ESTROGEN RECEPTOR-MEDIATED MODULATION OF INFLAMMATORY MARKERS IN HEPATITIS C VIRUS PATHOGENESIS

By

SARAH GROOVER

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MODULATION OF INFLAMMATORY MARKERS IN

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Dissertation Approved:

Dr. Rashmi Kaul

Dissertation Adviser

Dr. Gerwald A. Koehler

Dr. Anil K. Kaul

Dr. Alexander J. Rouch

Dr. B. Bavette Miller

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Abstract: Hepatitis C virus (HCV) is a global health concern whose pathogenesis depends largely on chronic inflammation; chronic infection with HCV is strongly linked to fibrosis/cirrhosis, liver failure, and hepatocellular carcinoma (HCC). About 80% percent of people infected with HCV will go on to develop chronic infection, and polymorphisms in the *IFNL4* gene have been shown to be strong predictors for both clearance as well as response to treatment. Clinical observations have established sex-based differences in HCV infection with the disease progressing more severely and more rapidly in males and postmenopausal females compared to premenopausal females, suggesting that estrogens and their receptors may play an important role in hepatic defenses and development of HCV-mediated cirrhosis and HCC. However, the precise mechanism of estrogen protection is poorly understood. In the current study, the role of ERs in modulating innate immune responses was investigated *in vitro* using a liver hepatoma (Huh7) cell line, and *ex vivo* using human liver tissues with HCV/cirrhosis and HCV/HCC. The results of the *in vitro* experiments revealed that treatment of Huh7 cells with ER agonists (E2 and DPN) and antagonists (ICI) led to changes in expression of ERβ, and inflammatory markers TNFα, CD55, IL-33. These results indicate that estrogens may confer protection in HCV by increasing complement activation (CD55) and decreasing the damaging effects of long-term inflammation (TNFα, IL-33). The lack of response from PPT stimulation indicates that ERβ, not ERα, appears to be the governing ER in liver cells. In the *ex vivo* study, ERβ was reported for the first time to have a greater mRNA expression than ERα in normal liver. In addition, ERβ mRNA expression was found to be decreased in diseased livers, while TNFα expression was increased. ERβ mRNA expression was also decreased in livers with the *IFNL4*-ΔG/ΔG genotype. Further analysis of the data revealed sex-specific correlations between ERα and ERβ. All together, these findings indicate that changes in ERβ expression are associated with worsening disease, and may be one of the sex-dependent factors in HCV pathogenesis, particularly the differential interactions of ERα and ERβ between males and females.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SUPPLEMENTARY FIGURES

ABBREVIATIONS

Akt	protein kinase B
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AP-1	activator protein 1
AST	aspartate aminotransferase
cAMP	cyclic adenosine monophosphate
CMV	cytomegalovirus
CRP	complement regulatory protein
DAA	direct-acting antiviral
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DPN	diarylpropionitrile
E2	17β-estradiol
ER	estrogen receptor
ERE	estrogen response element
FBS	fetal bovine serum
GGT	gamma-glutamyl transferase
GPER	G-protein coupled estrogen receptor
GUSB	beta-glucuronidase
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HRT	hormone replacement therapy
HSC	hepatic stellate cell
ICI 182, 780	fulvestrant
IFN	interferon
IRES	internal ribosomal entry site
IRF	interferon regulatory factor
ISG	Interferon-stimulated gene
JNK	c-Jun N-terminal kinase
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral-signaling protein
MELD	Model for End-stage Liver Disease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MyD88	myeloid differentiation primary response 88 protein
NF-κB	nuclear factor kappa B
NNPI	NS5B non-nucleoside polymerase inhibitor
NPI	NS5B nucleoside polymerase inhibitor
NS	nonstructural
PAMP	pathogen-associated molecular pattern
PEG-IFN	pegylated interferon alpha
PI	NS3/4A protease inhibitor
PI3K	phosphoinositide 3-kinase
PKR	protein kinase R
PPT	propylpyrazole triol
PRR	pattern recognition receptor
Rb	retinoblastoma
RIG-I	retinoic acid inducible gene-I
ROS	reactive oxygen species
RT-qPCR	quantitative reverse transcription PCR
SERD	selective estrogen receptor downregulator
SOCS	suppressor of cytokine signaling
SRSF4	serine and arginine-rich splicing factor 4
ST2	serum stimulation-2
STAT	signal transducer and activator of transcription
SVR	sustained viral response
T_H	T helper cell
TLR	toll-like receptor
TNFR	TNF receptor
TNFα	tumor necrosis factor alpha
TRIF	Toll/IL-1 receptor domain–containing adapter inducing IFN-β
UTR	untranslated region
wt	wild-type

CHAPTER I

INTRODUCTION

Background

Hepatitis C virus (HCV) is a global health concern that indiscriminately affects individuals in both underdeveloped and industrialized countries; over 2 million people in the United States alone are infected with the virus (Hofmeister, Rosenthal et al. 2019). HCV is adept at manipulating the innate immune response to avoid recognition of infected hepatocytes and thus controlling the development of robust adaptive immune responses needed for clearing the virus (Horner and Gale 2013). Chronic infection with HCV causes affected individuals to be highly susceptible to liver damage, liver failure, and cancer; HCV is the most common indication for liver transplantation in developed countries (Dubuisson and Cosset 2014). Notably, HCV-associated diseases such as cirrhosis and hepatocellular carcinoma (HCC) are usually not the result of direct cytotoxic factors by the virus itself; rather, these complications manifest from the long-term inflammation created by the host immune system in response to the persistent pathogen (Wynn 2008, Hoshida, Fuchs et al. 2014).

Only about 20% percent of people infected with HCV are able to spontaneously clear the virus without treatment (Liang, Rehermann et al. 2000). Spontaneous clearance of HCV infection depends on epidemiological, virus, and host factors (Micallef, Kaldor et al. 2006, O'Brien, Yang

et al. 2018). For example, genetic variants, such as *IFNL4* gene polymorphisms, have been shown to be strong predictors for both clearance as well as response to treatment (Aka, Kuniholm et al. 2014, Meissner, Bon et al. 2014, O'Brien, Pfeiffer et al. 2015), but the mechanism of action is not fully understood. Although there are several successful direct-acting antivirals (DAA) on the market today that can effectively cure an individual with HCV, they are extremely expensive, costing patients anywhere from $25,000 to $150,000 for the minimum recommended 12 weeks of treatment (Tabano 2018). The minimal side effects of DAAs greatly contrast the harsh interferon-based regimens that preceded them, but they are not without consequence. Like their name suggests, DAAs are designed to target specific HCV proteins, and as a result of the high genetic variability of the virus, patients undergoing treatment may develop a resistance to a particular DAA (Li and Chung 2019). Patients with advanced liver disease have an even shorter list of DAAs that are safe for treatment (US Food and Drug Administration 2019). DAAs have also been associated with a decrease in hormonal contraception efficacy (Baden, Rockstroh et al. 2014). Taken together, these studies highlight the need for alternative targets and the development of novel therapies.

Previous research has established that, compared to premenopausal females, both males and postmenopausal females are at a greater risk for developing HCV-associated diseases, and that their disease progression tends to be faster and more severe (Poynard, Bedossa et al. 1997, Chiaramonte, Stroffolini et al. 1999, Khan, Farrell et al. 2000, Poynard, Ratziu et al. 2001, Di Martino, Lebray et al. 2004, Corsi, Karges et al. 2016, Ryerson, Eheman et al. 2016). This suggests that estrogens and their receptors may play an important role in disease progression (Kalra, Mayes et al. 2008). Several epidemiological studies have reported that in females with HCV infection, lower circulating levels of estrogen are associated with a decreased response to treatment, increased expression of proinflammatory cytokines, more severe and faster rate of fibrosis, and greater risk of developing HCC (Yu, Chang et al. 2003, Di Martino, Lebray et al.

2004, Codes, Asselah et al. 2007, Villa, Karampatou et al. 2011, Hassan, Botrus et al. 2017). Estrogen has also been shown to downregulate proinflammatory cytokine expression (Ralston, Russell et al. 1990, Rogers and Eastell 2001, Evans, Lai et al. 2002), inhibit HCV entry and production (Hayashida, Shoji et al. 2010, Ulitzky, Lafer et al. 2016, Magri, Barbaglia et al. 2017), and decrease collagen deposition (Yasuda, Shimizu et al. 1999, Xu, Gong et al. 2002), but causal factors linking estrogen to disease protection remain speculative. Estrogens exert their action primarily by activating nuclear estrogen receptors (ERs), of which there are two subtypes, ERα and ERβ. Previous studies in our lab have shown differential expression of ERα and ERβ in the livers of both males and females and across HCV-related disease severity (Iyer, Kalra et al. 2017). Further, the diseased livers showed significantly higher expression of inflammatory marker nuclear factor-κB (NF- κB) and oncogenic marker cyclin D1.

Current Study

Based on the results of our previous studies on ER regulation of HCV-related pathogenesis, it was hypothesized that estrogen and ERs play a role in modulating inflammation caused by chronic HCV infection. Thus, this dissertation work was undertaken to evaluate the association between innate immune inflammatory markers (CD55, TNFα, and IL-33) and estrogen and/or ERs in HCV pathogenesis. The association of *IFNL4* polymorphisms with ER and inflammatory markers was also investigated. There were two primary aims of this study:

Specific Aim 1. To determine whether estrogen treatment affects the expression of ER subtypes and/or inflammatory markers in the human hepatoma cell line Huh7

For this, Huh7 cells were treated with varying levels of 17β-estradiol (E2) and the change in mRNA and protein expression of ERα and ERβ, and innate immune markers CD55, TNFα, IL-33 was measured. The results were verified by treating the cells with ER agonists PPT and DPN and ER antagonist ICI 182,780.

Specific Aim 2. To determine whether ER expression and/or *IFNL4* genotype is correlated with the expression of inflammatory markers in patients with HCV-associated diseases. For this, mRNA and protein expression of ERα, ERβ, TNFα, CD55, and IL-33 was measured in human liver tissues from patients with HCV/cirrhosis and HCV/HCC, and samples were genotyped for *IFNL4* variants.

Understanding the link between sex, estrogen receptors, and inflammation in HCV pathogenesis will help identify new targets for therapy, further personalize treatment strategies, and recognize individuals who are at a higher risk for HCV-related HCC disease progression.

CHAPTER II

REVIEW OF LITERATURE

Hepatitis C Virus

Chronic infection with hepatitis C virus (HCV) affects approximately 2% of the world's population, amounting to over 170 million total individuals (Shiffman and Benhamou 2015, Petruzziello, Marigliano et al. 2016). While less developed countries in Africa and Asia tend to have higher rates of infection compared to more developed countries, such as those located in North America and Europe, HCV is the most common blood borne infection in the United States, affecting over 2 million people across the nation (Hofmeister, Rosenthal et al. 2019). Due to advanced diagnostic tests and drug treatment regimens, the incidence of HCV is decreasing in developed countries, but because of the latent nature of the virus and the persistence of the disease, it will take decades to decrease HCV-related mortality rates. Up to 5% of all individuals ever infected with HCV will die from cirrhosis or cancer (Zein 2000, Dubuisson and Cosset 2014).

Initially described in 1975 as non-A, non-B hepatitis virus, HCV was the first virus discovered without the direct use of biologic methods (Zein 2000). Instead, in a collaboration between Chiron Corporation and the Centers for Disease Control and Prevention, scientists cloned HCV cDNA by extracting plasma from a chimpanzee infected with non-A, non-B hepatitis (Choo, Kuo

et al. 1989, Kuo, Choo et al. 1989). The host immune response to infection is believed to be the primary cause of HCV-associated liver injury and disease, as opposed to direct cytotoxic factors from the virus. While acute inflammation is necessary for effectively clearing pathogens from the body, if the stimulus persists then it may develop into chronic inflammation. Chronic inflammation kills host cells and damages DNA and is generally considered detrimental to the host organism. HCV pathogenesis is a complex process because the acute immune mechanisms employed to eliminate the virus are chronically detrimental to host tissues and organs.

Immunobiology of HCV-Related Diseases

Initial Infection with HCV. HCV is primarily spread through exposure to infected blood, such as through blood transfusions, injection drug use, or occupational exposures; there have also been reports of HCV infection via high-risk sexual contact and mother-to-child transmission (Alter 2007, Ghasemi, Rostami et al. 2015, Shiffman and Benhamou 2015). HCV virions are small, 50–80 nm in diameter, and consist of an icosahedral nucleocapsid, which includes the single-stranded, positive-sense RNA genome and core protein, surrounded by a lipid membrane viral envelope. HCV RNA consists of a single open reading frame and two untranslated regions (UTRs), one on each side of the coding sequence. Within the 5' UTR is an internal ribosomal entry site (IRES), and after the virus travels through the bloodstream and enters a hepatocyte, the IRES facilitates initiation of translation of the HCV genome into a single polyprotein composed of 3100 amino acids (Dubuisson and Cosset 2014, Ploen and Hildt 2015). Viral and cellular proteases cleave the viral polyprotein into mature structural proteins (core, E1, E2), which form

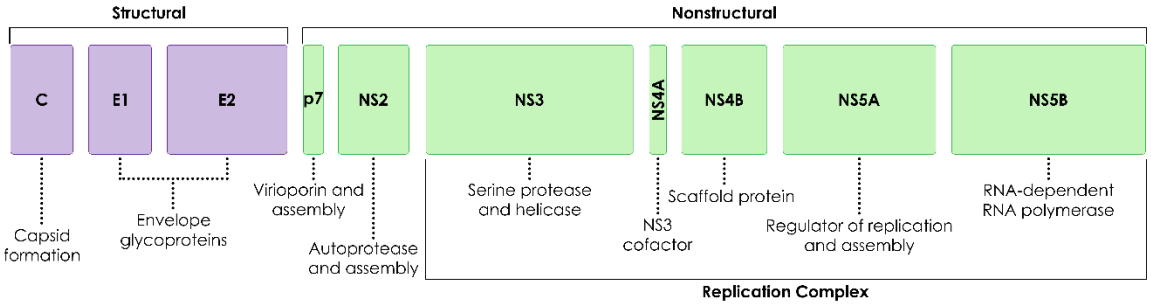


Figure 1: Hepatitis C virus genome organization and protein functions

NS: nonstructural

the viral particle, and nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B), which are subsequently involved in genome replication and protein assembly (Figure 1). The assembled virus particles then bud off the endoplasmic reticulum and are transported out of the cell in association with host lipoproteins. This unique association with lipoproteins in addition to the variability of HCV, particularly in the hypervariable region of structural protein E2, may aid in evasion of the host immune response (Bankwitz, Steinmann et al. 2010, Prentoe, Jensen et al. 2011). In fact, HCV has a number of mechanisms that contribute to its ability to establish a chronic infection with a high success rate.

Innate Immune Response. Humoral responses against the highly conserved HCV core protein are among the first reactions to infection in a patient. During the viral replication process, pattern recognition receptors (PRRs) in hepatocytes detect and bind endosomal and cytosolic pathogen-associated molecular patterns (PAMPs) from the HCV particles (Table 1). PRR recognition of HCV PAMPs initiates the activation of innate and adaptive immune responses via signaling proteins including mitochondrial antiviral-signaling protein (MAVS), Toll/IL-1 receptor domain–containing adapter inducing IFN-β (TRIF), and myeloid differentiation primary response 88 protein (MyD88) (Yoneyama and Fujita 2009, Park and Rehermann 2014, Lee, Tian et al. 2015). Ultimately, these pathways lead to the activation of downstream effector molecules, including nuclear factor-κB (NF-κB) and interferon (IFN) regulatory factors (IRFs) (Yoneyama and Fujita 2009, Horner and Gale 2013). NF-κB is a major transcription factor involved in the downstream production of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), and IRFs stimulate the production of IFNs (Yoneyama and Fujita 2009, Lee, Tian et al. 2015). One factor that can contribute to chronicity is the ability for HCV NS3/4A proteases to block PRR signaling by cleaving signaling proteins such as MAVS and TRIF, thereby mitigating host antiviral defenses (Foy, Li et al. 2003, Foy, Li et al. 2005, Li, Foy et al. 2005, Li, Sun et al. 2005).

Table 1: Summary of pattern recognition receptors

PRR (location)	PAMP	Signaling Protein	Effector Molecules
RIG-I (cytosol)	ssRNA, short dsRNA	MAVS	NF-κB, IRF-3
PKR (cytosol)	dsRNA (HCV IRES)	MAVS	NF-κB, IRF-3
TLR3 (endosome)	long dsRNA	TRIF	NF-κB, IRF-3
TLR7/8 (endosome)	ssRNA	MyD88	NF-κB, IRF-7

IRES: internal ribosomal entry site; IRF: interferon regulatory factor; MAVS: mitochondrial antiviral signaling protein; MyD88: Myeloid differentiation primary response 88; NF-κB: nuclear factor-κB; PKR: protein kinase R; RIG-I: retinoic acid inducible gene-I; TLR: toll-like receptor; TRIF: Toll/IL-1 receptor domain–containing adapter inducing IFN-β

Tumor necrosis factor alpha

TNFα is a pleiotropic cytokine that is involved in a variety of functions in the liver such as proliferation, metabolic activation, inflammatory responses, and apoptosis. TNFα facilitates the activation of several diverse signaling cascades, depending on its ligation to either TNF receptor 1 (TNFR1) or TNFR2. TNFR1 signaling can mediate the cleavage and activation of pro-apoptotic caspase-8; TNFR2 initiates the anti-apoptotic, pro-proliferative protein kinase B (Akt) pathways; and both receptors are involved in the activation of pro-apoptotic, pro-proliferative c-Jun N-terminal kinases (JNKs) and the regulation of the proinflammatory, anti-apoptotic NF-κB pathway (Liedtke and Trautwein 2012, Kalliolias and Ivashkiv 2016). TNFR1 plays the leading role in liver physiology (Liedtke and Trautwein 2012).

Because TNFα can initiate both cytoprotective and apoptotic responses, its role in HCV pathogenesis is unclear. Individuals with *TNFA* genotypes that trigger increased production and secretion of TNFα were associated with having an increased risk of fibrosis and cirrhosis (Dai, Chuang et al. 2006), possibly due to the action of HCV proteins suppressing NF-κB activation and enhancing TNFα-induced cell death (Park, Kang et al. 2012). Clinical studies have also suggested that overexpression of TNFα increases cellular levels of reactive oxygen species (ROS) and ultimately causes resistance to IFN-α treatments (Larrea, Garcia et al. 1996).

Interferons

IFNs are divided into three types (I, II, and III) depending on their associated receptor. In the case of HCV, type I (IFN-α and -β) and type III (IFN-λ) predominate (Metz, Reuter et al. 2013). Type I IFNs are well-known cytokines that respond to viral infections. When IFNs are produced, they

bind to their respective receptors to initiate the JAK/STAT signal transduction pathway that in turn upregulates interferon-stimulated genes (ISGs) (Metz, Reuter et al. 2013). Hundreds of ISGs have been identified and they are generally associated with antiviral capabilities such as inhibition of individual steps in the virus life cycle (Metz, Reuter et al. 2013). However, HCV core protein upregulates suppressor of cytokine signaling (SOCS)3 and SOCS1 expression, which are inhibitors of the JAK/STAT pathway (Miyoshi, Fujie et al. 2005, Yao, Waggoner et al. 2005, Zhang, Ma et al. 2011, Collins, Ahmed et al. 2014).

A polymorphism in a recently discovered type III IFN, *IFNL4*-ΔG/TT (rs368234815), has been strongly associated with HCV clearance and response to treatment (Prokunina-Olsson, Muchmore et al. 2013). Contrary to the typical antiviral function of INFs, it is individuals who carry the *IFNL4*-ΔG deletion allele, and therefore generate the full IFN-λ4 protein, who have impaired HCV clearance and response to treatment (Prokunina-Olsson, Muchmore et al. 2013, Aka, Kuniholm et al. 2014, Meissner, Bon et al. 2014, O'Brien, Pfeiffer et al. 2015). In chronic HCV infection, hepatic expression of IFN-λ4 protein by ΔG-carrying patients induces high levels of antiviral ISGs and low levels of suppressive ISGs (Noureddin, Rotman et al. 2015, Murakawa, Asahina et al. 2017) suggesting that overwhelming the IFN response pathways prevents such patients from further upregulating ISGs in their livers upon treatment with IFN-based therapies, thus allowing the virus to persist (Chinnaswamy 2014). Ramamurthy et al. offered an explanation for the paradoxical role of the IFN-λ4 protein: the difference in genotypes may not be necessarily in the specific activity of the molecules, but rather in the regulation of the HCV-induced

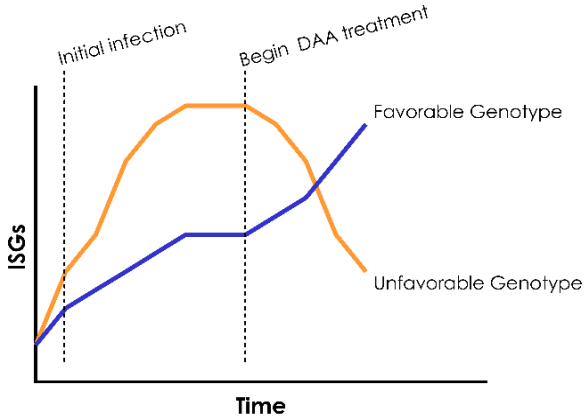


Figure 2: Model of hypothesized mechanism for ISG regulation by IFN-λ4 after initial infection with HCV and no spontaneous clearance

as proposed by Ramamurthy, Marchi et al. (2018)

ISG: interferon-stimulated gene

immune pathways at different viral load levels (Ramamurthy, Marchi et al. 2018) (Figure 2). While it would be impractical for physicians to genotype all HCV-positive patients for *IFNL4* (since the results would not necessarily change or inform a treatment plan), studying the molecular pathways involved may lead to a better understanding of why only a fraction of individuals are able to clear the virus on their own.

Complement Regulation. The complement system is a multifaceted component of the innate immune response that functions to recognize and eliminate both foreign pathogens as well as damaged self-cells. The complement system can be activated via the classical, lectin, or alternative pathways. All three pathways have a cascade of events that lead to the cleavage of C3 by C3 convertase into C3a and C3b fragments, which can go on to promote inflammation, opsonize pathogens, or initiate the formation of membrane attack complexes (MACs). Initiation of the classical and lectin pathways is a straightforward on/off process, where antigen-antibody complexes trigger the former and the binding of mannose-binding lectin to carbohydrates on the surface of pathogens triggers the latter (Ricklin, Hajishengallis et al. 2010). The alternative pathway, on the other hand, is a bit more complicated. Most C3 molecules are relatively inert, but a small percentage of the molecules can spontaneously hydrolyze or "tick-over" to $C3_{H2O}$ (Lachmann and Nicol 1973, Pangburn, Schreiber et al. 1981). In the absence of any regulatory proteins, factor B protease binds $C3_{H2O}$, and this complex is cleaved to form C3 convertase that activates complement by cleaving C3 into C3a and C3b. The complement system is tightly regulated in order to prevent autoimmune damage. Membrane-associated complement regulatory proteins (CRPs) that are expressed on most human cell types include CD35, CD46, CD55, and CD59 (Zipfel and Skerka 2009). They act at three critical points of the complement system: CD35 and CD55 accelerate the decay of convertases, CD35 and CD46 act as cofactors for factor I-mediated cleavage of C3b, and CD59 inhibits MAC formation (Pio, Corrales et al. 2014).

Many viruses take advantage of the complement regulation system in order to evade the host immune response. Vaccinia virus, for example, secretes a structural homolog to the CRPs that allows it to inhibit the formation of and accelerate the decay of C3 convertase (Kotwal, Isaacs et al. 1990, McKenzie, Kotwal et al. 1992). Cytomegalovirus (CMV) has been shown to upregulate CD55 and CD46 on infected cells (Spiller, Morgan et al. 1996). Vaccinia virus, CMV, human immunodeficiency virus (HIV), and influenza virus have all been reported to escape complement attack by acquiring and incorporating CRPs into their viral envelopes (Saifuddin, Parker et al. 1995, Spear, Lurain et al. 1995, Saifuddin, Hedayati et al. 1997, Vanderplasschen, Mathew et al. 1998, Shaw, Stone et al. 2008). HCV has also been observed to avoid MAC-mediated lysis by incorporating CD59 into its viral envelope (Amet, Ghabril et al. 2012). This alone, however, would still leave the virus susceptible to opsonization and phagocytosis. Alas, HCV can evade this function of the complement system too, using its NS3/4A proteases to cleave C4, therefore preventing the formation of C3 convertase in classical and lectin pathways (Mawatari, Uto et al. 2013).

Various complement components have been observed to have both anti-tumor and pro-tumor functions. Complement activation has been associated with liver steatosis, necrosis, and fibrosis (Rensen, Slaats et al. 2009, Vasel, Rutz et al. 2014) and blocking complement activation can diminish hepatic inflammation (Chang, Yeh et al. 2009). On the other hand, CD55 and other CRPs can act to promote carcinogenesis by inhibiting the action of complement on tumor cells (Nowicki, Nowicki et al. 2001, Zhao, Zhu et al. 2009, Wang, Yang et al. 2017).

Adaptive Immune Response. Unlike innate immune responses to HCV which are generated within hours to days after infection, it takes approximately 6-8 weeks before adaptive immune responses become detectable, corresponding to the onset of hepatitis (Thimme, Oldach et al. 2001, Thimme, Bukh et al. 2002). Although most infected individuals will produce neutralizing antibodies and begin to mount T cell responses, up to 80% will develop into chronic infection

(Seeff 2009). The virus is highly adept at escaping attack, so effective clearance of HCV requires a strong and sustained CD4+ and CD8+ T cell-mediated response (Diepolder, Zachoval et al. 1995, Missale, Bertoni et al. 1996, Cooper, Erickson et al. 1999, Lechner, Wong et al. 2000, Thimme, Oldach et al. 2001). HCV can suppress effector T cells by impairing antigen presentation in dendritic cells (O'Beirne, Mitchell et al. 2009) and by inducing IL-10, which promotes regulatory T cell functions (Keoshkerian, Hunter et al. 2016, Ren, Zhao et al. 2016).

HCV can also inhibit T cell responses in a complement-dependent pathway. The classical pathway is activated when complement subcomponent C1q (part of the C1 complex) binds to antigen-antibody complexes. The gC1qR protein is a membrane protein that can inhibit complement activation by binding to C1q (Ghebrehiwet and Peerschke 1998). Expression of gC1qR can be found on most cell types, and upon activation, can inhibit cell proliferation (Ghebrehiwet, Habicht et al. 1990, Chen, Gaddipati et al. 1994). Like C1q, HCV core protein can also bind to gC1qR expressed on T cells to inhibit proliferation by downregulating both IL-2 and its receptor, which are critical for T cell growth and differentiation (Kittlesen, Chianese-Bullock et al. 2000, Yao, Nguyen et al. 2001). Core protein binding to gC1qR expressed on human monocyte/macrophages upregulates expression of negative immune regulator PD-1 and inhibits IL-12 cytokine production, to further block T cells at various stages of response (Eisen-Vandervelde, Waggoner et al. 2004, Ma, Ni et al. 2011, Zhang, Ma et al. 2011).

HCV-Associated Diseases

Acute and Chronic Hepatitis. After initial contact with HCV, the infected hepatocytes will undergo an incubation period of 6–10 weeks (Manickam and Reeves 2014). Some symptoms of HCV infection include fever, fatigue, loss of appetite, nausea, joint pain, and jaundice, but most cases of both acute and chronic infection tend to be asymptomatic, hence it is rarely diagnosed early. However, should the patient undergo a routine blood test that measures liver function and enzymes, increased alanine aminotransferase (ALT) levels can be observed within 8–12 weeks of

exposure (Manickam and Reeves 2014). In 2020, the US Preventive Services Task Force updated HCV screening recommendations to all adults from 18 to 79 years (Force, Owens et al. 2020).

Liver Fibrosis and Cirrhosis. Chronic infection with HCV and prolonged inflammation cause hepatocytes to die and the liver begins to repair itself by accumulating excess connective tissue and extracellular matrix. Over several decades, this can cause fibrosis, irreversible liver damage, and carcinogenesis (Boyer and Marcellin 2000, Arzumanyan, Reis et al. 2013). Fibrosis is facilitated by perisinusoidal hepatic stellate cells (HSCs), which are the primary collagen producers in the liver. Activation of HSCs occurs through hepatocyte infection and cell death, and the subsequent immune response from both events. The most advanced stage of fibrosis is cirrhosis, characterized by degeneration of the normal hepatic architecture and formation of structurally abnormal nodules, at which point reversal is very difficult (Anthony, Ishak et al. 1978, Mormone, George et al. 2011). An estimated 15-20% of adults will develop cirrhosis within 20 years of developing a chronic HCV infection (Thein, Yi et al. 2008). HCV accounts for approximately a quarter of cirrhosis cases worldwide (Perz, Armstrong et al. 2006) with other causes including hepatitis B virus, alcohol, obesity, and diabetes. In developed countries, cirrhosis caused by chronic HCV infection is the sixth most common cause of death and the most common indication for liver transplantation, with up to 50% of all liver transplant patients testing positive for anti-HCV (Curry 2004, Brown 2005). While biopsy is the gold standard for diagnosing and staging fibrosis/cirrhosis, physical examinations, radiologic imaging tests, and serologic data can offer less invasive ways to estimate the amount of hepatic fibrosis present (Ansaldi, Orsi et al. 2014).

Serologic tests for liver function include enzyme tests and serum bilirubin. Enzyme tests include ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT). While liver enzymes and bilirubin are usually elevated in patients with cirrhosis, they can also be elevated in other disease pathologies, and normal liver enzyme results

do not rule out the possibility of liver disease (Goldberg and Chopra 2016). Because kidney failure is a major complication of advanced liver disease and is associated with poor prognosis, tests for kidney function, such as serum creatinine, may also be employed (Giannini, Botta et al. 2004, Goldberg and Chopra 2020).

Interleukin-33

Interleukin-33 (IL-33), an IL-1 family nuclear cytokine, is a crucial mediator in many inflammation-driven pathologies, playing both a pro- and anti-inflammatory role depending on the disease and model (Molofsky, Savage et al. 2015, Liew, Girard et al. 2016, Cayrol and Girard 2018). Because IL-33 lacks a signal sequence, it is thought to be released from cells following cellular damage or mechanical injury and serve as an alarmin. Once in the extracellular space, it regulates immune responses by binding to serum stimulation-2 (ST2) (Lingel, Weiss et al. 2009, Liu, Hammel et al. 2013, Tominaga, Ohta et al. 2016) which triggers the activation of NF-κB and mitogen-activated protein kinases (MAPKs) in a MyD88-dependent manner (Schmitz, Owyang et al. 2005, Andrade, Iwaki et al. 2011). ST2 is a transmembrane protein expressed on most myeloid and lymphoid immune cells, allowing IL-33 to support both type 1 and type 2 immune responses as well as aid in tissue repair. IL-33 observes strict nuclear localization and is constitutively expressed in humans by endothelial, epithelial, and fibroblast-like cells (Carriere, Roussel et al. 2007, Moussion, Ortega et al. 2008). An *in vivo* study using a knock-in mouse model described how deletion of the N-terminal nuclear domain of IL-33 resulted in elevated levels of IL-33 in the serum and ST2-dependent inflammation-induced lethality, suggesting that nuclear localization of IL-33 may have been naturally selected in order to restrict its own harsh proinflammatory activity (Bessa, Meyer et al. 2014, Liew, Girard et al. 2016).

IL-33 has been implicated in fibrotic diseases of the lung (Luzina, Pickering et al. 2012, Luzina, Kopach et al. 2013, Li, Guabiraba et al. 2014), skin (Rankin, Mumm et al. 2010, Yanaba, Yoshizaki et al. 2011, Zhang, Yan et al. 2014), and pancreas (Masamune, Watanabe et al. 2010,

Nishida, Andoh et al. 2010), so its role in liver is of much interest. In normal livers, the main source of IL-33 is sinusoidal endothelial cells, and in fibrotic livers, activated HSCs (Marvie, Lisbonne et al. 2010). Hepatic expression of IL-33 has been directly observed to be a driver in liver fibrosis by activating HSCs and enhancing hepatic inflammation (McHedlidze, Waldner et al. 2013, Tan, Liu et al. 2018).

Both intracellular and serum IL-33 levels tend to be higher in patients with hepatic fibrosis or cirrhosis, and lower in normal patients or those with liver cancer (Marvie, Lisbonne et al. 2010, Wang, Zhao et al. 2012, Yang, Wang et al. 2016, Tan, Liu et al. 2018). However, there are some studies that have found no difference in IL-33 expression between normal and cirrhosis patients (Yang, Wang et al. 2016), or found an increase in hepatic expression in neoplastic tissues compared to non-tumor tissues (Zhang, Liu et al. 2012, Bergis, Kassis et al. 2013).

Hepatocellular Carcinoma. Chronic fibrotic diseases account for nearly 45% of all deaths in industrialized nations, and this cycle of cell death and regeneration can facilitate the accumulation of mutations, transforming hepatocytes to malignant cells (Wynn 2008). Primary hepatic cancer is the second leading cause of cancer death in males worldwide, and cancer of the hepatocytes, otherwise known as hepatocellular carcinoma (HCC), is the most common type of liver cancer (Rusyn and Lemon 2014). HCV accounts for 25% of HCC cases worldwide (Alter 2007).

Unlike other carcinogenic viruses such as papillomaviruses, which incorporate their genomes into nuclear DNA to directly impair normal cell cycle checkpoints, HCV replicates in the cytoplasm and therefore cannot integrate into the host genome. Instead, the primary cause of HCV-associated HCC is believed to be the host immune response to chronic infection.

HCV does possess some cellular and molecular mechanisms that may directly contribute to carcinogenesis. When the virus infects the host cell, retinoblastoma (Rb) protein inhibits cell growth and proliferation until the environment is favorable; HCV protein NS5B has been shown

to bind to cytoplasmic Rb to reinitiate cell proliferation (Munakata, Nakamura et al. 2005, Walters, Syder et al. 2009). NS3, NS5A, and core protein are also thought to interfere with well-known tumor suppressor p53 (McGivern and Lemon 2011). It has also been shown that HCV protein NS4B activates a cascade of events leading to deregulation of cell transformation and apoptosis (Li, Zhang et al. 2012).

Current Treatments

Patient response to treatment is measured by sustained viral response (SVR), which is characterized by the absence of detectable HCV RNA in blood serum for at least 12 weeks after discontinuing treatment; achieving SVR has been referred to as being "cured," as recurrence is found in fewer than 1% of patients over the following 5-8 years (Shiffman and Benhamou 2015). Achieving SVR decreases the risk of both HCC and death by 75-85% (Morgan, Baack et al. 2013, Kanwal, Kramer et al. 2017, Backus, Belperio et al. 2019). Genotype must be taken into account when developing a drug treatment regimen. HCV genotype 1 (HCV-1) followed by genotype 3 (HCV-3) are the most common genotypes, accounting for 46.2% and 30.1%, respectively, of all cases worldwide (Ghasemi, Rostami et al. 2015). Seventy percent of HCV infections in the US are HCV-1 (Manickam and Reeves 2014).

Interferon-Based Treatments. Interferon-based drug regimens have been the foundation for HCV treatment for over 30 years (Hoofnagle, Mullen et al. 1986). Pegylated interferon alpha (PEG-IFN) in conjunction with ribavirin will lead to diminished HCV RNA levels and can induce SVR, but efficacy is relatively low. SVR is achieved in only 40% of HCV-1 patients, with higher rates in HCV-2 and HCV-3 patients (80% and 70%, respectively) (Shiffman, Suter et al. 2007). Furthermore, up to 80% of individuals undergoing the PEG-IFN/ribavirin combination therapy experience adverse side effects such as anemia, neutropenia, flu-like symptoms, and respiratory tract complications (McHutchison, Gordon et al. 1998, Dubuisson and Cosset 2014).

Direct-Acting Antivirals. Direct-acting antivirals (DAAs) were recently developed to address the shortcomings of interferon-based therapies; comparatively, DAAs have higher SVR rates and attenuated side effects (Ghasemi, Rostami et al. 2015). There are four classes of DAAs: NS3/4A protease inhibitors (PIs), NS5B nucleoside polymerase inhibitors (NPIs), NS5B non-nucleoside polymerase inhibitors (NNPIs), and NS5A inhibitors. By inhibiting the nonstructural viral proteins, DAAs directly target HCV viral replication. Different classes of DAAs are used in combination to create the most effective therapy; for example, NPI sofosbuvir in combination with either PI simeprevir or NS5A inhibitor ledipasvir achieves a 95% SVR rate in HCV-1. Sofosbuvir with ribavirin is an option for individuals with HCV-2, 3, 4, 5 and 6 with high SVR rates in all except for HCV-3, for unknown reasons (Jacobson, Gordon et al. 2013).

Although IFN-based regimens are relatively unsuccessful at achieving SVR, reports from the time observed that patients with HCV/cirrhosis who achieved SVR from IFN-based therapies had a significantly lower risk of developing HCC compared to those who did not (annual risk 0.7-1.2% compared to 2.1-5.9%, respectively) (Bruno, Stroffolini et al. 2007, Cardoso, Moucari et al. 2010, van der Meer, Feld et al. 2017). When DAAs arrived on the market, the outlook was positive as SVR rates consistently surpassed 80%, even in cases with decompensated cirrhosis. It was therefore both shocking and confusing when several reports were published shortly after suggesting "unexpectedly" high rates of HCC recurrence in patients with a history of cured HCC, who had later been treated with DAAs (Conti, Buonfiglioli et al. 2016, Reig, Marino et al. 2016, Yang, Aqel et al. 2016). Subsequent studies looking at *de novo* HCC in HCV patients reported different rates of occurrence within a year of finishing a DAA regimen ranging from 3.2-9.1% (Cardoso, Vale et al. 2016, Conti, Buonfiglioli et al. 2016, Kozbial, Moser et al. 2016, Ravi, Axley et al. 2017). At best, these numbers represent a similar risk of HCC to that of untreated patients; at worst, they indicate an increased risk. There are several limitations and concerns with these reports, however. All of the studies lack a control group and draw their conclusions using

relatively small, highly heterogeneous patient groups. Several are retrospective (Cardoso, Vale et al. 2016, Conti, Buonfiglioli et al. 2016, Ravi, Axley et al. 2017) and/or facilitate selection bias by only recruiting patients from a single center (Cardoso, Vale et al. 2016, Kozbial, Moser et al. 2016, Yang, Aqel et al. 2016, Ravi, Axley et al. 2017). Large-scale studies were initiated in response to these alarming claims. Multiple analyses representing multinational cohorts confirmed that achieving SVR from DAAs greatly reduced the risk of both recurrent and *de novo* HCC, and they did not find any evidence that DAAs might facilitate development of HCC (ANRS collaborative study group on hepatocellular carcinoma 2016, Cheung, Walker et al. 2016, Ioannou, Green et al. 2017, Kanwal, Kramer et al. 2017). More recently, two prospective, multicenter studies offered an explanation to the apparent increase in HCC incidence by controlling for confounders such as age and liver function and finding no significant increase in risk of HCC associated with DAA use (Nahon, Layese et al. 2018, Romano, Angeli et al. 2018). This view is further supported by two separate researchers who employed propensity scores to provide one-to-one matches between their cases and controls, accounting for variables such as age, sex, BMI, liver function, and disease severity (Ikeda, Kawamura et al. 2017, Cabibbo, Celsa et al. 2019). A meta-analysis of 26 studies compared IFN-based therapies to DAAs and found no difference in either risk of recurrent or *de novo* HCC (Waziry, Hajarizadeh et al. 2017). Any increase in overall risk of HCC occurrence/recurrence following DAA treatment must take into account that IFN-based regimens are contraindicated for patients with advanced disease due to the low SVR rates and difficult side effects, while DAAs are well suited for HCV-related diseases of all stages.

HCV Vaccine. There is currently no vaccine available for HCV. Some of the challenges for HCV vaccine development coincide with the challenges of studying long-term HCV-associated liver diseases; that is, extraordinary genetic variability, lack of adequate animal models, and limitations of human studies. Furthermore, the difficulties in vaccine development are compounded by the

many mechanisms HCV has in place to evade the immune system (Liang 2013, Ghasemi, Rostami et al. 2015).

Sex Differences in HCV

Although males are only slightly more likely to acquire HCV infection compared to females (Alter, Kruszon-Moran et al. 1999), it is well established that males are significantly less likely to spontaneously clear the virus and more likely to go on to develop HCV-associated diseases (Poynard, Bedossa et al. 1997, Hayashi, Kishihara et al. 1998, Poynard, Ratziu et al. 2001, Fattovich, Stroffolini et al. 2004, Micallef, Kaldor et al. 2006, van den Berg, Grady et al. 2011, Grebely, Page et al. 2014) (Figure 3). In addition to higher disease incidence, multiple studies have shown that compared to premenopausal females, males and postmenopausal females tend to have a greater severity of HCV-associated fibrosis/cirrhosis (Poynard, Bedossa et al. 1997, Di Martino, Lebray et al. 2004, Corsi, Karges et al. 2016) and HCC (Chiaramonte, Stroffolini et al. 1999, Khan, Farrell et al. 2000, Ryerson, Eheman et al. 2016), even after adjusting for age.

The disparity between the sexes is thought to be due in part to lifestyle differences, such as alcohol consumption and weight distribution, but there is overwhelming evidence to point to the importance of estrogens and their receptors influencing all stages of HCV infection, from the initial host immune response to disease progression to treatment. Males and postmenopausal females have similar levels of estrogen in the blood, which can be up to 10 times less than the amount in premenopausal females (Haldeman-Englert and Taylor), suggesting that estrogens and their receptors may play a protective role in disease progression.

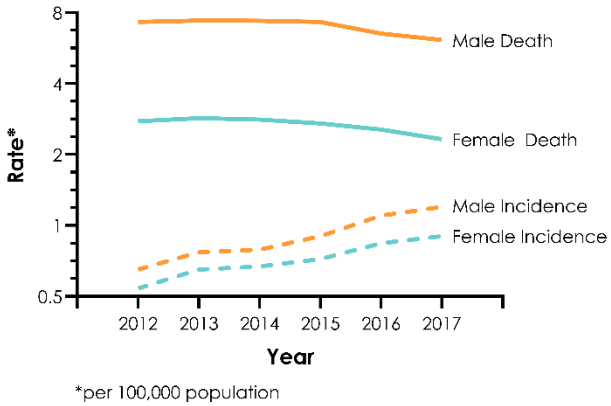


Figure 3: HCV Incidence and Deaths, 2012-2017

data from the CDC, National Notifiable Diseases Surveillance System and National Center for Health Statistics, Multiple Cause of Death 1999–2017 on CDC WONDER Online Database.

Estrogen and Estrogen Receptors. There are four major estrogens in the body, the most potent and prevalent of which is 17β-estradiol (E2). All estrogens act by binding to nuclear estrogen receptor alpha (ERα) or beta (ERβ) or transmembrane receptor G-protein coupled estrogen receptor (GPER) to mediate gene transcription. In addition to endogenous estrogens, other compounds can exert estrogenic actions including environmental contaminants, dietary estrogens, and synthetic agonists/antagonists (Figure 4) (Heldring, Pike et al. 2007, Paterni, Granchi et al. 2014). Synthetic ligands are useful because many of them were created to preferentially bind to either ERα or ERβ in order to investigate ER subtype-specific interactions. Since it was identified in 2000, propylpyrazole triol (PPT) has held the top spot for ERα-selective agonists with a 410-fold relative binding affinity for ERα over ERβ (Stauffer, Coletta et al. 2000). Diarylpropionitrile (DPN) is the most widely utilized ERβ-selective agonist with a 70-fold affinity for ERβ over ERα (Meyers, Sun et al. 2001). As a negative control, fulvestrant (ICI 182,780) was identified in 1991 to be an effective ER antagonist with a binding affinity closest to that of E2 (Wakeling, Dukes et al. 1991).

Activated ERs can function as traditional transcription factors by binding to estrogen response elements (EREs) on DNA, but they can also regulate gene expression in indirect ways via protein-protein interactions with other DNA-binding transcription factors such as activator protein 1 (AP-1) (Figure 4) (Bjornstrom and Sjoberg 2005). Estrogen can also control gene transcription without directly interacting with DNA, by binding to membrane-localized ERs to activate various signaling pathways, such as protein kinases. ERs have diverse patterns of expression in various tissue and cells types, thus triggering distinct, sometimes conflicting, cascades of events upon activation. There is also evidence to suggest that certain ligands can induce ER-specific gene regulation via differential recruitment of coregulators by ERα and ERβ complexes (Chang, Charn et al. 2008, Liang and Shang 2013).

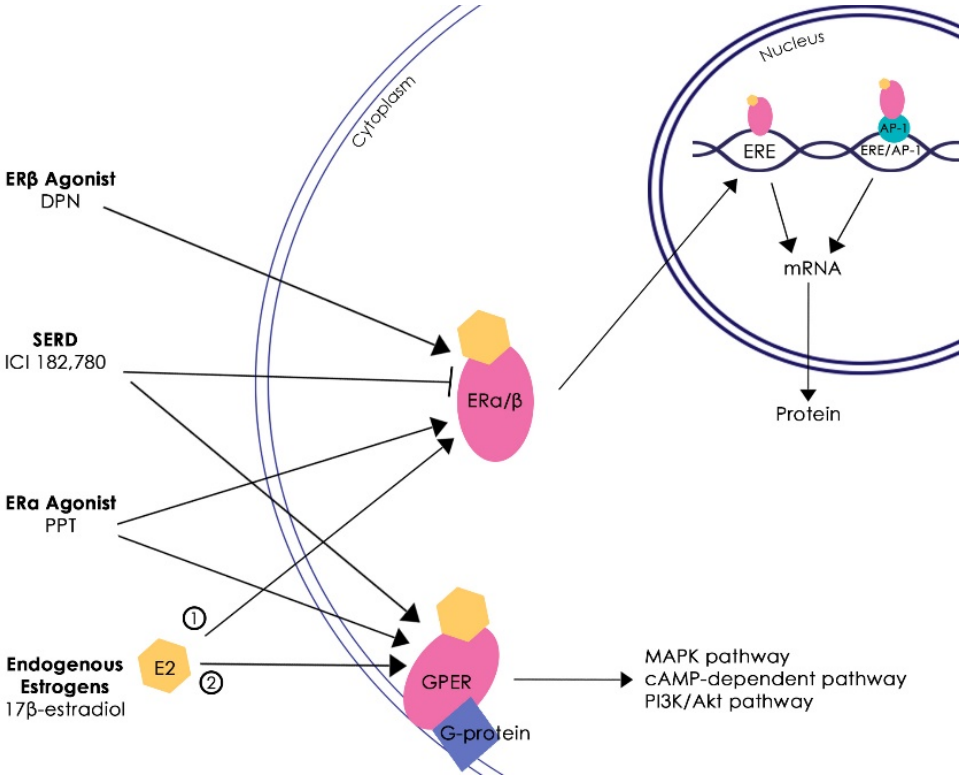


Figure 4: Estrogen receptor ligands and action

Agonists (arrows) and antagonists (t-bars) of ERα/β and GPER. Note that ICI 182,780 functions as an ERα/β antagonist but as an agonist of GPER. (1) Ligand-activated ERα/β regulates gene expression by translocating to the nucleus and binding to DNA at a sequence containing an ERE or by interacting with transcription factors such as AP-1. (2) E2 can also bind to and activate GPER at the plasma membrane to initiate downstream signaling via MAPK, cAMP, and PI3K/Akt pathways. Modified from Kalra, Mayes et al. (2008) Prossnitz and Barton (2014).

Akt: protein kinase B; AP-1: activator protein 1; cAMP: cyclic adenosine monophosphate; E2: 17β-estradiol; ERE: estrogen response element; GPER: G-protein coupled estrogen receptor; MAPK: mitogen-activated protein kinase; PI3k: phosphoinositide 3-kinase; SERD: selective estrogen receptor downregulator

Estrogens may confer protection by serving as an antiviral agent against HCV. Although there is no evidence that estrogens are "direct-acting antivirals," several studies support the idea that they may inhibit specific steps in the virus life cycle. Hayashida et al. demonstrated in a cell model of HCV infection that E2 inhibits the production of HCV infectious particles in an ERα–dependent manner, without affecting HCV RNA replication or protein synthesis (Hayashida, Shoji et al. 2010). Ulitzky et al. showed that E2 can downregulate HCV infection in Huh7.5 cells by

facilitating the cleavage of occludin, an HCV receptor that is required for viral entry (Ulitzky, Lafer et al. 2016). Magri et al. confirmed the previous two studies in their cell model of HCV infection and suggested that E2 can also reversibly impair viral assembly and/or release (Magri, Barbaglia et al. 2017).

Estrogen and Inflammation. The protective nature of estrogen may also have to do with the anti-inflammatory effects of the hormone (Straub 2007, Shi, Feng et al. 2014, Kovats 2015). There is evidence to suggest that the decrease in estrogen production during menopause is associated with spontaneous increases in proinflammatory cytokines such as TNFα (Pfeilschifter, Koditz et al. 2002), and treatment with E2 can inhibit TNFα expression and/or release in certain cells (Ralston, Russell et al. 1990, Rogers and Eastell 2001). Using both *in vivo* and *in vitro* techniques, estrogens have been shown to downregulate expression of other proinflammatory cytokines, including those from the IL-1 family (Kilbourne and Scicchitano 1999, Evans, Lai et al. 2002). Accordingly, estrogen has also been implicated as a mediator of fibrosis progression: in a rat model of fibrosis, males and ovariectomized females had a more severe disease presentation compared to control females, and E2 treatment decreased production and deposition of collagen as well as inhibited HSC activation (Shimizu, Mizobuchi et al. 1999, Yasuda, Shimizu et al. 1999, Xu, Gong et al. 2002). Epidemiological studies also support a protective role of estrogen in liver fibrosis by demonstrating associations between postmenopausal status and nulliparity with a higher rate of fibrosis, and hormone replacement therapy (HRT) with a lower rate of fibrosis (Di Martino, Lebray et al. 2004, Codes, Asselah et al. 2007). E2 has been shown to upregulate CRP CD55 in other tissues (Song, Deng et al. 1996, Sartini, Moussawi et al. 2004), so it may dampen complement-mediated attack in the liver as well. Long-term exposure to exogenous estrogen, such as from oral contraceptives and HRT, is a definite risk factor for various cancers (Liang and Shang 2013); in the liver, however, HRT is associated with a decreased risk of developing HCC, later disease onset, and increased survival times (Hassan, Botrus et al. 2017).

Role of ERs in the Liver While the exact mechanism of protection is unknown, it is widely supported that both ERα and ERβ receptors are differentially expressed in both diseased and normal livers (Porter, Elm et al. 1983, Iavarone, Lampertico et al. 2003, Liu, Yeh et al. 2009, Iyer, Kalra et al. 2017). Previous studies from our lab have shown that protein expression of ER subtypes and ERα:ERβ ratios differ significantly in normal male livers compared to females. Also, in livers with HCV-associated HCC, both ER subtypes are positively correlated with oncogenic marker cyclin D1 expression, and negatively correlated with activated inflammatory markers NF-κB and IKK (Iyer, Kalra et al. 2017). Several groups are proponents for the particular importance of ERα in HCV pathogenesis. Cengiz et al. (2014) showed that in humans with HCV infection, ERα expression was inversely correlated with stage of fibrosis and was an independent predictor for fibrosis progression. ERα may also promote HCV replication (Watashi, Inoue et al. 2007, Hillung, Ruiz-López et al. 2012).

Wild-type (wt)ERα seems to lose function as disease progresses, while variants of ERα (ERα46 and ERα36) are upregulated; this upregulation is associated with a worse prognosis for HCC (Miceli, Cocciadiferro et al. 2011). The presence of ERα variant transcripts in liver tumors has been shown to be the strongest negative predictor of survival in HCC compared to commonly used clinical scoring systems (Villa, Moles et al. 2000, Villa, Colantoni et al. 2003). The important role of ERα in the liver is further supported by several studies using rodent models of liver inflammation and fibrosis. Evans et al. (2002) demonstrated that estrogen treatment decreases proinflammatory gene expression in an ERα-dependent manner.

There has been significantly more research looking at ERα compared to ERβ, as ERβ was discovered more recently. However, that does not diminish its importance. ERβ expression was shown to be significantly greater than that of ERα in cultured rat hepatocytes, and when the cells were undergoing oxidative stress, E2 enhanced antiapoptotic activity via ERβ (Inoue, Shimizu et al. 2003). Patients with chronic liver disease tend to have higher levels of ERβ compared to those

with HCC (Iavarone, Lampertico et al. 2003). The ratio of ERα to ERβ expression is also of interest, as variations in ERα:ERβ expression have been reported in other cancers suggesting that coregulation of expression may play a role in mediating response to estrogen (Leygue, Dotzlaw et al. 1998, Shang 2006, Thomas and Gustafsson 2011).

Summary

Many of these *in vitro* and *in vivo* animal studies on HCV pathogenesis need to be confirmed directly in human liver cells or liver tissues. The challenge to be addressed by the current research is the lack of understanding behind the reasons why premenopausal females seem to be better protected from HCV-associated liver diseases compared to postmenopausal females and males. In order to fully appreciate the role of estrogen and ERs in the development of HCV-associated diseases, it is imperative to understand the relationship between ERs and markers of inflammation in the context of HCV infection. Three inflammatory markers of immediate concern are IL-33, TNFα, and CD55, all of which have been shown to be associated with HCV disease pathogenesis.

CHAPTER III

MATERIALS AND METHODS

In Vitro Methods

Cell Culture. Human hepatoma Huh7 cells were obtained from Dr. Clifford J. Steer, Department of Medicine, University of Minnesota. Huh7 is a well-differentiated cell line originating in 1982 from hepatocytes taken from a liver carcinoma in a 57-year-old Japanese male. Cells were cultured and maintained in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (29.2 mg/mL). Cell cultures were incubated at 37°C in a humidified atmosphere (85%) containing 5% CO_2.

Hormone Treatment. Huh7 cells were plated at 300,000 cells per well in 6-well plates and were allowed to grow overnight. The cells were then subjected to serum starvation for 24 hours followed by drug treatment for another 24 hours. The stock solutions of the hormonal drugs E2, PPT, DPN, and ICI 182,780 were prepared in ethanol or dimethyl sulphoxide (DMSO) and diluted to the required working concentrations in serum-free media (Table 2**).** The control wells included cells cultured in serum-free media with ethanol or DMSO (0.01% vol/vol).

Table 2: Drug treatment in Huh7 cells

Drug	Catalog #	Drug Action	Treatment Doses	Dilution medium
E2	MilliporeSigma E4389	ER agonist	0.001 μM, 0.01 μM, 0.05 μM, 0.1 μM	Ethanol
PPT	Cayman Chemical	ERα selective agonist	0.01 μM, 0.1 μM, 1 μM	Ethanol
DPN	Cayman Chemical	ERβ selective agonist	0.01 μM, 0.1 μM, 1 μM	Ethanol
ICI 182,780	Tocris 1047	ER antagonist	0.1 μM, 1 μM, 10 μM	DMSO

MTT Assay for Cell Toxicity. Huh7 cells were grown overnight in 96-well plates (15,000 cells/well). The cells were then subjected to serum starvation for 24 hours followed by treatment with different concentrations of hormonal drugs in serum-free media for 24 hours. The cells were then treated with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dissolved in serum-free DMEM media for 2 hours at 37°C. The formazan compound formed in the cells was solubilized in a 3:1 mixture of DMSO and serum-free DMEM media and the OD was measured at 540 nm. The non-toxic drug doses that were selected for the treatment of cells in experimental assays were as follows: E2 (0.001 μM, 0.01 μM, 0.05 μM and 0.1 μM), PPT (0.01 μM, 0.1 μM, and 1 μM), DPN (0.01 μM, 0.1 μM, and 1 μM), and ICI 182,780 (0.1 μM, 1 μM, and 10 μM).

Ex Vivo Methods

Liver Tissues. Explant liver tissues from deidentified patients with end stage liver disease due to either HCV-related cirrhosis or HCV-related HCC were obtained from the National Institutes of Health Liver Tissue and Cell Distribution System at the University of Minnesota, Minneapolis, MN. Normal liver tissues with no diagnosis of HCV or HCC were included as controls. Exclusion criteria for the study included patients co-infected with HIV or hepatitis B virus or with a history of alcohol consumption or drug use. Liver explants were aseptically collected and snap frozen in liquid nitrogen and stored at -80°C until further use. The study received approval from the Institutional Review Board at the University of Minnesota under Exemption IV. The study was

conducted at Oklahoma State University Center for Health Sciences under Institutional Review Board guidelines.

Genotyping. Total genomic DNA was extracted from liver tissue samples using a phenol chloroform method modified from previously published protocols (Sambrook, Fritsch et al. 1989). The total amount and purity of DNA for each extraction method was assessed by spectrophotometry (NanoDrop 1000 Full Spectrum UV/Vis Spectrophotometer; Wilmington, DE, USA). The total amount of DNA was obtained in ng/μL, and the A260/280 ratio was calculated for protein impurities. DNA was considered viable for analysis when A260/280 ratio value was from 1.7 to 2.0. Human liver tissue samples were genotyped for *IFNL4* variants rs12979860 and rs368234815 by personnel in the Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics (NCI/NIH, Bethesda, MD, USA; Acting Chief: Dr. Ludmila Prokunina-Olsson) with methods described previously (Prokunina-Olsson, Muchmore et al. 2013). Briefly, custom TaqMan allelic discrimination genotyping assays were performed using Genotype Master Mix (Qiagen) on ABI SDS 7700 (Applied Biosystems) under standard conditions.

Quantitative Analysis of mRNA Expression

RNA Isolation. Total RNA was extracted from all cells and human liver tissues using TRIzol reagent (Cat. no. 15596026; Invitrogen) and cleaned using the QIAGEN RNeasy Mini Kit (Cat. no. 74106) per the manufacturer protocol.

Quantitative Reverse Transcription PCR. Approximately 2 μg of total RNA was reverse transcribed using Moloney-Murine Leukemia Virus Reverse Transcriptase (Cat. no. M1701; Promega) and random primers per the manufacturer protocol. Quantitative reverse transcription PCR (RT-qPCR) was performed using custom primers from Invitrogen/Thermo Fisher Scientific (Table 3). Reactions were carried out in triplicate with PowerUp SYBR Green Master Mix (Cat. no. A25742; Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR system.

Samples were analyzed using Applied Biosystems 7500 Software v.2.0.6. Relative differences in gene expression between groups were calculated using cycle threshold values (C_t). The expression of target genes in liver tissues was normalized to the expression of endogenous control genes beta-glucuronidase (*GUSB*) and serine and arginine-rich splicing factor 4 (*SRSF4*) (Waxman and Wurmbach 2007, Congiu, Slavin et al. 2011, Yamaguchi, Matsumoto et al. 2013); the expression of target genes in Huh7 cells was normalized to the expression of *SRSF4* (Liu, Qin et al. 2017). The gene expression of the treatment group was further normalized to the experimental controls using the $\Delta\Delta C_t$ method ($\Delta\Delta C_t = \Delta C_t$ target of treatment group $- \Delta C_t$ target of experimental control) and expressed as fold change $\left(2^{-\Delta\Delta C_t}\right)$. Reaction efficiency for all targets was assessed to be 102.2%±4.0% (n=2) by titrating samples and using the integrated tool of the Applied Biosystems software.

Table 3: Primer sequences used for RT-qPCR

Target Gene	Primer Sequence (5'-3')		Amplicon Size (bp)	NCBI Accession
ESR1	Forward	CCAACCAGTGCACCATTGAT	105	NM_000125.3
	Reverse	GGTCTTTTCGTATCCCACCTTT		
ESR2	Forward	GGCAGAGGACAGTAAAAGCA	151	NM_001437.2
	Reverse	GGACCACACAGCAGAAAGAT		
IL-33	Forward	GCCTTCACTGAAAACAGGTAGA	85	NM_033439.3
	Reverse	GGCAAAGCACTCCACAGTAG		
CD55	Forward	TTTCCAGGACAACCAAGCATT	95	NM_000574.4
	Reverse	ACACGTGTGCCCAGATAGA		
TNFα	Forward	TGTAGCCCATGTTGTAGCAAAC	155	NM_000594.4
	Reverse	AGAGGACCTGGGAGTAGATGA		
GUSB	Forward	GCCATCGTGTGGGTGAATG	86	NM_000181.4
	Reverse	TGGACCAGGTTGCTGATGTC		
SRSF4	Forward	TGAAGACAAGCCAGGTTCCA	75	NM_005626.5
	Reverse	CTTCGAGAGCGAGACCTTGA		

Analysis of Protein Expression

Protein Isolation. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Cat. no. 23225). RIPA lysis buffer was used to prepare total protein extracts from cells. Representative liver tissues were selected from HCV/cirrhosis, HCV/HCC, and normal patient

groups. Cytoplasmic, nuclear, and total protein fractions were prepared from liver tissues using the Thermo Fisher Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (Cat. no. 78833) or T-PER™ Tissue Protein Extraction Reagent (Cat. no. 78510) as per the manufacturer's instructions.

Western Blotting. Equivalent protein (25-50 μg) per sample was loaded and separated on a 4%-12% gradient Bis-Tris polyacrylamide gels. Gels were then wet transferred to a nitrocellulose membrane, stained with REVERT Total Protein Stain (Cat. no. 926-11015; LI-COR) to verify complete transfer of proteins, and imaged for loading control. Blots were rinsed and blocked with Intercept Blocking Buffer in TBS (Cat. no. 927-60001, LI-COR). Blots were incubated overnight at 4°C while shaking with the indicated primary antibody (Table 4). Blots were rinsed and incubated for 1 hour at room temperature while shaking with the appropriate secondary antibody. Blots were rinsed and imaged on an Odyssey CLx Near-Infrared Fluorescence Imaging System (LI-COR). Image capture and densitometry were performed using Image Studio Lite v.5.2 software. Data were normalized to total protein (Moritz 2017, Kirshner and Gibbs 2018). Anti-Histone H3 and anti-GAPDH antibodies were used to confirm purity of cytoplasmic and nuclear fractions (Supplementary Figure 3). Uncropped western blot images are shown in the Supplementary Materials.

Table 4: Antibodies and positive controls used for western blot

Antibody	Catalog #	Host / Class / Isotype	Concentration	Observed Size (kDa)	
				Human Liver	Huh7 Cells
Anti-CD55	Abcam ab133684	Rabbit / monoclonal / IgG	1:10000 (1:20000 2nd)	70	70
Anti-ERβ	Abcam ab3576	Rabbit / polyclonal / IgG	1:1000 (1:30000 2nd)	70	70
Anti-TNFα	Abcam ab1793	Mouse / monoclonal / IgG1	1:1000 (1:10000 2nd)	45	18
Anti-ERα	Invitrogen MA5-13191	Mouse / monoclonal / IgG1	1:500 (1:10000 2nd)	37	37
Anti-IL-33	Abcam ab207737	Rabbit / monoclonal / IgG	1:1000 (1:30000 2nd)	35	25
Anti-Histone H3	Abcam ab1791	Rabbit / polyclonal / IgG	1:1000 (1:20000 2nd)	15	N/A
Anti-GAPDH (0411)	Santa Cruz sc-47724	Mouse / monoclonal / IgG1	1:1000 (1:10000 2nd)	50	N/A
Anti-Rabbit IRDye® 800CW	LI-COR 926-32213	Donkey / IgG	Varies	N/A	
Anti-Mouse IRDye® 800CW	LI-COR 926-32212	Donkey / IgG	Varies	N/A	
Recombinant Human TNFα	Abcam ab55237	N/A	10 ng/well	25	25
Recombinant Rat IL-33	Abcam ab207126	N/A	10 ng/well	20	20

Statistical Analysis

Data obtained from the experiments were analyzed statistically and graphed using GraphPad Prism version 8 (GraphPad Software Inc, La Jolla, CA, USA). Characteristics of the study population were compared using the chi-squared test. Statistical significance between appropriate groups in *in vitro* data was determined by parametric one-way ANOVA, and post-hoc analyses were performed using Dunnett's multiple comparisons test. *Ex vivo* data were analyzed using non-parametric methods that included the Kruskall-Wallis test and Mann Whitney U test; post-hoc analyses were performed using Dunn's Multiple Comparison test. Correlations between ER subtype expression and expression of inflammatory markers in liver tissues were analyzed by using the Spearman's rank correlation test. $P \leq 0.05$ was considered statistically significant.

CHAPTER IV

RESULTS

Specific Aim 1: To determine whether estrogen treatment affects the expression of ER subtypes and/or inflammatory markers in the human hepatoma cell line Huh7

MTT assay for hormonal drug toxicity in Huh7 cells

Drug toxicity for ER agonists and antagonist in Huh7 cells was tested by MTT assay to determine the appropriate non-toxic doses to use for the studies (Supplementary Figure 1A-D). Although statistically, cell viability did not differ significantly from the control in any of the doses tested, the highest E2 and PPT doses were not used due to the apparent cytotoxic effect. The non-toxic drug doses that were selected for the treatment of cells in experimental assays were as follows: E2 (0.001 μM, 0.01 μM, 0.05 μM and 0.1 μM), PPT (0.01 μM, 0.1 μM, and 1 μM), DPN (0.01 μM, 0.1 μM, and 1 μM), and ICI 182,780 (0.1 μM, 1 μM, and 10 μM).

Treatment of Huh7 cells with ER agonists/antagonists altered the expression of ERβ, but not ERα

To assess the regulation of ER subtype expression in the liver, Huh7 cells were treated with various concentrations of ER agonists and antagonists, and ERα and ERβ mRNA and protein expression were measured. No significant difference in ERα expression was observed at either the transcriptional or translational level; however, treatment with ER agonist E2 appears to downregulate *ESR1* mRNA expression (Figure 5A) and upregulate ERα protein expression (Figure 6A).

Treatment of cells with ER antagonist ICI followed by addition of 0.01 μM E2 had similar results on ERα, with an insignificant decrease in mRNA (Figure 5B) and increase in protein expression (Figure 6B). Treatment with ERα agonist PPT resulted in an increase in both ERα mRNA and protein expression (Figure 5C, 6C), while ERα protein expression trended down upon treatment with ERβ agonist DPN (Figure 6D).

ESR2 mRNA expression, on the other hand, was significantly decreased in Huh7 cells by treatment with all concentrations of both E2 and ICI+E2 (Figure 7A, B). This result is somewhat counterintuitive since E2 is an ER agonist, and ICI is an ERα/β antagonist. However, it is important to note that ICI is a selective estrogen receptor downregulator (SERD) in that it functions as an antagonist of ERα/β but as an agonist of GPER (Prossnitz and Barton 2014). DPN treatment significantly increased *ESR2* expression at the lowest dose, and expression trended downward as concentration increased (Figure 7D). ICI, PPT, and DPN all appear to upregulate ERβ protein expression in a dose dependent manner; however, the results were not significant (Figure 8B-D).

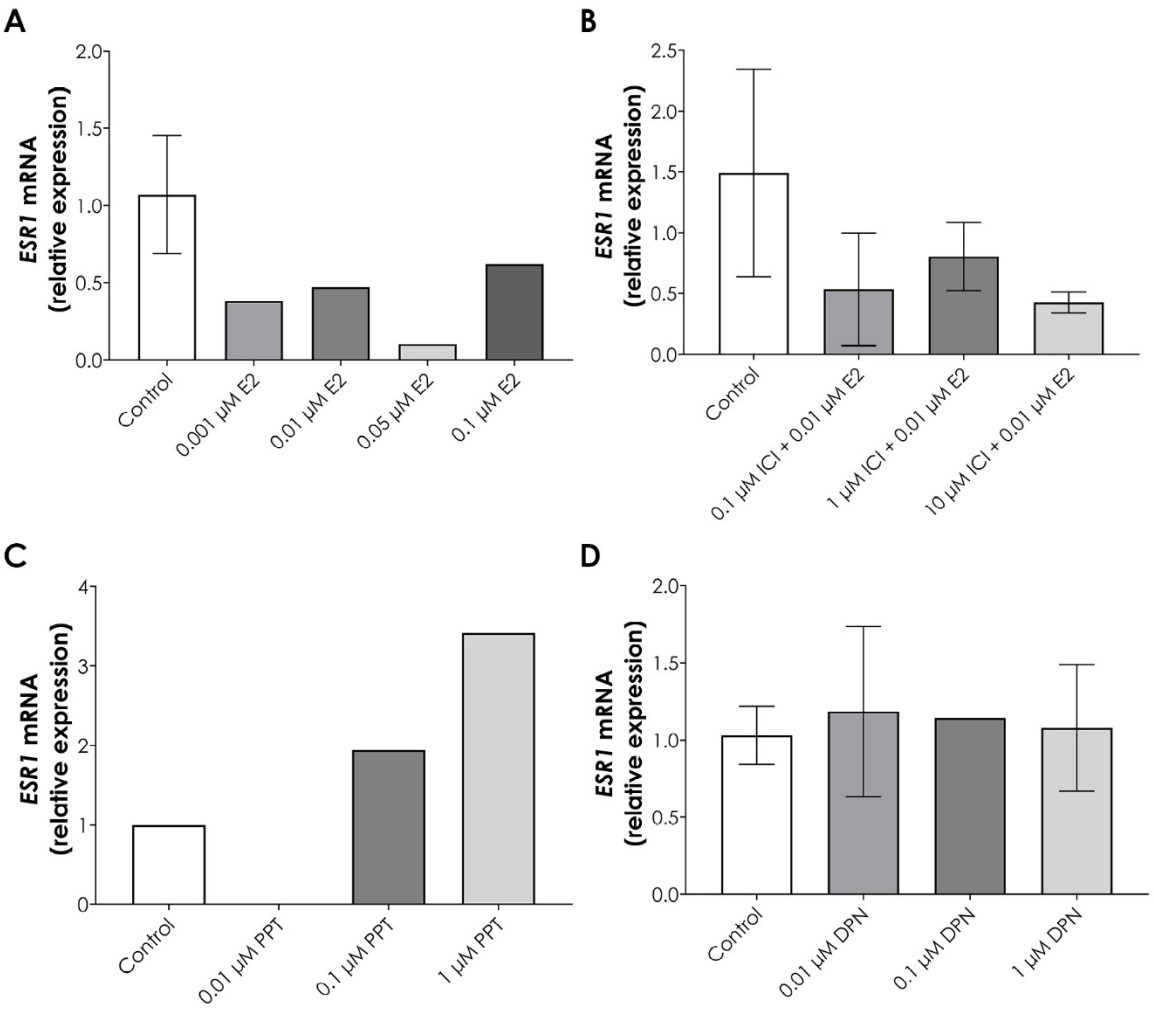


Figure 5: mRNA expression of ERα in Huh7 cells after treatment with various ER agonists/antagonists

ERα mRNA expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. *ESR1* transcript levels were measured using RT-qPCR, normalized to *SRSF4* expression, and compared using the ΔΔCt method. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 µM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.
Column bars represent the mean ± SEM. Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Groups with no column bar, or no SEM, indicate inconclusive qPCR reactions due to no amplification in sample.

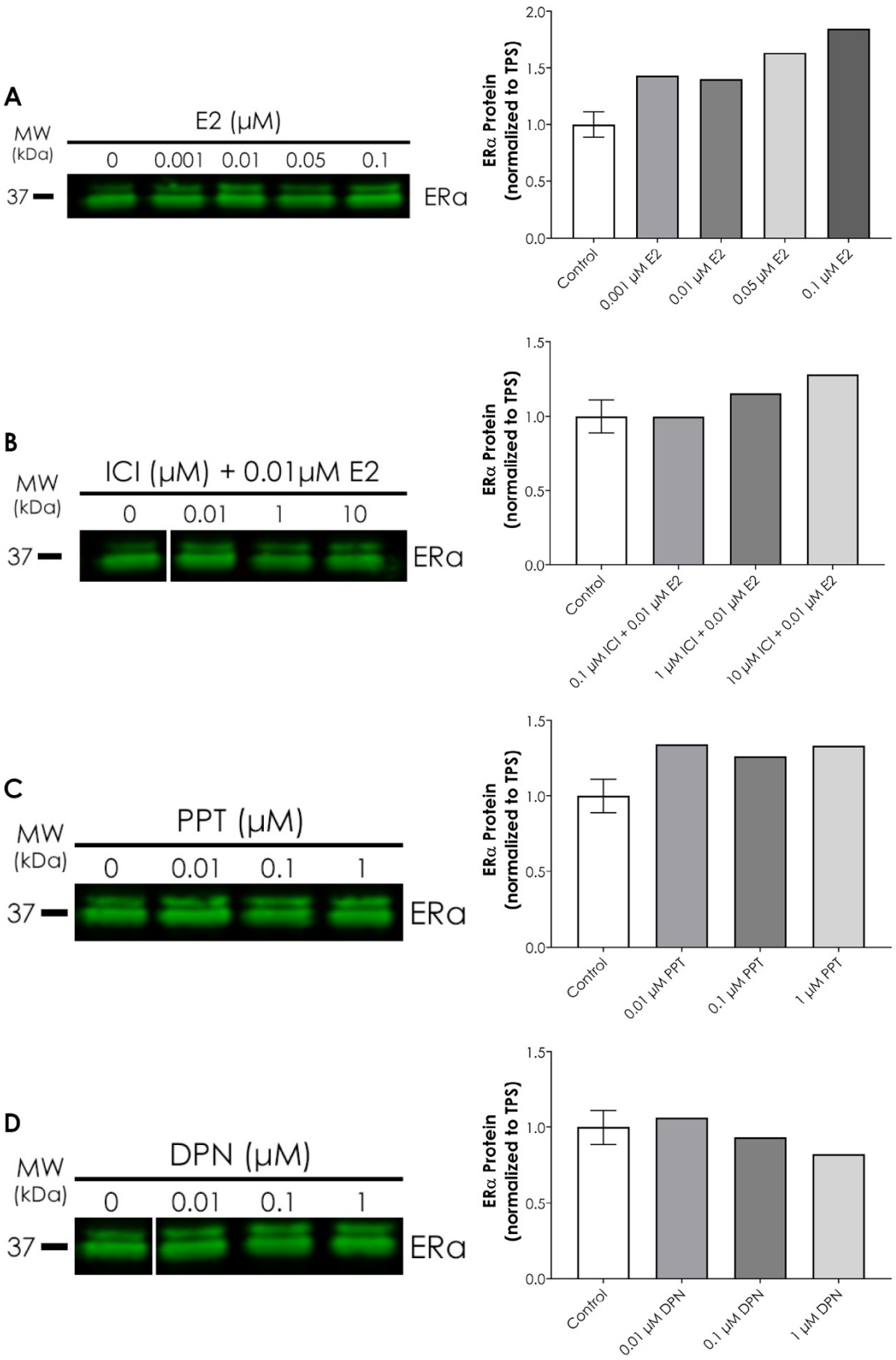

A
MW
(kDa)
E2 (μM)
0
0.001
0.01
0.05
0.1
37
ERa
ERα Protein
(normalized to TPS)
2.0
1.5
1.0
0.5
0.0
Control
0.001 μM E2
0.01 μM E2
0.05 μM E2
0.1 μM E2
B
MW
(kDa)
ICI (μM) + 0.01μM E2
0
0.01
1
10
37
ERa
ERα Protein
(normalized to TPS)
1.5
1.0
0.5
0.0
Control
0.1 μM ICI + 0.01 μM E2
1 μM ICI + 0.01 μM E2
10 μM ICI + 0.01 μM E2
C
MW
(kDa)
PPT (μM)
0
0.01
0.1
1
37
ERa
ERα Protein
(normalized to TPS)
1.5
1.0
0.5
0.0
Control
0.01 μM PPT
0.1 μM PPT
1 μM PPT
D
MW
(kDa)
DPN (μM)
0
0.01
0.1
1
37
ERa
ERα Protein
(normalized to TPS)
1.5
1.0
0.5
0.0
Control
0.01 μM DPN
0.1 μM DPN
1 μM DPN

Figure 6: Protein expression of ERα in Huh7 cells after treatment with various ER agonists/antagonists

ERα protein expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. ERα protein expression was analyzed by Western blot using whole cell lysates and normalized to total protein. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 μM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM (*n*=1, with 2 control lanes per target). Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 5). TPS, total protein stain.

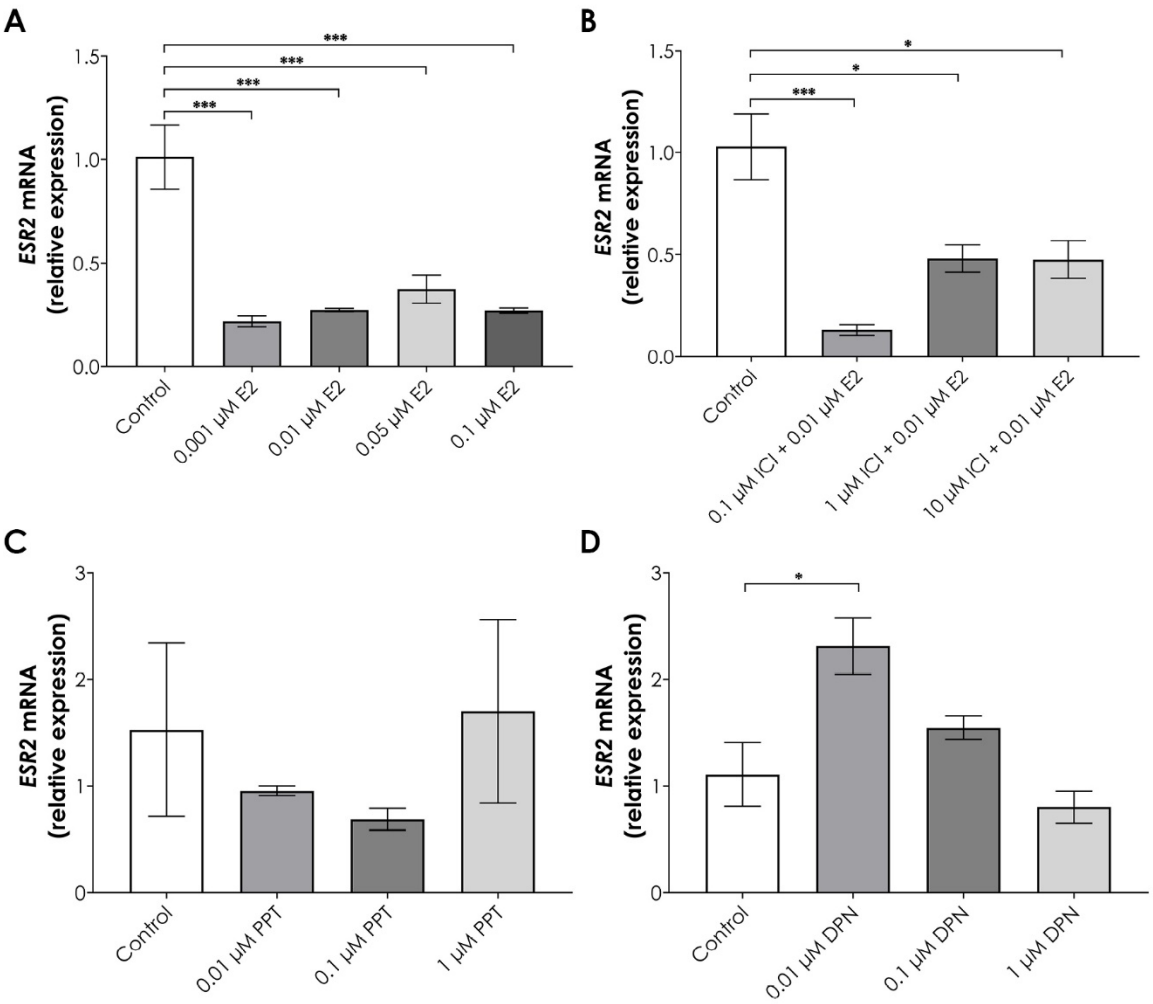


Figure 7: mRNA expression of ERβ in Huh7 cells after treatment with various ER agonists/antagonists

ERβ mRNA expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. *ESR2* transcript levels were measured using RT-qPCR, normalized to *SRSF4* expression, and compared using the ΔΔCt method. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 µM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM. Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where *$P \leq 0.05$, **$P \leq 0.01$.

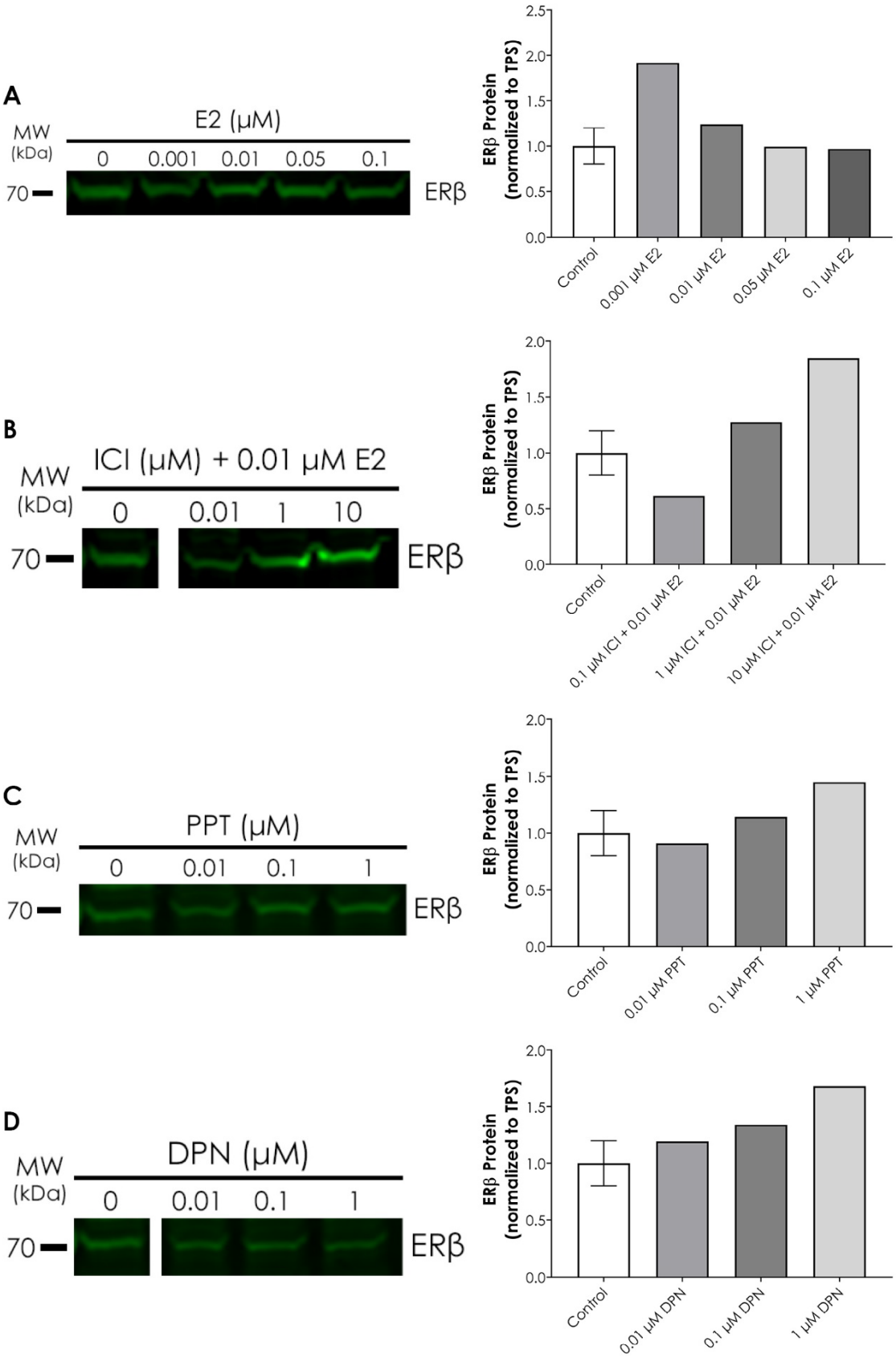

A
MW
(kDa)
E2 (μM)
0
0.001
0.01
0.05
0.1
70
ERβ
ERβ Protein
(normalized to TPS)
2.5
2.0
1.5
1.0
0.5
0.0
Control
0.001 μM E2
0.01 μM E2
0.05 μM E2
0.1 μM E2
B
MW
(kDa)
ICI (μM) + 0.01 μM E2
0
0.01
1
10
70
ERβ
ERβ Protein
(normalized to TPS)
Control
0.1 μM ICI + 0.01 μM E2
1 μM ICI + 0.01 μM E2
10 μM ICI + 0.01 μM E2
C
MW
(kDa)
PPT (μM)
0
0.01
0.1
1
70
ERβ
ERβ Protein
(normalized to TPS)
Control
0.01 μM PPT
0.1 μM PPT
1 μM PPT
D
MW
(kDa)
DPN (μM)
0
0.01
0.1
1
70
ERβ
ERβ Protein
(normalized to TPS)
Control
0.01 μM DPN
0.1 μM DPN
1 μM DPN

Figure 8: Protein expression of ERβ in Huh7 cells after treatment with various ER agonists/antagonists

ERβ protein expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. ERβ protein expression was analyzed by Western blot using whole cell lysates and normalized to total protein. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 μM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM (*n*=1, with 2 control lanes per target). Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 6). TPS, total protein stain.

ER agonists/antagonists mediate TNFα expression in Huh7 cells

The purpose of the next analysis was to compare the potential modulation of potent proinflammatory cytokine TNFα by ER agonists and antagonists. The effects of E2 and ICI+E2 on *TNFA* mRNA expression are similar to those of *ESR2*, where expression was significantly decreased, but only in the higher doses (Figure 9A, B). TNFα protein expression was sharply increased with the lowest dose of E2 treatment while the higher doses were closer to the control, although the results were not significant (Figure 10A). All DPN treatment concentrations significantly upregulated *TNFA* mRNA expression in a dose dependent manner (Figure 9D); protein expression was not significant, but trended in the same direction (Figure 10D). PPT also seemed to upregulate *TNFA* mRNA expression, though the results were insignificant (Figure 10C).

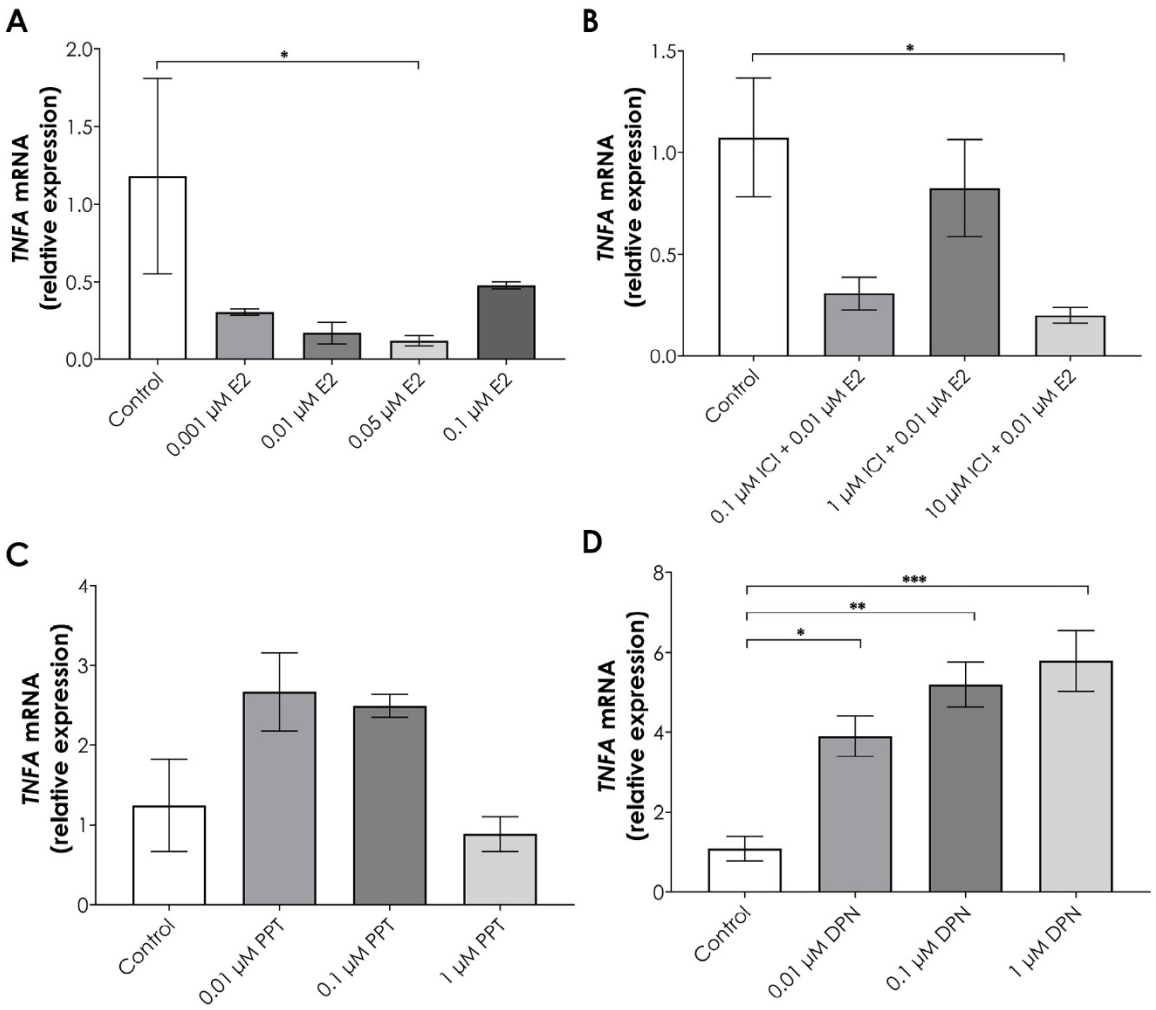


Figure 9: mRNA expression of TNFα in Huh7 cells after treatment with various ER agonists/antagonists

TNFα mRNA expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. *TNFA* transcript levels were measured using RT-qPCR, normalized to *SRSF4* expression, and compared using the ΔΔCt method. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 µM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM. Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $*P \leq 0.05$, $**P \leq 0.01$.

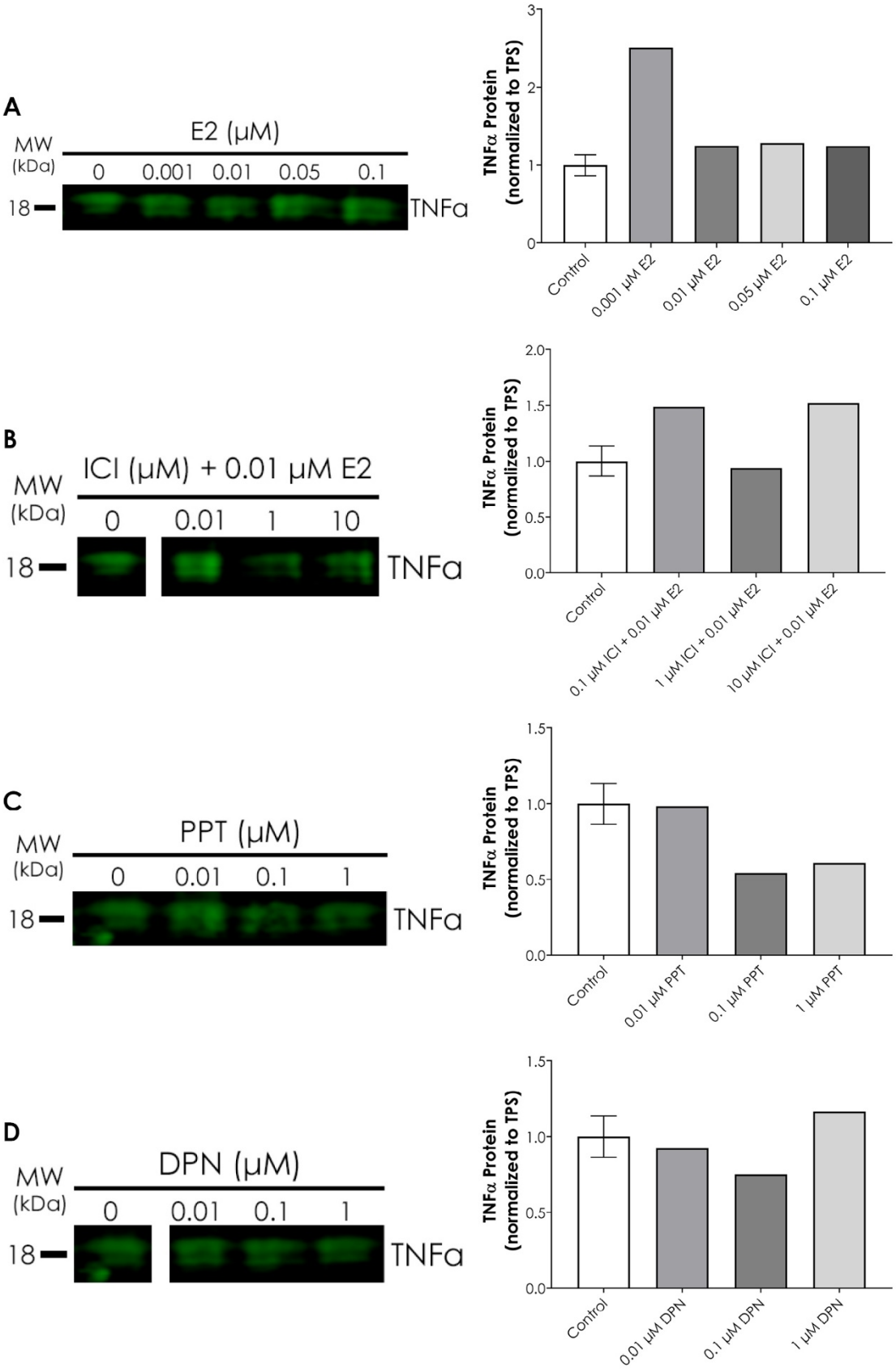

A
MW
(kDa)
E2 (μM)
0
0.001
0.01
0.05
0.1
18
TNFa
TNFα Protein
(normalized to TPS)
Control
0.001 μM E2
0.01 μM E2
0.05 μM E2
0.1 μM E2
B
ICI (μM) + 0.01 μM E2
0
0.01
1
10
0.1 μM ICI + 0.01 μM E2
1 μM ICI + 0.01 μM E2
10 μM ICI + 0.01 μM E2
C
PPT (μM)
0.01 μM PPT
0.1 μM PPT
1 μM PPT
D
DPN (μM)
0.01 μM DPN
0.1 μM DPN
1 μM DPN

Figure 10: Protein expression of TNFα in Huh7 cells after treatment with various ER agonists/antagonists

TNFα protein expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. TNFα protein expression was analyzed by Western blot using whole cell lysates and normalized to total protein. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 μM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM (*n*=1, with 2 control lanes per target). Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 7). TPS, total protein stain.

CD55 expression is altered by treatment with ER agonists/antagonists

CD55 mRNA expression was significantly decreased, whereas protein expression was unchanged, by all concentrations of E2 (Figure 11A, 12A). CD55 mRNA and protein expression was also downregulated by ICI+E2 treatment; however, the results were only significant in mRNA at 1 μM ICI (Figure 11B, Figure 12B). All concentrations of DPN significantly upregulated *CD55* mRNA expression (Figure 11C); protein expression sharply increased with the lowest dose of DPN treatment and then progressively decreased with higher concentration, although the results were not significant (Figure 12D). PPT also seemed to upregulate *CD55* mRNA and protein expression, though insignificantly (Figure 11C, 12).

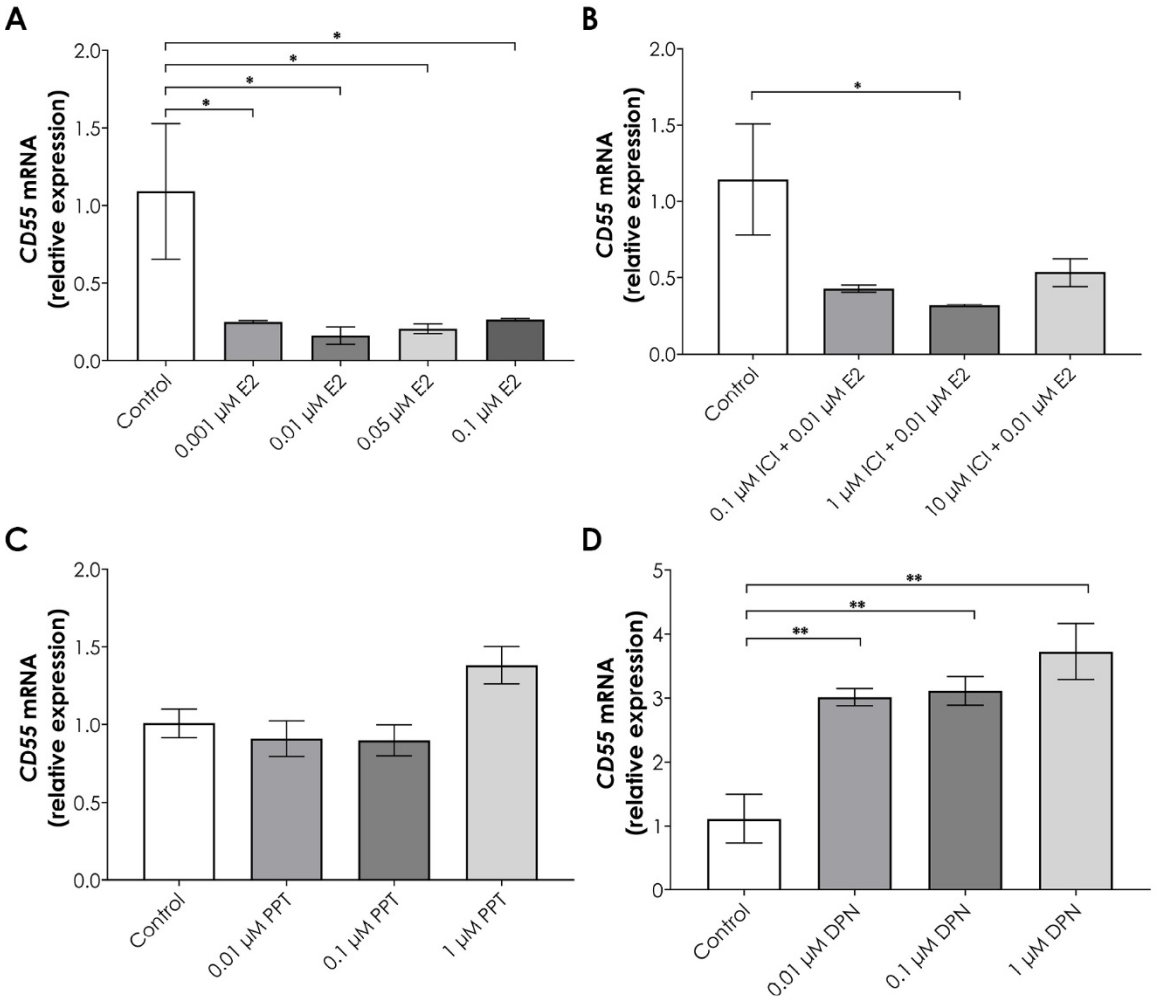


Figure 11: mRNA expression of CD55 in Huh7 cells after treatment with various ER agonists/antagonists

CD55 mRNA expression was analyzed in Huh7 cells following treatment with ER agonists/ant agonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. *CD55* transcript levels were measured using RT-qPCR, normalized to *SRSF4* expression, and compared using the ΔΔCt method. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 µM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM. Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where *$P \leq 0.05$, **$P \leq 0.01$.

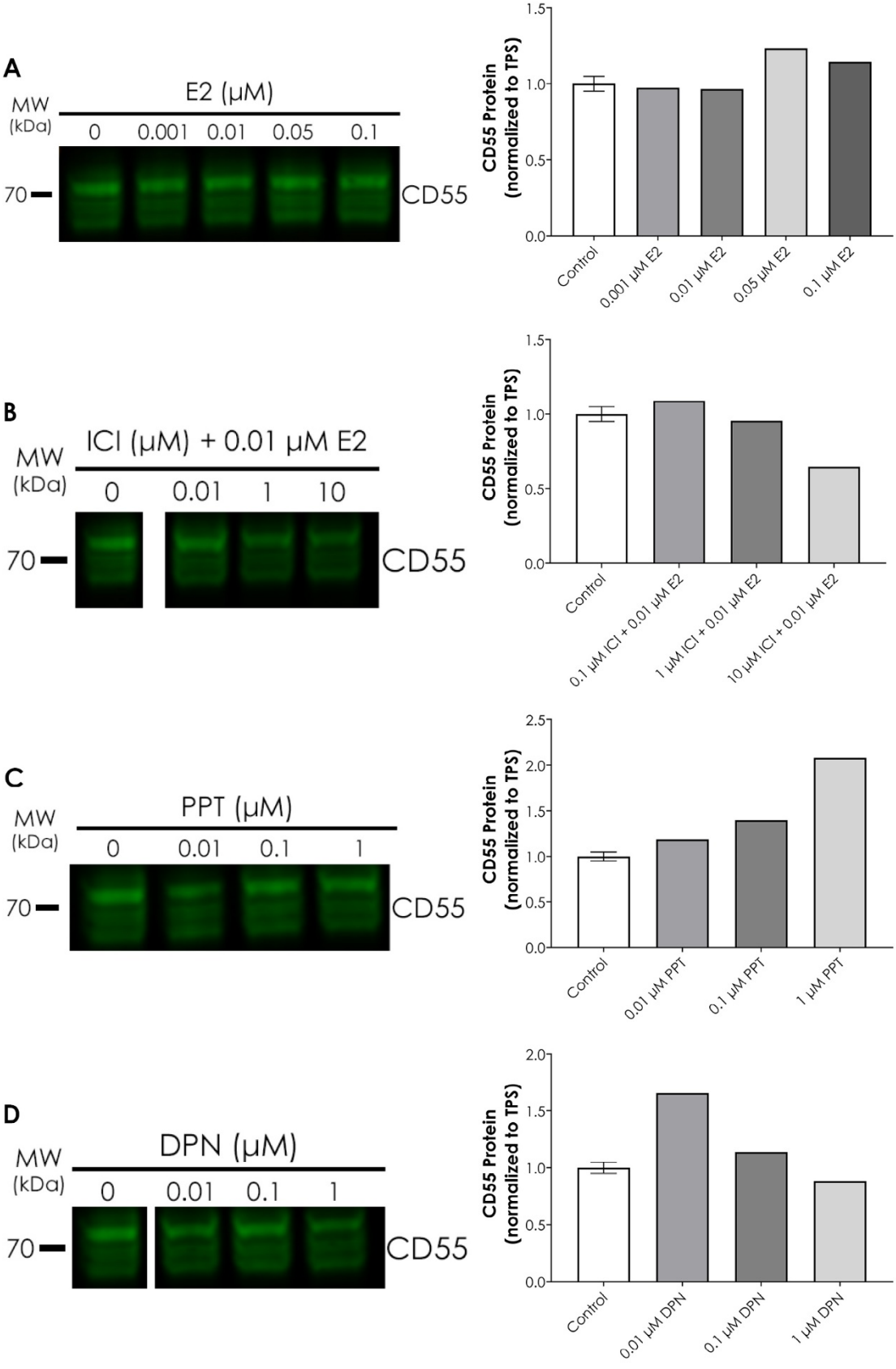

A
MW
(kDa)
E2 (μM)
0
0.001
0.01
0.05
0.1
70
CD55
CD55 Protein
(normalized to TPS)
1.5
1.0
0.5
0.0
Control
0.001 μM E2
0.01 μM E2
0.05 μM E2
0.1 μM E2
B
MW
(kDa)
ICI (μM) + 0.01 μM E2
0
0.01
1
10
70
CD55
CD55 Protein
(normalized to TPS)
1.5
1.0
0.5
0.0
Control
0.1 μM ICI + 0.01 μM E2
1 μM ICI + 0.01 μM E2
10 μM ICI + 0.01 μM E2
C
MW
(kDa)
PPT (μM)
0
0.01
0.1
1
70
CD55
CD55 Protein
(normalized to TPS)
2.5
2.0
1.5
1.0
0.5
0.0
Control
0.01 μM PPT
0.1 μM PPT
1 μM PPT
D
MW
(kDa)
DPN (μM)
0
0.01
0.1
1
70
CD55
CD55 Protein
(normalized to TPS)
2.0
1.5
1.0
0.5
0.0
Control
0.01 μM DPN
0.1 μM DPN
1 μM DPN

Figure 12: Protein expression of CD55 in Huh7 cells after treatment with various ER agonists/antagonists

CD55 protein expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. CD55 protein expression was analyzed by Western blot using whole cell lysates and normalized to total protein. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 μM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM (*n*=1, with 2 control lanes per target). Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 8). TPS, total protein stain.

IL-33 expression is modulated by ER agonists/antagonist in Huh7 cells

All concentrations of E2 and ICI downregulated *IL33* mRNA expression (Figure 13A, B). All doses of E2, but only the lowest dose of ICI, upregulated protein expression (Figure 14A, B); interestingly, the highest concentration of ICI actually downregulated IL-33 protein expression (Figure 14B). DPN and PPT treatment appears to downregulate *IL33* mRNA expression, although no statistically significant difference between the means was found (Figure 13C, D). However, IL-33 protein expression was significantly different from the control after treatment with both PPT and DPN (Figure 14C, D).

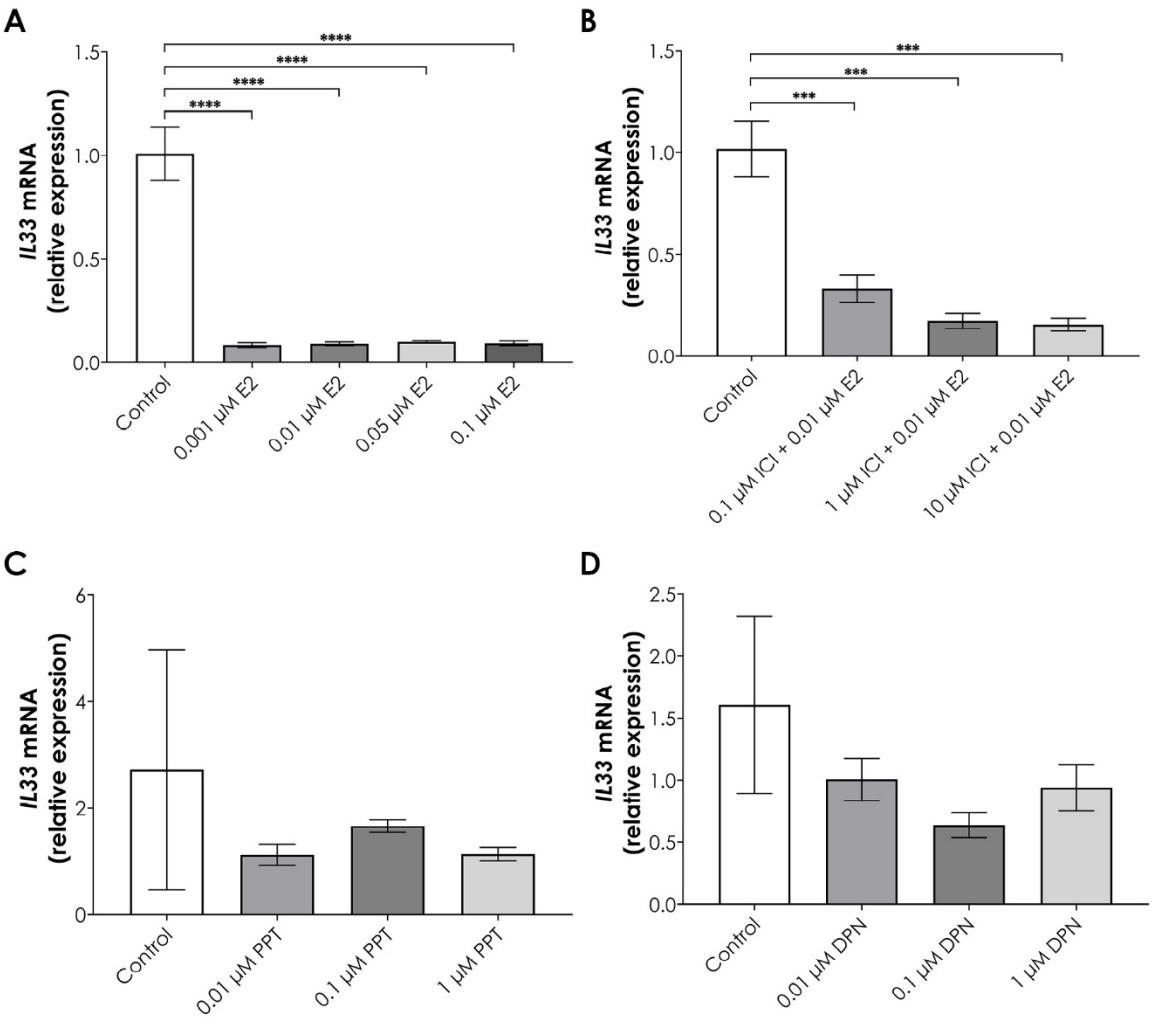


Figure 13: mRNA expression of IL-33 in Huh7 cells after treatment with various ER agonists/antagonists

IL-33 mRNA expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. *IL33* transcript levels were measured using RT-qPCR, normalized to *SRSF4* expression, and compared using the ΔΔCt method. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 µM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM. Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where $*P \leq 0.05$, $**P \leq 0.01$.

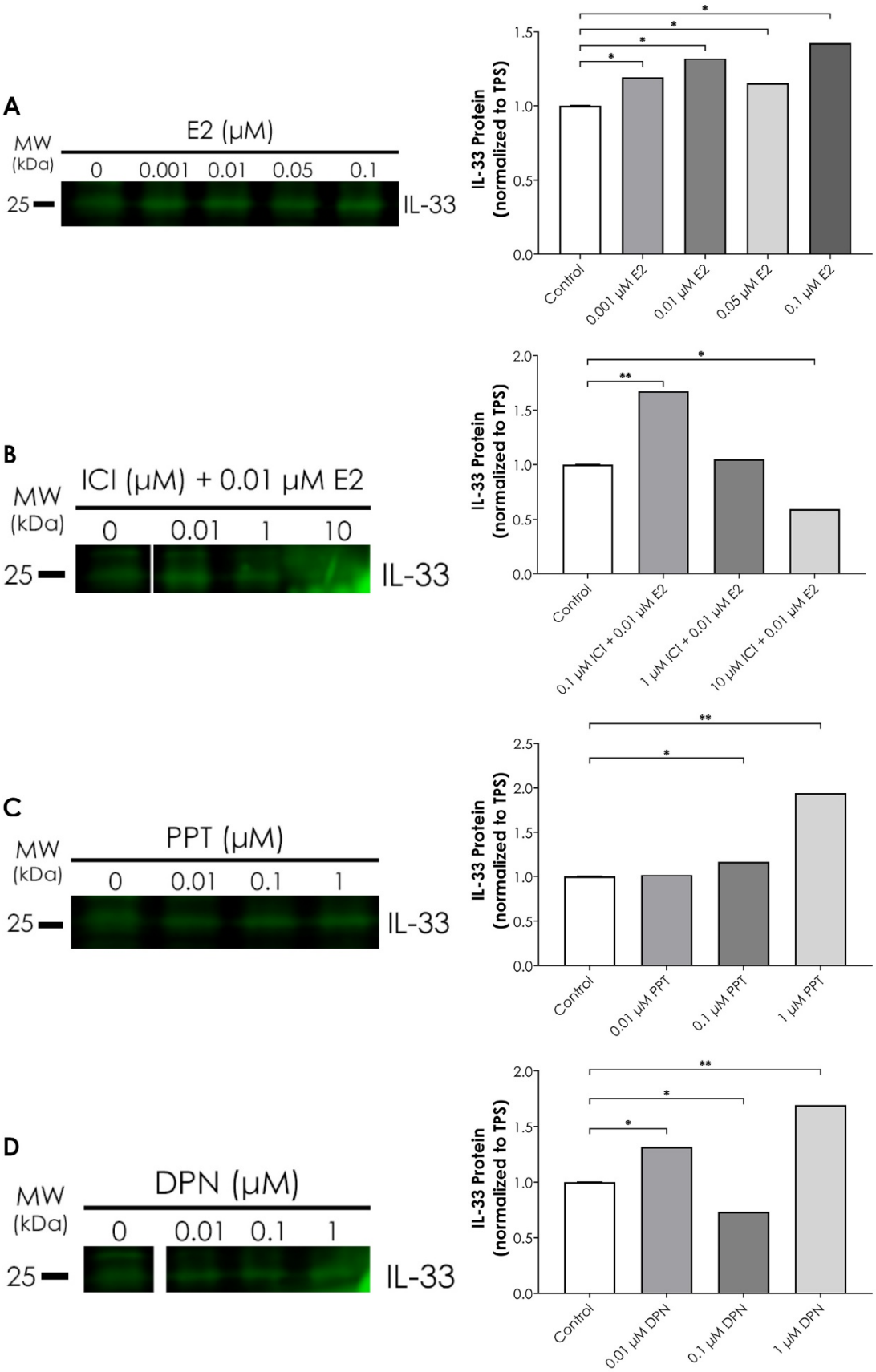

A
MW
(kDa)
E2 (μM)
0
0.001
0.01
0.05
0.1
25
IL-33
IL-33 Protein
(normalized to TPS)
Control
0.001 μM E2
0.01 μM E2
0.05 μM E2
0.1 μM E2
B
ICI (μM) + 0.01 μM E2
0
0.01
1
10
0.1 μM ICI + 0.01 μM E2
1 μM ICI + 0.01 μM E2
10 μM ICI + 0.01 μM E2
C
PPT (μM)
0.01 μM PPT
0.1 μM PPT
1 μM PPT
D
DPN (μM)
0.01 μM DPN
0.1 μM DPN
1 μM DPN

Figure 14: Protein expression of IL-33 in Huh7 cells after treatment with various ER agonists/antagonists

IL-33 protein expression was analyzed in Huh7 cells following treatment with ER agonists/antagonists and compared to control cells treated with vehicle. Cells were treated for 24 hours with varying concentrations of drug. IL-33 protein expression was analyzed by Western blot using whole cell lysates and normalized to total protein. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 μM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Column bars represent the mean ± SEM (*n*=1, with 2 control lanes per target). Data were analyzed by ordinary one-way ANOVA with Dunnett's Multiple Comparisons posttest where *$P \leq 0.05$, **$P \leq 0.01$. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 9). TPS, total protein stain.

Specific Aim 2. To determine whether ER expression and/or *IFNL4* genotype is correlated with the expression of inflammatory markers in patients with HCV-associated diseases.

Study Population

A total of 55 cases (HCV/cirrhosis, *n*=32; HCV/HCC, *n*=23) and 36 normal controls were included in the analyses (Table 5). Cases and controls did not differ significantly in the distribution of age, sex, or race. Normal and cirrhosis males were slightly younger than their female counterparts, whereas HCC females were, on average, 10 years older than HCC males (Table 6). However, the medians were not significantly different between the groups.

The frequency of the *IFNL4*-ΔG allele was 42.9% among the normal patients, 39.7% among patients with cirrhosis, and 47.7% among the patients with HCC (Table 5). In each group, the distribution of *IFNL4*-ΔG (rs368234815) genotypes was consistent with expectations under Hardy–Weinberg equilibrium. Compared to females with HCV, males were slightly less likely to have the ΔG/TT and ΔG/ΔG genotype (OR, 0.72, 0.88) but the results were not significant (Table 7).

Information regarding total bilirubin, creatinine, and liver enzymes AST and ALP were available for most HCV/cirrhosis and HCV/HCC patients. AST and creatinine levels did not differ significantly between the groups, but ALP and total bilirubin levels were significantly different between HCV/cirrhosis and HCC groups (Table 5).

Table 5: Characteristics of the study population

Characteristics	Normal (*n* =36)		Cirrhosis (*n* =32)		HCC (*n* =23)		
Age, y	*n*	(%)	*n*	(%)	*n*	(%)	*P* -value
<49	10	(27.8)	12	(37.5)	6	(26.1)	0.2593
50-59	15	(41.7)	17	(53.1)	10	(43.5)	
≥60	11	(30.6)	3	(9.4)	7	(30.4)	
Mean age ± SD, y	52.0 ± 14.6		52.2 ± 6.8		55.5 ± 7.1		0.3087
Sex							
Male	18	(50.0)	17	(53.1)	18	(78.3)	0.0765
Female	18	(50.0)	15	(46.9)	5	(21.7)	
Race							
White	18	(50.0)	22	(68.8)	15	(65.2)	0.4398
African American	2	(5.6)	1	(3.1)	2	(8.7)	
Other/Unknown	16	(44.4)	9	(28.1)	6	(26.1)	
Mean AST ± SD, U/L	N/A		187.6 ± 389.3		114.2 ± 62.4		0.4542
Mean ALP ± SD, U/L	N/A		158.5 ± 150.1		189.3 ± 110.4		0.0369
Mean Total Bilirubin ± SD, mg/dL	N/A		8.6 ± 11.7		2.8 ± 6.8		<0.0001
Mean Creatinine ± SD, mg/dL	N/A		1.8 ± 1.5		2.1 ± 2.9		0.3134
***IFNL4* -ΔG genotype**							
TT/TT	11	(30.6)	11	(34.4)	7	(30.4)	0.8405
ΔG/TT	18	(50.0)	13	(40.6)	9	(39.1)	
ΔG/ΔG	6	(16.7)	5	(15.6)	6	(26.1)	
Undetermined	1	(2.8)	3	(9.4)	1	(4.3)	
***IFNL4* -ΔG allele frequency, %**	42.9		39.7		47.7		

Table 6: Mean age of males and females in each disease group

	Mean age ± SD, y			
	Normal	**Cirrhosis**	**HCC**	*P* -value
Male	50.8 ± 18.3	50.7 ± 5.0	53.3 ± 5.9	0.0716
Female	53.1 ± 10.4	54.0 ± 8.3	63.3 ± 5.1	

Table 7: Number and proportions of HCV-positive patients who were male, by *IFNL4*-ΔG genotype

	Total	Male, %	OR	95% CI	*P-value*
TT/TT	18	66.7	Reference		
ΔG/TT	22	59.1	0.72	0.22-2.62	0.745
ΔG/ΔG	11	63.6	0.88	0.17-3.53	>0.9999

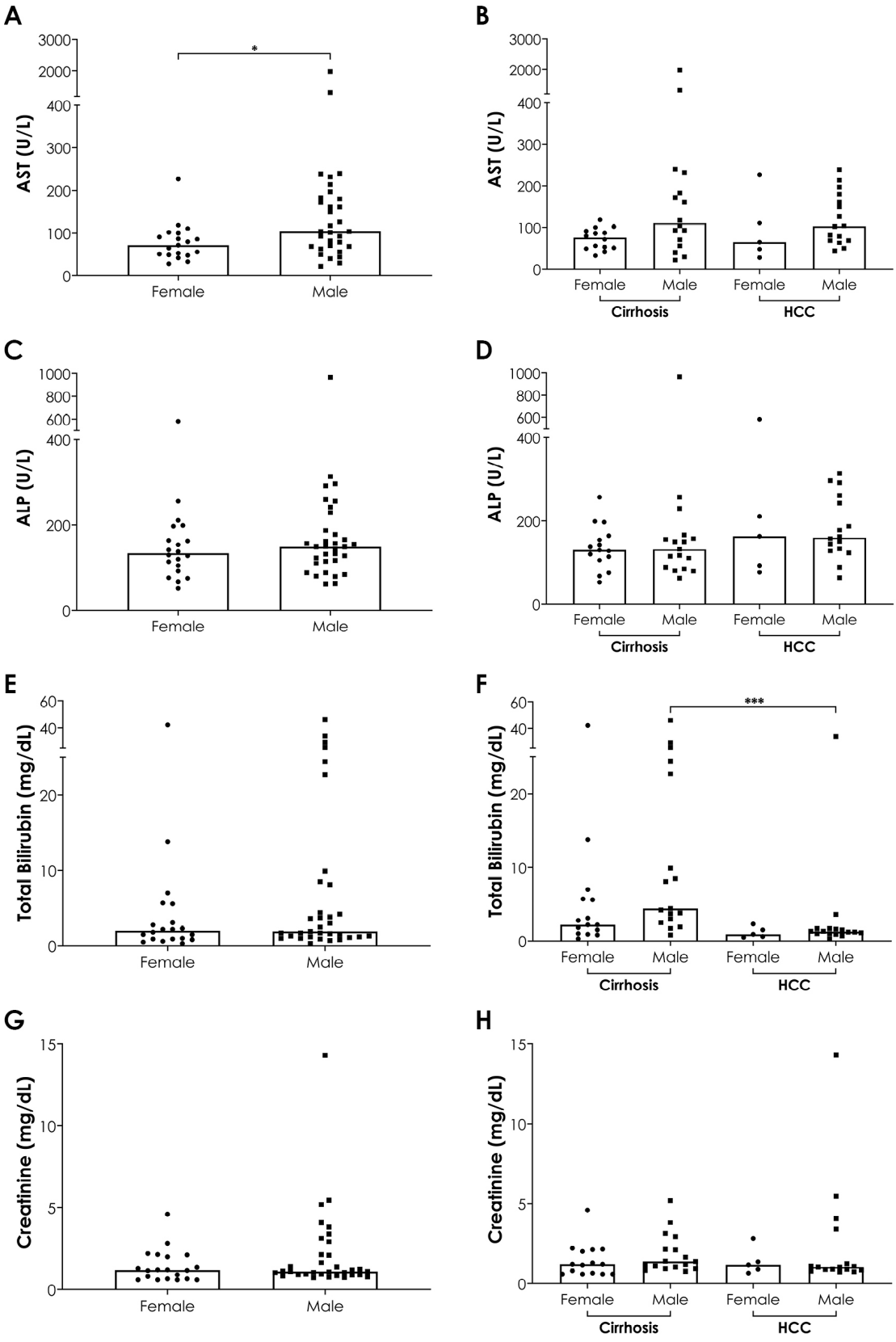

A
AST (U/L)
3000
2000
400
300
200
100
0
*
Female
Male
B
AST (U/L)
3000
2000
400
300
200
100
0
Female
Male
Female
Male
Cirrhosis
HCC
C
ALP (U/L)
1000
800
600
400
200
0
Female
Male
D
ALP (U/L)
1000
800
600
400
200
0
Female
Male
Female
Male
Cirrhosis
HCC
E
Total Bilirubin (mg/dL)
60
40
20
10
0
Female
Male
F
Total Bilirubin (mg/dL)
60
40
20
10
0
***
Female
Male
Female
Male
Cirrhosis
HCC
G
Creatinine (mg/dL)
15
10
5
0
Female
Male
H
Creatinine (mg/dL)
15
10
5
0
Female
Male
Female
Male
Cirrhosis
HCC

Figure 15: Comparison of clinical parameters by sex and disease

Clinical parameters from HCV/cirrhosis and HCV/HCC subjects were analyzed to determine if there were any sex differences between the groups. (A-B) AST; (C-D) ALP; (E-F) total bilirubin; (G-H) creatinine. Column bars represent the median. Each symbol represents one individual. Data were analyzed by Mann–Whitney *U* or Kruskal-Wallis test where $*P \leq 0.05$, $***P \leq 0.001$.

When the data were stratified by sex and disease, it was found that males, on average, had higher AST levels than females (Figure 15A). The difference in total bilirubin levels between cirrhosis and HCC subjects was only found in males (Figure 15F). No significant differences were found between the groups for ALP and creatinine (Figure 15C-D, G-H). These sex-based differences suggest that HCV may have differential pathogenesis in males and females.

Spearman's Correlation coefficient was determined using RT-qPCR data from HCV/cirrhosis and HCV/HCC livers to compare the degree of association between the mRNA expression of estrogen receptors and inflammatory markers (*ESR1, ESR2, ESR1:ESR2*, *TNFA, CD55,* and *IL33*) and clinical parameters (AST, ALP, bilirubin, and creatinine) (Tables 8-11). AST and total bilirubin were negatively correlated with *ESR1* mRNA expression (R = -0.39, P = 0.0042, Table 8; R = -0.58, $P < 0.0001$, Table 10, respectively). When segregated by disease, sex, and *IFNL4* genotype, AST was found to only have significant negative correlation with *ESR1* in HCV/cirrhosis (R = -0.40, P = 0.0294), female (R = -0.46, P = 0.0487), and ΔG allele (R = -0.46, P = 0.0098) groups (Table 8). Bilirubin maintained strong negative correlation with *ESR1* expression in both males and females (R = -0.60, P = 0.0002; R = -0.56, P = 0.0110, respectively), in HCV/cirrhosis subjects (R = -0.65, $P < 0.0001$), and in both TT/TT and ΔG allele groups (R = -0.69, P = 0.0030; R = -0.52, P = 0.0022, respectively) (Table 10). ALP was also significantly negatively correlated with *ESR1* in HCV/HCC patients (R = -0.53, P = 0.0143) (Table 9). Bilirubin was positively correlated with ESR2 expression in females overall, and in particular females with the ΔG allele (R = 0.72, P = 0.0067) (Table 10). As expected, similar patterns of correlation were found between the applicable clinical parameters and the *ESR1:ESR2* expression ratio. Bilirubin was

positively correlated with *CD55* expression in males overall, probably influenced by the strong positive correlation in males with the ΔG allele ($R = 0.60$, $P = 0.0070$) (Table 10). Creatinine had a strong negative correlation with CD55 in females with HCC ($R = -1.00$, $P = 0.0167$) (Table 11).

Table 8: Spearman's correlations between AST levels and mRNA expression of estrogen receptors and inflammatory markers (HCV-infected patients only)

Aspartate aminotransferase		*ESR1*		*ESR2*		*ESR1:ESR2*		*TNFA*		*CD55*		*IL33*	
		R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
All HCV	**Together (n=51)**	-0.39	(0.0042)	0.26	(0.0690)	-0.49	(0.0003)	0.13	(0.3667)	-0.04	(0.7645)	-0.10	(0.4894)
	Males (n=32)	-0.28	(0.1255)	0.22	(0.2311)	-0.43	(0.0135)	0.01	(0.9547)	0.16	(0.3695)	0.05	(0.7986)
	Females (n=19)	-0.46	(0.0487)	0.43	(0.0675)	-0.42	(0.0726)	0.27	(0.2665)	-0.05	(0.8529)	-0.53	(0.0206)
HCV/Cirrhosis	**Together (n=30)**	-0.40	(0.0294)	0.35	(0.0566)	-0.50	(0.0047)	0.07	(0.6937)	-0.12	(0.5371)	-0.20	(0.2821)
	Males (n=16)	-0.20	(0.4579)	0.10	(0.7107)	-0.31	(0.2449)	-0.24	(0.3590)	0.15	(0.5839)	-0.14	(0.6111)
	Females (n=14)	-0.33	(0.2530)	0.58	(0.0318)	-0.52	(0.0591)	0.42	(0.1324)	0.03	(0.9155)	-0.22	(0.4542)
HCV/HCC	**Together (n=21)**	-0.41	(0.0622)	0.12	(0.6039)	-0.48	(0.0276)	0.18	(0.4368)	-0.01	(0.9577)	-0.04	(0.8800)
	Males (n=16)	-0.37	(0.1570)	0.32	(0.2266)	-0.54	(0.0333)	0.28	(0.2919)	0.14	(0.5956)	0.32	(0.2311)
	Females (n=5)	-0.70	(0.2333)	-0.40	(0.5167)	0.00	(>0.9999)	0.10	(0.9500)	-0.60	(0.3500)	-1.00	(0.0167)
TT/TT	**Together (n=16)**	-0.48	(0.0626)	-0.09	(0.7483)	-0.37	(0.1570)	-0.11	(0.6863)	-0.25	(0.3504)	-0.31	(0.2449)
	Males (n=10)	-0.36	(0.2992)	-0.20	(0.5759)	-0.30	(0.3999)	-0.15	(0.6881)	0.33	(0.3527)	0.00	(>0.9999)
	Females (n=6)	-0.77	(0.1028)	0.37	(0.4972)	-0.37	(0.4972)	-0.03	(>0.9999)	-0.83	(0.0583)	-0.77	(0.1028)
ΔG	**Together (n=31)**	-0.46	(0.0098)	0.22	(0.2325)	-0.50	(0.0040)	0.13	(0.4910)	-0.05	(0.7785)	0.19	(0.3139)
	Males (n=19)	-0.37	(0.1195)	0.24	(0.3160)	-0.43	(0.0643)	-0.14	(0.5787)	0.08	(0.7398)	0.33	(0.1653)
	Females (n=12)	-0.37	(0.2367)	0.36	(0.2464)	-0.45	(0.1404)	0.41	(0.1845)	0.17	(0.5881)	-0.31	(0.3310)

<0.05
<0.01
<0.001

Table 9: Spearman's correlations between ALP levels and mRNA expression of estrogen receptors and inflammatory markers (HCV-infected patients only)

Alkaline phosphatase		*ESR1*		*ESR2*		*ESR1:ESR2*		*TNFA*		*CD55*		*IL33*	
		R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
All HCV	**Together (n=53)**	-0.05	(0.7200)	0.16	(0.2382)	-0.14	(0.3209)	0.19	(0.1684)	-0.16	(0.2586)	-0.18	(0.1945)
	Males (n=33)	0.12	(0.5173)	0.24	(0.1720)	-0.01	(0.9691)	0.24	(0.1813)	-0.22	(0.2116)	-0.16	(0.3754)
	Females (n=20)	-0.24	(0.3163)	0.12	(0.6269)	-0.16	(0.5021)	0.06	(0.8011)	0.06	(0.7865)	-0.22	(0.3456)
HCV/Cirrhosis	**Together (n=32)**	0.12	(0.5082)	0.21	(0.2401)	-0.05	(0.7941)	0.11	(0.5618)	-0.07	(0.7227)	-0.21	(0.2377)
	Males (n=17)	0.28	(0.2750)	0.37	(0.1461)	0.04	(0.8889)	0.32	(0.2057)	-0.39	(0.1234)	-0.19	(0.4591)
	Females (n=15)	-0.15	(0.5844)	0.12	(0.6669)	-0.14	(0.6115)	-0.10	(0.7337)	0.21	(0.4421)	-0.35	(0.2012)
HCV/HCC	**Together (n=21)**	-0.53	(0.0143)	0.20	(0.3911)	-0.62	(0.0026)	0.35	(0.1191)	-0.08	(0.7371)	-0.44	(0.0465)
	Males (n=16)	-0.44	(0.0937)	0.18	(0.5050)	-0.56	(0.0266)	0.30	(0.2583)	-0.01	(0.9694)	-0.52	(0.0410)
	Females (n=5)	-0.90	(0.0833)	0.30	(0.6833)	-0.90	(0.0833)	0.20	(0.7833)	-0.30	(0.6833)	-0.40	(0.5167)
TT/TT	**Together (n=17)**	-0.18	(0.4846)	0.37	(0.1500)	-0.40	(0.1086)	0.27	(0.2942)	-0.43	(0.0835)	-0.29	(0.2552)
	Males (n=11)	0.15	(0.6731)	0.32	(0.3415)	-0.07	(0.8385)	0.46	(0.1546)	-0.39	(0.2366)	-0.18	(0.5950)
	Females (n=6)	-0.49	(0.3556)	0.49	(0.3556)	-0.49	(0.3556)	-0.20	(0.7139)	0.14	(0.8028)	-0.66	(0.1750)
ΔG	**Together (n=32)**	0.09	(0.6342)	0.06	(0.7370)	0.06	(0.7362)	0.19	(0.3060)	-0.03	(0.8860)	-0.18	(0.3338)
	Males (n=19)	0.21	(0.3847)	0.17	(0.4836)	0.18	(0.4589)	0.11	(0.6445)	-0.02	(0.9403)	-0.22	(0.3609)
	Females (n=13)	-0.09	(0.7784)	0.02	(0.9639)	-0.02	(0.9639)	0.26	(0.3939)	0.01	(0.9929)	-0.13	(0.6827)

<0.05
<0.01
<0.001

Table 10: Spearman's correlations between total bilirubin levels and mRNA expression of estrogen receptors and inflammatory markers (HCV-infected patients only)

	Total bilirubin	*ESR1*		*ESR2*		*ESR1:ESR2*		*TNFA*		*CD55*		*IL33*	
		R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
All HCV	**Together (n=53)**	-0.58	(<0.0001)	0.17	(0.2378)	-0.62	(<0.0001)	0.17	(0.2370)	0.23	(0.0985)	-0.14	(0.3279)
	Males (n=33)	-0.60	(0.0002)	0.00	(>0.9999)	-0.67	(<0.0001)	0.10	(0.5627)	0.41	(0.0175)	-0.09	(0.6304)
	Females (n=20)	-0.56	(0.0110)	0.47	(0.0377)	-0.53	(0.0163)	0.27	(0.2575)	0.29	(0.2090)	-0.27	(0.2506)
HCV/Cirrhosis	**Together (n=32)**	-0.65	(<0.0001)	0.13	(0.4871)	-0.62	(0.0002)	-0.02	(0.9278)	-0.14	(0.4470)	0.04	(0.8180)
	Males (n=17)	-0.59	(0.0142)	-0.29	(0.2552)	-0.57	(0.0197)	-0.29	(0.2594)	-0.05	(0.8611)	0.12	(0.6390)
	Females (n=15)	-0.57	(0.0281)	0.51	(0.0565)	-0.58	(0.0258)	0.25	(0.3585)	0.19	(0.5002)	-0.09	(0.7406)
HCV/HCC	**Together (n=21)**	-0.39	(0.0791)	0.12	(0.6171)	-0.45	(0.0406)	0.39	(0.0830)	0.16	(0.4883)	-0.23	(0.3180)
	Males (n=16)	-0.33	(0.2166)	0.07	(0.7897)	-0.39	(0.1362)	0.41	(0.1139)	0.23	(0.3947)	-0.04	(0.8712)
	Females (n=5)	-0.30	(0.6833)	0.40	(0.5167)	0.00	(>0.9999)	0.40	(0.5167)	0.10	(0.9500)	-0.50	(0.4500)
TT/TT	**Together (n=17)**	-0.69	(0.0030)	-0.19	(0.4644)	-0.42	(0.0902)	0.15	(0.5506)	-0.16	(0.5347)	-0.42	(0.0912)
	Males (n=11)	-0.73	(0.0137)	-0.30	(0.3740)	-0.43	(0.1887)	0.15	(0.6580)	0.19	(0.5804)	-0.48	(0.1386)
	Females (n=6)	-0.38	(0.4722)	0.17	(0.7500)	-0.17	(0.7500)	-0.03	(0.9833)	-0.20	(0.7111)	-0.41	(0.4333)
ΔG	**Together (n=32)**	-0.52	(0.0022)	0.38	(0.0322)	-0.67	(<0.0001)	0.24	(0.1796)	0.44	(0.0112)	-0.02	(0.8970)
	Males (n=19)	-0.55	(0.0149)	0.17	(0.4947)	-0.66	(0.0022)	0.14	(0.5539)	0.60	(0.0070)	0.23	(0.3432)
	Females (n=13)	-0.66	(0.0171)	0.72	(0.0067)	-0.74	(0.0051)	0.52	(0.0693)	0.47	(0.1085)	-0.23	(0.4390)

<0.05
<0.01
<0.001

Table 11: Spearman's correlations between creatinine levels and mRNA expression of estrogen receptors and inflammatory markers (HCV-infected patients only)

	Creatinine	*ESR1*		*ESR2*		*ESR1:ESR2*		*TNFA*		*CD55*		*IL33*	
		R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
All HCV	**Together (n=53)**	-0.12	(0.4017)	0.01	(0.9390)	-0.08	(0.5630)	-0.04	(0.7542)	0.07	(0.6214)	-0.14	(0.3304)
	Males (n=33)	-0.08	(0.6465)	-0.10	(0.5755)	0.01	(0.9742)	-0.25	(0.1579)	0.15	(0.4002)	-0.26	(0.1396)
	Females (n=20)	-0.18	(0.4367)	0.12	(0.6230)	-0.14	(0.5590)	0.12	(0.6051)	0.06	(0.7929)	0.03	(0.8969)
HCV/Cirrhosis	**Together (n=32)**	0.10	(0.5902)	0.11	(0.5480)	0.03	(0.8882)	0.21	(0.2455)	0.12	(0.5130)	0.07	(0.6994)
	Males (n=17)	0.38	(0.1324)	-0.11	(0.6611)	0.34	(0.1765)	-0.08	(0.7708)	0.17	(0.5103)	-0.04	(0.8740)
	Females (n=15)	-0.12	(0.6752)	0.21	(0.4586)	-0.21	(0.4427)	0.43	(0.1145)	0.27	(0.3264)	0.14	(0.6235)
HCV/HCC	**Together (n=21)**	-0.38	(0.0893)	-0.16	(0.4872)	-0.14	(0.5329)	-0.44	(0.0461)	-0.14	(0.5422)	-0.65	(0.0015)
	Males (n=16)	-0.38	(0.1480)	-0.20	(0.4564)	-0.09	(0.7545)	-0.38	(0.1514)	-0.09	(0.7462)	-0.67	(0.0056)
	Females (n=5)	-0.60	(0.3500)	-0.50	(0.4500)	-0.10	(0.9500)	-0.70	(0.2333)	-1.00	(0.0167)	-0.60	(0.3500)
TT/TT	**Together (n=17)**	0.08	(0.7557)	-0.37	(0.1465)	0.41	(0.1046)	-0.10	(0.7126)	-0.33	(0.2008)	-0.15	(0.5727)
	Males (n=11)	0.21	(0.5432)	-0.31	(0.3442)	0.65	(0.0339)	-0.51	(0.1154)	0.06	(0.8656)	-0.04	(0.9185)
	Females (n=6)	0.21	(0.7333)	-0.39	(0.4500)	0.39	(0.4500)	0.27	(0.6167)	-0.64	(0.2000)	0.21	(0.7333)
ΔG	**Together (n=32)**	-0.19	(0.2852)	0.17	(0.3536)	-0.23	(0.2132)	-0.02	(0.9262)	0.30	(0.0921)	-0.16	(0.3911)
	Males (n=19)	-0.14	(0.5568)	-0.12	(0.6215)	-0.04	(0.8641)	-0.26	(0.2864)	0.22	(0.3592)	-0.25	(0.3108)
	Females (n=13)	-0.43	(0.1413)	0.43	(0.1469)	-0.45	(0.1228)	0.16	(0.6000)	0.40	(0.1769)	0.07	(0.8166)

<0.05
<0.01
<0.001

IL33 mRNA expression was negatively correlated with AST in overall females (R = -0.53, P = 0.0206); when stratified by disease and sex, the association was only seen in HCC females despite the small sample size (R = -1.00, P = 0.0167) (Table 8). *IL33* was also correlated with ALP and creatinine in HCC males (R = -0.52, P = 0.0410, Table 9; R = -0.67, P = 0.0056, Table 11, respectively). Creatinine was weakly negatively correlated with TNFA expression in HCC subjects (R = -0.44, P = 0.0461) (Table 11).

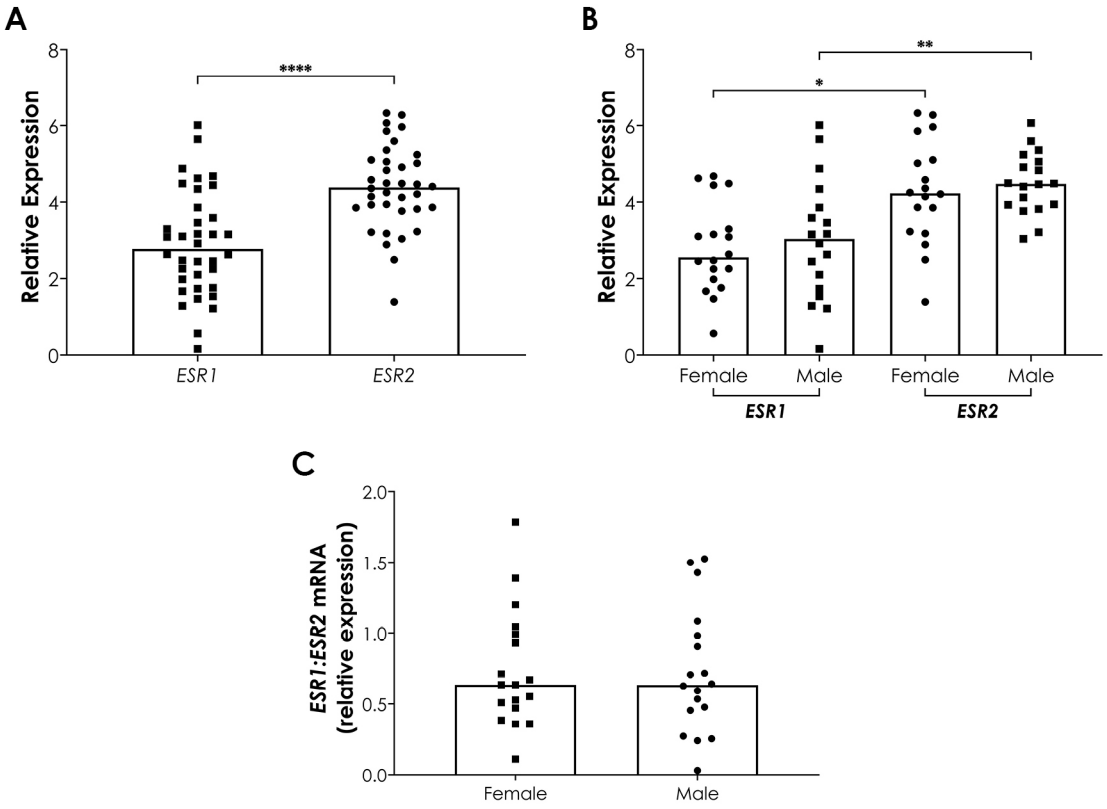


Figure 16: mRNA expression of ERα and ERβ in human liver tissues from control subjects

(A) ER subtype expression was analyzed in human liver tissues by RT-qPCR using gene specific primers. Target gene expression was normalized to *GUSB* and *SRSF4* expression using the ΔCt method and plotted. (B) Stratification of (A) by sex. (C) Ratio of *ESR1* to *ESR2* mRNA expression in controls stratified by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Mann–Whitney *U* or Kruskal-Wallis test where *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$.

Expression of ERβ, but not ERα, is altered in patients with HCV-associated diseases

To investigate the role of ERs in HCV-associated diseases, the mRNA expression of ERα and ERβ was first measured in normal livers using RT-qPCR. *ESR2* expression levels were significantly higher than *ESR1* levels when the data were pooled (Figure 16A) and when they were segregated by sex (Figure 16B). Since ERα and ERβ are known co-regulators of physiological outcomes, the *ESR1:ESR2* expression ratio was compared between males and females and it was found to be comparable (Figure 16C). The expression of ER subtypes in HCV/cirrhosis and HCV/HCC liver explants was then measured and compared to controls using RT-qPCR and Western blotting. At the transcriptional level, there was an apparent increase in *ESR1* expression in HCV/HCC livers, though the results were not statistically significant (Figure 17A-B). ERα protein expression decreased in HCV-related diseased livers, but again, the results were not statistically significant (Figure 17D-E).

ESR2 mRNA expression was significantly lower in the HCV/HCC group as compared to the control (Figure 18A). As expected, the *ESR1:ESR2* ratio expression was significantly increased in HCV/HCC livers compared to normal (Figure 18C). No significant differences in *ESR2* expression or in *ESR1:ESR2* ratio was found when results were stratified by both disease and sex (Figure 18B, D). *ESR1:ESR2* ratio in males with HCV/cirrhosis did show opposite trend of expression compared to females; however, it did not reach significance (Figure 18D).

There was a significant increase in the liver protein expression of ERβ in HCV/cirrhosis and HCC as compared to normal in both cytoplasmic and nuclear fractions (Figure 19A-B). When results were stratified by sex, the increase in nuclear ERβ was only significant in cirrhosis females and the increase in cytoplasmic ERβ was only significant in HCC females (Figure 19D-E). Although no significant differences were found at the transcriptional level, at the translational level, when the ratio of ERα total protein expression to ERβ cytoplasmic protein expression was analyzed, there was a significant decrease in both HCV/cirrhosis and HCC subjects compared to controls

(Supplementary Figure 4C). These results suggest that infection with HCV may alter the expression of ER subtypes, specifically ERβ.

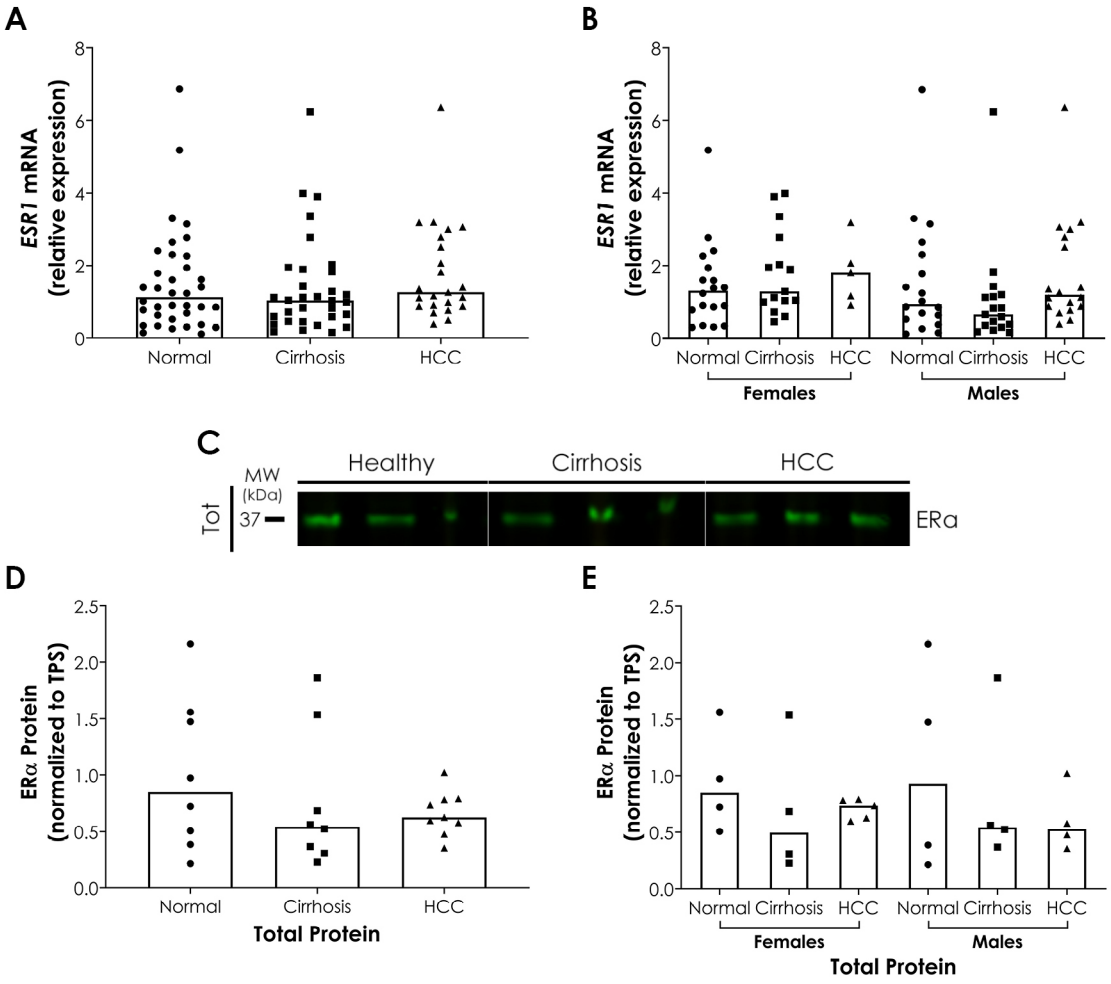


Figure 17: Expression of ERα in human liver tissues with HCV-related diseases

ERα mRNA and protein expressions were analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) *ESR1* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) ERα protein expression by Western blot using whole liver tissue lysates (tot). (D) Quantification of (C) by densitometry from multiple blots and normalized to total protein. (E) ERα protein expression in human liver tissues stratified by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 10). TPS, total protein stain.

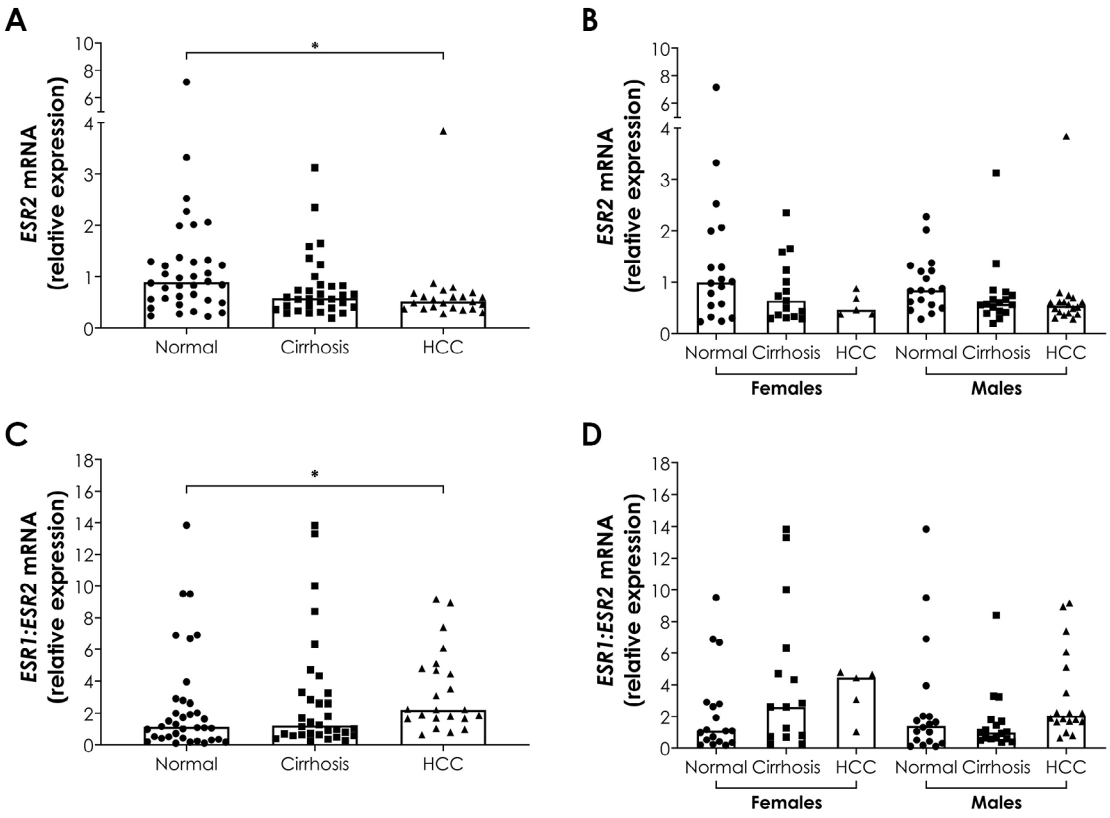


Figure 18: mRNA expression of ERβ in human liver tissues with HCV-related diseases

ERβ mRNA expression was analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) *ESR2* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) Ratio of *ESR1* to *ESR2* mRNA expression (D) Stratification of (C) by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant.

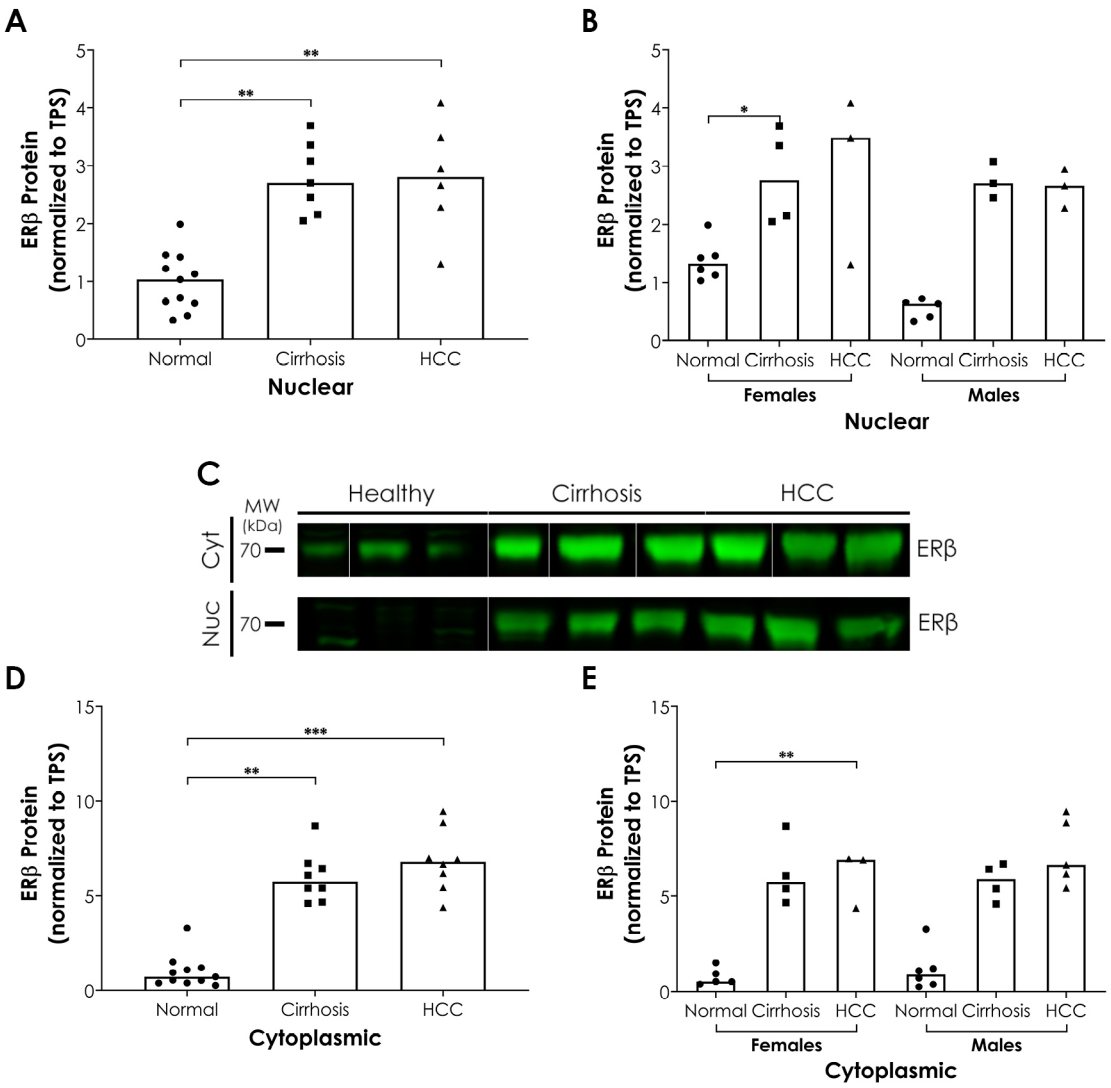


Figure 19: Protein expression of ERβ in human liver tissues with HCV-related diseases

ERβ protein expression was analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) ERβ protein expression by Western blot in human liver tissues fractionated by nuclear (nuc) and cytoplasmic (cyt) protein. (B-C) Quantification of (A) by densitometry from multiple blots and normalized to total protein. (D-E) ERβ protein expression in nuclear and cytoplasmic fractions of human liver tissues and stratified by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 11, 12). TPS, total protein stain.

TNFα mRNA expression is increased in patients with HCV-associated diseases

Because HCV pathogenesis relies primarily on inflammation to cause damage, the expression of proinflammatory cytokine TNFα in this cohort was analyzed. *TNFA* mRNA expression was significantly increased in both HCV/cirrhosis and HCV/HCC groups compared to controls (Figure 20A). When results were stratified by sex, *TNFA* was only significantly higher in HCV/cirrhosis livers of both males and females (Figure 20B). Although the results were not statistically significant, TNFα protein expression followed a similar trend as mRNA expression (Figure 20C-E).

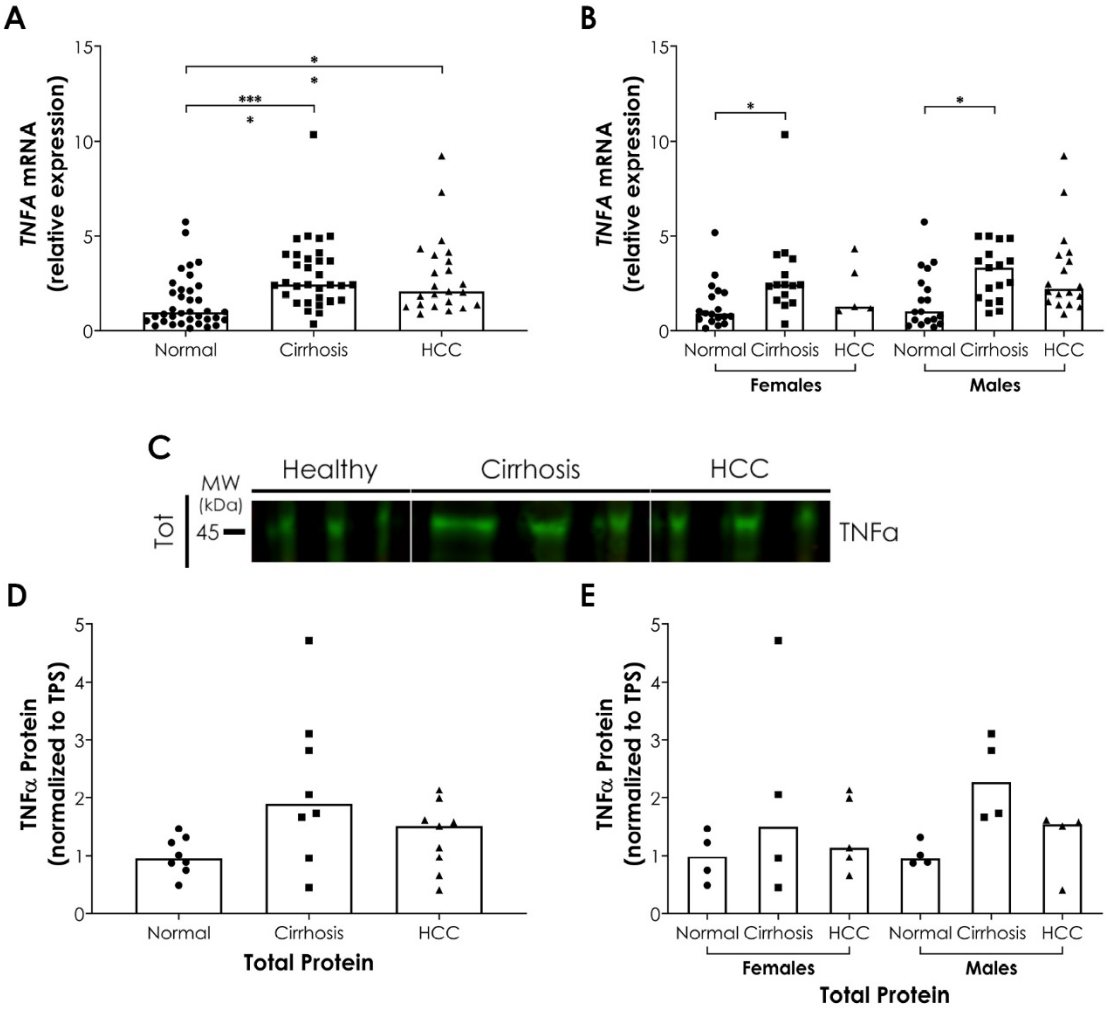


Figure 20: Expression of TNFα in human liver tissues with HCV-related diseases

TNFα mRNA and protein expressions were analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) *TNFA* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) TNFα protein expression by Western blot using whole liver tissue lysates. (D) Quantification of (C) by densitometry from multiple blots and normalized to total protein. (E) Stratification of (D) by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $*P \leq 0.05$, $**P \leq 0.01$, $****P < 0.0001$. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 13). TPS, total protein stain.

Differential expression of CD55 in patients with HCV-related cirrhosis and HCC

Complement regulatory proteins have been thought to play a role in carcinogenesis, so the expression of CD55 mRNA and protein during HCV pathogenesis was of interest. CD55 mRNA and nuclear and total protein expression was decreased in HCV/HCC livers, though the results were not significant (Figure 21A, 22B, F). Looking at Figure 21B as well as Supplementary Figure 2, it is apparent that females have higher *CD55* expression compared to males in each disease group, although no statistically significant difference between the groups was evident. Cytoplasmic protein expression was comparable between groups (Figure 22D-E). Because CD55 is a membrane protein, the protein expression was then measured in whole tissue lysates. Similar to the mRNA and nuclear protein results, CD55 expression appears to decrease in HCV/HCC livers, but the results were not significant (Figure 22F-G). Notably, nuclear and total CD55 protein expression saw an increase in cirrhosis subjects, particularly male cirrhosis subjects (Figure 22B-C, F-G).

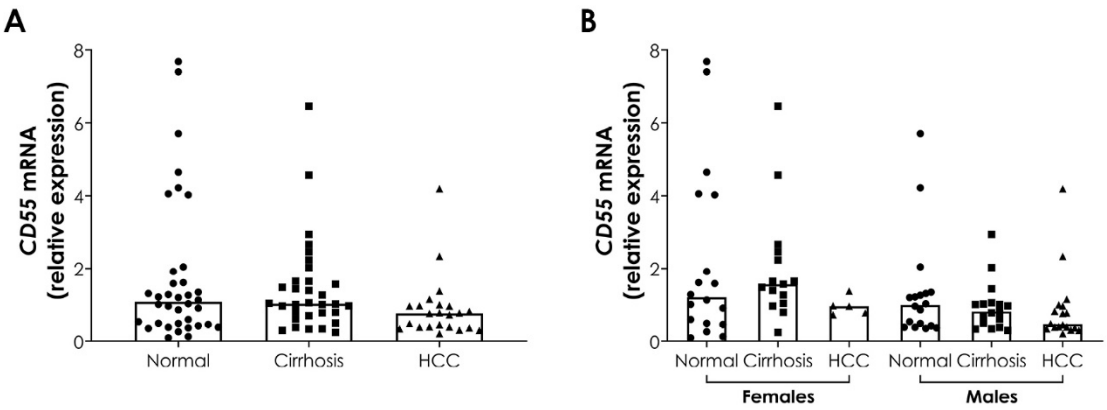


Figure 21: mRNA expression of CD55 in human liver tissues with HCV-related diseases

CD55 mRNA expression was analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) *CD55* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where P≤0.05 was considered significant.

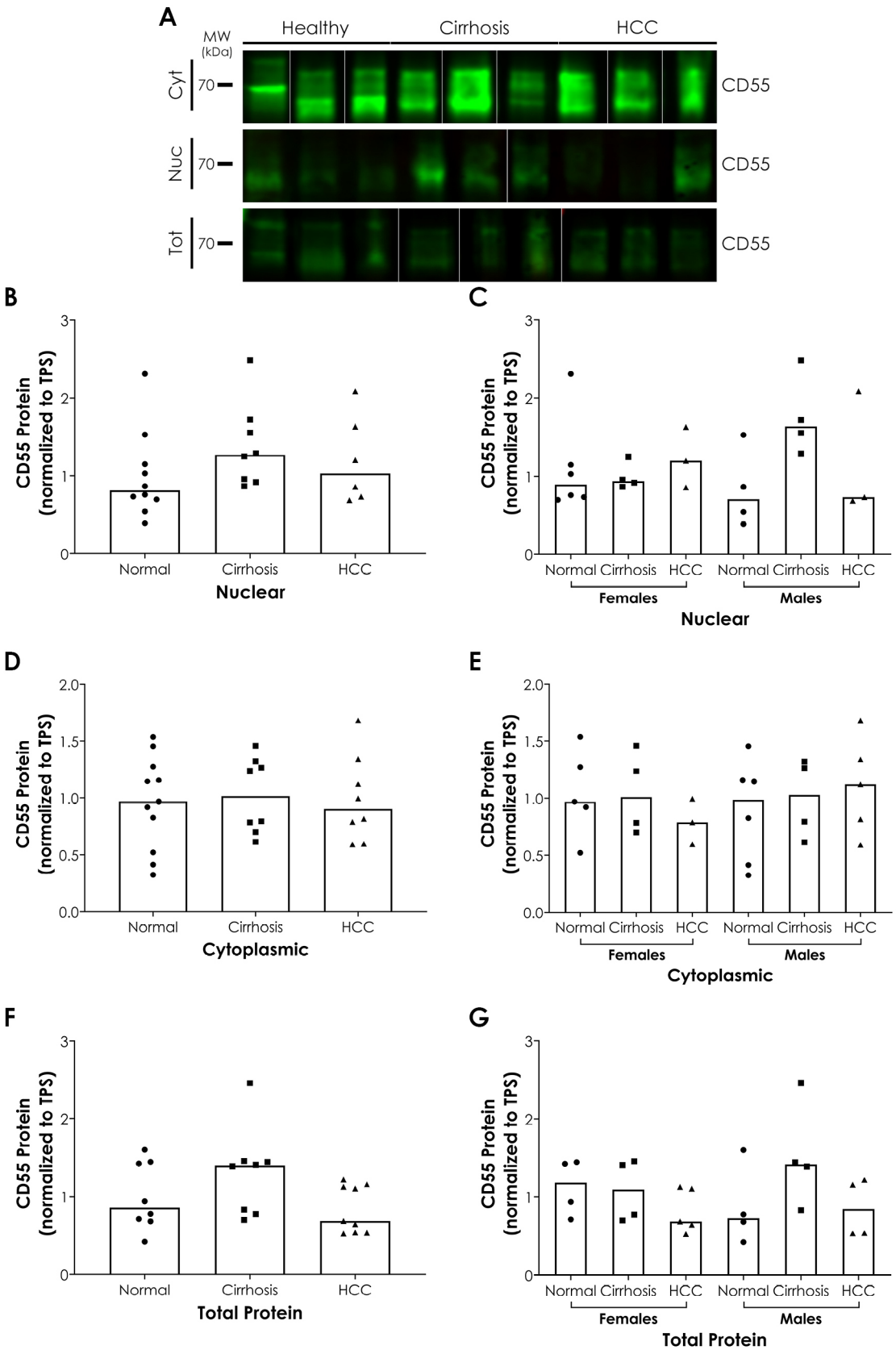

A
MW (kDa)
Healthy
Cirrhosis
HCC
Cyt
Nuc
Tot
70
CD55
B
C
D
E
F
G
CD55 Protein (normalized to TPS)
Normal
Cirrhosis
HCC
Females
Males
Nuclear
Cytoplasmic
Total Protein

Figure 22: Protein expression of CD55 in human liver tissues with HCV-related diseases

CD55 protein expression was analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) CD55 protein expression by Western blot in human liver tissues using whole tissue lysate (tot) and fractionated by nuclear (nuc) and cytoplasmic (cyt) protein. (B,D,F) Quantification of (A) by densitometry from multiple blots and normalized to total protein. (C,E,G) Stratified by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 14-16). TPS, total protein stain.

No significant difference in IL-33 expression was found

The expression of IL-33 mRNA and protein in HCV-related cirrhosis, HCC, and normal livers is depicted in Figure 23. HCV-related HCC livers showed increased expression of IL-33 at the mRNA and nuclear protein levels, but the results were not significant (Figure 23A-E). IL-33 protein expression showed a decreased trend in males with HCV-related cirrhosis but it did not reach significance (Figure 23E). Because of the apparent inverse correlation between the nuclear protein expression of IL-33 and CD55, Spearman's correlation analysis was performed between the groups but no significant association was found (Figure 23F). IL-33 expression was not measured in cytoplasmic extracts because it is strictly a nuclear protein (Cayrol and Girard 2018).

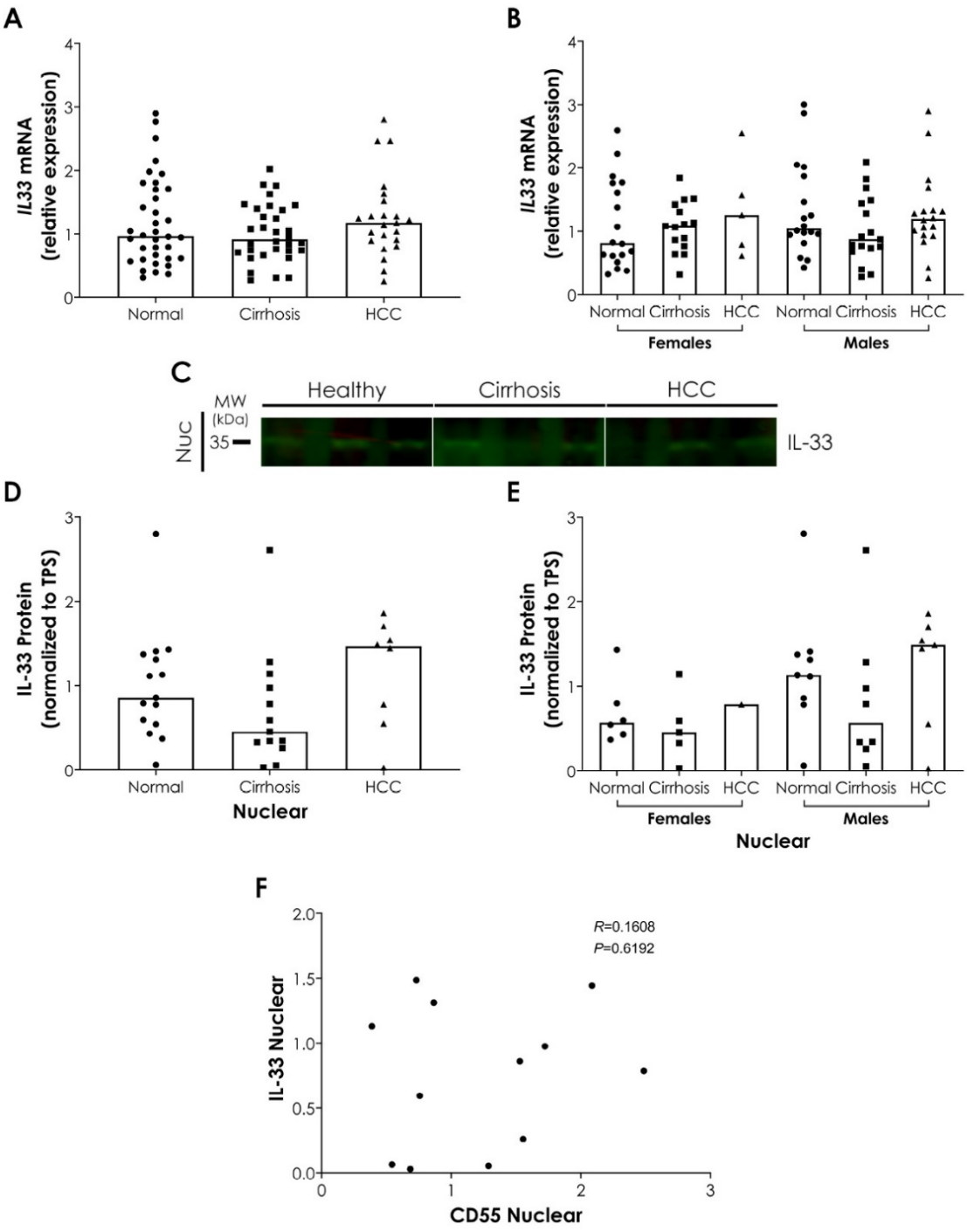

A
IL33 mRNA (relative expression)
Normal
Cirrhosis
HCC
B
IL33 mRNA (relative expression)
Normal Cirrhosis HCC Normal Cirrhosis HCC
Females
Males
C
Healthy
Cirrhosis
HCC
MW (kDa)
Nuc
35
IL-33
D
IL-33 Protein (normalized to TPS)
Normal
Cirrhosis
HCC
Nuclear
E
IL-33 Protein (normalized to TPS)
Normal Cirrhosis HCC Normal Cirrhosis HCC
Females
Males
Nuclear
F
R=0.1608
P=0.6192
IL-33 Nuclear
CD55 Nuclear

Figure 23: Expression of IL-33 in human liver tissues with HCV-related diseases

IL-33 mRNA and protein expressions were analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) *IL33* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) IL-33 protein expression by Western blot using nuclear fractions. (D) Quantification of (C) by densitometry from multiple blots and normalized to total protein. (E) IL-33 protein expression in human liver tissues stratified by sex. (F) Spearman's correlation between IL-33 nuclear protein and CD-55 nuclear protein

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. Uncropped Western blot images for both total protein and target protein can be found in the supplementary materials (Supplementary Figure 17). TPS, total protein stain.

Correlation Analyses by Disease

Spearman's Correlation coefficient was determined using RT-qPCR data from HCV/cirrhosis, HCV/HCC, and normal livers to compare the degree of association between the mRNA expression of estrogen receptors (*ESR1, ESR2,* and *ESR1:ESR2*) and inflammatory markers (*TNFA, CD55,* and *IL33*) (Table 12). Upon stratifying by sex within each disease group, *ESR1* was found to be negatively correlated with *ESR2* in females with HCV/cirrhosis (R = -0.84, P = 0.0002). Interestingly, males with HCV/cirrhosis were found to instead have a significant positive correlation between *ESR1* and *ESR2* (R = 0.57, P = 0.0197) (Table 12).

ESR2 mRNA expression had a significant positive correlation with *TNFA* in both HCV/cirrhosis (R = 0.61, P = 0.0002) and HCV/HCC patients (R = 0.45, P = 0.0293) (Table 12). When stratified by sex within each disease group, *ESR2* was found to maintain the significant positive correlation with *TNFA* in both HCV/cirrhosis males and females (R = 0.58, P = 0.0162; R = 0.64, P = 0.0116 respectively) but for HCV/HCC, only males had any significant correlation (R = 0.60, P = 0.0083) (Table 12). Interestingly, normal females also showed a significant positive correlation (R = 0.54, P = 0.0220). Despite the correlations found between *ESR2* and *TNFA*, the ratio of *ESR1* to *ESR2* was found to be correlated with *TNFA* only in HCV/HCC patients (R = -0.57, P = 0.0049), but upon stratifying by sex, negative correlations were found between *TNFA* and

ESR1:ESR2 in HCV/HCC males (R = -0.56, P = 0.0151) and HCV/cirrhosis females (R = -0.57, P = 0.0286) (Table 12).

CD55 mRNA expression was positively correlated with *IL33* in normal (R = 0.34, P = 0.0410) and HCC subjects (R = 0.44, P = 0.0377); however, when stratified by sex, the only significant correlation between *IL33* and *CD55* was in normal females (R = 0.49, P = 0.0399) (Table 12). Total females also had a positive correlation between *IL33* and *ESR1* (R = 0.33, P = 0.0450), but no correlation was found when disease groups were stratified by sex (Table 12).

Table 12: Spearman's correlations between mRNA expression of estrogen receptors and inflammatory markers, by disease and sex (all subjects)

			ESR1		*TNFA*		*CD55*		*IL33*	
			R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
Normal	Together (n=36)	***ESR1***	-	-	0.20	(0.2422)	-	-	-	-
		ESR2	-0.19	(0.2772)	0.18	(0.2972)	0.08	(0.6523)	-0.07	(0.6642)
		ESR1:ESR2	-	-	0.01	(0.9501)	0.06	(0.7497)	0.25	(0.1493)
		CD55	0.20	(0.2422)	0.14	(0.4067)	-	-	-	-
		IL33	0.26	(0.1211)	0.32	(0.0546)	0.34	(0.0410)	-	-
	Males (n=18)	***ESR1***	-	-	0.34	(0.1708)	-	-	-	-
		ESR2	-0.42	(0.0861)	-0.11	(0.6508)	0.36	(0.1445)	-0.35	(0.1496)
		ESR1:ESR2	-	-	0.35	(0.1600)	-0.01	(0.9579)	0.43	(0.0731)
		CD55	0.24	(0.3280)	0.24	(0.3322)	-	-	-	-
		IL33	0.38	(0.1234)	0.45	(0.0590)	0.24	(0.3451)	-	-
	Females (n=18)	***ESR1***	-	-	0.05	(0.8293)	-	-	-	-
		ESR2	0.01	(0.9579)	0.54	(0.0220)	-0.14	(0.5871)	0.08	(0.7664)
		ESR1:ESR2	-	-	-0.38	(0.1190)	0.14	(0.5701)	0.14	(0.5701)
		CD55	0.05	(0.8548)	0.13	(0.6042)	-	-	-	-
		IL33	0.22	(0.3900)	0.14	(0.5701)	0.49	(0.0399)	-	-
HCV/Cirrhosis	Together (n=32)	***ESR1***	-	-	0.09	(0.6165)	-	-	-	-
		ESR2	-0.10	(0.6038)	0.61	(0.0002)	0.02	(0.8954)	-0.14	0.4339
		ESR1:ESR2	-	-	-0.19	(0.3017)	0.15	(0.4197)	-0.03	0.8891
		CD55	0.25	(0.1761)	-0.11	(0.5491)	-	-	-	-
		IL33	-0.10	(0.5955)	0.06	(0.7257)	0.31	(0.0896)	-	-
	Males (n=17)	***ESR1***	-	-	0.42	(0.0934)	-	-	-	-
		ESR2	0.57	(0.0197)	0.58	(0.0162)	-0.38	(0.1333)	-0.40	(0.1132)
		ESR1:ESR2	-	-	0.07	(0.8020)	0.01	(0.9811)	-0.25	(0.3319)
		CD55	-0.04	(0.8835)	-0.24	(0.3517)	-	-	-	-
		IL33	-0.44	(0.0780)	-0.10	(0.7013)	0.22	(0.3934)	-	-
	Females (n=15)	***ESR1***	-	-	-0.30	(0.2708)	-	-	-	-
		ESR2	-0.84	(0.0002)	0.64	(0.0116)	0.08	(0.7925)	0.00	(>0.9999)
		ESR1:ESR2	-	-	-0.57	(0.0286)	-0.05	(0.8626)	0.16	(0.5667)
		CD55	0.04	(0.9031)	-0.13	(0.6575)	-	-	-	-
		IL33	0.32	(0.2425)	0.32	(0.2425)	0.24	(0.3820)	-	-
HCV/HCC	Together (n=36)	***ESR1***	-	-	-0.21	(0.3397)	-	-	-	-
		ESR2	0.08	(0.7301)	0.45	(0.0293)	0.09	(0.6833)	0.00	0.9857
		ESR1:ESR2	-	-	-0.57	(0.0049)	0.01	(0.9786)	0.13	0.5622
		CD55	0.28	(0.1897)	0.09	(0.6701)	-	-	-	-
		IL33	0.31	(0.1496)	0.03	(0.8932)	0.44	(0.0377)	-	-
	Males (n=23)	***ESR1***	-	-	-0.11	(0.6627)	-	-	-	-
		ESR2	0.14	(0.5814)	0.60	(0.0083)	0.05	(0.8293)	0.01	(0.9838)
		ESR1:ESR2	-	-	-0.56	(0.0151)	-0.13	(0.6157)	0.03	(0.8997)
		CD55	0.20	(0.4331)	-0.03	(0.8979)	-	-	-	-
		IL33	0.22	(0.3717)	0.07	(0.7914)	0.46	(0.0540)	-	-
	Females (n=5)	***ESR1***	-	-	0.00	(>0.9999)	-	-	-	-
		ESR2	-0.10	(0.9500)	0.30	(0.6833)	0.50	(0.4500)	0.40	(0.5167)
		ESR1:ESR2	-	-	-0.10	(0.9500)	0.10	(0.9500)	0.00	(>0.9999)
		CD55	0.60	(0.3500)	0.70	(0.2333)	-	-	-	-
		IL33	0.70	(0.2333)	-0.10	(0.9500)	0.60	(0.3500)	-	-

<0.05
<0.01
<0.001

IFNL4-ΔG genotype is associated with decreased *ESR2* expression in patients with HCV-associated diseases

Genomic DNA (gDNA) from the study population was extracted and genotyped for *IFNL4* variant rs12979860 and analyzed with previously acquired mRNA and protein data. In order to control for selection bias, only patients with either HCV-associated cirrhosis or HCV-associated HCC were included in these analyses. *ESR2* mRNA levels were found to be significantly decreased in patients with ΔG/ΔG genotype compared to those with TT/TT genotype (Figure 25A). Of note, when genotype data were stratified by sex, only female patients with ΔG/ΔG genotype had a decrease in *ESR2* expression compared to females with ΔG//TT genotype (Figure 25B). ERβ protein expression in cytoplasmic fractions is also decreased in the ΔG/ΔG group, but the results are not significant (Figure 25D). Nuclear protein expression of ERβ is comparable between groups, as is the ratio of *ESR1:ESR2* mRNA expression (Figure 25C, E-F).

No significant associations were found between *IFNL4*-ΔG genotype and expression of ERα (Figure 24), CD55 (Figure 27), or IL-33 (Figure 28). Although not significant, TNFα mRNA expression was interestingly decreased in individuals with ΔG/ΔG genotype (Figure 26A-B). However, when stratified by sex, the significance decrease was only seen between the ΔG/ΔG and the ΔG/TT group in females, suggesting that any differences may be due to something other than *IFNL4*-ΔG genotype.

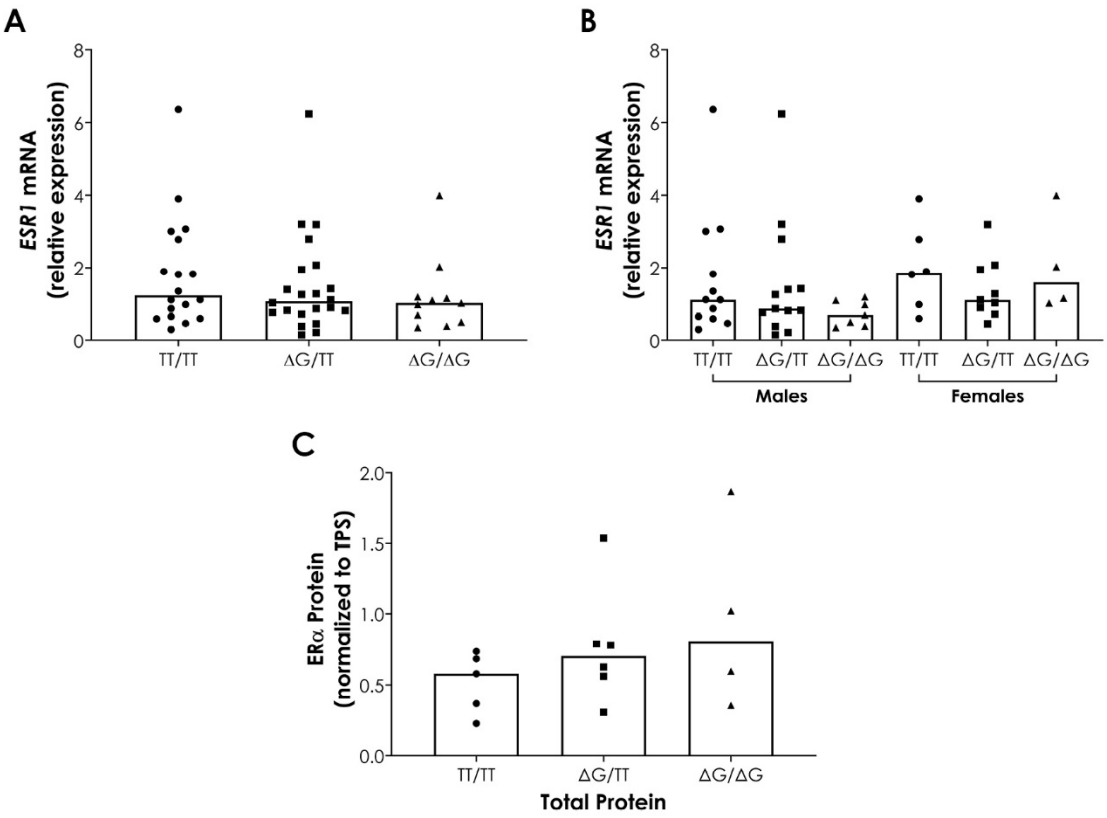


Figure 24: Association between ERα and *IFNL4* genotype in human liver tissues from subjects infected with HCV

HCV-related cirrhosis and HCV-related HCC liver tissues were stratified by *IFNL4* genotype and ERα expression was analyzed. (A) *ESR1* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) ERα protein expression by Western blot using whole liver tissue lysates quantified by densitometry and normalized to total protein.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. TPS, total protein stain.

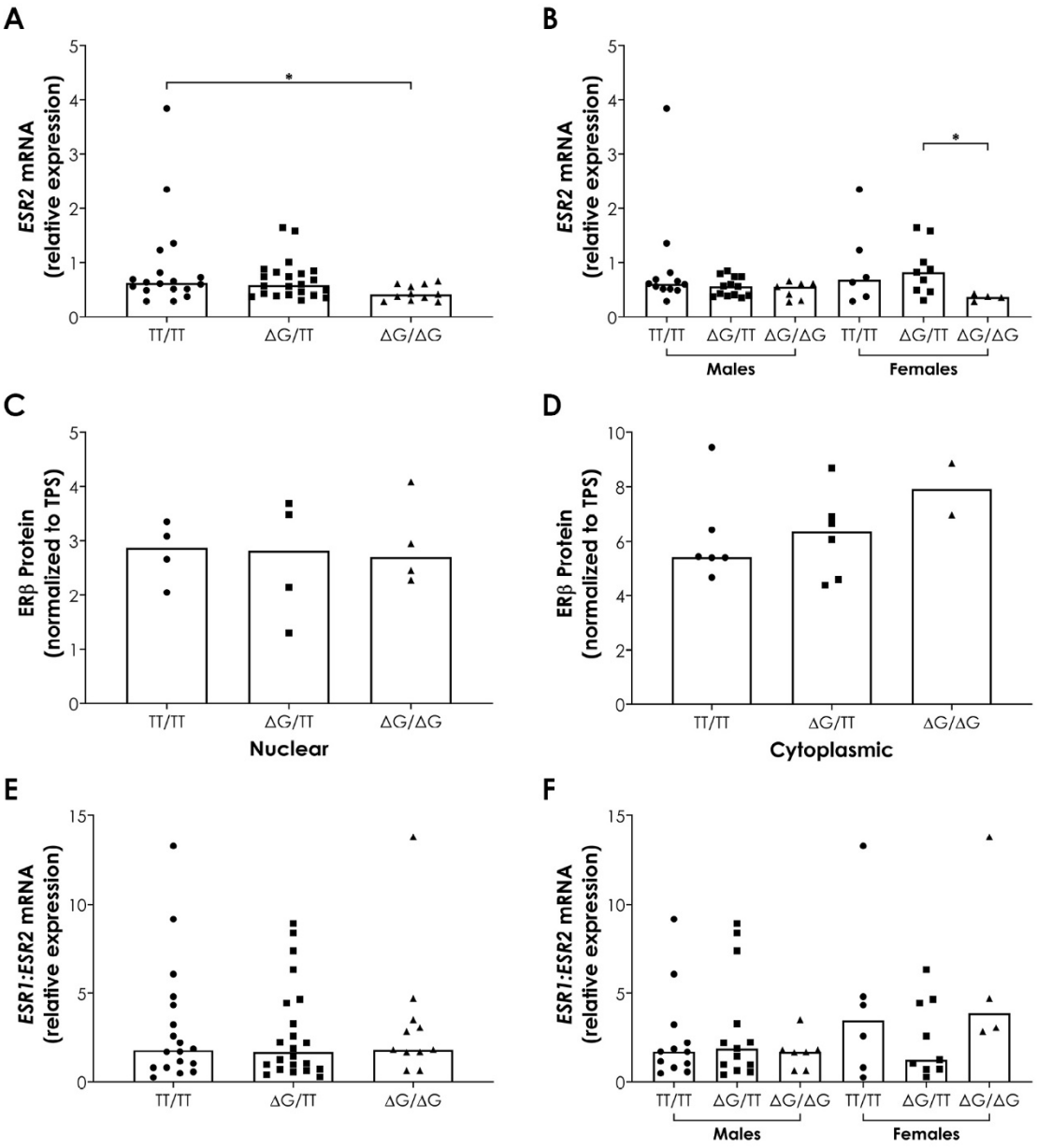


Figure 25: Association between ERβ and *IFNL4* genotype in human liver tissues from subjects infected with HCV

HCV-related cirrhosis and HCV-related HCC liver tissues were stratified by *IFNL4* genotype and ERβ expression was analyzed. (A) *ESR2* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C-D) ERβ protein expression by Western blot in human liver tissues fractionated by nuclear and cytoplasmic protein quantified by densitometry and normalized to total protein. (E) Ratio of *ESR1* to *ESR2* mRNA expression. (F) Stratification of (E) by sex.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where *$P \leq 0.05$. TPS, total protein stain.

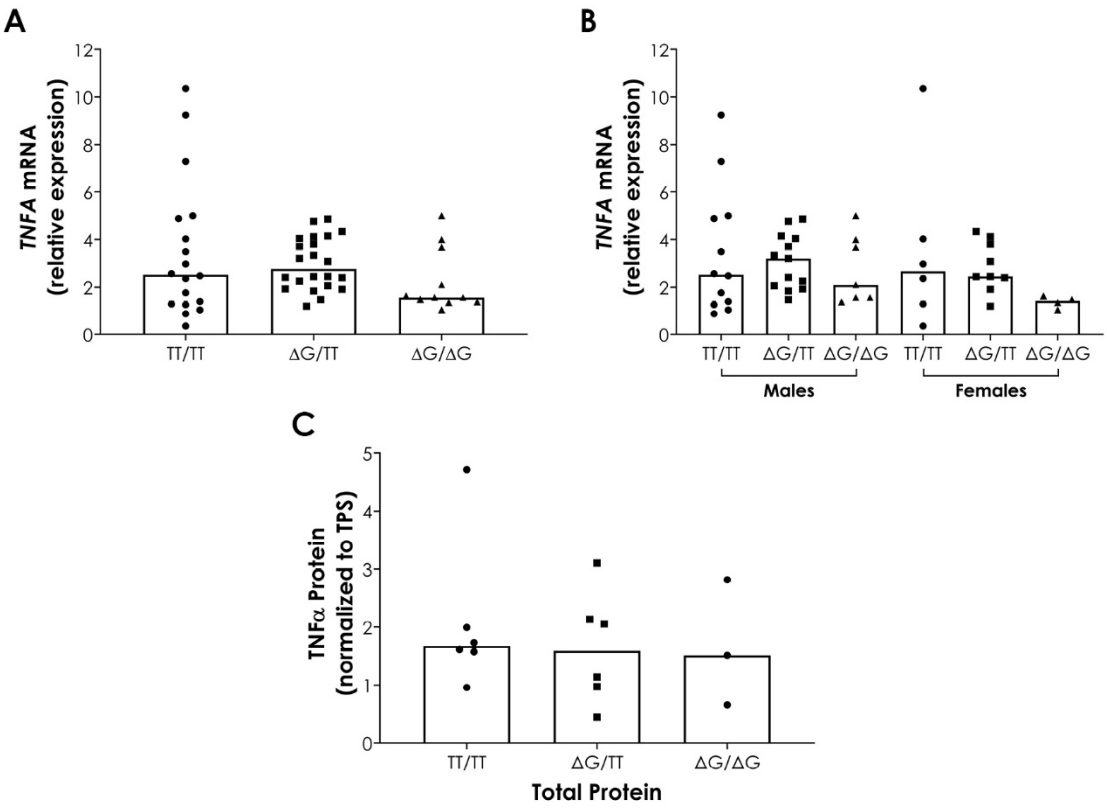


Figure 26: Association between TNFα and *IFNL4* genotype in human liver tissues from subjects infected with HCV

HCV-related cirrhosis and HCV-related HCC liver tissues were stratified by *IFNL4* genotype and TNFα expression was analyzed. (A) *TNFA* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) TNFα protein expression by Western blot using whole liver tissue lysates quantified by densitometry and normalized to total protein.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. TPS, total protein stain.

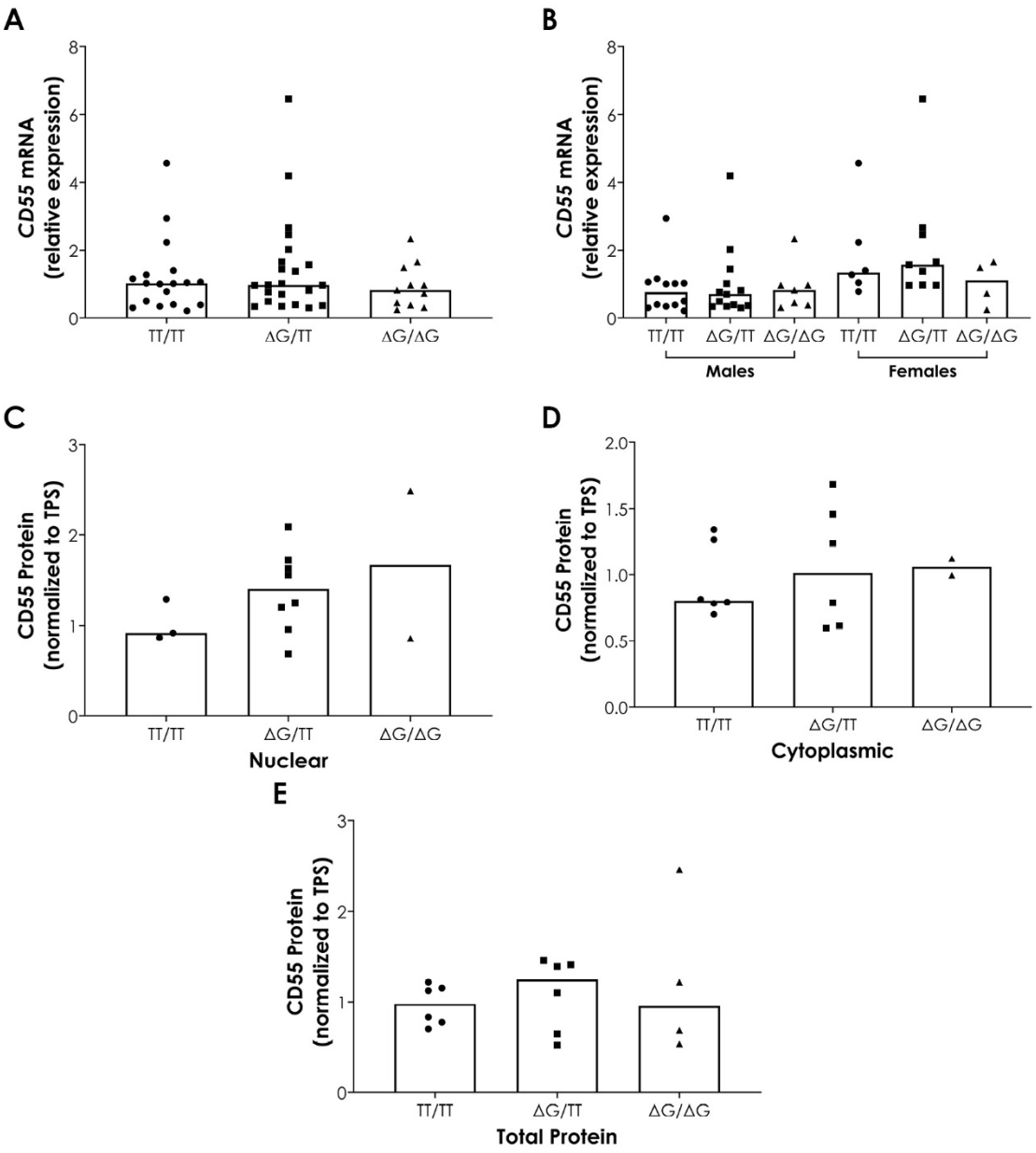


Figure 27: Association between CD55 and *IFNL4* genotype in human liver tissues from subjects infected with HCV

HCV-related cirrhosis and HCV-related HCC liver tissues were stratified by *IFNL4* genotype and CD55 expression was analyzed. (A) *CD55* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C-E) ERβ protein expression by Western blot in human liver tissues using whole tissue lysates and fractionated by nuclear and cytoplasmic protein quantified by densitometry and normalized to total protein.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. TPS, total protein stain.

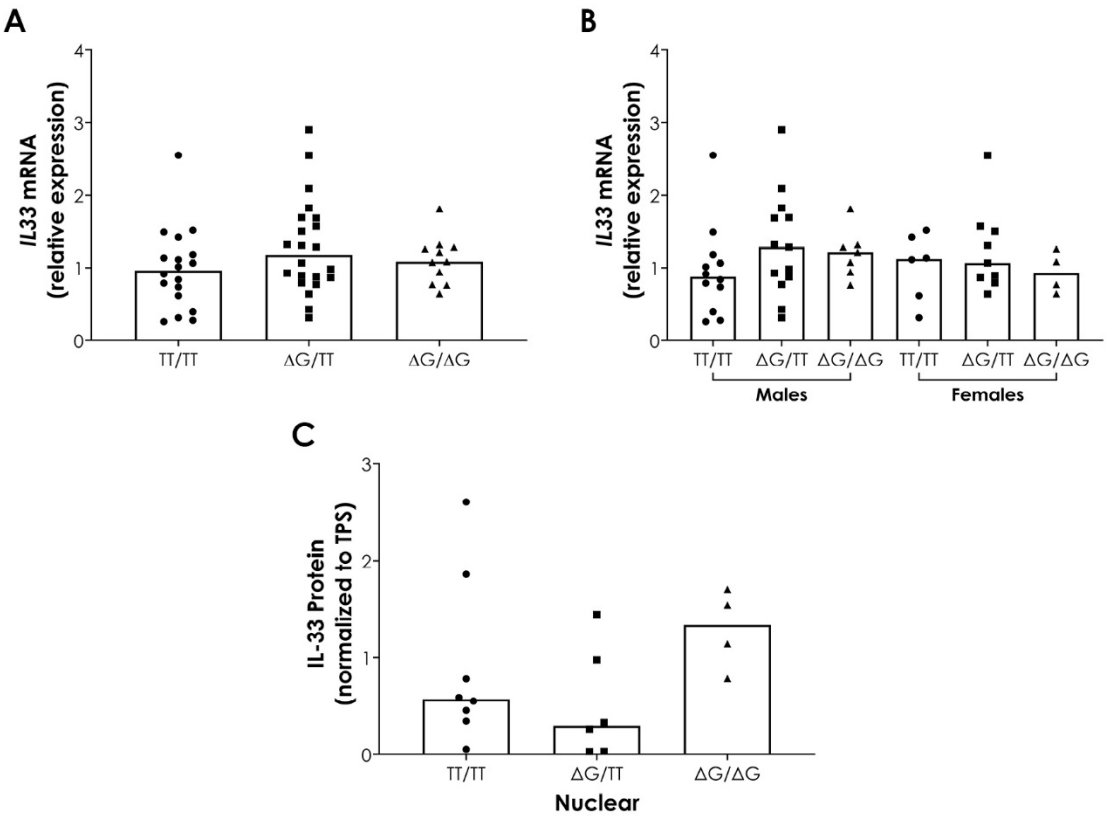


Figure 28: Association between IL-33 and *IFNL4* genotype in human liver tissues from subjects infected with HCV

HCV-related cirrhosis and HCV-related HCC liver tissues were stratified by *IFNL4* genotype for and IL-33 expression was analyzed. (A) *IL33* transcript levels by RT-qPCR normalized to *GUSB* and *SRSF4* expression and compared using the ΔΔCt method. (B) Stratification of (A) by sex. (C) IL-33 protein expression by Western blot in human liver tissues using nuclear fractions quantified by densitometry and normalized to total protein.

Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant. TPS, total protein stain.

Correlation Analyses by *IFNL4* Genotype

Spearman's Correlation coefficient was determined using RT-qPCR data and *IFNL4* genotype information from HCV/cirrhosis and HCV/HCC livers to compare the degree of association between the mRNA expression of estrogen receptors (*ESR1, ESR2,* and *ESR1:ESR2*) and inflammatory markers (*TNFA, CD55,* and *IL33*) (Table 13). In order to have more confidence in the effect of *IFNL4* genotype on marker expression, ΔG/ΔG and ΔG/TT subjects were grouped together and classified as ΔG allele. *ESR1* was found to be negatively correlated with *ESR2* in females with the ΔG allele (R = -0.64, P = 0.0221) (Table 13). *TNFA* mRNA expression was positively correlated with *ESR2* in both genotype groups (TT/TT R = 0.60, P = 0.0083; ΔG allele R = 0.62, P = 0.0001) (Table 13). However, upon stratifying by sex within each genotype group, *TNFA* only maintained negative correlation with *ESR2* in ΔG allele males and females (R = 0.74, P = 0.0002; R = 0.58, P = 0.0402, respectively). Significant negative correlations were found between *TNFA* and *ESR1:ESR2* in the overall ΔG allele group only (R = -0.36, P = 0.0392) (Table 13).

A weak association was found between *CD55* mRNA expression and *IL33* in the ΔG allele group (R = 0.32, P = 0.0147) and it became even stronger upon stratification by sex, in the males only (R = 0.47, P = 0.0071).

Table 13: Spearman's correlations between mRNA expression of estrogen receptors and inflammatory markers, by *IFNL4* genotype and sex (HCV-infected patients only)

			ESR1		*TNFA*		*CD55*		*IL33*	
			R	*P value*	*R*	*P value*	*R*	*P value*	*R*	*P value*
TT/TT	Together (n=18)	***ESR1***	-	-	-0.10	(0.6927)	-	-	-	-
		ESR2	-0.04	(0.8676)	0.60	(0.0083)	0.02	(0.9514)	0.33	(0.1765)
		ESR1:ESR2	-	-	-0.42	(0.0810)	0.09	(0.7170)	0.20	(0.4233)
		CD55	0.25	(0.3155)	-0.09	(0.7231)	-	-	-	-
		IL33	0.51	(0.0303)	0.09	(0.7231)	0.29	(0.2431)	-	-
	Males (n=12)	***ESR1***	-	-	-0.01	(0.9739)	-	-	-	-
		ESR2	0.25	(0.4303)	0.57	(0.0591)	-0.13	(0.6999)	0.41	(0.1928)
		ESR1:ESR2	-	-	-0.43	(0.1616)	-0.17	(0.5881)	0.05	(0.8861)
		CD55	0.05	(0.8861)	-0.12	(0.7160)	-	-	-	-
		IL33	0.42	(0.1767)	-0.13	(0.6832)	0.02	(0.9560)	-	-
	Females (n=6)	***ESR1***	-	-	-0.09	(0.9194)	-	-	-	-
		ESR2	-0.83	(0.0583)	0.49	(0.3556)	-0.20	(0.7139)	-0.31	(0.5639)
		ESR1:ESR2	-	-	-0.49	(0.3556)	0.20	(0.7139)	0.31	(0.5639)
		CD55	0.49	(0.3556)	-0.26	(0.6583)	-	-	-	-
		IL33	0.71	(0.1361)	0.43	(0.4194)	0.49	(0.3556)	-	-
ΔG	Together (n=33)	***ESR1***	-	-	-0.13	(0.4594)	-	-	-	-
		ESR2	-0.17	(0.3526)	0.62	(0.0001)	0.17	(0.3478)	-0.09	(0.6240)
		ESR1:ESR2	-	-	-0.36	(0.0392)	-0.08	(0.6626)	-0.12	(0.5132)
		CD55	0.11	(0.5536)	0.17	(0.3401)	-	-	-	-
		IL33	-0.12	(0.5168)	0.16	(0.2275)	0.32	(0.0147)	-	-
	Males (n=20)	**ESR1**	-	-	0.04	(0.8551)	-	-	-	-
		ESR2	0.08	(0.7241)	0.74	(0.0002)	-0.03	(0.8949)	-0.37	(0.1069)
		ESR1:ESR2	-	-	-0.19	(0.4162)	-0.25	(0.2796)	-0.11	(0.6496)
		CD55	-0.19	(0.4199)	0.24	(0.3007)	-	-	-	-
		IL-33	-0.19	(0.4199)	0.02	(0.9143)	0.47	(0.0071)	-	-
	Females (n=13)	**ESR1**	-	-	-0.31	(0.3063)	-	-	-	-
		ESR2	-0.64	(0.0221)	0.58	(0.0402)	0.27	(0.3734)	0.29	(0.3436)
		ESR1:ESR2	-	-	-0.54	(0.0611)	-0.12	(0.7097)	-0.05	(0.8776)
		CD55	0.01	(0.9782)	0.36	(0.2240)	-	-	-	-
		IL-33	0.24	(0.4257)	0.22	(0.2760)	0.34	(0.0891)	-	-

<0.05
<0.01
<0.001

CHAPTER V

CONCLUSIONS

Summary and Discussion

Over 2 million people in the United States remain infected with HCV despite the recent advances in therapy (Hofmeister, Rosenthal et al. 2019). HCV is adept at manipulating the innate and adaptive immune responses to facilitate the development of chronic infection in about 80% of infected individuals (Liang, Rehermann et al. 2000, Horner and Gale 2013). Many factors predicate whether an individual will clear the virus spontaneously or develop chronic infection; *IFNL4* gene polymorphisms have been shown to be one of the strongest predictors for both clearance as well as response to treatment (Aka, Kuniholm et al. 2014, Meissner, Bon et al. 2014, O'Brien, Pfeiffer et al. 2015). HCV-infected hepatocytes and liver macrophages produce proinflammatory cytokines and chemokines during disease progression. Chronic infection with HCV and the associated long-term inflammation causes affected individuals to be highly susceptible to liver complications such as fibrosis, cirrhosis, and HCC (Wynn 2008, Hoshida, Fuchs et al. 2014). One study found that cirrhotic patients with HCV are three times more likely to develop HCC compared to those with alcoholic liver disease or nonalcoholic fatty liver disease (Ioannou, Green et al. 2018).

It is also well established that male sex is a major risk factor for complications associated with chronic HCV infection (Poynard, Bedossa et al. 1997, Chiaramonte, Stroffolini et al. 1999, Khan, Farrell et al. 2000, Poynard, Ratziu et al. 2001, Di Martino, Lebray et al. 2004, Corsi, Karges et al. 2016, Ryerson, Eheman et al. 2016). Compared to females, males are three times more likely to develop liver cancer and four times more likely to die from cancer (El-Serag and Rudolph 2007, Tapper and Parikh 2018). Previous studies in our lab have shown differential expression of ER subtypes in the livers of males and females with HCV-associated diseases (Iyer, Kalra et al. 2017); however, there remains a huge gap in knowledge regarding the contribution of ER subtypes in HCV-induced inflammation and further sex differences in these immune responses. This study set out to investigate the sex-based association between ER subtypes with innate immune inflammatory markers (CD55, TNFα, and IL-33) in subjects with chronic HCV infection-related cirrhosis and HCC.

It has been demonstrated that females with higher circulating levels of estrogen are more likely to clear the virus, and if they do go on to develop chronic HCV infection, they tend to have less severe disease (Yu, Chang et al. 2003, Di Martino, Lebray et al. 2004, Codes, Asselah et al. 2007, Villa, Karampatou et al. 2011, Hassan, Botrus et al. 2017). In HCC, estrogen has been shown to downregulate the production of key inflammatory markers as well as inhibit the activation of tumor-associated macrophages (Naugler, Sakurai et al. 2007, Yang, Lu et al. 2012, Wei, Chen et al. 2016), which suggests that estrogen may directly contribute to the lower HCC incidence among females. Individuals infected with HCV who go on to spontaneously clear the virus exhibit a T helper 1 cell (T_H1)-dominated response, versus T_H2 (Reiser, Marousis et al. 1997, Tsai 1997, Fan, Liu et al. 1998, Sarih, Bouchrit et al. 2000). Estrogen has been demonstrated to enhance IFN-γ production, the defining cytokine of T_H1 cells, which in turn causes more undifferentiated CD4+ cells to differentiate into T_H1 cells (Grasso and Muscettola 1990, Karpuzoglu-Sahin, Hissong et al. 2001). However, more is not always better; the higher levels of

estrogen experienced during pregnancy have been observed to shift the immune response from T_H1 to T_H2 (Sabahi, Rola-Plesczcynski et al. 1995, Marzi, Vigano et al. 1996, Matalka 2003) although pregnancy seems to augment protection from HCV in females (Di Martino, Lebray et al. 2004). Estrogens are involved in different aspects of the innate and adaptive immune responses, and their effects are concentration dependent, as illustrated by the T_H1/T_H2 shift during pregnancy (Ruggieri, Gagliardi et al. 2018). The *in vitro* results presented in the current study illustrate the importance of estrogen concentration on downstream pathways; *ESR2* expression in Huh7 cells increased significantly following treatment with a low concentration of ERβ agonist DPN, but with successive increase in DPN concentration, *ESR2* expression declined to controls levels. IL-33 protein expression behaved similarly following treatment with ERα/β antagonist ICI, as did *TNFA* mRNA expression following treatment with ERα agonist PPT.

Estrogens act by binding to ERα, ERβ or GPER to mediate gene transcription either by directly binding to DNA or indirectly via protein-protein interactions. ERα and ERβ have a similar affinity to estrogen and can bind to most of the same EREs, producing some overlapping effects. However, they have often been observed to have unique, even antagonist functions due to a number of factors, including differing relative expression in various tissues and physiological states, interactions with diverse transcription factors and co-activators, and individual splice variant isoforms (Khan and Ansar Ahmed 2015, Hua, Zhang et al. 2018).

This is the first study reporting the mRNA levels of ERα and ERβ in normal human livers from a US population. In our cohort, *ESR2* transcript expression was greater than *ESR1* expression. This is in contrast to a study from 2000 that used immunohistochemistry to analyze the distribution of ER subtypes in various human tissues (Taylor and Al-Azzawi 2000). In liver tissues, they reported positive staining for both ERα and ERβ in portal vein, but only ERα in hepatocytes; these results have been since interpreted to mean that ERα is the primary ER in the liver (Nilsson, Koehler et al. 2011, Qiu, Vazquez et al. 2017). However, the paper suffers from a limited sample

size, as it describes using “at least” two samples for their analyses, and it failed to specify sample demographics or pathology. ER subtype expression can vary greatly depending on sex, age, disease conditions, and other variables, and two samples are not enough to make general conclusions.

Although the signaling mechanisms have been well-characterized, little is known about the epigenetic factors or posttranscriptional regulation of ERα and ERβ expression in the liver. The sex-based differences in these processes, if any, need further investigation. However, extensive research has associated changes in the expression of ERα and ERβ with the progression of numerous types of cancerous tissues, such as breast and ovarian (Segovia-Mendoza and Morales-Montor 2019). While the function and activity of ER subtypes relies heavily on the type of tissue and disease state, expression of ERα is generally thought to promote carcinogenesis while ERβ acts as a tumor suppressor (Hua, Zhang et al. 2018); however, much of the work looking at ER expression in cancer has been in breast tissues.

There is not yet a consensus on whether hepatic expression of either ER subtype is beneficial or detrimental in HCV pathogenesis. Some studies report a protective effect of ERα expression (Shimizu, Inoue et al. 2001, Cengiz, Ozenirler et al. 2014), others describe associations between ERα expression and worse prognosis (Iyer, Kalra et al. 2017), and still others report no correlations (Erkan, Yilmaz et al. 2013). Although no significant differences in expression of ERα mRNA or protein was found in either HCV/cirrhosis or HCV/HCC groups compared to controls, it was determined that in this cohort, *ESR1* is negatively correlated with AST and bilirubin (cirrhosis only) and with ALP (HCC only). These results support the conclusion that *ESR1* expression is associated with better liver function. However, using primers/antibodies for only wtERα and wtERβ only gives us partial data as there are several ER variants or isoforms with diverse tissue-specific expression patterns and functions. As liver function worsens, there is a decrease in wtERα expression and ERα variants (ERα46 and ERα36) are upregulated; this

upregulation is associated with a worse prognosis for HCV-associated diseases (Villa, Camellini et al. 1995, Villa, Dugani et al. 1998, Wang, Lee et al. 2006, Miceli, Cocciadiferro et al. 2011). In addition, this project only looked at the expression of markers in extratumoral tissues, as tumor biopsies were not available for all samples.

Since ERβ was discovered more than a decade after ERα, there are limited data on the role of ERβ in the liver; however, the results from this study make the case for its importance in HCV pathogenesis. Previous work from our lab reported significantly increased expression of *ESR2* mRNA in HCV-associated diseased livers compared to controls (Iyer, Kalra et al. 2017). In contrast, Iavarone et al. (2003) reported a loss of *ESR2* expression in patients with HCC compared to those with chronic liver disease. The present study found that while *ESR2* mRNA expression was significantly lower in the HCV/HCC group compared to control, cytoplasmic and nuclear protein expression was increased in both HCV/cirrhosis and HCV/HCC groups. The conflicting results observed at the translational level versus the transcriptional level may be attributed to the understanding that protein expression may not correspond linearly to its mRNA expression, due to possible disparities in translation efficiency or the half-life of the molecules (Omoto, Kobayashi et al. 2002). Studies to further reveal the function of ERβ and its variants in HCV pathogenesis are warranted in order understand these differences in mRNA and protein expression.

As expected, *TNFA* expression levels were highest in cirrhosis and HCC livers, since these diseases are primarily driven by chronic inflammation and TNFα is a potent proinflammatory cytokine. Notably, expression in HCC appears to be lower than in cirrhosis. This corresponds with other studies that reported a decrease in TNFα protein expression in more advanced HCV-related disease (Farinati, Cardin et al. 2006). *TNFA* was not associated with any clinical parameters except for a slight negative correlation with serum creatinine in HCC livers. The role of TNFα in HCV pathogenesis is decidedly complex. While its proinflammatory properties may

be useful for clearing the virus, if HCV establishes a long-term infection, TNFα may be a major player in the chronic inflammation that ultimately leads to liver damage. During initial infection, TNFα plays an antiviral role that can be suppressed by HCV p7 protein by activating signal transducer and activator of transcription (STAT)3 to induce of SOCS3 (Convery, Gargan et al. 2019). Consequently, STAT3 activation may help promote HCC development (He and Karin 2011) even without the apoptotic activity of TNFα. Estrogen has been shown to be associated with a decrease in TNFα expression (Ralston, Russell et al. 1990, Rogers and Eastell 2001, Pfeilschifter, Koditz et al. 2002), which is supported by the *in vitro* results demonstrating that E2 treatment significantly downregulated expression of *TNFA* mRNA. Estrogen downregulation of TNFα may help explain the less severe disease presentation in females compared to males. However, treatment of Huh7 cells with ERβ agonist DPN led to a significant increase in *TNFA* mRNA expression, and in human liver tissues, *ESR2* was positively correlated with *TNFA* in all disease/sex groups except for normal males and HCC females. While the lack of correlation in HCC females may be attributed to small sample size, the same cannot be said of the normal males.

Similarly to TNFα, CD55 appears to play a multifaceted role in HCV pathogenesis. Although the results were not significant, CD55 mRNA and protein expression was slightly downregulated in HCC livers compared to cirrhotic livers, which contradicts the understanding that CD55 expression is generally increased in carcinogenesis (Geller and Yan 2019). These results may represent the initial inhibition of complement by HCV to establish chronicity, and a later reduction of CRPs to allow activation of complement, facilitating the constant turning over of cells and malignancy (Chang, Yeh et al. 2009, Rensen, Slaats et al. 2009, Vasel, Rutz et al. 2014). *CD55* was also found to have a significant positive correlation with total bilirubin in males with the *IFNL4*-ΔG allele, and a strong negative correlation in HCC females. These observations are supported by a study where abnormal bilirubin levels in patients had increased markers of HCC

tumor aggressiveness (Carr, Guerra et al. 2014). *In vitro* treatment of Huh7 cells with E2 led to a significant reduction in *CD55* mRNA expression, while treatment with ERβ agonist DPN led to a significant increase in expression, suggesting that estrogen may play a dual role in counteracting the inhibition of complement by HCV and by mitigating the risk of cancer. In studies of transgenic mice expressing HCV core protein, Chang et al. (2009) found that the resulting inflammation and fibrosis could be ameliorated by administration of CD55. These results as well as the data on TNFα expression draw attention to the importance of considering time as a critical variable; in other words, the difference in outcomes may not be necessarily due to the distinct activity or interactions of the molecules, but rather in the regulation of immune pathways at different stages of HCV infection, namely fibrosis and HCC progression.

CD55 was positively correlated with *IL33* in HCC livers, normal female livers, and those with the *IFNL4*-ΔG allele. Increased IL-33 protein levels have been observed in serum and liver tissues from HCC patients with metastasis (Zhang, Liu et al. 2012); however, IL-33 has been described to have a dual role in cancer development, showing both pro-and anti-tumorigenic functions by inducing differential signaling on various immune and epithelial cells (Bergis, Kassis et al. 2013, Yang, Wang et al. 2016, Fournie and Poupot 2018). There is little published information on the relationship between IL-33 and estrogen. Although no significant differences were found in IL-33 expression in HCV-infected human liver tissues, *in vitro* treatment of Huh7 cells with E2 significantly downregulated *IL33* mRNA expression, and upregulated IL-33 protein expression. Treatment with PPT and DPN also upregulated protein expression, suggesting that a greater focus on the interaction between estrogen/ERs and IL-33 could produce interesting findings that explain its role, if any, in HCV pathogenesis.

In addition to looking at the individual associations between ER subtypes and immune markers, another objective of the project was to investigate the relationship between hepatic ERα and ERβ and their distinctive roles in the liver. The relative expression of ERα and ERβ in a given

environment is a significant determinant of their response to both endogenous and synthetic ligands, and a number of studies have observed ERβ-mediated modulation of ERα activity (Ogawa, Inoue et al. 1998, Pettersson, Delaunay et al. 2000, Lindberg, Moverare et al. 2003, Kansra, Yamagata et al. 2005, Bakas, Liapis et al. 2008). Upon binding with a ligand, ERs will dimerize prior to translocating to the nucleus, and relative concentrations of ERα and ERβ determine whether they will homodimerize or heterodimerize. ER homodimers regulate a different set of genes than that of ERαβ heterodimers (Monroe, Secreto et al. 2005). One of the more compelling findings in the present study was the positive correlation of *ESR1* with *ESR2* in cirrhosis males, and the negative correlation in cirrhosis females and *IFNL4*-ΔG allele females. *ESR1* and *ESR2* or their isoforms are known to coregulate the activity of each other (Zhao, Matthews et al. 2007, Williams, Edvardsson et al. 2008, Charn, Liu et al. 2010, Lu and Katzenellenbogen 2017) and our results offer some evidence that coregulation may be, at least in part, dependent on sex.

Genetics may very well play a role too; in patients with *INFL4*-ΔG/ΔG genotype, specifically female patients, hepatic expression of *ESR2* was found to be significantly decreased. This finding is consistent with the observation that ERβ expression is protective in some cancers (Hua, Zhang et al. 2018). Furthermore, a study in a Chinese population reported a polymorphism in *ESR2* that was associated with an increased susceptibility to HCV infection in males but a significantly reduced risk of HCV infection in females (Cai, Gao et al. 2014).

Liver function tests can indicate the presence of hepatic injury, and they are often used by physicians in the differential diagnosis of hepatic disease. One of the goals of this study was to determine if there were any disease- or sex-specific differences in the routinely used liver function tests among those with HCV-related diseases. ALP and total bilirubin levels were significantly different between HCV-related cirrhosis and HCC groups, and the difference in total bilirubin levels between cirrhosis and HCC subjects was only found in males. Males with HCV-

related cirrhosis and HCC had higher AST levels than females. In a previous study in normal populations, increased total bilirubin and AST levels were found in males compared to females (Adiga 2016). These results are contrary to that of Erkan et al. (2013) who found no difference in AST levels between males and females with HCV-related disease. Instead, they reported that males had higher levels of ALT and GGT, two liver enzymes whose values were not documented for our cohort.

Because all of the patient cases in this study were recipients of a liver transplant, it is important to understand the criteria to qualify for transplantation. Model for End-stage Liver Disease (MELD) scores are used to determine transplant candidacy in cirrhotic patients; in general, patients can qualify for liver transplantation once their MELD score is ≥15 (Dove and Brown 2020). Compared to males, females are less likely to undergo evaluation/receive liver transplantation (Bryce, Angus et al. 2009) and more likely to stay longer on the transplant waiting list and have a greater chance of dying before receiving transplant (Klassen, Klassen et al. 1998). The implementation of the MELD score for organ allocation worsened the systemic bias against females (Fink, Berry et al. 2007, Moylan, Brady et al. 2008, Mathur, Schaubel et al. 2011), partially due to the fact that females tend to have lower creatinine levels, a main determinant of MELD score (Rodriguez-Castro, De Martin et al. 2014).

Patients with a MELD score <15 may still become eligible if they have certain conditions or complications, such as HCC (Dove and Brown 2020). Patients with HCC can qualify for liver transplantation if they have either a single tumor ≤5 cm or up to three separate lesions all <3 cm and no evidence of vascular invasion or metastasis. Because males have a greater incidence of HCC compared to females, the use of HCC as an exception to MELD scores leads to an even greater sex disparity in liver transplantation. To summarize, in order to qualify for transplantation, the cirrhotic patients in our cohort needed to present with more advanced cirrhosis while the HCC patients were required to have less severe cancer.

Significance and Future Directions

HCV remains a problem of scientific and clinical significance due to the genetic variability of the virus, lack of robust early detection methods, and challenges of vaccine development. This project will be the basis for future work investigating the factors that are responsible for the sex-based differences in HCV disease pathogenesis. Studying the molecular pathways involved will lead to a better understanding of the reasons behind why only a fraction of individuals are able to clear the virus on their own, and will facilitate more personalized diagnosis and care.

Despite the recent development of drug treatments that can effectively cure a patient with HCV, they are not easily accessible due to cost. Realizing the molecular mechanisms involved in viral pathogenesis, inflammation, estrogen, and ERs is essential for the development of novel therapeutics. The results of this study provide evidence for the importance of ERβ in HCV pathogenesis, and future studies should concentrate on developing a deeper understanding of its role in inflammation and cancer using *in vitro* and *in vivo* models.

All of the targets of interest in this study have been described to have versatile, sometimes contradictory roles depending on the location, environment, and stimuli. A time-dependent study with more focus on how initial responses to HCV infection in both sexes may modulate the ER regulated inflammation determining later responses and outcome is therefore suggested.

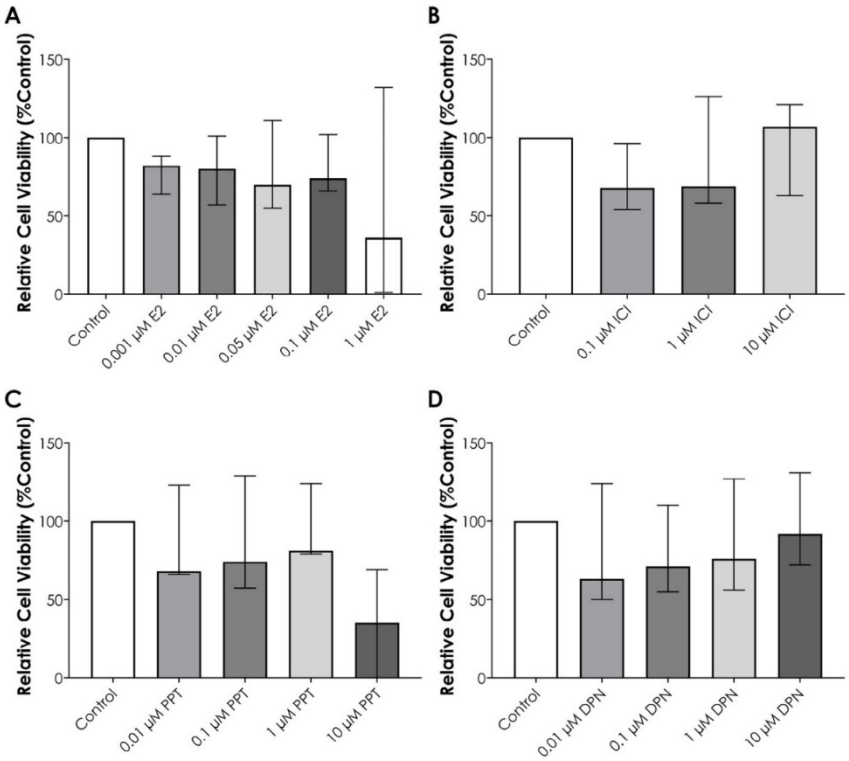
This study further confirms the conclusion from our previous study (Iyer, Kalra et al. 2017) that differential ER subtype expression is observed in males and females in both the premalignant and malignant stages of HCV-related disease progression. The *in vitro* results presented here using ERα- and β-specific agonists further supports ER subtype-mediated regulation of immune responses in the liver; the study should be repeated using Huh7 cells transfected with HCV full-length plasmids and/or viral protein plasmids. The idea that 17β-estradiol plays a protective role in the liver was also validated by showing downregulation of innate immune markers TNFα, CD55 and IL-33 at the gene transcription level; similar results were seen after treatment with

ERα/β antagonist, ICI 182,780, which may be due to the fact that it is an agonist of the membrane receptor, GPER. Further research in other inflammatory and oncogenic markers is, therefore, an essential next step in elucidating the immunomodulation by ER subtypes in the liver. In addition, since the focus of this study was on nuclear ERα and β, more research is needed to determine if the sex-based differences in HCV infection outcome are also mediated GPER or by isoforms of the nuclear receptors.

Something that also needs to be considered is the possibility that while estrogen may have inherent protective functions, the differences in HCV pathogenesis and disease progression between males and females may be exacerbated by the inhibitory effects of testosterone and progesterone on immune response (Klein and Flanagan 2016). For that reason, evaluating the role of other sex steroids and the expression and function of their receptors would be a fruitful area for further work.

CHAPTER VI

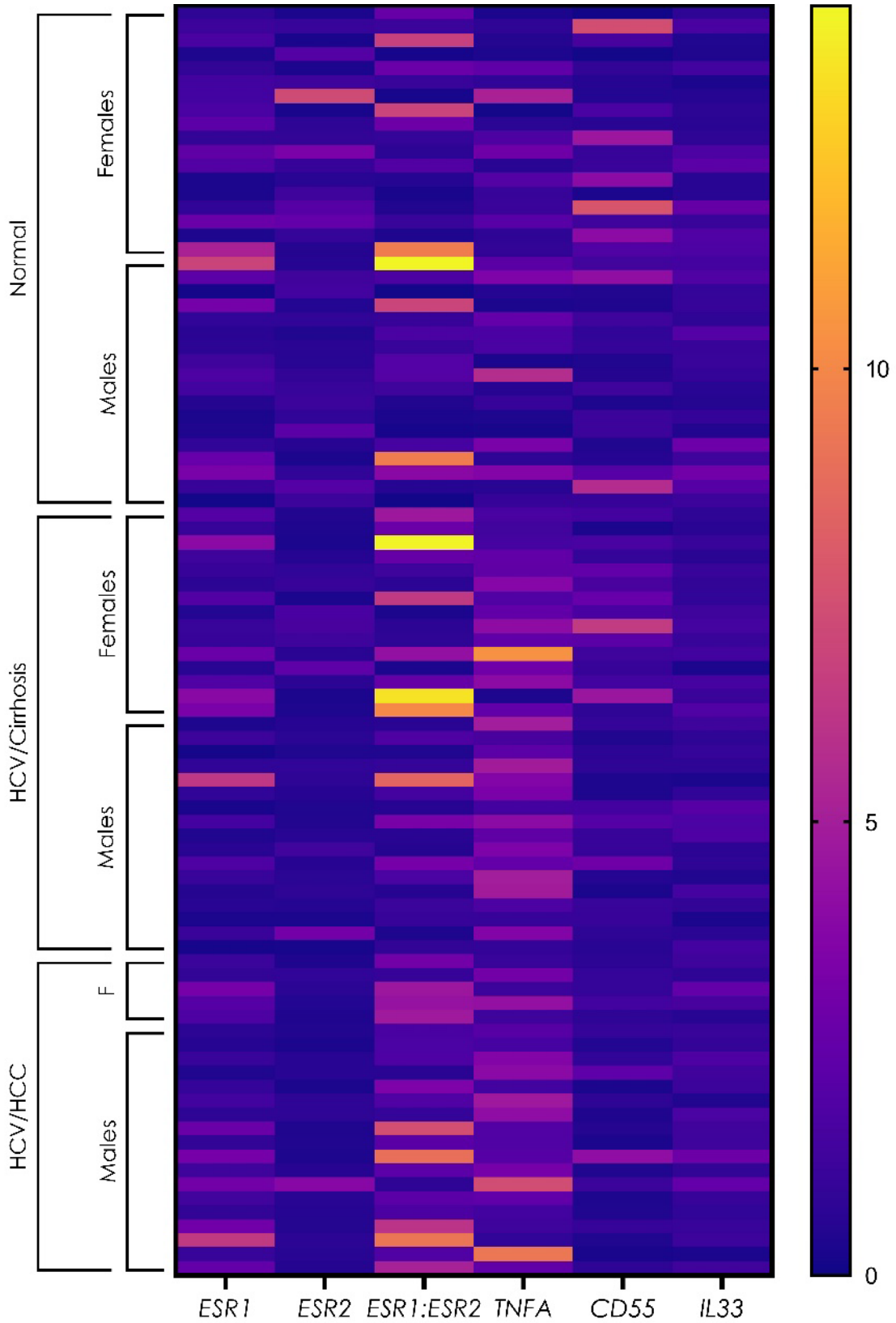
SUPPLEMENTARY MATERIALS



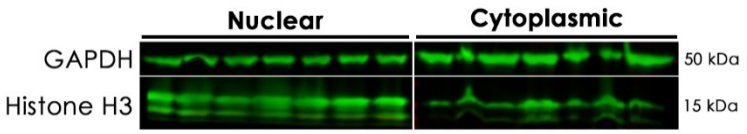
Supplementary Figure 1: MTT assay for drug toxicity in Huh7 cells

MTT assay was used to determine cell toxicity of ER agonists/antagonists in Huh7 cells. Cells were treated for 24 hours with varying concentrations of drug and compared to control cells treated with vehicle. (A) ERα/β agonist E2. (B) Pre-treatment with ERα/β antagonist ICI for 1.5 hours followed by addition of 0.01 µM E2. (C) ERα agonist PPT. (D) ERβ agonist DPN.

Cellular growth is represented as a relative percentage to the untreated control cells. Data are represented as mean ± SEM. Error bars represent data from at three independent experiments performed in quadruplicate. Data were analyzed by one-way ANOVA with Dunnett's Multiple Comparisons posttest where $P \leq 0.05$ was considered significant.

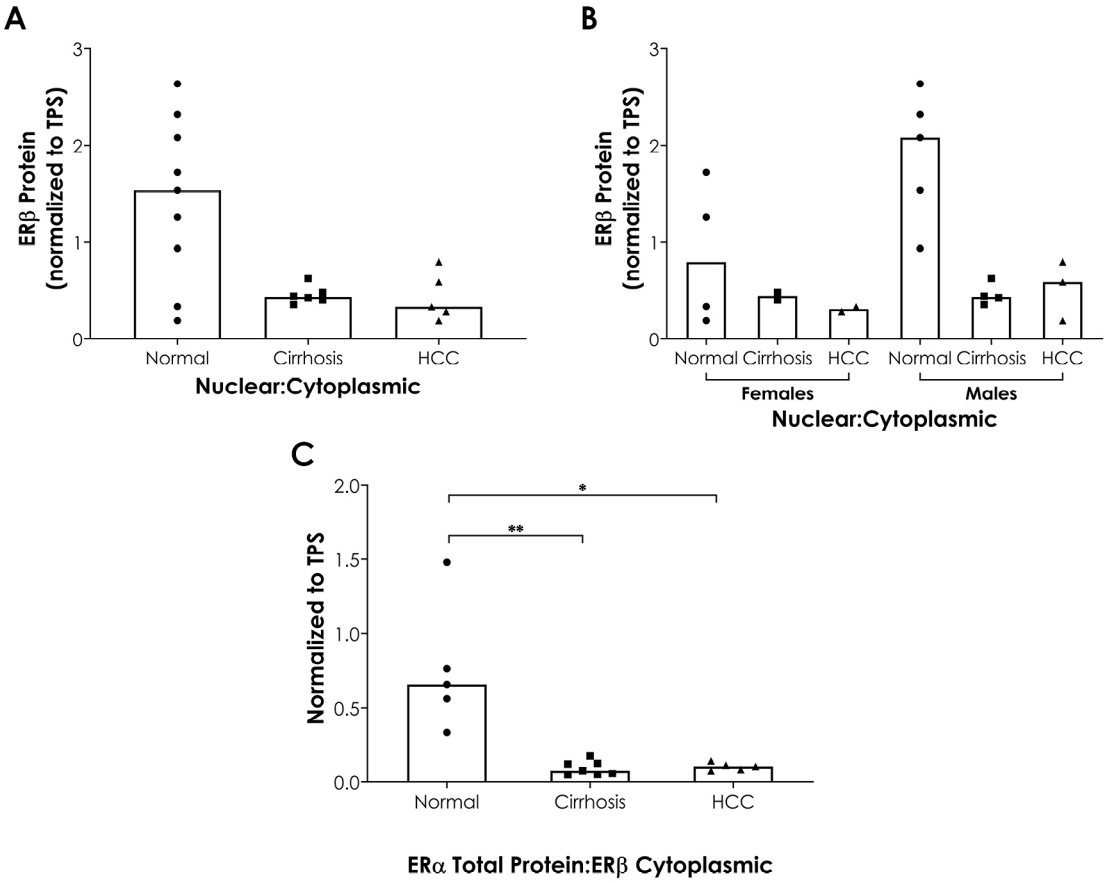


Supplementary Figure 2: Heat map diagram representing gene expression in the liver of the study population



Supplementary Figure 3: GAPDH and Histone H3 expression in the nuclear and cytoplasmic protein fractions of human liver tissue

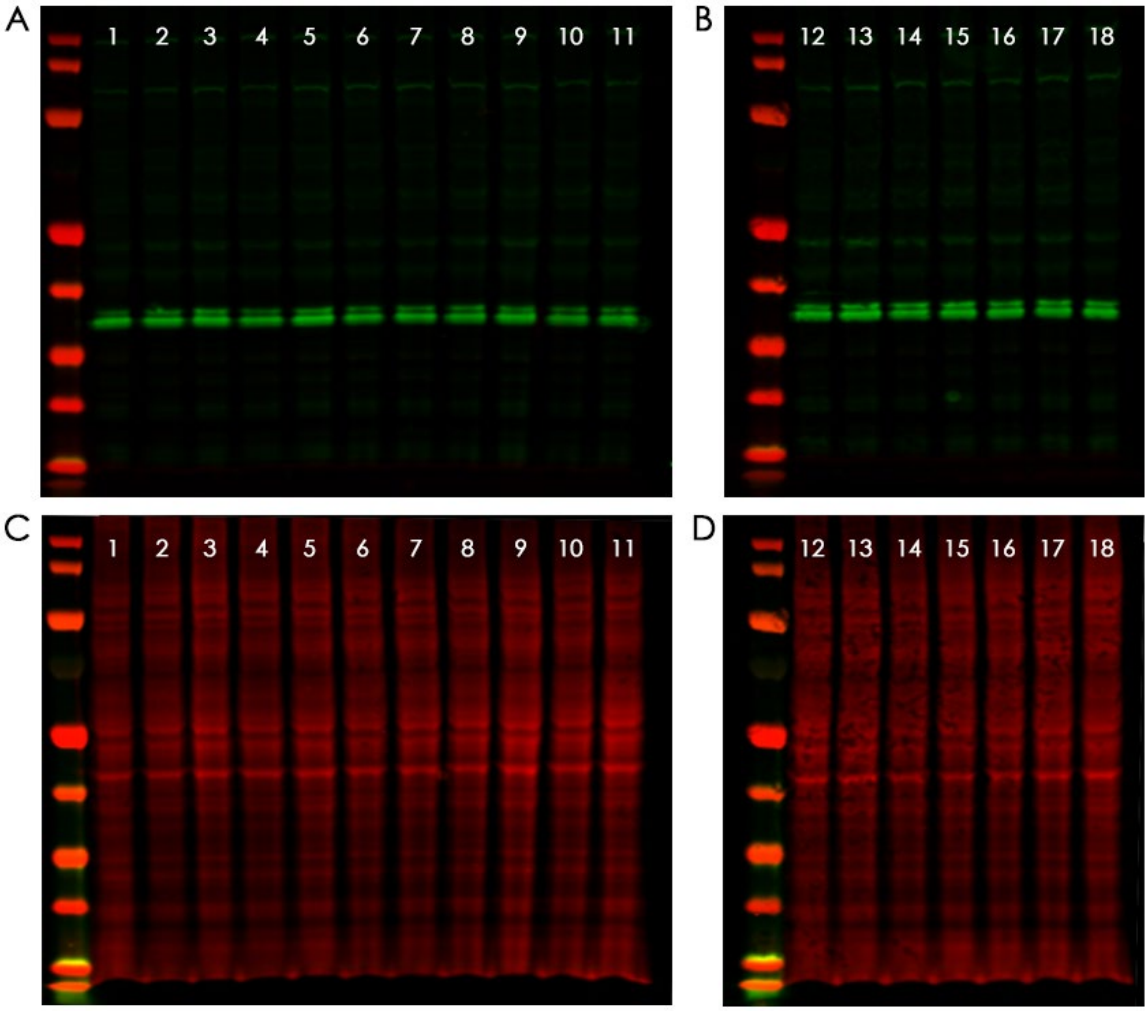
GAPDH and Histone H3 protein expression were used to monitor the purity of the nuclear and cytoplasmic fractions, respectively, in HCV-related cirrhosis and HCV-related HCC liver tissues.



Supplementary Figure 4: Protein expression of ER subtypes in human liver tissues with HCV-related diseases

ERα and ERβ protein expression was analyzed in HCV-related cirrhosis and HCV-related HCC liver tissues and compared to controls. (A) Ratio of ERβ nuclear to cytoplasmic protein expression. (B) Stratification of (A) by sex. (C) Ratio of ERα total protein to ERβ cytoplasmic protein expression.

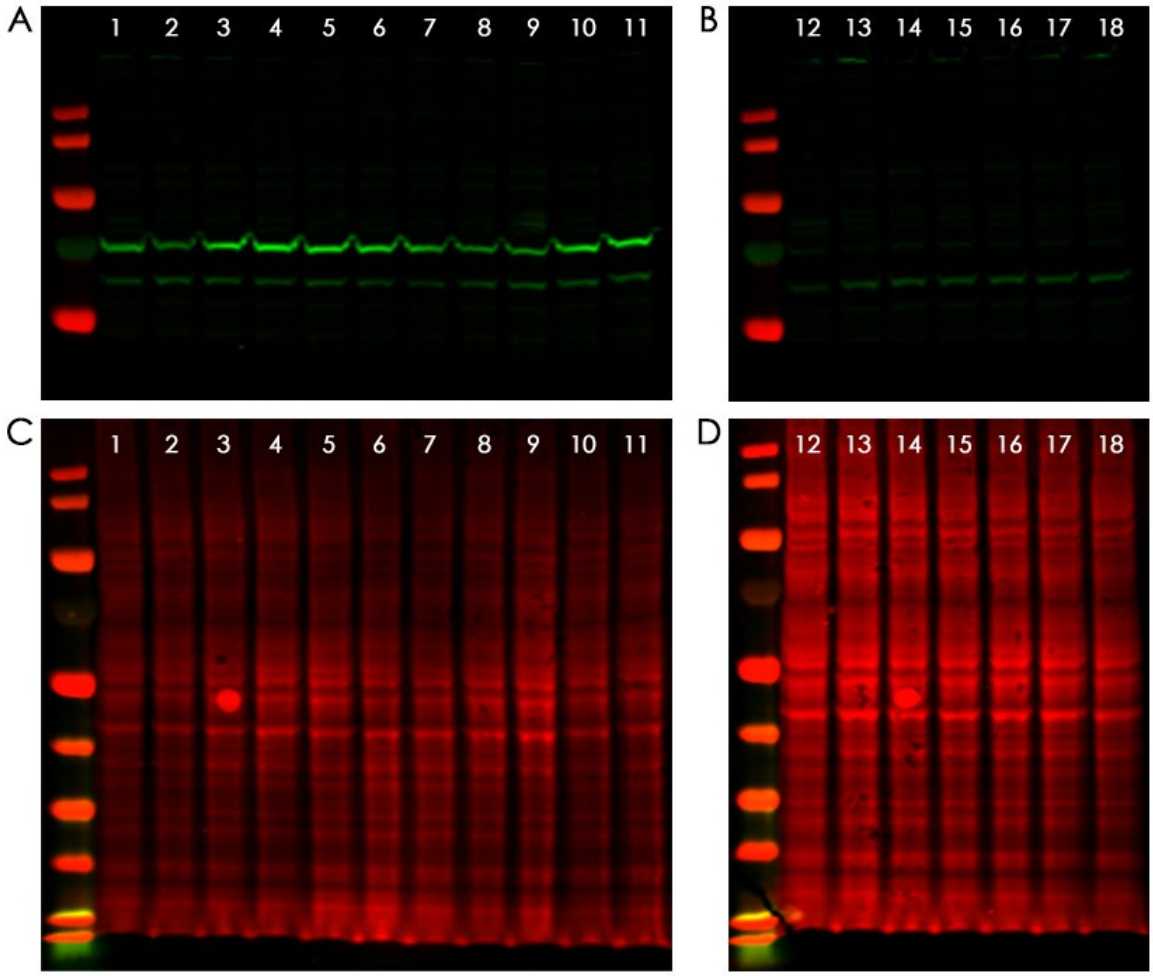
Column bars represent the median. Each symbol represents one individual. Data were analyzed by Kruskal-Wallis test with Dunn's Multiple Comparisons posttest where *P=0.05, **P=0.01, ***P=0.001. TPS, total protein stain.



Supplementary Figure 5: Uncropped Western blot images showing ERa total protein expression in Huh7 cells

(A-B) Western blots indicating protein of interest in Figure 6. (C-D) Total protein staining used as loading control.

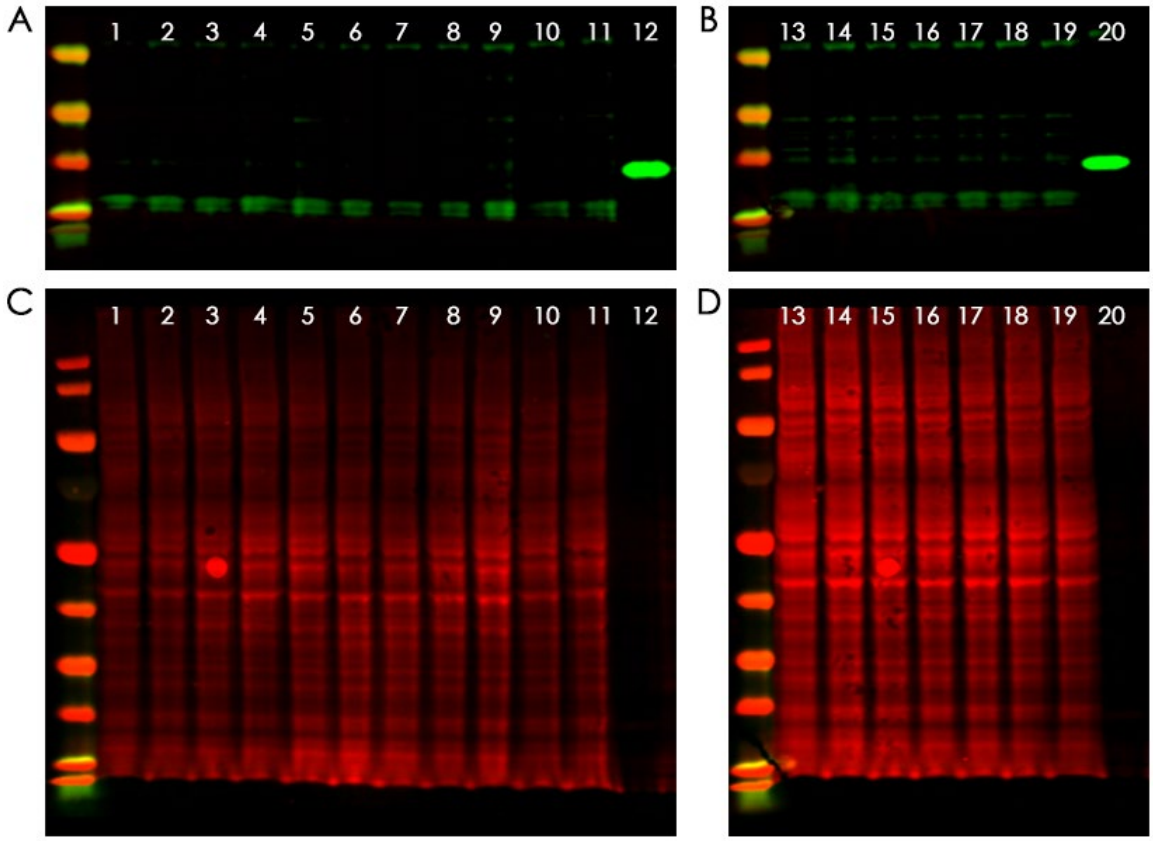
Treatment Control: 1, 12
0.001-0.1 µM E2: 2-5
0.1-10 µM ICI: 6-8
0.1-10 µM ICI + 0.01 µM E2: 9-11
0.01-1 µM PPT: 13-15
0.01-1 µM DPN: 16-18



Supplementary Figure 6: Uncropped Western blot images showing ERβ total protein expression in Huh7 cells

(A-B) Western blots indicating protein of interest in Figure 8. (C-D) Total protein staining used as loading control.

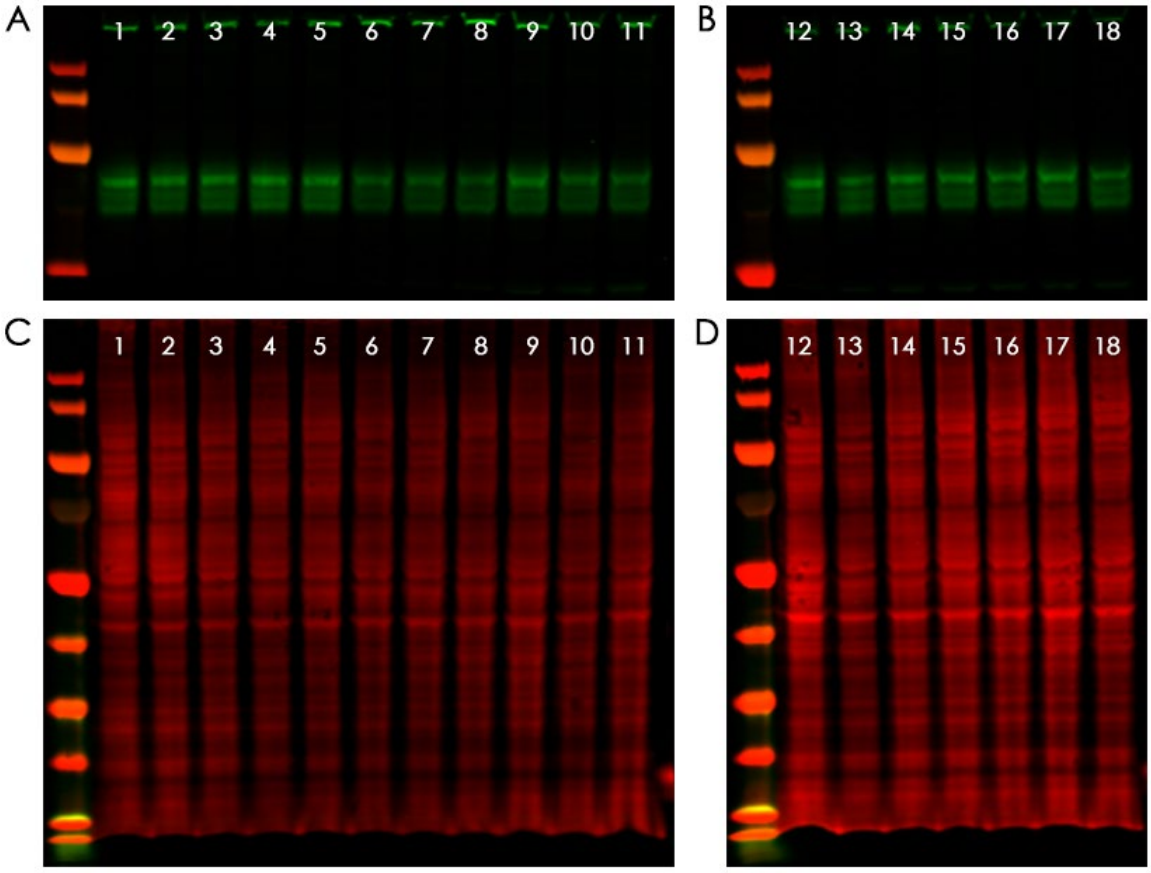
Treatment Control: 1, 12
0.001-0.1 μM E2: 2-5
0.1-10 μM ICI: 6-8
0.1-10 μM ICI + 0.01 μM E2: 9-11
0.01-1 μM PPT: 13-15
0.01-1 μM DPN: 16-18



Supplementary Figure 7: Uncropped Western blot images showing TNFa total protein expression in Huh7 cells

(A-B) Western blots indicating protein of interest in Figure 10. (C-D) Total protein staining used as loading control.

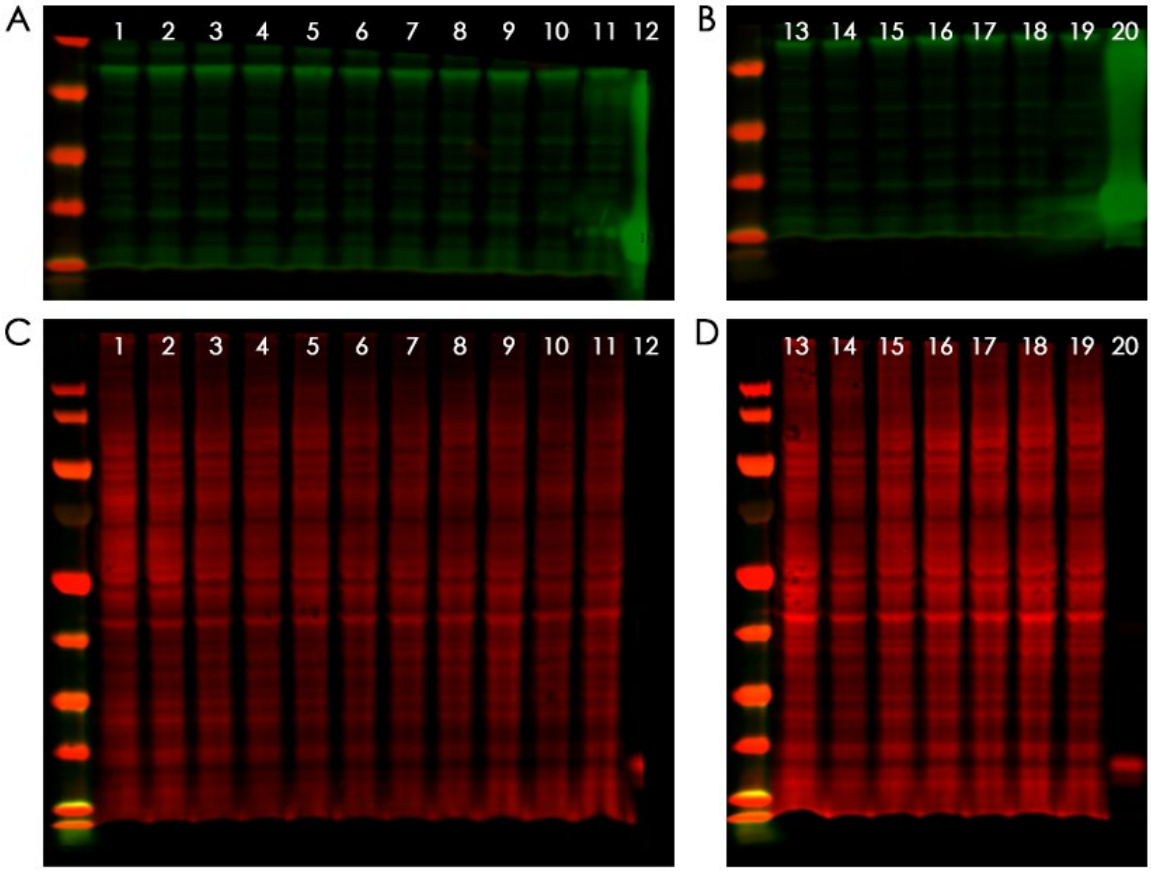
Treatment Control: 1, 13
Positive Control: 12, 20
0.001-0.1 µM E2: 2-5
0.1-10 µM ICI: 6-8
0.1-10 µM ICI + 0.01 µM E2: 9-11
0.01-1 µM PPT: 14-16
0.01-1 µM DPN: 17-19



Supplementary Figure 8: Uncropped Western blot images showing CD55 total protein expression in Huh7 cells

(A-B) Western blots indicating protein of interest in Figure 12. (C-D) Total protein staining used as loading control.

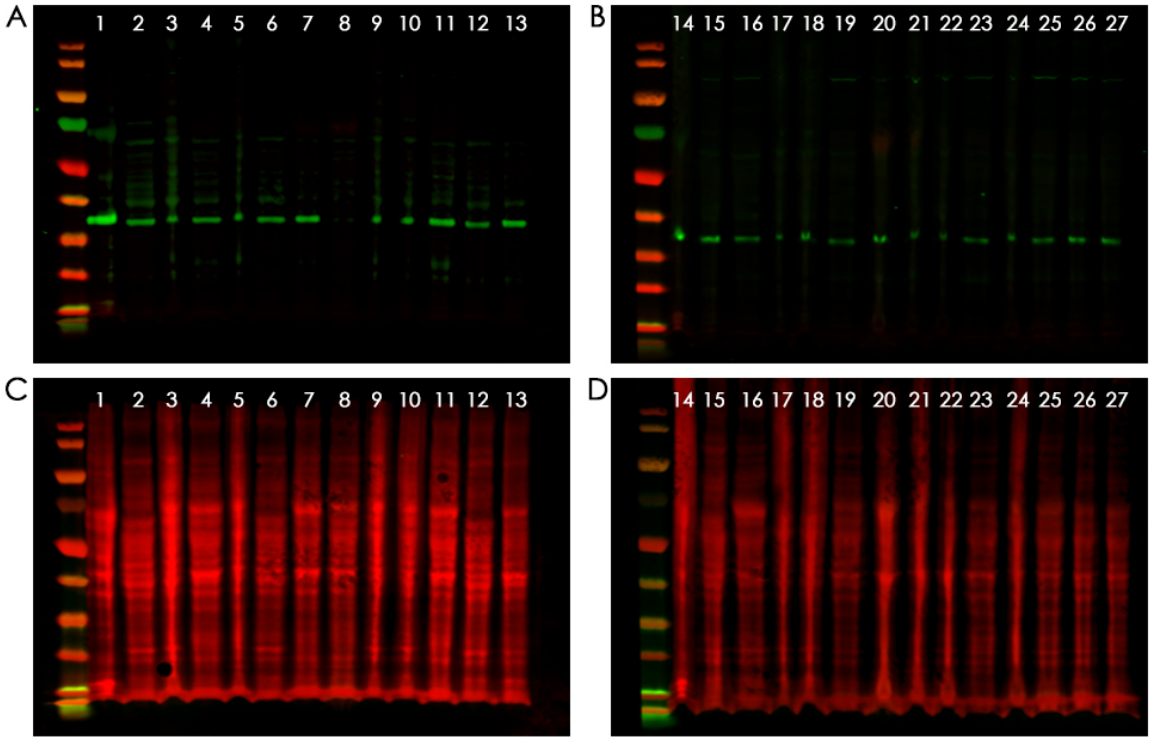
Treatment Control: 1, 12
0.001-0.1 µM E2: 2-5
0.1-10 µM ICI: 6-8
0.1-10 µM ICI + 0.01 µM E2: 9-11
0.01-1 µM PPT: 13-15
0.01-1 µM DPN: 16-18



Supplementary Figure 9: Uncropped Western blot images showing IL-33 total protein expression in Huh7 cells

(A-B) Western blots indicating protein of interest in Figure 14. (C-D) Total protein staining used as loading control.

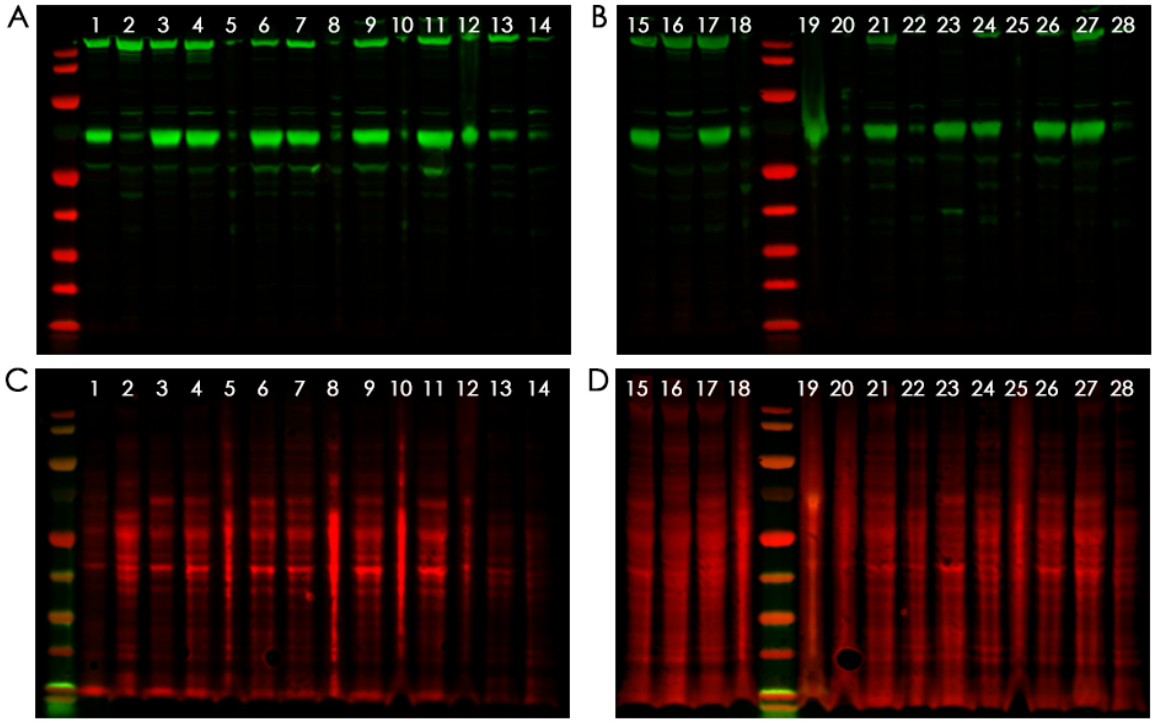
Treatment Control: 1, 13
Positive Control: 12, 20
0.001-0.1 µM E2: 2-5
0.1-10 µM ICI: 6-8
0.1-10 µM ICI + 0.01 µM E2: 9-11
0.01-1 µM PPT: 14-16
0.01-1 µM DPN: 17-19



Supplementary Figure 10: Uncropped Western blot images showing ERα total protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 17. (C-D) Total protein staining used as loading control.

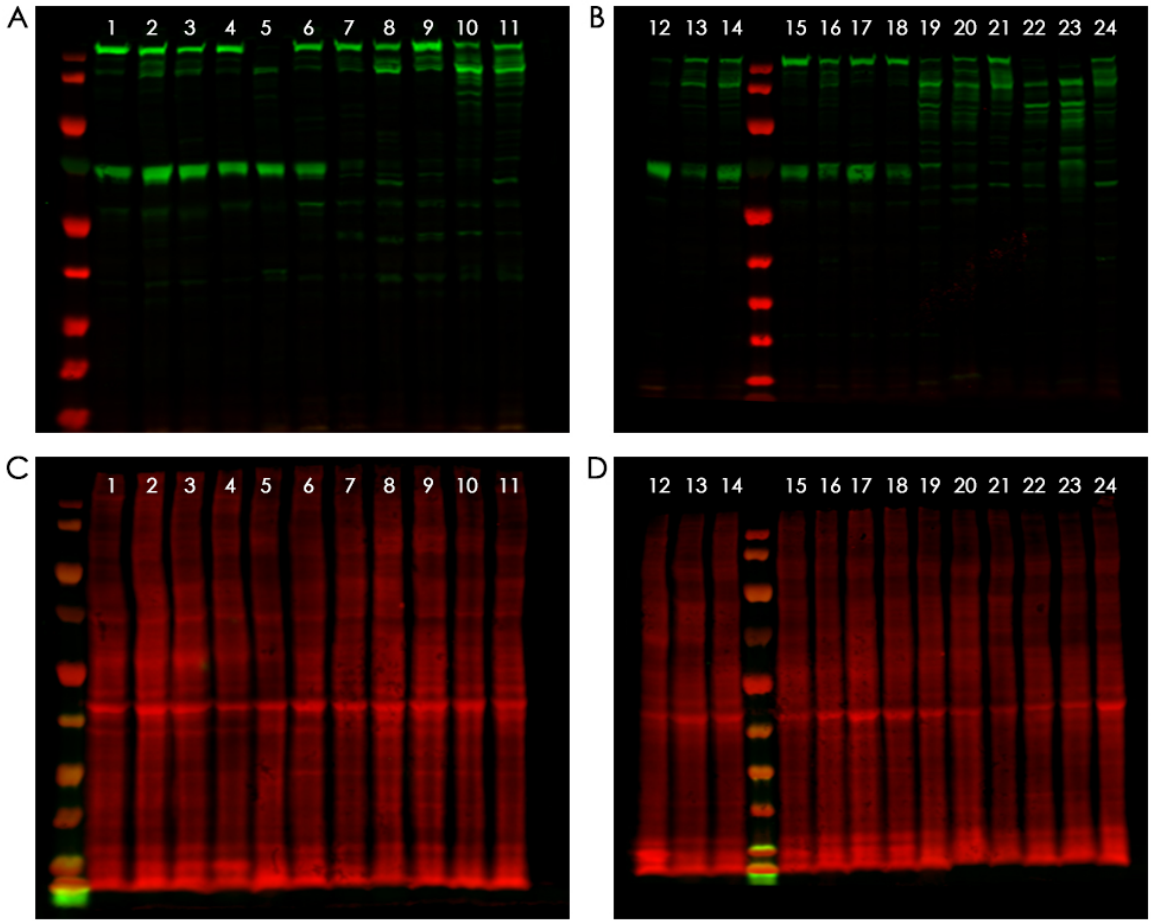
Positive Control: 1, 14
Normal: 2, 6, 12, 13, 15-18
HCV/Cirrhosis: 5, 7, 8, 11, 19-22
HCV/HCC: 3, 4, 9, 10, 23-27



Supplementary Figure 11: Uncropped Western blot images showing cytoplasmic ERβ protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 19. (C-D) Total protein staining used as loading control.

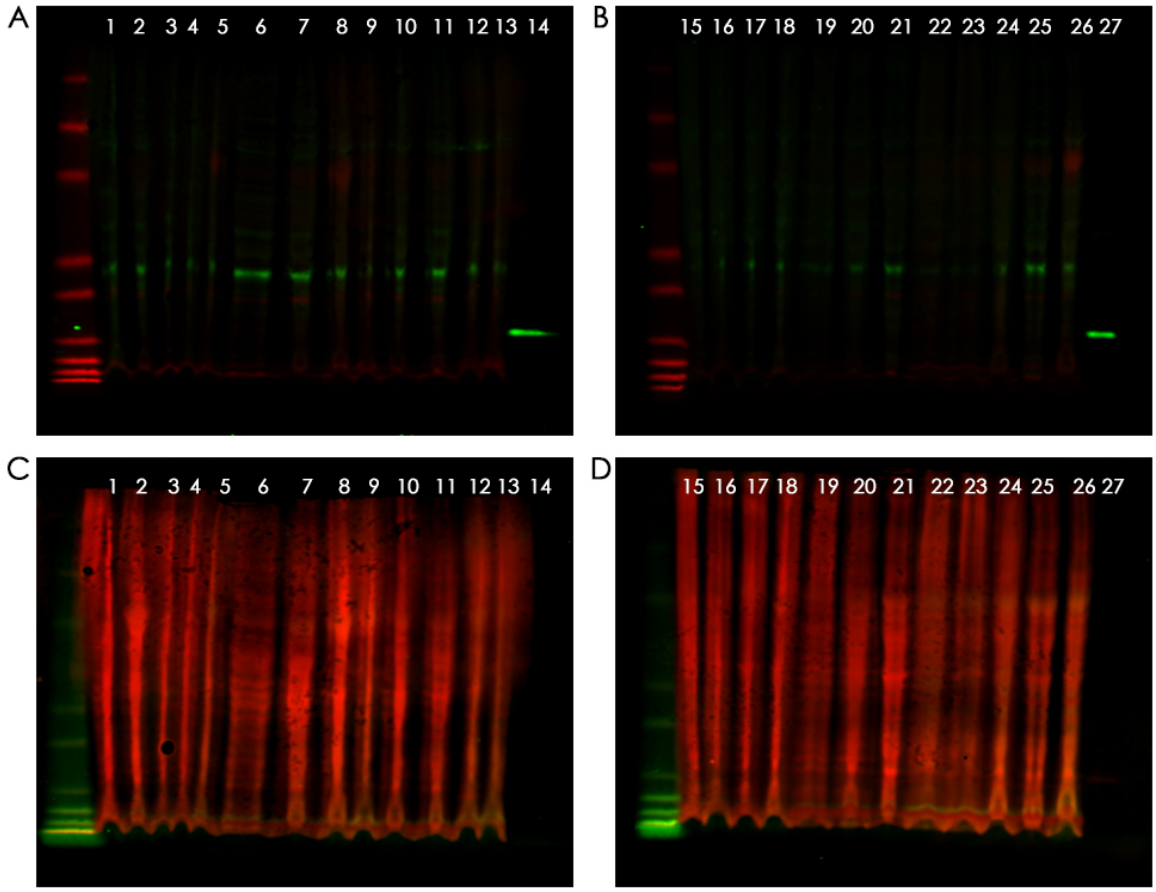
Normal: 2, 5, 8, 10, 13, 14, 16, 18, 20, 22, 25, 28
HCV/Cirrhosis: 1, 4, 7, 9, 11, 15, 21, 23
HCV/HCC: 3, 6, 12, 17, 19, 24, 26, 27



Supplementary Figure 12: Uncropped Western blot images showing nuclear ERβ protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 19. (C-D) Total protein staining used as loading control.

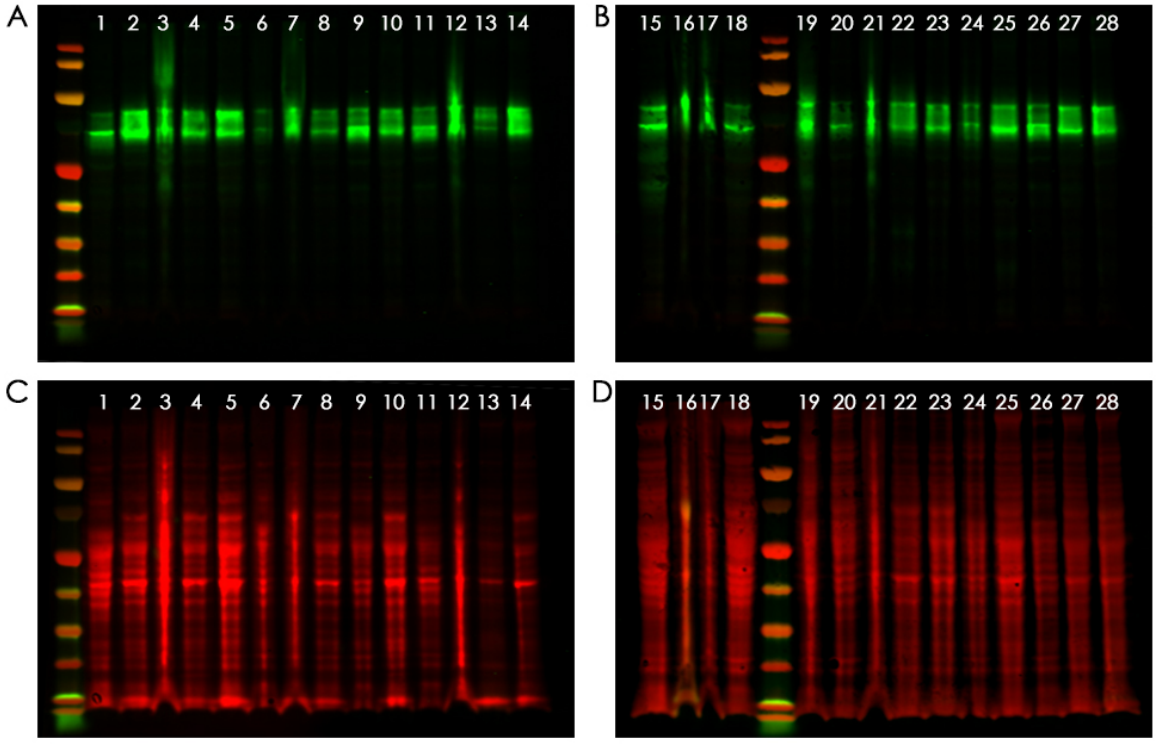
Normal: 7-11, 19-24
HCV/Cirrhosis: 4-6, 15-18
HCV/HCC: 1-3, 12-14



Supplementary Figure 13: Uncropped Western blot images showing TNFa total protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 20. (C-D) Total protein staining used as loading control.

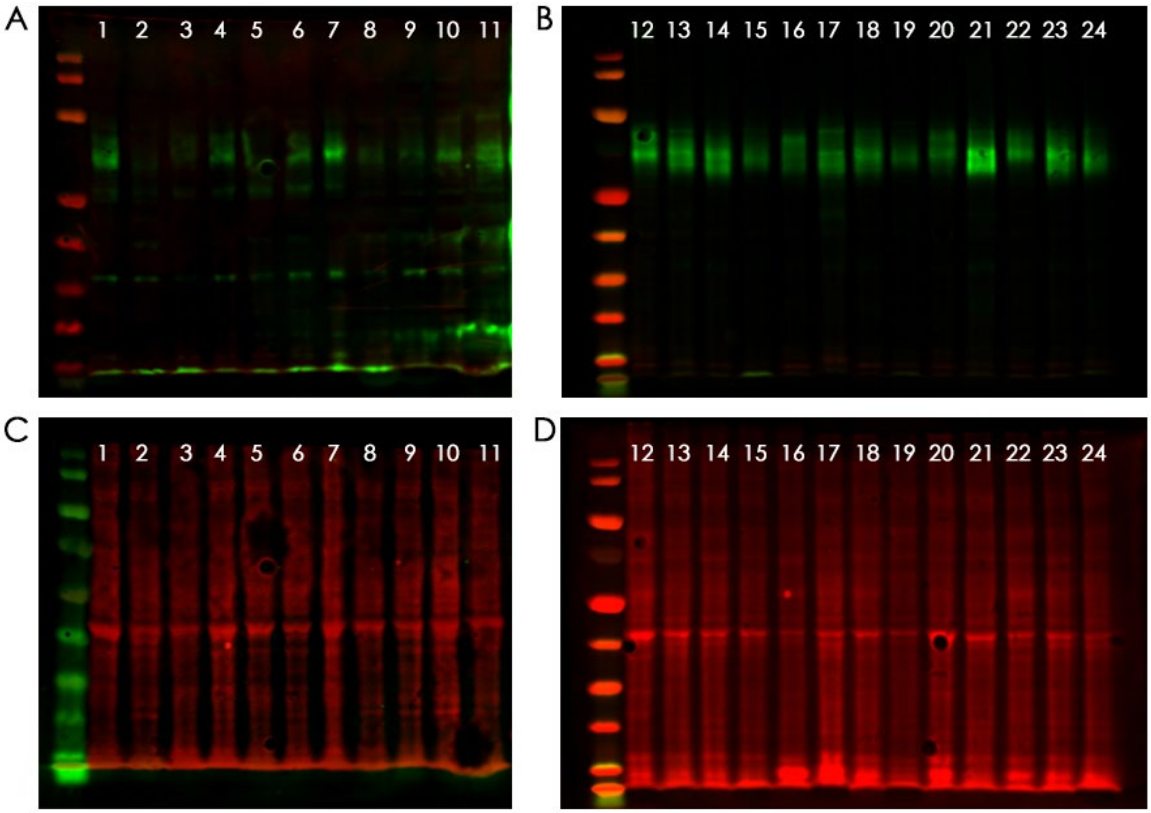
Positive Control: 14, 27
Normal: 1-4, 15-18
HCV/Cirrhosis: 5-8, 23-26
HCV/HCC: 9-13, 19-22



Supplementary Figure 14: Uncropped Western blot images showing cytoplasmic CD55 protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 22. (C-D) Total protein staining used as loading control.

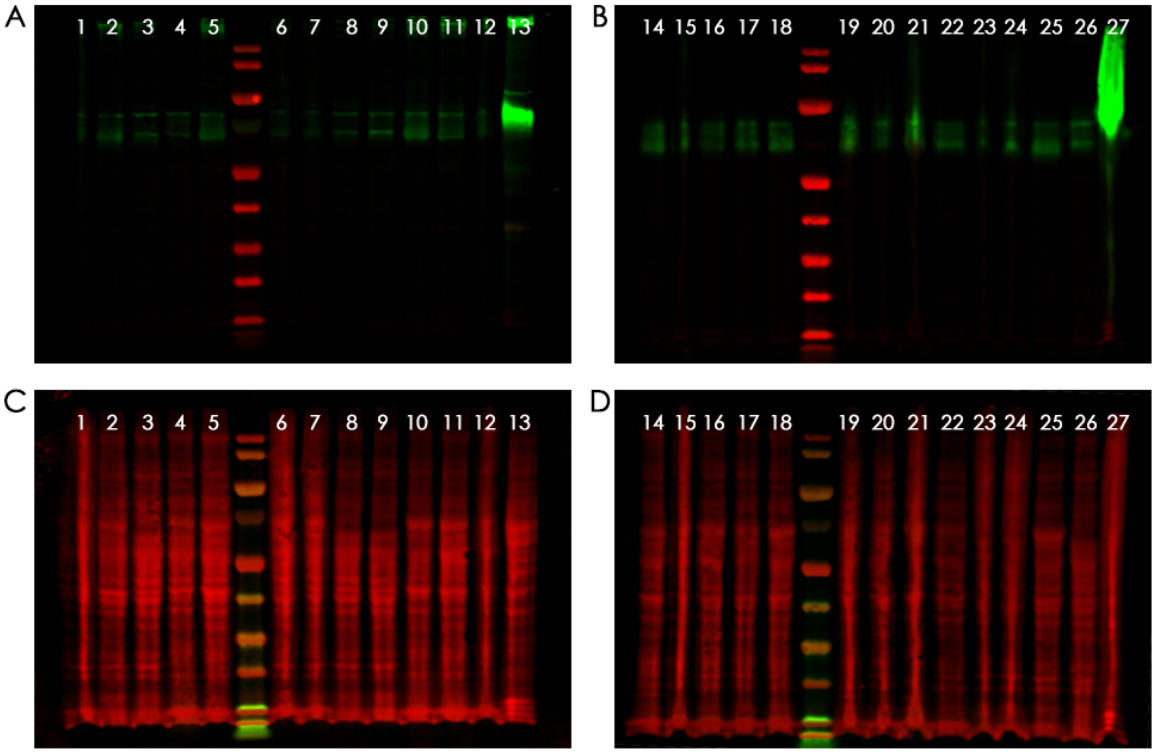
Normal: 1, 3, 6, 9, 11, 12, 15, 17, 19, 21, 24, 26
HCV/Cirrhosis: 2, 5, 8, 10, 13, 22, 25, 27
HCV/HCC: 4, 7, 14, 16, 18, 20, 23, 28



Supplementary Figure 15: Uncropped Western blot images showing nuclear CD55 protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 22. (C-D) Total protein staining used as loading control.

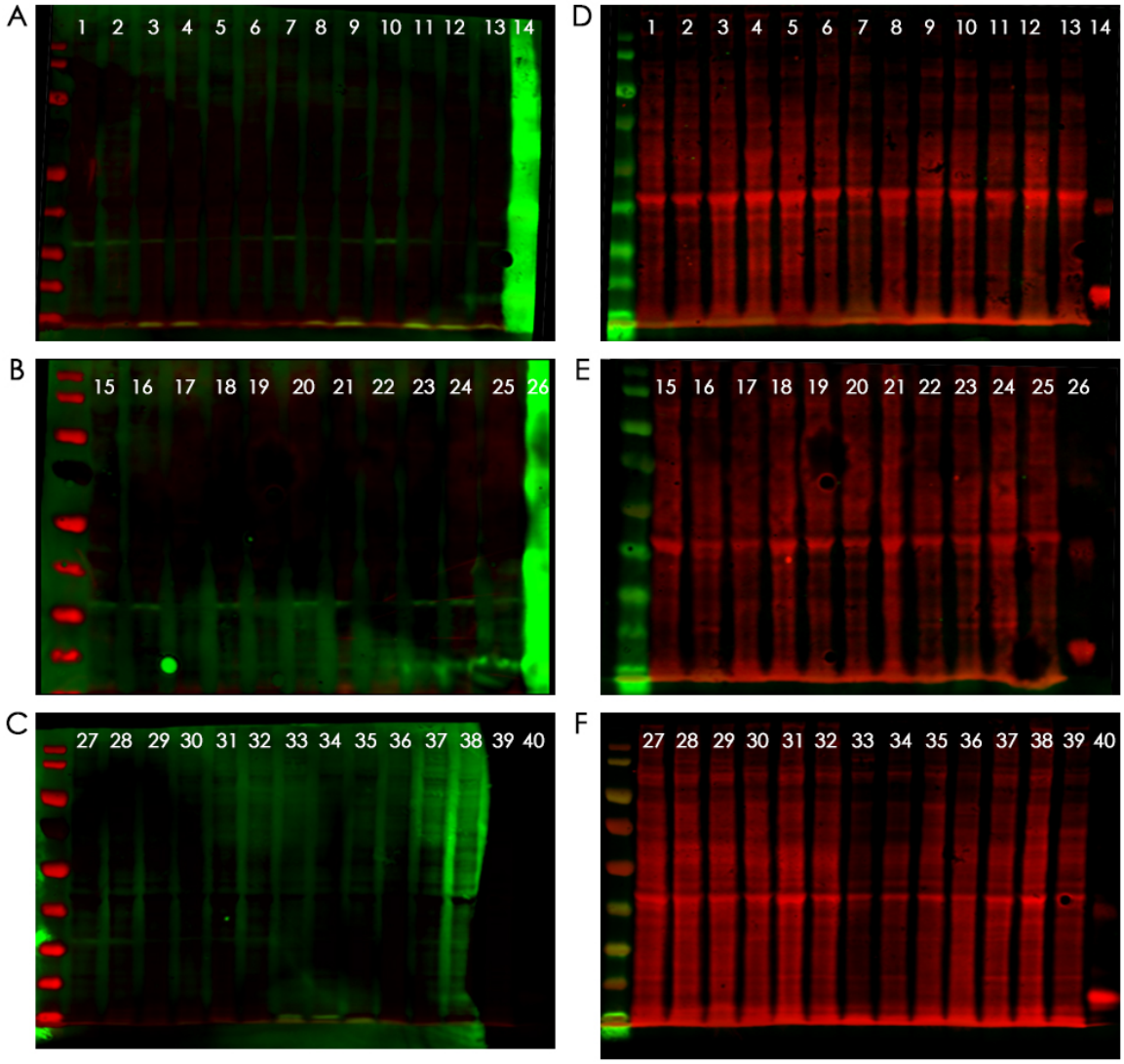
Normal: 8-11, 13, 15, 17, 19, 21, 24
HCV/Cirrhosis: 4-7, 12, 18, 20, 22
HCV/HCC: 1-3, 14, 16, 23



Supplementary Figure 16: Uncropped Western blot images showing CD55 total protein expression in human liver tissues

(A-B) Western blots indicating protein of interest in Figure 22. (C-D) Total protein staining used as loading control.

Positive Control: 13, 27
Normal: 2, 3, 8, 9, 23-26
HCV/Cirrhosis: 4, 10-12, 19-22
HCV/HCC: 1, 5, 6, 7, 14-18



Supplementary Figure 17: Uncropped Western blot images showing nuclear IL-33 protein expression in human liver tissues

(A-C) Western blots indicating protein of interest in Figure 23. (D-F) Total protein staining used as loading control.

Positive Control: 14, 26, 40
Normal: 9-13, 22-25, 28, 29, 31, 33, 34, 37
HCV/Cirrhosis: 5-8, 18-21, 27, 30, 35, 36, 38, 39
HCV/HCC: 1-4, 15-17, 32

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VITA

Sarah Groover

Candidate for the Degree of

Doctor of Philosophy

Thesis: ESTROGEN RECEPTOR-MEDIATED MODULATION OF INFLAMMATORY MARKERS IN HEPATITIS C VIRUS PATHOGENESIS

Major Field: Biomedical Sciences

Biographical:

Education:

Expected to complete the requirements for the Doctor of Medicine at the University of Colorado, Aurora, Colorado in May 2024.

Completed the requirements for the Doctor of Philosophy in Biomedical Sciences at Oklahoma State University, Stillwater, Oklahoma in July 2020.

Completed the requirements for the Bachelors of Science in Medical/Molecular Biology at Rogers State University, Claremore, Oklahoma in 2016.

Experience:

Graduate Research Assistant under Dr. Rashmi Kaul at Oklahoma State University, Center for Health Sciences from August 2016-July 2020.

Research Intern under Dr. Thomas O'Brien at the National Institutes of Health, National Cancer Institute from June 2017-August 2017.

Research Intern under Dr. Brett Langley at Burke Neurological Institute from May 2014-August 2014 and May 2015-August 2015.

Professional Memberships:

2019-2020 Vice President of the American Physician Scientists Association
Associate Member of the American Association for Cancer Research
Trainee Member of the American Association for the Study of Liver Diseases
Trainee Member of the Clinical Immunology Society