Predictors of Disease Progression and Outcome in Chronic Hepatitis C Virus Infection

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Abstract

Chronic HCV infection (CHC) is a significant cause of both liver related and non-liver related morbidity and mortality worldwide. Disease progression through to cirrhosis and hepatocellular carcinoma is highly variable, and once chronicity of infection has been established, the likelihood of spontaneous clearance without antiviral treatment is extremely low. Safe and highly effective oral antiviral therapy is now available for the treatment of CHC, however price and accessibility may limit the global use of these agents. Furthermore, concerns have been raised regarding the incidence of hepatocellular carcinoma in HCV-infected individuals receiving oral antiviral regimens, and there appears to be a 'point of no return' beyond which cirrhotic HCV-infected individuals fail to benefit from antiviral treatment. Thus, there remain a number of unanswered questions on the natural history of HCV infection.

Ageing of the immune system, or immunosenescence, appears to contribute to poorer clinical and treatment outcomes, however robust, non-invasive, clinically relevant biomarkers are lacking. MicroRNAs (miRNAs) are short, endogenous non-coding RNAs responsible for post-transcriptional control of host gene expression. Specific patterns of miRNA deregulation have been described in the serum, liver tissue and peripheral immune cells of HCV-infected subjects, and it is hypothesised that they may be suitable as both diagnostic and prognostic biomarkers.

We interrogated 3 patient cohorts (providing access to local and national clinical data) to identify patient factors associated with disease progression and both spontaneous and treatment-associated clearance of CHC. We found that chronological age and elevated BMI had the strongest association with hepatic cirrhosis. Co-morbid type 2 diabetes mellitus was associated with poor clinical outcomes during antiviral therapy. Spontaneous clearance of CHC occurred rarely (0.36 per 100 person-years follow up), and was associated with female gender, earlier age at infection, low HCV viral load and co-infection with HBV. Current injecting drug use was negatively associated with spontaneous clearance.

We also explored the use of miRNAs as biomarkers in these cohorts. We correlated miRNA expression with cellular markers of immunosenescence to identify novel prognostic biomarkers for disease outcomes in CHC. Our findings demonstrated that CHC was associated with a distinct miRNA signature in the serum and peripheral immune cells. Serum miR-21-5p, miR-122-5p and miR-885-5p levels correlated with the expression of previously described biomarkers of ageing, however these miRNAs performed poorly as biomarkers of cirrhosis in CHC. Elevated serum miR-21-5p expression was an independent predictor of virologic relapse following antiviral therapy, together with HCV genotype. MiR-21-5p also appeared to predict the likelihood of an adverse clinical event during treatment. We identified a further microRNA, miR-345-5p, elevated baseline expression of which correlated with negative clinical outcome during treatment, and was associated with the presence of both hepatic and extra-hepatic malignancy. We explored the regulation of miRNA expression in an *in vitro* model, and found that interferon-stimulated gene expression is necessary for IFN-induced miR-21-5p expression.

Finally, we performed pathway analysis for target genes regulated by miRNAs deregulated during CHC, and found that pathways in cancer were highly enriched. Pathway enrichment was similar between HCV-infected cirrhotic subjects and non-cirrhotic, immunosenescent subjects, suggesting that non-cirrhotic individuals with elevated biomarkers of immunosenescence may be at an increased risk of hepatocellular carcinoma and may benefit from enhanced surveillance and prioritisation for antiviral treatment. Overall, the wealth of clinical and molecular data provided the opportunity to explore possibilities for integrating novel biomarkers into clinical decision-making for monitoring liver-related disease in HCV-infected subjects.

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Author’s Declaration

All work presented in this thesis was obtained by the author’s own efforts, unless otherwise stated.

Definitions/Abbreviations

aa	Amino acid	1-29
ABC	ATP binding cassette transporter	1-73
AFP	Alpha-fetoprotein	4-152
AGO	Argonaute protein	1-103
AID	Activation-induced cytidine deaminase	1-109
AKT	Protein kinase B	1-52
ALT	Alanine aminotransferase	1-24
AMPK	AMP-activated protein kinase	4-197
APOB	Apolipoprotein B	1-29
APOC	Apolipoprotein C	1-29
APOE	Apolipoprotein E	1-29
APRI	Aspartate aminotransferase to platelet ratio	1-66
ARFP	Alternate reading frame protein	1-30
AST	Aspartate aminotransferase	1-66
AUC	Area under the ROC curve	2-137
BCL2	B-cell lymphoma 2	1-109
Bio-age	Biological age according to CDKN2A expression	4-154
BMI	Body mass index	1-59
BoA	Biomarkers of ageing	1-93
bp	Base pairs	1-27
BCRP	Breast cancer resistance protein	1-73
C/EBP	CCAAT-enhancer-binding protein	1-95
CARDs	Caspase recruitment domains	1-41
CCL2	CC-chemokine ligand 2	1-52
CCR2	CC-chemokine receptor 2	1-52
CDK	Cyclin dependent kinase	1-89
CDKN2A	Cyclin dependent kinase inhibitor 2A	1-89
CHC	Chronic HCV infection	1-30
CLDN1	Claudin-I	1-33
CMV	Cytomegalovirus	1-24
COX-2	Cyclo-oxygenase 2	1-29
CRP	C-reactive protein	1-109
CT	Cycle threshold	2-132

CXCL10/IP-10	Chemokine IFN-γ inducible protein-10	1-42
DAAs	Direct-acting antivirals	1-31
DBS	Dried blood spot	3-139
DDIT4	DNA damage inducible transcript 4	4-177
DDX3	DEAD box polypeptide 3	1-29
DDX5	DEAD box polypeptide 5	1-56
DGCR8	DiGeorge syndrome critical region 8	1-103
DM 2	Type 2 diabetes mellitus	1-60
DMSO	Dimethyl sulfoxide	4-180
dsRNA	Double-stranded RNA	1-41
EAP	NHS England Expanded Access Programme	1-78
EASL	European Association for the Study of the Liver	1-64
EBV	Epstein-Barr virus	1-24
ECM	Extracellular matrix	1-51
eIF	Eukaryotic initiation factor	1-34
eIF-2α	Initiation factor 2, alpha subunit	1-44
ELF	Enhanced Liver Fibrosis test	1-65
EOT	End of treatment	5-213
EOTR	End of treatment response	5-223
ER	Endoplasmic reticulum	1-29
ERK	Extracellular signal-regulated kinase	1-52
ESRF	End stage renal failure	1-76
FAK	Focal adhesion kinase	1-52
FDC	Fixed dose combinations	1-75
FDR	False discovery rate	2-136
GBV-B	GB virus B	1-25
GH	Growth hormone	1-95
GSK3B	Glycogen synthase kinase 3 beta	1-95
Gt	Genotype	1-37
GWAS	Genome wide association studies	1-44
HAV	Hepatitis A virus	1-24
HBc+	Hepatitis B core antibody	1-58
HBsAg	Hepatitis B surface antigen	1-59
HBV	Hepatitis B virus	1-24
HCC	Hepatocellular carcinoma	1-51

HCV	Hepatitis C virus	1-24
HCVRUK	HCV Research UK	5-214
HDAC1	Histone deacetylase 1	1-95
HE	Hepatic encephalopathy	5-236
HGF	Hepatocyte growth factor	1-113
HIF-1	Hypoxia-inducible transcription factor-1	4-197
HIV	Human immunodeficiency virus	1-33
HLA	Human leukocyte antigen	1-44
HMGB1	High-Mobility-Group-Protein B1	1-52
HPS	Health Protection Scotland	1-37
HSCs	Hepatic stellate cells	1-51
hTERC	Human telomerase RNA component	1-85
hTERT	Human telomerase reverse transcriptase	1-85
HTLV	Human T-cell lymphotropic virus	4-174
HVPG	Hepatic venous pressure gradient	1-60
HVR	Hypervariable region	1-30
IDU	Intravenous drug use	1-36
IGF2BP1	Insulin-like growth factor 2 mRNA-binding protein 1	1-111
IFITM1	Interferon-induced transmembrane protein 1	1-43
IFNAR	Type I IFN-α/B receptor	1-42
IFN	Interferon	1-32
IFN-α	Interferon-alpha	1-68
IGF-1	Insulin-like growth factor 1	1-95
IHHs	Immortalised human hepatocytes	1-110
IKKε	Inhibitor of kappa B kinase-related kinase	1-41
IL	Interleukin	1-40
IMPDH	Inosine monophosphatase dehydrogenase	1-69
INK4	Inhibitors of CDK4	1-89
IR	Insulin resistance	1-59
IRAK1	IL-1 receptor associated kinase 1	1-107
IRES	Internal ribosome entry site	1-29
IRF-3	Interferon regulatory factor 3	1-41
ISDR	Interferon sensitivity determining region	1-32
ISG	Interferon-stimulated gene	1-40
ISRE	Interferon-sensitive response element	1-42

JAK	Janus kinase	1-42
JNK	c-Jun terminal kinase	1-54
KCs	Kuppfer cells	1-52
KEGG	Kyoto Encyclopaedia of Genes and Genomes	2-135
KIR2DL3	Killer gene immunoglobulin-like receptor 2DL3	1-44
LCMV	Lymphocytic choriomeningitis virus	1-47
LDLr	LDL-receptor	1-33
LDLs	Low-density lipoproteins	1-29
LMICs	Low- and middle-income countries	1-38
LPS	Lipopolysaccharide	1-62
LT-BR	Lymphotoxin B receptor	1-29
LVPs	Lipoviroparticles	1-29
MAIT	Mucosal-associated invariant T cells	1-48
MAPK	Mitogen-activated protein kinase	1-92
MAVS	Mitochondrial antiviral signalling protein	1-41
MCM5	Mini-chromosome maintenance protein 5	4-177
Mda5	Melanoma differentiation-associated gene	1-41
MDM2	Mouse double minute 2 homolog protein	1-90
MELD	Model for end-stage liver disease	5-214
MHC	Major histocompatibility complex	1-57
MiR	MicroRNA	1-34
MiRNA	MicroRNA	1-66
MMP	Matrix metalloproteinase	1-29
MPTP	Mitochondrial permeability transition pore	1-100
mRNA	Messenger RNA	1-25
MSM	Men who have sex with men	1-37
MTCT	Mother to child transmission	1-37
mTOR	Mammalian target of rapamycin	4-177
MW	Membranous web	1-31
MyD88	Myeloid differentiation primary-response gene 88	1-41
NAFLD	Non-alcoholic fatty liver disease	1-57
NANBH	Non-A, non-B hepatitis	1-24
NASH	Non-alcoholic steatohepatitis	1-51
NF-κB	Nuclear factor-kappa B	1-41
NHSGGC	NHS Greater Glasgow & Clyde Health Board	3-139

NK cells	Natural killer cells	1-40
NPHV	Non-primate hepaciviruses	1-25
NPM	Nucleophosmin	1-90
NRES	National Research Ethics Service	5-214
NS	Non-structural	1-27
nt	Nucleotides	1-24
NTPase	Nucleoside triphosphatase	1-28
OCLN	Occludin	1-33
OMM	Outer mitochondrial membrane	1-29
ORF	Open reading frame	1-27
PAMPs	Pathogen associated molecular patterns	1-40
PBMC	Peripheral blood mononuclear cell	1-48
PD-1	Programmed cell death-1	1-48
PDGF	Platelet derived growth factor	1-52
PDGFR	Platelet derived growth factor receptor	1-52
PEG	Polyethylene glycol	1-70
pegIFN-α	PEGylated interferon-alpha	1-70
P-gp	P-glycoprotein	1-73
PKR	Protein kinase R	1-32
Poly (I:C)	Polyinosinic:polycytidylic acid	2-118
PPARγ	Peroxisome proliferator – gamma	1-53
Pre-miRNA	Precursor miRNA	1-103
PT	Prothrombin time	1-66
PTEN	Phosphatase and tensin homolog	4-173
PWID	Persons who inject drugs	1-36
RANKL	Receptor activator of NFκB ligand	1-66
RAS	Resistance associated substitutions	1-73
Rb	Retinoblastoma	1-89
RBV	Ribavirin	1-68
RdRp	RNA-dependent RNA polymerase	1-28
RIG-I	Retinoic acid-inducible gene I	1-41
RIN	RNA integrity numbers	2-122
RISC	RNA-induced silencing complex	1-103
RLRs	Retinoic acid-inducible gene-like receptors	1-41
RNA	Ribonucleic acid	1-24

ROC	Receiver operating characteristic	2-137
ROS	Reactive oxygen species	1-51
RR	Responder-relapsers	5-215
RSV	Respiratory syncytial virus	1-69
RT	Reverse transcriptase	1-33
SAE	Serious adverse event	5-215
SASP	Senescence-associated secretory phenotype	1-91
Sen-B-Gal	Senescence associated B-galactosidase	1-66
siRNA	Short interfering RNAs	1-105
SIRT1	Sirtuin 1	1-95
SMAD2	Sma- and Mad-related protein 2	1-109
SNP	Single nucleotide polymorphism	1-45
SoC	Standard of care	1-69
SOCS1	Suppressor of cytokine signalling 1	1-109
SOCS3	Suppressor of cytokine signalling 3	1-111
SR-B1	Scavenger receptor class B type 1	1-33
STAT	Signal transducer and activator of transcription	1-42
STI	Sexually transmitted infection	1-37
SVR	Sustained virologic response	1-45
SVR12	Sustained virologic response at 12 weeks	1-68
SVR24	Sustained virologic response at 24 weeks	1-68
TAB1	TGF-B activated kinase 1 binding protein 1	1-112
TAB2	TNF-B activated kinase 1 binding protein 2	1-109
TBK1	NF-κB activator binding kinase 1	1-41
TE	Transient elastography	1-65
TE	Tris-EDTA buffer	2-117
TGF-B	Transforming growth factor-B	1-52
TIMP	Tissue inhibitors of metalloproteinase	1-52
TLRs	Toll-like receptors	1-40
TNF-R1	Tumour necrosis factor receptor 1	1-29
TNF-α	Tumour necrosis factor-α	1-29
TRAF	TNF receptor-associated factor	1-41
TRBP	TAR RNA binding protein	1-103
Treg	Regulatory T cell	1-47
TRF	Telomere repeat binding factors	1-83

TRIF	TIR domain containing adaptor inducing interferon-B	1-41
TRIM25	Triple motif-containing protein 25	1-41
UGT	Uridine 5'-diphosopho-glucuronosyltransferase	1-74
ULN	Upper limit of normal	1-24
UTR	Untranslated region	1-25
VEGF	Vascular endothelial growth factor	1-109
VIF	Variance inflation factor	2-137
VLDLs	Very-low-density lipoproteins	1-29
WoSSVC	West of Scotland Specialist Virus Centre	3-139
XPO5	Exportin 5	1-103
αSMA	alpha-smooth muscle actin	1-52
B-ME	2-Mercaptoethanol	2-116
ΔCT	Normalised cycle threshold	2-134

1 Introduction

1.1 Background

1.1.1 'Non-A, Non-B' Hepatitis

'Non A, non B hepatitis (NANBH)' was first described in the 1970s as a form of transfusion-associated hepatitis which could not be identified with the use of serological tests for hepatitis A virus (HAV), hepatitis B virus (HBV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV) (Feinstone et al., 1975, Alter et al., 1975, Prince et al., 1974). Researchers postulated that it was caused by a third type of human hepatitis virus with an incubation period of two to 15 weeks (Feinstone et al., 1975, Hoofnagle et al., 1977).

Further research led investigators to propose that:

i) the epidemiology of NANBH resembled that of HBV infection
ii) an infectious carrier state must exist in relatively asymptomatic hosts
iii) it was more common in lower socio-economic groups
iv) it was significantly associated with the development of chronic liver disease (Feinstone and Purcell, 1978).

The causative agent was proven to be transmissible, as inoculation of chimpanzees with serum or plasma from patients with NANBH resulted in hepatitis, defined as a serum alanine aminotransferase (ALT) of greater than 3 times the upper limit of normal (ULN), histopathological changes consistent with viral hepatitis, or both (Shimizu et al., 1979, Yoshizawa et al., 1980).

Despite an active research programme, scientists struggled to identify specific viral antigens and antibodies with standard immunological methods and it was over a decade before the infectious agent was characterised. Choo and colleagues (1989) utilised elegant recombinant DNA cloning techniques to identify a positive-stranded ribonucleic acid (RNA) molecule of around 10,000 nucleotides (nt) which they termed the hepatitis C virus (HCV) (Choo et al., 1989).

1.1.2 Classification of HCV

HCV is now known to be an enveloped, positive-sense, single-stranded RNA virus. Based on these characteristics, it was initially presumed to be related to the *Togaviridae* or *Flaviviridae*. Members of the *Flaviviridae* family have virions composed of the following basic components:

1. A nucleocapsid containing a positive-sense, single-stranded RNA genome, 96000 – 123000 nt in length
2. An enveloped lipid bilayer, in which ≥ 2 envelope proteins are anchored.

The RNA genome serves as messenger RNA (mRNA) and, together with modified cell membranes as well as virus and host factors, acts as a template for replication through negative-strand full length intermediates.

Phylogenetic analysis ultimately classified HCV as a new member of the genus *Hepacivirus* within the family *Flaviviridae* (figure 1-1) (Simmonds et al., 1993). Other members of the *Hepacivirus* genus include GB virus B (GBV-B), a virus recovered from a tamarin killed in captivity (Simons et al., 1995), and the rodent, bat and non-primate hepaciviruses (NPHV) (Reuter et al., 2014, Lyons et al., 2014, El-Attar et al., 2015, Drexler et al., 2013). The list of hepaciviruses continues to grow with additional members recently identified in domestic cattle and catsharks (Hartlage et al., 2016).

HCV is divided into 7 separate phylogenetic groups, or genotypes, which display marked sequence diversity (figure 1-2). On average, the genotypes differ by 30-35% at the nucleotide sequence level over the complete genome (Smith et al., 2014) with most variability concentrated in regions such as the E1 and E2 envelope glycoproteins. The lowest variability between genotypes is seen in the 5' untranslated region (UTR). The genotypes can be further divided into more closely related subtypes, which diverge by around 20-25% of nucleotide sites. HCV subtypes are also epidemiologically distinct, demonstrating different distributions according to geographical range and risk factor group (chapter 1.1.6). There is considerable genetic diversity in those genotypes seen in sub-Saharan Africa and Southeast Asia, leading researchers to suggest that HCV has been endemic in these regions for periods spanning hundreds of years (Simmonds, 2001, Pybus et al., 2009).

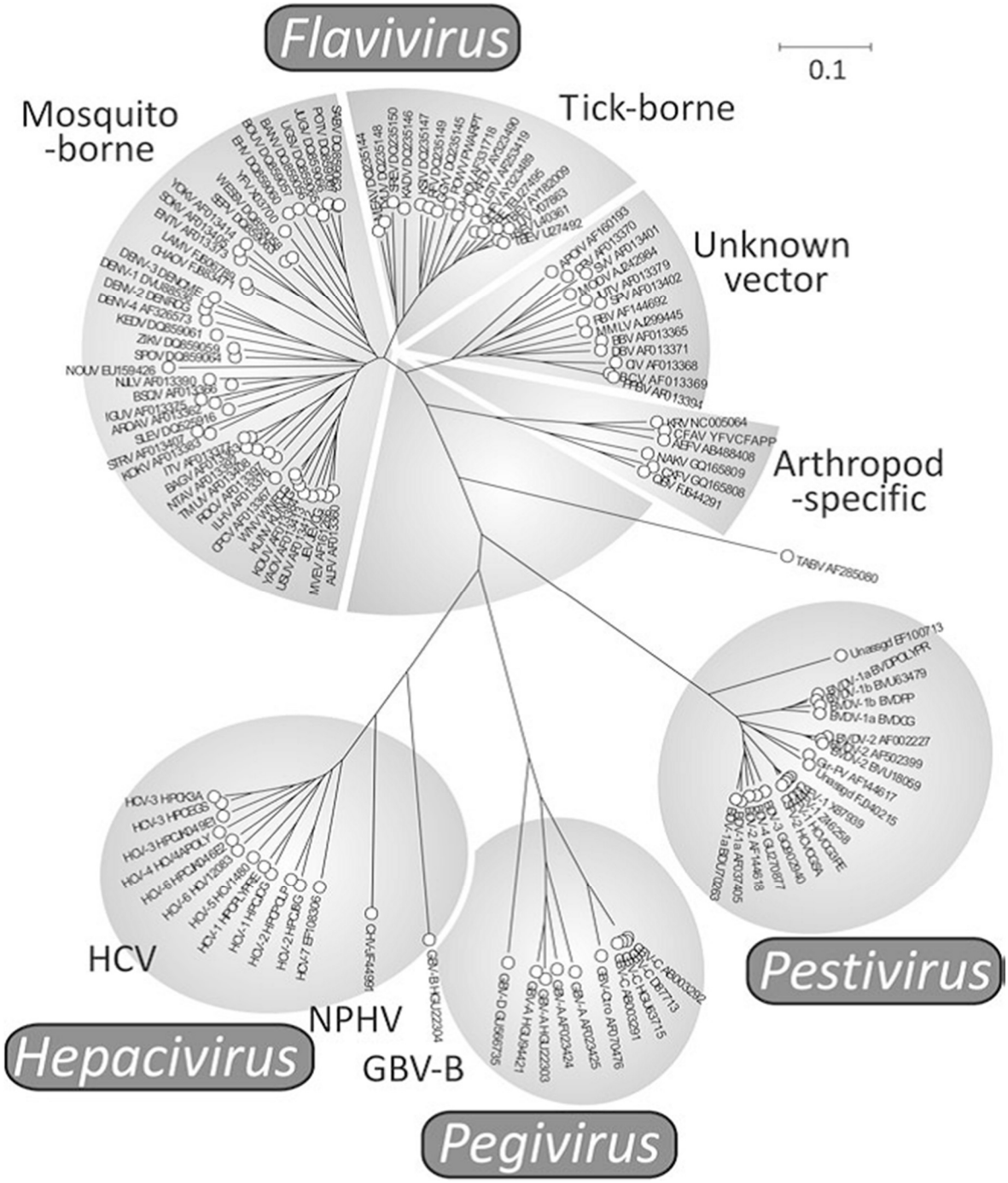


Figure 1-1: Phylogenetic tree of members of the family Flaviviridae. The scale bar depicts a divergence of 0.1 (10% amino acid sequence divergence). HCV, hepatitis C virus; NPHV, non-primate hepacivirus; GBV, GB virus B. Extracted from Simmonds (2013)

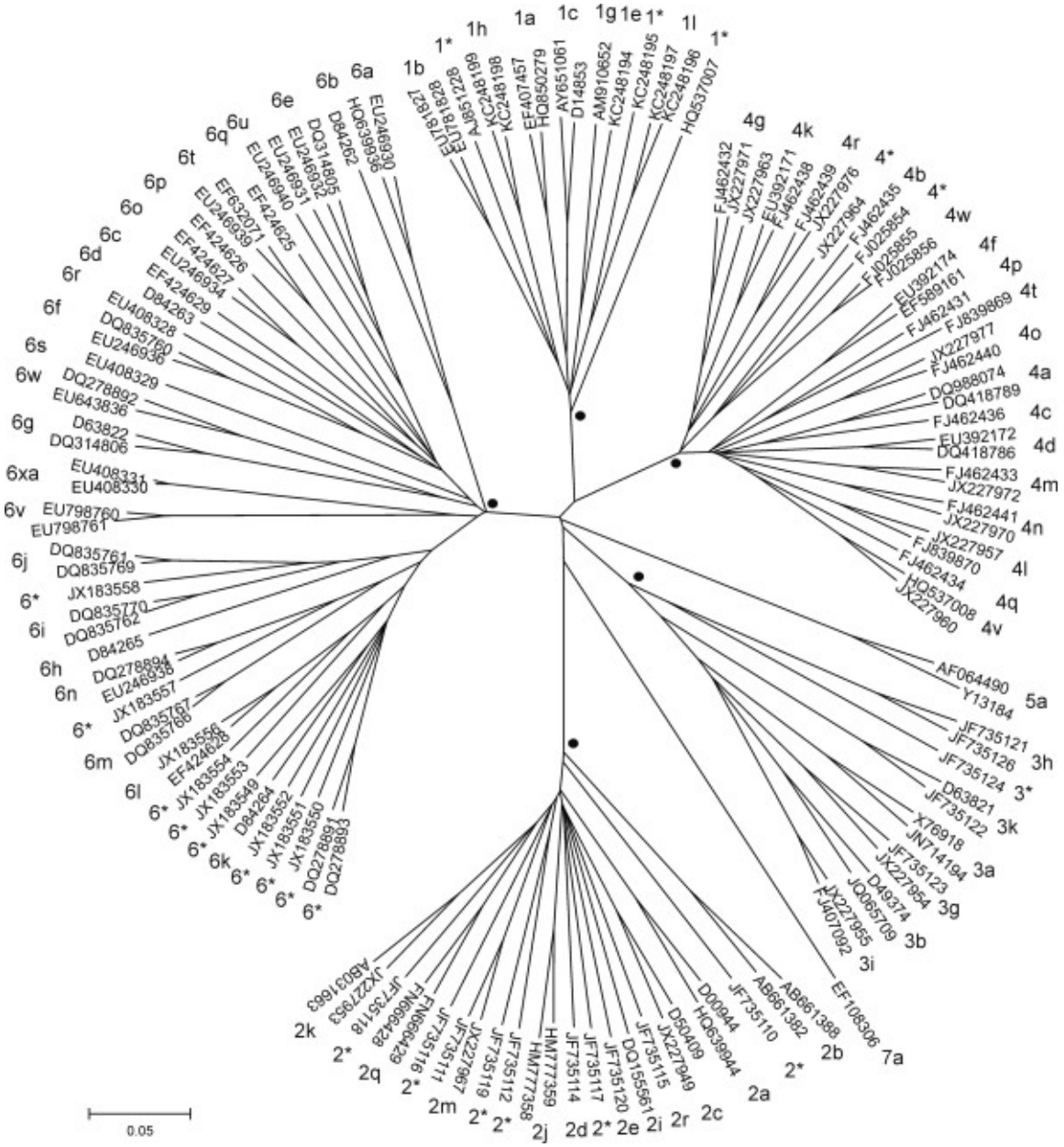


Figure 1-2: Evolutionary tree of NS5B sequences of HCV genotypes 1-7. The scale bar depicts an evolutionary distance of 0.05nt. Extracted from Smith et al. (2014)

1.1.3 Structure of the HCV virion

The length of the HCV genome is approximately 9600 base pairs (bp), consisting of a 5' UTR, a single open reading frame (ORF) and a 3' UTR (Moradpour and Penin, 2013, Choo et al., 1991, Kolykhalov et al., 2000). Most of the genome forms the ORF which encodes the entire HCV polyprotein, consisting of 3 structural (core, E1, E2) and 7 non-structural (NS) proteins (Simmonds, 2004). The 3 structural proteins are encoded by the amino-terminal segment of the

ORF. The NS proteins are encoded by the remainder, and include the p7 viroporin, the NS2 protease, the NS3-4A complex (nucleoside triphophatase (NTPase)/RNA helicase and protease activities), the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp) (Moradpour and Penin, 2013). The 5' and 3' UTRs contain the signals for replication and translation of the viral RNA (figure 1-3).

The 5' UTR is the most conserved part of the HCV genome, sharing 60% sequence homology with GBV-B. It is 341 nt long and contains 4 highly structured domains. Domains II, III and IV together with a segment of the core-coding region make up the internal ribosome entry site (IRES), consisting of 4 distinct stem loops (Honda et al., 1996).

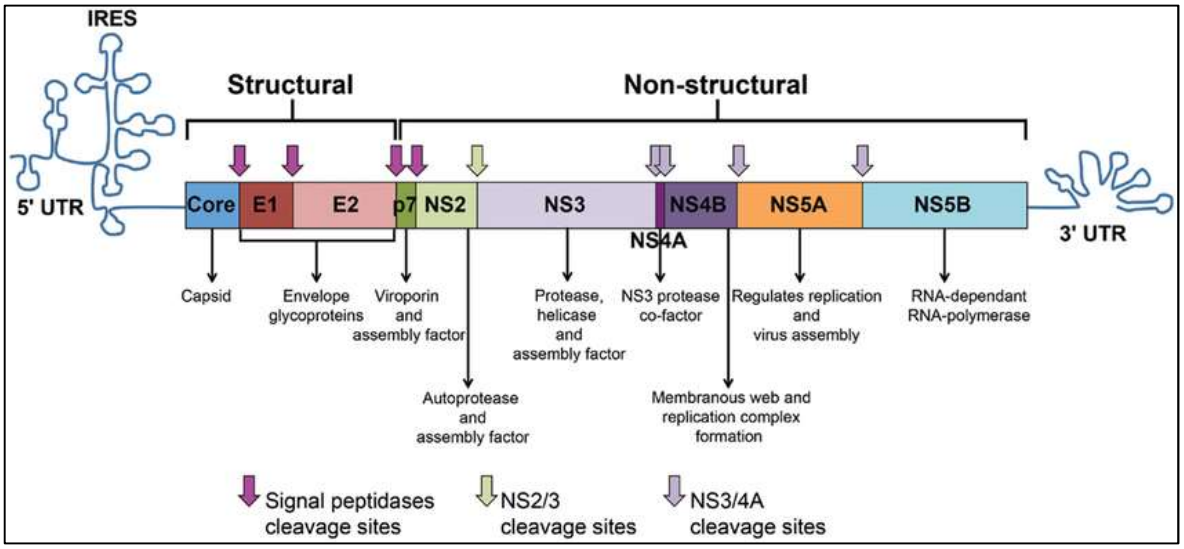


Figure 1-3: The HCV genome and gene products, extracted from Abdel-Hakeem and Shoukry (2014). Cleavage sites of the HCV polyprotein by host and viral proteases are indicated with arrows. The purple arrows indicate the cleavage sites for the host signal peptidases, the green arrow indicates auto-cleavage by NS2/3 and the lilac arrows indicate cleavage by the NS3 protease. IRES, internal ribosome entry site; UTR, untranslated region.

The viral genomic RNA together with the capsid protein (core) forms the nucleocapsid, which is spherical and approximately 30 nm in diameter. The nucleocapsid is surrounded by a lipid-containing envelope derived from host endoplasmic reticulum (ER) membranes. The morphological structure of HCV remains obscure as a consequence of the complex lipid envelope, however electron microscopy has demonstrated that the infectious virion is 40-100 nm in size (Gastaminza et al., 2006, Catanese et al., 2013). Virions circulate in

association with low-density lipoproteins (LDLs) and very-low-density lipoproteins (VLDLs) as lipoviroparticles (LVPs) (Catanese et al., 2013). These LVPs also contain apolipoproteins, including apolipoprotein B (APOB), apolipoprotein C (APOC) and apolipoprotein E (APOE).

1.1.4 Viral proteins

1.1.4.1 Core protein

The RNA-binding core protein forms the viral capsid, as described above. It contains 3 distinct domains: i) domain D1, an N-terminal predominantly hydrophilic domain of ~120 amino acids (aa), ii) domain D2, a 50 aa C-terminal hydrophobic domain, and iii) domain D3, a highly hydrophobic 20 aa domain serving as a signal sequence for E1 (McLauchlan, 2000). D1 is involved in RNA binding and nuclear localisation, whereas D2 is mainly responsible for binding with ER membranes, outer mitochondrial membranes (OMM) and lipid droplets.

Core protein directly interacts with a number of cellular proteins, including lymphotoxin ß receptor (LT-ßR), tumour necrosis factor receptor 1 (TNF-R1) and the DEAD box polypeptide 3 (DDX3), and plays an important role in diverse cellular processes. Core protein can promote oxidative stress, steatosis and apoptosis, leading to fibrogenesis independently of the immune response and inflammation (Missiha et al., 2008). Both core and NS5A proteins are able to upregulate expression of the pro-fibrogenic genes cyclo-oxygenase 2 (COX-2) and matrix metalloproteinase (MMP)-9 in hepatocyte-derived cell lines (Nunez et al., 2004). The effects of core protein on apoptosis are conflicting; both increased and reduced Fas-mediated apoptosis by core have been reported *in vitro* (McLauchlan, 2000) and it may have both pro- and anti-apoptotic functions. The picture is no clearer for the effect of core protein on tumour necrosis factor-α (TNF-α) or LT-ßR mediated apoptosis (Shimizu et al., 1996, Farci et al., 1996).

Core associates with lipid droplets, and transgenic mice expressing core have been shown to develop hepatic steatosis (defined as an accumulation of lipid droplets in the liver) from an early age (Moriya et al., 1997). These transgenic mice lines were subsequently found to develop hepatic tumours, with histology demonstrating adenomata initially followed by transformation into poorly-differentiated hepatocellular carcinomas (Moriya et al., 1998). Core protein has

previously been shown to regulate the transcription of the cellular oncogenes *c-myc* and *c-fos*, supporting a role in hepatic carcinogenesis (Ray et al., 1995).

1.1.4.2 E1 and E2 envelope glycoproteins

The type I transmembrane glycoproteins E1 and E2 are essential for virus attachment and entry (see Chapter 1.1.5.1). They consist of an N terminal domain (160 and 334 aa, respectively) and a short C terminal transmembrane domain of ~30 aa. Both E1 and E2 are highly glycosylated. E2 contains hypervariable region (HVR) 1, a 27 aa region at the N terminus which is the major HCV neutralising epitope, and HVR2, a 9 aa region downstream of HVR1. The hypervariable regions demonstrate the highest sequence variability between HCV genotypes and subtypes. The interaction between HVR1 and negatively charged molecules at the cell surface is thought to facilitate host cell recognition and attachment (Bartosch et al., 2005).

1.1.4.3 Frameshift protein

The frameshift protein or ARFP (alternate reading frame protein) is produced during chronic HCV infection (CHC). Specific antibodies proving the existence of this protein were first detected in 2001 (Walewski et al., 2001). It is encoded by a reading frame that overlaps the N-terminal core-encoding region of the HCV polyprotein and is generated by a -2/+1 ribosomal frameshift during translation, hence the name (Xu et al., 2003). Similarly to core protein, it appears to localise to the ER membrane but its exact role in the HCV life cycle is as yet undetermined.

1.1.4.4 P7 protein

P7 is an integral membrane-associated ion channel protein of approximately 63 aa. It is comprised of 2 transmembrane domains connected by a cytoplasmic loop. As p7 monomers are able to assemble into hexamers or heptamers, forming ion channels in lipid bilayers, it is thought to belong to the viroporin family (Gonzalez and Carrasco, 2003). P7 is now known to be critical for HCV infectivity as mutant polypeptides with deletions of all or part of p7 were not viable in a chimpanzee model (Sakai et al., 2003).

1.1.4.5 NS2 protein

NS2 is a non-glycosylated transmembrane protein which contains 2 internal signal sequences responsible for association with the ER membrane (Santolini et al., 1995). It is released from the HCV polyprotein by proteolytic cleavage; the N-terminus is separated from the E2/p7 polypeptide by host signal peptidase, whereas the C-terminus is cleaved at the NS2-3 junction by auto-proteolysis (figure 1-3). NS2 loses its protease activity after self-cleavage from the amino-terminal domain of NS3 and is quickly degraded by the proteasome (Franck et al., 2005).

1.1.4.6 NS3-NS4A complex

The NS3 protein contains a serine protease domain at the N-terminus, and a helicase/NTPase in the C-terminal region. The protease activity of NS3 is enhanced by an activator, NS4A. NS4A is a 54 aa protein which stabilises the NS3-NS4A complex (Bartenschlager et al., 1995). The NS3-4A protease is essential for productive infection, and consequently is a target for the new direct-acting antivirals (DAAs). It is responsible for cleavage at the NS3/4A, 4A/4B, 4B/5A and 5A/5B junctions (figure 1-3).

The NS3 helicase has NTPase and 3'-5' RNA unwinding functions, making it essential for HCV RNA synthesis (McGivern et al., 2015). It plays a critical role in separating template and nascent RNA strands during HCV replication (Scheel and Rice, 2013). Genetic evidence also implicates the NS3 helicase in early HCV particle assembly (Ma et al., 2008).

1.1.4.7 NS4B protein

NS4B is a 261 aa integral membrane protein which is essential for the organisation of the replication complex. It induces the formation of the membranous web (MW), consisting of vesicles in a membranous matrix, that harbours the replication complex (Egger et al., 2002). NS4B has also been shown to directly activate pro-oncogenic signalling pathways (Jiang et al., 2017), supporting a potential role in hepatocarcinogenesis.

1.1.4.8 NS5A protein

NS5A is an important regulator of HCV replication. It consists of a 30 aa amphipathic α-helix anchor at the N-terminus which is required for assembly of the replication complex and membrane localisation, together with a further 3 domains. Domain I contains a zinc-binding motif at the N-terminal end formed by 4 cysteine residues. Mutations which disrupted either membrane localisation or zinc binding were shown to block RNA replication (Tellinghuisen et al., 2004). The crystal structure of domain I was solved in 2005, and suggested potential protein, RNA and membrane binding sites, with significant implications for antiviral drug development (Moradpour et al., 2005).

The mechanism by which NS5A regulates HCV replication is not fully understood. Both domains I and II are known to be required for HCV replication, however the function of domain III was less clear. Subsequently, a deletion in domain III was shown to be able to disrupt virus production independently of replication. It was demonstrated that a serine residue within the deletion was responsible for the observed phenotype, and that phosphorylation at that position was required for early virion production (Tellinghuisen et al., 2008). Studies of inhibitors targeting NS5A have suggested that NS5A may have both phosphorylation dependent and independent functions in RNA replication: a *cis*-acting function in the context of the replication complex, and a further *trans*-acting function requiring hyperphosphorylation (Fridell et al., 2011).

NS5A interacts directly with cellular proteins, and is postulated to play a role in regulation of cellular growth and signalling, as well as response to interferon (IFN)-based therapy. The amino acid sequence of a region of NS5A, the interferon sensitivity-determining region (ISDR), has been shown to correlate with IFN sensitivity. Gale et al. (1998) were able to demonstrate that NS5A binds directly to the protein kinase catalytic domain of protein kinase R (PKR), which is a mediator of the IFN-induced antiviral response, and that the ISDR was required for this interaction (see chapter 1.2).

1.1.4.9 NS5B (RdRp)

NS5B is a tail-anchored protein, with a 21 aa α-helical transmembrane domain in the C-terminal region which may be involved in important protein-protein

interactions as well as acting as a membrane anchor. This membrane association is essential for HCV replication (Moradpour et al., 2004). The 530 aa N-terminal region forms a 'fingers, palm and thumb' polymerase structure, similar to the human immunodeficiency virus (HIV) reverse transcriptase (RT) (Ago et al., 1999). Uniquely, extensive interactions between the fingers and thumb result in a completely encircled enzyme active site (Lesburg et al., 1999). NS5B may also interact with other cellular proteins to regulate HCV replication. Similar to the NS3 protease and NS5A protein, NS5B is a target for the DAAs. Both nucleoside and non-nucleoside inhibitors potently block NS5B-directed HCV RNA synthesis (chapter 1.5.1.5).

1.1.5 HCV life cycle

1.1.5.1 Virus attachment and entry

HCV primarily infects and replicates within hepatocytes. The mechanism by which HCV enters the hepatocyte has not been fully elucidated, but involves interaction between the HCV envelope glycoproteins E1 and E2 and a number of host cell surface molecules. A major breakthrough came with the identification of the cell surface receptor CD81 as a binding partner for HCV in 1998 (Pileri et al., 1998). Subsequently, a number of further molecules have been identified with roles in HCV attachment and entry, including the LDL-receptor (LDLr) (Agnello et al., 1999), scavenger receptor class B type 1 (SR-B1) (Scarselli et al., 2002, Bartosch et al., 2003), claudin-I (CLDN1), occludin (OCLN) (Evans et al., 2007, Mensa et al., 2011), CD36 (Cheng et al., 2016) and the apolipoproteins.

Glycosaminoglycans and the LDLr appear to be involved in the initial binding step. E1-E2 then interacts with CD81 and SR-B1, and the co-receptor CD36. Proof-of-concept studies evaluating monoclonal antibodies directed against CD81 and SR-B1, and direct inhibition of CD36, have demonstrated that blockage of these receptors is associated with protection against HCV infection *in vivo*, confirming their essential role in the HCV life cycle (Meuleman et al., 2008, Meuleman et al., 2012, Cheng et al., 2016). Viral entry appears to be dependent on the tight junction factors CLDN1 and OCLN. The association between tight-junctions and HCV-receptor complexes facilitates cell-to-cell transmission; a combination of receptors is also involved in cell-free transmission.

Subsequent HCV entry into the hepatocyte results in clathrin-mediated endocytosis. The E1 envelope glycoprotein mediates fusion between the viral and endosomal membranes under acidic pH, facilitating release of the nucleocapsid into the cytoplasm.

1.1.5.2 Translation and replication

Following uncoating of the viral nucleocapsid, the positive-strand RNA genome is released into the cytosol where it serves as mRNA for the synthesis of the HCV polyprotein. Translation, which is initiated at the IRES on the 5' UTR, takes place at the ER membrane. The IRES is responsible for cap-independent translation of the genomic RNA, through the recruitment of cellular proteins, including the eukaryotic initiation factors (eIF) 2 and 3. The structural proteins are cleaved from the HCV polyprotein by two host proteases, signal peptide peptidase, responsible for maturation of core, and signal peptidase (McLauchlan et al., 2002). Viral peptidases (NS2-3 and NS3-4A) are responsible for the processing of the non-structural proteins, as described above (figure 1-3).

The HCV protein NS4B induces the formation of the ER-derived membranous web, rich in cholesterol, sphingolipids and fatty acids, which is essential for HCV replication (Bartenschlager et al., 2004). In the first step, negative-strand intermediates are synthesised from the RNA genome. The negative-strand RNA then serves as a template for the production of multiple positive-strand progeny for polyprotein translation, the synthesis of new template RNA strands or packaging into virus particles (Lohmann, 2013). NS5B is the key catalyst of RNA synthesis, and the RNA-binding NS5A protein is an essential part of the viral replication complex, the phosphorylation status of which may determine the balance between replication and virion production (as discussed above).

A number of other molecules have been shown to have important roles in the HCV life cycle. The liver specific microRNA-122 (miR-122) is able to bind to conserved sites in the IRES, and is essential for HCV replication (chapter 1.7.4). Cyclophilin A induces conformational changes in NS5A and NS5B which are required for HCV replication. The cholesterol and fatty acid synthesis pathways are also implicated in productive infection (Lindenbach, 2013).

1.1.5.3 Assembly and release

Our understanding of the mechanism of viral assembly and release is limited. Viral particle assembly is believed to be initiated by the interaction of core and NS5A with genomic RNA on the surface of lipid droplets. Biogenesis of VLDL occurs in the ER and Golgi apparatus, followed by secretion of mature VLDL particles through the Golgi secretory route (Hossain et al., 2014). HCV hijacks the VLDL production pathway, and fuses with APOB, APOE and other nascent VLDL particles in the ER to form LVPs. The LVPs bud off from the ER and are transported to the Golgi in COP-II vesicles (Syed et al., 2017) before being secreted through the Golgi secretory pathway.

1.1.6 Epidemiology and transmission of HCV

It is estimated that around 130-210 million individuals globally are anti-HCV antibody positive, i.e. roughly 3% of the world's population, although accurate determination of the true prevalence is problematic (Manns et al., 2017). The majority of individuals infected with HCV are asymptomatic and may be unaware of their infection, and groups at high risk of infection may be less likely to access clinical care. Additionally, the use of anti-HCV antibody testing to determine prevalence will not exclude those patients who have cleared the virus spontaneously or through treatment. The estimated prevalence of HCV viraemia is consequently lower, at around 0.8-1.14% worldwide ('Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study,' 2017).

The prevalence (figure 1-4) and risk factor for acquisition of HCV varies from country to country.

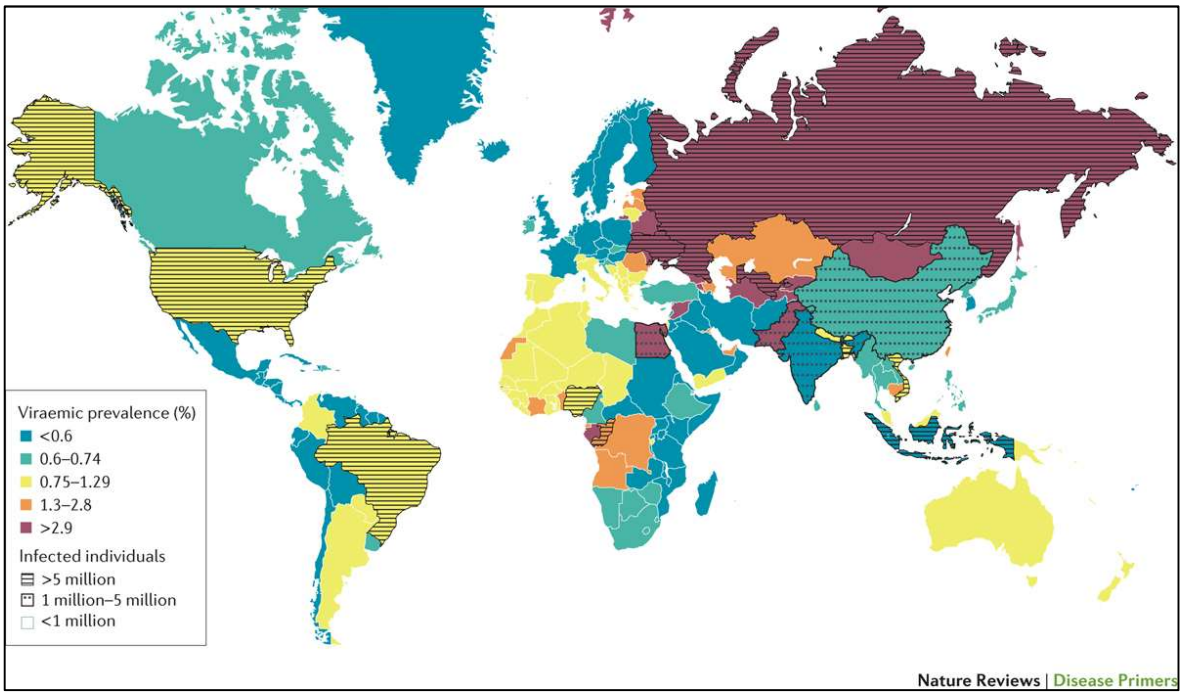


Figure 1-4: Geographical distribution of HCV infection. Extracted from Manns et al. (2017)

The major route of HCV transmission is parenteral - through blood and plasma product transfusion, the use of unsterilized medical equipment, vaccination programmes and intravenous drug use (IDU). Prior to the implementation of universal screening of blood products in 1991, a large cohort of patients contracted the virus through contaminated blood or blood products, e.g. haemophiliacs and patients receiving anti-rhesus D immunoglobulin (Power et al., 1995, CDC, 2014), and the highest prevalence of HCV infection is seen in countries with a high rate of iatrogenic infections. The re-use of glass syringes for injections remains a key risk factor for transmission in Pakistan (Qureshi et al., 2010), and in the Nile Delta the prevalence is > 10% of the population as a consequence of the re-use of needles during a widespread provision of intravenous tartar emetic to control *Schistosomiasis spp*. in the latter half of the 1900s (CDC, 2014, Frank et al., 2000). Increased HCV (and HIV) prevalence in China has also been attributed to procedural error during an officially encouraged plasma donation campaign (Lu et al., 2013).

In recent years, the predominant risk factor for the acquisition of HCV in the UK has been IDU. In Scotland, of those cases of HCV-antibody positivity in whom a risk factor was identified, 91% were among persons who inject drugs (PWID)

(HPS, 2016). Transmission through the sharing of equipment for intranasal drug use is also recognised, and this risk is often underappreciated by patients and care-providers (Karmochkine et al., 2006). A number of public health initiatives raising awareness of the disease and encouraging individuals to access testing have been developed, for instance, the Big Red C campaign in Scotland and 'Tune In to Hep C' in the US.

Transmission of HCV through mother to child transmission (MTCT) or sexual contact is uncommon in the absence of HIV co-infection (Wasley and Alter, 2000) and national guidelines do not recommend the use of barrier contraception for HCV-infected individuals (Bradshaw et al., 2013). However, in recent years an epidemic of acute HCV following sexual intercourse has been reported in industrialised countries amongst HIV-positive men who have sex with men (MSM) (Bradshaw et al., 2013, van der Helm et al., 2011, Wandeler et al., 2012). The reported incidence amongst HIV-negative MSM remains low, and the increase in incidence amongst HIV-infected individuals is thought to reflect both behavioural and biological factors, i.e. serosorting, mucosally traumatic practices, the use of mucosally administered recreational drugs, intercurrent sexually transmitted infections (STIs) and HIV itself (Bradshaw et al., 2013).

Health Protection Scotland (HPS) reported a cumulative number of HCV diagnoses in Scotland at 38577 cases (data to 31 Dec 2015), 17% of whom were known to have died (HPS, 2016). Infections in Scotland are primarily due to genotype (Gt) 1 and Gt3, as seen in Europe, Australia and the USA (McLeod A, 2014, Simmonds, 2004). The prevalence of each genotype varies according to geographical region, and has evolved over time with the changing epidemiology of infection. Interestingly, a recent phylogeographic reconstruction using Bayesian inference methods showed that HCV Gt1a lineage expansion in Scotland occurred between 1940 and 1965. The investigators hypothesised that this may have occurred through the introduction of independent Gt1a lineages to Scotland as a consequence of the mass population mixing during World War II, the first use of large scale parenteral treatments and the subsequent dissemination of HCV worldwide as individuals returned to their countries of origin (McNaughton et al., 2015).

Whilst Gt1 and Gt3 infections are the most prevalent worldwide, Gt4 and Gt5 are overrepresented in resource-limited countries (figure 1-5). In Europe, about 90% of infections are due to Gt1-3 (Messina et al., 2015, Esteban et al., 2008). Infection with subtypes classically associated with iatrogenic spread (1b, 2a and 2b) are found more commonly in the elderly, whereas subtypes 1a, 3a and 4d are more closely linked with IDU. Rare/novel strains of HCV may gain increasing prominence in the DAA era, with highly effective antiviral regimens now available for the more commonly seen genotypes. Recent data have suggested that rare subtypes and strains which cannot be assigned to known subtypes circulate in low- and middle-income countries (LMICs) and these may respond less effectively to DAA therapy (da Silva Filipe et al., 2017).

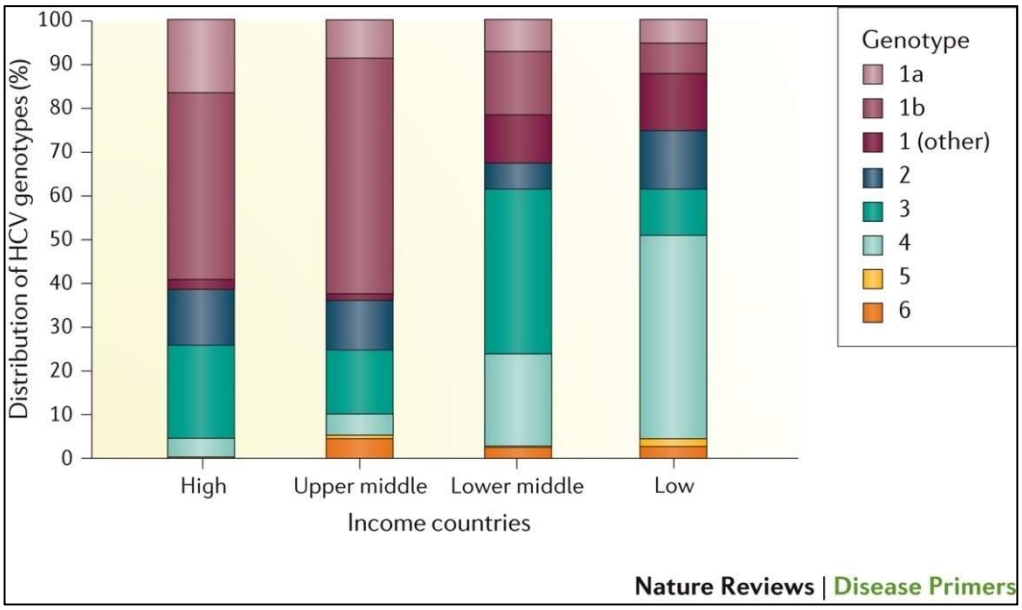


Figure 1-5: Distribution of HCV genotype by country according to economic status (extracted from Manns et al. (2017)).

1.1.7 Viral diversity and quasispecies

The diversity of HCV reflects both sequence drift over time and the rapid, adaptive changes the virus undergoes in a single individual in response to immunological pressures and antiviral therapy (chapter 1.5.2). Sequence drift is largely responsible for the diversity seen between different genotypes and is referred to as 'neutral' as it has little effect on phenotype and becomes fixed in the population by chance (Kimura, 1979, Simmonds, 2004). This differs from the

‘Darwinian’ theory of evolution in which changes result in improved viral fitness and are therefore sustained and ultimately predominate.

Evidence for both theories of evolution can be seen in the HCV genome sequences: rapid changes in HVR1 may allow the virus to evade the antibody response to infection, demonstrating ‘Darwinian’ evolution, whereas the sequence variability between geographically or epidemiologically distinct genotypes demonstrates marginal phenotypic change, consistent with neutral theory (Simmonds, 2004).

CHC results in a population of highly diverse variants, named ‘quasispecies’, which typically differ by 1-3%. A lack of proof-reading, coupled with a relatively high rate of virus production, results in the continuous generation of mutant virions, with implications for drug and vaccine development (Timm and Roggendorf, 2007). RNA copying by the HCV-encoded RNA polymerase is error prone, with error rates of between 1 in 10 000 and 1 in 100 000 bp copied (Domingo et al., 1996). This heterogeneity has already been shown to lead to viral escape during DAA treatment (Susser et al., 2009, da Silva Filipe et al., 2017). Genetic heterogeneity is found throughout the genome, however certain regions demonstrate more variability. HVR1 at the N-terminus of E2 is one such segment, with > 80% sequence variability between genotypes (Manns et al., 2017).

1.2 Immune response to HCV infection

The host immune response to infection consists of innate and adaptive components, the adaptive consisting of cellular and humoral responses. Innate immunity comprises physical barriers, cellular components (e.g. granulocytes, macrophages and natural killer (NK) cells), and soluble components (Neumann-Haefelin et al., 2005). Adaptive cellular immunity, mediated through CD4+ and CD8+ lymphocytes, is important in the control of viral infection.

1.2.1 Innate immune response

Interferons are members of the cytokine family which play a crucial role in the cellular defence against viral infection. Those of relevance in HCV infection include the type I IFNs (IFN-α subtypes and IFN-β), type II IFNs (IFN-γ) and type III IFNs (IFN-λ, otherwise known as interleukin (IL)-29, IL-28A and IL-28B). IFN-α and IFN-β induce the innate immune response, and can directly inhibit viral replication. Additionally, they are able to direct the adaptive immune response by priming cytotoxic T cells and T helper cells. IFN-γ has immunomodulatory activity and is secreted by NK cells and cytotoxic T cells (Lemon S, 2010).

HCV RNA replication induces type I IFN within 48 hours of infection in a chimpanzee model (Bigger et al., 2001). It is postulated that this early IFN response may restrict viral replication in acute infection (Neumann-Haefelin et al., 2005). However, despite this early response, type I IFN does not play a major role in viral clearance and HCV is largely eliminated by non-cytolytic effects through the IFN-γ driven interferon-stimulated gene (ISG) expression in hepatocytes. IFN-γ may also inhibit HCV infection by downregulating claudin-1 and CD81 (Wei et al., 2009).

The host innate immune response is induced upon recognition of conserved molecular patterns expressed on microbial and viral structures (pathogen associated molecular patterns; PAMPs) by resident membrane and cytosolic receptor molecules (Olivieri et al., 2013a). Amongst these molecules are the toll-like receptors (TLRs) which are transmembrane proteins of mammalian cells. In humans, viral nucleic acids are recognised by the endosomal TLRs -3, -7, -8 and -9, by PKR, and by the cytosolic retinoic acid-inducible gene-like receptors

(RLRs) retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene (Mda5).

HCV RNA contains specific PAMPs, including stem-loop double-stranded RNA (dsRNA) and poly-uridine motifs in the 3'-UTR (Tuplin et al., 2002). Cell culture studies have suggested that two distinct PAMP receptors, TLR-3 and RIG-I, direct the host response to HCV infection (Ishii and Koziel, 2008, Lemon, 2010). TLR-3 acts through the TIR domain containing adaptor inducing interferon-β (TRIF) molecule, whereas TLR-7, -8 and -9 act through myeloid differentiation primary-response gene 88 (MyD88) (Olivieri et al., 2013a). The association of TLR-3 with TRIF leads to the recruitment of TNF receptor-associated factor (TRAF) 6, TRAF3 and the nuclear factor-kappa B (NF-κB) activator binding kinase 1 (TBK1), resulting in phosphorylation of interferon regulatory factor 3 (IRF-3) by TBK1 and inhibitor of kappa B kinase-related kinase (IKKε). RIG-I is a DEAD box helicase able to sense short HCV replication intermediate dsRNAs with free 5'-triphosphatases. On recognition of the HCV PAMP, it is recruited to the mitochondrial surface where it interacts with MAVS (mitochondrial antiviral signalling protein, also known as IPS-1, VISA and Cardif) through shared caspase recruitment domains (CARDs) resulting in recruitment of TBK1 and IKKε. Both the E3 ubiquitin ligase triple motif-containing protein (TRIM) 25 and the mitochondrial targeting chaperone protein 14-3-3ε are involved in this process; TRIM25 mediates the ubiquitination of RIG-I at position Lys-63 which facilitates MAVS binding and is important for IFN production (Loo et al., 2006). RIG-I also interacts with apoptosis-associated speck like protein containing CARD resulting in activation of a caspase-1-dependent inflammasome (Nitta et al., 2013) (figure 1-6).

Cellular recognition of the HCV PAMPs by various PAMP receptor pathways leads to the activation and nuclear translocation of NF-κB and IRF-3. IRFs control the induction of IFN at the transcriptional level by binding to specific promotor elements. Although IRF-5 and IRF-7 are also important in the host response to viral infection, their role in HCV infection has not been characterised. Together with NF-κB and ATF2/c-Jun, IRF-3 forms an enhanceosome complex on the IFN-α/β promoter, resulting in IFN-β expression and secretion from the infected cell. IRF-3 also controls the transcription of the IFN-λs.

Both IFN-α and IFN-ß are recognised by the type I IFN-α/ß receptor (IFNAR) resulting in activation of the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signalling pathway. Following IFNAR binding, STAT1 and STAT2 are phosphorylated to form a heterodimer, resulting in recruitment of IRF9 and formation of the ISGF3 transcription factor complex. ISGF3 binds to the interferon-sensitive response element (ISRE) and results in high level of expression of ISGs (Ishii and Koziel, 2008, Lemon, 2010). Upregulation of ISGs has been demonstrated in the liver biopsy specimens of patients with CHC (Sarasin-Filipowicz et al., 2008), and is associated with an increase in circulating cytokines, including chemokine IFN-γ inducible protein-10 (CXCL10/IP-10) (Askarieh et al., 2010). Interestingly, Robinson and colleagues (2015) found that Gt1-infected biopsies demonstrated an upregulation of genes regulated by type I and III IFNs, whereas Gt3-infected biopsies displayed upregulation of IFN-γ induced transcripts.

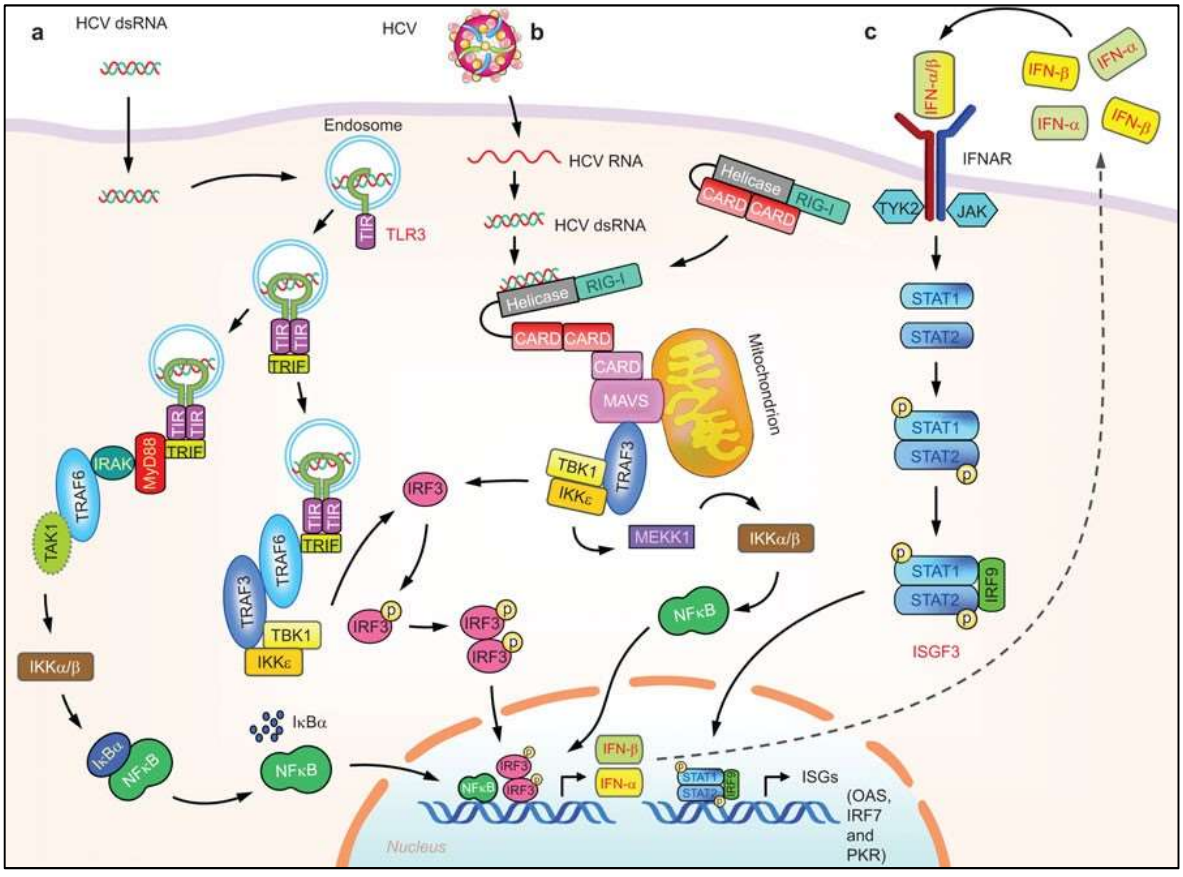


Figure 1-6: Induction of innate immunity by HCV in hepatocytes. Extracted from Wong et al. (2016). IFNAR, type 1 IFN-α/B receptor; TLR3; toll-like receptor 3; P, phosphorylated; ISGF3, interferon-stimulated gene factor 3.

The gene products of ISGs are able to limit HCV replication at different phases of the virus life cycle, as well as having direct effects on cellular processes. Many PAMP receptors and related molecules are also ISGs, for instance, RIG-I and PKR. Interferon-induced transmembrane protein 1 (IFITM1) is an important ISG effector, which is induced during IFN-α treatment and accumulates at hepatic tight junctions where it interacts with CD8 and OCLN to disrupt HCV entry (Wilkins et al., 2013).

ISG15 is another important ISG, which modulates host antiviral responses through 'ISGylation' of viral and host proteins and inhibition of virus release. IRF3, RIG-I and PKR are all modified by ISGylation, resulting in positive regulation of type I IFN signalling. ISG15 also targets many proteins involved in the innate immune response to viral infection, including JAK1, STAT1 and other ISGs (Morales and Lenschow, 2013). The stability of HCV NS5A protein is decreased by IFN-mediated ISGylation, leading researchers to suggest that ISG15 could be used as a therapeutic adjunct to IFN-α treatment in CHC (Kim and Yoo, 2010). Additionally, knockdown of ISG15 expression is associated with increased HCV replication in various cell lines (Jones et al., 2010, Domingues et al., 2015). Conversely, it has also been proposed that increased ISG15/ISGylation may promote HCV infection (Chen et al., 2010). Early PKR-dependent induction of ISG15 may inhibit RIG-I ubiquitination, blocking subsequent HCV RNA-mediated RIG-I activation and leading to negative regulation of the host immune response and promotion of HCV replication (Arnaud et al., 2011).

HCV utilises a number of additional strategies to evade host immune surveillance. Core protein is able to downregulate IFN signalling by blocking STAT1 phosphorylation, preventing heterodimerisation with STAT2 (Lin et al., 2006). Foy et al. (2003) demonstrated that the NS3/4A protein complex is able to block IRF-3 activity *in vitro* through preventing accumulation of the phosphorylated IRF-3 isoform, and the nuclear translocation of IRF-3, thus promoting viral persistence. Additionally, NS3/4A is able to cleave host proteins including TRIF and the adaptor protein MAVS, releasing it from the mitochondrial membrane and blocking its ability to signal (Lemon, 2010, Bowie and Unterholzner, 2008). Recent data suggest that immune evasion may also be mediated through NS3-4A targeting of importin β1 and related modulators of IRF3 and NF-κB nuclear transport (Gagne et al., 2017). Abe et al. (2007) showed

that the NS5A protein was able to inhibit TLR-MyD88-induced signalling in mouse macrophage cells by binding to the death domain of MyD88. Overexpression studies have suggested that the viral protein NS4B may also interfere with RIG-I signalling (Tasaka et al., 2007).

A key component of the IFN antiviral response is PKR. PKR phosphorylates serine 51 of the alpha subunit of the eukaryotic initiation factor 2 (eIF-2α) leading to blockage of protein synthesis initiation and inhibition of viral replication (Gale et al., 1998). PKR is also a mediator of stress-induced apoptosis and repression of PKR leads to malignant transformation of mammalian cells (Koromilas et al., 1992). The viral protein NS5A is able to bind to and inhibit PKR, blocking apoptosis through sequestration of p53, modulation of intracellular electrolyte levels, binding to growth factor receptor-bound protein 2 and induction of IL-8. The E2 protein also appears to bind to and inhibit PKR through molecular mimicry, as a result of sequence similarity to the phosphorylation domains of PKE and eIF-2α (Taylor et al., 1999).

NK and NKT cells are an important component of the innate immune response and contribute to suppression of viral replication through cytolysis of infected cells, production of immunomodulatory cytokines, such as IFN-γ, and activation of dendritic and T cells (Ishii and Koziel, 2008). It has been shown that certain NK cell receptor genes (killer cell immunoglobulin-like receptor 2DL3 (KIR2DL3) and human leukocyte antigen (HLA)-C1) are associated with resolution of HCV infection, suggesting that inhibitory NK cell interactions are important in determining outcome of infection (Khakoo et al., 2004). In CHC, NK cells demonstrate alterations in phenotype and function (Rehermann, 2013), with higher levels of STAT1, increased degranulation and decreased production of IFN-γ and TNF-α compared to NK cells from healthy controls. DAA therapy is associated with a rapid normalisation of NK cell cytotoxic effector functions, a decrease in the expression level of the ISG STAT1, and a decrease in serum levels of CXCL10/IP-10 and CXCL11 (Serti et al., 2015).

Several genetic polymorphisms have been shown to influence the outcome of HCV infection, namely those encoding IFN-λ, IFN-γ, and the NK receptor KIR2DL3 and its HLA ligand as described above (Lemon, 2010). Genome wide association studies (GWAS) found a strong correlation between single nucleotide

polymorphisms (SNPs) near the IL-28B (recently renamed the IFN-λ3 or IFNL3) gene (rs12979860 and rs8099917) and rates of spontaneous and IFN-based treatment-associated clearance of HCV (Ge et al., 2009, Rauch et al., 2010, Suppiah et al., 2009). HCV Gt1-infected patients with the unfavourable IFNL3 rs12979860 TT genetic polymorphism had significantly lower sustained virologic response (SVR) rates in response to IFN-based treatment than patients with the CC genotype. It has previously been demonstrated that patients with IFNL3 non-responder genotypes (non-CC) have higher hepatic ISG expression at baseline than those with responder genotypes (Urban et al., 2010), and that this correlates with a poorer response to IFN-α based therapy (Asselah et al., 2008). Furthermore, analysis of transcription factors in the peripheral blood of patients with CHC suggests that those with IFNL3 responder genotypes have a Th1/NK dominant state favouring viral clearance (O'Connor et al., 2016).

In 2013, a new polymorphism rs368234815 (previously ss469415590; TT or ΔG) upstream from the IFNL3 gene on chromosome 19q13 was found to result in a frameshift mutation, creating the ORF for a novel inducible protein-coding gene, IFNL4, and resulting in transient expression of the IFN analogue, IFN-λ4 (Prokunina-Olsson et al., 2013). The rs368234815-ΔG allele was found to correlate with the previously identified rs12979860-T allele, and more strongly predicts HCV clearance in Gt1 infection (O'Brien et al., 2015). These SNPs can result in the production of no IFN-λ4 protein (TT genotype), a mutant IFN-λ4 protein with reduced antiviral activity (called P70S), or the wild type IFN-λ4 protein. Counterintuitively, individuals with the rs368234815-ΔG allele who produce the active wild type IFN-λ4 protein have significantly lower rates of spontaneous viral clearance. IFN-λ4 induces STAT1 and STAT2 phosphorylation, and activates ISGs. This pre-activation of IFN-signalling may prevent further activation in response to viral infection, and may impair the response to exogenous IFN-α as described above (Prokunina-Olsson et al., 2013). In Gt1-infected biopsies, the presence of the IFNL4 ΔG variant was associated with higher expression of the ISGs IFIT1, ISG15, RSAD2 and USP18. However, IFNL4 genotype did not influence baseline ISG expression in Gt3-infected biopsies, perhaps explaining the reduced predictive power of IFNL3 genotype in Gt3 infection (Mansoor et al., 2016, Cariani et al., 2016).

1.2.2 Adaptive immune response

1.2.2.1 Humoral response to HCV infection

Circulating antibodies against various HCV proteins become detectable in the serum within several weeks of exposure to the virus (Ishii and Koziel, 2008, Neumann-Haefelin et al., 2005). The majority of antibodies have minimal relevant activity against HCV due to its high sequence variability, however rapid induction of virus-neutralising antibodies was associated with spontaneous clearance in one outbreak whilst chronic infection was associated with low titre or absent early phase antibody response (Pestka et al., 2007). Induction of cross-neutralising antibodies in the late phase of infection has been reported in chronically HCV-infected individuals, but is rarely associated with viral eradication (Alter et al., 1989).

The first detectable antibodies against HCV proteins usually target NS3 and core protein, followed by development of antibodies against NS4 and the glycoproteins E1 and E2. The HVR-1 of E2 is a major epitope for neutralising antibodies and the variability observed in this region may well reflect a viral mechanism evolved to evade B cell responses (Simmonds, 2004). HCV is also able to evade the antibody response through cell-to-cell spread via tight junctions between hepatocytes (Swadling et al., 2013).

Protective immunity to HCV remains a controversial subject. *In vivo* studies involving chimpanzees have demonstrated protection against HCV reinfection following vaccination or previous exposure (Farci et al., 1994, Farci et al., 1995). However, multiple re-infections with HCV have been reported in both immunocompetent and immunocompromised patients (Proust et al., 2000, Martin et al., 2013, Prince et al., 1992).

1.2.2.2 Cellular response to HCV infection

At 4-8 weeks following HCV infection, HCV-specific T cells are recruited to the liver. Both CD4+ and CD8+ T cell responses are crucial for an effective immune response to HCV infection. The HCV specific CD8+ T cell response and presence of neutralising antibodies is insufficient to suppress viraemia without the CD4+ T cell response (Kaplan et al., 2007). Without sustained CD4 T cell support, CD8 T

cells cannot control replication and escape mutations are able to develop (Ishii and Koziel, 2008).

CD4+ T cells act through secreting antiviral cytokines and through activation of viral specific B cells and CD8+ T cells. A fall in HCV RNA titre is associated with vigorous HCV-specific T cell responses and the induction of IFN-γ in liver and peripheral blood cells (Ishii and Koziel, 2008).

Studies have demonstrated that the hallmark of chronic infection is a weak, dysfunctional and narrowly targeted T cell response against a minimal number of epitopes (Neumann-Haefelin et al., 2005). As viral persistence is common in acute HCV, with around 80% of individuals progressing to chronic infection, a number of theories have been developed to explain this failure of the T cell response.

Data from the lymphocytic choriomeningitis virus (LCMV) model suggests that T cell 'exhaustion' may develop as a consequence of a high rate of viral replication (Ishii and Koziel, 2008). HCV is able to produce up to 10^{12} virions daily (Neumann et al., 1998), postulated to exceed the capacity of host immunity with resultant inability to maintain CD4+ T cells during acute infection. Additionally, in the chimpanzee model HCV-specific CD8+ T cells in the acute phase display a 'stunned' phenotype and are unable to secrete IFNγ in response to HCV infection (Ishii and Koziel, 2008).

Other groups have described impaired T cell maturation in chronic HCV, demonstrating that HCV-specific CD8+ T cells exhibit phenotypic changes characteristic of early stages of differentiation (CD28+ and/or CD27+) (Neumann-Haefelin et al., 2005) and are functionally impaired (Ishii and Koziel, 2008). HCV is also able to retard the normal maturation of non-HCV-specific cells. Lucas and colleagues (2004) demonstrated that in HCV-infected individuals, CMV specific CD8+ cells (which demonstrate a highly differentiated phenotype in CMV mono-infected subjects) also had higher expression of CD28 and CD27.

Rushbrook et al. (2005) demonstrated that marked CD4+ CD25+ FoxP3+ regulatory T cell (Treg) activity is present in patients with CHC, and this may contribute to weak CD8+ T cell responses. Ebinuma et al. (2008) observed an attenuated antigen-specific T cell proliferative response in FoxP3+ cells and

Cusick et al. (2011) demonstrated that depletion of CD4+ CD25+ cells restored peripheral blood mononuclear cell (PBMC) proliferative responses to the HCV NS3 protein. Additionally, Cabrera et al. (2004) found that HCV-specific IFN-γ was enhanced in PBMCs depleted of CD4+CD25+ and suppressed in PBMCs enhanced with CD4+ CD25+.

Interestingly, CD8+ T cell responses can be restored by blocking the programmed cell death-1 (PD-1)/PD-1 ligand pathway (Radziewicz et al., 2007). PD-1 is a cell surface receptor of the CD28 family and when bound to its ligand generates an immunomodulatory effect. Urbani et al. (2006) demonstrated that CD8+ lymphocyte expression of PD-1 was maintained at high levels in individuals demonstrating viral persistence, compared with rapid downregulation in those individuals who cleared the virus. Inhibition of this pathway was briefly under consideration as a therapeutic adjunct to HCV treatment (Urbani et al., 2008), however the efficacy of the new DAA regimes has rendered manipulation of this pathway largely obsolete in the HCV treatment landscape. Research into the use of PD-1 blockade for checkpoint inhibition has however gained pace in the field of chronic HBV infection (Liu et al., 2014).

Whilst early treatment of HCV with IFN may prevent the development of T-cell exhaustion (Abdel-Hakeem et al., 2010, Badr et al., 2008), IFN-based treatment of CHC is not associated with restoration of HCV-specific CD8+ T cell function (Barnes et al., 2002). It has been postulated that the IFN-based therapy itself contributes to the ongoing impairment of immune cell function through its inhibitory effect on the lymphocyte population. Conversely, viral eradication with an IFN-sparing regime was shown to be associated with a restoration of CD8+ T cell function, and a decrease in PD-1 expression on HCV-specific CD8+ T cells.

More recently, it has been shown that mucosal-associated invariant T cells (MAIT cells; innate-like effector T cells) are reduced in frequency in CHC, and display an activated phenotype (high expression of HLA-DR, granzyme B, PD-1 and CD69) with reduced response to antigen stimulation, and that this MAIT cell dysfunction is not reversed following successful DAA treatment (Hengst et al., 2016).

The importance of the cytotoxic T cell response in the clearance of HCV infection is further highlighted by the association between the HLA class I alleles, which are responsible for restricting the cytotoxic T lymphocyte response, and the likelihood of spontaneous and treatment-associated viral eradication. In a cohort of iatrogenically infected females with Gt1b infection, HLA-B*27 was found to be the strongest predictor of clearance, although A*03, and the class II alleles DRB1*04:01, DRB1*01:01 and DRB1*15, were all associated with viral eradication (McKiernan et al., 2004). Additionally, carriage of HLA-B*57 (associated with elite control in HIV infection (Asher et al., 2013)) has been associated with spontaneous clearance of HCV (Kim et al., 2011).

1.3 Natural history of HCV infection

1.3.1 Outcome following exposure to HCV

After exposure to HCV, there is a window period of 1-3 weeks before HCV RNA is detectable in the serum (Negro, 2012). Acute infection is only symptomatic in ~15% of individuals, complicating its diagnosis. If symptoms are present they tend to be non-specific, requiring a high index of suspicion from the healthcare provider and knowledge of an individual's risk exposure (table 1-1).

Table 1-1: Signs and symptoms associated with acute HCV infection

Weeks from exposure to HCV	Signs and symptoms
2-12	Fatigue Jaundice Flu-like symptoms Dyspepsia Abdominal pain
4-10	Seroconversion
4-12	Elevated or fluctuating liver enzymes

Clinical hepatitis coincides with the appearance of viral-specific T cells in the liver and blood (Missiha et al., 2008). Interestingly, if acute HCV infection is symptomatic there is a much higher chance of spontaneous clearance (approximately 50% compared with <20% in asymptomatic patients), presumably due to the more vigorous immune response. Subjects who spontaneously clear HCV infection remain HCV IgG antibody positive, but HCV RNA is undetectable.

Spontaneous clearance of HCV requires a broad, sustained CD4+ and CD8+ T cell response (chapter 1.2.2.2). Individuals who fail to mount a vigorous immune response develop CHC, defined as viral persistence beyond 6 months post exposure. Spontaneous clearance beyond 6 months infection occurs rarely and is poorly understood. In chronically infected individuals, the immune response is insufficient to eradicate the virus but results in chronic liver inflammation.

These individuals are at risk of progressive liver disease and hepatocellular carcinoma (HCC) (figure 1-7).

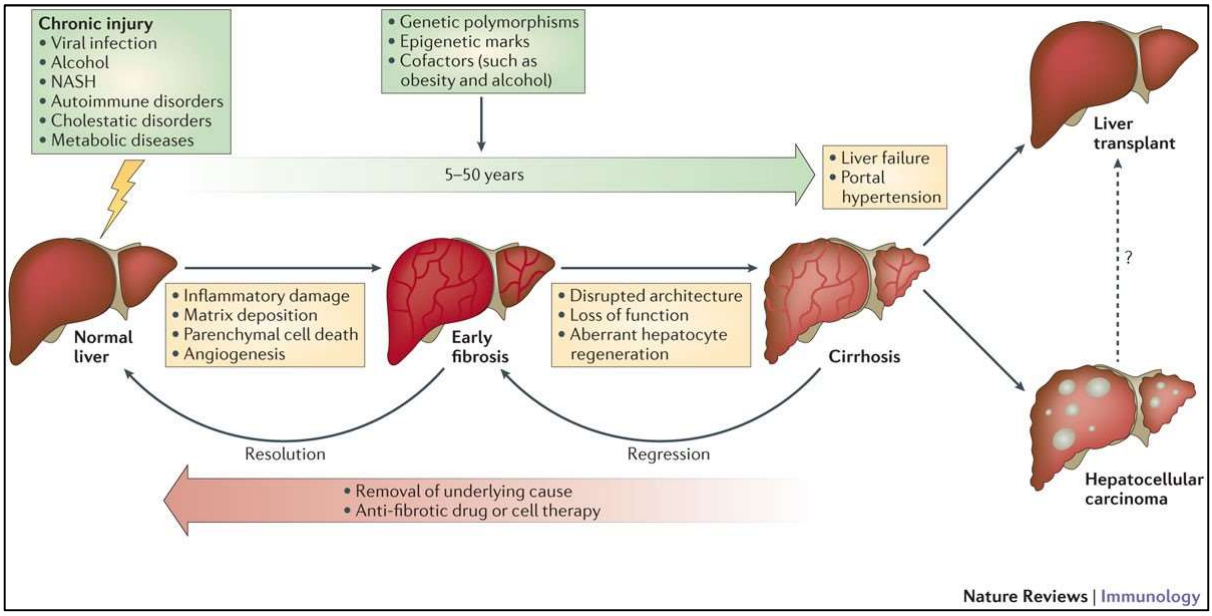


Figure 1-7: Natural progression of chronic liver disease. Extracted from Pellicoro et al. (2014). NASH, non-alcoholic steatohepatitis.

Chronic liver inflammation results in fibrosis, due to the accumulation in the liver of extracellular matrix (ECM) proteins including collagen, laminin, elastin and fibronectin. In liver disease secondary to chronic viral infection, fibrosis occurs mainly around portal tracts. It is a complex process, best viewed as a wound healing response to chronic liver injury (Sebastiani et al., 2014). Fibrosis may actually confer a protective benefit to hepatocytes as deposition of type I collagen protects the cells against toxic stimuli (Bourbonnais et al., 2012), however persistent inflammation results in scarring and ultimately loss of function.

Although multiple cell types contribute to the pathogenesis of liver cirrhosis, hepatic stellate cells (HSCs) are the primary cells responsible for fibrogenesis. HSCs are vitamin A storing cells which reside in the peri-sinusoidal space of Disse and are usually quiescent. Infection with HCV results in damage to liver parenchymal cells, including hepatocytes and cholangiocytes, and the release of reactive oxygen species (ROS) and other inflammatory mediators. Apoptosis both initiates and perpetuates the inflammatory cascade, and is mediated by several

stimuli including Fas and TNF-α. Oxidative stress results in cell death, lipotoxicity, immune infiltration and activation of HSCs, with transformation into proliferative myofibroblasts (Zoubek et al., 2017). These cells are the main source of ECM proteins, and are characterised by enhanced contractility, increased proliferation and migration, and the release of pro-inflammatory and pro-fibrogenic factors.

Monocytes are directed to the site of injury through the CC-chemokine ligand 2 (CCL2)-CC-chemokine receptor 2 (CCR2) pathway, whereas neutrophils are recruited by High-Mobility-Group-Protein B1 (HMGB1) (released following necroptosis) together with the chemokine CXCL12 (Kubes and Mehal, 2012). Phagocytosis of necrotic or apoptotic cells results in further release of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1B, recruitment of T cells and perpetuation of myofibroblast activation. Activated Kuppfer cells (KCs, specialised macrophages) are involved in activation of HSCs and KC-mediated inflammation is thought to aggravate hepatic injury and contribute to the development of cirrhosis (Lopez-Navarrete et al., 2011). Importantly, KCs can elicit expression of platelet derived growth factor receptor (PDGFR) on HSCs *in vitro* (Zhou et al., 2014). Platelet derived growth factor (PDGF) stimulation results in proliferation of myofibroblasts via extracellular signal-regulated kinase (ERK) dependent and independent pathways, whereas transforming growth factor B (TGF-B) released by stimulated immune cells promotes myofibroblast survival through activation of protein kinase B (AKT) and focal adhesion kinase (FAK). TGFB also upregulates type I collagen synthesis and alpha-smooth muscle actin (αSMA) by myofibroblasts leading to fibrogenesis. Interestingly, FAK inhibition reduces HSC migration and expression of αSMA and collagen, and manipulation of this pathway is under scrutiny for the development of anti-fibrotic therapies (Zhao et al., 2017).

The balance between ECM protein deposition and dissolution is dependent on the interplay between matrix metalloproteinase (MMP) enzymes and their inhibitors, tissue inhibitors of metalloproteinase (TIMP). An unfavourable MMP: TIMP ratio results in fibrogenesis and scarring, ultimately leading to cirrhosis. Hepatic myofibroblasts express TIMP1 which inhibits MMP activity and leads to the deposition of ECM proteins (Zoubek et al., 2017). Macrophages play important and opposing roles; restorative macrophages (characterised by low Ly6C

expression) display increased MMP-9 and MMP-13 production as well as expression of phagocytosis-associated receptors (Ramachandran et al., 2012) whereas pro-fibrotic macrophages ($Ly6C^{hi}$) promote hepatocellular injury through the release of pro-inflammatory cytokines as discussed above. The switch in macrophage phenotype from a fibrogenic to a restorative population is thought to occur in response to hepatic expression of CX_3CL1 and other anti-inflammatory signals (Pellicoro et al., 2014). Dendritic and NK cells are also involved in resolution of fibrosis, through promotion of MMP-9 dependent ECM degradation (Ramachandran et al., 2012).

Cytokines and their signalling pathways play important roles in hepatic fibrogenesis. PDGF, TGF-B and TNF-α have well described pro-inflammatory and pro-fibrogenic activity in the pathogenesis of chronic liver disease. A number of other molecules, including the adipokine leptin and connective tissue growth factor (CTGF), promote fibrogenesis through TGF-B1 signalling (Sebastiani et al., 2014). Leptin acts as a suppressor of the anti-fibrotic nuclear receptor peroxisome proliferator-γ (PPARγ), promoting HSC activation (Zhou et al., 2009).

IFNs are well recognised for their antiviral activity (chapter 1.2.1) and appear to also have anti-fibrotic activity. Leukocyte-derived IFN-B is able to inhibit the TGF-B and PDGF pathway in mice, resulting in decreased αSMA and collagen production by myofibroblasts (Rao et al., 2010). IFN-γ release by T cells may also reduce ECM deposition through the TGFB1/Smad3 pathway (Baroni et al., 1996).

Interleukins have both pro- and anti-fibrogenic activity, and can be produced by many cells types, including KCs and liver sinusoidal epithelial cells, in response to injury. IL-1 and IL-17 have pro-inflammatory roles; IL-1 directly activates HSCs and IL-17 is able to upregulate TNF-α, TGF-B1 and collagen via the STAT3 signalling pathway. Conversely, IL-10 downregulates the inflammatory response and modulates fibrogenesis through inhibition of HSC activity (Zhang et al., 2007). High PBMC expression of IL-10 has been associated with progression to chronicity after acute HCV infection, whereas low IL-10 expression is associated with clearance (Flynn et al., 2011).

The HCV viral proteins are able to directly trigger HSC activation. Incubation of NS3 and core protein with activated HSCs resulted in increased intracellular calcium concentration and reactive oxygen species (ROS) production (Bataller et al., 2004). The increased intracellular calcium concentration and ROS production results in the activation of cellular kinases and induction of osteopontin. Osteopontin activation results in phosphorylation of AKT and GSK-3B, promoting mesenchymal transformation of hepatocytes (Iqbal et al., 2013). The NS3 and NS5 proteins stimulate pro-inflammatory pathways (through NFκB and c-Jun terminal kinase (JNK) induction) whereas core protein was associated with increased cell proliferation through PI3K/AKT and Ras/ERK pathways. The E2 envelope glycoprotein is also able to promote fibrogenesis by activating MMP-2 upon binding to CD81 on HSCs, resulting in degradation of ECM, parenchymal damage and influx of inflammatory cells (Mazzocca et al., 2005). HCV infection also leads to HSC activation indirectly through the induction of immune responses and pro-inflammatory cytokines as previously described.

1.3.2 Factors contributing to disease progression in CHC

Liver disease progression in CHC is highly variable. Various modifiable and non-modifiable factors are postulated to influence the rate of disease progression; the most important factors identified appear to be gender, age at acquisition and alcohol consumption (Missiha et al., 2008). However, in a prospective study from the Trent Hepatitis C study group only age at biopsy and pre-existent fibrosis were found to correlate with increased progression (Ryder et al., 2004).

1.3.2.1 Non-modifiable factors

1. Age at acquisition and duration of infection:

   Older age at acquisition is independently associated with a faster progression to fibrosis, even when controlling for duration of infection (Missiha et al., 2008). The rate of disease progression following liver transplantation correlates with increasing age of the donor (Rayhill et al., 2007). Furthermore, children who are vertically infected appear to have a very slow progression to cirrhosis (Tovo et al., 2000).

Fibrosis progression accelerates with time, even after controlling for age (Poynard et al., 2001). A cohort study of Italian patients with bleeding disorders found that both age and duration of infection were associated with severity (Franchini et al., 2001). The reasons for the importance of age as a predictor of progressive fibrosis are undetermined, but may include changes in immune function and reduced hepatic blood flow. The relationship between ageing and HCV infection is reviewed in chapter 1.6.3.

2. Gender:

   Male sex is associated with a faster progression to cirrhosis, even when accounting for age, duration of infection, alcohol intake and metabolic factors. Fibrosis progression is known to accelerate following the menopause, and exogenous oestrogen exposure slows the rate of fibrosis in postmenopausal women, suggesting a hormonal explanation for these differences (Codes et al., 2007, Di Martino et al., 2004, Villa et al., 2012). *In vitro*, oestrogen inhibits proliferation and activity of HSCs, reducing fibrogenesis (Bissell, 1999). It has also been shown that the post-menopausal increase in progression of fibrosis may be due to steatosis (Codes et al., 2007). A further contribution may be made by a sex-specific IL-10 promoter polymorphism, leading to reduced fibrosis in females (Paladino et al., 2006).

3. Race:

   Black patients are less likely to progress to cirrhosis when compared with white patients, despite a higher incidence of HCC and increased liver-related mortality. They also have lower activity scores on biopsy, and lower ALT levels (Sterling et al., 2004, Wiley et al., 2002). Other racial differences are less robustly characterised, although Latino patients in the US demonstrate a faster progression to cirrhosis compared to whites (Missiha et al., 2008). North American Aboriginal populations have a higher rate of spontaneous clearance of the virus, thought to be secondary to a differing response to IFN-α and reduced production of IL-10 in response to viral core protein (Aborsangaya K, 2007, Rempel et al.,

2009). It is thought that differences in immune response may underlie racial variations.

4. Host genetic factors:

The 'favourable' IFNL responder genotype is associated with accelerated disease progression in CHC as a result of higher hepatic inflammatory activity (Bochud et al., 2012). It is also associated with a two-fold increased mortality, and an increased incidence of adverse clinical outcomes (Noureddin et al., 2013). Interestingly, this effect is more pronounced in Gt3-infected individuals (Eslam et al., 2015).

CHC is associated with increased serum and intrahepatic levels of IP-10/CXCL10 (Mascia et al., 2017). IP-10 levels have been shown to predict both fibrosis stage and outcomes with IFN-based HCV treatment (Askarieh et al., 2010, Harvey et al., 2003).

A number of additional host polymorphisms have been reported in association with disease progression in HCV infection, including polymorphisms of TGF-ß1, angiotensin, CCR5-δ32 and IL-10 promoter regions (Paladino et al., 2006, Powell et al., 2000, Patin et al., 2012). However, these results have not been consistent across studies. For instance, Powell and colleagues (2000) were unable to demonstrate any association between different IL-10 genotypes and severity of liver disease and Goyal et al. (2006) found that the CCR5-δ32 mutation did not affect susceptibility to infection or fibrosis stage. Historically, these studies have been limited by small size and failure to control for other factors influencing disease progression (Missiha et al., 2008).

A GWAS for functional single nucleotide polymorphisms (SNPs) was conducted by Huang et al. (2006) on 916 HCV-infected individuals and identified a SNP in the DEAD box polypeptide 5 (DDX5) gene (p68), an RNA helicase. DDX5 is able to bind to the HCV NS5B RNA polymerase, reducing HCV replication *in vitro* (Goh et al., 2004). Individuals with the DDX5 minor allele or DDX5-POLG2 haplotypes demonstrated more rapid fibrosis, although age and steatosis were not controlled for. Further polymorphisms identified through GWAS are currently under investigation,

including SNPs in genes that regulate apoptosis (Patin et al., 2012) and those within the major histocompatibility complex (MHC) on chromosome 6p21 (Urabe et al., 2013). Polymorphisms in the adiponutrin/patatin-like phospholipase domain-containing protein 3 which determines liver fat content have been shown to predict fibrosis progression in non-alcoholic fatty liver disease (NAFLD) as well as CHC (Tamaki et al., 2015). Richardson et al. (2005) have demonstrated that more rapid disease progression correlates with increasing number of fibrotic polymorphisms, highlighting the complexity of the role played by genetic variation in the pathogenesis of HCV-related fibrosis.

5. Viral factors:

   Assessing the effect of viral genotype on pathogenicity has proven challenging given the difficulties in estimating duration of infection and the bias towards recruitment of individuals with clinically apparent disease, thus the impact of viral factors remains controversial.

   Cohort studies of specific risk factor groups, for instance, haemophiliacs, have suggested that HCV Gt1 is more likely to establish persistent infection and to be associated with disease severity (Franchini et al., 2001, Yee et al., 2000). Conversely, Grebely (2014) described an increased likelihood of spontaneous clearance of acute infection associated with Gt1 infection, as has also been described in the chronic setting (Scott et al., 2006). In HBV co-infected patients, a higher incidence of HCC has been described with Gt1 infection, and in mixed infection with Gt 1 and Gt2 (Oh et al., 2012). However, Wright et al. (2003) found that non-1 genotypes were independently associated with disease progression. Gt3 infections are associated with a higher incidence of steatosis which may be associated with accelerated fibrosis progression (Rubbia-Brandt et al., 2000). As described above, the effect of IFNL genotype may also be more pronounced in Gt3-infected subjects.

   High quasispecies diversity is associated with elevated liver transaminases, and may contribute to accelerated fibrosis progression (Asselah et al., 2002).

6. Iron overload:

Iron overload develops in 30-40% of patients with HCV infection as a consequence of liver inflammation and is associated with increased fibrosis (Haque et al., 1996). It is attributed to deregulation of the iron regulatory hormone hepcidin, which is downregulated in response to HCV-induced oxidative stress (Sebastiani et al., 2014). Elevated serum ferritin, reduced hepcidin level and iron accumulation in the liver have all been associated with accelerated disease progression or poor outcome with IFN-based therapy (Tsochatzis et al., 2010, Lange et al., 2012).

1.3.2.2 Modifiable factors affecting disease progression in CHC

I. Alcohol consumption:

Chronic alcohol intake is associated with accelerated fibrosis progression, increased incidence of HCC and higher overall mortality in patients with HCV infection (Negro, 2012, Ostapowicz et al., 1998, Seeff et al., 2001, Wiley et al., 1998). Poynard et al. (2001) showed that patients who consumed over 50 g alcohol/day were more likely to develop progressive fibrosis. The effect of low level alcohol consumption on the history of HCV infection is as yet unknown. However, in cirrhotic patients even moderate alcohol consumption increases the incidence of HCC (Vandenbulcke et al., 2016).

II. Co-infection:

a. HBV

Co-infection with HBV leads to greater liver inflammation, accelerated progression to cirrhosis and a high risk of HCC (Gaeta et al., 2003, Zarski et al., 1998). Additionally, a retrospective cohort study performed by the International Interferon-α Hepatocellular Carcinoma Study Group ('Effect of interferon-alpha on progression of cirrhosis to hepatocellular carcinoma: a retrospective cohort study. International Interferon-alpha Hepatocellular Carcinoma Study Group,' 1998) demonstrated that hepatitis B core antibody (HBc+) positivity is associated with an ongoing

risk of HCC even in the absence of active infection (hepatitis B surface antigen (HBsAg) negative). HBV reactivation has been described in individuals undergoing HCV treatment with both IFN-based therapy (Potthoff et al., 2009) and DAAs (Bersoff-Matcha et al., 2017) and may result in a hepatitis flare. Patients should be monitored closely, and commenced on HBV nucleoside/nucleotide analogue therapy if HBV replication is detected.

b. Human Immunodeficiency Virus (HIV)

As with HBV infection, coinfection with HIV results in more rapid progression to cirrhosis and a higher incidence of HCC (Sulkowski et al., 2007, Garcia-Samaniego et al., 2001). Graham and colleagues (2001) showed that HIV-1 co-infection significantly increased the relative risk of cirrhosis and decompensated liver disease. Co-infection is also associated with reduced likelihood of spontaneous clearance and correlates with a poorer response to IFN-based therapy (Thomas and Seeff, 2005).

c. Schistosomiasis:

Coinfection with HCV and schistosomiasis is common in countries where both infections are endemic and is associated with increased inflammation and progression to fibrosis (Angelico et al., 1997).

III. Metabolic factors:

The relationship between HCV, obesity, fatty liver disease and insulin resistance (IR) is complicated. Non-alcoholic fatty liver disease incorporates both simple steatosis and steatohepatitis, the latter characterised by hepatocyte ballooning, lobular inflammation and progression to fibrosis (Firneisz, 2014). Steatohepatitis is thought to result from oxidative damage generated from fat accumulation in hepatocytes leading to influx of inflammatory cytokines and activation of stellate cells.

Steatosis is more common in HCV-infected patients when compared with the general population, and as described earlier, the incidence and severity may be increased in Gt3 infections (Rubbia-Brandt et al., 2004, Hwang et al., 2001). Body mass index (BMI) is an independent predictor of both steatosis and fibrosis in HCV-infected individuals (Friedenberg et al., 2003). However, steatosis in Gt3 infections can occur independently of obesity and IR and is thought to be secondary to core protein effects on lipid oxidation and LPL assembly. In non-Gt3 infections, incidence of fatty liver correlates with the usual metabolic risk factors (Monto et al., 2002, Rubbia-Brandt et al., 2004). Non-SVR in Gt1-infected individuals is associated with IR, steatosis, more advanced fibrosis and higher circulating levels of the adipokine lectin, which correlates with steatosis severity (Klok et al., 2007, Pavlidis et al., 2011). Interestingly, in Gt3-infected subjects, only fibrosis stage and IR predicted non-SVR. However, Westin and colleagues (2007) found that steatosis was associated with a slower fall in HCV viral load in response to treatment in both Gt3 and non-Gt3 infection.

Both viral and metabolic steatosis may occur in an individual patient, and have a different but additive effect on HCV disease (Adinolfi et al., 2013). In an analysis by Moucari et al. (2008) fibrosis was independently associated with severe steatosis. The presence of hepatosteatosis is associated with enhanced Fas-mediated hepatocyte apoptosis which correlates with increased disease severity (Ribeiro et al., 2004). Concordantly, Schwabl and colleagues (2017) found that hepatic steatosis and higher BMI were associated with increased mean liver stiffness, a lower hepatic venous pressure gradient (HVPG) decrease and persistent elevation of fibrogenic and angiogenic biomarkers following treatment in an HIV/HCV co-infected cohort.

The relationship between liver fibrosis and IR is difficult to assess given the role of the liver in carbohydrate metabolism and insulin degradation. However, studies have suggested that individuals with chronic HCV have an increased incidence of IR and type 2 diabetes mellitus (DM 2) when compared with healthy controls, regardless of cirrhosis (Allison et al., 1994, Fraser et al., 1996). Conversely, Friedenberg (2003) found that

when controlling for age, BMI and family history of diabetes this signal was lost. A later study by Moucari et al. (2008) found a higher frequency of IR in patients with HCV compared with the general population. Infection with HCV Gt1 or Gt4 was independently associated with IR. Serum HCV RNA levels also correlated with IR, suggesting a role for viral replication in the development of IR (Moucari et al., 2008). IR is also known to negatively impact response to HCV treatment (Romero-Gomez et al., 2005, EASL, 2011).

IV. Tobacco and cannabis smoking:

Tobacco smoking is well recognised as a risk factor for HCC, and may be independently associated with fibrosis progression (Hutchinson et al., 2005). The precise mechanisms are currently unknown although lifestyle factors such as smoking may be associated with accelerated cellular ageing.

A recent study by Ishida et al. (2008) also demonstrated that daily cannabis use was associated with moderate to severe fibrosis and they recommended that individuals with HCV should be dissuaded from cannabis use. Conversely, Brunet et al. (2013) were unable to demonstrate a significant association between cannabis use and disease progression in their HIV/HCV co-infected population.

The cannabinoid system consists of two receptors, CB1 and CB2, to which cannabinoids may bind. Depending on which receptor is expressed, cannabinoids can have opposite effects on the liver. Cannabis use leads to accelerated fibrosis through its effects via the CB1 receptor, promoting steatosis (Hezode et al., 2005, Hezode et al., 2008). In animal models, antagonism of the CB1 receptor reduced fibrosis whereas stimulation of the CB2 receptor demonstrated anti-inflammatory and anti-fibrogenic effects (Teixeira-Clerc et al., 2006). Levels of CB1 are 6 times higher in patients with CHC compared with healthy controls, and twice as high in individuals with cirrhosis compared to those with low level fibrosis suggesting that alteration of the CB1/CB2 ratio in HCV infection may

underlie the effect of cannabis ingestion on disease progression (van der Poorten et al., 2010).

V. Coffee intake:

Coffee consumption has been associated with lower mortality and reduced progression to fibrosis in patients with chronic liver disease (Masterton and Hayes, 2010). It is also associated with a reduction in the incidence of HCC regardless of aetiology (Gelatti et al., 2005). The underlying protective mechanism remains obscure, although a recent study has demonstrated that this beneficial effect may be mediated through a reduction in oxidative damage, which correlated with increased telomere length and lower collagen synthesis in patients with CHC (Cardin et al., 2013). Coffee may also reduce intestinal permeability, which may inhibit the promotion of fibrogenesis through bacterial lipopolysaccharide (LPS)-dependent TLR4 signalling (Schnabl and Brenner, 2014).

1.4 Assessment of liver disease severity in CHC

An important part of risk stratification is assessment of fibrosis stage and there are a number of methodologies in use in clinical practice. It is vital that patients with more advanced disease are offered surveillance for oesophageal varices and hepatocellular carcinoma, and where appropriate, are prioritised for antiviral treatment. All patients should receive interventions targeting modifiable factors influencing disease progression (chapter 1.3.2.2), for instance, counselling regarding lifestyle modifications (i.e. abstinence from alcohol, diet, exercise, weight loss), optimisation of glycaemic control and treatment of co-infection.

1.4.1 Invasive methods

Liver biopsy is the gold standard for assessing liver disease severity. Liver biopsy for fibrosis staging is usually performed under radiological guidance, and can be percutaneous or transjugular, with the transjugular route preferred for patients with ascites, or at high risk of complications. Laparoscopic liver biopsy is also performed. Liver biopsy is associated with a low mortality, however complications including pain, bleeding, infection and damage to other structures may occur (Grant and Neuberger, 1999). Histological scoring systems have been developed to report the grade (degree of inflammation) and stage (fibrosis), with Metavir (Bedossa and Poynard, 1996) and Ishak (Ishak et al., 1995) the most commonly used in clinical practice.

The Metavir activity score for chronic hepatitis grades hepatic inflammation into 3 categories, with A1 indicating minimal or mild disease, A2 indicating moderate hepatitis, and A3 reserved for severe disease. The principle observational features are the degree of periportal interface hepatitis (‘piecemeal necrosis’) and parenchymal injury (Goodman, 2007).

The stage of fibrosis is reported as below (figure 1-8):

Appearance	Ishak stage: Categorical description	ISHAK	METAVIR
	No fibrosis (Normal)	0	F0
	Fibrosis expansion of some portal areas ± short fibrous septa	1	F1
	Fibrosis expansion of most portal areas ± short fibrous septa	2	F2
	Fibrosis expansion of most portal areas with occasional portal to portal(P-P) bridging	3	
	Fibrosis expansion of portal areas with marked portal to portal(P-P) bridging as well as portal to central(P-C)	4	F3
	Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)	5	
	Cirrhosis, probable or definite	6	F4

Figure 1-8: Histological scoring systems for hepatic fibrosis severity using Sirius red staining for collagen. Extracted from Standish et al. (2006).

The European Association for the Study of the Liver (EASL) suggest that non-invasive methods can be used instead of liver biopsy, with examination of liver tissue reserved for cases of suspected mixed aetiology or in the case of contradictory results ('EASL Recommendations on Treatment of Hepatitis C 2015,' 2015).

1.4.2 Non-invasive methods

1.4.2.1 Transient elastography

Transient elastography (TE) is a non-invasive technique which uses ultrasound and low-frequency elastic waves to estimate liver fibrosis. It may be affected by age, obesity and necro-inflammatory activity, and although has excellent diagnostic accuracy for the diagnosis of cirrhosis, it is less discriminant at intermediate fibrosis stages (Friedrich-Rust et al., 2008). Liver stiffness values have been shown to correlate strongly with Metavir fibrosis stage in CHC (figure 1-9).

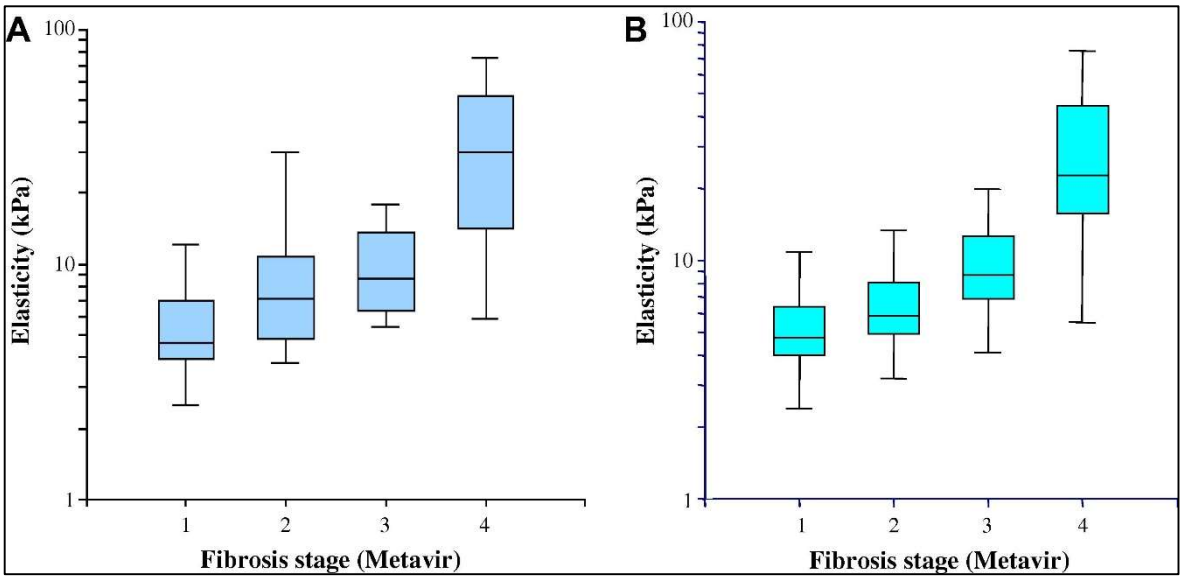


Figure 1-9: Box plots of liver stiffness values (kPa) for each Metavir fibrosis stage. Figure extracted from Castera et al. (2008). The vertical axis is in logarithmic scale. Panel A was adapted from Ziol et al. (2005) and panel B from Castera et al. (2005).

Researchers have suggested that diagnostic accuracy may be improved by combining TE with serum biomarker panels, particularly at the lower stages of fibrosis.

1.4.2.2 Biomarker panels

Well established panels of biomarkers are in use, for instance, Fibrotest™ and the Enhanced Liver Fibrosis Test (ELF™) ('EASL Recommendations on Treatment of Hepatitis C 2015,' 2015). These tests rely on a combination of biochemical and

haematologic parameters, such as aminotransferase level, bilirubin, prothrombin time (PT) and platelet count, with or without additional markers of liver fibrosis, including α2-macroglobulin, haptoglobin and apolipoprotein A1. Other factors influencing fibrosis progression, for instance, age and gender, are factored into several of the scoring systems.

Castera and colleagues (2008) found that a combination of TE and FibroTest™ offered the best diagnostic performance, and when concordant, matched the histological stage in > 90% of cases of severe fibrosis (≥F3) and cirrhosis (F4), and > 80% of cases with significant fibrosis (≥F2). When used individually FibroTest™ was shown to overestimate disease when compared with histology, whereas TE may underestimate fibrosis stage (Castera, 2015). The aspartate aminotransferase (AST) to platelet ratio (APRI) is a simple scoring tool which performs as well as TE in predicting advanced fibrosis (Wong et al., 2017).

Novel scoring panels consisting of angiogenic biomarkers have recently been developed for assessment of liver fibrosis in CHC. The Angio-Index panel includes angiopoietin-2, basic fibroblast growth factor, hepatocyte growth factor and endostatin, and was found to have an excellent predictive value for significant fibrosis (Toson et al., 2017). There has been considerable interest in the role of endogenous non-coding RNAs, such as microRNAs (miRNAs), as biomarkers of hepatic fibrosis and they will be discussed in chapter 1.7.

Interestingly, a number of biomarkers of cellular senescence also function as biomarkers of fibrosis in CHC. Replicative senescence has been associated with expression of senescence associated ß-galactosidase (Sen-ß-Gal) in liver cells of patients with CHC, and Paradis et al. (2001) showed that the presence of liver cells positive for Sen-ß-Gal correlated with age and increasing levels of fibrosis in HCV infection. Recently, the use of osteoprotegerin and collagen IV in a blood test to identify liver fibrosis has been shown to improve diagnostic accuracy (Bosselut et al., 2013). Osteoprotegerin is a member of the TNF receptor superfamily which acts as a decoy receptor for receptor activator of NFκB ligand (RANKL) and is known to be upregulated at senescence, making it an interesting marker for further study and supporting the association between ageing and faster progression to fibrosis. Additionally, chronically HCV-infected individuals have shorter CD4 T cell and hepatocyte telomere lengths compared with healthy

controls, and this has been shown to correlate with both advanced disease stage and poorer response to IFN-based therapy (Hoare et al., 2010b, Sekoguchi et al., 2007).

1.5 Treatment of CHC

1.5.1 History of HCV treatment

The primary goal of HCV therapy is cure, which was historically defined as undetectable HCV RNA 24 weeks after cessation of therapy (SVR24). Over 95% of patients who achieved SVR24 remained non-viraemic long term (Yoshida et al., 2015). More recently it has been demonstrated that an undetectable HCV RNA at 12 weeks post end-of-treatment (SVR12) is equivalent to SVR24 as an efficacy endpoint, and this has now become the standard marker of success in clinical trials (Yoshida et al., 2015). For many years, the standard of care (SoC) consisted of IFN and ribavirin (RBV), with treatment duration based on genotype and treatment response. However, the recent characterisation of the viral proteins and their role in the HCV life cycle has enabled the development of DAAs with marked improvements in tolerability and treatment response.

1.5.1.1 Aciclovir

The antiviral aciclovir was trialled for the treatment of chronic NANBH in 1985 in a small pilot study of only 5 patients (Pappas et al., 1985). Patients were treated with IV aciclovir at a dosage of 5mg/kg 8 hourly for 10 days. In 2 patients, serum aminotransferase levels decreased by more than 50%, however levels in the remainder were unchanged or increased relative to baseline. The 2 patients in whom aminotransferase levels improved were re-treated with a higher dose of aciclovir; aminotransferase levels rose in both patients. During follow up, no patient demonstrated lasting clinical or biochemical improvement, leading researchers to conclude that aciclovir was not effective in the treatment of chronic NANBH (Pappas et al., 1985).

1.5.1.2 Interferon-alpha

Human leukocyte interferon alpha (IFN-α) had been shown to be effective in the treatment of patients with chronic HBV infection and chronic active hepatitis as early as 1976 (Greenberg et al., 1976), and it was demonstrated subsequently that prolonged courses of recombinant IFN-α were associated with loss of HBsAg in a proportion of patients (Dusheiko et al., 1985). It was first used for the treatment of NANBH in 1986, before the identification of HCV as the aetiologic agent (Hoofnagle et al., 1986).

The use of thrice weekly IFN-α for 48 weeks was associated with 10-20% SVR (Poynard et al., 1998, McHutchison et al., 1998). Despite significant side effects and poor virologic response, IFN-α monotherapy remained the SoC for the best part of a decade.

1.5.1.3 Ribavirin

The antiviral activity of the synthetic guanosine analogue RBV was first described in 1972 (Witkowski et al., 1972). It has activity against both RNA and DNA viruses after intracellular phosphorylation, and was first approved for the treatment of severe respiratory syncytial virus (RSV) infection in children (Eggleston, 1987).

RBV was trialled in the treatment of CHC in the early 1990s, and was associated with a transient reduction in serum aminotransferases (Bodenheimer et al., 1997), however mean HCV RNA levels were not significantly decreased after 4 or 24 weeks of therapy (Tong et al., 1994), suggesting that RBV as monotherapy was insufficient. Subsequently, the combination of IFN-α and RBV was found to improve virologic outcomes, resulting in an SVR24 of 20-40%, although at the cost of a significant side effect profile (Bellobuono et al., 1997, McHutchison et al., 1998, Chemello et al., 1995). Older age, stage of liver disease, high pre-treatment HCV RNA and aminotransferase levels, and HCV genotype were found to influence treatment response (Chemello et al., 1995, Sherlock, 1995).

The antiviral mechanism of RBV against HCV is not fully understood. It is proposed to act by a) immunomodulation through induction of a shift from a Th2 to a Th1 immune response, b) inhibition of host inosine monophosphate dehydrogenase (IMPDH), c) direct inhibition of HCV replication and d) induction of mutagenesis (figure 1-10).

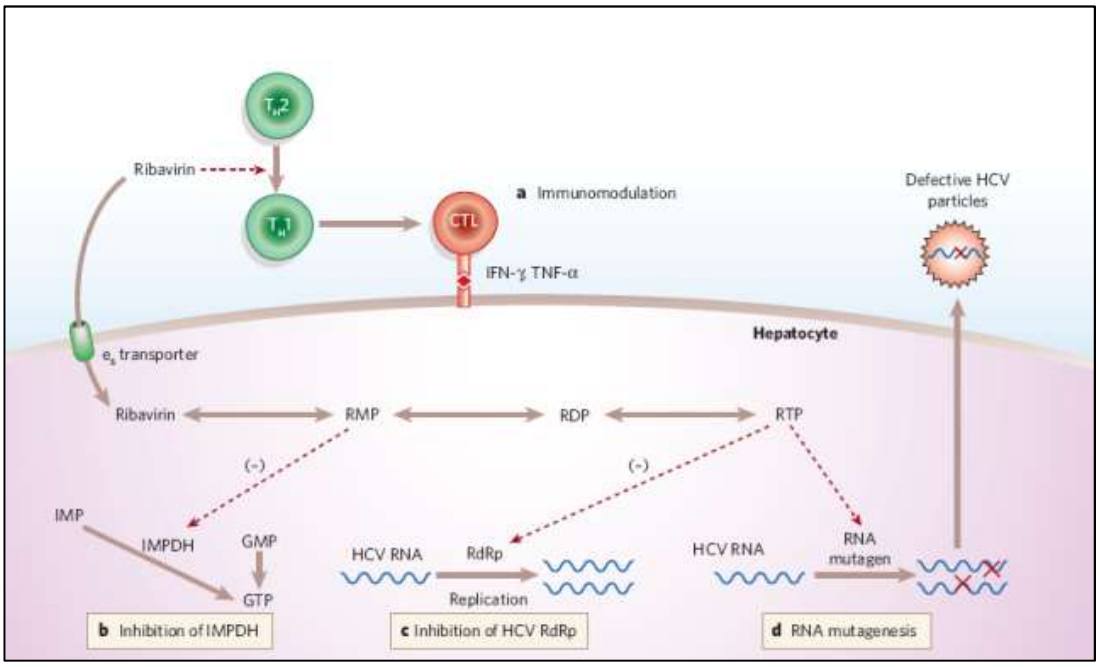


Figure 1-10: Sites of RBV action. Extracted from Feld and Hoofnagle (2005). Th, T-helper cell; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alfa; RMP, ribavirin monophosphate; RDP, ribavirin diphosphate; RTP, ribavirin triphosphate; IMP, inosine monophosphate; IMPDH, inosine monophosphate dehydrogenase; RdRP, RNA-dependent RNA polymerase; CTL, cytotoxic T-lymphocyte; e_s, equilibrative nucleoside transporter; GMP, guanosine monophosphate; GTP, guanosine triphosphate.

1.5.1.4 Pegylated IFN-α (pegIFN-α)

The next advance in the treatment of CHC came through the discovery that the half-life of IFN could be prolonged through PEGylation, the modification of a biological molecule through covalent conjugation with a polyethylene glycol (PEG) polymer.

The use of pegIFN-α in place of standard IFN-α monotherapy was associated with an improvement in SVR rate, but it was the combination of weekly pegIFN-α and daily RBV which became the SoC for the next decade. Two pegylated IFN-α molecules were developed, pegIFN-α2a and pegIFN-α2b. In their pivotal study, Fried and colleagues (2002) demonstrated that the combination of weekly pegIFN-α2a and daily RBV for 48 weeks was associated with an overall SVR24 of 46%, compared with 36% for thrice weekly non-pegylated IFN-α2b plus daily RBV, and 29% for weekly pegIFN-α2a alone. The alternative combination of pegIFN-α2b and RBV was associated with a slightly lower SVR rate of 42% (Manns et al., 2001).

The most important predictors of SVR with pegIFN-α and RBV were found to be HCV genotype, stage of hepatic fibrosis and host IFNL3 genotype, particularly in Gt1 infection. Patients with HCV Gt2 or Gt3 infection had an overall SVR rate of 65-80% with the combination of peg-IFNα and RBV for 24 weeks, compared with just 40-50% in HCV Gt1 infected patients receiving 48 weeks of therapy (EASL, 2011).

The combination of pegIFN-α and RBV was associated with significant side effects. The use of RBV is associated with the development of a dose-limiting reversible haemolytic anaemia (Bodenheimer et al., 1997), possibly as a consequence of oxidative damage to erythrocyte membranes (De Franceschi et al., 2000). Other haematological and biochemical side effects of combination therapy include neutropenia, thrombocytopenia and ALT flares. Thyroid function abnormalities are reported with pegIFN-α, and adequately controlled thyroid function is a prerequisite for treatment. Both pegIFN-α and RBV are contra-indicated in patients with severe pre-existing cardiac disease and electrocardiogram monitoring is recommended during their use. Neuropsychiatric side effects are also described secondary to both drugs, for instance, ‘riba rage’, and they are contra-indicated in individuals with severe psychiatric disorders. Additionally, their use in patients with depression and comorbid substance use disorders must be judged on an individual basis, under expert guidance. Importantly, RBV is teratogenic; pregnancy must be excluded before it is used, and effective contraception is essential during treatment and for 6 months beyond (EASL, 2011). Both drugs must be used with caution in patients with cirrhosis, and pegIFN-α is contra-indicated in decompensated disease.

As a consequence of the side effects and poor tolerability, the use of pegIFN-α is now rarely recommended as first line therapy for acute or chronic HCV infection, but the addition of pegIFN-α to a DAA combination may still be considered as salvage therapy for patients who have failed an IFN-sparing DAA regimen (Feld, 2014). One exception is in the treatment of IFN-eligible, treatment-naïve Gt2 HCV-infected individuals, in whom 16-24 weeks of pegIFN-α with RBV may still be considered due to the extremely high SVR rates seen with this combination (circa 90%) (Dillon et al., 2017). Additionally, the use of weight-based RBV in patients who have baseline predictors of poor response to DAA treatment is still

recommended ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016) (see chapter 1.5.2).

1.5.1.5 Direct-acting antivirals

Advancements in understanding of the HCV viral proteins and their role in the HCV life cycle (chapter 1.1.4) opened the door for the development of antiviral compounds active against these proteins. Four different classes of DAAs are now available, with activity against 3 essential viral proteins: the NS3/4A protease, the NS5A protein and the NS5B polymerase. Combinations of these DAAs (+/- RBV) now lead to cure rates of 90-100%, largely irrespective of genotype.

I. NS3/4A protease inhibitors

The first DAAs licensed for use in the treatment of CHC were the NS3/4A serine protease inhibitors, telaprevir and boceprevir. These first generation protease inhibitors were only licensed for use in Gt1 infection, and improved the SVR rate to 65-75% in combination with pegIFN-α and RBV (Lawitz et al., 2008, Jacobson et al., 2011, Kwo et al., 2010, Poordad et al., 2011, Zeuzem et al., 2011). Although these combinations were more effective, they were poorly tolerated and associated with significant adverse effects leading to an increased rate of discontinuation. Both were associated with dermatologic side effects including rash and pruritis, and telaprevir was particularly associated with the development of Stevens-Johnson syndrome and toxic epidermal necrolysis. Haematologic side effects including anaemia, leukopenia and neutropenia were commonly reported, as were gastrointestinal side effects including dysgeusia and proctalgia.

The next protease inhibitor to be approved for the treatment of chronic Gt1 HCV infection was simeprevir. The use of simeprevir in combination with pegIFN-α and RBV was associated with an SVR of 85-95% in treatment-naïve patients, and was associated with an improved side effect profile (Fried et al., 2013, Hayashi et al., 2014, Manns et al., 2014, Jacobson et al., 2014, Scott et al., 2015). This combination was also effective in patients who had previously failed pegIFN-α based treatment, demonstrating SVR rates of 61-80% compared to just 23% with pegIFN-α/RBV alone (Zeuzem et al., 2014a, Forns et al., 2014). The presence of

the resistance-associated substitution (RAS) Q80K in the NS3 protease sequence was found to be associated with significantly lower SVR rates.

In general, NS3/4 protease inhibitors have a high antiviral efficacy, but have a low barrier to resistance and must be prescribed as part of combination therapy. Currently, there are 3 NS3/4A protease inhibitors recommended as part of DAA regimens for the treatment of CHC in adults in Scotland: simeprevir, paritaprevir and grazoprevir (Dillon et al., 2017). A further protease inhibitor, voxilaprevir, is showing promise in the treatment of NS5A-inhibitor experienced patients in combination with sofosbuvir and velpatasvir (Bourliere et al., 2017).

All 3 currently available protease inhibitors are metabolised by cytochrome P450 3A4, and co-administration with drugs that induce or inhibit CYP3A4 should usually be avoided. This interaction is exploited however by the co-administration of paritaprevir with low dose ritonavir (a potent CYP3A4 inhibitor) which acts as a pharmacokinetic enhancer and allows once-daily dosing. Simeprevir and paritaprevir are inhibitors of the efflux transporter P-glycoprotein (P-gp) and the uptake transporter OATP1B1, and simeprevir and grazoprevir are mild inhibitors of CYP3A. Transient increases in bilirubin may be observed during treatment with simeprevir and paritaprevir (Zeuzem et al., 2014b) due to the inhibition of the bilirubin transporter OATP1B1. Both paritaprevir and grazoprevir additionally inhibit breast cancer resistance protein (BCRP), another ATP binding cassette (ABC) efflux transporter. All 3 drugs are transported by P-gp, OATP1B1 and OATP1B3 in addition to others. Drug interactions are common and should be actively sought prior to treatment, with consideration given to illicit recreational drug use ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016). Protease inhibitors should not be used in patients with advanced cirrhosis (Child-Pugh stage B or C) because of the substantially higher drug concentrations in this patient group.

II. NS5B polymerase inhibitors

Sofosbuvir, a nucleoside analogue inhibitor of the NS5A RdRp, was approved in January 2014. Sofosbuvir has pan-genotypic activity; the combination of sofosbuvir, pegIFN-α and RBV for 12 weeks for HCV Gt1, Gt4, Gt5 and Gt6 infection was evaluated in the NEUTRINO study. The study cohort consisted of

primarily Gt1 and Gt4 infected patients (98%) and the combination was associated with an SVR rate of ~90%. The combination was well tolerated, the most common side effects being fatigue and headache. Patients with cirrhosis did less well on treatment, with an overall SVR rate of 80% (Yoshida et al., 2015).

Sofosbuvir is not metabolised by CYP450 or uridine 5'-diphospho-glucuronosyltransferase (UGT), but it is transported by P-gp and should not be co-administered with drugs that inhibit P-gp, for instance, rifampicin. It does not inhibit or induce the drug transporters described previously and drug-drug interactions are minimal. One notable exception is amiodarone, co-administration of which may result in extreme bradycardia (Renet et al., 2015).

Dasabuvir is a non-nucleotide inhibitor of the HCV RdRp, which acts by changing the 3D conformation of the enzyme and inhibiting its function. It is metabolised in the liver by CYP2C8 and CYP3A4, and inhibits UGT1A4, BCRP and P-gp *in vivo*. It is co-prescribed with paritaprevir, ritonavir and ombitasvir +/- RBV for the treatment of HCV Gt1 infection. The response rate in patients with Gt1b infection was extremely high (97-100%), regardless of prior pegIFN-α experience or presence of compensated cirrhosis (Ferenci et al., 2014, Dore et al., 2016, Zeuzem et al., 2014b, Andreone et al., 2014, Feld et al., 2016). Individuals with HCV Gt1a infection had lower responses, but benefited from the inclusion of RBV (SVR rates of 90% and 97%, respectively) (Ferenci et al., 2014).

Failure to achieve an SVR with dasabuvir-containing regimens is associated with the development of RASs in the polymerase sequence. However, sofosbuvir has a high barrier to resistance and persistent, clinically relevant RASs have not been reported in treatment failure (Sarrazin, 2016). The S282T mutation, conferring 2.4-18.1 fold reduced susceptibility to sofosbuvir, is selected for *in vitro* (Gane et al., 2017a), but rarely emerges in clinical trials (Gane et al., 2013). It is therefore recommended that sofosbuvir forms the backbone of any re-treatment strategies.

III. NS5A replication complex inhibitors

There are 5 NS5A inhibitors currently recommended as part of DAA combinations in treatment guidelines ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016): daclatasvir, ledipasvir, ombitasvir, elbasvir and velpatasvir. The use of daclatasvir in the treatment of CHC was approved in 2014. It is a substrate of CYP3A4, and a substrate and inhibitor of P-gp. Daclatasvir has pan-genotypic activity, and the combination of sofosbuvir and daclatasvir was associated with high SVR rates in both treatment-naïve patients and those who had previously failed regimens containing first generation NS3/4A protease inhibitors (Sulkowski et al., 2014). The combination of daclatasvir, pegIFN-α and RBV was non-inferior to pegIFN-α/RBV (Dore et al., 2015, Hezode et al., 2015) but has been superseded by more efficacious regimens and is not recommended in international guidelines ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016).

Ledipasvir and velpatasvir are both administered as part of fixed-dose combinations (FDC) with sofosbuvir, with or without additional RBV depending on pre-treatment profile. Ledipasvir is a weak inducer of CYP3A4, CYP2C and UGT1A1 *in vitro*, and inhibits intestinal CYP3A4, UGT, P-gp and BCRP. Caution is therefore recommended when co-prescribing ledipasvir and P-gp substrates, for instance, digoxin. The solubility of ledipasvir is pH-dependent and real-world data suggest reduced SVR rates in patients receiving high dose proton-pump inhibitor therapy (Terrault et al., 2016). Velpatasvir is metabolised by CYP2B6, CYP2C8 and CYP3A4. It is a weak inhibitor of P-gp, OATP1B1 and OATP1B3, and a moderate inhibitor of BCRP, and is transported by the same.

The combination of ledipasvir and sofosbuvir for HCV Gt1 infection was studied in the ION Phase III trials. It was found to be extremely effective in treatment-naïve and treatment-experienced cohorts, and in patients with compensated cirrhosis (Afdhal et al., 2014a, Afdhal et al., 2014b, Kowdley et al., 2014).

The once daily, pangenotypic FDC of sofosbuvir and velpatasvir was investigated as treatment for CHC in the ASTRAL studies. ASTRAL-1 included treatment naïve and treatment-experienced patients with HCV genotypes 1, 2, 4, 5 and 6 infection, including patients with compensated cirrhosis. The combination was

found to be extremely effective, with an overall SVR of 99% (Feld et al., 2015). In the ASTRAL-2 and ASTRAL-3 studies, a 12 week course of sofosbuvir/velpatasvir was compared to the same duration of sofosbuvir/RBV in a cohort of both untreated and treatment-experienced patients with HCV Gt2 and Gt3 infection. For Gt2 infection, the SVR with sofosbuvir/velpatasvir was 99%, compared with 94% in the sofosbuvir/RBV arm. Individuals with HCV Gt3 infection had SVR rates of 95% and 80%, respectively (Foster et al., 2015). The combination was also studied in HCV Gt1-6 infected patients with decompensated cirrhosis (Child-Pugh B and above). Patients were assigned in a 1:1:1 ratio to receive sofosbuvir/velpatasvir for 12 weeks, sofosbuvir/velpatasvir plus RBV for 12 weeks or sofosbuvir/velpatasvir for 24 weeks. The overall SVR rates were 83% (95% CI, 74-90%), 94% (95% CI, 87-98%) and 86% (95% CI, 77-92%), respectively. Serious adverse events were similar between groups, and anaemia was common in patients receiving RBV (Curry et al., 2015). More recently, the combination of sofosbuvir/velpatasvir/RBV for 24 weeks has been studied in a cohort of HCV-infected patients who had previously failed a DAA containing regimen, and resulted in an overall SVR of 91% (95% CI, 82-97%) (Gane et al., 2017b). A 12 week course of sofosbuvir/velpatasvir is the preferred first line therapy for HCV Gt3 infected individuals in Scotland, with the consideration of additional RBV in those with cirrhosis (Dillon et al., 2017).

Finally, the NS5A inhibitor elbasvir is available as part of a FDC with the NS3/4A serine protease inhibitor grazoprevir for the treatment of HCV Gt1, Gt4 and Gt6 infection. Elbasvir is metabolised by CYP3A4 and inhibits P-gp and BCRP. It is transported by P-gp. Elbasvir/grazoprevir was shown to be highly effective in the treatment of CHC in both treatment-naïve and -experienced patients (Kwo et al., 2017, Zeuzem et al., 2015, Lawitz et al., 2015, Buti et al., 2016). The combination of elbasvir/grazoprevir for 12 weeks is the preferred first line therapy for individuals with HCV Gt1 infection in Scotland, with the addition of RBV for patients with compensated cirrhosis (Dillon et al., 2017). The duration should be extended to 16 weeks in patients with a high baseline HCV viral load and/or the presence of baseline NS5A RASs as these factors were found to determine treatment response (Komatsu et al., 2017). This combination is safe for use in patients with end stage renal failure (ESRF) (Roth et al., 2015),

including those on haemodialysis, but it is contraindicated in patients with decompensated cirrhosis (Child-Pugh stage B or C).

1.5.2 Factors influencing treatment response

1.5.2.1 IFN-α based treatment

In general, the modifiable factors influencing treatment response to IFN-based therapy mirror those affecting disease progression (chapter 1.3.2). Prior to the advent of the DAAs, the most important factors influencing response were HCV genotype, host IFNL3 genotype, and disease stage (Sherlock, 1995). It is now recognised that the functional variant lies within the INFL4 gene locus upstream of the IFNL3 locus on chromosome 19q13 (O'Brien et al., 2015). Expression of the wild type IFN-λ4 protein (associated with the unfavourable rs368234815-ΔG allele) is associated with increased basal antiviral ISG expression (see chapter 1.2.1) and a reduced response to exogenous IFN-α (Murakawa et al., 2017). Other genetic polymorphisms of interest include SNPs in SPP1, the gene encoding osteopontin. Osteopontin is an important protein with cytokine functions that is crucial for initiation of the Th-1 immune response. Serum osteopontin level was found to correlate with treatment response, and SNPs in the promotor region of SPP1 have been shown to predict SVR rates with IFN-based therapies (Naito et al., 2005, Shaker et al., 2013). Other predictors of response included baseline HCV RNA level, age, IR, gender, ALT level or co-infection with HIV or another hepatotropic virus (Manns et al., 2006).

1.5.2.2 DAA combination therapy

Whilst DAA combinations are extremely effective and associated with excellent SVR rates in the majority of patients, there remain a number of negative pre-treatment factors. An individualised approach to treatment is necessary, with consideration of HCV genotype/subtype, baseline resistance variants, presence of cirrhosis, prior treatment experience, and potential drug-drug interactions.

The presence of cirrhosis is associated with a poorer response to combination DAA therapy (Majumdar et al., 2015). The addition of RBV to sofosbuvir/velpatasvir was associated with a significant improvement in SVR in patients with decompensated cirrhosis (Curry et al., 2015). The real-world benefit of additional RBV in decompensated cirrhosis was also demonstrated in

the NHS England expanded access programme (EAP) (Foster et al., 2016). RBV may also be of value in the treatment of individuals with baseline RASs conferring reduced susceptibility to DAAs.

As previously described, RASs are amino acid substitutions in the viral RNA which confer resistance to DAA therapy. The first clinically important RAS identified in the DAA era was the naturally occurring Q80K mutation in the NS3 protease sequence which negated the effect of adding simeprevir to pegIFN-α/RBV in Gt1a HCV-infected individuals. Gt1a-infected subjects with a Q80K substitution had significantly lower SVR rates following treatment with pegIFN-α, weight-based RBV and simeprevir (overall SVR 58% in treatment-naïve patients, and 47% in IFN-α experienced) (Jacobson et al., 2014, Forns et al., 2014). This mutation was seen in ~13% of Gt1 infected, treatment naïve patients in a Scottish cohort (Shepherd et al., 2015). Additional NS3 resistance variants include the substitutions R155K and A156T/V (Tong et al., 2006, Halfon and Locarnini, 2011). Variants with natural RASs conferring resistance to DAAs may be present at low level as part of the circulating quasispecies population, but be selected for under antiviral pressure (Abdelrahman et al., 2015). Many RASs in the NS3 sequence confer a loss of replicative fitness (excepting Q80K) and are replaced quickly by wild-type virus when protease-based antiviral therapy is discontinued.

In contrast, RASs in the NS5A sequence do not appear to affect viral fitness and may persist long-term (Krishnan et al., 2015). Naturally occurring variants conferring resistance to NS5A inhibitors also appear to occur more frequently (Sarrazin, 2016, Welzel et al., 2017, Hirotsu et al., 2015).

Persistence of variants conferring resistance to sofosbuvir have not been described. The S282T variant results in markedly impaired viral fitness, however rapid reversion to wild-type virus is described after relapse, with the variant becoming undetectable 24-48 weeks post-sofosbuvir (Hedskog et al., 2015, da Silva Filipe et al., 2017, Gane et al., 2017a). However, naturally occurring variants associated with low-level resistance have been described, including C316N and S556G, and may persist long-term (Krishnan et al., 2015). S556G is the natural variant in HCV genotypes 2, 3, 4 and 5, explaining dasabuvir's genotype-specific activity (Di Maio et al., 2014).

International guidelines currently recommend sequencing only the NS5A region, and using a deep sequencing cut-off of 15% (i.e. only RASs present in >15% of generated sequences should be considered) ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016). A number of clinically important NS5A RASs have now been identified (table 1-2).

Table 1-2: Clinically important RASs

NS5A aa position	Ledipasvir RASs (Gt1a)	Elbasvir RASs (Gt1a)	Velpatasvir RASs (Gt3)
M28	M28A M28G M28T	M28A M28G M28T	
Q30	Q30E Q30G Q30H Q30K Q30R	Q30D Q30E Q30G Q30H Q30K Q30L Q30R	
L31	L31M L31V	L31F L31M L31V	
P32	P32L P32S		
H58	H58D	H58D	
Y93	Y93C Y93H Y93N Y93S	Y93C Y93H Y93N Y93S	Y93H

Whilst RASs may be associated with suboptimal efficacy of DAAs, treatment failure usually occurs only in the context of other negative predictive factors, for instance high baseline HCV viral load and advanced liver disease (Sarrazin,

2016). Extending the duration of treatment or intensification with the addition of RBV may negate the effect of baseline RASs on treatment outcomes (Sarrazin et al., 2016).

Interestingly, IFNL4 genotype may also be relevant in determining response to IFN-sparing regimens. Meissner and colleagues (2014) found that the IFNL4-ΔG allele was associated with slower early viral decay in response to sofosbuvir and RBV, and reduced treatment efficacy. The IFNL4 genotype may also influence the development of RASs: it is hypothesised that patients with the IFNL4-TT/TT genotype have a more efficient antiviral response to HCV infection, resulting in selection for viral adaptations (Akamatsu et al., 2015). In support of this, the IFNL4-TT/TT genotype has been shown to correlate with the presence of NS5A RASs at Y93H in HCV Gt1 infected subjects (Peiffer et al., 2016).

One major advantage of IFN-sparing combinations has been in the treatment of HCV in patients co-infected with HIV. Prior to the advent of DAAs, HIV/HCV co-infection was associated with a significantly poorer response to IFN-based treatment (Laguno et al., 2004, Moreno et al., 2005). The PRESCO trial found an overall SVR rate of 50% in response to pegIFN-α/RBV in HIV/HCV co-infected subjects (35% in HCV Gt1, and 72% in HCV Gt3) (Nunez et al., 2007). The combination was associated with significant side effects, including a reduction in CD4 count leading to increased susceptibility to opportunistic infection. HIV co-infection is no longer considered a negative predictor of response to treatment; HIV/HCV co-infected patients respond as well as HCV mono-infected patients to DAA combinations (Milazzo et al., 2016, Rockstroh et al., 2015, Schwabl et al., 2017, Sulkowski et al., 2015).

Older age, independent of fibrosis stage, does not appear to confer a negative prognostic impact on virologic outcomes with DAA treatment, however it may affect the incidence of adverse clinical events. Although age at baseline was not significantly associated with adverse outcome, Cheung et al. (2016) found that even in subjects with baseline albumin > 35g/L, only 50% of individuals aged ≥ 65 years remained free from adverse event during follow up (death, hospitalisation, transplantation, decompensation or sepsis), compared with 63.5% of patients < 65 years old.

It is clear that even in the DAA era, the relationship between ageing and negative clinical and treatment outcomes in CHC warrants further scrutiny.

1.6 Biological ageing in CHC

1.6.1 Biological ageing

Ageing is a complex physiological process that is mediated by genetic, epigenetic, cellular and environmental factors resulting in organ damage, an increased susceptibility to disease, decreased stress response and ultimately death (Deeks, 2011). Evidence is accumulating to suggest that cellular senescence may function as an essential mechanism to limit the proliferation of damaged cells, and that ageing may result partly from the efforts of the cellular stress response programme to reduce the risk of neoplastic change following insult (Hoare et al., 2010a, Kim and Sharpless, 2006). Cellular senescence may be triggered by telomere dysfunction, mitochondrial deterioration, DNA damage and chromatin instability, oxidative and oncogenic signals (Coppe et al., 2010, Hoare et al., 2010a) (figure 1-11).

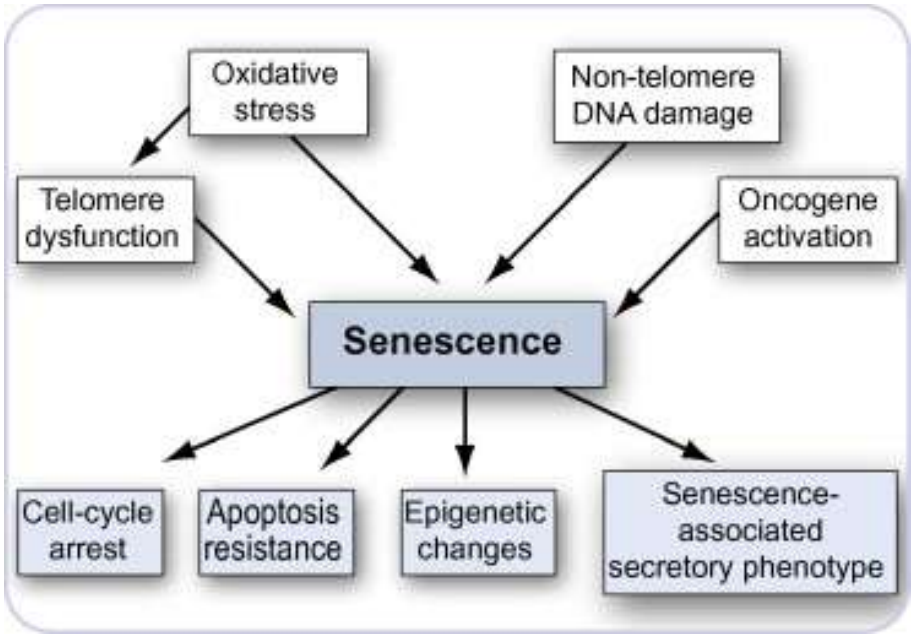


Figure 1-11: Overview of cellular senescence, extracted from Hoare et al. (2010a)

Depending on the level of stressors, cell populations will react in different ways with increasing levels of stress leading to senescence, apoptosis or necrosis. Senescent cells remain metabolically active, but display an altered phenotype and elevated Sen-ß-Gal activity.

Accelerated senescence is now recognised in a number of chronic medical conditions, and both elucidation of the pathways involved and identification of modifiable factors has come under intense scrutiny.

1.6.1.1 Cellular senescence

I. Telomere dysfunction

Telomeres are repeating hexanucleotide sequences with telomere binding protein complexes that function to cap the chromosomal ends and prevent chromosomal instability (Hoare et al., 2010a, von Figura et al., 2009). In humans, telomeres consist of tandem repeats of the DNA sequence TTAGGG over an area of 10-15 kb together with a 3' single strand overhang (Kitay-Cohen et al., 2008). The 3' overhang forms a T loop through insertion in the upstream double strand telomere sequence, protecting the telomere from developing a DNA damage response. The shelterin complex includes telomere repeat binding factors 1 and 2 (TRF1 and TRF2) and binds to the double-stranded telomere to protect and regulate this unique structure (figure 1-12).

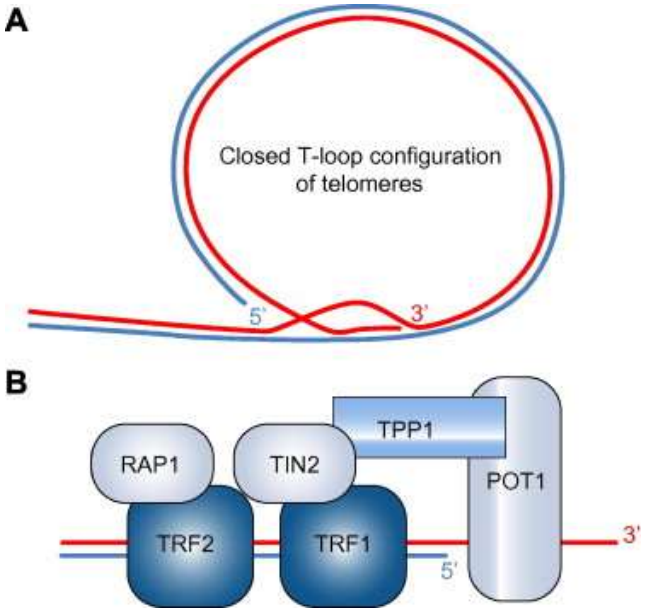


Figure 1-12: Structure of the human telomere. Extracted from Hoare et al. (2010a). RAP1, repressor/activator protein 1; TIN2, TRF1-interacting nuclear protein 2; TRF2, telomere repeat binding factor 2; TRF1, telomere repeat binding factor 1; POT1, protection of telomeres 1.

Telomeres act to protect chromosomes from recombination, end-to-end fusion and recognition by DNA damage checkpoints and contribute to the organisation of the chromosomes within the nucleus. They are also involved in the regulation of gene expression and control the replication capacity of the cell (Cong et al., 2002).

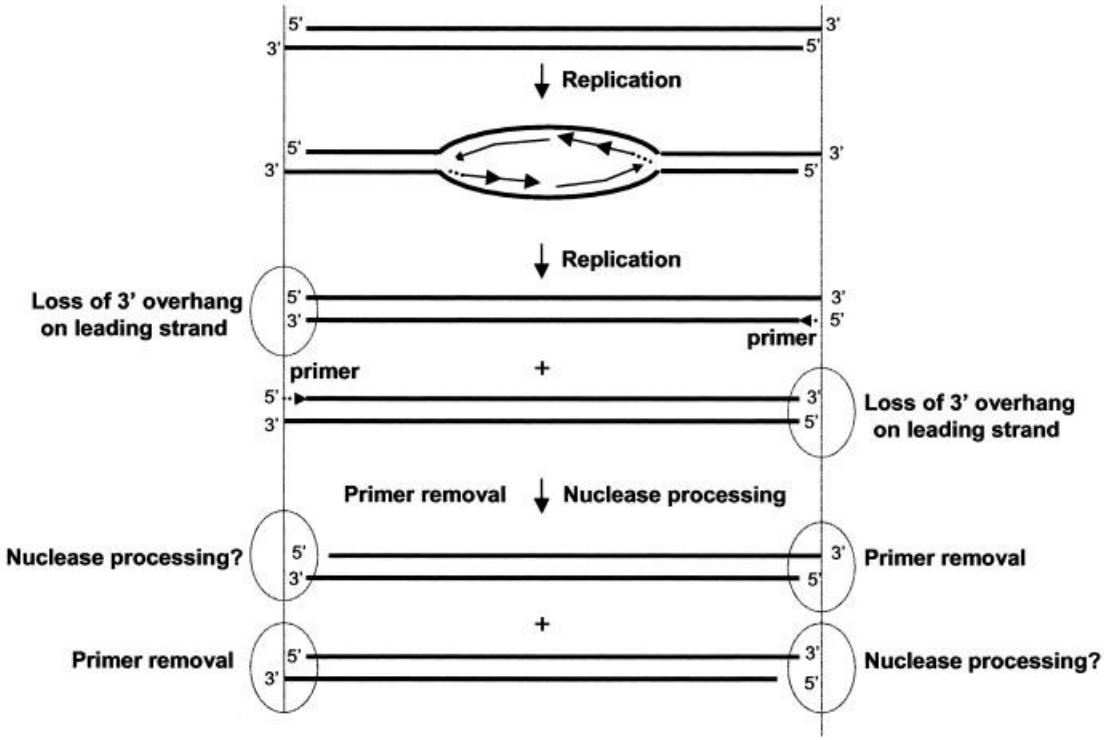


Figure 1-13: End replication problem, extracted from Cong et al. (2002). DNA replication by the conventional polymerase proceeds in the 5′-to-3′ direction. The newly synthesized leading strands would not generate overhangs, but the newly synthesized lagging strands would lose their extreme 3′ end after RNA primers are removed. Both parental strands may also be subject to nuclease processing.

In the absence of compensatory mechanisms, telomeres of proliferating cells shorten with each cell division (figure 1-13). On reaching a critical length, telomeres lose the ability to cap the chromosomal ends and DNA damage checkpoints are activated. Telomere dysfunction has been shown to activate p53/p21-dependent cell cycle arrest or apoptosis *in vivo*, dependent on cell type (von Figura et al., 2009). This was first formally described by Hayflick in 1965 as the process that limits the proliferation of human cells in culture and has thus been termed the 'Hayflick limit' (Hayflick, 1965) or cellular senescence (mortality stage I (M1)). Studies in mice revealed a cell cycle arrest response in liver cells, and an apoptotic response in lymphocytes (Satyanarayana et al., 2003).

Studies on fibroblasts have shown that interruption of p53-mediated senescence leads to an increased lifespan of cells, but that ongoing telomere shortening induces a second p53 independent checkpoint, named crisis or mortality stage II (M2). This stage is associated with marked chromosomal instability secondary to telomere dysfunction and ultimately cell death (Cong et al., 2002). Crisis results from a loss of inhibitors of DNA damage response elements with subsequent recruitment of the PI3 kinases ATM and ATR, phosphorylation of histone 2A at serine 139 (γ-H2AX) and stabilisation of p53 and p21. Cellular senescence and crisis are important functions to prevent the replication and growth of genomically unstable cells.

Telomere shortening has been described in many tissues during ageing, including peripheral blood cells, hepatocytes and lymphocytes (Jiang et al., 2007). It is postulated to contribute to the age-related accumulation of cytogenetic abnormalities recognised in human lymphocytes (Ramsey et al., 1995). Longer telomeres are associated with protection from age related diseases, improved cognition and better lipid profiles (Atzmon et al., 2010). Accelerated telomere shortening has also been described in a number of chronic inflammatory conditions, including cirrhosis, chronic HIV infection and inflammatory bowel disease (Deeks, 2011, Kinouchi et al., 1998, Kitada et al., 1995). Lifestyle factors commonly associated with ill health have also been shown to be associated with accelerated telomere shortening, for instance, smoking and a sedentary lifestyle (Song et al., 2010).

II. Telomerase activity

Telomerase is an RNA-dependent DNA polymerase that adds telomeric DNA to eukaryotic telomeres (Cong et al., 2002) to prevent telomere shortening with cell replication (von Figura et al., 2009). Telomerase consists of two essential components: a catalytic protein with reverse transcriptase activity (hTERT), and an RNA component (hTERC) which serves as the template for the synthesis of telomeric DNA. It includes several other proteins, including heat-shock protein 90 and dyskerin, which are known to be important for activity, stability and maturation of telomerase.

In human cells, activity of telomerase is tightly regulated. Expression of telomerase is high in cells with a high proliferative potential, such as germ cells and activated lymphocytes, yet most human somatic cells are telomerase negative. Overexpression of telomerase enables the cell to circumvent the DNA damage checkpoints M1 and M2, resulting in unlimited proliferation and 'immortalisation' of cell lines. It is recognised in approximately 90% of tumour samples (Cong et al., 2002, Kim et al., 1994). In support of this, whilst telomere dysfunction in telomerase knockout mice (Terc-/-) was associated with tumorigenesis secondary to chromosomal instability, tumour progression was impaired due to induction of cell cycle arrest and apoptosis (Lechel et al., 2007).

However, telomerase dysfunction is also associated with impaired organ maintenance and suppressed stem cell function (Rudolph et al., 1999, von Figura et al., 2009). Reduced telomerase function (as seen in dyskeratosis congenita) is associated with premature greying of the hair, osteoporosis, immune senescence and other conditions associated with frailty. More than 50% of cases of dyskeratosis congenita result as a consequence of mutations in genes encoding proteins that maintain telomere function, resulting in markedly shortened germ line telomeres (Cong et al., 2002, Savage and Alter, 2009).

1.6.1.2 Immunosenescence

Ageing results in changes to both the innate and adaptive immune system (figure 1-14). A number of characteristic changes are described, including i) reduced number and functionality of hematopoietic stem cells, ii) thymic involution, iii) increased frequencies of well-differentiated CD28-CD57+ T cells, iv) increased pro-inflammatory cytokines, v) reduced circulating naïve T cells and vi) decreased CD4/CD8 ratio (Deeks, 2011).

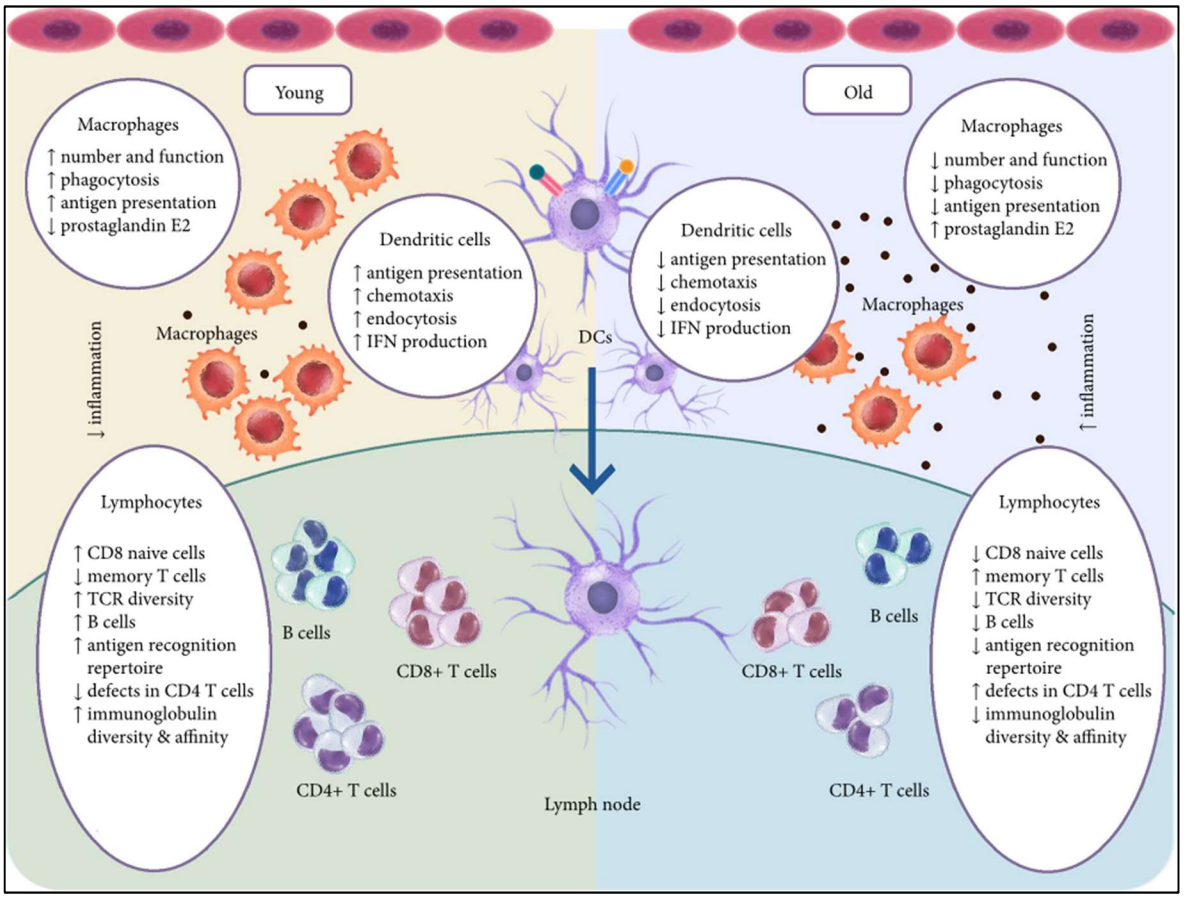


Figure 1-14: Changes in immune cell compartments with ageing. Ageing of the innate and adaptive immune system results in chronic low grade inflammation which drives immunosenescence. Extracted from Nguyen et al. (2017). DCs, dendritic cells.

Immune cells derive from bone marrow-derived haematopoetic stem cells (Nguyen et al., 2017). Senescent haematopoetic stem cells are less able to self-renew to prevent clonal exhaustion, and demonstrate myeloid skewing of differentiation and increased apoptosis (Janzen et al., 2006). Levels of the cyclin-dependent kinase inhibitor p16INK4A (chapter 1.6.1.3) accumulate in ageing stem cells, inhibition of which is associated with improved stress tolerance and reduced apoptosis (Janzen et al., 2006).

Ageing is associated with changes in humoral immunity resulting in the generation of less effective antibodies with poor affinity and reduced diversity. These changes occur due to alterations in the bone marrow environment with consequent defects in B cell development (Labrie et al., 2004). T cell maturation also demonstrates age-associated changes; T lymphocytes in the elderly show loss of functional capacity and cellular diversity, with reduced

immune response to antigens. There is an increase in the ratio of helper: cytotoxic T cells and ratio of memory: naïve T cells (Holcar et al., 2015). The age-related loss of naïve T cells is more marked in the CD8+ compartment, as is the expansion of the end-differentiated population. The thymus plays a crucial role in immunity, and is the primary organ facilitating T cell maturation. Ageing is associated with a dramatic reduction in the size of the thymus, characterised by reduced cellularity and epithelial space as well as fat infiltration and an increase in perivascular space (Li et al., 2003).

Telomere length and telomerase activity correlate with activation of B and T cells, and stage of differentiation. Ageing is associated with telomere attrition in T cells as a consequence of telomerase activity, naïve T cell percentage and health conditions (Lin et al., 2015). Loss of the costimulatory cell surface marker CD28 is a recognised marker of ageing T cells. Well differentiated CD4+ CD28- T cells are less able to proliferate and have shortened telomeres, and chronic antigen exposure and inflammation leads to dramatic clonal expansion of these cells. CD4+ CD28- T cells are pre-activated, and have a unique DNA methylation landscape, associated with higher expression of inflammasome related genes, release of IL-1B on activation and increased production of IFN-γ (Suarez-Alvarez et al., 2017), contributing to a pro-inflammatory state. The proportion of defective effector memory T cells also increases with age, and these cells are characterised by shorter telomeres and the expression of the cell surface receptor CD57 (Tarazona et al., 2000). Expression of CD57 is also associated with lack of proliferative ability and lower secretion of IL-2 (Manfras et al., 2004, Monteiro et al., 1996).

1.6.1.3 Regulation of INK4/ARF in ageing

The INK4/ARF locus on chromosome 9p21 contains genes encoding important tumour suppressor proteins, the cyclin dependent kinase inhibitors (CKIs) (Kim and Sharpless, 2006, Sherr, 2000). Cyclin dependent kinase inhibitor 2A (CDKN2A) encodes two transcripts which are translated in different open reading frames to yield two distinct proteins – p14ARF (also known as ARF) and p16INK4A, or CDKN2A (figure 1-15). In addition, the INK4/ARF locus encodes a third tumour suppressor protein, p15INK4B (CDKN2B), as well as a noncoding RNA, ANRIL (Broadbent et al., 2008, LaPak and Burd, 2014). ANRIL runs antisense

to CDKN2B and is thought to function as an epigenetic regulator of INK4/ARF gene transcription.

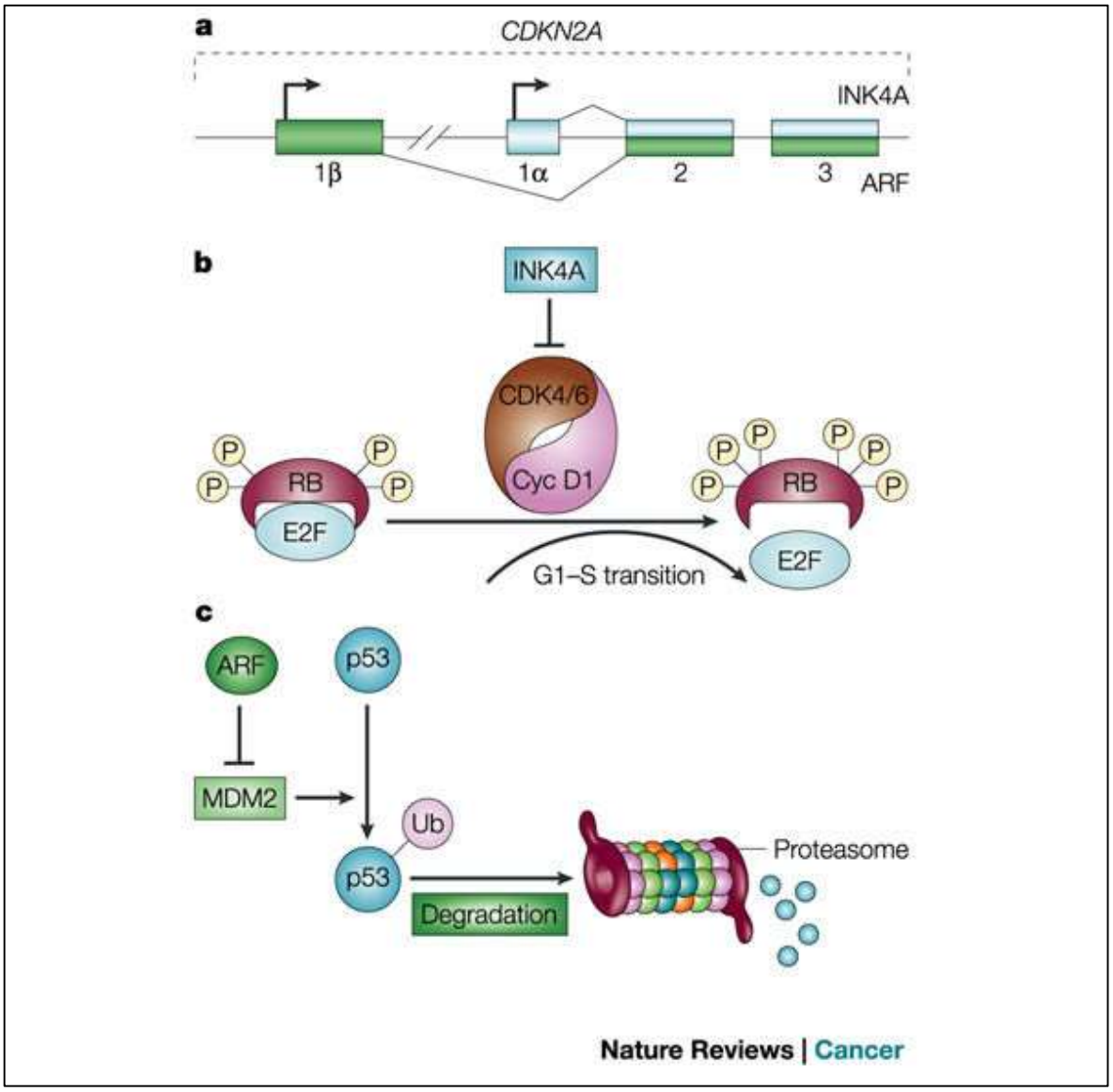


Figure 1-15: CDKN2A gene contains two upstream exons, 1α and 1B. The two exons are driven by distinct promoters, resulting in alternative transcripts. The transcript initiated from the proximal 1α promoter encodes INK4A (CKDN2A), whilst the transcript initiated by the 1B promoter encodes ARF. INK4 inhibits CDK4/6-cyclin-D-mediated hypophosphorylation of retinoblastoma family members, ultimately resulting in cell cycle arrest. ARF stabilises p53 levels through binding to and inactivating MDM2. Extracted from Chin (2003). RB, retinoblastoma; P, phosphoryl group

Both CDKN2A and CDKN2B are members of the **IN**hibitors of CD**K4** (INK4) class of CKIs and act by binding to and inactivating the cyclin-dependent kinases (CDKs) CDK4 and CDK6. CDK4 and CDK6 promote proliferation through binding to D-type cyclins, mediating subsequent phosphorylation of retinoblastoma (Rb) family members (Kim and Sharpless, 2006). The binding of CDKN2A and CDKN2B to the

cyclin dependent kinases therefore maintains the Rb-family proteins in a hypophosphorylated state resulting in E2F binding and cell cycle arrest. In humans, the induction of CDKN2A has been reported in association with critically short telomere length, and it is thought to exert a protective function in proliferative cells (Jacobs and de Lange, 2004, Wang et al., 2013).

Germline mutations of ARF are associated with melanoma and other malignancies (Murphy et al., 2004). ARF interacts with a number of targets to effect tumour suppression. One pathway is through the regulation of p53 in response to oncogenic stress signals or irregular proliferation. ARF binds to and inactivates the mouse double minute 2 homolog protein (MDM2), also known as E3 ubiquitin protein ligase, an important negative regulator of p53. MDM2 targets p53 for proteasomal degradation hence inactivation by ARF results in p53 stabilisation. ARF is able to interact with multiple other proteins, including E2F-1, topoisomerase I, MYC and nucleophosmin (NPM). Interaction of NPM with ARF causes sequestration of ARF in the nucleolus of the cell and prevents ARF from binding to MDM2. Mutant forms of NPM which are localised to the cytoplasm have been documented in approximately a third of cases of primary adult acute myelogenous leukaemia (Colombo et al., 2006) and are believed to compromise the ARF-p53 pathway by localising ARF to the cytoplasm where it is unable to interact with MDM2, decreasing its stability.

Recent evidence has suggested that the INK4/ARF locus may be implicated in ageing. Expression of CDKN2A increases with ageing in rodent, baboon and human tissues (Herbig et al., 2006, Nielsen et al., 1999, Zindy et al., 1997). Inactivation of CDKN2A but not ARF in a progeroid mouse strain is able to partially rescue certain age-related phenotypes (Baker et al., 2008). Additionally, Melzer et al. (2007) were able to demonstrate an association between SNPs near the INK4/ARF locus and physical function (Melzer et al., 2007). Increased expression of CDKN2A has been demonstrated in association with risk behaviours including smoking and physical inactivity, and is negatively correlated with telomere length (Liu et al., 2009). Furthermore, increased CDKN2A expression correlates with that of IL-6, a soluble biomarker of senescence.

1.6.1.4 Senescence associated secretory phenotype

Cellular senescence is an important tumour suppressor mechanism to limit replication in the presence of dysfunctional telomeres, chromatin instability, oncogenic signalling and expression of cell cycle inhibitors (Coppe et al., 2010). However, senescent cells may develop altered secretory phenotypes which influence the tissue microenvironment and paradoxically may promote tumorigenesis. This phenotype is termed the senescence-associated secretory phenotype (SASP). Senescent cells shown to develop this phenotype include HSCs (Schnabl et al., 2003) and endothelial cells (Shelton et al., 1999).

Senescent cells activate downstream signalling pathways resulting in the release of ROS, pro-inflammatory cytokines, chemokines and growth factors (Nelson et al., 2012). Nelson et al. (2014a) were able to demonstrate that co-culture of senescent fibroblasts with young, replication-competent fibroblasts resulted in a DNA damage response in the young cells. The number of surrounding cells positive for Sen-β-Gal and other markers of senescence was also higher following co-culture, indicating that senescent cells are able to induce senescence in adjacent cells *in vitro*. The authors also provided evidence for this phenomenon *in vivo* by using a marker of oxidative stress to demonstrate that senescent hepatocytes cluster in mouse liver.

SASP factors can be divided into soluble signalling pathways, proteases, and secreted insoluble proteins/ECM components (Coppe et al., 2010). The major cytokine of the SASP is IL-6. IL-6 is known to be associated with the DNA damage response (in particular, signalling through ATM and the protein kinase CHK2), and expression of IL-6 is able to induce senescence in neighbouring cells in a paracrine fashion. IL-1 is also overexpressed by senescent cells and acts through the IL-1 receptor/TLR family to trigger the NF-κB pathway. Other factors upregulated at senescence include prostaglandin E2, COX-2, colony-stimulating factors and osteoprotegerin. The IGF pathway and various chemokines are also thought to be important in the promotion of senescence. Proteases involved in the SASP include the MMPs, serine proteases and regulators of the plasminogen activation pathway. Fibronectin, an insoluble glycoprotein which interacts with cell surface receptors to promote adhesion, survival, migration and growth, is also upregulated in senescence.

The SASP is increasingly recognised as a major contributor to the spectrum of immunosenescence and to ‘inflamm-aging’, a term coined by Franceschi et al. (2000) to describe the upregulation of the inflammatory response with ageing. Inflamm-aging is a major driving force of frailty and has been described in a range of diseases, including chronic HIV infection and cardiovascular disease. For example, the SASP of senescent endothelial cells has been postulated to contribute to atherosclerosis development (Balistreri et al., 2013). It has been hypothesised that alteration of TLRs and/or their ligands could contribute to inflammation imbalance (Olivieri et al., 2013a).

Pattern recognition receptors, including TLRs, are able to detect altered endogenous ligands released by damaged, necrotic or senescent cells and PAMPs expressed by micro-organisms. A pro-inflammatory pathway is activated, resulting in the downstream activation of NF-κB, mitogen-activated protein kinases (MAPKs) and members of the IRF family (Olivieri et al., 2013a) This process is usually tightly regulated, and loss of this control can result in chronic inflammation (figure 1-16).

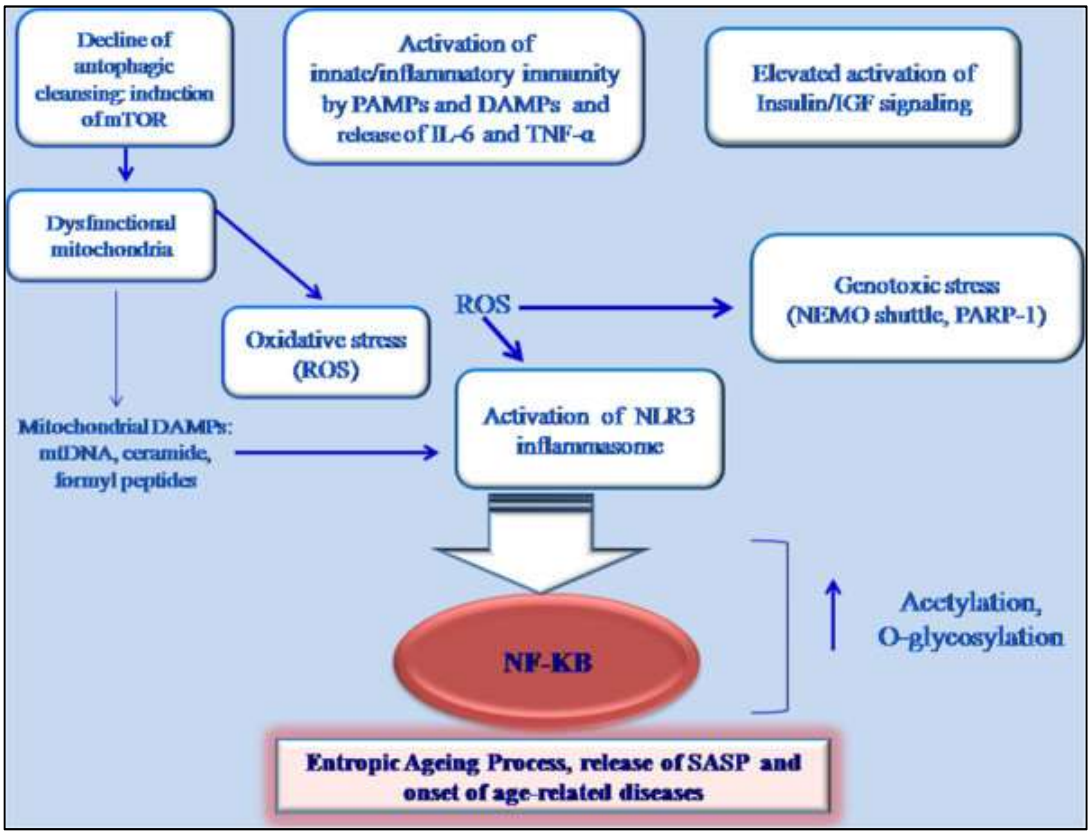


Figure 1-16: Activation of pro-inflammatory pathways by senescent cells. Extracted from Olivieri et al. (2013a). PAMPs, pathogen associated molecular patterns; DAMPs, damage associated molecular patterns; ROS, reactive oxygen species; SASP, senescence associated secretory phenotype

Interestingly, the SASP differs depending on the pathway driving cellular senescence. Cells induced to senescence through telomere dysfunction or replicative exhaustion express similar phenotypes, whereas senescence induced by CDKN2A overexpression is thought not to result in the full SASP despite being associated with other hallmarks of ageing and increased plasma levels of IL-6 (Coppe et al., 2010, Liu et al., 2009). The kinase inhibitor CDKN2A is an effective inhibitor of NF-κB which may partly explain why senescence induced by CDKN2A overexpression is not associated with development of the SASP.

1.6.1.5 Biomarkers of ageing

Biomarkers of ageing (BoA) have come under intense scrutiny in recent years. It is postulated that various biomarkers may be used to predict an individual's risk of age-related disease. As accelerated immune senescence is increasingly recognised in a number of chronic inflammatory diseases, such as HIV infection, cirrhosis and inflammatory bowel disease, these markers may also contribute to risk stratification and have a role in monitoring response to various therapies.

1.6.2 The ageing liver

The ageing process leads to functional and structural changes in the liver, affecting the liver's ability to withstand hepatic insults. Hepatocyte size and the number of binucleated cells increase with age, and there is a reduction in the number of mitochondria (Gan et al., 2011, Premoli et al., 2009). These changes are postulated to affect liver morphology and physiology in addition to oxidative capacity (Sheedfar et al., 2013) (figure 1-17).

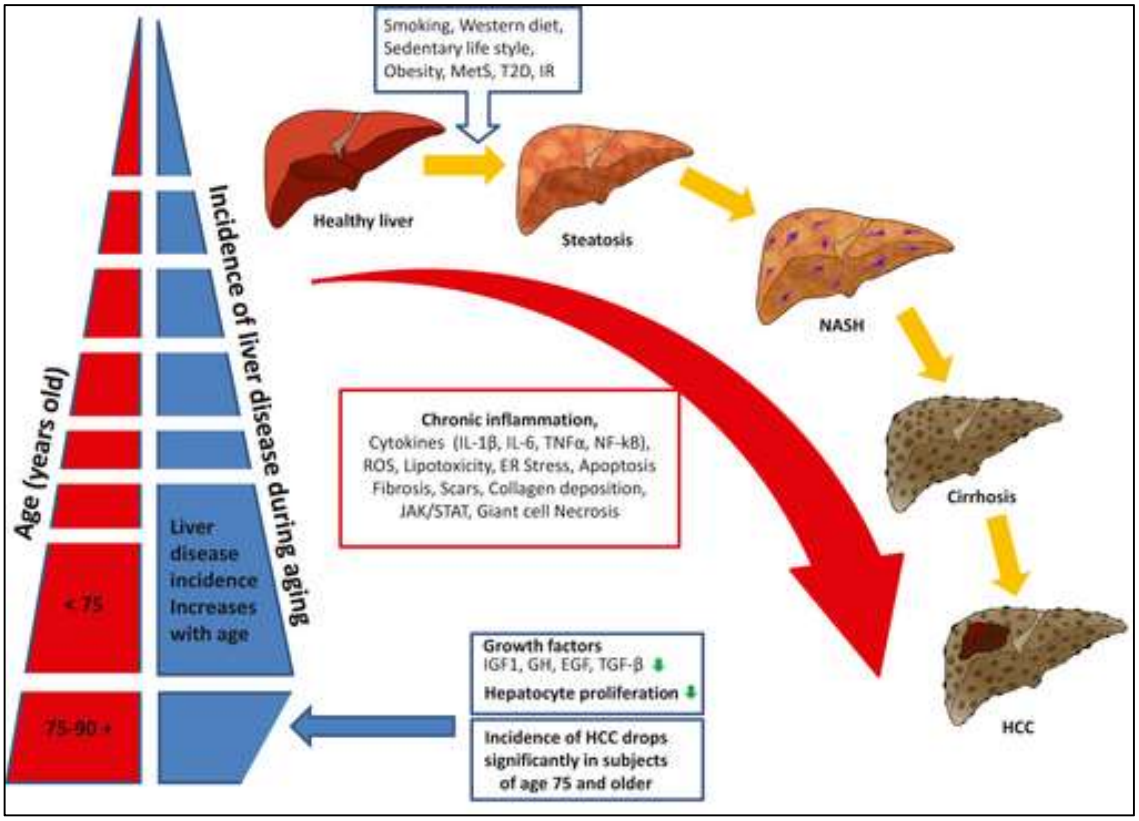


Figure 1-17: Schematic illustration of the pattern of liver disease progression during ageing, extracted from Sheedfar et al. (2013). NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma.

Macroscopically, there is approximately one-third loss of hepatic volume between the ages of 30 and 100 years, thought to be mediated by reduced perfusion with Wynne et al. (1989) reporting a 35% loss in hepatic blood flow in subjects over 65 when compared to subjects below 40 years.

A significant decrease in the regenerative property of the liver is seen with ageing (Hoare et al., 2010a, Sanz et al., 1999, Timchenko et al., 2006). Stocker & Heine (1971) demonstrated that in young rats, all hepatocytes are able to

enter the cell cycle after a 70% hepatectomy, whereas in aged rats only one-third did. More recent evidence suggests that there is an active age-related change in the regulation of hepatocyte proliferation and that the CCAAT-enhancer-binding protein (C/EBP) family members, glycogen synthase kinase 3 beta (GSK3β), histone deacetylase 1 (HDAC1) and sirtuin 1 (SIRT1) epigenetic and signalling pathways play an important role (Iakova et al., 2003, Sheedfar et al., 2013). C/EBP family members are known to have a profound impact on fat cell differentiation (Chiu et al., 2004). In addition, C/EBPα represses hepatocyte proliferation through inhibition of CDKs.

Downregulation of C/EBPα is reported after hemi-hepatectomy, resulting in hepatic regeneration. However, in aged rats the chromatin remodelling protein Brm is expressed at higher levels and interacts with C/EBPα leading to the formation of a high molecular weight complex also containing HDAC1, HP1α, E2F4 and Rb. This 'repression switch' from CDKs to E2F-promoted genes results in impaired proliferation following hepatectomy (Iakova et al., 2003). A recent paper by Zhu et al. (2014a) also found that expression of CDKN2A was significantly higher in older patients undergoing hepatectomy for liver cancer, and that it negatively correlated with liver regeneration in these patients.

An important feature of ageing is the reduction in circulating growth hormone (GH) and insulin-like growth factor 1 (IGF-1). Components of the IGF-1 pathway such as GSK3β and PI3K/AKT are important in hepatocyte senescence. Age associated elevation of C/EBPα-Brm complex is mediated by hyperphosphorylation of C/EBPα at S193 which occurs following activation of CDK4 (Wang et al., 2006). CDK4 is activated by increased levels of cyclin D3 which are present in aged mice due to protein stabilisation and declining levels of GSK3β (Jin et al., 2009). This senescence phenotype has been mimicked through inhibition of GSK3β in human liver-derived Chang cells (Seo et al., 2008). Further support for the role of C/EBP in the ageing process was provided by Chiu et al. (2004) who demonstrated that transgenic mice expressing C/EPBB in place of C/EPBα do not develop fatty liver and are longer lived.

Targeting GSK3β, HDAC1 and SIRT1 pathways has also been reported to improve the course of age-related diseases. HDAC1 is responsible for deacetylating

histone H3 on E2F-dependent promoters and appears to have a critical role in the regulation of liver regeneration in aged rodents. Aged livers contain high levels of the RNA binding protein CUGBP1 in a complex with eIF2. This complex is able to bind to the 5' regions of several mRNAs including HDAC1 and C/EBPα, resulting in increased translational activity (Willis-Martinez et al., 2010). Overexpression of HDAC1 in the livers of old rodents appears to lead to suppression of gene expression via epigenetic silencing (figure 1-18).

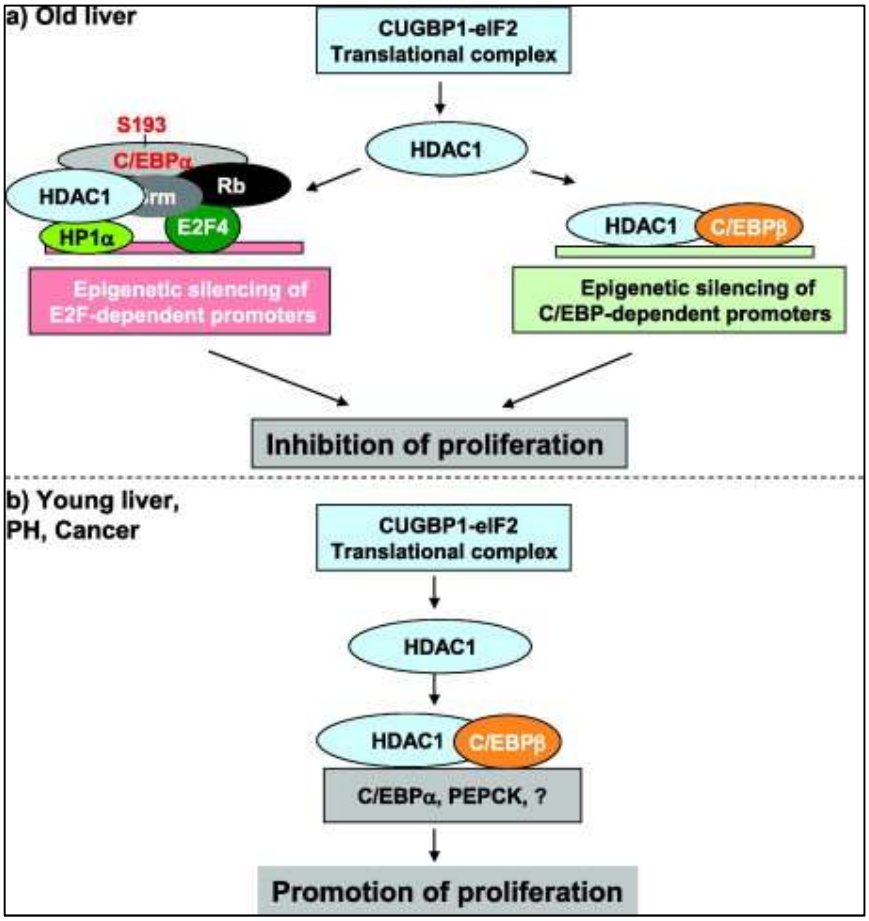


Figure 1-18: The dual function of HDAC1 in young and old livers is regulated by association with different protein complexes. Extracted from Willis-Martinez et al. (2010)

Ageing is associated with increased lipid deposition in the liver, and it is worth noting that overexpression of HDAC1 also leads to the development of hepatic steatosis, potentially through interaction with C/EBP (Willis-Martinez et al., 2010) (figure 1-18). Accumulation of the lipid in the liver is strongly associated with IR, and oxidative stress is thought to play a role in this 'lipotoxicity' (Slawik and Vidal-Puig, 2006). The commonest histological change on liver biopsy with ageing is the cytoplasmic accumulation of highly oxidised insoluble proteins,

known as lipofuscin (Jung et al., 2007) as a consequence of oxidative stress. Lipofuscin appears to promote free radical formation through the trapping of metallic cations (Jolly et al., 1995) and thus contributes to further oxidative damage.

As discussed previously, there is often a trade-off between cellular senescence and restriction of tumorigenesis. The development of hepatic senescence, whilst associated with failure of cellular proliferation and regeneration, may be protective against the development of HCC. Uncontrolled activation of cell proliferation through the C/EBPα pathway is associated with development of HCC. The decline of P13K/AKT and GSK3 axis with ageing drives the cell to enter a protective mode against HCC (Wang et al., 2004). The age associated increase in C/EBPα/Brm complex can be corrected by the use of GH (Timchenko et al., 2006) but upregulation of GH/IGF-1, whilst allowing for better recovery from injury, may lead to HCC development.

1.6.3 Ageing and CHC

CHC is associated with elevated expression of a number of biomarkers of cellular senescence, leading researchers to suggest that HCV infection may result in an aged phenotype. CHC is associated with increased mortality from both hepatic and extra-hepatic disease suggesting increased susceptibility to disease (Lee et al., 2012) – a recognised consequence of the normal ageing process. Ageing itself also appears to have a negative impact on the history of HCV infection (chapter 1.3.2.1); older patients experience more rapid disease progression and a poorer response to IFN-based treatment (Hoare et al., 2010a). Additionally, the morbidity and mortality associated with HCV infection increases exponentially with age (Grebely and Dore, 2011).

1.6.3.1 Cellular senescence in CHC

CHC is associated with telomere shortening in numerous cell types (Kitay-Cohen et al., 2008, Sekoguchi et al., 2007). In chronic liver disease, hepatocyte cell cycle turnover results in hepatocyte telomere shortening and induction of replicative senescence (Wiemann S, 2002), linked to progressive fibrosis and cirrhosis. Moreover, chronic inflammation is associated with the generation of

ROS with attendant effects upon telomere length. Sekoguchi et al. (2007) demonstrated that active HCV infection was associated with hepatocyte telomere shortening, but that this signal was lost in patients with normal ALT levels. Telomere shortening also correlated with lower platelet counts and higher levels of serum hyaluronic acid, markers of increased hepatic fibrosis. As Sekoguchi and colleagues (2007) were unable to identify hepatocytes with evidence of oxidative damage independent of telomere length, the investigators proposed that the primary cause of cellular senescence in CHC was telomere shortening due to cell turnover. They also observed more hepatocytes demonstrating morphological features of senescence suggesting the rate of senescence is increased in CHC (Sekoguchi et al., 2007). Although oxidative stress was not considered the main mechanism of cellular senescence, it was postulated to contribute to accelerated telomere shortening in advanced fibrosis.

Interestingly, Hartmann et al. (2011) demonstrated that telomerase gene mutations were more frequent in cirrhotic patients than in healthy controls, or HCV-infected patients with indolent disease, leading them to postulate that telomere shortening may represent a causal factor accelerating fibrosis and impairing liver regeneration in response to chronic liver injury.

1.6.3.2 Immunosenescence in CHC

Both HCV and ageing are associated with effects on the immune system. Chronic immune activation may result in the development of T cells that are capable of cytokine secretion yet demonstrate failure to proliferate. CD57 is a cell marker commonly expressed on such T cells and correlates with senescence (Kern et al., 1996). Manfras et al. (2004) found that increased oligoclonality and expression of CD57 on peripheral lymphocytes was associated with increased levels of fibrosis and reduced response to treatment in patients with HCV infection, perhaps explaining the poorer treatment outcomes classically seen in older patients (Sherlock, 1995).

HCV-infected individuals also have shorter lymphocyte telomeres (characteristic of immunosenescence) in comparison with healthy controls, and subjects in whom HCV has been eradicated (Kitay-Cohen et al., 2008, Grady et al., 2016). In contrast to the findings in hepatocytes, Kitay-Cohen and colleagues (2008)

demonstrated similar cell turnover in HCV-infected and healthy individuals, suggesting lymphocyte telomere shortening is not a result of shorter cell cycles. They also demonstrated that rates of aneuploidy persisted despite normalisation of ALT, suggesting that genomic instability secondary to telomere shortening endures despite disease remission (Kitay-Cohen et al., 2008).

Chronically HCV-infected individuals have reduced CD4 T lymphocyte telomere lengths compared to healthy controls (Grady et al., 2016). Hoare and colleagues (2010b) also demonstrated this association, and estimated the overall difference in telomere length to be equivalent to 10 years additional ageing. Furthermore, they showed that CD4 T lymphocyte telomere length correlated with predictors of morbidity and mortality (increased fibrosis stage, increased portal tract inflammation, prolonged PT and increased bilirubin) and suggested that individuals with mild disease but shortened telomeres should be offered antiviral therapy at the earliest opportunity.

Reduced hTERT mRNA expression, which is reflective of telomerase activity, has been reported in the peripheral lymphocytes of patients with HCV infection, and is unrelated to activity or stage of disease. Reduced telomerase activity represents premature ageing, and Satra and colleagues (2005) have suggested that deregulated hTERT mRNA expression may be involved in the immunopathogenesis of HCV infection. In contrast to this, Hoare et al. (2010a) were unable to demonstrate a failure of telomerase induction in peripheral lymphocytes of patients with CHC.

1.6.3.3 INK4/ARF regulation in HCV

CKDN2A has been suggested as a potential BoA, expression of which correlates with ageing in a number of animal tissues (Herbig et al., 2006, Nielsen et al., 1999, Zindy et al., 1997) (chapter 1.6.1.3). CDKN2A accumulates with ageing in haematopoetic stem cells, and CDKN2A inhibition is associated with reduced apoptosis and improved stress tolerance (Janzen et al., 2006). Robinson and colleagues (2013) observed higher expression of CDKN2A mRNA and the related transcripts ARF and CDKN2B in the PBMCs of chronically HCV-infected subjects compared with matched controls, and found that expression of CDKN2A and ARF mRNA correlated with disease stage. Elevated expression of CDKN2A mRNA correlated with increased p16 protein levels. They also demonstrated a trend

towards shorter telomere length in the PBMCs of patients with CHC compared with healthy controls but this failed to reach statistical significance.

1.6.3.4 Oxidative stress in HCV

Conditions causing a mismatch between production and removal of ROS lead to a change in the redox state of the cell, known as oxidative stress. Oxidative stress promotes cellular senescence both directly and indirectly through effects on telomere function. Mitochondria are an important source of ROS, and it has been postulated that HCV may contribute directly to mitochondrial dysfunction (Finkel, 2011).

HCV viral proteins, notably core and NS3/4A, localise to the OMM (Kasprzak et al., 2005, Horner et al., 2011). A number of *in vitro* studies have reported an increase in ROS production and mitochondrial dysfunction in association with HCV proteins, particularly the core protein (Korenaga et al., 2005, Okuda et al., 2002). Korenaga et al. (2005) demonstrated that incubation of core protein with control mitochondria caused glutathione oxidation and increased ROS production. These changes are thought to relate to enhanced mitochondrial uptake of calcium ions (Korenaga et al., 2005, Piccoli et al., 2007, Quarato et al., 2013).

Barbaro et al. (1999) found a higher prevalence of ultrastructural alterations of mitochondria in Gt1b infected patients together with depletion of mitochondrial DNA, and postulated that impaired oxidative phosphorylation in these patients may promote hepatotoxicity.

Quarato and colleagues (2013) proposed the existence of a positive feedback loop with increased Ca^{2+} flux from the ER into the mitochondria leading to increased ROS production and enhanced opening of the mitochondrial permeability transition pore (MPTP). Activation of the MPTP leads to further alteration of the redox state with subsequent effects on ER-mitochondrial Ca^{2+} homeostasis. Opening of the MPTP results in depletion of the low molecular weight molecules (carnitine, coenzyme A (CoA) and NAD+) required to import long chain acyl-CoA for ß-oxidation. Accumulating acyl-CoA molecules are thus forced towards triglyceride synthesis leading to hepatic lipid deposition and steatosis, with consequent effects on fibrosis progression (chapter 1.3.2.2).

Differing levels of oxidative stress appear to lead to different pathogenic outcomes; low production of ROS reportedly favours pro-survival adaptation, whereas high levels of ROS lead to permanent opening of the MPTP and resultant osmotic swelling, rupture of the OMM and ultimately, cell death.

1.6.3.5 Lifestyle factors

Studies have demonstrated a high prevalence of various risk behaviours amongst HCV-infected individuals. For instance, Zani et al. (2009) gathered self-reported data on smoking and alcohol use amongst 229 patients attending a clinic in Italy. Over half of the male patients consumed alcohol and around 44% were current smokers. Of the female patients, approximately 35% consumed alcohol and 27% were current smokers. Additionally, PWIDs make up the majority of the HCV-infected population in the UK (HPS, 2015).

Shorter telomere length has been reported in association with alcohol use, substance misuse and smoking (Pavanello et al., 2011, Song et al., 2010, Yang et al., 2013). Smoking also accelerates the age-dependent accumulation of cytogenetic abnormalities in lymphocytes (Ramsey et al., 1995). Grady et al. (2016) found that HCV-infected PWIDs had significantly reduced CD4+ T lymphocyte telomere lengths in comparison to healthy controls (Grady et al., 2016). This accumulation of DNA damage and accelerated cellular senescence (Song et al., 2010) may lead to faster disease progression and HCC development in individuals with HCV (chapter 1.3.2.2).

1.7 The role of microRNAs in HCV pathogenesis

MiRNAs are short endogenous non-coding RNAs approximately 22 nt in length which are responsible for post-transcriptional gene regulation. Specific alterations in circulating miRNA expression have been described in a number of diseases (Edwards et al., 2010) and these signatures are increasingly being exploited as biomarkers for diagnosis and staging as well as demonstrating prognostic implications (Calin and Croce, 2006, Witwer et al., 2012, Fognani et al., 2013).

1.7.1 MiRNA biogenesis and function

In 1993, Lee and colleagues (1993) discovered a gene involved in the timing of *C. elegans* larval development (lin-4) that did not code for a protein but rather produced a pair of short RNA transcripts 22 nt and 61 nt in length, with the longer transcript thought to be the precursor of the shorter. It was noted that both transcripts had antisense complementarity with multiple sites in the 3' UTR of the lin-14 gene, and it was subsequently shown that base pairing of the lin-4 RNA at these sites resulted in down-regulation of lin-14 translation (Wightman et al., 1993). The shorter 22nt transcript is now known to be a member of the miRNA family, a class of endogenous, non-coding RNAs involved in post-transcriptional gene regulation. MiRNAs are widespread in nature, having been described in both plants (Emery et al., 2003) and animals (Leaman et al., 2005, Pasquinelli et al., 2000). Currently, the human miRNA family includes 2588 mature miRNAs (miRBase v21.0, http://www.mirbase.org/). Each miRNA is able to regulate many different mRNAs and thus they exhibit crucial regulatory functions in diverse biological processes including immune response, cellular differentiation and aging (Bartel, 2004).

MiRNAs are transcribed as long primary transcripts (pri-miRNA), typically hundreds to thousands of nucleotides in length, by RNA polymerase II and RNA polymerase III. They may be transcribed from dedicated genes, or processed from introns of other genes. Pri-miRNAs then undergo cleavage in the nucleus into shorter 70-110 nt hairpin structures by the action of a microprocessor complex containing the RNAase III ribonuclease Drosha (Lee et al., 2003), also known as ribonuclease 3, and the microprocessor complex subunit DiGeorge

syndrome critical region 8 (DGCR8) (Kim, 2005) (figure 1-19). DGCR8 stabilises the pri-miRNA and determines the cleavage site for Drosha. Following cleavage, the shortened molecule is referred to as a precursor miRNA (pre-miRNA). The pre-miRNA is exported into the cytoplasm by exportin 5 (XPO5) where it is cleaved by another RNAse III nuclease called Dicer, generating a partially dsRNA structure. Dicer and its interaction-domain protein (TAR RNA binding protein, TRBP) dissociate from the miRNA to form the RNA-induced silencing complex (RISC). The dsRNA structure is unwound, leaving a mature functional strand which is complementary to the target mRNA and is incorporated into the RISC together with a member of the Argonaute (AGO) protein family, AGO2 (Chendrimada et al., 2005). The other strand is designated miRNA* and is degraded. The mature strand then guides RISC to target mRNA to inhibit translation or promote degradation. The specificity of miRNA to the target mRNA is determined by its seed sequence, a 6-8 nt sequence in the 5' region of the miRNA which binds to a complementary sequence in the 3' UTR of the target gene (Bartel, 2009). The degree of complementarity between the sequences determines the outcome of RISC binding; imperfect complementarity generally leads to impaired translation whereas perfect complementarity results in cleavage (Sullivan and Ganem, 2005).

The extracellular circulating miRNA population is heterogeneous; miRNAs can be carried in association with membrane vesicles (including exosomes, apoptotic bodies and shedding vesicles), packaged into high density lipoprotein particles, or bound to Argonaute family proteins (Turchinovich et al., 2012). It is thought that miRNAs contained within membrane vesicles may be specifically sorted and may aid communication between cells or play a role in regulation of gene expression in target cells (Shrivastava et al., 2015). In contrast, protein-bound miRNAs are thought to be released passively into the circulation on cell death.

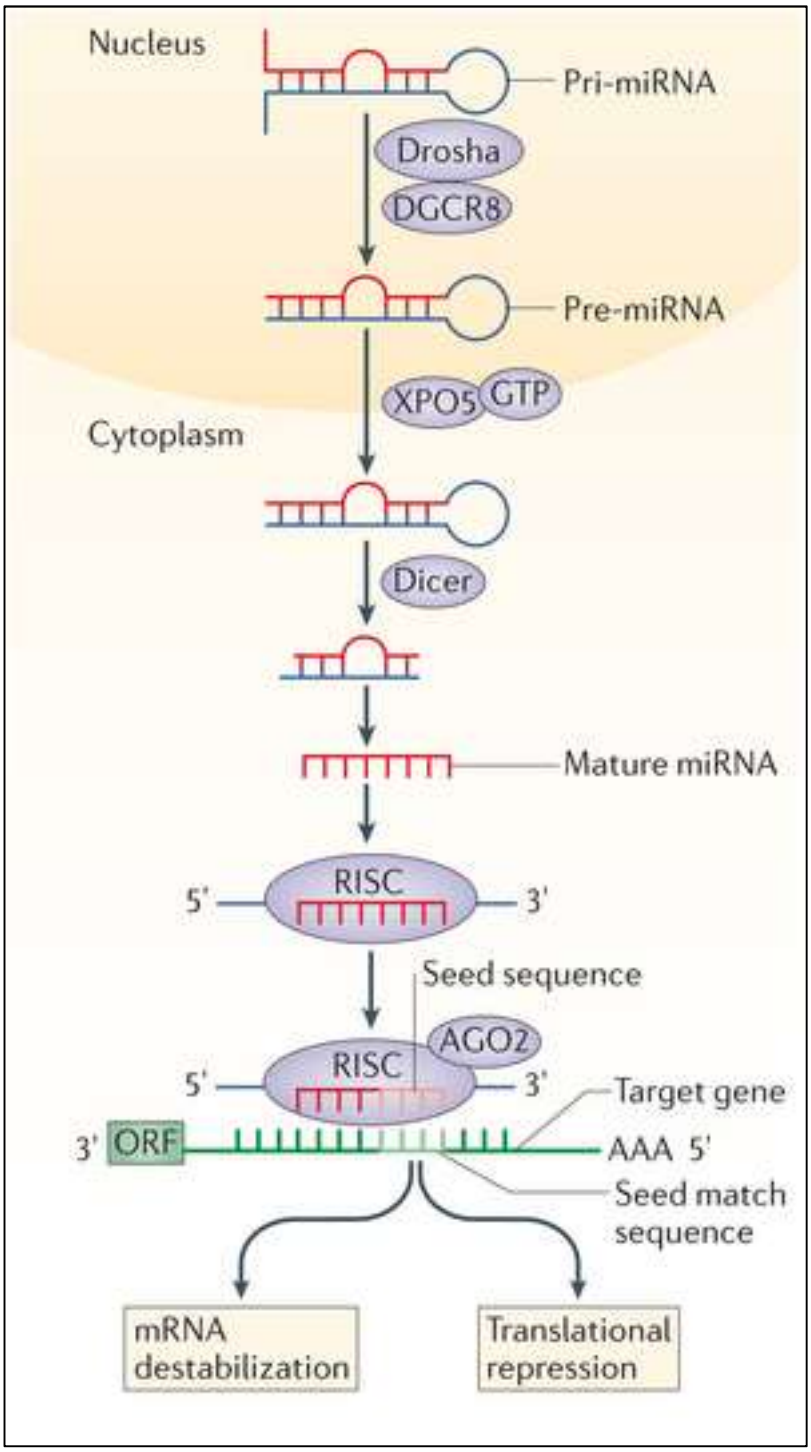


Figure 1-19: MiRNA biogenesis, extracted from Issler and Chen (2015). DGCR8, DiGeorge syndrome critical region 8; XPO5, exportin 5; GTP, guanosine triphosphate; RISC, RNA-induced silencing complex; AGO2, argonaute-2; ORF, open reading frame

1.7.2MiRNA nomenclature

A uniform system for miRNA annotation was developed to ensure that miRNA could be distinguished from other small RNAs including short interfering RNAs (siRNAs). MiRNAs are identified using a combination of expression and biogenesis criteria (Ambros et al., 2003):

Expression criteria:

A. Detection of a distinct ~22 nt RNA transcript by hybridisation to a size-fractionated RNA sample
B. Identification of the sequence in a cDNA library made from size-fractionated RNA, which must precisely match the genomic sequence of the organism from which it was cloned

Biogenesis criteria:

C. Prediction of a fold-back precursor structure containing the ~22nt miRNA sequence within 1 arm of the hairpin
D. Phylogenetic conservation of the ~22nt miRNA sequence and its predicted fold-back precursor secondary structure
E. Detection of increased precursor accumulation in organisms with depleted Dicer function

Novel miRNAs should meet both biogenetic and expression criteria. If the miRNA cannot be detected, a candidate gene can still be annotated as a miRNA gene if the phylogenetically conserved hairpin precursor can be detected, and it accumulates in organisms with reduced Dicer function (Ambros et al., 2003).

MiRNAs are named using the ‘miR’ prefix and assigned numbers sequentially according to date of identification, and identical miRNA are given the same number regardless of organism. Pre-miRNA are differentiated by using the uncapitalized prefix ‘mir’. The species in which the miRNA was observed is distinguished by a 3 letter annotation, for example, hsa-miR-21 in *Homo sapiens*. MiRNAs with closely related mature sequences are annotated with lower case letters (for example, miR-26a and miR-26b). If the mature sequences are

generated from distinct precursor sequences or genomic loci then this is indicated with an additional number, e.g. miR-26a-1.

MiRNA sequences which originate from the same predicted precursor in equal abundance are denoted with a '-3p' or '-5p' suffix, for example, miR-21-5p, to indicate whether the sequence is originating from the 3' or 5' end. Alternately, if the relative abundancies indicate that there is a predominant miRNA then it is assigned a name in the form miR-56, and the opposite arm is designated miR-56*.

1.7.3 'Inflamma-miRs' and 'onco-miRs'

The inflammatory response against pathogens must be tightly controlled: the production of inflammatory mediators is essential to clear infection, however unrestrained inflammation leads to tissue damage. MiRNAs play pivotal roles in regulating immune responses, and nearly all the miRNA involved in immune regulation demonstrate altered expression in ageing (Olivieri et al., 2013b). These miRNA have been termed 'inflamma-miRs' and act mainly as negative regulators of pro-inflammatory signalling pathways.

MiRNAs are suggested to modulate TLR signalling through several different mechanisms (figure 1-20). MiRNAs are able to directly activate the RNA-sensing TLR or to target components of the TLR signalling pathway. Additionally, miRNAs are directly regulated by TLRs. Evidence suggests that a number of miRNAs are involved in regulating this process, in particular, miR-155, miR-21 and miR-146a (Olivieri et al., 2013a, Quinn and O'Neill, 2011).

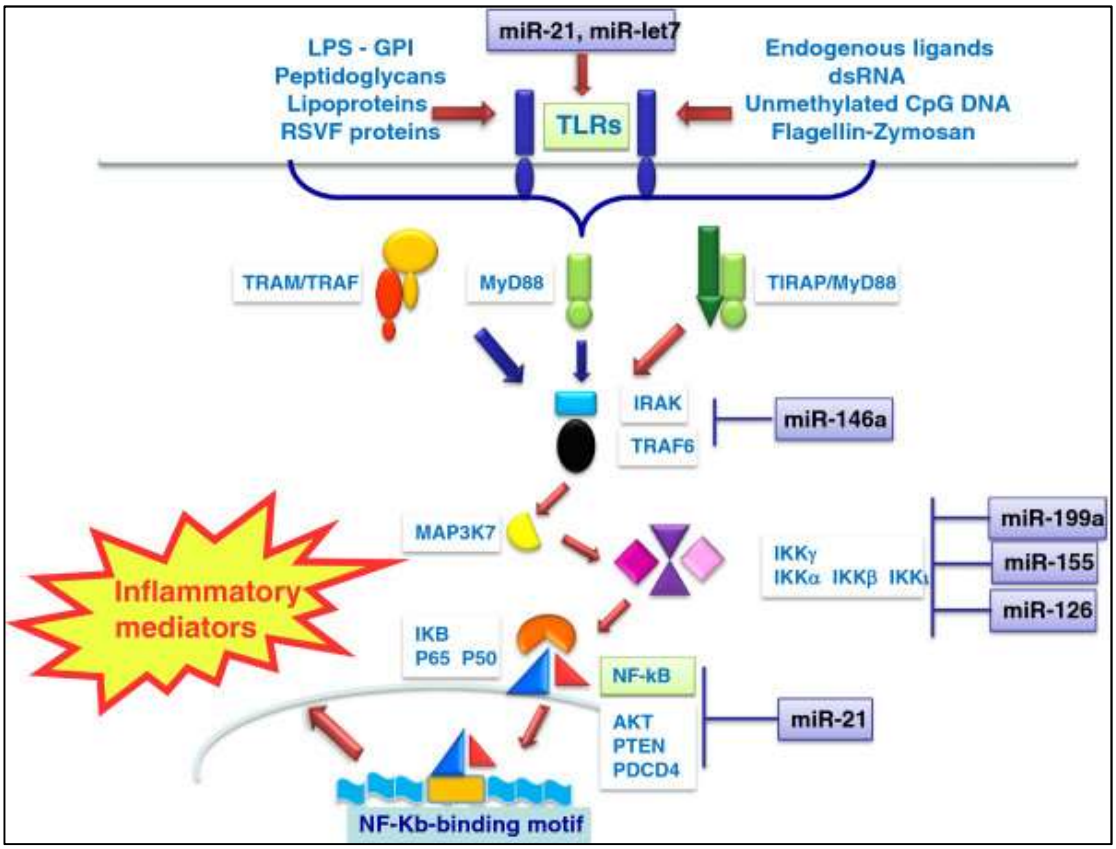


Figure 1-20: MiRNAs modulating TLR pathways, extracted from Olivieri et al. (2013a). LPS, lipopolysaccharide; GPI, glycosylphosphatidylinositol; RSVF proteins, respiratory syncytial virus fusion proteins; TLRs, toll-like receptors

MiR-146a is highly expressed in aged mice and higher circulating levels have been described in certain diseases of ageing, including cardiovascular disease, Alzheimer's dementia and osteoarthritis (Olivieri et al., 2013a). Induction of miR-146a by TNF-α and IL-1B is dependent on NF-κB. MiR-146a then acts as a negative feedback regulator by targeting IL-1 receptor associated kinase 1 (IRAK1) and TRAF6, resulting in downregulation of NF-κB activity (Taganov et al., 2006, Bhaumik et al., 2008). MiR-146a expression in primary human fibroblasts was also shown to downregulate IRAK1 as well as levels of the SASP mediators IL-6 and IL-8. Levels of miR-146a are increased in senescent fibroblasts, suggesting miR-146a induction may be part of a compensatory mechanism to modulate inflammation (Bhaumik et al., 2009). MiR-146a also has an important regulatory role in oncogenesis through reducing the metastatic potential of cancerous cells (Bhaumik et al., 2008).

MiR-21 is a well described 'onco-miR', aberrant expression of which is described in numerous cancers (Fouad et al., 2017, Supic et al., 2017, Nakka et al., 2017).

It is central to a number of inflammatory pathways, including TLR signalling (Olivieri et al., 2012, Quinn and O'Neill, 2011). MiR-21 is upregulated in PBMCs, macrophages and fibroblasts in response to bacterial LPS and is induced by IFN stimulation in a number of cell lines (Sheedy et al., 2010, Pedersen et al., 2007). IFN stimulation does not result in miR-21 induction in cell lines with a genetic deletion of the STAT3 locus, suggesting that STAT3 is an important regulator of miR-21 expression. There are several potential binding sites for NF-κB within the miR-21 promoter region, and IFN-induction of miR-21 has previously been shown to be NF-κB dependent. Importantly, IFN-induced NF-κB binding to the miR-21 promoter was abrogated by STAT3 knock down, suggesting crosstalk between the signalling pathways (Yang et al., 2015). MiR-21 plays a dual role, inhibiting as well as inducing TLR signalling. Transfection of cells with a precursor of miR-21 has been shown to suppress NF-κB activity and to promote secretion of the anti-inflammatory IL-10 in response to LPS stimulation. Induction of miR-21 results in a decrease in levels of the tumour suppressor PDCD4 protein, acting as a molecular switch from a pro-inflammatory to an anti-inflammatory response (Sheedy et al., 2010). However, miR-21 can also function as an agonist of single-stranded RNA-binding TLRs, resulting in NF-κB activation (Fabbri et al., 2012). Circulating levels of miR-21 have been shown to correlate with C-reactive protein (CRP) and fibrinogen and are positively associated with ageing, making miR-21 an attractive biomarker of ageing.

Elevated miR-155 expression is associated with diseases of ageing and inflammation, including acute myocardial infarction and atherosclerosis (Olivieri et al., 2013a). Like miR-21, it is a well characterised onco-miR, with deregulated expression in various cancers. It was first described in chickens suffering from avian leucosis virus-associated lymphoma and is now recognised to promote B-cell lymphomas, in particular Hodgkin lymphoma and diffuse large B-cell lymphoma, as well as being associated with solid organ tumours (Eis et al., 2005). MiR-155 appears to exert both positive and negative regulatory effects on NF-κB activity. MiR-155 is upregulated as part of the inflammatory response to LPS stimulation, and is associated with increased production of TNF-α (Tili et al., 2007). It is also downregulated by the anti-inflammatory cytokine IL-10, in contrast to miR-146a and miR-21 (McCoy et al., 2010). However, Ceppi et al. (2009) showed that miR-155 also exerts negative feedback through targeting

TGF-B activated kinase 1 binding protein 2 (TAB2), a signal transduction molecule in the TLR/IL1-R pathway, leading to inhibition of TAK1, and consequently NF-κB and MAPK. MiR-155 may also downregulate MyD88, as well as Ikkε, Sma- and Mad-related protein 2 (SMAD2) and Fas-associated death domain protein (Xiao et al., 2009), as well as targeting the suppressor of cytokine signalling 1 (SOCS1) gene.

MiR-16, miR-195, miR-15 and miR-457 are related miRNA sequences from the miR-15 family. MiR-16 has tumour suppressor functions and has been shown to bind to both B-cell lymphoma 2 (BCL2) (Cimmino et al., 2005) and vascular endothelial growth factor (VEGF) (Hua et al., 2006), resulting in suppression of anti-apoptotic and angiogenic signalling. Downregulation of miR-16 is recognised in many tumour types (Lu et al., 2005). Interestingly, unstimulated human B cells from elderly individuals demonstrate increased miR-155 and miR-16 expression compared with young subjects, and this is associated with elevated serum and intrinsic B cell TNF-α expression (Frasca et al., 2015). This pre-activated B cell phenotype is associated with the suboptimal responses to exogenous antigens and vaccines classically seen in immunosenescence. Frasca and colleagues (2015) found that miR-155 and miR-16 are able to target the transcription factor E47 and activation-induced cytidine deaminase (AID), contributing to the age-associated decline in B cell function.

Other miRNAs implicated in the ageing process include MiR-200, known to show markedly increased expression when exposed to ROS. This upregulation is known to involve p53 and retinoblastoma proteins, essential in the mechanisms underlying cellular senescence (Magenta et al., 2011).

1.7.4MiRNA deregulation in CHC

MiRNAs have been implicated in various stages of HCV pathogenesis. HCV viral proteins may directly modulate miRNA expression: the NS3 complex is able to upregulate miR-27a and downregulate miR-335 and miR-150 (Khanizadeh et al., 2017), whilst HCV core protein suppresses miR-138. MiR-138 targets hTERT, thus inhibiting cellular senescence and promoting proliferation and oncogenesis (Shiu et al., 2017).

Cellular miRNA play important roles in regulating HCV replication and pathogenicity. Sequestration of the endogenous liver-specific miRNA miR-122 was shown to lead to a significant reduction in HCV RNA (Jopling et al., 2005). MiR-122 binds to 2 adjacent sites in the HCV IRES in association with AGO2 forming an oligomeric complex. The miR-122 binds to the 5'UTR of the HCV RNA, protecting it from nucleolytic degradation and promoting stability (Shrivastava et al., 2015). Cell culture studies demonstrated the key role that miR-122 plays in the HCV life cycle, as exogenous miR-122 was able to rescue infectious virion production in a non-permissive cell line (Narbus et al., 2011). MiR-122 also demonstrates tumour suppressor activity through inhibiting cell proliferation and neoplastic transformation of hepatocytes (Hu et al., 2012), and target analysis suggests it may bind to the HCV receptor OCLN. Antagonists of miR-122 including miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide able to sequester mature miR-122, reached clinical trials as therapeutic adjuncts for the treatment of CHC (Janssen et al., 2013), although the efficacy of newer DAA combinations has rendered them largely redundant. There had been additional safety concerns as miR-122 knock-out in mice was associated with the development of hepatic steatosis and tumorigenesis (Tsai et al., 2012).

Several other cellular miRNAs are able to bind directly to the HCV genome, but in contrast to miR-122 they appear to attenuate HCV replication (figure 1-21).

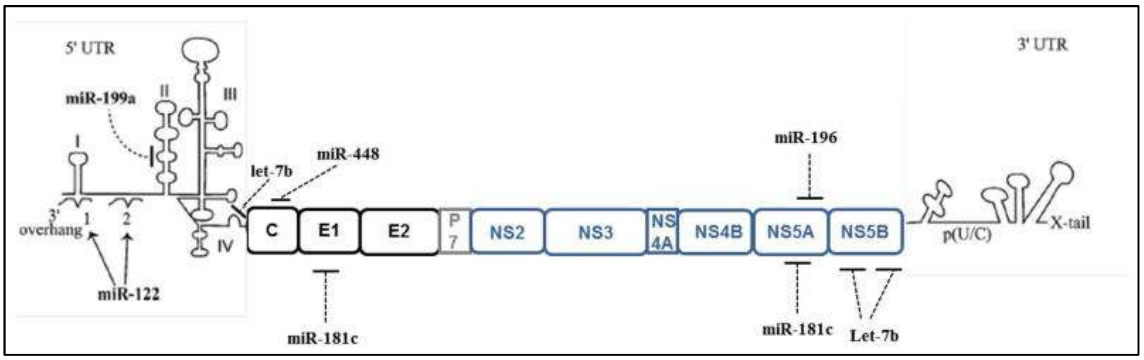


Figure 1-21: Cellular miRNAs targeting the HCV genome. MiR-122 binds to 2 sites within the 5'UTR, enhancing viral replication. Let-7b also binds at the 5'UTR as well as the NS5A coding site. The binding of miR-199a at the 5'UTR inhibits replication. MiR-448 targets core and miR-181c is able to inhibit viral replication through binding at E1 and NS5A coding regions. Extracted from Shrivastava et al. (2015)

Murakami et al. (2009) found that overexpression of miR-199a inhibited HCV genome replication in immortalised human hepatocytes (IHHs), and inhibition

was associated with accelerated replication. MiR-199a has been shown to bind directly to the 5'UTR of the HCV genome, and may have both direct and indirect antiviral effects. MiR-196 and miR-448 are induced by IFN in hepatoma cell lines, and synthetic mimics of these miRNA have been shown to inhibit HCV replication *in vitro* (Pedersen et al., 2007).

As well as directly interacting with the HCV genome, cellular miRNA are able to regulate genes involved in the innate immune response to HCV infection. For instance, let-7b is able to bind to the HCV genome at the 5'UTR and NS5A sequences, but is additionally able to target insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) resulting in inhibition of HCV replication and viral protein translation (Cheng et al., 2013).

Shrivastava et al. (2015) showed that miR-130a expression is upregulated in liver tissue from HCV-infected individuals, and in HCV-treated IHH. Knockdown of miR-130a was associated with upregulation of IFITM1 (Bhanja Chowdhury et al., 2012). As IFITM1 is an important ISG effector which interacts with CD81 and OCLN to disrupt HCV entry, researchers postulated that induction of miR-130a represents a strategy by which HCV is able to evade the IFN-signalling pathway (Bhanja Chowdhury et al., 2012).

HCV infection induces miR-155 expression, resulting in hepatocyte proliferation and oncogenesis through Wnt signalling (Zhang et al., 2012). The onco-miR miR-21 is also upregulated in IHH in response to HCV infection. MiR-21 is elevated in the liver tissue of individuals with CHC, and is strongly expressed in tumour biopsies from patients with HCC. Chen and colleagues (2013) showed that upregulation of miR-21 suppressed MyD88 and IRAK1 expression, abrogating the type I IFN-mediated antiviral response. Overexpression of miR-122 is also associated with disruption of the IFN-signalling pathway; MiR-122 overexpression is associated with reduced ISRE activity, whereas suppression of miR-122 downregulates suppressor of cytokine signalling 3 (SOCS3), resulting in enhanced IFN-induced ISRE activity. MiR-221 and miR-30 are also able to target SOCS3, and additionally SOCS1. SOCS1 and SOCS3 are members of the STAT-induced STAT inhibitor family, and are cytokine-inducible negative regulators of cytokine signalling. They are able to inhibit JAK tyrosine kinase activity and STATs in the JAK-STAT signalling pathway. The miR-30 cluster is downregulated in HCV-

infected Huh7.5 cells, and upregulated in response to IFN-α, and inhibition of miR-30c increased HCV RNA levels *in vitro* (Zhang et al., 2013a).

MiRNAs have both pro- and anti-fibrotic roles in the pathogenesis of chronic liver disease, depending on the nature of the stimuli and the cellular context (figure 1-22). MiR-29 is recognised as an important regulator of fibrosis, decreased expression of which is associated with fibrosis in many tissues. MiR-29a is downregulated following TLR4 stimulation, and has been shown to target important fibrotic genes, including TGF-B activated kinase 1 binding protein 1 (TAB1, the gene regulating TIMP-1) and PDGFR (O'Reilly, 2016, Ciechomska et al., 2014). The miR-29 family is also able to target the DNA methyltransferases, resulting in further epigenetic modification of genes involved in ECM regulation.

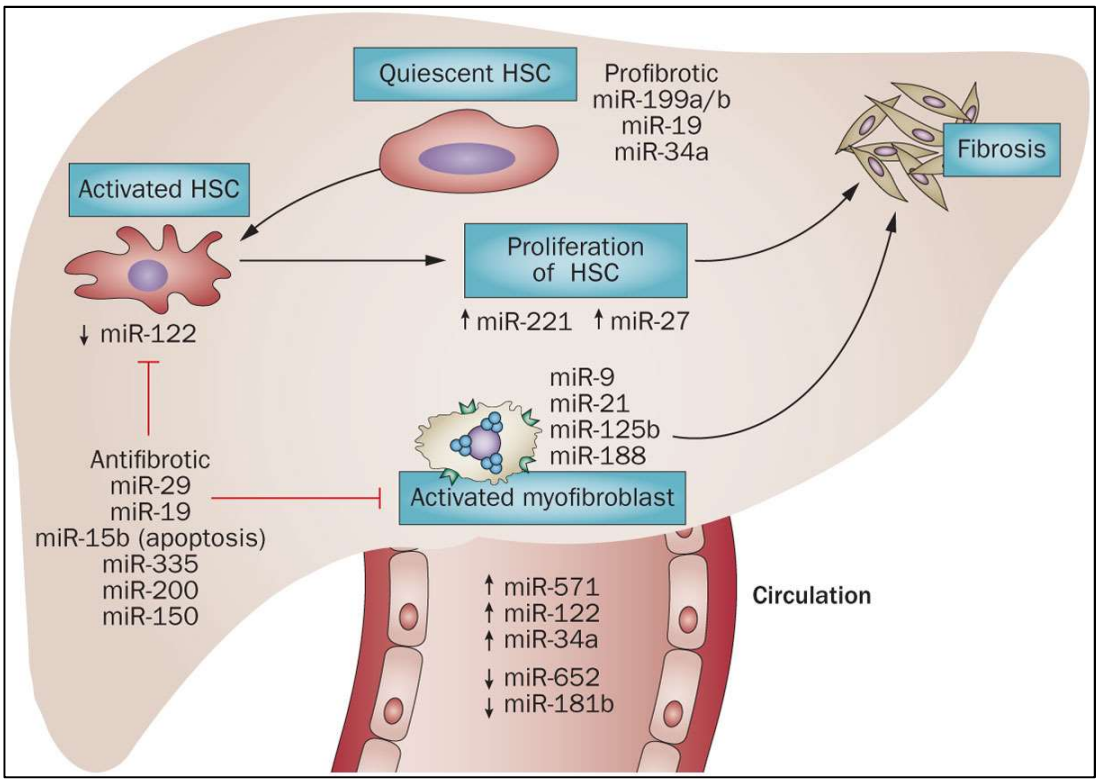


Figure 1-22: MiRNAs have both profibrotic and antifibrotic roles in liver disease. Extracted from Szabo et al. (2013). HSC, hepatic stellate cell.

As described previously, miR-21 is upregulated in the liver tissue of individuals with CHC and modifies the type I IFN inflammatory response. Downregulation of miR-21 is associated with suppression of TGF-B signalling and HSC activation (Zhang et al., 2013b). MiRNA target prediction analysis suggests that miR-21 may bind to the HCV receptor CLDN1.

The pro-fibrotic miRNA miR-181b is induced by TGF-B1, and regulates HSC proliferation through targeting the cell cycle regulator p27 (Wang et al., 2012). MiR-221 and miR-222 expression in liver tissue also correlates with fibrosis stage. MiR-222 binds to CDKN1B, also regulating the expression of p27, and is induced in response to TNF-α signalling (Ogawa et al., 2012). MiR-199a and miR-199b were also found to be upregulated in fibrotic liver tissue. MiR-199a has a role in regulating the TLR signalling pathway (figure 1-20) and a direct HCV antiviral effect. Murakami and colleagues (2011) found that overexpression of miR-199a was associated with upregulation of fibrosis related genes in HSCs in both humans and mouse models. MiR-16 is postulated to downregulate hepatocyte growth factor (HGF) and Smad7 expression levels, contributing to liver fibrosis (Zhu et al., 2015). MiR-221 is induced in response to apoptosis, and protects hepatocytes from apoptosis through binding to and regulating expression of pro-apoptotic genes. MiR-221 also regulates hepatocyte proliferation through targeting Arnt, resulting in an increased risk of HCC (Szabo and Bala, 2013).

A number of miRNAs have been shown to be upregulated in the sera of subjects with HCV infection and to correlate with necro-inflammation, including miR-122 and miR-21 (Bihrer et al., 2011b, Bihrer et al., 2011a). Trebicka and colleagues (2013) also showed that miR-122 expression positively correlated with enhanced inflammatory activity, but found that there was a negative correlation with fibrosis stage which they attributed to the loss of hepatocytes in advanced disease. Conversely, Cermelli et al. (2011) found that serum miR-122 levels positively correlated with disease severity. Elevated circulating miR-34a has been described in patients with liver fibrosis secondary to multiple aetiologies, including CHC and NAFLD (Cermelli et al., 2011, Salvoza et al., 2016). Increased serum miR-16 levels have also been described in both CHC and NAFLD (Cermelli et al., 2011).

Many of the miRNAs implicated in inflamm-aging are deregulated in HCV infection, and correlate with necro-inflammatory activity and fibrosis stage. We hypothesised that we could identify novel diagnostic and prognostic biomarkers for clinical and virologic outcomes in CHC by correlating miRNA expression with previously validated BoA.

1.8 Aims and objectives

Disease progression in CHC is highly variable and poorly understood, and there are little real-world data regarding predictors of treatment outcome with DAA therapy. Robust, non-invasive biomarkers able to identify HCV-infected subjects at high risk of progression to cirrhosis and HCC, or at risk of poor clinical and virologic outcomes with DAA therapy, are required. The primary aims of this research were:

1. To explore factors associated with spontaneous clearance of CHC in a large Scottish cohort

2. To gain further insights into modifiable and non-modifiable factors associated with disease progression and treatment outcomes in CHC

3. To explore the role of BoA as predictors of disease progression in CHC

4. To characterise patterns of miRNA deregulation during DAA treatment, in order to identify novel biomarkers associated with adverse clinical and virologic outcomes during DAA treatment

2 Materials and Methods

2.1 Materials

2.1.1 Cells

Table 2-1: Cell lines

Cells	Description	Source
HepaRG	Human hepatocyte cell line	Elihu Aranday Cortes
ISG15 KO HepaRG	Human hepatocyte cell line – ISG15 -/-	Elihu Aranday Cortes

2.1.2 Kits

Table 2-2: Kits

Kit	Source
exoRNeasy Serum/Plasma Starter	Qiagen
MiRNeasy Micro	Qiagen
MiRNeasy Serum/Plasma	Qiagen
Qubit® HS RNA Assay Kit	Thermo Fisher Scientific
Qubit® MicroRNA Assay Kit	Thermo Fisher Scientific
RNeasy Mini Plus	Qiagen
TaqMan® Advanced miRNA cDNA Synthesis Kit	Thermo Fisher Scientific
TaqMan® Array Human MicroRNA A+B Cards Set v3.0	Thermo Fisher Scientific
TaqMan® MicroRNA Reverse Transcription Kit	Thermo Fisher Scientific

2.1.3 Reagents

Table 2-3: Reagents

Reagent	Source
10X Poly A Buffer	Thermo Fisher Scientific
10X RT Buffer	Thermo Fisher Scientific
10X RT Enzyme Mix	Thermo Fisher Scientific
20X miR-Amp Primer Mix	Thermo Fisher Scientific
20X Universal RT Primer	Thermo Fisher Scientific
25X Ligation Adaptor	Thermo Fisher Scientific
2-Mercaptoethanol (B-ME)	Sigma-Aldrich
2X miR-Amp Master Mix	Thermo Fisher Scientific
50% PEG 8000	Thermo Fisher Scientific
5X Ligase Buffer	Thermo Fisher Scientific
5X RT Buffer	Thermo Fisher Scientific
ATP, 10mM	Thermo Fisher Scientific
Buffer RLT	Qiagen
Buffer RPE	Qiagen
Buffer RWT	Qiagen
Chloroform	Sigma-Aldrich
DNAZap™	Thermo Fisher Scientific
dNTPs with dTTP (100 mM)	Thermo Fisher Scientific
Ethanol	Sigma-Aldrich
hIFN-α	Invivogen
Megaplex™ PreAmp Primers – Human Pool A and B	Thermo Fisher Scientific
Megaplex™ RT Primers (10X) – Human Pool A and B	Thermo Fisher Scientific
$MgCl_2$ (25 mM)	Thermo Fisher Scientific
Multiscribe™ Reverse Transcriptase (50U/μL)	Thermo Fisher Scientific
Nuclease-free water	Thermo Fisher Scientific

Poly A Enzyme, 5 U/µL	Thermo Fisher Scientific
Poly(I:C)	Sigma-Aldrich
Qiazol® Lysis Reagent	Qiagen
RNA Ligase, 10 U/µL	Thermo Fisher Scientific
RNase Inhibitor (20 U/µL)	Thermo Fisher Scientific
RPMI-1640 Media	Sigma-Aldrich
TaqMan® Fast Advanced Master Mix	Thermo Fisher Scientific
TaqMan® PreAmp Master Mix (2X)	Thermo Fisher Scientific
TaqMan® PreAmp Primers (10X)	Thermo Fisher Scientific
TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2X	Thermo Fisher Scientific
Tris-EDTA (TE) Buffer pH 8	Ambion

2.1.4MiRNA assays

The TaqMan® Advanced miRNA assays were ordered from Thermo Fisher Scientific.

Table 2-4: MiRNA assays

Assay name	Mature miRNA sequence (5' – 3')
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG
hsa-miR-885-5p	UCCAUUACACUACCCUGCCUCU
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGU
hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-345	GCCCUGAACGAGGGGUCUGGAG
miR-146b-3p	UGCCCUGUGGACUCAGUUCUGG
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC
hsa-miR-574-3p	CACGCUCAUGCACACACCCACA
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU
cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG

2.1.5 MiRNA mimic

Table 2-5: MiRNA mimic

cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG

2.1.6 Cell culture growth media

All cell culture steps were performed by Dr. Elihu Aranday-Cortes, MRC-University of Glasgow Centre for Virus Research. HepaRG cell lines were cultured in William's E Medium (Invitrogen Life Technologies), supplemented with 10% fetal bovine serum gold (PAA), 2 mM L-glutamine, 100 IU/mL of penicillin, 100 µg/mL of streptomycin, 5 µg/mL insulin and 0.5 µM hydrocortisone. Cells were grown to confluency in 6 well plates before treatment. Cells were treated with recombinant hIFN-α2b (100 IU/mL), the TLR3 agonist polyinosinic: polycytidylic acid (poly (I:C)) (1 µg/mL) or mock-treated, and incubated for 24 hrs before being harvested.

2.1.7 Equipment

Table 2-6: Equipment

Equipment	Source
10/20 µL TipOne® Graduated Filter Tip	Starlab
1000 µL TipOne® Graduated Filter Tip	Starlab
200 µL TipOne® Graduated Filter Tip	Starlab
7500HT Fast Real-Time PCR System	Applied Biosystems
7900HT Fast Real-Time PCR System	Applied Biosystems
Eppendorf® Centrifuge 5424R	Sigma-Aldrich
Eppendorf® MiniSpin® Microcentrifuge	Sigma-Aldrich
GeneAmp® PCR System 9700	Applied Biosystems
Heraeus™ Megafuge™ 40R	Thermo-Fischer Scientific
MicroAmp® 8-Cap Strip	Applied Biosystems
MicroAmp® 8-Tube Strip, 0.2mL	Applied Biosystems
MicroAmp® Fast Optical 96-Well Reaction Plate	Applied Biosystems

MicroAmp® Optical Adhesive Film	Applied Biosystems
Sorvall® Legend RT Centrifuge	Thermo-Fischer Scientific
TaqMan® Array Microfluidic Card Sealer	Applied Biosystems
Vortex-Genie 2	Sigma-Aldrich

2.2 Methods

2.2.1 Manipulation of RNA

Samples of PBMCs, serum and plasma were available from pre-existing cohorts (see Chapter 4.2 and Chapter 5.2 for details). For the Outcome study (chapter 4), blood was processed on the day of venepuncture to ensure highest yield and quality of PBMCs. For the EAP study (chapter 5), samples were shipped to Glasgow overnight by courier before being stored at -70^{o}C.

2.2.1.1 Optimisation of serum miRNA extraction

In preliminary experiments, the yield of RNA from 150-200µL of serum was variable (6-22 ng/µL). Therefore, the following experiment was performed to compare the yield of RNA from different volumes of serum. The kit manufacturer (Qiagen) advised that processing serum volumes of > 200 µL can lead to contaminants which interfere with the purification process. Hence, the samples were separated into aliquots of 100 µL and then processed individually. The aqueous phases were subsequently pooled, and the washing steps were performed according to the manufacturer's protocol. RNA was eluted using 16 µL of RNase-free water, and quantified using a Qubit HS RNA kit.

Table 2-7: Yield of RNA according to starting volume of serum (µL)

Starting volume (µL)	Yield of RNA (ng/µL)
100	2.76
200	5.72
300	12.44
400	20.4
500	31.6

On the basis of this experiment, a starting volume of 500 µL serum, processed in 100 µL aliquots as above, was used for miRNA expression profiling with the TaqMan® MicroRNA Arrays (Chapter 2.2.2.1).

2.2.1.2 Extraction of miRNA from serum

Serum and plasma had been previously separated from whole blood using centrifugation and stored at -70^{o}C. For both the pilot and expanded Glasgow Outcomes Study cohorts (Chapter 4.2), a starting volume of 500 µL serum was used.

In the EAP analysis (Chapter 5), the starting volume was restricted by sample availability. A volume of 150 µL was used for the individual miRNA assays. Additionally, aliquots of 50 µL from each patient sample were pooled and homogenised, and 500 µL pooled sample per patient group was used for the TaqMan® MicroRNA Array. Starting volumes > 150 µL were processed in aliquots as described in Chapter 2.2.1.1.

Samples were thawed at room temperature prior to being mixed with 5 volumes of Qiazol and then incubated at room temperature for 5 minutes. At this stage, 3.5 µL of the exogenous control cel-miR-39-3p (MiRNeasy Serum/Plasma Spike-In Control, 1.6 x 10^{8} copies/µL) was added to a subset of samples. Next, 1 volume of chloroform was added, and the sample was mixed by shaking vigorously. The samples were incubated at room temperature for a further 2-3 minutes before being centrifuged at 4^{o}C at 12000G for 15 minutes. The upper aqueous phase was transferred to a new tube and mixed with 1.5 volumes of 100% ethanol. RNA was then extracted using the MiRNeasy Serum/Plasma column-based technique. On-column DNase treatment is not recommended for plasma/serum samples; these body fluids do not typically contain significant amounts of DNA and treatment may reduce recovery of small RNA. Additionally, assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. Washing steps were performed according to the manufacturer's protocol. The RNeasy spin column was then placed in a new collection tube, and a volume of 16 µL RNase-free water added to the membrane. The column was incubated at room temperature for 10 minutes before being centrifuged at >12000rpm for 1 minute

using the Eppendorf® MiniSpin® centrifuge. A second elution step was performed using the same eluate, resulting in a final volume of 14 µL.

RNA extracted from serum/plasma was analysed by spectrophotometry (NanoDrop™ 1000, Thermo Fisher Scientific) or Qubit™ fluorometric quantification (Thermo Fisher Scientific) according to starting volume of sample.

2.2.1.3 Extraction of miRNA from PBMCs

Samples of PBMCs were available from a pre-existing study (study details described in Robinson et al. (2013)). PBMCs had been collected following separation over a density gradient (Histopaque®-1077, Sigma Aldrich), suspended in freezing medium and stored in liquid nitrogen. For a subset of patients, aliquots of $5x10^6$ PBMCs previously lysed in 600 µL Buffer RLT (Qiagen) and stored at -70°C were also available.

Cryopreserved samples were thawed in a waterbath at 37°C before being resuspended in 2 volumes RPMI-1640 medium pre-warmed to ambient temperature. The diluted cell suspension was then added to 8 mL RPMI at room temperature. The supernatant was decanted following centrifugation at 250G for 10 minutes at 20°C, and the pellet was reconstituted in 600 µL Buffer RLT with 1% B-ME (Qiagen). Aliquots previously stored in Buffer RLT were thawed on ice.

Homogenised lysate was placed in a gDNA Eliminator spin column (RNeasy Mini Plus Kit, Qiagen) and centrifuged for 30 s at > 12000rpm using the Eppendorf® MiniSpin®. The flow-through was retained and mixed with 900 µL (1.5 volumes) of 100% ethanol by vortexing. Subsequent steps were performed according to the amended manufacturer's protocol (Appendix D), avoiding the use of Buffer RW1 which is incompatible with the extraction of small RNAs. RNA was eluted using 30 µL RNase-free water.

RNA extracted from PBMCs was analysed for quantity and purity using spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific). The average 260/280 ratio for the RNA extracted from cryopreserved PBMCs was 2.035 compared with a ratio of 2.025 for RNA extracted from PBMCs stored in lysis buffer. For a selection of samples, the quality of the RNA was also assessed by

Dr. Elihu Aranday-Cortes, University of Glasgow-MRC Centre for Virus Research using the Agilent 2200 TapeStation and the High Sensitivity R6K ScreenTape.

RNA integrity numbers (RIN) indicated that there was increased RNA degradation in the cryopreserved samples compared to the samples stored in lysis buffer (figure 2-1).

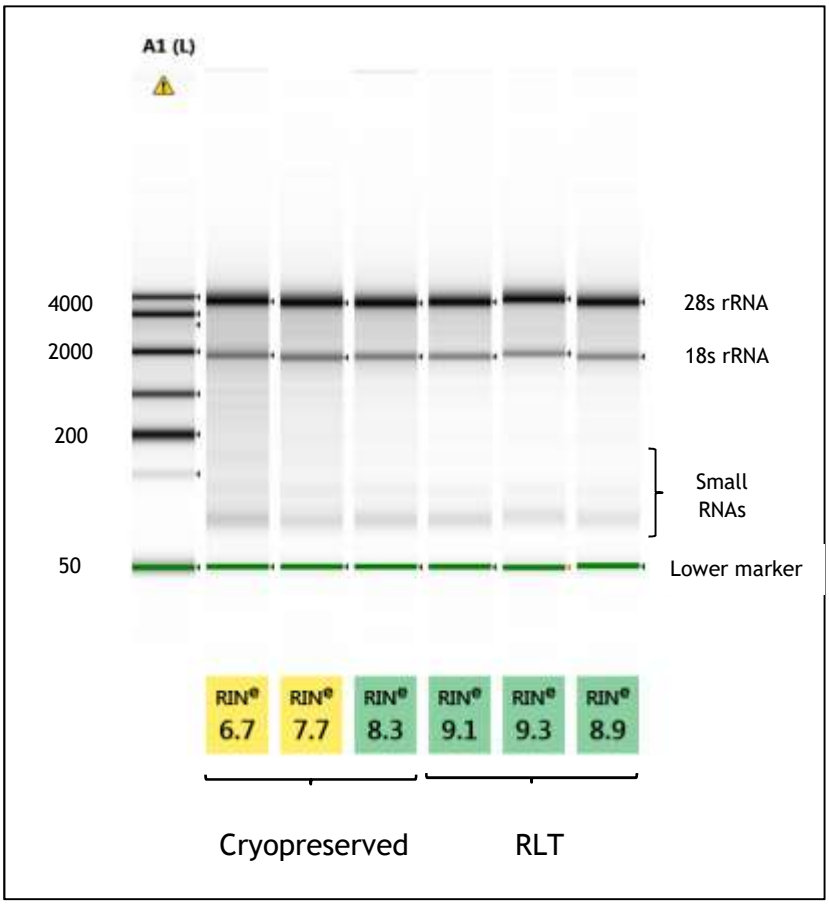


Figure 2-1: Comparison of RIN for RNA extracted from cryopreserved PBMCs and PBMCs in RLT lysis buffer. Results obtained by running different RNA samples on the R6K ScreenTape. The gel image shows the separation profile of each of the individual samples showing 28S, 18S, small rRNAs and lower marker

2.2.1.4 Extraction of miRNA from exosomes

For a subset of serum samples (n=8), miRNA was extracted from exosomes. Extraction was performed using the ExoRNeasy Serum/Plasma Midi Kit, suitable for starting volumes of 100 µL to 1 mL. Samples of 500 µL serum (stored at -70°C) were thawed at room temperature prior to being mixed with 1 volume Buffer XBP. The sample was mixed gently and transferred to the ExoEasy spin column before being centrifuged for 1 minute at 500G. The flow-through was

discarded and the sample was centrifuged for a further minute at 500G. The column was washed by adding 3.5 mL Buffer XWP and spinning for a further 5 minutes at 5000G. The spin column was then transferred to a fresh tube, and 700µL Qiazol added to the membrane. The sample was centrifuged at 5000G for 5 minutes to collect the lysate, which was subsequently vortexed and incubated at room temperature for 5 minutes. The lysate was incubated for a further 2-3 minutes at room temperature following the addition of 90 µL chloroform, and then centrifuged at 4^{o}C for 15 minutes at 12000G. The upper aqueous phase was mixed with 2 volumes 100% ethanol before being transferred to an RNeasy MinElute spin column. Washing steps with Buffer RWT and Buffer RPE were performed according to the manufacturer's instructions. As previously, 16 µL RNase-free water was added to the membrane and incubated at room temperature for 10 minutes before being centrifuged at >12000rpm using the Eppendorf® MiniSpin® for a further minute. A second elution step was performed using the same eluate, resulting in a final volume of 14 µL.

2.2.1.5 Extraction of miRNA from HepaRG cells

MiRNA was extracted from a monolayer of HepaRG cells (1x10^{6} cells) using a column based protocol, the MiRNeasy Micro kit (Qiagen). Firstly, 700 µL Qiazol was added to each cell culture dish; the lysate was mixed by vortexing for 1 minute and transferred to a tube to incubate at room temperature for 5 minutes. The lysate was incubated for a further 5 minutes at room temperature following the addition of 140 µL chloroform and then centrifuged at 4^{o}C for 15 minutes at 12000G. The aqueous phase was transferred to a new tube and mixed with 1.5 volumes of 100% ethanol. Washing steps were performed according to the manufacturer's protocol. A further 5 minute centrifugation step was performed to dry the spin column membrane. The spin column was then placed in a new collection tube, and 16 µL of RNase-free water was added to the membrane. The column was incubated at room temperature for 10 minutes before being centrifuged at 12000 rpm in the Eppendorf® MiniSpin® for 1 minute. A second elution step was performed using the eluate resulting in a final volume of 14 µL. Samples were then diluted with RNase-free water to a final concentration of 5 mg/µL for downstream cDNA synthesis.

2.2.2 MiRNA expression profiling

2.2.2.1 MiRNA expression profiling using TaqMan® MicroRNA Arrays

The TaqMan® MicroRNA Array consists of two 384-well microfluidic cards pre-loaded with TaqMan® primers and probes, allowing quantification of 754 individual miRNAs. Each card also contains 3 endogenous controls (the small nucleolar RNAs U6snoRNA, RNU44 and RNU48) and 1 negative control assay. Each card was used in conjunction with the matching Megaplex™ Pool of stem-looped RT primers.

In the Outcomes study (Chapter 4), the starting amount of total RNA was 133.2ng (44.4 ng/μL) for the PBMC analysis and 69 ng (23 ng/μL) for the serum analysis. In the EAP cohort (Chapter 5), the starting amount of total RNA was 104ng (34.7 ng/μL). In all cases, a pre-amplification step was included according to the manufacturer's recommendations. A control experiment was performed for each card type using 3 μL RNase-free water in place of sample to control for background contamination.

I. RT reaction

For the RT reaction, the following reagents were combined and gently mixed by inverting the microcentrifuge tube.

Table 2-8: RT reaction mix components

RT reaction mix component	Volume for 1 sample (μL)
Megaplex™ RT Primers (10X)	0.8
dNTPs with dTTP (100 mM)	0.2
MultiScribe™ Reverse Transcriptase (50 U/μL)	1.5
10X RT Buffer	0.8
$MgCl_2$ (25 mM)	0.9
RNase Inhibitor (20 U/μL)	0.1
Nuclease-free water	0.2
Total	4.5

A total of 4.5µL RT reaction mix was pipetted into a PCR tube, followed by 3µL total RNA. The tubes were sealed and incubated on ice for 5 minutes prior to cDNA synthesis. Thermal-cycling conditions on the GeneAmp® PCR System 9700 were as follows:

Table 2-9: Thermal cycling conditions for RT reaction

Stage	Temperature	Time
Cycle (40 cycles)	16°C	2 minutes
	42°C	1 minute
	50°C	1 s
Hold	85°C	5 minute
Hold	4°C	∞

II. Pre-amplification reaction

For the PreAmp reaction mix, the following reagents were thawed and combined:

Table 2-10: PreAmp reaction mix

PreAmp reaction mix component	Volume for 1 sample (µL)
TaqMan® PreAmp Master Mix (2X)	12.5
Megaplex™ PreAmp Primers (10X)	2.5
Nuclease-free water	7.5
Total	22.5

A total of 22.5 µL of the PreAmp reaction mix was added to 2.5 µL of the RT product from the previous reaction in a new PCR tube. The tubes were mixed gently, spun briefly and incubated on ice for 5 minutes. The following thermal cycling parameters were used for the preamplification reaction:

Table 2-11: Thermal cycling conditions for PreAmp reaction

Stage	Temperature	Time
Hold	95°C	10 minutes
Hold	55°C	2 minutes
Hold	72°C	2 minutes
Cycle (12 cycles)	95°C	15 s
	60°C	4 minutes
Hold	99.9°C	10 minutes
Hold	4°C	∞

Each 25µL reaction product was diluted with 75 µL 0.1X Buffer TE and stored at -20°C prior to running the real-time quantitative PCR.

III. Real-time quantitative PCR

In this step, the pre-amplified target cDNA was amplified by DNA polymerase using the sequence specific primers and probe on the TaqMan® MicroRNA Array. The PreAmp reaction product was thawed and mixed with TaqMan® Universal PCR Master Mix and nuclease-free water as follows.

Table 2-12: RT-qPCR reaction mix

PCR reaction mix component	Volume for 1 array (µL)
TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2X	450
Diluted pre-amp product	9
Nuclease-free water	441
Total	900

The TaqMan® Array cards were stored at 4°C; each card was allowed to equilibrate at room temperature for 15 minutes prior to use. Each port of the microfluidic card was then loaded with 98 µL of the PCR reaction mix, and the cards were then centrifuged for 2 minutes using the Heraeus™ Megafuge™ 40R at 331G before sealing.

The cards were run on the 7900HT Fast Real-Time PCR system using the thermal-cycling conditions described in Table 2-13:

Table 2-13: Thermal cycling conditions for RT-qPCR reaction

Stage	Temperature	Time
Hold	50°C	2 minutes
Hold	95°C	10 minutes
Cycle (40 cycles)	95°C	15 s
	60°C	1 minute
Hold	4°C	∞

2.2.2.2 MiRNA profiling using individual miRNA assays

The cDNA templates were prepared using the TaqMan® Advanced MiRNA cDNA Synthesis Kit. Mature miRNAs from total RNA are modified by adding a poly (A) tail to the 3' end of the transcript, then lengthening the 5' end by adaptor ligation to provide a forward-primer binding site for the miR-Amp reaction. The specificity of this system for miRNAs relies on the 5'adapter, which can only be ligated to the free phosphorylated 5' end of a miRNA molecule. Other cellular RNAs have 5' modifications that will not allow ligation of the adapter and so will not be reverse transcribed. The specificity is such that even other small RNAs such as small nuclear and small nucleolar RNAs, which often possess 5' modifications, are not recognised. As a consequence, the small RNA endogenous control assays used with the standard chemistry TaqMan miRNA assays are not compatible with the TaqMan Advanced miRNA workflow (B Revill 2018, personal communication, 28 March).

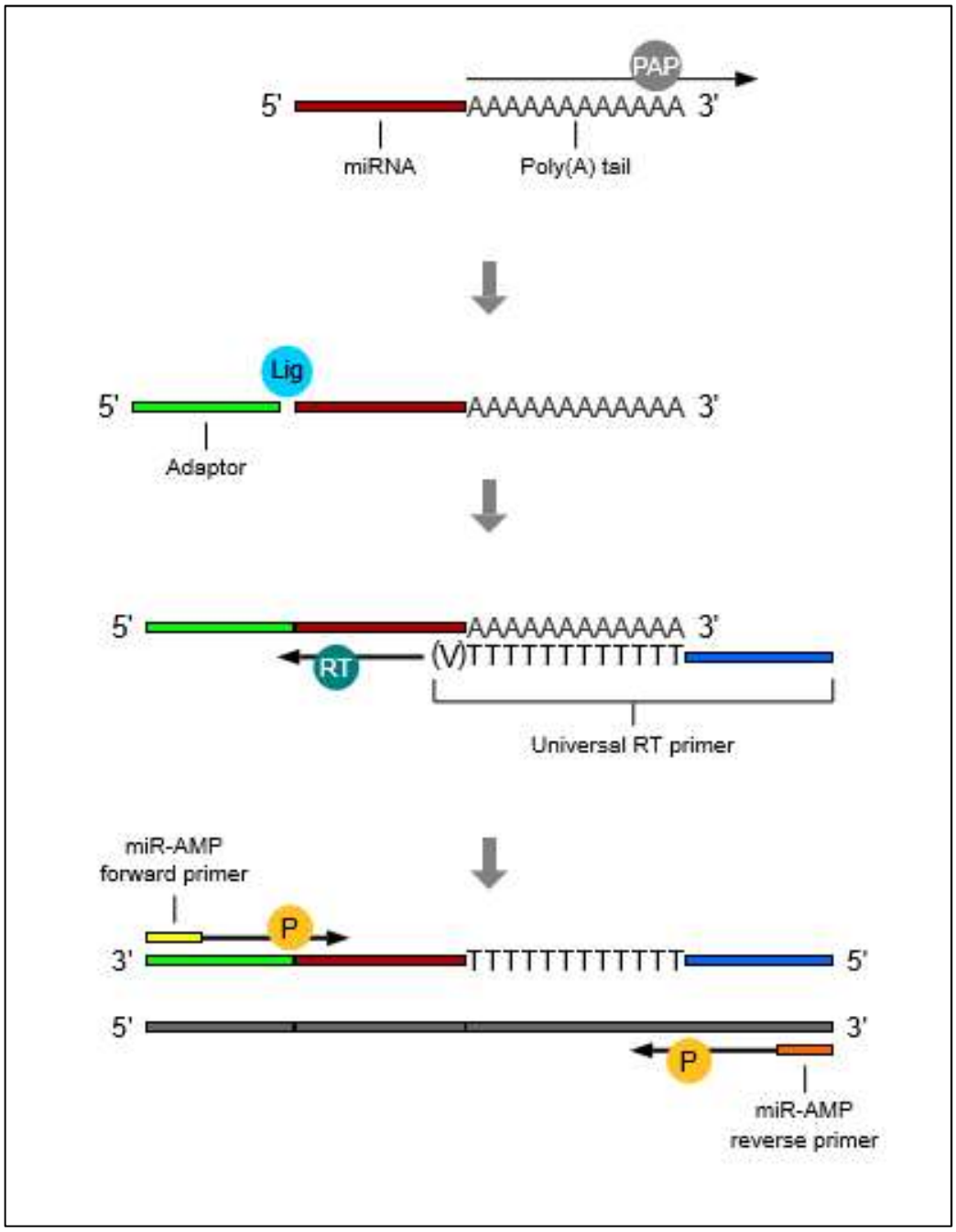


Figure 2-2: cDNA template preparation using TaqMan® Advanced MiRNA cDNA Synthesis Kit. PAP, poly (A) polymerase; Lig, ligase; RT, reverse transcriptase; P, hot-start DNA polymerase. Extracted from: Thermo Fisher Scientific (Inc, 2016).

I. 3' poly(A) tailing reaction

Samples and reagents were first thawed on ice. Two microlitres of sample was used for each reaction; for tissue samples a maximum of 10 ng total RNA was used. Reagents were mixed as follows before being added to each sample.

Table 2-14: Poly (A) tailing reaction mix

Poly(A) tailing reaction mix component	Volume for 1 sample (µL)
10X Poly (A) Buffer	0.5
ATP	0.5
Poly (A) Enzyme	0.3
RNase-free water	1.7
Total	3.0

The samples were incubated according to the following thermal-cycling settings:

Table 2-15: Thermal cycling conditions for poly (A) tailing reaction

Stage	Temperature	Time
Polyadenylation	37°C	45 minutes
Stop reaction	65°C	10 minutes
Hold	4°C	∞

II. Ligation reaction

The 5' ligation reaction mix was prepared as follows:

Table 2-16: Ligation reaction mix

Ligation reaction mix component	Volume for 1 sample (µL)
5X DNA Ligase Buffer	3.0
50% PEG 8000	4.5
25X Ligation Adaptor	0.6
RNA Ligase	1.5
RNase-free water	0.4
Total	10.0

The reaction mix was vortexed, centrifuged briefly, and transferred to the 5 µL poly (A) tailing reaction product. The reaction tubes were incubated according to the following settings:

Table 2-17: Thermal cycling conditions for ligation reaction

Stage	Temperature	Time
Ligation	16°C	60 minutes
Hold	4°C	∞

III. RT reaction

The RT reaction mix was prepared as follows:

Table 2-18: RT reaction mix

RT reaction mix component	Volume for 1 sample (µL)
5X RT Buffer	6.0
dNTP Mix (25mM)	1.2
20X Universal RT Primer	1.5
10X RT Enzyme Mix	3.0
RNase-free water	3.3
Total	15.0

The reaction mix was transferred to the reaction tube containing the adaptor ligation reaction product (30 µL total). The RT reaction was performed under the following thermal-cycling conditions:

Table 2-19: Thermal cycling conditions for RT reaction

Stage	Temperature	Time
Reverse transcription	42°C	15 minutes
Stop reaction	85°C	5 minutes
Hold	4°C	∞

IV. MiRNA amplification step

Table 2-20: MiR-Amp reaction mix

MiR-Amp reaction mix component	Volume for 1 sample (µL)
2X miR-Amp Master Mix	25.0
20X miR-Amp Primer Mix	2.5
RNase-free water	17.5
Total	45.0

The above reagents were combined in a microcentrifuge tube and mixed thoroughly. For each sample, 45 µL of the reaction mix was combined with 5 µL of the RT reaction product obtained in the previous step. The tubes were placed in a thermal cycler and incubated using the following settings and MAX ramp speed.

Table 2-21: Thermal cycling conditions for miR-Amp reaction

Stage	Temperature	Time	Cycles
Enzyme activation	95°C	5 minutes	1
Denature	95°C	3 s	14
Anneal/extend	60°C	30 s	
Stop reaction	99°C	10 minutes	1
Hold	4°C	∞	1

V. Real-time quantitative PCR

For the RT-PCR, a 1:10 dilution of cDNA template was prepared using 0.1X TE Buffer. The TaqMan® Advanced miRNA Assays were thawed on ice before being mixed with TaqMan® Fast Advanced Master Mix 2X and RNAse-free water as follows. Samples were run in quadruplicate.

Table 2-22: RT-qPCR mix

PCR reaction mix component	Volume for 1 reaction (µL)
TaqMan® Fast Advanced Master Mix (2X)	10
TaqMan® Advanced MiRNA Assay (20X)	1
RNase-free water	4
Total	15

The reaction mix components were mixed well by vortexing and centrifuged briefly. A total of 15 µL was transferred to each well of a PCR reaction plate followed by 5 µL diluted cDNA template, giving a total volume of 20 µL per reaction. The reaction plate was sealed with an optical adhesive cover and centrifuged for 1 minute at 1000G using the Sorvall® Legend RT centrifuge. The RT-qPCR was performed using the 7500 Fast Real-Time PCR system, with the following thermal cycling settings.

Table 2-23: Thermal cycling conditions for RT-qPCR

Stage	Temperature	Time	Cycles
Enzyme activation	95°C	20 s	1
Denature	95°C	3 s	40
Anneal/extend	60°C	30 s	

2.2.2.3 Analysis of miRNA expression profiling

I. Array-based profiling

Data from the TaqMan® MicroRNA Arrays were collected with the manufacturer's SDS software (SDS 2.4, Applied Biosystems). Thresholds, set automatically, were checked individually and corrected as required. Quantitative data analysis was performed using ExpressionSuite Software v1.0.3 (Applied Biosystems) which uses the comparative cycle threshold (CT) method (ΔΔCT). Global normalisation was applied as there was high variation of expression of the small RNA controls between samples. Global normalisation uses the median CT value of assays common to every sample as the normalisation factor, on a per sample basis and is appropriate for profiling a large number of miRNA targets (Mestdagh et al.,

2009). The maximum CT was set at 35, and wells were rejected if there was low amplification in the linear phase, bad passive reference signal, Cq confidence < 0.8, baseline algorithm failure, noise spikes or noise higher than others in the plate, and if there was no signal in the well or the fluorescence was offscale. TaqMan® MicroRNA Assays have an amplification efficiency of 100% ± 15% across 5 $logs_{10}$ and a dynamic range of ≥ 7 $logs_{10}$ with ≥ 0.98 linearity.

A control experiment was run with nuclease-free water in place of sample. MiR-601, miR-1290, miR-1243, miR-30a-5p, miR-151-3p, miR-520d-3p and miR-1274B were detected on control Card B (run with 3 µL nuclease-free water) and were excluded from all other analyses. No miRNAs were detected on Card A in the control experiment.

II. Identification of endogenous miRNA controls

Global normalisation is only appropriate when profiling a large number of genes (Mestdagh et al., 2009). Endogenous miRNA controls for the individual miRNA assays were therefore identified from the TaqMan® array data using ExpressionSuite software v1.0.3 (Applied Biosystems). All miRNAs were assessed for suitability as controls. CT values for each gene were plotted, and miRNAs showing the greatest stability of expression between samples were identified. Additionally, the average pairwise variation of each miRNA was calculated compared to all the other candidate miRNA to give a stability score (Vandesompele et al., 2002). Student's t-tests were then performed to confirm that there were no significant differences in expression of the endogenous controls between experimental groups.

For the PBMC analyses, miR-451a and miR-26a-5p were used as endogenous controls, and for the serum analyses, miR-574-3p was selected.

A further validation experiment was performed for the serum analyses, comparing the detection of the endogenous internal control miR-574-3p with spiked cel-miR-39-3p expression. This comparison demonstrated good correlation (R^2 = 0.829) between the endogenous and exogenous control (figure 2-3).

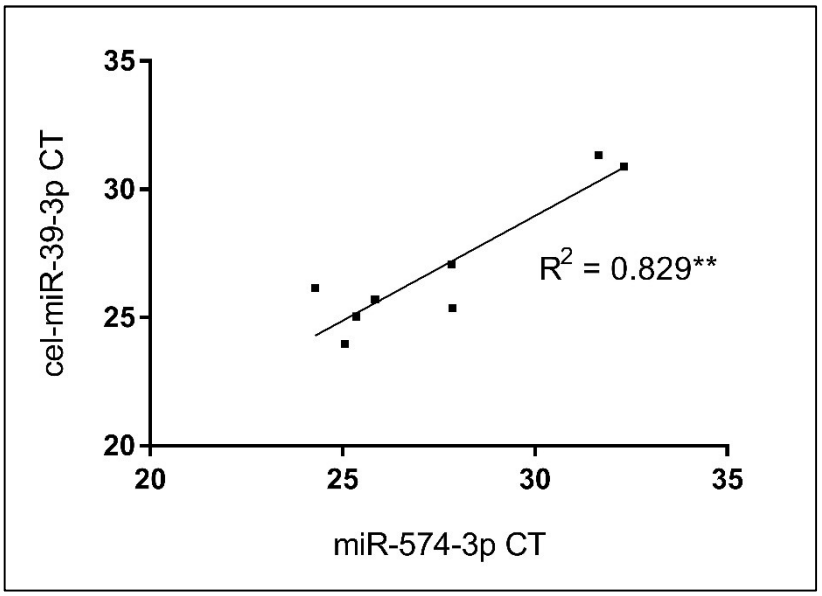


Figure 2-3: Correlation between endogenous miR-574-3p and spiked cel-miR-39-3p CT values in serum samples from subjects with CHC.

III. Validation

TaqMan® Advanced MiRNA Assays were performed in quadruplicate, according to the manufacturer's recommendations. Data from the assays were collected using the manufacturer's SDS software. Thresholds were set automatically, and individually reviewed. Mean CT values were accepted if the standard deviation (SD) was < 0.5; outliers were removed. If the CT SD remained > 0.5 despite the removal of 1 outlier, the experiment was repeated. The expression level of each miRNA was quantified using the mean normalised threshold cycle number (Δ*C*T) and the relative expression level was calculated as $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001, Schmittgen and Livak, 2008). As described above, the endogenous miRNAs miR-451a and miR-26a-5p were used as internal controls for PBMC samples, and miR-574-3p was used for the serum experiments.

2.2.2.4 Reproducibility of TaqMan® Advanced MiRNA Assays

An experiment was performed to assess the reproducibility of the individual miRNA assays over time. We analysed the expression of miR-21-5p, miR-122-5p, miR-885-5p and miR-574-3p in 4 serum samples. The experiment was repeated a week later on the same extracted RNA and under the same conditions.

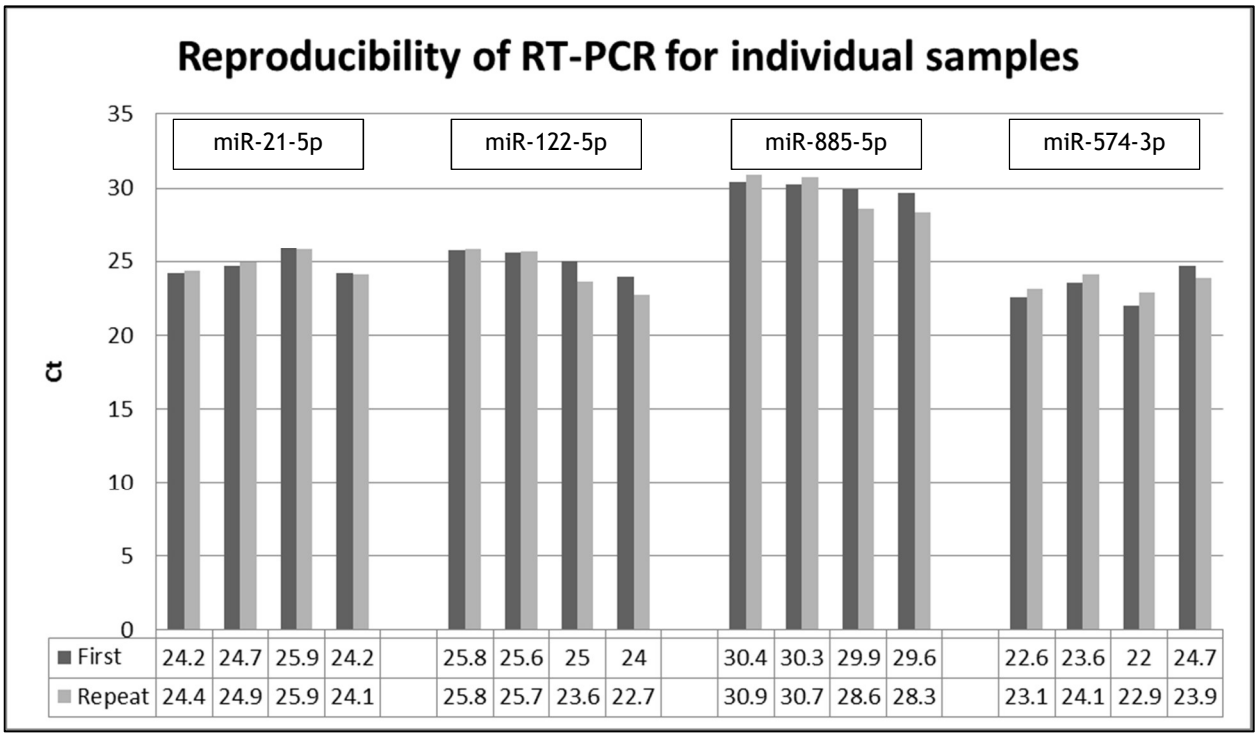


Figure 2-4: MiRNA assays demonstrate good reproducibility over time.

2.3 *In silico* target prediction and functional analysis

Validated targets for individual miRNAs were identified using MiRTarbase 2016 (http://mirtarbase.mbc.nctu.edu.tw/ (Chou et al., 2016)) and Tarbase v7.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index (Vlachos et al., 2015)). Analysis was restricted to targets appearing in both databases, and miRNA target genes validated only by weaker experimental methodology, i.e. microarray or pulsed stable isotope labelling by aa in cell culture, were excluded. STRING v10.5 (http://string-db.org/) was used for the functional analysis of validated or predicted targets of selected miRNAs (Szklarczyk et al., 2015). Interaction scores are calculated by the software by combining the probabilities from different evidence sources and correcting for the probability of randomly observing an interaction. Experimental data are extracted from 7 databases: BIND, DIP, GRID, HPRD, IntAct, MINT and PID, and curated data are extracted from a further 5 databases: Biocarta, BioCyc, GO, Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome. Only high confidence interactions (≥0.9) are included and figures are simplified by the removal of unconnected nodes from the interaction network. Up to 5 first shell interactors (i.e. proteins directly interacting with the input protein) were included to inform the pathway analysis. The Markov clustering algorithm was

applied with an inflation factor of 3 to extract relevant modules (Brohee and van Helden, 2006). Genes involved in the most significantly enriched KEGG pathway for each miRNA are highlighted in red for clarity.

Pathway analysis for complete miRNA datasets was performed using the online tool DIANA-miRPath (http://diana.imis.athena-innovation.gr/DianaTools/index.php). DIANA-miRPath performs an enrichment analysis of input datasets by comparing each set of genes to KEGG pathways utilising experimentally validated miRNA interactions derived from DIANA – TarBase v6.0 (Vlachos et al., 2012). The input dataset enrichment analysis is performed by a Pearson's chi-squared test ($x^2 = \Sigma[(O - E)^2/E]$), where O (Observed) is the number of genes in the input dataset found to participate in a given pathway and E (Expected) is the number of genes expected by chance, given the pathway and input list size, to be a member of that pathway (Papadopoulos et al., 2009). Correction for multiple testing was done using the Benjamini–Hochberg method and significantly enriched pathways were selected according to a false discovery rate (FDR) <0.001. The input dataset enrichment in each KEGG pathway is represented by the negative natural logarithm of the P-value ($-\ln P$). The comparison of pathway analyses was performed using Ingenuity® Pathway Analysis (IPA®, Qiagen).

2.4 Statistical analysis

Data from the TaqMan® MicroRNA Arrays were analysed using the ExpressionSuite Software v1.0.3 (Applied Biosystems). Only miRNA transcripts expressed in ≥ 2 biological replicates in each group were included in downstream analysis. Mathematical correction for multiple comparisons was not applied due to the small group size and the risk of increasing the type II error for those associations that were not null, and as all targets of interest underwent validation. Subsequent statistical analyses were performed using IBM® SPSS® Statistics v22 and GraphPad Prism® v7.03.

Continuous variables are expressed as medians and interquartile ranges, and categorical variables are recorded as number and percentages. Categorical variables were compared using chi-square testing and continuous variables were analysed using the exact Wilcoxon Mann-Whitney test or Student's t-test,

depending on distribution. Normality was assessed using the Shapiro-Wilk normality test. Differences between two paired sets of normally distributed variables were examined using the paired t-test, and differences between > 2 paired sets of variables were analysed with one-way ANOVA. Dunnett's multiple comparisons test was used to compare the mean of each set to the mean of the control for normally distributed data, whilst Dunn's multiple comparison's test was used for non-parametrically distributed data. Pearson correlation coefficients were calculated for pairs of variables; missing data were handled by pairwise deletion.

Where it was not possible to express miRNA expression with reference to a healthy control population, miRNA expression was dichotomised into 'positive' or 'negative' dependent on the ΔCT relative to the mean/median of the group. MiRNA expression was defined as positive when the ΔCT was lower than the mean/median (i.e. increased expression) and negative when the ΔCT was higher than the mean/median.

The relationship between biomarker expression, clinical characteristics and biochemical variables was assessed using binary logistic regression. Models were fitted using multiple logistic regression, starting with factors significant at the 10% level in univariate analysis. Collinearity diagnostics were performed; tolerance of <0.2 and variance inflation factor (VIF) > 2.5 were considered evidence of collinearity. Nagelkerke R^2 values are reported. Cook's distance was calculated to identify any cases unduly influencing the model. The diagnostic accuracy of each biomarker was evaluated using the area under the receiver operating characteristics (ROC) curve (AUC), and cut-offs were calculated according to the highest Youden index to maximise sensitivity and specificity.

P values are 2-sided and values of <0.05 were considered significant. For all statistical analysis other than calculation of correlation coefficients, missing variables were handled by listwise deletion, i.e. subjects with missing data were excluded.

3 Spontaneous clearance of CHC

3.1 Introduction

Spontaneous clearance of HCV during acute infection (< 6 months) occurs in 20-40% of people who acquire infection (Grebely et al., 2014, Vispo et al., 2014). Predictors of clearance are poorly understood, however there is concordance with factors known to be associated with improved clinical and treatment outcomes. Host factors, including gender (Grebely et al., 2014, Micallef et al., 2006, Hsi et al., 2014, van den Berg et al., 2011) and immune response (Lemon, 2010, Thomson et al., 2011), and viral factors, such as HCV genotype and quasispecies diversity (Grebely et al., 2014), are thought to influence disease progression and may also predict chance of spontaneous clearance (Westbrook and Dusheiko, 2014). Host genetics are relevant, and one of the strongest host factors associated with clearance (and response to HCV treatment) is a favourable IFNL3 gene polymorphism (chapter 1.2.1) (Grebely et al., 2014, Rembeck and Lagging, 2015).

Spontaneous clearance of HCV in the chronic phase is less well described (Watanabe et al., 2003). It has been reported in the literature following superinfection with HBV (Sagnelli et al., 2009, Gruener et al., 2002) or following HDV infection of HIV-HBV co-infected subjects (Maida et al., 2008). Additional studies have described clearance following the withdrawal of immunosuppressive medication (Somsouk et al., 2003), in the context of liver transplantation or surgery (Samonakis et al., 2005, Yoshikawa et al., 2001), following the development of hepatocellular carcinoma (Minami et al., 2014) and during pregnancy/parturition (Hattori et al., 2003, Zein et al., 2001). Additionally, spontaneous HCV RNA negativity has been described in HIV-HCV co-infected patients, including those with hepatic decompensation, following initiation or optimisation of antiretroviral therapy (Mora-Peris et al., 2015, Kaung et al., 2014, Stenkvist et al., 2014).

Host factors may be important predictors of clearance in the chronic phase as well as the acute phase; Raghuraman et al. (2012) reported a case of HCV clearance at 65 weeks post infection which was associated with reversal of T cell exhaustion and the appearance of neutralising antibodies, and two recent

studies looking at HIV-HCV co-infected patients found that late clearance was associated with a favourable IFNL3-CC genotype (Vispo et al., 2014, Stenkvist et al., 2014). However, interpretation of these studies is limited by the small number of cases.

In this study, the incidence and factors associated with spontaneous clearance of CHC were established amongst a large Scottish cohort.

3.2 Patient selection

3.2.1 Study design and population

The West of Scotland Specialist Virus Centre (WoSSVC) is part of the NHS Greater Glasgow & Clyde Health Board (NHSGGC) which serves a population of > 1 million individuals. Of the 35474 cases of HCV antibody positivity diagnosed in Scotland as of December 2013, 14076 (40%) reside within NHSGGC (McLeod A, 2014). The WoSSVC provides the majority of the diagnostic virology service for the West of Scotland and is the sole provider of HCV RNA testing. Data were obtained from the WoSSVC on HCV testing over a 20 year period between 1994 and 2013. The study followed a retrospective case-control design; cases were individuals who spontaneously resolved CHC, and controls were individuals who did not.

3.2.2 Identification of cases and controls

All patients included in the study must have been tested on either serum or dried blood spot (DBS) for HCV RNA as part of their clinical care. Patients with a minimum of 2 sequential samples positive for HCV RNA at least 6 months apart, followed by at least one negative test for HCV RNA, were identified. These patients were linked with national treatment data obtained from the Scottish Hepatitis C Clinical Database. This database is held by HPS and contains clinical and treatment data for HCV-infected patients attending outpatient specialist clinics across Scotland (McDonald et al., 2012). Patients with a history of HCV treatment were then excluded to create a cohort of individuals with potential spontaneous clearance of CHC. Clinical records of potential spontaneous clearers were reviewed to confirm the clinical scenario. Individuals in the spontaneous clearance group with > 1 negative HCV RNA sample were subcategorised as ‘confirmed’ clearers.

Patients with 2 positive HCV RNA samples at least 6 months apart with no subsequent negative samples were selected as our comparison group. To create a control group of chronically infected patients, individuals were randomly selected from the comparison group using number generation with a frequency of 4 controls per patient of interest.

3.2.3 Clinical, demographic and exposure data

Demographic patient data (age at infection, sex, ethnicity, alcohol intake, BMI, source of infection), HCV markers (liver enzymes, HCV genotype, IFNL3 genotype, HCV RNA and history of cirrhosis), and HIV, HBV and HDV serostatus were obtained from the Scottish Hepatitis C Clinical Database, augmented by case note review. Where available, biochemical and haematologic variables were recorded at the time of the last positive HCV RNA test for all patients, and concurrently with the first negative HCV RNA test for spontaneous clearers. The date of HCV clearance was estimated using the midpoint between the time at which the last positive HCV RNA and the first negative HCV RNA samples were collected. Duration of diagnosis (which serves as a proxy of duration of infection) was calculated as the interval between the first positive HCV RNA and the time of HCV clearance for spontaneous clearers; for the control group this was defined as the interval between the first positive and the last positive HCV RNA results. Follow up was censored at the last positive HCV RNA test for the control group. Clinical records for case patients were reviewed and data were collected on hospitalisations or acute events in the 12 months prior to clearance.

3.2.4 Incidence of spontaneous clearance of CHC

The incidence density rate of spontaneous clearance of CHC amongst untreated individuals was calculated as the number of cases of spontaneous clearance over the total number of person-years follow up.

3.3 Results

3.3.1 Clinical laboratory testing

All patients had been tested for HCV RNA as part of their clinical care. Viral load samples logged as ‘positive’ or ‘detectable’ were recorded as the upper limit of

sensitivity for the given assay. Patients also underwent HCV genotyping as part of their routine clinical care.

3.3.2 Derivation of sample set for analysis

A total of 25113 samples were positive for HCV RNA, relating to 10318 patients. Of these, 1430 patients had 2 sequential positive results followed by a negative result. Following linkage to the Scottish Hepatitis C Clinical Database 1314 patients were identified as treatment-experienced and were thus excluded, leaving 116 patients of interest. Ten patients were excluded following case note review as examination of full laboratory data showed that the HCV RNA positive samples were not sequential, suggesting fluctuating viraemia or 2 or more episodes of spontaneous clearance during acute infection rather than spontaneous clearance of CHC. A further 48 patients had exposure to HCV treatment that had not yet been recorded on the national database. For 7 patients, patient identifiers held in the database did not link with a clinical record. One patient had been incorrectly coded as negative, but on review of the laboratory data was found to have quantifiable HCV RNA. After these exclusions, 50 case patients remained and were included in downstream analysis, contributing 241 person-years follow up. Two patients were classified as spontaneous clearers solely on the basis of DBS testing, one of whom went on to have a positive serum HCV RNA test in the absence of ongoing risk exposure. A further two patients who were classified as spontaneous clearers on the basis of serum HCV RNA testing developed HCV RNA positivity > 1 year post probable clearance; one patient admitted to ongoing IDU. Twenty-seven patients went on to have at least 1 further negative HCV RNA test (26 serum samples and 1 DBS) and were subcategorised as ‘confirmed’ clearers.

For the comparison group, 3329 patients with 2 positive HCV RNA samples at least 6 months apart with no subsequent negative samples were identified of whom 955 were treatment-experienced. The remaining 2374 were untreated, contributing 13766 person-years follow up. Our control population comprised 200 randomly selected patients from this untreated cohort.

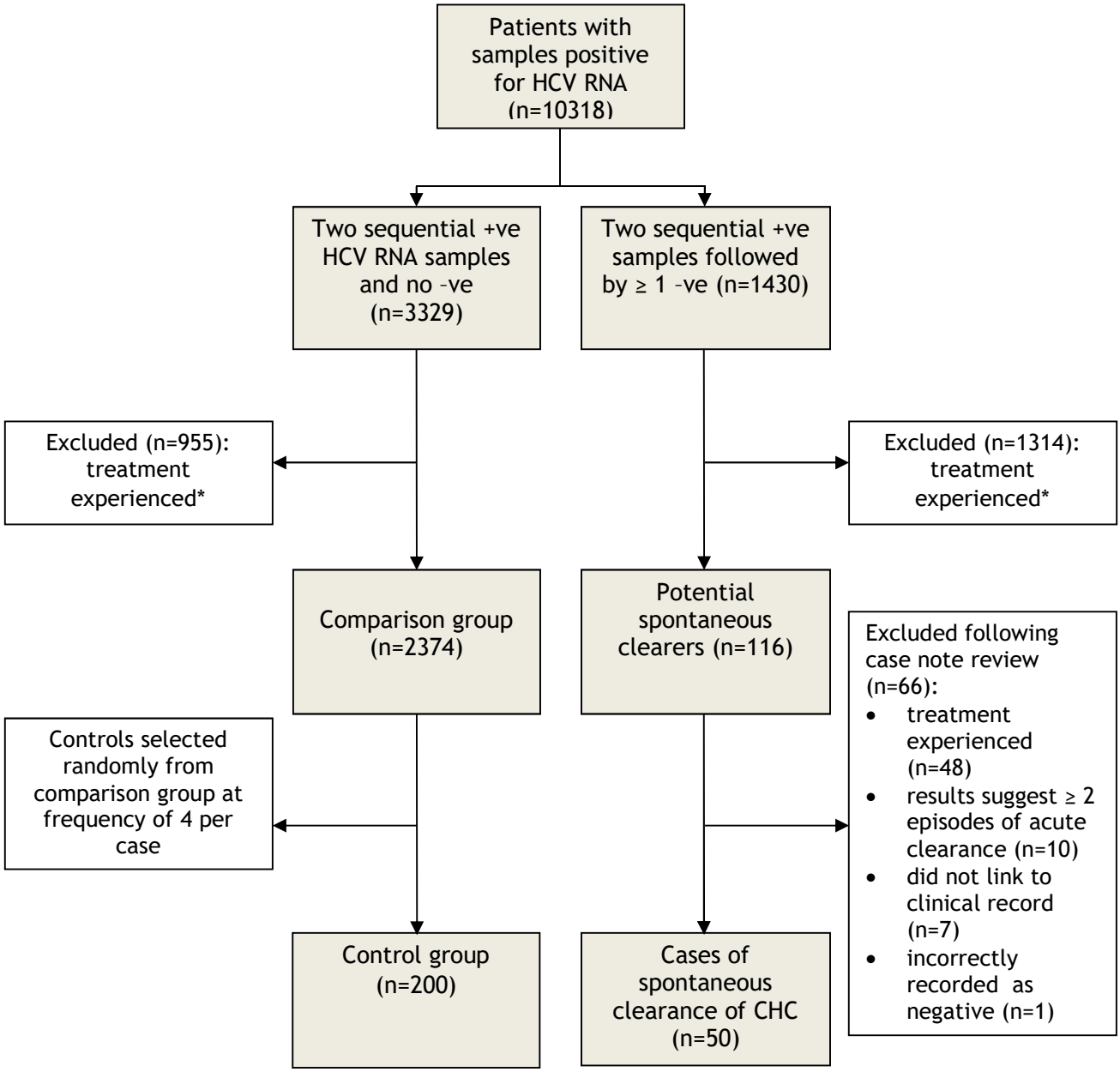


Figure 3-1: Derivation of final sample. *Patients excluded following linkage to treatment data held in the Scottish Hepatitis C Clinical Database

3.3.3 Incidence of spontaneous clearance of CHC

The overall incidence density rate of spontaneous clearance of CHC amongst the untreated patient population was 0.36 per 100-person-years follow up. When restricting the analysis to patients with 'confirmed' clearance, the incidence rate was 0.19 per 100-person-years follow up.

3.3.4 Characteristics of cases and controls

Table S1 summarises the main demographic and clinical characteristics of the study populations. The majority of patients were white, with a history of IDU as the risk factor for acquisition of HCV. There was a similar incidence of Gt1 and Gt3 infections. There were no significant differences in HCV genotype, ethnicity or risk group between the two populations. Ongoing IDU was positively associated with chronicity of infection (p=0.034).

Patients who spontaneously cleared CHC were more likely to be female (p = 0.001) and to have been diagnosed at a younger median age (28.5 years vs. 33 years, p = 0.022). Median age at diagnosis in females was not significantly different between the two groups (27 years vs. 31.5 years, p=0.144). The age at which males and females were diagnosed in each group was similar (cases, p=0.200; controls, p=0.108).

There was no difference in the distribution of duration of diagnosis between groups (median duration 50 months v 50 months, p= 0.854) (figure 3-2). The minimum duration of diagnosis in the spontaneous clearance group was 9 months and the maximum duration was 182 months, compared with 7 months and 195 months in the comparator group. As spontaneous clearance may be more likely in early infection, a subgroup analysis was performed for case patients (n=41) and control patients (n=144) with at least 24 months confirmed viraemia and showed identical findings.

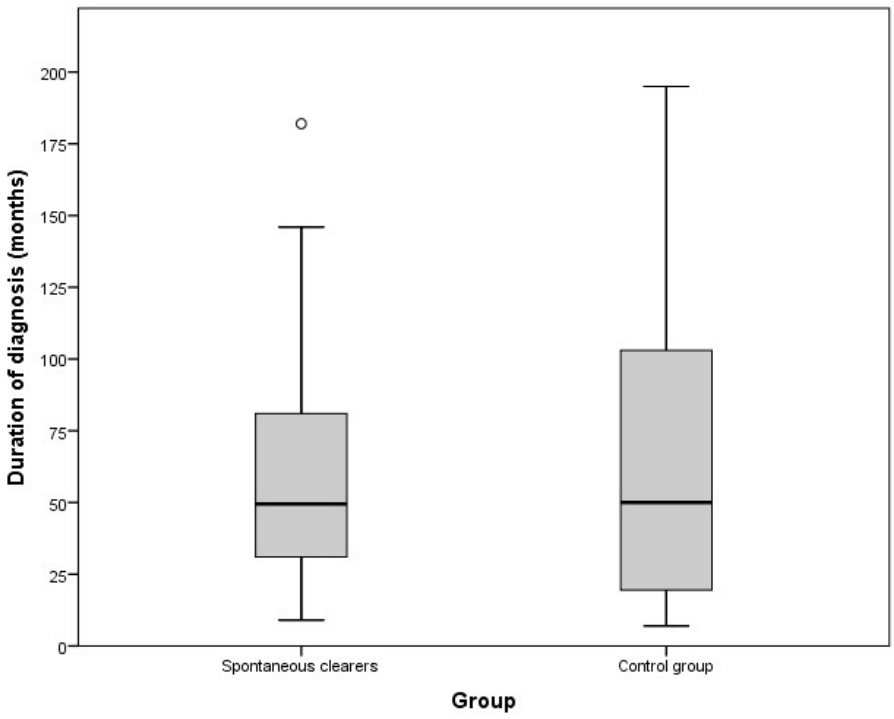


Figure 3-2: Duration of infection was not significantly different between spontaneous clearers and control patients. Data are represented as box-whisker plots; boxes represent the 25th and 75th percentile, whiskers the range, and horizontal lines represent the median. Outliers are shown as circles.

Median ALT levels were similar between cases and controls at the time of the last positive HCV RNA test (47.5 IU/L v 42.5 IU/L, p=0.560). There was a significant decrease in the ALT level between the last positive and the first negative HCV RNA test for case patients, providing further evidence of spontaneous clearance (47.5 IU/L v 20 IU/L, p<0.001).

Of those subjects who had been tested, quantitative HCV RNA levels were significantly lower amongst cases versus controls (p<0.001), however spontaneous clearance in the context of high-level viraemia (>100000 IU/mL) was observed in 7 patients. IFNL3 genotyping was performed on 1 case patient; this patient was found to carry the IFNL3-CC allele.

Amongst those tested, patients who spontaneously cleared CHC were significantly more likely to be hepatitis B surface antigen (HBsAg) positive (5/48 (10.4%) vs. 0/99 (0%), p<0.001). Eight case patients and 28 patients in the control group were positive for hepatitis B core antibody and negative for HBsAg indicating past infection. One HBsAg+ patient was co-infected with hepatitis delta virus. Rates of HIV IgG positivity were similar between the two groups (p=0.518).

Detailed review of medical notes was only possible for case patients due to time constraints. In 5 patients, 4 of whom had documentation of ongoing alcohol abuse, spontaneous clearance of CHC followed admission to hospital with decompensated liver disease. In 2 of these cases there was intercurrent sepsis and in 1 case the patient was admitted twice; once as a result of a staggered paracetamol overdose and several months later due to alcoholic hepatitis with queried spontaneous bacterial peritonitis. In the 4th case, the patient was admitted on 3 occasions with decompensation between the last detectable and first undetectable HCV viral load, on 2 occasions following intentional drug overdose and on 1 further occasion in the context of alcohol use. The abstinent patient decompensated due to gram negative bacteraemia. Of the decompensated patients, two had significant ALT rises (>5 times the upper limit of normal).

One patient underwent major abdominal surgery (hysterectomy) following the last positive HCV RNA PCR and prior to the first undetectable HCV RNA test. A further 3 patients underwent minor surgical procedures. Six patients (excluding those with sepsis and decompensation previously described) required hospital admission for various infections, including groin abscess, pneumonia, cellulitis and infective endocarditis.

Four patients had spontaneous vaginal deliveries during the period between their last detectable serum HCV RNA and the first negative test. A further individual underwent evacuation of retained products of conception following termination of pregnancy.

Twenty-seven of the cases had repeated negative RNA testing. Demographic and virologic characteristics of these are compared with controls in Table S2. On analysis of this more strictly defined cohort of spontaneous clearers, only female gender ($p=0.006$) and a lower median HCV viral load ($p=0.001$) remained significantly associated with clearance of CHC.

3.4 Discussion

This is the largest cohort of patients with evidence of spontaneous clearance of CHC studied to date. We have demonstrated that spontaneous clearance of CHC is rare, with an incidence rate of 0.19 – 0.36 per 100-person-years follow up. We found that spontaneous clearance of CHC was associated with female gender, HBsAg positivity, younger age at diagnosis and lower HCV RNA titres. It was negatively associated with current IDU. We observed that a proportion of cases occurred in the context of significant intercurrent illness and hepatic decompensation.

The incidence rate of spontaneous HCV clearance in our cohort is similar to that described in a previous Japanese study which demonstrated an annualized incidence rate of spontaneous CHC clearance of 0.5%/year/person and found that clearance was associated with milder liver disease (Watanabe et al., 2003). In contrast, a study by Scott et al. (2006) found that a significant percentage of Alaska Natives with CHC experienced HCV RNA negativity, corresponding to a rate of 1.15 per 100 persons per year. This variation in rate of spontaneous clearance may reflect the different genetic background of the study populations together with different incidences of factors associated with clearance of CHC. In addition, repeat HCV RNA testing in patients with established CHC in whom treatment is not immediately anticipated is performed rarely in our clinical practice, in accordance with international guidelines ('EASL Recommendations on Treatment of Hepatitis C 2015,' 2015). Therefore, infrequent repeat testing of HCV RNA may have led to an underestimate of the true incidence of spontaneous clearance in our cohort.

Concordant with our study, Scott et al. (2006) found that spontaneous HCV clearance was associated with a lower HCV viral load and a trend towards younger age at infection. Older age at acquisition is independently associated with a faster progression to fibrosis, even when controlling for duration of infection (Missiha et al., 2008), and children who are vertically infected appear to have a very slow progression to cirrhosis (Tovo et al., 2000). The presence of significant fibrosis is associated with a poorer response to HCV therapy ('EASL Recommendations on Treatment of Hepatitis C 2015,' 2015) and may be negatively associated with spontaneous clearance of CHC (Watanabe et al.,

2003). The reasons for the importance of age as a predictor of progressive fibrosis are undetermined, but may relate to changes in immune function and reduced hepatic blood flow (Sheedfar et al., 2013).

Female sex was significantly associated with spontaneous clearance of CHC in our study. This result remained significant when restricting the analysis to 'confirmed' clearers. These results mirror findings in the acute setting (Grebely et al., 2014, Vispo et al., 2014, Micallef et al., 2006, Hsi et al., 2014, van den Berg et al., 2011), and are supported by data from Scott et al. (2006) who found that all patients in their cohort with late sustained HCV RNA negativity were female. It has been postulated that gender-based differences in immunity may underlie the association between female sex and acute spontaneous clearance (Grebely et al., 2014), and the same may hold true for clearance in the chronic setting. Additionally, male sex is associated with a faster progression to cirrhosis, even after controlling for age, duration of infection, alcohol intake and metabolic factors (Poynard et al., 1997, Marabita et al., 2011). It is possible that male gender was associated with increased disease severity in our study, and therefore a lower rate of clearance. Furthermore, Grebely et al. (2014) demonstrated that the effect of IFNL3 and HCV genotype on clearance in the acute phase was greater among females than males. IFNL3-CC genotype has also been associated with spontaneous clearance of HCV in HCV-HIV co-infected patients (Vispo et al., 2014, Stenkvist et al., 2014), a finding we are unable to confirm due to infrequent testing amongst our cases.

Gt1 and Gt3 were equally distributed in our cohort, reflecting the distribution in Scotland (McLeod A, 2014). We did not find an increased likelihood of spontaneous clearance associated with Gt1 infection, as has previously been described in both the acute and the chronic setting (Grebely et al., 2014, Scott et al., 2006). However, as only a third of patients in our cohort had viral genotyping performed it is possible that this null result reflects limited statistical power.

Co-infection with HBV (HBsAg+) was significantly associated with spontaneous clearance of CHC ($p = 0.001$). HCV clearance in the context of HBV superinfection has been described in several case reports (Sagnelli et al., 2009, Gruener et al., 2002) and may occur as a bystander effect of antiviral cytokine

release. It has been suggested that release of type I IFNs from the liver during acute infection may contribute to clearance and that HBV may monopolise the synthetic machinery of the hepatocyte, thus interrupting the HCV replication cycle (Lauer and Kim, 2006).

Despite the negative association previously described between fibrosis and spontaneous clearance of CHC (Watanabe et al., 2003), one third of our case patients had a diagnosis of cirrhosis. Additionally, we identified a unique cohort of patients who cleared HCV following decompensation of their cirrhosis, most commonly in the context of alcohol excess and bacterial infection. The mechanisms underlying spontaneous clearance in this setting are unclear. Cirrhosis is associated with a reduction in the number of functional hepatocytes, potentially limiting viral replication and whilst HCV RNA quantification was performed too infrequently in our study to explore this hypothesis, patients with cirrhosis have been found to have lower HCV viral loads than non-cirrhotic subjects in a large Scottish mixed infection database (McNaughton A, personal communication, 2015). Furthermore, cirrhosis is associated with immune dysregulation and predisposition to bacterial infection (Thalheimer et al., 2005). Bacterial translocation occurs as a result of intestinal bacterial overgrowth and increased intestinal permeability, and results in endotoxaemia (Thalheimer et al., 2005, Giannelli et al., 2014). Bacterial LPS triggers production of inflammatory cytokines, including IFN-γ from hepatic lymphocytes, resulting in acute hepatic injury. Chronic alcohol ingestion enhances immune cell sensitivity to LPS resulting in increased production of inflammatory cytokines (Mandrekar et al., 2009). We hypothesise that HCV RNA clearance may occur in this setting as a result of non-specific stimulation of the immune system, perhaps on a background of lower baseline viral load. In support of this, two of the decompensated patients in our study had significant hepatitis flares preceding clearance suggesting the development of a vigorous Th1 cytopathic immune response. A similar mechanism may also explain spontaneous clearance of HCV in the context of major surgery, as described in the literature (Samonakis et al., 2005, Yoshikawa et al., 2001).

Clearance of HCV has been described in the literature during pregnancy/parturition (Hattori et al., 2003, Zein et al., 2001). In our study, we observed that 5 patients in the spontaneous clearance group became pregnant

during the last positive and first negative HCV RNA test, 4 of whom went on to have spontaneous vaginal deliveries and 1 of whom had a termination. Normal pregnancy is associated with distinct immunological phases (Mor and Cardenas, 2010). Late pregnancy is an immune-tolerant state and is associated with a Th2 dominant immune response induced by progesterone and adrenal corticosteroids. In contrast, parturition is a pro-inflammatory event associated with an influx of immune cells into the myometrium, and rebound of the Th1 response (Mor and Cardenas, 2010). Spontaneous clearance in this context is postulated to occur as a result of restoration of the immune system and HCV-specific T-cell response (Hattori et al., 2003).

Finally, we present the tentative finding that ongoing IDU is negatively associated with spontaneous clearance of CHC. PWIDs are at risk of superinfection with distinct HCV strains which may negatively impact the likelihood of spontaneous clearance (Blackard and Sherman, 2007, Herring et al., 2004). We also accept the possibility that a high HCV re-infection rate post clearance amongst PWIDs may mask the incidence of spontaneous clearance in this cohort (Micallef et al., 2006, Aitken et al., 2008). However, a recent study based in NHSGGC reported a trend towards a lower incidence of re-infection following spontaneous clearance (McDonald et al., 2012), as described in previous studies (Grebely et al., 2006).

There are a number of limitations to our study as a consequence of its observational and retrospective design. Our study is strengthened by the inclusion of patients presenting and followed up over two decades. Inherent in this however, is considerable variation in the utilisation of different laboratory tests over time, reflecting changing advice from clinical guidelines (EASL, 2011, Palfreeman et al., 2009) and the development and introduction of new technologies. As a consequence of missing data, multivariate analysis was not deemed appropriate and statistical inferences must be interpreted with caution.

We accepted HCV RNA testing on both serum and DBS in the study design to increase our study population. DBS testing may increase the uptake of screening in PWIDs, in whom venepuncture is often difficult and who may be less likely to attend clinic (Bennett et al., 2012, Hickman et al., 2008). However, HCV RNA testing on DBS has reduced sensitivity compared to the serum assay; one patient

in our cohort who was classified as a spontaneous clearer on the basis of DBS HCV RNA testing was found to have detectable HCV RNA on a subsequent serum sample. Additionally, the sensitivity of the serum quantitative HCV RNA assays varied over the course of follow up and earlier samples may have been more likely to be falsely negative. Moreover, fluctuating and low level viraemia is common in the early stages of infection. As we relied on only one negative HCV RNA for the definition of spontaneous clearance, it is possible that we misclassified these patients as spontaneous clearers. However, restricting the analysis to patients with at least 24 months confirmed infection did not change our findings and the normalisation of liver biochemistry provides further support for clearance.

Follow up of patients with presumed late spontaneous clearance was poor; only 60% of spontaneous clearers had follow up HCV RNA testing performed at any time point to confirm clearance. To address this limitation we performed an additional analysis of patients with persistent HCV RNA negativity over time and found that only female gender and low HCV viral load remained significant.

We used age at diagnosis as a surrogate marker for age at infection. Many patients self-identified as having been at risk of exposure to HCV many years before they were first tested and therefore it is likely that we overestimated the true age at infection. Also, for many case patients there was a considerable duration between the last positive and the first negative HCV PCR, making it difficult to ascertain the true date of HCV clearance.

As previously discussed, a detailed review of the medical notes was performed for all potential spontaneous clearers in order to confirm the clinical scenario. We were able therefore to obtain information on intercurrent illnesses, hospitalisations and pregnancies for the case patients. Due to the time constraints of the project, we were unable to obtain the same information for the control cohort. As a consequence, data on intercurrent illnesses and pregnancies in the case patients are presented qualitatively, and firm conclusions cannot be drawn regarding the association between these events and spontaneous clearance of chronic HCV.

Finally, HCV RNA testing rates may be subject to bias. Repeat HCV RNA testing in CHC is only recommended in patients for whom treatment is anticipated (EASL, 2011). Although we allowed testing on DBS to increase our study population, certain patient groups may have been less likely to have been tested, including patients with chaotic lifestyles who are not engaged in care, or patients with contraindications to therapy. However, despite the methodological drawbacks inherent in a retrospective study, the biological plausibility of our results and concordance with the precedent in the literature lead us to believe that our results are sound.

We conclude that spontaneous clearance of CHC is more common in females and patients with a low HCV viral load, and that previously described factors including superinfection with HBV and younger age at infection may play a role. This study reveals novel findings of a negative association with ongoing IDU, and describes a cohort of spontaneous clearance in the context of decompensated liver disease. Further work is required to identify the mechanisms underlying spontaneous clearance of chronic infection. Given that such clearance may occur after a prolonged duration of chronic infection, more regular serum HCV RNA monitoring may be warranted, particularly in females, HBV co-infected individuals, patients with low level viraemia and those with decompensated liver disease.

Excerpts from this chapter have now been published (Bulteel et al., 2016).

4 Circulating miRNA expression in patients with CHC: association with disease stage and biomarkers of ageing

4.1 Introduction

Disease progression in CHC is highly variable, and is dependent on both modifiable and non-modifiable factors (Chapter 1.2.2). At present, patients with more advanced disease are prioritised for antiviral treatment whilst a 'watchful waiting' approach may be taken in individuals with normal liver stiffness and no extra-hepatic complications of HCV infection. Assessment of liver fibrosis (chapter 1.4) is essential for risk stratification and to ensure that individuals with advanced fibrosis are offered appropriate surveillance for oesophageal varices and HCC.

It is recommended that individuals with ≥ F3 fibrosis undergo abdominal imaging +/- serum alpha-fetoprotein (AFP) monitoring at 6 monthly intervals to monitor for hepatoma development ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016, Ryder, 2003). However, studies have shown that in rare cases HCC may develop in HCV-infected patients without cirrhosis (Yeh et al., 2010). Additionally, data are emerging to suggest that delaying treatment results in an increased risk of liver-related death, and reduced treatment effectiveness (McCombs et al., 2014). Therefore, prognostic biomarkers able to identify individuals at high risk of progressing to end-stage liver disease or HCC during HCV infection are urgently required.

BoA are attractive candidates for prognostic biomarkers in CHC as immunosenescence has been associated with accelerated disease progression and poorer treatment outcomes with IFN-based therapy (Hoare et al., 2010b, Manfras et al., 2004). The cyclin dependent kinase inhibitor CDKN2A is a well characterised BoA, PBMC expression of which can be used to infer immunosenescence (chapter 1.6.1). Expression of CDKN2A in the liver tissue of patients with CHC has been previously shown to correlate with necro-inflammation (Bieche et al., 2005), and Robinson and colleagues (2013) found that higher expression of CDKN2A, and the related transcripts ARF and CDKN2B,

was observed in the PBMCs of HCV-infected subjects compared with healthy controls. The authors found that CDKN2A and ARF expression correlated with disease stage, and suggested that CDKN2 locus transcripts could serve as surrogate markers for advanced liver disease. They also hypothesised that expression of these transcripts could be used as prognostic biomarkers for the development of both hepatic and extra-hepatic disease manifestations of CHC (Robinson et al., 2013). However, PBMC separation can be time consuming which restricts their use in clinical practice. Furthermore, the use of RNA biomarkers has proven challenging as mRNA is subject to degradation resulting in alteration of gene expression *ex vivo* (Baechler et al., 2004).

Both membrane vesicle-associated and protein-bound miRNAs are remarkably stable in plasma and cell culture media (Turchinovich and Cho, 2014) compared with mRNA, making them excellent candidate biomarkers. Specific miRNA 'signatures' have been described in liver disease of multiple aetiologies, and they are increasingly being exploited as diagnostic and prognostic biomarkers, as an alternative to measuring differences in telomere length or gene expression (chapter 1.7).

In this chapter, we aimed to gain insights into the pathogenesis of HCV infection by characterising miRNA expression in the peripheral blood of subjects with CHC. The availability of matched serum and PBMC samples provided us with a unique opportunity to explore miRNA regulation at the level of the peripheral immune system as well as document serum miRNA expression. We postulated that we could identify non-invasive biomarkers for hepatic cirrhosis by correlating serum and PBMC miRNA expression with disease stage in HCV-infected subjects. Furthermore, we hypothesised that by correlating miRNA expression with PBMC CDKN2A expression (a biomarker of immunosenescence thought to have negative prognostic implications) we could identify novel prognostic biomarkers for non-cirrhotic HCV-infected individuals at high risk of progression to cirrhosis. As it is thought that miRNA within exosomes may play a role in cell-cell communication, and regulate gene expression in target cells, we also examined the expression of potential biomarkers within exosomes in order to determine whether a role in cell-cell signalling was supported. Finally, we studied the expression of a selected miRNA in a hepatoma cell line in response to TLR3 agonism and IFN-α

stimulation to explore this approach as a possible model for studying the role of miRNAs in the immune response to infection.

4.2 Patient selection

Matched serum and PBMC samples were available from a pre-existing cohort together with relevant clinical and demographic patient data. The Glasgow Outcomes Study cohort consisted of individuals with chronic HCV Gt1 and Gt3 infection (n=100) attending hospitals in Glasgow, together with healthy age and sex matched controls (n=25) who were negative for anti-HCV antibodies (Robinson M, 2013). Exclusion criteria included obesity (BMI> 30kg/m^2), current IDU, chronic alcohol excess, co-infection with HBV or HIV, and the use of lipid lowering medication. Patients with metabolic disorders, including DM 2, were also excluded. No participants were receiving HCV treatment at the time of sample collection.

The study protocol was approved by the NHS Greater Glasgow & Clyde Research Ethics Committee (REC) (REC project number 11/AL/0371; August, 2011). All study participants provided written consent for sample collection and subsequent analysis.

4.2.1 Pilot study cohort

For the pilot study, non-cirrhotic patients (n=16, liver stiffness < 7.1 kPa on TE (Boursier et al., 2012), F0-1 disease) and cirrhotic patients (n=9, liver stiffness > 12.5kPa, F4 disease) who had previously had their CDKN2A mRNA expression levels determined were selected from the pre-existing cohort (Robinson M, 2013). Healthy sex-matched controls (n=8) were also included in the pilot analysis.

As CDKN2A is a biomarker of cellular senescence, non-cirrhotic patients were sub-divided into ‘high biological age (bio-age)’ and ‘low bio-age’ according to the relative expression of PBMC CDKN2A mRNA, i.e. subjects with ≥ 2 fold higher expression of CDKN2A mRNA compared to healthy controls were classified as ‘high bio-age’, and those with < 2 fold change were classified as ‘low bio-age’.

Demographic patient data (age at enrolment, age at infection, sex, ethnicity, alcohol intake, current IDU, source of infection), HCV markers (liver enzymes, HCV genotype, IFNL3 genotype, HCV RNA, liver stiffness, history of cirrhosis), and data on co-morbidities had been previously obtained. Biochemical and haematologic variables were recorded at baseline. Duration of infection was calculated as the interval between age at infection and age at enrolment.

All patients were of white ethnicity. The median age of the cohort was 42 years (IQR 39-49) and the majority were male (n=24; 72.7%). Full baseline characteristics of the included patients are shown in Table S3.

Following the pilot study, promising results for serum biomarkers were validated in a larger patient cohort (chapter 4.2.2 below). The derivation of each patient group from the original Glasgow Outcomes study is shown in figure 4-1.

4.2.2 Expanded patient cohort

The expanded study consisted of a larger cohort of HCV-infected patients (n=51) together with healthy controls (n=10). Of the HCV-infected subjects, around a third had a liver stiffness >12.5kPa consistent with cirrhosis (n=15, 29.4%).

Data on CDKN2A, CDKN2B and ARF PBMC mRNA expression were only available for a subset of HCV-infected subjects (n=40, 78.4%), therefore bio-age could not be estimated and patients were simply grouped according to fibrosis stage, i.e. ‘cirrhotic’ and ‘non-cirrhotic’. Demographic and laboratory features of the patients are shown in Table S4.

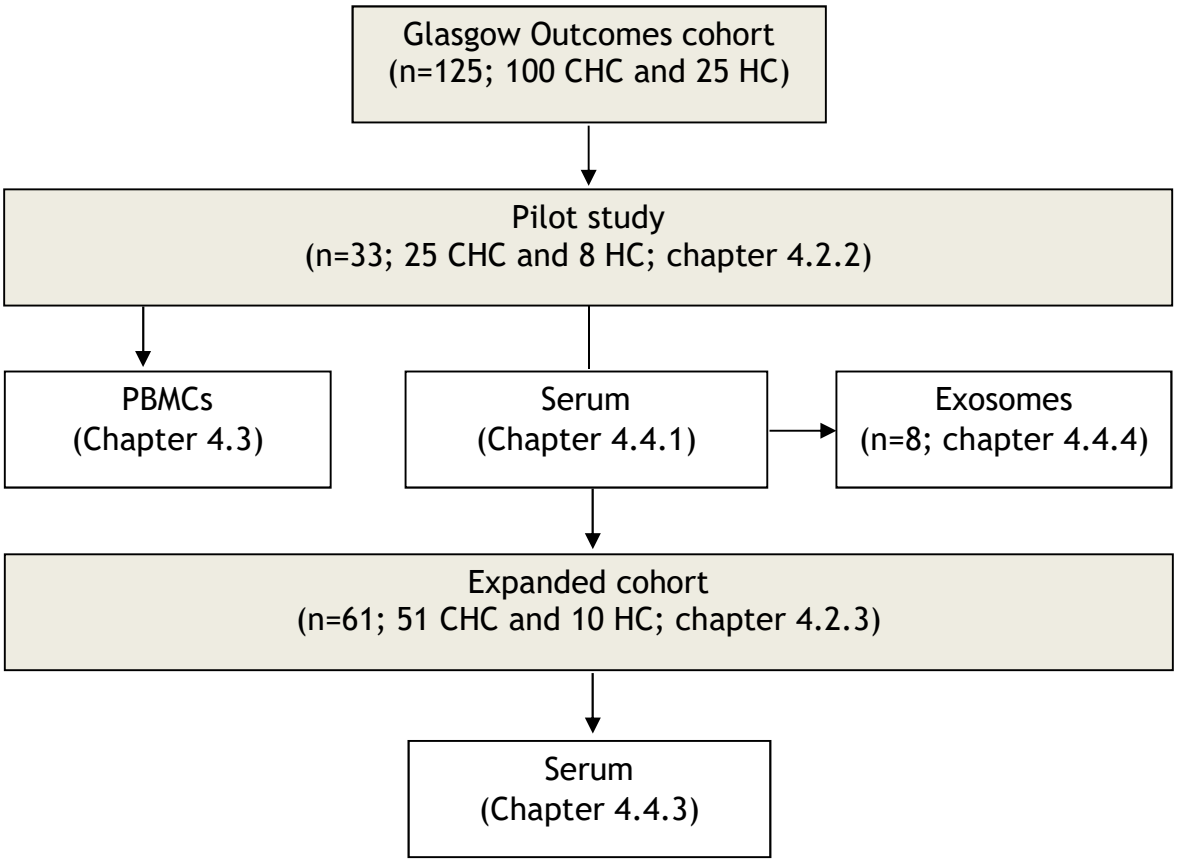


Figure 4-1: Derivation of each patient group for miRNA analysis from the original Glasgow Outcomes cohort. Matched PBMC and serum samples from HCV-infected subjects (n=25) and healthy controls (n=8) were analysed in the first instance. Promising results from the serum pilot study were validated in a larger bank of samples from HCV-infected subjects (n=51) and healthy controls (n=10). Exosomes were extracted from a subset of serum samples (n=8) from the pilot group. CHC, chronic hepatitis C virus infection; HC, healthy controls.

4.3 PBMC miRNA expression in CHC

4.3.1 Effect of PBMC storage method on miRNA expression profiling

It is often impractical to extract RNA from fresh PBMC fractions. PBMCs can be cryopreserved in liquid nitrogen or added to lysis buffer and stored at -70°C prior to RNA extraction. It is not known which storage method yields better results in downstream miRNA analysis.

Pre-separated PBMC samples were available for the Outcomes study cohort (Robinson et al., 2013). The PBMCs had been separated from fresh whole blood over a density gradient before being either suspended in 1 mL freezing medium (per $1x10^7$ cells) and cryopreserved in liquid nitrogen in aliquots, or lysed in 600 µL RLT buffer ($5x10^6$ cells) and stored at -70°C. We extracted RNA from paired PBMC samples stored using each method for 4 HCV-infected individuals. RNA was extracted from PBMCs as described previously (chapter 2.2.1.3).

We compared miRNA expression in PBMCs cryopreserved in liquid nitrogen with miRNAs from those lysed in RLT buffer and stored at -70°C (chapter 2.2.1.3). The average 260/280 ratio for the RNA extracted from cryopreserved PBMCs was 2.035 compared with a ratio of 2.025 for RNA extracted from PBMCs stored in lysis buffer. Gel electrophoresis was performed for RNA extracted from 3 of the cryopreserved samples using the Agilent TapeStation and demonstrated a mean RNA integrity number (RIN) of 7.6 indicating a degree of degradation (figure 2-1). In comparison, the mean RIN was 9.1 for the RNA extracted from PBMCs stored immediately in lysis buffer.

Each sample was run individually on a TaqMan® MicroRNA array card (chapter 2.2.2.1) and between group differences were explored. A total of 442 miRNA transcripts were reliably detected (i.e. expressed in ≥ 2 biological group replicates) in the PBMCs stored in RLT buffer, compared with only 274 transcripts in the cryopreserved PBMCs. Two hundred and sixty-five transcripts were expressed in both groups, and there was good overall correlation between CT values (figure 4-2; r=0.82, 95% CI: 0.78-0.86, p<0.0001).

Generally, miRNA expression levels were lower in cryopreserved PBMCs than in PBMCs stored in RLT buffer; 99 miRNAs were detected at significantly lower levels whereas only 11 were increased in abundance.

Although this analysis demonstrated reasonable overall correlation between miRNAs expressed in PBMCs stored in liquid nitrogen and frozen in RLT buffer, the data do suggest that there is a significant benefit to storing PBMCs immediately in lysis buffer for downstream RNA analysis. However, based on sample availability, further experiments were performed using PBMCs that had been cryopreserved in liquid nitrogen and the above findings should be taken into consideration when interpreting the data in the following section.

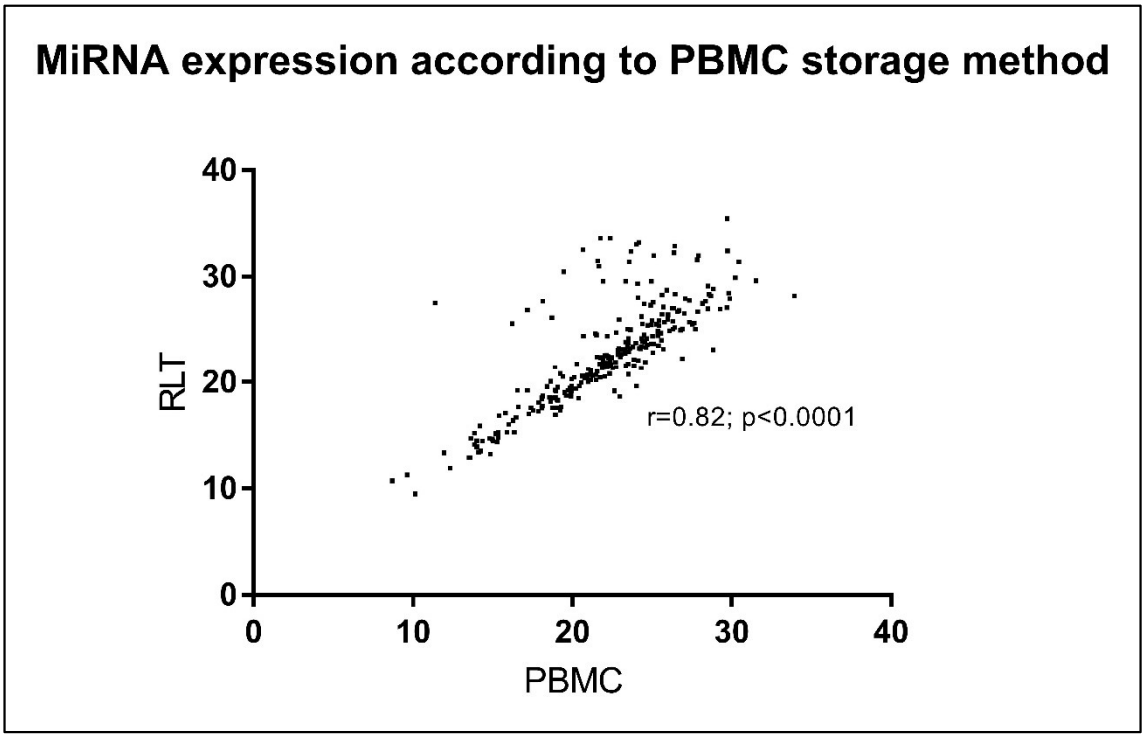


Figure 4-2: Correlation between CT values for miRNAs detected in PBMCs stored in RLT buffer compared with cryopreserved samples.

4.3.2 PBMC miRNA profile in CHC

The immune response to HCV infection is known to play an important role in disease progression in CHC (chapter 1.2) and PBMC miRNA expression has been shown to correlate with disease stage and treatment outcome (Hsi et al., 2014). It was therefore of interest to interrogate miRNA expression in the PBMCs of HCV-infected individuals within our cohort.

We examined global miRNA expression in the PBMCs of 25 HCV-infected individuals together with 8 healthy controls. We detected 450 of the 754 miRNAs examined in the PBMCs of HCV-infected subjects (present in ≥ 2 samples), compared with 353 in the healthy controls. Of the 242 miRNAs which could be compared across both groups, 26 demonstrated greater than two-fold change with respect to healthy controls; 14 miRNAs were significantly upregulated and 12 miRNAs were significantly downregulated (figure 4-3, table 4-1). We also found that miR-720 was significantly upregulated, however subsequent studies have demonstrated that the sequence annotated as miR-720 is likely to be a fragment of a tRNA, and it has been removed from the miRBase database (Schopman et al., 2010). It was therefore excluded from downstream analyses.

Table 4-1: MiRNA expression in the PBMCs of HCV-infected individuals compared with healthy controls

	MiRNA	Fold change	P value
Increased in CHC	miR-95-3p	2.095	0.011
	miR-500a-5p	2.117	0.037
	miR-140-3p	2.14	0.011
	miR-629-3p	2.191	0.01
	miR-1248	2.227	0.027
	miR-138-5p	2.271	0.034
	miR-629-5p	2.294	0.0001
	miR-589-3p	2.354	0.038
	miR-590-3p	2.404	0.023
	miR-16-5p	2.554	0.001
	miR-532-5p	2.698	0.009
	miR-146b-3p	2.708	0.002
	miR-193a-5p	2.774	0.001
	miR-1244	2.847	0.01
Decreased in CHC	miR-136-3p	0.072	0.0001
	miR-656-3p	0.184	0.001
	miR-323-3p	0.197	0.002
	miR-374a-3p	0.242	0.006
	miR-26a-1-3p	0.263	0.008
	miR-374b-3p	0.306	0.0001
	miR-410-3p	0.336	0.01
	miR-597-5p	0.334	0.049
	miR-151a-5p	0.346	0.028
	miR-376a-3p	0.349	0.012
	miR-744-3p	0.39	0.047
	miR-411-5p	0.415	0.037

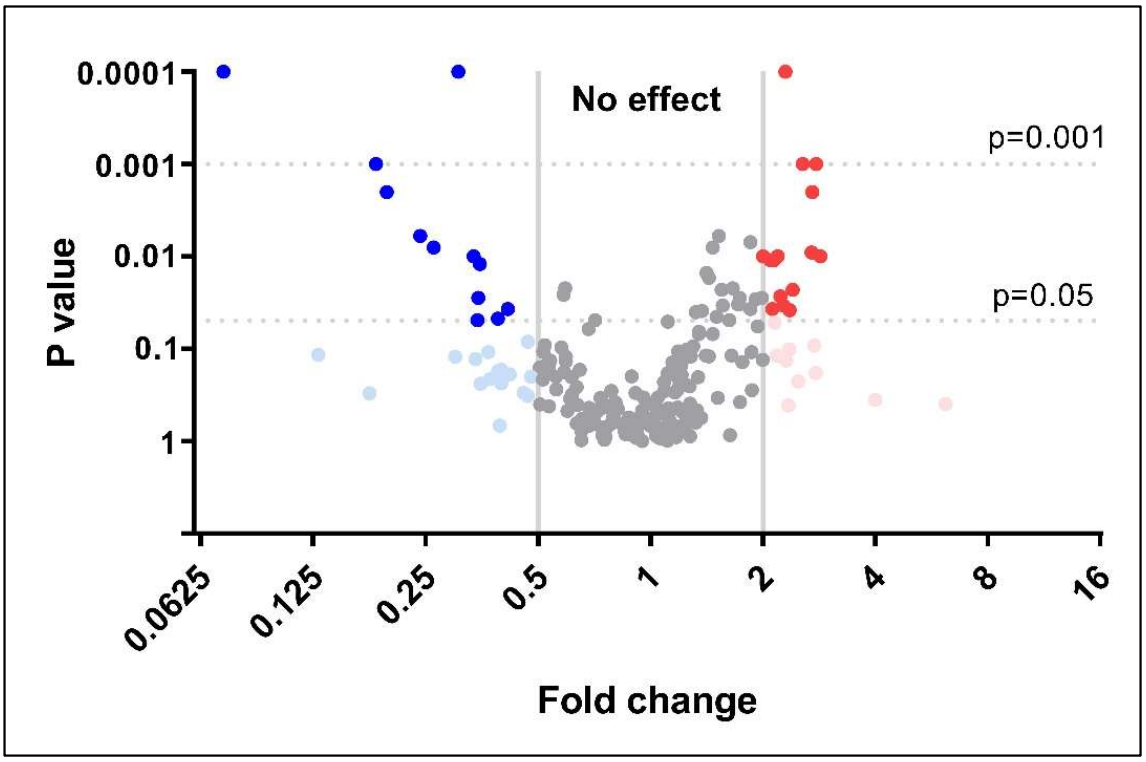


Figure 4-3: Volcano plot demonstrating p value against fold change for miRNAs deregulated in the PBMCs of subjects with CHC compared with healthy controls. Data points in red indicate miRNA upregulated compared with healthy controls and those in blue indicate downregulation. Fold change > 2 and p value < 0.05 were considered significant.

Pathway analysis was performed *in silico* using DIANA-miRPath for those miRNA significantly deregulated in the PBMCs of patients with CHC (figure 4-4). We found that cancer pathways were highly represented; microRNAs in cancer, viral carcinogenesis and proteoglycans in cancer were the most significantly enriched pathways. These data suggest an important role for miRNAs in regulating oncogenesis in CHC.

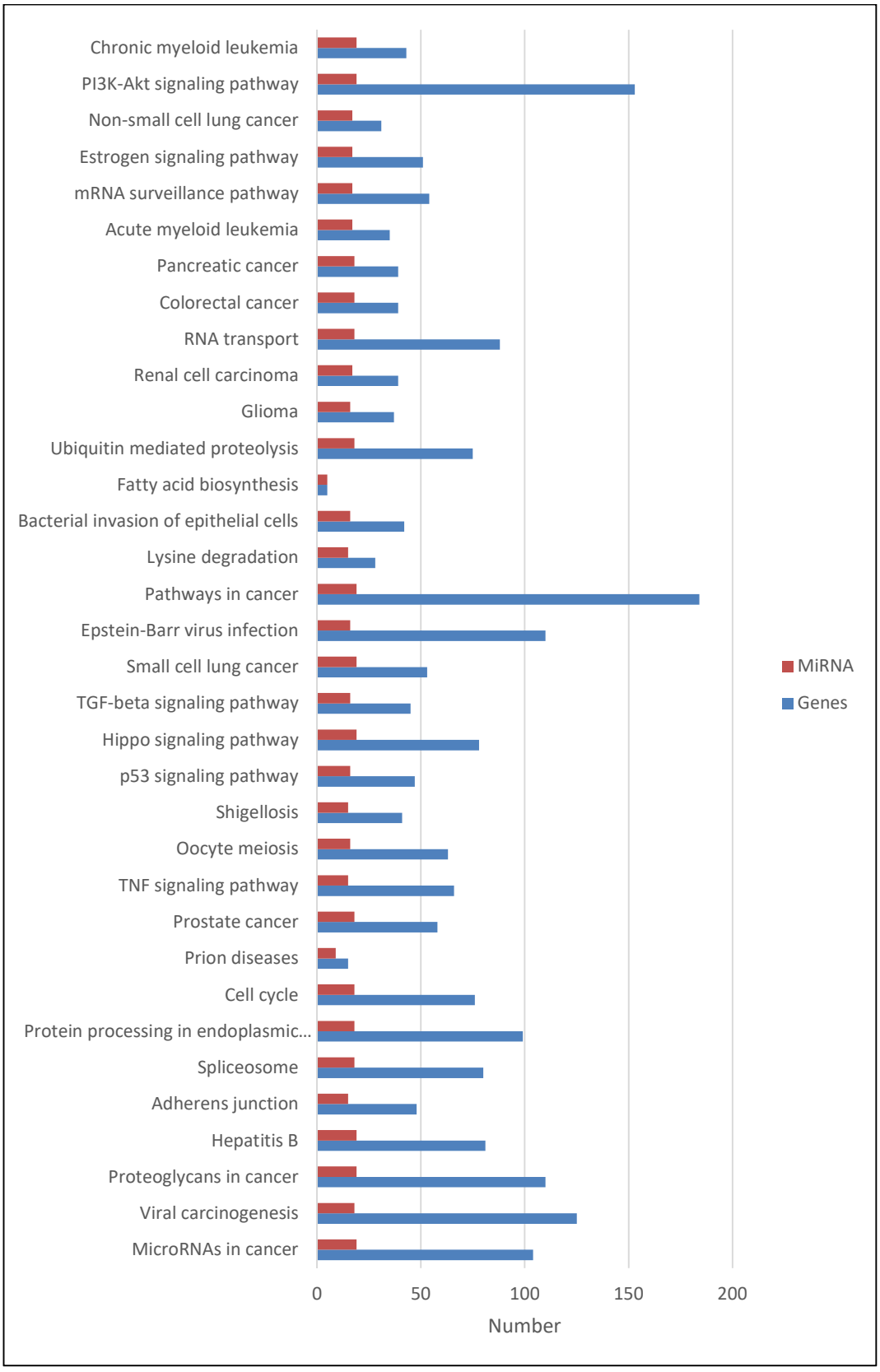


Figure 4-4: *In silico* pathway analysis using DIANA-miRPath for miRNAs significantly deregulated in the PBMCs of HCV-infected subjects. Red bars indicate number of miRNA involved in each pathway and blue bars indicate the number of genes. The y axis demonstrates significantly enriched KEGG pathways sorted according to a descending enrichment statistical score (-ln *P*).

4.3.3 Intergenotypic differences in PBMC miRNA expression

The effect of viral genotype on disease progression in CHC is unclear and data are conflicting (chapter 1.3.2.1). Some cohort studies have suggested that Gt1 infection is associated with increased disease severity and likelihood of viral persistence (Franchini et al., 2001, Yee et al., 2000). Conversely, an increased likelihood of clearance in both the acute and chronic stage has been described in Gt1 infection, although we were unable to demonstrate an association between HCV genotype and late spontaneous clearance in our Scottish cohort (Chapter 3). Gt3 infections are associated with a higher incidence of steatosis which may be associated with disease progression and increased incidence of HCC (Rubbia-Brandt et al., 2000). We hypothesised that profiling PBMC miRNA expression in Gt1 and Gt3 infection would provide important insights into the phenotypic differences between viral genotypes.

We detected 424 miRNA transcripts in ≥ 2 PBMC samples of Gt1-infected subjects (n=13) compared with 343 miRNA transcripts in Gt3 infection (n=12). We examined miRNA expression between Gt1- and Gt3-infected individuals and found that of the 173 miRNAs which could be directly compared, 49 miRNA were significantly upregulated in the PBMCs of Gt1- vs. Gt3-infected subjects. The full comparison is shown in Table S6. Only 8 miRNA were upregulated in HCV Gt3 infection compared to Gt1 infection (figure 4-5; table 4-2).

Table 4-2: Intergenotypic differences in PBMC miRNA expression

	MiRNA	Fold change	P value
Increased in Gt1 infection	miR-532-5p	29.41	0.0001
	miR-671-3p	5.43	0.0001
	miR-193b-3p	4.90	0.0001
	miR-193a-5p	3.61	0.0001
	miR-148a-3p	3.57	0.0001
	miR-324-3p	3.44	0.0001
	miR-423-5p	3.30	0.0001
	miR-328-3p	3.07	0.0001
	miR-502-3p	2.76	0.0001
	miR-744-5p	2.51	0.0001
	miR-30b-5p	2.39	0.0001
	miR-324-5p	2.08	0.0001
	miR-25-3p	2.03	0.0001
	miR-142-3p	6.33	0.001
	miR-296-5p	3.66	0.001
	miR-652-3p	2.79	0.001
	miR-106b-5p	2.16	0.001
	miR-451a	6.10	0.002
	miR-320a	3.53	0.002
	miR-331-3p	2.17	0.002
	miR-92a-3p	2.16	0.002
	miR-589-5p	2.06	0.002
	miR-629-5p	2.00	0.002
	miR-424-5p	3.95	0.003
	miR-221-3p	2.16	0.003
	miR-642a-5p	2.07	0.003
	miR-345-5p	2.61	0.004
	miR-484	2.07	0.005
	miR-19b-3p	2.27	0.007
	miR-142-5p	4.03	0.008
	let-7c-5p	2.68	0.008
	miR-374b-5p	4.31	0.009

	miR-375	3.09	0.011
	miR-148b-3p	2.54	0.011
	miR-21-5p	2.33	0.011
	miR-597-5p	2.72	0.012
	miR-574-3p	2.67	0.012
	miR-362-3p	4.42	0.013
	miR-181a-5p	2.26	0.013
	miR-150-5p	2.18	0.015
	miR-28-3p	8.40	0.016
	miR-101-3p	2.04	0.016
	miR-330-3p	2.03	0.019
	miR-339-3p	2.39	0.025
	miR-29a-3p	2.35	0.026
	miR-369-5p	5.95	0.028
	miR-140-5p	2.01	0.031
	miR-10a-5p	3.73	0.044
	miR-222-3p	2.24	0.048
Increased in Gt3 infection	miR-190a-5p	4.35	0.0001
	miR-195-5p	4.95	0.0001
	miR-548c-3p	5.65	0.001
	miR-483-5p	11.23	0.004
	miR-494-3p	3.175	0.005
	miR-422a	2.76	0.01
	miR-26b-5p	2.50	0.041
	miR-1-3p	3.64	0.047

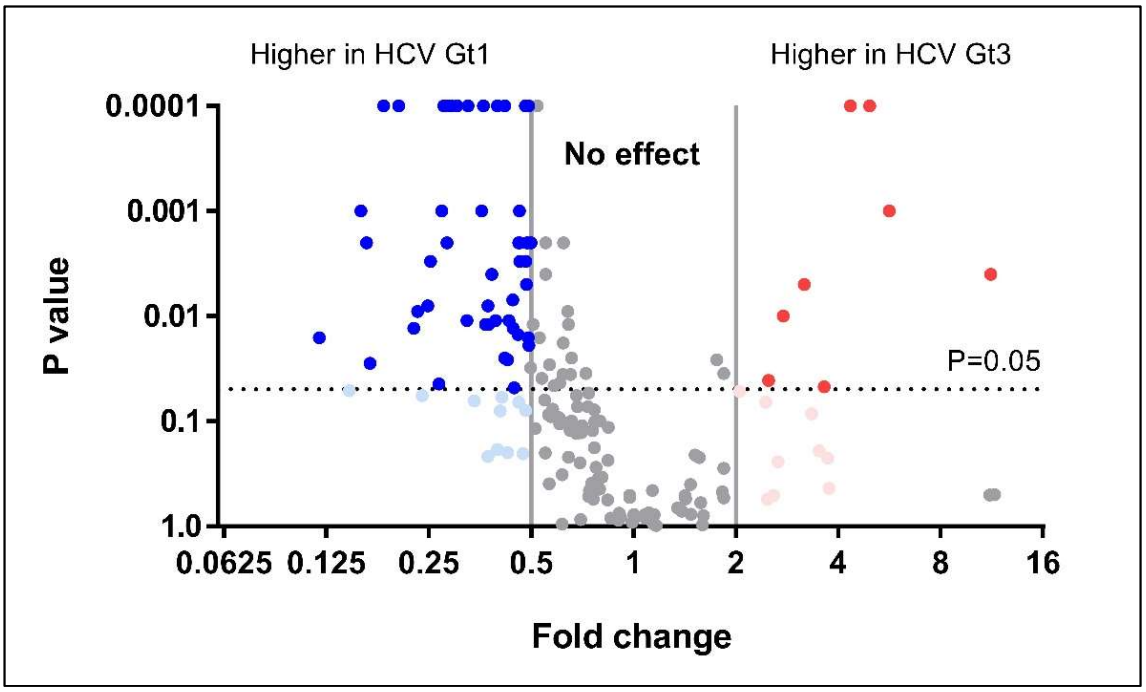


Figure 4-5: Volcano plot demonstrating p value against fold change for miRNAs deregulated in the PBMCs of Gt1- compared with Gt3-infected subjects. Data points in red indicate miRNAs upregulated in Gt3 infection compared with Gt1 infection, and those in blue indicate downregulation. Fold change > 2 and p value < 0.05 were considered significant.

We next compared the PBMC miRNA expression in each genotype to miRNA expression in the PBMCs of healthy controls. We found that 50 miRNAs were significantly upregulated in the PBMCs of HCV Gt1-infected subjects compared to healthy controls, and 27 were significantly downregulated. In Gt3-infected individuals only 8 miRNAs were significantly upregulated whilst 13 were significantly downregulated.

Eight miRNAs were significantly deregulated in both genotypes, miR-597-5p, miR-376c-3p, miR-151-5p, miR-376a-3p, miR-126-5p, miR-411-5p, miR-138-5p and miR-16-5p. All were downregulated apart from miR-138-5p and miR-16-5p which demonstrated > 2 fold upregulation compared to healthy controls.

It is difficult to interpret the difference in miRNA expression between genotypes given the lower total number of miRNAs detected overall in the PBMCs of Gt3- compared with Gt1-infected subjects. However, the number of deregulated miRNAs in the PBMCs of Gt3-infected subjects does appear to be fewer, even accounting for the difference in global miRNA expression.

Based on biological relevance (chapter 1.7.3), we selected miR-21-5p, miR-195-5p and miR-221-3p for validation with quantitative RT-PCR. We found that miRNA expression was highly variable between individual samples, particularly for miR-21-5p, and we were unable to reproduce the differences in miRNA expression between genotypes seen in the miRNA array. We identified considerable individual biological variance in PBMC miRNA expression.

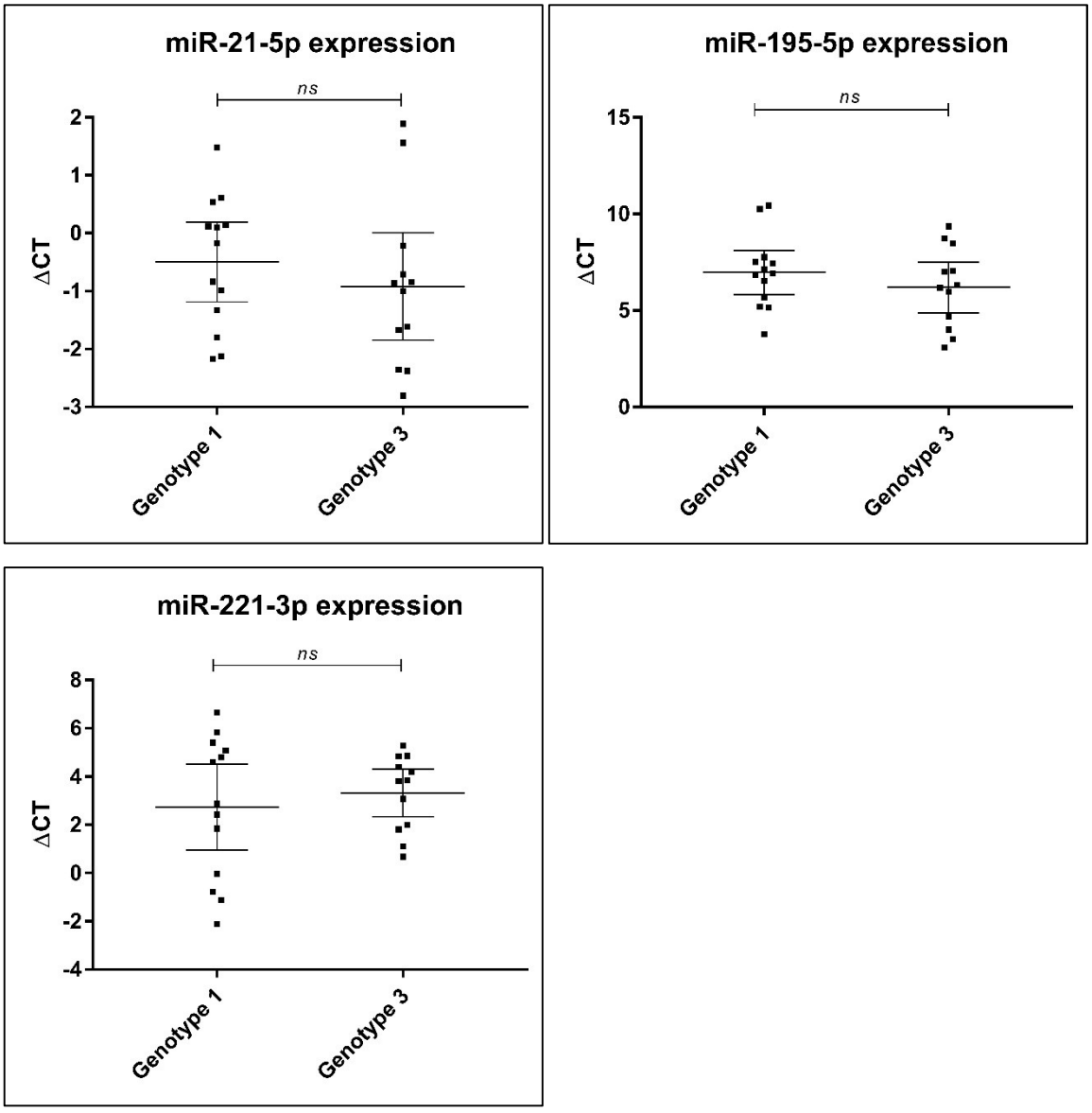


Figure 4-6: PBMC expression of miR-21-5p, miR-195-5p and miR-221-3p is not significantly different between Gt1 and Gt3 HCV-infected patients. Dot plot graphs depict mean/median expression +/- 95% CI; ns=non-significant.

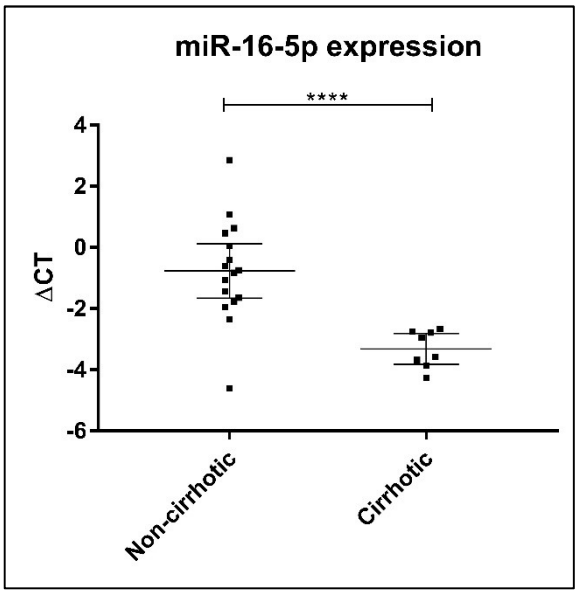
There were no significant differences in miR-21-5p, miR-195-5p or miR-221-3p expression between genotypes (figure 4-6). This highlights an issue with interpretation of the TaqMan® MicroRNA Array data – as fold changes are calculated based on the average ΔCT value for biological replicates, transcripts are only required to be present in ≥ 2 samples to be included. We have observed significant variance in miRNA expression between biological replicates. The small group size combined with high inter-individual variability in miRNA expression may explain why we are unable to reproduce the differences in miRNA expression observed between groups in the TaqMan® MicroRNA Array data, and highlights the importance of validating preliminary findings using individual assays.

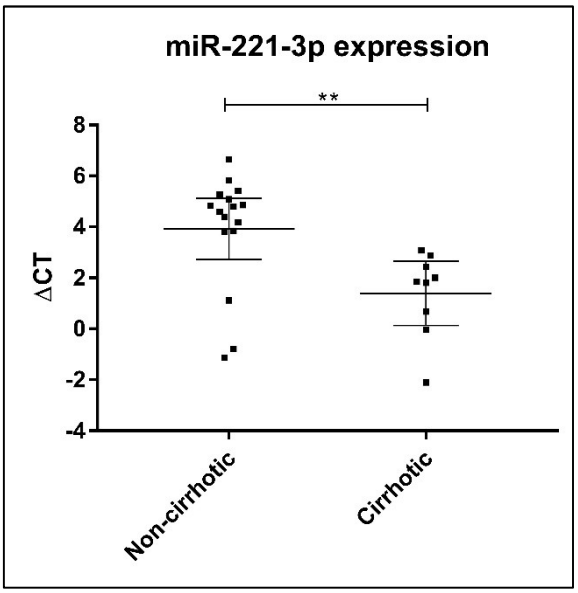
4.3.4 Correlation between PBMC miRNA expression and disease stage in CHC

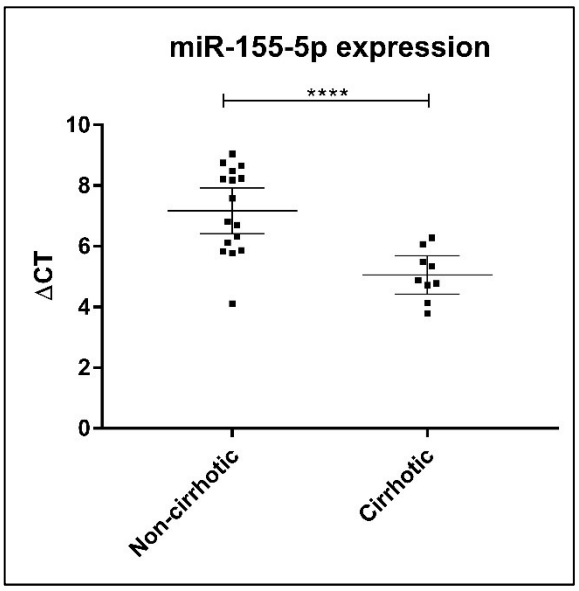
We hypothesised that we could identify non-invasive biomarkers for hepatic cirrhosis by correlating PBMC miRNA expression with disease stage. In our initial miRNA screen, we found that the well-characterised biomarker miR-16-5p (chapter 1.7.3) was significantly upregulated in the PBMCs of HCV-infected subjects compared to healthy controls (table 4-1 & table S5). We therefore selected this miRNA for validation with quantitative RT-PCR. We also examined a panel of related miRNAs present in the PBMCs of HCV-infected individuals which were known to be associated with inflamm-aging, as well as additional miRNAs that we had identified during our preliminary serum screen (chapter 4.4.3).

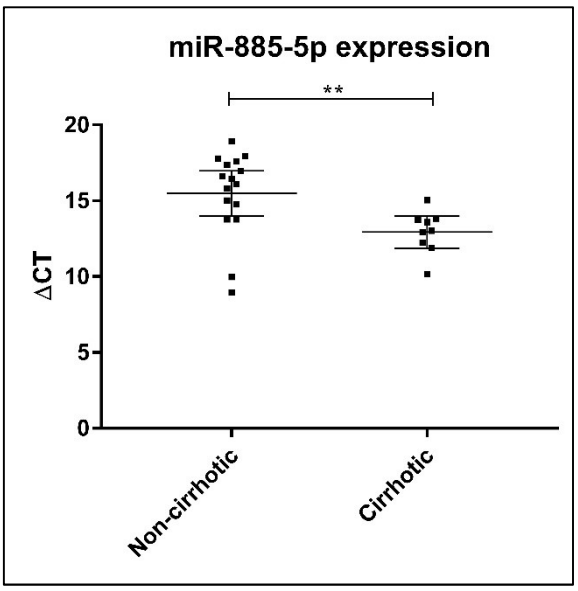
Between-group differences are shown in Table S7. There were no significant differences in selected miRNA expression in the PBMCs of non-cirrhotic individuals classified as 'high bio-age' and 'low bio-age' (chapter 4.2.2) and the data are therefore grouped together under 'non-cirrhotic' (n=16). Cirrhotic patients (n=9) were significantly older at study enrolment than HCV-infected non-cirrhotic patients (49 v 40.5 years, p=0.012) and had a higher BMI (27.4 v 24.6 kg/m^2, p=0.027). There were no differences between groups in terms of gender, age at infection or duration of infection. As published previously, individuals with HCV-related cirrhosis have elevated PBMC CDKN2A mRNA expression (Robinson et al., 2013).

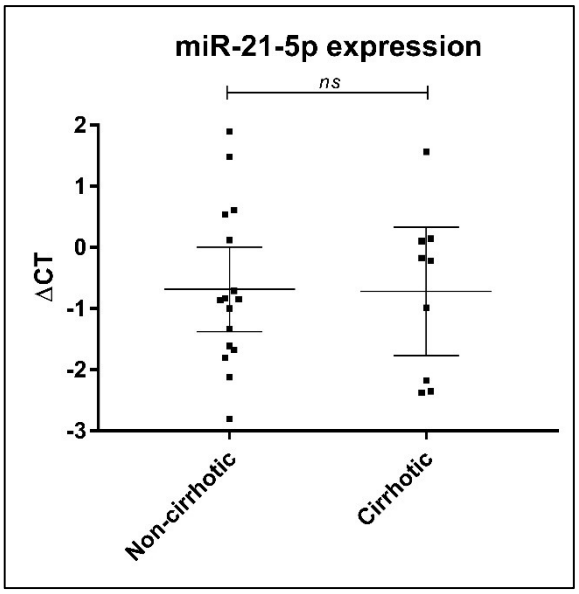
We found that miR-16-5p (p=0.001), miR-155-5p (p=0.002), miR-885-5p (p=0.002) and miR-221-3p (p=0.001) were significantly upregulated in the PBMCs of patients with cirrhosis compared with non-cirrhotic HCV-infected patients (figure 4-7). There were no significant differences in PBMC miR-21-5p and miR-195-5p expression between cirrhotic and non-cirrhotic patients.


miR-16-5p expression
****
ΔCT
4
2
0
-2
-4
-6
Non-cirrhotic
Cirrhotic


miR-221-3p expression
**
ΔCT
8
6
4
2
0
-2
-4
Non-cirrhotic
Cirrhotic


miR-155-5p expression
****
ΔCT
10
8
6
4
2
0
Non-cirrhotic
Cirrhotic


miR-885-5p expression
**
ΔCT
20
15
10
5
0
Non-cirrhotic
Cirrhotic


miR-21-5p expression
ns
ΔCT
2
1
0
-1
-2
-3
Non-cirrhotic
Cirrhotic

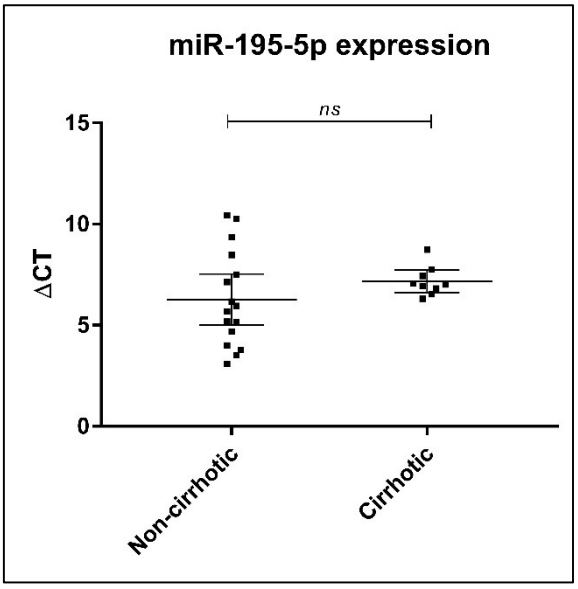

miR-195-5p expression
ns
ΔCT
15
10
5
0
Non-cirrhotic
Cirrhotic

Figure 4-7: Expression of miR-16-5p, miR-221-3p, miR-155-5p, miR-885-5p, miR-21-5p and miR-195-5p in the PBMCs of HCV-infected patients with and without cirrhosis. Dot plot graphs depict mean/median expression +/- 95% CI; **p<0.01, ****p<0.0001, ns=non-significant.

We found significant correlations between CDKN2A mRNA expression and the expression of miR-16-5p (r=-0.652, R^2=0.425, p<0.001), miR-221-3p (r=-0.561, R^2=0.315, p=0.004), miR-155-5p (r=-0.643, R^2=0.414, p<0.001), miR-885-5p (r=-0.684, R^2=0.468, p<0.001) and BMI (r=0.486, R^2=0.237, p=0.016) (figure 4-8).

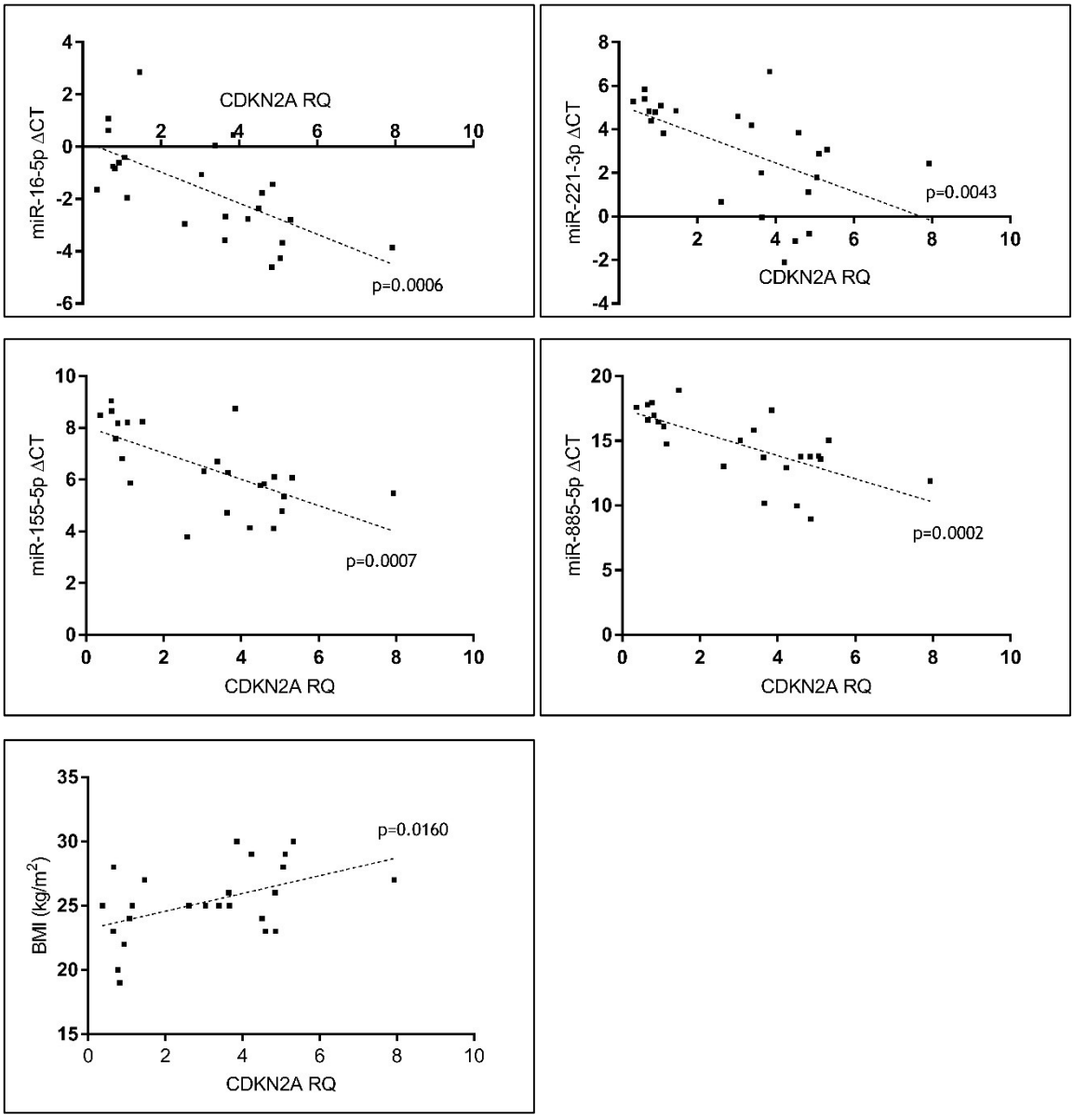


Figure 4-8: CDKN2A expression is positively correlated with expression of miR-16-5p, miR-221-3p, miR-155-5p and miR-885-5p, and BMI (kg/m2). Panels show XY correlation plots with linear regression curve fitted.

4.3.5 Functional analysis of selected miRNAs *in silico*

We analysed protein-protein interactions for genes targeted by the miRNA significantly upregulated in the PBMCs of cirrhotic HCV-infected subjects to gain insights into potential *in vivo* functions. The 4 miRNAs which were significantly differentially expressed in the PBMCs of cirrhotics vs. non-cirrhotics (miR-16-5p, miR-155-5p, miR-221-3p and miR-885-5p) are well described in the literature.

Using miRTarbase 2016 (Chou et al., 2016) and TarBase v7.0 (as described in chapter 2.3) we identified 37 experimentally validated target genes for miR-16-5p (figure 4-9). Predicted protein-protein interaction analysis (from STRING v10.5) suggested that cell cycle regulation and protein phosphorylation are the most significantly enriched biological processes. The most significantly enriched KEGG pathway was the PI3K/AKT pathway. Pathways in cancer and the p53 signalling pathway were also significantly enriched. AKT3, CCND1, CDK4, CDK6, FGFR1, IGF1R and TP53 are involved in both the p53 signalling pathway and the PI3K/AKT pathway. The PI3K/AKT pathway is an important senescence associated pathway, and *in vitro* studies suggest that it is directly regulated by HCV core protein (Tacke et al., 2011). Target proteins and first shell interactors involved in the PI3K/AKT pathway are highlighted in red in the following illustration (figure 4-9).

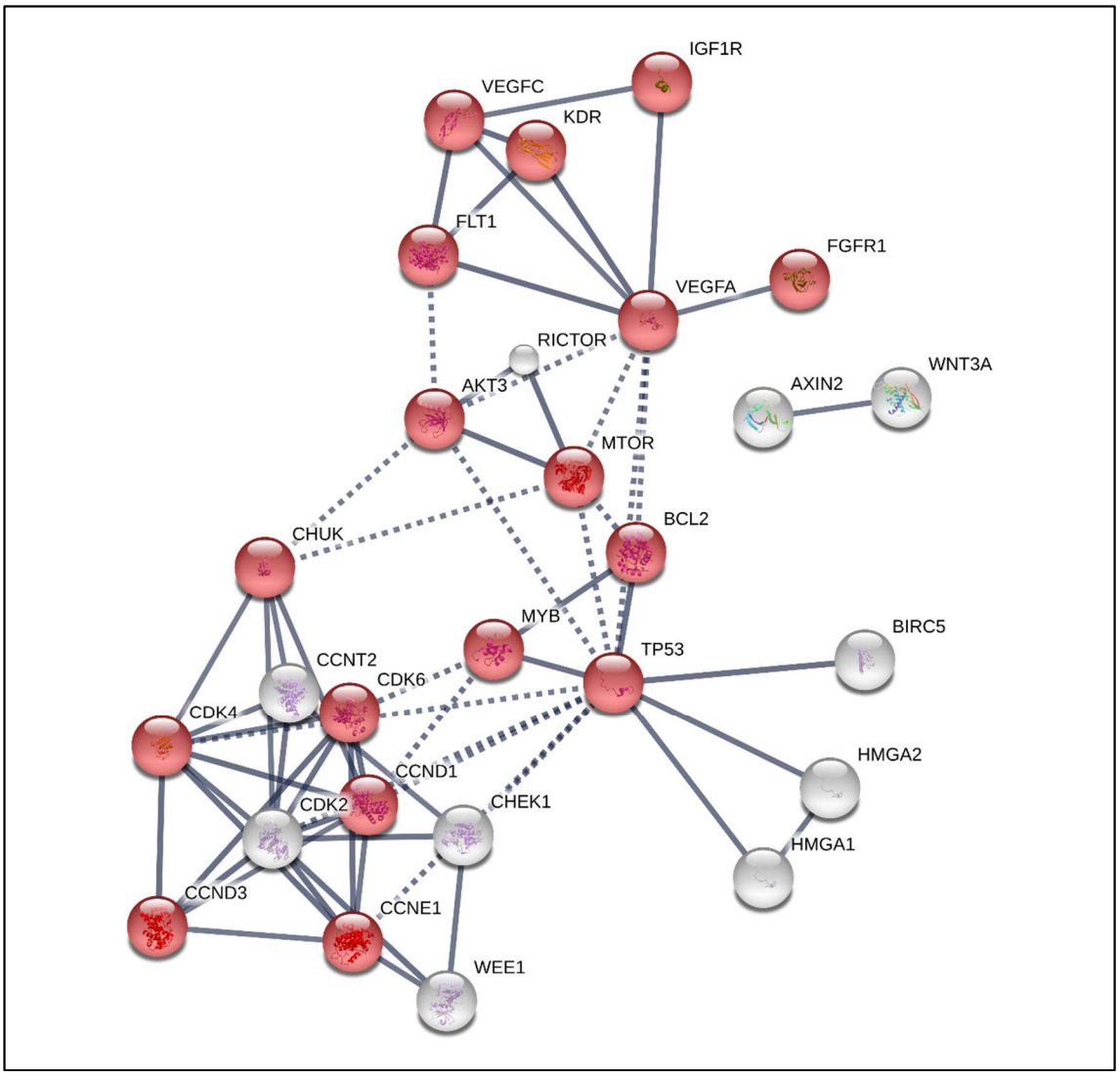


Figure 4-9: Predicted protein-protein interactions for miR-16-5p target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Genes involved in the PI3K/AKT pathway are highlighted in red.

We next explored potential functions of miR-221-3p. MiR-221-3p has 43 validated targets, including phosphatase and tensin homolog (PTEN) and TP53 (figure 4-10). The most significantly enriched biological process is negative regulation of cellular processes. As with miR-16-5p, pathways in cancer are significantly enriched; the 5 most significantly enriched KEGG pathways are microRNAs in cancer, pathways in cancer, hepatitis B, Epstein-Barr virus infection and cell cycle.

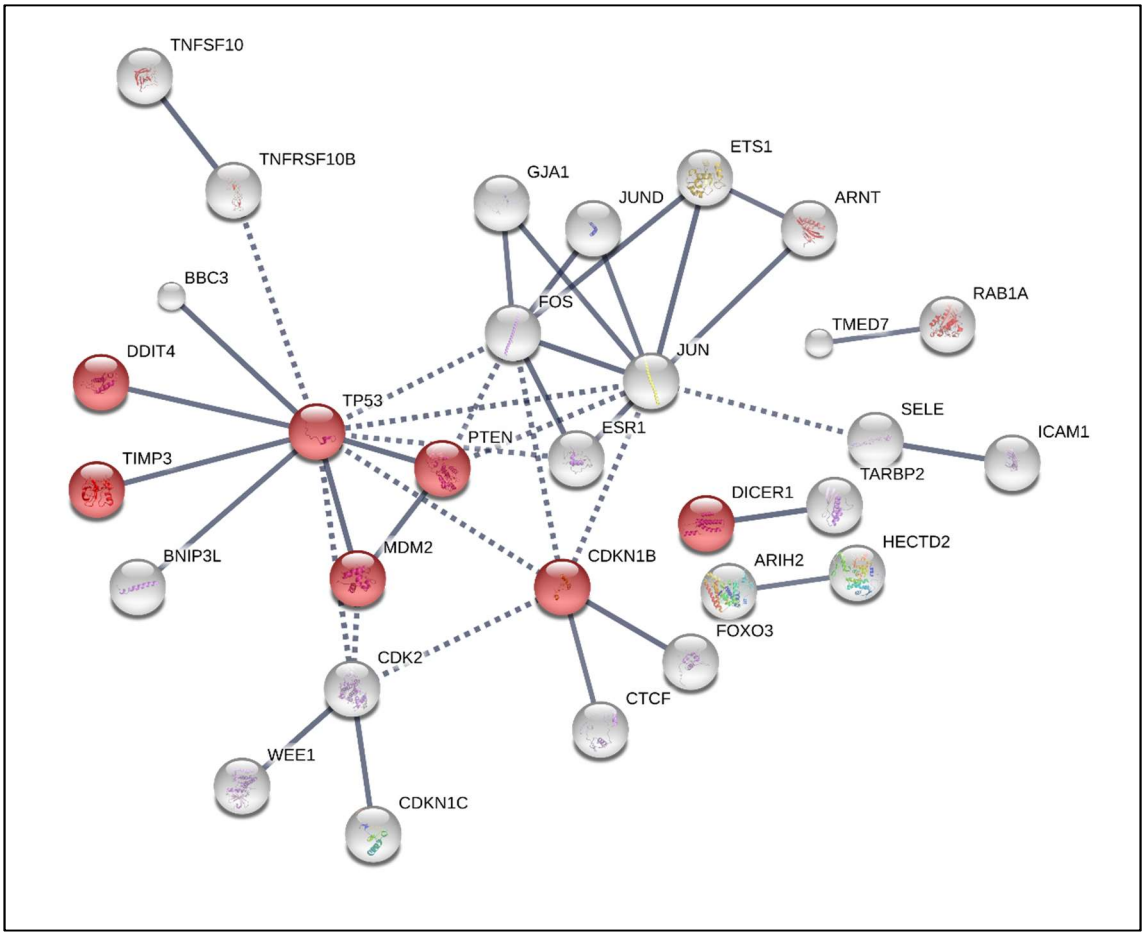


Figure 4-10: Predicted protein-protein interactions for miR-221-3p target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Proteins involved in microRNAs in cancer are highlighted in red.

For miR-155-5p, we identified 60 experimentally validated target genes (figure 4-11). Functional analysis of protein – protein interactions showed that positive regulation of cellular metabolic process was the most significantly enriched biological process. The 5 most significantly enriched KEGG pathways were human T-cell lymphotropic virus (HTLV) type 1 infection, non-small cell lung cancer, cell cycle, PI3K/AKT signalling pathway and the NF-κB signalling pathway. As previously, proteins involved in the most significantly enriched KEGG pathway are highlighted in red.

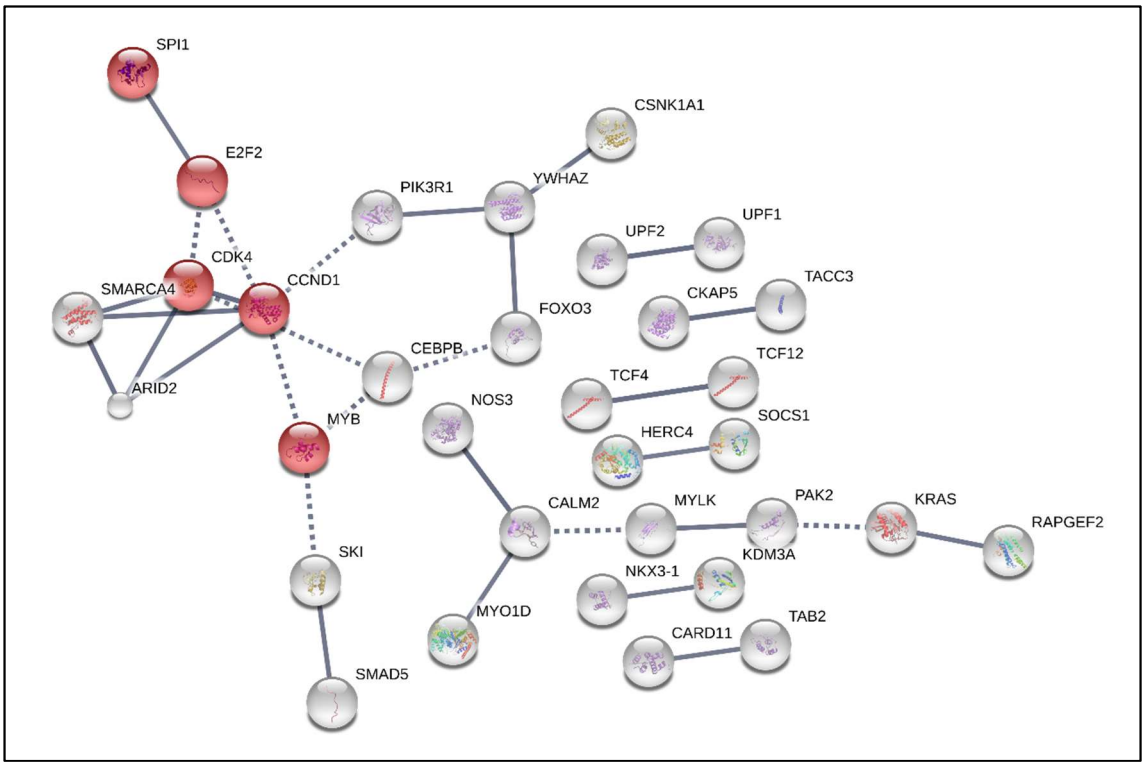


Figure 4-11: Predicted protein-protein interactions for miR-155 target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Proteins involved in the HTLV infection pathway are highlighted in red.

Only 3 validated targets for miR-885-5p were identified: CDK2, MCM5 and CASP3. Predicted protein-protein interactions with the identified nodes and first shell interactors are shown in figure 4-12. DNA replication and cell cycle were the most significantly enriched KEGG pathways, and DNA helicase activity was the most significantly enriched biological process.

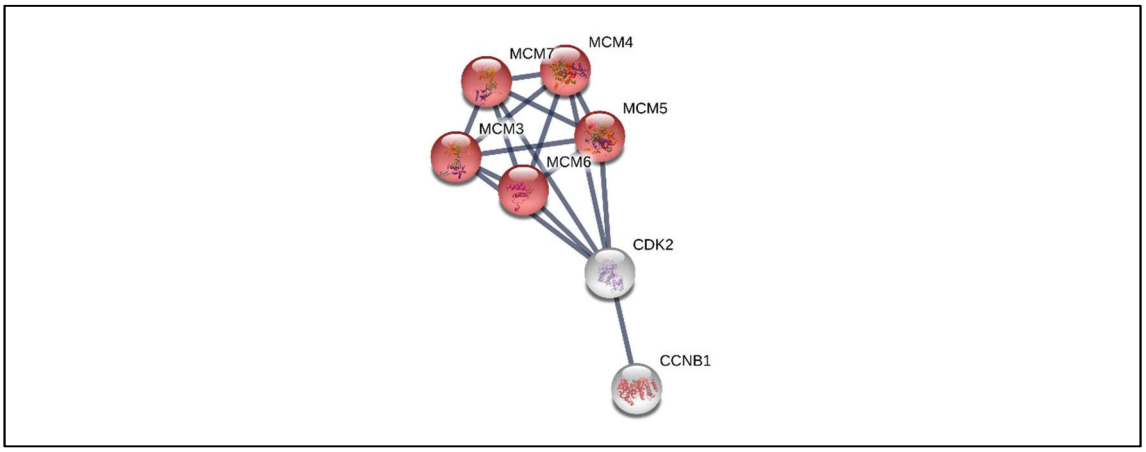


Figure 4-12: Predicted protein-protein interactions for miR-885-5p target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Proteins involved in DNA replication are highlighted in red.

4.3.6 Discussion

In this pilot study, we performed PBMC miRNA expression profiling on a cohort of chronically HCV-infected subjects, together with healthy controls. This has potentially allowed us to gain insights into the pathogenesis of CHC at the level of the peripheral immune system.

The risk of developing HCC is increased 15 to 17-fold in HCV-infected patients compared to uninfected individuals, and the risk of developing HCC in a patient with cirrhosis secondary to HCV is around 2-6% per year (El-Serag et al., 2009). The major determinant of the risk of HCC development is stage of liver disease, although a variety of modifiable and non-modifiable factors may contribute to disease progression (chapter 1.3.2). CHC is also associated with the development of haematological malignancy, particularly non-Hodgkin's lymphoma, in addition to solid organ tumours and skin cancers (Mahale et al., 2017).

The data show that miRNAs deregulated in the peripheral immune cells of patients with CHC are highly represented in cancer pathways; 19 of the 26 deregulated miRNAs are involved in various cancer pathways, with roles in angiogenesis, proliferation and evasion of apoptosis. Understanding the mechanisms underlying cancer development in HCV infection is vital for the development of targeted anti-cancer therapies, as well as guiding the monitoring of patients for early stage cancers.

We found that miR-16-5p, miR-221-3p, miR-155-5p and miR-885-5p were significantly upregulated in the PBMCs of HCV-infected subjects with cirrhosis compared to non-cirrhotic patients. We performed *in silico* analysis of predicted protein-protein interactions for target genes of each miRNA, and found that all 4 miRNAs targeted genes implicated in the cell cycle and cancer pathways. Upregulation of miR-155 in intra-hepatic immune cells (KCs) is associated with an activated state and the release of TNF, promoting inflammation (Szabo and Bala, 2013). Alteration of the miRNAs in the peripheral immune cells of HCV-infected individuals may mirror changes in the intrahepatic immune cells, reflecting an inflammatory, pro-fibrotic cellular environment secondary to CHC.

MiR-155-5p is a well characterised onco-miR which is known to be increased in the liver tissue, serum and monocytes of HCV-infected patients compared with

healthy controls (Bala et al., 2012). HCV infection has been shown to upregulate miR-155-5p expression in hepatocytes, monocytes and KCs *in vitro*, and this elevated expression is thought to be mediated indirectly through enhanced NF-κB transcription (Zhang et al., 2012, Bala et al., 2012). MiR-155-5p targets the tumour suppressor gene APC, responsible for inactivating Wnt/B-catenin signalling, promoting cellular proliferation and inhibiting apoptosis. Zhang and colleagues (2012) concluded that the HCV/NF-κB/miR-155/Wnt axis is an important pathway linking hepatic inflammation and tumorigenesis. Interestingly, higher miR-155 expression in the liver tissue is associated with SVR and the favourable IFNL3-CC genotype, and negatively correlates with ISG15 expression. Additionally, transfection of KCs *in vitro* with a miR-155 mimic was shown to abrogate the immunomodulatory effect of IL-10 and TGF-B on TLR-3 signalling (Jiang et al., 2014). It appears that miR-155-5p may have important pro-inflammatory and antiviral activities in CHC.

Overexpression of miR-885-5p was associated with downregulation of CDK2 and mini-chromosome maintenance protein (MCM5) in a neuroblastoma cell line (Afanasyeva et al., 2011), and it is thought to have tumour suppressor functions in a number of malignancies. MiR-885-5p may also modulate the Wnt/B-catenin signalling pathway; miR-885-5p overexpression results in inhibition of Wnt/B-catenin signalling, suppressing metastasis of malignant cells (Zhang et al., 2016). Functional analysis of predicted protein-protein interactions suggests that miR-885-5p functions as a regulator of the cell cycle and DNA replication, supporting a role in regulation of oncogenesis.

MiR-221-3p is upregulated by HCV infection in an NF-κB dependent manner *in vitro* (Ding et al., 2015). It is elevated in the serum of HCV-infected subjects and correlates with serum aminotransferase levels and miR-122-5p expression. MiR-221-3p is a well described onco-miR, and is highly upregulated in HCC compared to normal liver tissue (Pineau et al., 2010). It has been shown to target the CKI p27 and may also downregulate DNA damage inducible transcript 4 (DDIT4), a modulator of the mammalian target of rapamycin (mTOR) pathway, promoting tumorigenesis. MiR-221 overexpression in myeloid dendritic cells is associated with downregulation of pro-apoptotic ISGs and SOCS1, promoting cell survival (Sehgal et al., 2015). Interestingly, IFN-α has been shown to downregulate miR-

221 in dendritic cells via inhibition of STAT3, with consequent antiproliferative and immunomodulatory effects (Sehgal et al., 2015).

MiR-16-5p functions as a tumour suppressor (chapter 1.7.3) (El-Abd et al., 2015). It binds to both BCL2 (Cimmino et al., 2005) and VEGF (Hua et al., 2006), resulting in apoptosis and inhibition of angiogenesis. Overexpression *in vitro* is associated with inhibition of PI3K and AKT phosphorylation, resulting in suppression of proliferation and metastasis of HepG2 cells, which are derived from HCC (Wu et al., 2015). Elevated miR-16-5p has been described in various tissues along with ageing-associated disease (Li et al., 2015), and reflects the development of senescence as an important mechanism to limit cellular proliferation and neoplastic change. We found that miR-16-5p was elevated in the PBMCs of non-cirrhotic as well as cirrhotic HCV-infected individuals compared to healthy controls, supporting the association between CHC and immunosenescence.

Elevated expression of both miR-16 and miR-155 is seen in the B cells of elderly individuals, and is associated with a pro-inflammatory state and reduced B cell function (Frasca et al., 2015). Additionally, both miR-16-5p and miR-155-5p are elevated in the PBMCs of individuals with HCV-related lymphomas compared to healthy controls, HCV-infected subjects and individuals with HCV-related HCC (Piluso et al., 2015) indicating that they may have a role as diagnostic biomarkers for HCV-related haematological malignancy as well as providing insights into the pathogenesis of HCV-related cancers.

The effect of genotype on risk of HCC occurrence is unclear, and data are conflicting. Increased risk of HCC development has been described with both Gt1 and Gt3 infection and further work is needed to elucidate the relationship between HCV genotype and carcinogenesis (Bruno et al., 1997, Rubbia-Brandt et al., 2004). Chang and colleagues (2014) found that expression levels of miR-16, miR-193b, miR-199a, miR-222 and miR-324 were significantly higher in the PBMCs of HCV-infected patients compared to healthy controls and suggested that these miRNAs could be used as diagnostic biomarkers. They also demonstrated significant differences in PBMC miRNA expression between Gt1 and non-Gt1 infected individuals. In our initial miRNA screen we found that a greater number of miRNAs were expressed in the PBMCs of patients with HCV Gt1 infection than

in Gt3 infected patients with respect to healthy controls, and of those a greater number were significantly deregulated. Concordantly with Chang et al. (2014) we found that miR-193b, miR-222 and miR-324 were upregulated in PBMCs from Gt1 compared to Gt3 HCV-infected subjects in our initial screen. However, in subsequent experiments we were unable to demonstrate significant differences in expression between genotypes for any of the selected miRNAs. This may be due to the small sample size studied and high individual biological variance in miRNA expression. Additionally, mathematical correction for multiple comparisons was not applied to the initial screen, to avoid the risk of increasing the type II error for those associations that were not null. Naturally, this will have led to an increased incidence of type I error which may also explain our null findings.

Reflecting our larger patient cohort, cirrhotic patients were older at enrolment, and had a significantly higher BMI. Both older age and elevated BMI have been shown to accelerate fibrosis progression in CHC (chapter 1.3.2) and contribute to immunosenescence. It is possible that these baseline demographic factors contributed to the elevated miRNA expression in the PBMCs of HCV-infected subjects with cirrhosis independently of HCV infection. A cohort of age- and BMI-matched controls with chronic liver disease secondary to NAFLD would have been a preferable control group. The feasibility of setting up such a cohort was explored to control for these factors, but ultimately was not felt possible within the time frame of the project.

PBMCs are a heterogeneous population of cells, reflected in the variability of miRNA expression and lack of consistency in published miRNA studies involving PBMCs. The identification of endogenous miRNA controls unaffected by disease stage or genotype proved challenging. For instance, to aid analysis all TaqMan® MicroRNA Array cards are pre-loaded with 3 endogenous small non-coding RNA controls - RNU44, RNU48 and U6 sRNA. However, questions have been raised about the reliability of these endogenous controls and it has been demonstrated that expression of these housekeeping genes may be as variable as miRNA expression in tumour samples (Gee et al., 2011, Schwarzenbach et al., 2015). Levels of U6 sRNA have been shown to be highly variable in the serum, and are downregulated in the serum of patients with hepatic fibrosis (Marabita et al., 2016, Benz et al., 2013). Concordantly, we found that their expression was

significantly altered in the context of CHC and cirrhosis, precluding their use. We were unable to identify a reliably expressed endogenous control miRNA that was not differentially expressed in the PBMCs of patients with CHC compared with healthy controls; all candidate control miRNAs suggested by the manufacturer demonstrated significant between-group differences, and the pilot study did not yield an alternative. This discovery was not made until after the RNA had been extracted, at which stage an exogenous 'spike-in' control could not be utilised. We were able to utilise global normalisation for the miRNA array, however this technique is only appropriate when profiling a large number of genes and could not be used for validation in the pilot cohort (Mestdagh et al., 2009). Consequently, although miRNA expression in the PBMCs of HCV-infected subjects could be quantified and compared using endogenous miRNAs, we were unable to include healthy controls in our validation analysis.

It is known that high concentrations (>10%) of the cryoprotectant dimethyl sulfoxide (DMSO) found in freezing media can reduce Taq DNA polymerase activity by up to 50% and it is possible that this contributed to the poorer miRNA transcript yield in the cryopreserved group (chapter 4.3.1). We also found that storage method affected the miRNA expression profile, although overall correlation between detected transcripts was acceptable. RNA extracted from the cryopreserved PBMCs demonstrated more advanced degradation in comparison to RNA extracted from PBMCs frozen in lysis buffer. Our results suggest that there is a marked benefit to suspending PBMCs intended for subsequent miRNA analysis immediately in lysis buffer prior to storage at -70^{o}C rather than cryopreserving in liquid nitrogen. Additionally, the storage method utilised should be taken into consideration when interpreting miRNA arrays. We were required to use the cryopreserved samples for our pilot study, and this may have led to an underestimation of fold changes in our analysis. It is also possible that differences in the quality of RNA between cryopreserved samples led to an overestimation of between group differences. This may explain why we were unable to reproduce differences in PBMC miRNA expression between genotypes in the validation analysis.

Finally, isolation of PBMCs requires separation from whole blood using density centrifugation and is time consuming. This limits their appeal as a source of biomarkers for use in clinical practice. As a consequence of these limitations and

despite interesting preliminary findings, further analysis using a larger bank of PBMC samples was not prioritised during this time period.

Specific serum miRNA signatures have been described in CHC, and as with PBMC miRNA expression, have been associated with stage of disease and treatment outcome (Shrivastava et al., 2015). Serum is easily collected by centrifugation of whole blood that has been allowed to clot, making it an attractive and clinically relevant medium to study. We therefore next examined miRNA profiles in the serum of patients with CHC compared to healthy controls.

4.4 Serum miRNA expression in CHC

4.4.1 Serum miRNA profile in CHC

We first examined global miRNA expression in the serum of patients with CHC compared to healthy controls using the TaqMan® MicroRNA Array Cards (chapter 2.2.2.1).

We detected 423 of the 754 miRNAs examined in the serum of HCV-infected subjects (present in ≥ 2 biological replicates), compared with 344 in the healthy controls. Of the 272 miRNA transcripts which could be compared between the groups, we found that 47 were significantly differentially expressed in the serum of CHC patients with respect to healthy controls, and of those 27 demonstrated greater than two-fold change. Twenty-two miRNAs were significantly upregulated and 5 were significantly downregulated (figure 4-13, table 4-3).

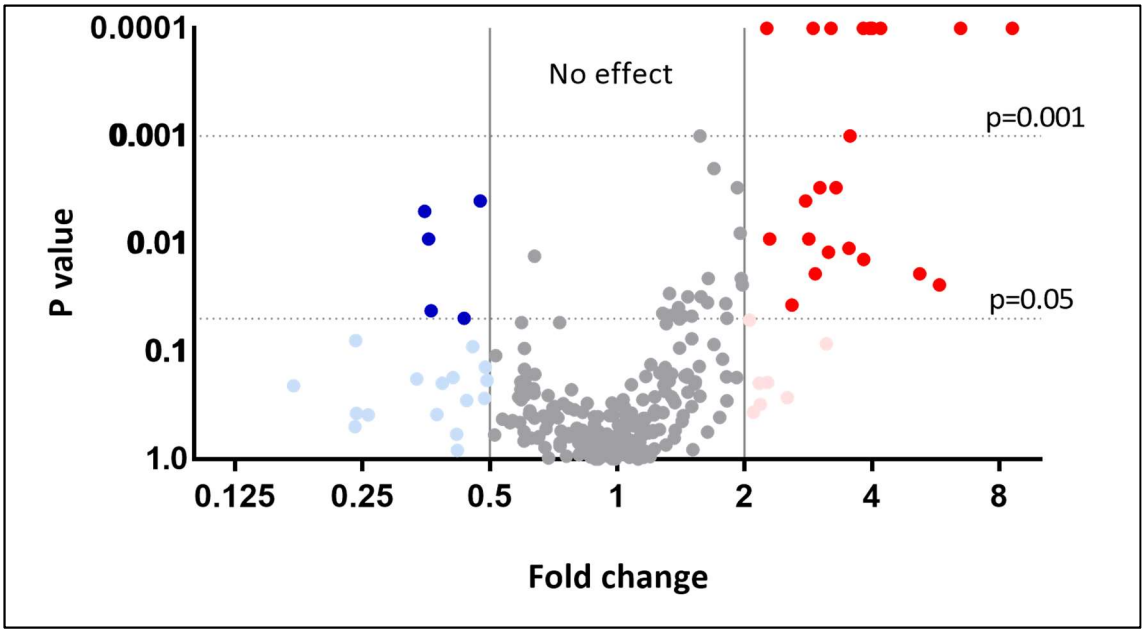


Figure 4-13: Volcano plot demonstrating p value against fold change for miRNAs deregulated in the serum of chronically HCV-infected subjects compared with healthy controls. Data points in red indicate miRNAs upregulated in CHC, and those in blue indicate downregulation. Fold change > 2 and p value < 0.05 were considered significant.

Table 4-3: MiRNA expression in the serum of HCV-infected subjects compared with healthy controls

	MiRNA	Fold change	P value
Increased in CHC	miR-885-5p	8.57	0.0001
	miR-193a-5p	2.90	0.0001
	miR-193b-3p	3.86	0.0001
	miR-21-5p	2.25	0.0001
	miR-122-5p	4.19	0.0001
	miR-192-5p	4.00	0.0001
	miR-30d-5p	3.20	0.0001
	miR-34a-3p	3.95	0.0001
	miR-875-5p	6.47	0.0001
	miR-375	3.55	0.001
	miR-455-5p	3.29	0.003
	miR-1291	3.01	0.003
	miR-625-5p	2.79	0.004
	miR-99b-3p	2.83	0.009
	miR-224-5p	2.29	0.009
	miR-200b-3p	3.53	0.011
	miR-146b-3p	3.16	0.012
	miR-1244	3.82	0.014
	miR-126-5p	5.18	0.019
	miR-26a-1-3p	2.94	0.019
	miR-483-5p	5.77	0.024
	miR-182-5p	2.59	0.037
Decreased in CHC	miR-671-3p	0.48	0.004
	miR-411-3p	0.35	0.005
	miR-654-3p	0.36	0.009
	miR-486-5p	0.36	0.042
	miR-93-3p	0.44	0.049

To explore potential biological processes underlying the observed changes in extracellular miRNA expression between HCV-infected subjects and healthy controls, significantly deregulated miRNAs were analysed for pathway enrichment (figure 4-14). As with PBMC miRNA expression, we found that pathways in cancer were the most significantly enriched by extracellular miRNAs deregulated in CHC. MicroRNAs in cancer had the highest enrichment in HCV-infected samples, followed by proteoglycans in cancer, colorectal cancer, pathways in cancer and viral carcinogenesis.

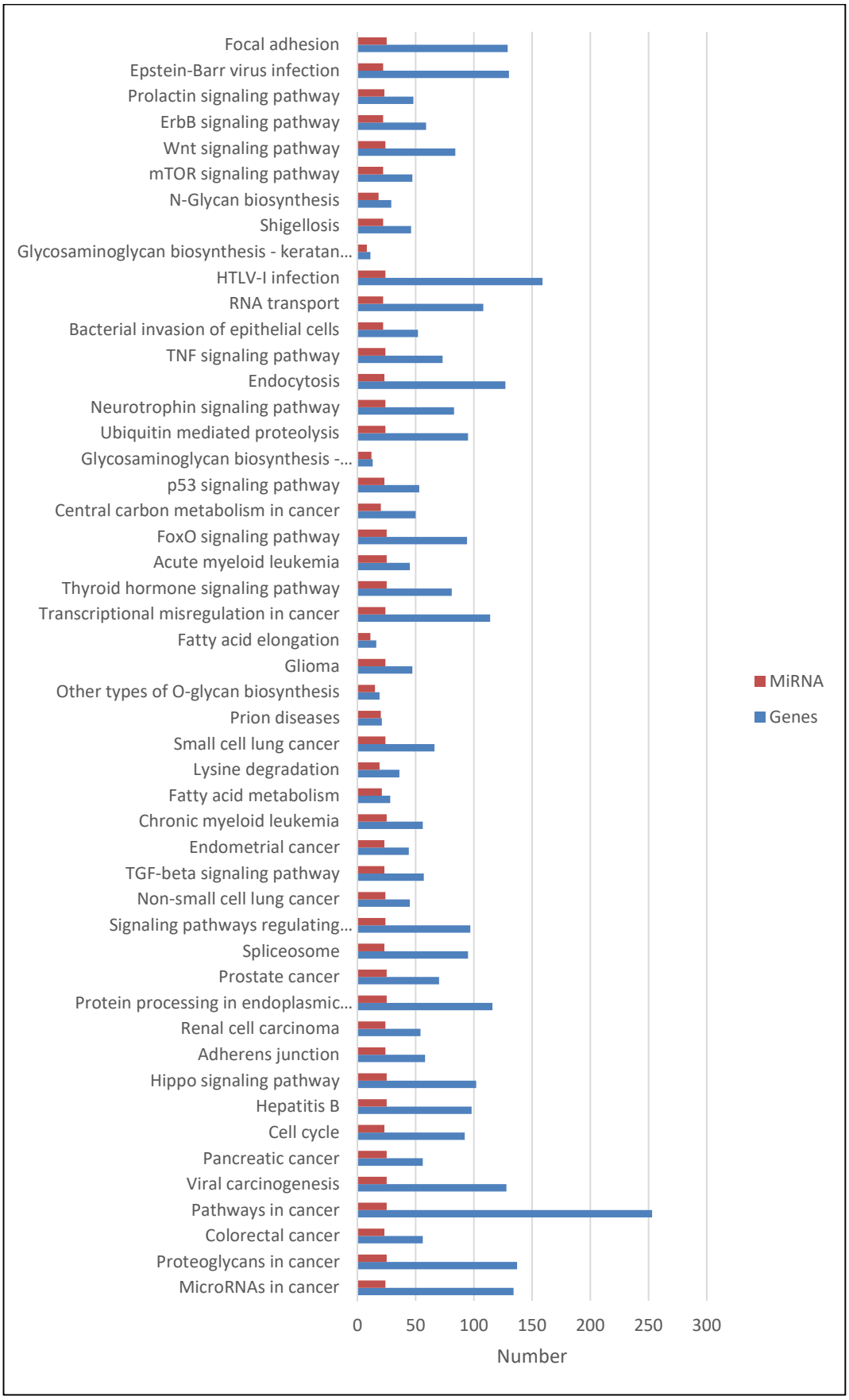


Figure 4-14: *In silico* pathway analysis for miRNAs significantly deregulated in the serum of HCV-infected subjects. Red bars indicate number of miRNAs involved in each pathway and blue bars indicate the number of genes. The y axis demonstrates significantly enriched KEGG pathways sorted according to a descending enrichment statistical score (-ln *P*).

4.4.2 Intergenotypic differences in serum miRNA expression

Previous studies have demonstrated significant differences in serum and PBMC miRNA expression between HCV Gt1 and Gt3 infected patients (Shwetha et al., 2013, Chang et al., 2014). However, we were unable to identify intergenotypic differences using the PBMCs in this study (chapter 4.3.3). We hypothesised that differences in miRNA regulation detected in serum rather than PBMCs from our patient cohort may explain phenotypic differences between genotypes.

We detected 394 miRNA transcripts in the serum of Gt1-infected subjects compared with 360 miRNA transcripts in Gt3 infection. We compared miRNA expression between Gt1- and Gt3-infected individuals and found that 5 miRNAs were significantly upregulated in Gt3 infection compared with Gt1 infection: miR-145-5p, miR-196b-5p, miR-500a-5p, miR-140-3p and miR-186-5p. A further 4 miRNAs were significantly downregulated: miR-130a-3p, miR-142-3p, miR-151-5p and miR-145-3p (table 4-4).

Table 4-4: Intergenotypic differences in serum miRNA expression

	MiRNA	Fold change	P value
Increased in Gt1 infection	miR-130a-3p	2.28	0.002
	miR-142-3p	2.06	0.005
	miR-151-5p	4.46	0.009
	miR-145-3p	2.02	0.043
Increased in Gt3 infection	miR-145-5p	2.49	0.007
	miR-196b-5p	2.29	0.01
	miR-500a-5p	2.29	0.02
	miR-140-3p	3.56	0.02
	miR-186-5p	5.72	0.04

We next subdivided the HCV infected subjects by genotype, and compared each group to the healthy controls to identify genotype-specific extracellular miRNA expression patterns.

We found that 23 miRNAs were significantly upregulated in the serum of HCV Gt1 infected patients (n=13) compared to healthy controls (n=8): miR-21-5p, miR-

193a-5p, miR-885-5p, miR-375, miR-660-5p, miR-455-5p, miR-483-5p, miR-193b-3p, miR-29c-3p, miR-122-5p, miR-100-5p, miR-182-5p, miR-101-3p, miR-130a-3p, miR-192-5p, miR-362-3p, miR-362-5p, miR-30d-5p, miR-875-5p, miR-34a-3p, miR-126-5p, miR-26a-1-3p and miR-99b-3p. A further 4 miRNAs were significantly downregulated: miR-671-3p, miR-486-5p, miR-654-3p and miR-93-3p.

Sixteen miRNAs were significantly upregulated in the serum of Gt3 infected patients (n=12): miR-192-5p, miR-193b-3p, miR-885-5p, miR-625-5p, miR-193a-5p, miR-200b-3p, miR-122-5p, miR-186-5p, miR-146b-3p, miR-224-5p, miR-375, miR-365a-3p, miR-30d-5p, miR-34a-3p, miR-1291 and miR-875-5p. Two miRNAs were significantly downregulated: miR-654-3p and miR-486-5p.

Eleven miRNAs were significantly deregulated in both genotypes, and demonstrated similar patterns of expression (miR-193a-5p, miR-885-5p, miR-375, miR-486-5p, miR-654-3p, miR-193b-3p, miR-122-5p, miR-192-5p, miR-30d-5p, miR-875-5p and miR-34a-3p).

Serum miRNA expression was similar between genotypes in the cohort. As a consequence, further exploration of inter-genotypic differences in extracellular circulating miRNA expression was not deemed to be worth pursuing with more samples.

4.4.3 Correlation between serum miRNA expression, disease stage and biomarkers of ageing in CHC

One objective of this study was to identify candidate miRNAs which could be used as alternative prognostic biomarkers in CHC. Cellular and immune senescence is associated with increased disease severity and poorer response to treatment in HCV-infected individuals (Hoare et al., 2010b, Manfras et al., 2004). We hypothesised that by correlating miRNA expression with biomarkers of immunosenescence we could identify novel prognostic biomarkers for non-cirrhotic HCV-infected individuals at higher risk of disease progression.

We therefore stratified miRNA expression by disease stage (presence or absence of cirrhosis) and bio-age (as described in section 4.2.1). We examined patterns of expression, specifically looking for miRNAs that demonstrated differential regulation in the cirrhotic and high bio-age groups compared to the low bio-age and healthy control patients.

Five miRNAs were significantly elevated in all HCV-infected patient groups compared to healthy controls: miR-885-5p, miR-122-5p, miR-193a-5p, miR-193b-3p and miR-30d-5p. A further 12 miRNAs were significantly upregulated in the serum of cirrhotic patients (miR-21-5p, miR-625-5p, miR-375, miR-150-5p, miR-186-5p, miR-140-3p, miR-99b-3p, miR-1291, miR-320a, miR-146b-3p, miR-192-5p and miR-34a-3p) and 3 miRNAs were significantly downregulated (miR-454-5p, miR-16-1-3p and miR-411-3p) compared with healthy controls. Five miRNAs were significantly elevated in the cirrhotic but not the non-cirrhotic patients (miR-625-5p, miR-150-5p, miR-186-5p, miR-140-3p and miR-320a).

Excluding the 5 miRNAs elevated in all patient groups, a further 10 miRNAs were significantly upregulated in the serum of ‘high bio-age’ patients (miR-21-5p, miR-146b-3p, miR-192-5p, miR-34a-3p, miR-200b-3p, miR-183-5p, miR-363-3p, miR-19a-3p, miR-126-5p and miR-875-5p) and 5 were downregulated (miR-671-3p, miR-28-3p, miR-191-3p, miR-181a-2-3p and miR-93-3p). Three miRNAs were significantly altered in both the cirrhotic and the high bio-age group (miR-146b-3p, miR192-5p and miR-34a-3p).

Finally, 3 additional miRNAs were significantly upregulated in the low bio-age group (miR-875-5p, miR-455-5p and miR-182-5p) and 4 miRNAs were downregulated (miR-576-3p, miR-93-3p, miR-590-5p and miR-411-3p) compared to the control group. Only 1 miRNA was common to the cirrhotic and low bio-age group (miR-411-3p); it was significantly downregulated in both.

Both miR-21-5p and miR-146b-3p were significantly elevated in the cirrhotic and the high bio-age group, but not the low bio-age group. Additionally, miR-885-5p expression was significantly increased in the cirrhotic and high bio-age groups (RQ 14.7 and 6.6 respectively), although it was also elevated in the low bio-age group (RQ=5.6). MiR-122-5p was significantly upregulated in all CHC patients compared to healthy controls, and showed a negative correlation with disease stage and biological age (table 4-5). These miRNAs were identified as potential biomarkers and selected for validation with quantitative RT-PCR.

Table 4-5: Fold change of selected miRNAs in the serum of HCV-infected subjects stratified by disease stage, compared to healthy controls

	Fold change compared to healthy controls		
	Cirrhosis	**High bio-age**	**Low bio-age**
miR-885-5p	14.66	6.55	5.63
miR-122-5p	3.19	3.87	6.40
miR-21-5p	2.45	2.47	1.96
miR-146b-3p	4.93	3.38	1.90

As proof of principle and to explore potential biological processes underlying the observed differences in extracellular miRNA expression according to bio-age between non-cirrhotic HCV-infected subjects, and between cirrhotic and non-cirrhotic individuals, we performed *in silico* pathway analysis for the miRNAs significantly deregulated in each patient group (figure 4-15). We observed that canonical pathway enrichment was similar between cirrhotic subjects and non-cirrhotic, immunosenescent individuals with potential implications for the clinical management of these subjects.

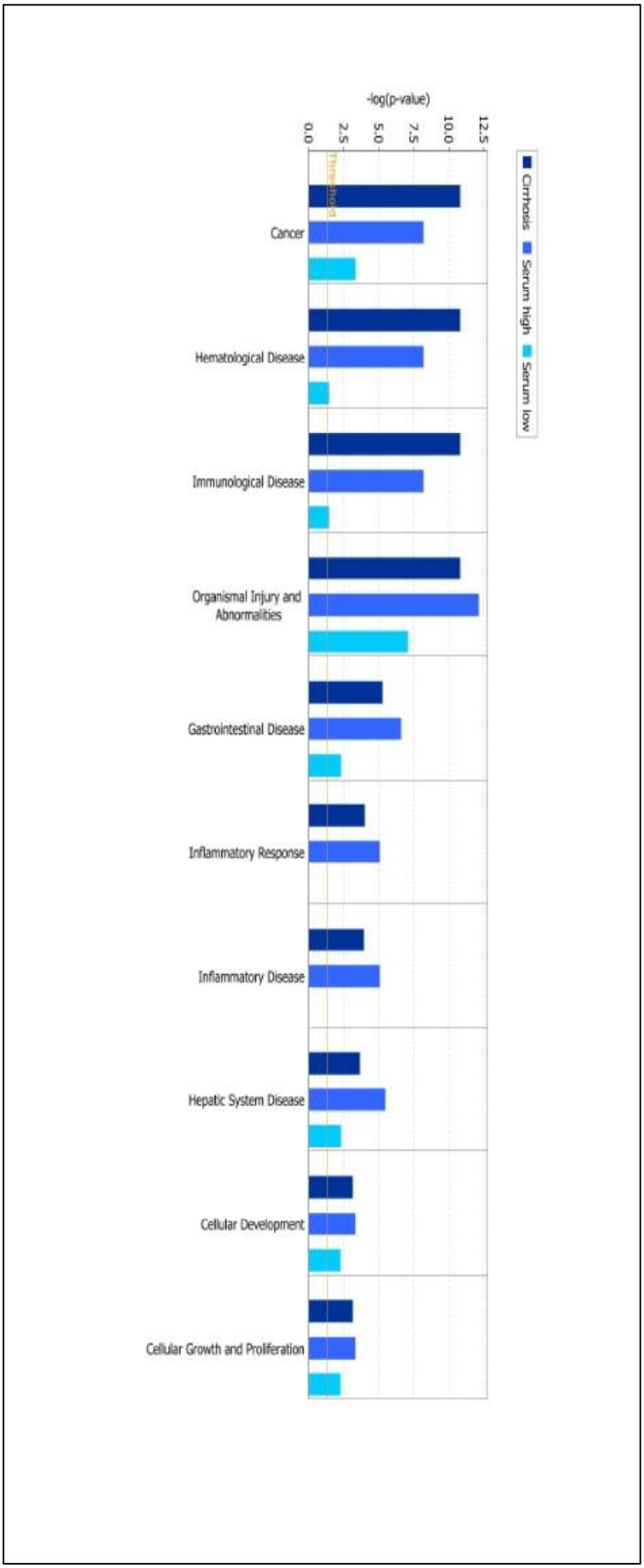


Figure 4-15: Comparison of pathway analyses for miRNAs deregulated in the serum of cirrhotic (dark blue), high bio-age (light blue) and low bio-age (turquoise) subjects.

In our pilot cohort, miR-885-5p showed significant upregulation in both cirrhotic (p=0.0008) and high bio-age patients (p=0.0132). MiR-122-5p was significantly upregulated in all patient groups compared to healthy controls (figure 4-16). As in the PBMC analysis, we observed high individual biological variance in serum miRNA expression.

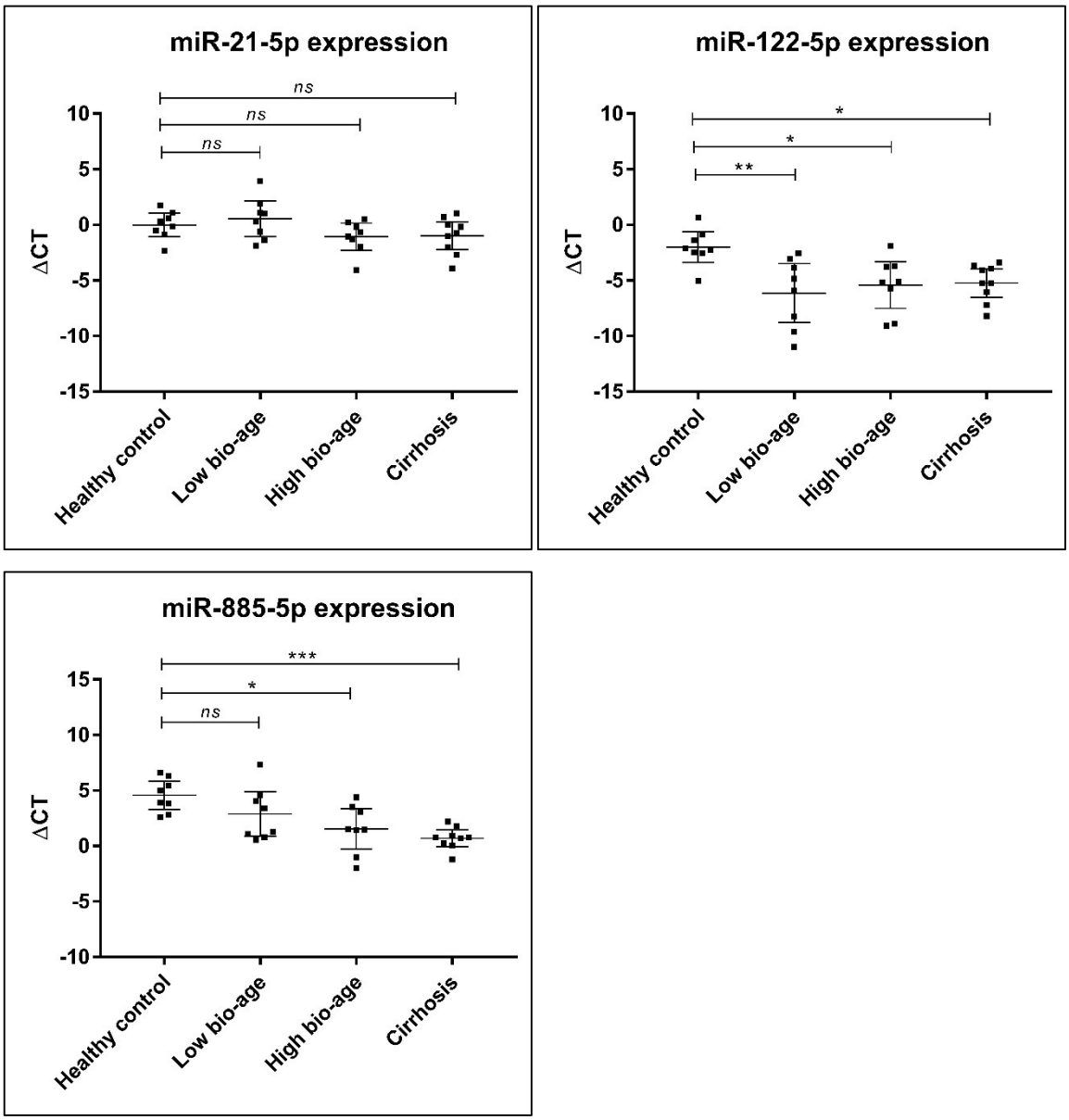


Figure 4-16: Expression of miR-21-5p, miR-122-5p and miR-885-5p in the serum of healthy controls and HCV-infected subjects with low bio-age, high bio-age and cirrhosis. Dot plot graphs depict mean/median expression +/- 95% CI; *p<0.05, **p<0.01, ***p<0.001, ns=non-significant.

MiR-21-5p showed a non-significant trend towards upregulation in cirrhotic and high bio-age patients compared to healthy controls. However, miR-21-5p was significantly higher when comparing all high risk patients (cirrhotic and high bio-age patients) to low risk patients (low bio-age and healthy controls) (p=0.0232, figure 4-17).

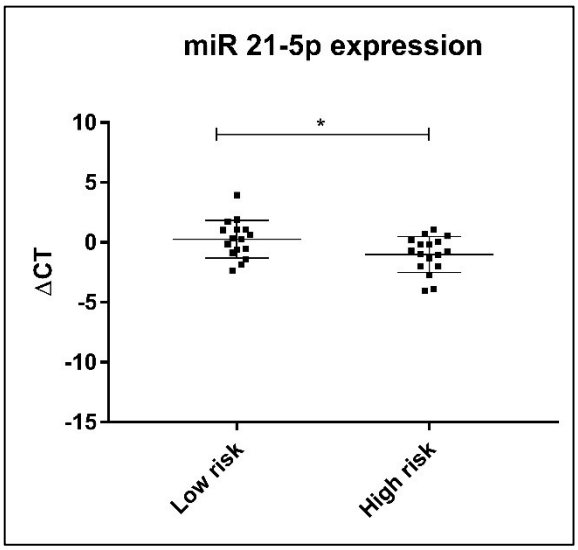


Figure 4-17: Expression of miR-21-5p in the serum of 'low risk' (HCV-infected low bio-age patients and healthy controls) and 'high risk' (HCV-infected cirrhotic and high bio-age patients) subjects. Dot plot graphs depict mean expression +/- 95% CI; *p<0.05.

Although miR-146b-3p correlated with disease stage and biological age in the initial miRNA screen, amplification was inconsistent in the validation analysis and this miRNA was not pursued further as a biomarker.

Next, we examined the use of miR-21-5p, miR-122-5p and miR-885-5p as biomarkers of cirrhosis in a larger bank of patient samples (described in section 4.2.2).

MiR-122-5p and miR-885-5p were significantly elevated in the serum of patients with cirrhosis secondary to HCV infection, and miR-885-5p was also elevated in non-cirrhotic HCV-infected patients compared with healthy controls (figure 4-18). Although there was a trend towards higher expression of miR-885-5p in cirrhotics vs. non-cirrhotics, we did not demonstrate significant differences in miRNA expression between cirrhotic and non-cirrhotic HCV-infected patients in this cohort (figure 4-18).

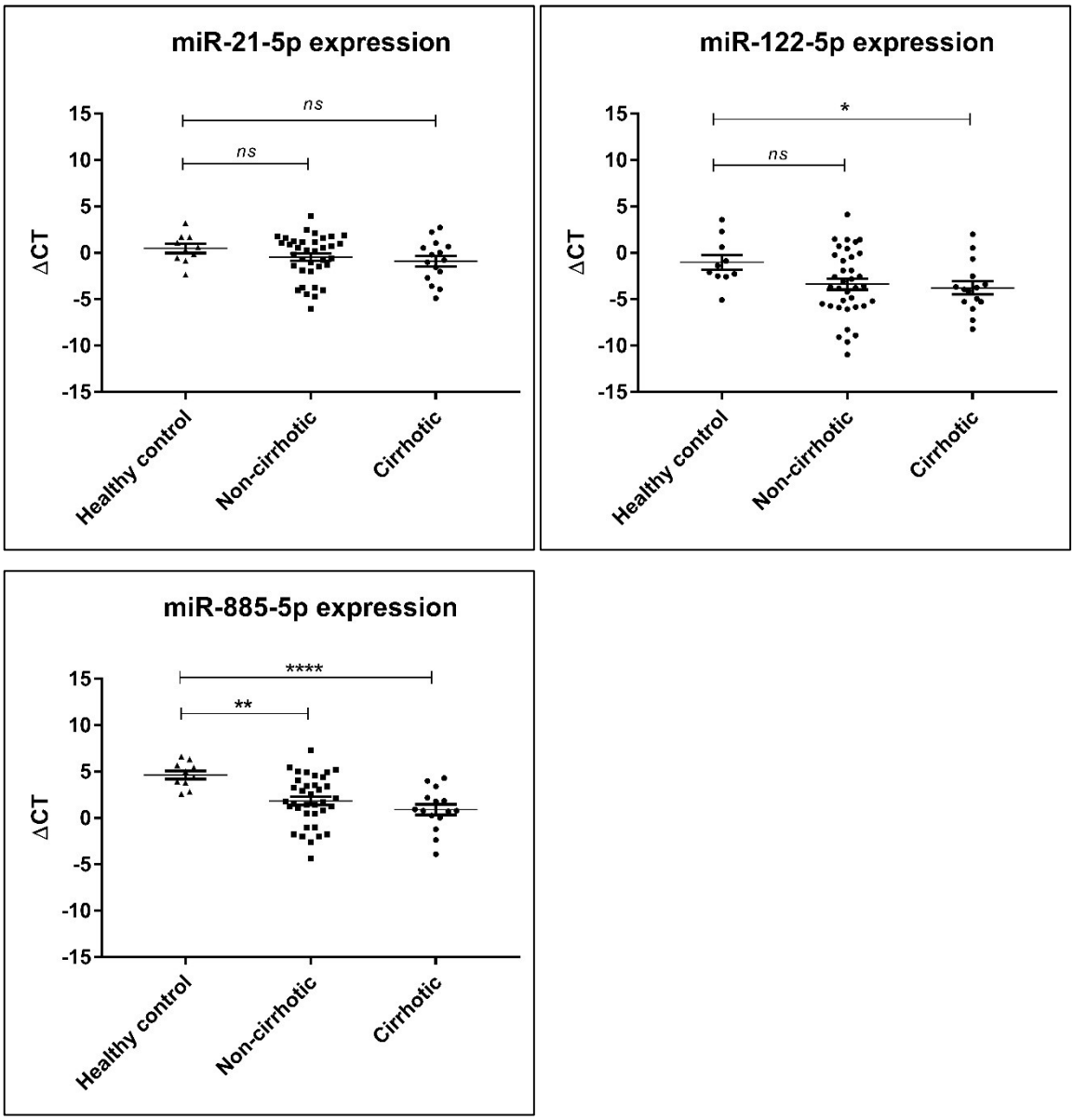


Figure 4-18: Expression of miR-21-5p, miR-122-5p and miR-885-5p in the serum of healthy controls (n=10) and HCV-infected subjects with (n=15) or without (n=36) hepatic cirrhosis. Dot plot graphs depict mean/median expression +/- 95% CI; *p<0.05, **p<0.01, ****p<0.0001, ns=non-significant.

Cirrhotic patients were found to be significantly older at enrolment (48yrs vs 41yrs, p=0.004) and to have higher BMI (27.4kg/m^2 vs 24.3kg/m^2, p=0.004). CDKN2A and ARF mRNA expression was significantly elevated in the PBMCs of HCV-infected subjects with cirrhosis, as known (Robinson et al., 2013).

The relationship between miRNA expression, CDKN2A expression, liver stiffness and additional laboratory variables was examined further in correlation analyses. As previously described (Robinson et al., 2013), we found that CDKN2A mRNA expression correlated significantly with that of the related transcripts CDKN2B (r=0.636, p<0.001) and ARF (r=0.907, p<0.001).

MiR-21-5p expression correlated with that of miR-122-5p (r=0.599, p<0.001) and miR-885-5p (r=0.777, p<0.001). There was also significant correlation between miR-122-5p and miR-885-5p expression (r=0.646, p<0.001).

All 3 miRNAs correlated weakly but significantly with CDKN2A, CDKN2B and ARF mRNA expression (figure 4-19). Of the 3 miRNAs, miR-885-5p correlated most strongly with CDKN2A mRNA expression (r=-0.369, p=0.008); miR-885-5p also correlated significantly with ALT (r=-0.355, p=0.005).

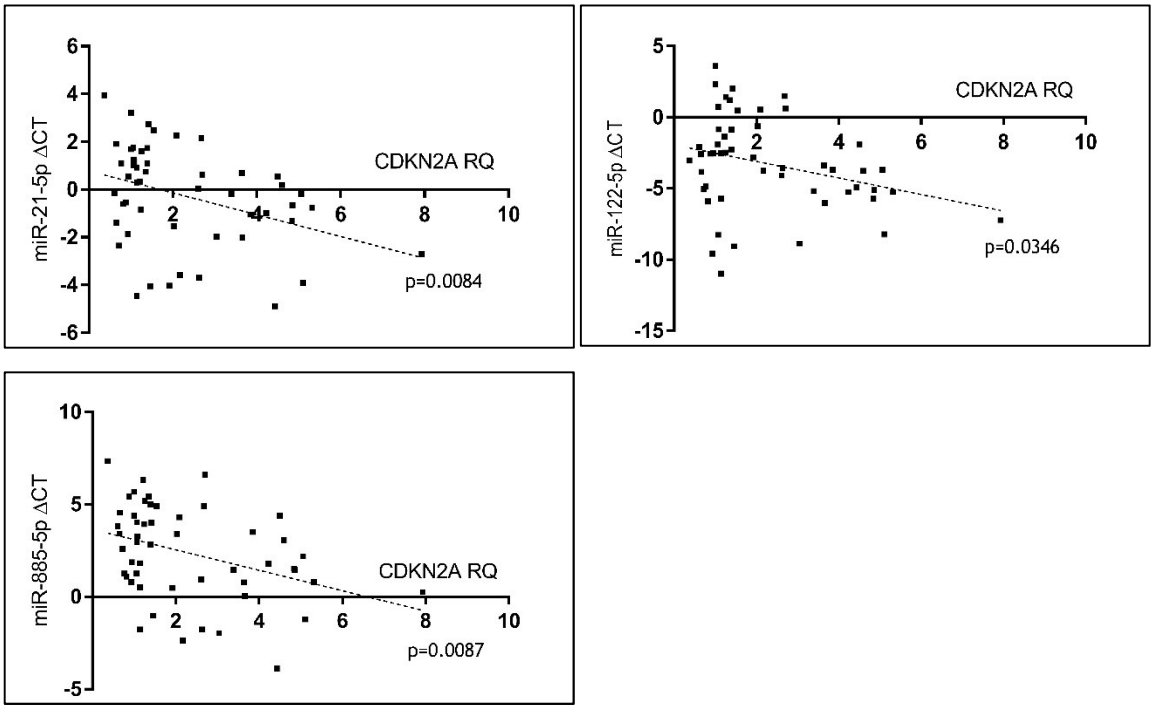


Figure 4-19: PBMC CDKN2A expression significantly correlates with serum miR-21-5p, miR-122-5p and miR-885-5p expression. Panels show XY correlation plots with linear regression curve fitted.

Liver stiffness correlated with age (r=0.388, p=0.006) and expression of CDKN2A (r=0.406; p=0.009), CDKN2B (r=0.489, p=0.001) and ARF (r=0.495, p=0.001). We also found that CDKN2A mRNA expression correlated weakly but significantly with age (r=0.283, p=0.044) and BMI (r=0.313, p=0.029).

We did not find significant correlation between miRNA expression and liver stiffness in this expanded cohort. Additionally, there were no significant differences in the expression of selected miRNAs between viral or host IFNL3 genotypes.

Interestingly, in a more strictly controlled comparison in which missing data were handled by listwise deletion (n=39), miR-122-5p expression positively correlated with HCV RNA (r=-0.373, p=0.019). This is consistent with findings in the literature: MiR-122 is required for HCV replication (chapter 1.1.5.2) and miR-122 level has been shown to correlate with HCV viral load during DAA therapy (Waring et al., 2016).

We used a two stage approach to identify factors associated with cirrhosis in our cohort. Univariate statistical tests were performed to assess key risk factors for potential associations (chapter 2.3). Models were then fitted for factors significant at the 10% level using multiple logistic regression analysis and reviewed.

As data on CDKN2 locus transcription were only available for a limited number of patients in the expanded cohort and to avoid bias in the model, 2 analyses were performed. The first analysis included all HCV-infected patients (n=51) and CDKN2 transcript expression was excluded. In the univariate analysis, older chronological age (p=0.004) and higher BMI (p=0.005) were significantly associated with the presence of cirrhosis (table S9). These factors remained significantly associated with cirrhosis in multivariate analysis. A model containing chronological age and BMI correctly classified 81.6% of individuals and explained 40.4% of variation in the dependent variable. The model was statistically significant (X^2 = 16.5, p<0.001). The inclusion of ALT (significant at the 10% level in univariate analysis) did not significantly improve the model (p=0.279) (table S10). A ROC curve analysis was performed using predicted probabilities for this model and demonstrated an AUC of 0.839 (95% CI: 0.717-0.962, p<0.001).

The second analysis included only patients who had had their CDKN2 locus status determined (n=40). In the univariate analysis, older chronological age (p=0.002), higher BMI (p=0.003), higher ALT levels (p=0.012), and both elevated PBMC CDKN2A mRNA (p=0.007) and ARF mRNA (p=0.006) expression were significantly associated with cirrhosis (table S11). Given the correlation between CDKN2A and ARF expression and to avoid collinearity bias, models were fitted using each transcript independently and in combination (unified into a single variable ‘CDKN2 transcript expression’; high transcript expression was classified as either

CDKN2A expression ≥ 2 fold higher than healthy controls, or ARF expression ≥ 2 fold higher than healthy controls, or both).

A model containing chronological age, BMI and ALT correctly classified 92.1% of cases, and explained 69.4% of the variation in the dependent variable. The model was statistically significant (X^2 = 26.5, p<0.001). Neither the inclusion of CDKN2 transcript expression nor the individual biomarkers significantly improved the diagnostic accuracy of the model (p=0.200) (table S12). Although inclusion of ALT improved the overall fit of the model, it was not significantly associated with cirrhosis when controlling for age and BMI (p=0.052). A ROC curve analysis was also performed using predicted probabilities for the final model (containing chronological age, BMI and ALT) and demonstrated an AUC of 0.935 (p<0.001, 95% CI 0.849 – 1.000).

Validated miRNAs were explored as potential diagnostic biomarkers using ROC curve analysis. We first examined the accuracy of each miRNA for the diagnosis of HCV infection. The AUCs for miR-21-5p, miR-122-5p and miR-885-5p were as follows: 0.620 (95% CI: 0.453 – 0.787, p=0.235), 0.733 (95% CI: 0.593 – 0.874, p=0.02) and 0.855 (95% CI: 0.751 – 0.959, p<0.001) respectively.

We next compared individual miRNAs with ALT and CDKN2 transcript expression as potential diagnostic biomarkers for the presence of cirrhosis in CHC. None of the selected miRNAs nor ALT performed well as diagnostic biomarkers for cirrhosis (table S13). CDKN2A and ARF mRNA expression appeared to have better diagnostic potential. A fold change of ≥ 1.97 in PBMC CDKN2A mRNA expression was associated with a sensitivity of 84.6% and specificity of 66.7% in the diagnosis of cirrhosis in CHC, whilst a fold change of ≥ 7.19 in PBMC ARF mRNA expression was associated with 61.5% sensitivity and 85.1% specificity (cut-offs calculated according to highest Youden index).

4.4.4 Functional analysis of selected miRNAs *in silico*

We analysed protein-protein interactions for the genes targeted by miR-122-5p and miR-21-5p to gain insights into potential *in vivo* functions. The predicted protein-protein interactions for miR-885-5p target genes have been documented previously (chapter 4.3.5).

MiR-122-5p is a well characterised miRNA. We identified a total of 248 validated target genes (figure 4-20). We found that viral carcinogenesis was the most significantly enriched canonical pathway, including 10 of the validated miR-122 target genes: BAX, CDK4, CREB1, GTF2B, GTF2H2, PKM, RAC1, RHOA and SRF. The hypoxia-inducible transcription factor-1 (HIF-1) signalling pathway (through genes AKT3, ALDOA, HMOX1, IGF1R, PFKFB2 and VHL), AMP-activated protein kinase (AMPK) signalling pathway (AKT3, CREB1, G6PC3, GYS1, IGF1R, PFKFB2 and PRKAB1) and insulin signalling pathway (through AKT3, G6PC3, GYS1, PHKA1, PRKAB1, PTPN1 and SOCS1) were also significantly enriched. Insulin resistance is thought to be more common in CHC, and has been shown to correlate with serum HCV RNA level, suggesting that viral replication promotes IR (Moucari et al., 2008). Additionally, HIFs are thought to regulate hepatic lipogenesis although the exact role of each factor is unclear, and data are conflicting (Jungermann and Kietzmann, 2000). It has also been suggested that HIF-dependent changes in hepatocyte metabolism may favour viral transmission and replication (Wilson et al., 2014).

In the case of miR-21-5p, we identified 83 experimentally validated target genes, including BCL2, BMPR2, CDC25A, E2F1, EGFR, ERBB2, HNRNPK, MARCKS, PDCD4, PTEN, RECK, SERPINB5, SPRY2, STAT3, TIMP3, TP63, VEGFA. Target genes connected to a network are shown on the diagram below (figure 4-21). The most significantly enriched biological pathway is regulation of molecular function, and pathways in cancer are again highly represented. The 5 most significantly enriched KEGG pathways are microRNAs in cancer, pathways in cancer, pancreatic cancer, proteoglycans in cancer and the FoxO signalling pathway.

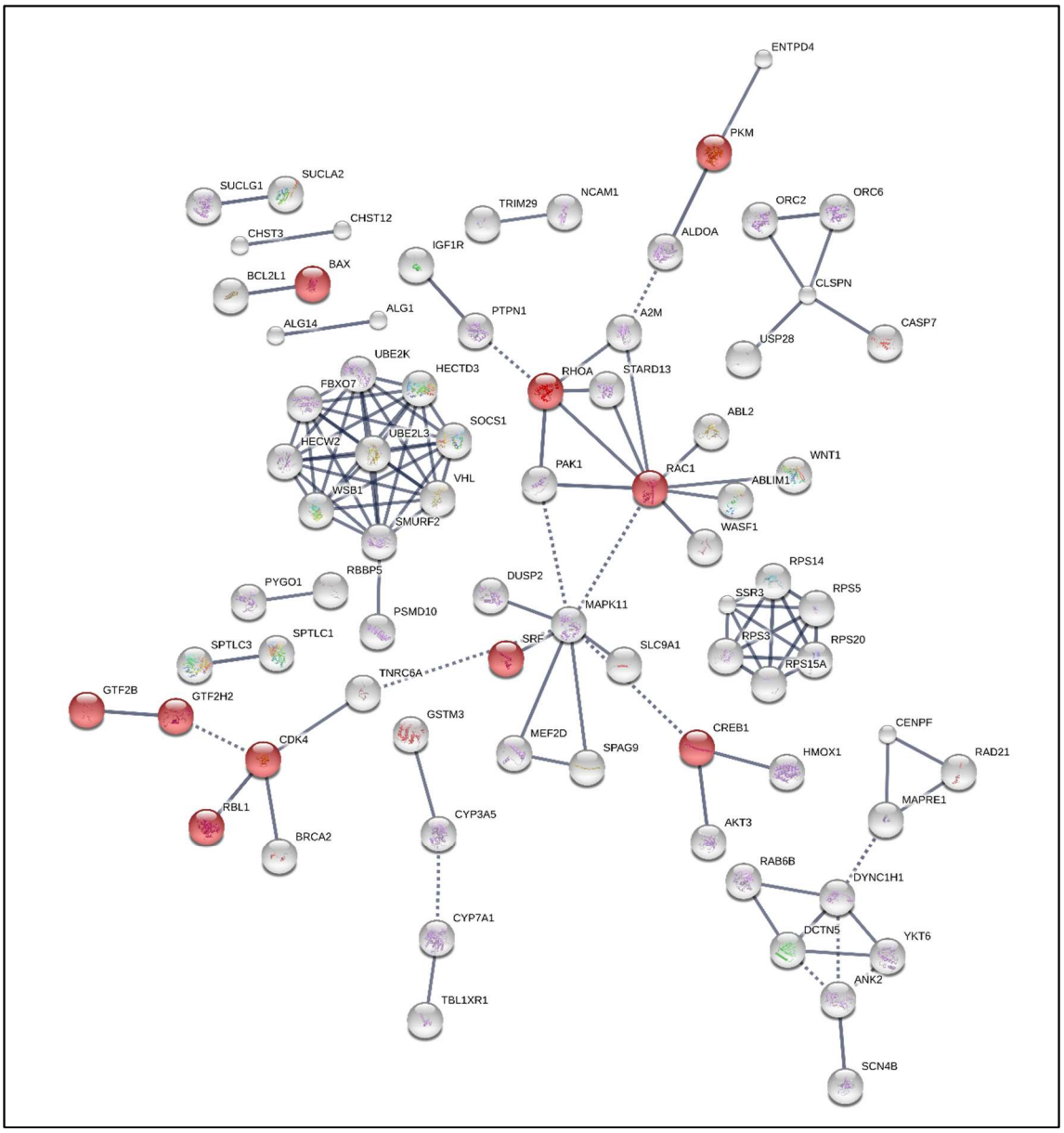


Figure 4-20: Predicted protein-protein interactions for miR-122-5p target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Proteins involved in the viral carcinogenesis pathway are highlighted in red.

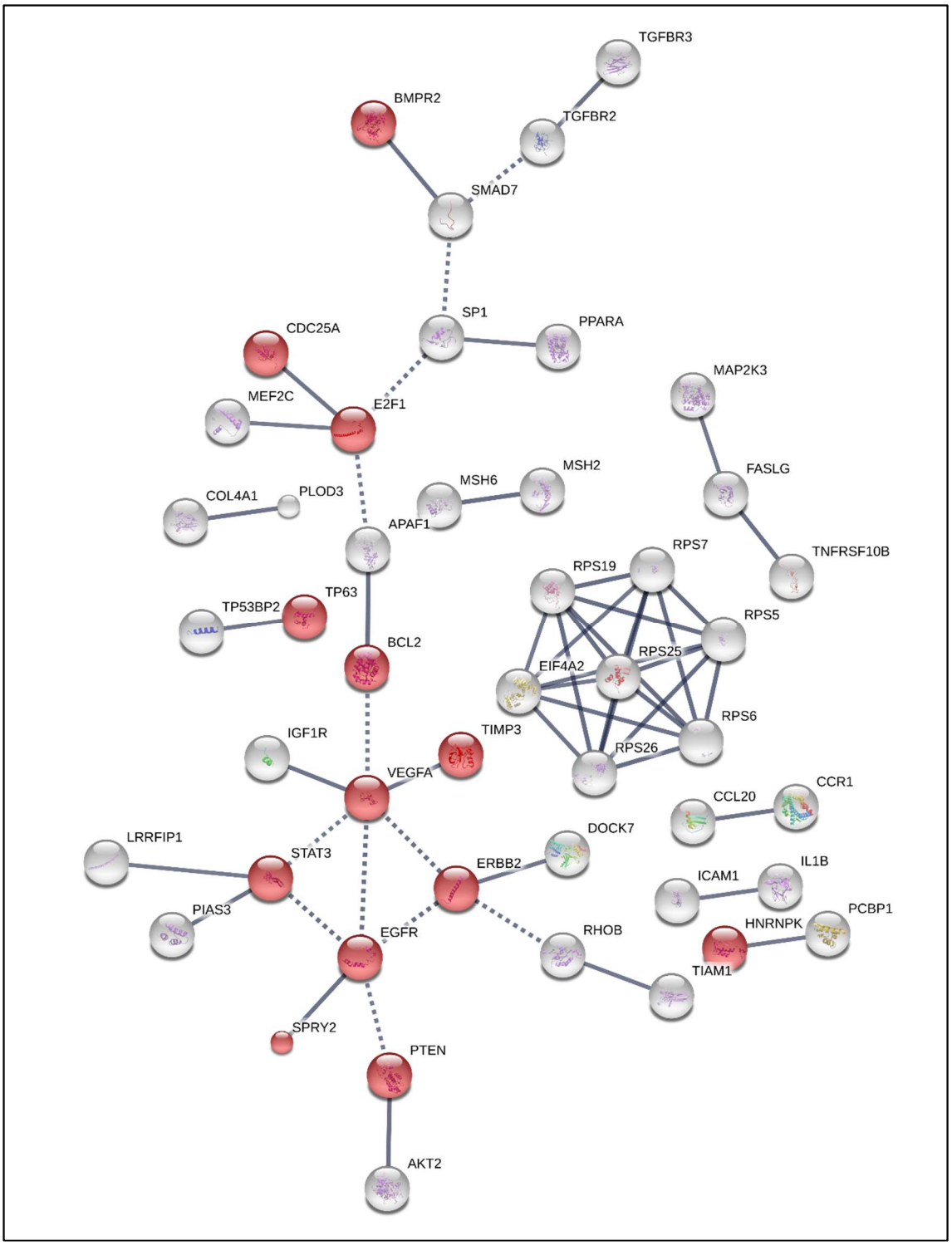


Figure 4-21: Predicted protein-protein interactions for miR-21-5p target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Proteins involved in the microRNAs in cancer pathway are highlighted in red.

4.4.5 MiRNA expression in the exosomes of patients with CHC

We examined the expression of miR-21-5p, miR-122-5p and miR-885-5p in the exosomes of matched serum samples in order to identify whether our candidate biomarker miRNAs were associated with membrane vesicles.

Quantitative analysis was not possible as the endogenous control miR-574-3p had not been validated for use with exosomes and therefore unadjusted CT values are shown. All 3 miRNAs were detected within the exosomes of patients with chronic HCV. MiR-122-5p and miR-885-5p were expressed at significantly lower levels in the exosome fractions compared to whole serum, however miR-21-5p expression was not significantly different between groups (figure 4-22).

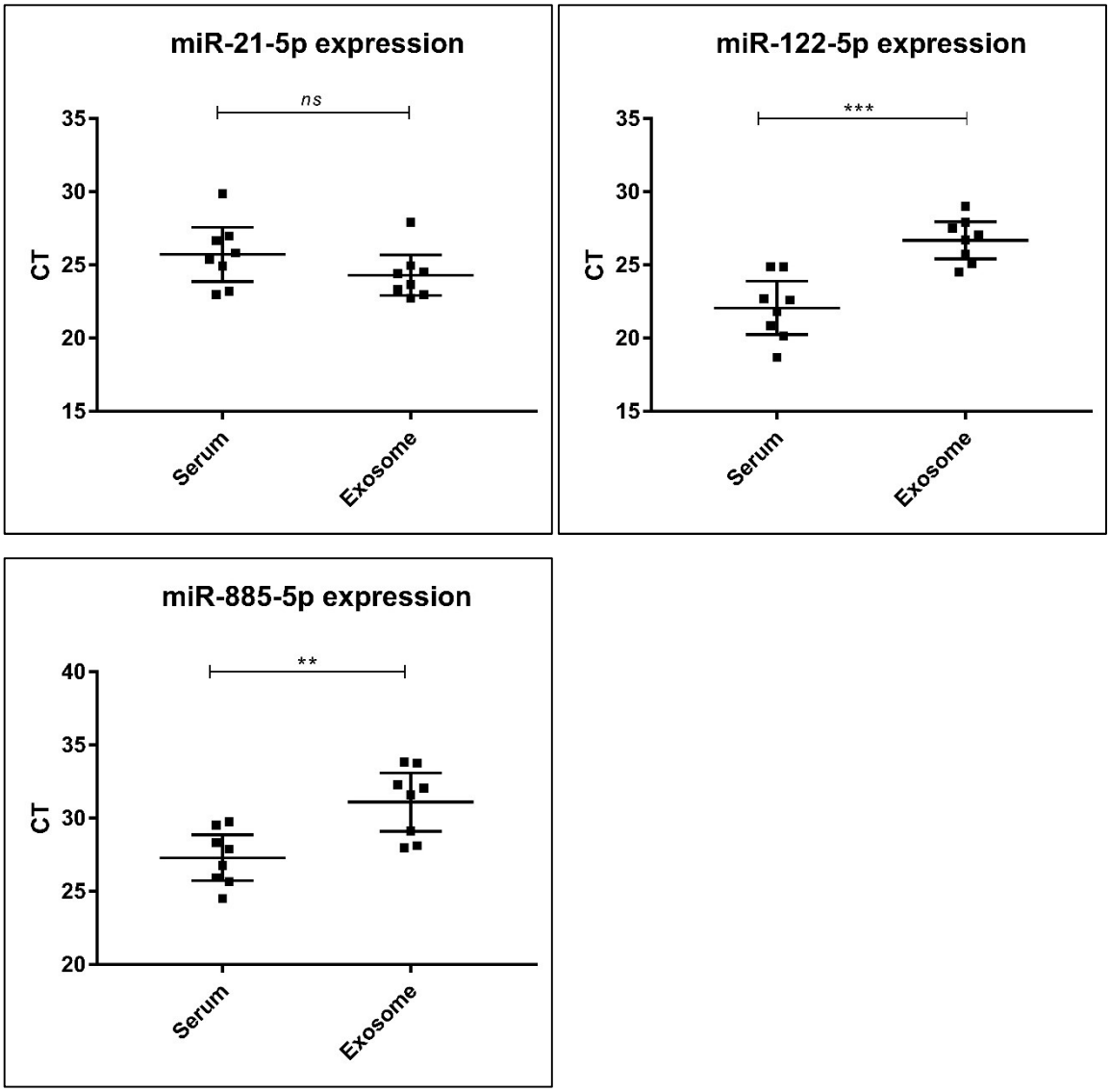


Figure 4-22: Expression of miR-21-5p, miR-122-5p and miR-885-5p expression in the serum and exosomes of 8 HCV-infected patients. Dot plot graphs depict mean expression +/- 95% CI; **p<0.01, ***p<0.001, ns = non-significant.

4.4.6 Discussion

In our pilot study, we identified a panel of miRNAs that showed significantly elevated expression in the serum of patients with CHC. Of these, we identified 3 miRNAs that correlated with disease stage and bio-age which we hypothesised could be used to identify individuals at higher risk of disease progression: miR-21-5p, miR-122-5p and miR-885-5p. Consistent with previous studies (Waring et al., 2016), we did not demonstrate significantly different miR-122-5p expression between Gt1 and Gt3 infection. MiR-21-5p and miR-885-5p levels were also similar between genotypes, suggesting that they are suitable for use as biomarkers in both Gt1 and Gt3 HCV-infected patients.

We found that miR-21-5p, miR-122-5p and miR-885-5p were all expressed within exosomes. The extracellular circulating miRNA population is heterogeneous, and both immune and non-immune cells are thought to release miRNAs into extracellular environments. MiRNAs may be released actively or passively, and it is thought that miRNAs contained within exosomes play a role in cell-cell communication and act as 'hormones' to regulate gene expression in target cells, whereas protein-bound miRNAs are released passively into the circulation on cell death. Interestingly, miR-122-5p and miR-885-5p were downregulated in the exosome fraction compared to whole serum whilst miR-21-5p was expressed at the same level in both groups. This may indicate that miR-21-5p is predominantly exosome-bound in the serum whereas miR-122-5p and miR-885-5p are mainly released passively into the circulation following cell death. The correlation between ALT and miR-885-5p adds some support to this hypothesis, although the same association was not seen with miR-122-5p. Of note, miR-885-5p demonstrates the same pattern of deregulation in the PBMC compartment and serum of individuals with CHC. This is intriguing, and suggests that PBMCs may be an additional source of circulating miR-885-5p in CHC.

We have demonstrated that miR-885-5p is upregulated in the serum of HCV-infected individuals, regardless of disease stage. We identified a trend towards increased miR-885-5p expression in cirrhotic compared with non-cirrhotic HCV-infected patients, as seen in the PBMCs (chapter 4.3.4), but this failed to reach significance (fold change 9.8 vs 5.0; p=0.160). We also found that miR-885-5p correlated with both ALT and CDKN2 transcript expression.

MiR-885-5p has tumour suppressor functions (chapter 4.3.6), and elevated circulating miR-885-5p expression has previously been described in association with liver cirrhosis and HCC (Gui et al., 2011). In CHC, HCC is thought to develop as a consequence of chronic inflammation and liver cell proliferation resulting from oxidative stress and cytokine signalling (chapter 1.3.1). Cellular proliferation is usually tightly regulated, and tumour suppressor genes function to limit inappropriate cell growth and metastasis. Mutations in these genes or alterations in cancer signalling pathways may result in oncogenesis.

There is increasing evidence to support a direct role for HCV proteins in modulating cancer pathways (Levrero, 2006). Transfection of a hepatoma cell line with HCV core protein resulted in marked upregulation of genes involved in oncogenic signalling, particularly Wnt-1 and its downstream target gene WISP-2, leading to increased proliferation, cell cycle progression and DNA synthesis (Fukutomi et al., 2005). MiR-885-5p is able to inhibit Wnt/B-catenin signalling (Zhang et al., 2016) highlighting the importance of its role as a tumour suppressor in chronic liver disease, particularly in the context of HCV infection.

Hepatocarcinogenesis has also been associated with deregulation of a number of other pathways regulating cell death or proliferation, including p53 and TGF-B. Mutations and hypermethylation of the p16INK4A gene have also been described in HCC (Liew et al., 1999). The risk of HCC is increased by factors including alcohol ingestion, metabolic liver disease and co-infection with other hepatotropic viruses (chapter 1.3.2).

The onco-miR miR-21 is thought to play a critical role in suppressing IFN-induced apoptosis through downstream targeting of PTEN and AKT. Upregulation of miR-21 results in a suppression of PTEN expression and increased phosphorylation of FAK, a downstream target of PTEN, with consequent deregulation of cell cycle progression and cell migration (Meng et al., 2007). Bihrer et al. (2011b) found that serum miR-21-5p expression correlated with ALT level, markers of liver synthetic expression and hepatic necro-inflammatory activity in CHC. In our pilot study, we found that serum miR-21-5p expression was increased in high risk vs. low risk subjects. However, we were unable to demonstrate differences in miR-21-5p expression between groups in our expanded cohort.

MiR-122-5p is also upregulated in the serum of cirrhotic HCV-infected individuals and appears to weakly correlate with HCV viral load, as described by Kumar et al. (2014). Expression was not significantly altered between HCV-infected non-cirrhotic patients and healthy controls. MiR-122-5p is a commonly described biomarker of liver injury, serum expression of which has been shown to correlate with hepatic necro-inflammation in both HBV and HCV infection (Waidmann et al., 2012b, Bihrer et al., 2011a).

Similarly to miR-885-5p, miR-122-5p is thought to have tumour suppressor properties. MiR-122-5p knockdown in a mouse model results in the development of hepatosteatosis and liver tumours resembling HCC (Hsu et al., 2012). MiR-122 binds to PKM2, resulting in increased apoptosis and reduced cell migration and invasion *in vitro* (Xu et al., 2015). Consistent with the mouse model, miR-122 is significantly downregulated in tumour tissue when compared to para-cancerous tissue, and low miR-122 expression in HCC is independently associated with poor 3-year survival (Xu et al., 2015).

The association of circulating miR-122-5p expression with disease stage is less clear; Bihrer and colleagues (2011a) found no association with fibrosis stage whereas other groups have described a negative correlation between serum miR-122 expression and fibrosis stage, as seen in our pilot cohort (Waidmann et al., 2012c, Marquez et al., 2010, Trebicka et al., 2013). There appears to be a two-phase pattern of circulating miR-122-5p expression during fibrosis progression; miR-122 is elevated during early fibrosis when there is high necro-inflammatory activity, and decreased in more advanced 'burnt-out' cirrhosis (Trebicka et al., 2013). It is hypothesised that in advanced cirrhosis, reduced serum miR-122 expression may reflect a loss of hepatocytes and less functional hepatic capacity.

Analysis of protein-protein interactions suggested that the HIF signalling pathway, insulin signalling pathway and the AMPK signalling pathway were significantly enriched by genes targeted by miR-122-5p. Hypoxia is a common feature of solid tumours, and results in induction of the transcription factor HIF-1, promoting cell migration and invasion (Ye et al., 2017). Transfection of HCV core into a hepatoma cell line resulted in overexpression of HIF-1α and induction of VEGF, an important regulator of angiogenesis in neoplasia (Zhu et al., 2014b).

Loss of miR-122 in the liver (which correlates with increased serum expression) also results in an upregulation of HIF-1α (Ambade et al., 2016), supporting its role as a tumour suppressor in CHC.

HCV infection is known to inhibit the insulin signalling pathway, and promotes IR through suppression of PTEN (possibly mediated by increased miR-21-5p expression) and increased phosphorylation of IRS-1 (Gao et al., 2015). Transfection of Huh7.5 cells with the viral NS5A alone was associated with increased phosphorylation of IRS-1 and enhanced expression of molecules involved in gluconeogenesis (Parvaiz et al., 2015). IRS-1 phosphorylation results in activation of the downstream PI3K and MAPK pathways and suppression of apoptosis (Tanaka et al., 1997), promoting cell survival and oncogenesis.

One striking observation was that non-cirrhotic subjects with higher biological age (i.e. increased PBMC CDKN2A expression) behaved similarly to the cirrhotic subgroup in terms of pathway enrichment. Our findings suggest that these subjects may be at an increased risk of oncogenesis. Consequently, a watchful waiting approach may not be appropriate in HCV-infected individuals with a high bio-age regardless of liver stiffness. Ongoing clinical monitoring is not currently recommended for individuals with normal liver stiffness in whom treatment is successful, and who have no additional liver-related comorbidity. It is possible that subjects with high biological age may be at ongoing risk of hepatic and extra-hepatic disease despite HCV eradication, and may benefit from a period of surveillance post treatment. Further study is needed to determine whether markers of immunosenescence improve with viral eradication, and whether elevated BoA correlate with adverse clinical outcomes.

We did not identify significant differences between selected miRNA expression in the serum of HCV-infected subjects with and without cirrhosis. Additionally, we did not demonstrate a difference in miR-21-5p expression between healthy controls and HCV-infected subjects with or without cirrhosis. This may reflect limited statistical power due to sample size; our study would benefit from the inclusion of a greater number of cirrhotic patients and healthy controls. However, we were reliant on a previously characterised cohort with limited biological material available.

We found that the chronological age and BMI were significantly associated with cirrhosis. Schwabl and colleagues (2017) previously showed that the presence of hepatic steatosis was associated with persistent histological necro-inflammation and elevation of fibrogenic and angiogenic biomarkers following SVR in HIV/HCV infected subjects. In our study, both BMI and age correlated with CDKN2A transcript expression. Our data support previous studies which have demonstrated that chronological age has a negative impact on disease progression in CHC (chapter 1.3.2.1) (Grebely and Dore, 2011). Interestingly, although we had excluded subjects with obesity (BMI > 30kg/m^2) and features of the metabolic syndrome, we still found that BMI was an independent predictor of fibrosis in CHC. This finding is in concordance with that of Friedenberg et al. (2003) who demonstrated that BMI was an independent risk factor for fibrosis. The finding that even a moderate increase in BMI is significantly associated with cirrhosis when compared to BMI within the normal range highlights the importance of weight reduction interventions in clinical practice. Furthermore, the correlation of BMI with PBMC CDKN2A expression supports the association between obesity and immunosenescence.

Obesity is associated with chronic immune activation, promoting inflamm-aging. An increase in adipose tissue is associated with IR, telomere shortening and p53 activation, leading to an increased incidence of age-associated illness, including cancer (Renehan et al., 2008) and atherosclerosis (Ellulu et al., 2017). Obesity is also associated with B cell dysfunction and reduced antibody responses, as seen with increasing age. Regardless of age, B cells from obese individuals demonstrate a pre-activated phenotype and secrete more pro-inflammatory TNF-α and IL-6, and less IL-10, in comparison to B cells from lean individuals (Frasca et al., 2017). We did not previously find a correlation between BMI and PBMC miR-16-5p or miR-155-5p expression as may have been expected given the correlation with B cell dysfunction (Frasca et al., 2015), but this may again reflect limited statistical power due to sample size, and the exclusion of subjects with BMI > 30 kg/m^2.

Higher BMI is associated with a poorer virologic response to IFN and RBV therapy (Bressler et al., 2003), although Todorovska and colleagues (2017) found that this signal disappeared when controlling for age, genotype, IR and inflammatory change in the liver. The effect of BMI on treatment outcomes with DAAs is less

clear, and data are conflicting. Fox and colleagues (2017) failed to demonstrate an association between BMI and response rate, whereas BMI > 30kg/m^2 was found to be significantly associated with virologic failure in the EAP study (Cheung et al., 2016). Our findings suggest that immunosenescence secondary to chronic immune activation may contribute to the poorer treatment responses seen in individuals with a high BMI. Weight loss has been shown to reduce the number of senescent cells (Sen-B-Gal positive) in white adipose tissue in a mouse model (List et al., 2016), and caloric restriction has been shown to slow measures of biological ageing in humans independent of changes in weight (Belsky et al., 2017). Further studies are warranted to explore whether weight reduction results in reversal of immunosenescence in individuals with CHC.

4.5 Cellular miRNA response to IFN stimulation *in vitro*

We have identified changes in the abundance of miRNAs in serum and peripheral immune cells that are likely surrogates for changes in the liver. The mechanism underlying the higher levels of some miRNAs is not known, but could result from HCV infection, modifiable factors and non-modifiable factors.

It is possible that the host response to infection may drive miRNA expression. One of the key host responses to infection is the IFN response (chapter 1.2); HCV infection results in the expression of higher levels of ISG transcripts, and it is possible that this may drive circulating and hepatic miRNA expression. Given that ISG mRNA transcription is altered by the IFN response to infection, we developed a model to examine whether miRNAs are regulated by IFN in liver-derived cells.

We selected the onco-miR miR-21-5p for the pilot study to explore the feasibility of this approach as it is known to be induced by type I IFNs *in vitro* (Yang et al., 2015). We also explored whether ISG15, a gene involved in post-translational modification, affected the response to IFN stimulation.

4.5.1 Induction of miR-21-5p by IFN-α in a hepatoma cell line is inhibited by ISG15 knockdown

We analysed miR-21-5p expression in a hepatoma cell line in response to IFN-α and poly (I:C) stimulation. We found that expression of miR-21-5p was significantly increased in IFN-α and poly (I:C) stimulated cells in comparison to mock-treated cells (figure 4-23). This corresponds with previous reports demonstrating that miR-21 expression is induced by IFN-α stimulation in a number of cell lines (Yang et al., 2010). Interestingly, when we performed the same experiment in an ISG15 knockout HepaRG cell line as proof of principle, we found that the increase in miR-21-5p in response to IFN-α was abrogated, and the response to poly (I:C) treatment was markedly reduced (figure 4-23).

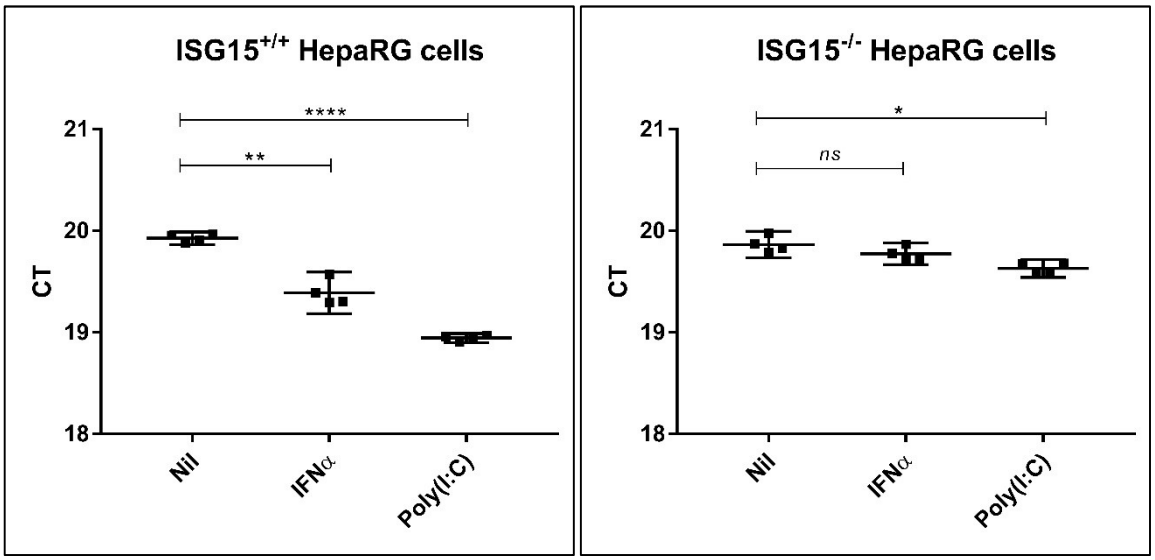


Figure 4-23: Expression of miR-21-5p in wild type HepaRG and ISG15-/- HepaRG cell lines in response to mock-treatment, IFN-α treatment and poly (I:C) stimulation. Dot plot graphs depict mean expression +/- 95% CI; *p<0.05, **p<0.01, ****p<0.0001, ns = non-significant.

Our data indicate that miR-21 induction is partly dependent on ISG15 signalling, as miR-21 induction in response to IFN-α and poly (I:C) stimulation was largely abrogated in the ISG15 KO cell line.

4.5.2 Discussion

This pilot study has proven the feasibility of studying the miRNA response to IFN signalling *in vitro*, and may be a useful tool for exploring miRNA deregulation in host processes.

ISG15 modulates host antiviral responses through 'ISGylation' of viral and host proteins and inhibition of virus release, and is thought to result in positive regulation of type I IFN signalling. ISG15 also targets many proteins involved in the innate immune response to viral infection, including RIG-I, JAK1, STAT1 and other ISGs (Morales and Lenschow, 2013). It is dependent on STAT3 for induction, similarly to miR-21 (Mahony et al., 2017). In support of an antiviral role for ISG15, $ISG15^{-/-}$ mice have been shown to be susceptible to influenza A and influenza B, herpes simplex virus and Sindbis virus (Lenschow et al., 2007). However, the same susceptibility has not been described with lymphocytic choriomeningitis virus or vesicular stomatitis virus (Osiak et al., 2005).

Counterintuitively, individuals born with inactivating ISG15 mutations do not present with increased susceptibility to viral infection, but are susceptible to

environmental mycobacterial infection as a consequence of impaired IFN-γ immunity, and demonstrate an autoimmune phenotype (Zhang et al., 2015, Bogunovic et al., 2012). Cells from ISG15 deficient patients demonstrated prolonged induction of ISGs in response to infection with highly pathogenic viruses, supporting a proviral role for ISG15 in humans (Speer et al., 2016).

The role of ISG15 in the response to HCV infection is unclear. Various groups have shown that knockdown of ISG15 expression is associated with increased HCV replication in a number of cell lines (Jones et al., 2010, Domingues et al., 2015). In contrast, Broering and colleagues (2010) found that short- or long-term knockdown of ISG15 was associated with suppression of HCV replication, and it has been proposed that increased ISG15/ISGylation may promote HCV infection (Chen et al., 2010). Early PKR-dependent induction of ISG15 may inhibit RIG-I ubiquitination, blocking subsequent HCV RNA-mediated RIG-I activation and leading to negative regulation of the host immune response and promotion of HCV replication (Arnaud et al., 2011).

Upregulation of miR-21 (Chen et al., 2013) is able to abrogate the type I IFN-mediated antiviral response through suppression of MyD88 and IRAK1 expression, whereas knockdown of miR-21 expression has been shown to sensitise cells to IFN-induced antiviral activity against vesicular stomatitis virus (Yang et al., 2015). MiR-21 blocks PDCD4, a negative regulation of IL-10, resulting in a switch to an anti-inflammatory and immunoregulatory environment (Sheedy et al., 2010). The use of IL-10 therapy has been shown previously to have a proviral and anti-inflammatory role in CHC (Nelson et al., 2003). MiR-21 also negatively regulates TNF, resulting in regulation of excessive inflammation but facilitating cell proliferation and migration (Sheedy, 2015). Infection of hepatocytes with HCV has been shown to induce miR-21, which results in promotion of viral replication as well as immunomodulation in favour of viral persistence (Chen et al., 2013).

We found that ISG15 is required for IFN-α and poly (I:C) induced upregulation of miR-21-5p in a hepatoma cell line. Upregulation of miR-21-5p in liver cells is associated with promotion of an immunotolerant cellular environment, and may support a proviral role of ISG15 in HCV infection.

4.6 Summary

In this chapter, we have demonstrated that CHC is associated with a distinct miRNA signature in the serum and peripheral immune cells.

We found that pathways in cancer were significantly enriched by target genes of miRNAs differentially regulated in the PBMCs of HCV-infected individuals. Of the miRNAs upregulated in CHC, we identified a panel that are significantly upregulated in HCV-infected subjects with cirrhosis. MiR-16-5p, miR-155-5p, miR-885-5p and miR-221-3p correlate with disease stage and markers of immune ageing, and are involved in the regulation of clinically relevant signalling pathways.

We also demonstrated that pathways in cancer were significantly enriched by the target genes of miRNAs deregulated in the serum of subjects with CHC. Moreover, we found that pathway enrichment was similar between HCV-infected cirrhotic patients and patients with a normal liver stiffness but high biological age. This suggests that decisions regarding treatment prioritisation should not be made on the basis of fibrosis stage alone, but should consider factors contributing to cellular senescence.

We found that miR-122-5p and miR-885-5p were significantly elevated in the serum of HCV-infected subjects with cirrhosis compared with healthy controls. We also showed that circulating miR-885-5p and miR-122-5p performed well as biomarkers for CHC, but not HCV-related cirrhosis. Furthermore, chronological age and BMI were more significantly predictive of cirrhosis than the selected miRNA biomarkers, highlighting the importance of weight loss interventions in routine clinical practice. Of the individual biomarkers tested, PBMC CDKN2 transcript expression was the most significantly associated with the presence of cirrhosis.

We explored an *in vitro* model to identify the mechanism for altered levels of miRNAs. We found that miR-21-5p expression was induced by TLR3 agonism and IFN-α stimulation in a hepatoma cell line. We found that this induction was largely abrogated in an ISG15 knockout cell line, suggesting that upregulation of miR-21-5p in response to IFN-α stimulation is ISG15 dependent, providing further tentative insights into the function of ISG15.

Our data add to the body of literature surrounding the use of miRNA signatures as biomarkers in CHC. We found that PBMC miRNA expression showed promise in the diagnosis of cirrhosis in CHC. Analysis of protein-protein interactions for the genes targeted by miRNAs significantly deregulated in the PBMCs of patients with cirrhosis added mechanistic support to their role in HCV-related haematological malignancy. We found that CHC was associated with a distinct extracellular miRNA signature, and that serum levels of miR-885-5p and miR-122-5p show potential as diagnostic biomarkers for HCV infection. Although serum expression of miR-885-5p, miR-122-5p and miR-21-5p correlated with CDKN2A expression, we failed to demonstrate differences in expression between cirrhotic and non-cirrhotic subjects.

We hypothesised that although the selected extracellular miRNAs did not perform well as diagnostic biomarkers for cirrhosis, they may still confer a prognostic benefit. We therefore next explored miRNA expression in HCV-infected subjects with cirrhosis receiving antiviral treatment.

5 MiRNAs as prognostic biomarkers for virologic and clinical outcomes during treatment of CHC

5.1 Introduction

In the absence of treatment, CHC leads to progressive liver fibrosis with an increased risk of hepatic decompensation and HCC. Data suggest that eradication of HCV is associated with improved clinical outcomes in patients with HCV-related cirrhosis (Cheung et al., 2016). Treatment may be beneficial even in patients with end-stage liver disease; previous studies of IFN-free therapy in HCV infected patients have shown that SVR is associated with a reduction in HVPG (Mandorfer et al., 2016), and allows delisting of a significant proportion of patients on the waiting list for liver transplantation (Belli et al., 2016). However, not all patients with cirrhosis benefit from achieving an SVR, leading researchers to suggest that there is a 'point of no return' in very advanced disease (Foster et al., 2016).

Schwabl et al. (2017) found that over 1/3 of HIV/HCV co-infected patients receiving DAA therapy had persistent histological necro-inflammatory activity despite achieving an SVR. Mortality in HCV-infected individuals who achieve SVR also remains higher than that in the general population; Innes et al. (2017) found that Scottish patients who achieve SVR had a mortality rate up to 6-fold greater than that of the general population, largely driven by deaths due to drug related causes and HCC. They were however less likely to be hospitalised or die for another liver-related reason (Innes et al., 2017). Worryingly, a number of recent studies have described an increased incidence of both *de novo* and recurrent HCC in cirrhotic patients who achieve SVR with DAAs (Conti et al., 2016, Reig et al., 2016). Whilst more recent studies have failed to demonstrate this association ('Lack of evidence of an effect of direct-acting antivirals on the recurrence of hepatocellular carcinoma: Data from three ANRS cohorts,' 2016), it remains a concern for many health care providers.

Finally, a proportion of patients will fail to respond to treatment or will relapse following treatment. Poor response to treatment is associated with a number of factors, including advanced liver fibrosis and cellular senescence (chapter 1.5.2).

Robust, non-invasive biomarkers to help characterise individuals at risk of poor virologic and clinical outcomes during DAA treatment are required. It is known that serum and hepatic miRNA expression differs significantly between patients who achieve an SVR with an IFN-α based regimen and those who relapse or fail to respond (Butt et al., 2016). The role of miRNAs in predicting response to IFN-sparing therapy is less well defined (Waring et al., 2016).

We have identified a panel of miRNAs that are elevated in the serum of patients with cirrhosis secondary to CHC, expression of which correlate with previously described biomarkers of cellular senescence (chapter 4.4). Other groups have shown that serum levels of these miRNAs are upregulated in a number of human cancers, including HCC (Gui et al., 2011, Xu et al., 2015, Meng et al., 2007).

We sought to compare miRNA expression in HCV-infected individuals at baseline and end of treatment (EOT) to identify whether the miRNA profile changes during DAA therapy. Additionally, we compared baseline miRNA expression in individuals who achieved SVR12 and those who relapsed to identify whether miRNAs signatures could be used to predict response to DAAs. Finally, we examined miRNA expression at baseline in subjects who experienced an adverse clinical outcome during DAA treatment despite HCV eradication and those who did not, to elicit whether miRNAs could be used to identify individuals who may not benefit from antiviral therapy.

5.2 Patient selection

5.2.1 Study design and population

The NHS England EAP was set up to provide DAA therapy for HCV-infected patients with severe liver disease who were at 'significant risk of death or irreversible damage within 12 months due to hepatic or extrahepatic manifestations', irrespective of viral genotype. Eligible patients included those with past or current decompensated cirrhosis (defined as ascites, variceal bleeding or encephalopathy), Child Pugh score ≥ B7 or extrahepatic manifestations of HCV likely to lead to irreversible damage within 12 months. Patients were also required to have been intolerant of, or non-responders to, IFN-based therapies. Exceptional cases were reviewed by a multidisciplinary panel, in line with the Clinical Commissioning Policy Statement. Patients received sofosbuvir combined with an NS5A inhibitor (ledipasvir or daclatasvir) with or without RBV for a fixed duration of 12 weeks. Choice of NS5A inhibitor and inclusion/exclusion of RBV was at physician discretion, with local multidisciplinary team input. Patients were enrolled into the HCV Research UK (HCVRUK) registry for prospective data collection. Ethical approval for HCVRUK was given by the National Research Ethics Service (NRES) committee East Midlands – Derby 1 (REC reference 11/EM/0314). Informed consent was obtained from all patients included in the study – patients who declined data collection were treated but excluded from analysis (Foster et al., 2016).

In this retrospective, observational cohort study, patients were selected from the NHS England EAP cohort in a 1:1:1 ratio. Group 1 consisted of patients who achieved SVR12, whose MELD score was unchanged or improved (i.e. decreased) from baseline at 12 weeks post therapy and who had no adverse clinical outcomes during the study period (SVR12). Group 2 included patients who achieved SVR12 but who had an adverse clinical outcome (SVR12-AE), and Group 3 consisted of responder-relapsers (RR), regardless of clinical outcome. A serious adverse event (SAE) was defined as MELD score increase ≥ 2, new decompensation event, hospitalisation, death or the development of malignancy between EAP baseline and 12 weeks post treatment. Data and clinical samples were requested from the HCV Research UK registry. All patients who received 12 weeks of sofosbuvir + ledipasvir/daclatasvir +/- RBV were eligible for inclusion.

Patients were required to have model for end-stage liver disease (MELD) scores available at baseline, EOT and 12 weeks post EOT. Additionally, patients were required to have stored serum samples available from two time points – baseline and EOT. Exclusion criteria included co-infection with HBV (HBsAg +ve) or HIV, non-compliance with study medication, and patients with a history of liver transplantation or who received a liver transplant during the study period.

In this pilot study the expected difference between the group means for individual miRNAs was not known. Based on sample size calculations, we required 20 patients in each group (i.e. ≥ 40 patients for each comparison) to have 90% power of demonstrating a difference between groups that was significant at the 5% level if the true difference was at least 1.5.

5.3 Results

5.3.1 Patient characteristics

Baseline characteristics of patients (n=59) are shown in table S14. Only 19 HCV-infected individuals met the criteria for inclusion in the SVR12-AE group. The majority of the patients were male (n = 40, 67.9%) and of white ethnicity (n=47, 79.7%). Most patients were Gt1- (n=26, 44.8%) or Gt3-infected (n=30, 51.7%) although Gt2 (n=1, 1.7%) and Gt4 (n=1, 1.7%) were also represented. HCV genotype data was missing for 1 subject. The median age on starting treatment was 54 years (IQR 48 - 61 years). The majority of patients received a DAA regimen containing ledipasvir (n=41, 69.5%) and RBV (n=52, 88.1%). The median ALT level was 54 IU/mL (IQR 37.8 – 70.3) and the median baseline MELD score was 11 (IQR 8.8 – 13.3). ALT level at baseline was not significantly different between genotypes (p=0.217). Co-morbid DM 2 was common (n=18, 30.5%).

Characteristics at EOT were also examined. There was a statistically significant decrease in ALT over the course of treatment (median ALT 23 IU/mL (IQR 17 – 30) vs 54 IU/mL (IQR 37.8 – 70.3), p<0.0001). However, there was no significant change in MELD score (median MELD score 11 (IQR 10 - 14) at EOT vs 11 (IQR 8.8 – 13.3) at baseline, p=0.09) (figure 5-1).

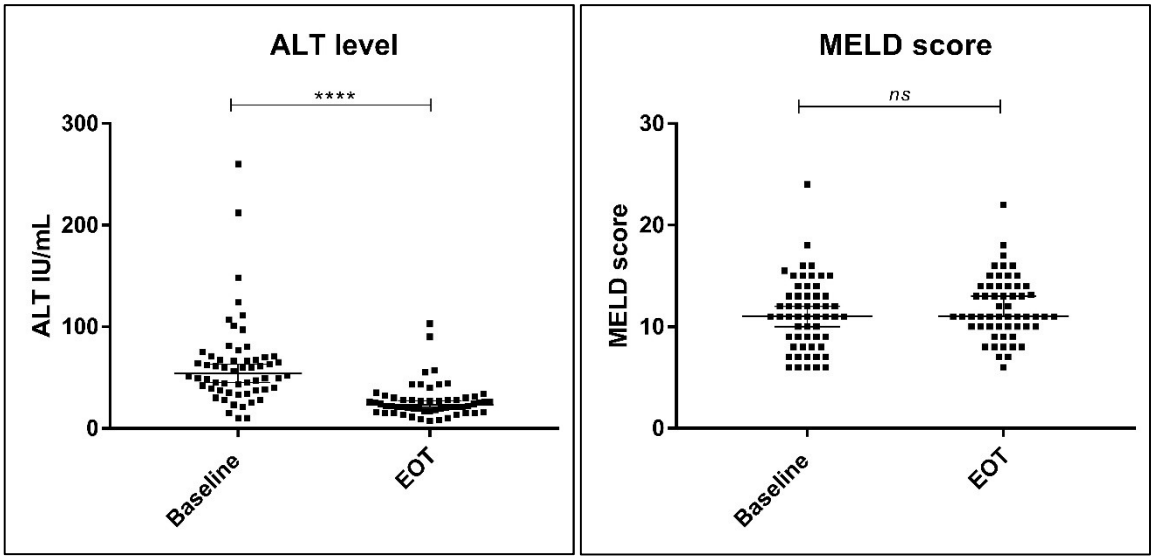


Figure 5-1: Serum ALT level and MELD score at baseline and end of 12 weeks' treatment (EOT) with DAAs. Dot plot graphs depict median expression +/- 95% CI; ****p<0.0001, ns=non-significant.

5.3.2 Serum miRNA expression is significantly altered by DAA therapy

We have previously demonstrated that serum levels of miR-21-5p, miR-122-5p and miR-885-5p correlate with markers of immune senescence in CHC (chapter 4). Immunosenescence is known to be associated with a poorer response to IFN-based treatment (Hoare et al., 2010b), and we hypothesised that it would be associated with poorer outcomes following DAA therapy. We therefore chose to explore whether expression levels of these miRNAs were altered during DAA treatment, and whether they could be used as prognostic biomarkers for HCV treatment-related outcomes.

We first examined inter-genotypic differences in expression of miR-21-5p, miR-122-5p and miR-885-5p at baseline. Only Gt1 (n=26) and Gt3 (n=30) infected subjects were compared, as they constituted the bulk of the cohort. Gt2 (n=1) and Gt4 (n=1) infected subjects were excluded and information on HCV genotyping was missing for 1 patient.

We did not demonstrate significant differences in miR-122-5p or miR-21-5p expression at baseline between Gt1 and Gt3 infection. However, miR-885-5p was significantly higher in Gt3 infection ($p=0.009$). This contrasts with our findings in the Outcomes cohort (chapter 4.4), in which all 3 miRNAs were expressed at similar levels between genotypes. One patient was diagnosed with Gt2 infection; this individual had markedly elevated expression of miR-122-5p (ΔCT -8.28) at baseline.

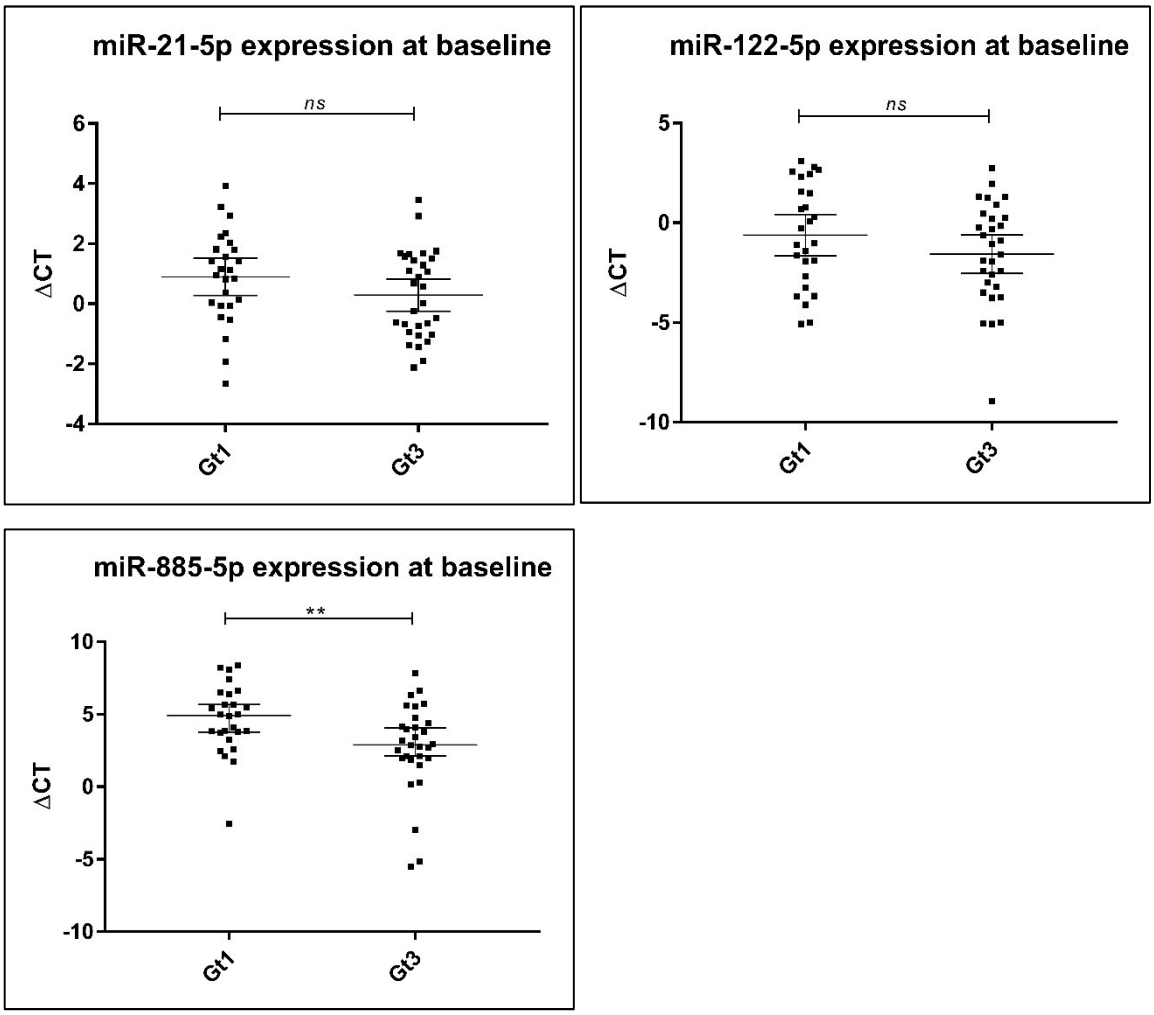


Figure 5-2: Baseline expression of miR-21-5p, miR-122-5p and miR-885-5p in the serum of HCV-infected subjects according to HCV genotype. Dot plot graphs depict mean/median expression +/- 95% CI; **p<0.01, ns=non-significant.

Next, we examined the expression of selected miRNAs at baseline and EOT for all patients (n=59). We found that miR-122-5p and miR-885-5p were significantly downregulated during DAA therapy (p<0.001). MiR-21-5p was not significantly altered over the course of treatment (figure 5-3).

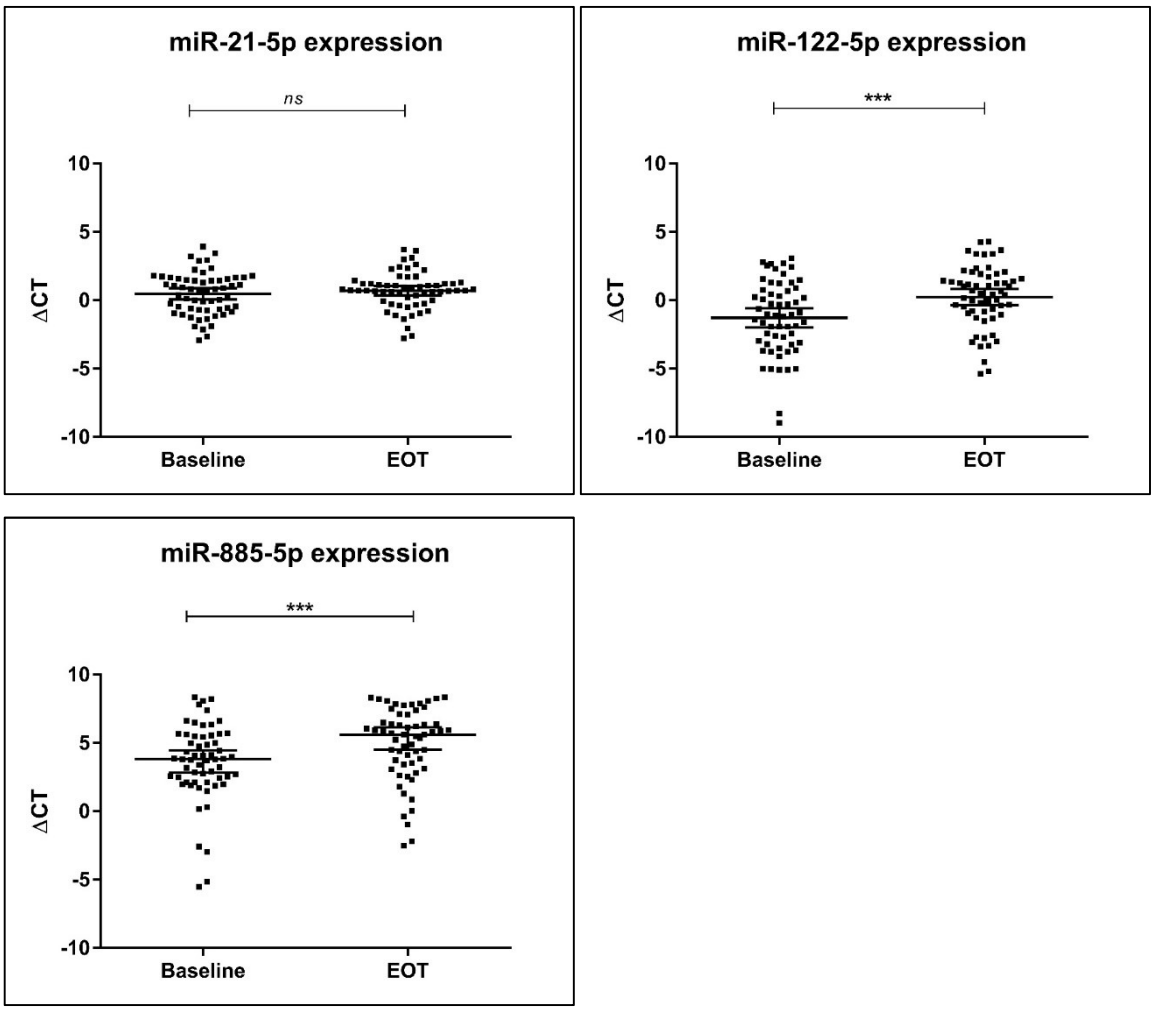


Figure 5-3: Expression of miR-21-5p, miR-122-5p and miR-885-5p in the serum of HCV-infected subjects at baseline and end of 12 weeks' treatment (EOT) with DAAs. MiR-122-5p and miR-885-5p were significantly downregulated following DAA treatment. Dot plot graphs depict mean/median expression +/- 95% CI; ***p<0.001, ns=non-significant.

We then stratified the HCV-infected subjects according to treatment outcome, and examined the pattern of miRNA deregulation over the course of treatment. We found that miR-122-5p and miR-885-5p were significantly downregulated at EOT in subjects who achieved viral eradication (SVR12, figure 5-4). MiR-21-5p was unchanged over the course of treatment in this group (p=0.168).

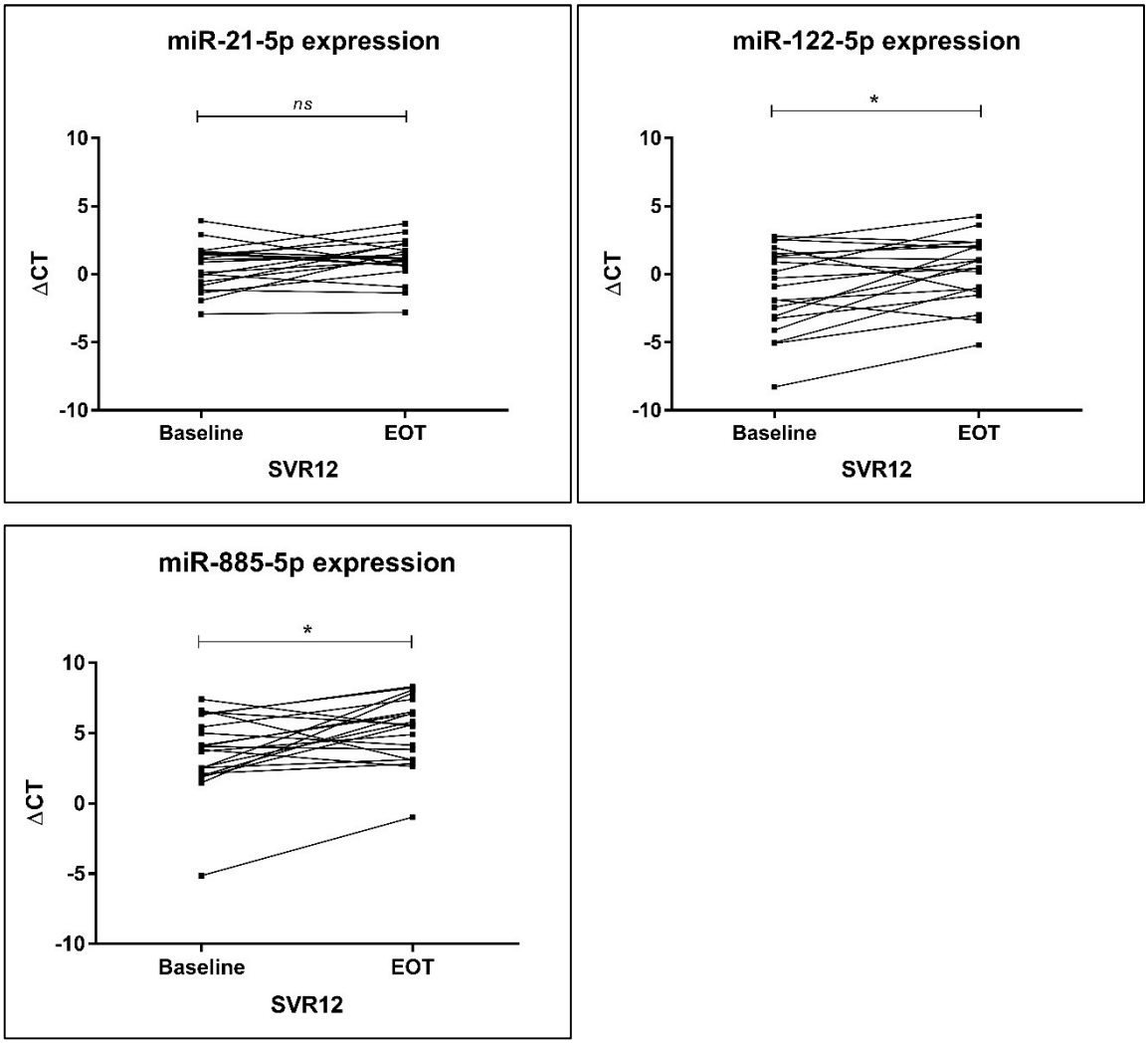


Figure 5-4: Before-after chart demonstrating expression of miR-21-5p, miR-122-5p and miR-885-5p at baseline and EOT in the serum of HCV-infected subjects who achieved viral eradication (SVR12). There was a significant decrease in miR-122-5p and miR-885-5p expression during treatment in patients who cleared HCV. MiR-21-5p expression was unchanged during treatment. *p<0.05; ns = non-significant.

We next explored changes in miRNA expression during treatment in subjects who experienced a negative clinical outcome despite viral eradication (SVR12-AE, figure 5-5). Neither miR-122-5p, nor miR-885-5p were significantly altered during treatment in these subjects. In contrast, miR-21-5p was significantly upregulated (p=0.029). However, the pattern of miRNA regulation over the course of treatment appeared highly variable in this group.

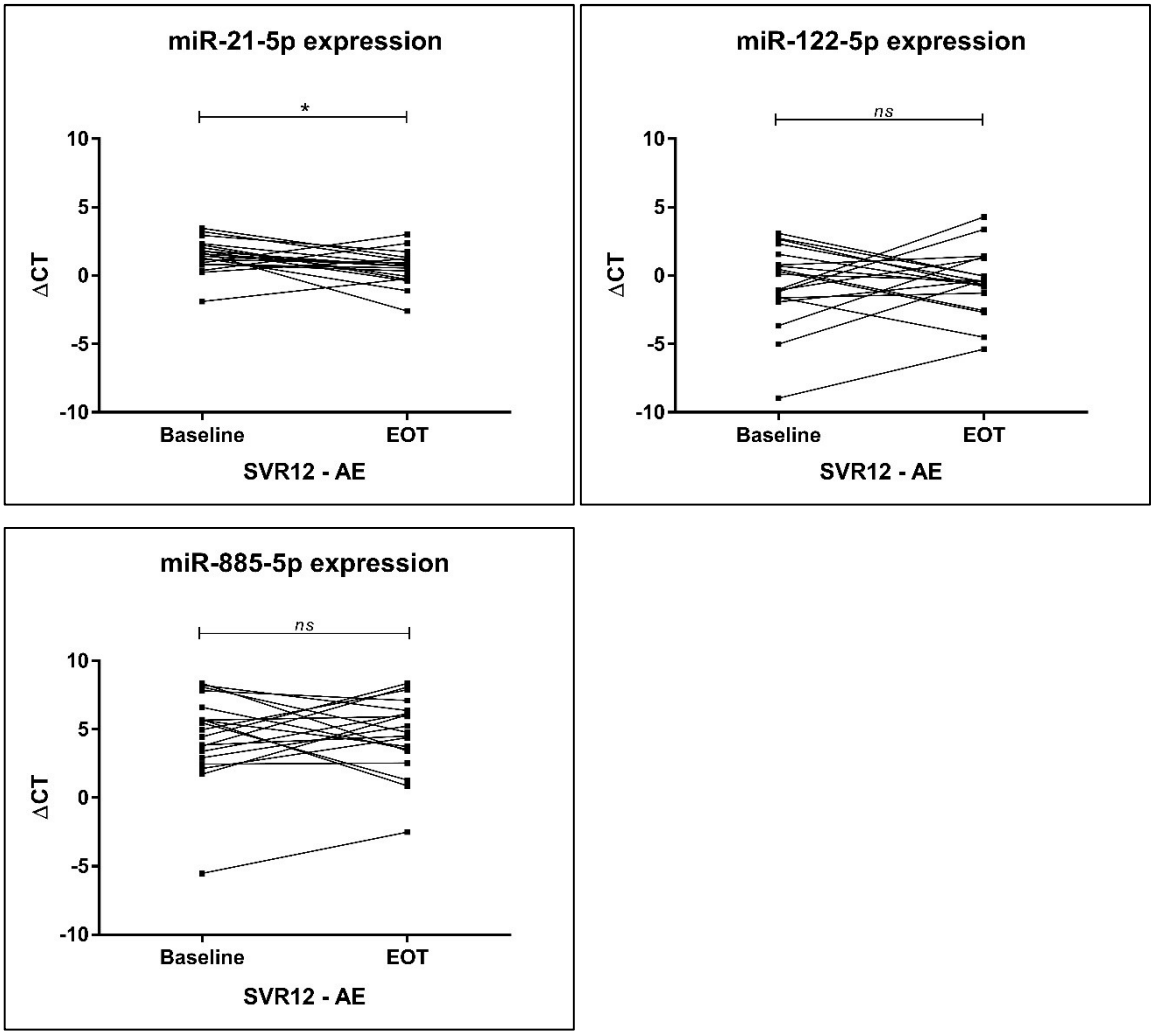


Figure 5-5: Before-after chart demonstrating expression of miR-21-5p, miR-122-5p and miR-885-5p at baseline and EOT in the serum of HCV-infected subjects who experienced an adverse clinical event during treatment (SVR12-AE). There was a significant increase in miR-21-5p expression during treatment in patients who experienced a negative clinical outcome. *p<0.05; ns = non-significant.

All 3 miRNAs were significantly downregulated between baseline and EOT in subjects who subsequently relapsed (figure 5-6), and the pattern of miRNA deregulation appeared more consistent.

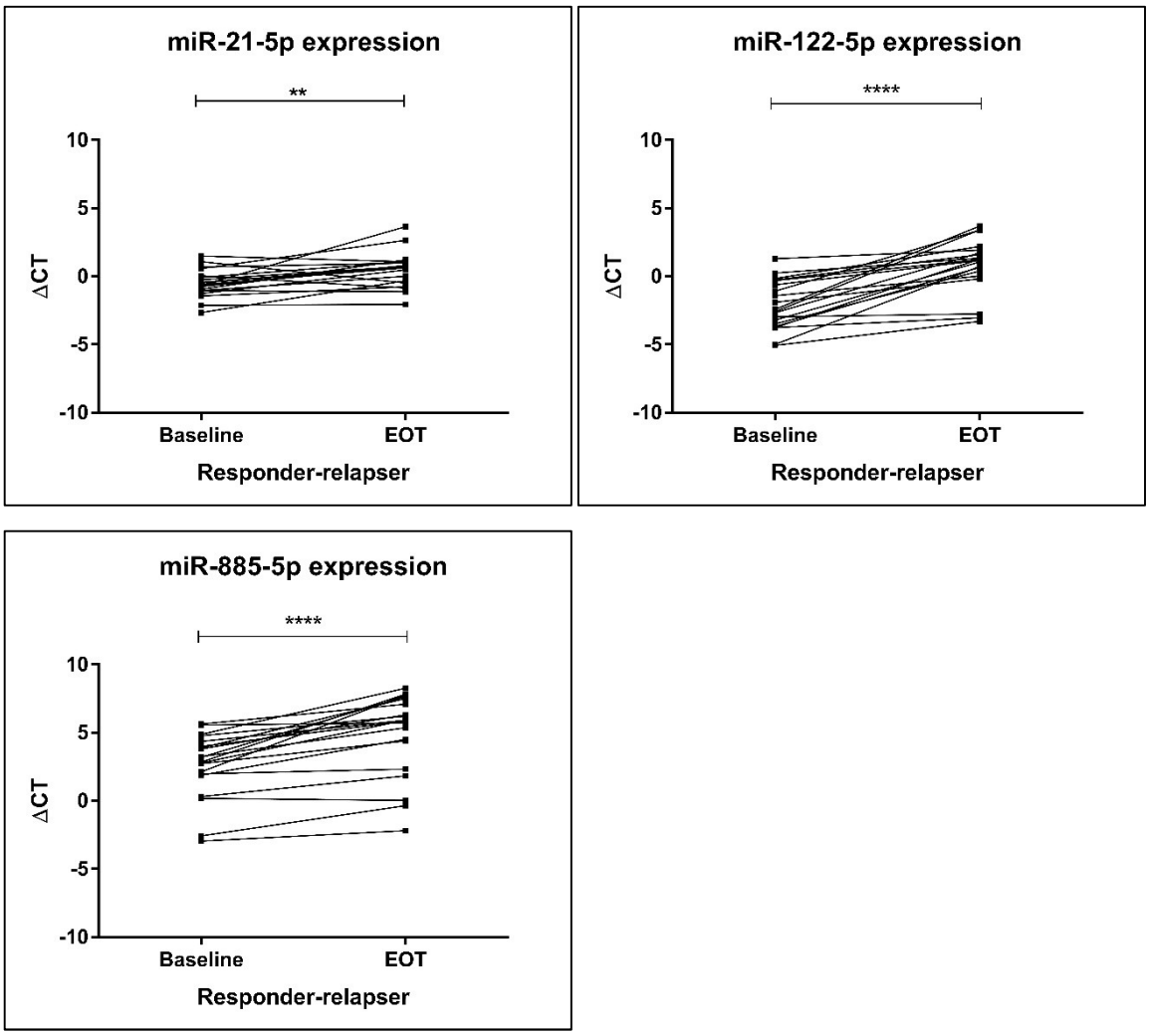


Figure 5-6: Before-after chart demonstrating expression of miR-21-5p, miR-122-5p and miR-885-5p at baseline and EOT in the serum of HCV-infected subjects who relapsed following EOT. There was a significant decrease in miR-21-5p, miR-122-5p and miR-885-5p expression during treatment in subjects who subsequently relapsed. *p<0.05; ****p<0.0001.

5.3.3 Baseline miRNA expression predicts virologic relapse

We next compared the expression of the selected miRNAs at baseline in patients who achieved an SVR12 regardless of clinical outcome (n=39), and subjects who achieved an EOT response (EOTR) then relapsed (n=20). We found that RRs had significantly higher levels of miR-21-5p, miR-122-5p and miR-885-5p at baseline compared with patients who achieved virologic clearance (figure 5-7).

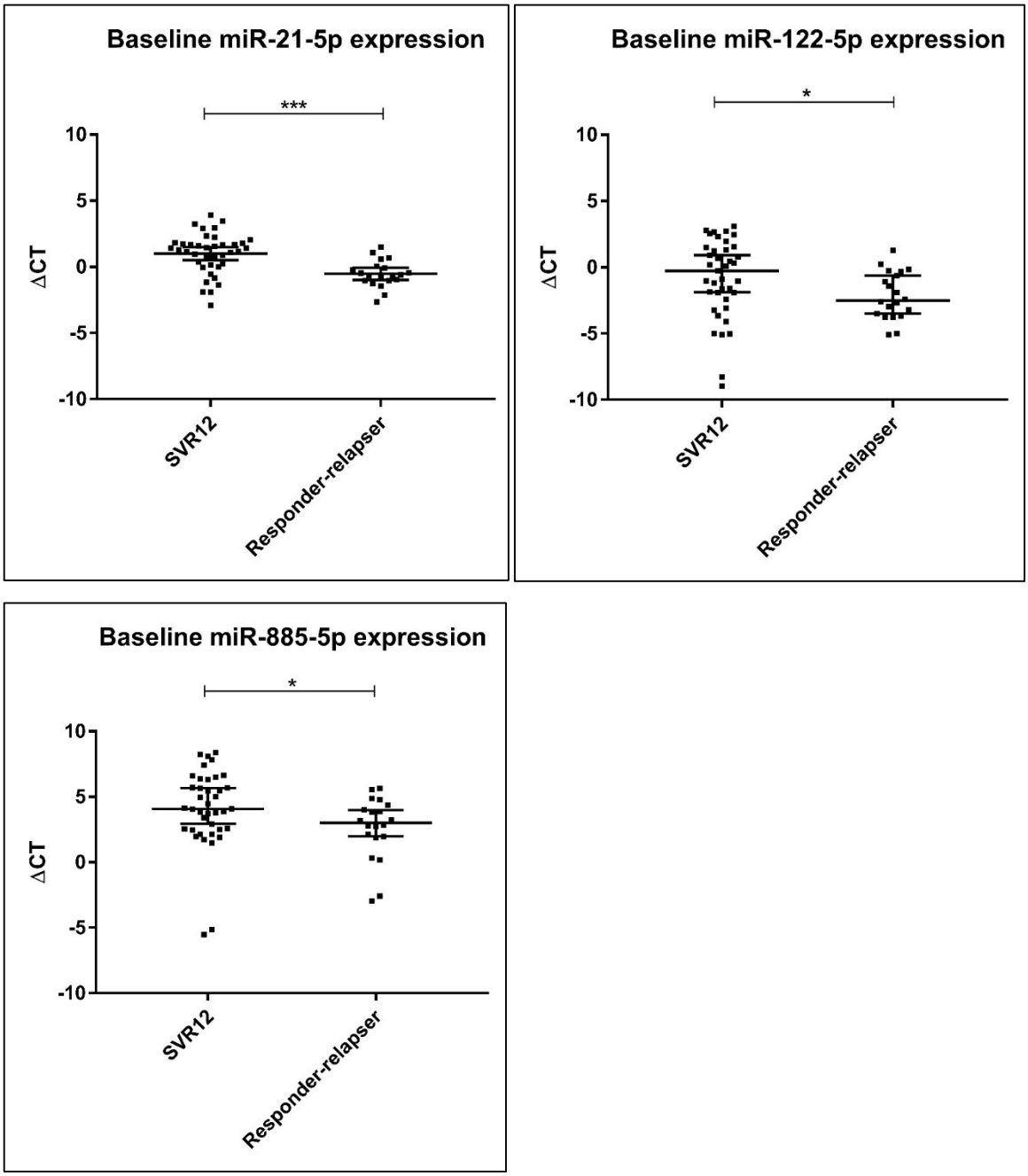


Figure 5-7: Expression of miR-21-5p, miR-122-5p and miR-885-5p at baseline in the serum of HCV-infected subjects who subsequently achieved a sustained virologic response (SVR12; n=39) or relapsed post end-of-treatment (n=20). All miRNAs demonstrated increased baseline expression in subjects who experienced virologic relapse post treatment. Dot plot graphs depict mean/median expression +/- 95% CI; *p<0.05, ***p<0.001.

As previously described (chapter 2.4), we used a two stage approach to identify factors associated with relapse in our cohort. Univariate statistical tests were performed to assess key risk factors for potential associations (chapter 2.3). Multiple logistic regression analyses were then performed for factors significant at the 10% level in univariate analysis and models reviewed. As it was not possible to express miRNA expression with reference to a healthy control population, miRNA expression is expressed as either 'positive' or 'negative' dependent on the ΔCT relative to the mean/median of the cohort. MiRNA expression is defined as positive when the ΔCT was lower than the mean/median (i.e. increased expression) and negative when the ΔCT is higher than the mean/median.

Choice of NS5A inhibitor and use of RBV did not differ significantly between groups (SVR and RR). Additionally, the prescription of DAAs was similar when stratified by genotype. The majority of the subjects in each group received a ledipasvir containing regimen: of the Gt3-infected subjects in the RR group (n=16), 37.5% received daclatasvir (n=6), whereas of the Gt3-infected patients who achieved SVR (n=15), 40% received daclatasvir (n=6). Of the Gt1-infected individuals who achieved SVR (n=23), and who relapsed (n=4), only a minority received daclatasvir (n=4, 17.4% and n=1, 25%, respectively).

In univariate analysis (table S15), infection with HCV Gt2 or Gt3 was significantly associated with likelihood of relapse when compared to infection with Gt1 or Gt4 (80% vs 20%; p=0.003). Higher baseline MELD score (median score 13 vs 11, p=0.006) was also significantly associated with relapse. Finally, baseline miR-21-5p positivity was significantly associated with chance of relapse (80% in the RR group vs 20% in the SVR12 group, p<0.001). There was a non-significant trend towards increased chance of relapse with miR-885-5p and miR-122-5p positivity at baseline (p=0.081). There were no differences in gender, age at start of treatment, ethnicity or HCV viral load between groups.

There was significant correlation between miR-21-5p, miR-885-5p and miR-122-5p expression, all of which were significant at the 10% level in univariate analysis. To avoid collinearity bias, models were fitted using each miRNA independently and in combination, and found that inclusion of miR-21-5p resulted in the best fit, as would be expected from the univariate analysis. A

model containing baseline miR-21-5p positivity and HCV genotype correctly classified 81% of cases and was statistically significant (X^2 26.1, p<0.001), explaining 50% of the variation in treatment outcome. Inclusion of baseline MELD did not significantly improve the fit of the model (p=0.404); baseline miR-21-5p and HCV genotype remained significantly associated with relapse when accounting for MELD and viral genotype (table S16).

In conclusion, Gt2- or Gt3-infected patients were significantly more likely to relapse than Gt1- or Gt4-infected subjects (OR 7.90, 95% CI: 1.7 – 36.2, p=0.008). Positive miR-21-5p expression at baseline was highly associated with relapse post EOTR (OR 16.9, 95% CI: 3.5 – 80.9, p<0.001). A ROC curve analysis was performed using predicted probabilities for the final model and demonstrated an AUC 0.861 (95% CI: 0.766 – 0.956, p<0.001).

Our data suggest that baseline miR-21-5p expression, independent of HCV genotype (and MELD), is highly predictive of virologic relapse following DAA therapy and could be used to guide decisions regarding treatment length or intensity. It would be interesting to see retreatment data on these patients to ascertain whether negative baseline predictive factors could be overcome with treatment prolongation or intensification, as is seen with baseline RASs (Sarrazin, 2016).

5.3.4 MiRNA expression predicts adverse clinical outcome during DAA therapy

Given the concerns about the risk of *de novo* and recurrent HCC development in subjects with HCV-related cirrhosis undergoing DAA treatment, and the idea of a 'point of no return' beyond which subjects fail to benefit from antiviral therapy, we were keen to identify a prognostic biomarker to identify such patients. To that end, we compared baseline expression of miR-21-5p, miR-122-5p and miR-885-5p in patients who achieved an SVR12, and those who achieved an SVR12 but experienced a negative clinical outcome. A negative clinical outcome was defined as worsening MELD score (≥ 2 points), a decompensation event, hospitalisation, the development of a malignancy or death.

We did not find any significant differences in baseline expression of miR-21-5p, miR-122-5p or miR-885-5p between the two groups (figure 5-8). However, we had previously shown that there is a significant increase in miR-21-5p ($p=0.029$) expression over the course of treatment in subjects who experienced an adverse clinical event (SVR12-AE). In contrast, miR-885-5p ($p=0.888$) and miR-122-5p ($p=0.837$) did not change significantly over the course of treatment in this group (figure 5-5). This in itself is interesting, as both miR-885-5p and miR-122-5p were significantly downregulated during treatment in the other patient groups (SVR12 and RR; figure 5-4 and figure 5-6), and may indicate that changes in the expression of miR-21-5p, miR-122-5p and miR-885-5p are driven by liver injury rather than HCV replication. However, the change in miRNA expression over the course of treatment in subjects who experienced negative clinical outcomes was highly variable, perhaps reflecting the amalgamated criteria used for the definition of SAE.

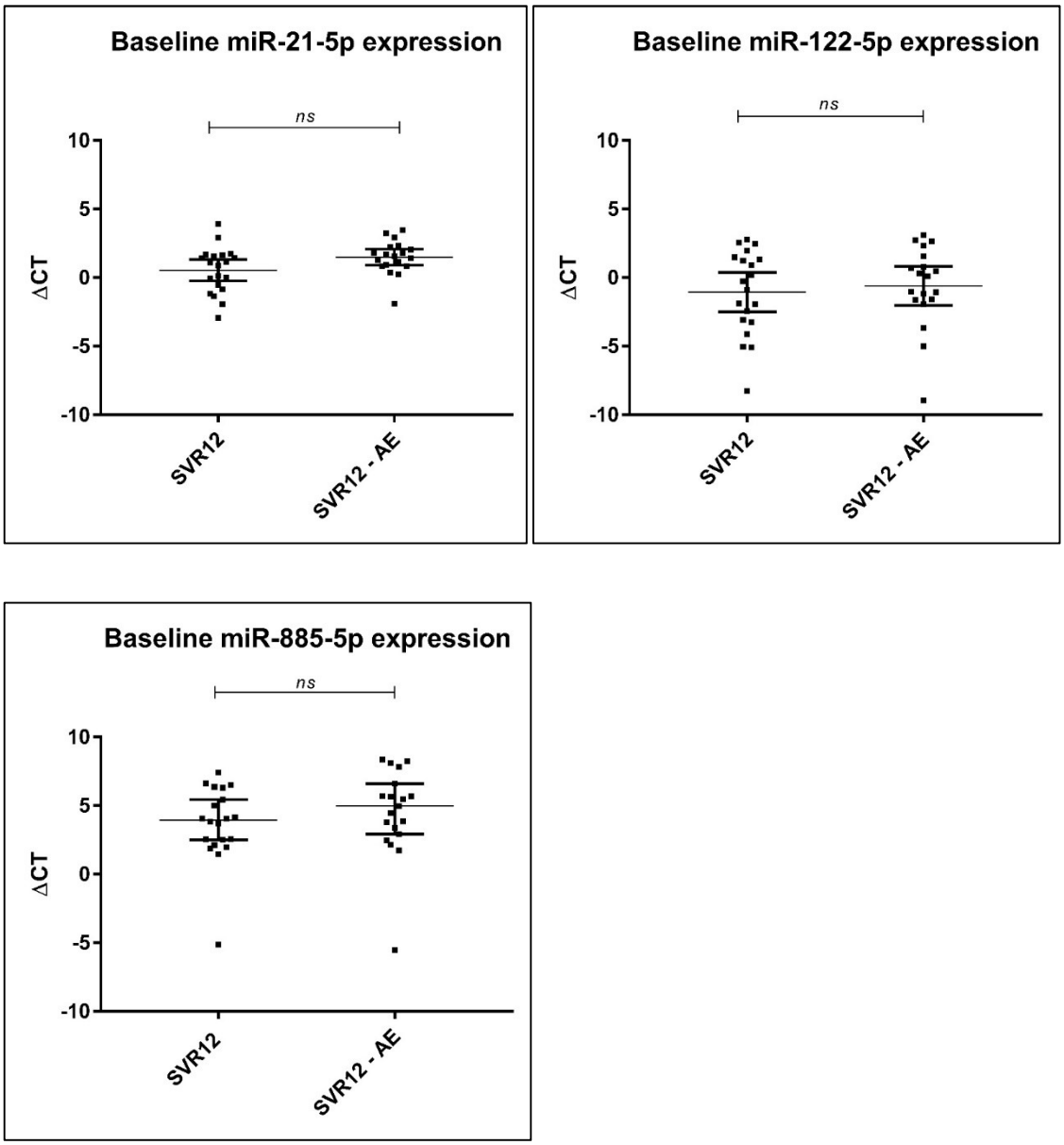


Figure 5-8: Expression of miR-21-5p, miR-122-5p and miR-885-5p in the serum of HCV-infected subjects who achieved an SVR12 compared with those who achieved an SVR12 but experienced an adverse clinical event during treatment. There were no differences in baseline miRNA expression between groups. Dot plot graphs depict mean/median expression +/- 95% CI; ns=non-significant.

In order to identify further potential prognostic biomarkers, we pooled sera from patients who achieved an SVR (SVR12, n=20), and those who achieved virologic suppression but experienced an adverse event (SVR12-AE, n=19), and used TaqMan® MicroRNA Arrau Cards to profile baseline miRNA expression in each group. Only 1 additional miRNA (miR-139-5p; RQ 3.96, p=0.031) was found to be significantly deregulated at baseline in the serum of patients who experienced an adverse outcome (figure 5-9). However, miR-345-5p was also upregulated (RQ 3.10, p=0.326) and a decision was made to explore both these miRNAs further as biomarkers.

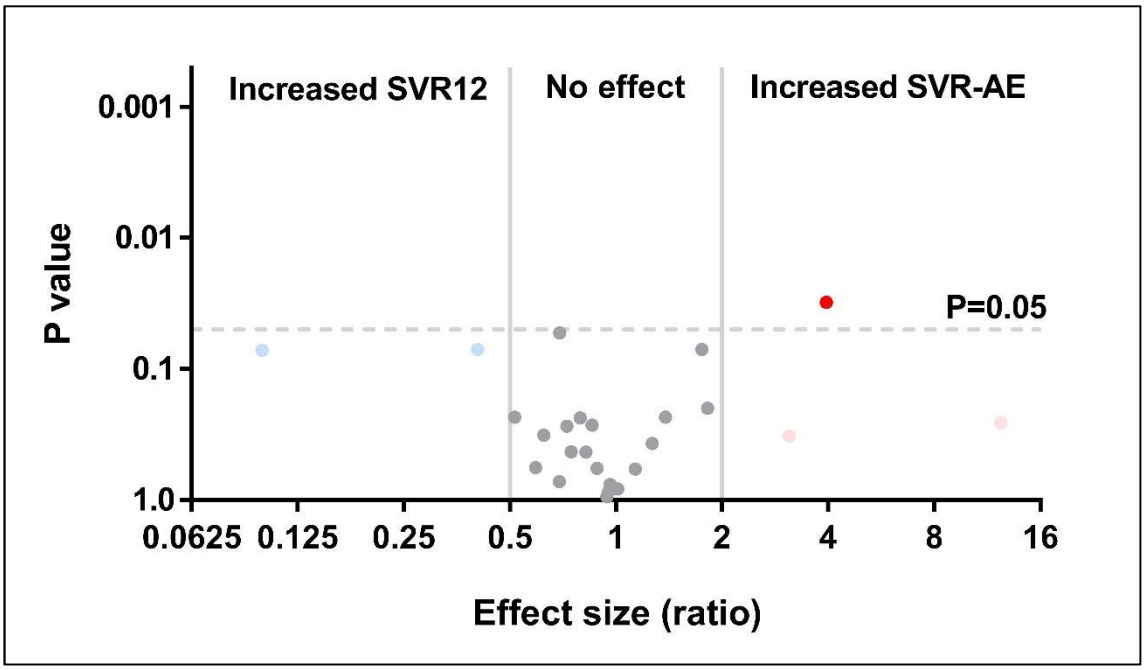


Figure 5-9: Volcano plot demonstrating p value against fold change for miRNA deregulated in the serum of chronically HCV-infected subjects who experienced a negative clinical outcome during treatment compared with subjects who achieved SVR12 with no adverse event. Data points in red indicate miRNAs upregulated > 2 fold in CHC, and those in blue indicate > 2 fold downregulation. Only miR-139-5p was significantly upregulated.

MiR-139-5p was not reliably expressed during the validation experiments and further investigation was abandoned. However, baseline miR-345-5p expression was shown to be significantly higher in patients who experienced a negative clinical outcome with treatment (figure 5-10).

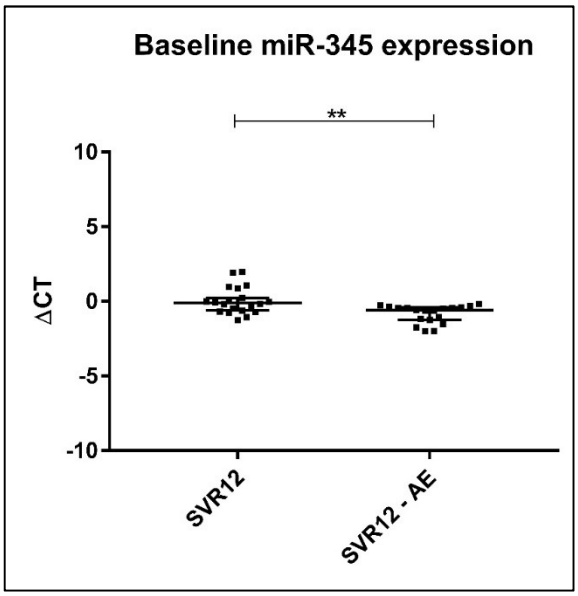


Figure 5-10: Baseline expression of miR-345 in the serum of HCV-infected subjects who achieved an SVR12 compared with those who achieved an SVR12 but experienced an adverse clinical event during treatment. MiR-345 was significantly increased at baseline in the serum of subjects who experienced a negative clinical outcome. Dot plot graph depicts mean/median expression +/- 95% CI; **p<0.01.

We went on to explore baseline factors associated with negative clinical outcomes in more detail. In univariate analysis (table S17), the presence of DM 2 was significantly associated with an adverse outcome during treatment (p=0.022). Baseline miR-21-5p negativity (i.e. expression lower than the cohort mean) was also significantly associated with adverse outcome (p=0.048). However, we found that low miR-21-5p expression at baseline was significantly associated with upregulation of miR-21-5p over the course of treatment (p=0.004), suggesting an interaction between the two variables. Neither miR-122-5p nor miR-885-5p expression at baseline was significantly associated with individual or global adverse outcome.

Higher miR-345 expression was significantly associated with adverse outcome during treatment (p=0.037). When looking at individual outcomes in more detail, we found that this finding was driven by the association between miR-345-5p and the development of malignancy (X^2=4.234, p=0.04). There was no association between baseline miR-345-5p expression and either MELD increase (X^2=0.672, p=0.412) or decompensation (X^2=1.699, p=0.428).

All patients who developed a malignancy (both HCC and non-HCC) during treatment in the SVR-AE group (n=4) were positive for miR-345-5p at baseline.

We also observed an upregulation of miR-21-5p during treatment in these patients. Our findings indicate that miR-345-5p may be a more sensitive biomarker of malignancy than miR-21-5p, although monitoring the expression of miR-21-5p during treatment may also aid diagnosis.

Interestingly, omission of RBV from the treatment regimen was significantly associated with adverse outcome (p=0.03). There were no significant differences in age, gender, ethnicity or HCV VL between groups. Choice of NS5A inhibitor was similar between groups. More detail on significant adverse events (SAEs) and baseline miRNA expression are shown in Table S18.

Our analysis suggested that an interaction between miR-345 and miR-21-5p was influencing the model. Models were therefore tested using each miRNA individually, and in combination, with baseline miR-21-5p negativity and miR-345-5p positivity indicating a positive event. This composite variable was more significantly associated with adverse outcome than either of the independent variables in univariate analysis (p=0.006). A crosstabulation was performed for the composite miRNA variable and the independent adverse events which demonstrated that the association between miRNA expression and the development of malignancy was again driving this association (X^2=7.131, p=0.008). There was no association between miRNA expression and either MELD increase (X^2=0.154, p=0.694) or decompensation (X^2=3.630, p=0.163).

A model containing composite baseline miRNA expression (OR 35.9, 95% CI: 3.5 – 366.6, p=0.003) and co-morbid DM 2 (OR 43.4, 95% CI: 3.4 – 561.2, p=0.004) correctly classified 82.1% of cases (table S19). This model was statistically significant (X^2=20.9, p<0.001) and explained 55.3% of variation in treatment outcome. The inclusion of RBV use did not significantly improve the model (p=0.206). A ROC curve analysis was performed using predicted probabilities for the final model including miRNA expression and DM 2, and demonstrated an AUC 0.845 (95% CI: 0.717 – 0.972, p=0.001).

MiR-345-5p and miR-21-5p were explored as individual biomarkers using ROC curve analysis. The AUC for miR-345-5p was 0.779 (95% CI: 0.630 – 0.928, p=0.003) and for miR-21-5p was 0.692 (95% CI: 0.524 – 0.860, p=0.04). This suggests that miR-345-5p may be a useful marker of adverse clinical outcome

during antiviral treatment, and is a more accurate predictor of SAE than miR-21-5p.

In conclusion, measuring baseline miR-345-5p and miR-21-5p expression may aid in risk stratification of patients at risk of adverse clinical outcomes during DAA therapy, specifically the development of malignancy, in addition to previously described co-morbidities. This is intriguing and suggests that monitoring miRNA expression during treatment may aid in the early diagnosis of cancer.

5.3.5 Functional analysis of miR-345-5p *in silico*

MiR-345-5 is not well defined in the literature. We identified only 3 validated target genes: CDKN1A, ABCC1 and NTRK3. Predicted protein-protein interactions are demonstrated below (figure 5-11). As with the other selected miRNAs, we found that KEGG pathways in cancer were highly enriched. The 5 most significantly enriched KEGG pathways are the cell cycle, hepatitis B, p53 signalling pathway, bladder cancer and glioma.

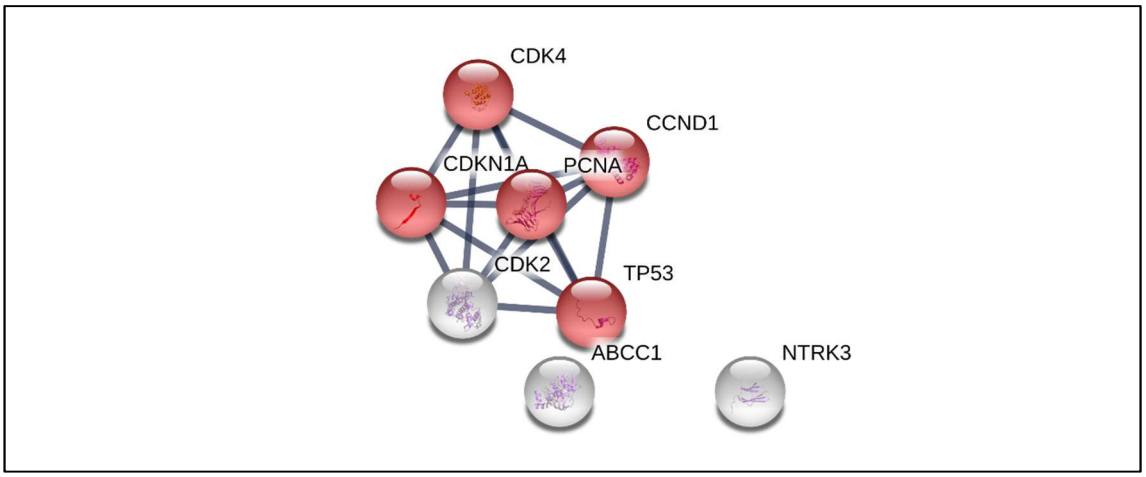


Figure 5-11: Predicted protein-protein interactions for miR-345-5p target genes. Networks are clustered according to the Markov cluster algorithm (inflation factor 3); strength of association is shown by the line thickness: dotted lines indicate weaker support. Proteins involved in the cell cycle pathway are highlighted in red.

5.4 Discussion

The objective of this study was to explore changes in extracellular miRNA expression during treatment of CHC with oral DAA regimens, and to identify novel prognostic biomarkers for adverse clinical and virologic outcomes during the treatment of CHC with DAAs. Overall, we found that the expression of miR-122-5p and miR-885-5p was significantly downregulated over the course of antiviral treatment, whereas miR-21-5p was unchanged. In contrast, when we looked only at patients who experienced negative clinical outcomes despite viral eradication, we found that the downregulation in miR-122-5p and miR-885-5p was abrogated, and miR-21-5p was upregulated.

We demonstrated that baseline serum levels of miR-21-5p, miR-885-5p and miR-122-5p were significantly higher in individuals who experienced viral rebound when compared to those who achieved SVR12. We found that a model containing miR-21-5p expression and HCV genotype accurately predicted virologic relapse following DAA treatment. Finally, we showed that upregulation of miR-21-5p together with baseline comorbid DM 2 and elevated miR-345-5p expression was significantly associated with the development of an adverse clinical outcome during antiviral therapy.

Elevated serum miR-885-5p expression has been proposed as a biomarker for cirrhosis and HCC in HCV-infected subjects (Gui et al., 2011). We previously observed elevated expression of miR-885-5p in the serum of HCV-infected individuals with cirrhosis, and found that miR-885-5p expression positively correlated with ALT (chapter 4.4). Serum miR-122-5p has also been shown to correlate with histologic necro-inflammatory activity and ALT (Dubin et al., 2014, Su et al., 2013), although we failed to demonstrate this association in our cohort. IFN-free antiviral therapy is associated with significant improvement in biochemical parameters, as well as a reduction in liver stiffness as measured by TE (Knop et al., 2016). The rapid improvement in liver stiffness measures by TE over the 12 week period of treatment suggest that these findings are largely due to resolution of necro-inflammation, and to a lesser extent regression of fibrosis. Improvement in histological necro-inflammatory activity has also been described in the HIV/HCV population following DAA therapy (Schwabl et al., 2017). The decrease in miR-885-5p and miR-122-5p levels detected during antiviral

treatment correlated with a significant reduction in ALT, and is concordant with a reduction in necro-inflammatory activity. The change in miR-122-5p expression during treatment may also correspond with the fall in HCV viral load, given the positive correlation previously observed (chapter 4.4.3). Conversely, neither MELD score nor miR-21-5p fell significantly over the course of treatment. MiR-21-5p has been described as a biomarker of fibrosis in a number of organs (Lorenzen et al., 2015, McClelland et al., 2015, Zhao et al., 2014), whilst the MELD score gives a reflection of liver synthetic function. A significant regression of cirrhosis would not be expected over 12 weeks, and the fact that these biomarkers remain static supports the earlier statement that the change in liver stiffness by TE predominantly reflects a reduction in inflammatory activity.

It has been demonstrated previously that SVR with an IFN-containing regime is associated with a significant decrease in serum miR-122-5p levels (Dubin et al., 2014). Concordant with our study, Waring et al. (2016) also found that miR-122 was significantly downregulated during DAA therapy, but that levels returned to baseline in subjects who relapsed or experienced virologic breakthrough. This is in line with our previous observation regarding the association between HCV viral load and miR-122-5p expression (chapter 4.4.3). The association between baseline miR-122-5p expression and treatment response is complex, and data are conflicting. Elevated serum miR-122 has been shown to correlate with the favourable INFL3 rs8099917 TT genotype, and to predict SVR with IFN-based therapy independently of host INFL3 genotype (Su et al., 2013), whilst lower hepatic miR-122-5p levels are described in non-responders to IFN-based therapy (Sarasin-Filipowicz et al., 2009). In contrast, Waidmann et al. (2012a) failed to demonstrate an association between serum miR-122-5p levels and treatment response. Waring and colleagues (2016) did not find that baseline serum miR-122-5p was predictive of SVR for either Gt1 or Gt3 infection, although the trend was for lower baseline serum miR-122 expression in subjects who achieved SVR, in line with our findings. However, they did find a significant positive association between serum miR-122-5p levels and SVR in Gt2 infected patients. Only one patient in our cohort was Gt2-infected; interestingly, this patient achieved an SVR and had very high baseline miR-122-5p expression (ΔCT -8.28). The association between HCV genotype, serum miR-122-5p expression and response to DAA therapy warrants further investigation in a larger cohort.

We found that Gt3 infection was significantly associated with relapse following EOTR. Our data mirror the findings in the wider EAP cohort; Foster and colleagues (2016) found that viral clearance was achieved in 209 of 231 Gt1-infected subjects (90.5%) compared with only 132 of 192 Gt3-infected subjects (68.8%). The association between Gt3 infection and poor outcome is unclear, but may relate to a combination of host and viral factors. Steatosis is more common in Gt3 infection, where it is thought to relate to direct effects of HCV core protein on lipid oxidation and LDL assembly (Rubbia-Brandt et al., 2000). Steatosis was found to be an independent predictor of relapse following IFN-based therapy in Gt3-infected subjects (Restivo et al., 2012), and may have contributed to relapse rates in our cohort. It is possible that the choice of NS5A inhibitor influenced the likelihood of virologic relapse: the majority of individuals in our cohort received a ledipasvir-containing combination, which is no longer recommended for individuals with Gt3 infection as a consequence of poorer treatment outcomes in comparison to other agents ('EASL Recommendations on Treatment of Hepatitis C 2016,' 2016). The use of less potent antiviral medications may have led to the development of NS5A resistance. However, the proportion of Gt3-infected subjects receiving each regimen was similar between treatment responders and RRs, so it is unlikely that choice of NS5A inhibitor contributed significantly to the observed association between genotype and relapse rates in this cohort.

In our study, we found that baseline miR-21-5p expression was highly predictive of virologic relapse post EOTR, even accounting for HCV genotype and MELD score. We have previously shown that miR-21-5p is induced by IFN-α and poly (I:C) stimulation, and that this signal was lost in an ISG15 knockout cell line. It has also been shown that higher basal ISG expression with higher levels of endogenous IFN (as seen with the unfavourable IFNL4 genotype ΔG) is associated with treatment failure in response to both IFN-containing and IFN-sparing antiviral regimens (Murakawa et al., 2017, Meissner et al., 2014). It is possible that the higher miR-21-5p reflects high basal ISG (and ISG15) expression, explaining the correlation between miR-21-5p expression and treatment outcomes. It has also been demonstrated that miR-21 has a proviral and immunomodulatory activity in CHC, promoting viral replication (Chen et al., 2013). Knockdown of miR-21 expression has been shown to sensitise cells to IFN-

induced antiviral activity against vesicular stomatitis virus (Yang et al., 2015), whereas upregulation of miR-21-5p is associated with promotion of an immunotolerant cellular environment. This may also contribute to the increased likelihood of virologic relapse in subjects with higher baseline miR-21-5p expression. Finally, as described previously, miR-21-5p is a recognised biomarker of fibrosis, and elevated levels may reflect more advanced hepatic fibrosis with consequent effect on treatment outcome.

We also explored baseline factors associated with the incidence of adverse clinical outcomes during treatment. We found that low baseline miR-21-5p expression, high miR-345-5p expression, comorbid DM 2 and omission of RBV from the treatment regimen were significantly associated with the development of an SAE during treatment in univariate analysis. The association between baseline miRNA expression and SAEs during treatment was driven by the association between baseline miR-345-5p and miR-21-5p and the development of malignancy.

MiR-345-5p is a promising prognostic biomarker. Upregulation of miR-345-5p has been described in association with a number of human cancers, including HCC (Cervigne et al., 2009, Guled et al., 2009). HCV core protein has been shown to induce miR-345 expression in a hepatoma cell line, resulting in suppression of p21 gene expression (Shiu et al., 2013). Deregulation of p21 expression is a frequently observed event in human cancers; p21 acts as tumour suppressor through negative regulation of the cell cycle. Protein p21 is transcriptionally activated by p53, and is able to bind to and inhibit the CDKs, resulting in cell cycle arrest (Abbas and Dutta, 2009). Interestingly, p21 may have both pro- and anti-apoptotic activity depending on cellular stresses, with paradoxical oncogenic activity. Functional analysis of protein-protein interactions for genes targeted by miR-345 showed that the p53 pathway was significantly enriched, and indicated an important role for miR-345 in regulation of the cell cycle. We found that miR-345 demonstrated increased abundance at baseline in all individuals who developed a cancer over the course of treatment, supporting its role as a biomarker of malignancy. We also observed an upregulation in miR-21-5p expression over the course of treatment in all patients who were diagnosed with malignancy, consistent with its role as an onco-miR in the literature (chapter 1.7.3).

The association between low baseline miR-21-5p expression and negative clinical outcome is less easy to rationalise, particularly as miR-345 and miR-21 are often co-expressed in tumour tissue (Cervigne et al., 2009). It is possible that low baseline serum miR-21-5p expression (reflecting low hepatic miR-21 expression) was associated with enhanced inflammatory activity in response to antiviral treatment. However, we also found that low miR-21-5p expression at baseline was significantly associated with an upregulation of miR-21-5p during treatment, suggesting an interaction between these variables.

The pattern of miR-21-5p deregulation was significantly different between treatment groups; miR-21-5p was upregulated over the course of treatment in 79% of subjects in the SVR12-AE group, compared with only 40% in the SVR12 group. MiR-21-5p upregulation has been described in the cerebral cortex of mice with hepatic encephalopathy (HE) secondary to acute liver failure (Vemuganti et al., 2014), and it has been proposed as a biomarker for inflammatory neurological disease in other animal models (Gaitero et al., 2016, Yelamanchili et al., 2015). It is possible that extracellular serum miR-21-5p levels may reflect aberrant miRNA expression in the brain tissue of subjects with HE. However, a rise in miR-21-5p during treatment was only observed in 2 of the 4 patients who developed encephalopathy during treatment, suggesting that serum miR-21-5p expression is an unreliable biomarker of encephalopathy. The association between miR-21, fibrosis and malignancy has been described in detail (chapter 1.7.3), and an upregulation in miR-21-5p was demonstrated in all subjects who developed a malignancy during treatment. The monitoring of miR-21-5p expression may improve the sensitivity of current HCC surveillance strategies in CHC, and warrants further exploration in a larger cohort.

We found that DM 2 was significantly associated with adverse clinical outcome during treatment, when controlling for miRNA expression and RBV use. Co-morbid DM 2 is significantly associated with the development of cirrhosis and decompensation in CHC (Huang et al., 2014). It has also been associated with an increased prevalence of HE in decompensated cirrhosis (Butt et al., 2013), as well as increased risk of HCC. In line with our study, Nahon and colleagues (2017) found that co-incident metabolic syndrome was associated with ongoing risk of HCC in HCV-infected subjects despite viral eradication. There is a high prevalence of NAFLD and non-alcoholic steatohepatitis (NASH) among individuals

with DM 2 (Firneisz, 2014), and it is conceivable that this contributed to the poorer clinical outcomes observed in diabetic subjects. Studies have shown that fasting glucose declines rapidly in diabetic HCV-infected subjects receiving DAAs (Pavone et al., 2016), and it is also possible that these metabolic changes may provoke hepatic decompensation events.

We found that omission of RBV from the treatment regimen was significantly associated with adverse clinical outcome in univariate analysis. However, RBV is contraindicated in subjects with decompensated cirrhosis and significant blood dyscrasia (Sulkowski et al., 2011). The omission of RBV may in fact have been a surrogate marker of advanced disease stage, which may explain the poorer clinical outcomes in these subjects.

As regards to the limitations of this study, it is possible that we have failed to adjust our model for other prognostic factors associated with adverse clinical and virologic outcomes. Our study would have benefited from the inclusion of data on BMI, given the correlation between BMI and disease progression identified in our earlier work (chapter 4.4.3). Information on the grade of hepatic steatosis would also have been valuable. It is possible that the association between co-morbid DM 2 and adverse clinical outcomes identified in this study is partly contributed to by an increased incidence of obesity and NAFLD in these patients, given the usual association of these features within the metabolic syndrome (Magliano et al., 2006). Information on host IFNL4 genotype and baseline RASs would also strengthen the study, and we hope to obtain these data in the future.

This study is retrospective and observational, and subjects were selected according to known treatment outcomes. Whilst we have attempted to control for confounding factors, the possibility of selection bias must be taken into consideration, and promising findings ideally require validation in a randomised controlled trial. As described previously (chapter 4), there was considerable inter-individual variance in miRNA expression, which together with the relatively small group sizes may have led to an underestimation of between-group differences. This also explains the wide confidence intervals seen in the statistical analysis. Finally, we were only able to identify 19 subjects in the SVR12-AE group who met the inclusion criteria. As the study was designed to

compare groups of ≥ 20 individuals, the final analysis of SAEs is underpowered, and findings must be interpreted with caution.

In conclusion, miRNAs may prove valuable prognostic biomarkers for treatment outcomes with DAA therapies. Measurement of selected miRNAs may aid in patient stratification prior to DAA initiation, and monitoring the pattern of miRNA deregulation over the course of treatment may provide useful information on underlying liver disease. Finally, we tentatively present miR-345 as a novel biomarker of both hepatic and extra-hepatic malignancy in CHC.

6 General discussion

In this study, we aimed to explore factors associated with disease progression in CHC using both local and national clinical data. We interrogated 3 cohorts of HCV-infected subjects together with healthy controls to identify factors associated with spontaneous clearance of CHC, progression of fibrosis, and clinical and virologic outcome in response to DAA therapy. We also characterised miRNA expression in the PBMCs and serum of HCV-infected individuals in order to explore the biological mechanisms underlying HCV pathogenesis, and to identify novel biomarkers for cirrhosis and for treatment outcomes with DAAs.

We found that spontaneous clearance of CHC occurred rarely in our local patient population, with an incidence rate of < 0.5 per 100-person-years follow up. In line with previous reports, clearance was associated with female gender, co-infection with HBV, younger chronological age at infection, and lower HCV RNA titres (Sagnelli et al., 2009, Scott et al., 2006, Gruener et al., 2002). We present the novel finding that spontaneous clearance was negatively associated with current IDU. We observed that a proportion of cases occurred in the context of significant intercurrent illness and possibly in the context of pregnancy, as had been described in the literature (Hattori et al., 2003, Zein et al., 2001, Mora-Peris et al., 2015).

We confirmed the findings in the literature that CHC is associated with a distinct miRNA expression profile in the serum and immune cells (Shrivastava et al., 2015, Chang et al., 2014), and observed that pathways in cancer were highly enriched by genes targeted by these miRNAs. Importantly, the data indicated that non-cirrhotic individuals with elevated biomarkers of immunosenescence behaved similarly to cirrhotic subjects, with implications for clinical practice and surveillance.

We demonstrated that HCV-infected subjects with cirrhosis had elevated PBMC levels of miR-16-5p, miR-155-5p, miR-221-3p and miR-885-5p compared to non-cirrhotic patients. MiR-16-5p and miR-155-5p are of particular interest, given their association with B cell dysfunction (Frasca et al., 2015) and haematological malignancy (Eis et al., 2005, Piluso et al., 2015). However, we also identified a number of issues with the PBMC analysis which limited our interpretation of the

data. We observed that PBMC storage method affected the quality of the RNA; RNA extracted from PBMCs cryopreserved in liquid nitrogen had evidence of increased degradation compared to RNA from PBMCs lysed in RLT buffer. We also found that a number of miRNAs were expressed at comparatively lower levels in cryopreserved PBMCs. Due to limited sample availability, we were required to use cryopreserved PBMCs for our analysis, and we hypothesise that poor RNA quality may have affected our miRNA expression analysis and contributed to our failure to identify a suitable endogenous control miRNA. The source of the problem with the cryopreserved PBMCs is not clear, but could arise from the presence of cellular RNAses in the samples. It is possible that our findings could also impact on transcriptomic studies with cryopreserved PBMCs, again resulting in data that may be difficult to interpret. We also observed considerable inter-individual variance in miRNA expression, which combined with the small cohort size, may have limited our ability to demonstrate between-group differences. Given the aforementioned limitations, a decision was made not to pursue further analysis using a larger bank of PBMC samples. It would be interesting to repeat the experiment in a larger cohort using freshly separated PBMCs which had been stored directly in lysis buffer, and an exogenous spike-in control.

We observed that miR-21-5p, miR-122-5p and miR-885-5p were upregulated in the serum of HCV-infected subjects compared to healthy controls. Unfortunately, we did not find that these miRNAs performed well as serum biomarkers of cirrhosis, although miR-885-5p demonstrated a trend towards increasing expression in cirrhotic subjects. We did however find that all of the selected miRNAs correlated weakly but significantly with CDKN2A mRNA expression, and we tentatively suggest that these miRNAs could be explored further as BoA in CHC.

Of the biomarkers tested, CDKN2 mRNA transcript expression was most significantly associated with cirrhosis; ALT level (a marker of hepatic inflammatory activity) was also significantly associated with cirrhosis. However, we found that chronological age and BMI were more strongly associated with disease stage than any of the biomarkers explored. Although these findings were ultimately disappointing, they remain important for clinical practice. Hepatic steatosis and BMI are associated with persistent hepatic necro-inflammatory activity in HIV/HCV co-infected subjects following HCV eradication (Schwabl et

al., 2017), and hepatic steatosis, together with age and hepatic fibrosis, has been associated with an increased incidence of HCC post SVR (Tanaka et al., 2007). At present, lifestyle modification and dietary advice represent the only interventions for hepatic steatosis outwith specific treatment for metabolic disorders (Nascimbeni et al., 2013), and should be implemented aggressively. One concern regarding the use of short course oral antiviral treatment for HCV (as opposed to 24-48 weeks IFN-based therapy) is that healthcare providers have less interaction with the patient, and have less opportunity to recommend public health interventions. Our findings highlight the need to counsel HCV-infected individuals to maintain a healthy weight; elevated BMI remained associated with cirrhosis in our cohort even having excluded obesity (chapter 4).

Interestingly, we also found that metabolic disorders were predictors of adverse clinical outcomes during antiviral treatment; co-morbid DM 2 was significantly associated with the development of an SAE. The presence of DM 2 may indicate more severe baseline liver disease, given its association with other features of the metabolic syndrome, including obesity and NAFLD, which have been associated with increased hepatic necro-inflammatory activity and incidence of HCC (Schwabl et al., 2017, Tanaka et al., 2007). Additionally, it is conceivable that decompensation/encephalopathy may be provoked by metabolic changes during treatment.

Circulating miR-122-5p and miR-885-5p levels were significantly downregulated over the course of DAA treatment, in concordance with serum ALT level. Neither miR-21-5p expression nor MELD score were significantly altered. However, in subjects who experienced a negative clinical outcome despite viral eradication, miR-122-5p and miR-885-5p levels were unchanged, and miR-21-5p was upregulated. This may indicate that serum miRNA expression is driven by host rather than viral factors, and prompted us to explore the use of models to study miRNA regulation *in vitro* (chapter 4.5). Interestingly, lower miR-21-5p expression at baseline was associated with the development of an SAE during treatment in univariate analysis. MiR-345-5p was also associated with adverse clinical outcome during treatment, and all individuals diagnosed with a malignancy during treatment (both HCC and extra-hepatic disease) were positive for miR-345-5p at baseline. This finding corresponds with reports in the literature describing upregulation of miR-345-5p in a number of human cancers

(Cervigne et al., 2009, Guled et al., 2009), and is supported by our functional analysis which indicates an important role for miR-345 in cancer pathways. Upregulation of miR-21-5p was also associated with the development of an SAE, and was observed in all patients subsequently diagnosed with cancer.

We used TaqMan® Advanced MicroRNA Assays to quantify selected serum and PBMC miRNA expression, and the RT-PCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems). TaqMan® RT-PCR reagents are used routinely in clinical laboratories together with the 7500 machine (Espy et al., 2006), and serum is readily obtainable from whole blood. As only 150 µL serum is required for RNA extraction (which can be automated), sufficient sample could be acquired in 1 draw at the pre-treatment clinic appointment when baseline bloods and HCV RNA levels are obtained. The use of miRNAs as biomarkers of virologic relapse or malignancy could therefore be easily incorporated into clinical practice. For instance, HCV-infected subjects with elevated miR-21-5p at baseline could be considered for treatment intensification or a prolonged treatment duration, and subjects with elevated miR-345-5p expression at baseline could be referred for more detailed liver imaging or enhanced hepatoma surveillance. Thus, monitoring of selected miRNAs could form part of routine surveillance, to aid in the early diagnosis of hepatic decompensation events.

The development of newer DAAs has revolutionised the treatment landscape in CHC, and treatment is associated with high SVR rates even in previously ‘hard-to-treat’ patient groups. As a consequence, biomarkers of treatment response in CHC may become increasingly redundant. However, there is ongoing concern regarding the risk of HCC post SVR with DAAs, as discussed previously (chapter 5.1). Data have suggested that there is an increased risk of *de novo* and recurrent HCC with all-oral DAA combinations, thought to relate to the loss of immune surveillance previously provided by IFN-containing regimens (Robert, 2016, Conti et al., 2016). Additionally, it has been demonstrated that a number of HCCs are missed through the current surveillance policies (Irving WL, personal communication, Aug 7, 2017). Although more recent meta-analyses have failed to demonstrate an association between DAA therapy and HCC incidence ('Lack of evidence of an effect of direct-acting antivirals on the recurrence of hepatocellular carcinoma: Data from three ANRS cohorts,' 2016), early

biomarkers of malignancy are urgently needed. MiR-345-5p was found to be elevated in all patients at baseline who subsequently developed a malignancy, and this finding warrants exploration in a larger patient cohort.

The miRNAs we have identified do not appear to be specific to HCV-related chronic liver disease. They may be relevant to other viral infections, as well as playing a role in non-viral liver disease. Pathway analysis suggested that the HBV, HTLV and EBV canonical pathways are significantly enriched by genes targeted by miRNAs deregulated in CHC. HBV can integrate into the host genome, driving oncogenic transformation and hepatocarcinogenesis (Zhao et al., 2016). MiR-21-5p is upregulated by HBV X protein, resulting in downregulation of PDCD4 and promoting oncogenesis (Qiu et al., 2013). MiR-122-5p (Wang et al., 2016) and miR-885-5p (Gui et al., 2011) are also upregulated in the serum of HBV-infected individuals. It would be of interest to explore whether these miRNAs correspond with disease severity and outcomes in non-HCV related liver disease.

There are a number of questions still to be answered before miRNA biomarkers are transferable as diagnostic tools ‘from bench to bedside’. For instance, this study would be strengthened by the inclusion of a larger cohort of healthy control patients. If miRNAs are to be developed as biomarkers for virologic and clinical outcomes during treatment, then a greater understanding of the ‘normal’ range of expression is required. There is significant inter-individual variation in both PBMC and miRNA expression, thus larger banks of patient samples are required. The UK Biobank (http://www.ukbiobank.ac.uk/about-biobank-uk/) is a rich repository of clinical and genetic data as well as serum samples for miRNA profiling, and represents one possible source of further samples. Additionally, since the EAP was introduced, the recommendations for CHC treatment in Scotland have been updated, with sofosbuvir-velpatasvir and elbasvir-grazoprevir now first line as treatment options (Dillon et al., 2017). It remains to be seen whether baseline miR-21-5p expression remains significantly associated with virologic relapse following EOTR with the newer DAAs. A similar question can be asked of the association between miR-345-5p and miR-21-5p expression and the risk of significant adverse event during treatment.

There were a number of limitations to the studies presented here. We were reliant on cohorts established by other researchers, with incomplete data and limited sample material available. In our study of spontaneous clearance of CHC (chapter 3), the volume of missing data meant that multivariate analysis was not deemed appropriate and statistical analysis must be interpreted with caution. Additionally, in the Outcomes study (chapter 4), the healthy control population consisted partly of non-HCV-infected subjects recruited from Glasgow hospitals. Although individuals taking lipid-lowering medications were excluded, we cannot exclude the possibility that other co-morbid conditions and lifestyle factors may have contributed to the miRNA profile in these subjects. This might have contributed to an underestimation of changes in miRNA expression in the HCV-infected population. In the same study, we found that cirrhotic patients were significantly older, and had significantly higher BMIs at baseline. Therefore, we cannot exclude the possibility that these baseline demographic factors contributed to the elevated miRNA expression in the PBMCs and serum of HCV-infected subjects with cirrhosis independently of HCV infection. We had explored the possibility of setting up a matched cohort of non-HCV infected individuals with chronic liver disease secondary to NAFLD to ascertain the contribution of HCV infection itself to the miRNA deregulation, but this was not deemed possible within the time constraints of the project. It would be of interest in the future to interrogate such a cohort, to establish whether miRNA deregulation in CHC was driven by HCV infection or chronic liver disease. The lack of miRNA response to viral eradication in subjects who experienced complications of their liver disease during treatment suggests the latter. Finally, as discussed earlier, cohorts in the EAP study (chapter 5) were categorised according to known treatment outcomes, and this may have resulted in selection bias. Ideally, the findings require validation in a larger, prospective, randomised controlled trial in which the investigators are blinded as to study outcome.

Our study lacks long-term follow up data. In my opinion, one of the most powerful observations of this study was that non-cirrhotic individuals with elevated biomarkers of immunosenescence demonstrated similar pathway enrichment to cirrhotic subjects. It would be of considerable interest to obtain long-term outcomes data for these patients to identify whether elevated biomarkers of immunosenescence were associated with more rapid disease

progression, or an increased incidence of HCC or extra-hepatic malignancy. Similarly, we would be keen to ascertain whether persistent elevation of miR-122-5p, miR-21-5p and miR-885-5p in the EAP cohort was associated with subsequent adverse clinical outcome. MiR-345-5p appears to be a promising biomarker for malignancy, and requires further investigation in a larger patient cohort. The STOP-HCV consortium (http://www.stop-hcv.ox.ac.uk/about-stop-hcv) was established with the aims of deriving patient stratification models to enhance clinical decision making, and of understanding disease mechanisms that define patient strata. Work Strand 5 of the STOP-HCV consortium focuses on the identification of serum biomarkers associated with treatment response and cirrhosis progression, and represents an exciting potential resource through which to explore these miRNAs further.

The association between chronological age at infection and likelihood of spontaneous clearance of CHC suggests that senescence may negatively impact likelihood of spontaneous clearance, as well as treatment outcomes and disease progression (Hoare et al., 2010b). It would be of interest to explore the association between immunosenescence, miRNA expression and spontaneous clearance in the future. Given the concordance between factors that influence disease progression and those we have found to be associated with spontaneous clearance of CHC, the prognostic biomarkers we have identified may also have a role in predicting likelihood of spontaneous clearance.

Finally, having developed an *in vitro* model to study the effect of IFN signalling on miRNA expression, it would be interesting to explore the effects of IFNα and poly (I:C) stimulation on expression levels of other selected miRNAs. This may provide further insight into the mechanisms underlying miRNA deregulation in CHC and the role played by host responses to infection. It is known that HCV-infected patients demonstrate constitutive induction of IFN-regulated genes (Robinson et al., 2015), which may also alter miRNA regulation. The preliminary study presented in chapter 4.5.1 is the first step in such an analysis. It provides an opportunity to link the IFN response to miRNA deregulation, and to explore the mRNA targets for deregulated miRNAs. This would allow validation of the *in silico* pathway analyses presented here and open new avenues to explore the underlying mechanism for host response to infection. Moreover, there is increasing availability of rich host genetic data on HCV-infected subjects (Ansari

et al., 2017), which could also be linked to miRNA profiles from our study and allow analysis of the impact of host genetic variation on miRNA deregulation.

In conclusion, we have identified a number of host and viral factors associated with disease progression and virologic outcomes in CHC. We found that host factors, including older chronological age and elevated BMI, were associated with the development of cirrhosis in CHC. Metabolic factors were also important in determining outcome following DAA therapy; a diagnosis of DM 2 was significantly associated with SAEs during treatment. Serum miR-21-5p expression and viral genotype were the most important predictors of DAA-related viral clearance of CHC, whilst patient demographic factors (female gender and younger chronological age at infection) were significantly associated with spontaneous clearance. Co-infection with HBV and low HCV RNA levels were also significantly associated with spontaneous clearance of CHC. We observed that all patients who were diagnosed with cancer in the EAP cohort had elevated miR-345-5p expression at baseline, and experienced an increase in miR-21-5p abundance during treatment. We suggest that monitoring selected miRNAs may aid in the early diagnosis of HCV-related malignancy, or hepatic morbidity, and may help identify HCV-infected individuals who require treatment prolongation or intensification. Our data suggest that non-cirrhotic HCV-infected individuals with high bio-age (as determined by PBMC CDKN transcript status) should be managed similarly to cirrhotic patients, and that lifestyle factors contributing to immunosenescence, for instance, elevated BMI and metabolic disorders, should be targeted aggressively as part of a multi-disciplinary approach to patient care.

Tables

Table S 1: Univariate analysis between case-control status and demographic/clinical factors

	Late spontaneous clearance (n=50)	Chronic infection (n=200)	P value
Male sex [n (%)]	19 (38)	129 (65)	**0.001**
Median age at diagnosis [years (IQR)]	29 (25-36)	33 (28-38)	**0.022**
Ethnic group [n (%)]			0.719
White	48 (96)	194 (97)	
Asian	2 (4)	6 (3)	
Risk group [n (%*)]			0.789
Intravenous drug use	41 (89)	161 (90)	
Other	5 (11)	17 (10)	
Unknown	4	22	
HCV GT [n (%*)]			0.713
1	7 (41)	61 (52)	
2	1 (6)	5 (4)	
3	9 (53)	52 (44)	
Unknown	33	82	
Serum HIV IgG [n(%*)]			0.518
Positive	2 (5)	3 (3)	
Negative	36 (95)	98 (97)	
Not tested	12	99	
Serum HBsAg [n (%*)]			**0.001**
Positive	5 (10)	0 (0)	
Negative	43 (90)	99 (100)	
Not tested	2	101	
Current IDU [n (%*)]			**0.034**
Yes	15 (38)	97 (56)	
No	25 (62)	76 (44)	
Unknown	10	27	
History of alcohol excess/ALD [n (%*)]			0.236
Yes	21 (47)	64 (36)	
No	24 (53)	109 (64)	
Unknown	5	27	
Cirrhosis [n (%*)]			0.238
Yes	13 (34)	34 (25)	
No	25 (66)	104 (75)	
Unknown	12	62	
Median duration of infection [months (IQR)]	50 (31-81)	50 (19-103)	0.854
HCV VL (IU/ml)			**<0.001**
Median	1000**	341142**	
Interquartile range	1000 - 83293	59496 - 1517864	

***Percentage related to the actually recorded data; missing data handled by listwise deletion.**
****Data on HCV VL only available for 19/50 (38%) patients and 138/200 (69%) patients respectively**

Table S 2: As per Table S1, but where cases are confined to 'confirmed clearers'

	'Confirmed' clearance (n=27)	Chronically infected (n=200)	P value
Male sex [n (%)]	10 (37)	129 (65)	**0.006**
Median age at diagnosis [years (IQR)]	29 (25-37)	33 (28-38)	0.142
Ethnic group [n (%)]			0.362
White	27 (100)	194 (97)	
Asian	0 (0)	6 (3)	
Risk group [n (%*)]			0.803
IDU	23 (92)	161 (90)	
Other	2 (8)	17 (10)	
Unknown	2	22	
HCV GT [n (%*)]			0.784
1	6 (55)	61 (52)	
2	0 (0)	5 (4)	
3	5 (45)	52 (44)	
Unknown	16	82	
Serum HIV IgG [n (%*)]			0.765
Positive	1 (4)	3 (3)	
Negative	23 (96)	98 (97)	
Not tested	3	99	
Serum HBsAg [n (%*)]			0.055
Positive	1 (4)	0 (0)	
Negative	26 (96)	99 (100)	
Not tested	0	101	
Current IDU [n (%*)]			0.126
Yes	9 (39)	97 (56)	
No	14 (61)	76 (44)	
Unknown	4	27	
History of alcohol excess/ALD [n (%*)]			0.500
Yes	11 (44)	64 (36)	
No	14 (56)	109 (64)	
Unknown	2	27	
Cirrhosis [n (%*)]			0.638
Yes	7 (29)	34 (25)	
No	17 (71)	104 (75)	
Unknown	3	62	
Median duration of diagnosis [months (IQR)]	46 (29-76)	50 (19-103)	0.593
HCV VL (IU/ml)			**0.001**
Median	1000**	341142	
Interquartile range	763 - 131242	59496 - 1517864	

***Percentage related to the actually recorded data; missing data handled by listwise deletion.**
****Data on HCV VL only available for 10/27 (37%) patients**

Table S 3: Baseline demographics of the Outcomes pilot study cohort

	Low bio-age (n=8)	High bio-age (n=8)	Cirrhotic (n=9)	Healthy control (n=8)
Male sex [n (%)]	7 (87.5)	6 (75.0)	5 (55.6)	6 (75)
Age [years (IQR)]	39.0 (33.0 – 42.0)	43.5 (38.8 - 47.5)	49.0 (41.5 – 55.0)	41.0 34.5 – 51.5)
Age at infection [years (IQR)]	17.0 (15.0 - 28.5)	18.0 (9.5 – 23.0)	24.0 (16.3 – 44.0)	-
Ethnicity [n (%)]				
White	8 (100)	8 (100)	9 (100)	8 (100)
Asian	0	0	0	0
Source [n (%*)]				-
IDU	6 (75.0)	4 (57.1)	5 (55.6)	
Other	2 (25.0)	3 (42.9)	1 (11.1)	
Unknown	0	1	3	
HCV Gt [n (%)]				-
1	4 (50)	4 (50)	5 (55.5)	
3	4 (50)	4 (50)	4 (44.5)	
BMI (kg/m^2)	23.9 (20.3 - 25.1)	24.9 (23.6 – 26.5)	27.4 (25.1 – 29.3)	24.1 (21.6 – 25.5)
Current alcohol excess [n (%)]	1 (12.5)	0 (0)	0 (0)	3 (37.5)
HCV VL [log_{10} IU/ml (IQR)]	6.2 (6.0 – 6.5)	6.2 (5.5 – 6.6)	5.7 (5.2 – 6.2)	-
ALT [IU/mL (IQR)]	74.5 (44.0 - 88.8)	76.0 (26.5 – 107.8)	89.0 (69.5 – 137.0)	21.5 (17.5 – 38.2)
Liver stiffness [kPa (IQR)]	6.2 (5.4 – 6.6)	5.1 (4.2 – 6.1)	31.4 (21.8 – 36.6)	-
CDKN2A [RQ (IQR)]	0.8 (0.7 - 1.0)	4.2 (3.1 – 4.7)	4.6 (3.6 – 5.3)	1.2 (0.8 – 1.4)
CDKN2B [RQ (IQR)]	1.0 (0.7 – 3.2)	4.5 (3.2 - 8.3)	7.9 (3.6 – 9.4)	1.3 (0.6 – 2.9)
ARF [RQ (IQR)]	0.8 (0.5 – 1.2)	7.6 (5.3 – 9.7)	9.7 (8.7 – 9.7)	1.3 (0.7 – 1.8)

***Percentage related to the actually recorded data; missing data handled by listwise deletion. IQR, interquartile range; IDU, injecting drug use; Gt, genotype; RQ, fold change.**

Table S 4: Baseline demographics of the expanded Outcomes study cohort

	HCV infected (n=36)	Cirrhotic (n=15)	Healthy control (n=10)
Male sex [n (%)]	30 (83)	9 (60)	6 (60)
Age [years (IQR)]	41.0 (37.3 – 46.8)	48.0 (41.0 – 54.0)	42.0 (33.0 – 48.0)
Age at infection [years (IQR)]	21.5 (17.0 -27.5)	21.5 (16.3 – 34.5)	-
Ethnicity [n (%)]			
White	35 (97.2)	14 (93.3)	6 (60)
Asian	1 (2.8)	1 (6.7)	4 (40)
Source [n (%*)]			-
IDU	26 (83.9)	8 (72.7)	
Other	5 (16.1)	3 (27.3)	
Unknown	5	4	
HCV Gt [n (%)]			-
1	20 (55.6)	7 (46.7)	
3	16 (44.4)	8 (53.3)	
BMI [kg/m^2 (IQR)]	24.3 (21.4 – 26.0)	27.4 (24.7 – 30.0)	24.3 (22.2 – 25.6)
Current alcohol excess [n (%)]	3 (8.3)	0 (0)	3 (30)
HCV VL [log$_{10}$ IU/ml (IQR)]	6.1 (5.2 – 6.5)	5.7 (5.2 – 6.3)	-
ALT [IU/mL (IQR)]	70.5 (45.0 – 98.8)	92.0 (77.0 – 144.0)	24.0 (17.0 – 44.0)
Liver stiffness [kPa (IQR)]	6.1 (5.0 -7.3)	27.2 (18.7 – 38.5)	-
CDKN2A [RQ (IQR)]	1.3** (1.1 – 3.0)	3.6*** (2.1 – 5.1)	1.1 (0.8 – 1.4)
CDKN2B [RQ (IQR)]	2.4** (1.1 – 4.6)	5.7*** (2.0 – 9.4)	1.1 (0.9 – 2.3)
ARF [RQ (IQR)]	1.4** (0.9 – 5.5)	8.8*** (2.5 – 9.7)	1.2 (0.9 – 1.7)

***Percentage related to the actually recorded data; missing data handled by listwise deletion. **Data only available for 27/36 (75%) patients ***Data available for 13/15 (86.7%) patients. IQR, interquartile range; IDU, injecting drug use; Gt, genotype; RQ, fold change with respect to healthy controls.**

Table S 5: Expression of miRNA in the PBMCs of HCV-infected subjects compared with healthy controls

MiRNA	Fold change	P-value
hsa-miR-136-3p	0.072	0.0001
hsa-miR-374b-3p	0.306	0.0001
hsa-miR-629-5p	2.294	0.0001
hsa-miR-16-5p	2.554	0.001
hsa-miR-193a-5p	2.774	0.001
hsa-miR-656-3p	0.184	0.001
hsa-miR-146b-3p	2.708	0.002
hsa-miR-323-3p	0.197	0.002
hsa-miR-155-5p	1.523	0.006
hsa-miR-374a-3p	0.242	0.006
hsa-miR-193b-3p	1.849	0.007
hsa-miR-26a-1-3p	0.263	0.008
hsa-miR-548c-5p	1.466	0.008
hsa-miR-532-5p	2.698	0.009
hsa-miR-1244	2.847	0.01
hsa-miR-410-3p	0.336	0.01
hsa-miR-629-3p	2.191	0.01
hsa-miR-720	*2.002*	*0.01*
hsa-miR-140-3p	2.14	0.011
hsa-miR-95-3p	2.095	0.011
hsa-miR-376a-3p	0.349	0.012
hsa-miR-520f-3p	1.414	0.015
mmu-miR-93-5p	1.434	0.017
hsa-miR-199a-3p	0.591	0.022
hsa-miR-642a-5p	1.66	0.022
hsa-miR-362-5p	1.551	0.023
hsa-miR-590-3p	2.404	0.023
hsa-miR-1270	0.585	0.026
hsa-miR-1248	2.224	0.027
hsa-miR-148b-5p	1.732	0.028
hsa-miR-151-5p	0.346	0.028
hsa-miR-365a-3p	1.987	0.028
hsa-miR-628-3p	1.915	0.029

hsa-miR-146b-5p	1.717	0.033
hsa-miR-222-3p	1.73	0.033
hsa-miR-345-5p	1.738	0.033
hsa-miR-138-5p	2.271	0.034
hsa-miR-502-3p	1.56	0.034
hsa-miR-29a-3p	1.853	0.037
hsa-miR-411-5p	0.415	0.037
hsa-miR-500a-5p	2.117	0.037
hsa-miR-589-3p	2.354	0.038
hsa-miR-548d-5p	1.374	0.039
hsa-miR-20b-5p	1.322	0.04
hsa-miR-29b-2-5p	1.503	0.045
hsa-miR-744-3p	0.39	0.047
hsa-miR-597-5p	0.344	0.049
hsa-miR-9-5p	0.711	0.049
hsa-miR-140-5p	1.624	0.049
hsa-miR-28-3p	1.113	0.051
hsa-miR-548J-5p	2.15	0.052
hsa-miR-34a-3p	1.938	0.057
hsa-miR-301a-3p	0.682	0.061
hsa-miR-574-3p	1.349	0.065
hsa-miR-223-3p	1.468	0.069
hsa-miR-24-3p	1.346	0.069
hsa-miR-16-1-3p	0.469	0.084
hsa-miR-616-5p	0.521	0.091
hsa-miR-449a	2.745	0.092
hsa-miR-589-5p	1.299	0.094
hsa-miR-139-5p	0.577	0.097
hsa-miR-17-5p	1.253	0.101
hsa-miR-645	2.351	0.101
hsa-miR-184	1.186	0.106
hsa-miR-329-3p	0.516	0.107
hsa-miR-27a-5p	1.863	0.108
hsa-miR-30a-3p	0.368	0.109
hsa-miR-191-5p	1.252	0.11
hsa-miR-376a-5p	0.129	0.116

hsa-miR-192-3p	1.407	0.118
hsa-miR-29c-3p	2.172	0.118
hsa-miR-454-3p	1.648	0.118
hsa-miR-181a-5p	1.281	0.12
hsa-miR-1300	*1.434*	*0.121*
hsa-miR-145-3p	0.3	0.122
hsa-miR-27b-3p	0.594	0.122
hsa-miR-146a-5p	1.239	0.123
hsa-miR-26a-2-3p	0.34	0.129
hsa-miR-378	1.998	0.132
hsa-miR-127-3p	0.539	0.134
hsa-miR-451a	2.308	0.134
hsa-miR-126-3p	0.594	0.137
hsa-miR-550a-5p	1.763	0.139
hsa-miR-103a-3p	1.143	0.141
hsa-miR-495-3p	0.528	0.144
hsa-miR-505-5p	1.207	0.15
hsa-miR-376b-3p	1.198	0.152
hsa-miR-551b-3p	0.505	0.16
hsa-miR-369-3p	0.535	0.164
hsa-miR-152-3p	0.647	0.168
hsa-miR-370-3p	0.399	0.169
hsa-miR-15a-3p	0.391	0.174
hsa-miR-199b-5p	0.587	0.178
hsa-miR-942-5p	1.134	0.179
hsa-miR-372-3p	2.766	0.182
hsa-miR-20a-5p	1.112	0.184
hsa-miR-154-3p	0.42	0.189
hsa-let-7g-3p	1.212	0.192
hsa-miR-515-3p	0.891	0.199
hsa-miR-30d-3p	0.479	0.2
hsa-miR-337-5p	0.558	0.201
hsa-miR-487b-3p	0.615	0.202
hsa-miR-890	1.341	0.202
hsa-miR-10a-5p	1.186	0.209
hsa-miR-411-3p	0.372	0.213

hsa-miR-432-5p	0.515	0.214
hsa-miR-302b-3p	2.484	0.226
hsa-miR-361-5p	1.085	0.23
hsa-miR-624-5p	0.399	0.235
hsa-miR-335-3p	0.351	0.239
hsa-miR-324-3p	1.197	0.246
hsa-miR-330-3p	1.25	0.248
hsa-miR-652-3p	1.272	0.256
hsa-miR-543	0.635	0.259
hsa-miR-1a-3p	0.559	0.276
hsa-miR-192-5p	1.18	0.278
hsa-miR-93-3p	1.87	0.28
hsa-miR-27a-3p	0.786	0.287
hsa-miR-671-3p	1.086	0.288
hsa-miR-339-3p	1.163	0.297
hsa-miR-630	0.912	0.299
hsa-miR-181a-2-3p	0.458	0.3
hsa-miR-367-3p	0.177	0.302
hsa-miR-29a-5p	0.617	0.312
hsa-miR-17-3p	0.469	0.324
rno-miR-29c-5p	0.959	0.335
hsa-let-7c-5p	1.045	0.338
hsa-miR-635	1.514	0.339
hsa-miR-125a-3p	0.734	0.34
hsa-miR-409-3p	0.609	0.347
hsa-miR-377-5p	3.994	0.356
hsa-miR-221-3p	0.742	0.371
hsa-miR-548E-3p	1.735	0.379
hsa-miR-409-5p	0.805	0.381
hsa-miR-489-3p	0.987	0.388
hsa-miR-18a-3p	1.277	0.391
hsa-miR-320a	1.006	0.392
hsa-miR-542-5p	6.165	0.396
hsa-miR-376c-3p	0.506	0.399
hsa-miR-766-3p	0.637	0.402
hsa-miR-135b-5p	2.34	0.411

hsa-miR-580-3p	0.535	0.419
hsa-miR-503-5p	0.788	0.428
hsa-miR-590-5p	0.748	0.433
hsa-miR-491-5p	1.182	0.433
hsa-miR-206	0.685	0.435
hsa-miR-128-3p	1.117	0.439
hsa-let-7f-2-3p	0.818	0.447
hsa-miR-618	1.053	0.455
hsa-miR-99b-5p	0.721	0.455
hsa-miR-204-5p	0.715	0.462
hsa-miR-31-3p	1.332	0.462
hsa-miR-494-3p	0.948	0.466
hsa-miR-744-5p	1.132	0.467
hsa-miR-1282	0.599	0.469
hsa-miR-570-3p	1.074	0.473
hsa-miR-218-5p	1.21	0.476
hsa-miR-493-3p	0.717	0.479
hsa-miR-224-5p	0.694	0.497
hsa-miR-1227-3p	1.322	0.528
hsa-miR-374a-5p	1.03	0.533
hsa-miR-106b-5p	1.263	0.545
hsa-miR-30d-5p	0.794	0.548
hsa-miR-195-5p	0.938	0.549
hsa-miR-125b-5p	0.875	0.554
hsa-miR-186-5p	1.367	0.56
hsa-miR-19b-3p	1.25	0.566
hsa-miR-190a-5p	0.656	0.569
hsa-miR-381-3p	0.671	0.575
hsa-miR-520c-3p	1.119	0.577
hsa-miR-30c-5p	0.975	0.578
hsa-miR-92a-1-5p	0.67	0.59
hsa-miR-1271-5p	0.705	0.601
hsa-miR-545-3p	0.8	0.601
hsa-miR-21-5p	1.321	0.626
hsa-miR-203-3p	0.637	0.631
hsa-let-7e-3p	0.634	0.644

hsa-miR-424-5p	1.008	0.648
hsa-let-7i-3p	0.781	0.65
hsa-miR-185-5p	0.904	0.655
hsa-miR-320B	1.128	0.658
hsa-miR-340-3p	0.84	0.66
hsa-miR-875-5p	0.395	0.675
hsa-miR-182-5p	1.031	0.677
hsa-miR-133a-3p	0.686	0.678
hsa-miR-331-3p	0.998	0.68
hsa-miR-19b-1-5p	1.01	0.693
hsa-miR-30b-5p	1.181	0.696
hsa-miR-28-5p	1.148	0.705
hsa-miR-29b-3p	0.739	0.711
hsa-miR-654-3p	0.923	0.711
hsa-miR-221-5p	1.077	0.731
hsa-miR-425-5p	0.914	0.732
hsa-miR-144-5p	0.659	0.733
hsa-miR-100-5p	0.879	0.739
hsa-miR-101-3p	1.241	0.742
hsa-miR-1254	1.126	0.745
hsa-miR-340-5p	0.911	0.757
hsa-miR-302a-3p	0.857	0.762
hsa-miR-19a-3p	0.851	0.767
hsa-miR-425-3p	0.756	0.767
hsa-miR-539-5p	0.652	0.772
hsa-miR-190b	1.073	0.786
hsa-miR-125a-5p	0.913	0.811
hsa-miR-197-3p	0.858	0.835
hsa-miR-375	0.888	0.853
hsa-miR-130b-3p	1.124	0.857
hsa-miR-598-3p	1.088	0.862
hsa-miR-876-3p	0.922	0.863
hsa-miR-142-3p	1.632	0.864
hsa-miR-518f-3p	0.744	0.87
hsa-miR-18a-5p	1.033	0.871
hsa-miR-362-3p	1.277	0.884

hsa-miR-181a-3p	0.758	0.903
hsa-miR-148a-3p	1.171	0.904
hsa-miR-30e-3p	1.079	0.905
hsa-miR-20b-3p	1.05	0.915
hsa-miR-20a-3p	0.916	0.924
hsa-miR-548b-5p	1.186	0.926
hsa-miR-26b-5p	1.062	0.927
hsa-miR-550a-3p	0.752	0.964
hsa-miR-19a-5p	0.652	0.98
hsa-miR-142-5p	1.11	0.988
hsa-miR-191-3p	0.949	0.997

Table S 6: PBMC miRNA expression in HCV Gt3 infection compared with HCV Gt1 infection

MiRNA	Fold change	P-value
hsa-miR-202-3p	0.004	0.09
hsa-miR-515-3p	0.004	0.127
hsa-miR-532-5p	0.034	0.0001
hsa-miR-28-3p	0.119	0.016
hsa-miR-323a-3p	0.146	0.051
hsa-miR-142-3p	0.158	0.001
hsa-miR-451a	0.164	0.002
hsa-miR-369-5p	0.168	0.028
hsa-miR-671-3p	0.184	0.0001
hsa-miR-193b-3p	0.204	0.0001
hsa-miR-362-3p	0.226	0.013
hsa-miR-374b-5p	0.232	0.009
hsa-miR-886-3p	0.239	0.057
hsa-miR-142-5p	0.248	0.008
hsa-miR-424-5p	0.253	0.003
hsa-miR-10a-5p	0.268	0.044
hsa-miR-296-5p	0.273	0.001
hsa-miR-193a-5p	0.277	0.0001
hsa-miR-148a-3p	0.28	0.0001
hsa-miR-320a	0.283	0.002
hsa-miR-324-3p	0.291	0.0001
hsa-miR-423-5p	0.303	0.0001
hsa-miR-375	0.324	0.011
hsa-miR-328-3p	0.326	0.0001
hsa-miR-551b-3p	0.34	0.064
hsa-miR-652-3p	0.358	0.001
hsa-miR-502-3p	0.362	0.0001
hsa-miR-597-5p	0.367	0.012
hsa-let-7c-5p	0.373	0.008
hsa-miR-31-5p	0.373	0.216
hsa-miR-574-3p	0.375	0.012
hsa-miR-345-5p	0.383	0.004
hsa-miR-148b-3p	0.393	0.011

hsa-miR-744-5p	0.398	0.0001
hsa-miR-886-5p	0.398	0.185
hsa-miR-369-3p	0.405	0.08
hsa-miR-376c-3p	0.41	0.059
hsa-miR-30b-5p	0.418	0.0001
hsa-miR-339-3p	0.418	0.025
hsa-miR-29a-3p	0.426	0.026
hsa-miR-197-3p	0.426	0.199
hsa-miR-21-5p	0.43	0.011
hsa-miR-19b-3p	0.441	0.007
hsa-miR-181a-5p	0.443	0.013
hsa-miR-222-3p	0.446	0.048
hsa-miR-150-5p	0.458	0.015
hsa-miR-331-3p	0.46	0.002
hsa-miR-532-3p	0.46	0.066
hsa-miR-106b-5p	0.462	0.001
hsa-miR-92a-3p	0.462	0.002
hsa-miR-221-3p	0.463	0.003
hsa-miR-372-3p	0.473	0.203
hsa-miR-324-5p	0.481	0.0001
hsa-miR-642a-5p	0.482	0.003
hsa-miR-146b-5p	0.482	0.079
hsa-miR-484	0.484	0.005
hsa-miR-589-5p	0.486	0.002
hsa-miR-101-3p	0.49	0.016
hsa-miR-25-3p	0.491	0.0001
hsa-miR-330-3p	0.492	0.019
hsa-miR-140-5p	0.498	0.031
hsa-miR-629-5p	0.499	0.002
hsa-miR-598-3p	0.507	0.012
hsa-miR-409-5p	0.514	0.117
hsa-miR-125b-5p	0.521	0.0001
hsa-miR-485-3p	0.528	0.016
hsa-miR-146a-5p	0.537	0.039
hsa-miR-27a-3p	0.548	0.063
hsa-miR-367-3p	0.55	0.201

hsa-miR-301a-3p	0.551	0.002
hsa-miR-3633p	0.552	0.004
hsa-miR-182-5p	0.562	0.087
hsa-miR-431-5p	0.565	0.395
hsa-miR-18a-5p	0.566	0.029
hsa-miR-223-3p	0.571	0.09
hsa-miR-127-3p	0.578	0.077
hsa-miR-196b-5p	0.584	0.046
hsa-miR-491-5p	0.603	0.092
hsa-miR-29c-3p	0.606	0.043
hsa-miR-200c-3p	0.606	0.106
hsa-miR-128-3p	0.616	0.102
hsa-miR-495-3p	0.616	0.323
hsa-miR-100-5p	0.617	0.954
hsa-miR-93-5p	0.619	0.036
hsa-miR-24-3p	0.621	0.018
hsa-miR-30c-5p	0.622	0.002
hsa-miR-185-5p	0.64	0.009
hsa-miR-342-3p	0.643	0.22
hsa-miR-103a-3p	0.644	0.012
hsa-miR-192-5p	0.651	0.123
hsa-miR-15b-5p	0.653	0.036
hsa-let-7d-5p	0.658	0.025
hsa-miR-20a-5p	0.658	0.1
hsa-miR-95-3p	0.674	0.115
hsa-miR-155-5p	0.679	0.13
hsa-miR-106a-5p	0.68	0.057
hsa-let-7e-5p	0.685	0.073
hsa-miR-381-3p	0.69	0.114
hsa-miR-545-3p	0.695	0.249
hsa-miR-152-3p	0.699	0.864
hsa-let-7g-5p	0.702	0.128
hsa-miR-191-5p	0.705	0.11
hsa-miR-199a-3p	0.725	0.035
hsa-miR-186-5p	0.734	0.073
hsa-miR-28-5p	0.738	0.054

hsa-miR-301b-3p	0.738	0.519
hsa-miR-654-3p	0.741	0.454
hsa-miR-138-5p	0.755	0.389
hsa-miR-17-5p	0.758	0.123
hsa-miR-361-5p	0.761	0.553
hsa-miR-16-5p	0.766	0.078
hsa-miR-758-3p	0.766	0.179
hsa-miR-370-3p	0.768	0.101
hsa-miR-376b-3p	0.776	0.274
hsa-miR-890	0.789	0.345
hsa-miR-27b-3p	0.794	0.443
hsa-miR-362-5p	0.797	0.1
hsa-miR-329-3p	0.808	0.339
hsa-miR-425-5p	0.839	0.559
hsa-miR-374a-5p	0.84	0.235
hsa-miR-20b-5p	0.842	0.238
hsa-miR-454-3p	0.843	0.114
hsa-miR-579-3p	0.857	0.836
hsa-miR-125a-5p	0.903	0.871
hsa-miR-335-5p	0.907	0.75
hsa-miR-29b-3p	0.916	0.813
hsa-miR-224-5p	0.973	0.529
hsa-miR-337-5p	0.974	0.507
hsa-miR-708-5p	0.99	0.914
hsa-miR-26a-5p	1.003	0.772
hsa-miR-548c-5p	1.016	0.811
hsa-miR-655-3p	1.077	0.778
hsa-miR-411-5p	1.087	0.839
hsa-miR-487b-3p	1.11	0.742
hsa-miR-410-3p	1.136	0.455
hsa-miR-493-3p	1.141	0.945
hsa-miR-340-5p	1.151	0.772
hsa-miR-218-5p	1.165	0.978
hsa-miR-140-3p	1.349	0.667
hsa-miR-518f-3p	1.387	0.714
hsa-miR-139-5p	1.417	0.512

hsa-miR-126-3p	1.422	0.548
hsa-miR-376a-3p	1.472	0.401
hsa-miR-135a-5p	1.474	0.769
hsa-miR-19a-3p	1.516	0.209
hsa-miR-590-5p	1.561	0.221
hsa-miR-193a-3p	1.576	0.596
hsa-miR-628-5p	1.595	0.969
hsa-miR-539-5p	1.605	0.793
hsa-miR-548d-5p	1.757	0.026
hsa-miR-302b-3p	1.826	0.468
hsa-miR-618	1.843	0.035
hsa-miR-627-5p	1.843	0.281
hsa-miR-338-3p	1.844	0.535
hsa-miR-520f-3p	2.05	0.052
hsa-miR-548b-5p	2.45	0.066
hsa-miR-889-3p	2.477	0.55
hsa-miR-26b-5p	2.496	0.041
hsa-miR-133a-3p	2.576	0.508
hsa-miR-141-3p	2.662	0.242
hsa-miR-422a	2.756	0.01
hsa-miR-494-3p	3.175	0.005
hsa-miR-302a-3p	3.339	0.085
hsa-miR-576-3p	3.518	0.192
hsa-miR-1-3p	3.637	0.047
hsa-miR-145-5p	3.723	0.225
hsa-miR-184	3.754	0.436
hsa-miR-190a-5p	4.346	0.0001
hsa-miR-195-5p	4.947	0.0001
hsa-miR-548c-3p	5.645	0.001
hsa-miR-136-5p	11.159	0.502
hsa-miR-483-5p	11.225	0.004
hsa-miR-885-5p	11.522	0.499

Table S 7: Univariate analysis between clinical factors and PBMC biomarkers in cirrhotic and non-cirrhotic HCV-infected subjects in the pilot study cohort

	Non-cirrhotic (n=16)	Cirrhotic (n=9)	P value
Male sex [n (%)]	13 (81.3)	5 (55.6)	0.170
Age [years (IQR)]	40.5 (38.3 – 45.8)	49 (41.5 – 55.0)	**0.012**
Age at infection [years (IQR)]	17 (12.5 – 25.5)	24 (16.3 – 44.0)	0.179
Duration of infection [years (IQR)]	22 (16.3 – 38.5)	30 (13.5 – 48)	0.677
Source [n (%*)]			0.688
IDU	10 (66.7)	5 (83.3)	
Other	5 (33.3)	1 (16.7)	
Unknown	1	3	
BMI [kg/m^2 (IQR)]	24.6 (23.4 – 25.5)	27.4 (25.1 – 29.3)	**0.027**
HCV VL [log_{10} IU/ml (IQR)]	6.2 (5.9 – 6.5)	5.7 (5.2 – 6.2)	0.057
ALT [IU/mL (IQR)]	74.5 (37.8 – 96.0)	89 (69.5 – 137)	0.207
CDKN2A [RQ (IQR)]	1.3 (0.8 – 4.3)	4.6 (3.6 – 5.3)	**0.007**
CDKN2B [RQ (IQR)]	3.4 (0.9 – 4.6)	7.9 (3.6 – 9.4)	**0.019**
ARF [RQ (IQR)]	2 (0.8 – 7.9)	9.7 (8.7 – 9.7)	**0.006**
miR-16-5p positive [n (%)]	5 (31.2)	8 (100)*	**0.001**
miR-155-5p positive [n (%)]	6 (37.5)	9 (100)	**0.002**
miR-221-3p positive [n (%)]	3 (18.8)	8 (88.9)	**0.001**
miR-21-5p positive [n (%)]	11 (68.8)	4 (44.4)	0.234
miR-885-5p positive [n (%)]	4 (25)	8 (88.9)	**0.002**
miR-195-5p positive [n (%)]	10 (62.5)	2 (22.2)	0.053

***Percentage related to the actually recorded data; missing data handled by listwise deletion. IQR, interquartile range; IDU, injecting drug use; Gt, genotype; RQ, fold change with respect to healthy controls.**

Table S 8: Expression of miRNA in the serum of HCV-infected subjects compared with healthy controls

MiRNA	Fold change	P-value
hsa-miR-122-5p	4.186	0.0001
hsa-miR-192-5p	4.003	0.0001
hsa-miR-193a-5p	2.9	0.0001
hsa-miR-193b-3p	3.816	0.0001
hsa-miR-21-5p	2.254	0.0001
hsa-miR-30d-5p	3.2	0.0001
hsa-miR-34a-3p	3.954	0.0001
hsa-miR-875-5p	6.47	0.0001
hsa-miR-885-5p	8.571	0.0001
hsa-miR-320a	1.569	0.001
hsa-miR-375	3.55	0.001
hsa-let-7c-5p	1.694	0.002
hsa-miR-1291	3.009	0.003
hsa-miR-363-3p	1.921	0.003
hsa-miR-455-5p	3.288	0.003
hsa-miR-625-5p	2.787	0.004
hsa-miR-671-3p	0.475	0.004
hsa-miR-411-3p	0.351	0.005
hsa-miR-125b-5p	1.952	0.008
hsa-miR-224-5p	2.289	0.009
hsa-miR-654-3p	0.358	0.009
hsa-miR-99b-3p	2.834	0.009
hsa-miR-200b-3p	3.53	0.011
hsa-miR-146b-3p	3.155	0.012
hsa-miR-720	*0.637*	*0.013*
hsa-miR-1244	3.823	0.014
hsa-miR-126-5p	5.183	0.019
hsa-miR-26a-1-3p	2.938	0.019
hsa-miR-16-5p	1.64	0.021
hsa-miR-362-3p	1.963	0.021
hsa-miR-100-5p	1.975	0.024
hsa-miR-483-5p	5.771	0.024
hsa-miR-423-5p	1.328	0.029

hsa-miR-26b-5p	1.468	0.031
hsa-miR-429	1.579	0.031
hsa-miR-424-3p	1.634	0.035
hsa-miR-362-5p	1.807	0.036
hsa-miR-182-5p	2.586	0.037
hsa-miR-130b-3p	1.396	0.039
hsa-miR-486-5p	0.363	0.042
hsa-miR-19a-3p	1.28	0.044
hsa-miR-133a-3p	1.309	0.046
hsa-miR-590-5p	1.349	0.046
hsa-miR-150-5p	1.429	0.047
hsa-miR-186-5p	1.499	0.047
hsa-miR-194-5p	1.817	0.049
hsa-miR-93-3p	0.435	0.049
hsa-miR-598-3p	1.402	0.05
hsa-miR-365a-3p	2.053	0.051
hsa-miR-191-3p	0.594	0.054
hsa-miR-328-3p	0.731	0.054
hsa-miR-95-3p	1.305	0.055
hsa-miR-660-5p	1.501	0.076
hsa-miR-454-5p	0.241	0.079
hsa-miR-181c-5p	3.119	0.085
hsa-miR-190a-5p	1.692	0.086
hsa-miR-645	0.456	0.09
hsa-miR-183-5p	1.404	0.093
hsa-miR-576-3p	0.604	0.094
hsa-miR-214-5p	0.516	0.109
hsa-miR-148a-3p	1.773	0.118
hsa-miR-22-5p	1.2	0.132
hsa-miR-152-3p	1.563	0.137
hsa-miR-485-3p	0.488	0.14
hsa-miR-195-5p	1.298	0.141
hsa-miR-16-1-3p	0.604	0.146
hsa-miR-345-5p	1.26	0.158
hsa-miR-505-5p	1.343	0.161
hsa-miR-181a-2-3p	0.639	0.163

hsa-miR-296-5p	1.285	0.163
hsa-miR-101-3p	1.466	0.164
hsa-miR-301a-3p	1.457	0.165
hsa-miR-629-5p	1.445	0.169
hsa-miR-155-5p	1.167	0.17
hsa-miR-134-5p	1.442	0.17
hsa-miR-203a-3p	1.812	0.172
hsa-miR-184	1.463	0.173
hsa-miR-1300	*0.409*	*0.174*
hsa-miR-511-5p	1.916	0.174
hsa-miR-1274A	*0.336*	*0.18*
hsa-miR-1227-3p	0.493	0.185
hsa-miR-487b-3p	0.605	0.188
hsa-miR-130a-3p	1.327	0.189
hsa-miR-10a-5p	1.528	0.192
hsa-miR-424-5p	0.592	0.192
hsa-miR-616-3p	2.263	0.194
hsa-miR-29c-3p	2.164	0.197
hsa-miR-590-3p	0.386	0.197
hsa-miR-548a-3p	1.526	0.2
hsa-miR-185-5p	1.08	0.203
hsa-miR-146a-5p	1.288	0.205
hsa-miR-486-3p	0.172	0.208
hsa-miR-605-5p	0.634	0.222
hsa-miR-26b-3p	0.592	0.224
hsa-miR-103a-3p	0.78	0.227
hsa-miR-190b	1.298	0.236
hsa-miR-27b-3p	1.469	0.239
hsa-miR-616-5p	0.636	0.244
hsa-miR-148b-3p	0.687	0.256
hsa-miR-628-5p	0.604	0.258
hsa-miR-206	1.57	0.261
hsa-miR-29a-3p	1.227	0.264
hsa-miR-335-3p	1.343	0.265
hsa-miR-145-3p	0.585	0.266
hsa-miR-338-3p	2.519	0.269

hsa-miR-597-5p	0.486	0.272
hsa-miR-320b	1.356	0.277
hsa-miR-139-3p	1.149	0.28
hsa-miR-9-3p	1.032	0.28
hsa-miR-370-3p	0.593	0.281
hsa-miR-18a-3p	0.441	0.286
hsa-miR-30d-3p	1.816	0.287
hsa-miR-339-3p	1.369	0.298
hsa-miR-579-3p	0.972	0.301
hsa-miR-491-5p	0.85	0.302
hsa-let-7e-5p	0.745	0.306
hsa-miR-548d-5p	2.178	0.31
hsa-miR-574-3p	1.008	0.311
hsa-miR-10b-3p	1.027	0.319
hsa-miR-548c-5p	1.5	0.324
hsa-miR-886-3p	*0.711*	*0.326*
hsa-miR-629-3p	1.039	0.328
hsa-miR-1262	0.767	0.335
hsa-miR-18a-5p	0.704	0.336
hsa-miR-223-3p	0.708	0.347
hsa-miR-744-5p	0.792	0.349
hsa-miR-301b-3p	1.228	0.353
hsa-miR-636	0.621	0.357
hsa-miR-31-5p	1.222	0.366
hsa-miR-411-5p	0.829	0.367
hsa-miR-886-5p	*2.099*	*0.367*
hsa-miR-652-3p	1.282	0.371
hsa-miR-484	1.081	0.374
hsa-miR-1285-3p	0.242	0.376
hsa-miR-543	0.62	0.378
hsa-miR-548b-5p	0.375	0.384
hsa-miR-93-5p	1.044	0.386
hsa-miR-218-5p	1.221	0.389
hsa-miR-515-3p	0.258	0.389
hsa-miR-30e-3p	1.431	0.393
hsa-miR-140-3p	0.959	0.395

hsa-miR-140-5p	0.977	0.395
hsa-miR-19b-1-5p	0.67	0.396
hsa-miR-642a-5p	0.9	0.403
hsa-miR-766-3p	0.883	0.403
hsa-miR-379-5p	1.745	0.409
hsa-miR-136-3p	0.724	0.413
hsa-miR-28-3p	0.638	0.414
hsa-miR-502-3p	1.165	0.418
hsa-miR-452-5p	1.153	0.42
hsa-miR-197-3p	1.06	0.424
hsa-miR-1303	0.536	0.425
hsa-miR-758-3p	1.06	0.428
hsa-miR-15b-5p	0.566	0.438
hsa-miR-1180-3p	0.685	0.444
hsa-miR-223-5p	1.422	0.444
hsa-miR-376b-3p	1.088	0.453
hsa-miR-132-3p	0.589	0.454
hsa-miR-92a-1-5p	1.153	0.456
hsa-miR-589-3p	0.558	0.458
hsa-miR-432-5p	0.884	0.472
hsa-miR-342-3p	1.007	0.483
hsa-miR-28-5p	1.046	0.484
hsa-miR-27a-3p	1.096	0.491
hsa-miR-1267	0.24	0.499
hsa-miR-340-5p	1.149	0.499
hsa-miR-376c-3p	0.802	0.514
hsa-miR-523-3p	0.679	0.522
hsa-miR-30a-3p	1.257	0.532
hsa-miR-495-3p	0.882	0.533
hsa-miR-19b-3p	1.137	0.537
hsa-miR-1254	1.37	0.538
hsa-miR-374b-5p	0.828	0.538
hsa-miR-191-5p	1.215	0.543
hsa-miR-199a-5p	0.736	0.546
hsa-miR-221-3p	0.903	0.552
hsa-miR-222-3p	1.038	0.554

hsa-miR-361-5p	0.602	0.554
hsa-miR-550a-5p	1.187	0.556
hsa-miR-181a-3p	1.634	0.559
hsa-miR-338-5p	1.214	0.566
hsa-miR-146b-5p	1.189	0.567
hsa-miR-205-5p	0.767	0.577
hsa-miR-500a-5p	0.824	0.579
hsa-miR-516b-3p	0.859	0.579
hsa-miR-204-5p	0.946	0.584
hsa-miR-542-3p	0.417	0.586
hsa-miR-125a-3p	0.875	0.593
hsa-miR-24-3p	1.15	0.594
hsa-miR-124-3p	0.513	0.594
hsa-miR-151-5p	0.889	0.6
hsa-miR-505-3p	0.792	0.607
hsa-miR-127-3p	0.767	0.619
hsa-miR-324-3p	0.653	0.619
hsa-miR-425-3p	0.986	0.62
hsa-miR-577	0.627	0.638
hsa-miR-628-3p	0.876	0.638
hsa-let-7e-3p	1.362	0.64
hsa-miR-15a-3p	0.649	0.64
hsa-miR-126-3p	1.123	0.641
hsa-miR-143-3p	0.996	0.651
hsa-miR-26a-5p	0.911	0.653
hsa-miR-494-3p	0.93	0.656
hsa-miR-138-5p	1.07	0.662
hsa-miR-196b-5p	0.966	0.674
hsa-miR-17-5p	0.834	0.676
hsa-miR-409-3p	0.602	0.677
hsa-miR-1-3p	0.866	0.683
hsa-miR-181a-5p	0.88	0.685
hsa-miR-211-5p	0.864	0.685
hsa-miR-409-5p	0.993	0.692
hsa-miR-422a	1.027	0.696
hsa-let-7d-5p	1.13	0.698

hsa-miR-889-3p	0.733	0.704
hsa-miR-200c-3p	0.811	0.707
hsa-miR-1271-5p	0.906	0.709
hsa-miR-376a-3p	0.993	0.73
hsa-miR-493-3p	1.072	0.734
hsa-miR-942-5p	1.302	0.741
hsa-miR-576-5p	1.062	0.745
hsa-miR-128-3p	1.023	0.747
hsa-let-7g-5p	1.03	0.75
hsa-miR-433-3p	0.732	0.753
hsa-miR-92a-3p	0.938	0.772
hsa-miR-451a	0.997	0.774
hsa-miR-890	0.943	0.778
hsa-miR-27a-5p	0.675	0.782
hsa-miR-517a-3p	0.854	0.786
hsa-miR-532-5p	0.894	0.799
hsa-miR-25-3p	0.925	0.801
hsa-miR-1255b-5p	1.223	0.804
hsa-miR-425-5p	0.921	0.806
hsa-miR-142-3p	0.956	0.81
hsa-miR-584-5p	1.13	0.814
hsa-miR-548c-3p	1.508	0.817
hsa-miR-148b-5p	0.419	0.83
hsa-miR-625-3p	0.911	0.835
hsa-miR-20b-5p	0.959	0.839
hsa-miR-331-3p	0.945	0.841
hsa-miR-1197	0.881	0.846
hsa-miR-337-5p	1.167	0.848
hsa-miR-324-5p	0.908	0.853
hsa-miR-144-5p	1.029	0.856
hsa-miR-340-3p	1.067	0.857
hsa-miR-30c-5p	1.048	0.862
hsa-miR-655-3p	0.819	0.868
hsa-miR-378	0.975	0.869
hsa-miR-518f-3p	0.853	0.872
hsa-miR-769-5p	0.853	0.875

hsa-miR-20a-5p	0.911	0.888
hsa-miR-20a-3p	1.136	0.892
hsa-miR-374a-5p	0.901	0.896
hsa-miR-29c-5p	0.853	0.896
hsa-miR-330-3p	1.026	0.901
hsa-miR-410-3p	0.812	0.909
hsa-miR-106b-5p	0.947	0.929
hsa-miR-139-5p	1.075	0.934
hsa-miR-519a-3p	0.758	0.936
hsa-miR-532-3p	1.088	0.937
hsa-miR-183-3p	1.195	0.947
hsa-miR-30b-5p	1.1	0.947
hsa-miR-329-3p	0.865	0.948
hsa-miR-34b-3p	1.154	0.957
hsa-miR-539-5p	1.067	0.958
hsa-miR-125a-5p	0.911	0.964
hsa-miR-591	0.688	0.972
hsa-miR-142-5p	0.972	0.975
hsa-miR-106a-5p	0.973	0.99
hsa-miR-199a-3p	0.989	0.994
hsa-miR-454-3p	0.888	0.995
hsa-miR-99b-5p	0.903	0.996
hsa-miR-145-5p	1.12	0.997

Table S 9: Univariate analysis between clinical factors and biomarkers in cirrhotic and non-cirrhotic HCV-infected subjects in the expanded Outcomes cohort (n=51)

	Non-cirrhotic (n=36)	Cirrhotic (n=15)	P value
Male sex [n (%)]	30 (83)	9 (60)	0.073
White ethnicity [n (%)]	35 (97.2)	14 (93.3)	0.514
Age [years (IQR)]	41.0 (37.3 – 46.8)	48.0 (41.0 – 54.0)	**0.004**
Age at infection [years (IQR)]	21.5 (17.0 -27.5)	21.5 (16.3 – 34.5)	0.566
Source [n (%*)]			0.647
IDU	26 (83.9)	8 (72.7)	
Other	5 (16.1)	3 (27.3)	
Unknown	5	4	
HCV Gt [n (%)]			0.562
1	20 (55.6)	7 (46.7)	
3	16 (44.4)	8 (53.3)	
IFNL3 Gt [%*)]			0.529
CC	9 (26.5)	4 (36.4)	
Non CC	25 (73.5)	7 (63.6)	
Unknown	2	4	
BMI [kg/m^2(IQR)]	24.3 (21.4 – 26.0)	27.4 (24.7 – 30.0)	**0.005**
Alcohol excess [n (%)]	3 (8.3)	0 (0)	0.249
HCV VL [log_{10} IU/ml (IQR)]	6.1 (5.2 – 6.5)	5.7 (5.2 – 6.3)	0.408
ALT [IU/mL (IQR)]	70.5 (45.0 – 98.8)	92.0 (77.0 – 144.0)	0.082
miR-21-5p [RQ (IQR)]	0.7 (0.4 – 2.8)	1.4 (0.5 – 5.6)	0.408
miR-122-5p [RQ (IQR)]	2.1 (0.2 – 8.9)	2.6 (0.9 – 6.5)	0.664
miR-885-5p [RQ (IQR)]	5.0 (1.1 – 12.1)	9.8 (3.7 – 16.6)	0.160

***Percentage related to the actually recorded data; missing data handled by listwise deletion. IQR, interquartile range; IDU, injecting drug use; Gt, genotype; RQ, fold change with respect to healthy controls.**

Table S 10: Multiple logistic regression for variables significant at the 10% level in the univariate analysis between clinical factors and biomarkers in cirrhotic and non-cirrhotic HCV-infected subjects in the expanded Outcomes cohort (n=51). *p<0.05

	Model 1			Model 2		
Variable	B	SE	OR	B	SE	OR
Constant	-17.01	5.34	0.00	-18.45	5.89	0.00
Age	0.17*	0.07	1.19	0.19*	0.08	1.20
BMI	0.34*	0.14	1.40	0.36*	0.15	1.42
ALT				0.00	0.00	1.00
-2LL		43.84			42.93	
Nagelkerke R2		40.4%			42.3%	
	X^2 = 16.52; df 2; p<0.001			X^2 = 17.43; df 3; p=0.001		
Hosmer & Lemeshow		p=0.721			p=0.234	
Classification accuracy		81.6%			85.7%	

Table S 11: Univariate analysis between clinical factors and biomarkers in non-cirrhotic and cirrhotic HCV-infected subjects; analysis restricted to subjects with data available on CDKN2 locus transcript expression (n=40)

	Non-cirrhotic (n=27)	Cirrhotic (n=13)	P value
Male sex [n (%)]	22 (81.5)	8 (61.5)	0.172
White ethnicity [n (%)]	27 (100)	12 (92.3)	0.144
Age [years (IQR)]	41.0 (38.0 – 46.0)	48.5 (41.0 – 54.3)	**0.002**
Age at infection [years (IQR)]	20.0 (16.3 -26.8)	18.0 (16.3 – 33.8)	0.660
Source [n (%*)]			0.648
IDU	20 (80)	6 (66.7)	
Other	5 (20)	3 (33.3)	
Unknown	2	3	
HCV Gt [n (%)]			0.145
Gt1	17 (63.0)	5 (38.5)	
Gt3	10 (37.0)	8 (61.5)	
IFNL3 Gt [n (%*)]			0.120
CC	5 (18.5)	4 (44.4)	
Non CC	22 (81.5)	5 (55.6)	
Unknown	0	4	
BMI [kg/m^2(IQR)]	24.9 (22.7 – 26.3)	27.5 (25.2 – 30.0)	**0.003**
Alcohol excess [n (%)]	3 (11.1)	0 (0)	0.211
HCV VL [log_{10} IU/ml (IQR)]	6.0 (5.4 – 6.4)	5.8 (5.2 – 6.4)	0.289
ALT [IU/mL (IQR)]	70.0 (39.0 – 85.0)	90.5 (75.5 – 133.5)	**0.012**
CDKN2A [RQ (IQR)]	1.3 (1.1 – 3.0)	3.1 (1.9 – 5.0)	**0.007**
CDKN2B [RQ (IQR)]	2.4 (1.1 – 4.6)	4.1 (1.1 – 9.4)	0.142
ARF [RQ (IQR)]	1.4 (0.9 – 5.5)	8.0 (1.6 – 9.7)	**0.006**
CDKN2 expression			**0.009**
High	11 (40.7)	11 (85.7)	
Low	16 (59.3)	2 (14.3)	
miR-21-5p [RQ (IQR)]	0.7 (0.3 – 2.2)	1.2 (0.6 – 3.8)	0.360
miR-122-5p [RQ (IQR)]	2.2 (0.6 – 8.9)	2.6 (0.8 – 14.7)	1.000
miR-885-5p [RQ (IQR)]	4.8 (0.8 – 9.7)	9.4 (3.2 – 14.8)	0.168

***Percentage related to the actually recorded data; missing data handled by listwise deletion. IQR, interquartile range; IDU, injecting drug use; Gt, genotype; RQ, fold change with respect to healthy controls.**

Table S 12: Multiple logistic regression for variables significant at the 10% level in the univariate analysis between clinical factors and biomarkers in cirrhotic and non-cirrhotic HCV-infected subjects in the expanded Outcomes cohort; analysis restricted to subjects with data available on CDKN2 locus transcript expression (n=40). *p<0.05

	Model 1			Model 2		
Variable	B	SE	OR	B	SE	OR
Constant	-34.76	12.76	0.00	-33.66	13.02	0.00
Age	0.28*	0.13	1.32	0.25	0.32	1.28
BMI	0.73*	0.30	2.07	0.71*	0.32	2.03
ALT	0.03	0.01	1.03	0.03*	0.01	1.03
CDKN2				1.54	1.27	4.67
-2LL		22.31			20.71	
Nagelkerke R2		69.4%			72.3%	
	X^2 = 26.5; df 3; p<0.001			X^2 = 28.1; df 4; p<0.001		
Hosmer & Lemeshow		p=0.532			p=0.992	
Classification accuracy		92.1%			84.2%	

Table S 13: Diagnostic power of individual biomarkers for cirrhosis in CHC

Biomarker	AUC	95% CI	P value
ALT	0.656	0.483 – 0.828	0.082
CDKN2A RQ	0.764	0.609 – 0.918	**0.008**
CDKN2B RQ	0.645	0.444 – 0.846	0.141
ARF RQ	0.765	0.612 – 0.918	**0.007**
miR-21-5p RQ	0.574	0.402 – 0.706	0.206
miR-122-5p RQ	0.539	0.373 – 0.705	0.912
miR-885-5p RQ	0.626	0.467 – 0.785	0.088

Table S 14: Baseline demographics of the EAP study population (n=59)

	All (n=59)	SVR12 (n=20)	SVR-AE (n=19)	RR (n=20)
Male sex [n (%)]	40 (67.9)	12 (60)	13 (68)	15 (75)
Age [years (IQR)]	54 (48 - 61)	51.5 (45.5 – 60.8)	59 (47 – 62)	54 (49.3 – 58.8)
Ethnicity [n (%*)]				
White	47 (83.9)	15 (83.3)	14 (77.8)	18 (90)
Asian	9 (16.1)	3 (16.7)	4 (22.2)	2 (10)
Unknown	3	2	1	0
HCV Gt [n (%*)]				
1	26 (44.8)	9 (45)	13 (72.2)	4 (20)
2	1 (1.7)	1 (5)	-	-
3	30 (51.7)	9 (45)	5 (27.8)	16 (80)
4	1 (1.7)	1 (5)	-	-
Unknown	1	0	1	0
DM 2 [n (%)]	18 (30.5)	2 (10)	8 (42)	8 (40)
HCV VL [log_{10} IU/ml (IQR)]	5.5 (4.9 – 6.0)	5.6 (4.3 – 6.1)	5.2 (4.8 – 6.0)	5.4 (5.0 – 6.0)
Use of RBV [n (%)]	52 (88.1)	20 (100)	15 (78.9)	17 (85)
NS5Ai [n (%)]				
Ledipasvir	41 (69.5)	14 (70)	14 (73.7)	13 (65)
Daclatasvir	18 (30.5)	6 (30)	5 (26.3)	7 (35)
ALT [IU/mL (IQR)]	54.0 (37.8 – 70.3)	61.0 (45.0 – 80.0)	47.0 (27.3 – 71.0)	60.0 (39.3 – 67.0)
MELD [n (IQR)]	11 (8.8 – 13.3)	10.5 (8.0 – 12.0)	11.0 (7.0 – 12.0)	13.0 (11.3 – 15.0)

***Percentage related to the actually recorded data. SVR12, sustained virologic response 12 weeks post end of treatment; AE, adverse event; IQR, interquartile range; Gt, genotype; RBV, ribavirin; NS5Ai, NS5A inhibitor; ALT, alanine aminotransferase; MELD, model for end-stage liver disease.**

Table S 15: Comparison of the baseline clinical characteristics, laboratory parameters and biomarkers in treatment responders (n=39) and RRs (n=20).

	SVR12 - all (n=39)	RR (n=20)	P-value
Male sex [n (%)]	25 (64.1)	15 (75)	0.396
Age [years (IQR)]	55 (47 – 61)	54 (49.3 – 58.8)	0.317
Ethnicity [n (%*)]			0.356
White	29 (80.6)	18 (90)	
Asian	7 (19.4)	2 (10)	
Unknown	3	0	
HCV Gt [n (%*)]			**0.003**
1 or 4	23 (60.5)	4 (20)	
2 or 3	15 (39.5)	16 (80)	
Unknown	1	0	
DM 2 [n (%)]	10 (25.6)	8 (40)	0.257
HCV RNA	5.5	5.4	0.543
[log_{10} IU/mL (IQR)]	(4.8 – 6.0)	(5.0 – 6.0)	
NS5Ai [n (%)]			0.592
Ledipasvir	28 (71.8)	13 (65)	
Daclatasvir	11 (28.2)	7 (35)	
Use of RBV [n (%)]	35 (89.7)	17 (85)	0.594
ALT [IU/mL (IQR)]	51.5 (33.8 – 72.5)	60 (39.3 – 67)	0.525
MELD score [n (IQR)]	11 (7.8 – 12)	13 (11.3 – 15)	**0.006**
miR-21-5p +ve [n (%)]	12 (30.7)	17 (85)	**<0.001**
miR-122-5p +ve [n (%)]	16 (41.0)	13 (65)	0.081
miR-885-5p +ve [n (%)]	16 (41.0)	13 (65)	0.081

***Percentage related to the actually recorded data; missing data handled by listwise deletion. SVR, sustained virologic response 12 weeks post end of treatment; AE, adverse event; IQR, interquartile range; Gt, genotype; NS5Ai, NS5A inhibitor; RBV, ribavirin; ALT, alanine aminotransferase; MELD, model for end-stage liver disease**

Table S 16: Multiple logistic regression for variables significant at the 10% level in the univariate analysis between baseline clinical factors and biomarkers in treatment responders (n=39) and RRs (n=20) in the EAP cohort. **p<0.01, ***p<0.001

	Model 1			Model 2		
Variable	B	SE	OR	B	SE	OR
Constant	-3.56	0.90	0.03	-4.71	1.55	0.01
miR-21-5p	2.83***	0.80	16.88	2.90**	0.85	18.22
HCV Gt 2 or 3	2.07**	0.78	7.90	2.18**	0.83	6.85
MELD score				0.10	0.12	1.10
-2LL		48.65			44.64	
Nagelkerke R2		50.0%			55.2%	
	X^2 = 26.1; df 2; p<0.001			X^2 = 29.2; df 3; p<0.001		
Hosmer & Lemeshow		p=0.706			p=0.932	
Classification accuracy		81%			82.5%	

Table S 17: Comparison of baseline clinical characteristics, laboratory parameters and biomarkers in treatment responders who did (SVR-AE, n=19) and did not (SVR12, n=20) experience adverse clinical outcomes in the EAP cohort

	SVR12 (n=20)	SVR12 - AE (n=19)	P-value
Male sex [n (%)]	12 (60)	13 (68.4)	0.584
Age [years (IQR)]	51.5 (45.5 – 60.8)	59 (47 – 62)	0.461
Ethnicity [n (%*)]			0.674
White	15 (83.3)	14 (77.8)	
Asian	3 (16.7)	4 (22.2)	
Unknown	2	1	
HCV Gt [n (%*)]			0.162
1 or 4	10 (50)	13 (72.2)	
2 or 3	10 (50)	5 (27.8)	
Unknown	0	1	
DM 2 [n (%)]	2 (10)	8 (42.1)	**0.022**
HCV RNA [IU/mL (IQR)]	5.6 (4.3 – 6.1)	5.2 (4.9 – 5.8)	0.409
NS5Ai [n (%)]			0.798
Ledipasvir	14 (70)	14 (73.7)	
Daclatasvir	6 (30)	5 (26.3)	
Use of RBV [n (%)]	20 (100)	15 (78.9)	**0.030**
ALT [IU/mL (IQR)]	61 (45 – 80)	47 (27.3 – 71)	0.196
MELD score [n (IQR)]	10.5 (8 – 12)	11 (7 – 12)	0.806
miR-21-5p +ve [n (%)]	9 (45)	3 (15.8)	**0.048**
miR-122-5p +ve [n(%)]	9 (45)	7 (36.8)	0.605
miR-885-5p +ve [n (%)]	9 (45)	7 (36.8)	0.605
miR-345 +ve [n (%)]	7 (35)	13 (68.4)	**0.037**
Composite miR [n (%)]	4 (20)	12 (63.2)	**0.006**

***Percentage related to the actually recorded data; missing data handled by listwise deletion. SVR, sustained virologic response 12 weeks post end of treatment; AE, adverse event; IQR, interquartile range; Gt, genotype; NS5Ai, NS5A inhibitor; RBV, ribavirin; ALT, alanine aminotransferase; MELD, model for end-stage liver disease**

Table S 18: Details of significant adverse events in the SVR12-AE group (n=19).

Age	Gender	Ethnicity	Gt	ALT	DM 2	Baseline MELD	MELD ↑	HCC	Ca	Decomp	Other	miR-345	miR-21	miR-122	miR-885	miR-21↑
62	Female	Asian	Gt3	30	Yes	7	Yes	No	No	Past	Anaemia	Pos	Neg	Neg	Neg	Yes
58	Male	White	Gt1	34	No	11	No	No	No	Current	HE	Pos	Neg	Neg	Neg	Yes
55	Female	White	Gt3	21	No	13	No	No	No	Current	Ascites	Pos	Neg	Pos	Pos	Yes
59	Male	White	Gt1	52	Yes	6	Yes	No	No	Current	NA	Neg	Neg	Neg	Neg	Yes
47	Male		Gt1	15	No	15	No	No	Yes	Past	HE	Pos	Neg	Neg	Neg	Yes
38	Male	White	Gt1	212	Yes	11	No	No	No	Never	Endoscopy	Neg	Pos	Pos	Pos	No
61	Female	Asian	Gt3	71	Yes			No	No	Past	HE	Pos	Pos	Pos	Pos	No
60	Male	White	Gt1	33	No	9	Yes	No	No	Never	NA	Pos	Neg	Neg	Neg	Yes
53	Male	White	Gt1	51	No	11	No	No	No	Never	Variceal bleed	Pos	Neg	Pos	Pos	Yes
64	Male	White	Gt3	148	No	7	No	Yes	Yes	Past	Ascites	Pos	Neg	Neg	Pos	Yes
61	Female	White	Gt3	28	No	6	Yes	No	No	Current	NA	Neg	Neg	Neg	Neg	Yes
59	Female	White	Gt1	10	No	6	No	No	No	Past	NA	Pos	Neg	Neg	Pos	Yes
75	Male	White	Gt1	25	No	11	No	No	Yes	Current	NA	Pos	Neg	Pos	Pos	Yes
50	Female	Asian		71	Yes	12		No	No	Current	HE	Pos	Pos	Neg	Neg	No
38	Male	White	Gt1	101	No	12	No	No	No	Past	NA	Pos	Neg	Neg	Neg	No
62	Male	White	Gt1	43	Yes	24	No	No	No	Current	Ascites	Pos	Neg	Neg	Neg	Yes
39	Male	Asian	Gt1		Yes	11	Yes	No	No	Current	Ascites	Neg	Neg	Neg	Neg	Yes
40	Male	White	Gt1	56	No	10	No	Yes	Yes	Past	NA	Pos	Neg	Neg	Neg	Yes
67	Male	White	Gt1	61	Yes	8	Yes	No	No	Current	NA	Neg	Neg	Pos	Neg	Yes

Gt, genotype; DM 2, diabetes mellitus type 2; MELD, model for end stage liver disease; HCC, hepatocellular carcinoma; Ca, malignancy other than HCC; Decomp, decompensation event; Pos, positive; Neg, negative; HE, hepatic encephalopathy; NA, not applicable

Table S 19: Multiple logistic regression for variables significant at the 10% level in the univariate analysis between treatment responders who did (SVR12-AE, n=19) and did not (SVR12, n=20) experience adverse clinical outcomes in the EAP cohort. *p<0.05, **p<0.01

	Model 1			Model 2		
Variable	B	SE	OR	B	SE	OR
Constant	-2.66	1.03	0.07	-2.66	1.03	0.07
DM 2	3.77**	1.31	43.36	3.08*	1.37	21.74
Composite miR	3.58**	1.19	35.92	3.58**	1.19	35.91
RBV				20.30	18153	655306328
-2LL		33.13			30.87	
Nagelkerke R2		55.3%			59.7%	
	X^2 = 20.91; df 2; p<0.001			X^2 = 23.17; df 3; p<0.001		
Hosmer & Lemeshow		p=0.991			p=0.991	
Classification accuracy		82.1%			82.1%	

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