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**Impairments in Signaling Cascades Mediating the Progression
of Liver Disease from Chronic Hepatitis to Hepatocellular
Carcinoma in Animal and Human Models**

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Thesis Presented for the Degree of
DOCTOR OF PHILOSOPHY
in the Department of Internal Medicine
UNIVERSITY OF CAPE TOWN

August 2011

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DECLARATION

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CONTENTS

Declaration ii

List of tables and figures vii

Foreword xiv

Acknowledgements xvi

Acronyms and abbreviations xviii

ABSTRACT xxiv

1. CHAPTER 1

Introduction

1.1 Purpose of the study 1

1.2 Objectives 5

1.3 Specific aims 6

1.4 Background 7

1.5 Alcoholic liver disease 9

1.6 Hepatocellular carcinoma (HCC) 16

1.7 General mechanisms of cancer 18

1.8 The molecular basis of HCC: signaling impairments 25

1.8.1 Insulin/Insulin growth factor (IGF) signaling 25

1.8.2 Wnt/ β -catenin signaling 27

1.8.3 Notch signaling in HCC 29

1.9 Cross-talk of signaling cascades 32

References 35

2. CHAPTER 2

Limited Therapeutic Effect of N-Acetylcysteine on Hepatic Insulin Resistance in an Experimental In Vivo Model of Alcohol-Induced Steatohepatitis

2.1 Introduction 54

2.2 Materials and methods	57
2.3 Results	68
2.4 Discussion	88
<i>References</i>	<i>94</i>
3. CHAPTER 3	
Cytokine Profile and Effects of N-Acetylcysteine Treatment in a Rat Model of Alcohol-Induced Steatohepatitis	
3.1 Introduction	108
3.2 Materials and methods	111
3.3 Results	112
3.4 Discussion	118
<i>References</i>	<i>121</i>
<i>Appendix</i>	<i>125</i>
4. CHAPTER 4	
Ceramide inhibitors ameliorate alcohol-induced steatohepatitis in an ex-vivo liver slice culture model	
4.1 Introduction	130
4.2 Materials and methods	135
4.3 Results	140
4.4 Discussion	155
<i>References</i>	<i>159</i>
5. CHAPTER 5	
Re-activated Wnt signaling is crucial in the pathogenesis of HCC in double transgenic mouse model that constitutively over-expresses the HBx and IRS-1 genes in liver	
5.1 Introduction	169

5.2 Materials and methods	174
5.3 Results	176
5.4 Discussion	194
<i>References</i>	198
6. CHAPTER 6	
Ceramide-Induced Lipotoxicity Promotes Insulin Resistance in Alcoholic Steatohepatitis in Humans	
6.1 Introduction	207
6.2 Materials and methods	210
6.3 Results	218
6.4 Discussion	229
<i>References</i>	233
7. CHAPTER 7	
Triad of dysregulated signaling in human hepatocellular carcinoma (HCC) suggests a multi-pronged approach is needed for therapeutic targeting	
7.1 Introduction	243
7.2 Materials and methods	247
7.3 Results	249
7.4 Discussion	262
<i>References</i>	267
8. CHAPTER 8	
Conclusion	
8.1 Conclusion	274

8.2 Future directions	282
8.3 Publications arising from this work	284

University of Cape Town

LIST TABLES AND FIGURES

Tables

Table 1:	Composition of diets	59
Table 2:	Liver histopathology scoring system	60
Table 3:	Rat primer pairs used for qRT-PCR assays	64
Table 4:	Effects of ethanol and NAC on markers of hepatocellular injury	68
Table 5:	Effects of ethanol and NAC on hepatic CYP2E1 activity and oxidative stress	69
Table 6:	Effects of ethanol and NAC on hepatic hydroxyproline content and expression of mRNAs associated with fibrosis and matrix remodelling	71
Table 7:	Effects of chronic ethanol feeding and treatment with NAC on pro-Sphingolipid gene expression in liver	77
Table 8:	Influence of chronic ethanol feeding and NAC on pro-inflammatory cytokine levels in liver	81
Table 9:	Contributions of “important” cytokines to immunologic responses	125
Table 10:	Ceramide inhibitors used in experiment	136
Table 11:	Human primer pairs used for qRT-PCR assays	212

Figures

- Figure 2.1: Effects of chronic ethanol feeding 73
- Figure 2.2: Lipid and ceramide accumulations in liver 75
- Figure 2.3: Role of sphingomyelinase activity as a mediator of increased hepatic ceramide levels 78
- Figure 2.4: Effects of chronic ethanol and NAC treatment on pro-inflammatory cytokine and CYP2E1 gene expression 82
- Figure 2.5: Effects of chronic ethanol and NAC treatment on insulin/IGF signaling network genes 84
- Figure 2.6: Effects of chronic ethanol and NAC treatment on upstream mediators of insulin/IGF signalling 85
- Figure 2.7: Effects of chronic ethanol and NAC treatment on downstream mediators of insulin/IGF signalling 87
- Figure 3.1: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on pro-inflammatory cytokine profile (non-significant) 113
- Figure 3.2: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on pro-inflammatory cytokine profile (significant) 114
- Figure 3.3: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on anti-inflammatory cytokine profile (non-significant) 115
- Figure 3.4: Effects of control- or ethanol-containing TEN feeding and N-

acetylcysteine (NAC) on chemokine and growth factor profile
(significant) 116

- Figure 4.1: Schematic representation of sphingolipid metabolism including the de novo, hydrolysis and salvage pathways 131
- Figure 4.2: Effects of chronic ethanol feeding and ceramide inhibitor treatment on steatohepatitis 141
- Figure 4.3: Effects of chronic alcohol feeding and ceramide inhibitor treatment on neutral lipid and ceramide accumulation at 48 and 72 hours post-culture 143
- Figure 4.4: Effects of chronic alcohol feeding and ceramide inhibitor treatment on triglyceride accumulation 144
- Figure 4.5: Effects of chronic alcohol feeding and ceramide inhibitor treatment on cytotoxicity 145
- Figure 4.6: Effects of chronic alcohol feeding and ceramide inhibitor treatment on ER stress signaling network genes 147
- Figure 4.7: Effects of chronic alcohol feeding and ceramide inhibitor treatment on downstream mediators of ER stress signaling at 48 hours 149
- Figure 4.8: Effects of chronic alcohol feeding and ceramide inhibitor treatment on downstream mediators of ER stress signaling at 72 hours 150
- Figure 4.9: Effects of chronic alcohol feeding and ceramide inhibitor treatment on upstream insulin signaling network genes

152

Figure 4.10: Effects of chronic alcohol feeding and ceramide inhibitor treatment on upstream mediators of insulin/IGF signalling

153

Figure 4.11: Effects of chronic alcohol feeding and ceramide inhibitor treatment on downstream mediators of insulin/IGF signalling

154

Figure 5.1: mRNA transcript fold difference of Wnt ligand signaling genes in mice that did not develop tumours over time 177

Figure 5.2: mRNA transcript fold difference of Wnt receptor signaling genes in mice that did not develop tumours over time 178

Figure 5.3: mRNA transcript fold difference of Wnt downstream signaling genes in mice that did not develop tumours over time 179

Figure 5.4: Gene expression of Wnt ligand signaling genes in WT and DT mice with tumours 181

Figure 5.5: Gene expression of Wnt receptor signaling genes in WT and DT mice with tumours 182

Figure 5.6: Gene expression of Wnt downstream signaling genes in WT and DT mice with tumours 184

Figure 5.7a: Gene expression of Wnt ligand signaling genes in WT and DT mice represented in bar graphs 185

Figure 5.7b: Gene expression of Wnt ligand signaling genes in WT and DT mice represented in bar graphs 186

Figure 5.8a:	Gene expression of Wnt receptor signaling genes in WT and DT mice represented in bar graphs	188
Figure 5.8b:	Gene expression of Wnt receptor signaling genes in WT and DT mice represented in bar graphs	189
Figure 5.9a:	Gene expression of Wnt downstream signaling genes in WT and DT mice represented in bar graphs	191
Figure 5.9b:	Gene expression of Wnt downstream signaling genes in WT and DT mice represented in bar graphs	193
Figure 6.1:	Lipid and ceramide accumulations in the liver of patients with alcoholic liver disease (ALD) or controls	218
Figure 6.2:	Gene expression levels of mediators of pro-ceramide signaling, measured in control and ALD human liver tissues	220
Figure 6.3:	Gene expression levels of mediators of the insulin/IGF signaling cascade, measured in control and ALD human liver tissues	222
Figure 6.4:	Protein expression levels of upstream polypeptides of the insulin/IGF signaling cascade, measured in control and ALD human liver tissues	222
Figure 6.5:	Protein expression levels of the downstream polypeptides of the insulin/IGF signaling cascade, measured in control and ALD human liver tissues	224
Figure 6.6:	Gene expression levels of mediators of the ER stress signaling cascade measured control and ALD human liver tissues	

- Figure 6.7: Cytokine expression levels measured in control and ALD human liver tissues 228
- Figure 7.1: Gene expression levels of mediators of the Insulin/IGF signaling cascade, measured in control and peri-tumour human liver tissues 250
- Figure 7.2: Gene expression levels of mediators of the Insulin/IGF signaling cascade, measured in peri-tumour and HCC human liver tissues 251
- Figure 7.3: Protein expression levels of upstream polypeptides of the Insulin/IGF signaling cascade, measured in peri-tumour and HCC human liver tissues 252
- Figure 7.4: Protein expression levels of the downstream polypeptides of the insulin/IGF signaling cascade, measured in peri-tumour and HCC human liver tissues 254
- Figure 7.5a: Gene expression levels of mediators of the Wnt/ β -catenin signaling cascade, measured in peri-tumour and HCC human liver tissues 256
- Figure 7.5b: Gene expression levels of mediators of the Wnt/ β -catenin signaling cascade, measured in peri-tumour and HCC human liver tissues 257
- Figure 7.6: Gene expression levels of mediators of the Notch signaling cascade, including AAH/Humbug measured in peri-tumour and

	HCC human liver tissues	259
Figure 7.7:	Cytokine expression levels measured in peri-tumour and HCC human liver tissues	260
Figure 8.1:	Mechanisms of progression to ASH	277
Figure 8.2:	Triad of signaling impairments and contribution to HCC	281

University of Cape Town

FOREWORD

Intensive research has been undertaken in recent times to understand the pathophysiologic mechanisms of chronic hepatitis and HCC. Newer therapeutic agents that inhibit receptor tyrosine protein kinases have been developed as a result of targeting specific proteins in signaling cascades or molecular events within cells, thus demonstrating the importance of understanding the pathological regulation of cellular and molecular events, as those that occur in chronic hepatitis and preceding the development of HCC. Therefore using this approach is useful in identifying critical steps in signaling that are important to link these two disease processes. In this way signaling pathways that influence growth or generate anti-apoptotic signals can be profiled so that aberrant signaling within the cascade can be readily isolated and characterized, and inhibitors or activators can later be tested to assess effect on outcomes with respect to tumour growth. Alternatively the expression of key target molecules or proteins can potentially be identified, that may be subsequently used as serum/tissue biomarkers to either predict or diagnose early disease and provide information regarding prognosis.

The aim of this work was to use novel approaches in an attempt to understand the pathogenesis of chronic hepatitis and its progression to HCC. The first chapter discusses the overall aims and objectives and provides the significance of and rationale for the research. Included therein is a background section that places the research in the context of clinical disease. Chapters two, three, four, five, six and seven are stand-alone chapters that deal with individual

but related elements on objectives of this research. Each of these chapters has its own introduction/background, materials and methods, results and discussion sections. Chapter eight is the conclusion and integrates the findings of the previous chapters and finally, it discusses proposed future directions of this research. It also includes a list of publications emanating from this work.

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ACKNOWLEDGEMENTS

I would like to thank my supervisors for their guidance and patience throughout the process of completing this work. Specifically I am grateful to Professor Michael Kew who encouraged me to undertake basic science research, something I had not previously considered and which I believe has added a unique but critically important aspect to my training as a physician. I performed all my experiments at the Liver Research Center (at the Alpert Medical School of Brown University), which is under the directorship of Dr Jack Wands and am humbled and grateful to have had the opportunity to work at his laboratory. I thank him for his intellectual input and constant encouragement and have great respect for the work that is done at this laboratory. None of this work would have been possible without the dedication of my mentor Dr Suzanne de la Monte. I am indebted to her for the comprehensive tutelage I received under her supervision. I have learned not only how to technically conduct well-thought out translational and relevant research, but also how to manage the many diverse aspects of research including managing and analyzing data, dealing with and teaching the younger laboratory members, and learning about the nuances and logistics of potentially setting up a laboratory in the future. As my mentor, colleague and woman she has imparted an immense amount of knowledge (which cannot be found in textbooks or through formal learning) about the practice of medicine that will carry me throughout my career. All members of the laboratory are gratefully acknowledged for their help and support, in particular Lisa Longato and Dr Ming Tong who patiently taught me every aspect of performing the experiments. I

would also like to acknowledge Professor Bongani Mayosi who has played and continues to play a key role in influencing my career, for his guidance and unfailing support. A special note of heartfelt gratitude is due to Dr Chris Kassianedis and Karin Fenton, for caring and always advocating for my welfare, well beyond the call of duty. They never gave up on me. Finally I would like to thank family i.e. my parents Sibisi and Quinn Setshedi, and siblings Moabi Setshedi and Motshabi Nomvethé for their unwavering support and encouragement, not only in the last three years, but always.

This work was supported by the Gastroenterology Foundation of South Africa, the South African Medical Association (SAMA), the National Research Fund (NRF) and the Department of Medicine of the University of Cape Town.

This thesis is dedicated to my parents, niece and nephews.

ACRONYMS AND ABBREVIATIONS

β -NGF	Beta-Nerve Growth Factor
4-HNE	4-Hydroxynonenal
4-MUP	4-Methylumbelliferyl phosphate
AAH	Aspartyl-(Asparaginyl)-Hydroxylase
ADH	Alcohol Dehydrogenase
ALD	Alcoholic Liver Disease
ALDH	Aldehyde Dehydrogenase
ALT	Alanine aminotransferase
AMPK	Adenosine Monophosphate Kinase
APC	Adenomatosis Polyposis Coli
ASH	Alcoholic Steatohepatitis
ASMase	Acid Sphingomyelinase
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
ATX	Hepatitis B X Transgenic Mice
BAX	Bcl-2-Associated X Protein
BCA	Bicinchoninic Assay
BSA	Bovine Serum Albumin
CAMs	Cell-to-Cell Adhesion Molecules
CERD	Ceramidase
CERS	Ceramide Synthase
CHOP	C/EBP-homologous Protein

CLDs	Chronic Liver Diseases
CRC	Colorectal Cancer
CYP2E1	Cytochrome P450 2E1
DNA	Deoxyribonucleic Acid
DNMTs	DNA Methyltransferases
DNs	Dysplastic Nodules
DSH	Disheveled Protein
E2F	Family of Transcription Factors
EDEM	ER-Degradation-Enhancing-Mannosidase-Like Protein
ELISAs	Enzyme-Linked Immunosorbent Assays
EMT	Epithelial Mesenchymal Transition
ER	Endoplasmic Reticulum
ER Stress	Endoplasmic Reticulum Stress
ERAD	ER-Associated Degradation
ERK	Extracellular Signal-Regulated Kinase
ETC	Electron Transport Chain
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FRP	Frizzled-related protein
FZD	Frizzled
G-CSF	Granulocyte Colony-Stimulating Factor
G6PD	Glucose-6-Phosphate Dehydrogenase
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor

GRP78	Glucose-Regulated Protein 78
GSH	Glutathione
GSK-3	Glycogen Synthase Kinase-3
H&E	Haematoxylin and Eosin
HBV	Hepatitis B Virus
HBx	Hepatitis B X Protein
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HES/HERP	Hes-Related Repressor Protein
HEY	Hairy and Enhancer of Split
HPRT	Hypoxanthine Phosphoribosyltransferase
HRP	Horseradish Peroxidase
IGF	Insulin Growth Factor
IgG	Immunoglobulin G
INS/IN	Insulin
IP-10	Interferon Gamma-Induced Protein 10 kDa
IRB	Institutional Review Board
IRE	Inositol Requiring Enzyme
IRS	Insulin Receptor Substrate
JNK	c-Jun N-Terminal Kinases
LPS	Lipopolysaccharide
LRP	Low-Density Lipoprotein Receptor Related Protein
LSC	Liver Slice Culture

MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MDA	Malondialdehyde
MIP-1 α	Macrophage Inflammatory Protein 1 alpha
MPT	Mitochondrial Permeability Transition
mTOR	Mammalian Target of Rapamycin
NAC	N-Acetylcysteine
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NAFLD	Non-Alcoholic Fatty Liver Disease
NICD	Notch Intracellular Domain
NSMase	Neutral sphingomyelinase
P58IPK	Inhibitor of PERK
P706SK	70-kDa Ribosomal Protein S6 Kinase
PCLSC	Precision-Cut Liver Slice Culture
PERK	RNA-activated Protein Kinase-Like ER Kinase
PI3-K	Phospho-inositol 3 kinase
PRAS40	Proline-Rich Akt Substrate of 40 Kilodaltons
pRb	Retinoblastoma Protein
PTEN	Phosphatase and Tensin Homolog
q-PCR	Quantitative Polymerase Chain Reaction
QT	Quantitative
R-	Receptor

RANTES	Regulated Upon Activation Normal T Cell Expressed and Secreted
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPLPO	Large Ribosomal Protein
RT	Reverse Transcriptase
S phase	Synthesis Phase
SEM	Standard Error of Measurement
SMPD	Sphingomyelinase
SPTLC	Serine Palmitoyl Transferase
SREBP-1	Sterol-Binding Regulatory Element Protein-1
SSA	Sub-Saharan Africa
TCF/ LEF	T-Cell Factor/Lymphoid Enhancing Factor
TEN	Total Enteral Nutrition
TGF	Transforming Growth Factor beta
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor alpha
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UGCG	UDP-Glucose Ceramide Glucosyltransferase
UPR	Unfolded Protein Response
US	United States
VEGF	Vascular Endothelial Growth Factor
VEGF	Vascular Endothelial Growth Factor
WISP-1	Wnt1 Inducible Signaling Pathway

XBP-1 X-box-Binding protein 1

ABSTRACT

The most common risk factors for chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC) include chronic alcohol abuse and infection with hepatitis B (HBV) or hepatitis C (HCV) virus. Growing evidence from human studies and experimental models suggests that pre-degenerative and pre-malignant abnormalities include disturbances in intracellular signaling and ongoing injury with oxidative stress, inflammation, and lipotoxicity. The major signal transduction pathways affected in both degenerative and neoplastic disease states in liver include: insulin/IGF, Wnt/ β -catenin, and others. The role of Notch signaling in HCC is an emerging area. Chronic alcohol exposure, which causes cirrhosis but is also a co-factor for HCC development, inhibits insulin signaling thus resulting in insulin resistance, increased DNA damage, and impaired hepatocellular growth, viability, and energy metabolism. HCC is associated with increased activation and dysregulation of insulin/IGF, Wnt/ β -catenin, and probably Notch signaling, together with increased DNA damage and increased cell turnover. In this work, the goal was to compare factors that contribute to the progression of liver disease towards cirrhosis on the one hand, and the subsequent development of a malignant neoplastic state, i.e. dysplasia and HCC, on the other hand, focusing on the roles of abnormal signaling through insulin/IGF, Wnt/ β -catenin and Notch pathways. The research plan was organized under two specific aims. The first aim was to examine the effects of chronic ethanol exposure on the expression of genes that regulate insulin/IGF signaling, toxic lipid accumulation/lipotoxicity, ER stress, and inflammation, and

evaluate the therapeutic effects of N-acetylcysteine and ceramide inhibitors on these indices of chronic alcohol-induced liver disease. The second aim was to examine the role of dysregulated Wnt/ β -catenin signaling in the pathogenesis of HCC in double-transgenic mouse model that constitutively over-expresses the HBx and IRS-1 genes in liver. Results were correlated with progression of liver disease in humans from stages of chronic injury with hepatic steatosis, to dysplasia, and finally HCC. For our purposes we utilized human tissue samples and robust experimental animal models. The experimental approaches included qRT-PCR analysis, multiplex and targeted array analysis of gene expression, multiplex ELISAs, histopathologic studies, lipid biochemistry, precision cut slice culture ex vivo models, and biostatistics. These same approaches could be used in future patho-molecular-epidemiological translational studies of human disease.

CHAPTER 1

INTRODUCTION

1.1 Purpose of the study

Alcoholic liver disease (ALD) progresses from normal hepatocytes, chronic inflammation, fibrosis and ultimately cirrhosis, which is a chief risk factor (4-5 fold) for the development of HCC in this context. However with chronic Hepatitis B and C infection (HBV and HCV) the risk is much higher (30-40 fold). The cellular mechanisms responsible for either progression from chronic hepatitis to cirrhosis or cirrhosis to a malignant phenotype are many, but not fully understood with respect to ALD. These mechanisms include the role of the innate immune system, oxidative stress, transcription factors and the activation of stellate cells and their interaction with signaling events that modulate these functions (Mandrekar & Szabo, 2009). Aberrant signal transduction is involved in promoting injury and progression of liver disease. Insulin signaling is required for many cellular functions including energy metabolism, regeneration and ultimately cell survival (Banerjee, Mohr, Wands, & de la Monte, 1998; Carter & Wands, 1985; Li, Liu, & Yanoff, 1995; Mohr, Tanaka, & Wands, 1998; Sasaki et al., 1994). By disrupting lipid metabolism and homeostasis, and impairing regenerative capabilities of injured hepatocytes, impaired insulin signaling contributes to the pathogenesis of ALD. Free fatty acids and triglycerides have traditionally been posited to be the culprits mediating lipid accumulation and its consequences of lipotoxicity. However, more recently the role of ceramides (in

both alcoholic and non-alcoholic steatohepatitis) in promoting lipotoxicity in animal models has been described. In addition to being bi-lipid layer components, ceramides affect multiple signaling pathways including the insulin pathway. Studies performed in our laboratory as well as others have shown that alcohol impairs insulin signaling at many levels but mainly by inhibiting insulin receptor binding (de la Monte et al., 2008; Patel, D'Arville, Iwahashi, & Simon, 1991) and suppressing the function of insulin receptor tyrosine kinases (Banerjee et al., 1998; Mohr et al., 1998; Sasaki & Wands, 1994), thus causing insulin resistance.

HCC may be the end-result of many years of cirrhotic liver disease due to any aetiology. HCC has tumour initiation and promotion stages, and progresses in a step-wise fashion influenced in part by cellular signaling mechanisms. Dysregulation of signaling pathways has been observed at even the earlier stages of disease, thus identification of aberrant signaling polypeptides that could be useful as novel biomarkers to predict the presence of pre-malignant disease. Previous studies in humans and experimental animal models indicate that three distinct signaling pathways in particular, that crosstalk at various levels, are frequently dysregulated in HCC. These include: 1) insulin/IGF, insulin receptor substrate (IRS) (Longato et al., 2009), and downstream mediators including PI3K/Akt and Erk/MAPK (Banerjee et al., 1998; Huynh et al., 2003); 2) Wnt/ β -catenin (Merle et al., 2004); and 3) Notch/Hes/Hes (Cantarini et al., 2006) pathways. In addition, malignant transformation is associated with increased cell

turnover, oxidative stress, adduct formation, and increased DNA damage (Seitz & Stickel, 2007). Consequences of dysregulation of these pathways, combined with increased hepatocyte injury and turnover, followed by enhanced motility, and invasive potential, leads to the transformed phenotype and the development of HCC.

Several independent studies have demonstrated variable degrees to which increased activation of one or more of these major signaling pathways is associated with HCC development and/or its progression. Studies in transgenic mice have shown that simple up-regulation of any given signaling pathway is not sufficient to cause HCC, despite increased growth and cell turnover in the liver. The challenge in our understanding of HCC pathogenesis stems from the lack of sufficient information about progressive shifts in gene expression and signaling that occur prior to and during the transformation process. Without a robust experimental animal model, it is not possible to dissect these mechanisms because human studies are mainly cross-sectional in nature, or at best employ two or three longitudinal time points of tissue analysis. Our laboratory succeeded in developing a double-transgenic mouse model (ATX) in which the hepatitis B x (HBx) and IRS-1 genes were over-expressed in liver under the control of liver-specific promoters. The rationale for generating this model was that previous studies suggested that HBx protein, a viral regulatory protein found expressed in acute and chronic hepatitis B virus infection mediates trans-activation of HBV transcription and activates growth factor signaling pathways in the chronic HBV infected liver. In addition, HBx promotes cellular injury, oxidative stress, and

DNA damage. A second point is that in most cases of human HCC examined, there is striking up-regulation of the IRS-1 gene, resulting in increased signaling downstream through PI3kinase/Akt and Erk/MAPK, which regulate growth and hepatocyte survival. Initial studies using this model demonstrated that there was no increase in HCC development in mice carrying a single transgene e.g. IRS-1. In contrast, progressive hepatocellular injury and turnover, dysplasia, and eventually HCC development occurred in male mice, beginning at 15 months of age, in double transgenic mice that expressed both HBx and IRS-1 transgenes (Longato et al., 2009). Using targeted PCR arrays, we also demonstrated that the expression levels of several epigenetic regulatory genes shifted over time and with increasing age, which are of intense interest in understanding the molecular pathogenesis of this devastating disease.

To summarize, both ALD and chronic HBV and HCV are common serious conditions with significant morbidity, mortality and economic cost and lead to or contribute to the development of HCC. Impaired signaling is operational and thus we aim to utilize animal and human models and tissues to interrogate the role of differential signaling in these liver diseases and how they participate in hepatic oncogenesis.

1.2 Objectives

To understand the role of impaired signaling in the liver, one major goal of this work was to determine the degree to which chronic ethanol exposure adversely affects insulin-signaling pathways and to determine the mediators of insulin resistance since it is associated with oxidative stress and DNA damage, which may participate in the development of HCC. We examined such factors in a robust experimental animal model of alcoholic steatohepatitis and in an ex vivo system as well. In addition, we used the same molecular approaches to determine how chronic alcohol abuse in humans alters constitutive insulin and Notch signaling pathways in the liver. The human studies allowed us to draw direct comparisons between humans and experimental animal models of chronic alcoholic liver disease in relation to these critical signaling alterations.

The second major goal was to characterize the aging and time-dependent shifts in Notch signaling-related genes in the ATX (HBx overexpression) model of HCC development. Since it takes approximately 15 months for the double transgenic mice (HBx/IRS-1) to develop HCC, despite constitutive overexpression of both HBx and IRS-1 from birth, we proposed that aging and chronic injury may be important factors that lead to continuous oxidative stress, cell turnover, and together with adduct formation and ultimately DNA damage which are major risk factors for hepatic oncogenesis. We characterized changes in Wnt gene and protein expression in liver and correlated responses with the histologic development of dysplasia, which may progress to HCC. Currently, there is no information on the time-dependent changes in expression of the IRS-

1 transgene, as well as endogenous insulin and IGF signaling molecules. We analyzed both Notch and insulin/IGF signaling pathways to better understand the molecular events associated with HCC development. A desired analysis of oxidative stress-induced adduct formation was beyond the scope of this work, however may be a relevant extension to this research in the future.

1.3 Specific aims

Aim 1: To examine the effects of chronic ethanol exposure on the expression of genes that regulate insulin/IGF signaling, toxic lipid accumulation and lipotoxicity, ER stress, and inflammation, and evaluate the therapeutic effects of N-acetylcysteine (NAC) and ceramide inhibitors on these indices of chronic alcohol-induced liver disease using in vivo and ex vivo systems respectively. Additionally, to correlate our findings with a similar analysis of liver obtained from patients with ALD.

Hypothesis: Alcoholic liver disease is associated with inhibition of insulin/IGF signaling, and increased oxidative stress mediated by lipotoxicity, particularly excess ceramide generation. Treatment with anti-oxidants and ceramide inhibitors may be effective in restoring insulin resistance and reducing lipotoxicity in the liver.

Aim 2: To examine the role of dysregulated Wnt and Notch signaling in the pathogenesis of HCC. In the first instance we used a double-transgenic (HBx/IRS-1) mouse model that constitutively over-expresses the HBx and IRS-1

genes in liver to study Wnt signaling. Results were correlated with progression of liver disease from stages of chronic injury with hepatic steatosis, to dysplasia, and finally HCC. This analysis was followed by a repeat of tests of Wnt and Notch signaling, this time using human liver tissue.

Hypothesis: Constitutive over-expression of the HBx and IRS-1 genes in liver accelerates aging-associated increases in mediators of oxidative stress leading to increased lipid peroxidation, DNA damage, and possibly adduct formation. These secondary but fundamental pre-malignant abnormalities mediate the eventual dysregulation of Wnt/ β -catenin signaling pathways, and appear fundamental to the pathogenesis of HCC.

1.4 Background

Chronic liver diseases (CLDs) including cirrhosis constitute a large proportion of disease burden worldwide, and are in the top twenty of all causes of mortality (World Health Organization, 2003). CLDs encompass a spectrum of disease pathology, that progress through various stages in a similar fashion, irrespective of aetiology. Classically, from the normal liver the disease will progress to a mild, followed by a more severe chronic inflammatory state characterized by a lymphocytic inflammatory infiltrate (in the case of chronic viral hepatitis) and altered architecture of the hepatic lobule. This is followed by progression to end-stage disease characterized by regenerative nodules, surrounded by bands of scar tissue and cirrhosis. Cirrhosis is associated with severe morbidity and

mortality and is the chief risk factor for the development of hepatocellular carcinoma (HCC).

The commonest risk factors for the development of chronic liver diseases are alcohol, hepatitis B (HBV) and C (HCV) infections, obesity, and in some parts of the Asia and Africa aflatoxins. HCV is a significant cause of CLD and HCC especially in the United States and Japan; the incidence of hepatocellular carcinoma has risen 3-fold in the last 30 years as a result of an increasing number of chronic HCV infections (Altekruse, McGlynn, & Reichman, 2009; Armstrong et al., 2006). HBV is more prevalent in East Asia and Sub-Saharan Africa (SSA), and is the commonest cause for HCC in these parts of the globe, with higher incidences of HCC than elsewhere. Worldwide 80-90% of HCC are associated with chronic HBV and HCV infection (El-Serag & Rudolph, 2007; Nordenstedt, White, & El-Serag, 2010). Consequently, currently HCC is the fifth most common cancer in the world with the 3rd-highest cause of all cancer-related mortality (Parkin, Bray, Ferlay, & Pisani, 2005). Alcoholic liver disease (ALD) is also a major health problem; other than the social problems it causes, it results in alcoholic hepatitis that has significant mortality, progresses to cirrhosis in 25-50% of individuals, accounts for 7-15% of HCCs (Poynard et al., 1991; Schafer & Sorrell, 1999) and is the second most common liver disease for orthotopic liver transplantation (Starzl et al., 1988).

Currently, although effective treatments for chronic liver disease exist, they are generally expensive and not universally available. Thus the overall quality of the life for most patients with severe liver disease is poor. With respect

to HCC, prognosis remains exceptionally poor as a result of late diagnosis, and chemoresistance to current treatments that are available. Therefore, there is a need to understand underlying pathophysiologic mechanisms that can aid in either identifying biomarkers whereby disease can be detected early, or find novel molecular targets to develop more effective treatments is crucial.

1.5 Alcoholic liver disease

Alcoholic liver disease (ALD) is the most common and the deleterious consequences of chronic alcohol consumption results in up to 12,000 liver-related deaths per annum in the US (Centers for Disease Control Prevention). ALD encompasses a spectrum of histologic features, which can progress from simple steatosis, to more serious stages of steato-hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Adachi & Brenner, 2005; Tilg & Day, 2007). Steatosis (i.e. the accumulation of lipids in liver cells) occurs almost universally with alcohol consumption. This stage is considered to be the first, relatively “benign” stage of liver injury (first hit). This is because in most cases steatosis is reversible with cessation of alcohol intake (Maher, 2002; Siegmund, Dooley, & Brenner, 2005). More than 50% of those consuming more than six drinks per day will develop steato-hepatitis (Bellentani et al., 2000; Maher, 2002) although even in those who cease consumption may develop cirrhosis in 18% (Teli, Day, Burt, Bennett, & James, 1995). Many factors are contributed to the development of steatosis. These include but are not limited to the metabolism of alcohol, which causes an alteration in the redox potential, in favour of a reduced state of

Nicotinamide adenine dinucleotide phosphate (NADP), thus causing increased production and esterification of fatty acids. Additionally alcohol suppresses β -oxidation of fatty acids by mitochondria; therefore both these factors potentiate the accumulation of lipids. Furthermore, the accumulation of intracellular ectopic fat is mediated by up-regulation of sterol-binding regulatory element protein-1 (SREBP-1) and the down-regulation of adenosine monophosphate kinase (AMPK) and peroxisome proliferator-activated receptors (PPARs) signaling pathways (Crabb, 2004; Galli, Pinaire, Fischer, Dorris, & Crabb, 2001; Nanji, Dannenberg, Jokelainen, & Bass, 2004; You, Fischer, Deeg, & Crabb, 2002; You, Matsumoto, Pacold, Cho, & Crabb, 2004). Impaired signaling by alcohol in both cascades, results in increased lipogenesis and steatosis. Other mechanisms involved in lipid accumulation include the role of cytokines; TNF- α causes an increase in free fatty acid release from adipocytes adjacent to the liver and thus increases lipogenesis and inhibits β -oxidation of fatty acids (Feingold & Grunfeld, 1987; Hardardottir, Doerrler, Feingold, & Grunfeld, 1992; Nachiappan, Curtiss, Corkey, & Kilpatrick, 1994).

Up to one third of patients who continue to drink heavily develop alcoholic steatohepatitis (ASH) (McCullough & O'Connor, 1998). ASH is characterized by steatosis associated with a chronic inflammatory infiltrate, ballooning degeneration of cells, apoptosis, Mallory's hyaline (Brunt, 2002) and portends a grave prognosis in half the patients. Although not fully understood, the pathogenesis of ASH is mediated by a so-called "second hit" or insult. The "second hit" mechanisms include genetic and environmental factors that result in

oxidative stress, lipotoxicity, DNA damage, and pro-inflammatory cytokine activation (Carter-Kent, Zein, & Feldstein, 2008; Farrell & Larter, 2006). Progression of alcohol-related liver disease (ALD) is partly mediated by impaired lipid metabolism and homeostasis, which lead to ectopic hepatic accumulation of mainly triglycerides and subsequent lipotoxicity, defined as a state where the cell's storage and metabolic capabilities are overwhelmed. Lipotoxic states as the name suggests are toxic to hepatocytes; initially the accumulating hepatic fat causes rupture of the cell membrane, and results in lipid peroxidation of membranes. Consequences of lipid peroxidation include the formation of acetaldehyde lipid and hybrid adducts. Moreover, toxic lipids can activate anti-survival signaling pathways, thereby affecting cell functioning and stimulation of pro-cytokine activation that results in activation of stellate cells, which lay down scar tissue. These repeated cycles of cell injury followed by repair are in the long-term damaging to the liver, and are the cause of the hepatitis-fibrosis-cirrhosis-cancer sequence.

The detoxification and elimination of consumed alcohol is primarily performed in the liver via a series of oxidative chemical reactions known as oxidative metabolism (Thiele, Klassen, & Tuma, 2008; Tuma & Casey, 2003). This process consists of three major steps: the reversible oxidation of ethanol to acetaldehyde, the non-reversible metabolism of the toxic acetaldehyde to acetate, and finally the breakdown of acetate to water and carbon dioxide. Alcohol dehydrogenase (ADH), the main oxidizing enzyme, with a high affinity for alcohol (Freeman et al., 2005) breaks down ethanol in the cytoplasm of

hepatocytes. A second pathway, CYP2E1 (inducible by chronic alcohol consumption) functions to form acetaldehyde in the peroxisome, and catalase oxidizes ethanol in microsomes (Barry & Williams, 1988; Crabb, 2004). The second step of alcohol metabolism, the breakdown of acetaldehyde, is carried out by mitochondrial aldehyde dehydrogenase (ALDH). However, the metabolism of alcohol (particularly by CYP2E1) results in the generation of reactive oxygen species (ROS). In addition Kupffer cells produce ROS during ethanol metabolism (Arteel, 2003) and in response to lipopolysaccharide (LPS) (Spolarics, 1998). Further, alcohol suppresses mitochondrial function, and together with acetaldehyde toxicity facilitate depletion of radical scavengers i.e. glutathione (GSH). These effects combined, result in increased oxidative stress, the consequences of which are to induce liver injury (Arteel, 2003; Hoek & Pastorino, 2002; Wheeler et al., 2001).

In addition, secondary to alcohol metabolism are the formation of various acetaldehyde-protein and DNA adducts, which impair protein function in the former (reviewed by Niemela, 1999) and can stimulate potent immunological response in the latter (Hoerner, Behrens, Worner, & Lieber, 1986; Niemela, 1999; Niemela et al., 1987) further causing hepatocyte injury. Additionally, acetaldehyde-DNA adducts are implicated in triggering DNA replication errors and mutations in oncogenes or onco-suppressor genes (Seitz & Stickel, 2007) that mediate transformation to cancer.

Tied in with the metabolic effects of alcohol leading to oxidative stress is the suppression of key organelle function. Alcohol directly impairs mitochondrial

function, thereby the ability for fatty acid oxidation and ATP synthesis (Bailey, 2003) and suppression of key mitochondrial metabolism enzymes, including ALDH (Coleman & Cunningham, 1991; Venkatraman et al., 2004). Mitochondria are also inhibited by acetaldehyde during alcohol metabolism, and result in a decrease in the availability of mitochondrial GSH, in addition to being major sources of ROS production (Boveris & Chance, 1973). Moreover, acetaldehyde damages the electron transport chain (ETC) of mitochondria, causing mitochondrial permeability transition (MPT), thereby inducing pro-apoptotic signaling (Kim, He, & Lemasters, 2003). The endoplasmic reticulum (critical for protein maturation and lipid synthesis) is an organelle which is extremely sensitive to oxidative stress and the increased NADPH/NADH ratio that occurs with alcohol. Conversely the ER is also a significant source of ROS, where singlet oxygen is generated by leakage of electrons from the NADPH cytochrome P450 reductase (Cross & Jones, 1991). Changes at trans-membrane level are detected by inositol requiring enzyme (IRE), activating transcription factor 6 (ATF-6) and RNA-activated protein kinase-like ER kinase (PERK) (Ji & Kaplowitz, 2006; Marciniak & Ron, 2006; Schroder & Kaufman, 2005). As a result, in response to endoplasmic reticulum (ER) stress, these trans-membrane sensors activate an unfolded protein response (UPR) comprising an attenuation of protein synthesis, and transcription of degradation chaperones and genes that up-regulate ER-associated degradation (ERAD) and improve protein folding to restore homeostasis. Specifically glucose-regulated protein 78 (GRP78), an intraluminal ER chaperone is displaced resulting in the release and activation of

the trans-membrane receptors; activated IRE-1 α cleaves X-box-binding protein 1 (XBP-1) mRNA, thereby activating it; this causes up-regulation of genes important for ERAD, and a degradation of genes encoding for secretory and membrane proteins (Hollien & Weissman, 2006). ATF-6 translocates to the nucleus to up-regulate chaperones such as GRP78 and others that increase protein folding of the ER (Ji, Chan, & Kaplowitz, 2006; Marciniak & Ron, 2006; Schroder & Kaufman, 2005). Activated PERK on the other hand phosphorylates eukaryotic translation initiation factor 2 α (eIF-2 α). However with prolonged ER stress, apoptotic signals transmitted through IRE-1 α -mediated signaling are activated, and these act through Bax/Bak, JNK, and ATF-4 (which increases expression of C/EBP-homologous protein (CHOP). CHOP is a transcription factor that along with GRP78 is the hallmark of the UPR and stress response (Ji, 2008) and is associated with ER-stress-induced apoptosis.

Another important component of progression of alcohol-related liver injury to steatohepatitis is mediated by cytokine activation. Tumour necrosis factor- α (TNF- α), the prototypical cytokine, along with other inflammatory mediators (e.g. interleukins 1, 6 and 8) is produced by macrophages, in response to LPS-mediated stimulation via Toll-like receptor 4 (TLR-4) and MyD88 complex (Szabo & Bala, 2010). In the same vein, anti-inflammatory cytokines interleukins 4, 10 and 13 are produced, to counter the effects of their pro-inflammatory counterparts. Therefore it is the imbalance in favour of pro-inflammatory cytokines that results in liver injury in the early stages of ALD. Activation of cytokines sets off an inflammatory cascade of events that lead to cell death,

failure of cell regeneration and ultimately progression of disease to cirrhosis, through stellate cell activation (Crews et al., 2006). Interestingly, recent evidence points to the fact that progression of disease to fibrosis/cirrhosis in chronic alcohol consumption occurs as a result of the switch from a pro-inflammatory Th-1 to an anti-inflammatory Th-2 response, via activation of transforming growth factor-beta (TGF- β) (Crews et al., 2006).

The only effective treatment to prevent progression of ALD is complete and sustained abstinence. However, only one third of patients on alcohol rehabilitation programs are able to consistently abstain from alcohol. Therefore therapeutic agents to limit liver injury secondary to ALD are necessary. Currently medications to treat craving, decompensated disease and to improve nutritional status of patients are used to treat ALD. Many of these drugs reduce cravings, frequency of drinking and improve outcomes in the short-term; their long-term effects of survival however are less clear. Therefore a search of newer agents operating on different mechanisms would be welcomed. Hepatic insulin resistance mediated by ceramide-induced lipotoxicity is a key feature in alcoholic steatohepatitis (ASH), as such novel treatments such as ceramide inhibitors may provide a useful way in which ALD can be targeted.

Therefore to sum up, the pathophysiologic process of progression of alcoholic liver disease from “simple” steatosis to steatohepatitis is rooted in the key roles of oxidative stress, cytokine activation and organelle dysfunction; this has been shown in both animal models and human studies. With prolonged chronic alcohol consumption the ensuing metabolic stresses overwhelm the cell's

ability to regenerate, thus leading to cell death, tissue destruction and subsequent tissue/organ failure.

1.6 Hepatocellular carcinoma (HCC)

HCC is the most common form of primary liver cancer. The majority of patients present with advanced disease and thus HCC is generally diagnosed late. Consequently, the prognosis is generally poor (only a few months). More concerning, are the mortality rates from HCC, which are equivalent to incidence rates. Thus despite recent advances in therapies for HCC, the impact is significant. The development and progression of cancer is considered to be a multi-step process, from a premalignant chronic inflammatory to a neoplastic stage. This occurs in a similar fashion to the well-described adenoma-carcinoma sequence of colorectal cancer (CRC). Chronically inflamed or cirrhotic hepatocytes undergo abnormal proliferation, and become dysplastic, particularly noted with HBV and HCV infections (Libbrecht, Craninx, Nevens, Desmet, & Roskams, 2001; Makino et al., 2000) and these lesions are definite risk factors for virus-related HCC (Makino et al., 2000; Terasaki, Kaneko, Kobayashi, Nonomura, & Nakanuma, 1998). A different type of lesion with different pathological features similarly occurs in the background of cirrhosis and represents a pre-malignant condition (Ferrell, Crawford, Dhillon, Scheuer, & Nakanuma, 1993; Roncalli, 2004). This lesion is the dysplastic nodule (DN) and is divided into low or high grade based on the histopathological features. The natural progression of these lesions is controversial, however, with some

researchers showing evidence that the majority (80%) of DNAs disappear or remain stable (Seki et al., 2000) whereas others definitively predicting malignant transformation/hepatocarcinogenesis (Borzio et al., 2003). The next distinctive histological lesion is HCC, which is divided into early and late HCC. By definition early HCC is well-differentiated with an obscure tumour margin, and must be distinguished from a high grade DN. Early HCC carries a good prognosis, regrettably few patients have symptoms and will therefore not usually present at this early stage. Late HCC by contrast carries with it a grave prognosis, and survival of only a few months.

There are several key and interrelated mechanisms that result in cancer formation. These include gene mutations of oncogenes and tumour suppressor genes, loss of heterozygosity (LOH) of chromosome 8p, epigenetic changes, the epithelial mesenchymal transition (EMT) and role of stem cells. A comprehensive review of all these mechanisms is beyond the scope of this work however, in the next section, the key tenets of cancer formation at molecular and cellular level that have relevance to our second aim will be highlighted.

1.7 General mechanisms of cancer (Hanahan & Weinberg, 2000)

The result of any type of cancer is a consequence of six critical acquired alterations in cell regulatory circuits viz: autonomous or self-sustaining growth signals, insensitivity to anti-growth signals, evasion of inherent protective mechanisms i.e. apoptosis, unlimited ability of cells to replicate, sustained angiogenesis (which promotes tumor invasiveness) and ability of cells to invade and replace normal tissue. All these mechanisms have to act in concert and over prolonged periods to induce malignant transformation.

Self-sustaining growth signals

For cells to proliferate normally, they need growth signals to be able to transition from a quiescent to an active state. These signals are transmitted by transmembrane receptors (that bind various messenger molecules) to the interior of the cell. The difference between normal and malignant cells is that the latter are capable of circumventing heterotypic signaling (whereby growth factors made by one type of cell can stimulate the proliferation of another type of cell), in that way obviating their dependence on extrinsic growth factors, thus they become more autonomous in their responses to in-coming signals. Cell surface receptors are also targets of deregulation by cancer cells, usually by overexpression and selection of the most efficient of these at the cell surface, making the cancer cell overly responsive to messages. This modification may be ligand-dependent or independent. Downstream the most important cascade linking received messages and outcome is the SOS-Ras-Raf-MAPK system. While this approach

to carcinogenesis is simplistic i.e. by not taking into account various interconnections between signaling cascades, and their target cells within an organ, it makes for a useful model to understand some of the basic tenets of malignant transformation.

Insensitivity to anti-growth signals

Soluble growth inhibitors exist to maintain homeostasis within normal tissues, and similar to pro-growth signals, these anti-growth inhibitory factors work through ligand-transmembrane-intracellular signaling interactions. These inhibitory signals can be disrupted resulting in tumor cells having a growth advantage. Most of these anti-proliferative signals are co-ordinated with the G1 phase of the cell cycle, and are mediated mainly through the retinoblastoma protein (pRb). Normally pRb blocks proliferation of cells by sequestering and altering the function of E2F transcription factors that control the expression of genes necessary for progression from G1 into the S phase (Hanahan & Weinberg, 2000). Thus disruption of pRB releases E2F thereby allowing proliferation of cells. The best-studied molecule by which this mechanism operates is through transforming growth factor beta (TGF- β), which can be disrupted by losing its responsiveness or that of its downstream targets, as a result of down-regulation, mutations, and sequestration by viral oncoproteins (i.e. E7) to name but a few. Thus its regulation on pRb is lost in tumours, allowing the malignant cells to proliferate. In addition, malignant cells acquire an ability to avoid being driven into the post-mitotic state (which forces the cells to become

differentiated), but to promote growth by causing over-expression of the c-Myc oncoproteins. Essentially many effectors are operational that have not as yet been elucidated, and in combination with the cell cycle machinery facilitate avoidance of transmission of these anti-proliferative signals.

Evasion of apoptosis

Apoptosis or programmed cell death is the cell's inherent protective mechanism whereby abnormal cells can be lost, allowing for the proliferation of new normal cells as replacement. Therefore cell proliferation must be balanced by cell loss to maintain homeostasis and well-functioning systems. Currently there is ample evidence from animal and human studies that the ability to resist apoptosis is perhaps the sine qua non of all types of cancer. The apoptotic machinery includes cell surface receptors that bind either survival (e.g. IGF-1 and IGF-2 through IGF-1R) or death factors (e.g. Fas ligand binding Fas receptor and TNF- α and its receptor). These apoptotic signals work through mitochondria, which release cytochrome C, a potent catalyst of apoptosis. Members of the Bcl-2 family of proteins have either pro-apoptotic (Bax, Bak, Bid, Bim) or anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W) properties, which govern mitochondrial signaling through cytochrome release. Therefore, for instance p53, a prototypical tumor suppressor promotes apoptosis by up-regulating the expression of Bax in response to sensing DNA damage; Bax in turn stimulates the release of cytochrome C. Every signal requires an effector; in the case of apoptosis proteases termed caspases in particular caspases 8 and 9 are activated by death receptors such

as Fas or cytochrome release by mitochondria respectively. Downstream these function with other effector caspases to effect apoptosis of subcellular structures, organelles and genomes (Hanahan & Weinberg, 2000). Apoptosis-evading strategies include loss of pro-apoptotic regulators by way of mutations; a good example of this is the p53 tumor suppressor gene. This results in inactivation of the p53 protein, and this is observed in more than fifty percent of all human cancers. Other strategies include the inactivation of anti-apoptotic survival mechanisms, such as those mediated by the PI3 kinase-AKT/PKB pathway, by inhibition of IGF-1/2 signaling, or loss of PTEN tumor suppressor function.

Unlimited ability of cells to replicate

While interruption of cell signaling is important to cause cancer, there is sufficient data to show that this alone is not sufficient to cause cancer. Inherently cells have mechanisms to prevent unregulated growth i.e. cells have finite replicative properties; therefore in order to cause tumors, this ability must also be impaired. This property is called senescence, which can be disabled by a mutation or inactivation of for example a tumor suppressor gene. This would result in the cell multiplying until it reaches a crisis state, characterized by massive cell death, karyotypic disarray, and even the emergence of a variant with an acquired ability to multiply indefinitely. This function is termed immortalization, and represents a key ability of malignant cells. However even these cells' replicative ability is undermined by apoptosis, accounting for a lot of attrition. This occurs because of the inability of DNA polymerase to completely replicate the 3' ends of

chromosomal DNA during the S phase. Thus the unprotected chromosomal ends can cause chromosomal fusions, yielding the karyotypic disarray associated with the crisis state. This too however, can be prevented by telomerase maintenance (seen in up to ninety percent of tumors). This occurs by up-regulation of the expression of the telomerase enzyme, which adds six base pair repeats to the ends of telomeric DNA, thus preventing cell loss. This evidence was gained from cell culture/line studies, or transgenic mice lacking telomerase function.

Sustained angiogenesis

To function and proliferate normally, cells require nutrients supplied by blood vessels; as a result many cells reside very close to capillaries. Once the tissue is formed, new capillaries are formed (termed angiogenesis), however this is a closely regulated process. As a result incipient malignant cells have to acquire angiogenic ability in order to survive. Pro- or anti-angiogenic signals are transmitted by soluble factors and their receptors on the surface of endothelial cells. The prototypical growth factors are vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) 1 and 2. There are angiogenic growth inhibitors as well i.e. thrombospondin-1. Other important components of angiogenic signaling include the role of integrins and adhesion molecules that mediate cell-matrix and cell-to-cell interactions respectively. Inactive cells express one type of integrin, whereas malignant cells express a different type. Evidence suggests that neovascularization is a pre-requisite of clonal expansion as abnormalities in angiogenesis can be appreciated in the pre-malignant

phases, and this was shown in pre-malignant lesions of human cervical, breast and skin cancer. Tumors achieve disruption of signaling by altering the balance of pro- and anti-angiogenic factors, commonly done by altering gene expression. Therefore for instance many tumors evidence increased expression of VEGF and/or FGFs compared to their normal counterparts, by incompletely understood mechanisms. However this can occur by inhibition of p53 activity, or proteases, which can regulate the bioavailability of angiogenic activators or inhibitors, by either sequestering or making available these factors.

Tissue invasion and metastases

The last requirement for tumor cells to exert their dominance over normal cells is the ability to not only spread from the primary site, but to invade normal tissue at a secondary site. Metastases are the cause of ninety percent of tumor-related deaths in humans. Similar to the formation of the original tumor, for metastases to occur the same five acquired abilities are necessary. However the mechanisms by which this occurs are complex and not well known. Certain proteins that are involved in 'adhering' cells to their surroundings in tissue are altered in metastatic/invasive cells. These proteins include the cell-to-cell adhesion molecules (CAMs) i.e. cadherins and integrins, which mediate cell to extracellular matrix substrates. The most widely noted alteration to this signaling in cancer involves E-cadherin expressed on epithelial cells. Normally E-cadherin results in the transmission of antigrowth signals through the interaction with β -catenin cytoplasmic contacts with intracellular signaling circuits that include the

LEF/TCF transcription factor. Thus, loss of function of E-cadherin results in the majority of epithelial cell cancers by mechanisms that include mutational inactivation of E-cadherin or β -catenin genes, transcriptional repression or proteolysis of the extracellular cadherin domain. Forced expression in cultured cells and transgenic mice impairs and interference with E-cadherin function in both enhances tumor invasiveness and metastatic potential. Shifts in the spectrum of integrin α or β subunits in tumor cells are also a key feature of the tumor cells' ability to successfully colonize secondary tissues. For example cancerous cells facilitate invasion by shifting their expression of integrins from those that favour the ECM present in normal epithelium to the integrins that preferentially bind degraded stromal components produced by extracellular proteases. These proteases are also necessary in this acquired ability of cancer cells to metastasize. Protease genes are either upregulated, or protease inhibitors down-regulated or inactive zymogen forms of proteases are converted into active enzymes. The mechanism by which this occurs is thought to be through the docking of active proteases on the cell surface, which can facilitate invasion of cancer cells into nearby stroma, across blood vessel walls and through normal epithelia cell layers. In summary, the activation of extracellular proteases and the altered binding specificities of cadherins, CAMs and integrins are central to the ability of cancer cells to invade tissue and metastasize.

1.8 The molecular basis of HCC: signaling impairments

As has already been stated in preceding sections, pathogenic mechanisms of HCC include cirrhosis and mutations of oncogenes and/or tumour suppressor genes. These mechanisms are linked with alterations in key cellular signaling pathways, including the insulin/IGF, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), Wnt/ β -catenin, transforming growth factor- α /epidermal growth factor, RAF/MEK/ERK pathways (Whittaker, Marais, & Zhu, 2010) to mention but a few. Additionally, the key tenets of the development of cancer involve cell-to-cell interactions mediated by signaling mechanisms. For our purposes we will limit the ensuing discussion to the three pathways of interest that are frequently dysregulated in HCC, i.e. the insulin/IGF, Wnt/ β -catenin pathways and the Notch pathway, the role of which is not yet clearly delineated.

1.8.1 Insulin/Insulin growth factor (IGF) signaling

Normal signaling: This pathway has been conclusively shown to be involved in the growth and survival of multiple solid tumours (Cheng, Lindsley, Cheng, Yang, & Nicosia, 2005). Activation of this cascade requires the binding of insulin, IGF-1 or 2 to the transmembrane insulin receptor, causing a conformational change in the intracellular portion of the receptor. This results in autophosphorylation of a number of tyrosine kinases present on the receptor of the β subunit. So-called adaptor or docking proteins i.e. the insulin receptor substrate proteins IRS-1, 2 and 4 recognize residues on tyrosine kinases and as a result of receptor

activation, these IRS proteins are recognized by a lipid kinase, the p85 regulatory subunit of phospho-inositol-3 kinase (PI3-K), and PI3-K phosphorylates phosphatidylinositol 3,4 biphosphate to 3, 4, 5 triphosphate. Akt is a key downstream effector of phosphatidylinositol 3,4,5 triphosphate, which recruits cytosolic enzymes to the cell membrane. Active Akt enters the cytoplasm where it leads to phosphorylation and subsequent inactivation of glycogen synthase kinase-3 (GSK-3). Inhibition of GSK-3 inactivates inhibition of glycogen synthesis, and thereby promotes glycogen storage. In addition to carbohydrate metabolism through the action of Akt insulin signaling regulates both protein and lipid by promoting their anabolic properties, by increasing the uptake of fatty lipids and amino acids, whilst simultaneously preventing their catabolism.

Impaired signaling in HCC: In HCC the insulin/IGF pathway is activated via overexpression of IGF-2 acting through the IGF-1R (Breuhahn et al., 2004; Desbois-Mouthon et al., 2009; Nussbaum et al., 2008; Sohda, Yun, Iwata, Soejima, & Okumura, 1996; Tovar et al., 2010) and these changes are associated with proliferation of tumours and/or metastases. In fact activation of IGF-2 may be the mechanism by which aflatoxin B1 exerts its pro-tumourigenic properties in HCC cell lines (Ubagai, Kikuchi, Fukusato, & Ono, 2010). Even in an in vivo system of IRS-1 overexpression, in association with another insult, i.e. alcohol exposure, there is up-regulation of IGF-2 similar to that seen in HCC (Longato, de la Monte, Califano, & Wands, 2008). Conversely, selective blockade of IGF-1R signaling results in a dose-dependent inhibition of proliferation and

enhanced apoptosis in HCC cells (Yao, Liu, Sheng, & Huang, 2011), decreased cell viability and proliferation in human HCC (Tovar et al., 2010) and reduced tumour cell motility and migration (Nussbaum et al., 2008). This effect has been interrogated in a study where salirasib, a ras/mTOR inhibitor, caused inhibition of HCC cell lines in vitro, and tumour growth in vivo (Charette et al., 2010), and with blockade of IGF1-receptor tyrosine kinase, with resultant antineoplastic effects (Hopfner et al., 2006). Impairments in HCC are also observed in the response of HCV to treatment. For instance, chemoresistance mechanisms are activated via the insulin signaling (Chen et al., 2011; Desbois-Mouthon et al., 2009). Thus signaling via IGF-2 and IGF1-R appears to be critical in HCC and therefore inhibition of IGF-1R can be potentially useful for the treatment of HCC.

1.8.2 Wnt/ β -catenin signaling

Normal signaling: The Wnt/ β -catenin pathway has important roles in motility, proliferation, morphology and embryogenesis, but in the adult or cancerous state, either up-regulates or represses genes important for cancer growth (Cadigan & Nusse, 1997; Korinek et al., 1998; Miller, 2002; Polakis, 2000). In the inactivated state, β -catenin is phosphorylated by GSK-3 β and thus targeted for degradation by the APC complex consisting of adenomatosis polyposis coli (APC), glycogen synthase (GSK) 3- β , and Axin. Contrastingly, when activated, a Wnt ligand binds to the receptor, which binds to the frizzled (FZD)/ low-density lipoprotein receptor related protein (LRP) complex (Bhanot et al., 1996; Tekmal & Keshava, 1997; Wodarz & Nusse, 1998), activating disheveled (DSH) (Willert, Brink, Wodarz,

Varmus, & Nusse, 1997). DSH in turn phosphorylates the APC complex, which becomes deactivated, resulting in intracellular accumulation of β -catenin, which translocates to the nucleus (Behrens et al., 1996) to bind to T-cell factor (TCF)/Lymphoid enhancing factor (LEF) family of DNA binding proteins leading to activation of transcription of Wnt target genes i.e. c-myc, cyclin D1 (Behrens et al., 1996; He et al., 1998; Tetsu & McCormick, 1999) and WISP-1. Therefore when activated, the transcription of oncogenic growth factors is enhanced. In fact, activation/deregulation of this pathway has been observed in cancer including HCC.

Impaired signaling in HCC: The Wnt/ β -catenin pathway is a major pathway that is dysregulated in HCC resulting in increased proliferation and differentiation of tissue. Dysregulation occurs in about one third of HCCs (Takigawa & Brown, 2008), and two thirds of HCC have increased levels of β -catenin (Wong, Fan, & Ng, 2001). The general mechanisms of Wnt activation in HCC include mutations of the APC complex (small proportion of HCC) or Axin1, promoter epigenetic changes that cause APC inactivation or dysregulation by overexpression of various ligands, receptors or polypeptides of Wnt signaling (Wong & Ng, 2008), Activation of Wnt signaling was observed even in Farnesoid X receptor knock-out mice (Wolfe et al., 2011), which spontaneously normally develop HCC, suggesting a strong independent risk of Wnt signaling activation. In HBV infection one mechanism for Wnt activation is via displacement of Wnt from the APC complex, thus causing its accumulation and activated transcription of target

genes (Hsieh, Kim, Lim, Yu, & Jung, 2011), or via the transactivating protein HBx, which acts via Wnt 1 to suppress GSK-3 β (Cha, Kim, Park, & Ryu, 2004). However, activation of Wnt occurs more readily in HCV-related HCC than in HBV-related HCC (Hsu et al., 2000; Huang et al., 1999; Thorgeirsson & Grisham, 2002). Ligand-receptor interactions were evaluated in a study, which showed that Wnt 3 and Frizzled 7 interacted to result in increased β -catenin nuclear transcriptional activity and that of its downstream target genes (Kim et al., 2008). And similar to the “two-hit” hypothesis of ALD and NAFLD, in a study where both IRS-1 and Hepatitis B x proteins were overexpressed in a double transgenic mouse model, dysplasia and HCC occurred with a higher frequency than in the single transgenic mice (Longato et al., 2009). In this study, there were also high levels of activation of Wnt 3 and Frizzled 7 in the double transgenics. Furthermore, similar to insulin/IGF signaling, activation of Wnt is associated with chemoresistance to combination therapy for HCC (Noda et al., 2009), thus showing evidence that it is a multi-functional oncoprotein that acts on different mechanisms not only to promote but to worsen the outcome of treated HCC.

1.8.3 Notch signaling in HCC

Normal signaling: Notch genes were discovered more than 30 years following the discovery of mutant *Drosophila* with a notched wing phenotype. Subsequently it was discovered that this gene is highly conserved in various species, including *C elegans* and indeed humans. Notch signaling consists of cell membrane receptors, Delta and Jagged (in humans), ligands, an intracellular

Notch domain and key downstream effectors. The main function of Notch signaling is the regulation of cell fate determination and differentiation that determines organ phenotype. Signaling through Notch is dependent on both canonical and non-canonical mechanisms, whereby in the former, two adjacent cells communicate with each other and with neighbouring or similar cells. The direction of the communication is dependent on which of two adjacent cells possesses the receptor. Under normal conditions one of the Notch ligands (Jagged-1, Jagged-2, DII-1, DII-3 and DII-4) which are cell surface proteins, and include numerous EGF-like repeats within their extracellular domains, bind to the transmembrane receptors (Notch1, 2, 3 and 4), thereby causing proteolytic cleavage of the receptors by a gamma secretase and metalloproteinase complex. This results in the liberation and dissociation of the Notch intracellular domain (NICD) from the cell membrane (F. Wang et al., 2010). NICD translocates to the nucleus to activate transcription factors of the Notch target genes including helix-loop-helix transcription factors of the hairy and enhancer of split (Hes) family and the Hes-related repressor protein (Hey) family (Artavanis-Tsakonas, Matsuno, & Fortini, 1995; Bray, 2006). The end-result of this activation is pro-survival and proliferative signals; this was evident in a partial hepatectomy rat model where activation of the NICD was associated with an increase in hepatocyte DNA synthesis (Kohler et al., 2004).

Impaired signaling in cancer: Dysregulated Notch signaling is implicated in a variety of both epithelial and solid tumours. In general, impaired signaling

functions as an oncogene in a variety of human cancers such as T-cell leukaemia, pancreatic, prostate and breast cancer (Wang et al., 2010). However, the role of abnormal Notch signaling in HCC has not been extensively studied, may be either up or downregulated in promoting HCC and therefore deserves further attention. Up or downregulation of Notch, particularly Notch-1 is context and cell-type specific. Whereas expression of Notch-1 was suppressed in HCC 66% compared to adjacent peri-tumour and normal tissue (Wang et al., 2009) and by up-regulating apoptosis by sensitizing TRAIL in HCC cells by inhibiting p53-mediated degradation (Wang et al., 2009), thus turning down oncogenic potential, some studies have shown upregulation of Notch1 and HES-1 in HCC compared to adjacent non-tumour liver (Cantarini et al., 2006; Gao et al., 2008; Qi et al., 2003). Similarly studies have yielded divergent results relating to the role of Notch 3 and Notch 4. In one study that evaluated the expression of Notch receptors (3 and 4) and their downstream targets (Hes-1 and Hes-6), while Notch receptors were abnormally expressed in HCC tissue 78% and 68% respectively, Notch-3 mRNA was upregulated in 95% of HCC and Notch-4 mRNA down-regulated in 80% HCC. HES-6 mRNA expression was higher in HCC than cirrhotic tissue, corresponding to increased Notch 3 upregulation (Gramantieri et al., 2007). In a contrasting study, which evaluated the role of cytoplasmic and nuclear components of Notch receptors, while nuclear Notch 4 was upregulated ($p < 0.05$) in HCC compared to peri-tumour tissue, cytoplasmic Notch 3 and 4 were similar in both types of tissue. To summarize, the role of Notch signaling in carcinogenesis appears to be dependent on several factors:

cell and tissue type, molecular context, the cellular localization of Notch receptor i.e. cytoplasmic or nuclear and acts as both as an oncoprotein and tumour suppressor. Thus its role in hepatocarcinogenesis deserves further study, as it may uncover mechanisms that could prove useful in detecting and/or treating HCC.

1.9 Cross-talk of signaling cascades

Cell-to-cell and cell-to-matrix communications are critical to how cells of different tissues function as a unit; therefore the role of signaling cascades is important in this context. It is therefore intuitive that different cascades would act in concert to either nullify or amplify a desired effect. Several lines of evidence show that some signaling cascades do in fact intersect at various points to have an effect on outcome. This is particularly evident in haematologic contexts, and the selection of stem and progenitor cells to more differentiated cell lines. For instance haemopoetic differentiation involves integrated, balanced and integrated Wnt and Notch signalling (Zhou et al., 2010). In HCC as stated previously several signalling cascades are dysregulated, and the majority of papers have evaluated each of these in isolation, with only a few exceptions where two pathways were simultaneously studied (Cantarini et al., 2006; M. Wang et al., 2009; Zhou et al., 2010). Although the insulin/IGF, Wnt/ β -catenin and Notch signalling pathways are implicated in hepatocarcinogenesis individually, there are converging points in their pathways where they could potentially interact. Furthermore, aspartyl- (asparaginy)- β -hydroxylase (AAH) is an enzyme that is involved in cell growth,

differentiation and migration (Lasky & Wu, 2005; Yoon & Gaiano, 2005) and is overexpressed in many cancers (Yang et al., 2010), including HCC and is associated with adverse outcomes (Wang et al., 2010) AAH is linked to both Notch and insulin signaling by hydroxylating and enhancing the expression of Notch and Jagged, but is itself stimulated by insulin/IGF via the ERK/MAPK and PI-3K/Akt pathways (Cantarini et al., 2006; de la Monte et al., 2006; Lahousse, Carter, Xu, Wands, & de la Monte, 2006). In a paper by Cantarini MC et al, overexpression of aspartyl-(asparagyl)-beta-hydroxylase (AAH), a cell membrane enzyme resulted in an increase in the mRNA transcripts of IGF-1 and IGF-2, as well as Notch-1 and HES-1 and an interaction between AAH and Notch and Jagged (Cantarini et al., 2006), suggesting that there is a complex interaction between AAH/Notch and insulin signaling cascades. As reviewed above, insulin signaling is transmitted downstream by the effector kinase PI-3K, which phosphorylates and thereby inactivates GSK-3 β , to result in glycogen storage. More interestingly, inhibition of GSK-3 β increased AAH protein probably by inhibiting the phosphorylation of AAH (Carter et al., 2008). Additionally, when activated, Wnt signaling phosphorylates and thereby de-activates GSK-3 β thus avoiding ubiquitination and accumulation of β -catenin. It is possible that deactivation of GSK-3 β by Wnt could also activate AAH, in this way connecting the Wnt and AAH/Notch pathways.

In a paper by Cantarini (2006), overexpression of aspartyl-(asparagyl)-beta-hydroxylase (AAH), a cell membrane enzyme resulted in an increase in the mRNA transcripts of IGF-1 and IGF-2, as well as Notch-1 and HES-1 and an

interaction between AAH and Notch and Jagged (Cantarini et al., 2006), suggesting that there is bi-directional regulation between AAH and insulin. Therefore we hypothesize that the interaction points linking this triad of signaling pathways, is via AAH and GSK-3 β . Further lines of evidence for cross-talk and interactions in signaling include the HBx protein, which is known to activate several signaling pathways including Ras-Raf-MAPK, SAPK/JNK, PI3 K-Akt and Janus kinase/STAT pathways (Doria, Klein, Lucito, & Schneider, 1995; Lee & Yun, 1998; Natoli et al., 1994). Therefore conceivably, HBx, a promiscuous transactivating protein, may also interact with the insulin/IGF cascade via PI3 K-Akt. While this hypothesis is attractive, there is a study showing that in HBV-related liver disease, down-regulation of Notch was associated with up-regulation of β -catenin ($r = -0.125$, $p = 0.023$) showing an inverse relationship (M. Wang et al., 2009). Therefore the interactive role of these three pathways is still unclear and needs further elucidation.

In summary, the progression of CLD of any aetiology through the stages of chronic hepatitis, cirrhosis and subsequent transformation to HCC which occurs in a step-wise stage is critical to our understanding of defining which patients are at risk of progression, the molecular factors underlying this progression and the identification of early HCC. This can be achieved by examining dysfunctional signaling pathways especially at points of convergence such that multiple cascades can be potentially targeted simultaneously utilizing a single inhibitor directed against a key target molecule of the pathway.

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CHAPTER 2

Limited Therapeutic Effect of N-Acetylcysteine on Hepatic Insulin Resistance in an Experimental In Vivo Model of Alcohol-Induced Steatohepatitis

ABSTRACT

Background: Alcohol-related steatohepatitis is associated with increased oxidative stress, DNA damage, lipotoxicity, and insulin resistance in liver.

Hypothesis: Since inflammation and oxidative stress can promote insulin resistance, effective treatment with anti-oxidants, e.g. N-acetylcysteine (NAC), may restore ethanol-impaired insulin signaling in the liver.

Methods: Adult male Sprague-Dawley rats were fed for 130 days with liquid diets containing 0% or 37% ethanol by caloric content, and simultaneously treated with vehicle or NAC. Chow-fed controls were studied in parallel. Liver tissues were used for histopathology, cytokine activation, and insulin/IGF-1 signaling assays.

Results: There was a significant positive trend of increasing severity of steatohepatitis ($P=0.016$) with accumulation of neutral lipid ($P=0.0002$) and triglycerides ($P=0.0004$) from chow to control, to the ethanol diet, irrespective of NAC treatment. In ethanol-fed rats, NAC reduced inflammation, converted the steatosis from a predominantly micro-vesicular to a mainly macro-vesicular histological pattern, reduced pro-inflammatory cytokine gene expression, ceramide

load and acid sphingomyelinase activity, and increased expression of IGF-1 and IGF-2 receptors and IGF-2 in liver. However, it did not abrogate ethanol-mediated impairments in signaling through insulin/IGF-1 receptors, IRS-1, Akt, GSK-3 β , or p70S6K, nor did it significantly reduce pro-ceramide or GM3 ganglioside gene expression in liver.

Conclusions: Anti-oxidant treatments reduce the severity of chronic alcohol-related steatohepatitis, possibly due to decreased expression of inflammatory mediators and ceramide accumulation, but they do not restore insulin/IGF-1 signaling in liver. Effective treatment of alcohol-related steatohepatitis most likely requires dual targeting of oxidative stress and insulin/IGF resistance.

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2.1 INTRODUCTION

Acute alcohol exposure causes simple hepatic steatosis, but with continued alcohol abuse, this relatively benign state of lipid (mainly triglyceride) accumulation can progress through stages of steatohepatitis, followed by fibrosis, cirrhosis, and finally liver failure (de Alwis & Day, 2008). In addition, chronic alcohol abuse can lead to hepatocellular carcinoma (HCC). Progression of alcohol-related liver disease (ALD) is partly mediated by impaired lipid metabolism and homeostasis, which lead to lipotoxicity, oxidative stress, pro-inflammatory cytokine activation, and insulin resistance (Carter-Kent, Zein, & Feldstein, 2008; Farrell & Larter, 2006). Lipotoxic states themselves activate pro-apoptotic, anti-growth, and anti-survival pathways, and they compromise reparative and regenerative mechanisms in the liver (Day, 2006; Day & James, 1998).

Growing evidence suggests that ceramides are key mediators of lipotoxicity with attendant increased inflammation, oxidative stress, and insulin resistance in both ALD and non-alcoholic fatty liver disease (Chavez et al., 2003; Fernandez-Checa, Colell, Mari, & Garcia-Ruiz, 2005; Holland et al., 2007; Pickersgill, Litherland, Greenberg, Walker, & Yeaman, 2007; Schutze et al., 1992). Ceramides constitute a family of lipids generated from fatty acid and sphingosine (Liu, Obeid, & Hannun, 1997; Reynolds, Maurer, & Kolesnick, 2004; Summers, 2006; Tilg & Hotamisligil, 2006). Ceramides are distributed in cell membranes, and in addition to their structural functions, regulate signaling pathways that mediate growth, proliferation, motility, adhesion, differentiation, senescence, and apoptosis. Ceramides are generated biosynthetically through the actions of ceramide synthase and serine palmitoyltransferase (Hannun, Luberto, & Argraves, 2001; Hauck et al., 2000; Laviad et al., 2008; Mizutani, Kihara, & Igarashi, 2005; Shah et al., 2008), or they can be formed catabolically by neutral or acidic

sphingomyelinase activity (Holland et al., 2007; Reynolds et al., 2004; Shah et al., 2008). Sphingomyelinases are activated by pro-inflammatory cytokines, e.g. TNF- α (Carter-Kent et al., 2008; Summers, 2006), and pro-apoptotic stimuli including ionizing radiation, Fas, and trophic factor withdrawal (Hannun & Obeid, 2002; Liu et al., 1997; Marchesini & Hannun, 2004; Reynolds et al., 2004). Ceramides impair cellular functions and cause apoptosis by: 1) modulating the phosphorylation states of various proteins, including those that regulate insulin signaling (Silveira et al., 2008); 2) activating enzymes such as interleukin-1 β converting enzyme (ICE)-like proteases, which promote apoptosis (Smyth et al., 1996); and 3) inhibiting Akt phosphorylation and kinase activity (Chalfant et al., 1999; Summers, Garza, Zhou, & Birnbaum, 1998; Zhou, Summers, Birnbaum, & Pittman, 1998) through activation of protein phosphatase 2A (Chalfant et al., 1999; Dobrowsky, Kamibayashi, Mumby, & Hannun, 1993).

Free fatty acid accumulation in hepatocytes stimulates lipogenesis, and factors related to increased stress promote lipotoxicity, which leads to oxidative stress and cell death (Marra, Gastaldelli, Svegliati Baroni, Tell, & Tiribelli, 2008). Oxidative stress itself plays a key role in the progression of simple steatosis to steatohepatitis, and the eventual activation pro-fibrogenic cascades (Albano, 2008; Dowman, Tomlinson, & Newsome, 2010). Susceptibility to oxidative and radical injury rests on maintaining a balance between pro-oxidant and anti-oxidant factors. Glutathione (GSH) is a radical scavenger and key attenuator of oxidative stress in liver, and hepatic glutathione levels are reduced in both ALD and non-alcoholic fatty liver disease (Das, Balakrishnan, Mukherjee, & Vasudevan, 2008; Videla et al., 2004). N-acetylcysteine (NAC), one of the anti-oxidants used to manage acute liver failure caused by acetaminophen poisoning (Marzullo, 2005), acts by donating cysteine residues for GSH synthesis (Aruoma, Halliwell, Hoey, & Butler, 1989;

Atkuri, Mantovani, & Herzenberg, 2007). Therefore, NAC therapy may reduce liver injury and progression from simple steatosis to steatohepatitis in ALD. Reduced inflammation and severity of steatohepatitis effectuated by NAC or other anti-inflammatory agents could lead to decreased generation of toxic lipids, including ceramides, and consequently improve hepatic insulin sensitivity. The present study utilizes a rat model of chronic ethanol feeding to evaluate the potential use of NAC for reducing the severity of alcohol-related steatohepatitis, lipotoxicity, pro-inflammatory cytokine activation, and insulin resistance in the liver.

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2.2 MATERIALS AND METHODS

Materials: The bicinchoninic acid (BCA) kit to measure protein concentration was purchased from Pierce Chemical Co (Rockford, IL). Histochoice fixative was purchased from Amresco, Inc (Solon, OH). Nile Red fluorescent dye, the Amplex Red Cholesterol Assay Kit, Amplex UltraRed soluble fluorophore, and the Akt Pathway Total and Phospho 7-Plex panels were purchased from Invitrogen (Carlsbad, CA). Triglyceride Reagent Kit was purchased from Analox Instruments Ltd (London, UK). The rat cytokine Milliplex xMAP ELISA kit was purchased from Millipore (Billerica, MA). Maxisorp 96-well plates used for enzyme-linked immunosorbant assays (ELISAs) were from Nunc (Thermo Fisher Scientific; Rochester, NY). Superblock-TBS, horseradish peroxidase conjugated antibodies, and SuperSignal Enhanced Chemiluminescence Reagent was from Pierce Chemical Co (Rockford, IL). Monoclonal anti-ceramide and synthetic oligonucleotides used in quantitative polymerase chain reaction (qPCR) assays were purchased from Sigma-Aldrich Co (St. Louis, MO). All other antibodies and immunodetection reagents were purchased from Abcam (Cambridge, MA), Upstate (Billerica, MA), Vector Laboratories (Burlingame, CA), Invitrogen (Carlsbad, CA) or Chemicon (Temecula, CA). Fine chemicals were purchased from CalBiochem (Carlsbad, CA), or Sigma-Aldrich (St Louis, Mo). QIAzol Lysis Reagent for RNA extraction and QuantiTect SYBR Green PCR Mix were obtained from Qiagen, Inc (Valencia, CA). The AMV 1st Strand cDNA Synthesis Kit was purchased from Roche Applied Science (Indianapolis, IN).

Experimental Model: We utilized the Tsukamoto and French intra-gastric feeding model of total enteral nutrition (TEN) to administer the control or ethanol-containing liquid diets (Baumgardner, Shankar, Hennings, Badger, & Ronis, 2008; Ronis et al., 2005; Ronis, Korourian, Blackburn, Badeaux, & Badger, 2010). With this

model, the entire range of pathological changes, including steatosis, steatohepatitis, and fibrosis, can be produced in rodents (Nanji & French, 2003). In brief, adult male Sprague Dawley rats were fitted with intra-gastric cannulae to infuse isocaloric TEN liquid diets (187 kcal/kg/day) containing 0% (N=18) or 37% (N=14) ethanol (caloric content) (Table 1). Ethanol was substituted for carbohydrates (12 g/kg/day). In subsets of control (N=9) and ethanol (N=7) fed rats, NAC (1.2 g/kg/day) was added to the diets. Chow-fed controls (*ad libitum*) were studied in parallel (N=7). Diets were adjusted weekly to maintain similar weights among the experimental groups, and after 130 days of feeding, rats were sacrificed and livers were harvested for study (Ronis et al., 2010). Liver tissue was immersion fixed in Histochoice and embedded in paraffin for histopathological studies. Fresh snap frozen tissue was used for biochemical and molecular analyses. Our experimental protocol was approved by the 'Institutional Animal Care and Use Committee' of the University of Arkansas for Medical Sciences. The protocol was compliant with NIH guidelines provided in the *Guide for the Care and Use of Laboratory Animals*.

Table 1: Composition of diets

Diet	Name	Composition
1	Chow	Chow ad libitum
2	Control TEN	Diet containing 41% carbohydrates and 43% fat
3	Control TEN+	Diet containing 41% carbohydrates, 43% fat and NAC (1.2 g/kg/day)
4	EtOH TEN	Diet containing 36% carbohydrates from ethanol and 43% fat
5	EtOH TEN+NAC	Diet containing 36% carbohydrates from ethanol, 43% fat and NAC (1.2 g/kg/day)

*Abbreviations: TEN, Total Enteral Nutrition; NAC, N-Acetylcysteine; EtOH, Ethanol

Histopathology: Histological sections (5 µm thick) were stained with Haematoxylin and Eosin (H&E) and scored under code by a pathologist using a semi-quantitative scale (1-4) to assess severity and extent of macro- and micro-steatosis, inflammation, and necrosis (including apoptotic bodies) (Table 2). The scoring system was graded as follows: 1=no significant abnormalities, i.e. expected cord-like architecture, minimal or absent inflammation, necrosis, apoptosis or anisocoria; 2=mild pathological changes, i.e. micro- and macrosteatosis affecting less than 25% hepatocytes, slight derangement of the cord-like architecture, rare scattered foci of inflammation near portal areas; and absent necrosis/apoptosis; 3=moderate steatohepatitis with 25-50% micro- or macrosteatosis, moderate disruption of the cord-like architecture, inflammation extending into lobules, and conspicuous foci of apoptosis; and 4=severe steatohepatitis with micro- and macro-steatosis involving greater than 50% of parenchyma, marked architectural

distortion, and severe inflammation with evidence of apoptotic bodies and foci of necrosis.

Table 2: Liver histopathology scoring system

Grade	Classification	Features
1	Normal	Normal liver cords, absent or minimal steatosis, inflammation, necrosis or apoptosis.
2	Mild	Slight derangement of the cord-like architecture, micro- or macrosteatosis affecting < than 25% hepatocytes, rare scattered foci of inflammation near portal areas and absent necrosis/apoptosis.
3	Moderate	Moderate disruption of the cord-like architecture, 25-50% hepatocytes with micro- or macrosteatosis, inflammation extending into lobules, and conspicuous foci of apoptosis.
4	Severe	Marked architectural distortion, micro- and/or macrosteatosis involving > than 50% of parenchyma, severe inflammation with evidence of apoptotic bodies and foci of necrosis.

*Adapted from Brunt (1999). This semi-quantitative grading scheme was used to evaluate liver histopathology in Sprague-Dawley adult male rats that were subjected to chronic feeding with control or ethanol-containing total enteral nutrition (TEN) diets and concomitant treatment with vehicle or NAC. Chow fed controls were simultaneously studied. The liver histopathology was reviewed under code by a pathologist.

Lipid studies: Lipids were extracted from fresh frozen liver with 2:1 chloroform-methanol (Lyn-Cook et al., 2009). Lipid content was measured using the Nile red fluorescence-based microplate assay as described (Lyn-Cook et al., 2009). Cholesterol was measured with the Cholesterol Amplex Red kit, and triglycerides were measured using the Analox Instruments GM7 Analyzer. Results were normalized to sample weight. Ceramide immunoreactivity was measured in lipid extracts using a direct-binding ELISA (Brade, Vielhaber, Heinz, & Brade, 2000). In brief, duplicate 50 μ l samples were dried onto the bottom surfaces of 96-well Polysorb plates. Samples were blocked for 1 hour at room temperature with Superblock-TBS. The samples were incubated with mouse monoclonal anti-ceramide (2.0 μ g/ml) overnight at 4°C with gentle platform agitation. Immunoreactivity was detected with horseradish peroxidase-conjugated anti-mouse IgG (1:10000) and enhanced chemiluminescence substrate. Luminescence was quantified in a Topcount machine. Between steps, the wells were washed 3 times with PBS (Brade et al., 2000). C6 synthetic ceramide (500 ng) served as a positive control. Negative controls included reactions in which the primary or secondary antibody was omitted.

Multiplex ELISA: We used bead-based multiplex ELISAs to examine the integrity of insulin and IGF-1 signaling networks, or measure immunoreactivity to selected pro-inflammatory cytokines and chemokines in liver tissue. With regard to insulin/IGF-1 signaling, we measured immunoreactivity to the insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, glycogen synthase kinase 3 β (GSK-3 β), p70S6 kinase (p70S6K), and proline-rich Akt substrate 40 (PRAS40), and their phosphorylated counterparts ^{pYpY1162/1163}-IR, ^{pYpY1135/1136}-IGF-1R, ^{pS312}-IRS-1, ^{pS473}-Akt, ^{pS9}-GSK3 β , ^{pTpS421/424}-p70S6K, and ^{pT246}-PRAS40 according to the

manufacturer's protocol. Regarding pro-inflammatory cytokine and chemokine activation, we measured immunoreactivity to interleukin (IL) 1 β , IL-2, IL-4, IL-6, TNF- α , GM-CSF, GRO/KC, and VEGF. Liver tissues were homogenized in buffer containing 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM Na₄P₂O₇, 2 mM Na₃VO₄, and protease and phosphatase inhibitors (Lyn-Cook et al., 2009). Samples containing 200 ng protein were incubated with the beads, and capture antigens were detected with biotinylated secondary antibody and phycoerythrin-conjugated Streptavidin. Plates were read in a Bio-Plex 200 system (Bio-Rad, Hercules, CA). Data are expressed as fluorescence light units corrected for protein concentration.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Assays: Liver tissue was homogenized in QIAzol reagent. RNA isolated using the EZ1 RNA Universal Tissue Kit and the BIO Robot EZ1 (Qiagen Inc., Valencia, CA), was reverse transcribed with random oligodeoxynucleotide primers and the AMV First Strand cDNA synthesis kit. The cDNAs were used to measure gene expression by qPCR with gene-specific primer pairs (Table 3). Primers were designed using MacVector 10 software (MacVector, Inc., Cary, NC) and target specificity was verified using NCBI-BLAST (Basic Local Alignment Search Tool). The Master ep realplex instrument and software (Eppendorf AG, Hamburg, Germany) were used to detect amplified signals from triplicate reactions. Relative mRNA abundance was calculated from the ng ratios of specific mRNA to 18S measured in the same samples (Moroz, Tong, Longato, Xu, & de la Monte, 2008). Template-free reactions served as negative controls.

Statistical Analysis: Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers), while tabulated data reflect means \pm SEMs for each group. Intergroup comparisons were made using one-way

analysis of variance (ANOVA) with Tukey post-hoc tests. In addition, we performed linear trend analysis to gauge the impact of ethanol relative to chow and control TEN with regard to severity of steatosis, lipid/triglyceride accumulation, and ceramide immunoreactivity in liver. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Significant P-values (<0.05) are shown within the panels or tables.

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Table 3: Rat Primer Pairs Used for qRT-PCR Assays

Primer	Direction	Sequence (5'→3')	Position (mRNA)	Amplicon Size (bp)
Insulin/IGF signaling				
Insulin	For	TTC TAC ACA CCC AAG TCC CGT C	145	135
Insulin	Rev	ATC CAC AAT GCC ACG CTT CTG C	279	
Insulin Receptor	For	TGA CAA TGA GGA ATG TGG GGA C	875	129
Insulin Receptor	Rev	GGG CAA ACT TTC TGA CAA TGA CTG	1003	
IGF-1	For	GAC CAA GGG GCT TTT ACT TCA AC	65	127
IGF-1	Rev	TTT GTA GGC TTC AGC GGA GCA C	191	
IGF-1 Receptor	For	GAA GTC TGC GGT GGT GAT AAA GG	2138	113
IGF-1 Receptor	Rev	TCT GGG CAC AAA GAT GGA GTT G	2250	
IGF-2	For	CCA AGA AGA AAG GAA GGG GAC C	763	95
IGF-2	Rev	GGC GGC TAT TGT TGT TCA CAG C	857	
IGF-2 Receptor	For	TTG CTA TTG ACC TTA GTC CCT TGG	1066	91
IGF-2 Receptor	Rev	AGA GTG AGA CCT TTG TGT CCC CAC	1156	
IRS-1	For	GAT ACC GAT GGC TTC TCA GAC G	604	134
IRS-1	Rev	TCG TTC TCA TAA TAC TCC AGG CG	737	
IRS-2	For	CAA CAT TGA CTT TGG TGA AGG GG	255	109
IRS-2	Rev	TGA AGC AGG ACT ACT GGC TGA GAG	263	
IRS-4	For	ACC TGA AGA TAA GGG GTC GTC TGC	2409	132

IRS-4	Rev	TGT GTG GGG TTT AGT GGT CTG G	2540	
Cytokine signaling				
IL-1 β	For	CAC AGT TGC TGG CTC ATC AT	233	60
IL-1 β	Rev	CTT CTC CAC AGC CAC AAT GA	292	
IL-2	For	CAC TTG GAA GAC GCT GGA A	309	111
IL-2	Rev	CAC AGT TGC TGG CTC ATC AT	419	
IL-6	For	ATG TTG TTG ACA GCC ACT GC	116	51
IL-6	Rev	GTC TCC TCTCCG GAC TTG TG	166	
TNF- α	For	ATG TGG AAC TGG CAG AGG AG	26	84
TNF- α	Rev	AGA AGA GGC TGA GGC ACA GA	109	
Ceramide signaling				
SPTLC-1	For	CTA ACC TTG GGC AAA TCG AA	2581	96
SPTLC-1	Rev	TGA GCA GGG AGA AGG GAC TA	2676	
SPTLC-2	For	GGA CAG TGT GTG GCC TTT CT	1823	50
SPTLC-2	Rev	TCA CTG AAG TGT GGC TCC TG	1872	
SMPD-1	For	CAG TTC TTT GGC CAC ACT CA	1443	65
SMPD-1	Rev	CGG CTC AGA GTT TCC TCA TC	1507	
SMPD-3	For	TCT GCT GCC AAT GTT GTC TC	2704	98
SMPD-3	Rev	CCG AGC AAG GAG TCT AGG TG	2801	
CERS-1	For	TGC GTG AAC TGG AAG ACT TG	947	98
CERS-1	Rev	CTT CAC CAG GCC ATT CCT TA	1044	
CERS-2	For	CTC TGC TTC TCC TGG TTT GC	698	82
CERS-2	Rev	CCA GCA GGT AGT CGG AAG AG	779	
CERS-4	For	CGA GGC AGT TTC TGA AGG TC	1240	72
CERS-4	Rev	CCA TTG GTA ATG GCT GCT CT	1311	

UGCG	For	GAT GCT TGC TGT TCA CTC CA	2682	67
UGCG	Rev	GCT GAG ATG GAA GCC ATA GG	2748	
CERD2	For	TCC TAA TCC ACA CGG GAG AG	1523	50
CERD2	Rev	ATC TGG TTG CCA AGG ATG AG	1572	
CERD3	For	AGG TGT GAC AAT GTG CGT GT	590	53
CERD3	Rev	CAC CAA AGG CCA GAG AAG AG	642	
GM3	For	TGT CGG ACG CTG AAT ACT ACG C	1237	142
SYNTHASE				
GM3	Rev	TCT GCC ACC TGC TTC CAA AAG	1378	
SYNTHASE				
ATF-4	For	GAG TCC TAC CTG GGC TCT CC	712	90
ER Stress signaling				
ATF-4	Rev	AGA ACC ACG AGG AAC ACC TG	801	
GRP78	For	AGC CCA CCG TAA CAA TCA AG	1430	96
GRP78	Rev	CAG GAG GGA TTC CAG TCA GA	1525	
CHOP	For	CAT GAA CTG TTG GCA TCA CC	669	96
CHOP	Rev	CAG CAT GTG CAC TGG AGA TT	764	
18S rRNA	For	GGA CAC GGA CAG GAT TGA CA	1278	50
18S rRNA	Rev	ACC CAC GGA ATC GAG AAA GA	1327	

*Abbreviations: qPCR, Quantitative Polymerase Chain Reaction; IGF, Insulin Growth Factor; IRS, Insulin Receptor Substrate; SPTLC, Serine Palmitoyltransferase; CERS, Ceramidase Synthase; SMPD, Sphingomyelin Phosphodiesterase; CERD, N-acylsphingosine Amidohydrolase (Ceramidase); UGCG, UDP-Glucose Ceramide Glucosyltransferase; IL, Interleukin; TNF, Tumour Necrosis Factor; GM3SYN, GM3 Synthase; ATF-4, Activating transcription factor 4; CHOP, C/EBP-

homologous protein; GRP78, glucose regulated protein 78; rRNA, ribosomal RNA; For, forward primer; Rev, reverse primer

2.3 RESULTS

General characteristics of the model (Ronis et al., 2010): Previous studies using samples from this model demonstrated significantly increased serum levels of alanine aminotransferase (ALT) activity and hepatic apoptosis indices and endonuclease G immunoreactivity in ethanol-exposed relative to chow and TEN-fed controls (Table 4). Treatment with NAC significantly reduced serum ALT and hepatic apoptosis and endonuclease G staining in ethanol-fed rats.

Table 4: Effects of ethanol and NAC on markers of hepatocellular injury

Group	ALT ¹ (serum)	Apoptosis (TUNEL ²)	Endonuclease G ³
Chow	42.50 ± 4.10 ^a	0.055 ± 0.05 ^a	5500 ± 1000 ^a
Control TEN	45.40 ± 3.40 ^a	0.135 ± 0.03 ^b	8000 ± 1000 ^{a,b}
Control TEN+NAC	48.88 ± 6.90 ^a	0.160 ± 0.04 ^b	7800 ± 3000 ^{a,b}
EtOH TEN	105.37 ± 23.0 ^b	0.197 ± 0.05 ^b	15100 ± 8100 ^b
EtOH TEN+NAC	64.20 ± 16.80 ^a	0.070 ± 0.02 ^a	12000 ± 3500 ^b

¹ALT = alanine aminotransferase activity (U/L), ²TUNEL as measure by % positive cells,

³Endonuclease G measured the staining intensity per cell. Data are represented as mean ± SEM.

Column means without a common superscript letter are significantly different (P<0.05) by one-way ANOVA followed by Student-Neuman-Keuls post-hoc analysis.

Chronic ethanol exposure significantly reduced hepatic glutathione and increased bovine serum albumin adducted with malondialdehyde and lipid peroxide, while concomitant treatment with NAC inhibited these effects of alcohol on oxidative stress mediators. The levels of thiobarbituric-reactive products in liver were unaffected by ethanol exposure or NAC treatment (Table 5).

Table 5: Effects of ethanol and NAC on hepatic CYP2E1 activity and oxidative stress

Group	Glutathione ¹	TBARS ²	BSA-MDA ³	BSA-LOOH ⁴
Chow	27.34 ± 4.54 ^b	0.59 ± 0.30	0.30 ± 0.02 ^b	0.19 ± 0.02 ^a
Control TEN	22.50 ± 2.24 ^b	0.55 ± 0.50	0.22 ± 0.01 ^a	0.24 ± 0.01 ^a
Control TEN+NAC	26.59 ± 6.70 ^b	0.70 ± 0.50	0.24 ± 0.02 ^a	0.22 ± 0.02 ^a
EtOH TEN	18.20 ± 0.78 ^a	0.75 ± 0.80	0.44 ± 0.02 ^c	0.42 ± 0.03 ^b
EtOH TEN+NAC	31.44 ± 3.35 ^b	0.74 ± 0.40	0.34 ± 0.01 ^b	0.29 ± 0.02 ^a

¹Glutathione (GSH) measured in nmol GSH per mg protein, ²TBARS = thiobarbituric acid-reactive products/g liver in nmol, ³BSA-MDA = Antibody titer against bovine serum albumin (BSA) adducted with malondialdehyde, ⁴BSA-LOOH = Antibody titer against BSA adducted with lipid peroxide. Data are represented as mean ± SEM. Column means without a common superscript letter are significantly different (P<0.05) by one-way ANOVA followed by Student-Neuman-Keuls post-hoc analysis.

Chronic ethanol exposure increased the expression of pro-fibrogenesis genes (mRNAs), but did not significantly alter hepatic hydroxyproline content. Quantitative RT-PCR analysis detected significantly elevated levels of α -1collagen Types I and III, and matrix metalloproteinase (MMP) 3, MMP-9, and MMP-13, but not TIMP-1 in ethanol-exposed relative to control livers (Table 6). Concomitant NAC treatment significantly reduced hepatic expression of MMP-9 in ethanol-exposed livers, but had no significant effect on the expression of the other pro-collagen genes examined.

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Table 6: Effects of ethanol and NAC on hepatic hydroxyproline content and expression of mRNAs associated with fibrosis and matrix remodelling

Group	Hydroxy-proline ¹	α -1 collagen Type I mRNA ²	α -1 collagen Type III mRNA ²	MMP-3 mRNA ²	MMP-9 mRNA ²
Chow	1.70 \pm 0.40	1.00 \pm 0.25 ^a	1.00 \pm 0.07 ^a	1.00 \pm 0.14 ^a	1.00 \pm 0.50 ^a
Control TEN	2.32 \pm 0.50	1.29 \pm 0.11 ^a	1.23 \pm 0.09 ^a	1.67 \pm 0.27 ^a	1.42 \pm 0.07 ^a
Control TEN+NAC	2.02 \pm 0.40	1.30 \pm 0.12 ^a	1.34 \pm 0.27 ^a	5.72 \pm 1.97 ^a	5.17 \pm 2.32 ^a
EtOH TEN	2.97 \pm 1.15	2.62 \pm 0.65 ^b	1.97 \pm 0.21 ^b	4.82 \pm 1.42 ^{a,b}	7.51 \pm 3.25 ^{a,b}
EtOH TEN+NAC	1.96 \pm 0.60	2.59 \pm 0.70 ^b	2.82 \pm 0.49 ^b	6.52 \pm 1.47 ^b	2.78 \pm 3.14 ^b

¹Hydroxyproline measured in % per protein content, ²mRNA data as measured by RT-PCR expressed as gene expression normalized to expression of 18S. Data are represented as mean \pm SEM. Column means without a common superscript letter are significantly different (P<0.05) by one-way ANOVA followed by Student-Neuman-Keuls post-hoc analysis.

Effects of ethanol and NAC on steatohepatitis: H&E stained sections of chow-fed livers demonstrated the expected cord-like architecture with minimal or absent portal lympho-mononuclear inflammatory cell infiltrates, and scattered hepatocytes with macrosteatosis (Figures 2.1 A, F, K). Control-TEN livers exhibited micro-vesicular steatosis that was predominantly distributed in Zone 2, but extended close to Zones 1 and 3. In addition, control-TEN livers contained small foci of lobular lympho-mononuclear cell inflammation and scattered apoptotic bodies and, the regular cord-like architecture was mildly disrupted (Figures 2.1 B, G, L). Treatment of control-TEN rats with NAC converted the predominantly micro-steatosis to macrosteatosis, and minimized the inflammation. However, the presence of scattered apoptotic bodies and mildly disrupted hepatic cord architecture persisted despite NAC treatment (Figures 2.1 C, H, M).

Chronic ethanol feeding caused moderate to severe steatohepatitis that was pan-lobular, i.e. distributed from Zone 1 through Zone 3, and mainly micro-vesicular in nature (Figures 2.1 D, I, N). Inflammation was prominent, multi-focal, and nodular. Apoptotic bodies were abundant, and the hepatic architecture was markedly disorganized. Co-treatment with NAC reduced the inflammation and converted the predominantly micro-vesicular steatosis to mainly macro-vesicular steatosis (Figures 2.1 E, J, O). Semi-quantitative grading of the liver histology under code, using the 1 to 4 scale (see Methods) revealed a significant trend of increasing severity of steatohepatitis, with minimal injury observed in chow-fed rats, to mild or moderate disease in the control-TEN group, to severe liver injury in the ethanol-TEN fed rats ($P=0.0157$; Figure 2.2 A). Overall, there were no discernible effects of the NAC treatments on the mean scores assigned to livers of control- or ethanol-TEN fed rats.

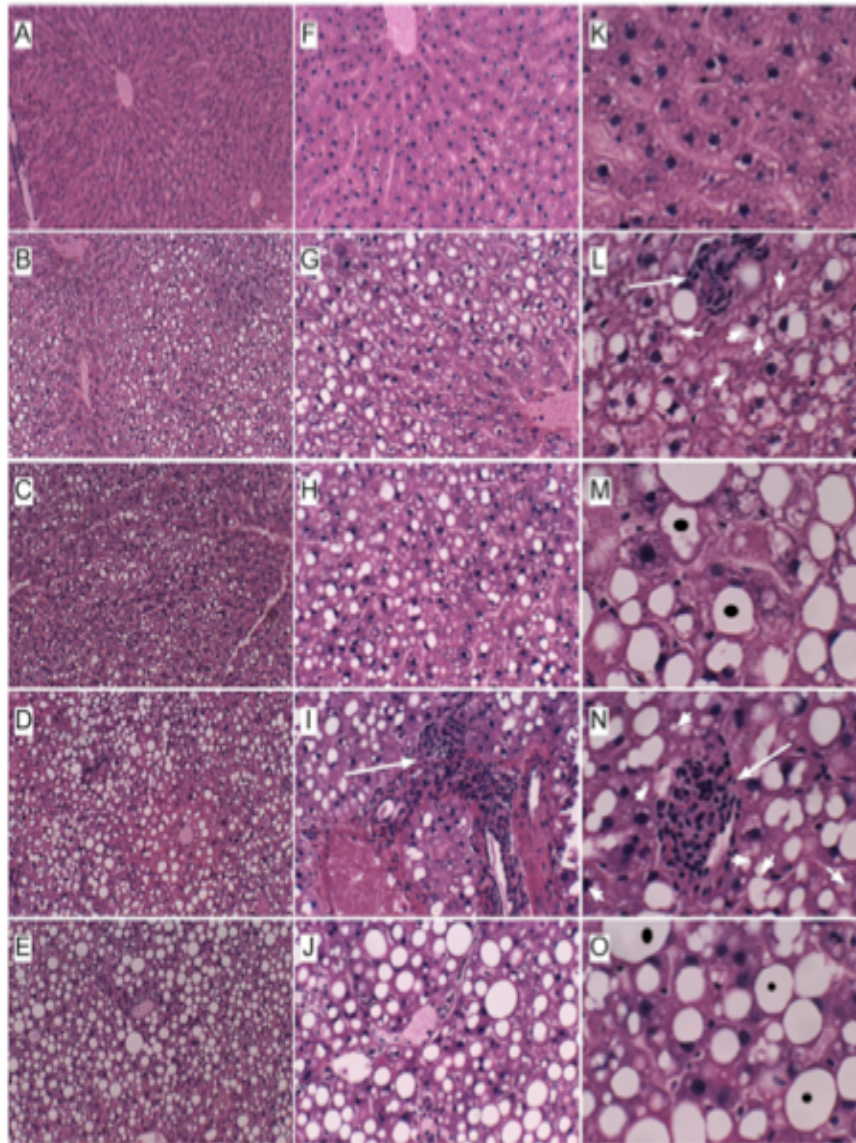


Figure 2.1: Effects of chronic ethanol feeding and NAC treatment on steatohepatitis. Adult male Sprague-Dawley rats were fed for 130 days with chow (A, F, K), control TEN (B, G, L), control TEN supplemented with NAC (C, H, M), ethanol TEN (37% by caloric content; D, I, N), or ethanol TEN supplemented with NAC (E, J, O). Paraffin-embedded histological sections (5 μm thick) of liver were stained with H&E and photographed at 40x (A-E), 160x (F-J), and 400x (K-O) magnifications. Note reduced inflammation and shift from microvesicular steatosis to macrovesicular steatosis associated with NAC treatments, but persistence of hepatic cord architecture disarray in the control liquid with ethanol livers despite NAC treatment.

Hepatic steatosis was further characterized by measuring neutral lipid, triglyceride, cholesterol, and ceramide content. The Nile Red assay demonstrated generally higher mean levels of neutral lipids in livers of TEN-fed relative to chow-fed rats. A borderline significant trend was observed indicating increased hepatic neutral lipid accumulation with respect to diet and ethanol feeding, i.e. from chow, to control-TEN, and then ethanol-TEN feeding ($P=0.05$; $R^2=0.097$). In addition, chronic ethanol feeding, with or without NAC treatment, significantly increased hepatic neutral lipid content relative to chow-fed but not TEN-fed controls (Figure 2.2 B). With regard to triglycerides, a significant positive trend for increasing hepatic triglyceride content with respect to treatment group (Chow < Control TEN < Ethanol TEN) was demonstrated ($R^2=0.29$; $P=0.0004$). The mean hepatic triglyceride content was significantly higher in the control-TEN and ethanol-TEN relative to chow fed rats, irrespective of NAC treatment (all $P<0.01$; Figure 2.2 C). In contrast, mean hepatic cholesterol levels were similar in all 5 groups (Figure 2.2 D). This result differs from findings in other studies in which chronic ethanol feeding significantly increased hepatic cholesterol levels (Wang, Yao, & Song, 2010). However, our result could be due to the use of TEN rather than a standard Lieber-De Carli diet. Finally, although no significant trend relating diet or treatment to hepatic ceramide content was observed, ANOVA tests demonstrated significant inter-group differences ($P=0.007$) with the highest level in the ethanol-TEN group, and significant differences in the mean levels between ethanol-TEN and ethanol-TEN+NAC ($P<0.05$; Figure 2.2 E). This indicates that ethanol-induced increases in hepatic ceramide can be reduced by NAC treatment.

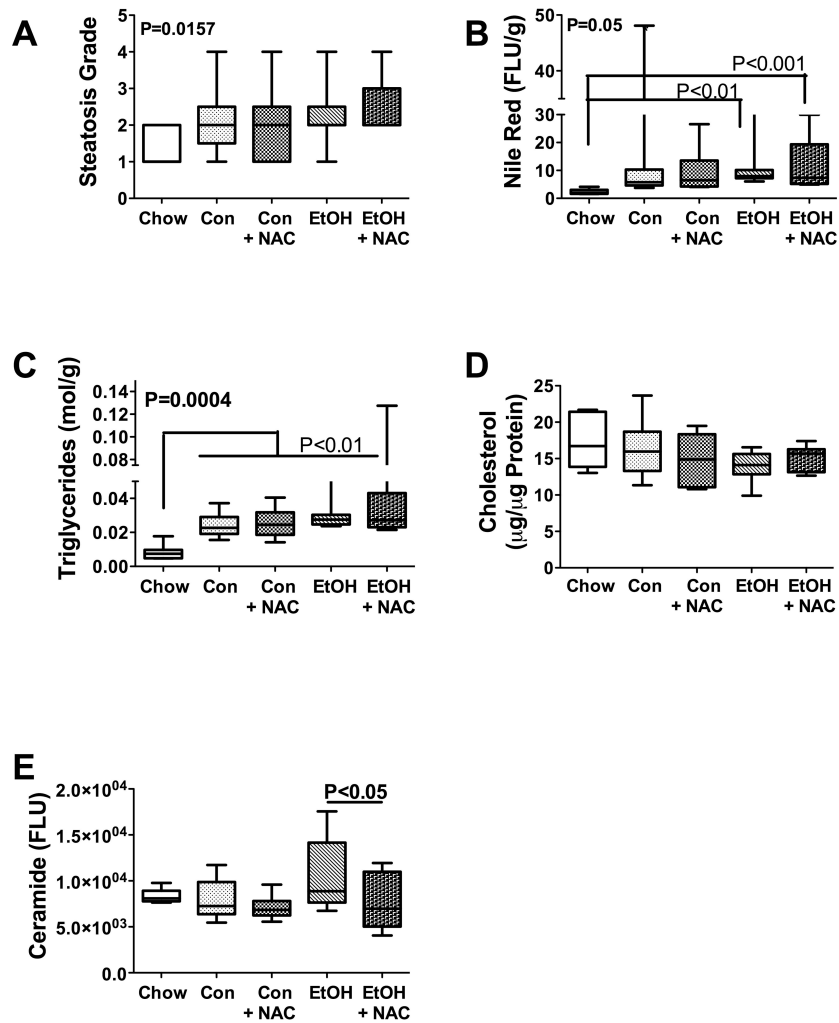


Figure 2.2: Lipid and ceramide accumulations in liver following chronic ethanol and NAC treatment. (A) Severity of hepatic steatosis observed in H&E stained histological sections was graded blind on a scale from 1 to 4. (B-E) Lipid extracted from fresh frozen liver tissue was analyzed for (B) neutral lipid content using the Nile Red assay, (C) triglycerides, (D) cholesterol, or (E) ceramide immunoreactivity (see Material and Methods), and values were normalized to tissue sample weight or protein content. Box plots depict medians (horizontal bars), 95% confidence interval limits, and range (whiskers). Inter-group comparisons were made using one-way ANOVA with the Tukey multiple comparison and linear trend post hoc tests. Significant P-values are indicated within the panels. Significant post-hoc linear trend analysis results are shown in the upper left quadrant of each panel.

To determine if increased hepatic ceramide was mediated by increased pro-ceramide gene expression or sphingomyelinase enzymatic activity, we measured mRNA levels of ceramide synthases 1, 2, and 4 (CERS), ceramidase 2 and 3 (CERD), serine palmitoyl transferase 1 and 2 (SPTLC), and sphingomyelinase 1 and 3 (SMPD), UDP-glucose ceramide glucosyltransferase (UGCG), and GM3 synthase by qRT-PCR analysis (Table 7), and acid (ASMase) and neutral (NSMase) sphingomyelinase activities using a commercial assay (Invitrogen, Carlsbad, CA) (Figure 2.3). The genes selected for analysis were based on previous studies demonstrating which mRNA transcripts were modulated with increasing steatohepatitis in animal models (de la Monte, Tong, Lawton, & Longato, 2009; Lyn-Cook et al., 2009; Shah et al., 2008). However, in the present study, similar mean levels of CERS2, CERD2, SPTLC2, and SMPD1 were measured in all experimental groups (Table 7). On the other hand, ASMase activity was significantly elevated in livers from control-TEN+NAC, ethanol-TEN, and ethanol-TEN+NAC relative to chow fed rats (Figure 2.3 A). Moreover, ethanol-TEN feeding significantly increased ASMase activity relative control-TEN, while NAC treatment abolished that difference. The mean levels of NSMase activity were similar in the five study groups (Figure 2.3 B). These findings suggest that NAC treatment may reduce hepatic ceramide accumulation in ALD through inhibition of ASMase activity.

Table 7: Effects of Chronic Ethanol Feeding and Treatment with NAC on pro-Sphingolipid Gene Expression in Liver

GENE	Chow	Con TEN	Con TEN + NAC	EtOH TEN	EtOH TEN + NAC	F-Ratio	p-Value
CERS1	0.02521 ^a ± 0.00196	0.02434 ^b ± 0.00135	0.03277 ^{b*} ± 0.00217	0.02970 ± 0.0022	0.03398 ^{a****;} b** ± 0.00199	18.85	0.0008
CERS2	0.2929 ± 0.069	0.6555 ± 0.1658	0.2738 ± 0.1064	0.4751 ± 0.1121	0.5806 ± 0.1323	7.297	NS
CERS4	0.02588 ^a ± 0.002796	0.02517 ^a ± 0.0009467	0.02825 ^b ± 0.002721	0.03106 ± 0.001883	0.04335 ^{a****;} b* ± 0.003032	27.00	<0.0001
CERD2	0.0006725 ± 0.000228	0.0009465 ± 0.0002361	0.0004346 ± 0.0001164	0.001056 ± 0.00025	0.001797 ± 0.001044	4.137	NS
CERD3	0.2567 ^a ± 0.0234	0.2740 ^b ± 0.0249	0.5914 ^{a**, b**} ± 0.0909	0.5428 ^{a****;} b*** ± 0.0482	0.4960 ^{a**;} b*** ± 0.0328	38.30	<0.0001
SMPD1	0.1037 ± 0.0215	0.1749 ± 0.0367	0.1015 ± 0.0331	0.1404 ± 0.0237	0.1345 ± 0.0347	3.198	NS
SMPD3	0.05598 ^a ± 0.005982	0.09191 ^{a*} ± 0.00748	0.1036 ^{a**} ± 0.0105	0.09724 ^{a*} ± 0.00846	0.08731 ± 0.00896	4.04	0.0049
SPTLC1	0.0000187 ^a ± 0.0000059	0.00000847 ± 0.0000022	0.0000095 ± 0.0000010	0.0000043 ± 0.0000006	0.0000073 ± 0.0000021	8.354	0.079
SPTLC2	0.001612 ± 0.000984	0.001213 ± 0.000299	0.001224 ± 0.000418	0.00172 ± 0.000561	0.001217 ± 0.000395	1.271	NS
UGCG	1.484 ± 0.3261	0.7152 ± 0.0666	0.9944 ± 0.1719	0.9889 ± 0.2521	0.8111 ± 0.0894	6.097	NS
GM3SYN	1.185 ^a ± 0.136	1.212 ^a ± 0.083	1.396 ^b ± 0.094	3.436 ^{a****;} b*** ± 0.207	2.327 ^{a****} ± 0.169	55.92	<0.0001

*Abbreviations: Con, Control; TEN, Total Enteral Nutrition; NAC, N-Acetylcysteine; EtOH, Ethanol; CERS, Ceramide Synthase; CERD, N-acylsphingosine Amidohydrolase (Ceramidase); SPTLC, Serine Palmitoyltransferase; SMPD, Sphingomyelin Phosphodiesterase; UGCG, UDP-Glucose Ceramide Glucosyltransferase; GM3SYN, GM3 Synthase. Gene expression was measured by qPCR using gene-specific primers (Table 3) and levels were normalized to 18S rRNA measured in the same samples. Results depict mean ± SEM of mRNA/18S rRNA (x10⁻⁶) calculated for each group. Inter group statistical comparisons were made by one-way ANOVA with the Kruskal-Wallis post-hoc test. Superscripted letters indicate inter-group differences that are statistically significant, *p < 0.05; **p < 0.01; ***p < 0.001 relative to group with superscripted letter but no asterisk.

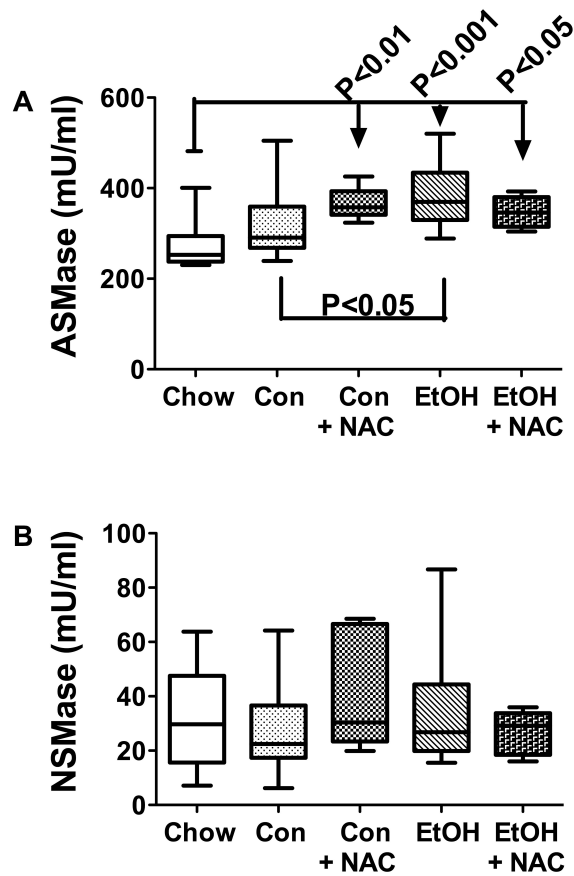


Figure 2.3: Role of sphingomyelinase activity as a mediator of increased hepatic ceramide levels following chronic ethanol feeding, and therapeutic effects of NAC. (A) Acid (ASMase) and (B) neutral (NSMase) sphingomyelinase activities were measured using a standard fluorometric assay and results were normalized to protein concentration. Inter-group comparisons were made using one-way ANOVA with the Tukey multiple comparison tests. Significant P-values are indicated within the panels.

Effects of ethanol and NAC treatments on cytokine levels in liver: Activation of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β , is a common feature of steatohepatitis (Carter-Kent et al., 2008; Larter & Farrell, 2006; McClain, Barve, Deaciuc, Kugelmas, & Hill, 1999; McClain & Cohen, 1989; Tilg & Hotamisligil, 2006). In addition, ALD is associated with increased levels of GRO/KC (Maltby, Wright, Bird, & Sheron, 1996; Ohki et al., 1998; Shiratori et al., 1993), which is a small CXC cytokine with mitogenic properties that mediates inflammation and angiogenesis (Haskill et al., 1990). We measured hepatic levels of these cytokines, plus IL-2, IL-4, GM-CSF, and VEGF by multiplex ELISA to determine the degree to which chronic ethanol feeding stimulates pro-inflammatory states and angiogenesis, and whether NAC treatment reduces cytokine activation. The studies demonstrated similar mean levels of TNF- α , IL-4, IL-6, and VEGF overall among the five study groups (Table 8; Figure 2.4). IL-1 β expression was significantly higher in livers of control-TEN (P=0.036) but not in ethanol-TEN relative to chow fed rats (Figure 2.4 A). IL-2 expression was significantly higher in livers of ethanol-TEN \pm NAC treated compared with chow-fed rats (P<0.05). Although the expression levels of GM-CSF and GRO/KC were generally higher in livers of control- and ethanol-TEN fed relative to chow-fed rats, significant differences were only observed between the chow and control-TEN fed groups (Table 4, P=0.001; and P=0.012 respectively). In aggregate, we observed significantly increased IL-2 immunoreactivity in ethanol-TEN fed rats relative to controls, but minimal or no significant modulation of pro-inflammatory cytokine expression mediated by the NAC treatments (Figure 2.4 B). mRNA levels of CYP2E1 were generally higher in the ethanol compared to the vehicle TEN group, however NAC had no effect in reducing these levels (Figure 2.4 E).

Effects of ethanol and NAC on insulin and IGF signaling mechanisms in liver:
Previous studies demonstrated that steatohepatitis produced by high fat diet or chronic ethanol feeding impairs insulin and IGF signaling in the liver (de la Monte et al., 2008; Svegliati-Baroni et al., 2006; Yeon, Califano, Xu, Wands, & De La Monte, 2003). We re-investigated this concept and also interrogated the potential therapeutic effects of NAC on ethanol-mediated inhibition of insulin/IGF signaling by measuring mRNA levels of insulin, IGF-1, and IGF-2 polypeptides, their corresponding receptors, and IRS-1, IRS-2, and IRS-4 by qRT-PCR analysis (Figure 2.5), and IR, ^{pYpY1162/1163}-IR, IGF-1R, ^{pYpY1135/1136}-IGF-1R, IRS-1, ^{pS312}-IRS-1, Akt, ^{pS473}-Akt, GSK-3 β , ^{pS9}-GSK3 β ,

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Table 8: Influence of Chronic Ethanol Feeding and NAC on Pro-Inflammatory Cytokine Levels in Liver

Protein	Chow	Con TEN	Con TEN+	EtOH TEN	EtOH TEN+	F-Ratio	p-Value
			NAC		NAC		
IL-4	17.97 ± 2.545	40.19 ± 11.3	25.59 ± 5.391	36.16 ± 17.56	25.97 ± 3.068	4.25	NS
GM-CSF	36.07 ^a ± 7.817	195.1 ^{a***} ± 36.58	82.28 ± 12.83	142.1 ± 43.76	83.83 ± 10.96	18.05	0.001
GRO/KC	98.33 ^a ± 7.55	199.9 ^a ± 34.37	142.4 ± 14.39	180.7 ± 43.24	123.5 ± 5.058	12.90	0.012
VEGF	11.3 ± 1.889	21.5 ± 5.301	14.59 ± 2.562	27.81 ± 8.942	14.02 ± 0.8307	2.23	NS

*Abbreviations: C, Control; TEN, Total Enteral Nutrition; IL-4, Interleukin 4; GM-CSF, Granulocyte Macrophage Colony-Stimulating Factor; GRO/KC, CXC Chemokine; VEGF, Vascular Endothelial Growth Factor. Effects of chronic ethanol and NAC treatment on pro-inflammatory cytokine expression in liver. Liver protein homogenates were used to measure immunoreactivity to IL-4, GM-CSF, GRO/KC and VEGF by multiplex ELISA. Immunoreactivity was normalized to protein concentration and data reflect mean ± SEM of fluorescence light units (arbitrary). Inter-group comparisons were detected with the post-hoc Kruskal-Wallis multiple comparison tests. Superscripted letters indicate inter-group differences that are statistically significant, *p < 0.05; **p < 0.01; ***p < 0.001 relative to group with superscripted letter but no asterisk.

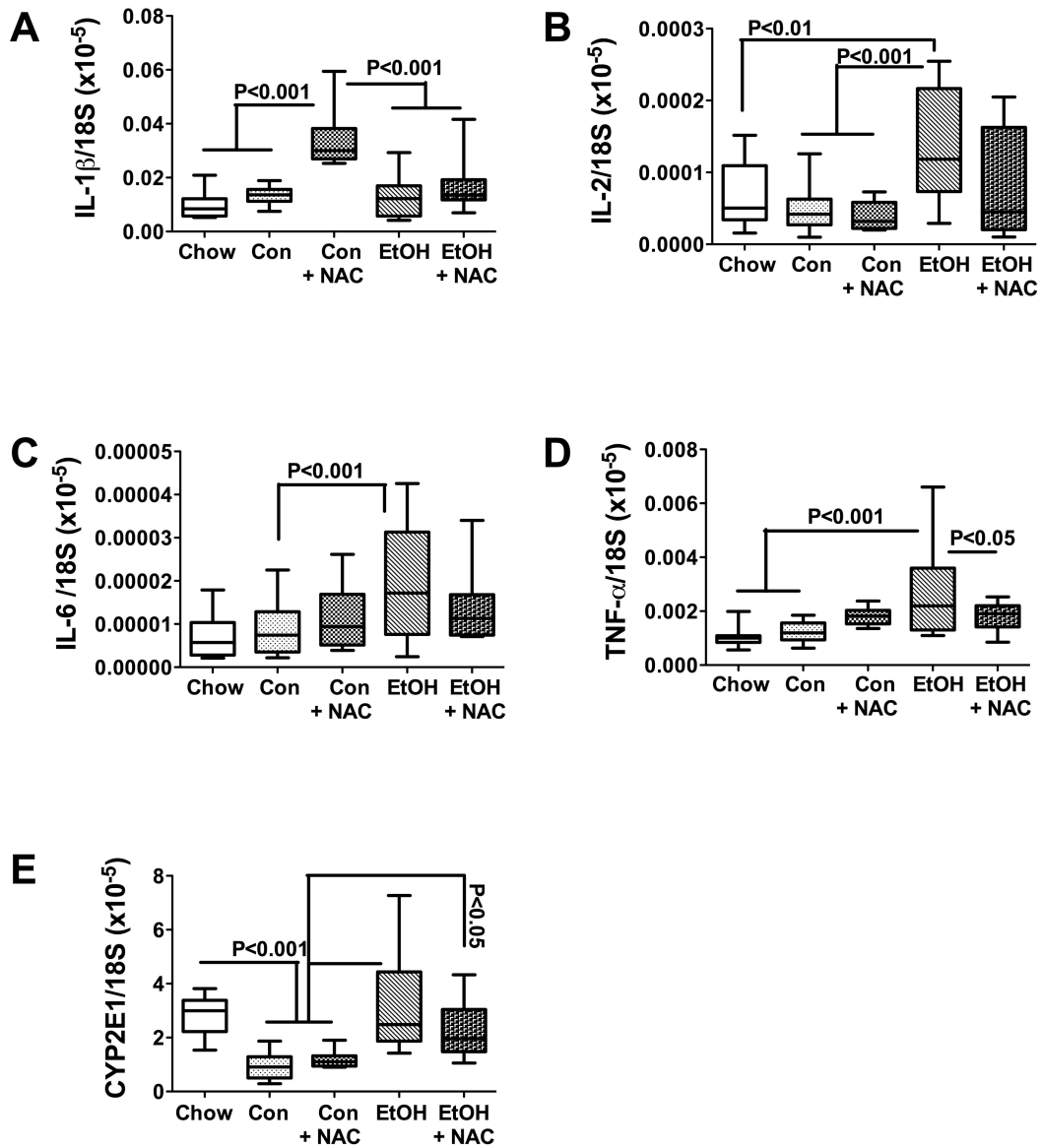


Figure 2.4: Effects of chronic ethanol and NAC treatment on pro-inflammatory cytokine and CYP2E1 gene expression in liver. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure to (A) IL-1 β , (B) IL-2, (C) IL-6, (D) TNF- α , and (E) CYP2E1 by qPCR using gene-specific primers (Table 3). Inter-group comparisons were made using one-way ANOVA with the Tukey multiple comparison tests. Significant P-values are indicated within the panels.

p70S6K, p^{TpS421/424}-p70S6K, PRAS40, and p^{T246}-PRAS40 immunoreactivities by multiplex ELISA (Figures 2.6 and 2.7). The qRT-PCR analyses demonstrated similar mean levels of insulin, IGF-1, IR, IRS-1, and IRS-2 expression in all groups. Hepatic IGF-2 polypeptide (P<0.05) and IGF-1R (P<0.01) mRNA levels were significantly higher in ethanol-TEN+NAC than in chow-fed rats (Figures 2.5 E and C). In addition, IGF-2R expression was significantly higher in livers of ethanol-TEN+NAC compared with chow and control-TEN fed rats (Figure 2.5 D). Finally, with regard to IRS-4, the mean hepatic mRNA levels were similar for chow, control- and ethanol-TEN fed rats, but the levels were significantly reduced by the NAC treatments relative to the corresponding control- and ethanol-TEN groups (Figure 2.5 H).

Analysis of the upstream signaling networks using multiplex ELISAs revealed similar mean levels of IR, IGF-1R, and IRS-1 in all groups (Figures 2.6 A-C). The mean hepatic levels of p^{YpY1162/1163}-IR (Figure 2.6 D) and the p^{YpY1162/1163}-IR/Total IR ratio (Figure 2.6 G) were significantly reduced in ethanol-TEN ± NAC relative to control-TEN fed rats (both P<0.01). The mean hepatic levels of p^{YpY1135/1136}-IGF-1R were significantly lower in ethanol-TEN (P<0.05) and ethanol-TEN+NAC (P<0.001) relative to control-TEN fed rats (Figure 2.6 E). Finally, the mean hepatic p^{S312}-IRS-1 and the p^{S312}-IRS-1/total IRS-1 ratio were significantly reduced in ethanol-TEN+NAC relative to control-TEN ± NAC fed rats (Figures 2.6 F and I). Therefore, chronic ethanol feeding suppressed hepatic signaling through IR, IGF-1R, and IRS-1, with no demonstrated protective effects of the NAC treatments.

Downstream signaling through insulin/IGF-1 in liver, showed similar mean levels of Akt, GSK-3β, p70S6K, and PRAS40 in all five groups (Figures 2.7 A-D). Hepatic p^{S473}-pAkt and the p^{S473}-pAkt /total Akt ratio were significantly reduced by chronic

ethanol-TEN relative to control-TEN feeding, and NAC treatment had no significant effect concerning the responses to ethanol (Figures 2.7 E and I). The mean

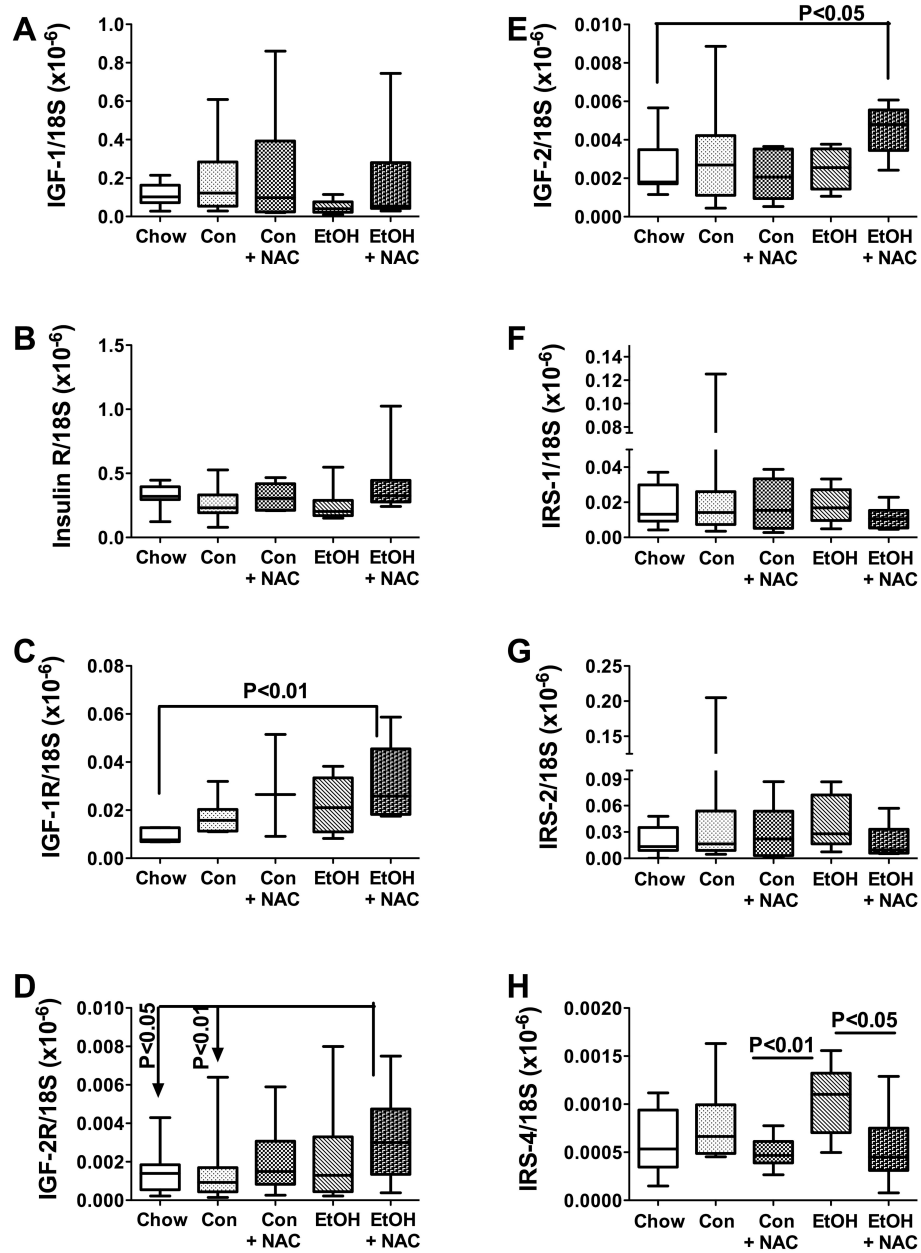


Figure 2.5: Effects of chronic ethanol and NAC treatment on insulin/IGF signaling network genes. RNA extracted from liver was reverse transcribed to measure (A) IGF-1, (B) insulin receptor, (C) IGF-1 receptor, (D), IGF-2 receptor, (E) IGF-2, (F) IRS-1, (G) IRS-2, and (H) IRS-4

mRNA expression by PCR. Inter-group comparisons were made using one-way ANOVA with the Tukey multiple comparison tests. Significant P-values are indicated within the panels.

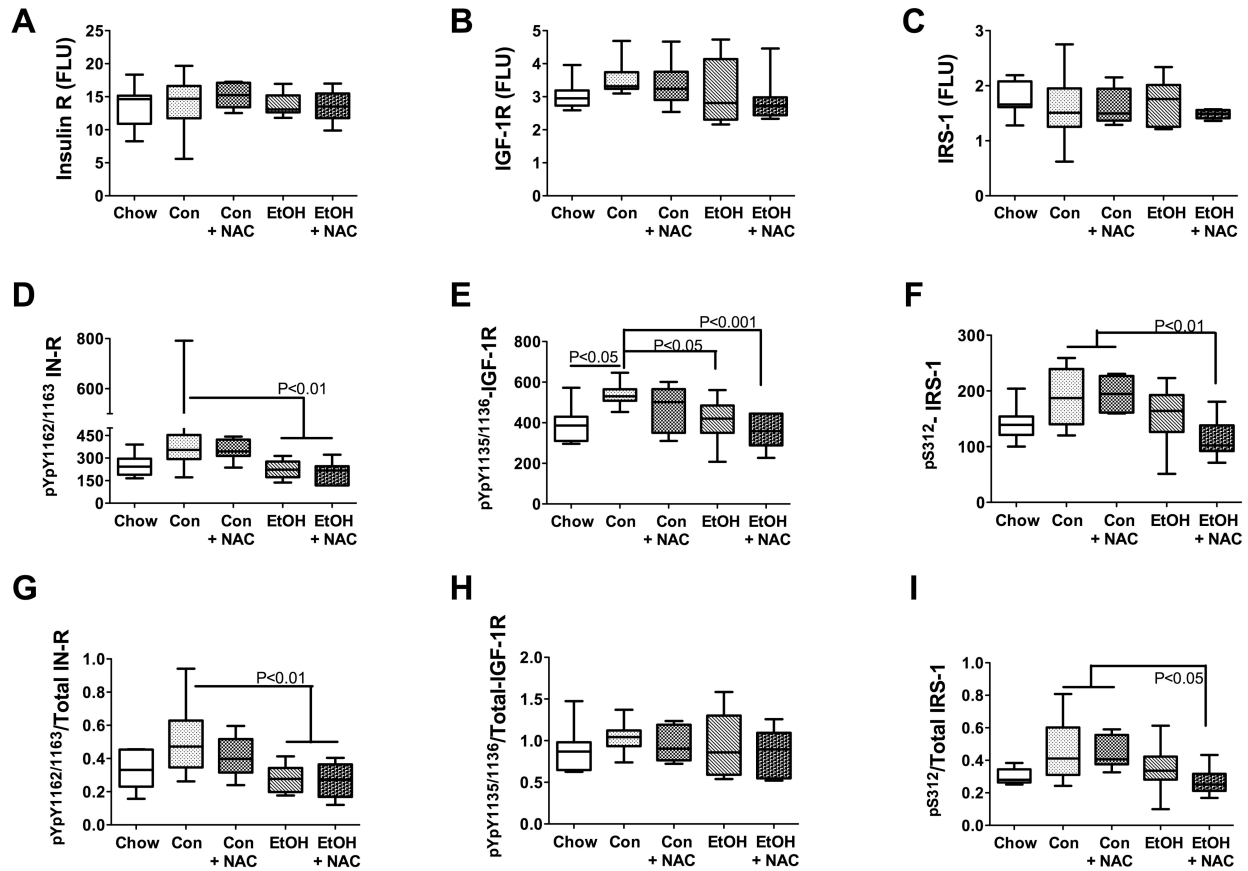


Figure 2.6: Effects of chronic ethanol and NAC treatment on upstream mediators of insulin/IGF signaling. Liver protein homogenates were used to measure immunoreactivity corresponding to (A) insulin receptor (R), (B) IGF-1R, (C) IRS-1, (D) pYpY1162/1163-IR, (E) pYpY1135/1136-IGF-1R, (F) pS312-IRS-1 with a bead-based Multiplex ELISA platform (see Methods and Methods). (G-I) In addition, the phosphorylated/total protein ratios for were calculated to assess relative levels of phosphorylation of each protein. Data were analyzed statistically using one-way ANOVA with the Tukey multiple comparison tests. Significant P-values are indicated within the panels.

hepatic levels of p^{S9} -GSK-3 β were significantly reduced in the ethanol-TEN relative to control-TEN fed rats (Figure 2.7 F), and the mean p^{S9} -GSK-3 β /GSK-3 β ratio was significantly lower in ethanol-TEN+NAC fed relative to all control groups ($P < 0.05$; Figure 2.7 J). Signaling through p70S6K was also impaired by ethanol as demonstrated by the significantly lower mean levels $p^{TpS421/424}$ -p70S6K in ethanol-TEN relative to control-TEN, particularly with respect to the control-TEN+NAC treated group (Figure 2.7 G). The mean ratio of $p^{TpS421/424}$ -p70S6K/total p70S6K was significantly lower in the ethanol-TEN+NAC relative to the control-TEN+NAC ($P < 0.01$), and ethanol-TEN ($P < 0.05$) fed groups (Figure 2.7 K). Finally, the mean hepatic levels of p^{T246} -PRAS40 and the p^{T246} -PRAS40/total PRAS40 ratio were significantly reduced in livers of ethanol-TEN+NAC treated relative to control-TEN.

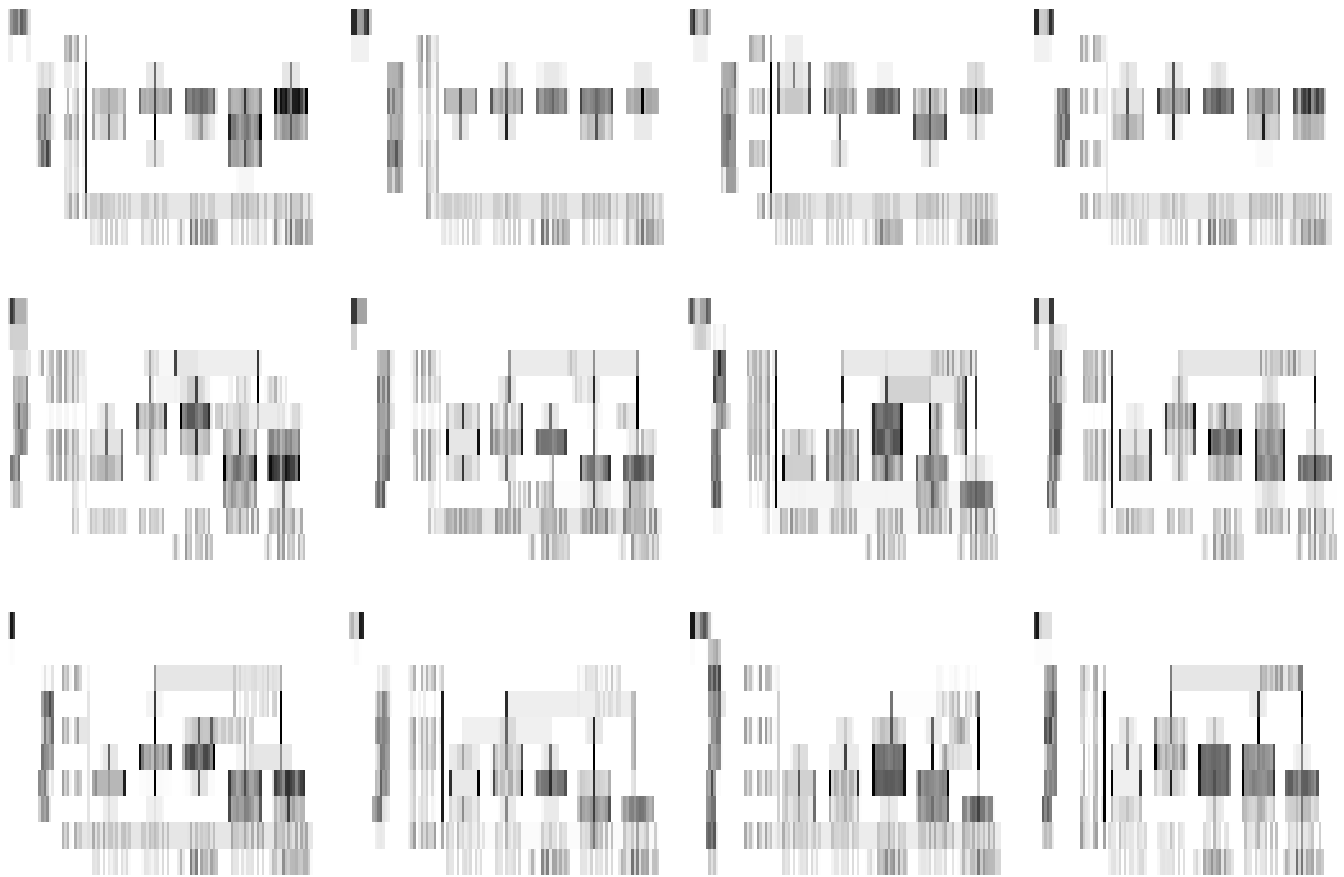


Figure 2.7: Effects of chronic ethanol, and NAC treatment on the downstream mediators of insulin/IGF signaling. Liver protein homogenates were used in bead-based Multiplex ELISAs to measure (A) Akt, (B) GSK-3 β , (C) p70S6K, (D) PRAS40, (E) p^{S473}-Akt, (F) p^{S9}-GSK3 β , (G) p^{TPS421/424}-p70S6K and (H) p^{T246}-PRAS40 immunoreactivities. (I-L) In addition, the phosphorylated/total protein ratios for were calculated to assess relative levels of phosphorylation of each protein. Data were analyzed statistically using one-way ANOVA with the Tukey multiple comparison tests. Significant P-values are indicated within the panels.

2.4 DISCUSSION

Total enteral nutrition (TEN) diets caused hepatic steatosis with mild inflammation, whereas superimposed chronic ethanol feeding strikingly increased the severity of steatohepatitis. Biochemical studies confirmed that the TEN diets increased hepatic lipid content (mainly triglycerides) relative to chow feeding, and that the addition of ethanol to the diets caused only modest further increases total hepatic lipid over TEN alone. Therefore, factors other than simply lipid load must play critical roles in mediating severity of steatohepatitis. One consideration is that ethanol may alter the composition of lipids accumulated in hepatocytes, and perhaps render them cytotoxic. It is noteworthy that the lipid droplets found in ethanol-TEN fed rat livers were mainly micro-vesicular and associated with prominent inflammation, apoptosis, necrosis, and disarray of the hepatic cord architecture, while in control-TEN fed rats, the lipid droplets were mixed micro- and macrovesicular, and the inflammation and cell injury were less severe. Given the similar overall mean triglyceride contents in livers of control-TEN and ethanol-TEN fed rats, other factors must account for the striking differences in the levels of inflammation and injury. Corresponding with earlier findings (Lyn-Cook et al., 2009), it appears that the micro-vesicular pattern of steatosis may mark a pathological state, whereas the macro-vesicular pattern of steatosis may be more benign. In this regard, it is noteworthy that the major effects of NAC were to reduce inflammation and shift hepatic steatosis from a micro-vesicular to mainly macro-vesicular pattern with accompanying reductions in apoptosis, necrosis, and architectural disarray in both groups, yet total hepatic lipid content was not significantly reduced by NAC.

Oxidative stress contributes to progression of simple steatosis to steatohepatitis (Albano, 2008; Dowman et al., 2010), while radical scavengers

attenuate oxidative stress. Since hepatic glutathione levels in ALD and non-alcoholic liver disease are reduced (Das et al., 2008; Videla et al., 2004), we investigated the therapeutic effects of NAC, an anti-oxidant that promotes glutathione synthesis (Aruoma et al., 1989; Atkuri et al., 2007), on the severity of alcohol-induced steatohepatitis. In the present study, NAC reduced inflammation, but not lipid content (Nile Red and triglyceride assays) in liver. However, NAC treatment shifted the sizes of intra-hepatic lipid droplets, converting them from microvesicular to mainly macrovesicular in nature. These findings correspond with results obtained in an earlier study showing that with progression of non-alcoholic steatohepatitis, the histopathology shifted from predominantly macro-vesicular to micro-vesicular steatosis, and was associated with increased inflammation, apoptosis, disorganization of the hepatic lobular architecture, and increased ceramide levels in liver (Lyn-Cook et al., 2009). However, contrary to findings in other models (Cai et al., 2005; McClain et al., 1999; Ronis et al., 2008; Wang et al., 2010), we did not observe a significant role for NAC inhibition of pro-inflammatory cytokines in relation to the reduced severity of alcohol-related steatohepatitis. Moreover, we did not detect significant ethanol-associated increases in pro-inflammatory cytokines in liver. Although the latter observation is not in agreement with previous reports (Thakur, Pritchard, McMullen, & Nagy, 2006), the discrepancies could be explained by the fact that in those studies, cytokine activation was measured in isolated Kupffer cells, whereas our assays were performed with liver homogenates. The use of tissue homogenates probably masked any differences present in Kupffer cells, which represent a relatively small percentage of the liver cell population.

This study demonstrated that an important consequence of the TEN diets was to increase hepatic ceramide levels. Moreover, the increased levels of

ceramide in TEN fed rats, with or without superimposed ethanol and/or NAC treatments, were likely mediated by increased acid sphingomyelinase activity, rather than increased expression of pro-ceramide genes, indicating that these responses may have been effectuated by post-transcriptional mechanisms. In a recent study, increased sphingomyelinase activity was associated with hepatic steatosis caused by chronic high fat feeding (Chocian et al., 2010). Mechanistically, sphingomyelinase activity can be induced by activation of hepatic stellate cells (Moles et al., 2010), and sphingomyelinases are activated by oxidative stress, reactive oxygen species, and reactive nitrogen species, and inhibited by glutathione and other anti-oxidants (Ichi et al., 2009; Levy, Castillo, & Goldkorn, 2006; Navas et al., 2002; Won & Singh, 2006). Therefore, increased oxidative stress was probably an important factor contributing to the increased levels of acid sphingomyelinase activity, and the attendant increased ceramide levels in livers of ethanol fed rats.

As ceramides can cause cytotoxicity, inflammation, apoptosis, and insulin resistance (Hannun & Obeid, 2008; Holland & Summers, 2008; Morales, Lee, Goni, Kolesnick, & Fernandez-Checa, 2007; Summers, 2006), the NAC-associated improvements in alcohol-induced steatohepatitis could have been mediated by reduced oxidative stress-stimulated sphingomyelinase activity, and consequential reductions in ceramide accumulation. Mechanistically, anti-oxidant treatments may help stabilize membranes of large lipid globules, preventing their rupture and release of cytotoxic lipids, including ceramides. In addition, recent preliminary studies using a precision-cut slice culture model revealed that chemical inhibition of ceramide synthases significantly reduces oxidative stress, lipotoxicity and insulin resistance in ethanol exposed livers (Setshedi et al., 2010). Therefore, inhibition of ceramide accumulation by reducing sphingomyelin degradation or ceramide

synthesis could be therapeutically effective for chronic alcoholic liver disease, although inhibition of ceramide synthesis may be more efficacious in restoring hepatic insulin responsiveness.

Gangliosides are glycosphingolipids that contain sialic acid and are expressed on mammalian plasma membranes. Gangliosides, like ceramides, are important signaling molecules that can promote insulin resistance (Holland et al., 2007; Yamashita et al., 2003) particularly in response to inflammatory mediators such as TNF- α (Tagami et al., 2002). Insulin resistance induced by TNF- α was shown to be associated with increased GM3 and reduced insulin receptor accumulation in detergent-resistant membrane microdomains, together with uncoupling of insulin receptor-IRS-1 signaling (Kabayama et al., 2005; Kabayama et al., 2007). Correspondingly, chemical inhibition of GM3 restores insulin responsiveness in leptin-deficient, insulin-resistant states (van Eijk et al., 2009). Our studies demonstrated striking and significantly increased expression of GM3 synthase in ethanol-fed rats, with only modest reductions in GM3 synthase gene expression in livers of the NAC-treated rats. Therefore, the combined effects of increased ceramide and GM3 ganglioside levels in the liver likely mediated ethanol-induced hepatic insulin resistance. The limited reductions in hepatic ceramide levels and GM3 expression effectuated by NAC treatment correspond with the modest rescue effects of NAC on the ethanol-induced hepatic insulin resistance.

Steatohepatitis produced by ethanol or chronic high fat feeding leads to impaired insulin and IGF signaling in liver (de la Monte et al., 2008; Svegliati-Baroni et al., 2006; Yeon et al., 2003). Included among the factors contributing to this phenotype are reduced hepatic expression of insulin/IGF polypeptides, their receptors, and IRS genes (de la Monte et al., 2008), and reduced phosphorylation

of receptor tyrosine kinases and IRS proteins (de la Monte et al., 2008; Yeon et al., 2003). In addition, chronic ethanol exposure causes insulin resistance with inhibition of signaling downstream through growth and survival pathways (He, Butler, Liu, & McNamara, 2006; Mohr, Tanaka, & Wands, 1998; Pang et al., 2009; Ronis et al., 2007; Sasaki & Wands, 1994). Correspondingly, in the present study, the main effects of chronic ethanol feeding were to inhibit phosphorylation and signaling through the insulin receptor, IGF-1 receptor, and IRS-1, as well as through Akt and p70S6K, while increasing activation of GSK-3 β .

Reduced levels of p^{S473}-pAkt indicate constitutive inhibition of growth and survival signaling in liver (Nawano et al., 1999), while the reduced levels of p^{S9}-GSK-3 β reflect constitutively increased GSK-3 β activity, and this also inhibits cell survival (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995). The findings of reduced Akt and increased GSK-3 β activities in ethanol-exposed livers are consistent with previous reports (de la Monte et al., 2008; Yeon et al., 2003). In addition to the impairments in survival and carbohydrate metabolism, we demonstrated significant ethanol inhibition of signaling downstream of PI3 kinase-Akt through p70S6K, and mTOR-dependent PRAS40 pathways, indicating that chronic ethanol feeding inhibits protein synthesis (Berven & Crouch, 2000) (Nascimento & Ouwens, 2009). Ethanol inhibition of p70S6K phosphorylation could have been mediated by inhibition of Akt, mTOR, or both, as the multiplex-ELISA detected p^{TpS421/424}-p70S6K; the T421 and S424 residues on p70S6K are phosphorylated by Akt and mTOR (Guillen, Navarro, Robledo, Valverde, & Benito, 2006). Since none of the inhibitory effects of ethanol on insulin/IGF/Akt signaling were abrogated by NAC treatment, anti-oxidant therapy alone would probably not be effective in restoring hepatic function or reducing progression of alcohol-related steatohepatitis.

In conclusion, this study demonstrates that NAC treatment has a role in reducing the severity of alcohol-induced steatohepatitis, but the therapeutic benefits are limited due to persistence of insulin/IGF signaling impairments in liver. In addition to decreasing inflammation, NAC reduced ceramide levels in liver. This effect was mediated by reductions in sphingomyelinase activity rather than changes in gene expression. We hypothesize that the NAC-mediated reductions in oxidative stress (Ronis et al., 2005; Samuhasaneeto, Thong-Ngam, Kulaputana, Patumraj, & Klaikeaw, 2007) led to improved membrane stabilization and attendant reductions in lipid breakdown with release of toxic sphingolipids, including ceramides. We propose that the histological correlate of this phenomenon is the NAC-associated shift in the pattern of steatosis from micro-vesicular to macro-vesicular. The findings herein reinforce our hypothesis that ethanol-induced steatohepatitis has dual independent but interactive pathogenic mechanisms: oxidative stress/inflammation and insulin/IGF resistance. While NAC and other anti-oxidant and radical scavenger treatments may aid in reducing the severity of oxidative stress/inflammation, additional therapeutic agents such as insulin sensitizers are probably needed to arrest the insulin/IGF-1 resistance arm of this disease, as previously suggested (de Oliveira et al., 2008; Pang et al., 2009).

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CHAPTER 3

Cytokine Profile and Effects of N-Acetylcysteine Treatment in a Rat Model of Alcohol-Induced Steatohepatitis

ABSTRACT

Background: Alcoholic steatohepatitis (ASH) is associated with pro-inflammatory cytokine activation, which correlates with progression and severity of alcoholic liver disease (ALD). The roles of tumor necrosis factor- α (TNF- α) and interleukins (IL-) 2, 6, 8 and 10 have been thoroughly investigated, but the contribution of other cytokines that may be important in the pathogenesis of oxidative stress and tissue injury with progression of ALD requires further study.

Hypothesis: Hepatic expression of pro-inflammatory and anti-inflammatory cytokines and chemokines is dysregulated by chronic ethanol exposure. Anti-oxidant treatment may reduce hepatocellular injury by normalizing inflammation and pro-inflammatory cytokine/chemokine expression in liver.

Methods: Adult male Sprague Dawley rats fed by total enteral nutrition (TEN) for 130 days, and were divided into 4 groups: control+vehicle (n=13); control+NAC (n=6); ethanol+vehicle (n=9); and ethanol+NAC (n=7). Ethanol comprised 37% of the caloric content in the liquid diets. Livers were used to measure cytokine and chemokine expression with a commercially prepared 24-plex bead-based ELISA panel.

Results: Pro-inflammatory TNF- α , IFN- γ , IL-1 β , IL-2, IL-6, and IL-12, and anti-inflammatory IL-4, IL-10 and IL-13 cytokines and the CXCL1, CCL11, CCL5 chemokines were similarly expressed in livers of control and ethanol TEN-fed rats. However, IL-1 α expression was significantly increased in the ethanol-exposed relative to controls, although NAC treatment did not decrease hepatic IL-1 α expression in either group. GM-CSF, G-CSF, MCP-1, and MIP-1 α were more abundantly expressed in alcohol TEN-fed relative to the control TEN-fed rats and the effect of NAC was to further increase G-CSF expression in both groups. Leptin on the other hand was not different among the study groups.

Conclusion: In this model cytokine/chemokine activation were in favor of a pro-inflammatory state, corresponding to the degree of hepatocyte inflammation. Anti-oxidant therapy had limited effect in modulating these effects, suggesting that the inflammatory response mechanisms may be independent of oxidative stress. Therefore other treatments are required to treat alcohol-induced liver injury mediated by inflammation.

3.1 INTRODUCTION

Alcoholic liver disease (ALD) encompasses a spectrum of pathological features, which can progress from simple steatosis, to more serious stages of steatohepatitis, cirrhosis and hepatocellular carcinoma (HCC) (Adachi & Brenner, 2005; Tilg & Day, 2007). Alcoholic steatohepatitis (ASH) the malign form of ALD, is characterized by hepatocyte necrosis and apoptosis, degeneration, and is associated with significant mortality (Sandahl, Jepsen, Thomsen, & Vilstrup, 2011). Central to the development of alcoholic steatohepatitis is endotoxin-mediated inflammatory response of Kupffer cells to alcohol (Adachi, Moore, Bradford, Gao, & Thurman, 1995; Nanji, Khettry, Sadrzadeh, & Yamanaka, 1993). Chronic alcohol exposure sensitizes CD14/Toll-like receptors on the surface of Kupffer cells to gut-derived lipopolysaccharides (LPS) (Nagy, 2003), which stimulate the production of cytokines. The functions of pro-, anti-inflammatory cytokines and chemokines are described in Table 9.

Tumor necrosis factor alpha (TNF- α), IL-1 β and IL-6 are the predominant pro-inflammatory cytokines implicated in ASH (Crews et al., 2006), leading to inflammation and hepatic necrosis. On the other hand Th2-mediated responses aim to counteract the effects of pro-inflammatory cytokine activation; therefore, it is the imbalance in favor of pro-inflammatory to anti-inflammatory cytokine milieu that mediates progression of alcoholic liver disease. Indeed in patients with ALD, serum (Bird, Sheron, Goka, Alexander, & Williams, 1990; McClain & Cohen, 1989) and adipose tissue (Naveau et al., 2010) TNF- α levels are elevated and correlate with degree of severity of liver disease and mortality. TNF- α elevations have also been

reported in the bloods of rats and mice exposed to ethanol (Thurman et al., 1998). Similarly, other lines of evidence show that IL-6, IL-8 and IL-12 levels correlate with ongoing alcohol consumption and disease severity (Huang et al., 1996; Tung et al., 2010), including clinical features of portal hypertension (Swiatkowska-Stodulska & Bakowska, 2004; Swiatkowska-Stodulska, Bakowska, & Drobinska-Jurowiecka, 2006) and mortality (Dominguez et al., 2009; Swiatkowska-Stodulska & Bakowska, 2004).

While the roles of pro- and anti-inflammatory cytokines in mediating injury in ALD have been studied, the role of chemokines, growth factors and leptin that could be potentially important has not been adequately assessed.

Simultaneously, the metabolism of alcohol contributes significantly to the pathogenesis of alcoholic steatohepatitis by promoting oxidative stress. One of the mechanisms of increased oxidative stress is depletion of glutathione (GSH), a radical scavenger and key attenuator of oxidative stress in liver. Alcohol feeding sensitizes hepatocytes to TNF- α as a result of reduced availability of mitochondrial glutathione (mGSH) (Fernandez-Checa & Kaplowitz, 2005). This observation was reproduced *in vitro* by selective pharmacological depletion of mGSH, which sensitized hepatocytes to TNF-mediated cell death (Colell et al., 1998). Moreover, oxidative stress also contributes to LPS-mediated stimulation of extracellular signal-regulated kinase (ERK) (Thakur, Pritchard, McMullen, & Nagy, 2006), further perpetuating the inflammatory response. In another study the prevalence of abnormal TNF- α was 5-fold higher in heavy drinkers with oxidative stress-induced IgG than in those without (Vidali et al., 2008). Thus oxidative stress itself plays a

key role in TNF-mediated alcoholic liver injury. Susceptibility to oxidative and radical injury however, rests on maintaining a balance between pro-oxidant and anti-oxidant factors.

Therefore, NAC therapy may reduce liver injury associated with pro-cytokine and chemokine inflammation and progression from simple steatosis to steatohepatitis in ALD. The present study utilizes a rat model of chronic ethanol feeding to evaluate the cytokine milieu and the potential use of NAC for reducing the severity of alcohol-related pro-inflammatory cytokine activation in the liver.

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3.2 MATERIALS AND METHODS

Materials: The protein concentration was measured as before using the bicinchoninic acid (BCA) kit and the rat cytokine Milliplex xMAP kit was utilized for the multiplex ELISAs (see Chapter 2).

Experimental Model: The same model as that described in Chapter 2 was used for cytokine measurements.

Multiplex ELISA: We used the same methods as those previously described to measure immunoreactivity to a panel of 24 selected pro-inflammatory cytokines and chemokines in liver tissue.

Statistical Analysis: Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers), while tabulated data reflect means \pm SEMs for each group. Intergroup comparisons were made using the Kruskal-Wallis one-way analysis of variance (ANOVA) with the Dunn's multiple comparisons test. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Significant P-values (<0.05) are shown within the panels.

3.3 RESULTS

Effects of ethanol and NAC treatments on cytokine levels in liver: To determine the degree to which chronic ethanol feeding stimulates pro-inflammatory states and whether NAC treatment reduces cytokine activation we used a bead-based 24-panel cytokine multiplex ELISA system. Thus to profile cytokine activation in this model we measured hepatic levels of the following cytokines: pro-inflammatory (TNF- α , IFN- γ , IL-1 α , IL-1 β , IL-2, IL-5, IL-6, IL-9, IL-12, IL-17, IL-18); anti-inflammatory (IL-4, IL-10, IL-13); chemokines (MIP-1 α , MCP-1, RANTES, GRO/KC (CXCL-1), IP-10 and eotaxin), leptin and growth factors (G-CSF, GM-CSF, and VEGF).

Pro-inflammatory cytokines: Hepatic levels of TNF- α , IFN- γ , IL-1 β , IL-2, IL-5, IL-6, IL-9, and IL-12p were similarly expressed in the livers of control- and alcohol-TEN fed rats, irrespective of treatment with NAC ($p > 0.05$) (Figure 2.8). However the levels of IL-1 α ($p = 0.048$) were higher in ethanol-TEN fed rats as compared to control-TEN fed rats, with no obvious benefit of treatment with NAC (Figure 3.1 A). IL-17 (Figure 3.1 B) and IL-18 (Figure 3.1 C) are both involved in microbial defense; in particular IL-18 induces cell-mediated immunity following injection with lipopolysaccharide (LPS). Although both IL-17 and IL-18 were expressed at similar levels in control- and alcohol-TEN fed rats,

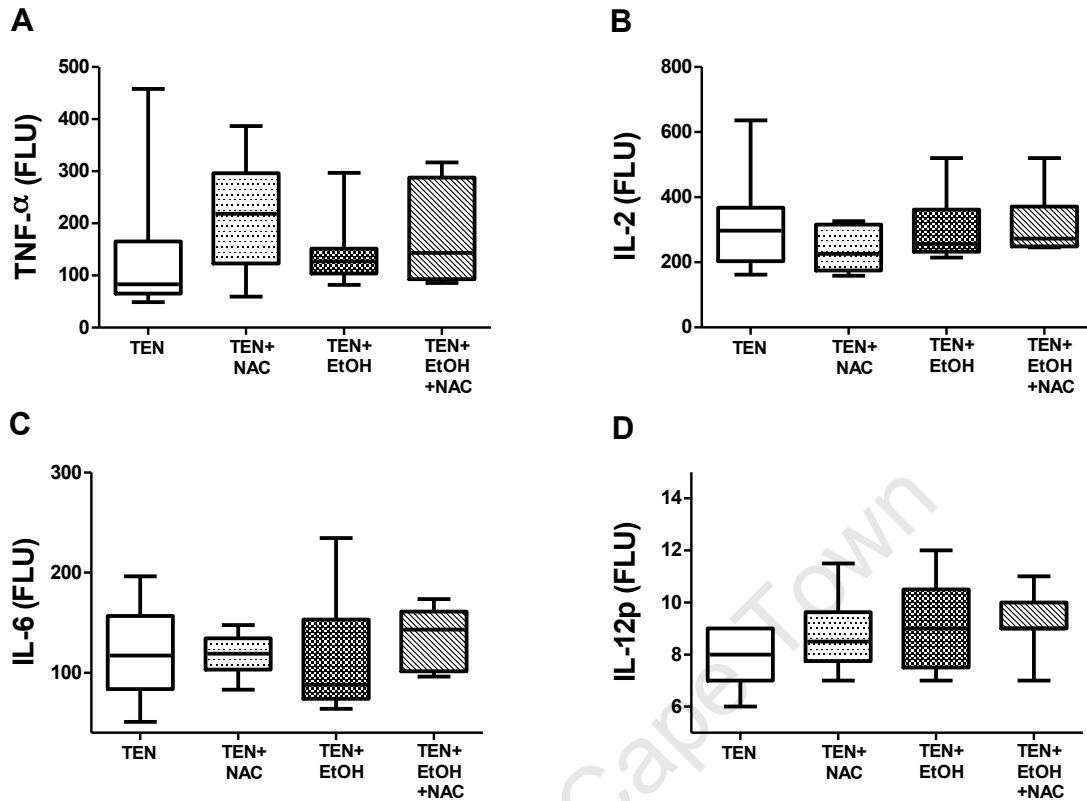


Figure 3.1: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on pro-inflammatory cytokine profile (non-significant). A bead-based multiplex ELISA was used to measure immunoreactivity (see Methods and Materials). Immunoreactivity to (A) TNF- α , (B) IL-2, (C) IL-6 and (D) IL-12p were measured. Liver tissues were homogenized in Tris-HCl buffer containing protease and phosphatase inhibitors. Samples containing 200mg protein were incubated with the beads, and capture antigens were detected with biotinylated secondary antibodies and phycoerythrin-conjugated Streptavidin. Data are expressed as fluorescence light units corrected for protein concentration. Inter-group comparisons were made using the Kruskal-Wallis one-way ANOVA with the Dunn's multiple comparison tests.

treatment with NAC served to further up-regulate these levels with the biggest difference between control-TEN and NAC-treated alcohol-TEN fed rats ($p < 0.01$) in the former, suggesting that treatment with NAC was not effective.

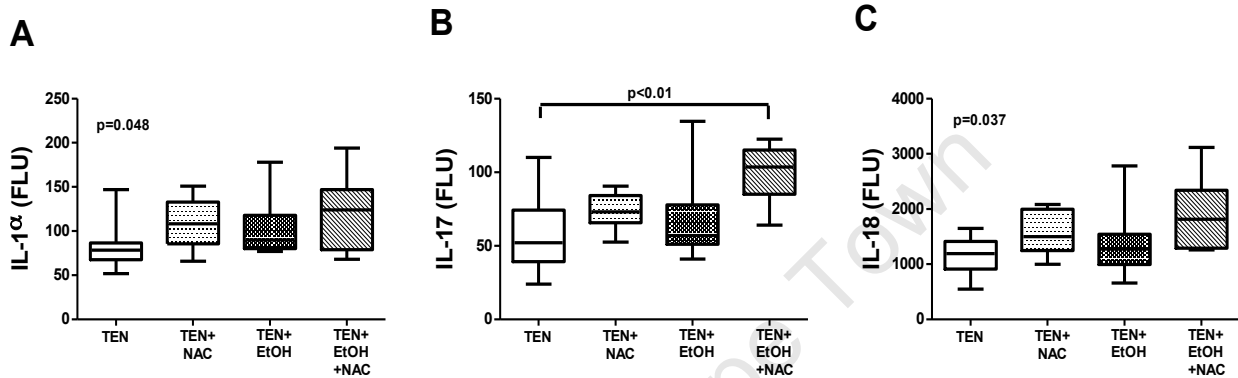


Figure 3.2: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on pro-inflammatory cytokine profile (significant). A bead-based multiplex ELISA was used to measure immunoreactivity (see Methods and Materials). Immunoreactivity to (A) IL-1 α , (B) IL-17 and (C) IL-18 were measured. Liver tissues were homogenized in Tris-HCl buffer containing protease and phosphatase inhibitors. Samples containing 200mg protein were incubated with the beads, and capture antigens were detected with biotinylated secondary antibodies and phycoerythrin-conjugated Streptavidin. Data are expressed as fluorescence light units corrected for protein concentration. Inter-group comparisons were made using the Kruskal-Wallis one-way ANOVA with the Dunn's multiple comparison tests. Significant P-values are indicated within the panels.

Anti-inflammatory cytokines: There were no differences observed in the hepatic expression levels of IL-4, IL-10 and IL-13 between the control- and alcohol-TEN fed rats. Similarly, co-administration of NAC had no significant effect (Figure 3.3).

Chemokines: GRO/KC, eotaxin, and IP-10 expression were essentially identical in livers of all rats in the study, with no beneficial effects of NAC. The expression levels of MCP-1 and MIP-1 α were generally low in control- and ethanol-TEN fed rats, and were upregulated in both groups with NAC treatment, in essence, showing no significant modulation of chemokine expression with treatment (Figure 3.4).

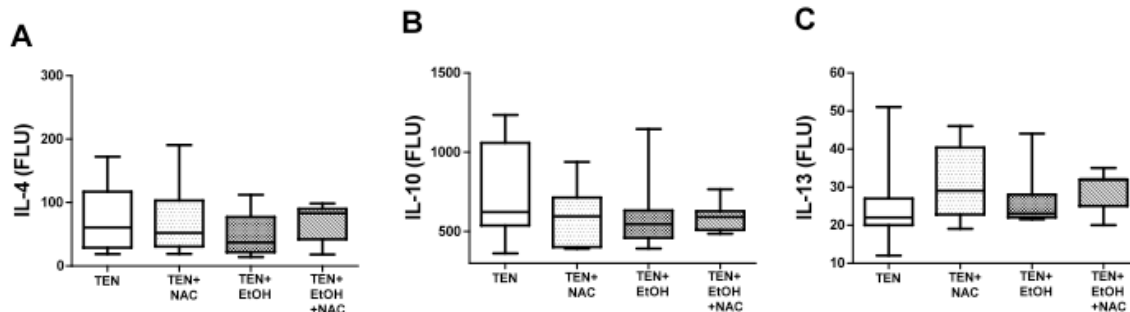


Figure 3.3: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on anti-inflammatory cytokine profile (non-significant). A bead-based multiplex ELISA was used to measure immunoreactivity (see Methods and Materials). Immunoreactivity to (A) IL-4, (B) IL-10 and (C) IL-13 were measured. Liver tissues were homogenized in Tris-HCl buffer containing protease and phosphatase inhibitors. Samples containing 200mg protein were incubated with the beads, and capture antigens were detected with biotinylated secondary antibodies and phycoerythrin-conjugated Streptavidin. Data are expressed as fluorescence light units corrected for protein concentration. Inter-group comparisons were made using the Kruskal-Wallis one-way ANOVA with the Dunn's multiple comparison tests.

Growth factors: Hepatic expression levels of VEGF and GM-CSF were essentially similar in control- and alcohol-TEN fed rats, with no effect of NAC co-administration. G-CSF hepatic levels were upregulated in alcohol-TEN fed compared to control-TEN fed rats ($p < 0.05$). In both groups co-treatment with NAC ameliorated these levels (Figure 2.11 C).

Leptin: In human subjects serum leptin correlates with degree of alcoholic steatosis, therefore we wanted to profile leptin levels with steatohepatitis and assess the therapeutic benefits of NAC. Leptin levels were similar in control- and ethanol-TEN fed rats, with no effect of NAC treatment (data not shown).

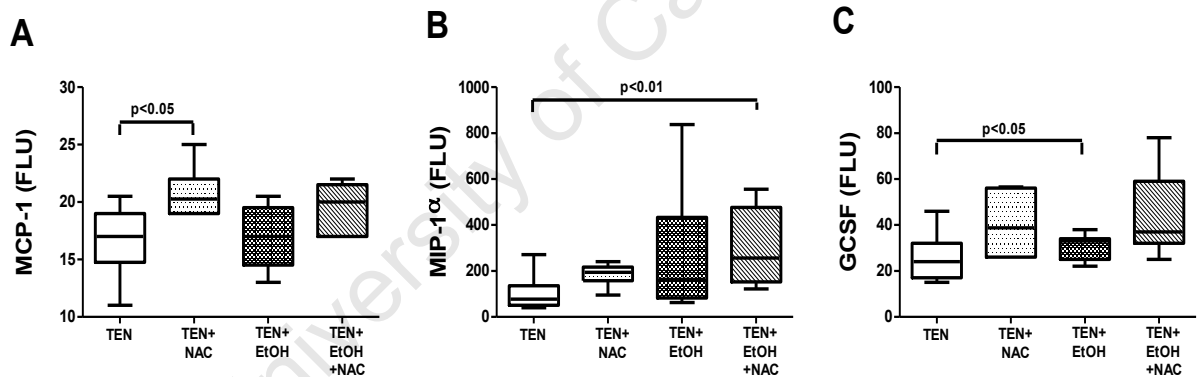


Figure 3.4: Effects of control- or ethanol-containing TEN feeding and N-acetylcysteine (NAC) on chemokine and growth factor profile (significant). A bead-based multiplex ELISA was used to measure immunoreactivity (see Methods and Materials). Immunoreactivity to (A) MCP-1, (B) MIP-1 α and (C) GCSF were measured. Liver tissues were homogenized in Tris-HCl buffer containing protease and phosphatase inhibitors. Samples containing 200mg protein were incubated with the beads, and capture antigens were detected with biotinylated secondary antibodies and phycoerythrin-conjugated Streptavidin. Data are expressed as fluorescence light units corrected for

protein concentration. Inter-group comparisons were made using the Kruskal-Wallis one-way ANOVA with the Dunn's multiple comparison tests. Significant P-values are indicated within the panels.

3.4 DISCUSSION

The objective of the present work was to primarily profile key cytokines that mediate inflammation and subsequent tissue destruction with alcohol-mediated liver injury. Secondly the study aimed to determine other less well-studied cytokines, chemokines and growth factors that may also be of value in determining likely worse outcomes in alcoholic steatohepatitis and to determine the effects of anti-oxidant therapy in reducing the injurious consequences of alcohol.

The effects of alcohol-TEN feeding in this model were increased inflammation associated with apoptosis, necrosis and architectural disarray. In addition there was corresponding increased triglyceride and ceramide accumulation (see previous chapter). Finally, the effect of NAC was to primarily reduce the inflammatory response. These findings are described more extensively in the preceding section.

The degree of inflammation is directly proportional to the amounts of pro-inflammatory relative to anti-inflammatory cytokines. Traditionally measurements of cytokines are done in serum, although measurement in other tissues particularly adipocytes (Naveau et al., 2010) has been described. Cytokines are signaling molecules that have systemic immunomodulating functions, i.e. endocrine properties, therefore we hypothesized that hepatic cytokines spill over into the bloodstream thus, measurement of hepatic cytokines would be reflective of serum cytokines. In this model, pro-inflammatory cytokines IL-1 α , IL-17 and IL-18 were upregulated from basal levels with NAC treatment. Thus anti-oxidant treatment resulted in a heightened pro-inflammatory response. A possible explanation for this

paradox could be that in early disease (i.e. before the onset of fibrosis as is the case in this model) a predominant pro-inflammatory is appropriate as an immune response to LPS-mediated endotoxaemia, and this response is improved by treatment, however with prolonged insult and aberrant stimulation of the immune response, this results in cell necrosis and subsequent fibrosis. Our model is not that of acute alcoholic hepatitis, but can be characterized as a sub-acute injury model given the period of exposure to alcohol and lack of fibrosis. Furthermore, IL-1 α has almost identical functions to TNF- α , which may suggest that either is sufficient to induce an inflammatory response. Therefore the lack of significant up-regulation of TNF- α in this model, as has been reported by others (Bird et al., 1990; Gobejishvili et al., 2006; Kishore, McMullen, Cocuzzi, & Nagy, 2004; Latvala et al., 2005; McClain, Hill, Song, Deaciuc, & Barve, 2002), may be explained on this basis. An alternative explanation (as stated in the previous chapter) could be that previous studies measured TNF- α in isolated hepatic Kupffer cells, whereas we measured total hepatocyte TNF- α levels; which could have masked actual differences between the groups. In contrast none of the anti-inflammatory cytokines were significantly different stratified by diet or treatment. One would have expected alcohol to downregulate anti-inflammatory cytokines and attenuation by NAC treatment. The reasons for this are not clear. Chemo-attractant ability was enhanced with co-treatment with NAC (upregulation of MCP-1 and MIP-1 α), and a similar effect was noted with levels of G-CSF. This finding may suggest that their down-regulation with TEN feeding with or without alcohol has a mechanism dependent on oxidative stress. Furthermore the upregulation of G-CSF with NAC

could be indicative of the reparative process undertaken by injured hepatocytes in an attempt to regenerate.

The pathogenesis of alcoholic liver disease is very similar to that of non-alcoholic steatohepatitis; in this context the role of leptin in alcoholic steatohepatitis would be informative in further understanding pathogenesis in ALD. Leptin, in addition to endocrine functions, modulates the immune system. Its effect is that of pro-inflammatory cytokine activation, thus promoting a Th1 response (Fernandez-Riejos et al., 2010; Tsochatzis, Papatheodoridis, & Archimandritis, 2006). In this study there was no difference in hepatic expression of leptin levels in control- and alcohol-TEN fed rats. Perhaps this is not surprising noting that leptin is produced in adipocytes, therefore hepatic measurements are not likely to be representative or give reliable results.

To summarize, this model of ethanol-induced steatohepatitis in a rat showed evidence of steatosis and inflammation associated with an imbalance in favor of pro-inflammatory cytokine and chemokine activation. Whilst the administration of anti-TNF agents have to date yielded mixed results in both animal models and humans, the quest for other agents that can be targeted against dysregulated immune function remains important and therefore, understanding the role of key cytokines is an important step towards achieving this goal. The inflammatory response in this model was not sufficiently robust; hence an accurate assessment of the utility of anti-oxidant therapy was not possible.

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APPENDIX

Table 9: Contributions of “important” cytokines to immunologic responses

Cytokine	Produced by	Functions
IL-1	Macrophage Many cells	Causes fever Induces other cytokines Causes T-cell stimulation Induces metalloproteinases and prostaglandins Increases adhesion molecule expression
IL-2	T cells	Increases T-cell proliferation Activates B-cells
IL-4 (anti)	T cells	Signal for immunoglobulin switch and increases IgE Decreases production of pro-inflammatory cytokines Suppresses delayed type hypersensitivity (Th1 cells)
IL-5	T cells Possibly mast cells	Triggers activated B-cell and eosinophil differentiation and proliferation Regulates expression of genes involved in proliferation, cell survival and maturation of B-cells and eosinophils
IL-6	Many cells	Stimulates B- and T-cell proliferation Acute phase reactant Induces natural protease inhibitor (TIMP)
IL-12 (p70)	Macrophages	Increases interferon-gamma production and Th1 cell differentiation
IL-13	T cells	Similar to IL-4

IL-17	Many cells	Implicated in many autoimmune and inflammatory diseases Role in microbial defense
IL-18	Macrophages	Together with IL-12, induces cell-mediated immunity following infection with e.g. LPS Stimulates natural killer cells and certain T-cells to release interferon gamma that activates other macrophages, IFN gamma and IL-12 Inhibits IL4-dependent IgE and IgG1 production
TNF-alpha	T cells Macrophages	Induces cachexia Induces other cytokines (i.e. RANTES) Stimulates T-cells Induces metalloproteinases and prostaglandins Increases adhesion molecule expression
IFN-gamma	T cells	Increases adhesion molecules Promotes HLA-DR expression, activation of T-cells, NK cells, and macrophages
Colony stimulating factors		
GM-CSF	Macrophages Fibroblasts T cells	Myeloid differentiation, and macrophage activation
G-CSF	Macrophages	Granulocyte differentiation and activation
Chemokines		
CXCL1 (previously GRO/KC)	Macrophages Neutrophils Epithelial cells	Attracts neutrophils Is involved in the acute inflammatory response
CXCL10 (IP-10)	Monocytes	Attracts activated T-lymphocytes,

	Endothelial cells Fibroblasts	NK cells and dendritic cells Promotes T-cell adhesion to endothelial cells
CCL11 (Eotaxin)	Epithelial cells Endothelial cells Smooth muscle cells Many cells	Chemotactic to eosinophils
CCL2 (MCP-1)	Monocytes Macrophages Dendritic cells	Chemotactic to monocytes, memory-T and dendritic cells
CCL3 (MIP-1 α)	Dendritic cells NK cells Other cells	Mediates monocyte and neutrophil chemotaxis
CCL5 (Rantes)	T-cells and T-cell clones, but not T-cell lines	Chemotactic for T-cells, eosinophils, basophils Recruits leucocytes Activates eosinophils Increases adherence of monocytes to endothelial cells
Growth factors		
VEGF	Many cells	Fibroblast proliferation, and angiogenesis

Adapted from 2011 UpToDate 18.3 (<http://www.uptodate.com/contents/role-of-cytokines-in-the-immune-system>)

CHAPTER 4

Ceramide inhibitors ameliorate alcohol-induced steatohepatitis in an ex-vivo liver slice culture model

ABSTRACT

Background: Steatohepatitis with attendant activation of pro-inflammatory cytokines, insulin resistance, and increased levels of reactive oxygen species (ROS) caused by mitochondrial dysfunction, endoplasmic reticulum (ER) stress, lipotoxicity, and DNA damage, play pivotal roles in the pathogenesis of chronic alcohol-related liver disease (ALD). Recent studies demonstrated roles for increased expression of pro-ceramide genes and ceramide accumulation in relation to severity of both ALD and non-alcoholic liver disease (NAFLD), since cytotoxic ceramides cause insulin resistance and lipotoxicity in liver.

Hypothesis: Severity of hepatic steatosis, cytotoxicity, and ER dysfunction can be abated by treatment with chemical inhibitors of ceramide synthesis.

Methods: Adult male Long Evans rats were pair-fed with isocaloric liquid diets containing 0% or 37% ethanol by caloric content for 8 weeks. Precision-cut fresh liver slices (150 μm thick) were cultured and treated with vehicle, 25 μM fumonisin B1, 10 μM myriocin, or 50 μM desipramine for up to 72 h. Cultured liver slices

were subjected to histological studies, measurement of cytotoxicity (G6PD release), insulin and ER stress signaling.

Results: Ethanol-exposed cultures exhibited steatohepatitis with significantly higher mean levels of triglyceride accumulation and G6PD release relative to control. Desipramine significantly reduced neutral lipid and ceramide levels, and ceramide inhibitor treatments in general lessened the severity of steatohepatitis, reduced ethanol-mediated up-regulation of ER stress markers, and restored insulin network signaling. While PERK was downregulated by ceramide inhibitor treatment, insulin signaling molecules were unaffected.

Conclusions: Reversing the histopathological, biochemical, and molecular indices of chronic ALD will likely require a multi-pronged approach aimed at minimizing lipotoxicity, ER stress, and insulin resistance. The ex vivo precision cut slice culture model is an excellent tool for effectively evaluating novel treatments for ALD.

4.1 INTRODUCTION

Chronic alcohol consumption is a major cause of chronic liver disease. The progression of liver disease through the stages of chronic hepatitis, fibrosis and cirrhosis and ultimately end-stage liver disease including hepatocellular carcinoma is well described (de Alwis & Day, 2008). In contrast the progression of relatively benign alcoholic steatosis to the malign steatohepatitis is less well understood. Several factors associated with progression of disease include impaired lipid metabolism and homeostasis (lipotoxicity), oxidative stress, pro-inflammatory cytokine activation, and insulin resistance (Carter-Kent et al., 2008; Farrell & Larter, 2006).

The role of ceramides as key mediators of lipotoxicity with attendant increased inflammation, oxidative stress, and insulin resistance in both ALD and NAFLD has been discussed in the second chapter. Additionally the structural and functional roles of ceramides, particularly in promoting insulin resistance were similarly reviewed. Finally ceramide synthetic and degradation pathways were outlined in the second chapter; as such the reader is referred to it. However, for revision, a figure depicting the metabolic pathways of sphingolipids is included in this chapter (Figure 4.1). In this chapter we investigate the role of ceramides in attenuating lipotoxic-associated insulin resistance by employing an ex vivo slice culture model of ASH, and by simultaneously administering ceramide inhibitor treatment. Our work in Chapter 2 showed evidence that ceramide accumulation as a result of upregulated ASMase activity likely induced the lipotoxicity and attendant inflammation in a rat model of ASH. Conceptually therefore, administering

inhibitors of ceramide synthesis will ameliorate these effects. In fact, in rodent and human experimental conditions of lipid infusions the administration of myricocin, inhibitor of serine palmitoyl transferase, improved glucose uptake and inhibition of hepatic gluconeogenesis (Holland, Brozinick et al., 2007).

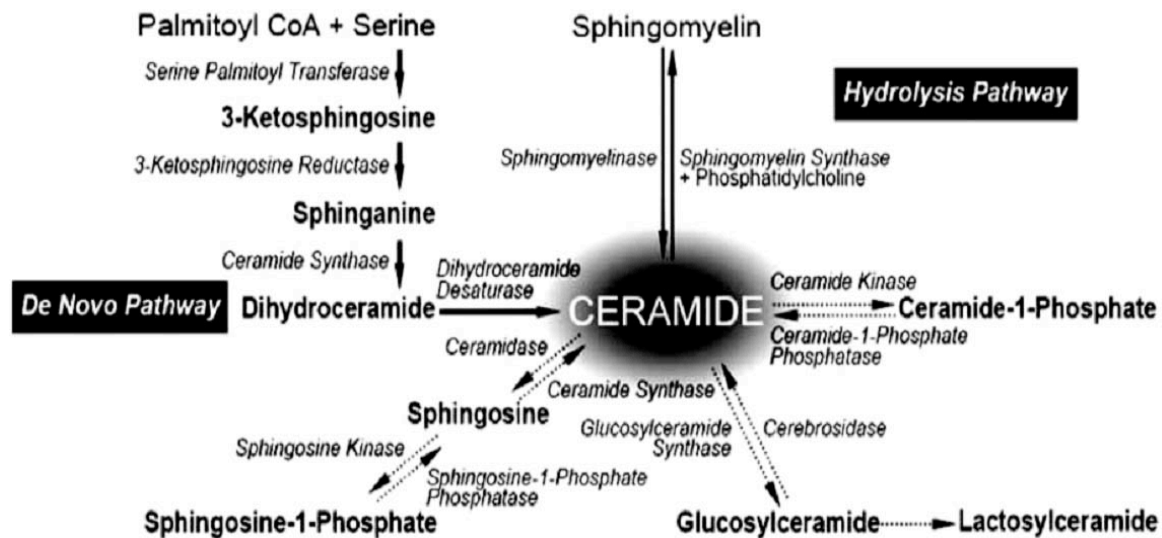


Figure 4.1: Schematic representation of sphingolipid metabolism including the de novo, hydrolysis and salvage pathways (Source: Lin, C. F., Chen, C.L., & Lin, Y.S, 2006)

Ceramide inhibitors work by blocking enzymes in the synthetic or degradation pathways of ceramide synthesis. For our purposes, we selected three ceramide inhibitors that have been used in both animal and human experiments, based on published data. Fumonisin B1 is a mycotoxin produced from *F. moniliforme*, a prevalent fungus of corn and other grains (Gelderblom et al., 1988). It functions as an inhibitor of ceramide synthase (sphingosine N-acyltransferase) (Balsinde, Balboa & Dennis, 1997). The inhibition of ceramide synthase blocks the

formation of ceramide from sphinganine and fatty acyl-CoA via de novo synthesis or from sphingosine via ceramidase-mediated ceramide degradation. The consequences of ceramide synthase inhibition include increased tissue and serum concentrations of sphinganine, sphingosine, and their 1-phosphate metabolites as well as decreases in complex sphingolipids downstream of ceramide (Riley et al., 1999). Myriocin is an amino fatty acid antibiotic derived from *Mycelia sterilia*. It is a potent immunosuppressant, but its relevance in this context is that it is a potent inhibitor of serine palmitoyltransferase ($K_i = 0.28$ nM), the enzyme that catalyzes the first step of sphingolipid biosynthesis (Hidari, Ichikawa, Fujita, Sakiyama & Hirabayashi, 1996). Serine palmitoyltransferase inhibition by myriocin results in a decrease of de novo synthesis of free sphingoid bases and ceramide (Miyake, Kozutsumi, Nakamura, Fujita, & Kawasaki, 1995). Therefore, myriocin has been shown to have a potential effect of being used as a therapeutic agent to treat fumonisin-related diseases based on its ability to block fumonisin B1-induced accumulation of free sphingoid bases (Riley et al., 1999). Lastly, desipramine, a tricyclic anti-depressant, induces irreversible intracellular proteolytic degradation that is specific to the acid sphingomyelinases and therefore, has been widely used as a specific acid sphingomyelinase inhibitor (Albouz et al., 1981; Hurwitz, Ferlinz & Sandhoff, 1994). Therefore, it inhibits the generation of ceramide via the degradation pathway. Thus the use of a combination of both ceramide de novo synthesis and catalytic pathway inhibitors, would allow us to evaluate the broad roles of ceramide inhibition in this model, and compare the effectiveness of each of these inhibitors.

Moreover alcohol causes organelle dysfunction particularly that of mitochondria (reviewed by Hoek JB et al) and the endoplasmic reticulum (ER) (Hoek, Cahill, & Pastorino, 2002). The metabolism of alcohol causes altered redox potential, oxidative stress and acetaldehyde adducts, all of which are implicated in inducing ER stress. The unfolded protein response (UPR), upregulation of protein chaperones and with prolonged insult induction of apoptosis are the key regulatory mechanisms involved in reducing ER stress. An interesting and recent development is the role of the ER in ALD, particularly as is mediated by homocysteinemia (Hamelet, Demuth, Paul, Delabar, & Janel, 2007; Ji & Kaplowitz, 2003; Werstuck et al., 2001). Its contribution has been demonstrated in various animal models, with resultant altered expression of genes of the UPR (Esfandiari, Villanueva, Wong, French, & Halsted, 2005; Ji & Kaplowitz, 2003; Ji, Mehrian-Shai, Chan, Hsu, & Kaplowitz, 2005; Tazi et al., 2007). Notwithstanding, the role of ER stress in the pathogenesis of alcoholic liver disease has not been completely elucidated and thus deserves further study.

Studying the metabolic effects of alcohol and that of administered therapy requires a model that elegantly represents human disease. While this has been achieved *in vivo*, with the various animal models of ALD, the ability to do controlled experiments at different time points and under varying conditions is expensive and time-consuming. Precision-cut liver slices (PCLS) are ideally suited for this purpose. PCLS have been used successfully in drug metabolism and toxicity studies (Ekins, 1996; Gandolfi, Wijeweera, & Brendel, 1996; Groneberg, Grosse-Siestrup, & Fischer, 2002; Parrish, Gandolfi, & Brendel, 1995) and chemically -

induced hepatocarcinogenesis (Godoy, Diaz Gomez, & Castro, 1983, 1984; Goodall, 1984; Mirvish, Ji, Makary, Schut, & Krokos, 1987). The advantage of this system in addition, is in its ability to retain tissue organization and maintain cell-to-cell matrix interactions (Groneberg et al., 2002) and therefore closely recapitulates the in- vivo situation.

Therefore we hypothesized that the use of rat model of alcoholic steatohepatitis in an in-vivo system whereby chemical inhibitors of ceramide synthesis or degradation could be directly administered to the liver would be ideally suited to test the utility of PCLS and simultaneously the effects of treatment on the metabolic consequences of alcohol.

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4.2 MATERIALS AND METHODS

Materials: The rats were purchased from Harlan Sprague Dawley Inc (Indianapolis, Indiana) and the diet in powder form from BioServ (Frenchtown, NJ). Dulbecco's modified Eagle's medium used for culture was from Sigma-Aldrich (St Louis, Mo) and the medium was enriched with amino acids, penicillin and streptomycin from Lonza (Basel, Switzerland). Precision-cut slices of liver tissue were obtained using the Mcllwain Tissue Chopper from Ted Pella Inc (Redding, CA). The same methods and protocols described in Chapter 2 were utilized for this experiment for the measurement of protein, lipids and the performance of PCR and ELISAs. Additionally, to measure cytotoxicity levels the Vybrant™ Cytotoxicity Assay Kit from Molecular Probes Inc (Eugene, OR) was used.

Experimental Model: Adult male Long Evans rats were fed a liquid control- or ethanol-containing diet (37% by caloric content), housed and monitored as described previously (de la Monte et al., 2008) for a period of 5 months. Livers harvested at sacrifice were cut into smaller 8mm cores that were used to generate precision-cut slice cultures (150um thick) using the Mcllwain Tissue Chopper. This was done under a hood following strict aseptic techniques. The liver slices were placed in 300µl full medium in 24-well multiwell culture plates (Greiner Bio-One, Monroe, NC) and separated with fine-needle forceps under a dissection microscope. The slices were subsequently maintained in a culture medium supplemented with vehicle, 25 µM fumonisin B1, 10 µM myriocin, or 50 µM desipramine (Table 10), in an incubator for up to 72 h. In addition, at harvest a piece of liver tissue was immediately immersion fixed in Histochoice for

histopathological analysis in order to serve as a control for the histology from the liver slices. After intervals of 24, 48 and 72 hours each of incubation, liver slices were homogenized in weak lysis buffer or TRIzol for protein and RNA extraction respectively, or immersion fixed in Histochoice. The experiments were performed in accordance with protocols approved by Institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital, and comply with the National Institutes of Health guidelines provided in the *Guide for the Care and Use of Laboratory Animals*.

Table 10: Ceramide inhibitors used in experiment

Name	Abbreviation	Formula weight	Enzyme	Final concentration
Fumonisin B1	Fumo	721.8	Ceramide Synthase	25 μ M
Myriocin	Myr	401.54	Serine Palmitoyl Transferase	10 μ M
Desipramine HCL	Desi	302.84	Acid Sphingomyelinase	50 μ M

* μ M = micromolar

Histopathology: The same histopathological criteria that were used in the in-vivo ASH model were used to analyze both the sliced and unsliced “control” liver tissue. (See Chapter 2)

Lipid studies: Lipids were measured from protein homogenates of liver tissue. Lipid content was measured using the Nile red fluorescence-based microplate assay as described (Lyn-Cook et al., 2009) and triglycerides were measured using the Serum Triglyceride determination Kit was purchased from Sigma-Aldrich Co (St Louis, MO). Results were normalized to protein concentration. Ceramide immunoreactivity was measured in protein extracts using a direct-binding ELISA as previously described.

Cytotoxicity assay: Following incubation of liver slices after 24, 48 and 72 hour time points, and prior to homogenization, 50µl of the full medium bathing the slices was aspirated and transferred to a white Optiplate for cytotoxicity (G6PD release) measurements. We added 50µl of the 2X Resazurin/reaction mixture and incubated the plate at 37°C for 30 minutes. Fluorescence was read in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA) at an absorbance of 530 nm and emission of 590 nm. The assay was performed according to the manufacturer’s protocol. Results were normalized to protein concentration.

Duplex Enzyme-Linked Immunosorbant Assay (ELISA): To quantify ER stress markers, we measured immunoreactivity to protein kinase RNA-like endoplasmic reticulum kinase (PERK), C/EBP homologous protein (CHOP), eukaryotic

inhibitory factor 2- α (eIF2- α) and 78 kiloDalton glucose-regulated protein 78 (GRP78) using duplex ELISA. Tissue homogenates were prepared in radio-immunoprecipitation assay buffer containing protease and phosphatase inhibitors, as previously described (Moroz et al., 2008). Thus the control antibody (large ribosomal protein or RPLPO) was measured in the same well as the antibody of interest, giving a more reliable result. Briefly, 50 μ l of protein was adsorbed to the bottom of 96-well Maxisorp plate overnight at 4°C. After blocking with a 1% BSA-Tris buffered saline solution for 3 hours, the samples were incubated with mouse monoclonal primary antibodies (0.1-1.0 μ g/ml) for 1 hour at room temperature. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies (1:10000) and Amplex Red soluble fluorophore substrate were used to detect immunoreactivity (Cohen, Tong, Wands, & de la Monte, 2007). Amplex Red fluorescence was measured (Ex 530 nm/Em 590 nm) in a M5 microplate reader. To detect the biotin-labelled RPLPO we used anti-RPLPO-biotin antibodies conjugated with streptavidin-alkaline phosphatase and 4-MUP substrate, and measured the fluorescence at an excitation of 360 nm and emission of 450 nm. Negative controls included reactions in which the primary or secondary antibody was omitted.

Multiplex ELISA: We used bead-based multiplex ELISAs to examine the integrity of insulin and IGF-1 signaling networks as before.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Assays: Liver tissue was homogenized in QIAzol reagent. RNA isolated using the EZ1 RNA Universal Tissue Kit and the BIO Robot EZ1 (Qiagen Inc., Valencia, CA),

was reverse transcribed with random oligodeoxynucleotide primers and the AMV First Strand cDNA synthesis kit. The cDNAs were used to measure gene expression by qPCR with universal probe-based primer pairs (Roche Diagnostics Corporation, Indianapolis, IN). The Lightcycler Roche instrument and software (Roche Diagnostics Corporation, Indianapolis, IN) were used to detect amplified signals from triplicate reactions. Relative mRNA abundance was corrected to actin measured in the same well as the samples in a duplex system. Template-free reactions served as negative controls.

Statistical Analysis: Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers), while tabulated data reflect means \pm SEMs for each group. Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Significant P-values (<0.05) are shown within the panels or tables.

4.3 RESULTS

Effects of ethanol and ceramide inhibitors on steatohepatitis: H&E stained sections of liver slices of control-fed rats demonstrated the expected cord-like architecture, normal hepatocyte nuclei and absent portal lympho-mononuclear inflammatory cell infiltrates, and scattered hepatocytes with macrosteatosis i.e. grade 1 (Figure 4.2 A-D, right panel). In contrast, in vivo chronic ethanol exposure caused steatohepatitis; ethanol-fed livers exhibited a disordered architecture with micro-vesicular steatosis that was predominantly distributed in Zone 2, wide sinusoids, and loss of nuclei grades 2-3 (Figure 4.2 E-H, right panel). Treatment with ceramide inhibitors led to an improvement in the histological appearance of alcoholic steatohepatitis compared to vehicle, with the most effect seen with fumonisin B1 (Figure 4.2 G, right panel). In addition, histological slides were obtained from liver tissue harvested de novo from control (Figure 4.2 A-C, left panel) and ethanol-fed (Figure 4.2 D-F, left panel) rats i.e. the livers were not sliced. These were used to serve as controls for the histological appearance and quality of liver slices. The architectural integrity of the liver slices at histology was maintained and the same detail as in the quality-control slides was seen. Overall, there were clearly discernible effects of ceramide inhibitor treatments, particularly fumonisin B1 on the histological appearance of the control and ethanol-fed liver slices.

Hepatic steatosis was further characterized by measuring neutral lipid, triglyceride, and ceramide content in liver homogenates. Nile red measured at 48

hours demonstrated a higher mean level of neutral lipids in livers of ethanol- relative to control-fed rats treated with vehicle, however the difference was not

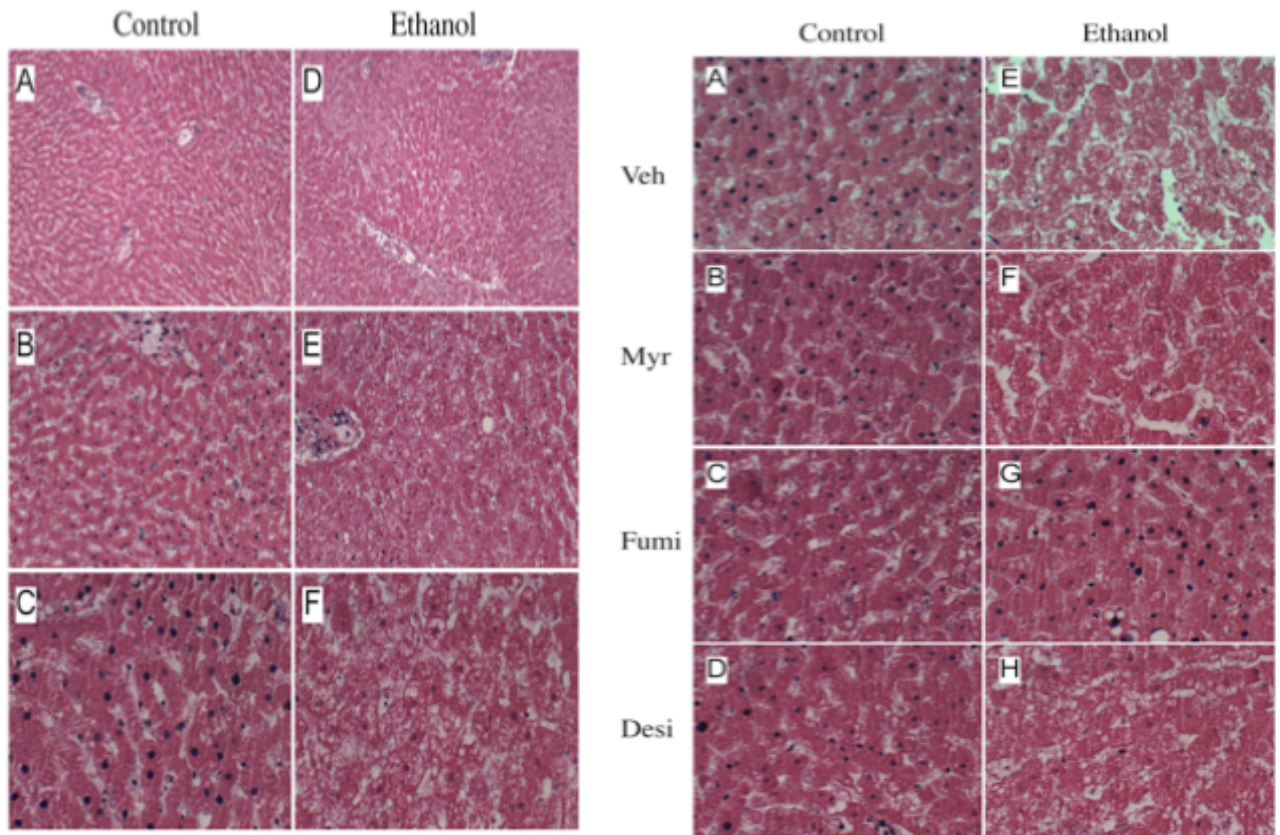


Figure 4.2: Effects of chronic ethanol feeding and ceramide inhibitor treatment on steatohepatitis (72 hours). Adult male Long Evans rats were fed for 5 months with a control or ethanol diet (37% by caloric content), and treated with vehicle or ceramide inhibitors viz: fumonisins B1 (fumi), myriocin (myr) and desipramine (desi). Paraffin-embedded histological sections (5 μ m thick) of whole pieces of liver (left panel) or liver slices (right panel) were stained with H&E and photographed at 40x (A, D), 160x (B, E), and 400x (C, F) magnifications in left panel and 400x magnification in right panel (A-H).

marked (data not shown). In control-fed rats, treatment with ceramide inhibitors resulted in similar or reduced levels of neutral lipids as in the vehicle-treated group, suggesting that these agents have a lipid-lowering effect under basal conditions. However, at 48 hours post-incubation (Figure 4.3 A) ceramide inhibitors did not reduce ethanol-induced elevations of neutral lipid accumulation, as evidenced by a near doubling of neutral lipid accumulation in ethanol-fed rats ($p < 0.001$). Similarly at 24 hours (Figure 4.4 A) triglyceride accumulation in ethanol-fed rats was higher than that in control-fed counterparts more so with fumonisin B1 ($p < 0.05$) and myriocin ($p < 0.001$) treatments than desipramine. Similar or lower levels were seen with ceramide inhibitors in control-fed rats. Ceramide inhibitors did not lead to a reduction of triglyceride levels as shown by increasing ratios of ethanol to control levels. At 72 hours hepatic neutral lipid (Figure 4.3 C) in livers of vehicle-treated rats was comparably lower to that observed at 48 hours in both the control- and ethanol-fed rats. Although neutral lipid levels were lower in the control-fed rats treated with ceramide inhibitors relative to the 48-hour time point, neutral lipid levels were marginally increased with myriocin and desipramine. In ethanol-fed rats however, treatment with ceramide inhibitors led to marked reductions in neutral lipid with the most effects seen with myriocin and desipramine. At 48 hours (Figure 4.3 B) ceramide inhibitor treatment reduced ethanol-induced elevations of triglycerides, most effective was myriocin ($p < 0.01$).

Ceramides were measured at 48 and 72 hours. At 48 hours (Figure 4.3 B), ethanol resulted in markedly increased levels of ceramide versus those in control-fed rats ($p < 0.01$). Ceramide levels in control-fed ceramide inhibitor-treated rats were

similar or lower than in vehicle-treated rats. However, after 48 hours ceramide inhibitors did not significantly reduce ethanol-induced elevations of ceramide; the least effective agent was fumonisin B1 ($p < 0.001$). At 72 hours (Figure 4.3 D) ceramide levels were reduced in both control- and ethanol-fed rats with ceramide inhibitor treatments, and fumonisin and desipramine were most effective in reducing ethanol-induced increases in ceramides.

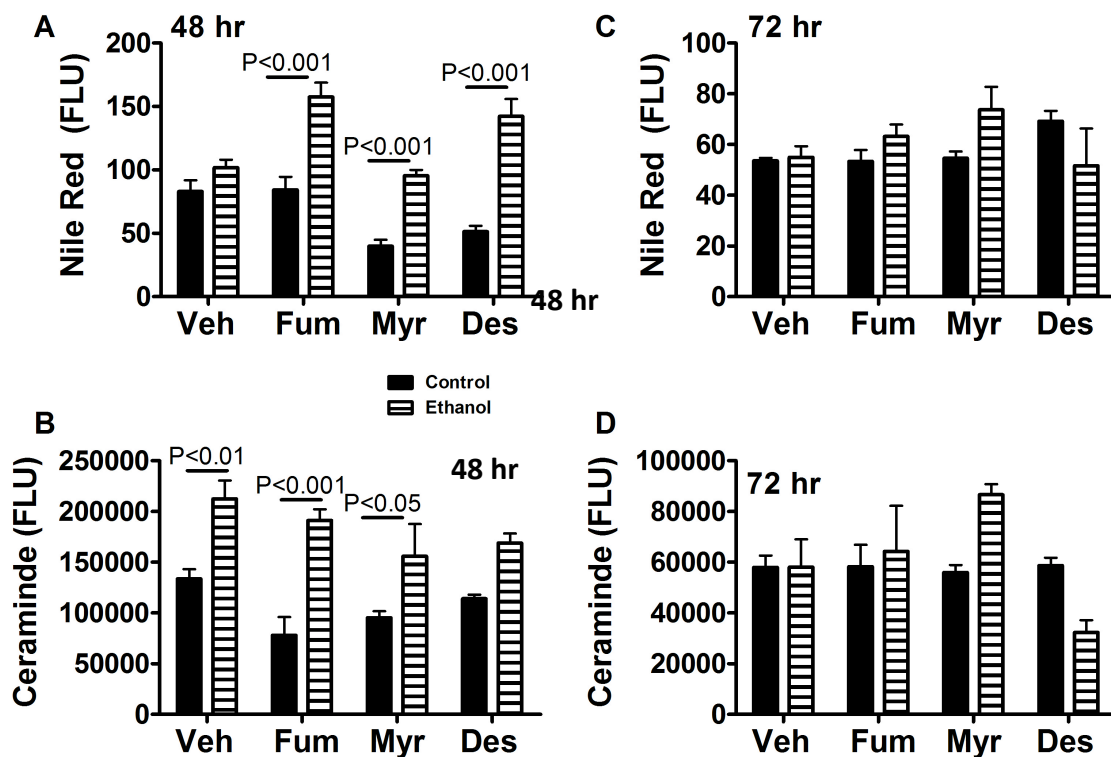


Figure 4.3: Effects of chronic alcohol feeding and ceramide inhibitor treatment on neutral lipid and ceramide accumulation at 48 and 72 hours post-culture. Liver slices from culture were homogenized in weak lysis buffer, and the protein extract was used to measure (A) neutral lipid at 48 hours and (C) at 72 hours and (B) ceramide at 48 hours and (D) at 72 hours, using the Nile Red stain and ceramide ELISA respectively. The results were normalized to protein concentrations (as

measured by the BCA method). Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

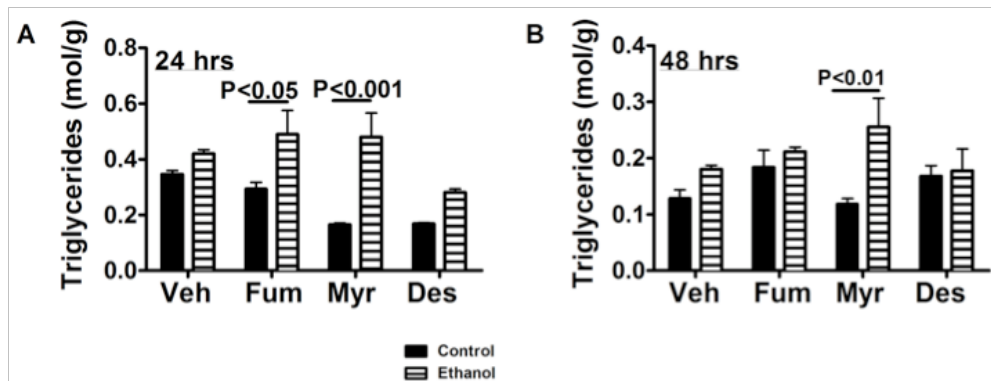


Figure 4.4: Effects of chronic alcohol feeding and ceramide inhibitor treatment on triglyceride accumulation. Liver slices from culture were homogenized in weak lysis buffer, and the protein extract was used to measure triglyceride at (A) 24 hours and (B) 48 hours using a commercially available kit (see Material and Methods). The results were normalized to protein concentrations (as measured by the BCA method). Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

Effects of ethanol and ceramide inhibitor treatments on cytotoxic levels in liver: The cytotoxicity assay used measures the release of cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) from damaged cells. The fluorescent signal detected (proportional to G6PD) correlates with the number of dead cells in the sample. Measurements at 24 hours (Figure 4.5 A), exhibited higher levels of GP6D release in ethanol- compared to control-fed rats. In control- and ethanol-fed

rats treatment with all ceramide inhibitors marginally reduced ethanol-induced increases towards control levels to a similar degree. At 48 hours (Figure 4.5 B), the ratio of G6PD release in ethanol- versus control-fed rats was higher than at 24 hours; myriocin ($p < 0.001$) and desipramine ($p < 0.01$) were the least effective in reducing ethanol-induced elevations. In contrast at 72 hours (Figure 4.5 C) G6PD release is at similar levels in fumonisin B1- and vehicle- treated rats, but is lower in ethanol-fed rats treated with desipramine ($p < 0.0001$) than any other group.

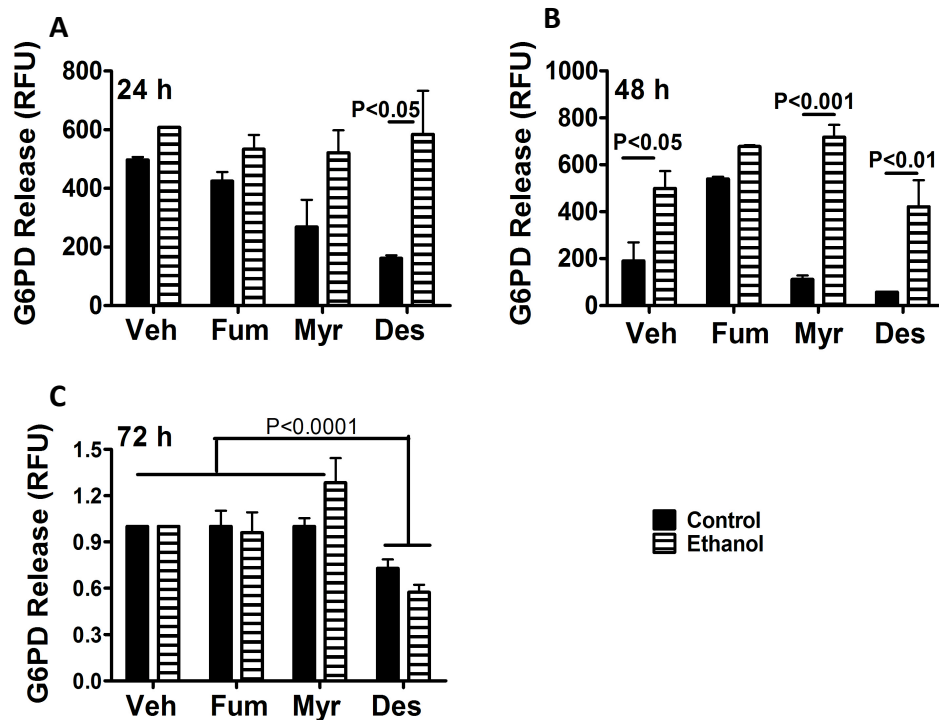


Figure 4.5: Effects of chronic alcohol feeding and ceramide inhibitor treatment on cytotoxicity. Liver slices from culture were homogenized in weak lysis buffer, and the protein extract was used to measure cytotoxicity at (A) 24 hours, (B) 48 hours and (C) 72 hours using a

commercially available kit (see Material and Methods). The results were normalized to protein concentrations (as measured by the BCA method). Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

Effects of ethanol and ceramide inhibitor treatments on ER stress markers in liver:

To investigate the role of ER stress in this model we measured hepatic mRNA levels of activating transcription factor-4 (ATF-4) (Figure 4.6 A), GRP78 (Figure 4.6 B) and CHOP (Figure 4.6 C) at 48 hours. Levels of ATF-4, GRP78 and CHOP were similarly low in vehicle-treated control- and ethanol-fed rats whereas control-fed rats treated with ceramide inhibitors had upregulated levels of ATF-4, GRP78 and CHOP compared to their ethanol-fed counterparts. The effects of ceramide inhibitor treatments (myriocin and desipramine more than fumonisin B1) were significant with marked reductions in ethanol-fed rats by at least 40%. Therefore treatment with ceramide inhibitors had a downregulating effect on ER stress mRNA levels associated with long- term ethanol feeding.

Correlative protein expression of ER stress was assayed using duplex ELISAs. We measured PERK and eIF2- α levels and their corresponding phosphorylated proteins at 48- and 72-hour time points. At 48 hours total levels of PERK (Figure 4.7 A) and eIF2- α (Figure 4.7 D) in vehicle and treated rats were not significantly different in control- and ethanol-fed rats, except for eIF2- α levels that were lower in ethanol-fed rats compared to control ($p < 0.01$) with desipramine

treatment. Although levels of phosphorylated PERK (Figure 4.7 B) and eIF2- α (Figure 4.7 E) were reduced by both myriocin ($p < 0.001$) and desipramine ($p < 0.001$) in ethanol-fed rats, a calculated ratio of phosphorylated to total levels was only significant for PERK with fumonisin B1 treatment ($p < 0.01$). At 72 hours total PERK and eIF2- α levels were similar in vehicle-treated rats. In treated rats total PERK (Figure 4.8 A) levels in ethanol-fed rats were reduced to similar levels as in control-fed rats with myriocin ($p < 0.01$) and desipramine ($p < 0.001$) treatments.

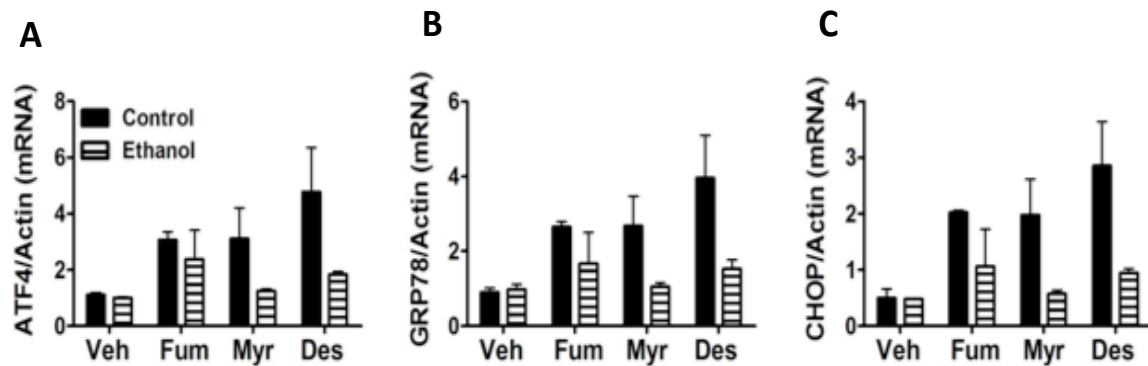


Figure 4.6: Effects of chronic alcohol feeding and ceramide inhibitor treatment on ER stress signaling network genes. Total RNA extracted from cultured liver slices was reverse transcribed (see Materials and Methods) and the resulting cDNAs were used as templates to measure (A) ATF-4, (B) GRP78 and (C) CHOP. mRNA abundance was corrected for using Actin as the control gene. Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

By contrast total eIF2- α levels (Figure 4.8 D) in control- and ethanol-fed rats were similar with fumonisin B1 and desipramine treatments, and higher in ethanol-fed rats with myriocin treatment ($p < 0.01$). Phosphorylated eIF2- α levels (Figure 4.8 E) were higher levels in ethanol- compared to control-fed rats with desipramine treatment ($p < 0.05$). A calculated phosphorylated/total PERK ratio (Figure 4.8 C) showed higher levels in all groups in control- relative to ethanol-fed rats. A calculated phosphorylated/total eIF2- α level (Figure 4.8 F) showed a reduction in ethanol-induced rises with myriocin treatment ($p < 0.05$) but a further increase with desipramine ($p < 0.001$). Collectively therefore, at 48 hours fumonisin B1 was effective in reducing active PERK levels in ethanol-fed rats, however at 72 hours there was no effect of all ceramide inhibitors on PERK levels as the profile was almost identical between the vehicle and treated groups. In contrast myriocin was effective at 72 hours in reducing active eIF2- α levels in ethanol-fed rats.

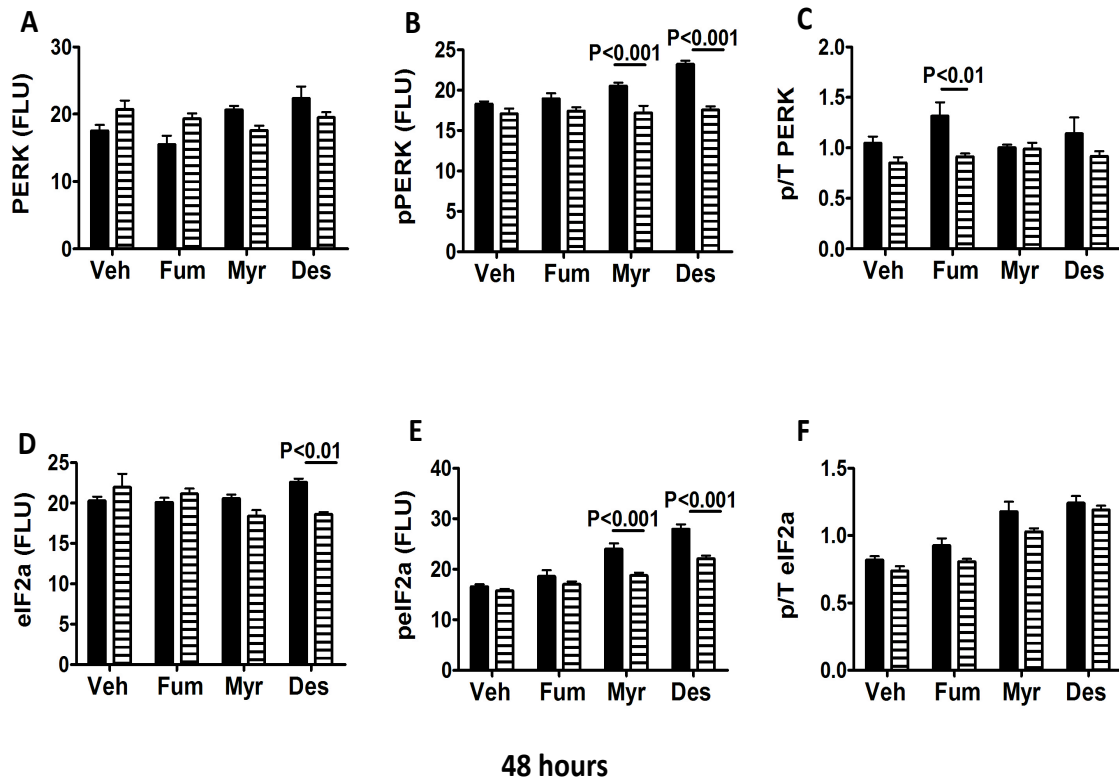


Figure 4.7: Effects of chronic alcohol feeding and ceramide inhibitor treatment on downstream mediators of ER stress signaling at 48 hours. Liver protein homogenates were used in bead-based Multiplex ELISAs to measure the immunoreactivities of (A) total PERK, (D) eIF2a and their phosphorylated counterparts (B, E respectively). In addition, the phosphorylated/total protein ratios (C, F respectively) were calculated to assess relative levels of phosphorylation of each protein. Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

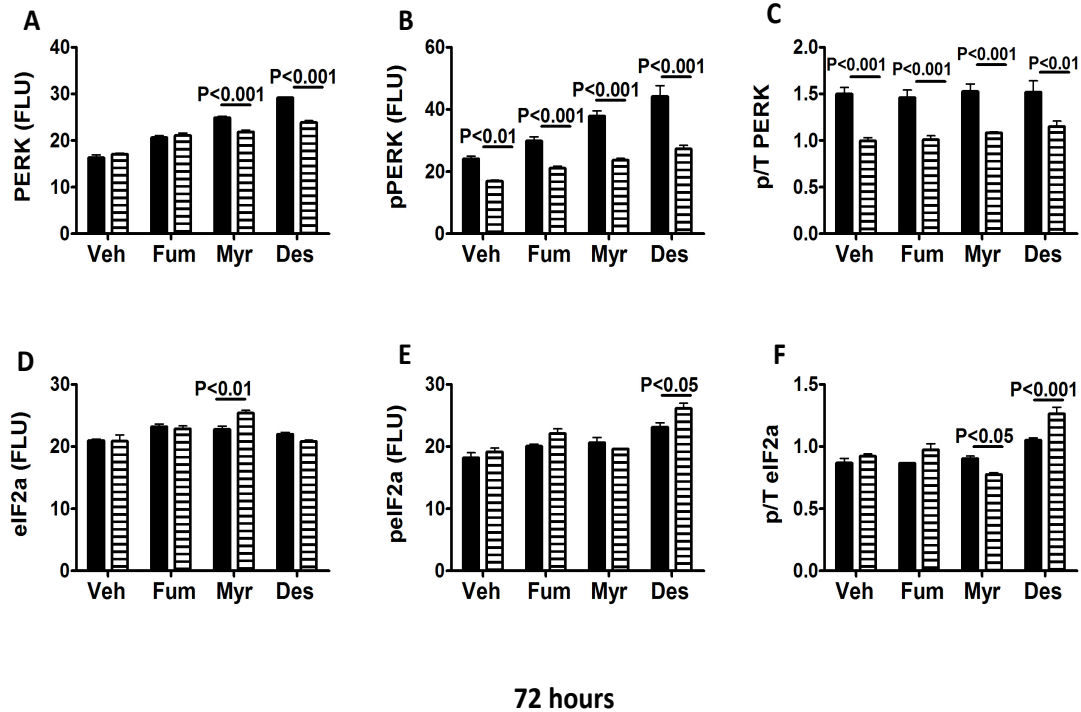


Figure 4.8: Effects of chronic alcohol feeding and ceramide inhibitor treatment on downstream mediators of ER stress signaling at 72 hours. Liver protein homogenates were used in bead-based Multiplex ELISAs to measure the immunoreactivities of (A) total PERK, (D) eIF2a and their phosphorylated counterparts (B, E respectively). In addition, the phosphorylated/total protein ratios (C, F respectively) were calculated to assess relative levels of phosphorylation of each protein. Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

Effects of ethanol and ceramide inhibitor treatments on insulin and IGF signaling mechanisms in liver: Previous studies demonstrated that steatohepatitis produced by high fat diet or chronic ethanol feeding impairs insulin and IGF signaling in the liver. We re-investigated this concept and also interrogated the potential therapeutic effects of ceramide inhibitors on ethanol-mediated inhibition of insulin/IGF signaling by measuring mRNA levels of insulin, IGF-1, and IGF-2 polypeptides, their corresponding receptors, and IRS-1 and IRS-4 by qRT-PCR analysis (Figure 4.9). The qRT-PCR analyses demonstrated similarly low mean levels of measured polypeptides in vehicle-treated rats except of IGF-2 receptor and IRS-4, which were lower in rats fed ethanol compared to control diet. Furthermore levels of insulin, IGF-1, IGF-2, insulin and IGF-1receptors, IRS-1 and IRS-4 were lower in ethanol- versus control-fed rats with all ceramide inhibitors.

Analysis of the upstream signaling networks using multiplex ELISAs revealed similar mean levels of total and phosphorylated IN-R, IGF-1R, and IRS-1 in all groups (Figure 4.10). With respect to phosphorylated/total ratios the only significant difference was observed with IN-R, with a lower mean in ethanol- relative to control-fed rats ($p < 0.05$) with vehicle. Therefore, chronic ethanol feeding lead to a suppression of hepatic signaling through IN-R and thus no protective effects of the ceramide inhibitor treatments could be demonstrated.

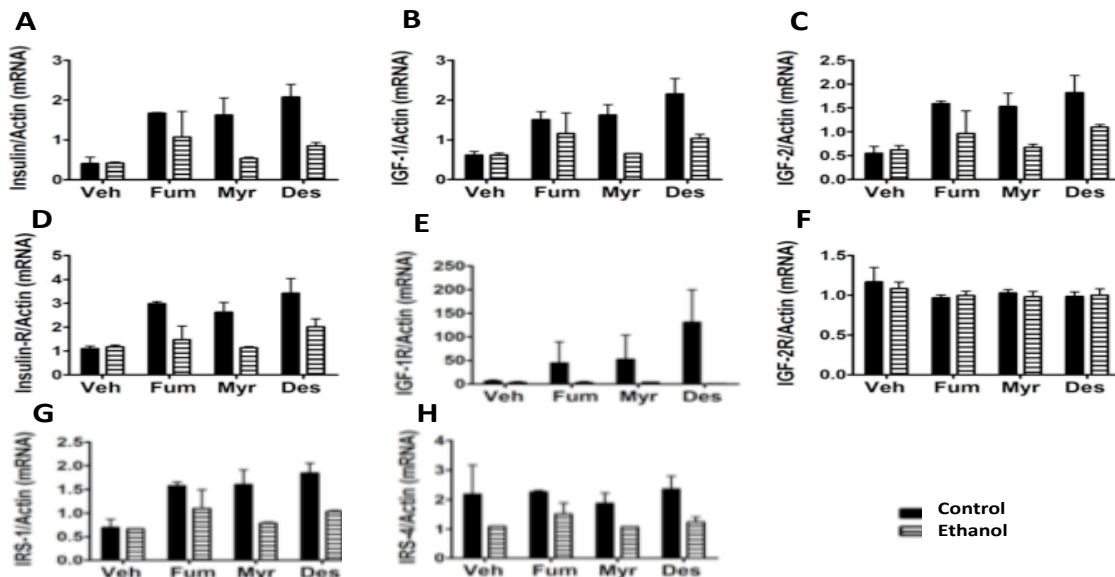


Figure 4.9: Effects of chronic alcohol feeding and ceramide inhibitor treatment on upstream insulin signaling network genes. Total RNA extracted from cultured liver slices was reverse transcribed (see Materials and Methods) and the resulting cDNAs were used as templates to measure (A) insulin, (B) insulin-like growth factor 1, (C) insulin-like growth factor 2 and their corresponding receptors (D, E, F respectively), as well as (G) IRS 1 and (H) IRS 4. mRNA abundance was corrected for using Actin as the control gene. Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

Downstream signaling through insulin/IGF-1 in liver (Figure 4.11) showed similar mean levels of total GSK-3 β , PRAS40 and p70S6K, in all groups. Signaling through Akt on the other hand was impaired in ethanol- relative to control-fed rats treated with vehicle ($p < 0.001$), signified by lower mean hepatic levels in the former. There was no difference between either diet or ceramide inhibitor treatment in the levels of the phosphorylated

insulin signaling polypeptides. Hepatic p^{S473} -pAkt /total Akt ratio ($p < 0.01$), p^{T246} -PRAS40/total PRAS40 ratio ($p < 0.05$) and $p^{TpS421/424}$ -p70S6K/total p70S6K ratio ($p < 0.01$) were significantly reduced by chronic ethanol- relative to control-feeding with vehicle, and ceramide inhibitor treatment had no significant effect in ameliorating the effects of ethanol. The mean p^{S9} -GSK-3 β /GSK-3 β ratio was unchanged in all groups.

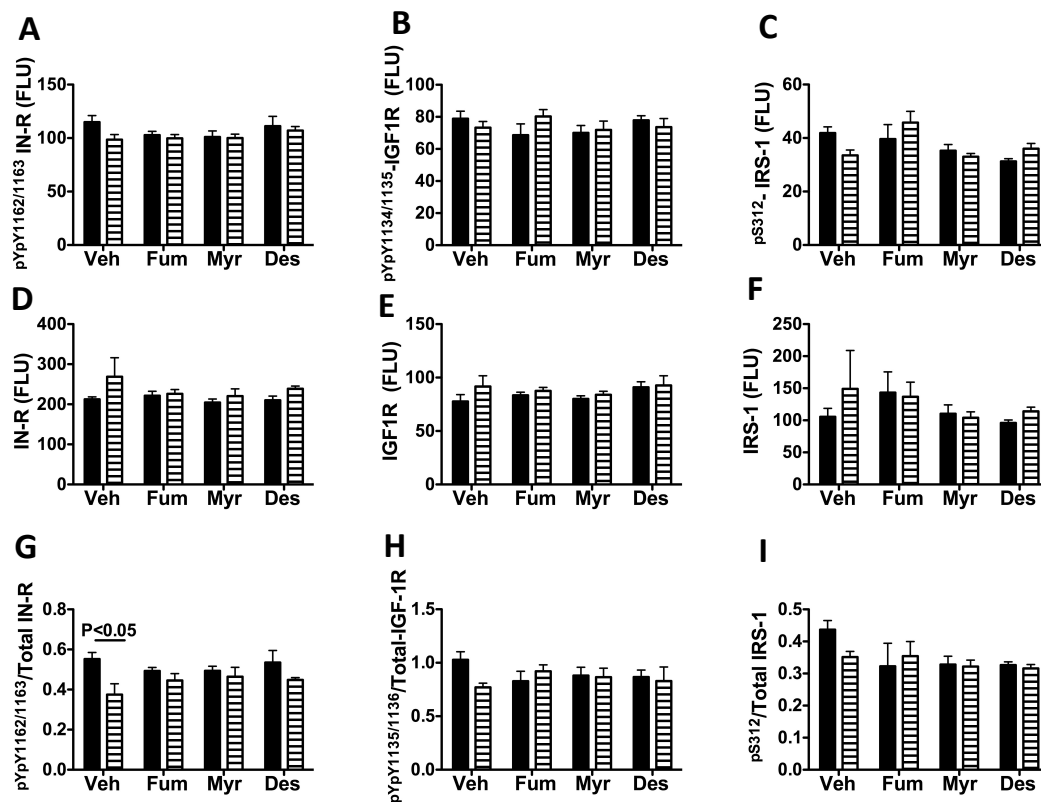


Figure 4.10: Effects of chronic alcohol feeding and ceramide inhibitor treatment on upstream mediators of insulin/IGF signaling. Liver protein homogenates were used to measure immunoreactivity corresponding to (D) insulin receptor (IN-R), (E) insulin-like growth factor 1 receptor (IGF-1R), (F) IRS-1, and their corresponding phosphorylated proteins $p^{Y1162/1163}$ -IN-R, $p^{Y1135/1136}$ -IGF-1R, and p^{S312} -IRS-1 (A, B, C) with a bead-based Multiplex ELISA platform. In addition, the phosphorylated/total protein ratios (G, H, I) were calculated to assess relative levels of phosphorylation of each protein. Data depicted in box

plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

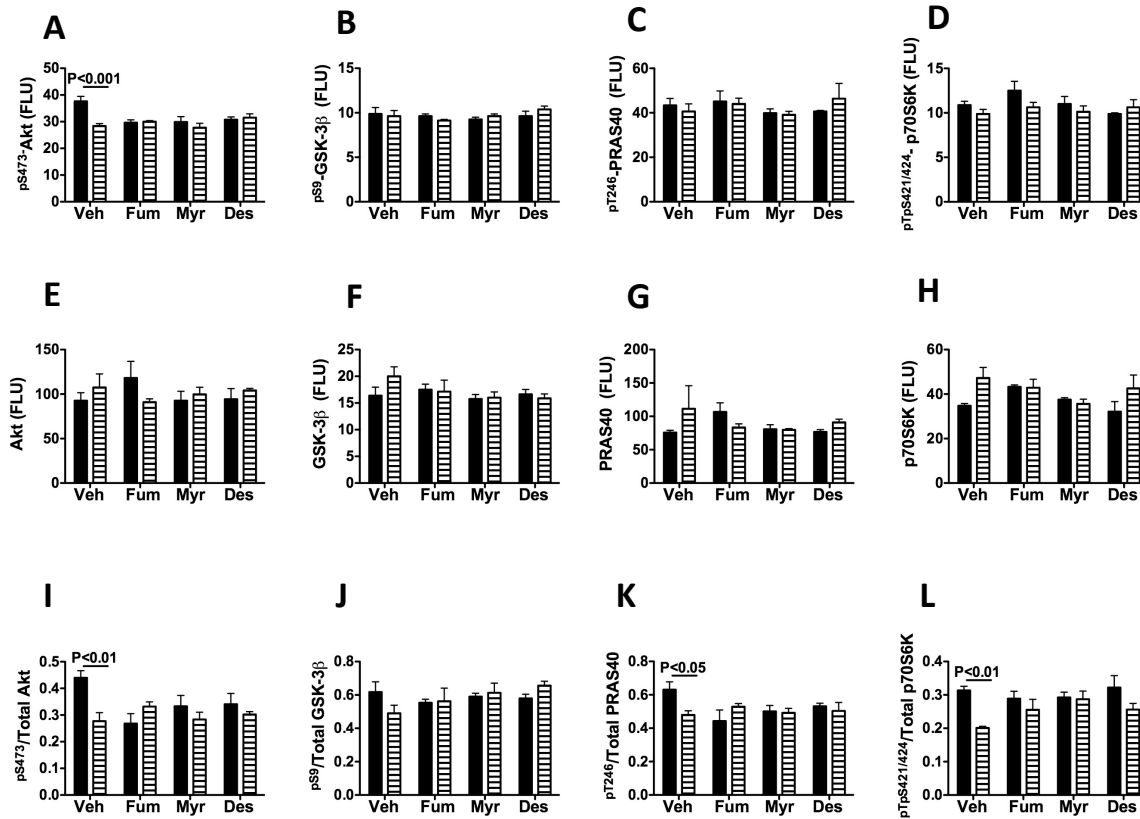


Figure 4.11: Effects of chronic alcohol feeding and ceramide inhibitor treatment on downstream mediators of insulin/IGF signaling. Liver protein homogenates were used to measure immunoreactivity corresponding to (E) Akt, (F) GSK-3 β , (G) PRAS40, (H) p70S6K, and their corresponding phosphorylated proteins p^{S473} -Akt, p^{S9} -GSK3 β , p^{T246} -PRAS40 and $p^{TpS421/424}$ -p70S6K (A-D) with a bead-based Multiplex ELISA platform. In addition, the phosphorylated/total protein ratios (I-L) were calculated to assess relative levels of phosphorylation of each protein. Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Inter-group comparisons were made using two-way ANOVAs with post-hoc Bonferroni tests. Significant P-values are indicated within the panels.

4.4 DISCUSSION

The present study sought to evaluate primarily the effects of ceramide inhibitor treatment on ethanol-induced steatohepatitis, particularly in reference to cytotoxicity, ER stress and impairments in insulin signaling. Secondary we aimed to study the utility and reliability of this in-vivo slice culture system as an alternative approach to studying the therapeutic applications and implications of a myriad of liver diseases. Our hypothesis is based on evidence that mediators of alcoholic steatohepatitis include ceramide-induced lipotoxicity and insulin resistance, oxidative stress and pro-cytokine activation. To achieve this we utilized PCLS as these have been shown to be useful on toxicology and pharmaceutical studies (Parrish et al., 1995), and would be well suited for our purposes.

Effects of ethanol: The main findings were that the full pathophysiological spectrum of alcoholic liver disease expected in rodent models of disease was seen as evidenced by the histological features, corroborated by the accumulation of lipid, including ceramide in ethanol-treated rats. As expected, ethanol-fed rats exhibited higher cytotoxic levels consequent to the attendant inflammation and possibly lipid peroxidation. Furthermore we observed IGF-2-mediated transcriptional and Akt-, PRAS40- and p70S6K-mediated post-transcriptional impairments of insulin signaling. Ethanol- or obesity-induced impairments in insulin signaling have been shown in our previous studies (de la Monte et al 2008, Denucci, SM 2010) and other laboratories (Svegliati-Baroni et al., 2006; Yeon et al., 2003). Moreover, in addition to the impairments in carbohydrate

metabolism, we demonstrated significant ethanol inhibition of signaling downstream of PI3 kinase-Akt through p70S6K, and mTOR-dependent PRAS40 pathways, indicating that chronic ethanol feeding inhibits protein synthesis (Berven & Crouch, 2000; Nascimento & Ouwens, 2009).

Effects of ceramide inhibitors: Our results showed that the administration of fumonisin B1 was most effective in improving the histological appearance of the liver slices. The effect of ceramide inhibitors on lipid accumulation was mirrored in quantitative reductions in neutral lipid accumulation at 72 hours with myriocin and desipramine treatments, and triglyceride accumulation at 48 hours with myriocin treatments. The 'dominant' effects of fumonisin and myriocin suggest that inhibition of ceramide synthesis may be more important than inhibition of ceramide degradation, in ameliorating ceramide-induced lipotoxicity. Although the half-life of these inhibitors has not been tested in in-vivo systems, it is likely that the half-life is longer than 48 hours, as these agents were more effective after longer incubations. On the other hand, at the 24 hour and 48 hour time points, neutral lipid and triglyceride accumulations respectively were not different in the control and ethanol-fed rats; this could be explained by the fact that the slices were not maintained in an alcohol chamber during incubation, thus resulting in an "alcohol off" effect. It will therefore be crucial in future experiments to include the use of an alcohol chamber to control for this.

With regard to cytotoxicity, in the initial phases post-incubation there is no difference in G6PD release in control and ethanol-fed rats. This may be due to the effects of the mechanical trauma sustained by the liver slices, irrespective of

diet or treatment. After 48 hours incubation, the 'true' effects of diet and treatment can be appreciated, and these correlate well with the effects seen on lipid accumulation, with desipramine resulting the most effective reduction in levels of cytotoxicity. Once again ceramide inhibitors were most effective after longer incubation periods.

Most evident in this model was the up-regulating effect of alcohol on markers of ER stress, and the effectiveness of fumonisin on PERK levels at 48 hours and myriocin on eIF2- α at 72 hours, at both transcriptional and post-transcriptional levels in reducing alcohol-induced ER stress. Alcohol causes ER stress not only by the altered redox potential generated with its metabolism, but also as a result of the associated metabolic stress, and steatosis. Therefore, the effect of ceramide inhibitors on ER stress proves the principle of the key role of ER stress in producing steatohepatitis with chronic alcohol feeding.

The in-vivo system: Historically PCLSC is fraught with problems of intra- and inter-laboratory variability not least in our hands. In our experiments we did not use a rotating platform during incubation, which has been reported by some to give superior results (Hashemi, Dobrota, Till & Loannides, 1999). However given that after 48 hours lipid studies and cytotoxicity levels showed a correlation it is likely that viability of cells was not negatively impacted by not using a rotating platform. In addition, the mechanical stress suffered by the slice culture technique and subsequent incubation did not impair the architectural integrity of the liver slices (see histology). As mentioned an area of improvement in future studies would be the use of an alcohol chamber during incubation, in order to

maintain high concentrations of ethanol exposure on the slices, to offset the “ethanol-off” effect that was evident in our model.

Thus we provide evidence that PCLSC is a robust reliable model for studying and understanding the pathophysiology of alcoholic steatohepatitis. Furthermore, notwithstanding technical improvements in the experimental procedures that can be implemented, it provides a user-friendly alternate in-vitro system by which the metabolic effects of toxic insults and therapeutic effects can be studied. This utility can have wide-ranging impact in the understanding of disease processes and applicable treatments.

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CHAPTER 5

Re-activated Wnt signaling is crucial in the pathogenesis of HCC in a double transgenic mouse model that constitutively over-expresses the HBx and IRS-1 genes in liver

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is associated with aberrant growth factor signaling. Both insulin/IGF and Wnt/ β -catenin signaling pathways have been individually implicated in the pathogenesis of HCC. Previous data have shown that crossing the single genes to create a double transgenic (ATX and IRS-1) mouse resulted in HCC associated with upregulation of IGF1 and Wnt3 and Frizzled7 mRNA transcripts. To date despite active research in the field, there are no therapeutic molecules targeting this pathway, therefore, identifying the target genes of Wnt signaling remains critical for understanding β -catenin-mediated carcinogenesis.

Aim: The purpose of this work is to characterize alterations of the Wnt/ β -catenin signaling pathway, and the gene expression pattern of components of canonical and non-canonical Wnt signaling in a double transgenic mouse model of HCC.

Methods: We analyzed liver tissue from double transgenic (ATX and IRS-1) mice sacrificed at 3, 6, 9, 12, 15, 18, 21 and >21 months (n=12 per time point) by qRT-PCR using a PCR array panel. Wild type mice were used as controls.

Results: Tumours developed only in the male mice generally after 15-18 months. The majority of canonical and non-canonical Wnt ligands, receptors and downstream targets including target genes were upregulated in mice without tumours at early time points. However, some genes including Wnt 1, 2, 3, 4, 6, 7b, 11 and Fzd8, 2, 3 and Fzdb were also upregulated in HCC (occurring at later time points), suggesting re-activation of Wnt signaling in HCC. Specifically Wnt5b and Wnt7b were significantly upregulated in double transgenic mice with tumours, suggesting they may be key regulators in HBV-related HCC. Surprisingly, some of the Wnt negative regulators, Wif1, Sfrp1, 2 and 4 were upregulated, which may be indicative of epigenetic regulation; however further studies are required to examine this.

Conclusions: Our study identified several candidate genes of Wnt signaling, particularly Wnt5b and 7b that are dysregulated and appear to be carcinogenic in mouse HCC. These genes may serve as useful potential therapeutic targets for the treatment of HCC.

5.1 INTRODUCTION

Cirrhosis is associated with decreased hepatocyte proliferation and is the chief risk factor for the development of hepatocellular carcinoma (HCC). Progression of liver disease to HCC occurs in a step-wise fashion, initially with the development of pre-neoplastic dysplastic nodules (DN), followed by well-differentiated HCC associated with more molecular abnormalities (Farazi & DePinho, 2006). Many factors are involved in the process of promoting hepatocarcinogenesis once cirrhosis is established. These include alterations in the genetic sequence coding for proto-oncogenes or tumour-suppressor genes important for growth or anti-growth signals respectively, commonly by way of mutations (Hussain, Hofseth, & Harris, 2003). Mutations of p53, p16, and β -catenin have been described in HCC (Herath, Leggett, & MacDonald, 2006), however do not account for all cases of HCC. There are other molecular events that predispose a cirrhotic liver to transform to HCC, and these include resistance to apoptosis, activation of telomerase and of oncogenic pathways i.e. Wnt/ β -catenin, PI3K/Akt and others (El-Serag & Rudolph, 2007).

Furthermore, changes in gene expression as a result of heritable changes of genes, albeit not of the sequence itself, that influence phenotype are involved in the pathogenesis of HCC. These are referred to collectively as epigenetic modifications, which fall into 3 broad groups: changes in methylation status, histone deacetylation (linked with transcriptional repression), and micro-RNA regulations. Typically with HCC there is global hypomethylation of the genome, which is associated with activation of proto-oncogenes e.g. c-Jun and c-Myc

(Calvisi et al., 2007) but hypermethylation of the promoter regions of genes with a high content of cytosine-guanosine nucleotides (CpG islands). Hypermethylation of CpG islands results in transcriptional repression of tumour suppressor genes, thus promoting proliferation, evasion of apoptosis and eventual cancer. Additionally, although normal ageing can be associated with hypermethylation (Esteller, 2008), hypomethylation appears to be the predominant mechanism of gene expression regulation in aging (Rodriguez-Rodero, Fernandez-Morera, Fernandez, Menendez-Torre, & Fraga, 2010). As many malignant diseases including HCC are associated with aging, the investigation of age-related epigenetic modifications being responsible for HCC is interesting. We hypothesized that impairments in Wnt signaling at various time points in the progression to HCC can uncover critical clues about which components of activated or downregulated signaling are important for hepatocarcinogenesis. The relevance of this study could be the potential use of activators or inhibitors of Wnt signaling that can be targeted for the treatment of HCC.

HBV accounts for over half of virus-related HCC cases worldwide (Parkin, Bray, Ferlay, & Pisani, 2005) but more so in the East and Africa. While the mechanism of HBV hepatocarcinogenesis is still being sought, a small protein, the hepatitis B x protein (HBx), encoded for by the hepatitis B x gene has been shown in many studies to affect many pathways involved in the promotion of cancer. HBx is an oncogenic multifunctional protein that up-regulates proto-oncogenes (c-myc, N-myc and c-jun), transcription factors (AP-1, NF- κ B and ATF/CREB) (Colgrove, Simon, & Ganem, 1989; Lucito & Schneider, 1992; Seto,

Mitchell, & Yen, 1990), and is involved in the regulation of apoptosis (Benn & Schneider, 1995; Gottlob, Fulco, Levrero, & Graessmann, 1998; Marusawa et al., 2003). More importantly HBx regulates a variety of pathways including Ras-Raf/MAPK, SAPK/JNK, PI3K/Akt (Doria, Klein, Lucito, & Schneider, 1995; Y. H. Lee & Yun, 1998; Natoli et al., 1994) including the Wnt pathway. HBx can activate Wnt/ β -catenin signaling by inhibiting GSK-3 β via several mechanisms or by stabilizing β -catenin independently of GSK-3 β (Cha, Kim, Park, & Ryu, 2004; Q. Ding et al., 2005; Jung, Kwun, Lee, Arora, & Jang, 2007).

Wnt/ β -catenin signaling is a highly conserved pathway that has roles in proliferation, cell fate determination, cell cycle, apoptosis and motility. Wnt signaling is quiescent in normal cells and is expressed at very low levels in liver. With canonical signaling, in this inactive state, intracellular β -catenin is primed for ubiquitin-proteasome degradation by the degradation complex (APC/Axin/GSK-3 β). In the active state, Wnt ligands bind to Frizzled receptors at the cell membrane, and these co-operate with LRP, to transmit the signal to Dishevelled, which associates with the degradation complex, thereby phosphorylating and inactivating GSK-3 β , thus allowing for the accumulation of β -catenin. β -catenin then translocates to the nucleus to increase transcription of target genes that promote carcinogenesis by increasing cell proliferation, angiogenesis, anti-apoptosis and the laying down of extracellular matrix (Avila, Berasain, Sangro, & Prieto, 2006). The role of β -catenin in HCC is evident in the fact that 17-40% of human HCC show a nuclear accumulation of β -catenin (Hsu et al., 2000; Nhieu et al., 1999; Prange et al., 2003; Wong, Fan, & Ng, 2001). There are two other

pathways of Wnt signaling i.e. planar cell polarity (PCP) pathway (that regulates the cytoskeleton and cell motility particularly during embryogenesis) and Wnt/Calcium pathway (that promotes the transcription of nuclear factor of activated T cells (NFAT), independently of β -catenin).

Insulin signaling favours cell growth and survival, and in human HCC the insulin signaling pathway is dysregulated as a result of increased expression of IGF-2 (Breuhahn et al., 2004; de La Coste et al., 1998; Longato et al., 2009; Sohda, Yun, Iwata, Soejima, & Okumura, 1996), or IRS-1 (Alexia, Fallot, Lasfer, Schweizer-Groyer, & Groyer, 2004; Breuhahn, Longerich, & Schirmacher, 2006). Indeed IRS-1 is overexpressed in 90% of human HCC, and is associated with increased signaling downstream through PI3kinase/Akt and Erk/MAPK, which regulate growth, motility and invasiveness, and IRS-1 correlates significantly with tumour size (Ito, Sasaki, & Wands, 1996; Tanaka, Mohr, Schmidt, Sugimachi, & Wands, 1997).

Initial studies in our laboratory using this model demonstrated no increased HCC development in mice carrying the IRS-1 single transgene (Mohr, Banerjee, Kleinschmidt, Bartolome Rodriguez, & Wands, 2008; Tanaka et al., 1997). However when IRS-1 was cross-bred with the hepatitis B x (ATX) transgene there was progressive hepatocellular injury and turnover, dysplasia, and eventually HCC development over time, beginning at 15 months of age, and predominantly in males, in double transgenic mice (Longato et al., 2009). In the same study there was upregulation of Wnt 1, Wnt 3 and AAH, all of which are associated with oncogenesis.

In this longitudinal mouse model of HCC, where the mice could be followed for at least 3 months to over a 2 year period, we aimed to characterize a broader spectrum of Wnt signaling components (i.e. ligands, receptors, target molecules including negative regulators) at the various time points in wild type and double transgenic mice, as well as to correlate these signaling changes with the possible role of aging in promoting the progression of disease through dysplasia and ultimately HCC.

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5.2 MATERIALS AND METHODS

Transgenic Mouse Models: The mice were purchased from and bred at Charles River laboratories, and heterozygous mice expressing the IRS-1 gene were mated with heterozygous mice expressing HBx (ATX gene). Transgenic expression was achieved by using a liver-specific promoter, the human alpha-1 antitrypsin regulatory region for the ATX mice and albumin promoter for IRS-1 (T. H. Lee et al., 1990; Tanaka et al., 1997). Thus there were 4 groups of mice viz: wild type (WT), ATX single transgenic, IRS-1 single transgenic and IRS-1/ATX (double transgenic). Non-transgenic littermates were used as controls. In this study 2 groups of animals viz: wild type and ATX/IRS-1 (double transgenic) were compared. To map out time course of histological and molecular changes, mice were sacrificed at time points 3, 6, 9, 12, 15, 18, 21 or more months and liver samples were collected for qRT-PCR analysis of Wnt pathway genes. During the course of breeding, the mice were monitored; food, activity levels and hydration were checked daily.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Assays: Liver tissue was homogenized in QIAzol Lysis Reagent. RNA was isolated using the EZ1 RNA Universal Tissue Kit and the BIO Robot EZ1 (Qiagen Inc., Valencia, CA), was reverse transcribed with random oligodeoxynucleotide primers and the AMV First Strand cDNA synthesis kit purchased from Roche Applied Science (Indianapolis, IN). The cDNAs were used to measure gene expression by qPCR with a Wnt Signaling Pathway RT² Profiler PCR Array kit from SABiosciences (Frederick, MD) to measure components of canonical, non-

canonical and Wnt target genes. The Roche instrument and software were used to detect amplified signals from triplicate reactions. Relative mRNA abundance was calculated from the ng ratios of specific mRNA to control genes [(hypoxanthine phosphoribosyltransferase1 (HPRT1), ribosomal protein L13a (RPL13A), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), actin beta (ACTB) and beta-2 microglobulin (B2M)] supplied with the kit. Template-free reactions served as negative controls.

Statistical analysis: The comparison was made between the wild type versus the double transgenic mice. Similarly we did an analysis of the gene expression fold change between double transgenic and wild type by calculating a ratio. The data was also compared over time using a line chart. To compare pre-malignant disease and HCC the data was plotted in bar graphs depicting the time points 3 to ≥ 21 months (pre-malignant phase) and HCC.

5.3 RESULTS

Gene expression patterns over time in WT and DT mice without tumours

To compare patterns of gene expression over time in mice that did not develop tumours i.e. under baseline conditions, we analyzed the data at individual time points from 3 to greater than 21 months. We measured the fold change in gene expression by plotting a ratio of DT expression levels over WT (Figures 5.1, 5.2, 5.3). There were three observed distinct patterns viz: some genes were highly expressed at 3 months (5-40 fold higher in DT than WT), and over time gradually declined either precipitously or in a step-wise fashion to very low levels. These were the Wnt ligands Wnt1, Wnt3, Wnt8a, Wnt8b and Wnt9a, the frizzled and related receptors Fzdb, Fzd3, Sfrp2 and Sfrp4 and downstream genes Dvl1, GSK-3 β , Tcf4, Pygo1, Sox17, Foxn1 and Fos1. The second pattern observed was that of genes that were upregulated (2 to 15-fold) at 3 months, rose to higher levels (at 6-12 months), and then steadily declined to lower levels by about 18 months. These include ligands Wnt3a, Wnt6, Wnt7a, Wnt7b, Wnt10a, Wnt11 and Wif1, Wisp1 and further downstream genes Dkk1, Lef1, Pitx2, Fgf4, Pitx2 and Fshb. The third group are those genes that from 3 to 24 months were essentially unchanged, and these are ligands Wnt2, Wnt4, Wnt5b, and Wnt16 and receptors Fzd2, Fzd6, Fzd7, Fzd8 and Sfrp1, and further downstream Tcf3, Nlk and Tle2. Wnt2b was different in that it had very low levels of expression until 17 months, when it is upregulated albeit marginally.

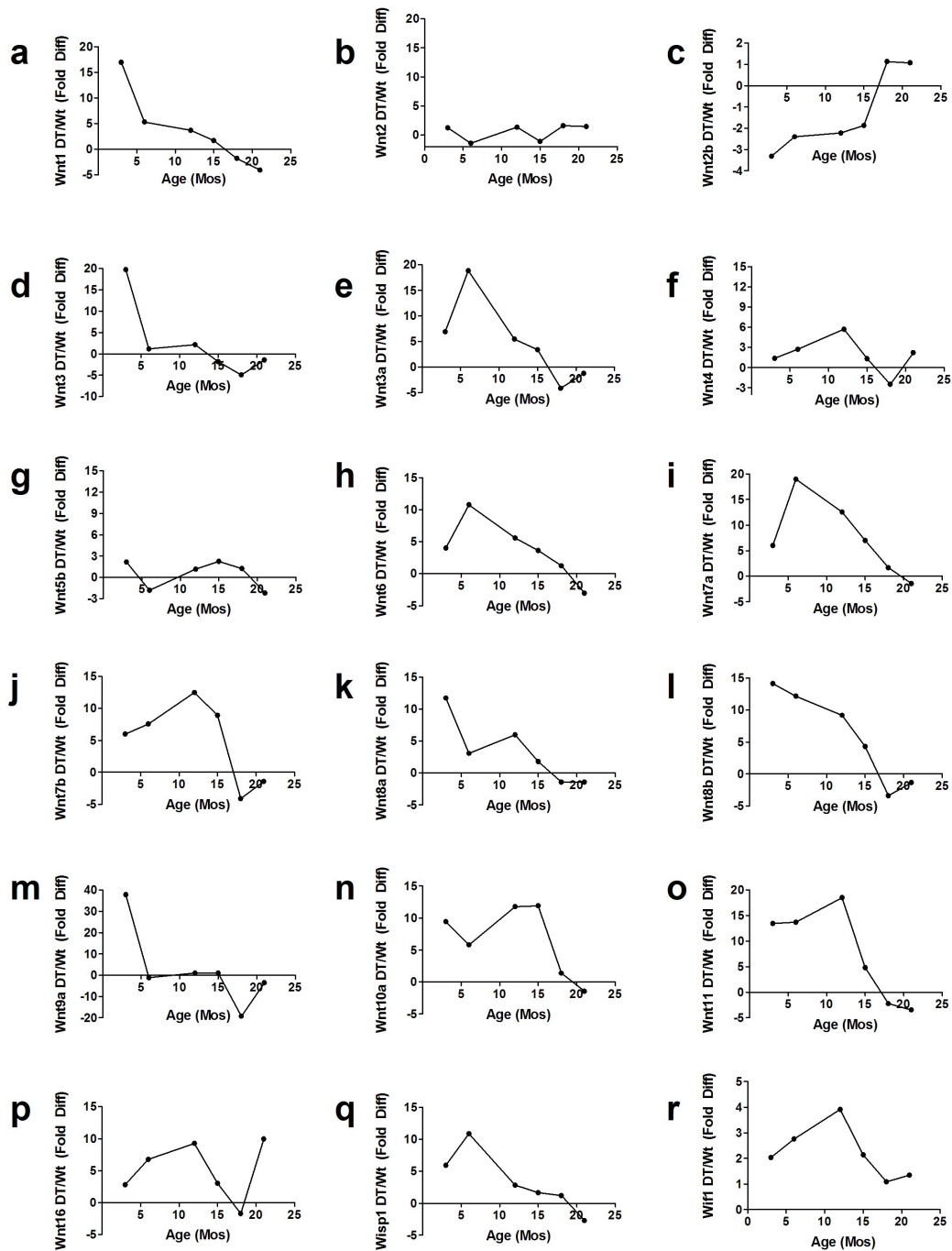


Figure 5.1: Analysis of the relative mRNA transcript fold difference of Wnt ligand signaling genes in mice that did not develop tumours over time from time points 3 to > than 21 months (represented in a line chart as a ratio of double transgenic (DT) mice over the wild type (WT) mice. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Wnt1, (B) Wnt2, (C) Wnt2b, (D) Wnt3, (E) Wnt3a, (F) Wnt4, (G) Wnt5b, (H) Wnt6, (I) Wnt7a,

(J) Wnt7b, (K) Wnt8a, (L) Wnt8b, (M) Wnt9a, (N) Wnt10a, (O) Wnt11, (P) Wnt16, (Q) Wisp1 and (R) Wif1 by qPCR using a PCR Array Kit (see Materials and Methods).

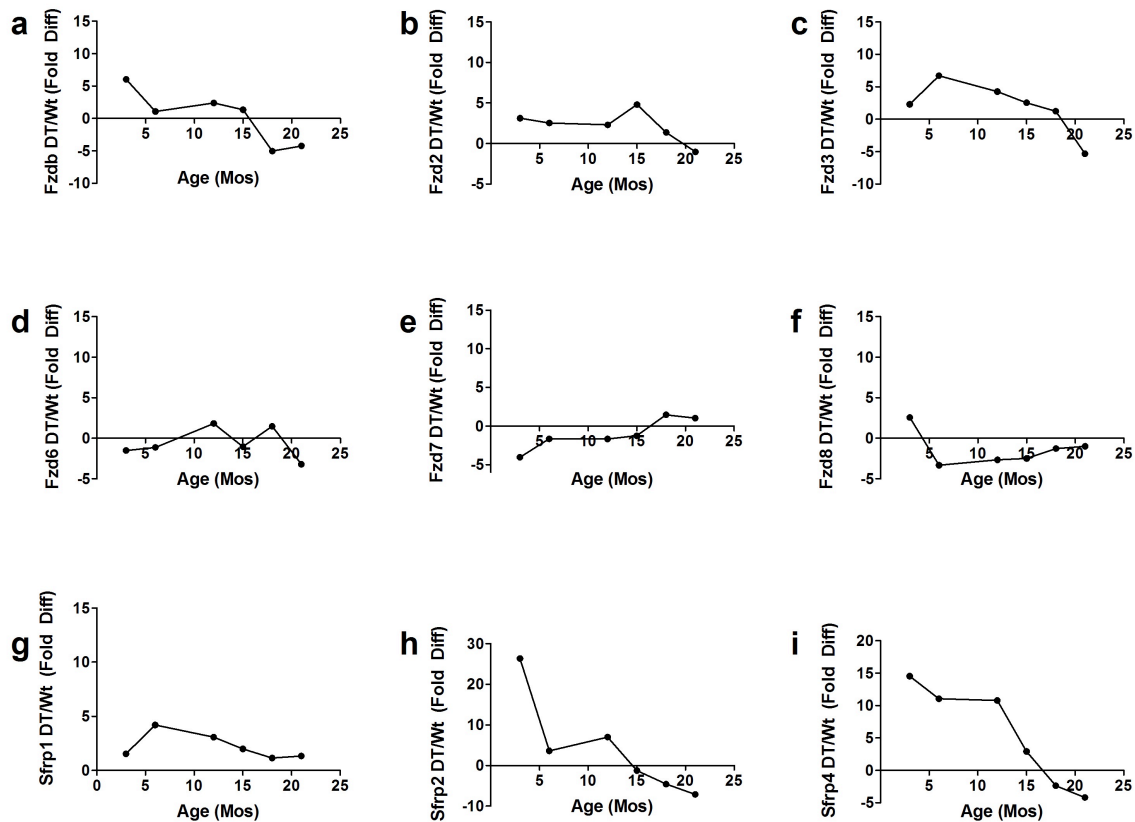


Figure 5.2: Analysis of the relative mRNA transcript fold difference of Wnt receptor signaling genes in mice that did not develop tumours over time from time points 3 to > than 21 months (represented in a line chart as a ratio of double transgenic (DT) mice over the wild type (WT) mice. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Fzdb, (B) Fzd2, (C) Fzd3, (D) Fzd6, (E) Fzd7, (F) Fzd8, (G) Sfrp1, (H) Sfrp2 and (I) Sfrp4 by qPCR using a PCR Array Kit (see Materials and Methods).

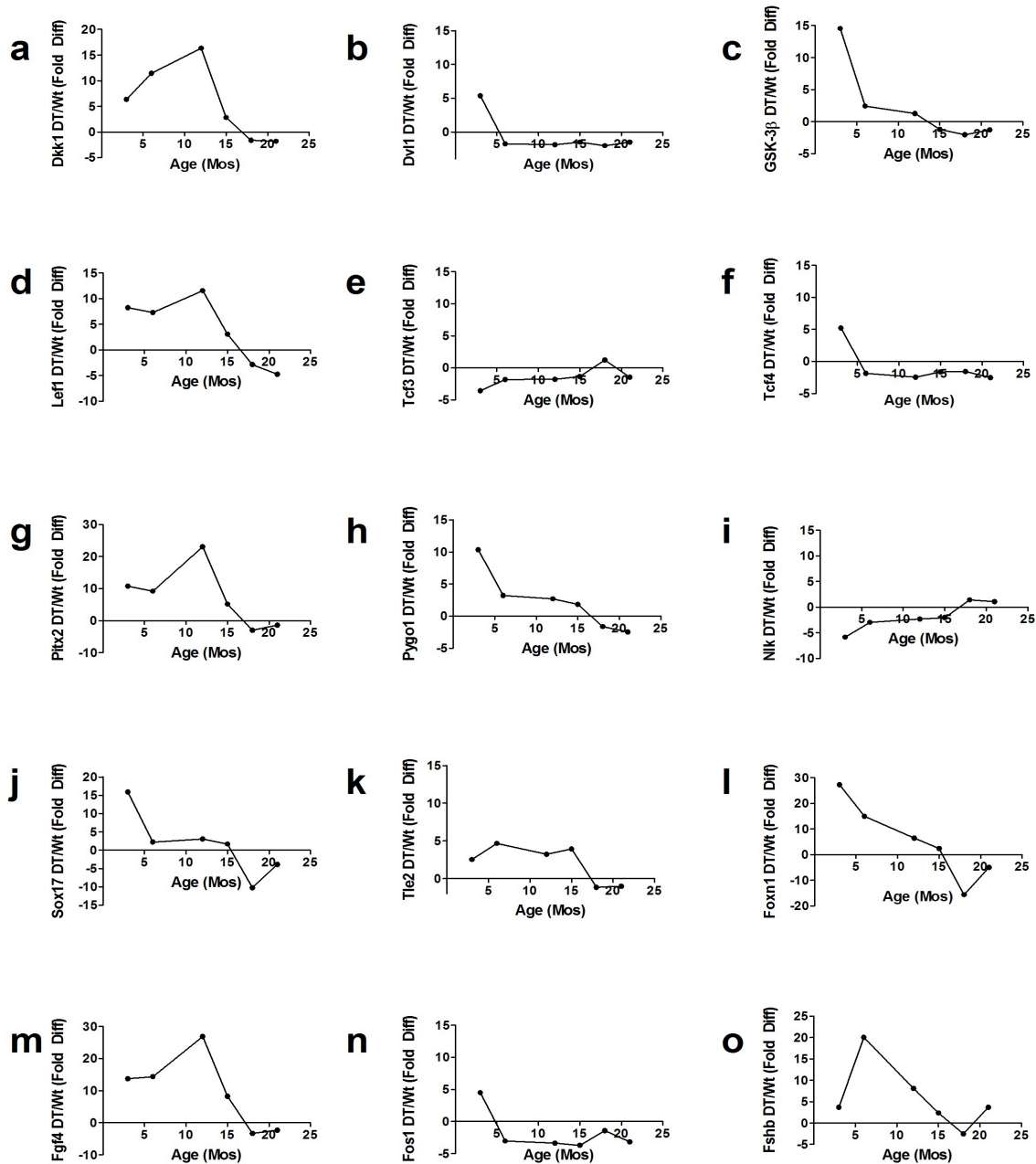


Figure 5.3: Analysis of the relative mRNA transcript fold difference of Wnt downstream signaling genes in mice that did not develop tumours over time from time points 3 to > than 21 months (represented in a line chart as a ratio of double transgenic (DT) mice over the wild type (WT) mice. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Dkk1, (B) Dvl1, (C) GSK-3 β , (D) Lef1, (E) Tcf3, (F) Tcf4, (G) Pitx2, (H) Pygo1, (I) Nik, (J) Sox17, (K) Tle2, (L) Foxn1, (M) Fgf4, (N) Fos1 and (O) Fshb by qPCR using a kit.

Tumours in wildtype (WT) versus tumours in double transgenic (DT) mice

Gene expression of Wnt ligands: Analysis of gene expression of Wnt ligands (calculated relative to housekeeping control gene) in wild type (WT) (n=4) and double transgenic (DT) mice (n=8) that developed tumours showed that out of a panel of 18 Wnt genes studied, the majority of them were either significantly or moderately upregulated in DT compared to WT, and unlike two genes that were higher in WT (Figure 5.4). Gene expression of Wnt5b was greater than 20-fold and that of Wnt7b greater than 15-fold higher in DT compared to WT. Wnt1, Wnt3, Wnt3a, Wnt6, Wnt7b, Wnt8b, Wnt9a, Wnt10a, Wnt11, Wif1 and Wisp1 mRNA transcripts were at least between 5 and 12-fold higher in DT than WT and those of Wnt2, Wnt4, Wnt7a and Wnt16 were 2 to 3-fold higher. Contrastingly, Wnt2b and Wnt8a had lower gene expression in DT compared to WT.

Gene expression of Frizzled and Sfrps: All measured inhibitors of frizzled i.e. sfrps were upregulated in DT relative to WT (Figure 5.5). The highest was sfrp4 (upregulated 20-fold), followed by sfrp2 (4-fold) and sfrp1, which was 1.5-fold upregulated in DT compared to WT. Fzd8 was significantly upregulated (4-fold) compared to other frizzled proteins i.e. Fzdb, Fzd2, Fzd3 with more modest upregulation (1 to 2.5-fold increases) in DT compared to WT. By contrast Fzd6 and Fzd7 were both 1 to 2-fold downregulated in DT relative to WT.

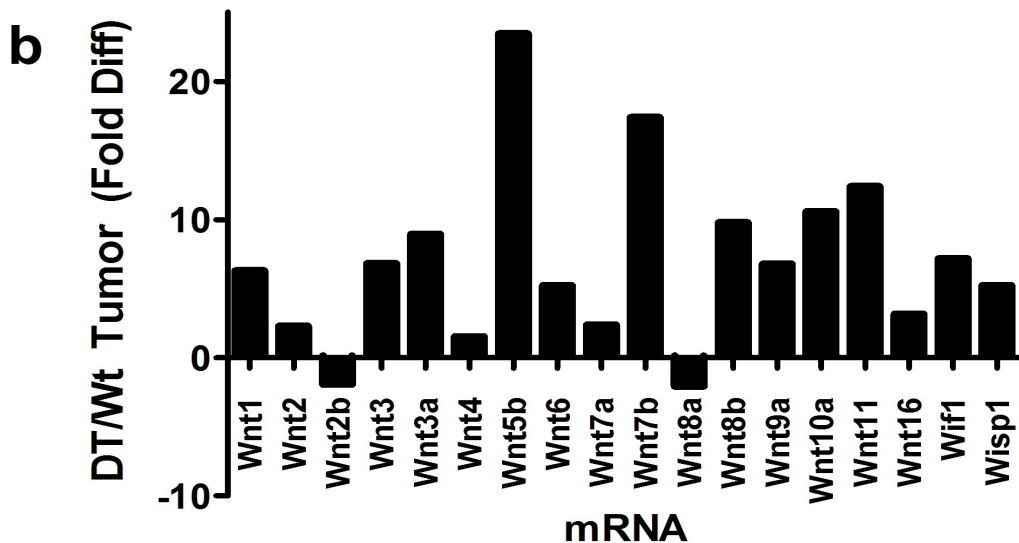
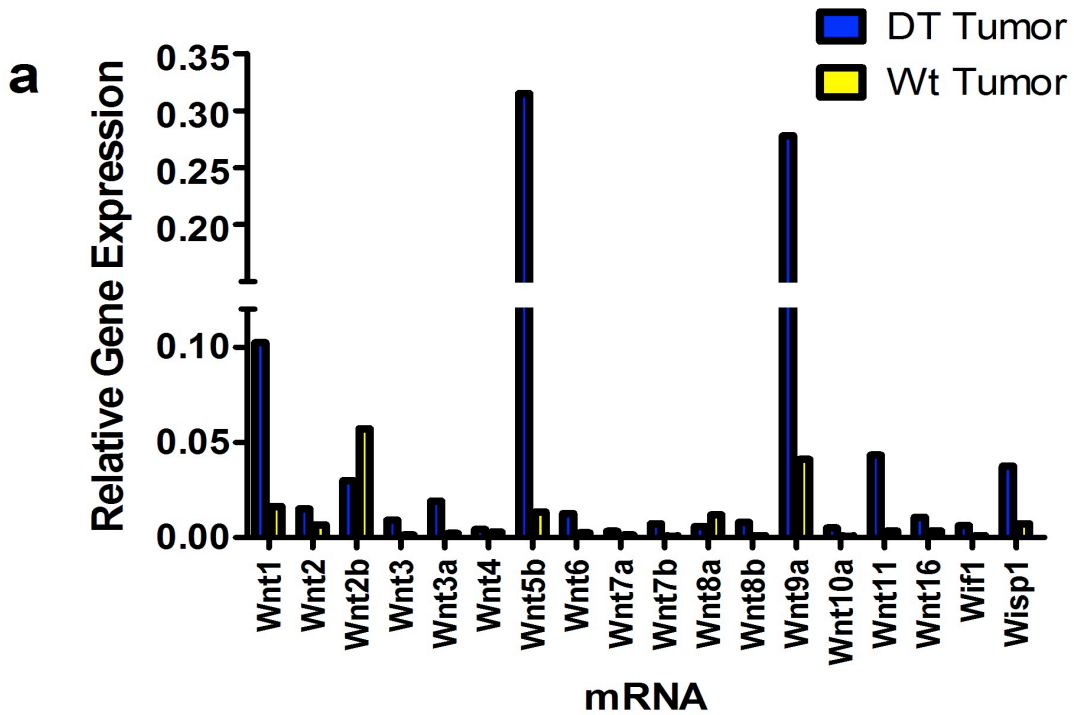


Figure 5.4: Analysis of the relative gene expression of Wnt signaling genes in WT and DT mice with tumours. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) the 18 afore-mentioned Wnt ligands by qPCR using a PCR Array Kit (see Materials and Methods). In (B) gene expression is expressed as a fold difference calculated as a ratio of gene expression in DT over that in WT mice.

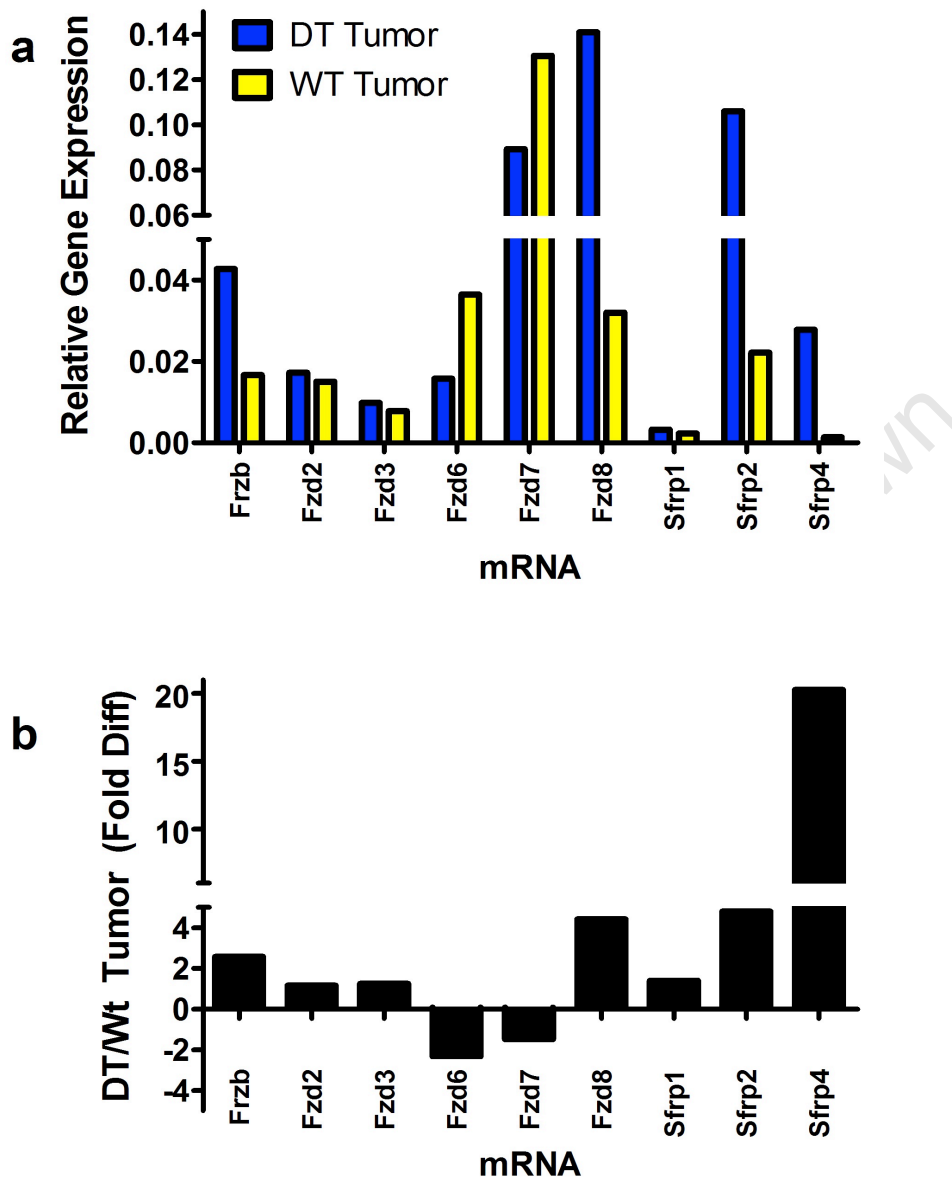


Figure 5.5: Analysis of the relative gene expression of Wnt signaling genes in WT and DT mice with tumours. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) the 9 afore-mentioned Wnt receptors and related proteins by qPCR using a PCR Array Kit (see Materials and Methods). In (B) gene expression is expressed as a fold difference calculated as a ratio of gene expression in DT over that in WT mice.

Gene expression of downstream genes of Wnt signaling: Genes of canonical signaling were significantly upregulated i.e. Foxn1 and Lef1 (\geq than 20-fold increase) and compared to other genes in DT versus WT (Figure 5.6). Other canonical genes that were more moderately upregulated (2 to 10-fold) in DT compared to WT include Ctbp2, Dkk1, Dvl1, GSK-3 β , Porcn and Pygo1. The target genes of Wnt signaling, Fosl1, Myc and Pitx2 were marginally upregulated (at least 1-fold) in DT compared to WT, and the genes involved in developmental processes such as growth and proliferation that were modestly upregulated were Fgf4, and Fshb and those downregulated were Rhou and Slc9a3r1 in DT relative to WT. Finally the remainder of the panel measured genes that are negative regulators of Wnt signaling i.e. Nik and Tle2, and these were downregulated whereas Ppp2r5d was upregulated in DT compared to WT.

Gene expression in pre-malignant versus HCC lesions

Wnt ligands: To understand more clearly the differences in signaling that occur at different stages of progression to HCC, we stratified the data according to the different time points from 3 to 21 months (representing the pre-malignant phase) versus HCC (Figure 5.7). Wnt1, Wnt3, Wnt3a, Wnt5b, Wnt9a, Wnt11 and Wisp1 had essentially similarly low levels of gene expression in the WT mice at 3, 6, 12, 15, 18, 21 months and in HCC with some fluctuations e.g. higher levels of Wnt9a in WT in HCC.

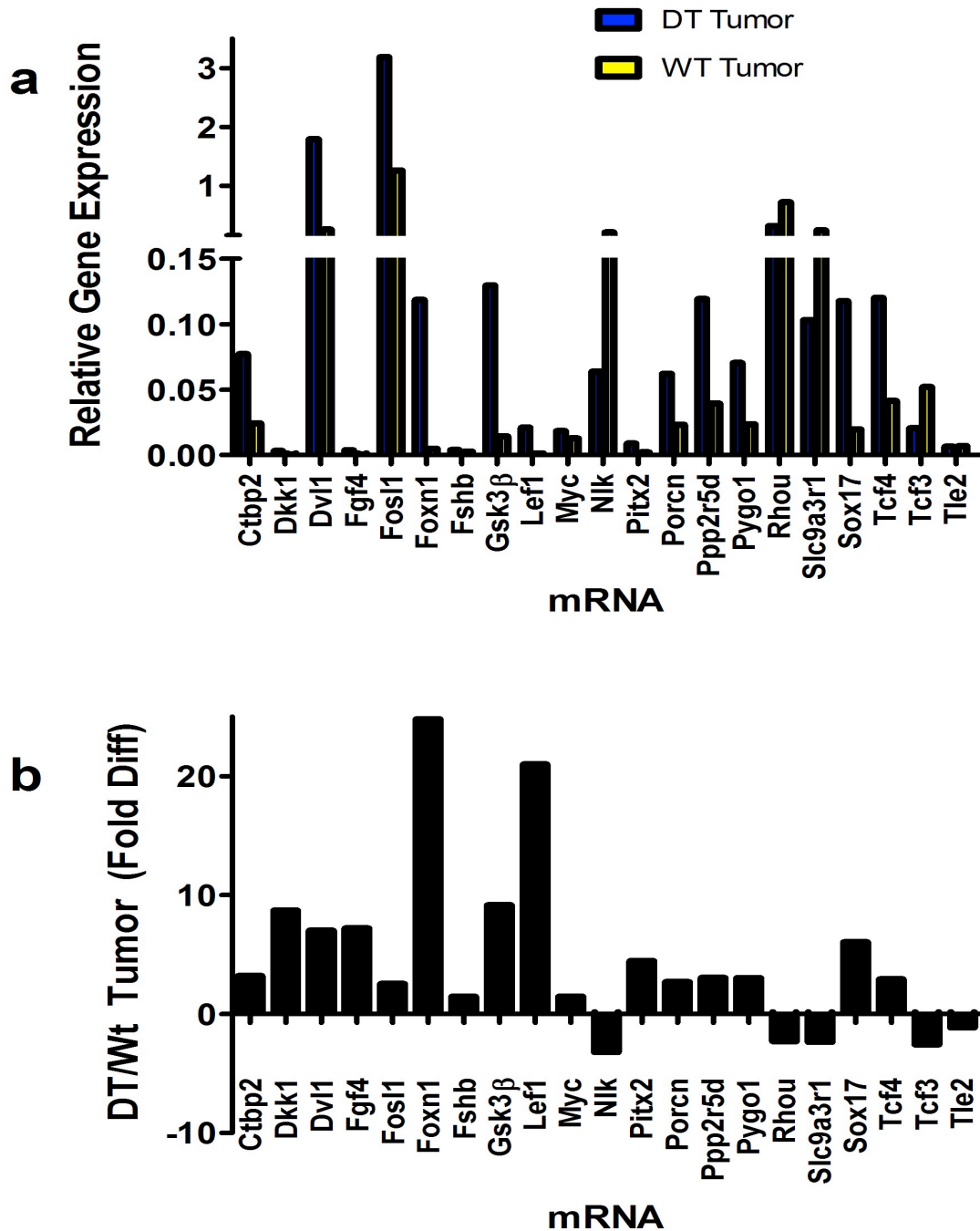


Figure 5.6: Analysis of the relative gene expression of Wnt signaling genes in WT and DT mice with tumours. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) the 21 afore-mentioned Wnt downstream targets by qPCR using a PCR Array Kit (see Materials and Methods). In (B) gene expression is expressed as a fold difference calculated as a ratio of gene expression in DT over that in WT mice.

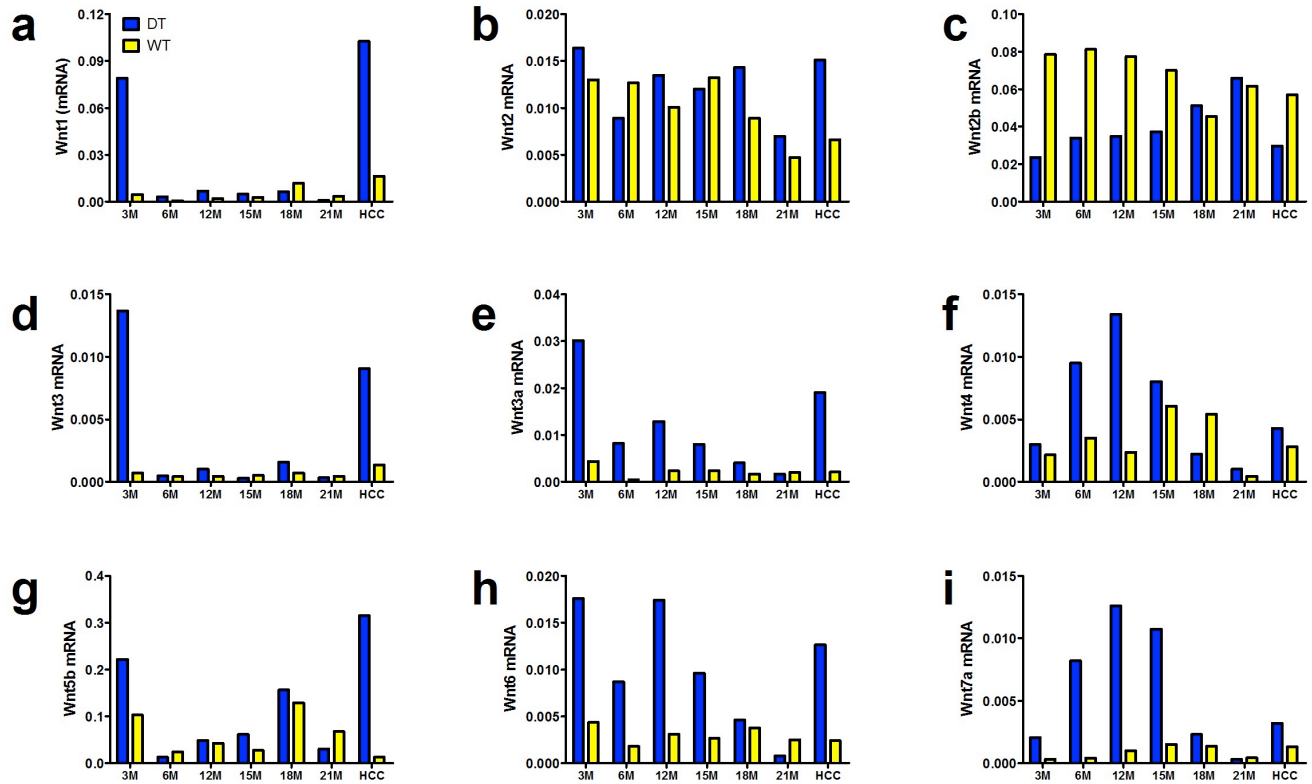


Figure 5.7a: Analysis of the gene expression of Wnt signaling genes in WT and DT mice represented in bar graphs. The time points 3, 6, 12, 15, and 21 months represent the pre-neoplastic phase, before the mice developed HCC. For comparison of Wnt ligand gene expression HCC is represented on its own. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Wnt1, (B) Wnt2, (C) Wnt2b, (D) Wnt3, (E) Wnt3a, (F) Wnt4, (G) Wnt5b, (H) Wnt6, (I) Wnt7a, (J) Wnt7b, (K) Wnt8a, (L) Wnt8b, (M) Wnt9a, (N) Wnt10a, (O) Wnt11, (P) Wnt16, (Q) Wif1 and (R) Wisp1 by qPCR using a PCR Array Kit (see Materials and Methods).

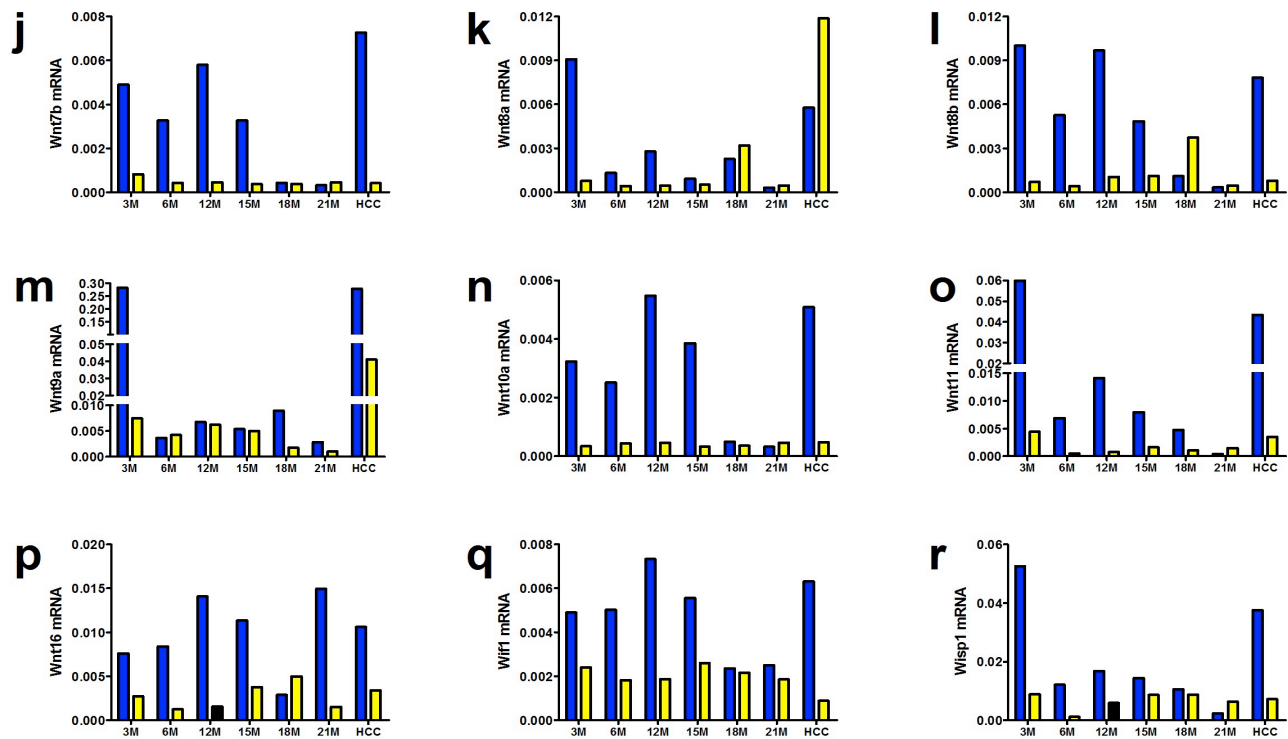


Figure 5.7b: Analysis of the gene expression of Wnt signaling genes in WT and DT mice represented in bar graphs. The time points 3, 6, 12, 15, and 21 months represent the pre-neoplastic phase, before the mice developed HCC. For comparison of Wnt ligand gene expression HCC is represented on its own. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Wnt1, (B) Wnt2, (C) Wnt2b, (D) Wnt3, (E) Wnt3a, (F) Wnt4, (G) Wnt5b, (H) Wnt6, (I) Wnt7a, (J) Wnt7b, (K) Wnt8a, (L) Wnt8b, (M) Wnt9a, (N) Wnt10a, (O) Wnt11, (P) Wnt16, (Q) Wif1 and (R) Wisp1 by qPCR using a PCR Array Kit (see Materials and Methods).

In DT mice however, gene expression levels were markedly upregulated at 3 months and in HCC, suggesting a re-activation of Wnt signaling in HCC. Gene expression levels of Wnt4, Wnt6, Wnt7a, Wnt7b, Wnt8b, Wnt10a and Wif1 were generally similar with mild fluctuations at all time points including HCC in WT, but were upregulated at 3, 6, 12 and 15 months, but not 18 and 21 months, and in HCC in DT, showing evidence of activation at earlier time points which wanes but is reactivated in HCC. Wnt16 had a similar profile with the exception that gene expression levels were high at 18 months and in HCC in DT. Gene expression levels of Wnt 8a were low at all time points in WT mice except in HCC where they were higher than in DT in HCC, whereas expression levels in DT were the highest in DT at 3 months, but lower in HCC. Finally Wnt2 and Wnt2b had similar profiles in premalignant stages as in HCC with DT mice having overall higher levels than WT in the former, and WT having at least 2-fold higher levels than DT in the latter.

Gene expression of Frizzled and Sfrps: The gene expression of the receptors frizzled and related proteins showed some similar profiles to Wnt ligands. Fzd2, Fzd8, sfrp2 and sfrp4 exhibited low mRNA transcript levels in WT mice at all time points, whereas DT mice had elevated levels at 3 months only and in HCC (Figure 5.8). Fzd3 and sfrp1 were expressed at similar levels in WT mice but had higher expression at 6, 12 and 15 months than at 3 months in DT mice, in the premalignant stages than in HCC. The remainder of measured receptors viz: Fzd1, Fzd4, Fzd5, Fzd6 and Fzd7 had overall similar levels of gene

expression and profiles in premalignant and HCC livers.

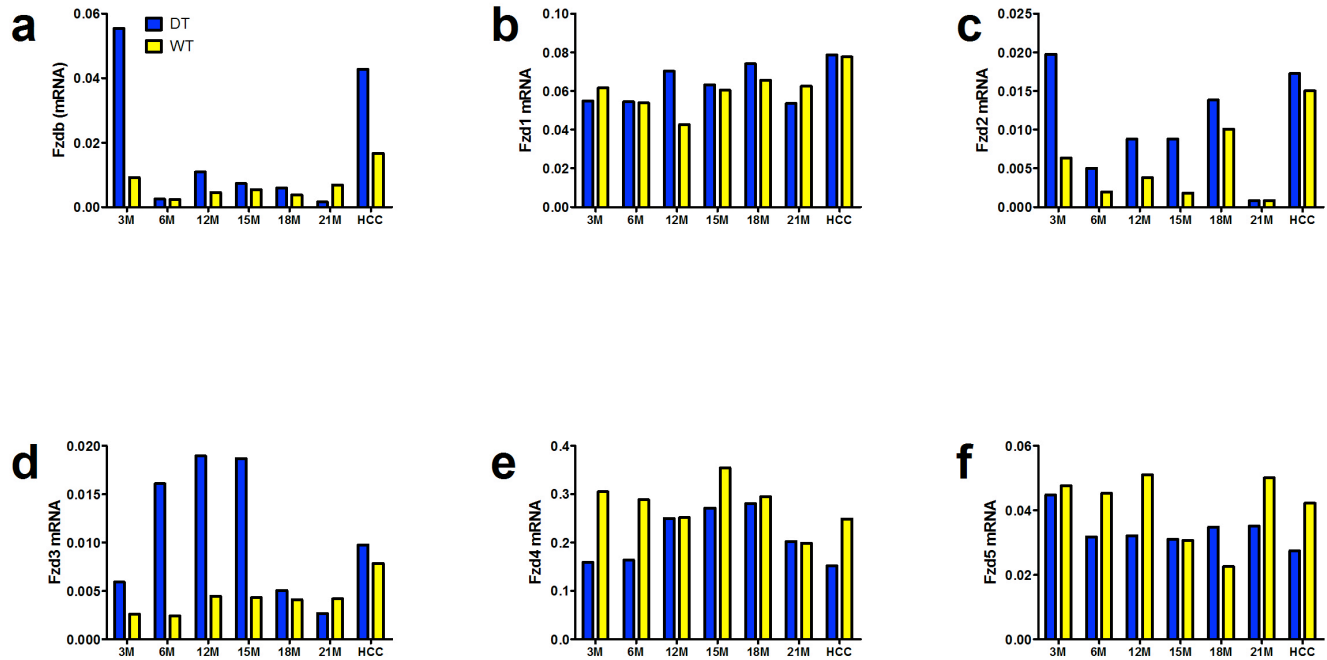


Figure 5.8a: Analysis of the gene expression of Wnt signaling genes in WT and DT mice represented in bar graphs. The time points 3, 6, 12, 15, and 21 months represent the pre-neoplastic phase, before the mice developed HCC. For comparison of Wnt ligand gene expression HCC is represented on its own. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Fzdb, (B) Fzd1, (C) Fzd2, (D) Fzd3, (E) Fzd4, (F) Fzd5, (G) Fzd6, (H) Fzd7, (I) Fzd8, (J) Sfrp1, (K) Sfrp2 and (L) Sfrp4 by qPCR using a PCR Array Kit (see Materials and Methods).

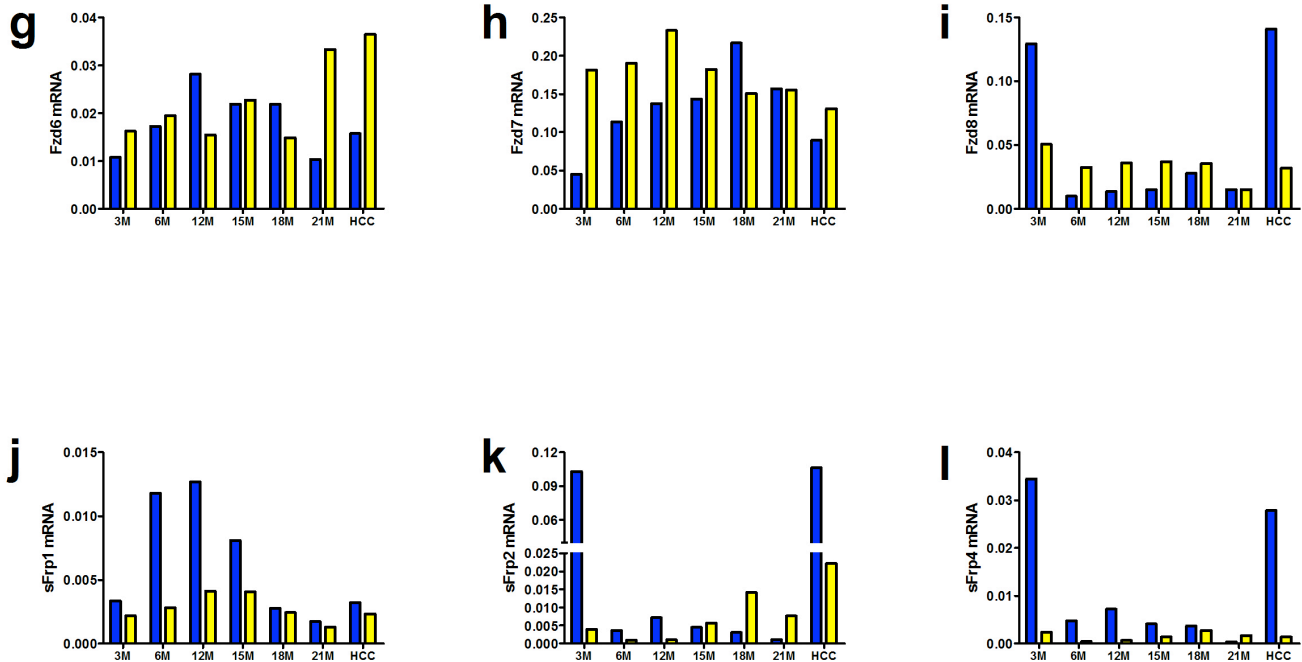


Figure 5.8b: Analysis of the gene expression of Wnt signaling genes in WT and DT mice represented in bar graphs. The time points 3, 6, 12, 15, and 21 months represent the pre-neoplastic phase, before the mice developed HCC. For comparison of Wnt ligand gene expression HCC is represented on its own. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Fzdb, (B) Fzd1, (C) Fzd2, (D) Fzd3, (E) Fzd4, (F) Fzd5, (G) Fzd6, (H) Fzd7, (I) Fzd8, (J) Sfrp1, (K) Sfrp2 and (L) Sfrp4 by qPCR using a PCR Array Kit (see Materials and Methods).

Gene expression of downstream genes of Wnt signaling: The gene expression of Dvl1, GSK-3 β , Lef1, Porcn, Ppp2r5d, Pygo1, Fosl1, Foxn1, Sox17 and Tcf4 was at low levels in WT mice at all time points, however in DT mice, was at higher levels at 3 months and in HCC suggesting similar activation patterns early in development and later in carcinogenesis (Figures 5.9 a and b). Other genes i.e. Dkk1, Fgf4 and Pitx2 had low expression in WT mice, but higher levels of expression in the earlier time points of 3, 6, 12 (peak levels) and 15 months, with a decline in HCC in DT mice. Axin, Ctbp1, Ep300, Ccnd1, Lrp6, Nkd1 and Nik had lower gene expression levels at all time points including HCC in DT compared to WT, with increasing levels from 3 months and mostly peaking at 15 months. Lrp5 and Myc mRNA transcripts were higher in the WT mice at earlier time points until 12-15 months, where the levels in DT are higher, including in HCC. Finally Bcl9 and Ctbp2 gene expression levels were higher in DT mice than WT at all time points including HCC, with a much higher level of Ctbp2 in HCC. However for both, the profile is similar in preneoplastic liver tissue and HCC.

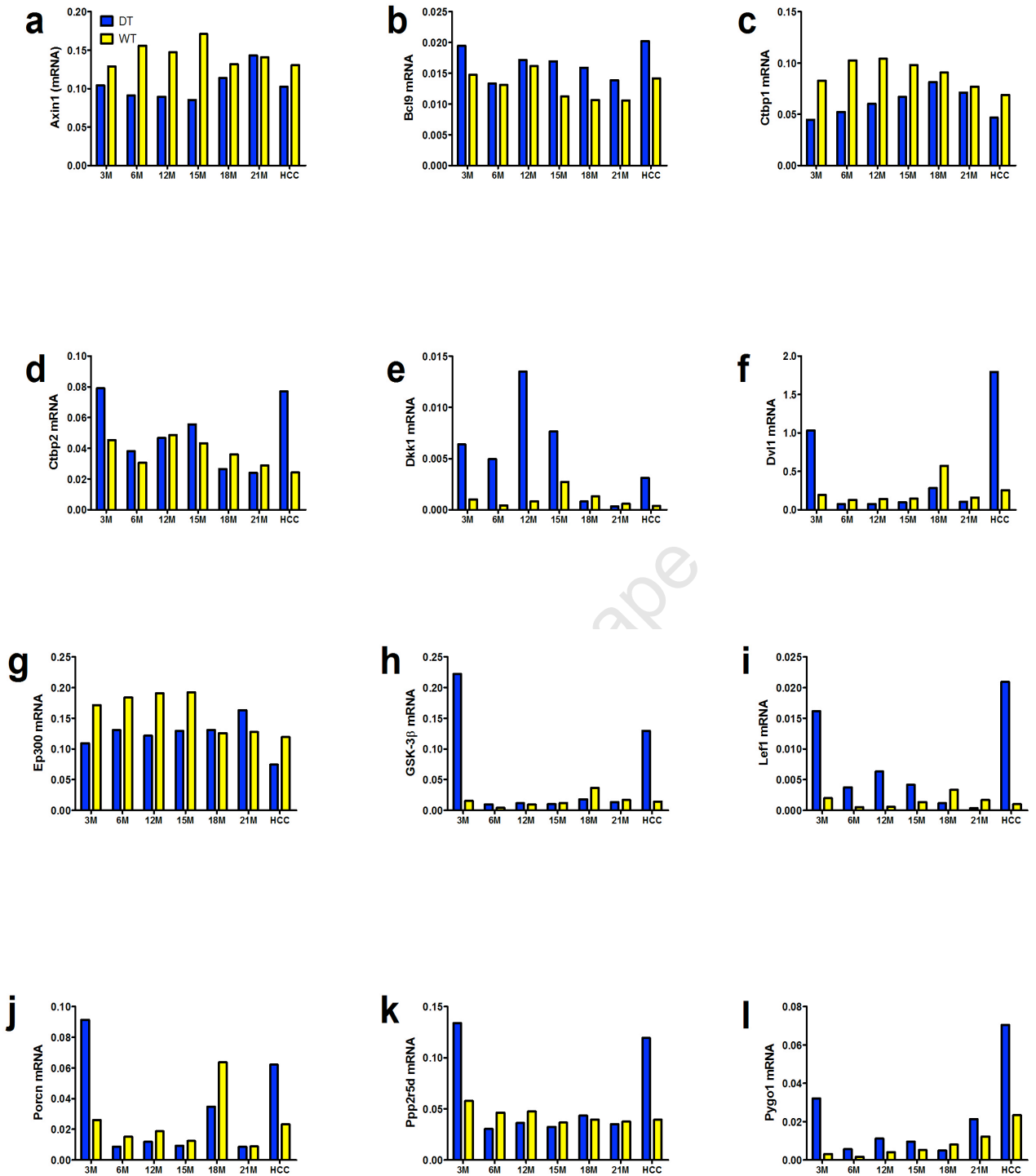


Figure 5.9a: Analysis of the gene expression of Wnt signaling genes in WT and DT mice represented in bar graphs. The time points 3, 6, 12, 15, and 21 months represent the pre-neoplastic phase, before the mice developed HCC. For comparison of Wnt ligand gene expression HCC is represented on its own. RNA extracted from liver was reverse transcribed and

the cDNAs were used to measure (A) Axin1, (B) Bcl9, (C) Ctbp1, (D) Ctbp2, (E) Dkk1, (F) Dvl1, (G) Ep300, (H) GSK-3 β , (I) Lef1, (J) Porcn, (K) Ppp2r5d and (l) Pygo1 by qPCR using a PCR Array Kit (see Materials and Methods).

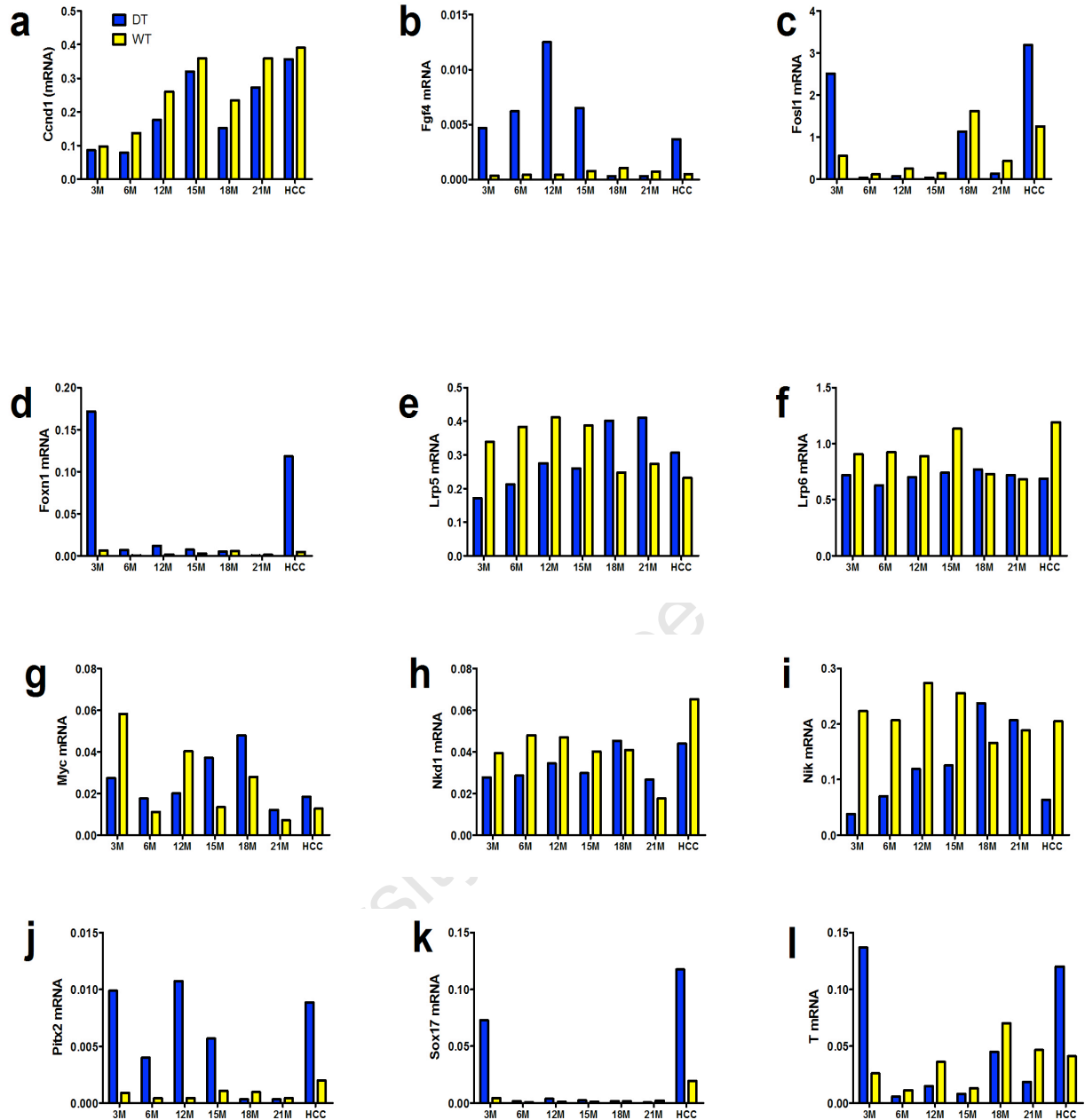


Figure 5.9b: Analysis of the gene expression of Wnt signaling genes in WT and DT mice represented in bar graphs. The time points 3, 6, 12, 15, and 21 months represent the pre-neoplastic phase, before the mice developed HCC. For comparison of Wnt ligand gene expression HCC is represented on its own. RNA extracted from liver was reverse transcribed and the cDNAs were used to measure (A) Ccnd1, (B) Fgf4, (C) Fosl1, (D) Foxn1, (E) Lrp5, (F) Lrp6, (G) Myc, (H) Nkd1, (I) Nik, (J) Pitx2, (K) Sox17 and (L) Tcf4 by qPCR using a PCR Array Kit (see Materials and Methods).

5.4 DISCUSSION

Previous data from this model reported by our laboratory demonstrated that the combination of the ATX and IRS-1 transgenes caused premalignant changes in the liver, with three cases showing lesions typical of HCC and increased β -catenin immunoreactivity (Longato et al., 2009). In the same study only Wnt3, Fzd3 and Fzd7 were evaluated, and these were significantly upregulated in the double transgenic mouse compared to other groups. The aim of this study was to characterize components of all three Wnt signaling pathways (canonical and non-canonical) at various time points in the progression to HCC with the purpose of uncovering critical molecules of activated or downregulated Wnt signaling that are important for hepatocarcinogenesis, particularly in HBV-related HCC. These signaling molecules can be potentially targeted in designing therapeutic agents against HCC. In DT mice without tumour, 58% of measured Wnt ligands, receptors and downstream genes were activated compared to WT mice. Activation of Wnt signaling was dominant particularly at an early time point (3 months), in some cases with Wnt activation peaking at 6 to 12 months time point, and then declining at the later stages. It is noteworthy that overall, the mice that developed tumours were male and at time points beyond 15-18 months. In this context, it is interesting that various components of canonical and non-canonical Wnt signaling were upregulated in non-tumour tissue at between 3-12 months and also in HCC suggesting that re-activation of these genes is necessary for the development of HCC, as these levels had declined beyond 12 months at baseline. Notwithstanding the key role of Wnt re-activation in HCC, hepatic

overexpression of normal or mutant β -catenin is insufficient to cause tumorigenesis in experimental models (Nejak-Bowen & Monga). Furthermore, HBx in association with activated Wnt1-activated Wnt signaling was shown to be necessary to stabilize β -catenin in hepatoma cells (Cha et al., 2004). Moreover, previous studies using either of the transgenes alone did not result in HCC (Mohr et al., 2008; Tanaka et al., 1997), therefore our study highlights the critical role of not only the two transgenes (ATX and IRS-1), but a transactivator and signaling molecule co-operating together to cause cancer, because this re-activation was not observed in the WT mice. In DT mice that developed tumours 89% of Wnt ligands were upregulated, with 50% having at least a 5-fold increase compared to WT mice with tumours. The most striking upregulations were those of Wnt5b and Wnt7b. This latter finding places these two signaling components as attractive potential signatures for HBV-related HCC versus non-HBV HCC and could be targeted when seeking for therapies for HCC. Corresponding to activated Wnt signaling, the target genes *Fos1* and *Myc* were similarly upregulated. Conversely, *Wnt 2*, *Wnt2b* and *Wnt8a* were downregulated in DT compared to WT mice, inferring that the binding of these ligands to receptors may be inhibitory and therefore protective. Quite surprisingly some inhibitors e.g. *Wif1*, *Sox17*, *sfrp4* and *sfrp2*, which typically antagonize Wnt signaling, were upregulated in DT tumours. A possible explanation for these opposite effects may be that these inhibitory modulators may have been epigenetically regulated with resultant transcriptional activation rather than suppression in these mice. Hypermethylation of *sfrps* (Shih et al., 2007; Shih et al., 2006; Takagi et al.,

2008) and Wif1 (Z. Ding, Qian, Zhu, & Xiong, 2009) occurs in HCC and is associated with downregulation of these molecules, therefore in our study there may have been promoter hypomethylation resulting in transcriptional activation rather than repression. The epigenetic contribution to our findings in this study was not pursued and is a potential future area of study to further explain these findings. By contrast Fzd6 and Fzd 7 were downregulated in DT versus WT mice with tumours. This finding is in sharp contrast to previous studies reporting overexpression of Fzd7 in HCC and subsequent accumulation of β -catenin (Longato et al., 2009; Merle et al., 2004; Merle et al., 2005). Considering that our study utilized the same tissue as that used by Longato and Merle, this finding is incongruous. An explanation for this may be the small number of samples (N=12 per group) used in our study, and sampling error compared to the larger numbers (N=30) used in other studies. Downregulation of Fzd6 and Fzd7 may be explained on the basis of ligand binding specificity in Wnt signaling; there are 19 Wnt ligands that can bind to 10 different frizzled receptors, or other co-receptors including LRP, therefore the combinations of ligand-receptor interactions are diverse and complex, and may be context-specific. Regarding the remainder of Wnt components measured, in both WT and DT mice with tumours, canonical Wnt downstream signaling genes including GSK-3 β were for the most part upregulated and negative regulators downregulated in DT relative to WT tumours. GSK-3 β is a key molecule in Wnt signaling because with Wnt binding, it is phosphorylated and thus deactivated, thus allowing β -catenin to avoid the destruction complex and translocate to the nucleus to activate transcriptional

activation of target genes. The upregulation of GSK-3 β mRNA transcripts in our study is consistent with its oncogenic potential and subsequent HCC development because overexpression of GSK-3 β is associated with the accumulation of β -catenin ($p < 0.05$) (Ban, Singh, Krishnan, & Seow, 2003), which is critical for tumorigenesis.

Recent advances in molecular techniques have allowed for efficient high throughput screening of molecular pathways that are responsible for many disease processes. This understanding is crucial in HCC, as newer treatment targets are urgently needed, either as monotherapy or dual therapy with sorafenib. The importance of this study is in its identification of potential disease markers for HBV-related HCC and therefore potential therapeutic targets within the Wnt signaling cascade. The disadvantage of PCR arrays however, is that in analyzing multiple genes at once, many genes are upregulated and without correlative protein determinations, it is difficult to interpret the significance of this regulation. To further understand the findings of this study, an extension to this study will require analysis of the epigenome, to characterize the role of age-associated epigenetic changes that likely played a role in this study.

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CHAPTER 6

Ceramide-Induced Lipotoxicity Promotes Insulin Resistance in Alcoholic Steatohepatitis in Humans

ABSTRACT

Background: Alcoholic steatohepatitis (ASH) has high mortality and mechanistically is associated with lipotoxicity-associated hepatic insulin resistance, pro-cytokine activation and increased endoplasmic reticulum (ER) stress.

Hypothesis: Ceramides are important mediators of insulin resistance, ER stress, and cytokine activation in the progression of alcoholic liver disease (ALD).

Methods: Human liver tissue from normal subjects (n=8) and those with clinical and laboratory features of ALD (n=8) were analyzed for lipid content, insulin, pro-ceramide and ER stress signaling and pro-cytokine activation using both ELISA and qT-RT-PCR assays.

Results: Neutral lipids (p=0.03) and ceramide (p=0.01) levels were higher in the ALD group of patients, and in the latter case there was a correlation with upregulation of ceramide synthesis and degradation pathway gene expression. In the alcohol group, due to suppressed signaling, there was a compensatory upregulation of the mRNA transcripts of the insulin receptor (p<0.0001), IGF-1 receptor (p<0.0001) and IGF-2 receptors (p=0.008) and insulin and IGF-1

receptors at protein expression level. In addition, we observed up-regulation of ER stress genes that are involved in both the UPR and ER stress-induced apoptosis, including CHOP ($p=0.013$) and BAX ($p=0.0003$). However, pro-cytokine activation was not evident; on the contrary, IL-6 was downregulated in the alcohol group, which may infer alcohol-induced loss of its hepato-protective effect.

Conclusions: The role of ceramides in alcoholic steatohepatitis is increasingly being recognized. Ceramides are potent cell signaling molecules, which also play a role in inducing the insulin resistance and ER stress implicated in the pathogenesis of ALD. Therefore the potential for future use of ceramide inhibitors in clinical settings is immense, and they may become an important part of the treatment strategy for ALD.

6.1 INTRODUCTION

Chronic alcohol abuse is pervasive in all societies. Alcohol is responsible for up to 50% of all cause end-stage liver disease and alcoholic liver-related deaths account for close to 60% of all deaths from alcohol use (Beier & McClain, 2010). Alcoholic liver disease (ALD) encompasses a potentially progressive spectrum of disease that is characterized by histopathological features ranging from simple fat accumulation in the liver (steatosis), to a malign condition typified by steatosis with inflammation and apoptosis (steato-hepatitis). More concerning however, is that consumption levels of about 80g/day are associated with hepatocellular carcinoma (HCC) (Hassan et al., 2002; Schoniger-Hekele et al., 2000). Alcoholic steato-hepatitis (ASH) is associated with high mortality rates in the short-term, up to 7% during an average hospital stay of 6.5 days (Liangpunsakul, 2010). The critical question is the mechanism(s) of progression from simple steatosis to steato-hepatitis, as not all patients progress in this manner. While active intense research has been on going, the mechanisms remain elusive. Current treatments for ALD are limited, and/or ineffective, therefore an understanding of newer mechanisms of progression will allow for the potential development of newer targeted treatments for this common and growing condition.

Progression of ALD is mediated by multiple factors including but not limited to, oxidative stress, pro-inflammatory cytokine activation and dysregulation of clotting (Arteel, 2003; Beier et al., 2008; Beier et al., 2009; de la Monte, Longato, Tong, DeNucci, & Wands, 2009; Imuro, Gallucci, Luster, Kono, & Thurman, 1997). Lipotoxicity has been extensively studied, in particular in diet-induced fatty liver

disease and contributes to the pathogenesis and progression of liver injury (Farrell & Larter, 2006; Ussher et al., 2010). Although the role of impaired lipid metabolism and homeostasis with resultant lipotoxicity in ALD has been less well characterized, there is some evidence for its role in the progression of disease (de la Monte, Longato et al., 2009). Lipotoxic states impair either binding or downstream signal transduction of insulin signaling thereby causing insulin resistance (de la Monte et al., 2008; Denucci et al., 2010; Pang et al., 2009) which compromises reparative and regenerative mechanisms in the liver, as intact signaling is required for liver remodeling and repair following injury (de la Monte & Wands, 2005; Giovannone et al., 2000).

Our group has studied the effects of ceramide inhibition on steatosis, ER stress and insulin resistance, with an improvement in all indices, however these studies were performed in animal models of ALD (Setshedi, Tong et al., 2010). Therefore one of the aims of this study was to evaluate the role of ceramides in promoting IR in human liver tissue of alcoholics.

Much of the liver injury of ALD is thought to be due to the serial oxidation and metabolism of alcohol to acetate. These reactions result in the generation of reactive oxygen species, an altered redox ratio and other metabolic effects, which are implicated in alcohol-induced endoplasmic reticulum (ER) stress. The unfolded protein response (UPR), upregulation of protein chaperones and with prolonged insult, induction of apoptosis is the key regulatory mechanisms involved in inducing ER stress. Furthermore, sphingolipids as emerging bio-effector molecules are also implicated in inducing various context-specific ER stress responses, which typically

depend on the fatty acid chain length (Prieur, Roszer, & Ricote, 2010; Senkal, Ponnusamy, Bielawski, Hannun, & Ogretmen, 2010; Spassieva, Mullen, Townsend, & Obeid, 2009).

Moreover the role of pro-cytokine activation effected mainly via toll-like receptor 4-MyD88 complex and downstream, TNF- α is well known (Szabo & Bala, 2010). More interestingly is the role of sphingolipids in TNF-mediated hepatocyte apoptosis (reviewed by Fernandez-Checa et al, 2005) (Fernandez-Checa, Colell, Mari, & Garcia-Ruiz, 2005), thus linking pro-cytokine activation and ceramides in the pathogenesis of ALD (Nikolova-Karakashian & Rozenova, 2010). Therefore possibly the individual independent drivers of advanced stages of ALD combine either additively or synergistically to amplify liver injury. Our study aimed to interrogate the role of and relationships between ceramides and its link to insulin resistance, ER stress, and cytokine activation in worsening the severity of disease.

6.2 MATERIALS AND METHODS

Patients and specimens: The samples utilized for the analysis comprise of liver tissue from normal subjects (n=8) who had no discernible liver disease, designated “controls” and liver from those with a significant history of alcohol consumption with clinical and laboratory features of ALD (n=8). These tissue samples were gratefully donated to our laboratory, the Liver Research Center of Brown University (a collaborative effort) from the Coro laboratory facility, a collaborator. The liver tissues were coded and had been de-identified, therefore were exempt from Human Ethics approval at our institution, the Lifespan IRB committee as no specific sensitive patient information was available.

Materials: The same materials described in Chapter 2, to process and analyze tissue for protein (ELISA) and RNA (qRT-PCR) applications were applied; as such the reader is referred to that chapter.

Lipid studies: Lipids were measured directly from the protein homogenates and the readings corrected for by sample protein concentration as measured by BCA.

Multiplex ELISA: We used bead-based multiplex ELISAs to examine the integrity of insulin and IGF-1 signaling networks, or measure immunoreactivity to selected pro-inflammatory cytokines and chemokines in liver tissue as previously outlined. Regarding pro-inflammatory cytokine and chemokine activation, we measured immunoreactivity to interleukin (IL) 1 β , IL-6, IL-8, IL-18, TNF- α , IFN- γ , IP-10, MIP-1, β -NGF and TRAIL.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Assays: The same method as previously described was used, with the exception that primers reactive to human tissue were used (Table 11). Relative mRNA abundance was calculated from the ng ratios of specific mRNA to 18S for the insulin genes and hypoxanthine phosphoribosyltransferase 1 (HPRT) for the pro-ceramide and ER stress genes, measured in the same samples (Moroz, Tong, Longato, Xu, & de la Monte, 2008). Template-free reactions served as negative controls.

Statistical Analysis: Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers). Intergroup comparisons were made using Student t-tests. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Significant P-values (<0.05) are shown within the panels or tables.

Table 11: Human Primer Pairs Used for qRT-PCR Assays

Primer	Direction	Sequence (5'→3')	Position (mRNA)	Amplicon Size (bp)
Insulin/IGF signaling				
Insulin	For	TTC TAC ACA CCC AAG TCC CGT C	189	134
Insulin	Rev	ATC CAC AAT GCC ACG CTT CTG C	322	
Insulin	For	GGT AGA AAC CAT TAC TGG CTT	1037	125
Receptor		CCT C		
Insulin	Rev	CGT AGA GAG TGT AGT TCC CAT	1161	
Receptor		CCA C		
IGF-1	For	CAC TTC TTT CTA CAC AAC TCG	1049	130
		GGC		
IGF-1	Rev	CGA CTT GCT GCT GCT TTT GAG	1178	
IGF-1	For	TAC TTG CTG CTG TTC CGA GTG G	295	101
Receptor				
IGF-1	Rev	AGG GCG TAG TTG TAG AAG AGT	395	
Receptor		TTC C		
IGF-2	For	CTG ATT GCT CTA CCC ACC CAA G	1024	76
IGF-2	Rev	TTG CTC ACT TCC GAT TGC TGG C	1099	
IGF-2	For	CAC GAC TTG AAG ACA CGC ACT	403	132
Receptor		TAT C		
IGF-2	Rev	GCT GCT CTG GAC TCT GTG ATT TG	534	
Receptor				

IRS-1	For	TGC TGG GGG TTT GGA GAA TG	4527	46
IRS-1	Rev	GGC ACT GTT TGA AGT CCT TGA CC	4572	
IRS-2	For	AAA ATT GGC GGA GCA AGG C	753	64
IRS-2	Rev	ATG TTC AGG CAG CAG TCG AGA G	816	
IRS-4	For	CCG ACA CCT CAT TGC TCT TTT	570	74
		C		
IRS-4	Rev	TTT CCT GCT CCG ACT CGT TCT C	643	
Pro-Ceramide signaling				
CERS-1	For	CAA TGT GGG CAT CCT TGT T	669	61
CERS-1	Rev	GGT GAA CTC AAG CTG CAC G	729	
CERS-2	For	ACT TCT GGT GGG AAC GTC TG	370	73
CERS-2	Rev	ACA CGT CCA TCT CGG TCT TC	442	
CERS-4	For	GCA GGT GAT ACA CCA CTT CG	944	91
CERS-4	Rev	TAA CAG CAG CAC CAG AGA GAG	1034	
		GC		
UGCG	For	GAT CAG GTG GAC CAA ACT ACG	1109	71
UGCG	Rev	AGC ATT CTG AAA TTG GCT CAC	1179	
CERD2	For	GAC CAT GTC TGG ACG AAG ACT TC	1626	77
CERD2	Rev	CAA CAG TCA TGT TCT GCA TCC C	1702	
CERD3	For	GGC AGT GGC TAT AGC ATA TGG	367	85
CERD3	Rev	AGA CCA GGC GGA TGA ACT G	451	
GM3	For	TGC TGC TGA TAC TGG GTC TG	1710	77
synthase				
GM3	Rev	CAA CAG AGA AGG GTT GTG ACC	1786	

synthase

SPTLC-1	For	CGC GCT ACT TGG AGA AAG A	1324	77
SPTLC-1	Rev	CTC TGT TTG TTC CAC CGT GA	1400	
SPTLC-2	For	ATC GGT GTC GTT GTG GTT G	1659	82
SPTLC-2	Rev	TGG TAT GAG CTG CTG ACA GG	1740	
SMPD-1	For	GCA CAC CTG TCA ATA GCT TCC	1142	78
SMPD-1	Rev	TGG CCA TCG CTT CAT AGA G	1219	
SMPD-3	For	AGG ACC TGC TGT CTG CTG	3956	76

TC

SMPD-3	Rev	TTG CAG CTC AAT GGG TGA C	4031	
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ER Stress Signaling

BAX	For	TGA CCC CTG ACC TCA CTG T	763	82
BAX	Rev	TGA GCA ATT CCA GAG GCA GT	844	
EDEM	For	GCT GAG TGA TTA CCT TAG CCA CA	5504	72
EDEM	Rev	TGG ACA GGA AAG CTC AGG AT	5575	
CHOP	For	GTC ATT GCC TTT CTC CTT CG	192	61
CHOP	Rev	GTC CTC ATA CCA GGC TTC CA	252	
P58IPK	For	CTG AAG GAG CAA GAG ACC ATC	2182	76
P58IPK	Rev	TTA ATC CAA AGT CTA AGC CTG	2257	

GAT A

GRP78	For	TGA AGG TGA AAG ACC CCT GA	1654	107
GRP78	Rev	CAA AGG TGA CTT CAA TCT GTG G	1760	
ATF4	For	AAG GAG TTC GAC TTG GAT GC	1156	78
ATF4	Rev	ATC CAA CGT GGT CAG AAG GT	1233	

HERP	For	ACT GTC GTT GCA GAG ATT GC	322	107
HERP	Rev	GTT CGG TCT CGG ACT CCA T	428	
WARS	For	CTG GAC GGG TGG ACT TAG C	2667	77
WARS	Rev	GAG GCC TGC CTA GAC ATC TG	2743	
Wnt Signaling				
Wnt1	For	CGA ACC TGC TTA CAG ACT CCA A	329	84
Wnt1	Rev	TCA GAC GCC GCT GTT TGC G	412	
Wnt3	For	ACT TCG GCG TGT TAG TGT CC	607	130
Wnt3	Rev	CAT TTG AGG TGC ATG TGG TC	736	
Wnt4	For	GGA GGA GAC GTG CGA GAA AC	221	68
Wnt4	Rev	CCA GGT TCC GCT TGC ACA TCT	288	
Wnt5a	For	TTC TCC TTC GCC CAG GTT GTA A	418	79
Wnt5a	Rev	CTT CTG ACA TCT GAA CAG GGT	496	
Wnt5b	For	CCA ACT CCT GGT GGT CAT TAG C	251	68
Wnt5b	Rev	CTG GGC ACC GAT GAT AAA CAT C	318	
Sfrp1	For	AGG CGG ATT TCC CTG GTA GT	2359	64
Sfrp1	Rev	TAG GGC AAC CAC GGA CTC TT	2422	
Sfrp5	For	GTC AAA ATG CGC ATC AAG GA	776	65
Sfrp5	Rev	TTC TGG GCT CCA ATC AGC TT	840	
Fzd3	For	TGG CTA TGG TGG ATG ATC AAA G	2172	78
Fzd3	Rev	TGG AGG CTG CCG TGG TA	2247	
Fzd6	For	ACA AGC TGA AGG TCA TTT CCA	1926	76

AA

Fzd6	Rev	GCT ACT GCA GAA GTG CCA TGA T	2001	
Fzd7	For	CAACGGCCTGATGTACTTTAAGG	731	121
Fzd7	Rev	CATGTCCACCAGGTAGGTGAGA	851	
AAH	For	GGG AGA TTT TAT TTC CAC CTG	1647	257
		GG		
AAH	Rev	CCT TTG GCT TTA TCC ATC ACT GC	1903	
Humbug	For	GCT GGG TTG ATT GAG GAT GTG	1873	302
		TG		
Humbug	Rev	GCA GGG GGA AAA AGT CAC CTT	2174	
		ATC		
Notch1	For	AGG ACC TCA TCA ACT CAC ACG C	6035	117
Notch1	Rev	CGT TCT TCA GGA GCA CAA CTG C	6151	
Jagged1	For	TGT CTG TGT CCC ACT GGT TTC	2047	148
		TC		
Jagged1	Rev	AGT TCT TGC CCT CAT AGT CCT	2194	
		CG		
HES1	For	CCA AAG ACA GCA TCT GAG CA	318	91
HES1	Rev	TCA GCT GGC TCA GAC TTT CA	408	

*Abbreviations: qPCR, Quantitative Polymerase Chain Reaction; For, Forward Primer; Rev, Reverse Primer; mRNA, Messenger Ribonucleic Acid; rRNA, Ribosomal Ribonucleic Acid; IGF, Insulin Growth Factor; IRS, Insulin Receptor Substrate; CERS, Ceramide Synthase; UGCG, UDP-Glucose Ceramide Glucosyltransferase; CERD, N-Acylsphingosine Amidohydrolase (Ceramidase); GM3 Synthase, Ganglioside Monosialo Synthase; SPTLC, Serine Palmitoyltransferase; SMPD, Sphingomyelin Phosphodiesterase; BAX, Bcl-2-Associated X Protein; EDEM, Endoplasmic Reticulum Degradation-Enhancing α -Mannosidase-like Protein; CHOP, C/EBP Homolog Protein; P58IPK, PKR Inhibitor (58 kiloDalton); GRP78, 78 kiloDalton Glucose-Regulated Protein; ATF, Activating

Transcription Factor; HERP, Homocysteine-Inducible Endoplasmic Reticulum Stress Protein; WARS, Tryptophanyl-tRNA Synthetase.

6.3 RESULTS

Hepatic steatosis was further characterized by quantification of lipids. We measured neutral lipid, triglyceride and ceramide content. The Nile Red assay demonstrated higher mean levels of neutral lipids in the alcohol relative to control group of patients ($p=0.03$) (Figure 6.1 A). Triglyceride levels (mol/g) on the other hand, were not significantly different between the two groups (Figure 6.1 B). Similar to neutral lipid accumulation, hepatic ceramide levels were higher in the alcohol versus to the control group ($p=0.01$) (Figure 6.1 C).

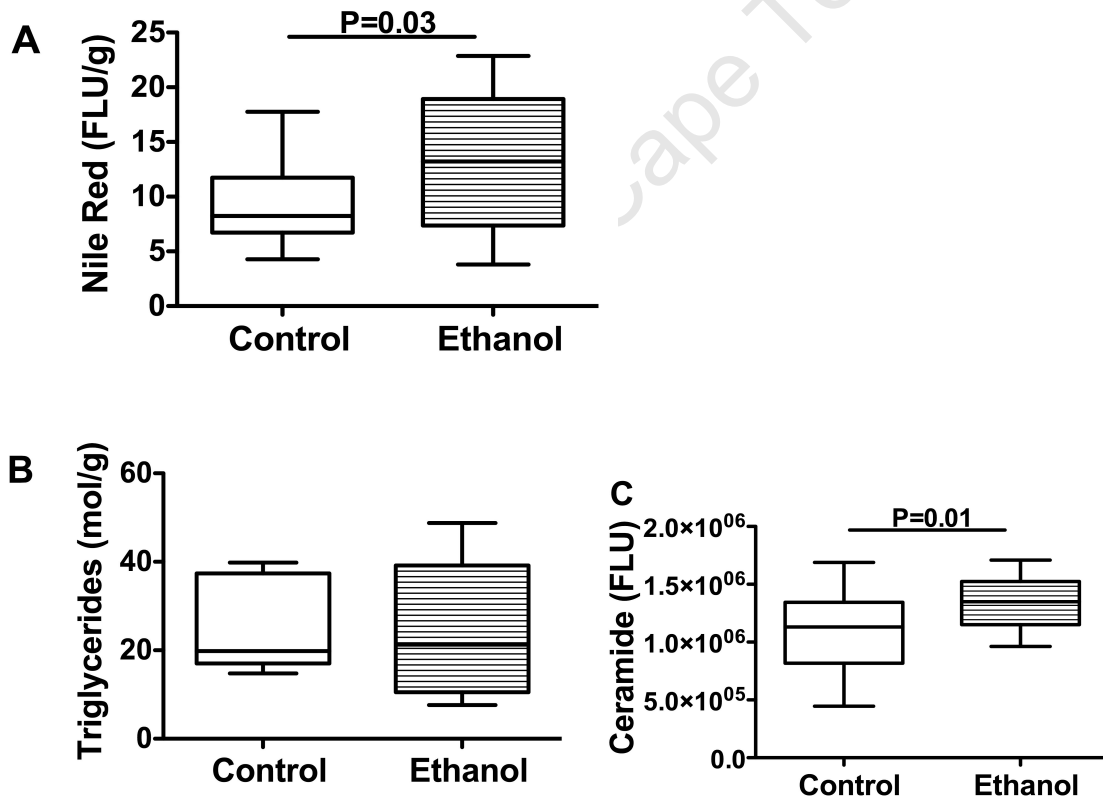


Figure 6.1: Lipid and ceramide accumulations in the liver of patients with alcoholic liver disease (ALD) or controls. Measurements were made from protein homogenates of fresh frozen liver tissue obtained from these patients. We measured (A) neutral lipid content using the Nile Red assay, (B) triglycerides and (C) ceramide immunoreactivity (see Material and Methods), and values

were normalized to tissue protein content. Box plots depict medians (horizontal bars), 95% confidence interval limits, and range (whiskers). Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

Hepatic gene expression levels of ceramide pathway genes: To determine if increased hepatic ceramide was mediated by increased pro-ceramide gene expression or sphingomyelinase enzymatic activity, we measured mRNA levels of ceramide synthases 1, 2, and 4 (CERS), UDP-glucose ceramide glucosyltransferase (UGCG), ceramidase 2 and 3 (CERD), ganglioside monosialo synthase (GM3), serine palmitoyl transferase 1 and 2 (SPTLC), and sphingomyelinase 1 and 3 (SMPD) by qRT-PCR analysis (Figure 6.2). The genes selected for analysis were based on previous studies, which demonstrated the mRNA transcripts that were modulated with increasing steatohepatitis; however these experiments were conducted in animal models (de la Monte, Longato et al., 2009; de la Monte, Tong, Lawton, & Longato, 2009; Lyn-Cook et al., 2009; Shah et al., 2008). The levels of (A) CERS1 ($p=0.025$), (D) UGCG ($p=0.0006$), (G) GM3 synthase ($p=0.006$), (H) SPTLC1 ($p=0.0001$), (J) SMPD1 ($p=0.036$) and (K) SMPD3 ($p=0.019$) were upregulated in alcoholic patients compared to controls. In contrast, however, levels of (E) CERD 2 were markedly down-regulated in the alcohol compared to the control group ($p=0.04$), while mean levels of (B) CERS2, (C) CERS4, (F) CERD3 and (I) SPTLC2 were similar.

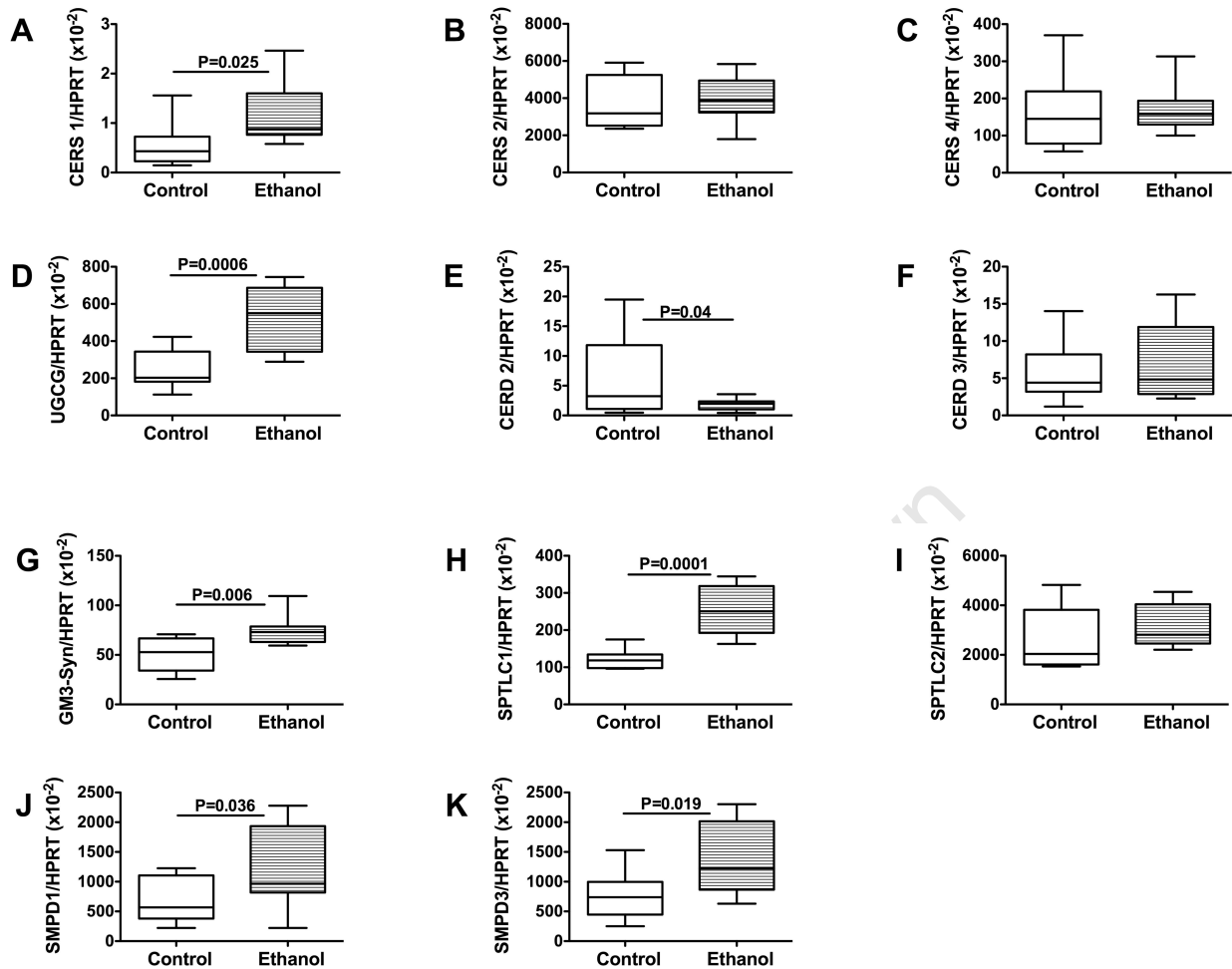


Figure 6.2: Gene expression levels of mediators of pro-ceramide signaling, measured in control and ALD human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) CERS1, (B) CERS2, (C) CERS4, (D) UGCG, (E) CERD2, (F) CERD3, (G) GM3-synthase, (H) SPTLC1, (I) SPTLC2, (J) SMPD1 and (K) SMPD3 mRNA expression by PCR. mRNA abundance was corrected for using HPRT as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

Effects of ethanol on Insulin and IGF signaling mechanisms in liver: Based on the fact that previous studies demonstrated that steatohepatitis produced by high fat diet or chronic ethanol feeding impairs insulin and IGF signaling in the liver in animal models (de la Monte et al., 2008; Svegliati-Baroni et al., 2006; Yeon, Califano, Xu, Wands, & De La Monte, 2003) we re-investigated this concept in human liver tissue. We measured mRNA levels of insulin, IGF-1, and IGF-2 polypeptides, their corresponding receptors, and IRS-1, IRS-2, and IRS-4 by qRT-PCR analysis (Figure 6.3), and IR, ^{pYpY1162/1163}-IR, IGF-1R, ^{pYpY1135/1136}-IGF-1R, IRS-1, ^{pS312}-IRS-1 (Figure 6.4), Akt, ^{pS473}-Akt, GSK-3 β , ^{pS9}-GSK3 β , p70S6K, ^{pTpS421/424}-p70S6K, PRAS40, and ^{pT246}-PRAS40 (Figure 6.5) immunoreactivities by multiplex ELISA. The qRT-PCR analyses demonstrated similar mean levels of (A) insulin, (B) IGF-1, (G) IRS-1, and (H) IRS-2 expression in all groups. Hepatic (D) insulin receptor ($p < 0.0001$), (E) IGF-1R ($p < 0.0001$), and (F) IGF-2R ($p = 0.008$) mRNA levels were significantly higher in the alcohol group. Contrastingly however, (C) IGF-2 polypeptide ($p < 0.0027$) and (I) IRS-4 ($p = 0.04$) expression was lower in livers of alcohol compared with control patients.

At protein expression level (Figure 6.4), analysis of the upstream signaling networks using multiplex ELISAs revealed similar mean levels of (A) IR, even though its phosphorylated counterpart (D) ^{pYpY1162/1163}-IR was increased in the alcohol group ($p = 0.0005$). This null effect correlates with the calculated (G) phosphorylated to total IR levels seen. Signaling via the IGF-1R was upregulated as evidenced by increased mean levels of (B) IGF-1R ($p = 0.0003$). However on

correcting phosphorylated IGF-1R by total levels (H), alcohol results in suppressed levels ($p=0.0008$) as compared to those observed in the control group.

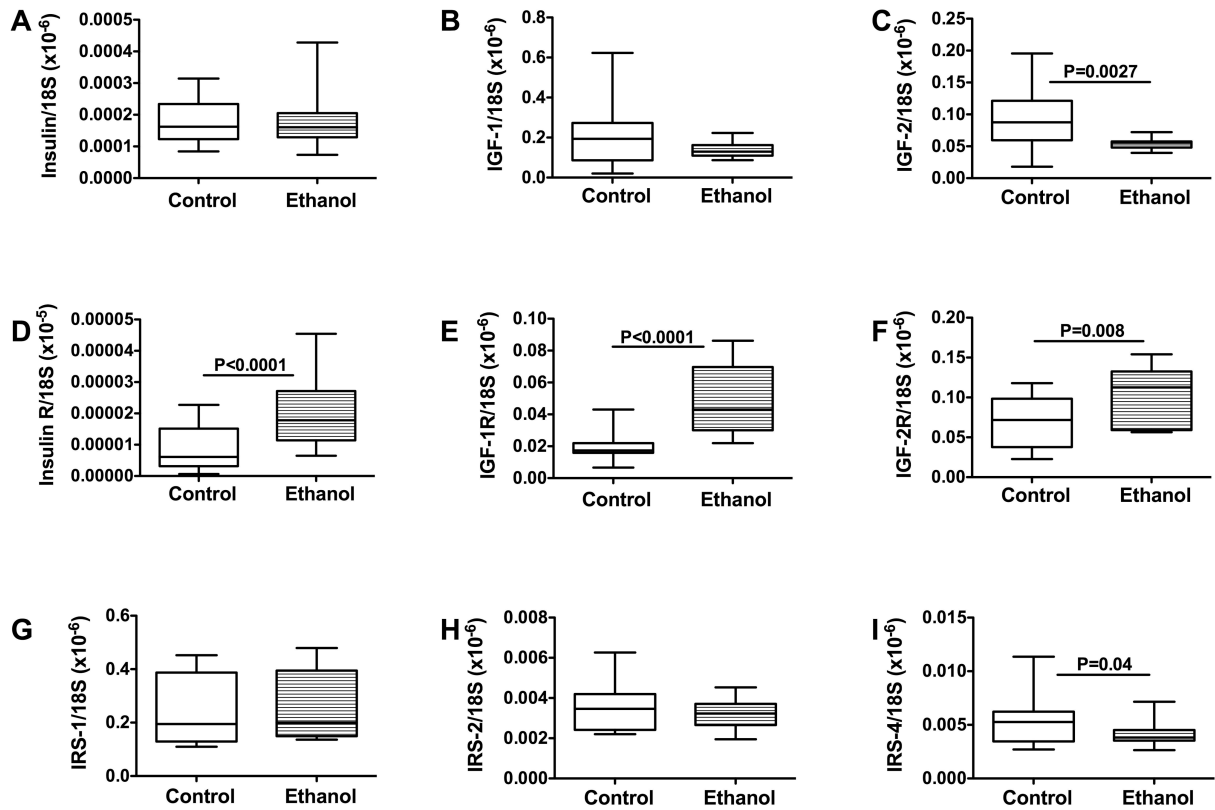
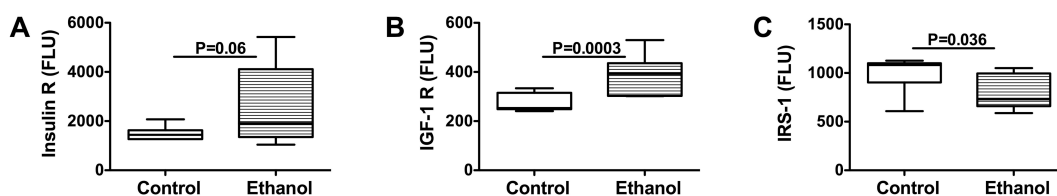


Figure 6.3: Gene expression levels of mediators of the insulin/IGF signaling cascade, measured in control and ALD human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) insulin, (B) IGF-1, (C) IGF-2, (D) insulin receptor, (E) IGF-1 receptor, (F) IGF-2 receptor, (G) IRS-1, (H) IRS-2 and (I) IRS-4 mRNA expression by PCR. mRNA abundance was corrected for using 18S as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.



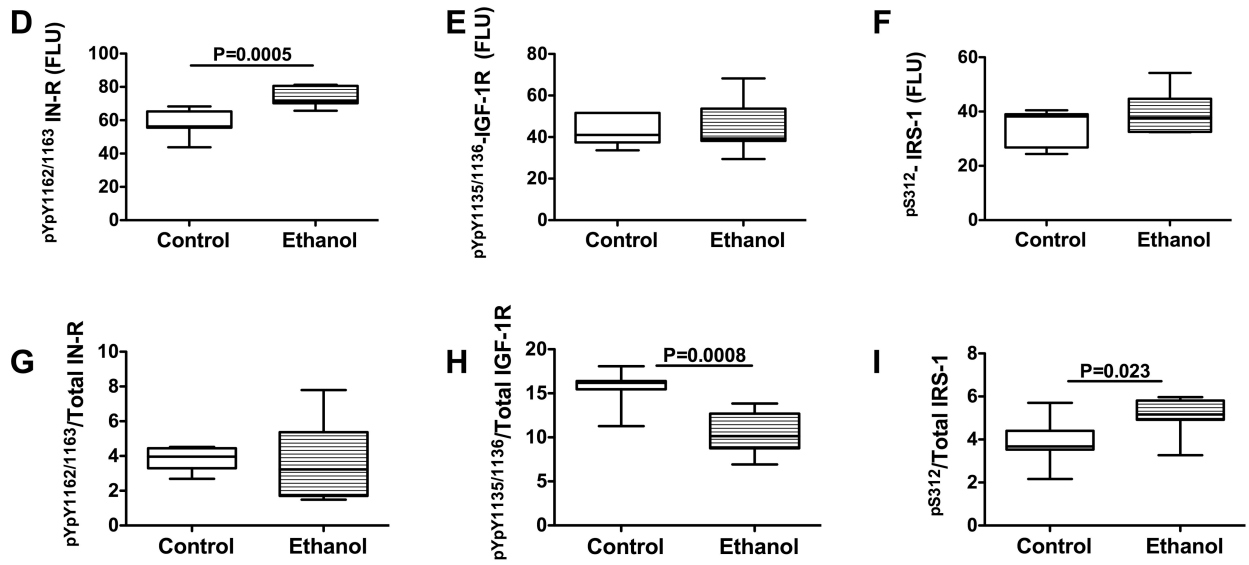


Figure 6.4: Protein expression levels of upstream polypeptides of the insulin/IGF signaling cascade, measured in control and ALD human liver tissues. Liver protein homogenates were used to measure immunoreactivity in fluorescence units corresponding to (A) insulin receptor, (B) IGF-1R, (C) IRS-1, (D) pYpY1162/1163-IR, (E) pYpY1135/1136-IGF-1R, (F) pS312-IRS-1 with a bead-based Multiplex ELISA platform (see Methods and Methods). (G-I) In addition, the phosphorylated/total protein ratios for were calculated to assess relative levels of phosphorylation of each protein. Data were analyzed statistically using t-tests. Significant P-values are indicated within the panels.

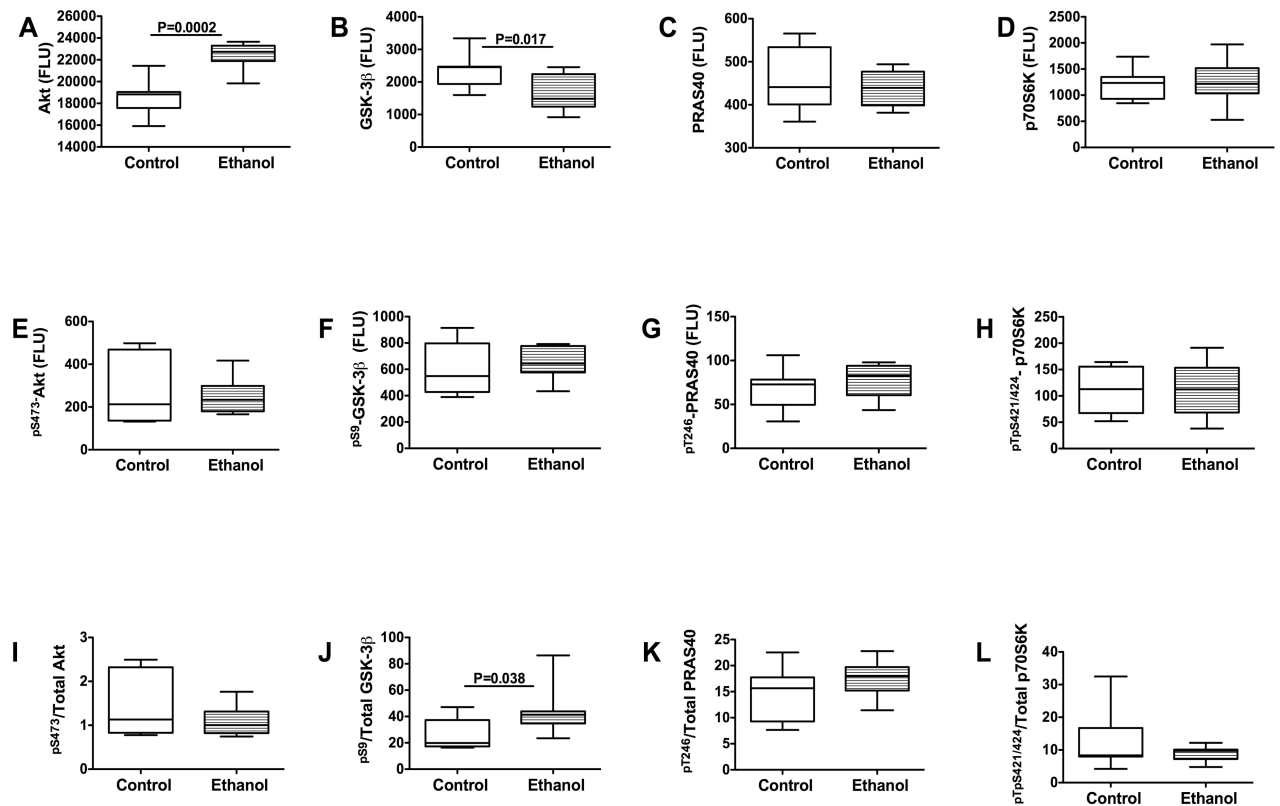


Figure 6.5: Protein expression levels of the downstream polypeptides of the insulin/IGF signaling cascade, measured in control and ALD human liver tissues. Liver protein homogenates were used to measure immunoreactivity in fluorescence units corresponding to (A) Akt, (B) GSK-3 β , (C) PRAS40, (D) p70S6K, (E) p^{S473}-Akt, (F) p^{S9}-GSK3 β , (G) p^{T246}-PRAS40 and (H) p^{TpS421/424}-p70S6K with a bead-based Multiplex ELISA platform (see Methods and Methods). (I-L) In addition, the phosphorylated/total protein ratios for were calculated to assess relative levels of phosphorylation of each protein. Data were analyzed statistically using t-tests. Significant P-values are indicated within the panels.

Furthermore although total levels of (C) IRS-1 were lower in the alcohol compared to the control group ($p=0.036$), the calculated (I) phosphorylated to total ratio was higher in the alcohol group ($p=0.023$).

Downstream signalling, through insulin/IGF-1 in liver, showed similar mean levels of PRAS40 and p70S6K, and in both groups (Figure 6.5). Mean total hepatic (A) Akt levels were significantly higher in the alcohol compared to control group ($p=0.0002$). In contradistinction, while total (B) GSK-3 β were downregulated in the alcohol group ($p=0.017$), the calculated (J) phosphorylated to total ratio was higher in the alcohol versus the control group ($p=0.038$).

Effects of ethanol on markers of endoplasmic reticulum (ER) stress: We measured the mRNA transcripts of several genes involved in the ER stress response using qRT-PCR (Figure 6.6). Overall there was an upregulation of these proteins in the livers of patients with ALD compared to controls. Specifically, mean mRNA levels of (A) BAX ($p=0.0003$), (D) CHOP ($p=0.013$), (G) GRP78 ($p=0.0006$), (H) ATF4 ($p<0.0001$), (J) HERP ($p=0.024$) and (K) WARS ($p=0.044$) were all higher in the alcohol relative to the control group. The mean gene expression levels of (B) EDEM and (E) P58IPK on the other hand were similar in both groups.

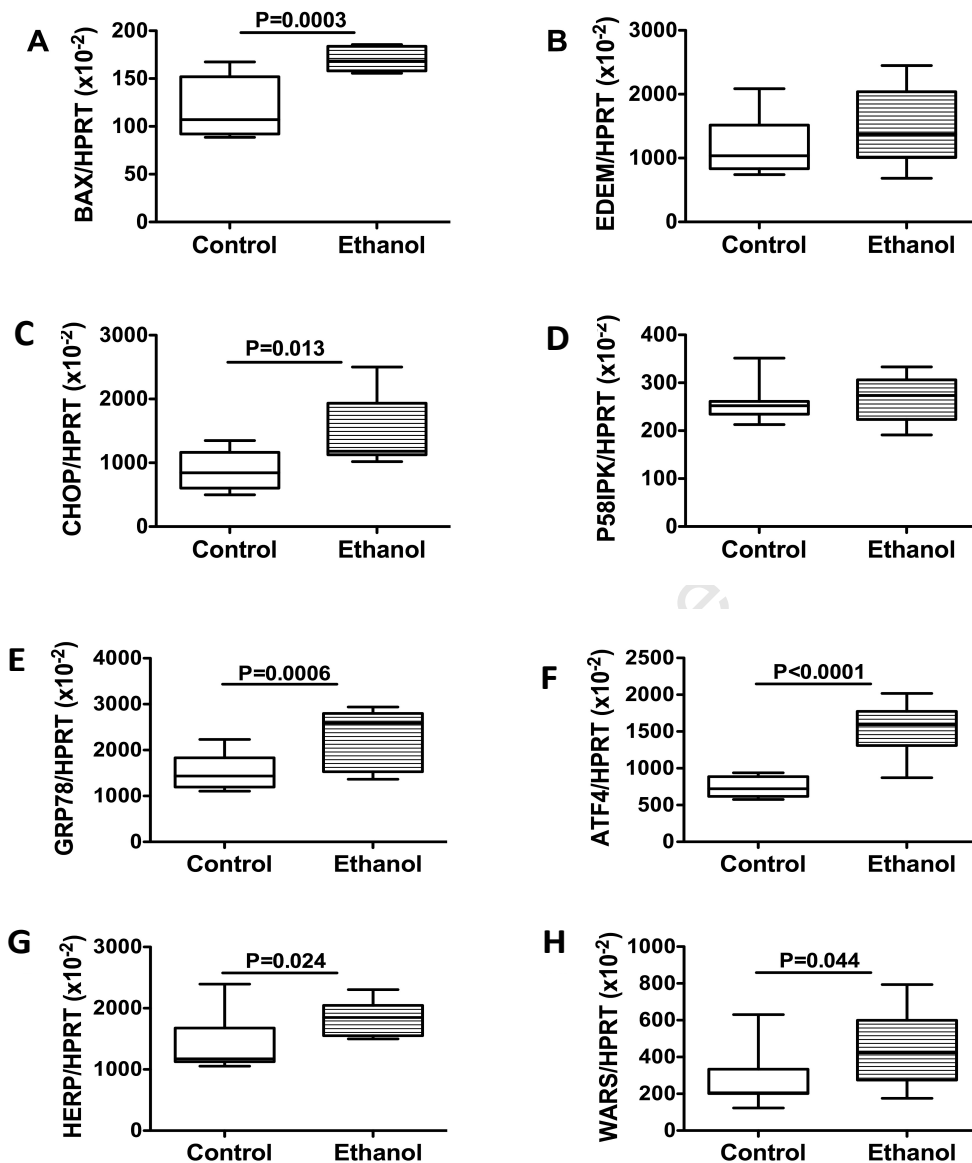


Figure 6.6: Gene expression levels of mediators of the ER stress signaling cascade measured control and ALD human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) BAX, (B) EDEM, (C) CHOP (D) P58IPK, (E) GRP78, (F) ATF4, (G) HERP and (H) WARS mRNA expression by PCR. mRNA abundance was corrected for using HPRT as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

Effects of ethanol on cytokine levels in liver: Activation of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β , is a common feature of steatohepatitis (Carter-Kent, Zein, & Feldstein, 2008; Larter & Farrell, 2006; Tilg & Hotamisligil, 2006). We measured hepatic levels of these cytokines, including IP-10, MIP-1, IL-18, β -NGF, IL-8, IFN- γ and TRAIL by multiplex ELISA to determine the degree to which alcohol stimulates pro-inflammatory cytokine activation (Figure 6.7). All measured cytokines were similarly expressed in both study groups (A-F, I); except for (G) IL-1 β ($p=0.028$), (H) IL-6 ($p=0.009$) and (J) TRAIL ($p=0.01$) that had lower immunoreactivities in the alcohol compared to the control group.

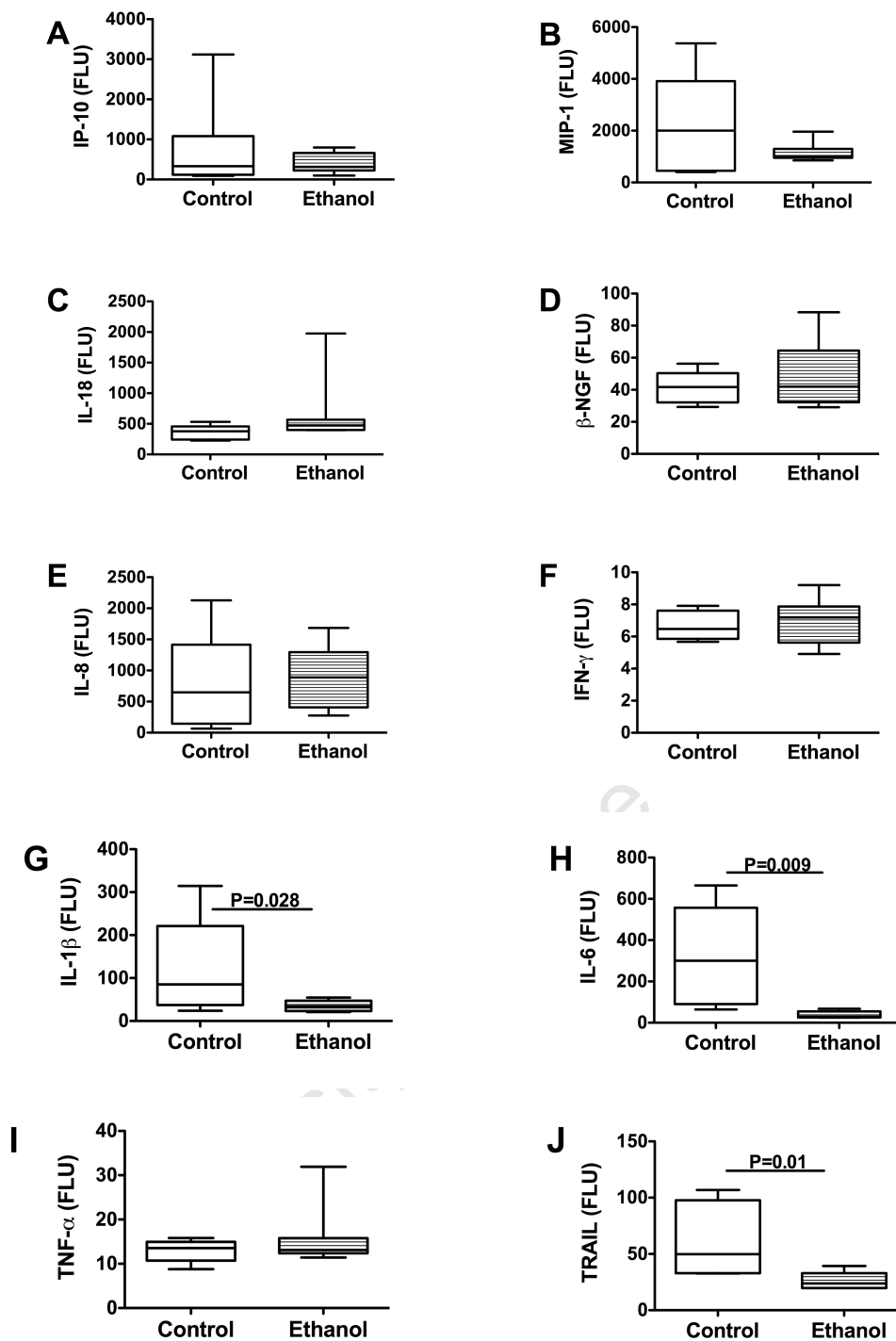


Figure 6.7: Cytokine expression levels measured in control and ALD human liver tissues. Liver protein homogenates were used to measure immunoreactivity in fluorescence units corresponding to (A) IP-10, (B) MIP-1, (C) IL-18, (D) β -NGF, (E) IL-8, (F) IFN- γ , (G) IL-1 β and (H) IL-6, (I) TNF- α and (J) TRAIL with a bead-based Multiplex ELISA platform (see Methods and Methods). Data were analyzed statistically using t-tests. Significant P-values are indicated within the panels.

6.4 DISCUSSION

The pathogenesis of ALD in terms of progression of disease from relatively 'benign' states, to more aggressive disease with worse outcomes has enjoyed much attention in the literature in part because it is as yet not fully elucidated. A clearer understanding of underlying disease mechanisms will be useful to prevent or limit progressive liver disease. The literature supports the roles of pro-cytokine inflammation, oxidative stress, lipotoxicity-induced insulin resistance and other related mechanisms; however the majority of these studies were performed in animal models. In this study of human ALD we aimed to evaluate the contribution of each of these mechanisms to disease, and thus by extension the reliability and applicability of utilized animal models.

The main findings were an elevation of neutral lipids and more importantly, ceramides in the alcohol group. The measurement of neutral lipid encompasses some measurement of triglycerides, however the finding of unelevated triglycerides in this study is interesting, as these lipids have been touted as the primary cause of hepatic steatosis, and this was evident in a previous study from our laboratory utilizing a rat model of alcoholic steatohepatitis (Denucci et al., 2010; Setshedi, Longato, Ronis, Wands, & De La Monte, 2010). Another explanation for similar levels of triglycerides in the study groups could be that the 'controls' in fact had some degree of steatosis. Information about their ultrasound examinations is not known. In this context therefore, it may be that ceramides play an important role in not just the steatosis, but also the ensuing steatohepatitis, as they are signaling effectors. The importance of ceramides is borne out in the expression levels of pro-

ceramide gene expression. Interestingly, the data supports that both de novo synthesis and degradation pathways of ceramide are involved in generating increased levels, and moreover more complex sphingolipids i.e. glucosylceramide (catalyzed by increased UGCG), and GM3 synthase (catalyzing the formation of GM3 ganglioside). These complex sphingolipids are important for many biological actions including cell differentiation, senescence, apoptosis, and proliferation. In this context however, their up-regulation could potentially explain the insulin resistance (Aerts et al., 2007; Bijl et al., 2009; Summers, 2010; Tagami et al., 2002; Yamashita et al., 2003; Yew et al., 2010; Zhao et al., 2009) seen in the alcohol group of patients, mechanistically by impairing IR binding due to their accumulation in cell membrane lipid rafts (Inokuchi, 2007). Analysis of the mRNA transcripts of insulin signaling revealed that the receptors (IR, IGF-1R and IGF-2R) were all upregulated, probably signaling a compensatory response to the prevailing insulin resistance. At protein expression level, mean levels of IGF-1R and Akt were reduced and similar when using the ratio respectively, in the alcohol group. Total GSK-3 β levels were similarly lower in the alcohol group, thus overall, suggesting suppressed insulin signaling by alcohol. It is established that alcohol suppresses insulin signaling both at receptor level and downstream, by affecting various components of signaling (de la Monte et al., 2008; Svegliati-Baroni et al., 2006; Yeon et al., 2003). Suppression of insulin signaling is detrimental to the ability of injured hepatic cells to regenerate and recover, thus resulting in cycles of injury, cell death and fibrosis, and ultimately organ failure.

There was a widespread up-regulation of genes of ER stress in the alcohol relative to the control group. More important was the up-regulation of BAX and CHOP which signify apoptotic signaling, a late consequence of ER-induced stress. This finding suggests that alcohol consumption in this model was either moderate to severe or sufficiently prolonged to induce apoptosis. Probably the most anomalous of the data was in the cytokine expression measurements. There was no difference in the levels of measured cytokines, except for IL-1 β , IL-6 and TRAIL, which were downregulated. The lack of up-regulation of the prototypical pro-inflammatory cytokines e.g. TNF- α in this study may be explained on the basis that these measurements were done on whole liver tissues and not isolated Kupffer cells, thus potentially masking true cytokine levels. Furthermore, the unreliability of our measurements could be because typically cytokines are measured in serum. Finally, another possibility could be that cytokine elevation following the acute alcoholic hepatitis phase is probably not marked; this study was performed in liver tissues of patients who had chronic hepatitis at least and potentially fibrosis/cirrhosis. The role of IL-6 in promoting a pro-inflammatory response and thereby causing hepatocyte injury is controversial. Earlier studies suggested its role to be mediate injury (Hill et al., 1992) whereas more recent studies have proved it to be hepato-protective (El-Assal, Hong, Kim, Radaeva, & Gao, 2004; Fukumura et al., 2007; Hong et al., 2002; Hong et al., 2004; Yamaguchi et al., 2011) in animal *in vivo* and *in vitro* models. Contrastingly in a human study of serum IL-6 levels, these were elevated and correlated with severity of disease (Swiatkowska-Stodulska & Bakowska, 2004). Thus alcohol consumption

in this study possibly led to an abrogation of the protective effects as was evident in the alcohol group.

To summarize, our results confirmed that ceramides are novel toxic lipids key to the pathogenesis of human ALD. Mechanistically, increased ceramide levels occur as a result of upregulation of pro-ceramide synthetic and degradation genes and the result thereof is impaired insulin signaling. Consequently, the use of ceramide inhibitors may in future become part of the management armamentarium of ALD. This is crucial as currently there are not as yet FDA-approved treatments for ALD (Frazier, Stocker, Kershner, Marsano, & McClain, 2011). The results of this study correlate well with our own work done previously, in animal models. Therefore the animal models in use today are truly representative of human disease, and therefore are critical in testing hypotheses, that are crucial for the translation of research from the bench to the bedside.

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CHAPTER 7

Triad of dysregulated signaling in human hepatocellular carcinoma (HCC) suggests a multi-pronged approach is needed for therapeutic targeting

ABSTRACT

Background: Previous studies highlighted potential roles of aberrantly increased activation of Insulin/Insulin Receptor Substrate (IRS), canonical Wnt/ β -Catenin, or Notch-Jagged-Aspartyl-(asparaginyl)- β -hydroxylase (AAH) as mediators of HCC pathogenesis or progression. In addition, a role for pro-inflammatory mediators of cell injury, turnover, and DNA damage is under investigation in HCC development. To re-focus the fundamental question, which has direct relevance to future diagnostic and therapeutic strategies, we examined the extents to which each these 3 pathways were upregulated in HCC versus peri-tumour (pre-malignant) liver tissue, and also evaluated the potential contributions of pro-inflammatory cytokines as mediators of progressive liver injury and DNA damage.

Methods: Paired surgically resected HCCs (n=8) and tumour-free peri-HCC livers (n=8) were used to measure gene expression by qRT-PCR analysis, and immunoreactivity by either multiplex or duplex ELISAs.

Results: *Insulin Pathway:* HCC was associated with significantly upregulated expression of the IGF2 receptor, IRS1, IRS2, and IRS4 genes, increased

signaling through IGF-1R ($p=0.001$) and IRS1 ($p=0.015$), and inhibition of GSK-3 β activity relative to peri-tumour liver tissue. *Wnt/ β -catenin*: HCCs had significantly increased mRNA expression of Wnt 3 ($p<0.0001$), Wnt5A ($p=0.004$), and Wnt5B ($p=0.01$) ligands, and Frizzled-6 receptor ($p=0.02$), and decreased expression of sFRP1 ($p=0.01$) and sFRP5 ($p=0.008$) relative to peri-tumour tissues. *Notch*: HCCs had significantly increased expression of multiple Notch pathway-related genes and proteins, including AAH, humbug, Notch-1, Jagged-1 and HES-1, relative to peri-tumour tissues. *Inflammation*: Multiplex ELISAs used to measure 24 different cytokines/chemokines, demonstrated increased expression of only IFN- γ , and significantly reduced levels of IL-1b, IL-6, TRAIL, MIP-1 and b-NGF in HCC relative to peri-tumour liver tissue.

Conclusions: HCCs are associated with simultaneous dysregulation of 3 major signaling pathways that likely crosstalk through various mechanisms, including GSK-3 β . The prominent down-regulation of several pro-inflammatory cytokines suggests that compromised host surveillance mechanisms rather than inflammation-mediated cell injury and DNA damage may contribute to the pathogenesis or progression of HCC. Altogether, the data suggest that effective therapeutic targeting of HCCs will require multi-pronged approaches that abrogate dysregulation of the Insulin/IRS, Wnt/ β -catenin, and Notch pathways and fortify host immune surveillance mechanisms.

7.1 INTRODUCTION

Chronic hepatitis secondary to viral infections hepatitis B and C (HBV and HCV), alcohol, and obesity are the commonest causes combined for the development of cirrhosis-driven hepatocellular carcinoma (HCC). HCC is the fifth most common malignancy, with an estimated half million new cases diagnosed per year globally (Llovet, Burroughs, & Bruix, 2003). It has the third highest cause for cancer-related death (Parkin, Bray, Ferlay, & Pisani, 2005), due to late discovery, thus rendering the prognosis particularly dismal (median survival 1-2 months) (Lau & Lai, 2008), and while therapeutic options are available for early localized tumours, these are fraught with challenges: only about a third of patients are suitable for curative procedures and there is chemo-resistance to available drugs. Correspondingly, the impact of HCC globally is significant. In this context, it is critical to understand the dominant molecular factors resulting in HCC so that the development of newer diagnostic markers and/or therapeutic agents might be possible.

For malignant transformation cells have to acquire the ability to become insensitive to anti-growth signals and have self-sustaining growth signals, in addition to having the propensity to replicate and avoid apoptosis (Hanahan & Weinberg, 2000). In animal and human studies, many signaling pathways (e.g. insulin/IGF, Wnt/ β -catenin, Transforming Growth Factor- α /Epidermal Growth Factor and others) that are involved in survival and/or anti-apoptosis including chemoresistance to sorafenib (Chen et al., 2011) are dysregulated in HCC. Arguably the best studied of these is the insulin/IGF pathway. There is ample

data to show that Insulin/IGF signaling is upregulated in HCC (Breuhahn et al., 2004; Cantarini et al., 2006; Lund, Schubert, Niketeghad, & Schirmacher, 2004; Schirmacher et al., 1992; Sohda, Yun, Iwata, Soejima, & Okumura, 1996; Tovar et al., 2010). Similarly, dysregulated Wnt/ β -catenin signaling occurs in HCC (Hsu et al., 2000; Nhieu et al., 1999; Prange et al., 2003; Wong, Fan, & Ng, 2001) and results in increased transcription of transcription factors T-cell factor (TCF) and Lymphoid enhancer factor (LEF) that promote carcinogenesis (Aoki, Hecht, Kruse, Kemler, & Vogt, 1999; Korinek et al., 1998). In addition, dysregulated activation of Wnt/ β -catenin, seen in 80% of HCC is associated with the development of tumor and progression (Lustig & Behrens, 2003; Reya & Clevers, 2005; Vincan, 2004).

The Insulin/IGF pathway favours growth, survival and energy metabolism (de la Monte & Wands, 2005) by transmitting intracellular signals via the binding of a ligand to transmembrane receptors. Binding causes a conformational change of the intracellular receptor, activates and phosphorylates tyrosine kinases that act on downstream targets such as the insulin receptor substrate (IRS) polypeptides, and Akt.

The Wnt/ β -catenin pathway is evolutionarily conserved and is critical for multiple functions particularly cell fate, which determines whether a cell progresses to or avoids differentiation; the latter promotes carcinogenesis (Hanahan & Weinberg, 2000). In the active state Wnt ligand binds to its transmembrane receptor (Frizzled/Dishevelled) and through a series of signaling steps results in the inactivation of the GSK-3 β /APC/Axin complex. Subsequently

β -catenin escapes phosphorylation and enters the nucleus to attach to TCF /LEF transcription factors, to effect increased cell motility, which impacts on tumor invasiveness and migration.

Less well studied however, in regulating cell fate determination is the role of Notch signaling, and even fewer studies evaluate its role in hepatocarcinogenesis. Notch signaling involves several catalytic reactions and occurs when ligands (Jagged and Delta-like) bind Notch 1-4 transmembrane receptors, resulting in the liberation of the intracellular domain of Notch-1 (NICD) which translocates to the nucleus to associate with the CSL for transcriptional activation of Notch target genes i.e. hairy enhancer of split (HES) and HES-related repressor protein (HERP/HEY). Notch signaling is upregulated in HCC and is particularly vital for cell motility, and therefore invasiveness and potentially metastasis.

These three pathways intersect at many levels for instance, Notch and Jagged are hydroxylated by AAH, an enzyme regulated by insulin/IGF and all three are upregulated in many cancers (HCC, cholangiocarcinoma and pancreatic cancer).

Therefore we elected to evaluate the roles of insulin/IGF, Wnt/ β -catenin and Notch signaling in promoting human HCC at different stages of disease, to gain a varied understanding of the molecular factors that act in concert to promote HCC. Since the molecular drivers of HCC are complex, a multi-pronged approach to therapy is essential; hence a determination of a potential molecular marker where the three pathways converge would be desirable because by

targeting it, many pathways that promote cancer cell survival could be simultaneously interrupted. To our knowledge there are no studies that have evaluated these pathways together. Finally, since cytokine activation associated with chronic HBV and HCV contributes to the development of HCC (Budhu & Wang, 2006), we wanted to evaluate the role of these cytokines in this model.

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7.2 MATERIALS AND METHODS

Patients and specimens: The samples utilized for the analysis were liver tissue obtained from patients who had undergone hepatic resection for HCC. Informed consent was obtained prior to surgery. They comprise of liver tissue from the HCC (n=8) and liver from the surrounding area, referred to as the peri-tumor tissue (n=8). These tissue samples were gratefully donated to our laboratory, the Liver Research Center of Brown University (a collaborative effort) from Korea and Italy. The majority of patients had either HBV or HCV infection or alcohol-related HCC. With respect to the normal tissue used as controls, this tissue was obtained from similarly aged patients who presented at Brown University Medical School for abdominal exploratory surgery for a possible solitary lesion in the liver (n=6). The liver tissue was obtained from normal areas in the periphery of the liver. In the first part of the analysis we compared liver tissue from normal subjects to that in adjacent peri-tumour liver tissue. In the second part of the analysis we compared peri-tumour tissue versus HCC. Our experimental protocol was exempted from Human Ethics approval by our institution, Lifespan IRB committee because the liver tissue was coded and de-identified by the collaborators, thus no specific sensitive patient information was available.

Materials: Similar materials and methods as those previously described in chapter 2 were used.

Multiplex ELISA: We measured immunoreactivity to the insulin signaling components as previously described. For cytokine and chemokine assessments we measured IP-10, IL-18, IL-8, IL-1 β , tumor necrosis factor alpha (TNF- α), MIP-

1, β -nerve growth factor (β -NGF), interferon gamma (IFN- γ), IL-6 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Assays: This was performed as previously described.

Statistical Analysis: Data depicted in box plots reflect group median, 95% confidence interval limits and range (whiskers), while tabulated data reflect means \pm SEMs for each group. Intergroup comparisons were made using paired t-tests. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Significant P-values (<0.05) are shown within the panels or tables.

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7.3 RESULTS

Insulin/IGF signaling in control versus peri-tumor tissue and peri-tumor versus HCC

Gene expression: The expression of mRNA transcripts of IGF-1 ($p < 0.0001$), IGF-2 ($p < 0.0001$) and their corresponding receptors ($p = 0.04$) and $p < 0.0001$ respectively were down-regulated in the peri-tumour tissue as compared to normal tissue. By contrast, insulin, insulin receptor, and IRS-1, 2 and 4 were not significantly different between the control and peri-tumour tissues (Figure 7.1 A-I). When comparing peri-tumour and HCC tissues, mRNA transcripts of IGF-1, insulin receptor and its receptor IGF1R were similar (Figure 7.2 B, D, E). Significantly however, IGF-2 mRNA in HCC was significantly lower than that in the paired surrounding tissue ($p = 0.03$), while its receptor IGF-2R was upregulated ($p < 0.0001$) (Figure 7.2 C, F). Comparing normal to peri-tumour tissues, gene expression of IRS-1, 2 and 4 was similar (Figure 7.1 G, H). Contrastingly however, IRS 1 ($p = 0.0018$), 2 ($p = 0.002$) and 4 ($p = 0.0005$) were upregulated in HCC compared to peri-tumour tissue, and this effect was more marked with IRS-4 (Figure 7.2 G, H, I).

Protein expression: Upstream insulin signaling polypeptides were measured using a bead-based multiplex ELISA. Total insulin receptor levels were higher in HCC than in adjacent tissue ($p = 0.01$), whereas its phosphorylated counterpart showed similar levels in both groups (Figure 7.3 A, D). To accurately assess relative levels of the active signaling polypeptides we measured a ratio of phosphorylated to total protein levels. Thus quite significantly ($p = 0.005$) relative

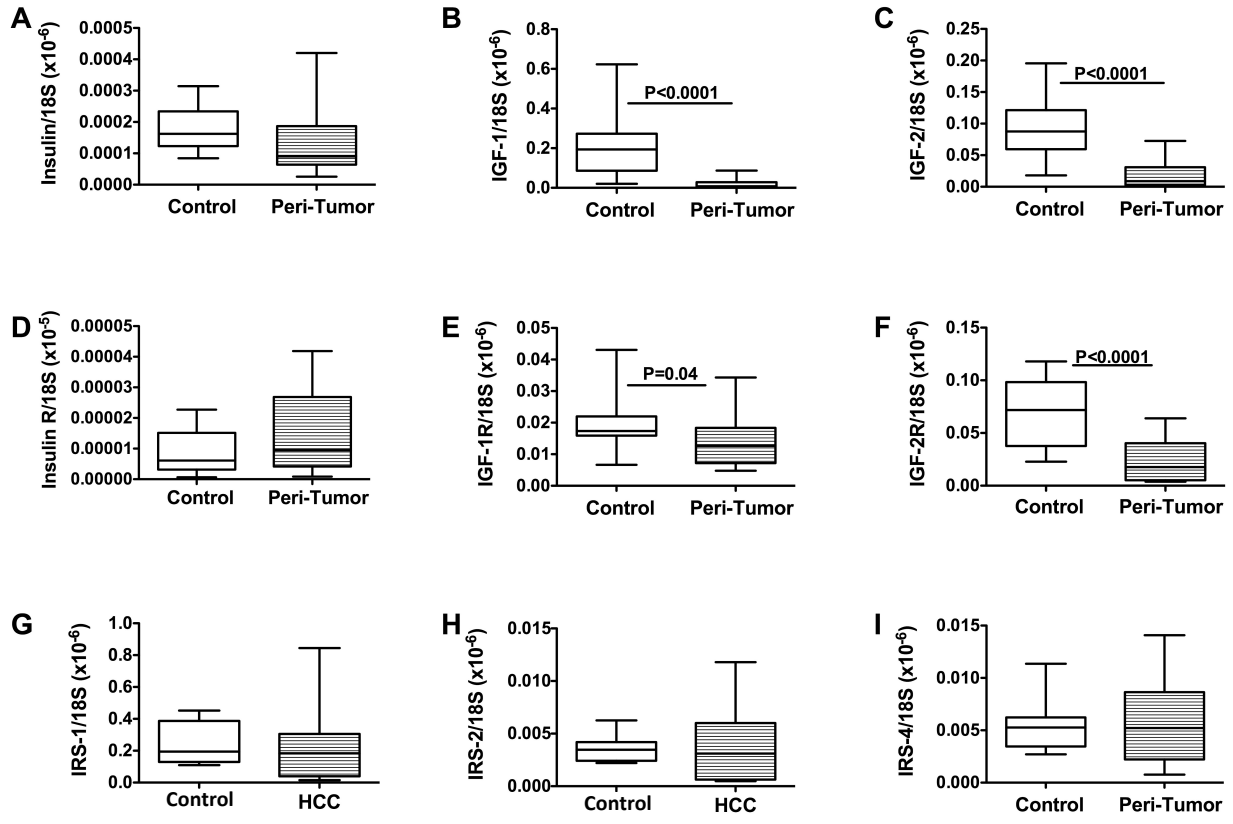


Figure 7.1: Gene expression levels of mediators of the Insulin/IGF signaling cascade, measured in control and peri-tumour human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) insulin, (B) IGF-1, (C) IGF-2, (D) insulin receptor, (E) IGF-1 receptor, (F) IGF-2 receptor, (G) IRS-1, (H) IRS-2 and (I) IRS-4 mRNA expression by PCR. mRNA abundance was corrected for using 18S as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

phosphorylated insulin receptor levels were downregulated in HCC versus peri-tumour tissue (Figure 7.3 G). Contrastingly while total IGF-1R levels were lower in HCC than peri-tumour tissue ($p=0.008$), the calculated phosphorylated to total levels were higher in HCC relative to peri-tumour tissue ($p=0.001$) (Figure 7.3 H).

Total IRS-1 levels in HCC were higher than in peri-tumour tissue ($p=0.046$), however with a calculated ratio, there was a reversal with HCC having lower levels than those observed in peri-tumour tissue ($p=0.015$) (Figure 7.3 C, I).

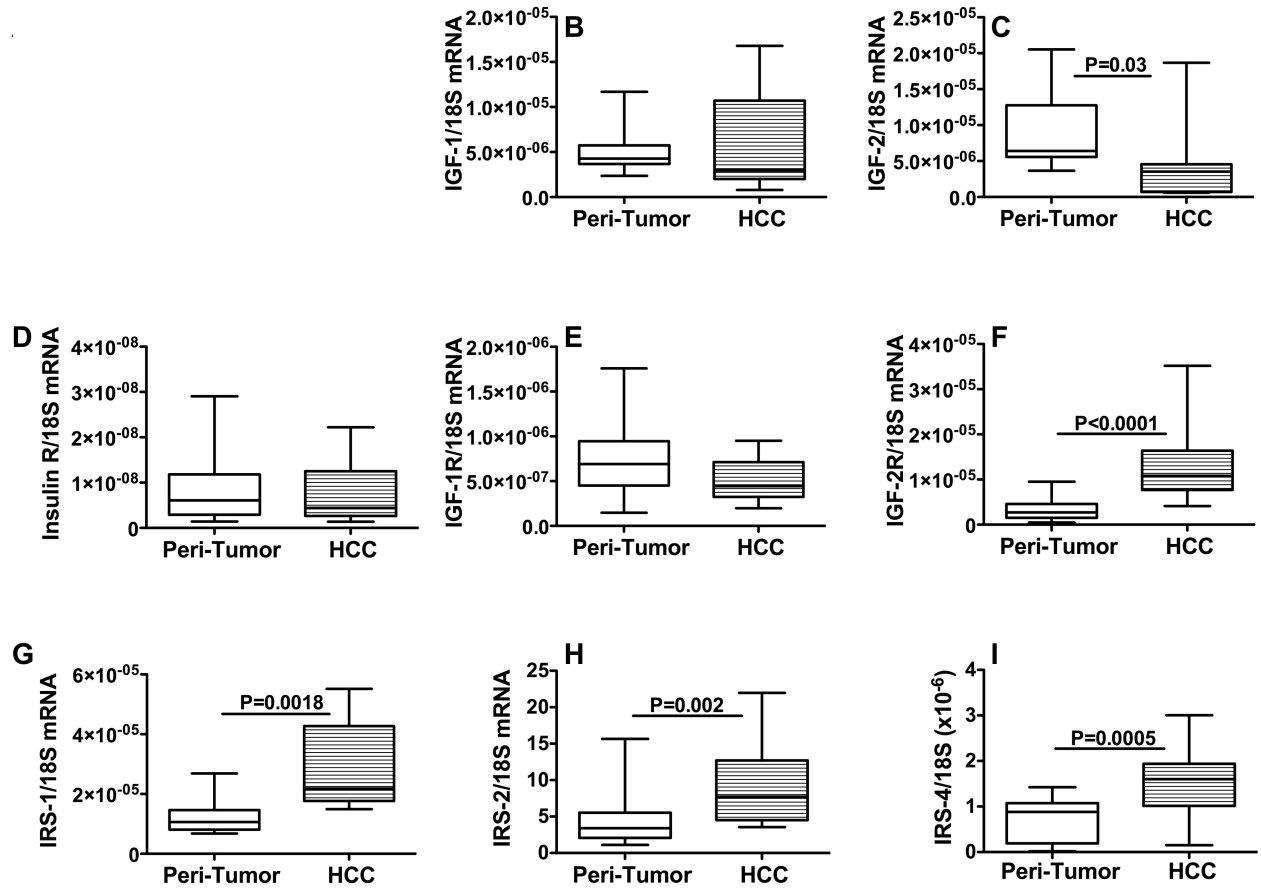


Figure 7.2: Gene expression levels of mediators of the Insulin/IGF signaling cascade, measured in peri-tumour and HCC human liver tissues. RNA extracted from liver was reverse transcribed to measure (B) IGF-1, (C) IGF-2, (D) insulin receptor, (E) IGF-1 receptor, (F) IGF-2 receptor, (G) IRS-1, (H) IRS-2 and (I) IRS-4 mRNA expression by PCR. mRNA abundance was corrected for using 18S as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

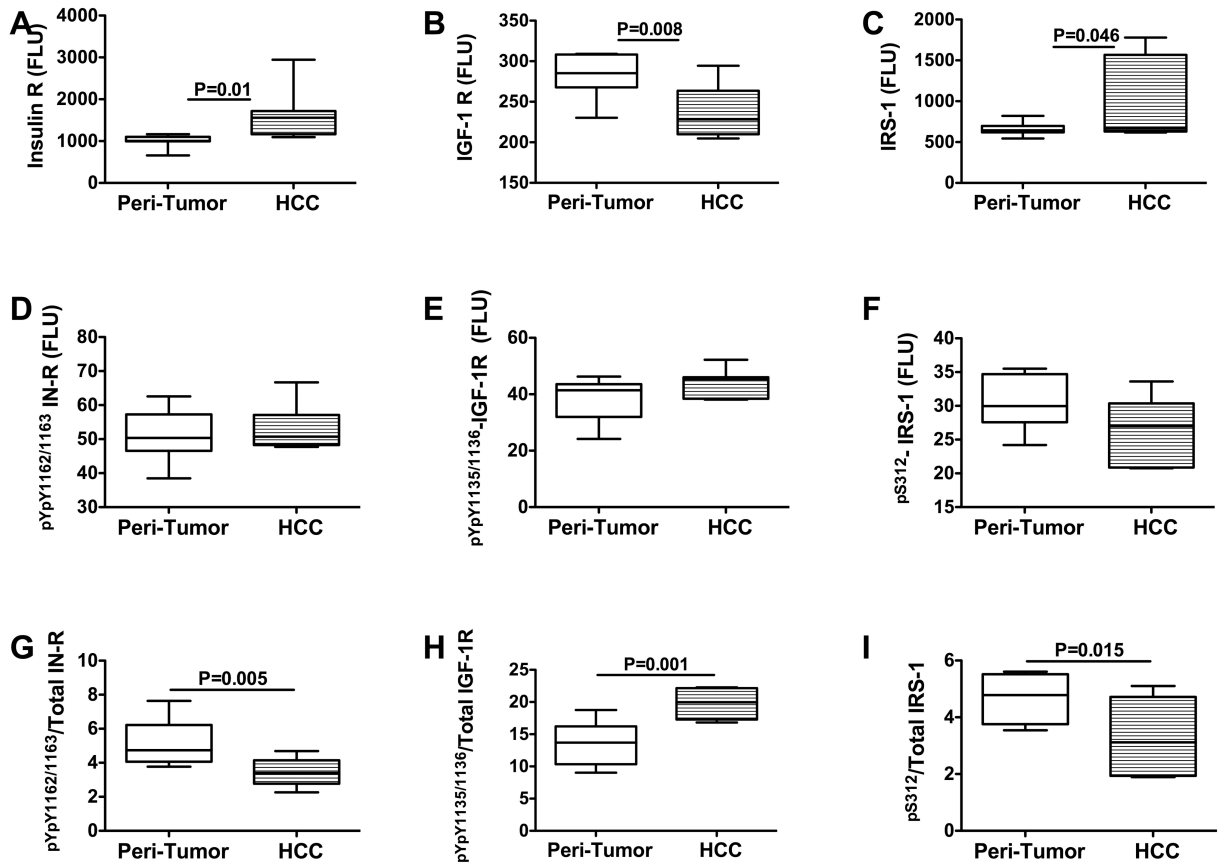


Figure 7.3: Protein expression levels of upstream polypeptides of the Insulin/IGF signaling cascade, measured in peri-tumour and HCC human liver tissues. Liver protein homogenates were used to measure immunoreactivity in fluorescence units corresponding to (A) insulin receptor, (B) IGF-1R, (C) IRS-1, (D) $pYpY1162/1163$ -IR, (E) $pYpY1135/1136$ -IGF-1R, (F) $pS312$ -IRS-1 with a bead-based Multiplex ELISA platform (see Methods and Methods). (G-I) In addition, the phosphorylated/total protein ratios (G-I) for were calculated to assess relative levels of phosphorylation of each protein. Data were analyzed statistically using t-tests. Significant P-values are indicated within the panels.

Downstream signaling signaling through Akt (Figure 7.4 A, E, I) and PRAS40 (Figure 7.4 C, G, K) was not significantly different in peri-tumour and HCC tissues. However whereas signaling through GSK-3 β was markedly up-regulated in HCC relative to peri-tumour tissues when measuring total ($p=0.0002$) and phosphorylated ($p=0.002$) levels, a ratio of phosphorylated to total GSK-3 β levels showed a significant down-regulation ($p=0.005$) in HCC tissues (Figure 7.4 B, F, J). p70S6K is a serine/threonine kinase that acts downstream of mammalian target of rapamycin (mTOR) and promotes protein synthesis when activated. In HCC although total levels of p70S6K (Figure 7.4 D) were higher than those in peri-tumour tissues ($p=0.039$), when corrected for by a ratio calculation of phosphorylated to total levels (Figure 7.4 L), p70S6K levels were lower in HCC than in surrounding tissue ($p=0.029$).

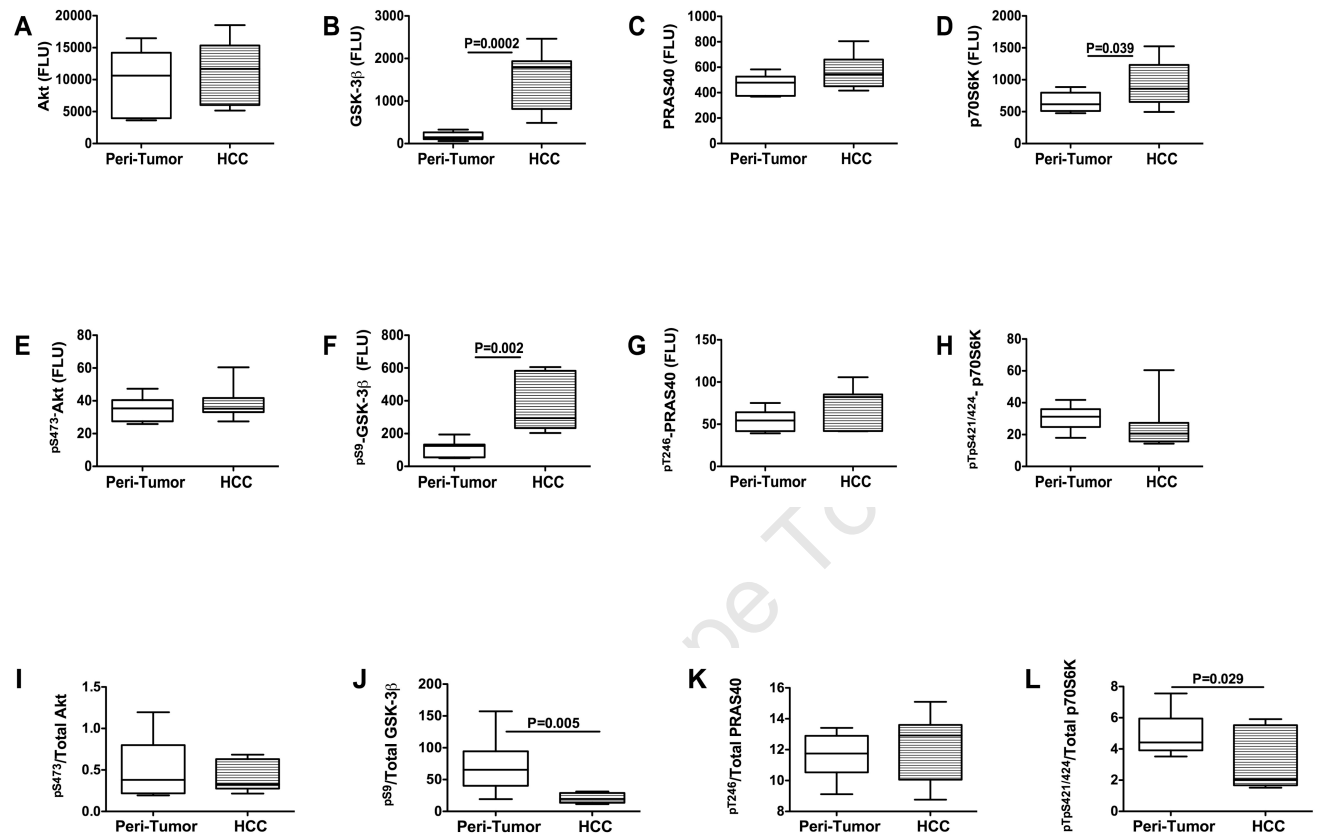


Figure 7.4: Protein expression levels of the downstream polypeptides of the insulin/IGF signaling cascade, measured in peri-tumour and HCC human liver tissues. Liver protein homogenates were used to measure immunoreactivity in fluorescence units corresponding to (A) Akt, (B) GSK-3 β , (C) p70S6K, (D) PRAS40, (E) p^{S473}-Akt, (F) p^{S9}-GSK3 β , (G) p^{TpS421/424}-p70S6K and (H) p^{T246}-PRAS40 with a bead-based Multiplex ELISA platform (see Methods and Methods). (I-L) In addition, the phosphorylated/total protein ratios for were calculated to assess relative levels of phosphorylation of each protein. Data were analyzed statistically using t-tests. Significant P-values are indicated within the panels.

Wnt/ β -catenin signaling in control versus peri-tumour tissue and peri-tumour versus HCC

Using qT-RT-PCR, we measured mRNA levels of components of the Wnt signaling cascade. Of the measured ligands Wnt 1, 3, 4, 5A and 5B, Wnt 3 ($p < 0.0001$), Wnt 5A ($p = 0.004$) and Wnt 5B ($p = 0.01$) mRNA transcripts were significantly upregulated in HCC versus peri-tumour tissues (Figure 7.5 B, D, E). Both transcripts of secreted Frizzled-related protein (FRP-1) ($p = 0.01$) and sFRP-5 ($p = 0.008$) were lower in HCC than peri-tumour tissues (Figure 7.5 F, G). While levels of Frizzled-3 (Fzd-3) and Fzd-7 were similarly expressed (Figure 7.5 H, J), those of Fzd-6 were higher in HCC than in peri-tumour tissues ($p = 0.02$) (Figure 7.5 I). Therefore enhanced Wnt signaling in HCC is mediated via Wnts 3 and 5 and frizzled-6 and downregulated inhibited Frizzled's soluble counterparts (which can interfere with binding between Wnt and FZD), thus amplify signaling through FZD.

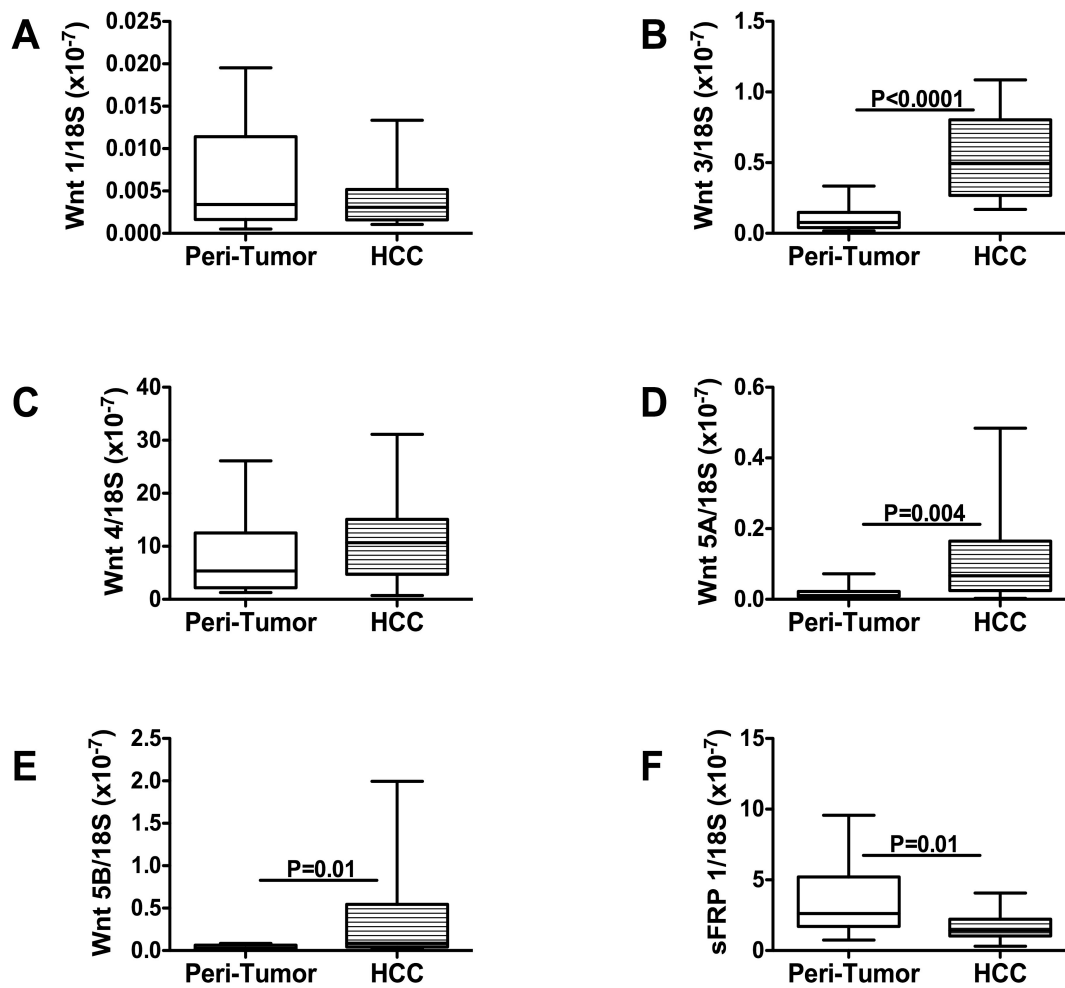


Figure 7.5a: Gene expression levels of mediators of the Wnt/ β -catenin signaling cascade, measured in peri-tumour and HCC human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) Wnt 1, (B) Wnt 3, (C) Wnt 4 (D) Wnt 5A, (E) Wnt 5B, (F) sFRP1, (G) sFRP5, (H) Fzd 3, (I) Fzd 6 and (J) Fzd 7 mRNA expression by PCR. mRNA abundance was corrected for using 18S as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

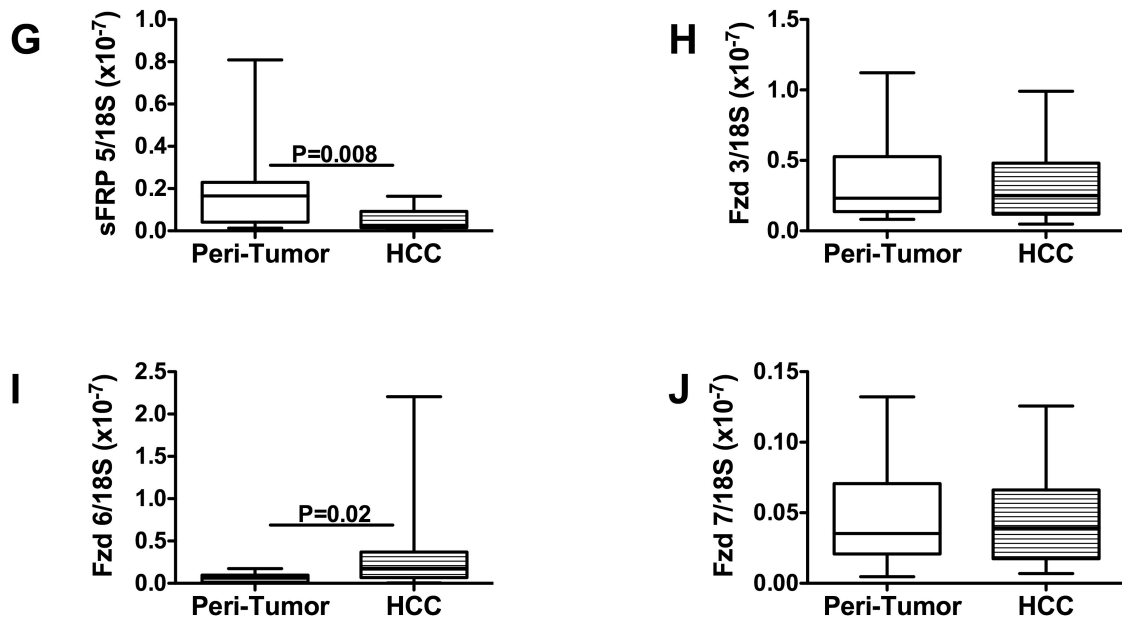


Figure 7.5b: Gene expression levels of mediators of the Wnt/ β -catenin signaling cascade, measured in peri-tumour and HCC human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) Wnt 1, (B) Wnt 3, (C) Wnt 4 (D) Wnt 5A, (E) Wnt 5B, (F) sFRP1, (G) sFRP5, (H) Fzd 3, (I) Fzd 6 and (J) Fzd 7 mRNA expression by PCR. mRNA abundance was corrected for using 18S as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

Notch signaling in peri-tumor versus HCC:

Cell motility is enhanced by the hydroxylation of Notch by aspartyl-(asparaginyl)- β -hydroxylase (AAH), and both these molecules are involved with tissue invasiveness and motility (de la Monte et al., 2006; Ince, de la Monte, & Wands, 2000; Lavaissiere et al., 1996; Maeda et al., 2003). AAH ($p=0.0014$) and Humbug ($p<0.0001$) mRNA transcripts were higher in HCC than adjacent peri-tumour tissue (Figure 7.6 A and B), suggesting similar biological activities. Humbug,

which encodes a truncated isoform of AAH is thought to promote malignant progression of various types of tumors (Lee, 2008; Maeda et al., 2004; Wang et al., 2007). At the cell membrane, Notch-1 ($p < 0.0001$) and jagged-1 ($p = 0.0022$) were significantly upregulated in HCC compared to peri-tumour tissue (Figure 7.6 C and D). Downstream signaling through HES-1 was similarly upregulated ($p < 0.0001$) in HCC as compared to peri-tumour tissue (Figure 7.6 E).

Cytokine activation in peri-tumor versus HCC tissues

Protein expression of IP-10, IL-18, IL-8 and TNF- α were similarly expressed in peri-tumour as compared to HCC tissue (Figure 7.7 A, C, E, I). Protein levels of IL-1 β ($p = 0.01$), MIP-1 ($p = 0.001$), β -nerve growth factor (β -NGF) ($p = 0.004$), IL-6 ($p = 0.044$), and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) ($p = 0.05$) were downregulated in HCC relative to peri-tumour tissues (Figure 7.7 G, B, D, H, J). Levels of IFN- γ by contrast were marginally higher in HCC compared to peri-tumour tissue ($p = 0.032$) (Figure 7.7 F).

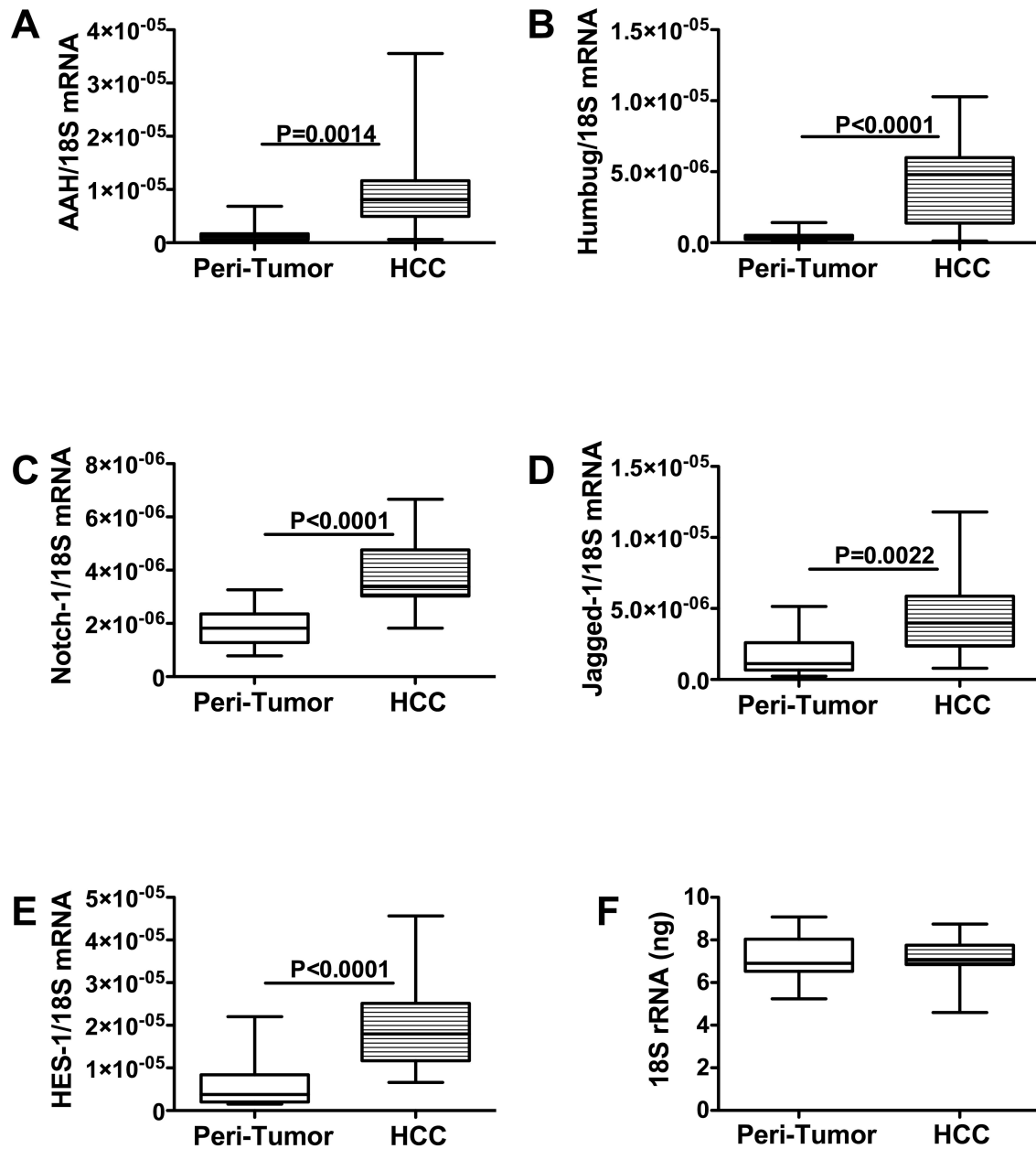


Figure 7.6: Gene expression levels of mediators of the Notch signaling cascade, including AAH/Humbug measured in peri-tumour and HCC human liver tissues. RNA extracted from liver was reverse transcribed to measure (A) AAH, (B) Humbug, (C) Notch-1 (D) Jagged-1 and (E) HES-1 mRNA expression by PCR. mRNA abundance was corrected for using 18S as the housekeeping gene. Inter-group comparisons were made using t-tests. Significant P-values are indicated within the panels.

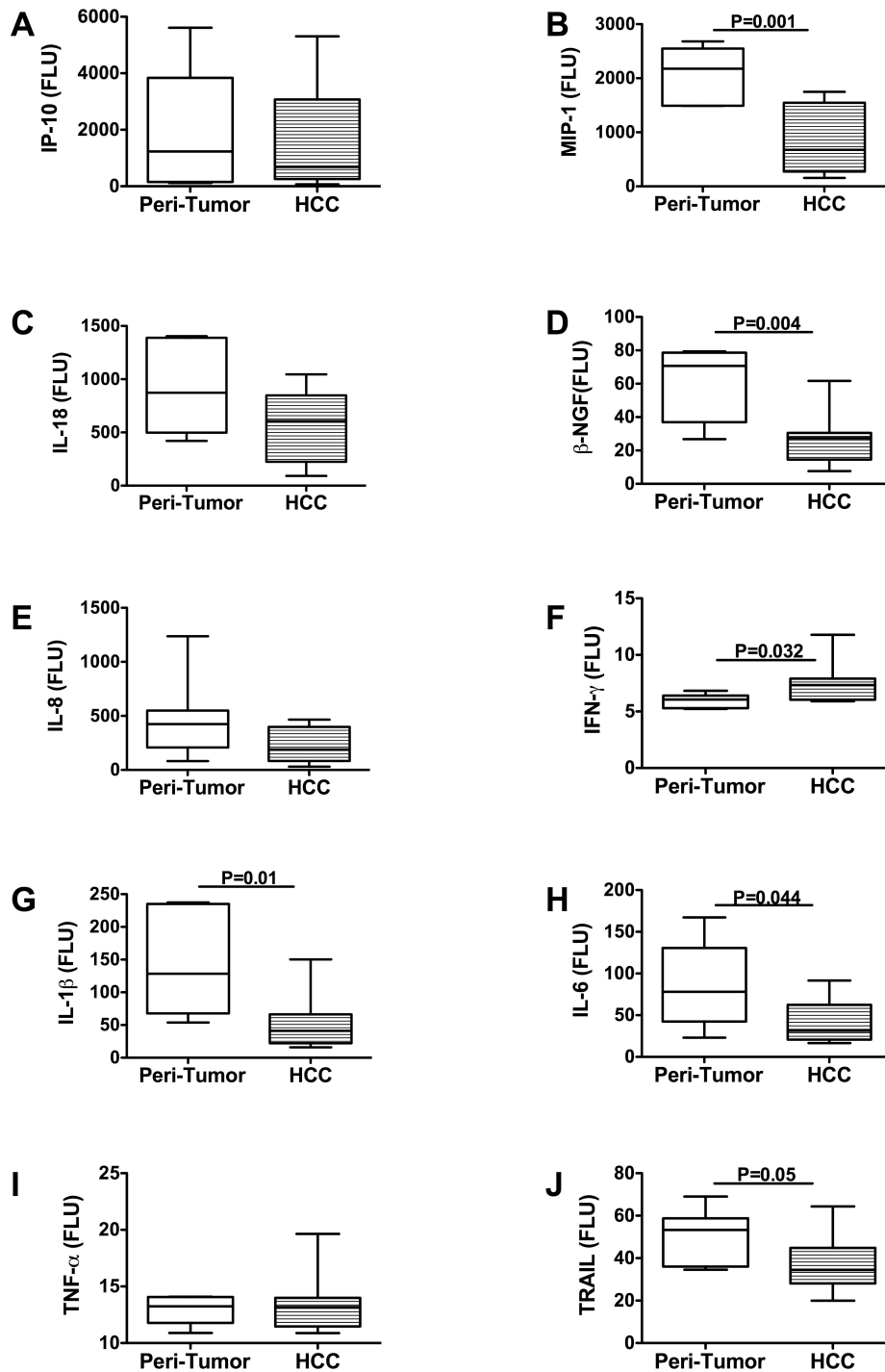


Figure 7.7: Cytokine expression levels measured in peri-tumour and HCC human liver tissues. Liver protein homogenates were used to measure immunoreactivity in fluorescence units corresponding to (A) IP-10, (B) MIP-1, (C) IL-18, (D) β -NGF, (E) IL-8, (F) IFN- γ , (G) IL-1 β and (H) IL-6, (I) TNF- α and (J) TRAIL with a bead-based Multiplex ELISA platform (see Methods and

Methods). Data were analyzed statistically using t-tests. Significant P-values are indicated within the panels.

7.4 DISCUSSION

Stepwise progression of liver disease of any aetiology from normal, to pre-malignant and ultimately a malignant phenotype is governed by many factors, not least of which are signaling cascade impairments that modulate growth, proliferation and apoptosis. Analysis of the differences in signaling impairments, if any, at various stages of liver disease may give key clues to the molecular aspects of disease that might lead to the identification of diagnostic markers that can be used for detecting early disease or indeed of additional therapeutic agents to target HCC. Our study aimed to evaluate the differences in Insulin/IGF, Wnt, and Notch signaling in normal, peri-tumour and tumour human liver tissue and to consider how in concert, these pathways may mediate hepatocarcinogenesis.

The findings were that in peri-tumour tissue, as compared to normal liver gene expression for ligand availability and binding was downregulated as evidenced by lower mRNA transcripts of IGF-1 and 2, and their corresponding receptors. When comparing peri-tumour to HCC tissues on the other hand, although there is no difference in the expression of IGF-1 mRNA transcripts and that of IGF-1R, IGF-2 transcripts are lower, while those of IGF-2R are higher in the latter suggesting a compensatory overexpression of IGF-2R as a result of reduced gene expression for IGF-2 ligand. A similar finding of overexpression of IGF-2R in 100% of HCC was reported in a previous paper from our laboratory (Cantarini et al., 2006). Correlative protein expression data suggests that IGF-2 signaling in HCC is suppressed in favor of signaling through insulin, via the

insulin receptor and IGF-1R because IGF-2R does not transmit signals, rather it functions to sequester IGF-2. Upregulated Insulin/IGF signaling in HCC compared to normal or peri-tumour tissues was evidenced in this study by the universal increased gene expression of IRS-1 to 4 polypeptides; similar results to those shown by others (Boissan et al., 2005; Cantarini et al., 2006; Nishiyama & Wands, 1992) thereby promoting increased protein expression for the survival of HCC cells.

Signaling through IGF-1R in HCC in this study is significant for two reasons: it was shown to be involved in tumour dissemination (Chen, Boyartchuk, & Lewis, 2009), and to be the sole receptor through which pro-tumourigenic effects are seen (Nussbaum et al., 2008). Correspondingly, signaling through IGF-1R was operative in this study.

Furthermore, more downstream, upregulated insulin signaling was mediated by GSK-3 β and p70S6K thus promoting glycogen and protein accumulation respectively that is needed for cell survival and regeneration. GSK-3 β is an important substrate of Wnt signaling, in inactive states, the GSK-3 β /APC/Axin complex phosphorylates and thereby marks β -catenin for degradation, thus GSK-3 β is a negative regulator of oncogenesis. However overexpression of GSK-3 β has been shown in many different types of tumours, suggesting its role as a tumour promoter. In fact overexpression of GSK-3 β was noted in cancerous versus normal mouse liver tissue (Gotoh, Obata, Yoshie, Kasai, & Ogawa, 2003). Collectively therefore, insulin signaling was upregulated

both upstream and downstream in HCC to promote the pro-survival and proliferation of transformed neoplastic cells.

For many causes of liver disease a solitary insult does not seem sufficient to result in significant disease, for instance, overexpression of IRS-1 in combination with alcohol in a transgenic mouse model of HCC resulted in significantly more impairments of insulin and Wnt signaling, including the expression of AAH, effects typically seen in HCC (Longato, de la Monte, Califano, & Wands, 2008). Similarly IRS-1 overexpression by itself did not result in tumours (Mohr, Banerjee, Kleinschmidt, Bartolome Rodriguez, & Wands, 2008), therefore showing the need for interactive signaling.

Upregulation of the Wnt/ β -catenin signaling was mediated by ligands Wnt3, 5A and B and as expected, the secreted frizzled proteins were downregulated, because these antagonize the action of the Wnt ligands (Leyns, Bouwmeester, Kim, Piccolo, & De Robertis, 1997; S. Wang, Krinks, Lin, Luyten, & Moos, 1997). Downstream signaling was mediated via frizzled-6; this finding, contrasts with other reports where frizzled-7 was the dominant downstream effector associated with beta-catenin nuclear accumulation (Merle et al., 2005). Moreover, the quoted study showed that Wnt3 interacts with and is likely activated by Fzd-7, thereby enhancing Wnt signaling and tumour cell migration. This discrepancy with previous data may be due to the fact that there are many Wnt receptors, the ligand specificity of which has not been fully elucidated. Therefore in different types of tissues, differences in ligand-receptor binding combinations may be responsible for these discrepancies. All measured

components of Notch signaling including its upstream regulator AAH were upregulated in HCC relative to peri-tumour tissues. Although AAH has been shown by others to be upregulated in HCC (Cantarini et al., 2006), the role of Notch signaling is less clear with divergent results from different laboratories (Gao et al., 2007; Gao et al.; Gramantieri et al., 2007; Wang et al., 2009; Wang et al.; Wang et al., 2009). One explanation of this could be that Notch signaling was evaluated in different in vivo and in vitro systems in these studies, and it is well known that signaling is dependent on the tissue and cell type, and the required function at the time. Upregulation of Notch-1 and Jagged in our study suggests that in human HCC Notch signaling is activated and is therefore key in promoting malignant transformation.

Finally in virus- and/or alcohol-associated HCC there is attendant inflammation, which is induced by cytokine activation. Therefore HCC is associated with cytokine activation. Various cytokines can have opposing effects depending on the cellular state, thus there is no consistency across the available data on activation of individual cytokines. The majority of measured cytokines were either unchanged or downregulated in HCC compared to peri-tumour tissue except for IFN- γ . IFN- γ is used successfully in the treatment of viral hepatitis, and thus its upregulation in HCC may be suggestive of its role in attempting to clear hepatocytes of the virus and/or of transformed abnormal cells. However additional mechanistic studies evaluating this concept are necessary.

To summarize this study was novel in that all 3 significant pathways involved in HCC were studied in parallel. In this model there was upregulation of

components of all three pathways. We posit that because a single “hit” seems insufficient to cause significant disease, the combination of insulin/IGF, Wnt/ β -catenin and Notch signaling results in growth, proliferation of abnormal cells and avoidance of apoptosis likely by the upregulation of Notch by AAH and the pro-oncogenic effects of GSK-3 β , stimulated by both Insulin/IGF and Wnt signaling. Relevance of this study could be in the targeting of respective signaling pathways either through the use of specific tyrosine kinase inhibitors or neutralizing antibodies. The pro-tumourigenic effects of IGF-II such as proliferation, anti-apoptosis, and migration are transmitted through its receptor IGF-1R, therefore selective inhibition of this tyrosine kinase by small molecule compounds might reduce IGF-II-driven tumour growth. It has already been shown that an antibody to Wnt-1 reduced viability and proliferation of cell lines likely by promoting apoptosis and decreasing the transcriptional activities of beta-catenin (Wei, Chua, Grepper, & So, 2009), therefore this approach is promising.

Alternatively, specific molecules within the respective pathways could be identified as potential early disease tumour markers, i.e. AAH and targeted.

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CHAPTER 8

8.1 CONCLUSION

Chronic liver disease and its complications of cirrhosis and hepatocellular carcinoma (HCC) are serious conditions with high morbidity and mortality, not to mention economic burden. In particular, other than the increasing prevalence of obesity, chronic hepatitis infection (B and C) and alcohol are the main aetiological factors. The underlying mechanisms of progression of disease of any aetiology to more severe phenotypes including steato-hepatitis, cirrhosis and HCC are incompletely understood, despite active on-going research in this field. Notwithstanding, the role of aberrant signaling cascades in these disease processes has come to the fore in recent times and since proteins that contribute to or are responsible for activating these pathways can be identified and isolated, this approach to studying disease mechanisms is exciting, feasible and may likely advance the understanding of these diseases. Thus the broad aim of this work was to characterize signaling impairments in two major pathways that are known to contribute, to varying degrees, to alcoholic liver disease (ALD) and HCC viz: insulin/IGF and Wnt/ β -catenin signaling. In addition, we sought to evaluate the role of the less well-characterized Notch signaling pathway in HCC.

Previous studies conducted in our laboratory have shown that alcoholic steatohepatitis in a rat model is associated with insulin resistance, lipotoxicity and oxidative and ER stress. While triglycerides are the main lipid responsible for hepatic steatosis, ceramides have emerged as the lipids responsible for many of

the features of ASH, the malign form of ALD. In this work we have shown both in *in vivo* and *ex vivo* experiments of ASH and in human ALD tissue that ceramide-induced lipotoxicity accounts for the insulin resistance that is implicated in progression of ALD. The mechanism for the insulin resistance is increased acid sphingomyelinase activity. Furthermore, in interrogating possible therapeutic options we showed that although oxidative stress is an important element of the pathophysiology of ALD, and is likely partly responsible for the ceramide-mediated lipotoxicity, treatment with anti-oxidants alone is not sufficient to quell the attendant insulin resistance. However, our *ex vivo* studies showed that treatment with ceramide inhibitors ameliorated the ER stress associated with ALD, suggesting therefore, the important role of ER stress as a mediator of disease progression (as seen in the human ALD study). Together these findings attest to the complex pathophysiological mechanism of ALD and therefore the necessity of a multi-pronged approach to treating this disease. In this context, our work contributes to current knowledge in both the pathophysiological mechanisms of progression to ASH (specifically oxidative and ER stress), and the potential therapeutic strategies that can be employed to treat this condition (Figure 8.1).

Consequently, the clinical implications of these findings firmly place ceramides as culprits that can be directly targeted in combination with other treatments to treat ALD, particularly ASH. This treatment would be a novel addition to the hitherto lack of effective or registered treatments for ALD. This however, was not tested in this project; therefore studies examining the effect of

the administration of ceramide inhibitors in patients with ALD are urgently needed.

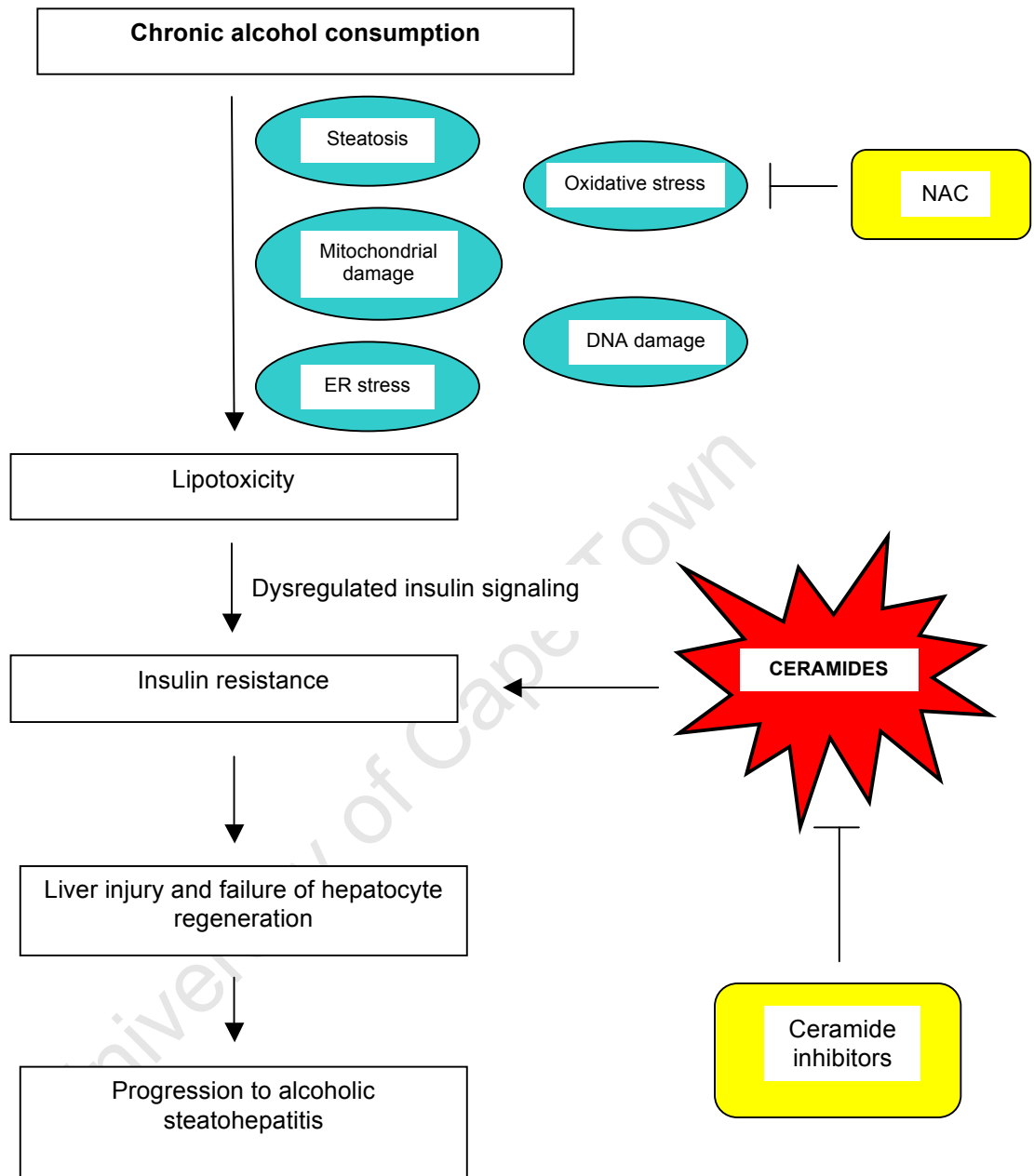


Figure 8.1: Mechanisms of progression to alcoholic steatohepatitis. The mechanisms of progression of alcoholic steatosis to steatohepatitis are thought to be mediated by oxidative and endoplasmic reticulum (ER) stress, DNA and mitochondrial dysfunction and lipotoxicity, to mention a few. Ceramides are toxic lipids that modulate signaling. In this model of alcoholic steatohepatitis, ceramides resulted in insulin resistance (IR) that ultimately impairs hepatocyte

growth and regeneration. The administration of anti-oxidant therapy i.e. N-acetylcysteine (NAC) and/or ceramide inhibitors led to an amelioration of the effects of chronic alcohol consumption.

Perhaps the most unexpected results from our experiments were the lack of pro-inflammatory cytokine activation in the rat model of ASH and in human HCC liver tissue. This finding contrasts quite strikingly with published reports. In terms of the animal ASH model, these findings are perhaps less surprising when looked at in the context of the fact that this model did not show significant inflammation histologically. Pro-cytokine inflammation is generally implicated in acute/sub-acute models of alcoholic hepatitis, which was not the case in this study, which was of chronic steatohepatitis. In human HCC, previous reports have yielded varying results as to the role and importance of cytokine activation in HCC. The role of cytokine activation seems to be important particularly in virus-related HCC i.e. HBV or HCV. We did not have any specific information about the aetiology of our HCC tissue and likely there are other unknown factors at play that determine the extent to which cytokine activation plays a role in HCC.

The most interesting data were that from the double transgenic mouse model of HCC and human HCC tissue. Our study was novel for the fact that in the former two specific Wnt signaling mRNA transcripts were identified as being markedly upregulated in the double transgenic mice, thus marking potential HBV-related HCC signatures that can be utilized in the identification of early pre-malignant disease. In the latter, the three signaling pathways i.e. insulin/IGF, Wnt/ β -catenin and Notch were simultaneously studied in pre-neoplastic and HCC

tissue. These were found to be independently aberrant in HCC; our results suggest that the cross-talk and co-activation of these pathways collectively determines the fate of chronically inflamed and scarred liver tissue, to either arrest progression of disease, i.e. at the cirrhosis stage, or to further progression to the malignant phenotype, HCC. AAH is linked to both Notch and insulin signaling by hydroxylating and enhancing the expression of Notch and Jagged, but is itself stimulated by insulin/IGF via the ERK/MAPK and PI-3K/Akt pathways (Cantarini et al., 2006; de la Monte et al., 2006; Lahousse, Carter, Xu, Wands, & de la Monte, 2006). Additionally, overexpression of aspartyl-(asparagyl)-beta-hydroxylase (AAH), upregulated both insulin/IGF and Notch signaling (Cantarini et al., 2006), suggesting that there is a complex interaction between AAH/Notch and insulin signaling cascades. Notably, inhibition of GSK-3 β increased AAH protein probably by inhibiting the phosphorylation of AAH (Carter et al., 2008), and it is known that when activated, Wnt signaling phosphorylates and thereby de-activates GSK-3 β . Correspondingly, it is possible that deactivation of GSK-3 β by Wnt could also activate AAH, in this way connecting the Wnt and AAH/Notch pathways. Therefore, we hypothesize that the interaction points linking this triad of signaling pathways, are likely via AAH and GSK-3 β . Upregulation of components of these three pathways that ultimately result in increased levels of AAH and GSK-3 β probably interact to promote cancer formation (Table 8.2). These findings are crucial to the understanding of hepatocarcinogenesis. More importantly, are the practical implications of this knowledge, and that is that certain key molecules (described in Chapter 5) can be useful targets for either

neutralizing antibodies, or tyrosine kinase inhibitor therapy, that can be used as adjunct therapy for HCC or indeed, as alternative therapy for this devastating disease.

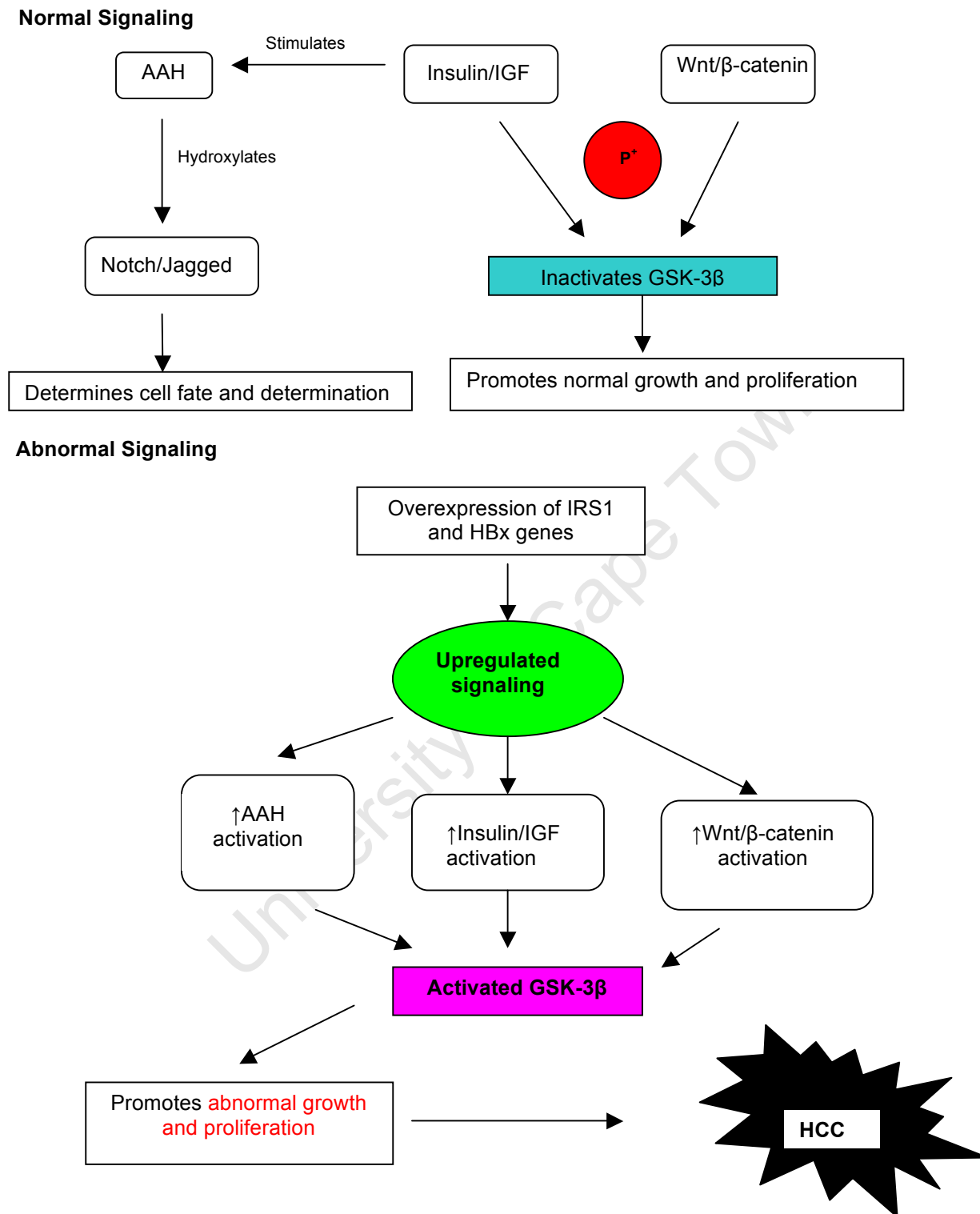


Figure 8.2. Triad of signaling dysregulation and the contribution to HCC.

8.2 Future Directions

This body of work has provided key insights into the pathogenesis of ALD and HCC. However many mechanistic questions that were beyond the scope of this work remain unanswered. One of the mechanisms involved in alcoholic steatosis is the downregulation of PPARs. Thus we aim to test the functional effects of PPAR agonists in ASH using the PCLSC model, which is amenable to such a study. In this way, if PPAR agonists reverse a different pathophysiologic mechanism of ASH, they can potentially be combined with ceramide inhibitors for their effect in ALD.

Furthermore, in the double transgenic mouse we hypothesized that to some extent age-related epigenetic regulation was responsible for the aberrant signaling changes. In fact, compared to the human HCC tissue, frizzled receptor inhibitors, i.e. sfrps were upregulated in the double transgenic mice, which is contrary to what would be expected. We hypothesize that this may be due to age-related epigenetic regulation in the double transgenic mouse. Thus in the next phase of this study we aim to do in the first instance, a methylation-specific PCR array analysis, to determine if there is an alteration of methylation that can explain the changes in gene expression of Wnt/ β -catenin observed at the different stages of liver disease. Methylation changes are the most common and better-studied epigenetic regulatory modifications, however if this analysis does not yield results, we will extend the study to examine the role of histone modifications in promoting hepatocarcinogenesis. Secondly it would be important to measure Wnt molecules at protein level and β -catenin levels in liver tissue, and to correlate them with the observed signaling changes in gene expression.

This analysis will shed light as to the functional consequences of Wnt activation at cellular level, and on disease phenotype.

Finally, in terms of the human HCC tissue analyzed and since preliminary results support our hypothesis, it would be desirable to increase the sample number to increase the power of our study in future studies. Therefore, firstly, we will repeat measurements of gene expression of all three signaling pathways in larger samples. Secondly, we will do correlative protein analysis of the signaling pathways, including a measurement of β -catenin, to see if it correlates with activated signaling. Additionally, to uncover the cross-talk mechanisms of these pathways underlying the additive or synergistic promotion of the development of HCC, we will do co-immunoprecipitation experiments between each of the three signaling pathways and AAH and GSK-3 β . The implications of positive results from this latter aim are enormous, because this suggests that targeting either AAH or GSK-3 β alone, can potentially target the three incriminated pathways simultaneously. The clinical importance of this finding cannot be overstated. Subsequent experiments could involve administering inhibitors against AAH or GSK-3 β , remeasuring gene and protein expression as proof of principle of the functions and effects of AAH and GSK-3 β in promoting HCC.

The potential of this and future studies, as outlined provides an exciting prospect for the understanding and possible therapeutic discoveries for ASH and HCC, both devastating diseases.

8.3 Publications arising from this work

1. Limited Therapeutic Effect of N-Acetylcysteine on Hepatic Insulin Resistance in an Experimental Model of Alcohol-Induced Steatohepatitis.

Setshedi M, Longato L, Petersen DR, Ronis M, Chen WC, Wands JR, de la Monte SM. *Alcohol Clin Exp Res*. 2011 Jul 25. doi: 10.1111/j.1530-0277.2011.01569.x. [Epub ahead of print] PMID:21790669

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