helpful in identifying the presence of the possible virus.

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