Investigation of Hepatitis C Virus (HCV) interactions with host lipid metabolism: a translational research study

Dr David Anthony Sheridan

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Newcastle University

Faculty of Medical Sciences

Institute of Cellular Medicine

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Abstract

Research into chronic hepatitis C virus (HCV) infection (CHC) is needed because only ~50% of patients with CHC are cured with existing treatments. HCV interacts with metabolism of cholesterol and very low density lipoproteins (VLDL) for replication, assembly, secretion and entry. Patients with CHC exhibit a dyslipidemia characterised by low LDL cholesterol (LDL-C) and insulin resistance. This translational study characterised the dyslipidemia apparent in CHC in retrospective and prospective HCV cohorts. Distinct metabolic phenotypes were defined between HCV genotypes 1 and 3. LDL-C was markedly reduced in HCV-G3, which exerted a greater effect than apoE genotype on LDL-C levels. Prospective analysis of non-cholesterol sterol intermediates established that disordered cholesterol synthesis in HCV was mediated predominantly via the lathosterol pathway. In HCV-G3, levels of Proprotein Convertase Subtilisin Kexin type 9 (PCSK9) were low compared to healthy controls, suggesting increased LDL clearance. Low LDL-C and high triglyceride/HDL ratio were found to be predictive of poor response to anti-viral therapy. Collaboration in a genome wide association study revealed that SNPs in IL28B rather than in lipid regulating genes are the major host genetic determinants of treatment response. Analysis of HCV lipoviral particles (LVP) by iodixanol density gradient ultracentrifugation revealed correlations between insulin resistance and triglycerides (TG) with LVP in HCV-G1. Determination of apoB in VLDL1, VLDL2, IDL and LDL fractions confirmed fasting TG are predominately in the VLDL1 fraction, implying that HCV-G1 preferentially associates with the VLDL1 pathway. Interferon γ inducible protein 10 (IP10), a marker of hepatic interferon stimulated gene expression correlated with LDL-C and HCV LVP ratio in HCV-G1, explaining the association between low LDL-C and poor treatment response. A randomised pilot trial in 60 CHC non-responders indicated that 12 weeks of treatment with Fluvastatin can lower total viral load in HCV-G1 & G4 and that low dose n3 PUFA's improved IP10 levels.

Dedication

This thesis is dedicated to Susan and Thomas.

Declaration

I declare that this thesis is my own work and that I have appropriately acknowledged the contributions of work made by others, which is summarised below. No material offered herein has been previously submitted for a degree or other qualification in this or any other University.

This work has been the result of an MRC Experimental medicine grant awarded to Prof M F Bassendine (MFB), Professor G L Toms (GLT) and Dr RDG Neely (DN) to investigate the interactions of hepatitis C virus and lipids. My role has been as the Clinical Research Fellow in the group, supervised by the aforementioned principle investigators. The content of this thesis therefore inevitably includes work conducted by or in collaboration with others. My specific independent contributions are listed below as are those contributions made by others.

Specific independent work performed by DS:

Retrospective HCV cohort – Identified patients, determined clinical phenotypes from hospital records; established Microsoft® Access database combining DNA with clinical phenotypes that were used for the genome wide association study (GWAS).

Prospective HCV cohort – Wrote the approved protocol for HCV Host Interactions, patient information sheet and GP letter with MFB; obtained ethics and hospital trust R&D approvals with MFB; established the 'Symphony' database and obtained Caldicott approval; recruited all patients; performed all clinical assessments; performed liver stiffness measurements using Fibroscan®.

Performed therapeutic venesections in four HCV & haemochromatosis patients used for LVP characterisation work by Dr Soren Nielsen.

Performed clinical protocol and sample collection for post prandial study (see publications arising from this thesis);

Intervention Trial – Obtained ethics, R&D and MHRA approval approvals for six protocol amendments with MFB; recruited patients from the clinic with MFB; performed clinical evaluation of patients at study visits; coordinated pharmacy supply, re-packaging and labelling of investigational drugs; co-ordinated the day to day running of the trial between two centres;

Performed all data analysis and statistics.

Laboratory – Undertook specific training to work in a Containment level III (CLIII) facility (appendix); prepared plasma and serum samples for further analysis of viral and biochemical parameters.

Performed all measurements of PCSK9 and IP10 by ELISA; developed an in house apoB ELISA; fractionated VLDL1, VLDL2, IDL and LDL using salt flotation ultracentrifugation; performed genomic DNA extractions from whole blood and viral RNA extractions. Wrote standard operating procedures and BioCOSHH risk assessments for all above CLIII laboratory procedures.

Contributions of others

I am indebted to the following individuals who provided specific experiments, reagents, and data that is included in this thesis:

Dr Simon Bridge – performed iodixanol gradient ultra centrifugations, RNA extractions and qRT PCRs on fractions; performed apoE genotyping.

Dr Daniel Felmlee - performed apoB western blots in the LVP assay development.

Dr Soren Nielsen – Performed iodixanol gradients, size exclusion chromatography and immuno-precipitation experiments on plasma obtained from haemochromatosis patients used for LVP assay development.

Dr Kim Bartlett, Dept Biochemistry, RVI – performed sterol analysis.

Clinical Biochemistry, RVI – performed lipid profiles and glucose / insulin measurements

Prof Simon Taylor Robinson, Prof Howard Thomas and Mary Crossey, Imperial College, London – were responsible for recruitment and running of the clinical trial at the St Mary's site.

Prof Jacob George and Vijay Suppiah, University of Sydney, Australia – performed genome wide association study; genotyped Newcastle patients for IL28B haplotypes.

Dr Nicola Harman and Dr Bruce Griffin, University of Surrey – provided comparator group A data for sterol profiles.

Dr Genevieve Dubuc, Nabil Seidah and Prof Jean Davignon, University of Montreal, Quebec, Canada – provided PCSK9 ELISA antibodies, standards and controls and comparator group B data.

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Abbreviations

ADH	Autosomal Dominant Hypercholesterolaemia
AE	Adverse Event
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
AR	Adverse Reaction
BMI	Body Mass Index
CETP	Cholesteryl Ester Transfer Protein
CHC	Chronic Hepatitis C
CR	Chylomicron remnant
CRF	Clinical Research Facility
eCRF	Electronic Case Report Form
CV	Coefficient of Variation
ER	Endoplasmic Reticulum
ELISA	Enzyme Linked Immunosorbent Assay
EVR	Early Virological Response
FAS	Fatty Acid Synthase
FFA	Free Fatty Acids
GGPP	Geranylgeranyl diphosphate
HCV	Hepatitis C Virus
HCVcc	Hepatitis C Virus cell culture
HCVpp	Hepatitis C Virus pseudoparticle
HDF	High Density Fraction (density >1.07g/ml)
HDL	High Density Lipoprotein
HOMA IR	Homeostasis Model Assessment of Insulin Resistance

HSPG	Heparin Sulphate Proteoglycans
IDL	Intermediate Density Lipoprotein
IFN	Interferon
IMP	Investigational Medicinal Product
ISG	Interferon Stimulated Genes
JAK	Janus Kinase
JFH1	Japanese Fulminant Hepatitis C virus 1
LCAT	Long Chain Acetyl Co A transferase
LDF	Low Density Fraction (density <1.07g/ml)
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein Receptor
LPL	Lipoprotein Lipase
LRP	LDL receptor like protein
LSM	Liver Stiffness Measurement
LVP	Lipoviral particle
LXR	Liver X Receptor
MHRA	Medicines and Healthcare Regulatory Authority
MTP	Microsomal Triglyceride Transfer Protein
NAFLD	Non Alcoholic Fatty Liver Disease
NK	Natural Killer cells
NKT	Natural Killer T cells
NR	Non-Responder
NEFA	Non Esterified Fatty Acids
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween 20
PCSK9	Proprotein Convertase Subtilisin Kexin Type 9
PIL	Patient Information Leaflet
PPAR	Peroxisome Proliferator Activated Receptor
REC	Research Ethics Committee
SAA	Serum Amyloid A
SAE	Serious Adverse Event
SOCS	Suppressor of Cytokine Signalling
SRB1	Scavenger Receptor B1
SREBP	Sterol Regulatory Element Binding Protein
STAT	Signal Transducer and Activator of Transcription
SUSAR	Suspected Unexpected Serious Adverse Reaction
SVR	Sustained Virological Response
TG	Triglyceride
TRL	Triglyceride rich lipoprotein
VLDL	Very Low Density Lipoprotein

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1 Chapter 1. Introduction

1.1 HCV Epidemiology, natural history and lifecycle

1.1.1 Epidemiology

Hepatitis C Virus was first discovered in the search for the infectious aetiological agent of non-A non-B Hepatitis (NANBH) in 1989. This landmark discovery was made by cloning the genome from a cDNA library of NANBH cases without prior characterisation of the NANBH agent (Choo, Kuo et al. 1989). The ~10,000 nucleotide positive strand RNA clone that was isolated was termed hepatitis C virus (HCV). The recombinant peptide was then used to capture circulating viral antibodies from NANBH sera, confirming that antibodies to HCV were present in cases of NANBH throughout the world (Kuo, Choo et al. 1989). Subsequently HCV infection has been found in over 170 million people worldwide (Heintges and Wands 1997), 2% of the worlds population (Shepard, Finelli et al. 2005). In the United Kingdom at least 185,000 people are affected (Health_Protection_Agency 2009). HCV is a blood borne virus and therefore transmission occurs parenterally. Globally, unsafe injection practices are the most common source of infection. In the UK, injecting drug use accounts for the majority of new infections and there are also a significant number of patients who received contaminated blood transfusions prior to screening of blood products in 1991 that contribute to the HCV epidemic (Health_Protection_Agency 2009). At present there is no effective vaccine to protect from HCV.

1.1.2 Natural History and factors involved in liver disease progression

The natural history of HCV is variable between individuals. Following exposure to HCV, 20-30% spontaneously clear the infection (Thomas, Astemborski et al. 2000), but 70 - 80% will develop chronic infection. Of those with chronic infection, 20-30% will progress to liver cirrhosis within 20 years and 1-3% of these may develop liver failure or liver cancer (Seeff 1997). Progression to cirrhosis is also variable between individuals. Factors known to be associated with more rapid progression include older age at time of exposure, male sex, immune suppression, co-infection with hepatitis B and / or HIV, high alcohol intake and diabetes. Additionally it is now recognised that

chronic HCV infection is associated with a variety of non-liver related conditions including cryoglobulinaemia, B cell lymphoma, anxiety and depression, insulin resistance and diabetes (Medina, Garcia-Buey et al. 2004).

1.1.3 HCV lifecycle

HCV is a single strand RNA virus and member of the *Flavividae* family of viruses, for which humans are the only natural host. It is of the genus Hepacivirus. The only other Hepacivirus is GBV-B. There are six HCV genotypes and several subtypes that vary by as much as 35% in sequence. The HCV genotypes also vary in the global distribution, with genotype 1a and 1b the most prevalent in the USA, genotypes 1b and 2 in Japan, genotype 3 in injecting drug users in Europe, genotype 4 in north Africa, and genotypes 5 & 6 in South Africa and Hong Kong (Zein 2000). A study looking at the relative evolutionary ages of the various HCV genotypes produced a cladogram indicating that the youngest HCV genotype is G1 followed by 4, 5, 3, 6 and 2. This corresponds to the likelihood of response to interferon based anti-viral therapy and suggests a possible mechanism of immune selection driving evolution and divergence of the HCV genotypes (Pang, Planet et al. 2009).

The major breakthrough that permitted further investigation of the intracellular HCV lifecycle was the discovery of a HCV genotype 2a strain cloned from an individual with fulminant hepatitis (JFH1) that was able to replicate efficiently in cell culture and was infectious in Chimpanzees (Wakita, Pietschmann et al. 2005). This has been termed HCVcc. Following cell entry, the genome of the virus particle is uncoated and its 9.6 kb single strand of RNA is directly translated to a single polyprotein precursor ~3000 amino acids long (Moradpour, Penin et al. 2007). The HCV polyprotein is then cleaved by viral and host proteases into structural and non structural proteins. The structural proteins are the envelope glycoproteins E1 and E2 and the capsid or core protein. These form the structural components of new virions. The core protein has other functional properties in replication, for example the C terminal domain binds to and localises the core to lipid droplets. The envelope glycoproteins, E1 and E2 assemble as a heterodimer to form the main protein components of the viral envelope. The non structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) are not incorporated into the virion but are involved in viral replication. A negative strand of RNA is made first to act as a template for new progeny positive strand virions. The

ER membrane is the site of HCV replication where all the non-structural proteins are tethered to the ER membrane to form a replication complex which is the factory for new virions (Moradpour, Penin et al. 2007). HCV replicates using an RNA dependent RNA polymerase which has no proof editing mechanism. Therefore a high rate of spontaneous mutations can arise, resulting in ever changing progeny quasispecies. This is clinically very important because it means that there is a very high potential for anti-viral resistance mutations to develop against drugs used to inhibit viral replication (STAT-C's) without complete termination of replication or continued sensitivity to interferon-α (Pawlotsky, Chevaliez et al. 2007).

1.2 Anti-viral therapy and factors important in determining outcome

1.2.1 Anti-viral therapy

Standard therapy for chronic HCV infection is a combination of pegylated interferon-α (PegIFN-α) and ribavirin. Long term prognosis is improved in those that achieve a sustained virological response (SVR), defined as undetectable HCV RNA by PCR more than 6 months after completion of anti-viral therapy (Manns, McHutchison et al. 2001; Davis and Lindsay 2005). Unfortunately this therapy is associated with numerous and sometimes significant side effects such as flu-like symptoms, depression, nausea, and cytopaenias. Duration of treatment differs depending on viral genotype. In the UK, HCV genotypes 1 (HCV G1) and genotype 3 (HCV G3) are the most common, accounting for >90% of patients presenting to the clinic. The most important viral factor for determining treatment outcome is HCV genotype, whereby genotypes 1 & 4 are associated with poor response. Individuals with HCV G3 infection achieve SVR's of 70-80% with 24 weeks of therapy whereas those with HCV G1 require at least 48 weeks of therapy with only approximately 50% achieving SVR. Currently there are no licensed treatment options for individuals who do not achieve an SVR. Host factors that adversely influence response to therapy include male gender, African ethnicity, older age, insulin resistance, obesity (Gao, Hong et al. 2004; Conjeevaram, Fried et al. 2006; Walsh, Jonsson et al. 2006) and advanced fibrosis (Everson, Hoefs et al. 2006). Interestingly total HCV RNA viral load correlates very poorly with host factors known to adversely affect outcome, and in a study of 2,472 HCV genotype 1 patients, HCV RNA correlated only weakly with age, male sex and BMI >27. In a multiple regression model these factors accounted for

less than 4.6% of the HCV RNA differences between individuals (Ticehurst, Hamzeh et al. 2007). There is currently no way to predict accurately in advance of anti-viral therapy those who will not respond. Instead the most reliable predictor of outcome is a quantitative PCR for HCV RNA after 12 weeks of treatment. Those that achieve non-detectable HCV RNA at 12 weeks are considered complete early virological responders (EVR). This is associated with SVR rates in excess of 80%. The other extreme is those who have less than 2-log reduction in total HCV RNA, who are considered null responders and for these individuals continuation of therapy is considered futile. Those in the middle that achieve at least a 2-log drop in HCV RNA but are still detectable are considered partial EVR's. A significant proportion of these individuals can still relapse and so recent emphasis has been on optimising and individualising treatment for example by weight based dosing and extending duration of therapy (Ghany, Strader et al. 2009).

Interferon therapy activates the innate immune system. During therapy with pegIFNα a number of phases of decline in HCV RNA have been described. The first phase decline occurs in the first 48 hours due to inhibiting production and release of new virions and has been reported to correlate with viral genotype (2&3 greater decline than 1), and is inversely related to fibrosis, steatosis, GGT and HOMA IR levels (Durante-Mangoni, Zampino et al. 2009). This is followed by the second phase decline lasting up to 28 days which is slower and predicted by viral load, ALT and the rate of the first phase decline (Durante-Mangoni, Zampino et al. 2009).

In recent years a number of new potent anti-HCV drugs that target viral replication have been developed. Collectively these new drugs have been termed 'Direct acting anti-virals (DAAV)' or 'specifically targeted anti-viral therapy for HCV (STAT-C's)'. These agents are either small molecules that target and inhibit the HCV NS3-NS4A protease (e.g. telepravir and bocepravir) or nucleoside or non-nucleoside analogues that target the NS5B RNA dependent RNA polymerase and are currently in phase II/III clinical trials (Asselah, Benhamou et al. 2009). Whilst these new drugs are promising they are by no means likely to be a panacea for all HCV patients. This is because they still need to be used in combination with PegIFN-α and ribavirin (i.e. triple therapy) with all the associated side effects. Moreover the development of anti-viral resistance remains a concern. In the published trials to date, viral resistance to VX950 (telepravir) was common but these resistant quasispecies fortunately remained

sensitive to PegIFN-α. These trials were done in treatment naïve and previous relapse patients, so the great concern is that protease inhibitors when given to interferon-α null responders may pose a high risk for long term anti-viral resistance (Pawlotsky, Chevaliez et al. 2007). Therefore targeting host factors may be an important therapeutic strategy particularly for the interferon null responders.

1.2.2 The innate immune response to HCV

HCV is remarkable in its ability to establish chronic infection in at least 70% of those exposed. It is likely that virally mediated modulation of innate antiviral mechanisms permits HCV chronicity. Innate anti-viral defences include direct intracellular mechanisms and innate immune cells such as NK, monocyte macrophages and NKT cells. Interferons are part of the innate anti-viral immune response. Interferon-α is a type 1 interferon and is used as the mainstay of treatment for HCV; however there are also endogenously produced β, γ and λ interferons. Interferons (IFN) produced in response to viral infections modulate the expression of hundreds of genes known as IFN stimulated genes (ISGs). ISGs code for proteins which confer an antiviral state within the cell. IFNs act by a specific receptor mediated pathway to ultimately promote ISG transcription. Following binding to the specific IFN cell surface receptor, the signalling cascade of the JAK STAT pathway is activated. Tyrosine kinase phosphorylation of Jak1 and Tyk2 activates STAT1 and STAT2, which then translocate to the nucleus and bind to specific promoter regions in the DNA of ISGs. As well as being antiviral, ISGs are also involved in lipid metabolism and other cellular processes (de Veer, Holko et al. 2001). Evidence suggests that HCV is able to interfere with the IFN / ISG system. Patients with elevated pre-treatment ISG's in liver biopsy specimens are more likely to be non-responders to IFNα therapy, suggesting a refractory state in the IFN signalling pathway in non-responders. Conversely those with low ISG levels pre IFNα therapy were more likely to be responders (Chen, Borozan et al. 2005; Sarasin-Filipowicz, Oakeley et al. 2008). Interferon-γ inducible protein 10kDa (IP10, also known as CXCL10) is a CXC chemokine that targets T-lymphocytes, NK cells, and monocytes but not neutrophils. IP10 is a serum marker of hepatic ISG activation. High baseline serum IP10 levels have been reported in non-responders to anti-viral therapy and low IP10 levels are predictive of sustained response (Diago, Castellano et al. 2006). In one report, a

baseline pre treatment IP10 level of >600 pg/ml was predictive of non-response with a 79% negative predictive value (Lagging, Romero et al. 2006). IP10 levels are also higher in those with more advanced fibrosis (Romero, Lagging et al. 2006). A recent study has indicated that IP10 levels are predictive of the first phase decline of HCV RNA during PegIFNα therapy and ribavirin rather than the second phase decline (Askarieh, Alsio et al. 2010). The clinical significance of ISGs has also been highlighted by a study measuring IP10, MIG and MCP1 as a serum markers of ISG activation, and identified that addition of ribavirin promotes IFN signalling (Feld, Lutchman et al. 2010).

1.2.3 Targeting HCV interactions with the host

The limitations of existing anti-viral therapy with PegIFN-α and ribavirin and potential limitations of the new STAT-C's in terms of side effects and low barrier to anti-viral resistance, makes targeting host factors an important therapeutic strategy. A key interaction of HCV with the host is in lipid pathways. Understanding these essential interactions in the viral lifecycle highlights potential targets that are amenable to therapeutic modulation.

1.2.4 Clinical correlates of HCV lipid interactions

Chronic HCV has been reported to influence serum lipid profiles. A community-based study of 11,239 individuals reported that lower serum cholesterol is associated with chronic HCV infection (Dai, Chuang et al. 2008). This may be particularly pronounced in HCV genotype 3 infection (Serfaty, T et al. 2001; Siagris, Christofidou et al. 2006). Higher baseline LDL cholesterol levels may be associated with SVR, in both HCV mono-infected and HCV/HIV co-infected patients (Gopal, Johnson et al. 2006; del Valle, Mira et al. 2008; Economou, Milionis et al. 2008). Conversely low cholesterol is a significant predictor of reduced likelihood of HCV genotype 1 patients achieving a SVR (Backus, Boothroyd et al. 2007).

In cohort studies of spontaneously resolved HCV (i.e. Anti-HCV antibody positive, RNA PCR negative), high serum triglycerides are associated with spontaneous viral clearance (Marzouk, Sass et al. 2007). Additionally there may be an association between the Apo E3 allele (encoding wild-type isoform with normal LDLr binding) and viral persistence (Price, Bassendine et al. 2006). Although this epidemiological

evidence supports a key role for lipids in determining clinical outcomes of patients with HCV infection, the mechanisms are not yet understood.

1.3 Rationale for investigating HCV interactions with lipid metabolism

HCV is variable in its ability to cause progressive liver disease and in the response to anti-viral therapy. Both viral factors such as HCV genotype and host factors are important in determining these important clinical outcomes. The next section discusses the large body of evidence that is now indicating that HCV directly interacts with host lipid metabolism as part of its life-cycle. The rationale for investigating lipid metabolism in HCV patients is therefore to further understand how these interactions are appreciably different between HCV genotypes 1 and 3 given their different sensitivity to anti-viral treatment; to investigate whether HCV-lipid interactions are important predictors of response to anti-viral therapy and to evaluate whether adjunctive therapies could be targeted to impact HCV-lipid interactions that would potentially improve existing therapy.

1.4 HCV interacts with VLDL metabolism

Overwhelming *in-vitro* data now indicates that HCV utilises interactions with lipids at multiple stages of the virus lifecycle; in viral replication, assembly, in the circulation and for viral entry. An association of HCV with serum lipoproteins was first described by Thomssen et al in 1993, who observed that HCV RNA was heterogeneous in density. Thomssen used sucrose density gradients to fractionate patient sera and found in some individuals that HCV was restricted to low densities between 1.03 and 1.08 g/ml, whereas in other individuals HCV was found distributed over the whole density gradient with additional peaks at higher densities. This observation was followed up using immuno-precipitation with anti-beta lipoprotein antibodies which found that HCV RNA could be completely co-precipitated in 8/20 and partially co-precipitated in 9/20 patients (Thomssen, Bonk et al. 1992), suggesting an association with apolipoprotein B. Subsequently the heterogeneity of density was identified to be due at least in part to variable association with immunoglobulins (Thomssen, Bonk et al. 1993). The low density lipoprotein associated HCV particles have been termed ‘Lipoviral particles, (LVP)’. That the heterogeneity in density of HCV may be

clinically important was recognised from a comparative study of acute and chronic infection in immunodeficient and immunocompetent patients. In this study, low density HCV was identified more often in acute infection, and high density HCV in chronic infection. In cases of acute infection acquired from the same batch of HCV G1a infected human immunoglobulin, markedly different clinical courses were noted. One individual with persistent low density HCV rapidly progressed to decompensated liver disease, whereas others with persistence of high density HCV had clearance with α-interferon or only mild disease (Watson, Bevitt et al. 1996).

It is therefore apparent that HCV interactions with lipids are not only critical in the viral lifecycle but also likely to be clinically important. These complex interactions have been further characterised in the HCV cell culture system and will be discussed further in detail in later sections. In order to understand these interactions with the host, an appreciation of lipoprotein metabolism is essential, which is reviewed in this next section.

1.4.1 Apolipoprotein B (apoB)

The liver synthesises and exports cholesterol and triglycerides as an energy supply to peripheral tissues through secretion of very-low-density lipoproteins (VLDL) (Olofsson, Asp et al. 1999; Davis and Hui 2001). Each VLDL particle consists of a neutral core of lipids (triglycerides (TG) and cholesterol esters) surrounded by a single non-transferable molecule of apolipoprotein B100 (apoB). On the surface of the VLDL are transferable lipoproteins including apolipoprotein E (apoE) and apoC's and phospholipids. Apolipoprotein B100 is the structural protein of VLDL synthesised in the liver and is the full length (100%) 4536 amino acid polypeptide. ApoB48 is the truncated form of apoB (48%) and is the structural protein of chylomicrons, synthesised by the small intestine. In the intestine, RNA editing converts the Gln^{2153} to a stop codon, producing a truncated form of only 48% of the protein from the N terminus (apoB48). In humans this occurs only in the intestine and not the liver. In some rodents the liver produces both apoB100 and apoB48.

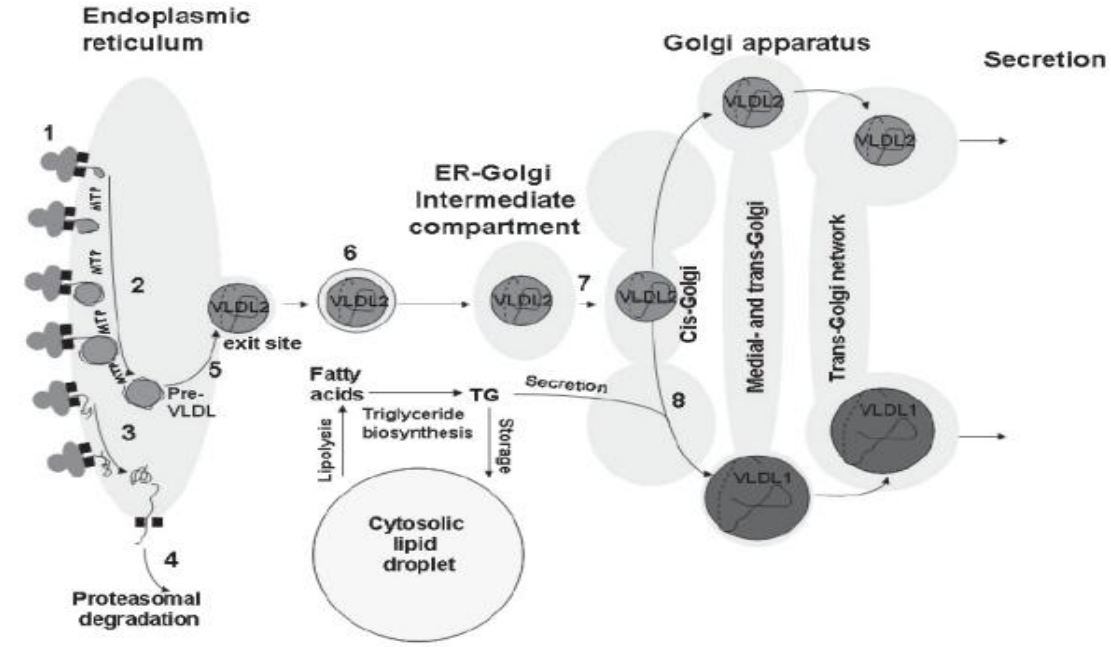
ApoB100 is a ligand for the LDL receptor (LDLr). The receptor binding domain is in the C terminal and therefore apoB48 cannot bind to LDLr. Chylomicrons therefore depend on apoE to bind the LDLr and LRP1 for clearance. ApoB100 has 5 domains (BetaAlpha1, Beta1, Alpha2, Beta2 and Alpha3). The globular BetaAlpha1 domain

extends away from the particle surface. There are no membrane spanning domains but there are hydrophobic beta sheet regions and hydrophilic regions. It is therefore amphipathic and able to form emulsions with neutral lipids in the particle core. This facilitates the transport of hydrophobic lipids in the aqueous environment of the serum.

1.4.2 VLDL synthesis

VLDL are synthesised by hepatocytes in a two stage process (see figure 1). Newly synthesised apoB are essentially pre-VLDL particles and contain small amounts of lipid (1). The apoB translocates across the membrane of the rough endoplasmic reticulum (ER) to reach the ER lumen (2). The rate determining step of apoB secretion is exit from the rough ER. Translocation across the ER membrane results in a steady state supply of membrane associated apoB. The first lipidation stage occurs in the lumen of the ER where microsomal triglyceride transfer protein (MTP) loads triglycerides onto the nascent apoB molecule (2). MTP associates with the N terminus of ApoB as a 'lipid pocket'. Unlipidated apoB100 is ubiquitinated and targeted to the proteasome for degradation (3&4). It is the extent of lipid transfer to these pre-VLDL particles that determines the size of the secreted VLDL particle. The second step fuses the partially lipidated VLDL with a triglyceride rich lipid droplet, again facilitated by MTP (6&7). Lipid droplets are intracellular storage compartments of triglycerides and cholesterol esters, surrounded by a phospholipid monolayer. Lipid droplets form as lenses in the ER before budding off as discrete organelles. The mature VLDL is then transported via the golgi for secretion by ER transport vesicles (8). VLDL are secreted by exocytosis. Up to 10^{18} VLDL are secreted per day.

Figure 1 Assembly of VLDL



Adapted from Martin S & Parton RG. Nature Reviews; Molecular Cell Biology 2006,7:373

Since apoB is constitutively expressed and its degradation is regulated, the number of VLDL particles that are secreted is dependent on the amount of apoB degradation. ApoB is degraded by two mechanisms. The first is endoplasmic reticulum associated degradation (ERAD) which occurs when TG availability is low and the apoB is ubiquitinated and targeted to the proteasome. The second is post-ER, pre-secretory proteolysis of apoB (PERPP). This occurs even when TG availability is normal and is stimulated by omega3 polyunsaturated fatty acids (n-3 PUFA's) such as eicosapentanoic acid and docosahexanoic acid. N-3 PUFA induction of PERPP occurs in the Golgi which then directs the apoB to autophagosomes. The PERPP pathway is also sensitive to insulin, whereby acute increases in insulin decrease hepatic VLDL and apoB secretion. *In vivo*, hepatic insulin resistance is associated with decreased apoB degradation by the PERPP pathway, hence VLDL overproduction (Ginsberg and Fisher 2009).

1.4.3 Metabolism of VLDL in plasma

There are two metabolically distinct classes of VLDL, VLDL1 and VLDL2, defined by their flotation rates in salt gradients. VLDL1 are large and TG rich (Sf60-400) and VLDL2 are smaller and cholesterol rich (Sf 20-60). VLDL1 and VDLD2 are regulated independently (Packard and Shepherd 1997). Plasma TG variation is mainly accounted for by VLDL1 (Tan, Foster et al. 1995). VLDL2 is the important determinant of low-density lipoprotein (LDL) concentration (Packard, Demant et al. 2000), and quantitiatively most of the serum apoB and cholesterol is in the LDL pool.

Secreted VLDL2 in the serum are the precursors of LDL. The conversion of VLDL to LDL is a dynamic process facilitated by lipoprotein Lipase (LPL) which resides on the luminal surface of the vascular endothelium and hydrolyses the triglyceride core of triglyceride rich lipoprotein (TRL's i.e. VLDL and chylomicrons) (Adiels, Olofsson et al. 2006). This produces free fatty acids (FFA) which are delivered to muscle and adipose tissue. LPL then dissociates from the endothelium onto the TRL and mediates subsequent targeting to the liver, acting as a bridge between lipoproteins and heparin-sulphate proteoglycans (HSPG) (Williams, Fless et al. 1992). VLDL1 are catabolised predominantly by LPL and then are rapidly cleared as remnant particles via apoE binding to LDLr. VLDL2 are catabolised by both LPL and hepatic lipase (HL) (Packard and Shepherd 1997). The TG depleted VLDL remnant becomes an intermediate density lipoprotein (IDL). The IDL can be cleared by the liver or become LDL. Interferon-α, used in the treatment of HCV, decreases LPL, HL and CETP activities, leading to elevated VLDL triglycerides and low HDL cholesterol (Shinohara, Yamashita et al. 1997).

1.4.4 HCV assembly utilises the VLDL secretion pathway

There is now substantive evidence indicating that HCV co-assembles with VLDL. Suppression of apoB100 and apoE inhibit both VLDL and HCVcc production (Huang, Sun et al. 2007; Gastaminza, Cheng et al. 2008). Moreover further inhibition of HCV was observed by inhibiting MTP, the key enzyme involved in VLDL assembly (Huang, Sun et al. 2007; Gastaminza, Cheng et al. 2008). In the HCV subgenomic replicon system, NS5A interacts with apoB and reduces MTP transcription and activity (Domitrovich, Felmlee et al. 2005). Interestingly Naringerin

which also inhibits VLDL secretion, reduced HCV secretion from infected cells by 80% (Nahmias, Goldwasser et al. 2008).

HCV and VLDL secretion are co-inhibited when expression of long chain acyl coA synthetase 3 (ACSL3) is inhibited by interference RNA (RNAi) (Yao and Ye 2008). ACSL3 is another enzyme crucial for VLDL assembly which conjugates fatty acids to co-enzyme A to allow them to be incorporated into TG, cholesterol ester, and phospholipids e.g. phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

The HCV core protein recruits HCV non-structural proteins and replication complexes to lipid droplet associated membranes for virus assembly (Miyanari, Atsuzawa et al. 2007). The hydrophobic domain D2 of HCV core protein permits the interaction and co-localisation with lipid droplets (Hope and McLauchlan 2000; Shavinskaya, Boulant et al. 2007). At this site, apoE appears to interact with NS5A. Silencing of apoE reduced production of infectious virus particles but did not affect viral entry or replication (Benga, Krieger et al. 2010). The lipid droplets redistribute around the perinuclear region (Boulant, Douglas et al. 2008). This re-distribution of the lipid droplets to the sites of HCV replication may permit an association between new virus particles and VLDL, at the second stage of VLDL assembly (Boulant, Montserret et al. 2006). Importantly in all these studies although HCV secretion was co-inhibited with VLDL secretion, viral replication was unaffected. Even when VLDL is inhibited there remains continued production of high density HCV, suggesting that the association with VLDL occurs at a maturation step for secretion (Gastaminza, Cheng et al. 2008).

The HCV envelope glycoproteins may be able to associate with apoB directly. A study using stable expression of HCV E1 and E2 in caco-2 cells, an intestinal cell line, was dependent on production of apoB. HCV envelope glycoproteins were only detectable in the apoB containing density fractions and were reduced by addition of an MTP inhibitor. The authors believe this data support an intrinsic capacity to the HCV envelope glycoproteins to utilise apoB synthesis for production and proposed a controversial model of ‘empty LVP’ which are HCV envelopes bound to apoB in the absence of nucleocapsids and RNA (Icard, Diaz et al. 2009).

1.4.5 HCV in serum can associate with host lipoproteins.

As outlined above, the heterogeneity of density of HCV in vivo first described by Thomssen et al in 1993 is accounted for by variable association of HCV with lipoproteins and antibodies (Thomssen, Bonk et al. 1992, Thomssen, 1993 #81; Thomssen, Bonk et al. 1993). The association with antibody has been used as the basis of a method of purifying lipoviral particles by Andre et al (Andre, Komurian-Pradel et al. 2002). This method prepared low density fractions of HCV plasma (VLDL, IDL and LDL) by sequential ultracentrifugation. Three fractions (densities <1.0063, 1.006 - 1.025 and 1.025 - 1.055 g/ml, adjusted with NaBr) were defined and these fractions were mixed with protein A magnetic beads. Protein A non-specifically binds IgG and IgM. After being passed down a magnetic column the Protein A immunoprecipitated particles were defined as HCV LVP. The methodology to fractionate HCV according to density was further refined by Nielsen et al by demonstration that the use of iodixanol, which is isosmotic and isotonic with blood was superior to sucrose or sodium bromide gradients in preserving the integrity of the lipoviral particles (Nielsen, Bassendine et al. 2006). Serum was fractionated from a patient without antibodies using iodixanol density gradients and Nielsen et al demonstrated that 91.8% of the HCV RNA could be immuno-precipitated with anti-apoE and 95% of HCV RNA with anti-apoB. The association of HCV with apoB was very strong and was not dissociated even after detergent treatment, whereas apoE was removed by detergent treatment. Further characterisation of this serum using immunopreciptitaion by Nielsen et al suggests that apoC1 is another important component of HCV LVP. Further *in vitro* studies have demonstrated that cell culture derived HCV (HCVcc) could be efficiently immuno-precipitated by anti-apoC1 and these antibodies also neutralized over 75% of infectious HCVcc particles (Meunier, Russell et al. 2008).

Since characterisation of HCV lipoviral particles with immuno-precipitation has indicated that in addition to ApoB, exchangeable lipoproteins including apoE and apoC1 are present on the LVP, the functional properties of these exchangeable lipoproteins are discussed next.

1.4.6 Apolipoprotein E (apoE)

Apolipoprotein E (apoE) is an exchangeable lipoprotein. During lipoplysis of TRLs (Chylomicrons and VLDL) by LPL to become remnant particles, the LPL remains associated with the remnant particle, which also become enriched with apoE derived from HDL. TRL remnants are then trapped in the *space of Disse* by interacting with heparin sulphate proteoglycans (HSPG) and are then internalised into hepatocytes. ApoE and LPL bind to the LDL receptor related protein 1 (LRP1) and the LDLr. Both these receptors are involved in the clearance of TRL remnants, however LRP1 is more important in the clearance of chylomicron remnants (Veniant, Zlot et al. 1998). Following internalisation of CM remnants, the apoE re-associates with HDL and is recycled back out of the hepatocyte (Heeren, Beisiegel et al. 2006). In order for apoE to recycle back onto HDL, HDL has to dock onto the hepatocyte. This is done via Scavenger Receptor class B1 (SRB1) and is discussed in more detail in 1.5.4.

A meta-analysis confirmed that polymorphisms in the *APOE* gene have a major influence on serum cholesterol levels (Bennet, Di Angelantonio et al. 2007). 7% of the variation of total cholesterol in healthy Caucasian individuals is related to three different isoforms of the apoE protein (Davington, Gregg et al. 1988). The wild type protein is *ε3* (apoE3 Cys112 and Arg158), and the two variants are *ε2* (ApoE2 Cys112 and Cys 158) and *ε4 (*apoE4 Arg112 and Arg158) (Mahley and Rall 2000). The *ε2* isoform has low affinity for LDLr and is associated with lower cholesterol and apoB (Utermann 1987). However in those with apoE2 there is defective hepatic clearance of remnant particles via LDLr and LRP1, leading to elevated remnant levels in plasma. ApoE4 has high affinity for LDLr and LRP1. In those with apoE4 there is enhanced uptake of apoE4 TRL remnants but defective recycling of TRL derived apoE4 back to HDL. This leads to accumulation of hepatic intracellular cholesterol and subsequent down regulation of LDLr. This down regulation of LDLr causes elevated LDL cholesterol levels, low HDL_E levels and decreased CR clearance resulting in post prandial hypertriglyceridaemia in those with apoE4. Therefore ApoE4 correlates with high LDL cholesterol, elevated TG and low HDL and has been associated with atherosclerosis (Davington, Gregg et al. 1988) and Alzheimer's disease (Roses 1996).

1.4.7 ApoC's

The apoC's (C1, CII and CIII) are exchangeable lipoproteins that play differing roles in the modulation of lipoproteins in the circulation, including lipolysis, remnant uptake via apoE and interactions with HDL via CETP and LCAT. The genes for apoE, apoC1 and apoCII form a gene cluster on chromosome 19 (Jong, Hofker et al. 1999). The genes for apoA1, CIII and AIV are clustered together on chromosome 11 (Groenendijk, Cantor et al. 2001).

ApoC1 inhibits the uptake of TRL's, particularly by LRP1. ApoC1 may prolong the circulatory time of lipoproteins to facilitate conversion of VLDL to LDL. ApoC1 is also a potent activator of LCAT but inhibits CETP.

Apolipoprotein CII is a 79 amino acid exchangeable lipoprotein which activates LPL. Lack of apoCII leads to severe hyper-triglyceridaemia. The site for activation of LPL resides in the C-terminal one-third of the apoCII molecule. ApoC1 and apoCII both inhibit apoE mediated binding or TRL's to LDLr and LRP1.

ApoCIII is synthesised by the liver and small intestine and is also an exchangeable lipoprotein on TRL's and HDL. ApoCIII is a non-competitive inhibitor of LPL. Over-expression of CIII leads to hyper-triglyceridaemia. There is a functional relationship between apoCIII and apoE on TRL's. Particles with high apoCIII have reduced apoE hence decreased clearance of TRL's. CII and CIII inhibit LCAT by displacing LCAT activators on HDL and ApoCIII also activates CETP.

1.4.8 Low density HCV particles are more infectious.

A hypothesis for the functional significance of HCV associating with host lipoproteins is that it enhances infectivity by facilitating binding and entry to hepatocytes via lipoprotein receptors. However a major difficulty for understanding the pathophysiology of HCV is the lack of an assay to study infectivity of patient derived virus. Development of the HCV cell culture (HCVcc) system was a major breakthrough allowing characterisation of steps of viral replication and permitting screening of potential anti-viral agents *in vitro*. JFH1 is a genotype 2a infectious clone that replicates in cell culture (Wakita, Pietschmann et al. 2005). However the cell line used in this model, Huh 7.5 cells, are derived from human hepatocellular carcinoma and although able to assemble and secrete apoB containing particles, they are known

to not make fully triglyceride loaded VLDL1 sized particles (Podevin, Carpentier et al. 2010). Additionally the JFH1 strain of the HCV is adapted for the cell culture system. Nevertheless in the HCVcc system lipoproteins are still required for HCVcc infectivity, and 70% of HCVcc infection can be blocked by anti-apoB antibodies (Andreo, Maillard et al. 2007). Notwithstanding the limitations of the available model systems, there is evidence that apoB associated and low density HCV LVP are more infectious. The majority of HCVcc particles have a high density (d) ~ 1.15 g/ml, whereas the peak of infectivity is at lower density ~ 1.12 g/ml (Miyanari, Atsuzawa et al. 2007). Although low density HCVcc has greater infectivity than high density HCVcc, it is noteworthy that both these densities are higher than some low density HCV particles found *in vivo*. HCVcc is able to establish long term infection in Chimpanzees and mice containing human liver grafts. When virus was recovered from these animals it showed improved infectivity for cell culture. These animal derived HCV particles of improved infectivity showed a lower buoyant density than HCVcc suggesting that the properties which make the particles of lower density also increase infectivity (Lindenbach, Meuleman et al. 2006). Chang et al analysed particles from HCVcc and found the peak of infectivity at d<1.12 g/ml and that higher density fractions were not infectious. Additionally apoB and apoE were detected on the infectious virions by immune precipitation. ApoE correlated particularly well with infectivity. Monoclonal antibodies specific to apoE efficiently neutralised infectivity in a dose dependent manner, and small interfering RNA (siRNA) to apoE reduced both intracellular and secreted HCV. This suggests that not only is apoE important for infectivity of secreted virus particles, but also that apoE is important in intra-cellular virus assembly (Chang, Jiang et al. 2007)

Another study indicated that low density HCVcc (d 1.02-1.12g/ml) had not only higher infectivity but also higher membrane fusogenicity indicating that lipids associated with HCV also promote membrane fusion (Haid, Pietschmann et al. 2009).

Compelling infectivity data *in vivo* comes from a case report of sexually transmitted acute resolving HCV infection that was acquired from an individual with chronic infection. Analysis of NS3 sequences in the chronically infected individual and the acutely infected patient identified quasispecies of transmitted viral particles in the acute patient that were very similar to quasispecies from the low density fraction

(<1.055 g/ml) of the contaminating patient. This suggests that only HCV from the low density fraction was transmitted (Diaz, Cubero et al. 2008).

1.5 HCV utilises lipoprotein receptors for attachment and entry

The mechanism by which natural HCV binds to and infects hepatocytes is not fully understood. HCV is thought to enter hepatocytes by clathrin-dependent endocytosis followed by fusion of the viral and host cell membranes in the low pH endosomal compartment. Additionally cell-cell transmission has been proposed (Timpe, Stamataki et al. 2008) but the relative importance of this mechanism *in vivo* is unknown. Several candidate receptors have been identified including tetraspanin CD81, scavenger receptor class B1 (SRB1), and the adhesion molecules DC-SIGN and L-SIGN and the tight junction components claudins 1, 6 and 9 which may permit cell-cell transmission (Evans, von Hahn et al. 2007; Zheng, Yuan et al. 2007). Additionally the low density lipoprotein receptor (LDLr) has long been considered important for attachment and entry of HCV *in vivo* (Agnello, Abel et al. 1999; Dubuisson, Helle et al. 2008). Given the close interaction of HCV and lipoproteins, the lipoprotein receptors LDLr and SRB1 will be discussed in detail here.

1.5.1 LDL receptor (LDLr) – physiology and regulation

The Low Density Lipoprotein Receptor (LDLr) is central to maintaining serum cholesterol levels. Expression of LDLr is tightly regulated and coupled to cellular cholesterol levels through the action of sterol regulatory element binding protein (SREBP) transcription factors. When cellular cholesterol levels fall, SREBP's mature and are activated to increase LDLr transcription. This is discussed in more detail later in section 1.6.

LDLr is a seven region trans-membrane glycoprotein, the primary ligand for which is LDL. LDL contains a single apoB and accounts for 65-70% of plasma cholesterol. LDLr also binds apoE containing lipoproteins such as VLDL and chylomicron remnants. Expression of LDLr on hepatocytes is inversely related to serum cholesterol levels (Brown and Goldstein 1986) because of clearance of LDL via LDLr.

LDLr removes LDL particles from the circulation by endocytosis of the LDL/LDLR complex within clathrin coated pits. Internalisation into hepatocytes is controlled by LDL adapter protein 1; the carboxy terminal region interacting with clathrin, the amino terminal region interacts with the LDLr. The extracellular region of the LDLr has a ligand binding domain, an EGF precursor homology domain and an O-linked

sugar rich domain. After LDL binds to the ligand binding domain of the LDLr, the LDLr-ligand complex is internalised to the endosome. Acid pH in the endosome unfolds the LDLr and displaces the LDL particle. The LDL moves to the lysosome where cholesterol esters are hydrolysed to form FFA and free cholesterol and apoB100 is degraded to amino acids. Each LDLr is then recycled back to the cell surface completing about 150 cycles before it is eventually degraded. Increased recycling of the LDLr therefore causes a fall in serum LDL cholesterol levels. LDLr may also act intra-cellularly in the regulation of apoB secretion. After exit from the ER, the VLDL particle is susceptible to bind LDLr via apoB or apoE to inhibit secretion of small, unlipidated VLDL particles (Blasiole, Oler et al. 2008). In this manner LDLr may play a quality control function, diverting nascent VLDL from the late secretory pathway to the endocytic pathway.

It was the work of Brown and Goldstein investigating the cause of Familial Hypercholesterolaemia (FH) that led to the discovery of the LDLr (Goldstein and Brown 2009). Understanding of the LDLr pathway has led to the concept of receptor mediated endocytosis, selective sorting of proteins within the plasma membrane and the concept of feedback regulation of receptors. To date over 1100 separate mutations in LDLr have been identified in patients with the FH phenotype (Goldstein and Brown 2009). FH is therefore due to any number of possible mutations in the LDLr gene. Additionally Autosomal Dominant Hypercholesterolaemia (ADH) can be caused by defects in other genes in the LDL pathway. A genetic defect in apoB100, the structural protein of LDL and ligand of the LDLr also causes the ADH phenotype. Five mutations in the LDLr binding domain of apoB100 have been reported (Boren, Ekstrom et al. 2001). The third gene defect to cause ADH is in a protein responsible for recycling of LDLr called Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) (Abifadel, Varret et al. 2003). The function of PCSK9 is discussed in the next section 1.5.2.

1.5.2 PCSK9 is important in the regulation of LDLr

Proprotein convertase subtilisin kexin-9 (PCSK9) is key regulator of LDLr expression in hepatocytes (Lopez 2008; Horton, Cohen et al. 2009). Secreted PCSK9 in the blood competes with LDL particles for binding to the LDLr. Usually following LDL binding to LDLr, the complex is internalised by receptor mediated endocytosis and the

majority of LDLr is recycled back to the cell surface as described previously 1.5.1. However, secreted PCSK9 can competitively bind to LDLr instead of LDL. The protease of PCSK9 interacts with the LDLr at a highly conserved leucine residue (leu 318) located between two cysteines (Cys 317 and 319) in the EFG precursor homology domain or the LDLr. Once bound, the PCSK9/LDLr complex enters the endosomal pathway where the LDLr is targeted to the lysosome for degradation. Catalytic activity is essential for activation and secretion of PCSK9 but not required for LDLr degradation. PCSK9 therefore influences the number of LDLr molecules expressed at the cell surface. At least 15 functional mutations in the PCSK9 gene have been identified. Some of these are gain of function mutations which result in more LDLr degradation, and others are loss of function mutations associated with increased LDLr recycling. Over-expression of PCSK9 in mice results in 9 fold increase in serum LDL cholesterol. This is only partially explained by reduced LDLr expression since mice lacking LDLr only have a 2 fold increase in LDL cholesterol. Instead it appears that PCSK9 also directly decreases apoB100 secretion (Lopez 2008) in this system.

Not only is PCSK9 important for the regulation of LDLr expression, but also its serum levels in men have been shown to inversely correlate with apoB fractional catabolic rate (i.e. clearance of LDL) because of its direct role in regulating expression of LDLr (Chan, Lambert et al. 2009). Therefore serum PCSK9 levels are an inverse marker of LDL clearance, and correlates positively with LDL cholesterol levels (Dubuc, Tremblay et al. 2009).

1.5.3 LDLr and HCV

There is evidence that the LDLr is important for attachment and entry of HCV *in vivo*. A study using primary human hepatocytes isolated from liver lobectomy specimens from 22 HCV negative patients, modulated expression of LDLr with a statin (squalestatin) which up-regulates LDLr, or 25-hydroxycholesterol which down regulates LDLr. These primary hepatocytes were shown to behave in a physiologically relevant manner and were then infected by HCV positive serum. By modulating LDLr, parallel changes in HCV infection were demonstrated. Furthermore, a soluble LDLr peptide encompassing the entire LDL binding domain markedly inhibited HCV infection whereas shorter peptides did not. Additionally anti-

LDLr monoclonal and polyclonal antibodies markedly inhibited HCV infection (Molina, Castet et al. 2007) .

Another study found an association between LDLr expression on monocytes in HCV infected patients and HCV viral load. Cell surface LDLr expression was found to be significantly associated with both LDL cholesterol and HCV viral load (Petit, Minello et al. 2007).

LDLr may be less important for cell culture adapted HCV however. For example there was no inhibition of HCVpp infection of Huh-7 cells by LDL or anti-LDLr antibodies and no inhibition of HCV-LP entry into MOLT4 cells by anti-LDLr antibodies (Triyatni, Saunier et al. 2002).

Interestingly interferon, which induces several intracellular anti-viral proteins, can also induce extracellular secretion of soluble LDLr as an anti-viral response (Fischer, Tal et al. 1993). Since LDLr is a candidate receptor for HCV, it is interesting to speculate that patients with defective or reduced expression of LDLr such as those with FH and high LDL cholesterol levels may be resistant to HCV infection. The only report of a combined HCV / FH heterozygote case indicates a reduction in LDL cholesterol levels from 7.2 mmol/l pre HCV infection to 1.6 mmol/l following infection by HCV genotype 3, highlighting the considerable cholesterol lowering ability of the virus (Bima, Hooper et al. 2009)

Recently PCSK9 has been shown to have an antiviral effect in HCVcc by down-regulating both CD81 and LDLr (Labonte, Begley et al. 2009).

1.5.4 Reverse cholesterol transport.

Scavenger receptor B1 (SRB1) has also been proposed as a receptor for HCV. SRB1 is important in the process of reverse cholesterol transport, i.e. transporting excess cholesterol from peripheral tissues back to the liver. High density Lipoprotein (HDL) is the mediator of reverse cholesterol transport. ApoA1 is the major protein of HDL, synthesised by the liver and small intestine. In the serum secreted ApoA1 interacts with phospholipids to form nascent discoidal HDL particles. Reverse cholesterol transport by HDL utilises two different pathways. The first is hepatic uptake of cholesterol ester (CE) from HDL which is mediated by SRB1 on the surface of the hepatocyte without internalisation of the apoA1 (Acton, Rigotti et al. 1996). The

second pathway is transfer of CE from HDL to apoB containing lipoproteins in the serum mediated by the actions of Cholesterol Ester Transfer Protein (CETP), and subsequent transport of cholesterol back to the liver in LDL (Tall 1993).

In the first pathway of reverse cholesterol transport, Scavenger receptor B1 has a number of functions. Firstly in peripheral tissues there is cholesterol efflux from macrophages and fibroblasts in the sub endothelial space to the nascent HDL. Cholesterol efflux to HDL is facilitated by SRB1, ATP binding cassette transporter A1 (ABCA1) and cavelolins which transport cholesterol from ER membranes to the plasma membrane. The free cholesterol is then esterified by lecithin-cholesterol acyltransferase (LCAT). ApoA1 is an essential co-factor for LCAT. SRB1 mediates a bidirectional process whereby cholesterol esters are exchanged for excess free cholesterol which is removed from the cell. This transfer of cholesterol to / from the HDL is initially with the plasma membrane and the uptake of cholesterol ester is enhanced by apoE (Bultel-Brienne, Lestavel et al. 2002). Secondly, SRB1 directly binds to lipoproteins apoA1, AII and CIII. Thirdly SRB1 mediates uptake of cholesterol ester from HDL in the liver where it is highly expressed in regions of the hepatocyte plasma membrane called lipid rafts.

In the second process of reverse cholesterol transport, CETP mediates transfer of cholesterol ester from HDL to apoB containing lipoproteins, in exchange for equal molar amounts of TG from the apoB lipoproteins. The cholesterol esters are therefore transferred from HDL to LDL. By the action of CETP, HDL can become TG enriched and is then processed by hepatic lipase to smaller, denser particles. TG enriched HDL are rapidly cleared by the liver. The actions of CETP accounts for the reciprocal relationship between high plasma TG's and low HDL, for example as seen in the metabolic syndrome. CETP inhibitors have therefore been developed as agents to raise HDL, and lower LDL cholesterol, as potential therapy for atherosclerosis (Chapman, Le Goff et al.).

1.5.5 SRB1 and HCV

SRB1 has been proposed as a co-receptor for HCV entry. Uptake of HCV pseudo particles (HCVpp), is mediated by the hypervariable region 1 of the HCV envelope glycoprotein E2 interacting with SRB1 (Bartosch, Vitelli et al. 2003). SRB1 appears to function cooperatively with CD81 in HCVcc to mediate HCV entry (Kapadia,

Barth et al. 2007). HCVcc and HCVpp infectivity had been observed to be enhanced in the presence of HDL (Bartosch, Verney et al. 2005). HDL is a reservoir of apoC1 in serum, and it was shown that apoC1 mediated the effects of HDL enhancing infectivity by interacting with the hypervariable region 1 of the HCV E2 glycoprotein and promoting membrane fusion (Dreux M 2007).

Another study using CHO cells, a hepatoma cell line that does not express the putative HCV receptor CD81, examined binding and uptake of natural HCV from patient sera. This study showed that natural HCV was interacting with SRB1 but that this interaction was not directly with the HCV E2 glycoprotein but was mediated indirectly through an interaction with VLDL which could be inhibited by anti-apoB antibodies and competition from VLDL (Maillard, Huby et al. 2006). The same group also examined the role of lipoprotein lipase (LPL) in the CHO cell system, and found that the amount of bovine LPL added to the system increased the amount of HCV RNA bound to the cells in a dose dependent manner and binding was inhibited by an anti-LPL monoclonal antibody. This interaction with LPL was dependent on the presence of HSPG on the cell surface and the catalytic activity of LPL. However catalytically active LPL inhibited HCVcc binding to Huh 7.5 cells (Andreo, Maillard et al. 2007).

Serum Amyloid A (SAA) is an acute phase protein secreted by the liver and is primarily associated with HDL3 (d 1.12g/ml) (Cabana, Feng et al. 2004). SAA is another ligand for SRB1 thereby inhibiting the interaction of HDL and SRB1. SAA was able to block HCV entry (Lavie, Voisset et al. 2006) but not attachment of HCVcc to Huh 7.5 cells (Cai, Cai et al. 2007). SAA is able to dissociate apoE from HDL in CSF and this effect was more evident in apoE4 than apoE3 or apoE2 carriers (Miida, Yamada et al. 2006). Interferon-α inhibits SRB1 expression through the STAT1/STAT2 interferon signalling pathway in HepG2 cells (Murao, Imachi et al. 2008). These contrasting data on the role played by SRB1 in natural HCV and HCVcc may indicate that different entry mechanisms are utilised *in vivo* and *in vitro* and that varying interactions with lipoproteins may modulate these routes of entry.

1.6 HCV replication and cholesterol pathways

In order to understand the interactions of HCV and cholesterol metabolism, some appreciation of the regulation of cholesterol and lipid homeostasis is required. Intracellular cholesterol levels are tightly regulated to maintain cellular homeostasis. Cholesterol is utilised in membranes, and is the precursor of steroid hormones, vitamin D and bile acids. Intermediates in the cholesterol biosynthetic pathway are also important for protein prenylation, a post translational modification that targets proteins to cell membranes. Cholesterol and triglycerides are both formed from two-carbon Acetyl CoA building blocks. Cholesterol is formed from the Mevalonate pathway. TG are formed from the malonyl-CoA pathway. The rate limiting step in the endogenous cholesterol biosynthetic pathway is the activity of HMG CoA reductase and the production of mevalonate. HMG CoA reductase is the enzyme which catalyses the rate limiting step of cholesterol production from acetyl CoA to a complex 27-carbon 4-ring structure of cholesterol in a series of 26 enzymatic reactions (figure 2). HMG CoA reductase is the target of statins, drugs used to lower serum cholesterol in the prevention of cardiovascular disease.

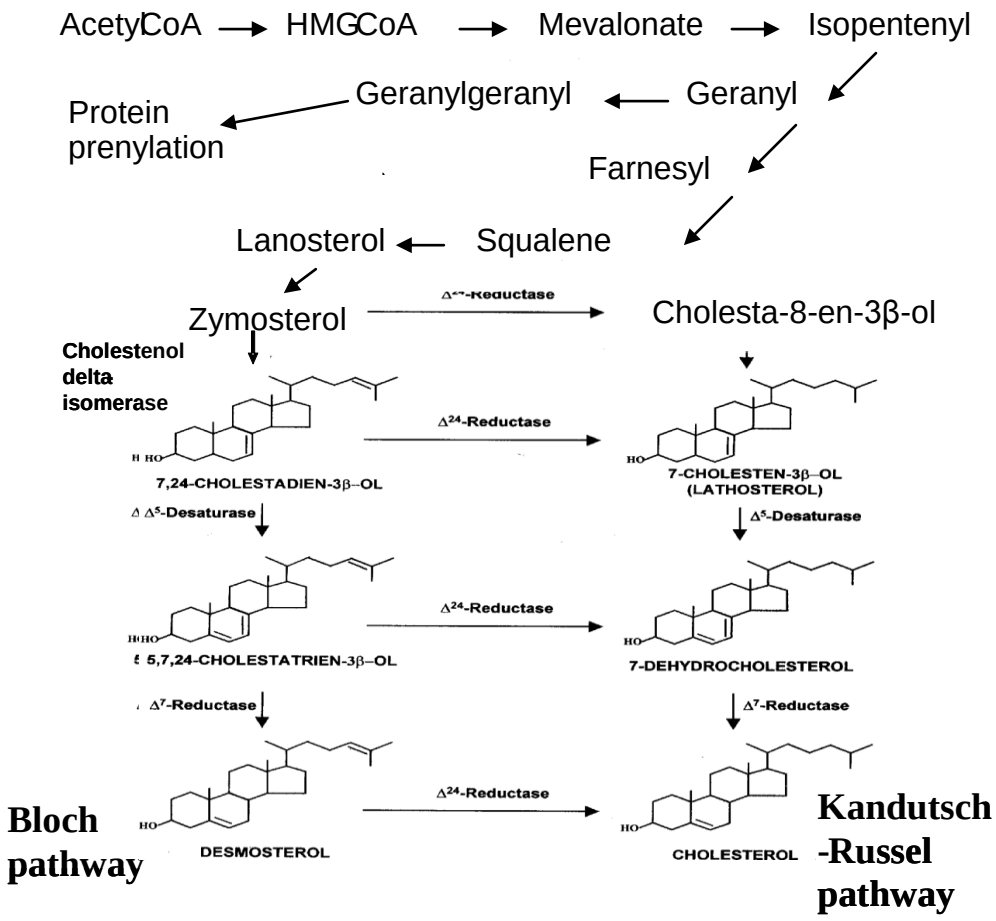


Figure 2 Schematic of the endogenous cholesterol biosynthetic pathway
(Based on Kegg pathway http://www.kegg.com/kegg-bin/show_pathway?rn00900)

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1.6.1 SREBP2 – when intracellular cholesterol is too low

The sterol regulatory element binding proteins (SREBP) act as cholesterol sensors which can switch on or off genes involved in cholesterol metabolism. SREBP's are bound to the ER membrane and when activated travel to the nucleus to act as transcription factors. There are three known members of the SREBP family, SREBP-1a, SREBP-1c and SREBP-2. SREBP-2 has 50% homology with SREBP-1. SREBP-1a activates all SREBP target genes. SREBP-1c activates target genes involved in fatty acid metabolism and SREBP-2 activates genes in cholesterol metabolism (Horton, Goldstein et al. 2002).

When intracellular cholesterol is normal, SREBP-2 is inactive and retained in the endoplasmic reticulum (ER) bound to two ER retention proteins, Insig-1 and Insig-2, and a SREBP cleavage activating protein (SCAP). Statins lower intracellular cholesterol by inhibiting HMG CoA reductase, and when intracellular cholesterol levels fall this is sensed by SREBP-2 which is activated by a series of steps. Firstly the SREBP-2/SCAP complex dissociates from Insig and moves to the golgi. In the golgi the SCAP is cleaved to activate SREBP-2 which then localises to the nucleus to up-regulate transcription of genes in cholesterol biosynthesis (DeBose-Boyd 2008). Because HMG CoA reductase is enzymatically inhibited by statins, intracellular cholesterol production does not increase, but SREBP-2 activation causes increased transcription and translation of LDLr causing increased uptake of exogenous cholesterol from LDL. SREBP-2 not only regulates endogenous cholesterol production but also up-regulates LDLr and PCSK9. SREBP-2 activation therefore responds to intracellular cholesterol depletion by increasing both endogenous cholesterol production and import of exogenous cholesterol via LDLr. This serves to lower the serum LDL cholesterol pool by increasing clearance of circulating LDL particles in an attempt to maintain intracellular cholesterol homeostasis.

1.6.2 Liver X Receptor (LXR) – when intracellular cholesterol is too high

The liver X receptors, LXRα (NR1H3) and LXRβ (NR1H2) are sterol dependent nuclear receptors that respond to cholesterol excess. LXR acts as a cholesterol sensor and is highly expressed in hepatocytes and activated by oxysterols (derivatives of cholesterol). LXR induces expression of genes involved in cholesterol efflux and removal of excess cholesterol in bile acids. LXR target genes include ABCA1 and ABCG1 to promote cholesterol efflux from the cell (Zelcer and Tontonoz 2006). LXR can also suppress LDL uptake by promoting ubiquitination of the LDLr cytoplasmic domain by an E3 ubiquitin ligase called Idol (inducible degrader of the LDLr) (Zelcer, Hong et al. 2009). In addition LXR can induce SREBP-1c to generate fatty acids needed for esterification of cholesterol to buffer the excess free cholesterol concentration. LXR binds to LXR element sequences of the SREBP-1c promoter which activates SREBP-1c transcription.

1.6.3 Lathosterol and endogenous cholesterol production

Cholesterol contained in LDL particles comes from either LDL precursors (i.e.VLDL) that have been secreted by the liver or acquired from HDL in exchange for triglycerides, mediated by CETP. Ultimately the cholesterol has either been endogenously synthesised *de novo* by the mevalonate pathway in the liver and packaged into secreted VLDL, or absorbed exogenously from diet in chylomicrons by the small intestine, and subsequently delivered to the liver in remnant particles. Total body cholesterol therefore represents a balance between endogenous cholesterol synthesis and dietary cholesterol absorption (Matthan and Lichtenstein 2004). Non-cholesterol sterols are also transported in LDL, and although present in small quantities, their measurement in serum gives an indication of the relative contributions of endogenous cholesterol synthesis and dietary absorption to the serum LDL cholesterol pool. Serum levels of non-cholesterol sterols are remarkably stable over time, one study reporting variation over a 48 week period of < 4% (Berge, von Bergmann et al. 2002). Lathosterol and desmosterol are precursors in the endogenous cholesterol biosynthetic pathway (figure 2). Absolute serum lathosterol or desmosterol levels and levels relative to total serum cholesterol (e.g. lathosterol : total cholesterol ratio) reflect endogenous cholesterol biosynthesis (van Himbergen, Matthan et al. 2009).

1.6.4 Cholesterol absorption, biliary excretion & the enterohepatic circulation

Sitosterol is an exogenous plant sterol which is not synthesised endogenously, but derived exclusively from diet by intestinal absorption (Miettinen, Tilvis et al. 1990). Serum sitosterol levels reflect dietary cholesterol absorption by the small intestine mediated by Neiman Pick C1-like 1 protein expression (Huff, Pollex et al. 2006). About one third of the cholesterol absorbed from the small intestine comes from diet. The remaining two thirds of cholesterol absorbed from the small intestine derives from endogenous synthesis by the liver and secreted in bile. Cholestanol is an endogenously produced sterol secreted into the bile. Its levels therefore reflect both cholesterol production and absorption – i.e. the entero-hepatic circulation.

The liver eliminates excess cholesterol as bile acids. Primary bile acids are cholate (CA) and chenodeoxycholate (CDCA) and are synthesised from cholesterol,

conjugated with taurine or glycine to increase their water solubility. Bacteria in the gut act on the primary bile acids to make secondary bile acids, lithocholate (LCA) and deoxycholate (DCA). These are reabsorbed in the ilium and transported back to the liver. Bile acids regulate gene expression in the liver through nuclear receptors FXR and PXR. FXR increases PPARα gene expression and inhibits SREBP-1c. (Watanabe, Houten et al. 2004). FXR target genes can therefore decrease plasma TG concentrations, control liver growth and regeneration and inhibit IFN sensitivity by inhibiting the activation of STAT1. In one study, bile acids enhanced HCV replication and antagonising FXR reduced HCV replication (Scholtes, Diaz et al. 2008).

1.6.5 Statins can inhibit HCV replication

The interactions and requirements of HCV for cholesterol are complex. Up regulation of cholesterol metabolism may be required to permit alterations in membranes required for replication and cell entry. Cholesterol enriched micro domains in the ER membranes called lipid rafts are required for viral replication (Dubuisson, Penin et al. 2002). Moreover, in the plasma membrane, components of the HCV receptor complex CD81 and SRB1 localise to cholesterol enriched micro domains called caveoli, where many cellular proteins including viral receptors are aggregated. Depletion of membrane cholesterol disrupts this localisation and inhibits CD81 mediated entry (Kapadia, Barth et al. 2007).

HCV core protein induces the formation and redistribution of lipid droplets (Boulant, Douglas et al. 2008). This interface between ER membranes and lipid droplets facilitates replication and particle assembly (Miyanari, Atsuzawa et al. 2007).

Replication of HCVcc requires geranylgeranylation of a host protein, FBL2 to permit interaction with the viral non-structural protein, NS5A (Wang, Gale et al. 2005). Geranylgeranyl diphosphate (GGPP) is a host lipid required for protein prenylation, a post translational modification that targets proteins to cell membranes. GGPP is produced from an intermediate in the cholesterol biosynthetic pathway, downstream of mevalonate but upstream of squalene (figure 1). HMG CoA reductase inhibitors (statins), have been shown to have an anti-viral effect on HCV *in vitro*. Lovastatin suppresses HCV RNA levels in genomic and subgenomic HCV replicons by 22-fold (Ye, Wang et al. 2003), but this inhibitory effect is overcome by the addition of

geranylgeranyl (Kapadia and Chisari 2005) indicating that an adequate supply of geranylgeranyl is essential for HCV replication (Kapadia and Chisari 2005).

1.7 HCV is associated with steatosis

Steatosis is the accumulation of hepatocyte lipid droplets and is a common histological feature in chronic hepatitis C. Multiple host and viral factors promote steatosis in HCV and the relative importance of each is not well understood. In genotypes 1 and 4 infection, steatosis is related primarily to host factors such as obesity, waist circumference and diabetes (Negro and Sanyal 2009), whereas HCV G3 appears to have specific virally mediated steatogenic properties. These differences are clinically important because 'metabolic' steatosis correlates with poor response to peg IFNα in HCV genotype 1 but not with 'viral' steatosis in HCV G3 (Romero-Gomez, Viloria et al. 2005). There is evidence of steatosis in up to 70% of those with HCV genotype 3 infection (Hui, Kench et al. 2002; Negro 2004). This appears to be directly related to HCV G3 virus protein expression, resolving with successful eradication of HCV following anti-viral therapy (Mirandola, Realdon et al. 2006). In order to understand the mechanisms of steatosis, some appreciation of the regulation of fatty acid metabolism is essential which is reviewed here.

1.7.1 Regulation of fatty acids – SREBP-1c

The main form of body energy storage is in the form of lipids in adipose tissue. These lipids derive from both diet (exogenous) and *de novo* synthesis from non lipid substrates including glucose and free fatty acids. Lipogenesis takes place in the liver. D*e novo* lipogensis only contributes a relatively small fraction (2-5%) of triglyceride to VLDL normally. This is increased to 20-30% in individuals with diets high in sugar and alcohol, and in NAFLD and infections.

Sterol regulatory element binding protein 1c (SREBP-1c) is the transcription factor that induces expression of genes involved in lipogenesis (endogenous synthesis of lipids). Like SREBP-2, SREBP-1c is synthesised as a precursor that resides in an inactive form in ER membranes before undergoing post-translational modification to become active. Proteolytic maturation and activation of SREBP-1c is stimulated by insulin, accounting for the lipogenic effects of chronic hyper-insulinaelmia, such as occurs in insulin resistant states for example the metabolic syndrome. Insulin induces

the transcription of SREBP-1c in liver, adipose tissue and muscle leading to an increase in expression of ER membrane bound and precursor forms of SREBP-1c. Gene transcription targets of SREBP-1c include glucokinase which is activated by insulin and inhibited by glucagon. Glucokinase phosphorylates glucose to Glucose-6-phosphate which is the first step of glycogen synthesis and glycolysis. Lipogenic genes such as L-PK, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are also activated by SREBP-1c and lead to a marked increase in hepatic triglyceride content. There is diurnal variation in expression of the lipogenic genes which are controlled in part by plasma insulin concentration and food intake.

Increased activity of SREBP-1c has been described in several models of insulin resistance. SREBP-1c levels are increased in fatty livers from obese, insulin resistant hyperinsulinaemic ob/ob mice. Alcohol promotes SREBP-1c expression and causes ER stress. ER stress can further induce the cleavage and hence activation of SREBP-1c. Ultimately SREBP-1c results in increased synthesis of fatty acids and their incorporation into triglycerides and phospholipids, resulting in lipid accumulation in the liver and steatosis.

1.7.2 PPAR's

Peroxisome-proliferator-activated receptor alpha (PPARα) is a transcription factor of the nuclear hormone receptor family. PPARα is a sensor for free fatty acids (FFA) and up-regulates genes involved in the β oxidation and transport of fatty acids (Staels, Dallongeville et al. 1998). PPARα regulates oxidation of fatty acids in mitochondria and peroxisomes. It is expressed in liver, heart, skeletal muscle and brown adipose tissue. It also mediates the effects of leptin in adipose tissue. When fatty acid concentrations increase, PPARα activation increases FFA uptake and oxidation. PPARγ is expressed predominantly in adipose tissue and when activated increases insulin sensitivity and enhances lipid storage in adipose tissue. Glitazones are PPARγ agonists. Therefore activation of PPARα and PPARγ lowers lipid levels. PPARα is also activated by poly-unsaturated fatty acids (PUFA) and fibrates. Fish oil contains omega3 PUFA's (n3 PUFA), docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) which bind to and activate PPARα.

PPAR's have an N-terminal DNA binding domain and a C-terminal ligand binding domain. Ligand binding causes a conformational change allowing recruitment of a co-

activator called retinoid X Receptor (RXR) and the PPAR/RXR complex can bind to target genes. Levels of PPARα are lower in HCV patients with steatosis than those without steatosis (Dharancy, Malapel et al. 2005), and are lower in liver biopsies from patients with HCV genotype 3 than genotype 1. A PPARα antagonist dispersed HCV replication complexes away from the membranous web environment (Lyn, Kennedy et al. 2009). A hypothesis that is explored in results chapter 5 is therefore that n3 PUFA may improve HCV related steatosis.

1.7.3 Mechanisms of steatosis in HCV

There are at least four potential mechanisms implicated for virally mediated steatosis in HCV G3. Firstly HCV induces *de novo* lipogenesis by up-regulating SREBP-1c and fatty acid synthase (Waris, Felmlee et al. 2007). Expression of HCV G3 core up-regulated the FAS promoter in an SREBP-1c dependent manner, and had a stronger effect on FAS activation than HCV G1b core. A single amino acid residue (phenylalanine^{164}) in the D2 region of HCV core which binds core to lipid droplets was critical for the steatogenic effect in HCV G3 (Hourioux, Patient et al. 2007; Jackel-Cram, Babiuk et al. 2007). Secondly HCV is associated with reduced mitochondrial oxidation of fatty acids. This is thought to be mediated by PPARα which induces oxidative enzymes and FA import into mitochondria by carnitine palmitoyl acyl-CoA transferase 1 (CPT1A). There is evidence that PPARα is inhibited by HCV (Dharancy, Malapel et al. 2005). Thirdly HCV core protein can reduce triglyceride export by impairing VLDL secretion via inhibition of MTP to induce steatosis (Perlemuter, Sabile et al. 2002). In liver biopsy specimens, MTP mRNA levels were significantly lower in HCV G3 than HCV G1 (Mirandola, Realdon et al. 2006). Fourthly there may be increased reactive oxygen species and increased lipid peroxidation. This has been demonstrated when HCV core protein is expressed in Huh7 cells (Okuda, Li et al. 2002).

1.8 HCV is associated with Insulin Resistance

Chronic hepatitis C infection is associated with insulin resistance. An understanding of insulin action and the methods used to evaluate insulin sensitivity are important to evaluate insulin resistance in HCV.

Insulin is secreted by the pancreatic β-cells in response to an increase in plasma glucose. Insulin promotes peripheral glucose uptake in muscle and fat and suppresses hepatic glucose production. Insulin sensitivity is the relative capacity of insulin to promote a decrease in blood glucose. Insulin resistance is defined as decreased sensitivity or responsiveness to the metabolic actions of insulin. Insulin resistance can occur in any target tissue and is influenced by genetic and environmental factors e.g. obesity and exercise. Measuring insulin sensitivity in the clinical situation is a subject of debate in the diabetes literature. The 'Gold Standard' method is the euglycaemic hyperinsulinaemic clamp. In this method, after an overnight fast, a continuous insulin infusion is set up to establish a new steady state insulin level above the fasting level. This increases glucose disposal in skeletal muscle and fully suppresses hepatic glucose production. A simultaneous 20% dextrose infusion is given to maintain glucose concentrations in the normal range, thereby establishing a new steady state plasma insulin, glucose and glucose infusion rate. Under these conditions the glucose infusion rate is equal to the glucose disposal rate in peripheral tissues because hepatic glucose production is completely suppressed. Additionally radio labelled glucose tracers can be used under clamp conditions to estimate hepatic glucose production. Under these conditions it is possible to measure hepatic vs. peripheral (predominantly skeletal muscle) insulin sensitivity. However the euglycaemic hyperinsulinaemic clamp is a time consuming, labour intensive method with technical difficulties and is inappropriate for large scale applications. Therefore surrogate measures of insulin sensitivity are used that measure fasting insulin and glucose levels. Elevations in fasting insulin correspond to increased insulin resistance. The Homeostasis Model Assessment of Insulin Resistance (HOMA IR) is a simple surrogate index of insulin resistance, calculated as follows: HOMA IR = fasting insulin (μU/ml) x fasting glucose (mmol/l)/22.5. The denominator of 22.5 is a normalising factor based on a "normal" healthy fasting plasma insulin level of 5μU/ml and glucose of 4.5 mmol/l. Therefore a normal HOMA IR = 1. HOMA IR correlates reasonably well to the

glucose clamp method, but is unreliable in patients with impaired β-cell function. The Quantitative insulin sensitivity check index (QUICKI) is also derived from the fasting glucose and insulin measurements as follows: QUICKI = 1/ (log fasting insulin μU/ml + log fasting glucose mg/dl). QUICKI may be more appropriate when populations include diabetics where fasting glucose is inappropriately high and insulin inappropriately low, and in this situation has a better correlation with glucose clamp estimates than HOMA IR (Muniyappa, Lee et al. 2008). As insulin resistance increase, so QUICKI scores tend to decrease.

1.8.1 Insulin receptor and signaling pathway

The insulin receptor (IR) has two extracellular α-subunits and 2 transmembrane β-subunits that have intracellular tyrosine kinase activity. Insulin binds to the extracellular domain of the insulin receptor. The insulin receptor has substrate tyrosine kinase activity which initiates phosphorylation reactions. This triggers a second messenger cascade which diverges and involves multiple other pathways. Insulin reduces the concentration of insulin receptors by increasing internalisation and degradation. Seven tyrosine residues in the cytoplasmic β-subunit domain are the sites of auto-phosphorylation. Substrates of the insulin receptor are IRS1 and IRS2 and Shc and APS. Phosphatidyinositol-3-kinase binds to IRS1. This promotes translocation of Glut 4 vesicles to the plasma membrane and increases the rate of glucose uptake. Cell growth and protein synthesis are associated with Ras/MAPK pathways which are activated by Shc phosphorylation. In the state of insulin resistance, the ability of insulin to initiate these phosphorylation cascades is reduced. IRS1 mediated signalling is dependent on auto-phosphorylation but IRS2 signalling is not. In type 2 diabetes, insulin receptor auto-phosphorylation is reduced in muscle following insulin infusion. This is also observed in obese patients and correlates with percent body fat. Diets high in fat and fructose reduce insulin receptor auto-phosphorylation and physical activity improves insulin sensitivity.

Glucagon is secreted by the pancreatic α-cells in the fasting state and opposes the actions of insulin, stimulating hydrolysis of adipose tissue triglyceride and releasing free fatty acids (FFA) and glycerol into the blood. FFA undergo beta oxidation in hepatocyte mitochondria and are converted to ketones in the fasting state. The ketones

are used as a source of energy for skeletal muscle, sparing glucose for use by the brain.

1.8.2 Hepatitis C and Insulin Resistance

Insulin resistance is common in patients with CHC and adversely affects fibrosis progression (Hui, Sud et al. 2003) and response to anti-viral therapy (Poustchi, Negro et al. 2008). In a detailed study of non-obese males with CHC using the euglycaemic hyperinsulinaemic clamp method, insulin resistance was found principally in peripheral tissues rather than due to impaired hepatic insulin action. FFA could be suppressed by low dose insulin, suggesting that the peripheral insulin resistance is in skeletal muscle rather than adipose tissue (Milner, Van Der Poorten et al. 2009). This study also found that CHC patients had higher levels of Lipocalin-2 and glucagon. Lipocalin 2 is an adipocytokine that antagonises the pro-inflammatory effects of TNFα on inflammation and up-regulates PPARγ in adipocytes (Zhang, Wu et al. 2008). Insulin resistance in HCV has been correlated with viral load (Moucari, Asselah et al. 2008). Moreover insulin sensitivity as measured by HOMA IR improves following clearance of HCV with anti-viral therapy (Kawaguchi, Ide et al. 2007). This suggests that HCV is either having a primary effect on insulin signalling, or a secondary effect through virally induced adipocytokines such as TNFα.

A primary effect by which HCV may be promoting insulin resistance may be mediated by viral proteins enhancing expression of Suppressors of Cytokine Signalling (SOCS). SOCS are a family of 8 proteins that regulate the cellular response to cytokines in a negative feedback manner. SOCS expression is induced by cytokines such as TNFα. SOCS inhibit the Janus Kinase-signal transducer and activator of transcription (JAK-STAT) pathway. SOCS were originally shown to attenuate the ability of JAKS to phosphorylate downstream STATS. SOCS proteins bind phosphorylated tyrosine residues in cytokine receptors and then inhibit or cause degradation of the receptor. SOCS-1, SOCS-3 and SOCS-6 can bind to the insulin receptor. The SH2 domain of SOCS binds to the insulin receptor, resulting in decreased insulin stimulated auto-phosphorylation of IRS-1 and IRS-2. Additionally the ‘SOCS box’ binds to IRS1 and IRS2 directly thereby targeting IRS1 and IRS2 for ubiquitination and proteasomal degradation. SOCS3 also inhibits leptin receptor signalling by inhibiting leptin receptor tyrosine phosphorylation of JAK2 and

consequently inhibits the STAT3 signal. Over-expression of SOCS1 and SOCS 3 causes insulin resistance and increased SREBP-1c in a mouse model of metabolic syndrome and steatosis (Ueki, Kondo et al. 2004). HCV core enhances SOCS3 expression leading to ubiquitination and degradation of IRS1 and IRS2 in hepatoma cell lines (Howard and Flier 2006), and may contribute to primary hepatic insulin resistance and steatosis.

A secondary effect of CHC on insulin resistance may be mediated through adipocytokines in a similar way to obesity related insulin resistance. Adipocytokines including adiponectin, leptin and resistin are produced by adipocytes. Adiponectin functions as an insulin sensitiser, increasing free fatty acid oxidation and glucose uptake. It has anti-inflammatory properties induces IL10 and inhibits IL6 and TNFα. Low levels of adiponectin are seen in type 2 diabetes and obesity. Leptin influences neuroendocrine function by acting on the hypothalamus. In obesity, leptin levels are high indicating leptin resistance. Resistin antagonises the effects of insulin in adipocytes and induces SOCS3 to inhibit the insulin receptor.

Low levels of adiponectin are associated with hepatic steatosis in non-alcoholic fatty liver disease and insulin resistance (Whitehead, Richards et al. 2006). However in HCV patients with insulin resistance, leptin and adiponectin levels are similar to those of age and BMI matched controls (Cua, Hui et al. 2007). Although TNFα is elevated in CHC, TNFα levels did not correlate with insulin action in the euglycaemic clamp study (Milner, Van Der Poorten et al. 2009). Furthermore there is no increased insulin resistance in hepatitis B where TNFα is also elevated, indicating that a secondary effect of HCV on insulin resistance mediated via adipocytokines is less important than a primary effect.

Insulin resistance is a key feature of the metabolic syndrome which is associated with high triglyceride levels. Insulin resistance is important in determining plasma TG levels in the metabolic syndrome. In normal states insulin up-regulates lipoprotein lipase activity in adipose tissue, inhibits release of FFA from adipose tissue and directly suppresses hepatic VLDL production. However in insulin resistance, the normal suppressive effect of insulin on VLDL is reversed. VLDL are metabolically heterogeneous. In a study of normo-glycaemic insulin resistant middle-aged men and women, HOMA IR correlated strongly with VLDL1 production rate and VLDL1 apoB pool size, but not with VLDL2 (Gill, Brown et al. 2004). LPL activity generally

decreases with insulin resistance, so the TG component of large VLDL1 are hydrolysed more slowly. In contrast hepatic lipae (HL) increases in insulin resistance leading to increased catabolism of VLDL2 to IDL and LDL. In the normal state insulin can down-regulate MTP gene expression, which reduces transfer of cytosolic TG to pre VLDL, resulting in more apoB degradation. In the insulin resistant state, the inhibitory effect of insulin on MTP is impaired, so more TG are loaded and larger VLDL1 are produced.

In summary, this thesis is translational research that stems from work funded by an MRC experimental medicine grant. The translational research cycle begins with the clinical problem. For patients with chronic HCV in 2010 there are still many challenges, particularly for those who have not responded to PegIFα and ribavirin or are intolerant of its side effects. Further understanding of the mechanisms of non-response is therefore essential to improve treatment options for these individuals. The intimate interaction of HCV with host lipid metabolism in the viral lifecycle makes this an important area of research. In particular the close relationship between HCV and VLDL in assembly of infectious viral particles and the requirement of cholesterol pathways for HCV replication suggests that further understanding of these processes may be clinically important. It is apparent that HCV is promoting a dyslipaemia in chronic infection characterised by low lipids, steatosis and insulin resistance. How these metabolic disturbances relate to the viral lifecycle and likelihood of responding to anti-viral therapy is not known. The observational studies presented here aimed to shed new light on the interaction between HCV and lipids. Further understanding of these interactions begins to indicate potential mechanisms that need to be elucidated to develop new therapeutic strategies. The pilot intervention trial using lipid modulating therapy tests some of these concepts to complete a translation research cycle.

1.9 Aims of the study

The aims of this study were:

1. To characterise in detail abnormal lipid profiles and metabolic parameters in patients with chronic hepatitis C.

2. To determine the relative importance of the influence of viral factors such as HCV genotype and viral load on lipid profiles verses other host factors known to influence lipid profiles such as apoE genotype.

3. To evaluate differences in lipid and metabolic parameters before and after viral eradication in sustained virological responders compared to viral persistence in non-responders to anti-viral therapy.
   These first 3 aims are addressed in chapter 3.

4. To understand further the lipid abnormalities present in HCV in terms of cholesterol production, absorption and clearance. This is addressed in detail in chapter 4.

5. To assess the influence of lipid profiles as determinants of anti-viral treatment outcome and possible mechanisms for the association. This is considered in chapter 5.

6. To understand how altered lipids and metabolism influence the formation of HCV lipoviral particles (LVP). This is addressed in Chapter 6 with the development of a novel LVP assay, which permitted futher evaluation of the metabolic determinants of LVP. The clinical utility of the LVP assay was evaluated prospectively in patients treated with anti-viral therapy.

7. To test whether lipid modulating therapy can alter the lipid abnormalities in HCV that impact on HCV LVP. This is addressed in the clinical trial in chapter 7.

2 Chapter 2. Materials and Methods

2.1 Retrospective HCV cohort

2.1.1 Data collection and establishing a DNA database

The retrospective HCV cohort was collected by identifying patients with known anti-viral treatment outcomes from a search of C-Base, a clinical database which is used routinely for audit, existing treatment lists provided by the viral hepatitis nurses at Freeman Hospital, and a previous dataset collected by Dr Ashley Price for a previous study in which ApoE genotyping had been performed using a standard PCR-RFLP method (Price, Bassendine et al. 2006). The combined data sets were entered into a new Microsoft® Access database and duplications identified and removed. Clinical phenotype data was collected including age, sex, HCV genotype, viral load, treatment outcome and whether or not they had previously provided a DNA sample. Participants had previously provided written consent for collection and analysis of DNA. Additionally non-fasting lipid profiles that had been taken pre and at least 6 months post anti-viral treatment were recorded where available. Complete data was available in 129 CHC patients who had undergone antiviral treatment, 72 of whom achieved an SVR and 57 were non-responders. Additionally there were 121 patients identified from the clinical treatment outcomes records in whom paired pre and post treatment lipid profiles were available, but in whom DNA was not available. This combined retrospective data was analysed for the study of lipid profiles in chapter 3 and treatment outcomes study in results chapter 5.

2.1.2 Non fasting lipid profiles

In the retrospective HCV cohort, total cholesterol, HDL cholesterol, non-HDL cholesterol and triglycerides had been measured by standard automated enzymatic methods (Olympus Diagnostics UK Ltd). Non-fasting serum lipid profiles (total cholesterol, triglycerides, HDL) were measured pre- and 24 weeks post treatment, and where possible paired data was collected thereby allowing each patient to act as their own control. Since these were non fasting samples, LDL-cholesterol, estimated by the Friedwald calculation (Bairaktari, Seferiadis et al. 2005) was not assessed. Therefore

apolipoprotein B associated cholesterol was calculated on each patient by subtracting HDL-cholesterol from total cholesterol level (non-HDL cholesterol). Paired pre- and post- treatment cholesterol levels were available in 100 patients achieving an SVR (90 HCV genotypes 1 and 3) and 66 non-responders (63 HCV genotypes 1 and 3). The remaining patients had incomplete or unpaired data.

2.2 Prospective HCV cohort

The prospective study consisted of three separate but related sub-studies, each with its own ethics approval and registration with UKCRN. These sub-studies were titled as follows:

1. Analysis of HCV host lipid interactions in chronic infection; UKCRN 6313; ethics approval from Northumberland REC 07/H0902/45.

2. A randomised controlled, factorial pilot study investigating Omacor and / or Fluvastatin in patients who have not responded to standard anti-viral therapy. UKCRN 4622; ethics approval from Fife and Forth Valley REC 07/S0501/21.

3. Application of a novel method to determine hepatitis C virus and very low density lipoprotein (VLDL) kinetics UKCRN 6863 (Intralipid Kinetics study); ethics approval from Newcastle 2 REC 09/H0907/17.

Another part of the HCV host lipid interactions study was a post prandial study in 10 CHC patients. The laboratory work for the post prandial and Intralipid kinetics studies was performed by Dr Dan Felmlee and thus is not further included in this thesis. I (DS) recruited and performed the clinical part of the post prandial and Intralipid studies and helped with experimental design and data analysis, hence I have included the post prandial study in the appendix of publications arising from this thesis.

2.2.1 Prospective study recruitment

The prospective HCV cohort was recruited from the viral hepatitis clinic at the Freeman Hospital, Newcastle upon Tyne, UK. The Newcastle upon Tyne Hospitals NHS Foundation trust acted as sponsor. Participants in the prospective study were invited to attend for fasting blood samples and clinical assessment during routine clinic review. Each participant was given the patient information leaflet (PIL) for the

relevant sub-study. After participants had read the PIL written informed consent was provided.

2.2.2 Inclusion and exclusion criteria

Prospective study participants that had provided consent were unselected apart from the following conditions: age ≥ 18, positive HCV RNA by PCR for >6 months (including treatment naïve patients and previous non responders to combination interferon-α and ribavirin antiviral therapy), no lipid modulating agent for 3 months prior to the study. Patients with hepatitis B virus (HBV), hepatitis delta virus (HDV) or human immunodeficiency virus (HIV) co-infection, alcohol dependency or concurrent lipid lowering therapy were excluded from the study. Other study specific inclusion and exclusion criteria for the clinical trial are indicated in the relevant chapters.

2.2.3 Healthy volunteers

Two non-HCV comparator groups were used in this study. Comparator group A was a group of healthy volunteers aged 18-55 that were recruited to a dietary intervention study by the University of Surrey in response to adverisements in local Newspapers. These subjects had no medical conditions known to affect lipid profiles, and refrained from eating cholesterol lowering functional foods such as those containing plant sterols 2 weeks prior to lipid measurements. Additionally this group excluded those that had been on a weight reduction diet or lost >3 kg in weight in the preceding 2 months. All the participants gave written informed consent and the study was approved by the University of Surrey's ethics committee. The lipid and sterol profiles data on these 45 healthy volunteers was provided by Dr Nicola Harman from the University of Surrey as a comparator for sterol profiles in the HCV cohort. The data on this comparator Group A has been published previously (Harman, Leeds et al. 2008).

Comparator group B data was provided by Dr G Dubuc and Prof J Davignon, University of Montreal, Quebec, Canada and included fasting lipid profiles, age, sex and BMI and PCSK9 measurements. These healthy volunteers were over 18 years of age and were not taking any medication for hyperlipidemia, hypertension, or diabetes. Samples were taken from 254 healthy volunteers (117 males and 137 females) and all

subjects gave informed written consent. The Institut de recherches cliniques de Montréal (IRCM) ethics committee approved this protocol. The data on this cohort has been published previously (Dubuc, Tremblay et al. 2009).

These data sets were used as the comparator groups for lipid profiles of the HCV patients and are detailed in the relevant chapters.

2.2.4 Prospective HCV cohort study groups

The prospective HCV cohort was sub-divided into study groups for the purposes of sample identification and data collection. Participants in the clinical trial were initially allocated a screening code, either NS 1-32 for those from the Newcastle cohort or LS 1-32 for those from the St Mary's Hospital, London cohort. Subsequently those screened for the trial were allocated a trial code from groups A to F, based on randomisation group. Those that were screen fails were allocated to Group G. Additionally patients that attended prospectively for fasting blood tests and clinical assessment but not in the clinical trial were allocated to group H. Those taking part in the 6 hour post-prandial study were allocated to Group I, and those in the Intralipid study to group IL.

2.2.5 'Symphony' database

Data from the prospective HCV cohort was captured on a web based clinical trials software package called 'Symphony' (http://newcastlectu.powertrial.com). Participants were anonymous in the database but identified by trial codes depending on groups. The software was designed for purpose and captured data in several domains. These included inclusion and exclusion criteria, demographic information (age, sex, ethnicity), clinical assessment (BP, waist hip circumference), NMR lipidomics dietary questionnaire, viral and fibrosis assessment (HCV genotype, viral load, previous liver biopsy), Fibroscan and MRI, oral glucose tolerance test, haematology (full blood count), biochemistry (U&E, LFT, CK, fasting lipid profile, glucose and insulin), virology, genetic tests (apoE genotype and storage of DNA), additional lipid tests (sterols, apoA1, B, E) additional metabolic tests (PCSK9), lipoprotein profiling tests and post prandial study. All databases had Caldicott approval from Newcastle upon Tyne Hospitals NHS Foundation Trust.

2.2.6 Clinical assessment

Participants in the prospective study had a full clinical assessment when they attended the Clinical Research Facility (CRF) for fasting blood tests. The following data was collected: sex, age, weight (kilograms), height (meters), waist and hip circumference (centimetres) and blood pressure. Body mass index (BMI) was calculated as weight divided by the square of the height (kg/m^2).

2.3 Laboratory Methods

2.3.1 Fasting lipid profiles and metabolic analysis

In the prospective study, participants attended the Clinical Research Facility following a 12 hour fast. Serum samples were taken for total cholesterol, LDL cholesterol, non-HDL cholesterol, triglycerides and glucose which were measured by standard automated enzymatic methods as above (Olympus Diagnostics UK Ltd). Additional biochemical analysis included liver function tests (alanine and aspartate transaminase and gamma glutamyl transferase), and renal function (urea, electrolytes and creatinine). Apolipoprotein A1 and B were measured by automated rate nephelometric methods (BNII, Dade Behring Ltd). Plasma glucose was measured from fluoride oxalate tubes using an automated glucose oxidase method (Olympus Diagnostics UK Ltd). Insulin was measured by ELISA (Linco Research Inc). Insulin resistance was be estimated from measured fasting glucose and insulin by calculation of the HOMA IR score (glucose x insulin / 22.5) (Matthews, Hosker et al. 1985). All of the above tests were performed by the Clinical Biochemistry department, Royal Victoria Infirmary.

2.3.2 Sterol Analysis

All participants in the prospective HCV cohort had a fasting sterol profile. Lathosterol, desmosterol, cholestanol and sitosterol were measured by gas chromatography mass spectrometry (GCMS) as described previously (Kelley 1995) with some modifications and were performed by Dr Kim Bartlett, Dept of Clinical Biochemistry, Royal Victoria Infirmary. In brief, the analysis was carried out as follows. 50µL of serum was mixed with 50µL 1mmol/L 5β-cholestan-3α-ol (Epicoprostanol (EPIC)) in ethanol and the mixture then saponified in 1ml 4% (w/v) KOH in ethanol, vortexed for 30 seconds and incubated for 60 mins at 64°C. After

saponification the samples were mixed with 1ml H_2O and vortexed for 30 seconds. The samples were then extracted in Hexane by adding 3.5ml of Hexane, vortexing the mixture vigorously for 2 mins then left to stand at room temperature for 5 mins. The upper phase was transferred to a 10ml 'Chromacol' screw cap tube. The hexane extraction was repeated twice, the extracts combined and the solvent evaporated under nitrogen to dryness at 40°C. 20µl anhydrous pyridine was added to the dried extract and mixed by vortexing. The samples were protected from light and left at room temperature overnight. Formation of the trimethylsilyl ether derivatives was achieved by addition of 80µl of BSTFA+1%TMCS, vortexing and heating at 64°C for 60min. Samples were cooled to room temperature and 40µl transferred to a GC autosampler vial prior to analysis by gas chromatography mass spectrometry (GCMS).

GCMS analysis was achieved using an Agilent MSD 5975 mass spectrometer fitted with an Agilent 6890 GC and autosampler. Samples (1µl) were introduced by means of splitless injection onto a J&W® DB5, 30m (0.25µm x 0.32mm id) column and subjected to temperature programming (150ºC isothermal for 2min, 15 ºC.min-1 to 260 ºC, 8 ºC.min-1 to 290 ºC, 290ºC isothermal for 15min). The carrier gas was helium (0.9 mL.min-1). Sterols were detected by selected ion monitoring [cholesterol (m/z 368), cholestanol (m/z 445), epicoprostanol (m/z 355), desmosterol (m/z 372), lathosterol (m/z 458), sitosterol (m/z 396), campesterol (m/z 382), squalene (m/z 341)] and quantitated by reference to the internal standard and calibration curves, constructed by analysis of standard mixtures of sterols.

2.3.3 DNA extraction and quantitation.

DNA was extracted from whole blood using the QIAamp blood midi kit (spin protocol) according to the manufacturer's instructions. Briefly 200µl of QIAGEN protease was added to a 15ml centrifuge tube with 1-2 mls of whole blood or cell pellets and mixed briefly. The volume was made up to 2ml by addition of sterile PBS. Lysis buffer AL (2.4 mls) was added and mixed thoroughly by inverting the tube 15 times, followed by vigorous shaking for at least 1 minute. The sample was incubated at 70 °C for 10 minutes. 2 mls of ethanol (96-100%) was added to the sample and mixed by inverting 10 times followed by additional vigorous shaking. Half of the solution was transferred onto the QIAamp midi column placed in the 15mls centrifuge tube and spun at 3000 rpm for 3 mins. The midi column was removed and the filtrate

discarded. This was repeated with the remainder of the solution transferred onto the column and centrifuged again at 300 rpm for 3 mins. Two washing steps were performed by adding 2 mls of buffer AW1 to the QIAamp midi column and centrifuged at 5000 rpm for 15mins, followed by 2mls buffer AW2 and centrifuged at 5000 rpm for 15 mins. The washed QIAamp midi column was then transferred to a clean 15 mls centrifuge tube for elution. Elution was performed by adding 300µl of buffer AE to the membrane of the midi column, incubated at room temperature for 5 mins and centrifuged at 5000 rpm for 2 minutes. A second elution with another 300 µl of AE onto the membrane was repeated for maximum yield. The eluate was transferred to a clean sterile labelled eppendorf and stored at -20 °C until used. Extracted DNA was quantitiated using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and DNA concentrations (ng/µl) recorded on the DNA database.

2.3.4 ApoE genotyping

In the prospective HCV cohort ApoE genotype was determined in using an automated dual fluorescent melting curve technique on genomic DNA isolated from EDTA whole blood using a commercial kit method (Nucleon DNA Extraction Kit, Amersham Life Sciences). ApoE genotyping was performed by the Clinical Biochemistry department, Royal Victoria Infirmary.

2.3.5 Venous plasma preparation for HCV LVP analysis.

A variable proportion of HCV RNA in the plasma of immunocompetent patients is precipitated on freezing (Nielsen, Bassendine et al. 2006). This is likely to be due to association of HCV with cryoglobulins (Agnello 1997). To minimise this, in the prospective HCV cohort blood samples were collected into EDTA vacutainer tubes (BD Biosciences) maintained at 37 °C and processed immediately for LVP assessment. The plasma was separated by centrifugation at 3000 rpm for 10 minutes at 37°C in a Rotanta 460R Hettich centrifuge (DJB Labcare, Buckinghamshire, UK). Complete protease inhibitor cocktail (Roche) was added to the plasma to prevent protein degradation. The plasma sample was flash frozen in liquid nitrogen for storage at -75 °C until used.

2.3.6 Iodixanol density gradient ultracentrifugation.

0.5mL of plasma was added to 9.5 mL 12.5% iodixanol solution (2.1ml iodixanol (60%) (Optiprep, Axis-Shield), 200 μL 100mM EDTA, pH 8.0, 200 μL 0.5M Tris-HCL pH 8.0, 7.45 ml 0.25M sucrose, 25 μL 2M $MgSO_4$, 25 μL 2M $MgCl_2$) in polycarbonate 16 x 76 mm centrifuge tubes (Beckman) and inverted several times to mix thoroughly before centrifuging at 50,000 RPM for 24 hours at 4°C in a type Ti50 rotor and a L8-7M ultracentrifuge (Beckman). Gradients were harvested according to whether they were from the LVP assay development cohort or the validation cohort. For the LVP assay development cohort (N=7 healthy volunteers and 9 CHC patients), the gradients were harvested from the top manually by collecting 20 x 500μL fractions. Each fraction was then used for measurement of apoB by western blot to determine the distribution of apoB in the density gradient. The detailed results are discussed in chapter 4. The data from the development cohort defined the distribution of apoB in the iodixanol gradients residing at a density <1.07 g/ml. Therefore for the validation cohort, the gradient was harvested into 2 fractions for HCV RNA quantitiation: a top 3.5mL low density fraction (LDF) and a bottom 6.5mL high density fraction (HDF) with a cutoff at d1.07 g/ml. The density between the fractions was determined using a refractometer (Atago) [the aliquot taken for density measurements was returned to the HDF]. The iodixanol density gradients were performed by Dr Simon Bridge.

2.3.7 SDS-PAGE and apoB western blotting.

Iodixanol fraction samples were prepared by boiling in Laemmli buffer (4% sodium dodecyl sulphate [SDS], 20% glyercol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125M Tris HCl) for 10 minutes. Proteins were separated by 3-18% SDS-polyacrylamide gel electrophoresis (PAGE) on a Bio-Rad Protean II system and transferred to a Hybond polyvinylidene difluoride membrane (Amersham Biosciences, UK) and detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, UK) and polyclonal anti-apoB antibody (DAKO, UK). The apoB western blots were performed by Dr Daniel Felmlee.

2.3.8 HCV RNA extraction.

HCV RNA was extracted from whole and fractionated plasma by QIAamp MinElute Virus Spin Kit according to the manufacturer's protocol (QIAGEN, UK). HCV RNA was extracted from 200 μL of EDTA plasma or iodixanol LDF/HDF and was eluted into 100 μL of buffer AVE.

2.3.9 HCV RNA quantitation by real-time RT-PCR

Extracted HCV RNA was quantitated by two-step real-time RT-PCR for HCV RNA as described previously (Nielsen, Bassendine et al. 2004) using primers NCR-3 (5'-ACCACAAGGCCTTTCGCGACCCAAC-3') and NCR-5 (5'-CCCCCCCTCCCGGGAGAGCCAT-3') plus a fluorescent probe SN1 (5'-FAM-ATTCCGGTGTACTCACCGGTTCCGCAGA-TAMRA-3'). Primers NCR-3 and -5 anneal between nucleotides 120 and 290 of the highly conserved internal ribosome entry site of the HCV genome. Reverse transcription was performed using the NCR-3 primer and AMV reverse transcriptase (Promega). The HCV positive-strand assay was calibrated against the WHO 3rd international standard for HCV RNA (National Institute of Biological Standards and Controls). Real-time PCR was conducted using an ABI Prism 7000 with Taqman Universal PCR Master Mix (Applied Biosystems). Determinations of duplicate tests were averaged. The qRT PCRs were performed by Dr Simon Bridge.

2.3.10 IP10 ELISA

Serum IP10 levels were measured using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions. High levels of IP10 are found in saliva, so particular care was made to avoid saliva contamination. Briefly the protocol was as follows: The IP10 standard was reconstituted in 1ml of distilled water to make a stock solution of 5000 pg/ml. 100μl of stock IP10 standard were pipetted into 900μl of calibrator dilutent RD6Q to make a 500 pg/ml concentration which served as the high standard. 500μl of calibrator dilutent RD6Q was pipetted into each of another 6 eppendorfs to prepare the 250, 125, 62.5, 31.2, 15.6 and 7.8 pg/ml standards. The dilution series was made by pipetting 500μl of the high standard into the next tube, ensuring that each tube was mixed thoroughly before the next transfer. The microplate strips were pre-coated with an IP10 specific monoclonal antibody.

75µl of assay dilutent RD1-56 were added to each well using a multichannel pipette. Then 75µl of standard and serum samples were added to each well according to the pre-determined plate plan in duplicates. The plate was sealed with the adhesive strip provided and incubated at room temperature for 2 hours. Each well was aspirated and washed for a total of 4 washes using 400µl of wash buffer from a squirt bottle. After the last wash the plate was inverted and blotted against clean paper toweling. 200µl of IP10 conjugate were added to each well and incubated for a further 2 hours. The plate was washed a further four times. 200µl of substrate solution was added to each well and incubated for 30 minutes at room temperature. The plate was covered and protected from light. 50µl of stop solution was added to each well causing a colour change from blue to yellow. The optical density (OD) of each well was measured immediately using a BIO RAD 680 microplate reader set to 450nm with wavelength correction set to 570nm. Standard curves were plotted on a Microsoft® excel spreadsheet and OD readings corrected for background. The average OD for each sample was calculated from the duplicates and adjusted for background. A co-efficient of variation (CV) value was also calculated for each duplicate pair of readings from the standard deviation / mean. IP10 levels were calculated from the average OD value using the regression equation of the standard curve, adjusted for background. Samples that had OD values outside the linear range of the standard curve were repeated after 1:1 dilution in calibrator dilutent RD6Q. The dilution factor was multiplied into calculation of the final concentration. The average intra-assay co-efficient of variation (CV) was 3.7% and inter-assay CV was 6.1%.

2.3.11 PCSK9 ELISA

Fasting PCSK9 levels were measured using a sandwich ELISA in both HCV patients and controls. The PCSK9 ELISA was developed and validated in a group of 254 healthy individuals using methods described previously (Dubuc, Tremblay et al. 2009). This data was used as the control group for this study. Briefly Nunc Maxisorp (NUNC Denmark) ELISA plates were coated with 0.55µg/well of immuno-purified anti-PCSK9 antibody (110µl of 5.0µg/ml stock antibody) in 1X carbonate coating buffer (prepared from 10X stock containing 9.539g sodium carbonate and 17.64g of sodium bicarbonate in 600µl of distilled H_20, pH 9.5). The coated plates were sealed and incubated at 37° for 3 hours then overnight at 4°C. The plate was washed six

times with washing buffer (PBST: 10mM sodium phosphate, 0.15M NaCl (PBS), pH 7.4 containing 0.5ml/L of Tween 20) and then incubated at room temperature for 1 hour with 200 µl of blocking buffer (PBS, 0.1% casein, 0.01% merthiolate). A standard curve was prepared using serial dilutions of recombinant PCSK9 in dilution buffer (PBS, 1.8M urea, 0.5 ml/L Tween 20 and 0.001% merthiolate). Plasma samples and controls (CTL-L 116.3 ng/ml and CRTL-H 154.3 ng/ml) were diluted 1 in 20 in dilution buffer without BSA. The samples, controls and standard curve were incubated for 30 mins in a water bath at 46°C prior to application in duplicates to the ELISA plates according to the pre-determined plate plan. The plates were incubated overnight at 37°C. The plates were washed six times followed by addition of 100 µl per well of hPCSK9 HRP conjugated antibody, diluted 1 in 750 in blocking buffer (PBS, 0.1% casein, 0.01% merthiolate) and incubated for 3 hours at 37°C. After four further washes in PBST, 100µl per well of substrate (TMB) were added for 15 minutes. The reaction was stopped by addition of 100µl of 0.25 M HCl and chemi-luminescence read at OD450 nm on a BIO RAD 680 microplate reader.

Standard curves were plotted on an excel spreadsheet and OD readings corrected for background. The average OD for each sample was calculated from the duplicates and adjusted for background. A co-efficient of variation (CV) value was also calculated for each duplicate set of readings from the standard deviation / mean. PCSK9 levels were calculated from average OD value using the regression equation of the standard curve adjusted for background.

The intra-assay coefficient of variation for the PCSK9 ELISA was 3.6%.The inter-assay CV was 9.3%.

2.3.12 Isolation of VLDL1, VLDL2, IDL & LDL from plasma by swing-out ultracentrifugation

VLDL1 (Sf 60-400), VLDL2 (Sf 20-60), IDL (Sf 12-20) and LDL (Sf 0-12) were isolated from plasma by cumulative density gradient ultracentrifugation. Stock solutions at density 1.006 g/ml and 1.182 g/ml were prepared as follows:

Solution A, d=1.006 g/ml solution - 22.8g NaCl and 0.2g Na2 EDTA were dissolved in 1 litre of water, 2ml 1N NaOH were added and made up to 2L with H_20. The final NaCl concentration was 0.195M. Solution B, d=1.182 g/ml solution - 249.8g NaBr were dissolved in 1litre of d 1.006 g/ml solution, NaCl concentration 0.195M, NaBr concentration 2.44M

These two density solutions (A and B) were mixed to give the following density gradient solutions.

Solution number	**Density (g/ml)**	**Solution B ml (d 1.006 g/ml)**	**Solution A ml (d 1.182 g/ml)**	**wt (g) of 100mls**
(1)	1.0988	50	55.78	109.88
(2)	1.0860	50	41.66	108.60
(3)	1.0790	75	53.16	107.90
(4)	1.0722	75	46.50	107.22
(5)	1.0641	75	36.93	106.41
(6)	1.0588	100	42.92	105.88

The density of the solutions was checked by measuring the weight in g of 100mls.

Fasting EDTA plasma samples that had been stored at -80 °C were used and initially adjusted to d 1.118 g/ml by adding 0.342g NaCl to 1 ml plasma and 1 ml of d1.006 g/ml solution. The sample was mixed gently but well ensuring that the NaCl had dissolved.

The discontinuous gradient prepared in Beckman Ultra clear tubes 14x95mm and was layered as follows:0.5 ml 1.182 at the bottom, over layered with the 2 ml adjusted plasma sample (d 1.118 g/ml), 1 ml d1.0988, 1ml d 1.0860, 2mls d1.0790, 2 mls d 1.0722, 2mls d 1.0641 and 2 mls d 1.0588.

The tubes were gently dropped into the buckets and caps screwed using ‘spinkote’ to ensure a good seal. The buckets were placed on an SW40 Swing out rotor (6 bucket).

Centrifugation was carried out at 23°C for the times given in the table below.

Fraction (Sf)	Speed (rpm)	Time h:m	ώ2t	sample vol removed
VLDL1 (60-400)	39K	1:38	$9.81x10^{10}$	1.0ml
VLDL2 (20-60)	18.5K	15:41	$2.12x10^{11}$	0.5ml
IDL (12-20)	39K	2:35	$4.65\ x10^{11}$	0.5ml
LDL (0-12)	30K	21:10	$7.52x10^{11}$	1.0ml

Rotors were decelerated without the brake. Fractions were removed carefully from the top of the tube, and temporarily stored at -20 degrees C for subsequent apoB quantitation.

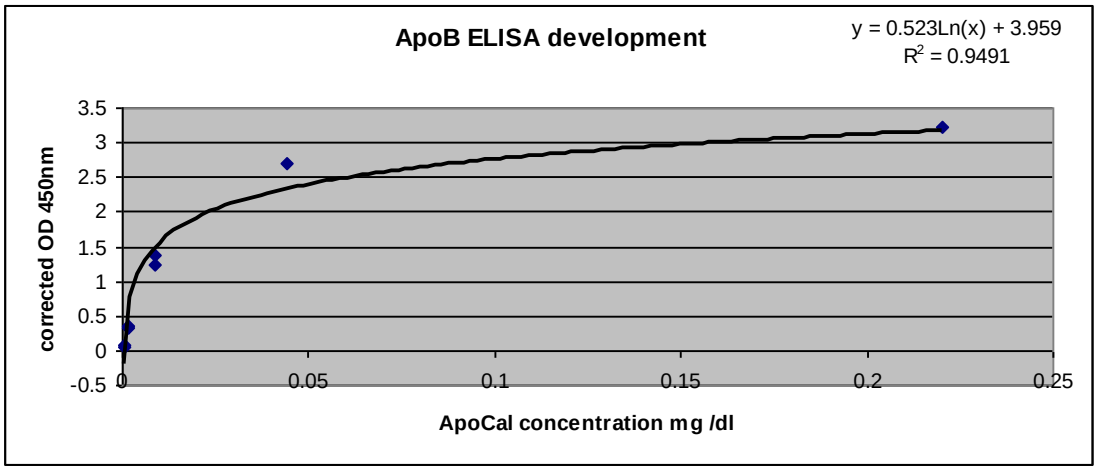
2.3.13 ApoB ELISA

ApoB was quantitiated from the VLDL1, VLDL2, IDL and LDL fractions by a sandwich ELISA. The ELISA protocol was developed and optimised initially by preparing a chequerboard to determine optimal dilutions of coating and capture antibodies. Nunc Maxisorp 96 well plates were used. The capture antibody was polyclonal Rabbit anti-human ApoB antibody (Dako, Denmark, Q0497), diluted in 1X carbonate coating buffer (prepared from 10X stock containing 9.539g sodium carbonate and 17.64g of sodium bicarbonate in 600µl of distilled H_20, pH 9.5), in the following dilutions: 1 in 100, 1 in 500, 1 in 1000, 1 in 2000, 1 in 4000, 1 in 6000, 1 in 8000 and 1 in 10,000. 50µl per well was used and the plate was covered and left for 1 hour at 4° C. Blocking of non-specific binding was achieved by adding 200µl of blocking buffer (3% bovine serum albumin in PBS Tween20), covered and left for a further 1 hour at room temperature. After washing twice in PBST, 50µl per well of HRP conjugated polyclonal Goat anti-human apoB100 detector antibody (Academy Bio-medical Co. Inc, Houston, Texas) was added in the following dilutions: 1 in 500, 1 in 1000, 1 in 2000, 1 in 4000, 1 in 6000, 1 in 8000 and 1 in 10,000.

In the preliminary apoB ELISA development stage, an apolipoprotein calibrator (APO CAL, Catalogue LP 3023 RANDOX) was used in a 1 in 500 dilution in blocking buffer BSA, 50µl per well. In the second step, three standard curves were prepared using 1 in 1000, 1 in 2000 and 1 in 4000 coating antibody concentrations with 1 in

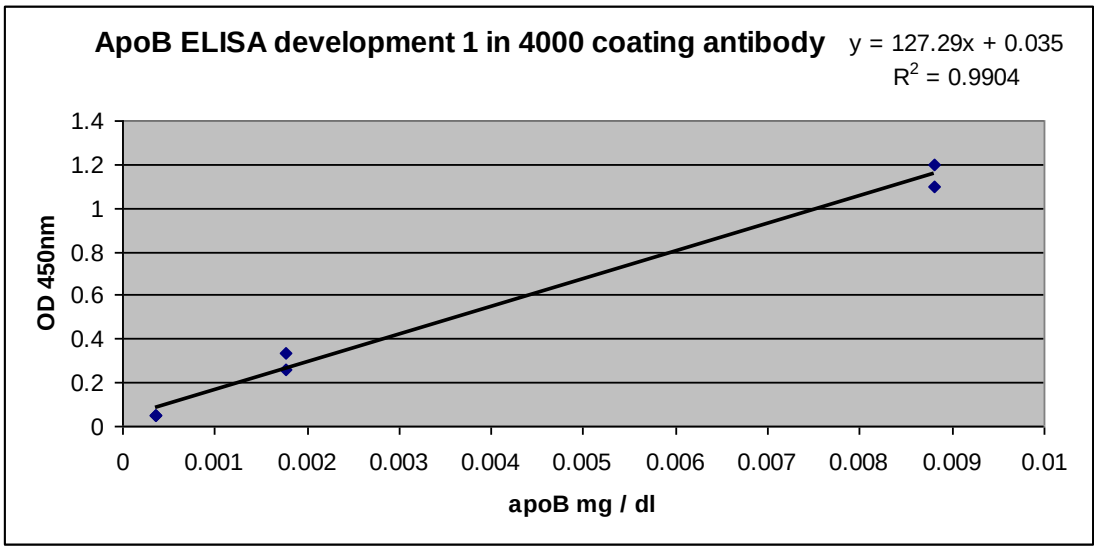
2000 detector antibody concentrations and a 5 fold dilution series of APO CAL to determine the point of plateau indicating saturation of the standard curve. (Figure 3)

Figure 3 ApoB ELISA development – saturation of standard curve



A coating antibody dilution of 1 in 4000 was found to be optimal with 1 in 2000 dilution of detector antibody. These coating and detector antibody dilutions were used in all subsequent experiments. The plateau of the standard curve was reached and the linear range was found at APO CAL concentrations <0.0088mg/dl (1 in 3125 dilution) figure 4.

Figure 4 ApoB ELISA development linear range of standard curve



For subsequent apoB quantitations, a standard curve was prepared from a dilution series of APO CAL starting from a 1 in 5000 dilution as the high end standard, as shown in table 2-1.

Table 2-1 Dilution series for ApoB ELISA standard curve

APO CAL Standard dilution	Apo B concentration mg/dl	Volume (of previous dilution) + volume BB/BSA (µl)
neat	220	
1 in 10	22	10 + 90
1 in 100	2.2	10 +90
1 in 1000	0.22	50 + 450
Dilutions used for standard curve		
1 in 5,(000)	0.044	100 + 400
1 in 20,(000)	0.011	100 (of 1:5) +300
1 in 25,(000)	0.088	100 (of 1:5) + 400
1 in 30,(000)	0.00733	100 (of 1:5) + 500
1 in 50,(000)	0.0044	200 (of 1:25) + 200
1 in 100,(000)	0.0022	100 (of1:50) + 100
1 in 125,(000)	0.00176	100 (of 1:25) + 400
1 in 250,(000)	0.00088	200 (of 1:125) + 200
1 in 625,(000)	0.000352	100 (of 1:125) + 400

For each plate two additional control sera were used (Wako control serum 1 (normal, WAKO code no. 410-00102) containing 76.6 mg/dl of apoB (range 61.3 – 91.9) and control serum II (abnormal, WAKO code no. 416-00202), containing 119 mg /dl apoB (range 95.2 – 143). Further experiments indicated the optimal dilution of sera and lipoprotein fractions which is shown in the table 2-2 before being loaded onto the plate.

Table 2-2 Dilutions of lipoprotein fractions and sera for apoB ELISA quantitation

Fraction	Dilution required
Whole serum / Control sera 1 and II	1 in 8000
VLDL 1	1 in 200
VLDL 2	1 in 750
IDL	1 in 8000
LDL	1 in 8000

50µl per well of appropriately diluted standards, samples and controls were loaded on to the coated ELISA plate according to pre determined plate plans in triplicates. The plate was incubated at 37°C for 2 hours then washed four times in PBST (PBST: 10mM sodium phosphate, 0.15M NaCl (PBS), pH 7.4 containing 0.5ml/L of Tween 20) . Detection was performed by measurement of chemi-luminescence following addition of TMB solution 100µl per well. The reaction was allowed for 15 minutes then terminated by addition of 100µl of 250 mM HCl. Optical density (OD) at 450nm was detected on a BIO-RAD 680 microplate reader. Standard curves were plotted on an excel spreadsheet and OD readings corrected for background. The average OD for each sample was calculated from the triplicates and adjusted for background. A co-efficient of variation (CV) value was also calculated for each triplicate set of readings from the standard deviation / mean. ApoB were calculated from average OD value using the regression equation of the standard curve adjusted for background. The intra-assay co-efficient of variation (CV) for the apoB ELISA on VLDL1, VLDL2, IDL and LDL fractions was 4.9%.

3 Chapter 3. The influence of chronic HCV infection on lipid profiles

3.1 Demographics

The aim of this study was to determine the influence of HCV on lipid profiles. Two HCV populations were characterised; a retrospective cohort with non-fasting lipid profiles (table 3-1), and a prospective cohort with fasting lipid profiles (table 3-2). The analysis of anti-viral treatment outcomes from the retrospective cohort compared Sustained Virological Responders (SVR) (i.e. those that were HCV RNA not detected at least six months after completion of therapy) to non-responders (NR). The non responders group included those that had either a complete or partial early virological response but subsequently relapsed and those that did not achieve a 2 log reduction in HCV RNA at 12 weeks (null responders).

Table 3-1 Baseline characteristics: Retrospective HCV cohort

HCV genotype	**1**	**2**	**3**	**4**	**5**	**6**	**Unidentified**
N=	109	12	113	5	4	1	7
Age (yrs)	45.9 ± 11.5	47.3 ± 10.0	43.3 ± 10.0	51 ±15.4	46.5 ± 21.5	62	44.7
Male (%) / Female (%)	72 (56%) 37 (44%)	6 (50%) 6 (50%)	72 (64%) 40 (36%)	4 (80%) 1 (20%)	2 (50%) 2 (50%)	0 1	5 (71%) 2 (29%)
SVR (%)	56 (51%)	10 (83%)	87 (77%)	2 (40%)	3 (75%)	1	6
NR (%)	53 (49%)	2 (17%)	26 (23%)	3 (60%)	1 (25%)	0	1

Table 3-2 Baseline characteristics of prospective (fasting) HCV and non-HCV comparator groups

	Comparator Group A	Comparator Group B	HCV G1	HCV G3
N=	45	254	61	28
Age (years)	44 ± 9.3	41.8 ± 12.5	47.6 ± 10.4	45.3 ± 10.5
BMI (kg/m^2)	29.0 ± 3.9	24.5 ± 4.4	25.3 ± 4.1	25.8 ± 3.2
Waist Circum. cm	94.7±10.2	Not available	89.4 ± 11.7	91.4 ± 9.3
Male (%) / Female (%)	13 (29%) / 32 (71%)	117 (46%) / 137 (54%)	43 (70%) / 18 (30%)	22 (81%) / 5 (29%)

(mean ± standard deviation)

3.2 Comparator group demographics and lipid profiles

Two separate non-HCV groups were used in these studies as comparators of lipid profiles to chronic HCV with kind permission of the study investigators. Recruitment of these comparator groups is described in 2.2.3. The baseline characteristics of the two comparator study cohorts (group A and group B) are shown in table 3-2. The first comparator group (Group A) data was provided by collaborators at the University of Surrey. In addition to fasting lipid profiles, group A comparators had measurement of non-cholesterol sterols (Sterol controls) performed by Clinical Biochemistry, Royal Victoria Infirmary, Newcastle upon Tyne. These otherwise healthy individuals were taking part in a dietary intervention study, hence had a higher mean BMI than the HCV patients, but were well matched for age. All the lipid profiles were taken at baseline prior to any intervention. However since comparator group A was a relatively small number (N=45, males =13) and these individuals were relatively overweight and hyperlipidaemic compared to the HCV patients, a second non-HCV comparator group was also evaluated.

The second non-HCV comparator group (Group B) consisted of a large group of 254 healthy volunteers with normal BMI, which was provided by University of Montreal. In this group fasting lipid profiles had been performed along with measurement of PCSK9. There were no significant differences between the HCV patients and group B

comparators in mean age and body mass index, but there were more males in both the retrospective (non-fasting) and prospective (fasting) HCV cohorts than comparator groups A and B. However in comparator group B it is important to note that there was no significant difference between total cholesterol level in males and females (p=0.549), but males had significantly higher non-HDL and LDL cholesterol levels than females (p=0.002), and females had significantly higher HDL cholesterol and TG/HDL cholesterol ratios than males (p=0.0001). Triglycerides were not significantly different between male and female group B comparators (p=0.1902). Therefore sex matched sub-group analyses were performed when comparing lipid parameters from group B comparators to the HCV groups which were predominantly male.

3.3 Comparison of HCV lipid profiles vs. non-HCV healthy subjects

Lipid profile summary statistics for the retrospective (non-fasting) HCV cohort and prospective (fasting) HCV cohort and both healthy comparator groups A and B are shown in tables 3-3 to 3-6 respectively. Total, LDL and non-HDL cholesterol conformed to a normal distribution and are summarised as mean ± standard deviation. For these normal variables, parametric 2-sample t tests were used to determine differences between groups. Triglycerides, HDL cholesterol and TG/HDL ratio were not normally distributed; therefore summary statistics of median and interquartile range are quoted. Non-parametric Mann-Whitney or Kruskall Wallace tests were used to determine differences in these variables between groups.

Patients in the retrospective HCV cohort had non-fasting lipid profiles performed as part of their routine clinic assessment. Therefore the numbers vary according to the available data which is shown in table 3-3. All HCV patients were viraemic at the time of lipid profiling and not concurrently receiving anti-viral therapy. Only lipid profiles from patients with HCV genotypes 1 and 3 were assessed owing to the small numbers of the other HCV genotypes.

Table 3-3 Non-fasting lipid profiles in HCV G1 & G3: Retrospective cohort

	N	HCV G1	N	HCV G3	P value
Total cholesterol#	93	4.70 ± 0.93	97	4.134 ± 0.903	<0.001
Non HDL cholesterol#	72	3.43 ± 0.93	78	2.71 ± 0.79	<0.001
Triglycerides*	56	1.67 ± 1.02 1.25 (1.10-1.95)	63	1.53 ± 1.01 1.20 (0.80-1.98)	0.204
HDL*	61	1.32 ± 0.41 1.30 (1.03-1.50)	68	1.30 ± 0.43 1.20 (1.00-1.60)	0.033
TG/HDL ratio*	47	1.40 ± 1.17 1.00 (0.55-1.89)	57	1.39 ± 1.09 1.00 (0.74-1.55)	0.964

Note variation in sample size depending on available data.

Table 3-4 Fasting lipid profiles in HCV G1 & G3: Prospective cohort

	HCV G1 N=61	HCV G3 N=28	P value
Total cholesterol#	4.56 ± 0.95	3.80 ± 0.92	0.001
Non HDL cholesterol#	3.30 ± 0.92	2.53 ± 0.80	<0.001
LDL cholesterol#	2.70 ± 0.83	2.11 ± 0.64	0.002
Triglycerides*	1.31 ± 0.72 1.20 (0.85-1.50)	1.04 ± 0.80 0.70 (0.60-1.13)	0.004
HDL cholesterol*	1.27 ± 0.35 1.20 (1.1-1.5)	1.26 ± 0.45 1.20 (0.9-1.65)	0.722
TG/HDL ratio*	1.17 ± 0.87 0.91 (0.60-1.33)	1.02 ± 1.06 0.54 (0.44-1.29)	0.052

*Units are mmol / l; # normally distributed data summarised by mean ± standard deviation, parametric 2-sample t-test; * non-normally distributed data summarised by median (Q1-Q3), non-parametric Kruskall-Wallace test*

Table 3-5 Comparator group A fasting lipid profiles

	All N=45	Female N= 32	Male N= 13	P value
Total cholesterol#	5.34 ± 1.06	5.29 ± 1.13	5.46 ± 0.89	0.634
Non HDL cholesterol#	4.20 ± 0.91	4.08 ± 0.93	4.50 ± 0.82	0.162
LDL cholesterol#	3.07 ± 0.71	3.03 ± 0.76	3.16 ± 0.58	0.590
Triglycerides*	1.19 (0.93-1.58)	1.14 (0.91-1.45)	1.29 (0.94-2.04)	0.341
HDL cholesterol*	1.10 (0.85-1.29)	1.16 (0.98-1.35)	0.93 (0.81-0.93)	0.010
TG/HDL ratio*	1.08 (0.76-1.52)	0.99 (0.74-1.33)	1.35 (1.05-2.33)	0.040

Table 3-6Comparator group B fasting lipid profiles

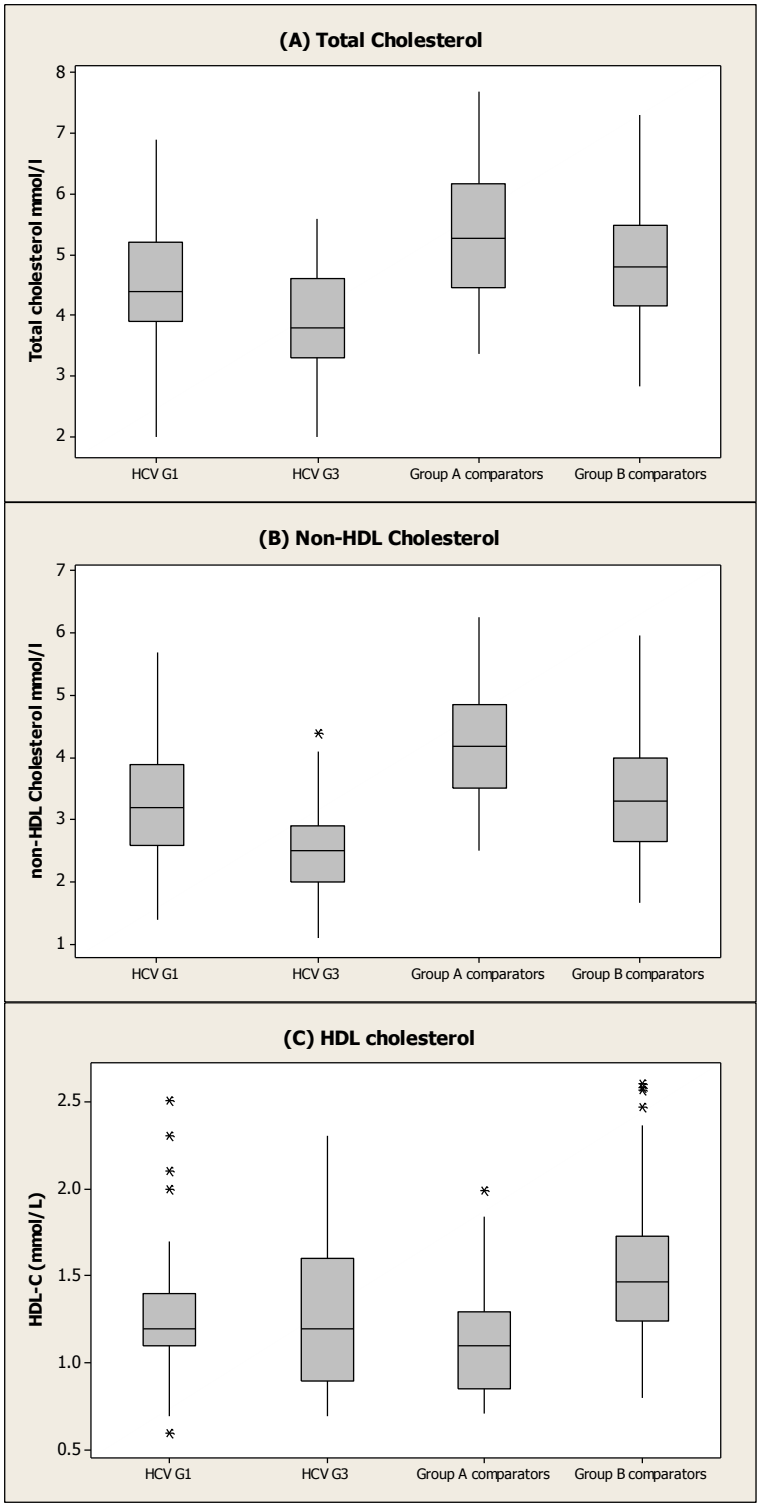
	All N=254	Female N= 137	Male N= 117	P value
Total cholesterol#	4.86 ± 0.89	4.83 ± 0.91	4.89 ± 0.63	0.549
Non HDL cholesterol#	3.36 ± 0.92	3.20 ± 0.92	3.56 ± 0.89	0.002
LDL cholesterol#	2.83 ± 0.80	2.69 ± 0.78	3.00 ± 0.80	0.002
Triglycerides*	1.03 (0.75-1.38)	0.94 (0.71-1.40)	1.08 (0.82-1.38)	0.1902
HDL cholesterol*	1.47 (1.24-1.74)	1.59 (1.37-1.88)	1.27 (1.07-1.54)	<0.0001
TG/HDL ratio*	0.69 (0.47-1.02)	0.61 (0.41-0.91)	0.84 (0.57-1.21)	0.0001

*Units are mmol / l; # normally distributed data summarised by mean ± standard deviation, parametric 2-sample t-test; * non-normally distributed data summarised by median (Q1-Q3), non-parametric Kruskall-Wallace test*

3.3.1 Cholesterol - total, HDL and non-HDL-C

In the prospective HCV cohort, total cholesterol was significantly lower in HCV patients than non-HCV control groups A and B in both HCV genotype 1 ($p=0.007$) and genotype 3 ($p<0.001$) Figure 5A. This remained significant when only male group B comparatorswere compared to males with HCV G1 ($p=0.004$) and HCV G3 ($p<0.001$). The difference in total cholesterol between HCV G1 and healthy male group B comparators was accounted for primarily by lower non-HDL cholesterol (figure 5B, $p=0.010$) rather then HDL cholesterol (figure 5C, $p=0.2022$). In contrast, the significantly lower total cholesterol in HCV G3 compared to male group B comparators was contributed by reductions in both HDL cholesterol ($p=0.0125$) and non-HDL cholesterol ($p<0.001$). The data from the retrospective (non-fasting) HCV cohort indicate that total cholesterol and non-HDL cholesterol were significantly lower in HCV G3 than HCV G1 ($p<0.001$). This was confirmed in the fasting prospective HCV cohort where total and non-HDL-C was significantly lower in HCV G3 than HCV G1 ($p<0.001$) (Table 3-4 and figure 5B) Although the HDL-C was significantly lower in HCV G3 than HCV G1 in the retrospective HCV cohort ($p=0.033$), this was not confirmed in the HCV prospective cohort ($p=0.722$) (figure 5C).

Figure 5 Cholesterol levels in HCV G1 & G3 (fasting prospective) and non-HCV comparators

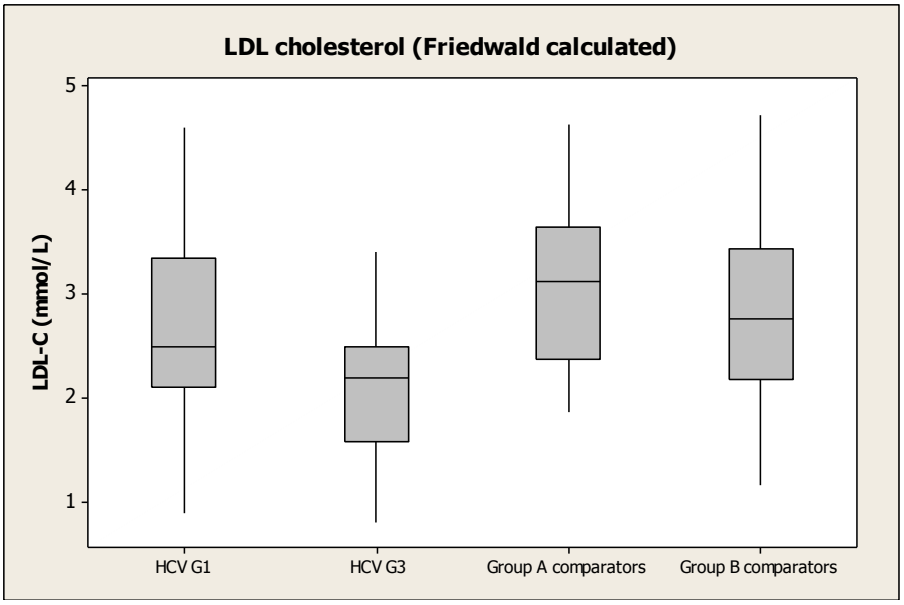


*(A) Total cholesterol (TC) was significantly lower in HCV G1 (p=0.007) and HCV G3 (p<0.001) than non-HCVcomparators. The difference was largely in non-HDL-C (non-HDL-C = TC – HDL-C) (B) which was significantly lower in HCV G3 than HCV G1 (p<0.001). HDL cholesterol (C) was not significantly different between groups. Boxes represent median value and interquartile range (Q1 – Q3). Lower whisker = Q1 - 1.5 (Q3-Q1) and the upper whisker = Q3 + 1.5 (Q3-Q1); * = outlying data points.*

3.3.2 Assessment of LDL cholesterol and non HDL cholesterol

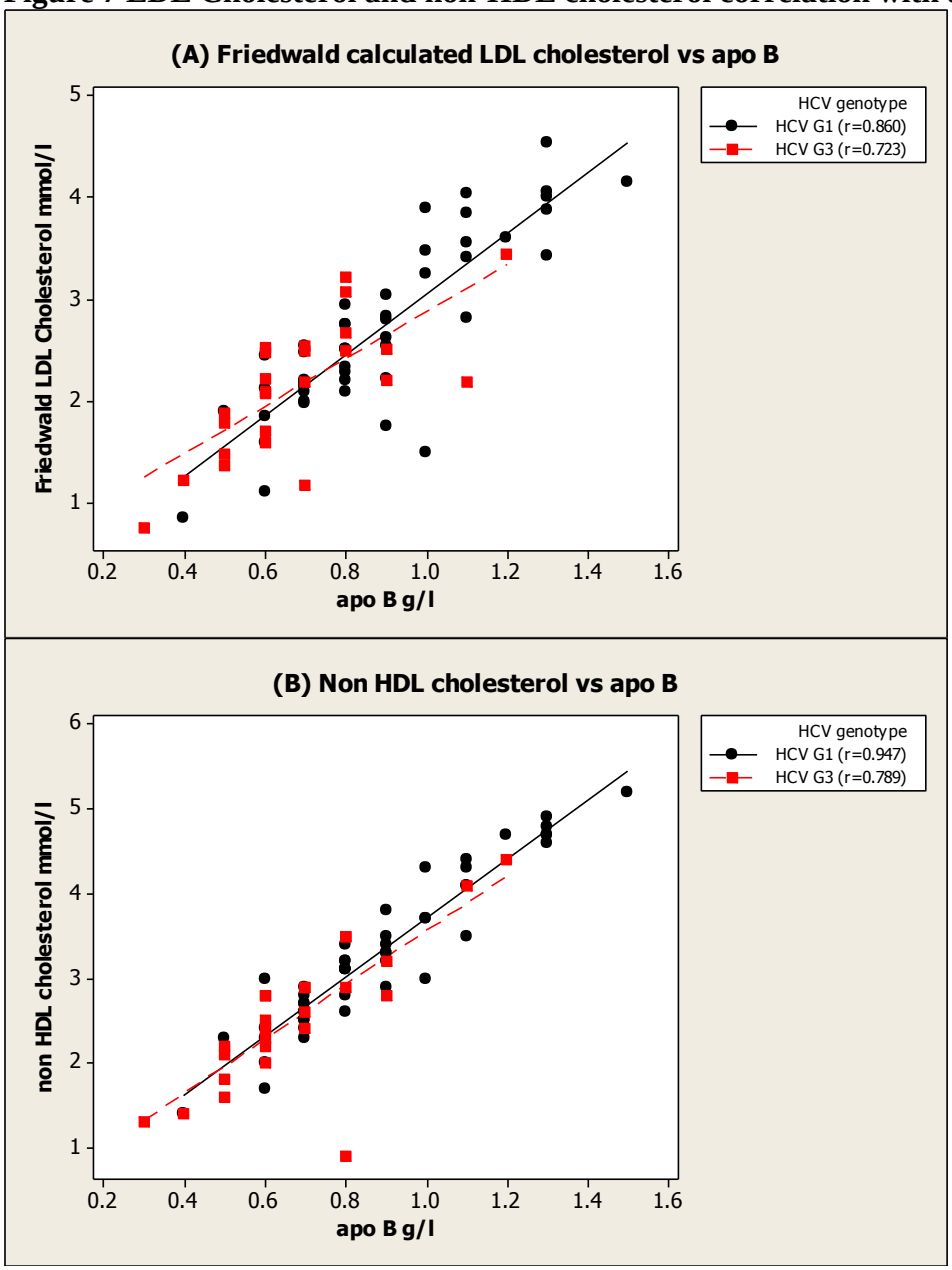
LDL cholesterol is usually estimated by the Friedwald calculation (LDL C = Total cholesterol – HDL cholesterol - (triglycerides/2.2)). Non HDL cholesterol is calculated simply (non-HDL C = total cholesterol – HDL cholesterol). LDL cholesterol levels calculated by the Friedwald equation in HCV patients and controls show the same differences as non HDL C and are shown in figure 6. Given that triglycerides are significantly lower in those with HCV G3 infection than in HCV G1 and controls (figure 8A), the predictive value of LDLC estimated by Friedwald equation vs. non-HDL cholesterol as an indirect measure of apoB was tested in the fasting prospective group serum. The correlations are shown in figure 7A and Figure 7B. In HCV G1 there was a stronger correlation between non HDL C and apoB ($r = 0.947$) than Friedwald calculated LDL cholesterol vs. apoB ($r = 0.860$). This was also apparent in HCV G3 where there was weaker correlation between the Friedwald LDL cholesterol vs. apoB ($r= 0.723$) compared to non-HDL cholesterol vs. apoB, ($r = 0.789$).

Figure 6 LDL cholesterol in HC1 G1 & G3 (fasting prospective) and non-HCV comparators



*LDL cholesterol calculated by the Friedwald equation was significantly lower in HCV G3 than in HCV G1 ($p<0.001$) and healthy comparator groups A and B ($p<0.001$). The median value and interquartile range (Q1 – Q3) shown. Lower whisker = Q1 - 1.5 (Q3-Q1) and the upper whisker = Q3 + 1.5 (Q3-Q1); * = outlying data points.*

Figure 7 LDL Cholesterol and non-HDL cholesterol correlation with apoB



Fasting LDL cholesterol calculated by the Friedwald equation (A) correlates less strongly with serum apoB than non-HDL cholesterol (B) in HCV G1 and HCV G3

3.3.3 Triglycerides

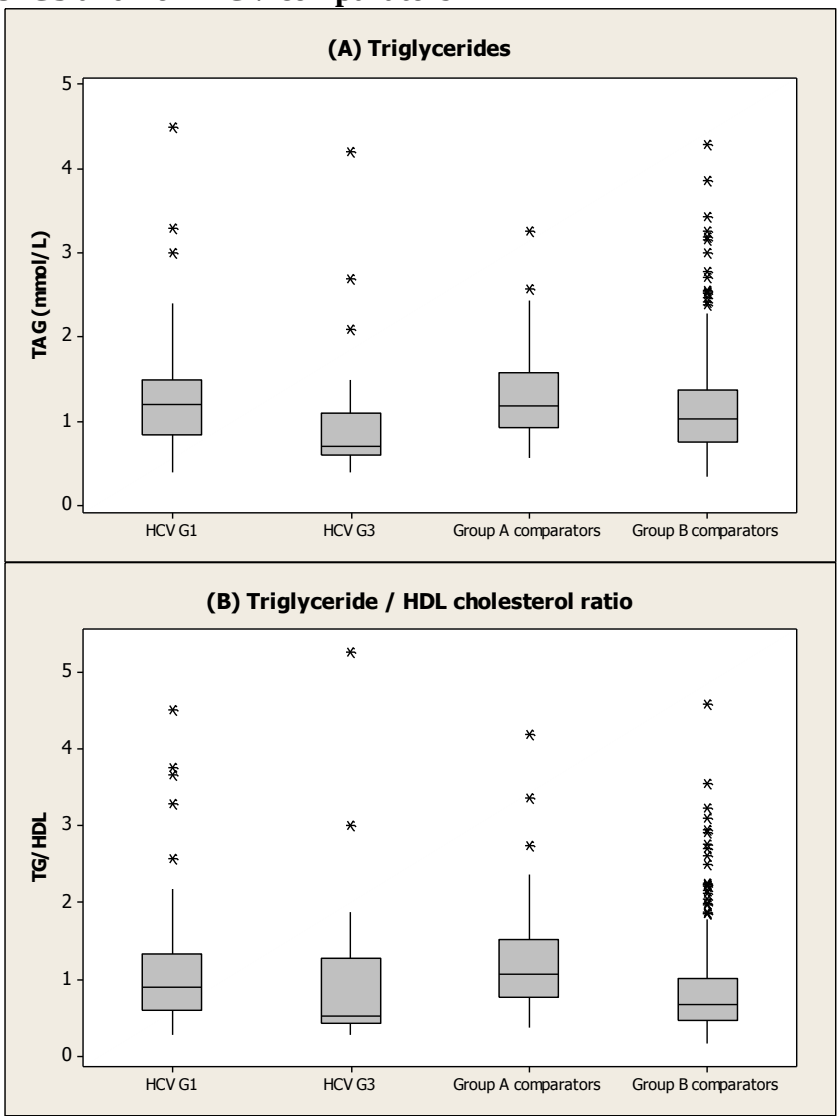
In the combined retrospective (non-fasting) & prospective (fasting) HCV cohorts, triglycerides were significantly higher in HCV G1 patients than comparators ($p=0.0003$), but not between HCV G3 and comparators ($p=0.7626$). However, since triglycerides are increased in the post prandial state, when only fasting data were considered, the difference between HCV G1 and male comparators was no longer significant ($p=0.3140$). In contrast fasting triglycerides in HCV G3 were significantly lower than in both male group A and group B controls ($p=0.008$) (figure 8A)

3.3.4 Triglyceride / HDL ratio

In group B comparators the TG/HDL ratio was significantly higher in males than females ($p=0.0001$) because males had lower HDL levels ($p<0.0001$) despite equivalent triglycerides ($p=0.1902$). In the combined retrospective (non-fasting) & prospective (fasting) HCV cohorts, patients with HCV G1 had significantly higher TG/HDL ratios than overall group B comparators ($p<0.001$). Also in the combined data set there was no significant difference in TG/HDL ratio between HCV G1 and HCV G3 ($p=0.2278$).

When the analysis was restricted to fasting samples in comparison to male group B comparators, the TG/HDL ratio was not significantly higher in HCV G1 than male group B comparators ($p=0.2356$) Although there was a trend to towards higher TG/HDL ratio in HCV G1 than HCV G3, this did not achieve statistical significance ($p=0.0528$). Likewise the fasting TG/HDL ratio in HCV G3 was not significantly different to male comparators ($p=0.1709$) (figure 8B). Therefore TG/HDL ratios are likely to be near normal in HCV.

Figure 8 Fasting triglycerides (TG) and triglyceride / HDL cholesterol ratio in HCV G1 & G3 and non-HCV comparators



*(A) Fasting triglyceride (TG) levels are significantly lower in HCV G3 than HCV G1 (p=0.004) and non-HCV comparators (p=0.008). However the TG/HDL ratios were not significantly different (B) (p=0.052), because HDL-C is the same. The median value and interquartile range (Q1 – Q3) is shown. Lower whisker = Q1 - 1.5 (Q3-Q1) and the upper whisker = Q3 + 1.5 (Q3-Q1); * = outlying data points.*

3.3.5 ApoB and ApoA1

The observation that HCV genotype 3 patients had significantly lower LDL cholesterol, non-HDL cholesterol and triglyceride levels than both HCV genotype 1 and healthy comparators ($p<0.001$) suggests that the levels of apoB containing lipoproteins (VLDL, IDL and LDL) are diminished in HCV G3. This was confirmed by measurement of serum apoB levels which were significantly lower in HCV G3 than HCV G1 ($p<0.001$) (table 3-7). ApoB was also measured in comparator group A(0.92 ± 0.22 g/l) which was significantly higher than in HCV G3 ($p<0.001$). However apoB in HCV G1 in the fasting prospective cohort was not significantly lower than group A comparators ($p=0.349$) (Figure 9).

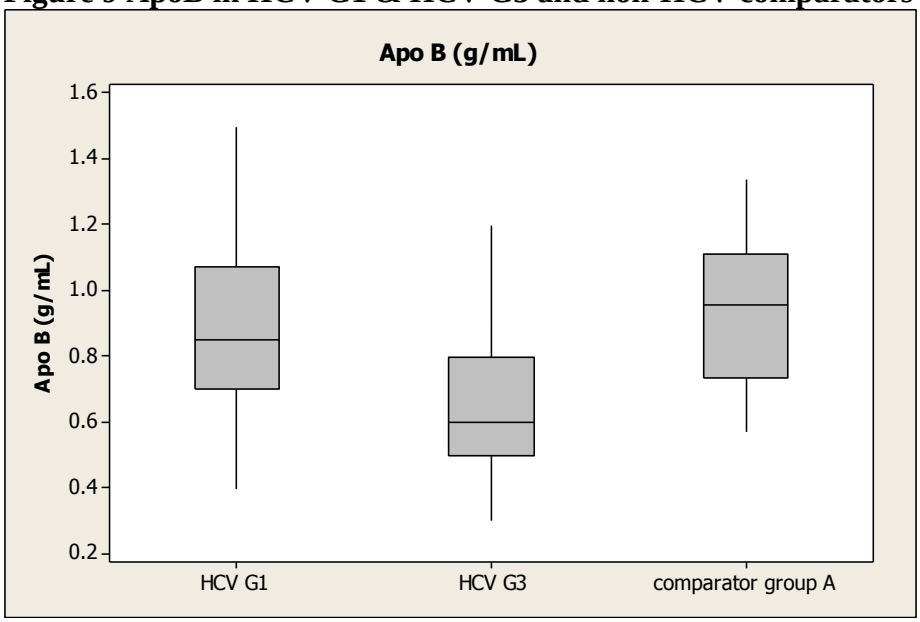
HDL cholesterol was not significantly different between HCV G1 and G3 ($p=0.845$). This was supported further by ApoA1 levels which were not significantly different between HCV genotypes 1 & 3 ($p=0.565$) Table 3-7.

Table 3-7 ApoB and ApoA1 (fasting prospective cohort)

	HCV G1 N=61	**HCV G3 N=27**	**P value**
ApoB mean ± SD	0.88 ± 0.25	0.66 ± 0.21	<0.001#
ApoA1 mean ± SD **Median (Q1-Q3)**	1.45 ± 0.30 1.40 (1.3-1.6)	1.38 ± 0.34 1.30 (1.0-1.6)	0.565*

#2-sample t-test; * Mann Whitney U-test

Figure 9 ApoB in HCV G1 & HCV G3 and non-HCV comparators



ApoB levels are significantly lower in HCV G3 then HCV G1 (p<0.001) and non-HCV comparators (p<0.001). There was no significant difference in apoB between HCV G1 and non-HCV controls (p=0.349).

3.4 Relationship between lipid profiles and HCV total viral load

Total HCV viral load and fasting lipid profiles were assessed at the same visit in all patients in the prospective cohort. In addition, paired viral load and lipid profiles were available in some of the retrospective cohort. The summary statistics of HCV total viral load for the combined cohorts for HCV G1 and HCV G3 is shown in the Table 3-8. The relationship between lipid profiles and viral load in the prospective fasting HCV cohort is covered in further detail in results chapter 4 on page 159.

Table 3-8 Total Viral Load summary statistics, combined cohorts

Total viral load Log10IU/ml	**HCV G1 (N=112)**	**HCV G3 (N=66)**
Mean (± SD)	5.81 ± 0.94	5.53 ± 0.60
Median (Q1-Q3)	5.86 (5.40 – 6.55)	5.66 (5.25 – 5.85)

Since log10 total HCV RNA viral load was not normally distributed, Spearman's Rank correlation analysis was used to test for associations between viral load and lipid parameters. In HCV G3 infection there was a significant inverse correlation between

total cholesterol and viral load ($r = -0.324$, $p = 0.010$). This significant negative correlation was most apparent in the HDL cholesterol fraction (figure 10, $r = -0.520$, $p < 0.001$) rather than non-HDL cholesterol (figure 11, $r = -0.195$, $p = 0.159$). Non-HDL cholesterol showed a significant negative correlation with viral load in fasting patients ($r= -0.541$, $p = 0.011$) and in the fasting group the HDL correlation was less strong ($r = -0.291$, $p = 0.201$) in HCV G3.

In HCV G1 infection there was no significant correlation between total HCV RNA viral load and total cholesterol ($r = 0.031$, $p = 0.755$). Likewise, there was no significant correlation of total viral load with HDL cholesterol ($r = 0.034$, $p = 0.744$) nor with non-HDL cholesterol ($r = 0.041$, $p = 0.693$) in HCV G1 patients.

The triglycerides did not correlate with total HCV RNA viral load in either HCV G1 ($r = -0.005$, $p = 0.964$) or HCV G3 ($r = 0.097$, $p = 0.476$). Total viral load showed no significant variability with host ApoE genotype

Figure 10 HCV G3 negative correlation between viral load and HDL cholesterol (combined cohort)

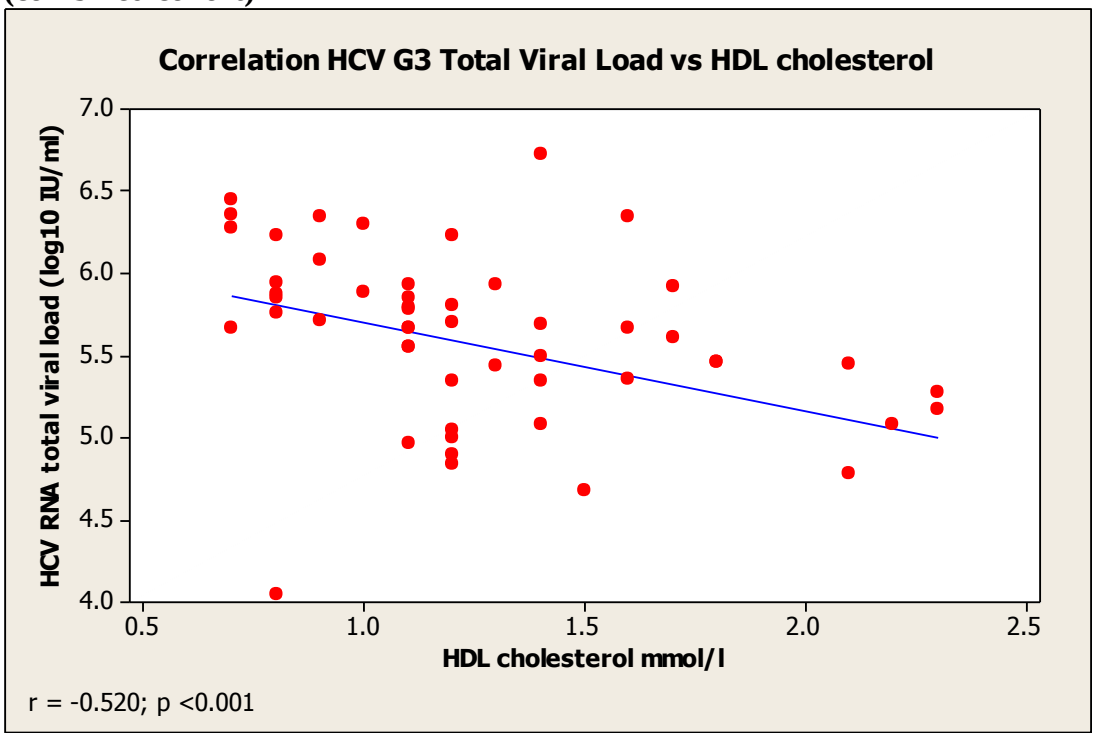
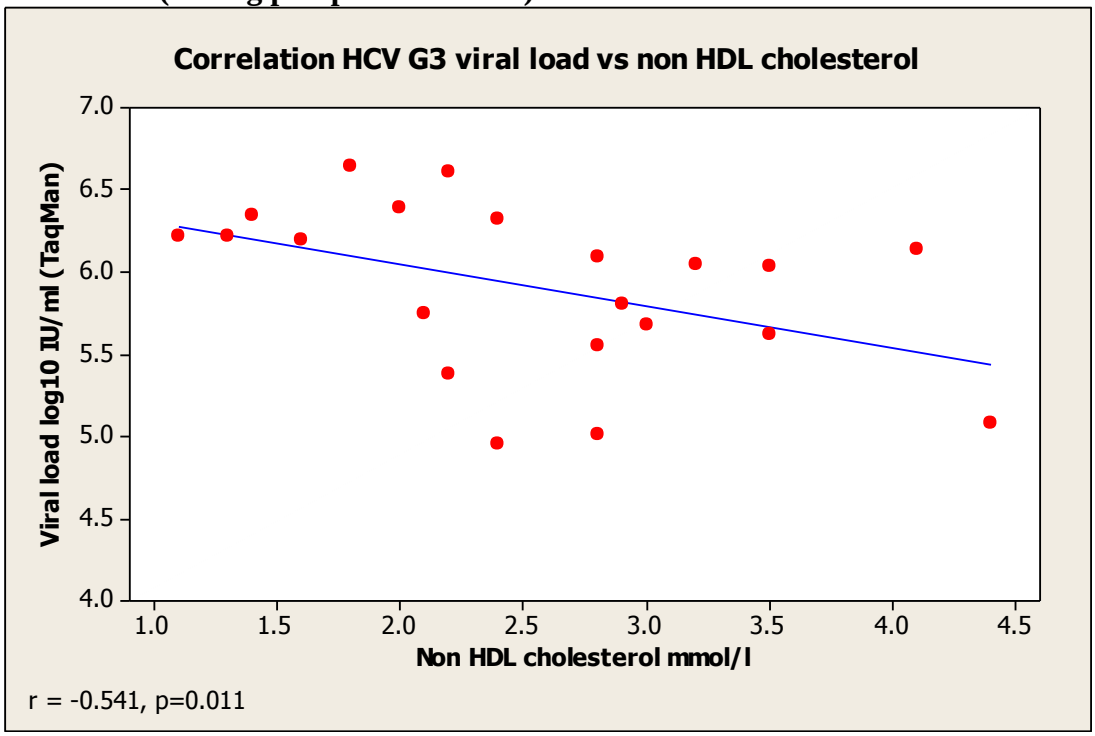


Figure 11 HCV G3 negative correlation between viral load and non-HDL cholesterol (fasting prospective cohort)



HDL cholesterol (figure 10) and non-HDL cholesterol (figure 11) both correlate inversely with viraemia in HCV G3.

3.5 The relative importance of host ApoE genotypes and HCV genotype on lipid profiles

The lipid profiles data presented thus far indicated that in patients with chronic HCV infection, non-HDL cholesterol levels were low. In those with HCV G3 in particular, chronic HCV infection was having a stronger effect on lowering cholesterol contained within the apoB lipoproteins (VLDL, IDL and LDL) than in HDL. It is well recognised that host ApoE genotype is an important determinant of LDL cholesterol, normally accounting for 7% of the variability of LDL cholesterol (Davington, Gregg et al. 1988). The expected pattern of serum cholesterol level is non HCV populations is E3/E4 > E3/E3 > E2/E3. The relative importance of host ApoE genotype on lipid profiles compared to HCV genotype was therefore investigated further.

3.5.1 Frequency of ApoE genotypes

The frequency distribution of ApoE genotypes in those with HCV G1 and G3 is shown in table 3-9. This combines ApoE genotypes from both the retrospective (non-fasting) and prospective (fasting) HCV cohorts. It is apparent that homozygote ApoE2/E2 and E4/E4 were very rare in the chronic HCV group, identified in only 1 each out of 227 patients genotyped The overall allele frequencies in the combined cohorts was ε2 0.068, ε3 0.782, ε4 0.015. This was not significantly different from the reported apoE allele frequencies from a meta-analysis of over 1000 individuals without coronary disease of ε2 0.07, ε3 0.82 and ε4 0.11 (Bennet, Di Angelantonio et al. 2007).

Table 3-9 Combined ApoE genotypes in retrospective and prospective cohorts

	HCV G1	HCV G3	Total (%)
E2/E2	1	0	1 (0.4)
E2/E3	16	9	25 (11)
E2/E4	3	1	4 (1.8)
E3/E3	77	57	134 (59)
E3/E4	38	24	62 (27.4)
E4/E4	1	0	1 (0.4)
Total (prospective / retrospective)	136	91	227

Lipid profiles were analysed in those with the most common ApoE genotypes i.e. E3/E3, E2/E3 and E3/E4. To increase statistical power, the lipid profiles for total cholesterol, non-HDL cholesterol and HDL cholesterol were combined from the retrospective (non-fasting) and prospective (fasting) cohorts.

3.5.2 Total Cholesterol, ApoE genotype and HCV genotype

Figure 12A indicates that the lower total cholesterol in HCV G3 compared to HCV G1 was more apparent in those with apoE3/E3 and E3/E4, than those with apoE2/E3, where cholesterol levels were similarly low. In those with apoE3/E4 total cholesterol was significantly lower than expected in those with HCV G3 ($p<0.001$).

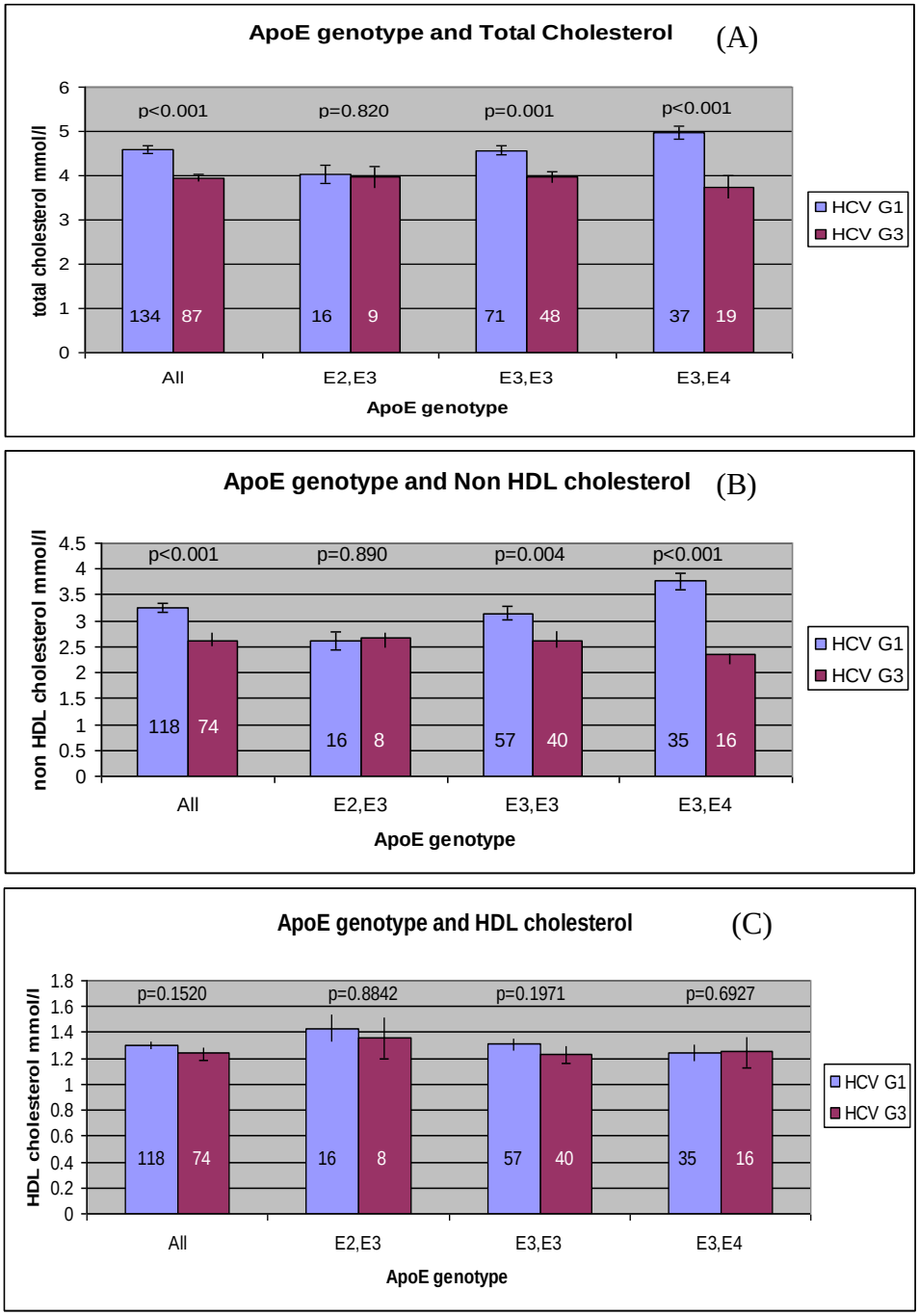
3.5.3 Non-HDL Cholesterol, ApoE genotype and HCV genotype

Figure 12B and figure 12C indicate that the difference in total cholesterol between HCV genotypes 1 & 3 was primarily in the non-HDL (i.e. apoB associated) fraction rather than in HDL. Non-HDL cholesterol in HCV G3 was significantly lower than expected for those with apoE3/E3 and apoE3/E4, but not significantly different in those with ApoE2/E3.

3.5.4 HDL-Cholesterol, ApoE genotype and HCV genotype

As expected, ApoE genotype did not appear to influence HDL cholesterol levels in either HCV G1 or HCV G3 (figure 12C).

Figure 12 ApoE genotype and Cholesterol (combined HCV cohorts)

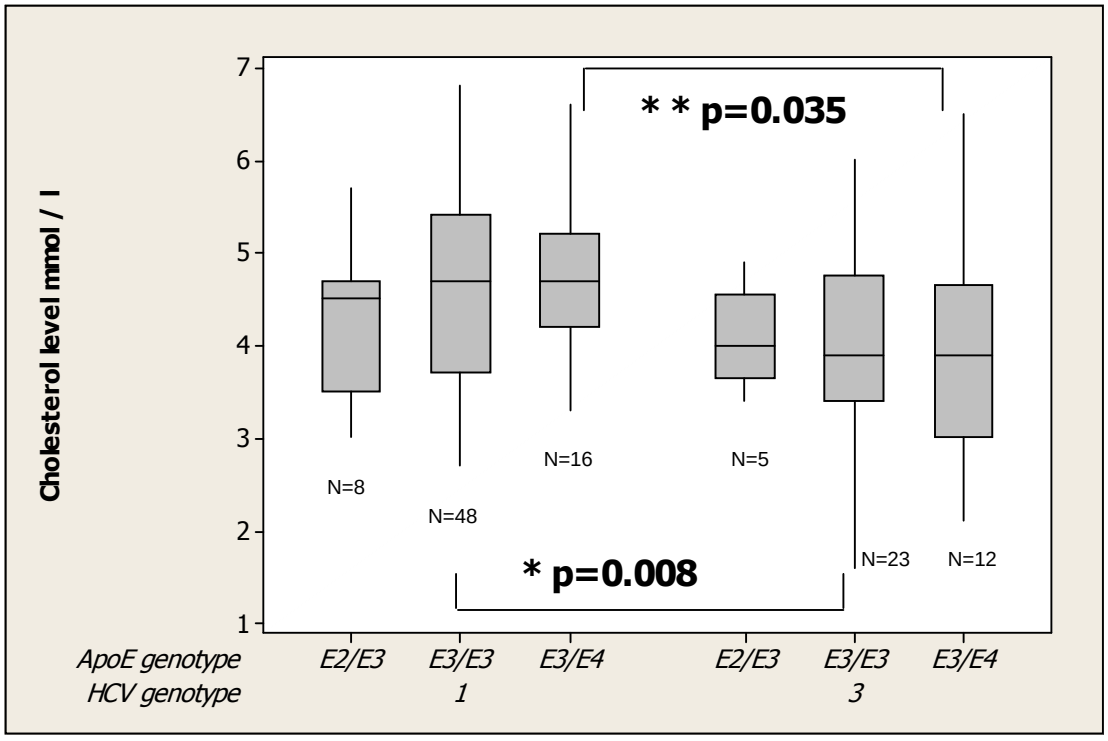


Total cholesterol (A) and non-HDL cholesterol (B) levels are significantly lower in HCV G3 than HCV G1 in those with apoE3/E3 and E3/E4 but not in apoE2/E3. HDL cholesterol is not affected by apoE genotype (C). Columns represent mean ± standard error. Numbers of each group are shown. 2-sample t-test was used to compare groups.

3.5.5 HCV G3 influences cholesterol levels more than apoE genotype

The expected pattern of serum cholesterol level is E3/E4 > E3/E3 > E2/E3. However, cholesterol levels were significantly lower in HCV G3 patients with apoE3/E3 and E3/E4 compared to HCV genotype 1 (* p =0.008; ** p = 0.035).
This suggests that HCV is exerting greater influence on cholesterol levels than apoE genotype in HCV genotype 3 infection (figure 13).

Figure 13 Effect of host apoE genotype on pre-treatment cholesterol in patients with chronic HCV infection.

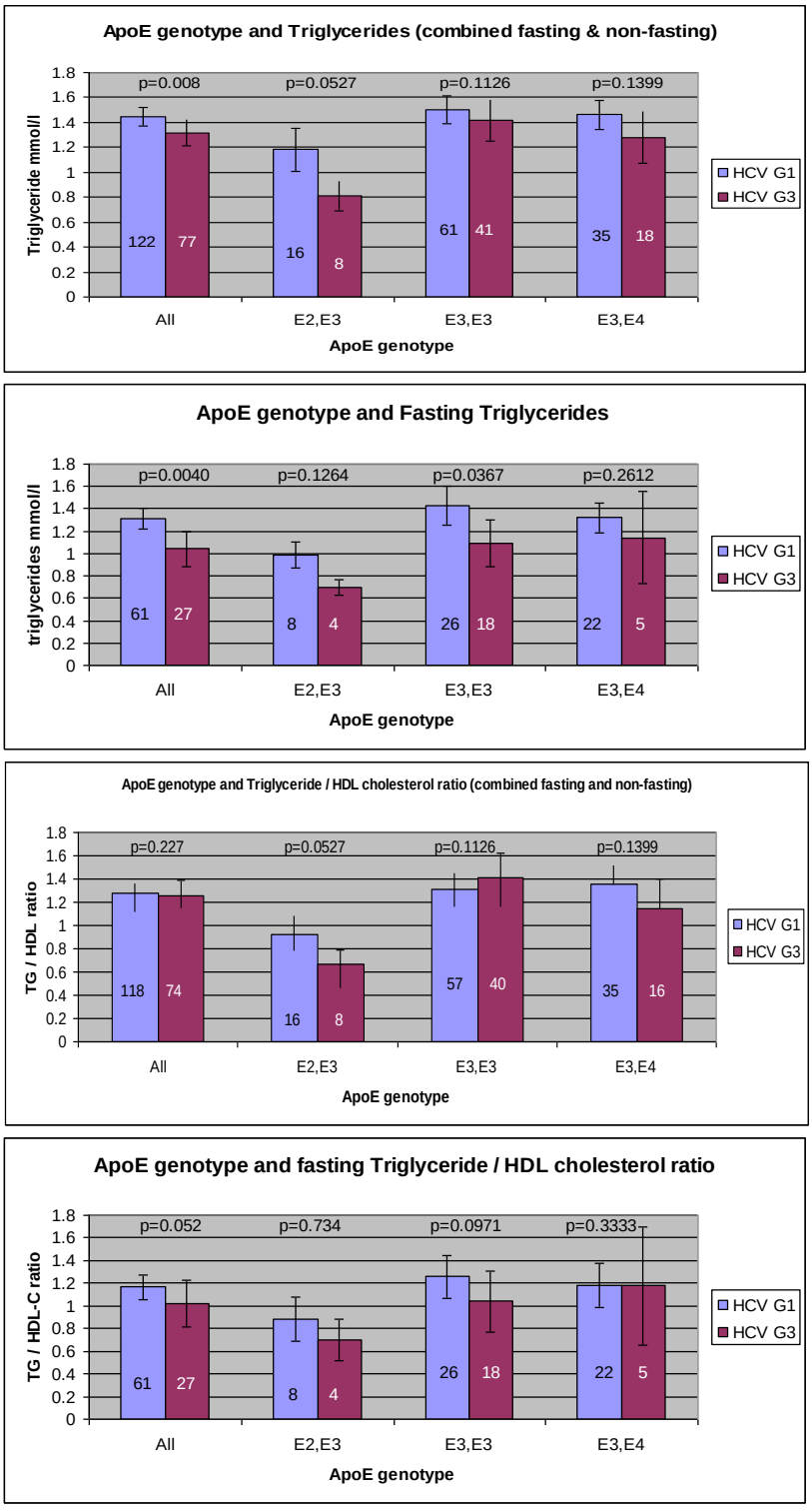


The normal variation in cholesterol levels with apoE genotype is respected in HCV G1 but overridden in HCV G3, where those with apoE3/E3 and E3/E4 have lower cholesterol levels than expected.

3.5.6 Triglycerides, ApoE genotype and HCV genotype

Overall HCV G3 patients had lower triglycerides than HCV G1. This was more significant in the fasting prospective cohort (Figure 14B p=0.004) than the combined cohort (figure 14A p=0.008). However there were not significant differences between HCV genotypes 1 & 3 when analysed according to apoE genotype. Instead the effects of apoE genotype on triglycerides were more in keeping with the expected effects, whereby those with apoE2/E3 had lower fasting triglycerides than those with E3/E3 and E3/E4 (figure 14A and 14B). This same pattern of apoE genotypes was observed with the TG/HDL ratio in the combined (figure 14C) and fasting only (figure 14D) cohorts. This would be expected given that the TG varied according to apoE genotype but the HDL did not.

Figure 14 ApoE genotype and Triglycerides (combined and fasting HCV cohorts)



Triglycerides (TG) levels are significantly lower in HCV G3 than HCV G1 in both the combined retrospective + prospective cohorts (A), and the fasting only HCV cohort (B). However TG levels did not vary significantly with apoE genotype. TG/HDL cholesterol levels showed a similar pattern in the combined (C) and fasting only (D) HCV cohorts.

3.6 The effect of antiviral treatment on lipid profiles in HCV

The majority of the patients in the retrospective cohort had undergone anti-viral therapy with combination pegylated interferon-α and ribavirin and long term treatment outcomes were identified retrospectively. Where available, paired non-fasting lipid profiles pre and post treatment in SVR's (Table 3-10) and non responders table 3-11 were recorded. The sample sizes shown in Table 3-10 and table 3-11 vary according to the availability of paired pre and post treatment data.

Total cholesterol, non-HDL cholesterol, triglycerides and TG / HDL-C ratio all increased significantly in individuals infected with HCV G3 who achieved an SVR. By contrast in those with HCV G1 infection who achieved SVR, the post treatment lipid profiles were not significantly different from pre treatment. HDL cholesterol level remained unchanged pre and post treatment in SVR's for both HCV genotypes 1 & 3.

In non-responders to therapy, there was no significant change in total cholesterol, non-HDL cholesterol, triglycerides, HDL or TG / HDL-C ratio pre- and post-treatment in either HCV genotype 1 or 3. These data strongly support the concept that it is chronic HCV infection *per se* that causes lower apoB associated cholesterol in HCV G3 infection because the lipid profiles normalise with successful eradication of the virus but persist in non-responders.

Table 3-10 Paired pre and post treatment lipid profiles SVR's

HCV Genotype 1	**N**	**Pre Treatment**	**Post Treatment**	**p-value**
Total Cholesterol	35	4.70 ± 1.06	4.73 ± 0.98	0.907
Non HDL Cholesterol	24	3.63 ± 1.06	3.65 ± 0.98	0.933
Log_{10} Triglycerides	29	0.16 ± 0.19	0.18 ± 0.22	0.771
Log_{10} HDL	29	0.10 ± 0.11	0.09 ± 0.10	0.782
TG/HDL ratio	19	1.31 ± 0.70	1.40 ± 0.99	0.725#
HCV Genotype 3	N	**Pre Treatment**	**Post Treatment**	p-value
Total Cholesterol	55	4.20 ± 0.85	5.09 ± 0.86	<0.001
Non HDL Cholesterol	42	2.80 ± 0.77	3.6 ± 0.75	<0.001
Log_{10} Triglycerides	44	0.06 ± 0.23	0.19 ± 0.23	0.010
Log_{10} HDL	43	0.11 ± 0.14	0.13 ± 0.14	0.538
TG/HDL ratio	18	1.24 ± 0.87	1.60 ± 1.20	0.028#

Table 3-11 Paired pre and post treatment lipid profiles non-responders

HCV Genotype 1	**N**	**Pre Treatment**	**Post Treatment**	**p-value**
Total Cholesterol	42	4.66 ± 0.82	4.63 ± 1.04	0.871
Non HDL Cholesterol	33	3.31 ± 0.80	3.19 ± 0.93	0.562
Log_{10} Triglycerides	42	0.17 ± 0.23	0.20 ± 0.24	0.630
Log_{10} HDL	38	0.09 ± 0.16	0.09 ± 0.15	0.986
TG/HDL ratio	26	1.35 ± 1.10	1.39 ± 1.19	0.890#
HCV Genotype 3	**N**	**Pre Treatment**	**Post Treatment**	p-value
Total Cholesterol	21	4.00 ± 1.20	3.63 ± 0.89	0.269
Non HDL Cholesterol	16	2.52 ± 0.99	2.31 ± 0.66	0.481
Log_{10} Triglycerides	22	0.18 ± 0.26	0.20 ± 0.26	0.769
Log_{10} HDL	19	0.06 ± 0.14	0.01 ± 0.19	0.274
TG/HDL ratio	17	1.88 ± 1.48	2.36 ± 1.74	0.082#

Data are in mmol / l and expressed as mean ± standard deviation. Paired t-test was used to compare pre and post treatment results. Abbreviations: SVR sustained virological response; HDL high density lipoprotein; Non-HDL calculated from total cholesterol – HDL # paired t test performed on log10 TG/HDL ratio

3.7 Discussion

This study has analysed the effect of HCV infection and apoE genotypes on serum lipid profiles and shown that HCV genotype 3 exerts a greater influence over baseline LDL / non-HDL cholesterol levels than host apoE genotype. The data confirms that HCV genotype 3 lowers serum cholesterol more than HCV genotype 1 which supports previously published data (Serfaty, T et al. 2001; Siagris, Christofidou et al. 2006; Dai, Chuang et al. 2008). Two lines of evidence support the concept that hypocholesterolaemia in HCV G3 is a directly virally mediated effect. Firstly, there is a negative correlation between HCV viral load and total cholesterol, in both the HDL and non-HDL cholesterol fractions in HCV G3, supporting a direct association between viraemia and lipid profiles. Secondly is the observation from the paired pre/post treatment data that the lipid profiles increase after successful eradication of the virus in those that achieve a sustained virological response (SVR) particularly in HCV G3 infection. This rebound increase in LDL cholesterol with HCV clearance post SVR may be clinically important in terms of atherosclerosis risk. A significant proportion of hypo-cholesterolaemic HCV patients when successfully treated experienced rebound increases in LDL cholesterol to levels warranting initiation of lipid lowering level to reduce risk of cardiovascular disease (Corey, Kane et al. 2009). Despite an apparently favourable lipid profile, a growing body of evidence suggests that chronic HCV infection may be associated with increased atherosclerosis risk. An important contributory factor that may increase atheroma risk may be the very high prevalence of smoking amongst the HCV population that have a history of injecting drug use (Basseri, Yamini et al. 2010). One study reported that HCV seropositivity was associated with more severe coronary atherosclerosis (Alyan, Kacmaz et al. 2008). Another study has reported more severe carotid intima-media thickness measurements (Mostafa, Mohamed et al. 2010). As part of assessment of cardiovascular risk, most clinical biochemistry laboratories estimate LDL cholesterol by the Friedewald equation (Bairaktari, Seferiadis et al. 2005). The calculation is LDL C = total cholesterol – HDL cholesterol – (Triglycerides / 2.2) (Friedewald, Levy et al. 1972). The Friedwald approximation assumes that total cholesterol is normally distributed across the VLDL, LDL and HDL fractions. Friedwald is known to be invalid when triglycerides are >4.5 mmol/l when it underestimates LDL-C. Because

the majority of triglycerides in fasting sera are in VLDL, Friedwald assumes normal VLDL secretion. However the assumptions behind the Friedwald calculation may not be valid in chronic hepatitis C because evidence suggests that HCV affects VLDL production by inhibition of microsomal triglyceride transfer protein (MTP) (Mirandola, Realdon et al. 2006). This study found stronger correlation coefficients for non-HDL cholesterol and apoB than Friedwald calculated LDL cholesterol and apoB, particularly in HCV G3 infection. Therefore using non-HDL cholesterol (total cholesterol – HDL cholesterol) may be more valid than LDL cholesterol in HCV infection because it includes all apoB associated cholesterol including VLDL, IDL and LDL. This may be clinically important because it indicates that in HCV infection in general, and HCV G3 in particular, LDL cholesterol as estimated by Friedwald, underestimates the apoB level. Therefore some HCV patients with high apoB/ LDL levels that may warrant lipid lowering therapy but may not be identified by conventional estimates of LDL cholesterol.

The new data from this study is the assessment of the relative importance of HCV genotype on lipid levels compared to host apoE genotype. A meta-analysis in non-HCV infected individuals confirmed that polymorphisms in the apoE gene have a major influence on serum cholesterol levels (Bennet, Di Angelantonio et al. 2007). 7% of the variation of total cholesterol in healthy Caucasian individuals is related to three different isoforms of the apoE protein (Davington, Gregg et al. 1988). The wild type protein is *ε3*, and the two variants are *ε2* and *ε4* (Mahley and Rall 2000). The *ε2* isoform binds poorly to LDLr and is associated with defective clearance of triglyceride rich lipoprotein (TRL) remnant particles. In apoE2 there is compensatory up-regulation of LDLr, causing lower LDL cholesterol and apoB (Utermann 1987). Those with apoE4 are thought to have high affinity uptake of remnant TRLs via apoE but defective recycling of apoE back to HDL. This leads to accumulation of intracellular cholesterol causing downregulation of LDLr and hence increased serum LDL-C levels. Therefore by affecting LDL clearance, those with the *E2* allele have lower LDL cholesterol and those with *E4* allele tend to have higher LDL cholesterol levels than the majority with the wild-type *E3* allele (Bennet, Di Angelantonio et al. 2007). This pattern was observed in those HCV patients infected by HCV genotype 1 but not in those infected by HCV genotype 3 where the observed cholesterol levels in those with apoE3/E3 genotype and apoE3/E4 genotype were lower than expected.

These observations demonstrate that HCV genotype 3 overides the influence of host apoE genotype on serum total and non-HDL cholesterol levels.

ApoE itself is important for production of infectious HCV particles. ApoE appears to be important in HCV assembly and associates with infectious viral particles *in vivo*. The importance of apoE in HCV assembly has been shown in HCV cell culture (HCVcc) where the viral non-structural protein NS5A recruits apoE after replication but before secretion (Jiang and Luo 2009). When apoE is silenced, infectious particle production is inhibited without affecting viral replication or entry (Chang, Jiang et al. 2007; Benga, Krieger et al. 2010). Further evidence suggests that apoE itself is a constituent of secreted infectious HCV 'lipoviral particles (LVP)'. Antibodies to apoE and apoB were able to immunoprecipitate >90% of HCV RNA from a immunodeficient HCV patient without natural antibodies in whom the majority of HCV RNA was detected in a low density fraction (Nielsen, Bassendine et al. 2006). Characterisation of HCV particles produced from cell culture (HCVcc) has also shown that some particles contain apoE, and that larger (>65nm) particles have higher infectivity (Gastaminza, Dryden et al. 2010). ApoE on secreted HCV particles may be important in cell entry via LDL receptor (Owen, Huang et al. 2009). Varitions in apoE genotype may therefore be important in the natural history of HCV *in vivo*. A previous study suggested that those with apoE3 were more likely to have chronic infection than those with apoE2 which was more associated with spontaneous resolution (Price, Bassendine et al. 2006). However this observation has not been reproduced in genome wide association studies of spontaneous resolution vs chronic infection (Thomas, Thio et al. 2009; Rauch, Kutalik et al. 2010). Another study suggested that apoE4 was associated with less fibrosis progression (Wozniak, Itzhaki et al. 2002), but more frequent neuropsychiatric symptoms on anti-viral therapy (Gochee, Powell et al. 2004), but again these findings have not been further validated.

It seems paradoxical that HCV genotype 3 lowers non-HDL cholesterol levels but responds better to anti-viral therapy than HCV genotype 1. Current evidence has only considered the effect of HCV genotype 3 on serum cholesterol in terms of VLDL production. This effect is probably exerted intracellularly, at the stage of virus maturation and secretion from the hepatocyte. HCV genotype 3 inhibits MTP transcription and activity (Mirandola, Realdon et al. 2006), reducing VLDL secretion and promoting liver steatosis. *In vitro* studies indicate that HCV secretion is

dependent on VLDL assembly (Huang, Sun et al. 2007) and silencing apoB messenger RNA in infected liver cells causes a 70% reduction in the secretion of both ApoB-100 and HCV (Nahmias, Goldwasser et al. 2008). The effect of HCV G3 on LDL clearance however is not known. However the observation that HCV G3 overrides the effect of apoE genotype on cholesterol levels may indicate abnormally upregulated LDLr mediated clearance of LDL, especially in those with the apoE3/E3 and E3/E4 genotypes. The next chapter explores further the differences in cholesterol metabolism between HCV genotypes 1 and 3 and healthy individuals in terms of markers of cholesterol synthesis and clearance.

4 Chapter 4. Is low apoB associated cholesterol in HCV G3 due to reduced synthesis or increased clearance?

4.1 Introduction

Hypocholesterolaemia is apparent in chronic HCV infection, particularly in HCV G3, however it is unknown whether this is due to reduced synthesis of apoB containing lipoproteins (VLDL, IDL and LDL) by the liver or increased clearance of LDL / cholesterol from the circulation, or a combination of both. To address this question, cholesterol metabolism was investigated further in CHC infection by measuring indirect serum markers of endogenous cholesterol synthesis, dietary absorption, entero-hepatic circulation, and LDL clearance.

Measurement of non-cholesterol sterols have been validated as markers of cholesterol metabolism. This is described in detail in the introduction (1.6.3 and 1.6.4). In brief, lathosterol and desmosterol levels are markers of endogenous *de novo* cholesterol biosynthesis. They are both pre-cholesterol intermediates in the late cholesterol biosynthetic pathway (Figure 15).

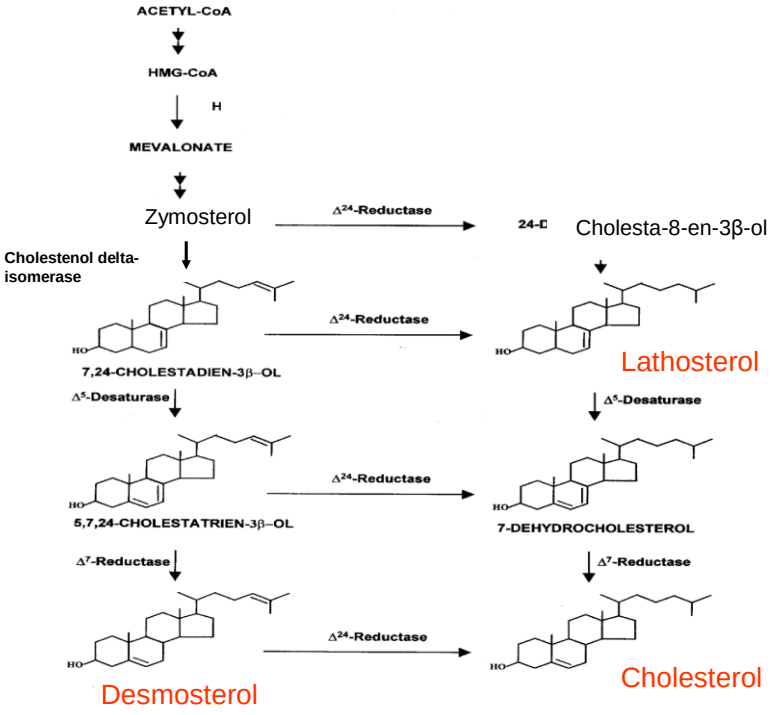


Figure 15 Late stages of endogenous cholesterol synthesis –sterol intermediates
The measured sterols (desmosterol & lathosterol) are shown in red.

Sitosterol is a plant sterol derived exclusively from diet. Sitosterol levels therefore reflect dietary cholesterol absorption through the small intestine. Cholestanol is endogenously synthesised from cholesterol but secreted into bile and therefore is also an absorption marker.

Proprotein Convertase Subtilisin Kexin 9 (PCSK9) is critical for regulating recycling of LDLr and serum PCSK9 levels have been shown to correlate with apoB fractional catabolic rate (Chan, Lambert et al. 2009). PCSK9 levels were therefore measured as an indirect determinant of LDL clearance.

4.2 Patients and Methods

4.2.1 Study population.

Blood samples were collected from the prospective HCV cohort following an overnight fast. All participants gave written informed consent and the study was approved by the Northumberland Research Ethics committee (REC number 07/H0902/45) and sponsored by Newcastle upon Tyne Hospitals NHS Foundation Trust. Participants were age ≥ 18, HCV RNA positive for >6 months, and not on a lipid modulating agent for 3 months prior to the study. Patients with hepatitis B, hepatitis delta, or HIV co-infection, or alcohol dependency were excluded.

Fasting sterol profiles had been performed by the Dept of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle upon Tyne on comparator group A, comprising 45 non-HCV patients as part of a dietary intervention study. This data was kindly shared with agreement of the investigators to act as the comparator for analysis of the HCV group sterol profiles. PCSK9 had been measured in a separate group of 254 non-HCV subjects (comparator group B) and data on this group was kindly provided by collaborators from the University of Montreal who also provided the PCSK9 antibodies and standards for measurement of PCSK9 by ELISA methods in the HCV group. The recruitment of these two comparator groups is described in section 2.2.3. The demographics (table 3-2) and lipid profiles of the two comparator groups (group A for sterol profiles, table 3-5 and group B for PCSK9, table 3-6) are summarised in the previous chapter.

Fasting sterol profiles were measured by gas chromatography mass spectrometry (GC MS) using the methods described in 2.3.2. PCSK9 was measured on fasting plasma samples using a sandwich ELISA described in 2.3.11.

4.2.2 Statistical analysis.

PCSK9 and all the sterol profiles were non-normally distributed. Therefore the data are summarised by median and interquartile ranges. Mann Whitney test was used to compare sterol profiles between groups. Spearman's rank r correlation coefficient was

used to determine relationships between continuous variables and $p<0.05$ was taken to indicate statistical significance. All statistical analysis was carried using Minitab version 15.

4.3 Fasting Sterol Profiles in HCV G1 and HCV G3

4.3.1 Assumptions

Interpretation of data in this study makes a number of assumptions. Firstly it assumes that absolute serum sterol levels reflect the size of the serum pool. The total serum cholesterol pool size is influenced by both inputs and outputs. Inputs are either from endogenous cholesterol synthesis (lathosterol and desmosterol) or dietary absorption (cholestanol and sitosterol). Outputs from the serum cholesterol pool represent clearance of LDL. In order to control for the influence of clearance, sterols were also considered as a proportion (ratio) of total cholesterol. The second assumption therefore is that the sterol/total cholesterol ratio indicates the relative contribution of the sterol source to total body cholesterol. Lathosterol/total cholesterol ratio and desmosterol/total cholesterol ratio therefore represent endogenous cholesterol synthesis. Sitosterol/total cholesterol ratio and cholestanol/total cholesterol ratio represent cholesterol absorption. The third assumption is that differences in relative serum sterol levels represent changes in flux through the particular pathways.

Summary statistics (median and interquartile ranges) for the overall sterol profiles in HCV G1 and HCV G3 are compared in table 4-1.

Table 4-1 Fasting absolute sterol profiles (prospective HCV cohort)

	HCV G1 N=61	HCV G3 N=28	p value
Lathosterol	2.60 (1.96 – 3.77)	2.04 (1.60 – 3.31)	0.0985
Desmosterol	1.19 (0.92 – 1.63)	1.26 (0.84 – 1.90)	0.9156
Cholestanol	5.55 (3.57 – 6.61)	4.48 (3.09 – 5.84)	0.0263
Sitosterol	4.49 (3.56 – 6.07)	5.74 (3.69 – 6.75)	0.5074

Levels are µmol / l; Median (Q1-Q3), Mann Whitney test

The relative contributions of each sterol to the total cholesterol pool in HCV G1 and G3 is summarised in table 4-2.

Table 4-2 Relative contribution of sterols to total cholesterol pool (Sterols/total cholesterol ratios)

(10^2 mmol/mol cholesterol)	**HCV G1 N=61**	**HCV G3 N=28**	**p value**
Lathosterol / TC ratio	61.3 (45.5 – 76.4)	63.6 (40.6 - 85.4)	0.7740
Desmosterol / TC ratio	25.8 (20.7 - 35.1)	30.7 (26.3 - 52.5)	0.0907
Cholestanol / TC ratio	116.8 (97.0 - 146.4)	126.5 (87.4 - 143.0)	0.9037
Sitosterol / TC ratio	103.3 (82.1 - 142.2)	140.0 (96.3 - 222.2)	0.0191

Levels are 10^2mmol / mol cholesterol; median (Q1-Q3), Mann Whitney test

4.3.2 Comparison of relative sterol profiles in HCV to non-HCV subjects

The lipid and sterol profiles in HCV G1 vs the non-HCVcomparator group A is shown in table 4-3. The corresponding data for HCV G3 vs the non-HCV comparators is shown in table 4-4.

Table 4-3 HCV G1 relative sterol profile vs non-HCV comparator group A

Sterol profile (10^2 mmol/mol cholesterol)	Non-HCV Comparators N=45	HCV G1 N=61	p value
Lathosterol	116.2 (79.1–146.8)	61.3 (45.5 – 76.4)	<0.001
Desmosterol	35.5 (28.2-48.3)	25.8 (20.7 - 35.1)	<0.001
Cholestanol	102.1 (87.8-125.2)	116.8 (97.0 - 146.4)	0.082
Sitosterol	103.1 (89.6-146.0)	103.3 (82.1 - 142.2)	0.504

Table 4-4 HCV G3 relative sterol profile vs non-HCV comparator group A

Sterol profile (10^2 mmol/mol cholesterol)	Non HCV comparators N=45	HCV G3 N=28	p value
Lathosterol	116.2 (79.1 – 146.8)	63.6 (40.6 - 85.4)	<0.001
Desmosterol	35.5 (28.2 - 48.3)	30.7 (26.3 - 52.5)	0.303
Cholestanol	102.1 (87.8 - 125.2)	126.5 (87.4 - 143.0)	0.232
Sitosterol	103.1 (89.6 - 146.0)	140.0 (96.3 - 222.2)	0.044

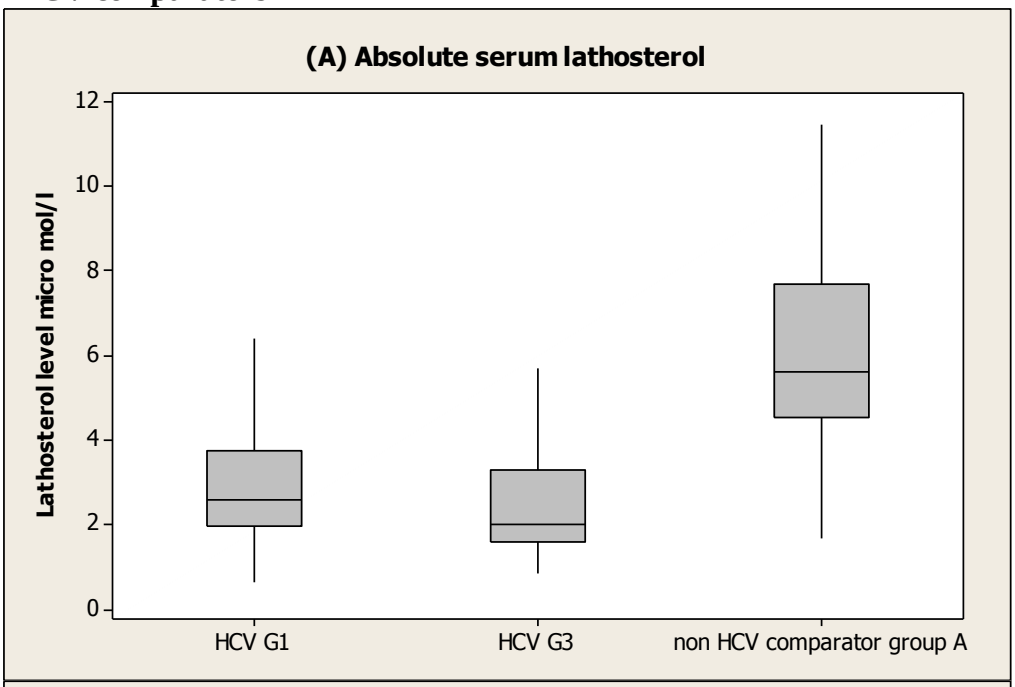
Levels of non-cholesterol sterols have been also shown to be affected by apoE genotype. In a previous study of healthy males, sterol synthesis markers were significantly higher in ApoE2 than E3 & E4, whereas absorption markers were higher in E3 & E4 than E2 (Nissinen, Gylling et al. 2008). Therefore in this study each sterol marker was sub-analysed according to ApoE genotype.

4.3.3 Endogenous Cholesterol synthesis - Lathosterol

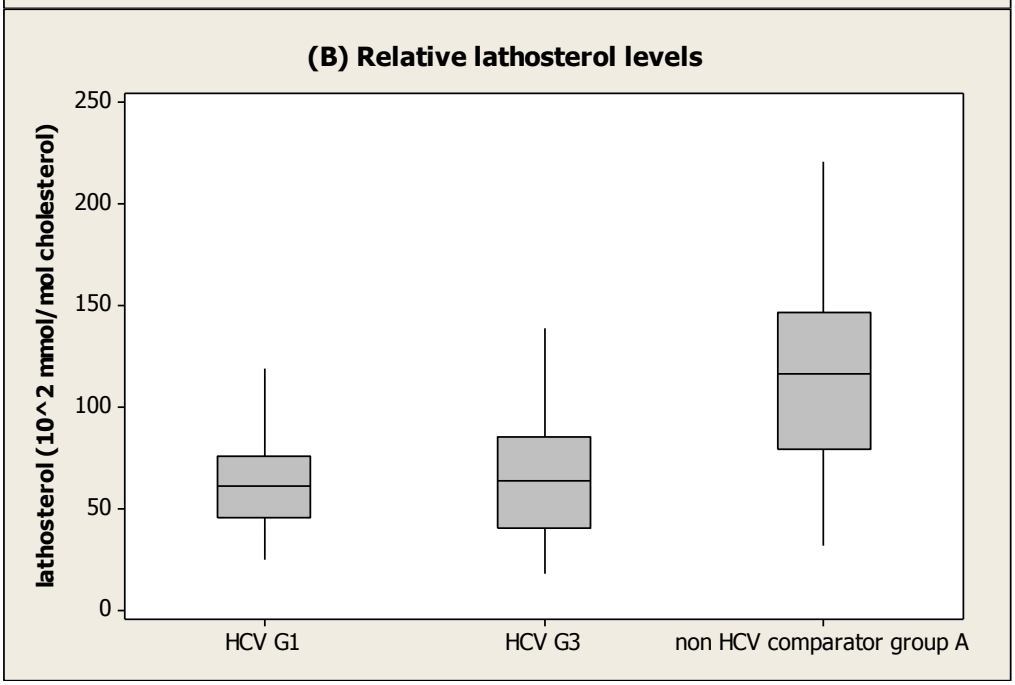
Both HCV G1 and HCV G3 had significantly lower absolute (figure 16A) and relative lathosterol (figure 16B) levels compared to non-HCV comparators. Relative lathosterol levels were not affected significantly by apoE genotype in non HCV comparators or HCV patients (figure 17A). Overall there was a tendency for those with HCV G3 to have lower absolute lathosterol levels than HCV G1 but this was not statistically significant (p=0.0985). However it is noteworthy that in those with apoE3/E4, the absolute serum lathosterol levels were significantly lower in HCV G3 than HCV G1 (p=0.0192) (figure 17B). Although absolute lathosterol levels were significantly lower in those with HCV G3 than HCV G1 with apoE3/E4 (figure 17B), the relative lathosterol levels were the same (p=0.7740) (figure 17C). This suggests that those with HCV G3 and apoE3/E4 have similar contributions of lathosterol to cholesterol synthesis but lower absolute lathosterol levels, likely related to abnormally up regulated clearance of LDL.

Figure 16 Absolute (A) and relative (B) lathosterol levels in HCV G1 & G3 and non-HCV comparators

A



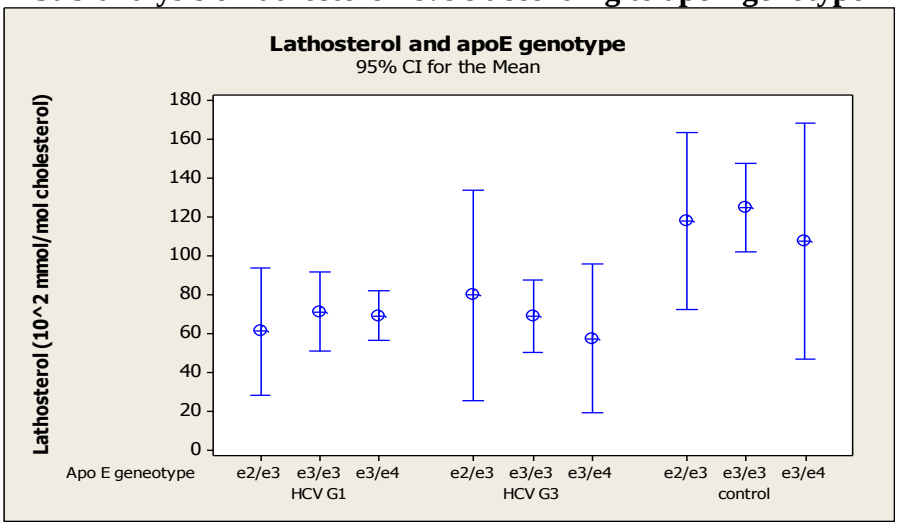
B



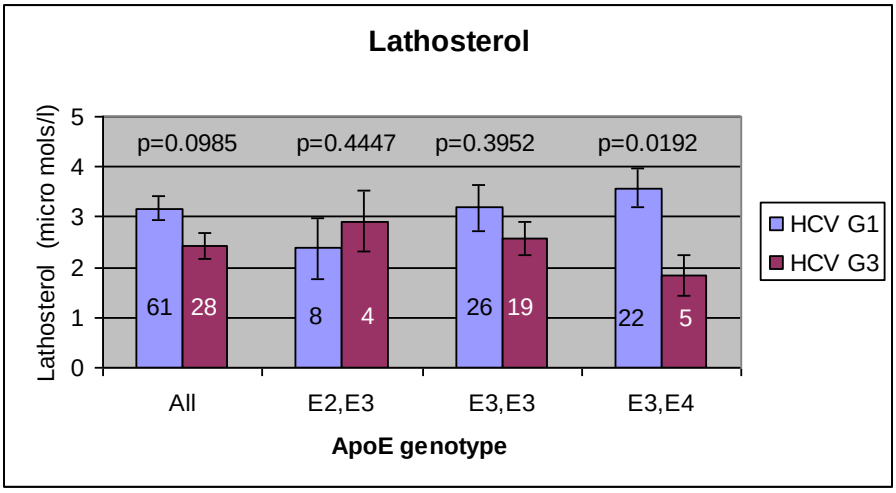
Absolute lathosterol (µmol/l) (A) and relative lathosterol to total cholesterol ratio (10^2 mmol/mol cholesterol) (B) are significantly lower in both HCV G1 and G3 than non-HCV comparators (p<0.001). There is a trend for absolute lathosterol to be lower in HCV G3 than HCV G1 (p=0.0985). The relative lathosterol levels between HCV G1 and G3 are not significantly different (p=0.7740).

Figure 17 Sub-analysis of lathosterol levels according to apoE genotype

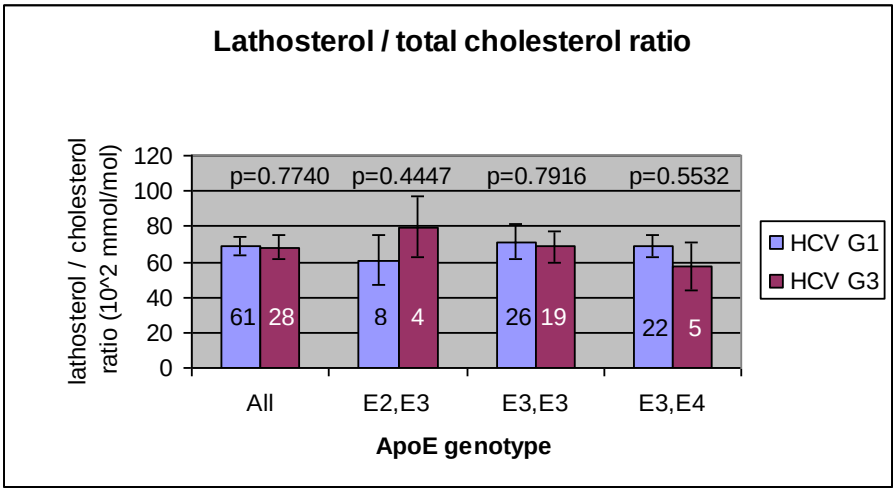
A



B



C



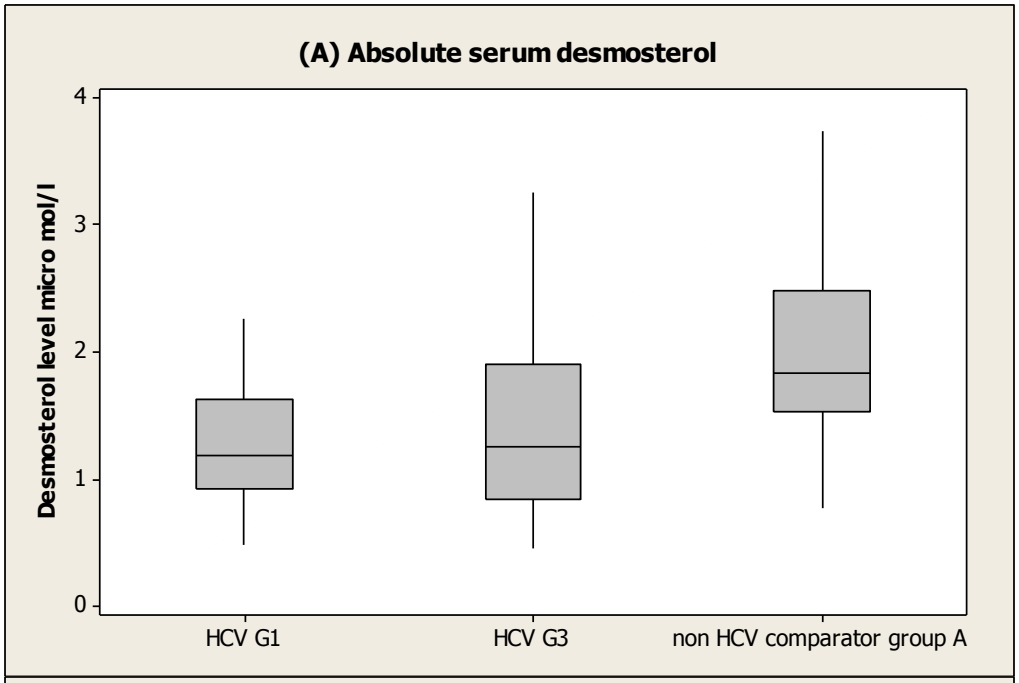
ApoE genotype did not significantly affect relative lathosterol levels in HCV G1, G3 or non HCV controls (A). Absolute lathosterol levels were significantly lower in HCV G3 patients with apoE3/E4 than HCV G1 patients with apoE3/E4 (B), but the relative lathosterol levels were the same (C).

4.3.4 Endogenous Cholesterol synthesis - Desmosterol

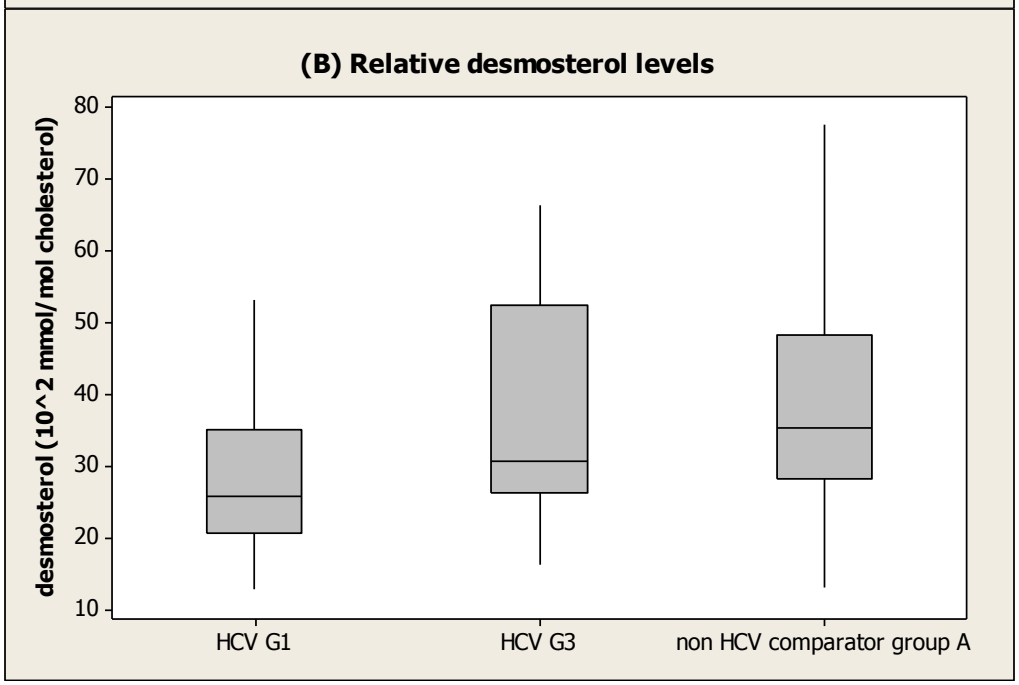
Desmosterol is another marker of endogenous cholesterol synthesis. Overall absolute desmosterol levels were lower in both HCV G1 & HCV G3 than non-HCV comparators (figure 18A). Relative desmosterol levels (desmosterol / total cholesterol) tended to be higher in HCV G3 than HCV G1 ($p=0.0907$) and closer to the normal values of the comparator group (figure 18B). Relative desmosterol levels showed more variation between apoE genotypes than relative lathosterol levels in HCV G3 patients Figure 19A. When sub-analysed according to apoE genotype, the absolute desmosterol level in those with apoE3/E3 was significantly higher in HCV G3 than HCV G1 ($p=0.0432$) (figure 19B). The relative desmosterol levels was significantly higher in HCV G3 than HCV G1 in those with apoE3/E3 ($p=0.0013$) but not E3/E4 (figure 19C).

Figure 18 Absolute and relative desmosterol levels in HCV G1 & G3 and controls

A

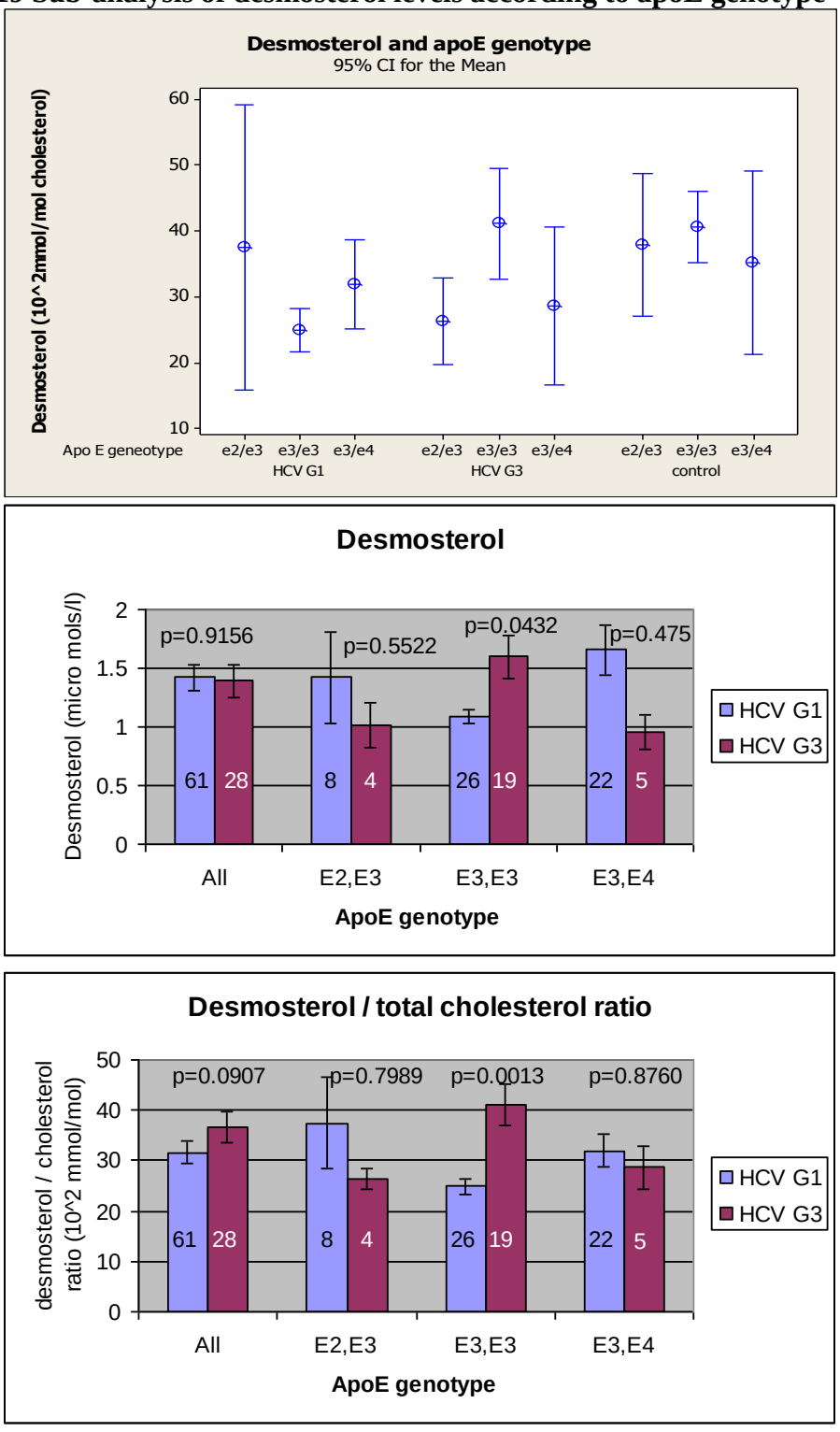


B



Absolute desmosterol levels (µmol/l) (A) were significantly lower in both HCV G1 and HCV G3 than non HCV comparators (p<0.001). The relative desmosterol to total cholesterol ratio (10^2 mmol/mol cholesterol) (B) tended to be higher in HCV G3 than HCV G1, being maintained at close to normal levels.

Figure 19 Sub-analysis of desmosterol levels according to apoE genotype

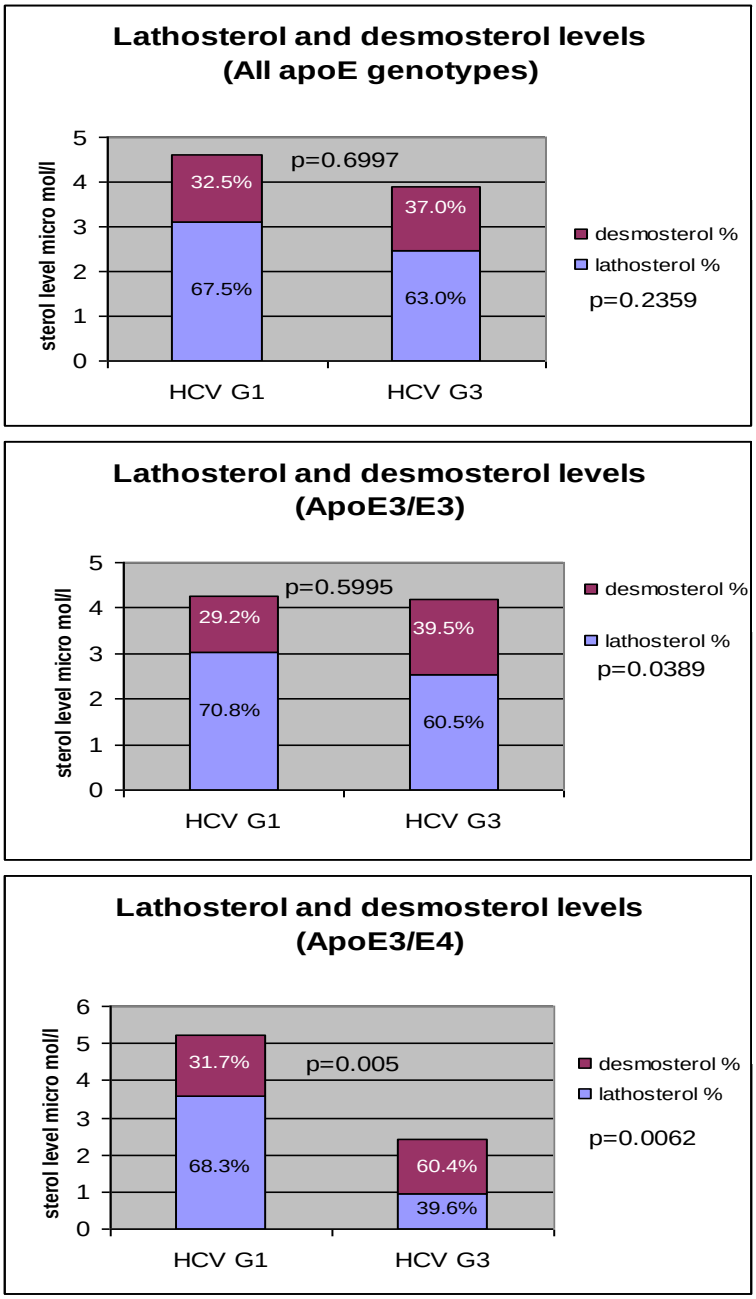


ApoE genotype was associated with more variation in relative desmosterol levels (10^2 mmol/mol cholesterol) in HCV patients than in non-HCV controls (A). Both absolute desmosterol (B) and relative desmosterol (C) were significantly lower in HCV G1 patients with apoE3/E3 than HCV G3 patients respectively. Those with HCV G3 and apoE3/E3 or E3/E4 had close to normal relative desmosterol levels.

In summary the observed relative desmosterol levels were unexpectedly discordant with the relative lathosterol levels in those with HCV G3 infection, given that they are both markers of endogenous cholesterol synthesis.

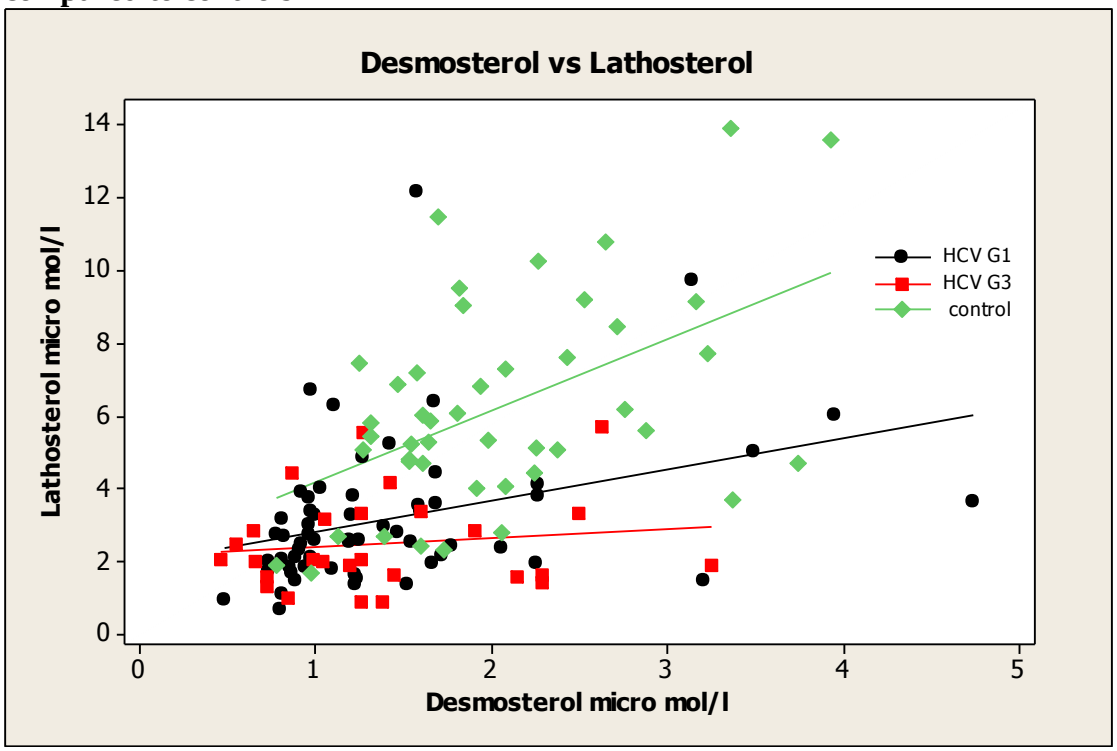
To further evaluate discordance in lathosterol and desmosterol as markers of endogenous cholesterol synthesis, the total amount of endogenous sterols (i.e. lathosterol + desmosterol) and the relative proportions (%) of lathosterol & desmosterol between HCV G1 and G3 were assessed according to apoE genotype (Figure 20). Overall there was no significant difference in total endogenous sterol levels (latho + desmo) between HCV G1 and G3. However, in those with apoE3/E3 the relative contribution of desmosterol was significantly higher in HCV G3 than HCV G1 (39.5 % vs 60.5%, p=0.0389). In those with apoE3/E4 the discordant endogenous sterol profiles in HCV G3 were even more marked. The discordance in endogenous sterols was largely a consequence of low lathosterol levels with relative sparing of desmosterol in HCV G3. Therefore the significant reductions in the total endogenous sterols could be attributed predominantly to reductions in lathosterol in HCV G3 (68.3% lathosterol in HCV G1 vs 39.6% in HCV G3). As a consequence, although the total endogenously derived sterol pool was reduced in HCV G3, the relative contribution of desmosterol was greatly increased, especially in those with apoE3/E4 (31.7% in HCV G1 vs 60.4% in HCV G3, p=0.0062). This is summarised by Figure 20 which indicates how the relative contribution of desmosterol is increased particularly in HCV G3.

Figure 20 Combined endogenous sterol synthesis levels (lathosterol + desmosterol), and relative contributions (%) in HCV G1 & HCV G3 by apoE genotype.



Lathosterol and desmosterol are both endogenous cholesterol synthesis precursors. The combined lathosterol + desmosterol level indicates total endogenous cholesterol synthesis. The p value between the columns is the comparison of differences in total levels of synthesis markers between HCV G1 and HCV G3. The p value in the legend is comparison of the relative % contribution of each sterol. Overall lathosterol contributes twice as much as desmosterol with no significant differences between HCV G1 and G3 (A). However in individuals with apoE3/E3, lathosterol is lower in HCV G3 than HCV G1, with an increase in desmosterol, changing the relative contributions of each sterol significantly (p=0.0389) (B). In those with apoE3/E4 and HCV G3, lathosterol is reduced further, with preservation of desmosterol levels but large relative (%) increase (p=0.0062) (C).

Figure 21 Discordance between desmosterol and lathosterol levels in HCV compared to controls



In non HCV healthy comparators there is concordant correlation between lathosterol and desmosterol (r=0.427, p=0.004). In HCV G1 lathosterol and desmosterol remained concordantly correlated (r=0.414, p=0.004) but with a reduced slope i.e for a given lathosterol level, desmosterol is higher than would be expected.. In HCV G3 there is no significant relationship between lathosterol and desmosterol (r=0.080, p=0.690) indicating discordance between the lathosterol and desmosterol pathways.

Furthermore when desmosterol levels were correlated with lathosterol levels, there was a significant correlation between desmosterol and lathosterol in non HCV controls (r = 0.427, p = 0.004). This would be expected given that they are both synthesis intermediates. In HCV G1 this correlation remained significant (r = 0.414, p = 0.001) but the slope was reduced indicating less flux via lathosterol. In HCV G3 the relationship between desmosterol and lathosterol was disrupted such that the two no longer correlated (r = 0.080, p=0.690) Figure 21.

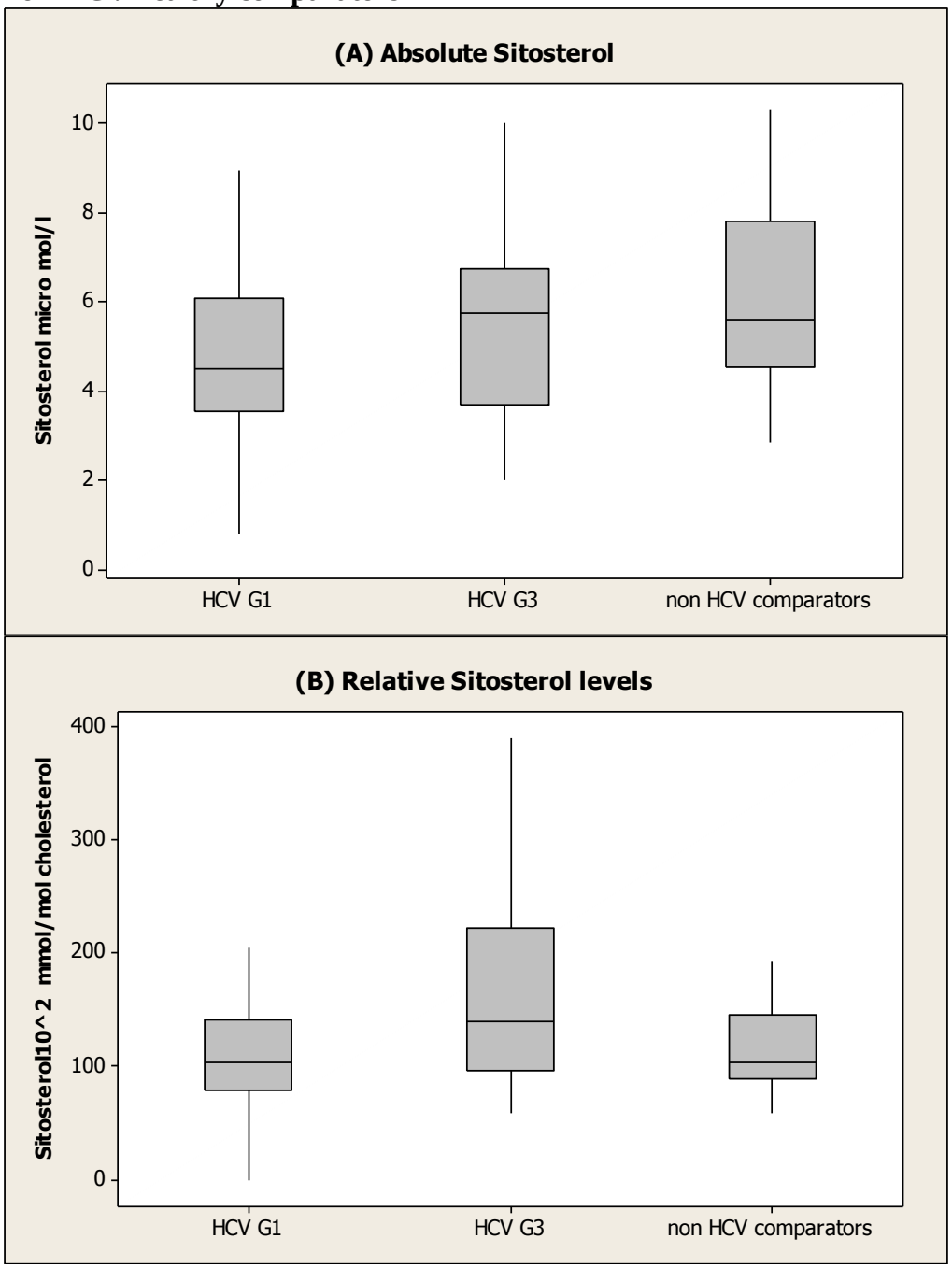
In summary, the data suggest that HCV reduces endogenous cholesterol synthesis predominantly via the lathosterol pathway and that in HCV G3 there is selective preservation of the desmosterol pathway, especially in those with apoE3/E4.

4.3.5 Dietary Cholesterol absorption (sitosterol)

Absolute sitosterol levels, a marker of dietary cholesterol absorption, were not significantly different between the HCV genotypes (p=0.5074) (figure 22A). However the relative sitosterol level, (i.e. sitosterol / total cholesterol ratio) was significantly higher in HCV G3 compared to HCV G1 (p=0.0191) and to non HCV comparators.(p=0.044) (figure 22B).

There was no significant variance in sitosterol according to apoE genotype but the higher relative sitosterol level apparent in HCV G3 was only significant in those with apoE3/E3 (p=0.0317).

Figure 22 Absolute (A) and relative (B) sitosterol levels in HCV G1 & G3 and non-HCV healthy comparators

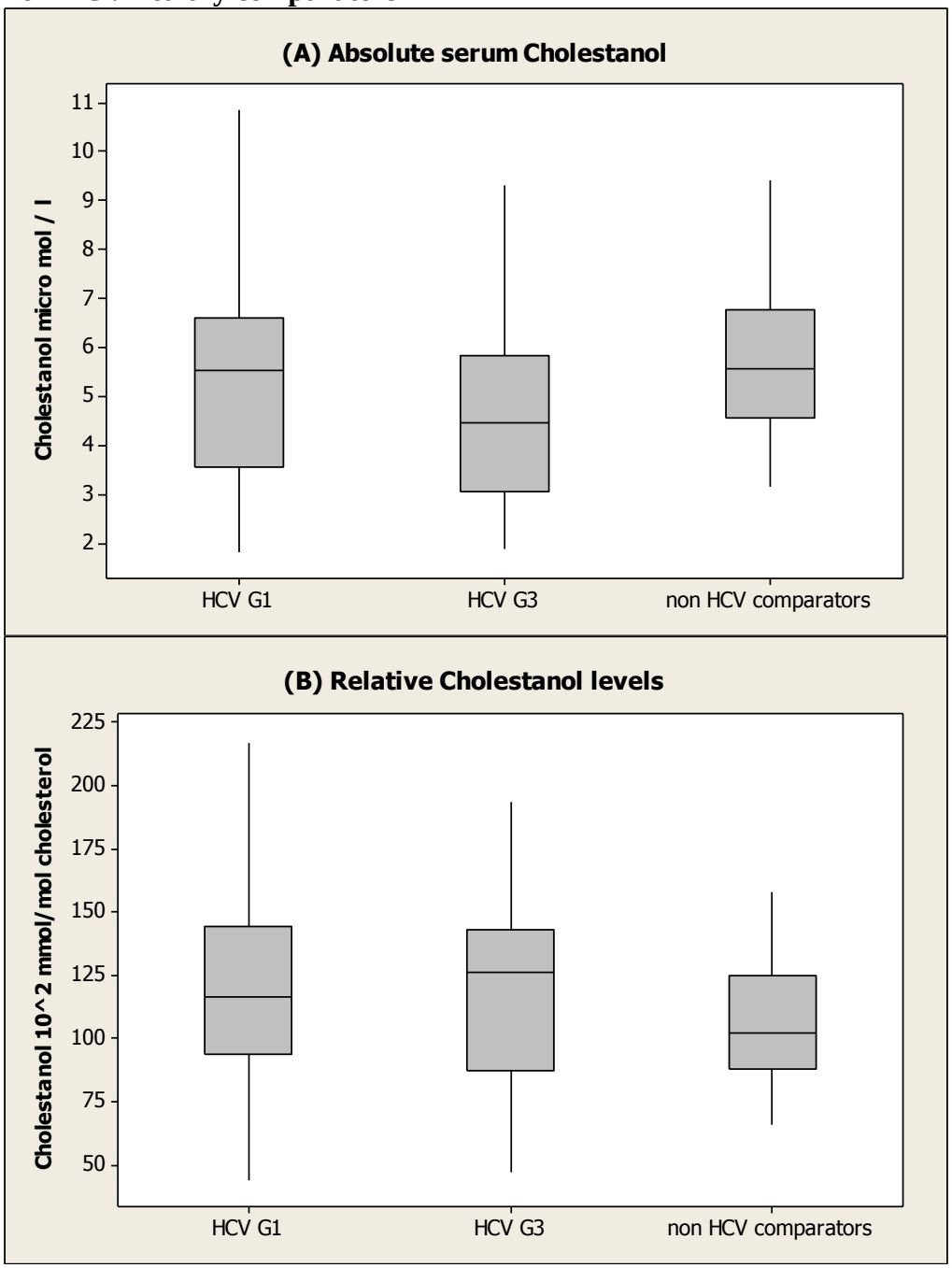


There were no significant differences in absolute sitosterol levels between HCV G1, G3 and non-HCV healthy comparators. However relative sitosterol levels were significantly higher in HCV G3 compared to HCV G1 (p=0.0191), and also higher than non-HCV comparators (p=0.044).

4.3.6 Cholestanol

Absolute cholestanol levels were significantly lower in HCV G3 than HCV G1 (p=0.0263) and HCV G3 vs non-HCV comparators (p=0.007) (figure 23A), but the difference was restricted only to those with apoE3/E4 genotype in HCV G3 (p=0.0192). Absolute cholestanol was also significantly lower in HCV G1 than non HCV comparators (p=0.042) (figure 23A). However the relative cholestanol levels were not significantly different between HCV G1 and non HCV comparators (p=0.494) and HCV G3 and non HCV comparators (p=0.220) (figure 23B). Likewise relative cholestanol was not significantly different between HCV G1 and HCV G3 overall (p=0.9037) or when subdivided by apoE genotypes. Since cholestanol is both a cholesterol synthesis and absorption marker and there were low absolute cholestanol levels in HCV G3 with apoE3/E4 but not significantly different relative cholestanol levels, this supports the concept of increased LDL clearance in those with HCV G3 and apoE/E4, rather than defective synthesis *per se*.

Figure 23 Absolute (A) and relative (B) cholestanol levels in HCV G1 & G3 and non HCV healthy comparators

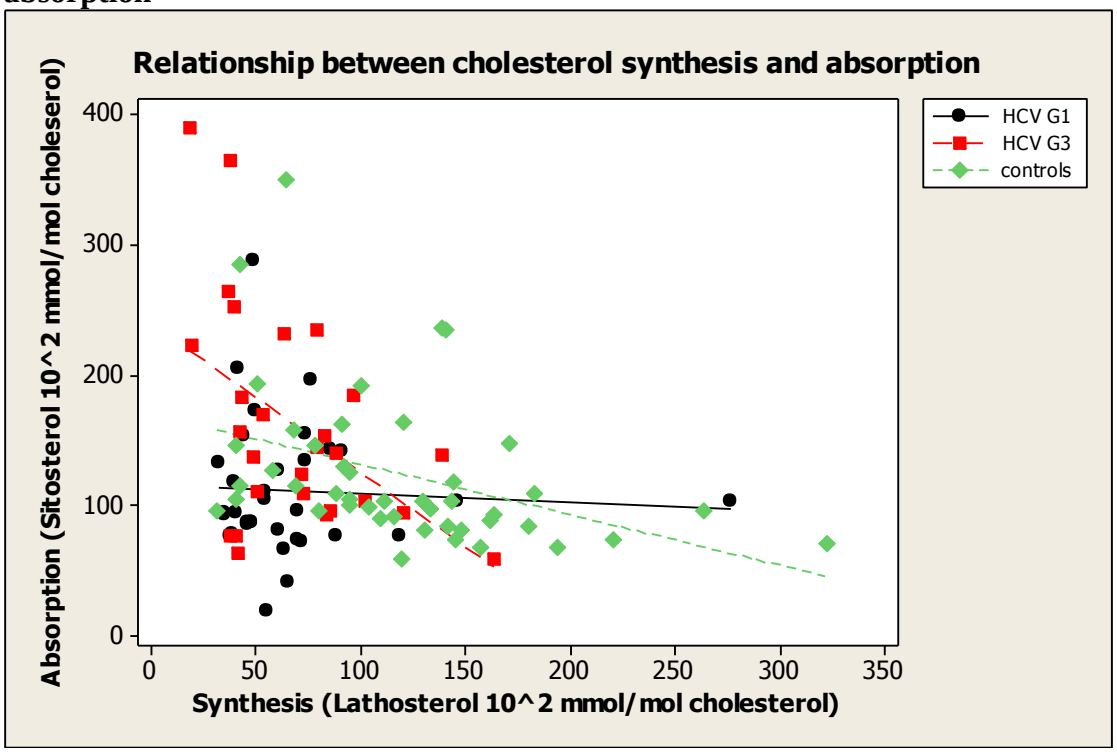


Absolute cholestanol levels were significantly lower in HCV G3 than HCV G1 (p=0.0263) and non HCV comparators (p=0.007) (A). However relative cholestanol (B) was not significantly different between HCV G3 and G1 (p=0.494) or HCV G3 and non HCV comparators (p=0.220).

4.3.7 Relationship between endogenous cholesterol synthesis and dietary cholesterol absorption.

In controls there was a close inverse relationship between endogenous cholesterol synthesis and dietary absorption (r = -0.537, p<0.001). In the HCV cohort this correlation was weakened overall (r= -0.296, p=0.005) but remained significant in those with HCV G3 (r = -0.422, p=0.032) but not in HCV G1 (r = -0.017, p = 0.926) (Figure 24). This indicates that although endogenous cholesterol synthesis is low in HCV G1, there is not the expected compensatory increase in cholesterol absorption.

Figure 24 Correlation *de novo* cholesterol production and intestinal cholesterol absorption



In non-HCV healthy comparators, as endogenous synthesis decreases, dietary cholesterol absorption increases (r=-0.537, p<0.001). In HCV G3 this relationship is weakend (r=-0.422, p=0.032) and is non-significant in HCV G1 (r=-0.017, p=0.926).(Spearmans Rank correlation)

4.3.8 Sterol profiles pre and post anti-viral therapy

Five patients (2 HCV G1 & 3 HCV G3) had paired fasting sterol profiles performed pre anti-viral therapy and at least 3 months post successful completion of treatment. The post treatment sterol profiles were taken when the participants were known to be HCV RNA PCR not detected, consistent with HCV eradication. Although the small numbers limits statistical power, it can be seen that lathosterol levels appear to increase (figure 25A), whilst desmosterol and sitosterol decrease with viral eradication (figure 25B and 25C). This reflects an increase in *de novo* cholesterol synthesis via lathosterol and reduction in absorption. Cholestanol levels did not change so significantly, possibly reflecting that cholestanol is both a synthesis and absorption marker (figure 25D). Interestingly NEFA's also appear to increase with viral clearance (figure 26).

Figure 25 Paired pre and post treatment sterol profiles

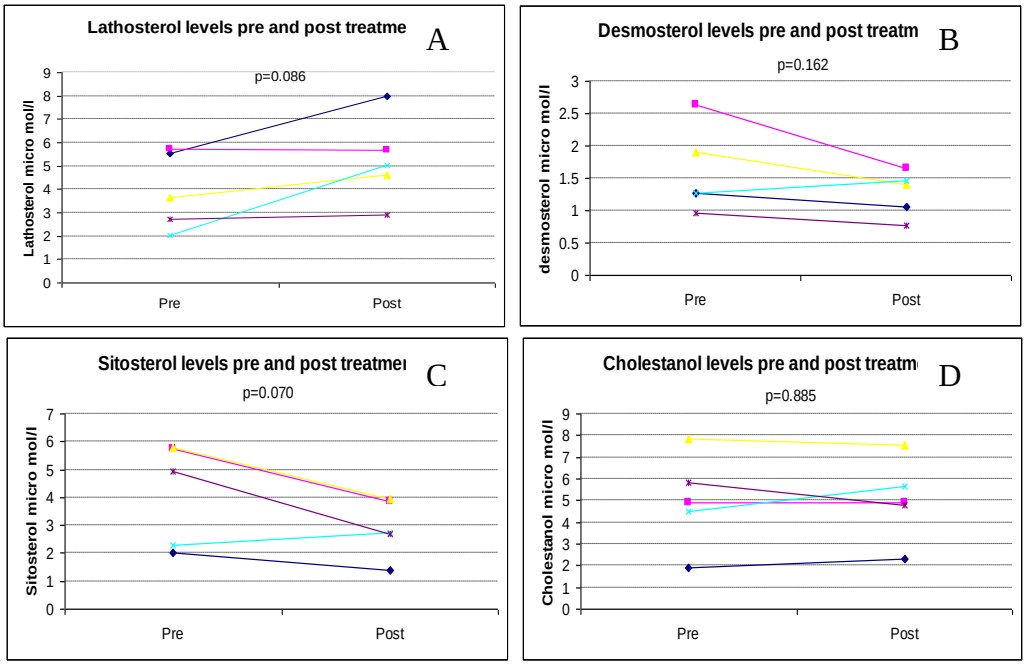


Figure 25*. Five HCV patients had paired fasting sterol profiles pre (viraemic) and post (non-viraemic) successful antiviral therapy. Lathosterol levels increased, and desmosterols and sitosterol levels decreased with HCV eradication.*

Figure 26 Paired pre and post treatment non-esterfied fatty acids (NEFA)

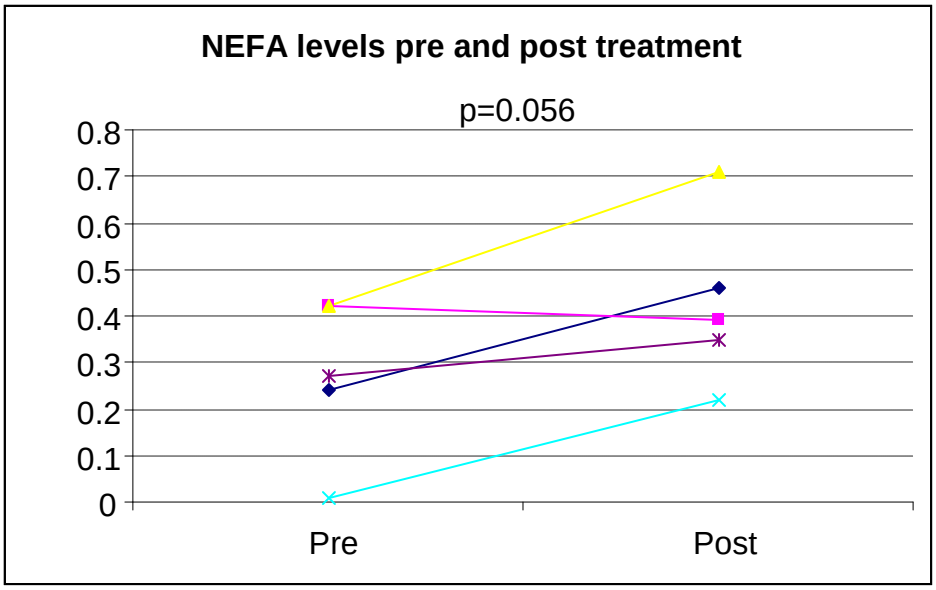


Figure 26. *NEFA levels increased with eradication of HCV post antiviral therapy*

4.3.9 Sterols and steatosis

Serum gamm-glutamyl-transpeptidase (GGT) is a liver enzyme that has been widely correlated with increasing steatosis in patients with hepatitis C and metabolic syndrome (Benini, Pigozzi et al. 2009). Relative desmosterol levels correlated significantly with GGT in HCV G3.

Table 4-5 Relative sterol levels and GGT

Correlation with GGT	HCV G1 N=61	HCV G3 N=28
Lathosterol / total cholesterol ratio	r = -0.138, p = 0.307	r = 0.034, p = 0.871
Desmosterol/ total cholesterol ratio	r = 0.185, p = 0.169	**r = 0.475, p = 0.014**
Cholestanol / total cholesterol ratio	r = -0.102, p = 0.455	r = 0.231, p = 0.256
Sitosterol / total cholesterol ratio	r = -0.079, p = 0.563	r = 0.223, p = 0.274

Spearman's Rank correlation analysis

4.3.10 Sterols and ALT

ALT, a marker of hepatic inflammation correlated negatively with relative lathosterol in HCV G1 but positively with relative desmosterol in HCV G3.

Table 4-6 Relative sterol levels and ALT

Correlation with ALT	HCV G1 N=61	HCV G3 N=28
Lathosterol / total cholesterol ratio	**r = -0.301, p = 0.023**	r = 0.102, p = 0.621
Desmosterol/ total cholesterol ratio	r = 0.221, p = 0.098	**r = 0.421, p = 0.032**
Cholestanol / total cholesterol ratio	r = -0.012, p = 0.933	r = 0.333, p = 0.096
Sitosterol / total cholesterol ratio	r = -0.037, p = 0.785	r = 0.385, p = 0.052

4.3.11 Sterols and liver stiffness

Liver stiffness measurement by transient elastography (Fibroscan ®) correlated significantly with relative desmosterol, cholestanol and sitosterol levels in HCV G3 but not in HCV G1. Liver stiffness measurements were not performed in all patients therefore note smaller sample size.

Table 4-7 Relative sterol levels and Liver stiffness measurements

Correlation with liver stiffness	HCV G1 N=53	HCV G3 N=24
Lathosterol / total cholesterol ratio	r = -0.120, p = 0.391	r = -0.273, p = 0.196
Desmosterol / total cholesterol ratio	r = 0.118, p = 0.398	**r = 0.470, p = 0.021**
Cholestanol / total cholesterol ratio	r = -0.019, p = 0.893	**r = 0.513, p = 0.010**
Sitosterol / total cholesterol ratio	r = 0.159, p = 0.261	**r = 0.429, p = 0.036**

4.3.12 Sterols and Insulin Resistance

In this prospective cohort, fasting glucose and insulin was measured and the HOMA IR calculated (glucose x insulin/22.5). Those with HOMA IR ≥2 were considered insulin resistant. 34% of HCV G1 and 40% of HCV G3 had insulin resistance by this definition (table 4-8).

Table 4-8 Metabolic parameters (fasting prospective cohort)

	HCV G1 N=61	HCV G3 N=28
Glucose	4.99 ± 0.64	5.44 ± 1.17
Insulin	8.51 ± 6.16	7.46 ± 3.51
HOMA IR	1.91 ± 1.63 1.61 (0.93 – 2.35)	1.90 ± 1.24 1.34 (1.02-2.53)
HOMA IR ≥ 2 N(%)	21 (34%)	11 (40%)
HOMA IR < 2 N (%)	40 (66%)	17 (60%)
NEFA	0.45 ± 0.31	0.45 ± 0.35

Data are mean ± standard deviation; HOMA IR = Homeostasis model assessment of insulin resistance, (glucose x insulin / 22.5).

Previous studies measuring sterol profiles in type 2 diabetes and normoglycaemic insulin resistant men, have indicated that insulin resistance is associated with increased cholesterol synthesis and decreased cholesterol absorption (Pihlajamaki, Gylling et al. 2004). For this reason, given that a significant proportion of HCV patients were insulin resistant, the relationship between cholesterol synthesis and absorption in insulin resistant HCV patients was explored.

In HCV patients overall, lathosterol correlated positively with BMI (r=0.256, p= 0.016) and waist circumference (r = 0.331, p=0.002) but not with insulin resistance as measured by HOMA IR (r = 0.153, p=0.152) and fasting insulin (r = 0.121, p=0.264).

However in HCV G1 there was significant correlation between relative desmosterol levels and insulin resistance.

Table 4-9 Relative sterol levels and insulin resistance correlations

Correlation with HOMA IR	HCV G1 N=61	HCV G3 N=28
Lathosterol / total cholesterol ratio	r = 0.017, p = 0.902	r = 0.015, p = 0.943
Desmosterol / total cholesterol ratio	**r = 0.385, p = 0.003**	r = 0.142, p = 0.489
Cholestanol / total cholesterol ratio	r = -0.030, p = 0.827	r = 0.061, p = 0.766
Sitosterol / total cholesterol ratio	r = 0.054, p = 0.693	r = 0.086, p = 0.677

4.4 PCSK9

4.4.1 PCSK9 levels indicate differences in LDL clearance

Absolute serum LDL cholesterol levels reflect the circulating pool size, which is determined by production and clearance. The sterol data indicates that cholesterol synthesis in HCV is reducedcompared to the non HCV group, but the relative contribution of lathosterol as a synthesis marker between HCV G1 and G3 was the same. However, HCV G3 patients had evidence of a smaller LDL pool size as indicated by significantly lower non-HDL cholesterol and apoB than HCV G1. This difference between HCV genotypes could be explained by increased clearance of LDL in HCV G3. Clearance of LDL is largely determined by LDLr expression. Therefore the hypothesis of increased LDL clearance in HCV G3 was tested by measuring PCSK9 levels, which are known to directly affect the expression of LDLr. PCSK9 alters the recycling and degradation of LDLr and is discussed in detail in 1.5.2. Serum PCSK9 levels have been shown to negatively correlate with apoB fractional catabolic rate, such that high PCSK9 is associated with low LDLr expression and slow clearance, and low PCSK9 is associated with high LDLr and rapid apoB clearance (Chan, Lambert et al. 2009). The PCSK9 summary data is shown in table 4-10.

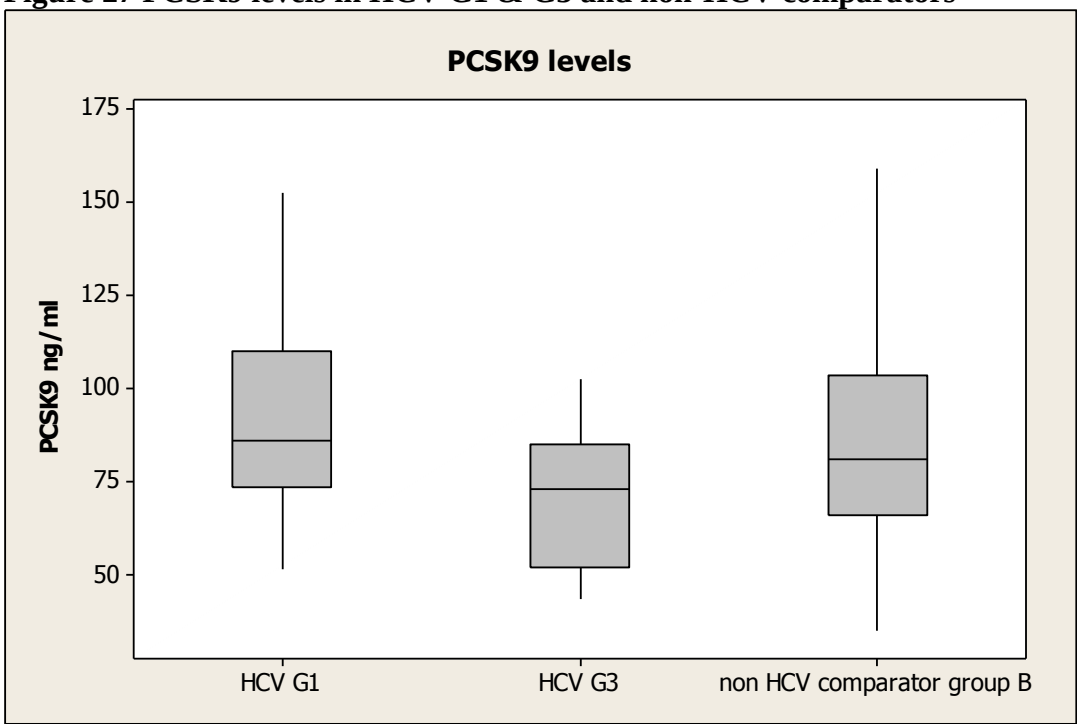
Table 4-10 PCSK9 levels in HCV G1, HCV G3 and non-HCV healthy comparators

PCSK9 ng / ml	HCV G1 N=41	HCV G3 N=18	Comparator group B N=254
All	95 ± 30 ng / ml	72 ± 22 ng / ml	89 ± 32 ng / ml
Male	93 ± 29 ng / ml	71 ± 18 ng / ml	85 ± 27 ng / ml
Female	101 ± 32 ng / ml	93 ± 16 ng / ml	93 ± 35 ng / ml
PCSK9:LDL cholesterol ratio #	38.3 ± 19.7	40.1 ± 17.4	31.1 ± 12.5

Data are mean ± standard deviation PCSK9. (# ng of PCSK9 /µmol LDL cholesterol)

Overall mean PCSK9 level was 88.4 ng / ml (SD ± 29.58) in CHC patients and 89.4 ± 31.9 ng / ml in non-HCV comparators (group B) (p=0.789). However the mean PCSK9 levels were significantly lower in HCV G3 (72.2 ± 22.02 ng /ml) than G1 (94.5 ± 29.6 ng/ml) (p=0.007). There was also significantly lower PCSK9 level in HCV G3 vs non HCV comparators (p=0.016), but not between HCV G1 vs non HCV comparators (p=0.789) (figure 27). PCSK9 levels were noted to be lower in males than females in both healthy non HCV comparators and HCV infected patients.

Figure 27 PCSK9 levels in HCV G1 & G3 and non-HCV comparators



PCSK9 levels were significantly lower in HCV G3 than non HCV comparators (p=0.016) and than HCV G1 (p=0.007). PCSK9 levels were not significantly different between HCV G1 and non HCV comparators (p=0.789)

4.4.2 PCSK9 Clinical and lipid profile correlations

Table 4-11 shows that PCSK9 levels correlated significantly with total, LDL & non HDL cholesterol, age and BMI in non-HCV healthy comparators but these correlations were not significant in HCV patients.

Table 4-11 PCSK9 correlations.

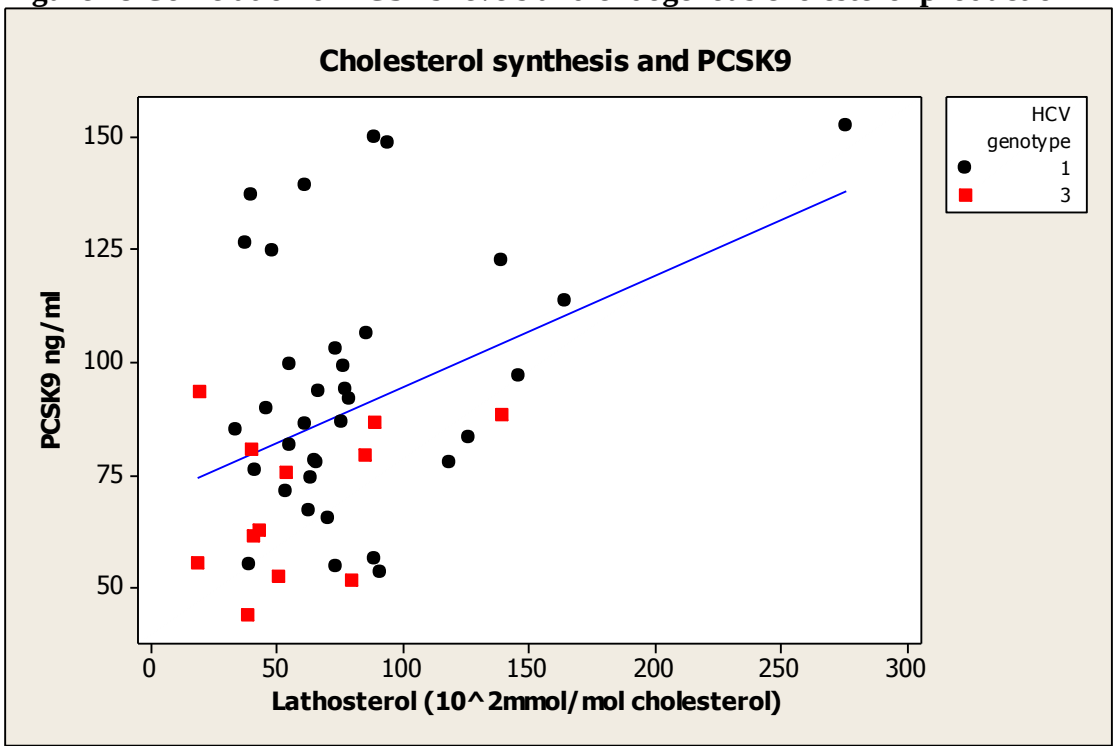
	Comparator Group B		**HCV all**	
	r	p-value	r	p-value
Total cholesterol	0.38	<0.001	0.09	0.446
Non HDL cholesterol	0.40	<0.001	0.01	0.372
LDL cholesterol	0.35	<0.001	0.06	0.633
age	0.38	<0.001	-0.15	0.170
BMI	0.26	<0.001	0.03	0.766

Pearson's r correlation co-efficient.

4.4.3 PCSK9 correlations with sterol markers of endogenous cholesterol production and dietary cholesterol absorption

Although PCSK9 did not correlate as expected with fasting lipid profiles in HCV patients, in male HCV patients, PCSK9 levels correlated significantly with relative lathosterol, and inversely with relative sitosterol levels. Figure 28 shows a significant positive correlation (r = 0.272, p = 0.046) between endogenous cholesterol synthesis (lathosterol/total cholesterol ratio) and PCSK9.

Figure 28 Correlation of PCSK9 levels and endogenous cholesterol production



PCSK9 levels in HCV correlate weakly with endogenous cholesterol synthesis via lathosterol (r=0.272, p=0.046).

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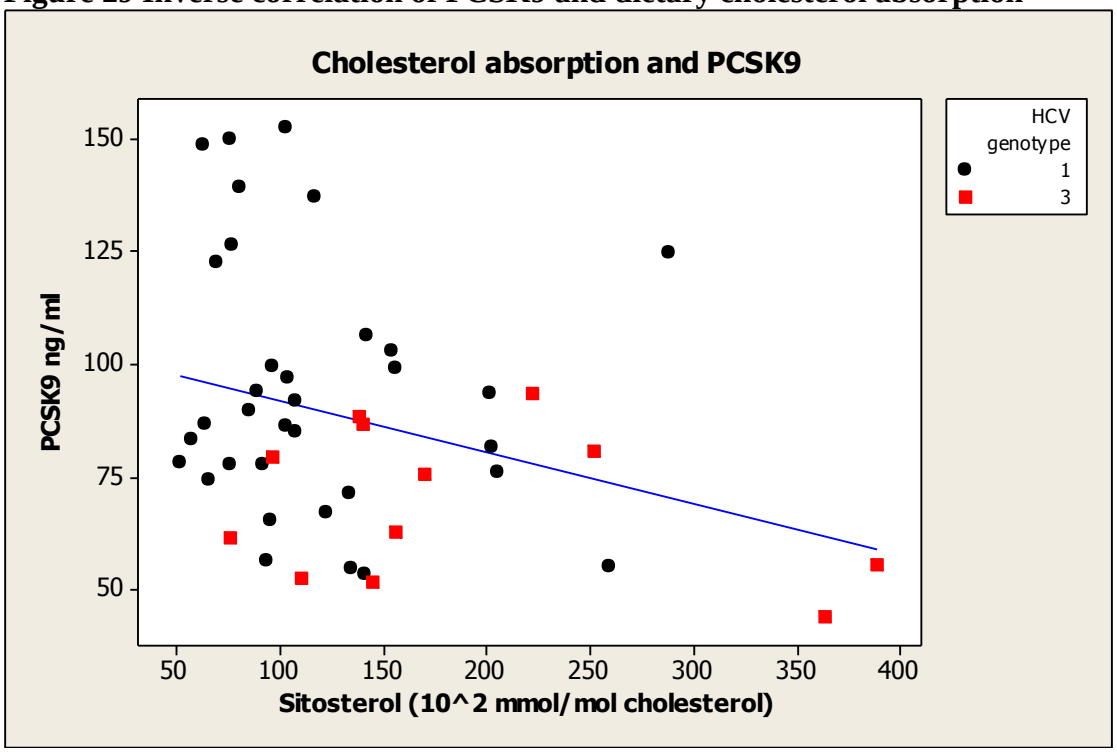
Figure 29 shows a significant inverse correlation (r = -0.284, p= 0.040) between intestinal cholesterol absorption (sitosterol/total cholesterol ratio) and PCSK9 in males with chronic HCV infection (both genotypes 1 & 3). This indicates that those HCV patients with reduced endogenous cholesterol production (i.e. low relative lathosterol) tend to have low PCSK9 levels consistent with high LDLr expression.

Figure 29 Inverse correlation of PCSK9 and dietary cholesterol absorption



PCSK9 levels in HCV weakly correlate inversely with cholesterol absorption (r=-0.284, p=0.040).

4.4.4 Other PCSK9 correlations

The PCSK9/LDL-C ratio was significantly higher in HCV G1 vs non HCV comparators ($p<0.001$) and HCV G3 vs non HCV comparators ($p<0.001$), but not significantly different between the two HCV genotypes ($p=0.686$). This implies that for a given level of LDL-C, the relative PCSK9 level in HCV patients is actually higher than would be expected for the same LDL-C level in a healthy individual.

Overall there was no correlation between HCV RNA viral load and PCSK9 ($r=0.046$, $p=0.986$) which was non-significant for both HCV G3 ($r=0.216$, $p=0.322$) and HCV G1 ($r=-0.029$, $p=0.835$).

There was no significant association of PCSK9 levels and treatment outcomes.

4.5 Discussion

4.5.1 HCV G3 is associated with increased LDL clearance

This study provides further evidence that chronic HCV infection causes a disorder in host lipid metabolism with distinct metabolic phenotypes for individuals infected by HCV G1 and HCV G3. The conclusions drawn assume that the sterol profiles in the non HCV comparator group A are "normal". It should be noted however that the comparator group A had higher BMI than both HCV G1 and G3 patients. BMI, insulin resistance (Hoenig and Sellke 2010) and hepatic steatosis in NAFLD (Simonen, Kotronen et al. 2011) have been shown to correlate with increasing cholesterol synthesis markers. The sterol data in the literature is largely drawn from hyperlipidaemic subjects to investigate atheroscerosis and metabolic syndrome. However the observed lathosterol levels in this study in the HCV patients were still lower than those reported in the literature in a group of healthy men with similar characteristics to the HCV patients in this study (mean age of 46 and BMI of 24.2) (Prinsen, Romijn et al. 2003). In that study lathosterol was 3.61 ±0.64 µmol/l vs 2.60 µmol/l in HCV G1 and 2.04 µmol/l in HCV G3, supporting the general conclusion that these markers are abnormally low in the HCV group.

The first important conclusion of this study is that HCV G3 infection is likely to be associated with increased LDL clearance. A number of lines of evidence support this conclusion. Firstly is that the apoE3/E4 genotype in non-HCV patients is usually associated with down regulation of LDLr and delayed LDL clearance compared to wild type apoE3/E3. In those with apoE3/E4 and HCV G3 however, this reduced LDL clearance is largely overcome and is manifest by much lower apoB and non-HDL cholesterol than controls. The up regulation of LDL clearance in HCV G3 therefore appears to be particularly marked in those with apoE3/E4 genotype. Secondly, the sterol profiles also suggest increased LDL clearance in HCV G3 which would account for the low absolute lathosterol and desmosterol levels, compared to HCV G1 and controls. However, because relative lathosterol and desmosterol ratios were similar in HCV G3 to HCV G1, this implies similar low relative rates of cholesterol synthesis.

The final piece of evidence supporting increased LDLr mediated clearance in HCV G3 is the significantly lower PCSK9 levels in patients with HCV G3 compared to

HCV G1 and non HCV comparators. Consistent with the known functions of PCSK9 in LDLr regulation, low PCSK9 levels will permit both reduced competition with LDL for LDLr binding and also cause less degradation of LDLr. Hence with low PCSK9, more LDLr can be recycled back to the cell membrane to increase LDLr mediated clearance of apoB containing lipoproteins.

Absolute PCSK9 levels are low in HCV G3 relative to non HCV comparatorsand non significantly increased in HCV G1. This implies normal or reduced LDL clearance in HCV G1 which would account for the relatively normal apoB levels in HCV G1, despite reduced endogenous cholesterol synthesis. However given the very low serum levels of LDL cholesterol in HCV G3, relative PCSK9 levels are also actually considerably higher than would be expected for a similar level of cholesterol in a non-HCV population (i.e. higher PCSK9/LDL cholesterol ratio). This is important because it suggests that the compensatory responses observed in non-HCV patients treated with statins as cholesterol lowering agents are mirrored in chronic HCV infection. In statin therapy, serum LDL cholesterol is lowered by inhibiting cholesterol synthesis and increasing LDLr expression. PCSK9 levels are also noted to increase in patients on statins because of secondary SREBP-2 activation (Dubuc, Tremblay et al. 2009). This same pattern is seen in patients with HCV G3 infection where the PCSK9/LDL cholesterol ratio is high. This suggests that HCV is behaving in a similar way to a statin by inhibiting cholesterol synthesis enzymatically, and enhancing SREBP activation. Given the inhibitory effect of statins on HCV replication *in vitro* it is possible that in HCV the defect in endogenous cholesterol synthesis occurs at a later step post-geranylgeranyl. SREBP-2 activation would also permit over expression of LDLr to facilitate viral entry, and trigger an increase in PCSK9 to counter act this as a compensatory response, hence increase the PCSK9/LDL cholesterol ratio. Since PCSK9 is also a competitive ligand for LDLr, absolute serum PCSK9 levels may also be lowered indirectly by removal via over-expressed LDLr rather than by direct viral inhibition of PCSK9 in HCV G3.

The concept of increased LDL clearance in HCV G3 provides a new insight into the mechanisms of hypocholesterolaemia in HCV G3 infection. This data therefore contributes further to the literature which to date has considered that the low levels of apoB and LDL cholesterol in HCV G3 are largely due to reduced VLDL production by viral inhibition of microsomal triglyceride transfer protein (MTP). These studies

indicated that HCV core protein reduces MTP activity (Perlemuter, Sabile et al. 2002), and reduced levels of MTP were identified in liver biopsies with steatosis (Mirandola, Realdon et al. 2006).

4.5.2 HCV reduces endogenous cholesterol synthesis

The second important conclusion from this study is that markers of endogenous cholesterol synthesis were reduced in both HCV G1 and HCV G3. Importantly the reduction in endogenous cholesterol synthesis was predominantly via the lathosterol pathway and was to a similar extent in both HCV genotypes as evidenced by significantly lower relative lathosterol levels (i.e. lathosterol/total cholesterol ratios) than non HCV comparators. Low lathosterol synthesis was apparent in HCV across all apoE genotypes. Unlike lathosterol however, desmosterol levels were largely normal in HCV G3, despite profound hypocholesterolaemia and varied more by apoE genotype. In those with wild type apoE3/E3, both the absolute and relative desmosterol levels were significantly higher in HCV G3 than G1. This suggests that in some HCV G3 patients, cholesterol synthesis via the desmosterol pathway makes a relatively larger contribution to total cholesterol synthesis compared to the lathosterol pathway which is consistently suppressed. The apparent substitution of desmosterol for cholesterol in some HCV G3 patients with profound hypocholesterolaemia may be functionally important. In this study in HCV G3 infection, relative desmosterol levels correlated with increasing GGT and liver stiffness, suggesting a possible association of desmosterol with hepatic steatosis. This hypothesis is biologically plausible given existing understanding of the regulatory roles of desmosterol. Desmosterol has been shown to be a potent activator of LXR. LXR is normally activated by cholesterol excess which up-regulates ABC Cassette transporter 1 to remove excess cholesterol, i.e. enhances reverse cholesterol transport. LXR can also activate SREBP-1c to switch on *de novo* lipogensis to buffer the toxic effects of excess free cholesterol. High relative desmosterol levels in HCV G3 may cause inappropriate lipogenesis and therefore contribute to the development of hepatic steatosis.

The interactions of HCV and the endogenous cholesterol biosynthetic pathway are complex. The enzymes responsible for *de novo* cholesterol biosynthesis are located in the ER membrane, which is also the site of HCV replication. The early rate limiting

step in the endogenous cholesterol biosynthetic pathway is the activity of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase and the production of mevalonate. Following production of mevalonate, the intermediate geranyl can branch off to produce geranalygeranyl. Geranylgeranylation of a host protein, FBL2 is essential for replication of HCVcc (Kapadia and Chisari 2005). This permits an interaction with HCV NS5A (Wang, Gale et al. 2005) and can be inhibited by HMG CoA reductase inhibitors (statins) (Ye, Wang et al. 2003). One explanation for the low lathosterol levels apparent *in vivo* may be that HCV replication is diverting pre-cholesterol intermediates towards geranylgeranyl production at the expense of cholesterol and the late precursors of cholesterol production such as lathosterol. Geranyl that is not used in prenylation is converted to farnesyl and subsequently to squalene. Squalene is converted to lanosterol which is the first sterol intermediate in the mevalonate pathway. Several enzymes then modify lanosterol to form cholesterol (Ikonen 2008). From lanosterol, cholesterol biosynthesis can proceed by two routes: via a desmosterol intermediate (Bloch pathway), or via a lathosterol intermediate (Kandutsch-Russel pathway). This is determined by the stage at which the carbon double bond at position 24 of the sterol side chain is reduced. In desmosterol production (Bloch pathway) the C24 double bond is retained until the final reaction, whereas in the formation of lathosterol and 7-dehydrocholesterol (Kandutsch-Russel pathway) the C24 double bond is reduced at an earlier step. The functional importance of this is not fully appreciated, but some evidence from Huh7 cells suggests that replacing cholesterol with desmosterol in plasma membrane lipid rafts can impair the formation of lipid raft domains and alter receptor function as evidenced by impaired insulin receptor signalling (Vainio, Jansen et al. 2006). Alternatively 7-dehydrocholesterol, the post lathosterol precursor of cholesterol from the Kandutsch-Russel pathway promoted plasma membrane lipid raft domains more strongly than cholesterol (Xu, Bittman et al. 2001). 7- dehydrocholesterol is converted via cholecalciferol to vitamin D by the action of UV light in the skin. HCV patients with low lathosterol levels may therefore be at increased risk of vitamin D deficiency. Indeed in a recent study, patients with HCV genotype 1 infection had significantly lower 25-hydroxyvitamin D levels than controls, and low vitamin D levels were associated with more severe fibrosis and less interferon response (Petta, Camma et al.).

This study has additionally identified that exogenous cholesterol from dietary sources contributes a relatively higher proportion of the serum cholesterol pool in HCV G3 than in HCV G1, as evidenced by the significantly higher relative sitosterol levels. This is likely to be a compensatory increase in absorption of dietary sterols in the context of diminished hepatic cholesterol synthesis in HCV G3, particularly those with apoE3/E3. This is the opposite sterol profile to that which is seen in obesity (Miettinen and Gylling 2000) and Type 2 diabetes (Simonen, Gylling et al. 2002) where endogenous cholesterol synthesis is high and fractional intestinal cholesterol absorption is low. Moreover in normoglycaemic males, insulin resistance as measured by the hyperinsulinemic euglycaemic clamp correlated with cholesterol synthesis as measured by desmosterol and lathosterol levels and decreased cholesterol absorption (Pihlajamaki, Gylling et al. 2004). In the HCV cohort however there was no significant correlation between relative lathosterol levels and HOMA IR. However relative desmosterol levels correlated with HOMA IR in HCV G1 infection. It is therefore possible that relative excess desmosterol may be promoting insulin resistance by impairing insulin receptor function by disrupting plasma membrane lipid rafts (Vainio, Jansen et al. 2006). Further disruption of membrane lipid rafts may also affect candidate HCV receptors such as SRB1 and CD81.

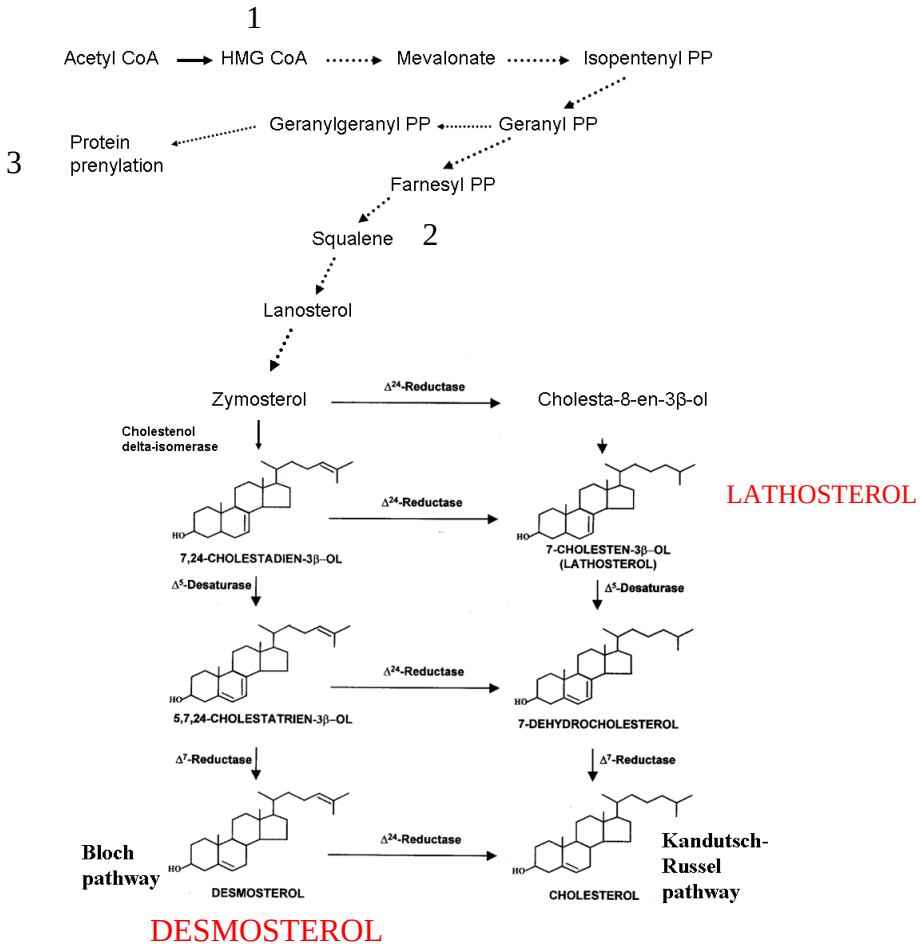


Figure 30 Schematic of the endogenous cholesterol biosynthetic pathway.
Cholesterol synthesis involves a complex series of enzymatic reactions from the 2 carbon acetyl coA to 27 carbon cholesterol. Late stages of cholesterol synthesis can occur via pathways involving either lathosterol or desmosterol intermediate. In HCV infection there is discordant cholesterol synthesis with downregulation of the lathosterol pathway and relative preservation of desmosterol, particularly in HCV G3. In vitro gene expression studies have shown down regulation of HMGCR (1) and SQLE (2) but up regulation of geranyl transfer proteins (3) (Blackham, Baillie et al. 2010), supporting the concept that HCV can differentially dysregulate the endogenous cholesterol biosynthetic pathway.

The mechanisms causing low cholesterol synthesis in HCV and the apparent discordance in lathosterol and desmosterol pathways in HCV G3 are not clear. There is evidence that HCV can directly and differentially alter expression of a number of genes involved in lipid metabolism. Gene expression profiling of Huh 7 cells infected by JFH-1 showed complex effects on gene expression in a number of pathways including cell defence mechanisms such as apoptosis, trafficking and anti-oxidants, lipid and protein metabolism and intracellular transport. The effects on lipid genes in this system are noteworthy. Genes in cholesterol biosynthesis such as SQLE and HMGCR were decreased. However genes involved in Geranyl transfer to cellular proteins from intermediates in the mevalonate pathway were increased (e.g. GGPS1 and PGGT1B), indicating differential regulation of branches of the cholesterol synthetic pathway. Genes involved in synthesis and transport of phospholipids were increased (SGPP1, SPTLC1, CHKA and ACSL3). Genes involved in oxidation and degradation of fatty acids were decreased (e.g. ACAT2 and ACADSB) whereas synthesis, transport (e.g. ELOVL4, ACSL3, VLDLR and FABP3), and regulation (e.g. PPARGC1A and TXNIP) of fatty acid metabolism were increased (Blackham, Baillie et al.). HCV may also be directly promoting *de-novo* lipogenesis by up-regulating fatty acid synthase (FAS) via SREBP-1c. HCV core protein can activate FAS in an SREBP-1 dependent manner and blocking FAS decreases HCV production (Yang, Hood et al. 2008). NS2 protein also increases SREBP-1c transcription, protein expression and proteolytic processing, and as a result FAS transcription is up regulated (Oem, Jackel-Cram et al. 2008). Another study in Chimpanzees of gene expression analysis of liver biopsies following infection with HCV genotype 1a showed up-regulation of a number of genes including SREBP, SCAP, Lipase A, ATP citrate lyase, and fatty acid synthease (FAS). Additionally there was down regulation of PPARα and hepatic lipase C in this system. Further experiments inducing SREBP activation with nystatin which sequesters cholesterol, increased HCV replication levels by nearly 100%. In contrast cerulenin which directly inhibits FAS and 25-hydroxycholesterol thereby inhibiting SREBP, inhibited HCV in a dose dependent manner (Su, Pezacki et al. 2002). *In vitro* data from transfection studies indicate that HCV core protein has a direct effects on expression of genes involved in lipid metabolism (Yamaguchi, Tazuma et al. 2005). HCV core and NS4B derived from genotype 3a have been shown to induce activation of SREBP-2 (Waris, Felmlee et al. 2007). These data suggest that SREBP-2 activation is required for HCV replication

and supports the concept that HCV is having differential effects on the endogenous cholesterol pathway and promoting *de novo* lipogenesis.

In conclusion, HCV has co-evolved with the host (Pang, Planet et al. 2009) such that it utilises the different steps of cholesterol metabolism for its own advantage. HCV may increase demand for cholesterol or cholesterol precursors involved in viral replication (e.g. geranylgeranyl), and activate SREBP-2 directly to meet this demand. However, later enzymatic steps of endogenous cholesterol biosynthesis appear to be inhibited by HCV *in vivo*. This study provides evidence to support the concept that HCV reduces endogenous cholesterol biosynthesis predominantly via the lathosterol pathway, and that this effect is conserved in both HCV G1 and HCV G3. However in HCV G3, cholesterol synthesis via the desmosterol pathway appears relatively preserved. The distinct metabolic phenotype associated with HCV G3 infection of low apoB and LDL cholesterol compared to HCV G1 is therefore largely due to increased clearance of LDL probably by over-expression of LDLr rather than differences in synthesis *per se*. HCV is known to associate with host lipoproteins as lipoviral particles (LVP) (Andre, Komurian-Pradel et al. 2002; Nielsen, Bassendine et al. 2006) and these LVP have increased infectivity *in vitro* (Miyanari, Atsuzawa et al. 2007). LDLr has long been considered an important receptor for HCV attachment and entry in vivo (Agnello, Abel et al. 1999; Owen, Huang et al. 2009). High expression of unbound LDLr in HCV G3 would favour attachment and entry of HCV lipo-viral particles via LDLr. This state of a high fractional catabolic rate of LDL in HCV G3 ideally needs to be confirmed by formal apoB kinetics studies. Taken together these observations may favour HCV on two levels, firstly to increase LDLr as a portal of entry and secondly to reduce the potentially anti-viral effect of PCSK9 which has been shown to inhibit viral entry via CD81 in vitro. This also suggests the potential use of recombinant PCSK9 as a novel anti-viral agent in those individuals with HCV induced hypocholesterolaemia.

5 Chapter 5. Do lipid profiles influence anti-viral treatment outcomes?

5.1 Introduction and aims

The primary aim of this study was to determine whether lipid profiles that are influenced by chronic hepatitis C (CHC) infection are important determinants of anti-viral treatment outcome with pegylated interferon-α and ribavirin. This was investigated by a retrospective analysis of lipid profiles in CHC patients, comparing pre treatment lipid profiles in those patients that had achieved a sustained virological response (SVR) (i.e. HCV RNA subsequently not detected > 6 months post treatment) to non-responders.

The second aim was to investigate host genetic polymorphisms associated with anti-viral treatment outcomes. Previous work from the Newcastle HCV research group had suggested carriers of the apoE2 allele were more likey to have spontaneous resolution of acute HCV infection than those with apoE3/E3 who were more likely to become chronic (Price, Bassendine et al. 2006). However this hypothesis driven, candidate gene approach to genetic determinants of treatment outcomes in small clinical cohorts has resulted in largely non-reproducable results. More recently more powerful non-hypothesis driven genome wide association study (GWAS) technology has become available. The GWAS technique is a powerful method of identifying susceptibility single nucleotide polymorphisms (SNP's) associated with disease or clinical outcomes. In order for a successful GWA study of HCV treatment outcomes, large case control cohorts are required to compare SVRs and non-responders, with DNA and matched clinical data. Effective research collaborations are therefore essential. The GWAS method utilises chip arrays with hundreds of thousands of SNP's distributed across the whole genome. Statistical analysis can determine whether there are significant differences in alleles, genes or haploytpes between cases and controls. The GWAS approach has the advantage of identifying many previously un-thought of and novel susceptibility genes. Another advantage of GWAS approach is identification of genes in a complex disease that have only modest effects on risk. GWA studies require large collaborative cohorts with DNA and well characterised clinical phenotypes. Detailed characterisation of HCV treatment outcomes,

demographics and clinical phenotypes with the establishment of a local DNA database enabled effective collaboration in a genome wide association study with the University of Sydney to investigate genetic determinants of anti-viral treatment outcome in patients with HCV G1 infection. The collaboration was a significant contribution to a landmark genetics study in HCV.

The third section of this chapter stems from the results of the lipid profiles and treatment outcome study and the GWAS to investigate the relationship between the innate interferon response and lipid profiles. Prospective analysis of interferon gamma inducible protein 10 (IP10), a serum marker of hepatic interferon stimulated gene expression in the prospective fasting HCV group was correlated against alterations in fasting lipid profiles. A mechanistic link between host genetics, interferon susceptibility and lipid profiles in the context of anti-viral treatment outcomes is proposed in the discussion.

5.2 Retrospective lipid profiles and treatment outcomes study

5.2.1 Demographics

129 HCV patients in the retrospective cohort had undergone anti-viral treatment with standard or pegylated interferon-α 2A or 2B and ribavirin and had documented outcomes more than 6 months after completion of therapy. Those that were HCV RNA PCR not detected > 6 months post treatment were considered sustained virological responders (SVR's). Those that were still HCV RNA detected were considered non-responders (NR). Relapsers, i.e. those that were HCV RNA not detected during or at the end of treatment but became HCV RNA positive again were included in the non-responders group for this binary analysis. In total there were 72 SVR's and 57 non responders. The summary demographics is shown in Table 5-1.

Table 5-1 Retrospective HCV cohort. Treatment outcomes and demographics

Parameter	**SVR**	**Non Responders**
N	72	57
Sex Male **Female**	63% 37%	65% 35%
Age years ± SD Male **Female**	42.05 ± 9.5 44.0 ± 11.7	48.7 ± 10.2 52.3 ± 11.9
HCV Genotype 1 N (%)	29 (47%)	33 (53%)
HCV Genotype 3 N (%)	32 (64%)	18 (36%)
Other HCV genotypes (2, 4, 5, 6 & unknown)	11 (65%)	6 (35%)
APOE*E3/E3	62.5%	64.9%
APOE*E3/E4	23.6%	29.8%
APOE*E2/E3	9.7%	3.5%
APOE*E2/E4	2.8%	0
APOE*E4/E4	1.4%	1.8%
APOE*E2/E2	0	0

5.2.2 Statistical analysis

The distribution of continuous data was assessed by normality tests. Age, total cholesterol and non-HDL cholesterol conformed to a normal distribution. Triglycerides and HDL cholesterol levels were positively skewed and therefore $\log_{10}$ transformed to normal distributions before parametric tests were applied. The F test was applied to test the assumption of equal variances and then paired t-tests were used to compare paired total cholesterol, $\log_{10}$ triglycerides, $\log_{10}$ HDL and non-HDL cholesterol levels pre and post treatment. A two-sample t-test was used to compare the same lipid parameters between SVR's and non responders.

Factors associated with achieving a SVR were assessed by a binary logistic regression model. The response was treatment outcome (SVR=1, NR =0). Continuous predictor factors in the model were total cholesterol, non HDL-cholesterol, $\log_{10}$ triglyceride, $\log_{10}$ HDL cholesterol and age. Categorical factors in the model were sex, HCV genotype and apoE genotype. All statistical analysis was performed in Minitab Version 15. Statistical significance was defined at the 5% level based on two-tailed test of the null hypothesis.

5.2.3 Results of logistic regression analysis

The primary aim was to examine whether pre-treatment non-fasting lipid levels were associated with treatment outcome. However, HCV genotype, age and sex are known to influence treatment outcome. HCV genotype and host apoE genotype are also known to influence lipid levels as indicated from results chapter 1. Therefore to control for these interactions and confounders, a binary logistic regression analysis in 88 patients was performed in whom complete data including apoE genotype was available. The results of this analysis are shown in table 5-2. The binary logistic regression analysis confirms the negative association of male sex (odds ratio 0.09, 95% CI 0.02-0.37, p=0.001) and increasing age (odds ratio 0.93, 95% CI 0.87-0.99, p= 0.021) with SVR. The important finding was an independent association between higher apoB associated cholesterol (i.e. non HDL cholesterol) and increased odds of achieving SVR (OR 2.14, 95% CI 1.19-3.83, p=0.011) and a negative association of TG/HDL ratio and likelihood of SVR (OR 0.56, 95% CI 0.32 - 0.95, p=0.033). There was no significant association of total cholesterol with SVR (OR 1.2, 95% CI 0.74-1.97, p= 0.459). Overall apoE genotype was not significantly associated with SVR. However patients with apoE2/E3 had an increased odds ratio of 4.93 of achieving SVR, but this was not statistically significant (95% CI 0.66-36.6, p=0.119) owing to the low frequency of this apoE genotype. Overall SVR rate in HCV genotype 1 was 45% and in genotype 3 was 63%. For those with wild type ApoE3/E3 the SVR rate was 44% for HCV genotype 1 and 68% for HCV genotype 3. Five patients with HCV genotype 3 were apoE2/E3 and all 100% (5/5) achieved an SVR compared to only 38% (5/13) in those with apoE3/E4 (Fishers Exact Test p = 0.0359).

Table 5-2 Results table binary logistic regression analysis

Variable	Odds Ratio (OR)	P value	95% Confidence interval of OR
Age	0.93	0.021*	0.87 - 0.99
Male (vs Female)	0.09	0.001*	0.02 – 0.37
HCV Genotype 3 (vs genotype 1)	3.05	0.025 *	1.15 – 8.05
APOE*E3/E4 (vs E3/E3)	0.72	0.611 (NS)	0.20 – 2.60
APOE*E2/E3 (vs E3/E3)	4.93	0.119 (NS)	0.66 – 36.61
Non HDL Cholesterol	2.14	0.011*	1.19 – 3.83
TG/HDL Ratio	0.56	0.033*	0.32 – 0.95
Total Cholesterol	1.2	0.459 (NS)	0.74 – 1.97

N=88 Multivariate binary logistic regression analysis for factors associated with treatment outcome. Results are the Odds Ratio of the baseline event of a sustained virological response (SVR).

The association of elevated non-HDL cholesterol with increased likelihood of SVR and high triglyceride / HDL ratio with non-response to anti-viral therapy is intriguing. One possible explanation is that host genetic polymorphisms associated with increased lipid levels are also important independent determinants of anti-viral treatment outcome. Although ApoE genotype was not independently associated with treatment outcome there was a tendency that those with ApoE2/E3 genotype had improved outcomes. The alternative hypothesis is that lipid profiles represent a surrogate marker of some other factor that determines outcome, such as activation of interferon stimulated genes (ISG's). Although a hypothesis that lipid modulating genes were important determinants of anti-viral treatment outcome has been made, collaboration with the University of Sydney in a GWA study permitted a more powerful non-hypothesis driven approach to investigate SNP's associated with anti-viral treatment response.

5.3 Genome wide association study of anti-viral treatment outcomes - Collaboration with University of Sydney

Collection of DNA and detailed clinical phenotyping of HCV patients that had undergone anti-viral therapy permitted a collaborative GWA study with the University of Sydney. The GWA study took a two-stage approach, a discovery phase and a replication phase. The University of Sydney performed a discovery genome wide association (GWA) study on 293 HCV G1 patients of northern European ancestry to identify candidate single nucleotide polymorphisms (SNP's) associated with treatment outcome. The Newcastle cohort contributed to the independent replication phase. DNA and clinical phenotypes data was collected for the Newcastle HCV cohort for 89 HCV G1 patients in whom anti-viral treatment outcomes were known. Additional replication cohorts were recruited in collaboration with groups from Germany and Italy. The demographics of the Australian GWAS discovery cohort, and the independent replication cohorts including the Newcastle cohort of 89 patients that made up GWA study replication cohort are summarised in in tables 5-3 and 5-4. It is noteworthy that the replication cohorts consistently had a higher proportion of females in the responder groups than non-responder groups.

Table 5-3 Demographic characteristics of the GWAS Australian discovery cohort and combined replication cohorts in HCV G1 (Suppiah, Moldovan et al. 2009).

	GWAS Australian cohort 1 (*n* = 293)		**Combined replication phase (*n* = 555)**	
Study	**Responders (131)**	**Non-Responders (261)**	**Responders (294)**	**Non-responders (162)**
Age (years)	40.9 (10.0)	40.8 (11.2)	46.5 (10.3)	43.9 (7.0)
Females	51 (38.9)	129 (49.4)	109 (37.1)	35 (21.6)
Males	80 (61.1)	132 (50.6)	185 (62.9)	127 (78.4)
BMI	26.9 (5.0)	24.9 (4.5)	25.9 (4.6)	27.5 (5.1)
Viral load (IU/ml)	NS		NS	

Table 5-4 Demographics of replication cohorts including the Newcastle cohort (UK) for the genome wide association study (Suppiah, Moldovan et al. 2009)

Replication phase										
	Berlin cohort (*n* = 298)		**Turin cohort (*n* = 93)**		**UK cohort (*n* = 89)**		**Bonn cohort (*n* = 43)**		**Australian cohort 2 (*n* = 32)**	
	Responders (143)	**NR (155)**	**Responders (50)**	**NR (43)**	**Responders (42)**	**NR (47)**	**Responders (13)**	**NR (30)**	**Responders (13)**	**NR(19)**
Age (years)	41.3 (10.4)	46.9 (10.1)	43.1 (13.0)	44.3 (10.1)	37.2 (11.4)	45.2 (11.9)	39.2 (12.8)	50.8 (10.9)	34.8 (9.9)	50.8 (4.9)
Females	76 (53.1)	67 (43.2)	27 (54.0)	15 (34.9)	14 (33.3)	11 (23.4)	6 (46.2)	10 (33.3)	6 (46.2)	6 (31.6)
Males	67 (46.9)	88 (56.8)	23 (46.0)	28 (65.1)	28 (66.7)	36 (76.6)	7 (53.8)	20 (66.7)	7 (53.8)	13 (68.4)
BMI	25.2 (4.5)	25.9 (3.9)	24.1 (3.4)	24.6 (3.5)	24.3 (5.8)	26.5 (6.4)	24.3 (3.5)	27.3 (4.6)	26.7 (5.3)	25.7 (6.3)

Table 5-5 The most associated SNPs identified in the GWAS and replication phases of the GWA study ((Suppiah, Moldovan et al. 2009)

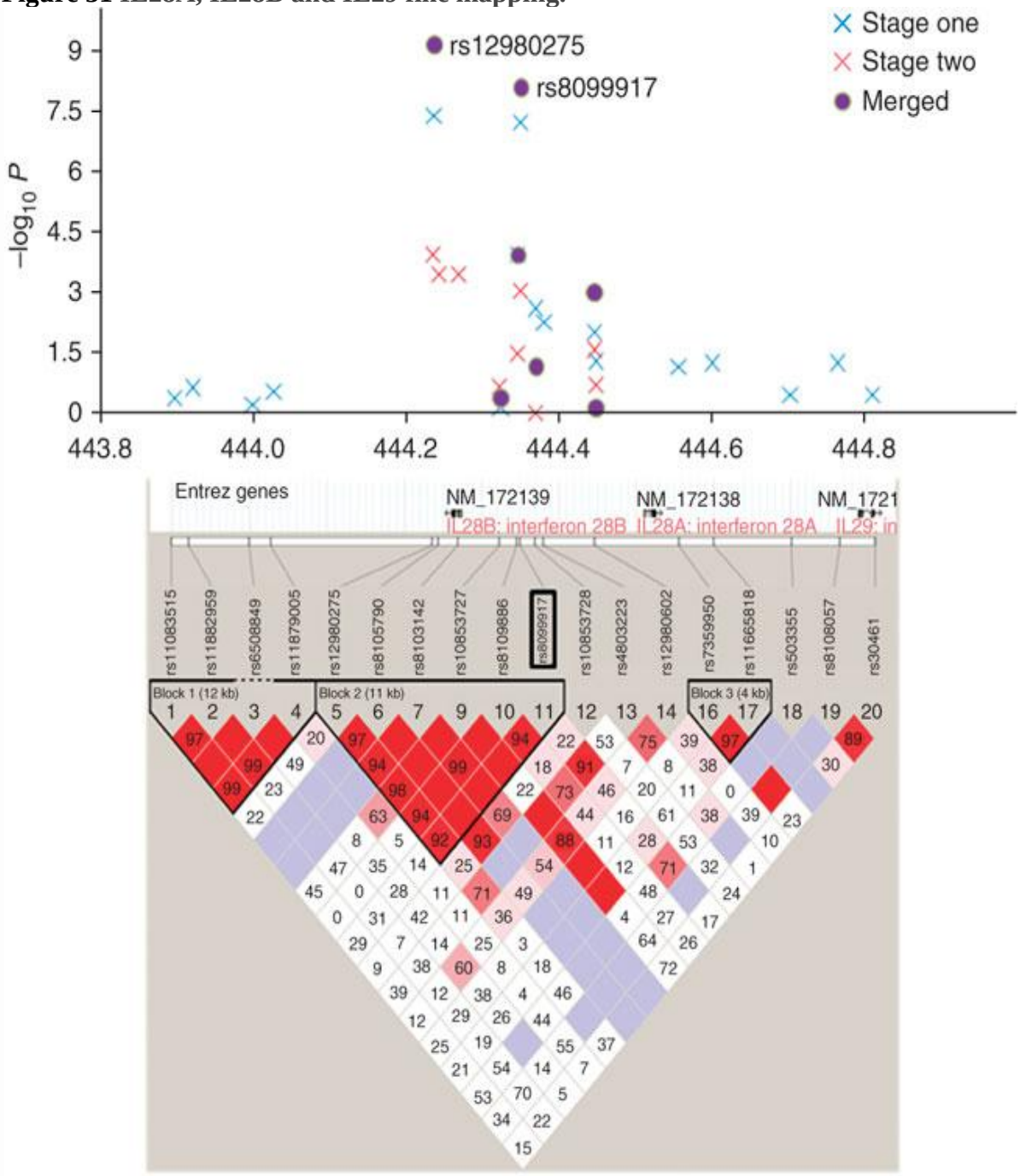
SNP	Location[a]	MAF (total)	Responder allele	Nonresponder allele	GWAS P value[b]	Replication P value[b]	Merged P value[b]	OR (95% CI)[c]	Gene[d]
rs8099917	Chr:19 44435005	0.268	T	G	7.06×10^{-8}	9.39×10^{-4}	9.25×10^{-9}	1.98 (1.57–2.52)	*IL28B*
rs6806020	Chr:3 54949198	0.303	T	C	3.81×10^{-5}	3.43×10^{-2}	3.98×10^{-5}	1.54 (1.25–1.90)	*CACNA2D3*
rs1931704	Chr:10 129229799	0.272	G	A	4.42×10^{-7}	2.76×10^{-1}	8.62×10^{-5}	1.54 (1.24–1.92)	*NPS*
rs7750468	Chr:6 118183677	0.080	A	G	1.48×10^{-5}	1.01×10^{-1}	8.91×10^{-5}	2.09 (1.44–3.05)	
rs1503391	Chr:11 104417668	0.158	C	A	4.07×10^{-3}	9.22×10^{-3}	1.53×10^{-4}	1.68 (1.28-2.20)	*CARD16*
rs2066911	Chr:6 23656329	0.261	C	T	9.03×10^{-6}	1.86×10^{-1}	2.58×10^{-4}	1.52 (1.21–1.91)	
rs557905	Chr:11 104403053	0.158	G	A	5.68×10^{-3}	1.28×10^{-2}	2.87×10^{-4}	1.64 (1.25–2.15)	*CASP1*
rs568910	Chr:11 104409780	0.158	G	T	5.68×10^{-3}	1.83×10^{-2}	4.30×10^{-4}	1.61 (1.23–2.11)	*CASP1*
rs3093390	Chr:16 27370949	0.227	T	C	1.84×10^{-3}	3.83×10^{-2}	4.47×10^{-4}	1.49 (1.19–1.87)	*IL21R*
rs7512595	Chr:1 27729758	0.093	G	A	2.72×10^{-4}	7.67×10^{-2}	5.34×10^{-4}	1.81 (1.29–2.55)	*WASF2* and *AHDC1*
rs1792774	Chr:11 104417763	0.103	T	C	1.39×10^{-3}	6.08×10^{-2}	6.71×10^{-4}	1.74 (1.26–2.41)	*CARD16*
rs17461620	Chr:9 88152828	0.180	T	C	1.17×10^{-3}	7.90×10^{-2}	9.47×10^{-4}	1.53 (1.19–1.97)	*ZCCHC6*

[a]From the Hapmap project data release 27. [b]*P* values based on Cochrane-Armitage trend test. [c]OR and 95% CI are calculated using logistic regression based on co-dominant model for inheritance. [d]Genes were within ±50 kb of the associated SNP. No gene listed indicates that no genes were within 50 kb of the SNP.

In the first stage of the GWA study, the University of Sydney analysed 293 samples from the Australian cohort (131 responders and 162 non responders) using an Illumina infinium HumanHap300 or the CNV370 Quad genotyping BeadChip with data on 311,159 SNP's. The threshold of genome wide significance was set at $\leq 1.6 \times 10^{-7}$. SNPs with p-values between 1.6×10^{-7} and 1.0×10^{-5} were considered to show a highly suggestive association. 172 candidate SNP's from the first stage were taken into the second replication stage. Of these, 14 SNPs achieved the threshold suggestive of an association with treatment outcome (table 5-5). The most associated SNP was rs8099917, located on chromosome 19. This SNP is upstream of the IL28B gene. rs8099917 was associated with a combined odds ratio for non-response of 1.98 (95% CI 1.57-2.52) $p = 9.25 \times 10^{-9}$.

This association was further refined by genotyping 20 tag SNP's in the IL28A, IL28B, IL29 region. Fine mapping identified rs8099917 to tag a six allele haplotype block comprising of rs12980275 ($p=7.74n \times 10^{-10}$), rs8105790, rs8103142, rs10853727, rs8109886 and rs8099917 (GCCTAG) (figure 31).

Figure 31 IL28A, IL28B and IL29 fine mapping.



(Figure from (Suppiah, Moldovan et al. 2009) showing fine mapping of the IL28A, IL28B and IL29 genes in the discovery (stage 1) and replication (stage 2) cohorts. The Newcastle HCV cohort contributed 89 patients to the replication (stage 2) group. -log$_{10}$P values are shown for all markers genotyped in stage 1 (blue crosses), fine mapping for all samples in stage 2 (red crosses) and merged P values (purple circles). Below is the haplotype block structure, shown for 18 genotyped SNPs in the IL28A, IL28B and IL29 gene regions. The block structure is based on criteria established previously (Gabriel, Schaffner et al. 2002) with the use of pairwise estimates of standardized Lewontin's disequilibrium coefficient (D').Regions with high D' values are dark red, and regions with low D' values are lighter shades of red.

The frequencies of the non-response haplotype in the Newcastle cohort is shown in table 5-6. It can be seen that the non-response haplotype is relatively infrequent in this cohort of 89 HCV G1 patients. This emphasises the importance of large collaborative studies that are required to reach adequate statistcal power for meaningful results of genetic associations.

Table 5-6 Frequency of the six allele non-response haplotype rs12980275, rs8105790, rs8103142, rs10853727, rs8109886 and rs8099917 (GCCTAG) in SVR's and non-responders in the Newcastle cohort

Haplotype **GCCTAG**	**SVR N(%)**	**Non-responder** **N(%)**	**p-value**
Yes	5 (15%)	4 (10%)	Chi square =0.341, P = 0.559
No	28 (85%)	34 (90%)	

5.4 Lipid profiles and IL28B polymorphisms

Given the association of low non-HDL-C and non response, and IL28B genotype and non-response, an intriguing question arises whether these two new findings are connected. Total cholesterol levels were only available in 65 Newcastle patients who had IL28B genotypes performed from the GWA study. Full lipid profiles including HDL cholesterol and triglycerides were only available in 57 of these patients overall. Lipid profile data was not available for the other cohorts in the GWAS. The small numbers therefore limits meaningful analysis of IL28B genotype and lipid profiles. Of those patients with lipid profiles available, there was a non significant trend towards higher total and non-HDL cholesterol and lower TG/HDL ratio in SVR's compared to non-responders (Table 5-7), supporting the findings of the larger logistic regression analysis but this has to be investigated further in larger numbers.

Table 5-7 Lipid profiles in SVR's and non-responders in HCV genotype 1 - the Newcastle genetics study replication cohort (mean ± standard deviation)

	SVR	**Non responders**	**P value**
Total Cholesterol mmol/l	N=27 4.68 ± 0.94	N=37 4.46 ± 0.87	0.324
Non HDL Cholesterol mmol/l	N=24 3.34 ± 0.90	N=35 3.05 ± 0.93	0.239
TG/HDL ratio	N=24 1.17 ± 0.64	N=35 1.49 ± 0.93	0.568

Lipid profiles were compared in those with and without the IL28B non-response haplotype (rs12980275, rs8105790, rs8103142, rs10853727, rs8109886 and rs8099917 (GCCTAG). No statistically significant differences in lipid profiles were found between those with and without the non-response haplotype because the numbers of patients with the non-response haplotype were too small (N=7). However there was a tendency for lower total cholesterol levels in those with the non-response haplotype (4.2 vs 4.6 mmol/l, p=0.226) and higher Triglyceride / HDL ratios (1.46 vs 1.06, p=0.075). There was no significant differences in non-HDL cholesterol when patients were compared according to IL28B haplotype (3.19 vs 3.28 mmol/l p = 0.798). Although not conclusive because of small numbers, these data suggest that there may be an association between the non-response IL28B haplotype with lower

cholesterol and higher triglycerides which will need to be investigated further in larger numbers.

5.5 IP10

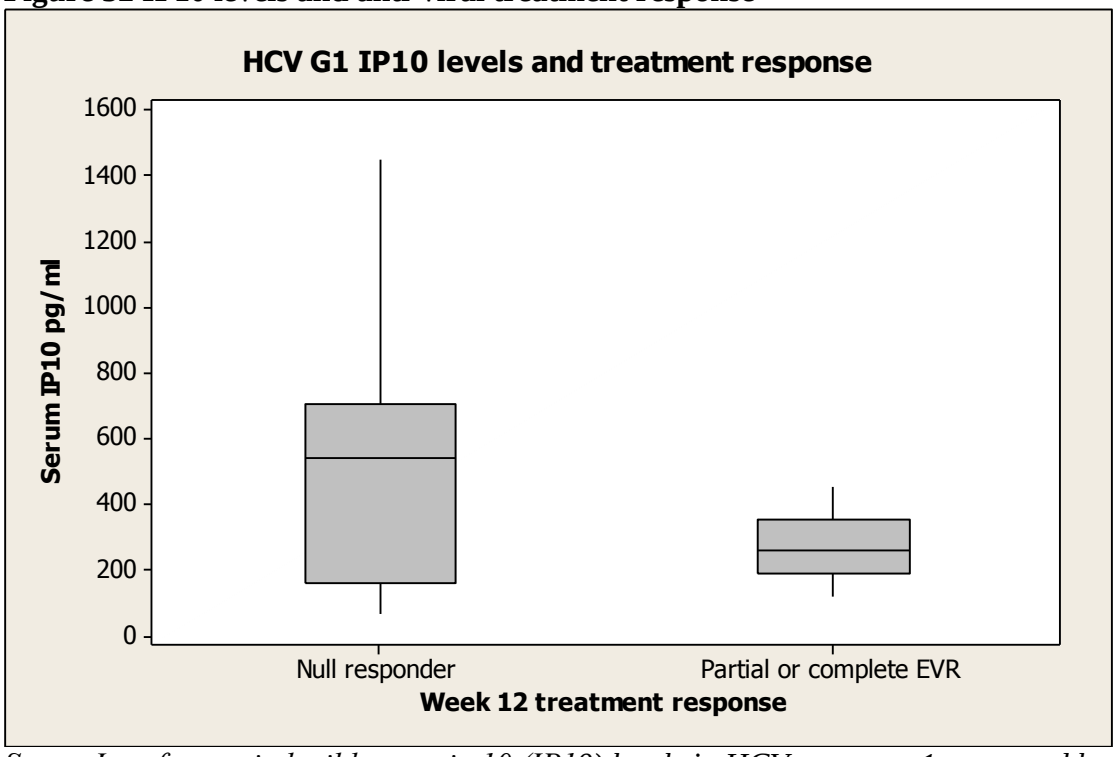
The alternative hypothesis to host genetic polymorphisms causing the association between lipid profiles and treatment outcome is that lipid profiles represent a surrogate marker of some other factor that determines outcome, such as activation of interferon stimulated genes (ISG's).

Interferon-gamma-inducible protein 10kDa (IP10) is a serum marker for interferon stimulated gene (ISG) expression (Askarieh, Alsio et al. 2010). Serum IP10 levels were measured using a commercially available ELISA (see methods) on fasting serum from the prospective fasting HCV cohort. IP10 levels were correlated with lipid parameters using Spearmans rank correlation analysis in 51 HCV G1 and 24 HCV G3 patients.

5.5.1 IP10 and anti-viral treatment outcomes

Previously published literature has shown high IP10 levels are associated with non-response to anti-viral therapy (Butera, Marukian et al. 2005; Diago, Castellano et al. 2006; Lagging, Romero et al. 2006). Small numbers limited analysis of IP10 and treatment outcomes in the prospective HCV study cohort which included both treatment naïve patients about to start anti-viral treatment and previous non-responders. Since SVR is defined as undectable HCV RNA >6 months post completion of 48 weeks of anti-viral treatment, full data on SVR was unavailable prospectively. Instead treatment response after 12 weeks of therapy was used to define those that had at least a 2 log reduction in HCV RNA (partial or complete early virological response (EVR)) and those that did not (null responders). The null responders were largely retrospective (i.e. had previously failed treatment before IP10 levels were measured) whereas the EVRs were prospectively treated after the IP10 was measured. In HCV G1, serum IP10 levels tended to be higher in null responders (NR) compared to EVR, but this was at borderline statistical significance (NR N=22, median IP10 542 pg/ml vs EVR, N=25, median IP10 262 pg/ml; p=0.172) (Figure 32), but supportive of the published literature. In HCV G3, numbers of non-responders were too small for meaningful analysis, but no large differences in IP10 levels between null responders and complete or partial EVR's were detected (NR N=5, median IP10 320 pg/ml vs EVR N=19, median IP10 327 pg/ml; p=0.804).

Figure 32 IP10 levels and anti-viral treatment response



Serum Interferon γ inducible protein 10 (IP10) levels in HCV genotype 1 measured by ELISA tended to be higher in those that failed to achieve ≥2 log10 reduction in total HCV RNA viral load at 12 weeks of treatment with PegIFN and ribavirin (null responders) than those that had ≥2 log10 reduction (partial and complete early virological response) but did not reach statistical significance (NR N=22, median IP10 542 pg/ml vs EVR, N=25, median IP10 262 pg/ml; p=0.172).

5.5.2 IP10 levels and lipid profiles

Serum IP10 levels were correlated with fasting lipid parameters. The results are summarized in Table 5-8.

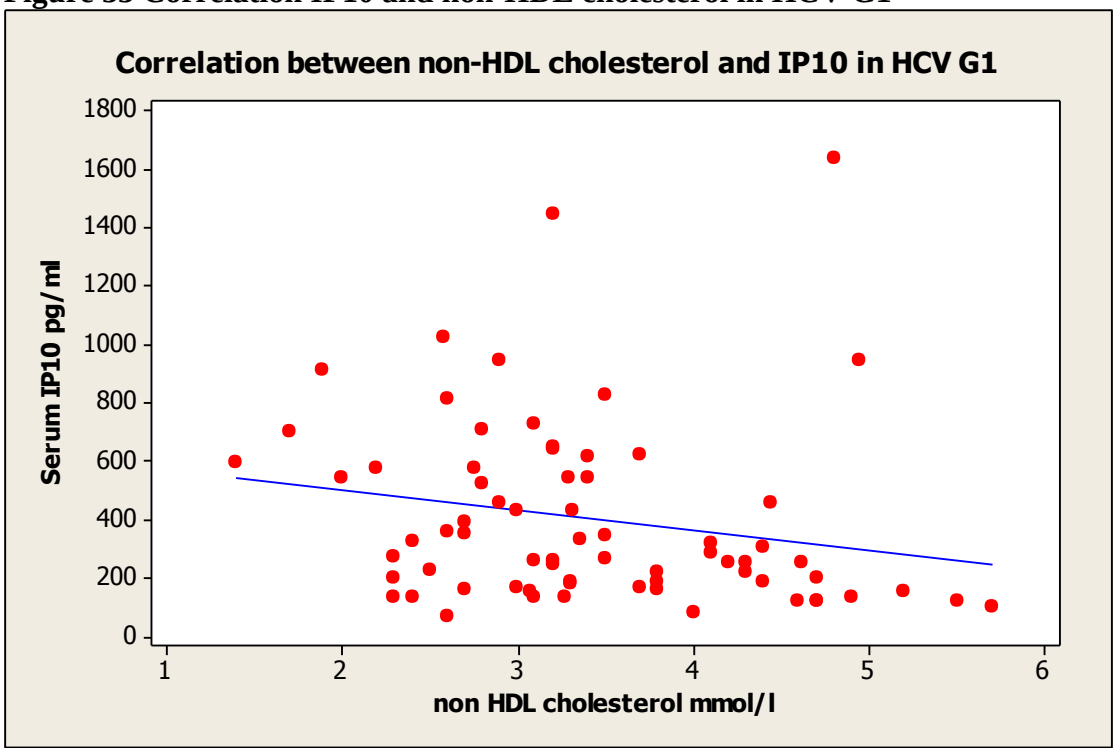
Table 5-8 IP10 metabolic and lipid correlations in HCV G1 and HCV G3

	HCV G1 N=51	**HCV G3 N=24**
Non-HDL cholesterol	**r = -0.319** **p = 0.007**	r = 0.102 p = 0.546
ApoB	**r = -0.313** **p = 0.017**	r = 0.132 p=0.519
Lathosterol	**r = -0.388** **p = 0.002**	r = 0.024 p = 0.906
Lathosterol/total cholesterol ratio	**r = 0.261** **p = 0.068**	r = 0.115 p = 0.577
PCSK9	**r = 0.400** **p = 0.006**	r = 0.006 p = 0.983
Triglycerides	r = -0.050 p= 0.730	r = 0.275 p = 0.175
Triglyceride / HDL ratio	r = 0.012 p = 0.936	r = 0.285 p = 0.159
HOMA IR	r= 0.096 p= 0.509	**r = 0.508** **p = 0.008**

Spearman's rank correlation analysis

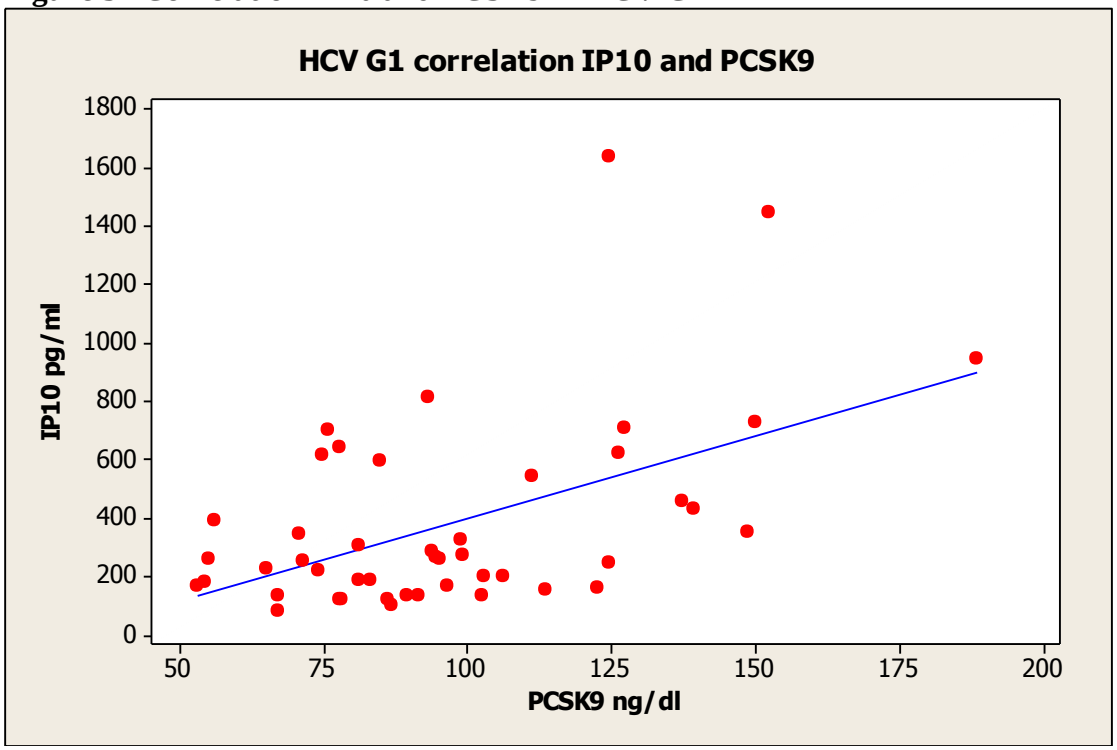
In HCV G1, there was a significant inverse correlation between serum IP 10 levels and non-HDL cholesterol (r = -0.319, p = 0.007) (figure 33) and apoB (r = -0.313, p=0.017). There was also a significant correlation between IP10 and absolute lathosterol (r = -0.388, p = 0.002). IP10 correlated less strongly with relative lathosterol / total cholesterol ratio however (r=-0.261, p=0.068). There was also a strong correlation between IP10 and PCSK9 in HCV G1 (r = 0.400, p=0.006) (Figure 34).

Figure 33 Correlation IP10 and non-HDL cholesterol in HCV G1



In HCV G1 there was a significant inverse correlation between serum IP10 levels and fasting non-HDL cholesterol (Spearmans Rank r=-0.319, p=0.007)

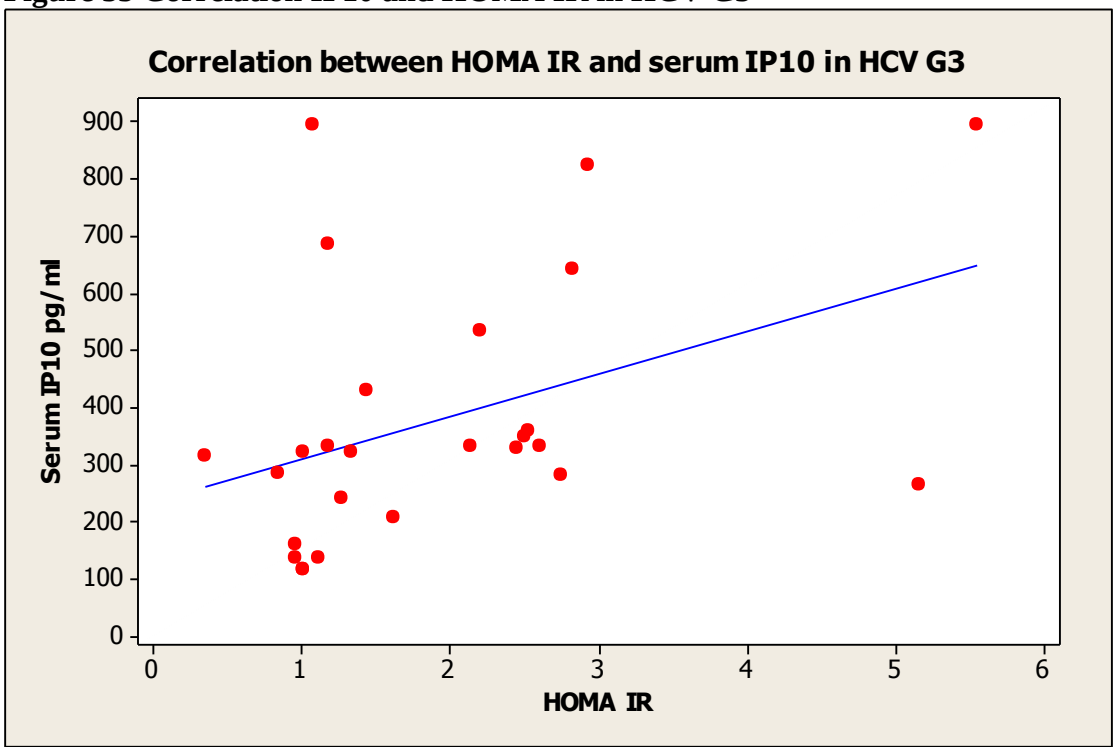
Figure 34 Correlation IP10 and PCSK9 in HCV G1



In HCV G1 there was a significant inverse correlation between serum IP10 and PCSK9 (Spearmans Rank r=0.400, p=0.006)

In HCV G3 infection, serum IP10 levels did not correlate with apoB and non-HDL cholesterol but did correlate strongly with HOMA IR (r = 0.508, p = 0.008) figure 35.

Figure 35 Correlation IP10 and HOMA IR in HCV G3



In HCV G3 there was a significant correlation between IP10 and insulin resistance as estimated by the Homeostasis Model Assessment (HOMA IR) (fasting glucose x insulin /22.5) (Spearmans Rank r=0.508, p=0.008).

This suggests that in individuals with high IP10 levels in HCV G1, there is a combination of low endogenous cholesterol synthesis and high PCSK9 which is likely to result in slower LDLr mediated clearance. In HCV G3 high IP10 levels are associated with insulin resistance.

5.6 Discussion

5.6.1 Lipid profiles influence anti-viral treatment outcome

This study shows that high non-HDL (i.e. apoB associated) cholesterol is positively associated with increased odds of achieving a SVR (OR = 2.14, P< 0.05). However because of incomplete retrospective data, the relative importance of non-HDL cholesterol to other factors known to influence SVR such as insulin resistance (Romero-Gomez, Viloria et al. 2005; Poustchi, Negro et al. 2008) and advanced fibrosis (Everson, Hoefs et al. 2006; Asselah, Estrabaud et al. 2010) was unable to be determined.

This was the first HCV treatment outcomes study to include apoE genotype as a covariate factor in the logistic regression analysis, which is important given that apoE genotype is the most common genetic variant known to influence lipid profiles. Prior to publication of this study (Sheridan, Price et al. 2009), four other retrospective studies had shown similar observations that high LDL cholesterol is associated with improved SVR in HCV mono-infected patients (Gopal, Johnson et al. 2006; Akuta, Suzuki et al. 2007; Backus, Boothroyd et al. 2007; Economou, Milionis et al. 2008). However these previous studies had not considering the potential confounding effects of apoE genotype, nor considered that LDL cholesterol as estimated by the Friedwald equation may not be valid in chronic HCV. A study in HIV-HCV co-infected patients has also shown independent association with LDL-cholesterol greater that 100mg/dl and SVR (OR 2.51) (del Valle, Mira et al. 2008). One large study of US veterans showed that low cholesterol was a significant predictor of decreased likelihood of an SVR in HCV genotype 1 patients, but this was not found in genotype 2 or 3 (Backus, Boothroyd et al. 2007). More recently since this work was published, data from two large prospective randomised controlled trials has now confirmed the association of high LDL cholesterol and improved SVR rates in HCV G1 infection (Harrison, Rossaro et al. 2010; Ramcharran, Wahed et al. 2010). The published studies finding an association of high LDL / non-HDL cholesterol and SVR are summarised in chronological order in table 5-9.

Table 5-9 Summary of published studies indicating an association of high LDL cholesterol in chronic HCV with sustained virological response

Author	N=	HCV Genotypes	Prospective or retrospective	Country	Association with SVR	p-value
(Gopal, Johnson et al. 2006)	99	49G1, 50G2/3	retrospective	USA	LDLC >130mg/dl OR 2.598	0.0092
(Backus, Boothroyd et al. 2007)	5944	G1, G2/3	retrospective	USA	Total cholesterol <130mg/dl OR 0.73	0.009
(Akuta, Suzuki et al. 2007)	114	G1	prospective	Japan	LDLC > 86mg/dl OR 12.8	0.005
(Economou, Milionis et al. 2008)	109	G1,2,3,4	retrospective	Greece	Total cholesterol OR 2.84 apoB OR 1.62	<0.001
(del Valle, Mira et al. 2008)	260	HIV co-infected G1,2,3,4	retrospective	Spain	LDL >100 OR 2.51	0.003
(Sheridan, Price et al. 2009)	88	G1 & 3	retrospective	UK	Non-HDLC OR 2.09	0.042
(Angelico, Francioso et al. 2009)	65	37 G1, 28 G2/3	prospective	Italy	Total cholesterol >165mg/dl OR of RVR 62.3	0.003
(Mawatari, Yoneda et al. 2010)	44	G1b	prospective	Japan	Higher cholesterol in VLDL >44.5 nm in SVR's vs NR	0.019
(Harrison, Rossaro et al. 2010)	1464	G1	prospective	USA	LDLC OR 1.6	<0.001
(Ramcharran, Wahed et al. 2010)	330	G1	prospective	USA	LDL RR of SVR 1.04	0.001

5.6.2 Host genetics are important determinants of anti-viral treatment outcome

Evidence is now clearly indicating that high LDL / non-HDL cholesterol in CHC is a factor independently associated with improved response to treatment with pegIFN and ribavirin. However a mechanistic explanation of this association has hitherto not been forthcoming. A hypothesis for the observation that high LDL / non-HDL cholesterol improves anti-viral treatment response is that common host genetic variants known to affect lipid profiles, such as apoE genotype also directly affect interferon responsiveness. Serum lipoprotein levels of the majority of individuals in the population are known to be influenced by several genes with common variants which influence levels of cholesterol and triglycerides around the median levels of the population. Genes with common variants have been identified from previous classical candidate gene studies and more recently genome wide association (GWA) studies (Kathiresan, Melander et al. 2008; Kooner, Chambers et al. 2008). Common variants that influence LDL cholesterol include ApoE, LDLr, PCSK9, ApoB, and HMGCR. Recently a large GWAS in >100,000 individuals found 95 loci with genome wide associations with variation in plasma lipids, including several previously uncharacterised SNP's that demand further investigation (Teslovich, Musunuru et al. 2010). Variants that influence HDL cholesterol include CETP and ABCA1and LPL. Triglycerides are influenced by LPL and apoA5 amongst others. Rare polymorphisms with a prevalence < 1 in 10^5 in the population affect the extreme ends of lipid distributions and often cause monogenic syndromes such as Familial Hypercholesterolaemia (Hegele 2009). The retrospective study was underpowered to detect significant differences in treatment response rates between ApoE genotypes given the low prevalence of apoE2/E3 in this study population. However, there was a trend for apoE2/E3 to be associated with improved interferon response which is intriguing. A previous study had shown that apoE2 allele was more prevalent in spontaneous resolvers and rarer in those with chronic infection than was observed in a control population, raising the hypothesis that the E2 allele may protect against viral persistence (Price, Bassendine et al. 2006). One possibility for this may be via defective binding of HCV lipoviral particles containing apoE2 to lipoprotein receptors

such as LDLr and SRB1 (Price, Bassendine et al. 2006; Catanese, Graziani et al. 2007; Van Eck, Hoekstra et al. 2008; Sheridan, Price et al. 2009).

Previous candidate gene studies of treatment outcomes in CHC have tended to be in small cohorts and have generated largely unreproducable findings, mainly focusing on candidate genes involved in pathways related to interferon signaling and immunomodulation (Lin, Hwang et al. 2006; Huang, Yang et al. 2007). Other approaches have studied gene expression in liver biopsies from responders and non-responders (Hwang, Chen et al. 2006). These approaches have now been superseded by non-hypothesis driven genome wide association study (GWAS) methodology which has been extensively reviewed (McCarthy, Abecasis et al. 2008). The GWAS method requires recruitment of suitable discovery and replication cohorts. Obtaining cohorts of suffiently large numbers of samples with DNA and clinical phenotypes required for a GWAS is rarely possible from a single centre, hence collaborations are essential (Karlsen, Melum et al. 2010). Careful collection of DNA coupled with clinical phenotyping of the retrospective HCV cohort permitted the use of this cohort for the replication phase of a GWA study in collaboration with the University of Sydney. This collaboration has been an important contribution to a seminal discovery of IL28B genotype influencing interferon response in genotype 1 CHC infection (Suppiah, Moldovan et al. 2009). Having established discovery and replication cohorts, the next step of the GWAS is to select and use a genotyping array that has adequate coverage of the genome. The international HapMap project characterised genetic variation in 3.1 million single nucleotide polymorphisms (SNPs) across the human genome (http://hapmap.ncbi.nlm.nih.gov). The theortical paradigm of a GWAS is linkage disequilibrium. This is where two alleles of single nucleotide polymorphisms (SNP's) on the same chromosome occur more frequently together than would be expected from their relative frequencies, because they are close together in a genetic region. If two SNPs are always found together there is perfect linkage disequilibrium (r^2 =1). There are ~2.8 million SNP's with minor allele frequency >5%. A proportion, (50-80%) of these SNPs have been captured in commercially available genotyping arrays and act as tag SNP's. The more SNPs covered by the array, the closer the coverage is to perfect linkage disequilibrium (r^2=1). In reality, r^2 thresholds of >0.8 are used. The Illumina HumanHap300 bead chip used for the Australian GWAS contains 317,000 tag SNP's within 10kb of genes

and has a threshold r^2 of 0.8. The third step of the GWAS is a quality control exercise, excluding mismatches, duplicates, ethnic outliers, or failed genotyping. Statistical analysis needs to be performed by groups with experience and expertise in the field. Because the analysis involves multiple tests, many associations can arise by chance. Therefore the threshold of genome wide significance is defined by the Bonferroni correction as 0.05 divided by the number of SNPs analysed. Typical genome wide significant p-values are therefore ususally $<10^{-6}$ to 10^{-8}. Replication of any associations defined in the discovery cohort is required to validate that the findings are not spurious associations. In the replication cohort, further genotyping can be performed to home-in on the causal variant.

The present GWA study identified that the most significant polymorphisms associated with anti-viral treatment outcome are in immune regulatory genes, in particular IL28B on chromosome 19, which codes for interferon lambda (IFNλ), a type III interferon. HCV has co-evolved with the human host over hundreds or thousands of years. During this time both the virus and host have developed adaptive mutations conferring either susceptibility or increasing resistance to the host innate immune response. The SNP rs8099917 in the IL28B promoter showed the strongest genome wide significance in the initial GWA study ($p=7.06 \times 10^{-8}$, OR 3.36, 95% CI = 2.15-5.35) for non-response to anti-viral therapy. This SNP is 8.9kb upstream of IL28B and 16kb from the end of IL28A. The Newcastle cohort participated as part of the replication phase, and rs8099917 remained the most significant association in the replication cohort ($p=9.39 \times 10^{-4}$, OR 1.59, 95% CI 1.21-2.11). In the combined overall analysis the G allele was associated with non-response; GG homozygotes OR's for non-response were 2.39 (95% CI 1.16-4.94) and GT heterozygotes OR 1.64, (95% CI 1.15-2.32) compared to non carriers (TT). For carriers of the non-response (GG) allele, the positive predictive value of failure to respond was 64%. Multivariate analysis in the overall group in the GWA study demonstrated that rs8099917 is an independent predictive factor, not confounded by viral load, age, gender or stage of fibrosis. The haplotype that distinguishes responders from non responders is tagged by rs8099917. The non-responder haplotype is carried by 27% of northern Europeans. 69% of homozygotes (GG) and 64% of heterozygote (GT) carriers fail to clear HCV with PEG IFNα and ribavirin therapy. The rs8099917 SNP appears to be functional because healthy individuals with rs8099917 TT have higher IL28A and IL28B

mRNA expression in peripheral blood than those with GT or GG (Suppiah, Moldovan et al. 2009).

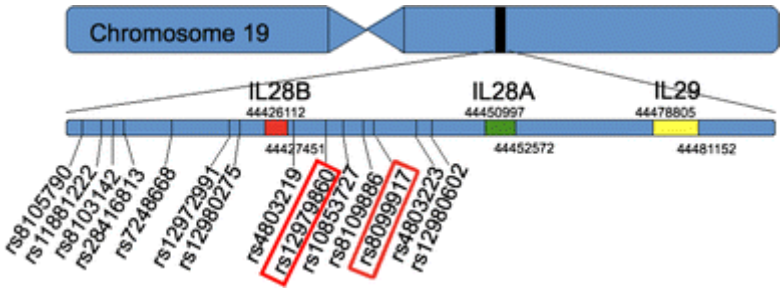
This GWAS was one of three landmark papers independently finding that IL28B SNP's were the predominant genetic determinant of anti-viral treatment outcome in HCV genotype 1. Two other independent GWA studies have independently replicated the findings (Ge, Fellay et al. 2009; Tanaka, Nishida et al. 2009). The first was a GWA study of 1600 individuals in the IDEAL trial which compared three different treatment regimens using PegIFN-α-2b (1.5 or 1 mcg / kg) and PegIFN-α-2a combined with ribavirin (Ge, Fellay et al. 2009). This trial demonstrated significantly lower treatment efficacy in African Americans than Americans of European ancestry. A different polymorphism in IL28B, rs12979860, was strongly associated with SVR with genome wide significance ($p=1.37 \times 10^{-28}$). The rs12979860 SNP is 3 kilobases upstream of the IL28B gene. The CC genotype was associated with twofold greater SVR than the TT genotype in Europeans (95% CI 1.8 – 2.3), threefold greater SVR in African Americans (95% CI 1.9-4.7) and twofold greater SVR in Hispanics (95%CI 1.4 – 3.2). This SNP exerts greater influence over likelihood of SVR than other previously known clinical factors such as high baseline viral load, advanced fibrosis or African ethnicity. African Americans with the favourable CC genotype had a 55.3% SVR, and did better than Europeans with the adverse TT genotype (33.3% SVR). The same group also genotyped the rs12979860 variant in 388 patients who had spontaneously resolved HCV and compared this to 620 patients with persistent infection (Thomas, Thio et al. 2009). They found that the CC genotype was also strongly associated with spontaneous resolution both among those of European and African ancestry. In those of European ancestry the C allele was present in 80.3% of those that spontaneously cleared HCV, compared to 66.7% of those with persistent infection. In those of African ancestry, the C allele was present in 56.2% of spontaneous resolvers compared to 37% of those with persistent infection ($p=1 \times 10^{-5}$). CC homozygotes were three times more likely to clear HCV spontaneously than CT or TT. However there was no significant difference in clearance between CT and TT genotypes suggesting that the protective effect of the C allele is recessive. Although in the US study, a different IL28B SNP (rs12979860) was the most significantly associated with SVR (combined $p = 1.38 \times 10^{-28}$), two other IL28B SNPs identified in the Australian GWA study were also replicated in the US cohort;

rs12980275 was second most significant (combined $p = 2.54 \times 10^{-27}$), and rs8099917 was third most significant ($p = 3.70 \times 10^{-26}$) (Ge, Fellay et al. 2009). An important observation is the population distribution of the favourable C allele of the rs12979860 SNP which is more frequent in Caucasian and Asian populations than in Africans. 90% of Chinese and Japanese carry this variant compared to only 30-50% of sub-Saharan Africans. This may help explain why African Americans respond less well to anti-viral therapy than Caucasians and Asians (Conjeevaram, Fried et al. 2006).

The other independent GWA study was from Japan. In the initial GWA scan, a chip array of ~900,000 SNP's in 82 non responders and 72 SVR's confirmed rs8099917 and rs12980275 as the most important SNP's associated with non-response (Tanaka, Nishida et al. 2009). Subsequently another independent GWA study from Switzerland of 1362 patients including 347 spontaneous resolvers and 1015 with persistent infection (Rauch, Kutalik et al. 2010) again found the strongest association was with rs8099917 (OR 2.31, 95% CI 1.74-3.06, $p=6.07 \times 10^{-9}$) and HCV persistence. It also remained significant in those with HIV / HCV co-infection. The frequency of rs8099917 TT was more common in spontaneous resolvers (78% vs 58%). Again male sex was independently but more weakly associated with HCV persistence. There was no significant direct association of rs8099917 with HCV RNA levels (OR 1.10, 95% CI 0.81-1.25, p=0.94). When SNP's associated with treatment outcome were assessed, rs8099917 minor G allele was associated with treatment failure (OR =5.19, 95% CI=2.90-9.30, $p=3.11 \times 10^{-8}$) in patients with HCV genotypes 1 & 4 only. The frequencies of TT, GT and GG in non-responders were 0.42, 0.51 and 0.07 compared to 0.68, 0.29 and 0.03 in SVR's. No significant variation in IL28B and response to therapy was found in those with HCV genotypes 2 and 3.

It should be noted that rs12979860 identified in the American study (Ge, Fellay et al. 2009) is ~3kb upstream of IL28B and is in linkage disequilibrium with rs8099917, found ~8kb upstream of IL28B in Europeans (figure 36). Also in the Swiss study, rs1297860 was not measured in 149 individuals genotyped with the Illumina 550 chip, but was measured in the remaining 1213 patients. They found that rs12979860 was also highly associated with chronic infection (OR1.95, 95% CI 1.54-2.48, $p=3.38 \times 10^{-8}$) and was in linkage disequilibrium with rs8099917 (Rauch, Kutalik et al. 2010).

Figure 36 Location of IL28B polymorphisms



Location of most relevant interleukin (IL) 28B polymorphisms on chromosome 19 relative to the interferon (IFN)-λ gene.) (figure from (Ahlenstiel, Booth et al. 2010).

The genes identified as important determinants of spontaneous resolution and IFN-α treatment response are in the promoter regions of lambda interferons (IFNλ) (figure 36). Humans have three forms of λ interferons, IL28A, IL28B and IL29. The genes are clustered together on chromosome 19. IL28A and IL28B show 96% sequence homology. IFNλ are type III interferons and signal through a specific receptor heterodimer (IL10Rβ and IL28Rα). This specific receptor has a narrower tissue distribution than IFNα receptors but both IFNλ and IFNα signal through common downstream phosphorylation of kinases. Because of the more selective tissue distribution of IFNλ receptors, therapeutic use of IFNλ may theoretically have fewer side effects. Indeed IL29 is currently in phase 2 clinical trials in HCV treatment.

Of the 172 SNP's identified from the initial GWA study from the University of Sydney collaboration that were taken into the replication cohort, only 10 were SNP's in genes involved in lipid metabolism. The most significant lipid gene was rs2830028 (p=0.0035003), on chromosome 21 which codes for the Amyloid beta A4 precursor protein. The majority of the remaining SNP's were involved in immune regulation. Neither ApoE nor other genes with known common variants that determine plasma lipoproteins were amongst those SNP's significantly associated with anti-viral treatment outcome. In the American GWA study, the most significant SNP possibly involved in lipid metabolism was in the gene for Low Density Lipoprotein Receptor like-protein 1B (LRP1B) (rs970600) which was the 26th most significant SNP associated with SVR ($p = 6.73 \times 10^{-05}$). However, the role of LRP1B in lipid metabolism is not fully understood. LRP1B is a member of the LDL receptor family and shares 59% DNA sequence homology and similar domain structure with LRP1, a major receptor involved in chylomicron remnant clearance via apoE. However, LRPs

are not only involved in lipoprotein metabolism but also have a multitude of other diverse functions in coagulation, cell adhesion, growth and differentiation. Whilst LRP is abundantly expressed in liver, LRP1B is not expressed in liver but detected in thyroid, salivary glands and brain where it is involved in neuronal growth and has been implicated in the pathogenesis of Alzheimer's Disease (Liu, Li et al. 2001). Both LRP1B and LRP have four extracellular ligand binding domains that can bind to several ligands including apoE, complexes of urokinase plasminogen activator, plasminogen activator inhibitor type-1, β-amyloid precursor protein and receptor associated protein (RAP) (Cam, Zerbinatti et al. 2004). When RAP binds to LRP it can inhibit binding of all other LRP ligands. Unlike LRP1, LRP1B also has an additional ligand binding repeat in domain IV, and an additional amino acid sequence in its cytoplasmic tail, not found in LRP. Functionally, endocytosis via LRP1B is slower than via LRP. LRP1B is frequently activated in non-small cell lung cancers and gastric cancer and is therefore a putative tumour suppressor gene (Lu, Wu et al.).

Given that no genes with known common variants that influence lipid metabolism were identified as strongly related to treatment outcomes in three independent GWA studies, an alternative explanation for the relationship between higher non-HDL cholesterol and SVR needs to be considered.

5.6.3 Lipid profiles are affected by the innate anti-viral response

The IL28B genes are not close to any known lipid regulating genes. An alternative hypothesis is that LDL / non-HDL cholesterol levels are a surrogate marker of the host endogenous interferon response to hepatitis C. Although in this study, no significant difference in lipoprotein profiles was noted between those with the favourable and unfavourable IL28B SNP's, the sample size was too small to draw definitive conclusions. There was however a tendency for those with the non-response haplotype to have higher triglyceride / HDL ratios and lower total cholesterol levels which demands further investigation. A recent large study examined LDL cholesterol and apoB levels in 651 HCV genotype 1 patients and found that those with the favourable CC genotypes in rs12979860 had significantly higher LDL cholesterol and apoB levels than those with the unfavourable TT or TC genotypes (Li, Lao et al.). The close relationship between serum IP10 levels and low non-HDL cholesterol and apoB suggests that the unfavourable low lipid profile associated with non-response relates

to interferon stimulated gene (ISG) activation. IP10 is a C-X-C chemokine (CXCL10) that is produced by hepatocytes and sinusoidal epithelial cells in the liver to attract T lymphocytes, NK cells and monocytes (Neville, Mathiak et al. 1997). IP10 is induced by type 1 interferons and is a ligand for the CXCR3 receptor on Th1 lymphocytes, along with other CXC chemokines including MIG (monokine induced by interferon gamma, CXCL9) and I-TAC (inducible T-cell α chemo-attractant, CXCL11) (Zeremski, Petrovic et al. 2007). IP10 may therefore mediate the inflammatory T cell response to HCV to cause lobular inflammation and promote fibrosis (Zeremski, Petrovic et al. 2008). Serum levels of IP10 reflect liver IP10 mRNA expression (Askarieh, Alsio et al. 2010). Several studies have reported high serum IP10 levels in patients with chronic hepatitis C, which are particularly elevated levels in non-responders (Butera, Marukian et al. 2005; Romero, Lagging et al. 2006). High levels of IP10 have also been associated with mixed cryoglobulinaemia and active vasculitis in CHC (Antonelli, Ferri et al.). Moreover IP10 has been shown to decrease with viral clearance but remain elevated in non-responders (Diago, Castellano et al. 2006). Therefore pre treatment IP10 levels appear to predict response to PegIFNα in HCV G1. High pre treatment IP10 levels >600 pg/ml have been associated with lack of RVR and EVR. Cut off values >600 pg/ml had a negative predictive value of 79% for SVR which is comparable to the negative predictive value of <2 log reduction in HCV RNA at 12 weeks of 86%. A low IP10 level <150 pg/ml in contrast had a positive predictive value for SVR of 71% (Lagging, Romero et al. 2006; Romero, Lagging et al. 2006). Further characterisation of viral kinetics demonstrated that low IP 10 predicted the first phase decline in HCV RNA during the initial 48 hours of therapy but not the slower second phase decline between day 8 and 29 or third phase between treatment day 29 and week 6 (Askarieh, Alsio et al. 2010).

The mechanism by which IP10 is induced in HCV is not fully appreciated. Interferon α, β and γ are all known inducers of IP10 via the STAT1 signalling pathway. However in hepatitis B the viral protein HBx also induces IP10 directly by a mechanism of viral protein interactions with cellular kinases and the NF kappa B nuclear transcription factor (Zhou, Wang et al.), raising the possibility of direct viral factors in HCV as well.

IP10 is a serum marker for interferon stimulated gene (ISG) expression. IFN-α non-responders appear to have a maximally induced hepatic IFN response (Sarasin-

Filipowicz, Oakeley et al. 2008). The effect of IL28B genotype on mediating non-response may lie in the relationship with hepatic ISG induction. A recent study evaluating gene expression profiles in liver biopsies identified that up-regulated ISG's were significantly associated with non-response to treatment and with the unfavourable IL28B (rs8099917) genotype (minor TG or GG alleles) (Honda, Sakai et al.). In this study, multivariate analysis showed that up-regulated ISGs were associated with low HCV RNA and low LDL cholesterol levels, supporting the concept that low LDLC in HCV G1 is a surrogate of increased hepatic ISG expression (figure 37). This hypothesis is consistent with the reported effect of interferon alpha (IFNα) on serum lipid profiles; serum triglyceride levels, largely derived from VLDL, significantly increase following IFNα treatment (Hamamoto, Uchida et al. 2005) due to a decrease in lipoprotein lipase activity (Shinohara, Yamashita et al. 1997). Likewise, given that it is known that therapy with IFNα increases triglyceride levels, a possible explanation for the apparent higher TG / HDL ratio in those with the non-response IL28B haplotype is that the higher TG's represent a marker of increased activation of ISGs, which is known from previous studies to be associated with non-response to anti-viral therapy (Sarasin-Filipowicz, Oakeley et al. 2008).

Another link between innate anti-viral responses and lipid metabolism occurs with micro RNA's. Recent reports indicate that a liver-specific microRNA (miR), miR-122, is a key regulator not only in cholesterol and fatty acid metabolism in adult liver (Esau, Davis et al. 2006) but also in hepatitis C viral replication. miRNA-122 is a highly abundant liver expressed miRNA which binds to the 5' non-coding region of the HCV genome causing increased HCV replication (Jopling, Schutz et al. 2008). MicroRNA-122 inhibition has been shown to decrease plasma cholesterol levels in a diet-induced obesity mouse model (Esau, Davis et al. 2006). Interestingly decreased levels of miR 122 in the liver of CHC patients (which would be expected to be associated with lower plasma cholesterol levels) has recently been reported to correlate with a poor response to treatment (Sarasin-Filipowicz, Oakeley et al. 2008). Although IL28B genotype is important in determining hepatic ISG expression, this is not inevitably irreversible. A study using a novel miRNA 122 inhibitor (SPC3649) as anti-HCV therapy in Chimpanzees significantly lowered HCV RNA and IP10 levels, implying restoration of IFN sensitivity (Lanford, Hildebrandt-Eriksen et al.).

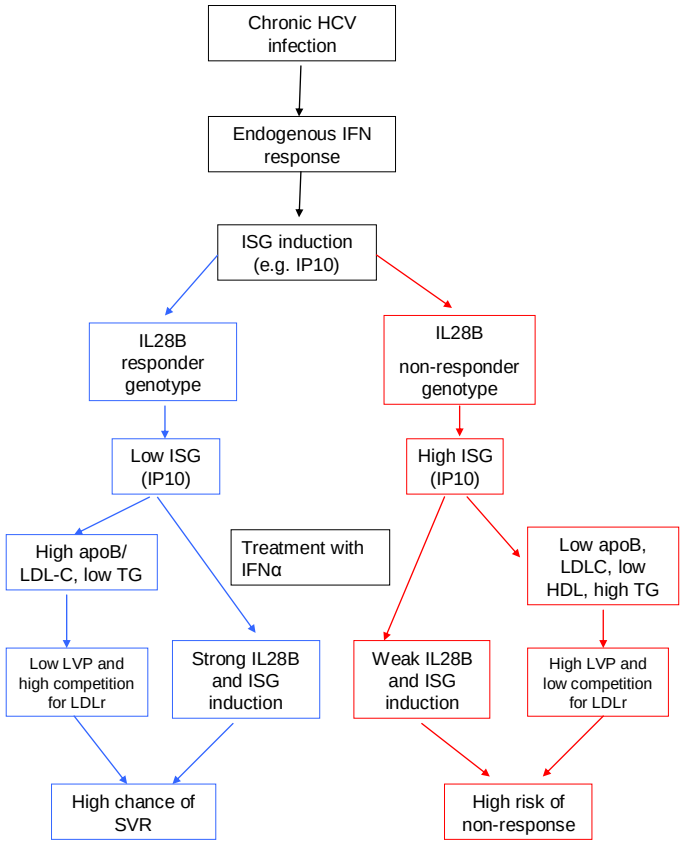
5.6.4 Competition hypothesis

Another hypothesis to consider is that the effect of higher apoB-B associated cholesterol on improved SVR may be exerted at the stage of HCV entry. Non-HDL cholesterol is an indirect method of quantifying apoB associated lipoprotein particles. The key constituents of VLDL are apolipoprotein B100 (one molecule per VLDL particle), apoE, and apoC's. Since HCV RNA in plasma has been shown to be associated with both apoB (Andre, Komurian-Pradel et al. 2002) and apoE at a buoyant density similar to that of VLDL as 'lipo-viral particles (LVP)' (Nielsen, Bassendine et al. 2006) it is possible that VLDL compete with HCV LVP for hepatocyte entry via shared receptors. It has long been proposed that the LDL receptor may be one of the receptors for HCV (Agnello, Abel et al. 1999; Monazahian, Bohme et al. 1999). Both apoB and apoE are ligands for the LDLr and it is thus possible that there is competition *in vivo* between LDL or VLDL remnants and HCV LVP for LDLr-mediated uptake, which has been demonstrated *in vitro* (Andre, Komurian-Pradel et al. 2002). ApoE also interacts with heparin sulphate-proteoglycans (HSPG) and can be transferred to LDL receptor-related protein (LRP) for internalization and apoC1 interacts with HSPG *in vitro* (Meunier, Russell et al. 2008), so apoC1 on HCV-LVP may use the HSPG-LRP pathway for viral entry. Furthermore SR-B1, which is implicated as another receptor for HCV (Catanese, Graziani et al. 2007), plays an important role in the metabolism of VLDL remnants (Van Eck, Hoekstra et al. 2008), so VLDL remnants may also compete with HCV LVP for hepatocyte uptake via SR-B1. Hence higher non-HDL cholesterol as a marker of the number of LDL and remnant particles may interfere with the HCV life-cycle by competing with HCV LVP for entry into hepatocytes via shared receptors.

In summary this study has shown that higher apoB associated cholesterol is a significant determinant of sustained virological response in CHC patients receiving anti-viral therapy with pegylated interferon-α and ribavirin. Polymorphisms of genes with common variants that influence lipid metabolism were not identified as predictors of anti-viral treatment response in a genome wide association study. Instead, the GWA studies have identified polymorphisms in IL28B, a type III interferon to be the most significant host genetic determinant of treatment response. Despite this, elevated LDL cholesterol remains associated with SVR, and elevated Triglyceride / HDL ratio with non-response. These lipid profiles are likely to be

surrogate markers of baseline innate immune responses mediated through endogenous interferons, ISG's and micro RNA's (figure 37). The clinical importance of these genetic studies is that advanced pre-treatment knowledge of the IL28B genotype could become an important part of future treatment algorithms. A genotyping test may be useful in identifying those who may respond better to new IL29 therapy, or indeed suggests the possibility of using IL28B as therapy. These studies also highlight the importance of the innate immune response on viral persistence and clearance and may point to a link between the innate immune system and regulation of lipid metabolism. Alternatively, the data is consistent with the hypothesis that the effect of higher apoB-associated cholesterol on improved SVR may be exerted at the stage of HCV entry, possibly due to competition between infectious low density HCV 'lipoviral particles' and LDL for hepatocyte entry via shared receptors. The relationships between HCV lipoviral particles, lipid profiles, metabolic and clinical parameters is explored further in the next chapter.

Figure 37 Model for the interactions of IL28B polymorphisms, lipid profiles and interferon stimulated genes (ISG) associated with treatment outcome in chronic hepatitis C (CHC) virus infection (adapted from (Ahlenstiel, Booth et al. 2010).



The IL28B responder genotype is associated with low-level ISG induction in CHC (Honda, Sakai et al. 2010) characterised by low IP10 levels and normal or high apoB / LDL cholesterol levels. High apoB / LDL results in high competiton for HCV lipoviral particles to infect hepatocytes via LDLr. When treated with IFN-α, there is strong ISG (Feld, Nanda et al. 2007) and IL28B induction. The IL28B may further enhance ISG induction, which may ultimately lead to a sustained virological response in the IL28B responder genotypes. In contrast, the IL28B non-responder genotype is associated with strong but ineffective ISG induction. High IP10 levels are a marker of high ISG. High IP10 correlates with low apoB and LDL cholesterol, which reduces competition for HCV lipoviral particles to infect hepatocytes via LDLr. When treated with IFN-α, there is suboptimal ISG response and IL28B induction. In these patients, ISG and therefore IFN responses are further impaired due to the activation of IFN inhibitory pathways secondary to the strong pretreatment ISG induction. High ISG levels correlate with low apoB / LDLC pre-treatment, and poor ISG induction on treatment is associated with non-response.

6 Chapter 6. Analysis of HCV Lipoviral particles: clinical, lipid & metabolic associations

6.1 Introduction and Aims

A large body of evidence now indicates that HCV is not only interacting with lipids at several stages of its life cycle but also that these interactions are likely to be clinically important. Previous characterisation of HCV in patient serum has demonstrated heterogeneity in the distribution of HCV RNA according to density. Low density HCV particles are associated with apolipoproteins B, E and C1 (Nielsen, Bassendine et al. 2006). These low density particles containing at least HCV RNA, core and apoB have been termed 'lipoviral particles (LVP) (Andre, Komurian-Pradel et al. 2002)'. Very Low Density Lipoprotein (VLDL) and its structural lipoprotein apoB in particular, appear to be essential for HCV assembly and maturation (Gastaminza, Kapadia et al. 2006; Huang, Sun et al. 2007). Silencing of apoB in HCV infected cells caused a 70% reduction in secretion of both apoB and HCV (Nahmias, Goldwasser et al. 2008). Both high and low density HCV particles *in vivo* are associated with antibodies (Thomssen, Bonk et al. 1993), although the ability of antibody mediated neutralisation is diminished in low density HCV compared to high density HCV (Grove, Nielsen et al. 2008). Low density cell culture derived HCV (HCVcc) had higher specific infectivity than high density HCVcc, despite being only a minority of HCV produced by this system (Miyanari, Atsuzawa et al. 2007), suggesting that the association with apolipoprotein B enhances infectivity.

Given the close association of HCV with lipoproteins and the effects of chronic HCV infection on lipid metabolism, it is likely that these interactions are also clinically important. It has been known for some time that poorer anti-viral treatment responses were associated with host factors such as age, male sex, advanced fibrosis and insulin resistance. The data presented in the previous chapter has contributed further to understanding these host characteristics, indicating that genetic polymorphisms in IL28B predicts non-response to pegylated interferon-α and ribavirin. Furthermore, low non-HDL cholesterol levels and high triglyceride / HDL ratios are also associated with non response and there is a relationship between lipid levels, insulin resistance and IP10 as a maker of interferon stimulated gene activation. However it is also

noteworthy that although HCV genotype is a major viral determinant of anti-viral treatment response, total HCV RNA viral load is not (Ticehurst, Hamzeh et al. 2007), only being predictive if it is very low at treatment initiation or the change in HCV RNA titre after 4 or 12 weeks of therapy.

The aim of this further work was to develop a clinically useful assay to measure apoB associated HCV 'lipoviral particles (HCV LVP) in patients. After developing the HCV LVP assay, the next aim was to investigate host factors that influence HCV LVP levels and characterise the clinical relevance of HCV LVP in terms of important clinical end points, such as anti-viral treatment response and liver fibrosis.

6.2 LVP Assay development

The LVP assay development experiments were a modification of the protocol described by Nielsen et al (Nielsen, Bassendine et al. 2006). This previous work by Nielsen et al had demonstrated that >90% of HCV RNA could be immunoprecipitated from a low density fraction using antibodies to apolipoproteins B and E. However this work had been performed in an unusual patient with combined immunodeficiency that had no circulating antibodies. In contrast, most patients with chronic HCV infection have considerable amounts of circulating antibody. Dr Nielsen was working on purifying LVP from patients using a two-stage purification protocol; fractionation by density using iodixanol gradient ultracentrifugation and then by size, using size exclusion chromatography. Two immune competent HCV patients in the prospective HCV cohort with co-existing genetic haemochromatosis (H01 and H06) underwent therapeutic venesections and the large volume of plasma obtained enabled further purification of LVP. The purified LVP fractions were defined as low density ($d<1.12$ g/ml) and large size (VLDL size or larger). Immune precipitation using capture anti-apoB or protein G coated to sepaharose beads from the purified fractions from two immune competent patients demonstrated that there was high variability. In patient H01, 93% of HCV RNA in the purified LVP fraction could be immune precipitated with anti-apoB, but in patient H06 only 35% was immune precipitated by anti-apoB. Instead, protein G which binds non-specifically to immune complexes precipitated 78% of the HCV RNA in patient H06, indicating that a considerable proportion of low density, large VLDL sized HCV particles are immune complexes (table 6-1). One reason that an anti-apoB immunoprecipitation approach to measuring LVP from

unfractionated plasma would not be reliable is because antibody coating of LVP may mask epitopes reacting with anti-apoB, and therefore underestimate the actual amount of apoB associated HCV present. Based on this data, an approach using immune precipitation using anti-apoB on unfractionated plasma was considered to be too variable to reliably estimate LVP. Moreover, multiple purification steps are too time consuming to be applicable to the large patient cohorts required to address important clinical questions regarding the clinical relevance of HCV LVP. Therefore a simpler method based purely on density distribution of apoB was developed.

Table 6-1 Percentage of HCV RNA immunoprecipitated from VLDL fraction purified by density (<1.12g/ml) and size

Beads coated with	Immunoprecipitation of VLDL	CHC patient H01	CHC patient H06	immuno-deficient patient
Protein G	no	41%	78%	4%
Anti- ApoB	yes	93%	35%	90%
Rabbit IgG Control	no	5%	4%	2%

(Data provided by Dr Soren Nielsen)

Previous work had shown the iodixanol was superior to density gradients formed with Sodium Bromide or Sucrose in terms of preserving the integrity of lipoprotein associations with HCV RNA (Nielsen, Bassendine et al. 2006).

A series of development experiments were performed by Dr Simon Bridge. A range of isotonic iodixanol density gradients were prepared with increasing concentrations of iodixanol: 6.25, 12.5, 18.75, 25, 31.25, 37.5, 43.75, 50% and the distribution of apoB within the density gradients was determined by Western blot. 12.5% iodixanol was found to be the optimum concentration because this formed the most linear distribution of apoB in the top fractions. Increasing the concentration of iodixanol above 12.5% reduced the density linearity and concentrated the apoB to the top of the gradient.

Having determined that 12.5% iodixanol was the most appropriate concentration, the next step was to determine the reproducibility of the density gradients and to determine a cut off density which captures all the apoB containing lipoproteins. Plasma from 7 healthy volunteers was fractionated by 12.5% iodixanol density gradient ultracentrifugation into 20 x 500µl fractions and the density of each was measured using a refractometer. ApoB was detected by Western blotting in all the donors in the top 7 fractions (3.5mls) at a density <1.07g/mL. The density and apoB distributions were consistently reproduced using plasma from 13 patients with chronic hepatitis C infection. ApoB was never detected in a fraction with density > 1.07g/mL (figure 38). Therefore a cut-off value was defined separating two fractions; a low density fraction with density <1.07g/mL (top 3.5mL = fractions 1-7 of the gradient) capturing apoB and a high density fraction with a density >1.07g/ml in the remaining

6.5mls (fractions 8-20) where apoB was undetected by western blot. HCV RNA was detected by real time PCR in the top 3.5 ml, low density fraction (d<1.07g/ml) was considered to represent 'lipoviral particles (LVP)'. HCV RNA detected in the high density fraction (d>1.07g/ml) was considered to represent non-LVP.

The ratio of LVP as a proportion of total HCV was calculated thus:

LVP ratio = LVP/ (LVP + non-LVP).

Reproducability of the calculated LVP ratio was enhanced by the using measured (LVP + non LVP) to represent total viral load, rather than using total viral load measured directly from the unfractionated plasma because of potential variability in the fractionation process and down stream PCR steps. Evaluation of the yield was calculated by comparing the measured total viral load from whole plasma to the calculated LVP + non-LVP (% recovery = total viral load / (LVP +non LVP)). The median recovery was 117.5% (Q1 61.6% - Q3 190%) indicating some variability in recovery yields. Recoveries >100% can be explained by the presence of PCR inhibitors in the whole plasma which have been previously described, which may be a particular issue in haemolysed samples. A number of factors including haemoglobin, lactoferrin and immunoglobulin G are the major inhibitors of diagnostic PCRs (Al-Soud and Radstrom 2001). The LVP assay dilutes the plasma and PCR inhibitors 1 in 20 which may account for the higher median recovery. The LVP calculation assumes that any losses (or gains by dilution of PCR inhibitors) from the fractionation process are distributed equally between the two (LVP and non-LVP) fractions, and that this gives the best estimate of total viral load as the denominator in the LVP ratio calculation

The two-fraction, LVP assay was applied to the prospective cohort of HCV patients. Clinical and metabolic correlates with HCV LVP, non-LVP, LVP ratio and total viral load were assessed in univariate analysis in two groups, those with HCV G1 and those with HCV G3.

Figure 38 Distribution of ApoB by western blot in 12.5% iodixanol density gradients in healthy volunteers and HCV patients

Fraction	d (g/mL)	HV1	HV2	HV3	HV4	HV5	HV6	HV7	P1	P2	P3	P4	P5	P6	P7	P8	P9
1	1.025	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++	+++	+	++	+++
2	1.035	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++
3	1.04	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	+++
4	1.048	+++	+++	++	+++	+++	++	++	++	+++	+++	++	+++	+		+	++
5	1.055	++	++	+	++	++		+	+	+	+++	++	+++				+
6	1.062		+		+	+					+	+	++				
7	1.069												+				
8	1.074																
9+10	1.08																
11+12	1.085																
13+14	1.093																
15+16	1.112																
17+18	1.15																
19+20	1.271																

Distribution of apoB detected by western blot in 12.5% iodixanol density gradients fractionated into 20 x 500µl fractions from 7 healthy volunteers and 9 HCV patients. Intensity of the western blot band is graded +, ++, +++, or not detected. ApoB was never detected at d >1.07 g/ml.

6.3 Patient baseline characteristics

The characteristics of the patients from the prospective HCV cohort that had determination of LVP are summarised in table 6-2. 51 patients were HCV G1 and 21 were HCV G3. There were no significant differences in age, BMI, waist / hip ratio or sex. Liver stiffness measurements (LSM) were assessed by transient elastography. Of the HCV G1 group, 29% had a LSM ≥ 13.0 kPa, consistent with established cirrhosis (Friedrich-Rust, Ong et al. 2008). Of the HCV G3 group, 37% had LSM ≥ 13.0 kPa. In the HCV G1 group, two patients were type 2 diabetic and receiving insulin replacement. For this reason, measurement of insulin resistance using QUICKI was determined in addition to HOMA IR. Applying the criteria of the International Diabetes Federation (Appendix A) to this cohort (Alberti, Zimmet et al. 2005) a total of 12 (23.5%) HCV G1 patients and 4 (19.0%) of HCV G3 patients were defined as having the metabolic syndrome.

Table 6-2 LVP assay - patient characteristics

	HCV G1 (N=51)	**HCV G3 (N=21)**
Age years	48 ± 10.4	44 ± 11.6
Sex male / female	37 / 14	17 /4
Body Mass Index Kg/m^2	25.4 ± 4.1	25.8 ± 3.4
Waist / hip ratio	0.96 ± 0.06	0.97 ± 0.04
Glucose mmol/l	4.97 ± 0.65	5.47 ± 1.29
Insulin	7.94 ± 4.14	7.90 ± 3.75
HOMA IR	1.71 ± 1.39	1.97 ± 1.40
QUICKI	1.21 ± 0.39	1.20 ± 0.38
NEFA	0.44 ± 0.30	0.52 ± 0.42
Liver Stiffness kPa	Mean 14.0 ± 15.4 Median 7.7 (5.43-16.88)	Mean 14.8 ± 13.3 Median 8.8 (5.8-19.2)
LSM ≥ 13 kPa (%)	29%	37%
Metabolic Syndrome (%)	23.5%	19%

Fasting lipid profiles in HCV G1 and HCV G3 groups that had prospective determination of LVP are shown in table 6-3. Lipid profiles were performed in tandem with the LVP analysis and so represent a true measurement of the lipid profile that was subsequently correlated with viral parameters. The lipid profiles data confirms previous observations of significantly lower total cholesterol, non-HDL cholesterol, triglycerides and apoB in HCV G3 than HCV G1. In addition HCV G1 patients had higher total cholesterol/HDL ratios and borderline significantly higher TG/HDL ratios.

Table 6-3 Lipid profiles in patients evaluated for LVP

	HCV G1 N=51	**HCV G3 N=21**	**P-value**
#Total cholesterol mmol/l	4.59 ± 0.99	3.84 ± 0.97	0.004
#Non-HDL cholesterol mmol/l	3.36 ± 0.95	2.55 ± 0.89	0.001
***HDL Cholesterol mmol/l**	1.2 (1.1-1.4)	1.2 (0.9-1.6)	0.699
***Triglycerides mmol/l**	1.20 (0.90-1.50)	0.80 (0.70-1.25)	0.032
***ApoA1 g/l**	1.4 (1.3-1.6)	1.4 (1.2-1.7)	0.813
#ApoB g/l	0.89 ± 0.25	0.66 ± 0.24	0.001
***TG/HDL ratio**	0.92 (0.69-1.40)	0.54 (0.44-1.51)	0.0580
***Total cholesterol / HDL ratio**	3.62 (3.07-4.42)	2.86 (2.31-3.86)	0.0103

 # normally distributed, summarised as mean ± standard deviation, 2-sample t test;
**non-parametric distribution summarised as median (Q1-Q3), Kruskall Wallace test.*

6.4 Variability of HCV LVP

Summary statistics for HCV RNA total viral load, LVP fraction (d≤ 1.07 g/ml), non-LVP fraction (d>1.07 g/ml) and the LVP ratio (calculated as LVP ratio = LVP / (LVP + non-LVP)) are shown in Table 6-4 for HCV G1 and HCV G3. There were no significant differences between HCV genotypes in any of these parameters.

Table 6-4 LVP summary statistics

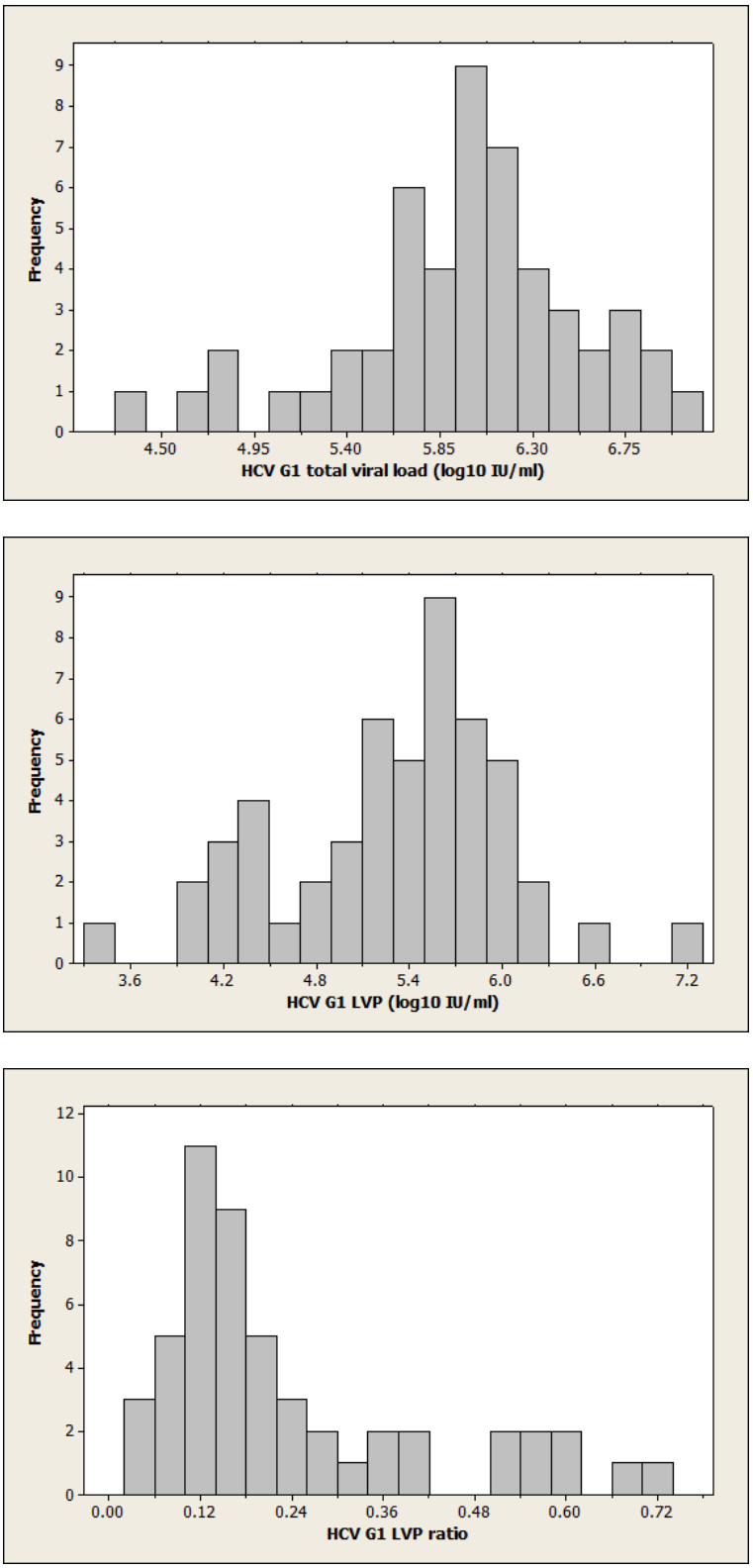
	HCV G1 N=51	**HCV G3 N=21**	**p-value #**
Total viral load (log_{10} IU/ml)	5.98 ± 0.57 6.04 (5.76-6.29)	5.91 ± 0.50 6.05 (5.89-6.27)	0.6736
LVP (log_{10} IU/ml)	5.29 ± 0.73 5.38 (4.81-5.82)	5.15 ± 0.81 5.35 (5.40-6.09)	0.5857
Non-LVP (log_{10} IU/ml)	5.88 ± 0.63 5.94 (5.52-6.42)	5.74 ± 0.63 5.92 (5.40-6.09)	0.3658
LVP ratio	0.24 ± 0.18 0.18 (0.13-0.31)	0.27 ± 0.27 0.14 (0.05-0.46)	0.4278

 # Mann Whitney U test

The distribution of HCV RNA total viral loads, LVP loads and LVP ratios are shown for HCV G1 in Figure 39 and for HCV G3 in Figure 40.

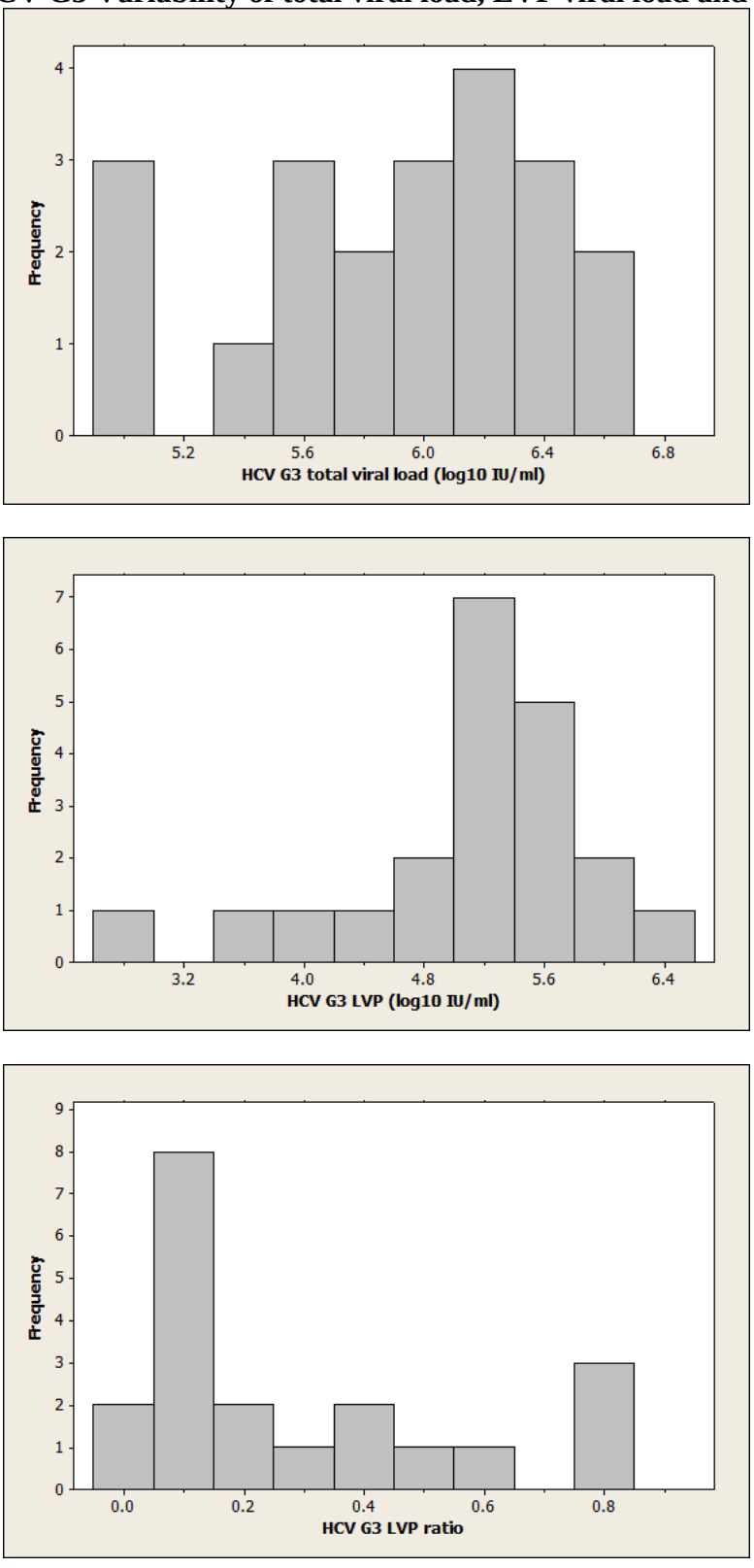
In both HCV G1 and HCV G3 LVP ratios varied widely. For HCV G1 the range of LVP ratios was 0.03 to 0.74 (mean 0.24, median 0.18). In HCV G3 patients the range of LVP ratios was 0.03 to 0.83 (mean 0.27, median 0.14).

Figure 39 HCV G1 Variability of total viral load, LVP viral load and LVP ratio



Distribution of viral parameters in HCV G1. (A) total HCV RNA viral load (log10 IU/ml). (B) LVP load (log10 IU/ml) in fraction density<1.07 g/ml. (C) LVP ratio (=LVP / (LVP + non LVP))

Figure 40 HCV G3 Variability of total viral load, LVP viral load and LVP ratio



Distribution of viral parameters in HCV G3. (A) total HCV RNA viral load (log10 IU/ml). (B) LVP load (log10 IU/ml) in fraction density<1.07 g/ml. (C) LVP ratio (=LVP / (LVP + non LVP))

6.5 Metabolic Determinants of LVP

To investigate the variation in total HCV viral load, LVP load, non-LVP load and LVP ratio, univariate Spearman's rank correlation analysis was used to determine metabolic and clinical correlates. The results of correlates for HCV G1 are shown in table 6-5 and for HCV G3 in table 6-7. Those correlations with $p<0.05$ were considered to be significant and were subsequently included in a multivariate linear regression model.

6.5.1 HCV G1 correlations

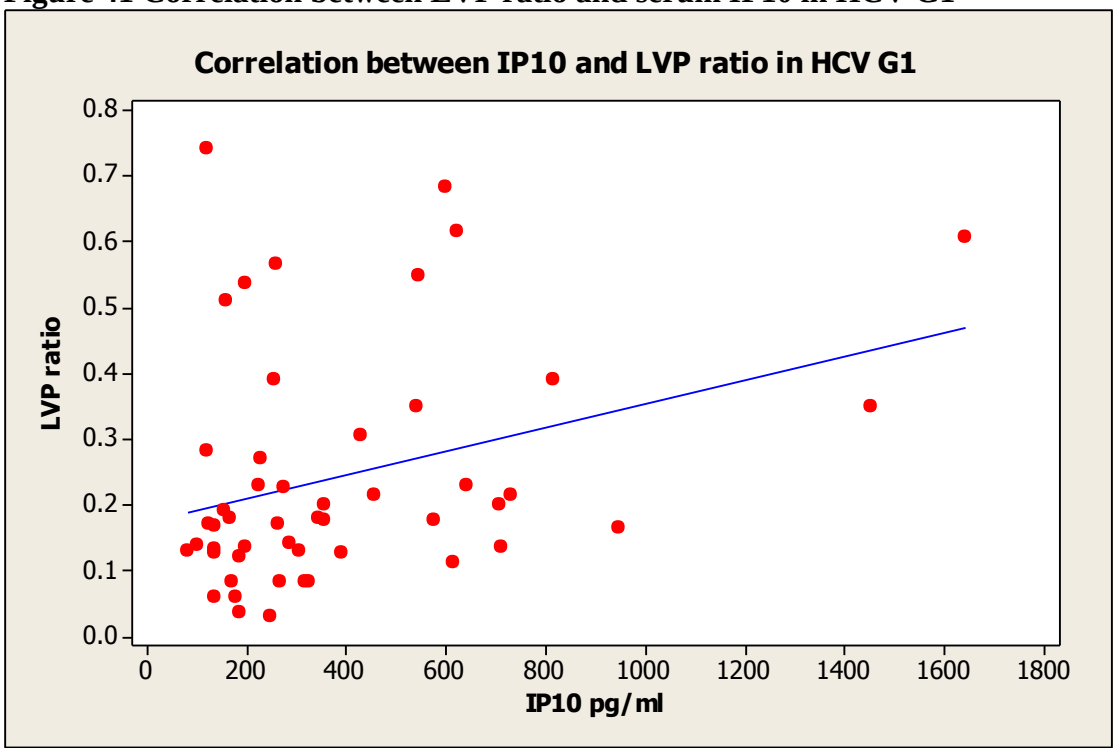
In HCV G1, LVP load IU/ml correlated significantly with glucose and insulin levels, and with the calculated markers of insulin resistance, HOMA IR (glucose x insulin / 22.5) and QUICKI score (1/(log insulin x log glucose)). Both HOMA IR and QUICKI calculations were performed because although the HOMA score is widely reported, the QUICKI score is more valid in populations that include patients with diabetes mellitus where fasting glucose is inappropriately high and insulin inappropriately low, and in this situation has a better correlation with glucose clamp estimates than HOMA IR (Muniyappa, Lee et al. 2008). As insulin resistance increases, so QUICKI scores tend to decrease.

LVP ratio (LVP/LVP+non-LVP) correlated significantly with triglycerides and the TG/HDL ratio in addition to insulin resistance as measured by HOMA IR and QUICKI in the univariate analysis. Additionally LVP ratio correlated with IP10 ($r=0.293$, $p= 0.039$). Multivariate linear regression analysis showed that HOMA IR was the main independent determinant of LVP load ($\log_{10}$ IU/ml) ($r^2=16.6\%$, $p=0.037$), and TG/HDL ratio was the main independent predictor of LVP ratio ($r^2=24.4\%$, $p=0.019$).

Table 6-5 HCV G1 correlations

Parameter	Total Viral Load (log10 IU/ml)	LVP (log10 IU/ml)	LVP Ratio	Non-LVP (log10IU/ml)
Age	r = 0.229 p = 0.106	r = 0.163 p = 0.253	r = 0.043 p = 0.765	r = 0.147 p = 0.303
Body mass index (kg/m^2)	r = 0.159 p = 0.265	r = 0.249 p = 0.078	r = -0.077 p = 0.592	r = 0.200 p = 0.160
Fibroscan score KPa	r = -0.086 p = 0.555	r = 0.124 p = 0.395	**r = 0.419** **p = 0.003**	r = -0.163 p = 0.263
Total cholesterol	r = 0.181 p = 0.203	r = -0.018 p = 0.899	r = -0.141 p = 0.324	r = 0.127 p = 0.374
Non-HDL cholesterol	r = 0.185 p = 0.194	r = 0.058 p = 0.687	r = -0.021 p = 0.884	r = 0.071 p = 0.621
HDL cholesterol	r = 0.178 p=0.216	r = -0.014 p= 0.921	r = -0.073 p = 0.615	r = 0.079 p = 0.584
Total cholesterol / HDL ratio	r = 0.085 p = 0.551	r = 0.056 p = 0.697	r = 0.121 p = 0.400	r = 0.065 p = 0.651
Apo B	r = 0.178 p = 0.216	r = -0.014 p = 0.921	r = -0.073 p = 0.615	r = 0.046 p= 0.755
ApoA1	r = -0.118 p = 0.416	r = -0.136 p = 0.347	r = -0.257 p = 0.072	r = 0.108 p = 0.457
Triglycerides	r = 0.101 p = 0.479	r = 0.248 p = 0.079	**r = 0.320** **p = 0.022**	r = 0.082 p = 0.567
TG /HDL ratio	r = 0.082 p = 0.568	r = 0.256 p = 0.070	**r = 0.392** **p = 0.004**	r = 0.000 p = 0.997
Glucose	r = 0.171 p = 0.231	**r = 0.307** **p = 0.028**	**r = 0.311** **p = 0.026**	r = 0.113 p = 0.430
Insulin	r = 0.121 p = 0.407	**r = 0.393** **p = 0.005**	**r = 0.393** **p = 0.005**	r = 0.177 p = 0.228
HOMA IR	r = 0.078 p = 0.585	**r = 0.383** **p = 0.005**	**r = 0.397** **p = 0.004**	r = 0.144 p = 0.314
QUICKI	r = -0.121 p = 0.414	**r = -0.387** **p = 0.007**	**r = -0.371** **p = 0.009**	r = -0.185 p = 0.209
IP10	r = -0.003 p = 0.984	r = 0.221 p = 0.122	**r = 0.293** **p = 0.039**	r = 0.043 p = 0.774

Figure 41 Correlation between LVP ratio and serum IP10 in HCV G1



There was a significant postitive correlation between serum IP10 levels (a marker of hepatic interferon stimulated genes) and LVP ratio in HCV Genotype 1 infection (N=51, r= 0.293, p=0.039). This remained significantly associated with LVP ratio after multi-variate linear regression analysis (p=0.048).

In multivariate linear regression analysis, the most significant independent determinant of LVP ratio was TG/HDL ratio (p=0.001) and the only other independent determinant of LVP ratio was IP10 (p=0.048) (figure 40). The combined r^2 was 26.4%.

When the HCV G1 patients were divided into two groups, those with a low LVP ratio (defined as below the median value of 0.177) (n = 25) and those with a high LVP ratio (defined as above the median value of 0.177) (n = 26), characteristic differences in metabolic profiles were noted (table 6-6). Those with a high LVP ratio had a significantly higher fasting glucose (p = 0.044), insulin (p = 0.005), and HOMA-IR (p = 0.008). Additionally those with high LVP ratios had a higher triglycerides (p = 0.015), lower HDL cholesterol (p = 0.015), hence higher TG/HDL ratios (p = 0.003) and larger waist circumferences (p = 0.037). These are all noted to be characteristics of the metabolic syndrome.

Table 6-6 Metabolic Syndrome characteristics of HCV G1 patients with high LVP ratio

	Low LVP ratio (N=25)	High LVP ratio (N=26)	p-value
Waist circumference cm	85.7 ± 11.1	92.7 ± 12.2	0.037
Triglycerides	1.08 ±0.46	1.62 ± 0.89	0.015
HDL cholesterol	1.36 ± 0.33	1.11 ± 0.25	0.015
Triglyceride / HDL	0.84 ± 0.4	1.62 ± 1.1	0.003
Glucose	4.84 ± 0.65	5.1 ± 0.64	0.044
Insulin	5.96 ± 2.75	9.56 ± 4.67	0.005
HOMA IR	1.25 ± 0.68	2.17 ± 1.3	0.008

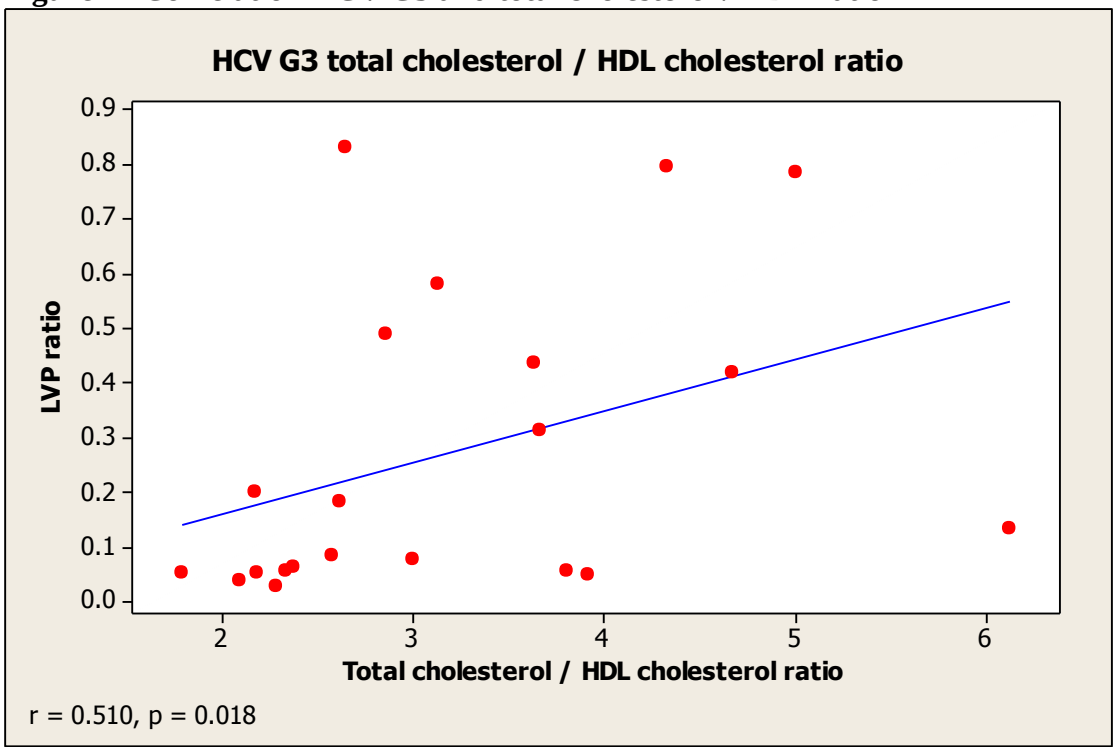
6.5.2 HCV G3 correlations

Table 6-7 shows univariate Spearman's rank correlation analysis that demonstrated that in HCV G3 there was a significant negative correlation between total cholesterol and total viral load ($r = -0.631$, $p = 0.002$). This negative correlation was accounted for by the non-LVP fraction ($r = -0.674$, $p = 0.001$) more than the LVP ($r = -0.402$, $p = 0.071$), and was related to the non-HDL cholesterol component ($r = -0.650$, $p = 0.001$) rather than HDL cholesterol ($r = -0.326$, $p = 0.149$), and was confirmed by the negative correlation with apoB ($r = -0.432$, $p = 0.050$). LVP had a significant negative correlation with HDL cholesterol ($r = -0.460$, $p = 0.036$), which was confirmed with the negative correlation with apoA1 ($r = -0.488$, $p = 0.025$). The LVP ratio was significantly correlated with the total cholesterol/HDL ratio ($r = 0.510$, $p = 0.018$) (figure 42). In contrast to HCV G1, there was no significant correlation of LVP or LVP ratio with triglycerides, TG/HDL ratio or insulin resistance.

Table 6-7 HCV G3 correlations

Parameter	Total Viral Load (log10 IU/ml)	LVP (log10 IU/ml)	LVP Ratio	Non-LVP (log10IU/ml)
Age	r = -0.195 p = 0.396	r = -0.295 p = 0.194	r = -0.145 p = 0.529	r = -0.156 p = 0.499
Body mass index (kg/m^2)	r = -0.187 p = 0.418	r = 0.008 p = 0.971	r = -0.078 p = 0.738	r = 0.115 p = 0.620
Fibroscan score KPa	r = 0.215 p = 0.378	r = -0.046 p = 0.853	r = -0.121 p = 0.623	r = 0.323 p = 0.178
Total cholesterol	**r = -0.631** **p = 0.002**	r = -0.402 p = 0.071	r = 0.162 p = 0.483	**r = -0.674** **p = 0.001**
Non-HDL cholesterol	**r = -0.541** **p = 0.011**	r = -0.056 p=0.810	r = 0.339 p = 0.113	**r = -0.650** **p = 0.001**
HDL cholesterol	r = -0.291 p=0.201	**r = -0.460** **p= 0.036**	r = -0.384 p = 0.086	r = -0.326 p = 0.149
Total cholesterol / HDL ratio	r = -0.184 p = 0.424	r = 0.265 p = 0.246	**r = 0.510** **p = 0.018**	r = -0.245 p = 0.285
Apo B	r = -0.416 p = 0.061	r = -0.041 p = 0.859	r = 0.267 p = 0.242	**r = -0.432** **p= 0.050**
ApoA1	r = -0.338 p = 0.134	**r = -0.488** **p = 0.025**	r = -0.411 p = 0.064	r = -0.238 p = 0.299
Triglycerides	r = -0.071 p = 0.760	r = 0.267 p = 0.242	r = 0.283 p = 0.213	r = -0.027 p = 0.908
TG /HDL ratio	r = 0.124 p = 0.594	r = 0.357 p = 0.112	r = 0.330 p = 0.144	r = 0.219 p = 0.340
Glucose	r = 0.305 p = 0.179	r = 0.115 p = 0.619	r = -0.015 p = 0.949	r = 0.348 p = 0.122
Insulin	r = 0.009 p = 0.969	r = 0.052 p = 0.823	r = -0.060 p = 0.797	r = 0.045 p = 0.847
HOMA IR	r = 0.179 p = 0.439	r = 0.023 p = 0.920	r = -0.140 p = 0.544	r = 0.266 p = 0.243
QUICKI	r = -0.043 p = 0.854	r = -0.053 p = 0.819	r = 0.068 p = 0.771	r =-0.066 p = 0.778
IP 10	r = 0.138 p = 0.610	r = 0.156 p = 0.564	r = 0.006 p = 0.983	r = -0.029 p = 0.914

Figure 42 Correlation HCV G3 and total cholesterol / HDL ratio



LVP ratio (=LVP / LVP + non LVP) correlates significantly with the total cholesterol / HDL ratio in HCV G3, (r=0.510, p=0.018)

6.6 Correlations VLDL1, VLDL2, IDL and LDL apoB

Given that HCV LVP in HCV G1 correlated most significantly with triglycerides, further experiments were performed to test the assumption that fasting triglycerides were predominantly contained within the VLDL1 fraction in HCV G1 patients. Additional fasting plasma was collected at the same time as the LVP plasma in 23 HCV G1 patients which was used for sequential density gradient flotation using salt gradients as described in the methods. ApoB was quantitated in each fraction using a quantitative ELISA as described in methods. Quantities of apoB in each fraction were then correlated against fasting whole plasma lipid parameters. Table 6-8 shows that VLDL1 apoB contributes only ~2% of the fasting apoB, yet correlates most significantly with fasting triglycerides ($r=0.712$, $p<0.001$) (table 6-9). In contrast VLDL2 accounts for ~10% of the total apoB and only accounts for a minority of fasting triglycerides. Therefore the assumption that fasting triglycerides are predominantly in the VLDL1 fraction appears to be valid in HCV G1.

Table 6-8 VLDL1, VLDL2, IDL and LDL ApoB sub fractions

Fraction	apoB mg / dl	% of total apoB
VLDL1 (S_f 60-400)	1.00 ± 0.35	2.01 ± 4.64
VLDL2 (S_f 20-60)	7.30 ± 2.44	10.59 ± 14.60
IDL (S_f 12-20)	23.42 ± 10.68	23.40 ± 12.09
LDL (S_f 0-12)	69.37 ± 27.70	64.00 ± 17.95

Mean ± standard deviation (N=23)

Table 6-9 VLDL apoB correlations

Correlations N=23	r	r^2	P value
VLDL1 apoB vs TG (log10)	0.712	0.51	<0.001
VLDL2 apoB vs TG (log10)	0.112	0.01	0.612
LDL apoB vs LDL chol (Friedwald calculated)	0.317	0.10	0.150
TG vs LVP ratio (ranks)	0.199	0.04	0.362
VLDL1 apoB vs LVP ratio (ranks)	0.198	0.04	0.366
VLDL2 apoB vs LVP ratio (ranks)	0.022	<0.01	0.922

6.7 HCV LVP associations with clinical outcomes

In HCV G1 the LVP assay indicated stronger associations with important clinical outcomes that total HCV viral load measurements. The two major clinical outcomes were estimation of liver stiffness by transient elastography (Fibroscan®), a non-invasive marker of hepatic fibrosis and anti-viral treatment outcomes.

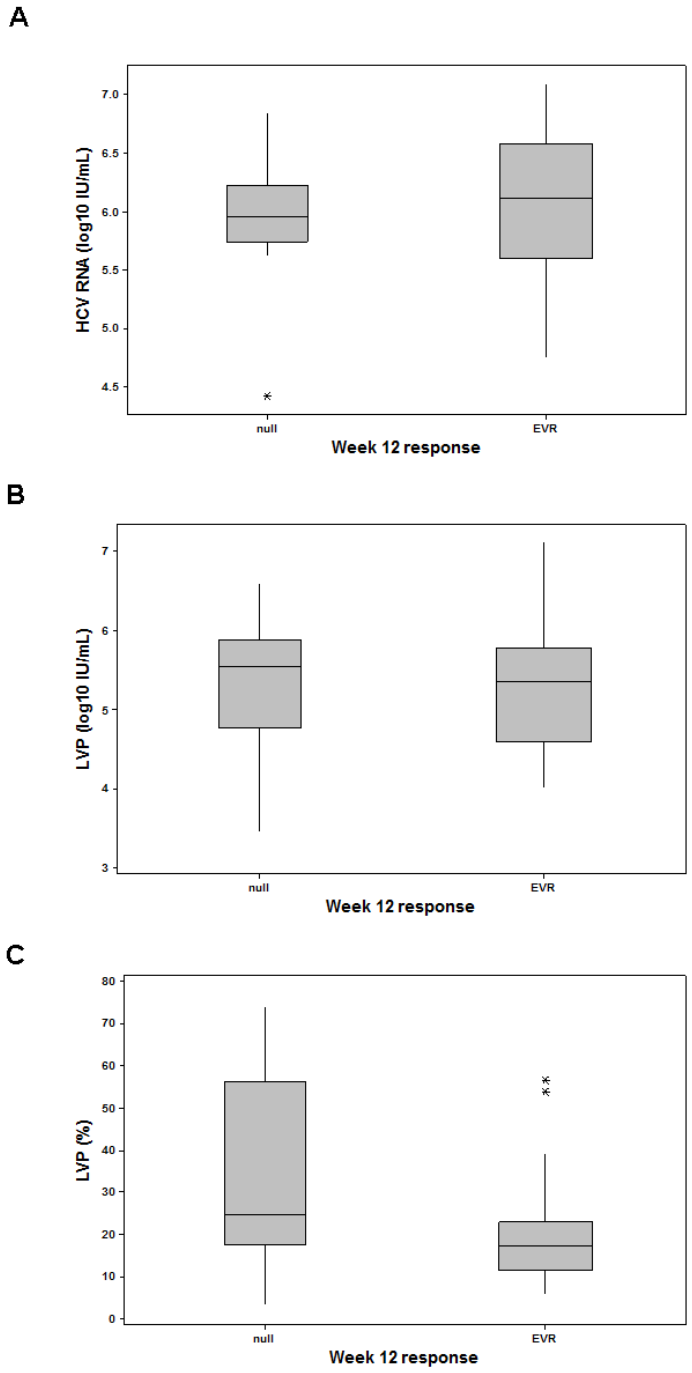
6.7.1 HCV LVP and liver stiffness measurements

The LVP ratio correlated significantly with liver stiffness measurement (LSM) scores ($r = 0.419$, $p = 0.003$). When HCV G1 patients were divided into those with LVP ratios above and below the median of 0.17, those with high LVP ratios were more likely than expected to have high LSM scores >13kPa (χ^2 5.95, $p = 0.015$).

6.7.2 LVP and anti-viral treatment outcomes

Of the 51 patients in the HCV G1 group, 42 had undergone antiviral therapy. 24 of these had received previous treatment with pegylated interferon-α and ribavirin at least six months before the LVP / lipids were assessed, hence the treatment data was retrospective. These patients were all relapsers or non-responders who remained viraemic. An additional 18 patients provided the LVP / lipid profiles prospectively prior to commencing anti-viral therapy. Given that time from initiation of therapy to determination of outcome six months post completion is at least 18 months, data on SVR was incomplete for these prospectively treated patients. Instead, assessment of HCV RNA as a response to therapy at week 12 (i.e. Early Virological Response (EVR)) was compared. 18 (43%) were non-responders at week 12, the remaining 24 (57%) were either complete or partial early virological responders (i.e. achieving at least >2 log reduction in HCV RNA viral load at 12 weeks compared to baseline). Non-responders at week 12 had significantly higher LVP ratios than the EVR group (median LVP ratio for non-responders 0.338 vs 0.201 for EVR, $p = 0.031$) (figure 43). Likewise when the HCV G1 group was divided above and below the median LVP ratio, those with low LVP ratios were more likely than expected to have had an EVR (χ^2 4.36, $p = 0.037$). Importantly, total HCV RNA viral load was not significantly different between non-responders and EVR's.

Figure 43 Total viral load, LVP viral load and LVP ratio (%) in null responders and early virological responders to pegylated interferon-α and ribavirin
(A)



(A) Total HCV RNA viral load was not significantly different between those achieving a complete or partial early virological response (EVR) vs those not (null responders). LVP was also not significant (B). LVP ratio was significantly lower in EVRs than null responders (p=0.031). The line in the box is the median value and the box represents the interquartile range. Lower whisker is Q1 + 1.5(Q3-Q1) and upper whisker represents Q3 + 1.5(Q3-Q1).

6.8 Discussion

6.8.1 LVP assay development and potential clinical utility

HCV can associate with apoB-containing lipoproteins as HCV 'lipoviral particles' (LVP), however the variability of LVP and host metabolic factors that influence variation in LVP levels is poorly understood. Moreover the relationship of LVP to clinically important outcomes has hitherto been unknown. In this study, LVP was measured in 51 patients with HCV G1 infection and 21 patients with HCV G3 using iodixanol density gradients which have previously been shown to maintain the integrity of the lipoprotein associations with HCV RNA (Nielsen, Bassendine et al. 2006). The density cut-off separating 'LVP' from 'non-LVP' fractions was determined by the distribution of apoB, which was never detected at densities higher than 1.07 g/ml by western blot in preliminary experiments in 9 HCV patients and 7 healthy volunteers. The novel assay measured HCV RNA in the low density (d<1.07g/mL) fraction that is likely to contain the vast majority of apoB. This was considered to represent the 'LVP' fraction. HCV RNA was also measured in the high density (>1.07 g/ml) fraction that was considered to be 'non-LVP' and contained little if any apoB. Lastly HCV RNA was measured in whole undiluted plasma by sensitive qRT- PCR methods. The proportion of total HCV RNA found in plasma as LVP (i.e. LVP ratio) was also calculated from the equation LVP ratio = LVP/(LVP+non-LVP). The denominator (LVP+nonLVP) was used instead of measured total viral load from whole plasma. This was to overcome variation in losses or gains from the dilution and fractionation process. Therefore LVP ratio assumes that losses or gains from fractionation are equally distributed between the LVP and non-LVP fractions. This study indicated that the novel HCV LVP method provided additional information over and above that from conventional measurement of total plasma HCV RNA viral load.

The metabolic associations of LVP in HCV G1 and HCV G3 appeared distinct. In HCV G1 those with high LVP ratios had characteristic features of the metabolic syndrome such as larger waist circumference raised triglycerides, low HDL and insulin resistance. LVP ratio in HCV G1 also correlated with IP10 and adverse clinical outcomes including more advanced fibrosis as measured by LSM and poorer anti-viral treatment response. In contrast, in HCV G3 the most significant metabolic

correlates of LVP were inverse relationships between LVP and HDL cholesterol, and non-LVP with apoB and non-HDL cholesterol. In this small cohort of HCV G3 patients there was no association of LVP with liver stiffness or treatment response but this needs to be investigated in larger numbers.

6.8.2 LVP in HCV G1 correlate with insulin resistance and triglycerides

In HCV G1 there was a strong correlation between LVP load and insulin resistance as determined by HOMA-IR and QUICKI scores. LVP ratio also correlated significantly with insulin resistance as measured by HOMA-IR and QUICKI. In multivariate analysis, HOMA-IR accounted for approximately 16% of the variability of LVP load. Additionally LVP ratio correlated with triglycerides and TG/HDL ratio. The association of LVP ratio and TG/HDL ratio provides a possible explanation for the association of high TG/HDL ratio and poor response to anti-viral therapy identified from the logistic regression analysis of treatment outcomes in results chapter 3. In contrast there was no association between total viral load and any host clinical or metabolic parameter. The lack of association of total viral load with host factors has been reported previously in a study of 2472 HCV G1 patients. In this study, host factors including age and BMI correlated weakly with total viral load and explained only <0.05% of the variability of HCV RNA (Ticehurst, Hamzeh et al. 2007). The strong correlations in this study between LVP, insulin resistance and TG/HDL ratio but not total viral load in HCV G1 indicate that the host factors known to be associated with adverse clinical outcomes impact largely on the LVP fraction.

The association of HCV G1 LVP and insulin resistance is particularly intriguing. Epidemiological evidence indicates that patients with chronic hepatitis C have a high risk for development of insulin resistance and diabetes mellitus (Mehta, Strathdee et al. 2001; Hui, Sud et al. 2003; Mehta, Brancati et al. 2003; Huang, Dai et al. 2007; Serfaty and Capeau 2009; Park, Cho et al. 2010). This risk is particularly associated with HCV genotypes 1 and 4 (Moucari, Asselah et al. 2008). Moreover, suppression or clearance of HCV results in improved insulin sensitivity (Delgado-Borrego, Jordan et al.).

HCV may promote insulin resistance through genotype-specific molecular mechanisms (Pazienza, Clement et al. 2007; Douglas and George 2009) although recent evidence suggests insulin resistance in chronic hepatitis C is mainly in

peripheral skeletal muscle rather than the liver (Milner, Van Der Poorten et al. 2009). The development of insulin resistance in HCV is clinically important because it is associated with more advanced fibrosis (Fartoux, Poujol-Robert et al. 2005; Muzzi, Leandro et al. 2005; Svegliati-Baroni, Bugianesi et al. 2007), increased liver stiffness (Merchante, Rivero et al. 2009), the development of oesophageal varices (Petta, Camma et al. 2008; Camma, Petta et al. 2009; Petta, Camma et al. 2009) and poorer response to anti-viral therapy (Romero-Gomez, Viloria et al. 2005; Poustchi, Negro et al. 2008).

6.8.3 LVP in HCV G1 correlate with IP10

This study found that the second most important independent determinant of LVP ratio after triglyceride / HDL ratio was the serum IP10 level. This is a potentially very significant finding. In the previous chapter, IP10 levels correlated inversely with non-HDL cholesterol and apoB and provided a possible link between the lipid abnormalities associated with poor anti-viral treatment response in HCV G1 (i.e. low LDL cholesterol) and high IP10 levels that have been reported to be associated with poor interferon response (Butera, Marukian et al. 2005; Lagging, Romero et al. 2006). The correlation of LVP ratio and IP10 further links the formation of LVP with the lipid abnormailites associated with chronic stimulation of endogenous interferons. This may be an evolutionary adaptation of HCV to effectively evade the innate immune response or represent increased activation of innante immune defenses by HCV LVP compared to non-LVP.

6.8.4 LVP may be important in anti-viral treatment outcome

Antiviral treatment outcomes were available in a subgroup of 42 HCV G1 patients. In this group, LVP ratio was significantly higher in non-responders compared with those with a complete or partial EVR. It is possible therefore that increased LVP fraction contributes to persistent infection. Furthermore HCV G1 patients with higher LVP ratios had increased liver stiffness. It remains to be tested whether reducing LVP can improve clinical outcomes such as fibrosis or anti-viral treatment response or whether insulin sensitisers can lower LVP. Therapeutic interventions with insulin-sensitizing drugs, used either before or in combination with pegylated interferon-α and ribavirin are being evaluated with some conflicting results (Khattab, Emad et al. ; Romero-

Gomez, Diago et al. 2009). The data from this study indicate that insulin resistance may be a surrogate marker of LVP load. Measurement of LVP may be more relevant in determining patients that may benefit for adjunctive therapies such as lipid modulators or insulin sensitisers in future clinical trials. The measurement of LVP in appropriately powered larger prospective studies would confirm whether it could be clinically useful in predicting patient responsiveness to standard antiviral therapy and identifying patients that would benefit from receiving insulin sensitizing or lipid modulating therapies.

6.8.5 Mechanisms of LVP production in HCV G1

The association of LVP ratio with triglycerides implies that HCV G1 preferentially associates with VLDL1 rather than VLDL2. These two sub fractions of VLDL are defined by different flotation rates in salt gradients. VLDL1 (Sf 60-400) are considered to be larger and normally account for the majority of triglycerides in the fasting plasma, but are only a minority of apoB containing lipoproteins. VLDL2 (Sf20-60) are smaller, contain relatively less triglycerides but more cholesterol and are the predominant precursors of LDL which contain the majority of apoB in the plasma. The relationship between VLDL1 apoB and triglycerides was confirmed in this study by salt gradient ultracentrifugation methods in 23 HCV G1 patients. VLDL1 apoB was more strongly correlated with fasting serum triglycerides than VLDL2 apoB.

Although there is a clear association of HCV LVP with triglyceride rich VLDL1 and insulin resistance, it is not apparent from this study whether this is cause or effect. However, it is possible that it is both. There is now a body of evidence that links insulin resistance to over production of VLDL1 (Adiels, Olofsson et al. 2006; Adiels, Olofsson et al. 2008; Verges 2010). HCV promotes insulin resistance which has the effect of increasing VLDL1 and TG availability, which then increases LVP. Therefore insulin resistance promoted by HCV G1 may be impacting on the VLDL1 pathway and promoting the formation of LVP.

In fasting patients with both HCV G1 and HCV G3, LVP ratio varied widely, from <0.03 to >0.74. However, on average, LVP contributed less than a fifth of total viral load (median HCV G1 18%, HCV G3 14%). Instead, in the fasting state the majority of HCV RNA resides in the ‘non-LVP’ fraction. The amount of HCV in a particular

fraction will be determined by rates of input and clearance from the fraction. Inputs can be from *de novo* HCV production, and / or movement of virus from an adjacent density fraction above or below due to alterations in lipid and antibody association. Clearance can be due to receptor mediated uptake (infectivity) or movement into an adjacent density fraction by lipolysis or transfer (i.e. like exchangeable apolipoproteins). Given these assumptions, it is likely that the non-LVP fraction must have higher inputs from production and / or slower outputs than the LVP fraction. The LVP fraction may represent a minority of total viral load because of more rapid clearance of HCV from the LVP fraction. In the HCVcc system, low density HCV showed higher specific infectivity than high density HCV but contributed a minority of viral particles (Miyanari, Atsuzawa et al. 2007). By analogy to the HCVcc system, LVP *in vivo* may represent a highly infectious and rapidly clearing fraction of HCV in plasma.

The evidence presented in the previous chapters suggests that the key differences between the HCV genotypes in terms of lipid profiles is that HCV G3 patients have less apoB containing lipoproteins overall, specifically less large triglyceride rich VLDL, and a rapidly turning over LDL compartment. Therefore the difference in LVP correlations between the HCV genotypes may reflect the differences in the availability of TRLs in the low density fraction.

HCV LVP could be derived either from *de novo* assembly and secretion of HCV directly associated with apoB containing lipoproteins or from transfer from the high density (non-LVP) fraction onto TRL's in the LVP fraction, or a combination of both processes. There is mounting evidence to suggest that HCV co-assembles with VLDL (Huang, Sun et al. 2007; Gastaminza, Cheng et al. 2008; Yao and Ye 2008). However there is now also new evidence from additional work undertaken by our group that HCV is able to transfer directly onto TRL's including chylomicrons following a post prandial surge in TRL'(Felmlee, Sheridan et al. 2010). Intravascular HCV transfer onto TRLs appears to explain much of the redistribution into very low density fractions after a high fat meal. Moreover HCV in these very low density fractions ($d<1.025$ g/ml) appears to be rapidly cleared rather than migrating to the adjacent higher density fraction by lipolysis. If transfer is the predominant mechanism determining the amount of HCV LVP post prandially, then availability of an acceptor TRL pool is the limiting factor determining the amount of LVP present at any one

time. Given that transfer of HCV onto TRLs may be rapid, in the fasting state it is likely that transfer has reached a steady state. Not all TRLs may be equally efficient HCV acceptors. Large VLDL or chylomicrons may be more efficient acceptors for HCV transfer than small cholesterol rich LDL. These important potential mechanisms need to be tested in further experimental studies.

6.8.6 Hypothesis - a unifying LVP model

In light of the above evidence, a unifying model is proposed for HCV LVP in the fasting state (figure 44). In the model, the majority of HCV is likely to be held in a 'non-LVP' fraction in the fasting state, potentially associated with ApoA1/HDL, lipid poor apoB, antibodies or as 'free virions'. A minority of HCV is low density, associated with apoB containing lipoproteins VLDL, IDL and LDL as LVP, detected at d<1.07g/ml. HCV LVP may be derived from both *de novo* production of HCV LVP co-assembled with VLDL and from transfer onto TRL's from the 'non-LVP' fraction. In the model HCV is cleared *in vivo* predominantly from the LVP fraction into the liver via lipoprotein receptors. Furthermore, within the LVP fraction, HCV associated with very large TRL's such as VLDL1 and chylomicrons and TRL remnants may be cleared more rapidly than HCV associated with smaller VLDL2 / IDL / LDL, which may subsequently be shifted down into the 'non-LVP' fraction if bound by antibody.

In the model, HCV in the 'non-LVP' fraction is slowly cleared but can preferentially move to the LVP fraction for rapid clearance by directly transferring onto TRL's in the presence of sufficient TRL acceptors. HCV G1 may promote VLDL1 / remnant mediated clearance, whereas HCV G3 may promote a high turnover LDL mediated clearance pathway. There may be a trade off between promoting sufficient acceptor TRL's and competition for hepatocyte entry via shared lipoprotein receptors.

In the fasting state, HCV transfer from 'non-LVP' to LVP is likely to have reached a steady state, its rate limited by the availability of acceptor TRL's. The mechanism of transfer is completely unknown, but candidate mediators include CETP or exchangeable lipoproteins such as ApoE or apoC's. In HCV G1, insulin resistance promotes the formation of large TG rich VLDL1. This favours HCV which is both co-assembled / secreted with VLDL1 and excess VLDL1 are also available as acceptors for HCV transferring from the 'non-LVP' fraction. This accounts for the positive correlations between LVP and TG & TG/HDL ratios in HCV G1. It also

accounts for the association of HCV G1 LVP and insulin resistance. However in HCV G3 infection, there is reduced VLDL production and limited TRL's available. Moreover there is a smaller LDL pool because of rapid LDL clearance. Therefore in HCV G3, the predominant determinant of LVP is ApoA1/HDL because this determines how much HCV can transfer from the predominant 'non-LVP' fraction into the LVP fraction for rapid clearance. In situations of high apoA1/HDL, less HCV can transfer to the LVP fraction for clearance, hence lower LVP levels. When there is reduced apoA1/HDL, more HCV can transfer to LVP, hence higher LVP levels. Likewise if there is an increase in the LDL acceptor pool (i.e. increased apoB and non-HDL cholesterol) then the non-LVP fraction gets smaller because more is being pulled up as LVP which is then rapidly cleared. Therefore in the model the balance between apoA1/HDL lipoproteins and apoB containing lipoproteins is critical for the relative distribution of HCV between the LVP and non-LVP fractions in the fasting state.

In summary this is the first accurate measurement of the LVP fraction of total hepatitis C viral load in a well-characterized cohort of HCV G1 and HCV G3 patients. The results show that LVP ratio varies at least 25-fold between patients and is strongly associated with metabolic parameters in both genotypes and important clinical outcomes in HCV G1. In particular, higher LVP ratio associates strongly with insulin resistance and higher TG levels in HCV G1, both components of the metabolic syndrome. Higher LVP ratios were also found in non-responders to antiviral treatment, higher IP10 levels and those with more advanced fibrosis. This study offers further insight into the life-cycle of HCV outside the liver in the circulation and suggests that HCV preferentially associates with triglyceride rich lipoproteins. In HCV G1 infection, the promotion of insulin resistance to increase VLDL1 like TRL's may drive the production of more infectious LVP, providing an explanation of why HCV G1 patients with insulin resistance have poorer response to therapy than those without insulin resistance and those with HCV G3.

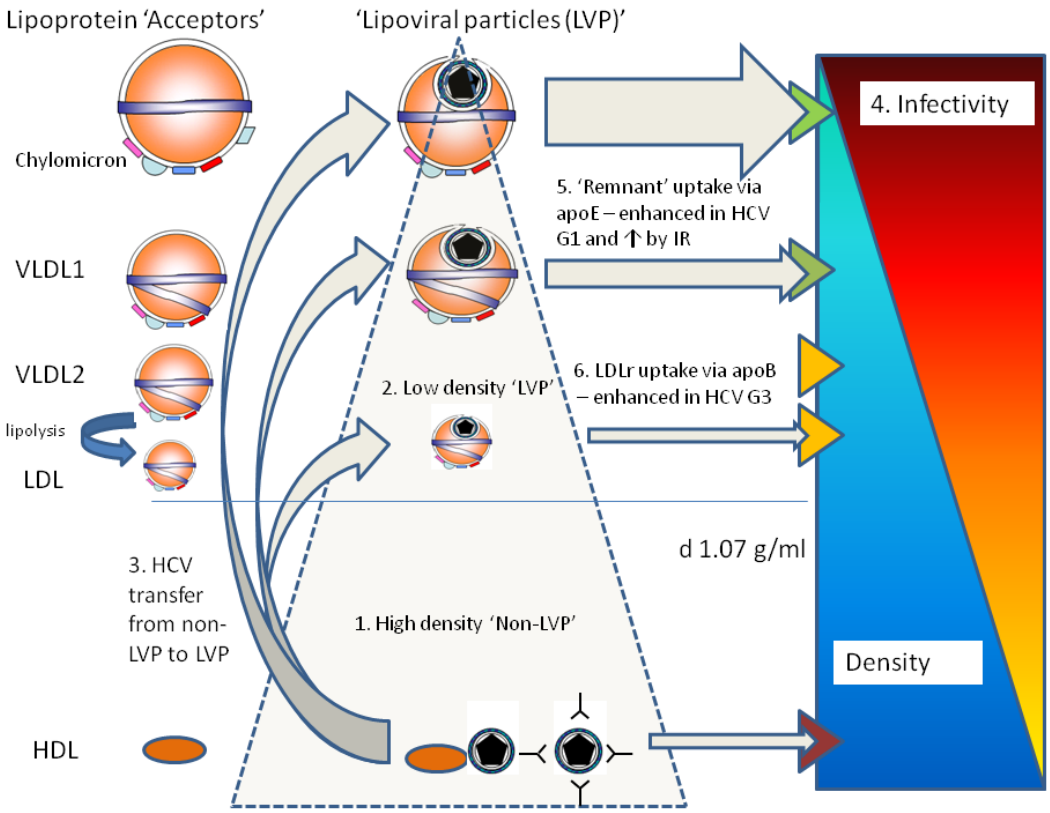


Figure 44 The'Lipoviral particle (LVP)' model

1. The majority of HCV particles in the fasting state are high density (d>1.07 g/ml) 'non-LVP', associated with HDL / ApoA1, lipid poor apoB, antibodies or 'free' virions. The 'non-LVP' represent the largest proportion of total viral load (represented by the dashed line triangle);2. A minority of total HCV in the fasting state is low density (d<1.07 g/ml), associated with apoB containing lipoproteins, VLDL, IDL and LDL as'LVP'; 3. 'Non-LVP' can transfer to a lipoprotein acceptor into the 'LVP' fraction to enhance clearance; 4. Low density 'LVP' are more rapidly cleared and of higher infectivity compared to the high density 'non-LVP'. 5. HCV G1 has selective adaptations that favour the VLDL1 / remnant uptake pathway via apoE mediated clearance. This is enhanced by insulin reistance; 6. HCV G3 in contrast promotes high turnover LDL clearance mediated via apoB binding to LDLr.

7 Chapter 7. A randomised, controlled, factorial pilot study investigating Omacor and/or Fluvastatin in patients with chronic hepatitis C who have not responded to standard combination anti-viral therapy

7.1 Introduction and aims

Current treatment options are limited for HCV patients that have previously not responded to combination PegIFNα and ribavirin therapy. In those that failed to achieve at least a 2 log reduction in HCV RNA (null responders), current guidelines do not advocate re-treatment because of very low response rates (Ghany, Strader et al. 2009). Although specifically targeted anti-viral therapies for hepatitis C (STAT-C's) are in phase III clinical trials, these new agents require the addition of PegIFNα and ribavirin to minimise breakthrough of resistant HCV strains (Thompson and McHutchison 2009; Chary and Holodniy 2010; Lange, Sarrazin et al. 2010; Michaels and Nelson 2010). Therefore alternative strategies are required for this group of difficult to treat patients. This pilot trial was designed to evaluate whether lipid modulating therapy in the form of Fluvastatin and Omega 3 polyunsaturated fatty acids (Omacor) could have a beneficial impact in non-responders to previous anti-viral therapy. Specifically, given the close relationship between HCV and VLDL metabolism for the production of HCV lipoviral particles (LVP) and the reliance of HCV on cholesterol pathways for viral replication (Ye 2007), this study aimed to determine whether lipid modulating therapy can reduce total viral load, or factors that influence HCV LVP such as triglycerides and IP10 (see results chapter 4), high levels of which are also associated with non-response to PegIFNα and ribavirin. HCV LVP is also being measured as an end-point but at the time of writing this data was not yet available.

The potential benefits of lipid modulating agents as adjunctive therapy for HCV is supported by a number of pre-clinical studies. Statins are a class of drug that are HMG CoA reductase inhibitors. In HCV genomic and subgenomic replicons, Lovastatin markedly suppressed HCV RNA levels (Ye, Wang et al. 2003). The inhibitory effect of Lovastatin was mediated by inhibiting geranylgeranylation of a

host protein FBL2 required for HCV replication (Kapadia and Chisari 2005). Different statins appear to have different effects on HCV replication, with Lovastatin showing the weakest anti-HCV activity, Pravastatin having no anti-HCV activity and Fluvastatin possessing the strongest anti-HCV activity (IC50: 0.9micro-molar) (Ikeda, Abe et al. 2006). In combination with interferon α (IFN), Fluvastatin possessed the strongest synergistic inhibitory activity (Ikeda, Abe et al. 2006), hence was the statin of choice for this pilot trial.

Several polyunsaturated fatty acids (PUFA's) including arachadonic acid (AA), docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) dramatically inhibit HCV replication *in vitro* using the HCV RNA replicon system (Kapadia and Chisari 2005). A strong synergistic anti-HCV effect has also been observed when n3 PUFA were used in combination with IFN (Leu, Lin et al. 2004). Data suggests that PUFA's may inhibit HCV replication *in vitro* by a mechanism that is independent of their ability to suppress lipogenic gene expression (Kapadia and Chisari 2005).

This trial utilised a factorial design. The major advantage of the factorial design is its efficiency such that two interventions can be investigated in a single trial. Analysis of the trial was undertaken according to the principles set out by McAlister et al (McAlister, Straus et al. 2003). This factorial trial was designed to be analysed "at the margins" by comparing outcomes of all patients treated with Fluvastatin (with or without Omacor) vs no Fluvastatin. The efficacy of Omacor was assessed by comparing all those taking Omacor to those not on Omacor (with or without Fluvastatin). When the two treatments act independently, addititve effects would be expected and this analysis "at the margins" is appropriate. However when the intervention effects are less than additive or more than additive (synergistic) then an interaction is occurring and appropriate analysis should be performed "inside the table" for each separate group. The aim of this pilot trial was to evaluate whether n3 PUFA fish oils [Omacor] and/or Fluvastatin, as monotherapy and combination therapy shows a trend towards efficacy in patients with chronic hepatitis C who have not had a SVR to standard combination anti-viral therapy [non-responders]. This was a pilot study looking at short-term treatment and was not expected to produce definitive results. This pilot study was open, that is both physicians and patients were aware of the treatment group to which patients have been allocated. However the

primary outcome variable was objective and was not expected to be influenced unduly by knowledge of the treatment group. A larger definitive study would be run blind.

7.2 Methods –study design and analysis.

7.2.1 Recruitment

Patients were recruited from two centers, Freeman Hospital, Newcastle upon Tyne and St Mary's Hospital, Imperial College, London. Patients were approached to take part in the study through normal follow-up clinics and had as long as they needed before coming to a decision whether or not to participate. Once informed consent had been signed, participants were scheduled for a screening visit to confirm inclusion and exclusion criteria. Participants were then randomised to one of 6 groups described below. The recruitment target was for 72 patients to be randomised, 12 in each of six groups.

7.2.2 Inclusion and Exclusion criteria

To be eligible for this study, participants had the following inclusion criteria: age≥18 years; positive Hepatitis C RNA for more than 6 months; elevated serum alanine transaminase (ALT) above normal limits, as defined by Prati D. et al. (Annals of Internal Medicine 2002: 137: 1-9); previous lack of sustained virological response (SVR) to treatment with standard combination anti-viral therapy (Standard interferon α and Ribavirin and/or Pegylated interferon α and Ribavirin); no lipid modulating agents for at least 3 months; negative urine pregnancy test (for women of child bearing potential) documented within the 48 hour period prior to the first dose of test drug. Additionally all participants had to ensure adequate contraception during and for one month after treatment.

The following exclusion criteria were applied: HBV, HDV or HIV co-infection; a medical condition associated with chronic liver disease other than viral hepatitis, specifically excluding non-alcoholic fatty liver disease by Body Mass Index (BMI) ≥ 30; clinical evidence of decompensated cirrhosis (ascites, portal hypertension with Grade 2 oesophageal varices, hepatocellular cancer); alcohol use in excess of safe limits [28 units per week for men and 21 units per week for women]; unable to conform to study protocol due to alcohol misuse or drug abuse; serum alphafoetoprotein ≥ 100; platelet count < 60,000 cells per/ml; any research study within previous 3 months; severe seizure disorder or concurrent phenytoin use; lactation; history of muscular toxicity secondary to statins or fibrates; hereditary

muscle disorder or family history of hereditary muscle disorder; concurrent anti-coagulant use.

7.2.3 Study Groups

This study was designed as a 3x2 factorial, randomised, open pilot trial. A factorial design was chosen for its efficiency allowing two treatments to be tested simultaneously, and for its ability to detect any additional effects of using both Omacor and Fluvastatin in combination. All patients received 12 weeks of either no active drug, Omacor in 2 different doses, Fluvastatin 40mg for 4 weeks, increasing to 80mg for a further 8 weeks or a combination of the active drugs in a factorial design (see Table 7-1). Group A received no active drug and acted as a control group.

Table 7-1 Factorial design of HCV lipid pilot study

Intervention	**No Omacor**	**Omacor 1 gram**	**Omacor 2-4 grams**
No Fluvastatin	Group 1 (A)	Group 2 (B)	Group 3 (C)
Fluvastatin	Group 4 (D)	Group 5 (E)	Group 6 (F)

Patients were randomised to:

Group A – No active drug for 12 weeks

Group B - Omacor 1g daily for 12 weeks

Group C - Omacor 2g daily for 4 weeks increasing to 1g q.d.s. from week 5-12

Group D - Fluvastatin 40mg daily for 4 weeks, then 80mg daily from week 5-12.

Group E - Omacor 1g daily for 12 weeks, combined with Fluvastatin 40mg daily for 4 weeks, then 80mg daily from week 5-12

Group F - Omacor 2 g daily for 4 weeks combined with Fluvastatin 40mg daily for 4 weeks, then Omacor 1 g q.d.s and Fluvastatin 80mg daily from week 5-12.

7.2.4 Randomisation

Randomisation was stratified according to HCV genotype [genotype 1 & other, or genotypes 2 & 3] as this has been shown to influence response to standard combination anti-viral therapy. A blocked allocation system was used to allocate patients to the 6 groups (block size was not disclosed to the investigators).

Randomisation was administered centrally via Newcastle Clinical Trials Unit using a secure web based system. www.ncl.ac.uk/random

7.2.5 Omacor

Omacor is a fish oil that contains Omega-3-acid ethyl esters. It is licensed for adjunctive therapy to diet for the reduction of elevated triglycerides and for secondary prevention post myocardial infarction. It was taken either in the dose of 1 capsule daily (Groups B and E) or in the recommended dose of initial treatment with 2 capsules daily, increased to 4 capsules daily at end of week 4 (Groups C and F).

Common side-effects encountered are predominantly gastro-intestinal (GI) and include nausea, dyspepsia, abdominal distension, abdominal pain, eructation and diarrhoea. If GI side effects were encountered the dose was reduced to the maximum tolerated.

7.2.6 Fluvastatin

Fluvastatin is licensed for adjunctive therapy to diet in mixed dyslipidaemia, in primary hypercholesterolaemia and for secondary prevention of coronary events post percutaneous coronary intervention. It was taken in the recommended starting dose of 40mg once daily and, as the maximum reduction in LDL-C at a given dose is seen within 4 weeks, the dose was adjusted at 4 weeks to the recommended maximum daily dose of 80mg. Common anticipated side-effects include myalgia, muscle pain and muscle weakness, GI symptoms such as flatulence, nausea, diarrhoea and allergic reactions such as rash and angioedema have been reported. If there was evidence of myopathy &/or creatine kinase levels >5x upper limit of normal the statin was discontinued. If GI side effects were encountered the dose was reduced to the maximum tolerated.

7.2.7 Study Visits

There were seven study visits in total. All participants had a screening visit up to 8 weeks prior to starting treatment. Following randomisation, participants received treatment for 12 weeks and were followed up at day 2, weeks 4, 8, 12 and week 24. The age, sex, BMI, waist circumference and blood pressure were recorded in all participants. As chronic HCV infection is associated with insulin resistance and

diabetes, all participants had a two-hour seven-sample oral glucose tolerance test (OGTT) to formally assess glucose tolerance status and insulin sensitivity prior to commencing treatment at the screening visit. All participants were well characterised by a questionnaire detailing dietary history, alcohol, smoking and exercise. Additionally a depression and anxiety questionnaire (HAD score) was used to detect any exacerbation in symptoms during therapy. Participants were seen at each visit after a 12 hour fast. Patients on medication for diabetes were given specific instruction to delay taking hypoglycaemic medication on the morning of each visit until after the fasting blood tests to avoid hypoglycaemia

7.2.8 Trial discontinuation criteria (withdrawal from study drug)

Participants were withdrawn from the study drug if any of the following occurred: serious or life-threatening adverse reaction; participant found to be in violation of the protocol eligibility criteria; failure to comply with the dosing, evaluations or other requirements of the study; request of the participants (participants had the right to discontinue treatment at any time for any reason); the investigator felt that discontinuation is in the best interest of the participant; pregnancy; myopathy &/or creatine kinase levels >5x upper limit of normal; ALT levels > 5 x the baseline level.

7.2.9 Concomitant medication and treatment

Systemic anti-viral, anti-neoplastic and immunomodulatory treatments (including steroids and radiation) were not allowed during the entire study period. Steroids given as replacement were permitted. Other investigational drugs and herbal or other remedies being taken for possible or perceived effects against HCV were excluded. A complete listing of all concomitant drugs received was recorded in the electronic case report form (eCRF).

7.2.10 Safety data - Definitions

An adverse event (AE) was defined as any untoward medical occurrence. An AE did not necessarily have a causal relationship with the treatment. "Treatment" included all investigational agents (including comparative agents) administered during the course of the study. Medical conditions/diseases present before starting study treatment were only considered adverse events if they worsened after starting study treatment.

An adverse reaction (AR) was defined as any untoward or unintended responses to an Investigational Medicinal Product (IMP) related to any dose administered - All AEs judged by either the reporting investigator or the sponsor as having reasonable causal relationship to a medicinal product qualified as adverse reactions. An assessment of causality was made using the definitions listed in table 7-2.

A Serious Adverse Event (SAE) or Serious Adverse Reaction: was defined as any untoward medical occurrence or effect that at any dose resulted in death, or was life-threatening, required hospitalisation or prolongation of existing inpatients' hospitalisation, resulted in persistent or significant disability or incapacity, or resulted in a congenital anomaly or birth defect.

Suspected, Unexpected Serious Adverse Reactions (SUSARs) were defined as an adverse reaction that was both unexpected and serious. An adverse reaction was 'unexpected' if its nature or severity was not consistent with the applicable product information.

Table 7-2 Adverse event causality definitions

Relationship	Description
Unrelated	No evidence of any causal relationship
Unlikely	Little evidence to suggest a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatment).
Possible	Some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments).
Probable	Evidence suggests a causal relationship and the influence of other factors is unlikely.
Definitely	Clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.
Not assessable	Insufficient or incomplete evidence to make a clinical judgement of the causal relationship.

7.3 Trial research governance

7.3.1 Data Monitoring

Monitoring of study conduct and data collected was performed by a combination of central review and site monitoring visits. Study site monitoring was undertaken by Newcastle Clinical Trials Unit. The main areas of focus were consent, serious adverse events, essential documents in study files and drug accountability & management.

7.3.2 Clinical Trial Authorisation (CTA)

The trial had Clinical Trials Authorisation from the UK Competent Authority; MHRA. Reference: 31008/0001/001-0001 and EudraCT reference 2006-004335-29.

7.3.3 Ethics Approval

The trial had approval from the Fife and Forth Valley Research Ethics Committee REC Ref: 07/S0501/21. The study was conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

7.4 Statistics and data analysis

The two primary outcome measures were a fall in HCV total viral load (log10 IU/ml) and ALT from pre-treatment (average of screening and baseline visits) to end of treatment week 12. The main hypothesis was that PUFAs [Omacor] as monotherapy or in combination with Fluvastatin would alter cholesterol and fatty acid biosynthesis pathways in the liver and that this will reduce the level of HCV replication and inflammation as assessed by HCV RNA and ALT level. These two primary outcomes were evaluated by intention to treat analysis in 56 patients that attended all study visits, including all available data even in those that did had to discontinue the study drugs prematurely due to side effects (figure 45). Separate analysis was performed in 50 patients that completed the full 12 weeks protocol of study drugs to evaluate direct treatment effects, although this may marginally bias against those that could not tolerate treatment due to side effects.

This was a pilot study and not expected to clear of HCV RNA in any participant but was looking for evidence of a treatment effect in decreasing HCV RNA and liver damage. If a treatment effect was found in any group, then larger studies would be needed. The FDA (Food and Drug Agency) recommend that 12 subjects per arm represents a sufficient number of subjects for a pilot study (FDA Guidance for industry (http://www.fda.gov/cder/guidance/5356fnl.pdf.).

The factorial design of this pilot study allowed 'at the margins analysis' of patients receiving no Fluvastatin (Groups A,B & C) *versus* receiving Fluvastatin (Groups D,E & F). Similarly analysis was performed comparing Omacor *versus* No Omacor and the 2 Omacor doses (No Omacor: Groups A & D *versus* Omacor 1 gram daily: Groups B & E *versus* Omacor 2-4 grams daily; Groups C & F). Lastly the effect of combination therapy could be assessed by comparing the combination Omacor and Fluvastatin (groups E & F) with Fluvastatin monotherapy (group D), Omacor monotherapy (groups B & C) or no drug (group A).

A formal power calculation for the pilot trial was not made. However, analysis of pre treatment HCV RNA viral loads measured routinely in the clinic by Roche Amplicor PCR in 152 patients demonstrated a mean total HCV RNA viral load of 5.413 $\log_{10}$ IU/ ml with a standard deviation of 0.694 $\log_{10}$ IU / ml. Therefore a minimum number of 48 patients (8 per group) would allow detection of a difference of 0.711 $\log_{10}$ in

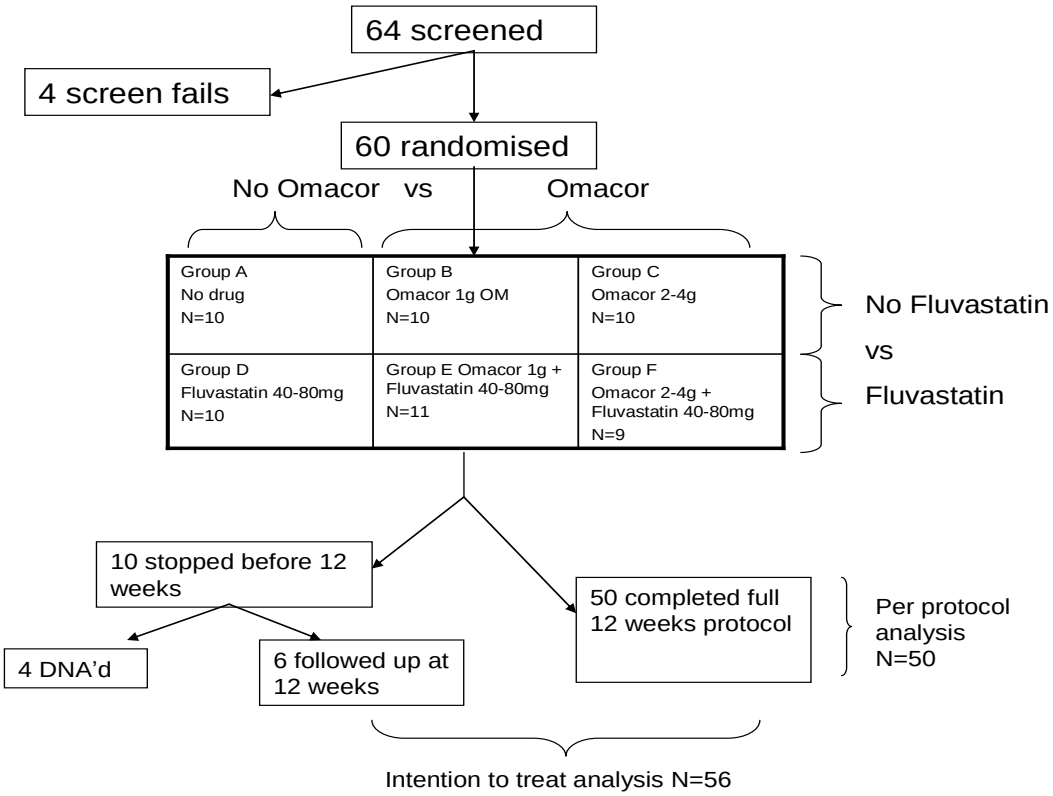
total HCV RNA with 80% power at a significance level (α) of 0.05 using a 2 sample t test, by combining groups together so that n is at least 16 in each group (i.e. those on low dose Omacor (Groups B+C) vs no Omacor (A+D) vs high dose Omacor (C+F) and Fluvastatin (D+E+F) vs no fluvastatin (A+B+C). This compares favourably to the only other published trial of Fluvastatin in 31 HCV patients which observed a maximum 1.75 $\log_{10}$ reduction in total HCV RNA (Bader, Fazili et al. 2008). The purpose of the analysis was to make a preliminary assessment of outcome in order to inform the planning of a larger trial; thus sample size calculations had not been performed and this study did not have the power to make a definitive conclusion.

The purpose of the factorial design of the trial was to allow evaluation of the treatment effect of two drugs (Fluvastatin and Omacor) to be tested simultaneously.

Fluvastatin - The effects of Fluvastatin were evaluated by comparing the combined groups randomized to Fluvastatin, either alone or in combination with Omacor (i.e groups D + group E + group F) and those not taking any Fluvastatin (i.e group A + group B + group C). Paired differences in total viral load, liver function tests, lipid profiles, liver stiffness and IP10 were calculated by subtracting the week 12 data from the average of the screening and baseiline visits. The null hypothesis of a paired difference of zero was tested using a 1 sample t-test.The data for this analysis is shown in table 7-5.

Omacor – The effects of Omacor, either alone or in combination with Fluvastatin were evaluated by combining all those on Omacor (groups B + C + E + F) and those not on Omacor (groups A + D). The effect of low dose Omacor was evaluated by combining group B + group E. The effect of high dose Omacor was evaluated by combining group C + group F and no Omacor by combining group A + group D. Paired differences in total viral load, liver function tests, lipid profiles, liver stiffness and IP10 were calculated by subtracting the week 12 data from the average of the screening and baseiline visits. The null hypothesis of a paired difference of zero was tested using a 1 sample t-test and the data is summaried in table 7-6.

Figure 45 Trial summary of screening, randomization and analysis.



64 HCV patients that were previous non-responders to Pegylated interferon-α and ribavirin therapy were screened. 60 were randomized to one of six treatment groups in a 3 x 2 factorial design. 10 patients did not complete the full 12 weeks of study drugs, of whom 4 did not attend further follow-up and 6 had adverse events. 50 patients completed the protocol.

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7.5 Results

7.5.1 Demographics / baseline characteristics

Sixty-four patients were consented and screened for the trial. There were four screen failures, hence 60 patients were randomized. The reasons for two of the screen fails was BMI>30; another screen fail was due to platelets <60,000, and the remaining screen failure was due to normal ALT and non-compliant with protocol. Of the 60 participants randomized, 10 did not complete the full 12 weeks of study drug. The reasons for withdrawal from the study drug are discussed later and given in table 7-8. Of the 10 not completing the protocol, 6 still attended for a week 12 visit (group B = 1, group C =1, group D = 1, group E = 2 and group F = 1), so viral load and ALT data from this visit was included in intention to treat analysis of the primary end-points. Separate completion of protocol only analysis was performed in those 50 participants that completed 12 weeks of the study drug to evaluate any treatment effect (summarized in figure 44).

The baseline characteristics of the 60 patients initially randomized is shown in table 7-3. Participants randomized to group D were older than those in group A (one way ANOVA p=0.05), but there was no difference in body mass index (p=0.523). There were no significant differences in ALT, AST or GGT levels between the trial groups. However, despite randomisation, lipid profiles were significantly different between trial groups, with lower total cholesterol and non-HDL cholesterol in groups C (high dose Omacor) and E (low dose Omacor + Fluvastatin) than in the other groups (table 7-3). When the groups were sub-analysed according to viral genotype - HCV G1&4, (HCV G1 n=33, + HCV G4 n= 6) and HCV G2&3 (HCV G2 n= 2, HCV G3 n=19), it is apparent that the difference in non-HDL cholesterol is accounted for by the HCV G3 patients rather than HCV G1 & G4. Comparison of trial groups for HCV G1 & G4 only, indicated no significant differences in total or non-HDL cholesterol table 7-4. However in the HCV G3 sub-population, total and non-HDL cholesterol remained significantly lower in groups C (high dose Omacor) and E (low dose Omacor + Fluvastatin) (one way ANOVA p=0.006). Pre-treatment triglycerides were lower in group B (low dose Omacor) and C (high dose Omacor) compared to group A (control) in HCV G1&4 (one way ANOVA p=0.040). HDL was higher in group B (low dose

Omacor) compared to E (low dose Omacor + Fluvastatin) in those with HCV G1&4 (one way ANOVA p=0.018).

Analysis of completed protocol was performed in those 50 randomised participants that completed 12 weeks of study drug. The analysis calculated the paired differences at week 12 vs the average of screening and baseline visits and compared this calculated difference to the null hypothesis of a mean difference of zero, using a 1 sample t-test. The significance level was defined at $p<0.05$. This approach comparing the paired differences with or without an intervention therefore minimised the statistical relevance of the differences between study groups in baseline lipid parameters in the subsequent analysis.

Table 7-3 HCV lipid trial baseline characteristics – Liver function tests and fasting lipid profiles

Group (N=60)	A (10)	B (10)	C (10)	D (10)	E (11)	F (9)	P value ANOVA (reason for difference)
Treatment	Controls	Omacor 1g	Omacor 2-4g	Fluvastatin	Omacor 1g + Fluvastatin	Omacor 2-4g + Fluvastatin	
Did not complete 12 weeks study drug (N=10)	2	1	1	2	3	1	
Age	47.9 ± 5.4	49.1 ± 6.3	50.0 ± 8.8	56.8 ± 7.4	50.8 ± 8.2	54.2 ± 7.1	0.05 (D>A)
Sex m/f	7/3	9/1	6/4	4/6	10/1	5/4	
BMI	25.5 ± 3.5	25.1 ± 2.6	24.9 ± 3.5	25.8 ± 3.1	26.6 ± 2.9	24.8 ± 2.4	0.523
HCV G1	7	5	6	6	5	4	
HCV G3	2	4	3	3	4	3	
other	1 (G2)	1 (G4)	1 (G4)	1 (G2)	2 (G4)	2 (G4)	
ALT	93 ± 53	93 ± 39	88 ± 72	78 ± 39	119 ± 91	70 ± 27	0.561
AST	82 ± 55	72 ± 36	74 ± 54	58 ± 25	87 ± 66	54 ± 22	0.572
GGT	99 ± 72	112 ± 59	104 ± 58	96 ± 72	91 ± 101	107 ± 83	0.985
Total cholesterol	5.18 ± 0.76	4.54 ± 0.89	3.45 ± 0.80	4.18 ± 0.98	3.80 ± 1.08	4.46 ± 0.74	0.003 (A>C&E)
Non HDL-Cholesterol	3.97 ± 0.76	3.02 ± 0.72	2.36 ± 0.61	2.87 ± 0.87	2.72 ± 1.13	2.94 ± 0.73	0.005 (A>C&E)
HDL cholesterol*	1.23 ± 0.29	1.50 ± 0.14	1.10± 0.25	1.28 ± 0.409	1.08 ± 0.23	1.58 ± 0.42	0.002 (A>B)
Triglyceride*	1.63 ± 0.77	0.87 ± 0.31	1.03 ± 0.46	1.16 ± 0.39	1.17 ± 0.97	1.05 ± 0.30	0.156
TG/HDL	1.51 ± 1.09	0.59 ± 0.25	0.97 ± 0.46	1.03 ± 0.54	1.15 ± 1.06	0.72 ± 0.31	0.114
HOMA IR	1.71 ± 0.48	1.40 ± 0.54	2.31 ± 0.58	2.06 ± 0.76	1.95 ± 1.28	2.03 ± 0.97	0.526

*Mean ± standard deviation *ANOVA performed on log10 triglyceride and. log10 HDL.*

Table 7-4 HCV G1 & 4 Baseline lipid profiles

	Group A Controls N=7	Group B Omacor 1g N=7	Group C Omacor 2-4g N=6	Group D Fluvastatin N=6	Group E Omacor 1g +Fluvastatin N=7	Group F Omacor 2-4g +Fluvastatin N=6	P value ANOVA
Total cholesterol	4.89 ± 0.63	4.54 ± 1.09	3.83 ± 0.46	4.31 ± 1.15	4.33 ± 0.97	4.66 ± 0.81	0.435
Non HDL cholesterol	3.81 ± 0.72	3.00 ± 0.86	2.62 ± 0.40	3.02 ± 1.08	3.25 ± 1.02	3.21 ± 0.75	0.283
Triglycerides*	1.79 ± 0.86	0.94 ± 0.34	0.92 ± 0.28	1.35 ± 0.35	1.63 ± 1.02	1.16 ± 0.23	0.040
HDL cholesterol*	1.08 ± 0.22	1.50 ± 0.15	1.21 ± 0.20	1.24 ± 0.37	1.08 ± 0.26	1.46 ± 0.25	0.018

*Mean ± standard deviation *ANOVA performed on Log10 triglycerides and log10 HDL cholesterol*

Table 7-5 Baseline to week 12 change in viral, biochemical and liver stiffness parameters in those completing 12 weeks of study drug –Effect of Fluvastatin

	Fluvastatin (groups D+E+F) N=24		**No Fluvastatin (groups A+B+C) N= 26**	
	Mean change (±SD)	p-value (95% CI)	Mean change (±SD)	p-value (95% CI)
Total viral load (log10 IU/ml)	-0.10 (±0.39)	0.249 (-0.27, 0.07)	+0.03 (±0.50)	0.788 (-0.21, 0.27)
ALT u/l	+17.0 (±37.1)	**0.035 (1.3, 36.6)**	+25.5 (±70.3)	0.096 (-4.9, 55.9)
AST u/l	+15.3 (±27.0)	**0.011 (3.9, 26.6)**	+16.5 (±55.6)	0.160 (-70, 40)
GGT u/l	+19.5 (±44.2)	**0.042 (0.8, 38.1)**	+16.0 (±51.2)	0.158 (-6.7, 38.6)
Total Cholesterol mmol/l	-0.77 (±0.55)	**<0.001 (-1.00, -0.54)**	+0.11 (±0.57)	0.359 (-0.14, 0.37)
Non-HDL cholesterol mmol/l	-0.81 (±0.72)	**<0.001 (-1.11, -0.51)**	+0.04 (±0.51)	0.702 (-0.183, 0.266)
HDL cholesterol mmol/l	+0.02 (±0.30)	0.761 (-0.11, 0.15)	+0.07 (±0.18)	0.095 (-0.01, 0.15)
Triglycerides mmol/l	-0.28 (0.38)	**0.002 (-0.44, -0.11)**	-0.19 (±0.37)	**0.018 (-0.34, -0.04)**
ApoB g/l	-0.17 (±0.12)	**<0.001 (-0.22, -0.11)**	+0.03 (±0.14)	0.366 (-0.03, 0.08)
Liver stiffness kPa	+0.2 (±8.4) N=19	0.916 (-3.8, 4.3)	-1.4 (±4.84) N=17	0.243 (-3.9, 1.1)
IP10 pg/ml	-18.6 (±128.7)	0.496 (-74.2, 37.1)	-79.7 (±187.5)	0.059 (-162.8, 3.4)

Table 7-6 Baseline to week 12 change in viral, biochemical and liver stiffness parameters in those completing 12 weeks of study drug – Effect of Omacor

	All Omacor Groups B+C+E+F N=34		**Low dose Omacor Groups B+E N= 17**		**High dose Omacor Groups C+F N=17**		**No Omacor Groups A+D N=16**	
	Mean change (±SD)	p-value (95% CI)	Mean change (±SD)	p-value (95% CI)	Mean change (±SD)	p-value (95% CI)	Mean change (±SD)	p-value (95% CI)
Total viral load (log10 IU/ml)	-0.07 (±0.45)	0.412 (-0.24, 0.10)	-0.13 (±0.37)	0.125 (-0.37, 0.05)	-0.02 (±0.51)	0.884 (-0.28, 0.32)	+0.02 (±0.45)	0.849 (-0.24,0.29)
ALT u/l	+12.6 (±31.3)	**0.030 (1.3, 23.8)**	+7.8 (±28.9)	0.300 (-7.6, 23.1)	+17.4 (±33.7)	0.057 (-0.6,35.4)	+39.5 (±86)	0.097 (-8.2, 87.1)
AST u/l	+8.2 (±23.4)	**0.053 (-0.10, 16.5)**	+4.3 (±18.1)	0.348 (-5.1, 13.5)	+12.4 (±28.0)	0.097 (-2.5, 27.3)	+32.7 (±67.6)	0.082 (-4.7, 70.2)
GGT u/l	+19.5 (±50.3)	**0.039 (1.1, 38.0)**	+15.0 (±49.0)	0.256 (-12.2, 42.2)	+23.8 (±52.6)	0.091 (-4.3, 51.8)	+14.2 (±41.5)	0.206 (-8.8, 37.2)
Total Cholesterol mmol/l	-0.27 (±0.75)	0.058 (-0.54, 0.01)	-0.27 (±0.80)	0.218 (-0.71, 0.18)	-0.26 (±0.73)	0.165 (-0.65, 0.12)	-0.52 (±0.61)	**0.006 (-0.86, -0.18)**
Non-HDL cholesterol mmol/l	-0.37 (±0.82)	**0.019 (-0.67, -0.07)**	-0.34 (±0.68)	0.070 (-0.72, 0.03)	-0.39 (±0.96)	0.126 (-0.90, 0.12)	-0.48 (±0.60)	**0.008 (-0.81, -0.14)**
HDL cholesterol mmol/l	+0.04 (±0.18)	0.181 (-0.02, 0.11)	+0.09 (±0.21)	0.116 (-0.03, 0.21)	+0.001 (±0.14)	0.986 (-0.07, 0.08)	+0.04 (±0.37)	0.694 (-0.16, 0.24)
Triglycerides mmol/l	-0.26 (±0.40)	**0.001 (-0.40, -0.12)**	-0.33 (±0.48)	**0.010 (-0.57, -0.09)**	-0.18 (±0.25)	**0.016 (-0.31=2,-0.04)**	-0.17 (±0.32)	0.066 (-0.35, 0.01)
ApoB g/l	-0.06 (±0.17)	0.052 (-0.13, 0.01)	-0.11 (±0.17)	**0.030 (-0.20, -0.01)**	-0.02 (±0.17)	0.647 (-0.11, 0.07)	-0.09 (±0.15)	**0.029 (-0.17, -0.01)**
Liver stiffness kPa	-1.6 (±6.0) (N=25)	0.201 (-4.0, 0.9)	-3.4 (±7.6)	0.157 (-8.2, 1.5)	+0.1 (±3.3) (N=13)	0.929 (-1.9, 2.1)	+1.7 (±8.6) (N=11)	0.525 (-4.1, 7.5)
IP10 pg/ml	-53.5 (±167)	0.085 (-114.7, 7.8)	-97.7 (±160)	**0.019 (-177.3, -18.1)**	+7.7 (±162.6)	0.867 (-90.5, 105.9)	-37.3 (±153.3)	0.380 (-125.8, 51.3)

7.6 Primary Outcomes

There were two primary oucome measures: 1) change in total HCV RNA viral load, 2) change in ALT after 12 weeks of study drug.

7.6.1 Total HCV RNA viral load

The primary outcome measure was a change in total HCV RNA viral load after 12 weeks of intervention. 'Within the table' analysis indicated that there was no significant reduction in total HCV RNA between baseline and week 12 in any of the treatment groups in the overall (figure 46). However this analysis is greatly limited by the small numbers in any individual treatment group. The factorial design enabled comparison of those on Fluvastatin (groups D+E+F) *vs* not on Fluvastatin (groups A+B+C) to be compared (table 7-7). There was no significant difference in total viral load at pre treatment or at week 12 of treatment with Fluvastatin compared to no Fluvastatin in either the 'intention to treat' analysis or only in those completing the protocol (table 7-7). Analysis of paired change in total viral load from baseline to week 12 in those that completed the protocol again revealed no significant change in total HCV viral load in either those taking Fluvastatin for 12 weeks (p=0.249) or those not taking Fluvastatin (p=0.788) (see table 7-5).

Table 7-7 Total viral load intention to treat and per protocol analysis

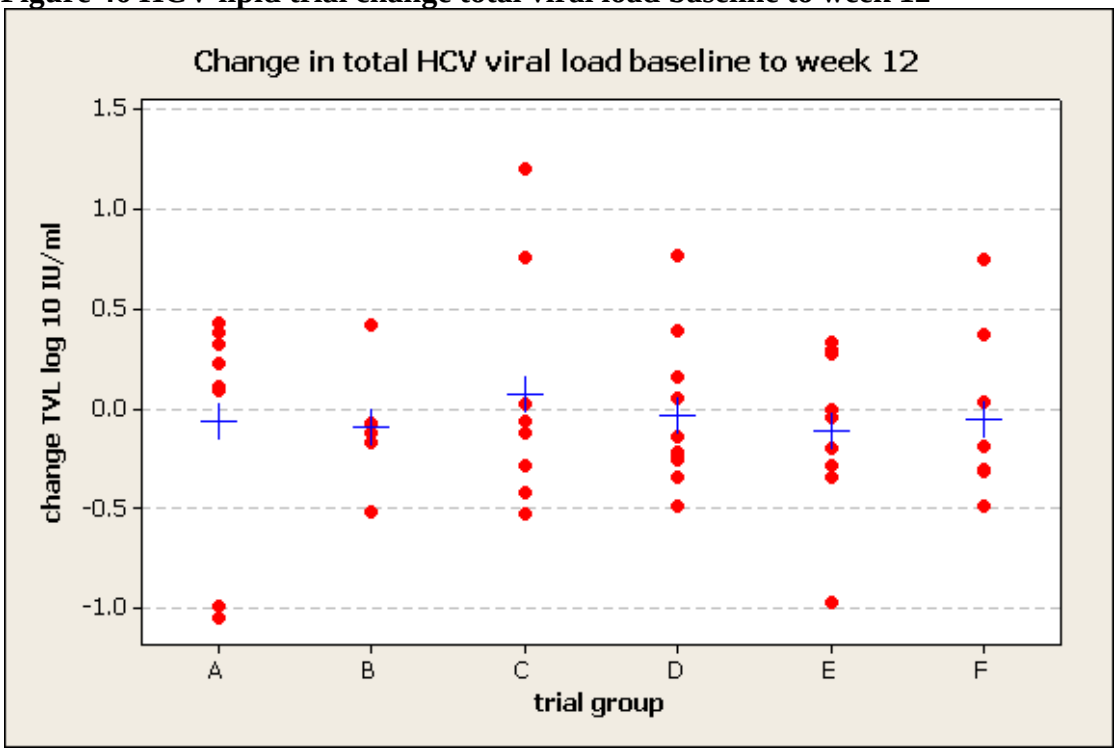
	Fluvastatin (groups D + E + F)	**No fluvastatin (groups A + B + C)**	**p-value**
Intention to treat N = 56 (28 / 28)			
Pre treatment	5.77 ± 0.79	5.87 ± 0.85	0.635
Week 12	5.71 ± 0.87	5.90 ± 0.89	0.460
Completed protocol N =50 (24 / 26)			
Pre treatment	5.73 ± 0.83	5.76 ± 0.86	0.539
Week 12	5.63 ± 0.86	5.88 ± 0.94	0.364

	Omacor (groups B + C + E + F)	**No Omacor (groups A + D)**	**p-value**
Intention to treat N = 56 (39 / 17)			
Pre treatment	5.67 ± 0.85	6.13 ± 0.66	0.048
Week 12	5.60 ± 0.90	6.09 ± 0.77	0.054
Completed protocol N =50 (34 / 16)			
Pre treatment	5.61 ± 0.86	6.01 ± 0.72	0.085
Week 12	5.56 ± 0.87	6.01 ± 0.87	0.058

Sub-group analysis according to HCV genotype, indicated that in HCV G1&4 only (N=32) total HCV RNA was modestly but significantly reduced in those taking Fluvastatin (groups D + E + F, N=15). The pre treatment total viral load on Fluvastatin (N=15) was 5.94 ± 0.60 log10 IU/ml. The week 12 total viral load fell to 5.70 ± 0.79 log10 IU/ml. The mean reduction in total viral load was -0.24 log10 IU/ml (95% CI -0.43, -0.05), p=0.019. In contrast there was no significant change in total viral load in those not taking Fluvastatin with HCV G1 & G4 (Groups A + B + C, N=17), pre treatment total HCV RNA viral was 5.79 ± 0.88 log10 IU/ml and at week 12 was 5.89 ± 1.01 log10 IU/ml. The mean change in total viral load in the non-Fluvastatin group was +0.11 log10 IU/ml (95% CI -0.21, 0.42), p=0.472) (figure 47).

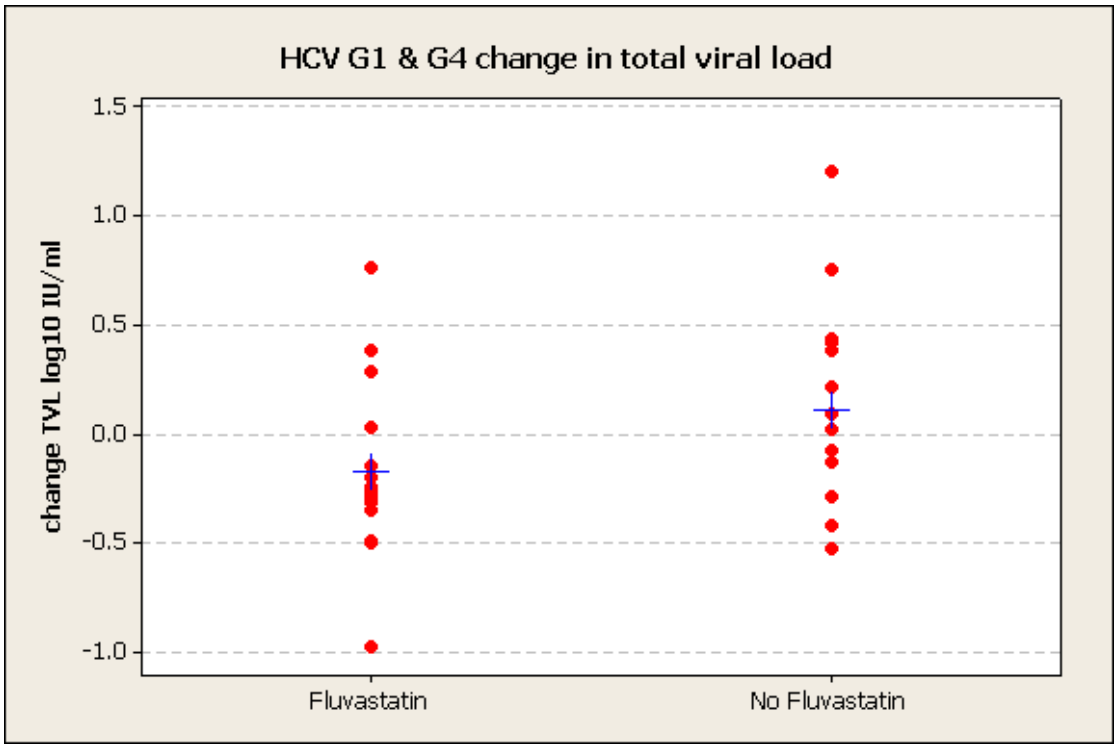
In HCV G2 & G3 patients only (N=18 overall), no significant change in total viral load was detected in those on Fluvastatin [(groups D + E + F, N=9); +0.14 log10 IU/ml (95% CI -0.10, 0.39) p=0.218], and not taking Fluvastatin [group A + B + C, N=9, mean change in total viral load -0.13 log10 IU/ml (95% CI -0.47, 0.20) p=0.393], but these numbers are too small to be conclusive.

Figure 46 HCV lipid trial change total viral load baseline to week 12



Dotplot representing change in total viral load log10 IU/ml calculated from paired week 12 – baseline total viral load (protocol completion anaysis N=50). Blue cross represents the mean value. Group A (control, N=8, p=0.733), group B (low dose Omacor, N=10, p=0.305), group C (high dose Omacor,N=8, p=0.510), group D (Fluvastatin, N=8, p=0.903), group E (low dose Omacor + Fluvastatin, N=8, p=0.301), group F (high dose Omacor + Fluvastatin, N=8, p=0.483).

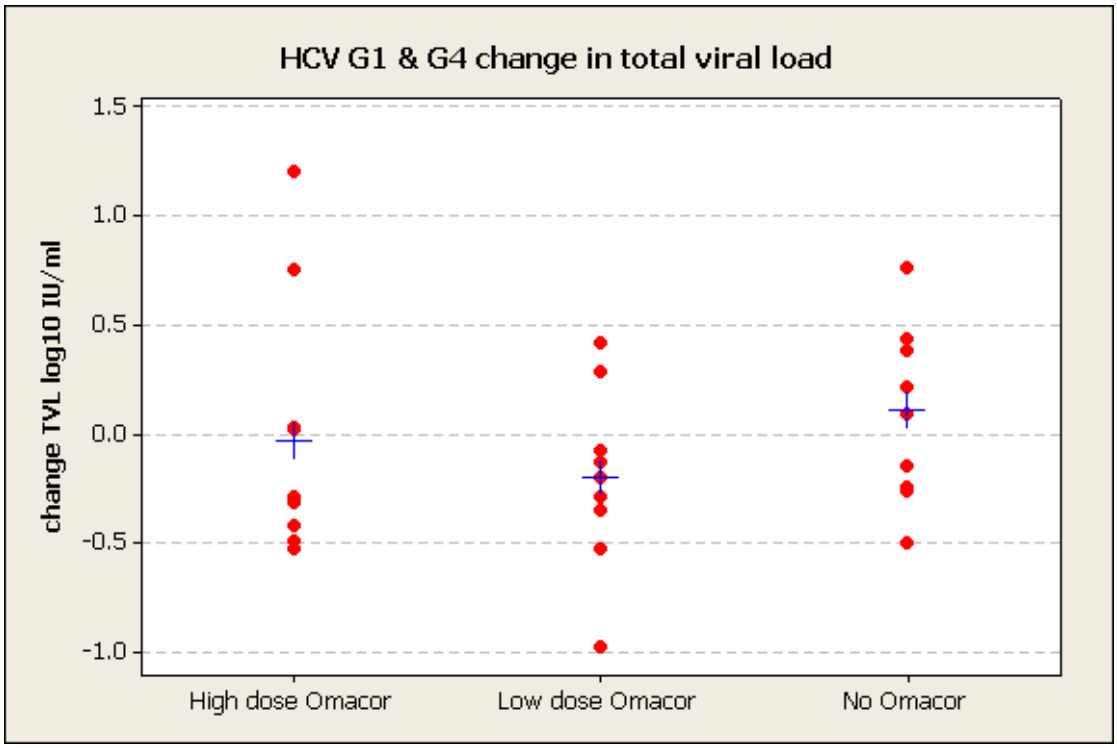
Figure 47 Change in total viral load HCV G1 & G4 Fluvastatin vs no Fluvastatin



*Dotplot representing change in total HCV RNA viral load from baseline to end of treatment in those completing 12 weeks of Fluvastatin therapy (N= 15 on Fluvastatin vs, no Fluvastatin N=17) with HCV genotypes 1 & 4 only. Total HCV RNA viral load decreased by -0.24 log10 IU/ml (**p=0.019**, 95% CI -0.43, -0.05) in those completing 12 weeks of Fluvastatin . In those not on Fluvastatin, mean change in total viral load was +0.11 log10 IU/ml (p=0.472, 95% CI -0.21, 0.42). The blue cross represents the mean value.*

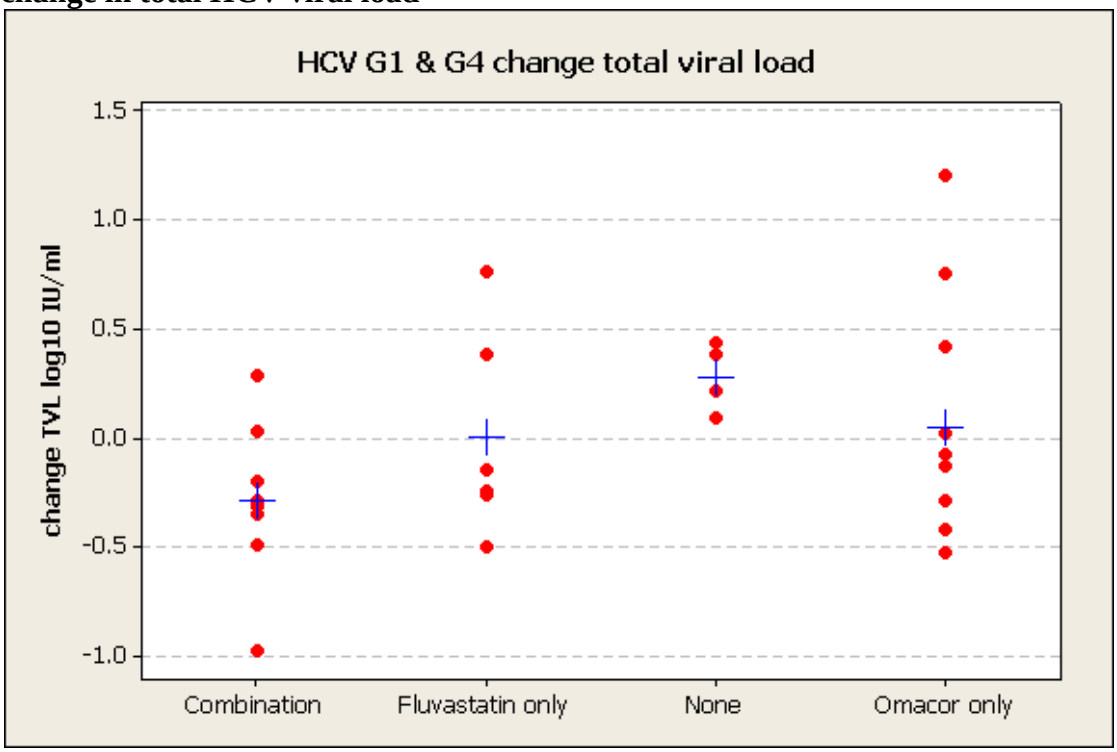
Omacor in high or low dose did not have a significant effect on change in total HCV viral load. Intention to treat analysis indicated pre treatment total viral load was lower at pre-treatment in the Omacor group compared to the no Omacor group but was not significantly different at week 12 (table 7-7). Completion of protocol analysis did not confirm these differences and there was no significant reduction in total viral load from baseline to week 12 in those taking Omacor overall, or in high dose Omacor (group C + F, p=0.884), low dose Omacor (groups B + E, p=0.125) or no Omacor (groups A + D, p=0.849) (table 7-6). Sub-group analysis for HCV G1 & G4 was suggestive of a non-significant trend towards reduction in viral load on low dose Omacor (groups B + E fall in viral load -0.23 ± 0.39 log10 IU/ml, p=0.080, 95% CI -0.49, 0.03 log10 IU/ml, figure 48). Additionally in HCV G1&4 there may be a small synergistic effect with a reduction in total viral load from taking Omacor in combination with Fluvastatin (-0.29 log10 IU/ml, 95%CI -0.55, -0.02 log10 IU/ml, p=0.038, figure 49).

Figure 48 Change in total viral load HCV G1 & G4, high dose Omacor vs low dose Omacor vs no Omacor



Dotplot representing the change in total HCV RNA viral load (TVL) between baseline and week 12 in those completing therapy with HCV G1&4 only. There was no significant difference in TVL between high, low or no Omacor, but a weak trend towards lower total viral load with low dose Omacor (group B + E, change in TVL -0.23 ± 0.39 log10 IU/ml, p=0.080) than high dose Omacor (group C + F, p=0.908) or no Omacor (group A + D, p=0.871).

Figure 49 HCV G1&4, combination Fluvastatin + Omacor vs mono therapy change in total HCV viral load



*Dotplot representing the change in total viral load (TVL) in HCV G1&4 only that completed 12 weeks of treatment.There was a significant reduction in TVL of -0.29 log10 IU/ml (95% CI -0.55, -0.02, **p= 0.038**) in those on combination Fluvastatin + Omacor (groups E + F, N=9). There was no significant change in TVL in those on Fluvastatin alone (group D, N=5, -0.15 log10 IU/ml, 95% CI -0.56, 0.26, p=0.366), Omacor alone (groups B + C, N=10, +0.05 log10 IU.ml 95% CI -0.37, 0.46, p=0.802) or no active drug. Blue cross represents the mean value.*

7.6.2 Liver transaminases – ALT, AST and GGT

The other primary endpoint of the trial was a reduction in liver transaminases. There was no significant change in ALT from baseline to week 12 in any of the individual trial groups overall. Instead there was a modest but statisticallly significant increase in liver transaminases in those that completed 12 weeks of Fluvastatin therapy (groups D + E + F) (table 7-5). There was also a modest but non-significant increase in ALT, AST and GGT in those not taking statins (groups A + B + C) (table 7-8). Omacor in low or high doses was also associated with small increases in liver transaminases (table 7-6) but were neither clinically or statisitically significant.

7.7 Secondary Outcomes

There were a number of secondary outcomes of this pilot study that were considered to be of value to aid design of future trials. Firstly was an evaluation of safety and tolerability of lipid modulating agents in chronic HCV with specific reference to withdrawals and whether changes in cholesterol impact on depression and anxiety symptoms. Secondly was to evaluate whether lipid modulating therapy affects liver stiffness. Thirdly was an evaluation of IP10 levels after 12 weeks of lipid modulating therapy, because of the relationship between this serum marker, hepatic interferon stimulated gene expression and non-response to pegylated interferon therapy.

7.7.1 Withdrawals from study drugs

Ten of the 60 participants that were randomized did not complete the full 12 weeks of study drug. Four were due to non-compliance / non-attendance at subsequent follow-up visits. The remaining six premature withdrawals from study drug were related to adverse events (table 7-8).

Table 7-8 Reasons for early withdrawals from study

Group	code	Genotype	Reason for premature withdrawal	Duration of study drug completed
A	NS14	1	Non-compliance – DNA's	0
A	NS20	1	Non-compliance – DNA'd	8 weeks
B	NS22	1	Non-compliance – DNA'd	4 weeks
C	LS05	3	AE's – GI symptoms and worsening depression	2 weeks
D	LS09	1	AE's Malaise and palpitations	10 weeks
D	LS18	3	AE's Headache and depression	5 days
E	NS31	1	Non-compliance – DNA'd	0
E	NS03	3	AE's Worsening Depression	5 weeks
E	NS29	4	AE's GI symptoms and worsening depression	1 week
F	NS26	1	AE's GI symptoms	11 days

7.7.2 Adverse events

All the adverse events (AEs) reported in the trial are listed in Table 7-9 and Table 7-10. Nine AEs were considered to be probably related to the study drugs (i.e adverse reactions AR). All these AR's were gastrointestinal (GI) side effects; three GI AR's were reported in each of groups C, E and F, and were attributed likely to be due to the fish oils. The severity GI ARs was mild in most and moderate in only two participants in groups E and F. Exacerbation of depression was reported in six participants, (1 in group B, 1 in group C, 1 in group D and 3 in group E). The only SAE was the worsening depression in one participant in group B which led to attempted suicide two weeks after completing the study drugs. The local investigators considered this to be reactive to the individual's circumstances and unlikely to be directly related to the study drug. Another participant in group E had a severe exacerbation of depression which led to discontinuation of the study drugs after 3 weeks, but again was thought by the local investigators to be reactive to life events.

Table 7-9 Trial all adverse events

Group	Adverse Event	Severity	Related to study drug?	Onset of AE	End of AE	Duration of AE	Study drug stopped?	Serious AE	Outcome	Duration of study drug completed
A	URTI	mild	unrelated	week 14	week 15	7 days	no	no	resolved	Completed
B	haematuria	mild	unrelated	week 6	week 6	1 day	no	no	resolved	Completed
B	flu syndrome	moderate	possible	week 7	week 8	7 days	no	no	resolved	Completed
B	depression	severe –suicide attempt	unlikely	week 14	ongoing	ongoing	no	yes	ongoing	Completed
C	dyspepsia	mild	probable	week 4	week 6	14 days	no	no	resolved	Completed
C	nausea	mild	probable	day 7	week 4	21 days	no	no	resolved	Completed
C	diarrhoea	mild	probable	week 2	week 3	7 days	yes	no	resolved	14 days
C	vomiting	mild	possible	week 2	week 3	7 days	yes	no	resolved	14 days
C	insomnia	moderate	possible	week 5	week 6	7 days	yes	no	resolved	14 days
C	anxiety	mild	possible	week 5	week 6	7 days	yes	no	resolved	14 days
C	depression	mild	possible	week 5	week 6	7 days	yes	no	resolved	14 days
D	malaise	moderate	possible	week 8	week 10	2 weeks	yes	no	resolved	10 weeks
D	palpitations	mild	possible	week 8	week 10	2 weeks	yes	no	resolved	10 weeks
D	rash	mild	possible	week 4	week 12	8 weeks	no	no	resolved	Completed
D	headache	severe	possible	day 3	day 8	5 days	yes	no	resolved	5 days
D	depression	severe	possible	day 3	day 8	5 days	yes	no	resolved	5 days
D	flu syndrome	mild	possible	week 4	week 4	3 days	no	no	resolved	Completed
D	vomiting	moderate	unrelated	week 9	week 9	1 day	no	no	resolved	Completed

Table 7-10 Trial all adverse events continued

Group	Adverse Event	Severity	Causality	Onset of AE	End of AE	Duration of AE	Study drug stopped?	Serious AE	Outcome	Duration of study drug
E	diarrhoea	mild	probable	day 4	week 2	10 days	no	no	resolved	Completed
E	itch	mild	possible	week 8	week 12	14 days	no	no	resolved	Completed
E	lethargy	moderate	possible	week 8	week 12	28 days	no	no	resolved	Completed
E	dyspepsia	mild	probable	week 2	week 3	7 days	no	no	resolved	Completed
E	depression	severe	possible	week 3	week 5	14 days	yes	no	resolved	5 weeks
E	fatigue	mild	possible	day 7	week 3	14 days	yes	no	resolved	7 days
E	diarrhoea	moderate	probable	day 2	week 3	19 days	yes	no	resolved	7 days
E	depression	moderate	possible	day 7	week 3	14 days	yes	no	resolved	7 days
E	headache	mild	possible	week 3	week 3	1 day	no	no	resolved	Completed
E	depression	mild	possible	week 23	week 24	1 weeks	no	no	ongoing	Completed
E	insomnia	mild	possible	week 13	week 19	6 weeks	no	no	resolved	Completed
E	chest pains	mild	unrelated	week 8	week 8	1 day	no	no	resolved	Completed
E	myalgia	mild	possible	week 4	week 5	7 days	no	no	resolved	Completed
E	flu syndrome	mild	possible	week 4	week 5	7 days	no	no	resolved	Completed
F	diarrhoea	moderate	probable	day 3	week 2	11 days	yes	no	resolved	11 days
F	ado pain	moderate	probable	day 3	week 2	11 days	yes	no	resolved	11 days
F	headache	mild	possible	day 2	week 5	3 days	no	no	resolved	Completed
F	diarrhoea	mild	probable	day 2	week 4	26 days	no	no	resolved	Completed
F	headache	mild	possible	day2	day 3	1 day	no	no	resolved	Completed

Depression and anxiey symptoms were recorded in each participant at each visit (except day 2) using the HAD depression and anxiety questionnaire. Table 7-11 indicates that both depression and anxiety symptoms are common in patients with chronic HCV infection. Overall 21.7% of the HCV cohort at screening had HAD depression scores ≥8 and 43% had HAD anxiety scores ≥8. Patients with HCV G3 tended to have more frequent depressive symptoms that other genotypes (HAD-D ≥8, 31.6% (G3) vs 17.1% G1, 2 & 4), but similar frequency of anxiety symptoms (HAD-A ≥8, 47.3% (G3) vs 41% G1, 2 & 4). Depressive symptoms were more frequent in those 10 patients that did not complete the 12 week protocol (40% vs 18%) table 7-12.

Table 7-11 Baseline Characterisitics depression and anxiety scores N=60

HCV Genotype (N)	**Depression score**	**HAD-D ≥ 8 N (%)**	**Anxiety score**	**HAD-A ≥ 8 N (%)**
G1 (N=33)	4 (1 – 6)	6/33 (18.2%)	6 (3-9.5)	14/33 (42.4%)
G2 (N=2)	2	0	2.5	0
G3 (N=19)	6 (1-8)	6/19 (31.6%)	6. (4-12)	9/19 (47.3%)
G4 (N=6)	5.5 (1.5-6.5)	1/6 (16.7%)	7.5 (5.5-9.5)	3/6 (50%)
Overall	4 (1.3-6.8)	13/60 (21.7%)	6 (3.3-10)	26/60 (43%)

Median (Q1-Q3) HAD depression (D) and anxiety (A) scores (maximum possible score 21 for each)

Table 7-12 Frequency of depression and anxiety symptoms in relation to completion of trial protocol

	HAD-D ≥ 8 (%)	**HAD-A ≥ 8 (%)**
Completed 12 weeks (N=50)	9 (18%)	21 (42%)
Drop outs (N=10)	4 (40%)	5 (50%)

7.7.3 Lipid profiles and relationship with depression and anxiety

As expected, those taking Fluvastatin (Groups D, E and F) showed significant reductions in total cholesterol, non-HDL cholesterol, apoB and triglyceride after 12 weeks of therapy (table 7-5). Low and high dose Omacor was associated with significant decrease in triglycerides (table 7-6).

Depression was more frequent in HCV G3 than in other genotypes at the screening visit. A possible explanation was that low cholesterol associated with HCV G3 infection was correlative with depression and / or anxiety symptoms. Fasting lipid profiles at screening visit were compared in those reporting depressive symptoms as indicated by HAD-D ≥8 and anxiety symptoms as indicated by HAD-A ≥8 in the overall cohort (N=60). This analysis indicated a tendency for lower total cholesterol in depressed HCV patients (HAD-D ≥8) than in non-depressed HCV patients (p=0.053) table 7-13. Although no significant differences in non-HDL cholesterol, HDL cholesterol and apoB were found between the depressed and not depressed groups, quantitiative apoE was significantly lower in the depressed group (p=0.029). Lipid profiles were not significantly different in those HCV patients with and without anxiety symptoms (table 7-14)

Table 7-13 Lipid profiles and HAD Depression – all HCV genotypes

	HAD D <8 (N=47)	HAD D ≥8 (N=13)	p-value
Total cholesterol mmol/l	4.4 ± 0.9	3.8 ± 1.2	0.053
Non HDL-C mmol/l	3.1 ± 0.9	2.7 ± 1.0	0.171
HDL-C# mmol/l	1.3 (1.1-1.5)	1.2 (1.0-1.3)	0.096
ApoB g/l	0.78 ± 0.23	0.67 ± 0.24	0.136
ApoE g/l	0.039 ± 0.013	0.030 ± 0.012	**0.029**

Table 7-14 Lipid profiles and HAD Anxiety - all HCV genotypes

	HAD A <8 (N=26)	HAD A ≥8 (N=34)	p-value
Total cholesterol mmol/l	4.3 ± 1.1	4.2 ± 0.8	0.801
Non HDL-C mmol/l	3.0 ± 1.0	2.9 ± 0.8	0.837
HDL-C# mmol/l	1.3 (1.1-1.5)	1.3 (1.0-1.5)	0.729
ApoB g/l	0.76 ± 0.26	0.75 ± 0.20	0.864
ApoE g/l	0.038 ± 0.012	0.036 ± 0.015	0.508

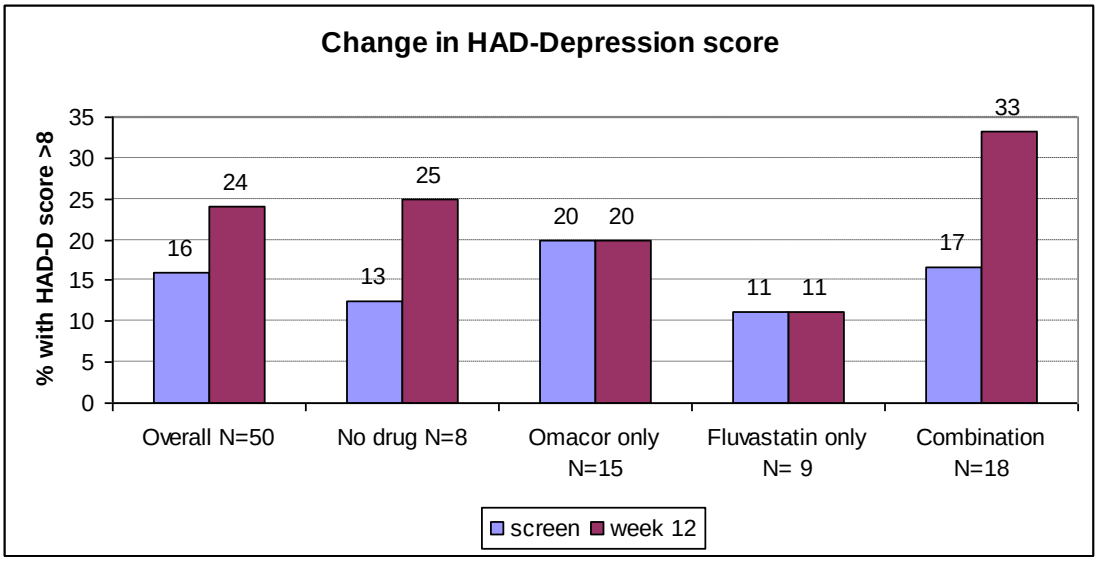
Mean ± standard deviation, comparison using 2-sample t-test. # Median (Q1-Q3) comparison using Kruskall Wallace test

7.7.4 Depression and anxiety – treatment effects

The change in HAD-Depression and anxiety scores from the screening visit to week 12 was evaluated those 50 participants that completed 12 weeks of the study protocol. The frequency of depressive symptoms as indicated by HAD-D ≥8 increased overall from 16% to 24% at week 12. This increase was greatest in those randomised to combination Fluvastatin and Omacor (groups E + F, 17% to 33%) compared to Fluvastatin only or Omacor only that had no change in HAD-D scores (figure 50). HAD-D scores ≥8 were more frequent in HCV G3 than other genotypes at screening and at week 12, but the increase in depressive symptoms on combination Fluvastatin and Omacor was observed in all HCV genotypes (figure 51).

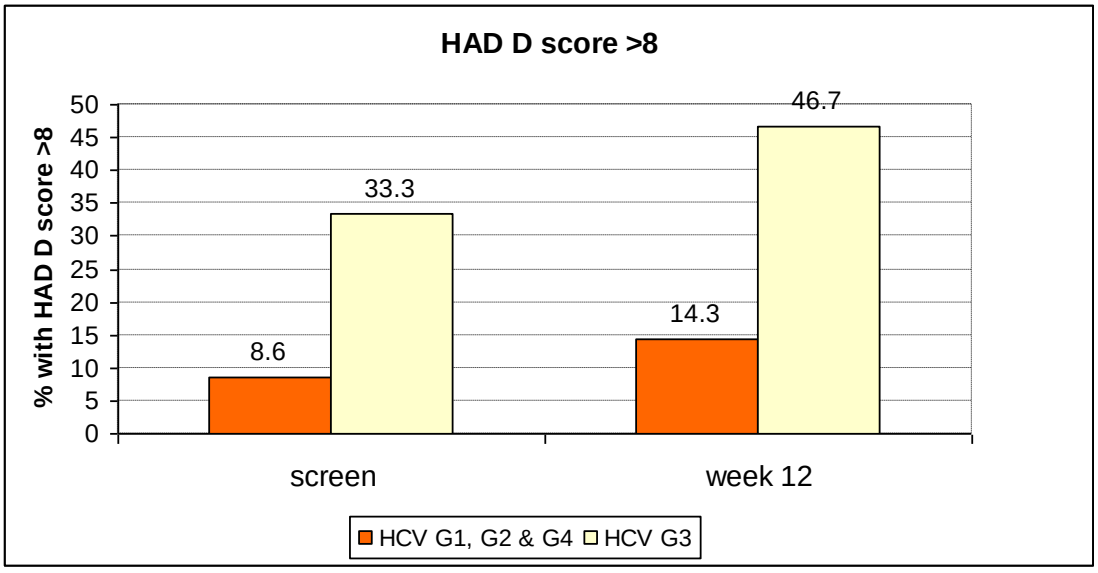
Anxiety symptoms as indicated by HAD-A score ≥8 decreased in frequency with 12 weeks of protocol completion from 40% at screening to 28% at week 12 overall (figure 52). This reduction in anxiety scores was observed largely in the combination therapy group (56% to 33%) rather than Fluvastatin or Omacor only groups. The reduction in HAD-A scores was observed in all HCV genotypes (figure 53).

Figure 50 Frequency of HAD depression scores ≥8 with 12 weeks Fluvastatin and/ or Omacor therapy



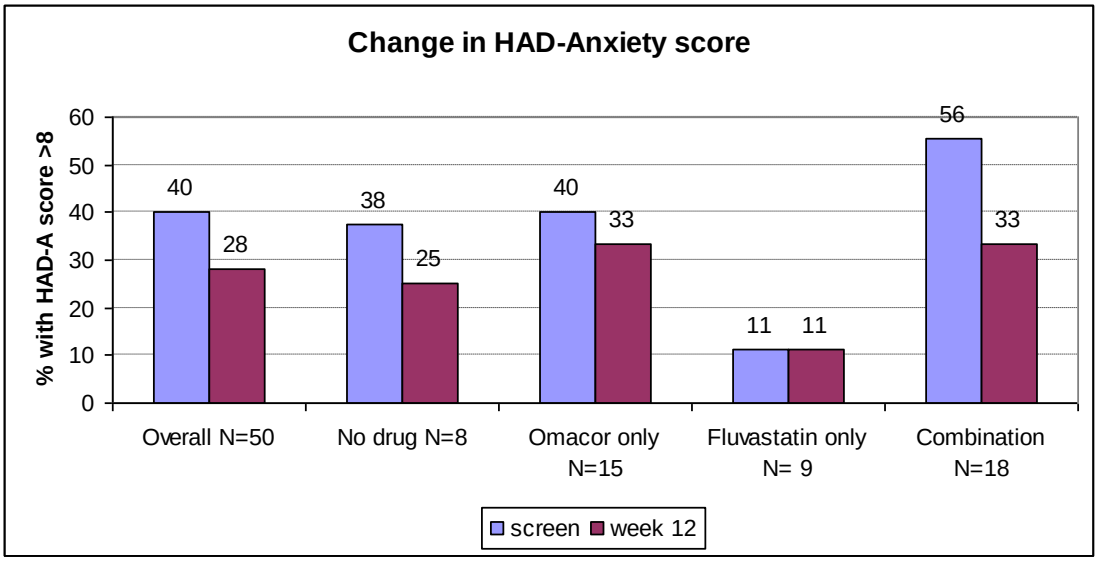
Depressive symptoms were defined by HAD-D score ≥8 measured at screening and week 12. Overall the frequency of those reporting depression increased from 16% to 24%, and was accounted for largely by those taking combination Omacor and Fluvastatin therapy (17% to 33%), rather than monotherapy.

Figure 51 Frequency of HAD depression scores ≥8 at screening and week 12 in HCV G3 and other genotypes (1,2 &4)



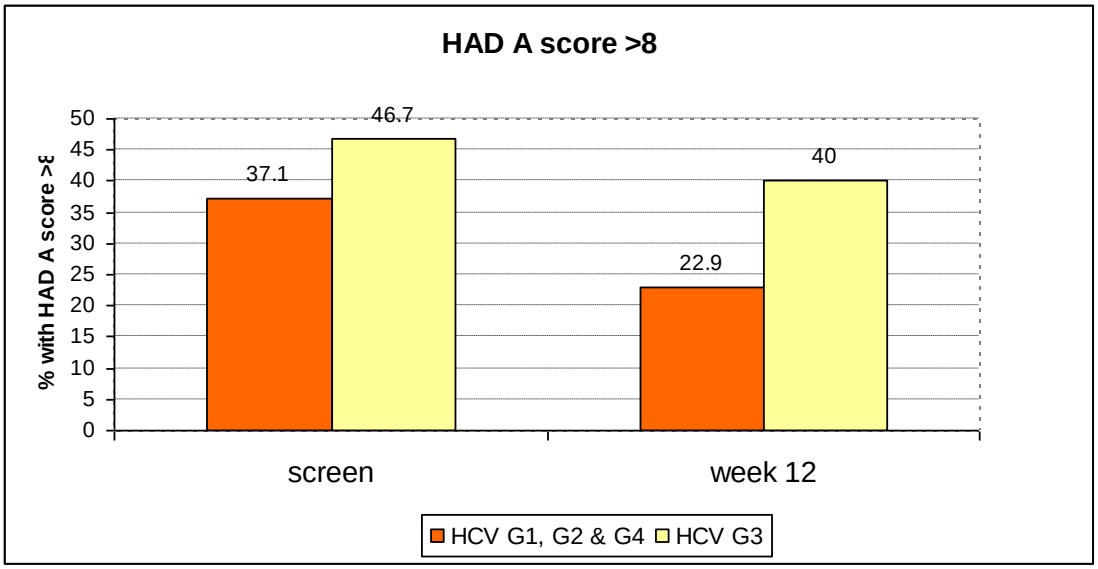
Depression (defined as HAD-D score ≥8) was more frequent in HCV G3 patients than in other genotypes (G1, 2 & 4). Depressive symptoms were more frequent at week 12 than screening for all genotypes.

Figure 52 Frequency of HAD anxiety scores ≥8 with 12 weeks Fluvastatin and/ or Omacor therapy



Anxiety symptoms were defined by HAD-A score ≥8 measured at screening and week 12. Overall the frequency of those reporting anxiety decreased from 40% to 28%, and was accounted for largely by those taking combination Omacor and Fluvastatin therapy (56% to 33%), rather than monotherapy.

Figure 53 Frequency of HAD anxiety scores ≥8 at screening and week 12 in HCV G3 and other genotypes (1,2 &4)



Anxiety (defined as HAD-A score ≥8) was more frequent in HCV G3 patients than in other genotypes (G1, 2 & 4). Anxiety symptoms were less frequent at week 12 than screening for all genotypes.

7.7.5 Liver stiffness

The majority, but not all of the trial participants underwent assessment of liver stiffness measurement (LSM) using Fibroscan ® which was performed between screening and baseline visits and repeated within 1 month of the week 12 visit. There was a non-significant tendency for a reduction in median LSM for those in trial group B after completion of 12 weeks of low dose Omacor, compared to a pre treatment LSM (table 7-15). Although not statistically significant because of small sample size, this was a potentially clinically important reduction in liver stiffness of 8.5 kPa and needs to be validated in future studies. The apparent improvement in LSM was greatest in HCV G1 & G4 rather than in G2 & G3 but the sample size was too small to draw definitive conclusions. Given that there was also a modest reduction in liver stiffness measurements in group E as well as B, when the two groups were combined the paired differences in pre / post treatment LSM reached borderline statistical significance (Combined Group B + E (N=13), pre 17.2 KPa, post 11.2 KPa, difference -6.0 K Pa, paired t-test p=0.076)

The mean reduction in LSM in those taking low dose Omacor (B+E) was -6.0 KPa compared to +1.75 KPa in those not taking any Omacor (groups A+D) p=0.059.

LSM did not appear to be affected by Fluvastatin.

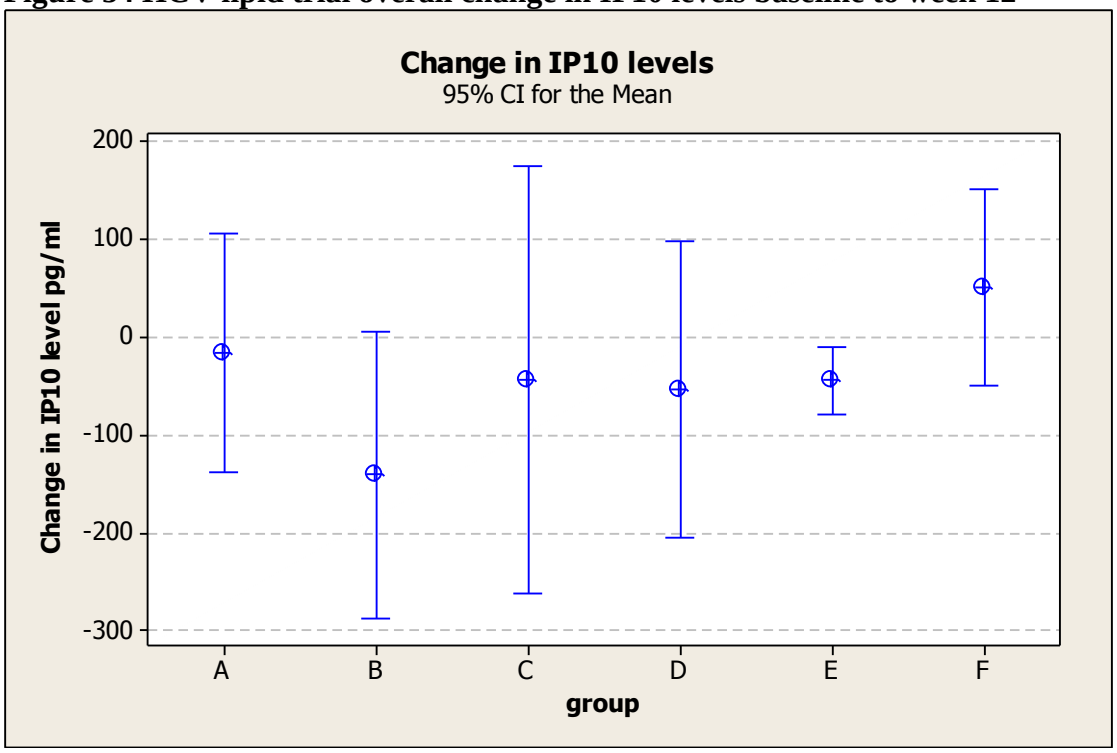
Table 7-15 Mean liver stiffness measurement (LSM) scores paired pre/post 12 weeks of intervention (paired t-test)

Group	N	Pre-screen LSM kPa	>Week 12 LSM kPa	Paried Difference kPa	p-value
A. Control	5	9.5	9.0	-0.5	0.819
B. low dose Omacor	7	15.6	7.1	-8.5	0.126
C. high dose Omacor	6	17.3	17.6	+0.3	0.857
D. Fluvastatin	7	10.9	14.2	+3.3	0.412
E. low dose Omacor + Fluvastatin	6	19.2	15.9	-3.3	0.452
F. high dose Omacor + Fluvastatin	7	8.7	8.6	-0.1	0.833

7.7.6 IP10

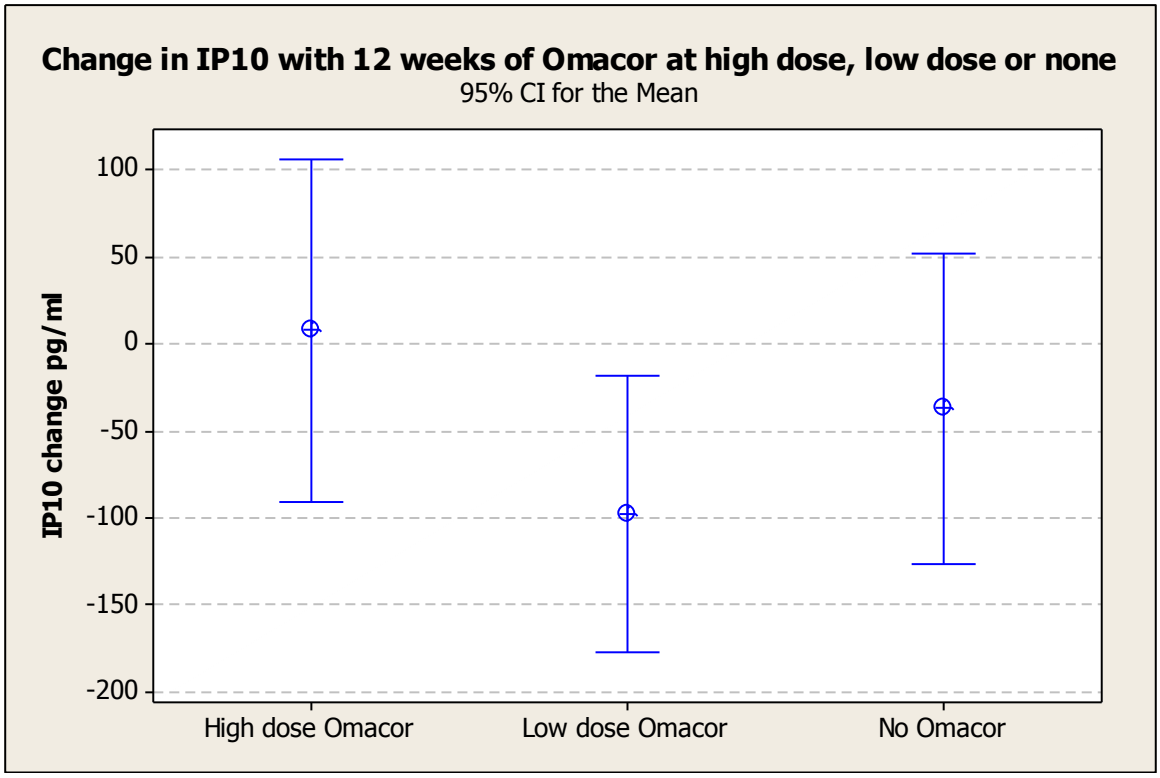
Interferon gamma inducible protein 10 (IP10) is a serum maker of interferon stimulated gene activation. Previous studies have shown high IP10 levels in non-responders to PegIFNα and ribavirin (Lagging, Romero et al. 2006). Because this trial selected previous non-responders, high IP10 levels were anticipated. Recent studies in Chimpanzees using a miRNA 122 inhibitor that efficiently blocked HCV replication showed a correlative reduction in IP10(Lanford, Hildebrandt-Eriksen et al. 2010). IP10 was therefore measured in all study visits on fasting serum using a commercially available ELISA kit (described in material & methods chapter). Analysis was performed on the week 12 – average of screen and baseline IP10 levels to determine whether there had been any significant change in IP10 with 12 weeks of therapy with Fluvastatin or Omacor. 'Within the table' analysis indicated a reduction in serum IP10 levels at week 12 compared to baseline in patients in trial groups B and E overall (figure 54). This fall in IP10 was apparent only in those with HCV G1 & G4, and not in those with HCV G2 & G3. 'At the margins' ananlysis for all those completing 12 weeks of low dose Omacor (groups B + E) indicated a mean reduction in IP10 of 97.7 pg/ml (95% CI -177.3, -18.1, p=0.0019) (table 7-6) but no significant change with high dose Omacor or no Omacor (figure 55). In contrast, in those on Fluvastatin (groups D + E + F) there was not a significant reduction in IP10 (p=0.049) (table 7-5 and figure 56).

Figure 54 HCV lipid trial overall change in IP10 levels baseline to week 12



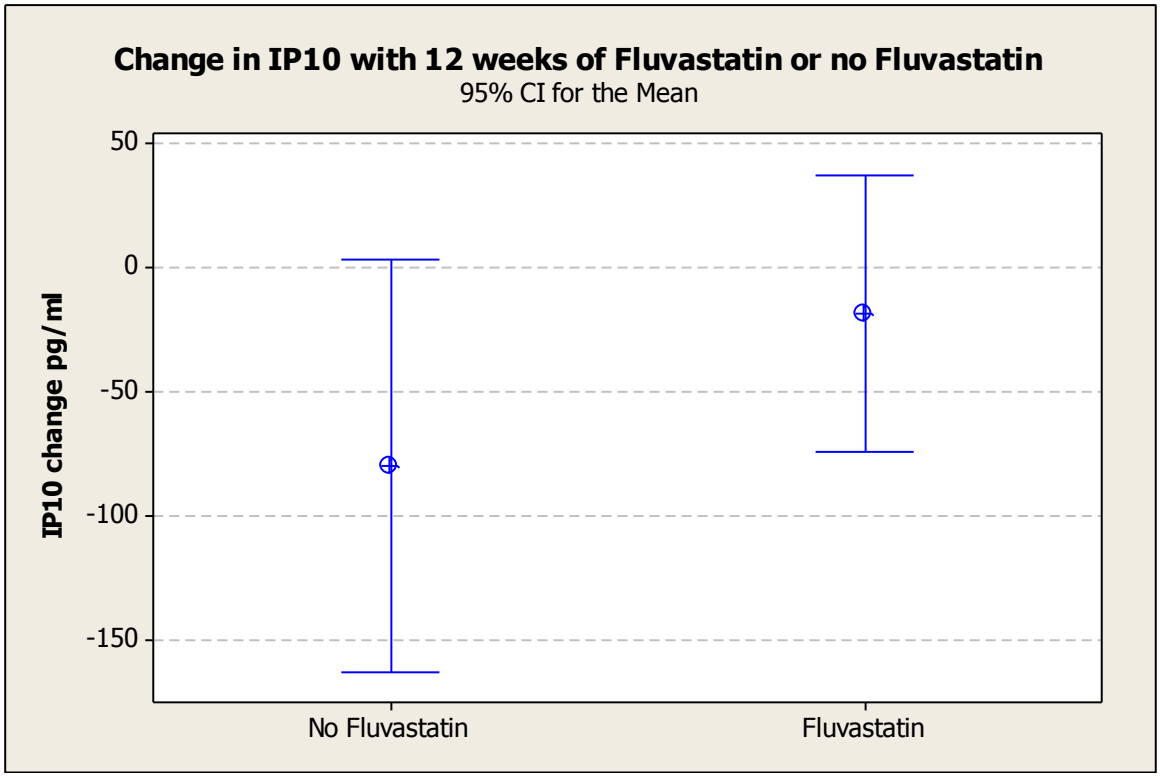
The mean reduction in serum IP10 in group B after 12 weeks of low dose Omacor was 156 pg/ml (95% CI 3 to 293 pg/ml; p=0.053). The mean reduction in IP10 in group E after 12 weeks of Omacor + Fluvastatin was 33.5 pg / ml (95% CI 17.9 to 80.5 pg/ml; p=0.013. Mean and 95% confidence intervals are shown.

Figure 55 Change in IP10 levels in those completing 12 weeks of study drug – Effect of Omacor dose



*Low dose Omacor [groups B + E (also on fluvastatin), N=18] was associated with a significant reduction in serum IP10 of -97.7 pg/ml after 12 weeks compared to baseline (**p=0.019**). IP10 levels did not change significantly in those on high dose Omacor [groups C + F (also on fluvastatin), N=16, p=0.867] or in those not receiving Omacor [(groups A (control) + D (fluvastatin only) N=16, p=0.380].*

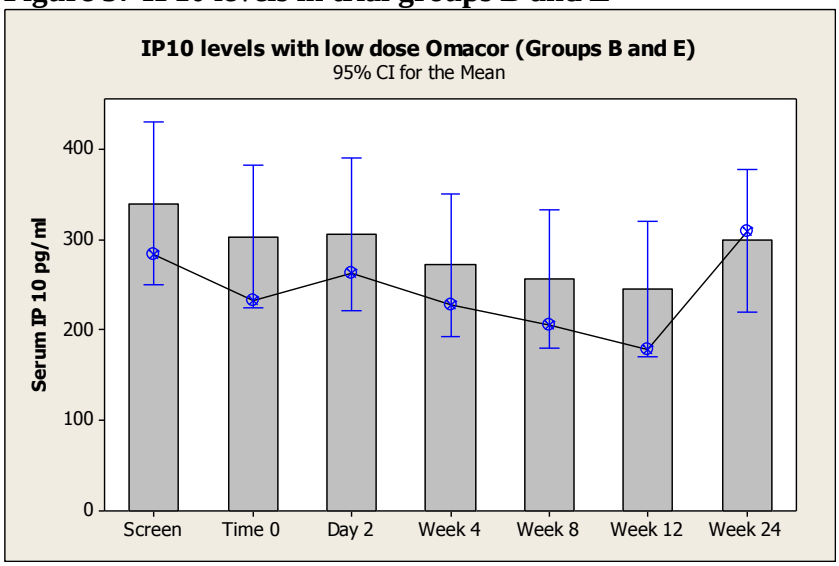
Figure 56 Change in IP10 levels in those completing 12 weeks of study drug – Effect of Fluvastatin



Fluvastatin treatment [groups D + E (also on low dose Omacor) + F (also on high dose Omacor) N=24] was not associated with any significant change in serum IP10 after 12 weeks compared to baseline (p=0.496). There was a non-significant trend towards decreased IP10 level in those not on Fluvastatin [groups A (control) + B (low dose Omacor) + C (high dose Omacor, N=26, p=0.059].

The timing of the reduction in IP10 in groups B and E started at week 4 and continued until week 12. Of those that had a fall in IP10 levels the effect was maximal at week 8 as indicated by one-way ANOVA analysis table 7-16 and figure 57. The reduction in IP10 was not sustained to week 24, supporting that the change is likely to be a drug response.

Figure 57 IP10 levels in trial groups B and E



IP10 levels during the trial for combined low dose Omacor (groups B + E). Shaded columns represent mean IP10 level pg/ml, interval bars are the 95% confidence interval. The line connects the median (*) values at each visit.

Table 7-16 Differences in IP10 levels between study groups during the trial – one way ANOVA analysis

Visit	**One-way ANOVA p-value**
Screen / Base average	0.238
Day 2	0.188
Week 4	0.035
Week 8	0.007
Week 12	0.074
Week 24	0.114

7.8 Discussion

This randomised pilot clinical trial has provided data that will be informative to the approach and design of further trials of lipid modulating therapies in chronic HCV. The data has suggested some potential benefits of Fluvastatin and n3 PUFA's in selected patients with chronic HCV that were previous non-responders to Peg-interferon-α and ribavirin therapy.

The primary outcome measure in the trial was a reduction in total HCV RNA. Overall, 12 weeks of therapy with Omacor and / or Fluvastatin was not associated with a clinically significant reduction in total HCV RNA, and these agents are clearly not potent anti-viral agents. However it was noteworthy that in a sub-group with HCV G1 & G4 infection there was a modest but statistically significant reduction in total HCV RNA viral load in those taking Fluvastatin. The numbers of HCV G2&3 randomised to each group were too small to draw any definitive conclusions about efficacy for these genotypes. N3 PUFA's did not have any apparent significant impact on total HCV viral load in this small trial. There was a suggestion of an additive synergistic effect of combination Fluvastatin + Omacor in HCV G1 & G4.

A viral suppressive effect of fluvastatin would be consistent with the effects shown on viral replication by statins in HCVcc. Statins are thought to exhibit an anti-HCV effect by inhibition of geranylation of the host protein FBL2, thereby disrupting a subsequent association with NS5A and suppressing HCV replication (Wang, Gale et al. 2005). Fluvastatin has been reported to have the strongest anti-HCV activity *in vitro* followed by atorvastatin and simvastatin having moderate effects and Lovastatin the weakest. Pravastatin had no HCV inhibitory effects (Ikeda, Abe et al. 2006). Statins have been shown to have synergistic additive anti-viral activity in combination with IFNα *in vitro* (Ikeda, Abe et al. 2006; Delang, Paeshuyse et al. 2009). Since Pegylated interferon was not used in this trial, synergistic activity of combination with Omacor or Fluvastatin was not evaluated. A recent in vitro study combining statins with a non-nucleoside inhibitor resulted in more profound anti-viral effects and reduced the selection of resistant HCV replicons (Delang, Paeshuyse et al. 2009) justifying further assessment of these agents in patients. However in pilot trials to date there have been variable responses to statin mono therapy in HCV, some showing modest reductions in viral load and others no difference in HCV

monoinfected patients. These previous studies have also been largely small, with poorly designed entry criteria and variable statin doses. An early study gave ten hyperlidaemic HCV patients (8 HCV G1, 1 G2, 1 G4) Atorvastatin for 12 weeks but found no significant reduction in HCV RNA (O'Leary, Chan et al. 2007). A later study using variable doses of fluvastatin up to 320mg as monotherapy for variable durations found a maximum reduction in HCV RNA of 1.75 log10 IU/ml. (Bader, Fazili et al. 2008). That study had a confusing study design and included 15 HCV G1 and 7 HCV G3 patients including treatment naive. Another small pilot study randomised 22 HIV/HCV G1 co-infected patients to Fluvastatin at high doses of 80mg QDS for 4 weeks but found a significant increase in total HCV viral load from 5.63 to 5.84 log10 IU/ml (Milazzo, Meroni et al. 2009). However with subsequent anti-viral treatment there was an improved RVR rate but no improved SVR (Milazzo, Caramma et al.). Another study from Japan used 20mg of Fluvastatin in combination with PegIFNα and ribavirin in 21 HCV G1 patients and achieved 67% SVR. This trial had no control group however (Sezaki, Suzuki et al. 2009). A retrospective study of 50 hyperlipidaemic patients receiving concurrent statin therapy found no change in total HCV viral load (Forde, Law et al. 2009). Recently sub-group analysis from the IDEAL trial indicated a beneficial effect of taking statins in combination with Pegylated IFNα in HCV G1 infection (Harrison, Rossaro et al.). In this study, 66 patients receiving a statin pre-interferon treatment had a higher SVR rates those not receiving statins (53.0% versus 39.3%, $p = 0.02$).This study also confirmed the observation that a high LDL cholesterol level and a low HDL level and statin use were all independently associated with SVR.

The present study adds to the literature concluding there is insufficient evidence of clinically significant efficacy to use Fluvastatin or n3PUFA's as mono-therapy in chronic HCV. However the data from this study suggest some modest anti-viral effect of Fluvastatin in patients with HCV G1 & G4 infection. Future trials should consider the combination of Fluvastatin with Pegylated interferon therapy in this selected group of patients. Overall Fluvastatin and Omacor were safe and reasonably well tolerated in patients with chronic HCV. The most commonly reported adverse event was GI upset but this was mild in most cases. Depression and anxiety are both common symptoms in chronic HCV, and were more prevalent in those with HCV G3 infection. Depression was associated with low serum cholesterol and apoE, and

depressed patients were more likey to stop the medication prematurely. Therefore this study does not support the use of Fluvastain or Omacor in HCV G3 patients particularly with hypocholesterolaemia and depression. Any future trials of lipid modulating agents in this HCV population should estimate a 15% drop out rate when making calulations of sample size.

7.8.1 n3-PUFA and decreased IP10

Secondary analysis of this pilot trial suggested a reduction in serum IP10 levels in those HCV G1 & G4 patients taking low dose n3 PUFAs. This raises the possibility of improving interferon sensitivity in this group of difficult to treat patients. Data from the previous chapters has already demonstrated an independent association of serum IP10 and HCV LVP ratio in genotype 1 infection (results chapter 4). IP10 levels also correlate inversely with lipid profiles in HCV (results chapter 3). In HCV G1 high IP10 levels are correlated with low non-HDL cholesterol and low apoB. In HCV G3 high IP10 levels correlate with HOMA IR. Therefore as a marker of interferon gene activation, IP10 provides a likely link between these lipid abnormalities and adverse response to interferon α treatment outcomes (results chapter 3).

The observation in this pilot trial that 12 weeks of low dose n3–PUFA may reduce serum IP10 levels is intriguing because it suggests an improvement in the unfavourable state of ISG activation in previous IFNα non-responders, and raises the possibility of restoration of interferon sensitivity. This needs to be evaluated in further studies, either in a Chimpanzee animal model or in future trials with n3 PUFA in HCV which should consider paired liver biopsies at baseline and 12 weeks to evaluate interferon stimulated gene expression profiles, prior to initiating further interferon therapy.

The mechanism by which n3-PUFA may lower IP10 levels remains to be elucidated. However n3 PUFA's are widely recognised as having anti-inflammatory properties. Two mechanisms are likely to be involved. Firstly n3 PUFA are incorporated into phospholipids at the expense of the n6 PUFA arachadonic acid (AA). AA is converted to pro-inflammatory prostaglandins by the action of cyclo-oxygenase (COX). The n3-PUFA's Docosahexanoic acid (DHA) and Eicosapentaeoic acid (EPA) are also substrates for COX and lipoxygenase (LO). N3 PUFAs are preferentially catabolised by COX-2 over COX-1. Hence both DHA and EPA compete with AA as substrates for COX and LO. (Schmoker, Weylandt et al. 2007). Eicosanoids produced from AA such as leukotriene B4 are more pro-inflammatory than those produced from n3-PUFA long chain fatty acid substrates such as leukotriene B5. Therefore the antagonism of AA by n3-PUFA's promotes anti-inflammatory properties (Mori and Beilin 2004).

N3 PUFAs may also lower IP10 by an effect on gene expression mediated by the action of n3 PUFA as PPAR activators (Huwiler and Pfeilschifter 2009). Previous data from the transgenic fat-1 mouse animal model of acute liver injury indicated a beneficial effect of n3-PUFA in dampening inflammation, by reducing hepatic gene expression of TNFα, IL1β, IFNγ and IL6. An alternative mechanism may be via PPARγ induced activation which has been shown to inhibit IFNγ induced expression of IP10 in human endothelial cells. (Marx, Mach et al. 2000). Increased levels of n3 PUFA in cell membranes reduces the production of inflammatory cytokines such as IL6, IL10 and TNFα. N3 PUFAs inhibit signalling through Toll –like receptor 2 and thereby suppress NF kappa B which has the effect of reducing transcription of pro-inflammatory cytokines (Lo, Chiu et al. 1999). DHA in particular has been shown to reduce IP10 production from Rhinovirus infected respiratory epithelial cells (Saedisomeolia, Wood et al. 2009). The IP10 gene has an NF kappa B response element, therefore decreased IP10- levels may be due to decreased NF Kappa B activation (Wu, Ohmori et al. 1994).

The data from this pilot study support a further prospective randomised trial using low dose n3-PUFA as lead in therapy in previous IFNα non-responders. This may be particularly important in those with the unfavourable IL28B genotype to evaluate whether pre-treatment with n3 PUFA as adjunctive therapy can improve interferon sensitivity.

7.8.2 n3 PUFA and liver stiffness

Another interesting secondary outcome from the trial was some reduction in liver stiffness in a number of participants receiving low dose Omacor. Given the magnitude of improved LSM with only a short 12 week intervention, it is likely that n3 PUFA are impacting on liver fat and / or inflammation rather than fibrosis. N3 PUFA may impact on steatosis by its action as a PPARα activator. PPARα is a transcription factor that up-regulates genes involved in the β oxidation and transport of fatty acids (Staels, Dallongeville et al. 1998). PPARα is expressed in liver, heart, skeletal muscle and brown adipose tissue and regulates oxidation of fatty acids in mitochondria and peroxisomes. When fatty acid concentrations increase, PPARα activation increases FFA uptake and oxidation.

Both n3 PUFA's docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) can bind to and activate PPARα. Levels of PPARα have been reported to be lower in HCV patients with steatosis than those without steatosis (Dharancy, Malapel et al. 2005), and lower in liver biopsies from patients with HCV genotype 3 than genotype 1 so it was anticipated that the greatest improvements would be found in those with HCV G3. There have been a few pilot studies in patients with NAFLD suggestive of reduced hepatic steatosis in those taking n3 PUFA (Masterton, Plevris et al. ; Capanni, Calella et al. 2006) and there are n3PUFA trials ongoing in NAFLD.

7.9 Summary, trial limitations and conclusions

This pilot trial had a number of limitations. The major limitation related to small numbers. Although a recruitment target was originally set for 72 pateints to be randomised to give 12 per treatment group, it was apparent during the trial that this would not be reached. One major factor for failing to reach the recruitment target was competing trials using new anti-protease inhibitors in combination with PegIFNα and ribavirin for HCV G1 non-responders. This trial did not include re-treatment with PegIFNα and hence offered participants no immediate prospect of a cure. Furthermore the *in-vitro* studies using Fluvastatin and n3 PUFA's in cell culture have indicated a synergistic effect when combined with PegIFNα, hence it may have been better to follow a monotherapy phase with combination treatment. However this would have greatly increased the cost, duration and likelihood of significant side effects.

The factorial design had the advantage of being able to compare two different treatments. However because this trial included two different doses of Omacor in a 3 x 2 design, the numbers in each individual group were very small. It may have been better to simplify the study with just one Omacor dose and one Fluvastatin dose as a 2 x 2 factorial study rather that a 3 x 2 study to increase the sample size for the individual groups.

Since the date when the trial protocol was written and initiated, mounting evidence is indicating that HCV G3 is metabolically distinct from HCV G1, and has profund effects on host lipid metabolism. Inclusion of small numbers of HCV G3 patients into each study group serves largely to skew lipid profiles data. However the numbers of patients with HCV G3 in each group were too small to make any meaningful sub-analysis for this particular genotype. In future trials it would be more appropriate to separate HCV genotype 3 completely in the inclusion criteria.

Of the 60 patients that were randomised, 10 did not complete the full 12 week duration of study drug for various reasons. In 4 /10 the reason for non-completion was non-attendance with further study visits. This is a practical difficulty of any trial in this population, many of whom have a history of injecting drug use and chaotic lifestyles. The other 6 that did not complete the full 12 weeks reported side effects. The major adverse events reported were gastrointestinal which are expected AE's from the study drugs. Because the study was not blinded, each participant was given

the manufacturer's patient information sheet about the medication to which they were randomised, on the recommendation of the research ethics committee. This may have contributed bias to an excess in reporting of anticipated adverse events. Depression was prevalent at screening, more frequent in HCV G3 than other genotypes and also more commonly reported on combination n3 PUFA / Fluvastatin lipid modulating therapy. Two individuals with a past history of depression had signifcant depressive exacerbations.These episodes were considered by the investigators to be reactive to their individual circumstances rather than directly related to the study drug. This study showed a significant reduction in anxiety symptoms in those taking high dose n3 PUFA's for 12 weeks.

Depression and anxiety are common symptoms of chronic HCV infection and have been reported in other studies to affect 20-30 % of HCV patients (Golden, O'Dwyer et al. 2005). Studies of patients that acquired HCV from contaminated anti-D immunoglobulin showed an increase in prevalence of anxiety and depression (Goulding, O'Connell et al. 2001) that suggests that HCV may have some biological relationship with cognitive symptoms. Cognitive and neuropsychological symptoms in HCV may be related to a direct biological effect of HCV infection in the CNS, causing immune activation of microglial cells (Forton, Taylor-Robinson et al. 2006; Forton, Hamilton et al. 2008). Depression has also been widely reported to be associated with low cholesterol in the psychiatric literature, although the mechanisms are not elucidated (Partonen, Haukka et al. 1999; Sansone 2008). It is possible that the combined effects of chronic HCV infection in the CNS coupled with low cholesterol in HCV G3 account for the high prevalence of depressive symptoms of this group in this trial. Depressive symptoms are also common side effects of interferon therapy and limit compliance affecting >30% of patients treated (Martin-Santos, Diez-Quevedo et al. 2008). Studying biological mediators of depression in HCV however is difficult because of the high prevalence of co-existing clinical depression and HCV amongst injecting drug users.

Polyunsaturated fatty acids may have a biological effect on cognitive function. The neurotransmitters glutamate, dopamine and serotonin are derived from arachadonic acid. It has been proposed that in depression there is enhanced AA signalling promoted by relative deficiency of n3-PUFA (Liperoti, Landi et al. 2009). EPA can also inhibit phospholipase A2, which is involved in releasing unesterified fatty acids

from phospholipids. EPA appears to have anti-depressant activity whereas DHA does not. The improved anxiety symptoms in those treated with high dose fish oil may be mediated through these central mechanisms.

Despite the limitations, the pilot trial justifies a further trial of Fluvastatin in combination with PegIFNα and ribavirin in patients with HCV G1 and 4. Additionally it needs to be evaluated in a further trial whether a lead in phase with 8 to 12 weeks of low dose Omacor can enhance interferon responsiveness in previous PegIFNα and ribavirin non-responders. The impact of these lipid modulating agents on HCV LVP and LVP ratio is also in progress.

Final Discussion and future work

The data presented in this thesis have provided further understanding of the interactions of HCV and lipid metabolism and how these interactions may impact on the response to anti-viral therapy.

The data in chapter 3 indicated that the clinical phenotype of dyslipidaemia in HCV appears to be distinct between HCV G1 and G3, whereby those with HCV G3 have particularly marked reductions in LDL. In chapter 4 the evidence presented indicates that endogenous cholesterol synthesis is reduced equally between HCV G1 and G3, predominantly via the lathosterol pathway. In HCV G3 there is relative sparing of the desmosterol pathway which appears to be discordantly up-regulated. These abnormalities appear to resolve with successful eradication of HCV after anti-viral therapy. A key difference between the HCV genotypes is low PCSK9 levels in HCV G3. This supports the concept that the major distinction between the metabolic phenotype of low LDL in HCV G3 compared to HCV G1 is largely due to increased LDL clearance in G3. The data indicating that low LDL in HCV G3 is due at least in part to increased LDL clearance require further validation by a formal stable label apoB kinetics study, ideally in a number of HCV G3 patients before and after successful anti-viral therapy. Profound hypocholesterolaemia in HCV G3 may be a factor in exacerbating depressive symptoms in this group of patients. This was evident in the clinical trial where depressive symptoms were common amongst those with HCV G3 infection and correlated with low LDL cholesterol and particularly low apoE. This is clincally very relevant because depression limits compliance with PegIFNα and RBV therapy in up to 30% of those treated (Golden, O'Dwyer et al. 2005). It is possible that recombinant PCSK9 may be of therapeutic value in HCV G3 patients with profound hypocholesterolaemia, potentially reducing LDLr expression to normal and restoring the dyslipidaemia.

The combination of low PCSK9, relatively high desmosterol and hepatic steatosis in HCV G3 also demands further investigation. The hypothesis is that HCV may modulate endogenous cholesterol synthesis preferentially via desmosterol rather than lathosterol pathway. One approach to address this hypothesis *in vitro* would be to use radio labelled C^{13} acetyl co-A, and monitor its incorporation into sterol intermediates in Huh7.5 cells before and after infection with HCVcc. It would also be important to evaluate the effects of relative desmosterol excess on *de novo* lipogenesis and fatty

acid oxidation. It would be interesting to compare sterol profiles in patients with HCV to patients with non-alcoholic fatty liver disease, and obese individuals without steatosis. The working hypothesis is that relative desmosterol excess maybe important in regulating the development of hepatic steatosis via inappropriate activation of *de novo* lipogenesis, and may be an important factor in determining which individuals with central obesity develop steatosis.

Chapter 5 showed an independent association of LDL cholesterol and anti-viral treatment outcome, independent of viral genotype. Individulas with low LDL-C tended to be less likely to respond to PegIFNα and ribavirin than those with high LDL cholesterol. This important observation has subsequently been corroborated in prospective clinical trials (Harrison, Rossaro et al. 2010). Also TG/HDL ratio was associated with poor response to anti-viral therapy. The most likely explanation for the association of LDL cholesterol and interferon treatment outcome is an interaction between the endogenous interfon response and lipid metabolism. Collaboration in a GWA study led to the discovery that the most important host genetic determinant of treatment outcome is IL28B genotype, a type III interferon involved in innate anti-viral defences. High endogenous IFN responses are associated with poor response to therapy and serum IP10 has been well documented as a serum marker of hepatic ISG activation (Sarasin-Filipowicz, Oakeley et al. 2008). The negative correlation between IP10 and LDL cholesterol links the innate interferon response to the lipid abnormalities associated with adverse treatment outcomes in HCV G1. This explains the apparent paradox that low LDL is associated with poorer treatment response, and yet HCV G3 which lowers LDL more than HCV G1 responds better to anti-viral therapy. A possible explanation is that in HCV G1, low LDL cholesterol is another marker of hepatic ISG activation, but in HCV G3 low LDL is likely to be predominantly caused by a different mechanism through independent genotype specific up-regulation of LDLr.

Development of a novel assay to measure HCV LVP has indicated that the LVP ratio is associated with poor treatment response and more advanced liver disease in HCV G1. The primary metabolic determinants of LVP in HCV G1 appear to be triglycerides and insulin resistance. The association between IP10 and LVP ratio in HCV G1 again links the innate anti-viral response and dyslipidaemia to the formation of potentially more infectious virions, favouring viral persistence in the host.

In future studies, genetic determinants of LVP need to be investigated. Any further measurement of LVP in future clinical cohorts should be combined with collection of genomic DNA and evaluated in the context of IL28B genotype. A GWAS approach in a large cohort of patients comparing those with high and low LVP may help characterise the specific host genetic determinants of LVP and provide further insight into the possible mechanisms of HCV interactions with lipid metabolism.

Finally this study has indicated that lipid modulating therapy with Fluvastatin may reduce HCV replication in HCV G1 & G4. Moreover low dose n3 PUFA may lower IP10 in HCV G1 & G4, raising the potential capacity of restoring interferon sensitivity. A priority for further work is to complete the measurement of LVP from the intervention trial, to elucidate whether n3-PUFAs and Fluvastatin are modulating LVP directly. Ideally further larger prospective intervention trials are required. The LVP assay in its existing form is rather too laborious and time consuming for application in a large multi-center clinical trial. The LVP assay will require further development for more routine applications. However the data from the pilot trial would support a larger randomised clinical trial in HCV G1 non-responders. The results from this pilot study would suggest testing an 8 to 12 week lead in phase comparing low dose n3 PUFA to placebo to determine whether a fall in IP10 can favourably impact on subsequent IFN sensitivity. This lead in phase could be followed by combination therapy with PegIFNα and ribavirin, with and without the addition of Fluvastatin. In addition to standard clinical end points such as RVR, EVR and SVR, assessment of LVP ratio would be important. The relationship between LVP and insulin resistance in HCV G1 also highlights the utility of measuring LVP in future trials using insulin sensitisers to determine whether these agents can favourably impact on LVP ratio. Use of LVP ratio in future practice may be helpful in discriminating which patients may benefit from such adjunctive therapies, possibly combined with a genetic test for IL28B genotype.

Patients with HCV G3 are at risk of being left behind with current anti-HCV drug developments. Although 70-80% of HCV G3 patients are cured with existing therapies, the options for the 20-30% that do not respond are limited. Clinical trials to date have indicated that the new generation of protease inhibitors have limited efficacy in HCV G3, and are unlikely to be licensed for use in HCV G3 in the first wave (Asselah, Benhamou et al. 2009). In this study, the major metabolic correlates

(insulin resistance and raised triglycerides) with LVP appeared to be in HCV G1 rather than HCV G3. In contrast in HCV G3, LVP correlated mainly with low HDL. The data for HCV G3 in particular need to be considered only preliminary because of small numbers of HCV G3 patients in this cohort so far. However, raising HDL may be a beneficial therapeutic strategy for both HCVG1 and G3, to potentially lower LVP directly in G3 and indirectly by reducing VLDL TG in HCV G1. Existing HDL raising drugs include Niacin and CETP inhibitors that could be piloted for efficacy in HCV.

Although much more research needs to be done in the field of HCV and host lipid interactions, the data presented in this thesis has clarified some of the important research questions and direction for future studies.

8 Appendices

8.1 Appendix A- International Diabetes Federation definition of Metabolic syndrome

Central Obesity - waist circumference, ethnicity specific (>80cm women, >94cm Europoid or >90 Asian)

Plus any two of:

Raised triglycerides >1.7mmol/l

Reduced HDL cholesterol <1.03 mmol/l in men; <1.29 mmol/l women

High blood pressure >130 mmHg systolic, >85 mmHg diastolic, or previously treated hypertension

Raised fasting glucose >5.6mmol/l

Previously diagnosed type 2 diabetes

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10 Publications