Novel Therapies in Chronic Liver Disease

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SUMMARY

This thesis will address the two foremost concerns in clinical hepatology today: finding a treatment for those with cirrhosis and treating the emerging epidemic of non-alcoholic fatty liver disease (NAFLD). These two issues represent the two ends of the chronic liver disease spectrum.

End-stage disease is a chronic fibrotic disease, the result of any number of aetiologies. In situations where the underlying cause cannot be treated or the treatment is unsuccessful, there is a need for an anti-fibrotic, liver regenerative treatment to address this cirrhotic end stage. Currently there are no successful therapies and the scarcity of donors and increase in demand means transplantation is not a viable treatment strategy for the majority of people. In Chapters 2 & 3 in my thesis I assessed a novel, readily available, and plentiful cell-based therapy, the amniotic epithelial cell (hAEC). I examined the mechanisms through which hAEC reduce liver scarring by affecting the cell involved in this process, the hepatic stellate cell (HSC). I have shown that hAEC release soluble factors *in vitro* that have an anti-fibrotic effect on HSC through reduced proliferation, activation, collagen production and increased collagenase activity. In an animal model of chronic liver disease I showed that both hAEC and conditioned media (CM) therapy reduced liver fibrosis. However, only hAEC tempered the expression of liver progenitor cell (LPC) markers in the diseased liver, a marker of disease severity. Furthermore I examined the effects of hAEC on the LPC to assist in the restoration of liver parenchyma in chronic liver disease. I have shown that *in vitro*, hAEC induce LPC proliferation and differentiation into hepatocytes through soluble factors. These chapters add to the growing body of evidence for the use of hAEC as a therapy in chronic liver disease, which one day may enter human trials.

NAFLD is emerging as the most common chronic liver disease in the world. Successful treatment strategies are needed to manage this condition before cirrhosis develops. There are no successful medical therapies for NAFLD, leaving lifestyle modification by diet and exercise the only successful intervention.

However, poor adherence to these regimens and poor long-term response necessitates the need for a new approach. In chapter 4 I have explained the results of a 12-week randomised controlled clinical trial (with subsequent 12-week cross over) of a novel lifestyle modification compared to standard care calorie restriction / exercise advice in the treatment of NAFLD and its associated metabolic conditions. I have shown that both intermittent fasting and standard care advice result in weight, total body fat mass and body mass index reduction with improvements in leptin and adiponectin. However, only intermittent fasting resulted in an improvement in transient elastography, waist circumference, visceral fat volume, blood pressure and insulin resistance. Intermittent fasting was also well tolerated and well adhered. When groups crossed over to the alternative arms, those who changed from standard care to intermittent fasting noted continued improvements in weight, waist circumference, total body fat mass and transient elastography. Whereas, those who changed from intermittent fasting to standard care advice gained weight, waist circumference, total body fat mass and developed insulin resistance. Through this pilot study I have shown that counting minutes not calories by adhering to intermittent fasting is a well tolerated, safe and cheap method that in addition to weight and total body fat loss appears to target the liver and visceral adiposity in a way that standard diet and exercise advice do not. I hope that the results of this pilot study can form the basis for further research in larger trials with liver histological endpoints.

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original papers published, 1 submitted in peer reviewed journals and 1 unpublished publications. The core theme of the thesis is novel therapies in the treatment of liver disease. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Centre for Inflammatory Diseases, Department of Medicine, under the supervision of Professor William Sievert and Dr. Gregory Moore.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 1 - 4 my contribution to the work involved the following:

[If this is a laboratory-based discipline, a paragraph outlining the assistance given during the experiments, the nature of the experiments and an attribution to the contributors could follow.]

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
1	Introduction and review	unpublised	100%
2	Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells	Published	Experimental design, optimisation of protocols, experimental analysis and drafting of manuscript 80%
3	Human amniotic epithelial cells produce soluble factors that decrease liver fibrosis an promote liver regeneration through effects on liver progenitor cells	unpublished	Experimental design, optimisation of protocols, experimental analysis and drafting of manuscript 80%
4	Non-alcoholic fatty liver disease Intermittent Fasting Time Intervention (NIFTI)	submitted for publication	Experimental design, conduct clinical trial, experimental analysis and drafting of manuscript 70%

I have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed:  **…… Date: ……08/10/2014……**

STRUCTURE OF THE THESIS

In compliance with Monash University Doctorate Regulations, this thesis consists of published, submitted and unpublished work relating to "Novel Therapies in Liver Disease".

- **Chapter 1 – Introduction and review.** Written as a chapter.

- **Chapter 2 – Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells.** Presented as a published manuscript from *Cytotherapy.*

- **Chapter 3 - Human amniotic epithelial cells promote liver regeneration and decrease in liver fibrosis through effects on liver progenitor cells by soluble factors.** Written as a chapter.

- **Chapter 4 - Non-alcoholic fatty liver disease Intermittent Fasting Time Intervention (NIFTI): A randomised pilot trial of fasting without calorie restriction versus standard care.** Presented as a submitted manuscript.

- **Chapter 5 – Discussion, future directions.** Written as a chapter.

PUBLICATIONS AND PRESENTATIONS ARRISING FROM THIS THESIS

Work from this thesis has been submitted and presented at national and international conferences as detailed below.

PEER REVIEWED JOURNAL PUBLICATIONS

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Hodge, A., Lourensz, D., Vaghjiani, V., Nguyen, H., Tchongue, J., Wang, B., et al. (2014). Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells. Cytotherapy, 16(8), 1132–1144. doi:10.1016/j.jcyt.2014.01.005

SUBMITTED

Hodge, A., Mack, A., Tuck, C., Tchongue, J., Holt, D., Sievert, W., Moore, G. T. (2014). Non-alcoholic fatty liver disease Intermittent Fasting Time Intervention (NIFTI): A randomised trial of fasting without calorie restriction versus standard care. Submitted

ABSTRACTS / PRESENTATIONS AT CONFERENCES

Hodge, A., Mack, A., Tuck, C., Tchongue, J., Holt, D., Sievert, W., Moore, G. T. Non-alcoholic fatty liver disease Intermittent Fasting Time Intervention (NIFTI): Fasting without calorie restriction improves hepatic transient elastography, visceral adiposity and insulin resistance compared to standard care. Oral presentation at Australian Gastroenterology Week 2014. Gold Coast, Queensland, October 22 – 24, 2014. Poster presentation at the 65th Annual meeting of the American Association for the Study of Liver Diseases, The Liver Meeting, Boston Massachussetts, USA, November 7 – 11, 2014.

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LIST OF ABBREVIATIONS

AASLD	American Association for the Study of Liver Disease
AFP	Alpha fetoprotein
α-SMA	Alpha smooth muscle actin
ALT	Alanine aminotransferase
BM	Bone marrow
BMI	Body mass index
BMSC	Bone marrow stem cells
BrdU	5-bromo-2'-deoxy-uridine
CAP	Controlled attenuation parameter
CCL_4	Carbon tetrachloride
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CK	Cytokeratin
CM	Conditioned media
CPS	Child's-Pugh score
CT	Computed tomograpy
CTGF	Connective tissue growth factor
DDP-4 inhibitor	Dipeptidyl peptidase-4 inhibitor
DEXA	Dual energy X-ray absorptiometry
DMEM/F12	Dulbecco's Modified Eagle's / Ham's F12 Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESLD	End-stage liver disease
ET-1	Endothelin-1
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum

GST	Glutathione transferase
hAEC	Human amniotic epithelial cell
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HOMA	Homeostasis model assessment
HRS	Hepatorenal syndrome
HSC	Hepatic stellate cell
IF	Intermittent fasting
IFN-γ	Interferon gamma
IGF-II	Insulin-like growth factor 2
IL-6	Interleukin-6
IR	Insulin resistance
ITS	Insulin-Transferrin-Selenium
IV	Intravenous
LOX	Lysyl oxidase
LOXL-2	Lysyl oxidase-like molecule 2
LPC	Liver progenitor cells
LSM	Liver stiffness measurement
MELD	Model for End-stage Liver Disease
MHTP	Monash Health Translation Precinct
MMP	Matrix-metallo proteinases
MNC	Mononuclear stem cells
MSC	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NAS	NASH activity score
NASH	Non-alcoholic steatohepatitis
PAF	Platelet activating factor
Pan-CK	Pan cytokeratin
PCR	Polymerase chain reaction

PDGF	Platelet derived growth factor
PPAR	Peroxisome proliferator-activated receptor
PTX	Pentoxifylline
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
rtPCR	Real time polymerase chain reaction
SBP	Systolic blood pressure
SC	Standard care
SD	Standard deviation
SEM	Standard error of the mean
siRNA	Small interfering RNA
SVR	Sustained virological response
TE	Transient elastography
TG	Triglyceride
TGF-β	Transforming growth factor beta
TIMPS	Tissue inhibitors of metalloproteinases
TNF-α	Tumour necrosis factor-alpha
TRF	Time restricted feeding
tTG	Tissue transglutaminase
WC	Waist circumference

CHAPTER 1

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1.1 GENERAL INTRODUCTION

This thesis addresses two major areas of unmet need in clinical hepatology today: finding effective and acceptable treatments for patients with advanced fibrosis/cirrhosis and for those with non-alcoholic fatty liver disease (NAFLD).

End-stage liver disease is a chronic fibrotic process, the result of a number of aetiologies, including NAFLD. In situations where the underlying cause cannot be treated or the treatment is unsuccessful, there is a need for an anti-fibrotic, liver regenerative treatment to address this cirrhotic end-stage. Currently there are no successful therapies and the scarcity of donors and increase in demand mean organ transplantation is not a viable treatment strategy for the majority of people. I will explore a novel, readily available and plentiful cell-based therapy, the human amniotic epithelial cell (hAEC). Using hAEC in an animal model for chronic liver disease and in *in vitro* experiments, I have assessed their anti-fibrotic and liver regenerative potential. The second concern in clinical hepatology is the need to develop successful strategies to address the most common chronic liver disease, NAFLD. Due to its high prevalence and associated metabolic conditions, there is a strong desire to manage and prevent this condition from progressing to cirrhosis. There are no medical therapies for NAFLD, leaving lifestyle modification by diet and exercise the only successful intervention. However, poor adherence to such regimens and poor long-term response requires the need for a new approach. I have devised and completed a clinical trial of a novel lifestyle modification compared with the standard care model to treat NAFLD and its associated metabolic conditions.

This thesis is comprised of four chapters. The first is an introduction and review of novel anti-fibrotic therapies for liver fibrosis currently in open clinical trials. The second and third chapters focus on a hAEC-based therapy for end-stage liver disease. Chapter 2 looks specifically at hAEC effects on the effector cell of liver fibrosis, the hepatic stellate cell (HSC). Chapter 3 compares the anti-fibrotic effects of hAEC and its soluble factors (cell-free therapy) in an animal model of chronic liver disease and then focuses on the effects of hAEC factors on liver progenitor cells (LPC), one of the drivers of liver regeneration. The data in

chapter 3 is being prepared for publication. Finally, chapter 4 comprises a randomised controlled trial of lifestyle modification (time-restricted feeding) compared to standard care diet/exercise advice for NAFLD. This has been submitted for publication and is currently under review.

1.2 HYPOTHESES AND AIMS

Chapter 2: Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells

Hypothesis: hAEC produces soluble factors that have anti-fibrotic effects on HSCs.

Aims: To determine if hAEC conditioned media

1. reduces HSC collagen production and increases collagen degradation
2. induces an anti-fibrotic phenotype in HSC
3. causes apoptosis of HSCs
4. contains proteins, lipids or particles that mediate effects on HSCs

Chapter 3: Human amniotic epithelial cells promote liver regeneration and decrease liver fibrosis through the effects of soluble factors on liver progenitor cells

Hypothesis: hAEC induce LPC proliferation and differentiation into hepatocytes

Aims: To determine if hAEC conditioned media

1. influences LPC proliferation
2. induces LPC differentiation into hepatocytes

Chapter 4: Non-alcoholic fatty liver disease Intermittent Fasting Time Intervention (NIFTI): A randomised pilot trial of fasting without calorie restriction versus standard care

Hypothesis: Three months of intermittent fasting without calorie restriction is non-inferior to standard care (diet and exercise) at improving non-invasive markers of liver fibrosis / steatosis and visceral adiposity.

Aims: To determine if intermittent fasting for 12 weeks in participants with NAFLD

1. reduces transient elastography markers of liver fibrosis and steatosis
2. reduces visceral adiposity
3. improves markers of liver inflammation and insulin resistance
4. is non-inferior to standard care diet and exercise at aims 1, 2 and 3.

1.3 NOVEL THERAPIES IN LIVER DISEASE

1.3.1 INTRODUCTION

According to a consensus report written by the Gastroenterological Society of Australia, in 2013, liver diseases affected more than one quarter of the population or 6 million Australians, with an annual cost to society of $50.7 billion (Dixon, 2013). The majority of this burden is due to the complications that arise from these conditions such as liver failure and hepatocellular carcinoma (HCC). The hallmark of chronic liver diseases is the progression of liver fibrosis, which is the end result of repeated liver injury and contributes to the development of liver failure and HCC. To properly address these burdensome diseases, we must have a two-pronged approach: to allow the liver to heal itself by treating or eliminating the cause of liver injury pre-fibrosis progression and to focus on anti-fibrotic treatments to reduce fibrosis and promote liver regeneration in cases where treatment of the primary injury is not possible or is unsuccessful.

This introductory chapter will outline the clinical features of chronic liver diseases and the cellular mechanisms of fibrosis. Following this, I will review the current anti-fibrotic therapies in clinical trials. Finally, I will discuss the most common chronic liver disease, NAFLD, and the evidence for lifestyle modification for its treatment.

1.3.2 INTRODUCTION TO AND CLINICAL FEATURES OF CHRONIC LIVER DISEASE

The liver is a vital intra-abdominal organ involved in the production of fundamental proteins and biochemical processes essential for life. It plays a vital role in metabolism, detoxification and digestion and our survival depends on its health. The liver has a tremendous ability to 'heal' itself and in most cases of acute injury, once the event passes, the liver is able to regain its pre-injury health. It is the only human organ capable of natural regeneration and as little as 25% of a remnant liver can regenerate itself in a short period of time (Michalopoulos & DeFrances, 1997). In chronic liver diseases, if this healing

process is persistent and unregulated, then progressive fibrosis or 'scarring' and eventual dysfunction of the liver results. There are a multitude of causes of liver injury, but regardless of the aetiology, chronic injury causes accumulation of collagen and other extracellular matrix molecules that distort the liver architecture and vasculature resulting in cirrhosis with nodule formation and organ contraction. This condition can instigate a number of life-threatening complications thereby necessitating liver transplantation. The speed at which this condition develops is dependent on underlying aetiology, environmental influences, co-morbidities and host genetics.

Individuals with liver fibrosis do not have symptoms alerting them to the state of their liver health. It is not until they develop cirrhosis that the symptoms and complications of the disease become apparent. If symptoms are present, they are often non-specific (Schuppan & Afdhal, 2008) subtle and may be overlooked. It has been estimated that 10 - 40% of people with liver cirrhosis are asymptomatic (Falagas, Vardakas, & Vergidis, 2007).

As cirrhosis progresses, liver dysfunction worsens and signs become more overt with the development of ascites, jaundice and encephalopathy. In some instances, the diagnosis is made on presentation of a life-threatening event such as variceal haemorrhage or spontaneous bacterial peritonitis. When an individual has one or more of these complications, their liver disease is categorised as 'decompensated liver disease' or 'end-stage liver disease' (ESLD). These complications are responsible for the high morbidity and mortality seen in cirrhotic individuals.

End-stage liver disease is a multi-system disease whose effects include, but are not limited to, the brain, blood, heart, kidneys and lungs. Through various mechanisms that remain unclear, ESLD renders patients susceptible to develop sepsis and sepsis-induced organ failure (Gustot, Durand, Lebrec, Vincent, & Moreau, 2009). Cirrhotic patients are at risk of both pro- and anti-thrombotic blood disorders, including portal or hepatic vein thrombosis and variceal haemorrhage (Roberts, Patel, & Arya, 2010). Renal impairment occurs

secondary to dehydration, medications and shock. Individuals with advanced liver disease with associated portal hypertension are at risk of developing hepatorenal syndrome (HRS), which is characterised by renal impairment precipitated by circulatory dysfunction. HRS occurs either slowly or rapidly and universally has a poor prognosis as individuals with rapid onset HRS have less than 10% chance of survival (Ginès, Guevara, Arroyo, & Rodés, 2003). Another condition unique to those with end-stage liver disease is hepatopulmonary syndrome, which affects <30% of cirrhotic individuals (Hoeper, Krowka, & Strassburg, 2004) but has a mortality rate as high as 41%. (Krowka, Dickson, & Cortese, 1993)

Alongside the renal and pulmonary complications, cardiac dysfunction such as cirrhotic cardiomyopathy is common and has been shown to affect up to 50% of cirrhotic patients undergoing liver transplantation (Zardi et al., 2010). Those with cirrhosis are susceptible to develop HCC, the most prevalent primary liver cancer, the fifth most prevalent cancer and the third most common global cause of cancer-related deaths (Shariff et al., 2009). Eighty percent of HCCs arise in cirrhotic livers (Simonetti et al., 1991) and the mortality rate from HCC is increasing despite regular surveillance programs to detect early cancers (Fattovich, Stroffolini, Zagni, & Donato, 2004).

End-stage liver disease is costly in terms of mortality and healthcare dollars. Liver cirrhosis and its corresponding complications are associated with poor survival; a 2-year survival of 60% in its early stages and 35% in later stages (Infante-Rivard, Esnaola, & Villeneuve, 1987). End-stage liver disease has a significant economical impact on the health-care system. In the United States, this condition accounts for more that 150,000 hospital admissions annually at a cost of $4 billion (U.S. dollars). It accounts for 40,000 deaths annually, which is equivalent to diabetes and more than kidney diseases (W. R. Kim, Brown, Terrault, & Serag, 2002; Talwalkar, 2006)

1.3.3 CELLULAR AND MOLECULAR MECHANISMS OF LIVER FIBROSIS

Despite differences in the aetiology and origin of the liver injury, there is a common process through which fibrosis, or extracellular matrix (ECM) accumulation occurs. Although other epithelial and bone marrow derived cells have been implicated, the resident cell at the centre of this process is the liver-specific pericyte, the HSC. The HSC resides in the space separating hepatocytes from the sinusoidal endothelial cells. Within this space also resides the ECM that provides the liver's structural support (Fig. 1). In the quiescent state, HSCs contain vitamin A, however in response to injury, HSCs activate, lose retinoid and develop a myofibroblast phenotype that expresses typical markers such as alpha smooth muscle actin. HSCs undergo a series of cellular changes perpetuated by apoptotic hepatocytes and inflammatory cells that drive the excess accumulation of ECM and proteins, including collagen, resulting in fibrosis (Friedman, 2000).

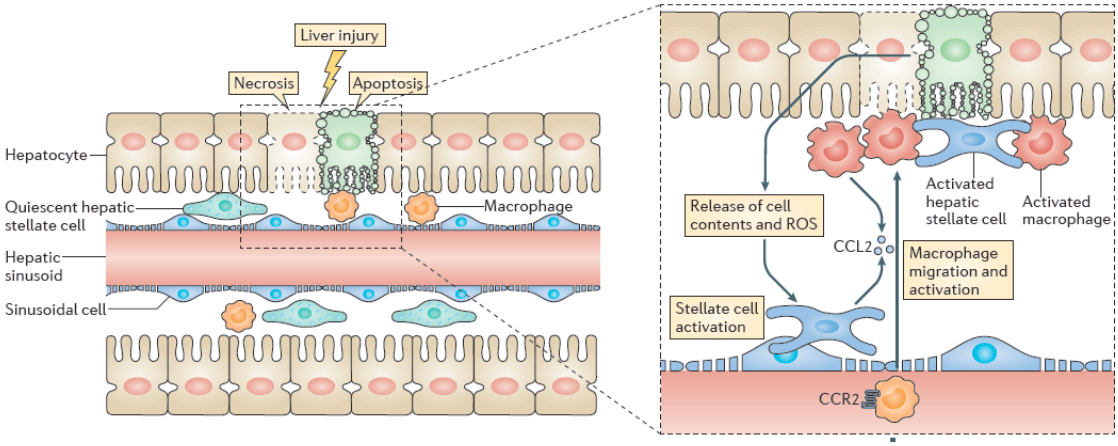

Figure 1.1: Events during hepatic fibrogenesis. Shows the location of the stellate cell in relation to the hepatocytes and sinusoidal space. Demonstration of hepatocyte necrosis/ apoptosis that activates stellate cell through release of reactive oxygen species and attracts macrophages that potentiate the inflammatory response. Resident macrophages in the sinusoid activate and migrate to the location of injury and stellate cell activation. Adapted from Pellicoro et al. (Pellicoro, Ramachandran, Iredale, & Fallowfield, 2014).

In response to liver injury, quiescent HSCs undergo changes enabling them to respond to stimuli from the surrounding injured hepatocytes and inflammatory cells such as lymphocytes, neutrophils and the resident hepatic macrophage, Kupffer cells. Proceeding this initial step are a series of HSC responses which include proliferation, fibrogenesis, cytokine release, contractility, matrix

degradation, and retinoid loss (Friedman, 2000). These HSC changes result in the ongoing accumulation of ECM or fibrosis.

There are a number of growth factors and cytokines that perpetuate these processes. The proliferation of HSCs is primarily driven by platelet derived growth factor (PDGF) (Pinzani, 2002) and fibrogenesis through transforming growth factor beta (TGF-β) and to a lesser extent connective tissue growth factor (CTGF). These growth factors are produced by HSCs (Bissell, Wang, Jarnagin, & Roll, 1995; Paradis et al., 1999) and other inflammatory cells. Other autocrine cytokines that regulate HSC activation include hepatocyte growth factor (HGF), endothelin-1 (ET-1) and platelet activating factor (PAF) (Friedman, 2008; Pinzani & Marra, 2001). During this process, increased HSC contractility via ET-1 leads to increased portal resistance and liver contraction (Reynaert, Thompson, Thomas, & Geerts, 2002). Perpetuation leads to a loss of HSC stored retinoid (vitamin A), but whether this is a result of or cause of activation is not known (Blaner et al., 2009). It is through these processes of perpetuation that the accumulation of ECM occurs.

The constituents of the ECM include collagen: basement membrane types IV & VI and fibril-forming types 1, III & V, proteoglycans, laminin, fibronectin and matricellular proteins (Hernandez-Gea & Friedman, 2010). In the non-fibrotic liver there is a regulated balance between ECM synthesis and degradation, which is tipped in favour of matrix formation in the case of chronic liver injury. Although collagen production is stimulated primarily through TGF-β, the interplay between collagen degrading enzymes known as matrix-metalloproteinases (MMP) and their inhibitors called tissue inhibitors of metalloproteinases (TIMPS) is equally important. HSCs are a key source of MMPs (MMP-2, -3, -9 and -13) (Hernandez-Gea & Friedman, 2010). In their activated states however, HSCs upregulate expression of TIMP-1 and TIMP-2, which serve to inhibit MMPs and result in ECM accumulation, fibrosis and eventual cirrhosis.

Resolution of hepatic fibrosis may occur provided the agent initiating injury is treated or withdrawn (Fig 1.2). There is an initial reduction in activated HSC number through programmed cell death, or apoptosis (Issa et al., 2001) while some activated HSCs may undergo reversion to a quiescent phenotype (D. Li & Friedman, 1999). In doing so, TIMP expression decreases and consequently MMP activity increases resulting in net collagen degradation (Iredale, Thompson, & Henderson, 2013). Despite the evidence for improvement in liver fibrosis following treatment of the underlying aetiology, the degree to which normal hepatic architecture can be restored is unclear. It has been proposed that in advanced cirrhosis, areas of hypocellularity and tissue transglutaminase (tTG) induced ECM cross-linking limit the degree of fibrosis regression (Issa et al., 2004). The importance of this cross-linking in limiting fibrosis resolution is controversial. Recent data suggests that although tTG activity is upregulated during hepatic fibrosis, the resulting ECM cross-linking does not contribute to fibrogenesis or stabilisation of collagen matrix (Popov et al., 2011). Nevertheless, we know that liver fibrosis is a two-way street and with the right conditions and stimuli, fibrosis in a cirrhotic liver can improve. The first step to is to identify and treat the cause of fibrosis.

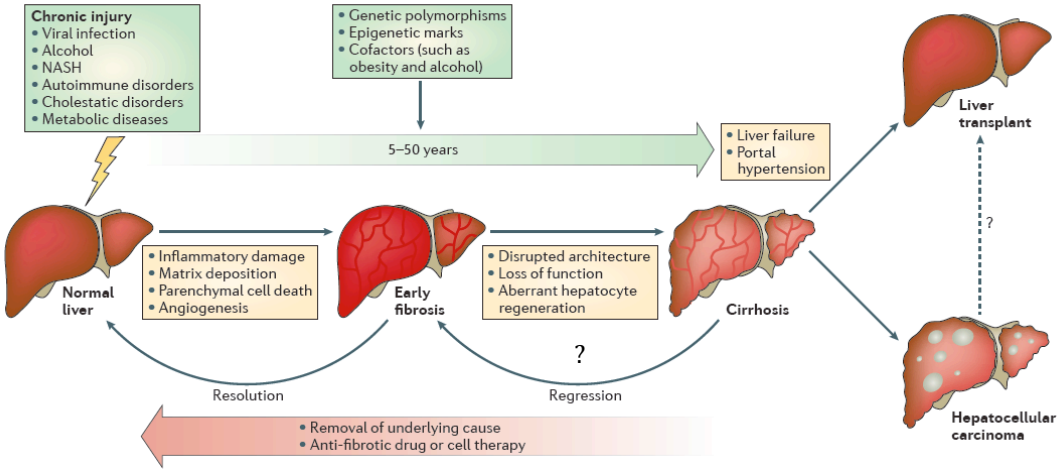

Figure 1.2: Possible causes and fate of chronic liver disease. Various aetiologies precipitate chronic liver injury buoyed by various genetic and environmental cofactors. With progressive liver injury comes increasing matrix deposition and architectural disruption. Degradation of matrix occurs in resolution as the underlying aetiology driving the disease is removed or treated. In end-stage disease (cirrhosis) improvements in liver fibrosis can still occur (regression) however to what degree improvement can be achieved is debatable. Adapted from Pellicoro et al. (Pellicoro et al., 2014)

1.3.4 CAUSES OF CHRONIC LIVER DISEASE

There are a multitude of conditions that can result in the hepatic inflammation or cholestasis required to initiate and potentiate the mechanisms of liver fibrosis. The most prevalent causes in developed countries include chronic viral hepatitis (hepatitis B, C), alcoholic liver disease and non-alcoholic steatohepatitis. The table below outlines the aetiologies of chronic liver disease (Table 1).

Most common causes:	Less common causes:	
Viral hepatitis (B, C)	Autoimmune hepatitis	Porphyria
Alcoholic liver disease	Medications (eg. amiodarone, methotrexate)	Infection (eg. echinococcosis, schistosomiasis)
Haemochromatosis	Genetic metabolic disease	Vascular abnormalities
Nonalcoholic steatohepatitis	Bile acid disorders	Veno-occlusive disease
	Wilson's disease	Right-sided heart failure
	Alpha-1 antitrypsin deficiency	Primary biliary cirrhosis
	Primary sclerosing cholangitis	

Table 1.1: Aetiologies of chronic liver disease. Modified from Heidelbaugh and Bruderly, AFP 2006 (Heidelbaugh & Bruderly, 2006)

1.3.5 INTRODUCTION TO THE TREATMENT OF CHRONIC LIVER DISEASE

The most effective way to treat liver disease and its fibrotic sequelae is to remove the offending stimulus thereby allowing the liver to repair and heal itself. There is evidence in a number of conditions including alcoholic, metabolic, autoimmune and viral liver diseases, that successful treatment prevents fibrosis progression and allows regression of established fibrosis. In chronic alcoholic liver disease, reducing the level of alcohol consumption may cease fibrosis progression (Lieber et al., 2003). In metabolic liver diseases, removal of the injurious agent also results in fibrosis regression. In the treatment of Wilson's disease, limited case series suggest that treatment with zinc sulfate can reverse liver fibrosis (Marcellini et al., 2005) and may result in the regression from

decompensated to compensated cirrhosis (Linn, Houwen, van Hattum, van der Kleij, & van Erpecum, 2009). Haemochromatosis, a prevalent metabolic liver disease, also responds to treatment. Individuals with haemochromatosis, including those with or without cirrhosis, demonstrated a regression in fibrosis with regular venesections (Falize et al., 2006). Fibrosis regression has also been demonstrated in individuals with chronic viral hepatitis receiving adequate antiviral therapy. Irrespective of the treatment offered, patients with chronic hepatitis C (HCV) who achieved undetectable HCV RNA at 6 months post treatment (sustained viral response or SVR) showed marked improvement in their liver histology with either interferon-alpha monotherapy (Lau et al., 1998; Marcellin et al., 1997) or pegylated interferon alfa-2b and ribavirin dual therapy (Poynard et al., 2002). Antiviral therapy encourages fibrosis regression in chronic hepatitis B (HBV). Patients with chronic HBV show regression of fibrosis at 9 months with interferon (Weng et al., 2005) and at 1, 3 and 5 years with nucleoside analogues (Bourlière, Kahloun, & Gascou-Tessonnier, 2009; T.-T. Chang et al., 2010; Marcellin et al., 2013). A study by Marcellin et al. demonstrated that up to 5 years of therapy with the nucleoside analogue, tenofovir, resulted in 176 of 348 (51%) having regression of fibrosis with 74% of those initially diagnosed with cirrhosis no longer classified as having this condition (Marcellin et al., 2013) The natural history of fibrotic liver disease can also be altered in autoimmune conditions. Immunosuppressive therapy in autoimmune hepatitis has shown to be effective in fibrosis regression, even in those with cirrhosis (Czaja & Carpenter, 2004; Dufour, DeLellis, & Kaplan, 1997). In primary biliary cirrhosis, treatment with ursodeoxycholic acid may confer a survival benefit (Parés, Caballería, & Rodés, 2006) and alter the natural history of fibrosis. Although fibrosis regression has not been demonstrated, those patients with a biochemical response to treatment exhibited lack of progression in their fibrosis (Kumagi et al., 2010).

The effectiveness of these treatments in limiting fibrosis progression relies on successful eradication or suppression of the underlying liver insult. In numerous cases however, treatment is unsuccessful or not possible. When the disease

process continues, fibrosis accumulates leading to cirrhosis and its associated complications. At this end-stage, a fibrosis specific treatment is sorely needed.

The remainder of this chapter will focus on two contemporaneous key issues in clinical hepatology:

1.) Treating fibrosis: addressing end-stage liver disease, or cirrhosis, when there is no treatment for the underlying aetiology, or treatment of the aetiology has failed. A review of current clinical trials of anti-fibrotic therapies for liver disease is discussed.
2.) Preventing fibrosis: treatment of the liver condition before the disease progresses and fibrosis develops. A review of lifestyle intervention as treatment for the most common liver disease, NAFLD, is presented.

1.3.6 TREATMENT OF FIBROSIS

There is a great need for specific anti-fibrotic therapies that target the mechanisms driving fibrosis irrespective of the underlying aetiology. This should be straightforward, in theory, since the molecular mechanisms involved in hepatic wound healing and fibrogenesis are common to most forms of injury rather than unique to a particular disease or condition. There are a multitude of such therapies that have been tried in cell culture and animal models with varying degrees of success. Many of the successful agents progress to human studies with some forming the basis for clinical trials. A search for clinical trials registered with 'clinicaltrials.gov' was performed on July 31st 2014. Using the search term 'liver fibrosis' and refining for 'open interventional studies' that had 'fibrosis' as an outcome measure yielded 59 studies. Of these studies, there were 11 separate therapies with anti-fibrotic potential. These will be discussed below.

Pentoxifylline

Pentoxifylline (PTX) is a competitive non-selective phosphodiesterase inhibitor that inhibits tumour necrosis factor-alpha (TNF-α). Its primary indication is in the treatment of chronic arterial claudication due to its ability to improve red

blood cell deformability and reduce blood viscosity (Ward & Clissold, 1987). PTX has shown beneficial effects in a number of chronic liver disease complications. Animal studies have demonstrated PTX's ability to decrease portal pressures (Soupison et al., 1996). Human studies have demonstrated its efficacy in managing hepatorenal syndrome (Akriviadis et al., 2000) and hepatopulmonary syndrome (Gupta et al., 2008; Sztrymf et al., 2004). In the latter, efficacy was demonstrated with a 3-month (Gupta et al., 2008) treatment. In the largest clinical trial assessing PTX and liver disease complications, Lebrec et al. randomized 335 patients with cirrhosis (Child-Pugh class C) to PTX or placebo and assessed mortality and complication rates at 2 and 6-months. Individuals treated with PTX had a significant reduction in liver-related complications but not mortality (Lebrec et al., 2010). PTX has also been used in clinical studies as an anti-fibrotic agent based on its inhibitory effects on HSC activation demonstrated in cell culture and animal studies (Desmoulière et al., 1999; Lee et al., 1997). As TNF-α serum levels are elevated in patients with non-alcoholic steatohepatitis (NASH) (Warne, 2003), NASH was chosen to study PTX's anti-fibrotic effects. Satapathy et al. studied 9 patients with histologically proven NASH treated for 12 months with PTX (Satapathy, Sakhuja, Malhotra, Sharma, & Sarin, 2007). This research group noted that 4 of the 6 patients who had end of treatment biopsies had a reduction in fibrosis. However a recent meta-analysis of 5 studies with a total of 147 patients found that although there was some histological and biochemical improvement in patients with NASH, there was no histological improvement in steatosis or fibrosis (Du, Ma, Yu, & Li, 2014). Currently, a placebo-controlled trial is recruiting patients with NASH which hopes to further address this question (http://clinicaltrials.gov; Clinicaltrials.gov Identifier NCT00267670).

Stem cell therapies

There is interest in the use of cell-based therapies, such as blood or bone marrow derived stem cells, to treat chronic liver disease. These therapies are based on the observation of bone marrow (BM) derived cells found in injured livers (Petersen et al., 1999). These cells demonstrate HSC characteristics (Baba et al., 2004) and in animal studies, up to 70% of the HSC population in a cirrhotic liver

can be BM derived (Russo, Alison, Bigger, & Amofah, 2006). Further animal studies have shown that transplantation of bone marrow derived mesenchymal stem cells (MSC) into rodent models of cirrhosis reduce mortality and cause regression of fibrosis (Sakaida et al., 2004; D.-C. Zhao et al., 2005). Zhao et al. demonstrated a 40 – 50% reduction in collagen deposition in rat models of cirrhosis treated with MSC derived from rat bone marrow (D.-C. Zhao et al., 2005). This ability of BM cells to treat fibrosis has been demonstrated in human cell infusions in an animal model. In a carbon tetrachloride rat model of fibrosis, Chang et al. infused human MSC which resulted in improvements in biochemical liver function with no accumulation of collagen (Y.-J. Chang et al., 2009).

The mechanism(s) through which stem cell therapy can improve liver function and reduce fibrosis has not been fully elucidated, however there are a number of hypotheses. 1.) Cellular plasticity: There is evidence that stem cells infused into a diseased individual home into the injured liver and undergo differentiation into hepatocyte-like cells (Jang, Collector, Baylin, Diehl, & Sharkis, 2004). Stem cells from various sources have also been differentiated into hepatocyte-like cells pre-transplantation (Farzaneh, Pakzad, Vosough, Pournasr, & Baharvand, 2014; Sancho-Bru et al., 2011; Zhang et al., 2014; Zhou et al., 2014) 2.) Fusion: After homing to the liver, the stem cells do not differentiate, but fuse with resident hepatocytes forming regenerative nodules of normal hepatocytes (Vassilopoulos, Wang, & Russell, 2003; X. Wang et al., 2003) or require direct cell contact to exert their anti-fibrotic effect (P.-P. Wang et al., 2012). 3.) Paracrine effects: Infused stem cells take up residence in the liver and other tissues subsequently releasing soluble factors that can influence different cell types within the liver and beyond. BM derived stem cells have been shown to direct the hepatic progenitor cell niche causing expansion and differentiation (J. K. Kim et al., 2010). Stem cells may also cause fibrosis resolution through the release of proteinases such as MMP-9 (Sakaida et al., 2004) or by the release of mediators that cause a reduction in proliferation and activation of HSC (Parekkadan, van Poll, Megeed, et al., 2007a). The notion that soluble factors are responsible for the effects observed in stem cell transplantation has encouraged trials of cell-free stem cell conditioned media as a therapy. Although this has not been used in humans,

animal studies using MSC soluble factors (conditioned media) in rodent models of acute liver injury have demonstrated significant survival benefit and improvement in liver function following these cell-free therapies (Parekkadan, van Poll, Suganuma, et al., 2007b; van Poll et al., 2008). The true mechanism(s) of how various stem cells have a reparative, anti-fibrotic effect is likely a combination of all three hypotheses depending on the cells used and the conditions present in the host at time of transplantation.

There have been human studies to assess the efficacy of bone marrow stem cell (BMSC) based therapy for chronic liver disease. BMSC types have varied with cells harvested from the bone marrow or mobilised and harvested from the peripheral blood, with the main types being: unsorted mononuclear stem cells (MNCs), haematopoietic stem cells and MSCs (Margini, Vukotic, Brodosi, Bernardi, & Andreone, 2014). A recent review of 18 prospective clinical trials of BMSC therapy for chronic liver disease revealed favourable outcomes (Margini et al., 2014) with improvements in survival, biochemical data and scoring systems for assessing severity of chronic liver disease: the Model For End-Stage Liver Disease (MELD) and Child's-Pugh Score (CPS). Studies have used patients with decompensated cirrhosis from diverse aetiologies with variations in cell treatment. Delivery methods include hepatic artery, portal vein, peripheral vein and intrasplenic injections. Cell numbers range from 10^6 to 10^9. Frequency of treatments, study lengths and endpoints vary from study to study. In a recent comprehensive systematic review of the literature on autologous stem cell therapy for liver disease, Moore et al. assessed 33 studies, with some studies overlapping those assessed by Margini et al (Moore, Stutchfield, & Forbes, 2014). Moore et al. found that despite the heterogeneity in studies with varying cell sources and doses, delivery methods, study durations and endpoints, this therapy was safe and generally resulted in an improvement in liver function tests. They concluded that further robust trials with standardised protocols are needed.

Further studies are being conducted to assess BM derived MNC and MSC in patients with decompensated cirrhosis with fibrosis assessed via histology as a

primary endpoint (http://clinicaltrials.gov; Clinicaltrials.gov Identifier: NCT01741090 and NCT01875081) and via elastography as a secondary endpoint (http://clinicaltrials.gov; Clinicaltrials.gov Identifier: NCT02171949). As we await the results of these trials, research needs to be continued to address the unresolved questions about mechanisms, optimal stem cell source for maximal efficacy and long term safety issues (Houlihan & Newsome, 2008; Meier, Müller, Morel, Gonelle-Gispert, & Bühler, 2013).

Other cell types have been studied as potential therapies. Bone-marrow derived macrophages have been shown in limited animal studies to reduce hepatic fibrosis (J. A. Thomas et al., 2011). However their role is more controversial as they are implicated in both fibrosis progress and resolution (Wynn & Barron, 2010).

Mammalian target of rapamycin (mTOR) inhibitor

mTOR inhibitors (sirolimus and everolimus) are potent immunosuppressive agents used in renal transplantation that have demonstrated the ability to stabilise or elicit regression of fibrosis in chronic allograft nephropathy (M. Liu et al., 2007; Pontrelli et al., 2008; Stallone et al., 2005). These anti-fibrotic effects have also been noted in models of liver fibrosis. Treatment with mTOR inhibitors in rat models of liver fibrosis have shown reduction in pro-fibrotic markers (Neef, Ledermann, Saegesser, Schneider, & Reichen, 2006; Patsenker et al., 2011). The mechanism of action may be through the drugs' effect on HSCs by reducing collagen production and attenuating activation (Piguet et al., 2014). In human liver transplant recipients, sirolimus given as a single agent appears to be an effective immunosuppressive agent with a favourable safety profile (S. J. F. Harper, Gelson, Harper, Alexander, & Gibbs, 2010; Vivarelli et al., 2010). Accelerated fibrogenesis in the transplanted liver has been a particular problem in the setting of recurrent hepatitis C infection. This makes mTOR inhibitors a desirable immunosuppressive agent due to their dual anti-fibrotic and immunosuppressive effect. However, to date there is no human data on their anti-fibrotic effects in the transplant setting. A single study of 60 patients receiving either everolimus or standard care calcineurin inhibitors did not

demonstrate any change in non-invasive markers of fibrosis, however, there was a decrease in serum TGF-β in the group receiving everolimus (Fernández-Yunquera et al., 2014). A clinical trial is currently underway to histologically assess the anti-fibrotic effect of everolimus in hepatitis C recurrence post transplant (http://clinicaltrials.gov; Clinicaltrials.gov Identifier: NCT01707849). This study will help clarify whether mTOR inhibitors dual anti-fibrotic and immunosuppressive effects seen in renal transplantation also occur in liver transplant recipients.

Peroxisome proliferator-activated receptor (PPAR) γ ligands

PPARs are nuclear receptors that regulate metabolic processes, inflammation and adipocyte differentiation. There are three types: α, β/δ, and γ. PPARγ1 is expressed in the liver and is important in the control of insulin sensitivity and glucose metabolism (Barroso et al., 1999). For this reason, thiazolidinedione derivatives (PPARγ agonists) have been used to treat individuals with type 2 diabetes mellitus (Stumvoll, Goldstein, & van Haeften, 2005). In the liver, the PPARγ receptor is thought to play a critical role in fibrogenesis through activation of HSCs (L. Yang et al., 2006). In animals models of NASH, the PPARγ agonist pioglitazone has been shown to ameliorate fibrosis and improve NASH liver histology (J.-S. Zhao, Zhu, Liu, Yang, & Chen, 2012).

Pilot studies in humans have shown promise in improving biochemical and histological parameters of NASH, however, the results are inconsistent. Two small studies demonstrated biochemical and histological improvements including fibrosis. Neuschwander et al. gave the PPARγ agonist rosiglitazone to 30 patients with biopsy proven NASH for 48 weeks and alanine aminotransferase (ALT) levels and insulin sensitivity improved. Liver histology demonstrated a significant improvement in hepatocellular ballooning and perisinusoidal fibrosis although the overall fibrosis score did not improve (Neuschwander-Tetri, Brunt, Wehmeier, Oliver, & Bacon, 2003). A second study gave pioglitazone (PPARγ agonist) to 18 biopsy-proven NASH patients and after 48 weeks of treatment, there was overall improvement in biochemical markers (insulin sensitivity and ALT) with histological improvements in inflammation,

fibrosis and steatosis in two thirds of patients (Promrat et al., 2004). Longer studies utilising PPARγ agonists for up to 2 years did not show improvements in fibrosis. In a placebo-controlled trial using pioglitazone, 26 patients were treated with this PPARγ agonist or placebo for 6 months (Belfort et al., 2006). Biochemical improvements (insulin sensitivity and ALT) were again noted, however, biopsies only revealed an improvement in steatosis, ballooning and inflammation without any improvement in fibrosis. This lack of anti-fibrotic effect was further demonstrated in a 1-year placebo-controlled trial of 66 NASH patients randomised to receive either rosiglitazone or placebo. At the end of the treatment period, liver biopsies did not show any improvement in fibrosis (Ratziu et al., 2008). An open-label extension of the trial was completed for an additional 2 years and despite revealing a decrease in serum insulin, ALT and homeostasis model assessment (HOMA) as a measure of insulin sensitivity, there was still no significant change in the histological parameters of ballooning, inflammation and fibrosis (Ratziu et al., 2010).

Despite the lack of consistent anti-fibrotic effects observed in previous studies, PPARγ agonists continue to be studied. Currently there is a clinical trial assessing 6 months of pioglitazone use in NASH patients (http://clinicaltrials.gov; Clinicaltrials.gov Identifier: NCT 01068444), with a measurable change in fibrosis as a secondary endpoint.

Angiotension pathway

The renin-angiotensin system, classically associated with blood pressure and fluid balance, also plays a key role in hepatic fibrosis through stellate cell activation and pro-inflammatory cytokine release (Bataller et al., 2003; Munshi, Uddin, & Glaser, 2011). To date, human studies have been inconsistent. Retrospective and observational studies have demonstrated an improvement in fibrosis. A large retrospective study of 284 hypertensive chronic hepatitis C patients found that those receiving angiotensin-blocking agents had less histological fibrosis than controls (Corey et al., 2009). A cross-sectional study of hypertensive NASH patients noted that individuals taking RAS blockers had less advanced hepatic fibrosis (Goh et al., 2014).

Prospective studies have been less favourable. A prospective study of 14 patients with chronic hepatitis C revealed that 18-months of losartan resulted in down-regulation of fibrotic genes (Colmenero et al., 2009). However, biopsies completed at baseline and after treatment did not show any significant change in fibrosis. Conversely, a smaller prospective study of 12 patients with HCV treated with candesartan showed improvements in non-invasive fibrosis indexes, however, biopsies were not completed as a comparison (Ueki et al., 2009). Further prospective studies are required to determine whether angiotensin blockade results in fibrosis regression. A 24-week prospective study with losartan treatment in NASH patients is currently underway (http://clinicaltrials.gov; Clinicaltrials.gov Identifier: NCT 01051219).

Connective-Tissue Growth Factor (CTGF) therapy

CTGF is involved in many signalling pathways, fibrogenesis and tissue remodelling (Blom, Goldschmeding, & Leask, 2002; Lipson, Wong, Teng, & Spong, 2012) Its expression correlates with the degree of liver fibrosis (Guo-Qiu et al., 2010). Post-transcriptional CTGF inhibition through the use of small interfering RNA (siRNA) has been successful in suppressing activated HSCs (G. Li et al., 2008) and decreasing liver fibrosis in an animal model (G. Li et al., 2006). In humans, monoclonal anti-CTGF antibodies are currently in or have recently finished phase 1 or 2 clinical trials for various fibrotic diseases including diabetic nephropathy (Adler et al., 2010). Based on these findings, a randomised double blind placebo controlled study will evaluate the efficacy of monoclonal anti-CTGF antibody therapy in reversing liver fibrosis in those with chronic HBV treated with entecavir (http://clinicaltrials.gov/; Clinical Trials.gov Identifiers: NCT01217632).

Galectin-3 inhibitor

Galectin-3 is a protein that binds to terminal galactose residues in glycoproteins (Di Lella et al., 2011). It is expressed in immune cells and is involved in acute and chronic inflammation (Henderson & Sethi, 2009). Galectin-3 expression is increased in fibrotic livers and is thought to be required for TGF-β mediated HSC

activation and collagen production. (Henderson et al., 2006). Blocking galectin-3 by inhibitor GR-MD-02, has been shown to be effective in reducing fibrosis in animal models (Traber et al., 2013). This limited but compelling data has formed the basis for a clinical trial using this inhibitor in NASH patients with advanced fibrosis (http://clinicaltrials.gov/; Clinical Trials.gov Identifiers: NCT01899859).

Omega-3 fatty acids

Omega-3 fatty acid intake reduces serum triglyceride and is an approved treatment for hypertriglyceridaemia (Kris-Etherton, Harris, Appel, AHA Nutrition Committee. American Heart Association, 2003), a condition associated with the metabolic syndrome and NAFLD. These associations have prompted the investigation of utilising omega-3 fatty acids in the treatment of NAFLD. Despite reducing hepatic steatosis, omega-3 fatty acid therapy has not been shown to alter the presence of fibrosis even after 18-months of therapy (Scorletti et al., 2014; Shapiro et al., 2011). Omega-3 fatty acid's ability to reduce liver fibrosis has only been demonstrated in one animal study (Shaaban, Shaker, Zalata, El-kashef, & Ibrahim, 2014). Despite the paucity of omega-3's anti-fibrotic effects in humans, human studies investigating its effects in NASH patients continue (http://clinicaltrials.gov/; Clinical Trials.gov Identifiers: NCT00941642).

MB-12066

There is very little information on this therapy and its use as a liver specific treatment. MB-12066 is a novel obesity drug that targets NADPH:quinone oxidoreductase 1 activation (Chugh & Sharma, 2012). It works centrally as an appetite suppressant (Chugh & Sharma, 2012) and may have an effect peripherally in the adipose tissue and liver, playing a role in glucose tolerance and liver steatosis (Palming et al., 2007). There is currently a phase 2 study underway to evaluate MB-12066's effect on the histological parameters in NASH (http://clinicaltrials.gov/; Clinical Trials.gov Identifiers: NCT02029586).

Phyllanthus niruri

Plants of the genus *Phyllanthus* are tropical flowering plants that have been used in Indian and Chinese folk medicine for years. There are many species of plant from this genus that have been used to treat a wide range of conditions including diabetes, diarrhoea, and liver disease (Levy, Seeff, & Lindor, 2004). *P. urinaria* treatment in an animal model of NASH resulted in improvement of steatohepatitis (Shen et al., 2008). However, a 24-week human study of *P. urinaria* in patients with NASH did not show any histological improvements after 24-weeks of treatment (V. W.-S. Wong et al., 2013). Similarly, a different species, *P. niruri* has shown promising anti-fibrotic effects in an animal model of liver fibrosis (Amin et al., 2012). We await the results of a trial of *P. niruri* in patients with NASH to determine whether unlike others in this genus, *P. niruri's* anti-fibrotic effects in animals translate into humans. (http://clinicaltrials.gov/; Clinical Trials.gov Identifiers: NCT01680003)

β-catenin blocker

β-catenin is the downstream effector of the Wnt signalling pathway. Wnt/ β-catenin pathway activation is involved in the pathogenesis of fibrotic disorders (Guo, Xiao, Sun, & Liu, 2012). In the liver, this pathway works in combination with TGF-β signalling to induce liver fibrosis (Miao et al., 2013). Inhibition of the Wnt signalling pathway reduces liver fibrosis through inhibition of HSC activation (Cheng et al., 2008). PRI-724 is a small molecule that selectively inhibits the β-catenin dependent pathway of Wnt signalling and is currently being used in a phase 1 study of individuals with HCV induced cirrhosis (http://clinicaltrials.gov/; Clinical Trials.gov Identifiers: NCT01899859).

Dipeptidyl peptidase-4 (DDP-4) inhibitor

DPP-4 inhibitors are a type of oral anti-hyperglycaemic medication that have been used in the management of type 2 diabetes, with sitaglipin being the most widely used (Plosker, 2014). The DPP-4 enzyme inactivates incretin hormones such as GLP-1. Treatment with DPP-4 inhibitors lead to increased GLP-1 levels which inhibit glucagon release and in turn increase insulin secretion, decrease gastric emptying and decrease blood glucose levels (McIntosh, Demuth,

Pospisilik, & Pederson, 2004). Due to the association between diabetes and NASH, DPP-4 inhibitors have been studied in the latter. Sitaglipin treatment in patients with diabetes and NASH has shown improvements in liver histology (ballooning but not fibrosis) and transaminases in one study (Yilmaz, Yonal, Deyneli & Celikel, 2012). In fatty liver animal models, sitagliptin improved liver histology including fibrosis (Jung et al., 2014; Kaji et al., 2014) A 3-year clinical trial is underway utilising sitagliptin in patients with type 2 diabetes and NASH with liver histology, including fibrosis, as a primary endpoint (http://clinicaltrials.gov/;Clinical Trials.gov Identifiers: NCT01260246).

Monoclonal antibody for lysyl oxidase-like molecule 2 (LOXL2)

Lysyl oxidase (LOX) is an enzyme that catalyses crosslinking of collagen, which provides strength and elasticity to connective tissue (Kagan, 1994). Its involvement in liver fibrosis was first described almost 40 years ago (Siegel, Chen, Greenspan, & Aguiar, 1978). Hepatic stellate cells and portal fibroblasts are the major liver source of this enzyme (Perepelyuk et al., 2013). One member of this family of enzymes, LOXL-2, is potentially involved in tumourogenesis, lung and liver fibrosis. Blocking LOXL-2 in animal models reduces myofibroblast number and formation of ECM (Barry-Hamilton et al., 2010). This evidence has formed the basis for clinical trials of Simtuzumab (Gilead), a monoclonal antibody to LOXL-2. Although this agent did not meet the inclusion criteria for the clinical trials search, it is one of few agents specifically targeting fibrosis irrespective of the underlying aetiology. It is currently or has recently been used in 5 clinical trials assessing its antifibrotic effect in primary sclerosing cholangitis, HCV/HIV or NASH induced liver fibrosis. It is also being used in idiopathic pulmonary fibrosis and colorectal and pancreatic cancer. (https://clinicaltrials.gov/ct2/results?term=simtuzumab&Search=Search).

1.3.7 PREVENTING FIBROSIS - TREATMENT OF EARLY DISEASE

To prevent the progression of fibrosis to cirrhosis, one has to be able to identify then treat the causative factor by removing or suppressing it. As stated in 1.3.4

this can be achieved to varying degrees of success in many liver conditions. There is one liver disease that has become an increasing problem and does not have a straightforward or successful therapy. This disease is NAFLD.

1.3.7.1 Introduction to NAFLD

NAFLD is defined as liver fat (hepatic steatosis) accumulation in >5% of hepatocytes in the absence of other causes of liver steatosis, namely alcohol, medications and genetic conditions (Torres, Williams, & Harrison, 2012). It describes a spectrum of diseases from simple liver steatosis without liver inflammation or hepatocyte injury, through to NASH, a condition with hepatic steatosis, hepatocyte ballooning (hepatocyte injury) and inflammation with or without fibrosis (Matteoni et al., 1999). In Western societies, hepatitis C and alcoholic liver disease are the two primary causes of liver cirrhosis but NASH is threatening to overtake them, with recent data suggesting that the prevalence of NASH is 5 to 6 times higher than chronic hepatitis C (Williams et al., 2011).

The prevalence of NAFLD in the community varies depending on the population studied but has been reported in up to 46% of the general population (Lazo & Clark, 2008; Torres et al., 2012). Most people with NAFLD are asymptomatic in terms of liver disease and are diagnosed on liver imaging, with ultrasound being the most commonly used modality. One of the most applicable studies to the general Australian or western population was performed by Williams et al. (Williams et al., 2011). They performed an abdominal ultrasound on 400 participants undergoing a general health check at a US military hospital and noted that 46% had ultrasound features of NAFLD. These individuals had confirmatory liver biopsies and it was revealed that 30% had NASH and 2.7% of the total ultrasound cohort had advanced fibrosis. This and other studies suggest NAFLD is a common disease, which raises significant public health concerns, due to its impacts on the liver and its strong association with other metabolic disorders.

NAFLD has a strong association with insulin resistance and is a significant risk factor for the development of metabolic disorders (Fig 1.2)(Fan et al., 2007),

including hypertension, dyslipidaemia and cardiovascular disease, with NAFLD being an independent risk factor for the latter (Donati et al., 2004; Hamaguchi et al., 2007; Ruhl & Everhart, 2003; Targher et al., 2007). NAFLD is increasingly becoming recognised as the hepatic manifestation of the metabolic syndrome (Table 1.2) (Tarantino & Finelli, 2013).

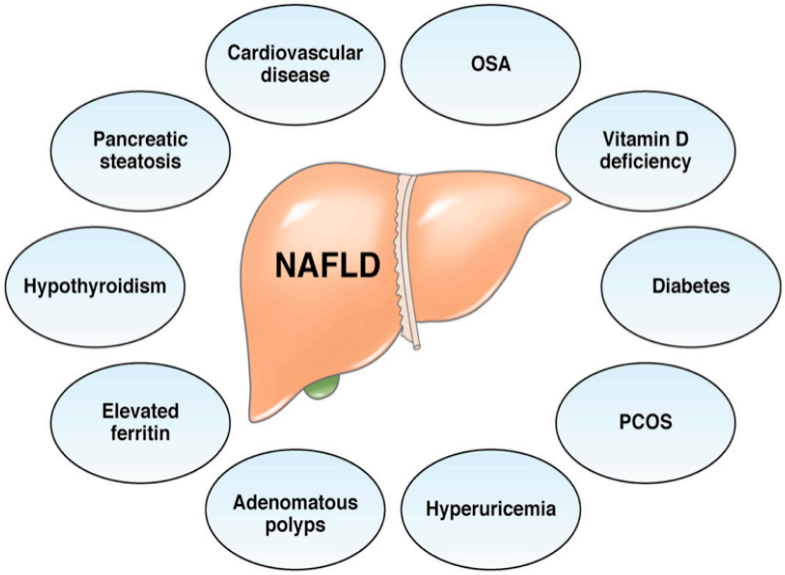

Figure 1.3 : Clinical conditions associated with NAFLD. Adapted from Torres et al. 2012 (Torres et al., 2012)

Central obesity (defined as waist circumference with ethnicity specific values OR body mass index > 30kg/m^2) **Plus** any two of the following factors:	
Raised triglycerides	≥ 1.7 mmol/L or specific lipid lowering treatment
Reduced HDL	< 1.03 mmol/L in males < 1.29 mmol/L in females or specific treatment
Raised blood pressure	Systolic BP ≥ 130 or diastolic BP ≥85 mm Hg
Raised fasting plasma glucose	≥ 5.6 mmol/L or previously diagnosed type 2 diabetes

Table 1.2: Metabolic syndrome. New International Diabetes Federation definition for metabolic syndrome adapted from Zimmet et al. (Zimmet, Alberti, & Serrano Ríos, 2005)

Those with NAFLD have up to a three-fold increased risk of developing diabetes (Choi et al., 2013; Fan et al., 2007) and those with diabetes and fatty liver disease have an increased rate of diabetic complications such as cardiovascular disease and chronic kidney disease (Targher & Byrne, 2013). Additionally, individuals

with diabetes and NAFLD have more progressive liver disease (Vernon, Baranova, & Younossi, 2011) and a higher risk of cirrhosis (Younossi, Gramlich, Matteoni, Boparai, & McCullough, 2004) and HCC (El-Serag, Tran, & Everhart, 2004). Obesity is associated with NAFLD as upwards of 90% of the morbidly obese population have NAFLD (Gholam, Flancbaum, Machan, Charney, & Kotler, 2007; Ong et al., 2005).

Like other chronic diseases of the liver, NAFLD can result in progressive liver fibrosis and cirrhosis. Liver transplantation as a consequence of NASH is an increasing trend (Kemmer et al., 2013) and is set to be the primary indication for transplantation in the future. However, most people with NAFLD will not develop cirrhosis. On average, 30% of individuals with NAFLD will have the inflammatory disease NASH (Williams et al., 2011). Of these people, the rate of fibrotic progression is such that 11% will develop cirrhosis over a 15-year period (Angulo, 2011). This means that on average, a person with NAFLD over a decade will have a 3% risk of developing cirrhosis. For the individual, this risk is much less that other liver diseases, however, as up to one in two adults has fatty liver disease, NASH cirrhosis will become an increasing burden on the community and our health care system. It is imperative we find successful treatment strategies for NAFLD to reduce the impact that NASH related cirrhosis would have on our society.

In order to develop treatment strategies we need to understand the pathophysiology of the disease. Although the risk factors for NAFLD / NASH have been elucidated, the pathophysiology is not completely understood. The initial theory was a 'two-hit' hypothesis. The first 'hit' is the accumulation of liver steatosis which sensitises the liver to the onset of inflammation by a second 'hit' that promotes oxidative stress causing NASH (Day & James, 1998). This model has been revised to recognise there are likely a combination of genetic and environmental factors, including excess caloric intake and inactivity, that generate combinations of second 'hits' that lead to NASH (Day, 2002; Farrell, van Rooyen, Gan, & Chitturi, 2012).

Liver steatosis develops from an imbalance between triglyceride (TG) acquisition and removal from the liver. These TGs are derived from excessive caloric intake, de novo synthesis and adipose tissue (Cohen, Horton, & Hobbs, 2011). Obesity is associated with increased adipocyte mass and insulin resistance with peripheral lipolysis leading to an increase in hepatic TG synthesis (Mittendorfer, Magkos, Fabbrini, Mohammed, & Klein, 2009).

The shift from non-inflammatory NAFLD to inflammatory NASH is not clearly understood, but it is thought there is a combination of local and systemic factors. There is growing evidence for the role of TNF-α and other inflammatory cytokines such as Interleukin-6 (IL-6) in the development of NASH (Feldstein, 2010). Serum levels of the key adipocyte derived cytokine or adipokine, adiponectin, is also implicated in NASH (Hui et al., 2004). Adiponectin has insulin-sensitising, anti-lipogenic and anti-inflammatory properties and hypoadiponectinaemia is thought to play a role in progression of NAFLD to NASH (Trauner, Arrese, & Wagner, 2010). Inflammatory cells are also implicated in the development of NASH. Macrophage infiltration into the liver is seen as an initiating event in the development from simple steatosis to NASH (Bieghs, Rensen, Hofker, & Shiri-Sverdlov, 2012). There are also important intestinal and visceral fat sources of inflammation. Increased intestinal permeability and translocation of bacterial endotoxins have been shown to drive hepatocyte apoptosis and fibrogenesis in fatty liver disease (Miele et al., 2009). Additionally, there is evidence that the metabolically active visceral fat is a source of systemic inflammation that likely contributes to the development of NASH (Fontana, Eagon, Trujillo, Scherer, & Klein, 2007).

1.3.7.2 Management of NAFLD – oral therapy

The increasing prevalence of NAFLD has led to an increased demand for an effective therapy. Therapies have been directed at treating NASH, as this end of the fatty liver spectrum is associated with inflammation and potential progression to cirrhosis. There are a number of medical therapies that have shown promise (Torres et al., 2012), but to date there is no clear effective

pharmacological therapy. I will briefly mention some of the more commonly trialled therapies

Vitamin E

In 2012, Practice Guidelines by the American Association for the Study of Liver Disease (AASLD) recommended the use of high-dose vitamin E for NASH (Chalasani et al., 2012). Vitamin E therapy has been shown to improve liver steatosis. Therapy with high-dose vitamin E for 2 years was shown to reduce ALT and improve liver histology, namely in steatosis and inflammation (Sanyal et al., 2010). However, there was no improvement in liver fibrosis and adverse outcomes such as increases in insulin resistance and serum triglyceride levels were noted (Musso, Cassader, Rosina, & Gambino, 2012; Sanyal et al., 2010). Vitamin E has been associated with an increased all-cause mortality (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; Miller et al., 2005) and prostate cancer in healthy men (E. A. Klein et al., 2011). These potential adverse outcomes limit this vitamin as a treatment.

Insulin sensitising agents

Metformin and thiazolidinediones have successfully been used in diabetes to improve insulin sensitivity and increase glucose utilisation (Bailey & Turner, 1996; Iwamoto et al., 1996). Despite favourable results in small limited trials, AASLD practice guidelines do not recommend these medications as first line treatments for NASH (Chalasani et al., 2012). A meta-analysis concluded that up to 12 months of metformin use did not improve biochemistry or liver histology in those with NASH (Vernon et al., 2011). The same meta-analysis demonstrated that thiazolidinediones were able to reduce liver steatosis and inflammation but had no effect on liver fibrosis (Vernon et al., 2011). Additionally, thiazolidinediones are associated with weight gain and increased risk of cardiac failure in diabetic patients (Lincoff, Wolski, Nicholls, & Nissen, 2007).

Coffee

Coffee as a hepatoprotective agent is gaining popularity as more and more studies come to light touting its benefits. Coffee consumption in a wide range of

liver diseases is associated with reduced risk of cirrhosis and lower incidence of hepatocellular carcinoma (S. Chen, Teoh, Chitturi, & Farrell, 2014). There is evidence that coffee consumption is beneficial in NAFLD. A dietary survey from 2001 – 2008 indicated that caffeinated coffee was independently associated with a decreased risk of developing NAFLD (Birerdinc, Stepanova, Pawloski, & Younossi, 2012). There are also 3 small case control studies that have shown coffee drinking is associated with less liver steatosis (Molloy et al., 2012), obesity and insulin resistance (Catalano et al., 2010; Gutiérrez-Grobe et al., 2012). Anty et al., showed that morbidly obese women with NAFLD undergoing bariatric surgery who drank filtered coffee (not espresso) had less liver fibrosis (Anty et al., 2012). Further studies are needed to determine the method of coffee preparation, recommended number of cups and whether decaffeinated coffee is equally efficacious. As coffee is readily available, inexpensive and well tolerated, recommendations for its consumption should be considered in those with NAFLD.

Other therapies

Other therapies have been investigated including omega-3 fatty acids, ursodeoxycholic acid, Chinese herbs, statins and probiotics. Despite the lack of conclusive evidence, many of these therapies have been used in clinical practice.

Omega-3 fatty acids have been shown to reduce hepatic steatosis, improve insulin sensitivity and improve biochemical markers of inflammation. These benefits have been demonstrated in animals studies but very few human studies have shown similar results (Masterton, Plevris, & Hayes, 2010; Parker et al., 2012). Further controlled studies of longer duration are needed to determine whether omega-3 fatty acids will play a role in the treatment of NAFLD.

Ursodeoxycholic acid is a naturally occurring bile acid and was initially thought to be a promising therapy for NASH, but a 2-year randomised controlled trial found it no better than placebo for patients with NASH (Lindor et al., 2004).

Chinese herb formulations have been used to treat a variety of aliments including liver diseases. To assess the efficacy of Chinese herbs in NAFLD, a large meta-analysis compared combinations of over 240 Chinese herbs with placebo, ursodeoxycholic acid, insulin sensitisers, lipid-lowering drugs and antioxidants (Shi et al., 2012). Almost six thousand patients from 62 randomised controlled trials were included. The results of this study suggested that Chinese herbs had a better effect on transaminases and improving radiological liver steatosis than the western counterparts, with Hawthorn fruit the most common herb found in the herbal formulations (Shi et al., 2012). Controlled trials are required to further study the effectiveness of herbs, particularly Hawthorn fruit.

Despite the effectiveness of statins in treating hypercholesterolaemia, studies have not shown any improvements in liver histology in NAFLD (Chalasani et al., 2012). There has been concern regarding the use of statins in individuals with NAFLD due to potential hepatoxicity, but statins are safe to use in this population and should be considered in the treatment of hypercholesterolaemia associated NAFLD, particularly in those individuals with concurrent cardiovascular disease.

There is emerging evidence for the role of the gut microbiome in the pathogenesis of NASH (L. Zhu et al., 2013). The manipulation of the intestinal flora through probiotics has been suggested to be beneficial by providing an anti-inflammatory effect. To date, studies using probiotics have not proved efficacious in the treatment of NASH (Iacono, Raso, Canani, Calignano, & Meli, 2011).

1.3.7.3 Management of NAFLD – lifestyle modification

Due to the close association between NAFLD / NASH and metabolic co-morbidities including obesity, hyperlipidaemia, insulin resistance and type 2 diabetes, a successful management strategy should address more than one of these factors. This is best accomplished by lifestyle modification through diet manipulation with or without exercise. The following is the evidence for lifestyle modification in NAFLD / NASH including its strengths and shortcomings.

There are no current recommendations on the preferred diet or lifestyle modification for individuals with NAFLD. Regardless of the lifestyle approach, there is considerable evidence supporting weight loss as the primary goal. AASLD practice guidelines suggest a 5 to 10% reduction in body weight as it correlates with an improvement in liver steatosis and inflammation (Chalasani et al., 2012). This recommendation is based on research that consistently demonstrates that weight loss results in improved liver histology. Promrat et al. studied 31 obese individuals with biopsy proven NASH and subjected them to a lifestyle intervention of diet and exercise for 48 weeks. Compared to the control group, those who lost ≥ 7% of their body weight had significant improvements in steatosis, lobular inflammation, ballooning injury and NASH activity score (NAS) but no change in fibrosis (Promrat et al., 2010). A 2012 systematic review of 23 studies of lifestyle intervention for NAFLD concluded that weight loss results in improvements in liver steatosis (Thoma, Day, & Trenell, 2012). Additionally, those studies with liver biopsies demonstrated a reduction in liver inflammation but only one showed an improvement in liver fibrosis. This study had the largest number of biopsies (30 biopsies per group) and assessed participants after a 6-month hypo-caloric diet and exercise program with or without an antioxidant (Vilar Gomez et al., 2009). Since this systematic review was conducted in 2012, a more recent study has shown weight loss improves fibrosis. Hoofnagle et al. conducted a *post hoc* analysis of the Pioglitazone vs. Vitamin E vs. Placebo in Non-alcoholic steatohepatitis (PIVENS) study. Over a 2 year period, those individuals who lost at least 2 kg of weight regardless of vitamin E usage, had improvements in liver fibrosis (Hoofnagle et al., 2013). Vitamin E use alone was not associated with fibrosis improvements. Conversely, weight gain was associated with worsening liver fibrosis.

Weight loss has benefits beyond the liver. It has been shown to improve intrahepatic triglyceride content and insulin sensitivity in the liver and in muscle in those with normal oral glucose tolerance (Vitola et al., 2009) and improves glycaemic control in those with diabetes (Rock et al., 2014). Weight loss is associated with improvements in liver enzymes (Suzuki et al., 2005) although changes in and overall elevation of liver enzymes do not accurately reflect liver

histology in NAFLD / NASH. (Wieckowska, McCullough, & Feldstein, 2007). Weight loss improves and is considered to be the first line treatment for hypercholesterolaemia according to the National Cholesterol Education Program expert panel (Jehle, 2002). Finally, weight loss improves blood pressure and vascular function in healthy individuals and those with metabolic syndrome or cardiovascular disease (Ades & Savage, 2014).

Weight loss can be achieved with a variety of diet and exercise combinations. There are specific dietary factors that have been associated with NAFLD. Diets rich in saturated fats, cholesterol and low in polyunsaturated fat, fibre and antioxidants are associated with NASH and insulin resistance (Musso et al., 2003). Excessive carbohydrate intake is associated with hepatic inflammation (Solga et al., 2004), with fructose linked to increased liver inflammation, steatosis and fibrosis (Vos & Lavine, 2013). In general, an overall excess energy intake leads to obesity and NAFLD so dietary modification focuses on reduction in total caloric intake (hypocaloric diet). Evidence for hypocaloric diets date back over 40 years ago when Drenick et al. demonstrated that 1 year of calorie restriction resulted in decreased liver steatosis (Drenick, Simmons, & Murphy, 1970). In 2010, a meta-analysis concluded that caloric restriction is the most important treatment goal for NASH to improve histology (Musso, Gambino, Cassader, & Pagano, 2010). The general recommendations for a hypocaloric diet are to reduce consumption of saturated fats, simple carbohydrates and sweetened drinks (Musso, Gambino, Pacini, De Michieli, & Cassader, 2009; Zivkovic, German, & Sanyal, 2007). Despite these generalities, no specific hypocaloric diet has been recommended for NAFLD in the AASLD guidelines (Chalasani et al., 2012).

Individuals with NAFLD are typically less physically active than their healthy counterparts (Zelber-Sagi et al., 2008). Conversely, regular exercise is associated with a reduced risk for development of NAFLD (Miyake et al., 2014). Physical activity is associated with a risk reduction of 35% for diabetes and 49% for cardiovascular disease (Bassuk & Manson, 2005; Kruk, 2007), both conditions associated with the metabolic syndrome and NAFLD. While exercise is typically

a component of most lifestyle interventions for NAFLD, exercise without concurrent dietary modification has also showed benefit in NAFLD. Keating et al. conducted a systematic review of 12 exercise studies and noted that physical activity resulted in a reduction in liver steatosis independent of weight reduction (Keating, Hackett, George, & Johnson, 2012). The addition of exercise to a diet regimen also potentiates the dietary benefits through further reductions in hepatic steatosis, insulin resistance and inflammation independent of weight loss (Oh et al., 2014). Regular exercise should be an essential facet of any lifestyle intervention for NAFLD as its benefits extend beyond the liver to address the associated metabolic conditions.

Despite most lifestyle modifications being both accessible and low-cost, the compliance and long term sustainability of such changes are often poor (Dombrowski, Knittle, Avenell, Araújo-Soares, & Sniehotta, 2014; Svetkey et al., 2008). Lifestyle programs need to be flexible, easy to follow and focussed at the community level. Home, community and work-place programs need to be addressed to successfully reduce the burden of NAFLD and associated metabolic diseases (Centis et al., 2013).

In summation, lifestyle modification through diet with or without exercise can improve features of the metabolic syndrome and NAFLD. The primary goal is weight loss, which improves the histological features of NAFLD although evidence for fibrosis resolution is still lacking. Dietary changes focus on caloric reduction although the optimum diet is unknown. At this time, vitamin E remains the only recommended oral therapy, although questions about its efficacy in reducing fibrosis and long-term safety issues limit its use. Coffee consumption appears to be beneficial as a preventive measure for the development of both liver steatosis and fibrosis, but more research on this agent is required before formal recommendations can be made. Over the next 15 years, it is projected that in Australia, those with NASH-related cirrhosis will number more than other liver diseases. To avert this course the need for a simple and cost effective therapy could not be greater. In Chapter 4, I will propose one such method.

I have shown that there are 2 key areas that need addressing in the treatment of chronic liver diseases. Firstly, in end-stage disease or cirrhosis, when treatment of the underlying disease is unsuccessful or unavailable, a targeted anti-fibrotic liver restorative treatment is needed. Chapters 2 and 3 will explore a novel cell-based therapy, hAEC, in the pre-clinical phase. The effects of hAEC in both cell and cell-free (CM) treatments will be explored in relation to their anti-fibrotic effects on HSCs (Chapter 2), and liver restorative effects on liver progenitor cells (Chapter 3). Chapter 4 will address the second key area in chronic liver diseases, namely the prevention of disease progression. This chapter will summarise the results of a controlled randomised single-blinded pilot study of a lifestyle intervention and standard care diet and exercise advice on individuals with the most prevalent liver disease, NAFLD.

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CHAPTER 2

SOLUBLE FACTORS DERIVED FROM HUMAN AMNIOTIC EPITHELIAL CELLS SUPPRESS COLLAGEN PRODUCTION IN HUMAN HEPATIC STELLATE CELLS

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2.3 SOLUBLE FACTORS DERIVED FROM HUMAN AMNIOTIC EPITHELIAL CELLS SUPPRESS COLLAGEN PRODUCTION IN HUMAN HEPATIC STELLATE CELLS 59

REFERENCE: Hodge, A., Lourensz, D., Vaghjiani, V., Nguyen, H., Tchongue, J., Wang, B., et al. (2014). Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells. Cytotherapy, 16(8), 1132–1144. doi:10.1016/j.jcyt.2014.01.005

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Minor Amendment:

What other cell populations are present in amnion membranes, other than hAECs? Could CM from these other cell populations have an effect on HSC?

Methods, Human AEC isolation: What is meant by "….. stage-specific . . . cells . . were recoverd. . "

There are amniotic mesenchemal stromal cells (hAMSCs) present in the amniotic membrane along with the hAECs. Both of these cells types express similar stem-cell markers and have been used in lung and liver mouse models of fibrosis (Caruso, Evangelista, & Parolini, 2012). *However in an amnion membrane hAECs are more plentiful and have not been found to be teratpagenic. The Stage-specific embryonic antigen (SSEA) or CD15 is a marker for pluripotent stem cells* . (Miki, 2011).

2.1 INTRODUCTION

Chronic liver diseases affect millions of people worldwide. Although there are many conditions that induce liver injury, over time they can all lead to fibrosis of the liver. Over time, fibrosis can progress to severe fibrosis (cirrhosis) and its complications. Many of the underlying causes can be treated, suppressed or managed in a way to reduce or eliminate progressive liver injury. In some cases this is not possible and the only curative therapy is liver transplant. Issues with donor shortages, high costs and lifelong immunosuppressive therapies mean alternative treatments for end-stage liver disease or cirrhosis are being sought. Cell therapies have been born out of this need.

To date, cell therapies have consisted of adult hepatocyte or undifferentiated stem cell (SC) transplantation. The latter includes embryonic and fetal SC, adult liver SC, and bone marrow or adipose derived mesenchymal SC. The amniotic epithelial cell is a placental derived cell that exhibits stem cell-like characteristics. It is being developed for use in a wide range of clinical applications from eye conditions to liver disease (Miki, 2011) and possesses many advantages over other cell types. Amniotic epithelial cells have no tumourigenicity, are easily isolated from placenta and yields are usually much higher than other cell types, limiting the need for expansion in cell culture (Miki, 2011).

The use of human amniotic epithelial cell (hAEC) in treating liver disease has not progressed to human trials, however the results from *in vivo* an *in vitro* studies are encouraging (Manuelpillai et al., 2012; 2010; Sakuragawa et al., 2000; Sant'Anna, Cargnoni, Ressel, Vanosi, & Parolini, 2011; Takashima, Ise, Zhao, Akaike, & Nikaido, 2004). We understand that hAEC have anti-fibrotic effects on fibrotic livers but through what mechanisms this occurs has yet to be unravelled. The driving force behind liver fibrogenesis is the hepatic stellate cell (HSC). For an anti-fibrotic therapy to be successful at reducing liver fibrosis it should be effective at modifying HSC behaviour. In this chapter I investigate the anti-fibrotic effects of soluble factors in conditioned media (CM) produced by hAECs on human HSC and investigate potential mechanisms through which this occurs.

Monash University

Declaration for Thesis Chapter 2

Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells *Cytotherapy*, 2014;16:1132-1144

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Experimental design, optimisation of protocols, experimental analysis and drafting of manuscript.	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Dinushka Lourensz	assisted with experiments	N/A
Vijesh Vaghjiani	assisted with amnion isolation	N/A
Huyen Nguyen	assisted with ELISAs	N/A
Jorge Tchongue	assisted with ELISAs	N/A
Bo Wang	assisted with ELISA	N/A
Padma Murthi	provided amnion	N/A
William Sievert	Drafting of manuscript and intellectual input	N/A
Ursula Maneulpillai	Drafting of manuscript and intellectual input	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 10 October 2014
Main Supervisor's Signature	[redacted]	**Date** 10 October 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Cytotherapy, 2014; 16: 1132–1144

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Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells

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Abstract

Background. Intravenous infusion of human amniotic epithelial cells (hAECs) has been shown to ameliorate hepatic fibrosis in murine models. Hepatic stellate cells (HSCs) are the principal collagen-secreting cells in the liver. The aim of this study was to investigate whether factors secreted by hAECs and present in hAEC-conditioned medium (CM) have anti-fibrotic effects on activated human HSCs. *Methods.* Human AECs were isolated from the placenta and cultured. Human hepatic stellate cells were exposed to hAEC CM to determine potential anti-fibrotic effects. *Results.* HSCs treated for 48 h with hAEC CM displayed a significant reduction in the expression of the myofibroblast markers α-smooth muscle actin and platelet-derived growth factor. Expression of the pro-fibrotic cytokine transforming growth factor-β1 (TGF-β1) and intracellular collagen were reduced by 45% and 46%, respectively. Human AEC CM induced HSC apoptosis in 11.8% of treated cells and reduced HSC proliferation. Soluble human leukocyte antigen–G1, a hAEC-derived factor, significantly decreased TGF-β1 and collagen production in activated HSCs, although the effect on collagen production was less than that of hAEC CM. The reduction in collagen and TGF-B1 could not be attributed to PGE2, relaxin, IL-10, TGF-B3, FasL or TRAIL. *Conclusions.* Human AEC CM treatment suppresses markers of activation, proliferation and fibrosis in human HSCs as well as inducing apoptosis and reducing proliferation. Human AEC CM treatment may be effective in ameliorating liver fibrosis and warrants further study.

Key Words: *amnion epithelial cells, hepatic stellate cells, liver cirrhosis, placental stem cells*

Introduction

Liver fibrosis represents a wound-healing response to chronic inflammation leading to the accumulation of collagen and other extracellular matrix (ECM) proteins that can result in distortion of liver architecture and vasculature, liver dysfunction and hepatocyte dysplasia. Inflammatory liver injury can be precipitated by viral, metabolic, toxin, autoimmune and other causes. The hepatic stellate cell (HSC) is the resident liver cell at the center of collagen deposition because its response to tissue injury initiates and modulates liver fibrosis. HSC activation represents a series of cellular processes resulting in transformation to a myofibroblast-like, pro-fibrotic phenotype. The driving forces leading to this transformation are hepatocyte apoptosis (1) and resident and recruited immune cells, which are sources of cytokines such as platelet-derived growth factor-β (PDGFβ), endothelin-1, connective tissue growth factor, epidermal growth factor and transforming growth factor-β (TGF-β) (2). This pro-fibrotic response results in ECM deposition and liver fibrosis (3). If the stimulus for liver injury is removed, activated HSCs may revert to a quiescent phenotype or decrease in number through spontaneous or directed apoptosis (4). The remaining HSCs express a number of enzymes and enzyme inhibitors whose net result is fibrolysis or ECM degradation leading to regression of liver fibrosis. If liver injury is perpetuated, the ongoing fibrogenesis can lead to cirrhosis and its associated complications.

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Recently, stem cell–based therapies have been used for their liver-regenerative and anti-fibrotic properties and have been shown to be efficacious in experimental (5–7) and human liver disease (8–10). Cell sources include embryonic stem cells, induced pluripotent stem cells, and adult hematopoietic and mesenchymal stromal cells. However, there are a number of issues including availability, tumor induction (11,12) and potential ethical constraints that diminish the clinical application of some types of stem cells. One cell source that overcomes many of these limitations is the placenta, which contains several stem or stem-like cells, including amniotic epithelial cells. These cells retain stem cell characteristics and express surface markers present on embryonic and germ-line cells (13,14). Placenta-derived cells also have properties that address the safety and availability concerns with other stem cells. Human amniotic epithelial cells (hAECs) do not form teratomas *in vivo* (13,14), as has been shown with embryonic stem cells (15). Human AECs express low levels of human leukocyte antigen class IA molecules (HLA-IA) and lack class II antigens (14,16), which partially accounts for the low immunogenicity and lack of acute rejection observed when transplanted into humans (17). Human AECs are also highly abundant, and approximately 150 million cells can be isolated from one placenta (18,19). Human placentas are routinely discarded after birth, which largely obviates the ethical concerns regarding their use in human disease. On the basis of their stem cell characteristics, accessibility and safety, hAECs are a promising source for cell-based therapy.

We have shown that hAECs have an anti-fibrotic effect after xenotransplantation in murine models of liver fibrosis. In a 4-week acute model and a 12-week chronic model of carbon tetrachloride (CCl_4)-induced liver fibrosis, mice given a single intravenous dose of hAECs showed significant reduction in liver fibrosis (20,21). This anti-fibrotic effect is mediated partially through a reduction in the number of activated HSCs and through reduction in pro-fibrotic cytokine expression. The reduction in fibrosis may be through an indirect effect after hAEC modulation of pro-fibrotic macrophages or through a direct effect of hAECs on HSCs (21). This notion is suggested by the observation that infused hAECs were found in the liver in close proximity to HSCs, suggesting that hAEC soluble factors may alter HSC collagen expression. In this study, we have sought to determine whether soluble factors from hAECs inactivate HSCs and inhibit collagen expression. We also explored potential mechanisms that might explain such changes.

Methods

Human AEC isolation

Amniotic membranes were collected from healthy women with a normal singleton pregnancy undergoing cesarean section at term (37–40 weeks' gestation). Informed written consent was obtained before surgery. The study was approved by the Royal Women's Hospital Human Research Ethics Committee.

Human AECs were isolated from amnion membranes, and purity was assessed as described previously (14,19). Briefly, amniotic membranes were cut up and digested twice for 40 min at 37°C in 0.05% Trypsin: ethylenediaminetetra-acetic acid (Gibco, Grand Island, NY, USA). After washing, red blood cells were lysed by incubation in a hypotonic solution. Viable stage specific embryonic antigen-positive cells were recovered by Percoll density gradient centrifugation. Purity was assessed by means of flow cytometry for cytokeratin 7 and 8/18 (Dako, Glostrup, Denmark), and batches that were ~98% positive for the cytokeratins and had typical cobblestone morphology in culture were used. Cells were cryopreserved in 90% fetal calf serum (FCS) with 10% dimethyl sulfoxide.

Human AEC–conditioned medium

Cryopreserved hAECs from four randomly selected donor placentas were revived, pooled together and grown in Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12) supplemented with 10% FCS, an antibiotic and an antimycotic, L-glutamine and 10 ng/mL epidermal growth factor (Life Technologies, Grand Island, NY, USA) on collagen IV–coated flasks (1 mg/mL; Roche, Mannheim, Germany). Flasks were kept at 37°C in a humidified 5% CO_2 atmosphere. For collection of conditioned medium (CM), confluent cells were serum-starved in DMEM/F12 and the CM was collected after 72 h. The CM was spun to remove cell debris and frozen at −80°C until needed. CM from three to five pools was used, with each pool taken from a culture of hAECs from four donor placentas. Before CM from pooled hAEC donors was used for planned experiments, individual and various combinations of pooled hAECs were assessed. Four randomly selected donors were chosen to assess for individual donor variability in regard to TGF-β1 production and Pro-line incorporation (collagen production); after this, 12 randomly chosen donors were cultured in three separate pools (four hAEC donors per pool) to obtain CM. Comparisons were made between individual donors and control and between individual pools and control. No significant difference was noted between

control and CM from individual donors or pooled donors regarding selected experimental outcomes (supplementary Figure 1); therefore, subsequent experiments were performed with the use of CM from hAEC cultures of four pooled donors.

HSC culture

LX-2 cells, which are immortalized human HSC, were maintained in DMEM/F12 with 5% FCS, unless stated otherwise, and 100 U/mL of penicillin and streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For this study, LX-2 cells will be referred to as HSCs. For each experiment, HSCs were plated in 6-, 12- or 24-well tissue culture plates at a density of 2×10^5, 1×10^5 or 5.3×10^4 cells per well, respectively, and grown to confluence unless otherwise indicated. Confluent HSCs in DMEM/F12 with FCS and 100 U/mL of penicillin and streptomycin served as controls. In the treatment groups, HSCs were cultured in 50% hAEC CM in DMEM/F12 supplemented with FCS. TGF-β (10 ng/mL, R&D Systems, Minneapolis, MN, USA) was used as an additional positive control in assays as indicated. After 48 h of culture, supernatants were removed and frozen at −80°C and the cells were processed. Experiments were set up in triplicate or quadruplicate and repeated more than once to ensure the independence and validity of results.

Addition of soluble HLA-G1 to HSCs

Soluble HLA-G1 (sHLA-G1) concentration in the 50% hAEC CM was determined from previous work to be approximately 25 ng/mL (16,22). Purified recombinant HLA-G1 protein (Origene, Rockville, MD, USA) was added to confluent HSCs grown in DMEM/F12 with FCS in concentrations of 25, 50 and 100 ng/mL, cultured for a further 48 h before the supernatant was removed and collagen synthesis in the cells was determined.

Collagen synthesis

[^{3}H] Proline (1 μCi; Perkin Elmer, Boston, MA, USA) was added to each well of confluent HSCs with or without CM. HSCs were washed with cold phosphate-buffered saline, followed by 30 min of incubation with 10% trichloroacetic acid on ice. Cells were then washed with cold trichloroacetic acid and incubated with 1 mol/L sodium hydroxide for 45 min at 37°C. Equal volumes of the cell lysate and 1 mol/L hydrochloric acid were added to 10 parts of Insta-gel plus scintillation fluid (Perkin Elmer, Waltham, MA, USA) in polyethylene scintillation vials (Sarstedt, Etten-Leur, The Netherlands). Each vial was vortexed and was then analyzed on a liquid scintillation counter for 60 s (1409 DSA Wallac liquid scintillation counter, Pharmacia, Turku, Finland). Results are expressed in counts per minute (cpm).

Determination of collagen content in supernatant

Confluent HSCs were treated with CM with FCS for 48 h, after which the supernatants were removed. The collagen content in the culture supernatant was measured with the use of the Sircol Sirius red colorimetric assay (Biocolor, Newtown Abbey, Northern Ireland) according to the manufacturer's instructions. Briefly, Sircol dye reagent was added to sample supernatants and mixed for 30 min to allow precipitation of collagen-dye complexes. Samples were spun at 12,000 rpm for 10 min to pellet collagen-dye, and tubes were inverted to remove excess dye. A sodium hydroxide alkali reagent (0.5 mol/L) was added to dissolve the bound dye into solution. Released dye was then measured on a spectrophotometer at 555 nm (Magellan, Tecan, Austria).

Enzyme-linked immunosorbent assay

TGF-β1 protein in cell culture supernatants was analyzed by means of enzyme-linked immunosorbent assay, according to the manufacturer's instructions (R&D Systems). Samples were activated with 1 mol/L hydrochloric acid before analysis, and plates were read with the use of a microplate reader at 450 nm (Magellan). TGF-β1 concentration was calculated from the standard curve generated in GraphPad Prism 5.0d for Mac OS X (GraphPad Software, La Jolla, CA, USA).

RNA isolation and quantitative real-time polymerase chain reaction analysis

Total RNA from HSCs was isolated with the use of the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and RNA concentration was measured with the use of the Nanodrop ND-100 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA (1 μg) was used to generate complementary DNA (cDNA) with the use of the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions. To examine changes in gene expression, real-time polymerase chain reaction analysis was performed with the use of Power Sybr Green (Life Technologies) on the Rotor Gene 3000 light cycler (Qiagen, Sydney, Australia). Complementary DNA (4 μL of a 1:20 dilution) was used in each 20-μL reaction. The following standard cycle was used: 95°C for 10 min,

Table I. Primer sequences used in quantitative polymerase chain reaction.

Primer ID	Sequence 5′−3′	Source
ACE1 F	CCA CGT CCC GGA AAT ATG AAG	PrimerBank ID: 307691163c3
ACE1 R	AGT CCC CTG CAT CTA CAT AGC	
ACE2 F	CAA GAG CAA ACG GTT GAA CAC	PrimerBank ID: 194306626c3
ACE2 R	CCA GAG CCT CTC ATT GTA GTC T	
AGT F	CAA CAC CTA CGT CCA CTT CCA A	GenBank Accession No.
AGT R	TGT TGT CCA CCC AGA ACT CCT	NM_000029
α-SMA F	TTC AAT GTC CCA GCC ATG TA	Primer 3
α-SMA R	GAA GGA ATA GCC ACG CTC AG	
BMP-7 F	ATC GAG AGT TCC GGT TTG ATC T	PrimerBank ID: 187608319b3
BMP-7 R	GTC TCA TTG TCG AAG CGT TCC	
MMP-9 F	TGT ACC GCT ATG GTT ACA CTC G	PrimerBank ID: 74272286b1
MMP-9 R	GGC AGG GAC AGT TGC TTC T	
PDGFβ F	CTC GAT CCG CTC CTT TGA TGA	PrimerBank ID: 208879462c1
PDGFβ R	CGT TGG TGC GGT CTA TGA G	
TIMP-2 F	AAG CGG TCA GTG AGA AGG AAG	PrimerBank ID: 73858577b1
TIMP-2 R	AGG GTT GCC ATA AAT GTC GTT	
18S F	GTA ACC CGT TGA ACC CCA TTC	Dr Eric Morand
18S R	GCC TCA CTA AAC CAT CCA ATC G	

followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The target gene of interest was analyzed relative to 18S. The sequences for the custom primers (Life Technologies) are listed in Table I. The results are expressed as a relative fold change compared with HSC controls through the use of the comparative cycle threshold method.

HSC proliferation assay

HSCs were seeded onto 96-well plates at a density of 5×10^3 cells per well and cultured until the cells reached 75% confluence. Human AEC CM or DMEM/F12, both with 1% FCS, were added to the experimental samples the following day, and cultures were maintained for a further 48 h. Proliferation of activated HSCs was assessed by means of the colorimetric 5-bromo-2′-deoxy-uridine (BrdU) assay (Roche). BrdU labeling medium (10 μmol/L) was incubated with the cells for 3.5 h and was then assayed according to the manufacturer's instructions.

Matrix metalloproteinase assay

Confluent HSCs were treated with CM or DMEM/F12, both with 1% FCS; the latter served as a control. After 48 h, supernatants were removed and analyzed for gelatinolytic activity by sodium dodecyl sulphate−polyacrylamide gel electrophoresis under non-reducing conditions as previously described (23). Briefly, samples were separated on 10% polyacrylamide gels containing 1 mg/mL gelatin at 4°C. After a brief wash with water, the gels were incubated in 2.5% Triton X-100 for 45 min at room temperature to remove sodium dodecyl sulphate, and gelatinolytic activity was developed by incubation in development buffer (50 mmol/L Tris, pH 7.4, 5 mmol/L $CaCl_2$, 200 mmol/L NaCl, 0.02% NaN_3, 1% Triton X-100) at 37°C for 16 h. Gelatinolytic bands were visualized by means of staining gels with 0.15% Coomassie blue R-250 in water/methanol/acetic acid solution (4.5:4.5:1 ratio). Serum-free cell culture medium from tissue plasminogen activator-stimulated human skin fibroblasts (Sigma-Aldrich, Saint Louis, MO, USA) was used as a qualitative positive control and reference. Semi-quantitative densitometric analysis was performed with the use of ImageJ software (National Institutes of Health, Bethesda, MD, USA).

HSC apoptosis assay

HSCs were seeded onto 12-well plates at a density of 1×10^5 cells per well and were cultured until confluent. Controls were maintained in DMEM/F12 with FCS, and experimental samples were treated with CM with FCS for 48 h. HSCs treated with 7.5 μmol/L of gliotoxin (Tocris Bioscience, Bristol, United Kingdom) for 24 h were used as a positive control. Cells were lifted with the use of TrypLE (Life Technologies, Naerum, Denmark) for 2.5 min at 37°C and stained with the use of the Annexin V: fluoresceine isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Propidium iodide (PI) was used to stain dead cells. Apoptotic and dead HSCs were identified by use of the BD fluorescence-activated cell sorting (FACS) CantoII Analyzer (BD Biosciences, San Jose, CA, USA) and analyzed with the use of FlowJo 8.7 software (Tree Star, Ashland, OR, USA).

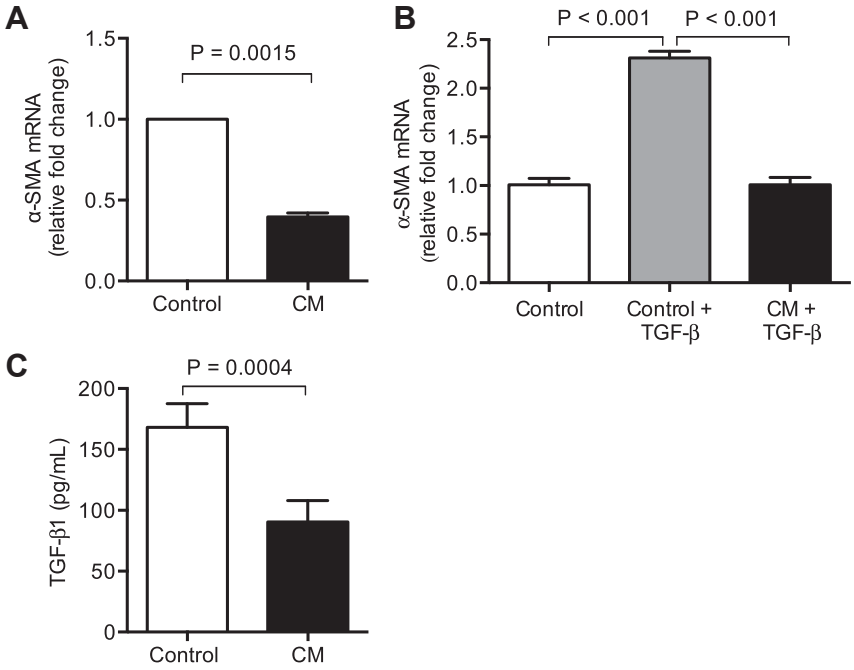

Figure 1. Suppression of HSC activation. Confluent HSCs were cultured for 48 h in hAEC CM or control medium with or without 10 ng/mL of TGF-β. (A) Results are expressed as change in α-SMA gene expression relative to HSC controls in DMEM/F12 with FCS. Human AEC CM significantly decreased α-SMA expression 2.5-fold compared with control ($P = 0.0015$). (B) The addition of TGF-β to controls resulted in a 2.3-fold increase in α-SMA gene expression ($P < 0.001$). CM attenuated this response, which resulted in α-SMA gene expression levels similar to that in the control ($P < 0.001$). Results represent combined data from two and three representative experiments, respectively. CM ($n = 3$) were used in triplicate. (C) HSCs treated with CM for 48 h showed a significant decrease in the release of TGF-β1 into the supernatant ($P = 0.0004$). Results represent combined data from eight experiments, with CM pools ($n = 4$) in triplicate. Data are expressed as mean ± standard error of the mean; data in A and C were analyzed by paired *t*-test and in B by analysis of variance followed by Newman-Keuls *post hoc* test.

Identification of potential soluble factors in CM

To identify specific soluble factors in hAEC CM, we chose a number of candidate molecules to investigate, on the basis of the published literature and our previous work (24–28). Prostaglandin E2 (PGE_2) was measured by means of enzyme immunoassay; relaxin, TGF-β3, bone morphogenetic protein-7 (BMP-7), interleukin-10 (IL-10), FAS ligand (FasL) and tumor necrosis factor–related apoptosis inducing ligand (TRAIL) were determined by means of enzyme-linked immunosorbent assay. BMP-7, angiotensin-converting enzyme 1 (ACE1), ACE2 and angiotensinogen (AGT) gene expression in hAECs was determined by real-time polymerase chain reaction. The sequences for the custom primers (Life Technologies) are listed in Table I.

Statistical analysis

Experiments were performed between one and eight times, and each experiment had at least three replicates. Statistical analysis was performed with the use of GraphPad Prism 5.0d for Mac OS X (GraphPad Software). Statistical significance was determined by one-way analysis of variance with Newman-Keuls *post hoc* test for multiple comparisons. The paired or unpaired *t*-test was performed for comparisons across experiments as appropriate. The data are shown as mean ± standard error of the mean.

Results

Human AEC CM reduces HSC activation

We examined whether hAEC CM altered human HSC expression of the pro-fibrogenic cytokine TGF-β1 and transformation to a myofibroblast phenotype. Human AEC CM treatment reduced α-smooth muscle actin (α-SMA) messenger RNA (mRNA) expression ($P = 0.0015$; Figure 1A) and attenuated the pro-fibrotic stimulus of exogenous TGF-β on α-SMA mRNA expression to levels similar to that in control ($P < 0.001$; Figure 1B). Furthermore, hAEC CM reduced TGF-β1 protein production by 46% in HSC cultures treated with CM (control, 168.3 ± 19.3 pg/mL vs CM-treated, 90.33 ± 17.7 pg/mL, $P = 0.0004$; Figure 1C).

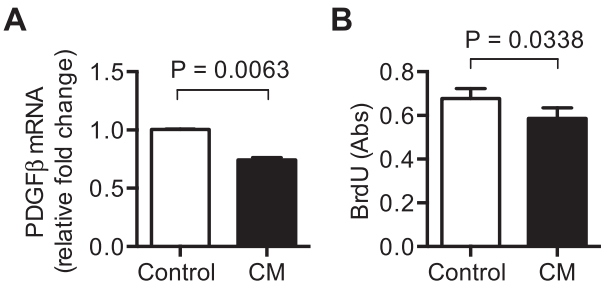

Figure 2. Effect of hAEC CM on HSC proliferation. HSCs were cultured for 48 h in CM or control medium. (A) Results are expressed as change in PDGFβ gene expression relative to the control. HSCs treated with CM for 48 h showed a 1.35-fold decrease in PDGFβ mRNA expression ($P = 0.0063$). Results represent combined data from three experiments, with CM pools ($n = 3$) in triplicate wells. (B) BrdU incorporation was used to determine HSC proliferation. After 48 h, BrdU incorporation was reduced in hAEC CM–treated HSCs by 1.16-fold ($P = 0.0338$). Results represent combined data from three experiments, with CM pools ($n = 5$) in triplicate. Results are expressed as mean ± standard error of the mean; data were analyzed by means of the paired *t*-test in A and B.

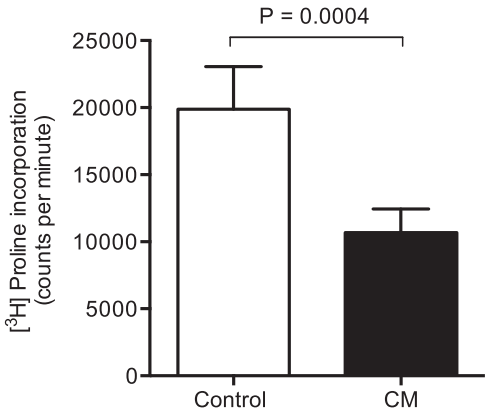

Figure 3. Reduction of collagen production in HSCs treated with hAEC CM. Confluent HSCs were cultured in hAEC CM or control medium containing 1 μCi [3H] Proline. After 48 h of culture, the supernatant was removed. Cells were lysed and [3H] Proline incorporation was determined. There was a 46% reduction in intracellular collagen expression in hAEC CM–treated HSCs (paired *t*-test, $P = 0.0004$). Results are expressed as mean ± standard error of the mean and represent combined data from eight experiments, with CM pools ($n = 4$) in three to six replicate wells.

Human AEC CM reduces HSC proliferation

HSC proliferation is a hallmark of myofibroblast transformation and is important in fibrogenesis. PDGFβ is an important mitogen driving this proliferation. PDGFβ mRNA expression was reduced 1.35-fold after exposure to hAEC CM compared with untreated controls ($P = 0.0063$; Figure 2A). We assessed the effect of hAEC CM on HSC proliferation through the use of BrdU incorporation. Sub-confluent HSC cultures treated with hAEC CM for 48 h displayed a 1.16-fold reduction in BrdU incorporation ($P = 0.0338$; Figure 2B).

Human AEC CM attenuates HSC collagen synthesis

To investigate the effect of hAEC CM on collagen production in activated, pro-fibrotic HSC, we determined intracellular collagen content by [^{3}H] Proline incorporation. A 46% decrease in collagen production was demonstrated in HSCs treated with hAEC CM compared with untreated controls (control, 19,891 ± 3173 cpm vs CM-treated, 10,683 ± 1775 cpm; $P = 0.0004$; Figure 3).

Human AEC CM stimulates collagen degradation through matrix metalloproteinase-9 expression

Next, we explored the notion of whether hAEC CM induces collagen degradation. Extracellular (supernatant) collagen was measured in control and CM before and 48 h after addition to activated HSC. The basal collagen levels in the culture media (control or hAEC CM) were subtracted from the collagen levels after a 48 h exposure to HSC. Control HSCs demonstrated an increase in supernatant collagen of 15.01 ± 2.17 μg after 48 h. HSCs treated with hAEC CM showed a reduction in supernatant collagen of 5.42 ± 2.05 μg after 48 h, which indicates collagen degradation. There was a mean 20.43 μg difference in supernatant collagen concentration between the control and hAEC CM–treated HSC ($P = 0.0002$; Figure 4A). Given the finding of active collagen degradation, we sought to determine whether hAEC CM stimulated matrix metalloproteinase (MMP) induction and/or suppression of tissue inhibitors of MMP (TIMP). Human AEC CM treatment resulted in a 2.74-fold increase in MMP-9 gene expression ($P = 0.0189$; Figure 4B). Gene expression of TIMP-2 was reduced by 1.35-fold, but this was not statistically significant ($P = 0.1187$; Figure 4C). Gelatin zymography of hAEC CM revealed that MMP-9 was the dominant active MMP that accounted for the collagen degradation seen in our experiments. Semi-quantitative densitometry was used to calculate MMP-9 activity in HSC cultures after basal MMP-9 activity in hAEC CM was subtracted. HSC controls had arbitrary densitometry units of 525.8 ± 43 pixels/cm^2 compared with 1257 ± 171 pixels/cm^2 in HSCs treated with hAEC CM ($P = 0.0533$; Figure 4D).

Human AEC CM enhances apoptosis of activated HSCs

Given the changes in collagen production observed after hAEC CM treatment, we explored whether

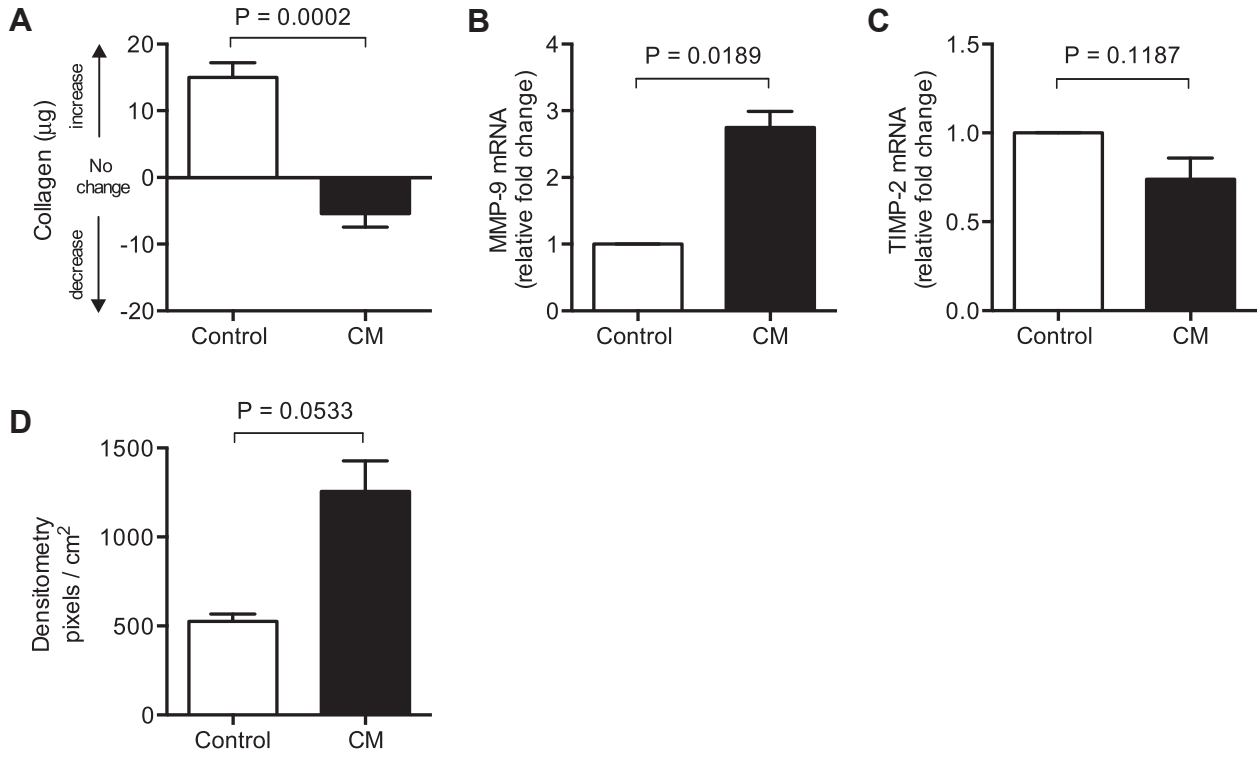

Figure 4. Human AEC CM stimulates collagen degradation through MMP-9 expression. Confluent HSCs were cultured for 48 h in hAEC CM or control medium. (A) Extracellular (supernatant) collagen content was determined by means of the Sircol colorimetric assay in control and hAEC CM at baseline and 48 h after treatment of HSCs. Extracellular collagen increased in control HSCs and decreased in hAEC CM−treated HSCs ($P = 0.0002$). Results represent combined data from two experiments, with CM pools ($n = 3$) in three or four replicates. (B) There was a significant increase in MMP-9 gene expression that increased 2.74 fold ($P = 0.0189$) in CM-treated HSCs relative to control. (C) TIMP-2 mRNA expression was reduced but did not reach statistical significance. Results for (B) and (C) represent combined data from three experiments, with CM pools ($n = 3$) in triplicate. (D) Gelatinolytic activity was determined by gel zymography. Densitometry area for gelatinolytic activity was determined after deduction of baseline activity. HSCs treated with CM had significant MMP-9 activity ($P = 0.0533$). Results represent one CM pool run in duplicate. Results are expressed as mean ± standard error of the mean in A−D; data were analyzed by paired *t*-test in A−C and unpaired *t*-test in D.

treated HSC underwent apoptosis. Confluent HSCs treated with hAEC CM were incubated with FITC-labeled Annexin V to detect cells undergoing early apoptosis and PI to stain late apoptotic or dead cells. HSCs treated with gliotoxin were used as a positive control (cell death). Significantly more HSCs underwent apoptosis after treatment with hAEC CM compared with untreated controls (11.8% ± 1.0% compared with 5.8 % ± 0.1%, respectively; $P = 0.0009$; Figure 5A,C). There was no difference in the number of late apoptotic or dead HSCs between the hAEC CM−treated and control groups at 48 h (Figure 5B).

Soluble HLA-G1 reduces TGF-β1 expression and collagen production

Human AECs produce sHLA-G1, a HLA molecule with immunosuppressive activity (22,29). We found that HSCs expressed CD160 mRNA (supplementry Figure 2), a receptor for sHLA-G1 (30). To determine whether sHLA-G1 was the factor responsible for the observed changes in HSC activation, confluent HSCs were treated with concentrations of sHLA-G1 ranging from 25−100 ng/mL. HSCs treated with DMEM/F12 and CM supplemented with 5% FCS served as controls. TGF-β1 protein in the culture supernatant was significantly reduced at all concentrations of sHLA-G1 tested to levels similar to that of hAEC CM (Figure 6A). Intracellular collagen content was significantly reduced by the addition of 100 ng/mL of sHLA-G1 ($P < 0.05$; Figure 6B) but did not reach a reduction similar to that seen with the hAEC CM.

Identification of other candidate factors

We examined other potential factors that might account for the effects of CM on HSCs that we observed. We determined that PGE_2 concentrations in various hAEC CM pools ranged from 43.7−105 pg/mL. HSCs produced smaller amounts of PGE_2 in culture (mean = 11.9 pg/mL). However, there was no effect of exogenous PGE_2 (50−100 pg/mL) on

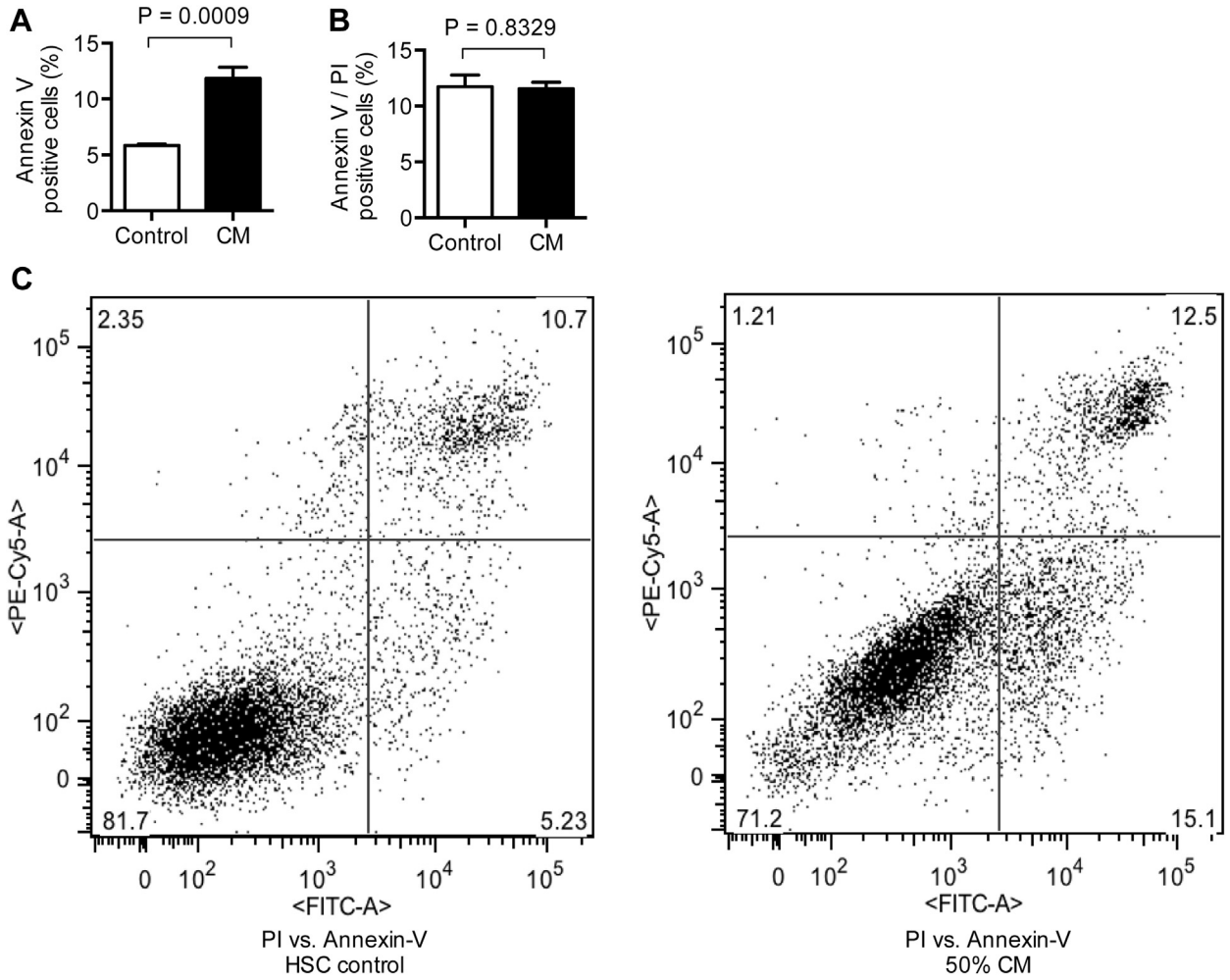

Figure 5. Effect of hAEC CM on apoptosis. Confluent HSCs were exposed to hAEC CM or control medium for 48 h and were then stained with FITC-Annexin V to detect cells undergoing apoptosis and PI to label dead cells. (A) There was a significant increase in apoptosis in HSCs treated with hAEC CM compared with that in control HSC ($P = 0.0009$). (B) There was no difference in the number of dead/late apoptotic cells between the groups. Results in A and B are expressed as mean ± standard error of the mean and represent combined data from two experiments, with CM pools ($n = 2$) run in triplicate or quadruplicate. Data were analyzed by means of paired *t*-test. (C) Representative dot plots for fluorescence-activated cell sorting are presented.

HSC collagen or TGF-β1 production (supplementary Figure 3). We also found TGF-β3 in hAEC CM from cultures of pooled and individual donors at an average concentration of 150 pg/mL. However, when HSCs were exposed to media to which TGF-β3 was added (150 pg/mL or 300 pg/mL), collagen production increased (supplementary Figure 4). We next found that hAECs expressed BMP-7 mRNA; however, protein was not detected in hAEC CM. IL-10 was not detected in hAEC CM, consistent with previous reports (16,22), or from HSCs exposed to hAEC CM. We did not find FasL present in hAEC CM. TRAIL was detected but below the quantifiable limit of the assay. Relaxin concentrations were measured and found to be absent in hAEC CM. We found that hAECs expressed ACE1, ACE2 and AGT mRNA, which are associated with angiotensin (Ang)-1−7 expression, a component of the renin-angiotensin (RAS) system with anti-fibrotic activity.

Discussion

We have previously shown in a murine model of CCl_4-induced liver fibrosis that hAEC therapy resulted in attenuation of fibrosis, decreased liver TGF-β1 levels, decreased macrophage and T-cell numbers and decreased α-SMA expression as a marker of HSC transformation to a myofibroblast phenotype (21). Furthermore, some hAECs were found in close proximity to HSCs in the liver. These results suggest that hAECs may have an effect on HSC activation through varying mechanisms: cell-to-cell contact, indirectly by influencing immune cells or through soluble factors. We explored the

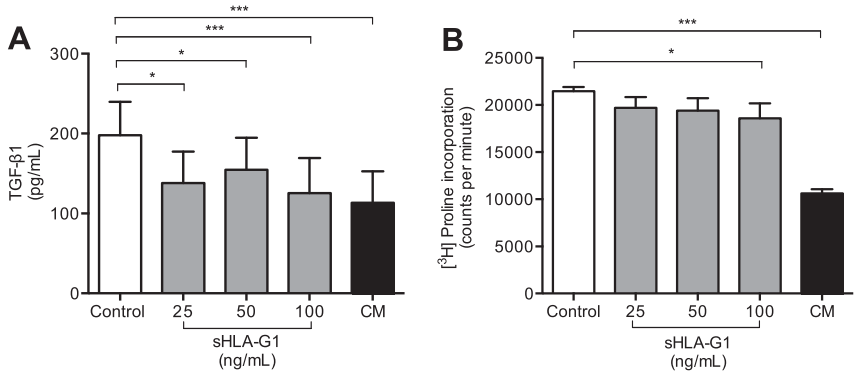

Figure 6. Soluble HLA-G1 affects TGF-β and collagen production in HSC. Soluble HLA-G1 was added to confluent HSCs grown in control medium to give final concentrations of 25, 50 and 100 ng/mL. HSC minus sHLA-G1 and HSC treated with hAEC CM served as controls. After 48 h, sHLA-G1 resulted in a significant decrease in (A) TGF-β1 protein for all concentrations of sHLA-G and in (B) collagen production at 100 ng/mL of sHLA-G1. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, respectively, by analysis of variance. Results are expressed as mean ± standard error of the mean and represent combined data from three and two experiments, respectively; each with CM pools ($n = 2$) run in quadruplicate.

latter notion and found that hAEC CM significantly reduced HSC activation and collagen expression, promoted HSC apoptosis and induced HSC-derived MMP-9 that could degrade collagen.

We derived CM from four randomly selected hAEC donors pooled together to form a single culture. Although there was variation between donors, as was expected, we determined that hAEC CM, whether from individuals or pooled, had the same degree of suppression on activated HSCs (supplementary Figure 1). The ability to pool donors eliminates any minor donor-to-donor variability and the need to screen each donor amnion to determine the most efficacious. If hAEC CM therapy progresses to human trials, pooling donors will simplify the transplantation process and rectify any issues with varying hAEC yield between donor amnions.

HSCs grown on culture ware express α-SMA and transform to a myofibroblast phenotype. These changes mimic those seen *in vivo* when HSCs undergo activation during episodes of liver inflammation/injury (31). The exposure of HSCs to hAEC CM resulted in a clear and significant reduction in pro-fibrotic markers of activated HSCs. We demonstrated reduced mRNA expression of α-SMA and PDGFβ and TGF-β1 protein, which are key markers of pro-fibrotic HSCs (32,33). PDGF is also a potent mediator of HSC proliferation (34); CM treatment resulted in significantly decreased HSC proliferation, although the magnitude of change was modest, suggesting that decreased HSC proliferation is not a major mechanism of the anti-fibrotic effect of hAECs. In addition to the suppression of TGF-β1 production, hAEC CM attenuated the effect of TGF-β1 on activated HSCs. When exogenous TGF-β1 was added to HSC cultures, hAEC CM decreased TGF-β1–induced α-SMA mRNA expression to levels similar to that in control cultures without added TGF-β1. We examined whether this response might occur by suppression of the TGF-β receptor-1, but we did not observe suppression of this receptor in hAEC CM–treated HSC cultures (supplementary Figure 5).

HSCs are the main matrix-producing cells in liver fibrogenesis (32). The success of an anti-fibrotic therapy is characterized by suppression of fibrogenesis and enhancement of collagen degradation. We found that hAEC CM dramatically reduced both HSC intracellular collagen synthesis and enhanced the degradation of extracellular collagen. We investigated whether changes in MMP-9 and TIMP-2 expression were responsible for this observation. MMP-2 and MMP-9 are collagen-degrading proteinases upregulated in hepatic fibrogenesis (35) and expressed in both HSCs and Kupffer cells (resident liver macrophages) (35–37). Human AECs also produce MMP-9 (38). We found that hAEC CM induced a non-significant increase in expression of HSC-derived MMP-2 mRNA (supplementary Figure 6) but significantly increased MMP-9 mRNA expression. We observed collagen degradation above the baseline activity of MMP-9 found in hAEC CM, which suggests additional MMP-9 production by HSCs (Figure 4A), although this difference did not reach statistical significance. The effect of increased activity of MMP-9 may be augmented by a decrease in TIMP-2. HSCs produce TIMP-2 (and TIMP-1), which inhibits the activity of MMPs, resulting in enhanced fibrosis degradation (35). TIMP expression is increased in fibrotic livers (36) and decreased in fibrosis resolution (39). We did not observe a statistically significant decrease in TIMP-2 mRNA expression; however, this may not reflect the activity of the protein in culture. In summary, we have shown

that collagen degradation is the result of increased MMP-9 activity derived from hAECs and HSCs after exposure to hAEC CM.

We further explored the hypothesis that the anti-fibrotic effects observed in hAEC CM–treated HSCs may be explained by the induction of apoptosis. Both spontaneous and induced apoptosis of activated HSCs are important in promoting fibrosis resolution (40). We found that hAEC CM induced apoptosis in pro-fibrotic HSCs, which is consistent with our findings of reduced α-SMA, PDGFβ, TGF-β1 and collagen noted in CM-treated HSCs. However, apoptosis occurred in a relatively small proportion of HSCs, which suggests that there are probably other factors contributing to the anti-fibrotic response.

We also sought to establish the factors in hAEC CM that are responsible for the effects we observed. We first examined HLA-G, a HLA class Ib molecule produced by hAECs (22,41) that modulates maternal and fetal immune cell function and protects the fetus from maternal rejection during pregnancy. HLA-G has both membrane-bound (HLA-G1, HLA-G2, HLA-G3 and HLA-G4) and soluble forms (HLA-G1, HLA-G5, HLA-G6 and HLA-G7) (42). HLA-G has inhibitory and anti-proliferative effects on natural killer cells, T cells and antigen-presenting cells (43). In endothelial cells, sHLA-G1 induces apoptosis through binding to CD160 receptor (30), but, to our knowledge, no published data exist on CD160 expression or sHLA-G1 effects on HSCs. Because we found a shift to early apoptosis in HSCs treated with hAEC CM, we hypothesized that this may be through sHLA-G1 binding to CD160. Although HSCs expressed CD160, exposure to sHLA-G1 did not induce apoptosis (supplementary Figure 7).

We examined the effect of exogenous sHLA-GI on fibrogenic outcomes. The concentration of sHLA-G1 contained in 50% hAEC CM was approximately 25 ng/mL, on the basis of our previous experiments (16,22); however, this concentration of sHLA-G1 had no effect on collagen production, although there was a modest reduction at 100 ng/mL. Addition of sHLA-G1 to HSC cultures resulted in a significant decrease in TGF-β1 protein to levels similar to that in hAEC CM treatment (Figure 6A). This indicates that the TGF-β1 decrease induced by sHLA-G1 does not entirely account for the collagen suppression seen. Our novel results show for the first time that sHLA-G1 has a suppressive effect on TGF-β1 production in HSCs that appears to be independent of the hAEC CM effect on collagen production.

Because sHLA-G1 did not cause a significant reduction in HSC collagen production, we considered other known hAEC-derived factors to explain our findings. PGE_2 is produced by hAECs in culture (24,44) and has been shown to inhibit TGF-β1–mediated collagen α1(I) expression in HSCs (45). However, we found no effect of PGE_2 on HSC production of either collagen or TGF-β. TGF-β3 is reported to have anti-fibrotic properties that suppress collagen synthesis in HSCs (46). Although we found this cytokine in hAEC CM, the addition of TGF-β3 to HSC did not result in collagen suppression. Instead, collagen production increased, similar to findings in fibroblasts (47). BMPs belong to the TGF-β superfamily and play an essential role in embryonic development (48). BMP-7 is a family member that has an anti-fibrotic effect in the liver and has been shown to suppress HSC activation and to antagonize TGF-β signaling (49). We verified that hAECs express the *BMP-7* gene; however, the protein was not detected in hAEC CM. Other members in this superfamily such as BMP-2 and BMP-4 are involved in HSC transdifferentiation (50) and may mediate HSC activation (51). Further work is required to examine the role that other BMPs may have.

IL-10 was investigated next. This anti-inflammatory cytokine is produced by a number of immune cells including macrophages, T-helper 2 cells, B cells and mast cells. It is also produced by activated HSCs and plays a role in ECM remodeling by working in an autocrine fashion to stimulate collagenase and inhibit collagen α1(I) expression (52). IL-10 has also been shown to decrease TGF-β1 (53) expression as well as to induce FasL-mediated apoptosis in HSCs (54). We were unable to find measurable IL-10 in hAEC CM, consistent with a previous report (16), or in CM-treated HSCs, which suggests that hAECs neither make this cytokine nor induce its production in HSCs. We conclude that the effects of hAEC CM on activated HSCs are independent of IL-10.

We observed hAEC CM–induced HSC apoptosis. We sought to determine whether two well-known apoptosis inducers, FasL and TRAIL, were responsible. Human AECs express FasL and TRAIL mRNA, which have been shown to induce apoptosis in HSCs (55,56). However, we did not find FasL in hAEC CM. TRAIL was present but below the quantification limit of the assay. We concluded that HSC apoptosis induced by hAEC CM occurs independent of FasL and TRAIL.

Ang-(1–7) is a metabolite of the RAS created by ACE2 conversion of angiotensin II. In pregnancy, there is a progressive upregulation of the RAS, and it is thought that in both early and late gestation Ang-(1–7) may be produced by the amniotic epithelium, which has been shown in rats. Ang-(1–7) has a variety of actions including vasodilation (57) and anti-hypertensive effects (58). It has also been associated with a reduction in liver fibrosis and reduced collagen production in HSCs (59). We determined

that hAECs express ACE1, ACE2 and AGT mRNA, components necessary to produce Ang-(1−7). Renin is also required in this pathway to form Ang-(1−7), and it is expressed in the human amnion (60), which suggests that Ang-(1−7) may be present in hAEC CM. Further work is needed to confirm this and elucidate whether Ang-(1−7) plays a role. Other potential hAEC factors that may be present in the CM include nerve growth factor (61) and relaxin (62). The latter reduces collagen production, TIMP secretion and α-SMA in HSCs (63,64); however, we did not identify relaxin in hAEC CM.

In summary, we have shown that hAECs produce soluble factors that have a potent anti-fibrotic effect on activated stellate cells; this may explain some of the observed effects of hAECs in an *in vivo* model of hepatic fibrosis. Human AEC CM treatment suppresses HSC transformation to a myofibroblast phenotype, proliferation and collagen production while enhancing collagen degradation and HSC apoptosis. The factors that mediate these findings have not been fully identified. The effect of sHLA-G1 on TGF-β1 expression in HSCs is a novel finding but does not explain all our observations. Furthermore, PGE_2, BMP-7, IL-10, FasL and TRAIL do not appear to be responsible for the effects we observed. The anti-fibrotic effects noted in studies of hAEC transplantation are likely to occur through the effects that hAECs have on various cell types, including macrophages (21,65), and may be explained by the direct effect of soluble factors secreted from transplanted hAECs on HSCs. We are currently investigating the efficacy and safety of hAEC CM as an anti-fibrotic therapy together with or in place of hAEC transplantation.

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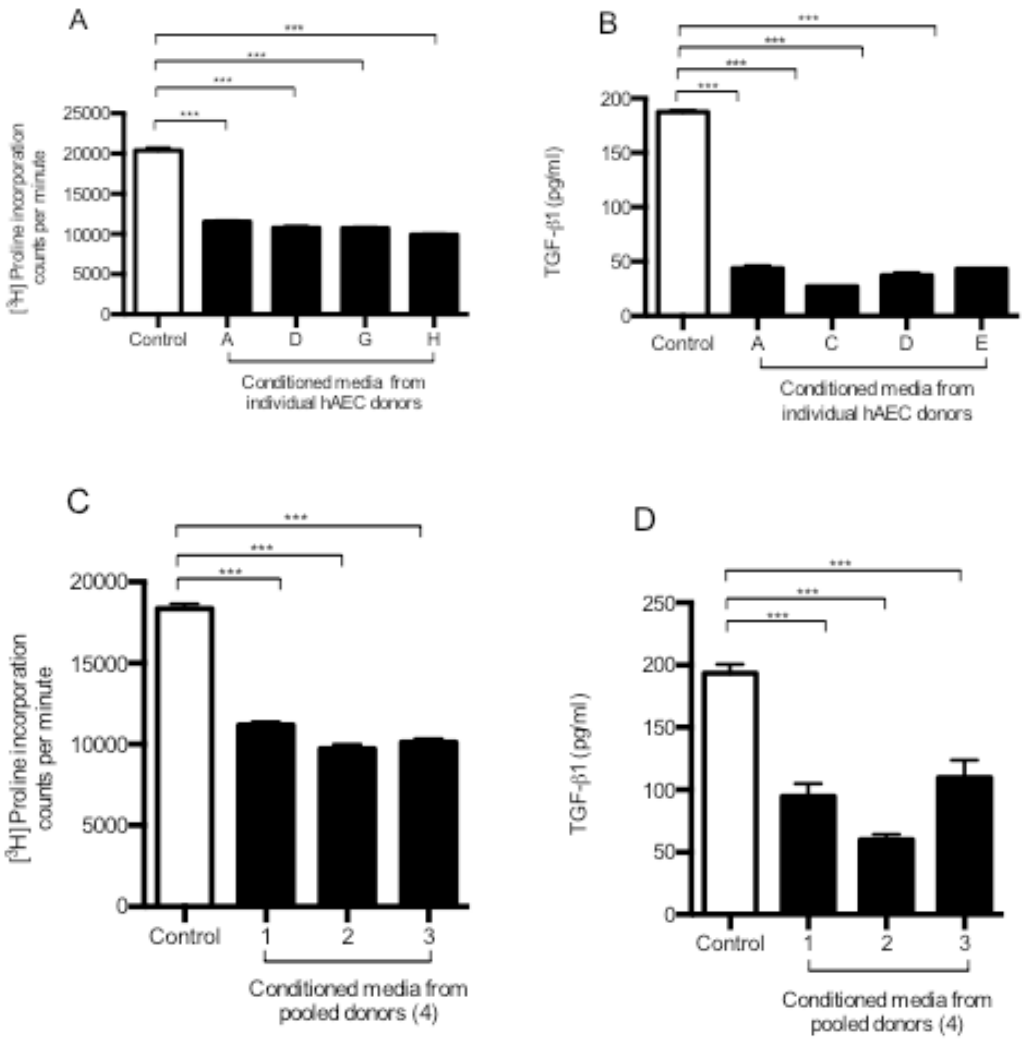
Supplementary data

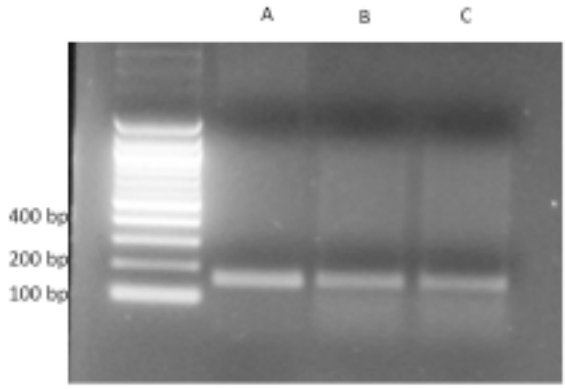
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcyt.2014.01.005

2.4 SUPPLEMENTAL DATA



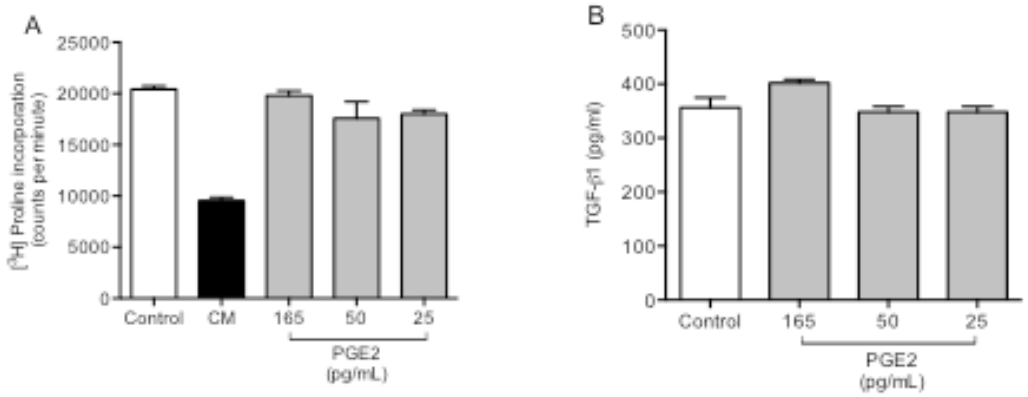
Supplemental Figure 1 - Reduction in collagen content in HSC treated with CM from individual and pooled hAEC donors.

Confluent HSC were cultured in hAEC CM or DMEM/F12 (controls) with 1 uCi [^{3}H] proline. After 48 h, culture supernatant was removed and TGF-β1 was analysed by ELISA. Cells underwent lysis and [^{3}H] proline incorporation was determined. CM from 4 individual hAEC cultures, were compared to control. hAEC CM from each individual culture had a similar effect on **(A)** collagen and **(B)** TGF-β1 production when compared to control (*** $P < 0.001$; one-way ANOVA with multiple comparisons). Twelve randomly selected donors were divided into 3 separate cultures (4 hAEC donors per culture). CM from each pooled culture was compared to control. Each pooled hAEC CM had the same effect on **(C)** collagen and **(D)** TGF-β1 production when compared to control (*** $P < 0.001$).



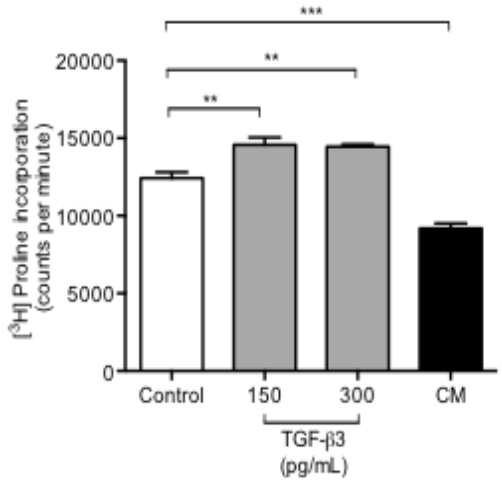
Supplemental Figure 2 - RT-PCR analysis for CD160

RT-PCR for CD160 was performed on **A**) peripheral blood mononuclear cells (as positive control); **B**) LX2 HSC and **C**) HepG2 (human transformed hepatocyte cell line). All three cell types showed amplicons specific for CD160.



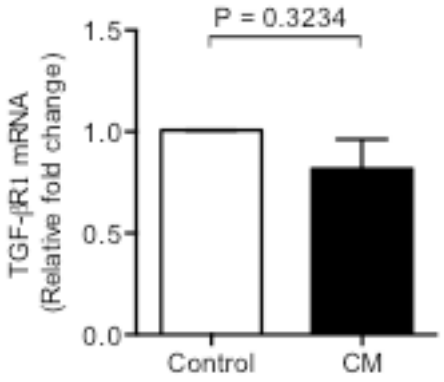
Supplemental Figure 3 - Prostaglandin E2 does not alter collagen or TGF-β1 production in HSC

Confluent HSC were cultured in hAEC CM or control medium containing 1 μCi [^{3}H] proline containing 0 pg/mL, 25 pg/mL or 165 pg/mL of Prostaglandin E2 (PGE2). After 48 h of culture the supernatant was removed. Cells were lysed and [^{3}H] proline incorporation was determined. **(A)** PGE2 treated HSC did not have a significant reduction in intracellular collagen production (one-way ANOVA with multiple comparisons). **(B)** There was no change in TGF-β1 supernatant in the control or PGE2 treated samples. Results represent one experiment run in quadruplicate. Data are expressed in mean ± SEM using CM made from four individual hAEC cultures.



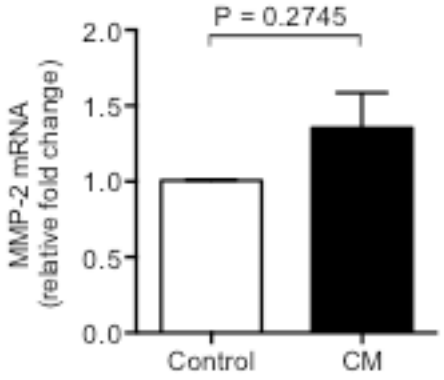
Supplemental Figure 4 - TGF-β3 does not reduce HSC collagen production

Confluent HSC were cultured in hAEC CM, DMEM/F12 (controls) or DMEM/F12 with TGF-β3 at concentrations of 100 pg/mL or 300 pg/mL, all with 1 uCi [^{3}H] proline. After 48 h, cells underwent lysis and [^{3}H] proline incorporation was determined. TGF-β3 treated HSC had a significant increase in collagen production. There was a significant decrease in CM treated HSC. ** and *** $P < 0.01$ and 0.001, respectively by ANOVA. Results represent one experiment run in triplicate. Data are expressed in mean ± SEM using CM made from n = 4 CM pools.



Supplemental Figure 5 - hAEC CM does not alter the expression of TGF-β-Receptor1

Confluent HSC were cultured in hAEC CM or control medium for 48 h. Results are expressed as a change in TGF-β receptor (R)-1 gene expression relative to HSC controls. There was no significant decrease in gene expression in HSC treated with CM (P = 0.3234; paired t-test). Results represent combined data from 3 experiments and data are expressed in mean ± SEM using n = 4 CM pools in triplicate.



Supplemental Figure 6 - hAEC CM does not affect MMP-2 expression

Confluent HSC were cultured in hAEC CM or control medium for 48 h. Results are expressed as a change in MMP-2 mRNA expression relative to HSC controls. There was a non-significant 1.35 fold increase in MMP-2 expression in CM treated HSC (P = 0.3467; paired t-test). Results represent combined data from 3 experiments using n = 4 CM pools in triplicate.

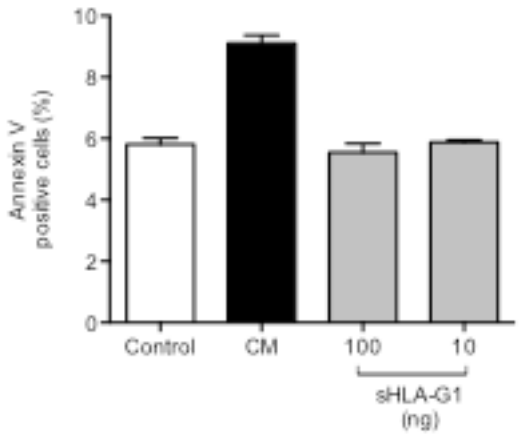


Figure 7 - sHLA-G1 does not modify HSC apoptosis

Confluent HSC were exposed to hAEC CM or control medium with 0 ng, 10 ng or 100 ng of soluble (s)HLA-G1 for 48 h and then stained with FITC-Annexin V to detect cells undergoing apoptosis and PI to label dead cells. There was no increase in the number of cells undergoing early apoptosis (Annexin V positive PI negative) when exposed to sHLA-G1 (one-way ANOVA with multiple comparisons).

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CHAPTER 3

HUMAN AMNIOTIC EPITHELIAL CELLS PRODUCE SOLUBLE FACTORS THAT DECREASE LIVER FIBROSIS AND PROMOTE LIVER REGENERATION THROUGH EFFECTS ON LIVER PROGENITOR CELLS

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3.1 INTRODUCTION

Progressive liver fibrosis eventually leads to cirrhosis and, in some patients, liver failure and / or hepatocellular carcinoma (HCC). However there is evidence supporting the notion that if the liver insult is removed or treated then fibrosis and liver function improve. This has been demonstrated in viral hepatitis (Chang et al., 2010; Marcellin et al., 2013) autoimmune disease (Dufour, DeLellis, & Kaplan, 1997) and biliary obstruction (Sikora et al., 2008). The mechanism appears to be through apoptosis and deactivation of HSC, phenotypic changes in macrophage populations and restoration of functioning hepatocytes.

In the preceding chapter I have shown that cell-free CM produced by hAEC is able to change HSC from a pro-fibrotic to an anti-fibrotic phenotype *in vitro*. Our group has shown that hAEC cell therapy in CCL_4 treated mice will reduce fibrosis (Manuelpillai et al., 2010), however, we had not addressed whether cell-free hAEC CM used in chapter 2 could, *in vivo*, exert a similar anti-fibrotic response as cells (hAEC therapy). I sought to determine whether cell-free CM, *in vivo*, was as efficacious in decreasing fibrosis as hAEC therapy. To do this I used an established CCL_4 mouse model of chronic liver disease which I treated with either CM or hAEC. Demonstrating a reduction in liver fibrosis is important, but the wound healing process also involves regeneration of hepatocytes, the major parenchymal cell of the liver, to restore the hepatocyte mass that was replaced by the extensive collagen accumulation that occurs in cirrhosis. Therefore, I assessed the capacity of hAEC, specifically CM containing hAEC-derived soluble factors, to expand and differentiate the liver progenitor cell population to regenerate and restore liver hepatocytes.

Liver progenitor cells (LPC) or “oval cells”, as they are referred to in rodents, are a rare population of immature epithelial cells that have the potential to expand and differentiate into both hepatocytes and cholangiocytes, the two main epithelial components of a functional liver. They are situated in the canal of Hering, a structure lined by both hepatocytes and cholangiocytes (Fig. 3.1). Under conditions of liver injury, such as viral hepatitis, non-alcoholic and

alcoholic fatty liver diseases in which normal hepatocyte replication is impaired these LPC can emerge, expand and differentiate into hepatocytes and cholangiocytes to aid in the regeneration of the damaged or lost liver tissue. This response is often referred to as "ductular reaction" (DR) (Fausto, 2004) which contains liver progenitor cells that are proliferating and differentiating as well as inflammatory cells and extracellular matrix (Roskams et al., 2004). The hallmark of the DR is liver progenitor cell proliferation, which I will refer to as the liver progenitor 'response'. The degree of DR correlates to the degree of liver injury (Lowes, Brennan, Yeoh, & Olynyk, 1999). There are two contrasting ideas regarding the relationship between LPC and fibrogenesis. The first notion is that DR may promote matrix deposition, partly through expression of tumour necrosis factor-like weak inducer of apoptosis (TWEAK) (Kuramitsu et al., 2013). The other possibility is that fibrosis precedes and occurs independently of the DR (Williams, Clouston, & Forbes, 2014). It is likely that both of the situations can occur dependent on the local environment (Williams et al., 2014). In addition to an association either directly or indirectly with fibrogenesis, LPC also possess a high proliferation rate and as such play a critical role in liver regeneration. Despite this benefit, the proliferation response in chronic liver diseases has been linked to HCC development (Fang, Gong, & Zhang, 2004; Lowes et al., 1999). It has been shown that LPC can be transformed in culture and, when given to immunodeficient mice, to develop tumours (Dumble, Croager, Yeoh, & Quail, 2002).

LPC are of epithelial cell lineage and display primitive cell markers: Thy-1, α-fetoprotein (AFP), CD133, MIC1-1C3 and pan-cytokeratin (Dorrell et al., 2008; 2011; Lemmer, Shepard, Blakolmer, Kirsch, & Robson, 1998; Masson et al., 2006; Yovchev, Grozdanov, Joseph, Gupta, & Dabeva, 2007). In rodents, oval cells also express the marker A6, which is a useful marker for proliferating oval cells and biliary epithelium (Petersen et al., 2002). As LPC differentiate they lose expression of these early markers and express markers more specific for hepatocytes: albumin, hepatocyte nuclear factor-4alpha, glucose-6-phosphatase (Hakoda et al., 2003; Nowak et al., 2005) and cholangiocytes: Cytokeratin (CK)-19, CK-7 (Gaudio et al., 2009).

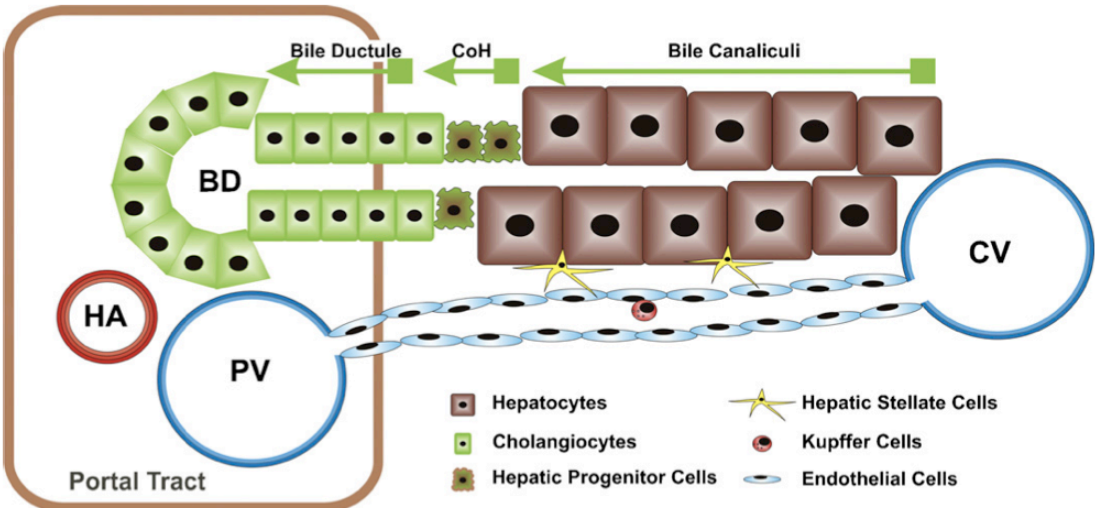

Figure 3.1: Location of hepatic progenitor cells within the liver. BD, bile duct; CoH, canals of Hering; CV, central vein; HA, hepatic artery; PV, portal vein. Adapted from Gaudio et al. (Gaudio et al., 2009).

This following chapter will investigate the *in vivo* efficacy of CM as a viable anti-fibrotic therapy and its effects at hepatic restoration through LPC augmentation.

3.2 MATERIALS AND METHODS

3.2.1 ETHICS

The Monash Health Human Research Ethics Committee approved the study for the collection and use of human amnion and informed written consent was obtained from each patient prior to surgery (Monash Health HREC approval numbers: 01067B, 12223B).

Mice were purchased from Monash Animal Services, Melbourne, Australia. All animal experiments were approved by the Animal Ethics Committee, Monash University (ethics approval MMCB 2011/26).

3.2.2 ISOLATION OF HAECS

hAEC were isolated from amnion membranes as described previously in Chapter 2. Briefly, placentas were collected from healthy women with normal term (37–40 weeks gestation) pregnancies by caesarean section. Amnions were stripped from the underlying chorion and digested twice for 60 mins with agitation at 37°C in 0.05% trypsin/EDTA (Gibco, Grand Island, NY, USA). Following

enzymatic removal of the cells from the membrane, the dispersed cells were washed in DMEM/F12 (Gibco) and passed through a 70 μm filter. Cells were frozen in FBS (Gibco) with 5% DMSO (Sigma Aldrich, Saint Louis, MO, USA) and later assessed for viability, and mycoplasma, bacterial and fungal contamination.

3.2.3 GENERATION OF HAEC CONDITIONED MEDIA (CM)

hAEC CM was generated as described in Chapter 2. hAECs from multiple donors were pooled (n = 4 - 5) and maintained in DMEM/F12 with 10 - 20% FCS, and 10 ng/ml EGF (Gibco) on rat tail collagen IV (1 mg/mL Roche, Mannheim, Germany) coated tissue culture flasks until greater than 90% confluence was achieved. Cells were then serum starved for 72 hours in DMEM/F12, and media collected, spun and passed through a 0.2 μm filter (Sartorius, Goettingen, Germany). CM was stored at -80^{o}C until needed.

3.2.4 MOUSE HEPATIC FIBROSIS MODEL

Six week old male C57/BL6 mice were given twice weekly intra-peritoneal injections with 1 μl/g body weight CCl_4 (Merck, Darmstadt, Germany) mixed with olive oil 1:10 as previously described (Manuelpillai et al., 2010). At 8 weeks animals received a single dose of 2 x 10^6 cells from a mixture of 5 hAEC donors or 350 μl hAEC CM three times weekly for four weeks via tail vein injections. Control groups consisted of mice given CCl_4 only, CCl_4 and DMEM/F12 (3 times weekly for 4 weeks beginning at week 8) and healthy mice that did not undergo any treatment. Animals were culled 4 weeks later (total of 12 weeks of CCl_4). Blood and liver tissues were collected.

3.2.5 IMMUNOHISTOCHEMISTRY: α-SMA, PAN-CK AND A6

Liver tissue was fixed in 10% neutral buffered formalin (NBF) (Australian Biostain, Victoria, Australia) overnight prior to tissue processing and embedding in paraffin. Then 4 μm tissue sections were cut, deparaffinised, and stained. 10 minutes of antigen retrieval was performed using 0.1 M tri-sodium citrate in the microwave for α-SMA, or Proteinase K (Dako, Victoria, Australia) at room temperature for pan-CK and A6. Subsequent to blocking with 1- 3% hydrogen peroxide, and either CAS protein block (Life Technologies, Camarillo, CA, USA) or

Dako's Serum free protein block, the following primary antibodies were applied: 1:500 mouse anti-mouse α-SMA (Sigma Aldrich) for 30 mins at room temperature, 1:400 Wide Spectrum Screening Cytokeratin (PanCK; Dako) overnight at 4˚C and 1:50 A6 (gift from Dr. V. Factor) overnight at 4˚C. For a-SMA and A6 the following secondary antibodies were used respectively: 1:300 biotinylated rabbit IgG2a (Life Technologies) for 30 mins at room temp or rat HRP secondary antibody (GE Life Sciences, NSW, Australia) for 1 hour at room temperature. VectaStain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for α-SMA only. For Pan-CK, staining was visualised using the Universal LSAB™ + Kit/HRP (Dako). Finally, slides were stained with DAB, counterstained with hematoxylin, mounted with DPX, and scored.
For α-SMA, manual scoring was done at 250 times magnification with the number of positive cells per high powered field. For PanCK and A6, slides were scanned at 40x using the Aperio® ScanScope digital slide scanner (Aperio Technologies, Vista, CA, USA) and were viewed and annotated using ImageScope (Aperio Technologies). Positive staining was quantified using the Positive Pixel count algorithm (version 9; Aperio Technologies).

3.2.6 MORPHOMETRIC ANALYSIS OF FIBROSIS

Fibrosis area corresponded to the collagen proportionate area determined by Sirius Red staining, which was performed as previously described (Manuelpillai et al., 2010). Briefly, sections were incubated for 90 mins in Picro Sirius Red [Direct Red 80 0.1% wt/vol in saturated picric acid (Sigma Aldrich)] and washed in acetic acid water (1:200). Fifteen consecutive non-overlapping fields were acquired from each mouse liver using Leica DMLB microscope (Leica Microsystems, Ltd, Heerbrugg, Switzerland) at 50 times magnification. Images were digitized and fibrosis area measured by computer-assisted morphometry using the Scion Image for Windows (vAlpha 4.0.3.2, Scion Corporation, Frederick, MD).

3.2.7 MURINE LIVER PROGENITOR CELL LINE

Liver progenitor cells were a kind gift from Dr. George C.T. Yeoh (University of Western Australia, Crawley, Australia), who created the Bipotential Murine

Embryonic Liver cell line (BMEL). Briefly cell lines were derived from livers of day 14 embryos of TAT-GRE lacZ transgenic mice by the 'plate and wait' method described by Strick-Marchand (Strick Marchand & Weiss, 2002). In this method cells undergo spontaneous immortalisation following prolonged culture maintenance. Under conditions promoting differentiation, cells express biliary and hepatic markers. Hepatic differentiation can also be confirmed measuring the expression of the lacZ transgene, which is under transcriptional control of the tyrosine aminotransferase (TAT) promoter element (Tirnitz-Parker, Tonkin, Knight, Olynyk, & Yeoh, 2007).

3.2.8 LPC CULTURE AND CM TREATMENT

Immortalised mouse LPC were seeded in tissue culture plates (6, 24 and 96 well) at a density of 8.3 X 10^3 cell per cm^2 in RPMI 1640 medium GlutaMAX (Gibco) with 10% FCS, 30 ng/ml IGF-II (Sino Biological Inc, Beijing, China), 50 ng/ml EGF, and 10 µg/ml insulin (Sigma Aldrich). The media was changed 3 days later and the cells were then exposed to either control medium (RPMI GlutaMax, 50% DPBS (Gibco), 5% FCS, 30 ng/ml IGF-II, 50 ng/ml EGF, 10 µg/ml insulin), hAEC CM (RPMI GlutaMax, 50% hAEC CM , 5%FCS, 30 ng/ml IGFII, 50 ng/ml EGF, 10 µg/ml insulin) or Differentiation media (RPMI GlutaMax, 50% DPBS, 5% FCS, 1X ITS (Gibco), 50 ng/ml EGF, 10 mM nicotinamine (Sigma Aldrich), 0.1 µM Dexamethasone (Sigma Aldrich)). This latter media drives LPC differentiation into hepatocytes (Schwartz et al., 2002). Cultures were maintained up to 24 days post treatment with media changes ever 3-4 days. Cells were grown with 100 U/ml penicillin and streptomycin (Gibco) at 37˚C in a humidified 5% CO_2 atmosphere.

3.2.9 LPC AND LX-2 CO-CULTURE

Immortalised human hepatic stellate cells (HSC) (LX-2 cells, a gift from Prof. Scott Friedman, Mt Sinai, New York) were seeded in 6 well plate transwell inserts (Corning, NY, USA) at a density of 1.2 x 10^5 cells per well in DMEM/F12 with 5% FCS. LPC's were seeded as described above into the receiver well. Once both cell types reached confluence, they were combined and the media conditions added: Control (RPMI GlutaMax, 50% DPBS, 5% FCS) and hAEC CM

(RPMI GlutaMax, 50% hAEC CM , 5% FCS). LPC only wells were used as a control. Cultures were maintained for up to 8 days with a media change at day 4. Cells were grown with 100 U/ml penicillin and streptomycin (Gibco) at 37˚C in a humidified 5% CO_2 atmosphere.

3.2.10 REAL TIME PCR AND MICROARRAY

LPC total RNA was isolated according to the manufacturers' instructions using the Qiagen RNeasy mini kit (Qiagen Pty Ltd, Hilden, Germany). Following elution, RNA concentration was determined using the Nanodrop ND-100 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 1 μg RNA was then used to generate cDNA, as per manufacturer's instructions, using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA). For Real Time PCR, changes in gene expression were assessed using Power Sybr Green (Life Technologies) on the Rotor Gene 3000 light cycler (Qiagen Pty Ltd, Sydney, Australia) using the following cycle conditions: 95 ˚C for 10 min, followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s. All changes in gene expression were represented relative to 18S. All mouse primers were purchased from MicroMon, (Victoria, Australia) and listed in Table 1.

Table 1: Real Time PCR primers

PRIMER ID	SEQUENCE	SOURCE or Reference
18S F	GTA ACC CGT TGA ACC CCA TTC	Dr Eric Morand
18S R	GCC TCA CTA AAC CAT CCA ATC G	Dr Eric Morand
AFP F	GGC GAT GGG TGT TTA GAA AG	(Strick Marchand & Weiss, 2002)
AFP R	CAG CAG CCT GAG AGT CCA TA	
ALB F	CTT AAA CCG ATG GGC GAT CTC ACT	(Strick Marchand & Weiss, 2002)
ALB R	CCC CAC TAG CCT CTC GCA AAA T	
CK19 F	CAT GGT TCT TCT TCA GGT AGG C	(Schwartz et al., 2002)
CK19 R	GCT GCA GAT GCA TTC AGA ACC	
GGT F	TTC AGG CTC ATA GTA GGC GG	Dr Rebecca Lim
GGT R	TGA CCC AAA GTT TGT CGA TG	

For microarray analysis, total RNA was analysed using the Agilent SurePrint G3 Mouse Gene Expression 8 x 60K slide at the MHTP Medical Genomics Facility. Results were analysed on the GeneSpring GX program (Agilent, Santa Clara, CA, USA). Array files were normalised against the brightness of the array from control (untreated) LPCs, using quantile normalisation such that each chip has the same intensity distribution. In order to assess differentially expressed genes, I applied an empirical Bayes analysis using a LIMMA package (Smyth, 2004) where the p-value was set to 0.01 and a 2-fold change in the expression of any gene was assumed to be significant. A hierarchical cluster analysis was then performed on Gostat (Beissbarth & Speed, 2004) the over represented genes with Benjamini correction for multiple testing. A heat map was generated using the freeware Gene-E program (Broad Institute Inc., Cambridge, MA)

3.2.11 HEPATOCYTE FUNCTIONAL ASSAYS

Urea

Cell culture supernatants from day 17 cultures were sent to Monash Medical Centre Biochemistry (Southern Cross Pathology) for analysis to determine urea content. All values represented had basal media urea content subtracted.

β-galactosidase (β-gal)

β-gal expression was assessed on day 17 and day 24 cultures using the Mammalian β-Gal Assay Kit (Pierce Biotechnology, Rockford, IL, USA) both as an on-plate assay according to the manufacturer's instructions and also following lifting cells and normalising against cell number. Absorbance was measured at 409nm on the Tecan microplate reader (Tecan Group, Seestrasee, Mannedor, Switzerland).

Periodic acid Schiff (PAS)

Day 16 cultures were stained for glycogen using PAS staining. Cells were fixed in 24 well plates with neutral buffered formalin for 10 mins, covered with 0.5% Periodic Acid Solution for 5 min, incubated with Schiff's Reagent DeTomasi (HD Scientific Supplies, NSW, Australia) for 15 min and counterstained with Hoechst dye 33342 (Molecular Probes, Eugene, Oregon, USA). Photographs of both stained and unstained cells were taken using the Olympus IX71 inverted microscope.

3.2.12 PROLIFERATION

LPC proliferation at day 2 and day 10 was assessed using the colorimetric bromodeoxyuridine (BrdU) ELISA (Roche) according to manufacturer's instructions. Cells were incubated with 10 μM BrdU labelling medium for 5.5 hours.

3.2.13 STATISTICAL ANALYSIS

Experiments were performed more than once and with more than 3 replicates per experiment. Murine studies were conducted with 4 to 7 animals in each group. Statistical analysis was performed using GraphPad Prism 6.0e for Mac OS

X (GraphPad Software, La Jolla, California, USA). Statistical significance was determined using unpaired t-test for comparisons across experiments as appropriate. One-way ANOVA with Newman-Keuls post hoc test was performed for multiple comparisons. The data are shown as mean +/- SEM.

3.3 RESULTS

In vivo studies

3.3.1 HAEC CM AMELIORATES FIBROSIS IN A MOUSE MODEL OF CHRONIC LIVER DISEASE

In chapter 2, hAEC CM was shown, *in vitro*, to influence HSC, the primary effector cells in liver fibrosis. I sought to determine whether CM would have similar anti-fibrotic effects *in vivo* in a 12 week CCl_4 murine model. Thrice weekly intravenous administration of hAEC CM from weeks 8 – 12 resulted in a 34.5% reduction in fibrosis area compared to controls (CM treated 3.33 ± 0.191 % vs. control media 5.09 ± 0.210 %; $P < 0.01$; Fig. 3.2A). A single treatment of hAEC at week 8 resulted in a 33.1 % reduction in fibrosis area at week 12 compared to controls (hAEC treated 3.03 ± 0.267% vs. control 4.53 ± 0.523%; $P < 0.01$; Fig. 3.2A). Thus, both cell and cell free therapy had a similar anti-fibrotic effect. The anti-fibrotic effect of CM was similar to what was observed with hAEC therapy (Fig 3.2A). CM treatment resulted in a 33.9% decrease in activated HSC (CM treated 24.66 ± 1.84 vs. control plus media 37.33 ± 5.88 α-SMA positive areas per field; $P < 0.05$; Fig. 3.2B). Mice given hAEC showed no change in α-SMA positive area compared to controls (Fig. 3.2B).

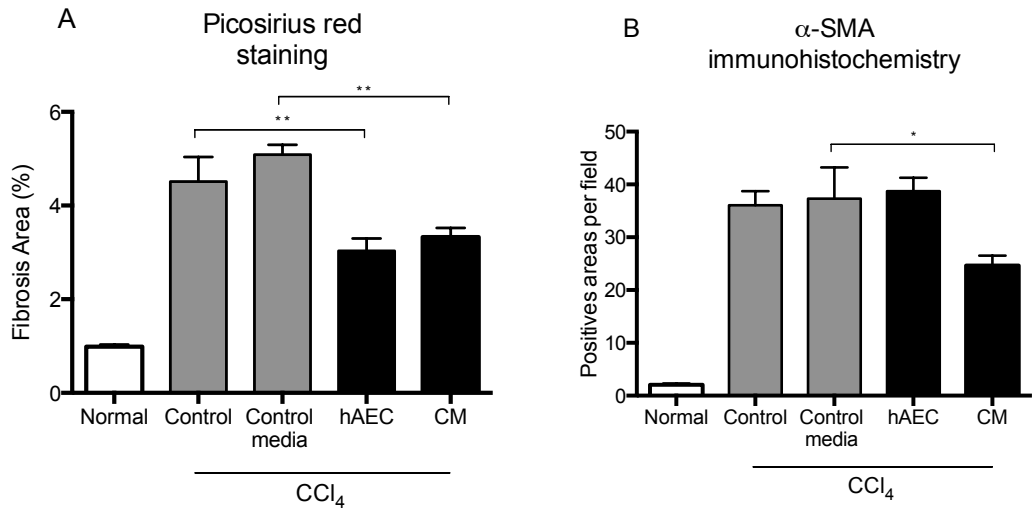

Figure 3.2: Anti-fibrotic effects of hAEC and CM *in vivo*. Quantification of Picrosirius red staining of collagen and α-SMA immunohistochemistry of HSC are shown. C57/BL/6 mice with established fibrosis induced by CCL_4 were given hAEC or CM (A) hAEC and CM treated mice had a significantly reduced fibrosis area. (B) CM resulted in fewer activated HSC. * $P < 0.05$, ** $P < 0.01$.

3.3.2 HAEC NOT CM DAMPENS THE CCL_4 INDUCED LPC RESPONSE

To investigate the regenerative capacity of hAEC therapy in chronic liver disease I examined the relative expression of LPC markers Pan-CK and A6 in control fibrotic and treated fibrotic groups. CCl_4 induced chronic liver disease resulted in an increase of markers of LPC, Pan-CK and A6 (Fig 3.3 A& B). hAEC therapy resulted in a 46.2% and a 49.0% reduction in Pan-CK and A6 expression respectively (hAEC treated 1.766 ± 0.40 vs. control 3.28 ± 0.62 Pan-CK percent positive pixels per field of view; $P < 0.05$; Fig 3.3A; hAEC treated 0.34 ± 0.09 vs. control 0.68 ± 0.9 A6 percent positive pixels per field of view; $P < 0.01$; Fig 3.3B). CM treatment did not significantly affect the LPC response in the CCl_4 induced chronic liver disease model (Fig 3.3 C & D).

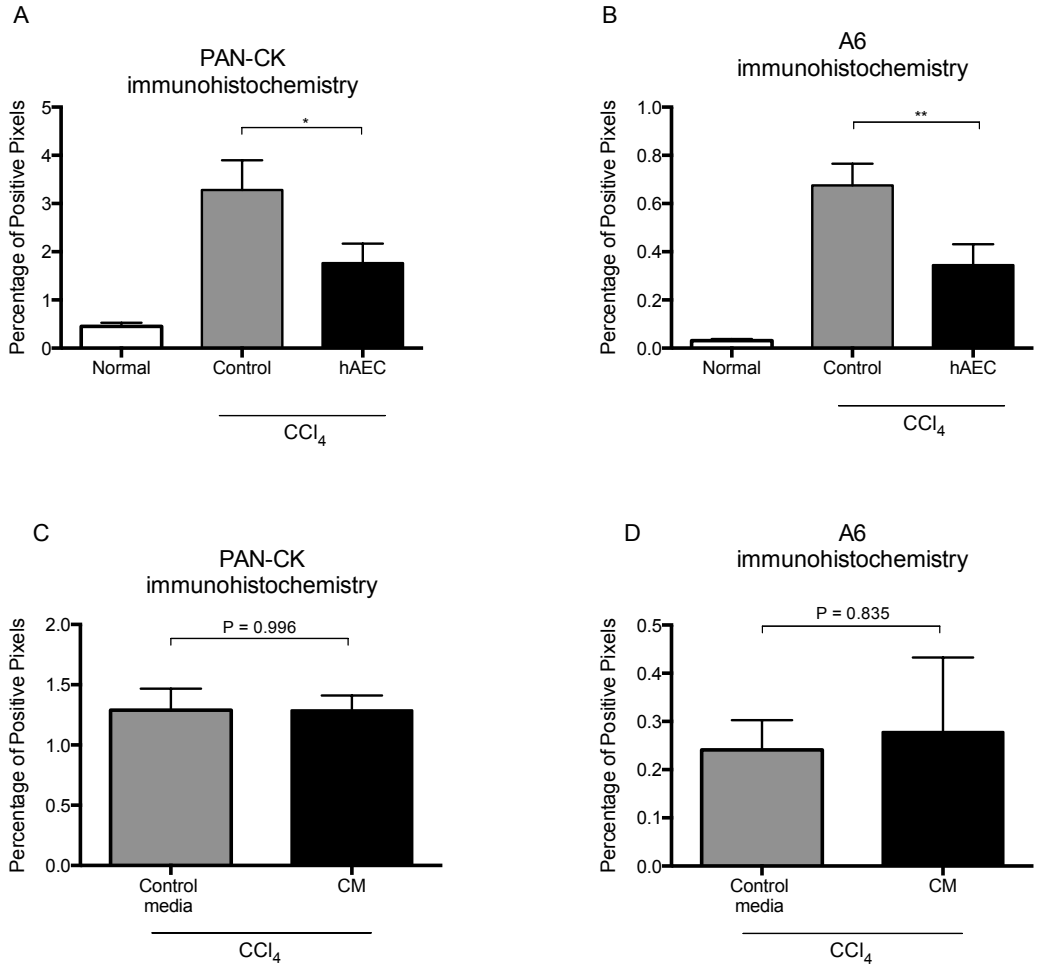

Figure 3.3: Effects of hAEC and CM on LPC *in vivo*. Quantification of Pan-CK and A6 immunohistochemistry of LPC are shown. C57/BL/6 mice with established fibrosis induced by CCL_4 were given hAEC or CM. (A & B) hAEC treated mice had a significantly reduced LPC response. (C & D) Treatment with CM did not reduce LPC expression. There was no change in Pan-CK ($P = 0.996$) or A6 expression ($P = 0.835$) between the CM treated and untreated fibrotic animals. * $P < 0.05$, ** $P < 0.01$.

In vitro studies

3.3.3 HAEC CM INDUCES LPC PROLIFERATION

In vitro assays were performed to investigate the LPC response to hAEC soluble factors over time. Human AEC CM treatment resulted in increased LPC proliferation measured by BrdU incorporation, at both early (day 3) and late (day 10) time points. At day 3 of CM exposure LPC demonstrated a 14% increase in BrdU incorporation similar to that seen in LPC treated with hepatocyte differentiation media ($P < 0.0001$; Fig 3.4 A). At day 10 of CM treatment, BrdU

incorporation in these mature cultures increased by 85% compared to the control ($P < 0.0001$; Fig 3.4 B) and CM augmented differentiation media increased BrdU incorporation by 96% compared to differentiation media alone ($P < 0.0001$; Fig 3.4 B).

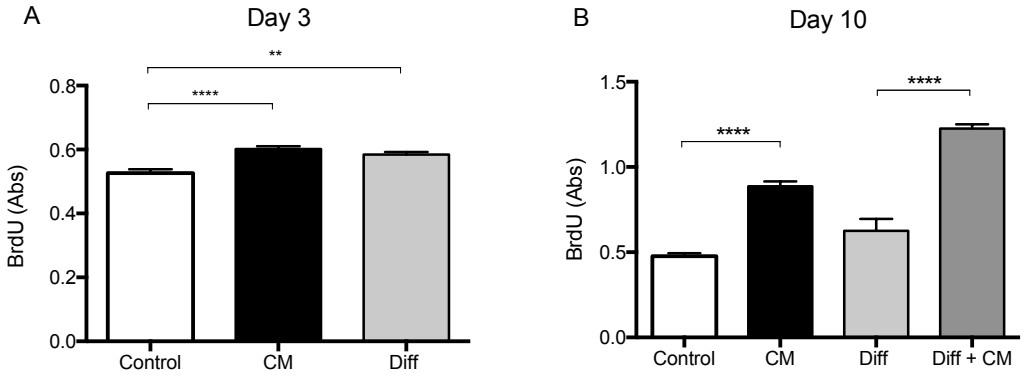

Figure 3.4: HAEC CM induces LPC proliferation. LPC were cultured from 3 to 10 days with control media, CM or differentiation media (diff), which preferentially differentiates LPC into hepatocytes. BrdU incorporation was used to determine LPC proliferation. (A) After 3 days BrdU incorporation was increased in CM treated and differentiation media treated LPC by 1.14 fold and 1.11 fold respectively. (B) By 10 days proliferation increased 1.86 fold in CM treated LPC. The addition of CM further enhanced the effects of differentiation media by 1.96 fold. ** $P < 0.01$, **** $P < 0.0001$.

3.3.4 HAEC CM INDUCES LPC HEPATOCYTE DIFFERENTIATION

LPC treated with hAEC CM expressed a significant increase in two markers of hepatocyte regeneration – AFP (Dabeva et al., 1998) and albumin. There was a 5.4 fold increase in AFP ($P <0.05$; Fig 3.5 A) and an 8.13 fold increase in albumin ($P < 0.05$; Fig 3.5 B) expression in LPC treated with CM after 8 days. Hepatocyte function was assessed by urea production as a surrogate marker for nitrogen metabolism. After 16 days of exposure to CM, the concentration of urea in the supernatant was 22 times that of the control (CM 0.367 ± 0.092 mmol/L vs. control 0.017 ± 0.017 mmol/L; $P < 0.01$; Fig. 3.5 C) and 5.5 times that of differentiation media (CM 0.367 ± 0.092 mmol/L vs. differentiation media 0.067 ± 0.042 mmol/L; $P < 0.01$; Fig. 3.5 C). The immortalised LPC line was derived from transgenic mice that expressed β-galactosidase (β-gal) in mature hepatocytes. The relative activity of this enzyme between control, CM treated

and differentiation media treated LPC was determined. At 17 days of culture there was no significant difference in β-gal activity. After 24 days of culture the β-gal activity increased by 13.8% in CM treated conditions compared to the control ($P < 0.05$; Fig 3.5 D) and 15.8% compared to differentiation media conditions ($P < 0.05$; Fig 3.5 D). At day 16 cultures were stained with PAS to show that cells were storing glycogen a marker of functional activity in differentiated hepatocytes (Fig. 3.5 E). PAS positive cells formed aggregates (Fig. 3.5 F & G). Cystic structures were also noted consistent with biliary epithelium (cholangiocytes) (Fig. 3.5 F& G). LPC grown in control media maintain the undifferentiated state with the absence of structures noted in LPC cultured with CM or differentiation media (Fig. 3.5 H).

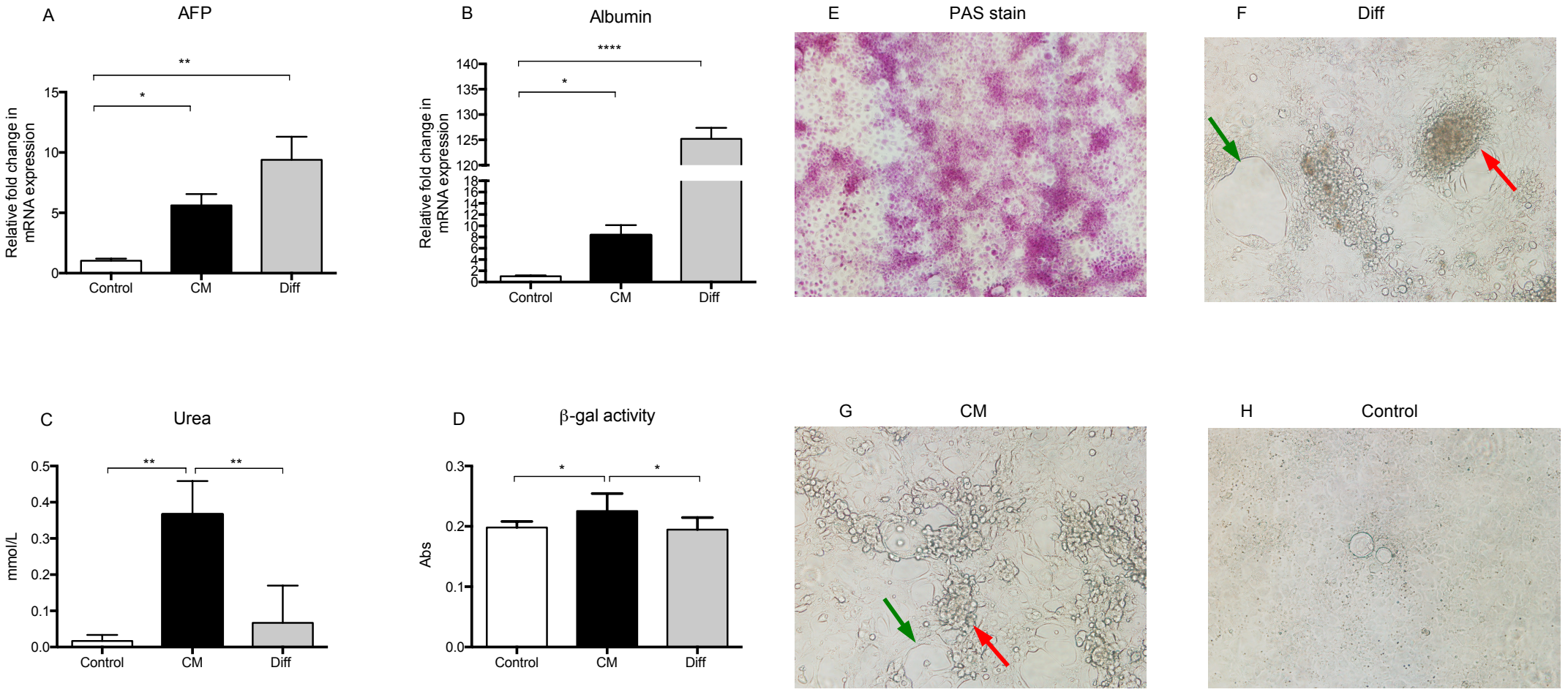

Figure 3.5: HAEC CM induces differentiation of LPC into hepatocytes. LPC were cultured with control media, CM and differentiation media (diff) up to 24 days. (A & B) alpha-fetoprotein (AFP) and albumin expression, compared to control were increased 5.4 and 8.13 fold respectively. (C) Urea excretion was increased 22 fold and 8.13 fold compared to the control and diff media treated LPC respectively. (D) β-gal activity, a marker of LPC differentiation to mature hepatocytes, was increased in CM treated by 13.8% compared to control and 15.8% compared to diff media treated. (E) PAS staining demonstrates glycogen storage (pink cells) as a marker of functional hepatocytes (10X magnification). (F, G) Bright field microscopy at day 17 (20 X magnification). Aggregates of cells (red arrow) represent cells that have differentiated to hepatocytes. Cystic structures (green arrows) have the characteristic appearance of biliary epithelial cells but require confirmatory CK-19 staining. (H) Undifferentiated LPC form cobblestone appearance in culture, lacking structures noted in G & H. * $P < 0.05$ ** $P < 0.01$, **** $P < 0.0001$.

3.3.5 HAEC CM MAINTAINS CHOLANGIOCYTE DIFFERENTIATION

CM treatment maintains but does not increase CK-19 expression, a marker of cholangiocyte differentiation, when compared to control LPC cultures at day 8. Hepatocyte differentiation media treatment results in a decrease in CK-19 expression ($P < 0.05$; Fig. 3.6). There was no change in CK-19 expression between control and CM treated LPC.

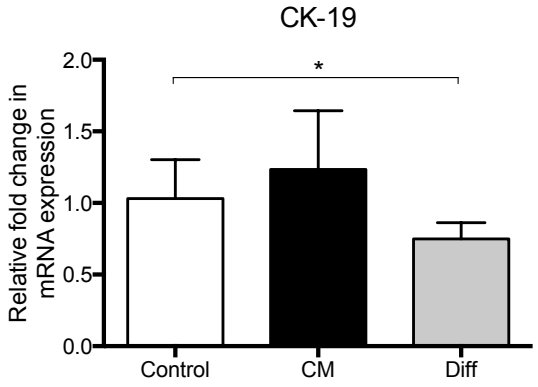

Figure 3.6: HAEC CM maintains cholangiocyte differentiation. CK-19 expression did not change when LPC were treated with CM. Differentiation media (diff) treatment resulted in 0.73 fold decrease in CK-19 expression. * $P < 0.05$

3.3.6 LPC HEPATOCYTE DIFFERENTIATION IS NOT INFLUENCED BY ACTIVATED HSC

There is evidence that activated HSC are involved in liver regeneration in chronic liver injury through their effects on LPC proliferation and differentiation, however the exact mechanism is unclear (Yin, Evason, Asahina, & Stainier, 2013). LX-2 cells when plated in tissue culture represent pro-fibrotic activated HSC (Xu et al., 2005). Eight days of co-culture with activated human HSC and LPC did not influence LPC differentiation, with or without the addition of CM. There was no significant change in LPC expression of AFP and albumin when co-cultured with HSC (Fig 3.7)

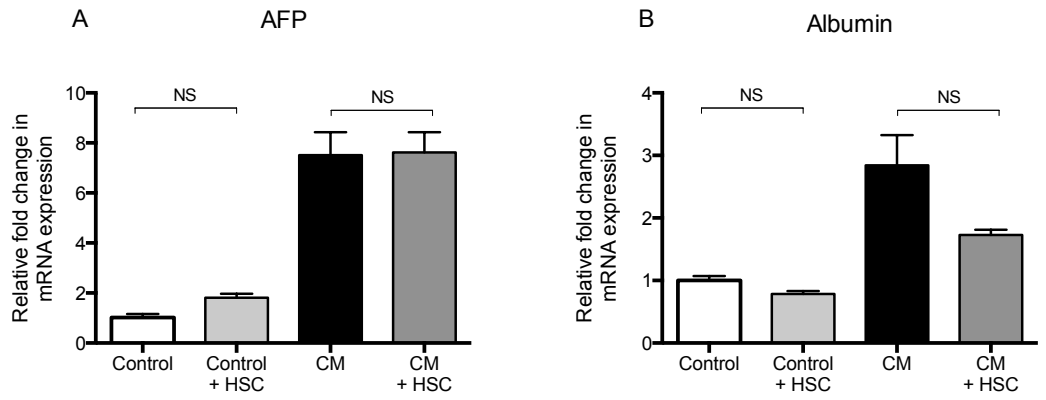

Figure 3.7: Activated human HSC do not influence LPC hepatocyte differentiation. LPC were cultured with control media and CM with or without exposure to activated human HSC through transwell co-culture systems. (A & B) alpha-fetoprotein (AFP) and albumin expression did not change when LPC were co-cultured with activated human HSC with or without CM. NS, non-significant.

3.3.7 HAEC CM INDUCES EXPRESSION OF GLUTATHIONE TRANSFERASES

Glutathione transferases (GST) are a group of enzymes best known for their role in drug metabolism (Di Simplicio, Jensson, & Mannervik, 1989). They are expressed in rodent liver progenitor cells (oval cells) and their expression is relevant for liver regeneration (Lowes et al., 1999; Lowes, Croager, Olynyk, Abraham, & Yeoh, 2003). A rat model of cirrhosis and regeneration has shown there is increased expression of the various subtypes of GST during regeneration (Liu et al., 2007). GST are thought to play a crucial role in hepatocyte survival and proliferation during liver regeneration (Pajaud, Kumar, Rauch, Morel, & Aninat, 2012). This may be through regulating the hepatocyte cell-cycle (Loyer, Corlu, & Desdouets, 2012). LPC were cultured for 16 days exposed to CM, control or differentiation media. Relative expression of GST was determined by microarray analysis and displayed as a heatmap (Fig 3.8 A). GST expression in LPC treated with CM or diff were significantly increased relative to controls except GSTm2 in CM treated LPC and GSTm4 in diff and CM treated LPC ($P < 0.0001$; Fig 3.8 B).

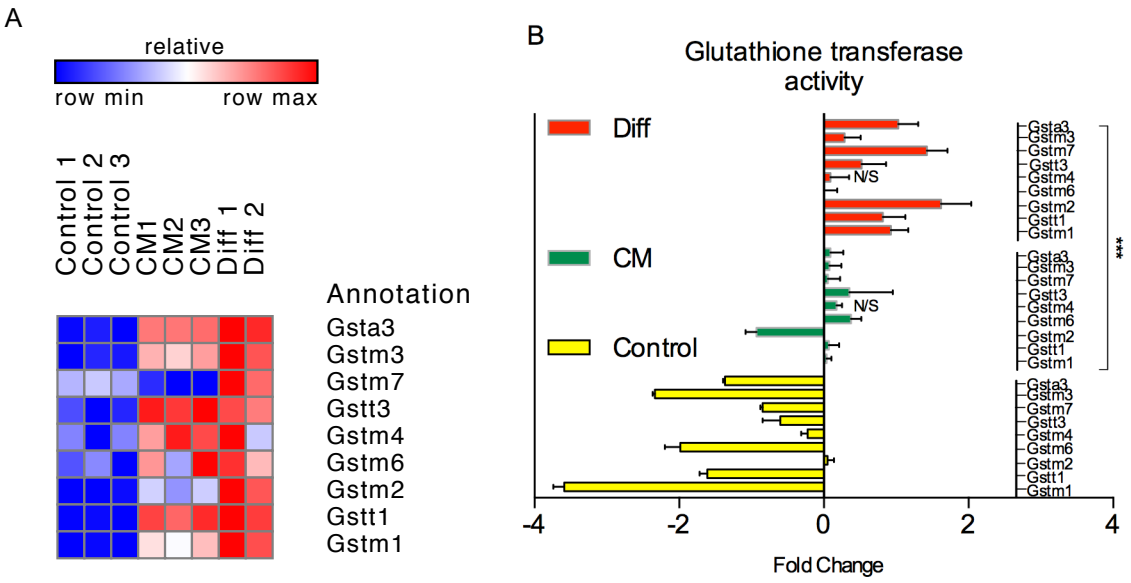

Figure 3.8: HAEC CM induces expression of glutathione transferases (GST). LPC were treated with CM, control, and differentiation (diff) media for 16 days. Results represent a single experiment. (A) Heat map representative of the GSTs activity. (B) GST expression in LPC treated with CM or diff was significantly increased relative to controls, except GSTm2 in CM treated LPC and GSTm4 in diff and CM treated LPC. *** $P < 0.0001$

3.4 DISCUSSION

The aim of this study was to confirm the *in vitro* findings described in Chapter 2, as well as assess the effects of hAEC CM on the LPC population. In Chapter 2 the ability of hAEC CM to suppress HSC activation, proliferation and collagen expression was described. Here I sought to assess the *in vivo* translation of these *in vitro* findings in a murine model of chronic liver fibrosis. I observed that 4 weeks of hAEC CM treatment resulted in an equivalent reduction in liver fibrosis to that observed with cell-based hAEC treatment. Additionally the effects of hAEC and hAEC CM on the liver's restorative capacity were determined by assessing LPC number. Human AEC treatment but not CM dampened the CCL_4 induced LPC response. Further *in vitro* experiments revealed hAEC produce soluble factors in CM that induce bipotential LPC proliferation, differentiation down the hepatocyte lineage and maintenance of cholangiocyte differentiation.

Findings from this chapter and those described in chapter 2 strongly indicate that hAECs are able to release potent anti-fibrotic factors. Furthermore, these secreted factors contained in conditioned medium are stable in -80°C storage and can be repeatedly administered over a period of weeks. Here I have shown that treatment with CM reduced CCl_4 induced liver fibrosis comparable to hAEC therapy. Further, CM reduced the percentage of activated stellate cells (myofibroblasts) as determined by α-SMA immunohistochemical staining. This was not observed in animals treated with hAEC, which was surprising as hAEC treatment consistently decreases HSC activation (decreased α-SMA staining) in the CCL_4 mouse model of chronic liver disease in both unpublished and published work from our laboratory (Manuelpillai et al., 2010; 2012). The difference between the CM and hAEC results and the relative inability of hAEC to reduce activated HSC may be explained by donor variability. Due to donor availability at the time of the experiments, hAEC donors used to produce CM were different than those pooled for cell transplantation. Although I have shown in chapter 2 (supplemental data) that there is no significant difference in the anti-fibrotic effect of the individual donors I tested *in vitro*; it is possible this was not the case for these *in vivo* experiments, and this may have explained these results. Further studies to determine donor-to-donor variability in regards to the potency of anti-fibrotic effect need to be addressed. This will be discussed further in chapter 5.

After establishing the potency of CM in reducing fibrosis both *in vitro* (chapter 2) and *in vivo,* I sought to determine the ability of CM to regenerate the damaged liver parenchyma by influencing LPCs (oval cells in rodents), the bipotential cells that comprise the liver stem cell niche. Expression of Pan-CK and A6, the primary markers of oval cells in mice, was determined to assess the overall LPC response to hAEC or CM therapy in the 12-week CCL_4 model of chronic liver disease. The degree of LPC response, or ductular reaction, correlates with the degree of liver injury (Lowes et al., 1999) and carbon tetrachloride induces LPC proliferation more so than other liver toxins (Petersen, Zajac, & Michalopoulos, 1998), which in turn is thought to enhance hepatic fibrosis (Pritchard & Nagy, 2010). Treatment with hAEC reduced the LPC response by 50% compared to the

CCL_4 controls. This may be due to a combination of LPC suppression and a greater proportion of LPC undergoing hepatocyte differentiation rather than proliferating. This reduction in LPC response is a desired effect. As I have stated in the introduction, an unbridled LPC response in liver injury models may result in HCC formation. Human AEC treatment was able to temper the LPC response. Knight et al. have shown in mice that reduced LPC proliferation leads to a decrease in the risk for liver tumour formation (Knight et al., 2000). A reduction in LPC response was not observed in CM treated animals. In fact CM had no effect on the overall LPC response. This could be explained by the pro-proliferative effect that CM had on LPCs (Fig 3.4). Human AECs were administered as a single IV bolus, 8 weeks into the 12-week CCl_4 dosing, while 12 doses of CM were administered from weeks 8 to 12. Repeated administration of hAEC CM over the 4-week period may have resulted in increased exposure to soluble factors that perpetuated the LPC response compared to the animals treated with a single hAEC dose. However, *in vivo*, it remains to be seen whether repeated CM treatment would result in HCC or would preferentially increase differentiation and restoration of functioning hepatocytes. The *in vitro* results suggest the latter rather than the former, however to confirm this *in vivo* would require cell tracking and lineage tracing experiments.

To further assess the extent to which hAEC CM could affect the LPC population, assays were performed to ascertain the impact of CM on hepatocyte and cholangiocyte differentiation. Differentiation status was assessed by gene expression analysis, phenotypic and functional assays. I observed that CM preferentially supported the differentiation of LPCs towards the hepatocyte lineage with increased expression of AFP and albumin, which are associated with hepatocyte proliferation during reparative and regenerating growth after and during liver injury. In addition differentiated LPC colonies also demonstrated glycogen and urea synthesis, which are important hepatocyte functions.

The potential of activated HSC to influence LPC hepatocyte differentiation was also assessed. I observed in a co-culture system of LPC and activated HSC treated with CM that the presence of HSC did not influence hepatocyte

differentiation of LPC. It is not known whether LPC proliferation was increased, as might be expected, however, this can be clarified in future experiments. Interestingly, CM administration was not accompanied by a marked reduction in the transcription of cholangiocyte marker, CK19. This may reflect the retention of a subpopulation of bipotential LPCs after exposure to hAEC CM. This finding is important since the maintenance of a bipotential progenitor cell niche is important in the restoration of normal liver parenchyma after injury. Finally, I was able to demonstrate that hAEC CM induces GST expression, which are *in vivo* markers of liver regeneration, although confirmation of these findings with PCR is required.

In summary, the findings from this study support the promise of hAECs as a modality for regenerative medicine, especially in the setting of liver fibrosis. It is worth noting that this is the first *in vivo* study comparing the effect on LPC of hAEC or hAEC CM. I have shown that hAEC treatment of an animal model of chronic liver disease results in both a reduction in fibrosis and a tempering of the LPC response, the latter in turn may limit the potential of HCC formation. Furthermore, hAEC produce soluble factors that induce LPC differentiation into hepatocytes while maintaining cholangiocyte differentiation. This suggests hAEC may be a potent driver of hepatocyte restoration. The use of cell-free treatment (CM) *in vivo* needs further study alongside hAEC therapy to determine the true fate of LPC in both conditions. I have shown that hAEC release soluble factors into CM that have anti-fibrotic effects on HSC in cell culture (chapter 2). In this chapter, I show for the first time that in an animal model of chronic liver disease, treatment with CM acts specifically on the stellate cells to reduce their activation and result in improvements in fibrosis similar to cell therapy with hAEC. These studies assert that hAEC release soluble factors that are efficacious in the treatment of chronic liver disease by having both a role in fibrosis resolution and hepatocyte regeneration. There still is much work to be done to elucidate and characterise the factors responsible for these important biological effects.

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CHAPTER 4

NON-ALCOHOLIC FATTY LIVER DISEASE INTERMITTENT FASTING TIME INTERVENTION (NIFTI)

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Monash University

4.1 Declaration for Thesis Chapter 4

Non-alcoholic fatty liver disease intermitted fasting time intervention (NIFTI): A randomised trial of fasting without calorie restriction versus standard care.
Submitted to journal

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Experimental design, conduct clinical trial, experimental analysis and drafting of manuscript	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Alexandra Mack	conduct clinical trial	25%
Caroline Tuck	analyse food diaries	2%
Jorge Tchongue	assist with ELISA	N/A
Darcy Holt	assist with CT analysis	2%
William Sievert	Drafting of manuscript and intellectual input	N/A
Gregory Moore	Drafting of manuscript and intellectual input	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 10 October 2014
Main Supervisor's Signature	[redacted]	**Date** 10 October 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

4.2 INTRODUCTION

Chapter 2 and 3 dealt with the exploration of a novel anti-fibrotic therapy using hAEC and hAEC derived CM. In chapter 2 I examined the effects of CM on the cell at the centre of liver fibrosis deposition, the HSC. I found CM treatment of HSC resulted in a shift from a fibrotic to an anti-fibrotic phenotype that resulted in the degradation of collagen *in vitro*. I went on to further examine the anti-fibrotic effects of CM *in vivo* and found that CM caused a similar reduction in fibrosis as did treatment with hAEC. This is evidence that soluble factors released from cells destined for therapeutic use may be as efficacious as the cells themselves in treating chronic liver diseases. Regression of fibrosis is essential in the treatment of chronic liver disease, but equally important is the restoration of normal hepatic function. In chapter 3 I described how CM causes proliferation and differentiation of *in vitro* LPC into functioning hepatocytes, while maintaining the same basal rate of cholangiocyte differentiation in this bipotential LPC population.

Chapter 4 now addresses the other end of the liver disease spectrum, the prevention of chronic liver disease. Here I target the most prevalent liver disease, NAFLD. I have shown in chapter 1, that lifestyle modification primary through dietary modification, is recommended as the first line treatment. With this in mind I sought to look at an alternative lifestyle intervention that would be both easy and more liver specific when compared to the standard recommended dietary and exercise advice.

To my knowledge, this is the first randomised controlled trial comparing time-related intermittent fasting, without calorie restriction, to standard advice (diet and exercise) in patients with non-alcoholic fatty liver disease (NAFLD). I feel that this is a novel study with clinical implications. Intermittent fasting, the controversy over the importance of eating breakfast and the timing of meals are ideas of considerable interest due to their topicality.

4.3

NON-ALCOHOLIC FATTY LIVER DISEASE INTERMITTENT FASTING TIME INTERVENTION (NIFTI): A RANDOMISED TRIAL OF FASTING WITHOUT CALORIE RESTRICTION VERSUS STANDARD CARE

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ABSTRACT

Objective

We assessed the effectiveness of intermittent fasting (IF) for 16 hours fasting with 8 hours feeding without calorie restriction, compared to standard care (SC) diet and lifestyle advice in improving liver fibrosis, steatosis and visceral adiposity in adults with non-alcoholic fatty liver disease (NAFLD).

Design

Participants with NAFLD were randomised to IF or SC for 12 weeks in this single blind crossover pilot study. Primary endpoints were changes in liver stiffness and steatosis. Secondary endpoints were changes in visceral fat, body fat, weight, waist circumference (WC), blood pressure, serum alanine aminotransferase, tumour necrosis factor-α, adiponectin, leptin, insulin, glucose, cholesterol, triglycerides and insulin resistance. Exploratory endpoints included caloric intake, adherence to dietary advice, and satiety. A further 12-week crossover study was offered to participants with similar endpoints.

Results

We enrolled 32 subjects with 28 completing the study. Baseline demographics were similar. In both SC and IF groups at 12 weeks, weight, BMI, body fat and leptin decreased significantly while adiponectin increased significantly. Only the IF group showed a significant decrease in liver stiffness, liver steatosis, WC and visceral fat. There was no difference in exploratory endpoints between the groups. A 12-week crossover study resulted in further improvements of liver steatosis, weight, WC and body fat in those changing from SC to IF.

Conclusions

IF may be a simple, well tolerated strategy to treat NAFLD with significantly greater improvement in liver stiffness and steatosis, waist circumference, visceral fat and insulin resistance compared to standard diet and exercise advice.

Words: 248

Ethics: Ethical approval was obtained from Monash Health Human Research Ethics Committee A (HREC 12298A).

Trial registration number: Australian New Zealand Clinical Trials Registry ACTRN12613000935730

Significance of this study

What is already known about this subject?

- Lifestyle modification through calorie reduction and increased activity improves non-alcoholic fatty liver disease (NAFLD) and features of the metabolic syndrome.
- Standard advice on diet and exercise has poor adherence and gains are often short lived.
- Intermittent fasting (IF) with *ad libitum* time restricted feeding has been shown to be effective in reducing weight and improving metabolic parameters.

What are the new findings?

- IF for 16 h followed by *ad libitum* caloric intake and standard care advice on diet and exercise both result in weight loss, total body fat reduction and improvements in the adipocytokines leptin and adiponectin.
- IF for 16 h followed by *ad libitum* caloric intake, but not standard care diet and exercise advice, results in additional improvement in markers of liver fibrosis and steatosis and visceral fat volume, waist circumference and blood pressure.
- IF is well tolerated in regard to overall satiety and diet satisfaction.

How might it impact on clinical practice in the foreseeable future?

- The results of this study suggest that lifestyle interventions focusing on time restricted feeding rather than caloric reduction may have better liver and visceral fat specificity.
- Point-of-care assessments of waist circumference and liver stiffness are clinically relevant and can be used to monitor metabolic and hepatic improvements during IF in patients with NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, affecting more than 46% of adults in developed nations.[1,2] NAFLD is a disease spectrum ranging from simple hepatic steatosis to hepatic steatosis accompanied by inflammation (non-alcoholic steatohepatitis, NASH). Progressive fibrosis can occur in 10 to 25% of NASH cases leading to cirrhosis and its complications.[1,3] Although cirrhosis from any cause is a risk factor for hepatocellular carcinoma, NAFLD may be an independent risk factor for the development of HCC in cirrhotic and non-cirrhotic livers. [4,5] NAFLD may be a potential cause of the metabolic syndrome [6]- a constellation of central obesity, impaired glucose tolerance, dyslipidaemia and hypertension.[7] NAFLD is associated with a wide range of health problems including obstructive sleep apnoea, cardiovascular disease, polycystic ovarian syndrome [1] and an increased risk of type 2 diabetes.[8] NAFLD patients with metabolic syndrome or diabetes have an increase in all-cause, liver–specific and cardiovascular mortality.[9-11]

Due to the high prevalence of NAFLD and its associated liver and non-liver complications, there is a pressing need for an effective treatment strategy; however, there are as yet no approved medical therapies.[12] As NAFLD is inextricably linked to the metabolic syndrome through over-nutrition,[13] treatment has focused on lifestyle modification through dietary changes with or without exercise. However, long-term compliance is poor and sustained weight loss results are disappointing.[14-16] Diet manipulation has mostly been in the form of calorie restriction (CR), a cornerstone of management of NAFLD as well as obesity and the metabolic syndrome. A prospective study in 1970 demonstrated that prolonged periods of fasting or a calorie restricted diet resulted in weight loss and improved liver steatosis.[17] Reducing caloric intake also has positive effects such as improved glucose tolerance and insulin sensitivity, hypertension, reduced oxidative damage, increased lifespan, reduced incidence of tumours and protection from neuronal degeneration and some forms of kidney disease.[18]

Unfortunately the majority of participants on low-calorie diets regain the weight they lose [19-21] as adherence to CR diets is often poor and inversely proportional to the severity of caloric restriction.[22] An alternative approach is to alter the timing of energy intake through reduced meal frequency or intermittent fasting (IF). One IF approach has been through alternate day fasting (ADF) regimens in which a day of *ad libitum* consumption is followed by a day of complete or partial caloric restriction. This approach can have weight, metabolic and cardiovascular benefits,[23-25] but some find adherence difficult.[24] Time restricted feeding (TRF) is another form of IF in which individuals consume *ad libitum* only within a set window of time each day (e.g. 8h) and fast the remainder of the day (e.g. 16h). An important point of difference between CR and IF is that the overall caloric intake with IF need not be reduced, only the frequency and timing of consumption are altered.[18,26] A recent review on the effects of TRF on body weight and metabolic risk factors found this regimen to be associated with reductions in body weight, lipids and inflammatory markers with improvements in insulin sensitivity.[27] Whether TRF could be a successful lifestyle strategy to address NAFLD is not known.

AIMS

The aim of this pilot study was to determine whether a 12-week period of IF, in the form of TRF in individuals with NAFLD, is an acceptable, sustainable form of dietary modification and to measure clinical and biochemical outcomes in comparison to individuals given standard lifestyle modification advice regarding CR and exercise.

METHODS AND ANALYSIS

Study design

NIFTI was a randomised, controlled, single-blind, pilot study to assess the effects of 12 weeks of IF versus standard lifestyle modification advice. The primary endpoint was change in a non-invasive marker of hepatic steatosis. All participants were seen at baseline (week 0), weeks 4, 8 and 12 weeks. Two-weekly complete 3-day food diaries assessed compliance to IF and caloric intake. The study was approved by Monash Health Human Research Ethics Committee A

(registration number: HREC 12298A) and registered at the Australian New Zealand Clinical Trials Registry (registration number: ACTRN12613000935730).

Sample size

Sample size estimates were based on a previous validation study of Fibroscan® controlled attenuation parameter (CAP) measurements for assessing liver steatosis.[28] We estimated a 10% decrease in CAP values by the end of 12 weeks. With a power assumption of 80% and a confidence interval of 95% we calculated that 14 participants were required in each group (28 total) to achieve this endpoint.

Subjects

Participants were recruited from the gastroenterology outpatient clinics of Monash Health and from general practitioner and specialist practices in Melbourne, Australia. Eligible subjects were adults between 18 – 75 years of age with an ultrasound diagnosis of fatty liver (determined by radiological features including increased hepatic parenchymal echotexture and / or vascular blurring or diaphragm definition).[29] Exclusion criteria included liver disease from causes other than NAFLD, alcohol intake or medications known to be associated with hepatic steatosis (Supplemental Table 1). Metformin, HMG CoA reductase inhibitors and vitamin D were permitted if the dose was stable for the preceding 4 weeks. All participants gave oral and written informed consent and were randomised 1:1 by blinded envelope to either IF or standard care.

Interventions

Intermittent fasting arm

Subjects allocated to the IF arm were instructed to withhold all food and energy containing drinks for 16 h from 8pm until 12 noon the following day. During this time, water, black tea and black coffee were permitted. Between the hours of noon and 8pm (8 h), subjects were able to consume food as desired, without any prescribed caloric restriction.

Standard dietary and exercise advice

Those allocated to the SC arm were instructed to follow the lifestyle and diet advice provided by the Gastroenterological Society of Australia.[30] These are general instructions for CR and increased exercise (Supplemental Table 2).

Primary endpoint

The primary endpoint was reduction in liver steatosis assessed by liver stiffness measurement (LSM) with simultaneous CAP determination. LSM and CAP were measured using the Fibroscan® M probe. All Fibroscan® studies were performed with participants having fasted for at least 2 hours. Only LSM and CAP studies with ≥ 10 valid readings and median measurement with interquartile range / median value of ≤ 30% were used in the analysis. Fibroscan® studies were performed at 0 and 12 weeks. The single operator was blinded to the participants study assignment.

Secondary endpoints

All secondary endpoints (listed in Table 1) were assessed as changes from baseline to week 12. Some values were recorded every four weeks; missing values were analysed using the 'last observation carried forward' method. Methods are provided in the supplemental material.

Table 1. Secondary endpoints

	Weeks				
	0	4	8	10	12
Visceral fat volume [Single slice (L4/5) abdominal CT, Slice-O-Matic analysis]	X				X
Whole body DEXA for fat (kg)	X				X
Physical examination (including: BP, weight, WC, BMI)	X	X	X	X	X
Faecal calprotectin	X				X
Serum ALT	X	X	X	X	X
Serum TNF-α	X				X
Serum adiponectin	X				X
Serum leptin	X				X
Serum insulin, glucose, HOMA-IR	X	X	X	X	X
Serum Cholesterol, TG	X		X	X	X

ALT, alanine aminotransferase; BP, blood pressure; BMI, body mass index; CT, computed tomography; DEXA, dual energy X-ray absorptiometry; TG, triglyceride; TNF, tumour necrosis factor; WC, waist circumference.

Exploratory Endpoints

Exploratory endpoints included diet, hunger, activity and quality of life monitoring. Dietary changes were measured with a validated 3-day food diary self-reported at baseline and every two weeks. Total daily calorie, fat, carbohydrate and protein intake were calculated using Foodworks Professional (Version 7, Xyris Software, Kenmore, Queensland, Australia). The average of the 3 days was used to estimate daily intake. Questionnaires were used throughout the study to determine the impact of IF and SC on hunger, activity and quality of life. Hunger, satisfaction, fullness and happiness with diet were assessed by a visual analogue scale (VAS).[31] The degree of physical activity of each study participant was determined via the World Health Organisation (WHO) Global Physical Activity Questionnaire (GPAQ).[32] The WHO quality of life (QOL) questionnaire was completed to measure general health and psychosocial aspects of each study participant during the length of the study.[33] Questionnaires were performed every 4 weeks. (see supplemental material for methods)

12-week crossover

At the end of 12 weeks participants were invited to crossover to the opposite arm (IF to SC or SC to IF) and continue for an additional 12 weeks. There was no washout period. The exploratory endpoints for the crossover study were to determine if changes in visceral fat and transient elastography markers of liver stiffness and hepatic steatosis achieved after 12 weeks of IF were maintained and whether additional benefits were achieved after transitioning from SC to IF.

Statistical analysis

Study participants who completed the first 12-weeks were analysed per protocol. The significance of normally distributed data between the two groups was analysed with a two-tailed t test; paired between the same groups and unpaired between different groups. Data not normally distributed was compared with a Wilcoxon test or Mann-Whitney U test for paired and unpaired data respectively. Means of several groups of normally distributed data were compared using one-way analysis of variance for paired and unpaired data. Data not normally distributed was compared with a Friedman or Kruskal-Walllis test for paired and unpaired data respectively. Spearman's correlation was performed to measure monotonic relationships between two non-parametric continuous variables. A p value < 0.05 was considered statistically significant. All values noted in tables are results of calculations which were then rounded to two decimal places in most intances.

RESULTS

Patient flow

Thirty-two subjects were enrolled in the study, 17 in the IF group and 15 in the SC group (Figure 1). Three discontinued the trial, 1 was lost to follow-up; 14 in each group were analysed at 12 weeks.

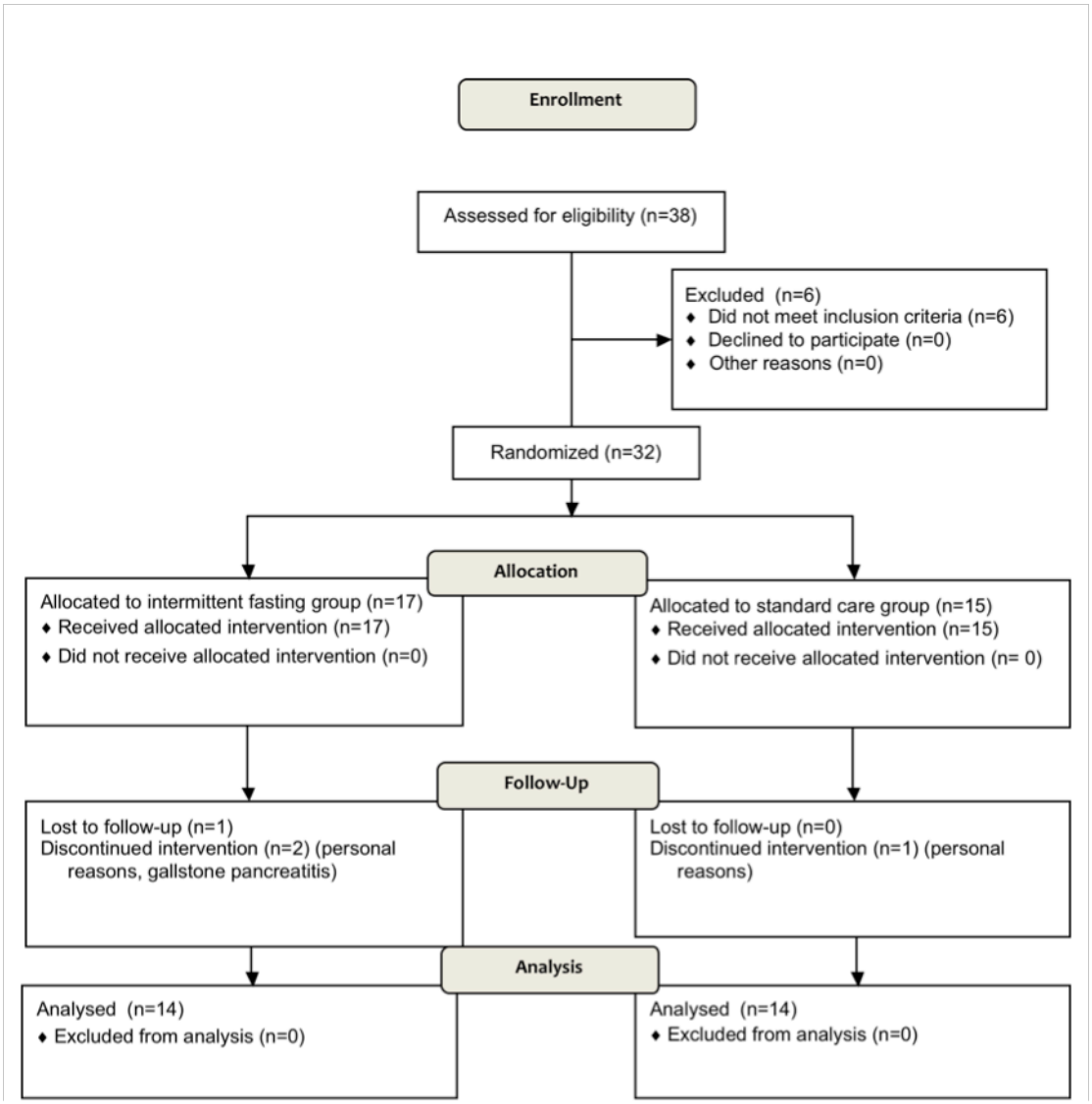

Figure 1: CONSORT patient flow diagram

Baseline characteristics

The demographic and anthropometric characteristics of each group were similar at baseline (Table 2). The mean age of the participants was 58.5 years (11.4 SD), weight 83 kg (12.9 SD), BMI 29.7 kg/m^2 (3.1 SD) and waist circumference (WC) 101.1 cm (9.1 SD).

Table 2: Baseline characteristics of participants by study group

	Intermittent fasting (n=17)	Standard care (n=15)	p Value*
Age (years)	61.9 (8.7)	54.7 (13.0)	0.079
Sex (M/F)	10/7	9/6	
Ethnicity (n)			
• Caucasian	13	10	
• South Asian	2	3	
• East Asian	2	0	
• Hispanic	0	2	
Weight (kg)	83.2 (14.4)	82.7 (11.5)	0.924
Height (cm)	167.4 (10.1)	166.4 (8.7)	0.771
BMI (kg/m²)	29.5 (3.0)	29.9 (3.3)	0.472
WC (cm)	101.8 (10.2)	100.4 (8.0)	0.674
• Males	105.45 (2.79)	100.89 (2.68)	0.26
• Females	96.57 (3.90)	99.67 (3.56)	0.51
Metabolic syndrome (%)†	8 (47%)	7 (47%)	
Baseline caloric intake (kJ) Δ	7747 (861)	7307 (1965)	0.455

Data expressed as mean ± standard deviation.
*p Values compare mean values between weeks 0 and 12.
† According to the International Diabetes Federation criteria for the metabolic syndrome [7]
Δ Average of 3-day total caloric intake.

Liver steatosis and stiffness

There was no significant change in overall CAP values at 12 weeks in either group and thus the primary endpoint of the study was not achieved. However, subgroup analysis of those with CAP values > 215 db/m, a proposed cut-off for significant steatosis,[34] demonstrated a significant decrease in CAP values after

12 weeks in the IF compared with SC participants. Liver stiffness decreased significantly in the IF group at week 12 but not in the SC group (Table 3).

Table 3: Transient elastography (Fibroscan®)

	Group(Δ)	Week 0	Week 12	p Value*	Change
Liver stiffness (kPa)	IF (12)	7.33 (3.1)	5.84 (1.9)	**0.0088**	- 17% (15)
	SC (12)	6.32 (1.7)	6.09 (2.2)	0.7305	- 1 % (35)
CAP (dB/m)	IF (14)	264 (62)	256 (49)	0.6307	- 2 % (38)
	SC (13)	256 (53)	270 (35)	0.7869	+ 13 % (46)
Significant liver steatosis (dB/m)†	IF (11)	287 (46)	263 (43)	**0.0120**	- 8 % (10)
	SC (12)	268 (34)	268 (36)	0.9807	NC

Data expressed as mean ± standard deviation.
* p Values compare the mean values between weeks 0 and 12 weeks.
† Participants with baseline CAP values defining significant liver steatosis > 215 dB/m
Δ Participants with valid studies
CAP, controlled attenuation parameter; IF, intermittent fasting; NC, no change; SC, standard care.

Anthropometry, blood pressure and fat mass

At week 12, weight, BMI, and whole body fat mass decreased significantly in both IF and SC groups. However, visceral fat volume, WC and systolic blood pressure (SBP) were significantly lower only in the IF group (Table 4).

Table 4: Anthropometric measurements and blood pressure

	Group(Δ)	Week 0 (n=28)	Week 12 (n=28)	p Value*	*Change*
Weight (kg)	IF	81.9 (13.7)	79.8 (13.1)	**0.0024**	- 2.5 % (2.4)
	SC	82.3 (11.7)	81.0 (11.9)	**0.0066**	-1.6 % (1.9)
BMI (kg/m²)	IF	29.0 (2.3)	28.3 (2.6)	**0.002**	-1.6 % (1.9)
	SC	29.5 (3.2)	29.1 (3.3)	**0.006**	-2.5 % (2.4)
WC (cm)	IF	102.0 (9.6)	99.1 (8.8)	**0.028**	- 3.0 cm (4.5)
	SC	100.2 (8.3)	99.7 (9.7)	0.441	- 0.5 cm (2.4)
Visceral fat volume (cm^2)	IF (12)	177 (43)	154 (46)	**0.0186**	- 13 % (15)
	SC (11)	161 (58)	153 (53)	0.2836	- 4 % (15)
Fat mass (kg)	IF	29.0 (7.1)	27.8 (7.4)	**0.0001**	- 4.7 % (3.6)
	SC	30.6 (7.8)	29.0 (7.8)	**0.0031**	- 5.3 % (5.5)
Systolic BP (mmHg)	IF	137 (13)	132 (12)	**0.0173**	- 6 (8)
	SC	133 (18)	128 (16)	0.3503	- 5 (19)
Diastolic BP (mmHg)	IF	85 (9)	82 (8)	0.1145	- 3 (8)
	SC	83 (13)	80 (9)	0.3874	- 3 (11)

Data expressed as mean ± standard deviation.
Δ n = 14 for both IF & SC unless indicated otherwise.
*p Values compare the mean values between weeks 0 and 12 weeks.
BMI, body mass index; IF, intermittent fasting; SC, standard care; BP, blood pressure, WC, waist circumference.

Metabolic and inflammatory biomarkers

While inflammatory markers [tumour necrosis factor (TNF)-α, ALT and faecal calprotectin] did not change, there was a significant change in the adipocytokines leptin and adiponectin in both groups. Leptin decreased by 15% and 28% in IF and SC groups, while adiponectin increased by 24% and 23% in IF and SC respectively. Although the absolute change in HOMA-IR values in IF and SC were not significant, comparison of the proportional change showed a significant reduction in insulin resistance in the IF group compared to the SC group (Table 5). Serum triglycerides were significantly lower in the SC group.

Table 5: Metabolic and inflammatory biomarkers

	Group(Δ)	Week 0 (n=28)	Week 12 (n=28)	p Value*
ALT (U/L)	IF	54.9 (31.7)	44.9 (21.2)	0.161
	SC	54.6 (36.1)	44.9 (17.0)	0.173
Glucose (mmol/L)	IF	5.5 (0.6)	5.4 (0.6)	0.369
	SC	5.7 (2.5)	5.7 (1.7)	0.132
Insulin (mU/L)	IF	13.8 (7.9)	11.9 (5.3)	0.241
	SC	9.1 (5.5)	9.5 (5.5)	0.414
HOMA-IR	IF	3.4 (2.3)	2.9 (1.5)	0.076
	SC	2.3 (1.5)	2.5 (1.7)	0.326
% change	IF		- 9.9 (20)	**0.039**
% change	SC		+ 2.5 (1.6)	
Total cholesterol (mmol/L)	IF	5.0 (1.2)	5.2 (1.2)	0.651
	SC	5.4 (1.4)	5.2 (1.5)	0.357
TG (mmol/L)	IF	1.5 (0.7)	1.6 (0.8)	0.726
	SC	1.7 (0.7)	1.4 (0.6)	**0.046**
TNF-α (pg/mL)	IF	2.25 (1.32)	1.82 (0.57)	0.173
	SC (13)	1.60 (1.02)	2.04 (1.21)	0.305
Leptin (ng/mL)	IF	8.27 (4.04)	7.35 (4.33)	**0.033**
	SC	7.04 (3.36)	5.53 (3.85)	**0.0004**
Adiponectin (μg/mL)	IF	15.20 (9.04)	17.93 (9.75)	**0.003**
	SC	16.71 (6.65)	19.58 (6.00)	**0.0003**
Faecal calprotectin (μg/mL)	IF (12)	84.7 (60.8)	160.5 (163.6)	0.129
	SC	98.0 (154.7)	138.5 (164.1)	0.296

Data expressed as mean ± standard deviation.
Positive sign (+) denotes an increase, Negative sign (-) denotes a decrease.
Δ n = 14 for both IF & SC unless indicated otherwise.
*p Values compare the mean values between weeks 0 and 12.
ALT, alanine transferase; IF, intermittent fasting; HOMA-IR, homeostatic model assessment – insulin resistance; SC, standard care; TG, triglycerides.

Diet, hunger, activity and quality of life

Total caloric intake did not change in either IF and SC groups (Figure 2). Additionally, there was no change in the percentage of energy obtained from total fat, saturated fat, protein or carbohydrate throughout the study or between the groups. VAS scores for hunger, satisfaction or happiness with diet did not change in either group over 12 weeks. There was also no change in the four WHOQOL domains or the total activity level of all participants before and during the study (Supplemental Table 3).

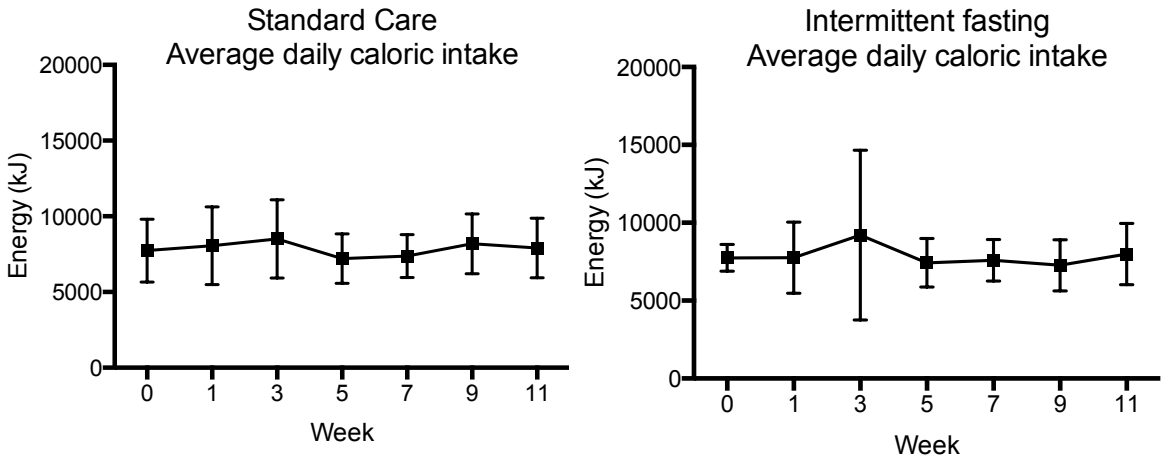

Figure 2: Total daily caloric intake
Average (±SD) caloric intake over 3 days from self-reported 3-day food diary completed every 2 weeks. Food diaries were analysed by a dietitian.

Twelve-week crossover

At 12 weeks participants were invited to crossover to the opposite group (SC to IF and IF to SC with no washout period) and to continue for a further 12 weeks. Of 14 in the IF group only 1 did not crossover, citing personal reasons. Three in the SC group did not crossover to the IF arm (due to gastro-oesophageal reflux, gout and personal reasons, 1 each). In total, 13 changed from IF to SC and 11 from SC to IF for a further 12 weeks. CAP values for liver steatosis fell by 3% in the group that crossed from SC to IF (SC/IF) and were numerically higher in the group that crossed from IF to SC (IF/SC). In addition weight, BMI, WC, and total fat mass all significantly decreased in the SC/IF group and increased in the IF/SC group. ALT significantly decreased in the SC/IF group and HOMA-IR significantly increased in the IF/SC group. Changing from SC to IF resulted in further improvements in factors associated with central adiposity (weight, BMI, WC, fat mass) but resulted in an increase in those features in those moving from IF to SC (Table 6), despite no overall change in caloric intake (Supplemental Figure 1). There was no significant change in visceral fat volume or liver stiffness in either group by the end of the crossover study at week 24.

Table 6: Crossover study

	Group Δ	Week 12	Week 24	p Value*	*Change*	p Value
Visceral fat volume (cm^2)	SC/IF (7) IF/SC (13)	153 (53) 154 (46)	154 (54) 164 (59)	0.4733 0.2578	- 5 % (14) + 8 % (22)	0.1768
Liver stiffness (kPa)	SC/IF (9) IF/SC (12)	5.87 (1.9) 6.68 (3.6)	6.48 (2.4) 7.9 (5.3)	0.4665 0.0522	+ 16 % (23) + 16% (39)	0.9774
CAP (dB/m)	SC/IF (11) IF/SC (13)	263 (47) 256 (52)	251 (47) 283 (55)	**0.0158** 0.34	- 3% (13) + 12%(15)	**0.0183**
Weight (kg)	SC/IF IF/SC	80.9 (11.5) 80.1 (13.6)	79.3 (11.0) 81.8 (14.3	**0.0369** **0.0012**	- 1.9 % (2.6) + 2.0 % (1.6)	**0.0001**
BMI (kg/m^2)	SC/IF IF/SC	29.2 (3.1) 28.2 (2.6)	28.7 (3.4) 28.7 (2.7)	**0.0398** **0.0006**	- 1.9 % (2.6) + 2.0 % (1.6)	**0.0001**
WC (cm)	SC/IF IF/SC	100.5 (7.9) 99.7 (9.6)	98.5 (8.2) 103 (9.6)	**0.0402** **0.0035**	- 2.0 cm (2.8) + 3.3 cm (2.8)	**<0.0001**
Fat mass (kg)	SC/IF IF/SC	28.2 (5.8) 26.8 (7.6)	26.7 (5.9) 27.9 (7.7)	**0.0277** **0.0136**	- 5.6 % (7.0) + 4.2 % (5.1)	**0.0009**
ALT	SC/IF IF/SC	39.8 (12.6) 46.1 (21.6)	30.5 (8.2) 49.3 (23.3)	**0.020** 0.487	- 19.4 % (24.1) + 8.8 % (26.7)	**0.0131**
HOMA-IR	SC/IF IF/SC	2.7 (1.7) 2.9 (1.5)	3.0 (3.0) 3.6 (1.8)	0.9658 **0.0215**	+ 4% (41.7) + 34 % (41)	0.0884

Data expressed as mean ± standard deviation.
*p Values compare the mean values between weeks 12 and 24.
Positive sign (+) denotes an increase, Negative sign (-) denotes a decrease.
Δ Participants with valid studies, n = 14 for both SC/IF & IF/SC unless stated otherwise. Groups are labeled as first 0 – 12 weeks / 12 – 24 weeks
ALT, alanine aminotransferase; CAP, controlled attenuation parameter; IF, intermittent fasting; HOMA-IR, homeostatic model assessment – insulin resistance; SC, standard care.

DISCUSSION

Due to the high prevalence and adverse long-term outcomes of NAFLD there is need for an effective and well-tolerated therapy. Recent data challenges the current dogma surrounding the importance of breakfast.[35] As such, there is increasing interest in IF and TRF with and without CR as a treatment for obesity. We have explored this regimen as a treatment for NAFLD and found that patients in both the IF and SC arms achieved a small but significant reduction in weight loss, total body fat mass and positive changes in adiponectin and leptin. Importantly, only IF patients showed significant reductions in WC, visceral fat

volume, SBP, liver stiffness and, in those with definite steatosis (CAP > 215 dB/m), a significant reduction in hepatic steatosis. Although there was no significant change in fasting insulin or glucose values in this short study, the relative change in HOMA-IR between IF and SC was significant.

This type of lifestyle modification is attractive because of its simplicity in restricting the time to eat rather than which foods to eat. A recent review by Rothschild *et al*. summarized all 11 human trials of TRF.[27] None of these cohort studies or case series had control groups and feeding windows ranged from 4 to 12 h with most not measuring caloric intake. Despite the heterogeneity among studies, they consistently demonstrate a decrease in total cholesterol, triglycerides, glucose and insulin although weight loss was only shown with the longer feeding windows (12 h). An animal study of time restricted feeding by Hatori *et al*. formed the basis for our 8 h feeding window. [36] The 12 to 8 pm feeding times were chosen as they were thought to be acceptable to most participants. To our knowledge ours is the first randomised controlled trial comparing time-related IF to standard advice regarding diet and exercise in patients with NAFLD. The main strength of our study is the accurate reflection of the average individual with NAFLD seen in the primary care and specialist setting. The methodology used, TE and anthropometry, are easily accessible point of care assessments that can be applied in the clinic with little cost and with immediate results to guide decision-making.

NAFLD treatment success is determined by a reduction in hepatic steatosis, inflammation and fibrosis. We used TE to determine liver steatosis as it measures the degree of ultrasound attenuation due to hepatic fat, referred to as CAP and represented in decibels/meter (dB/m). CAP has been shown to correlate well with the amount of steatosis [28,34,37,38] and values > 215 dB/m are ≥ 90% sensitive for steatosis in ≥ 10% of hepatocytes.[34] While we did not reach our primary endpoint and see a change in overall CAP values in either IF or SC group, when we analysed those with baseline CAP >215 dB/m there was a significant reduction of 8% noted in the IF group. It is possible that CAP values below 215 dB/m are unreliable in identifying fatty liver or changes in steatosis

over time. By the end of 12 weeks both TE and visceral fat were significantly reduced in the IF group but not the SC group suggesting that IF may be an effective way of targeting liver and visceral fat. We found that percentage change in WC was moderately associated with percentage change in CAP (Spearman's correlation $r=0.53$, $p<0.0001$). This suggests monitoring WC changes may be a simple way to monitor the effectiveness of therapies targeted at central adiposity and liver steatosis.

Hepatic fibrosis is the main predictor of NAFLD progression,[39] however, liver biopsy is an invasive, costly and undesirable way to monitor fibrosis progression or regression. Determination of LSM by TE is a non-invasive tool that correlates well with histological fibrosis stage in NAFLD.[40] While we found that LSM values in the IF group decreased by 17% after 12 weeks, in the absence of liver histology we are unable to verify a reduction in fibrosis, which would be relatively unlikely in 12 weeks. Similarly, as there was no change in serum TNF-α or ALT levels, decreased inflammation seems an unlikely cause of the LSM change. Improvements in steatosis and NASH histology have been seen with a 5 – 10% weight loss,[41] however the IF group only achieved a 2.5% weight loss by 12 weeks, suggesting that weight loss is also not the reason for the improvement in LSM. Likewise, total body fat reduction does not explain LSM improvements, as both SC and IF had similar decreases in fat mass determined by whole body DEXA. We suggest that LSM improvements may be due to the specific loss of fat in two key areas: visceral and liver fat. Improvements in visceral fat are independently associated with improvements in liver fibrosis.[42] In our study, only the IF group had significant improvements in these parameters. Visceral fat volume was reduced by 13% and WC, a marker of visceral fat, by 3cm. Similar results were not observed in the SC group. These measures of central adiposity and visceral fat in addition correlate with liver steatosis [43] which is also independently associated with liver fibrosis.[44]

We considered caloric reduction as an explanation for the significant metabolic and hepatic changes seen in the IF groups, however there was no change in total

energy consumption from baseline or through the study in either IF or SC participants. Each group on average consumed approximately 7500 kJ/day. Although the caloric intake did not change, the time during which calories were consumed did, which may explain why the IF regimen is liver and visceral fat specific. A recent randomized trial by Koopman *et al*. has shown that despite the same high caloric intake individuals who ate more often rather than consuming three large meals without snacking, had more liver steatosis and abdominal fat independent of weight gain.[45] This study suggests that snacking, or spreading the calories out over the day, contributes to obesity and fatty liver. In our trial, participants in the IF arm were instructed to eat for only 8 h each day, which limited snacking time between meals. Those in the SC arm were able to eat throughout the day. This difference may have contributed to the changes seen in visceral adiposity and liver steatosis.

There was no change in fasting insulin, glucose, cholesterol TNF-α or ALT over the 12 week study period in either IF or SC groups, which is probably because most participants had normal baseline biochemical markers and did not have the metabolic syndrome. However, change was noted in two important adipokines, adiponectin and leptin, which significantly increased and decreased respectively. In the liver, adiponectin increases insulin sensitivity and has anti-fibrotic and anti-inflammatory effects.[46] Low adiponectin levels are associated with more severe liver histology in NAFLD [47] while higher levels correlate with improved liver histology and lower cardiovascular risk.[47,48] Adiponectin levels increase as fat mass decreases;[46] which may explain our findings as we observed decreases in total fat mass in both SC and IF groups and subsequently significant increases in adiponectin levels. Conversely we observed significantly lower leptin levels in both SC and IF groups by 12 weeks. While obesity is associated with leptin resistance,[49] the role of leptin in NASH is controversial as some studies suggest no correlation with serum levels and fibrosis [50] while others have shown that leptin regulates inflammation and fibrosis progression in NASH.[51] Despite the controversy, weight loss results in leptin reduction,[52] which likely explains our findings. A decrease in leptin is thought to stimulate increased feeding [53] but this was not observed in our study.

An association between weight loss and BP reduction has been well established through CR with or without exercise.[54] We showed a reduction in SBP in both IF and SC groups although the reduction was statistically significant only in the IF participants. As both groups lost weight, the difference in SBP could be independent of weight loss or may be related to the small number of participants. Our observed reduction of 6 mmHg has the potential to prevent hypertension related clinical outcomes. Even a 3 mmHg reduction in SBP equates to an 8% reduction in stroke mortality and 5% reduction in mortality from coronary heart disease.[55]

Throughout the study, neither caloric intake nor activity levels significantly changed in either group or when groups crossed-over despite dietary and exercise advice given to the SC groups. Most IF participants (88%) adhered to the 8 h TRF. The 12% lack of compliance (52/422 days) was mainly due to adding milk to morning coffee. Both diets were well accepted with no change in hunger scores, happiness or diet satisfaction between the groups.

At the end of 12 weeks, most participants agreed to crossover for an additional 12 weeks. Those who switched from SC to IF showed further improvements beyond that achieved in the first 12 weeks of SC with significant reductions in weight, WC, total body fat mass and hepatic steatosis. Conversely those who switched from IF to SC had a significant increase in the same variables except hepatic steatosis. In addition there was a significant increase in HOMA-IR in this group. This suggests that beneficial changes incurred with IF are not sustained when IF discontinues, despite advice to adhere to the SC diet and exercise regimen. Conversely, IF can result in metabolic gains for those who have previously following the SC diet and exercise regime.

Our study had a number of limitations. NAFLD was diagnosed by ultrasound, the most commonly used diagnostic tool for detection of hepatic steatosis with a reported sensitivity of 60 – 94% and specificity 66 – 95%.[56] Thus participants may have had variable levels of hepatic steatosis. We used TE to determine liver stiffness and hepatic steatosis, however these outcomes were not confirmed with liver histology. Small participant numbers in each group did not allow for

sufficient power to determine some differences between SC and IF groups. Finally, there is often underreporting in diet histories [57] and our study relied on self-reporting to assess adherence to the time restricted feeding and total dietary intake so true adherence rates may have been lower than reported.

In conclusion, we found that IF and standard diet / exercise advice are equally effective in decreasing body weight and improving serum leptin and adiponectin values. However, IF may be superior in targeting the liver with improvements in transient elastograpy (liver stiffness, steatosis) and visceral adiposity in addition to decreased WC and visceral fat volume. These changes are clinically relevant and are novel findings for a short duration lifestyle intervention study. This pilot study provides sufficient evidence to support a larger randomised controlled trial in a well-defined NAFLD population.

AUTHORS' CONTRIBUTIONS:

All authors made substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. AH and GM conceived and designed the study and AH drafted the manuscript. AH also performed blinded outcomes. AM organised, instructed and collected data from participants. CT oversaw the collection and analysis of dietary data. JT performed protein assays. DH analysed visceral fat CT. AM, GM and WS contributed to the manuscript draft and revised it critically for important intellectual content. All authors approved the final version for publication.

COMPETING INTERESTS

The authors have no competing interests to declare.

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4.5 SUPPLEMENTAL MATERIAL

Table 1: Exclusion criteria

Current or prior (past 10 years) alcohol intake ≥30g/day in men and ≥20g/day in women.
Hepatitis B infection (detectable Hepatitis B surface antigen) or Hepatitis C infection (detectable HCV RNA).
Other liver diseases including: autoimmune hepatitis, primary biliary cirrhosis, Wilson's disease, primary sclerosing cholangitis
Poorly controlled Type 2 diabetes mellitus as defined by HbA1C >8.0%
Type 2 diabetes mellitus on insulin, sulphonylurea agents or other glucose lowering therapy
Pregnant or breast-feeding
Cirrhosis with current or a history of decompensated events
Current therapy with: a. Magnesium or aluminium containing antacids b. Anticonvulsants including primidone c. Barbiturates d. Calcitonin e. Bisphosphonates such as: etidronate and pamidronate f. Thiazide diuretics g. Cholestyramine h. Milk thistle i. Vitamin E j. Thiazolidenediones k. Corticosteroids l. Amiodarone m. Tamoxifen n. Methotrexate o. Oestrogen p. Selective serotonin re-uptake inhibitor (started within 4 weeks)

Table 2: Gastroenterological Society of Australia (GESA) guidelines for NAFLD management

1. If overweight, begin a weight management program of their choice with the goal of gradual 0.25 to 0.5 kg weight loss a week.
2. Aim for a waistline of 80 cm (or less) if female or 95 cm (or less) if male.
3. Exercise at least 5 days a week consisting of both aerobic and resistance exercise.
4. Eat a healthy diet low in fat and calories, high in fibre and pay attention to serving size.
   Participants were provided with the GESA information sheet on NAFLD for further reference and information.

Methods: secondary endpoints

Abdominal computer tomography (CT) for visceral fat volume

All CT were performed at Monash Medical Centre at 0, 12 and 24 weeks using a GE Lightspeed (General Electric Medical Systems, Milwaukee, WI, USA). A single abdominal slice was taken of the lumbar spine at the L4/5 level. The L4-5 level was analysed for visceral fat area (VFA), a single slice at this level has been shown to correlate highly with total visceral fat volume.[1] Digital Imaging and Communications in Medicine images were imported and analysed for body composition using SliceOmatic 4.3 (TomoVision, Montreal, Canada). Hounsfield unit (HU) ranges were used to differentiate between components of body composition on the images. Tissue from -30 to -190 HU was segmented as fat, and tissue from −30 to 150 HU was segmented as muscle. Fat inside the abdominal muscle layer was then differently segmented as visceral fat, and fat outside the abdominal muscle wall was considered as subcutaneous fat. Any fat inside muscle was segmented as inter-muscular fat. VFA was calculated from the sum of areas for the relevant segments. A single, experienced observer, blinded to the participants grouping performed all SliceOmatic segmentations. 12 out of 14 in the IF group and 11 out of 14 in the SC group had suitable images at baseline and week 12 for analysis. After the crossover, 7 of 11 in the IF group and 12 of 13 in the SC group had suitable images at 24 weeks for analysis.

Reference:

1. Yoshizumi T, Nakamura T, Yamane M, *et al*. Abdominal fat: standardized technique for measurement at CT. *Radiology*. 1999 Apr 1;211(1):283–6

Whole body dual-energy X-ray absorptiometry (DEXA)

Body composition for total body fat was assessed at Monash Medical Centre using the same GE lunar Prodigy DEXA on standard mode (software version 13.60).

Blood pressure

This was performed using a manual sphygmomanometer by the investigator blinded to the participant's grouping. A single reading was taken on the right arm with the patient seated.

Anthropometry

The study investigators performed anthropometric measurements. Participants were weighed in hospital gowns using the same Wedderburn DS-530 Handrail scale with weights recorded to the nearest 0.1kg. Height was measured using a stadiometer and recorded to the nearest 0.5cm. WC was measured at the midpoint between the superior aspect of the iliac crests and the inferior margin of the ribs, recorded to the nearest 0.5cm. Anthropometry measurements were performed by an investigator blinded to the participants grouping.

Faecal calprotectin

Faecal material was collected by individuals at home and frozen at -20°C before delivering to investigators. Samples were then stored at -80°C until the end of the study. Faecal samples were processed with Buhlmann Smart-Prep Extraction kit followed by Buhlmann Calprotectin ELISA following manufacturers instructions (Taylor Scientific, St Louis, USA). Analysis was done using a microplate reader at 450 nm (Magellan). Faecal calprotectin concentration was calculated from the standard curve generated in GraphPad Prism 5.0d for Mac OS X (GraphPad Software, La Jolla, CA, USA).

Serum markers: (alanine aminotransferase (ALT), insulin, glucose, total cholesterol, triglyceride, tumour necrosis factor (TNF)-α, adiponectin, leptin)

All participants were asked to fast for at least nine hours prior to venepuncture. Monash Health Pathology, using assays accredited by the National Association of Testing Authorities, processed blood samples for ALT, insulin, glucose, total cholesterol, triglyceride and stored serum at -80°C. Stored serum was used for TNF-α, adiponectin and leptin analysis by ELISA according to the manufacturer's instructions (R&D Systems Minneapolis, MN, USA & Cusabio, Wuhan, Hubei Province, China). Analysis was done using a microplate reader at 490 nm for TNF-α and 450 nm for adiponectin and leptin (Magellan). TNF-α, adiponectin and leptin concentrations were calculated from the standard curve generated in GraphPad Prism 5.0d for Mac OS X (GraphPad Software, La Jolla, CA, USA).

Methods: questionnaires

Visual Analogue Scale (VAS)

Subjective appetite sensations were determined using a VAS. The VAS assessed participant hunger, satisfaction and happiness with diet over the preceding 24 hours using scales 10 cm in length. Participants completed questionnaires by making a mark along each line to indicate how they were feeling, eg. "least hungry" to "most hungry". Results were expressed as centimetres from zero, recorded to the nearest 0.1 cm.

World Health Organization Quality of Life Questionnaire-BREF (WHOQOL-BREF)

The WHOQOL-BREF consists of 26 questions relating to health and psychosocial aspects of participant's life. It is an abridged version of the WHOQOL-100 questionnaire and covers four domains: physical, psychological, social and environmental. Participants were asked to consider the questions with respect to the preceding four weeks and score each question 1 to 5, with higher scores indicating better quality of life. Each domain score was then calculated by averaging the scores of the items in that domain. Calculation of domain scores and cleaning of data was performed in accordance with the WHOQOL-BREF user's manual.

Global Physical Activity Questionnaire (GPAQ)

Participants were asked to complete 19 questions assessing the time spent per week partaking in physical activity across three domains: work, transport and leisure. Within these domains, physical activity was further categorised into two sub-domains: vigorous or moderate activity. Based on the GPAQ responses the number of Metabolic Equivalent (MET-) minutes achieved by each patient per week were calculated. Calculation of MET-minutes and data cleaning were performed in accordance with the GPAQ Analysis Guide.

Table 3: Questionnaires

	Group	Week 0	Week 4	Week 8	Week 12	p Value
VAS score						
Hunger over last 24 hours	IF	4.4 (1.2)	4.0 (1.4)	3.7 (2.2)	4.8 (1.8)	NS
	SC	4.1 (2.3)	3.6 (1.7)	4.2 (2.2)	4.8 (2.0)	
Satisfaction over last 24 hours	IF	5.7 (1.8)	5.6 (1.7)	5.3 (1.7)	5.5 (1.8)	NS
	SC	7.0 (2.0)	6.7 (1.4)	6.1 (2.5)	6.3 (1.5)	
Happiness with diet over last 24 hours	IF	5.2 (1.3)	6.5 (1.8)	6.4 (1.9)	6.5 (2.1)	NS
	SC	5.7 (2.0)	6.2 (1.7)	5.7 (2.2)	6.0 (1.8)	
GPAQ						
Metabolic equivalent hours (kcal/kg) per week	IF	45.4 (44.6)	53.5 (52.8)	54.2 (66.9)	66.5 (90.9)	NS
	SC	40.6 (53.3)	52.4 (53.5)	37.3 (37.3)	45.1 (43.2)	
WHOQOL-BREF						
Physical	IF	15.4 (1.9)	15.6 (1.9)	15.4 (1.8)	15.4 (1.8)	NS
	SC	15.5 (2.1)	15.4 (2.2)	15.3 (2.2)	15.7 (2.4)	
Psychological	IF	14.8 (1.9)	15.3 (1.5)	15.4 (1.5)	15.6 (1.5)	NS
	SC	15.8 (1.7)	16.2 (1.8)	15.6 (1.6)	16.4 (1.6)	
Social	IF	14.3 (2.1)	14.8 (3.0)	14.4 (2.7)	14.2 (2.8)	NS
	SC	15.6 (2.7)	15.7 (2.5)	15.4 (2.6)	15.2 (2.4)	
Environmental	IF	16.2 (1.5)	15.7 (1.1)	16.1 (1.5)	15.9 (1.2)	NS
	SC	16.9 (1.6)	16.9 (1.8)	16.6 (2.0)	17.0 (1.9)	

Data expressed as mean ± standard deviation.
*Comparisons between means were done using ANOVA or t-test as appropriate.
IF, intermittent fasting; GPAQ, Global Physical Activity questionnaire; MET, metabolic equivalents; SC, standard care; VAS, visual analogue scale; WHQOL-BREF, World Health Organisation Quality-of-life-BREF

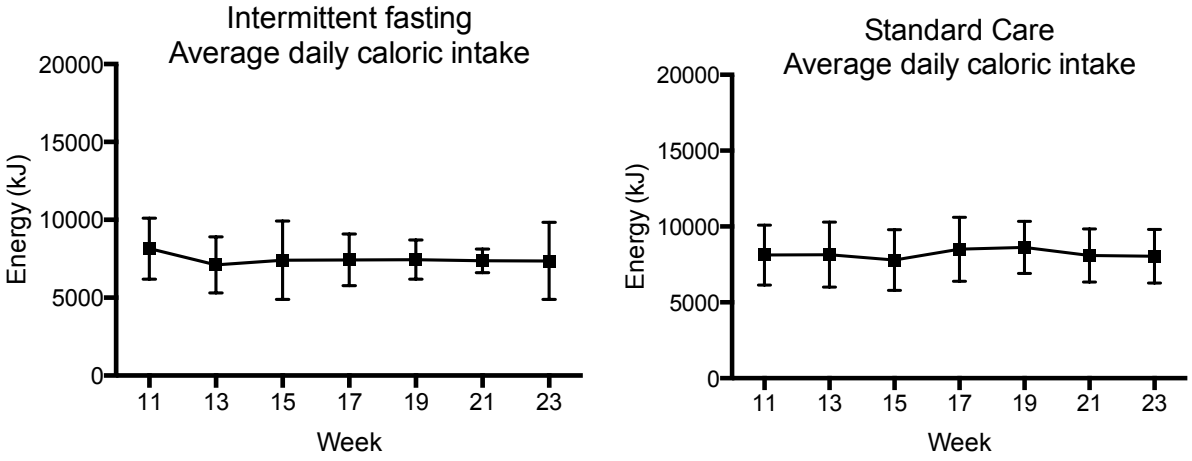

Figure 1: Total daily caloric intake in crossover 12 – 24 wks
Average (±SD) caloric intake over 3 days from self-reported 3-day food diary completed every 2 weeks. Food diaries were analysed by a dietitian.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

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5.1 hAEC as cell-based therapy for liver fibrosis and regeneration

This section of the thesis principally describes an experimental cell based therapy using hAECs or their soluble factors in conditioned medium (CM). The use of hAECs has numerous benefits over other cells types such as BM derived MSCs. Human AECs are derived from the fetus and easily retrieved from the amnion of the placenta. One placenta produces an average of 120 million viable hAECs. There is no shortage of placentas with over 350,000 live births per year in Australia (http://www.abs.gov.au/ausstats/abs@.nsf/mf/3301.0). Most placentas are discarded making hAEC utilisation the ultimate in bio-recycling. This reduces the ethical concerns and makes hAECs a significant cell source for therapeutic applications.

In previous publications from our laboratory, hAEC therapy was investigated in a CCl_4 mouse model of chronic liver fibrosis and we demonstrated that treatment with these cells results in improvement of liver fibrosis due in part to effects on hepatic stellate cells (HSC) and macrophages (Manuelpillai et al., 2012; 2010). This thesis adds to earlier work by clarifying the anti-fibrotic effects of hAEC on HSC and exploring the effects of hAEC on liver regeneration through liver progenitor cell (LPC) manipulation. These experiments were completed using hAEC CM. Human AEC CM is serum free media containing soluble factors released from hAEC after 72 hours of culture. Conditioned media was used to observe the effects of hAEC on HSC and LPC as co-culturing of hAEC with either cell type proved problematic. Attempts were made to culture these cells together in a direct culture or co-culture system but cell viability was unsatisfactory. Despite varying the conditions (differing cultureware, timing, seeding density and growth media), only co-culturing LPC with HSC using transwells was suitable and reproducible (chapter 3, 3.2.9).

The use of CM as a therapy may have specific benefits over the use of cells. Currently cell therapy for liver disease is impeded by the inability to monitor the fate of transplanted cells, safety concerns regarding tumourgenicity, and long term effects of the immune system on survival of the transplanted cells (Puglisi

et al., 2011; Yu et al., 2012). These issues may be overcome with the use of CM. Additionally it is evident from these experiments and those in other studies (Baglio, Pegtel, & Baldini, 2012; Lavoie & Rosu-Myles, 2013; Skalnikova, 2013) that stem cell medicine may not need to rely on cellular therapies *per se*. The bounty may, instead, lie in unravelling the specific components released from the cells. Others have shown that CM from cells such as mesenchymal stem cells have demonstrated anti-fibrotic effects (Ueno et al., 2013).

Results of experiments in chapters 2 and 3 indicate that hAECs exert their anti-fibrotic and liver regenerative effects through soluble factors. Potential factors that may have an anti-fibrotic effect on HSC include soluble human leukocyte antigen (sHLA)– G1, tumour necrosis factor (TGF)-β3, Angiotensin (Ang)-1-7 and prostaglandin E_2 (PGE_2). Human AEC are known to secrete soluble human leukocyte antigen G1 (sHLA-G1) (Pratama et al., 2011; Tee et al., 2013). HLA-G is a major histocompatibility complex class I molecule that is expressed in the placenta and is involved in maternal tolerance of the fetus. It has immunomodulatory properties and suppresses natural killer and cytotoxic T cells (González et al., 2012). I found that treating HSC with a range of sHLA-G1 concentrations had a dampening effect on TGF-β1 (protein and mRNA expression) but had no effect on HSC collagen production (chapter 2, Fig. 6). Prostaglandin E_2 is produced by hAEC (Liu et al., 2012; Tahara et al., 1995). *In vitro* studies suggest that prostaglandins are key immunomodulatory molecules released by BM derived MSCs (Rossi, Pianta, Magatti, Sedlmayr, & Parolini, 2012) and that they exert anti-fibrotic effects through induction of fibroblast apoptosis (Maher et al., 2010). Although PGE_2 has been shown to reduce collagen production in HSC (Hui et al., 2004), I did not find that it suppressed collagen from CM treated HSC (chapter 2, supplemental Fig. 3). Despite beneficial anti-fibrotic effects *in vitro*, in human liver disease, excess PGE_2 is thought to be detrimental by dampening the immune response associated with cirrhosis (O'Brien et al., 2014).

To exploit the benefits of hAEC CM and avoid potential adverse events, the soluble factors require detailed characterisation and accompanying dosage

studies. As an alternative to studying candidate molecules, it may be more useful to characterise the hAEC secretome, which includes all soluble factors released from the hAEC that are contained in the CM. Analysis of these components involves the use of protein digestion followed by mass spectrometry and peptide-mass fingerprinting. Many of the proteins are secreted in low abundance and can be difficult to detect. In this case, concentration of the CM or use of sensitive assays such as antibody microarrays can be employed. To determine the relevance of the protein identified, bioinformatics tools can be applied (Caccia, Dugo, Callari, & Bongarzone, 2013).

Two future considerations for the development of hAEC CM as a therapeutic agent are the optimal conditions for hAEC CM collection and hAEC donor variability. In regards to the former, there is evidence that stem cell priming is a critical step for therapeutic use. Hypoxia conditioning (Muscari et al., 2013), oxytocin treatment (Kim et al., 2012), inhibition of Rho kinase activity (Lee et al., 2014) and mechanical stimulation (Subramony, Su, Yeager, & Lu, 2014) have all been shown to differentially prime stem cells. However, priming regimens have never been assessed in hAECs. The optimal pre-conditioning regimen that hAECs should undergo prior to collecting CM is unknown. It may be useful to vary priming conditions depending on the disease being treated, for example, there may be cellular cross-talk between hAEC and specific 'target' cells, such as HSC or LPC, which alter the hAEC secretome. Our laboratory's method of CM production depended on hAEC confluence, priming with protein rich foetal calf serum, then washing and collecting CM after 72 hours in serum-free conditions. This 72 hour exposure window was determined after I set-up different times of serum-free exposure to confluent hAEC (24, 48, and 72 hours) and found that CM derived after 72 hours produced an optimal anti-fibrotic effect on HSC without a decrease in hAEC viability. Experimental results in this thesis may have been different had the cells been primed with different conditions.

The results of MSC studies with cytokine and hypoxia pre-treatments may provide a guide for future hAEC studies. Using MSC's primed with TNF-α and IL-1β, Ren et al. demonstrated increased release of chemokines and nitric oxide in

to the media, which had an enhanced immunosuppressive effect on T-cells. (Ren et al., 2008). An animal study of ischaemic heart disease by Luo et al. demonstrated that treatment of MSC with IL-1β and TGF-β increased their release of vascular endothelial growth factor (Luo et al., 2012). Interestingly, VEGF is involved in liver regeneration (Taniguchi, Sakisaka, Matsuo, Tanikawa, & Sata, 2001), and is beneficial in resolution of liver injury. Hypoxic conditions also influences MSCs. Wei et al. exposed rat MSC to an hypoxic environment and found that this induced the secretion of MMP-2 and MMP-9 (Wei et al., 2013). Both of these are potent collagenases and are involved in liver fibrosis resolution. Other investigators have found that exposing stem cells to non-damaging hypoxic conditions prior to transplantation increases the survival and differentiation of the cells upon transplantation which enhances their regenerative potential (Kavanagh, Robinson, & Kalia, 2014; Muscari et al., 2013). There is a paucity of knowledge on the effects of hAEC priming, but these studies suggest that altering hAEC conditions to produce CM or prior to cell transplantation may affect the efficacy of the therapy. Further attention should be given to this in the future.. Current stem cell banking practices ignore the need for cell priming and are focused on the safety of autologous applications. While safety is clearly important, optimising the conditions to achieve optimal hAEC and CM potency should not be ignored.

The second consideration regarding the development of hAEC CM is donor-to-donor variation. In chapter 2, I assessed the anti-fibrotic effects of CM from 4 individuals and from 3 different pools of CM with 4 individuals in each pool (chapter 2 supplemental material). When compared to the control, there was no significant difference in reduction of collagen content in HSC treated with CM from individuals or CM from pooled donors. Although the overall anti-fibrotic effect was the same, there was still a small degree of variability among donors. These results suggest that the use of separate or combined donors does not change the potency of treatment. Pooling donors would simplify transplantation in human trials and would resolve potential issues with varying hAEC yields from the isolation process. The ability to pool however, assumes that all donor

hAEC behave the same way. Donor variation may also be dependent of the clinical condition and complexity of the disease.

I tested the anti-fibrotic effects of single donor or pooled hAEC CM using proline incorporation as a marker of collagen production in HSC. This is a quick and reproducible test to screen for a donor's anti-fibrotic potential, however it is not known whether these *in vitro* results translate into similar findings in animal studies. It is also not known whether proline incorporation is the optimal screening test for the anti-fibrotic potential of hAEC. Other potential anti-fibrosis screening tests on HSC could include a combination of α-SMA staining (for HSC activation), Brd-U incorporation (proliferation assay) or colourimetric collagen assay for extracellular collagen (which represents the result of collagen production plus degradation). We also do not know what donor-to-donor variability exists for other applications such as liver regeneration or for macrophage specific effects. Determining donor variability may include staining LPC cultures treated with donor CM for albumin or AFP. To test the potential to affect macrophages, donor CM could be tested in its ability to increase collagenase production or phagocytic potential of macrophages. Screening would allow elimination of less potent donors and the potential to pool donors whose hAEC demonstrate potential efficacy for specific conditions. A screening platform for the suitability of a given donor for a given clinical application, incorporating different priming regimens, would best address these concerns.

Liver progenitor cells (LPC) are resident liver cells that reside within the Canal of Hering and remain dormant and in small number under normal physiological conditions. In the case of an acute injury hepatocytes replicate and facilitate regeneration of the liver. In chronic liver injury, persistent inflammation characterised by the presence of cytokines including IFN-γ and TGF-β inhibits hepatocyte induced regeneration but does not affect the reparative ability of the LPC (Brooling, Campbell, Mitchell, Yeoh, & Fausto, 2005; Nguyen et al., 2007). It is in this environment LPC take the lead role in liver repair and extensively proliferate and migrate into the liver lobules. These bipotent progenitors then differentiate into both hepatocytes and cholangiocytes in attempts to restore the

damaged liver parenchyma. The proliferative response of the LPC occurs independently of the underlying disease aetiology but parallels the severity of the chronic injury (Lowes, Brennan, Yeoh, & Olynyk, 1999).

LPC are closely situated with HSC, macrophages and the extracellular matrix, all of which influence LPC proliferation, migration and differentiation in the diseased liver. Activated HSC in fibrotic livers facilitate the LPC response (Pintilie et al., 2010). The infiltration of extrahepatic macrophages induce LPC proliferation through TWEAK, then differentiation into hepatocytes and cholangiocytes through the Wnt and Notch pathways respectively (Bird et al., 2013; Boulter et al., 2012). Laminin, one component of the extracellular matrix in the fibrotic liver, maintains the undifferentiated LPC state (Lorenzini et al., 2010). However, if there a stimulus for collagenase production and matrix remodelling begins, this degradation enables LPC to mediate regeneration (Kallis et al., 2011; M. J. Williams, Clouston, & Forbes, 2014). A successful anti-fibrotic therapy must induce matrix degradation and in turn facilitate LPC mediated liver restoration. However, the therapy should also be able to reduce activated HSC and macrophages which could potentially have the opposite effect to matrix degradation on the LPC population. The ideal therapy would be able to overcome these competing interests and result in a controlled LPC response. This notion was a major aim of the work in chapter 3, which was to determine whether hAECs would also be able to stimulate LPC proliferation and differentiation.

In chapter 3, I demonstrated that hAEC therapy reduced but did not eliminate the LPC proliferative response in a mouse model of chronic liver disease. *In vitro* experiments revealed that LPC exposed to hAEC CM increased proliferation and differentiation into hepatocytes and maintained cholangiocyte differentiation. To demonstrate the presence of cholangiocytes, I used a well characterised biliary epithelium marker, CK-19 (Stosiek, Kasper, & Karsten, 1990), which unlike other cholangiocyte markers, such as secretin receptor and CFTR , is expressed in all biliary epithelial cells regardless of size (Glaser et al., 2009). Cytokeratin-7 is another cholangiocyte marker whose expression usually

parallels that of CK-19, however I was unable to establish a working primer for rtPCR. Hepatocyte differentiation was confirmed by functional markers such as albumin expression and glycogen storage.

HSC and macrophages both support the LPC response. So I considered that hAEC therapy may suppress LPC proliferation and differentiation as studies have shown that hAEC therapy reduces inflammatory cytokines and reduces the number of hepatocyte macrophages and activated HSC (Manuelpillai et al., 2010; Murphy et al., 2012; 2011; Vosdoganes et al., 2011). In chapter 2, I also demonstrated that hAEC CM suppressed expression of the inflammatory cytokine, TGF-β, in HSC. Inflammatory cytokines such as TNF-α (Knight et al., 2000), TGF-β (Nguyen et al., 2007)and IFN-γ (Brooling et al., 2005) are necessary for LPC induced liver restoration in chronic liver injury.. Although there was a small reduction in LPC in hAEC treated animals, the *in vitro* work demonstrates the ability of hAEC to enhance LPC proliferation and hepatocyte differentiation. This is a novel and important finding as it suggests that despite the effect hAEC has on reducing inflammation and fibrosis, this does not appear to have a deleterious anti-proliferative effect on the LPC population. These findings provide support for the clinical use of hAEC therapy in patients with impaired liver regeneration.

Current and Future directions

Ongoing research is required if we are to see hAEC or CM therapy used in human trials. I am looking at three areas that need to be addressed: model specificity, characterisation of CM and the cellular mechanisms of the liver regenerative response. I have shown that hAEC and CM have anti-fibrotic effects on a CCl_4 induced murine model of chronic liver disease. To determine whether these effects are model specific, I sought to use hAEC and CM in alternate mouse models of chronic liver disease. I first attempted to develop a murine bile duct ligation model but due to technical issues, this model was not pursued. Due to high prevalence of NASH in the community I chose to develop a murine model of NASH. This is the most common cause of chronic liver disease in the community and development of hAEC as a successful anti-fibrotic therapy would also need to

show efficacy in this condition. I developed a diet-induced model adapted from the fast-food diet mouse developed by Charlton et al. (Charlton et al., 2011). I fed male C57/Bl6 mice a diet consisting of 21% fat, 2% cholesterol with drinking water containing 42g/L of a fructose sucrose mixture, which I labelled as the 'western diet' (WD). By 8-months, animals developed the characteristic chicken wire pattern of hepatic fibrosis seen in NASH. At this time point mice were divided into 3 treatment groups and 2 control groups. The 'single dose hAEC ' treatment group received a single IP injection of 4 million hAEC cells at 8 months. The second treatment group ('double dose hAEC') received an IP injection of 4 million hAEC cells at 8 months and a second IP injection of 4 million hAEC at 9 months. The third treatment group ('CM group') received CM given IP three times a week from 8 months. There were also two WD control groups, one given no treatment, the other given control media IP three times a week starting at 8 months. A control group of C57/Bl6 fed a standard chow diet was maintained concurrently with the WD model. At 10 months all animals were culled.

At 10 months WD mice treated with either single or double dose hAEC demonstrated a 40% reduction in fibrosis area compared to the WD control (assessed by Sirius red collagen staining using computer-assisted morphometric analysis) ($P<0.05$). Mice treated with CM demonstrated a 27% reduction in fibrosis compared to controls, but this did not reach statistical significance. Liver macrophages were stained using a well-recognised marker F4/80. Treatment of WD mice with single dose hAEC, double dose hAEC or CM demonstrated a reduction in hepatic macrophage density by 42%, 52% and 50% respectively compared to controls ($P<0.0001$). A marker of hepatocyte injury, serum ALT was measured in treated and control animals. Despite the improvements seen in macrophages numbers and fibrosis, treatment with hAEC or CM did not decrease the ALT elevation seen in WD animals. This outcome was surprising given the demonstrated anti-inflammatory effect of hAEC and CM treatment in the CCl4 model. I am investigating this further by analysing liver lysates for the inflammatory cytokines IFN-γ, TGF-β, TNF-α, IL-1β, IL-6 and IL-10. In addition, metabolic endpoints, liver and serum triglycerides and IP glucose tolerance

testing will be assessed. Finally a number of animals were culled for FACS analysis, which I am analysing for macrophage markers Ly6C and CD11b to determine whether hAEC or CM induces an anti-fibrotic phenotype $CD11b^{hi}$, $Ly6C^{lo}$, in hepatic macrophages. To date the results from this model are encouraging and suggest that the anti-fibrotic effects of hAEC are not model specific. Additional work is required to determine the difference in efficacy between hAEC and CM.

In regards to the characterisation of CM, our lab is working on various approaches to achieve this goal. We are using the proline assay described in chapter 2 as a screening tool to determine the anti-fibrotic efficacy of various derivations of CM on HSC. Another approach is to determine whether the soluble molecular candidates in CM are transported in exosomes or whether exosomes, independent of their cargo, affect HSC activation. Exosomes are 30 – 100 nm diameter microvesicles derived from the cell membrane that are shed through exocytosis and contain intracellular proteins and RNA. They are implicated in specialised areas such as signalling in various tissues including the liver (A. I. Masyuk, Masyuk, & Larusso, 2013). There is evidence that exosomes may play a role in HSC collagen production (Koeck et al., 2014). Members in our lab have isolated exosomes from CM and have treated HSC with both purified exosomes and exosome depleted CM. Preliminary results have revealed that exosome deplete CM has a greater effect at inhibiting HSC collagen production than either standard CM or an isolated exosome solution. This work is continuing and *in vivo* experiments are planned. We are also employing another approach, to analyse various protein fractions in CM based on size. A number of size specific fractionation columns are currently waiting to be tested. Following these tests, we will investigate which specific proteins are present in purified fractions and the exosome deplete CM by means of mass spectroscopy.

The liver progenitor work is in its infancy. The microarray data described in chapter 3 requires further bioinformatics analysis and confirmation of the findings with PCR. The view is to determine the pathways involved in LPC differentiation induced by CM. To further study this phenomenon *in vivo,* we

plan to isolate LPC by FACS from mice with CCl_4 induced chronic liver disease treated with hAEC or CM. These cell populations will then be single cell sorted and analysed in a microfluidics system (Fluidigm®) to identify genes involved in LPC proliferation, hepatocyte and cholangiocyte differentiation. This platform allows for comparison of cell populations from a complex organ such as the liver. Our particular set-up will enable us to assay 48 different genes in 48 individual cells. Using the Fluidigm® platform has benefits over conventional bulk isolation of cell types because of the ability to study individual cells from a sparse population in the liver, such as the LPC and also to distinguish potential subtle differences among cells with similar attributes, like macrophages. Further studies are planned to elucidate the interaction between macrophages, HSC and LPC using a combination of FACS / Fluidigm® analysis and multi-colour fluorescence confocal microscopy.

5.2 Intermittent fasting and NAFLD

There is an epidemic of NAFLD in our society. It crosses ethnic and socio-economic boundaries and is inextricably linked to the rise of obesity and the metabolic syndrome. Like other liver diseases that produce chronic liver injury, NAFLD, or more accurately the inflammatory form - NASH, can lead to liver cirrhosis and its complications. NAFLD can also lead to HCC in both cirrhotic and non-cirrhotic forms of the disease. The disease burden is not restricted to the liver. A number of other conditions are associated with NAFLD including: colorectal adenomas, obstructive sleep apnoea, polycystic ovarian syndrome, hypothyroidism, diabetes and cardiovascular disease (Torres, Williams, & Harrison, 2012). Most individuals with NAFLD do not die from liver disease but from the complications associated with cardiovascular disease and cancer.

At present time, there is no approved drug therapy for NAFLD leaving lifestyle intervention the cornerstone of management. This intervention centres on diet modification through calorie restriction with recommended moderate exercise and a target of 5 – 10% weight loss (Chalasani et al., 2012). However, there have been long-standing issues with adherence to lifestyle measures. Most people are

unable to sustain these measures long term, often regaining the weight initially lost. Alternative, simpler, more sustainable options are required. One such approach is through fasting with or without intended caloric restriction. In chapter 4, I investigated intermittent fasting (IF). This intervention employed a fasting window of 16 hours (between 8pm and 12 midday the following day), and a non-fasting window (between 12 midday and 8 pm). During this window, participants were allowed to eat *ad libitum*. Participants with NAFLD, based on ultrasound criteria, were randomised to receive advice for IF or standard diet and exercise advice [standard care (SC)] as per the Gastroenterological Society of Australia (GESA) guidelines. Each group was assessed over a 12-week span, then asked to crossover to the opposite group for an additional 12 weeks. At the end of 12 weeks both groups saw significant improvements in weight, BMI, body fat, leptin and adiponectin. Those in the IF group had a significant decrease in waist circumference, visceral fat and transient elastography (Fibroscan®) results of liver stiffness and steatosis. Crossover resulted in further improvements in liver steatosis, weight, waist circumference and body fat in those changing from standard care to IF. Despite repeated fasting there was no overall change in the visual hunger scale when participants moved onto the IF regimen nor was there any change in total caloric intake when people began IF.

To my knowledge, this is the first randomised controlled trial comparing IF without calorie restriction to standard lifestyle advice. I demonstrated that IF is an effective weight loss tool and may preferentially target liver and visceral fat compared to standard lifestyle advice. The beauty of the IF regimen lies in its simplicity. There are no complicated instructions on caloric intake, or on what to eat, only when to eat. Additionally, the majority of endpoints are easily obtained in a clinic setting so that individuals can receive immediate results. Fibroscan®, unlike liver biopsy, offers real time feedback on liver specific indices. On the spot feedback benefits patients as it is a motivational tool to encourage ongoing commitment to lifestyle interventions. As an intervention, IF was tolerated well and surprisingly did not significantly alter participants overall sensation of hunger. Some of the participants commented on the 'hunger bump' that was felt in the morning when they were unable to eat breakfast, but this did not

significantly affect adherence. Overall hunger, satisfaction and happiness with IF or SC were similar. The majority of people were able to adhere to the advised 8 hr 'eating window'. Few people consumed calories outside this window.

At the end of 12 weeks, measurements of liver and visceral fat were decreased in the IF compared to SC group. Those who crossed over from SC to IF continued to show improvements, while those who converted from IF to SC gained weight and waist circumference. These findings were observed despite the absence of calorie restriction. Although calories did not change, the time during which participants could consume calories did. This suggests that *when* you eat may be equally or more important than *what* you eat. Koopman *et al*. recently showed that a hypercaloric diet with high meal frequency increased abdominal and liver fat when compared to the same caloric intake consumed at standard meal times (Koopman et al., 2014). They concluded that the act of snacking or spreading calories throughout the day may have deleterious effects and be contributing to obesity and NAFLD. Participants in the IF group were limited from snacking due the 8 hr 'eating window', which was reflected in their food diaries. An animal study by Hatori *et al.* investigated the possible mechanisms responsible for the benefits gained with time restricted feeding (Hatori et al., 2012). They studied two groups of mice that consumed the same high fat diet, one during a restricted 'feeding window' and the other, *ad libitum*, throughout the day. Mice in the time-restricted group were protected from obesity, diabetes, liver steatosis and inflammation. Hatori determined that *ad libitum* feeding disturbs nutrient regulators such as mTOR and AMPK which resulted in increased fat mass and metabolic dysregulation such as insulin resistance (Hatori et al., 2012).

The timing of meals may also negatively influence the gut microbiota. There is emerging evidence that meal frequency may be related to systemic inflammation as increased meal frequency leads to increased gut derived bacterial endotoxin release (Piya et al., 2014). Gut flora can be influenced through caloric restriction which may result in health benefits such as increased longevity (Zhang et al., 2013). Manipulation of gut flora through lifestyle interventions has been shown to improve or prevent metabolic disorders such as obesity (Chen, He, & Huang,

2014; DiBaise et al., 2008). Additionally, faecal flora may be involved in pathogenesis of NAFLD (Chassaing, Etienne-Mesmin, & Gewirtz, 2013; Frasinariu, Ceccarelli, Alisi, Moraru, & Nobili, 2013). It is possible that IF without calorie restriction influences gut microbiota, which in turn may have accounted for some of the findings. Although faecal samples were collected at 0, 12 and 24 weeks, microbiota testing was not performed.

There were a number of limitations that need to be taken into account, many of which were described in chapter 4. This was a small study of a heterogeneous group of patients with NAFLD, the diagnosis of which was made via ultrasound. While this reflects a real world scenario, reliance on ultrasound diagnosis has inherent problems. Some participants in the study may not have had NAFLD and the degree of steatosis could not be confirmed at the start of trial as this would have required liver biopsies. Liver biopsies performed at baseline would have provided an accurate measurement of steatosis and fibrosis. Additional biopsies completed at the end of the study would have enabled an accurate depiction of steatosis and fibrosis change over the course of the study, acknowledging that fibrosis is unlikely to change significantly over 12 weeks. Despite the usefulness of a liver biopsy, it is invasive and is not without risk. Participants would have been more reluctant to participate in a study that required serial biopsies. Furthermore, the inherent risks of liver biopsy make for challenging ethical approval. Lack of metabolic syndrome in the participants was another limitation. Although the majority of participants had central adiposity, half of them did not have metabolic syndrome. The lack of metabolic dysfunction may have limited the ability to detect changes in many of the biochemical and cytokine markers.

Future directions

Future studies ideally should have a larger number of participants and a more homogenous participant group, such as those with NAFLD *and* metabolic syndrome. These factors may enable the observation of significant change in a greater number of endpoints. Histological endpoints from biopsies are largely accepted as the gold standard in trials involving liver diseases. Change in

steatosis based on computerised morphometric analysis of liver biopsies at baseline and at the study's conclusion would be a more accurate measurement of liver fat than Fibroscan®. An alternative non-invasive tool to assess hepatic steatosis is Hydrogen-1 MR spectroscopy as it correlates closely with the histological grade of steatosis (Georgoff et al., 2012). Further endpoints from liver biopsy would be change in percentage of fibrosis (based on morphometric analysis) and NASH activity score, graded by a pathologist. Additionally, faecal microbiota DNA could be extracted from stool samples and used to ascertain phenotypic changes over the course of the study.

Conclusion

Chronic liver disease is a progression of liver fibrosis secondary to a chronic inflammatory process caused by a number of possible aetiologies, the most common being NAFLD. This thesis sought to address both ends of the disease spectrum. In chapters 2 & 3, I explored a treatment for the disease at the end of the spectrum by focussing on improving fibrosis and liver injury. This was through the use of hAEC and CM on its anti-fibrotic effects on the HSC and liver regenerative effects on LPC. The focus of chapter 4 was on tackling the aetiology of chronic disease at the beginning of the disease spectrum, before it could progress. In this chapter, I addressed the most common liver disease, NAFLD through a novel lifestyle intervention.

The work presented in this thesis adds to the growing body of evidence advocating the effectiveness of hAEC as a therapy for chronic liver disease. Specifically, I showed the effectiveness of hAEC derived CM in exerting an anti-fibrotic effect on HSC *in vitro* and demonstrated that CM is equally effective at reducing fibrosis as hAEC cell therapy in a CCl_4 mouse model of chronic liver disease. However, this may not be the case in a diet-induced model of NASH as the reduction in fibrosis with CM did not reach statistical significance. In the CCl_4 model, I demonstrated that hAEC tempers the LPC response which is known to correlate with the degree of liver injury and if left uncontrolled thought to be a cause of HCC formation. This dampened response may have been due in part to the *in vitro* effects of hAEC CM observed on LPC. I*n vitro* studies revealed that

CM has the ability to induce LPC differentiation into functioning hepatocytes and maintain the cholangiocyte differentiation. These studies contribute to the understanding of the mechanisms behind the ability of hAEC to treat chronic liver disease and may move the field closer to human trials.

Secondly, I demonstrated in the first randomised controlled trial of IF (without caloric restriction) in NAFLD that IF results in improvements in visceral fat, central adiposity, liver stiffness and steatosis when compared to standard lifestyle advice. The beauty of this intervention is in its simplicity. There are no complicated rules to follow, it does not require a working knowledge of nutrition, and it has no financial or time costs. This pilot study could form the basis for larger studies and may lead to formal dietary recommendations for patients with NAFLD.

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